The meiotic cohesin subunit Rec8 in the pathogenic fungus *Cryptococcus neoformans*

A thesis submitted towards partial fulfilment of the degree for

Masters of Science

As a part of the Integrated PhD program

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DECLARATION

I do hereby declare that the work described here in this thesis entitled 'The meiotic cohesin subunit Rec8 in the pathogenic fungus, *Cryptococcus neoformans*' is original and has been carried out by myself under the guidance and supervision of Dr. Kaustuv Sanyal, Professor, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore-560064, India.

Bornika Roy

Dated:

CERTIFICATE

This is to certify that this thesis entitled 'The meiotic cohesin subunit Rec8 in the pathogenic fungus, *Cryptococcus neoformans*', submitted by Bornika towards the partial fulfilment of Integrated PhD Program, as part of the project work for Master of Science, at Jawaharlal Nehru Centre for Advanced Scientific Research, is based on the studies carried out by her under my supervision and guidance.

KaustuvSanyal
Professor

Dated:

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ABBREVIATIONS

° C – degree Celsius

ml – milli litre

µl – micro litre

µg – micro gram

ng – nano gram

bp – base pairs

kb – kilo base

mM – milli molar

BLAST- Basic Local Alignment Search Tool

DDK- Cdc7-Dbf4 kinase or Dbf4 Dependent Kinase

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

GFP – Green fluorescent protein

HIV/AIDS- Human Immunodeficiency Virus/ Acquired Immunodeficiency Syndrome

KT – Kinetochore

NAT – Nourseothricin

MSA- Multiple Sequence Alignment

mCh - mCherry

OD_{600} – Optical density at a wavelength of 600nm

ORF – Open reading frame

PCR – Polymerase chain reaction

PLK- Polo-Like Kinase

RNA – Ribonucleic acid

SC – Synaptonemal Complex

Sgo- Sugoshin

SLE- systemic lupus erythematosus

SMC- Structural Maintenance of Chromosomes

SPB – Spindle Pole Body

CHAPTER 1

INTRODUCTION

1.1 Meiosis

The term ‘meiosis’ originates from the Ancient Greek word **meíōsis**, which means “a lessening”. This is apt, given that the process of meiosis is a special mode of cell division wherein chromosome number or ploidy is halved, creating haploid cells from a diploid cell(Ohkura 2015). It is essential for sexual reproduction, which, in turn, is the most common means of generating genetic diversity. This genetic diversity increases the likelihood ofsurvival of at least some individuals of a population in the event of a calamity and reduces the incidences of unfavourable genetic traits on the whole. Meiosis can be considered one of the first and most important “innovations” of the eukaryotes. Indeed, it has been theorized that linear chromosomes that are seen in eukaryotes may have arisen because of the advent of meiosis (Goodenough and Heitman 2014).

Sexual reproduction is comprised of two broad steps: conjugation, or fertilisation; and the meiotic cell division itself. During the former, two genetically distinct cells fuse, nuclear fusion or karyogamy may or may not happen simultaneously, and the outcome of this is a diploid cell. In diploid organisms, fertilization occurs to restore a diploid state during or after meiosis required for gamete formation, but haploid organisms undergo the conjugation step prior to meiotic division.The concept of meiosis was first discovered by the German biologist Oscar Hertwig in 1876, when he observed the fusion of egg and sperm in the transparent Sea Urchin Egg, and concluded that the nuclei of the two cells contributed to the inherited traitspassed on to the offspring. It was describedeight years laterat the level of chromosomes inthe eggs of the roundworm *Ascaris,*by the Belgian zoologist Edouard Van Beneden(Hamoir 1992). However, its significance for genetic inheritance was onlyunderstood in 1890 by the German scientist August Weismann, who observed that two cell divisions wereimperative for the transformation of one diploid cell into four haploid cells,which, in turn, was needed to maintain thenumber of chromosomes or the ploidy.

Meiotic division occurs in two phases (Figure 1), with the first phase achieving reductional division and the second being an equational division. These are termed as meiosis I and II respectively. The mitotic cell cycle is broadly divided into interphase, wherein a cell prepares itself for division by increasing its size and necessary components required for division, and mitosis. Interphase is further divided into three stages: G1 (Gap 1), S (Synthesis) and G2

(Gap 2) phase. In G1, the cell synthesizes its vast array of proteins, including the enzymes and structural proteins it will need for growth. S phase is the stage in which replication doubles the DNA content of a cell. Mitotic phase is the stage when segregation of DNA occurs, and it is subdivided into prophase, metaphase, anaphase and telophase. In contrast, meiosis consists of two divisions- meiosis I and II, without an intervening S phase, which is essential for reducing the ploidy.

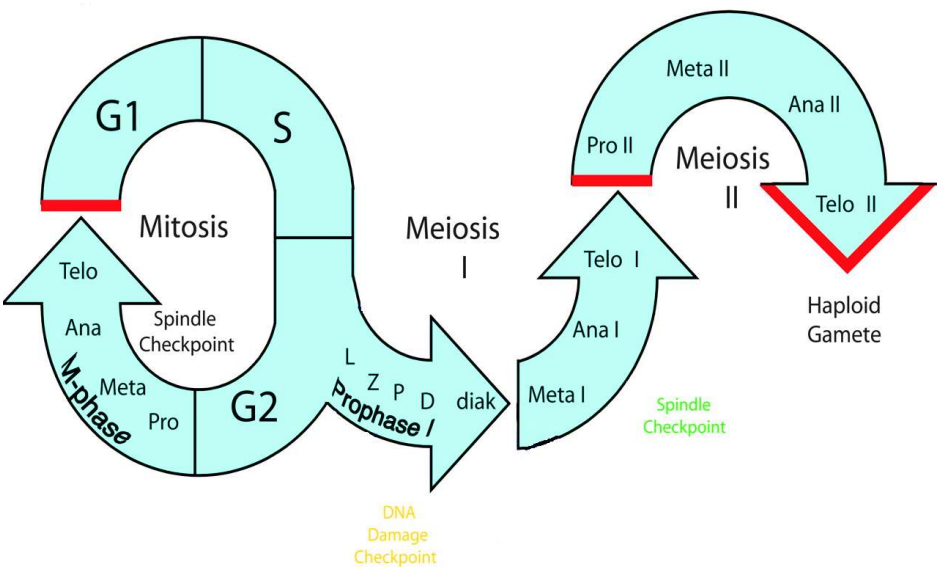


Figure 1 Making the switch from mitosis to meiosis. Adapted from (Morelli and Cohen 2005). Mitosis has two gap phases G1 and G2 intervened by a synthesis or S phase. The all-important spindle checkpoint occurs at the end of G2, just before the cell enters mitosis. On the other hand, the meiotic interphase of G1 and S is directly followedby Meiosis I, the prophase of which is subdivided into Leptotene, Zygotene, Pachytene, and diplotene. The spindle checkpoint occurs after the metaphase to anaphase transition of Meiosis I; there is no G2 phase. Meiosis I is followed by Meiosis II, the result of which are daughter cells with half the ploidy of the original parental cells.

The preparatory steps that lead up to meiosis are identical to G1 & S phases of the interphase of the mitotic cell cycle. The G_2 phase as seen before mitosis is not present in meiosis (Figure1) The prophase during the first meiotic division (Prophase I)is the most complex and time-consuming of the meiotic phases, accounting for majority of the total time taken by the entire process(Alberts, Bray et al. 2014). It is further divided into the stages Leptotene, Zygotene, Pachytene and Diplotene.(Snustad and Simmons 2008, Lewin, Krebs et al. 2011)

The name of the first stage isleptotene, derived from Greek words "**Lepto**" meaning narrow or fine, and "**tainia**" meaning bands or ribbons.It is also known as leptonema, from "nema", meaning thread. In this stage, individual chromosomes—each consisting of two sister

chromatids—become ‘individualized’due to the condensation and coiling of the chromatinfibres, to form visible strands within the nucleus (Figure 2). These are known as dyads.

The condensation of chromatin is achieved by condensins, which introduce positive super-helical tension into the double-stranded DNA in an ATP-hydrolysis-dependent manner. (Kimura and Hirano 1997). The sister chromatids themselves are held together by a related protein complex, called cohesin(Peters, Tedeschi et al. 2008). Homolog recognition and pairing happen at this step.

The second stage is zygonema or zygotene, derived from Ancient Greek ‘**zygosis**’ meaning joining or union. Here, the dyad pairs align to form tetrads or bivalents, a synapsis that is facilitated by a tripartite protein structure called a Synaptonemal Complex(SC)(Urry, Cain et al. 2017). The SC forms between homologous chromosomes (two pairs of sister chromatids) and thus ‘zips’ the homologues together (Figure 2). The SC starts to assemble towards the end of leptotene, and its assembly continues through zygotene, to be completed before entering the next sub-stage, called pachytene (from Greek ‘**pakhus**’ or thick). Due to their physical proximity, the nonsister chromatids of homologous chromosomes may exchange segments over regions of homology.This homologous recombination includes chromosomal crossovers, and occur at sites known as chiasmata(Cooper and Hausman 2016).

The SC is not indispensable for meiosis; certain organisms such as the protozoa *Tetrahymena thermophila*do not require the formation of SC for genetic recombination.(Chi, Mahe et al. 2014). Because individual homologous chromosomes cannot be distinguished from each other in the synaptonemal complex, the actual act of crossing over cannot be observed through the microscope, and thus chiasmata are not visible until the next stage- Diplotene (from the Greek word diplos, meaning double). In this stage, the synaptonemal complex breaks down,and homologous chromosomes separate from one another a little. The chromosomes themselves uncoil a bit, allowing for some amount of transcription. The homologous chromosomes of each bivalent, however, remain tightly bound at chiasmata. The chiasmata remain on the chromosomes until they are severed at the transition to anaphase I.

Diakinesis, from Greek words meaning moving through, sees sites of crossing over entangle together and overlap, making chiasmata clearly visible. The nucleoli disappear,and the meiotic spindle is formed, making it similar to the prometaphase stage of the mitotic cell cycle(Losos, Mason et al. 2008).

Simultaneously along with the homologous pairing and genetic re-assortment in Prophase I, the centrosomes or the spindle pole bodies function as microtubule organizing centres (MTOCs). They nucleate microtubules which attach to the chromosomes at the kinetochore (a protein complex that acts as the interface between the meiotic spindle and the centromere of the chromosomes) (Petronczki, Siomos et al. 2003). Thereby the tetrads of the chromosomes are pulled along the attached microtubule toward the originating centrosome. In some organisms, the nuclear envelope dissolves after diplotene,and in others, it may only lose its integrity towards the SPBs. In yet others, there is no dissolution of the nuclear membrane.

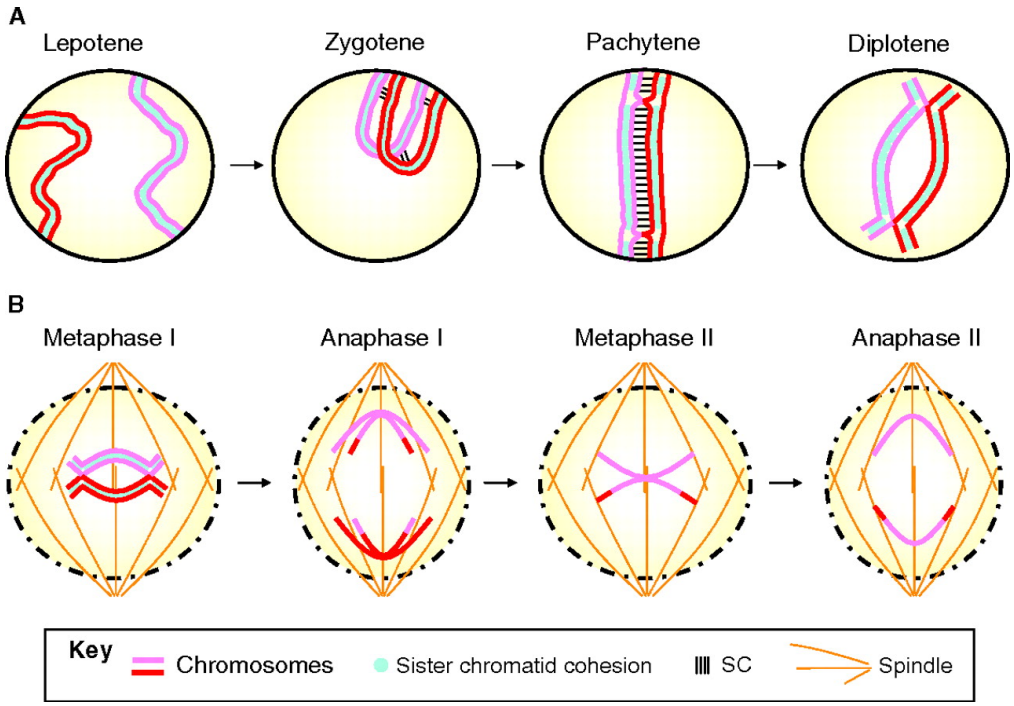


Figure 2Stages of meiosis. Adapted from (Tsai and McKee 2011)Panel A shows the various substages of Prophase I- Leptotene, Zygotene, Pachytene and Diplotene (described in text). Panel B shows the metaphase to anaphase transition in Meiosis I, where the homologous chromosomes separate but sister chromatids co-segregate, and in Meiosis II, where sister chromatids move to opposite poles.

By the time metaphase I commences, the centrioles/ SPBs have migrated to the opposite poles of the cell. The bivalents are now as tightly condensed as they will be for the rest of meiosis. Spindle fibres from each pole attach to the dyad closest to them; resulting in homologues being pulled to opposite poles. The position of each chromosome within these bivalents is random - bivalents from either parent can align on either side of the cell. This is important for the proper segregation of the bivalents in the next stage- anaphase I.

During mitosis and meiosis II, sister chromatids need to segregate into different daughter cells. Therefore, they are required to attach to spindle fibres emanating from opposite poles to facilitate this equational division. This is achieved by an amphitelic bi-orientation,i.e. the kinetochores of the sister chromatids face the opposite poles and are thus captured by microtubules emanating from different SPBs, which can pull the sister chromatids apart once all cohesion is lost. In contrast, meiosis I necessitatesthat sister chromatids co-segregate, and their homologous chromosome segregate to the opposite pole. Thus, unlike mitosis or meiosis II, cohesin is maintained at centromeres during anaphase of meiosis I. Kinetochores on sister chromatids face the same direction,i.e. they are mono-oriented — and are thus captured and pulled by spindle fibres from the same pole(Tachibana-Konwalski 2015)(Watanabe 2006) (Figure 3).

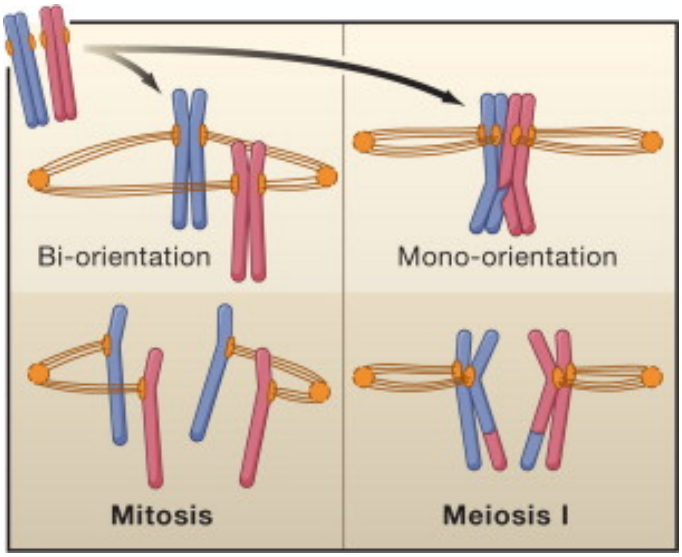


Figure 3Adapted from (Watanabe 2006). Biorientation of KT is required for the equational division in Mitosis and Meiosis II, wherein sister chromatids need to be segregated into two different daughter cells. Mono-orientation in Meiosis I, on the other hand, enables co-segregation of sister chromatids while separating homologous dyads to opposite poles.

To prevent mis-segregation, the cell must pass inspection at various surveillance points to ensure proper segregation of chromosomes during mitosis as well as meiosis. One such checkpoint is the Spindle Assembly Checkpoint or SAC, which in meiosis I occursbefore Anaphase I. This checkpoint ensures that the kinetochores are oriented in the fashion specific to whether it is in Meiosis I or Meiosis II/mitosis. The problem of aberrant chromosome distribution can betackled by delaying anaphase onset when defects are detected(Steuerwald, Cohen et al. 2001). Improperly attached kinetochores are some of the most likely sources of

the pause signals produced upon activation of the SAC. The kinetochore complex binds an evolutionarily conserved set of proteins (MAD1, MAD2, and MAD3, and BUB1 and BUB3)that are components of the checkpoint signalling apparatus (Jablonski, Chan et al. 1998). The checkpoint is then able to discern if the kinetochores are appropriately attached to microtubules by monitoring the tension generated across the centromere.

The SAC acts by negative regulation of CDC20, a co-factor of the ubiquitin ligase anaphase-promoting complex/cyclosome(APC/C). This prevents CDC20 from activating the APC/C-mediated polyubiquitylation of two key substrates, cyclin B and securin, which would otherwise have resulted in tagging them for proteasomal degradation. Securin is a stoichiometric inhibitor of a protease known as separase, which is required to cleave the α-kleisin subunit of the cohesinring that holds sister chromatids together, and cohesin cleavage is necessary to execute anaphase (Figure 4).This could be why mutations in the Rec8 expressing gene that codes for a meiosis-specific α-kleisin cohesin subunit, results in equational rather than reductional division at meiosis I(Watanabe and Nurse 1999).

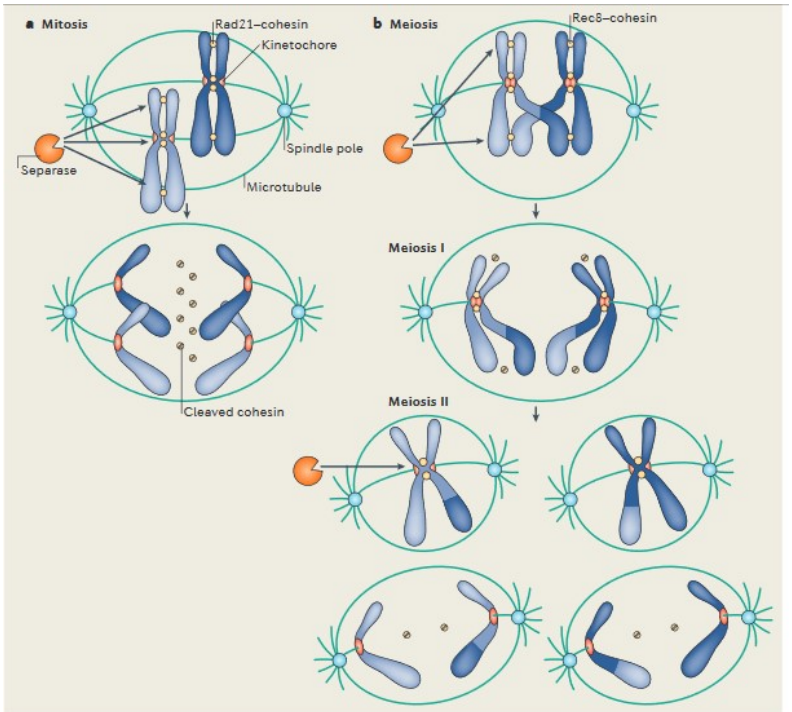


Figure 4The process of chromosomal segregation in a. Mitosis, and b. Meiosisfrom (Watanabe 2012). During mitosis as well as meiosis II, sister chromatids face opposite poles (bi-orientation) and hence, due to their kinetochores interacting with the microtubules emanating from these poles, segregate to different daughter nuclei. In meiosis I, on the other hand, the sister chromatids and hence their kinetochores, face the same pole

(mono-orientation), whereas the homologous chromosomes are bi-oriented, resulting in co-segregation of the sister chromosomes.

After successful passage through the SAC, the cell enters anaphase I, by the end of which homologous chromosomes separate and move to opposite poles. Unlike in mitosis, the centromeres do not split,and sister chromatids remain paired in anaphase I, as they are still bound by cohesins at the centromere. Protection of this centromeric cohesinis described in greater detail in the next section.

In telophase I, the homologs of each bivalent have arrived at opposite poles of the cell, and a new nuclear envelope forms around each set of chromosomes, if the nuclear envelope had dissolved in prophase I. Cytokinesis then divides the nucleus into two daughter nuclei. Each of the two daughter nuclei is now haploid (*n*), with half the number of chromosomes per nucleus as in meiosis I. In some species, the nuclear membrane briefly forms around the chromosomes, while in others it does not. The cell now proceeds into meiosis II, with the chromosomes remaining condensed.
The events of meiosis II are similar to that of mitosis, with the critical difference being that the genetic makeup of the resultant daughter cells are different from that of the original parent cells that fused to undergo meiosis I. Just like in mitosis, the nuclei undergo prophase followed by metaphase, at the end of which, upon successfully passing through the SAC, centromeric cohesin is degraded to result in separation of sister chromatids to different daughter cells. Finally, after telophase II, all fourdaughter cells, eachwith a ploidy of n (haploid) separate.

1.2 Cohesins

Successful propagation of genetic material in progeny is essential for the survival of any species. Mis-segregation of chromosomes can result in aneuploidy. Such defects in chromosome segregation during meiosis cause miscarriages, infertility and genetic diseases such as Down's syndrome(Chiang, Duncan et al. 2010, Lister, Kouznetsova et al. 2010). The cohesion of sister chromatids in a parent cell ensures their successful segregation,so that daughter cells inherit complete copies of their genome(Miyazaki and Orr-Weaver 1994). In eukaryotic cells, cohesion is mediated by a multi-subunit protein complex called cohesin. By linking the sister chromatids, the cohesin rings counteract the bipolar pulling force of mitotic

spindle microtubules, therefore ensuring the proper biorientation of chromosomes on the metaphase spindle, thus preventing the precocious segregation of sister chromatids (Ocampo-Hafalla and Uhlmann 2011). This cohesin complex is comprised of the four proteins: Smc1, Smc3, Scc1/Mcd1, (also called kleisin, it is the target of the protease separase), and Scc3/Irr1/STAG(Michaelis, Ciosk et al. 1997). The homolog of Scc1 in vertebrates is known as Rad21 in mitosis, and Rec8 in meiosis. Some higher eukaryotes haveanother type of α-kleisin subunit, called RAD21L which is thought to facilitate crossing over by bringing the arms of the homologues even closer (Lee and Hirano 2011, Polakova, Cipak et al. 2011).(Figure 5)

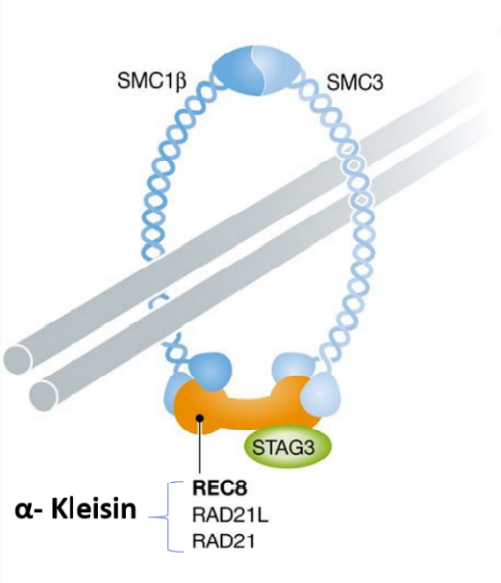


Figure 5Various components of the meiotic cohesin complex. Adapted from (Ishiguro and Watanabe 2016). The intramolecular coiled- coils of the SMC proteins come together at the hinge domain. Their head domains, which contain ABC type ATPase domains, are bridged by the kleisin subunit, which in the case of meiosis is Rec8. Rad21-like or Rad21L is another meiotic kleisinsubunit,but it is exclusive to metazoans. During mitosis, the SMC1 is of the α type, as opposed to β type in meiosis, and all kleisin subunits are Rad21. Some Rad21-containing cohesin is found even during mitosis. The entire ring of the cohesinis stabilized by an orthologue of the budding yeast Scc3 protein, which are of 3 different varieties in metazoans. In mice, these orthologues are called STAG1, 2 and 3, with the former two associating with mitotic cohesin and STAG 3 stabilizing all meiotic cohesin. In humans, the STAG proteins are called SA proteins.

SMC stands for Structural Maintenance of Chromosome, and the two SMC proteins form a heterodimer with intramolecular coiled-coils that help do just that. Observations from early biochemical experiments indicate that Smc1p and Smc3p hetero-dimerize via their hinge regions to form a V-shaped structure, and the α-kleisin subunit, be it RAD21 or

REC8,bridges the SMCs' globular ATPase domain heads to create a ring-like complex(Gruber, Haering et al. 2003). Scc3homologsassociate with the kleisinbut not the SMC subunits to form the final complex(Haering, Lowe et al. 2002), thereby stabilizing the whole structure by association (Hopkins, Hwang et al. 2014).Various topological models have been proposed to explain how exactly cohesin rings bring about sister chromatid cohesion. The main ones have been shown in the cartoon in Figure 6.

In the simple ring model, also known as the embrace model, a single cohesin ring embraces both the sister chromatids. In the handcuff model, two different cohesin rings embrace each of the sister chromatids, and the two rings themselves are associated through the Scc3 homolog(Zhang, Kuznetsov et al. 2008). A variation of this is the two-ring model, where each of the kleisin subunits interacts with both the rings,i.e.SMC1 of the first ring and SMC3 of the other. The two-gate model is proposed to be of a temporary, intermediate nature-slightly different from the one ring embrace model, despite also involving both strands being held by one ring(Skibbens 2015).One of the sister chromatids is contained within the pore created by the SMCs -this is the stable binding, and the other in the gap bordered by the head domains of the SMCs and the α kleisin subunit- this is temporary. Thiswas proposed because it has been experimentally determined that in situations where the cohesin has to release the sister chromatids temporarily,i.e. without degradation of the kleisin subunit(Guacci 2007), it does so by disassociation of the kleisin subunit from either of the SMCs.Thisis facilitated by the association of the cohesin ring with Wap1, the cohesin's "antagonist", thereby creating an 'exit gate' for the DNA.This is otherwise prevented by acetylation of Smc3 by the protein Eco1, which counteracts Wap1's anti-establishment activity(Chan, Roig et al. 2012, Murayama and Uhlmann 2015). Hence this also means that the 'entry' gate,i.e. the trappingof chromatin between the pore created by SMC1 and 3(Gruber, Arumugam et al. 2006), is different from the exit gate- the Wap1-dependent mechanism to release it.(Chan, Roig et al. 2012, Murayama and Uhlmann 2015)

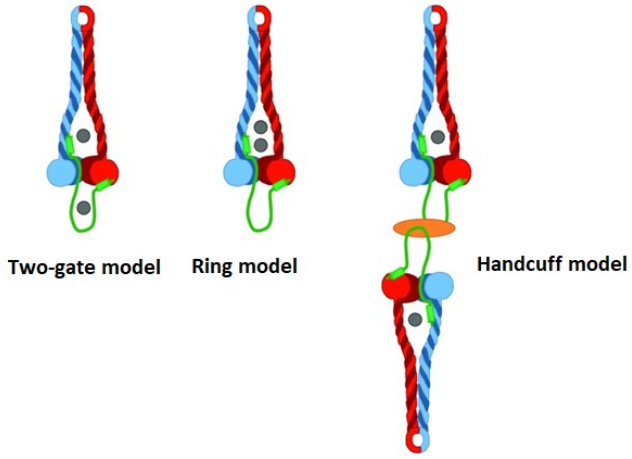


Figure 6 Various models proposed for the cohesin-chromatin interaction. Adapted from (Barrington, Finn et al. 2017) The blue and red represent the SMC proteins 1 and 3. The green represents the α- kleisin subunit, and the grey dots each represent one of the sister chromatids.The two- gate model, as explained in the main text, is a temporary situation, that ultimately results in the entrapment of DNA within a more stable cohesin topology. These could be either the ring or embrace model, where both sister chromatids are held together within one cohesin ring, or the two-ring handcuff model, where two different cohesin rings

The next important question to understand sister chromatid cohesion is, "How is this cohesion established?" ATP binding and hydrolysis by the ABC-type ATPase head domains in the SMC components appear to be necessary for the loading of cohesin onto chromosomes(Arumugam, Nishino et al. 2006, Lengronne, McIntyre et al. 2006) and may be functioning to facilitate DNA into the cohesinring.Additionally, a cohesin loader complex comprised of the proteins Scc2 and 4, or their homologs(Ciosk, Shirayama et al. 2000) is employed.This seems to be only required for the initial loading of cohesin, as evidenced by its dispensability during S and G2 phases (Ciosk, Shirayama et al. 2000). This makes sense in the light of the fact that in many organisms, cohesin plays additional roles independent of its function in the cell cycle, as evidenced by the requirement for cohesin in differentiated, post-mitotic cells in *Drosophila melanogaster*(Pauli, Althoff et al. 2008, Pauli, van Bemmel et al. 2010), or its effect on regulating transcriptional termination at co-transcribed convergent gene units in *Schizosaccharomyces pombe* (Gullerova and Proudfoot 2008).

The observation that many of the proteins essential for cohesion establishment were associated with the replication machinery indicated that replication and cohesion of DNA are closely linked (Kenna and Skibbens 2003).In fact, the cell cycle window where the cohesin complex is bound to chromatin varies depending on the organism, with the only commonality being that the loading of cohesinsprecedes DNA replication (Guacci 2007).

1.3 Rec8

Multiple genes have been identified to be expressed exclusively in cells undergoing meiosis; this gives us an inventory of "meiosis-specific genes" or the meiotic toolkit (Schurko and Logsdon 2008, Schurko, Neiman et al. 2009), which can be used as molecular markers of sexual reproduction. Rec8, the α kleisin subunit of cohesin specific to meiosis, is one such gene.It is comprised of two conserved regions (Figure 7)- the N terminal domain that binds to SMC3, and the winged helix domain containing the C-terminal domain, which interacts with the head of SMC1β (the meiosis-specific SMC1) (Nasmyth 2005). The C-terminal domain belongs to the winged helix superfamily.

Figure 7InterPro(Apweiler, Attwood et al. 2001)listing of the conserved domains in *S. pombe* Rec8. The conserved N-terminal Rad21/Rec8 like domainis found in Rec8 from any organism that possesses it. Most, but not all organisms, also have a conserved C-terminal region, which belongs to the winged helix superfamily, in their Rec8 domain architecture.

The name of the gene is derived from the word recombination, as it was one of the genes implicated in screens for mutations affecting meiotic recombination in *Schizosaccharomyces pombe* (Ponticelli and Smith 1989, De Veaux, Hoagland et al. 1992). Later studies showed that it was one of the genes that behaved in a region-specific way to activate meiotic recombination(DeVeaux and Smith 1994)with differing recombination frequencies at different sites. This pointed to a differential distribution of the gene product throughout the genome. Rec8 was then implicated in the formation of axial elements of synaptonemal complexes(Molnar, Bahler et al. 1995) and was shown to play a role in recombination between homologs, and sister chromatid cohesion (Klein, Mahr et al. 1999). Its localization was seen to vary during meiosis in a stage-specific manner, with it first being spread all over the chromosomes, to disappearing at all regions except for on and around the centromere at the end of meiosis I, and it persistance in the vicinity of the centromere till the onset of anaphase II(Klein, Mahr et al. 1999). Almost simultaneously, it was reported that Rec8 is necessary for reductional chromosomal segregation during meiosis (Watanabe and Nurse

1999). Further research showed that phosphorylation by various kinases such as DDK, PLK and HRR25 (homolog of mammalian Casein kinase 1δ)(Sumara, Vorlaufer et al. 2002, Brar, Kiburz et al. 2006, Katis, Lipp et al. 2010) tagged the kleisin subunits for proteolytic cleavage by the endopeptidase separin (Buonomo, Clyne et al. 2000) or separase (Hauf, Waizenegger et al. 2001, Kitajima, Miyazaki et al. 2003), and that securin prevented this degradation of Rec8 by keeping separase deactivated (Jallepalli, Waizenegger et al. 2001, Herbert, Levasseur et al. 2003, Huo, Zhong et al. 2006), thereby regulating degradation of the kleisin subunit .The question then arose, what signalled the removal of cohesins at the arm but maintained it at the centromere at the metaphase I to anaphase I transition? The mechanism by which cohesion is protected at the centromere, but not the arms (Moore, Page et al. 1998) was understood to involve a 'guardian spirit' that prevented this destruction of the kleisin subunit (Watanabe 2005, Ishiguro, Tanaka et al. 2010),which, along with Protein Phosphatase 2A (PP2A) recruits Mnd2 (an antagonist of the Anaphase Promoting Complex, that ubiquitinates securin, targeting it for degradation) and also dephosphorylates residues in the kleisin subunit, preventing the destructive action of separase, as described in Figure 8.

The irreversible loss of cohesin by cleavage of the kleisin subunit is not the only method to decrease sister chromatid cohesion; a pathway has been identified in mammals wherein cohesin is removed from the chromosomal arms (Waizenegger, Hauf et al. 2000, Buheitel and Stemmann 2013). However, this pathway has not yet been identified in lower eukaryotes

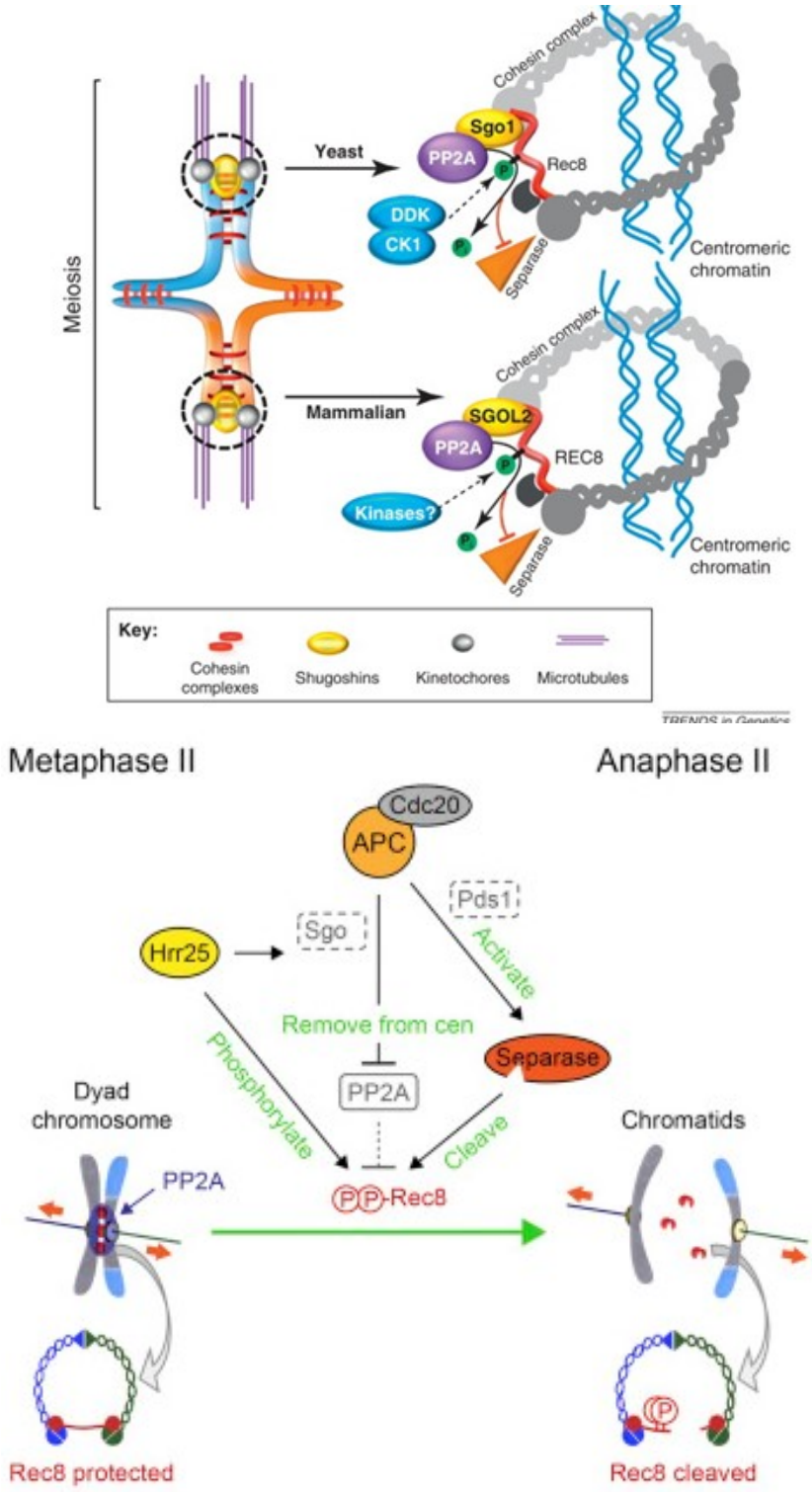


Figure 8Protection of cohesin at the centromere by shugoshin. Adapted fromtop:(Gutierrez-Caballero, Cebollero et al. 2012), and bottom: (Arguello-Miranda, Zagoriy et al. 2017). The metaphase to anaphase transition is brought about by the Anaphase Promoting Complex or APC. It separates securin or Pds1 from separase/separin or Esp1, thereby activating the latter, and freeing it up to cleave Rec8 in the unprotected

cohesin. However, the centromeric and pericentromeric cohesion is protected by Shugoshin, also called the 'guardian spirit', which recruits PP2A. Protein phosphatase 2A removes the phosphorylation marks made by kinases such as Hrr25, DDK and PLK, that would have tagged Rec8 for destruction by separase. However, in the metaphase to anaphase transition of meiosis II, Shugoshin is removed from the centromere, leaving the cohesin leftover from anaphase I vulnerable. Therefore, the sister chromatid cohesion is lost by the end of anaphase II.

1.4 *Cryptococcus neoformans* – an opportunistic fungal pathogen

Kingdom: Fungi
Phylum: Basidimycota
Subphylum: Basidimycotina
Order: Sporidiales
Family: Sporidiobolaceae
Genus: Cryptococcus (*)
Species:*Cryptococcus neoformans* (*)

(*)The filamentous form was earlier named *Filobasidiella neoformans*, and was placed in the genus Filobasidiella)

*Cryptococcus neoformans*was first found in fermented peach juice by Sanfelice and was identified to be a pathogen when found in skin lesions. It is a basidiomycete, and thus evolutionally divergent from the other common pathogenic fungi which are mostly ascomycetes (e.g.*Candidaalbicans*). The infection caused by *C. neoformans* is known as Cryptococcosis and affects important organs including the lungs and the central nervous system. Unlike most infectious diseases, cryptococcosis is not transmitted from person to person.Rather, it is caused by inhalation of the infectious propagules (the yeast cells or the spores) from environmental sources of the fungus (Figure 9, (Lin and Heitman 2006), such as soil contaminated with guano or feces of birds such as pigeons(Emmons 1955), munia, and canaries (Pal 1989), or from the litter of debris from various trees such as eucalyptus, tamarind (Gokulshankar, Ranganathan et al. 2004),java plum or jamun(Randhawa, Kowshik et al. 2006).

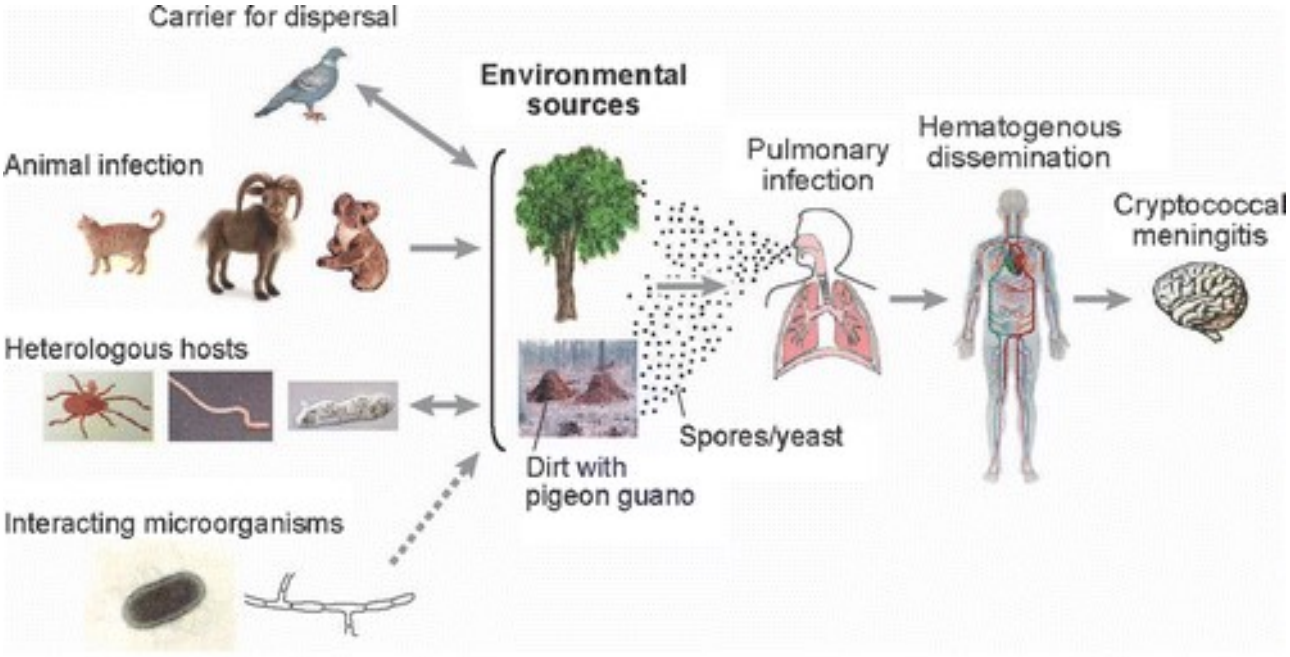


Figure 9How cryptococcosis spreads. From (Lin and Heitman 2006). Unlike most infections, cryptococcosis does not have a human to human mode of transmission. Instead, it is caused by the inhalation of the infectious agents- spores and yeast cells- from environmental sources. These sources could be soil contaminated with bird guano or tree litter. Apart from humans, cryptococcosis is also seen in animals such as cats, goats, and koala bears. There are also some heterologous hosts of *C. neoformans* include ticks and earthworms. It has been theorized that the virulence factors that make *C. neoformans* such a successful opportunistic pathogen evolved to defend against predation by other organisms such as amoebae in the environment. This theory, called accidental virulence, has been described in further detail in the main text.

*C. neoformans*enters the host through the respiratory system as dehydrated haploid yeast or basidiospores (Casadevall and Pirofski 2007) where they fit into the alveolar spaces inside the lungs. Once inside, they get rehydrated and develop a thick, gelatinous polysaccharide capsule in response to the environmental factors inside the host, such as humidity, low glucose, serum, 5% carbon dioxide, and low iron etc. (Velagapudi, Hsueh et al. 2009). In immunocompetent individuals, the initial infection by *C. neoformans* is usually asymptomatic- the pathogen remains dormant in a lymph node complex, much like tuberculosis. However, reactivation involving hitchhiking in host phagocytes to cause a systemic infection occurs in immunocompromised individuals, such as HIV/AIDS patients and solid organ transplant recipients, where the host doesn't have enough T-cell dependent immune function, resulting in approximately 1/3rd of the fatalities in such cases. This is why *C. neoformans* is called an opportunistic pathogen (Buchanan and Murphy 1998, Bahn, Cox et al. 2005, Lin and Heitman 2006), and is more prevalent in areas which see greater

incidences of diseases that are risk factors, such as HIV/AIDS (Figure 10)(Rajasingham, Smith et al. 2017)and SLE (Fang, Chen et al. 2016).

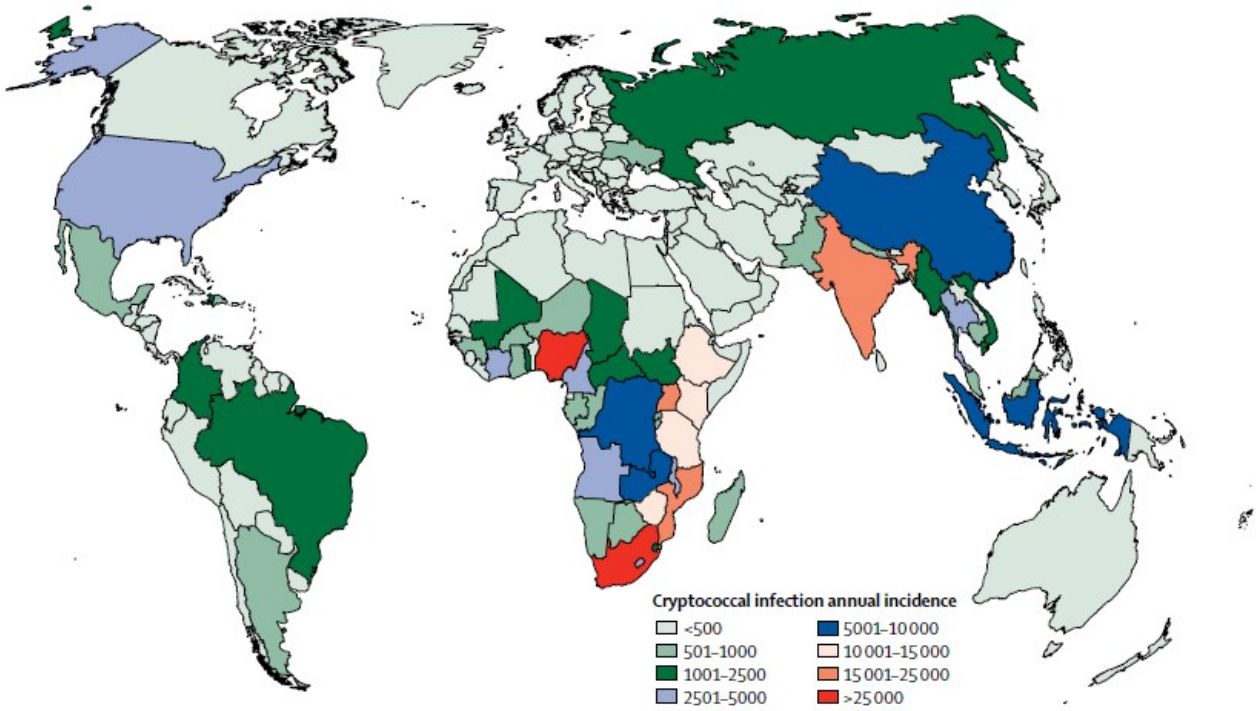


Figure 10From (Rajasingham, Smith et al. 2017) The global picture of Cryptococcosis in HIV/AIDS patients. A map has been colour coded for the average annual incidences of *C. neoformans* infections in various countries.

Why is *C. neoformans* an opportunistic pathogen? The answer may lie in its virulence factors, such as non-lytic escape from host cells (vomocytosis), complex polysaccharide capsule, laccase activity, and the ability to synthesize melanin;which are likely to offer protection against environmental pressures such as escape from predatory soil amoebae, desiccation, and exposure to ultraviolet light, or aid in the colonization of plant or animal hosts.This hypothesis that cryptococcal pathogenesis does not result from direct selection for virulence within a mammalian host, but rather by the evolution of traits (which happen to be advantageous in mammals) in response to other selective pressures in both environmental and animal niches, is termed 'accidental virulence' (Casadevall, Steenbergen et al. 2003, Casadevall and Pirofski 2007)

1.5 Asexual reproduction in *C. neoformans*

C. neoformans reproduces asexually by budding when inside the host which is why most isolates of the fungus are haploid andare found in tissues in the yeast form. Haploid basidiospores germinate into yeast, which divide by mitosis.*C. neoformans* has a haploid genome of approximately 19 Mb, comprised of 14 chromosomes (Loftus, Fung et al. 2005).

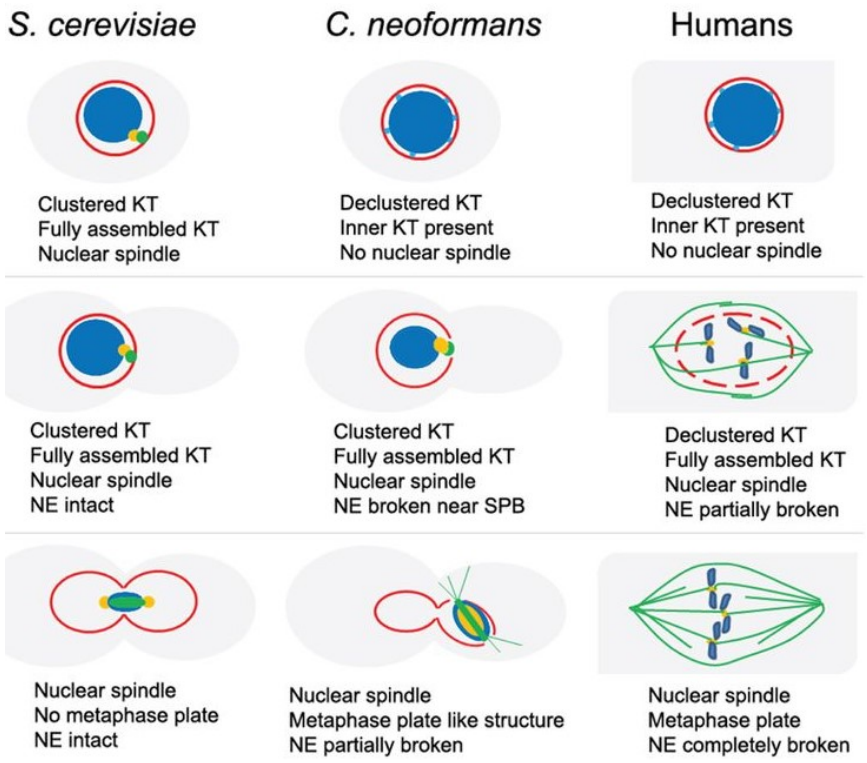


Table 1.Key differences between mitosis in budding yeast, *C. neoformans,* and humans. Adapted from (Kozubowski, Yadav et al. 2013). The kinetochores in *C. neoformans* are initially declustered and go on to be assembled entirely only in subsequent stages of mitosis. This is similar to the situation in human somatic cells, and unlike other lower fungi such as the budding yeast S. cerevisiae. Another important characteristic of mitosis in *C. neoformans* is that the integrity of the nuclear envelope is lost only partially- near the SPBs- due to disassembly of the nuclear pore complex during metaphase. This results in a semi-open mitosis, unlike the closed mitosis of *S. cerevisiae*, or the open mitosis in humans.

As is already known from a previous publication from the lab(Kozubowski, Yadav et al. 2013), mitotic events in *C. neoformans* represent an intermediate scenario between other well studied fungi, such as *S. cerevisiae*, and metazoans, such as humans (Table 1): The kinetochore, a protein complex that facilitates attachment of microtubules for faithful chromosome segregation, is not completely assembled until the commencement of mitosis in

C. neoformans, and a metaphase plate-like structure is observed, similar to humans. Secondly, similar to humans but atypical of yeasts, the centromeres are not clustered but positioned adjacent to the nuclear envelope in pre-mitotic *C. neoformans* cells. The centromeres gradually coalesce to a single cluster as cells progress toward mitosis. While the nuclear envelope remains intact throughout in *S. cerevisiae* (closed mitosis) and it completely breaks down in humans (open mitosis), it only loses its integrity partially in *C. neoformans* (Semi-open mitosis).

1.6 Sexual reproduction and Filamentation in *C. neoformans*

Besides a prevalent asexual life cycle, *C. neoformans* also presents a sexual life (Idnurm, Bahn et al. 2005, Kozubowski and Heitman 2012). It has a bipolar mating cycle with two mating types, MAT**a**andMATα, with the latter being the most prevalently isolated from hosts and the environment. Whereas other members of the genus *Cryptococcus* can also undergo unisexual mating or monokaryotic fruiting, *C. neoformans* has only been observed to undergo a dimorphic transition to filamentous growth form by the following differentiation pathway, called mating (Figure 11).α yeast cells secrete peptide pheromones that trigger cell-cell fusion of an '**a**' and 'α**'** cell. The resulting dikaryon initiates filamentous growth, and the 2-parent nuclei migrate into the hyphae. Septa are formed to separate the cells, and clamp cells are developed to facilitate the transfer of one nucleus from each dikaryotic hyphal compartment to the next one. During this hyphal growth, blastospores can bud out from the hyphae and divide mitotically. The apex hyphal cell forms a club-like structure called basidium. Here, the two nuclei fuse and undergo meiosis. The resultant daughter nuclei undergo mitosis to produce four haploid nuclei that bud out of the surface to form basidiospores.

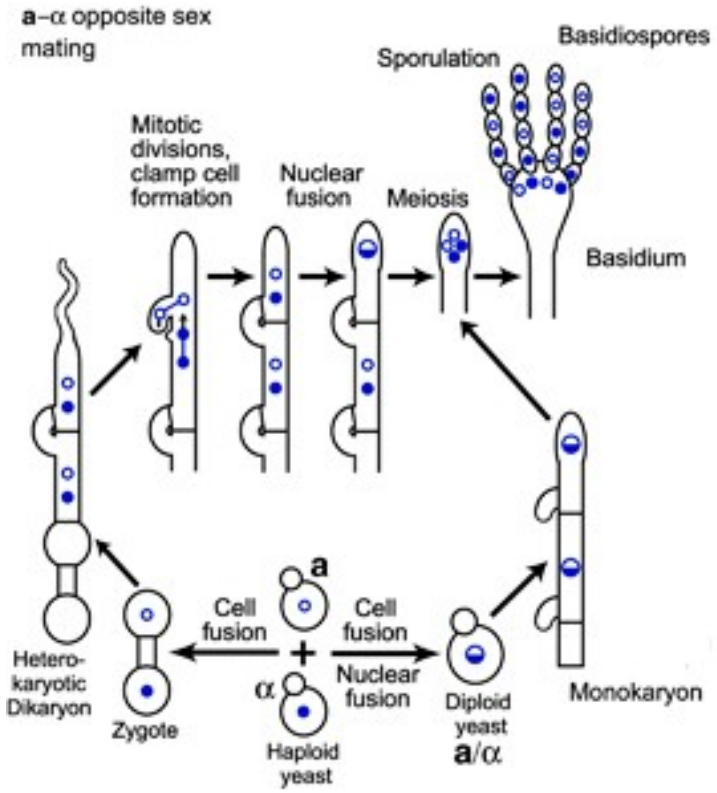


Figure 11Bisexual reproduction in *C. neoformans*. Adapted from (Kozubowski and Heitman 2012). If cells of the opposite mating type, a and α are present in close proximity, they can sense the pheromones released by the latter. This initiates a signalling cascade that results in cell-cell fusion, followed by formation of a hypha by one of the cells, to which the nuclei from both parental cells migrate. This hypha then divides mitotically to give a multi-segmented filament containing dikaryotic hyphal compartments. Clamp cells, a characteristic feature of basidiomycetes, are formed to facilitate movement of nuclei from the previous segment, to ensure that each compartment has one nucleus of each parental genotype. Finally, the apical segment swells up to form a club-like structure called the basidium, for which the phylum Basidiomycotais named. This basidium is the site of nuclear fusion, meiosis, and sporulation.

Optimal growth conditions for the yeast (asexual) and filamentous (sexual) forms of *C. neoformans* are very different, possibly to ensure efficient utilization of resources, and enhance the survival of the species. The yeast form grows more efficiently in liquid media whereas development of the filamentous structureis inhibited therein. When nutrients are abundant, the cellsgrow asexually in the yeast form, and all cells participate in mitotic division. This also increases population size exponentially(Lin 2009). In such situations, for example, inside a host, meiosis, and therefore filamentation, is rare. Thus, in the majority of the cases, yeast cells are the ones isolated from tissues during animal infections. In fact, many clinical isolates are unable to mate or to produce hyphae under laboratory conditions(Yan, Li

et al. 2002). On the other hand, hyphae are more effective in foraging for nutrients, which is why a morphogenetic switch to the filamentous form occurs under nutrient starvation conditions. Growing in a hyphal form involves only nuclei in the hyphal tips participating in active division, and those in the sub-apical hyphal compartments are dormant unless a new branch formation is initiated. This is necessary due to limited availability of nutrients. Because of the apical dominance, hyphae propagate at much lower rates comparing to yeast cells(Lin 2009)

There are two major signalling pathways knownto date that affect filamentation in *C. neoformans* – the MAP kinase cascade, and the cAMP signalling (Figure 12). Examples of molecules influencing filamentation through the former are Gpm1, Crz1, Hog1 and Ste50. Gpa1 and Crg2 are examples of molecules that affect filamentation through cyclical AMP signalling. Crg1 interestingly affects both the MAP kinase and cAMP signalling pathways.

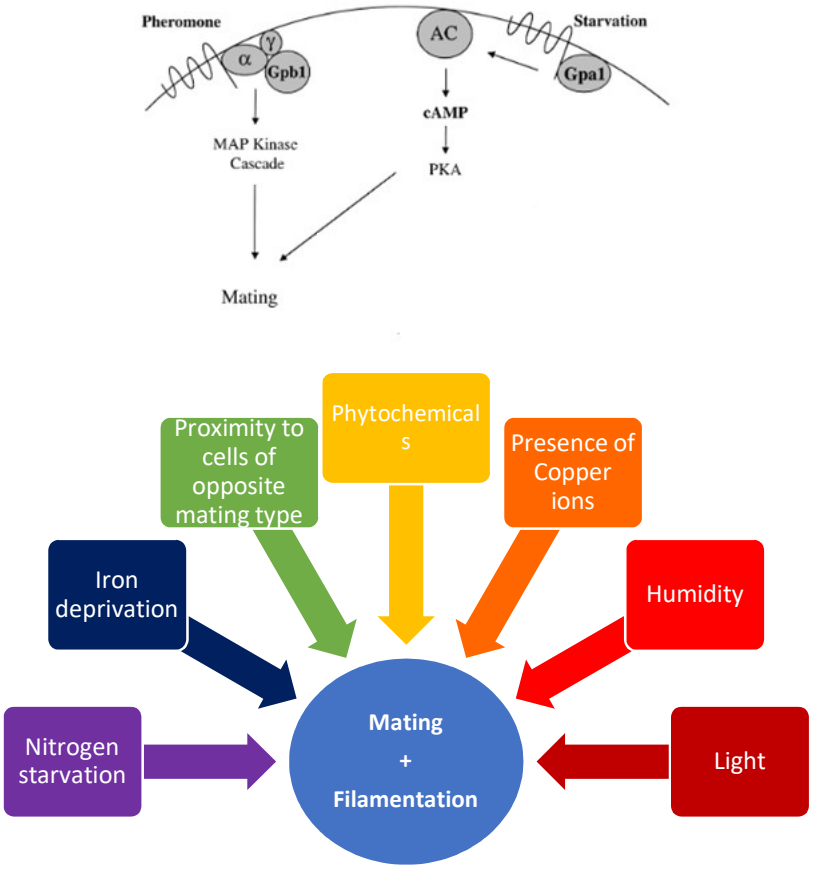


Figure 12Left:Signalling pathways that affect mating and other downstream processes such as filamentation. Adapted from (Alspaugh, Davidson et al. 2000). The two pathways- cyclic AMP as well as MAP Kinase – are triggered due to a variety of factors (Right panel)

1.7 Rationale and objectives for the research project

Rec8 in other organisms (such as the ascomycetes *S. pombe* and *S. cerevisiae*) has been used as a molecular marker for meiosis; also its changing localization is indicative of various sub-stages of meiotic division. Since not much is known about what goes on at the molecular level during meiosis in *C. neoformans*, it made sense to study Rec8 in the basidiomycetous opportunistic pathogen *C. neoformans*. Another reason to explore the process of meiosis in *C. neoformans*, in general, is that Rec8 deletion has proven to drastically reduce the viability of spores, which are one of the infectious agents of cryptococcosis. Since *C. neoformans* only infects the immunocompromised individuals, observations drawn from studying this organism can be extrapolated to the process of meiosis and the resulting basidiospores in *C. gatii*, a close relative of *C. neoformans* that is capable of affecting the immunocompetent. The findings from this project can also be extrapolated to the sister species *C. deneoformans*, for which sexual reproduction is better characterized and which can mate with *C. neoformans* to produce hybrids.

Therefore, this study was conducted with the objective to identify and localize Rec8 through a GFP tagged strain.Another aim was to study the function of Rec8 by deleting it in *C. neoformans*.

CHAPTER 2

OBSERVATIONS

AND

RESULTS

2.1 Rec8 is present in the *C. neoformans* genome

A BLASTP search was performed using the protein sequences of Rec8 in *S. cerevisiae* and *S. pombe*. By this method, the ORF CNAG_04404 on the 9th chromosome was identified as the homolog of Rec8 in *Cryptococcus neoformans*, with a BLAST score of 41.2 and 68.6 respectively. The only other hit with an E value <1 was an ORF whose transcript was annotated as Scc1 (the budding yeast orthologue of Rad21), with a score of 35 and 45.8 respectively. Hidden Markov Modelling, a reciprocal BLASTP search, and a search for the conserved N-terminus and C-terminus domains confirmed that CNAG_04404 was indeed the homolog of Rec8 in *S. pombe* and Rec8p in *S. cerevisiae*.The ORF is on the 9th chromosome and is ~3.1kb long. It shares a 22.39% identity with Rec8 in *S. pombe*, 19.05% identity with that in *S. cerevisiae*, 22.22% identity with mouse Rec8 and 33.33% identity with human Rec8.

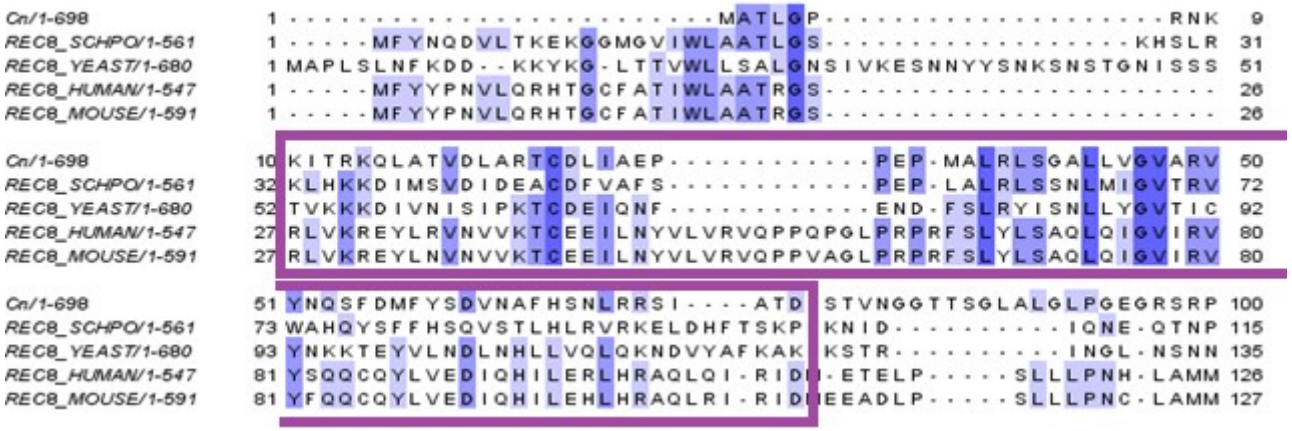


Figure 13Multiple Sequence Alignment using MAFFT showing the conserved N-terminal domain (outlined in purple) in Rec8 of *Cryptococcus neoformans, S. pombe, S. cerevisiae, H. sapiens,* and *M. musculus*

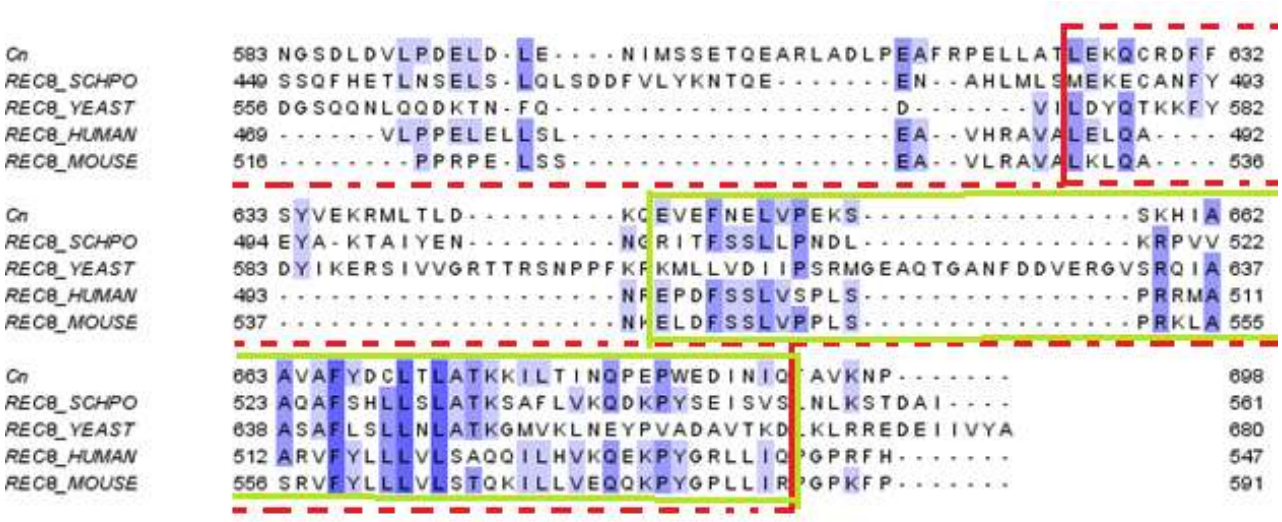


Figure 14 *MSA showing conserved winged helix (red dashed line) & C-terminal regions (green) of Rec8 of* Cryptococcus neoformans, S. pombe, S. cerevisiae, H. sapiens, *and* M. musculus

When BLAST is performed using protein sequence of ORF CNAG_04404 as the query, the ORF with the second lowest E value (of 0.003) is again CNAG_01023 annotated as the cohesin complex subunit Scc1. A pairwise alignment of the two sequences showed an identity score of 22.73%. When aligned, a greater degree of sequence conservation and fewer, shorter gaps were seen at the N and C-terminal regions of the two proteins than in a randomly selected portion from the middle (Figure 15)

Figure 15 Sequence conservation at the terminal regions where the conserved domains were predicted as compared to a randomly selected *region* in the middle. Alignment was done with MAFFT.

2.2 Checking Rec8 expression and localization

2.2.1 Rec8 is not expressed during mitosis

By observing the fluorescent signal in Rec8 GFP cells grown mitotically in YPD for approximately 8 hours, it was concluded that Rec8 GFP did not express in cells dividing mitotically, at least to levels detectable by fluorescent microscopy. GFP Cse4 was used as a positive control,and untagged H99 α WT (the genetic background for the Rec8 GFP strain) was used as the negative control. (Figure 16)

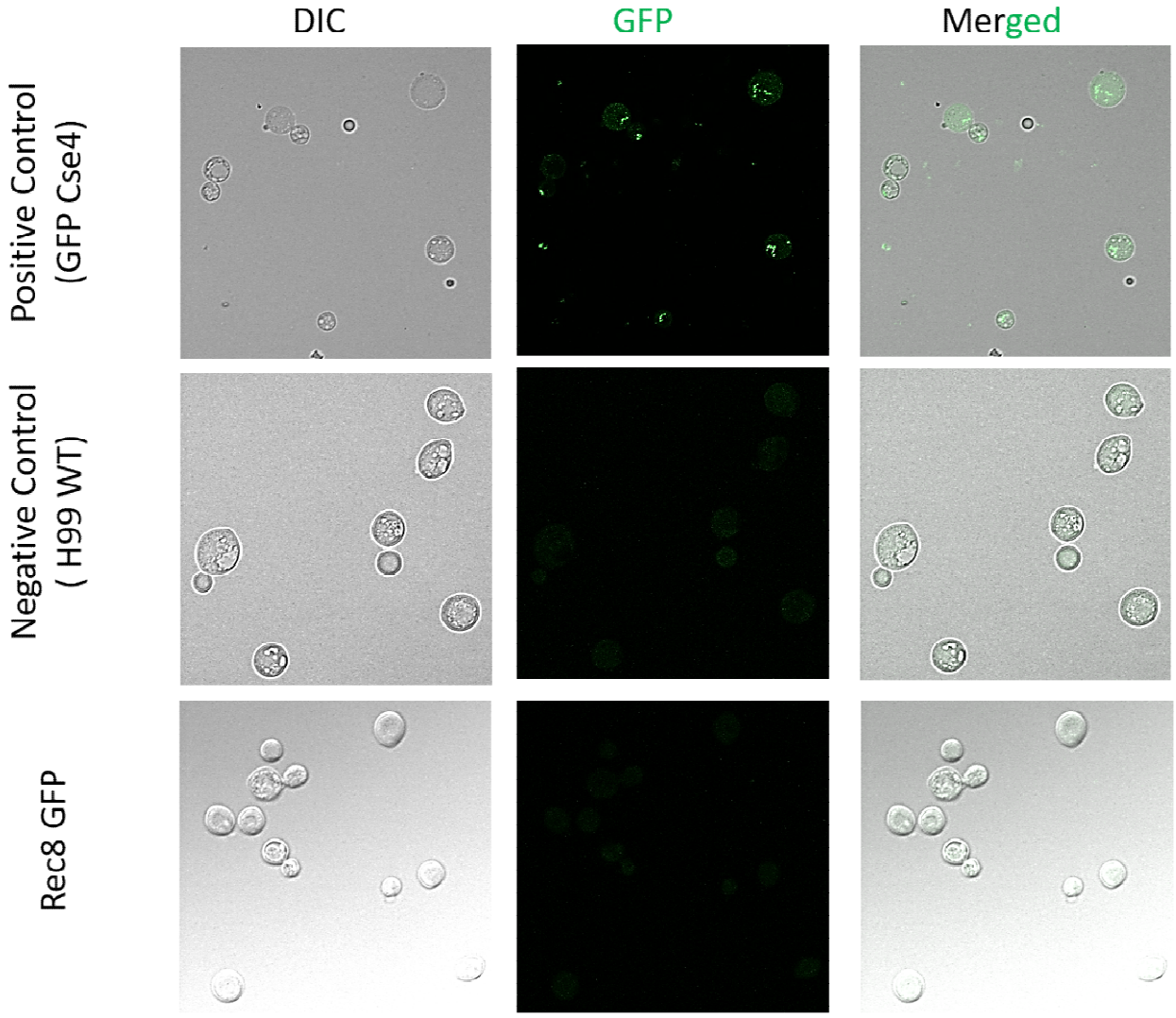


Figure 16 GFP signal in (top) Cse4 GFP, the positive control; (middle) untagged H99 WT, the negative control, and (bottom) Rec8 GFP cells grown in YPD,i.e. during mitosis.

2.2.2 Rec8 expression and localization during mating, meiosis and post sporulation

Rec8 GFP seems to colocalize with H4 mCh signal (Figure 17). Rec8 GFP is not expressed to a level observable by fluorescent microscopy in certain hyphae (Top panel, Fig.17) but seems to colocalize with H4 mCh signal in others. The signal is distinguishable from the background fluorescence after basidium formation and seems to persist after sporulation. Matingswere set up as described in materials and methods, section 4.2

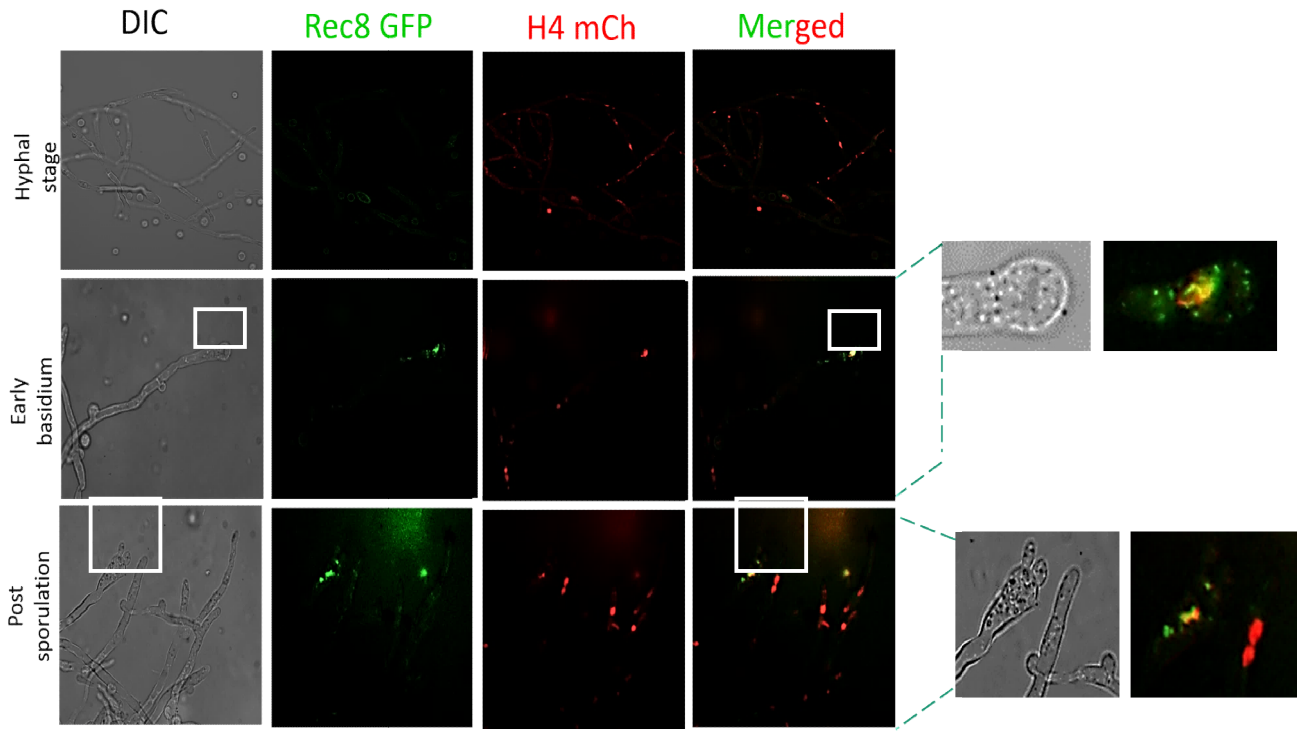


Figure 17 Rec8 GFP and H4 mCh signals in filaments arising from an H99 Rec8 GFP x KN99a H4mCh cross. Various stages imaged are (top) hyphal stage, (middle) during meiosis,i.e. soon after the formation of basidium, and (bottom) after sporulation. Rec8 GFP signal is seen in the third filament from the left, but not in the hyphae in the top pane. Insets show a zoomed in view of the regions highlighted by the boxes.

2.3 Phenotype of Rec8 null mutant

2.3.1 Rec8 is involved in filamentation

Rec8 seems to be implicated in filamentation (Figure 18 and 19), as evidenced by a reduction in the overall sizes and density (bushiness) of the patches of hyphae at the periphery of mating spots.

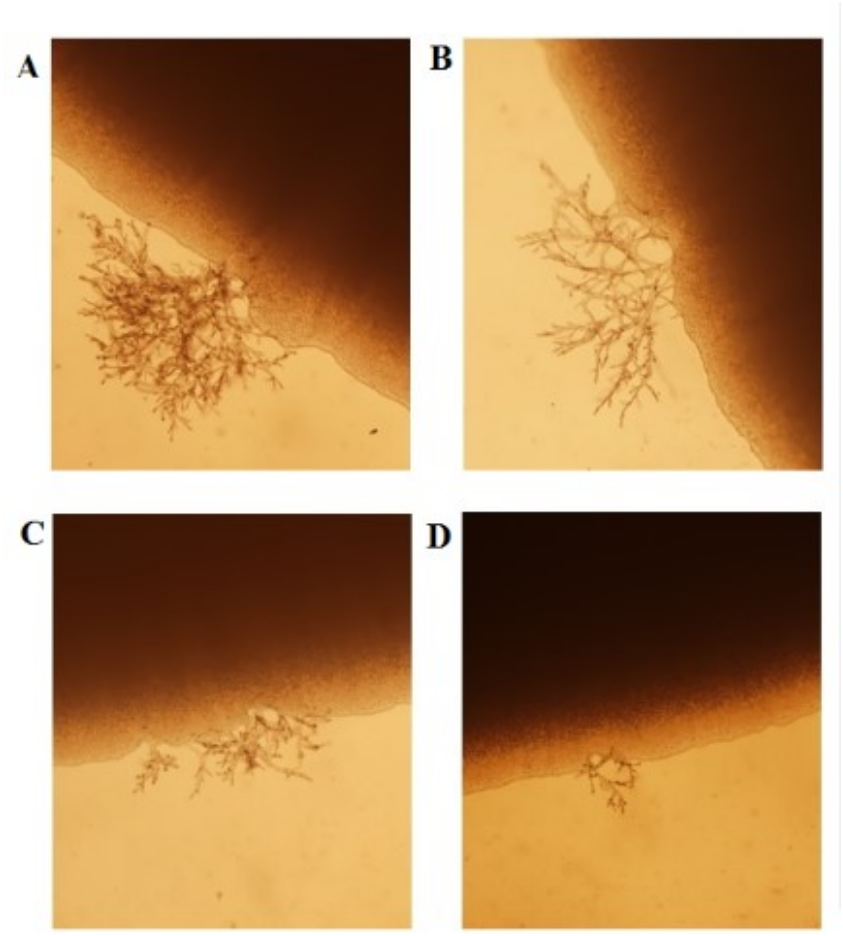


Figure 18 Rec8 mutants show reduced filamentation as observed by unilateral and bilateral crosses. ***A****: H99α WT x KN99**a** WT* ***B****: KN99**a** WT x H99α Rec8Δ* ***C****: H99αWT x KN99**a** Rec8Δ* ***D****: H99α Rec8Δ x KN99**a** Rec8Δ*

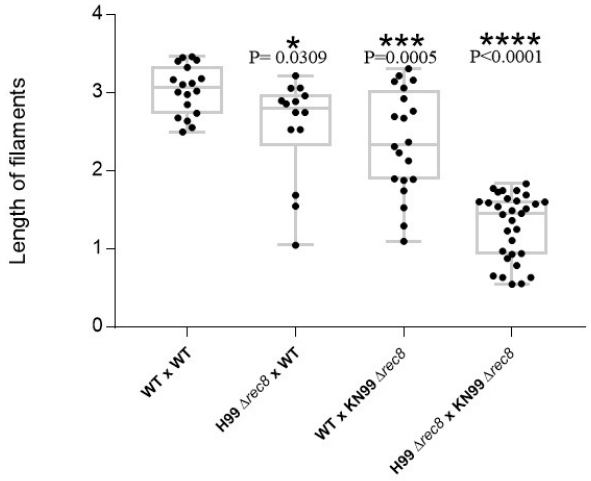


Figure 19Quantification of filamentation in Wild-type, unilateral, and bilateral crosses of *rec8* null mutants. Filament lengths were measured only for the hyphae that were traceable from the point of emergence to the tip. Measurements were made as described in materials and methods, section 4.3 for more than 15 filaments in 3 or more patches (N > 15 x 3 = 45)This experiment was repeated for three plates (n=3).One-way ANOVA performed to check significance, and graph created using GRAPH PAD Prism v7.04 for Windows

2.3.2 Hyperfilamentation of Δcrg1 cannot rescue the phenotype in Δrec8

Since crg1 null mutants show a hyperfilamentous phenotype whereas rec8 null mutants have decreased filamentation capability, and since Crg1 is a downstream effector in both of the main pathways (Wang, Cutler et al. 2004), we decided to check whether the hyperfilamentous ΔCrg1 mutant could rescue the decreased filamentation phenotype seen in a unilateral ΔRec8 cross.This we did by two methods: first, by checking for pheromone response in the confrontation assay, as described in the materials and methods section 4.5 (since crg1 is responsible for pheromone responsive mating) (Figure 20), and by quantifying the extent of filamentation (Figure 21) as described in materials and methods, section 4.3.

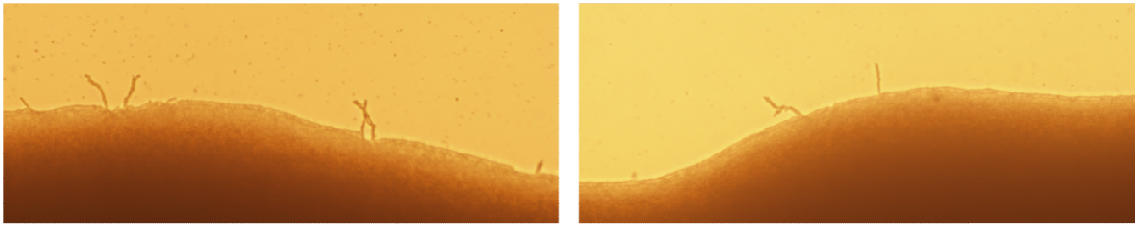


Figure 20 Conjugation tubes seen under 10x magnification when H4mCh (positive control) and KN99a *rec8* were streaked in close confrontation with *crg1*. Both the crosses showed roughly equal number of conjugation filaments.

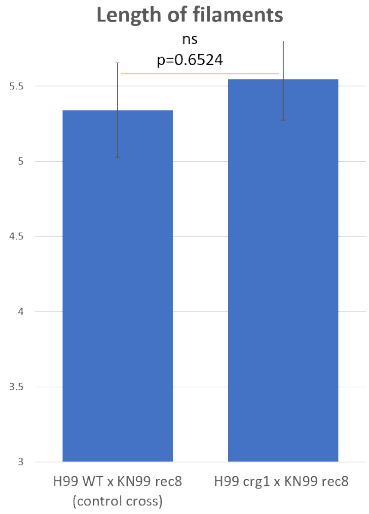


Figure 21The*crg1* hyperfilamentous mutant does not seem to rescue reduced filamentation phenotype in *rec8* null unilateral crosses (P values calculated using T-test on MEDCALC ®) as there is no significant difference in length.

2.2.3 Rec8 is needed for efficient sporulation.

As can be seen in Figure 22, the bilateral cross of H99 *rec8* x KN99 *rec8* shows a drastic decrease in the number of basidia bearing spores. Additionally, those basidia that sporulate do so 22-24 days after spotting on the mating media, as opposed to the 17-18-day mark for the wild-type and unilateral crosses. The number of spores that successfully separate from the basidia and germinate on the YPD agar plates when spotted is also less for the bilateral cross, though the difference between the unilateral and bilateral crosses does not seem to be so dramatic.

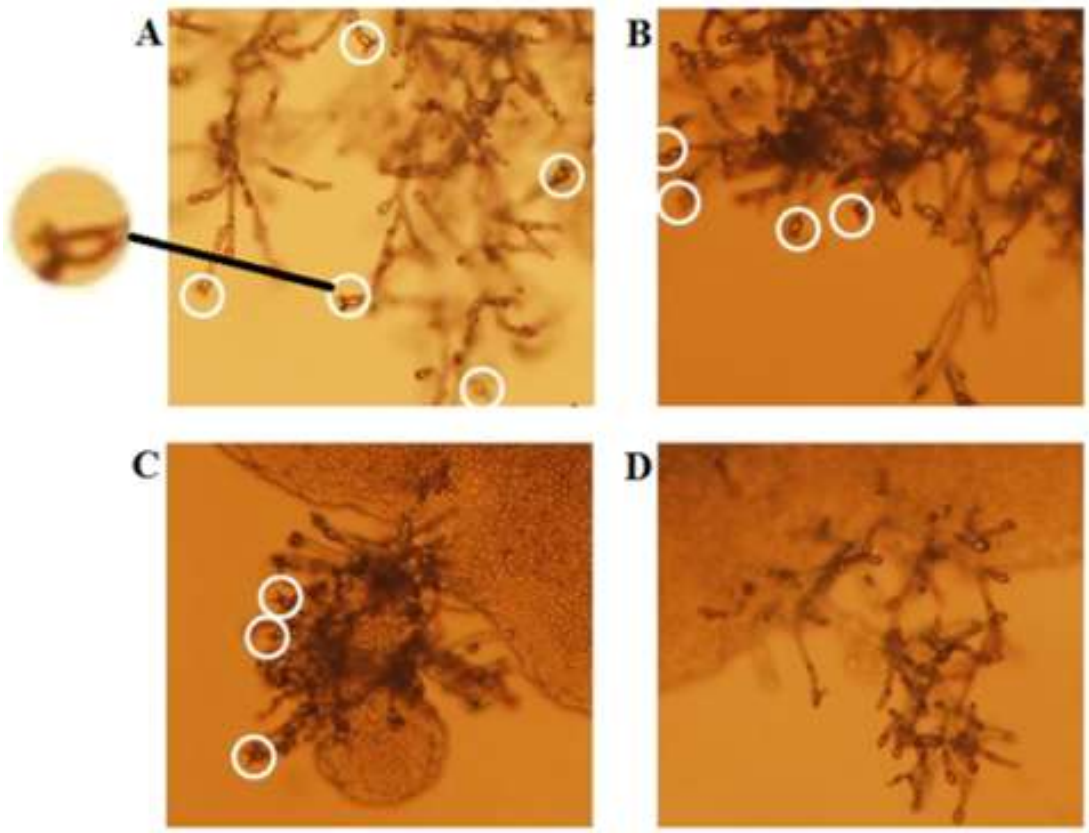


Figure 22A decrease in the number of basidia bearing spores in the bilateral cross (D), as compared to the unilateral crosses (B and C), and the wild-type control cross (A). The offset to the left of (A) shows what a single basidium bearing spores looks like.

***A**: H99α WT x KN99**a** WT* ***B**: KN99**a** WT x H99α Rec8Δ* ***C**: H99αWT x KN99**a** Rec8Δ* ***D**: H99α Rec8Δ x KN99**a** Rec8Δ*

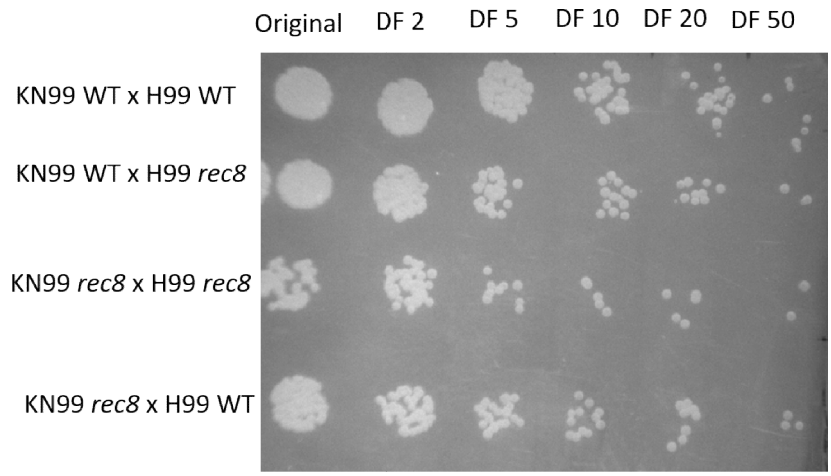


Figure 23A quantitative screenshot of sporulation from basidiospores randomly isolated from the periphery of mating spots.The original supernatant (obtained upon following the methodology described in section 4.4 of

materials and methods) as well as dilution of factor 2, 5, 10, 20 and 50, were spotted on a YPD plate.Imaging was done 3-5 days after incubation at 30º C. Normal yeast cells scraped off old plates and spotted in a similar fashion grow in ~1-2 days.

2.2.4 Rec8 null mutants do not display sensitivity or resistance to microtubule depolymerizing agents

The rec8 null mutants were also tested for any mitotic defects, such as slow growth (Figure 24) or susceptibility to drugs such as Thiabendazole or Benomyl (Figure 25). No such abnormalitieswere found.

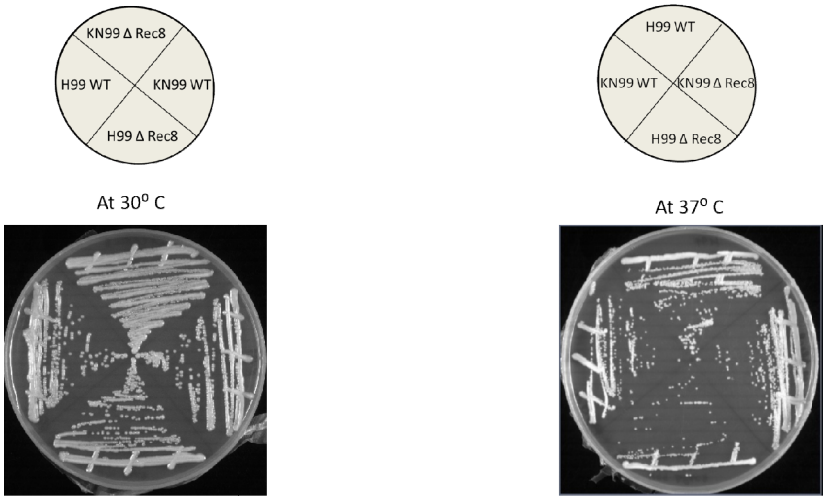


Figure 24 No defects in mitotic growth at 30 or 37ºCwere found upon streaking the cells on a YPD agar plate, as compared to the wild-type strains.

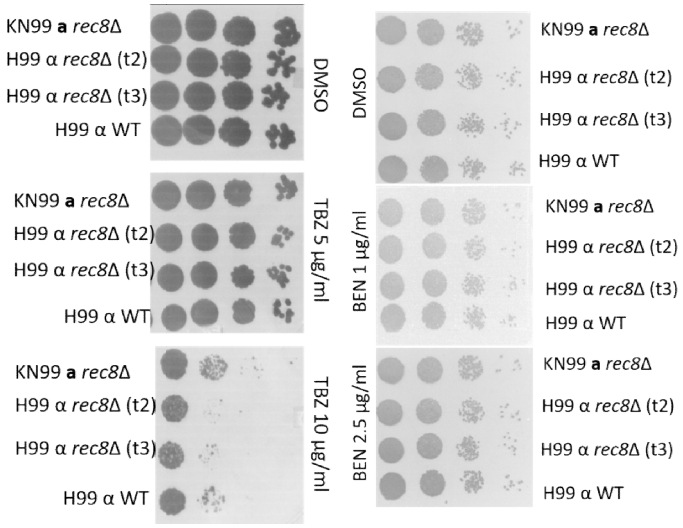


Figure 25*Δrec8*mutants show no altered sensitivity to the spindle toxins Benomyl and Thiabendazole as compared to WT

CHAPTER 3

DISCUSSION

&

FUTURE WORK

Rec8expression is not exclusively meiotic

The lack of fluorescent signal in Rec8 GFP cells grown in YPD indicates that Rec8 has no role to play during mitosis. Thisis supported in the light of the fact that RNAseq data from the lab (S. Sridhar, unpublished) shows that Rec8 expression is approximately 80-fold lower than that of various kinetochore proteins. However, given that Rec8 affects filamentation, which occurs before meiosis, and its expression persists in basidia even post-sporulation,i.e. after completion of meiosis, Rec8 must be playing non-canonical roles in *Cryptococcus neoformans.* (Figure 26)

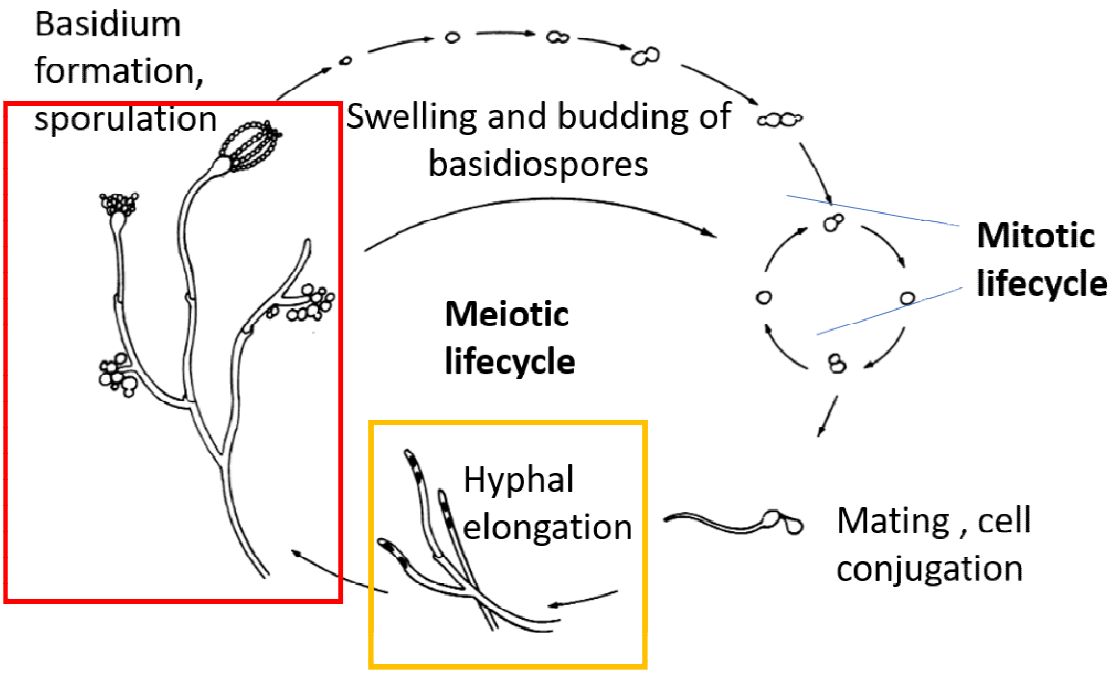


Figure 26 Rec8 influences the two major stages of sexual reproduction in *Cryptococcus neoformans*:filamentation (yellow) and sporulation (red). Adapted from (Erke 1976)

The influence of Rec8 on sporulation is to be expected, as Rec8 deletion in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* is known to cause a dramatic loss in viability or indeed, complete abolishment of sporulation, depending on the conditions. Further studies to characterize recombination and aneuploidy can be performed to understand the extent of segregation defects. These could be in the form of random basidiospore isolation from *rec8* null mutants in auxotrophic backgrounds(Idnurm 2010), fusion assay, etc. Co-localization studies of Rec8 with markers of karyogamy might help better understand the pre-meiotic roles of Rec8.

The mechanism by which Rec8 affects filamentation is still unknown; however, it can be at least concluded that it acts in a different pathway than Crg1. To further verify this, Rec8 needs to be deleted in a Δcrg1 null mutant background. Another possible pathway that affects filamentation is the one influenced by the phylogenetically divergent catalytic subunit of the ribonucleotide reductase (Zulkifli, Kaur et al. 2012). Whether Rec8 is involved in this pathway or not can easily be tested by setting up crosses on V8 media containing hydroxyurea.

Rec8 does not influence mitotic growth

That Rec8 does not affect mitotic division in *Cryptococcus neoformans* is evidenced by lack of growth defects even on knocking out of the gene.

Both Benomyl and Thiabendazole are spindle antagonists, as they interact with β tubulin and thereby prevent further polymerization of microtubules. The fact that *rec8* null mutants are not differentially influenced by these drugs as compared to wild-type proves that spindle attachment is not perturbed by deletion of Rec8.

Does Rec8 take over from Rad21 prior to meiosis?

One possible explanation for Rec8 affecting pre-meiotic division, in this case, filamentation, is that Rec8 might take over the responsibility of sister chromatid cohesion from Rad21 even before the cell enters meiosis.In order to prove this, colocalization studies must be conducted by mating the Rec8 GFP strain with Rad21 and Kar7 (a karyogamy marker) tagged strains.

A post-meiotic role for Rec8?

Sporulation indicates the completion of meiosis. The persistence of the Rec8 GFP signal even in basidia bearing spores might point to a post-meiotic role of the protein. However, no signalis seen in the basidiospores themselves. To verify whether Rec8 indeed has roles beyond meiosis in *C. neoformans*, the Rec8 GFP signal must be co-localized with vesicular markers; this will inform us whether the persistence of the signal was due to Rec8 expression even after sporulation, or the signal came from degraded cargo in the vesicles.

CHAPTER 4

MATERIALS

AND

METHODS

4.1 CREATION OF STRAINS

Two primary methods to study a protein are to knock it out or by tagging it. Hence twoprincipal strains were created for this project – Neomycin resistant Δ*rec8* mutants in both H99*α*and KN99 **a** backgrounds, and Nourseothricin resistant (NAT) Rec8 tagged with GFP at the C terminal.

To tag Rec8 with GFP at the native locus, a ~ 1.2 kb long fragment from the C-terminus of the ORF which did not include the stop codon was amplified from Wild-Type genomic DNA (Panel A from Figure 28). It was cloned into the plasmid pVY7 which already contained NAT resistance and GFP genes, such that the GFP would be in frame when the protein was finally tagged. The resultant plasmid was transformed into *E.coli*, and positive clones were identified by a shift in mobility of plasmids on the gel (as compared to the parental plasmid), and a particular digestion pattern by the restriction enzyme EcoRI (Panel B of Figure 28)

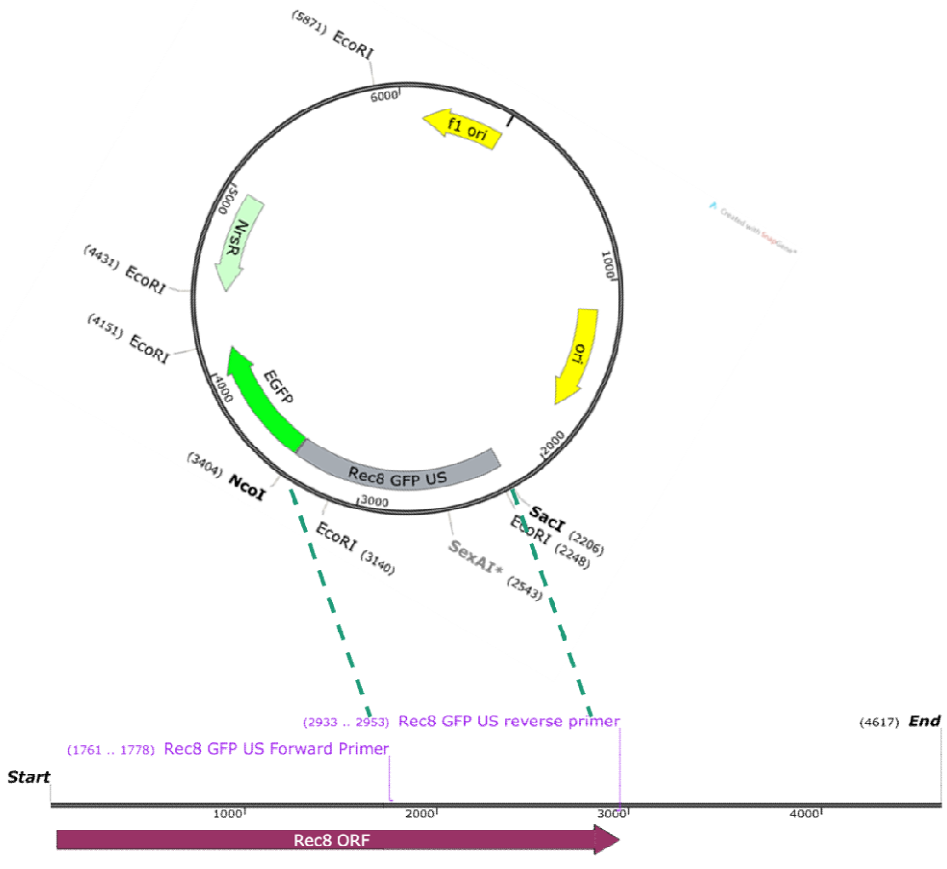
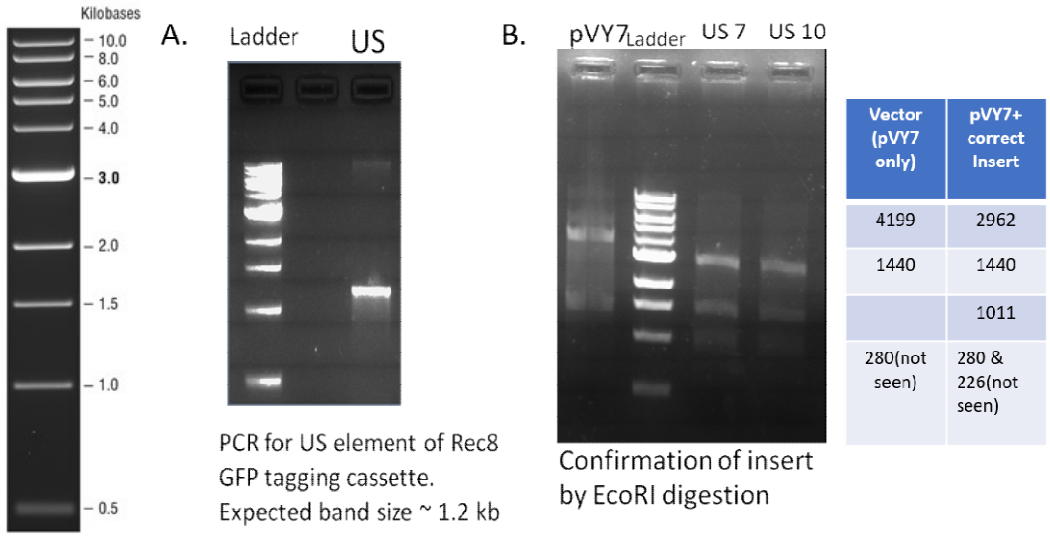


Figure 27Strategy for creating a plasmid to tag Rec8 with GFP at the native locus.



Vector (pVY7 only)	pVY7+ correct Insert
4199	2962
1440	1440
	1011
280(not seen)	280 & 226(not seen)

Figure 28 A. Amplification of an 'Upstream' element (US) from the genomic DNA of H99 WT. B. Confirmation of positive transformants by digestion of plasmid using EcoRI. The patterns of bands expected in case of parent plasmid and successful transformant are indicated in the table. To the left is an image of the band pattern in the NEB 1kb ladder which has been used as a reference throughout.

The plasmid thus obtained was transformed into Cryptococcus neoformans H99 α cells as described in the next section. The resulting transformants were screened by PCR, the schematic and a sample gel image for which is shown in Figure 29. 4 positive transformants were identified in this fashion and used for further experiments.

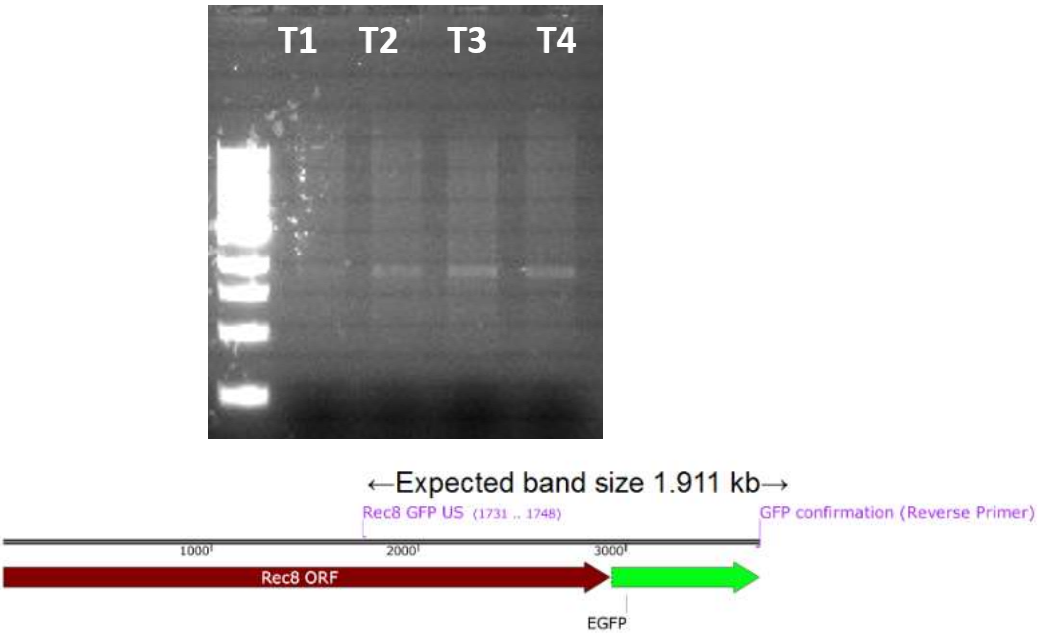


Figure 29 PCR confirmation of tagging. Schematic used is shown below the gel image. T2, T3 and T4 are true null mutants

For the deletion of Rec8 in *C. neoformans*, an overlap cassette was created comprising of a ~1.2 kb long region upstream of the Rec8 ORF, the Neomycin resistance gene, and a downstream homology region of approximately 1 kb (Figure 30 and 31). The US and DS homology regions were amplified from H99 WT genomic DNA, and the Neomycin resistance gene was amplified from the plasmid pLK25. The resulting overlap cassette was biolistically transformed into *C. neoformans* cells as before with the Rec8 GFP strain.

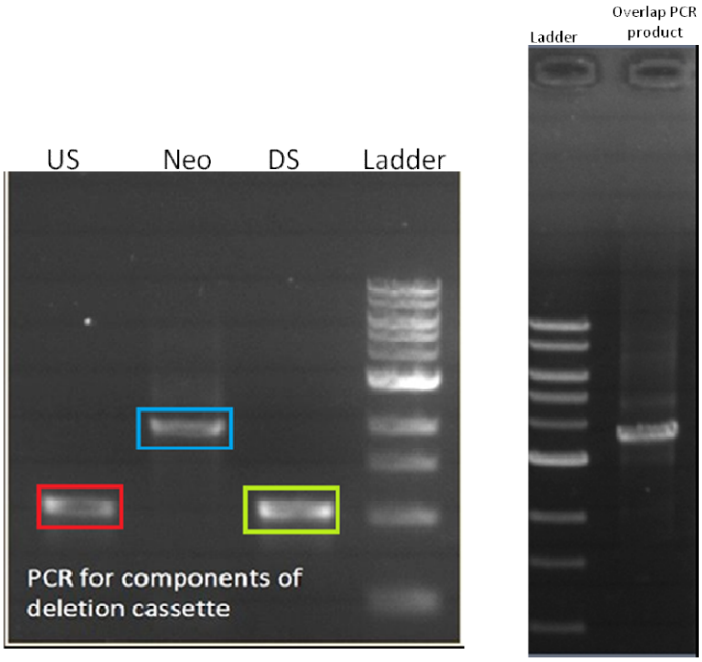


Figure 30 Components of the overlap cassette for Rec8 deletion (Left) and overlap PCR for the cassette (right). Expected band sizes for the US, Neo and DS are ~1.1 kb, ~ 1.9kb, and ~1kb. When put together in an overlap cassette, the expected size of the cassette is ~4kb.

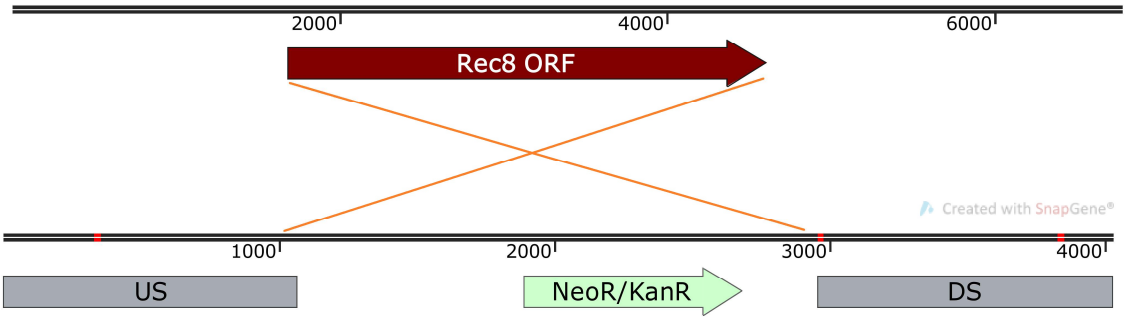


Figure 31 Strategy for deletion of Rec8 in C. neoformans

The colonies obtained after transforming *C. neoformans* in this manner were screened by two different PCRs- an ORF PCR, wherein a positive transformant (i.e. a true null mutant) should not show any band, and a PCR for the deletion product. In the latter, a primer was selected from beyond the Rec8 ORF's 5' terminus, and a reverse primer from inside the neomycin resistance gene was used. Thus, a band will be obtained if and only if the overlap cassette has integrated at the right locus. The schematics and sample results of such PCRs are shown in Figure 32

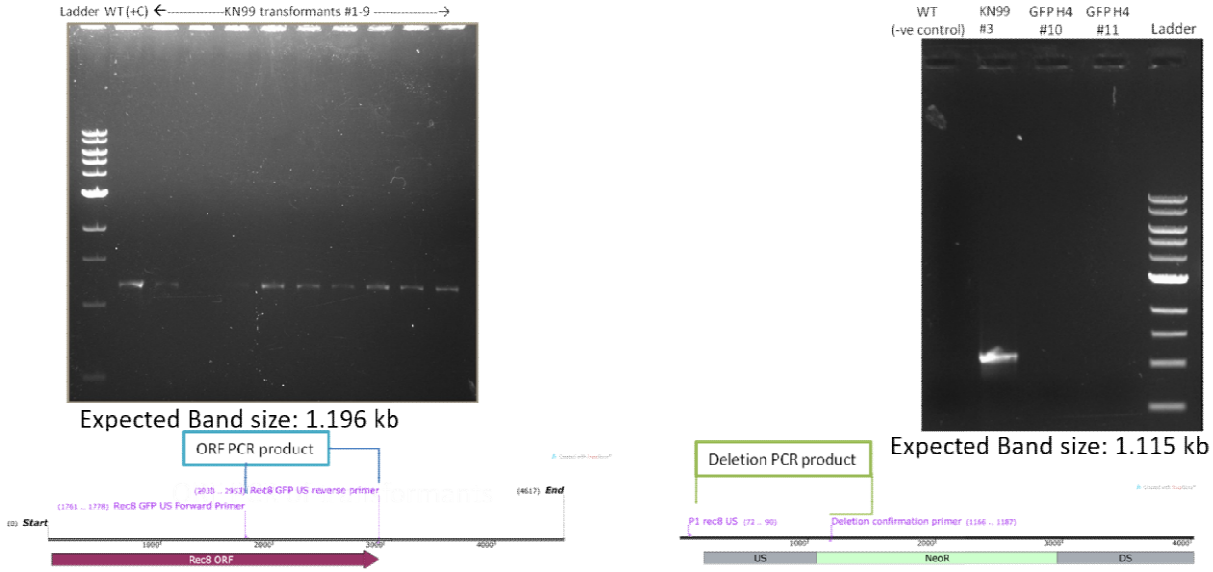


Figure 32 Schematics and sample results of the ORF and deletion confirmation PCRs.

4.1.1 BIOLISTIC TRANSFORMATION

The transformation of DNA into *C. neoformans* cells was done as described in (Toffaletti, Rude et al. 1993) using PDS-1000 He Biolistic Particle Delivery System. Briefly, 5ml of overnight cultures (in YPD media: 2% D-Glucose+ 2% Peptone+1% Yeast extract) of the *C. neoformans* strains were pelleted by centrifuging at 4000 rpm for 5minutes to completely remove the media. The pellet was resuspended in 200-300µl autoclaved dH_2O and spread uniformly at the centre of a YPD+1M Sorbitol plate. The plated cells were allowed to dry in the hood. Meanwhile, the gold beads stock (60mg/ml) was vortexed vigorously for approximately 15 minutes. 10µl of the gold bead suspension was taken in a 1.5ml Eppendorf tube and mixed well with 2-3 µg of the DNA. To this, 10µl of 2.5M $CaCl_2$ and 2µl of 1M Spermidine free base was added, and the mixture was vortexed for1minute and allowed to stand at room temperature for 5 minutes. The beads were pelleted, and the supernatant was discarded. These beads were then resuspended in 500 µl 100% ethanol. This was then

vortexed for 15s and pelleted down. The supernatant was discarded. The pellet was resuspended in 10µl 100% ethanol. The macrocarrier membranes and stopping screen were dipped in 70% ethanol and allowed to dry in the hood. The chamber and holders were also sterilized with 70% ethanol. The DNA-microcarrier (Gold beads) resuspension was spotted on a macrocarrier membrane and allowed to dry. The biolistic transformation was carried out using the gene gun. Subsequently, the cells were allowed to recover by incubating at 30℃ for approximately 6 hours. Then, 1ml of dH_2O was used to scrape off cells and make a resuspension of the same. The resuspension was spread on the selection plate, allowed to dry, and incubated at 30℃ for 4-5 days.

4.2 MATING ASSAYS

Mating crosses were set up in the following manner (Figure 33): Cells were streaked on a YPD plate (1% Yeast extract, 2% Peptone, 2% dextrose, 2% agar) and allowed to grow for approximately 24 hours. An approximately equal number of cells were resuspended in 500µl autoclaved dH_2O,and their O.D. at 600nm was measured. Equal $O.D._{600}$s of the cells for each of the crosses, assuming 1 $O.D._{600}$ to contain 1 x 10^7cells (Wild-type H99 α x KN99 a; Wild-type H99 α x Rec8Δ KN99 a; *rec8*Δ H99 α x Wild-type KN99a; and *rec8*Δ H99 α x *rec8*Δ KN99 **a**) were mixed, spun down at 13000 rpm for 5 minutes, and resuspended in 200µl autoclaved dH_2O. Next, Multiple spots of 1-5 µlwere made on V8 plates (5% V8 juice clarified by centrifugation, 0.05% KH_2PO_4, 4% agar at pH 5-5.3) and allowed to dry. The plates were then incubated in an upright position in the dark at 25℃ for 12 days to 5 weeks. Silica gel bags were used to maintain humidity if necessary. The periphery of the spots were monitored under 10x magnification regularly for the formation of hyphae, basidia and spores.

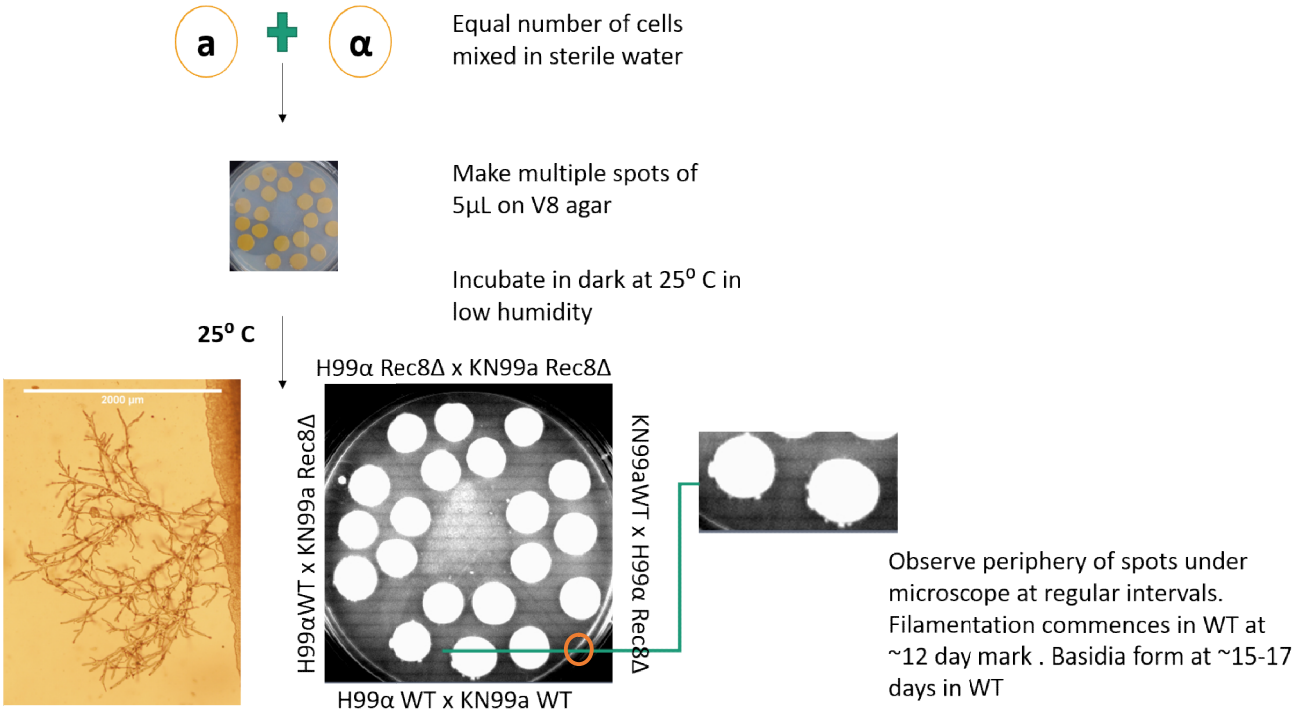


Figure 33Methodology for setting up mating crosses. Evidence of filamentation is seen by the naked eye as irregularities on the periphery of the mating spot, as opposed to the smooth periphery of a spot without filamentation (example outlined in red)

4.3 QUANTIFICATION OF FILAMENTATION

Filamentation was quantified from images captured using the same microscope under a 10x objective. The polyline option in the software Image J or FIJI was used to map and measure the length of each individual traceable filament from the hyphal tip to the point of emergence from the mating spot (Figure 34). The lengths thus measured were averaged,and their standard error of the meanwere calculated as the error bars for the histograms. Statistical significance was commented upon by performing a one-way ANOVA on GraphPad Prism v7.04 for Windows.

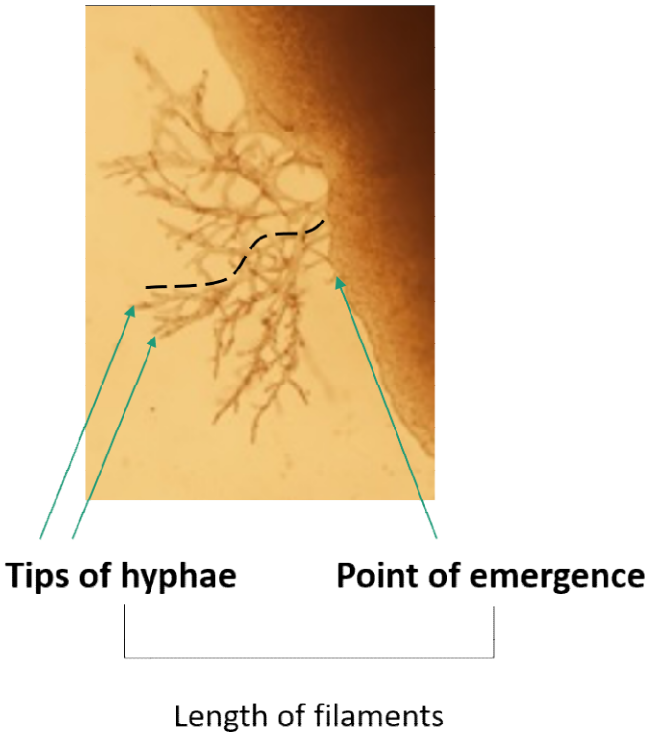


Figure 34Measuring individual traceable filaments with Image J

4.4 QUANTIFICATION OF SPORULATION

In order to quantify the viability of basidiospores from a particular mating patch that showed the presence of spores under the microscope, a sterile cut P20-200 tip was used to punch out a section of the mating media showing filamentation, not too far from the central spot but not too close either (to prevent contamination by the yeast cells).This punched out agar was added to an Eppendorf containing 200µL autoclaved distilled water and vortexed vigorously for 2-5 minutes. 5µL of this original suspension was spotted onto a YPD plate, and dilutions of factors 2 (1:1), 5(1:4), 10 (1:1 of the previous dilution), 20 (1:1 of the previous dilution) and 50 (1:4 of the DF 10 suspension) were also spotted.Thiswas repeated for the rest of the crosses on the mating plate. Growth was typically seen 2-3 days later, as opposed to a healthy opaque growth of yeast cells in approximately one day. A graphic explaining this procedure is shown in Figure 35.

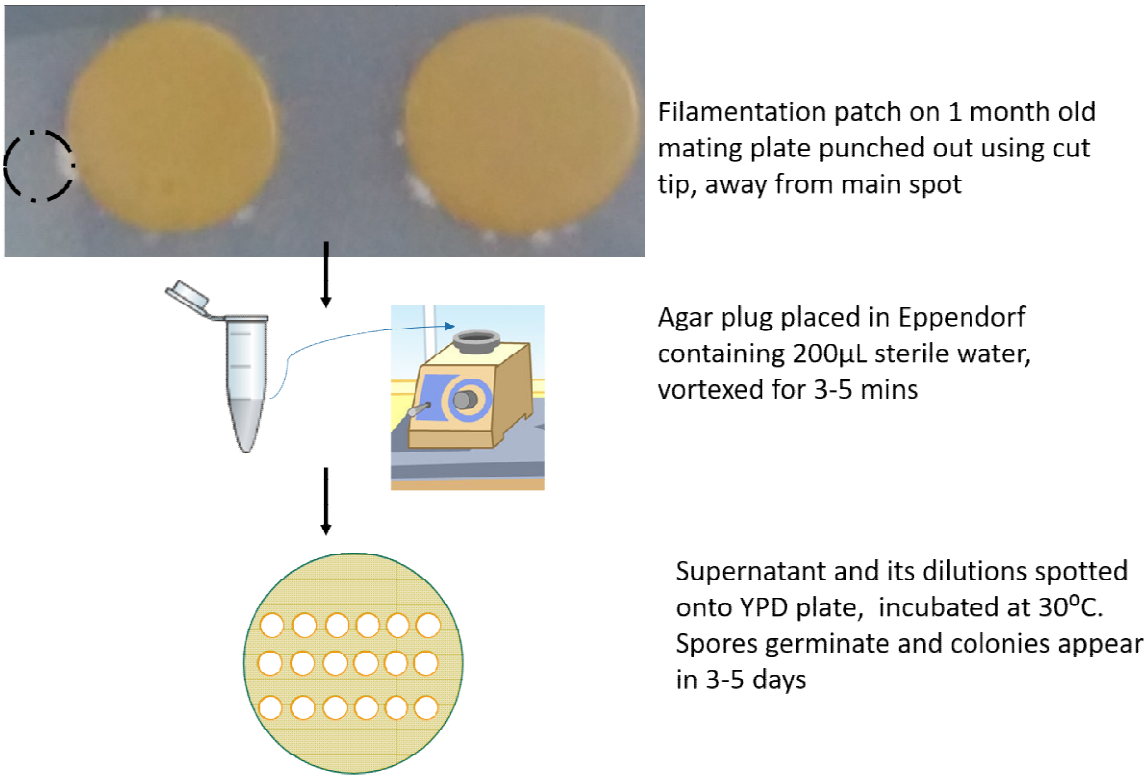


Figure 35 Methodology for taking a quantitative snapshot of sporulation

4.5 CONFRONTATION ASSAY

The confrontation assay was done by streaking the cells of the opposite mating type in close confrontation to each other (~1-5 mm apart) on V8 plates and incubating them in the dark at 25ºC for 5-12 days with regular monitoring under the microscope.

4.6 DRUG RESISTANCE/ SENSITIVITY ASSAY

The drug plates were prepared by dissolving the required amount of the working stock (10mg/ml in DMSO) in YPD before pouring. Since Benomyl has a tendency to precipitate but is quite thermally stable, it was added to a 50 ml Falcon tube containing YPD which was then put in a boiling water bath intermittently and inverted to mix. 2 O.D.600 (approximately 2x 107 cells) of a fresh culture were spun down and resuspended in 1ml of autoclaved distilled water.5 µL of this was spotted onto the drug plate as well as a spotting control containing an equal volume of DMSO as the drug plate. 5 µL of serial dilutions were also spotted. Three mutants as well as a Wild-type controlwere spotted in this fashion, the spots allowed to dry before incubating the plates at 30ºC for 2-3 days.

LIST OF PRIMERS USED (Table 2)

NAME	SEQUENCE	PRIMER LENGTH
Rec8 deletion primers (underlined portions anneal to the genome**)**		
P1 rec8 US	GCAGCTCGCTACTGTGGAC	19
P1 full rec8	GCCACGGAATCGCTTGTG	18
P3 rec8	ATGACTATGATATTGGCTGCGAGGATGTGA GCTGGAGAGC	40 (20+20)
P2 rec8 US	GCTCTCCAGCTCACATCCTCGCAGCCAATA TCATAGTCAT	40 (20+20)
P5 rec8 DS	CGTGTTAATACAGATAAACCCGGTCGTCCT GATCTCTATG	40 (20+20)
P4 rec8	CATAGAGATCAGGACGACCGGGTTTATCTG TATTAACACG	40 (20+20)
P6 full rec8	AGTCGCCAGCAACTCTGGAC	20
P6 rec8 DS	CTGGCACTAGCTCGTTGAATTC	22
Neo confirmation RP	TTGTTGTTACCATCATCCTCTC	22
Rec8 GFP tagging primers (underlined portions anneal to the genome**)**		
Rec8-GFP.US.FP	ATATGAGCTCGACCTCGGTCAACACAAC	28
Rec8-GFP.US.RP	CGATCCATGGCCGGGTTTTTGACAGCAAAC TG	32
GFP/mCh confirmation primer	CTTGTACAGCTCGTCCATGCC	21

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