Characterization and essentiality of the fungal specific Dam1 complex in pathogenic yeast *Candida tropicalis*

A thesis submitted for the partial fulfilment of the degree of

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as a part of integrated Ph.D. program by

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DECLARATION

I do hereby declare that the work described here in this thesis entitled 'Characterization and essentiality of the fungal specific Dam1 complex in pathogenic yeast *Candida tropicalis*' has originally been carried out by myself under the guidance and supervision of Prof. Kaustuv Sanyal, Assistant Professor, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore-560064, India**.**

S. Sundar Ram

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CERTIFICATE

This is to certify that this thesis entitled 'Characterization and essentiality of the fungal specific Dam1 complex in pathogenic yeast *Candida tropicalis*' submitted by S Sundar Ram towards the partial fulfilment of Integrated PhD Program, as part of Project for Master of Science, at Jawaharlal Nehru Centre for Advanced Scientific Research, is based on the studies carried out by him under my supervision and guidance.

Prof. Kaustuv Sanyal

Date:

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ABBREVIATIONS

°C	degree Celsius
µg	micrograms
µL	micro litre
bp	base pair
DAPI	4’,6-Diamino-2-phenylindole
DNA	Deoxyribonucleic acid
GFP	Green fluorescent protein
h	hour
kb	kilo basepairs
kDa	kilo Daltons
KT	kinetochore
Mb	Mega basepairs
MBP	Maltose binding protein
min	Minutes
ml	milli litre
mM	milli molar
MT	microtubules
NAT	Nourseothricin
ng	nanogram
OD_{600}	Optical density at 600nm
ORF	Open reading frame
PCR	Polymerase chain reaction
s	Seconds
EM	Electron microscopy
SPB	Spindle pole body

Introduction

INTRODUCTION

1. Chromosome segregation and the key players involved.

Mitotic cell cycle ensures that the progeny cell receives a complete complement of the genome from the parent. Whenever growth supportive conditions exist, the cell senses and commits itself to the process of cell division that can be temporally delineated as the **G1** (Gap1) phase, **S** (synthesis) phase, **G2** (Gap2) phase and **M** (mitotic). Checkpoints are placed at G1/S, G2/M and metaphase-anaphase transitions the to ensure that key processes such as DNA replication, repair and chromosome segregation are completed at the desired phase. One of the key processes central to cell division is the segregation of sister chromatids, ensuring the daughter cells receive an equal genetic complement. The process of segregation is not initiated unless all the chromosomes are properly bi-oriented to spindles emanating from spindle pole bodies (SPBs). Premature segregation is avoided by sister chromatid cohesion and also by the spindle assembly checkpoint (SAC) that senses improper/ unattached kinetochore(KT) and arrests the cell cycle prior to anaphase (Lara-Gonzalez et al., 2012).

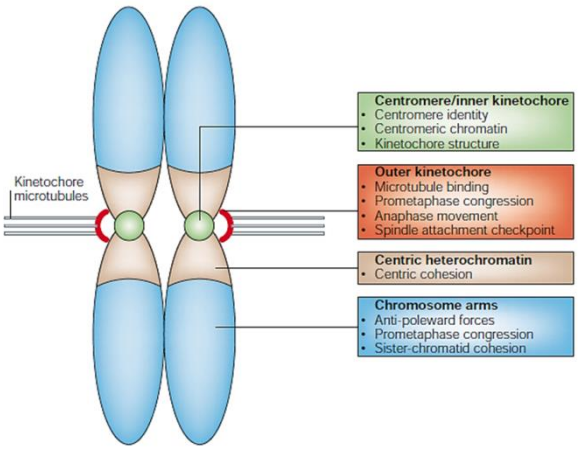


Fig 1: Schematic representation of a chromosome showing the architecture of a kinetochore (Sullivan *et al*., 2001)

By doing so, the system is given more time to resolve such problems so that there are no errors in this key process. The consequences of perturbation of these processes are catastrophic. Aneuploidy, a condition wherein a cell gains or loses chromosomes or parts of a chromosome leading to abnormal chromosomal complement is a classic example. These conditions are known have a direct effect on the survival of a cell. For instance, genomic instability has been well related to disease conditions such as cancer and cell death (Draviam et al., 2004; Jallepalli and Lengauer, 2001). Thus, the process of segregation is required for completion of cell cycle and survival of the cell itself. The centromere forms a hub where most of the cellular processes pertaining to segregation occur. Assembly of kinetochores (KTs) on the centromere DNA, interaction of KTs with the microtubules (MTs) and the activation/inactivation of SAC are the three key processes that facilitate accurate chromosome segregation.

2. Centromeres

From the perspective of chromosome segregation and cell cycle, one can define a centromere as a unique DNA locus on the chromosome that recruits and assembles the KT over it. By doing so, a MT attachment site is generated, facilitating the process of segregation through the dynamic interactions between them. This makes the specification and formation of centromeres very critical for cell survival. Despite the conserved functions a centromere serves, remarkable variations in their architecture have been reported (Roy and Sanyal, 2011). By virtue of the size and organization, they can be classified as point, small regional and large regional centromeres. Point centromeres are a characteristic feature of *S. cerevisiae* and other yeasts like *Candida glabrata, Kluyveromyces lactis* (Meraldi et al., 2006). The centromeres of this kind span a short length (< 400 bp) and are genetically defined. On the contrary, regional centromeres span longer stretches of DNA. Centromeres of humans and certain fungi like

Schizosaccharomyces pombe and *Neurospora crassa* fall under this category. Placed in between these two classes are the small regional centromeres (4-6 kb in length) that were recently discovered in *Candida* species (Padmanabhan et al., 2008; Sanyal et al., 2004). The regional centromeres are not strictly sequence dependent, rather they are defined epigenetically. The centromeric protein-A (CENP-A), a histone H3 variant found exclusively in the centromeric nucleosome, is the candidate for the epigenetic mark till date. By virtue of its restricted localization, the CENP-A/Centromeric chromatin earmarks the site for the assembly of kinetochores.

3. Kinetochores

The term kinetochore literally means '**movement place**'. A classical definition of kinetochore, dating back to 1940s reads as follows: *"the specialized region of the chromosome where the property of active mobility on the spindle is manifested irrespective of the number or extension of these regions in the chromosome body and of other properties that may be associated with the region of active mobility"* (Lima-de-Faria, 1949). The proteins of the KT complex are classified based on their proximity to centromeres or the MTs as the inner, middle or the linker and the outer KT layer, with the inner being the most proximal to centromeric chromatin. Some of the key protein components of this multi-protein complex and their functions are discussed below.

3.1 Centromeric chromatin and the inner kinetochore layer

With the site for assembly of the kinetochore earmarked by centromere/centromeric chromatin, the foundation is laid by a group of about 16 proteins that are collectively known as the **c**onstitutively **c**entromere **a**ssociated **n**etwork (CCAN) (Foltz et al., 2006; Takeuchi and Fukagawa, 2012). Along with CENP-A and CENP-C, these proteins

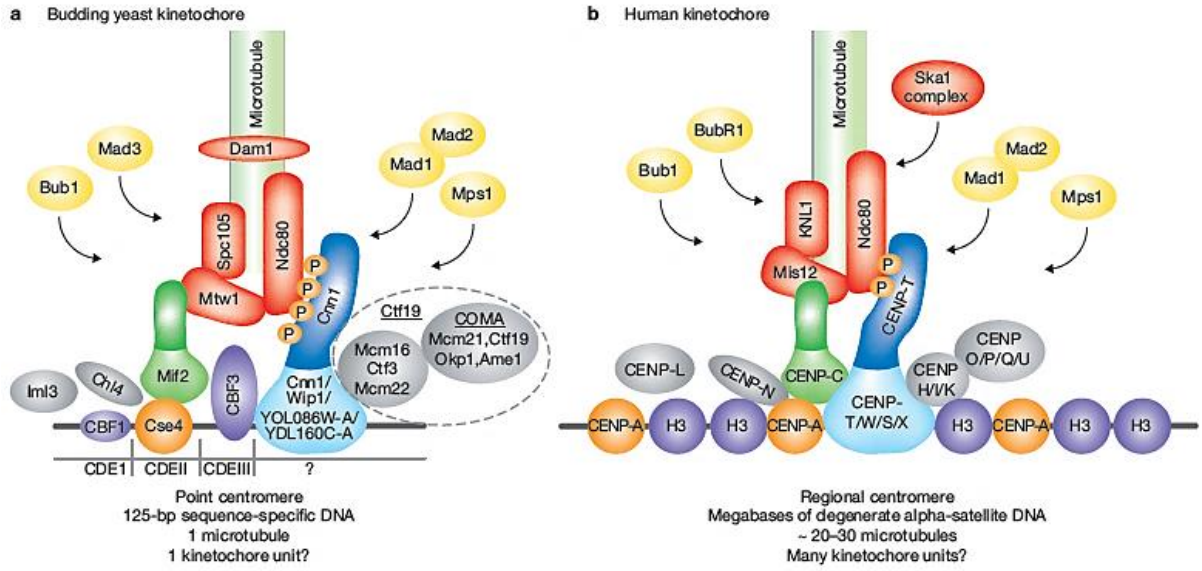


Fig 2: Schematic representation of the kinetochore structure from budding yeast and humans (Gascoigne and Cheeseman, 2012).

assemble as distinct sub complexes (CENP-H/I/K, CENP-L/M/N, CENP-O/P/Q/R/U, CENP-T/W/S/X) and associate themselves with the CENP-A nucleosomes. Three key proteins in this complex are described below. CENP-A or Cse4 in budding yeast is one of the most characterized of all the kinetochore proteins. The protein contains a CENP-A targeting domain (CATD) in its histone fold domain responsible for its targeting to the centromeric chromatin (Black et al., 2004). Most of the known kinetochore proteins show a dependency on CENP-A for proper localization. In addition, any perturbation to this protein resulted in severe mitosis defects and loss of viability, making it an essential protein (Perpelescu and Fukagawa, 2011). CENP-C or Mif2 is another essential kinetochore protein shown to interact with CENP-A nucleosomes. This protein requires CENP-A and the Mis12 complex for proper localization (Westermann et al., 2003). By their mutual interaction, these proteins facilitate the assembly of the outer kinetochore proteins like the Ndc80 complex. Another receptor for the outer kinetochore proteins is the recently identified CENP-T/W/S/X complex. By virtue of the HFD like structure at

the C terminus they form an integral part of the CCAN, while using the highly disordered N-term tail, they serve as receptors of the Ndc80 complex (Nishino et al., 2013). It has also been shown that the requirement of CENP-A can be bypassed upon ectopic expression of CENP-T and CENP-C complex (Gascoigne et al., 2011). On the contrary, ectopic expression of Cse4 is sufficient to drive kinetochore assembly in non-native loci in *Drosophila melanogaster* (Mendiburo et al., 2011). Interesting reports like these make it debatable whether the role of the CENP-A/ CCAN components is restricted to specify the site of KT assembly or it extends to forming a structural platform. It should be noted that proteins of the CCAN are greatly conserved in fission yeast and to some extent in plants as well. However, homologs of most of these proteins are absent in case of *S. cerevisiae*. Having genetically defined centromeres, proteins complexes that are specific to these point centromeres have been found. The CBF3 complex in budding yeast an example of this kind (Lechner and Carbon, 1991). Homologs of most of the CCAN proteins in *C. elegans* (holocentric architecture) are not found, the reasons for which are still not very clear (McAinsh and Meraldi, 2011).

3.2 The linker layer

As mentioned earlier, the proteins of this complex link the MT binding outer KT complex and the *cen* bound inner KT complex. It mainly comprises of the KMN supercomplex (also known as NMS complex in yeasts) which can be broken down into three sub complexes namely **K**nl1 (**S**pc105) complex, **M**is12/**M**tw1 complex and the **N**dc80 complex. The Mis12 complex, also called the 'keystone' complex, forms a stable platform by interacting with the inner layer through CENP-C or CENP-T to host the other two complexes in this network (Hori and Fukagawa, 2012; Petrovic et al., 2010). It consists of four globular proteins (Nnf1, Mis12, Dsn1 and Nsl1) of which the C-terminus of the Nsl1 has been shown to interact with the subunits of the Ndc80 and the Knl1

complex while the identity of CENP-C interacting partners are unclear (Maskell et al., 2010; Petrovic et al., 2010; Screpanti et al., 2011). The Knl1/Spc105 complex consists of an elongated Spc105 protein that binds to the Mis12 through its C-terminal (Cheeseman et al., 2006). The N-terminus of this molecule is known to have weak microtubule binding activity *in vitro.* However the major function of the Spc105 is playing host to the components of the SAC. The extended N-terminus of this protein has been shown to recruit SAC proteins Bub1 and Bub3 which initiate the STOP signal for anaphase transition, reviewed in (Foley and Kapoor, 2013; Lara-Gonzalez et al., 2012). Consistent with the aforementioned roles of these complexes, depletion of any subunit of these complexes led to defects in KT-MT interaction, bi-orientation and segregation (Kline et al., 2006). Positioned at the heart of the segregation machinery, the Ndc80 complex is the main load bearing complex with microtubule binding ability. This complex, in metazoans is classified as a part of the outer KT complex, while in yeasts it constitutes the linker layer. It consists of four subunits viz. Ndc80, Nuf2, Spc24 and Spc25 assembling in a 1:1:1:1 stoichiometry. It assumes a dumbbell shape with two globular domains separated by an extended coiled coil structure, that gives it the ability to span the entire length from the inner KT layer to the MT binding interface (Ciferri et al., 2005). While the Spc24-25 subunits are known to bind the Mis12 complex, the MT binding domains reside in the Ndc80-Nuf2 sub complex. The globular domains of these two proteins possess Calponin Homology Domain (CHD), rich in positive charged residues that facilitates an electrostatic interaction with the E-Hooks of tubulin subunits rich in aspartate and glutamate residues (Ciferri et al., 2008). Another structural feature of this region is the N-terminal disordered tail. Bivalent interactions of this tail region have been shown, (1) with tubulin subunit and (2) with the adjacent Ndc80 head, leading to a clustering effect. These interactions are under the surveillance of the Aurora B kinase. Apart from the structural support it gives, a major role of this complex is to recruit the Mps1 kinase with the help

of Aurora B kinase (Santaguida et al., 2010). Yet another unique feature of the Ndc80 complex is the presence of a looped structure whose function was not very clear until recent studies. It has now been shown that the looped region is essential for the binding of Dam1 complex (in *S. cerevisiae*) and its functional homolog Ska1 complex in case of vertebrates (Maure et al., 2011; Zhang et al., 2012). While the role of this loop in functioning of SpDam1 complex is not known, its effect on the function of microtubule associated proteins Dis1, Alp7 that stabilize and regulate spindle architecture is reported. Strains with mutations in this loop show improper MT binding and profound segregation defects (Nilsson, 2012; Tang and Toda, 2013).

3.3 The outer kinetochore complex and the KT-MT interactions.

The process of segregation occurs in a small window of time where the sister chromatids have to be pulled apart to the opposite poles. This function has been assigned to the KT complex and the driving force is the dynamic MTs (Koshland et al., 1988) . Although the Ndc80 complex is the major load bearing complex of the KT, its microtubule binding is supported by the third layer of KT called the outer kinetochore complex, which in yeasts is called the Dam1 complex. Although the KT subunit composition and the functions remain conserved across species, the outer KT complex shows striking differences and has always been an exciting avenue for research (Cheeseman and Desai, 2008; Westermann et al., 2007). Unlike the other layers, the outer KT complex constituted by the Dam1 complex in fungi has no clear homologs in metazoans.

The first components of the complex, Duo1 and Dam1, were identified over a decade ago in classical genetic screens for spindle/ microtubule defects and were later localized to the KT. Mutants studies revealed a severe defects in spindle structure, segregation and loss of viability, leading to their classification as members of the KT complex (Cheeseman et al., 2001a; Cheeseman et al., 2001b; Hofmann et al., 1998). Next to be identified was the Dad1 protein, that was found in screen for Dam1/Duo1 interacting proteins (Enquist-

Newman et al., 2001). Subsequent studies dissected out the complex further and now we know that the Dam1 complex is a hetero-decameric complex, consisting of ten subunits viz. Dad1-4, Dam1, Duo1, Ask1, Spc19, Spc34, Hsk3 (Cheeseman et al., 2002; Janke et al., 2002; Li et al., 2005; Li et al., 2002).

The impetus for extensive research on the structure of this complex was provided by reports (Miranda et al., 2005; Westermann et al., 2005) in which the complex was first purified *in vitro* and was shown to assemble into a ring like structure. As a 16-mer (each monomer contains all the ten subunits), the complex was shown to form a ring encircling the MTs, making it an ideal candidate for a MT base coupler to support Ndc80 function. The ring was shown to have an inner diameter of 35nm and interactions with the MTs are facilitated by the protrusions from each monomer towards the MT by the dam1 complex subunits. Cryo-EM studies using MBP-labelled complex has yielded information on the relative positions of these subunits in the monomer (Ramey et al., 2011). For the function the complex is expected to perform, a ringed structure is the well suited as it gains support from similar architecture of proteins like the PCNA, which slides down the linear DNA molecule.

The purified Dam1 complex possesses intrinsic ability to attach to and track the depolymerizing ends of the microtubules. *In vitro* studies show that it can traverse several micrometres when tethered to MTs without detaching from the lattice (Asbury et al., 2006; Westermann et al., 2006). In addition, the study also showed the ability of these purified complexes to transport cargo in the form of beads, mimicking the case of segregating chromatids. Yet another interesting property of the complex is its ability to preferentially bind to GTP-tubulin subunit. This gives insight into the tip tracking ability or in other words, the selectivity to the plus end of the microtubules which drives the process of segregation(Westermann et al., 2005). Despite the attractive results with the *in*

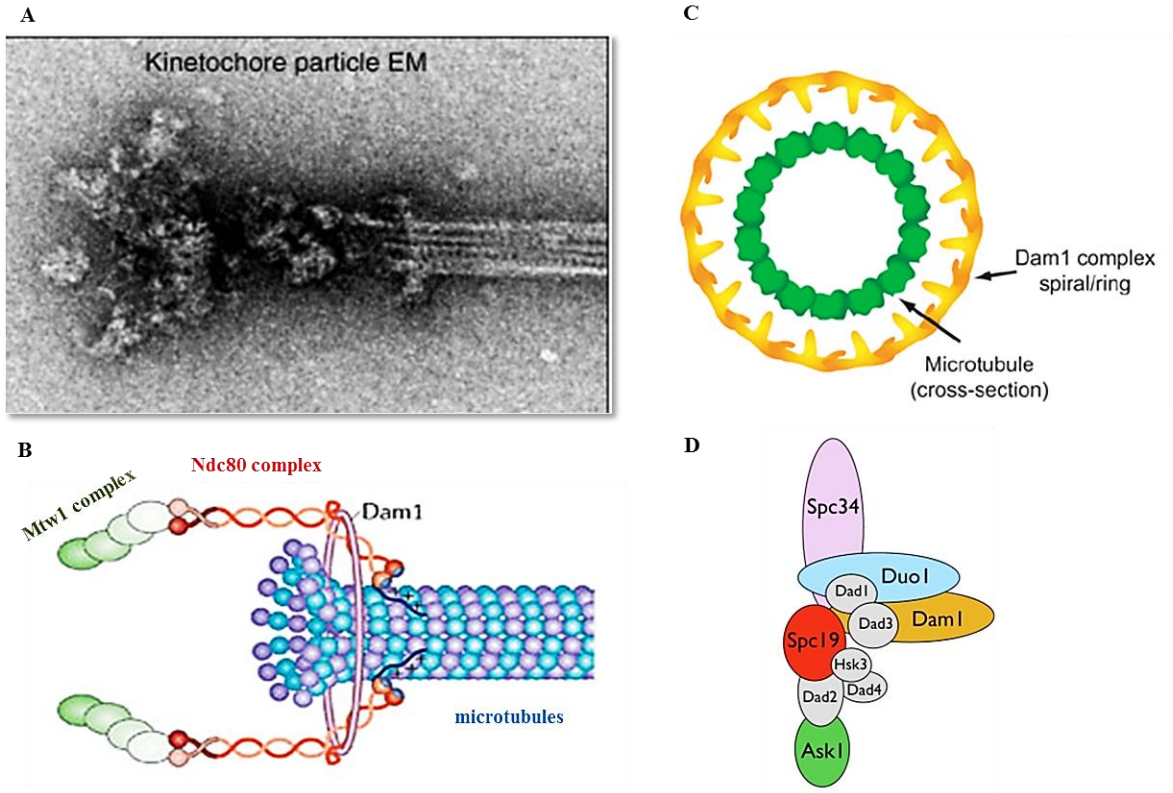


Fig 3: The subunit organization and structure of the scDam1 complex. (A) Electron microscopy (EM) image of a single KT particle showing a ring structure around the MTs. (B) A schematic of the KT-MT interface with protein interactions between the Mtw1, Ndc80 and Dam1 complex. (C) Graphical reconstruction of the cross section view of Dam1 rings around MTs from the EM images. (D) The ring structure requires the assembly of 16 monomers each containing the ten subunits. The subunit organisation in each monomer is schematically represented in D. (Gonen S. *et al* (2012); Ramey, V.H. *et al* (2011))

vitro ring structure, concrete statements cannot be made on the *in vivo* relevance of this structure as no reports indicating the existence of such a structure *in vivo* are available. However, the closest report in this regard estimates the copy number of constituent kinetochore proteins by quantitative fluorescence microscopy. Using the GFP tagged versions of the protein from the native promoter, the copy number of Dam1 complex subunits have been approximated to 16-20 copies during metaphase in case of budding yeasts *S. cerevisiae* and *C. albicans* (Joglekar et al., 2008; Joglekar et al., 2006). It is well known that one KT/MT stoichiometry exists in *S. cerivisiae*. Thus 16-20 molecules of Dam1 subunits per MT can translate into a ring structure *in vivo*. Such a correlation

cannot be made in *C. albicans* due to lack of definite data regarding the MT per KT stoichiometry. In fission yeast *S. pombe*, known to support 2-3 MTs/KT, the number of Dam1 complex subunits ranged from 2-3, making a ring structure improbable (Joglekar et al., 2008). Supporting this report, a non-ring form of the Dam1 complex, called the 'Dam1 speckle' has been shown to exist in *S. pombe* with properties similar to ScDam1 complex (Gao et al., 2010).

The Dam1 complex function is negatively regulated by phosphorylation by the kinase Aurora-B/ Ipl1. The subunits of the Dam1 complex especially the Dam1 subunit itself harbour multiple Ipl1 phosphorylation sites in the C-terminus, that map to the MT proximal protrusion domain (Biggins et al., 1999; Cheeseman et al., 2002). In cases of improper KT-MT interactions, the affinity for MT binding is reduced and this transient detachment occurs by Ipl1 assisted phosphorylation. This gives the system another chance to make a proper KT-MT interaction. Apart from interaction with MTs, the self assembly of the subunits into rings is under phospho-regulation by Ipl1. It has been hypothesised that the Dam1 complex assists the primary load bearing Ndc80 complex in segregation by enhancing its MT binding and tip tracking properties. This cooperative binding between the two complexes is also under Aurora-B phospho-regulation (Lampert et al., 2010; Tien et al., 2010).

In humans, a functional homolog of the Dam1 complex, called the Ska complex has been identified (Guimaraes and Deluca, 2009; Welburn et al., 2009). Although no sequence conservation exists, the Ska complex shows similar properties as the Dam1 complex, such as tracking depolymerizing ends of MTs. It has three subunits viz. Ska1, Ska2 and Ska3 all of which are essential for complex formation. The structure of the complex has been solved. It was found to be a dimer with 4 MT binding sites, assuming a W shaped structure. The Ska needs the members of the KMN network for its localization. Mutants of the subunits of this complex showed defects in MT-KT attachment, chromosome

segregation. Interactions of this complex with the MTs and other KT proteins are under the surveillance of Aurora-B kinase. Given these similarities, it is believed to be the functional counterpart of the Dam1 complex found in yeasts (Jeyaprakash et al., 2012; Varma and Salmon, 2012).

4. Kinetochore assembly and regulation in fungi

With the exception of few species specific complexes, the proteins of various layers of KT remain conserved to a greater extent. However it is interesting to see differences in some aspects like their assembly and regulation. Proteins of different layers of the KT remain clustered and are constitutively present across the cell cycle in case of *S. cerevisiae* and *C. albicans* (Roy et al., 2011; Thakur and Sanyal, 2011). The constitutive localization of the KT at the centromere DNA is independent of tubulin in case of *C. albicans* but in *S. cerevisiae*, a spindle dependent loading of the Dam1 complex has been reported (Li et al., 2002; Thakur and Sanyal, 2011). Quite different from this is the case in *S. pombe* where the outer kinetochore proteins (except Dad1) are loaded only during mitosis stage (Liu et al., 2005). An ordered assembly of the middle and outer layers of KT proteins in the mitosis phase has recently been reported in a basidiomycetous yeast *Cryptococcus neoformans* (Kozubowski et al., 2013).

The point centromere protein Ndc10 dictates the localization and assembly of other kinetochore proteins in budding yeast, thereby acting as a master regulator for KT assembly in this species. In *S. pombe*, the Mis6 protein governs the localization of various other kinetochore proteins along with Spc7 [reviewed in (Roy et al., 2013)]. Interestingly, proteins of different layers of the KT show an interdependency for their localization in case of pathogenic yeast *C. albicans* (Thakur and Sanyal, 2012), unlike the hierarchical nature of dependency observed till date. The necessity of these differences in the regulation especially of the outer kinetochore, from an evolutionary perspective remains unclear. Further studies on this process across different species might provide some

insights in identifying the branch point for this trend.

5. Essentiality of the Dam1 complex

All the subunits of the complex are essential for viability in the budding yeast (Enquist-Newman et al., 2001; Hofmann et al., 1998; Janke et al., 2002; Li et al., 2005). A similar result was seen in the case of pathogenic yeast *C. albicans* (Burrack et al., 2011; Thakur and Sanyal, 2011). Mutant strains showed a multitude of defects ranging from abnormal spindle morphology, growth arrest and defects in nuclear segregation subsequently leading to loss of viability. In case of fission yeast, defects in segregation were seen at an augmented rate in the Dam1 complex mutants, however, the complex was dispensable for viability (Liu et al., 2005). As mentioned earlier, despite the process of cell division being conserved, minor differences do exist across species. In these two species- *S. cerevisiae* and *S. pombe* where the essentiality of the Dam1 complex is different, few differences like the centromere architecture, stoichiometry of MTs/KT and the temporal association of the Dam1 complex with centromeres are well known. Studies on *C. albicans*, an organism in placed in between these two fungi, revealed that the complex is essential for viability (Burrack et al., 2011; Thakur and Sanyal, 2011). It should be noted that *C. albicans* has repeatless small regional centromeres , hence the role of centromere architecture in governing the essentiality was ruled out. The hypothesis that the essentiality is limited to the presence of 1MT per KT stoichiometry was also tested in *C. albicans* by a system where Cse4 levels were overexpressed. In such conditions, it was found that the essentiality of the Dam1 complex was compromised to some extent (Burrack et al., 2011). This gives an interesting lead to test for the essentiality of the Dam1 complex in systems where one can expect variations in these properties.

6. *Candida tropicalis*

C. tropicalis is an asexual budding yeast that one of the etiological agents of candidiasis. It is normally a part of commensal microbiota, acts as an opportunistic pathogen causing

the disease in immunocompromised conditions.

6.1 General features

In nature, *C. tropicalis* can be found as a commensal in the gastrointestinal and the reproductive tract. It is a member of the CTG clade, a subgroup of species that translate the CTG encoding leucine as serine. The organism can exist in three physiological states viz. the yeast form, the hyphal and the pseudohyphal form. Classic yeast forms are ellipsoid in shape and they form round and smooth colonies. Pseudohyphal cells show unipolar budding pattern and forms chains of cells attached to one another as they do not separate from one another after cytokinesis. Hyphal cells are elongated and show tube like morphology. Recently, a parasexual cycle, similar to *C. albicans* was identified in *C. tropicalis* where diploid yeasts of **a** and **α** mating type form tetraploids. Later by concerted chromosome loss they revert back to diploidy completing the parasexual cycle (Seervai et al., 2013). *C. tropicalis* is predicted to have a 30 Mb genome organised into 7 pairs of chromosomes. Repeat associated centromeres, much similar to that of fission yeast *S. pombe* has been identified in this species (Chatterjee et al., unpublished results).

6.2 .Classification

The taxonomic classification scheme for *C. tropicalis* is as follows

Kingdom	Fungi
Phylum	Ascomycota
Subphylum	Saccharomycotina
Class	Saccharomycetes
Order	Saccharomycetales
Family:	Saccharomycetaceae
Genus	*Candida*
Species	*Candida tropicalis*

6.3 Clinical relevance

Reports indicating the rise of *non-Candida albicans* species resulting in candidiasis are increasing in the last decade. Three major species in this category include *C. tropicalis*, *C. glabrata* and *C. parapsilosis* (Pfaller et al., 2010; Silva et al., 2012). In tropical countries like India, *C. tropicalis* is found to hold a major share in causing systemic and nosocomial candidiasis (up to 70%) (Kothavade et al., 2010). Results from the ARTEMIS antifungal surveillance study has indicated that *C. tropicalis* is the second most causative agent isolated from infected specimens in Asia-Pacific. The study also shows a rise in number of azole- resistance strains owing to unprecedented use of antifungals for prophylaxis (Pfaller et al., 2010). In addition to immunocompromised cases, the incidence of *C. tropicalis* infections is also higher in patients with neutropenia and other haematological malignancies. It is also interesting that *C. tropicalis* has been shown to be more invasive than *C. albicans* (Wingard et al., 1980).

7. Basis of this study

The role of KTs in the process of chromosome segregation and in turn the survival of a cell is well established. The outer KT Dam1 complex is an integral part of this machinery that is found exclusively in the fungal kingdom. Playing a critical role in cell division, this complex presents itself as an attractive target for development of antifungals against pathogenic fungi. Understanding the essentiality of this complex in pathogenic fungi like *C. tropicalis* would open up new targets for therapeutic intervention. In addition, the species has an advantage with its place in the phylogenetic tree. With centromere architecture close to *S. pombe* unlike the other *Candida* members, and other properties like the dynamics of KT assembly, the stoichiometry of MT/KT unexplored, this species makes an ideal organism to study the course of evolution of this complex.

8. Specific objectives

With the above premise, the specific objectives of this study are laid down below

- Identification of the homologs of Dam1 complex in *C. tropicalis*, and their characterization (of four representative subunits Dad1, Dad2, Dam1 and Ask1).
- Identification and characterization of *MET3* promoter in *C. tropicalis.*
- Studying the essentiality of the Dam1 complex using the *MET3* promoter.

Results

1. The outer kinetochore complex in *C. tropicalis.*

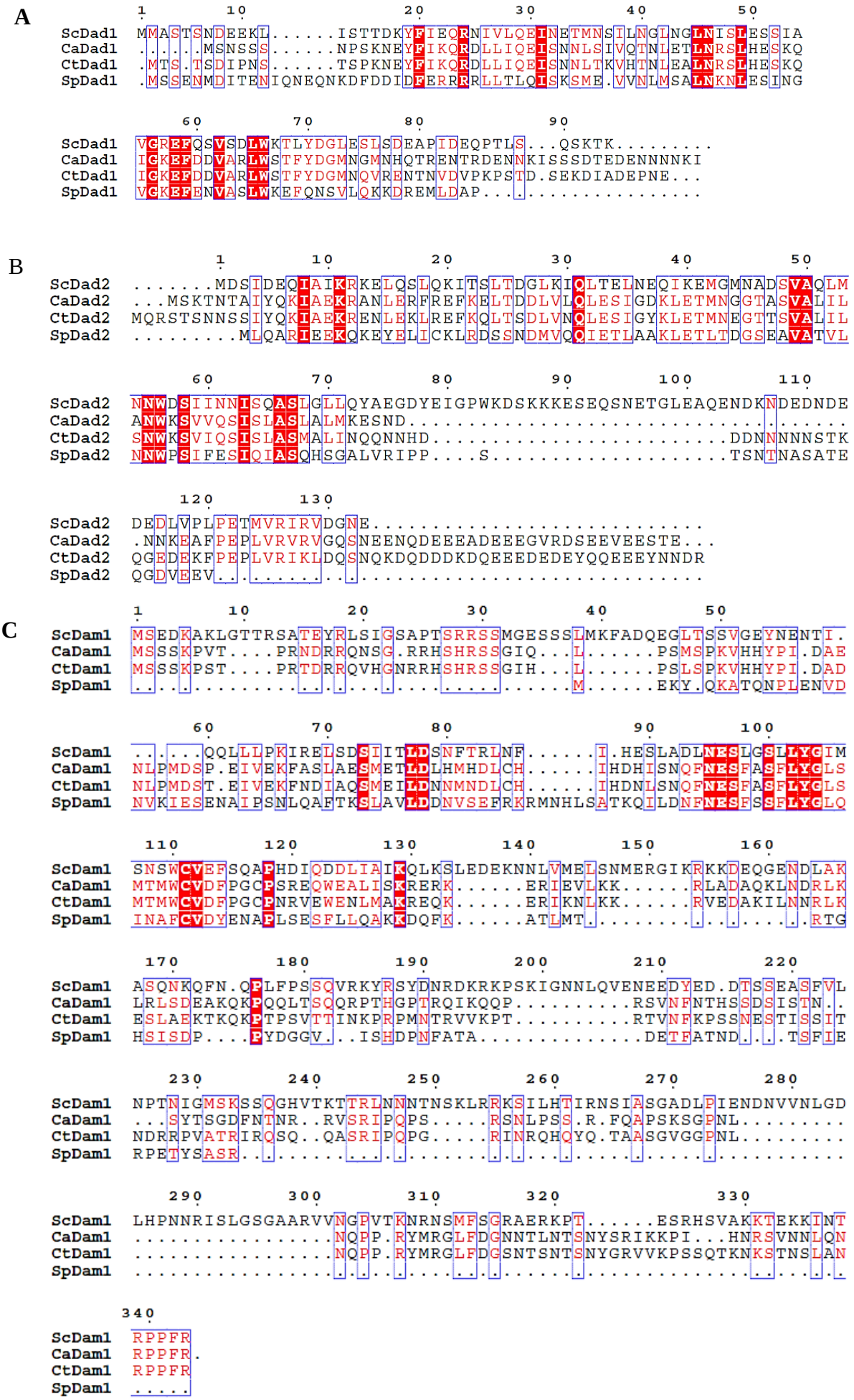
1.1 Identification of the Dam1/DASH complex subunits in *C. tropicalis.*

Using the amino acid sequences of the subunits of the Dam1 complex of the closely related *C. albicans* species as the query, the homologs of all except Dad4 was obtained by BLAST analysis. Despite the genetic relatedness among the two species, the degree of homology was found to be less, evident from the sequence alignment of proteins from yeasts *S. cerevisiae, C. albicans* and *S. pombe* along with *C. tropicalis* (Fig 4). This could be a result of the evolution of these proteins along with the centromere sequences (discussed later). Table 1 summarizes the biochemical properties of the proteins of this complex along and the extent of conservation with *C. albicans*.

Table 1: Dam1 complex subunits in *C. tropicalis.*

Protein	ORF # (CTRG_#)	Length (No. of amino acids)		Homology with *C. albicans* protein (%)	Mol. weight (kDa)	Predicted pI of the protein
		C. tropicalis	*C. albicans*			
Dad1	03625	99	99	71	11.83	4.68
Dad2	01487	143	125	61	17.16	4.34
Dad3	00628	150	138	70	18.00	4.17
Dam1	03385	287	277	57	34.44	10.35
Duo1	05744	163	171	53	19.55	4.76
Ask1	00035	456	594	41	54.72	5.59
Hsk3	01265	98	106	87	11.76	5.58
Spc19	04775.3	173	175	49	20.76	9.89
Spc34	05730	268	247	46	32.16	4.70

http://www.broadinstitute.org/annotation/genome/candida_group/Blast.html


A
ScDad1
CaDad1
CtDad1
SpDad1
B
ScDad2
CaDad2
CtDad2
SpDad2
C
ScDam1
CaDam1
CtDam1
SpDam1

D

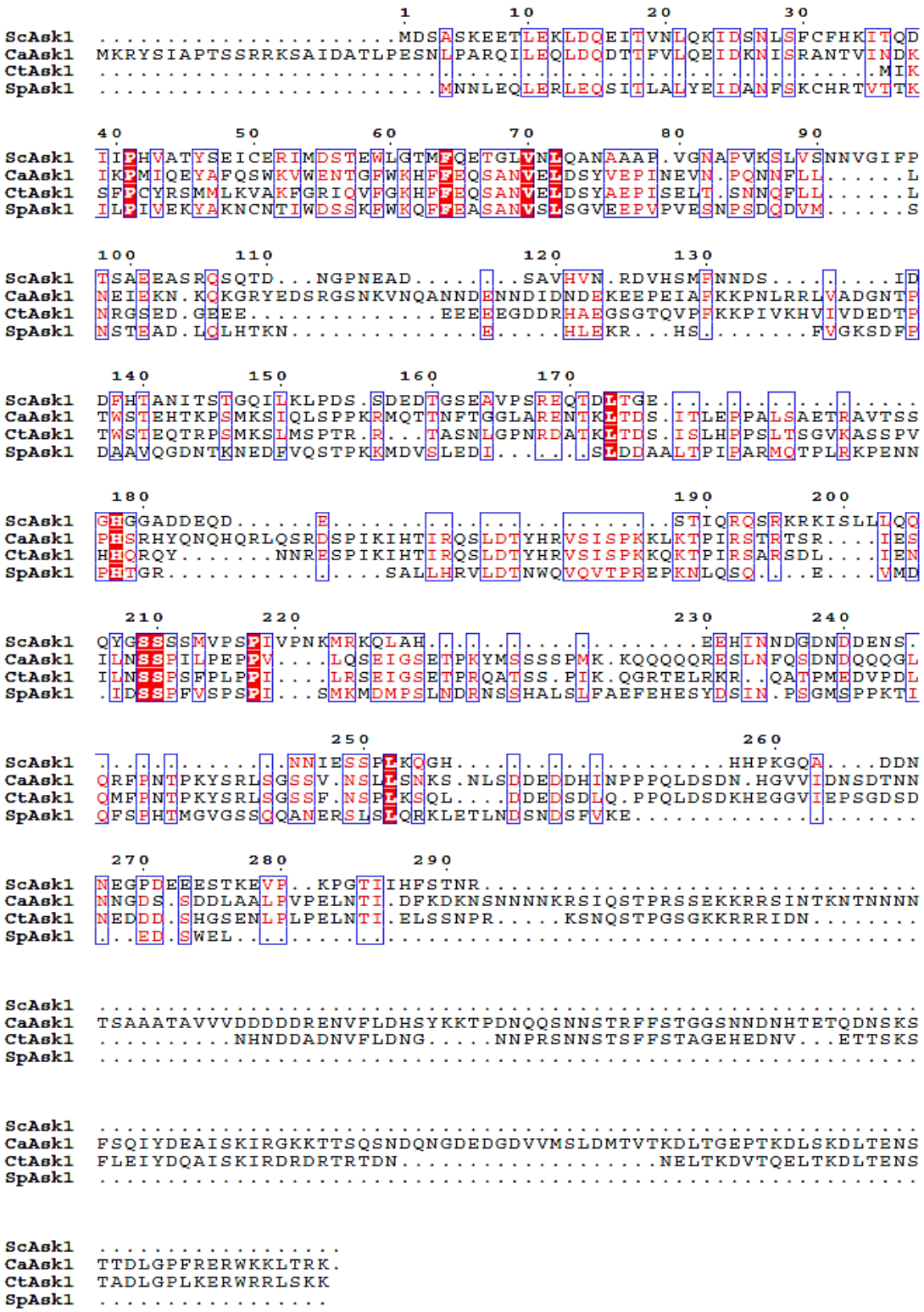


Fig 4: Alignment of sequences of (A) Dad1, (B) Dad2, (C) Dam1 and (D) Ask1 subunits of the Dam1 complex from four yeasts: *S. cerevisiae, C. albicans, C. tropicalis and S. pombe.*

1.2 Epitope tagging of 4 subunits of the Dam1 complex.

To characterize the subunits of the outer kinetochore complex in this species, four from the above identified nine subunits namely Dad1, Dad2, Dam1 and Ask1 were taken ahead for further experiments. Each of these four proteins was tagged at the C-terminus with a Tandem Affinity Purification (TAP) tag.

The TAP tag contains a Protein A tag and a Calmodulin Binding Protein (CBP) sequence that are separated by a Tobacco Etch Virus (TEV) protease cleavage site. Both the tags can be used in tandem to pull down native protein complexes by using affinity matrices from biological systems and hence the name TAP tag. A schematic representation of TAP tag and the cassette constructed for the TAP tagging proteins are shown in Fig 5. The TAP tagging cassette for these four proteins was used to transform the strain CTKS107. The transformants were selected for uracil prototrophy, subsequently

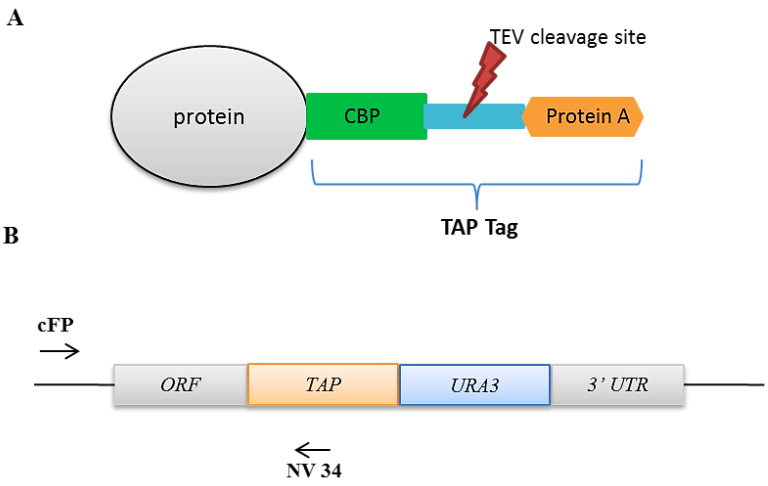


Fig 5: Schematic representation of the Tandem Affinity Purification (TAP) tag and the cassette constructed for tagging the protein of interest. (A) Two epitopes- Protein A and Calmodulin Binding Peptide (CBP) separated by a Tobacco Etch Virus (TEV) protease site constitute the TAP tag used in this study. (B) Cassette for homologous recombination was constructed using the ORF and the 3'UTR sequences. Arrows indicate the locations of confirmatory Forward Primer (cFP, placed outside the cassette) and reverse primer (within the cassette) used for PCR confirmation of transformants.

confirmed by PCR (see Materials and Methods).The expression of these fusion proteins were checked by western blotting using the total cell lysates from strains CTSR100 (*DAD1/DAD1-TAP*), CTSR200 (*DAD2/DAD2-TAP*), CTSR300 (*DAM1/DAM1-TAP*) and CTSR400 (*ASK1/ASK1-TAP*). The following results were obtained upon probing these lysates with anti-Protein A antibody. Lysate from CTSR100 showed a single band of size 33.5 kDa, the expected size of the Dad1-TAP fusion protein (Fig 6A). Similarly, bands at the expected sizes of 38 kDa and 52.6 kDa were obtained from the CTSR200 and CTSR300 lysates corresponding to Dad2-TAP and Dam1-TAP fusion proteins indicating that the proteins are indeed expressed (Fig 6B and 6C). The Ask1-TAP fusion protein was picked up as a single band of ~100 kDa size (Fig 6D). However, the expected size of this protein is 71.7 kDa. One reason for this anomalous behaviour could be the intrinsic negative charge/ low pI of this protein.

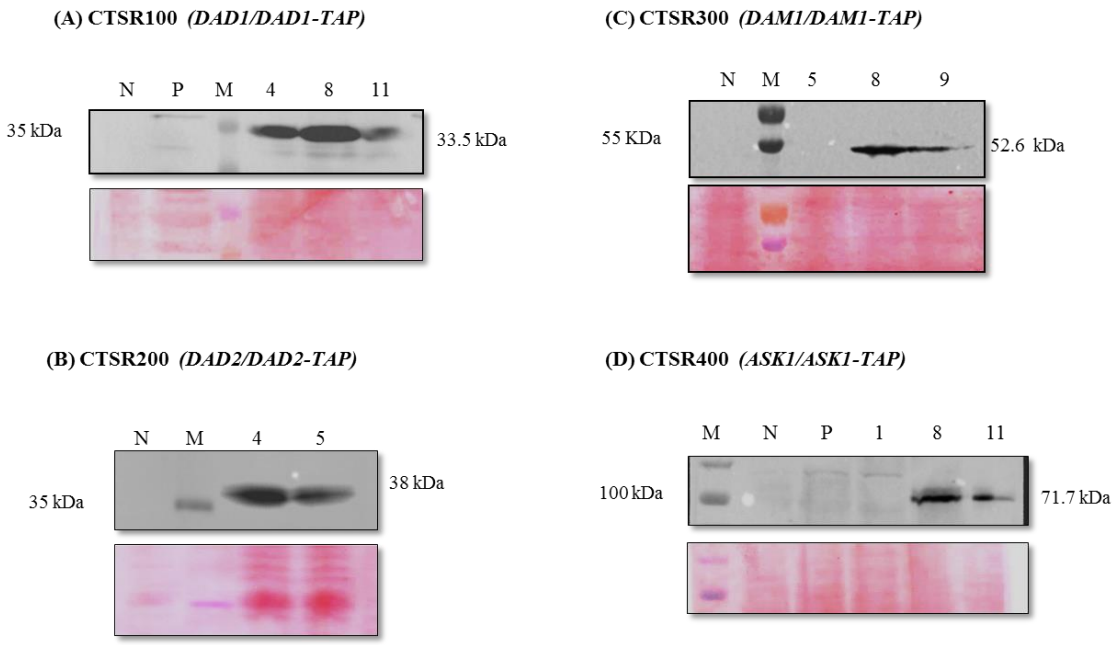
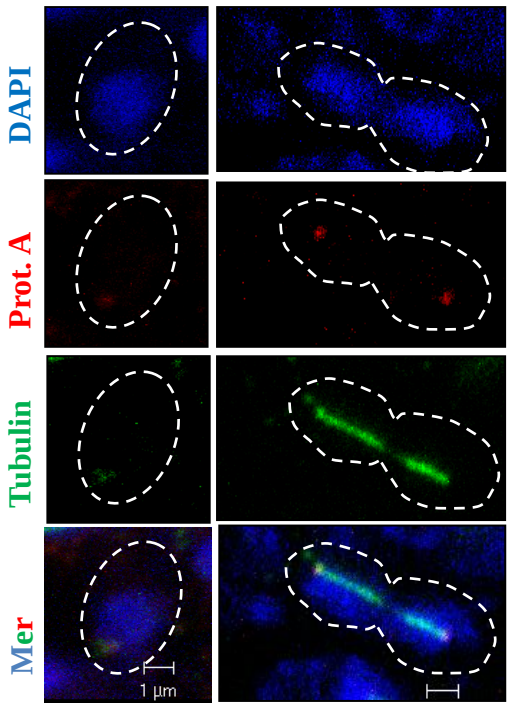


Fig 6: Confirmation of TAP tagged proteins by western blot. Total cell lysates from the strains CTSR100, CTSR200, CTSR300 and CTSR400 were prepared by TCA method and probed with anti-Protein A antibody. (a) The fusion protein Dad1-TAP was detected at the expected size of 33.5 kDa. (b) The Dad2-TAP protein with an expected size of 38 kDa was picked up as a single band at this size. (c) A 52.6 kDa band corresponding to Dam1-TAP fusion protein was detected from CTSR300 lysate. (d) Ask1-TAP was detected as a single band from CTSR400 lysates with a size close to 100 kDa (higher than the expected 71.7 kDa for the fusion protein). Lanes N- negative control, P- positive control, M- molecular weight marker, 1- 11: transformant number.

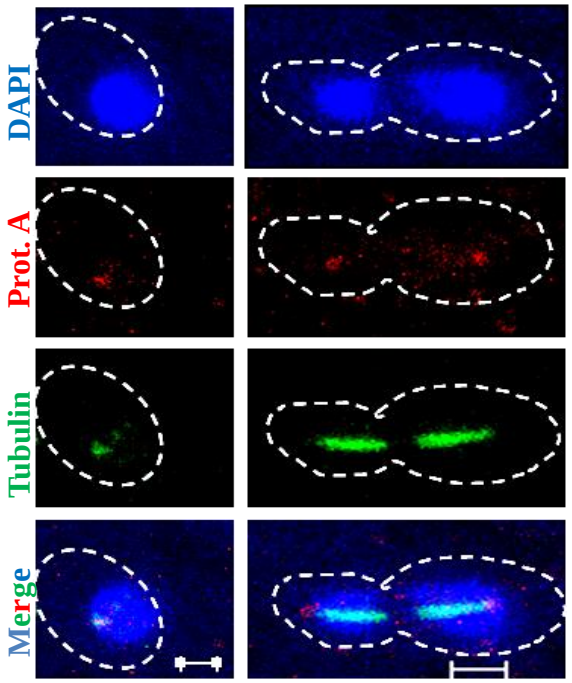
1.3 The tagged outer KT proteins show localization similar to the *C. albicans* homologs.

The localization of outer kinetochore proteins are well known in the budding yeast and the closely related pathogenic yeast *C. albicans* (Thakur and Sanyal, 2011). Subcellular localization of these four proteins from *C. tropicalis* was studied by indirect immunofluorescence using the strains CTSR100, CTSR200, CTSR300 and CTSR400. Cells at log phase were harvested, fixed and stained with anti-Protein A and anti-tubulin antibodies along with a nuclear stain DAPI. When observed under fluorescence microscope, distinct dot like signals close to the SPBs and located at the periphery of the nuclear mass was evident in unbudded cells. In the case of large budded cells, two distinct dots, one at each bud connected by the mitotic spindle were evident along with the segregated nuclear mass (Fig 7). Such a localization pattern suggests that these proteins are clustered and they localize to the KT throughout the cell cycle as observed in the case of *C. albicans*.

(A) CTSR100- *(DAD1/DAD1-TAP)*



(B) CTSR200- *(DAD2/DAD2-TAP)*



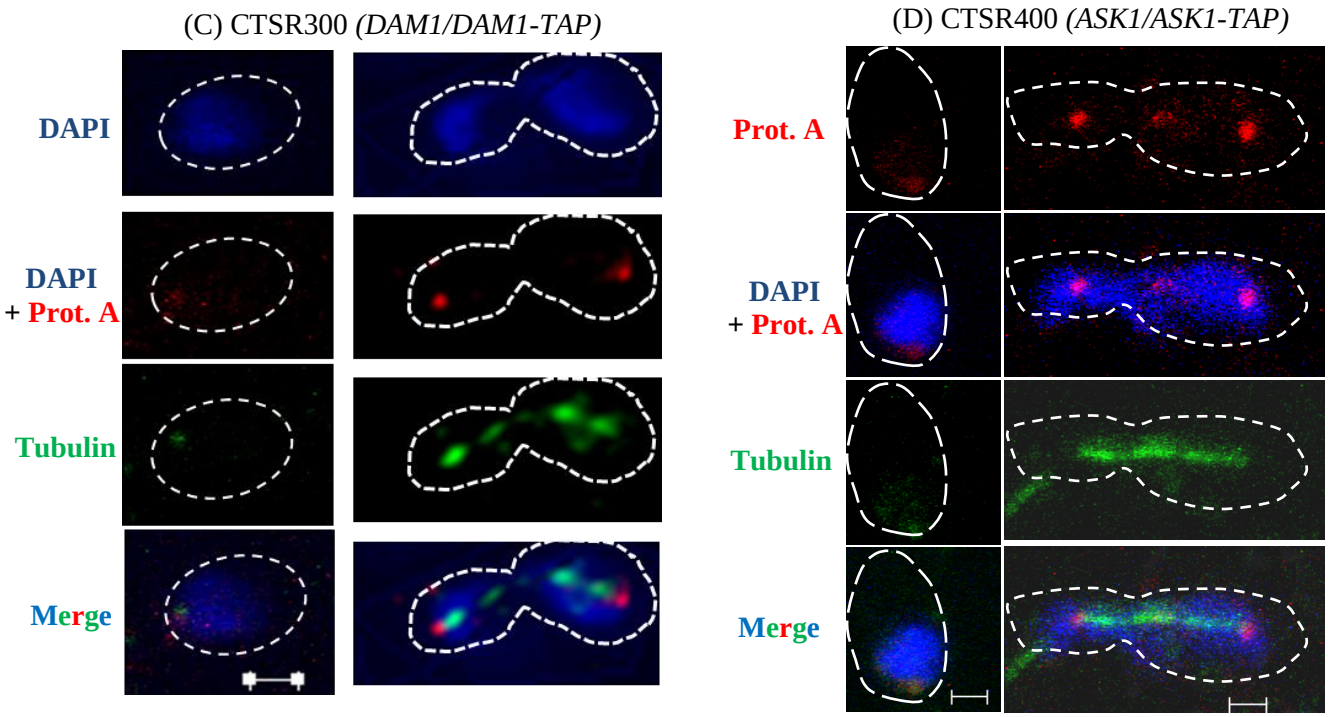


Fig 7: Subunits of the Dam1 complex localize to the centromeres in *C. tropicalis*. CTSR100, CTSR200, CTSR300, CTSR400 strains were fixed and stained with DAPI, anti Protein-A and anti-tubulin antibody. Distinct dot like signals were detected in both unbudded and large budded cells for (A) Dad1-TAP, (B) Dad2-TAP, (C) Dam1-TAP and (D) Ask1-TAP. This indicated that the Dam1 complex is constitutively localized to the KT in this species. Bar, 1μm.

2. Characterization of the *MET3* promoter in *C.tropicalis*.

2.1 Background

The *MET3* promoter is one of the well characterized promoters in yeasts that is commonly used to shut off the transcription on gene under it upon addition of methionine and cysteine. The *MET3* gene codes for an enzyme ATP sulphurylase, acts in the first step of the inorganic sulphur assimilation pathway for biosynthesis of sulphur containing amino acids. The transcription of enzymes in this pathway is controlled by a regulator Met4. This protein has three domains viz. the activation domain, the inhibitory domain and the auxiliary domain. Methionine and cysteine upon assimilation by the cell leads to formation of S-adenosyl methionine (SAM). This binds to the inhibitory domain of the transcription factor to negatively regulate the transcriptions (Marzluf, 1997).

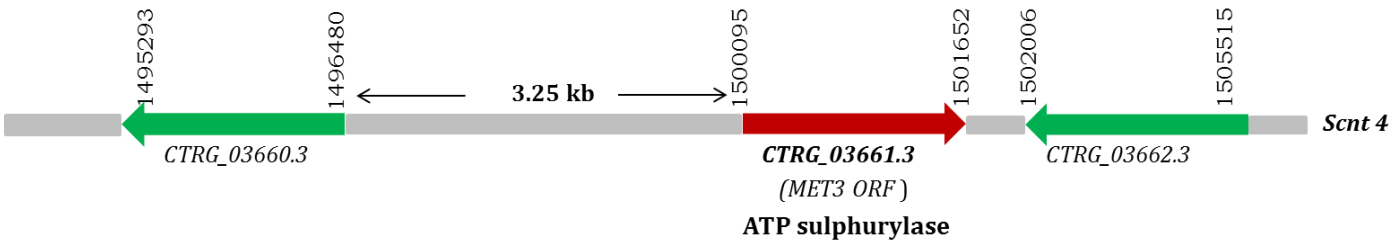
2.2 Constructs to identify the promoter length for maximum efficiency

The homolog of this protein was found in *C. tropicalis* using the sequence of the *C. albicans MET3* gene as the query for BLAST search. The DNA locus CTRG_03661.3 was shown as the putative homolog for the *MET3* gene. The sequence alignment of the two ORFs is shown in Fig 8A. The intergeneic region between the *MET3* ORF and the previous ORF spans 3.25 kb (Fig 8B). For optimising the length of the region showing the promoter activity, two plasmid constructs were made, (a) one with sequences of the entire intergeneic region ($MET3pr_{long}$) and (b) one with sequences of 1.4 kb upstream from the ATG of the *MET3* ORF ($MET3pr_{short}$). The *DAD1* ORF was cloned under these two promoters to measure the promoter activity. A schematic representation of different steps involved in making a conditionally expressing strain is shown in Fig 8C.

A


CaMET3
CtMET3

B


1495293
1496480
3.25 kb
1500095
1501652
1502006
1505515
Scnt 4
CTRG_03660.3
CTRG_03661.3
(MET3 ORF)
ATP sulphurylase
CTRG_03662.3

C

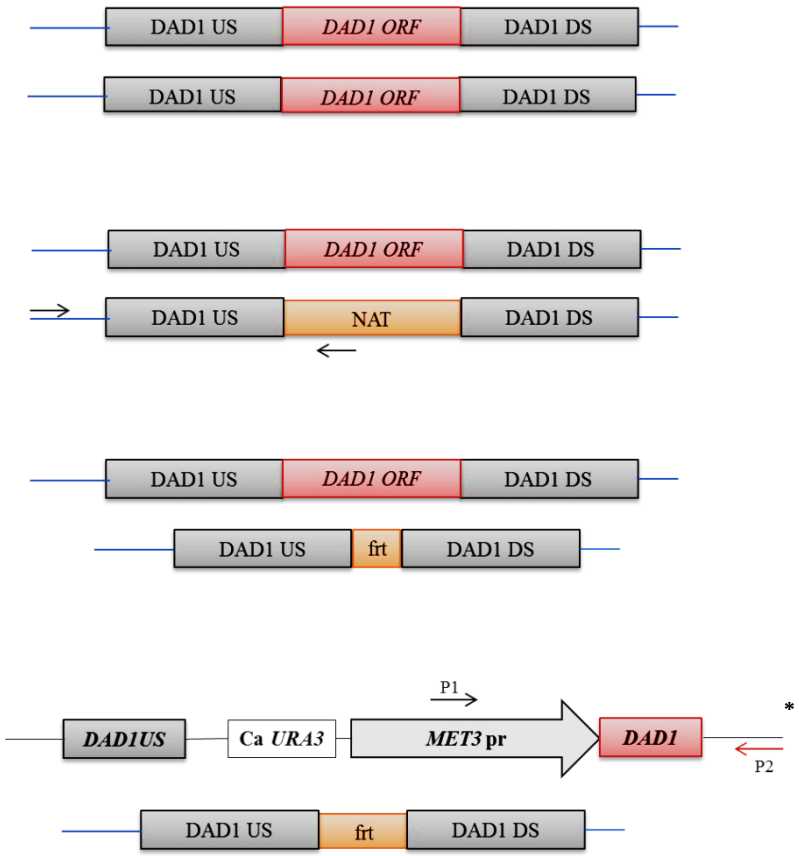


Fig 8: *MET3 in C. tropicalis*. (A) Sequence alignment of *MET3* sequences from *C. albicans* and *C. tropicalis* show 88% conservation between therm. (B) A schematic representation of the *MET3* locus in the genome, showing the flanking *ORF*s and the intergenic regions. (C) The stepwise process in generating a conditional mutant of Dad1 is illustrated: in the first step, one allele is deleted to generate a NAT^R heterozygous strain. The marker is then recycled after which the other copy is placed under the *MET3* promoter. Asterisk (*): schematic of the promoter replacement cassette. Two constructs with varying lengths of *MET3* promoter were made ($MET3_{long}$ and $MET3_{short}$ promoter respectively).

2.3 The full length *MET3* promoter is required for complete repression of Dad1

In order to test the promoter efficiency in these sequences, the only copy of the *DAD1* ORF should be placed under these promoters. Since *C.tropicalis* is an obligate diploid species, one of the two alleles of the gene has to be deleted. In this regard, a deletion cassette was constructed such that sequences (~500 bp) upstream and downstream of the *DAD1* ORF were cloned using the sites flanking the Ca*SAT* marker present in the plasmid pSFS2a. This cassette was used to transform the strain CTKS107. The transformants were selected for Nourseothricin (NAT) resistance.

The two *MET3prDAD1*constructs were then used to transform CTSR103 strain (*dad1/DAD1; CSE4/CSE4-GFP*) to place *DAD1* under the *MET3* promoter. The strains with *DAD1* under 1.4 kb *MET3promoter* are named CTSR104 while those where *DAD1* is under full length MET3 promoter are named CTSR105. The transformants were confirmed by PCR using the primers VR49 and SR28.

Confirmed transformants were then streaked for single colonies on plates containing different concentrations of met and cys (0, 1, 2.5 and 5 mM each) to check for the promoter efficiency. From the plate pictures shown below (Fig 9), it is evident that there is no drastic inhibition in growth of the transformants in plates up to 5 mM methionine and cysteine in the strain with 1.4 kb *MET3* promoter. On the contrary, the transformants in which *DAD1* is under full length *MET3* promoter showed inhibited growth in concentrations as low as 1mM met and cys. From this observation, it can be stated that the full length intergeneic region is required for optimal promoter function, to achieve desired levels of repression

.

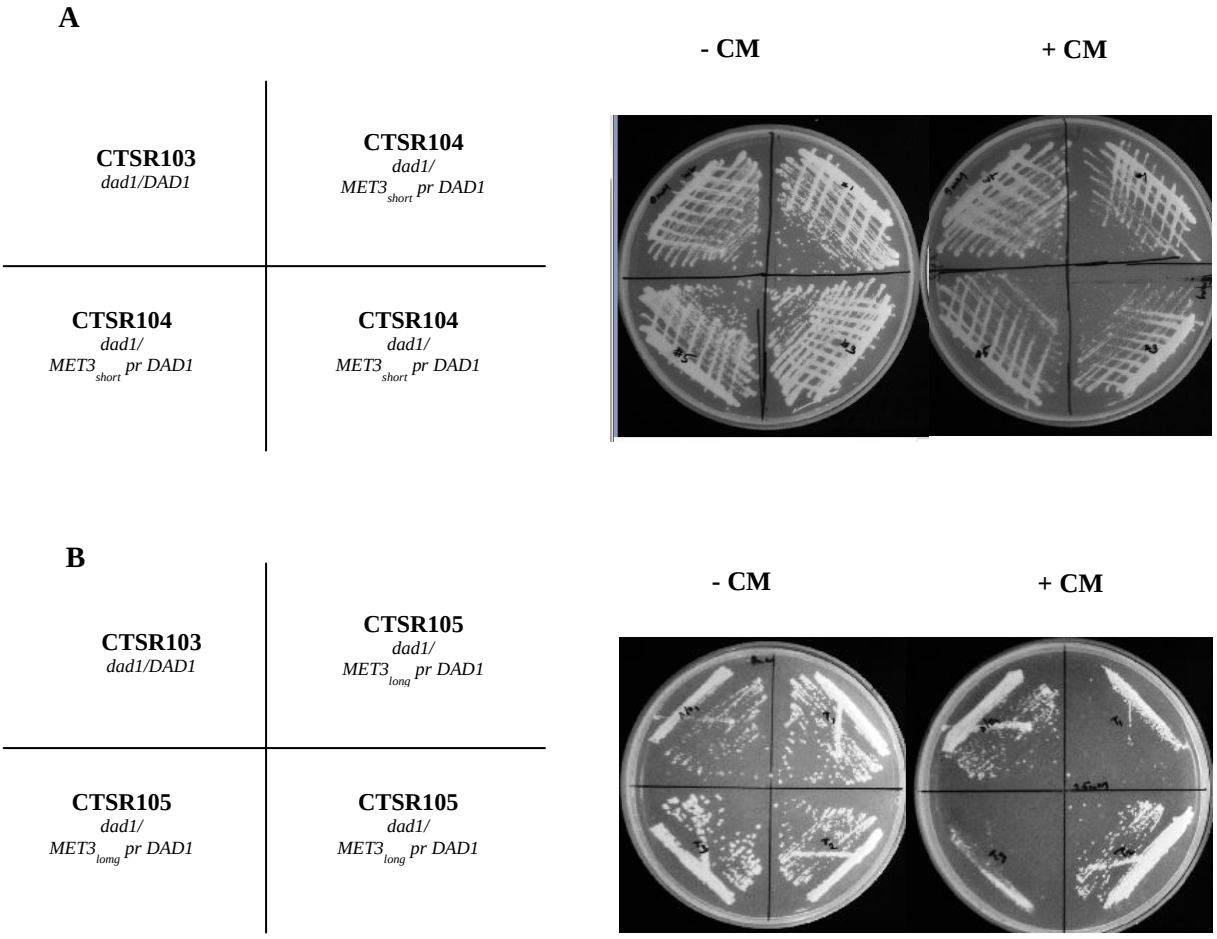


Fig 9: Analysis of promoter efficiency. (A) The efficiency of the short promoter were checked by streaking CTSR104 strain along with the parent CTSR103 in permissive (-CM: no met and cys) and repressive (+CM: 1mM met and cys) media. (B)The efficiency of the full length promoter were checked by streaking CTSR105 strain along with the parent CTSR103 in permissive (-CM) and repressive (+CM) media. The pattern of streaking is shown schematically in the left side. Plates were photographed after 48 h incubation. On comparing the growth of CTSR104 and CTSR105 strains in repressive conditions, it was inferred that the full length intergeneic sequence is required for optimum promoter activity.

3. Effect of Dad1 depletion on growth of *C. tropicalis.*

The following experiments were performed to study the effect of outer kinetochore protein (Dad1) depletion on the cell cycle of *C.tropicalis*

3.1 Dad1 is essential for growth and viability of *C.tropicalis*

CTSR105 strains, with *DAD1* under the full length *MET3* promoter along with the parent strain CTSR103 were streaked on permissive (-CM: CM-met-cys) and repressive plates (+CM: CM+1mM met and cys). The plates were observed for growth of single colonies after 48 h incubation. While that parent strain (*dad1/DAD1*) grew on both permissive and repressive media to form single colonies, the CTSR105 transformants did not show growth beyond the main streak (Fig 10). The inability of these transformants to grow in repressive conditions indicated that Dad1 is an essential protein, indispensable for growth and viability of *C.tropicalis.*

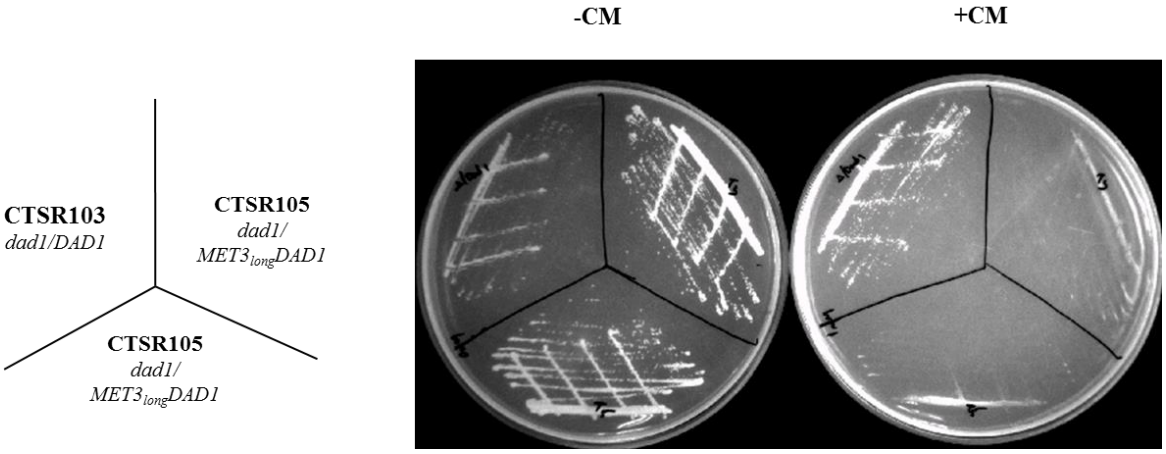


Fig 10: Dad1 is essential for viability of *C. tropicalis.* CTSR105 strains along with the parent CTSR103 strain were streaked on permissive (-CM) and repressive media (+CM) and imaged after 48 h incubation. The streaking pattern is depicted in the left side. The inability of the CTSR105 strains to grow upon depletion of Dad1 in repressive media indicates that Dad1 is indispensable for viability of this species.

3.2 Phenotypic changes associated with Dad1 depletion.

Candida species are known to propagate in different forms viz. the yeast form, the hyphal and the pseudohyphal form. Deletion of one of the two alleles of *DAD1* did not show any visible phenotype or growth differences. Upon depleting the protein under non permissive conditions, CTSR105 strains showed gradual accumulation of large budded cells (indicating a growth arrest) which later progressed into elongated budded structures (Fig 11). The figure shows increase in the proportion of large budded cells up to 6 hours and accumulation of cells with elongated bud in the subsequent time points.

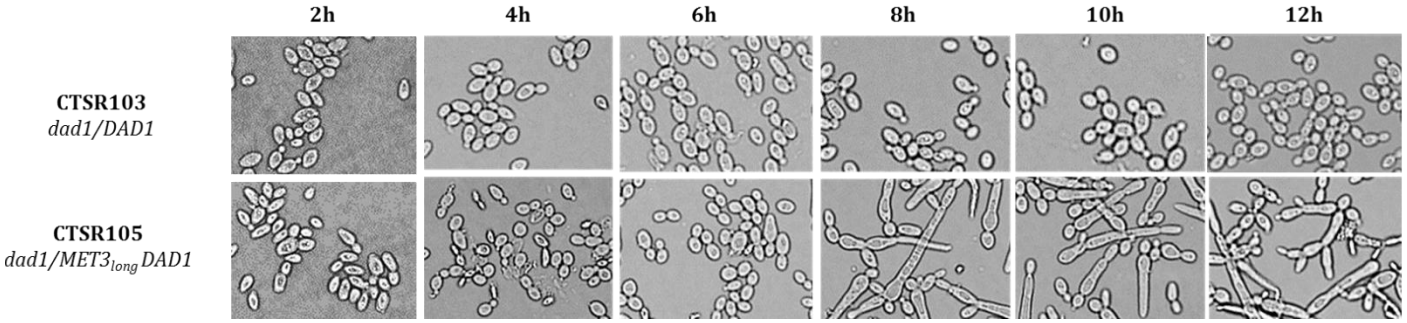


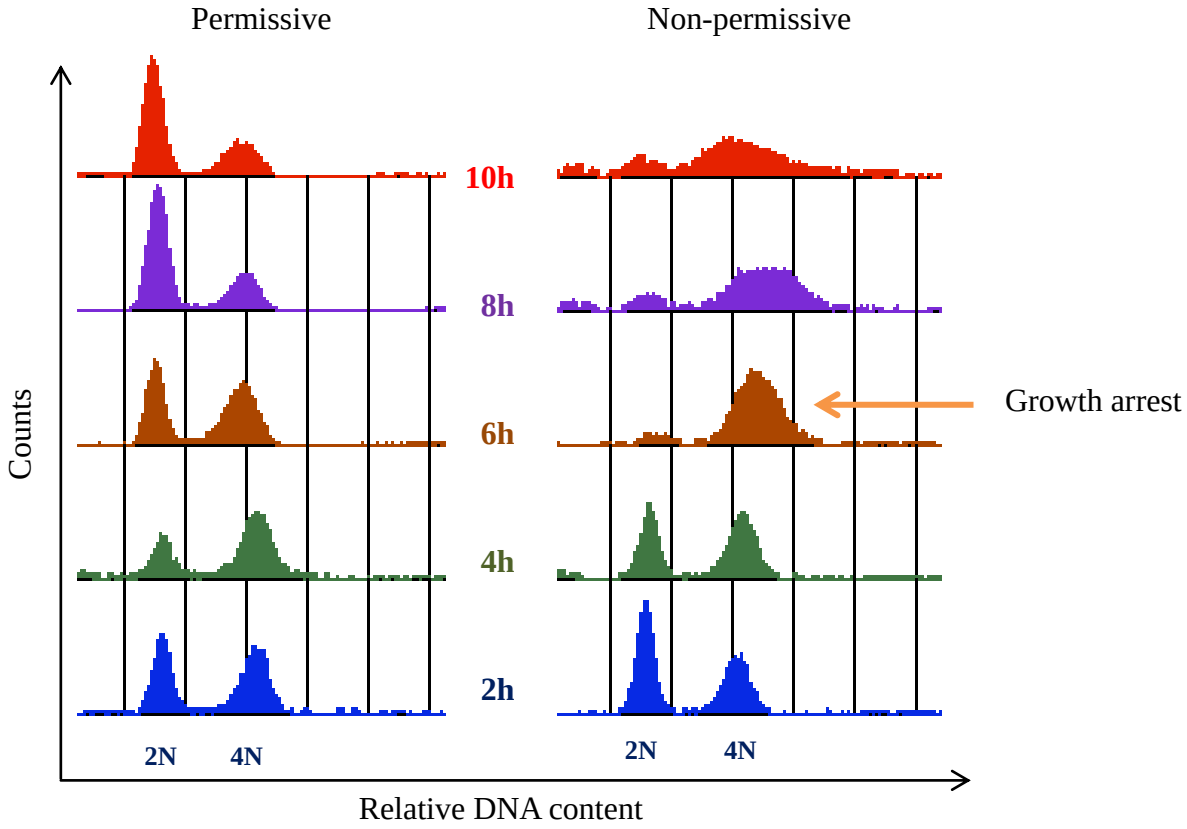
Fig 11: Dad1 depletion causes accumulation of large budded and cells with elongated buds. CTSR103 and CTSR105 strain was grown in non -permissive condition (+CM) to observe for any visible phenotype defects. Accumulation of large budded cells was seen at 6 h time point that progressed into elongated budded cells at later time points. The control strain, with a single intact allele of *DAD1* showed no such defect up to 12 h growth in the same media.

3.3 Role of Dad1 in cell cycle progression in *C. tropicalis*

To study the cell cycle progression upon depleting Dad1, the following experiment was performed. CTSR103 and CTSR105 strains were grown in permissive media (-CM) and incubated for 12-14 h. From this preinoculum, cells were seeded to 0.1 OD_{600} into permissive (-CM) and repressive media (+CM). Cells were harvested at every 2 h intervals up to ten hours, processed and analysed by flow cytometry. The CTSR104 (*dad1/MET3pr*$_{short}$ *DAD1*) strain was also included in this study to check the difference in the efficiency between these two constructs. Accumulation of cells in the G2/M stage can be seen after 6 h of growth in repressive conditions while the population of cells in permissive media showed no such arrest up to 12 h growth period. The results of this experiment are shown in Fig 12.

From the histogram, gradual accumulation of large budded cells (rise in the G2/M peak) up to 6 h can be seen when the Dad1 protein was depleted using non permissive media. At this time point, there is almost a complete suppression of G1 peak indicating that the major population of cells are in the large budded stage. No such growth arrest was evident when the same cells were grown in permissive media (Fig 12, left panel). This showed that Dad1 is required for proper progression and completion of cell cycle in *C. tropicalis*. The broad and stunted peaks seen at the later time points upon repression (10 h time point) could be due to the phenotype changes (accumulation of cells with elongated buds) associated with Dad1 depletion as evident from light microscopy images of cells at these time points.

In addition, when the CTSR104 cells, with 1.4 kb *MET3* promoter were grown in non-permissive conditions, no G2/M arrest was observed. This further validated our conclusion that the 1.4 kb promoter is insufficient to exert complete repression (Fig 13).



CTSR105 *(dad1/MET3 pr$_{long}$ DAD1)*

Fig 12: Dad1is required for proper cell cycle progression in *C. tropicalis*. Flow cytometry analysis was performed on fixed CTSR105 cells stained with PI after growth in non-permissive conditions to study cell cycle progression. Accumulation of cells with 4N DNA content indicated a large bud (G2/M) arrest upon repression for 6 h, while no such effect was seen in permissive conditions. This showed that Dad1 is required for proper progression of cell cycle in this species.

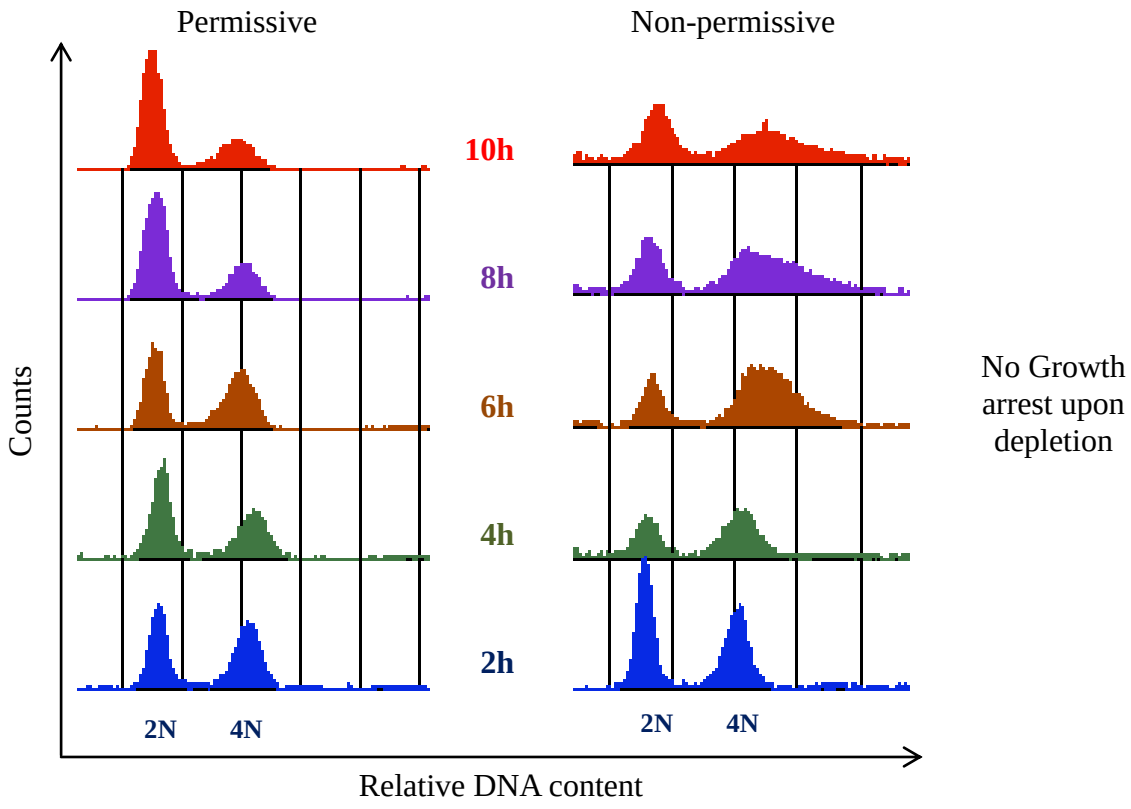
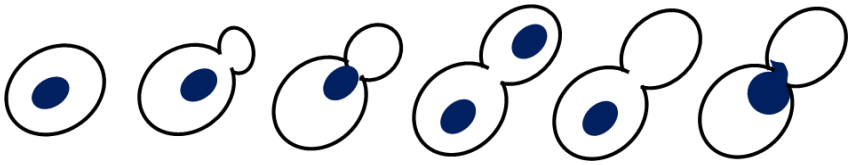


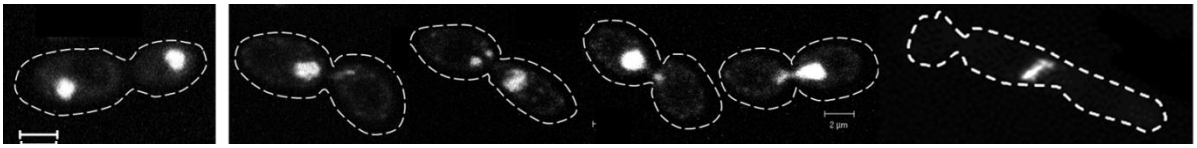
Fig 13: The 1.4 kb *MET3* promoter is insufficient for optimal promoter activity. Flow cytometry analysis was performed on fixed CTSR104 cells stained with PI after growth in non permissive conditions to test the efficiency of this promoter. A slight increase in proportion of cells with large buds were evident, however Dad1 depletion using this promoter did not result in a complete suppression of G1 peak as observed with the full length promoter. This indicated that the 1.4 kb promoter is insufficient to exert the levels of repression required to deplete a KT protein.

3.4 Effect of Dad1 depletion on chromosome segregation

Depletion of a KT protein is known to cause defects in segregation. To study this process in the case of Dad1 depletion, CTSR105 cells, grown in permissive media for 12 h were seeded into permissive and repressive media and incubated for 6 h. The cells were then fixed and stained with DAPI. By fluorescence microscopy, it was observed that large budded cells accumulated upon repression and they had abnormal nuclear morphology. Most of them had an undivided single nuclear mass stuck at the bud neck (90% of the large buds, 68% of the total), while other patterns like unsegregated nuclei at one of the buds, unequally segregated nuclei were found at lower frequency. Only a meagre population (less than 10%) of cells showed proper segregated nuclear mass upon repression (Fig 14). Cells with elongated buds with the nuclear mass in the bud, started to appear at this stage (7% of the total).However, the control population (CTSR105 cells grown in permissive media)showed proper distribution of unbudded, small and large budded cells typical of an asynchronous culture with no defect in nuclear segregation. Such a profound rise in segregation defects could be the reason for the drop in viability upon depletion.

Table 2: Effect of Dad1 depletion on nuclear segregation

Culture conditions	Percentage of cells with indicated segregation pattern					
						
Permissive (N=154)	**35**	**65**	**10**	**12**	**-**	**<1**
Non-permissive (N=142)*	**10**	**7**	**-**	**6**	**2**	**68**



Representative DAPI images of the observed nuclear morphologies.
Asterisk (*), 7% of the population were elongated budded cells as shown in the last DAPI image. Bar, 2µm

Discussion

Discussion

1. The Dam1 complex is conserved in *C. tropicalis*.

All the subunits of the Dam1 complex, except Dad4 were identified by BLAST analysis using the sequences of *C. albicans* homologs as the query sequence. Only a modest level of conservation of proteins between the two closely relate species *C. albicans* and *C. tropicalis* (see Table 1) was observed. The same can be observed from the sequence alignment of proteins of the Dam1 complex from four species (*S. cerevisiae, C. albicans, C. tropicalis, and S. pombe*) across the fungal kingdom (Fig 4). The KT proteins are amongst the most rapidly evolving proteins in the genome , driven by the rapidly evolving nature of centromeric sequences. Evolution of centromeric sequences in various organisms is well documented. Recently, such an observation was made in the centromeres of two closely related species *C. albicans* and *Candida dubliniensis* (Padmanabhan et al., 2008). *C. tropicalis* was recently found to have repeat associated centromeres similar to *S. pombe* and not like *C. albicans* centromeres (Chatterjee et al., unpublished data). The low level of conservation between proteins in these two closely related species could be attributed as an adaptation to their divergent centromeres. In addition, there are instances where there is insertion/deletion of a stretch of amino acids from the protein. This is well seen in the case of the Dam1 protein-SpDam1 protein does not have a stretch of amino acids in its C-term that is known to harbour sites for MT binding (Fig 4C). Deletion of this domain from ScDam1 has shown to reduce its MT binding capacity (Grishchuk et al., 2008). However, it should be noted that various biochemical properties like the pI, sequences with tendency to form coiled coil regions, distribution of charged residues are conserved. In case of Dad4, it could be possible that the protein has diverged to an extent where one cannot identify its homolog solely based on the amino acid sequence. Rather, conservation could be at the level of the structure of the protein. Besides these bioinformatic predictions, affinity purification of the entire outer KT complex

using any of the TAP tagged proteins generated in this study will shed light on the actual composition of this complex *in vivo*.

Remarkable differences in the behaviour of KT proteins across the cell cycle have been observed. In budding yeast, the proteins of all the three KT layers remain associated with the centromeres and the spindle MTs throughout the cell cycle (Cheeseman et al., 2001a; Enquist-Newman et al., 2001; Janke et al., 2002). Further, the loading of the Dam1 complex is also dependent on the spindles (Li et al., 2002). A similar trend has been shown in the case of pathogenic yeast *C. albicans*. The KTs of this species are localized to centromeres throughout the cell cycle. Studies using nocodozole treated cells showed that the localization of the Dam1 complex is not dependent on the spindle unlike the case of *S. cerevisiae* (Thakur and Sanyal, 2011). On the contrary, a role of Dad2 in maintenance of spindle morphology in interphase cells has been shown. The scenario is much different in the case of fission yeast *S. pombe*. While the inner and middle layer KT proteins localize constitutively to the centromeres, the outer KT proteins (except Dad1) are loaded only on the mitosis stage (Liu et al., 2005). This scheme of assembly is similar to vertebrate systems which show a hierarchical assembly of KT protein complexes. To study how this process has evolved in *C. tropicalis*, we have expressed four proteins from the Dam1 complex as TAP fusion proteins. Upon immunostaining with anti-Protein A and anti-tubulin antibodies, we show that the Dam1 complex is associated with the KT throughout the cell cycle as in the case of *S.cerevisiae* and *C. albicans*. Signals corresponding to all the four tagged proteins were detected in both unbudded and large budded cells unlike *S. pombe* where only Dad1 is constitutively localized (Fig 6). A single dot like signal also shows that the *C. tropicalis* centromeres remain clustered throughout the cell cycle, facilitating proper MT-KT attachment and efficient segregation. Our results indicate that the Dam1 complex is conserved in this species and functions similar to its *C. albicans* homologs. Further characterization like *in vivo* association of these proteins to centromeres (ChIP assay)

requirement of spindle for localization will provide concrete proof for the above argument.

2. The *MET3* promoter in *C.tropicalis*

In order to study essentiality of a genes and the function of an essential gene, one requires a controllable expression system so that the effect of expression or depletion of a gene product can be tracked over time and studied. One of the well worked out promoter systems in the case of repression of a gene is the *MET3* promoter system. The main advantage of this system is that it does not require a change of carbon source, which generally could lead to other growth and phenotype changes. Rather, it requires only the addition of two amino acids- met and cys, to the growth media. This promoter has been characterized in other fungi like *S. cerevisiae, Pichia pastoris,* and *Ashbia gossypii* and also in *C. albicans* (Care et al., 1999; Delic et al., 2013; Dünkler and Wendland, 2007; Mao et al., 2002). In *C. albicans*, of the 4 kb intergeneic region between the *MET3* ORF and the preceding ORF, sequences 1.36 kb upstream of the ATG of *MET3* was shown to have optimum promoter activity. However, from our characterization, it is evident that the full length intergenic region is essential for optimum promoter activity. This promoter will be a useful tool to study gene function in this unexplored organism. However, the need still exists for identification of other controllable promoters like *PCK1* promoter, *MAL* promoter etc. (gene expressed in the presence of succinate and maltose) as one gets a freedom of studying two or more proteins in tandem. Unlike the *MET3* promoter, these are inducible promoters that increase the transcription manifold upon change of carbons source. Recently, a *Candida* adapted tetracycline induced gene expression cassette was optimized for *C. albicans*. Being a member of the CTG, one can expect the promoter to function in this species as well (Park and Morschhäuser, 2005).

3. Dad1 is essential for viability in *C. tropicalis*.

The importance of KT proteins for faithful segregation of sister chromatids is well known. The outer KT plays an important function of anchoring the MTs and the other KT layers bound to the *CEN*. Perturbation of any protein in this complex is expected to have defective

nuclear segregation and hence growth. In *S. cerevisiae,* mutants of any of the Dam1 subunit proteins showed severe mitotic defects (Cheeseman et al., 2002; Cheeseman et al., 2001a; Janke et al., 2002). Temperature sensitive Dad1 mutants in this species showed a wide variety of spindle defects such as short spindles, elongated or bent spindles and broken spindles. In addition, when grown at restrictive temperatures, the missegregation frequency increased to 30% and there was a drop in viability if these strains (Enquist-Newman et al., 2001). Similar results have been reported for other proteins from this complex. Using conditional expression system, proteins of the outer KT have been shown essential in case of pathogenic yeast *C. albicans*. Upon repression of the outer KT proteins (Dad2, Dam1, Ask1 and Spc19), cells arrested at G2/M stage, showed profound nuclear segregation defects and also defects in spindle morphology (Thakur and Sanyal, 2011). The Dad1 subunit was shown to be essential in this species using a tetracycline based expression system (Burrack et al., 2011; Roemer et al., 2003). However the mutants were not characterized further.

In this study, we developed a conditionally expressing mutant of Dad1 under the *MET3* promoter in *C. tropicalis*. The depletion of Dad1 for 6 h resulted in accumulation of cells at the large budded stage as seen in the microscopy images. This is well supported by the flow cytometry result that indicates a clear G2/M arrest. Further repression led to the formation of elongated budded cells that are a result of polarized call growth. In *C. albicans,* cases such as NOC treatment that arrests cells in mitotic stage, cells still elongate despite their inability to divide resulting in structures that are similar to pseudohyphal cells. The nuclear mass migrates to the elongating bud. Unlike the other phenotypes known, this is a terminal phenotype i.e cells do no divide further and they eventually die (Berman, 2006). This could be the phenomenon happening in *C. tropicalis* upon depleting Dad1. Much like the description above, we see a rise in population of elongated budded cells at time points after 6 h repression. Results from DAPI staining also corroborate well with these observations. At 6 h repression, greater than 90% of the large budded cells show defective segregation

phenotypes (unsegregated nuclei at one of the buds or stuck at the bud neck). Further, close to 8% of the population at this time point show elongated bud with a single undivided nuclear mass. The proportion of cells with such morphology is strikingly higher in later time points (evident from light microscopy images). A cumulative effect of all these results could be the reason for cells not able to grow on conditions that repress Dad1 levels. Being a part of the protein complex governing KT-MT interaction, depletion of Dad1 is expected to impede the same which can be sensed by the SAC (Lara-Gonzalez et al., 2012). Upon SAC activation, the anaphase transition is halted, resulting in a cell cycle arrest which is precisely what we observe. One experiment that can be done in this regard is to check if the cell cycle arrest is bypassed in the absence of Mad2 upon Dad1 depletion. From our results, we can conclusively say that the predicted Dad1 ORF is indeed an outer KT protein and it is essential for viability of *C. tropicalis.*

Studies on the outer KT complex from *S. cerevisiae, S. pombe and C. albicans* have supported the hypothesis of essentiality of this complex for viability only in cases of one MT/KT in the cell (Cheeseman et al., 2002; Cheeseman et al., 2001a; Liu et al., 2005; Thakur and Sanyal, 2011). In conditions where multiple MTs/KT is facilitated in *C. albicans*, the essentiality has been shown to be compromised to some extent (Burrack et al., 2011). Our results indicate that the Dam1 complex is essential for viability in *C. tropicalis*, a species placed between *C. albicans* and *S. pombe*. Studies on the ratio of MTs/KT in this species would be a good test for the hypothesis, to verify whether 1MT/KT governs the essentiality of the Dam1 complex.

The regulation of kinetochore assembly shows interesting differences across various species in fungi. Ndc10 plays the role of master regulator governing the assembly of most of the KT proteins in *S. cerevisiae,* while Mis6 and Spc7 play a similar role in *S. pombe*. Strikingly different from these, the proteins of different layers of KT show interdependency for their localization and function in case of *C. albicans.* Depletion of a protein from any layer of the

KT resulted in disintegration of the entire KT complex. It would be interesting to see if such a phenomenon exists in *C. tropicalis* as well. If such a regulatory pattern exists, one can use the disintegration of the KT complex as the readout to develop screens for Dam1 complex inhibitors. *C. tropicalis* is one of the most commonly isolated organisms from candidiasis cases in tropical countries like India. Rise of *non C. albicans* species in such cases have grown from 15% to 60% over the past decade (Pfaller et al., 2010). In addition, cases of infection by resistant strains have increased indicating a need to identify new targets for therapeutic intervention. Common changes that an organism does to counter the effects of drugs include modification of the enzymes or pathways that the drug targets, increasing the number of efflux pumps to avoid drug build up within the cell etc. However, this adaptation becomes very difficult when a core process where the cell cannot afford drastic changes is targeted. This makes the fungal specific outer KT an attractive and efficient target.

Materials and Methods

Materials and Methods

1. Media and growth conditions

C. tropicalis strains used in this study were grown in YPDU (2% dextrose, 2% peptone, 1% yeast extract and 0.01% Uracil) and incubated at 30°C at 180 rpm. Conditional expression strains were propagated in complete media (CM) without methionine and cysteine as permissive condition. Methionine and cysteine were added to a final concentration of 1mM in CM for repression. Transformation of *C. tropicalis* was performed as detailed in (Geitz and Woods, 2002). Selection of transformants was based on prototrophy for the metabolic markers used. In case of antibiotic marker NAT, selection was done in YPDU media supplemented with 100 μg/mL nourseothricin (ClonNAT; CAS 96736-11-7, Werner Bioagents, Jena, Germany). Recycling of NAT marker was done by growing the nourseothricin resistant strains in YPMU (2% Maltose, 2% peptone, 1% yeast extract and 0.01% Uracil).

2. Strain construction

2.1 Construction of strains expressing Protein-A tagged outer KT proteins.

Four subunits of the outer kinetochore complex were tagged with protein-A in this study.

***DAD1/DAD1-TAP* strain:**

The *DAD1* ORF with some upstream sequences and 500bp of the 3'UTR were amplified from genomic DNA using primer pairs Dad1 FP and Dad1 RP, Dad1 UTR FP and Dad1 UTR RP respectively. The TAP-URA fragment was amplified from the plasmid pPK335 using the primers Dad1 TAP FP and Dad1 TAP RP. An overlap PCR was setup using these fragments to generate the 2.9 kb DAD1-TAP-URA cassette.

***DAD2/DAD2-TAP* strain:**

The *DAD2* ORF and 500 bp of the 3'UTR were amplified from genomic DNA using primer pairs Dad2 FP and Dad2 RP, Dad2 UTR FP and Dad2 UTR RP respectively. The TAP-URA fragment was amplified from the plasmid pPK335 using the primers Dad2 TAP FP and Dad2 TAP RP.An overlap PCR was setup using these fragments to generate the 2.9 kb DAD2-TAP-URA cassette.

DAM1/DAM1-TAP and ASK1/ASK1-TAP:

The TAP fragment from pPK335 was released and cloned as *Bam*HI-*Apa*I fragment into pBS-Ura plasmid, giving rise to pBS TAP-Ura plasmid. The Dam1 and Ask1 ORFs were amplified (using the primer pairs SR41- SR42 and SR45- SR46 respectively) from genomic DNA and cloned into pBS TAP Ura plasmid as *Spe*I-*Bam*HI fragments to give plasmids pRAM1 and pRAM3. The 3'UTR sequences were amplified (using the primer pairs SR 43- SR44 and SR47- SR48 respectively) and cloned as *Apa*I-*Kpn*I fragments in pRAM1and pRAM3 to give pRAM2 and pRAM4. The cassette for transformation was released by *Spe*I-*Kpn*I digestion and used for the transformation.

All the cassettes for TAP tagging were used to transform CtKS107 to obtain *ORF/ORF-TAP* strains. The transformants obtained were selected on CM-ura plates. The CTSR100 transformants were confirmed by PCR by a forward primer SR49.2 upstream of the ORF and a reverse primer NV34 from the protein-A sequence. A 1023 bp amplicon indicated that the cassette integrated at the correct loci (Fig 14A). The CTSR200 transformants were screened by PCR with SR29 and NV34 primers. Correct transformants were identified by an amplicon of 1.23 kb with these primers (Fig 14B). CTSR300 transformants were confirmed by PCR using primers SR50 and NV34 to give a 1036 bp amplicon in the case of proper integration. The expected band was picked up in

three transformants (Fig. 14C). CTSR400 strains were tested by PCR for proper integration of TAP tag cassette using the primer pairs SR51 and NV34. Amplicon of 932 bp in length indicated the transformant has the cassette in the correct locus (Fig 14D)

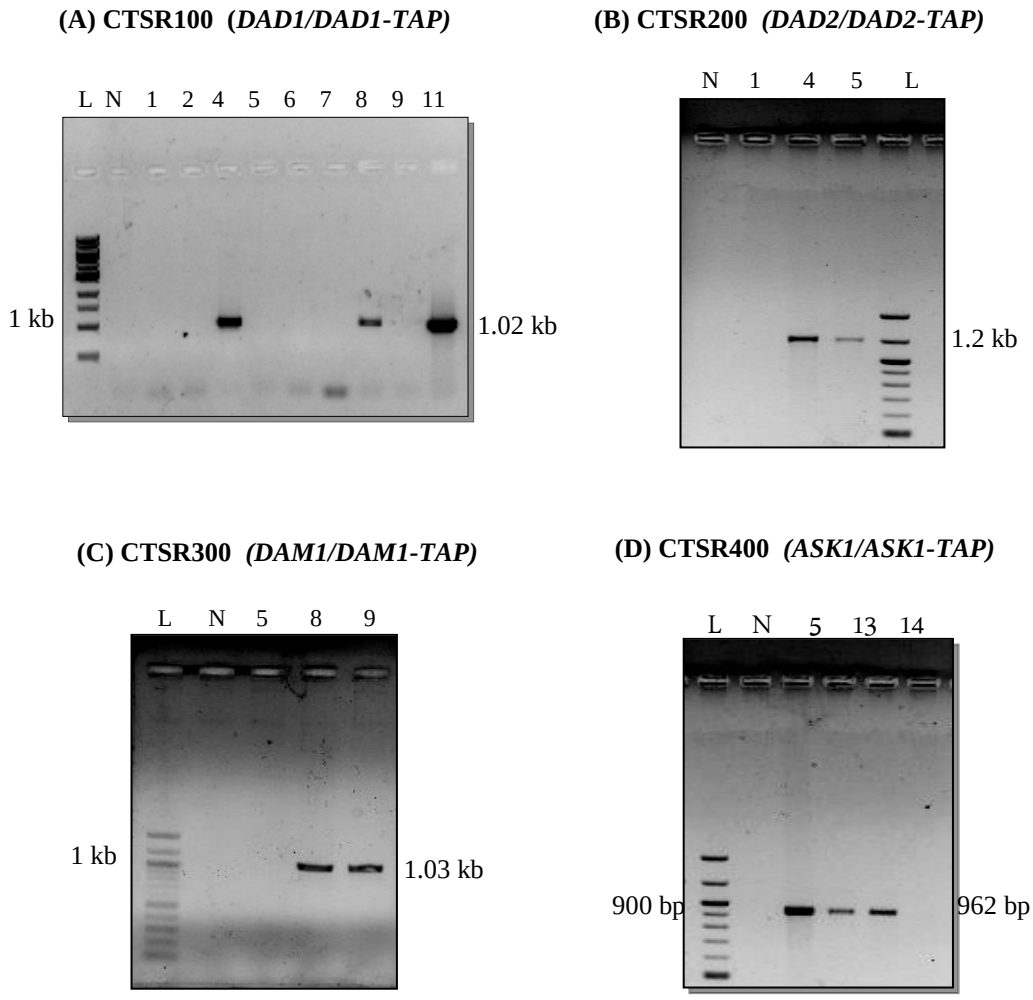


Fig 14: PCR confirmation of TAP tagged strains. (A) PCR using the genomic DNA from CTSR100 with primers SR49.2 and NV34 resulted in 1023bp amplicon, confirming that the *DAD1* ORF was tagged properly. (B) PCR using the genomic DNA from CTSR200 with primers SR29 and NV34 resulted in 1.2 kb amplicon, confirming the DAD2 TAP tag. (C) PCR using the genomic DNA from CTSR300 with primers SR50and NV34 resulted in 1036 bp amplicon, confirming the proper tagging of Dam1. (D) PCR using the genomic DNA from CTSR400 with primers SR51 and NV34 resulted in 962 bp amplicon, indicating the correct integration of Ask1 TAP cassette.

2.2 Construction of a Dad1 conditional expressing strain.

The deletion cassette for deleting one allele of *DAD1* was constructed using the plasmid pSFS2a. Sequence upstream of the Dad1 ORF was amplified (using SR25 and SR 26) and cloned as *Sac*I-*Sac*II fragments into pSFS2a generating the plasmid pRAM5. The sequence downstream of the DAD1 ORF was amplified (using SR27 and SR 28) and cloned as a*Xho*I-*Kpn*I fragment into pRAM5, giving the pRAM6 plasmid. The deletion cassette for transformation was obtained by *Sac*I-*Kpn*I digestion this plasmid. The NAT^R transformants were confirmed by PCR using a forward primer outside the cassette (SR65) and reverse primer from NAT gene (SR80). After recycling the marker, the NAT^S transformants were again confirmed by PCR with primers SR65 and SR82.

The MET3 promoter fragment were amplified using primers (VR49 and VR 35) as *Cla*I-*Sal*I fragments, which were cloned using the same sites into pBSUra to generate pMET1. Sequence upstream of *Dad1ORF* was amplified (using SR71- SR72) and cloned as *Bam*HI-*Pst*I fragment into pMET1 to generate pMET2 plasmid. Dad1 ORF was amplified (using SR73- SR74) and cloned as *Sal*I-*Kpn*I fragment into this plasmid to give the final construct pMET3. Full length promoter was amplified using the primer pairs Met3.3FP and VR35. The 1.4 kb promoter from the plasmid pMET3 was replaced by the full length promoter using *Cla*I-*Sal*I sites to obtain the plasmid pMET4. The promoter replacement cassette was released by *Bam*HI-*Kpn*I digestion and used to transform CTSR103 strain. The transformants were selected on CM-Ura plates devoid of methionine and cysteine. These strains were confirmed by PCR using VR49 forward primer and SR26.

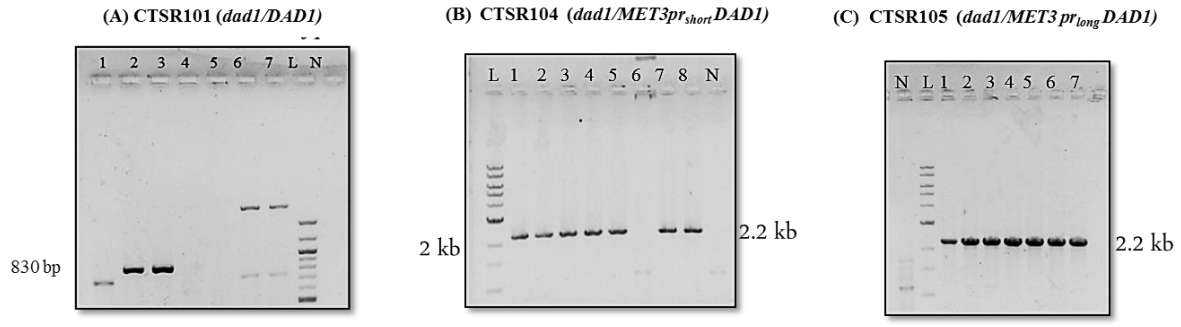


Fig 15: PCR confirmation CTSR103, CTSR104 and CTSR105 strains. (A) PCR using the genomic DNA from CTSR103 with primers SR65 and SR80 resulted in 830 bp amplicon, confirming that the *DAD1* ORF was deleted. (B) PCR using the genomic DNA from CTSR104 with primers VR49 and VR35 resulted in 2.2 kb amplicon, confirming the *DAD1* ORF was placed under the $MET3_{short}$ promoter. (C) PCR using the genomic DNA from CTSR105 with primers VR39 and VR35 gave a 2.2 kb amplicon, confirming the correct integration of $MET3_{long}$ promoter cassette

3. Subcellular localization by Indirect immunofluorescence

Subcellular localization was performed as per the protocol detailed before (Sanyal and Carbon, 2002). Rabbit anti-Protein A antibody (P3775, Sigma) and Rat anti-tubulin antibody (YOL1/34, InVitrogen) were used at a dilutions 1:1000 and 1:30 respectively. The Fluorochrome conjugated secondary antibodies viz. Alexa Fluor Goat anti rabbit IgG 568 (A11011, InVitrogen) and Alexa Fluor goat anti-rat IgG 488(A11006,InVitrogen) were used at 1:500 dilution each. Images were captured using LSM510 META software using a laser confocal microscope (Carl Zeiss, Germany). with the following lasers for specific flurophores: Ar laser (bandpass, 500 to 550 nm) for Alexa Fluor 488, He/Ne laser (band pass, 565 to 615 nm) for Alexa fluor 568, and a two-photon laser near infrared (band pass, ~780 nm) for DAPI. Z stacks were collected at 0.4- to 0.5 μm intervals, and stacked projection images were further processed using LSM software.

4. Protein lysate preparation and Western blot

Protein lysates for western blot were prepared by the TCA method. From overnight grown cultures, $3OD_{600}$ equivalent cells were harvested, washed and resuspended in 400 μL of 12.5 % ice cold TCA solution. The suspension was vortexed briefly and stored at -20°C for 12 h. The suspension was thawed on ice, pelleted at 14000 rpm for 10 min and washed twice with 350 μL of 80 % Acetone (ice cold). The washed pellets were air dried completely and resuspended in desired volume of lysis buffer (0.1N NaOH+1% SDS). Rabbit anti-Protein A antibody (P3775, Sigma) and the HRP conjugated Goat anti-Rabbit secondary antibody, both were used at 1:5000 dilution in 2.5 % Skim Milk powder in 1X-PBS. The blots were developed using Supersignal Chemiluminescent substrate (Pierce) and imaged using Syngene G-Box gel doc system.

5. Flow cytometry for cell cycle analysis

Cells were harvested at the required time points and processed as described in(Sanyal and Carbon, 2002). Prior injection of sample into the flow cytometer, the cell suspension was sonicated briefly (30% amplitude, 7 s pulse). The sonicated sample was diluted to desired cell density using 1X-PBS and injected into the flow cytomer (BD FACSCalibur) for analysis. The output was analysed using BD CellQuestPro software.

6. DAPI staining

Cells grown in permissive and repressive media were harvested, washed and resuspended in 300 µL sterile distilled water. These cells were fixed by adding 700 µL absolute ethanol and incubated at room temperature for 1h. After fixing, the cells were washed with 1mL water twice and resuspended to obtain desired cell density. Cells were allowed to rehydrate in water prior imaging. To 5 µL cell suspension, 3 µL DAPI (100 ng/mL, D9542 Sigma) was added in the glass slide, mixed gently by pipetting and then the cover slip was placed. After 5 min incubation, the cells were imaged using a fluorescence microscope (Olympus BX51).

Table 3: List of primers used in this study

Primer code	Sequence (5' – 3')	Purpose
DAD1 FP	TTCAGCAGCTCTTGCCTTC	Amplification of Dad1 ORF
DAD1 RP	CTTTTTCCATCTTCTCTTTTCTTCATTTGGTTCGTCTGC	
DAD1-TAP FP	GCAGACGAACCAAATGAAGAAAAGAGAAGATGGAAAAAG	Amplification of TAP-Ura
DAD1-TAP RP	GTCCATCTATTGTCCACCATACGACTCACTATAGGGCGAATTG	
DAD1-3UTR FP	CAATTCGCCCTATAGTGAGTCGTATGGTGGACAATAGATGGAC	Amplification of DAD1 3'UTR
DAD1-3UTR RP	AGATTATGTTACGTTGCTTTAG	
DAD2 FP	ATGCAGAGATCAACAAGTAAC	Amplification of Dad2 ORF
DAD2 RP	CTTTTTCCATCTTCTCTTTTCTCGATCATTATTATATTCCTCTTC	
DAD2-TAP FP	GAAGAGGAATATAATAATGATCGAGAAAAGAGAAGATGGAAAAAG	Amplification of TAP-Ura
DAD2-TAP RP	CAATTTAATGAATCTTTAATCATGATACGACTCACTATAGGGCGAATTG	
DAD2-3UTR FP	CAATTCGCCCTATAGTGAGTCGTATCATGATTAAAGATTCATTAAATTG	Amplification of DAD1 3'UTR
DAD2-3UTR RP	TGAAATTGGAAGAATTGAAG	
SR 25	CCAGAGCTCGACCTTCATTTACTTCACCAC	Amplification of Dad1 US with *Sac*I -*Sac*II sites
SR 26	TCCCCGCGGGATTGATAATGGGAGTAAATAAAGAATATCTAAGG	
SR 27	GCCGCTCGAG GTACTATCAAAACTAAGCGACTTTTCTTC	Amplification of Dad1 DS with *Xho*I -*Kpn*I sites
SR 28	GGGGTACCGTTTGGAAGCTGCTGGTATTG	
SR 29	CCAGAGCTCCAGTCGCAAGTTTTGAATGAATC	Amplification of Dad2 US with *Sac*I -*Sac*II sites
SR 30	TCCCCGCGGGGTGGTTTATAATAGTTGTTATTGTTGTTG	
SR 31	GCCGCTCGAGCTAGTATTTCATTACACCCTATTTATAGTCTAG	Amplification of Dad1 DS with *Xho*I- *Kpn*I sites
SR 32	GGGGTACCCAACTATAAAACCATCACTACTTAATAAAGAATCC	
SR43	GCGGGCCCCCACCATATATAAGCTGAAGTTTAAGTTCG	Amplification of Dam1 3'UTR with *Apa*I-*Kpn*I sites
SR44	GGGGTACCATCAGTTGCTTGACCTTTAGCC	
SR45	GGACTAGTCTACAACCGCCGCAATTG	Amplification of AskI ORF with *Spe*I-*Bam*HI sites
SR46	CGGGATCCTTTTTTTGGATAATCTTCTCCATCTCTC	
SR47	GCGGGCCCAACAATGTGCATAAATTATAAAGATTAATTAG	Amplification of AskI 3'UTR with *Apa*I-*Kpn*I sites
SR48	CAGAGCAAACCATTATTATATTAGTG	
SR49.2	CTAACATGACCTTCATTTACTTCACCACC	Forward primer to confirm Dad1TAP
SR50	CGTCATCTGCATAGATCTTCAGG	Forward primer to confirm Dad2TAP
SR51	CCCTACCTCCTATCTTGAGATCAG	Forward primer to confirm Ask1TAP
NV 34	GGTAAATGTAAGATCTCATAGAACGA	Reverse primer from TAP

SR65	CACCAGATGGATGAGAACACATG	Forward primer to confirm Dad1 deletion
SR82	GCAAACCTCTTCTCAATCATCTGC	Reverse primer to confirm Dad1 deletion
SR80	GGTTCTCGGGAGCACAGG	Reverse primer from NAT gene
MET3Pr. 3K FP	CCATCGATGATTAAATAGGTATTTATTATTG	Forward primer for full length MET3 promoter with *Cla*I site
SR71	CGCGGATCCCCAAACTGCCATTCTATGACCTG	Amplification of Dad1 5'UTR with *Bam*HI-*Pst*I sites
SR72	GCCCTGCAGGGAGAAGATGAAGAAGATGATGATATTG	
SR73	GACCGTCGACATGACATCTACATCAGACATTCC	Amplification of Dad1 ORF with *Sal*I-*Kpn*I sites
SR74	CGTGGTACCGTCCATCTATTGTCCACCAC	
SR118	CAGAATCCAAGACAATACCGGTG	Primer for amplifying probe for *MET3prDAD1* southern confirmation
SR119	GTACTAAAAGTTTATGAATACTAACTCCCG	

Table 4: List of *C. tropicalis* strains made/used in this study

Strain Name	**Genotype**
CTKS107	*ura:flp/ura:flp his:flp/his:flp*
CTSR100	*ura:flp/ura:flp his:flp/his:flp DAD1/DAD1-TAP*
CTSR101	*ura:flp/ura:flp his:flp/his:flp DAD1/dad1::NAT-flp*
CTSR102	*ura:flp/ura:flp his:flp/his:flp DAD1/dad1::flp*
CTSR103	*ura:flp/ura:flp his:flp/his:flp DAD1/dad1::flp CSE4/CSE4-GFP*
CTSR104	*ura:flp/ura:flp his:flp/his:flp dad1/MET3 pr$_{short}$ DAD1 CSE4/CSE4-GFP*
CTSR105	*ura:flp/ura:flp his:flp/his:flp dad1/MET3 pr$_{long}$ DAD1 CSE4/CSE4-GFP*
CTSR200	*ura:flp/ura:flp his:flp/his:flp DAD2/DAD2-TAP*
CTSR300	*ura:flp/ura:flp his:flp/his:flp DAM1/DAM1-TAP*
CTSR400	*ura:flp/ura:flp his:flp/his:flp ASK1/ASK1-TAP*

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