Sister-chromatid cohesion mediated by the alternative RF-C$^{Ctf18/Dcc1/Ctf8}$, the helicase Chl1 and the polymerase-α-associated protein Ctf4 is essential for chromatid disjunction during meiosis II

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Summary

Cohesion between sister chromatids mediated by a multisubunit complex called cohesin is established during DNA replication and is essential for the orderly segregation of chromatids during anaphase. In budding yeast, a specialized replication factor C called RF-C$^{Ctf18/Dcc1/Ctf8}$ and the DNA-polymerase-α-associated protein Ctf4 are required to maintain sister-chromatid cohesion in cells arrested for long periods in mitosis. We show here that *CTF8, CTF4* and a helicase encoded by *CHL1* are required for efficient sister chromatid cohesion in unperturbed mitotic cells, and provide evidence that Chl1 functions during S-phase. We also show that, in contrast to mitosis, RF-C$^{Ctf18/Dcc1/Cft8}$, Ctf4 and Chl1 are essential for chromosome segregation during meiosis and for the viability of meiotic products. Our finding that cells deleted for *CTF8, CTF4* or *CHL1* undergo massive meiosis II non-disjunction suggests that the second meiotic division is particularly sensitive to cohesion defects. Using a functional as well as a cytological assay, we demonstrate that *CTF8, CHL1* and *CTF4* are essential for cohesion between sister centromeres during meiosis but dispensable for cohesin's association with centromeric DNA. Our finding that mutants in fission yeast *ctf18* and *dcc1* have similar defects suggests that the involvement of the alternative RF-C$^{Ctf18/Dcc1/Ctf8}$ complex in sister chromatid cohesion might be highly conserved.

Supplemental data available online

Key words: Sister-chromatid cohesion, Mitosis, Meiosis

Introduction

The propagation of our genomes from one generation to the next requires the accurate segregation of chromosomes during both mitotic and meiotic divisions. Errors during mitosis contribute to oncogenesis and those during meiosis give rise to aneuploid gametes. In mitotic cells, sister chromatids are held together from their generation during S-phase until the metaphase-to-anaphase transition (Guacci et al., 1994). This phenomenon, called sister-chromatid cohesion, enables cells to determine which chromatids are sisters and which must therefore be pulled towards opposite poles at the onset of anaphase.

Work over recent years has identified a conserved multiprotein complex called cohesin that holds sister chromatids together until the onset of anaphase (Haering and Nasmyth, 2003; Hirano, 2002). In budding yeast, cohesin consists out of four core subunits, Smc1, Smc3, Scc1 and Scc3. It is tightly associated with chromosomes from S-phase until metaphase, particularly around centromeres but also at discrete locations along chromosome arms (Guacci et al., 1997; Michaelis et al., 1997; Orr-Weaver, 1999). Biochemical and structural analyses have shown that cohesin forms a ring-like structure in which Scc1 bridges ABC-like ATPase heads situated at the termini of a V-shaped Smc1-Smc3 heterodimer (Anderson et al., 2002; Gruber et al., 2003; Haering et al., 2002). This ring-like structure has led to the hypothesis that cohesin could hold sister chromatids together by trapping sister DNA molecules within its ring (Haering et al., 2002).

The segregation of sister chromatids to opposite poles at anaphase requires that sister kinetochores be attached to microtubules emanating from opposite spindle poles (a state called biorientation or amphitelic attachment). Cohesin promotes biorientation by ensuring that tension is generated when microtubules attach in an amphitelic manner by resisting the tendency of microtubules to pull sisters apart (He et al., 2000; Tanaka et al., 2000). This tension stabilizes kinetochore-microtubule attachments, which are otherwise disrupted through the action of the Ipl1/Aurora-B protein kinase (Tanaka et al., 2002). In the absence of sister chromatid cohesion (as in cohesin mutants) chromosomes are unable to biorient and segregate at random (Guacci et al., 1997; Michaelis et al., 1997; Tanaka et al., 2000).

Cleavage of cohesin's Scc1 subunit by a cysteine protease called separase triggers the metaphase-to-anaphase transition and the segregation of sister chromatids to opposite poles (Uhlmann et al., 1999; Uhlmann et al., 2000). Separase is kept inactive for most of the cell cycle by binding to securin (Pds1 in budding yeast) (Ciosk et al., 1998; Uhlmann et al., 1999). Destruction of securin at the hands of a ubiquitin protein ligase called the anaphase-promoting complex or cyclosome (APC/C) (Cohen-Fix et al., 1996; Funabiki et al., 1996) at the metaphase-to-anaphase transition occurs when every single chromosome has bioriented. Unaligned chromosomes inhibit the APC/C through activating Mad2 (Musacchio and Hardwick, 2002).

A major variation of this theme enables the production of haploid gametes from diploid somatic cells during meiosis (Petronczki et al., 2003), which involves two successive rounds of chromosome segregation, termed meiosis I and II, preceded by a single round of DNA replication. The two meiotic divisions differ in important but distinct respects from mitosis. Homologous and not sister centromeres are pulled in opposite directions during meiosis I. This is made possible by reciprocal recombination between homologous chromatids that lead to the interconnection of maternal and paternal centromeres through chiasmata and by the action of 'monopolin' proteins like Mam1, which suppress biorientation and promote mono-orientation of sister kinetochores (Rabitsch et al., 2003; Toth et al., 2000). Chiasmata interconnect homologous centromeres by virtue of sister-chromatid cohesion distal to crossovers and they are therefore resolved by the destruction of this cohesion, an event that triggers the first meiotic division. Unlike mitosis, sister-chromatid cohesion in the vicinity of centromeres is preserved at this point and so it can be used to biorient sister centromeres during meiosis II. Meiosis II is more similar to mitosis but differs from it by the absence of any DNA replication. Meiosis II chromosome segregation is triggered by destruction of the cohesion between sister centromeres inherited from meiosis I.

In many eukaryotes, cohesin's Scc1 subunit is largely replaced during meiosis by a meiosis-specific variant called Rec8 (Klein et al., 1999; Watanabe and Nurse, 1999). In yeast, the first meiotic division is triggered by separase-mediated cleavage of Rec8 along chromosome arms, an event caused by the destruction of securin by the APC/C (Buonomo et al., 2000; Kitajima et al., 2003; Salah and Nasmyth, 2000). Protection of Rec8 in the vicinity of centromeres from separase at this point requires a kinetochore protein called Sgo1, which is related to *Drosophila* Mei-S332 (Katis et al., 2004; Kitajima et al., 2004; Marston et al., 2004; Rabitsch et al., 2004). Destruction of securin that had reaccumulated after the first meiotic division leads to a second round of separase activation (Salah and Nasmyth, 2000), causing cleavage of centromeric Rec8 and thereby the onset of anaphase II (Kitajima et al., 2003).

We do not currently understand how sister-chromatid cohesion is established. It is nevertheless clear that cohesin must be present during S-phase. When expressed artificially after DNA replication, cohesin complexes tightly associate with chromosomes but fail to establish functional connections between sister chromatids (Uhlmann and Nasmyth, 1998; Watanabe et al., 2001). This has led to the notion that cohesion arises either from the passage of DNA replication forks through cohesin rings or from a hitherto obscure process facilitated by the physical proximity of the newly synthesized DNA molecules. Establishment of sister-chromatid cohesion during S-phase but neither its maintenance later nor the association of cohesin with chromosomes requires a protein called Eco1/Ctf7 (Skibbens et al., 1999; Toth et al., 1999). The notion that an Eco1-dependent event distinct from mere cohesin loading occurs during S-phase is supported by the finding that overexpression of the DNA polymerase processivity factor and sliding clamp proliferating cell nuclear antigen (PCNA) partially suppresses the lethality of *eco1/ctf7* mutants (Skibbens et al., 1999). Furthermore, deletion of the non-essential *CTF4* gene, whose product binds DNA polymerase α in vitro (Miles and Formosa, 1992), causes detectable but not lethal defects in sister-chromatid cohesion (Hanna et al., 2001), as do deletions of *CTF18*, *DCC1* and *CTF8*, whose gene products along with Rfc2, Rfc3, Rfc4 and Rfc5 form an alternative replication factor C (RF-$C^{Ctf18/Dcc1/Ctf8}$) (Mayer et al., 2001; Naiki et al., 2001). The canonical RF-C^{Rfc1} (containing Rfc1 instead of Ctf18/Dcc1/Ctf8) is an integral part of the replication fork and loads PCNA onto DNA, thereby facilitating the switch from the primosome to the replicative DNA polymerase δ (Diffley and Labib, 2002).

Despite elevated chromosome loss and a Mad2-dependent anaphase onset delay (Hanna et al., 2001; Kouprina et al., 1993; Mayer et al., 2001; Spencer et al., 1990), the physiological significance of sister-chromatid cohesion defects detected in mutants lacking Ctf4 or RF-$C^{Ctf18/Dcc1/Ctf8}$ is unclear for two reasons. First and foremost, neither *CTF4* nor *CTF18/DCC1/CTF8* are essential genes and deletion mutants clearly possess sufficient sister-chromatid cohesion for chromosome biorientation. Second, their cohesion defects have only been measured in cells arrested in M-phase for long periods by treatment with spindle poisons (Hanna et al., 2001; Mayer et al., 2001). It has never been established whether sister-chromatid cohesion in cycling cells is also compromised by the mutations.

We describe here the isolation of *CHL1* as a high-copy-number suppressor of a *ctf8Δ* cells. We show that *CHL1*, *CTF8* and *CTF4* are all required for efficient sister-chromatid cohesion in cycling mitotic cells and provide evidence that the helicase encoded by *CHL1* is required for the establishment of cohesion during S-phase. We demonstrate that Chl1, Ctf4 and RF-$C^{Ctf18/Dcc1/Ctf8}$ are essential for chromosome segregation during meiosis and, using a functional assay, show that they make a major contribution to the establishment of sister-chromatid cohesion in the vicinity of centromeres. Finally, we show that fission-yeast mutants *ctf18* and *dcc1* have similar meiotic defects to those observed in budding yeast, suggesting a conserved role for the alternative RF-$C^{Ctf18/Dcc1/Ctf8}$ in sister-chromatid cohesion.

Materials and Methods

Aneuploidy of mutant strains

The high proportion of cells with two *URA3*-GFP signals we observed in mutant strains for the experiments shown in Figs 1, 3 are not a consequence of hyperploidy of the strains for the green-fluorescent-protein (GFP) labeled chromosome V but rather of defects in sister-chromatid cohesion. We determined the degree of hyperploidy of the GFP-labeled chromosome V by counting the number of GFP signals in securin-negative unbudded mitotic cells for Fig. 1 and in unbudded

G1 cells prior to entry into meiosis for Fig. 3. Consistent with data from other publications, the proportion of mutant cells hyperploid for *URA3*-GFP never exceeded 4% and therefore does not influence the interpretation of the results.

Yeast strains, deletion and tagging of genes

The genotypes of all yeast strains used in this study are provided in supplementary Table S1 (http://jcs.biologists.org/supplemental/). Tagging, promoter replacement and deletion of genes were performed using the one-step PCR method (Knop et al., 1999; Longtine et al., 1998; Wach et al., 1994). The *URA3*-GFP system was described previously (Michaelis et al., 1997).

High-copy-number suppressor screen of c*tf8Δ* spore lethality

A diploid *ctf8Δ* yeast (K12359) strain was transformed with a 2μ high-copy-number genomic library constructed in YEp13 (Nasmyth and Tatchell, 1980). 50,000 transformants were grown on plates lacking leucine for 72 hours, replica plated for 48 hours onto plates lacking leucine and regenerated on YEPD for 24 hours at 30°C. Colonies were transferred onto sporulation medium, incubated for 72 hours at 30°C, replica plated onto YEPD glass plates and immediately treated with diethyl ether (Rockmill et al., 1991) to kill all non-sporulated cells. Colonies were germinated and grown for 48 hours at 30°C. Clones showing increased colony growth compared with the parental strain were picked. The library plasmids were recovered and retested for suppression of spore lethality by transformation into K12359. Sequencing of both ends of the insert in the library plasmids revealed the genes responsible for suppression.

α-Factor arrest and induction of *GAL1-10–HA3–CHL1*

Synchronization of strain K12131 by α factor was performed as described previously (Amon, 2002). The experiment was carried out at 25°C in YEP containing 2% raffinose, with or without the addition of 2% galactose at the indicated time points. Samples for flow cytometry and protein extracts, taken every 10 minutes, and samples for in situ immunofluorescence, taken 90 minutes after the release from α factor, were processed as described below.

Candidate deletion screen

For the list of genes selected for deletion, see supplementary Table S2 (http://jcs.biologists.org/supplemental/). Genes were deleted in the diploid strain K8409, processed and analysed as described previously for a different screen (Rabitsch et al., 2001).

Other techniques

Flow cytometry of DNA content and the preparation of protein extracts were performed as described previously (Klein et al., 1999; Piatti et al., 1996). For immunoblotting, hemagglutinin (HA)-tagged proteins were detected using a monoclonal rat anti-HA 3F10 antibody (Eurogentec) at 1:2000 and the loading control Swi6 was detected using a polyclonal rabbit anti-Swi6 antibody at 1:5000 (Taba et al., 1991). Sporulation time courses were performed as described previously (Buonomo et al., 2000). In situ immunofluorescence was performed as described previously (Pringle et al., 1991) on cells that were fixed overnight on ice in 3.7% formaldehyde. The following primary antibodies were used: monoclonal rat anti-α-tubulin YOL1/34 1:100 (Serotec), monoclonal mouse anti-Myc 9E10 1:5 (Evan et al., 1985), polyclonal rabbit anti-Myc 1:500 (Gramsch) and monoclonal mouse anti-HA 16B12 1:500 (Eurogentec). Chromosome spreads were performed as described previously (Nairz and Klein, 1997) and stained with a monoclonal rat anti-HA 3F10 antibody (Eurogentec) at 1:500 and a rabbit anti-Zip1 serum at 1:500 (kindly provided K. Schmekel). Highly cross-adsorbed secondary antibodies conjugated to Alexa488 (Molecular Probes) (1:500), Cy3 (1:500) or Cy5 (Chemicon) (1:100) were used. DNA was visualized by staining with DAPI. Cells sporulated on plates at 30°C were fixed in ethanol at –20°C, stained with DAPI and scored for the segregation of *URA3*-GFP under the fluorescence microscope. Image acquisition was performed on an Axioplan 2 Imaging microscope (Zeiss) equipped with a CoolSNAP fx camera (Roper Scientific, Photometrics). Spore viability was determined by microdissection of tetrads sporulated on plates at 30°C for 48 hours and subsequent germination and growth at 30°C for 4 days on YEPD.

Schizosaccharomyces pombe experiments

The genotypes of all fission yeast (*Schizosaccharomyces pombe*) strains used in this study are listed in supplementary Table S1 (http://jcs.biologists.org/supplemental/). The *ura4+* cassette was inserted near the N-terminal end of the *dcc1* open reading frame (ORF) (180 bp of 1050 bp, SPAC31A2.15c) and in the middle region of the *ctf18* ORF (1603 bp of 2883 bp, SPBC902.02c). Sporulation and the analysis of chromosome segregation using *cen1*-GFP was performed as described previously (Watanabe and Nurse, 1999).

Results

CHL1 is a high-copy-number suppressor of *ctf8Δ* spore lethality

Deletion of genes encoding subunits specific to RF-$C^{Ctf18/Dcc1/Ctf8}$ causes massive chromosome mis-segregation during meiosis, leading to low spore viability (see below). To identify factors that ameliorate this phenotype, we screened for genes that, when present at high copy number on 2μ plasmids, increased the viability of *ctf8Δ* spores. In addition to plasmids encoding *CTF8* and *CTF18*, we recovered multiple clones containing the entire *CHL1* gene. High-copy-number vectors containing *CHL1* were able to increase *ctf8Δ* spore viability from 5% to 20%. *CHL1* belongs to an evolutionary conserved family of DEAH-box helicases and has been shown to be important for the fidelity of chromosome transmission during mitosis in budding yeast (Gerring et al., 1990; Kouprina et al., 1993; Shiratori et al., 1999; Spencer et al., 1990). The defect responsible for chromosome mis-segregation in *chl1Δ* mutants was nevertheless unknown. Our results raise the possibility that the *CHL1* helicase might be involved in generating sister-chromatid cohesion.

CHL1 is required for efficient sister-chromatid cohesion during mitosis along with *CTF8* and *CTF4*

RF-$C^{Ctf18/Dcc1/Ctf8}$ and *CTF4* are required to maintain efficient sister-chromatid cohesion during a prolonged cell-cycle arrest induced by nocodazole treatment (Hanna et al., 2001; Mayer et al., 2001). It has, however, never been established whether these proteins or Chl1 have such a role in cycling cells in the presence of microtubule pulling forces. To address this, we analysed wild-type, *chl1Δ*, *ctf8Δ* and *ctf4Δ* strains expressing a Myc-tagged version of securin (Pds1-Myc18) and carrying a tandem array of tetracycline operators integrated at the *URA3* locus 35 kbp away from the centromere of chromosome V (Michaelis et al., 1997). Expression of a tet-repressor–GFP fusion protein enabled us to visualize sister-chromatid cohesion at the *URA3* locus (*URA3*-GFP). Cycling cells were fixed and stained with DAPI and antibodies against tubulin and

the Myc epitope. We identified metaphase cells on the basis of their bipolar spindles, large buds and high levels of nuclear securin, and determined the proportion of these cells containing one or two *URA3*-GFP signals (Fig. 1A). In contrast to wild-type cells, which never split their replicated *URA3* loci into two GFP signals before the onset of anaphase, precocious separation of *URA3* loci along the spindle axis was observed in 43% of *chl1Δ*, 56% of *ctf4Δ* and 45% of *ctf8Δ* metaphase cells (Fig. 1A). We noted that the proportion of cells in metaphase was 13% in wild-type, but 31% in *chl1Δ* and 28% in *ctf8Δ*, asynchronous cultures. The simplest explanation for the increase in metaphase cells in *chl1Δ* and *ctf8Δ* cells is that their defective sister-chromatid cohesion causes a transient metaphase arrest induced by the Mad2-dependent mitotic checkpoint, consistent with previous findings of a Mad2-dependent increase of mutant cells with a 2*C* DNA contents (Li and Murray, 1991; Mayer et al., 2001). We conclude that Chl1, Ctf8 and Ctf4 are necessary for efficient sister-chromatid cohesion in cycling cells as well in cells arrested for long periods in a metaphase-like state by nocodazole.

Despite their striking cohesion defects, the frequency of mis-segregation of sister chromatids during anaphase in *ctf8Δ*, *ctf4Δ* and *chl1Δ* mutant cells is very low (<2%, data not shown), which is consistent with their high viability. The mutant cells clearly generate sufficient cohesion along chromosome arms to biorient most sister kinetochores despite splitting of sequences at least up to 35 kbp away from the centromere.

Chl1 is required for establishment of sister-chromatid cohesion during S phase

Membership of Ctf18, Dcc1 and Ctf8 as part of an alternative RF-C and Chl1's homology to helicases suggest that this class of proteins might have a role in the establishment of sister-chromatid cohesion

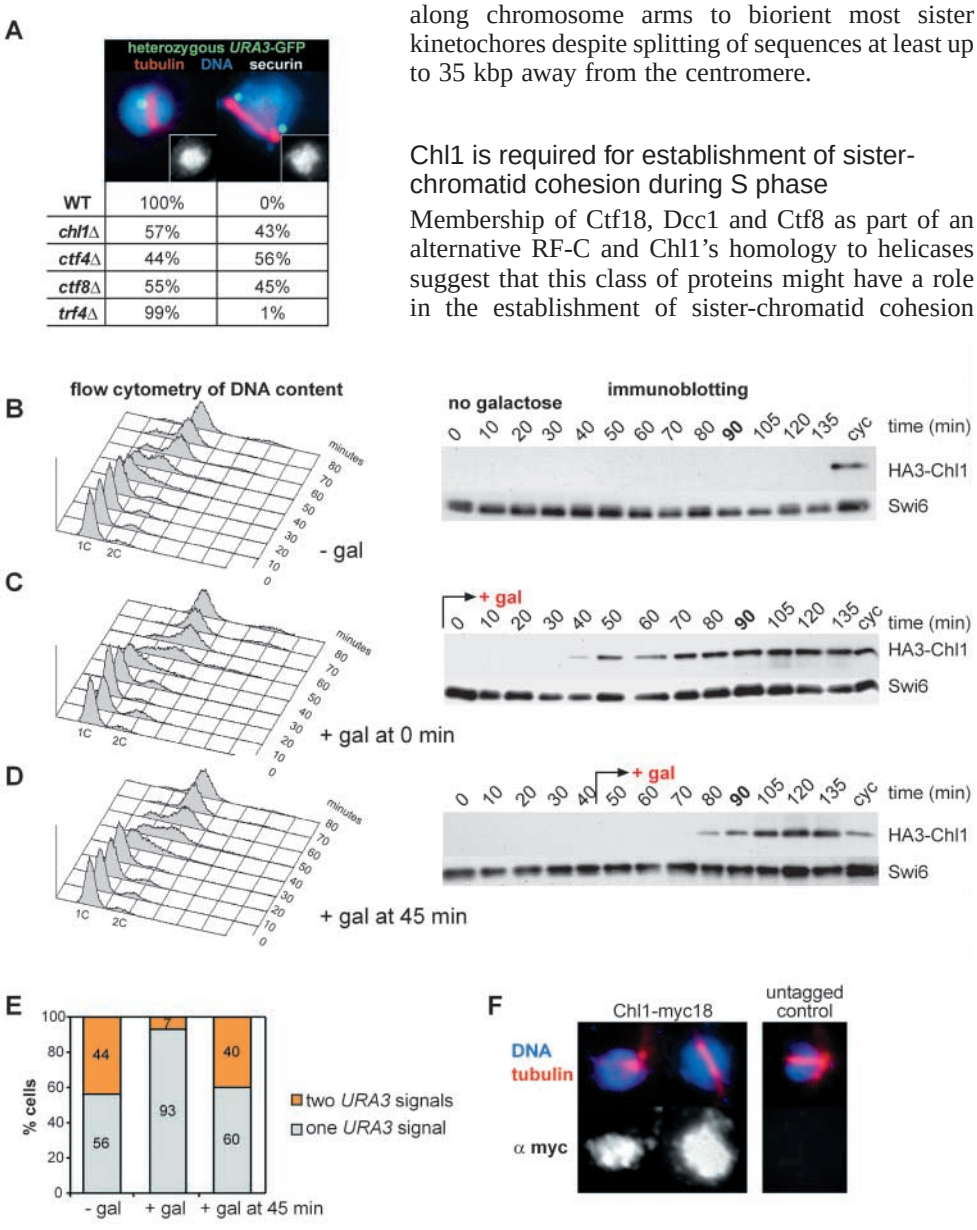


Fig. 1. (A) *CHL1*, *CTF8* and *CTF4* are required for sister-chromatid cohesion during mitosis. Cycling cultures of wild-type (K10003), *chl1Δ* (K11652), *ctf4Δ* (K11692), *ctf8Δ* (K10576) and *trf4Δ* (K12661) strains expressing Pds1-Myc18 (securin) and carrying chromosome V marked by GFP at the *URA3* locus 35 kbp away from the centromere were fixed and stained for DNA and with antibodies against tubulin and the Myc epitope. Fluorescence images of a wild-type (left) and *chl1Δ* (right) metaphase cell showing *URA3*-GFP (green), tubulin (red) and DNA (blue) in the large frame and securin (white) of the respective cell above in the small inserts. The proportions of securin-positive cells with a bipolar spindle containing one or two URA3-GFP signals in the different strains are given below the images. 100 metaphase cells were scored for each strain in this experiment. (B-E) Chl1 is required during S phase to support sister-chromatid cohesion. A haploid yeast strain (K12131) with chromosome V marked by GFP at the *URA3* locus and expressing Pds1-Myc18 (securin) in which the endogenous *CHL1* ORF was put under regulation of the *Gal1-10* promoter and three HA tags were fused to the N-terminus was arrested with α factor and released in medium containing raffinose (B) with no addition of galactose, (C) with the addition of galactose at 0 minutes (min) or (D) with addition of galactose at 45 minutes. At the indicated time points, DNA replication was monitored by flow cytometry of DNA content and expression of HA3-Chl1 by immunoblotting with an anti-HA antibody and a loading control (Swi6). (E) Samples from all three cultures taken at 90 minutes were fixed and stained with antibodies against tubulin and the Myc tag of securin to asses the ratio of securin-positive cells with a bipolar spindle containing one or two *URA3*-GFP signals. 100 metaphase cells were scored for all three cultures in this experiment. (F) Chl1 localizes to the nucleus. Cycling cells of a strain expressing Chl1-Myc18 (K11770) (left) and of an untagged control strain (K8378) (right) were fixed and stained for DNA and with antibodies against tubulin and the Myc epitope. Fluorescence microscopy images of a G1 (left) and metaphase (right) cell showing tubulin (red) and DNA (blue) in the upper panel and anti-Myc staining of the respective cells above in the lower panel.

during S phase. To determine whether Chl1 must act during S phase or whether it can also act during G2 or M phase, the endogenous *CHL1* ORF was placed under control of the galactose-inducible *GAL1-10* promoter and its N-terminus was fused to three HA epitopes to monitor protein expression. Yeast cells grown in medium containing raffinose (*GAL1-10* off) were first arrested in G1 phase with α factor. After release from this arrest, the culture was split into three aliquots. To one culture, no galactose was added (Fig. 1B); to the second, galactose was added immediately upon release (0 minutes; Fig. 1C); to the third, galactose was added 45 minutes after release (Fig. 1D). DNA replication was monitored by flow cytometry and expression of HA3-Chl1 by immunoblotting. DNA replication occurred with similar kinetics in all three cultures. After 90 minutes, when most cells had entered metaphase, we determined the proportion of metaphase cells (with bipolar spindles and high securin levels) with split *URA3*-GFP signals (Fig. 1E). In the absence of galactose, 44% of metaphase cells had two distinct *URA3*-GFP loci (Fig. 1E), which is comparable to the proportion in *chl1Δ* cells (Fig. 1A). Addition of galactose at 0 minutes led to the accumulation of HA3-Chl1 protein to normal levels at 40-50 minutes and before bulk DNA replication. This expression was sufficient for efficient sister-chromatid cohesion because only 7% of metaphase cells had split *URA3*-GFP signals (Fig. 1E). Addition of galactose at 45 minutes resulted in significant accumulation of HA3-Chl1 by 80 minutes, by which time all cells had completed DNA replication. Unlike early expression, this was not sufficient for efficient sister chromatid cohesion and sister *URA3*-GFP signals were split in 40% of metaphase cells (Fig. 1E). These data are consistent with the helicase Chl1 being required during S phase.

To analyse the localization of Chl1, we performed in situ immunofluorescence on mitotic cells expressing Chl1-Myc18 from its endogenous locus. Consistent with a function in sister-chromatid cohesion, we found that Chl1-Myc18 localizes to the nucleus (Fig. 1F).

RF-C$^{Ctf18/Dcc1/Ctf8}$, the helicase *CHL1* and *CTF4* are essential for chromosome segregation in meiosis and for spore viability

RF-C$^{Ctf18/Dcc1/Ctf8}$, *CTF4* (Hanna et al., 2001; Mayer et al., 2001) and *CHL1* (this work) are required for sister-chromatid cohesion in mitotic cells (Fig. 1A). Nevertheless, *ctf8Δ*, *chl1Δ* and *ctf4Δ* mutants are viable and usually disjoin sister chromatids to opposite poles in anaphase (see above). Whether these genes have a more important function in meiosis is unknown. Using a candidate-gene approach based on functional genomic data (see below), we identified two subunits of the alternative RF-C, *DCC1* and *CTF8* as being essential for meiotic chromosome segregation.

Parallel phenotypic analysis of sporulation in budding yeast has identified 158 genes that are not required for spore formation but appear to be required for efficient spore germination (Deutschbauer et al., 2002). We reasoned that deletion of genes required for meiotic chromosome segregation would result in aneuploidy of spores, low spore viability and hence inefficient germination. We therefore selected 55 of the 158 germination-defective genes [see supplementary Table S2 (http://jcs.biologists.org/supplemental/)], excluding genes with known functions during meiosis (e.g. *SPO11*) or clearly assigned roles other than chromosome segregation (e.g. *FAR1*), and deleted them in a sporulation-proficient diploid yeast strain in which both homologs of chromosome V were tagged with GFP (homozygous *URA3*-GFP, 35 kbp away from CEN5). This allowed us to monitor the segregation of chromosome V in tetrads following meiosis. Out of the 55 selected genes, deletion of three genes *CTF8*, *DCC1* and *YGL214W* resulted in severe chromosome segregation defects during meiosis. In wild-type cells, each spore contains one GFP signal corresponding to one chromatid of chromosome V (Fig. 2). More than half of *ctf8Δ* and *dcc1Δ* tetrads carry at least one spore without a copy of chromosome V and one with two copies (Fig. 2). *YGL214W* overlaps with C-terminus of the *SKI8* ORF located on the complementary strand. *SKI8* is known to be involved in meiotic recombination (Gardiner et al., 1997), which would account for the mis-segregation phenotype of *ygl214wΔ*. In addition to Ctf8 and Dcc1, Ctf18 is the third subunit specific to RF-C$^{Ctf18/Dcc1/Ctf8}$ that has been shown to be required for sister-chromatid cohesion in mitosis (Hanna et al., 2001; Mayer et al., 2001). Deletion of *CTF18* resulted in severe chromosome mis-segregation during meiosis similar to *ctf8Δ* and *dcc1Δ* cells (Fig. 2).

We next analysed chromosome segregation in strains containing only a single homolog of chromosome V tagged with GFP (heterozygous *URA3*-GFP). In the wild type, the two GFP-labeled chromatids always segregated into opposite spores during meiosis II (Fig. 2). In all three deletion strains, sister GFP signals segregated into the same spore in about 30% of tetrads (Fig. 2). This high incidence of meiosis II non-disjunction (Fig. 2) compared with the low frequency of meiosis I homolog non-disjunction (see below, Fig. 3E,F) suggests that meiosis II errors are largely responsible for the

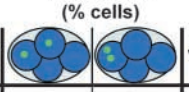
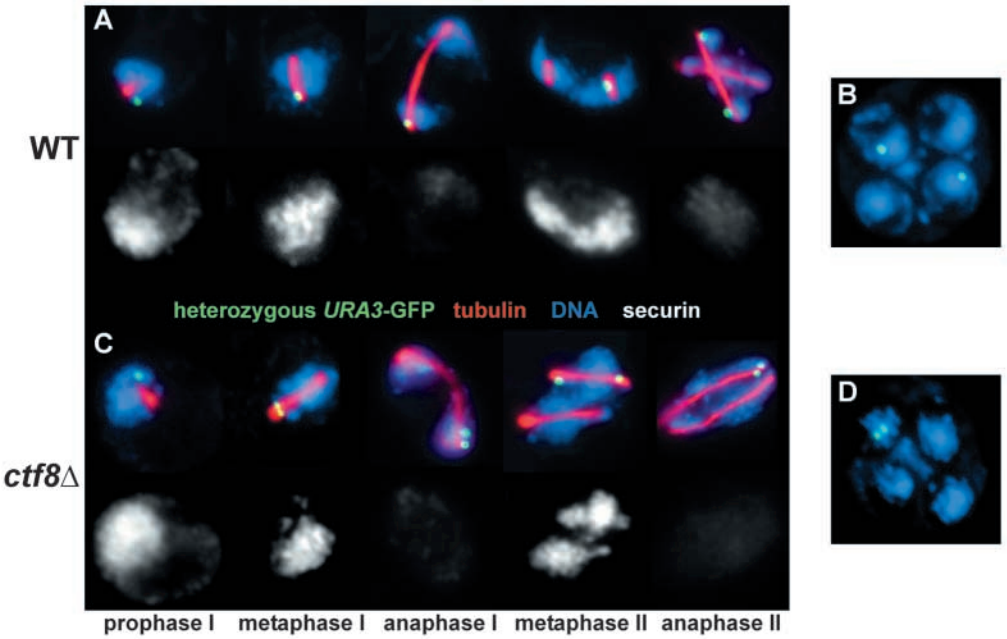
	homozygous *URA3*-GFP (% cells)				heterozygous *URA3*-GFP (% cells)		
							spore viability (%)
WT	100	0	0	0	100	0	95
***ctf18*Δ**	40	44	16	0	72	28	3
***dcc1*Δ**	45	45	10	0	71	29	6
***ctf8*Δ**	46	41	13	0	67	33	5
***chl1*Δ**	42	44	12	2	73	27	13
***ctf4*Δ**	43	47	8	2	70	30	6
***trf4*Δ**	96	2	2	0	97	3	n.d.

Fig. 2. The alternative RF-C$^{Ctf18/Dcc1/Ctf8}$, *CHL1* and *CTF4* are required for chromosome segregation during meiosis and for spore viability. Diploid wild-type (K8409), *ctf18Δ* (K10488), *dcc1Δ* (K10117), *ctf8Δ* (K10092), *chl1Δ* (K11603), *ctf4Δ* (K11681) and *trf4Δ* (K12384) strains in which both homologs of chromosome V were marked by GFP (homozygous *URA3*-GFP) were sporulated and chromosome segregation in tetrads was scored under the fluorescence microscope. Spore viability (n=100) was determined by dissection. Diploid wild-type (K10003), *ctf18Δ* (K10608), *dcc1Δ* (K10352), *ctf8Δ* (K10576), *chl1Δ* (K11652), *ctf4Δ* (K11692) and *trf4Δ* (K12661) strains in which only one copy of chromosome V was marked by GFP (heterozygous *URA3*-GFP) were sporulated and chromosome segregation in tetrads was scored under the fluorescence microscope. More than 200 tetrads of each strain were scored for *URA3*-GFP segregation. n.d., not determined.



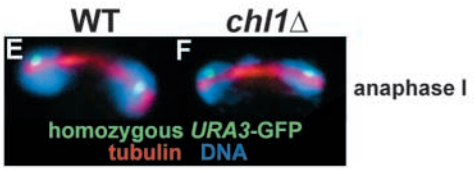


Fig. 3. (A-D) *CTF8* is required for sister-chromatid cohesion during meiosis. Diploid wild-type (K10003) (A,B) and *ctf8Δ* (K10576) (C,D) strains expressing Pds1-Myc18 (securin) and carrying one homolog of chromosome V marked by GFP (heterozygous *URA3*-GFP) were analysed in a meiotic time course experiment. Cells were fixed and stained for DNA and with antibodies against tubulin and the Myc epitope. Fluorescence microscopy images of various meiotic cell cycle stages (indicated below the images) of wild-type (A) and *ctf8Δ* (C) cells showing *URA3*-GFP (green), tubulin (red) and DNA (blue) are arranged in the upper panels as a simulated time course. The lower panels in (A,C) display the securin staining of the cells above them. Fluorescent-microscopy pictures of a sporulated wild-type (B) and *ctf8Δ* (D) tetrad stained for DNA (blue) and showing heterozygous *URA3*-GFP (green). (E,F) Deletion of *CHL1* does not cause frequent meiosis I non-disjunction of homologues. Diploid wild-type (K8409) (E) and *chl1Δ* (K11603) (F) strains carrying both homologs of chromosome V marked by GFP (homozygous *URA3*-GFP) were analysed in meiotic time course experiment. Cells were fixed and stained for DNA and with antibodies against tubulin. Wild-type (E) and *chl1Δ* (F) anaphase I cells displaying URA3-GFP (green), tubulin (red) and DNA (blue) are shown. For each strain, 100 anaphase I cells were scored in this experiment.

chromosome mis-segregation observed in the mutant cells. Consistent with severe aneuploidy, spores from *ctf18Δ*, *dcc1Δ* and *ctf8Δ* diploids have a low viability (Fig. 2).

The identification of *CHL1* as a high-copy-number suppressor of *ctf8Δ* spore lethality (see above) together with the fact that *chl1Δ* cells have sister-chromatid cohesion defects comparable to *ctf8Δ* cells during mitosis led us to investigate meiosis in *chl1Δ* diploids. We found that deletion of *CHL1* caused massive chromosome mis-segregation, with a high frequency of meiosis II non-disjunction and low spore viability (Fig. 2). Deletion of *CTF4* had a very similar effect (Fig. 2).

The severe meiosis II chromosome mis-segregation observed in *ctf8Δ*, *chl1Δ* and *ctf4Δ* strains cannot be a consequence of a failure to repair DNA double-strand breaks induced during meiotic recombination because deletion of the recombination-initiating endonuclease *SPO11* (Keeney et al., 1997) did not alter the meiosis II non-disjunction (data not shown). We conclude that, in contrast to the situation in mitosis, Chl1, Ctf4 and RF-C$^{Ctf18/Dcc1/Ctf8}$ are essential for chromosome segregation during meiosis II and for the production of viable gametes.

ctf8Δ and *chl1Δ* cells lose sister-chromatid cohesion during meiosis

To address whether chromosome mis-segregation during meiosis in *ctf8Δ* and *chl1Δ* cells might be caused by a loss of sister-chromatid cohesion, we used in situ immunofluorescence to analyse diploids in which one copy of chromosome V was tagged with GFP (heterozygous *URA3*-GFP) and that expressed securin tagged with Myc18 (Pds1-Myc18) and Rec8 tagged with HA3 (Rec8-HA3) (Fig. 3). *chl1Δ* cells proceeded through meiosis with kinetics similar to the wild type. Premeiotic DNA replication, accumulation of securin and expression of Rec8 in *chl1Δ* cells were very similar to wild-type cells (data not shown). *ctf8Δ* cells proceeded through meiosis more slowly and less efficiently but, nevertheless, half of them underwent both meiotic divisions (data not shown). In the wild type, centromere-linked *URA3* sequences marked by GFP remain closely associated until the onset of anaphase II. They invariably move to the same pole at anaphase I, remain bound together during metaphase II and move to opposite poles only at anaphase II, ending up in separate spores (Fig. 3A,B). Precocious separation of sister *URA3* sequences occurred in a large proportion of *ctf8Δ* and *chl1Δ* cells. During metaphase I,

sisters were split in 15% of *ctf8Δ* and 36% of *chl1Δ* cells (Fig. 3C). At this stage, most split GFP signals were found close to one of the two spindle poles. Despite the precocious splitting, most sister *URA3* sequences segregated to the same pole in *ctf8Δ* (93%) and *chl1Δ* (88%) anaphase I cells. Thus, *URA3*-GFP signals were segregated to opposite poles in only 7% of *ctf8Δ* and 12% of *chl1Δ* cells. When sister *URA3* sequences had co-segregated at the first division, the *URA3*-GFP signals were found as two distinct spots in 10% of *ctf8Δ* and 28% of *chl1Δ* anaphase I cells (Fig. 3C), a phenotype never observed in the wild type. In metaphase II, sister *URA3*-GFP loci were split along the same spindle axis in 56% of *ctf8Δ* and in 50% of *chl1Δ* cells in which sister centromeres had segregated to the same pole at meiosis I (Fig. 3C). During the ensuing anaphase II, sister chromatids mis-segregated to the same pole in 30% of *ctf8Δ* and 31% of *chl1Δ* cells (Fig. 3C), and, as a consequence, were found in a single spore of tetrads (Fig. 3D). These frequencies of meiosis II non-disjunction are consistent with the mis-segregation of heterozygous *URA3*-GFP observed in tetrads (Fig. 2).

The above findings demonstrate that deletion of *CTF8* and *CHL1* leads to defects in sister-chromatid cohesion throughout meiosis, but this appears to be accompanied by massive chromosome mis-segregation only during meiosis II, when cohesion is confined to centromeric regions. As might be expected of cells with reduced cohesion between sister centromeres, metaphase II spindles were elongated and nuclei stretched in 52% of *ctf8Δ* and 64% of *chl1Δ* cells that had not yet destroyed securin (Fig. 3A,C). In some cases, chromatids had already segregated into two DNA masses despite the presence of securin (Fig. 6D). Cohesion between sister chromatids cannot be entirely absent in *ctf8Δ* and *chl1Δ* cells during meiosis II because we detected only 30% and not 50% sister-chromatid non-disjunction at anaphase II. Nevertheless, up to 60% of chromatids segregated at random during meiosis II in the mutant cells. Our results not only confirm that RF-$C^{Ctf18/Dcc1/Ctf8}$ is involved in sister-chromatid cohesion but also demonstrate that this complex and the Chl1 helicase have a crucial role in this process during meiosis.

To test whether removal of *CTF8* or *CHL1* affects the segregation of homologous chromosomes to opposite poles during meiosis I, we analysed diploid wild-type, *ctf8Δ* and *chl1Δ* strains carrying both copies of chromosome V tagged with GFP (homozygous *URA3*-GFP) by in situ immunofluorescence in a meiotic time-course experiment (Fig. 3E,F). Wild-type cells invariably segregated homologous chromosomes to opposite poles during anaphase I (Fig. 3E). Meiosis I homolog non-disjunction to the same pole was observed in 4% of *chl1Δ* and 5% of *ctf8Δ* cells in anaphase I. The vast majority of mutant cells (96% for *chl1Δ* and 95% for *ctf8Δ*) segregated the *URA3*-GFP signals to opposite poles (Fig. 3F). This finding supports our conclusion that frequent meiosis II errors rather than meiosis I homolog non-disjunction predominantly contribute to the meiotic chromosome mis-segregation phenotype observed in the mutant cells. However, the sister-chromatid cohesion defect in *ctf8Δ* and *chl1Δ* cells appears to result in a significant proportion of meiosis I mis-segregation owing to the equational segregation of sister chromatids and non-disjunction of homologous chromosomes.

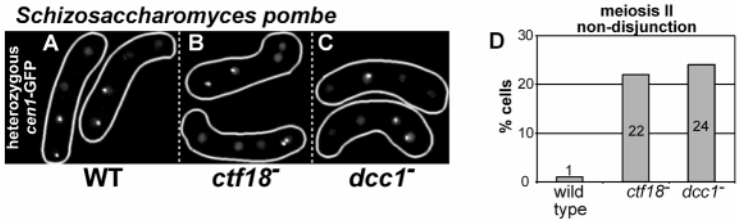


Fig. 4. Mutations in the fission yeast *ctf18* and *dcc1* homologs cause meiosis II non-disjunction. *Schizosaccharomyces pombe* wild-type (PY796×JY333) (A), *ctf18*$^{-}$ (PX101×PX131) (B) and *dcc1*$^{-}$ (PX100×PX128) (C) strains carrying one homolog of chromosome 1 marked by GFP (heterozygous *cen1*-GFP) were sporulated and analysed under the fluorescence microscope. *cen1*-GFP is shown in white and the asci are highlighted using margins. (D) Diagram showing the percentages of *cen1*-GFP meiosis II non-disjunction in fission yeast wild type, *ctf18*$^{-}$ and *dcc1*$^{-}$ tetrads. More than 200 tetrads were scored for each strain.

Mutations in the *S. pombe* homologs of *DCC1* and *CTF18* lead to meiosis II non-disjunction

In a random insertion mutagenesis screen for meiotic chromosome mis-segregation mutants in *S. pombe* (S. Yokobayashi and Y. Watanabe, unpublished), we identified insertions in the fission yeast orthologs of *DCC1* and *CTF18* that disrupted their ORFs. To analyse chromosome segregation during meiosis, we used strains in which *cen1* was marked by GFP on one of the two homologs. Although there was a low but significant increase in segregation to opposite poles, sister *cen1*-GFP loci segregated to the same pole during meiosis I in most mutant cells as in the wild type (data not shown). As in budding yeast (Fig. 2, Fig. 3C,D), the predominant phenotype caused by the *dcc1* and *ctf18* insertions was meiosis II sister-chromatid non-disjunction (Fig. 4). These results suggest that RF-$C^{Ctf18/Dcc1/Ctf8}$ might have an important role in sister-chromatid cohesion during meiosis in a wide range of eukaryotes.

Deletion of *CTF8*, *CTF4* and *CHL1* rescues the nuclear division block of *mam1Δ* cells

Although the precocious separation of sister *URA3*-GFP sequences in budding yeast *chl1Δ* and *ctf8Δ* mutants is consistent with the notion that defective cohesion between sister centromeres is responsible for their high rate of meiosis II non-disjunction (Fig. 2, Fig. 3C,D), we cannot be certain merely from observing precocious separation of *URA3* sequences that defective sister-centromere cohesion is responsible for the meiosis II non-disjunction. For example, similar cohesion defects during mitosis are not accompanied by high levels of non-disjunction (Fig. 1A).

What is required is a method of assaying the functionality of cohesion between sister centromeres. Mutants defective in the monopolin complex provide such an assay. Deletion of *MAM1*, which encodes monopolin's meiosis-specific subunit, causes the attachment of sister kinetochores to opposite poles at meiosis I. As a consequence, *mam1Δ* cells attempt to split and segregate sister centromeres to opposite poles following the destruction of securin and the removal of Rec8 cohesin complexes from chromosome arms in anaphase I but are prevented from doing so by the persistence of cohesion

between sister centromeres (Toth et al., 2000). As a consequence, the spindle fails to elongate and nuclear division fails to take place (‘abortive’ anaphase I; Fig. 5C). If cohesion between sister centromeres were severely compromised in the absence of *CTF8*, *CHL1* and *CTF4* then deletion of these genes should permit *mam1Δ* cells to undergo anaphase I, albeit separating sister centromeres to opposite poles (anaphase I; Fig. 5F). To test this, we used in situ immunofluorescence to compare meiotic chromosome segregation in wild-type, *mam1Δ*, *ctf8Δ*, *chl1Δ*, *ctf4Δ* and double mutant combinations with *mam1Δ* (Fig. 5). All strains expressed a tagged version of securin (Pds1-Myc18) and had one of the two homologs of chromosome V marked by GFP (heterozygous *URA3*-GFP).

We selected meiosis I cells with bipolar spindles that had clearly degraded securin and scored whether they had segregated their chromosomes and extended their spindles (Fig. 5G). Whereas wild-type, *ctf8Δ*, *chl1Δ* and *ctf4Δ* cells readily elongated their spindles and segregated their chromosomes after securin destruction in meiosis I (Fig. 5G), the vast majority of *mam1Δ* cells failed to segregate chromosomes and accumulated as mononucleated cells with short metaphase-I-like spindles (‘abortive’ anaphase; Fig. 5A-C,G). Deletion of *CTF8*, *CHL1* or *CTF4* almost completely restored meiosis I chromosome segregation to *mam1Δ* cells (Fig. 5D-G). The vast majority of *mam1Δ ctf8Δ*, *mam1Δ chl1Δ* and *mam1Δ ctf4Δ* double mutant cells that had destroyed securin possessed symmetrical DNA masses at either end of an elongated anaphase I spindle (anaphase; Fig. 5D-G). Whereas, in wild-type and single *ctf8Δ*, *chl1Δ* or *ctf4Δ* mutants, most sister *URA3*-GFP sequences segregated to the same pole (Fig. 3A,C), they segregated to opposite poles in 84% of *mam1Δ ctf8Δ* (Fig. 5E), in 86% of *mam1Δ chl1Δ* and in 82% of *mam1Δ ctf4Δ* double mutant cells (n=100). These observations imply that the cohesion between sister centromeres responsible for blocking the attempted equational division at meiosis I in *mam1Δ* mutants is largely dependent on *CTF8*, *CHL1* and *CTF4*. The corollary is that these proteins are crucially important for creating the cohesion between sister centromeres needed for meiosis II chromosome segregation as already suggested by the high incidence of meiosis II non-disjunction in mutant cells (Fig. 2, Fig. 3C,D). Our data do not imply that *CTF8*, *CHL1* and *CTF4* are more important for cohesion around centromeres than along chromosome arms. The exquisite sensitivity of meiosis II to the elimination of these proteins more probably stems from the fact that sister-chromatid cohesion is confined to a much smaller chromosomal region at meiosis II than at mitosis or meiosis I.

Retention of the meiotic cohesin Rec8 at centromeres until anaphase II is not affected by deletion of *CTF8* and *CHL1*

The restoration of chromosome segregation to *mam1Δ* mutants could be due either to a failure of *ctf8Δ*, *chl1Δ* or *ctf4Δ* mutants to generate cohesion at premeiotic S phase or to a failure to protect centromeric Rec8 from separase at the onset of anaphase I. For example, Scc1 expressed ectopically instead of Rec8 during meiosis is not protected from separase cleavage at the onset of anaphase I. It is entirely removed from chromosomes at this stage, and this is sufficient to rescue the *mam1Δ* nuclear division block (Toth et al., 2000). To address whether Rec8 is first loaded onto chromosomes normally and subsequently protected from separase in the vicinity of centromeres, we used chromosome spreads and in situ immunofluorescence to analyse the distribution of Rec8 (tagged with three HA epitopes) during meiosis in wild-type, *ctf8Δ* and *chl1Δ* mutant cells.

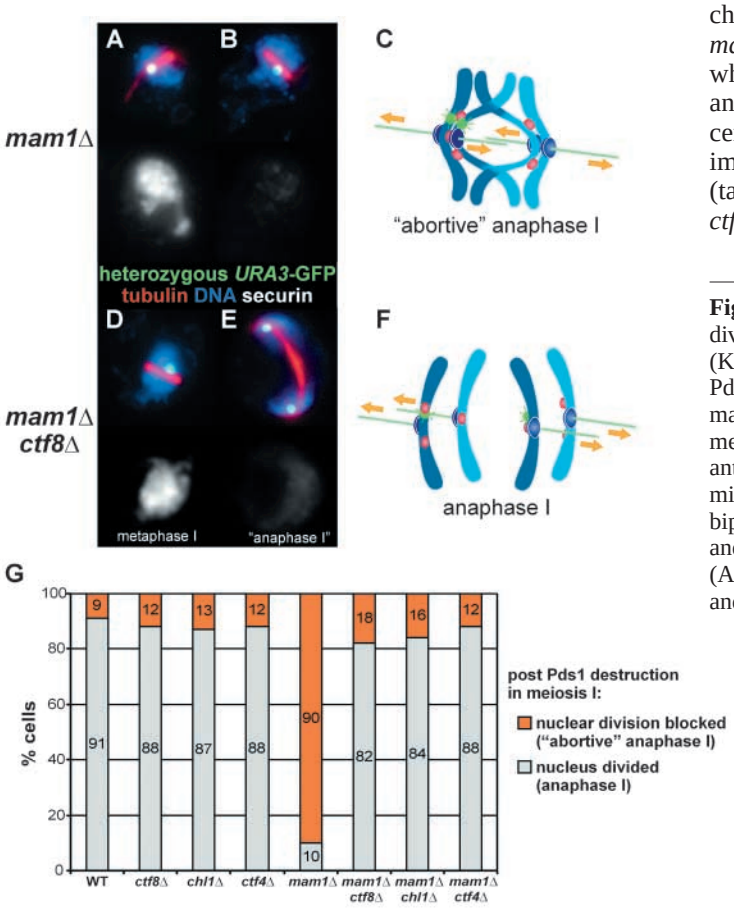


Fig. 5. Deletion of *CTF8*, *CHL1* and *CTF4* rescues the nuclear division block of *mam1Δ* cells in anaphase I. Diploid *mam1Δ* (K8923) (A,B) and *mam1Δ ctf8Δ* (K10572) (D,E) strains expressing Pds1-Myc18 (securin) and carrying one homolog of chromosome V marked by GFP (heterozygous *URA3*-GFP) were analysed in a meiotic time course. Cells were fixed and stained for DNA and with antibodies against tubulin and the Myc epitope. Fluorescent-microscopy images of securin-positive metaphase I cells with a bipolar spindle (A,D), a securin-negative ‘abortive’ anaphase I (B) and a securin-negative elongated anaphase I (E) are shown. (A,B,D,E) The upper panels show *URA3*-GFP (green), tubulin (red) and DNA (blue); the lower panels show securin staining (white) of the cells above them. Schematic drawings illustrating an ‘abortive’ anaphase of *mam1Δ* cells (C) and the rescued anaphase in *mam1Δ ctf8Δ* cells (F) showing meiotic cohesin in red and *URA3*-GFP in green. (G) The percentage of nuclear division and spindle elongation in wild-type (K10003), *ctf8Δ* (K10576), *chl1Δ* (K11652), c*tf4Δ* (K11692), *mam1Δ* (K8923), *mam1Δ ctf8Δ* (K10572), *mam1Δ chl1Δ* (K11703) and *mam1Δ ctf4Δ* (K11828) cells following the destruction of securin in anaphase I. 100 securin-negative cells with a bipolar metaphase I or anaphase I spindle were scored for each strain in this experiment.

During pachytene in meiotic prophase, Rec8-HA3 was associated along the entire length of synapsed chromosomes in wild-type as well as in *ctf8Δ* and *chl1Δ* cells (Fig. 6A,B, data not shown). Deletion of *CTF8* and *CHL1* had little or no effect on chromosome synapsis and condensation at that stage (data not shown). Following cleavage of chromosome arm Rec8 at anaphase I, centromeric Rec8-HA3 staining was observed in the center of the metaphase II spindles in 100% of wild-type cells (Fig. 6C). The fraction of *ctf8Δ* and *chl1Δ* cells staining positive for Rec8-HA3 in metaphase II was very similar, namely 92% and 94%, respectively (Fig. 6D). The metaphase II spindles in *ctf8Δ* and *chl1Δ* mutants were much longer than those in the wild type, and centromeric Rec8-HA3 was distributed along the spindle axes in mutant cells indicating that kinetochores had attached to microtubules but had not properly bioriented (Fig. 6D). These data imply that *CTF8* and *CHL1* are neither required for the stable association of Rec8 with chromosomes nor for the protection of centromeric Rec8 from separase cleavage at the onset of anaphase I. The meiosis II non-disjunction observed in the mutants cannot therefore be caused merely by an absence of centromeric cohesin but rather by a failure of cohesin complexes to generate cohesion between sister DNA molecules.

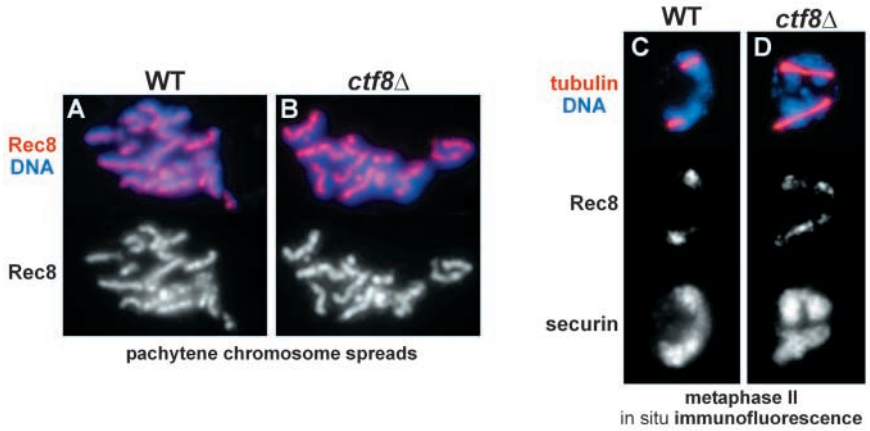


Fig. 6. Association of Rec8 with chromosomes and its retention at centromeres in meiosis II are not affected in the absence of *CTF8*. Diploid wild-type (K10003) (A,C) and *ctf8Δ* (K10572) (B,D) strains expressing Rec8-HA3 and Pds1-Myc18 (securin) were analysed in a meiotic time-course experiment by chromosome spreading (A,B) and in situ immunofluorescence (C,D). Chromosome spreads were stained for DNA and with antibodies against the HA tag. Pachytene-stage chromosome spreads of wild-type (A) and *ctf8Δ* (B) cells showing Rec8 (red) and DNA (blue) in the upper panels and Rec8 alone (white) in the lower panels. Fixed cells were stained for DNA and with antibodies against tubulin, the HA and Myc epitope, and analysed by fluorescence microscopy. Wild-type (C) and *ctf8Δ* (D) metaphase II cells showing tubulin (red) and DNA (blue) in the upper panel, Rec8 (white) in the middle panel, and securin (white) in the lower panel. 100 cells were scored for each strain in this experiment.

DNA polymerase σ, Trf4, is not required for sister-chromatid cohesion in mitosis or meiotic chromosome segregation

The budding yeast DNA polymerase σ, Trf4, has been reported to be involved in sister-chromatid cohesion during mitosis (Wang et al., 2000) and proposed to be loaded onto DNA by RF-$C^{Ctf18/Dcc1/Ctf8}$ to regulate cohesion (Carson and Christman, 2001). If this hypothesis were correct and the major function of RF-$C^{Ctf18/Dcc1/Ctf8}$ in mediating sister-chromatid cohesion is to load Trf4 onto DNA, deletion of *TRF4* should result in mitotic sister-chromatid cohesion defects and severe chromosome mis-segregation during meiosis comparable to mutations in the alternative RF-C subunits. Contrary to this prediction, we found that deletion of *TRF4* had a very minor if any effect on sister-chromatid cohesion in mitotic metaphase cells and on meiotic chromosome segregation (Fig. 1A, Fig. 2). Splitting of *URA3*-GFP signals occurred in only 1% of *trf4Δ* cells with a bipolar spindle and high levels of nuclear securin (Fig. 1A). Furthermore, deletion of *TRF4* did not lead to the characteristic increase in metaphase cells in an asynchronous mitotic culture that we observed for mutations in *CTF8* and *CHL1* (data not shown). Consistent with the lack of a cohesion defect in the absence of Trf4, this result indicates that *trf4Δ* cells do not delay anaphase onset through the activation of the spindle checkpoint. During meiosis, only 4% and 3% of *trf4Δ* tetrads showed mis-segregation of homozygous and heterozygous *URA3*-GFP, respectively (compared with 54% and ~30% or more in alternative RF-C mutants) and the vast majority of *trf4Δ* tetrads displayed wild-type segregation (Fig. 2). Therefore, our data in both mitosis and meiosis do not support the notion that the alternative RF-$C^{Ctf18/Dcc1/Ctf8}$ loads the DNA polymerase σ, Trf4, onto DNA and thereby facilitates the generation of sister-chromatid cohesion.

Discussion

The isolation of mutants with defective chromosome segregation has been a rich source of proteins involved directly or indirectly in sister-chromatid cohesion (Guacci et al., 1993; Kouprina et al., 1993; Michaelis et al., 1997; Spencer et al., 1990; Toth et al., 1999). However, the defective sister-chromatid cohesion of some mutants could be due to secondary consequences of inactivating physiological processes that have little direct bearing on this process. Of all the proteins required for sister-chromatid cohesion, the cohesin complex is the one most directly implicated in holding sister chromatids together. Cohesin is found along interchromatid axes and, crucially, cleavage of cohesin's Scc1 or Rec8 subunit appears to trigger the onset of anaphase in mitotic and meiotic cells (for a review, see Petronczki et al., 2003). No other cohesion protein has been implicated in the actual process of cohesion in such a direct manner, but some have been found either to be associated with cohesin or to be required for its association with chromosomes. Thus, a sizeable proportion of the Scc2-Scc4 complex has been found to be bound to cohesin and to be essential for its association with chromosomes (Arumugam et al., 2003; Ciosk et al., 2000). Likewise, the *Caenorhabditis elegans* TIM1 protein, which is a homolog of the protein encoded by the *Drosophila* and mammalian clock gene *TIMELESS*, is found associated with cohesin and is required for the stable

association with meiotic chromosomes of cohesin's Rec8 subunit (Chan et al., 2003). We noted that the budding yeast *TOF1* gene is the *TIMELESS* ortholog of *Saccharomyces cerevisiae*. Consistent with very recent studies (Mayer et al., 2004; Warren et al., 2004), we find that deletion of *TOF1* results in a modest defect in mitotic sister-chromatid cohesion and meiotic chromosome segregation (B.C. and M.P., unpublished).

Most cohesion proteins have, by contrast, not yet been directly implicated in cohesin function. Some, like Eco1/Ctf7, are essential proteins that have potentially indispensable functions in sister-chromatid cohesion (Skibbens et al., 1999; Toth et al., 1999), whereas others, like Ctf4 and RF-$C^{Ctf18/Dcc1/Ctf8}$, are not essential for mitosis (Hanna et al., 2001; Mayer et al., 2001). Sister-chromatid cohesion is compromised but not eliminated in their absence. Another problem with the interpretation of the cohesion defects in this class of mutants is that measurements have largely been confined to mutant cells induced to arrest for prolonged periods in a metaphase-like state by incubation in spindle poisons.

It is therefore desirable to apply other more physiological assays to assess sister-chromatid cohesion in these types of mutant strain and, in particular, to find circumstances in which their cohesion defects have clear biological consequences. In this study, we describe three new assays to evaluate the roles of Ctf4 and RF-$C^{Ctf18/Dcc1/Ctf8}$. We show that both are required for efficient sister-chromatid cohesion in unperturbed mitotic cells and that both are essential for orderly sister-chromatid disjunction during meiosis II and for the cohesion between sister centromeres that prevents the first meiotic division in monopolin mutants. Our work demonstrates that meiosis is particularly suitable for detecting cohesion defects because, during meiosis II, sister-chromatid cohesion is confined to the vicinity of centromeres. We suggest that cohesion defects have a disproportionate effect at this stage because the reduced cohesion cannot be compensated for by cohesion along chromosome arms.

Sister chromatids segregate to the same pole at meiosis II in 30% of *ctf8Δ* mutant cells, which suggests that segregation might be random for 60% of chromosomes, a level of non-disjunction that causes 95% of spores to be inviable. This enabled us to screen for genes that can, when overexpressed, partially suppress the inviability of spores derived from *ctf8Δ* diploids. Our aim was to isolate genes that either facilitated the maintenance of sister-centromere cohesion or helped to improve the establishment of cohesion. By this means, we identified a gene called *CHL1* whose overexpression increased the viability of spores derived from *ctf8Δ* diploids. *CHL1* had previously been identified in two independent screens for mitotic chromosome transmission fidelity mutants (Kouprina et al., 1993; Spencer et al., 1990). We showed that *CHL1* has a function in sister-chromatid cohesion that is of similar importance to those of Ctf4 and RF-$C^{Ctf18/Dcc1/Ctf8}$: a non-essential role in establishing cohesion during mitosis but a crucial role in avoiding a disastrous level of sister-chromatid non-disjunction during meiosis II. Our findings are consistent with the very recent finding that *chl1Δ* mutations cause sister-chromatid cohesion defects at centromeres and chromosome arms in nocodazole-arrested cells (Mayer et al., 2004). The previous observation that *chl1Δ* cells mis-segregate small artificial chromosomes during mitosis at a much higher frequency than larger ones (Holloway, 2000) supports the notion that meiosis II is more sensitive to cohesion defects than mitosis because this division relies on sister-chromatid cohesion within a more limited chromosomal interval.

Our finding that deletion of *CTF8*, *CHL1* and *CTF4* suppresses the centromeric-cohesion-dependent nuclear division block of *mam1Δ* cells in meiosis I demonstrates for the first time in a functional assay that all three genes are essential for centromeric sister-chromatid cohesion. Despite the strong cohesion defects and high incidence of non-disjunction in meiosis II, *ctf8Δ* and *chl1Δ* cells retain association of the meiotic cohesin subunit Rec8 with centromeres during meiosis II. The mutants also appear to have little or no defect in the loading of Rec8 onto chromosome arms before or during pre-meiotic DNA replication. This indicates that these genes are required for neither the association of cohesin with centromeres nor the protection of centromeric Rec8 from cleavage by separase in meiosis I.

RF-$C^{Ctf18/Dcc1/Ctf8}$, Chl1 and Ctf4 are all evolutionarily conserved proteins, and orthologs can be found in most eukaryotic genomes (Amann et al., 1997; Hanna et al., 2001; Mayer et al., 2001; Merkle et al., 2003; Shiratori et al., 1999; Williams and McIntosh, 2002). We show here that mutations in fission yeast orthologs of *CTF18* and *DCC1* cause meiotic chromosome mis-segregation phenotypes similar to those observed in budding yeast mutants. Therefore, the role of RF-$C^{Ctf18/Dcc1/Ctf8}$ in regulating sister-chromatid cohesion is presumably an evolutionarily conserved feature of this protein complex. Data on human orthologs are consistent with this notion. The CTF18, DCC1 and CTF8 proteins from humans interact with each other and with canonical RF-C subunits (Merkle et al., 2003). In addition, human RF-$C^{Ctf18/Dcc1/Ctf8}$ is found in the nucleus and hCTF18 appears to bind chromatin preferentially during S phase (Merkle et al., 2003).

CHL1 encodes a conserved helicase belonging to the DEAH family (Shiratori et al., 1999). The human homolog hCHLR1 has DNA-dependent ATPase and DNA-helicase activities (Hirota and Lahti, 2000). Mutations in budding yeast *CHL1* predicted to inhibit the protein's enzymatic function abolish its ability to support accurate chromosome segregation (Holloway, 2000), suggesting that Chl1's helicase activity is important for the establishment of efficient sister-chromatid cohesion. hCHLR1 accumulates within nuclei in proliferating cells upon their entry into S phase but not in quiescent cells (Amann et al., 1997), which is consistent with a role in establishing sister-chromatid cohesion. It is conceivable that mutations in human RF-$C^{Ctf18/Dcc1/Ctf8}$ or hCHLR1 could lead to chromosomal instability in mitotic cells, a hallmark of most solid cancers (Jallepalli and Lengauer, 2001), or to infertility owing to the production of aneuploid gametes during meiosis.

The underlying molecular functions of RF-$C^{Ctf18/Dcc1/Ctf8}$, the Chl1 helicase and Ctf4 in regulating sister-chromatid cohesion remain unclear. All three factors are either similar to or interact with replication-related proteins, suggesting a function in the establishment of cohesion at the replication fork during S phase. We provide evidence that Chl1 is indeed required during this period of the cell cycle. Whether Ctf4 and

RF-C$^{Ctf18/Dcc1/Ctf8}$ also act during S phase has not been addressed. It is nevertheless worth noting that mutations that eliminate RF-C$^{Ctf18/Dcc1/Ctf8}$, Chl1 or Ctf4 do not greatly if at all affect replication of bulk DNA during S phase (Hanna et al., 2001; Mayer et al., 2001) (this study). We used affinity purification and mass spectrometry to investigate potential protein partners, and confirmed the composition of RF-C$^{Ctf18/Dcc1/Ctf8}$ but failed to find any stable partners of either Chl1 or Ctf4 (B.C. and M.P., unpublished). It therefore remains unclear whether Ctf4 really does interact with DNA polymerase α in vivo.

Neither we nor others have been able to detect any stable physical association between RF-C$^{Ctf18/Dcc1/Ctf8}$ and cohesin (Mayer et al., 2001; Merkle et al., 2003). Human RF-C$^{Ctf18/Dcc1/Ctf8}$, by contrast, has been shown to interact with PCNA (Merkle et al., 2003; Ohta et al., 2002) and is capable of loading this polymerase clamp onto DNA in vitro, albeit with a lower efficiency than the canonical RF-C^{Rfc1} (Bermudez et al., 2003). It has been suggested that RF-C$^{Ctf18/Dcc1/Ctf8}$ promotes efficient cohesion by loading a specialized DNA polymerase onto cohesin-rich regions of DNA and coordinates their replication with the establishment of cohesion (Carson and Christman, 2001). One candidate for such an enzyme is the putative DNA polymerase σ Trf4, because *trf4* mutants have been reported to be defective in sister-chromatid cohesion in nocodazole-arrested cells (Wang et al., 2000). Our observations that deletion of *TRF4* had little or no effect on sister-chromatid cohesion in an unperturbed cell cycle or on chromosome segregation during meiosis do not support this model. In addition, our *TRF4* results stress the importance of analysing cohesion phenotypes under more physiological conditions rather than in a prolonged mitotic arrest. Although our data strongly question whether Trf4 plays a role in sister-chromatid cohesion, it is still possible that defects in *trf4Δ* cells are masked by redundancy between *TRF4* and its paralog *TRF5* (Edwards et al., 2003; Wang et al., 2000). Nevertheless, the main function of RF-C$^{Ctf18/Dcc1/Ctf8}$ in mediating sister-chromatid cohesion is unlikely to be only the recruitment of Trf4 via PCNA. PCNA can act as a platform for the interaction with many different proteins in addition to serving as a sliding clamp for replicative DNA polymerases (Maga and Hubscher, 2003). It remains to be determined whether RF-C$^{Ctf18/Dcc1/Ctf8}$ loads PCNA or a related clamp onto DNA in vivo and, if so, which factors are subsequently recruited by the clamp.

In conclusion, our work now leaves little doubt that RF-C$^{Ctf18/Dcc1/Ctf8}$, the helicase Chl1 and Ctf4 have crucial roles in sister-chromatid cohesion. Future studies will have to focus on the function of these proteins at the replication fork and how they influence the generation of cohesive structures that bind sister chromatids together.

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Table S1. Yeast strains used in this study

Strain	Genotype
K8378	SK1 *MAT**a** lys2 ho_::LYS2 ura3 leu2 his3 trp1*
K8409	SK1 *MAT**a**/MAT_ HO URA3::tetO224 LEU2::tetR-GFP REC8-HA3::URA3 lys2 his3 trp1*
K8923	SK1 *MAT**a**/MAT_ ura3/URA3::tetO224 LEU2::tetR-GFP REC8-HA3::URA3 PDS1-myc18::klTRP1 lys2 ho_::LYS2 mam1Δ::HIS3MX 6his3 trp1*
K10003	SK1 *MAT**a**/MAT_ ura3/URA3::tetO224 LEU2::tetR-GFP REC8-HA3::URA3 PDS1-myc18::klTRP1 lys2 ho_::LYS2 his3 trp1*
K10092	SK1 *MAT**a**/MAT_ HO URA3::tetO224 LEU2::tetR-GFP REC8-HA3::URA3 ctf8Δ::HIS3MX6 lys2 his3 trp1*
K10117	SK1 *MAT**a**/MAT_ HO URA3::tetO224 LEU2::tetR-GFP REC8-HA3::URA3 dcc1Δ::HIS3MX6 lys2 his3 trp1*
K10348	SK1 *MAT**a**/MAT_ ura3/URA3::tetO224 LEU2::tetR-GFP REC8-HA3::URA3 PDS1-myc18::klTRP1 ho_::LYS2 spo11Δ::KanMX4 lys2 his3 trp1*
K10350	SK1 *MAT**a**/MAT_ ura3/URA3::tetO224 LEU2::tetR-GFP REC8-HA3::URA3 PDS1-myc18::klTRP1 ho_::LYS2 spo11Δ::KanMX4 ctf8Δ::HIS3MX6 lys2 his3 trp1*
K10352	SK1 *MAT**a**/MAT_ ura3/URA3::tetO224 LEU2::tetR-GFP REC8-HA3::URA3 PDS1-myc18::klTRP1 lys2 ho_::LYS2 dcc1Δ::HIS3MX6 his3 trp1*
K10353	SK1 *MAT**a**/MAT_ ura3/URA3::tetO224 LEU2::tetR-GFP REC8-HA3::URA3 PDS1-myc18::klTRP1 ho_::LYS2 spo11Δ::KanMX4 dcc1Δ::HIS3MX6 lys2 his3 trp1*
K10488	SK1 *MAT**a**/MAT_ HO URA3::tetO224 LEU2::tetR-GFP REC8-HA3::URA3 ctf18Δ::HIS3MX6 lys2 his3 trp1*
K10572	SK1 *MAT**a**/MAT_ ura3/URA3::tetO224 LEU2::tetR-GFP REC8-HA3::URA3 PDS1-myc18::klTRP1 lys2 ho_::LYS2 mam1Δ::HIS3MX6 ctf8Δ::HIS3MX6 his3 trp1*
K10576	SK1 *MAT**a**/MAT_ ura3/URA3::tetO224 LEU2::tetR-GFP REC8-HA3::URA3 PDS1-myc18::klTRP1 lys2 ho_::LYS2 ctf8Δ::HIS3MX6 his3 trp1*
K10608	SK1 *MAT**a**/MAT_ ura3/URA3::tetO224 LEU2::tetR-GFP REC8-HA3::URA3 PDS1-myc18::klTRP1 lys2 ho_::LYS2 ctf18Δ::HIS3MX6 his3 trp1*
K11603	SK1 *MAT**a**/MAT_ HO URA3::tetO224 LEU2::tetR-GFP REC8-HA3::URA3 chl1Δ::HIS3MX6 lys2 his3 trp1*
K11652	SK1 *MAT**a**/MAT_ ura3/URA3::tetO224 LEU2::tetR-GFP REC8-HA3::URA3 PDS1-myc18::klTRP1 lys2 ho_::LYS2 chl1Δ::HIS3MX6 his3 trp1*
K11681	SK1 *MAT**a**/MAT_ HO URA3::tetO224 LEU2::tetR-GFP REC8-HA3::URA3 ctf4Δ::HIS3MX6 lys2 his3 trp1*
K11692	SK1 *MAT**a**/MAT_ ura3/URA3::tetO224 LEU2::tetR-GFP REC8-HA3::URA3 PDS1-myc18::klTRP1 lys2 ho_::LYS2 ctf4Δ::HIS3MX6 his3 trp1*
K11703	SK1 *MAT**a**/MAT_ ura3/URA3::tetO224 LEU2::tetR-GFP REC8-HA3::URA3 PDS1-myc18::klTRP1 lys2 ho_::LYS2 mam1Δ::HIS3MX6 chl1Δ::HIS3MX6 his3 trp1*
K11770	SK1 *MAT**a** CHL1-myc18::klTRP1 lys2 ho_::LYS2 ura3 leu2 his3 trp1*
K11828	SK1 *MAT**a**/MAT_ ura3/URA3::tetO224 LEU2::tetR-GFP REC8-HA3::URA3 PDS1-myc18::klTRP1 lys2 ho_::LYS2 mam1Δ::HIS3MX6 ctf4Δ::HIS3MX6 his3 trp1*
K11831	SK1 *MAT**a**/MAT_ ura3/URA3::tetO224 LEU2::tetR-GFP REC8-HA3::URA3 PDS1-myc18::klTRP1 ho_::LYS2 spo11Δ::KanMX4 chl1Δ::HIS3MX6 lys2 his3 trp1*
K11834	SK1 *MAT**a**/MAT_ ura3/URA3::tetO224 LEU2::tetR-GFP REC8-HA3::URA3 PDS1-*

	myc18::klTRP1 ho_::LYS2 spo11Δ::KanMX4 ctf4Δ::KanMX4 lys2 his3 trp1
K12131	*MAT**a** URA3::tetO336 HIS3::tetR-GFP PDS1-myc18 ::klTRP1 ho KanMX4::GAL1-10-HA3-CHL1 leu2 trp1 lys2*
K12359	SK1 *MAT**a** lys2 ho_::LYS2 URA3::HO ctf8Δ::HIS3MX6 leu2 his3 trp1*
K12384	SK1 *MAT**a**/MAT_ HO URA3::tetO224 LEU2::tetR-GFP REC8-HA3::URA3 trf4Δ::HIS3MX6 lys2 his3 trp1*
K12661	SK1 *MAT**a**/MAT_ ura3/URA3::tetO224 LEU2::tetR-GFP REC8-HA3::URA3 PDS1-myc18::klTRP1 lys2 ho_::LYS2 trf4Δ::HIS3MX6 his3 trp1*
JY333	*S. pombe h⁻ leu1 ade6*
PY796	*S. pombe h⁺ lys1⁺<<lacO his7⁺<<*P_{dis1}*-GFP-lacI-NLS leu1*
PX100	*S. pombe h⁺ dcc1::ura4⁺ lys1⁺<<lacO his7⁺<<*P_{dis1}*-GFP-lacI-NLS leu1 ade6*
PX101	*S. pombe h⁺ ctf18::ura4⁺ lys1⁺<<lacO his7⁺<<*P_{dis1}*-GFP-lacI-NLS leu1 ade6*
PX128	*S. pombe h⁻ dcc1::ura4⁺*
PX131	*S. pombe h⁻ ctf18::ura4⁺*

Table S2. List of 55 *Saccharomyces cerevisiae* genes and open reading frames selected for deletion based on parallel phenotype analysis of sporulation and postgermination growth (Deutschbauer et al., 2002). Deletion of genes highlighted in bold caused strong chromosome missegregation during meiosis.

ADR1	*UBP6*	*YJR142W*
ARP1	*YAK1*	*YKL033W-A*
CTF8	*YAP3*	*YKL076C*
CUE1	*YBL046W*	*YKL171W*
DCC1	*YBL083C*	*YLL032C*
DIG1	*YBR027C*	*YLR358C*
END3	*YBR255W*	*YML117W*
FPR1	*YDL133W*	*YMR306C-A*
GIS1	*YDL183C*	*YNR040W*
HSL7	*YDL213C*	*YPL166W*
JEM1	*YGL152C*	*YPL180W*
JNM1	***YGL214W***	
KAR9	*YGR106C*	
PAK1	*YHL044W*	

PAT1	*YIL029C*
PFD1	*YIL041W*
PIN2	*YJL192C*
SHE1	*YJR054W*
SOH1	*YJR111C*
SSD1	*YML013C-A*
SST2	*YML013W*
TRF4	*YML014W*

Reference:

Deutschbauer, A. M., Williams, R. M., Chu, A. M. and Davis, R. W. (2002). Parallel phenotypic analysis of sporulation and postgermination growth in Saccharomyces cerevisiae. *Proc Natl Acad Sci USA* **99**, 15530-5.