universität wien

DISSERTATION

Titel der Dissertation

Construction and Characterization of an Artificial Kinetochore in Budding Yeast

angestrebter akademischer Grad

Doktor/in der Naturwissenschaften (Dr. rer.nat.)

Verfasserin / Verfasser:	Eva Kiermaier
Matrikel-Nummer:	0746179
Dissertationsgebiet (lt. Studienblatt):	Molekulare Biologie
Betreuerin / Betreuer:	Dr. Stefan Westermann

Wien, am 22. März 2010

ABSTRACT V

ZUSAMMENFASSUNG VI

1 INTRODUCTION 1

1.1 THE BUDDING YEAST CELL CYCLE 1
1.1.1 MITOTIC EVENTS 2
1.1.2 PRINCIPLES OF MITOTIC REGULATION 3
1.2 KINETOCHORES 3
1.2.1 KINETOCHORE SPECIFICATION IN BUDDING YEAST 3
1.2.2 MOLECULAR COMPOSITION OF KINETOCHORES 4
1.2.3 BI-ORIENTATION 8
1.2.4 THE SPINDLE ASSEMBLY CHECKPOINT 9
1.3 CONSTRUCTION OF AN ARTIFICIAL KINETOCHORE 11
1.3.1 THE TETR-*TETO* SYSTEM 11
1.3.2 AIM OF THIS PROJECT 12

2 RESULTS 14

2.1 FUNCTIONALITY OF KINETOCHORE-TETR FUSION PROTEINS 14
2.2 MITOTIC STABILITY OF GENERATED MINI-CHROMOSOMES 15
2.2.1 RESCUE OF CENTROMERE-COMPROMISED MINI-CHROMOSOMES 16
2.2.2 STABILIZATION OF ACENTRIC MINI-CHROMOSOMES 17
2.3 INTEGRITY OF THE DAM1 COMPLEX 19
2.4 VISUALIZATION OF MINI-CHROMOSOMES 20
2.5 QUANTIFICATION OF MINI-CHROMOSOME STABILITY 21
2.5.1 LIVE CELL MICROSCOPY OF MINI-CHROMOSOME SEGREGATION 22
2.6 FUNCTIONAL CHARACTERIZATION OF THE ARTIFICIAL KINETOCHORE 23
2.6.1 DEPENDENCY ON STRUCTURAL KINETOCHORE COMPONENTS 24
2.6.2 DEPENDENCY ON REGULATORY KINETOCHORE ELEMENTS 25
2.7 SEGREGATION OF NATIVE CHROMOSOMES BY THE ARTIFICIAL KINETOCHORE 27

3 DISCUSSION 29

3.1 DISRUPTION OF THE NATIVE CENTROMERE AND STABILIZATION OF CDEII-COMPROMISED MINI-CHROMOSOMES 29
3.2 CONSTRUCTION OF A KINETOCHORE WITHOUT A NATIVE CENTROMERE 31
3.3 RECRUITMENT OF MULTIPLE KINETOCHORE PROTEINS 33
3.4 MOLECULAR COMPOSITION OF THE ARTIFICIAL KINETOCHORE 34
3.5 INTEGRITY OF THE DAM1 COMPLEX 35
3.6 ESTABLISHMENT OF BI-ORIENTATION 35
3.7 ALTERNATIVE MECHANISMS OF ACENTRIC MINI-CHROMOSOME PROPAGATION 36
3.8 COMPLEXITY OF NATIVE KINETOCHORES 36
3.9 OUTLOOK 37

4 ADDITIONAL RESULTS – CHARACTERIZATION OF THE SPC105 COMPLEX 39

4.1 THE CENTRAL KINETOCHORE 39
4.1.1 THE EUKARYOTIC KMN NETWORK 39
4.1.2 THE BUDDING YEAST SPC105 COMPLEX 40
4.2 RECONSTITUTION OF THE SPC105 COMPLEX 41
4.2.1 PURIFICATION OF SPC105 COMPLEX FROM YEAST EXTRACTS 41
4.2.2 IDENTIFICATION OF PHOSPHO-SITES 42
4.2.3 PURIFICATION OF OVER-EXPRESSED SPC105P FROM YEAST EXTRACTS 42
4.2.4 PURIFICATION OF SPC105P-MYC FROM WHOLE CELL EXTRACTS BY SIZE EXCLUSION CHROMATOGRAPHY 44
4.3 PHOSPHO-REGULATION OF THE SPC105 COMPLEX 45
4.3.1 *IN VITRO* PHOSPHORYLATION OF SPC105P BY CDC28P-CLB2P 45
4.3.2 *IN VIVO* ANALYSIS OF SPC105 PHOSPHO-MUTANTS 46
4.3.3 DISTURBED KINETOCHORE ORGANIZATION IN SPC105P PHOSPHORYLATION DEFICIENT MUTANTS 47
4.4 SPC105P BINDS TO MICROTUBULES *IN VITRO* 49
4.5 PURIFICATION OF SPC105P FROM BACTERIA 50
4.6 THE SPC105$^{1-411}$ CANNOT COMPLEMENT THE SPC105 KNOCK-OUT *IN VIVO* 51
4.7 OUTLOOK 52

5 MATERIAL AND METHODS 54

5.1 MICROBIAL TECHNIQUES 54
5.1.1 YEAST CELL CULTURING 54
5.1.2 YEAST CROSSING 54
5.1.3 YEAST STRAIN CONSTRUCTION 55
5.1.4 TRANSFORMATION OF YEAST CELLS 55
5.1.5 SPOT ASSAY 55
5.2 GENERATION OF PLASMIDS 55
5.2.1 TET-REPRESSOR (TETR)-INTEGRATION PLASMID 55
5.2.2 MINI-CHROMOSOMES USED FOR STABILITY ASSAYS 56
5.2.3 MINI-CHROMOSOMES USED FOR VISUALIZATION 56
5.3 DETERMINATION OF MITOTIC STABILITY 56
5.4 VISUALIZATION OF MINI-CHROMOSOMES 57
5.4.1 FIXATION OF YEAST CELLS FOR MICROSCOPY 57
5.4.2 PREPARATION OF COVER SLIPS 57
5.4.3 LIVE CELL IMAGING 57
5.4.4 MICROSCOPY AND IMAGE ACQUISITION 58
5.4.5 ANALYSIS IN TEMPERATURE SENSITIVE YEAST STRAINS 58
5.5 TAP-TAG PURIFICATION OF DAM1 COMPLEX 58
5.6 SEGREGATION OF NATIVE CHROMOSOMES 59
5.7 ADDITIONAL METHODS – CHARACTERIZATION OF THE SPC105 COMPLEX 59
5.7.1 YEAST CELL CULTURING 59
5.7.2 STRAIN GENERATION 60
5.7.3 PREPARATION OF WHOLE CELL EXTRACTS (WCE) 60
5.7.4 SPOT ASSAY 60
5.7.5 GENERATION OF PLASMIDS 60
5.7.6 EXPRESSION OF SPC105$^{1-411}$ IN BACTERIA 61
5.7.7 AFFINITY PURIFICATION 61
5.7.8 PURIFICATION OF OVER-EXPRESSED SPC105P FROM A GAL-PROMOTER 61
5.7.9 SIZE EXCLUSION CHROMATOGRAPHY 62
5.7.10 CDC28 KINASE ASSAY 62
5.7.11 IMAGING OF SPC105 PHOSPHO-MUTANTS 62
5.7.12 MICROTUBULE-BINDING ASSAY 63
5.7.13 EXPRESSION OF SPC105$^{1-411}$ IN BACTERIA 63
5.7.14 COMPLEMENTATION OF SPC105 KNOCK-OUT 64

6 APPENDIX 65

6.1 KIERMAIER E, WOEHRER S, PENG Y, MECHTLER K, WESTERMANN S., *NAT CELL BIOL*. 2009 SEP;11(9):1109-15. A DAM1-BASED ARTIFICIAL KINETOCHORE IS SUFFICIENT TO PROMOTE CHROMOSOME SEGREGATION IN BUDDING YEAST. 65

6.2 ABBREVIATIONS 66

6.3 TABLE OF FIGURES 68

6.4 REFERENCES 70

6.5 CURRICULUM VITAE 79

6.6 ACKNOWLEDGMENTS 81

Abstract

To maintain their genetic integrity, eukaryotic cells must segregate their chromosomes accurately to opposing spindle poles during mitosis. Errors during the process of chromosome segregation can lead to aneuploidy - a hallmark of human cancers. Kinetochores are large multi-protein structures, which connect the chromosomes to the mitotic spindle throughout the process of chromosome segregation. Kinetochores are organized into distinct protein sub-complexes, which assemble in a hierarchical manner on centromeric DNA[2]. In contrast to higher eukaryotes, the budding yeast centromere spans over only 125 bp and is connected to just a single microtubule which makes it a suitable tool to study kinetochore function[3]. Nevertheless, even the relatively simple budding yeast kinetochore comprises more than 70 different proteins impeding the analysis of individual proteins or sub-complexes.

During my PhD work I developed a system that allows to artificially recruit individual kinetochore proteins to yeast circular mini-chromosomes via the TetR-*tetO* transcriptional system and ask whether they can provide any aspect of kinetochore function *in vivo*. I could show that recruitment of members of the Dam1 complex is sufficient to attach, bi-orient and segregate acentric mini-chromosomes on the mitotic spindle. This “artificial kinetochore” bypasses the functional requirement for conserved inner and linker kinetochore complexes[4]. It cannot, however, overcome segregation defects imposed by mutants of the conserved kinase AuroraB/Ipl1 and it is not under the control of the spindle assembly checkpoint. Furthermore, the system can be transferred to native yeast chromosomes where it can direct the segregation of a chromosome whose native centromere has been inactivated. These data provide evidence that a simplified eukaryotic chromosome segregation system can be reconstituted *in vivo* by directly recruiting microtubule force-transducing components to DNA, a strategy, which has been already used by primitive bacteria to distribute their genomic DNA[5].

Zusammenfassung

Eukaryotische Zellen teilen ihre duplizierten Chromosomen waehrend der Mitose praezise zwischen den beiden Spindelpolen auf. Dieser Prozess gewaehrleistet die Integritaet des genetischen Materials ueber viele Generation. Sollte es dennoch vorkommen, dass Chromosomen falsch segregieren, kann dies zu Aneuploiditaet fuehren – ein charakteristisches Merkmal vieler humaner Tumore. Im Laufe der Zellteilung muessen die Chromosomen mit dem Spindelapparat verknuepft werden. Dies geschieht mit Hilfe von Kinetochoren. Kinetochore sind makromolekulare Maschinen, die an einer definierten Chromosomenregion, dem so genannten Centromer assemblieren und so als eine Art Plattform fuer die Plus-Enden der Spindelmikrotubuli dienen. Kinetochore bestehen aus einer Vielzahl an Proteinen, welche weiters in verschiedene Subkomplexe untergliedert werden. Diese Komplexe bauen sich hierarchisch auf dem Centromer auf und bilden so eine definierte Topologie die gewoehnlich als trilaminare Kinetochorstruktur bezeichnet wird. Im Gegensatz zu hoeheren Eukaryoten erstreckt sich das Centromer in *S. Cerevisiae* ueber einen Bereich von 125 Basenpaaren und ist mit nur einem einzelnen Mikrotubulus verknuepft. Diese vereinfachenden Umstaende machen die Hefe zu einem idealen Modelorganismus um die Organisation und Funktion von komplexen Kinetochoren zu untersuchen. Dennoch bestehen selbst in *S. Cerevisiae* die einzelnen Kinetochore aus mehr als 80 verschiedenen Proteinen was die genaue Charakterisierung von individuellen Subkomplexen erschwert.

Waehrend meiner Doktorarbeit entwickelte ich ein System, mit Hilfe dessen individuelle Kinetochorproteine artifiziell durch das TetR-*tetO* Transkriptionssystems zu einem Hefen Mini-Chromosom rekrutiert werden koennen. Dies erlaubt die Beantwortung der Frage, ob die artifiziell rekrutierten Proteine in der Lage sind, die Funktion eines nativen Kinetochores zu uebernehmen. Im Zuge dessen konnte ich zeigen, dass die artifizielle Rekrutierung des Dam1 Komplexes ausreicht, um ein azentrisches Mini-Chromosom fehlerfrei zu segregieren – unabhaengig von inneren und zentralen Kinetochorproteinen. Dieses artifizielle Segregationssystem benoetigt die konservierte Kinase Ipl1, aber unterliegt nicht mehr der Kontrolle des allgemeinen Spindel Checkpoints. Des weiteren konnte das System effizient auf native Hefechromosomen uebertragen werden. Meine Daten zeigen, dass ein einfaches eukaryotisches Chromosomensegregationssystem kuenstlich nachgebaut werden kann, indem man Microtubuli-bindende Proteine rekrutiert, die die mechanische Energie, die waehrend der Depolymerisierung der MT entsteht, in Bewegung von Chromosomen umwandeln koennen. Diese Strategie wird auch von primitiven Bakterien genutzt, um ihre Erbinformation auf zwei Zellen aufzuteilen.

1 Introduction

Cell division is a fundamental process of life as every cell arises by division of an existing cell. One of the most important issues during the process of cell replication is the transmission of genetic information into a pair of daughter cells. To ensure faithful chromosome segregation over many generations the series of events leading to cell reproduction are tightly regulated during the cell cycle.

1.1 The budding yeast cell cycle

Unicellular eukaryotes like the budding yeast *Saccharomyces cerevisiae* are valuable model organisms to study the cell cycle as they harbor small, fully defined genomes, which are easily accessible for genetic manipulation. Furthermore principal features of the cell cycle have been conserved throughout evolution between a wide range of organisms.

The cell cycle can be subdivided into discrete stages, which are typically defined on the basis of chromosomal events (**Figure 1a**). In a first step most of the cell components are duplicated and then equally distributed into the two daughter cells. In *G1 phase*, cells are unbudded containing a haploid set of chromosomes and a single spindle pole body (SPB; **Figure 1b**). If the cell is committed to division, DNA replication and SPB duplication are initiated at the G1/S-transition (START)[6]. The replication of DNA content occurs in *S phase,* which is morphologically characterized by the emergence of a small bud. During *S phase* the bud grows and the SPBs separate from each other to form a bipolar spindle. Chromosome segregation and nuclear division (mitosis) follow in *M phase*, which ends with cell division (cytokinesis). In budding yeast the mitotic spindle can assemble even before DNA synthesis is completed suggesting that *S*- and *M phase* may partially overlap[7]. Therefore these cells lack a clearly defined *G2 phase* between *S* and *M phase*.

Nevertheless the cell-cycle machinery of all eukaryotes comprises a robust and reliable control system, which regulates the timing and order of cell cycle events.

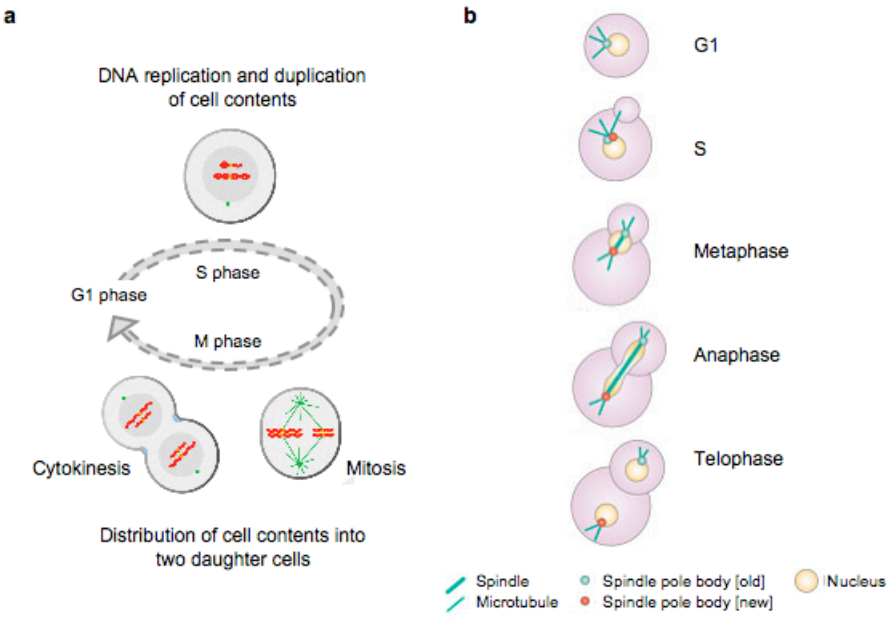


Figure 1: a) Events during the budding yeast cell cycle (adapted from David O Morgan, The Cell Cycle; 2007). b) Stages of *M phase* in budding yeast (adapted from Tanaka, Nature reviews, 2005).

1.1.1 Mitotic events

The major goal of mitosis is the separation and segregation of replicated chromosomes ensuring that each daughter cell inherits a full complement of the genome. Budding yeast undergoes a closed mitosis which is characterized by a lack of nuclear envelope breakdown[8].

Upon chromatin condensation during prophase duplicated chromosomes are present as tightly connected sister chromatids. These connections arise from DNA catenation and specialized protein complexes called cohesins[9]. Furthermore, microtubules (MTs) – polymers of α/β-tubulin dimers - nucleate from the duplicated SPBs which are embedded in the nuclear envelope and assemble a bipolar spindle-shaped structure. In metaphase, spindle microtubules emanating from opposing spindle poles attach to specialized regions on the sister chromatids called centromeres. These connections are bridged by kinetochores - huge proteinacious structures that assemble on centromeric DNA and form the connections between chromosomes and the mitotic spindle. If all sister kinetochores are attached to microtubules extending from opposing spindle poles, a state referred to as bi-orientation, the machinery that dissolves the cohesion links between sister chromatids is activated allowing the separation of chromatids to opposite spindle poles in anaphase[1]. This cell cycle stage can be subdivided into two parts, which both contribute to the segregation of sister chromatids: in anaphase A kinetochore MTs disassemble thereby pulling the sister chromatids to the opposing spindle poles. Anaphase B is characterized by an increase in

the pole-to-pole distance, by moving the spindle poles farther apart from each other thus completing the segregation of sister chromatids. In telophase, the final stage of mitosis, chromosomes are decondensed and the mitotic spindle is disassembled. In budding yeast the elongated anaphase nucleus is pinched into two daughter nuclei during cytokinesis[10].

1.1.2 Principles of mitotic regulation

Mitotic events as described above follow a strict temporal order and have to be tightly controlled by the cell cycle control system. Major players in this system are cyclin dependent kinases (Cdks) and their regulatory subunits cyclins. The enzymatic activity of all Cdks requires the binding of cyclins leading to the phosphorylation of target proteins such as structural and regulatory components[11]. While intracellular Cdk concentrations are generally very high and do not vary significantly, cyclin levels oscillate during the cell cycle driving the formation of active cyclin-Cdk complexes. In budding yeast, the major cell cycle kinase Cdc28, is controlled by two groups of mitotic cyclins that govern progression through mitosis[12]. Clb5 and 6 levels rise at the beginning of *S phase* and function in the control of DNA replication; Clb1-4 increase in mid *S phase* controlling mitotic events[13].
Furthermore Cdks can activate the Anaphase Promoting Complex (APC) which leads to the destruction of cohesion that holds sister chromatids together (see 1.2.4).

1.2 Kinetochores

Kinetochores play a crucial role during the cell cycle by forming a physical link between the centromeres of the chromosomes and the spindle apparatus. In budding yeast, a single microtubule is attached to each sister kinetochore whereas higher eukaryotes contain a fiber of 20-40 microtubules per kinetochore[14]. Even though kinetochore proteins show limited homology at least on the level of their primary amino acid sequence, major principles in kinetochore composition and regulation are conserved throughout eukaryotes.

1.2.1 Kinetochore specification in budding yeast

Kinetochore assembly is directed by specialized, short chromosomal regions termed centromeres consisting of three conserved centromere DNA elements CDEI, II and III[15] (**Figure 2**).

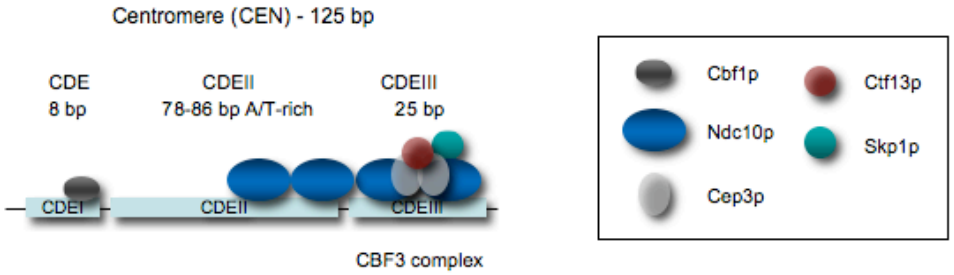


Figure 2: Centromere organization in budding yeast. Centromeric DNA elements (CDEI-III) and their corresponding binding proteins are depicted.

CDEI is bound by a homodimer of Cbf1p but neither CDEI nor the bound Cbf1 protein are essential for centromere function[16]. CDEII elements from all 16 yeast chromosomes are conserved in length (78-87 bp), high levels of A and T base composition (>90%) and short repeated tracts of A or T residues[17]. CDEIII is bound by the CBF3 complex and contains a highly conserved central region, which is absolutely required for centromere function. Single mutations within this region completely abolish centromere function due to the loss of CBF3 binding[18].

The budding yeast centromere spans over only 125 bp and is therefore referred to as a point centromere. In contrast to that, centromeres of higher eukaryotes range from a few kilobases to several megabases and are considered as regional centromeres[19]. One common feature of all eukaryotic centromeres is the replacement of histone H3 from the nucleosome by an evolutionary conserved H3 variant Cse4p (Cenp-A in mammals)[20]. Current models propose that the centromeric DNA is wrapped around a single nucleosome thereby leading to the compaction of pericentric DNA[21,22]. One appealing feature of this model is the resulting width of the chromatin fiber, which would be approximately 22nm, roughly the diameter of a single microtubule (25nm).

De novo kinetochore formation requires the protein Chl4p (the budding yeast Cenp-N counterpart) whereas it is not essential for the duplication of existing kinetochores. In the absence of Chl4p, Cse4p and Ndc10p are lost from centromeres and dicentric mini-chromosomes, which are usually very unstable due to breakage derived from opposing pulling forces, are stabilized[23]. Since Chl4p is a non-essential component of the kinetochore it implies that *de novo* kinetochore formation is usually not taking place within the cell but rather propagation of pre-existing kinetochores.

1.2.2 Molecular composition of kinetochores

Budding yeast kinetochores consist of more than 70 different proteins, which are organized in defined sub-complexes and assembled in a hierarchical manner from the chromatin to the outer

kinetochore[24]. Electron microscopy studies in eukaryotic cells and tandem-affinity purifications from yeast extracts have revealed a trilaminar morphology, dividing the kinetochore into three distinct regions: the inner kinetochore which forms the interface with chromatin, the outer kinetochore, a disc-shaped structure that embeds the plus-ends of microtubules and the central or linker kinetochore which bridges the former two layers (**Figure 3**)[2,25].

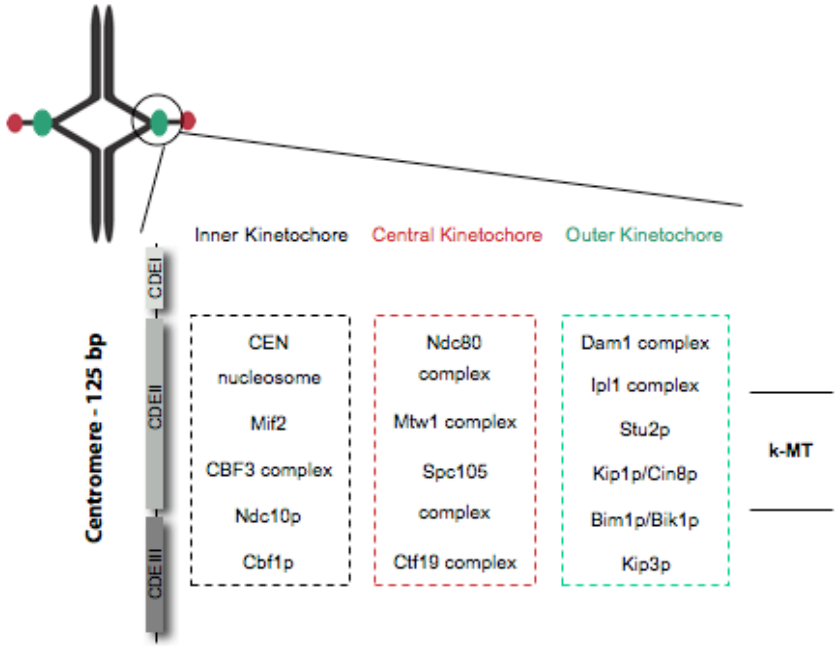


Figure 3: Architecture of the budding yeast kinetochore. Protein sub-complexes are assigned to one of the three layers, which bridge between the centromeres and the kinetochore MTs (k-MT).

According to their relative position to DNA, the individual kinetochore sub-complexes are assigned to one of these three layers.

1.2.2.1 DNA interface

The first step in kinetochore assembly is the binding of the 4-protein CBF3 complex (Ndc10p, Cep3p, Ctf13p and Skp1p) to the CDEIII element of the centromere[26]. Recruitment of all other kinetochore complexes depends on the presence of this complex emphasizing its role in specifying kinetochore assembly. The major DNA binding activity of the CBF3 complex is provided by a zinc-finger motif within Cep3p[27].

Together with two evolutionary conserved proteins Cse4p and Mif2p (the budding yeast CenpA and CenpC homologues) these proteins form the interaction surface with chromosomes as judged by chromatin immunoprecipitation and tandem affinity purifications[25,28]. Cse4p replaces

the canonical histone H3 in centromeric nucleosomes and interacts with Mif2p. The latter one plays an important role in linking the inner complexes to the central kinetochore as it interacts with Cse4 containing nucleosomes as well as with the central Mtw1 complex[29].

1.2.2.2 Central kinetochore/linker complexes

Proteins of the kinetochore linker layer need to connect the inner complexes, which do not possess any microtubule-binding activity, to the various MT associated proteins of the outer kinetochore. Furthermore they play important roles in regulatory pathways, for instance, by recruiting spindle assembly checkpoint effector proteins.
In addition to the Mtw1 complex, three other major complexes – Ctf19, Ndc80 and Spc105 – are considered to be linker complexes. Proteins of the Mtw1- and Ctf19 complexes are constitutively present at kinetochores whereas others are more transiently associated. Tandem-affinity purifications from yeast extracts led to the isolation of the 12-protein Ctf19 complex. The role of its individual proteins is not completely clear to date but only two of them are essential for viability in budding yeast[30]. Recent studies have implicated the Mis12 complex, the human counterpart of the Mtw1 complex, in specifying kinetochore assembly at specific sites downstream of Cse4p[31,32]. Together with KNL-1 (Spc105p in budding yeast) and the Ndc80 complex, these three complexes form the KMN network, which constitutes the core microtubule attachment site of the kinetochore[33] (see 1.2.2.3 and 4.1.1). The latter two components harbor an intrinsic microtubule-binding affinity, which is synergistically increased after they associate with each other. Furthermore both complexes display concentration dependent microtubule binding, arguing that they associate in a cooperative manner on the MT lattice[33].

1.2.2.3 Kinetochore-microtubule interface

One essential function of the kinetochore is to stably attach chromosomes to the mitotic spindle and either generate or transduce the forces, which are required for chromosome segregation. Various microtubule-binding proteins are involved in creating a stable attachment site and coupling kinetochore movements to disassembling microtubules[34].
As mentioned above, the Ndc80 complex plays a crucial role in microtubule binding. It consists of four proteins, which form a rod-like structure with two globular heads at each end and a long coiled-coiled stretch in between[35]. The N-terminus comprises Ndc80p and Nuf2p, which point towards the outer kinetochore and are responsible for MT-binding[36]. The other end of the rod is composed of Spc24p and Spc25p and is more closely oriented towards the inner kinetochore[37]. Even though the Ndc80 complex on its own harbors a low affinity for microtubules, a series of

such weak binding sites would allow attachments to remain dynamic in response to growing and shrinking microtubules[38]. Quantitative measurements of fluorescently labeled Ndc80 molecules revealed that there are likely to be eight Ndc80 complexes per microtubule supporting the model of multivalent interactions per microtubule[39]. Furthermore the Ndc80 complex was suggested to have implications in recruiting checkpoint proteins such as Mad2p and Bub1p[40,41].

In budding yeast the ten-subunit Dam1 complex plays a major role in creating stable kinetochore-microtubule attachments. It localizes to kinetochores in an Ndc10-, Ndc80 and microtubule dependent manner[42]. All ten proteins are essential for viability and copurify as a stable heterodecamer, consisting of one copy of each subunit. Temperature-sensitive mutations in any of the subunits show severe defects in chromosome segregation emphasizing its role in connecting kinetochores to the mitotic spindle[43]. Studies by electron microscopy revealed that 16 Dam1 complexes oligomerize into ring-like structures around microtubules *in vitro* (**Figure 4a**)[44,45]. In contrast to the Ndc80 complex, the Dam1 complex does not show any molecular footprint on the microtubule surface allowing individual rings to slide laterally along the lattice (**Figure 4b**)[46]. This feature makes the Dam1 complex an ideal device to transform the energy, which is generated during microtubule depolymerization into the movements of chromosomes (**Figure 4c**).

Indeed it has been shown, that beads coupled to the Dam1 complex can move with depolymerizing microtubules *in vitro*[46]. Similar experiments revealed that beads coated with Dam1 complex undergo assembly- and disassembly driven motility sustaining a force of 0.5-3 pN[47].

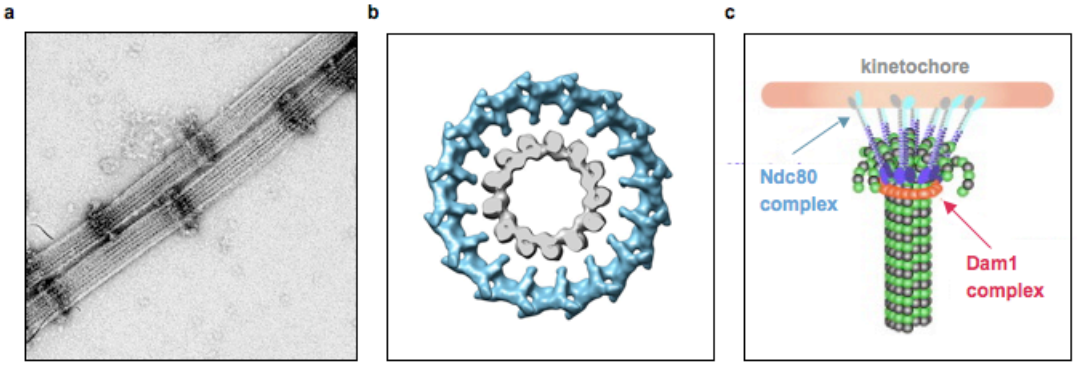


Figure 4: The budding yeast Dam1 complex. a) Negatively stained MTs are encircled by individual Dam1 rings[44]. b) Reconstruction of the complex around a MT emphasizing the gap between them. Adapted from Wang et al., Nature Struct. Mol. Biol, 2007. c) Mechanism of force generation by the Dam1 complex. Adapted from Welburn et al., Dev. Cell, 2009.

So far, no homologues of the Dam1 complex have been found outside fungi raising the possibility that its essential function in budding yeast is related to the requirement to tightly connect a single MT to kinetochores.

Recently the 3-subunit Ska complex was identified in metazoan cells as a new microtubule binding complex[48,49,50]. Depletion of the complex by RNAi leads to attachment defects and severe chromosome missegregation similar to phenotypes observed in *dam1* mutants. Furthermore its kinetochore localization depends on the presence of the Ndc80 complex suggesting that the Ska complex may be a functional homologue of the Dam1 complex[50]. However, further investigations are required to evaluate this hypothesis.

1.2.3 Bi-orientation

Prior to the onset of chromosome segregation in anaphase the cell has to ensure that all sister kinetochores attach to microtubules extending from opposing spindle poles – a state, which is termed bi-orientation or amphitelic kinetochore-microtubule attachment (**Figure 5a**). Initially kinetochores attach to the lateral side of a single MT which provides a much larger surface area for contact than the MT tips[51]. This initial encounter occurs by a search-and-capture process between the growing and shrinking MTs and the kinetochores[52,53]. Once bound to the MT lattice, kinetochores are transported polewards, which is promoted by the minus end-directed motor protein Kar3p[51,54]. Since MTs remain dynamic at the plus ends during kinetochore transport, they often catch up with sliding kinetochores converting the lateral attachments to the MT ends (called end-on pulling)[54].

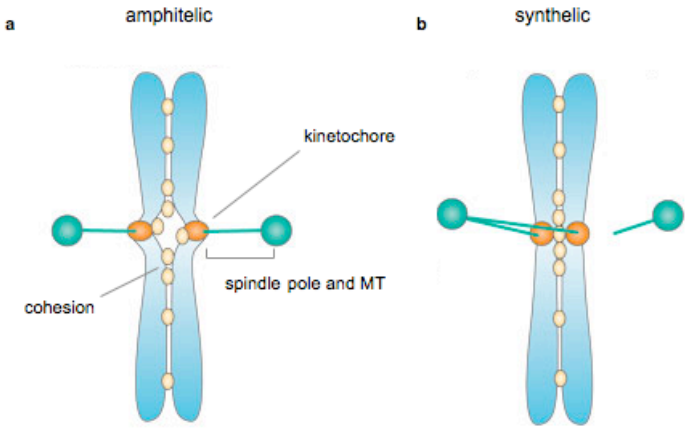


Figure 5: a) Sister chromosome bi-orientation. Kinetochores are attached to MTs emanating from opposing spindle poles. The cohesin complex holds the two sisters together resisting the pulling forces exerted by the MTs. b) Both sister kinetochores are connected to the same SP (synthelic attachment). Adapted from Tanaka, Nature reviews, 2005.

This end-on attachment is more stable than lateral association and can therefore sustain the tension applied by the mitotic spindle. As kinetochores approach the spindle poles, each sister kinetochore eventually attaches to MTs extending from the same (synthelic; **Figure 5b**) or opposite spindle poles (amphitelic). If both sister kinetochores attach to the same spindle pole they need to be re-oriented until proper bi-orientation is achieved[55]. Two kind of mechanisms have been proposed to favor amphitelic attachments: a geometry-dependent and a tension-dependent mechanism[56,57]. The first one relies on a back-to-back position of sister kinetochores due to cohesion between sister centromeres. After attaching to one spindle pole the remaining kinetochore can only be captured by a microtubule emanating from the opposite spindle pole due to geometry restraints[58]. In contrast to that the tension-dependent mechanism is thought to correct improper attachments, relying on tension applied on these attachments. One important player in promoting correction is the conserved kinase Aurora B (termed Ipl1 in budding yeast). In *ipl1-2* temperature sensitive mutants unreplicated, dicentric mini-chromosomes (with two centromeres opposite to each other) fail to bi-orient and change their spindle pole association in the absence of tension[59]. Therefore Ipl1 was suggested to establish proper kinetochore-microtubule attachments by eliminating those that do not generate tension. One proposed model how this process could be regulated is based on the spatial separation of the kinase from its substrates[60,61]. Ipl1 localizes to the inner centromere in pro- and metaphase where it phosphorylates target proteins such as Ndc80 or the Dam1 complex thereby lowering their affinity to microtubules and creating unattached kinetochores. Once proper connections are established tension is applied pulling the substrates away from the kinase to the spindle poles. Furthermore Ipl1 relocalizes from kinetochores to the spindle midzone upon anaphase onset[62]. Phosphatases like Glc7/PP1 and Cdc14 oppose Ipl1's function by removing phosphates from the target proteins therefore promoting exit from mitosis[63,64,65].

1.2.4 The spindle assembly checkpoint

To ensure the fidelity of chromosome segregation the spindle assembly checkpoint (SAC) monitors defects in kinetochore-microtubule attachments. Since only productive attachments lead to the segregation of chromosomes the cell must recognize connections, which are not amphitelic. Proper attachments are characterized by two hallmarks, which are both necessary to satisfy the spindle assembly checkpoint: kinetochores must be associated with a microtubule and this attachment has to generate tension.

In an unperturbed mitosis the proteins securin (Pds1 in budding yeast) and cyclin B (Clb2) are targeted for degradation through the 26S proteasome prior to anaphase onset by APC/C-mediated polyubiquitylation (**Figure 6**)[66].

Securin serves as an inhibitor of the protease separase (Esp1), which is required to cleave the cohesin rings that hold sister chromatids together[67]. Destruction of securin releases separase thereby allowing sister chromatid dissociation and initiation of anaphase. Furthermore the proteolysis of cyclin B inactivates the major mitotic kinase Cdk1 (Cdc28), which promotes exit from mitosis[68].

Unattached kinetochores contribute to the assembly of the mitotic checkpoint complex (MCC), which consists of the proteins Mad2p, Mad3p, Bub3p and Cdc20p[69]. This complex negatively regulates the ability of Cdc20 to activate the APC/C by occupying its substrate binding sites thereby preventing the polyubiquitylation of securin and cyclin B[70]. Securin, as described above, inhibits the protease separase to avoid precocious sister chromatid separation by prolonging prometaphase until all kinetochores become bi-oriented[68].

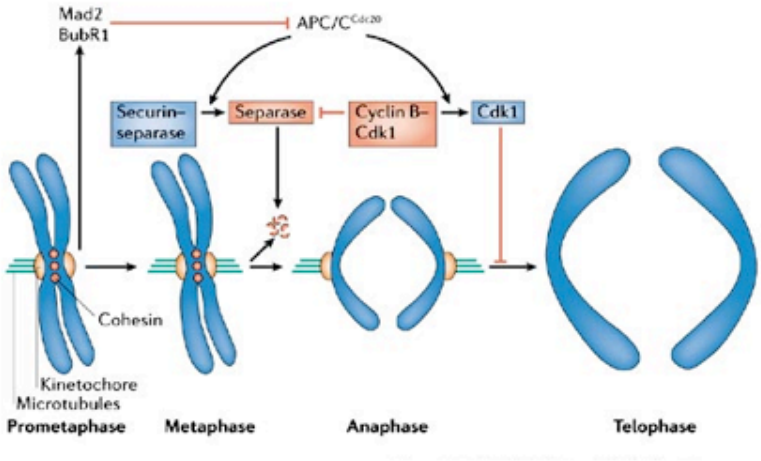


Figure 6: Activation of the SAC by unattached kinetochores. Adapted from Peters, Nature reviews; 2006.

Several studies have shown that all SAC proteins localize to unattached kinetochores supporting the idea that kinetochores provide a platform which contributes to MCC formation[71,72]. In particular proteins of the kinetochore linker layer have been suggested in recruiting SAC proteins, like the Ndc80- or Spc105 complex[41,73]. Additionally, a number of kinases have been implicated in recruiting effector proteins and generating robust SAC activity including Mps1 and Ipl1[74,75]. Ipl1 is believed to detect and destabilize faulty kinetochore-MT interactions generating unattached kinetochores (see 1.2.3), which are sensed by the SAC[76].

In contrast to higher eukaryotes, the spindle assembly checkpoint is dispensable for chromosome segregation in an unperturbed cell cycle in budding yeast. Deletion of MCC proteins has little effect on cell viability and fidelity of chromosome segregation[77]. This difference probably results from the fact that in budding yeast bi-orientation is established much more rapidly than in

metazoan cells making a delay in anaphase onset by the SAC dispensable for chromosome segregation. Nevertheless the SAC becomes essential when MT-depolymerizing drugs, which disrupt the mitotic spindle, disturb the cell cycle[78].

1.3 Construction of an artificial kinetochore

The complexity in kinetochore composition and its contribution to different regulatory pathways, as described above, make the analysis of individual kinetochore proteins a challenging task. Therefore it would be extremely helpful to reduce the complexity of a native budding yeast kinetochore to a more primitive segregation system, which is simpler in its composition. Ideally such an artificial kinetochore should still be able to act like a wildtype kinetochore in terms of chromosome segregation thereby allowing functional studies.

1.3.1 The TetR-*tetO* system

Tetracycline (tc) is a broad family of antibiotics indicated for use against many bacterial infections. It binds to the 30S subunit of the ribosome thereby blocking protein synthesis by preventing peptide chain elongation. Nevertheless many gram-negative bacteria have developed a resistance which is mainly based on the efflux of the drug from the cell[79]. The expression of tc resistance genes is regulated by the Tet-Repressor (TetR), a dimeric protein which binds with high specificity to the palindromic *tet operator* (*tetO*) sequence in the absence of tetracycline (association constant $K_a \sim 10^{11} M^{-1}$)[80]. This binding inhibits the transcription of the *tetR* and *tetA* genes. After tc enters the cell it chelates the divalent Mg^{2+} ion forming the biologically active $[MgTc]^+$ complex. This complex binds to the TetR inducing an allosteric conformational change, which releases it from DNA. This event allows for the transcription of *tetR* and *tetA* genes, which code for the Tet-Repressor and the TetA antiporter membrane protein, respectively. TetA protein couples the export of $[MgTc]^+$ from the cell with the import of H^+. The rise of TetAp and TetR in the cell quickly reduces cytoplasmic levels of tc, thus restoring repression of the *tetA* and *tetR* genes[81]. Importantly the affinity of the $[MgTc]^+$ inducer complex is three orders of magnitude higher for the TetR than for the ribosome ensuring the export of tc before it can bind to and interfere with the ribosome[80]. Due to the high efficiency of the TetR-*tetO* transcriptional system, it has been adapted as a transcriptional regulator in eukaryotic cell lines as well as in *Saccharomyces cerevisiae*[82].

1.3.2 Aim of this project

To reduce the complexity of native budding yeast kinetochores we decided to fuse individual kinetochore proteins to the Tet-Repressor, described above, and guide them to circular yeast mini-chromosomes via the *tetO* sequence (**Figure 7**). The high affinity of the TetR to the *tetO* DNA should allow efficient recruitment of the respective kinetochore protein.

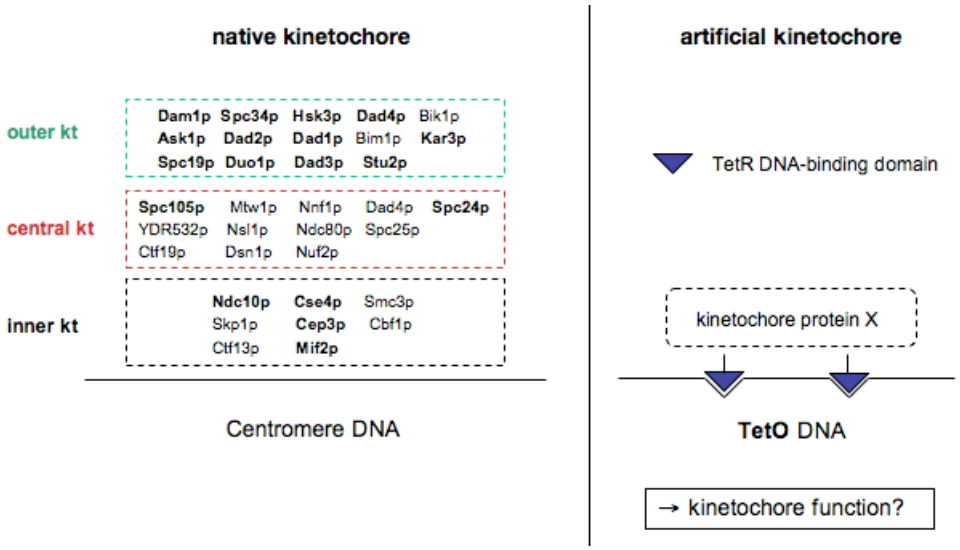


Figure 7: Comparison between a native (left panel) and the artificial (right panel) kinetochore. Kinetochore proteins are recuited to *tetO* DNA by fusing them to the Tet-Repressor.

Yeast mini-chromosomes are small circular plasmids, which contain an autonomously replicating sequence (ARS) site and an auxotrophic marker to allow for selection on minimal medium. In addition, they harbor a centromere that directs the assembly of the kinetochore therefore maintaining them stably over many generations. Nevertheless they are lost more frequently from growing cultures than native chromosomes (10^{-2} per division in contrast to 10^{-5} per division for native chromosomes)[83]. Mini-chromosomes have been used in the past in various studies to investigate protein-DNA interactions between centromeres and the mitotic spindle or mechanisms of bi-orientation[59,84].

These two features - their small size and their dispensability for normal cell growth - make them an ideal tool to design an artificial segregation system: by tethering individual kinetochore proteins to yeast mini-chromosomes we can ask whether this artificial recruitment is sufficient to either rescue a CEN-compromised mini-chromosome or even allow segregation of an acentric plasmid. By using this system, we can analyze to what extent individual kinetochore proteins contribute to the event of mini-chromosome segregation.

Additionally, the elucidation of the minimal requirements for chromosome segregation would be helpful in order to understand the organization and function of native kinetochores.

2 Results

2.1 Functionality of kinetochore-TetR fusion proteins

The Tet-Repressor (TetR) was integrated at the endogenous gene locus of the respective kinetochore protein by homologous recombination via a one-step PCR method generating C-terminal TetR fusion proteins. Expression of the fusion protein under its own promoter should guarantee endogenous protein levels, which is particularly important for kinetochore proteins that are assembled into multiprotein complexes. Furthermore it has been reported that over-expression of CID, the drosophila homolog of Cse4p, leads to the formation of functional ectopic kinetochores[85]. In addition, a flexible linker of 13 amino acids was introduced between the kinetochore protein and the TetR ensuring its flexibility and efficient binding to the *tetO* array. To exclude the possibility that the TetR compromises the functionality of the appropriate kinetochore protein viability of the haploid TetR strains was monitored at 30°C and 37°C, respectively (**Figure 8**).

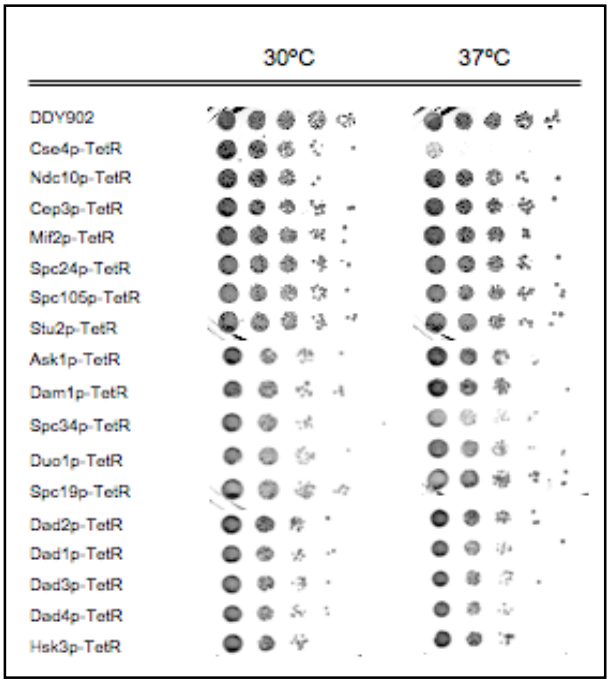


Figure 8: Spot assay of TetR-tagged kinetochore proteins at 30°C and 37°C. All strains are viable except Cse4-TetR, which displays temperature-sensitivity at 37°C.

All strains - except Cse4-TetR - show normal growth at every temperature similar to the wildtype strain background (upper lane; DDY902) suggesting that the TetR-fusion does not interfere with the functionality of the proteins. Only Cse4-TetR (second lane from the top) exhibits compromised

growth at 34°C and 37°C, respectively. Nevertheless at 30°C there seems to be no obvious growth defect which implies that the protein can fulfill its normal function since it is an essential component of the kinetochore. This allows the usage of the strain for further experiments which are carried out at 30°C. Additionally it is important to mention that all TetR strains exhibit similar growth rates excluding differences in cell doubling times, which could complicate the calculation of plasmid stabilities.

2.2 Mitotic stability of generated mini-chromosomes

Circular as well as linear mini-chromosomes are lost more frequently from growing cultures than native yeast chromosomes. Usually a CEN ARS plasmid is two to three orders of magnitude less stable than a native chromosome[3]. If cells containing a mini-chromosome are cultured in rich medium, the absence of selection pressure leads to an accumulation of cells lacking mini-chromosomes. To determine the mitotic stability of the generated mini-chromosomes, the strains were grown for a certain time period under non-selective conditions and consecutively plated on selective and non-selective agar plates to estimate their mitotic stability. Under these conditions a wildtype mini-chromosome containing a native centromere (wt CEN) displays a mitotic stability of 67%±5% in contrast to an acentric plasmid, which is rapidly lost (stability of 1.5%±1%; **Figure 9**).

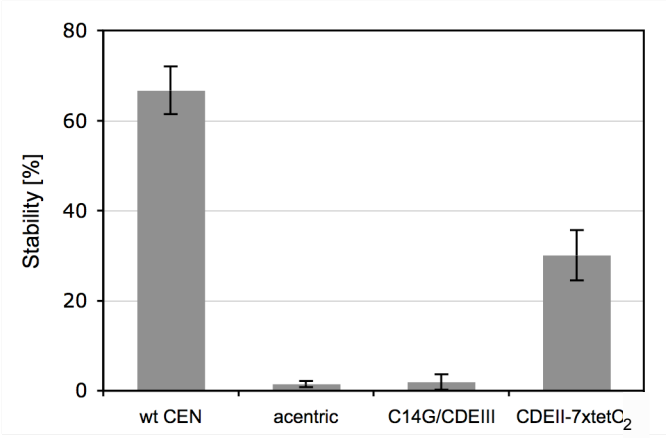


Figure 9: Mitotic stabilities of wildtype, acentric and mutant mini-chromosomes in a wildtype background strain (DDY902). Error bars denote ±standard deviation (±sd) of seven independent experiments.

Introduction of a point mutation into the CDEIII element of the wildtype mini-chromosome (C14G/CDEIII) completely abolished centromere function, arguing that this conserved base in CDEIII is essential for a fully active mitotic centromere[86].

The *7xtetO$_2$* insertion into the CDEII element of the centromere (CDEII-*7xtetO$_2$*) also significantly decreased the stability of the wildtype mini-chromosome to approximately 50% of the wildtype level (mitotic stability of 30%±6%; P<0.001). Nevertheless there is residual centromere activity allowing mini-chromosome propagation to a certain extent.

2.2.1 Rescue of centromere-compromised mini-chromosomes

To address whether the recruitment of individual kinetochore proteins is able to rescue the CDEII-*7xtetO$_2$* compromised mini-chromosome, the same assay as described above was carried out to determine the mitotic stability in the background of individual kinetochore-TetR fusion strains. For better comparison, the stability of the wildtype mini-chromosome in the appropriate TetR background strain was normalized to 100% and the stability of the CDEII mutant mini-chromosome adjusted relative to that (**Figure 10**).

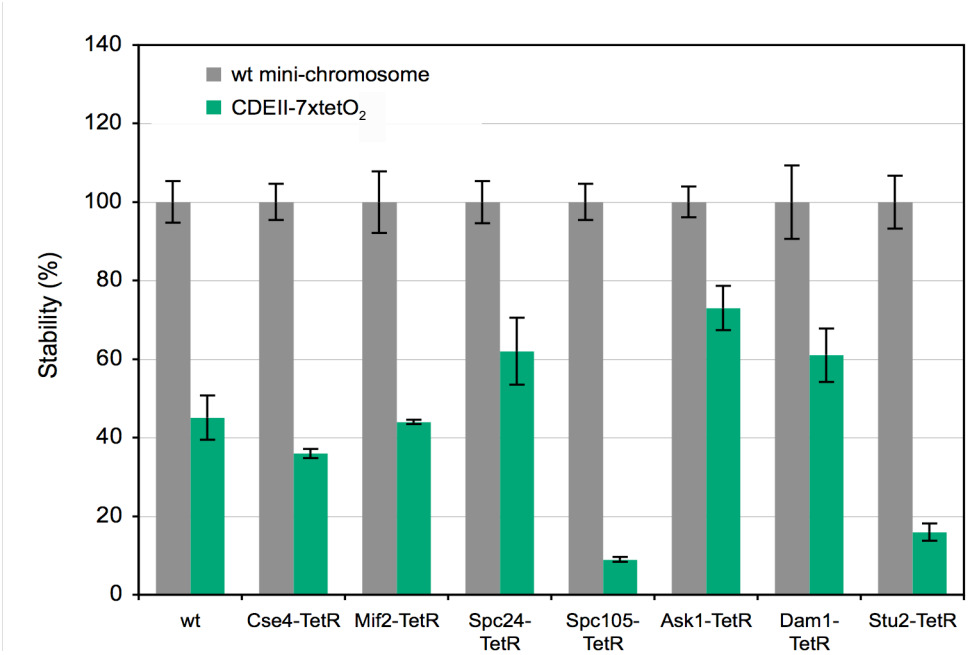


Figure 10: Mitotic stabilities of wildtype and CDEII-*7xtetO$_2$* mutant mini-chromosomes in different TetR strains. Erros bars denote ±sd of four independent experiments.

From this experiment the recruitment of three proteins seems to significantly stabilize the mutant mini-chromosome: Spc24-, Dam1- and Ask1-TetR. Spc24p is a member of the 4-protein Ndc80 complex and the latter two of the 10-protein Dam1 complex, both involved in microtubule binding (see 1.2.2.3). However two other proteins, which harbor an intrinsic MT-binding affinity – Spc105p and Stu2p – do not show any significant rescue of the CDEII-7xtetO$_2$ compromised mini-

chromosome suggesting that not any kind of MT-binding protein can act as stabilizer. Interestingly, also none of the inner kinetochore proteins (Cse4p and Mif2p) was able to increase the stability of the CDEII-*7xtetO*$_2$ compromised mini-chromosome. To further characterize the requirements for mini-chromosome stabilization, the CDEII-*7xtetO*$_2$ insertion was combined with the C14G/CDEIII point mutation.

Combination of both did not lead to the rescue of the mini-chromosomes in the Dam1-TetR background suggesting that an intact CDEIII element is required for proper mini-chromosome segregation under these conditions (**Figure 11**).

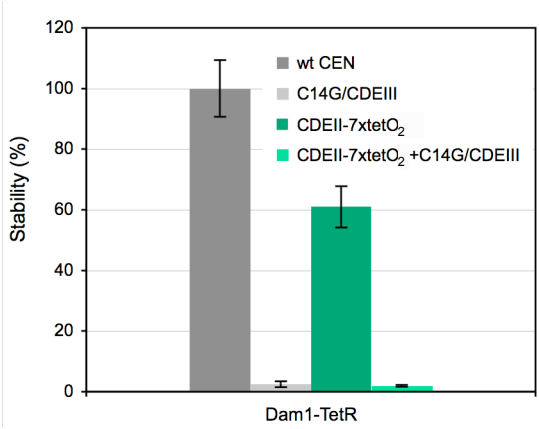


Figure 11: Mitotic stabilities of wt and mutant mini-chromosomes in a Dam1-TetR background. CDEIII is required to rescue the CDEII-*7xtetO*$_2$ compromised mini-chromosome by Dam1-TetR.

Furthermore this experiment excludes the possibility, that the *tetO* mini-chromosomes are propagated by a hitchhiking process on native chromosomes, which incorporate the kinetochore TetR fusion protein.

2.2.2 Stabilization of acentric mini-chromosomes

Since the centromere function is not completely abolished by the CDEII-7xtetO$_2$ insertion, it is difficult to distinguish between the kinetochore defect, which arises due to the *tetO* insertion and the rescuing effect by artificial recruitment of kinetochore TetR fusion proteins. To overcome this problem the system was used to monitor the segregation of acentric mini-chromosomes lacking any centromeric sequence by artificial recruitment of individual kinetochore TetR-fusion proteins. Therefore either plasmids without centromeric sequence or with a *10xtetO*$_2$ array were generated and their mitotic stabilities determined as described above. As expected, the acentric plasmids are rapidly lost, regardless of the strain background. Surprisingly, however, the stability of the

10xtetO$_2$ mini-chromosome is significantly increased in the Dam1- and Ask1-TetR background (**Figure 12**).

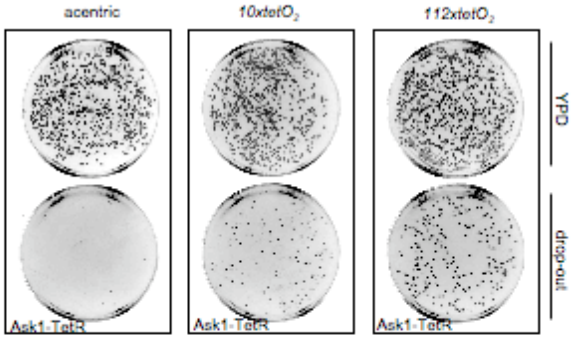


Figure 12: Plating assay of acentric mini-chromosomes in the Ask1-TetR background. Upper plates show growth on rich medium (YPD); lower plates growth under selective conditions (drop-out) upon re-plating after a period of non-selective growth.

Extension of the *tetO* array to 112 repeats even strengthened this effect; in particular for the Dam1-TetR fusion the mitotic stability of the *112xtetO$_2$* mini-chromosome increased three fold compared to the *10xtetO$_2$* array. Furthermore, recruitment of Ndc10- and Cep3-TetR, two members of the inner CBF3 complex, led to a statistically significant stabilization of the 112xtetO2 mini-chromosome albeit to a lesser extent than the recruitment of Dam1- or Ask1-TetR. In contrast to that, no stabilization could be observed for any other microtubule-binding protein (Spc24p, Spc105p or Stu2p; **Figure 13**).

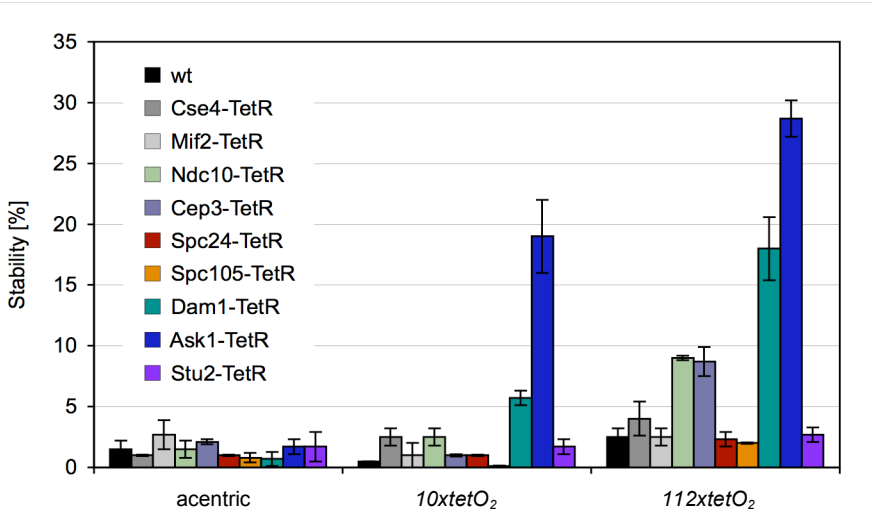


Figure 13: Mitotic stabilities of acentric and *tetO* mini-chromosomes in different TetR strains. Error bars denote ±sd of three independent experiments.

Since the two proteins that were able to segregate the acentric *tetO* mini-chromosomes are two members of the 10-protein Dam1 complex we systematically tagged the remaining 8 subunits with the Tet-Repressor and monitored their ability to stabilize the *112xtetO*$_2$ mini-chromosome. In addition to Dam1- and Ask1-TetR three other members of this complex (Spc34-, Spc19- and Dad1-TetR) are able to confer segregation capacity to the acentric *112xtetO*$_2$ mini-chromosome (**Figure 14**).

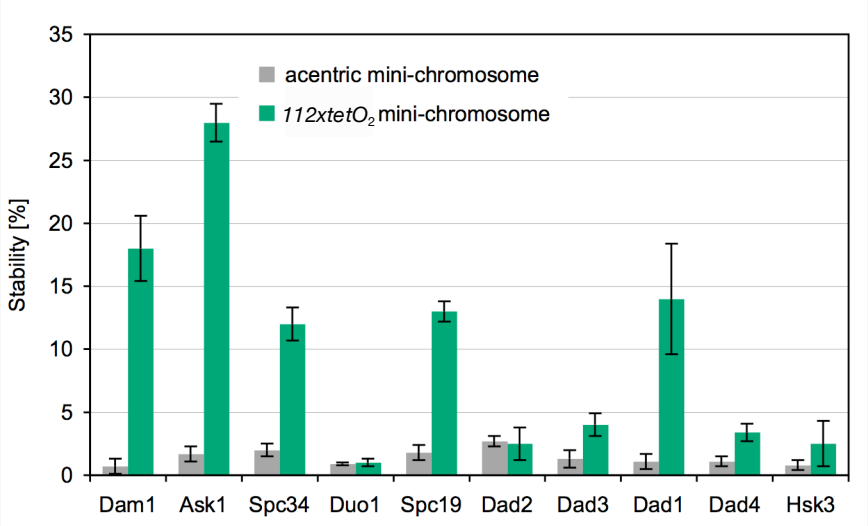


Figure 14: Mitotic stabilities of acentric and *tetO* mini-chromosomes conferred by the members of the Dam1 complex. All subunits were tagged with the Tet-Repressor. Error bars denote ±sd of three independent experiments.

In summary, these experiments show that artificial recruitment of members of the Dam1 complex is sufficient to bring segregation capacity to a plasmid lacking any centromere sequence.

2.3 Integrity of the Dam1 complex

To address whether the TetR-fusions function as part of the full Dam1 heterodecamer, the complex was purified from either a wildtype or an Ask1-TetR strain background. Therefore the Dad1p subunit was tagged with a TAP-tag, which allows tandem affinity purification from whole cell extracts. The samples were resolved on an SDS-gel and analyzed by silver staining and mass spectrometry, respectively (**Figure 15**). Inspection of the band pattern reveals no differences between the wildtype and Ask1-TetR strain except an up-shift in the migration behavior of Ask1p due to the TetR fusion (green boxes).

In both samples all 10 subunits of the Dam1 complex could be detected by mass spectrometry (data not shown) suggesting that the Tet-Repressor does not interfere with proper complex

assembly and that the TetR-fusions likely function in the context of the entire Dam1 complex. In addition to the ten subunits of the Dam1 complex, the regulatory kinase Ipl1p, a known interacting partner and regulator of the complex, could be co-purified as detected by mass spectrometry.

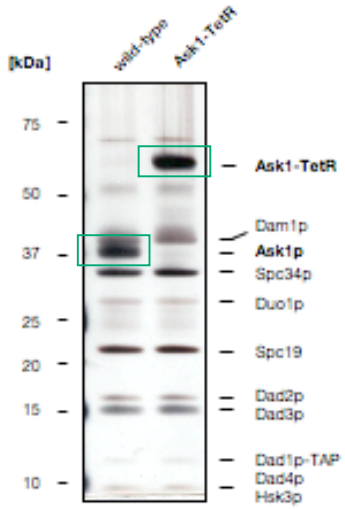


Figure 15: Affinity purification of the Dam1 complex from either a wildtype strain (left lane) or a strain expressing Ask1-TetR (right lane). Respective subunits of the Dam1 complex are assigned on the left side. Ask1p and Ask1-TetR are outlined in green boxes.

2.4 Visualization of mini-chromosomes

To follow the mini-chromosome segregation in real time, mini-chromosomes with a *256xlac operator* array were generated and transformed into a yeast strain expressing LacI-GFP. The binding of LacI-GFP to the *lac operator* (*lacO*) allows the visualization of mini-chromosomes (**Figure 16**)[7,87]. Spindle poles were tagged with Spc42-mCherry to monitor cell cycle stages according to spindle size. Wildtype mini-chromosomes appear as cohesed pairs in *S phase* (upper left panel) and get separated to opposing spindle poles in anaphase (left lower panel) whereas acentric mini-chromosomes show a strong bias for the mother cell (middle upper panel). Due to the expression of either Dam1-TetR or Ask1-TetR, the *10xtetO$_2$* acentric mini-chromosomes get equally distributed between mother and daughter cell (middle lower and right panel). However, in contrast to a wildtype mini-chromosome, they are not tightly associated with the spindle pole bodies but reside proximal to the mitotic spindle.

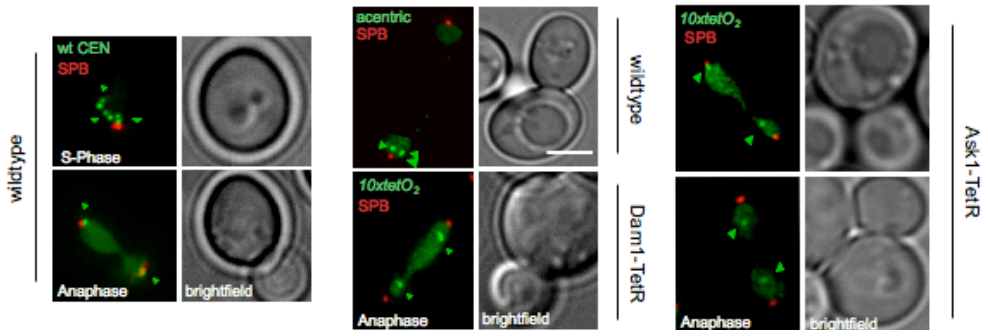
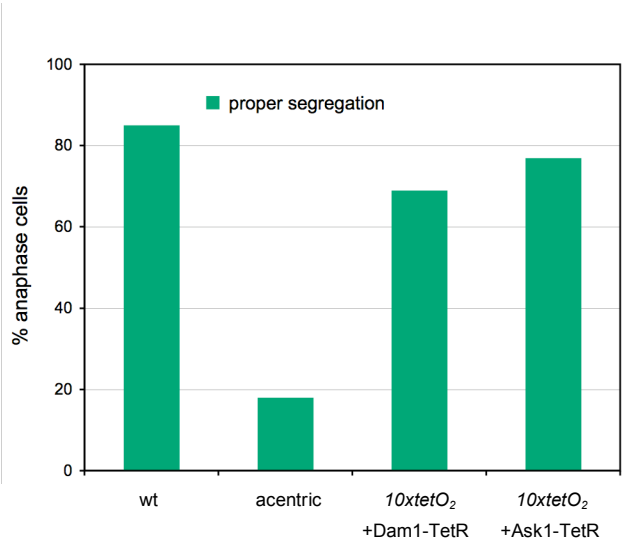


Figure 16: Visualization of wildtype (left panel) and acentric (middle and right panels) mini-chromosomes in a wildtype, Dam1-TetR or Ask1-TetR background. Scale bar denotes 2µm.

2.5 Quantification of mini-chromosome stability

Unlike the plating assay, the visualization of mini-chromosomes allows to follow their segregation in individual cells in real time. To quantify the mitotic stability of the respective mini-chromosomes, the percentage of anaphase cells that show proper segregation of the appropriate



mini-chromosomes was assessed (**Figure 17**).

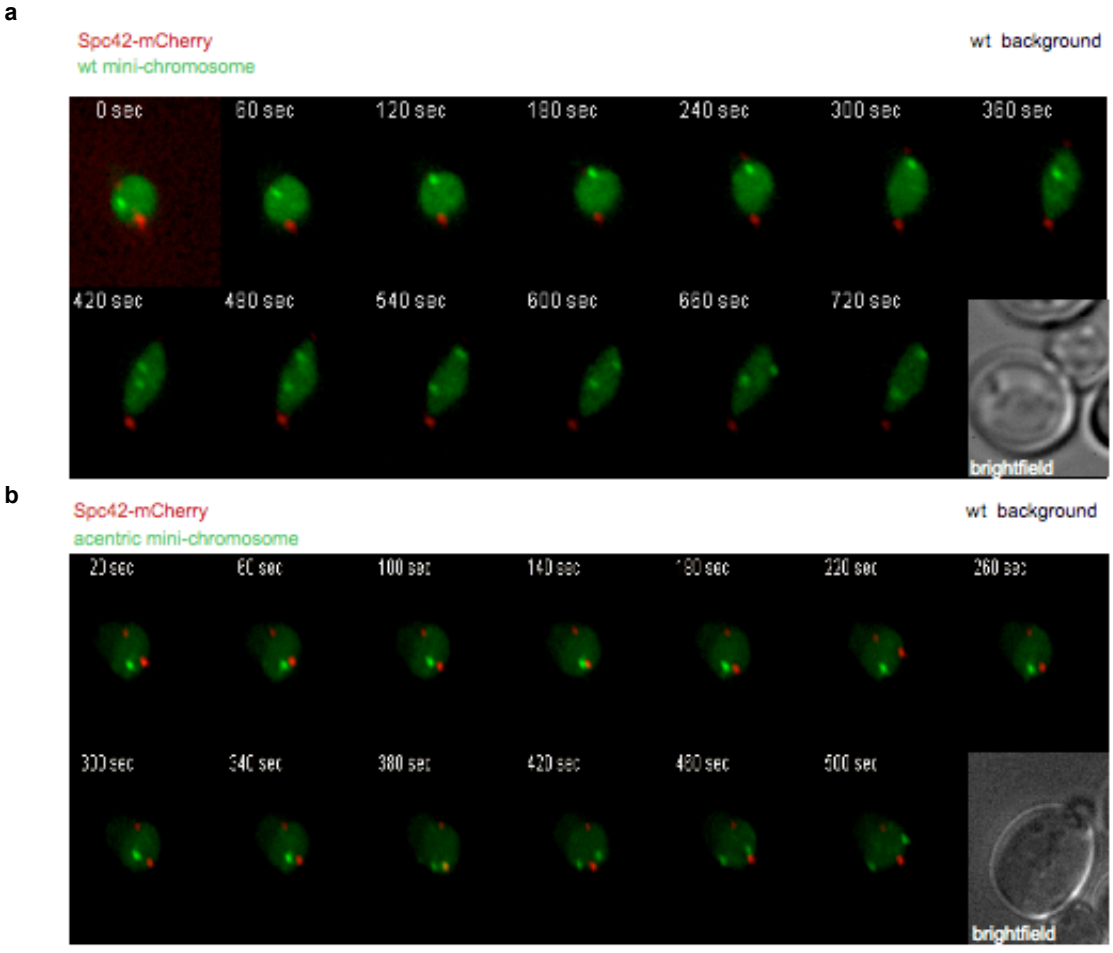
Figure 17: Quantification of anaphase cells showing proper mini-chromosome segregation as judged by fluorescence microscopy. n: 100 cells.

Whereas the acentric mini-chromosomes accumulate in the mother cell (18% proper segregation) wildtype mini-chromosomes are usually equally distributed between mother and daughter cells

(85% proper segregation). A similar distribution of the *tetO* acentric mini-chromosome can be observed in the Dam1- and Ask1-TetR background (69% and 77% proper segregation, respectively) confirming the data derived from the plating assay. However the mitotic stability as judged by microscopy seems to be significantly higher than in the plating assay. This discrepancy arises due to different numbers of cell cycles for the individual experiments: mini-chromosome segregation monitored by microscopy includes only one or two cell cycles whereas the plating assay comprises 10-12 cell cycles.

2.5.1 Live cell microscopy of mini-chromosome segregation

To follow the mini-chromosome segregation over time, cells were recorded throughout the course of mitosis. Wildtype mini-chromosomes align and bi-orient along the spindle axis in metaphase before they get pulled apart to opposing spindle poles in anaphase (**Figure 18a**).



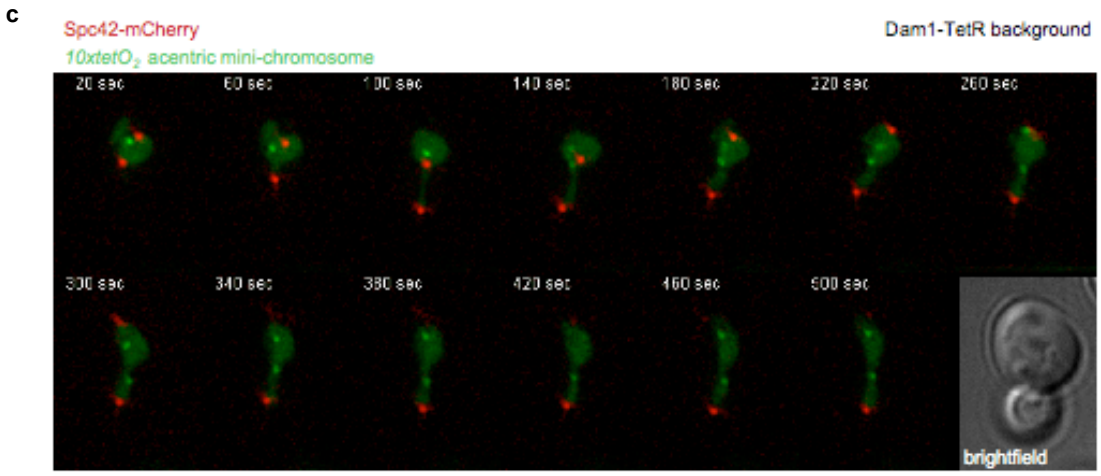


Figure 18: Time-lapse live-cell microscopy showing segregation of a wildtype (a) acentric (b) and *10xtetO$_2$* mini-chromosome (c; green) in a Dam1–TetR strain background.

In contrast to that acentric mini-chromosomes do not show any directed movements (**Figure 18b**). Instead they randomly move within the nucleus without being attached to the spindle poles. Expression of Dam1-TetR leads to proper bi-orientation and subsequent segregation of the *10xtetO$_2$* plasmid similar to wildtype mini-chromosomes (**Figure 18c**). Overall, the data derived from the live cell experiments confirm that the artificial kinetochore allows bi-orientation and proper segregation of acentric *tetO* mini-chromosomes in the Dam1- and Ask1-TetR background.

2.6 Functional characterization of the artificial kinetochore

To analyze the functional dependency of the artificial segregation system on other kinetochore components, acentric mini-chromosome segregation was observed in the background of different kinetochore mutants. If the system operates independently of the respective kinetochore components acentric mini-chromosome segregation should not be affected in these mutants unlike a wildtype mini-chromosome carrying a native centromere. In contrast to that, if the ask1-based segregation system depends on other kinetochore components, wildtype as well as acentric *tetO* mini-chromosomes will fail to segregate under conditions, which disrupt native kinetochores. To address the above-mentioned question different temperature-sensitive alleles of selected kinetochore proteins were combined with the artificial recruitment system and mini-chromosome segregation monitored at the restrictive temperature.

2.6.1 Dependency on structural kinetochore components

As a control for successful inactivation of the respective kinetochore protein, wildtype mini-chromosome segregation was analyzed in fixed anaphase cells at the restrictive temperature. Mutants like *ndc10-2* are known to disrupt native kinetochores leading to complete detachment of microtubules[88]. After shifting the cells to the restrictive temperature, the wildtype mini-chromosomes fail to segregate and instead accumulate in the mother cell (**Figure 19a,b**; green arrow heads; grey bars) emphasizing the role of Ndc10p for proper kinetochore assembly and mini-chromosome segregation. Expression of Ask1-TetR significantly increased the percentage of anaphase cells showing proper *10xtetO*$_2$ mini-chromosome segregation to almost wildtype levels at the non-permissive temperature (**Figure 19a,b**; first two bars). Similar results were obtained for two other temperature-sensitive alleles of the CBF3 complex (*ctf13-30, cep3-1*) and two conserved inner kinetochore proteins (*cse4-1, mif2-3*, **Figure 19b**).

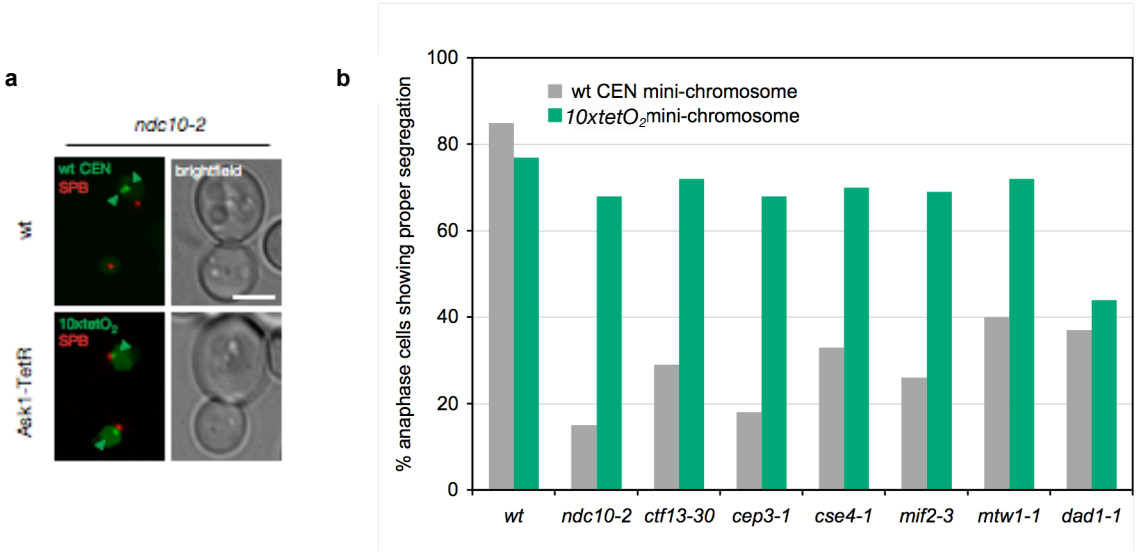


Figure 19: a) Mini-chromosome segregation in an *ndc10-2* temperature sensitive strain in the presence or absence of Ask1-TetR. Cells were monitored at the restrictive temperature. Scale bar denotes 2µm. b) Quantification of wildtype (grey bars) and *10xtetO*$_2$ (green bars) mini-chromosome segregation in different temperature sensitive strains expressing Ask1-TetR. wt (first two bars) represents cells expressing Ask1-TetR at the non-permissive temperature.

These results confirm that the artificial dam1-/ask1-based kinetochore bypasses the functional requirement for the inner CBF3 complex (Ndc10p, Ctf13p and Cep3p) as well as two evolutionary conserved proteins Cse4p and Mif2p. In addition to that, one of the linker complexes - Mtw1 – is not required for artificial mini-chromosome segregation. Inactivation of one of the subunits of the Dam1 complex, Dad1p, leads to the breakdown of the artificial segregation system suggesting

that not single Dam1p subunits account for proper mini-chromosome segregation but rather the entire complex being responsible in fulfilling this function.

2.6.2 Dependency on regulatory kinetochore elements

2.6.2.1 Chromosomal passenger complex (CPC)

Mini-chromosome segregation was analyzed in temperature-sensitive alleles of two different members of the CPC (*ipl1-2* and *sli15-3*). For both alleles approximately 40% of cells containing wildtype mini-chromosomes show impaired segregation at the restricted temperature (**Figure 20**); the segregation defects are less severe than in the *ndc10-2* mutant since MTs do not completely detach from kinetochores in these mutants[89].

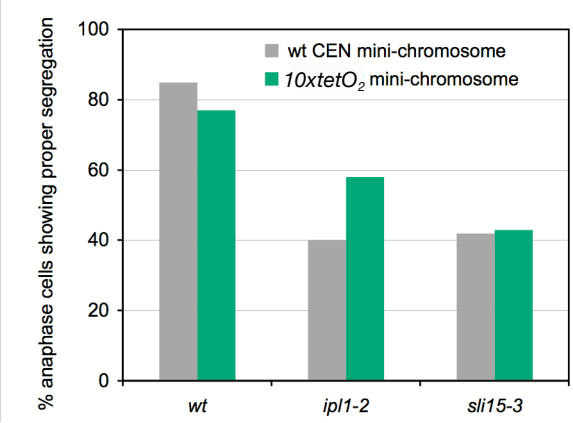


Figure 20: Quantification of wildtype (grey bars) and *10xtetO₂* mini-chomosome (green bars) segregation in mutant alleles of the chromosomal passenger complex at the restricted temperature. All strains express Ask1-TetR.

However recruitment of Ask1-TetR does not significantly advance the segregation of the *10xtetO₂* mini-chromosome in these mutants. Even though the percentage of anaphase cells that show proper *10xtetO₂* mini-chromosome segregation is slightly increased in the *ipl1-2* mutant/Ask1-TetR background, the segregation defects are still comparable to what was has been reported previously for native yeast chromosomes (60% missegregation in *ipl1-321* mutants)[63]. Similarly to the *ipl1-2* mutants the segregation of wildtype and *tetO* acentric mini-chromosomes was strongly impaired in the *sli15-3* background (58% and 57% missegregation, respectively). These results imply that the artificial segregation system requires the conserved kinase Ipl1p/AuroraB and its co-activator Sli15/INCENP to mediate proper mini-chromosome segregation. Additionally, a sub-stoichiometric amount of Ipl1 kinase was co-purified with the Ask1–TetR containing Dam1

complex in the previous affinity purification (see 2.3), suggesting that regulation by Aurora B kinase might also apply to the artificial segregation system.

2.6.2.2 Spindle assembly checkpoint (SAC)

The artificial dam1-/ask1-based kinetochore allows proper segregation of acentric *tetO* mini-chromosomes over the course of few cell cycles but it is clearly less effective than a wildtype kinetochore (**Figure 9**). One possible explanation could be the absence of an active spindle assembly checkpoint (SAC), which under native conditions monitors proper kinetochore-MT attachments. To address this issue, the stability of a wildtype mini-chromosome under checkpoint *on*- and *off* conditions was compared to the stability of the *tetO* acentric mini-chromosome under the same checkpoint conditions but in the presence of Ask1-TetR (**Figure 21**).

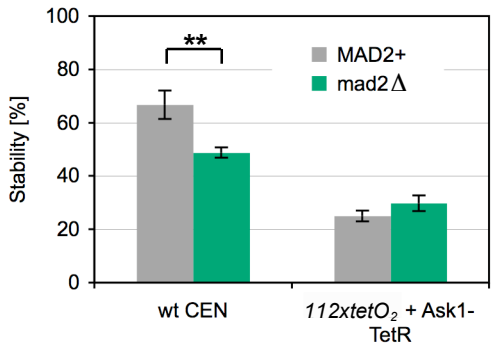


Figure 21: Mitotic stability of mini-chromosomes under spindle assembly checkpoint *on* (grey bars)- and *off* (green bars) conditions. **: P<0.001.

For wildtype mini-chromosomes, deletion of *MAD2* (checkpoint *off*; green bars), one of the checkpoint effector proteins, leads to a significantly decreased mitotic stability due to elevated missegregation (P<0.001). By contrast, there is no such difference detectable for the artificial kinetochore under checkpoint *on*- or *off* conditions arguing that the SAC is not activated by the tether-based system. Similar results were obtained for the artificial kinetochore in the Dam1-TetR background.

Taken together, the functional characterization of the dam1-/ask1-based kinetochore has revealed that it bypasses the requirement for the DNA-binding components of natural kinetochores. It may physically lack structural components like the inner CBF3 complex as well as regulatory elements such as the spindle assembly checkpoint. On the other hand, it still requires the conserved kinase Ipl1 and its co-activator Sli15, which are important components for the process of chromosome bi-orientation.

2.7 Segregation of native chromosomes by the artificial kinetochore

In the experiments described so far centromere-independent segregation was only achieved on a yeast mini-chromosome. To ask whether the artificial kinetochore can be transferred to a native yeast chromosome, the endogenous centromere of chromosome V was conditionally inactivated by inserting a galactose inducible promoter immediately upstream of CEN5[90]. Transcription from the Gal-promoter sterically inhibits the assembly of the kinetochore therefore switching off the native centromere in galactose-rich medium. Additionally, an *112xtetO$_2$* array was introduced 35kbp upstream of the conditional centromere (**Figure 22**), which can be bound by either TetR-GFP to visualize chromosome V and/or Ask1-TetR to form an artificial kinetochore.

After growth for 6 hours in galactose rich medium, more than 90% of cells containing the conditional centromere showed severe chromosome missegregation in the absence of Ask1-TetR. Upon expression of Ask1-TetR however correct segregation of chromosome V was observed in 83% of the cells showing that the artificial kinetochore is able to rescue the missegregation phenotype to almost wildtype levels (**Figure 23a,b**).

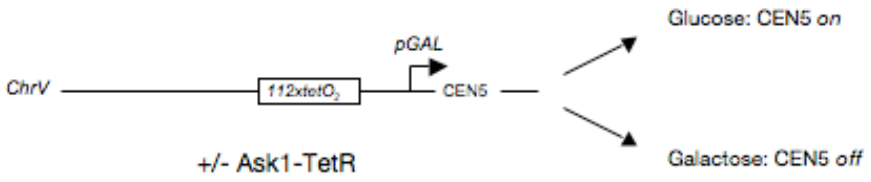


Figure 22: A strategy to test artificial kinetochore function on a native yeast chromosome. A galactose inducible promoter was placed upstream of the centromere of chromosome V, allowing its inactivation by shifting the cells to galactose rich medium. A *tetO* array was introduced 35 kbp away from the conditional centromere to allow visualization and artificial kinetochore formation.

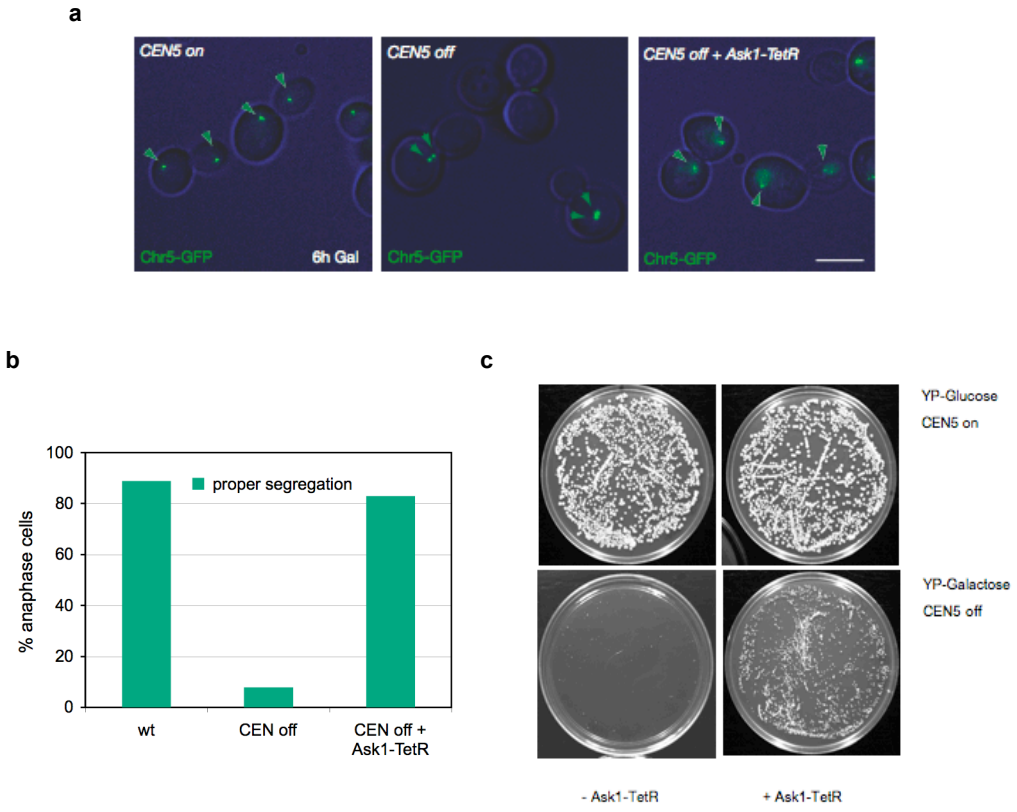


Figure 23: Segregation of native yeast chromosomes. a) Visualization of chromosome V (Chr5-GFP) by binding of TetR-GFP to the *112xtetO$_2$* array. b) Quantification of anaphase cells showing proper segregation of chromosome V under CEN *on* and *off* conditions. c) Plating assay to monitor the mitotic stability of chromosome V under CEN *on* and *off* conditions.

Furthermore, plating equal numbers of cells on galactose rich medium revealed that expression of Ask1–TetR leads to colony formation under CEN *off* conditions (**Figure 23c**). In summary, these results demonstrate that artificial recruitment of the Dam1 complex can promote segregation of a native yeast chromosome under conditions in which the endogenous kinetochore is inactivated.

3 Discussion

3.1 Disruption of the native centromere and stabilization of CDEII-compromised mini-chromosomes

Centromeres act as loading platforms on which kinetochores assemble upon entry into *M phase*[1]. Two of the three centromere DNA elements (CDEI and CDEIII) are conserved between all 16 yeast chromosomes and function as sequence-specific elements by the criterion that single point mutations abolish centromere function[18]. The non-essential protein Cbf1p binds to CDEI whereas CDEIII recruits the inner CBF3 complex which directs the assembly of the remaining kinetochore complexes[16]. By contrast, CDEII is not conserved in sequence but in its approximate length of 76-84 bp and A/T content (~90%)[91]. Centromeric DNA is thought to wrap around a centromere specific nucleosome, in which histone H3 is replaced by a conserved centromeric H3 variant, Cse4p (CenpA in mammals). Most of the contact area between the centromere specific nucleosome and the DNA is provided by CDEII thereby creating a platform which allows the assembly of the inner kinetochore complexes[92]. The DNA wrapped around the Cse4p nucleosome leads to a direct interaction between Cbf1p and the CBF3 complex thereby acting as a protein clamp[19]. The resulting broad interaction surface may be important for stable centromere binding or to provide a large binding surface for more distal kinetochore proteins, or both.

To construct an artificial segregation system we first sought to disrupt a native centromere by inserting an array of *tetO* repeats into the CDEII element. We reasoned that the defect in centromere activity might be easier to overcome by recruiting individual kinetochore proteins to a compromised centromere than tethering them to acentric plamids, which completely lack any centromere sequence.

Insertion of the *7xtetO*$_2$ array into the CDEII element significantly decreased the stability of the respective mini-chromosomes compared to wildtype plasmids but does not completely abolish centromere function like observed for single point mutations in CDEIII (**Figure 9**). The *tetO* insertion extends the length of the CDEII element most likely leading to interferences in the assembly of inner kinetochore complexes, which usually contact this area. These perturbations might affect the overall architecture of kinetochore complexes resulting in weak kinetochore-microtubule attachments, which are prone to break (**Figure 24b**). The recruitment of Ask1- and Dam1-TetR is able to restore strong kinetochore-microtubule interactions probably by tightly connecting the MTs to the inner centromere thereby overcoming the defects in kinetochore geometry induced by the *tetO* insertion (**Figure 10 and 24c**). Similarly, the recruitment of Spc24p is able to restore proper kinetochore architecture.

Introduction of a point mutation within CDEIII abolished centromere function completely (**Figure 9**). A combination of both – *tetO* insertion bound by Dam1-TetR and point mutation within CDEIII – was not sufficient to segregate the plasmid suggesting that an intact CDEIII element is required for proper segregation (**Figure 11**). Most likely the inner CBF3 complex is still bound to the centromere at the CDEII-*7xtetO*$_2$ compromised mini-chromosome.

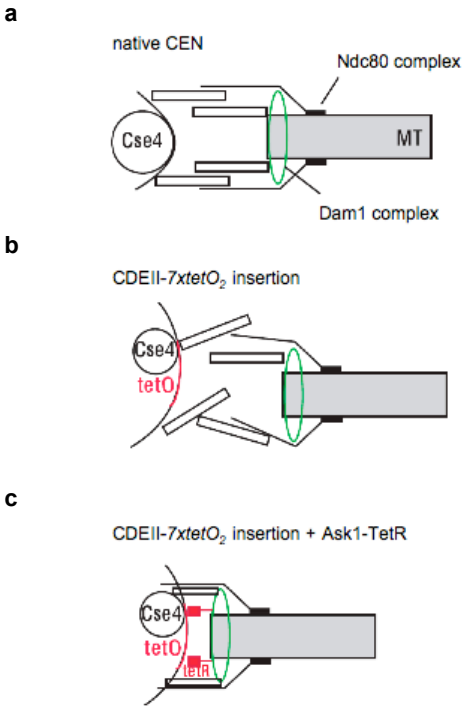


Figure 24: Mode of interaction between the CDEII-*7xtetO*$_2$ insertion and disruption of kinetochore architecture. a) Unperturbed kinetochore-DNA interaction. b) Disruption of kinetochore architecture by insertion of the *tetO* array. c) Recruitment of Ask1-TetR restores the native architecture. Adapted from Westermann, unpublished data.

Mutation of a single base is sufficient to disrupt this interaction thereby preventing the assembly of other core kinetochore and linker complexes as well as the connections to the MTs. Nevertheless it is difficult to predict the precise molecular effect of the *tetO* insertion on kinetochore composition and architecture or analyze which aspects the TetR-fusion restores. Therefore we decided to simplify the system by recruiting the appropriate kinetochore proteins to 'naked' DNA.

3.2 Construction of a kinetochore without a native centromere

Centromeres usually direct the assembly of native kinetochores thereby connecting the chromosomes to the plus-ends of spindle MTs. Remarkably these short sequences get dispensable in budding yeast if MT-force transducing components such as the Dam1 complex are tethered directly to naked DNA (**Figure 13 and 14**). Nevertheless the efficiency of such an artificial segregation system is much lower than for a native kinetochore (**Figure 9 and 13**) since it lacks important core and linker kinetochore complexes (**Figure 19**).

Surprisingly targeting of other kinetochore complexes, which are involved in force-generation, did not lead to the stabilization of acentric *tetO* mini-chromosomes. Spc24p constitutes a subunit of the 4-protein Ndc80 complex, which forms a dimer with Spc25p. The C-terminal globular domains of Spc24p/Spc25p point towards the inner centromere whereas their N-terminal coiled-coil rods interact with Nuf2p and Ndc80p, respectively[93]. The latter two bind to MTs via their calponin-homology (CH) domains albeit with relatively low affinity (Lampert et. al., in press). Recently it has been shown, that the Ndc80 complex is a component of the KMN network constituting the main attachment site for spindle microtubules. The Ndc80 complex alone binds to MTs only with a low affinity under physiological salt conditions but together with KNL1 (the vertebrate counterpart of Spc105p) the binding affinity gets synergistically increased[33].

While Spc24p was able to stabilize the CDEII-*7xtetO*$_2$ compromised mini-chromosomes such an effect could not be detected for the acentric plasmids. Assuming that Spc24-TetR binds to the *tetO* array equally well as the Dam1 complex, one could speculate that the weak binding affinity of the Ndc80 complex is not sufficient to stably connect the acentric *tetO* plasmids to the growing and shrinking ends of the MTs. In support of that there is evidence that the Ndc80 complex requires the Dam1 complex to tightly connect to dynamic MTs (Lampert et. al., in press). Presumably the Dam1 complex does not get recruited along with the Ndc80 complex to the artificial kinetochore in this situation, therefore failing to establish robust kinetochore-MT interactions, which are able to resist the pulling forces of the mitotic spindle. In contrast to that the Dam1 complex is able to stably tether the mini-chromosomes to the plus-ends of spindle MTs while at the same time providing enough flexibility for assembling and disassembling tubulin dimers.

Structural studies on the Dam1 complex have elucidated that it self-assembles into ring-like structures around the MT surface *in vitro*[44,45]. This suggests that the mode of interaction is primarily based on electrostatic forces between the acidic C-terminal tails of α-and β-tubulin and the basic surface of individual Dam1 subunits (mainly of Dam1p and Duo1p). Dam1 rings can slide laterally along the MT lattice and track with disassembling MTs *in vitro* and *in vivo*[46,51]. Therefore the Dam1 complex has been suggested to harness the energy derived from

protofilament peeling and transduce it into the movements of chromosomes during anaphase (**Figure 25a**).

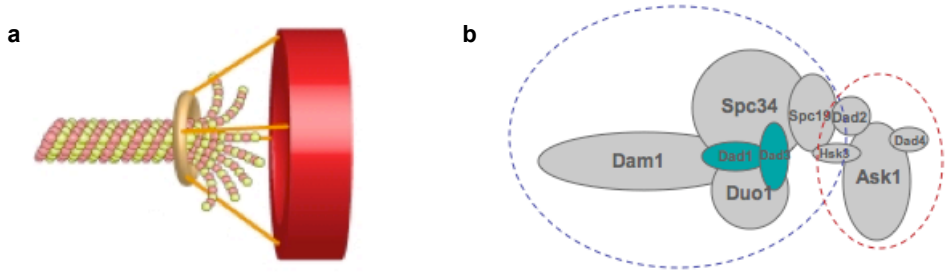


Figure 25: a) Model for coupling chromosomes to dynamic MTs. The Dam1 complex acts as a force-coupler by forming rings, which move with the disassembling tips of MTs when protofilaments curl. Adapted from Santaguida et al., EMBO reviews, 2009. b) Hypothetical architecture of the Dam1 complex. Beside the two stable sub-complexes encircled by dashed lines, Dad1p and Dad3p form a stable dimer (green filling). Adapted from Nogales et al., Journal of Cell Science, 2009.

This model of the Dam1 complex acting as a force-coupler could explain why the recruitment of Dam1- and Ask1-TetR leads to the efficient stabilization of acentric *tetO* mini-chromosomes: by encircling the MTs, the mini-chromosomes get robustly tethered to the plus-ends of dynamic MTs. In contrast to the Ndc80 complex, the Dam1 complex is able to resist the pulling forces exerted by the mitotic spindle and creates stable end-on attachments between the mini-chromosomes and the MT tips. However with the performed experiments we cannot address whether the Dam1 complex assembles into ring-like structures encircling the MTs or if single complexes account for the stabilization of the *tetO* mini-chromosomes. Generally the properties of the Dam1 complex in forming load-bearing attachments to dynamic MTs do not strictly depend on ring formation[94].

Besides Dam1- and Ask1 three other subunits of the Dam1 complex are able to confer segregation capacity to an acentric mini-chromosome (Spc34p, Spc19p, Dad1; **Figure 13**), which are - except Dad1p - among the bigger subunits of the Dam1 complex. One reason for that could be, that the Tet-Repressor is not perfectly accessible when bound to the remaining subunits hindering the binding to the *tet operator* within the mini-chromosome.

Deletion of specific subunits from co-expression vectors results in the formation of a number of sub-complexes leading to a hypothetical architecture of the Dam1 complex (**Figure 25b**)[95]. For instance, depletion of Hsk3 results in the formation of two stable sub-complexes consisting of Ask1p-Dad2p-Dad4p and Dam1p-Duo1p-Spc34p-Spc19p-Dad1p-Dad3p, respectively (dashed lines). From this low-resolution map subunits like Dam1p, Spc34, Ask1p and Duo1p emerge to be more exposed to the surface of the complex whereas smaller subunits bridge between the two sub-complexes and are therefore buried inside. The degree of accessibility of individual subunits

from that theoretical architecture correlates approximately to the ability of the respective TetR-fusion protein to stabilize the acentric mini-chromosomes.

In contrast to the outer Dam1 complex, recruitment of inner kinetochore proteins like Cse4p and Mif2p did not lead to a significant stabilization of the *tetO* mini-chromosomes. At centromeres, the CenH3 variant Cse4p replaces canonical histone H3 in centromeric nucleosomes[20]. Recent data suggest that Cse4p containing nucleosomes induce positive DNA supercoils. This behavior is in contrast to canonical nucleosomes, in which the left-handed DNA wrapping leads to induction of negative supercoils[96]. This right-handed configuration gives rise to an intramolecular loop which projects away from the chromatin and might provide essential interaction domains for kinetochore proteins to assemble on centromeric DNA. In our artificial targeting system, Cse4-TetR may not be able to introduce these changes in chromatin structure and is therefore insufficient in stabilizing the acentric mini-chromosomes under our assay conditions. Even tough it has been shown that acentric mini-chromosomes tend to form more negatively supercoiled DNA after depleting the centromere this does not induce intramolecular loop formation due to the lack of torsional stress (for instance positive super-coiling)[97]. Most likely kinetochores need this specialized chromatin structure and organization to assemble on centromeric DNA.

3.3 Recruitment of multiple kinetochore proteins

Besides the Dam1 complex, the recruitment of Ndc10p and Cep3p – two members of the inner CBF3 complex - seemed to be able to stabilize the *tetO* acentric mini-chromosomes, albeit to a lesser extent (**Figure 13**). Presumably the recruitment of inner kinetochore proteins does not lead to the assembly of a fully native kinetochore so that linker and/or outer complexes are missing, which could establish robust kinetochore-MT interactions. Assuming that the artificial kinetochore bypasses these inner proteins we were wondering whether the simultaneous recruitment of both – inner and outer kinetochore proteins – could enhance mini-chromosome segregation. Therefore strains with two distinct kinetochore-TetR proteins have been generated. A complication that arises is that many combinations appeared to be synthetic lethal or sick (for instance Spc24- and Ask1-TetR). Only the combined tagging of Ask1- and Ndc10-TetR gave viable spores but did not lead to a significant increase in mini-chromosome stability as judged by the plating assay: for the *112xtetO*$_2$ plasmid the mitotic stability was similar to those in the Ask1-TetR background alone (23%±1.2%). Another complication of these experiments is that the two proteins compete with each other for the *tetO* binding sites most likely leading to suboptimal stabilization.

3.4 Molecular composition of the artificial kinetochore

Our data indicate that the recruitment of the Dam1 complex to DNA confers segregation capacity to an acentric mini-chromosome (**Figure 13 and 14**). This raises the question if the artificial tethering system requires additional kinetochore components, which assist in mini-chromosome propagation or whether it works independently of these components. In budding yeast kinetochore-MT interactions are maintained throughout the cell cycle, except for a short time period of 1-2 min during *S phase*. Upon centromere DNA replication, kinetochores are transiently disassembled, causing centromere detachment from MTs[100]. Subsequently they are reassembled and interact with MTs again. This reassembly process occurs rapidly due to the organization of kinetochore proteins into defined sub-complexes, most of which do not disassemble upon DNA replication. Similarly, the artificial kinetochore could be assembled on mini-chromosomes by recruitment of the Dam1 complex leading to the reconstitution of a native kinetochore rather than to an artificial kinetochore.

Analysis in temperature sensitive strains of different inner and linker kinetochore proteins revealed that the artificial segregation system works independently of the CBF3 complex as well as two evolutionary conserved proteins Cse4p and Mif2p (**Figure 19**). This suggests that the inner layer is bypassed by the artificial kinetochore and does not assemble upon binding of the Dam1 complex. Furthermore the function of one of the linker proteins, Mtw1p, which is part of the 4-protein Mtw1 complex, is dispensable for artificial mini-chromosome segregation.

Recently it has been reported that the Ndc80 complex, as well as the Dam1 complex, is able to form load-bearing attachments to dynamic MT tips[38]. Therefore we tried to address whether the Ndc80 complex assists the dam1-/ask1-based kinetochore in segregating acentric mini-chromosomes. However due to severe spindle defects in these temperature sensitive mutants (*ndc80-1; ndc80-2*), which became even stronger due to synthetic effects between the tagged Dam1 subunits and the Ndc80 mutants, we could not draw any conclusions from these experiments.

Overall, our studies in temperature-sensitive kinetochore mutant strains confirm, that the artificial segregation system - mediated by the Dam1 complex – exhibits a different and simpler functionality than a native kinetochore. Major components of inner and linker proteins are not required suggesting that it is indeed a simplified, more primitive segregation system. Whether it is also a minimal kinetochore consisting exclusively of the Dam1 complex, remains to be answered in the future.

3.5 Integrity of the Dam1 complex

Biochemical purification of the Dam1 complex in the absence or presence of Ask1-TetR led to the *in vitro* reconstitution of the entire hetero-decameric complex even under stringent washing conditions (400 mM KCl; **Figure 15**) confirming that all subunits are tightly associated with each other. So far there is no evidence that single subunits of the Dam1 complex function outside the context of the complex. One of the most potent proteins in stabilizing the acentric *tetO* mini-chromosomes – Ask1p – does not harbor an intrinsic MT-binding affinity by itself[95], suggesting that other subunits of the Dam1 complex fulfill this function. Therefore we assume that the entire Dam1 complex assembles at the site of the *tetO*-TetR interaction rather than individual subunits. This hypothesis is further strengthened by the fact that inactivation of Dad1p - one of the Dam1 subunits - leads to failure of the artificial segregation system (**Figure 19**).

3.6 Establishment of bi-orientation

The distribution of sister chromatids into a pair of daughter cells in anaphase depends on the prior capture of sister kinetochores by MTs emanating from opposing spindle poles (amphitelic attachment or bi-orientation)[58]. The conserved kinase Ipl1p was suggested to promote chromosome bi-orientation by altering kinetochore-spindle pole connections in a tension dependent manner. It is involved in detecting and destabilizing faulty kinetochore-MT attachments, which are characterized by lack of tension due to missing pulling forces exerted by MTs with opposing orientations[59,63].

Analysis in temperature sensitive strains revealed that Ipl1/AuroraB and its co-activator Sli15p/INCENP are still required for proper mini-chromosomes segregation mediated by the artificial kinetochore (**Figure 19**), suggesting that regulation by Ipl1p might also apply for the artificial segregation system. This hypothesis is further strengthened by the co-purification of sub-stoichiometric amounts of Ipl1p during tandem affinity purifications of the Ask1-TetR containing Dam1 complex (**Figure 15**). The presence and function of Ipl1p could explain why acentric *tetO* mini-chromosomes bi-orient efficiently along the spindle axis in the Dam1-/Ask1-TetR background as observed by live cell imaging (**Figure 18c**). Acentric *tetO* sister mini-chromosomes are still held together by cohesion even though they do not harbor any centromeric sequence, which constitutes the major site of cohesion[101]. Possibly, the residual amount of cohesion is sufficient to establish enough tension, which can be recognized by Ipl1p. Faulty mini-chromosome-MT attachments, which do not generate tension across the sisters, could be efficiently corrected presumably by Ipl1p-mediated phosphorylation of the Dam1 complex thereby lowering its affinity for MT-binding. However the process of recapturing unattached mini-chromosomes has to happen within a short time interval since they do not activate the spindle assembly checkpoint,

which is supposed to delay the cell cycle in the presence of erroneous attachments (**Figure 21**). In budding yeast the spindle assembly checkpoint is dispensable in an unperturbed mitosis arguing that there is enough time provided for the cell to correct improperly attached kinetochores. This circumstance might explain why Ipl1p is capable of correcting errors even though the cell cycle does not halt upon SAC activation. Furthermore Ipl1p/Sli15p might act as a structural component of the artificial kinetochore tightening the interactions between the Dam1 complex and the MTs.

In order to study the role of Ipl1p in mediating mini-chromosome bi-orientation in more detail, a system was generated, which allows arresting cells in metaphase by shutting of the expression of Cdc20p, an activator of the APC. By comparing the metaphase behavior of wildtype, acentric and *tetO* mini-chromosmes in the Ask1-TetR background, we can address whether *tetO* acentric mini-chromosomes bi-orient in a manner similar to wildtype mini-chromosomes. These experiments should clarify the role of Ipl1p/Sli15p during mini-chromosome bi-orientation and error correction.

3.7 Alternative mechanisms of acentric mini-chromosome propagation

Non-centromeric plasmids exhibit a strong bias for the mother cell[98]. This asymmetric segregation pattern was suggested to rely on the anchoring of acentric plasmids to nuclear pore complexes (NPCs) which are retained in the mother cell due to a diffusion barrier within the nuclear envelope[99]. Theoretically, the dam1-/ask1 based segregation system could interfere with such a tethering mechanism and simply eliminate the bias of these plasmids to stay in the mother cell. However there is no evidence that the Dam1 complex shows any interactions with nuclear pore proteins. Furthermore, acentric mini-chromosomes do not appear to be tethered to the nuclear envelope as judged by live cell imaging. Over-expression of LacI-GFP leads to an excess of GFP, which allows the visualization of the whole nucleus. We could not observe a strong bias of mini-chromosomes for the nuclear rim as expected for anchored plasmids. Instead they were moving randomly within the nucleus (**Figure 16 and 18b**). By recruiting Dam1- or Ask1-TetR, mini-chromosomes aligned along the spindle axis and showed directed movements relative to the spindle poles (**Figure 18c**). Therefore it seems rather unlikely that the TetR-fusion simply interferes with a mechanism, which anchors non-centromeric plasmids to the nuclear envelope.

3.8 Complexity of native kinetochores

Our data suggest that the recruitment of a MT-binding complex is sufficient to promote chromosome segregation in budding yeast. This mechanism – connecting DNA to a dynamically instable polymer – has been used already by primitive prokaryotes like enteric pathogens to partition drug-resistance plasmids[5]. The *par* operon encodes for a minimal spindle consisting of

three components: *parC*, ParR and ParM. *parC* appears as a stretch of centromeric DNA which includes the *par* pomoter and is bound by the repressor protein ParR[102]. ParM is an actin homolog that polymerizes in an ATP-dependent manner into two-stranded helical filaments, which bind specifically to the ParR-*parC* complex[103,104]. Polymerization of ParM has been suggested to generate enough force to push the plasmids to opposite poles of the cell.

If a primitive segregation system like that described above allows efficient distribution of genomic material, why did such complicated structures like kinetochores arise during evolution?

Even though it seems to be sufficient to tether MT-force transducing elements, like the Dam1 complex, to DNA, this segregation system is more susceptible to errors occurring during the cell cycle. One important feature of native kinetochores is the recruitment of checkpoint proteins, which activate the SAC during a perturbed mitosis. This allows the cell to gain additional time to establish proper attachments between kinetochores and MTs. While the artificial segregation system is able to robustly connect mini-chromosomes to spindle MTs it does not provide additional time for error correction mediated by the SAC (**Figure 21**). Simple segregation systems like the dam1-/ask1-based system suffice to propagate acentric plasmids for a number of cell cycles. However to stably maintain genomic material, the cell needs to integrate the mechanical state of kinetochores with cell cycle progression through signaling pathways like the SAC.

3.9 Outlook

Assuming that the artificial segregation system bypasses inner and linker kinetochore proteins, the question still remains whether it also represents a minimal segregation system consisting exclusively of the Dam1 complex. To address this issue, mini-chromosomes can be purified from yeast cultures using antibody-coupled magnetic beads as previously reported[105]. Subsequent mass spec analysis could reveal possible interacting partners, which assist the Dam1 complex in mediating acentric mini-chromosome segregation. This approach could shed light on the presence of the Ndc80 complex and whether it is involved in the artificial segregation system. Furthermore, the behavior of purified mini-chromosomes can be observed by total internal reflection microscopy (TIRF). Reconstitution experiments *in vitro* could shed light on the mode of interaction between the components of the artificial segregation system with dynamic MTs.

In addition to the purification of mini-chromosomes, native yeast chromosomes harboring the dam1-/ask1-based artificial kinetochore can elucidate the precise protein composition of this primitive segregation system. Chromatin immunoprecipitation (ChIP) on native chromosomes is commonly used to identify DNA interacting components. Together with biochemical preparations of yeast mini-chromosomes the composition of the artificial kinetochore can be analyzed in more detail and shed light on the architecture and function of this primitive segregation system.

Furthermore the system is well suited to characterize the artificial kinetochore *in vivo* and compare it to a native yeast kinetochore. Chromosome tagging by the *tetO*/TetR or *lacO*/LacI system allows the simultaneous visualization of native chromosomes and chromosomes carrying the artificial kinetochore within the same cell. Live cell imaging offers the opportunity to compare the mode of segregation in real time and might reveal differences in terms of timing between chromosomes carrying the artificial and native kinetochore.

Finally, as mentioned above the artificial kinetochore is a well-suited system to study the process of bi-orientation during metaphase and in particular the role of Ipl1p and its co-activator Sli15p, which are still required for proper *tetO* mini-chromosome segregation.

4 Additional results – Characterization of the Spc105 complex

4.1 The central kinetochore

4.1.1 The eukaryotic KMN network

One fundamental role provided by kinetochores is the generation and transduction of motile force, which leads to the segregation of chromosomes. The KMN network (**N**dc80 complex, **M**is12 complex and **K**NL-1 complex) was suggested to constitute a highly conserved core microtubule-binding network of proteins, mediating the interactions between DNA and the plus-ends of kinetochore MTs[33,104]. It's major role in that process is to couple the force, which is generated during MT-depolymerization into the movements of chromosomes. In addition to the KMN network, the Dam1 complex is essential to ensure proper chromosome segregation in yeast. Since it was discussed in detail in 1.2.2.3, this section will focus on the former network.

The best-characterized complex of the KMN network is the Ndc80 complex (see 1.2.2.2 and 1.2.2.3), which constitutes the principle MT-attachment site at the kinetochore in all eukaryotes. *In vitro* studies have shown that the mode of interaction between MTs and individual Ndc80 complexes is based primarily on electrostatic interactions between the basic N-terminal tails of Ndc80p and the acidic C-terminal tails of $\alpha\beta$-tubulin subunits[105]. The N-terminal tail is also subject of Ipl1p mediated phosphorylation, which lowers the binding affinity of Ndc80p to MTs[106]. Surprisingly, this N-terminal tail is not essential in budding yeast[107]. Additionally, the two calponin-homology (CH)- domains in the Ndc80p/Nuf2p-subunits are thought to participate in MT-binding[105]. The Ndc80 complex connects to the inner kinetochore via the filamentous Mis12/-Mtw1 complex (see 1.2.2.2).

In contrast to the Ndc80 complex, relatively little is known about the KNL-1 protein (the Spc105p counterpart in budding yeast). RNAi mediated depletion of KNL-1 in *C.elegans* results in a **k**inetochore **n**u**l**l (knl1) phenotype, which arises from a failure to form the MT-binding outer domain of the kinetochore[108]. In human cells, depletion of hSPC105 (also termed Blinkin or AF15q14) by RNAi causes detachment of sister chromatids from the spindle without loss of the human NDC80 complex[72]. In mammalian cells as well as in *C.elegans*, hSPC105/KNL-1 has important implications in recruiting checkpoint proteins such as Bub1, BubR1 and Zwint[72,108] to the kinetochore. Recently it has been shown that in Drosophila loss of Spc105 activity leads to aberrant kinetochore assembly and inability of sister chromatids to align along the spindle axis. Interestingly the spindle checkpoint is active in these cells lacking Spc105 activity[109].

4.1.2 The budding yeast Spc105 complex

Budding yeast Spc105p was first identified as a protein that co-purified with components of enriched spindle pole body extracts[110]. These spindle pole preparations also contained centromere and kinetochore components due to the close proximity of these structures to SPBs during most of the cell cycle. ProteinA tagging of individual identified subunits revealed biochemical interactions between Spc105p and YDR532Cp, which are thereupon referred to as the Spc105 complex[111]. Furthermore the complex was shown to co-purify with subunits of the Mtw1- and COMA complex[24]. Fluorescence and electron microscopy demonstrated that both proteins are kinetochore components, which localize to CEN DNA in an Ndc10p dependent manner[111]. Depletion of Spc105p leads to cell death and temperature sensitive mutants fail to segregate their chromosomes properly due to defective kinetochore-MT attachments similarly to the depletion of KNL-1 in *C.elegans* and Blinkin in mammalian cells[72]. In contrast to that, the interacting partner YDR532Cp is annotated to be non-essential for viability in the S288C strain background. However in a different genetic background (W303), it was also described to be essential for cell growth[112].

Biochemical analysis demonstrated that Spc105p and YDR532Cp form a 1:2 heterotrimeric complex, which exhibits an elongated shape. Its recruitment to CEN DNA depends exclusively on the presence of the CBF3 complex as judged by chromatin immunoprecipitation but not on other DNA-binding or linker complexes[112]. Furthermore the Spc105p/YDR532Cp complex is required to recruit several kMAPs and kinesins to kinetochores including Bim1p, Bik1p, Cin8p and Kar3p.

Similar to the vertebrate KNL-1, scSpc105p binds to MTs *in vitro*, albeit with a low affinity (K_D: 1.2±0.6μM) compared to other MT-binding proteins (K_D of the Ndc80 complex: 0.2-0.8μM).

In addition, Spc105p is required in budding yeast to recruit checkpoint proteins such as Mad2p and Bub1p like observed for human Blinkin[112].

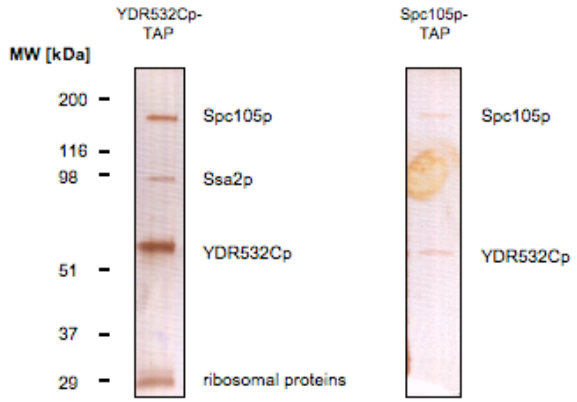
In contrast to the *C.elegans* KMN network, little is known about the corresponding budding yeast components, how they interact with each other or how they connect to the inner core kinetochore. Therefore it is important to characterize individual components as well as their behavior in the context of the whole network. One approach to answer this question is to biochemically purify single sub-complexes and reconstitute them *in vitro*. Furthermore this approach could shed light on some regulatory aspects of how the assembly of the KMN network is organized. Protein modifications such as phosphorylation contribute to the regulation of protein interactions by altering, for example, binding affinities. This work concentrates exclusively on the reconstitution and characterization of the Spc105 complex since it is the most poorly described complex of the KMN network.

4.2 Reconstitution of the Spc105 complex

4.2.1 Purification of Spc105 complex from yeast extracts

In order to search for interacting proteins and detect posttranslational modifications, the Spc105 complex was isolated from yeast extracts by tandem affinity purification of either an Spc105p-S-tag-TEV-ZZ or YDR532Cp-S-tag-TEV-ZZ tagged strain and the protein composition analyzed by mass spectrometry (**Figure 26**).

a



b

Protein	Molecular weight [kDa]	Number of peptides
Spc105p	104.8	30
YDR532Cp	44.7	6
Ssa2p	69.4	11
M Chain M, Structure Of The Ribosomal 80s-Eef2-Sordarin Complex	15.4	8
Protein component of the large (60S) ribosomal subunit	27.4	5
Ribosomal protein 59 (rp59) of the small (40S) ribosomal subunit,	14.6	7

Figure 26: Tandem affinity purification of the Spc105 complex. a) Silver stained SDS gels of isolated proteins from YDR532Cp-TAP and Spc105p-TAP purifications. Corresponding proteins are assigned on the right side. b) List of identified proteins from in solution digest of a). Only hits with the 7 most abundant peptides are listed. Numbers of identified peptides are indicated in the right column.

Purification of YDR532Cp-S-tag-TEV-ZZ led to a much higher yield of protein eluted from the beads (**Figure 26a**; left lane) than the corresponding purification of Spc105p-S-tag-TEV-ZZ (right lane). For that reason mass spec analysis was carried out only on the YDR532C sample (**Figure 26b**). Nevertheless, in both purifications the full complex could be isolated, albeit in a relatively low yield. In addition to Spc105p and YDR532Cp the heat shock protein Ssa2, which acts as a chaperon and some ribosomal subunits co-purify with the complex. Since the chaperon Ssa2 is also present after the isolation of Spc105p over-expressed from a galactose-inducible promoter (see next section), it might be required to ensure proper folding of the complex thus avoiding its aggregation.

Unfortunately, no other KMN network components or novel interaction partners could be detected in the eluates. Generally the employed purification procedure is a rather stringent method for protein isolation since it comprises two tags (ZZ-tag and S-tag) allowing repeated coupling of proteins to specific antibody coupled beads. This two-step purification procedure might lead to the

loss of weakly interacting partners. Furthermore, the purification was carried out under relatively high salt conditions (300mM KCl), which may eliminate weak protein-protein interactions. Nevertheless even under less stringent purification conditions (150mM KCl) no other kinetochore protein could be detected by mass spectrometry.

4.2.2 Identification of phospho-sites

Protein modifications, for instance phosphorylation, have important implications in many regulatory and signaling pathways. Therefore phospho-peptides of Spc105p and YDR532Cp have been identified from the previous affinity purification by mass spectrometry after trypsin digest. For YDR532Cp, no phospho-peptides could be detected. However, the sequence coverage – the number of identified peptides of a certain protein relative to the total number of respective peptides – did not exceed 53% leaving the possibility that some of the remaining peptides might be phosphorylated. In contrast to that, seven phospho-peptides could be identified for Spc105p. Similar to the YDR532Cp sample, the sequence coverage for Spc105 was only 33%, leaving open the possibility that addional phosphorylation sites exist. Nevertheless, three out of the seven identified phospho-sites match the consensus site of the major yeast cell cycle kinase Cdc28p (S/T*PxK/R).
To increase the yield of purified protein and facilitate the analysis of eventual phospho-sites, the protein was isolated after overexpression from a galactose (GAL)-inducible promoter and subsequent affinity purification.

4.2.3 Purification of over-expressed Spc105p from yeast extracts

To increase the protein yield, Spc105p was over-expressed from a GAL-promoter on a multi-copy plasmid. A C-terminal TEV-cleavage site prior to a myc-tag allows release of the isolated protein from anti-myc coated beads. By using this system, the protein can be expressed in reasonable amounts and efficiently coupled to α-myc coated beads (**Figure 27**; left lane). However, only a small fraction of bound protein is eluted efficiently from the beads together with the heat shock protein Ssa2, which has been identified in the previous TAP-tag purification. One possible explanation for the low yield could be that the protein adheres non-specifically to the beads after TEV-cleavage due to hydrophobic interactions.
Furthermore the yield of eluted protein decreases due to proteolysis during TEV-cleavage since a faster migrating protein band just below the full-length protein can be identified. Western blot and mass spec analysis identified the lower band as C-terminal Spc105p-truncation (Spc105Δ).

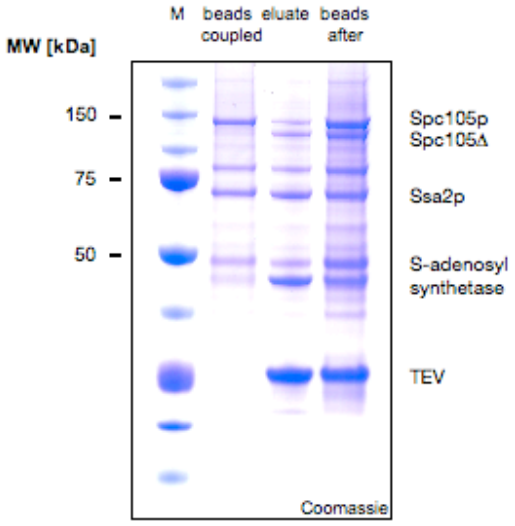


Figure 27: Purification of Spc105p after over-expression from a galactose-inducible promoter. Left lane: total amount of bound protein to the beads; middle lane shows the eluate after TEV cleavage and the right lane a fraction of beads after TEV cleavage. Corresponding proteins are assigned on the right side as identified by mass spectrometry.

Similarly to the previous TAP-tag purification, no known or novel interacting partners could be identified - including YDR532Cp. Attempts to further purify the protein by applying the eluted fraction to a size exclusion column failed due to the big size and low amounts of Spc105p in the eluates. Phospho-peptide mapping of trypsin-digested samples revealed a couple of additional phosphorylation sites, which are summarized in **Figure 28**. Due to the increased amount of Spc105p after over-expression from the Gal-promoter, every possible peptide derived from the trypsin digest could be identified (sequence coverage of 100%).

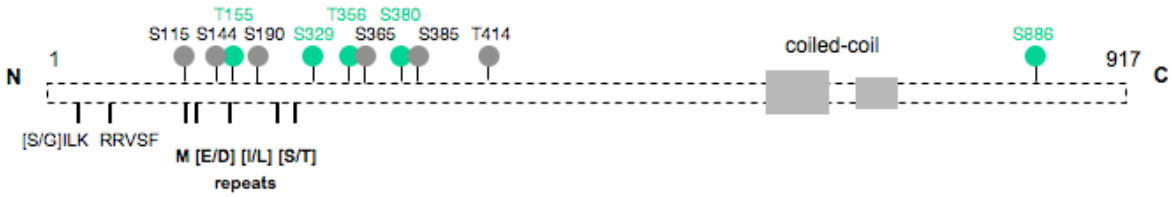


Figure 28: Spc105p protein organization and mapped phospho-sites. Besides a C-terminal coiled-coiled domain, which favors the oligomerization and interaction of proteins, the N-terminus contains two additional motifs [S/G] ILK and RRVSF as well as several MELT repeats. These motifs are conserved between human, vertebrate and yeast Spc105p[104]. Phospho-sites are derived from Tap-tag purification and from galactose induced over-expression. Green circles indicate cdc28 specific phosphorylation sites.

In total, 11 different phosphorylation sites could be identified, most of which are clustered in the N-terminal half of the protein. Among them, five match the consensus site of Cdc28p, the CDK1 homolog in budding yeast (depicted as green circles). Only one phosphorylated serine was found close to the C-terminus, which is most likely a target of Cdc28p.

Overall, Spc105p harbors six cdc28 consensus sites of which five were found to be phosphorylated *in vivo* raising the possibility that Spc105p is a target of this major cell cycle kinase in budding yeast. Furthermore, quantitative proteomic analysis of purified yeast kinetochores recently showed, that even the cdc28 consensus site S109, which was not detected in our analysis, can be phosphorylated *in vivo*[103].

4.2.4 Purification of Spc105p-myc from whole cell extracts by size exclusion chromatography

We sought to further purify the overexpressed Spc105 protein by applying whole cell extracts directly on a gelfiltration column (**Figure 29a**).

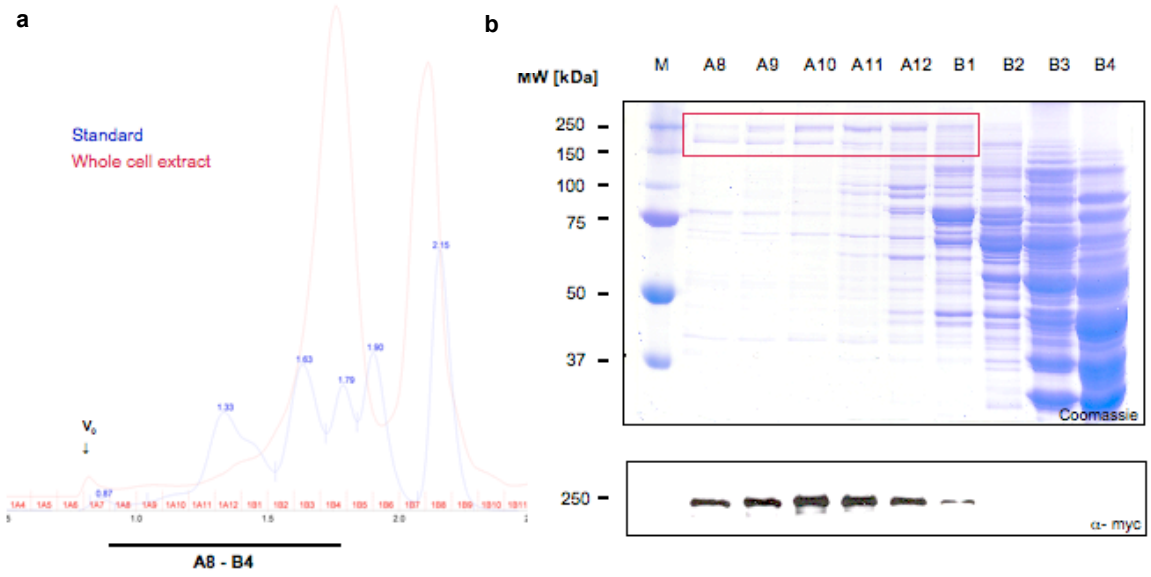


Figure 29: Size exclusion chromatography of yeast whole cell extracts. a) The extract was applied to a Superose6 column collecting 100µl fractions. V_0 indicates the dead volume of the appropriate column. b) Fractions A8-B4 were loaded on a 10% SDS-gel and stained with Coomassie to analyze its protein composition. α-myc Western blotting confirmed the presence of Spc105p.

The red curve depicts the elution profile of the WCE compared to standard proteins (blue curve). After the void volume (V_0), a small peak eluted whose fractions were analyzed on an SDS-protein

gel by Coomassie staining and Western blotting (**Figure 29b**). Interestingly, these early-eluting fractions did not contain the bulk of cellular protein, but a considerable amount of Spc105p. Additionally, the chaperon Ssa2 co-eluted in a stochiometric manner just as observed in the previous purifications, suggesting that they form a tight complex. The interacting partner YDR532Cp could not be detected even by mass spectrometry. Immunoblotting against the myc-tag showed that the protein does not elute as a sharp peak but over a broader range of five fractions. Nevertheless most of the protein peaks in fractions A10 and A11.

The early elution position suggests that multiple Spc105p molecules may oligomerize into a larger complex, or that the protein exhibits an elongated shape, which prevents its permeation into the gel matrix of the column. Additionally, a combination of both could be possible.

Hydrodynamic analysis of the budding yeast Spc105p has suggested a trimeric complex consisting of one subunit Spc105p and two subunits of YDR532Cp with an apparent molecular weight of 218kDa[112]. However such a trimeric complex cannot explain the elution profile since the standard protein thyroglobulin, which possesses a molecular weight of 660kDa, exhibits a similar elution pattern arguing for an elongated stretched protein shape of Spc105p. Other yeast kinetochore complexes, including the Ndc80 complex, have similar elution profiles and have been shown by electron microscopy to form rod-like structures[35].

4.3 Phospho-regulation of the Spc105 complex

4.3.1 *In vitro* phosphorylation of Spc105p by Cdc28p-Clb2p

To investigate the functional importance of the identified phosphorylation sites within Spc105p, they were mutated to alanine and aspartic acid, to either prevent or mimic phosphorylation, respectively. Since only five of the eleven identified phospho-sites follow a clear consensus pattern, we focused on the six cdc28 sites – five of which have been mapped *in vivo* - and generated Spc105^{6A} and Spc105^{6D} mutants, in which all possible cdc28 phosphorylation sites are manipulated. *In vitro* kinase assay using purified protein confirmed Spc105p as a substrate of Cdc28p (**Figure 30**).

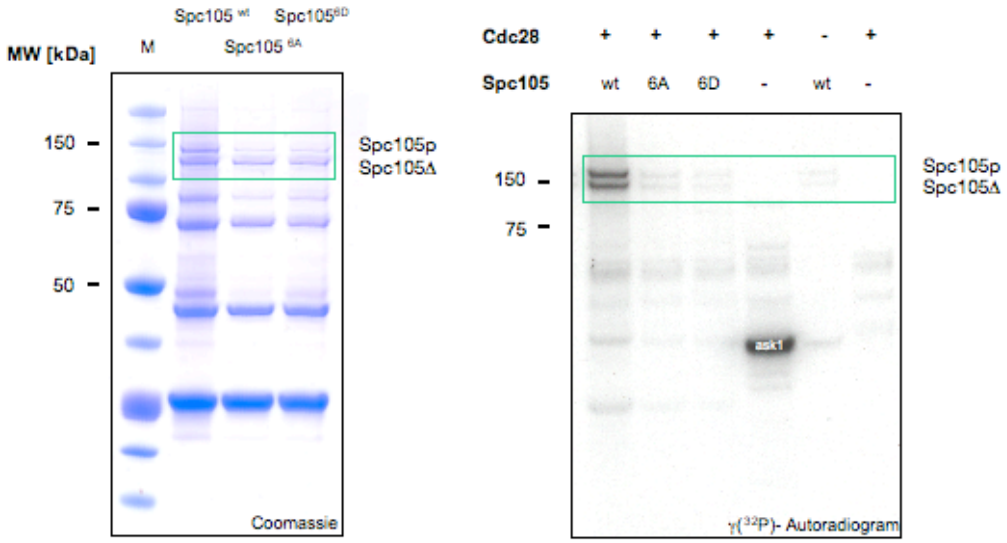


Figure 30: ***In vitro*** **kinase assay of purified Spc105p variants with Cdc28p. Left panel shows Coomassie stained gel of purified proteins; right panel the autoradiogram after introduction of radioactively labeled phosphate [γ(^{32}P)]. Cdc28p was purified as a complex with its corresponding activator Clb2p.**

While wildtype Spc105p is efficiently phosphorylated by purified Cdc28p-Clb2p, the two phospho-mutants show reduced signals confirming Spc105p as a Cdc28p target *in vitro*. Purified Dam1 complex served as a positive control since the subunit Ask1p is a known target of Cdc28p. As expected, also the C-terminal truncation Spc105Δ is efficiently phosphorylated by cdc28 since only one site (S886) is missing (see **Figure 28**).

4.3.2 *In vivo* analysis of Spc105 phospho-mutants

To further characterize the role of cdc28-mediated phosphorylation of Spc105p the generated phospho-mutants were introduced into a heterozygous Spc105 knock-out strain and subjected to tetrad analysis. All four spores are viable and did not show a severe growth phenotype at 37°C. However the Spc105^{6A} mutant displays slightly compromised growth at 37°C (**Figure 31**; red rectangle) compared to Spc105^{wt}, whereas the Spc105^{6D} mutant does not show any noticeable growth phenotype. Furthermore, growing the strains on plates supplemented with Thiobendazol (TBZ; right panel), a microtubule-destabilizing drug, did not compromise cell growth as observed for checkpoint mutants. Strains lacking *MAD2* for instance (*mad2Δ*), fail to activate the spindle assembly checkpoint in the presence of MT poisons like TBZ leading to severe chromosome missegregation. Normal growth on TBZ plates at 30°C implies, that the Spc105 phospho-mutants are checkpoint proficient thus allowing proper chromosome segregation even when microtubules

are destabilized. Activation of the spindle assembly checkpoint normally leads to a cell cycle delay, providing enough time for the cell to establish stable kinetochore-MT attachments during a perturbed mitosis. The activation of the SAC could account for the slightly decreased growth of the Spc105^{6A} mutant at 37°C.

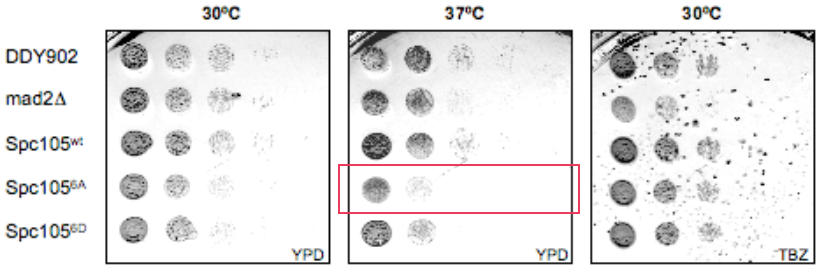


Figure 31: Spot assay of Spc105 wildtype and phospho-mutants on YPD- at 30°C and 37°C (left panels) and TBZ plates at 30°C (right panel). The corrsponding strain background is indicated at the left side.

4.3.3 Disturbed kinetochore organization in Spc105p phosphorylation deficient mutants

The spindle assembly checkpoint monitors defects in kinetochore-MT attachments, which could be the result of a disturbed kinetochore organization. To address this issue, a kinetochore marker (Mtw1p-GFP) was introduced into the appropriate Spc105p phospho-mutant strains, which allows visualization of the kinetochore configuration in fixed metaphase cells (**Figure 32**).

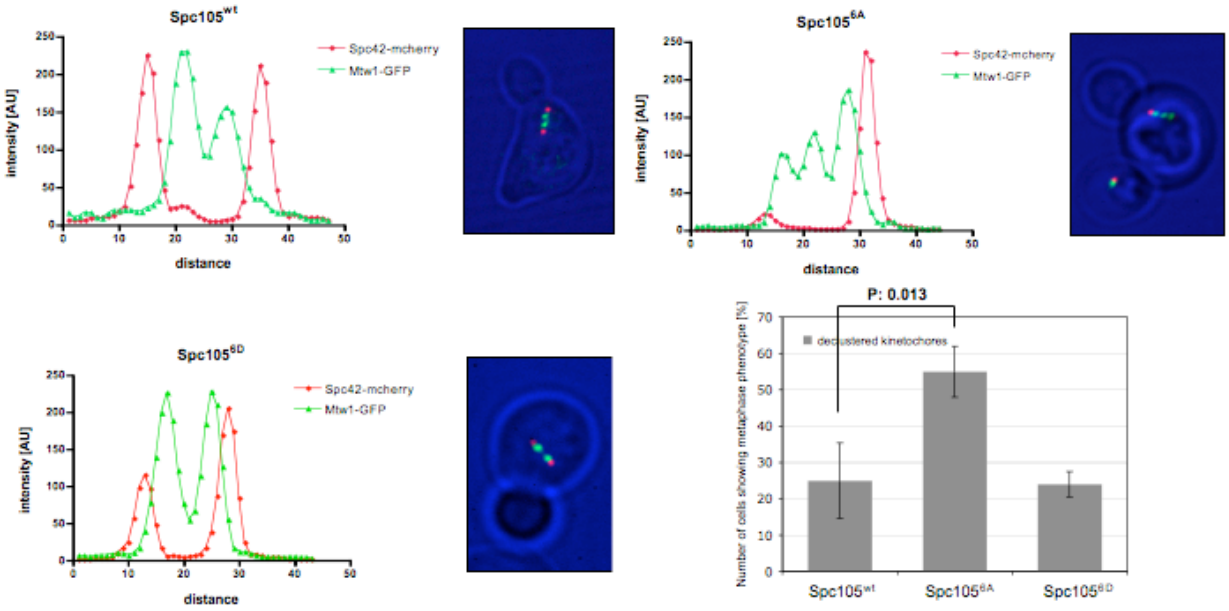


Figure 32: Visualization of kinetochore organization in Spc105 wildtype and phospho-mutant strains. Line scans were obtained from 2D projections of the adjacent picture. Bars depict the number of metaphase cells that show declustered kinetochores. Error bars denote ± s.d. of 3x100 cells.

In addition to Mtw1p-GFP, the spindle pole bodies are tagged with Spc42-mCherry to allow the visualization of the mitotic spindle. In wildtype cells, kinetochores cluster into their characteristic bilobed metaphase configuration (upper left panel) with two distinct foci between the two spindle pole bodies. In contrast to that, the bilobed clustering is disturbed in the Spc105^{6A} mutant as judged by supernumerary GFP foci (upper right panel). Line scans along the spindle axis illustrate the kinetochore configuration based on the Mtw1p-GFP signal. Unlike the Spc105^{6A} mutant, the Spc105^{6D} mutant resembles the kinetochore configuration of an Spc105 wildtype strain with kinetochores resolved into two distinct foci located between the two spindle pole bodies (lower left panel).

A similar phenotype has recently been described for deletion of the kinesin-5 family member Cin8p[113]. The authors suggest that Cin8p is involved in establishing or maintaining a normal metaphase configuration by bundling k-MTs. Furthermore Spc105p is thought to recruit several kMAPs like Slk19p, Bim1p, Bik1 and Cin8p to kinetochores[112]. To address whether in the Spc105^{6A} mutant Cin8p fails to localize to kinetochores and thus could account for the observed phenotype, the protein was visualized as a Cin8p-redstar fusion protein (**Figure 33**).

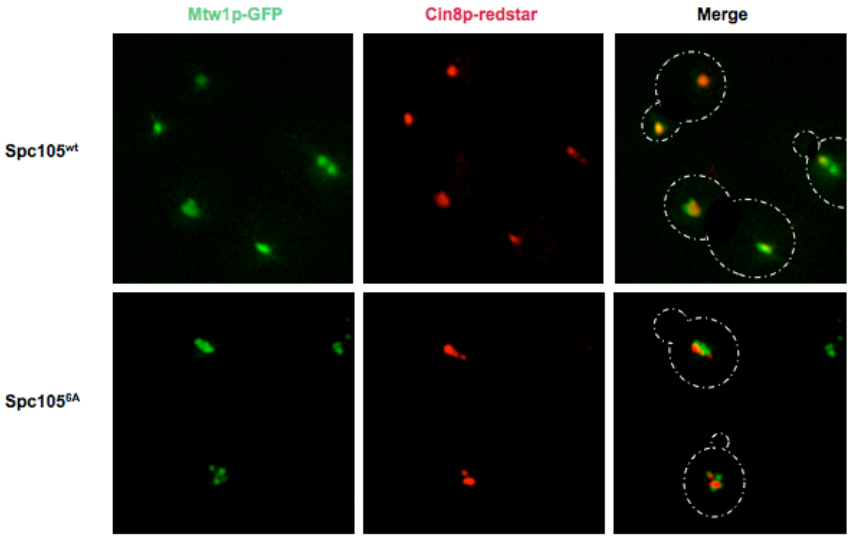


Figure 33: Visualization of Cin8p localization in Spc105^{wt} (upper panel) and Spc105^{6A} mutant (lower panel) strains. Right panels show merged signals of Mtw1p-GFP and Cin8p-redstar images.

Due to the faint signal of the fusion protein it is difficult to quantify the amount of Cin8p at kinetochores. Nevertheless, even when kinetochores were declustered in the Spc105^{6A} mutant, some cells clearly showed co-localization of Cin8p-redstar with the Mtw1-GFP signal, making it unlikely that a failure in Cin8p recruitment accounts for the observed phenotype. By contrast, it seems more plausible that Spc105p by itself is required to establish and maintain a proper metaphase configuration, similar to the human homolog Blinkin and the vertebrate KNL1, which have been suggested to establish proper chromosome alignment at the metaphase plate[72,114].
In addition to the declustered kinetochore configuration in the Spc105^{6A} mutant, several cells showed only a faint signal for one of the spindle poles (**Figure 32**; upper right panel). This phenomenon could be observed preferentially for the new SP, which stays in the mother cell. Presumably this arises from a delay in the maturation of the respective SPB in the Spc105^{6A} mutant.

4.4 Spc105p binds to microtubules *in vitro*

To address whether Spc105p harbors an intrinsic binding affinity for MTs, the purified protein was incubated with taxol-stabilized MTs and subsequently pelleted in an ultracentrifuge. Analysis of supernatants and pellets on an SDS-gel revealed, that a considerable amount of protein co-pelleted with MTs indicating that Spc105p is indeed able to bind to taxol stabilized MTs *in vitro* (**Figure 34**). In the absence of MTs the protein remains in the supernatant (left lane; - MTs). Increasing amounts of taxol-stabilized MTs shift the protein to the pellet fractions in a

concentration dependent manner. In addition to Spc105p, the heat shock protein Ssa2 is transferred to the pellet fractions presumably because it is required to ensure proper protein folding. Whether the interacting partner YDR532Cp assists in binding to MTs could not be addressed since the protein did not co-purify with Spc105p after overexpression.

Recent studies have shown that phosphorylation can lower the binding affinity of certain kinetochore components like the Ndc80 complex to MTs due to the introduction of negative charges. The phosphorylation state did not seem to influence Spc105 binding to MTs, as the phospho-mutants bound to MTs with similar efficiency (data not shown). Thus, the declustering of kinetochores in metaphase is probably not caused by disrupted kinetochore-MT attachments in the Spc105^{6A} phospho-mutant.

Similar to budding yeast Spc105p, vertebrate KNL-1 harbors an intrinsic binding affinity for MTs suggesting that this property of Spc105p is evolutionary conserved between different species[33].

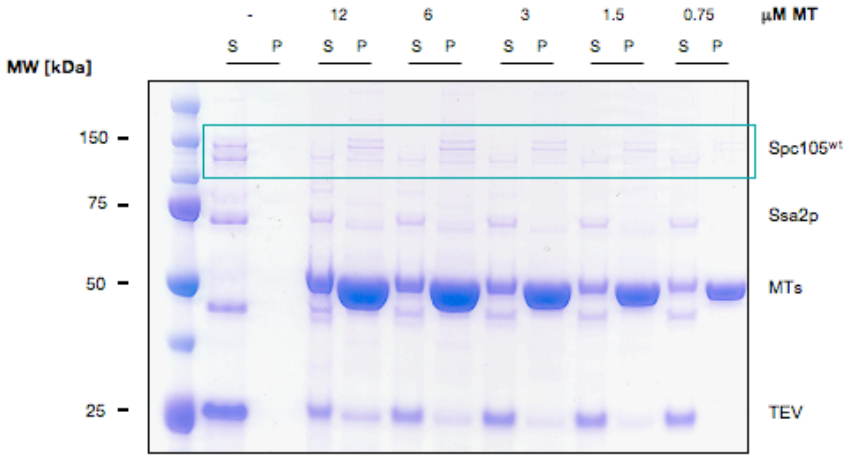


Figure 34: Microtubule-binding assay with purified Spc105p. Equal amounts of protein were mixed with taxol stabilized MTs and centrifuged in an ultracentrifuge. Supernatant and pellets were separated and loaded on a 10% SDS-gel. Spc105p fractions are highlighted with a blue frame.

4.5 Purification of Spc105p from bacteria

In addition to the over-expression of Spc105p in yeast cells, other systems have been tried to obtain recombinant protein. Several attempts to express either the full-length protein or different Spc105p fragments in *E.coli* failed, probably due to the large size and hydrophobicity of the protein. Nevertheless we were able to express an N-terminal portion of Spc105p, which comprises aminoacids 1-411 fused to an N-terminal his-tag (Spc105$^{N\text{-terminus}}$). Induction with IPTG led to the expression of a protein migrating at the expected size of 75kDa (**Figure 35a**). Western blotting with an α-his antibody confirmed the presence of Spc105$^{N\text{-terminus}}$ (not shown). The N-

terminal his-tag allows the purification via a nickel-NTA column, which results in already quite pure protein fractions (**Figure 35b**, left lane). Subsequent size exclusion chromatography further increased the purity of the sample (**Figure 35b,c**). The purified protein was applied to a MT-binding assay as described above, but surprisingly the protein remained exclusively in the supernatant even at higher MT concentrations (**Figure 35d**). This implies that the N-terminal part of Spc105p does not contribute to MT-binding as has been suggested for KNL1[33]. However since we were not able to express the C-terminal half of Spc105p, it is difficult to conclude that this missing part of the protein is responsible for MT-binding. Alternatively, the integrity of the full-length protein may be required to bind efficiently to MTs *in vitro*.

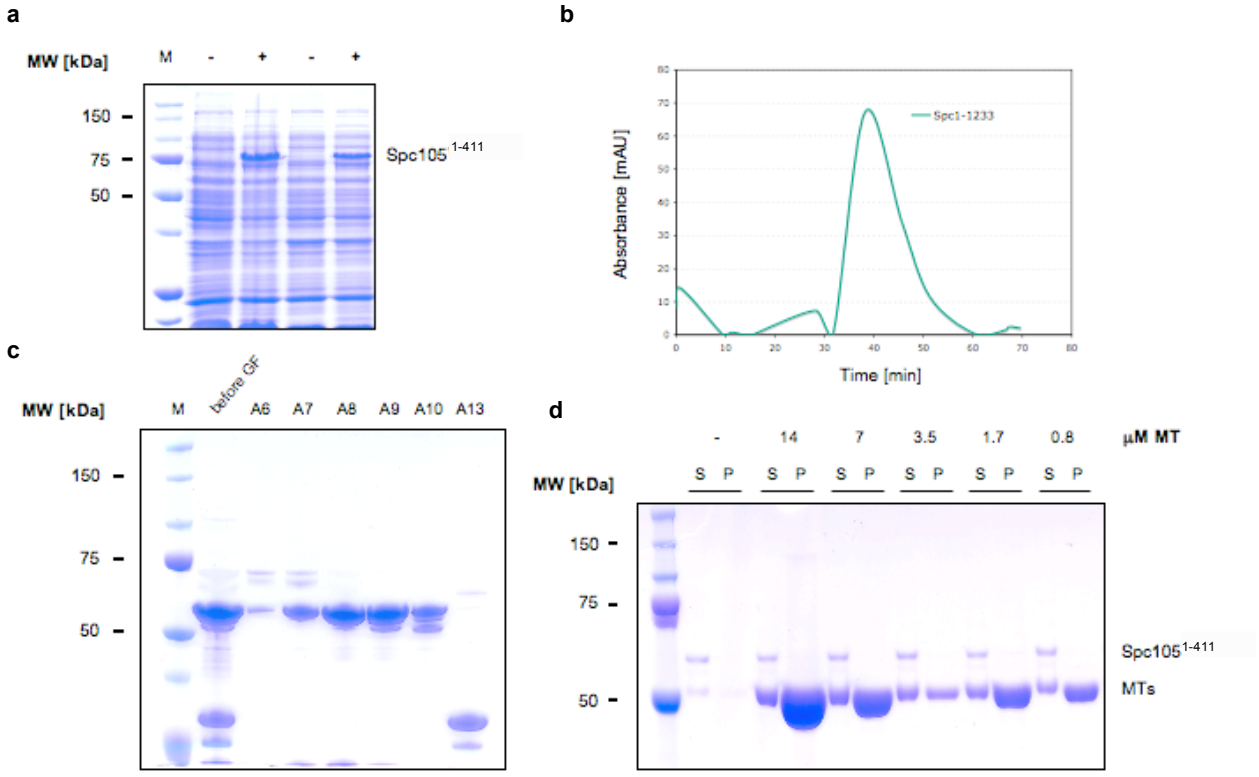


Figure 35: Expression and purification of Spc105p from bacteria. a) Induction with IPTG leads to the expression of Spc105$^{N\text{-terminus}}$. +/- indicates induction/non-induction with IPTG. b) Purification of Spc105$^{N\text{-terminus}}$ via nickel-NTA agarose followed by size exclusion chromatography. c) Elution profile after gelfiltration. d) MT-binding assay of Spc105$^{N\text{-terminus}}$ with taxol stabilized MTs.

4.6 The Spc105$^{1\text{-}411}$ cannot complement the Spc105 knock-out *in vivo*

To address the role of the N-terminus of Spc105p *in vivo,* Spc105$^{1\text{-}411}$ was introduced into the heterozygous Spc105 knock-out strain. Tetrad analysis after sporulation revealed a 2:2 segregation pattern indicating that only two of the spores are viable (**Figure 36**). Neither of the

viable stores contained the Spc105 knock-out. This shows that the N-terminal part (amino acids1-411) of Spc105p is not sufficient to rescue the knock-out phenotype, but that the C-terminus must have essential functions. Indeed, the C-terminus of Spc105p/KNL-1 was suggested to interact with components of the Mtw1/Mis12- complex via its coiled-coil domain in budding yeast and human cells[72,111]. KNL-1 alone exhibits only a weak binding affinity for MTs whereas the Mtw1-complex does not bind to MTs at all. However, together with the Ndc80 complex the Mtw1 complex is capable of binding to taxol stabilized MTs *in vitro* arguing that the binding affinity is synergistically increased within the KMN network. Disruption of these interactions due to the missing C-terminus of Spc105p could lead to defects in kinetochore-MT attachments, which cannot be compensated by any other MT-binding complex.

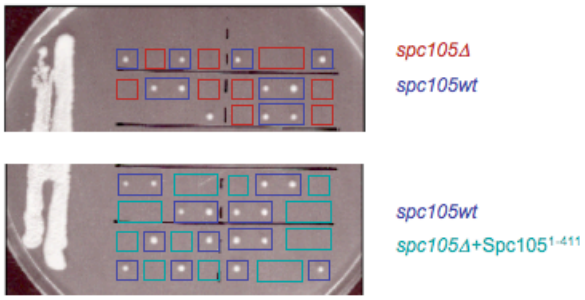


Figure 36: Tetrad analysis of Spc105 knock-out complemented with the N-terminus of Spc105p. Two tetrads are dissected horizontally and separated by a black dashed line.

4.7 Outlook

Biochemical purification of Spc105p should allow the reconstitution of the complex *in vitro*. However in addition to expression in budding yeast and bacteria, the complex could be isolated from insect cells, which display another powerful tool for recombinant protein expression. The major goal would be to isolate sufficient amounts of protein and reconstitute the entire KMN network. It is still not clear how the components of the KMN network are associated with inner kinetochore complexes, for instance the COMA complex or DNA-binding proteins such as Mif2p. Furthermore there is evidence that the Mis12/Mtw1 complex may play a role in the transient disassembly of the entire kinetochore upon DNA replication. Phosphorylation events can trigger disassembly processes by lowering binding affinities within individual proteins and sub-complexes. Therefore it would be interesting to analyze the phosphorylation status of individual components of the KMN network over the cell cycle and ask how these events influence the composition of the KMN network *in vitro* and *in vivo*. Since the components of the KMN network are highly conserved from yeast to human this might also shed light on the regulatory processes

governed by this network such as the spindle assembly checkpoint. Studying the KMN network in budding yeast might therefore give insights into the constitution and regulation of kinetochores in higher eukaryotes including human cells.

5 Material and Methods

5.1 Microbial techniques

All generated yeast strains used for this study are derived from the S288C background unless otherwise stated.

5.1.1 Yeast cell culturing

Liquid cultures were set up in complete YEP medium (1% bacto-yeast extract, 2% bacto-peptone) containing 2% glucose (YPD) and incubated at 30°C in a shaker. For microscopy SD synthetic minimal media (0.67% bacto-yeast nitrogen base without amino acids) containing 2% glucose and supplemented with all growth factors except leucine (doLeu: 2% adenine sulphate, 2% uracil, 2% l-tryophan, 2% l-histidine, 3% l-lysine) was used to avoid auto fluorescence of the YEP media. For growing strains on solid medium, YPD agar (1% bacto-yeast extract, 2% bacto-peptone, 2% bacto-agar) containing 2% glucose was used. To select for certain markers or mini-chromosomes the appropriate supplement was excluded from the media.

5.1.2 Yeast crossing

Cells from two different strains were streaked across each other and mated overnight on YPD plates at 25°C. Zygotes were picked with a micro needle according to their characteristic dumb-bell shape and incubated for 3-4 days on YPD plates at 25°C. For sporulation, 1ml of saturated overnight culture was washed once with distilled water, resuspended in 5ml sporulation medium (1% KAc, 0.1% bacto-yeast extract, 0.05% glucose, 0.67% adenine sulphate, 0.67% uracil, 0.67% l-tryophan, 0.67% l-histidine, 1% leucine, 1% l-lysine) and incubated at 25°C in a rotor for 3-7 days (depending on the yeast strains used for mating). To allow tetrad dissection, 250µl of sporulation culture was harvested, washed once with distilled water and subsequently incubated with 37.5µl of 1mg/ml Zymolase at 37°C for 3-4min to digest the cell wall. To stop the reaction 200µl of 20mM NaP_i pH 7.2 was added. Spores were separated with a micro-needle on YPD plates using a dissecting microscope and incubated for 3-5 days at 25°C. Single spores were diluted in SD synthetic minimal media in a 96-well plate and transferred to selective agar plates to determine the corresponding genotype.

5.1.3 Yeast strain construction

All kinetochore-TetR strains were generated by a PCR-based integration system[116]. The Tet-Repressor was amplified from plasmid EKY17 with primers containing overhanging sequences for the appropriate kinetochore protein allowing integration by homologous recombination at the endogenous gene locus. As background strain for kinetochore protein TetR-tagging DDY902 was used. Proper integration was confirmed by PCR and sequencing, respectively. Knock-out and TAP-tagged strains were generated by the same technique.
All temperature sensitive yeast strains are derived from the lab collection and were crossed to EKY6 and selected for temperature sensitivity at 37°C. TetR-tagging of Dam1p and Ask1p in these strains was carried out as described above.

5.1.4 Transformation of yeast cells

Yeast cells were transformed using the lithium acetate procedure as described previously[117]. An overnight culture was diluted back to OD_{595} 0.2 in 50ml YPD medium and grown to mid log phase (OD_{595} 0.6-0.8). Cells were harvested by centrifugation, washed twice with distilled water and resuspended in 1.5ml LiAc/1xTE. To facilitate DNA uptake, 20µl salmon sperm was heated for 5min at 95°C and used as carrier DNA. 200µl of cell suspension and 1.2ml PEG solution were added and rotated for 30min at room temperature following a 12min heat shock at 42 °C in a water bath. Cells are centrifuged, resuspended in 150µl water and spread on agar plates selective for the transformed DNA. After 2-4 days of growth at 25°C colonies were re-streaked again on selective agar plates.

5.1.5 Spot assay

Overnight cultures were adjusted to an OD_{595} of 0.4 in 100µl YPD media. Starting from that, a 1:10 dilution series was set up to a final OD_{595} of $0.4x10^{-4}$ in a 96-well plate. A pinner was used to spot the cell suspension on individual YPD agar plates, which were incubated at the indicated temperatures for two days. Images were acquired using a gel documentation machine.

5.2 Generation of plasmids

5.2.1 Tet-Repressor (TetR)-integration plasmid

The TetR-integration plasmid was generated by amplifying the Tet-Repressor from plasmid pCM174 introducing a flexible linker $(SG4)_2$ at its N-terminus. The GFP sequence in pFA6a–

GFP(S65T)-KanMX6 was released by *Pac*I/*Asc*I[116] digest and replaced by the linker+TetR sequence. Proper integration was confirmed by sequencing. Positive transformants were selected on YPD plates supplemented with 250µl of 50mg/ml G418 solution.

5.2.2 Mini-chromosomes used for stability assays

As wildtype mini-chromosome the pRS313 plasmid containing the centromeric sequence of chromosome VI (CEN6), an origin of replication ArsH4 and the HIS3 gene to select for positive transformants was used. The *tetO* sequence was amplified from pCM189new and inserted into the Swa1 site of CDEII to generate the CDEII-*7xtetO*$_2$ mutant mini-chromosome[118]. Point mutations in CDEIII were introduced by PCR-directed mutagenesis using the Quick-change mutagenesis kit (Stratagene) according to the manufacturers instructions.
The acentric mini-chromosomes are derived from plasmid pRS303 by amplifying the ArsH4 element from pRS313 and introducing it into the Xho1 site of pRS303. To construct the *10xtetO*$_2$ acentric mini-chromosome the *tetO* sequence was amplified from pCM189new and cloned into the BamH1 site of ArsH4/pRS303. The *112xtetO*$_2$ mini-chromosome was generated by cloning the ArsH4 element from pRS313 into the BamH1 site of pRS306-*tetO*$_2$*x112*, which in addition contains the URA3 gene for selection.

5.2.3 Mini-chromosomes used for visualization

The wildtype mini-chromosome was constructed by cloning the CEN6/ArsH4 element from pRS313 into the Xho1 site of pAFS59 (LEU2$^+$) that contains an array of *256lacO* repeats. Due to the big size of this plasmid (~15kDa) all bacteria strains had to be cultured at 30°C instead 37°C to avoid recombination events within the repetitive LacO array. The acentric and *10xtetO*$_2$ mini-chromosomes were generated by subcloning the ArsH4 and *10xtetO*$_2$/ArsH4 elements from ArsH4/pRS303 and *10xtetO*$_2$/ ArsH4/pRS303 into the BamH1 site of pAFS59.

5.3 Determination of mitotic stability

Kinetochore TetR strains were freshly transformed with mini-chromosomes generated for the stability assay (see above). Cells were grown at 30°C for 24h in YPD medium. An OD_{595} of 0.6 was adjusted and diluted 1.000-fold in SD synthetic minimal medium containing 2% glucose. An aliquot (80 µl) of diluted cell suspension was spread on YPD and doHIS or doURA plates, respectively and incubated for 2 days at 30°C. The total number of colonies was counted and the loss rates determined according to the percentage difference of colonies on doHIS/doURA and

YPD plates. Plates were analyzed in duplicates and all experiments were performed three times. Statistical analysis (Student's two-tailed t-test) was carried out using origin and excel software.

5.4 Visualization of mini-chromosomes

For all experiments the appropriate yeast strains were transformed freshly with mini-chromosomes generated for visualization (see above). Cells were cultured in SD synthetic minimal media containing 2% glucose and supplemented with all growth factors except leucine to ensure mini-chromosome maintenance. An overnight culture was diluted 1:3 with fresh media and grown for 2-3 hours at 25°C. Expression of LacI-GFP was induced by adding a final $CuSO_4$ concentration of 50μM for 15min.

5.4.1 Fixation of yeast cells for microscopy

For fixation 500μl of diluted cell suspension was harvested, washed once with 0.1M K_2PO_4/1.2M Sorbitol and resuspended in 100μl 4% p-formaldehyde (PFA). After 5min of incubation at room temperature the PFA was removed and the cells were washed and resuspended in 50μl 0.1M K_2PO_4/1.2M Sorbitol. For imaging, 5-10μl of fixed cells were spread on ConA-coated cover slips (see below) and incubated for 15min at room temperature. Unattached cells were removed by washing the cover slips carefully with 0.1M K_2PO_4/1.2M Sorbitol. Cover slips were mounted with nail polish to avoid evaporation of cells.

5.4.2 Preparation of cover slips

To attach the cells to the cover slips, they were pre-incubated with 50μl of 0.1mg/ml ConA solution for at least 15min and subsequently washed with SD synthetic medium containing 2% glucose.

5.4.3 Live cell imaging

Cells were grown overnight and diluted back as described above. After growing them for 2-3 hours at 25°C in a rotor, 50-100μl of diluted cell suspension was spread on ConA-coated cover slips and incubated for at least 15min at room temperature to allow adherence. After washing the cover slips carefully to remove unattached cells, they were sealed with vacuum grease and immediately used for microscopy.

5.4.4 Microscopy and image acquisition

For image acquisition, a DeltaVision microscope (AppliedPrescision), equipped with an UPlanSApo 100 objective lens (Olympus; NA 1.40), and a CoolSnap HQ CCD camera (Photometrics) was used. For single images, 32-36 *z* sections (0.2μm apart) were acquired and subsequently deconvoluted (aggressive mode) using SoftWoRx software (AppliedPrescision). Single images were projected by maximal intensity to two-dimensional images and assembled using ImageJ software. Time-lapse microscopy was carried out at 30°C taking 12–14 *z* sections of 0.5μm distance.

5.4.5 Analysis in temperature sensitive yeast strains

Cells were grown overnight in SD synthetic minimal medium containing 2% glucose and selective for leucine, diluted 1:3 with fresh medium the next day and grown for 2–3 hours at 25°C. After transferring the cells to the restrictive temperatures for 1h (34°C for *ndc10-2* mutants and 37°C for all other mutants), LacI–GFP expression was induced for 15 min in the presence of 50μM $CuSO_4$. Fixation procedure was carried out as described in 2.4.1.

5.5 TAP-tag purification of Dam1 complex

Overnight cultures were diluted to OD_{595} 0.15 in 12L YPD medium and grown at 30°C to an OD_{595} of 1.2. Cells were harvested by centrifugation in an SLC-6000 rotor and washed once with distilled water. The pellets were resuspended in a minimal volume of water (~3ml) and drop frozen in liquid nitrogen. Cell lysis was performed by grinding the beads in a Warring Blender using liquid nitrogen. For tandem-affinity purification 30g of powder was dissolved in 30ml 2x Hyman buffer and quickly thawed in a warm water bath. To avoid protein degradation, a Protease Inhibitor cocktail (Calbiochem; 1:100 dilution) and PMSF to a final concentration of 1mM were added. In addition, a final concentration of 1% Triton-X was supplemented to facilitate cell membrane lysis. The lysates were sonicated (setting 4) for 30sec and centrifuged for 25min at 10.000rpm in a SS-34 rotor followed by another centrifugation step in a Ti70 rotor for 30min at 45.000rpm. All centrifugation steps were carried out at 4°C. The cleared lysate was passed over a CL-6B sepharose column that was pre-equilibrated with 1x Hyman buffer. 1.2ml of pre-washed IgG Sepharose was added to the cleared lysate and incubated by gentle rotation for 3h at 4°C. The IgG beads were collected in a Biorad disposable column and washed 15ml 1xHyman buffer containing 400mM KCl and 25ml 1xHyman buffer +400mM KCl+1mM DTT and 0.1% Tween20. To allow cleavage from the IgG beads 40μl TEV protease was added and rotated overnight at 4°C. The next day, beads were washed with 600μl 1xHyman buffer+400mM KCl+1mM

DTT+0.1% Tween20. 100µl 1:1 slurry of pre-washed Protein S Agarose beads was added and rotated for 3h at 4°C. Beads were collected in a Biorad disposable column and washed with 5ml 1xHyman buffer +400mM KCl+1mM DTT+0.1% Tween20. Protein elution was carried out by adding 10mM glycin pH 2.0 and gentle shaking at room temperature for 30min. In order to analyze the samples by SDS-PAGE, the pH was adjusted with 5µl 1M Tris pH 8.0. Proteins were stained with silver as described previously[119].

5.6 Segregation of native chromosomes

Chromosome V was placed under the control of a galactose inducible promoter and the 112xtetO_2 array inserted 35kbp upstream of it. Cells were grown overnight in YEP medium containing 2% raffinose. After washing them three times with YEP containing 2% galactose they were adjusted to an OD_{595} of 0.2 and grown for six hours at 25°C in galactose rich medium. Cells were imaged on a Zeiss Axiovert microscope, equipped with a 100 objective (Zeiss) and a Coolsnap CCD (Photometrics) camera. Images are maximum intensity projections of 8x 0.6µm stacks. To determine growth on YEP-galactose plates, cells were adjusted to OD_{595} of 0.7 and a 100-fold dilution was plated on YEP-2% dextrose and YEP–2% galactose plates. Images were taken after 3 days of growth at 30°C.

5.7 Additional methods – Characterization of the Spc105 complex

5.7.1 Yeast cell culturing

Cells were grown in complete YEP medium supplemented with 2% glucose unless otherwise stated. For expression of proteins using the Gal-TEV-myc system, an overnight culture was set up in 100ml SD synthetic minimal media containing 2% glucose and supplemented with all growth factors except uracil (doURA medium). The inoculum was added to 2L doURA medium supplemented with 2% raffinose and grown to an OD_{595} of 1.0 at 30°C. Protein expression was induced by adding 2% galactose and 200ml bacto-peptone/yeast extract solution (40g bacto-peptone, 20g yeast extract) and incubated for 8 hours at 30°C. Cells were harvested by centrifugation for 5min at 6000rpm in a SLA-3000 rotor followed by a washing step with 20ml of distilled water in a 50ml falcon tube. Pellets were resuspended in 3-5ml distilled water and drop frozen in liquid nitrogen.

5.7.2 Strain generation

All strains were generated by either PCR-based integration or crossing of two strains as described in 4.1.3 and 4.1.2, respectively.

5.7.3 Preparation of whole cell extracts (WCE)

Cells were grown overnight in 5ml YPD medium to saturation. $2OD_{595}$ were harvested, washed once with 1xPBS and resuspended in 60µl 4xSDS-sample buffer. After boiling for 3min at 95°C, acid washed glass beads were added to 2/3 of the total volume leaving enough liquid to cover the beads. Samples were vortexed for 2min before adding 60µl 2xSDS-sample buffer followed by an additional incubation step at 95°C for 2min. Supernatants were transferred into a fresh eppendorf tube and boiled for 5min at 95°C before loading on a SDS-gel.

5.7.4 Spot assay

Spot assays were carried out as described in 4.1.5.

5.7.5 Generation of plasmids

5.7.5.1 Spc105p phospho-mutants

The *spc105* gene was amplified by PCR from genomic DNA together with its endogenous promoter (200bp upstream of coding sequence) and cloned into the Xho1 site of pRS306. Phosphorylated amino acids - as detected by mass spectrometry - were mutated to alanine using the Quick-Change Mutagenesis kit (Stratagene) according to the manufacturers instructions.

5.7.5.2 Over-expression of Spc105p phospho-mutants

The gene was amplified from the appropriate pRS306 plasmids by PCR and cloned into the BamH1/Not1 site of pDD1183 (Gal-TEV-myc site) derived from the lab collection. Sequence integrity was confirmed by sequencing for all generated constructs.

5.7.6 Expression of Spc105$^{1-411}$ in bacteria

Different truncation constructs of the *spc105* gene were amplified from genomic DNA and cloned into the Nco1/BamH1 site of pET28a(+) to generate N-terminal his-tag fusion proteins.

5.7.7 Affinity purification

Tandem-affinity purification of YDR532c-TAP and Spc105-TAP has been carried out as described in 4.5 except that 300mM KCl have been used. The starting material was 50g lysed yeast powder for Spc105-TAP and 34g of YDR532c-TAP, respectively.

5.7.8 Purification of over-expressed Spc105p from a Gal-promoter

5.7.8.1 Preparation of α-myc beads

500µl of Protein G Sepharose 1:1 slurry was washed three times with TBS+0.5% TritonX before adding 100µl myc-antibody (9E10) and 800µl TBS+0.5% TritonX and rotated overnight at 4°C. Beads were washed twice with TBS+0.5% TritonX and three times with 0.2M Sodium Borate pH 9. For cross-linking, 6mg DMP were dissolved in 1ml 0.2M Sodium Borate pH 9.0, immediately transferred to the beads and incubated for 1 hour at room temperature. To quench the reaction, beads were spun down and 200µl supernatant removed before adding 200µl 1M Tris pH 8.0. Afterwards beads were washed three times in 2x HEK buffer (50mM HEPES pH 7.5, 1mM EDTA pH 8.0, 200mM KCl)+0.5% TritonX and stored at 4°C.

5.7.8.2 Protein isolation

For small-scale purifications, 3g of lysed yeast powder were thawed in 3ml 2x HEK buffer in a 15ml falcon by inverting the tube gently. All steps were carried out on ice to avoid protein degradation. 60µl of Calbiochem Yeast Protease Inhibitor Cocktail were added and the mixture transferred to four TLA100.1 centrifugation tubes (Beckmann) and centrifuged at 80000rpm for 20min at 4°C in an ultracentrifuge. The high speed supernatant was removed carefully to avoid the upper lipid phase and the lower detritus and transferred into a 1.5ml eppendorf tube. For 3g of yeast powder, 60µl 1:1 bead slurry were added and incubated overnight at 4°C in a lab quake.
The next day, beads were pelleted for 30sec at 3000rpm to remove the high-speed supernatant and washed 4 times with 1.5ml cold 1x HEK buffer. To remove unspecific bound protein from the beads, two additional washing steps with 1x HEK+0.5M NaCl+0.1% Tween20 followed. To cleave

the protein off the beads, 25µl TEV-protease in 50µl buffer (1x HEK+0.2M NaCl+0.1% Tween20+1mM DTT) were added and incubated for 3 hours at 22°C under gentle shaking. Beads were spun down and the supernatant (~50µl) carefully removed to avoid the beads. To analyze the protein composition, 15µl of supernatant were mixed with 5µl 4xSDS-sample buffer and loaded on a 4-12% gradient SDS-gel.

5.7.9 Size exclusion chromatography

Clear cell extracts were prepared from 3g frozen yeast powder as described in 4.7.8.2 and concentrated 10-times in a 15ml centricon tube (MW cut-off: 10kDa) to a final volume of 500µl. The concentrated extract was centrifuged for 10min at 13000rpm to clear the lysate and get rid of insoluble cell detritus. 50µl sample was applied to a Superose6 column, which was washed before with 3ml 1xgelfiltration buffer (50mM Bis-Tris Propane pH 7.0, 150mM KCl, 5mM EDTA, 5mM β-glycerolphosphate). 100µl fractions were collected in a 96-well plate and analyzed on a 10% SDS-gel and by Western blotting, respectively.

5.7.10 Cdc28 kinase assay

Spc105p was isolated freshly from 3g frozen yeast powder as described in 4.7.8.2. Kinase reaction was carried out in a final volume of 40µl using equal amounts of protein for each reaction, 1x Cdc28 kinase buffer (20mM HEPES-NaOH pH 7.4, 100mM NaCl, 1mM $MgCl_2$, 25mM β-glycerolphosphate) 25µM cold ATP, 1µl hot ATP, 7µl purified Cdc28/clb2 kinase. The reaction was incubated for 30min at 25°C and stopped by adding 15µl 4xSDS-sample buffer. 35µl were loaded on a 4-12% gradient SDS-gel, which was vacuum dried between 2 cellophane sheets for 1hour. The dried gel was exposed to X-ray films for 7 hours and overnight, respectively.

5.7.11 Imaging of Spc105 phospho-mutants

Cells were cultured overnight in 5ml SD synthetic medium containing 2% glucose and supplemented with all growth factors. The next morning, a 1:5 dilution was prepared in 5ml fresh medium and incubated for 3-4 hours at 25°C in a rotor. 200µl of cell suspension was harvested, washed once with 1xPBS and fixed with 100µl 4% p-formaldehyde for 5min at room temperature. Cells were washed and resuspended in 50µl 0.1M K_2PO_4/1.2M Sorbitol. For imaging, 10µl of fixed cells were spread on ConA-coated cover slips as described in 4.4.2. 32-36 *z*-sections (0.2µm apart) were acquired and subsequently deconvoluted (aggressive mode) using SoftWoRx

software (AppliedPrescision). Single images were projected by maximal intensity to two-dimensional images and assembled using ImageJ software.

5.7.12 Microtubule-binding assay

40µl of porcine tubulin (56µM) was pre-cleared by centrifugation at 13000rpm for 5min and transferred to a fresh eppendorf tube. 1µl of 100mM GTP and 40µl G-PEM buffer (80mM Pipes-KOH pH 6.8, 1mM EGTA, 1mM $MgCl_2$, 50% glycerol) were added and incubated for 30min at 36°C to allow microtubule assembly. To stabilize the MTs, taxol was added to a final concentration of 10µM with a cut-off tip to avoid shearing forces and mixed gently. After incubating the reaction for 10min at room temperature, a 1:2 MT-dilution series was prepared using G-PEM buffer. To check for MT-binding activity, 20µl of purified protein was mixed with 20µl MTs at different concentrations and incubated for 20min at 25°C. The mixture was overlaid on 100µl G-PEM and centrifuged for 25min at 60000rpm and 25°C in an ultracentrifuge. Supernatants (~40µl) were carefully removed into fresh tubes and the rest of the glycerol cushion discarded. Pellets were resuspended in 40µl PEM+10mM $CaCl_2$. 10µl of 4xSDS-sample buffer were added and 10µl sample loaded on a 4-12% gradient SDS-gel.

5.7.13 Expression of $Spc105^{1-411}$ in bacteria

Cells were grown overnight at 37°C in 50ml LB_0 medium (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl) supplemented with 50µg/ml Kanamycin and 34µg/ml Chloramphenicol. The cell suspension was used to inoculate a 2L culture the next day, which was grown at 37°C to an OD_{595} of 0.8-1.0. Protein expression was induced by adding IPTG to a final concentration of 0.25mM and grown overnight at 18°C. Cells were harvested by centrifugation in a SLA-3000 rotor at 4000rpm for 15min, washed once with 1xPBS in a 50ml falcon and pellets frozen at -80°C.
For protein isolation, pellets were thawed on ice and resuspended in 20ml lysis buffer (50mM sodium phosphate, 300mM NaCl, 10mM Imidazol, 5mM β-glycerolphosphate). 4 tablets of protease inhibitor cocktail, 0.1mg/ml lysozyme and 0.5% TritonX were added and incubated on ice for 10min. After sonicating the cell suspension three times for 30sec (setting 4) it was centrifuged in a SS34 rotor for 20min at 15000rpm to obtain a clear lysate.
1ml 1:1 nickel-NTA agarose was washed 3-4 times with lysis buffer before adding the clear lysate and incubated for 2hours at +4°C to allow protein binding. Unbound protein was removed by washing the beads three times with washing buffer (50mM Na_2PO_4, 300mM NaCl, 20mM Imidazol, 5% glycerol, 5mM β-mercaptoethanol). Afterwards beads were transferred to a Biorad disposable column and incubated with 3ml elution buffer (50mM NaH_2PO_4, 300mM NaCl, 5%

glycerol, 250mM Imidazol, 5mM β-mercaptoethanol) for 10min before collecting 0.5ml eluate fractions. Protein composition was analyzed on a 10% SDS-gel.
For further purity, one fraction (~0.4ml) was injected to a Superdex200 column and 0.5ml samples collected. Separation of proteins was analyzed on a 4-12% gradient SDS-gel.

5.7.14 Complementation of Spc105 knock-out

$Spc105^{1-411}$ was amplified from genomic DNA and cloned into the Xho1 site of pRS306. Positive clones were confirmed by analytical digest and sequencing, respectively. The plasmid was linearized with Nco1 and transformed into SWY117 (heterozygous Spc105 knock-out) as described in 4.1.3. Positive clones were selected on doURA plates and restreaked after 4 days of growth. Overnight cultures in YPD liquid medium were prepared for sporulation and dissected after 1 week of incubation at 25°C in a rotor.

6 Appendix

6.1 Kiermaier E, Woehrer S, Peng Y, Mechtler K, Westermann S., *Nat Cell Biol*. 2009 Sep;11(9):1109-15. A Dam1-based artificial kinetochore is sufficient to promote chromosome segregation in budding yeast.

nature cell biology

A Dam1-based artificial kinetochore is sufficient to promote chromosome segregation in budding yeast

Eva Kiermaier[1], Sophie Woehrer[1], Yutian Peng[2], Karl Mechtler[1] and Stefan Westermann[1,3]

Kinetochores are large multiprotein complexes that mediate chromosome segregation in all eukaryotes by dynamically connecting specialized chromosome regions, termed centromeres, to the plus-ends of spindle microtubules[1,2]. Even the relatively simple kinetochores of the budding yeast *Saccharomyces cerevisiae* consist of more than 80 proteins, making analysis of their respective roles a daunting task[3]. Here, we have developed a system that allows us to artificially recruit proteins to DNA sequences and determine whether they can provide any aspect of kinetochore function *in vivo*. We show that artificial recruitment of the microtubule-binding Dam1 complex to a plasmid lacking any centromere DNA is sufficient to confer mitotic stabilization. The Dam1-based artificial kinetochores are able to attach, bi-orient and segregate mini-chromosomes on the mitotic spindle, and they bypass the requirement for essential DNA-binding components of natural kinetochores. Thus, we have built a simplified chromosome segregation system by directly recruiting a microtubule force-transducing component to DNA.

Despite the small size of their centromere DNA (125 bp), kinetochores of *S. cerevisiae* are complex structures, consisting of a dynamic assembly of various multiprotein complexes, most of which are conserved throughout evolution. So far, analysis of kinetochore function has relied mainly on the use of loss-of-function mutants or on biochemical approaches to isolate and understand the function of individual kinetochore proteins and complexes[1]. A different approach to study kinetochore function is to define minimal modules of proteins that can recapitulate or reconstitute aspects of kinetochore function. Kinetochores of *S. cerevisiae* are especially well suited for such *in vivo* reconstitution approaches, as their centromere DNA sequence is well defined and amenable to genetic manipulation. *S. cerevisiae* centromeres contain three sequence elements (CDEI, II and III) of which CDEIII serves as the binding site for the essential CBF3 complex, whereas CDEII, which is conserved in length and rich in AT-content, is thought to wrap around a single nucleosome containing the histone H3 variant CENP-A[4,5]. Furthermore, kinetochore function in *S. cerevisiae* can be analysed using mini-chromosomes, circular plasmids that contain a yeast origin of replication (*ARS*) and a centromere (*CEN*)[6–8].

A number of different microtubule motors and plus-end binding proteins are associated with centromeres, and parts of the conserved KMN protein network, such as the Ndc80 complex, show microtubule-binding activity[9,10]. In addition, the ten-protein Dam1 complex is of special importance for the generation and maintenance of kinetochore–microtubule interactions in *S. cerevisiae*[11–13]. Biochemical analysis has revealed a number of unique properties: Dam1 complexes can oligomerize to form a stable, 16-fold ring that topologically embraces the microtubule[14–16]. The ring is diffusible on the microtubule lattice, stays attached to the end of a disassembling microtubule and can translate the force generated by microtubule depolymerization into the movement of a microbead *in vitro*[17–19]. Whether this complex is indeed an essential chromosome coupler *in vivo* remains to be established. Chromosomes do not detach completely from microtubules in *dam1* temperature-sensitive mutants, and analysing the function of the complex is complicated by its additional role in maintaining the integrity of the yeast mitotic spindle[20].

To construct a system that allows us to analyse the contributions of individual proteins to kinetochore function, we artificially tethered candidate proteins to a mini-chromosome and assessed their ability to improve chromosome segregation *in vivo* (Fig. 1a). We introduced two different arrays of tet operator repeats ($10xtetO_2$ or $112xtetO_2$) on a yeast circular mini-chromosome, which lacks a native centromere (Fig. 1b). By fusing the tetracyclin-repressor protein (TetR) — the corresponding binding domain of the tet-operator sequence — to the carboxy-termini of selected inner, central and outer kinetochore proteins, these proteins could be recruited directly to the acentric mini-chromosomes. We then assayed the maintenance of the mini-chromosomes after 24 h of non-selective growth and examined whether this artificial recruitment of kinetochore proteins is sufficient to provide mitotic stabilization (Fig. 1c).

As expected, acentric mini-chromosomes were extremely unstable in every strain background (Fig. 1d). Although most of the TetR fusions were unable to improve the stability of the $10xtetO_2$ mini-chromosome, we detected a remarkable level of stabilization in the Ask1–TetR and,

[1]Research Institute of Molecular Pathology (IMP), Dr. Bohr-Gasse 7, 1030 Vienna, Austria. [2]Department of Molecular and Cell Biology, University of California, Berkeley, 16 Barker Hall, 94720-3202 Berkeley, USA.
[3]Correspondence should be addressed to S.W. (e-mail: westermann@imp.ac.at)

Received 3 April 2009; accepted 16 June 2009; published online 16 August 2009; DOI: 10.1038/ncb1924

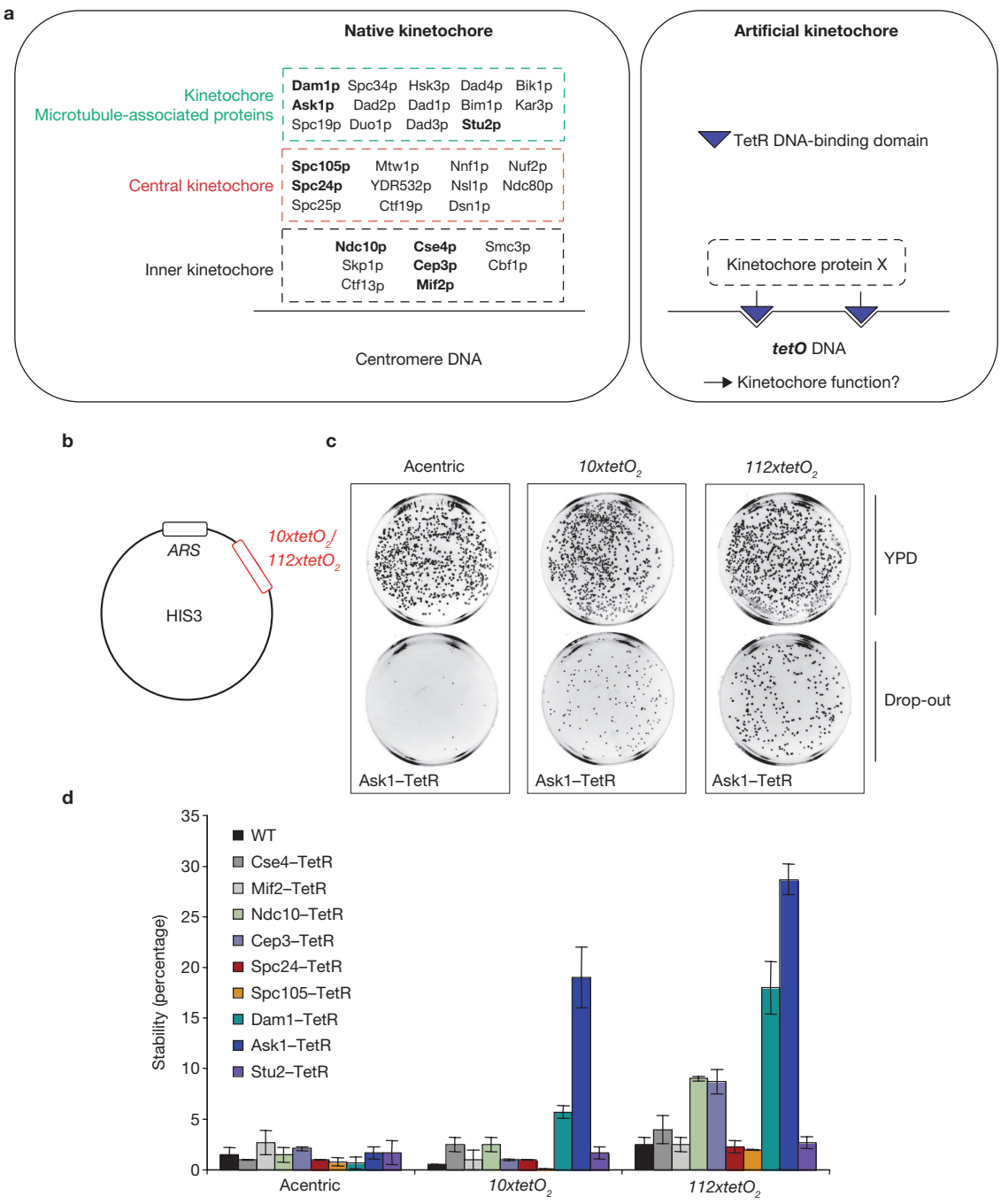


Figure 1 A strategy to construct artificial kinetochores in *S. cerevisiae*. (**a**) Schematic representation of a native kinetochore (left panel) and assignment of selected proteins to different layers. Kinetochore proteins in bold letters were tested as TetR fusion proteins in this study. Individual kinetochore–TetR fusion proteins were recruited to *tetO* DNA on a yeast mini-chromosome and tested for kinetochore function (right panel), (kt, Kinetochore, MAPs, Microtubule-associated proteins) (**b**) Acentric mini-chromosomes containing two different arrays of *tetO* repeats. (**c**) Mitotic stabilization of mini-chromosomes in the Ask1–TetR background. After 24 h of non-selective growth, equal numbers of cells were plated on YPD or drop-out medium. (**d**) Quantification of mini-chromosome stability in different TetR-fusion strains. Stabilization of *tetO* mini-chromosomes in the Dam1–TetR and Ask1–TetR background occurred in a length-dependent manner. Data are mean ± s.d. of three independent experiments.

to a lesser extent, in the Dam1–TetR strain (Fig. 1d). Increasing the size of the array to *112xtetO*$_2$ improved the stabilizing effect. After 12 generations of non-selective growth, approximately 30% of the colonies retained the mini-chromosome in the Ask1–TetR strain. By contrast, a wild-type mini-chromosome showed 67 ± 5.3% (mean ± s.d.) stability under the same assay conditions.

The stabilizing effect of Ask1–TetR and Dam1–TetR did not seem to be a consequence of tethering any kind of microtubule-binding protein to acentric mini-chromosomes, as recruitment of Stu2p, the budding yeast homologue of XMAP215 (ref. 21), of the Ndc80 complex (Spc24–TetR) or of the KNL-1 homologue Spc105p were ineffective in our assay (Fig. 1d). We note that fusions to Ndc10p and Cep3p, the DNA-binding

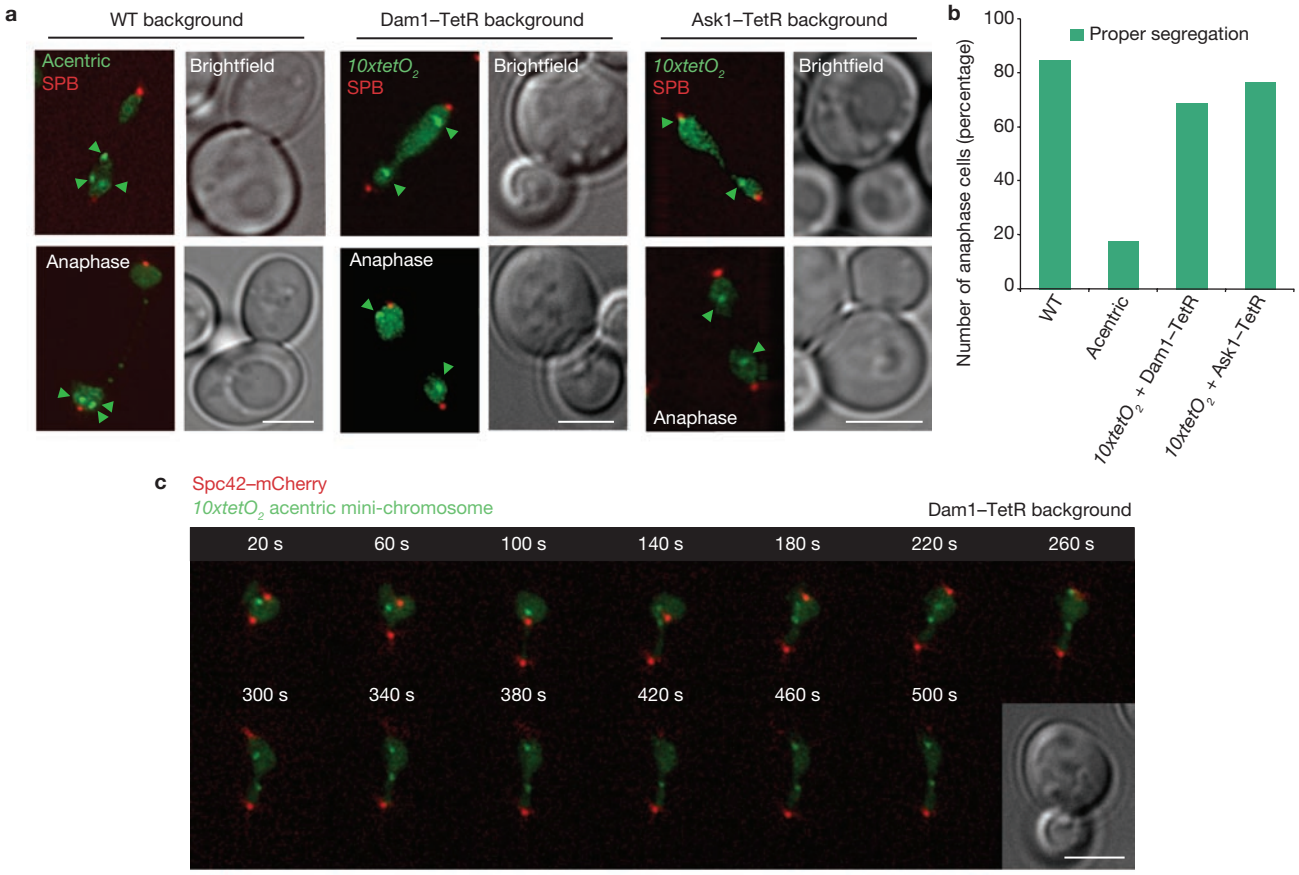


Figure 2 Live-cell imaging of mini-chromosome segregation mediated by Ask1–and Dam1–TetR. (**a**) Live-cell microscopy of acentric mini-chromosomes (green arrowheads) in a wild-type (WT) strain; the spindle poles were labelled with Spc42–mCherry (red). Acentric mini-chromosomes failed to segregate and instead accumulated in the mother. In the presence of Dam1–TetR and Ask1–TetR, the *10xtetO*$_2$ acentric mini-chromosomes segregated properly between mother and bud. Scale bars, 2 μm. (**b**) Quantification of mini-chromosome segregation in WT, Dam1–TetR and Ask1–TetR background ($n = 100$ cells per strain) (**c**) Time-lapse live-cell microscopy showing segregation of an acentric *10xtetO*$_2$ mini-chromosome (green) in a Dam1–TetR strain background. Spindle pole bodies were visualized by Spc42–mCherry (red). See also Supplementary Information, Movie 3. Scale bar, 2 μm.

components of native kinetochores, provided some degree of stabilization to the *112xtetO*$_2$ plasmid (Fig. 1d), but were clearly less effective than the Ask1–TetR and Dam1–TetR proteins.

To address whether the improved stability of the acentric mini-chromosomes was attributable to proper segregation during mitosis, we visualized the mini-chromosomes using a *lacO*–LacI–GFP system (Fig. 2a). In a wild-type strain only 18% of the observed anaphase cells contained acentric mini-chromosomes that had segregated successfully between mother and daughter cell. Instead, we observed a strong bias for these mini-chromosomes to accumulate in the mother cell, as previously reported for *ARS* plasmids[22] (Fig. 2a). By contrast, the percentage of anaphase cells with correctly segregated *10xtetO*$_2$ acentric mini-chromosomes was markedly increased to 77% and 69% in the Ask1–TetR and Dam1–TetR background, respectively (Fig. 2b). Time-lapse microscopy revealed that wild-type mini-chromosomes aligned along the spindle axis and were equally distributed between mother and bud as the cell entered anaphase (Supplementary Information, Movie 1). By contrast, the movements of acentric mini-chromosomes, were uncoupled from the spindle pole bodies (Supplementary Information, Movie 2). Remarkably, expression of Dam1–TetR or Ask1–TetR allowed the *10xtetO*$_2$ acentric mini-chromosomes to bi-orient and travel to opposing spindle pole bodies similarly to a wild-type mini-chromosome (Fig. 2c; Supplementary Information, Movie 3).

To address whether any of the remaining eight subunits of the Dam1 complex are also able to confer segregation capacity to an acentric mini-chromosome, we constructed TetR fusions to these proteins and scored the mitotic stability of the corresponding plasmids. We found that, in addition to Ask1–TetR and Dam1–TetR, fusions to Spc34p, Spc19p and Dad1p improved the segregation of the *112xtetO*$_2$ mini-chromosome, albeit to a lesser extent (Fig. 3a).

To determine whether the TetR fusion protein is part of the entire Dam1 complex, we purified the complex from yeast extracts using a tandem-affinity tag (TAP) fused to the Dad1p subunit, either in the wild-type or in the Ask1–TetR background. Comparison of the purified complexes on a silver-stained gel revealed an identical pattern of protein bands for both strains, except for a shift in the Ask1 migration consistent with the addition of the TetR (Fig. 3b, right lane). Moreover, mass spectrometry analysis confirmed the presence of all ten subunits of the Dam1 complex in both samples (data not shown). If the Ask1–TetR fusion works as part of the Dam1 complex we would expect that its stabilizing activity might be compromised in mutants of other Dam1 complex subunits. Analysing mini-chromosome segregation in the *dad1-1* mutant showed that this was indeed the case: segregation of mini-chromosomes with a wild-type *CEN* or with the artificial kinetochore, was compromised to the same degree in the *dad1-1* mutant (Fig. 3d). Taken together,

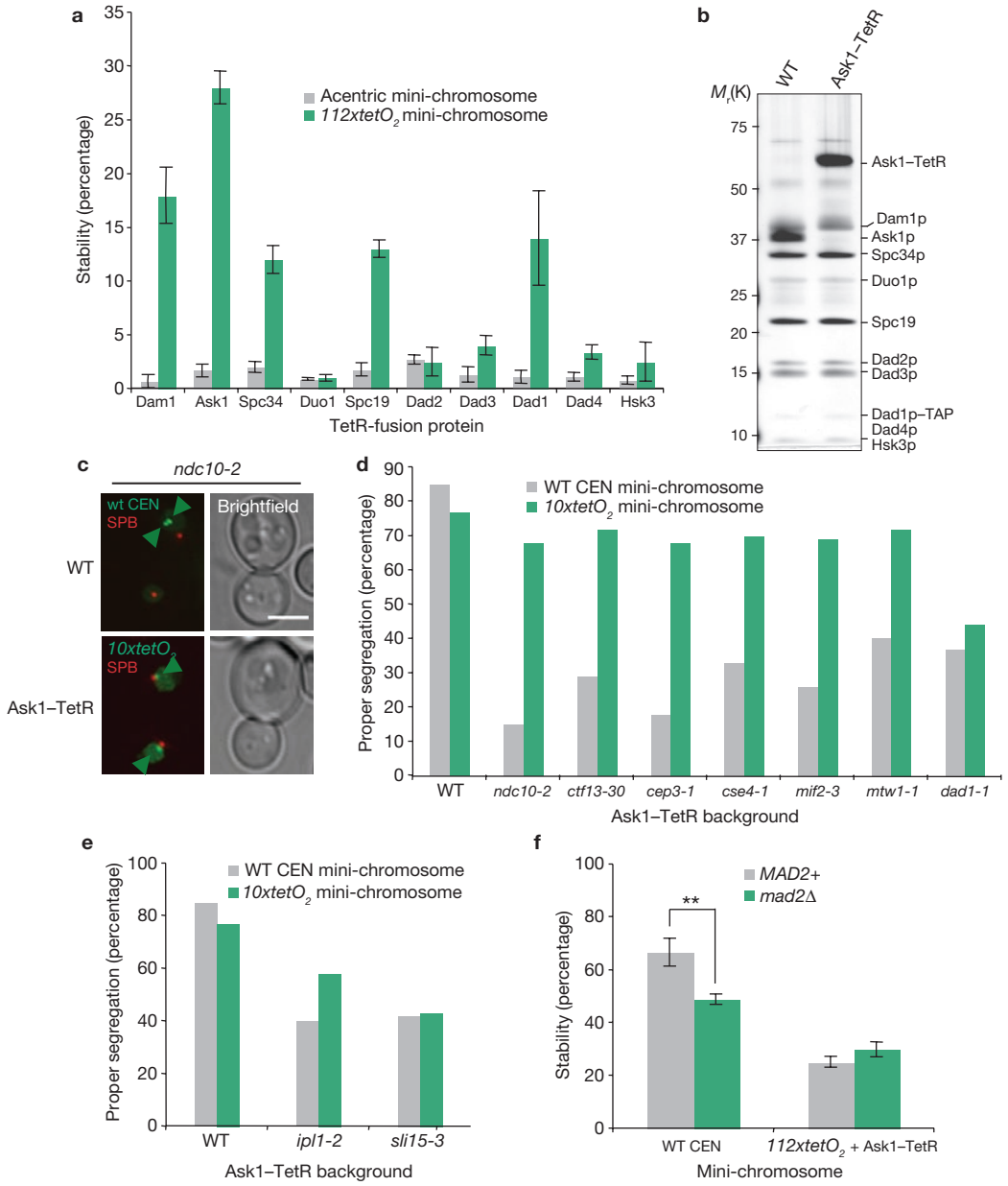


Figure 3 Analysis of artificial kinetochore function. (**a**) Quantification of *tetO* mini-chromosome segregation mediated by TetR fusions to individual subunits of the Dam1 complex. Note the stabilization in the Spc34–TetR, Spc19–TetR and Dad1–TetR strains. Data are mean ± s.d. of three independent experiments. (**b**) Silver-stained gel of purified Dam1 complex in a wild-type (WT) or Ask1–TetR background. Ask1 position is shifted in the right lane because of addition of the TetR fusion protein. (**c**) Analysis of mini-chromosome segregation in the *ndc10-2* mutant. Live-cell imaging of WT mini-chromosomes or *10xtetO*$_2$ mini-chromosomes (green arrowheads) in an Ask1–TetR background. Scale bar, 2 μm. (**d**) Analysis of acentric mini-chromosome segregation mediated by Ask1–TetR in various temperature-sensitive kinetochore mutants (n = 100 cells per strain). (**e**) Mini-chromosome segregation in *ipl1-2* and *sli15-3* temperature-sensitive mutants (n = 100 cells per strain). (**f**) Mini-chromosome stability mediated by a WT or an artificial kinetochore was compared in checkpoint-proficient (*MAD2*) and checkpoint-deficient (*mad2Δ*) cells. Data are mean ± s.d. of three independent plating assays (**$P < 0.001$ unpaired two-tailed t-test).

these findings strongly suggest that the observed stabilization of mini-chromosomes is due to recruitment of the entire Dam1 complex.

To address whether the Dam1-based segregation system represents a simplified kinetochore, we scored the mini-chromosome segregation in mutants that disrupt all native kinetochores. We therefore combined the TetR fusion strains with a temperature-sensitive allele of *NDC10*. Temperature-sensitive *ndc10* mutants fail to assemble a kinetochore at the restrictive temperature and show complete detachment of chromosomes from the mitotic spindle. After 1.5 h at the restrictive temperature, more than 85% of anaphase cells showed missegregation of wild-type mini-chromosomes in the *ndc10-2* mutant (Fig. 3c), demonstrating that native kinetochore function is indeed abolished under these conditions. Remarkably, a large fraction (68%) of *10xtetO*$_2$ mini-chromosomes segregated successfully in the Ask1–TetR (Fig. 3c, d) and Dam1–TetR (Supplementary Information, Fig. S2) background. Acentric mini-chromosome segregation mediated by Ask1–TetR

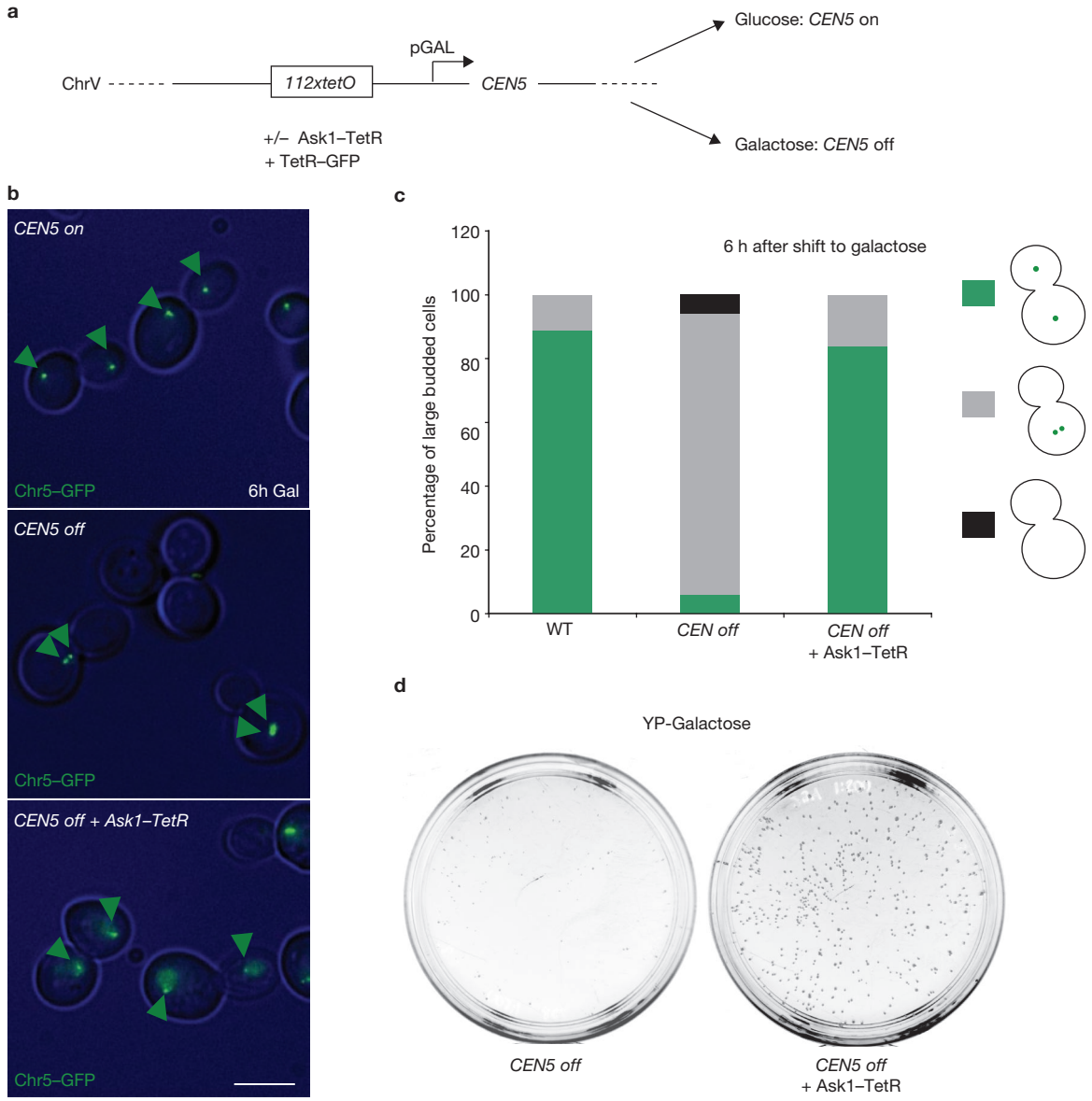


Figure 4 The artificial kinetochore can direct the segregation of a native yeast chromosome. (**a**) A strategy to analyse artificial kinetochore function on a native yeast chromosome. The centromere of chromosome V was placed under the control of a galactose-inducible promoter, inactivating native kinetochore function in galactose-containing medium. (**b**) Live-cell imaging of wild-type chromosome V (*CEN5 on*, upper panel, green arrowheads), chromosome V with a regulatable centromere (*CEN5 off*, middle panel) and chromosome V with a regulatable centromere in the Ask1–TetR background (*CEN5 off* + Ask1–TetR, lower panel), 6 h after transfer to galactose-containing medium. Note that the chromosome signal is decreased in the *CEN5 off* + Ask1–TetR strain because of competition between TetR–GFP and Ask1–TetR for binding to the *tetO* array. The corresponding brightfield image is pseudo-coloured in blue to show the cell outlines. Scale bar, 5 μm. (**c**) Quantification of chromosome V segregation, 6 h after shifting the culture to galactose-containing medium ($n = 100$). (**d**) Equal numbers of logarithmically growing cells in YP-raffinose were plated on YP-galactose. Expression of Ask1–TetR allows colony formation on YP-galactose under *CEN5 off* conditions.

and Dam1–TetR was also unaffected by *ctf13-30* and *cep3-1* mutants, which represent alleles in two additional subunits of the CBF3 complex (Fig. 3d; Supplementary Information, Fig. S2). These results demonstrate that the Dam1-based artificial kinetochore bypasses the requirement for the essential CBF3 complex. Furthermore, it excludes the possibility that mini-chromosomes segregate by 'hitchhiking' on native chromosomes, which fail to segregate in these mutants.

A specialized nucleosome containing the histone H3 variant CENP-A (Cse4p in *S. cerevisiae*) and the presence of the inner kinetochore protein CENP-C (Mif2p) are hallmarks of the DNA–kinetochore interface in all kinetochores. We therefore analysed the segregation of wild-type and *tetO* mini-chromosomes in *cse4-1* and *mif2-3* mutants. Although segregation of wild-type mini-chromosomes was severely compromised in both mutants, artificial mini-chromosome segregation occurred with the same efficiency in these mutants as in the wild-type background (Fig. 3d). Thus, the artificial kinetochore also bypasses the functional requirements for evolutionarily conserved inner kinetochore proteins.

The conserved KMN (KNL-1/Mtw1/Ndc80) network represents an important part of the kinetochore–microtubule interface in all eukaryotes[9]. We examined segregation of wild-type and *tetO* mini-chromosomes

in the temperature-sensitive *mtw1-1* allele. Although segregation of mini-chromosomes containing a wild-type *CEN* was compromised at the restrictive temperature, segregation mediated by Ask1–TetR did not seem to be affected (Fig. 3d). We also attempted to analyse acentric mini-chromosome segregation in temperature-sensitive alleles of the Ndc80 complex (*ndc80-1, ndc80-2*); however a substantial amount of spindle damage at the restrictive temperature made it impossible to analyse whether the Dam1-based segregation was dependent on *NDC80* function (data not shown).

Bi-orientation of kinetochores is dependent on the activity of the mitotic kinase Aurora B/Ipl1p and its activator protein INCENP/Sli15p. A sub-stoichiometric amount of Ipl1p/Aurora kinase was co-purified with the Ask1–TetR-containing Dam1 complex in the previous TAP purification (data not shown), suggesting that regulation by Aurora B kinase might also apply to the artificial segregation system. We therefore combined the Ask1–TetR fusion with temperature-sensitive alleles of *SLI15* and *IPL1* (Fig. 3e). After 2 h at the restrictive temperature, 60% of anaphase cells showed missegregation of wild-type mini-chromosomes, consistent with the defects observed on depletion of Sli15p[23]. The same amount of missegregation was observed for *tetO* mini-chromosomes segregated by Ask1–TetR at the restrictive temperature of *sli15-3*. In the *ipl1-2* mutant, mini-chromosomes containing the artificial kinetochore showed a slight improvement in segregation, but were clearly still affected by the mutation (Fig. 3e). Thus, the artificial kinetochore bypasses essential DNA-binding components of native kinetochores, but it cannot overcome segregation defects imposed by the *sli15-3* or *ipl1-2* mutants. This suggests that the artificial system bi-orients in an Ipl1-dependent manner. Alternatively, the microtubule binding capacity of the Ipl1–Sli15 complex could contribute directly to the coupling activity of the Dam1 complex.

Centromere–microtubule interactions mediated by native kinetochores are under surveillance by the mitotic checkpoint, which enhances the fidelity of chromosome segregation by preventing the onset of anaphase until all chromosome–microtubule connections have been properly established. To determine whether the Dam1-mediated segregation system is under the control of the mitotic checkpoint, we compared mitotic stability of wild-type mini-chromosomes with mini-chromosomes segregated by Ask1–TetR in *MAD2* and *mad2Δ* cells (Fig. 3f). Stability of mini-chromosomes carrying a wild-type centromere was significantly reduced in a *mad2Δ* strain (67 ± 5.3% compared with 49 ± 2.1%, P <0.001). By contrast, under our assay conditions, stabilization of the acentric *112xtetO*$_2$ mini-chromosomes conferred by Ask1–TetR, was unaffected by *mad2Δ* (25 ± 2% compared with 30 ± 3%, P = 0.06). This suggests that the artificial kinetochore is not under the control of the mitotic checkpoint.

Finally, we examined whether the functionality of artificial kinetochores is restricted to small circular mini-chromosomes or whether it can be transferred to native yeast chromosomes. To test this, we placed the endogenous centromere of chromosome V under the control of a galactose-inducible promoter, which allowed us to conditionally inactivate centromere function by transferring the cells from glucose to galactose-containing medium (Fig. 4a). We inserted an array of *112xtetO*$_2$ operators 35 kb upstream of the conditional centromere and analysed the segregation of chromosome V in strains that express TetR–GFP and either wild-type Ask1p or Ask1–TetR. Six hours after shifting the cells to galactose-containing medium, we observed substantial missegregation of the labelled chromosome V under *CEN off* conditions (Fig. 4b, middle panel). By contrast, 84% of the large budded cells expressing Ask1–TetR were able to segregate chromosome V correctly under *CEN off* conditions (Fig. 4b, c). Furthermore, plating equal numbers of cells on galactose-containing medium revealed that expression of Ask1–TetR allowed colony formation under *CEN off* conditions (Fig. 4d). Taken together, these results demonstrate that artificial recruitment of the Dam1 complex can promote segregation of a native yeast chromosome under conditions in which the endogenous kinetochore is inactivated.

The common design principle of kinetochores, which even extends to primitive plasmid segregation mechanisms found in prokaryotes[24], is to couple specific DNA regions to force-generating polymers through proteins that allow insertional polymerization and depolymerization mechanisms. Using an *in vivo* reconstitution approach we have established not only that the Dam1 complex is necessary for chromosome segregation in *S.cerevisiae*, but also that its artificial recruitment to an acentric plasmid is sufficient to confer mitotic stabilization. Our results suggest general mechanisms underlying the organizational principles of kinetochores: the ability of outer kinetochore proteins, such as the Dam1 complex, to translate microtubule force into chromosome movement is not strictly coupled to the presence of inner kinetochore proteins but is instead an inherent property of the complex itself. In that sense the organization of the kinetochore as a macromolecular machine is modular: if the specification of assembly is provided by alternative means (for example, artificial targeting), outer kinetochore complexes are still able to function according to their native context. Our study demonstrates that, in principle, it is possible to build a kinetochore by directly recruiting a microtubule-binding component to DNA. However, why are 'real' kinetochores so much more complex? Although it might be possible to build a simple attachment site that provides a linkage to dynamic microtubules, the additional complexity of kinetochores that arose during evolution probably comes from the necessity to regulate kinetochore assembly spatially and temporally and to integrate the mechanical state of kinetochores with cell-cycle progression through signalling cascades such as the mitotic checkpoint. We envision that 'artificial' kinetochores, as described in this study, will be useful for further analysis of kinetochore function in the future. With protocols for the purification of mini-chromosomes available[25], different artificial kinetochores can be purified, analysed by mass spectrometry to identify interacting proteins and used for *in vitro* assays to study their association with dynamic microtubules. Ultimately, this offers the possibility to dissect functions of individual kinetochore proteins in reconstituted systems for chromosome bi-orientation and anaphase movements *in vitro*.

Note added in proof: a related manuscript by Lacefield et al. (Nature Cell Biol. ***11,*** *doi: 10.1038/ncb1925; 2009*) *is also published in this issue.*

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturecellbiology/

Note: Supplementary Information is available on the Nature Cell Biology website.

ACKNOWLEDGEMENTS

The authors wish to thank all members of the Westermann lab for discussions, and Jan-Michael Peters and Barry Dickson for critical reading of the manuscript. We thank Soni Lacefield and Andrew Murray for communicating results before publication, and the Nasmyth and Drubin/Barnes labs for strains and plasmids.

Research in the laboratory of S.W. was supported by the European Research Council under the European Community's Seventh Framework Programme (FP7/2007-2013)/ERC starting grant agreement no. [203499], and by the Austrian Science Fund FWF (SFB F34-B03).

AUTHOR CONTRIBUTIONS

S.W. and E.K. designed the experiments and wrote the manuscript. E.K. performed most of the experiments with help from So. W., Y.P. and S.W; K.M. contributed the mass spectrometry analysis.

COMPETING INTERESTS

The authors declare that they have no competing financial interest.

Published online at http://www.nature.com/naturecellbiology/
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

1. Cheeseman, I. M. & Desai, A. Molecular architecture of the kinetochore-microtubule interface. *Nature Rev. Mol. Cell Biol.* **9,** 33–46 (2008).
2. Tanaka, T. U. & Desai, A. Kinetochore-microtubule interactions: the means to the end. *Curr. Opin. Cell Biol.* **20,** 53–63 (2008).
3. Westermann, S., Drubin, D. G. & Barnes, G. Structures and functions of yeast kinetochore complexes. *Annu. Rev. Biochem.* **76,** 563–591 (2007).
4. Meluh, P. B., Yang, P., Glowczewski, L., Koshland, D. & Smith, M. M. Cse4p is a component of the core centromere of *Saccharomyces cerevisiae*. *Cell* **94,** 607–613 (1998).
5. Furuyama, S. & Biggins, S. Centromere identity is specified by a single centromeric nucleosome in budding yeast. *Proc. Natl Acad. Sci. USA* **104,** 14706–14711 (2007).
6. Clarke, L. & Carbon, J. Isolation of a yeast centromere and construction of functional small circular chromosomes. *Nature* **287,** 504–509 (1980).
7. Megee, P. C. & Koshland, D. A functional assay for centromere-associated sister chromatid cohesion. *Science* **285,** 254–257 (1999).
8. Dewar, H., Tanaka, K., Nasmyth, K. & Tanaka, T. U. Tension between two kinetochores suffices for their bi-orientation on the mitotic spindle. *Nature* **428,** 93–97 (2004).
9. Cheeseman, I. M., Chappie, J. S., Wilson-Kubalek, E. M. & Desai, A. The conserved KMN network constitutes the core microtubule-binding site of the kinetochore. *Cell* **127,** 983–997 (2006).
10. Ciferri, C. *et al.* Implications for kinetochore-microtubule attachment from the structure of an engineered Ndc80 complex. *Cell* **133,** 427–439 (2008).
11. Cheeseman, I. M. *et al.* Implication of a novel multiprotein Dam1p complex in outer kinetochore function. *J. Cell Biol.* **155,** 1137–1145 (2001).
12. Cheeseman, I. M. *et al.* Phospho-regulation of kinetochore-microtubule attachments by the Aurora kinase Ipl1p. *Cell* **111,** 163–172 (2002).
13. Tanaka, K., Kitamura, E., Kitamura, Y. & Tanaka, T. U. Molecular mechanisms of microtubule-dependent kinetochore transport toward spindle poles. *J. Cell Biol.* **178,** 269–281 (2007).
14. Miranda, J. J., De Wulf, P., Sorger, P. K. & Harrison, S. C. The yeast DASH complex forms closed rings on microtubules. *Nature Struct. Mol. Biol.* **12,** 138–143 (2005).
15. Westermann, S. *et al.* Formation of a dynamic kinetochore-microtubule interface through assembly of the Dam1 ring complex. *Mol. Cell* **17,** 277–290 (2005).
16. Wang, H. W. *et al.* Architecture of the Dam1 kinetochore ring complex and implications for microtubule-driven assembly and force-coupling mechanisms. *Nature Struct. Mol. Biol.* **14,** 721–726 (2007).
17. Westermann, S. *et al.* The Dam1 kinetochore ring complex moves processively on depolymerizing microtubule ends. *Nature* **440,** 565–569 (2006).
18. Asbury, C. L., Gestaut, D. R., Powers, A. F., Franck, A. D. & Davis, T. N. The Dam1 kinetochore complex harnesses microtubule dynamics to produce force and movement. *Proc. Natl Acad. Sci. USA* **103,** 9873–9878 (2006).
19. Grishchuk, E. L. *et al.* The Dam1 ring binds microtubules strongly enough to be a processive as well as energy-efficient coupler for chromosome motion. *Proc. Natl Acad. Sci. USA* **105,** 15423–15428 (2008).
20. Cheeseman, I. M., Enquist-Newman, M., Muller-Reichert, T., Drubin, D. G. & Barnes, G. Mitotic spindle integrity and kinetochore function linked by the Duo1p/Dam1p complex. *J. Cell Biol.* **152,** 197–212 (2001).
21. He, X., Rines, D. R., Espelin, C. W. & Sorger, P. K. Molecular analysis of kinetochore-microtubule attachment in budding yeast. *Cell* **106,** 195–206 (2001).
22. Shcheprova, Z., Baldi, S., Frei, S. B., Gonnet, G. & Barral, Y. A mechanism for asymmetric segregation of age during yeast budding. *Nature* **454,** 728–734 (2008).
23. Sandall, S. *et al.* A Bir1-Sli15 complex connects centromeres to microtubules and is required to sense kinetochore tension. *Cell* **127,** 1179–1191 (2006).
24. Garner, E. C., Campbell, C. S. & Mullins, R. D. Dynamic instability in a DNA-segregating prokaryotic actin homolog. *Science* **306,** 1021–1025 (2004).
25. Ivanov, D. & Nasmyth, K. A topological interaction between cohesin rings and a circular minichromosome. *Cell* **122,** 849–860 (2005).

DOI: 10.1038/ncb1924

METHODS

Yeast genetics and molecular biology. To construct acentric mini-chromosomes, the ArsH4 element was amplified from pRS313 and cloned into the *Xho*I site of pRS303. The *10xtetO*$_2$ array was amplified from pCM189new[26] and cloned into the *Bam*HI site of pRS303+*ArsH4*. For the *112xtetO*$_2$ mini-chromosome, the *ArsH4* element was amplified as described above and cloned into the *Bam*HI site of pRS306-*tetO*$_2$*x112*. For visualization, the *TetO/ArsH4* array was subcloned into the *Bam*HI site of pAFS59 (ref. 27). Yeast cells were transformed using the lithium acetate procedure.

Integration of the Tet-Repressor (TetR) was accomplished by one-step PCR from a TetR-integration plasmid. The TetR sequence was amplified from plasmid pCM174 (ref. 26) with a flexible linker $(SG_4)_2$. The GFP sequence in pFA6a–GFP(S65T)-KanMX6 (ref. 28) was replaced with TetR by *Pac*I/*Asc*I digest. Proper fusion of TetR to target proteins was confirmed by DNA sequencing and western blotting (Supplementary Information, Fig. S1).

Plating assay and data analysis. Cells were grown at 30°C for 24 h in YP medium containing 2% glucose. An optical density of 0.6 was adjusted and diluted 1,000-fold in SD minimal medium containing 2% glucose. An aliquot (80 µl) of diluted cell suspension was spread on YPD and doHIS or doURA plates, respectively and incubated for 2 days at 30°C. The total numbers of cells were counted and the loss rates determined according to the percentage difference of colonies on doHIS/doURA and YPD plates. Plates were analysed in duplicates and all experiments were performed three times. Statistical analysis was carried out using origin software.

Dam1 complex purification. Tandem-affinity purification of the Dam1 complex and identification of proteins by mass-spectrometry was performed as described previously, except that 400 mM KCl was used throughout the study[11].

Microscopy. For image acquisition, we used a DeltaVision microscope (AppliedPrescision), a UPlanSApo ×100 objective lens (Olympus; NA 1.40), SoftWoRx software (AppliedPrescision) and a CoolSnap HQ CCD camera (Photometrics). For single images, we acquired 32-36 (0.2 µm apart) *z* sections, which were deconvoluted (aggressive mode) and projected to two-dimensional images. Time-lapse microscopy was carried out at 30°C taking 12–14 *z* sections of 0.5 µm distance. Image analysis was performed using SoftWoRx software.

Analysis in temperature-sensitive mutants. Cells were grown overnight in SD minimal medium containing 2% glucose, diluted 1:3 with fresh medium the next day and grown for 2–3 h at 25°C. After transferring the cells to restrictive temperatures for 1 h (34°C for *ndc10-2* mutants and 37°C for all other mutants), LacI–GFP expression was induced for 20 min in the presence of 50 µM $CuSO_4$. An aliquot (500 µl) of cell suspension was fixed with 4% paraformaldehyde and resuspended in 100 µl of 0.1M K_2HPO_4/1.2M sorbitol. For imaging, 5 µl of fixed cells were spread on ConA-coated cover slips and incubated for 15 min at room temperature. Images were acquired as described above.

To test for artificial kinetochore function on a native yeast chromosome, strains SWY294, SWY297 and SWY298 were grown overnight in YP-2% raffinose. They were washed three times with YP-2% galactose, adjusted to 0.2 OD and grown for 6 h at 30°C in YP-2% galactose. Cells were imaged on a Zeiss Axiovert microscope, equipped with a ×100 objective (Zeiss) and a Coolsnap CCD (Photometrics) camera. Images are maximum intensity projections of 8 × 0.6 µm stacks. To determine growth on YP-galactose, cells from strains SWY294 and SWY298 growing logarithmically in YP-raffinose were counted, adjusted to 0.7 OD and a 100-fold dilution was plated on YP-2% dextrose and YP–2% galactose plates. Photographs of the plates were taken after 3 days of growth at 30°C.

26. Gari, E., Piedrafita, L., Aldea, M. & Herrero, E. A set of vectors with a tetracycline-regulatable promoter system for modulated gene expression in Saccharomyces cerevisiae. *Yeast* **13,** 837–848 (1997).
27. Straight, A. F., Belmont, A. S., Robinett, C. C. & Murray, A. W. GFP tagging of budding yeast chromosomes reveals that protein-protein interactions can mediate sister chromatid cohesion. *Curr. Biol.* **6,** 1599–1608 (1996).
28. Longtine, M. S. *et al*. Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* **14,** 953–961 (1998).

nature cell biology

DOI: 10.1038/ncb1924

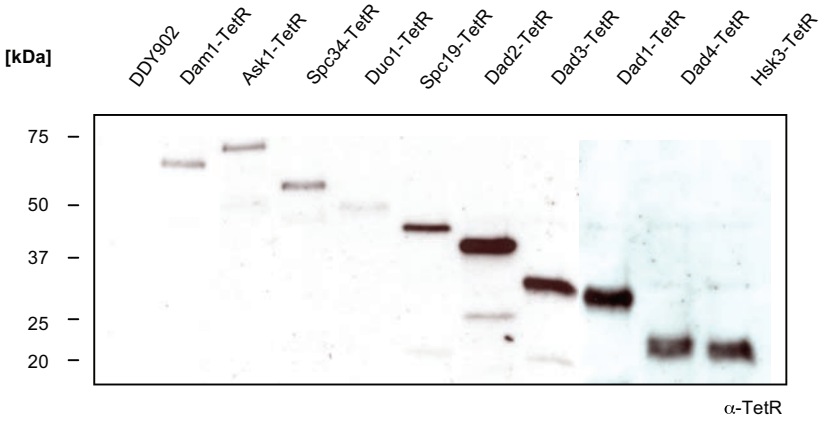


Figure S1 Western Blot analysis of the TetR fusion proteins. Whole cell extracts from a wild-type strain (DDY902), and strains expressing the Dam1 complex subunits fused to the Tet-Repressor were separated by SDS-PAGE, blotted and probed with an anti–TetR antibody. The TetR fusion proteins are detected at the expected sizes.

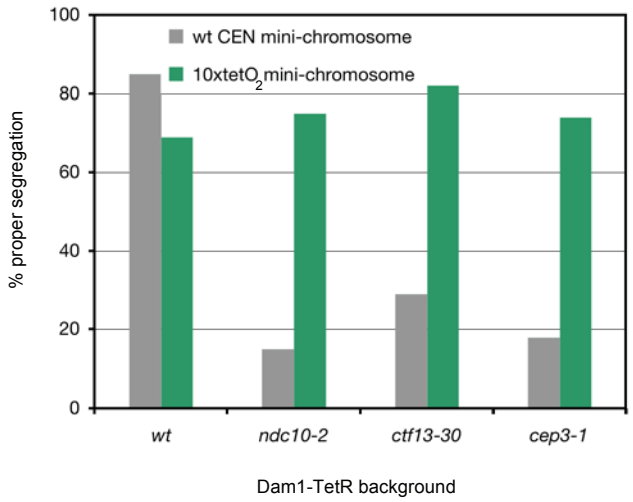


Figure S2 Segregation of wild-type mini-chromosomes or 10xtetO$_2$ mini-chromosomes bound by Dam1-TetR analyzed in temperature-sensitive mutants of the CBF3 complex. n= 100 cells per strain.

Supplementary Movie Legends

Movie S1 Movements of wild-type mini-chromosomes (green) and spindle pole bodies (Spc42-mChrerry, red) in a dividing yeast cell upon entering anaphase.

Movie S2 Movements of acentric mini-chromosomes (green) and spindle pole bodies (Spc42-mCherry, red) in a wild-type strain background observed by Deltavision microscopy. Note that movements of mini-chromosomes are uncoupled from movement of spindle pole bodies.

Movie S3 Movements of an acentric mini-chromosome (green) containing a $10xtetO_2$ array and spindle pole bodies (Spc42-mChrerry, red) in a Dam1-TetR strain background observed by Deltavision microscopy. Note successful segregation of the acentric $10xtetO_2$ mini-chromosomes in the Dam1-TetR strain.

Supplementary Table 1:

Yeast strains used in this study

Name	Genotype
EKY4	*Matα, his3Δ200, ura3-52, leu2-3, pCu-LacI-GFP::HIS*
EKY6	*Matα, his3 Δ200, ura3-52, leu2-3, pCu-LacI-GFP::HIS, SPC42-mCherry::URA3*
DDY902	*Mata, ade2-1, leu2-3,112, his3 Δ200, ura3-52*
EKY56	*Mata, ade2-1, leu2-3,112, his3 Δ200, ura3-52, CSE4-TetR::KanMX, ts at 37°C*
EKY63	*Mata, ade2-1, leu2-3,112, his3 Δ200, ura3-52, SPC24-TetR::KanMX*
EKY70	*Mata, ade2-1, leu2-3,112, his3 Δ200, ura3-52, ASK1-TetR::KanMX*
EKY77	*Mata, ade2-1, leu2-3,112, his3 Δ200, ura3-52, DAM1-TetR::KanMX*
EKY79	*Matα, his3Δ200, ura3-52, leu2-3, pCu-LacI-GFP::HIS, SPC42-mcherry::URA3, DAM1-TetR::KanMX*
EKY71	*Matα, his3Δ200,ura3-52, leu2-3, pCu-LacI-GFP::HIS,SPC42-mCherry::URA3, ASK1-TetR::KanMX*
EKY84	*Mata, ade2-1, leu2-3,112, his3Δ200, ura3-52, Spc105-TetR::KanMX*
EKY91	*Mata, ade2-1, leu2-3,112, his3Δ200, ura3-52, Ndc10-TetR::KanMX*
EKY94	*Mata, ade2-1, leu2-3,112, his3Δ200, ura3-52, Cep3-TetR::KanMX*
EKY98	*Mata, ade2-1, leu2-3,112, his3Δ200, ura3-52, Stu2-TetR::KanMX*
EKY105	*Mata, leu2-3,112, his3Δ200, ura3-52, Mif2-TetR::KanMX*
EKY162	*Mata, his3Δ200, ura3-52, leu2-3, HIS::pCu-LacI-GFP, Spc42-mcherry::URA3, ndc10-2 (ts at 34 and 37°C)*
EKY161	*Mata, his3Δ200, ura3-52, leu2-3, HIS::pCu-LacI-GFP, URA::Spc42-mcherry, Dam1-TetR::KanMX, ndc10-2 (ts at 34 and 37°C)*
EKY176	*Mata, his3Δ200, ura3-52, leu2-3, HIS::pCu-LacI-GFP, URA::Spc42-mcherry, Ask1-TetR::KanMX, ndc10-2 (ts at 34 and 37°C)*
EKY179	*Matα, ade2-1, lys2-801, his3Δ200, ura3-52, leu2-3, HIS::pCu-LacI-GFP, Spc42-mcherry::URA, ctf13-30 (ts at 37°C)*
EKY180	*Mata, lys2-801, his3 Δ200, ura3-52, leu2-3, HIS::pCu-LacI-GFP, URA::Spc42-mcherry, Ask1-TetR::KanMX, ctf13-30 (ts at 37°C)*
EKY181	*Matα, ade2-1, lys2-801, his3Δ200, ura3-52, leu2-3, HIS::pCu-LacI-GFP, Spc42-mcherry::URA3, Dam1-TetR::KanMX, ctf13-30 (ts at 37°C)*
EKY185	*Mata, ade2-1, lys2-801, his3Δ200, ura3-52, leu2-3, HIS::pCu-LacI-GFP, Spc42-mcherry::URA3, cep3-1 (ts at 37°C)*
EKY186	*Mata, his3Δ200, ura3-52, leu2-3, HIS::pCu-LacI-GFP, Spc42-mcherry::URA3, Dam1-TetR::KanMX, cep3-1 (ts at 37°C)*
EKY187	*Mata, ade2-1, lys2-801, his3Δ200, ura3-52, leu2-3, HIS::pCu-LacI-GFP, Spc42-mcherry::URA3, Ask1-TetR::KanMX, cep3-1 (ts at 37°C)*
EKY188	*Mata, ade2-1, lys2-801, his3Δ200, ura3-52, leu2-3, HIS::pCu-LacI-GFP, Spc42-mcherry::URA3, cse4-1 (ts at 37°C)*

EKY189	*Mata, ade2-1, his3Δ200, ura3-52, leu2-3, HIS::pCu-LacI-GFP, Spc42-mCherry::URA3, Dam1-TetR, cse4-1 (ts at 37°C)*
EKY190	*Mata, ade2-1, his3Δ200, ura3-52, leu2-3, HIS::pCu-LacI-GFP, Spc42-mcherry::URA3, Ask1-TetR, cse4-1 (ts at 37°C)*
EKY191	*Matα, lys2-801, his3Δ200, ura3-52, leu2-3, HIS::pCu-LacI-GFP, Spc42-mcherry:: URA3, mif2-3 (ts at 37°C)*
EKY192	*Mata, his3Δ200, ura3-52, leu2-3, HIS::pCu-LacI-GFP, Spc42-mCherry::URA3, Dam1-TeR::KanMX, mif2-3 (ts at 37°C)*
EKY193	*Mata, his3Δ200, ura3-52, leu2-3, HIS::pCu-LacI-GFP, Spc42-mcherry::URA3, Ask1-TeR::KanMX, mif2-3 (ts at 37°C)*
EKY194	*Mata, lys2-801, his3Δ200, ura3-52, leu2-3, HIS::pCu-LacI-GFP, Spc42-mCherry::URA3, mtw1-1 (ts at 37°C)*
EKY195	*Mata, ade2-1, lys2-801, lys2-801, his3Δ200, ura3-52, leu2-3, HIS::pCu-LacI-GFP, Spc42-mcherry::URA3, Dam1-TetR, mtw1-1 (ts at 37°C)*
EKY196	*Mata, his3Δ200, ura3-52, leu2-3, HIS::pCu-LacI-GFP, Spc42-mcherry::URA3, Ask1-TetR, mtw1-1 (ts at 37°C)*
EKY200	*Matα, lys2-801, his3Δ200, ura3-52, leu2-3, HIS::pCu-LacI-GFP, Spc42-mcherry::URA3, dad1-1 (ts at 37°C)*
EKY201	*Mata, lys2-801, his3Δ200, ura3-52, leu2-3, HIS::pCu-LacI-GFP, Spc42-mCherry::URA3, Ask1-TetR::KanMX, dad1-1 (ts at 37°C)*
EKY203	*Mata, lys2-801, his3Δ200, ura3-52, leu2-3, HIS::pCu-LacI-GFP, Spc42-mCherry::URA3, ipl1-2 (ts at 34°C and 37°C)*
EKY204	*Mata, lys2-801, his3Δ200, ura3-52, leu2-3, HIS::pCu-LacI-GFP, Spc42-mCherry::URA3, Dam1-TetR::KanMX ipl1-2 (ts at 34°C and 37°C)*
EKY205	*Mata, lys2-801, his3Δ200, ura3-52, leu2-3, HIS::pCu-LacI-GFP, Spc42-mCherry::URA3, Ask1-TetR::KanMX, ipl1-2 (ts at 34°C and 37°C)*
EKY206	*Mat a, lys2-801, his3Δ200, ura3-52, leu2-3, HIS::pCu-LacI-GFP, Spc42-mCherry::URA3, sli15-3 (ts at 37°C)*
EKY207	*Mata, his3Δ200, ura3-52, leu2-3, HIS::pCu-LacI-GFP, Spc42-mCherry::URA3, Dam1-TetR::KanMX, sli15-3 (ts at 37°C)*
EKY208	*Mata, his3Δ200, ura3-52, leu2-3, HIS::pCu-LacI-GFP, Spc42-mCherry::URA3, Ask1-TetR::KanMX, sli15-3 (ts at 37°C)*
EKY209	*Mata, ade2-1, leu2-3,112, his3Δ200, ura3-52, Spc34-TetR::KanMX*
EKY210	*Mata, ade2-1, leu2-3,112, his3Δ200, ura3-52, Duo1-TetR::KanMX*
EKY211	*Mata, ade2-1, leu2-3,112, his3Δ200, ura3-52, Spc19-TetR, KanMX*
EKY212	*Mata, ade2-1, leu2-3,112, his3Δ200, ura3-52, Dad2-TetR::KanMX*
EKY213	*Mata, ade2-1, leu2-3,112, his3Δ200, ura3-52, Dad3-TetR::KanMX*
EKY214	*Mata, ade2-1, leu2-3,112, his3Δ200, ura3-52, Dad1-TetR::KanMX*
EKY215	*Mata, ade2-1, leu2-3,112, his3Δ200, ura3-52, Dad4-TetR::KanMX*
EKY216	*Mata, ade2-1, leu2-3,112, his3Δ200, ura3-52, Hsk3-TetR::KanMX*
EKY217	*Mata, ade2-1, leu2-3,112, his3Δ200, ura3-52, mad2Δ::URA3*
EKY218	*Mata, ade2-1, leu2-3,112, his3Δ200, ura3-52, mad2Δ::URA3, Ask1-TetR::KanMX*

EKY219	*Mata, ade2-1, leu2-3,112, his3Δ200, ura3-52, Ask1-TetR::KanMX, dam1-19::KanMX (ts at 37°C)*
SWY276	*Mata, ade2-1, leu2-3,112, his3Δ200, ura3-52, Dad1-S-tag-TEV-ZZ::HIS3, Ask1-TetR::KanMX*
SWY277	*Mata, ade2-1, leu2-3,112, his3Δ200, ura3-52, Dad1-S-Tag-TEV-ZZ::HIS3*
SWY258	*Mata, his3Δ200, ura3-52, leu2-3,112, tetOx112::URA3, TetR-GFP::LEU2*
SWY285	*Mata, his3Δ200, ura3-52, leu2-3,112, HIS3::pGAL-CEN5, tetOx112::URA3, TetR-GFP::LEU2, Ask1-TetR::KanMX*
SWY286	*Matα, his3Δ200, ura3-52, leu2-3,112, HIS3::pGAL-CEN5, tetOx112::URA3, TetR-GFP::LEU2*
SWY294	*MATa, leu2-3,112, his3Δ200, ura3-52, tetOx112::URA3, tetR-GFP-LEU2, HIS3::pGAL-CEN5, Ask1-TetR::KanMX6, cdc15-1*
SWY297	*MATa, leu2-3,112, his3Δ200, ura3-52, tetOx112::URA3, tetR-GFP-LEU2, cdc15-1*
SWY298	*MATa, leu2-3,112, his3Δ200, ura3-52, tetOx112::URA3, tetR-GFP-LEU2, HIS3::pGAL-CEN5, cdc15-1*

Supplementary Table 2:

Plasmids used in this study

Plasmid#	Name	Description
pEK1	wt pRS313	CEN6/ArsH4 in pRS313
pEK2	ArsH4 pRS303	ArsH4 amplified from pRS313 and subcloned into Xho1 site of pRS303
pEK3	$tetO_{10}$/ArsH4 pRS303	10x$TetO_2$ cloned into BamH1 site of ArsH4 pRS303
pEK4	$tetO_2$x112/ArsH4 pRS306	ArsH4 cloned into BamH1 site of pRS306 containing ($tetO_2$x112)
pEK6	wt pAFS59	CEN6/ArsH4 cloned into pAFS59
pEK9	ArsH4 pAFS59	acentric -/ArsH4 cloned into Xho1 site of pAFS59
pEK10	$tetO_{10}$/ArsH4 pAFS59	10x$TetO_2$ cloned into BamH1 site of -/ArsH4 pAFS59
pEK17	C-terminal linker-TetR tagging	linker+TetR cloned into Pac1/Asc1 site of pFA6a-GFP(S65T)-KanMX

6.2 Abbreviations

APC/C	anaphase promoting complex/cyclosome
bp	basepair
Cdc	cell division cycle
Cdk	cyclin dependent kinase
C.elegans	*Caenorhabditis elegans*
CEN	centromere
CH	calponin homology
ChIP	chromatin immunoprecipitation
Da	Dalton
do	drop-out
DTT	dithiothreithol
DNA	deoxyribonucleic acid
E.coli	Escherichia coli
EDTA	ethylenediamin tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
g	gram
G418	geneticin
GAL	galactose
GFP	green fluorescent protein
his	histidine
IPTG	isopropyl β-D-1-thiogalactopyranoside
KanMX	geneticin resistance
kDa	kilo Dalton
k-MT	kinetochore microtubule
ko	knock-out
LacI	lactose-inhibitor
lacO	lactose-operator
LB	Luria-Bertani
leu	leucine
mg	milligram
min	minute
ml	milliliter
µl	micro liter
MT	microtubule
MW	molecular weight

myc	human c-myc epitope
nm	nanometer
Noc	nocodazol
NTA	nitrilotriacetic acid
OD	optical density
PAGE	polyacrylamid gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylenglycole
RNA	ribonucleic acid
RNAi	RNA interference
SDS	sodium dodecyl sulphate
PMSF	phenylmethylsulphonyl fluoride
RAF	raffinose
rpm	revolutions per minute
SAC	spindle assembly checkpoint
S.c.	Saccharomyces cerevisiae
sd	standard deviation
SPB	spindle pole body
TAP	tandem affinity purification
TBS	tris-buffered saline
TBS-T	tris-buffered saline with Tween20 detergent
TBZ	thiobendazole
tet	tetracycline
tetO	tetracycline-operator
TetR	tetracycline-repressor
TEV	tobacco etch virus
Ura	uracil
v_0	voit volume
WCE	whole cell extract
wt	wildtype
YEP	yeast extract peptone
YPD	yeast extract peptone dextrose

6.3 Table of Figures

Figure 1: Events during the budding yeast cell cycle. 2

Figure 2: Centromere organization in budding yeast. 4

Figure 3: Architecture of the budding yeast kinetochore. 5

Figure 4: The budding yeast Dam1 complex. 7

Figure 5: Sister chromosome bi-orientation. 8

Figure 6: Activation of the SAC by unattached kinetochores. 10

Figure 7: Comparison between a native and the artificial kinetochore. 12

Figure 8: Spot assay of TetR-tagged kinetochore proteins at 30°C and 37°C. 14

Figure 9: Mitotic stabilities of wildtype, acentric and mutant mini-chromosomes in a wildtype background strain. 15

Figure 10: Mitotic stabilities of wildtype and CDEII-7xtetO_2 mutant mini-chromosomes in different TetR strains. 16

Figure 11: Mitotic stabilities of wt and mutant mini-chromosomes in a Dam1-TetR background. 17

Figure 12: Plating assay of acentric mini-chromosomes in the Ask1-TetR background. 18

Figure 13: Mitotic stabilities of acentric and tetO mini-chromosomes in different TetR strains. 18

Figure 14: Mitotic stabilities of acentric and tetO mini-chromosomes conferred by the members of the Dam1 complex. 19

Figure 15: Affinity purification of the Dam1 complex from either a wildtype strain or a strain expressing Ask1-TetR. 20

Figure 16: Visualization of wildtype and acentric mini-chromosomes in a wildtype, Dam1-TetR or Ask1-TetR background. 21

Figure 17: Quantification of anaphase cells showing proper mini-chromosome segregation. 21

Figure 18: Time-lapse live-cell microscopy of wildtype acentric and 10xtetO_2 mini-chromosome in a Dam1–TetR strain background. 23

Figure 19: Dependency on structural kinetochore components 24

Figure 20: Dependency on regulatory kinetochore components 25

Figure 21: Mitotic stability of mini-chromosomes under spindle assembly checkpoint on- and off conditions. 26

Figure 22: A strategy to test artificial kinetochore function on a native yeast chromosome. ____ *27*

Figure 23: Segregation of native yeast chromosomes. _____ *28*

Figure 24: Mode of interaction between the CDEII-7xtetO_2 insertion and disruption of kinetochore architecture. _____ *30*

Figure 25: Model for coupling chromosomes to dynamic MTs and hypothetical architecture of the Dam1 complex. _____ *32*

Figure 26: Tandem affinity purification of the Spc105 complex. _____ *41*

Figure 27: Purification of Spc105p after over-expression from a galactose-inducible promoter. _ *43*

Figure 28: Spc105p protein organization and mapped phospho-sites. _____ *43*

Figure 29: Size exclusion chromatography of yeast whole cell extracts. _____ *44*

Figure 30: In vitro kinase assay of purified Spc105p variants with Cdc28p. _____ *46*

Figure 31: Spot assay of Spc105 wildtype and phospho-mutants on YPD- at 30°C and 37°C and TBZ plates at 30°C. _____ *47*

Figure 32: Visualization of kinetochore organization in Spc105 wildtype and phospho-mutant strains. _____ *48*

Figure 33: Visualization of Cin8p localization in Spc105^{wt} and Spc105^{6A} mutant strains. _____ *49*

Figure 34: Microtubule-binding assay with purified Spc105p. _____ *50*

Figure 35: Expression and purification of Spc105p from bacteria. _____ *51*

Figure 36: Tetrad analysis of Spc105 knock-out complemented with Spc105p$^{N\text{-}terminus}$ _____ *52*

6.4 References

1 Cheeseman, I. M. & Desai, A. Molecular architecture of the kinetochore-microtubule interface. *Nat Rev Mol Cell Biol* **9**, 33-46, doi:nrm2310 [pii] 10.1038/nrm2310 (2008).

2 Dong, Y., Vanden Beldt, K. J., Meng, X., Khodjakov, A. & McEwen, B. F. The outer plate in vertebrate kinetochores is a flexible network with multiple microtubule interactions. *Nat Cell Biol* **9**, 516-522, doi:ncb1576 [pii] 10.1038/ncb1576 (2007).

3 Clarke, L. & Carbon, J. The structure and function of yeast centromeres. *Annu Rev Genet* **19**, 29-55, doi:10.1146/annurev.ge.19.120185.000333 (1985).

4 Kiermaier, E., Woehrer, S., Peng, Y., Mechtler, K. & Westermann, S. A Dam1-based artificial kinetochore is sufficient to promote chromosome segregation in budding yeast. *Nat Cell Biol* **11**, 1109-1115, doi:ncb1924 [pii] 10.1038/ncb1924 (2009).

5 Garner, E. C., Campbell, C. S. & Mullins, R. D. Dynamic instability in a DNA-segregating prokaryotic actin homolog. *Science* **306**, 1021-1025, doi:306/5698/1021 [pii] 10.1126/science.1101313 (2004).

6 Hartwell, L. H., Culotti, J., Pringle, J. R. & Reid, B. J. Genetic control of the cell division cycle in yeast. *Science* **183**, 46-51 (1974).

7 Goshima, G. & Yanagida, M. Establishing biorientation occurs with precocious separation of the sister kinetochores, but not the arms, in the early spindle of budding yeast. *Cell* **100**, 619-633, doi:S0092-8674(00)80699-6 [pii] (2000).

8 Winey, M. & O'Toole, E. T. The spindle cycle in budding yeast. *Nat Cell Biol* **3**, E23-27, doi:10.1038/35050663 (2001).

9 Nasmyth, K. & Haering, C. H. Cohesin: its roles and mechanisms. *Annu Rev Genet* **43**, 525-558, doi:10.1146/annurev-genet-102108-134233 (2009).

10 Morgan, D. O. (ed Eleanor Lawrence) (New Science Press Ltd, 2007).

11 Nurse, P. Universal control mechanism regulating onset of M-phase. *Nature* **344**, 503-508, doi:10.1038/344503a0 (1990).

12 Fitch, I. *et al.* Characterization of four B-type cyclin genes of the budding yeast Saccharomyces cerevisiae. *Mol Biol Cell* **3**, 805-818 (1992).

13 Richardson, H., Lew, D. J., Henze, M., Sugimoto, K. & Reed, S. I. Cyclin-B homologs in Saccharomyces cerevisiae function in S phase and in G2. *Genes Dev* **6**, 2021-2034 (1992).

14 Winey, M. *et al.* Three-dimensional ultrastructural analysis of the Saccharomyces cerevisiae mitotic spindle. *J Cell Biol* **129**, 1601-1615 (1995).

15 Fitzgerald-Hayes, M., Clarke, L. & Carbon, J. Nucleotide sequence comparisons and functional analysis of yeast centromere DNAs. *Cell* **29**, 235-244, doi:0092-8674(82)90108-8 [pii] (1982).

16 Baker, R. E. & Masison, D. C. Isolation of the gene encoding the Saccharomyces cerevisiae centromere-binding protein CP1. *Mol Cell Biol* **10**, 2458-2467 (1990).

[17] Cumberledge, S. & Carbon, J. Mutational analysis of meiotic and mitotic centromere function in Saccharomyces cerevisiae. *Genetics* **117**, 203-212 (1987).

[18] McGrew, J., Diehl, B. & Fitzgerald-Hayes, M. Single base-pair mutations in centromere element III cause aberrant chromosome segregation in Saccharomyces cerevisiae. *Mol Cell Biol* **6**, 530-538 (1986).

[19] Cleveland, D. W., Mao, Y. & Sullivan, K. F. Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling. *Cell* **112**, 407-421, doi:S0092867403001156 [pii] (2003).

[20] Meluh, P. B., Yang, P., Glowczewski, L., Koshland, D. & Smith, M. M. Cse4p is a component of the core centromere of Saccharomyces cerevisiae. *Cell* **94**, 607-613, doi:S0092-8674(00)81602-5 [pii] (1998).

[21] Keith, K. C. & Fitzgerald-Hayes, M. CSE4 genetically interacts with the Saccharomyces cerevisiae centromere DNA elements CDE I and CDE II but not CDE III. Implications for the path of the centromere dna around a cse4p variant nucleosome. *Genetics* **156**, 973-981 (2000).

[22] Furuyama, S. & Biggins, S. Centromere identity is specified by a single centromeric nucleosome in budding yeast. *Proc Natl Acad Sci U S A* **104**, 14706-14711, doi:0706985104 [pii] 10.1073/pnas.0706985104 (2007).

[23] Mythreye, K. & Bloom, K. S. Differential kinetochore protein requirements for establishment versus propagation of centromere activity in Saccharomyces cerevisiae. *J Cell Biol* **160**, 833-843, doi:10.1083/jcb.200211116 jcb.200211116 [pii] (2003).

[24] De Wulf, P., McAinsh, A. D. & Sorger, P. K. Hierarchical assembly of the budding yeast kinetochore from multiple subcomplexes. *Genes Dev* **17**, 2902-2921, doi:10.1101/gad.1144403 1144403 [pii] (2003).

[25] Westermann, S. *et al.* Architecture of the budding yeast kinetochore reveals a conserved molecular core. *J Cell Biol* **163**, 215-222, doi:10.1083/jcb.200305100 jcb.200305100 [pii] (2003).

[26] Pietrasanta, L. I. *et al.* Probing the Saccharomyces cerevisiae centromeric DNA (CEN DNA)-binding factor 3 (CBF3) kinetochore complex by using atomic force microscopy. *Proc Natl Acad Sci U S A* **96**, 3757-3762 (1999).

[27] Espelin, C. W., Kaplan, K. B. & Sorger, P. K. Probing the architecture of a simple kinetochore using DNA-protein crosslinking. *J Cell Biol* **139**, 1383-1396 (1997).

[28] Ortiz, J., Stemmann, O., Rank, S. & Lechner, J. A putative protein complex consisting of Ctf19, Mcm21, and Okp1 represents a missing link in the budding yeast kinetochore. *Genes Dev* **13**, 1140-1155 (1999).

[29] Meluh, P. B. & Koshland, D. Evidence that the MIF2 gene of Saccharomyces cerevisiae encodes a centromere protein with homology to the mammalian centromere protein CENP-C. *Mol Biol Cell* **6**, 793-807 (1995).

[30] Ghosh, S. K., Poddar, A., Hajra, S., Sanyal, K. & Sinha, P. The IML3/MCM19 gene of Saccharomyces cerevisiae is required for a kinetochore-related process during chromosome segregation. *Mol Genet Genomics* **265**, 249-257 (2001).

[31] Goshima, G., Kiyomitsu, T., Yoda, K. & Yanagida, M. Human centromere chromatin protein hMis12, essential for equal segregation, is independent of CENP-A loading pathway. *J Cell Biol* **160**, 25-39, doi:10.1083/jcb.200210005 jcb.200210005 [pii] (2003).

[32] Kline, S. L., Cheeseman, I. M., Hori, T., Fukagawa, T. & Desai, A. The human Mis12 complex is required for kinetochore assembly and proper chromosome segregation. *J Cell Biol* **173**, 9-17, doi:jcb.200509158 [pii] 10.1083/jcb.200509158 (2006).

[33] Cheeseman, I. M., Chappie, J. S., Wilson-Kubalek, E. M. & Desai, A. The conserved KMN network constitutes the core microtubule-binding site of the kinetochore. *Cell* **127**, 983-997, doi:S0092-8674(06)01345-6 [pii] 10.1016/j.cell.2006.09.039 (2006).

[34] Akhmanova, A. & Steinmetz, M. O. Tracking the ends: a dynamic protein network controls the fate of microtubule tips. *Nat Rev Mol Cell Biol* **9**, 309-322, doi:nrm2369 [pii] 10.1038/nrm2369 (2008).

[35] Wei, R. R., Sorger, P. K. & Harrison, S. C. Molecular organization of the Ndc80 complex, an essential kinetochore component. *Proc Natl Acad Sci U S A* **102**, 5363-5367, doi:0501168102 [pii] 10.1073/pnas.0501168102 (2005).

[36] DeLuca, J. G. *et al.* Hec1 and nuf2 are core components of the kinetochore outer plate essential for organizing microtubule attachment sites. *Mol Biol Cell* **16**, 519-531, doi:E04-09-0852 [pii] 10.1091/mbc.E04-09-0852 (2005).

[37] Wei, R. R. *et al.* Structure of a central component of the yeast kinetochore: the Spc24p/Spc25p globular domain. *Structure* **14**, 1003-1009, doi:S0969-2126(06)00223-1 [pii] 10.1016/j.str.2006.04.007 (2006).

[38] Joglekar, A. P., Bouck, D. C., Molk, J. N., Bloom, K. S. & Salmon, E. D. Molecular architecture of a kinetochore-microtubule attachment site. *Nat Cell Biol* **8**, 581-585, doi:ncb1414 [pii] 10.1038/ncb1414 (2006).

[39] Gillett, E. S., Espelin, C. W. & Sorger, P. K. Spindle checkpoint proteins and chromosome-microtubule attachment in budding yeast. *J Cell Biol* **164**, 535-546, doi:10.1083/jcb.200308100 jcb.200308100 [pii] (2004).

[40] McCleland, M. L. *et al.* The highly conserved Ndc80 complex is required for kinetochore assembly, chromosome congression, and spindle checkpoint activity. *Genes Dev* **17**, 101-114, doi:10.1101/gad.1040903 (2003).

[41] Enquist-Newman, M. *et al.* Dad1p, third component of the Duo1p/Dam1p complex involved in kinetochore function and mitotic spindle integrity. *Mol Biol Cell* **12**, 2601-2613 (2001).

[42] Cheeseman, I. M., Enquist-Newman, M., Muller-Reichert, T., Drubin, D. G. & Barnes, G. Mitotic spindle integrity and kinetochore function linked by the Duo1p/Dam1p complex. *J Cell Biol* **152**, 197-212 (2001).

[43] Westermann, S. *et al.* Formation of a dynamic kinetochore- microtubule interface through assembly of the Dam1 ring complex. *Mol Cell* **17**, 277-290, doi:S1097276505010014 [pii] 10.1016/j.molcel.2004.12.019 (2005).

[44] Miranda, J. J., De Wulf, P., Sorger, P. K. & Harrison, S. C. The yeast DASH complex forms closed rings on microtubules. *Nat Struct Mol Biol* **12**, 138-143, doi:nsmb896 [pii] 10.1038/nsmb896 (2005).

[45] Westermann, S. *et al.* The Dam1 kinetochore ring complex moves processively on depolymerizing microtubule ends. *Nature* **440**, 565-569, doi:nature04409 [pii] 10.1038/nature04409 (2006).

[46] Asbury, C. L., Gestaut, D. R., Powers, A. F., Franck, A. D. & Davis, T. N. The Dam1 kinetochore complex harnesses microtubule dynamics to produce force and movement. *Proc Natl Acad Sci U S A* **103**, 9873-9878, doi:0602249103 [pii] 10.1073/pnas.0602249103 (2006).

[47] Hanisch, A., Sillje, H. H. & Nigg, E. A. Timely anaphase onset requires a novel spindle and kinetochore complex comprising Ska1 and Ska2. *EMBO J* **25**, 5504-5515, doi:7601426 [pii] 10.1038/sj.emboj.7601426 (2006).

[48] Gaitanos, T. N. *et al.* Stable kinetochore-microtubule interactions depend on the Ska complex and its new component Ska3/C13Orf3. *EMBO J* **28**, 1442-1452, doi:emboj200996 [pii] 10.1038/emboj.2009.96 (2009).

[49] Welburn, J. P. *et al.* The human kinetochore Ska1 complex facilitates microtubule depolymerization-coupled motility. *Dev Cell* **16**, 374-385, doi:S1534-5807(09)00039-2 [pii] 10.1016/j.devcel.2009.01.011 (2009).

[50] Tanaka, K. *et al.* Molecular mechanisms of kinetochore capture by spindle microtubules. *Nature* **434**, 987-994, doi:nature03483 [pii] 10.1038/nature03483 (2005).

[51] Kirschner, M. & Mitchison, T. Beyond self-assembly: from microtubules to morphogenesis. *Cell* **45**, 329-342, doi:0092-8674(86)90318-1 [pii] (1986).

[52] O'Connell, C. B. & Khodjakov, A. L. Cooperative mechanisms of mitotic spindle formation. *J Cell Sci* **120**, 1717-1722, doi:120/10/1717 [pii] 10.1242/jcs.03442 (2007).

[53] Tanaka, K., Kitamura, E., Kitamura, Y. & Tanaka, T. U. Molecular mechanisms of microtubule-dependent kinetochore transport toward spindle poles. *J Cell Biol* **178**, 269-281, doi:jcb.200702141 [pii] 10.1083/jcb.200702141 (2007).

[54] Hauf, S. & Watanabe, Y. Kinetochore orientation in mitosis and meiosis. *Cell* **119**, 317-327, doi:S0092867404009961 [pii] 10.1016/j.cell.2004.10.014 (2004).

[55] Ault, J. G. & Rieder, C. L. Chromosome mal-orientation and reorientation during mitosis. *Cell Motil Cytoskeleton* **22**, 155-159, doi:10.1002/cm.970220302 (1992).

[56] Loncarek, J. *et al.* The centromere geometry essential for keeping mitosis error free is controlled by spindle forces. *Nature* **450**, 745-749, doi:nature06344 [pii] 10.1038/nature06344 (2007).

[57] Tanaka, T. U., Stark, M. J. & Tanaka, K. Kinetochore capture and bi-orientation on the mitotic spindle. *Nat Rev Mol Cell Biol* **6**, 929-942, doi:nrm1764 [pii] 10.1038/nrm1764 (2005).

[58] Dewar, H., Tanaka, K., Nasmyth, K. & Tanaka, T. U. Tension between two kinetochores suffices for their bi-orientation on the mitotic spindle. *Nature* **428**, 93-97, doi:10.1038/nature02328 nature02328 [pii] (2004).

[59] Fuller, B. G. *et al.* Midzone activation of aurora B in anaphase produces an intracellular phosphorylation gradient. *Nature* **453**, 1132-1136, doi:nature06923 [pii] 10.1038/nature06923 (2008).

[60] Liu, D., Vader, G., Vromans, M. J., Lampson, M. A. & Lens, S. M. Sensing chromosome bi-orientation by spatial separation of aurora B kinase from kinetochore substrates. *Science* **323**, 1350-1353, doi:1167000 [pii] 10.1126/science.1167000 (2009).

[61] Ruchaud, S., Carmena, M. & Earnshaw, W. C. The chromosomal passenger complex: one for all and all for one. *Cell* **131**, 230-231, doi:S0092-8674(07)01266-4 [pii] 10.1016/j.cell.2007.10.002 (2007).

[62] Tanaka, T. U. *et al.* Evidence that the Ipl1-Sli15 (Aurora kinase-INCENP) complex promotes chromosome bi-orientation by altering kinetochore-spindle pole connections. *Cell* **108**, 317-329, doi:S0092867402006335 [pii] (2002).

[63] Stegmeier, F. & Amon, A. Closing mitosis: the functions of the Cdc14 phosphatase and its regulation. *Annu Rev Genet* **38**, 203-232, doi:10.1146/annurev.genet.38.072902.093051 (2004).

[64] Francisco, L., Wang, W. & Chan, C. S. Type 1 protein phosphatase acts in opposition to IpL1 protein kinase in regulating yeast chromosome segregation. *Mol Cell Biol* **14**, 4731-4740 (1994).

[65] Buschhorn, B. A. & Peters, J. M. How APC/C orders destruction. *Nat Cell Biol* **8**, 209-211, doi:ncb0306-209 [pii] 10.1038/ncb0306-209 (2006).

[66] Ciosk, R. *et al.* An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. *Cell* **93**, 1067-1076, doi:S0092-8674(00)81211-8 [pii] (1998).

[67] Clute, P. & Pines, J. Temporal and spatial control of cyclin B1 destruction in metaphase. *Nat Cell Biol* **1**, 82-87, doi:10.1038/10049 (1999).

[68] Sudakin, V., Chan, G. K. & Yen, T. J. Checkpoint inhibition of the APC/C in HeLa cells is mediated by a complex of BUBR1, BUB3, CDC20, and MAD2. *J Cell Biol* **154**, 925-936, doi:10.1083/jcb.200102093 154/5/925 [pii] (2001).

[69] Herzog, F. *et al.* Structure of the anaphase-promoting complex/cyclosome interacting with a mitotic checkpoint complex. *Science* **323**, 1477-1481, doi:323/5920/1477 [pii] 10.1126/science.1163300 (2009).

[70] De Antoni, A. *et al.* The Mad1/Mad2 complex as a template for Mad2 activation in the spindle assembly checkpoint. *Curr Biol* **15**, 214-225, doi:S0960982205000734 [pii] 10.1016/j.cub.2005.01.038 (2005).

[71] Howell, B. J. *et al.* Spindle checkpoint protein dynamics at kinetochores in living cells. *Curr Biol* **14**, 953-964, doi:10.1016/j.cub.2004.05.053 S0960982204003860 [pii] (2004).

[72] Kiyomitsu, T., Obuse, C. & Yanagida, M. Human Blinkin/AF15q14 is required for chromosome alignment and the mitotic checkpoint through direct interaction with Bub1 and BubR1. *Dev Cell* **13**, 663-676, doi:S1534-5807(07)00346-2 [pii] 10.1016/j.devcel.2007.09.005 (2007).

[73] Weiss, E. & Winey, M. The Saccharomyces cerevisiae spindle pole body duplication gene MPS1 is part of a mitotic checkpoint. *J Cell Biol* **132**, 111-123 (1996).

[74] Biggins, S. & Murray, A. W. The budding yeast protein kinase Ipl1/Aurora allows the absence of tension to activate the spindle checkpoint. *Genes Dev* **15**, 3118-3129, doi:10.1101/gad.934801 (2001).

[75] Pinsky, B. A., Kung, C., Shokat, K. M. & Biggins, S. The Ipl1-Aurora protein kinase activates the spindle checkpoint by creating unattached kinetochores. *Nat Cell Biol* **8**, 78-83, doi:ncb1341 [pii] 10.1038/ncb1341 (2006).

[76] Warren, C. D. *et al.* Distinct chromosome segregation roles for spindle checkpoint proteins. *Mol Biol Cell* **13**, 3029-3041, doi:10.1091/mbc.E02-04-0203 (2002).

[77] Yamaguchi, A., Iwasaki-Ohba, Y., Ono, N., Kaneko-Ohdera, M. & Sawai, T. Stoichiometry of metal-tetracycline/H+ antiport mediated by transposon Tn10-encoded tetracycline resistance protein in Escherichia coli. *FEBS Lett* **282**, 415-418, doi:0014-5793(91)80527-A [pii] (1991).

[78] Orth, P., Schnappinger, D., Hillen, W., Saenger, W. & Hinrichs, W. Structural basis of gene regulation by the tetracycline inducible Tet repressor-operator system. *Nat Struct Biol* **7**, 215-219, doi:10.1038/73324 (2000).

[79] Speer, B. S., Shoemaker, N. B. & Salyers, A. A. Bacterial resistance to tetracycline: mechanisms, transfer, and clinical significance. *Clin Microbiol Rev* **5**, 387-399 (1992).

[80] Saenger, W., Orth, P., Kisker, C., Hillen, W. & Hinrichs, W. The Tetracycline Repressor-A Paradigm for a Biological Switch. *Angew Chem Int Ed Engl* **39**, 2042-2052, doi:10.1002/1521-3773(20000616)39:12<2042::AID-ANIE2042>3.0.CO;2-C [pii] (2000).

[81] Clarke, L. & Carbon, J. Isolation of a yeast centromere and construction of functional small circular chromosomes. *Nature* **287**, 504-509 (1980).

[82] Koshland, D., Kent, J. C. & Hartwell, L. H. Genetic analysis of the mitotic transmission of minichromosomes. *Cell* **40**, 393-403, doi:0092-8674(85)90153-9 [pii] (1985).

[83] Hegemann, J. H., Shero, J. H., Cottarel, G., Philippsen, P. & Hieter, P. Mutational analysis of centromere DNA from chromosome VI of Saccharomyces cerevisiae. *Mol Cell Biol* **8**, 2523-2535 (1988).

[84] Straight, A. F., Belmont, A. S., Robinett, C. C. & Murray, A. W. GFP tagging of budding yeast chromosomes reveals that protein-protein interactions can mediate sister chromatid cohesion. *Curr Biol* **6**, 1599-1608, doi:S0960-9822(02)70783-5 [pii] (1996).

[85] Kopski, K. M. & Huffaker, T. C. Suppressors of the ndc10-2 mutation: a role for the ubiquitin system in Saccharomyces cerevisiae kinetochore function. *Genetics* **147**, 409-420 (1997).

[86] Biggins, S. *et al.* The conserved protein kinase Ipl1 regulates microtubule binding to kinetochores in budding yeast. *Genes Dev* **13**, 532-544 (1999).

[87] Hill, A. & Bloom, K. Genetic manipulation of centromere function. *Mol Cell Biol* **7**, 2397-2405 (1987).

[88] Cheeseman, I. M., Drubin, D. G. & Barnes, G. Simple centromere, complex kinetochore: linking spindle microtubules and centromeric DNA in budding yeast. *J Cell Biol* **157**, 199-203, doi:10.1083/jcb.200201052 jcb.200201052 [pii] (2002).

89 Stoler, S., Keith, K. C., Curnick, K. E. & Fitzgerald-Hayes, M. A mutation in CSE4, an essential gene encoding a novel chromatin-associated protein in yeast, causes chromosome nondisjunction and cell cycle arrest at mitosis. *Genes Dev* **9**, 573-586 (1995).

90 Ciferri, C. *et al.* Implications for kinetochore-microtubule attachment from the structure of an engineered Ndc80 complex. *Cell* **133**, 427-439, doi:S0092-8674(08)00401-7 [pii] 10.1016/j.cell.2008.03.020 (2008).

91 Gestaut, D. R. *et al.* Phosphoregulation and depolymerization-driven movement of the Dam1 complex do not require ring formation. *Nat Cell Biol* **10**, 407-414, doi:ncb1702 [pii] 10.1038/ncb1702 (2008).

92 Miranda, J. J., King, D. S. & Harrison, S. C. Protein arms in the kinetochore-microtubule interface of the yeast DASH complex. *Mol Biol Cell* **18**, 2503-2510, doi:E07-02-0135 [pii] 10.1091/mbc.E07-02-0135 (2007).

93 Furuyama, T. & Henikoff, S. Centromeric nucleosomes induce positive DNA supercoils. *Cell* **138**, 104-113, doi:S0092-8674(09)00510-8 [pii] 10.1016/j.cell.2009.04.049 (2009).

94 Bloom, K. S. *et al.* Chromatin conformation of yeast centromeres. *J Cell Biol* **99**, 1559-1568 (1984).

95 Murray, A. W. & Szostak, J. W. Pedigree analysis of plasmid segregation in yeast. *Cell* **34**, 961-970, doi:0092-8674(83)90553-6 [pii] (1983).

96 Shcheprova, Z., Baldi, S., Frei, S. B., Gonnet, G. & Barral, Y. A mechanism for asymmetric segregation of age during yeast budding. *Nature* **454**, 728-734, doi:nature07212 [pii] 10.1038/nature07212 (2008).

97 Kitamura, E., Tanaka, K., Kitamura, Y. & Tanaka, T. U. Kinetochore microtubule interaction during S phase in Saccharomyces cerevisiae. *Genes Dev* **21**, 3319-3330, doi:21/24/3319 [pii] 10.1101/gad.449407 (2007).

98 Powers, A. F. *et al.* The Ndc80 kinetochore complex forms load-bearing attachments to dynamic microtubule tips via biased diffusion. *Cell* **136**, 865-875, doi:S0092-8674(09)00023-3 [pii] 10.1016/j.cell.2008.12.045 (2009).

99 Onn, I., Heidinger-Pauli, J. M., Guacci, V., Unal, E. & Koshland, D. E. Sister chromatid cohesion: a simple concept with a complex reality. *Annu Rev Cell Dev Biol* **24**, 105-129, doi:10.1146/annurev.cellbio.24.110707.175350 (2008).

100 Dam, M. & Gerdes, K. Partitioning of plasmid R1. Ten direct repeats flanking the parA promoter constitute a centromere-like partition site parC, that expresses incompatibility. *J Mol Biol* **236**, 1289-1298, doi:0022-2836(94)90058-2 [pii] (1994).

101 Moller-Jensen, J., Jensen, R. B., Lowe, J. & Gerdes, K. Prokaryotic DNA segregation by an actin-like filament. *EMBO J* **21**, 3119-3127, doi:10.1093/emboj/cdf320 (2002).

102 Moller-Jensen, J. *et al.* Bacterial mitosis: ParM of plasmid R1 moves plasmid DNA by an actin-like insertional polymerization mechanism. *Mol Cell* **12**, 1477-1487, doi:S1097276503004519 [pii] (2003).

[103] Akiyoshi, B., Nelson, C. R., Ranish, J. A. & Biggins, S. Quantitative proteomic analysis of purified yeast kinetochores identifies a PP1 regulatory subunit. *Genes Dev* **23**, 2887-2899, doi:gad.1865909 [pii] 10.1101/gad.1865909 (2009).

[104] Cheeseman, I. M. *et al.* A conserved protein network controls assembly of the outer kinetochore and its ability to sustain tension. *Genes Dev* **18**, 2255-2268, doi:10.1101/gad.1234104 18/18/2255 [pii] (2004).

[105] Miller, S. A., Johnson, M. L. & Stukenberg, P. T. Kinetochore attachments require an interaction between unstructured tails on microtubules and Ndc80(Hec1). *Curr Biol* **18**, 1785-1791, doi:S0960-9822(08)01484-X [pii] 10.1016/j.cub.2008.11.007 (2008).

[106] DeLuca, J. G. *et al.* Kinetochore microtubule dynamics and attachment stability are regulated by Hec1. *Cell* **127**, 969-982, doi:S0092-8674(06)01408-5 [pii] 10.1016/j.cell.2006.09.047 (2006).

[107] Kemmler, S. *et al.* Mimicking Ndc80 phosphorylation triggers spindle assembly checkpoint signalling. *EMBO J* **28**, 1099-1110, doi:emboj200962 [pii] 10.1038/emboj.2009.62 (2009).

[108] Desai, A. *et al.* KNL-1 directs assembly of the microtubule-binding interface of the kinetochore in C. elegans. *Genes Dev* **17**, 2421-2435, doi:10.1101/gad.1126303 17/19/2421 [pii] (2003).

[109] Schittenhelm, R. B., Chaleckis, R. & Lehner, C. F. Intrakinetochore localization and essential functional domains of Drosophila Spc105. *EMBO J* **28**, 2374-2386, doi:emboj2009188 [pii] 10.1038/emboj.2009.188 (2009).

[110] Wigge, P. A. *et al.* Analysis of the Saccharomyces spindle pole by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. *J Cell Biol* **141**, 967-977 (1998).

[111] Nekrasov, V. S., Smith, M. A., Peak-Chew, S. & Kilmartin, J. V. Interactions between centromere complexes in Saccharomyces cerevisiae. *Mol Biol Cell* **14**, 4931-4946, doi:10.1091/mbc.E03-06-0419 E03-06-0419 [pii] (2003).

[112] Pagliuca, C., Draviam, V. M., Marco, E., Sorger, P. K. & De Wulf, P. Roles for the conserved spc105p/kre28p complex in kinetochore-microtubule binding and the spindle assembly checkpoint. *PLoS One* **4**, e7640, doi:10.1371/journal.pone.0007640 (2009).

[113] Tytell, J. D. & Sorger, P. K. Analysis of kinesin motor function at budding yeast kinetochores. *J Cell Biol* **172**, 861-874, doi:jcb.200509101 [pii] 10.1083/jcb.200509101 (2006).

[114] Cheeseman, I. M., Hori, T., Fukagawa, T. & Desai, A. KNL1 and the CENP-H/I/K complex coordinately direct kinetochore assembly in vertebrates. *Mol Biol Cell* **19**, 587-594, doi:E07-10-1051 [pii] 10.1091/mbc.E07-10-1051 (2008).

[115] Daniel, J. A., Keyes, B. E., Ng, Y. P., Freeman, C. O. & Burke, D. J. Diverse functions of spindle assembly checkpoint genes in Saccharomyces cerevisiae. *Genetics* **172**, 53-65, doi:genetics.105.046441 [pii] 10.1534/genetics.105.046441 (2006).

[116] Longtine, M. S. *et al.* Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. *Yeast* **14**, 953-961 (1998).

[117] Ito, H., Fukuda, Y., Murata, K. & Kimura, A. Transformation of intact yeast cells treated with alkali cations. *J Bacteriol* **153**, 163-168 (1983).

[118] Gari, E., Piedrafita, L., Aldea, M. & Herrero, E. A set of vectors with a tetracycline-regulatable promoter system for modulated gene expression in Saccharomyces cerevisiae. *Yeast* **13**, 837-848 (1997).

[119] Merril, C. R., Goldman, D., Sedman, S. A. & Ebert, M. H. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* **211**, 1437-1438 (1981).

6.5 Curriculum vitae

PERSONAL DETAILS

Name: Eva Kiermaier, M.Sc.
Date of birth: 9.12.1981
Place of birth: Dachau
Gender: female
Languages: English, French, German

EDUCATION

Feb 2007 – present PhD student	*Research Institute of Molecular Pathology (I.M.P.) –Vienna* PhD Thesis: Reconstitution of a Kinetochore-Microtubule Interface *in vitro* and *in vivo*
Mar 2006 – Dec 2006 Master student	*Helmholtz Zentrum Munich, Neuherberg* Master Thesis: Functional Consequences of Mutations in the p27 Tumor Suppressor Gene
Oct 2004 – Dec 2006 Graduate Studies	*Technical University of Munich, Germany* Master in Biochemistry
Aug - Nov 2004:	*Eidgenoessische Technische Hochschule Zuerich (ETH), Zuerich* Summer student: The Role of N-linked Glycoproteins of *C. Jejuni*
Oct 2001 – Aug 2004 Undergraduate Studies	*Technical University of Munich, Germany* Bachelor in Biochemistry
Aug – Oct 2003:	*Institute of Immunology, Heidelberg* Summer student: Comparison of Cytotoxic Assays for Multiple Myeloma Immunotherapy
Sep 1992 – Jul 2001 High School	*Gymnasium Unterschleißheim, Germany* Abitur in May 2001

PUBLICATIONS

Kiermaier E, Woehrer S, Peng Y, Mechtler K, Westermann S.
A Dam1-based artificial kinetochore is sufficient to promote chromosome segregation in budding yeast. *Nat Cell Biol*. 2009 Sep;11(9):1109-15. Epub 2009 Aug 16.

Sara Molatore, **Eva Kiermaier**, Christian Jung, Elke Pulz, Heinz Höfler, Michael J. Atkinson and Natalia S. Pellegata. Characterization of a naturally occurring p27 mutation predisposing to multiple endocrine tumors. submitted to *Molecular Cancer*. 2009 Dec

Kowarik M, Numao S, Feldman MF, Schulz BL, Callewaert N, **Kiermaier E**, Catrein I, Aebi M. N-linked glycosylation of folded proteins by bacterial oligosaccharyltransferase.
Science. 2006 Nov 17;314(5802):1148-50.

6.6 Acknowledgments

I would like to thank Stefan for giving me the chance to work on these interesting projects and his constant support and supervision during my PhD. Thanks for being there whenever I needed you and for all the helpful discussions concerning my work.
Furthermore I would like to thank all the nice people in the lab – Tom, Kathi, Fabi, Peter, Sophie and Chrissi - for creating such a nice working atmosphere and helping me with sharing their experiences with me. I really enjoyed spending my time with you inside and outside the lab. You all became really good friends to me, which I will miss a lot.
In particular, I would like to thank Sophie for helping me so much during her time in the lab. It was a real pleasure for me to work with such a talented and smart person.

I am very grateful to Branka, Linda, Anne and Bori for all their advices and patience in listening to my problems. Thank you so much for always supporting me and the wonderful time we had. I will never forget the endless hours of chatting and laughing together.

I would also like to thank Julia and Caro for always believing in me and giving me all the strength during the past years. You are the best online WG ever.

Finally I would like to thank my family, especially my Dad, for all their help and support during my time in Vienna. You always gave me the feeling of being loved.