Efficient Chromosome Biorientation and the Tension Checkpoint in *Saccharomyces cerevisiae* both Require Bir1[▽][†]

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Accurate chromosome segregation requires the capture of sister kinetochores by microtubules from opposite spindle poles prior to the initiation of anaphase, a state termed chromosome biorientation. In the budding yeast *Saccharomyces cerevisiae*, the conserved protein kinase Ipl1 (Aurora B in metazoans) is critical for ensuring correct chromosomal alignment. Ipl1 associates with its activators Sli15 (INCENP), Nbl1 (Borealin), and Bir1 (Survivin), but while Sli15 clearly functions with Ipl1 to promote chromosome biorientation, the role of Bir1 has been uncertain. Using a temperature-sensitive *bir1* mutant (*bir1-17*), we show that Bir1 is needed to permit efficient chromosome biorientation. However, once established, chromosome biorientation is maintained in *bir1-17* cells at the restrictive temperature. Ipl1 is partially delocalized in *bir1-17* cells, and its protein kinase activity is markedly reduced under nonpermissive conditions. *bir1-17* cells arrest normally in response to microtubule depolymerization but fail to delay anaphase when sister kinetochore tension is reduced. Thus, Bir1 is required for the tension checkpoint. Despite their robust mitotic arrest in response to nocodazole, *bir1-17* cells are hypersensitive to microtubule-depolymerizing drugs and show a more severe biorientation defect on recovery from nocodazole treatment. The role of Bir1 therefore may become more critical when spindle formation is delayed.

Accurate chromosome segregation during anaphase is vital for ensuring the maintenance of genome integrity during cell division and, in turn, depends critically on the correct attachment of sister chromatids to kinetochore microtubules. For high-fidelity chromosome segregation, kinetochores must capture spindle microtubules such that sister chromatids are connected to opposite spindle poles (termed amphitelic attachment or chromosome biorientation), ensuring that they are pulled in opposite directions during the subsequent anaphase.

In the budding yeast *Saccharomyces cerevisiae*, the majority of sister chromatids remain attached to microtubules from a single pole (mono-oriented) without the intervention of a correction mechanism to promote amphitelic attachment (36), a key element of which is the Ipl1 protein kinase. Ipl1 has been proposed to promote the detachment of incorrect microtubule-kinetochore connections so that correct attachments subsequently can form (35). In the absence of Ipl1 function, at the point of anaphase onset around two-thirds of sister chromatids remain mono-oriented, attached to microtubules originating from a single pole to which they then cosegregate (35). Kinetochore proteins such as Dam1 and Ndc80 have been proposed as key Ipl1 substrates for their role in promoting chromosome biorientation (6, 41). Ipl1 kinase also is required for cells to activate the spindle checkpoint in the absence of tension on kinetochore-microtubule attachments, and hence *ipl1* mutant cells fail to delay anaphase despite their many mono-oriented

chromosomes (2). Depending on the circumstances, the checkpoint role of Ipl1 involves either the generation of unattached kinetochores (26) or the phosphorylation of the checkpoint protein Mad3 (19). Ipl1 also is required in the absence of the BimC family kinesin Cin8p, probably reflecting a role in spindle assembly (9, 21), and is involved in regulating spindle disassembly following anaphase (5).

Ipl1 kinase is highly conserved, and its metazoan ortholog (Aurora B) is involved in both chromosome biorientation and the spindle assembly checkpoint, forming part of the chromosomal passenger complex that also contains INCENP, Survivin, and Borealin (27, 40). The chromosomal passenger complex is so called because although these proteins colocalize throughout the cell cycle, their location changes dynamically from the chromosome arms in G_1 to centromeres in prometaphase and finally to the central spindle in anaphase. Such coordinated behavior is consistent with the recent crystal structure of the complex between INCENP, Survivin, and Borealin, in which they interact via tightly entwined helical domains (16).

In budding yeast, Ipl1 interacts with Sli15, Bir1, and Nbl1, which have been proposed to be orthologs of INCENP, Survivin, and Borealin, respectively (6, 18). All three proteins are the products of essential genes. Like INCENP, Sli15 has a conserved C-terminal domain (the IN-box) that is required for Ipl1 kinase activation, and *sli15* mutants have a phenotype that is very similar to that of *ipl1* mutants (17, 18). Although yeast cells with reduced Bir1 function show chromosome instability, the first-described *bir1* mutants failed to reveal a chromosome biorientation defect but instead conferred defects in septin dynamics during anaphase (38). Bir1 interacts with Ndc10 and is responsible for taking Ndc10 to the anaphase spindle (38, 42, 43), a role that may be linked to this septin defect (4). Yeast Bir1 is much larger than its metazoan counterpart (Survivin)

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TABLE 1. Yeast strains

Strain[a]	Genotype	Source or reference
AKY4	*MAT***a** *bir1::HIS3MX6* [YCplac33-*BIR1*]	A. Kozak
AKYD3	*MAT***a** /*MAT*α *BIR1/bir1::HIS3MX6* [YCplac33-*BIR1*]	A. Kozak
AY925	*MAT***a**	K. Arndt
BP635	*MAT***a** *dam1-S265A,S292A,S257A*	T. Tanaka
K5043	*MAT***a** *CFIII (CEN3.L.YPH278) URA3 SUP11*	24
PKY63	*MAT***a** *ipl1-321*-myc$_{13}$::*KANMX6*	P. Keating
T2608	*MAT***a** *IPL1*-myc$_{12}$::*URA3*	T. Tanaka
T2997	*MAT***a** *cen3::pGAL-CEN3-tCYC1-tetOs::ura3 leu2::tetR-GFP::LEU2 trp1::YFP-TUB1::TRP1 cdc20::pMET3-CDC20::TRP1 ask1-3*	T. Tanaka
T5241	*MAT***a** *IPL1-GFP::KANMX6 CTF19-3ECFP-SkHIS3MX6 NDC80-3ECFP-SkHIS3MX6*	T. Tanaka
VMY1	*MAT***a** *bir1-17*	This study
VMY5	*MAT***a** *IPL1*-myc$_{12}$::*URA3 bir1-17*	This study
VMY7	*MAT***a** *bir1-36*	This study
VMY14	*MAT***a** *IPL1-GFP::KANMX6 ctf19::CTF19-3ECFP-SkHIS3MX6 ndc80::NDC80-3ECFP-SkHIS3MX6 bir1-17*	This study
VMY33	*MAT***a** *CEN3-tetO$_{336}$::URA3 leu2::tetR-GFP::LEU2 trp1::YFP-TUB1::TRP1 cdc20::pMET3-CDC20::TRP1*	This study
VMY34	*MAT***a** *CEN3-tetO$_{336}$::URA3 leu2::tetR-GFP::LEU2 trp1::YFP-TUB1::TRP1 cdc20::pMET3-CDC20::TRP1 bir1-17*	This study
VMY36	*MAT***a** *scc1-73 leu2::tetR-GFP::LEU2 pds1::PDS1*-myc$_{18}$::*LEU2 ura3::tetOs::URA3*	This study
VMY48	*MAT***a** *pds1::PDS1*-myc$_{18}$::*LEU2 ura3-1::tetOs::URA3 leu2-3::tetR-GFP::LEU2 bir1-17::NATRMX*	This study
VMY56	*MAT***a** *Pds1::PDS1*-myc$_{18}$::*LEU2 ura3-1::tetOs::URA3 leu2-3::tetR-GFP::LEU2*	This study
VMY113	*MAT***a** *bir1-17::NATRMX CFIII (CEN3.L.YPH278) URA3 SUP11*	This study
VMY137	*MAT***a** *pds1::PDS1*-myc$_{18}$::*LEU2 ura3::pGAL-CDC6::URA3*	This study
VMY132	*MAT***a** *pds1::PDS1*-myc$_{18}$::*LEU2 bir1-17::NATRMX ssc1Δ::TRP1 leu2::LEU2::pGAL-SCC1*	This study
VMY134	*MAT***a** *pds1::PDS1*-myc$_{18}$::*LEU2 ssc1Δ::TRP1 leu2::LEU2::pGAL-SCC1*	This study
VMY139	*MAT***a** *pds1::PDS1*-myc$_{18}$::*LEU2 ura3::pGAL-CDC6::URA3 bir1-17::NATRMX*	This study

[a] All strains are in the W303 background: *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 ssd1-d2* Gal^{+}.

and shows little sequence conservation outside the conserved BIR domain, yet this region is nonessential in yeast (42) and therefore unlikely to be involved in chromosome biorientation. Conversely, metazoan Borealin proteins are much larger than yeast Nbl1, which consists of little more than the helical region proposed to form the tight interaction with INCENP/Sli15 and Survivin/Bir1 complexes. Furthermore, a significant fraction of both Sli15 and Bir1 are present in a complex that lacks Ipl1 (29, 38) and that recent work has shown to contain Nbl1 (25), bringing into question the importance of Bir1 for chromosome biorientation. The extent to which Bir1 and Survivin function in conserved or analogous ways within the chromosomal passenger complexes of yeast and metazoans therefore was unclear at the start of our work.

The Sli15-Bir1 complex has been proposed to interact both with microtubules (via the central domain of Sli15) and with kinetochores (through the Bir1-Ndc10 interaction) and through these interactions to function as a tension sensor, relaying information concerning the state of microtubule-kinetochore connections to Ipl1 kinase. Thus, when chromosomes are mono-oriented, the Bir1-Sli15-Nbl1 complex might activate Ipl1 in the absence of tension so as to promote chromosome biorientation by detaching incorrect microtubule attachments (29). This model predicts an essential role for Bir1 in promoting chromosome biorientation, but such evidence has been lacking. By generating a temperature-sensitive *bir1* allele (*bir1-17*) and showing that it confers a profound defect in chromosome biorientation, we demonstrate that Bir1 does play a key role in the correction process needed to ensure that all yeast chromosomes become correctly aligned on the mitotic spindle. Furthermore, since the *bir1-17* mutant fails to activate the spindle assembly checkpoint properly in response to reduced sister kinetochore tension, like Ipl1 it forms part of the

tension checkpoint mechanism. Our data therefore are consistent with a role for Bir1 in conferring tension responsiveness on Ipl1 function.

MATERIALS AND METHODS

Yeast strains and general methods. All yeast strains used in this study (Table 1) are derivatives of W303-1a (37) and have the following markers unless indicated to the contrary: *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 ssd1-d2* Gal^{+}. Basic yeast methods, growth media, and routine recombinant DNA methodology were performed as previously described (1, 10, 28). For creating temperature-sensitive *bir1* mutants, error-prone PCR, gapped plasmid repair, and plasmid shuffling were used as previously described (32). The region selected for mutagenesis encompassed amino acids 489 to the end of the protein (954) and was defined by the two PCR primers used (5'-GGCATAAATACAGACACAA AAGGAGC-3' and 5'-AAGCATCAGGAACGCCCG-3'). For the gap repair step, YCplac22-*BIR1* was digested with PpuMI and NsiI to generate a suitable fragment missing the C-terminal *BIR1* coding region. To make YCplac22-*BIR1*, a 3.7-kb genomic PvuII fragment starting 484 bp upstream of *BIR1* first was inserted into pSP72 (Promega) and then moved as an XhoI-HindIII fragment into YCplac22 (11) cut with SalI and HindIII. The shuffle strain (AKY4) was generated by the sporulation and tetrad dissection of AKYD3, a strain heterozygous for a *bir1::KANMX6* knockout and carrying YCplac33-*BIR1*, a plasmid identical to YCplac22-*BIR1* except for its YCplac33 backbone (11). Plasmids carrying potential temperature-sensitive *bir1* alleles were verified by plasmid rescue and retesting by plasmid shuffling, and then the sites of mutations were located by DNA sequencing. To integrate the *bir1-17* allele using the pop in-pop out method (32), the *bir1-17* fragment from YCplac22-*bir1-17* was excised and cloned into the *URA3* plasmid YIplac204 (11). This construct was targeted to the *BIR1* locus in AY925 by linearizing it at its unique SwaI site prior to transformation. Following selection on 5-fluoroorotic acid for recombination between the repeated *BIR1* sequences thus generated, colonies were screened for temperature sensitivity, and a strain in which the *bir1-17* allele had replaced the wild-type sequence was identified by the DNA sequencing of a suitable genomic PCR fragment encompassing the mutated region. The temperature sensitivity of strains was assessed by diluting mid-exponential-phase cultures of cells in yeast extract-peptone-dextrose (YPD) to an optical density at 600 nm (OD$_{600}$) of 0.1 and by spotting 5 μl of this and of five 10-fold serial dilutions. Plates were photographed after growth for 2 days at the indicated temperature.

Biochemical and immunological techniques. For the synchronization of cells in G_1, 1.25 μg/ml α-factor was used. Standard assays were performed as previously described (26). This involved α-factor treatment for 2 h, followed by the release of the α-factor arrest by filtration and a wash with prewarmed medium, followed by being shaken in fresh YPD medium at 26 or 37°C as indicated. To prevent cells from entering the next cell cycle, α-factor was added back (7.5 μg/ml) when small buds appeared in the majority (>80%) of cells. This procedure was modified for the depletion of cohesin (using p*GAL-SCC1*) or *CDC6* (using p*GAL-CDC6*) as described elsewhere (19). Briefly, cells were grown in YP medium containing 2% raffinose and 2% galactose at 26°C, treated with α-factor for 50 min, filtered as described above and transferred to fresh YPD medium containing α-factor for a further 2 h, and finally released into fresh YPD at 37°C as described above. Where cells were released into YPD containing nocodazole, the drug was used at a concentration of 30 μg/ml. Cell cycle progression was studied by monitoring budding and Pds1 levels in strains containing *PDS1-myc*$_{18}$. Samples were taken during a 180-min time course for immunoblotting, lysing cells either by using glass beads as described in King et al. (19) or by using sequential NaOH and trichloroacetic acid treatment (23). Pds1-myc$_{18}$ was detected with an anti-*myc* antibody (c-*myc* A-14; sc-789; Santa Cruz Biotechnology). Histone H3 phosphorylated on serine-10 was detected with an anti-phospho-histone H3 (Ser10) from Upstate Biotechnology (no. 06-570), total histone H3 was detected using ab-1791 (Abcam), and Cdc28 was detected with an anti-Cdc28 antibody from Santa Cruz Biotechnology (sc-6709).

Immunoprecipitation and protein kinase assay. Cultures of mid-log-phase cells grown at 37°C were collected and lysates were prepared and immunoprecipitated, and where indicated, in vitro kinase assays were carried out as previously described (5). Ipl1-*myc* was immunoprecipitated using an anti-*myc* antibody (sc-789; Santa Cruz Biotechnology), and recombinant glutathione S-transferase (GST)–Dam1 was used as a kinase substrate at 5 μg/reaction. Immunoprecipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to Immobilon-P membranes (Millipore), and then treated with the indicated antibodies (typically at a 1:1,000 dilution), followed by an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody. The detection of HRP-labeled secondary antibodies utilized enhanced chemiluminescence that was detected by exposing the membrane to X-ray film. Kinase assays were processed similarly, and the incorporated ^{32}P was detected by exposure to X-ray film. Films from either Western blotting or kinase assays were scanned using an Epson V750 scanner in transmissive mode, and band intensities were quantified using Image J (http://rsb.info.nih.gov/ij/; National Institutes of Health, Bethesda, MD).

Microscopy. The time-lapse fluorescence microscopy of live cells for monitoring chromosome biorientation was performed as previously described (34). Time-lapse images of cells released from G_1 (α-factor) to metaphase arrest (Cdc20 depletion) at the restrictive temperature for the *bir1-17* mutant (37°C) were collected for 4 min using a DeltaVision RT microscope (Applied Precision), a UplanSApo ×100 objective lens (numeric aperture, 1.40; Olympus), SoftWoRx software (Applied Precision), and a CoolSnap HQ (Photometrics) charge-coupled device camera. Seven Z sections (0.3 μm apart) were acquired and subsequently deconvolved, projected as two-dimensional images, and analyzed with SoftWoRx software. Yellow fluorescent protein (YFP) and green fluorescent protein (GFP) signals were visualized with a JP3 filter set, and cyan fluorescent protein (CFP) and GFP signals were visualized with an ET filter set. Sister *CEN3* centromeres that remained unseparated at one end of the metaphase spindle were scored as mono-oriented, whereas sister *CEN3* centromeres that showed dynamic separation and reassociation or that remained separated for the duration of the time-lapse sequence were scored as bioriented. When fixed cells were imaged to monitor biorientation, samples of cells were fixed in 2% paraformaldehyde for 2 min at room temperature and then resuspended in 0.1 M potassium phosphate buffer (pH 6.6). Images were collected as described above, except that cells were immobilized on an agarose pad and a single exposure was taken from each field. Sister *CEN3* centromeres on a metaphase spindle were scored as either unseparated or separated. For imaging live cells without time lapse, a setup identical to that described above was used. Statistical analysis of microscopy data was carried out using Fisher's exact test, and all *P* values are two tailed.

Chromosome loss assay. The chromosome loss assay was performed by monitoring the loss of a previously described chromosome III fragment (CFIII) carrying *URA3* and *SUP11* (31) in a colony-sectoring assay, where the loss of CFIII (and, hence, *SUP11*) leads to a red colony color due to the failure to suppress the *ade2-1* mutation. K5043 (*BIR1* wild-type strain) and its *bir1-17* derivative (VMY113) were grown in minimal medium lacking uracil to mid-log phase at 26°C, and then cells were washed and resuspended in YPD medium lacking additional adenine. After suitable dilutions were plated on unsupple-
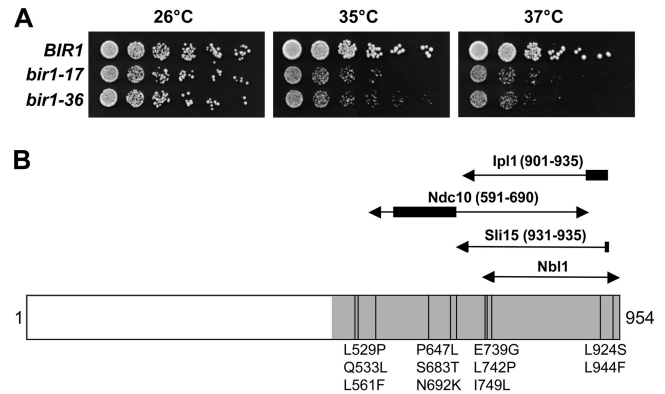
FIG. 1. Characterization of the *bir1-17* allele. (A) Temperature sensitivity of the *bir1-17* and *bir1-36* alleles. Equivalent 10-fold dilutions of wild-type *BIR1* (AY925), *bir1-17* (VMY1), and *bir1-36* (VMY7) strains were spotted onto YPD agar and grown for 2 days at 26, 35, or 37°C. (B) Location of the amino acid substitutions in *bir1-17*. The region of the 954-amino-acid Bir1 open reading frame that was mutagenized is shaded, and the 11 amino acid substitutions are indicated. The limits to the regions proposed to interact with Ndc10, Sli15, Nbl1, and Ipl1 from the two-hybrid analysis of Thomas and Kaplan (38) and Nakajima et al. (25) are indicated (the regions denoted by thicker lines abolish the indicated interactions when deleted or mutated). Only residues 876 to 954 are essential, provided a nuclear localization signal is included (42). Unlike the other substitutions, L924S and L944F affect residues that are highly conserved in Bir1 orthologs from other fungi, such as *Ashbya gosypii*, *Saccharomyces bayanus*, and *Kluyveromyces lactis* (not shown).

mented YPD agar, CFIII loss was monitored visually after growth at 26°C for 6 days by the production of red sectors. The CFIII loss rate per cell division was calculated by dividing the number of half-sectored red colonies (i.e., at least 50% red) by the total number of colonies (excluding any completely red colonies derived from cells that already had lost CFIII before plating).

RESULTS

A conditional *bir1* allele conferring chromosome instability and showing genetic interactions with *ipl1* and *sli15* mutants. To address whether Bir1 is required for efficient chromosome biorientation, we generated temperature-sensitive *bir1* mutants by error-prone PCR, focusing mutagenesis on the C-terminal domain that had been shown previously to be sufficient for providing the essential function of Bir1 (42). Two alleles (*bir1-17* and *bir1-36*) were obtained that supported normal proliferation at 26°C but showed a reproducible temperature-sensitive lethal phenotype at 37°C on centromeric plasmids, which was retained when these alleles were used to replace the genomic copy of *BIR1* (Fig. 1). However, *bir1-17* was chosen for study because of its stronger temperature-sensitive phenotype. Of the amino acid substitutions encoded by *bir1-17* (Fig. 1), two (L924S and L944F) fall within the minimal region required to provide the essential function of Bir1 (residues 876 to 954 [42]). Both of these substitutions affect conserved positions, and one of them (L924S), which also was identified in a *bir1* allele recently described by Shimogawa et al. (30), changes a residue predicted to contribute directly to the triple-helical interaction between Nbl1, Sli15, and Bir1 (25). To examine chromosome stability in a *bir1-17* strain, we made use of a colony-sectoring assay in which the loss of a supernumerary chromosome III fragment could be quantitated. Table 2 shows

TABLE 2. Chromosome loss rates

Strain	No. of red colonies	No. of half-sectored colonies	Total no. of colonies	Chromosome loss rate per cell division[a] (10^3)	Fold increase
Wild type (K5043)	3	2	2,380	0.84	
bir1-17 (VMY113)	35	39	2,540	15.6	18.5

[a] Calculated as half-sectored/(total − red).

that chromosome loss at 26°C in the *bir1-17* strain was elevated 18.5-fold relative to that of the control, which is consistent with a role for Bir1 in promoting efficient chromosome segregation.

The *bir1-17* allele next was crossed with several strains carrying mutations in genes encoding cell cycle regulators, and then tetrad analysis was performed to test for synthetic lethal interactions. Double *bir1-17 mad2Δ* and *bir1-17 mad3Δ* mutants were viable, indicating that *bir1-17* strains do not require a functional spindle assembly checkpoint for survival. However, *bir1-17* showed synthetic lethal interactions with both *sli15-3* and *ipl1-321*, which is consistent with a close functional relationship between Bir1 and these other components of the yeast chromosomal passenger complex. *SLI15* originally was isolated through its synthetic lethality with *ipl1* mutants (18), so mutations affecting all three pairwise combinations of *SLI15*, *BIR1*, and *IPL1* are inviable. Like *ipl1* mutants (9), *bir1-17* was inviable in the absence of functional Cin8, a BimC kinesin involved in yeast mitotic spindle assembly and spindle pole separation (12).

bir1-17 cells are defective in the establishment but not the maintenance of bioriented chromosomes. To examine the potential role of Bir1 in chromosome biorientation, we generated control and *bir1-17* strains in which we could visualize both microtubules (YFP-*TUB1*) and sister centromeres on chromosome III by virtue of *tetR*-GFP binding to an array of *tet* operators inserted at *CEN3* (designated *CEN*-dot). In such strains, bioriented chromosomes can be scored by the dynamic splitting and reassociation of the two sister *CEN*-dots in pre-anaphase cells, whereas mono-oriented chromosomes appear as a single, unresolved *CEN*-dot that localizes toward one of the two spindle poles (35). These strains also were dependent on the expression of *CDC20* from the methionine-repressible *MET3* promoter, enabling cells to be arrested in metaphase by Cdc20 depletion in the presence of methionine (39). This experimental design monitors biorientation directly in real time in metaphase rather than relying on failed sister chromatid segregation following anaphase as a readout, which also could result from other types of defect. Cells of the control and *bir1-17* strains were arrested in G_1 with α-factor, shifted to 37°C, and then released from the α-factor arrest into methionine-containing medium. Once the majority of cells had reached metaphase, chromosome biorientation was monitored by the time-lapse imaging of *CEN*-dot behavior in the metaphase cells. Figure 2 shows representative images for the control and mutant cells, and representative time-lapse movies are included in the supplemental material. Our data show that *bir1-17* cells have a marked, statistically significant ($P < 0.0001$) defect in chromosome biorientation such that sister
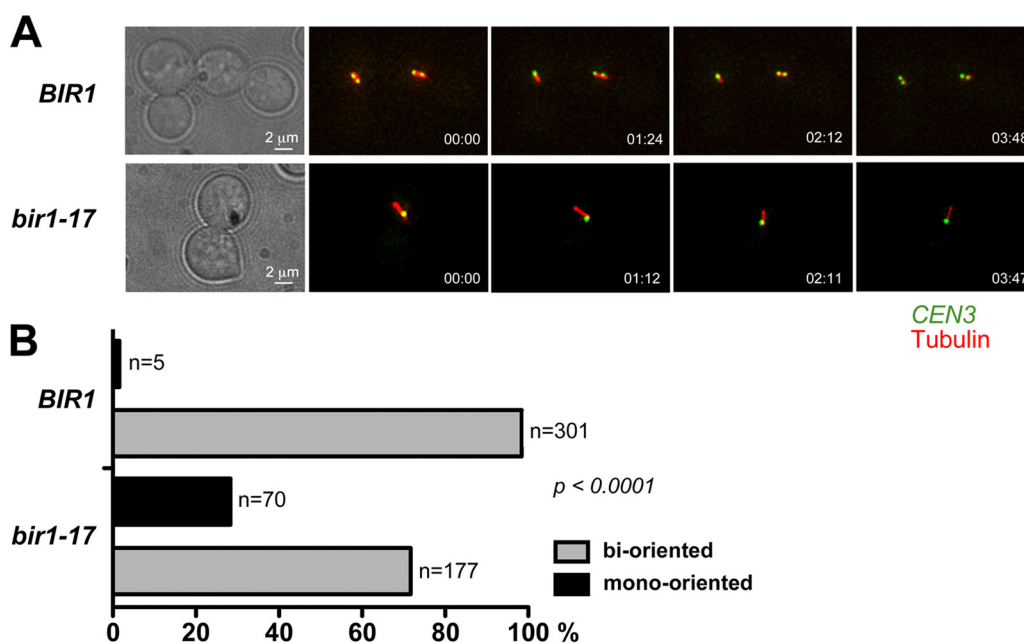

FIG. 2. *bir1-17* mutant cells show mono-oriented attachment of sister centromeres to microtubules during metaphase. Wild-type *BIR1* (VMY33) and *bir1-17* (VMY34) cells containing *CEN3*-(*tetO*)$_{336}$, *tetR*-GFP, YFP-*TUB1*, and p*MET3-CDC20* were arrested in G_1 with α-factor at 26°C and then released to a metaphase block in rich medium (containing 2 mM methionine to deplete Cdc20) at 37°C for 2.5 h. (A) Representative time-lapse images taken from live cells during 4 min. Bioriented chromosomes show the dynamic splitting and reassociation of sister *CEN3* centromeres, whereas mono-oriented chromosomes show continuously unresolved sister *CEN3* centromeres localized to one end of the spindle. Green, *CEN3* labeled with *tetR*-GFP; red, YFP-tubulin. (B) Quantification of chromosome biorientation in metaphase-arrested cells from multiple time-lapse fields (*n* is the number of cells scored in each category). Examples of wild-type and *bir1-17* time-lapse movies can be found in the supplemental material.
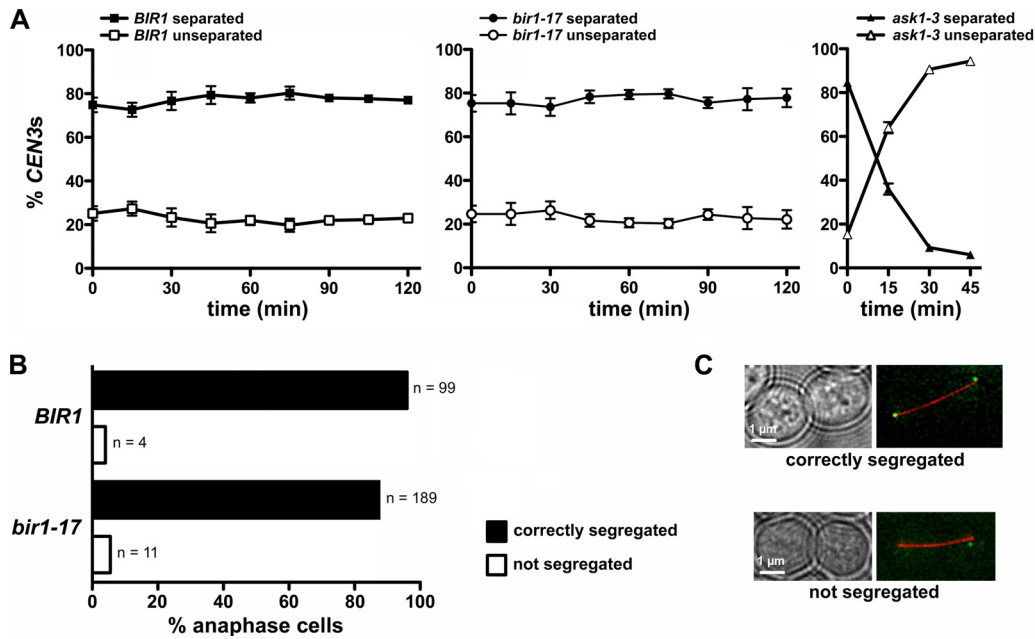
FIG. 3. Bir1 is not required for maintenance of biorientation. (A) Wild-type *BIR1* (VMY33), *bir1-17* (VMY34), and *ask1-3* (T2997) cells with *CEN3*-(*tetO*)$_{336}$, *tetR*-GFP, YFP-*TUB1*, and p*MET3*-*CDC20* were arrested in G$_1$ with α-factor, incubated for 2.5 h at 26°C in rich medium containing 2 mM methionine (to deplete Cdc20), and then shifted to 37°C in the same medium (defined as time zero). Samples were fixed with paraformaldehyde at the indicated time points. The graphs show the percentage of cells with separated and unseparated sister *CEN3* centromeres on the bipolar spindle, plotting the mean values from three separate experiments (error bars indicate standard errors of the means). Sister *CEN3* centromere separation was not scored after 45 min in *ask1-3* cells, as they exhibited a collapsed spindle at later times. Note that while essentially all separated sister *CEN3* centromeres should represent instances where chromosome III is bioriented, the unseparated sister *CEN3* category will contain both mono-oriented chromosomes and bioriented chromosomes where the sister *CEN* centromeres had transiently reassociated at the point of fixation. (B) Metaphase-arrested cells at 37°C from panel A were released at 37°C in methionine-free medium, and the distribution of sister *CEN3* centromeres was scored on elongated, anaphase spindles observed within the first 30 min. Cumulative data from two experiments are shown. (C) Representative images of cells to indicate the phenotypes scored in panel B. Red, tubulin (YFP-*TUB1*); green, *CEN3* (*tetR*-GFP).

CEN3-dots remained mono-oriented in almost 30% of metaphase-arrested cells. Thus, Bir1 clearly is required for efficient chromosome biorientation. Given that haploid yeast cells contain 16 chromosomes, the magnitude of the *bir1-17* defect implies that under restrictive conditions virtually no cells in the population will have a complete set of bioriented chromosomes when they undergo anaphase.

Although required for promoting chromosome biorientation, Ipl1 function is not needed to maintain chromosomes in the amphitelic state once it has been attained (35). We therefore tested whether Bir1 similarly is dispensable for the maintenance of chromosome biorientation by shifting metaphase-arrested control and *bir1-17* cells to 37°C and monitoring the behavior of sister *CEN3* centromeres during the following 2 h in samples of fixed cells. Neither control nor mutant cells showed any significant decline in the proportion of cells with two clearly separated *CEN*-dots, which is indicative of bioriented sister *CEN3* centromeres (Fig. 3A, left and center). In contrast, an *ask1-3* kinetochore mutant showed rapid loss of chromosome biorientation in this assay (Fig. 3A, right). Furthermore, when these metaphase-arrested cells subsequently were released into anaphase at the restrictive temperature, chromosomes segregated with normal efficiency in the *bir1-17* mutant compared to the rate of segregation of the control strain (Fig. 3B, C). Thus, Bir1 is required for the establishment but not the maintenance of bioriented chromosomes, which segregate normally during anaphase in the absence of *BIR1* function.

bir1-17 cells respond normally to nocodazole but show a tension checkpoint defect. In normal cells, the spindle assembly checkpoint can respond to both unattached kinetochores and incorrect microtubule-kinetochore attachments by delaying anaphase, allowing time for all chromosomes to become correctly bioriented on the mitotic spindle (22). To determine whether the defect in *bir1-17* cells results in spindle assembly checkpoint activation, we monitored the kinetics of Pds1 degradation, a marker for anaphase entry (8), in cells synchronized in G$_1$ using α-factor and then released into a new cell cycle at 37°C. Control and *bir1-17* cells budded with similar kinetics at 37°C following release from α-factor (not shown) and initiated anaphase after 100 to 120 min, with little or no delay in the mutant cells compared to data for the wild type (Fig. 4A). In contrast, Pds1 was stabilized in the *scc1-73* cohesin mutant in which the lack of sister chromatid cohesion relaxes the tension applied to sister kinetochores through their microtubule attachments, indicating that anaphase was delayed as expected. Thus, *bir1-17* does not lead to significant checkpoint activation, consistently with the lack of synthetic lethality between *bir1-17* and either *mad2*Δ or *mad3*Δ (as described above). We next tested whether the *bir1-17* mutation affected the normal checkpoint-dependent arrest seen in response to microtubule depolymerization using nocodazole. Figure 4B shows that at 37°C, control and *bir1-17* cells both showed a robust and prolonged stabilization of Pds1 in response to nocodazole treatment compared to results for untreated cells.
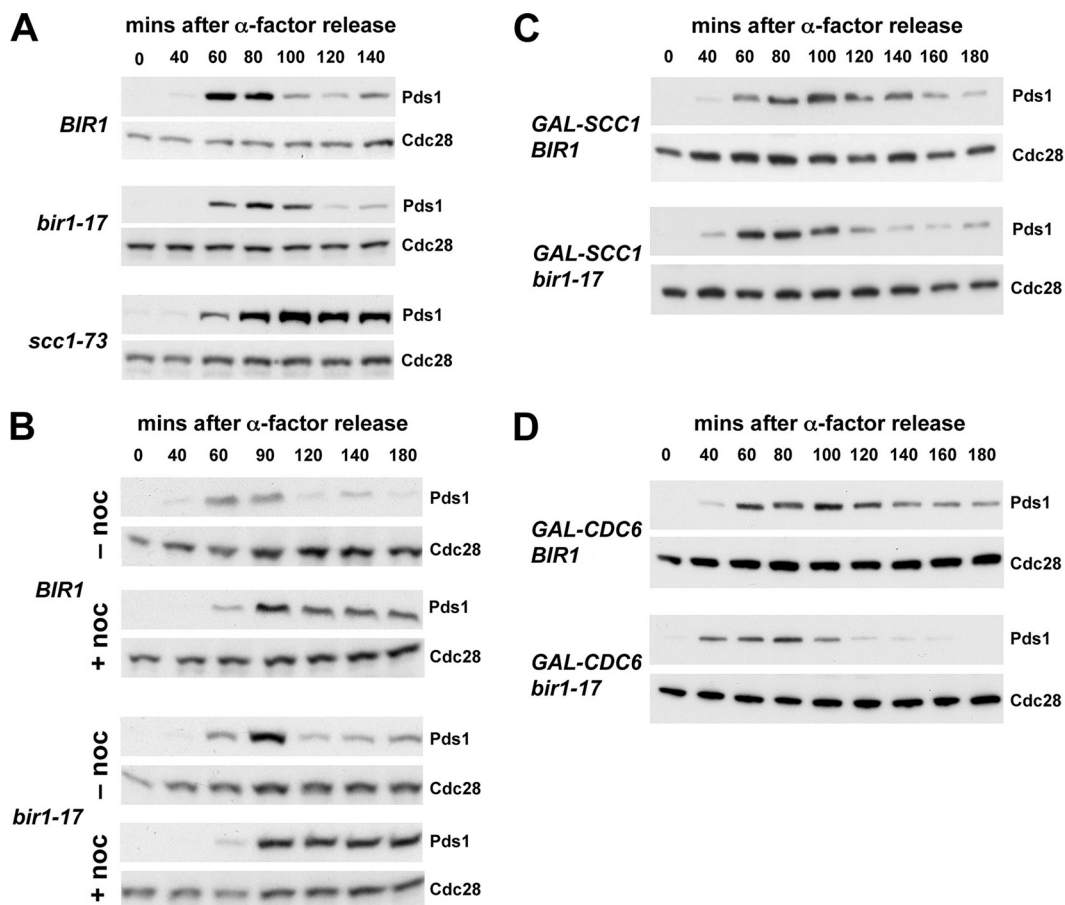
FIG. 4. Bir1 is required for the checkpoint response to reduced cohesion. (A) Wild-type *BIR1* (VMY56), *bir1-17* (VMY48), and *scc1-73* (VMY36) strains expressing Pds1-myc_{18} were arrested in G_1 with α-factor and synchronously released into YPD medium at 37°C (the restrictive temperature for *bir1-17*), and we collected samples at the indicated times. Levels of Pds1-myc_{18} (Pds1) and Cdc28 (loading control) were monitored by immunoblotting using anti-*myc* and anti-Cdc28 antibodies, respectively. (B) Wild-type *BIR1* (VMY56) and *bir1-17* (VMY48) mutant cells were synchronized as described for panel A, except that cells were released in the presence (+ noc) or absence (− noc) of 30 μg/ml nocodazole at 37°C. (C) Wild-type p*GAL-SCC1 BIR1* (VMY134) and p*GAL-SCC1 bir1-17* (VMY132) cells expressing Pds1-myc_{18} were arrested with α-factor for 2 h in medium containing galactose and then released at 37°C in medium containing glucose to repress p*GAL-SCC1*. Pds1 and Cdc28 (loading control) were monitored as described for panel A. Pds1 levels declined faster in p*GAL-SCC1 bir1-17* cells than in p*GAL-SCC1 BIR1* cells, indicating that Bir1 is required for the checkpoint arrest following p*GAL-SCC1* shutoff. (D) Wild-type p*GAL-CDC6 BIR1* (VMY137) and p*GAL-CDC6 bir1-17* (VMY139) cells were arrested with α-factor for 2 h in medium containing galactose and then released at 37°C in medium containing glucose to repress p*GAL-CDC6*. Pds1 and Cdc28 (loading control) were monitored as described for panel A. Pds1 levels declined faster in p*GAL-CDC6 bir1-17* cells than in p*GAL-CDC6 BIR1* cells, indicating that Bir1 is required for the checkpoint arrest in response to unreplicated sister chromatids.

However, since *ipl1* mutants specifically are defective in the tension checkpoint (i.e., the activation of the spindle assembly checkpoint mechanism in response to reduced sister kinetochore tension), we next examined whether the *bir1-17* mutant affected the tension checkpoint by examining the response to cohesin depletion using strains dependent on p*GAL-SCC1* for Scc1 expression. Synchronous cultures of such strains show a profound anaphase delay when grown on glucose to repress *SCC1* expression due to the activation of the spindle checkpoint (15), and this appears to be mediated through the phosphorylation of Mad3 by Ipl1 kinase (19). Figure 4C shows that following cohesin depletion, *bir1-17* cells failed to stabilize Pds1 to anywhere near the same extent as control cells with normal *BIR1*, indicating that they are defective in spindle checkpoint activation under these conditions. Similar results were obtained using the *scc1-73* mutant as an alternative to cohesin depletion using

p*GAL-SCC1* (data not shown). We also used strains dependent on p*GAL-CDC6* to reduce tension on microtubule-kinetochore attachments by a different means. When such strains are released from α-factor arrest into medium containing glucose, the failure to express Cdc6 blocks DNA replication initiation and causes cells to undergo a spindle checkpoint-dependent arrest in metaphase with unreplicated chromosomes (33). Figure 4D shows that while Pds1 is stabilized in control Cdc6-depleted cells, indicating that the spindle checkpoint has been activated, *bir1-17* cells enter anaphase and degrade Pds1. Thus, *bir1-17* cells are defective in restraining anaphase through the activation of the spindle assembly checkpoint when tension on microtubule-kinetochore attachments is reduced, either by interfering with sister chromatid cohesion or by blocking DNA replication. This defect is specific for tension checkpoint activation, because *bir1-17* cells arrest normally in response to unattached kinetochores gener-

ated by treatment with the microtubule-depolymerizing drug
nocodazole (Fig. 4B).

Ipl1 localization in the *bir1-17* mutant. Since Ipl1 kinase is
thought to promote chromosome biorientation by phosphory-
lating proteins at the kinetochore-microtubule interface, either
the failure to localize Ipl1 kinase correctly or a reduced ability
to activate Ipl1 kinase could explain the chromosome biorien-
tation defect in the *bir1-17* mutant. We therefore examined the
effect of the *bir1-17* mutation on Ipl1 localization by generating
wild-type and mutant *bir1-17* cells in which Ipl1 was tagged
with GFP (Ipl1-GFP) and that expressed CFP-tagged Ndc80
and Ctf19 as kinetochore markers.

In both wild-type and *bir1-17* cells after 1 h at 37°C, Ipl1-
GFP fluorescence was closely associated with the single kinet-
ochore cluster in unbudded cells, while in anaphase cells Ipl1
was localized along the anaphase spindle (data not shown) as
previously described (5, 38). In metaphase cells (defined by two
kinetochore clusters showing up to ~1.6 μm separation), most
wild-type and the majority of *bir1-17* cells showed kinetochore-
associated Ipl1-GFP (Fig. 5A). However, in contrast to wild-
type cells, no Ipl1-GFP was evident at either of the kinetochore
clusters in around one-third of *bir1-17* cells (Fig. 5A). This
difference between wild-type and *bir1-17* populations was
statistically significant (*P* < 0.0001) (Fig. 5B), but since a
similar difference was apparent in *bir1-17* cells at 26°C (data
not shown), it seems unlikely that it is solely responsible for
the chromosome biorientation defect seen specifically at the
higher temperature. We did not observe any bright foci of
Ipl1-GFP at the nuclear periphery seen in a different *bir1*
mutant (30), possibly due to allele-specific differences or to
the use of different fluorescent protein tags in the two
studies.

While carrying out this analysis, we noticed that in meta-
phase cells of both wild-type and mutant strains, although
Ipl1-GFP was often in close proximity to one or both kineto-
chore clusters defined by Ndc80 and Ctf19 fluorescence, it was
seldom coincident with the kinetochore clusters. The Ipl1-GFP
signal frequently localized to one side of each cluster, often
with significant fluorescence surrounding the kinetochore clus-
ters or located between them. When examined by time-lapse
microscopy, Ipl1 localization was observed to change consid-
erably from frame to frame and showed larger frame-to-frame
differences than the two kinetochore clusters themselves; a
representative time-lapse movie is included in the supplemen-
tal material. Thus, while Ipl1 clearly is associated with the
kinetochore clusters, it behaves differently from a bone fide
kinetochore protein.

Ipl1 kinase activity is reduced in the *bir1-17* mutant. We
next adopted two different but complementary approaches to
determine whether Ipl1 protein kinase activity shows depen-
dence on Bir1p. First, we generated Ipl1 immune precipitates
from both wild-type and *bir1-17* cell extracts prepared from
cells grown at either 26 or 37°C, and we measured their ability
to phosphorylate recombinant GST-Dam1 in an in vitro pro-
tein kinase assay, taking care to normalize the activity mea-
sured to the Ipl1-*myc* input. Figure 6A shows that the specific
activity of Ipl1 kinase recovered from the *bir1-17* strain was
reduced when cells were grown at either temperature. How-
ever, the defect was more severe when Ipl1 was precipitated
from cells grown at 37°C, with a specific protein kinase activity
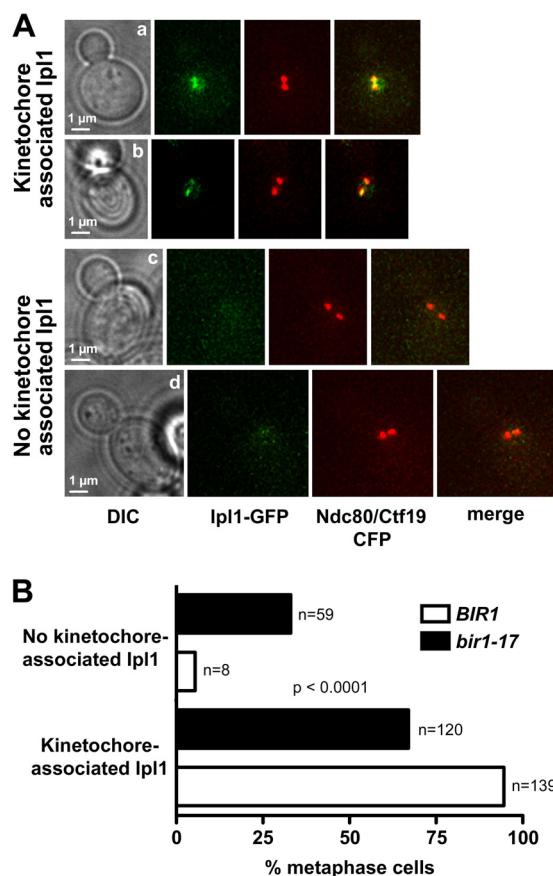

FIG. 5. Effect of *bir1-17* on the cellular localization of Ipl1 kinase in
cells in metaphase. Asynchronous, exponentially growing cultures of
wild-type *BIR1* (T5241) and *bir1-17* (VMY14) mutant cells expressing
Ipl1-GFP and both CFP-tagged Ctf19 and Ndc80 as kinetochore mark-
ers were shifted to 37°C for 1 h, and then images were acquired.
(A) Representative differential interference contrast (DIC), Ipl1-GFP
(green), Ctf19/Ndc80 (red), and merged live cell images from two
wild-type cells showing kinetochore-associated Ipl1-GFP (a and b) and
two *bir1-17* cells where no kinetochore-associated Ipl1-GFP was evi-
dent (c and d). Note, however, that both populations contained exam-
ples of both types of localization. (B) Quantitation of kinetochore-
associated Ipl1-GFP in metaphase-arrested cells. *n* is the number of
cells scored in each category.

under these conditions of only around one-third of that of Ipl1
from control cells. Second, we examined the phosphorylation
of ser-10 on histone H3 in synchronous cultures of control and
bir1-17 cells. In yeast, histone H3 phosphorylation on this res-
idue is Ipl1 dependent (13) (Fig. 6C) and represents an in vivo
readout of Ipl1 kinase activity. Compared to that of control
cells, *bir1-17* cells showed greatly reduced histone H3 phos-
phorylation at 37°C, with a much smaller, but noticeable, re-
duction in cells grown at 26°C (Fig. 6B). Thus, two different
approaches both indicate much lower levels of Ipl1 protein
kinase activity in *bir1-17* cells at the restrictive temperature.

**The *bir1-17* biorientation defect increases in severity on
recovery from nocodazole treatment.** Despite showing a nor-
mal metaphase arrest in response to microtubule depolymer-
ization (Fig. 4B), we noticed that *bir1-17* cells nonetheless were
hypersensitive to benomyl (Fig. 7A). We therefore examined
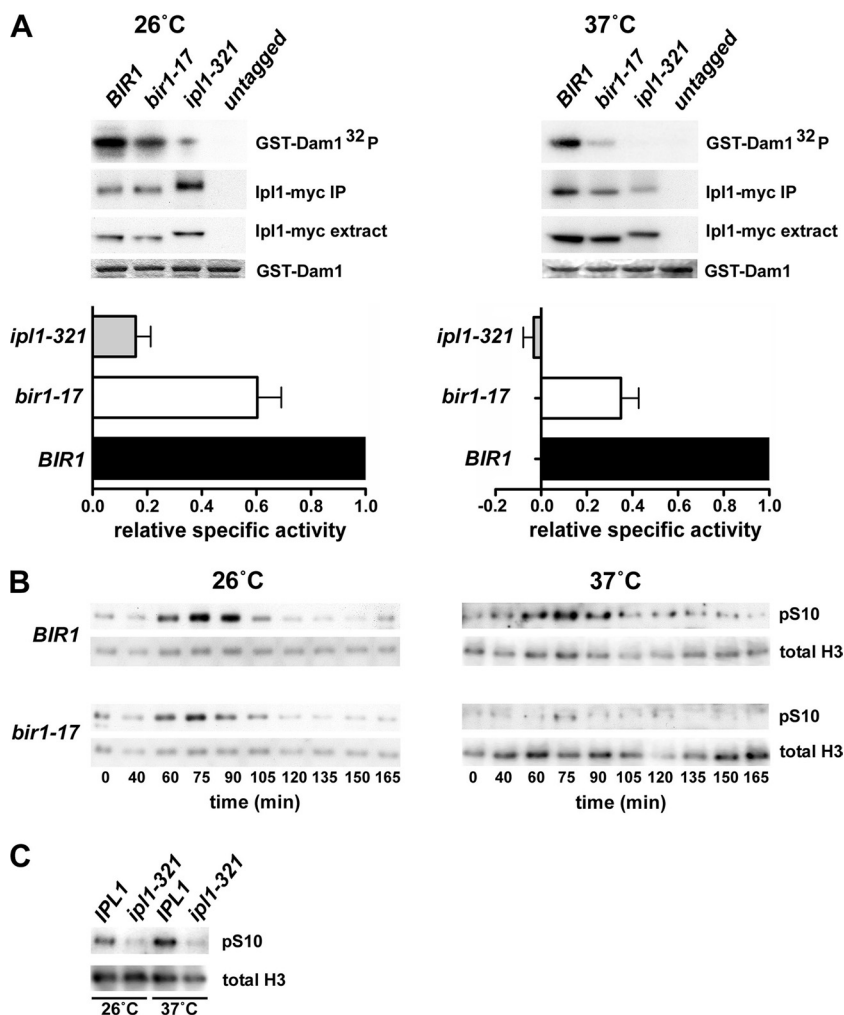how proficiently *bir1-17* cells could generate bioriented chro-

FIG. 6. Ipl1 kinase activity is significantly reduced in *bir1-17* mutant cells. (A) *IPL1 BIR1* (AY925; untagged), *IPL1-myc*$_{12}$ *BIR1* (T2608), *IPL1-myc*$_{12}$ *bir1-17* (VMY5), and *ipl1-321-myc*$_{13}$ *BIR1* (PKY63) cells were cultured at 26 or 37°C for 2 h as indicated, and the *myc*-tagged Ipl1 was recovered by immune precipitation. The level of tagged Ipl1 in each extract (Ipl1-*myc* extract) and recovery of Ipl1 (Ipl1-*myc* IP) was quantified by Western blotting. The in vitro protein kinase activity of the immunoprecipitates was measured by the incorporation of radiolabel from [γ-^{32}P]ATP into recombinant GST-Dam1 as a substrate. Kinase assays were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (GST-Dam1-^{32}P). GST-Dam1 input was visualized by Coomassie staining (GST-Dam1). Histograms show the relative specific activity of Ipl1 kinase in the different immune precipitates. ^{32}P incorporation was normalized to the level of Ipl1-*myc* recovered in each immunoprecipitate, and the values for the *bir1-17* and *ipl1-321* strains are expressed as a fraction of the value obtained for the wild-type strain (set to 1.00). Values for the two mutant strains represent the means ± standard errors of the means for four (37°C) or six (26°C) experiments. (B) In vivo phosphorylation of histone H3 is severely reduced in the *bir1-17* mutant at 37°C. Wild-type *BIR1* (VMY56) and *bir1-17* (VMY48) cells were synchronized in G$_1$ using α-factor and then released at either 26 or 37°C as indicated. Samples were analyzed across the first cell cycle by Western blotting to detect the phosphorylation of histone H3 serine 10 and total histone H3 levels. (C) Serine 10 phosphorylation of histone H3 is an Ipl1-dependent phosphorylation event, as confirmed by the use of an *ipl1-321* mutant strain.

mosomes when released from nocodazole arrest at 37°C rather than during a normal cell cycle. This experiment was performed using p*MET3-CDC20*-dependent strains in the presence of methionine such that cells remained arrested in metaphase following nocodazole removal. Surprisingly, we found that under these conditions the biorientation defect was far more severe, with around 80% of mutant cells persistently failing to achieve biorientation during a 120-min time course after nocodazole removal (Fig. 7B, C). In the majority of control *BIR1* cells, the marked chromosome became bioriented within 30 to 60 min (Fig. 7B, C). Thus, cells have a greater need for Bir1 when they are forced to attach their sister kineto-

chores to microtubules during metaphase rather than immediately following *CEN* replication (20), as in a normal cell cycle.

DISCUSSION

Despite its known interactions with Sli15 and Ipl1, both of which are required for effective yeast chromosome biorientation, evidence to support a role for Bir1 in promoting chromosome biorientation has been lacking. However, our analysis of the *bir1-17* mutant provides strong support for such a role and is consistent with a need for Bir1 to support Ipl1 function. Thus, despite their lack of obvious conservation outside the
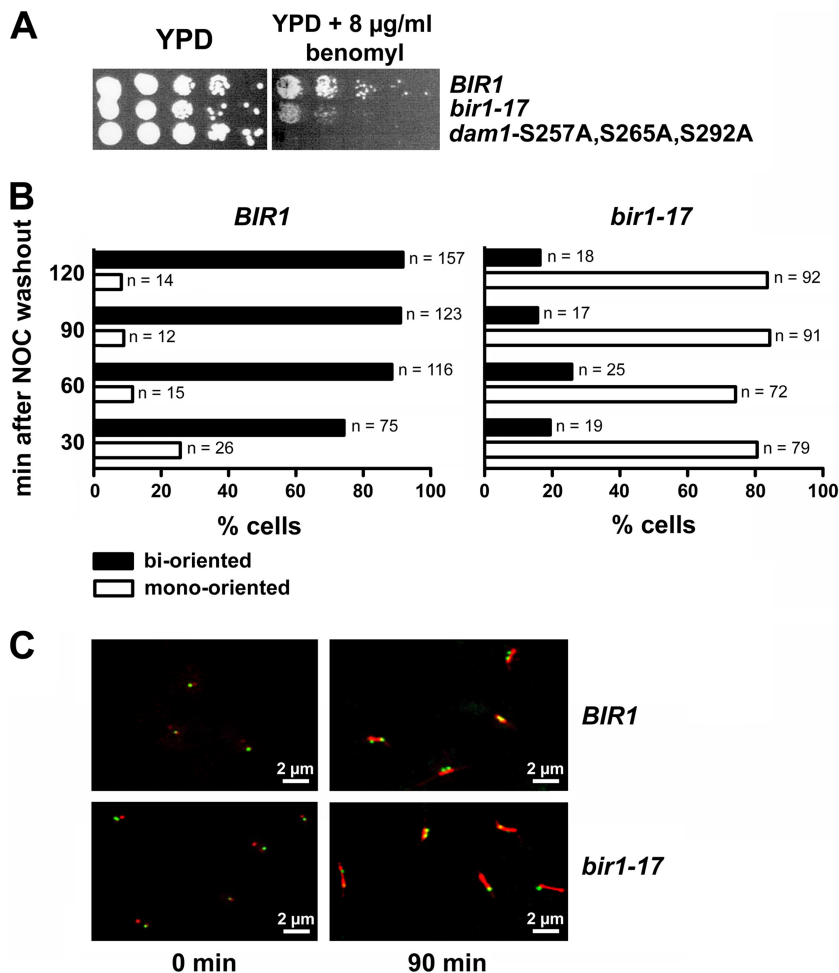
FIG. 7. Bir1 inactivation causes a severe biorientation defect following nocodazole (NOC) treatment. (A) Equivalent 10-fold dilutions of wild-type (AY925), *bir1-17* (VMY1), and *dam1-S257A,S265A,S292A* (BP635; nocodazole-hypersensitive control) cells were plated on YPD medium containing 8 μg/ml benomyl. (B) *BIR1* (VMY33) and *bir1-17* (VMY34) cells containing *CEN3-(tetO)*$_{336}$, *tetR*-GFP, *YFP-TUB1*, and p*MET3-CDC20* were arrested in G$_1$ with α-factor at 26°C and then released for 2.5 h to a metaphase block in rich medium containing 2 mM methionine (to deplete Cdc20) and nocodazole (30 μg/ml; to depolymerize microtubules). Subsequently, nocodazole was removed and the temperature concurrently shifted to 37°C (to inactivate Bir1). Cells showing a clear bipolar spindle were scored for mono-oriented or bioriented sister *CEN3* genes based on 2-min time-lapse movies taken during a 30- to 120-min time course (*n* is the number of cells scored). (C) Examples of cells from panel B at the point of nocodazole release (0 min) showing the absence of spindles and at 90 min showing bipolar spindles. Red, YFP-Tub1; green, sister *CEN3* centromeres (*tetR*-GFP).

BIR domains, which are not required for chromosome biorientation in yeast, Bir1 and its metazoan counterpart Survivin may fulfill similar roles. Our findings therefore concur with those reported recently in a concurrent study (30). Although we find that functional Bir1 is required for the efficient establishment of chromosome biorientation, it clearly is dispensable for maintaining the bioriented state either during metaphase or anaphase and so, like Ipl1, its role appears specifically to be in promoting the reorientation of chromosomes that have acquired monopolar (syntelic) attachment.

Although the *bir1-17* mutation confers a significant biorientation defect, the phenotype is less severe than that of the *ipl1-321* mutant analyzed in the same manner, which shows 60 to 70% mono-orientation (35 and data not shown). This may reflect fortuitous differences in allele strength between *ipl1-321* and *bir1-17* but also may have deeper significance. Both the *bir1-107* allele described by Shimogawa et al. (30) and the

nbl1-6 allele (25) have relatively mild phenotypes compared to those of *ipl1* mutants, whereas *sli15-3* (affecting the IN-box required for Ipl1 kinase activation) has a stronger biorientation phenotype that is comparable to that of *ipl1-321* (35). Furthermore, it is possible to isolate very-slow-growing, polyploid Bir1- and Nbl1-deficient cells (25, 29), whereas such survivors are not observed following the deletion of *IPL1* or *SLI15* (our unpublished data). Perhaps this reflects the difference between mutations that largely abolish Ipl1 catalytic activity (*ipl1-321* and *sli15-3*) and mutations that reduce Ipl1 activity or interfere with its correct targeting (*bir1* and *nbl1* mutations).

Sandall et al. (29) have proposed that a Bir1-Sli15 complex forms a tension-sensitive linkage at the kinetochore-microtubule interface such that when tension is reduced, the Sli15 IN-box is able to bind to and activate Ipl1 kinase. This complex presumably contains Nbl1, because the Bir1-Sli15 interaction is Nbl1 dependent (25). Our data showing the Bir1 dependence

of Ipl1 kinase activity and a requirement for Bir1 to activate the tension checkpoint are consistent with its involvement in a tension-responsive mechanism. However, if Bir1 has a role in switching off Ipl1 activity once a chromosome has bioriented and its sister kinetochores are under tension, *bir1* loss-of-function mutants might fail to inactivate Ipl1, leading to the destabilization of kinetochore-microtubule attachments. However, since *bir1-17* cells can maintain their chromosomes in the bioriented state when shifted to 37°C, it seems unlikely that Bir1 functions to restrain Ipl1 kinase activity once chromosomes are bioriented. Our finding that Bir1 functions positively for Ipl1 kinase activity also is inconsistent with such a role. Thus, while the tension sensor model is attractive, it may be an oversimplification.

How might Bir1 affect the reorientation of syntelically attached sister chromatids? Our finding that the protein kinase activity of Ipl1 clearly is reduced by the *bir1-17* mutation both in vitro and in vivo provides a possible explanation. Bir1 might activate Ipl1 kinase through a direct protein-protein interaction within the chromosomal passenger complex or may support Ipl1 kinase activity through stabilizing the chromosomal passenger complex as proposed for Nbl1 (25), but in either case the reduced kinase activity would compromise the cell's ability to detach incorrectly attached microtubules from kinetochores. Alternatively, Bir1 may be required to localize Ipl1 to tensionless kinetochore-microtubule connections so as to promote their reorientation, and Shimogawa et al. (30) have provided evidence that Bir1 localization responds to the tension status of such connections. Although we find a significant defect in the localization of Ipl1 to the vicinity of clustered kinetochores in the *bir1-17* mutant, because this defect is observed at both the restrictive and permissive temperatures, it is not easy to see how it could account for a chromosome biorientation defect that becomes significant only at the restrictive temperature. If the Bir1-dependent localization of Ipl1 is important, then perhaps the need for the Ipl1-dependent reorientation of mono-oriented chromosomes is greater at 37°C than at 26°C. The reduced kinetochore association of Ipl1 in *bir1-17* therefore might be tolerable at 26°C but become limiting for efficient biorientation at higher temperatures. However, since the *bir1-17* mutation adversely affects Ipl1 kinase activity more severely at 37°C, particularly in vivo, it seems likely that the effect of the mutation on Ipl1 kinase activity forms an important part of the biorientation defect. Such a role for Bir1 in Ipl1 kinase activation is consistent with previous work showing that Survivin is needed to promote the full activation of Aurora B in metazoans, at least in vitro (3, 7).

We also have found that Bir1 is a key element in the tension checkpoint, in which the spindle assembly checkpoint mechanism is activated in response to a lack of sister kinetochore tension. Ipl1 may activate the tension checkpoint either by the generation of unattached kinetochores (26) or by the phosphorylation of Mad3 (19), and our observation that Ipl1 is associated with, but not stably and precisely colocalized at, the two clusters of bioriented sister kinetochores is entirely consistent with the recent findings of Shimogawa et al. (30) that the chromosomal passenger complex is delocalized from sister kinetochores under tension. Both the Bir1 requirement for full Ipl1 activity and the failure of Ipl1 to associate with clustered

kinetochores in a significant fraction of *bir1-17* mutant cells may contribute to the tension checkpoint defect.

The chromosome biorientation defect in *bir1-17* cells is enhanced following release from nocodazole arrest, demonstrating a greater dependence on Bir1 under these conditions. This phenotype is highly reminiscent of that shown by the *sgo1-100* mutant. Yeast Sgo1 has been proposed to act in the pathway used to monitor sister chromatid tension (15), yet it is a nonessential gene, which is inconsistent with an obligatory role for Sgo1 in normal chromosome biorientation during an unperturbed S phase and contrasts with Bir1, Sli15, Nbl1, and Ipl1, all of which are essential. However, Sgo1 becomes essential for chromosome biorientation following release from nocodazole arrest (14). Chromosomes normally reattach to spindle microtubules very soon after the replication of the centromeric DNA (20). Since Bir1 is more critical for chromosome biorientation upon release from nocodazole, this may indicate that cells have a greater requirement for the Ipl1-dependent correction of mono-oriented chromosomes when spindle formation is delayed relative to replication.

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