universität wien

DIPLOMARBEIT

Molecular characterisation of an artificial kinetochore in budding yeast

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag. rer.nat.)

Verfasserin:	Sophie Wöhrer
Matrikel-Nummer:	0403560
Studienrichtung:	A490 Molekulare Biologie
Betreuerin / Betreuer:	Dr. Stefan Westermann

Wien, im September 2009

Contents

Zusammenfassung

Während der Zellteilung ist es entscheidend, dass die genomische Information der sich teilenden Zelle zu gleichen Teilen an die Tochterzellen weitergegeben wird. Dafür ist ein spezieller Apparat nötig, der eine feste Verbindung zwischen den Chromosomen und den Mikrotubuli des Spindelapparates bereitstellt. Die komplizierte Aufgabe, eine kraftübertragende Verbindung zu dynamischen Mikrotubuli aufzubauen, wird vom Kinetochor, einem komplizierten Multiproteinkomplex, erfüllt. In der Bäckerhefe *Saccharomyces cerevisiae*, der Modelorganismus der in der folgenden Arbeit verwendet wurde, bindet jedes Kinetochor an einen einzelnen Mikrotubulus.

Da das Kinetochor eine sehr komplizierte Struktur darstellt, ist eine genaue Analyse der einzelnen Komplexe oder gar individueller Proteine eine gewaltige Aufgabe. Die Etablierung eines Systems, das die Komplexität des Kinetochors auf minimale Notwendigkeiten reduziert, wäre hilfreich um die funktionellen Eigenschaften einzelner Proteine zu analysieren. Dies wurde erreicht, indem einzelne Kinetochorproteine durch Fusion mit einer DNA-Bindungsdomäne direkt an die DNA rekrutiert wurden. Daraufhin konnten jene an eine Abfolge von entsprechenden Erkennungssequenzen auf kleinen, zirkulären Minichromosomen binden. Untereinheiten des Dam1 Komplexes, vor allem das Protein Ask1p, zeigten in dieser Versuchsanordnung eine signifikante mitotische Stabilisierung dieser Minichromosomen. Die hier gezeigten Daten deuten darauf hin, dass mit diesem System der gesamte Dam1 Komplex an die DNA rekrutiert wird. In nativen Kinetochoren spielt dieser Proteinkomplex eine wesentliche Rolle im Aufbau der Verbindungen zu den Mikrotubuli und in der Weiterleitung von Kräften während der Segregation von Chromosomen.

Die funktionelle Charakterisierung des auf diese Weise konstruierten artifiziellen Kinetochors in temperatur-sensitiven Hefemutanten ergab, dass seine Funktion in der Segregation der Minichromosomen unabhängig von einigen essentiellen Kinetochorproteinen ist. Die Eigenschaft, eine kraftübertragende Verbindung zwischen dynamischen Mikrotubuli und dem Kinetochor aufbauen zu können, ist also ein intrinsisches Merkmal des Dam1 Komplexes.

Es bleibt noch zu klären, wie das artifizielle Kinetochor reguliert wird, damit es korrekt an Mikrotubuli der Spindel bindet und an beide Tochterzellen weitergegeben werden kann. Die direkte Visualisierung lässt vermuten, dass Minichromosomen, die unter der Kontrolle des artifiziellen Kinetochors stehen, weit weniger Kohäsion aufbauen können, als jene mit einer Zentromersequenz und einem nativen Kinetochor. Es wird interessant sein zu sehen, wie das artifizielle Kinetochor, trotz minimaler Kohäsion zwischen den Schwesterchromatiden, eine korrekte Segregation der Minichromosomen während der Mitose vermitteln kann.

Zusammenfassend lässt sich sagen, dass das artifizielle Kinetochor ein nützliches Werkzeug zur Untersuchung der Eigenschaften eines einzelnen Kinetochor-Komplexes darstellt.

Abstract

During the process of cell division it is crucial for a cell to segregate its genomic material equally. Therefore a machinery is required, which establishes a firm connection between chromosomes and microtubules of the mitotic spindle. The challenging task is to assemble load-bearing attachments to the highly dynamic microtubule plus-ends. This is achieved by the kinetochore, a huge structure composed of several protein subcomplexes. In *Saccharomyces cerevisiae*, the model organism used for the following study, a single microtubule is connected to each kinetochore.

Since the kinetochore displays a very difficult structure, detailed analysis of individual complexes or even proteins is a very daunting task. In order to identify minimal requirements for chromosome segregation *in vivo*, it would be helpful to work with a system that allows to investigate functional properties of individual proteins. This was achieved by artificially tethering individual kinetochore proteins directly to DNA by fusing them to a DNA binding domain. This allowed them to bind to an array of the according recognition site on a small circular minichromosome. Subunits of the Dam1 complex, especially Ask1p, achieved significant mitotic stabilisation of minichromosomes in this assay. Our data suggested, that the entire Dam1 complex is tethered to the minichromosomes in this system. In native kinetochores the complex is known to play a role in microtubule attachments and force transduction during chromosome segregation.
Functional characterisation of the artificial kinetochore in temperature-sensitive yeast mutants revealed that it functions independently of several essential kinetochore proteins. Thus, the property to form load-bearing attachments is an intrinsic characteristic of the Dam1 complex. How processes like biorientation are achieved for the artificial kinetochore remains to be established. Judged by live cell imaging experiments, it seems that there is much less cohesion established on minichromosomes which are under the control of the artificial kinetochore, than on their wildtype counterparts, carrying a native centromeric sequence. It will be interesting to investigate if bi-orientation can also be established with a minimal amount of cohesion.
In summary, the artificial kinetochore provides a useful tool for investigating the properties of an individual kinetochore complex.

1 Introduction

1.1 Cell cycle & mitosis

The ability of a cell to divide and to pass on its genetic material to a new emerging cell is one of the basic processes for life. One of the most important aspects is the equal distribution of chromosomes between both daughter cells during cell division. Therefore highly conserved machineries and mechanisms control, coordinate and perform several cell cycle events in order to achieve the correct outcome of this process.

1.1.1 The cell cycle of *Saccharomyces cerevisiae*

The budding yeast *Saccharomyces cerevisiae* is a small, unicellular eukaryote that provides a perfect tool for studying mitotic events and underlying processes. It is easy to access with genetic as well as biochemical experimental approaches. Budding yeast is a rapidly growing organism that can alternate between a haploid and a diploid state. In the former two mating types exist, which allow cells from different types to mate and generate diploid zygotes. Upon starvation diploid cells can sporulate and form four haploid cells. The cell cycle can be divided into several phases, characterised by specific events (Figure 1) (Morgan 2007).

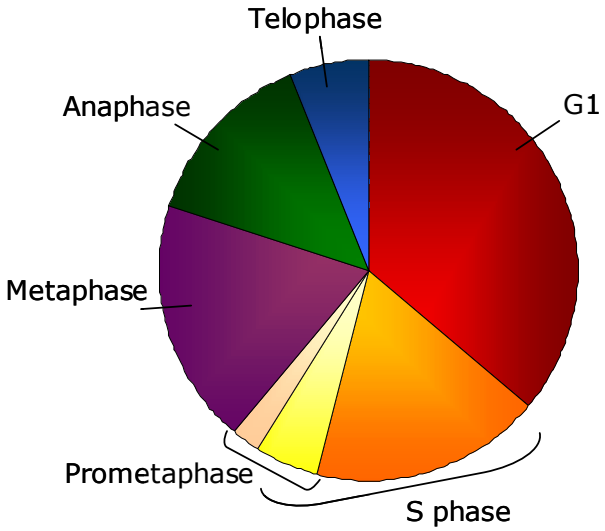


Figure 1: Budding yeast cell cycle (adapted from Tanaka et al. 2005)

1.1.2 Mitotic events

Mitosis can be divided into several stages, representing a defined order of important processes (Figure 1). In metazoan cells the boundaries between these phases are set more clearly than in budding yeast. Key components of the cell-cycle control system are cyclin-dependent kinases (Cdk) which act as molecular switches. They become activated upon binding of their positive regulator subunits called cyclins, whose levels oscillate during the cell cycle in order to trigger a special process at the right time. In budding yeast all cell cycle events are controlled by Cdk1, activated by different cyclins. Furthermore Cdk function is regulated by phosphatases and inhibitory kinases. The first transition is called Start and regulates the entry from G1 into S phase. Metaphase to anaphase transition leads to activation of the anaphase promoting complex (APC) and triggers completion of mitosis and cytokinesis (Morgan 2007).

In *S phase* the cell grows in size and the 16 chromosomes are duplicated. Emerging sister chromatids are held together by cohesion. This is established by the cohesin protein complex, which consists of four subunits: Smc1, Smc3, Scc1 and Scc3. These proteins form a ring around sister chromatids, holding them together (Morgan 2007).
The yeast kinetochore, the proteinaceous structure which links chromosomes to spindle microtubules to ensure their correct segregation, is only disassembled for the short time of subjacent DNA replication. As soon as the kinetochore is intact again, it interacts with microtubules extending from the spindle pole body (SPB). Another event in S phase is the formation of the new spindle pole body. When it becomes mature later in S phase, the two SPBs separate in order to build up the bipolar mitotic spindle (Figure 2). Both spindle pole bodies remain embedded in the nuclear envelope, which does not break down like in higher eukaryotes. Microtubules nucleating from the SPBs are extending to perform different functions. Astral microtubules project towards the cell membrane where they perform an important function by generating forces that orient the spindle. Intranuclear microtubules give rise to the mitotic spindle, which consists of 32 kinetochore microtubules and 2-4 overlapping polar microtubules (Tanaka et al. 2005; Morgan 2007).

The phase during which chromosomes are bound by microtubules, partly in a “search and capture” process, and transported to the spindle via interaction between kinetochores and

microtubules is defined as the *prometaphase*. In yeast an accurate boundary between S phase and prometaphase is hard to define (McAinsh et al. 2003; Tanaka et al. 2005).

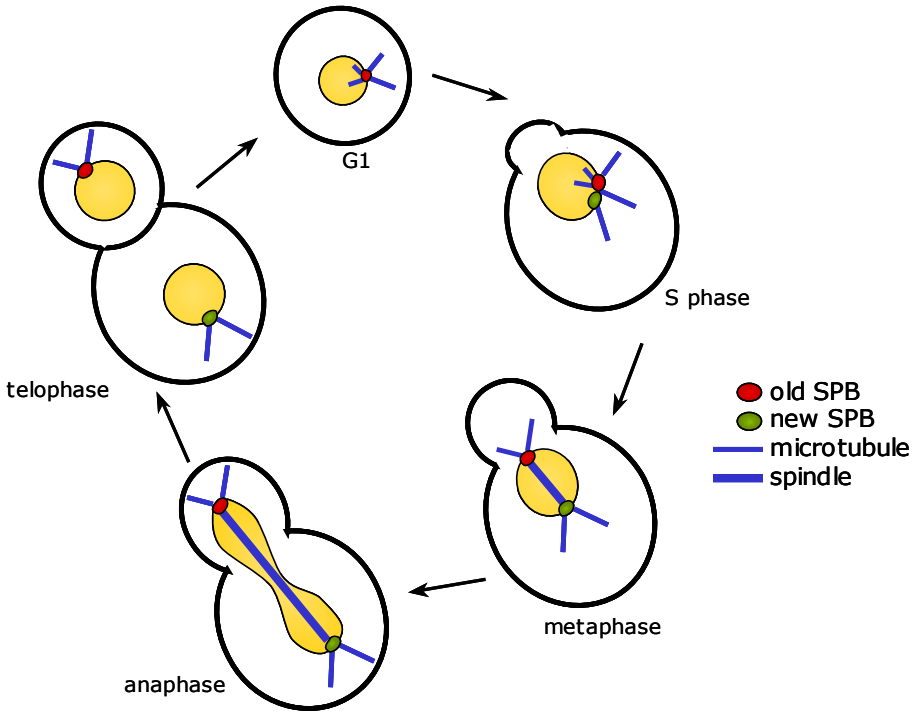


Figure 2: Cell growth and spindle formation during cell cycle (adapted from Tanaka et al. 2005). The new SPB is assembled in S phase. In metaphase the mitotic spindle is built up between the two separated SPBs. At the end of anaphase the nucleus and the spindle are elongated. At telophase the spindle reaches its maximum length and subsequently it is disassembled, nucleus and cell are finally divided.

During *metaphase* cells prepare the duplicated sister chromatids for a proper division. Unlike in higher eukaryotes, where chromosomes align at the so called metaphase plate between the spindle poles, yeast chromosomes are distributed along the metaphase spindle. Fluorescent labelling of a single chromosome reveals a signal that moves back and forth along the spindle long axis. Depending on the position of the fluorescent marker this can also be detected as a stretched signal. The reason for this is precocious splitting of sister chromatids at their centromeric region while chromatid arms are still held together. This process is also known as biorientation, since kinetochores of two sister chromatids have to attach to spindle microtubules emanating from opposite SPBs (Straight et al. 1997; Tanaka et al. 2000).

Once the checkpoints monitoring the processes of correct kinetochore-microtubule attachments are satisfied, the cell is ready for the metaphase to *anaphase* transition and to separate the sister chromatids. In a so far not fully understood signalling cascade the anaphase promoting complex (APC) gets activated by Cdc20 and can therefore ubiquitinate its targets. One of the outcomes is the release of the protease separase from its inhibitor securin which leads to cleavage of Scc1 and therefore removal of cohesin from sister chromatids (Morgan 2007; Peters et al. 2008). After this release, they rapidly move to opposite spindle poles in anaphase A. Anaphase B is characterised by the following separation of the poles and elongation of the mitotic spindle (Straight et al. 1997).
Telophase is defined as the stage when the spindle reaches its maximum length and is subsequently disassembled. It is finished when the cell and the nucleus are pinched in two during cytokinesis (Tanaka et al. 2005; Morgan 2007).

1.2 The kinetochore

In order to guarantee correct segregation of chromosomes a machinery is required which tightly links chromatids to spindle microtubules. This function is achieved by a huge protein structure, the kinetochore. It is capable to connect chromosomes to the plus ends of highly dynamic microtubules. Building up a stable connection is a challenging task, since microtubules show a behaviour called “dynamic instability”. This means they switch between phases of growth and shrinkage, a property which is needed for the capture of chromosomes, their biorientation and for force generation during sister chromatid segregation. But kinetochores do not only provide a firm linkage to microtubules, they are also able to influence their dynamics (Westermann et al. 2007).

Vertebrate kinetochores exhibit a very complex and dynamic structure, which binds to the kinetochore fiber, a bundle of microtubules. In *S. cerevisiae* in contrast, each kinetochore forms a stable attachment to a single microtubule. This property makes the biochemical description of the kinetochore-mictrotubule attachment site more realisable than in higher eukaryotes. A lot of essential kinetochore proteins are highly conserved structurally as well as functionally from yeast to human. Moreover, the overall function of this machinery in metaphase and anaphase is similar in all eukaryotic cells, and also its regulatory system seems to be highly conserved. This conservation of constituent parts suggests the “repeat

subunit" model: larger kinetochores of higher eukaryotes are probably assembled in a way that repeats the basic module present in yeast. Therefore, research on the molecular architecture and functional description of the kinetochore in budding yeast is valuable to understand its function in higher eukaryotes, including humans (Kitagawa & Hieter 2001; Joglekar et al. 2006; Westermann et al. 2007; Santaguida & Musacchio 2009).

In the following paragraphs major properties of the yeast kinetochore will be summarised:

- where and how the site of assembly is specified;
- the overall structure of the kinetochore;
- regulatory processes monitoring the state of attachment.

1.2.1 Specifying the location for the assembly of a kinetochore

The site of attachment for kinetochore proteins on the chromosome has to be specified in order to ensure correct segregation. The signal for assembly can be restricted to one site (monocentric chromosomes) or extended over the whole length of a chromosome (holocentric chromosomes, e.g. *C.elegans*) (Cheeseman & Desai 2008). In budding yeast a relatively short stretch of 125 base pairs (bp) is necessary and sufficient to direct the assembly of a kinetochore. This defined sequence is referred to as the centromere. In comparison, in fission yeast and vertebrates centromeres are spread over several kilo- or megabases (regional centromeres). This difference makes the centromere of budding yeast much more accessible for genetic manipulations. The 125bp long sequence is composed of three distinct elements – CDE I, II and III. CDEI consists of ~15bp and recruits a dimer of the Cbf1 protein. The ~80bp long sequence of CDEII is highly enriched for A and T and interacts with a Cse4-nucleosome and the CBF3 component Ndc10p. CDEIII forms an imperfect palindrome where CBF3 binds in a sequence specific way (McAinsh et al. 2003; Santaguida & Musacchio 2009).

1.2.2 Structure of the kinetochore in budding yeast

The budding yeast kinetochore consists of more than 65 proteins, which are assembled in a hierarchical order as several multiprotein subcomplexes. They are often grouped into three categories, according to their relative position within the protein assembly:

- inner kinetochore proteins, which are able to directly interact with centromeric DNA, and therefore providing a kind of platform for other components;
- outer kinetochore proteins, which directly bind to microtubules;
- linker components serve as a platform to connect these two former layers.

(Westermann et al. 2007)

This classification is consistent with a model that suggests that the kinetochore is build up from the centromere towards the microtubule attachment site in an inside-out manner (Santaguida & Musacchio 2009). Nevertheless, these allocations only provide an approximate position and sometimes it is hard to assign protein complexes strictly to one of these layers. According to these categories individual protein complexes are described in more detail in the following paragraphs and an oversimplified scheme of the kinetochore architecture is given in Figure 3.

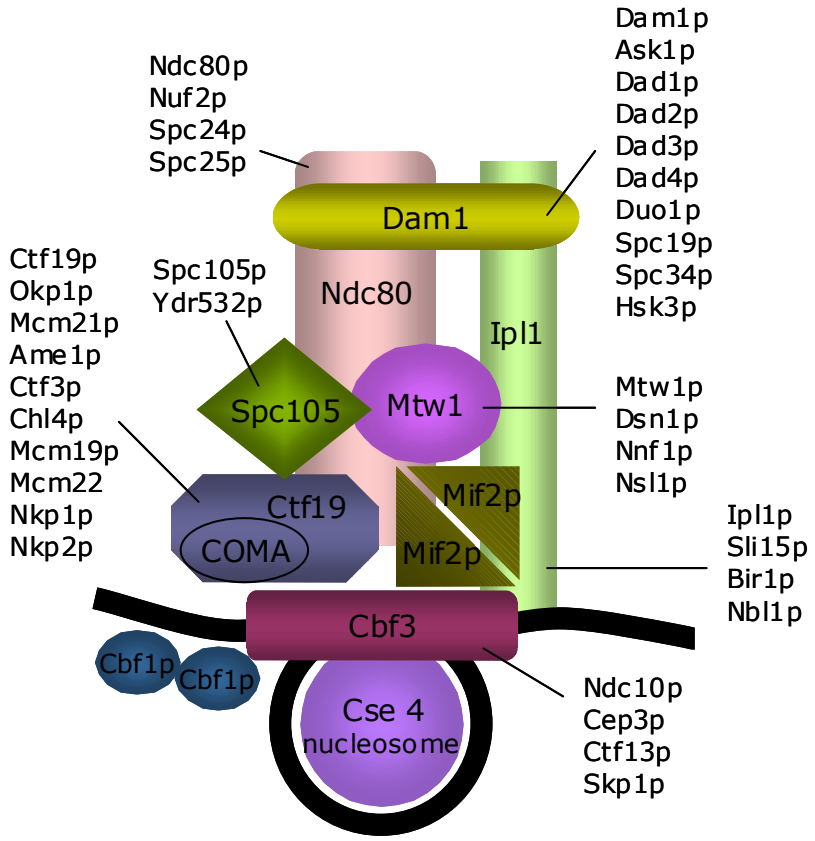


Figure 3: Schematic drawing of a budding yeast kinetochore.

(adapted from Santaguida & Musacchio 2009)

In budding yeast the kinetochore is assembled almost throughout the whole cell cycle. Only during the brief period when the replication machinery is passing the centromeric region, the structure disassembles. It is thought that some of the proteins stay in close proximity to the DNA, to allow quick and easy reassembly shortly after replication (Westermann et al. 2007). Fluorescence recovery after photobleaching (FRAP) experiments revealed that kinetochore proteins show a very low recovery rate, suggesting a high stability for the proteins and almost no turnover once the kinetochore is built up (Joglekar et al. 2006).

1.2.2.1 Inner kinetochore complexes

Eukaryotic DNA is organized into nucleosomes as it is wrapped around octameric histone core particles (H2A, H2B, H3 and H4). Various post-translational modifications or variants of these proteins have the potential to influence chromatin structure and accessibility. At centromeric nucleosomes the canonical histone H3 is replaced by a centromere specific variant, which is crucial for the recruitment of other kinetochore proteins. The function of a nucleosome containing CenH3 – Cse4p in budding yeast, CENP-A in humans – in serving as one of the primary determinants for kinetochore assembly is highly conserved throughout evolution, although the different proteins are very diverged. There seems to exist a single Cse4p containing nucleosome per centromere, containing two copies of the protein. Recent data also suggest a structural difference concerning DNA supercoiling around centromeric nucleosomes which may represent some kind of epigenetic mark for defining centromeric loci (Furuyama & Henikoff 2009; Cheeseman & Desai 2008; Santaguida & Musacchio 2009; Westermann et al. 2007).

One of the key events in kinetochore assembly is the interaction of the CBF3 complex with the CDEIII element of centromeres. This four protein complex, consisting of Ndc10p, Cep3p, Ctf13p and Skp1p, is one of the primary determinants for constitution of a kinetochore, since all analysed budding yeast kinetochore proteins depend on CBF3 for association with the centromere (Westermann et al. 2007). Although Skp1p was reported to be functionally conserved between yeast and human, the occurrence of a CBF3 like protein complex seems to be restricted to fungi with a sequence-defined centromere (Kitagawa & Hieter 2001). In addition it was reported, that Ndc10p alone can also bind to CDEII. It is

suggested that this interaction serves as a foundation for recruiting other kinetochore proteins to the centromere (Espelin et al. 2003).

The recruitment of all other kinetochore proteins is dependent on the CBF3 complex and the Cse4-nucleosome (Santaguida & Musacchio 2009).

1.2.2.2 Complexes forming the kinetochore linker layer

The relatively uncharacterised Ctf19 complex consists of more than 10 proteins, identified by TAP-tag purifications. Four of these proteins, Ctf19p, Okp1p, Mcm21p and Ame1p, behave as a subcomplex, known as COMA. Only Okp1p and Ame1p are required for viability and the exact role for Ctf19 in kinetochore function has to be investigated further (Westermann et al. 2007). Recent data claim a role for the Ctf19 complex in loading of cohesion on centromeric and pericentromeric regions (Fernius & Marston 2009). A functional equivalent to the Ctf19 complex in higher eukaryotes is probably the CCAN (constitutive centromere-associated network) (Santaguida & Musacchio 2009).

The budding yeast protein Mif2p is recruited to CBF3 bound CDEIII. Recent data describe its structural and functional properties. The mammalian orthologue is CENP-C and the strongest conservation lies within the “CENP-C signature motif” in the middle of Mif2p. This region together with a DNA binding domain is required for normal growth. It was suggested that the protein binds as a dimer to CBF3 occupied CDEIII (Cohen et al. 2008).

The Spc105 complex consists of Spc105p and YDR532c and is poorly described in budding yeast (Westermann et al. 2007). Homologs in different eukaryotes are known as Spc7, KNL-1 or Blinkin. It was shown that this complex tightly interacts with two other complexes (Mis12/Mtw1 and Ndc80) and with components of the spindle assembly checkpoint (SAC). Studies of the *D. melanogaster* Spc105 protein suggest a role in providing a platform within the outer kinetochore upon which various other kinetochore proteins can assemble (Schittenhelm et al. 2009).

The budding yeast proteins Mtw1p, Dsn1p, Nnf1p and Nsl1p form the Mtw1-complex. Temperature sensitive mutants in complex subunits result in chromosome missegregation

and are not able to produce sufficient tension between sister kinetochores. Therefore they show defects in biorientation. The Mtw1 complex interacts with Cse4p, Mif2p and Ndc80. It is conserved from yeast to human, where it is referred to as the Mis12 complex (Westermann et al. 2007).

One of the best characterised kinetochore components is the Ndc80 complex. It consists of four subunits: Ndc80p (Hec1 in humans), Nuf2p, Spc24p and Spc25p, which form a long rod-like structure with globular domains at the ends and an elongated coiled-coil domain in between. The complex is connected with other kinetochore proteins via its Spc24p/Spc25p head and it was shown to bind to microtubules with its Ndc80p/Nuf2p component. Therefore it was suggested to form a load bearing microtubule attachment site. While Ndc80 has a low binding affinity for microtubules on its own, this property can be enhanced upon interactions with other proteins (Cheeseman & Desai 2008; Joglekar & DeLuca 2009; Powers et al. 2009; Joglekar et al. 2006; Joglekar et al. 2009). From force clamp studies with Ndc80 coated beads a model describing a biased-diffusion mechanism for Ndc80 movement was suggested. In such a model several low affinity binding sites would allow attachments to dynamic microtubules (Powers et al. 2009; Joglekar & DeLuca 2009). Quantitative fluorescence measurements revealed that there are approximately eight Ndc80 molecules present at a yeast kinetochore (Joglekar et al. 2006). Recent results from a study, in which the exact positions of various kinetochore proteins were measured, showed that the Ndc80 complex is spanning over a long distance across the kinetochore, interacting with inner proteins, as well as with microtubules (Joglekar et al. 2009).

In other eukaryotes homologs of the last three described protein complexes (Ndc80, Mtw1 and Spc105), were shown to interact tightly and thereby forming the so-called KMN network (KNL1, Mis12, Ndc80). The combined protein network was shown to bind with higher affinity to microtubules than the single complexes. This network was supposed to form a microtubule binding site and thereby attaching the kinetochore to these dynamic polymers (Tanaka & Desai 2008).

1.2.2.3 Proteins directly interacting with microtubules

The budding yeast Dam1 complex consists of ten proteins: Dam1p, Ask1p, Dad1p, Dad2p, Dad3p, Dad4p, Duo1p, Spc19p, Spc34p and Hsk3p (Westermann et al. 2007). Since the mutant phenotype of individual components is similar, it is suggested that the entire complex is required for its correct function. The complex has an important role in mediating kinetochore-microtubule interactions and chromosome segregation by establishing and maintaining end-on connections. Mutants show a high rate of missegregation. Dam1 was shown to be a target of the Ipl1p kinase, which plays an important role in the correction of improper kinetochore-microtubule attachments (Cheeseman et al. 2001; Li et al. 2002; Westermann et al. 2007). Biochemical analysis and *in vitro* studies revealed the formation of a ring around a microtubule formed by 16 single heterodecameric Dam1 complexes. It is thought that electrostatic interactions between the negatively charged C-termini of tubulin and the overall positively charged Dam1 complex play a role in this assembly. The oligomerisation around microtubules includes conformational changes of the complex. It is suggested, that microtubules enhance ring formation by interacting with the complex (Westermann et al. 2005; Wang et al. 2007). An important feature of the ring is that it can diffuse along the microtubule lattice and moreover it was shown to move processively with a depolymerising end of a microtubule. This could be explained by a translation of the mechanical energy, generated by outward protofilament peeling upon GTP hydrolysis and depolymerisation, into movement along the lattice (Figure 4).

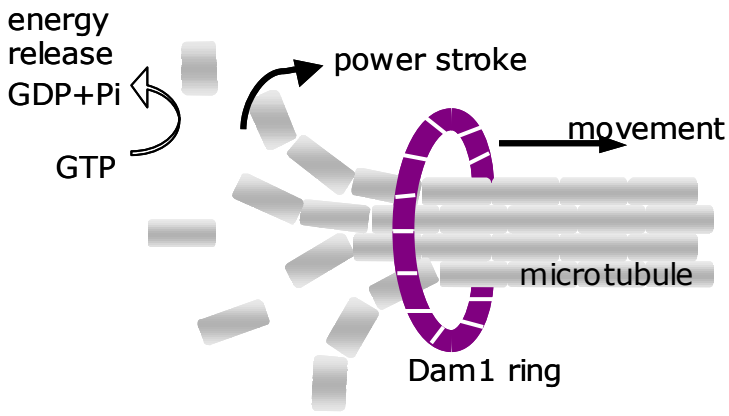


Figure 4: Model of processive movement of a Dam1 ring along the microtubule lattice. After hydrolysis of GTP the energy is stored in the microtubule and released as a power stroke of curling protofilaments. This mechanical energy can than be translated into movement of the Dam1 ring (adapted from Westermann et al. 2007).

Such a model offers the possibility that a Dam1 ring acts as the force-coupling device connecting a microtubule to the kinetochore during chromosome segregation in yeast (Westermann et al. 2006). In a bead assay, similar to that described for Ndc80, Dam1 was shown to be a processive coupler and force transducer (Asbury et al. 2006; Grishchuk et al. 2008). Nevertheless, so far the existence of a Dam1 ring could not be proven *in vivo*. But it was shown, that 16-20 Dam1 complexes are present at one attachment site, consistent with the required number for the assembly of a single ring (Joglekar et al. 2006).
The described properties of the Dam1 complex imply a major role in faithful chromosome segregation. So far a metazoan homolog has not been identified. The recently characterised Ska1 complex is suggested to play a corresponding role at the vertebrate kinetochore. (Welburn et al. 2009)

Microtubule associated proteins (MAPs) and motor proteins play additional roles in attachments and segregation. These MAPs include Stu2p (XMAP215), Bik1p (CLIP170) and Bim1p (EB1), which recognize and bind to plus ends of microtubules and probably regulate their dynamics. Motor proteins like Kar3p, Kip1p, Kip3p or Cin8p play a role in chromosome segregation by influencing and organizing chromosome movements. But their exact function during mitotic processes is not fully understood. (McAinsh et al. 2003; Westermann et al. 2007).

1.2.2.4 Checkpoints and regulatory elements

Several checkpoint mechanisms and regulatory processes have to guarantee correct segregation of the genetic material during cell division. The spindle assembly checkpoint (SAC) monitors the attachment status of kinetochores and retains the cell in metaphase as long as a single unattached kinetochore is present. Key players in this pathway are Mad1p, Mad2p, Mad3p (BubR1 in humans), Bub1p and Bub3p. Since the state of attachment is monitored, the function of the SAC is closely connected to kinetochores. The majority of the involved proteins is recruited there, probably by proteins of the KMN network. Once the checkpoint is satisfied, and all kinetochores are properly attached to microtubules, SAC signals are shut off and transition to anaphase is triggered (Musacchio & Salmon 2007; Santaguida & Musacchio 2009).

Attachment alone does not guarantee faithful chromosome segregation. Sister chromatids have to attach to opposite SPBs with each kinetochore in order to ensure equal distribution of DNA to both cells. Therefore a mechanism is needed, which can discriminate between correct and incorrect microtubule attachments. One of the essential components in this correction process is the protein kinase Ipl1p (AuroraB) (Santaguida & Musacchio 2009). It forms a complex with its activator Sli15p (INCENP), Bir1p (Survivin) and Nbl1p (Borealin) and this assembly is referred to as the chromosomal passenger complex (CPC). Several models exist which try to explain how this complex is able to distinguish between correct and incorrect attachments and how it stabilises the former while destabilising the latter. Intra-kinetochore tension, generated by pulling forces of microtubules, is thought to play a critical role. According to this, a mechanism was proposed, in which the physical distance between the kinase and its substrates is a key regulator. Kinetochores which are under tension become stretched and the substrates are no longer in the vicinity of the kinase, which is anchored at the inner kinetochore. In contrast, at kinetochores which are not under tension, Aurora B can reach its substrates and upon phosphorylation the interaction of the kinetochore with the microtubule is weakened (Liu et al. 2009; Kelly & Funabiki 2009). Since error correction results in unattached kinetochores, which are also not under tension, the question arises if and how Ipl1p (Aurora B) is involved in the SAC (Santaguida & Musacchio 2009).
A lot of future work will have to be invested to reveal the complex pathways of regulatory mechanisms at kinetochores.

1.3 Minichromosomes

Yeast circular minichromosomes were described some thirty years ago in different studies, which aimed to characterise yeast centromeric DNA sequences and sites of replication initiation. When the centromeric region from a native yeast chromosome was cloned into a plasmid containing an ARS site (autonomously replicating sequence) it behaved like a native chromosome. These minichromosomes were stable in mitosis and in first meiotic divisions. For this stability the centromeric sequence together with the replication site was necessary. Nevertheless, the minichromosome constructs were lost more frequently than

wildtype chromosomes (10^{-2} per division for minichromosomes, 10^{-5} per division for native chromosomes). Already in one of the first studies it was suggested, that such engineered minichromosomes would serve as a tool to study protein-DNA interactions which occur between the centromere and the spindle (Clarke & Carbon 1980; Koshland et al. 1985).

In a mutant screen minichromosomes were used to identify genes, which are involved in the mitotic stability of these plasmids in yeast. The study revealed several mutants organized into 16 complementation groups and referred to them as minichromosome maintenance (Mcm) genes or proteins, respectively (Maine et al. 1984).

2 Aim of the project

Since the kinetochore is a very complex structure, detailed analysis of individual complexes or even proteins is a very daunting task. Therefore it would be helpful to work with a system that allows to investigate functional properties of individual proteins in the context of chromosome segregation *in vivo*. In previous work in the lab a system was established, in which single kinetochore proteins are artificially tethered to a minichromosome and their ability in terms of correct segregation can be monitored. Two promising candidates obtained from these studies have been Ask1p and Dam1p, both members of the Dam1 complex. When recruited directly to a minichromosome, both are able to generate considerable levels of mitotic stabilization (Kiermaier et al. 2009). In the course of this work, I have functionally characterised the Dam1-based segregation system in more detail.

Small circular plasmids containing a native yeast centromere sequence and a chromosomal replicator (ARS) are known to behave like functional chromosomes. Such minichromosomes are easy to manipulate and thus perfectly suited as a tool for the artificial kinetochore system. Instead of building up a kinetochore structure on a native centromere region, kinetochore proteins are tethered individually to the minichromosome. This recruitment is achieved by utilising the interaction between the bacterial tetracycline-repressor protein (TetR) and its DNA binding motif, the tetracycline-operator (*tetO*).

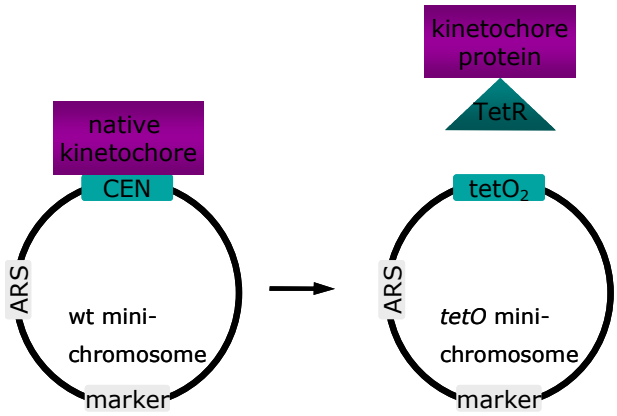


Figure 5: The artificial kinetochore system. Core components of a wt minichromosome: an ARS, a genetic marker and a native centromere sequence, where a kinetochore can assemble and segregate the plasmid as native chromosomes. A *tetO*-minichromosome is lacking any native centromeric sequence. Individual kinetochore proteins are tethered to the plasmid and mitotic segregation of the construct is analysed.

The mitotic stabilisation of such *tetO*-minichromosomes was analysed in plating assays and furthermore by live cell microscopy (both systems are described below). Comparisons were conducted between wildtype-minichromosomes, containing a native centromere sequence (CEN6), and acentric minichromosomes, containing neither a CEN nor a *tetO* array. As expected, the wildtype-minichromosome was stable over many cell cycles, whereas the acentric one was rapidly lost. From these studies it was shown, that Ask1p- as well as Dam1p-TetR fusions were able to stabilize the maintenance of a minichromosome, containing a 10x$tetO_2$ or a 112x$tetO_2$ insertion, to a remarkable degree. In comparison, most of the tested TetR-fusion strains were unable to perform this function (e.g. Cse4p-, Mif2p-, Spc24p-, Spc105p-, Stu2p-TetR), suggesting further that this system does not work with any microtubule binding protein, but requires special properties like force transduction (Kiermaier et al. 2009).

In order to understand the function of this artificial kinetochore in more detail, several questions are addressed in this work:

- → Is the whole Dam1 complex recruited to the minichromosome?
- → Which other factors are necessary for the function of the artificial kinetochore?
- → Do processes like kinetochore biorientation still work for the *tetO*-minichromosomes?

3 Results and Discussion

3.1 Recruitment of the Dam1 complex to minichromosomes

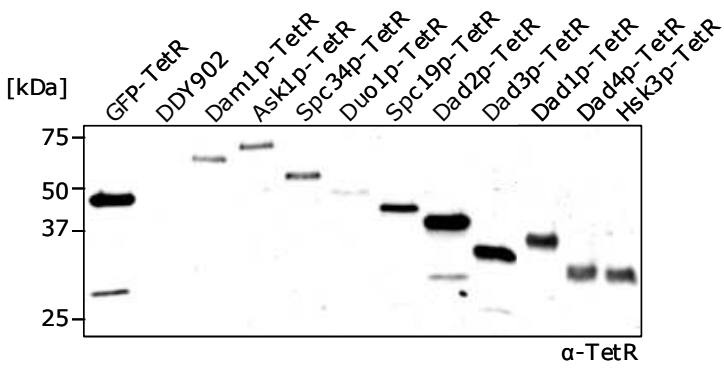
Since Ask1p-TetR and Dam1p-TetR are capable to trigger correct segregation of a *tetO*-minichromosome during mitosis, it is important to know whether this function is achieved by recruiting just individual subunits or the whole Dam1 complex to DNA. The complex consists of ten proteins, which interact very tightly and behave as one unit as far as microtubule binding *in vitro* is concerned (Cheeseman et al. 2001). Furthermore, it is suggested, that the entire complex is needed for performing its correct function (Li et al. 2002). Therefore, tethering individual subunits to a minichromosome may lead to the recruitment of the entire complex. Experiments, which should help to verify this assumption, included a functional as well as a biochemical approach (systematic tagging and complex formation).

3.1.1 Systematic TetR fusions to all Dam1 subunits

Based on the hypothesis, that the entire Dam1 complex is recruited to a minichromosome by directly tethering Ask1p or Dam1p to DNA, I first asked whether TetR-fusions to additional subunits are also able to stabilise a minichromosome in this system.

Therefore the proteins Dad1p, Dad2p, Dad3p, Dad4p, Duo1p, Spc19p, Spc34p and Hsk3p were tagged with the same strategy as Ask1p and Dam1p. A $(SG_4)_2$ linker sequence plus TetR, fused to KanMX as a marker, was amplified from the appropriate plasmid with primers containing overhangs with homology to the genomic target sequence (Figure 22). The resulting PCR product was transformed into yeast cells (DDY902), and via homologous recombination between the targeted genomic locus and the 60bp overhangs on each side of the PCR product, the gene can be tagged. The plasmid and primers were designed to fuse the TetR domain to the C-terminus of the respective Dam1 subunit. After transformation the cells were spread on YPD plates and then replica plated onto YPD + G418. Single colonies were observed after a few days incubation at 25°C. Candidate colonies were restreaked and tested for correct integration by PCR. To this end a forward

primer, homologous to a sequence at the 3' end of the gene of interest, and a reverse primer, matching the KanMX sequence, were designed and the PCR product was sequenced. Furthermore the clones were checked for expression of the TetR tagged gene by Western Blotting, using an antibody against TetR (Figure 6).



Protein	size[kDa]
Dam1p	38.4
Ask1p	32.1
Spc34p	34.1
Duo1p	27.5
Spc19p	18.9
Dad2p	15.1
Dad3p	10.8
Dad1p	10.5
Dad4p	8.2
Hsk3p	8.1
TetRp	23.7
GFPp	27.0

Figure 6: Western blot analysis of TetR fusion proteins. Whole cell extracts from two control strains (GFP-TetR as positive and DDY902 as negative control) and strains expressing a subunit of the Dam1 complex tagged with TetR, were loaded on a gel. Following SDS-PAGE, proteins were blotted onto a nitrocellulose membrane and probed with an antibody recognizing TetR. All fusion proteins were detected at the expected size (Ask1p migrates more slowly, because of a low pI of the protein).

To ensure that the cells displayed normal growth characteristics in the presence of the tagged protein, growth curves were recorded (Figure 7). This is of special interest for subsequent experiments assessing the stability of the minichromosome, since differences in the growth speed of individual strains would make a comparison very difficult.

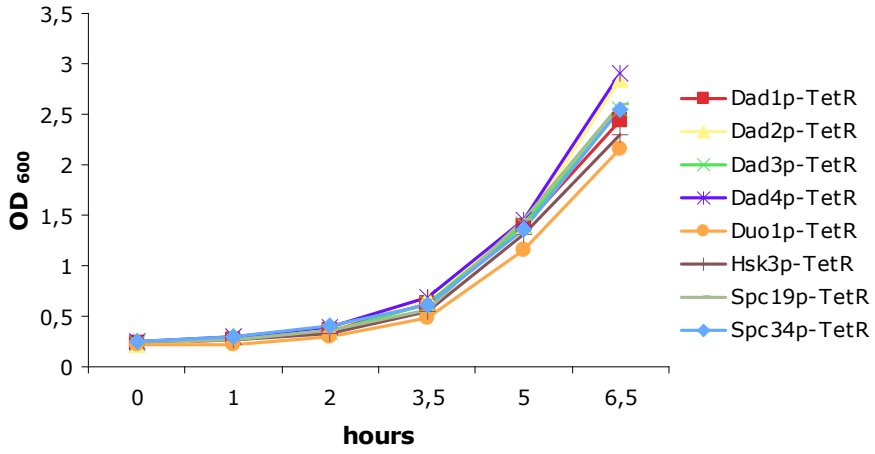


Figure 7: Growth curves of TetR fusion strains. O/N cultures in YPD were diluted to an OD_{600} of 0.15 and incubated at 25°C. After the indicated time points the OD_{600} was measured. Values were plotted against time.

Furthermore a spot assay was performed to check if the strains show compromised growth at higher temperatures (Figure 8). From both, the growth curves and the spot assay, the TetR-fusion strains displayed wildtype growth rates, and they were clearly not temperature sensitive. By comparison, a fusion to Cse4p (the yeast CENP-A protein) is temperature sensitive at 37°C (Figure 8).

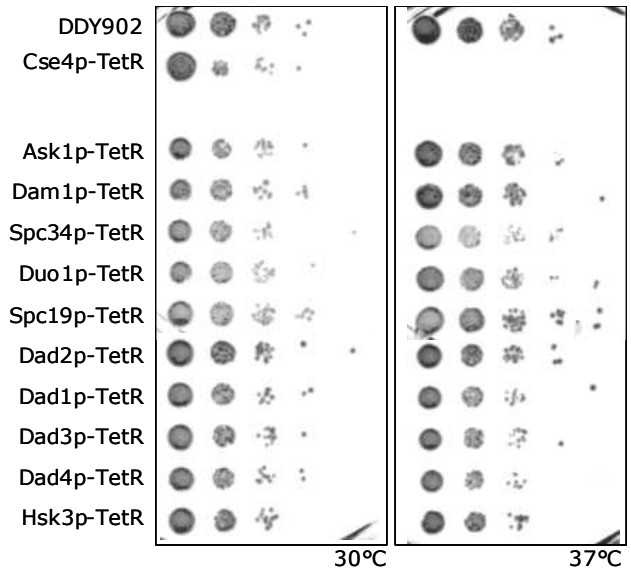


Figure 8: Spot assay of TetR fusion strains. O/N cultures of two control strains (DDY902 is not temperature sensitive, Cse4p-TetR is inviable at high temperature) and strains with a subunit of the Dam1 complex tagged with TetR were diluted. Equal amounts of liquid from a dilution series was pinned on YPD plates and incubated at different temperatures. TetR fusion strains (except Cse4p-TetR) are viable at 37°C.

After successful testing for correct integration of the TetR-tag and fitness of the strains, they were examined for their ability to stabilise a *tetO*-minichromosome during mitotic cell divisions. To this end the plasmid containing 112x$tetO_2$ repeats and an acentric plasmid without any $tetO_2$ sequence as control were transformed into the TetR-strains, and single colonies were restreaked. For the plating assays they were grown for 24 hours (equates approximately 12 generations) under non-selective conditions in liquid culture and a defined dilution was spread onto selective and non-selective plates. The percentage difference of colonies grown on selective and non-selective medium was calculated and defined as the loss rate. This value subtracted from 100% resulted in the relative stability of the minichromosome in a certain strain background. An acentric plasmid was lost very rapidly and showed a stability of a maximum 2.7 ± 0.4% (in Dad2p-TetR experiment). In

contrast, the *tetO*-minichromosome was stabilised to a significant degree in certain strain backgrounds. In addition to Ask1p- and Dam1p-TetR also Spc34p-, Spc19p- and Dad1p-TetR showed a clear stabilising effect (Figure 9). The stability mediated by Ask1p- and Dam1p-TetR are taken from identical experiments already performed in the lab. Under the same assay conditions a wildtype minichromosome showed a stability of 67 ± 5.3% (Kiermaier et al. 2009).

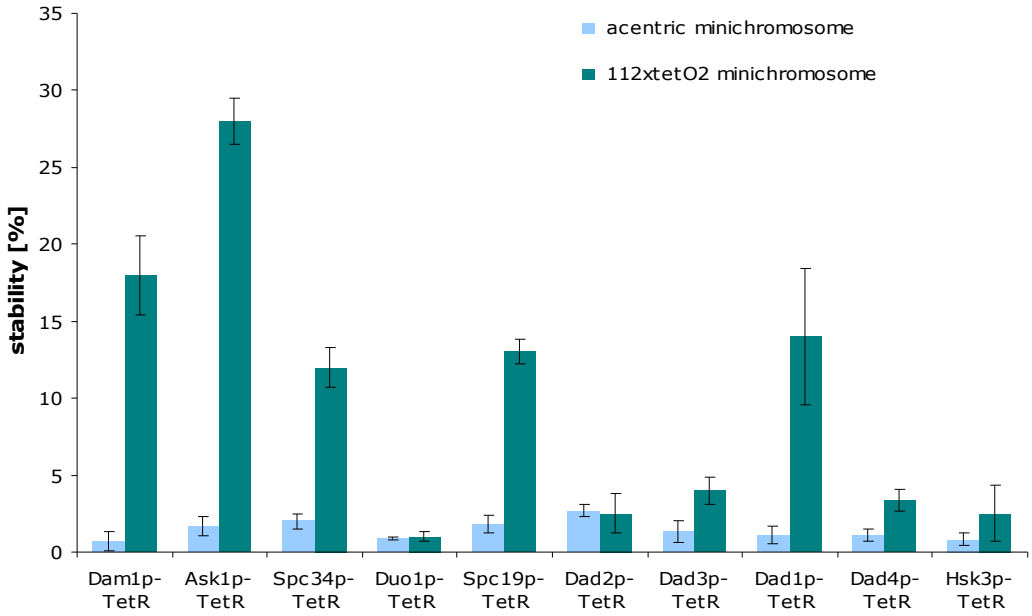


Figure 9: Stability of minichromosomes in TetR fusion strains. Plating assays were performed to analyse the stability of a 112x*tetO*$_2$ minichromosome over approximately 12 generations in strain backgrounds containing a subunit of the Dam1 complex tagged with TetR. Stabilisation was detected for Dam1p-, Ask1p-, Spc34p- Spc19p- and Dad1p-TetR. The bars represent data from three independent experiments, error bars denote the standard deviation.

The experiment was performed with a minichromosome containing 112x*tetO*$_2$ repeats. Nevertheless, it was shown that also a plasmid containing just 10x*tetO*$_2$ repeats could be stabilised by the Ask1p- and Dam1p-TetR fusion proteins, although to a lower degree, suggesting an influence of the array size.

Taken together, this experiment showed, that five out of ten subunits are able to stabilise the *tetO*-minichromosome during mitotic cell divisions.

3.1.2 Purification of the Dam1 complex

The formation of the entire Dam1 complex is a prerequisite for its correct function. Therefore it is important to obtain evidence that the assembly of the complex is not compromised *in vivo* by the Ask1p-TetR fusion protein. In a Tandem Affinity Purification (TAP) the ability of the TetR-tagged Ask1p protein to be incorporated into the complex should be verified. The experiment was performed in parallel with two strains (SWY276 and SWY277), both carrying a tandem affinity tag C-terminally fused to Dad1p, a subunit of the Dam1 complex. One of the strains contained the tagged Ask1p fusion protein (SWY276), whereas the other did not and was therefore referred to as the control (wt Ask1p, SWY277). In general, the purification was performed as described previously (Cheeseman et al. 2001). After a pre-cleaning step the lysate was bound to IgG sepharose via interaction with the ZZtag (Protein A IgG binding motif). Due to a cleavage site for TEV protease, proteins bound to IgG sepharose could be cleaved off specifically. In a second purification step the cleaved protein-complex can be bound to Protein S Agarose via the S-tag. After elution of the bound proteins, the samples were loaded on an SDS gel to check for the integrity of the complex via subsequent silver staining.

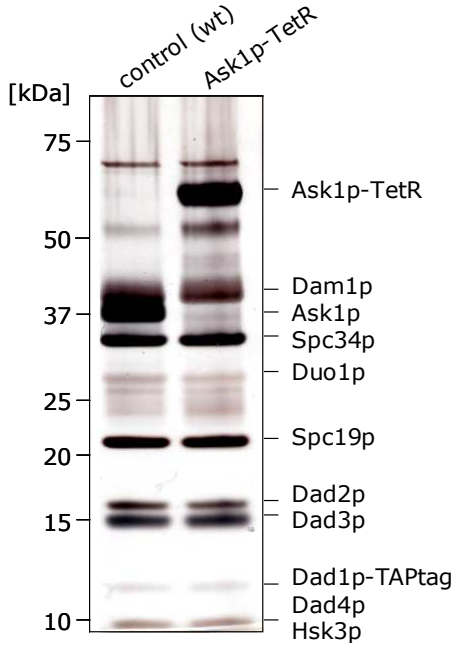


Figure 10: Purification of the Dam1 complex. The complex was purified from a wt control strain and a strain containing Ask1p tagged with TetR. Samples were separated via SDS-PAGE and the gel was silver stained. All ten complex subunits can be identified in both purifications. In the right lane the position of Ask1p is shifted according to the TetR tag.

When the patterns of the purified complexes, derived from the control and the Ask1p-TetR strain, were compared, they both show ten distinct bands which can be assigned to the subunits of the Dam1 complex according to their molecular weight. The only difference results from an up-shift of Ask1p, caused by the TetR fusion (Figure 10).
In addition, both samples were analysed by mass spectrometry and the presence of all ten subunits was confirmed. Interestingly, in addition to Dam1 subunits, the kinase Ipl1p, a potential regulator of Dam1, was detected by mass spectrometry.
The biochemical purification confirms, that the TetR fusion to Ask1p does not interfere with the correct assembly of an entire Dam1 complex.
Taken together, these data strongly suggest that the Ask1p-TetR fusion protein works as a part of the entire Dam1 complex, which is recruited to the minichromosome and can somehow function as a linker between DNA and microtubules. Furthermore, in an experiment described below, it was shown that the artificial kinetochore is not functional in a *dad1* temperature sensitive mutant. This result is consistent with previous work, suggesting that all subunits are needed for the integrity of a functional complex (Li et al. 2002).

3.2 Functional dependency of Dam1-mediated minichromosome segregation

The experiments described above suggested a recruitment of the entire Dam1 complex upon tethering Ask1p to a minichromosome. There, it is able to connect the DNA to microtubules and improve the segregation of the minichromosome. However, it is not clear how this function is achieved. Since it was proposed, that a Dam1 ring would be a perfect device for transduction of microtubule-generated pulling forces (Westermann et al. 2006), it is conceivable that the Dam1-based minichromosome-segregation system works independently of other kinetochore proteins. On the other hand, the recruited Dam1 complex may depend on other kinetochore proteins to stabilize the minichromosomes. To distinguish between these possibilities, experiments were in demand that would allow to analyse the functional dependency on other kinetochore proteins.

The function of the Dam1-TetR fusions were analysed in a microscopical approach. The Ask1p- or Dam1p-TetR tagged proteins were first combined with a variety of temperature

sensitive (ts) mutant alleles of different kinetochore proteins. All of the tested mutant alleles are known to be essential for correct function of the kinetochore. As a result, wildtype-minichromosomes are expected to show missegregation in each of these ts strains at the restrictive temperature. By contrast, this phenotype should be rescued for a *tetO*-minichromosome in an Ask1p- or Dam1p-TetR background strain, if the artificial kinetochore is independent of the protein function of the respective ts-mutant allele.

Since the question should be answered in a microscopical approach, the system required some features for visualisation. New minichromosomes were designed, containing a 256x*lacO* array in addition to a CEN sequence or a 10x*tetO*$_2$ array, respectively. Although the smaller *tetO* array size led to lower stabilities in the plating assay, it was chosen here because of the large size of the *lacO* plasmid (~14kbp) necessary for visualisation. The yeast strains for microscopy expressed LacI-GFP, controlled by a copper-inducible promotor, which binds to the *lacO* array on the plasmid (Figure 11). In addition the spindle poly body (SPB) protein Spc42p was tagged with mCherry. This combination enabled visualisation of a minichromosome and its relative position to the SPB. Furthermore this allowed to check for the integrity of the mitotic spindle, which often shows defects when kinetochore proteins are mutated. These tools were already available in the lab.

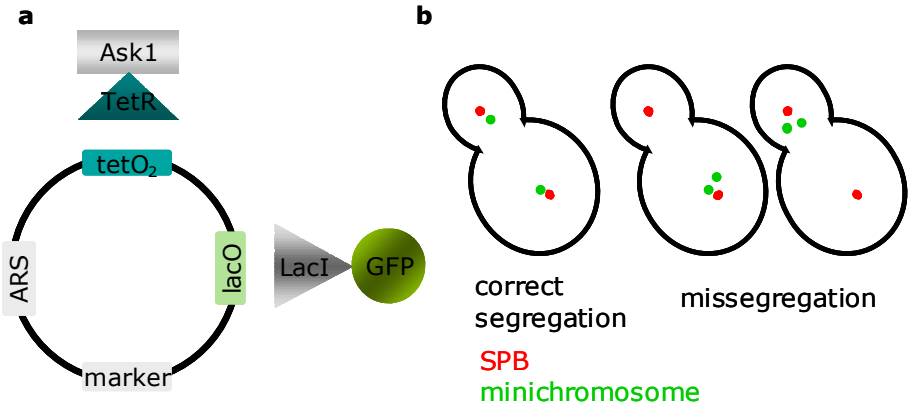


Figure 11: Visualisation of the minichromosome for microscopy. (**a**) The minichromosome constructs for visualisation contained a *256xlacO* array. This feature was combined with acentric, wt CEN and 10x*tetO*$_2$ arrangements. Strains for microscopy contain Cu-inducible LacI-GFP, which can bind to minichromosomes and makes them visible. (**b**) For ts experiments the SPB protein Spc42p was fused to mCherry. Only cells with an intact mitotic spindle (separated SPBs) were considered for analysis. Minichromosomes either segregated correctly (a signal in mother cell and bud) or they showed missegregation (no discrimination between both signals in mother or in bud).

In a first step of this experiment a number of new strains was generated. In order to combine the TetR tagged proteins with the ts-mutants, the appropriate strains were crossed. After sporulation and dissection of tetrads, haploid spores with the desired combination of markers were selected. This resulted in strains with and without the TetR tagged Ask1p fusion protein (for some cases also the Dam1p-TetR strains were available) carrying one of the following ts mutant alleles: *dad1-1*, *ndc10-2*, *ctf13-30*, *cep3-1*, *cse4-1*, *mif2-3*, *ndc80-1*, *ndc80-2*, *mtw1-1*, *ipl1-2* or *sli15*-3. Next, wildtype and 10x*tetO*$_2$ minichromosomes for visualisation were transformed into these strains. In addition the plasmids were transformed into a control strain (wt) without a ts mutant allele.

Images to analyse the behaviour of the minichromosomes were acquired from fixed cells. Before fixing, cells were shifted to the restrictive temperature (37°C for most ts alleles, 34°C for *ipl1-2* and *ndc10-2*) and LacI-GFP expression was induced. For every cell with an intact mitotic spindle the segregation pattern of the minichromosomes was scored. Unsegregated SPBs, often located in the middle of mother and bud, although the nucleus was already enlarged, were classified as compromised spindles and those cells were not further considered. In analysing the segregation pattern of minichromosomes it was not discriminated between those cases with both of them located in the mother cell or both in the bud (Figure 11). For each experiment 100 cells with an intact mitotic spindle were analysed.

3.2.1 Dependency of minichromosome segregation on other Dam1 complex subunits

In addition to the experiments described in the previous section, which should reveal whether or not the entire Dam1 complex is recruited to the minichromosomes, Dam1-mediated segregation in a temperature sensitive mutant of a complex subunit was scored. Analysing the segregation of wildtype- and *tetO*-minichromosomes in the *dad1-1* mutant background strain yielded similar results. As expected, the wildtype minichromosome was missegregated in more than 60% of the cells. Segregation of the *tetO*-minichromosome in the Ask1p-TetR strain was affected to a similar degree in the *dad1-1* mutant (Figure 12). This result supports the conclusion that the functionality of the entire Dam1 complex is required for segregation of the *tetO*-minichromosomes.

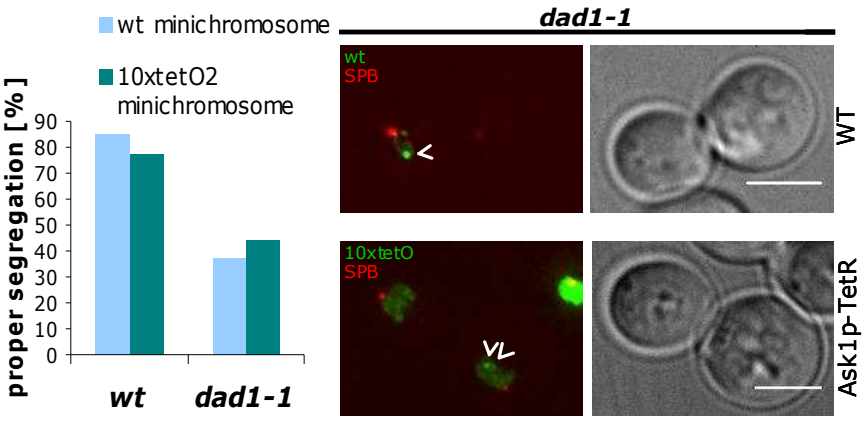


Figure 12: Minichromosome segregation in the *dad1-1* mutant. A wildtype-minichromosome and a *tetO*-minichromosome in an Ask1p-TetR background show similar segregation defects in a *dad1-1* mutant (left panel). In the *dad1-1* mutant wt minichromosomes show missegregation. This phenotype cannot be rescued for a *tetO*-minichromosome in Ask1p-TetR background. n=100; Representative pictures are shown on the right. The arrow heads are indicating minichromosome signals. Scale bar 2µm.

3.2.2 Dependency of Dam1-based minichromosome segregation on the CBF3 complex

Next, the dependency of the artificial kinetochore on members of the DNA binding CBF3 complex was examined. This complex is known to play a major role in building up a functional kinetochore by acting as the primary determinant of its assembly. In this experiment the segregation of minichromosomes in *ndc10-2*, *ctf13-30* and *cep3-1* mutant strain backgrounds was analysed at the restrictive temperature. Since they fail to assemble a functional kinetochore, these mutants are known to disrupt all native kinetochores. As a consequence chromosomes cannot be attached to microtubules from the mitotic spindle and a severe missegregation phenotype is observed. In accordance with these facts, wildtype-minichromosomes failed to segregate correctly under our assay conditions. However, this phenotype was rescued when Ask1p-TetR or Dam1p-TetR was directly tethered to the *tetO*-minichromosome. Although the cells were incubated at the restrictive temperature and consequently Ndc10p, Ctf13p or Cep3p, respectively, was not functional, segregation of minichromosomes with the artificial kinetochore was not affected (Figure 13).

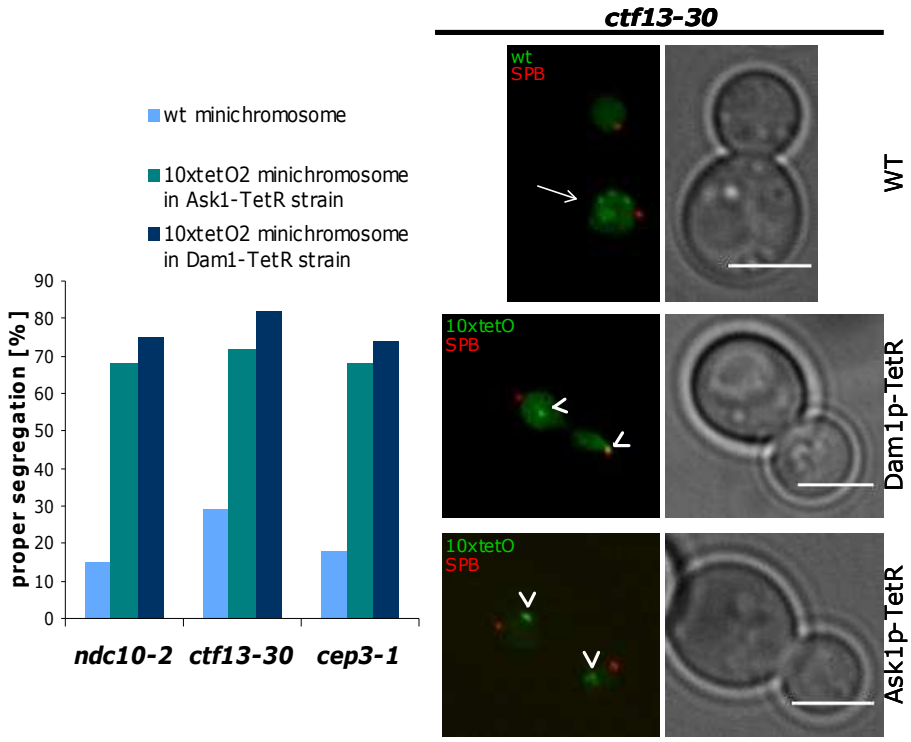


Figure 13: Minichromosome segregation in CBF3 complex mutants. Wildtype minichromosomes show a high rate of missegregation in *ndc10-2*, *ctf13-30* and *cep3-1* strains. In comparison, *tetO*-minichromosomes were properly segregated in these mutants when Ask1p or Dam1p was fused to TetR. n=100; Representative pictures are shown on the right. There is an accumulation of signals to see for the wt minichromosome (indicated by the arrow), which frequently missegregated. This phenotype was also observed in other mutants. Arrow heads are indicating minichromosome signals. Scale bar 2µm.

This leads to the conclusion, that the Dam1-based artificial kinetochore bypasses the functional requirement for the essential CBF3 complex. Moreover, these experiments provide evidence that the *tetO*-minichromosomes do not segregate by "hitch-hiking" on native chromosomes, since they cannot segregate correctly in these mutants.

3.2.3 Dependency of Dam1-based minichromosome segregation on conserved inner kinetochore proteins

The requirement for two other inner kinetochore proteins was examined by performing the microscopy experiment with ts alleles of *cse4* and *mif2*. Both of these proteins are hallmark features of kinetochores in all eukaryotes. Cse4p is the centromere specific histone H3 variant and Mif2p plays an important role at the interface between the inner and central kinetochore layers (Westermann et al. 2007).

As expected, by shifting cells to the restrictive temperature the formation of an intact kinetochore and therefore correct (mini)chromosome segregation was abolished in *cse4-1* or *mif2-3* mutants. In contrast to this, the *tetO*-minichromosome showed proper segregation in almost 70% of the cells in these two mutants in the presence of Ask1p-TetR (Figure 14). This indicates that the requirement for Cse4p and Mif2p is bypassed by the artificial kinetochore.

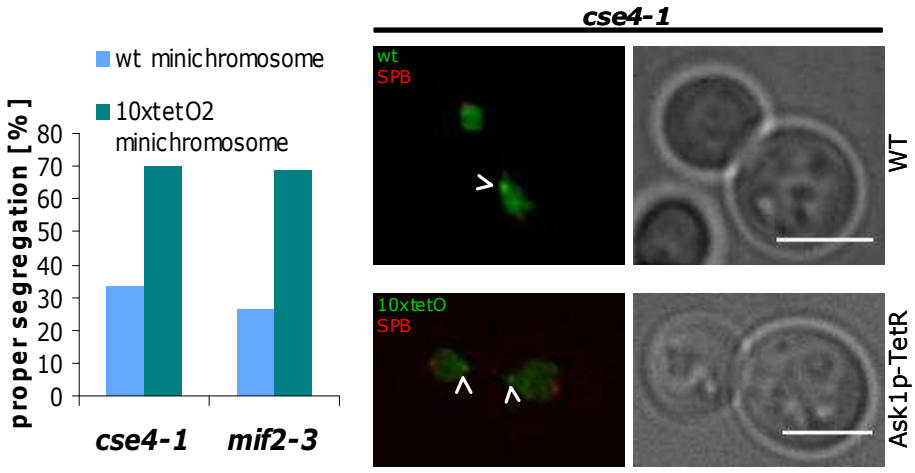


Figure 14: Minichromosome segregation in *cse4* and *mif2* mutants. Correct segregation of wt minichromosomes was abolished in *cse4-1* and *mif2-3* mutants. By contrast, a minichromosome under the control of the artificial *tetO*/TetR kinetochore was stabilised significantly in these mutants. n=100; Representative pictures are shown on the right. The arrow heads are indicating the minichromosome signals. Scale bar 2µm.

3.2.4 Dependency of minichromosome segregation on structural elements like the KMN network

An important structural feature of native kinetochores is the so called KMN network. This conserved group of proteins is thought to play a central role at the outer kinetochore and in building up a machinery that binds to microtubule ends (Tanaka & Desai 2008). The influence of two components, Ndc80p and Mtw1p, on the artificial kinetochore function should be investigated here. The former is a member of the four protein Ndc80 complex, which is thought to be important for microtubule attachment. Recently it was suggested to play a major role in establishing and maintaining the load-bearing attachment of kinetochores to microtubules (Powers et al. 2009).Unfortunately it was not possible to analyse the influence of Ndc80p on the artificial kinetochores. Both ts mutant strains tested

in this experiment (*ndc80-1* and *ndc80-2*) showed such a severe spindle defect when shifted to the restrictive temperature, that analysis of minichromosome segregation was not possible. In case of a broken mitotic spindle it is not feasible to draw any conclusion whether or not the artificial kinetochore depends on the function of Ndc80p.

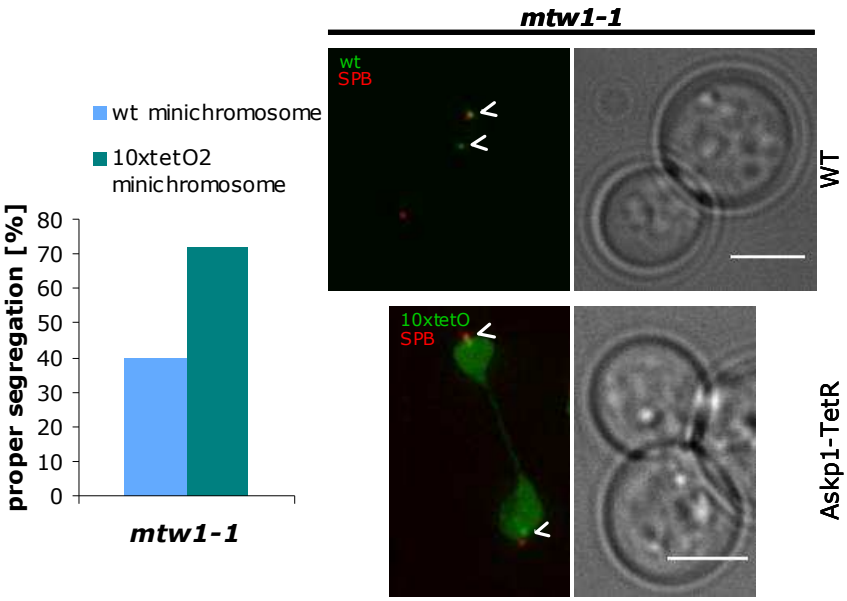


Figure 15: Minichromosome segregation in the *mtw1-1* mutant. Again, the wildtype minichromosome failed to segregate correctly in the majority of the cells in the *mtw1-1* mutant. This phenotype was rescued when Ask1p-TetR was directly tethered to the *tetO*-minichromosome and therefore mediated its segregation independent of Mtw1p. n=100; Representative pictures are shown on the right. Arrow heads are indicating minichromosome signals. Scale bar 2µm.

In the *mtw1-1* strain background the spindle defects were not as extensive and the segregation could be analysed. While wildtype-minichromosomes were missegregated, *tetO*-minichromosomes could be properly segregated in more than 70% of the cells carrying Ask1p-TetR (Figure 15). These data indicate a functional independency of the artificial kinetochore from Mtw1p. Unfortunately with this experiment alone the conclusion cannot be expanded to the whole KMN network and analysis of additional mutant alleles is necessary.

3.2.5 Dependency of minichromosome segregation on regulatory proteins

To ask whether the artificial kinetochore is still under a regulatory control, it was interesting to analyse the requirement of Ipl1p and Sli15p for correct segregation. These are yeast homologs of the Aurora B kinase and its acivator protein INCENP and are necessary for the correction of erroneous attachments of kinetochores to microtubules (Kelly & Funabiki 2009).

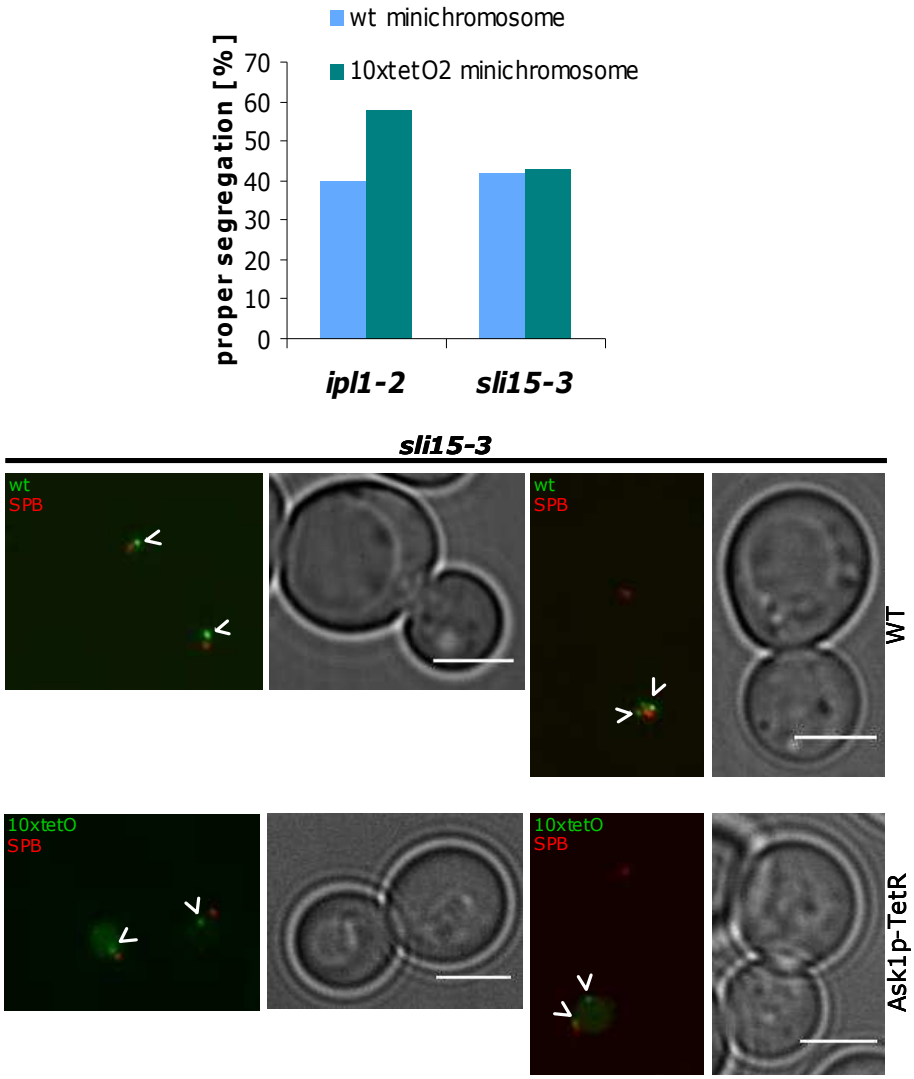


Figure 16: Minichromosome segregation in *ipl1-2* and *sli15-3* mutants. As expected for these mutants the wt-minichromosome was segregated properly only in ~50% of the cells. The same was true for the *tetO*-minichromosome in the Ask1p-TetR stain (segregation in *ipl1-2* was less compromised). n=100; Representative picture for proper segregation and missegregation in the *sli15-3* mutant (each for wt and Ask1p-TetR) are shown. Arrow heads are indicating minichromosome signals. Scale bars 2µm.

Two hours after shifting the strains to the restrictive temperature, missegregation was observed in approximately 60% of the cells carrying a wildtype-minichromosome. The *tetO*-minichromosome in the Ask1p-TetR strain showed the same degree of missegregation in the *sli15-3* background (Figure 16). For *ipl1-2* the phenotype was less severe, but still the missegregation failure could not be rescued by the Ask1p-TetR fusion to the level of an *ipl1* wt strain (Figure 12). These results suggest, that Ipl1p and Sli15p are still required in the context of a Dam1-based artificial kinetochore.

3.3 Live cell microscopy to observe the metaphase behaviour of minichromosomes

In order to achieve correct chromosome segregation, many regulatory processes have to occur in metaphase. Each sister-chromatid has to attach to microtubules emanating from opposite SPBs. The process in which this is achieved is called biorientation. Two important factors during this event is the protein kinase Ipl1p and its regulatory subunit Sli15p. Since the ts experiments, performed above, suggested a functional dependency of the artificial kinetochore on these two proteins, further investigations into the regulatory mechanism would be interesting.

To study single events of minichromosome segregation in more detail, a system was available in the lab that allows to arrest cells in metaphase by shutting off the expression of Cdc20p, the activator of the anaphase promoting complex. In a modified strain *CDC20* was placed under the control of a galactose inducible promoter. Therefore cells display normal growth in medium containing raffinose and galactose, but by switching the conditions to glucose containing medium *CDC20* expression is shut off and cells get arrested in metaphase. The *CDC20* allele was combined with a strain that expressed mCherry labelled tubulin for visualisation of the mitotic spindle and copper-inducible LacI-GFP to detect *lacO*-containing minichromosomes. In this way the behaviour of wt- as well as *tetO*-minichromosomes, in an Ask1p-TetR strain background, in metaphase (and in metaphase to anaphase transition) could be visualised directly.

A readout for proper biorientation is the so called centromeric breathing. This phenomenon results from pulling forces generated from microtubules attached to kinetochores of both sister chromatids. A high level of tension is build up when these microtubules emanate from opposite SPBs. Consequently sister centromeres split precociously and chromosomes

are stretched, since cohesion holds their arms still together. Cohesion is a prerequisite for proper biorientation. The observation of chromosomal stretching provides an assay for cohesive forces and biorientation (Tanaka et al. 2000).

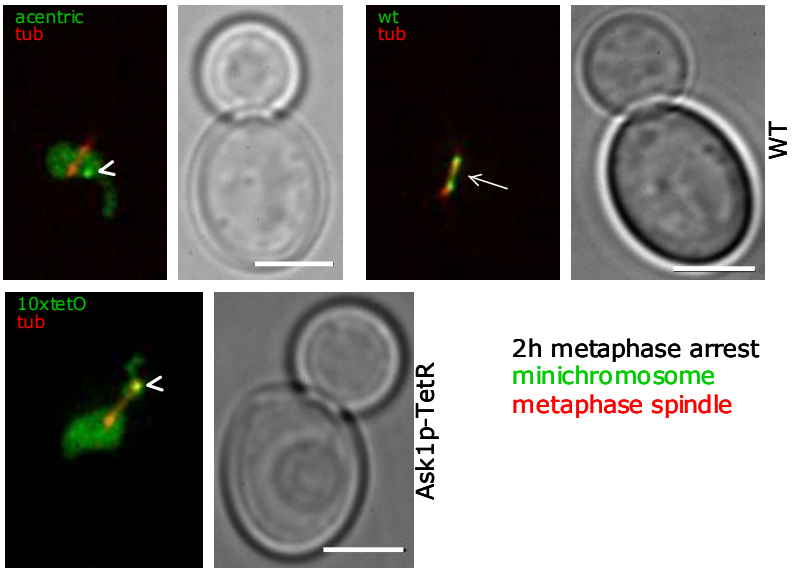


Figure 17: Live cell imaging of metaphase arrested cells. Cells were arrested in glucose containing medium for 2h. Time lapse movies were taken by recording stacks every 20sec for 20min. Images from single timepoints are shown here. Acentric minichromosomes moved throughout the nucleus most of the time and failed to interact with the spindle. Constructs containing a wt CEN sequence showed strechting (indicated by the arrow) and *tetO*-minichromosomes were attached to a SPB most of the time, but no stretching was observable. Arrow heads are indicating minichromosome signals. Scale bars 2µm.

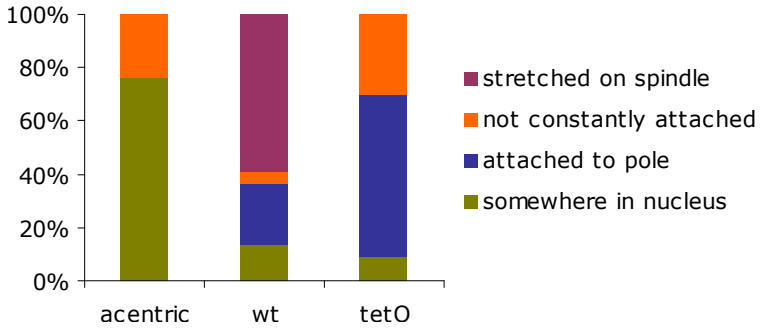


Figure 18: Quantification of minichromosome behaviour in metaphase arrest. Time laps movies were analysed and classified into four different phenotypes. n=20.

Live cell imaging was carried out after the cells were arrested for two hours in glucose containing medium (Figure 17). The metaphase arrest revealed the expected phenotype for the wildtype and the acentric minichromosome: Plasmids carrying a native CEN could properly bi-orient on a metaphase spindle, which was observable as chromosome

stretching along the longitudinal spindle axis. Acentric minichromosomes never showed this phenotype, instead they moved throughout the nucleus most of the time. Nevertheless, in some cases there was still some degree of movement detected together with the spindle movements. Maybe this observation resulted from the fact, that the movement was analysed only in two dimensions. The *tetO*-minichromosome displayed a different phenotype. It was neither stretched along the spindle, nor the majority was completely detached from the spindle. Instead, it was often attached close to one spindle pole body. In some cases a minichromosome-signal was to detect at both SPBs, but it was difficult to say, whether this reflected a separated pair, or two unseparated minichromosomes. The observed phenotypes were classified into four groups and approximately 20 movies were analysed for each plasmid construct (Figure 18). Also after just one hour of metaphase arrest the *tetO*-minichromosome showed no stretching on the spindle. Therefore it was concluded, the artificial kinetochore did not show the typical bi-orientation process of natural kinetochores.

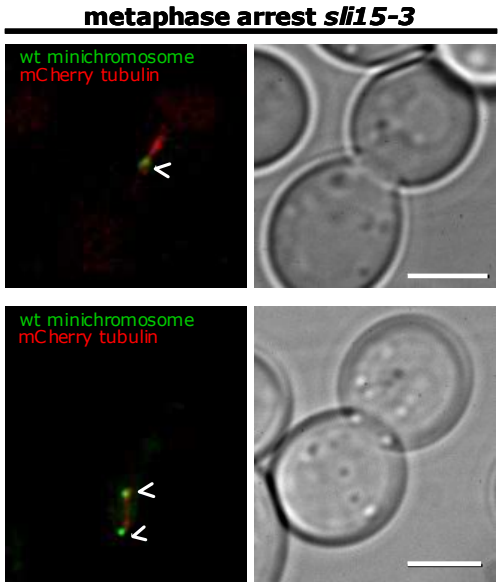


Figure 19 Metaphase behaviour of wt minichromosomes in a *sli15-3* mutant. Wt-minichromosomes displayed no stretching along the spindle in a *sli15-3* mutant. Instead they were attached to the SPBs, similar to *tetO*-minichromosomes in the strain with functional Sli15p. Arrow heads are indicating minichromosome signals. Scale bars 2µm

Furthermore, the strain for visualisation and metaphase arrest experiments was combined with the temperature sensitive *sli15-3* allele. The medium switch for arresting the cells was

performed simultaneously with a shift to the restrictive temperature (37°C) for two hours. After this procedure wildtype-minichromosomes did not show the stretching phenotype any more, verifying the stretching to be a readout for Sli15 dependent biorientation (Figure 19). Moreover, in case of abolished Sli15 function, wt minichromosomes demonstrated the same phenotype as *tetO* minichromosomes in the *sli15* wt background. These data suggest that *tetO*-minichromosomes segregated by Ask1p-TetR cannot bi-orient in a wildtype manner. One reason for this is may be a strongly reduced cohesion between sister minichromosomes.

A recovery experiment was performed to check whether the cells could be released onto anaphase after the metaphase arrest. After two hours of metaphase arrest in glucose, a drop of cell suspension was placed on a coverslip as usual. After ten minutes allowing the cells to adhere to the surface, they were washed extensively with raffinose and galactose containing medium. This should allow the cells to express Cdc20p again and proceed normally throughout the cell cycle. Indeed, the arrest could be abrogated, the cells progressed through anaphase and segregated their minichromosomes correctly. Time lapse movies were acquired for wildtype- (Figure 20) and *tetO*-minichromosomes (Figure 21).

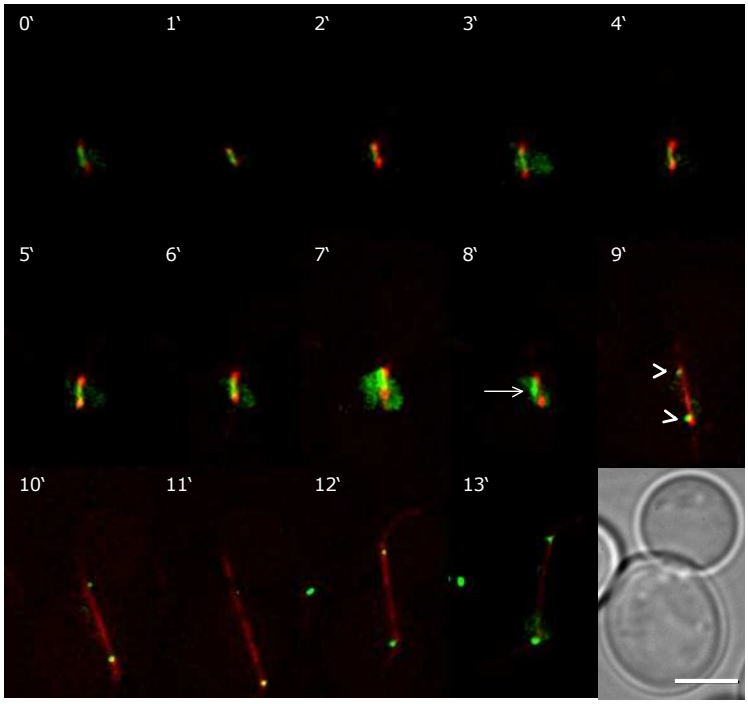


Figure 20: Anaphase transition after arrest in metaphase for wt-minichromosome. After 2h metaphase arrest in glc medium, cells were washed into raff/gal medium directly on a coverslip. Time lapse movies of live cells were recorded. After stretching on the metaphase spindle, the separation and transition to anaphase proceeds (from timepoint 8 to 9). Arrow heads are indicating minichromosome signals. Scale bar 2µm.

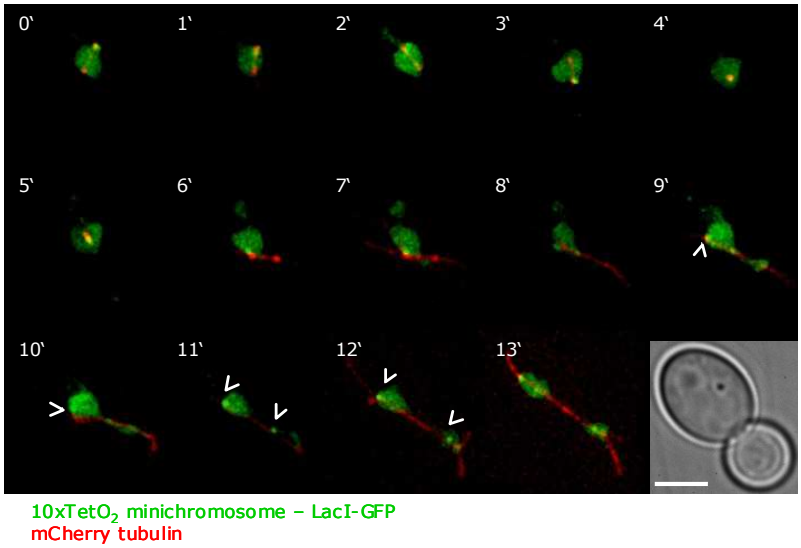


Figure 21: Anaphase transition after arrest in metaphase for *tetO*-minichromosome in Ask1p-TetR strain. After 2h metaphase arrest in glucose medium, cells were washed into raff/gal medium directly on a coverslip. Time lapse movies of live cells were recorded. Although no stretching is detectable, the cell manages to properly segregate the minichromosomes (entry into anaphase between timepoint 10 and 11). Arrow heads are indicating minichromosome signals. Scale bar 2µm.

4 Conclusions

The principle of chromosome segregation is to connect DNA to highly dynamic microtubules which are able to generate pulling forces. This connection has to be firm enough to ensure stable attachments and to transduce the generated forces to chromatids. In addition this connection still has to allow microtubules to behave as dynamic polymers. The kinetochore, a macromolecular structure composed of several protein complexes, is able to fulfil these requirements. The establishment and maintenance of such kinetochore-microtubule attachments has to be highly regulated in space and time during the cell cycle. To elucidate the minimal requirements a faithful segregation system needs would be helpful for understanding the function of a native kinetochore.

An artificial segregation system was presented and characterised in this work. Tethering Ask1p or Dam1p directly to a small circular minichromosome resulted in a significant degree of mitotic stabilisation. In addition to the experiments carried out with minichromosomes, it was also shown that Ask1p-TetR is able to stabilise native yeast chromosomes with an inactivated centromere but carrying a *tetO* array (Kiermaier et al. 2009). Another study revealed similar results when Ask1p was tethered to native chromosomes via the interaction between *lacO* and LacI (Lacefield et al. 2009).

In this work I could show that three additional subunits of the Dam1 complex are also able to fulfil this function and that Ask1p-TetR is part of a fully assembled Dam1 complex. These results lead to the conclusion, that the entire Dam1 complex is recruited to the minichromosomes. However, five out of ten Dam1 subunits do not stabilise a *tetO*-minichromosome when fused to TetR. One reason could be that TetR cannot bind to *tetO* when the fusion protein is incorporated into the complex because of a wrong orientation. Another possibility could be that the TetR tag interferes with a correct formation of the complex, if it is fused to certain proteins. The presented experiments do not address if this recruitment results in Dam1 ring formation or if enough complexes to form a ring are present at the attachment site. In general, elucidating the composition and function of the Dam1 based artificial kinetochore will also help to understand the functional properties of the complex in the context of a native kinetochore.

Dam1 was shown to be a processive force coupler *in vitro* (Asbury et al. 2006; Grishchuk et al. 2008). The results from this study verify this role *in vivo*. Experiments revealing the dependency of the artificial segregation system on essential kinetochore proteins, showed that the function of key components like the CBF3 complex, Cse4p, Mif2p and Mtw1p is bypassed by the artificial system. Thus, the ability of the Dam1 complex to translate microtubule forces into chromosome movement is independent of inner kinetochore proteins. This leads to the conclusion, that force transduction is an intrinsic property of the complex *in vivo*.

Native yeast kinetochores consist of several protein complexes and most of them are necessary for viability of the cells. What is the advantage then of building up a complicated multi-modular structure instead of just recruiting one component which is able to transmit forces from dynamic microtubules (like in the artificial system)? Probably the answer lies in the need to regulate the structure and function of a kinetochore in space and time according to the cell cycle stages. Furthermore this more complicated architecture provides several platforms for fine-tuning its functional properties.

This study shows that the Dam1-based kinetochore is at least partly dependent on the function of Ipl1p and Sli15p. However, it is not clear, which functional aspect of the artificial kinetochore is disturbed in these mutants. Ipl1p is known to directly phosphorylate Dam1 and therefore the kinase influences the kinetochore-microtubule attachment (Cheeseman et al. 2002). Maybe Ipl1p regulates the function of the artificial kinetochore in a similar manner. The other possibility would be, that some microtubule binding activity of Sli15p is required for segregation mediated by the artificial kinetochore.

Live cell imaging of cells was performed to observe the behaviour of minichromosomes in metaphase arrested cells. A stretched signal of a fluorescent marker, which serves as a read-out for biorientation of kinetochores, was not detectable for minichromosomes with an artificial kinetochore. The reason may be that *tetO*-minichromosomes fail to establish enough cohesion, which is necessary for proper biorientation. This is consistent with the fact, that cohesin is enriched at native centromeric sequences (Onn et al. 2008), which are missing on these minichromosomes.

Additional information about the exact composition of the artificial kinetochore would be helpful. The experiments, in which minichromosome segregation was analysed in mutants of essential kinetochore proteins, already revealed some functional independencies. Furthermore, some pilot experiments were already carried out to purify the minichromosomes with the aim to analyse the artificial kinetochore by mass spectrometry. The protocol for purification is based on previously described experiments (Ivanov & Nasmyth 2005). The purification strategy takes advantage of the already established system that was used for microscopy. LacI-GFP binds to *tetO* minichromosomes containing a *lacO* array. With an antibody against GFP coupled to beads, it was tried to purify the minichromosome, on which the artificial kinetochore should be built up. So far the experiment was not successful and the protocol is still in need of optimisation. So far, enrichment of the plasmid seems to be insufficient. While it could be detected by PCR in several aliquots taken during the purification procedure, it was lost in the final centrifugation step, which should separate genomic DNA from plasmids.

Once the molecular composition of the artificial kinetochore is known, this information can be used for *in vitro* studies. Reconstitution of dynamic microtubules and associated minichromosomes carrying artificial kinetochores could reveal the mechanism of regulatory processes like biorientation and would help to understand the function of individual kinetochore proteins.

5 Materials and Methods

5.1 Strains, plasmids and media

5.1.1 Yeast strains

Name	*Genotype*
DDY902	*Mat a, ade2-1, leu2-3, his3Δ200, ura3-52*
EKY161	*Mat a, leu2-3, his3Δ200, ura3-52, pCu-LacI-GFP::HIS, Spc42-mCherry::URA3, Dam1-TetR::KanMX, ndc10-2 (ts at 34°C)*
EKY162	*Mat a, leu2-3, his3Δ200, ura3-52, pCu-LacI-GFP::HIS, Spc42-mCherry::URA3, ndc10-2 (ts at 34°C)*
EKY176	*Mat a, leu2-3, his3Δ200, ura3-52, pCu-LacI-GFP::HIS, Spc42-mCherry::URA3, Ask1-TetR::KanMX, ndc10-2 (ts at 34°C)*
EKY179	*Mat α, ade2-1, leu2-3, his3Δ200, ura3-52, lys2-801, pCu-LacI-GFP::HIS, Spc42-mCherry::URA3, ctf13-30 (ts at 37°C)*
EKY180	*Mat a, leu2-3, his3Δ200, ura3-52, lys2-801, pCu-LacI-GFP::HIS, Spc42-mCherry::URA3, Ask1-TetR::KanMX, ctf13-30 (ts at 37°C)*
EKY181	*Mat α, ade2-1, leu2-3, his3Δ200, ura3-52, lys2-801, pCu-LacI-GFP::HIS, Spc42-mCherry::URA3, Dam1-TetR::KanMX, ctf13-30 (ts at 37°C)*
EKY185	*Mat a, ade2-1, leu2-3, his3Δ200, ura3-52, lys2-801, pCu-LacI-GFP::HIS, Spc42-mCherry::URA3, cep3-1 (ts at 37°C)*
EKY186	*Mat a, leu2-3, his3Δ200, ura3-52, pCu-LacI-GFP::HIS, Spc42-mCherry::URA3, Dam1-TetR::KanMX, cep3-1 (ts at 37°C)*
EKY187	*Mat a, ade2-1, leu2-3, his3Δ200, ura3-52, lys2-801, pCu-LacI-GFP::HIS, Spc42-mCherry::URA3, Ask1-TetR::KanMX, cep3-1 (ts at 37°C)*
EKY188	*Mat a, ade2-1, leu2-3, his3Δ200, ura3-52, lys2-801, pCu-LacI-GFP::HIS, Spc42-mCherry::URA3, cse4-1 (ts at 37°C)*
EKY190	*Mat a, ade2-1, leu2-3, his3Δ200, ura3-52, pCu-LacI-GFP::HIS, Spc42-mCherry::URA3, Ask1-TetR::KanMX, cse4-1 (ts at 37°C)*
EKY191	*Mat α, leu2-3, his3Δ200, ura3-52, lys2-801, pCu-LacI-GFP::HIS, Spc42-mCherry::URA3, mif2-3 (ts at 37°C)*
EKY193	*Mat a, leu2-3, his3Δ200, ura3-52, pCu-LacI-GFP::HIS, Spc42-mCherry::URA3, Ask1-TetR::KanMX, mif2-3 (ts at 37°C)*
EKY194	*Mat a, leu2-3, his3Δ200, ura3-52, lys2-801, pCu-LacI-GFP::HIS, Spc42-mCherry::URA3, mtw1-1 (ts at 37°C)*
EKY196	*Mat a, leu2-3, his3Δ200, ura3-52, pCu-LacI-GFP::HIS, Spc42-mCherry::URA3, Ask1-TetR::KanMX, mtw1-1 (ts at 37°C)*
EKY200	*Mat α, leu2-3, his3Δ200, ura3-52, lys2-801, pCu-LacI-GFP::HIS, Spc42-mCherry::URA3, dad1-1 (ts at 37°C)*
EKY201	*Mat a, leu2-3, his3Δ200, ura3-52, lys2-801, pCu-LacI-GFP::HIS, Spc42-mCherry::URA3, Ask1-TetR::KanMX, dad1-1 (ts at 37°C)*
EKY203	*Mat a, leu2-3, his3Δ200, ura3-52, lys2-801, pCu-LacI-GFP::HIS, Spc42-mCherry::URA3, ipl1-2 (ts at 34°C)*

EKY205	*Mat a, leu2-3, his3Δ200, ura3-52, lys2-801, pCu-LacI-GFP::HIS, Spc42-mCherry::URA3, Ask1-TetR::KanMX ipl1-2 (ts at 34°C)*
EKY206	*Mat a, leu2-3, his3Δ200, ura3-52, lys2-801, pCu-LacI-GFP::HIS, Spc42-mCherry::URA3, sli15-3 (ts at 37°C)*
EKY208	*Mat a, leu2-3, his3Δ200, ura3-52, pCu-LacI-GFP::HIS, Spc42-mCherry::URA3, Ask1-TetR::KanMX, sli15-3 (ts at 37°C)*
EKY209	*Mat a, ade2-1, leu2-3, his3Δ200, ura3-52, Spc34-TetR::KanMX*
EKY210	*Mat a, ade2-1, leu2-3, his3Δ200, ura3-52, Duo1-TetR::KanMX*
EKY211	*Mat a, ade2-1, leu2-3, his3Δ200, ura3-52, Spc19-TetR::KanMX*
EKY212	*Mat a, ade2-1, leu2-3, his3Δ200, ura3-52, Dad2-TetR::KanMX*
EKY213	*Mat a, ade2-1, leu2-3, his3Δ200, ura3-52, Dad3-TetR::KanMX*
EKY214	*Mat a, ade2-1, leu2-3, his3Δ200, ura3-52, Dad1-TetR::KanMX*
EKY215	*Mat a, ade2-1, leu2-3, his3Δ200, ura3-52, Dad4-TetR::KanMX*
EKY216	*Mat a, ade2-1, leu2-3, his3Δ200, ura3-52, Hsk3-TetR::KanMX*
EKY220	*Mat a, leu2-3, his3Δ200, ura3-52, lys2-801, pCu-LacI-GFP::HIS, mCherry-Tubulin::URA3, cdc20::TRP1::GAL1/10-CDC20*
EKY221	*Mat a, leu2-3, his3Δ200, ura3-52, lys2-801, pCu-LacI-GFP::HIS, mCherry-Tubulin::URA3, cdc20::TRP1::GAL1/10-CDC20, Ask1-TetR::KanMX*
EKY70	*Mat a, ade2-1, leu2-3, his3Δ200, ura3-52, Ask1-TetR::KanMX*
EKY77	*Mat a, ade2-1, leu2-3, his3Δ200, ura3-52, Dam1-TetR::KanMX*
SWY276	*Mat a, ade2-1, leu2-3, his3Δ200, ura3-52, Dad1-S-tag-TEV-ZZ::HIS3, Ask1-TetR::KanMX*
SWY277	*Mat a, ade2-1, leu2-3, his3Δ200, ura3-52, Dad1-S-tag-TEV-ZZ::HIS3*

5.1.2 Plasmids

Name	*Description*	*Cloning*
pRS313	wt minichromosome for plating assays; CEN6/ArsH4	
pEK2	acentric minichromosome for plating assays	ArsH4 was amplified from pRS313 and cloned into Xho1 site of pRS303
pEK3	10x*tetO*$_2$ minichromosome for plating assays	10x*tetO*$_2$ was cloned into BamH1 site of pEK2
pEK4	112x*tetO*$_2$ minichromosome for plating assays	ArsH4 was amplified from pRS313 and cloned into BamH1 site of 112x*tetO*$_2$ plasmid based on pRS306
pEK6	wt minichromosome for visualisation	CEN6/ArsH4 was amplified from pRS313 and cloned into Xho1 site of pAFS59
pEK9	acentric minichromosome for visualisation	ArsH4 was cloned into Xho1 site of pAFS59
pEK10	*tetO* minichromosome for visualisation	10x*tetO*$_2$ was cloned into BamH1 site of pEK9
pEK17	PCR template for C-terminal linker-TetR tagging	linker + TetR was cloned into Pac1/Asc1 site of pFA6a-GFP(S65T)-KanMX

5.1.3 Bacterial strain

DH5α (chemically competent *E.coli* cells): fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17

5.1.4 Media

All recipes for yeast media are indicated here for 1l total volume. After dissolving the ingredients in ddH_2O, media were autoclaved and sugar was added afterwards. Depending on the strain used for the experiment, glucose or raffinose and galactose were added. All sugar solutions were provided with a concentration of 20% and were diluted 1:10 with the medium (YEP or minimal medium).

YEP: 10g yeast extract, 20g bacto-peptone

minimal medium: 80g yeast nitrogen base w/o amino acids

drop out stock solutions (100x):

doLEU: 2g adenine sulphate, 2g uracil, 2g l-tryptophan, 2g l-histidine, 3g l-lysine

doHIS: 2g adenine sulphate, 2g uracil, 2g l-tryptophan, 3g l-leucine, 3g l-lysine

doURA: 2g adenine sulphate, 2g l-tryptophan, 2g l-histidine, 3g l-leucine, 3g l-lysine

keep at RT, wrapped in foil

sporulation medium: 1% potassium acetate, 0.1% bacto yeast extract, 0.05% dextrose, freshly added SC 1:300

SC (synthetic complete): 0.2g adenine sulphate, 0.2g uracil, 0.2g l-tryptophan, 0.2g l-histidine, 0.3g l-leucine, 0.3g l-lysine

For pouring plates 22g bacto-agar was added to 1l medium.

Geneticin (G418): 50mg/ml in ddH_2O, 250µl of stock spread on YPD plate

LB medium (1l): 10g tryptone, 5g yeast extract, 10g NaCl, adjusted to pH 7.0 with NaOH

2xTY (1l): 16g tryptone, 10g yeast extract, 5g NaCl, adjusted to pH 7.0 with NaOH

Ampicillin: 50mg/ml in H_2O, stored at –20°C, diluted 1:1000

SOC medium from Invitrogen

5.2 Biochemistry

5.2.1 SDS-PAGE

For electrophoretic separation of proteins according to their molecular mass, SDS-PAGE was performed. Pouring of gels and electrophoresis were carried out with a Biorad Protean Mini Cell system. Samples were mixed with 4xSDS loading buffer and boiled for 5min before loading on the gel. As a marker Precision Plus Protein Dual Coloured Protein marker from Biorad was used.

Gel recipes (for 2 small gels) and buffers:

separating gel	10%	12,5%
dH_2O	4.1ml	3.24ml
30% Acrylamide/Bis (Biorad)	3.3ml	4.16
4x Separating buffer	2.5ml	2.5ml
10% APS	80µl	60µl
TEMED	8µl	8µl

stacking gel	
dH_2O	2.85ml
30% Acrylamide/Bis (Biorad)	0.85ml
4x Stacking buffer	1.25ml
10% APS	40µl
TEMED	4.5µl

4x Separating buffer: 1.5M Tris pH 8.5, 0.4% SDS

4x Stacking buffer: 0.5M Tris pH 6.8, 0.4% SDS

4x SDS Sample buffer: 250mM Tris pH6.8, 40% (v/v) glycerol, 8% (w/v) SDS, 0.04mg/ml bromphenol blue; 80µl β-ME added to 1ml 4x buffer right before use

1x Running buffer: 25mM TRIS pH8.5, 52mM glycine, 0.05% (w/v) SDS

For some experiments electrophoresis was carried out using the Invitrogen NuPAGE system with Novex Tris-Acetate Mini Gels (4-12% gradient gels), according to the supplier's protocol.

5.2.2 Whole cell extracts from yeast for SDS-PAGE

Two OD from an O/N culture were pelleted at 2500rpm for 3min and washed once with water. The pellet was resuspended in 60µl 4xSDS sample buffer and boiled for 3min at 95°C. Acid-washed glass beads were added and the sample was vortexed for 2min. 60µl of 2xSDS sample buffer were added and the sample was boiled again for 2min. After

spinning down, the supernatant could be transferred into a fresh tube and was ready to be loaded on a SDS gel.

5.2.3 Silver staining of protein gels

All solutions were prepared with ddH_2O. The SDS gel was incubated for 30min at RT in 30% (v/v) EtOH, 10% (v/v) acetic acid for fixation. For sensitising, the gel was incubated for 30min at RT in 30% EtOH, 0.1% (w/v) sodium thiosulfate, 0.1M sodium acetate pH6.8. Afterwards the gel was washed in water 3 times for 10min. To stain the proteins, the gel was incubated in 0.1% (w/v) silvernitrate, 0.1% (v/v) formaldehyde, so silver ions can bind to the side chains of amino acids. After a quick water wash the gel was developed in 2.5% (w/v) sodium carbonate, 0.2% (v/v) formaldehyde. The protein bands were becoming visible by the reduction of silver ions at basic pH. To stop the staining reaction the gel was put into 1% (v/v) acetic acid. The gels were dried between two sheets of cellophane.

5.2.4 Western blotting

After separation by SDS-PAGE the proteins were transferred onto a nitrocellulose membrane (GE Healthcare) in a wet blotting apparatus. The blot was conducted in the cold room at 100V for 2 hours. To avoid unspecific binding of the antibody to the membrane, it was blocked in 5% (w/v) milk in TBST. Usually the membrane was incubated with the first antibody diluted in 5% milk TBST O/N at 4°C, with gentle shaking. After washing the membrane 3 times for 5min with TBST, it was incubated with the secondary antibody, coupled to HRP, for 1hour at RT. After that the membrane was washed again 3 times 5min with TBST. Protein bands could be visualised by adding ECL-solution (GE Healthcare) to the membrane and detect the emitted light produced by the HRP with a Hyperfilm.

If incubation with another antibody was needed, the membrane was incubated with stripping buffer (Thermo scientific) for 20min at RT, re-blocked and new antibodies were bound.

Transfer buffer:	25mM Tris, 190mM Glycin, 20% (v/v) MeOH, 0.02% (w/v) SDS
TBS:	10mM Tris, 150mM NaCl, pH7.6
TBST:	TBS + 0.1% (v/v) Tween20

5.2.5 TAP tag purification

Overnight cultures of cells were grown in YPD (4x100ml) and diluted to an OD_{600} of 0.15 (6x2l). Cells were grown at 30°C to an OD_{600} ~1.15 and harvested by centrifugation at 5000rpm for 10min in an SLC-6000 rotor. The pellet was resuspended in 10ml water and transferred into a 50ml Falcon tube. After centrifugation at 4000rpm for 10min (Heraeus Biofuge Premium) the pellet was resuspended in 0.2vol water to drop freeze the yeast in liquid nitrogen. Beads were stored at –80°C.

To lyse the cells, frozen beads were grinded in a Warring Blender with liquid nitrogen. 30g of yeast powder were weighed out and after adding 30ml 2xHyman Buffer it was quickly thawed in a warm water bath. From this point on the samples were kept on ice. To avoid protein degradation Protease Inhibitor Cocktail Set IV (Calbiochem) was added at a 1:100 dilution, PMSF was added to a final concentration of 1mM and Triton X-100 to 1%. This lysate was sonicated in Corex glass tubes for 30sec and afterwards centrifuged in a SS-34 rotor (Sorvall) at 10000rpm for 25min at 4°C. The lysate was pelleted again by ultracentrifugation in a Ti70 rotor at 45000rpm for 30min at 4°C. This highspeed-supernatant (HSS) was passed over a CL-6B sepharose column (15ml 1:1 slurry), pre-equilibrated with 1xHyman Buffer. In this step the same base matrix was used as for the IgG sepharose in the next step, in order to pre-clean the HSS.

1.2ml 1:1 slurry of IgG Sepharose were collected in a Biorad disposable column and washed with 10ml TST, 1ml 0.5M NH_4OAc pH3.4, 5ml TST, 1ml 0.5M NH_4OAc pH3.4 and 5ml 1xHyman Buffer 400mM KCl. Washed IgG Sepharose was added to the HSS and gently rotated at 4°C for 3 hours.

After the incubation time the beads were collected in a Biorad disposable column (flow through was discarded) and washed with 15ml 1xHyman Buffer 400mM KCl and 25ml 1xHyman Buffer 400mM KCl + 1mM DTT + 0.1% Tween20. The beads were resuspended in a total volume of 2ml with 1xHyman Buffer 400mM KCl + 1mM DTT + 0.1% Tween20 and transferred to a 2ml Eppendorf tube. 40µl TEV protease were added and the solution was rocked O/N at 4°C.

The next day beads were spun down and the supernatant was collected in a new tube. The beads were washed once with 600µl 1xHyman Buffer 400mM KCl + 1mM DTT + 0.1% Tween20. 100µl 1:1 slurry of Protein S Agarose beads – washed 3 times with 1xHyman Buffer 400mM KCl + 1mM DTT + 0.1% Tween20 (in tube) – were added. The HSS was rocked with the Protein S beads for 3 hours at 4°C.

After the incubation the beads were collected on a Biorad disposable column and washed with ~5ml 1xHyman Buffer 400mM KCl + 1mM DTT + 0.1% Tween20. The beads were transferred into a fresh tube. Proteins were eluted with glycine by resuspending the beads in 50µl 10mM glycine pH2.0 and shaking them gently at RT for 30min. After spinning down, the eluate was taken off and the elution procedure was repeated two more times. Before loading on a SDS gel, the pH had to be adjusted with 1 M Tris pH 8.0.

Buffers for TAP:

1xHyman Buffer	
50mM	Bis-Tris Propane pH 7.0 - HCl
100mM	KCl
5mM	EGTA
5mM	EDTA
10%	Glycerol
40mM	β-Glycerolphosphate (add freshly)

TST	
50mM	Tris-HCl pH 7.4
150mM	NaCl
0.1%	Tween20

5.2.6 Purification of minichromosomes

For purification of the wildtype- or the *tetO*-minichromosome O/N cultures (100ml) were grown at 25°C in minimal doLEU medium. The cells were diluted in 1l YPD medium to an OD_{600} of 0.2 and incubated at 30°C. After induction of LacI-GFP by adding $CuSO_4$ to a final concentration of 250µM for 15min/7min, cells were harvested at an OD_{600} of ~0.9 by centrifugation at 5000rpm for 5min in a SLC-6000 rotor. Cells were washed twice with ice cold water and transferred to a 50ml Falcon tube. The pellet was resuspended in 50ml spheroblasting buffer and incubated at RT for 15min. The cells were harvested by centrifugation at 5000rpm for 4min at 4°C (SS34 rotor). The pellet was washed with ice cold water and resuspended in 50ml cell wall digestion buffer. After incubation for 30min at 30°C, spheroblast formation was checked under the microscope. Spheroblasts were centrifuged at 6000rpm for 6min at 4°C (SS34 rotor) and washed with 1M cold sorbitol. The pellet was resuspended carefully in 2ml 0.4M cold sorbitol and transferred to a 50ml Falcon tube. 8ml of lysis buffer were added and an incubation on ice for 30min followed. The lysate was further homogenized by stroking 3 to 5 times with a glass douncer and a motor driven Teflon pestle. To clarify the lysate it was centrifuged for 15min at 10000rpm (SS34 rotor).

Aliquots taken during the procedure were purified by mixing 1:1 with PIC (phenol:chloroform:isoamylalcohol 25:24:1) and centrifugation for 5min at maximum speed. The aqueous phase on top was transferred into a new tube. This procedure was repeated once. To precipitate DNA, 2vol 95% EtOH were added and the samples were centrifuged for 10min at 13000rpm. The pellet was washed 2 times with 70% EtOH and resuspended in ~200µl TE. In order to get a cleaner DNA solution, the samples were purified with a Qiagen nucleotide removal kit.

Buffers for minichromosome purification:

Spheroblasting buffer	
0.1M	Tris-Base pH9.2
10mM	DTT

Cell wall digestion buffer	
50mM	Tris-HCl pH 7.65
1M	sorbitol
1mM	$CaCl_2$
1mM	$MgCl_2$
20µg/ml	lyticase

Lysis buffer	
25mM	HEPES/NaOH
50mM	KCl
10mM	$MgSO_4$
10mM	sodium citrate
25mM	sodium sulfite
0.25%	TritonX-100
1mM	PMSF
3mM	DTT
0.1mg/ml	RnaseA

5.3 Molecular biology techniques

5.3.1 DNA Gel-electrophoresis

To dissolve 1% (w/v) agarose in TAE buffer, the solution was boiled in the microwave oven. For subsequent staining of the DNA ethidium bromide was added to a final concentration of 0.5µg/ml (10mg/ml stock solution) and the gel was poured. DNA was mixed with 6xloading buffer (Fermentas) and loaded into the pockets of the gel. Electrophoresis was carried out at 100V in 1xTAE. Ethidium bromide is intercalating with DNA and therefore DNA bands could be detected under UV light.

If necessary, gel bands were cut out to purify DNA with a Qiagen kit according to the supplier's protocol.

1xTAE buffer: 40mM Tris pH 8.0, 1mM EDTA, 20mM acetic acid

5.3.2 Cloning

Insert (PCR product) and vector were cut with the appropriate restriction enzymes. Procedures were performed as recommended in the particular supplier's protocol. Often fast digest enzymes from Fermentas were used. In order to avoid religation of the vector, it was treated with calf intestine phosphatase (Roche). Vector and insert were purified with a QIAGEN nucleotide removal kit. Ligations were performed with a vector to insert ratio of ~1:3 with HC T4 ligase (Invitrogen) at RT for 10min to 1h, or at 16°C O/N.

5.3.3 Bacterial transformation and plasmid amplification

For transformation either 2µl or 15µl ligation mix were added to 100µl competent DH5α cells. The cells were kept on ice for 30min and a heat shock was performed for 15sec at 42°C in the waterbath. For recovery 300µl SOC medium were added and the cells were incubated at 37°C for 1h. Afterwards they were spread on LB-Amp plates and incubated at 37°C. The plasmid (~2µl from miniprep) was transformed into competent DH5α cells as described above, and cells were incubated at 37°C. If plasmids with a bigger array (e.g. LacO) were amplified, cells were incubated at 30°C. Single colonies were inoculated in LB medium (O/N) or 2xTY medium (few hours). Miniprep of plasmid was performed with a Qiagen kit according to the supplier's protocol.

5.3.4 Transformation of yeast cells

The transformation protocol is based on the study from Schiestl & Gietz, 1989.
In the morning an O/N culture was diluted to an OD_{600} of ~0.1 in 50ml YPD medium and incubated shaking at 30°C in a waterbath. At an OD_{600} of 0.6-1.0 cells were harvested by centrifugation at 3000rpm for 3min. After washing the cells with ~15ml water, the pellet was resuspended in 1ml water and transferred to an Eppendorf tube. Cells were centrifuged at 3000rpm for 2min and resuspended in 1.5ml 1xTE, 0.1M LiAc in H_2O and kept on ice until further use.

Transformation mix	
20µl	ssDNA 2mg/ml (salmon sperm as carrier DNA)
3-5µl / 70µl	plasmid / PCR product
200µl	competent yeast cells
1.2ml	1xTE, 0.1M LiAc in 50% PEG

The transformation mix was kept at RT for 30min, gently rotating. Afterwards a heat shock was performed for 12min in a 42°C waterbath. The cells were spun down at 3000rpm for 30sec and resuspended in 150µl water, so that they could be plated on the appropriate medium.

5.3.5 Preparation of genomic DNA from yeast

1.5 OD of cells from an O/N culture in YPD were harvested by centrifugation at 3000rpm for 2.5min. After washing the cells once with water, they were resuspended in 0.5ml lysis buffer. To open up the cells ~0.6g of 0.5mm acid-washed glass beads were added and the suspension was vortexed 3 times for 1min each. Between the vortex steps, the lysate was kept on ice for 1min. Afterwards the lysate was incubated at 70°C for 10min. 200µl 5M KOAc and 150µl 5M NaCl were added and incubation on ice for 10min followed. To separate large cell debris and precipitated protein, the lysate was centrifuged at 13000rpm for 10min. The supernatant was transferred into a fresh tube and 2vol 95% EtOH were added to precipitate the DNA. The sample was centrifuged at 13000rpm for 15min. The DNA pellet was washed with 70% EtOH and carefully dried after centrifugation to be resuspended in 49µl TE and 1µl 10mg/ml RNaseA. After dissolving of the pellet 200µl of TE were added.

Lysis Buffer	
50mM	Tris-HCl pH 7.5
20mM	EDTA pH 8.0
1%	SDS
	in H_2O

5.3.6 PCR

To confirm genomic integrations PCR was performed using Bio-X-Act polymerase (bioline). The reaction mixture of 25µl volume contained 1µl DNA (genomic DNA prep), 2.5µl 10xbuffer, 2mM $MgCl_2$, 200µM dNTPs, 1µM each primer and 1U polymerase. Instead of genomic DNA colony PCRs were performed in some cases. A bit of a yeast colony was directly put into a PCR tube and heated in the microwave for 1min. The lysed yeast cells were then resuspended in the PCR-master mix.

For amplifications of inserts or PCR products for gene tagging, PCR was performed with Phusion polymerase (Finnzymes). A 50µl PCR mixture contained 1µl genomic DNA or

0.5µl plasmid DNA as a template, 10µl 5xBuffer, 0.5µM each primer, 200µM dNTPs and 1U Phusion polymerase.

Standard protocols for PCR:

Bio-X-Act polymerase	
98°C	5’
98°C	1’
52°C-58°C	1’
68°C	~1.5’/kb
68°C	5’
4°C	
	30 cycles

Phusion polymerase	
98°C	1’
98°C	20’’
52°C-58°C	20’’
72°C	max 30’’/kb
72°C	5’
4°C	
	32 cycles

5.3.7 Tagging strategy for C-terminal TetR fusions

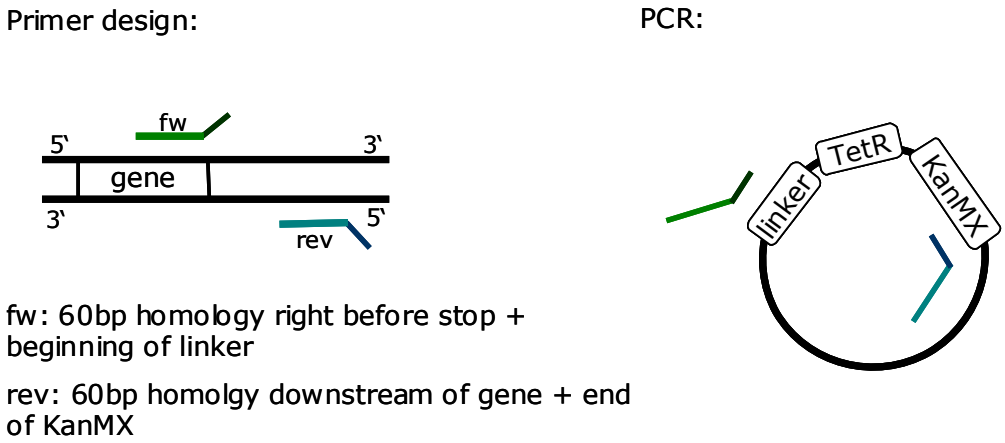


Figure 22: Primer design and PCR for generation of C-terminal TetR fusions. Fw primers were designed by linking 60bp homology of gene of interest right before the stop codon (without stop) and the start of the linker sequence from the plasmid. Rev primers were designed by linking 60bp ~100bp reverse complement homology sequence downstream of stop of gene of interest and the end of KanMX sequence from the plasmid (incl. stop, also reverse complement). This PCR product was able to integrate into the genome via homologous recombination between the genomic locus and the overhangs from the PCR product, which were homologous to this region.

5.4 Yeast assays and genetics

5.4.1 Spot assay

From an O/N culture in YPD a dilution series was made in a 96-well plate. The first dilution was adjusted to an OD_{600} of 0.4 and diluted further 1:10 until an OD_{600} of $4x10^{-4}$ was reached. With a pinning device ("Frogger"), fitting into the cavities of the 96-well plate, equal drops of the dilutions were spotted onto YPD plates. The plates were incubated at different temperatures to compare the growth of individual yeast strains under different conditions.

5.4.2 Plating assay

In this assay the stability of minichromosomes during mitotic divisions was determined. Cells carrying a minichromosome were grown in 5ml YPD, under non-selective conditions, at 30°C for 24 hours (equates to ~12 generations). In minimal medium + 2% glc an OD_{600} of 0.6 was adjusted and diluted further to an OD_{600} of $6x10^{-4}$. An 80µl aliquot of this cell suspension was spread onto YPD and drop-out plates, according to the genetic marker on the particular minichromosome. On YPD plates every cell could grow and form a colony, whereas on the drop-out plates, providing selective conditions, only cells still carrying the minichromosome were able to form a colony. The stability of the minichromosome resulted from the percentage difference between the colony numbers under selective and non-selective conditions. The plates were analysed in duplicates and the assay was performed three times for each strain.

5.4.3 Yeast crosses

In a first step two strains (one with mating type a, the other with alpha) to be crossed were streaked across each other on a non-selective plate. They were allowed to mate for 5 hours to O/N at 25°C. With a dissecting microscope zygotes, which could be recognized by their dumbbell shape, were picked and grown to single colonies.

In order to be able to separate single haploid spores, diploid cells have to sporulate. Therefore single diploid colonies were inoculated and grown O/N in appropriate non-

selective medium. From 1ml of this saturated culture cells were harvested, washed with water and resuspended in 5ml of sporulation medium + SC. In glass tubes, gently rotating, they were allowed to sporulate for approximately 4 days at 25°C. Tetrad formation was checked under the microscope.

As soon as tetrads were visible, they were dissected to isolate haploid spores. In order to lyse the cell wall, 250µl of sporulation culture were harvested and resuspended in 37,5µl 1mg/ml zymolase. For proper digestion the solution was incubated at 37°C for 3min. 200µl 20mM NaPi pH 7.2 were added to stop the reaction, and cells were kept on ice until use. Under the dissection microscope single spores from tetrads were separated, using a microneedle, and placed on a non-selective plate.

As soon as haploid colonies were growing on the plate, they were resuspended in minimal medium and spotted on different plates, for testing their ability to grow under certain conditions. In this way the marker-combination of every single spore from a tetrad could be analysed.

5.5 Microscopy

5.5.1 Microscope and settings

Microscopy and image acquisition was performed with a DeltaVision microscope (AppliedPrescision), equipped with a UplanSApo x100 objective lens (Olympus; NA 1.40), SoftWoRx software (AppliedPrescision) and a CoolSnap HQ CCD camera (Photometrics).

For microscopy on fixed cells images were acquired by recording 30-36 z sections, 0.2µm apart. After deconvolution (aggressive mode) and maximum intensity projection images were assembled using ImageJ.

Time-lapse movies from living cells were acquired by recording 5-7 z sections, 0.7µm apart, every 20 seconds for a total time of 20 minutes in case of metaphase arrested cells. For recovering cells after metaphase arrest pictures were taken every minute for 1 hour. After deconvolution (aggressive mode) and projection, images and movies were assembled with MetaMorph software.

5.5.2 Fixation of cells for imaging

An O/N culture of cells in minimal doLEU medium + 2% glucose was diluted ~1:5 and cells were incubated for ~2hours at 25°C. Then they were shifted to the restrictive temperature (37°C) for 1hour (2hours for *sli15-3* and *ipl1-2* strains). $CuSO_4$ was added to the cells to a final concentration of 250µM in order to induce expression of LacI-GFP, which can bind to the minichromosomes and thereby makes them visible. Induction was conducted for 15min to 18min. Cells from 500µl to 1ml of culture were harvested and fixed by resuspending them in 100µl 4% PFA. After 5min incubation at RT PFA was removed, cells were washed with KPO_4/sorbitol and resuspended in 50µl to 100µl KPO_4/sorbitol. Fixed cells could be stored at the fridge for up to one month.
Coverslips were prepared as described for live cell imaging. Instead of mounting the coverslip with vacuum grease, nail polish was used in case of fixed cells.

4% PFA:	0.4g paraformaldehyde dissolved in less than 10ml H_2O, few drops of NaOH added to dissolve it completely, 0.34g sucrose added, filled up to 10ml
KPO_4/sorbitol:	10ml 1M KPO_4 pH7.5, 60ml 2M sorbitol, 30ml H_2O

5.5.3 Live cell imaging

For investigating the behaviour of minichromosomes in metaphase, cells were inoculated in minimal doLEU medium + 2% raff/gal and grown O/N at 25°C. Depending on the density of the culture 500µl to 1ml were harvested and washed twice with water. They were resuspended in 5ml of minimal doLEU medium + 2% glc and incubated for 2hours at 25°C, gently rotating. Due to the glucose containing medium expression of Cdc20p was repressed and cells were arrested in metaphase (due to the relative short time of medium switch not all of the cells were completely arrested). The minichromosomes were visualized by bound LacI-GFP. Its expression was induced by adding $CuSO_4$ to a final concentration of 250µM for 15min to the cells. In the meantime coverslips were prepared by placing a 50µl drop of ConA solution (1mg/ml stock diluted 1:10) in the center and incubating it for 10min. After washing the coverslip with water or medium, 50µl of cell suspension was placed on the center and allowed to adhere for 10 to 15 min. The coverslip was washed carefully with medium (w/o $CuSO_4$) to remove unattached cells. In case of

recovery experiments, cells were washed with medium containing 2% raff/gal. With vacuum grease a square in the size of the coverslip was marked on a glass slide. With foreceps the coverslip was carefully placed upside down on the glass slide, so that the vacuum grease was sealing the edges. The coverslip was then carefully pressed on the slide and extra liquid was removed with a tissue. After this cells were ready for imaging.

6 References

Asbury, C.L. et al., 2006. The Dam1 kinetochore complex harnesses microtubule dynamics to produce force and movement. *Proceedings of the National Academy of Sciences of the United States of America*, 103(26), 9873-9878.

Cheeseman, I.M. et al., 2001. Implication of a novel multiprotein Dam1p complex in outer kinetochore function. *The Journal of Cell Biology*, 155(7), 1137-1145.

Cheeseman, I.M. et al., 2002. Phospho-regulation of kinetochore-microtubule attachments by the Aurora kinase Ipl1p. *Cell*, 111(2), 163-172.

Cheeseman, I.M. & Desai, A., 2008. Molecular architecture of the kinetochore-microtubule interface. *Nature Reviews. Molecular Cell Biology*, 9(1), 33-46.

Clarke, L. & Carbon, J., 1980. Isolation of a yeast centromere and construction of functional small circular chromosomes. *Nature*, 287(5782), 504-509.

Cohen, R.L. et al., 2008. Structural and functional dissection of Mif2p, a conserved DNA-binding kinetochore protein. *Molecular Biology of the Cell*, 19(10), 4480-4491.

Espelin, C.W. et al., 2003. Binding of the essential Saccharomyces cerevisiae kinetochore protein Ndc10p to CDEII. *Molecular Biology of the Cell*, 14(11), 4557-4568.

Fernius, J. & Marston, A.L., 2009. Establishment of cohesion at the pericentromere by the Ctf19 kinetochore subcomplex and the replication fork-associated factor, Csm3. *PLoS Genetics*, 5(9), e1000629.

Furuyama, T. & Henikoff, S., 2009. Centromeric nucleosomes induce positive DNA supercoils. *Cell*, 138(1), 104-113.

Grishchuk, E.L. et al., 2008. The Dam1 ring binds microtubules strongly enough to be a processive as well as energy-efficient coupler for chromosome motion. *Proceedings of the National Academy of Sciences of the United States of America*, 105(40), 15423-15428.

Ivanov, D. & Nasmyth, K., 2005. A topological interaction between cohesin rings and a circular minichromosome. *Cell*, 122(6), 849-860.

Joglekar, A.P., Bloom, K. & Salmon, E.D., 2009. In vivo protein architecture of the eukaryotic kinetochore with nanometer scale accuracy. *Current Biology: CB*, 19(8), 694-699.

Joglekar, A.P. et al., 2006. Molecular architecture of a kinetochore-microtubule attachment site. *Nature Cell Biology*, 8(6), 581-585.

Joglekar, A.P. & DeLuca, J.G., 2009. Chromosome segregation: Ndc80 can carry the load. *Current Biology: CB*, 19(10), R404-407.

Kelly, A.E. & Funabiki, H., 2009. Correcting aberrant kinetochore microtubule attachments: an Aurora B-centric view. *Current Opinion in Cell Biology*, 21(1), 51-58.

Kiermaier, E. et al., 2009. A Dam1-based artificial kinetochore is sufficient to promote chromosome segregation in budding yeast. *Nature Cell Biology*, 11(9), 1109-1115.

Kitagawa, K. & Hieter, P., 2001. Evolutionary conservation between budding yeast and human kinetochores. *Nature Reviews. Molecular Cell Biology*, 2(9), 678-687.

Koshland, D., Kent, J.C. & Hartwell, L.H., 1985. Genetic analysis of the mitotic transmission of minichromosomes. *Cell*, 40(2), 393-403.

Lacefield, S., Lau, D.T.C. & Murray, A.W., 2009. Recruiting a microtubule-binding complex to DNA directs chromosome segregation in budding yeast. *Nature Cell Biology*, 11(9), 1116-1120.

Li, Y. et al., 2002. The mitotic spindle is required for loading of the DASH complex onto the kinetochore. *Genes & Development*, 16(2), 183-197.

Liu, D. et al., 2009. Sensing chromosome bi-orientation by spatial separation of aurora B kinase from kinetochore substrates. *Science (New York, N.Y.)*, 323(5919), 1350-1353.

Maine, G.T., Sinha, P. & Tye, B.K., 1984. Mutants of S. cerevisiae defective in the maintenance of minichromosomes. *Genetics*, 106(3), 365-385.

McAinsh, A.D., Tytell, J.D. & Sorger, P.K., 2003. Structure, function, and regulation of budding yeast kinetochores. *Annual Review of Cell and Developmental Biology*, 19, 519-539.

Morgan, D.O., 2007. *The cell cycle*, New Science Press.

Musacchio, A. & Salmon, E.D., 2007. The spindle-assembly checkpoint in space and time. *Nature Reviews. Molecular Cell Biology*, 8(5), 379-393.

Onn, I. et al., 2008. Sister chromatid cohesion: a simple concept with a complex reality. *Annual Review of Cell and Developmental Biology*, 24, 105-129.

Peters, J., Tedeschi, A. & Schmitz, J., 2008. The cohesin complex and its roles in chromosome biology. *Genes & Development*, 22(22), 3089-3114.

Powers, A.F. et al., 2009. The Ndc80 kinetochore complex forms load-bearing attachments to dynamic microtubule tips via biased diffusion. *Cell*, 136(5), 865-875.

Santaguida, S. & Musacchio, A., 2009. The life and miracles of kinetochores. *The EMBO Journal*. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19629042 [Accessed August 21, 2009].

Schiestl, R.H. & Gietz, R.D., 1989. High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Current Genetics*, 16(5-6), 339-346.

Schittenhelm, R.B., Chaleckis, R. & Lehner, C.F., 2009. Intrakinetochore localization and essential functional domains of Drosophila Spc105. *The EMBO Journal*, 28(16), 2374-2386.

Straight, A.F. et al., 1997. Mitosis in living budding yeast: anaphase A but no metaphase plate. *Science (New York, N.Y.)*, 277(5325), 574-578.

Tanaka, K. et al., 2005. Molecular mechanisms of kinetochore capture by spindle microtubules. *Nature*, 434(7036), 987-994.

Tanaka, T. et al., 2000. Cohesin ensures bipolar attachment of microtubules to sister centromeres and resists their precocious separation. *Nat Cell Biol*, 2(8), 492–499.

Tanaka, T.U. & Desai, A., 2008. Kinetochore-microtubule interactions: the means to the end. *Current Opinion in Cell Biology*, 20(1), 53-63.

Tanaka, T.U., Stark, M.J.R. & Tanaka, K., 2005. Kinetochore capture and bi-orientation on the mitotic spindle. *Nature Reviews. Molecular Cell Biology*, 6(12), 929-942.

Wang, H. et al., 2007. Architecture of the Dam1 kinetochore ring complex and implications for microtubule-driven assembly and force-coupling mechanisms. *Nature Structural & Molecular Biology*, 14(8), 721-726.

Welburn, J.P.I. et al., 2009. The human kinetochore Ska1 complex facilitates microtubule depolymerization-coupled motility. *Developmental Cell*, 16(3), 374-385.

Westermann, S. et al., 2005. Formation of a dynamic kinetochore- microtubule interface through assembly of the Dam1 ring complex. *Molecular Cell*, 17(2), 277-290.

Westermann, S., Drubin, D.G. & Barnes, G., 2007. Structures and functions of yeast kinetochore complexes. *Annual Review of Biochemistry*, 76, 563-591.

Westermann, S. et al., 2006. The Dam1 kinetochore ring complex moves processively on depolymerizing microtubule ends. *Nature*, 440(7083), 565-569.

Acknowledgements

I would like to thank all those people who accompanied me with their constant support during my studies and the work on this project.
First of all I am really grateful to Evi for supervising my work in the lab and being there with words and deeds whenever I needed help. With endless patience for all my problems she was guiding me through this year. Many thanks to Stefan for giving me the opportunity to work in his lab on this interesting project, for scientific advisory and the hard work of correcting my thesis. Due to a great working atmosphere I really enjoyed this year in the lab. Therefor many thanks to Fabi, Chrissi, Kathi, Tom and Peter. All of them where supporting me and sharing their experience and knowledge with me. Besides from technical assistance and scientific discussions I would also like to thank for the “off-topic” discussions. My colleagues became wonderful friends to me.

Many thanks to the girls Doris, Sandra, Johanna, Ivi, Janja, Barbara and Eva for hours of chatting, laughing, eating, drinking coffee, partying, having fun, discussing the past and looking ahead…

I am so grateful for all the help I got from my family. The constant support and love from my parents, my sister and my grandparents, is the basement for everything I have achieved so far. They are there for me, whenever I need them.

I want to thank Thomas for his endless patience, especially during the last weeks, for sharing my sorrows and happiness, for being there for me and for his unremitting love. Thank you for being my best friend.

Curriculum Vitae

Family name	Wöhrer
First name	Sophie
Date and place of birth	19.06.1986, Mödling
Nationality	Austria

Academic career:

10/2004 – 10/2009	Studies of Molecular Biology University of Vienna, Austria
07-08/2008	International Biology Undergraduate Summer School BUSS2008 University of Zurich, ETH Zurich, Switzerland
07/2006	CEEPUS Biomedicine Student's Council, one week Summer University Zadar, Croatia
09/1996 – 06/2004	Real- und Aufbaugymnasium Don Bosco Gymnasium, Unterwaltersdorf, Austria

Publications:

Eva Kiermaier, **Sophie Woehrer**, Yutian Peng, Karl Mechtler and Stefan Westermann. A Dam1-based artificial kinetochore is sufficient to promote chromosome segregation in budding yeast. *Nature Cell Biology*, 11(9), 1109-1115 (2009).