Premature Silencing of the Spindle Assembly Checkpoint Is Prevented by the Bub1-H2A-Sgo1-PP2A Axis in *Saccharomyces cerevisiae*

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ABSTRACT The spindle assembly checkpoint (SAC) monitors mistakes in kinetochore-microtubule interaction and its activation prevents anaphase entry. The SAC remains active until all chromosomes have achieved bipolar attachment which applies tension on kinetochores. Our previous data in budding yeast *Saccharomyces cerevisiae* show that Ipl1/Aurora B kinase and a centromere-associated protein, Sgo1, are required to prevent SAC silencing prior to tension generation, but we believe that this regulatory network is incomplete. Bub1 kinase is one of the SAC components, and Bub1-dependent H2A phosphorylation triggers centromere recruitment of Sgo1 by H2A in yeast and human cells. Although yeast cells lacking the kinase domain of Bub1 show competent SAC activation, we found that the mutant cells fail to maintain a prolonged checkpoint arrest in the presence of tensionless attachment. Mutation of the Bub1 phosphorylation site in H2A also results in premature SAC silencing in yeast cells. Previous data indicate that Sgo1 protein binds to $PP2A^{Rts1}$, and we found that *rts1*Δ mutants exhibited premature SAC silencing as well. We further revealed that *sgo1* mutants with abolished binding to H2A or $PP2A^{Rts1}$ displayed premature SAC silencing. Together, our results suggest that, in budding yeast *S. cerevisiae*, the Bub1-H2A-Sgo1-$PP2A^{Rts1}$ axis prevents SAC silencing and helps prolonged checkpoint arrest prior to tension establishment at kinetochores.

KEYWORDS Bub1 kinase; PP2A; SAC silencing; Sgo1; spindle assembly checkpoint

THE spindle assembly checkpoint (SAC) monitors defects in kinetochore-microtubule interaction and an active SAC blocks anaphase onset, thus this checkpoint is crucial for faithful chromosome segregation. The SAC components include Bub1, Bub3, Mad1, Mad2, Mad3/BubR1, and Mps1 (Stukenberg and Burke 2015). Recent evidence from budding yeast *Saccharomyces cerevisiae* and fission yeast *S. pombe* indicates that SAC proteins Bub1 and Bub3 bind to a kinetochore protein Spc105/Knl1, and protein kinase Mps1-mediated phosphorylation of Spc105 promotes the interaction of its MELT domains with Bub3 (Shepperd *et al.* 2012; Primorac *et al.* 2013). Kinetochore-associated Bub3-Bub1 complexes further recruit Mad1 and Mad2, where Mad2 is converted to a closed form that prevents the activation of anaphase-promoting complex/cyclosome and blocks the degradation of anaphase inhibitor Pds1 (Luo *et al.* 2000; London and Biggins 2014).

The SAC has to be silenced to allow anaphase onset, but the regulation of SAC silencing is poorly understood. One model is that kinetochore-microtubule attachment removes the SAC kinase Mps1 from kinetochores to silence the SAC, which is supported by recent observations in mammalian cells (Hiruma *et al.* 2015; Ji *et al.* 2015). Research work in budding yeast supports the model that the interaction of microtubule-associated Dam1 complex with the Ndc80 kinetochore complex separates Ndc80-associated Mps1 from its substrates to trigger SAC silencing (Aravamudhan *et al.* 2015). On the other hand, protein phosphatase PP1 has been shown to be essential for SAC silencing likely through its dephosphorylation of Mps1 kinase substrates at the kinetochore (London *et al.* 2012). The yeast kinetochore protein Spc105 binds to PP1 through a conserved motif and mutation of this motif blocks anaphase entry, resulting in lethality. Interestingly,

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this cell cycle arrest and lethality are rescued by deletion of a SAC gene, indicating that Spc105-PP1 interaction is essential for SAC silencing (Rosenberg *et al.* 2011). Furthermore, PP1 overexpression causes premature SAC silencing in the presence of detached kinetochores (Pinsky *et al.* 2009; London *et al.* 2012). In addition to Spc105, PP1 also binds to another yeast kinetochore protein, Fin1 (Akiyoshi *et al.* 2009). Our recent data demonstrate that the Fin1-PP1 complex promotes the removal of Bub1 from the kinetochore. Because Fin1 localizes to the kinetochore after anaphase entry, this regulation is not essential for SAC silencing, but untimely Fin1 kinetochore localization leads to premature SAC silencing (Bokros and Wang 2016; Bokros *et al.* 2016), indicating that PP1 negatively regulates SAC at multiple levels.

Ipl1/Aurora B kinase regulates the stability of kinetochore-microtubule interaction as well as the SAC silencing process (Cheeseman *et al.* 2002). In budding yeast, Ipl1 phosphorylates Dam1, a protein of a 10-subunit kinetochore complex which mediates Ndc80-microtubule interaction (Janke *et al.* 2002; Li *et al.* 2002). We found that phospho-deficient *dam1* mutants showed premature SAC silencing, while the phospho-mimetic *dam1* mutants exhibited delayed anaphase entry. This delay is mainly attributed to the failure of SAC silencing, whereas defective kinetochore attachment only plays a minor role (Jin and Wang 2013). Because Dam1 dephosphorylation is triggered by tension at kinetochores (Keating *et al.* 2009), Ipl1-dependent Dam1 phosphorylation likely prevents SAC silencing until chromosome bipolar attachment generates tension at kinetochores. Therefore, this mechanism links bipolar attachment and SAC silencing (Wang *et al.* 2014). *SGO1* encodes a centromere-binding protein (Indjeian *et al.* 2005), and *SGO1* deletion also leads to premature SAC silencing (Jin *et al.* 2012; Jin and Wang 2013). However, it is largely unknown how Sgo1 regulates SAC silencing at the molecular level.

In yeast and human cells, kinetochore-localized checkpoint protein Bub1 phosphorylates histone H2A to promote its association with Sgo1, resulting in the recruitment of Sgo1 to centromeres and pericentromeres (Kawashima *et al.* 2010). Interestingly, results from budding yeast indicate that the kinase domain of Bub1 is dispensable for SAC activation (Fernius and Hardwick 2007). In this report, we determine the role of the Bub1 kinase domain in the regulation of SAC silencing in budding yeast *S. cerevisiae*. We found that deletion of the Bub1 kinase domain or mutation of the Bub1 phosphorylation site in H2A leads to premature SAC silencing, without compromising SAC activation. Sgo1 interacts with protein phosphatase $PP2A^{Rts1}$ (Riedel *et al.* 2006; Eshleman and Morgan 2014), and we found that deletion of *RTS1* also led to premature SAC silencing. In addition, mutation of the H2A or $PP2A^{Rts1}$ binding motif in Sgo1 causes anaphase entry in the presence of tensionless chromosome attachment. Therefore, our results suggest that the Bub1-H2A-Sgo1-$PP2A^{Rts1}$ axis prevents SAC silencing until cells have achieved chromosome bipolar attachment.

Materials and Methods

Plasmids construction

The *SGO1* gene was cloned by PCR using the forward primer 5′-GATTCCCCGCGGGGACTACTTCGATTGGGTTATTGA-3′ and the reverse primer 5′-GATACCATCGATGGTAGGGACGTTAAAGACATTGA-3′. The *SGO1* DNA fragment from PCR was inserted into the pRS403 vector after digestion with *Cla*I and *Sac*II restriction enzymes. Using this constructed *SGO1* plasmid, site-directed mutagenesis was performed to generate the plasmid harboring the *sgo1-N51I* mutant gene, in which the PP2A binding motif was mutated. To generate the *sgo1-4A* plasmid, the DNA that encodes the H2A binding motif (KMRR) in *SGO1* gene was mutated to the sequence encoding four alanine residues.

Yeast strain and growth conditions

The relevant genotypes and the sources of the strains used in this study are listed in Supplemental Material, Table S1. All of the strains listed are isogenic to Y300, a derivative of W303. The Bub1 kinase deletion allele (*bub1-ΔK*) was constructed by inserting a *13myc-Sphis5*$^+$ or *3HA-Sphis5*$^+$ cassette after the 608 residue of Bub1 by a PCR-based method (Longtine *et al.* 1998). The resulting strains express truncated Bub1 lacking the fragment that encodes the amino acid residues from 609 to 1021. The *bub1-ΔK* mutants were verified by determining the protein size of the tagged protein fragment using Western blotting. The *rts1*Δ strain was also created using a PCR-based method. The *h2a-121A* and the H2A-wild type (WT) strains are a gift from the Watanabe Laboratory. The endogenous H2A gene was deleted in these strains, but the strains express WT H2A or mutated *h2A-S121A* from plasmids. The constructed plasmids containing *SGO1*, *sgo1-N51I*, and *sgo1-4A* genes were inserted into the genome of *sgo1*Δ strains at the endogenous locus to generate *SGO1*, *sgo1-N51I*, and *sgo1-4A* yeast strains.

The yeast cell growth, synchronization, and *CIK1-CC* overexpression were performed as described previously (Jin *et al.* 2012). Briefly, yeast cells with a control vector or P_{GAL}CIK1-CC plasmid were grown in synthetic medium containing raffinose to midlog phase and then arrested in G_1 phase with α factor. The arrested cells were released into medium containing galactose to induce *CIK1-CC* overexpression. For nocodazole treatment, G_1-arrested cells were released into YPD containing 20 μg/ml nocodazole and 1% of DMSO. We added 10 μg/ml of nocodazole every hour.

Western blot analysis

We collected 1.5 ml yeast cell culture and the cell pellets were resuspended in 100 μl H_2O and then 100 μl 0.2 N NaOH was added. The mixture was left at room temperature for 5 min. The pellet was resuspended in the loading buffer. For Pds1-18myc protein detection, we used 10% acrylamide gels for SDS-PAGE. For the detection of Mad1 modification, we used and 8% acrylamide gels. The anti-myc antibody (9E10) and anti-HA (16B12) (Covance Research Products) were used at a 1:1000 dilution.

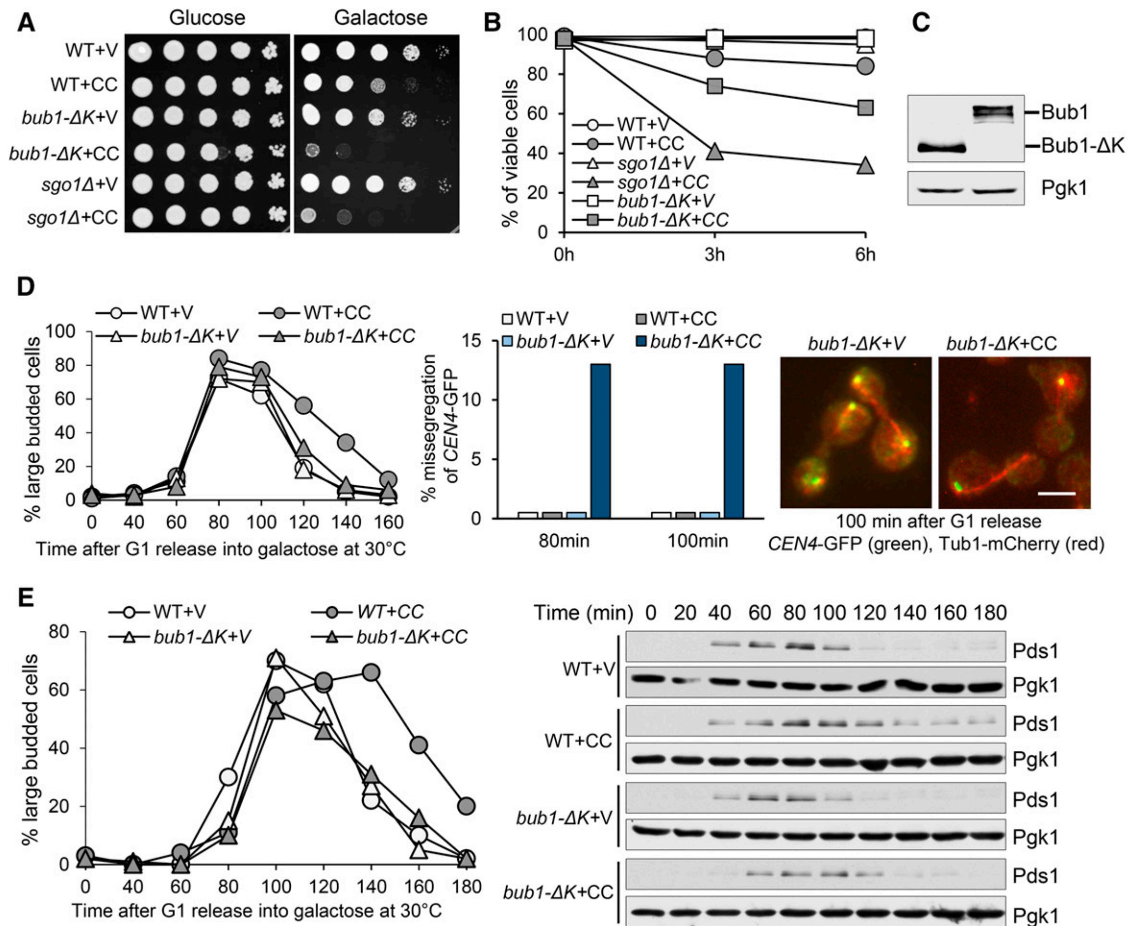

Figure 1 *bub1-ΔK* mutants exhibit premature SAC silencing in response to *CIK1-CC*-induced syntelic attachment. (A) Overexpression of *CIK1-CC* is toxic to *bub1-ΔK* cells. Saturated WT and mutant cells with a vector (V) or a P_{GAL}CIK1-CC plasmid (CC) were 10-fold serial diluted, spotted onto glucose and galactose plates, and incubated at 30° for 2 days. (B) The viability loss of *bub1-ΔK* cells after *CIK1-CC* overexpression. WT and *bub1-ΔK* cells with a vector or a P_{GAL}CIK1-CC plasmid were grown to log phase in raffinose medium and then released into galactose medium at 30°. Cells were collected at 0, 3, and 6 hr and spread onto YPD plates to determine the plating efficiency after overnight growth at 25° ($n \geq 200$). (C) The expression of truncated Bub1. *BUB1-13-myc* and *bub1-ΔK-13myc* cells in midlog phase were treated with 20 μg/ml of nocodazole for 120 min at 30°. The cells lysates were prepared for the examination of myc-tagged Bub1 and Bub1-ΔK. (D) *CIK1-CC* overexpression causes chromosome missegregation in *bub1-ΔK* mutant cells. A vector or a P_{GAL}CIK1-CC plasmid was introduced into WT and *bub1-ΔK* cells with *CEN4-GFP TUB1-mCherry*. The transformants were first arrested in G_1 phase in raffinose medium and then released into galactose medium at 30°. α-factor was restored after budding to block the second round of cell cycle. Cells were collected at the indicated time points for the examination of fluorescence signals. The percentage of *CEN4*-GFP missegregation is shown in the center panel; the distribution of *CEN4*-GFP and spindle morphology in some representative cells is shown on the right. The budding index is shown in the left panel. Bar, 5 μm. (E) *bub1-ΔK* mutation alleviates the delay of Pds1 degradation induced by *CIK1-CC* overexpression. G_1-arrested *PDS1-18myc* and *bub1-ΔK PDS1-18myc* cells with a vector or a P_{GAL}CIK1-CC plasmid were released into galactose medium and incubated at 30°. α-factor was restored after budding. Cells were collected at the indicated time points and protein samples were prepared for Western blotting. The budding index and Pds1 levels are shown. Pgk1 protein levels are used as a loading control.

Phosphoglycerate kinase 1 (Pgk1) antibody (Molecular Probes, Eugene, OR) was used at a 1:5000 dilution. Proteins were detected with ECL (Perkin-Elmar-Cetus, Norwalk, CT).

Chromosome segregation assays

Strains containing GFP-labeled chromosome *IV* (*CEN4*-GFP) and Tub1-mCherry were collected and fixed with 3.7% formaldehyde for 5 min at room temperature. After centrifugation, the pellet was resuspended in 1× PBS buffer. The fluorescence signals were analyzed in cells with an elongated spindle using a fluorescence microscope (EVOS from Life Technologies).

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

Results

Bub1 kinase activity is required for the cell cycle delay induced by syntelic chromosome attachment

Cik1 and Kar3 form a hetero-motor complex through their coiled-coil domains (Barrett *et al.* 2000). We demonstrate

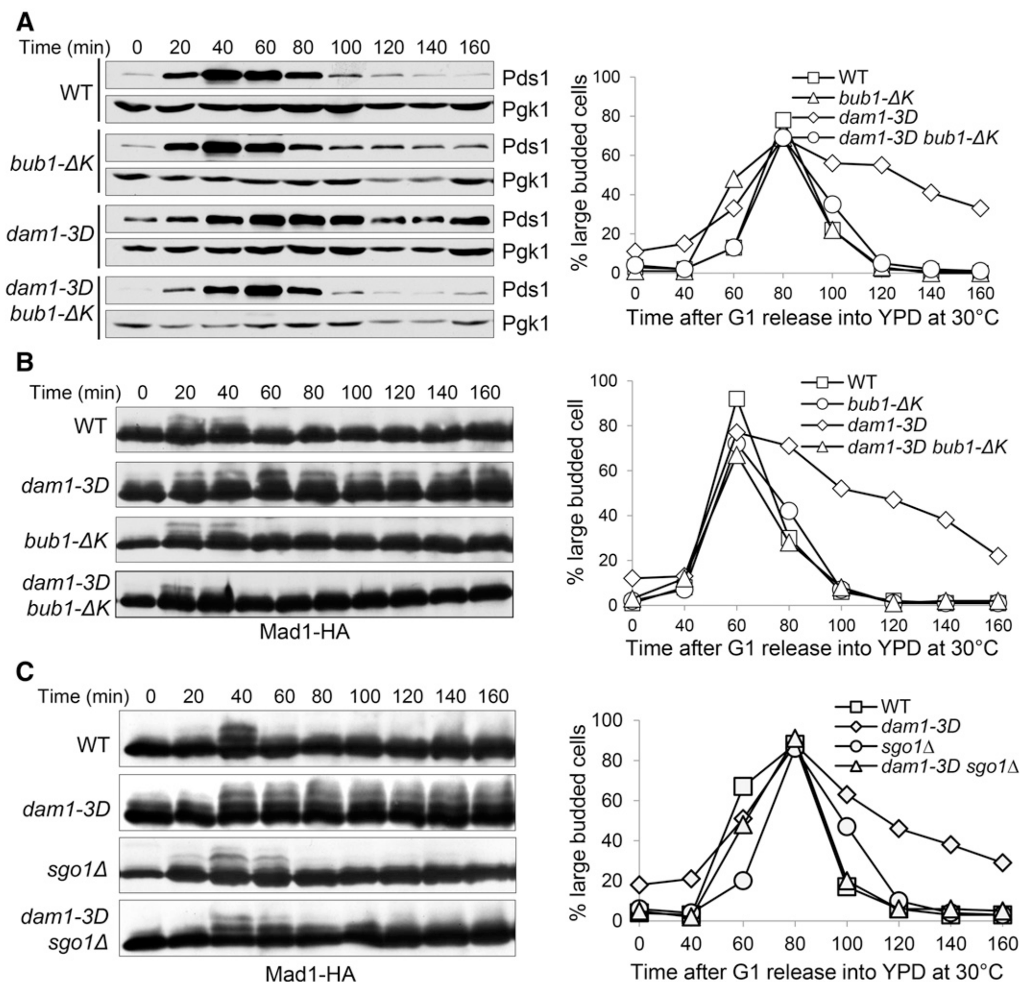

Figure 2 *bub1*-Δ*K* and *sgo1*Δ mutants suppress the anaphase entry delay in *dam1-3D* cells. (A) The delayed Pds1 degradation in *dam1-3D* cells is suppressed by *bub1*-Δ*K*. G_1-arrested WT and mutant cells with Pds1-18myc were released into YPD medium at 30°. α-factor was added after budding. Cell lysates were prepared at the indicated times for western blotting with anti-myc antibody. The budding index and Pds1 protein levels are shown. Pgk1, loading control. (B) The delay of Mad1 dephosphorylation in *dam1-3D* cells is suppressed by *bub1*-Δ*K*. G_1-arrested WT and mutant cells with Mad1-3HA were released into YPD medium at 30°. Cell lysates were prepared at the indicated time points for Western blotting with anti-HA antibody. The Mad1 modification is show in the left panel and the budding index is shown in the right panel. (C) The delay of Mad1 dephosphorylation in *dam1-3D* cells is suppressed by *sgo1*Δ. The cells were treated as described above. The Mad1 modification and the budding index are shown.

that overexpression of the coiled-coil domain of *CIK1* (*CIK1-CC*) disrupts Cik1-Kar3 interaction and causes chromosome syntelic attachment, *i.e.*, both sister kinetochores are attached by microtubules from the same spindle pole. We showed that Ipl1 kinase and Sgo1 are required to prevent SAC silencing in response to syntelic attachment induced by *CIK1-CC* overexpression (Jin *et al.* 2012; Jin and Wang 2013). Because the centromere localization of Sgo1 depends on H2A phosphorylation by Bub1 kinase (Fernius and Hardwick 2007; Kawashima *et al.* 2010; Nerusheva *et al.* 2014), Bub1 kinase activity might be required to prevent SAC silencing in response to syntelic attachment, although this kinase activity is dispensable for SAC activation (Fernius and Hardwick 2007).

To investigate the role of the Bub1 kinase domain in preventing SAC silencing, we first examined the sensitivity of yeast cells lacking this domain (*bub1*-Δ*K*) to *CIK1-CC* overexpression. In *bub1*-Δ*K* mutant, the C-terminal fraction of the *BUB1* gene that encodes 609–1021 amino acid residues was deleted to eliminate the kinase domain. We introduced P_{GAL} CIK1-CC plasmids, which contain *CIK1-CC* under the control of a galactose promotor (GAL), into *bub1*-Δ*K* cells. Similarly to *sgo1*Δ, *bub1*-Δ*K* cells grew a little slower than WT cells, but the mutant cells with P_{GAL}CIK1-CC plasmids failed to grow on galactose plates (Figure 1A). After *CIK1-CC* induction for 6 hr, 37% *bub1*-Δ*K* cells lost viability; compared to 15% viability loss in WT cells (Figure 1B). The expression of truncated Bub1 (Bub1-ΔK-13myc) was similar to that of Bub1-13myc (Figure 1C). Thus, the observed phenotype of *bub1*-Δ*K* mutant is unlikely to be attributed to decreased Bub1 expression.

The sensitivity of *bub1*-Δ*K* cells to *CIK1-CC* overexpression might be a result of chromosome missegregation. To test this idea, a control vector or P_{GAL}CIK1-CC plasmid was introduced into WT and *bub1*-Δ*K* mutants with the GFP-marked centromere of chromosome *IV* (CEN4-GFP) and mCherry-labeled spindle (Tub1-mCherry). The cells were arrested in G_1 phase in noninducible raffinose medium and then released into galactose medium to induce *CIK1-CC* overexpression. *CIK1-CC* overexpression caused a cell cycle delay in WT cells as indicated by the higher proportion of large-budded cells in later time points, but this delay was largely suppressed by *bub1*-Δ*K*. In anaphase cells, *CIK1-CC* overexpression resulted in 13% missegregation of chromosome *IV* in *bub1*-Δ*K* cells, as indicated by two *CEN4*-GFP dots in one daughter cell (Figure 1D). The cosegregation

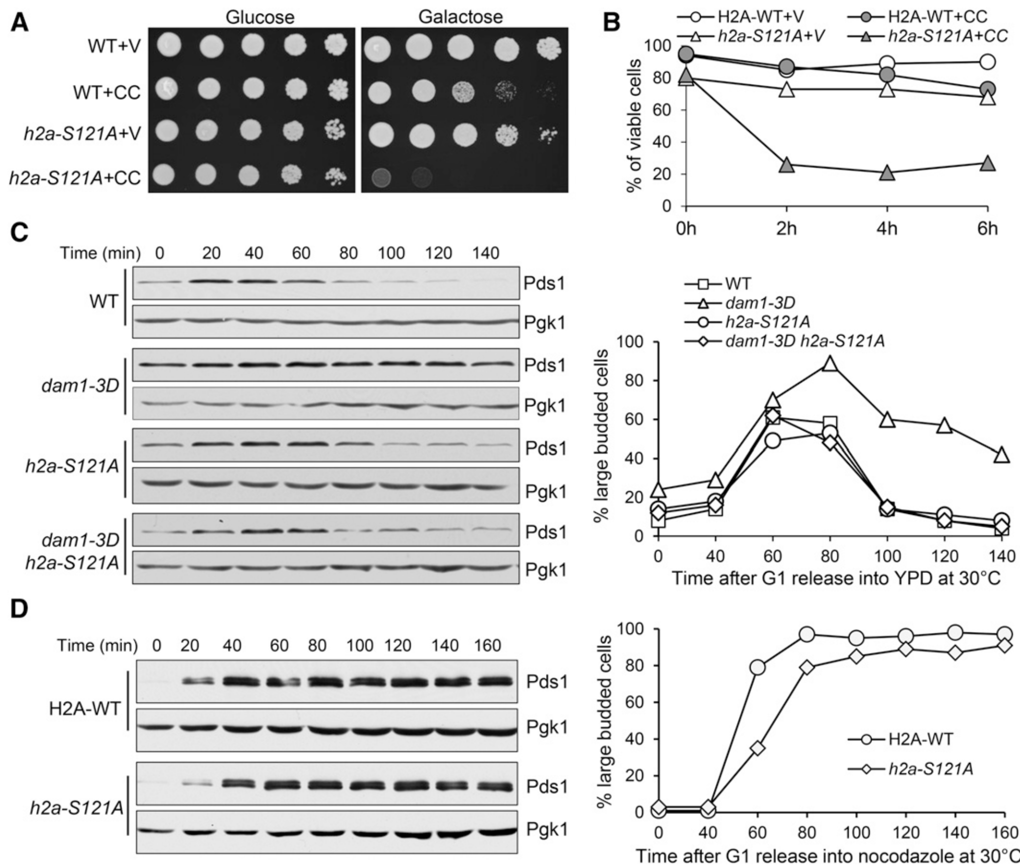

Figure 3 The abolishment of Bub1-dependent H2A phosphorylation leads to premature SAC silencing. (A) *h2a-S121A* mutant cells are sensitive to *CIK1-CC* overexpression. WT and *h2a-S121A* cells with a vector (V) or a P_{GAL} CIK1-CC (CC) plasmid were serial 10-fold diluted and then plated onto glucose and galactose plates for further incubation at 30° for 2 days. (B) *h2a-S121A* mutants lose viability after *CIK1-CC* overexpression. Cells with the indicated genotypes in raffinose medium were released into galactose medium at 30°. Cells were collected at 0, 2, 4, and 6 hr and spread onto YPD plates to examine the plating efficiency after overnight growth at 25° ($n \geq 200$). (C) *h2a-S121A* mutation abolishes the anaphase entry delay in *dam1-3D* mutants. Cells with the indicated genotypes were arrested in G_1 phase with α-factor and then released into cell cycle in YPD. Cells were collected over time to examine the Pds1 protein levels. Budding index and Pds1 protein levels are shown. Pgk1, loading control. (D) *h2a-S121A* mutant cells show efficient metaphase arrest in response to nocodazole treatment. G_1-arrested *PDS1-18myc* and *h2a-S121A PDS1-18myc* cells were released into YPD medium containing 20 μg/ml of nocodazole and incubated at 30°. Pds1 protein levels were determined after Western blotting. The Pds1 levels and budding index are shown. Pgk1, loading control.

of *CEN4*-GFP in *bub1*-Δ*K* cells indicates checkpoint arrest failure. Our previous study showed that overexpression of *CIK1-CC* in *sgo1*Δ cells caused >30% *CEN4*-GFP missegregation using the same analysis (Jin *et al.* 2012), which is higher than that of *bub1*-Δ*K* cells. One explanation is that Sgo1 is still functional enough in *bub1*-Δ*K* cells to prevent SAC silencing, but less efficient due to the failure of centromere localization.

To further demonstrate the premature SAC silencing in *bub1*-Δ*K* cells, we analyzed the degradation kinetics of the anaphase inhibitor Pds1 in cells overexpressing *CIK1-CC*, which marks anaphase entry. G_1-arrested *PDS1-18myc* and *bub1*-Δ*K PDS1-18myc* cells carrying either a vector or a P_{GAL}*CIK1-CC* plasmid were released into galactose medium. Overexpression of *CIK1-CC* induced moderate cell cycle delay in WT cells, as indicated by the budding index as well as by delayed Pds1 degradation. However, this delay largely disappeared in *bub1*-Δ*K* cells, indicating that this anaphase entry delay depends on the kinase domain of Bub1 (Figure 1E). Because previous research indicates that the kinase activity of Bub1 is dispensable for SAC activation, it is likely that this kinase domain is specifically required to prevent premature SAC silencing and maintain prolonged checkpoint arrest when tensionless attachment occurs.

The anaphase entry delay in dam1-3D mutants is alleviated by bub1-ΔK

The phosphorylation of kinetochore protein Dam1 by Ipl1 kinase destabilizes kinetochore-microtubule attachment to facilitate correction (Pinsky *et al.* 2006; Tien *et al.* 2010), but the delayed anaphase entry in phospho-mimetic mutant *dam1-3D* is primarily attributed to its inability to silence the SAC (Jin and Wang 2013). Thus, we tested if the anaphase entry delay in *dam1-3D* requires the kinase activity of Bub1. First, we compared the anaphase entry process in synchronized *dam1-3D* and *dam1-3D bub1*-Δ*K* cells by examining Pds1 protein levels. Consistent with our previous report, *dam1-3D* exhibited delayed Pds1 degradation and persistent appearance of large-budded cells, but these phenotypes were abolished in *dam1-3D bub1*-Δ*K* cells (Figure 2A). Therefore, the anaphase entry delay in *dam1-3D* cells depends on the kinase activity of Bub1. We noticed the presence of some large-budded cells and detectable Pds1 protein expression in *dam1-3D* mutant after α factor treatment, which is likely due to the difficulty in SAC silencing.

The SAC component Mad1 is phosphorylated in a SAC-dependent manner and its dephosphorylation indicates SAC silencing (Hardwick and Murray 1995; Mirchenko and Uhlmann 2010). *dam1-3D* cells exhibited persistent Mad1

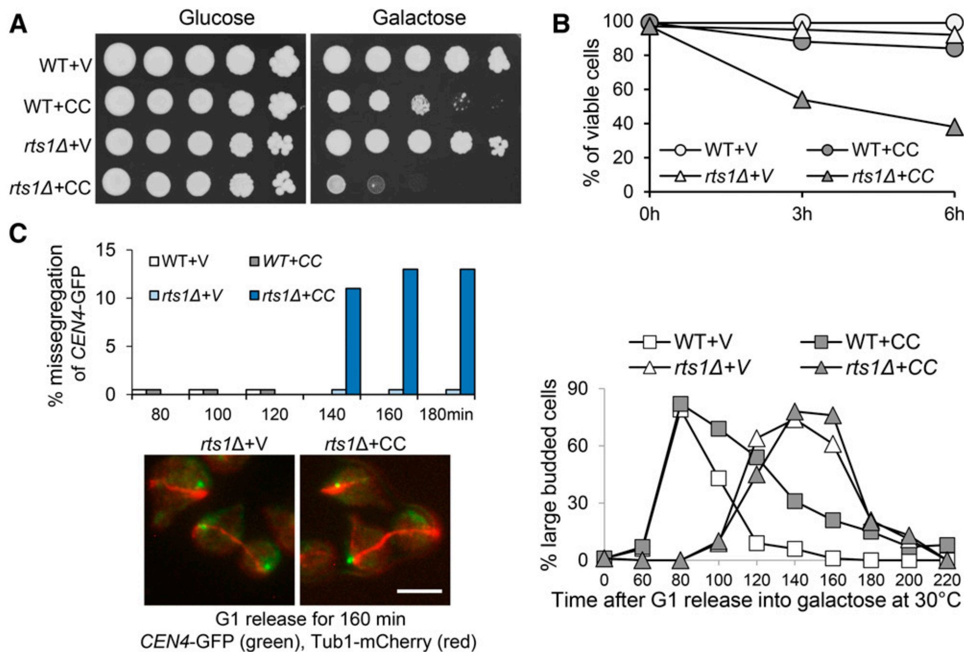

Figure 4 *rts1*Δ mutants are sensitive to *CIK1-CC* overexpression. (A) Overexpression of *CIK1-CC* is toxic to *rts1*Δ cells. WT and *rts1*Δ cells with a vector (V) or a P_{GAL}CIK1-CC (CC) plasmid were 10-fold serial diluted and spotted onto glucose and galactose plates for further incubation at 30° for 2 days. (B) *rts1*Δ cells lose viability after *CIK1-CC* overexpression. WT and *rts1*Δ cells with a vector or a P_{GAL}CIK1-CC plasmid were grown to log phase in raffinose medium at 30° and then galactose was added into the medium. Cells were collected at 0, 3, and 6 hr and spread onto YPD plates to assess the plating efficiency ($n \geq 200$). (C) *CIK1-CC* overexpression leads to chromosome missegregation in *rts1*Δ cells. *CEN4-GFP TUB1-mCherry* and *rts1*Δ *CEN4-GFP TUB1-mCherry* cells with a vector or a P_{GAL}CIK1-CC plasmid were arrested in G_1 phase in raffinose medium and then released into galactose medium at 30°. Cells were collected at indicated time points and fixed for the examination of fluorescence signals. The percentage of cells with missegregated sister *CEN4*-GFPs among all anaphase cells is shown on the top left ($n > 100$). The localization of CEN4-GFP as well as spindle morphology in some representative cells is shown at the bottom. The budding index is shown on the right. Bar, 5 μm.

phosphorylation, indicating compromised SAC silencing (Jin and Wang 2013). We compared Mad1 dephosphorylation kinetics in synchronized *dam1-3D* and *dam1-3D bub1-ΔK* cells. The delay of Mad1 dephosphorylation in *dam1-3D* was eliminated in the double mutant cells (Figure 2B). Similarly, *sgo1*Δ mutation also suppressed this delay (Figure 2C). Taken together, the premature SAC silencing in *bub1-ΔK* cells and the suppression of SAC silencing defect in *dam1-3D* mutants by *bub1-ΔK* support the conclusion that the Bub1 kinase activity is required to prevent premature SAC silencing.

The phosphorylation of H2A by Bub1 prevents SAC silencing

The phosphorylation of budding yeast H2A at serine 121 by Bub1 is essential for centromere localization of Sgo1, and phospho-deficient *h2a-S121A* mutant fails to recruit Sgo1 to centromeres (Kawashima *et al.* 2010; Nerusheva *et al.* 2014). Thus, we postulated that *h2a-S121A* mutants would show premature SAC silencing similar to *bub1-ΔK* and *sgo1*Δ mutants. To test the idea, we first examined the growth of *h2a-S121A* mutant cells overexpressing *CIK1-CC*. The growth defect of *h2a-S121A* cells with a control vector on galactose plates was noticeable but minor. The mutant cells with a P_{GAL}CIK1-CC plasmid, however, failed to grow on galactose plates (Figure 3A). Moreover, after 2 hr incubation in galactose medium, only 26% of *h2a-S121A* cells remained viable (Figure 3B). The viability loss is likely caused by chromosome missegregation, but the multiple selection markers in the *h2a-S121A* strain make it difficult to construct strains and examine chromosome segregation.

We have shown that *bub1-ΔK* and *sgo1*Δ mutations alleviate the anaphase entry delay in *dam1-3D* mutants (Figure 2). Therefore, we tested if this is also true for the *h2a-121A* mutant. G_1-synchronized *dam1-3D* and *dam1-3D h2a-S121A* cells were released into the cell cycle and the Pds1 protein levels were examined. It was clear that *h2a-S121A* suppressed the delayed Pds1 degradation in *dam1-3D* mutants (Figure 3C). Since this suppression could be caused by the failure of SAC activation, we further analyzed SAC function in *h2a-S121A* mutant cells. For this purpose, G_1-arrested WT and *h2a-S121A* mutant cells were released into a medium containing 20 μl/ml nocodazole, which activates the SAC to prevent Pds1 degradation. In both WT and *h2a-S121A* mutant, Pds1 protein levels were persistent in the presence of nocodazole, indicating that the SAC is competent in *h2a-S121A* mutant cells (Figure 3D). Therefore, our data support the conclusion that Bub1-dependent phosphorylation of H2A at S121 in budding yeast is required to prevent premature SAC silencing in the absence of kinetochore tension. This phosphorylation regulates SAC silencing likely through centromere recruitment of Sgo1.

Rts1 prevents SAC silencing in the presence of syntelic attachment

Rts1 and Cdc55 are the two PP2A B-regulatory subunits (Shu *et al.* 1997). Centromere-localized Sgo1 recruits $PP2A^{Rts1}$, condensin, and Ipl1 kinase complex to the centromere region (Riedel *et al.* 2006; Peplowska *et al.* 2014; Verzijlbergen *et al.* 2014). Although we found that Ipl1 is required to prevent premature SAC silencing in response to tension defects (Jin and Wang 2013), previous work shows that the centromere localization of Ipl1 is dispensable for this function (Campbell and Desai 2013). One possibility is that Sgo1's function in SAC silencing depends on its role in centromere recruitment

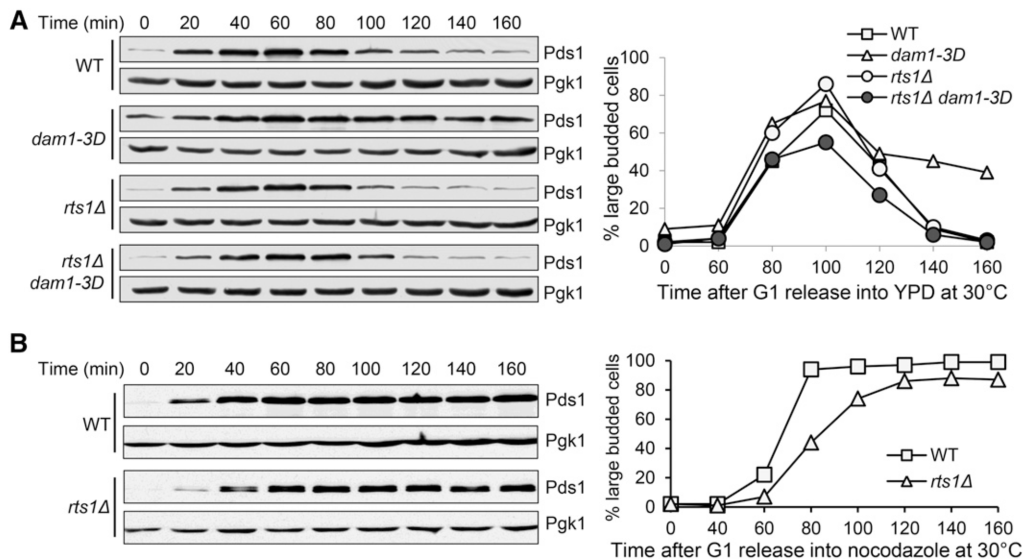

Figure 5 *rts1*Δ mutation suppresses the anaphase entry delay in *dam1-3D* cells without compromising SAC activation. (A) The delay of Pds1 degradation in *dam1-3D* cells is suppressed by *rts1*Δ. G_1-arrested WT and mutant cells with Pds1-18myc were released into YPD medium at 30°. α-factor was added back after budding. Cell lysates were prepared at the indicated time points for Western blotting with anti-myc antibody. The Pds1 levels and budding index are shown. Pgk1, loading control. (B) *rts1*Δ cells show intact SAC function. G_1-arrested *PDS1-18myc* and *rts1*Δ *PDS1-18myc* cells were released into YPD medium containing 20 μg/ml of nocodazole and incubated at 30°. Cells were collected every 20 min for the budding index and the determination of Pds1 protein levels. Pgk1, loading control. The Pds1 levels and the budding index are shown.

of PP2A^{Rts1}, but not Ipl1 kinase. Thus, we first examined the sensitivity of *rts1*Δ mutants to *CIK1-CC* overexpression. *rts1*Δ cells harboring P_{GAL}CIK1-CC plasmids grew very poorly on galactose plates (Figure 4A), which is consistent with a previous observation (Peplowska *et al.* 2014). Moreover, after 6-hr incubation in galactose medium, 62% of *rts1*Δ cells with P_{GAL}CIK1-CC plasmids were inviable, whereas only 8% of *rts1*Δ cells with a vector lost viability. The viability loss for WT cells with P_{GAL}CIK1-CC plasmids was much less significant (16%) (Figure 4B). We further assessed *CEN4*-GFP separation in *rts1*Δ cells overexpressing *CIK1-CC*. G_1-arrested cells were released into galactose medium and CEN4-GFP segregation was examined in cells with an elongated spindle. More than 10% of *rts1*Δ cells overexpressing *CIK1-CC* showed *CEN4*-GFP cosegregation at 140, 160, and 180 min; but no missegregation was observed in WT cells (Figure 4C). We noticed delayed cell cycle progression of *rts1*Δ cells in the synthetic galactose medium, which is consistent with a previous observation that loss of Rts1 causes delayed initiation of bud growth (Artiles *et al.* 2009). These results indicate that *rts1*Δ mutants are sensitive to the induction of syntelic attachment due to chromosome missegregation. However, the rate of missegregation is less significant compared to *sgo1*Δ mutant cells, indicating the presence of redundant pathways.

Next we asked if *rts1*Δ mutation suppresses the failure of SAC silencing in *dam1-3D* cells as *sgo1*Δ and *bub1-ΔK* do. *dam1-3D* and *dam1-3D rts1*Δ cells synchronized in G_1 were released into the cell cycle and Pds1 levels were monitored over time. The stabilized Pds1 protein level and cell cycle delay in *dam1-3D* cells were largely abolished by *RTS1* deletion (Figure 5A). To test if the suppression is due to defective SAC function in *rts1*Δ cells, we also examined the cell cycle arrest in *rts1*Δ cells treated with nocodazole, which activates the SAC. As a result, both WT and *rts1*Δ cells exhibited stabilized Pds1 protein levels in the presence of nocodazole, indicating a competent SAC in *rts1*Δ cells (Figure 5B). These results indicate that Rts1 is also required to prevent anaphase entry in the presence of tension defects, but Rts1 is dispensable for SAC activation in response to spindle damage.

The interaction of Sgo1 with either H2A or PP2A is required to prevent premature SAC silencing

Our observations support the possibility that the recruitment of Sgo1-PP2A^{Rts1} to the centromere region through Sgo1-H2A interaction prevents premature SAC silencing. To test this possibility, we examined the SAC silencing process in cells with disrupted association of Sgo1 with either H2A or PP2A. The Sgo1-H2A interaction depends on a conserved basic motif KMRR in Sgo1 (Kitajima *et al.* 2004). Thus, we mutated this motif to four alanine residues, generating a *sgo1-4A* plasmid. Previous works define the PP2A-binding domain in Sgo1 as well, and *sgo1-N51I* mutation abolishes this interaction (Xu *et al.* 2009; Peplowska *et al.* 2014). We also generated a *sgo1-N51I* plasmid. To construct *sgo1-4A* and *sgo1-N51I* yeast mutant strains, we inserted the integrating plasmids containing *SGO1*, *sgo1-4A*, and *sgo1-N51I* genes into the endogenous locus in a *sgo1*Δ strain. To compare the sensitivity of these *sgo1* mutants to *CIK1-CC* overexpression with *rts1* and *bub1* mutants, we performed the 10-fold dilution of all the mutants on the same plates. *sgo1-N51I* and *sgo1-4A* mutant cells harboring a control vector grew well on galactose plates, but the mutant cells with P_{GAL}CIK1-CC plasmids failed to grow on galactose plates. Their sensitivity to *CIK1-CC* overexpression is similar to *sgo1*Δ and *bub1-ΔK* cells, but *rts1*Δ cells are slightly less sensitive (Figure 6A). After *CIK1-CC* induction for 6 hr in galactose medium, 88% of cells with *SGO1* were viable, but the viability of *sgo1-4A* and *sgo1-N51I* mutants reduced to

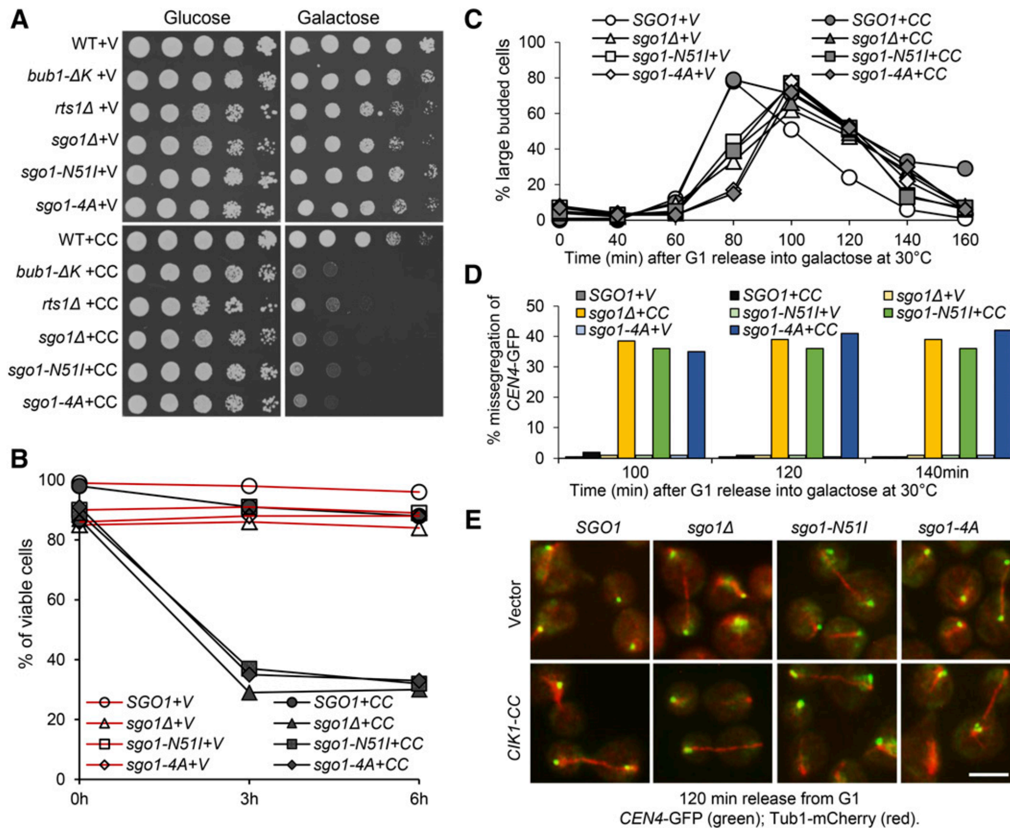

Figure 6 The binding of Sgo1 to H2A and PP2A is essential to prevent premature SAC silencing. (A) Overexpression of *CIK1-CC* is toxic to *sgo1* mutants with abolished binding to either H2A or PP2A. Saturated cells with a vector (V) or a P_{GAL}CIK1-CC plasmid (CC) were 10-fold serial diluted and spotted onto glucose and galactose plates, which were incubated at 30° for 2 days. (B) The viability loss of *SGO1*, *sgo1*Δ, *sgo1-N51I*, and *sgo1-4A* cells after *CIK1-CC* overexpression. Log-phase cells in raffinose were released into galactose medium at 30°. Cells were collected at 0, 3, and 6 hr and spread onto YPD plates to determine the plating efficiency ($n \geq 200$). (C–E) *CIK1-CC* overexpression causes chromosome missegregation in *sgo1* mutants. A vector or a P_{GAL}CIK1-CC plasmid was introduced into WT, *sgo1*Δ, *sgo1-N51I*, and *sgo1-4A* mutants with *CEN4-GFP TUB1-mCherry*. G_1-arrested cells were released into galactose medium at 30°. α-factor was restored after budding. Cells were collected at the indicated time points for the examination of fluorescence signals. (C) The budding index . (D) The percentage of anaphase cells that show cosegregated *CEN4*-GFP. (E) The distribution of *CEN4*-GFP and spindle morphology in some representative cells. Bar, 5 μm.

32 and 33%, respectively, which is comparable to the viability of *sgo1*Δ cells (30%) (Figure 6B).

To investigate whether the viability loss of these *sgo1* mutants resulted from chromosome missegregation, a vector and P_{GAL}CIK1-CC plasmid were introduced into *SGO1*, *sgo1*Δ, *sgo1-N51I*, and *sgo1-4A* cells with *CEN4*-GFP and Tub1-mCherry. G_1-arrested cells were released into galactose medium. *CIK1-CC* overexpression caused a cell cycle delay in cells with WT *SGO1*, but failed to do so in *sgo1*Δ, *sgo1-4A*, and *sgo1-N51I* mutant cells (Figure 6C). After G_1 release for 100 min, 36% of *sgo1-N51I* and 35% of *sgo1-4A* cells with an anaphase spindle showed *CEN4*-GFP cosegregation, which is similar to *sgo1*Δ cells (39%). The same is true for cells at 120 and 140 min. In clear contrast, no *CEN4*-GFP cosegregation was observed in cells with WT *SGO1* during *CIK1-CC* overexpression (Figure 6, D and E). These results suggest that abrogation of the interaction of Sgo1 with H2A or PP2A completely abolishes Sgo1's function in preventing premature SAC silencing.

Discussion and Conclusion

Using budding yeast as a model organism, recent studies indicate that centromere-associated Sgo1 protein is required to prevent SAC silencing until tension is generated at kinetochores by bipolar attachment (Keating *et al.* 2009; Jin and Wang 2013; Wang *et al.* 2014). Here we show that components acting up- and downstream of Sgo1 are also required to prevent premature SAC silencing. We first found that the kinase domain of Bub1 is essential to maintain SAC activation in cells lacking tension. Yeast Bub1 phosphorylates H2A at S121 to trigger the recruitment of Sgo1 to centromeres (Kawashima *et al.* 2010). We further demonstrated that mutation of the Bub1 phosphorylation site in H2A (*h2a-S121A*) or the H2A binding site in *SGO1* (*sgo1-4A*) resulted in SAC silencing in the presence of tensionless syntelic attachment. Therefore, Bub1-dependent centromere binding of Sgo1 is essential to prevent premature SAC silencing. In addition, we found that abolishment of Sgo1-PP2A binding in *sgo1-N51I* mutants as well as the deletion of *RTS1*, which encodes one of the PP2A regulatory subunits, also leads to premature SAC silencing. These results support the conclusion that Bub1-dependent centromere recruitment of Sgo1-PP2A^{Rts1} through H2A prevents premature SAC silencing. Therefore, our results uncover the role of the Bub1-H2A-Sgo1-PP2A^{Rts1} axis in the maintenance of a prolonged checkpoint arrest in response to tensionless chromosome attachment.

*sgo1*Δ cells display more significant viability loss than *bub1-ΔK* after the induction of syntelic attachment by *CIK1-CC*. However, mutation of the Bub1 phosphorylation site in H2A (*h2a-S121A*) causes comparable viability loss as *sgo1*Δ after *CIK1-CC* overexpression. One possibility is that the

h2a-S121A mutation, but not the deletion of the kinase domain of *BUB1*, abolishes Sgo1 centromere localization completely. Although we are unable to analyze chromosome missegregation in *h2A-S121A* strains due to multiple selection markers, we expect a high rate of missegregation. In addition, the *sgo1-4A* mutants with abolished Sgo1-H2A interaction exhibited a similar frequency of chromosome missegregation as *sgo1*Δ cells after *CIK1-CC* overexpression. These observations support the conclusion that the association of Sgo1 with H2A is essential for its function in preventing SAC silencing.

An important question is how Sgo1 regulates SAC silencing. Previous studies indicate that Sgo1 recruits Ipl1/Aurora B kinase complex to centromeres (Yamagishi *et al.* 2010; Peplowska *et al.* 2014). Because Ipl1 kinase prevents SAC silencing in response to tension defects (Biggins and Murray 2001; Jin *et al.* 2012; Jin and Wang 2013), one speculation is that Sgo1 prevents SAC silencing through Ipl1 kinase. However, the observation that the tension sensing by Ipl1 is independent of the centromere localization of Ipl1 complex argues against this possibility (Campbell and Desai 2013). Consistently, overexpression of the Ipl1 cofactor *SLI15* restores centromere Ipl1 localization in *sgo1*Δ mutants, but this fails to suppress *sgo1*Δ's sensitivity to *CIK1-CC* overexpression (Peplowska *et al.* 2014). Here, we further showed that *sgo1*Δ mutation alleviated the anaphase entry delay in the phospho-mimetic mutant *dam1-3D*. Because Dam1 is a well-defined substrate of Ipl1 kinase (Cheeseman *et al.* 2002), Sgo1 likely acts downstream of Ipl1.

In addition to Ipl1, Sgo1 also recruits PP2A and condensin to centromeres (Nerusheva *et al.* 2014; Peplowska *et al.* 2014), and the presence of $PP2A^{Rts1}$ at centromeres prevents cohesin cleavage by separase (Riedel *et al.* 2006). It is unlikely that centromere cohesion prevents SAC silencing, because cohesin mutants show delayed SAC silencing (Biggins and Murray 2001; Jin and Wang 2013). Since some condensin mutants are sensitive to *CIK1-CC* overexpression (Peplowska *et al.* 2014), another untested possibility is that Sgo1 regulates SAC silencing through condensin; but recent work supports the possibility that the condensin facilitates chromosome biorientation (Verzijlbergen *et al.* 2014). Our results support the notion that Sgo1 recruits PP2A to centromeres to prevent premature SAC silencing. First, mutation of the PP2A-binding motif in Sgo1 causes premature SAC silencing. Moreover, the absence of the PP2A regulatory subunit Rts1 also leads to premature SAC silencing. It is likely that $PP2A^{Rts1}$ dephosphorylates kinetochore proteins to prevent SAC silencing, and it will be our future interest to identify these PP2A substrates.

sgo1-N51I mutants show abolished interaction with PP2A (Xu *et al.* 2009). Of interest, we noticed that *sgo1-N51I* mutants exhibited more pronounced viability loss and chromosome missegregation than *rts1*Δ mutants after *CIK1-CC* overexpression. Because PP2A has two regulatory subunits, Rts1 and Cdc55, our explanation is that $PP2A^{Cdc55}$ and $PP2A^{Rts1}$ may show redundant function, which is supported by the demonstrated interaction of Sgo1 with both Rts1 and Cdc55 (Verzijlbergen *et al.* 2014). The *sgo1-N51I* mutation may abolish its interaction with both of them, thereby showing more pronounced phenotypes. Indeed, we found that the *cdc55* mutant also exhibits chromosome missegregation after *CIK1-CC* overexpression (Bokros *et al.* 2016), but further experiments are needed to test if this phenotype in *cdc55* mutant is independent of its role in mitotic exit (Wang and Ng 2006).

Recent data in mammalian cells indicate the spatial regulation of Sgo1 during mitosis. Cohesin and phosphorylated H2A specify two distinct pools of Sgo1-PP2A at inner centromere and kinetochores, respectively. The tension at kinetochores triggers the redistribution of Sgo1 from inner centromeres to kinetochores (Liu *et al.* 2013). It is unclear if yeast cells have a spatial Sgo1 regulation during mitosis. Previous data indicate that Chl4 and Iml3, two inner kinetochore proteins, are essential for the association of Sgo1 with the pericentric region in meiosis I (Kiburz *et al.* 2005), and a recent study shows the interaction between Sgo1 and these kinetochore proteins (Hinshaw and Harrison 2013). Thus, yeast Sgo1 could bind to both H2A and kinetochore proteins, but further experiments are needed to test this possibility.

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