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# Identification of Protein Complexes Required for Efficient Sister Chromatid Cohesion

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**Ctf8p is a component of Ctf18-RFC, an alternative replication factor C-like complex required for efficient sister chromatid cohesion in *Saccharomyces cerevisiae*. We performed synthetic genetic array (SGA) analysis with a *ctf8* deletion strain as a primary screen to identify other nonessential genes required for efficient sister chromatid cohesion. We then assessed proficiency of cohesion at three chromosomal loci in strains containing deletions of the genes identified in the *ctf8* SGA screen. Deletion of seven genes (*CHL1*, *CSM3*, *BIM1*, *KAR3*, *TOF1*, *CTF4*, and *VIK1*) resulted in defective sister chromatid cohesion. Mass spectrometric analysis of immunoprecipitated complexes identified a physical association between Kar3p and Vik1p and an interaction between Csm3p and Tof1p that we confirmed by coimmunoprecipitation from cell extracts. These data indicate that synthetic genetic array analysis coupled with specific secondary screens can effectively identify protein complexes functionally related to a reference gene. Furthermore, we find that genes involved in mitotic spindle integrity and positioning have a previously unrecognized role in sister chromatid cohesion.**

## INTRODUCTION

The maintenance of proper ploidy during cell division requires both the accurate replication of chromosomes and their faithful segregation during mitosis. The physical association of sister chromatids after DNA replication, or sister chromatid cohesion, is crucial for the proper segregation of sister chromatids at anaphase and is therefore critical for genome stability. In *Saccharomyces cerevisiae*, cohesion is mediated by a multisubunit protein complex called cohesin that is composed of at least four proteins: Smc1p, Smc3p, Mcd1p/Scc1p, and Irr1p/Scc3p (SA1 or SA2 in mammalian cells) (Guacci *et al*., 1997; Michaelis *et al*., 1997; Toth *et al*., 1999). Pds5p is also required for sister chromatid cohesion and its localization to chromatin requires Mcd1p/Scc1p (Hartman *et al*., 2000; Panizza *et al*., 2000). Before the onset of anaphase, Esp1p, a protease required for the separation of sister chromatids, is bound to its inhibitor Pds1p (Ciosk *et al*., 1998). At the onset of anaphase Pds1p is ubiquitinated and targeted for degradation by the anaphase promoting complex/cyclosome (APC/C) (Cohen-Fix *et al*., 1996). Degradation of Pds1p releases Esp1p, which then cleaves the cohesin subunit Scc1p resulting in sister chromatid separation (Uhlmann *et al*., 1999, 2000).

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Other proteins required for proper sister chromatid cohesion function during the establishment of cohesion, which takes place during S phase. Scc2p and Scc4p physically interact with each other but are not core components of the cohesin complex (Ciosk *et al*., 2000). Scc2p and Scc4p are, however, required for the association of cohesin with DNA (Ciosk *et al*., 2000). Eco1p/Ctf7p is also required for the establishment but not the maintenance of cohesion (Skibbens *et al*., 1999; Toth *et al*., 1999). In *eco1* mutants, the cohesin complex is able to associate with DNA, but proper sister chromatid cohesion is not established, indicating that the proper establishment of sister chromatid cohesion is likely to be a multistep process requiring both the association of the cohesin complex with DNA as well as the physical pairing of sister chromatids (Toth *et al*., 1999).

Deletion of *SMT4* does not seem to affect the association of the cohesin subunit Mcd1p/Scc1p with chromatin, but *smt4* strains are unable to maintain centromeric cohesion (Bachant *et al*., 2002). Smt4p is capable of reversing Smt3p/SUMO-1 modification (Li and Hochstrasser, 1999, 2000), and several observations suggest that the centromeric cohesion defect of *smt4* is partially due to defective Smt3p/SUMO-1 modification of Top2p. Overexpression of *TOP2* reduces the degree of defective centromeric cohesion in *smt4* strains, and *top2-4* mutants exhibit defective centromeric cohesion. Finally, expression of Top2p-SNM, in which the SUMO-1 modification sites of Top2p have been mutated, results in partial suppression of the *smt4* cohesion defect at centromeric loci (Bachant *et al*., 2002). These data suggest that Smt3p/SUMO-1 modification of Top2p may play a role in

establishing a modified chromosome structure or localized catenations that are required for efficient centromeric cohesion.

Many other observations have highlighted the important role that the DNA replication machinery plays in sister chromatid cohesion. Overexpression of *POL30*, which encodes the DNA polymerase processivity factor PCNA, rescues the temperature sensitivity of a *ctf7-203* allele (Skibbens *et al*., 1999). Ctf4p, which is required for efficient sister chromatid cohesion (Hanna *et al*., 2001), binds specifically to DNA polymerase $\alpha$ (Miles and Formosa, 1992a,b). *ctf4* also exhibits genetic interactions with *cdc17-1* (which encodes DNA polymerase $\alpha$) and is synthetically lethal with *rfc1/cdc44*, a component of the replication factor C (RFC) complex (Formosa and Nittis, 1999; Budd and Campbell, 2000). An additional link between DNA replication and the establishment of sister chromatid cohesion came with the discovery and analysis of Trf4p and Trf5p, redundant homologues that encode the nuclear DNA polymerase $\sigma$. Deletion of *TRF4* results in a sister chromatid cohesion defect (Wang *et al*., 2000).

Finally, components of Ctf18-RFC, an alternative RFC complex composed of Rfc2p, Rfc3p, Rfc4p, Rfc5p, Ctf18p, Ctf8p, and Dcc1p, are required for proper sister chromatid cohesion (Hanna *et al*., 2001; Mayer *et al*., 2001; Naiki *et al*., 2001). Because one function of the canonical RFC complex (composed of Rfc1p, Rfc2p, Rfc3p, Rfc4p, and Rfc5p) is to initiate a switch from DNA polymerase $\alpha$ to DNA polymerase $\delta$ during lagging strand DNA synthesis (reviewed in Waga and Stillman, 1998), these data suggest a model whereby Ctf18-RFC functions to initiate a polymerase switch to DNA polymerase $\sigma$ during DNA replication, which would be required for the efficient establishment of sister chromatid cohesion. The human Ctf8, Ctf18, and Dcc1 proteins were recently shown to interact with the p36, p37, p38, and p40 subunits of RFC, indicating that the Ctf18–RFC complex exists in human cells (Bermudez *et al*., 2003; Merkle *et al*., 2003). In addition, hCtf18 coimmunoprecipitated PCNA, and the seven-member hCtf18-RFC was shown to be able to load the PCNA clamp onto DNA in an ATP-dependent manner (Bermudez *et al*., 2003; Merkle *et al*., 2003).

To identify other genes required for sister chromatid cohesion, we performed synthetic genetic array (SGA) analysis to isolate genes that, when deleted, result in synthetic fitness defects in combination with a deletion of *CTF8*. We then assessed sister chromatid cohesion in strains carrying a deletion in genes found to be synthetically sick or lethal with *ctf8*Δ. Here we report that deletion of *VIK1*, *CTF4*, *CSM3*, *KAR3*, *TOF1*, *CHL1*, or *BIM1* results in inefficient sister chromatid cohesion. Using mass spectrometric analysis of immunoprecipitated complexes, we find that Vik1p and Kar3p, and Csm3p and Tof1p, physically interact in vivo. These data indicate that SGA analysis of a reference gene can be used to identify functionally relevant protein complexes. Of particular significance, we find that genes involved in mitotic spindle integrity and positioning are required for efficient sister chromatid cohesion. Thus, these pathways make previously uncharacterized contributions to accurate chromosome segregation via their effects on sister chromatid cohesion.

## MATERIALS AND METHODS

### *Synthetic Genetic Array Analysis*

SGA analysis was carried out as described previously (Tong *et al*., 2001). Briefly, a starting strain containing *ctf8*Δ (MAT$\alpha$ *ctf8*Δ*::natR can*Δ*1::MFA1pr-HIS3-MF*$\alpha$*1pr-LEU2 his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 met15*Δ*0*) was mated to the yeast genome deletion set (MATa *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0* with [*nonessential orf*]Δ*::kanMX6*). Diploids were sporulated and the resulting haploids were grown on selective plates to identify viable gene deletions that show synthetic genetic interactions with *ctf8*Δ. Genetic interactions were confirmed by tetrad analysis on YPD.

### *Assessing Sister Chromatid Cohesion*

Tet repressor-GFP/Tet operator repeat strains were as follows: wild-type (YPH1477, MATa *ade2-1 trp1-1 can1-100 his3-11,15 leu2::LEU2-TetR-GFP ura3::3xURA3-TetO112 PDS1-13Myc:TRP1*; Mayer *et al*., 2001), *chl1*Δ (YPH1614, YPH1615), *vik1*Δ (YPH1616, YPH1617), *csm3*Δ (YPH1618, YPH1619), *bim1*Δ (YPH1620, YPH1621), *tof1*Δ (YPH1622, YPH1623), *kar3*Δ (YPH1624, YPH1625), and *top1*Δ (YPH1626, YPH1627) strains were grown logarithmically in YPD and diluted to $OD_{600}$ = 0.150 before arresting in G2/M with 15 $\mu$g/ml nocodazole (Sigma-Aldrich, St. Louis, MO) or in G1 with 5 $\mu$g/ml alpha factor (Diagnostic Chemicals Limited, Charlottetown, Prince Edward Island, Canada) for 3 h at 25°C. Cells were fixed with an equal volume of fresh 4% paraformaldehyde for 10 min at room temperature, washed once with SK (1M sorbitol, 0.05 M $K_2PO_4$), and resuspended in SK for cohesion assessment.

Lac repressor-GFP/Lac operator repeat strains were as follows: wild-type (Y819, MATa *ade2-1 trp1-1::LacO-TRP1-LEU2 can1-100 his3-11,15::LacI-GFP-HIS3 leu2–3112 ura3-1*; Sanchez *et al*., 1999), *chl1*Δ (YPH1628, YPH1629), *vik1*Δ (YPH1630, YPH1631), *csm3*Δ (YPH1632, YPH1633), *bim1*Δ (YPH1634, YPH1635), *tof1*Δ (YPH1636, YPH1637), and *kar3*Δ (YPH1638, YPH1639), or wild-type (YPH1444, MATa *ade2-101 trp1-1 can1-100 his3-11,15::LacI-GFP(pAFS144, thermostable)-HIS3 leu2-3112 ura3-1 CEN15(1.8kb)-LacO-URA3*; Goshima and Yanagida, 2000, and references therein), *chl1*Δ (YPH1640, YPH1641), *vik1*Δ (YPH1642, YPH1643), *csm3*Δ (YPH1644, YPH1645), *bim1*Δ (YPH1646, YPH1647), *tof1*Δ (YPH1648, YPH1649), and *kar3*Δ (YPH1650, YPH1651) strains were grown logarithmically in YPD at 25°C and then collected by centrifugation and resuspended in synthetic complete media lacking histidine and containing 40 mM 3-aminotriazole (Sigma-Aldrich). Cells were grown in this media for 90 min at 25°C to induce the Lac repressor-GFP fusion protein that was expressed from the *HIS3* promoter. These cells were then collected by centrifugation, resuspended in YPD, and arrested in G2/M with nocodazole or in G1 with alpha factor for 3 h at 25°C. Cells were processed as described above, and the number of GFP signals in each cell was scored.

### *Epitope Tagging and Immunoprecipitations*

C-terminal epitope tagging of proteins at their endogenous loci was performed as described previously (Longtine *et al*., 1998). All tagged strains are derived from YPH1652 (MATa *ade2-101 trp1*Δ*63 his3*Δ*200 leu2*Δ*1 ura3-52 lys2-801*). For mass spectrometric analysis of immunoprecipitated complexes, immunoprecipitations of YPH1653 (MATa *CHL1–13Myc:TRP1), YPH1654 (MATa CSM3–13Myc:TRP1*), YPH1655 (MATa *VIK1-13Myc:TRP1*) and YPH1652 (untagged control) were performed as described previously (Lamb *et al*., 1994) from 500 to 700 mg of protein extract. For coimmunoprecipitation experiments, immunoprecipitations of YPH1656 (MATa *TOF1-3HA:kanMX6*), YPH1657 (MATa *TOF1-3HA:kanMX6 CSM3-13Myc:TRP1*), YPH1658 (MATa *TOP1-3HA:kanMX6*), YPH1659 (MATa *TOP1-3HA:kanMX6 CSM3-13Myc: TRP1*), YPH1654 (MATa *CSM3-13Myc:TRP1*), YPH1660 (MATa *TOF1-3HA: kanMX6 TOP1-13Myc:HIS3MX6*), YPH1661 (MATa *TOP1-13Myc:HIS3MX6*), and YPH1652 (untagged control) were performed as described previously (Tyers *et al*., 1992; Measday *et al*., 2002). Briefly, cells were lysed in 50 mM Tris-HCl pH 7.5, 250 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, and 0.1% NP-40 buffer containing protease inhibitors, and equal amounts of protein (at least 4 mg) were immunoprecipitated using anti-hemagglutinin (HA) or anti-Myc conjugated beads (Covance, Princeton, NJ). Forty micrograms of total lysate and 15% of the immunoprecipitated fractions were loaded on SDS-PAGE gels, and tagged proteins were detected on Western blots with anti-HA (12CA5) or anti-Myc (9E10) antibodies (Roche Diagnostics, Indianapolis, IN).

### *Pds1 Assay*

Green fluorescent protein (GFP) and Pds1-13Myc were detected by indirect immunofluorescence in nocodazole arrested, paraformaldehyde fixed cells by using anti-GFP (AbCam) and anti-Myc (Roche Diagnostics) antibodies, respectively. The presence or absence of Pds1-13Myc signal in cells containing two separated GFP dots was assessed. At least 20 two-dot cells were examined for each strain.

### *Mass Spectrometry Analysis*

The proteins contained in silver-stained bands were analyzed by capillary chromatography, electrospray ionization tandem mass spectrometry (LC-MS/MS) essentially as described previously (Gygi *et al*., 2000). Silver stained bands were excised and the proteins therein were trypsinized in situ. The generated peptide fragments were extracted and analyzed by LC-MS/MS by using an LCQ Classic ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with an in-house microelectrospray ionization source. Needle voltage was set at 2 kV. Ion signals above a predetermined threshold automatically triggered the instrument to switch from MS to MS/MS mode for

generating collision-induced dissociated spectra (data-dependent MS/MS). The generated collision-induced dissociated spectra were searched against the whole genome yeast sequence database using the computer algorithm SEQUEST (Eng *et al*., 1994).

## RESULTS

### *Genome-wide Synthetic Genetic Interaction Screen with ctf8Δ*

Previously, we have shown that a deletion of *CTF8* is synthetically lethal with mutations in genes required for sister chromatid cohesion including *smc3-42*, *scc1-73*, and *scc2-4* (Mayer *et al*., 2001). In contrast, deletion of *CTF8* is not synthetically lethal with deletions of other nonessential components of the Ctf18-RFC complex including *ctf18*Δ and *dcc1*Δ. These data suggested that *CTF8* becomes essential when genes that function in cohesion pathways distinct from that of the Ctf18–RFC complex are impaired. They also suggested that a synthetic lethal screen could be used to identify other nonessential genes required for efficient sister chromatid cohesion. We performed a synthetic genetic interaction screen using an array containing the ~4600 viable haploid yeast open reading frame deletion mutants, as described previously (SGA analysis) (Tong *et al*., 2001). The SGA screen was repeated three times and 55 deletion mutants were identified that displayed synthetic growth defects with *ctf8*Δ in at least two of the three screens (Table 1). Because *CTF4* is the only known nonessential gene required for efficient cohesion other than those in the Ctf18–RFC complex, isolation of *ctf4*Δ in the *ctf8*Δ SGA screen indicated that this approach was successful in identifying other genes required for efficient sister chromatid cohesion.

### *Assessing Sister Chromatid Cohesion in Mutants Synthetically Lethal with ctf8Δ*

We first analyzed sister chromatid cohesion directly in the 55 mutants identified in the *ctf8*Δ SGA screen (Table 1). To identify those genes required for efficient sister chromatid cohesion, we mated these mutants to a strain containing both a Tet repressor-GFP fusion and a Tet operator repeat located 35 kb from the centromere of chromosome V. The resulting diploids were sporulated and dissected, and two independent haploids containing the deletion of interest, as well as the Tet repressor-GFP fusion and the Tet operator repeats, were assayed for defective cohesion. Cells were grown logarithmically, arrested in G2/M with nocodazole for 3 h, fixed, and then scored to determine the number of GFP dots in each cell. *MAD1* and *MAD2* were excluded from this analysis because they encode mitotic checkpoint proteins required for proper G2/M arrest in response to nocodazole (Li and Murray, 1991). Four other genes (*BUD25*, *SMI1*, *GIM3*, and *SWI3*) were excluded from further analysis due to abnormal budding phenotypes or special growth requirements. The deletion of 10 genes (*ygl168w*Δ, *bim1*Δ, *cdh1*Δ, *chl1*Δ, *csm3*Δ, *ctf4*Δ, *kar3*Δ, *rpl13b*Δ, *tof1*Δ, and *vik1*Δ) increased the percentage of nocodazole-arrested cells with two GFP dots to at least 15% (wild type was 10.7%), indicative of either defective sister chromatid cohesion or polyploidy in these cells (Table 1). Further analysis of *rpl13b*Δ cells indicated that the high percentage of cells with two GFP signals in these strains was due to high levels of polyploidy, not defective sister chromatid cohesion. Because *CTF4* had previously been reported to be required for efficient sister chromatid cohesion (Hanna *et al*., 2001), we did not investigate the role of this gene in cohesion any further. We also did not pursue *CDH1* because it is a substrate specificity factor for the APC/C (Schwab *et al*., 1997; Visintin *et al*., 1997). The increased percentage of *cdh1*Δ cells with two GFP dots could be indicative of abrogated APC/C activity or high Clb2p levels in the cell, rather than a direct role of Cdh1p in cohesion. Finally, we did not pursue *ygl168w*Δ because, upon further analysis, this mutant did not consistently exhibit at least 15% of cells with two GFP signals in the cohesion assay (our unpublished data). Therefore, this initial analysis identified six genes (*VIK1*, *CSM3*, *TOF1*,

**Table 1.** Synthetic genetic interactions with *ctf8*Δ and initial sister chromatid cohesion analysis

| Systematic name | Gene name | Genetic interaction on YPD | % Two GFP dots: initial analysis |
|---|---|---|---|
| YDR332W | | No | 9 |
| YGL168W | | Sick | 18.5 |
| YML095C-A | | Sick | 9.8 |
| YMR166C | | No | 14.4 |
| YDR375C | BCS1 | No | 11.8 |
| YER016W | BIM1 | Lethal | 16.9 |
| YER014C-A | BUD25 | No | Not done |
| YGL003C | CDH1 | No germination | 19 |
| YPL008W | CHL1 | Lethal | 27.4 |
| YMR198W | CIK1 | No | 7.3 |
| YNL298W | CLA4 | Sick | 8.1 |
| YPR119W | CLB2 | Sick | 9.2 |
| YMR048W | CSM3 | Lethal | 21.1 |
| YPR135W | CTF4 | Lethal | 31.7 |
| YPL194W | DDC1 | No | 11 |
| YAL026C | DRS2 | No | 10 |
| YDR518W | EUG1 | No | 10.5 |
| YFL023W | FYV11 | No | 8.6 |
| YNL153C | GIM3 | Sick | Not done |
| YEL003W | GIM4 | Sick | 9.9 |
| YDR225W | HTA1 | Sick | 10 |
| YNL106C | INP52 | No | 8.7 |
| YPR141C | KAR3 | Lethal | 16.5 |
| YGL173C | KEM1 | Sick | 14.2 |
| YLR260W | LCB5 | No | 8 |
| YAL024C | LTE1 | No | 8 |
| YGL086W | MAD1 | Sick | Not done |
| YJL030W | MAD2 | Sick | Not done |
| YIR021W | MRS1 | No | 10.2 |
| YJR073C | OPI3 | No | 12 |
| YGR078C | PAC10 | Sick | 12.6 |
| YNL264C | PDR17 | No | 9.7 |
| YGL153W | PEX14 | No | 9.1 |
| YDR329C | PEX3 | No | 7.8 |
| YNL329C | PEX6 | No | 10.2 |
| YGL167C | PMR1 | Sick | 12.3 |
| YKL113C | RAD27 | No | 14.7 |
| YLR039C | RIC1 | No | 12.3 |
| YMR142C | RPL13B | No germination | 31 |
| YGL244W | RTF1 | Sick | 6.8 |
| YHR154W | RTT107 | No | 11.6 |
| YMR272C | SCS7 | No | 10 |
| YLR268W | SEC22 | No | 13.6 |
| YGL066W | SGF73 | Sick | 11.7 |
| YBL031W | SHE1 | No | 12 |
| YOR195W | SLK19 | Sick | 10.2 |
| YGR229C | SMI1 | Sick | Not done |
| YLR452C | SST2 | No | 9 |
| YPL057C | SUR1 | No | 8.3 |
| YJL176C | SWI3 | No | Not done |
| YNL273W | TOF1 | Lethal | 15.1 |
| YFR010W | UBP6 | No | 6.3 |
| YBR173C | UMP1 | Sick | 9.4 |
| YLR373C | VID22 | No | 8.6 |
| YPL253C | VIK1 | Sick | 19.3 |

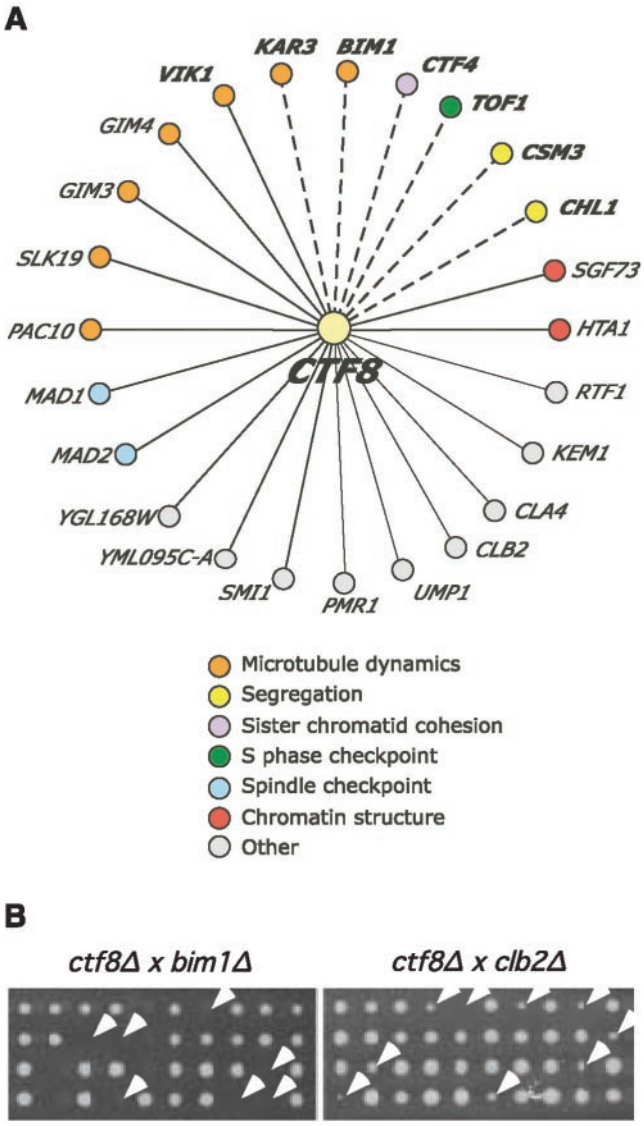



**Figure 1.** *CTF8* genetic interactions. (A) Results from SGA analysis with *ctf8*Δ presented as a genetic interaction map. Lines represent a confirmed synthetic sick interaction between the connected genes. Broken lines represent synthetic lethal interactions. Gene functions are indicated. Genes required for efficient sister chromatid cohesion are indicated in bold. (B) Examples of tetrad analyses for a synthetic lethal interaction (*ctf8*Δ *bim1*Δ) and a synthetic sick interaction (*ctf8*Δ *clb2*Δ). Double mutants are indicated by the arrowheads.

*BIM1*, *KAR3*, and *CHL1*) with previously undescribed roles in sister chromatid cohesion.

### *Confirmation of Genetic Interactions by Tetrad Analysis*

The synthetic genetic interactions between *ctf8*Δ and the gene deletions identified in the SGA screen were confirmed by tetrad dissection. A *ctf8*Δ strain was mated to a deletion of each gene identified in the SGA screen and the resulting diploid was sporulated and dissected on rich medium (YPD) (Figure 1B). Of the 55 mutants in the initial set, six mutants were lethal when combined with *ctf8*Δ on YPD, and 18 mutants were sick when combined with *ctf8*Δ on YPD (Table 1 and Figure 1A). Thus, 44% of the genetic interactions identified in the SGA screen were confirmed by tetrad analysis. The genetic interactions identified show an enrichment for genes involved in the spindle checkpoint, microtubule dynamics, chromosome segregation, and chromatin structure, consistent with the role of *CTF8* in sister chromatid cohesion. Of particular significance, the mutants that were synthetically lethal with *ctf8*Δ, namely, *chl1*Δ, *csm3*Δ, *tof1*Δ, *ctf4*Δ, *bim1*Δ, and *kar3*Δ, include six of the seven mutants that displayed defects in sister chromatid cohesion (Figure 1A). Thus, the severity of the fitness defect in the *ctf8*Δ double mutants served as an excellent predictor of biological function. These data further suggest that cumulative defects in sister chromatid cohesion pathways are likely to result in lethality.

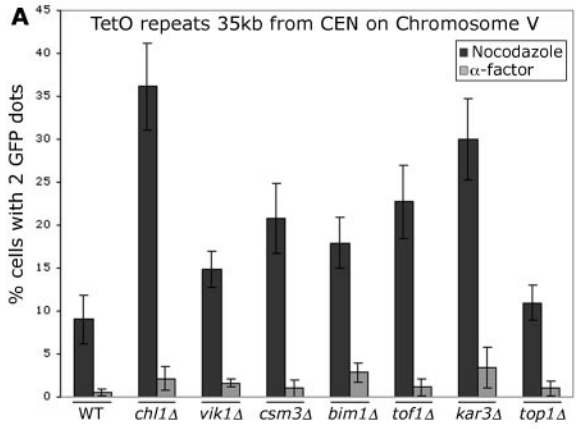
### *Analysis of Sister Chromatid Cohesion at a Locus 35 kb from CEN5*

We next proceeded with a detailed analysis of sister chromatid cohesion defects in the six novel cohesion mutants. Each gene was deleted in YPH1477, which contains a Tet repressor-GFP fusion as well as Tet operator repeats located 35 kb from the centromere of chromosome V, and the effect on sister chromatid cohesion at this arm locus was assayed. Strains containing each of the single gene deletions were grown logarithmically and arrested either in G2/M using nocodazole or in G1 using alpha mating factor for 3 h at 25°C. These cells were then fixed, and the number of GFP dots per cell was scored. Deletion of *VIK1*, *CSM3*, *TOF1*, *BIM1*, *KAR3*, or *CHL1* resulted in defective sister chromatid cohesion at this arm locus as evidenced by an increased percentage of G2/M-arrested cells with two GFP dots compared with the wild-type strain (Figure 2). The increased number of GFP dots could not be attributed to an increase in polyploidy because when these strains were arrested in G1 with alpha factor, the cells had either no increase or only a slight increase in the number of cells with more than one GFP dot compared with a wild-type strain arrested in G1. Furthermore, 80–90% of the nocodazole-arrested cells with separated sister chromatids had high levels of Pds1p, indicating that these cells had not broken through the nocodazole arrest and had not initiated anaphase (Figure 2B). Finally, we observed a range of cohesion defects, with *chl1*Δ displaying the greatest defect, followed by *kar3*Δ, *tof1*Δ, *csm3*Δ, *bim1*Δ, and then *vik1*Δ. The magnitudes of the cohesion defects in the *chl1*Δ and *kar3*Δ strains were similar to that observed in *ctf8*Δ mutants (Mayer *et al*., 2001). In contrast, the *vik1*Δ mutant exhibited a less pronounced cohesion defect than the other mutants (Figures 2–4), consistent with the weaker genetic interaction observed between *vik1*Δ and *ctf8*Δ in comparison to that of other mutants.

Finally, we measured cohesion in a *top1*Δ mutant, because Tof1p is reported to physically interact with Top1p (Park and Sternglanz, 1999) and because topoisomerase II has been implicated in sister chromatid cohesion (Bachant *et al*., 2002). We found no detectable defect in cohesion at this arm site in the *top1*Δ mutant (Figure 2).

### *Analysis of Sister Chromatid Cohesion at a Locus 12 kb from CEN4*

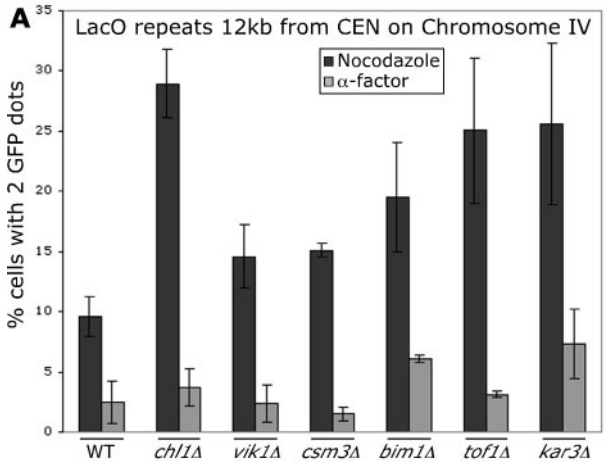
We assessed sister chromatid cohesion at a second arm locus in *chl1*Δ, *kar3*Δ, *vik1*Δ, *bim1*Δ, *tof1*Δ, and *csm3*Δ strains. The genes were individually deleted in strain Y819, which contains Lac operator repeats integrated 12 kb from the centromere of chromosome IV and expresses a Lac repressor-GFP fusion protein. As with the chromosome V locus, deletion of each of these genes resulted in a significant increase in defective sister chromatid cohesion at this locus (Figure 3). Although 9.6% of wild-type (WT) cells arrested in nocoda-




B

| Strain | % NZ-arrested cells with 2 GFP dots | % NZ-arrested cells with 2 GFP dots that had Pds1p |
|---|---|---|
| WT (YPH1477) | 9.0 ± 2.8 | 76% |
| *chl1Δ* | 36.1 ± 5.1 | 78% |
| *vik1Δ* | 14.9 ± 2.1 | 87% |
| *csm3Δ* | 20.8 ± 4.1 | 85% |
| *bim1Δ* | 18 ± 2.9 | 92% |
| *tof1Δ* | 22.7 ± 4.3 | 90% |
| *kar3Δ* | 30 ± 4.7 | 93% |
| *top1Δ* | 11 ± 2.0 | 88% |

**Figure 2.** (A) Assessing cohesion at an arm locus 35 kb from CEN5. *CHL1*, *VIK1*, *CSM3*, *BIM1*, *TOF1*, *KAR3*, and *TOP1* were deleted in strain YPH1477, which expresses a Tet repressor-GFP fusion protein and Pds1-13Myc, and contains a Tet operator repeat integrated 35 kb from the centromere of chromosome V. Two independent strains for each deletion were scored in two independent experiments for sister chromatid cohesion defects. At least 200 cells were scored for each strain. (B) Sister chromatid cohesion defects occur in the presence of Pds1p. The average percentage of nocodazole-arrested cells with two GFP signals from two strains in two independent experiments is given along with the SD. The presence or absence of Pds1-13Myc was also scored in nocodazole-arrested cells with two GFP signals. At least 20 two-dot cells were scored for Pds1p in each strain.




B

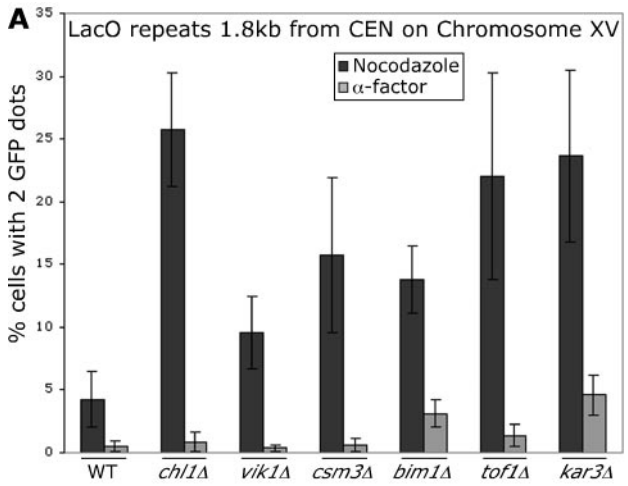
| Strain | % NZ-arrested cells with 2 GFP dots |
|---|---|
| WT (Y819) | 9.6 ± 1.7 |
| *chl1Δ* | 29 ± 2.9 |
| *vik1Δ* | 14.6 ± 2.6 |
| *csm3Δ* | 15.1 ± 0.55 |
| *bim1Δ* | 19.5 ± 4.6 |
| *tof1Δ* | 25.1 ± 6.0 |
| *kar3Δ* | 25.6 ± 6.7 |

**Figure 3.** (A) Assessing cohesion at an arm locus 12 kb from CEN4. *CHL1*, *VIK1*, *CSM3*, *BIM1*, *TOF1*, and *KAR3* were deleted in strain Y819, which expresses a Lac repressor-GFP fusion protein and contains a Lac operator repeat integrated 12 kb from the centromere of chromosome IV. The number of GFP signals in each cell was scored for two independent strains for each deletion in two independent experiments. At least 200 cells were scored for each strain. (B) Sister chromatid cohesion quantified. The average percentage of nocodazole-arrested cells with two GFP signals from two strains in two independent experiments is given along with the SD.

zole had two GFP dots, 29% of *chl1*Δ cells had two GFP signals (Figure 3B). This increase in the percentage of cells with two GFP signals could be attributed to defective sister chromatid cohesion and not to high levels of polyploidy because *chl1*Δ cells arrested in G1 had about the same percentage of cells with two GFP signals as did the WT strain (3.7% in *chl1*Δ and 2.5% in WT) (Figure 3A). Similarly, deletion of *CSM3*, *VIK1*, *TOF1*, *BIM1*, or *KAR3* resulted in an increased percentage of G2/M-arrested cells with two GFP dots (15.1, 14.6, 25.1, 19.5, and 25.6% respectively), and this increase was not due to excessive polyploidy in these cells (Figure 3).

### *Characterization of Sister Chromatid Cohesion at a CEN Locus*

Centromeres in *S. cerevisiae* have previously been found to have unique features with respect to sister chromatid cohesion. Mcd1p/Scc1p and Smc1p have been shown by chromatin immunoprecipitation experiments to bind to defined regions along the chromosome with increased abundance at and around centromeres (Blat and Kleckner, 1999; Megee and Koshland, 1999; Tanaka *et al*., 1999; Laloraya *et al*., 2000). Centromere DNA has also been shown to nucleate binding of the cohesin subunit Mcd1p/Scc1p, resulting in the association of Mcd1p/Scc1p with at least 2 kb of DNA flanking the centromere sequence (Megee and Koshland, 1999; Tanaka *et al*., 1999). To assess sister chromatid cohesion at a CEN locus, *KAR3*, *BIM1*, *CHL1*, *CSM3*, *TOF1*, and *VIK1* were individually deleted in YPH1444, which has a Lac operator repeat integrated 1.8 kb from the centromere of chromosome XV and expresses a Lac repressor-GFP fusion protein. Deletion of *CHL1* resulted in 25.8% of nocodazole-arrested *chl1*Δ cells having two GFP dots compared with only 4.3% in WT cells (Figure 4). When *chl1*Δ cells were arrested in G1, 0.83% of cells had two GFP dots, whereas 0.5% of WT cells arrested in G1 had two GFP dots, indicating that the high percentage of separated signals in nocodazole-arrested *chl1*Δ cells is not due to increased polyploidy. Thus, *CHL1* is required for efficient cohesion at a centromeric locus on chromosome XV. Likewise, deletion of *CSM3*, *VIK1*, *TOF1*, *BIM1*, or *KAR3* increased the percentage of nocodazole-arrested cells with two GFP dots (15.8, 9.6, 22, 13.8, and 23.7%, respectively), indicating that these genes are also important for efficient cohesion at this centromere proximal locus (Figure 4). Collectively, these data indicate that *KAR3*, *CHL1*, *CSM3*, *TOF1*,




B

| Strain | % NZ-arrested cells with 2 GFP dots |
|---|---|
| WT (YPH1444) | 4.3 ± 2.2 |
| *chl1Δ* | 25.8 ± 4.5 |
| *vik1Δ* | 9.6 ± 2.8 |
| *csm3Δ* | 15.8 ± 6.1 |
| *bim1Δ* | 13.8 ± 2.7 |
| *tof1Δ* | 22 ± 8.3 |
| *kar3Δ* | 23.7 ± 6.8 |

**Figure 4.** (A) Assessing cohesion at a CEN locus 1.8 kb from CEN15: *CHL1*, *VIK1*, *CSM3*, *BIM1*, *TOF1*, and *KAR3* were deleted in strain YPH1444, which expresses a Lac repressor-GFP fusion protein and contains a Lac operator repeat integrated 1.8 kb from the centromere of chromosome XV. The number of GFP signals in each cell was scored for two independent strains for each deletion in two independent experiments. At least 200 cells were scored for each strain. (B) Sister chromatid cohesion quantified. The average percentage of nocodazole-arrested cells with two GFP signals from two strains in two independent experiments is given along with the SD.

*BIM1*, and *VIK1* are required for efficient sister chromatid cohesion at both CEN and arm loci.

### *Identification of Protein Complexes Important for Cohesion*

Kar3p has been reported to interact with two accessory proteins, Vik1p and Cik1p (Page *et al*., 1994; Manning *et al*., 1999). Whereas *kar3*Δ, *cik1*Δ, and *vik1*Δ were identified in our initial screen as having synthetic genetic interactions with *ctf8*Δ, only the interactions with *kar3*Δ and *vik1*Δ could be confirmed by tetrad analysis, and only *kar3*Δ and *vik1*Δ were found to have mitotic cohesion defects. The reported physical association between Kar3p and Vik1p, two of six proteins found to have roles in sister chromatid cohesion in the course of this work, encouraged us to look for protein interactions with the six genes found to have a role in sister chromatid cohesion. We tagged Vik1p, Chl1p, and Csm3p with 13Myc epitopes at their C termini by integrating sequences encoding the tag into their respective chromosomal loci. Vik1-13Myc, Chl1-13Myc, and Csm3-13Myc were immunoprecipitated from cell extracts and the immunoprecipitates were run on a 10% polyacrylamide gel that was then silver stained (Figure 5A). Two specific proteins were evident in the Vik1-13Myc immunoprecipitate; one was Vik1–13Myc, whereas the other protein was identified as Kar3p using mass spectrometric analysis (Figure 5A). We were unable to identify any prominent specific bands in the Chl1-13Myc immunoprecipitate, other than Chl1-13Myc itself (Figure 5A). We identified two specific proteins in the Csm3-13Myc immunoprecipitate, one of which corresponded to Csm3-13Myc. Mass spectrometry was used to identify the other protein as Tof1p, one of the other gene products we found to be required for efficient sister chromatid cohesion (Figure 5A).

To confirm the physical interaction between Tof1p and Csm3p suggested by mass spectrometric analysis, we tagged Tof1p with 3HA epitopes at its C terminus. Tof1–3HA was expressed from its endogenous promoter. Immunoprecipitation of Tof1–3HA with anti-HA conjugated beads specifically coimmunoprecipitated Csm3–13Myc from extracts prepared from Tof1-3HA Csm3-13Myc cells (Figure 5B). The association of Tof1-3HA with Csm3-13Myc was also observed when the immunoprecipitation was carried out with anti-Myc–conjugated beads (our unpublished data). We conclude that Csm3p and Tof1p physically interact in vivo.

Tof1p was first identified as a topoisomerase-interacting protein by two-hybrid analysis using the central domain (not containing the active site) of topoisomerase I as bait (Park and Sternglanz, 1999). These data prompted us to investigate whether we could confirm a physical interaction between topoisomerase I (encoded by the *TOP1* gene) and Csm3p or Tof1p by coimmunoprecipitation. We tagged Top1p with either 13Myc or 3HA epitopes at its C terminus. We found no physical association between Top1-3HA and Csm3-13Myc or between Tof1-3HA and Top1-13Myc under the same experimental conditions used to coimmunoprecipitate Csm3-13myc and Tof1-3HA (Figure 5B).

## DISCUSSION

Because *ctf8* is synthetically lethal with mutations in essential genes required for cohesion, we hypothesized that a synthetic lethality screen using a *ctf8*Δ strain as a bait may identify other genes required for sister chromatid cohesion. The only other nonessential gene previously shown to be required for sister chromatid cohesion, other than the nonessential genes in the Ctf18–RFC complex, is *CTF4*. Isolation of *ctf4*Δ in the *ctf8*Δ SGA analysis indicated that this screen was successful in identifying other nonessential genes required for sister chromatid cohesion. In addition to *CTF4*, six other genes isolated in the *ctf8*Δ SGA screen were found to be required for efficient sister chromatid cohesion: *BIM1*, *CSM3*, *TOF1*, *VIK1*, *KAR3*, and *CHL1*. Warren *et al.* (2004; this issue) describe a similar screen, by using a chip-based method, to isolate genes that are synthetically lethal with *ctf4*Δ. From the list of interactors, they define 17 deletion mutants that exhibit cohesion defects, three of which were also found in our screen: *kar3*Δ, *tof1*Δ, and *csm3*Δ. Together, these results provide a list of 20 new nonessential genes in addition to *CTF4* that define pathways involved in proper sister chromatid cohesion. Moreover, our study shows that the proteins encoded by four of the six identified genes in our screen form two distinct complexes: Kar3p and Vik1p, which had been reported previously (Manning *et al*., 1999), and Csm3p and Tof1p. Thus, our data demonstrate that a synthetic genetic array screen followed by cohesion assays as a secondary screen was a successful approach to identify novel protein complexes involved in sister chromatid cohesion.

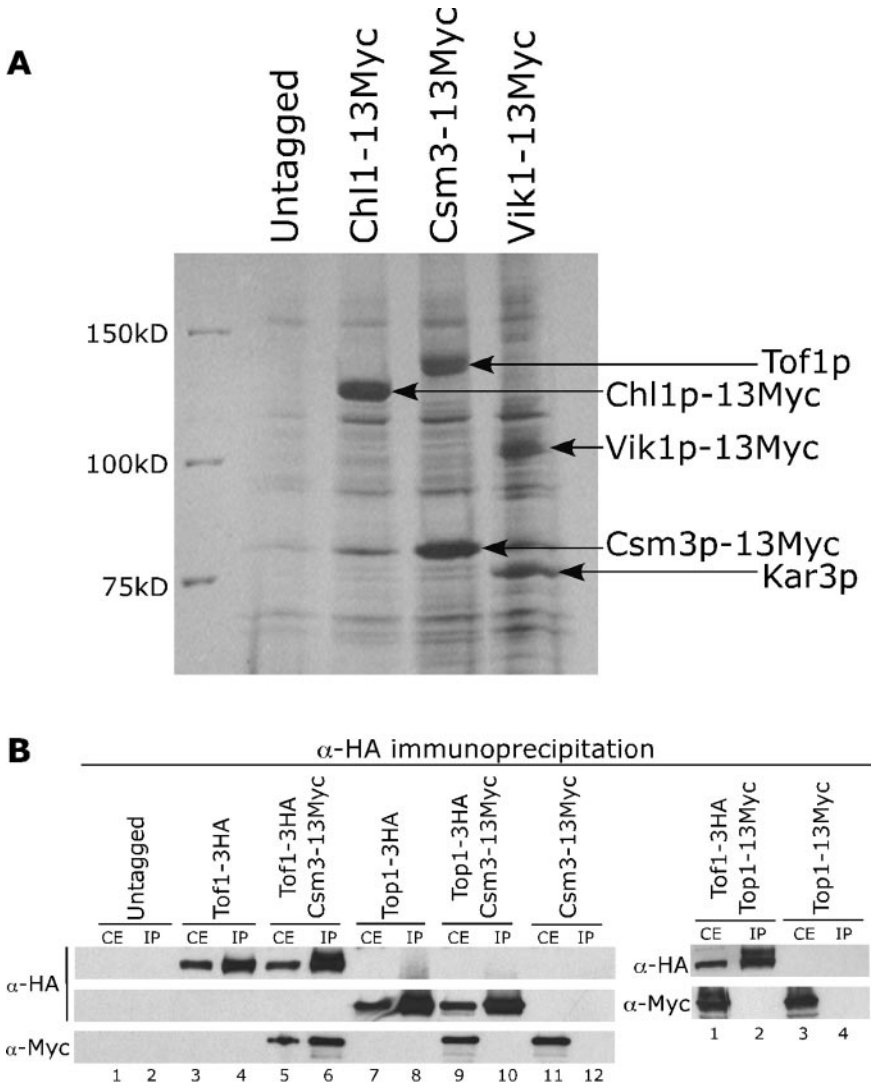



**Figure 5.** (A) Identification of proteins that physically interact with Chl1p, Csm3p, and Vik1p. Chl1p, Csm3p, and Vik1p were epitope tagged with 13Myc epitopes at their C termini under their endogenous promoter. Cellular extracts were prepared from these strains and an isogenic wild-type strain. Immunoprecipitations were performed from equal amounts of protein (500–700 mg), and the immunoprecipitates were run on a 10% SDS-PAGE gel, which was subsequently silver stained. The identity of unique protein bands was determined by mass spectrometry. (B) Confirmation of protein interactions. Tof1p, Csm3p, and Top1p were tagged at their C termini with either 13Myc or 3HA epitopes at the endogenous loci. Immunoprecipitations were performed from equal amounts of protein using anti-HA conjugated beads. Western blot analysis of the immunoprecipitate was performed using either anti-HA or anti-Myc monoclonal antibodies as indicated.

### *DNA Topology and Sister Chromatid Cohesion*

In all of our cohesion assays, *chl1* mutants consistently had cohesion defects of similar magnitude to that observed in *ctf8*, *dcc1*, and *ctf18* mutants; ~30% of nocodazole arrested *chl1* cells had 2 GFP signals. *CHL1* was previously identified in two screens designed to isolate mutations in genes required for high fidelity chromosome segregation (Spencer *et al*., 1990; Kouprina *et al*., 1993). Sequence analysis reveals that Chl1p is an evolutionarily conserved DEAH family helicase (Shiratori *et al*., 1999) and human Chl1 (hChlR1) has been shown to possess both ATPase and DNA helicase activities (Hirota and Lahti, 2000). Consistent with *CHL1* having a role in sister chromatid cohesion, we find that deletion of *CHL1* is synthetically lethal with *scc1-73*, *ctf7-203*, *smc1-259*, *scc2-4*, and *smc3-42* (our unpublished data). Furthermore, deletion of *CHL1* results in an accumulation of cells with G2/M DNA content that is dependent on the mitotic spindle checkpoint gene *MAD2* (Li and Murray, 1991; our unpublished data). Just as Smt3p/SUMO-1 modification of Top2p may play a role in establishing a modified chromosome structure or localized catenations that are required for efficient centromeric cohesion (Bachant *et al*., 2002), Chl1p's strong sequence homology to helicases suggests that Chl1p may function to alter DNA topology during the establishment or maintenance of sister chromatid cohesion. The observation that Chl1p is required for efficient sister chromatid cohesion adds to a growing body of evidence suggesting that DNA topology is critical to proper cohesion.

### *Identification of Protein Complexes Required for Efficient Sister Chromatid Cohesion*

Csm3p was previously found to be required for high-fidelity chromosome segregation in meiosis (Rabitsch *et al*., 2001). Consistent with the role of Csm3p in chromosome transmission, we find that *CSM3* is required for efficient sister chromatid cohesion in mitosis. Furthermore, we find that Csm3p physically interacts with Tof1p. A physical interaction between Csm3p and Tof1p had previously been suggested by two-hybrid analysis (Ito *et al*., 2001). We also isolated *tof1*Δ in the *ctf8*Δ SGA screen and found that *TOF1* is required for efficient cohesion. Tof1p was previously reported to interact in a two-hybrid assay with the central domain of topoisomerase I (Park and Sternglanz, 1999). Tof1-GST expressed in *Escherichia coli* has also been shown to physically associate with the central domain of topoisomerase I in an in vitro binding assay (Park and Sternglanz, 1999). Given the effect of

defective Smt3p modification of Top2p on centromeric cohesion (Bachant *et al*., 2002), we tested whether Top1p physically interacted with either Csm3p or Tof1p. We were unable to identify any physical interaction between Csm3-13Myc and Top1-3HA or between Top1-13Myc and Tof1-3HA by coimmunoprecipitation experiments. It is therefore unclear whether full-length Top1p physically interacts with Tof1p or Csm3p in vivo. Deletion of *TOP1* had no significant effect on sister chromatid cohesion at an arm locus 35 kb from CEN5. Thus, although the Csm3p–Tof1p protein complex seems to be required for efficient sister chromatid cohesion, we were unable to find any interaction between this complex and Top1p, and deletion of *TOP1* seemed to have no significant effect on cohesion.

Tof1p also functions in the checkpoints that respond to DNA damage or replication fork stalling during S phase and is important for activation of the Rad53 checkpoint protein kinase (Foss, 2001). Tof1p was recently shown to interact with Cdc45p, a component of the DNA replication machinery, and to move with the replication machinery as DNA synthesis proceeds during S phase. This suggested that Tof1p functions during checkpoint response in a replication-pausing complex that ensures that the replication complex does not uncouple from sites of DNA synthesis (Katou *et al*., 2003). The role of Tof1p in sister chromatid cohesion suggests a functional link between checkpoint pathways, DNA replication, and cohesion. It is currently unclear whether efficient cohesion requires intact checkpoint pathways, or whether Tof1p performs two separable functions. However, it is becoming increasingly evident that S phase checkpoint pathways are critical for replication fork integrity (Lopes *et al*., 2001; Tercero and Diffley, 2001; Sogo *et al*., 2002), and given the links between DNA replication and the establishment of sister chromatid cohesion, it is possible that checkpoint pathways contribute directly to efficient cohesion. Interestingly, Tof1p shows limited homology to a *C. elegans* protein of the TIMELESS family, TIM-1, that is essential for proper chromosome segregation in mitosis and meiosis and was recently shown to interact with cohesin, and hypothesized to facilitate cohesin loading or stability (Chan *et al*., 2003).

*KAR3* encodes a microtubule motor protein with minus end directionality (Endow *et al*., 1994). Kar3p functions in a range of cellular processes, including spindle integrity, nuclear fusion during mating (Meluh and Rose, 1990), and mitotic spindle positioning (Cottingham *et al*., 1999). Kar3p physically associates with two homologous accessory factors, Cik1p and Vik1p (Page *et al*., 1994; Manning *et al*., 1999). The Kar3p–Cik1p and the Kar3p–Vik1p complexes seem to be functionally distinct. For example, Kar3p complexed with Cik1p, but not Vik1p, has been implicated in spindle positioning (Cottingham *et al*., 1999) and karyogamy (Page *et al*., 1994; Manning *et al*., 1999). Although we identified *kar3*Δ, *vik1*Δ, and *cik1*Δ in the *ctf8*Δ SGA screen, we found that only *KAR3* and *VIK1* were required for efficient sister chromatid cohesion. It is therefore likely that the role of Kar3p in cohesion is mediated through its interaction with Vik1p rather than Cik1p. Interestingly, Kar3p has previously been reported to interact with the cohesin protein Smc1p in a two-hybrid assay (Newman *et al*., 2000).

### *BIM1 and Sister Chromatid Cohesion*

Bim1p is a microtubule binding protein that interacts with the plus end of microtubules and with the spindle itself (Schwartz *et al*., 1997; Tirnauer *et al*., 1999). Bim1p is a critical element of the Kip3p pathway that ensures correct orientation of the mitotic spindle (Tirnauer *et al*., 1999; Lee *et al*., 2000), and as such is important for polarized cell growth. SGA analysis with *bim1*Δ identified genetic interactions with spindle assembly checkpoint genes and genes encoding kinetochore components, suggesting that Bim1p might also be involved in the attachment of microtubules to the kinetochore (Tong *et al*., 2001). Although a role for Bim1p in sister chromatid cohesion had not previously been hypothesized, there are several links between Bim1p function and accurate chromosome segregation. The *bim1*Δ SGA identified genetic interactions with the Ctf18-RFC genes *DCC1* and *CTF8* (Tong *et al*., 2001), suggesting a functional relationship between *BIM1* and Ctf18-RFC. *bim1*Δ is also synthetically sick with *csm3*Δ (Tong *et al*., 2001), which we find is important for sister chromatid cohesion. Genetic interactions with the spindle checkpoint genes (Tong *et al*., 2001) could be due to defective cohesion in *bim1*Δ, rather than to defective microtubule attachment or spindle positioning. Finally, mutations in the *S. pombe* homologue of *BIM1*, *mal3*$^{+}$, cause a 400-fold increase in chromosome loss (Beinhauer *et al*., 1997). We find that deletion of *BIM1*, in addition to being synthetically lethal with *ctf8*Δ, confers a two- to threefold increase in premature separation of sister chromatids. This suggests that efficient sister chromatid cohesion might be important for establishing or maintaining the position of the mitotic spindle. Alternatively, the cohesion defect in *bim1*Δ might reflect a separate function for Bim1p, distinct from its role in spindle orientation. This additional function of Bim1p is of particular interest given the connections between the human Bim1p homologue EB1, the adenomatous polyposis coli tumor suppressor, and genetic instability in human cancers (Su *et al*., 1995).

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