Origin of chromatin anaphase bridges

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Abstract

Successful chromosome segregation is crucial for the survival of a cell and to avoid diseases such as cancer. Anaphase bridges are a type of segregation defect that can arises from structurally compromised chromosomes. Little is known about the mechanisms that normally prevent them. In this study I screened for genes that normally prevent anaphase bridges in order to learn more about their origin. I found anaphase bridges to arise in replication mutants and it is possible to trigger these bridges by exposing cells to replication stress. Thus, impaired replication is one cause for anaphase bridges. Further I identified a role for the mitotic exit network (MEN) in chromosome segregation. MEN mutants display anaphase bridges and I present evidence that these bridges arise from telomeric regions and may involve un-replicated DNA.

Resum

La correcta segregació dels cromosomes és esencial per la supervivencia de la cèl·lula i per evitar l'aparició de certes malalties com el càncer. Els ponts anafàsics són un tipus d'error de segregació que pot ser originat per defectes estructurals dels cromosomes. Es coneix molt poc sobre els mecanismes que eviten la formació d'aquests ponts anafàsics. En aquest estudi he fet un análisis global dels diferents gens que normalment eviten la formació d'aquests ponts, per abançar en la comprensió del seu origen. He vist que el ponts anaphasics es formen en mutants que tenen afectat el proces de replicació i que és posible de provocar la formació d'aquests ponts exposant les cèl·lules a estrés replicatiu. Per tant, els problemes en la replicació són una de les causes dels ponts d'anafase. He identificat el rol de "mitotic exit network (MEN)" en la segregació cromosómica. Els mutants per MEN formen ponts anafàsics i mostren evidències que aquests ponts probenen de regions telomèriques i podrien incloure DNA no replicat.

Inhalt

1. Introduction

The maintenance of genomic stability through cell division depends on the high fidelity of chromosome segregation. This process, although under extensive investigation, is still incompletely understood. The gaps in our understanding are owed to the complexity of the process and likely the incomplete list of factors involved. This work aims to extend this list, to find novel factors involved in the assembly of a mitotic chromosome, and to add to a more complete global picture of the events needed to bring chromosome segregation to fruition. To this end we used budding yeast as a model, exploiting its amenability to high throughput genetic manipulation and imaging. We performed a screen for mutants that display anaphase bridges. We found a novel role for the mitotic exit network (MEN) in chromosome segregation. Further genetic analysis suggested that the MEN is required for the segregation of telomere-proximal regions, through a process that may involve DNA replication. This work therefore describes an unexpected regulatory step for chromosome segregation by a late anaphase signalling cascade. These findings are interesting in light of the emerging novel mitotic roles of the MEN and its animal homologues of the Hippo Pathway.

1.1 Mitotic chromosome segregation

Chromosome segregation is one of the most fundamental processes of all domains of life[1-4]. It is the means by which a cell physically partitions two genomes, which it has previously generated by DNA replication, to two different locations in the cell. This gives rise to two complete daughter genomes, destined for the daughter cells. A failure to segregate chromosomes correctly can lead to aneuploidy or chromosome damage. Damaged chromosomes can undergo mutagenesis from which cancer-causing genomic lesions can emerge. Aneuploidy leads to altered gene dosage, which begets genomic instability, but also serves as a pathway for rapid adaption to mutation [5-16].

Understanding the causes of chromosome mis-segregation is of tremendous importance for the understanding of both, evolutionary processes and the etiology of diseases.

1.2 Eukaryotic chromosome segregation in the context of the cell cycle

1.2.1 The cell cycle

Chromosome segregation in eukaryotes takes place within the larger framework of the cell cycle. The cell cycle is a series of events that leads to the duplication of a cell. This series of events is grouped into the four distinct phases G1-, S-, G2- and M-phase, traversed in this order and resulting, after M-phase, in the generation of two daughter cells. S-phase is defined as the time of DNA replication. M-phase encompasses both chromosome segregation in mitosis and cell division by cytokinesis. S and M phases are separated by the gap-phases G1 and G2 which provide time for cell growth[17]. Budding yeast constitutes an exception to this general description. Its mitotic spindle is assembled before the completion of DNA replication but mitosis does not progress further until S-phase has completed[18].

Unidirectional progression through the phases of the cell cycle is enforced by the cell cycle control machinery and the correct execution of individual sub-processes is monitored by control mechanisms termed cell cycle checkpoints[19, 20].

The transition from G1 into S-phase and from G2 into M-phase are stages at which well-known checkpoints act to ensure the transition only happens when cells are ready to undertake the next phase. The transition from G1 to S phase is termed START in budding yeast or the restriction point in animal cells. START is thought to ensure sufficient cell size before entry into the cycle[21, 22]. It has been suggested to subdivide

the definition of the restriction point as it is composed of two temporally separable events, one dependent on the presence of sufficient nutrients, in analogy to START, the other dependent on growth factors[23-25]. Another well known checkpoint, the DNA damage checkpoint opposes entry into S-phase and M-phase in the presence of DNA lesions[26, 27].

During S phase, the genome of a cell, encoded in its DNA, undergoes semi-conservative replication. The duplex DNA of the double helix melts and the two single strands are used as template onto which two new strands of DNA are synthesized. This generates two copies of the original genome, each composed of one strand of the old genome and one newly synthesized strand[28]. The progression of DNA synthesis is monitored by the replication checkpoint which is able to sense the perturbation of DNA replication[29, 30].

M-phase begins with mitosis. Mitosis was described originally in animal cells as the cytological discernible sequence of events leading up to and including chromatin partitioning[31]. During mitosis the replicated genome is packaged into compact bodies, the mitotic chromosomes. The mitotic chromosomes each contain two identical sister chromatids which are the product of DNA replication. These are bioriented on the bipolar mitotic spindle, the poles of which will define the approximate location of the two daughter genomes after segregation. Biorientation along the spindle serves to sort the genome by targeting the two sister chromatids to opposite poles as discussed in more detail later. After all chromosomes are bioriented, the sister chromatids disengage and segregate towards the mitotic poles, completing the partitioning of the two genomes and thereby mitosis[1, 32-35].

Following mitosis, the last step of M-phase is the division of the parent cell into two daughter cells by cytokinesis. In fungi and animals, the plasma membrane of the cell constricts and undergoes fission in a plane that lies orthogonal to the axis connecting

the mitotic poles. Cytokinesis thus bisects the parent cell to generate two daughter cells which each now contain a complete genome[36-39].

1.2.2 General overview of Mitosis

Mitosis is generally subdivided into five sequential phases, prophase, prometaphase, metaphase, anaphase and telophase[2, 31].

During prophase, chromosomes begin to condense and become visible as distinct bodies of chromatin rather than a hazy nuclear meshwork. Chromosome condensation will continue into anaphase [40-43]. The breakdown of the nuclear envelope (NE) marks the onset of prometaphase[44, 45]. During prometaphase, the bipolar mitotic spindle assembles from microtubules (MTs) which is followed by progressive biorientation of the chromosomes on the spindle [35, 46-48].

MTs are assembled from polar tubulin dimers to give rise to polar filaments, one end of which is designated the plus end while the other designated the minus end[49-53]. A typical spindle in animal somatic cells and fungi is formed by three types of microtubules that emanate from the two spindle poles in which centrosomes reside as microtubule organizing centers. Interpolar microtubules (ipMTs), which form the two interdigitating bundles of the central spindle and link the two poles mechanically, astral microtubules (aMTs), which interact with the cortex and facilitate spindle positioning, and kinetochore microtubules (kMTs) to which the chromosomes attach to facilitate their segregation towards the spindle poles in anaphase[48].

One obstacle to faithful partitioning of the eukaryotic genome during mitosis is that it is distributed between multiple chromosomes that have no discerning features for the mitotic apparatus. Yet one copy of each sister needs to reach each daughter cell. This is elegantly solved by sorting sister chromatids through a process called biorientation. After their replication, sister chromatids are held together by the ring like protein

complex cohesin[54, 55]. Cohesin is loaded during replication and topologically entraps the two sister chromatids, linking them together[56-58]. While cohesin is largely evicted from chromosome arms in prophase it is retained at centromeres[59]. During prometaphase, chromosomes attach to kMTs via protein complexes termed kinetochores. The kinetochore assembles on a specific locus of a chromosome, the centromere. The kinetochore binds to kMTs with high affinity to create a load bearing attachment for subsequent chromosome segregation[60, 61]. Kinetochores harbor an active tension sensing mechanism of which the Aurora B kinase is an important component. Their attachment to kMTs is only stable under tension. In absence of tension Aurora B is thought to phosphorylate their MT binding proteins and trigger detachment[62-64]. Sister chromatid links by cohesin and the tension dependent attachment of the kinetochore form the basis of biorientation[65, 66]. Only attachments to kMTs from opposing spindle poles will generate tension between sister-chromatids which are linked together at their centromere by cohesin. Therefore the attachment of a chromosome to the spindle is only stable if the two sister chromatids are attached to kMTs of opposing poles. This form of attachment is termed bioirented. A non-bioriented chromosome will continuously destabilize its MT attachments until it has achieved stable biorientation[67].

Bioriented chromosomes move to the spindle equator. Each chromosome biorients individually and once all chromosomes have achieved biorientation and have congressed at the spindle equator, metaphase is reached. Metaphase can be defined as the time it takes from chromosome congression until anaphase initiation[68]. The metaphase to anaphase transition is the time in which a major cell cycle checkpoint, the spindle assembly checkpoint (SAC), acts. The SAC inhibits the transition into anaphase before all chromosomes have bioriented[69, 70]. It only permits anaphase entry when the last kinetochore has attached and is placed under tension[71].

The SAC prevents anaphase entry by inhibiting a ubiquitin ligase, the anaphase promoting complex/cyclosome (APC/C) (see section 1.6.2.). Once biorientation is

complete, the SAC is satisfied, inhibition of the APC/C is relieved and anaphase ensues. The APC/C ubiquitinates securin, the inhibitor of the protease separase[72-74]. Separase, after liberation from its inhibitor, permits sister chromatid segregation by cleaving the remaining centromeric cohesin[59]. During anaphase, sister chromatids move to their respective pole. Anaphase is subdivided in two phases, anaphase A and anaphase B. During anaphase A kMTs shorten while chromosomes remain attached by their kinetochores, pulling them towards the pole[75]. During anaphase B, the spindle extends, pushing the poles outward and dragging the attached chromatids along[76, 77]. Finally, during Telophase, the spindle disassembles and the NE reforms[45, 78-80]. This is brought about by destruction of another APC/C substrate, cyclin B (see section 1.3)

1.2.3 Mitosis in budding yeast

Mitosis in budding yeast follows a similar series of events but there are some important differences that need to be highlighted.

First, in contrast to animals, the plane of cytokinesis is not defined by the spindle but by the placement of the bud. Cytokinesis will occur in the bud neck and it is imperative that, prior to anaphase, the spindle is aligned along the mother bud axis so it can enter the bud during anaphase B to deliver the daughter genome [81-83]. Secondly, budding yeast undergoes a closed mitosis, its NE never breaks down. Its centrosome analogs, termed spindle pole bodies (SBP), reside in the NE. The aMTs project into the cytoplasm to interact with the cortex, while its spindle, the ipMTs and kMTs are located intra-nuclear[84-87]. During anaphase B the nuclear envelope extends, together with the spindle, and in late anaphase will form a thin tube between the two lobes of segregated chromatin[88]. During mitotic exit, the nuclear tube will undergo fission in a process that is not well understood and give rise to the two separate daughter nuclei[89]. Third, the spindle assembles early in the cell cycle, before DNA replication is complete[18]. Fourth, the Chromosomes of budding yeast do undergo mitotic

compaction but do not condense to become visible as distinct stainable bodies[90]. Therefore prophase is not defined. Similarly, the absence of NE breakdown and reformation, preclude an easy classification of prometaphase and telophase. The time of chromosome biorientation is sometimes defined as prometaphase[91, 92]. The majority of chromosome movement in anaphase derives from spindle extension during anaphase B as kMTs are already short during metaphase[93].

Finally, cohesin is not removed from chromosome arms before metaphase. Rather it is removed solely by seperase at the metaphase to anaphase transition. The late removal of chromosome arm cohesion might be responsible for the pronounced display of elastic behavior in the chromosome arms during anaphase. Alternatively progressive de-catenation of the chromosome arms in anaphase may be responsible (see section 1.5). Centromere proximal loci are pulled from the chromatin mass by the extending spindle earlier than distal loci leading to an initial stretch. When linkage between a given arm locus and its sister locus has been lost this locus will recoil from the spindle midzone towards the centromere located at the mitotic pole. It has been suggested that, as the arm regions are progressively drawn into the spindle midzone, cohesin is cleaved sequentially downwards along the arms, permitting segregation of loci progressively further away from the centromere. Alternatively the force applied by the mitotic spindle may facilitate progressive de-catenation down the chromosome arm. [94-96].

1.2.4 Cytokinesis

M-phase concludes with cell division by cytokinesis. In yeast and animal cells a contractile ring assembles and constricts the plasma membrane. This is followed by the fission of the plasmas membrane during abscission[[36-39].

In animal cells, the position of the contractile ring is defined by the mitotic spindle[97]. The two microtubule asters emanating from the centrosomes and the anaphase central spindle confine the recruitment of the components of the cytokinetic ring, including the GTPase RhoA, to a narrow band at the plasma-membrane which encircles a plane between the two mitotic poles[98, 99]. RhoA promotes the recruitment recruitment of further components such as actin, and myosin-II. The contraction of the ring is induced by two protein complexes, centralspindlin and the chromosome passenger complex which contains the Aurora B kinase. During ring contraction, the plasma-membrane invaginates to form the cleavage furrow[38, 39]. The spindle midzone matures into the midbody, a dense bundle of anti-parallel microtubules which are no longer connected to the mitotic spindle. The plasma membrane contracts onto the midbody which will orchestrate abscission. The completion of cytokinesis requires a number of factors in membrane trafficking and exocytosis and the last step is mediated by the ESCRT machinery which is involved in various membrane scission events [38, 39].

In budding yeast the position of the contractile ring is defined by the position of the bud neck which in turn is specified at the entry to S-phase[82, 83, 100]. After bud growth and segregation of the daughter genomes, cytokinesis closes the bud neck and completes cell division. Some of the earliest cytokinesis-factors to assemble at the bud site are septins which form a ring around the neck of the emerging bud. Septins are required for the recruitment of the type II myosin Myo1. A Myo1 ring co-localizes with the septin ring before mitotic exit[82, 100]. Although the Myo1 ring is established early, during at bud emergence, the contractile ring assembles only during cytokinesis when actin and the actin cross-linking protein Iqg1 are recruited to the bud neck[100].

The localization of both Myo1 and Iqg1 during cytokinesis depend on Mlc1, a myosin light chain that is also a light chain for Myo2 which has a role in vesicle and organelle transport[36, 37]. Mlc1 requires septins for its localization to the bud neck[37]. Upon mitotic exit cytokinesis initiates and the septin ring splits into two rings, separating from the centrally located actomyosin ring. It has been hypothesized that septin ring

splitting is required for the subsequent contraction of the Myo1 ring[101]. The motor domain of Myo1 contributes but is not essential for Myo1 ring contraction[102]. In budding yeast the type 2 myosin Myo1 is thought to act mainly as a scaffold for other factors in cytokinesis. Other factors contributing to cytokinesis in budding yeast are thought to be actin filament dynamics and the formation of the primary septum[37].

The primary septum is formed by deposition of extracellular chitin into the bud neck. Its formation is tightly linked with contraction of the actomyosin ring and mutants defective for septum formation show a defect in actomyosin ring contraction. Conversely, cells defective for chitin deposition display asymmetric acto-myosin ring contraction. A complex of Hof1, Inn1 and Cyk3 is thought to link ring contraction to septum formation, though their precise function is still incompletely understood. Timely contraction of the actomyosin ring requires components involved in exocytosis[36, 37, 103, 104].

1.3 The cell cycle control machinery

The cell cycle is a sequence of ordered events and the success each event depends on the correct execution of the previous. To give an example, mitosis without prior duplication of the genome would have disastrous consequences for the survival of the two daughter cells. The cell cycle control machinery ensures that the individual processes of the cell cycle are initiated in the correct temporal order. Importantly the progression through the cell cycle is gated by checkpoint controls to ensure that all processes required for the next step of the cycle have completed (see section 1.6).

1.3.1 CDK1 drives progression of the cell cycle from G1 to metaphase

The central factors that drive the ordered sequence of events during the cells cycle are the cyclin dependent kinases (CDKs). CDKs , directly or indirectly, trigger the hallmark events of cell division including DNA replication, assembly of a mitotic spindle and budding in yeast[105]. Budding and fission yeast utilize a single CDK, CDK1(cdc2), to trigger both S-phase and M-phase onset[21, 106-109]. Four mitotic CDKs have been described in animal cells, CDK6, CDK4, CDK2 and CDK1[110]. While these are classically thought to each trigger individual events of the animal cell cycle, more recent work demonstrates that mouse embryos that retain only CDK1 develop until mid-gestation. [111, 112]. CDK1 is sufficient to drive the mammalian cell cycle[112]. It has been suggested CDK6, CDK4 and CDK2 act as additional layers of control that have a more prominent function in specific cell lineages and cancer cell lines. Thus, like yeasts, animal cell cycles can be controlled with a single CDK1[111].

To allow control of the cell cycle by a single kinase, budding yeast CDK1 is embedded in a complicated regulatory network that controls its activity by multiple mechanisms. First and foremost, this regulation engenders the oscillation of CDK1 activity throughout the cell cycle. CDK1 activity is low in G1, rises during the cell cycle to when it triggers entry into S- and subsequently M-phase. After that, CDK1 is inactivated which promotes the completion of M-phase and the reentry into G1[113, 114].
The activation of CDK1 brought about by a combination of transcriptional and biochemical regulation[25, 115-118]. CDK1 is inert as kinase unless bound to activators termed cyclins[114, 119-123]. Individual cyclins are sequentially expressed and expression oscillates during the cell cycle. Budding yeast CDK1 is controlled by nine cyclins, the early G1 cyclin Cln3, the pair of late G1 cyclins Cln1 and Cln2 and the three pairs of B type cyclins, Clb5 and Clb6, clb3 and clb4, as well as Clb1 and Clb2. Cln3 and the four cyclin pairs are expressed sequentially, in this order[19, 124].

The sequentially appearing cyclin pairs trigger the timely initiation of different cycle events . Cln1 and Cln2 trigger bud formation, Clb5 and Clb6 trigger DNA replication,

Clb3 and Clb4 trigger spindle assembly and Clb1 and Clb2 are required for spindle elongation and chromosome segregation[83, 125-129]. This may suggest that the cyclins determine the target specificity of CDK1, which is true an extent as some cyclin domains that confer target specificity or control cyclin localization have been described[130-132]. However the fact that the presence of any one of the three G1 cyclins is required to maintain viability and that over-expression of either clb1 or clb6 can rescue the combined loss of all other B-type cyclins suggests considerable redundancy[133]. An alternative and not mutually exclusive model is that the cell cycle is ordered intrinsically and the sequential events require increasing threshold CDK1 activity to initiate[116, 134-136]. Supporting this the fission yeast cell cycle control machinery has recently been deconstructed to a single cyclinB-CDK1 fusion protein. Here the transition though G1, S, G2 and into M-phase are triggered solely by increasing CDK1 activity[137].

1.3.2 Regulation of anaphase progression and mitotic exit by FEAR and MEN

While CDK1 activity is required to drive a cell from G1 until the metaphase-anaphase transition, removal of the phosphorylation placed by CDK1 is important for anaphase spindle dynamics, chromosome segregation and cytokinesis[138-140]. To this effect, CDK1 needs to be inhibited and its targets need to be de-phosphorylated. These events are probably best understood in budding yeast

The initial step is the activation of the APC/C ubiquitin ligase. The APC/C is inhibited by the spindle assembly checkpoint until all chromosomes have achieved bi-orientation. Once relived from inhibition, the APC/C targets, among other factors, securin and B type cyclins for proteolysis. Destruction of securin liberates seperase, which in turn cleaves cohesin. Destruction of B-type cyclins on the other hand inactivates CDK1[141-145]. This is assisted by the inhibition of cyclin B-CDK1 complexes by CDK inhibitors, such as Sic1, which is expressed during mitotic exit[146-148].

The second step entails the dephosphorylation of CDK1 substrates. Cdc14 is the phosphatase that dephosphorylates critical CDK1 targets to bring about spindle elongation, chromosome segregation and cytokinesis[149]. The activity of Cdc14 itself is tightly regulated. From G1 until anaphase it is sequestered in the nucleolus through its association with Net1[150, 151]. Cdc14 is released at anaphase onset by two sequential pathways, the Cdc14 early release pathway (FEAR) and the mitotic exit network (MEN)[152-156].

In anaphase CDK1 and polo kinase phosphorylate Net1 to abolish its binding to Cdc14[157-159]. However until FEAR activation in anaphase, the phosphatase PP2A counteracts Net1 phosphorylation to retain Cdc14 in the nucleus. Separase, after its release from securin and in complex with Slk19 down-regulates PP2A activity towards Net1 through Zds1 and Zds2, by a mechanism independent of its protease activity[160-163]. This in turn leads to a partial release of Cdc14 into the nucleus[164].

After anaphase onset, firing of the MEN effects full release of Cdc14. The MEN is, at its core, a linear signaling cascade where the Tem1 GTPase is placed at the apex, the kinase Cdc15 is placed in center, and the kinase Dbf2 together with its activator Mob1 are placed at the terminus[155, 165]. Tem 1 is concentrated on the daughter-bound SBP of the elongating spindle. Active Tem1 recruits Cdc15 which in turn phophorylates the SPB scaffold protein Nud1 to recruit and activate Mob1-Dbf2, possibly by direct phosphorylation. A second pathway for Cdc15 recruitment to the SPB and its activation is provided by polo kinase[165-169]. After activation Mob1-Dbf2 effect full release of Cdc14 into the nucleus and, by phosphorylating the NLS of Cdc14, also into the cytoplasm[170].

Because the activation of FEAR depends on the libration of seperase from securin, it is activated in response to correct bi-orentation of all chromosomes. The MEN in turn is activated by correct spindle positioning. When the spindle elongates in early anaphase

and the daughter bound SBP enters the bud, Tem1 located at the SPB is activated [171, 172]. The activation of Tem1 then triggers MEN mediated full, sustained Cdc14 release. The position dependent activation of Tem1 will be discussed in more detail later (see section 1.3).

Because FEAR acts by permitting CDK1 mediated phosphorylation of Net1, its release of Cdc14 is transient. In absence of the MEN low CDK1 activity leads to re-sequestration of Cdc14 and a resurgence of CDK1 activity. However the early Cdc14 release by FEAR permits the MEN to activate by removing inhibitory CDK1 phosphorylation from Cdc15 and Mob1. The MEN in turn after correct spindle positioning can effect Cdc14 release in absence of CDK1 activity [152, 173-176].

Cdc14 release is required for spindle stabilization, chromosome segregation and cytokinesis. The microtubule crosslinker Ase1 is required to stabilize the anaphase spindle and it is recruited to the spindle after its CDK1 sites have been dephosphorylated by Cdc14. The same holds true for the chromosome passenger complex (CPC) which contains the aurora kinase. Cdc14 mediated recruitment of both factor is required for correct spindle elongation in anaphase B[177-179]. The role of Cdc14 on chromosome in chromosome condensation is likely the loading of condensin which facilitates de-catenation of chromosomal regions in anaphase, as will be discussed later[180-182]. Finally during cytokinesis, de-phosphorylation of Iqg1 by Cdc14 is promotes the formation of the contractile ring[183].

While the function of Cdc14 in mitotic exit of budding yeast is very prominent, it is by no means the only factor involved in mitotic exit. The disassembly of the mitotic spindle for example is thought to require the APC/C mediated destruction of microtubule cross-linkers[80]. Similarly cytokinesis requires the MEN independently of its function in Cdc14 release. Dbf2 phosphorylates both the Hof1 and the Chitin synthase Chs2 which is required for their function in actomyosin ring contraction and the formation of the primary septum[184-186].

The central role of Cdc14 in anaphase and mitotic exit is rather specific for budding yeast. In fission yeast and C. elegans, Cdc14 contributes to cytokinesis but are not required for Cdk1 inactivation or other aspects of mitotic exit. Furthermore, depletion of Cdc14 isoforms does not delay the progression of mitotic exit in human cells. De-phosphorylation CDK1 targets in higher organisms is attributed to the interplay of multiple kinases including PP1 and PP2A[187].

1.4 DNA replication

The machinery that promotes mitotic chromosome assembly acts on a DNA that has undergone replication. In fact there is evidence that proteins that organize a mitotic chromosome require DNA replication for their recruitment[188, 189]. On the other hand it is important that DNA replication completes before chromosome assembly. Classical experiments show that S-phase chromosomes are pulverized if forced into assembly[190].

1.4.1 Initiation and completion of DNA replication

DNA replication initiates from chromosomal loci termed origins. In budding yeast origins are defined by a consensus sequence, ARS (autonomously replicating sequence)[191]. Origins are recognized by the origin binding complex (ORC). In budding yeast this entails sequence specific binding to ARS regions. In higher organisms the ORC appears to binds DNA without sequence specificity and any DNA is replicated. The sites utilized for initiation are thought to result from preferential accessibility to ORC which is determined epigenetically by chromatin context[192]. Origins are grouped in early or late origins depending on whether they will fire at the onset of S-phase or later

during S-phase. It is thought that late origins constitute a back-up mechanism. If two converging forks collapse, the intervening DNA is unable to replicate. Reserving origins for later in S-phase affords the possibility to restart replication by activating a dormant origin in the intervening region[193].

The firing of replication origins is tightly regulated by the cell cycle control machinery. Origin activation outside S-phase is prevented through the subdivision of activation into two processes, origin licensing and origin priming[194]. Priming is only permitted in S-phase and entails the recruitment of the ORC which binds to the origin and in turn recruits the licensing factors Cdc6 and Cdt1. Cdt1 is recruited together with the Mcm2-7 complex that will form part of the replicative helicase[195].Licensed early origins fire when cells enter S-phase. The increase in CDK1 activity is required to initiate firing and CDK1 activity prevents further licensing by inhibiting helicase recruitment and targeting licensing factors for nuclear export and destruction[196-201].

Origin firing proceeds by the conversion of the Mcm2-7 complex into the active replicative helicase. This is brought about by the loading of Cdc45 and the GINS complex to the Mcm2-7 complex to assemble the CMG helicase. The Cdc45 and GINS are part of the helicase but a second group of proteins, TopBP1^{Dbp11}, Sld2, Sld3 and Sld7 are required as initiation factors.

Together with CDK1, the DKK kinase composed of Cdc7 and its regulatory subunit Dbf4, are essential for the activation of the helicase. DDK phosphorylation of the Mcm2-7 complex leads to recruitment of Cdc45, Sld3 and Sld7 already in G1. However CDK1 phosphorylation after the transition into S-phase is required to finalize CMG assembly by the recruitment of the GINS. Further, Cdk1 phosphorylated Sld3 recruits TopBP1^{Dpb11} and Sld2. This constitutes a major regulatory step in origin firing as CDK1 phospho-mimicking mutants render DNA replication independent of M-CDK1 activity. In addition to the Sld2-Sld3-TopBP1^{Dpb11} complex, DNA polymerase epsilon (DNA Polε) binds directly to the CMG and is required for origin firing prior to actual DNA synthesis.

Together with an as of yet incompletely understood function of Mcm10, these events lead to activation of the CMG helicase and origin unwinding. The Mcm2-7 complex is loaded as a head to head arranged double hexamer. After activation, the two CMG helicases assembled from the individual hexamers will unwind DNA as they move in opposite directions to generate two replication forks [193, 195].

One model for the unwinding of DNA by CMG helicase is that it functions as a wedge on double stranded DNA (dsDNA) by encircling a strand of single stranded DNA (ssDNA) and translocation along it. The stretches of ssDNA generated are bound by replication protein A (RPA) which keeps the ssDNA strands from re-annealing. DNA polymerase alpha-primase complex (DNA Polα) then synthesizes a RNA-DNA hybrid primer to initiate DNA polymerization. DNA Polα is evicted after primer synthesis, followed by the loading of the replication clamp PCNA by replication factor C (RFC) to the ss/dsDNA junction and the loading of the major replicative DNA polymerases, DNA polymerase delta (DNA Pol δ) and DNA Pol ε [193, 202].

DNA can only be synthesized in a polar direction, by the formation of a covalent bond between the phosphate moiety located at the 5´postion of the deoxyribose ring of one nucleotide and the 3´ hydroxyl group of another. Thus DNA has to be synthesized in a 5´-3´manner. The directions of the single strands in DNA are anti-parallel which creates an obstacle for a replication fork moving in one direction. One strand, the leading strand, can be continuously synthesized in direction of fork movement. The other strand, the lagging strand must be synthesized in the opposite direction of fork movement. This is accomplished by means of discontinuous synthesis along the lagging strand in Okazaki fragments. As the fork moves along the lagging strand, it is continuously re-primed and synthesized in small fragments of about 200bp that are aligned in the opposite direction of fork movement[203, 204].

The CMG helicase is thought to move down the leading strand, together with bound DNA Polε. Ample evidence suggests that DNA Polε is the preferential leading strand

DNA polymerase while DNA Polδ acts as the lagging strand polymerase. This is however not an absolute classification as the catalytic activity of DNA Polε is not essential in budding yeast and is thought to be replaceable by DNA Polδ. Recent in vitro reconstitution experiments suggest that DNA Pol δ can perform leading strand replication but is outcompeted when DNA Pol ε is present[202].

A number of factors move with the replication fork and are thought to be components of the active replisome. These include the CMG helicase, DNA Polα, DNA Polδ, DNA Polε, PCNA, Ctf4 and a complex of Mrc1, Csm3 and Tof1. The latter complex has a role replication checkpoint (see section 1.6) Ctf4 on the other hand is thought to tether DNA Polα to the CMG helicase. There is currently no evidence for a coupling of DNA Polδ to the CMG helicase. Sld2, Sld3, Sld7 and TopBP1^{Dpb11} are required for initiation but they do not move with the replisome[193, 205-208].

To complete DNA replication, the lagging Okazaki fragments have to mature and the replisome components have to be unloaded. DNA Polδ during discontinous synthesis of Okazaki fragments, synthesizes into existing fragments, displacing the original primer potion of the fragment. This flap is cleared by the Fen1 and Dna2 endonucleases and the junction between fragments is subsequently ligated to complete lagging strand synthesis. Two mechanisms have been described to effect removal of replication factors from chromatin. The RFC-like Elg1 unloads remaining PCNA from replicated chromatin which is linked to Okazaki fragment maturation. The CMG helicase, which forms the core of the replisome, is ubiquitinated by SCF^{Dia2} which likely leads to the disassembly of the replisome by the Cdc48 segregase[193, 209-212].

1.5 Assembly of a mitotic chromosome

The assembly of a mitotic chromosome as it is currently understood entails a profound restructuring of DNA in three dimensional space. This is necessary primarily to overcome two obstacles to chromosome segregation.

The first obstacle is the length of DNA. In many cases, the length of the DNA contained in a chromosome easily exceeds the length of the cell it is contained in. As an example, assuming a 0.33nm step per base-pair, the largest DNA molecule of budding yeast, chromosome 12 with around 2Mb, has a length of around 650µm[213]. An anaphase yeast cell on the other hand may reach a length of around 10µm. Yet, eukaryotic chromosome segregation commonly operates by attaching one locus, the centromere, to the spindle and pulling it to the spindle pole. This creates an obstacle for a segregation machinery; a large portion of the chromosome arm would be too long to follow. That obstacle is overcome through chromosome condensation, the three-dimensional packing of DNA into compact bodies by introduction of higher order structure.

The second obstacle to chromosome segregation is intrinsic to the structure of DNA and the chemistry of its replication. The DNA of two sister chromatids is intertwined or catenated after replication. This results from the double helical structure of DNA which undergoes semi-conservative replication. When the hydrogen bonds between complementary bases are broken to generate the two single stranded templates for DNA replication they are still topologically linked, winding around each other. After synthesis of the new strands, the resulting molecules retain the intertwines of the parent strands. These intertwines are termed catenations and they link the two sister chromatids together to resist the seperation of chromatids in anaphase. This obstacle is overcome by decatenation. One duplex of DNA is cleaved and the intact sister is passed through the break before it is sealed[214].

1.5.1 Chromosome condensation

The term chromosome condensation was initially used to describe the emergence of discernible bodies from the interphase chromatin[2]. As mentioned, this is thought to entail a major reorganization of DNA from its interphase state. In particular, somehow the path of DNA is arranged such that it will assume a defined higher order structure, a cylindrical body of chromatin. The path of the DNA in a chromosome is subject of multiple, not mutually exclusive models.

Early electron microscopy of histone extracted mitotic chromosomes show loops of DNA that are attached at their base to a central proteinacious scaffold [215]. However a load bearing proteinacious scaffold was never suggested and strong evidence against such an assumption comes from micromanipulation experiments in which chromosomes were extended along their axis. Digestion of either DNA or Protein affects the elastic response of a chromosome. Therefore the structural integrity of a chromosome relies on both, DNA and the associated proteins[216, 217].

A second classical view of mitotic chromosome organization is the hierarchical folding model. In this model, a 10nm fiber composed of DNA wrapped around nucleosomes is wound into chromatin fibers of increasing thickness, in the most recent work a 30nm, a 130nm, and a 250nm filament which is then curled into a chromosome of 0.5-0.75 µm thickness[41, 218, 219]. These models derive from the observation of domains of this size during chromosome condensation. While these observations should not be discounted, there is substantial evidence against a hierarchical folding of a metaphase chromosome as 30nm or 250nm fibers are not observed in metaphase chromosomes[220-223].

An attractive model is the organization of a mitotic chromosome into a series of consecutive loops of around 100kb in length. These loops would be formed by loop extruding factors (LEF) binding to one locus and spooling DNA upstream and downstream of their binding site through their extrusion site. LEFs or other factors

would then clamp the base of loops dynamically. Many loops form along the length of a chromosome to generate a central axis defined by consecutive loop bases and a corona of chromatin loops that spreads radially from the center[223, 224].

The last model is by no means the only remaining explanation. However it does accommodate both, a central chromosome axis that is dependent on protein and DNA for its integrity and a DNA loop based organization. It also attractive because the organization of a mitotic chromosome into sequential loops is suggested in budding yeast. This model may unify chromosome condensation into common principles, despite cytological differences, among species[224-227]. Such unified principles are anticipated as the proteins required for chromosome condensation are conserved among animals and yeasts.

A central and conserved factor required for chromosome condensation is the condensin complex[228]. Animal cells harbor two condensin complexes, condesin I and condesin II. Condensin II localizes to chromatin during replication and is required for the resolution of sister chromatids. Condensin I on the other hand associates with chromatin after nuclear envelope breakdown. Notably, both condensins localize to a central axis embedded in a metaphase chromosome. The two condensins are thought to mediate distinct aspects of condensation, owing to the distinct effect of their depletion on the structure of a metaphase chromosome. Depletion of condensin I renders metaphase chromosomes swollen while depletion of condensin II leads to a curly structure of the central chromosome axis[41, 189, 229-232]. Budding yeast harbors only a single condensin complex which is localized to the nucleus throughout the cell cycle but is enriched in the rDNA region specifically in anaphase in a Cdc14 dependent manner[181, 228, 233-235].

Condensin is a ring shaped SMC complex related to cohesin and has two known biochemical effects on DNA[228]. First its binding to DNA induces positive supercoiling [236-238]. Secondly it binds DNA and is able to topologically link to DNA strands

together[239]. Given its role in chromosome condensation and its localization to a central axis embedded in animal chromosomes, it is the prime candidate to act as a loop fastener for the organization of a chromosome into consecutive loops. Alternatively its activity in generating supercoils could be involved in the formation of a mitotic chromosome. However micromanipulation experiments suggest that supercoils are not a major factor required for the structural integrity of a mitotic chromosome[240].

Additional factors involved in chromosome segregation have been described. Histone H3 phosphorylation by Aurora B plays a role in multiple species. In budding yeast a pathway has been delineated in which Aurora B controls adaptive hypercondensation. Aurora B is localized to the spindle midzone in anaphase and phosphorylates histone H3 on S10 to recruit the deacetylase Hst1 which deacetlyate Histone H4 at K16 to permit condensation through internucleosome interactions. This allows a cell to adapt to artificially long chromosomes by increasing their condensation[43, 241-244]. Further the requirement of the N terminal tail of histone H2B for chromosome condensation in the Xenopus egg extract system indicates the presence of as of yet unidentified histone modifications that are required for chromosome condensation and placed by an unknown factor[245, 246]. Finally a number of seemingly unconnected factors have been suggested to facilitate chromosome condensation, including the yKU complex and the deacetylase Sir2 in yeast and titin in drosophila. Their function or relation to the established chromosome assembly machinery is poorly understood[247-249].

1.5.2 Chromosome decatenation

Decatenation of DNA depends mainly on type 2 topoisomerases. Topoisomerase 2 (Top2) catalyzes what is termed a strand passage reaction. It binds two strands of DNA. One strand is cleaved. The two ends of the break site are held in proximity by covalent bonds to the enzyme while the second strand is passed through the broken strand.

After passage of the second strand the broken strand is sealed. This activity allows topoisomerase 2 to decatenate sister chromatids which is required for their segregation[250]. Importantly, while topoisomerase can act in S-phase, it is still required during anaphase, suggesting that chromosomes are not completely decatenated when chromosome segregation begins. This holds true for both yeast and animal cells. A possible reason for why topoisomerase is unable to remove all chromosomal catenanes before anaphase onset is a subset of catenanes may be shielded from Top2 action by the cohesin ring. Thus, separase mediated cleavage would be required to complete catenation and permit chromosome segregation[96, 251, 252].

1.5.3 The interplay of topoisomerase 2 and condensin

Top 2 and condensin show partially dependent behavior for both chromosome condensation and decatenation.

In budding yeast and animal cells the localization of top2 after loss of condensin is perturbed. A loss of condensin function, in addition to its effects on chromosome segregation also perturbs chromosome arm resolution in anaphase. It has therefore been suggested that condensin in its potential function in ordering chromatin into loops promotes disentanglement of chromosome by Top 2[180, 253-255].

Conversely Top2 has also been described as required for chromosome condensation, however not as a structural component[256]. In micromanipulation experiments, addition of Top 2 would substantially reduce chromosome stiffness suggesting that entangled DNA is a structural competent of mitotic chromosomes[240]. In line with this, a sumoylation negative topoisomerase 2 mutant in budding yeast leads to a stretching of pericentromeric DNA of a bioriented chromosome, and not to its increased compaction as may be expected from its role in removing sister chromatid interlinks[257]. This may be due to the interplay of Top2 and condensin on the DNA

substrate. Condensin binding to DNA induces non-catalytic supercoiling. However in presence of Top2, this can be converted into stable knots[258].

1.6. Cell cycle checkpoints that preserve genomic stability through mitosis

The transition between different phases of the cell cycle is gated by checkpoints to ensure that all processes required for the next phase have been completed. However checkpoints exist that do not induce a rigid cell cycle arrest and this classical definition has changed in recent years. For example an essential function of the replication checkpoint is also to prevent replication forks from collapsing. Thus this checkpoint has targets beyond the cell cycle machinery that promote recovery from the insult that triggered it[259].

1.6.1 The replication checkpoint

The observation that cells arrest before mitosis after addition of DNA replication inhibitors or the inactivation of essential replication factors led to the discovery of the S-phase or replication checkpoint[260].

As is the case for all checkpoints, the most pertinent question about the replication checkpoint is what feature it uses to sense the replication stress. Early on it had been suggested that the replication checkpoint may sense un-replicated DNA, possibly by sensing unfired pre-RCs. However depletion of CMG helicase components, central components of the pre-RC do not promote exit from a cell cycle arrest that is induced by inhibition of replication with the ribonucleotide reductase inhibitor HU[261]. Conversely complete inactivation of replication be depletion of factors required for initiation or licensing, such as Cdc6 or the DDK kinase does not induce a cell cycle

arrest even though the whole genome is un-replicated. Thus it does not appear that there exists a replication-completion checkpoint that monitors un-replicated DNA or senses fully replicated DNA[262-267].

Because the replication checkpoint requires DNA replication to initiate in order to become active it is thought that the structures that arise at impaired replication forks constitute the signal that activates the checkpoint. More specifically the inhibition of DNA synthesis by can leads to uncoupling of the helicase from the DNA polymerization. The helicase in turn will continue to unwind DNA[268].

A pathway through which the replication checkpoint is activated has been delineated. The Replication checkpoint through the activation of the sensor kinase ATR^{Mec1}. ATR in complex with $ATRIP^{Ddc2}$ binds to RPA coated ssDNA. The recruitment of ATR^{Mec1} to RPA-ssDNA is thought to be insufficient for its activation. In addition response of the replication checkpoint depends on primer synthesis by Polα. Primer synthesis generates ss/dsDNA junctions when short fragments are synthesized onto the template. Importantly, in the egg extract system the combination of ssDNA and a ssDNA junction is sufficient to induce a checkpoint response[268-275].

Instead, ATR^{Mec1} is activated by TopBP1 which binds to RPA-ssDNA. This function is separable from its role in initiating replication. Another component in this pathway is the PCNA like 9-1-1 complex (Rad9-Rad1-Hus1). The 9-1-1 complex is recruited by TopBP1 to load on its preferred substrate, 5´ ss/dsDNA junctions. Binding of the 9-1-1 complex in turn induces a conformational change in TopBP1 that allows it to activate ATR^{Mec1}[276-278].

ATR^{Mec1} in turn activates effector kinaseses, $Rad53^{Chk2}$ in yeast or Chk1 in animal cells via the replication checkpoint mediator Mrc1, or its animal homologue claspin, which travels with the replisome. $Rad53^{Chk2}$ or Chk1 in turn signal a cell cycle arrest and promote stabilization of stalled forks[273, 274].

While this pathway is becoming increasingly understood the observation that inactivation of the Smc5/6 complex leads to anaphase entry in the presence of DNA structures that resemble replication intermediates raises the question to what extent the replication checkpoint is able to ensure the completion of DNA replication. It is clear that gross insults to DNA replication trigger the checkpoint but these may do so because they resemble DNA damage. Other perturbations, such as an experimentally induced stretch of un-replicated DNA by the placement of tandem fork-blocks appear to go unnoticed [279-281].

1.6.2 The spindle assembly checkpoint (SAC)

The SAC monitors the attachment of chromosomes via kinetochores to the spindle[69, 282]. More specifically, it monitors their biorientation, that is whether the sister-kinetochores have been targeted to opposing mitotic poles. Until all chromosomes have achieve biorientation, anaphase entry is prohibited[71]. The SAC acts directly on the APC/C and inhibits its ability to ubiquitinate Securin and B-cyclins by blocking the function of its co-activator Cdc20.

Unattached kinetochores generate a mitotic checkpoint complex (MCC) in which Cdc20 is prevented from interaction with the APC/C through binding to Mad2 and in which a Bub3-BubR1^{Mad3} dimer supplies a pseudosubstrate degron to block APC/C substrate recognition. Importantly this does not only lead to inhibition of Cdc20 function. BubR1^{Mad3} promotes the ubiquitination and destruction of Cdc20. This counteracts synthesis of Cdc20 and is required to maintain a SAC arrest[283-290]. However alternate mechanisms have been suggested such as phosphorylation of Cdc20 by Bub1[291].

One possible pathway by which unattached kinetochores may catalytically generate the MCC is by Mad2 templated assembly. Mad2 in turn is activated to bind Cdc20 by its

recruitment to unattached kinetochores through Mad1, where it undergoes a conformational change which depends on the checkpoint kinase Mps1. This conformational change allows binding to Cdc20 and promotes recruitment of Bub3-BubR1^{Mad3}, leading to the assembly of the MCC[292-294].

1.6.2 The spindle position checkpoint(SPOC)

Anaphase in budding yeast is unusual inasmuch as that the cleavage plane is not defined by the spindle but by the bud neck. A successful mitosis requires that the anaphase spindle delivers the daughter genome through the bud neck. To ensure this, the spindle position checkpoint (SPOC) monitors the successful entry of the daughter-bound SPB into the bud.

Until the daughter SPB has reached the bud, the SPOC inhibits mitotic exit by negatively regulating the MEN GTPase Tem1. Tem1 is inhibited by two GTPase activating proteins (GAPs), Bub2 and Bfa1. Polo kinase in turn phosphorylates Bfa1 to inhibit it thereby activating Tem1 and triggering mitotic exit. This system is able to sense spatial cues in the mother and daughter cell. A second kinase, Kin4 phosphorylates Bfa1 which renders it resistant to polo phopsphorylation, keeping Bfa1 active and thus the MEN inactive. Importantly, Kin4 is active only in the mother cell, it is inhibited in the bud by Lte1. When the daughter bound SPB enters the bud, Bfa1 is liberated from phosphorylation by Kin4. Polo kinase can now inactivate Bfa1 to activate Tem1, thereby triggering the MEN and ultimately mitotic exit[169, 172, 295-298].

1.6.3 The NoCut checkpoint

Defects in chromosome assembly can lead to the formation of anaphase bridges as detailed in the next section. Anaphase bridges form when the spindle extends in

anaphase but the attached chromosomes segregate incompletely, for example because they are uncondensed, catenated or incompletely replicated. Chromatin that remains at the site of impeding cell division is exposed to the advancing cytokinetic machinery.

In budding yeast, anaphase bridges that derive from the defects mentioned above trigger the NoCut checkpoint which in turn delays the final stage of cytokinesis, abscission. The NoCut is thought to sense anaphase bridges by an interaction between Aurora B and chromatin. Aurora B is recruited to the spindle midzone in anaphase and artificial tethering of Aurora B is sufficient to inhibit abscission. During unimpeded chromosome segregation, chromatin segregates away from the spindle midzone but when an anaphase bridge forms, chromatin remains in the vicinity of Aurora B which activates the checkpoint. The means by which Aurora B induces the abscission delay in yeast remains to be determined. In animal cells however, the inhibition of abscission entails a direct Aurora B mediated regulation of the abscission machinery through phosphorylation of the ESCRT-III subunit CHMP4C[299-303].

Anaphase bridges that are unable to resolve, such as those induced by inactivation of condensin or Top 2 are cut despite the activity of NoCut. However because the NoCut promotes viability and reduces DNA damage following cytokinesis in cells exposed to replication stress, it is thought that the NoCut checkpoint provides a window of time for anaphase bridges that can be resolved to complete segregation[299].

1.7 Anaphase bridges

Anaphase bridges are a form of chromosome mis-segregation. They are visible as lagging chromatin that spans the spindle midzone and connects the two masses of segregated anaphase chromosomes. Lagging chromatin can broadly be defined as chromatin that segregates later than the bulk of anaphase chromosomes and remains

in the vicinity of the spindle midzone. In contrast to whole lagging chromosomes, which often arise from spindle attachment defects, anaphase bridges are frequently comprised of only a portion of a chromosome and are thus more likely to be rooted in the structural features of a chromosome. Late segregation may be entirely normal and visible in unperturbed cells in some cases. In other cases they signify catastrophic segregation failures that will lead to DNA damage, genome instability and cell death. Anaphase bridges can be divided into two types, chromatin bridges and ultrafine bridges according to whether they are detectable with conventional DNA dyes or not.

1.7.1 Chromatin bridges

Chromatin bridges are clearly visible bridges of bulk chromatin that stain with DNA dyes and chromatin markers such as fluorescent histones. Chromatin bridges may arise spontaneously in unperturbed cells. They are observed up to 2% in untransformed cells such as cultured human fibroblasts and their incidence is elevated in tumor cells [304, 305]. Spontaneous lagging chromosomes can occur due to merotelic attachments in which one kinetochore is attached to microtubules from opposing poles. The mis-attached centromeres can be visualized on the lagging chromosome in conjunction with the bound microtubules[306-308]. Chromosome structure is far less amenable to microscopy than the mitotic spindle and therefore structural defects of a chromosome are not as readily correlated with an anaphase bridge. Consequently the contribution of chromosome structural defects to anaphase bridges is not well understood. One structural that has been implicated in the spontaneous formation of anaphase bridges is the formation of a dicentric chromosome. A dicentric chromosome contains two centromeres which can attach to opposite spindle poles, leading to the pulling of the two centromeres into opposite directions and stretching the intervening chromatin across the spindle midzone. The dicentric chromosomes can in turn arise from improper DNA repair, for example after telomere erosion[5, 6, 304, 309].

Chromatin bridges can be induced experimentally by gross perturbation of chromosome assembly. A loss of function of codensin or Top2 for example lead to anaphase bridges owing to a defective in shortening and decatenating of chromosome arms[254, 310-312]. In addition to that, the loss of factors that regulate these processes has similar effects. In budding yeast *cdc14* mutants exhibit defective condensin recruitment in anaphase and display chromatin bridges. One explanation for the defect in condensin loading observed in *cdc14* mutants is that condensin loading requires the shutoff of transcription which is mediated by Cdc14. A failure in loading condensin in turn would lead to under-condensed chromosomes but has also been reported to impair de-catenation as previously mentioned[181, 253, 313, 314].

1.7.2 Ultrafine bridges

The second type of anaphase bridges are ultrafine bridges (UFB), so termed because they are not stained by conventional DNA dyes and no histone signal is observed on them, was identified relatively recently in human cells. They initially identified indirectly, by visualizing proteins that localize to UFBs such as the PICH, the Bloom helicase or TopBP1. However the presence of DNA in these UFBs was confirmed by their ability to incorporate labeled nucleotides. Importantly, UFBs are a common feature of normal mitosis in untransformed human cell lines. They have been sub-classified further, based on the chromosomal loci they derive from[315-317].

The first subclass of UFBs are centromeric UFB´s. PICH and the Bloom helicase frequently label UFBs that contain centromeric DNA during anaphase in untransformed human cells[251]. Because these bridges are observable in most anaphases and progressively disappear during anaphase, they are believed to be a normal feature of anaphase. Specifically, it is thought that centromeric DNA that retains the cohesin complex in metaphase will have to undergo decatenation in anaphase. In line with this, Topoisomerase 2A is recruited to centromeric UFBs by TopBP1^{Dbp11} [251, 318, 319].

A second class of UFBs are fragile site associated bridges. Common fragile sites (CFS) are regions in the genome with which are intrinsically difficult to replicate. The exact reasons for this are not well understood but it has been suggested that the presence of secondary structures formed by repeating DNA sequences challenges the progression of the replication or the lower frequency of active replication origins in these regions[317]. Fragile site UFBs are rarely observed in normally dividing human cells but can be induced by treatment of cells with low doses of replication inhibitors. Importantly, these fragile site UFBs are different from the normal centromeric UFBs in terms of the proteins they recruit. In addition to PICH, the Bloom Helicase and TopBP^{Dbp11} the Replication Protein A (RPA) and the DNA repair proteins of the Fanconi anemia complex components have been implicated in protecting stalled replication forks from degradation[320-322]. FANC-D2/I localize into two foci at the bridge terminus flanking the Bloom helicase and RPA70 which localized onto the UFB. Because of this and the origin of these bridges are believed to be either unreplicated DNA or incomplete resolution of replication intermediates.

Finally a third class of UFBs are telomeric UFBs. It has been suggested that telomeres resemble fragile sites on the grounds that a loss of function of either TAZ1, a shelterin component of S.pombe, or TRF1, its mammalian homologue lead to replication forks stalling at the telomere[323, 324]. This generates the equivalent of ultrafine bridges in S.pombe, RPA coated anaphase bridges that were visible with a histone fluorescent tag only on overexposure[325]. The loss of TRF1 in human cells does not lead to telomeric UFBs but overexpression of the paralogue TRF2 does induce both telomeric replication fork stalling and RPA positive telomeric UFB´s[326].

1.7.3 Ultrafine bridges in budding yeast

Ultrafine bridges have been described very recently in budding yeast. Like their animal counterparts they recruit TopBP^{Dbp11}, the Bloom helicase and RPA. These UFBs recapitulate the behavior of human UFBs in as much as that they with common DNA dyes or recruit fluorescently labeled histones to a visible level. Budding yeast UFBs can be induced by perturbing replication but they arise at a lower frequency spontaneously. Surprisingly these bridges have been described in 40% of normal cell divisions, suggesting that they are normally resolved timely, before cytokinesis[327].

1.8 Goals

Many of questions regarding the origin of anaphase bridges remain unanswered. They can be experimentally induced by a variety of means such as the inactivation of factors essential for chromosome assembly. However their observation in untransformed cells and wild type yeast indicates that they can arise spontaneously and that these bridges are not necessarily lethal. This in turn suggests, together with the association of anaphase bridges with mutagenic events and their increased occurrence in transformed cells, that bridges may be driving factors in the formation cancer. It is thus of great interest to understand better the processes that normally act to prevent them.

Conversely the observation that anaphase bridges form after known chromosome assembly factors are lost suggests that spontaneous anaphase bridges hay have similar origins. Whether this may be due to impairment of known processes such as chromosome condensation or decatenation is an open question. Both are essential processes and their lack incompatible with life. While a number of factors are known to be required for the assembly of a mitotic chromosome our understanding of this process remains, in many respects, incomplete.

The question lingers. Do we know all the processes and factors required to make and segregate of a mitotic chromosome? Could other factors be required to prevent anaphase bridges?

To investigate this I decided to screen budding yeast for mutants that show display bridges in order to identify novel factors or processes that are required to prevent them. Among the mutants that displayed anaphase bridges, I chose two categories to investigate further, DNA replication mutants and mutants defective for the activation of the mitotic exit network (MEN) . First, I show that mild replication stress leads to anaphase bridges in wild type yeast. Secondly I identify members of the conserved mitotic exit network (MEN) as factors that are required to prevent anaphase bridges.

2. Results

2.1 Anaphase bridge screen

I performed a reverse genetic visual screen to identify mutants that fail to prevent anaphase bridges. We expected the genes involved to be essential, as this process is vital to genome maintenance. We therefore focussed on the essential genes of budding yeast.

Decades of yeast genetics have led to the generation of thermosensitive alleles for the majority of the essential genes of budding yeast. A thermosensitive allele is a tool that is used to conditionally inactivate the protein product of a gene in question. It generates a protein that performs its function normally at the permissive temperature, typically 22°C-25°C, while it becomes unstable and is degraded at the restrictive temperature, typically between 30°C-38.5°C. These alleles are available in mutant collections, or arrays, which I have used to screen for mutants that fail chromosome assembly[328, 329].

To identify mutants that display a chromosome assembly defect, I screened for a cytologically visible diagnostic phenotype, the appearance of anaphase bridges. An anaphase bridge in budding yeast presents itself as a connecting thread between the two lobes of segregating chromatin during anaphase. Morphologically this is indistinguishable from a late anaphase nucleus in which most of the chromatin has segregated and the remaining thread is about to complete segregation. The difference between a normal anaphase and a bridge lies in whether the connecting thread will be segregated on time, late, or not at all. One way to identify mutants that fail chromosome assembly is to find those that fail to segregate this connecting thread and therefore display an increased number of anaphase figures.

However, a chromatin connection that fails to clear the bud neck will be cut during cytokinesis. The inactivation of condensin, which severely compromises chromosome condensation, increases the life-time of an anaphase figure only by about ten minutes. (see Fig. 5).

We reasoned that, to be able to detect anaphase bridges in a population of cells, it would be necessary to stabilize them by preventing cytokinesis. Since this would have to be performed in a large number of samples in parallel, it was desirable to be able to block cytokinesis rapidly, through application of a simple treatment. The use of existing pharmacological agents such as the type 2 myosin inhibitor blebbistatin or latrunculin was not possible. Blebbistatin acts on the motor domain of type 2 myosins and the motor domain of Myo1 is not essential for cytokinesis in budding yeast. Depolymerisation of the actin cytoskeleton by latrunculin on the other hand disrupts a normal cell cycle[102, 330, 331].

2.1.1 Inhibition of cytokinesis using a thermosensitive iqg1-1 allele under control of a copper-repressible promoter

I first endeavoured to find a suitable means to inhibit cytokinesis and stabilize anaphase bridges. Conditional inactivation of the essential proteins Myo1 or Iqg1 was attempted by use of both temperature or auxin induced degrons[332, 333]. These degrons either led to hypomorphic alleles, rendering cells sick under permissive conditions, or were not lethal under restrictive conditions (not shown).

I turned to the previously described temperature sensitive allele *iqg1-1*[334]. This allele is lethal at restrictive temperature in the W303 background in which it was originally generated. However it is not lethal at 37°C in the BY4742 background in which the thermo-sensitive collections were constructed (Fig1). This suggested the incomplete inactivation of this essential protein.

In an attempt to inactivate Iqg1 more stringently, I characterized the behaviour of strains in which *IQG1* or *iqg1-1* had been placed under control of a copper repressible promoter *pCTR1* (Fig. 2A). The copper repressive elements (CuREs) contained in *pCTR1* are bound by the Mac1 transcription factor in response to high copper in the medium which leads to rapid promoter shutoff[335].

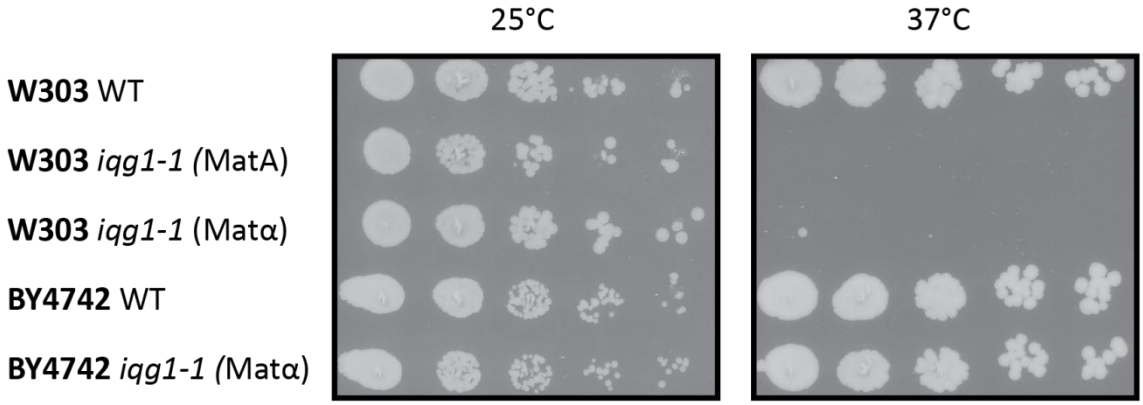
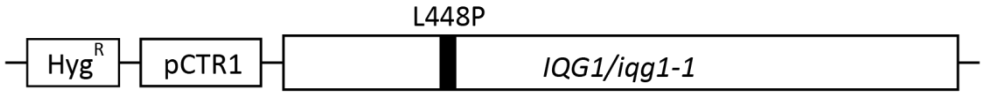


Figure 1) *Spot assay for viability of iqg1-1 mutants in W303 and BY4742 backgrounds at restrictive and permissive temperature.*

To evaluate the response of the *pCTR1-IQG1* and *pCTR1-iqg1-1* alleles, I added 100µM $CuSO_4$ as a restrictive copper concentration to YPDA in order to repress Iqg1 expression. Growth on YPDA or YPDA + 100µM BCS (a copper chelator) were tested as conditions permissive for growth (Fig. 2B). The *pCTR1* promoter leads to inhibition of growth at restrictive copper concentrations. This is not further enhanced by the *iqg1-1* mutation at 37°C, in accordance with the previous result. The growth inhibition by repression of Iqg1 was less pronounced at 37°C. Nevertheless there was a substantial effect on growth in strains harbouring *pCTR1-IQG1* or *pCTR1-iqg1-1* alleles, suggesting cytokinesis was at least strongly compromised by repression of Iqg1.

A)



B)

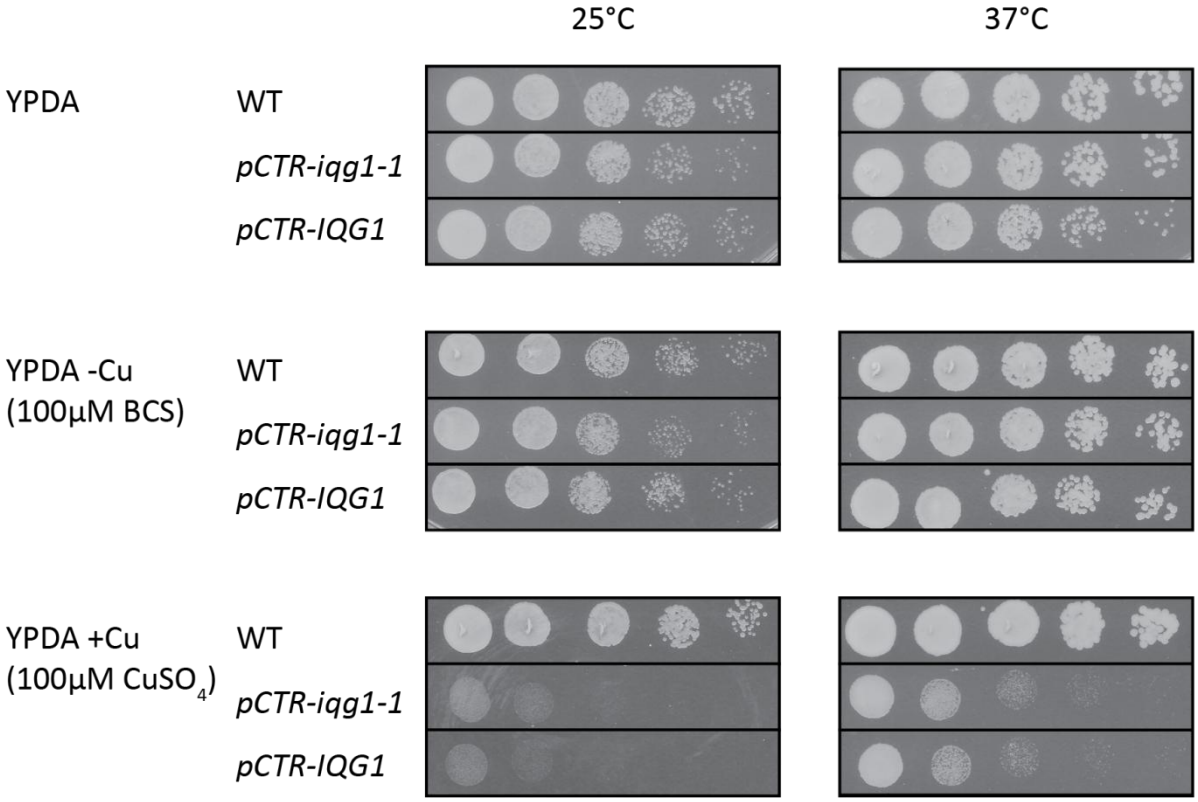


Figure 2A) *Schematic representation of the pCTR1-IQG1 and pCTR1-iqg1-1 alleles, depicting the L448P thermosensitive iqg1-1 mutation*[334]*.* ***2B)*** *Viability assays of clones harbouring either pCTR1-IQG1 or pCTR1-iqg1-1 alleles under permissive and restrictive conditions.*

2.1.2 Inhibition of cytokinesis stabilizes anaphase bridges induced by inactivation of condensin

After finding that both, the *pCTR-IQG1* and the *pCTR-iqg1-1* alleles were able to inhibit cell growth in response to copper, I tested whether they would stabilize anaphase bridges, as we had hypothesized earlier. To this end I introduced the thermosensitive condensin mutant allele *ycg1-2* into both strains in order to generate anaphase bridges. In addition, I fused a fluorescent tag to Histone H2B, Htb2-mCherry, to visualize chromatin.

The promoter shutoff and the temperature shift can be timed independently. Depending on the response time of *pCTR1*, there may be a sequence of copper addition and temperature shift that would maximize the stabilization of anaphase bridges. To test this I performed a small scale screen for treatments in which repression of *pCTR1* was triggered at different times before the temperature shift. Cells were fixed at the indicated time-points, 1, 2 or 3 hours after the temperature shift and then imaged. The percentage of anaphase figures over total number of nuclei was determined. Intriguing tripolar chromatin structures were found in cells that had two buds, indicating that these cells had entered a second anaphase after failing chromosome segregation in the first (Fig3A). These structures were also scored as anaphase nuclei. The *pCTR1-IQG1* construct did not appear to stabilize anaphase bridges under the conditions tested. In contrast, the *pCTR-iqg1-1 ycg1-2* double mutant allele showed an increase in anaphase figures. A maximum of about 50% nuclei in anaphase figure conformation were observed in the sample in which copper had been added one hour before it was shifted to 37°C for three hours (Fig. 3B).

To confirm this effect, further time course analysis was performed. There was a substantial increase in anaphase figures during the time of the experiment in the *pCTR1-iqg1-1 ycg1-2* double mutant. The *pCTR1-iqg1-1* and *ycg1-2* single mutants did not show this response. These results suggested that the *pCTR1-iqg1-1* allele had the desired effect of stabilizing anaphase bridges by inhibition of cytokinesis (Fig. 3C).

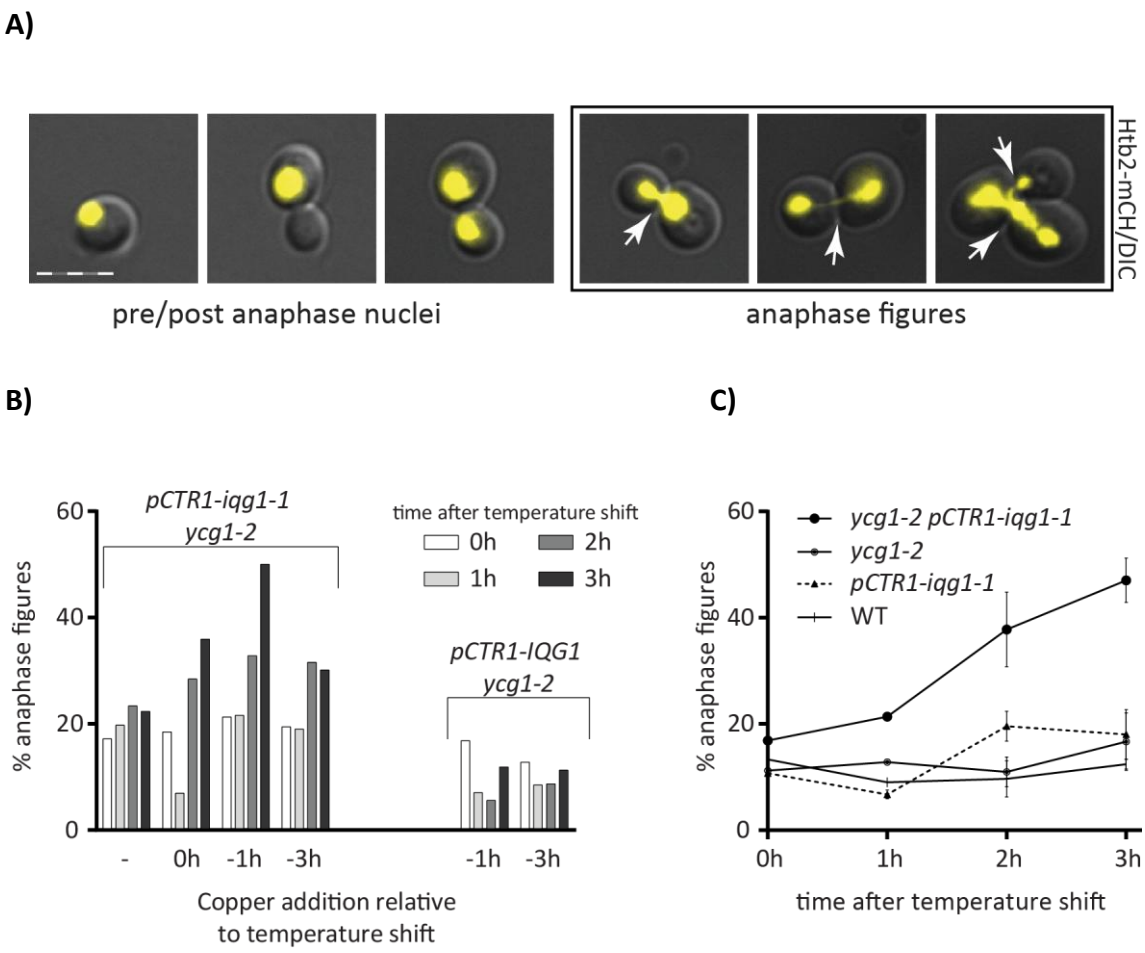


Figure 3A) *Nuclear conformations scored as anaphase figures in time course experiments (boxed).* ***3B)*** *Percentage of nuclei found in anaphase figure conformation after copper addition at different time-points prior to the temperature shift from 25°C to 37°C.* ***3C)*** *Time-course analysis of anaphase figures after addition of 100µM $CuSO_4$ one hour before temperature shift. Cultures were growing logarithmically. Error bars display the standard error between experiments*

It may be unexpected that the condensin single mutant does not show an increase in anaphase figures. Logarithmically growing cells were used in these experiments. I

attribute low number of anaphase figures in the condensin single mutant to cell death, which results from cut anaphase bridges and removes these cells from the cycling population.

2.1.3 Characterization of anaphase bridge life time by live cell time-lapse microscopy

To test whether my interpretation of the results of the fixed cell time courses were correct, I performed time-lapse microscopy to measure the lifetime of individual anaphase figures. Copper was added to logarithmically growing cells one hour before the samples were shifted from 25°C to 37°C by mounting them in a pre-warmed incubator on a spinning disk microscope.

While the wild-type undergoes rapid chromosome segregation, this is delayed in the condensin mutant. Disappearance of the connection between the nascent nuclei in the *ycg1-2* mutant often occurred after shortening of the elongate nucleus, creating the impression of a nucleus being cut (Fig. 4, second panel)[299].

It was interesting to observe that the *pCTR1-iqg1-1* strain undergoes subsequent rounds of re-budding and nuclear division to yield syncytium-like cells (Fig. 4, third panel). The tripolar structures observed previously in the time-courses of the *pCTR1-iqg1-1 ycg1-2* double mutant were indeed found to result from a second anaphase after a failure of chromosome segregation during the first (Fig. 4, fourth panel). This second anaphase could have one of two outcomes, either a tripolar anaphase, as depicted, or a comparatively normal bipolar anaphase figure in which one bud remains empty. This behaviour likely results from the assembly of two spindles during the second cell cycle and the outcome depends on whether the two spindles are targeted towards the same or towards different buds.

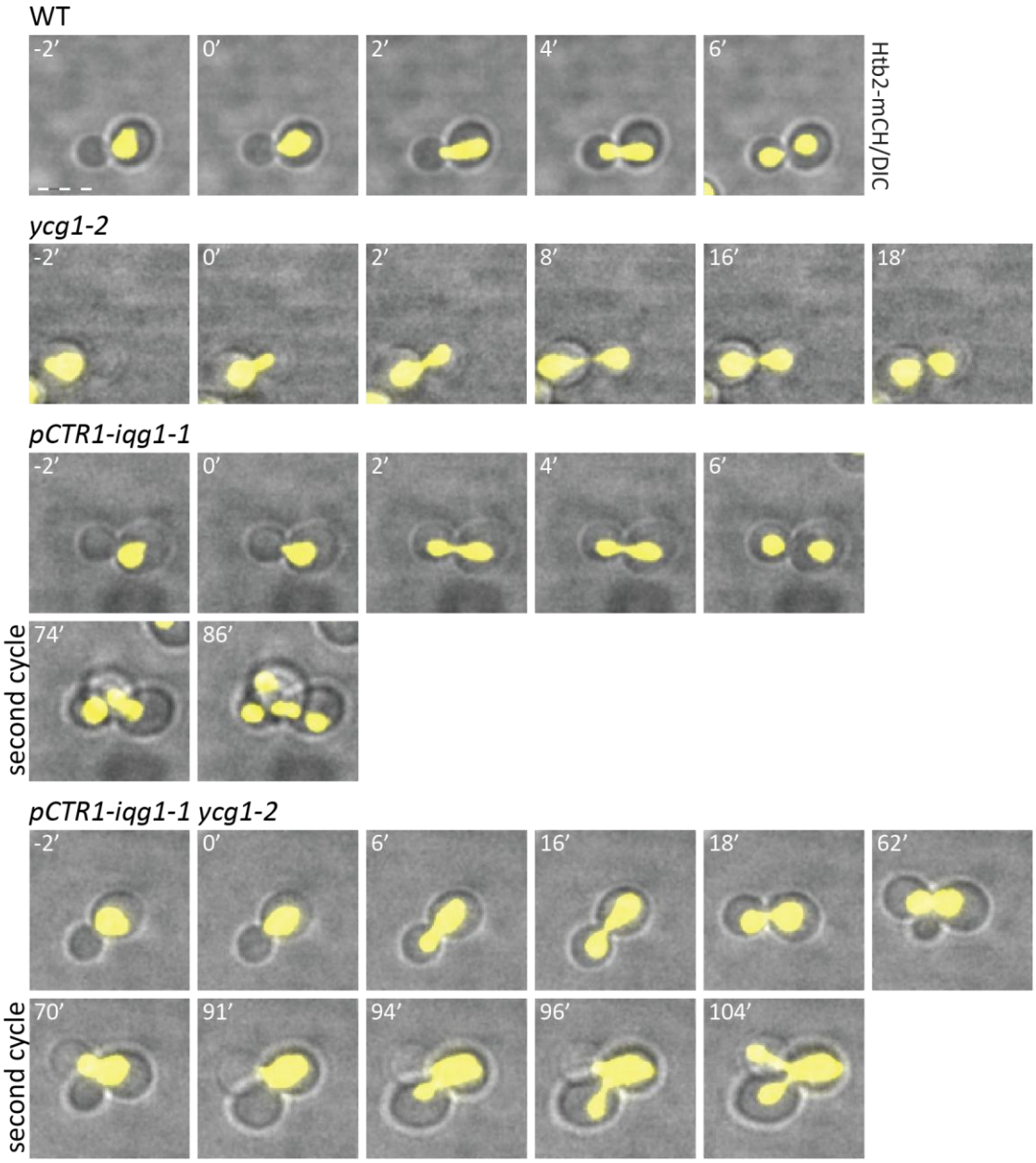


Figure 4) *Representative montages of wild type, ycg1-2, pCTR1-iqg1-1 and double mutant cells undergoing anaphase. Note the formation of 4 nuclei in the second cycle of a pCTR1-iqg1-1 mutant and the tripolar bridge in the second anaphase of pCTR1-iqg1-1 ycg1-2 double mutant. Zero minutes is the onset of anaphase as defined by the beginning of nuclear elongation.*

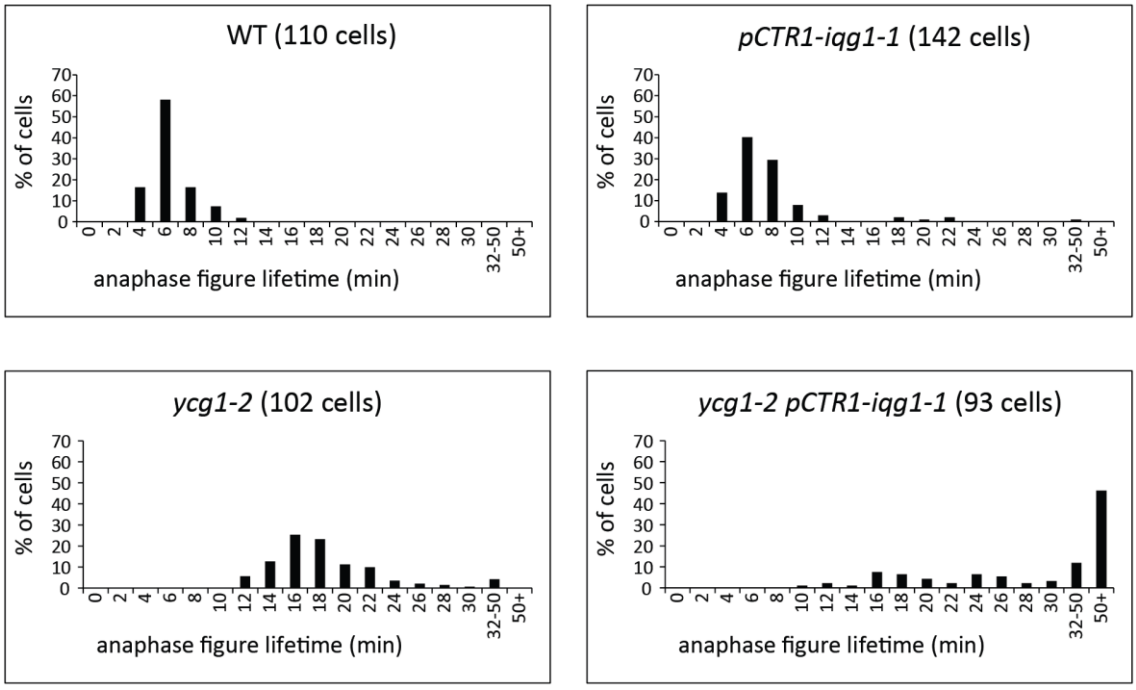


Figure 5) *Binned lifetime of anaphase figures, measured as described in the text, for wild type, ycg1-2, pCTR1-iqg1-1 and double mutant cells.*

The lifetime of anaphase figures was determined by measuring the time of appearance, as identified by the onset of nuclear elongation, until disappearance, as defined by a loss of a visible connection between the two nascent nuclei. At times, a connection transiently disappeared to reappear some frames later. This was attributed to a temporally low fluorescence intensity of these dynamic structures. These anaphase figures were scored as continuous until the final disappearance of the connection as it is unlikely that an anaphase bridge would reform after two nuclei have completed segregation. Under these conditions, the wild type and the *pCTR1-iqg1-1* strain complete anaphase with a mode of 6 minutes. While the lifetime of the anaphase figure was clearly increased in the *ycg1-2* mutant, it was visible for only about 16 minutes. Importantly, the lifetime of anaphase figures was substantially increased in the *pCTR1-iqg1-1 ycg1-2* double mutant, with about 50% of cells exhibiting anaphase figures that were stable for 50 minutes or longer (Fig. 5). This

analysis corroborates the results of the time-course analysis and demonstrates the efficacy of *pCTR1-iqg1-1* allele in stabilizing anaphase bridges.

2.1.4 Rescue of viability of a condensin mutant by the inhibition of cytokinesis

The previous analysis suggested that the inhibition of cytokinesis protects anaphase bridges from being cut and that polyploidy resulting from the inhibition of cytokinesis is not immediately lethal. This raised the question whether the inhibition of cytokinesis may actually rescue the lethality that results from the cleavage of an anaphase bridge. If correct, it may be possible to find conditions under which chromosome condensation is impaired partially and this is rescued by a delay in cytokinesis. To test this, I used the *pCTR-iqg1-1 ycg1-2* double mutant and the two single mutants in a growth assay under a semi-permissive temperature with and without the addition of Copper or BCS. Indeed, at 30 degrees, in absence of copper or in presence of BCS, a partial rescue of the viability of the condensin mutant by *pCTR-iqg1-1* is visible. This suggests that the lethality of the condensin mutation derives at least in part from the cleavage of anaphase bridges (Fig. 6).

2.1.5 Workflow of the anaphase bridge screen

With the *pCTR1-iqg1-1* allele in hand I proceeded to generate the strains to screen for anaphase bridges. As a starting collection of mutants I used two previously described thermosensitive mutant arrays[328, 329]. To introduce the *pCTR1-iqg1-1* allele into the two thermosensitive mutant arrays, I used high throughput cross technology. This technique relies on haploid selection and diploid counter-selection to obviate the necessity of micro-dissection for the isolation of haploids of the desired genotype from a cross. Along with the *pCTR1-iqg1-1* allele, I introduced *HTB2-mCherry* and *SHS1-GFP*

fluorescent fusion tags. The *SHS1-GFP* tag serves to visualize the septin ring at the bud neck which is used as a marker for the cell cycle stage (Fig. 7A).

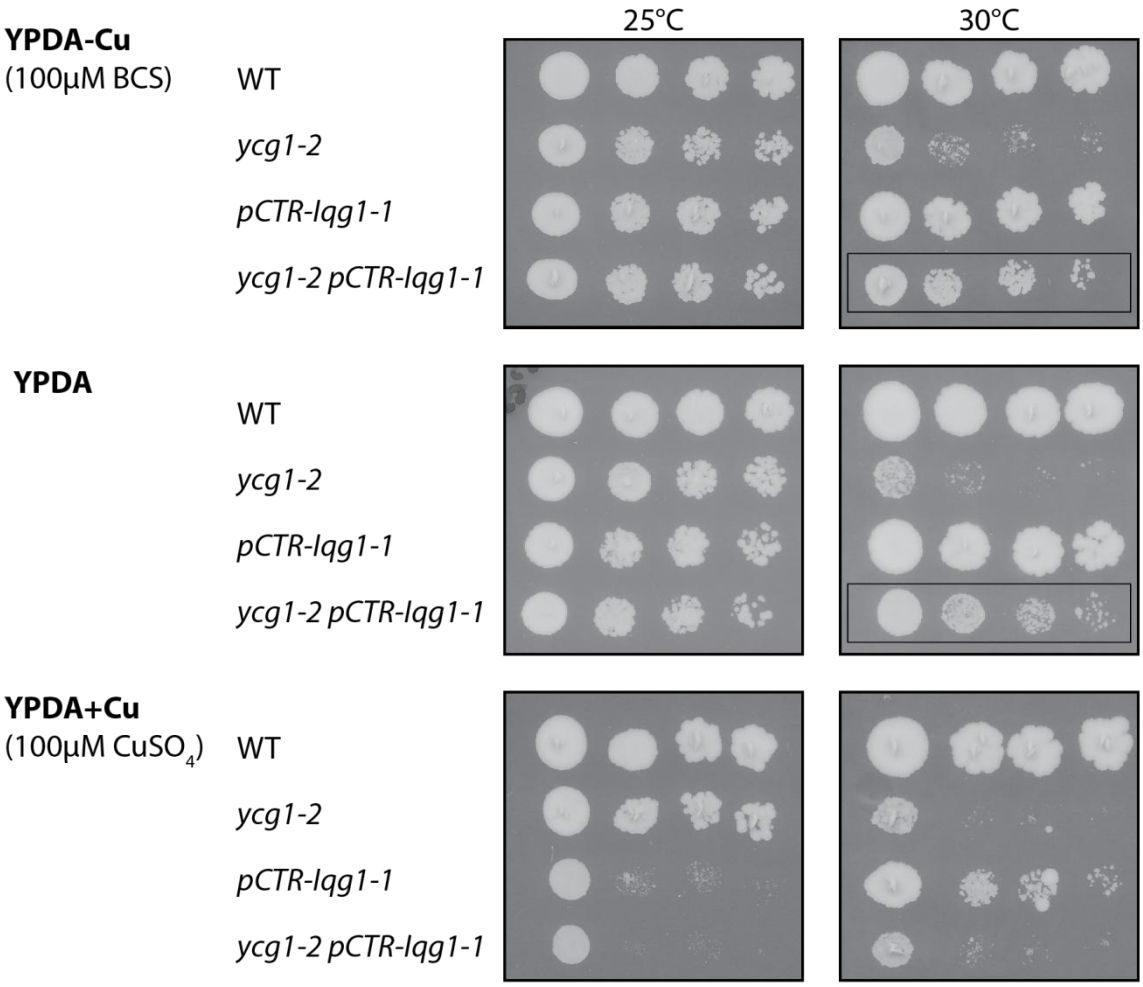


Figure 6) *Viability assay at semi permissive conditions. Rescue of ycg1-2 by pCTR-iqg1-1 boxed. Note that pCTR-iqg1-1 mutants show residual growth after 5 days at 30*°C.

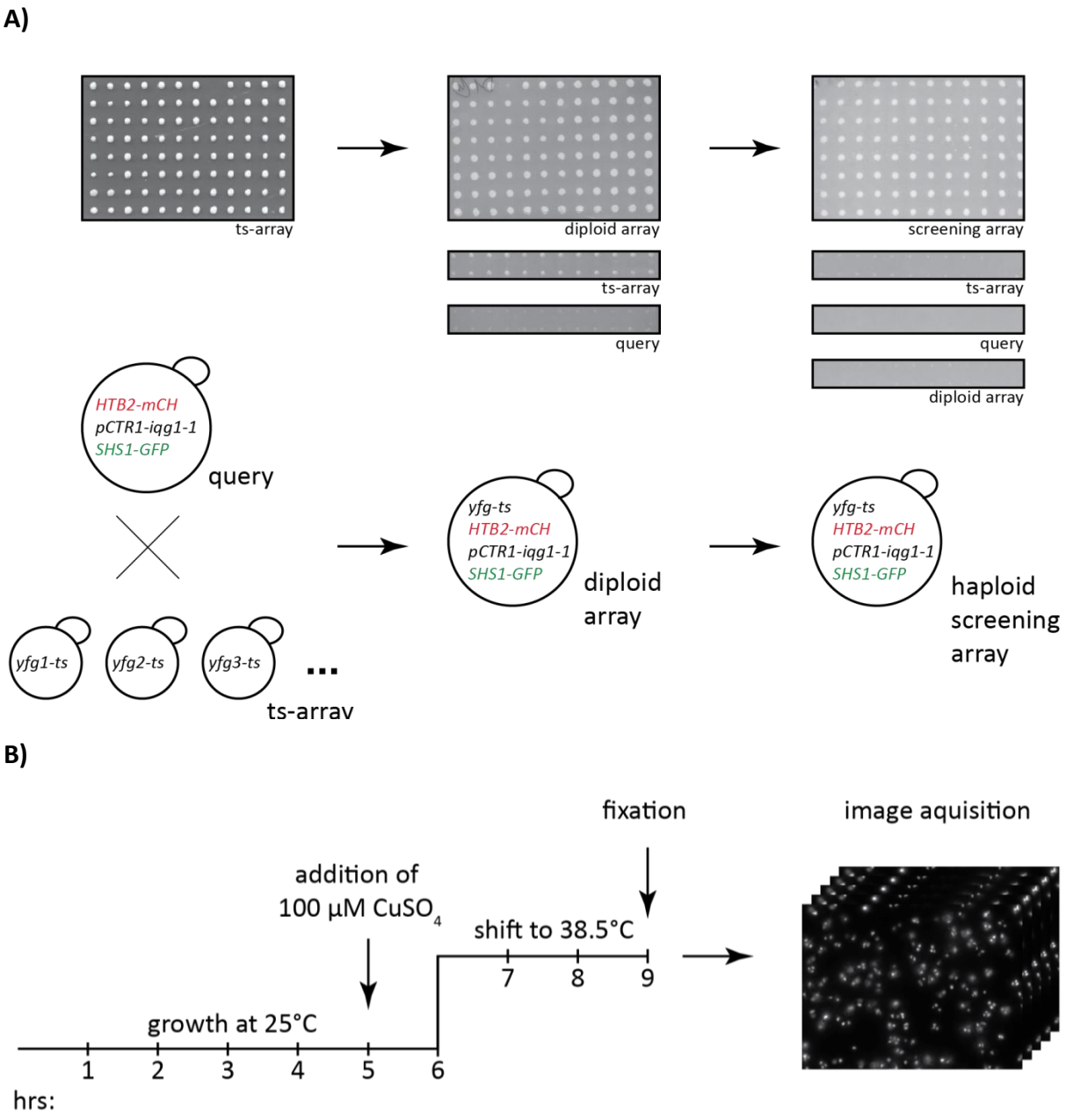


***Figure 7A**) The desired genetic features, pCTR1-iqg1-1, SHS1-GFP and HTB2-mCherry were introduced into the thermosensitive array by high throughput crossing. Representative plates are shown. The thermosensitive array is crossed with a "query" strain that contains the desired features and diploids are enriched by simultaneous selection for the markers present in both, the query strain and thermosensitive array. After sporulation and germination, haploids of the desired genotype are selected while diploids are counter-selected by use of the haploid specific selection markers[328]. **7B**)*

Sample treatment regime: Strains were grown for five hours at 25°C, copper was added for one hour before temperature shift to 38.5°C. Cells were fixed after three hours and processed for image acquisition

The septin ring undergoes a stereotypical morphological change during the cell cycle, when it splits from a single ring into a double ring, signifying mitotic exit[101]. This is later used to distinguish post-anaphase bridges from a normal anaphase figure (Fig. 8A).

After construction of the screening array, the strains were treated and processed for microscopy. All strains were grown in liquid medium and copper was added one hour before the cultures were shifted to 38.5°C. This temperature was chosen because this was the minimum restrictive temperature for some thermosensitive mutants in the original collection. After three hours, cells were fixed in 1% formaldehyde and the fixed cells were further processed for image acquisition (Fig. 7B).

2.1.6 Results of the anaphase bridge screen

An anaphase bridge can be defined as the prolonged co-localization of chromatin and septin rings as it indicates failed or delayed chromatin segregation. This can manifest in two ways. The first is a situation in which a mutant is defective in segregating chromatin away from the bud neck but competent for mitotic exit. In this case chromatin will be found traversing septin rings that have split during mitotic exit. These bridges will be termed post-anaphase bridges. A more severe display of such a mutant is the formation of a tripolar bridge. These are formed by two consecutive rounds of failed chromosome segregation (Fig. 8A, left and middle panels). A caveat here is that a similar situation may arise from a premature mitotic exit that precedes the completion of chromosome segregation. This may be expected of for example mutants that are defective in a cell cycle checkpoint.

A second way anaphase bridges may manifest is in mutants that are deficient for both chromosome segregation and mitotic exit. These mutants are likely to accumulate in high number in late anaphase with undivided chromatin traversing un-split septin rings.

In the *pCTR1-iqg1-1* query strain, chromatin traversing split septin rings is found exceptionally rarely. In fact only a single cell was found among all replicates of quantified *pCTR1-iqg1-1* controls that was reminiscent of a tri-polar bridge. Post-anaphase bridge structures were never seen in the *pCTR1-iqg1-1* query strain. In contrast about 30% of anaphase figures in the *pCTR1-iqg1-1 ycg1-2* double mutant are either of the post-anaphase bridge or tri-polar bridge type. This suggests that even though elevated temperature was necessary to inactivate the thermosensitive alleles screened, *pCTR1-iqg1-1* was still competent to stabilize anaphase bridges (Fig. 8B).

A) **B)**

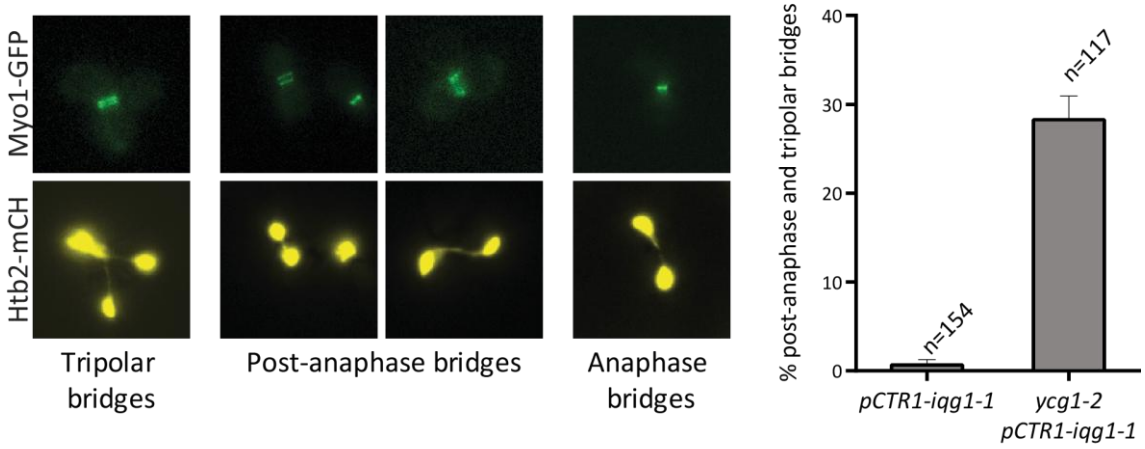


Figure 8A) *Classes of anaphase bridges that were found in the screening array.* ***8B)*** *Quantification of the percentage of post-anaphase bridges or tri-polar anaphases among anaphase figures in pCTR-iqg1-1 and pCTR-iqg1-1 ycg1-2 double mutants. Error bars display standard error between replicates.*

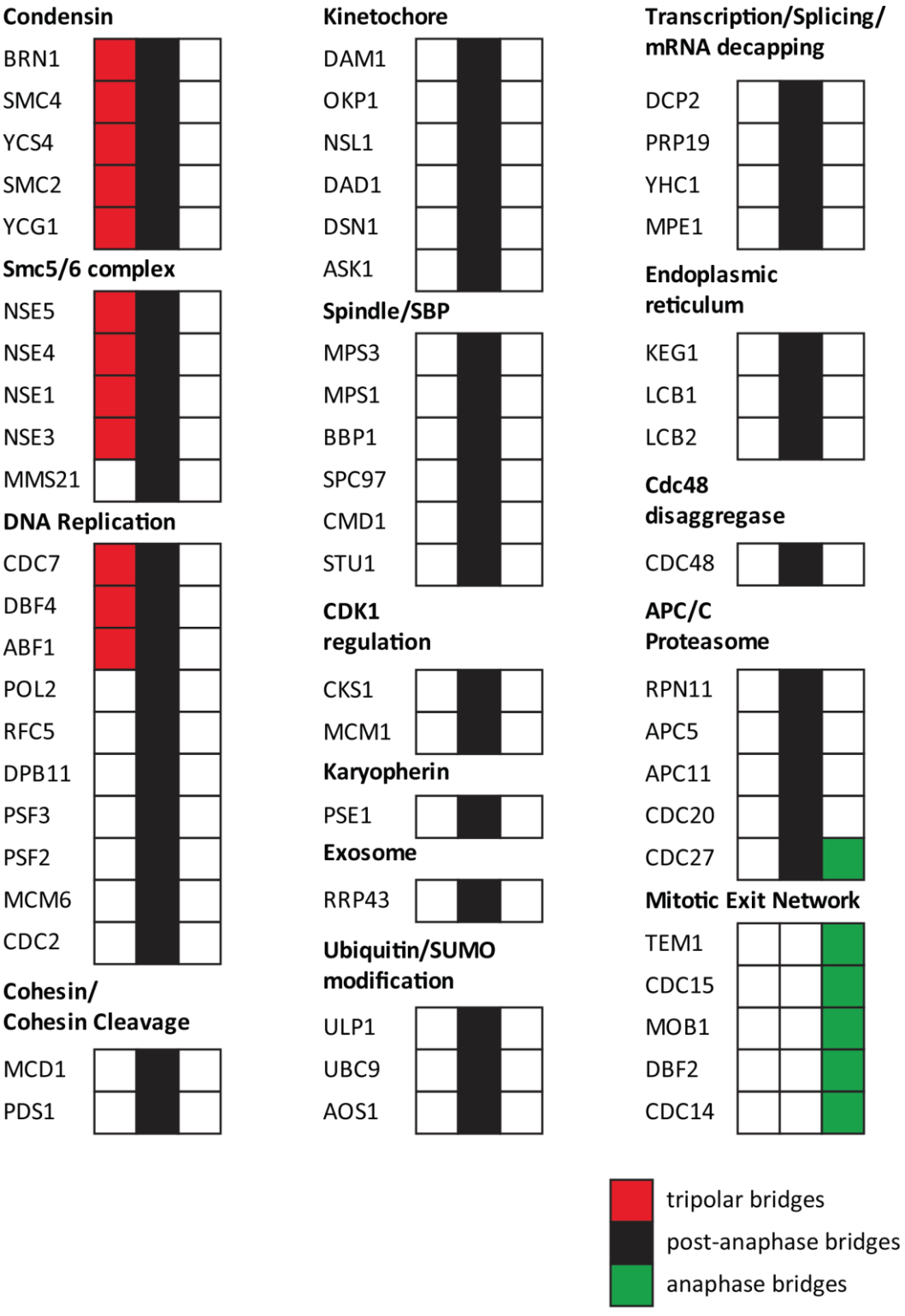


Table 1: *Summary of hits of the anaphase bridge screen. The colour code can be found in the legend. A filled box designates the presence the respective structure in both replicates of at least one thermosensitive allele of a tested gene.*

1085 individual alleles covering 765 individual genes or about 70% of the essential genes were screened in biological duplicates. For both biological replicates, 9 fields per strain were screened blind, manually, to identify strains that fall into one of two categories: those that display a chromosome segregation defect before or after mitotic exit. As the occurrence of post-anaphase bridges and tripolar bridges was exceedingly low in the *pCTR1-iqg1-1* query, mutants were classified as post-anaphase or tripolar bridge hits if two or more of these structures were found in both replicates. On the other hand mutants were only classified as anaphase bridge hits if they had an immediately apparent substantial increase in the number of late anaphase figures.

A summary of the hits is found in Table 1. Significantly, all condensin alleles contained in the collection were found as well as 15 of 17 Smc5/6 complex mutant alleles. Both have a known role in chromosome segregation and their identification demonstrates the efficacy of the screen. *mps1* likely is an example for a mutant that displays post-anaphase bridges as a result of a defective checkpoint and defective chromosome segregation. MPS1 is essential for both SPB duplication and the SAC and therefore likely exits mitosis in absence of chromosome segregation[336, 337]. *cdc14* on the other hand is an example for a mutant that fails both chromosome segregation and anaphase exit as both of these functions have previously been described.

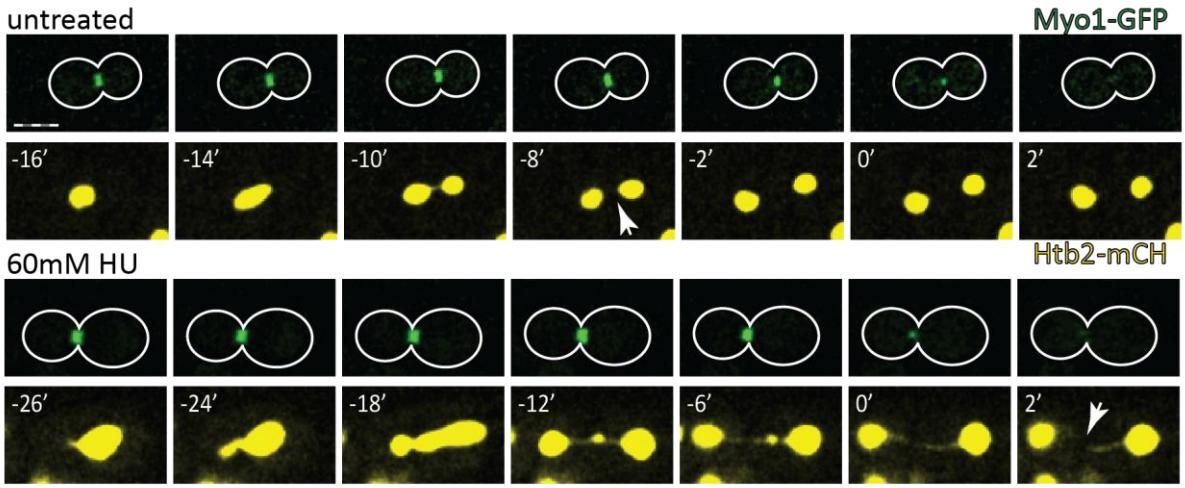
2.2. Replication stress induces chromatin anaphase bridges

2.2.1. Persistent replication stress induces Htb2-mCH positive anaphase bridges

Finding post-anaphase bridges in DNA replication mutants was intriguing as most DNA replication mutants have been described to arrest before anaphase[21].

I wondered whether it might be possible, not to inhibit, but to perturb DNA replication to the point where cells would enter anaphase in presence of bridges which may arise

A)



B)

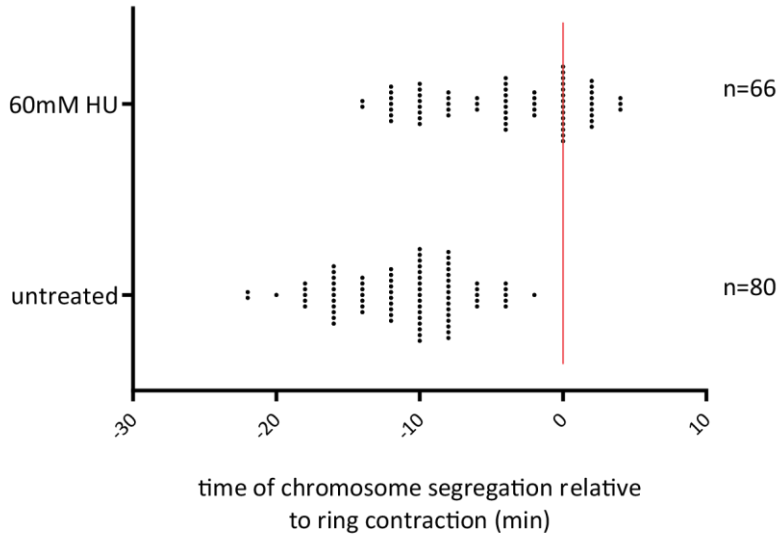


Figure 9A) *Cells growing in the presence of 60mM HU display histone-positive anaphase bridges which are cut by the contracting actomyosin ring. Arrows indicate the point at which a histone-positive anaphase bridge disappears.* ***9B)*** *Quantification of chromosome segregation relative to full ring contraction. Pooled plot of three independent experiments. Time zero is the time of maximal ring contraction, each dot indicates the first time-point in which the visible connection between two nuclei is lost.*

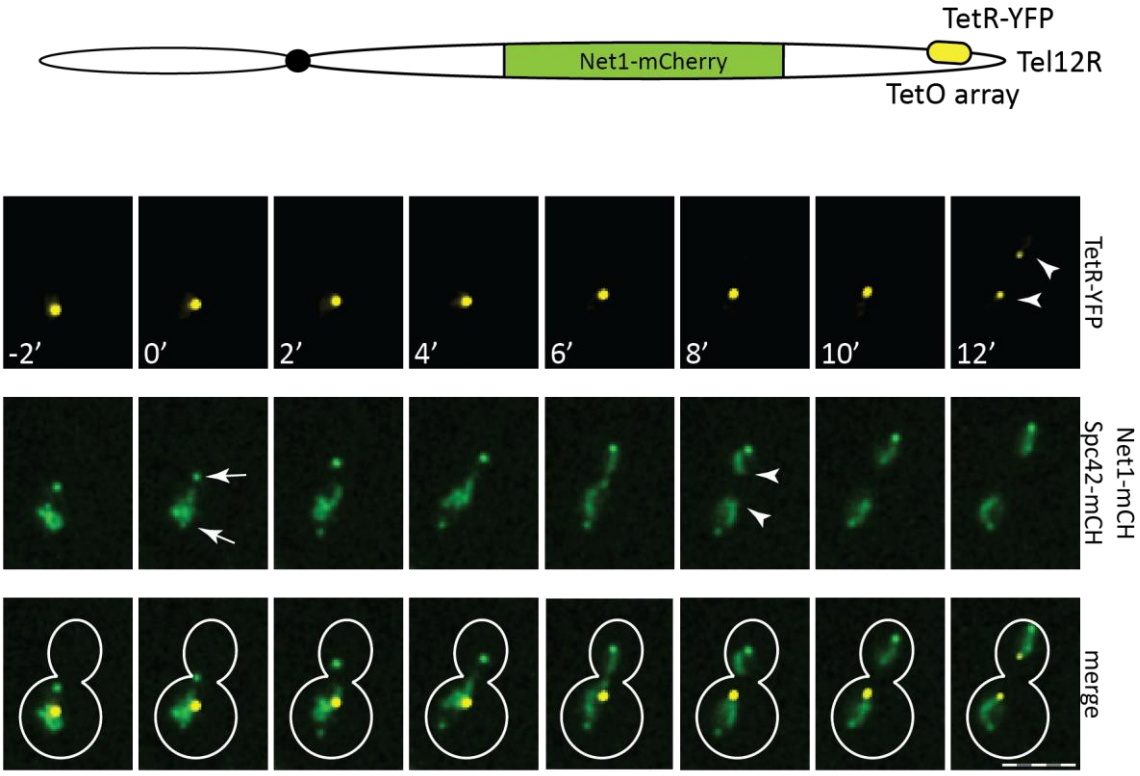
from remaining un-replicated DNA. Treatment of cells with the ribonucleotide reductase inhibitor hydroxyurea (HU) leads to a depletion of nucleotides during DNA replication and is routinely used to arrest budding yeast in S-phase.

A treatment with 100mM HU induces an S-phase arrest, therefore I tested a lower concentration. When adding HU to a concentration of 60mM, anaphase bridges were reproducibly observed in three experiments. In these experiments I analyzed a strain which harbors both a Htb2-mCherry fusion and a Myo1-GFP fusion by live time-lapse microscopy. HU was added to the sample immediately before imaging. An anaphase bridge is defined here as chromatin that is cut by the acto-myosin ring, i.e. when the acto-myosin reaches full contraction before or at the time of the disappearance of the connection between the two nuclei (Fig. 9A).

In the presence of 60mM HU, 40% of cells exhibit anaphase bridges as defined above. When plotting the timing of chromosome segregation relative to full ring contraction, the HU treated cells show bimodal distribution with one maximum at 10 minutes before ring contraction, similarly to the untreated cells, while the other maximum is found at ring contraction (Fig. 9B). Thus mild replication stress can induce anaphase bridges in at least a subpopulation of cells.

2.2.2. Replication stress does not lead to telomere 12R or rDNA mis-segregation

Telomeres and the rDNA in budding yeast are known to present obstacles to the replication fork [338, 339]. I wondered if these regions may be preferentially under-replicated after HU treatment and if it was possible to observe a resulting segregation defect. To this end I utilized a strain which contains a subtelomeric TetO array at telomere 12R, visualized by a TetR-YFP fusion, and a Net1-mCherry fusion to visualize



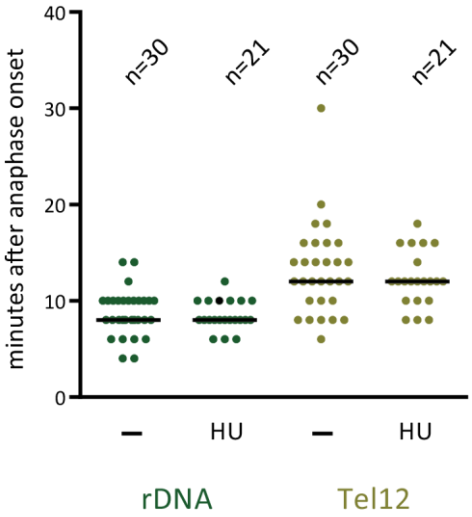


Figure 10A*) Schematic representation of the fluorescent loci on chromosome 12* [340] *.* ***10B****) Example of a HU treated cell completing segregation of the rDNA and telomere 12R. Arrows mark spindle elongation, arrowheads mark locus disjoining.* ***10C****) Quantification of the timing of locus disjoining relative to the onset of spindle elongation.*

the rDNA (Fig. 10A). In addition, this stain harbours a Spc42-mCherry fusion to visualize SPBs which allows to determine anaphase onset by spindle elongation. This strain was treated as in the experiment above, grown logarithmically before 60mM HU was added and time lapses were acquired. Segregation of the rDNA and telomere 12R were scored as the time from anaphase onset, as identified by spindle elongation, until the

time of disjoining of the respective loci (Fig. 10B). No segregation defects were observed either for the rDNA locus or for telomere 12R (Fig. 10C). It appears that the replication stress induced anaphase bridges are not concentrated in the loci tested here.

2.3. Anaphase bridges in MEN mutants

It was unexpected to find incompletely separated chromatin in mitotic exit network mutants. While Cdc14 is required for chromosome segregation, it is thought that sufficient Cdc14 function is provided by its early release through the FEAR pathway[181]. I thus chose to investigate this phenomenon in more detail.

2.3.1. MEN mutants fail to divide their chromatin

I first sought to confirm the failure of chromosome segregation in MEN mutants. To exclude the possibility that the observed defect arose from unrelated mutations that may have arisen during passaging of the strains in the arrays, I introduced the *tem1-3*, *cdc15-2*, *dbf2-2, mob1-ts* and *cdc14-1* alleles into a strain of the S288C background which harbours a Histone H2B-mCherry fusion tag.

To assess whether MEN mutants indeed arrest before they have completed chromosome segregation, I performed fixed cell time course analysis. Cells were grown to mid log-phase in YPDA and arrested for two hours with alpha factor. They were then released from the arrest into YPDA pre-warmed to 37°C and permitted to grow for a total of 3 hours. Aliquots of the cultures were fixed every twenty minutes and then imaged. The budding index and frequency of anaphase figures were determined from the acquired images (Fig. 11A).

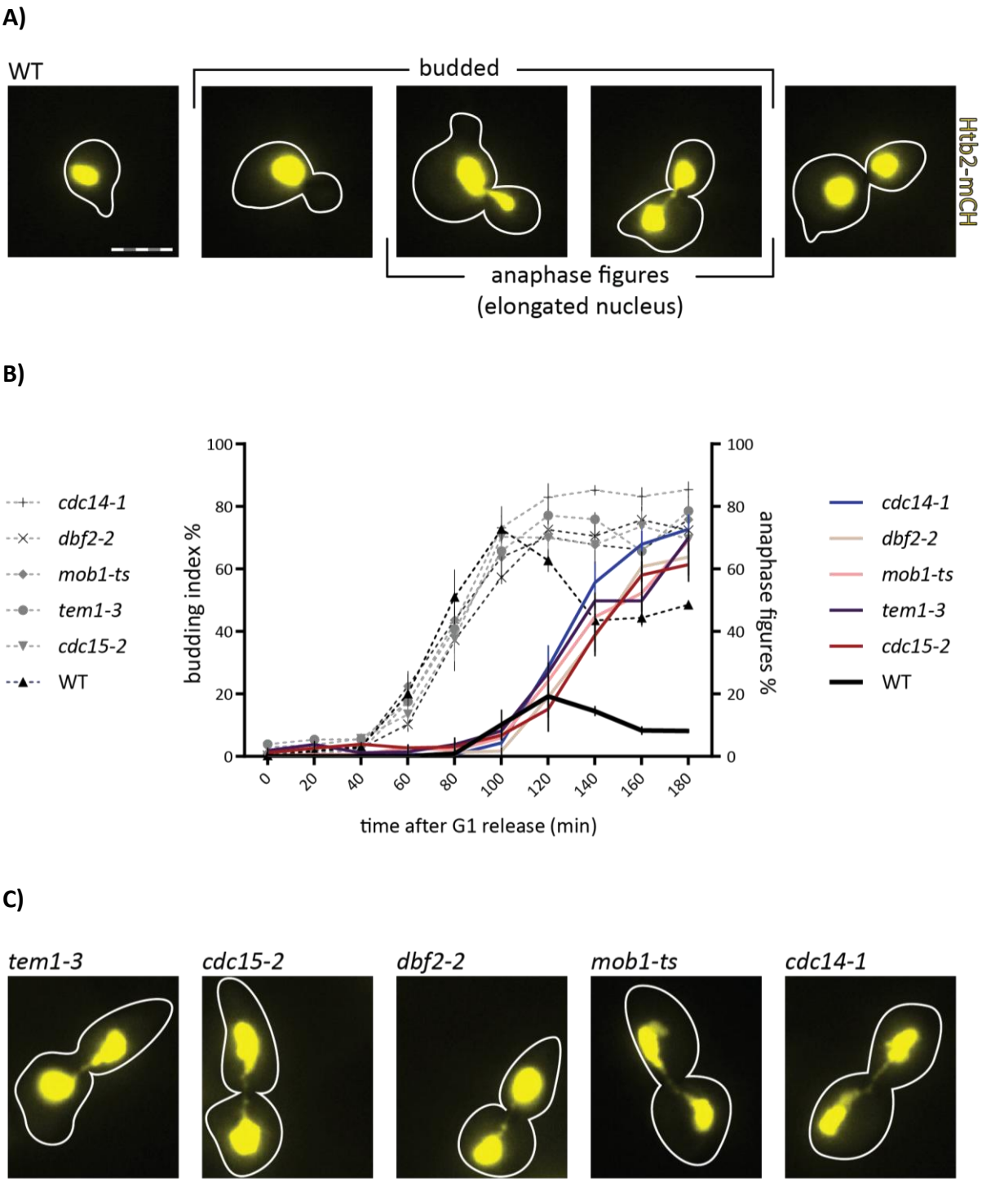


***Figure 11A**) Representative images of wild type cells at different stages during the cell cycle. Indicated are cells scored as budded and anaphase figures. **11B)** Quantification*

of budding index and anaphase figures after release from alpha factor and shift to 37°C. Error bars display standard error between experiments. **11*C)*** *Representative images of anaphase figures in MEN and CDC14 mutants.*

As expected, MEN and *cdc14-1* mutant strains arrested with large buds while the wild type completed one cell cycle and entered the next (Fig. 11B). The time of anaphase onset, as scored by the appearance of anaphase figures was similar for the wild type, the MEN mutants and the *cdc14-1* mutant. In the wild type, the percentage of anaphase figures began to drop two hours after release from alpha factor as chromosome segregation completed. MEN mutants displayed a continuous rise in the percentage of anaphase figures until the three hour time point when it reached about seventy percent (Fig. 11B). The anaphase figures in MEN mutants are almost exclusively late anaphase figures in which two separated nuclear lobes are connected by a thin bridge (Fig. 11C).

MEN mutants thus arrest in late anaphase and fail to complete chromosome segregation. The *cdc14-1* mutant behaves similarly and arrests in late anaphase with two connected nuclear lobes. Superficially, the connection between the nuclear lobe appears more pronounced in intensity and thickness in *cdc14-1* mutants when compared to MEN mutants.

2.3.2 Anaphase bridges in MEN mutants contain stainable DNA

The MEN mutant alleles used here are well studied and a role in chromosome segregation had heretofore not been ascribed to them. On the other hand budding yeast undergoes a closed mitosis which leads to the formation of a thin nuclear tube in late anaphase. This raised the possibility that bridges observed in MEN mutants correspond to unbound Htb2-mCherry diffusing through a chromatin free tube formed by the nuclear envelope. To exclude this possibility I endeavored confirm the presence of DNA in the bridge by staining it with a nucleic acid dye.

Initial attempts were confounded by a strong staining of mitochondrial DNA that would often localize in the vicinity of the presumptive bridge. I thus resorted to the use of a S288C strain that had spontaneously lost its mitochondrial DNA and become petite. Petite [ρ^0] stains arise spontaneously in budding yeast, at a comparatively high frequency in the S288C background[341].

If the petite strain is to be used as a background in which to assay for DNA segregation defects, it is necessary to confirm that this petite strain does not itself display DNA segregation defects. This is especially pertinent since the loss of mitochondrial DNA has been shown increase mutation rate and the behavior of the petite strain may differ from its parent[342]. To this end time course analysis as in Figure 11 was performed with the S288C wild type and its petite derivate. The petite strain was derived from an Htb2-mCherry carrying parent grande. After release from an alpha factor arrest and a temperature shift to 37°C, aliquots were taken and fixed every twenty minutes and the budding index and the percentage of anaphase figures was determined.

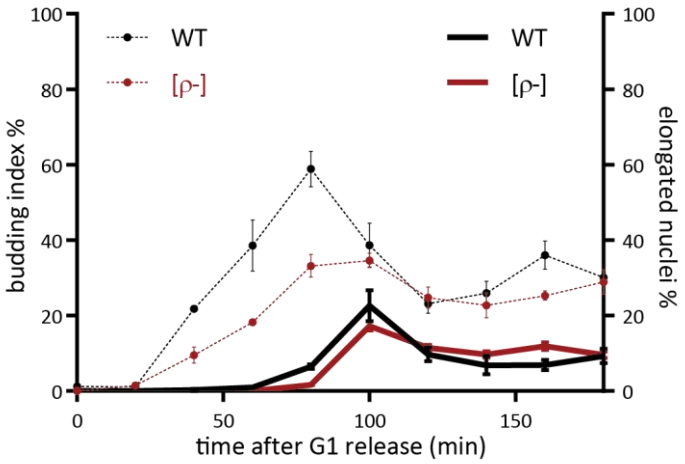


Figure 12) *Time course analysis of the petite strain used for construction of petite MEN mutants. The budding index and the percentage of anaphase figures are plotted. Error bars display standard error between experiments*

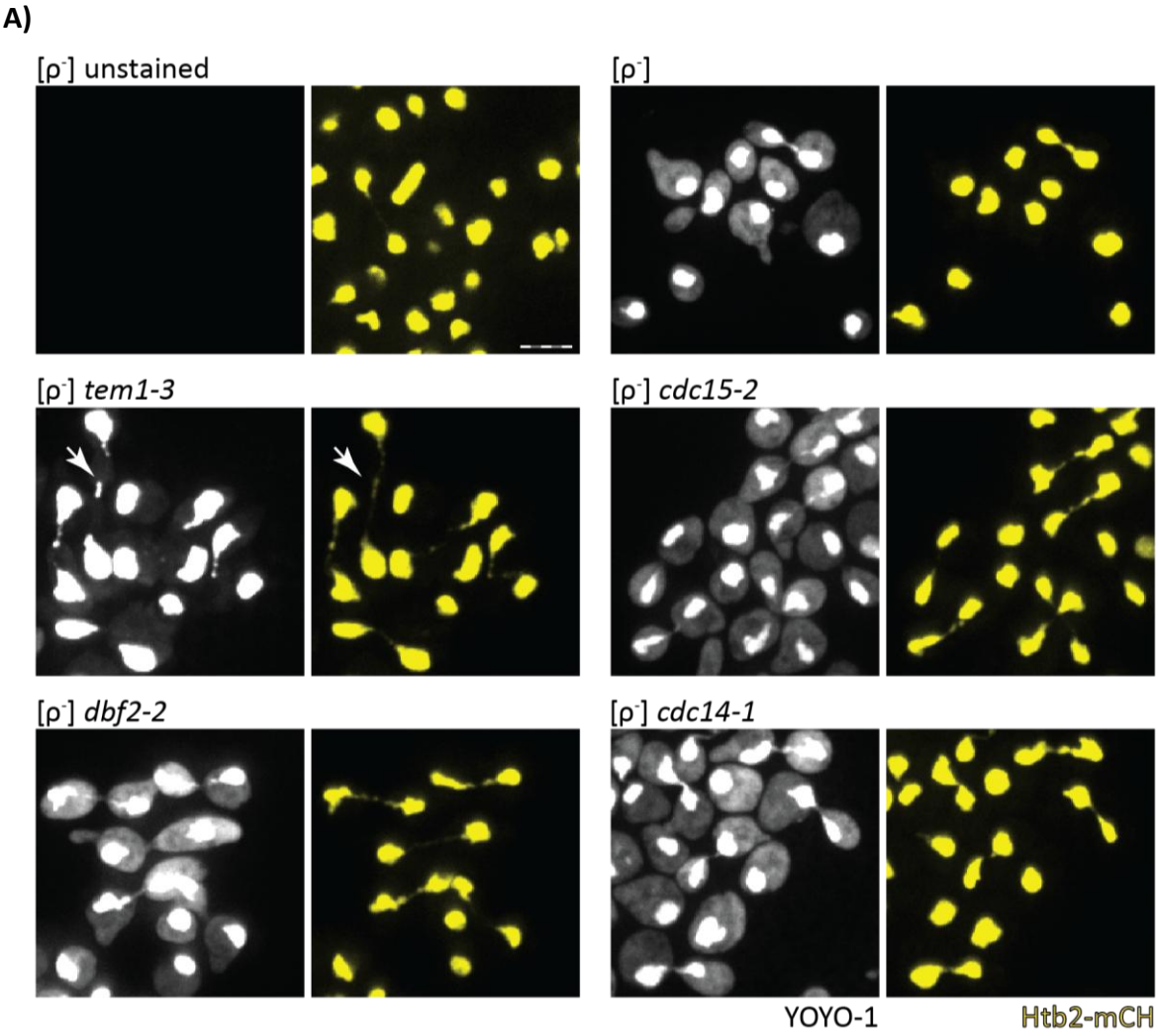

A)
[ρ-] unstained
[ρ-]
[ρ-] tem1-3
[ρ-] cdc15-2
[ρ-] dbf2-2
[ρ-] cdc14-1
YOYO-1
Htb2-mCH
B)
% anaphase figures staining for DNA
100
80
60
40
20
0
[ρ0]
tem1-3 [ρ0]
cdc15-2 [ρ0]
dbf2-2 [ρ0]
cdc14-1 [ρ0]
C)
% anaphase anaphase figures
80
60
40
20
0
[ρ0]
cdc15-2 [ρ0]
dbf2-2 [ρ0]
cdc14-1 [ρ0]
late anaphase not stained by YOYO-1
late anaphase stained by YOYO-1
early anaphase

Figure 13A) *Representative images of MEN and cdc14-1 mutants in which chromatin is labeled with Htb2-mCherry and YOYO-1* ***13B)*** *Quantification of anaphase figures, visualized with the fluorescent Htb2-mCH label, that stain with YOYO-1.Lagging chromatin marked by the arrow in the middle left panel was scored as co-localizing. Samples scored were fixed and stained after three hours of growth at 37°C following release from alpha-factor arrest. Error bars display standard error between experiments* ***13C)*** *Percentage of anaphase nuclei in the sample, average of three experiments.*

Passage through START in the petite strain appears less synchronous but at no point during the cell cycle was an overt increase of anaphase figures observed. Thus, the loss of mtDNA does not noticeably affect chromosome segregation on this level in this petite strain (Fig. 12).

Having confirmed that the petite strain does not exhibit anaphase bridges on its own, I introduced the *cdc15-2*, *dbf2-2*, and *cdc14-1* alleles into it. Wild type, MEN and *cdc14-1* mutants were harvested after three hours of growth at 37°C following a release from alpha factor. Cells were fixed in formaldehyde, digested with zymolyase and RNAse and stained with YOYO-1. Anaphase figures were first scored for whether staining by YOYO-1 is detectable on a bridge identified by Htb2-mCherry fluorescence. Anaphase figures were scored as co-localizing with YOYO-1 only when staining was complete or when there was detectable staining in the center of the bridge connecting two nuclear lobes (see Fig. 13A, middle left panel). A mass of DNA stain observed in the bridge without connection to the nuclear lobes is likely the result inaccessibility of DNA to the dye. An alternative explanation would be that inactivation of the MEN leads to detachment of chromosomes from the spindle but this was never observed in later experiments (see Figure 14). Between 40% and 50% of anaphase bridges are stained by YOYO-1 in all MEN mutant alleles tested. The anaphase figures in the *cdc14-1* mutant

stain well with YOYO-1, up to 80%, further suggesting that there may be more DNA in the anaphase bridge caused by Cdc14 inactivation (Fig. 13B).

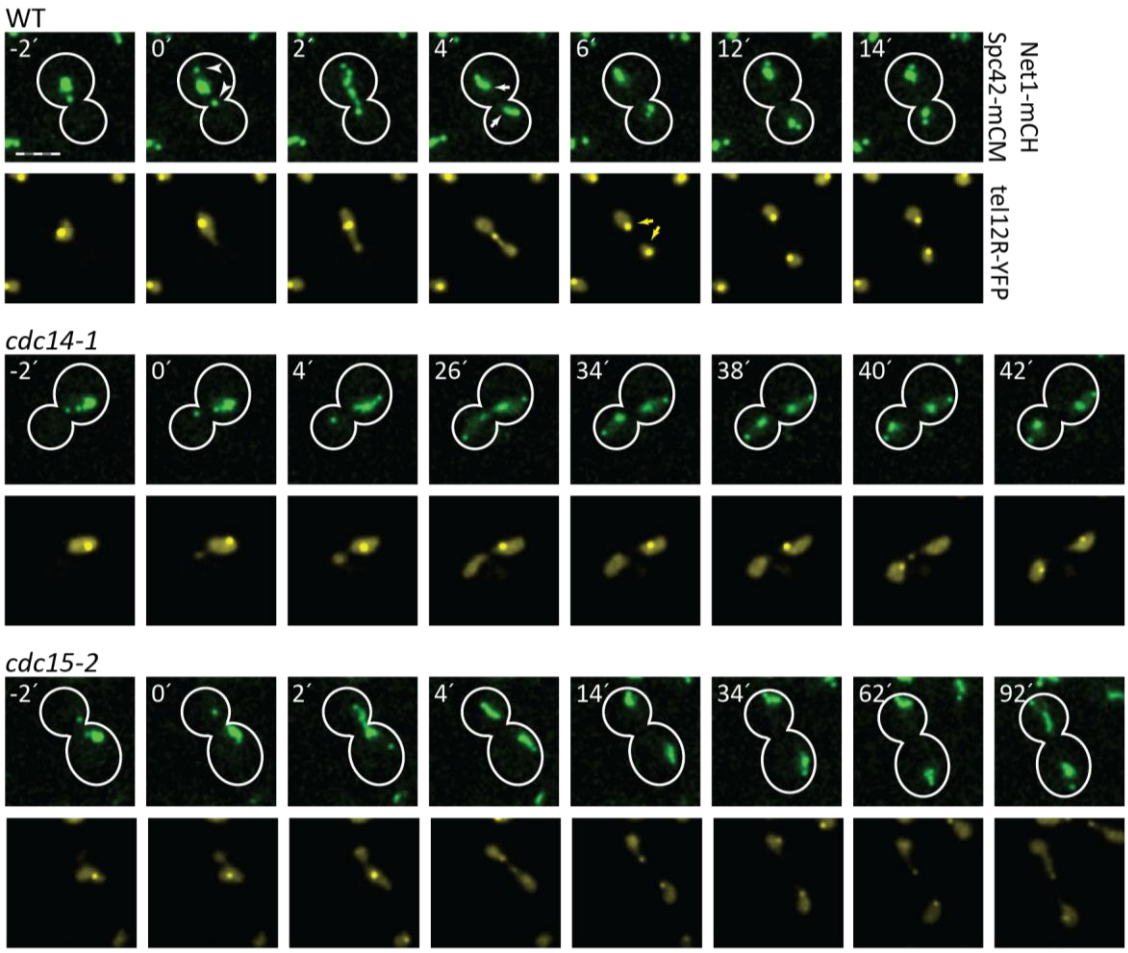
To ask whether an increase in anaphase bridges was visible by YOYO-1 staining alone, the fraction of anaphase nuclei relative to all nuclei in the sample was plotted. Three categories were considered, early anaphase nuclei which always were YOYO-1 positive, late anaphase nuclei positive for both Htb2-mCherry and YOYO-1 and late anaphase nuclei positive for Htb2-mCherry but lacking YOYO-1 staining along the connecting thread. For reasons unknown, sample preparation lead to strong cell fragmentation in the *tem1-3* mutant. The large number of fragmented cells prevented a meaningful comparison of the ratio of anaphase to total nuclei in this strain. The tem1-3 mutant is thus omitted from this plot (Figure 13C).

It is unclear if the anaphase bridges in MEN mutants that do not stain fail to do so because they do not contain DNA, because the DNA is not accessible to the dye or simply because there is too little DNA to generate a detectable signal. However the staining of almost half of the observed anaphase bridges of MEN mutants with a DNA dye corroborates the result that the MEN is required to complete chromosome segregation and indicates that these bridges contain DNA.

2.3.3 MEN and cdc14-1 mutants display distinct defects in respect to segregation of the rDNA and telomere 12R

Cdc14 has a known role in chromosome segregation. In *cdc14* mutants the separation of rDNA and telomeres is impaired[181]. Conceptually, anaphase bridges in MEN mutants may arise simply from defective Cdc14 release due to lack of MEN function. To test whether inactivation of the MEN phenocopies the segregation defects that have previously been described in Cdc14 mutants, I monitored the segregation of the rDNA and subtelomere 12R in these mutants.

A)


WT
-2´
0´
2´
4´
6´
12´
14´
Net1-mCH
Spc42-mCM
tel12R-YFP
cdc14-1
-2´
0´
4´
26´
34´
38´
40´
42´
cdc15-2
-2´
0´
2´
4´
14´
34´
62´
92´

B)

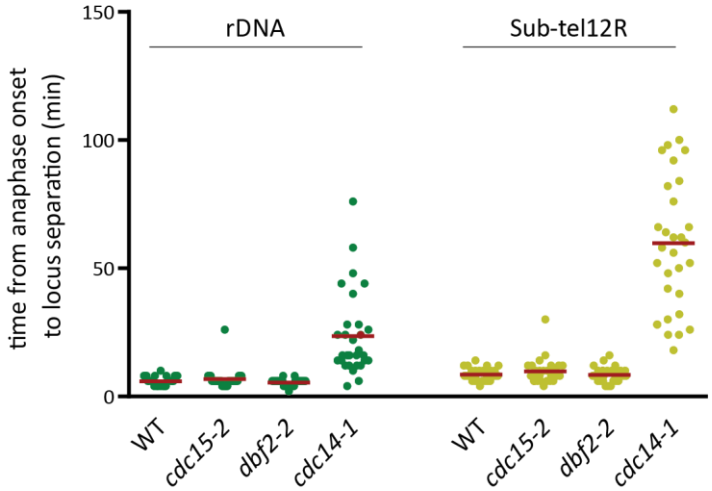
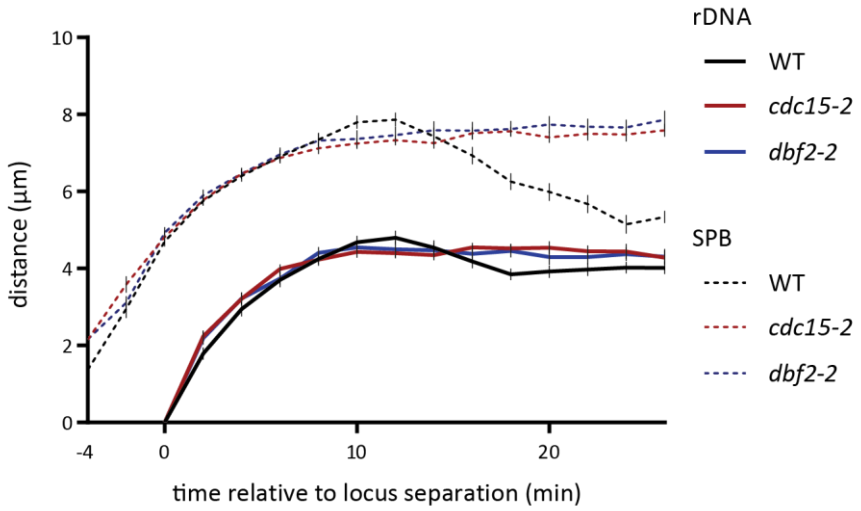

time from anaphase onset to locus separation (min)
150
100
50
0
rDNA
Sub-tel12R
WT
cdc15-2
dbf2-2
cdc14-1
WT
cdc15-2
dbf2-2
cdc14-1

Figure 14A) *Representative montages rDNA and telomere 12R segregation in wild type cells and cdc15-2 and dbf2-2 mutants. Arrowheads indicate the onset of spindle elongation, arrows indicate locus disjoining* ***14B)*** *Disjoining of the rDNA and telomere 12R relative to anaphase onset as identified by spindle elongation.*

The *cdc15-2, dbf2-2* and *cdc14-1* alleles were introduced into the previously described strain that harbors fluorescent labels at the rDNA and telomere 12R (Fig. 14A). Cells were grown to mid log-phase before they were mounted on a spinning disk microscope in an incubator that was pre-warmed to 38.5°C and time-lapses were acquired. The segregation of the two loci was analyzed by measuring the time of disjoining of the rDNA and telomere 12R relative to anaphase onset (Fig. 14A). The cdc14-1 mutant displayed the previously described segregation defect. Wild-type cells disjoin their rDNA and telomere 12R loci on average 6 and 9 minutes after anaphase onset. The *cdc14-1* mutant required on average 24 minutes and 60 minutes to disjoin the rDNA and telomere 12R respectively. (Fig. 14B). It should be noted that most of the *cdc14-1* cells completed segregation of the two loci eventually. MEN mutants display no such defect. The *cdc15-2* and *dbf2-2* mutants both separate these loci with a timing indistinguishable from wild-type cells.

I characterized the segregation kinetics of the sister-loci in more detail by measuring their distance from each other during segregation (Fig 15A/B). In MEN mutants, sister loci remained closer to each other when compared to wild type cells. Sister Telomere 12R loci on average reach a separation of around 5µm during a wild type anaphase, while this locus separates to only about 4µm during an anaphase in MEN mutants (Fig. 15 B). However this is likely accounted for by a concomitant decrease in spindle length at this time point in the *dbf2-2* and *cdc15-2* mutants (Fig15B). The same behavior is seen in the rDNA locus, albeit less pronounced (Fig. 15A). The coincident drop and the following increase in spindle length and telomere 12R distance found in the wild type signify spindle breakdown followed by nuclear reorientation in G1. Therefore, MEN mutants behave as wild types for the separation timing and kinetics of both rDNA and telomere 12 until spindle extension ceases.

A)



B)

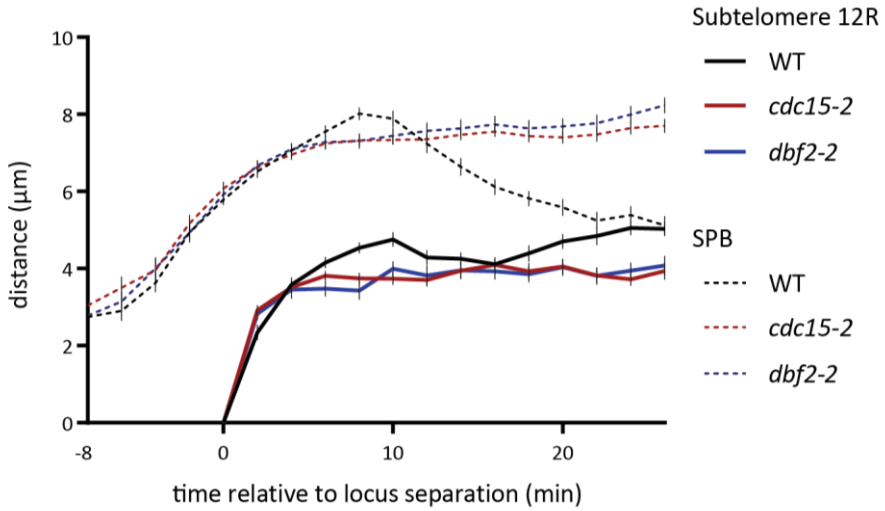
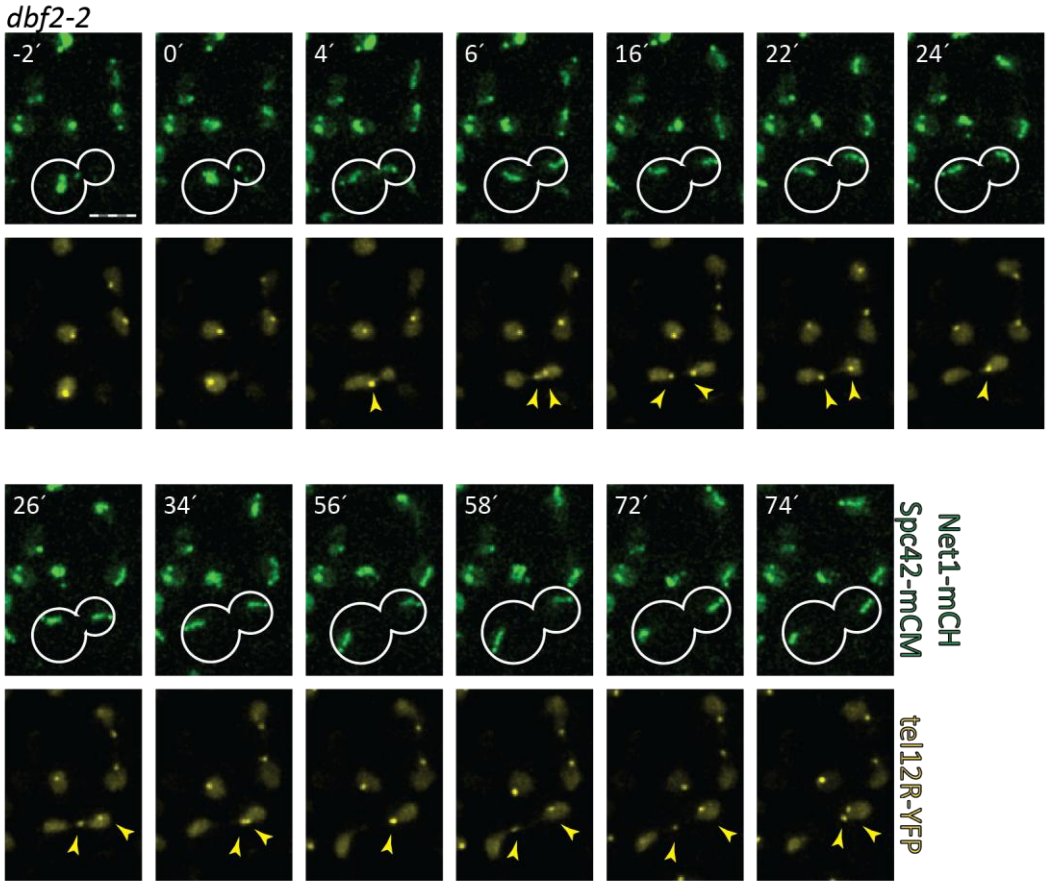


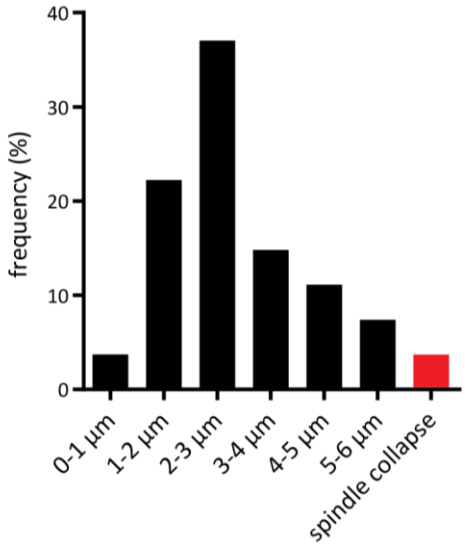
Figure 15) *Separation kinetics for the rDNA* ***(A)*** *and telomere 12R (**B**) loci. Plots are aligned to the onset of separation of the respective locus. Error bars depict standard error.*

Although telomere 12R separates on time and undergoes segregation initially there was a curious behavior of this locus at later time-points. The two sister-loci had a tendency to re-enter the nuclear tube or visit the opposite nuclear lobe, closely approaching each other and re-collapsing into a single dot in some cases (Fig. 16A). In most cases this behavior was observed in absence of an appreciable shortening of the

A)


dbf2-2
-2´
0´
4´
6´
16´
22´
24´
26´
34´
56´
58´
72´
74´
Net1-mCH
Spc42-mCM
tel12R-YFP

B)


frequency (%)
40
30
20
10
0
0-1 µm
1-2 µm
2-3 µm
3-4 µm
4-5 µm
5-6 µm
spindle collapse

C)

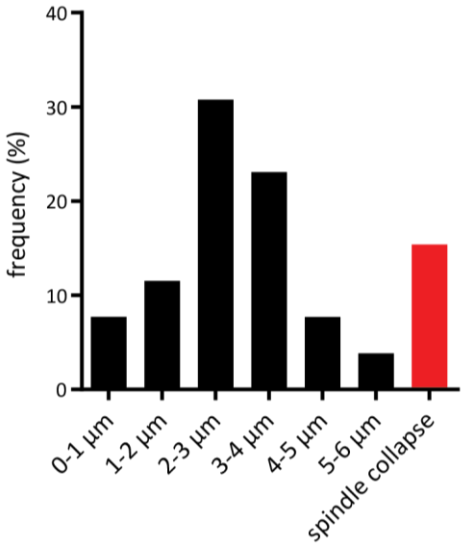

frequency (%)
40
30
20
10
0
0-1 µm
1-2 µm
2-3 µm
3-4 µm
4-5 µm
5-6 µm
spindle collapse

Figure 16A) *Telomere re-collapse example in a dbf2-2 mutant.* ***16B)*** *Binned distances of closest reproach of two telomeres after initial segregation in cdc15-2(**B**) and dbf2-2(**C**) mutants. Black bars are re-approaches in absence of appreciable spindle collapse. Red bars signify re-approaches concomitant with spindle collapse*

spindle (Fig 16A). The behavior telomere 12R varied between cells and an re-approach of the sister loci to 2µm or less was seen in about 20% of cells but a full collapse as in Figure 16A was only observed in a few individual cells (Fig.16 B/C).

2.3.4 Anaphase bridges in MEN mutants are resolved by inactivation of Cdc13 or DNA Polymerase alpha

Due to the curious telomere behavior described earlier, I wondered whether telomere-proximal sister chromatid links may be responsible for the formation of anaphase bridges in MEN mutants. Cdc13 is one component of the RPA like CST complex and in budding yeast promotes telomerase recruitment, telomere lagging strand synthesis and telomere capping functions. In coordinating these functions, it acts as a central factor in telomere maintenance[343].

The fact that MEN mutants not only display anaphase bridges but also arrest in anaphase affords the unique possibility to inactivate potential factors responsible for sister chromatid links and observe the immediate effect on the bridge. To test whether Cdc13 may be involved in the maintenance of anaphase bridges in MEN mutants, I constructed a *cdc15-as1* single and a *cdc13-1 cdc15-as1* double mutant which also contained fluorescent Htb2-mCherry. *The cdc15-as1* allele sensitizes Cdc15 kinase activity to inhibition by 1NA-PP1[344]. The combination of an analog sensitive allele of Cdc15 with the temperature sensitive Cdc13 mutation *cdc13-1* allows independent inactivation of the two proteins.

In order to test whether anaphase bridges in MEN mutants may arise from telomeric links, I inactivated Cdc13 in cells in which anaphase bridges had been generated by inhibition of Cdc15. The single and double mutant were both arrested by the addition of 2.5µM 1NA-PP1 for three hours to generate bridges. In the presence of the inhibitor, the cells were then shifted to 38.5°C by mounting them on a spinning disk microscope in a preheated incubator (Fig 17A). Live time-lapses were acquired and analyzed by monitoring the persistence of anaphase bridges. MEN bridges are largely stable over a period of three hours after the temperature shift with a resolution rate of about 10% in the *cdc15-as1* mutant (Fig. 18B).

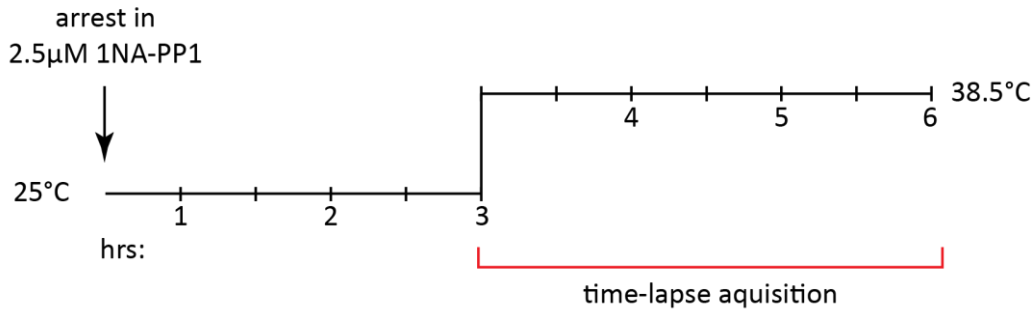


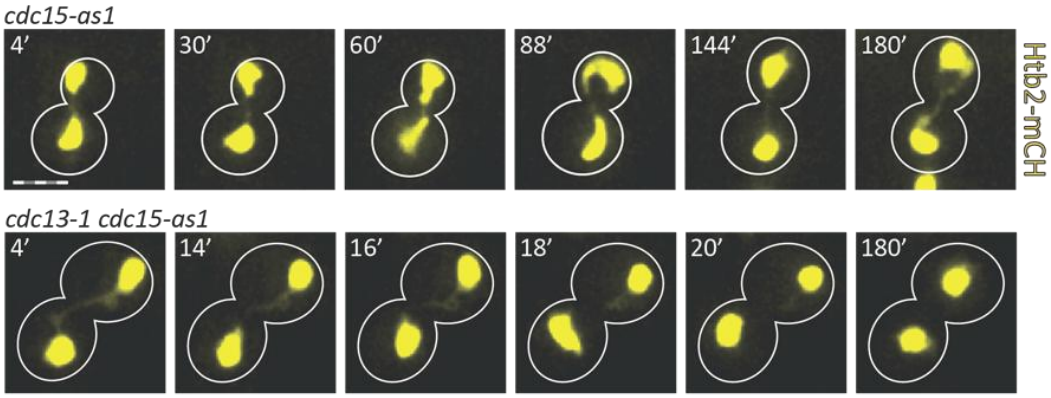
Figure 17) *Experimental protocol for sequential inactivation of Cdc15 and Cdc13*

However, in the *cdc15-as1 cdc13-1* double mutant 50% of the previously established anaphase bridges resolved after inactivation of Cdc13 (Fig 18A/B).

Inactivation of Cdc13 may have an effect on the MEN arrest itself. The observed disappearance of anaphase bridges may have been due an anaphase exit and cytokinesis rather than actual bridge resolution. To exclude this possibility, I introduced a Myo1-GFP fusion to be able to observe whether the actomyosin ring contracted during bridge disappearance. Bridge disappearance in the *cdc15-as1 cdc13-*

1 double mutant occurs in absence of ring contraction and therefore these cells undergo bona fide bridge resolution (Fig 19A/B). About 65% of bridges resolved within a three hour window, comparable to the previous results. At this point I need to anticipate one of the following sections. DNA Polα was tested initially for the requirement of replication for the resolution of anaphase bridges.

A)



B)

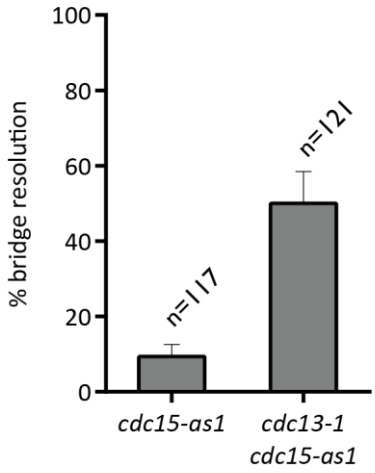
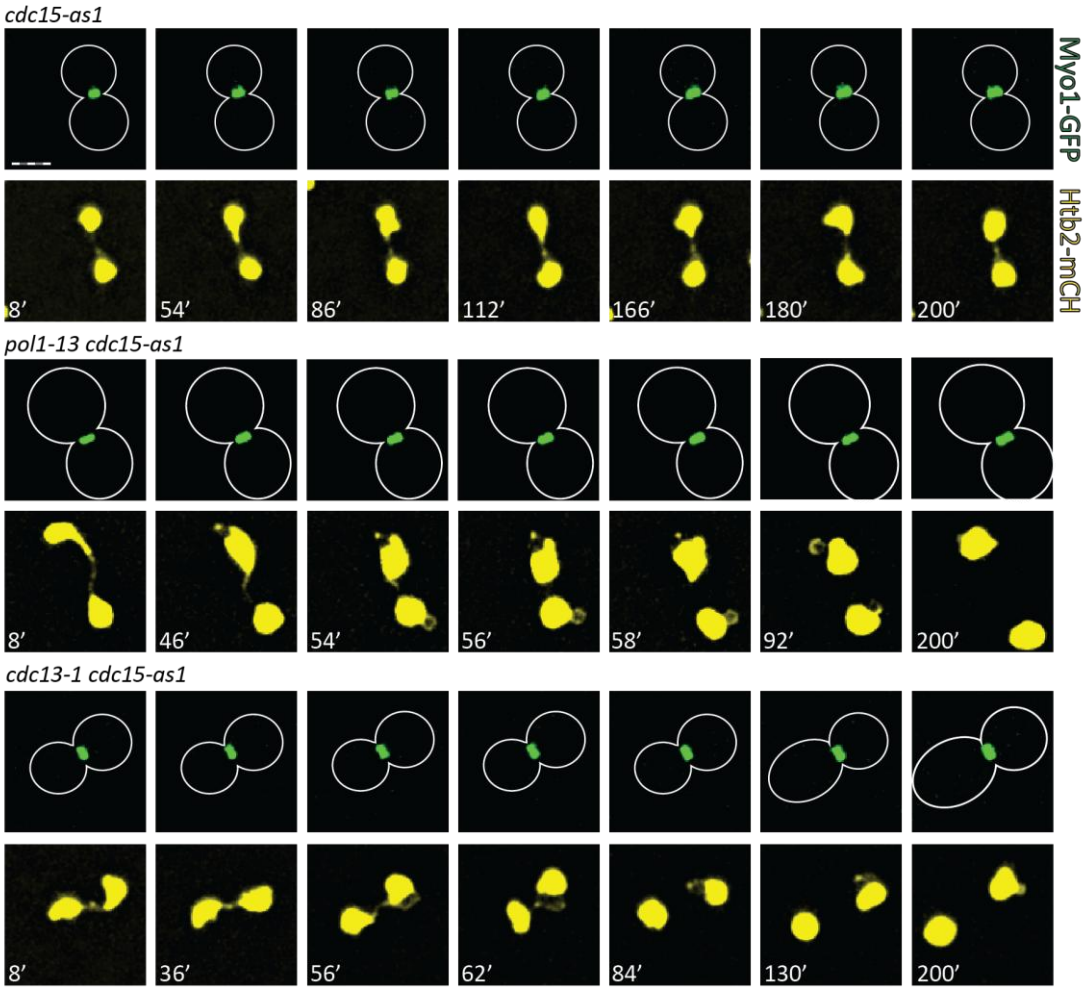


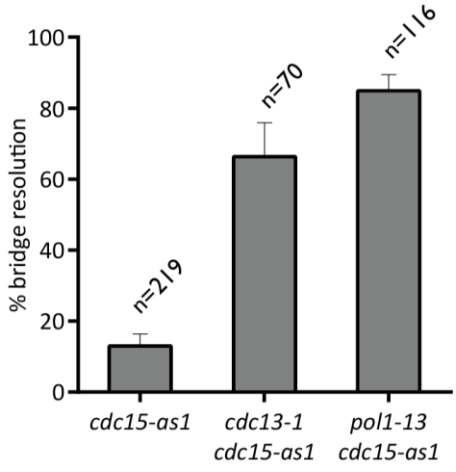
Figure 18A) *Representative montages of a stable anaphase bridge in the cdc15-as1 mutant and the resolution of a bridge in the cdc13-1 cdc15-as1 double mutant* ***18B)*** *Quantification of bridge resolution in the single and double mutant. Error bars display standard error between experiments.*

These experiments, as will be detailed later, require an early temperature shift, 30 minutes before image acquisition. In those experiments most cells of the *cdc15-as1 Pol1-13* (DNA Polα) double mutant were found in a state that suggested resolution had happened before image acquisition, with two separated, unlinked nuclei and an un-contracted actomyosin ring. This raised the possibility that inactivation of

A)


cdc15-as1
Myo1-GFP
Htb2-mCH
8′
54′
86′
112′
166′
180′
200′
pol1-13 cdc15-as1
8′
46′
54′
56′
58′
92′
200′
cdc13-1 cdc15-as1
8′
36′
56′
62′
84′
130′
200′

B)


% bridge resolution
100
80
60
40
20
0
n=219
n=70
n=116
cdc15-as1
cdc13-1 cdc15-as1
pol1-13 cdc15-as1

C)

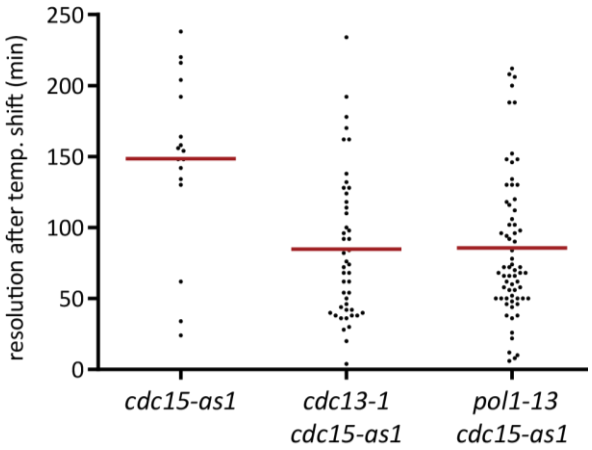

resolution after temp. shift (min)
250
200
150
100
50
0
cdc15-as1
cdc13-1 cdc15-as1
pol1-13 cdc15-as1

Figure 19A) *Representative montages of a stable anaphase bridge in the cdc15-as1 mutant and the resolution of anaphase bridges in absence of actomyosin ring contraction in the cdc13-1 cdc15-as1 and pol1-13 cdc15-as1 double mutants. Time zero indicates begin of acquisition immediately after temperate shift* ***19B)*** *Quantification of bridge resolution in the single and double mutant. Error bars display standard error between experiments* ***19C)*** *Time of bridge resolution relative to start of acquisition.*

DNA Polα may lead to anaphase bridge resolution, intriguing because of known physical and functional interactions between the CST complex and DNA Polα. In particular, the Pol1 subunit of DNA Polα binds to Cdc13 while the Pol12 subunit binds to Stn1, a second member of the CST complex. In addition DNA Polα in is required for telomere elongation[345-347].

It became necessary to test whether inactivation of DNA Polα alpha may also lead to anaphase bridge resolution. I thus repeated the same assay with a *cdc15-as1 pol1-13* double mutant. Like Cdc13 inactivation, DNA Polα inactivation leads to resolution of MEN bridges in absence of actomyosin ring contraction(Fig 19A/B). The kinetics of the resolution process are similar, with an average of about 80 minutes to resolution after the temperature shift (Fig. 19C).

2.3.5 Aurora B is not required for maintenance of MEN bridges

The MEN has been suggested to control Aurora B localization to the spindle midzone by antagonizing CDK1 phosphorylation of the CPC[348]. Aurora B on the other hand has been described to control telomere associated processes such as the eviction of telomerase[349]. Thus the effect of the MEN on chromosome segregation may be mediated indirectly by mis-regulation of Aurora B.

One possibility is that that Aurora B is involved in the formation of an anaphase bridge by controlling a process that maintains sister chromatid links. Here, the impaired recruitment of Aurora B to the spindle midzone may lead to its continued association with chromatin and a failure to remove sister chromatid links.

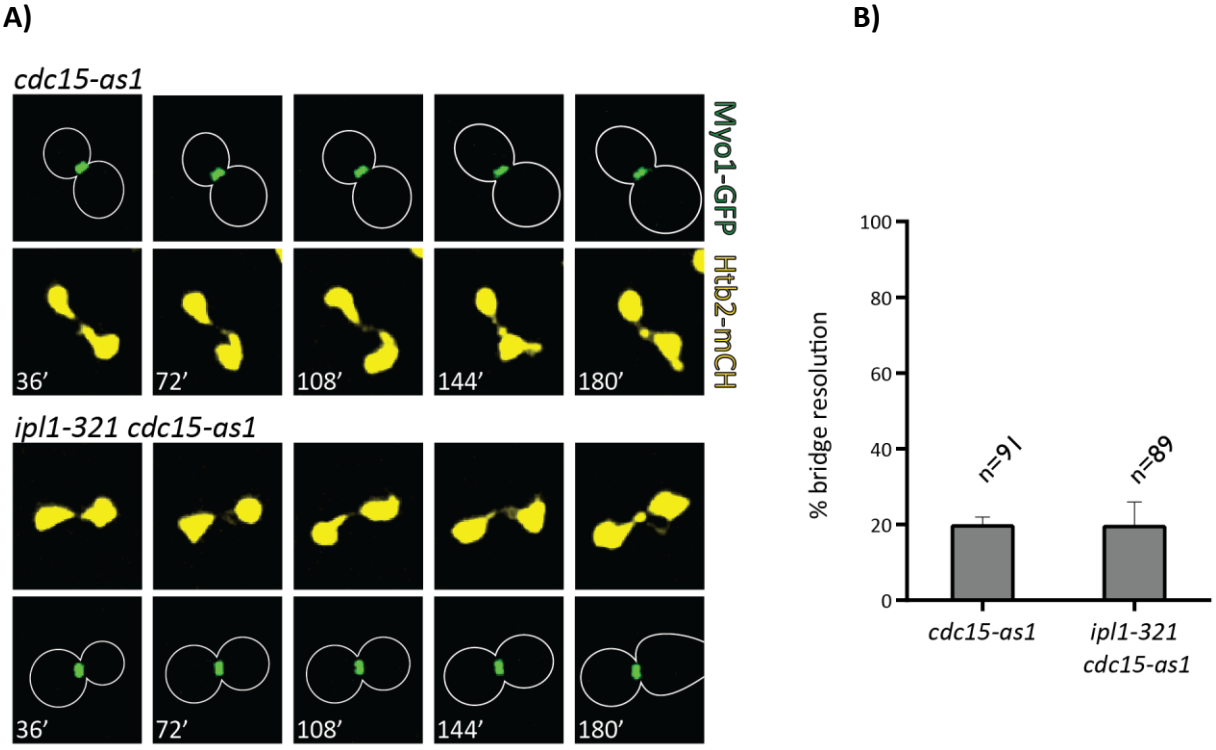


Figure 20A) *Representative montages of stable MEN bridges in cdc15-as1 and ipl1-321 cdc15-as1 double mutant. Time zero indicates begin of acquisition immediately after temperate shift.* ***20B)*** *Quantification of MEN bridge resolution in the single and double mutant. Error bars display standard error between experiments*

To test this, I asked whether inactivation of Aurora B would lead to resolution of anaphase bridges in the same assay previously utilized to test the role of Cdc13 and Polα. A temperature shift of the *cdc15-as1 ipl1-321* double mutant did not induce an increased resolution of anaphase bridges when compared to the *cdc15-as1* single mutant. Thus inactivation of Aurora B does not appear to promote anaphase bridge resolution. (Fig. 20 A/B)

2.3.6 Anaphase bridges in MEN mutants resolve independently of Topoisomerase 2

Budding yeast enters anaphase with catenated sister chromatids and topoisomerase 2 is required for chromosome segregation even after the metaphase-anaphase transition [96]. Persisting catenation of sister chromatids at the execution point of the MEN is another possible cause for MEN bridges. If this were the case, topoisomerase 2 should be required for resolution of a MEN bridge after MEN activity has been restored.

To test this, I analyzed the segregation of anaphase bridges after reactivation of Cdc15 in absence or presence of topoisomerase 2 function. The strains used harbored both Myo1-GFP and Htb2-mCherry. I expected anaphase bridges to resolve before the actomyosin had contracted after reactivating Cdc15. This was indeed the case in the majority of cells. Only a small fraction fully contracted their ring before chromosome segregation had completed and presented a cut phenotype (Fig. 22A/B). I found that a release into medium containing 0.25µM 1NA-PP1 instead of inhibitor free medium yielded less variability between experiments with respect to the prevalence in cut phenotypes. Thus in this and further Cdc15 release experiments, cells were released into medium that contained 0.25µM of the inhibitor.

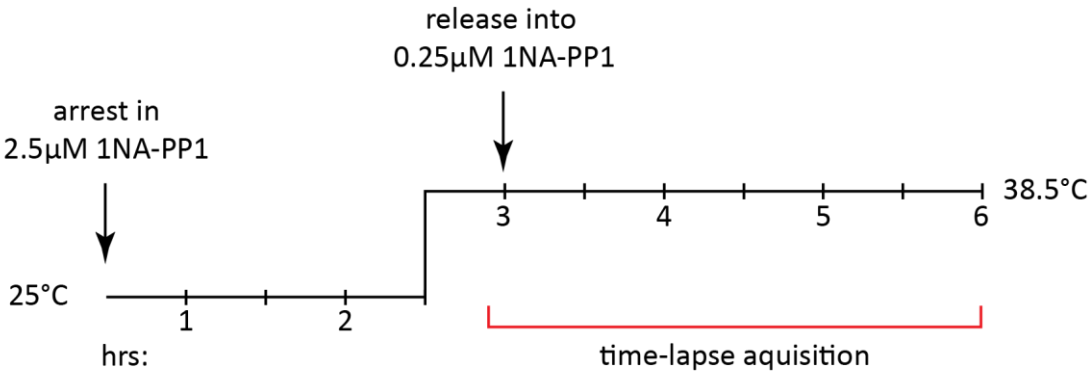


Figure 21) *Treatment regime for the inactivation of Top2 prior to reactivation of Cdc15.*

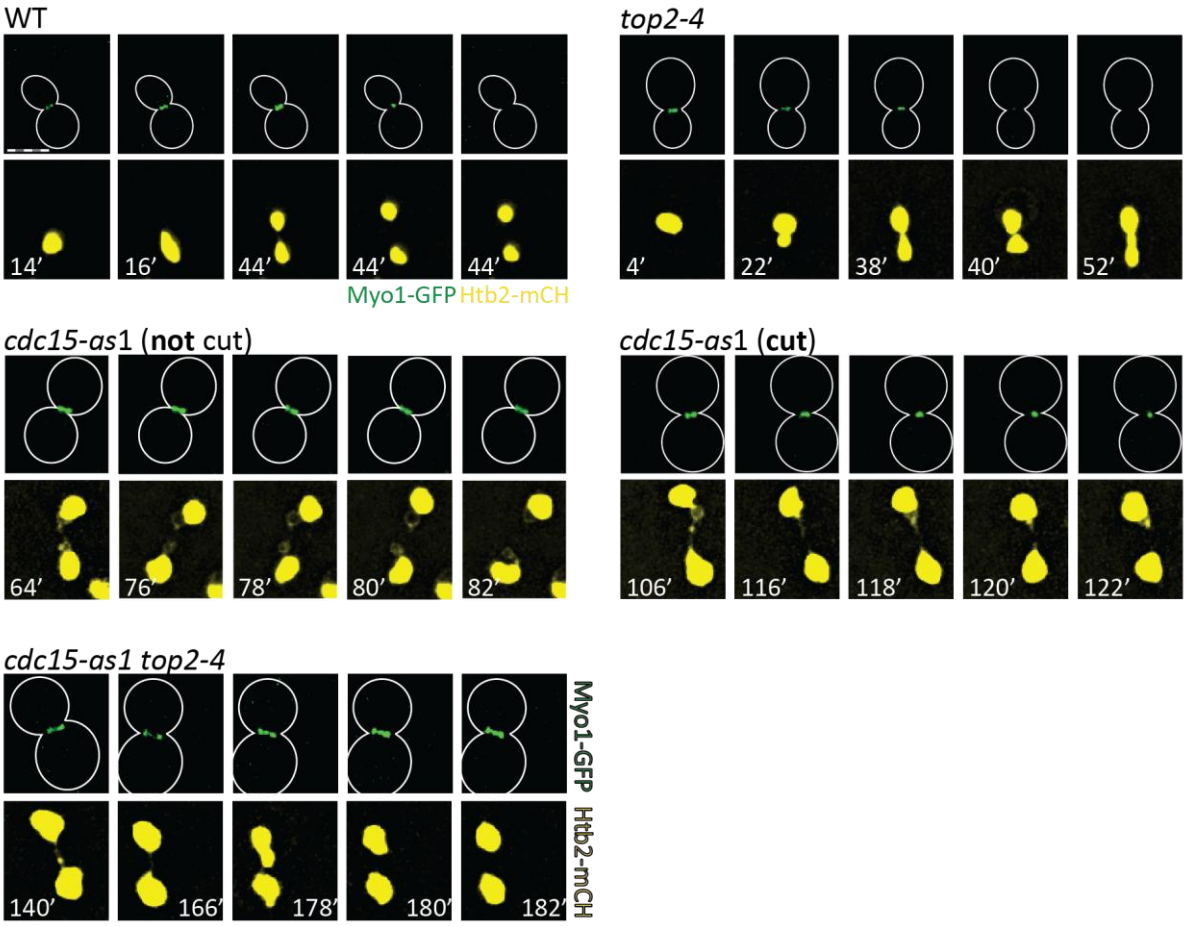
If topoisomerase 2 is required for the resolution of anaphase bridges after reactivation of Cdc15, then releasing cells from a Cdc15 arrest in absence of topoisomerase 2 function should lead to an increase in cut phenotypes. To test this, anaphase bridges were induced in *cdc15-as1* single and *top2-4 cdc15-as1* double mutants by addition of 1NA-PP1 for 2.5 hours. The cultures were then shifted to 38.5°C for thirty minutes to inactivate Top2-4 in the presence of the inhibitor. Finally cells were released from the arrest while maintaining them at restrictive temperature for *top2-4* to allow exit from the MEN arrest in absence of topoisomerase 2 function (Fig. 21). Time lapses were acquired and the time of resolution of the MEN bridge after release was monitored relative to the contraction of the actomyosin ring. The *top2-4* single mutant serves as control to confirm inactivation of Top2 under these conditions.

While there is no cell cycle arrest in response to the inhibitor, by the time of release, virtually all *top2-4* cells display a cut phenotype when they enter anaphase. This confirms the inactivation of Top2 at the beginning of the experiment. After release, the vast majority of MEN bridges resolve before the actomyosin ring has contracted. Inactivation of topoisomerase 2 after a MEN release does not lead to an increase in cut phenotypes suggesting that it is not required for chromosome segregation after the execution point of Cdc15 (Fig22 A/B).

2.3.7 Aurora B is not required for the resolution of anaphase bridges in MEN mutants

As detailed earlier, the MEN may be required for the resolution of anaphase bridges indirectly, through controlling the localization of Aurora B to the spindle midzone (see section 2.3.5). To investigate a potential requirement of Aurora B for MEN bridge resolution, I employed the MEN release assay. A *cdc15-as1 ipl1-321* was constructed and imaged alongside a wild type and the *cdc15-as1* and *ipl1-321* single mutants.

A)


WT
14′
16′
44′
44′
44′
Myo1-GFP Htb2-mCH
top2-4
4′
22′
38′
40′
52′
cdc15-as1 (not cut)
64′
76′
78′
80′
82′
cdc15-as1 (cut)
106′
116′
118′
120′
122′
cdc15-as1 top2-4
140′
166′
178′
180′
182′
Myo1-GFP
Htb2-mCH

B)

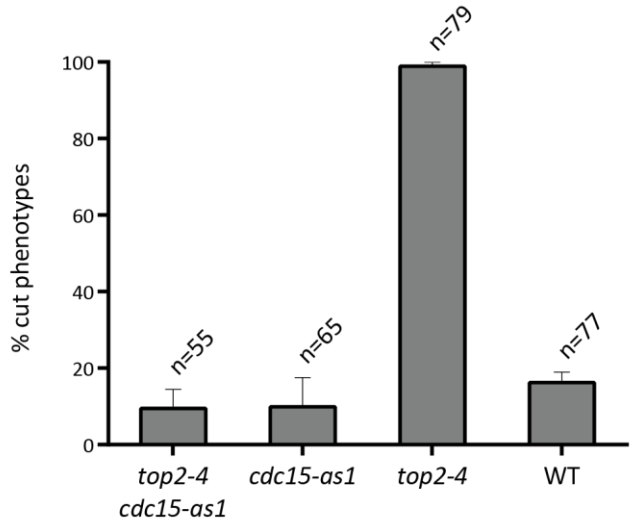

% cut phenotypes
100
80
60
40
20
0
n=55
n=65
n=79
n=77
top2-4 cdc15-as1
cdc15-as1
top2-4
WT

Figure 22A) *Representative montages of wild type, top2-4, cdc15-as1 and the top2-4 cdc15-as1 double mutant after inhibitor washout at 38.5°C. Chromosome segregation events scored as cut phenotypes are depicted in the top and middle right panel. Time zero indicates begin of acquisition immediately after release* ***17B)*** *Quantification of frequency of cut phenotypes. Error bars display standard error between experiments*

Inactivation of Aurora B under the experimental conditions was confirmed by comparison of the wild type and the *ipl1-321* single mutant. Aurora B is required for both the SAC and the biorientation of chromosomes on the spindle[350]. In absence of Aurora B function, chromosomes are partitioned unequally between daughters[351]. To confirm Aurora B inactivation, Htb2-mCherry fluorescence intensity was measured in the frame after the resolution of the last chromatin connection between the two nascent nuclei. The ratio between the fluorescence intensity of the two daughter nuclei was used to calculate chromatin asymmetry to demonstrates inactivation of Aurora B in this experiment (Fig. 23A/B/C).

The majority of MEN bridges are able to resolve before actomyosin ring contraction in absence of Aurora B function, but there is a slight increase in cut phenotypes when compared to the release of the *cdc15-as1* single mutant (Figure 20D/E). However a similar small increase in cut phenotypes was observed in the *ipl1-321* single mutant

2.3.8 DNA polymerase delta is required to resolve anaphase bridges in MEN mutants

Another possible origin of persisting chromatin bridges in MEN mutants may be un-replicated DNA. To test this, I asked whether MEN bridges require DNA polymerases to resolve. Of the two major replicative DNA Polδ and DNA Polε, only the activity of DNA DNA Polδ is essential, and it has been suggested that DNA Polδ can cover for the loss of a catalytic function in DNA Polε[352]. Therefore I chose to test whether

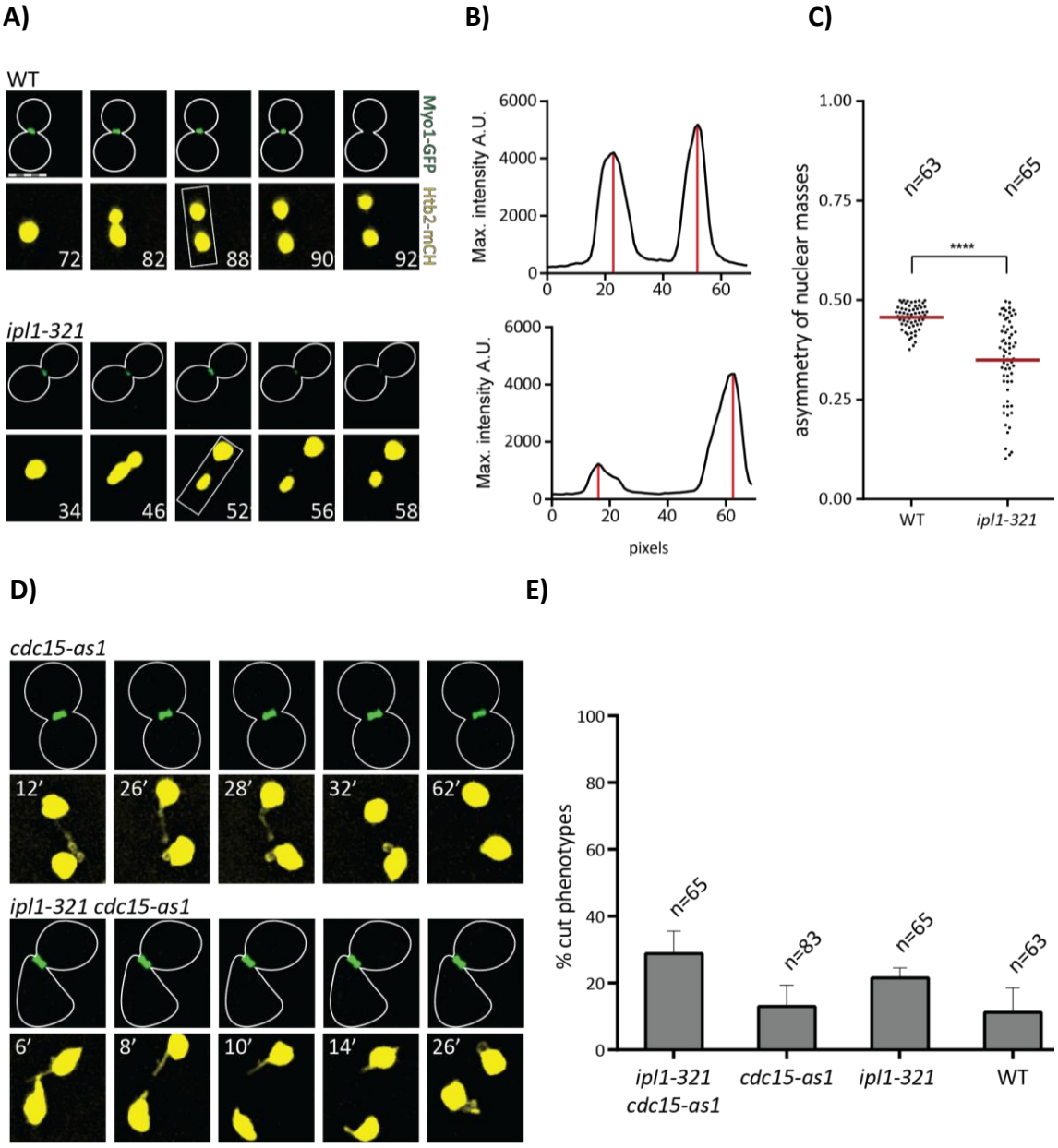


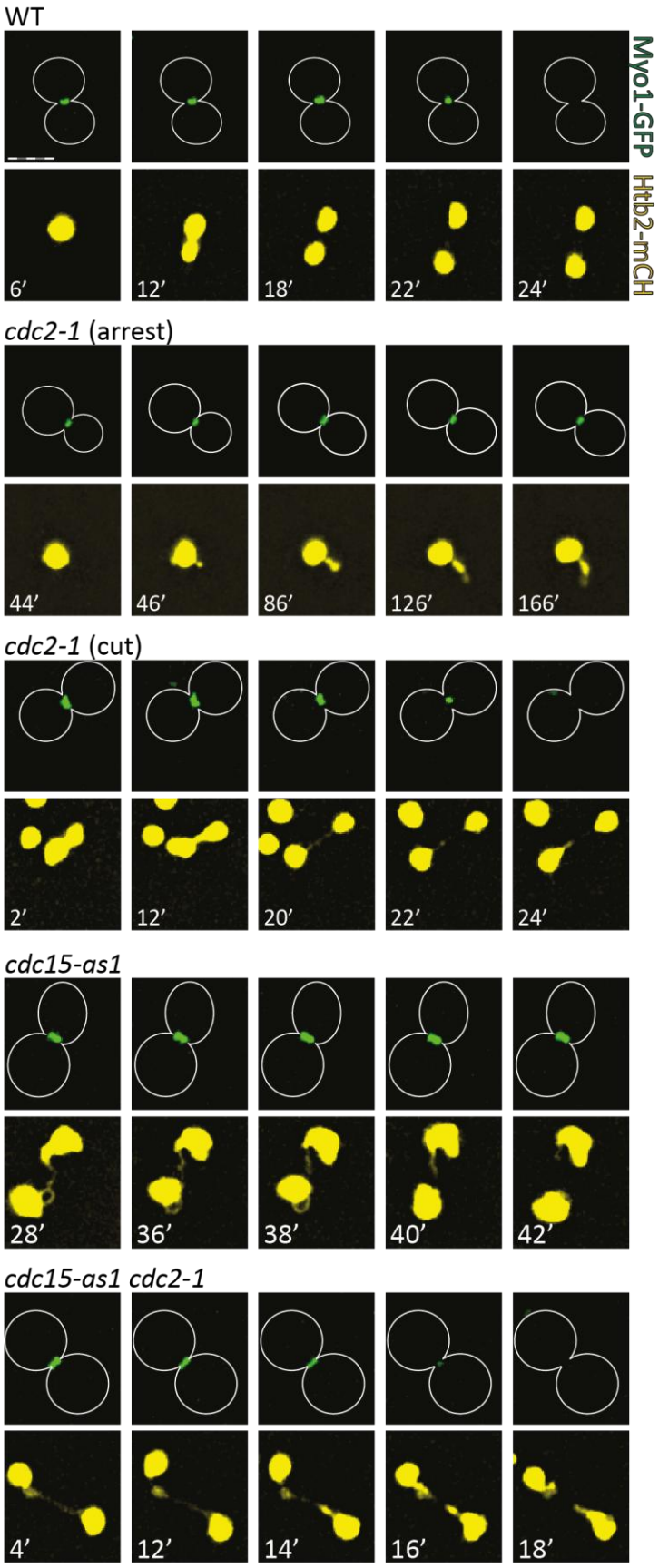
Figure 23A) *Representative montages of chromosome segregation in wild type and ipl1-321 cells after inhibitor removal.* ***23B)*** *Peak intensity measurement to confirm asymmetric chromatin segregation and Ipl1 inactivation in ipl1-321 mutant* ***23C)*** *Plot of the ratio of small nucleus fluorescence intensity relative to intensity of both daughter nuclei.* **23D)** *Representative montages of chromosome segregation in cdc15-as1 and cdc15-as1 ipl1-321 double mutants after release. Time zero indicates begin of acquisition immediately after release.* ***23E)*** *Quantification of cut phenotypes. Error bars display standard error between experiments*

DNA Polδ was required for the resolution of MEN bridges by employing the MEN release assay. Initially DNA Polα was tested for the same reason using a MEN release assay. The MEN release assay entails a temperature shift 30 minutes before MEN release and image acquisition. By the time image acquisition started, most *pol1-13 cdc15-as1* double mutants were found with open myosin rings but unconnected nuclei and as detailed before, a loss of Polα leads to resolution of anaphase bridges.

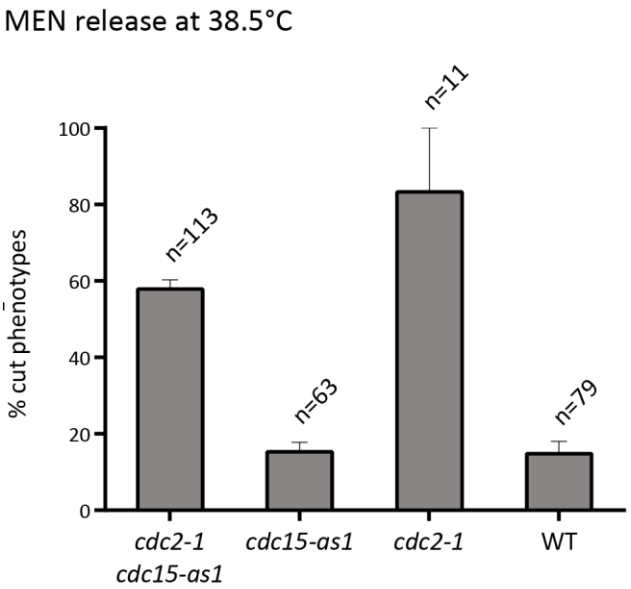
Employing the MEN release assay, I tested whether the inactivation of DNA Polδ delta (Cdc2) would affect bridge resolution upon release from a MEN arrest. As a control that *cdc2-1* was inactive, I acquired the *cdc2-1* single mutant in parallel and quantified the fraction of G2/M arrested nuclei (Fig 24A). More than 95% of cdc2-1 cells did not enter anaphase for the duration of acquisition and often displayed nucleopodia-like structures previously that are known to arise in a G2/M arrest in budding yeast[353] (Fig 24D). Of the few cdc2-1 mutant cells that did enter anaphase, the majority displayed a cut phenotype (Fig 24A/B). This indicates that DNA Polδ was inactive at the beginning of the experiment. Surprisingly, about 60% of *cdc2-1 cdc15-as1* double mutants displayed a cut phenotype after release from the MEN arrest on absence of DNA Polδ function(Fig 24A/B). This indicates that DNA replication is required for the resolution of MEN bridges.

It has been reported that *cdc14-1* mutants grown at permissive temperature traverse the cell cycle with a under-replicated DNA that is not detected by the replication checkpoint[354]. This raised the possibility that the resolution defects observed in the *cdc2-1 cdc15-as1* double mutant may arise from a hypomorphic behavior of the *cdc2-1* allele in the S-phase preceding the temperature shift and not from a requirement for Cdc2 after the MEN release. If this were the case, the defect in MEN bridge resolution in the double mutant should be observable at the permissive temperature for the *cdc2-1* allele. Thus the release experiments were repeated at room temperature.

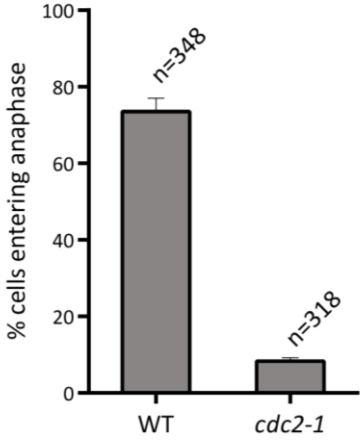
A)


WT
Myo1-GFP
Htb2-mCH
6′
12′
18′
22′
24′
cdc2-1 (arrest)
44′
46′
86′
126′
166′
cdc2-1 (cut)
2′
12′
20′
22′
24′
cdc15-as1
28′
36′
38′
40′
42′
cdc15-as1 cdc2-1
4′
12′
14′
16′
18′

B)



D)



C)

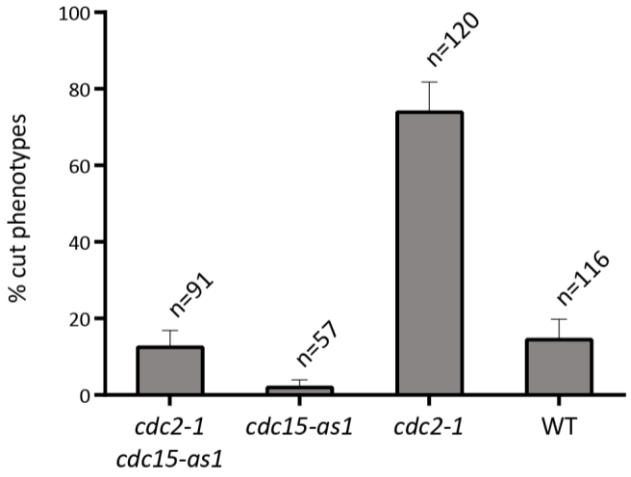


Figure 24A) *Representative montages of wild-type, cdc2-1, cdc15-as1 and cdc2-1 cdc15-as1 double mutants after inhibitor wash-out at 38.5°C. Time zero indicates begin of acquisition immediately after temperate shift.* ***24B)*** *Quantification of cut phenotypes after release at 38.5°C. Error bars display standard error between experiments.* ***24C)*** *Quantification of cut phenotypes after release at 25°C.* ***24D)*** *Quantification of cell cycle arrest in wt and cdc2-1 mutants. Plotted are percentage of cells that enter anaphase during the duration of acquisition. Error bars display standard error between experiments. Error bars display standard error between experiments.*

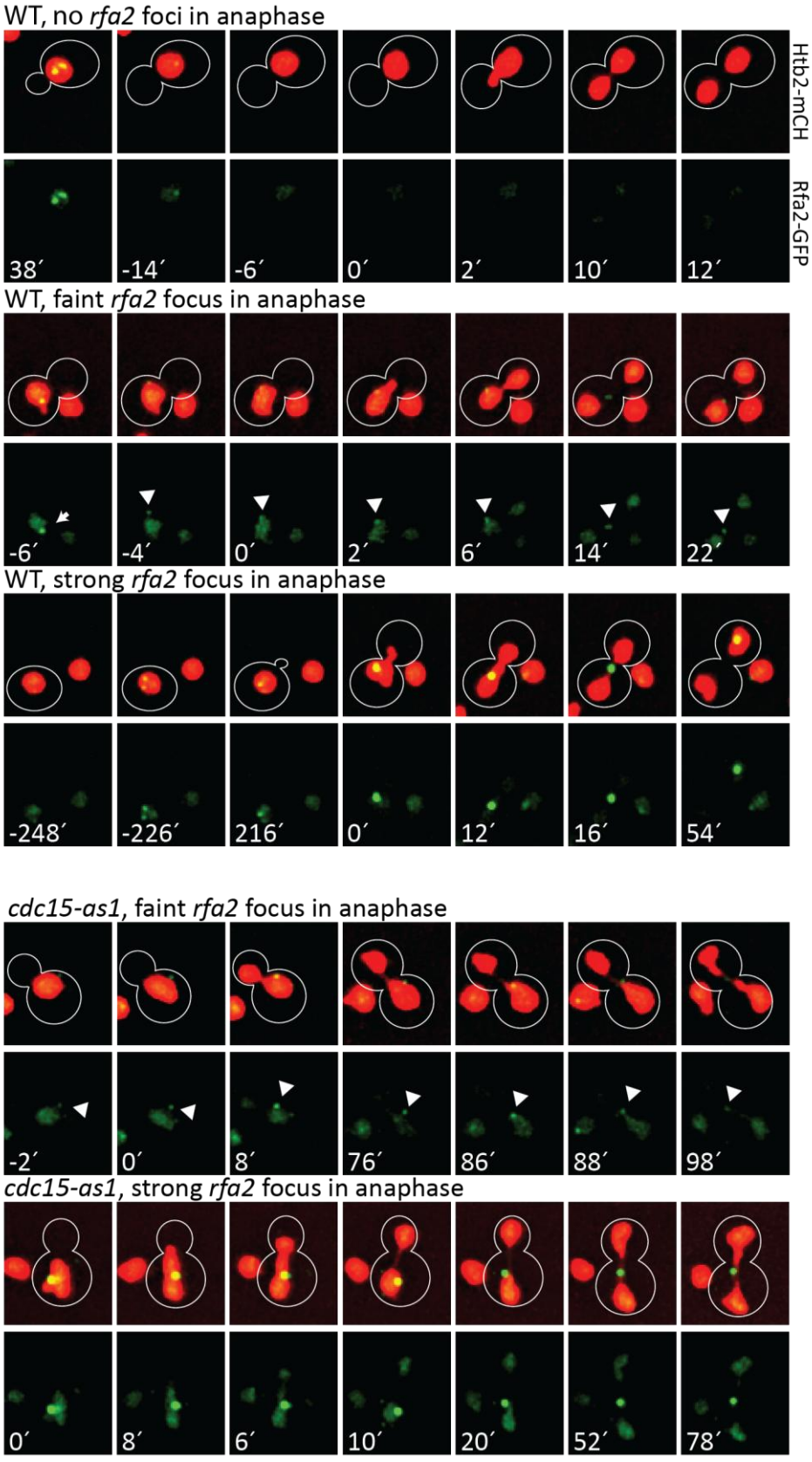
Without the temperature shift, the *cdc15-as1 cdc2-1* double mutant did not display an increase in cut phenotypes when compared to the c*dc15-as1* single mutant (Fig 24C).
The requirement of DNA Polα for the timely segregation of MEN bridges suggests the involvement of DNA replication. The lack of cut phenotypes after release of the *cdc2-1 cdc15-as1* double mutant at permissive temperature demonstrate that the cut phenotypes observed at the restrictive temperature do not originate from chronic defect of the *cdc2-1* allele at permissive temperature but from a requirement for Cdc2 after a MEN release.

2.3.9. RPA localizes to anaphase bridges in MEN mutants

The requirement for DNA Polδ for resolution of anaphase bridges in MEN mutants suggested that these may originate from un-replicated DNA. As a first step towards investigating this, I imaged the localization of the RPA subunit Rfa2 in *cdc15-as1* arrested cells.

To this end I introduced Htb2-mCherry and *cdc15-as1* into a strain that harbored a Rfa2-GFP fusion protein. This strain and a strain without the *cdc15-as1* allele were grown to log phase and then treated with 2.5µM 1NA-PP1 before time lapses were acquired. The behavior of Rfa2p-GFP foci in relation to the Htb2-mCherry labeled chromatin was analyzed during anaphase. Notably Rfa2 foci appeared concomitantly with the appearance of a bud (Fig. 25A, panel3) and would in the wild type in most cases disappear during anaphase. However in about 20% of wt anaphases, an Rfa2 focus would be persist. The *cdc15-as1* mutant arrests in anaphase. In almost 80% of cases, an Rfa2 focus was visible in these cells during the anaphase arrest eventually (Fig. 25B). Importantly however, these foci were often not visible immediately at anaphase onset but were later pulled out of the fluorescent background of one of the nuclear lobes into the bridge. Currently it is unclear if the Rfa2 foci in the *cdc15-as1* mutant are preexisting or form later in the anaphase arrest.

A)


WT, no *rfa2* foci in anaphase
Htb2-mCH
Rfa2-GFP
38´
-14´
-6´
0´
2´
10´
12´
WT, faint *rfa2* focus in anaphase
-6´
-4´
0´
2´
6´
14´
22´
WT, strong *rfa2* focus in anaphase
-248´
-226´
216´
0´
12´
16´
54´
cdc15-as1, faint *rfa2* focus in anaphase
-2´
0´
8´
76´
86´
88´
98´
cdc15-as1, strong *rfa2* focus in anaphase
0´
8´
6´
10´
20´
52´
78´

B)

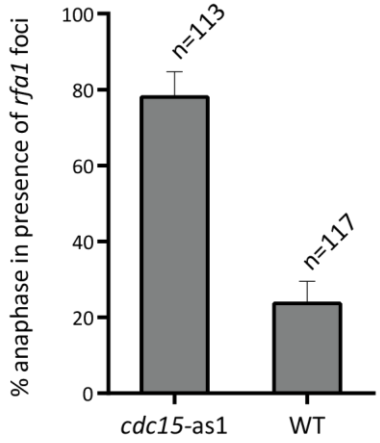


Figure 25A) *Representative montages of Rfa2-GFP foci wild-type, and cdc15-as1 cells exposed to 2.5* µM 1NA-PP1. Arrows designate faint Rfa2 foci.

25B) *Quantification of Rfa2-GFP foci in anaphase cells. Cell were monitored during anaphases entry and in the case of the cdc15-as1mutant during the anaphase arrest for the presence of Rfa2 foci. Error bars display standard error between experiments.*

It should not be concluded from this data that replication is unfinished in MEN mutants. Rfa2 foci could just as well signify ssDNA due to DNA damage. However the often late recruitment of Rfa2 into the bridge (see Fig. 25, panel 5) further corroborates the notion that anaphase bridges in MEN mutants contain DNA.

3.Discussion

In the present study I have identified novel factors that are required to complete the segregation of a mitotic chromosome. This was achieved through a visual screen for anaphase bridges among the essential genes of budding yeast. I identified replication stress as a cause for anaphase bridges. I also identified a role for the mitotic exit network in preventing anaphase bridges. In characterizing the anaphase bridges observed in MEN mutants further I found evidence for un-replicated DNA as a possible cause. The resolution of anaphase bridges that form in MEN mutants by inactivation of the CST complex suggests that these bridges may arise from telomere proximal regions.

3.1 Inhibition of cytokinesis stabilizes anaphase bridges

To stabilize anaphase bridges in the screen, I generated the *pCTR1-iqg1-1* allele. This allele allows inactivation of Iqg1 by a combined promoter shutoff and a thermosensitive mutation. The *pCTR1-iqg1-1* allele stabilized anaphase bridges, evident from the substantial increase in the lifetime of the anaphase figures formed in condensin mutants.

The fate of an anaphase bridge is an important issue due to the potential effects on genome stability. While the contraction of the actomyosin ring is an obvious candidate for a process that can break a bridge, it is not the only one that has been suggested. Alternative possibilities are a breaking of a bridge by spindle forces or by enzymatic cleavage, which could be mediated for example by Top2. In this specific case, the Iqg1 dependent loss of a *ycg1-2* induced anaphase bridge strongly suggests that it is ring contraction or a process dependent on ring contraction that cuts a bridge arising from de-condensed chromatin[307].

It was interesting to observe that *pCTR1-iqg1-1 can* partially rescue a *ycg1-2* mutation. One possible explanation is that a delay in cytokinesis provides sufficient time for some cells to segregate partially de-condensed chromosomes. An alternative explanation is that the inhibition of cytokinesis allows cells to undergo anaphase without cutting the bridge, thereby avoiding DNA damage and a lethal G2/M arrest. Cells that fail cytokinesis become polyploid and the observation of multinucleate syncytium-like cells indicates that this polyploidy is not immediately lethal. The additional time provided by the averted DNA damage could facilitate emergence of suppressor mutations during subsequent endocycle-like events.

3.2 Results of the anaphase bridge screen

I found anaphase bridges in 59 of the 765 screened genes. At this point it is important to restate the definition of an anaphase bridge in the context of the screen. This was either an overt increase in late anaphase figures, chromatin traversing a double ring of septins or tripolar bridges. It is important to recapitulate this definition because it encompasses any chromatin present in the bud neck after ring splitting. While this includes mutants in which chromatin segregation is delayed with respect to mitotic exit, it also includes mutants that advance mitotic exit with respect to chromatin segregation. This is likely the case in mps*1* mutants which fail to assemble a spindle but also fail to arrest at the spindle assembly checkpoint[336, 337]. In this context it is not possible to clearly discriminate between mutants that have undergone anaphase spindle elongation and failed to segregate chromatin away from the bud neck and those that have not attempted chromosome segregation and triggered mitotic exit. Both types of mutants are likely present among the hits.

SMC complex members

All 6 condensin mutant alleles contained in the array were found to give a strong post-anaphase bridge and tri-polar bridge phenotype. The severity of this phenotype was only paralleled by the Smc5/6 complex mutants. 15 of 17 Smc5/6 complex mutants were found, two alleles, *kre29-ts2* and *Nse1-ts* were not identified. Other Nse1 mutants were however scored as hits. Both the condensin complex and the Smc5/6 complex have known roles in chromosome segregation and the identification of 90% of these mutants suggests a good detection rate[232, 233, 340].

Cohesin and securin

The cohesin Scc1(Mcd1) subunit has been implicated in chromosome condensation, thus far uniquely in budding yeast[355]. Finding a securin mutant to give rise to anaphase bridges on the other hand was perplexing. One would not expect a situation that advances chromosome de-cohesion to generate to anaphase bridges. Loss of securin has however previously been reported to lead to onset of cytokinesis in absence of spindle elongation, a likely explanation for this observation[356].

Spindle and Kinetochore

While inactivation of some spindle components would have been expected to give rise to anaphase bridges, possibly due to delayed or incomplete segregation of chromosomes by a structurally impaired spindle, finding anaphase bridges in kinetochore mutants was unexpected. In higher organisms multiple microtubules attach to a kinetochore. In these organisms, anaphase bridges stemming from impaired kinetochore function could have been explained by means of merotelic attachment to the spindle[306]. However, budding yeast utilizes a point centromere that recruits only a single microtubule per kinetochore[87]. Therefore merotelic attachments are not likely. One possible explanation is that in these specific mutants, kinetochores

are compromised in a way that prevents a full SAC activation and cells enter anaphase prematurely with unattached or unstably attached chromosomes that are not fully segregated to the poles by the time of septin ring splitting.

CDK1-regulation

Two factors found in the screen, Cks1 and Mcm1, are known to regulate CDK1 activity. Cks1 phosphorylates Cdk1 to allow cyclin binding while Mcm1 is a transcription factor that is required for transcription of polo kinase and B-cyclins[357, 358]. Cdk1 and Polo kinase are known regulators of chromosome condensation and multiple other processes during mitosis[238, 359]. Thus the effects of the transcription factor Mcm1 and the Cks1 kinase are likely indirect.

DNA replication

A large group of hits encompasses genes involved in DNA replication. These include the major replicative DNA Polδ and Polε, three components of the CMG helicase Psf2, Psf3 and Mcm6, TopBP1^{DPB11}, the replication factor C subunit RFC5 and the DDK kinase subunits Cdc7 and Dbf4. All of these are essential components of the DNA replication machinery. TopBP1^{DPB11}, the DDK kinase and the CMG components are required for origin firing while the CMG helicase, the DNA Polδ and ε and RFC are components of the moving replisome[193]. Thus all of these mutants are very likely severely compromised for DNA replication, yet some cells exit mitosis with chromatin stretching over split septin rings. DNA replication is under surveillance by the replication checkpoint. This checkpoint requires a number of replisome components that appear to be involved in both, DNA replication and sensing of ongoing DNA replication. These include TopBP1^{DPB11}, RFC5, and DNA Pol ε [360-362]. Anaphase bridges in these mutants possibly arise from a combined defect in DNA replication and the replication checkpoint.

Components of the CMG helicase have previously been inactivated in an established HU induced arrest [261]. This did not lead to a loss of the checkpoint response suggesting that the CMG helicase is not a component of the checkpoint. The CMG mutants identified here are different from the ones previously used to assess the role of the CMG in the replication checkpoint. Thus these alleles may behave differently and potentially display a weakened checkpoint. A second possibility relates to the described dose dependent replication checkpoint response. Replication forks are required to sense replication stress. The replication checkpoint is weakened if fewer forks are active leading to a diminished response to under-replicated DNA [259, 363]. These CMG mutants may activate fewer forks which may lead to a diminished checkpoint response to under-replication and therefore to anaphase entry in presence of bridges.

APC/C and proteasome

A number of factors required to inactivate CDK1 at the end of mitosis were found to give rise to anaphase bridges when inactivated. Components of the APC/C, Cdc20 and the proteasome behaved very allele specific. The majority of APC/C, Cdc20 and proteasome alleles showed the expected metaphase arrest. The Cdc20-1, Cdc20-2, Cdc27-2, apc11-22, apc5-CA-PAps and rpn11-11 alleles however displayed anaphase bridges. As outlined earlier, a SAC arrest is the outcome of a balance between Cdc20 and MCC levels[285]. The MCC targets Cdc20 itself for ubiquitination and destruction by the APC/C. This mechanism maintains low levels of the continuously expressed Cdc20 during SAC activation. If in the mutants named above the degradation of Cdc20 were compromised, this could conceivably lead to anaphase entry before chromosome assembly or de-cohesion and thereby to anaphase bridges.

The mitotic exit network

All essential components of the mitotic exit network were found to be required for chromosome segregation. Unlike in the mutants discussed above, anaphase bridges in MEN mutants were not identified by chromatin traversing split septin rings. The MEN is required for splitting of the septin ring [101]. Rather MEN mutants were identified by an overt increase in late anaphase figures, suggesting that these mutants had arrested in anaphase but not yet completed chromatin segregation. The requirement of Cdc14 for chromosome segregation is known[181]. These functions are attributed the early anaphase functions of Cdc14 which are triggered by its FEAR mediated release from the nucleolus. Therefore the appearance of anaphase bridges in Cdc14 mutants was anticipated. The observation of anaphase bridges in MEN mutants was novel.

3.3 Replication stress induces anaphase bridges

The replication mutants found in the screen may display anaphase bridges because they are impaired in DNA replication or because they are impaired in the replication checkpoint. The majority of replication mutants did not display anaphase bridges, thus the presence of bridges in a subset of replication mutants may indicate a failure of the replication checkpoint. Alternatively the mutants that display anaphase bridges could be leaky and allow replication to proceed slowly during a replication checkpoint mediated cell cycle delay. If the there is a threshold for the activation of the replication checkpoint, then once slow replication has proceeded to the point when too few replisomes are left to signal ongoing replication, cells may be expected to enter anaphase in the presence of un-replicated DNA.

To test this, wild type cells were exposed to low persistent replication stress with the aim to slow but not inhibit DNA replication. HU treatment caused anaphase bridges in 40% of cells of cells. The observation of histone-positive bridges in cells growing in low doses of HU suggests that slowing replication leads to a situation in which replication

has proceeded to a point where it is not yet complete but this remaining un-replicated DNA eludes checkpoint detection.

3.4 Anaphase bridges in MEN mutants

Anaphase bridges in have not been described to arise in MEN mutants, even though the mutants they were found in here are well studied. An involvement of the MEN in chromosome segregation is not usually reported with one notable exception. Hartwells original analysis groups the CDC14 and CDC15 gene into the same category, required to complete late nuclear division [21, 364, 365]. More specifically, thin connections were observed between almost divided nuclei in the *cdc15-1* mutant in Giemsa-stained samples (strain 17-17D1 in [365]).

To ensure that the anaphase bridges observed were not artifacts from free fluorescent histone diffusing through the late anaphase nuclear tube I stained Htb2-mCherry labeled anaphase bridges with YOYO-1. The Htb2-mCherry label does not appear to induce bridges in our hands as at room temperature, in absence of any treatment with pharmacological compounds or temperature shifts, all cells complete chromosome segregation before actomyosin ring contraction (Fig 9). Yet it is formally possible MEN mutants only display anaphase bridges when sensitized by the Htb2-mCherry fusion. Thus the YOYO-1 stains need to be performed in MEN mutants that do not harbor Htb2-mCherry to exclude this possibility.

Additional confirmation that MEN bridges contain DNA comes from the observation of the telomere behavior in *cdc15-2* and *dbf2-2* mutants. The re-approach of sister sub-telomeres demonstrates that specific chromosomal loci can be present in MEN bridges at least transiently. However, in the case of telomere 12R they invariably reenter the bridge after initial segregation to the two nuclear lobes. The localization of RFA foci into up to 80% of MEN bridges further corroborates the presence of DNA in at least a

fraction of the histone-positive bridges. Of note, during the analysis of rDNA and telomere 12R segregation, co-segregation of the two rDNA sister loci into the same cell was never observed, arguing against whole chromosome mis-segregation occurring with a frequency comparable to the formation of anaphase bridges in MEN. Thus these bridges are unlikely to arise from faulty chromosome attachments to the spindle. What may be their origin?

The potential involvement of the MEN in chromosome segregation via Cdc14 release.

The MEN is required for sustained Cdc14 release. It is necessary resolve whether CDK1 inactivation can resolve anaphase bridges in MEN mutants and therefore whether the MEN is indirectly involved in chromosome segregation, through release of Cdc14, or whether it plays a direct role. The fact that anaphase bridges are observed in all MEN mutants and the MEN is a linear signaling cascade suggests that the required function for chromosome segregation is performed by the Mob1-Dbf2 kinase complex, although this still has to be demonstrated, for example by co-overexpression of Mob1 and Dbf2 in a *cdc15-2* mutant. It is interesting that more recently Cdc14 independent functions of the Mob1-Dbf2 kinase complex have described and these are not only limited to anaphase. Dbf2 phosphorylates Hof1 and Chs1 to promote cytokinesis and it phosophorylates Kar9 to maintain spindle asymmetry before anaphase onset [184-186, 366]. Experiments to attempt to resolve anaphase bridges in MEN mutants by over-expression of Sic1 and Cdc14 are ongoing.

Cdc14 has at least four suggested functions during in chromosome segregation, all of which are thought to be mediated by reversal of Cdk1 phosphorylation. These roles are origin priming, condensin loading, completion of decatenation and inhibition of RNA synthesis to allow completion of replication[180-182, 354]. The exact interplay of these functions is not completely clear but it has been reported that Cdc14 inactivates RNA polymerases and active transcription counteracts condensin loading. Condensin loading in turn is required for decatenation of at least the rDNA region.

Hypomorphic *cdc14-1* mutants have been suggested to accrue un-replicated DNA due to multiple reasons, including a transcription and import defect of replication factors [354]. In light of the recent reports on anaphase bridges arising from under-replication or unprocessed replication intermediates, it is possible that MEN bridges originate from chronic DNA under-replication stemming from a decreased Cdc14 release at permissive temperature. This may be the case if the MEN mutants that were used behaved as hypomorphs. However it is unlikely that all MEN mutants in the mutant array display a hyopomorphic behavior that induces a comparable number of anaphase bridges in the timecourse (Figure 11). A metaphase arrest rescued the rDNA segregation defects observed in the cdc14-1 mutant[354]. It remains to be tested whether a metaphase arrest could also rescue anaphase bridges arising in MEN mutants. However the resolution of anaphase bridges observed in anaphase arrested *cdc15-as1* cells was inefficient, at a rate about 10-20% during three hours of arrest.

The suggested function of Cdc14 in promoting de-catenation, specifically of the rDNA locus is unlikely to be responsible for MEN induced bridges[180]. First because the rDNA segregates in MEN mutants and secondly because the resolution of anaphase bridges in MEN mutants does not require topoisomerase 2. Similarly a requirement of Cdc14 for loading condensin, specifically to the rDNA, to allow its segregation is unlikely to be responsible for anaphase bridges in MEN mutants MEN mutants do not display a defect in segregating the rDNA. Furthermore condensin recruitment to the rDNA has been assayed and is not defective in *cdc15-2* mutants[181].

The final possibility by which a deregulation of Cdc14 by MEN mutants may cause anaphase bridges is by mis-regulation of Yen1. Yen1 is a structure specific nuclease that processes remaining DNA interlinks in anaphase to allow chromosome segregation. Importantly Yen1 is excluded from the nucleus by CDK1 phosphorylation until anaphase. Cdc14 is required for Yen1 activity and import. Cdc15 has been

suggested to be required for the maintenance of Yen1 in the nucleus, by maintaining Cdc14 released. However, Yen1 isolated from *cdc15* mutants is an active nuclease while Yen1 isolated from *cdc14* mutants is not, suggesting that the MEN is at least not required for activation of its catalytic activity [367-369]. It remains to be seen whether its altered localization, it's premature nuclear export in MEN mutants, may explain the presence of anaphase bridges.

Structure of anaphase bridges in MEN mutants

Given the previously mentioned classification scheme of anaphase bridges into chromatin and ultrafine bridges, and bearing in mind that this is purely a descriptive classification based on DNA staining and protein localization, where do anaphase bridges in MEN mutants fall? On the one hand they stain with a nucleic acid dye and are observable with fluorescent Htb2 and would therefore be termed a chromatin bridge. On the other hand they appear recruit RPA although this may be a later event. RPA localization has been described for fragile site UFBs and telomeric UFBs in humans and telomeric UFBs in fission yeast. Although the RPA foci are not interpreted as proof of unreplicated DNA in MEN mutants, it does suggest the presence of at least some ssDNA. It will be interesting to see whether TopBP1^{DPB11} localizes to anaphase bridges in MEN mutants and conversely to see if RPA can be found on other types of anaphase bridges, such as those caused by inactivation of condensin or topoisomerase 2 to further sub-classify MEN-mutant bridges. It is also possible that there is no clear cut distinction between chromatin and ultrafine bridges. Treatment of cells with 60mM HU lead to histone labeled chromatin bridges while treatment with 20mM HU was reported to induce histone-negative UFBs[327]. Conversely telomeric UFBs in fission yeast were reported to display faint histone staining[325]. Thus the distinction between chromatin bridges and UFBs may in some cases be confounded by sensitivity issues.

Supporting the idea that MEN bridges originate from incompletely replicated DNA and therefore may in some way be related the UFBs induced by impairing replication or arising from collapse replication forks in telomeres is the requirement of DNA pol δ (CDC2) for their timely resolution after MEN reactivation.

Telomeric nature of MEN bridges

MEN bridges resolve when Cdc13 or DNA Polα are inactivated. The latter was surprising as DNA Polδ is required for MEN bridge segregation, the opposite effect. The resolution of MEN bridges by inactivation of Cdc13 however is a clear indication for an involvement of telomeres in the formation of MEN bridges. Cdc13, together with Stn1 and Ten1 is part of the conserved CST complex. CST is structurally similar to RPA and has a role in telomere elongation and replication[370]. In budding yeast it recruits both telomerase and DNA Polα, the latter is required for lagging strand synthesis[343]. While the CST complex has an emerging role in chromosomal DNA replication in animals where it plays a role in dormant origin firing, no such role has been found in budding yeast and its functions in this organism seem purely telomeric[371]. Budding yeast Cdc13 has a role as single strand telomere repeat binding factor, telomerase recruiter and telomere capping [343, 372]. How would inactivation of the CST complex lead to MEN bridge segregation? I would like to speculate on two possible hypothesis.

The first is a direct role for Cdc13 in the origin of anaphase bridges in MEN mutants. Animal telomeres recruit a pool of cohesin that is apparently protected from cleavage by association with the shelterin complex[373, 374]. Telomerase in turn has been suggested to form a dimer in yeast and humans and act on two telomeres simultaneously, although this is subject to debate[343, 375, 376]. Further it has been suggested that the observed telomere cohesion in human cells mediated by the shelterin complex is important for telomere elongation by telomerase[377]. It is possible that telomere cohesion would persist longer than cohesion elsewhere in the

chromosome to for example allow processes such as telomere extension. The obvious question therefore is whether anaphase bridges in MEN mutants depend on cohesin. Preliminary results suggest that this is not the case but these need to be further substantiated before they warrant formal reporting. It is also conceivable that interaction between dimerized Cdc13 and telomeric ssDNA or the interaction between telomerase and Cdc13 physically link sister telomeres. Cdc13 is known to dimerize and it has nanomolar affinity for ssDNA. In addition, Cdc13 recruits telomerase by direct interaction and budding yeast telomerase in vitro remains stably bound to its product[347, 375, 378].

Telomerase is evicted by phosphorylation of Cdc13 on S314. Aurora B has been described as the kinase responsible for this process[349]. Conceptually telomerase eviction could be MEN dependent as the MEN is required to maintain Aurora B association with the spindle midzone during anaphase. However the loss of Aurora B function did not strongly affect bridge resolution after release from a MEN arrest. It is interesting to note here that Cdc13 contains three RXXS Dbf2 consensus sites [379, 380]. Importantly, one of these phosphosites is the S314 site suggested to be phosphorylated by Aurora B. The second putative Dbf2 site, S333 was independently demonstrated to be phosphorylated in vivo[381]. Characterization of Cdc13 phosphomutants is underway to test whether phospho-negative Cdc13 variants induce anaphase bridges and whether phospho-mimicking Cdc13 variants allow resolution of MEN bridges.

The above hypothesis suggests telomere cohesion by protein links, possibly the machinery that effects telomere elongation and highlights some possible bridging interactions. Bridge resolution by inactivation of DNA Polα in this model may be due to its physical binding to the ssDNA of replicating telomeres as well as its interaction with Stn1 and Cdc13[343]. Speculatively the CST complex, DNA Polα and telomerase may form a higher order complex to perform coordinated telomere elongation and lagging strand synthesis. Binding of this complex to two sister telomeres during telomere

elongation may resist telomere segregation. High molecular weight complexes up to 5Md in size containing both telomerase and DNA Polα have been purified from Euplotes[382].

The above model requires the postulation of CST dependent telomere cohesion. It also does not easily accommodate the dependence of MEN bridge resolution on DNA pol δ unless it is additionally postulated that CST complex or telomerase eviction depends on replication.

The second, more parsimonious explanation is that bridge resolution by inactivation of Cdc13 or DNA Polα constitutes a bypass. Both Cdc13 and the DNA Polα subunit Pol12 have a role in capping telomeres. Telomeres are capped to prevent them from being recognized as double strand breaks and being subjected to 5' resection by nucleases[343]. Upon inactivation of Cdc13, resection can be observed reaching up to 15kb into the chromosomes arm [383]. In this model, lack of MEN function would lead to un-replicated regions or persistent replication intermediates that form telomere proximal links between sister chromatids. These links would require DNA Polδ for their resolution and de-capping of telomeres by inactivation of Cdc13 or DNA Polα would lead to telomere resection, removing the links. It will be important to determine whether anaphase bridge resolution by Cdc13 or DNA Polα inactivation is still observable in cells in which *RAD24* or both *SGS1* and *SAE2* have been deleted as these deletions strongly impair telomere resection[384, 385]. The main shortcoming of this model is that there is not yet an obvious link between the MEN and the postulated DNA structures that impede chromosome segregation.

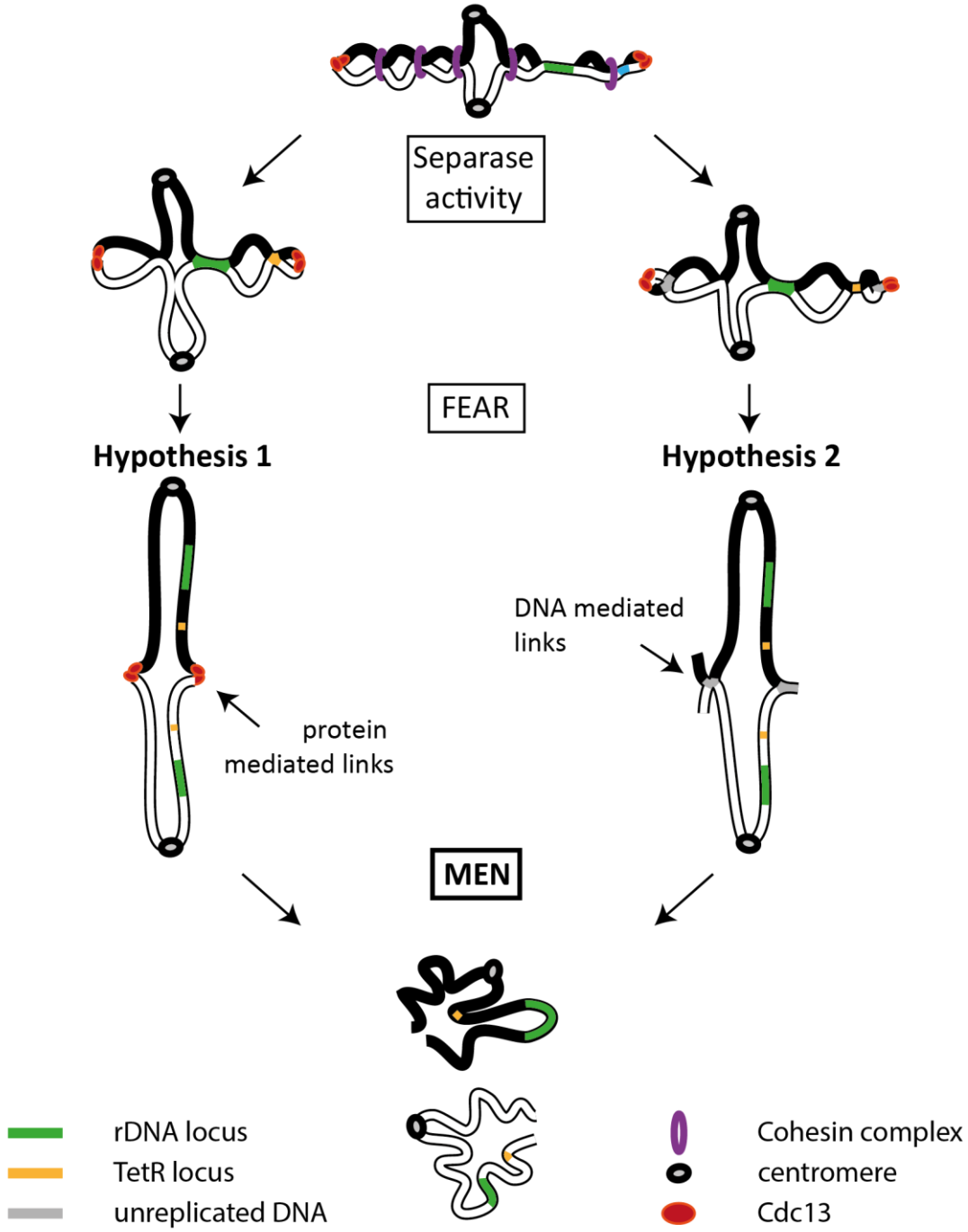


Figure 26) *Schematic representation of the two hypothesis discussed in the text. Hypothesis 1 postulates that seperase mediated cleavage of cohesin and FEAR mediated Cdc14 release removes all interlinks between chromosomes except telomeric cohesive complexes that include Cdc13. This hypothetic complex then requires the MEN*

for removal, to allow completion of chromosome segregation. Hypothesis 2 postulates that after cohesin cleavage and FEAR mediated Cdc14 release, there exist teleomeric proximal DNA mediated chromosome links. These may arise from unfinished replication or incomplete removal of replication intermediates. These hypothetical DNA mediated links are then removed in a MEN and DNA Polδ dependent manner.

4. Conclusions

The results of the anaphase bridge screen highlight the many processes that are involved in successful chromosome segregation and the prevention of anaphase bridges. As this was not a genome wide screen, other factors involved may yet be to be found.

A subset of replication mutants display anaphase bridges. What distinguishes the replication mutants that du display anaphase bridges from those that don't is currently unclear. One possibility is that the replication mutants that do display bridges are not complete loss of function alleles and may slowly complete replication. A fraction of cells exposed to sub-inhibitory doses of HU progress into anaphase and complete ring contraction in the presence of a bridge. This implies that the replication checkpoint is not able to mount an arrest that is sufficient to prevent anaphase onset in the face of incompletely replicated DNA or structures that arise from impaired replication.

Finally, the MEN is a novel regulator of chromosome segregation and acts to prevents anaphase bridges. MEN induced anaphase bridges appear to arise from neither defects in cohesin removal nor chromosome decatenation. Taken together with the previously described unperturbed localization of condensin in MEN mutants, this suggests that none of the major chromosome assembly or resolution pathways are involved in the formation of anaphase bridges in MEN mutants. Rather, the localization of RPA to the bridges and the requirement of Pol δ for their resolution implicate some aspect of DNA synthesis in their formation. MEN bridges are resolved by inactivation of the CST complex and in MEN- arrested mutants subtelomeric sister-loci have a tendency to re-approach, sometimes fully collapse. This is not observed for the rDNA region. The current data therefore suggests that these anaphase bridges arise preferentially from telomeric regions. An alternative explanation to the observed resolution of MEN bridges in response to CST inactivation is that the CST in some way mediates telomere interactions. Future experiments will address the nature of the primary cause of anaphase bridges in MEN mutants.

5. Materials and Methods

5.1. Strains used in this study

yMM	genotype	background
yMM1565	*MATa ; ura3-52; trp1D2; leu2-3_112; his3-11; ade2-1; can1-100 MatA*	W303
yMM2614	*iqg1-1 ade2-1; trp1-1; leu2-3,112(sic); ura3-52 MatA*	W303
yMM2634	*iqg1-1 ade2-1; trp1-1; leu2-3,112(sic); ura3-52 MatA*	W303
yMM2995	*Shs1-GFP::caUra3 Htb2-mCH::NAT can1Δ::STE2pr-his5 lyp1Δ::STE3pr-LEU2 ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 Matα*	BY4742
yMM3067	*HYG::iqg1-1 ade2-1; trp1-1; leu2-3,112(sic); ura3-52 MatA*	W303
yMM3068	*HYG::iqg1-1 Shs1-GFP::caUra3 Htb2-mCH::NAT can1Δ::STE2pr-his5 lyp1Δ::STE3pr-LEU2 ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 Matα*	BY4742
yMM2027	*pCTR-iqg1-1::Hyg can1Δ::STE2pr-his5 lyp1Δ::STE3pr-LEU2 ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 Matα*	BY4742
yMM2777	*pCTR-iqg1-1::Hyg can1Δ::STE2pr-his5 lyp1Δ::STE3pr-LEU2 ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 Matα*	BY4742
yMM2784	*pCTR-Iqg1::Hyg can1Δ::STE2pr-his5 lyp1Δ::STE3pr-LEU2 ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 Matα*	BY4742
yMM2995	*Shs1-GFP::caUra3 Htb2-mCH::NAT can1Δ::STE2pr-his5 lyp1Δ::STE3pr-LEU2 ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 Matα*	BY4742
yMM2855	*Htb2-mCH::NAT can1Δ::STE2pr-his5 lyp1Δ::STE3pr-LEU2 ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 Matα*	BY4742
yMM2492	*Ycg1-2::KanR Htb2-mCH::His3 ADE5,7::KanR; Htb2-mCH::His3 his3^1 leu2^0 ura3^0 met15^0 MatA*	BY4741
yMM2819	*pCTR-Iqg1-1::Hyg Ycg1-2::KanR Htb2-mCH::His3 can1Δ::STE2pr-his5 lyp1Δ::STE3pr-LEU2 ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 Matα*	BY4742
yMM2822	*pCTR-Iqg1::Hyg Ycg1-2::KanR Htb2-mCH::His3 can1Δ::STE2pr-his5 lyp1Δ::STE3pr-LEU2 ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 Matα*	BY4742
yMM2854	*pCTR-Iqg1-1::Hyg Htb2-mCH::Nat can1Δ::STE2pr-his5 lyp1Δ::STE3pr-LEU2 ura3Δ0 leu2Δ0 his3Δ1 met15Δ0MatA*	BY4741
yMM3458	*Shs1-GFP::KanR pCTR-Iqg1-1::Hyg Htb2-mCH::Nat can1Δ::STE2pr-his5 lyp1Δ::STE3pr-LEU2 ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 Matα*	BY4742
yMM3468	*pCTR1-Iqg1-1 Htb2-mCH::Nat Shs1-GFP::caURA3 can1Δ::STE2pr-his5 lyp1Δ::STE3pr-LEU2 ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 Matα*	BY4742
yMM1984	*Htb2-mCherry::URA Myo1-GFP::HIS ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63 MatA*	S288C
yMM3503	*NET1-mCH::Hyg Spc42-mCH::Nat bar1Δ leu2-3,112 ura3-52 his3-Δ200 trp1-Δ63 ade2-1 lys2-801 pep4 TetR-YFP ADE2TetO(5.6Kb)::1061Kb ChrXII HIS3 MatA*	AS499
yMM3420	*Htb2-mCH::His ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63 MatA*	S288C
yMM3424	*Tem1-3::Kan Htb2-mCH::His ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63 MatA*	S288C
yMM3270	*Cdc14-1::KanR Htb2-mCH::His ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63 MatA*	S288C
yMM3268	*Dbf2-2::KanR Htb2-mCH::His ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63 MatA*	S288C
yMM3267	*Cdc15-2::KanR Htb2-mCH::His ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63 MatA*	S288C
yMM3865	*Mob1-ts::URA Htb2-mCH::Hyg ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63his3-Δ200 MatA*	BY4741/ S288C
yMM3415	*Htb2-mCH::Hyg ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63 his3-Δ200 Matα*	S288C
yMM3363	*[ρ^0] Htb2-mCH::His ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63 MatA*	S288C

yMM3889	*[ρ^0] Cdc15-2::Kan Htb2-mCH::His ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63 MatA*	S288C
yMM3890	*[ρ^0] Dbf2-2::Kan Htb2-mCH::His ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63 MatA*	S288C
yMM3891	*[ρ^0] Tem1-3::Kan Htb2-mCH::His ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63 MatA*	S288C
yMM3894	*[ρ^0] Cdc14-1::Kan Htb2-mCH::His ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63 MatA*	S288C
yMM3504	*Cdc14-1::Kan Net1-mCH::Hyg Spc42-mCH::Nat bar1Δ leu2-3,112 ura3-52 his3-Δ200 trp1-Δ63 ade2-1 lys2-801 pep4 TetR-YFP ADE2 TetO(5.6Kb)::1061Kb ChrXII HIS3 MatA*	AS499
yMM3506	*Cdc15-2::Kan Net1-mCH::Hyg Spc42-mCH::Nat bar1Δ leu2-3,112 ura3-52 his3-Δ200 trp1-Δ63 ade2-1 lys2-801 pep4 TetR-YFP ADE2 TetO(5.6Kb)::1061Kb ChrXII HIS3 MatA*	AS499
yMM3507	*DBF2-2::Kan Net1-mCH::Hyg Spc42-mCH::Nat bar1Δ leu2-3,112 ura3-52 his3-Δ200 trp1-Δ63 ade2-1 lys2-801 pep4 TetR-YFP ADE2 TetO(5.6Kb)::1061Kb ChrXII HIS3 MatA*	AS499
yMM3579	*Cdc13-1::Kan Cdc15-as1::Nat Htb2-mCH::Hyg ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63his3-Δ200*	S288C/ BY4742
yMM3581	*Cdc15-as1::Nat Htb2-mCH::Hyg ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63his3-Δ200*	S288C/ BY4742
yMM3841	*Myo1-GFP::His Cdc13-1::Kan Cdc15-as1::Nat Htb2-mCH::Hyg ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63his3-Δ200*	S288C/ BY4742
yMM3843	*Myo1-GFP::His Cdc15-as1::Nat Htb2-mCH::Hyg ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63his3-Δ200*	S288C/ BY4742
yMM3859	*Cdc15-as1::Nat top2-4 Htb2-mCherry::URA Myo1-GFP::HIS ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63 MatA*	S288C
yMM2676	*top2-4 Htb2-mCherry::URA Myo1-GFP::HIS ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63 MatA*	S288C
yMM3858	*Cdc15-as1::Nat Htb2-mCherry::URA Myo1-GFP::HIS ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63 MatA*	S288C
yMM3936	*Cdc15-as1::Nat ipl1-321 Htb2-mCherry::URA Myo1-GFP::HIS ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63 MatA*	S288C
yMM2249	*ipl1-321 Htb2-mCherry::URA Myo1-GFP::HIS ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63 MatA*	S288C
yMM3854	*Myo1-GFP::Trp1 Cdc2-1::Kan (Pol3) Htb2-mCH::His ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63 MatA*	S288C
yMM3866	*Cdc15-as1::Nat Myo1-GFP::Trp Cdc2-1::Kan (Pol3) Htb2-mCH::His ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63 MatA*	S288C
yMM3856	*Myo1-GFP::Trp1 Pol1-13::Kan Htb2-mCH::His ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63 MatA*	S288C
yMM3868	*Cdc15-as1::Nat Myo1-GFP::Trp Pol1-13::Kan Htb2-mCH::His ura3-52 his3Δ200 leu2 lys2-801 ade2-101 trp1Δ63 MatA*	S288C
yMM3949	Htb2-mCH::Hyg RFA1-GFP::His3MX his3D1 leu2D0 met15D0 ura3D0	BY4741
yMM3964	Cdc15-as1::Nat Htb2-mCH::Hyg RFA2-GFP::His3MX his3D1 leu2D0 met15D0 ura3D0	BY4741

5.2. Strain construction and maintenance

Growth media

YPDA contains 2% bacto-peptone (Becton Dickinson, 211820), 1% yeast extract (Becton Dickinson, 212720) and 2% glucose (Sigma-Aldrich, G7528) with an added

0.004% adenine (Sigma-Aldrich, A9126). Filtered glucose and adenine were added after autoclaving the other components. If selection for antibiotic resistance was desired, the relevant antibiotic was added (100mg/l ClonNAT (Werner Bioagents), 200mg/l G418 (Invitrogen) or 300mg/l hygromycin B (Nucliber)). Synthetic minimal (SM) medium contains 0.67% Yeast Nitrogen Base w/o ammonium sulfate (Becton Dickinson, 291920)), 0.004% adenine (Sigma-Aldrich, A9126), 0.002% uracil (Sigma-Aldrich, U0750), 0.002% tryptophan (Sigma-Aldrich, T0254), 0.002% histidine (Sigma-Aldrich, 53319), 0.003% lysine (SigmaAldrich, 62840), 0.003% leucine (Sigma-Aldrich, 61820) and 0.002% methionine(Sigma-Aldrich, M9625). All components were sterilized by filtration. If selection for auxotrophic markers was desired, the relevant amino acids were omitted. Sporulation medium consists of 1% potassium acetate and 0.005% zinc acetate in water (Sigma-Aldrich, P1190). For solid YPDA media,2% agar was added to before autoclaving (Becton Dickinson, 214510). For solid SM medium, 2% agar was autoclaved in water before the filter-sterilized components were added. Solid YP-Glycerol was prepared analogous to solid YPDA, including adenine but omitting Glucose and adding 3% Glycerol instead.

PCR amplification of integration constructs and pCtr1-iqg1-1 and Cdc15-as1 alleles

All fluorescent fusions were constructed as previously described [386, 387] with the exception of –yeGFP::ca-Ura3 fusions which was amplified from pMM126 (pKT209)[388] The thermosensitive alleles, *cdc2-1, pol1-13, cdc14-1, cdc15-2, dbf2-2, tem1-3* were amplified from genomic DNA from the Boone lab thermosensitive collection and transformed into the S288C backgrounds with the published primers[328]. The alleles *cdc13-1* and *mob1-ts*, the latter from the Hieter lab thermosensitive array, were refractory to amplification with the published primers and introduced into the S288C background by cross[329]. The *cdc15-as1* allele was a kind gift from Dr. Fernando Monje Casas. The *cdc15-as1* allele was received in W303 background. A region containing the *CDC15* promoter and the analogue sensitive mutation L99G was amplified from genomic DNA using oMM1177/1178. A second fragment containing a NAT^R marker was amplified from pMM51 (*pFA6a–natNT2*) using oMM1175/1176. Both fragments were fused in a overlap extension PCR using oMM1175/1178. The resulting NAT^R-*cdc15-as1* allele could be amplified from the genomic DNA of the resulting yMM2664 strain and transformed into other strains as required. The *pCTR1-iqg1* and *pCTR1-iqg1-1* alleles were constructed in a three step overlap extension PCR. The original *iqg1-1* allele was received in the W303 background as a kind gift from Dr. Roberta Fraschini. The CTR1 promoter *pCTR1* and a 5´segment of the the *iqg1-1* allele containing the thermosensitive L448P mutation were amplified from genomic DNA using primers oMM2063/2061 and oMM2057/2059 respectively. The fragments were fused by overlap extension using oMM2059/2063. A third fragment containing a HYG^R marker was amplified from pMM52 (pFA6a-hphNT1) using primers oMM2064/1176. The third, HYG^R marker containing, fragment was fused to the *pCTR1-iqg1-1* fusion fragment in a second overlap extension PCR to yield HYG^R-

pCTR1-iqg1-1 by use of oMM2064/2059 and then transformed into the SGA query starting strain yMM2027.

For PCR and primer design given protocols were followed using published plasmids or genomic DNA of thermosensitive mutants ([328, 329, 386, 387]) Exceptions are listed below.

number	sequence	use
yMM1960	AAAAAAATGACACGTATACTGATTTAGCCTCTATTGCATCGGGTA GAGATGGTGACGGTGCTGGTTTA	Shs1-yeGFP-caURA3 5'
yMM1961	TATTTATTTATTTATTTGCTCAGCTTTGGATTTTGTACAGATACAA CTCA TCGATGAATTCGAGCTCG	Shs1-yeGFP-caURA3 3'
yMM1175	CTAGCGGCGAACTACTGTAATGTAATAATGTCCTCTGGCGTCG TGTATGGCGTACGCTGCAGGTCGAC	NAT^R 5'
yMM1176	ATCGATGAATTCGAGCTCG	NAT^R/HYG^R3'
yMM1177	CGAGCTCGAATTCATCGAT GGCATAATCAAAATTGGGCC	cdc15-as1 5'
yMM1178	GGTAGGGTGGATTCTTTGTGAGC	cdc15-as1 3'
yMM2057	ATGACAGCATATTCAGGCTCTCCTTCGAAACCAGGC	iqg1-1 5'
yMM2059	CTCTAAATTTATTTCCTCTTGGG	iqg1-1 3'
yMM2061	GCCTGGTTTCGAAGGAGAGCCTGAATATGCTGTCATTTTGAATGT CAAATATAATACAC	pCTR1 3'
yMM2063	CGAGCTCGAATTCATCGAT CCGCATTTTGAACCGTATTTTGCTC	pCTR1 5'
yMM2064	TTTATTGAAAATAAGAAAGTAGTTGGCCAAAACATAGATTATTTT TTTGA	HYG^R 5'

Yeast transformation

Overnight pre-cultures were diluted in YPDA to $OD_{(600)}$=0.2 and harvested at $OD_{(600)}$ =0.2 and harvested by centrifugation at $OD_{(600)}$=0.8 by centrifugation for 5 minutes at 500g. 15ml of culture were used per transformation. Harvested cells were washed in 1ml transformation buffer (YTB), then re-suspended in 72µl transformation buffer (100mM Lithium acetate, 10mM Tris, 1mM EDTA, pH 8). Salmon sperm was denatured 5 min at 95° C, then cooled on ice for 2min. 50µl of the PCR mix containing the fragment to transform, 11µl denatured salmon sperm and 650µl YTB+PEG (40% v/v PEG, 100mM Lithium acetate, 10mM Tris, 1mM EDTA, pH 8) were added to the cell suspension. The transformation mix was incubated on a tube rotator for 30min at room temperature before 72µl DMSO were added and the cell suspension was placed in a water bath warmed to 42°C for 15minutes. After the heat shock, cells were harvested by centrifugation, 2min at 500g, re-suspended in sterile water and plated directly onto the dropout medium in case of selection for auxotrophic markers or in YPDA in case of antibiotic resistance markers. To select for resistance against antibiotics, cell lawns grown on YPDA for three days were then replica-plated on YPDA with the desired antibiotic. All clones were subject to a second round of selection by re-streaking.

Yeast crosses

Yeast crosses were performed by mixing both strains on solid YPDA to permit mating over night. Diploids were placed in sporulation for 5-7 days. Tetrads were digested in 0.01mg/ml Zymolyase 100T for 10 minutes and then dissected onto solid YPDA using a Zeiss Axioskop 40 . Spores were genotyped by growth solid selection medium.

Yeast stocks

Yeast strains were stocked by suspending cells in YPDA +30% v/v glycerol and freezing them at -80°C

5.3. Microscopy

In addition to the imaging of the anaphase bridge screen samples with the Image Xpress automated microscope described below two microscopes were used. The time-courses for anaphase bridge stabilization by *pCTR1-iqg1-1* and for the appearance of anaphase bridges in MEN mutants were acquired on a Leica AF 6000 wide-field fluorescent microscope with an Andor DU-885K-CSO-#VP. All live-cell time lapses and the imaging of YOYO-1 stained cells were performed on an Andor spinning disk microscope equipped with a Andor Ixon 897E Dual Mode EM-CCD camera. Live cells were imaged in 8 well LabTek chambers that were coated with concanavalin A (ConA). For ConA coating, a 1mg/ml solution ConA was applied to the well for 15min, then removed and washed three times with the medium in which cells would be placed for image acquisition. Except for the analysis of anaphase figure life time increase in *pCTR1-iqg1-1* mutants, all time lapses were acquired in liquid SM medium (containing all of the above mentioned amino acids including tryptophan). The cultures for time lapse microscopy were prepared from overnight pre-cultures grown in YPDA. Cells were diluted in SM medium to an $OD_{(600))}$=0.2 and grown to log-phase, $0.5 \leq OD_{(600))} \leq 0.8$. Cultures were then placed in ConA coated wells and allowed to settle for 10 minutes before washing off un-adhered cells. In experiments in which cells were imaged in presence of hydroxyurea , HU was added to a final concentration of 60mM to the well. Cells were then mounted on the microscope either at room temperature or with the incubator pre-warmed to 38.5°C when inactivation of thermosensitive proteins was desired. In experiments in which thermosensitive proteins were inactivated in MEN arrested cells, the wells of the LabTek chamber was washed with SM medium + 2.5µM 1NA-PP1 (VWR international, 529579-1) and the un-adhered were washed off with SM medium + 2.5µM 1NA-PP1 before 200µl SM medium + 2.5µM 1NA-PP1 was added to the adhered cells. These samples were then placed in the pre-warmed incubator. In experiments in which the MEN was to be reactivated after inactivation of a thermosensitive protein, cultures for arrested for 2.5 hrs with 2.5µM 1NA-PP1 were shifted to 37°C for 15 minutes while a ConA coated LabTek chamber was washed with SM+2.5µM 1NA-PP1 and then warmed to 38.5°C. The

arrested culture was placed into the LabTek dish kept at 38.5°C in the incubator of the microscope for 15 minutes for cells to settle. Following this, the medium was removed and adhered cells gently washed once with pre-warmed SM+0.25µM 1NA-PP1. 200µl SM+0.25µM 1NA-PP1 were added to the adhered cells and image acquisition at 38.5°C was begun. Note that initially cells were released into inhibitor free SM but this gave larger inter-experiment variability with respect to the cut phenotypes in *cdc15-as1* single mutants. In all cases except the analysis of anaphase figure life time increase in *pCTR1-iqg1-1* mutants, all strains that are part of the experiment, that is all strains plotted in the same graph, were imaged in parallel and images were acquired from 2-4 wells with a time resolution of 2 minutes. Per field of view, 15 planes spaced 0.3µm apart were acquired. Time lapses to quantify anaphase figure life time increase in *pCTR1-iqg1-1* mutants were acquired in YPDA medium as *pCTR1-iqg1-1* cells did not grow in SM medium. Fixed YOYO-1 stained cells were mounted on microscope slides for imaging.

5.4. Screening array construction

The screening array was constructed according to high throughput cross technology according to the Boone laboratory protocol . In brief the thermosensitive array received from the Boone laboratory contains a KAN^R marker linked to all thermosensitive alleles while the thermosensitive array received from the Hieter laboratory contain a *URA3* marker linked to all thermosensitive alleles. The query strain thus was constructed to contain *pCTR1-Iqg1-1::Hyg*^{R}, *Htb2-mCH::Nat*^{R} and either *Shs1-GFP::Kan*^{R} or *Shs1-yeGFP::caURA3*, complementary to the intended target thermosensitive array. The query strain also contains *can1Δ::pSte2-HIS5* and *lyp1Δ::pSte3-LEU2*. These two markers allow for selection of haploids and counter selection against diploids. The *CAN1* deletion renders cells resistant against canavanine, the *LYP1* deletion renders cells resistant against thialysine, two toxic amino acid analogues that are normally imported by the two respective deleted transporters. On the other hand *pSte2* is a mating type A specific promoter while *pSte3* is a mating type α specific promoter. This allows for mating type specific selection, only haploid MatA cells will grow on dropout histidine medium, only haploid Matα strains will grow on dropout leucine medium, diploids will grow on neither as both promoters are repressed in diploids. Further diploids in contrast to the query strain will carry one copy of each, *CAN1* and *LYP1*, rendering them sensitive to canavanine and thialysine. Strain construction was performed manually using Re-Pads (Singer REP- 001) on solid medium in Plus Plates (PLU-003). The published method utilizes a solid synthetic complete medium w/o ammonium sulphate to allow selection by antibiotics. This medium will be referred to here as SGA SC and only the dropout amino-acids will be specifically mentioned. The query strain and the strains of the complementary thermosensitive array were pinned on top of each other and allowed to mate on YPDA for one day. Following mating, diploids were selected on SGA SC + KAN NAT HYG

doURA. 2 rounds of diploid selection were performed, each plate permitted to grow for 2 days at 22°C. Diploid colonies were pinned onto solid sporulation medium after the second round of selection and allowed to sporulate for 7 days at 22°C. After sporulation, spores were pinned onto haploid selection medium SGA SC doARG doLYS doHIS + 50mg/l canavanine +50mg/l thialysine (Sigma Aldrich, C1625 and A2636 repectively) . After germination and MatA haploid selection on this medium, cells were pinned onto full selection medium, SGA SC doARG doLYS doHIS + KAN +NAT +HYG + 50mg/l canavanine +50mg/l thialysine to select against anything but MatA haploids carrying the desired genotype *pCTR1-iqg1-1::Hyg*^{R}, *Htb2-mCH::Nat*^{R}, *Shs1-GFP::Kan*^{R}*(or Shs1-yeGFP::caUra3)* and *yfg-ts::Ura3* (or *yfg-ts::Kan*^{R}). After two rounds of selection of full selection medium, cells were pinned into liquid selection medium in 96 well plates and allowed to grow for 2 days before 15% glycerol was added and stocks were frozen.

5.5. Sample preparation for screen

Samples for the screen were prepared as follows: each plate of the screening array cryostock was pinned on solid full selection medium +100µM BCS. Colonies were moved to liquid full selection medium -canavanine -thialysine +100µM BCS where they were allowed to grow for two days at 22°C without shaking . Cells were re-suspended and moved to YPDA +100µM BCS, diluting them 1/10 in 200µl final culture. Cells were grown at room temperature for 5hrs shaking before adding 200µM $CuSO_4$. 1hr after copper addition cells were shifted to a shaker at 38.5°C and incubated for 3hrs. Cells were then fixed in 1% formaldehyde for 10 minutes at 38.5°C allowing them to sediment. The supernatant was removed and sedimented cells were washed twice with PBS. To prepare samples for imaging, optical plates (MoBiTech 5241-20) were coated for 15min with 1mg/ml ConA and wells were washed twice with PBS. Plates were flipped and a drop of 5µl Type F immersion oil (Leica biosystems, 11513859) was placed at the glass bottom of every well. Plates were turned back around and placed on an upward turned plate lid to support it at the edges but maintain the oil drops. Fixed cells suspended in PBS were placed in the wells and allowed to adhere for 15 minutes. Un-adhered cells were removed by 1-2 washes with PBS (depending on the density of cells on the glass bottom judged by eye). All operations were performed using Re-Pads (Singer REP- 001) and an 8 channel multi-pipettes in biological duplicate

5.6. Image acquisition screen

Images for the screen were acquired on an ImageXpress Micro Widefield High-Content Analysis System which was custom fitted with a 60x oil immersion lens. For both replicates 9 fields of view were acquired per well, with a stack of 20 images spaced 0.2µm to image Htb2-mCherry and 8 images spaced 0.5µm to image Shs1-GFP.

5.7. Image analysis

Image analysis was performed in ImageJ, data analysis in Microsoft Excel, data visualization in GraphPad Prism. Analysis was performed manually. Time lapses were analyzed in the maximal projection in 2D, temporal measurements according to frame number, distance measurements using the integrated manual measuring tool. For dot-like signals the distance was measured from the center of the dot. For the line-like rDNA, distance was measured between the centromere distal portions of the rDNA, i.e. the most proximal parts of the two sister loci during segregation. The number of cells scored is indicated in the respective graph. For fixed cell measurements, bridge stabilization, anaphase bridges in MEN mutants and YOYO-1 stains of anaphase bridges cells were analyzed manually in 3D following anaphase bridges through the z-stack. For every sample and time-point, at least 100 cells were scored. The images acquired for the anaphase bridge screen were scored as follows: the images of Shs1-GFP were projected in 2D and then copied 20 times and interleaved with the images of Htb2-mCherry. The resulting stacks were screened by eye with knowledge of well position but not mutant allele. Split septin rings were identified and queried for the presence of Htb2-mCherry signal. Tripolar bridges as described in the results section were searched among the fields and a general strong increase of anaphase figures indicative of anaphase bridges was noted when present. Presence of these features were individually recorded in an excel table for every well, wells were then converted to the corresponding gene names and only those wells which had scored positive for one of the features on both replicates were included in the hits.

5.8. Viability assays

For viability assays strains were grown in YPDA liquid overnight pre-culture diluted in YPDA to $OD_{(600))}$=0.1 and grown to $OD_{(600)}$ = 0.6-0.8. These cultures were diluted to $OD_{(600)}$ = 0.1 and then serially dilute 1/5 four times before spotting 2,5µl of each dilution on the indicated medium. In viability assays that included strains carrying *pCTR-iqg1-1* the pre-culture and log phase culture contained 100µM BCS (Bathocuproinedisulfonic acid, Sigma Aldrich B1125) . Before the dilution series, these cells were washed in PBS to remove the copper chelator. Images shown were acquired after three days, except those of the *pCTR1-iqg1-1 ycg1-2* rescue which were acquired after five days.

5.9. Anaphase bridge stabilization by pCTR1-iqg1-1 in fixed cells and MEN anaphase bridge time-course

For analysis of anaphase bridge stabilization by *pCTR1-iqg1-1,* cells were grown in YPDA+100µM BCS to log phase ($OD_{(600)}$ = 0.6-0.8). 200µM $CuSO_4$ were added at the indicated time-point relative to the temperature shift. Cells were shifted to 37°C. Aliquots were removed at the time-points indicated and fixed in 4% Formaldehyde for 10 minutes. Cells were washed 3 times and then re-suspended in PBS and imaged on the Leica AF6000. 16 planes spaced 0.3µM were acquired per field of view. For the analysis of anaphase bridges in MEN mutants by time-course, cells were grown to log-

phase $OD_{(600)}$ = 0.6-0.8 in YPDA and arrested with 15 µg/ml alpha factor for two hours. They were released by three washes with YPDA and placed at 37°C for three hours. Aliquots were removed every 20 minutes and fixed for 10 minutes in 4% Formaldehyde before stopping fixation by three washes in PBS. Cells were subsequently imaged on the AF6000 16 planes spaced 0.3µM were acquired per field of view.

5.10. Petite mutant isolation

Petite [ρ^0] arise spontaneously in the S288C background and identification is assisted by their inability to produce the red pigment that forms in an *ade2-101* mutant. To isolate yMM3363, S288C wt was spread on a YPD plate and small white single colonies were isolated. The petite phenotype was confirmed by a failure to grow on solid YP-Glycerol.

5.11. Anaphase bridge stain by YOYO-1

From an overnight pre-culture, cells were grown to mid-log phase $OD_{(600)}$ = 0.6-0.8 in YPDA and arrested in 15 µg/ml alpha factor for 2 hrs at 25°C. They were then released by 5 washes with YPDA and placed at 37°C for 3 hrs. At the three hour timepoint, cultures were fixed for 30min in 4% formaldehyde. Fixation was stopped by 3 washes in PBS. Fixed cells were spun down and re-suspended in 300yl of 5mg/ml Zymolase in P solution (1.2M Sorbitol, 0.1M potassium phosphate buffer pH6.2) for 1 minute. Cells were spun down in the last 30sec of digestion at 500g. The supernatant was removed and cells were re-suspended in 200µl P-Solution +0.2% Tween20 and 100µg/ml RNAse A. Cells were digested 1h at 37°C. The suspension was then spun down at 500g for 2 min. Cells were taken up in 10yl of P-Solution containing 25µM YOYO-1 (Thermo Fischer, Y3601) and imaged on the Andor spinning disk microscope acquiring 15 z-planes spaced 0.3µm per field of view.

6.References

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