

Sgt1 Dimerization Is Negatively Regulated by Protein Kinase CK2-mediated Phosphorylation at Ser³⁶¹*[§]

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The kinetochore, which consists of centromere DNA and structural proteins, is essential for proper chromosome segregation in eukaryotes. In budding yeast, Sgt1 and Hsp90 are required for the binding of Skp1 to Ctf13 (a component of the core kinetochore complex CBF3) and therefore for the assembly of CBF3. We have previously shown that Sgt1 dimerization is important for this kinetochore assembly mechanism. In this study, we report that protein kinase CK2 phosphorylates Ser³⁶¹ on Sgt1, and this phosphorylation inhibits Sgt1 dimerization.

The kinetochore is a structural protein complex located in the centromeric region of the chromosome coupled to spindle microtubules (1, 2). The kinetochore generates a signal to arrest cells during mitosis when it is not properly attached to microtubules, thereby preventing chromosome missegregation, which can lead to aneuploidy (3, 4). The molecular structure of the kinetochore complex of the budding yeast *Saccharomyces cerevisiae* has been well characterized; it is composed of more than 70 proteins, many of which are conserved in mammals (2).

The centromere DNA in the budding yeast is a 125-bp region that contains three conserved regions, CDEI, CDEII, and CDEIII (5, 6). CDEIII (25 bp) is essential for centromere function (7) and is bound to a key component of the centromere, the CBF3 complex. The CBF3 complex contains four proteins, Ndc10, Cep3, Ctf13 (8–15), and Skp1 (14, 15), all essential for viability. Mutations in any of the CBF3 proteins abolish the ability of CDEIII to bind to CBF3 (16, 17). All of the kinetochore proteins, except the CDEI-binding Cbf1 (18–20), localize to the kinetochores in a CBF3-dependent manner (2). Thus, CBF3 is a fundamental kinetochore complex, and its mechanism of assembly is of great interest.

We have previously found that Sgt1 and Skp1 activate Ctf13; thus, they are required for assembly of the CBF3 complex (21). The molecular chaperone Hsp90 is also required to form the active Ctf13-Skp1 complex (22). Sgt1 has two

highly conserved motifs that are required for protein-protein interaction: the tetratricopeptide repeat (21) and the CHORD protein and Sgt1-specific motif. We and others have found that both domains are important for the interaction of Sgt1 with Hsp90 (23–26), which is required for assembly of the core kinetochore complex. This interaction is an initial step in kinetochore activation (24, 26, 27), which is conserved between yeast and humans (28, 29).

We have recently shown that Sgt1 dimerization is important for Sgt1-Skp1 binding and therefore for kinetochore assembly (30). In this study, we have found that protein kinase CK2 phosphorylates Sgt1 at Ser³⁶¹, and this phosphorylation inhibits Sgt1 dimerization. Therefore, CK2 appears to regulate kinetochore assembly negatively in budding yeast.

EXPERIMENTAL PROCEDURES

Yeast Strains and Medium—Table 1 lists the genotypes of yeast strains used in this study. The medium for yeast growth and sporulation was prepared using previously described methods (31). Yeast transformation was done according to the method of Ito *et al.* (32). Strains that expressed tagged proteins were generated according to the procedure of Longtine *et al.* (33). Regions that encoded Myc tags were inserted at the 3'-end of the endogenous locus.

Plasmid Construction and Primers—Table 2 lists the plasmids used in this study. Details about their construction (34) and primer sequences are available upon request.

Antibodies—Anti-Skp1, anti-Sgt1, and anti-Hsp82 antibodies were used as previously described (21, 24, 35). Anti-hemagglutinin (HA²; Roche Applied Science), anti-Myc (Roche Applied Science), anti-GST (Abcam), and anti-His₆ (Qiagen) antibodies were purchased.

Protein Expression and Immunoprecipitation—Immunoprecipitation using yeast lysates was performed as described previously (24). His₆-Sgt1 and GST-Sgt1 proteins were expressed and purified according to the manufacturer's instructions, as previously described (24).

Two-dimensional Gel Electrophoresis—Myc-tagged Sgt1 was immunoprecipitated from yeast cell lysates using an anti-Myc antibody. Isoelectric focusing was performed with a 17-cm immobilized pH 3–10 gradient strips (Bio-Rad), following the manufacturer's instructions. Gel electrophoresis was performed in a Bio-Rad PROTEAN Plus Dodeca cell.

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² The abbreviations used are: HA, hemagglutinin; GST, glutathione S-transferase.

TABLE 1
Yeast strains used in this study

Strain	Genotype	Reference ^a
YPH499	<i>Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1</i>	49
Y1681	<i>Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS414-3myc-SGT1 pRS416-3HA-SGT1</i>	
Y1682	<i>Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS414-3myc-sgt1-S361A pRS416-3HA-sgt1-S361A</i>	
Y1684	<i>Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS414-3myc-sgt1-S361D pRS416-3HA-sgt1-S361D</i>	
Y1686	<i>Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 sgt1-S361A:LEU2 CFIII(CEN3.L.YPH983) TRP1 SUP11</i>	
Y1687	<i>Matα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 sgt1-S361D:LEU2 pRS416-SGT1 URA/CEN</i>	
Y1734	<i>Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 CKA1-myc</i>	
Y1736	<i>Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 cka1Δ::His3MX6 PRS414-3HA-Sgt1 pRS415-3myc-Sgt1</i>	
Y26	<i>Matα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 SGT1:LEU2 CFIII(CEN3.L.YPH983) TRP1 SUP11</i>	
CDY26	<i>MATaANB1-UB-R-lacI-4HA-SGT1::kan pACE1-LIBR1 pACE1-ROX1 trp1-Δ1 ura3-Δ52 leu2::PET56 ade2-101</i>	25
YKK54	<i>Matα ura3-52 trp1Δ63 his3Δ200 leu2Δ1 lys2-801 ade2-101 sgt1-3:LEU2 CFIII(CEN3.L.YPH983) TRP1 SUP11</i>	21
Y1773	<i>Matα ura3-52 trp1Δ63 his3Δ200 leu2Δ1 lys2-801 ade2-101 cka1Δ::His3MX6 CFIII(CEN3.L.YPH983) TRP1 SUP11</i>	
Y1775	<i>Mata ura3-52 trp1Δ63 his3Δ200 leu2Δ1 lys2-801 ade2-101 cka1Δ::His3MX6 sgt1-3:LEU2</i>	
Y1870	<i>Matα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 sgt1Δ::HIS3-MX6 pRS415-3myc-Sgt1</i>	
Y1871	<i>Mat α ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 sgt1Δ::HIS3-MX6 pRS415-3myc-sgt1-S361A</i>	
Y1872	<i>Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS415-3myc-Sgt1</i>	
Y1873	<i>Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 cka1Δ::His3MX6 pRS415-3myc-Sgt1</i>	
Y1874	<i>Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 cka1Δ::His3MX6 pRS416-3HA-sgt1-S361A, pRS414-3myc-sgt1-S361A</i>	

^a Strains for which no reference is given were generated during this study.**TABLE 2**
Plasmids used in this study

Plasmid	Relevant characteristics	Reference ^a
B1354	<i>pRS416-3HA-SGT1 URA CEN</i>	30
B180	<i>pRS414-3HA-SGT1 TRP CEN</i>	30
B1367	<i>pRS415-3myc-SGT1 LEU CEN</i>	30
B940	<i>pDEST17-HIS-SGT1</i>	30
B942	<i>pDEST15-GST-SGT1</i>	
B1657	<i>pDEST15-GST-sgt1-S361A</i>	
B824	<i>pDEST15-GST-hSGT1</i>	
BPB452	<i>pDEST15-GST-hsgt1A-S299A</i>	
B191	<i>pRS414-3myc-SGT1 TRP CEN</i>	30
B1374	<i>pRS414-3myc-sgt1-S361A TRP CEN</i>	
B1375	<i>pRS414-3myc-sgt1-S361D TRP CEN</i>	
B1377	<i>pRS416-3HA-sgt1-S361A URA CEN</i>	
B1378	<i>pRS416-3HA-sgt1-S361D URA CEN</i>	
B1699	<i>pRS415-3myc-sgt1-S361A LEU CEN</i>	

^a Plasmids for which no reference is given were generated during this study.

After two-dimensional gel electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane, and immunoblotting was performed using anti-Myc antibodies.

CK2 Phosphorylation Assay—Phosphorylation of recombinant GST-Sgt1 or His-Sgt1 by human protein kinase CK2 was performed in a reaction mixture containing 100 ng to 1 μg of GST-Sgt1 or His-Sgt1 protein and 2 μCi of [γ -³²P]ATP in 30 μl of reaction buffer (20 mM HEPES (pH 7.5), 50 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, 1 mM CaCl₂, and 0.2 mM ATP). The reactions were started by adding 250 units of CK2 (New England Biolabs) and incubated for 30 min at 30 °C. Reactions were stopped by adding 10 μl of 4× SDS buffer. Reaction samples were separated on a 4–15% SDS-polyacrylamide gel and stained with SYPRO Ruby protein stain (Molecular Probes). After images were taken, the gels were dried and autoradiographed. Phosphorylation signals were quantified by a PhosphorImager.

RESULTS

Phosphorylation of Ser³⁶¹ on Sgt1—Matrix-assisted laser desorption time-of-flight mass spectrometry revealed that an oligopeptide, ³⁴⁸AGADPDKRAMMKSE³⁶², is phosphorylated *in vivo* (Fig. S1). Serine 361 is within the consensus site that is phosphorylated by CK2 (36), and this residue is conserved in humans (see Fig. 2B). Therefore, we tested by two-dimensional gel electrophoresis whether Ser³⁶¹ is phosphorylated *in vivo*.

Immunoprecipitated Sgt1-Myc appeared as two major spots on the two-dimensional gel (Fig. 1A, *top left*), and phosphatase treatment substantially diminished the low pH spot (Fig. 1A, *top right*), indicating that the low pH spot corresponds to phosphorylated forms. An unphosphorylated mutant sgt1-S361A protein showed a reduced low pH spot, indicating that Ser³⁶¹ is phosphorylated *in vivo* (Fig. 1A, *bottom left*). Phosphatase treatment reduced the low pH spot of sgt1-S361A protein, suggesting that there are other phosphorylation sites.

We also found that GST-Sgt1 and His₆-Sgt1 were phosphorylated by human CK2 *in vitro* (Fig. 1B and Figs. S2 and S3). Sgt1 was phosphorylated with 0.6 mol of phosphate/mol of substrate (Fig. 1C). The K_m and V_{max} of CK2 for the phosphorylation of Sgt1 were 288 μM and 4.8 μmol/min/mg (Fig. 1D), respectively, which are within the same range as that for known CK2 substrates (37–39), indicating that Sgt1 is a suitable substrate for CK2 *in vitro*.

CK2 did not efficiently phosphorylate the sgt1-S361A mutant protein (Fig. 2A), indicating that the S361A on Sgt1 is a phosphorylation site for CK2 *in vitro*.

Serine 361 is within the consensus site that is phosphorylated by CK2 (Fig. 2B), and this residue is conserved in humans and corresponds to serine 299 in human SGT1A (Fig. 2B). Nowotny *et al.* (40) reported that an oligopeptide containing this serine residue, human SGT1A-(263–333), is phosphorylated by CK2 *in vitro*. A search at PhosphoBase (41) revealed that in human SGT1A-(263–333), residue Ser²⁹⁹ can be phosphorylated by CK2. Human SGT1A-(263–333) oligopeptide containing the E302K mutation was also efficiently phosphorylated, suggesting that the CK2 phosphorylation site might be different from that identified by computer prediction. However, whether Ser²⁹⁹ is a CK2 phosphorylation site has not been directly tested. Therefore, we made the human SGT1A-S299A protein and found that CK2 did not phosphorylate the mutant protein as efficiently as the wild-type protein (Fig. 2C). This finding strongly suggests that Ser²⁹⁹ is a CK2 phosphorylation site *in vitro*.

Inhibition of Sgt1 Dimerization by Phosphorylation of Ser³⁶¹—To examine the possible effect of phosphorylation of Ser³⁶¹, we

CK2 Inhibits Sgt1 Dimerization

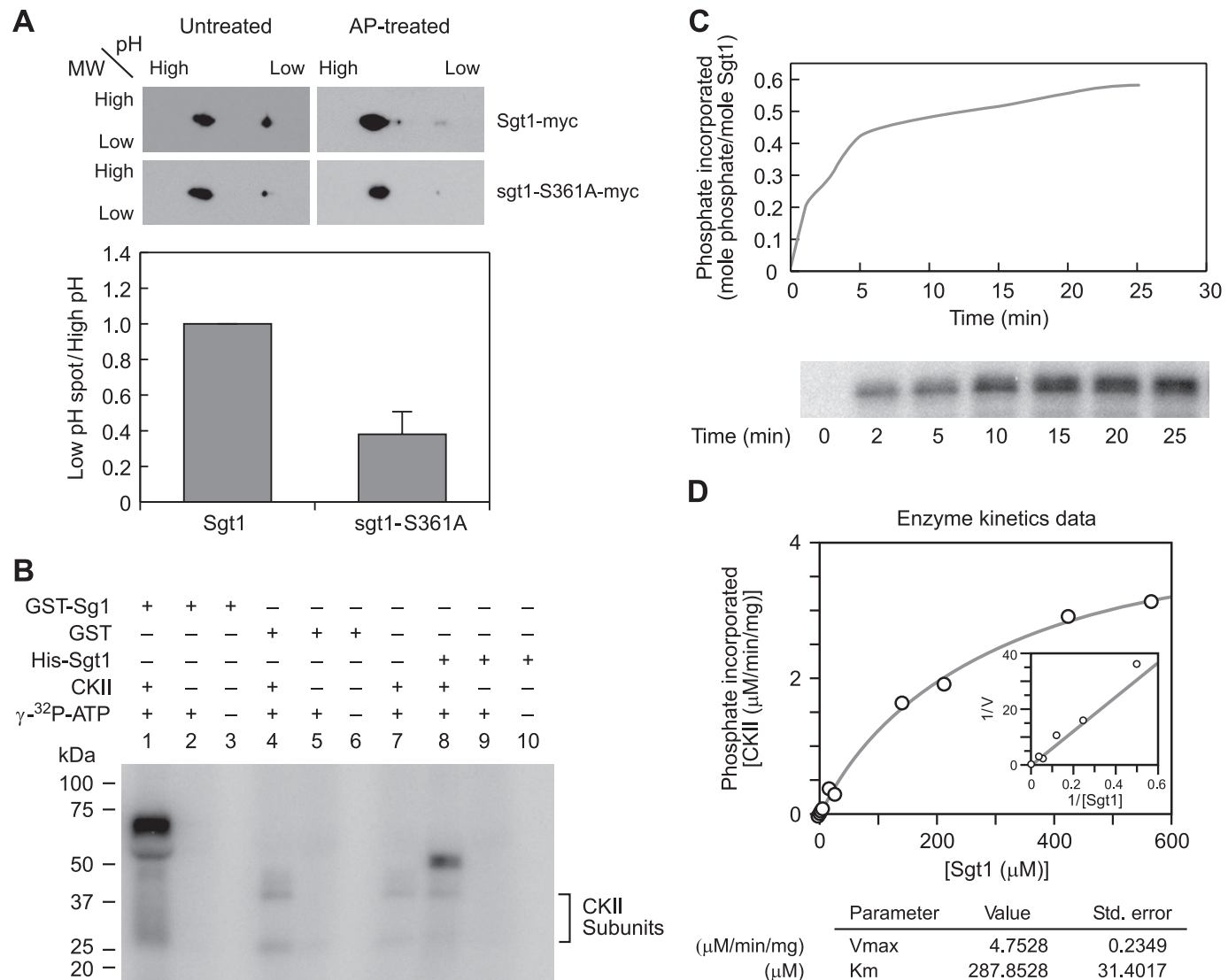


FIGURE 1. Sgt1 is phosphorylated at serine 361. *A* (top), Sgt1 is phosphorylated at Ser³⁶¹ *in vivo*. The 3Myc-Sgt1 (Y1870) or 3Myc-sgt1-S361A (Y1871) was immunoprecipitated from cycling cells, and immunoprecipitates were treated with alkaline phosphatase (AP-treated). Immunoprecipitates were analyzed by two-dimensional gel electrophoresis. An anti-Myc antibody was used to detect Sgt1-Myc on the immunoblots. (bottom) The signal intensity was quantified, and the ratio of phosphorylated Sgt1 (toward low pH) to unphosphorylated Sgt1 (toward high pH) in untreated immunoprecipitates is shown (gray bars). *B*, recombinant human CK2 phosphorylates Sgt1 *in vitro*. Kinase activity was determined by assaying phosphorylation levels of GST-Sgt1 (lanes 1–3), GST only (lanes 4–6), or His-Sgt1 (lanes 8–10), incubated with [γ -³²P]ATP, as described under “Experimental Procedures.” The arrowhead indicates autophosphorylated CK2. *C*, CK2-mediated phosphorylation of Sgt1. His-Sgt1 (1.6 pmol) was mixed in the reaction buffer with CK2 (710 pmol) and incubated with 0.5 μ Ci of [γ -³²P]ATP for the indicated time periods at 30 °C. The protein was then analyzed by SDS-PAGE. Phosphorylated Sgt1 bands were quantified with a STORM 860 PhosphorImager and ImageQuant software (Amersham Biosciences), and data were converted into pmol units of phosphate relative to that of the control [γ -³²P]ATP. The level of Sgt1 phosphorylation was calculated by dividing the pmol units of phosphate by the pmol units of Sgt1 used in the reaction. *Bottom*, the phosphor image of phosphorylated Sgt1. *D*, kinetics of Sgt1 phosphorylation by CK2. Reactions were performed in 15 μ l of solution containing 20 mM HEPES (pH 7.5), 50 mM NaCl, 10 mM MgCl₂, 0.2 mM ATP, 0.5 μ Ci of [γ -³²P]ATP, 1 mM dithiothreitol, 1 mM CaCl₂, 35 units of CK2, and varying concentrations of recombinant His-Sgt1 at 30 °C. Reactions were stopped after 10 min with SDS buffer, and proteins were resolved by SDS-PAGE. Phosphorylated Sgt1 was quantified using a STORM 860 PhosphorImager and ImageQuant software (Amersham Biosciences). *Bottom*, the kinetic properties V_{max} and K_m of Sgt1 phosphorylation were determined from Michaelis-Menten plots using GraFit (Erithacus Software Ltd.).

generated a nonphosphorylated mutant (S361A) strain and a phosphorylation-mimic mutant (S361D) strain. The S361A mutant protein bound to itself and Skp1 *in vivo* (Fig. 3A). However, the S361D protein did not bind to either itself or Skp1 efficiently, suggesting that Ser³⁶¹-phosphorylated Sgt1 cannot form dimers effectively. Interestingly, the *sgt1-S361D* but not *sgt1-S361A* mutation was lethal when expressed *in vivo* as the sole version of Sgt1 (Fig. 3B). These results suggest that the phosphorylation of Ser³⁶¹ inhibits Sgt1 dimerization, Sgt1

dimerization is essential for viability, and thus that CK2 negatively regulates Sgt1 dimerization.

To confirm this hypothesis *in vivo*, we examined whether the CBF3 complex could be formed in *sgt1-S361D* mutant cells. Because the *sgt1-S361D* mutation is lethal, we used a conditional *SGT1*-null mutant by using N-degron-4HA-Sgt1 expressed under the control of a repressible promoter (25) to remove the N-degron-wild-type protein but not the Myc-tagged mutant protein from cells that were used for the CBF3

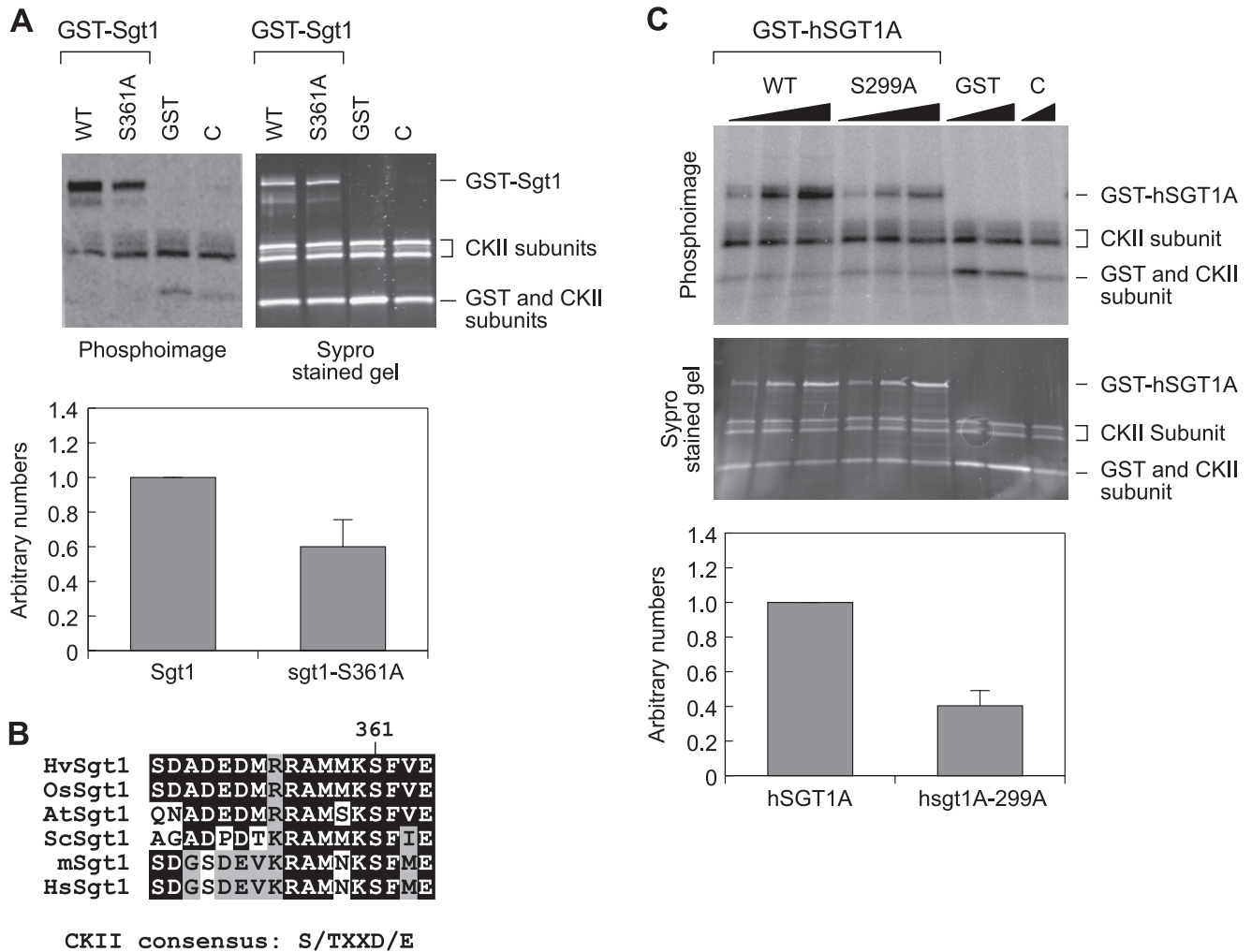


FIGURE 2. A, CK2 phosphorylates Sgt1 at S361 *in vitro*. Top, 100 ng of recombinant GST-Sgt1 and GST-sgt1-S361A mutant proteins were used for the *in vitro* kinase assay (left, a phosphor image; right, a SYPRO Ruby-stained image). Bottom, phosphorylation signals were quantified by a PhosphorImager. Input Sgt1 (top bands on the gel) in the reaction was quantified from the SYPRO Ruby-stained gel image, and the phosphorylation signals were normalized. GST-Sgt1 was given the arbitrary value of 1. Numbers on the y axis are arbitrary. Signals were quantified from three independent experiments. C, a negative control without substrates. B, Sgt1 Ser³⁶¹ is conserved from yeast to humans. A short peptide sequence around Ser³⁶¹ in yeast Sgt1 is aligned with the amino acid sequence of the corresponding region in Sgt1 homologs of several other organisms, as indicated: barley (*HvSgt1*), rice (*OsSgt1*), *Arabidopsis* (*AtSgt1*), *S. cerevisiae* (*ScSgt1*), mouse (*mSgt1*), and human (*HsSgt1*). C, human SGT1A is phosphorylated by CK2 *in vitro*. Recombinant GST-hSGT1A, GST-hSGT1A-S299A (hSGT1A-S299A corresponds to yeast Sgt1-S361), and GST alone were used as substrate for the CK2-dependent *in vitro* kinase assay. WT, wild type.

band shift assay (8). In this system, copper induces the expression of Ubr1 and Rox1. Ubr1 is a ubiquitin ligase that binds proteins containing an N-degron and facilitates their ubiquitination and subsequent destruction by the 26 S proteasome. Rox1 represses the transcription of the *ANB1* promoter controlling the expression of N-degron-Sgt1. Thus, the addition of copper both blocks the expression of N-degron-Sgt1 and triggers its proteolysis. The levels of N-degron-Sgt1 decreased after CuSO₄ was added and were substantially lower after a 4-h incubation, but the levels of Myc-tagged Sgt1 and Myc-tagged sgt1-S361D mutant protein did not change (Fig. 3C, right). The cells in which Sgt1-Myc or sgt1-S361D-Myc were expressed and N-degron-Sgt1 was depleted were used for the CBF3 band shift assay. Although the viability after an 8-h incubation was substantially reduced, that after a 4-h incubation appeared to be high enough to assess CBF3 activity (Fig. 3D). The CBF3 complex was not formed efficiently in cell lysates containing only sgt1-S361D-Myc (Fig. 3C, left),

suggesting that the phosphorylation of Ser³⁶¹ inhibits the formation of the CBF3 complex.

CK2 Phosphorylates Sgt1 *in Vivo*—Next, we examined whether Sgt1 is phosphorylated by CK2. We found that Sgt1 interacted with Cka1 (a subunit of yeast CK2) *in vivo* (Fig. 4A) and that the low pH spot diminished in *cka1Δ* (Fig. 4B). These results strongly suggest that CK2 phosphorylates Sgt1 *in vivo*.

The phosphorylation of Ser³⁶¹ appears to negatively regulate Sgt1 dimerization; thus, if Ser³⁶¹ is phosphorylated by CK2 *in vivo*, then the amount of Sgt1 dimers should be greater in yeast casein-kinase mutants than in wild-type cells. Indeed, Sgt1 dimerization increased in *cka1Δ* mutant cells (Fig. 4C), and Skp1 binding also increased (Fig. 4C). Furthermore, sgt1-S361A dimerization or Skp1 binding did not increase in *cka1Δ* mutant cells, indicating that the stimulation of dimerization by reduction of CK2 activity is dependent on Ser³⁶¹ phosphorylation (Fig. 4C). Consistent with these results, the deletion of *cka1* suppressed the benomyl sensitivity of the *sgt1-3* mutant (Fig.

CK2 Inhibits Sgt1 Dimerization

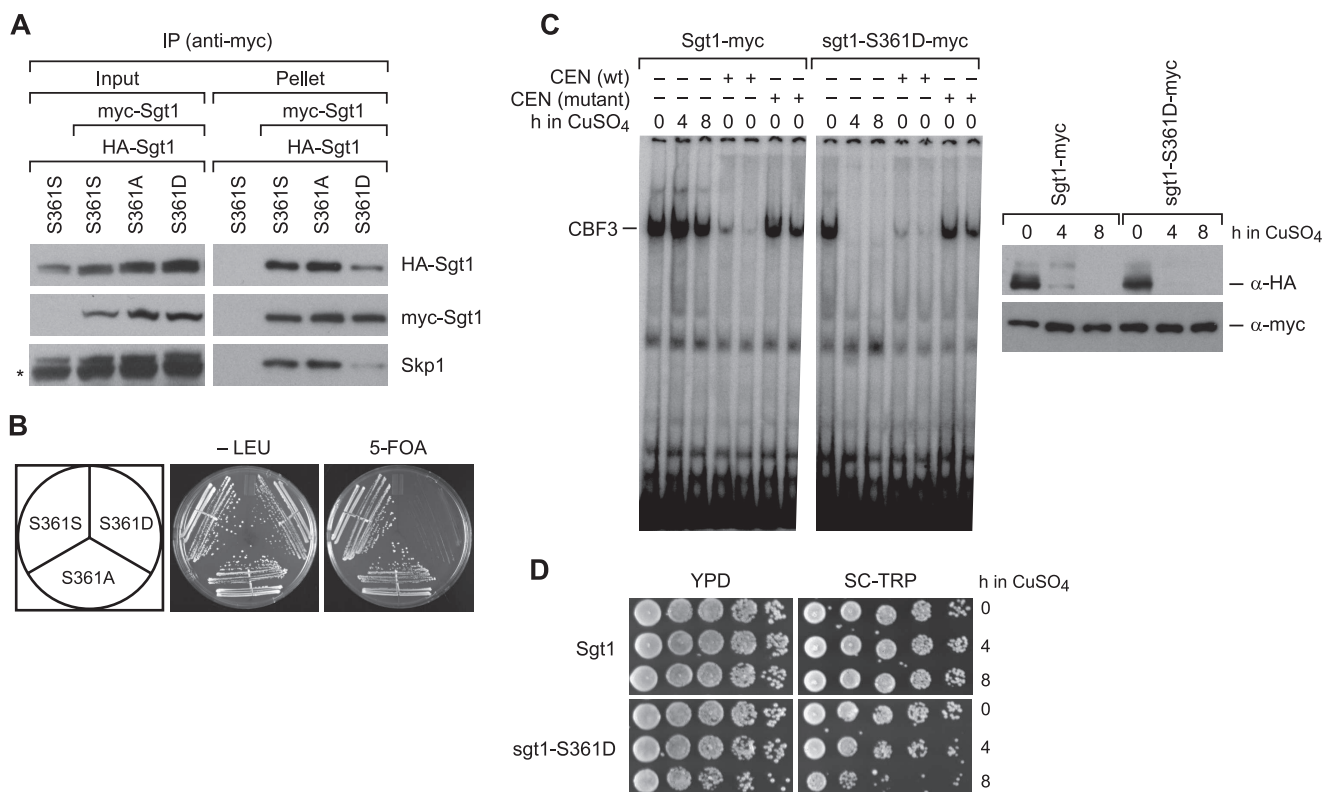


FIGURE 3. Phenotypes of the *sgt1-S361D* mutant. *A*, mimic of Sgt1 phosphorylation at Ser³⁶¹ inhibits Sgt1-Sgt1 and Sgt1-Skp1 binding. Sgt1-S361S (wild type), Sgt1-S361A, or *sgt1-S361D* was expressed as HA-tagged and Myc-tagged proteins from two different plasmids (pRS416-HA and pRS416-Myc). Sgt1-S361S was expressed in the Y1681 strain, Sgt1-S361A in the Y1682 strain, and *sgt1-S361D* in the Y1684 strain. (All three strains were derived from the YPH499 strain.) The YPH499 strain carrying only pRS416-HA-SGT1-S361S was used as a negative control (*leftmost lane*). *B*, mimic of Sgt1 phosphorylation at Ser³⁶¹ is lethal. Yeast cells that were haploid for *SGT1* (Y26), *sgt1-S361A* (Y1686), or *sgt1-S361D* carrying *SGT1* on a URA/*CEN* plasmid (Y1687) were streaked onto plates that contained Sc+ 5-fluoroorotic acid (5-FOA) or Sc-Leu. Plates were incubated at 25 °C for 3 days. *C* (*right*), the addition of 0.5 mM CuSO₄ to strain CDY26 (N-degron-Sgt1) induced the proteolysis of N-degron-4HA-Sgt1 after either a 4- or 8-h incubation with CuSO₄, as determined by immunoblotting with anti-HA antibodies, but Myc-tagged Sgt1 or *sgt1-S361D* was not affected. *Left*, protein extracts of the indicated strains were collected at the indicated time points after adding CuSO₄. Cell extracts (40 μg) were subjected to the band shift assay to examine the CBF3 assembly activity. Competitor *CEN* DNA (100 or 200 μg) or mutant *CEN* DNA (CCG motif at CDEIII region is mutated to CCC) was added to CuSO₄-untreated extracts of each cell type. *D*, survival of cells after a 4- or 8-h incubation with 0.5 mM CuSO₄, as determined by a dilution-spotting assay. The numbers of cells that were spotted onto the indicated plates were $\sim 1.25 \times 10^6$, 2.5×10^5 , 5×10^4 , 1×10^4 , and 2×10^3 . Plates were incubated at 30 °C for 2 days. *IP*, immunoprecipitation.

4D) (21, 30). These results strongly suggest that CK2 phosphorylates Sgt1 at Ser³⁶¹ and negatively regulates Sgt1 dimerization *in vivo*.

DISCUSSION

In this study, we have shown that Sgt1 dimerization, which is required for kinetochore assembly in budding yeast, is regulated negatively by CK2-mediated phosphorylation.

CK2 Negatively Regulates Sgt1 Dimerization—CK2 is a serine/threonine protein kinase that is ubiquitous in eukaryotes (42, 43). The enzyme is composed of a catalytic subunit α and a regulatory subunit β that form a native $\alpha_2\beta_2$ holoenzyme (42, 43). CK2 of the budding yeast consists of two catalytic subunits, α and α' , and two regulatory subunits, β and β' , which are encoded by the *CKA1*, *CKA2*, *CKB1*, and *CKB2* genes, respectively (44). Individual deletion of either *CKA1* or *CKA2* does not show any significant phenotypes, but their simultaneous deletion is lethal (45).

To demonstrate that Ser³⁶¹ is phosphorylated by CK2 *in vivo*, we attempted to generate antibodies against phosphorylated Ser³⁶¹. However, unfortunately, antibodies produced using a phosphorylated peptide were not specific for the phosphorylated form and recognized the unphosphorylated

form even after affinity purification. Therefore, we used indirect approaches to answer this question. Two-dimensional gel analysis revealed that Sgt1 is phosphorylated by CK2. When CK2 activity was altered by deletion of *Cka1* but not *Cka2* or *Ckb2*, Sgt1 dimerization and Skp1 binding were stimulated (data not shown). It has been previously reported that different combinations of subunits exhibit properties typical for CK2 but differ in substrate specificity and sensitivity to inhibitors, which suggests that each CK2 isomer may regulate different processes or may have different mechanisms of regulation (46). Thus, the regulation of Sgt1 dimerization may be a specific function of *Cka1*.

Because the *sgt1-S361D* mutant is lethal, we had to perform coimmunoprecipitation experiments to test the dimerization activity of the *sgt1* mutant proteins in the presence of endogenous wild-type Sgt1 (Fig. 3A). Since the *sgt1-S361D* protein presumably binds more efficiently to the wild-type Sgt1 than to *sgt1-S361D*, the dimerization activity detected by co-immunoprecipitation might be an underestimation.

The stimulation of Sgt1 dimerization by deletion of *Cka1* was dependent on Ser³⁶¹. The alteration of CK2 activity by deletion

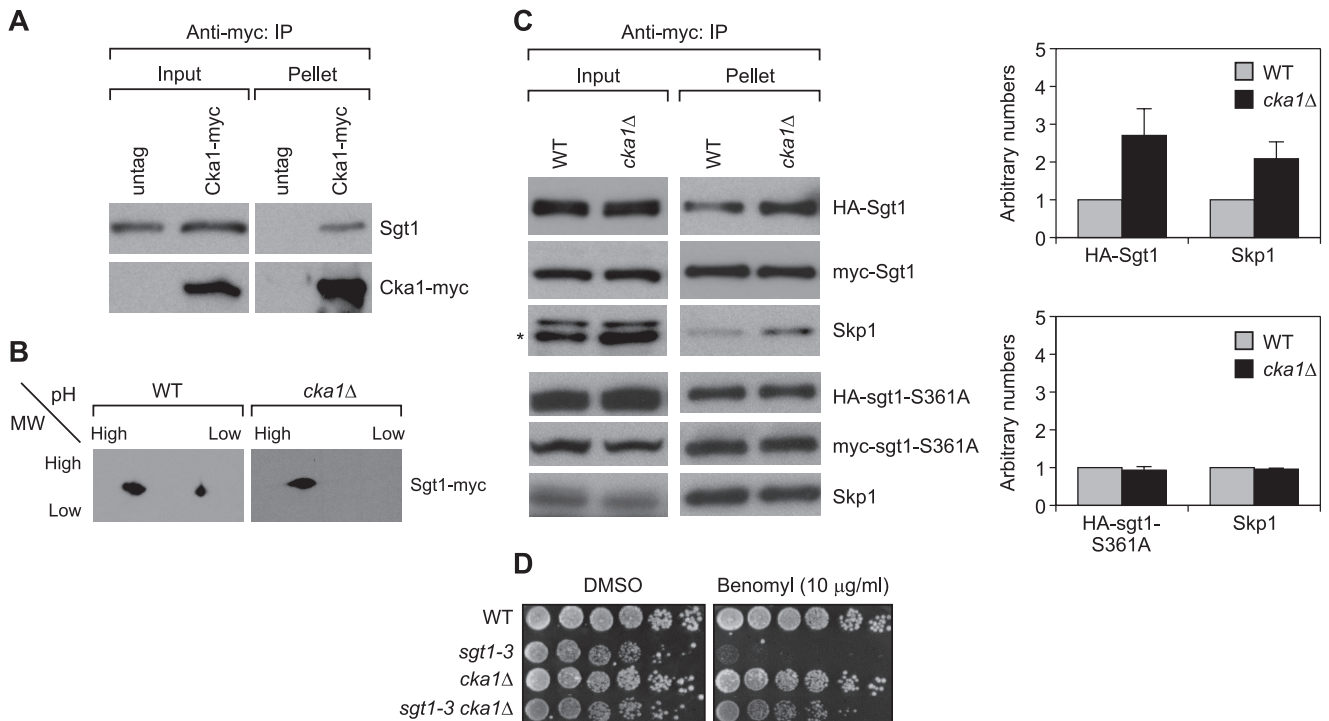


FIGURE 4. CK2 phosphorylates Sgt1 *in vivo*. *A*, Cka1, a subunit of CK2, associates with Sgt1. Cell lysates obtained from cycling untagged wild-type (*untag*; strain YPH499) and Cka1-Myc (strain Y1734) cells were immunoprecipitated by using anti-Myc antibodies. Sgt1 was detected with anti-Sgt1 antibodies, and Cka1 was detected with anti-Myc antibodies. An untagged strain was used as a negative control. *B*, phosphorylated Sgt1 diminished in *cka1Δ* cells. 3Myc-Sgt1 was immunoprecipitated from wild-type (*WT*; Y1872) cells or *cka1Δ* cells (*cka1Δ*; Y1873) harboring 3Myc-Sgt1. Immunoprecipitates were analyzed by two-dimensional gel electrophoresis, and immunoblotting was performed by using anti-Myc antibodies. *C* (top left panels), Sgt1 dimerization increases in *cka1Δ* cells. Immunoprecipitation was carried out on cell lysates from wild-type (*WT*; Y1681) cells or *cka1Δ* cells (*cka1Δ*; Y1736) harboring 3HA-Sgt1 and 3Myc-Sgt1. Bottom left panels, Sgt1 dimerization increases in *cka1Δ* cells, which is dependent on serine 361. Data are from experiments similar to those above, except that cell lysates were prepared from cells expressing HA-sgt1-S361A and Myc-sgt1-S361A in wild-type (Y1682) and *cka1Δ* cells (*cka1Δ*; Y1874). HA-tagged Sgt1 (wild type or mutant) was identified by anti-HA antibodies, and Myc-tagged Sgt1 (wild type or mutant) was identified by anti-Myc antibodies. Right panels, signals of HA-Sgt1 and Skp1 in Myc-Sgt1 immunoprecipitates were quantified from two independent experiments. Quantification was performed by giving an arbitrary value of 1 to HA-Sgt1 or Skp1 in the wild-type cells. *D*, the benomyl sensitivity of the *sgt1-3* mutant was suppressed by the deletion of *cka1*. The indicated strains (wild type (YPH499), *sgt1-3* (YKK54), *cka1Δ* (Y1773), and *sgt1-3 cka1Δ* (Y1775)) were grown on yeast extract-peptone-dextrose plates containing 10 μg/ml benomyl or DMSO. The numbers of cells that were spotted onto each plate (left to right) were $\sim 1.25 \times 10^6$, 2.5×10^5 , 5×10^4 , 1×10^4 , 2×10^3 , and 4×10^2 . Plates were incubated at 30 °C for 3 days. *IP*, immunoprecipitation.

of Cka1 suppressed the benomyl sensitivity of the *sgt1-3* mutant, and Sgt1 associated with Cka1 *in vivo*. Therefore, we conclude that CK2 negatively regulates Sgt1 dimerization by phosphorylating Ser³⁶¹, which in turn represses kinetochore assembly. However, the biological significance of why Sgt1 dimerization needs to be negatively regulated is unknown and needs further analyses. A monomer form might have a different function.

Another interesting observation is that Ser³⁶¹ is outside the Sgt1 dimerization domain but within the SGS domain (amino acids 307–395). Our binding analysis suggested that an inhibitory region may be present between amino acids 270 and 312 (30). The phosphorylation of Ser³⁶¹ may enhance this internal inhibitory activity by affecting the structure of the SGS domain.

Highly Conserved Kinetochore Function of Sgt1—The human homolog of Sgt1 can rescue the yeast *sgt1*-null mutant (21), suggesting that the function of Sgt1 is conserved between humans and yeast. Sgt1 interacts with Hsp90 in yeast and human cells (28, 47). CENP-I, CENP-F, and Hec1, but not CENP-C, are absent from kinetochores in human cells depleted of SGT1 or HSP90 or treated with 17-AAG (an HSP90 inhibitor) (28, 29). This result strongly suggests that the human SGT1-HSP90 complex is crucial for assembly of

the human kinetochore. Therefore, the kinetochore function of Sgt1 is evolutionarily conserved between humans and yeast.

However, Nyarko *et al.* (48) have shown by gel filtration chromatography that human SGT1A does not form dimers efficiently. Our recent analytical ultracentrifuge experiments have also revealed that human SGT1A exists mainly as a monomer (30). Further studies are required to determine whether human SGT1 is phosphorylated by CK2 *in vivo* and, if so, what role phosphorylation of this protein plays.

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REFERENCES

- Cheeseman, I. M., and Desai, A. (2008) *Nat. Rev. Mol. Cell Biol.* **9**, 33–46
- Westermann, S., Drubin, D. G., and Barnes, G. (2007) *Annu. Rev. Biochem.* **76**, 563–571
- Sudakin, V., and Yen, T. J. (2007) *Biodrugs* **21**, 225–233
- Weaver, B. A., Silk, A. D., Montagna, C., Verdier-Pinard, P., and Cleveland, D. W. (2007) *Mol. Cell Biol.* **27**, 1000–1010

CK2 Inhibits Sgt1 Dimerization

- D. W. (2007) *Cancer Cell* **11**, 25–36
5. Fitzgerald-Hayes, M., Clarke, L., and Carbon, J. (1982) *Cell* **29**, 235–244
 6. Hieter, P., Pridmore, D., Hegemann, J. H., Thomas, M., Davis, R. W., and Philippsen, P. (1985) *Cell* **42**, 913–921
 7. Hegemann, J. H., Shero, J. H., Cottarel, G., Philippsen, P., and Hieter, P. (1988) *Mol. Cell. Biol.* **8**, 2523–2535
 8. Lechner, J., and Carbon, J. (1991) *Cell* **64**, 717–725
 9. Doheny, K. F., Sorger, P. K., Hyman, A. A., Tugendreich, S., Spencer, F., and Hieter, P. (1993) *Cell* **73**, 761–774
 10. Goh, P. Y., and Kilmartin, J. V. (1993) *J. Cell Biol.* **121**, 503–512
 11. Jiang, W., Lechner, J., and Carbon, J. (1993) *J. Cell Biol.* **121**, 513–519
 12. Lechner, J. (1994) *EMBO J.* **13**, 5203–5211
 13. Strunnikov, A. V., Kingsbury, J., and Koshland, D. (1995) *J. Cell Biol.* **128**, 749–760
 14. Connelly, C., and Hieter, P. (1996) *Cell* **86**, 275–285
 15. Stemmann, O., and Lechner, J. (1996) *EMBO J.* **15**, 3611–3620
 16. Sorger, P. K., Doheny, K. F., Hieter, P., Kopski, K. M., Huffaker, T. C., and Hyman, A. A. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 12026–12030
 17. Kaplan, K. B., Hyman, A. A., and Sorger, P. K. (1997) *Cell* **91**, 491–500
 18. Baker, R. E., and Masison, D. C. (1990) *Mol. Cell. Biol.* **10**, 2458–2467
 19. Cai, M., and Davis, R. W. (1990) *Cell* **61**, 437–446
 20. Cai, M. J., and Davis, R. W. (1989) *Mol. Cell. Biol.* **9**, 2544–2550
 21. Kitagawa, K., Skowyr, D., Elledge, S. J., Harper, J. W., and Hieter, P. (1999) *Mol. Cell* **4**, 21–33
 22. Stemmann, O., Neidig, A., Köcher, T., Wilm, M., and Lechner, J. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 8585–8590
 23. Azevedo, C., Sadanandom, A., Kitagawa, K., Freialdenhoven, A., Shirasu, K., and Schulze-Lefert, P. (2002) *Science* **295**, 2073–2076
 24. Bansal, P. K., Abdulle, R., and Kitagawa, K. (2004) *Mol. Cell. Biol.* **24**, 8069–8079
 25. Dubacq, C., Guerois, R., Courbeyrette, R., Kitagawa, K., and Mann, C. (2002) *Eukaryot. Cell* **1**, 568–582
 26. Lingelbach, L. B., and Kaplan, K. B. (2004) *Mol. Cell. Biol.* **24**, 8938–8950
 27. Catlett, M. G., and Kaplan, K. B. (2006) *J. Biol. Chem.* **281**, 33739–33748
 28. Niikura, Y., Ohta, S., Vandenbeldt, K. J., Abdulle, R., McEwen, B. F., and Kitagawa, K. (2006) *Oncogene* **25**, 4133–4146
 29. Steensgaard, P., Garrè, M., Muradore, I., Transidico, P., Nigg, E. A., Kitagawa, K., Earnshaw, W. C., Faretta, M., and Musacchio, A. (2004) *EMBO Rep.* **5**, 626–631
 30. Bansal, P. K., Nourse, A., Abdulle, R., and Kitagawa, K. (2009) *J. Biol. Chem.* **284**, 3586–3592
 31. Rose, M. D., Winston, F., and Hieter, P. (1990) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory Press, New York
 32. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) *J. Bacteriol.* **153**, 163–168
 33. Longtine, M. S., McKenzie, A., 3rd, Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J. R. (1998) *Yeast* **14**, 953–961
 34. Kitagawa, K., and Abdulle, R. (2002) *BioTechniques* **33**, 288–292
 35. Kitagawa, K., Abdulle, R., Bansal, P. K., Cagney, G., Fields, S., and Hieter, P. (2003) *Mol. Cell* **11**, 1201–1213
 36. Pinna, L. A. (1990) *Biochim. Biophys. Acta* **1054**, 267–284
 37. Carmichael, D. F., Geahlen, R. L., Allen, S. M., and Krebs, E. G. (1982) *J. Biol. Chem.* **257**, 10440–10445
 38. Fritz, G., and Kaina, B. (1999) *Oncogene* **18**, 1033–1040
 39. Mckenzie, J. A., and Strauss, P. R. (2003) *Anal. Biochem.* **313**, 9–16
 40. Nowotny, M., Spiechowicz, M., Jastrzebska, B., Filipek, A., Kitagawa, K., and Kuznicki, J. (2003) *J. Biol. Chem.* **278**, 26923–26928
 41. Kreegipuu, A., Blom, N., and Brunak, S. (1999) *Nucleic Acids Res.* **27**, 237–239
 42. Pinna, L. A., and Meggio, F. (1997) *Prog. Cell Cycle Res.* **3**, 77–97
 43. Poole, A., Poore, T., Bandhakavi, S., McCann, R. O., Hanna, D. E., and Glover, C. V. (2005) *Mol. Cell Biochem.* **274**, 163–170
 44. Glover, C. V., III (1998) *Prog. Nucleic Acids Res. Mol. Biol.* **59**, 95–133
 45. Padmanabha, R., Chen-Wu, J. L., Hanna, D. E., and Glover, C. V. (1990) *Mol. Cell. Biol.* **10**, 4089–4099
 46. Domańska, K., Zieliński, R., Kubiński, K., Sajnaga, E., Masłyk, M., Bretner, M., and Szyszka, R. (2005) *Acta Biochim. Pol.* **52**, 947–951
 47. Lee, Y. T., Jacob, J., Michowski, W., Nowotny, M., Kuznicki, J., and Chazin, W. J. (2004) *J. Biol. Chem.* **279**, 16511–16517
 48. Nyarko, A., Mosbahi, K., Rowe, A. J., Leech, A., Boter, M., Shirasu, K., and Kleanthous, C. (2007) *Biochemistry* **46**, 11331–11341
 49. Sikorski, R. S., and Hieter, P. (1989) *Genetics* **122**, 19–27