

## Retinoblastoma Protein Enhances the Fidelity of Chromosome Segregation Mediated by hsHec1p

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**Retinoblastoma protein (Rb) plays important roles in cell cycle progression and cellular differentiation. It may also participate in M phase events, although heretofore only circumstantial evidence has suggested such involvement. Here we show that Rb interacts, through an IxCxE motif and specifically during G<sub>2</sub>/M phase, with hsHec1p, a protein essential for proper chromosome segregation. The interaction between Rb and hsHec1p was reconstituted in a yeast strain in which human *hsHEC1* rescues the null mutation of *scHEC1*. Expression of Rb reduced chromosome segregation errors fivefold in yeast cells sustained by a temperature-sensitive (*ts*) *hshec1-113* allele and enhanced the ability of wild-type hsHec1p to suppress lethality caused by a *ts smc1* mutation. The interaction between Hec1p and Smc1p was important for the specific DNA-binding activity of Smc1p. Expression of Rb restored part of the inactivated function of *hshec1-113p* and thereby increased the DNA-binding activity of Smc1p. Rb thus increased the fidelity of chromosome segregation mediated by hsHec1p in a heterologous yeast system.**

Genetic instability is one of the most important hallmarks of cancer. It occurs at two different levels. On one level, increased mutation rates result from defective repair of damaged DNA or replication errors, which leads to missense, nonsense, or other small but functionally important mutations in several types of cancer. On another level, improper segregation of whole chromosomes or pieces of chromosomes during mitosis leads to aneuploidy or translocations, traits commonly observed in cancers (35). Chromosome segregation is controlled by a large group of proteins that together coordinate M phase progression (43, 44, 48, 58). Loss of function of key proteins important for the structure and dynamics of mitotic chromosomes would be expected to lead to cell death and thus to prevent passage of mutations of such fundamental proteins to daughter cells. Loss of function of proteins that play subtler regulatory roles in mitosis, however, may not be immediately lethal but instead may lead to high frequencies of chromosome abnormalities and to neoplasia.

Associations of oncoproteins or tumor suppressors with the process of chromosome segregation provide possible links between carcinogenesis and chromosomal instability. Recent studies suggest that both p53 and retinoblastoma protein (Rb) play important roles in the prevention of aneuploidy in human and rodent cells (12, 28, 34, 56). When treated with microtubule-destabilizing agents, cells lacking functional Rb or p53 do not finish mitosis properly but nonetheless enter a new cell cycle, leading to hyperploidy (28, 34). p53 has been found to be associated with centrosome duplication activity (15) and mitotic or postmitotic checkpoint control (18, 34), loss of these functions would result in aberrant mitosis and contribute to the observed increase in ploidy. Similarly, the propensity of Rb-deficient cells to become hyperploidy is most likely due to the loss of a novel function of Rb in M phase of the cell cycle, although supportive evidence remains scarce.

Study of the function of Rb has been centered on the progression of G<sub>1</sub> phase (17, 23, 52). However, accumulating evidence has suggested potential functional roles for Rb during other phases of the cell cycle (27, 31, 46), especially during M phase. First, the functional, hypophosphorylated form of Rb is present at this phase of the cell cycle (8, 38). Second, hypophosphorylated Rb is associated with at least three cellular proteins that have crucial functions in M phase progression (50). One example is the human H-nuc2 (also called hCDC27) protein (9), a subunit of the anaphase-promoting complex that controls the onset of sister chromatid separation and metaphase-anaphase transition by degradation of specific substrates (30, 32). Another Rb-associated protein, protein phosphatase 1 $\alpha$  catalytic subunit (13), is important for kinetochore function, chromosome segregation, and M phase progression, as demonstrated by the abnormal phenotype resulting from the mutational inactivation of its yeast homolog (2, 3, 49, 51). Lastly, mitosin (also called CENP-F), a kinetochore protein (60), also interacts specifically with Rb during M phase.

However, the mechanisms by which Rb plays a role in chromosome segregation and M phase progression remain elusive. The current approach of counting total chromosome numbers by karyotyping or detecting a specific chromosome by fluorescent in situ hybridization can only display the status of chromosome instability, which may not necessarily be a direct consequence of a certain gene defect in mammalian cells. To determine whether the loss of a gene function is responsible for improper chromosome segregation, a method for monitoring the dynamic transmission of a specific chromosome marker is required. Any attempt to select for mammalian cells carrying an integrated exogenous chromosome marker, however, bears the risk of immortalizing a primary cell line or making the genetic content of a tumor cell line even more unpredictable. Studies of chromosome segregation in mammalian cells are therefore complicated. On the other hand, methods for monitoring the dynamic transmission of chromosomes in yeast cells are feasible, and the genetic manipulation of a given gene in yeast can be accomplished without affecting the rest of the gene population and chromosome structures. Moreover, the

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basic machinery for chromosome segregation is conserved between mammals and yeast (42).

In this study, a yeast assay system for investigating the role of Rb in chromosome segregation was established, based on the study of hsHec1p. hsHec1p, isolated from a screen for proteins interacting with Rb (10, 13), is a coiled-coil protein crucial for proper mitosis (10, 11, 59). Inactivation of hsHec1p leads to disruption of M phase progression (10). The homolog of hsHec1p in *Saccharomyces cerevisiae*, scHec1p (also called Ndc80 or Tid3), has a similar essential function (57, 59), and hsHEC1 is able to rescue the lethality caused by the null mutation of scHEC1 (59). Yeast cells carrying a mutant allele of human or yeast HEC1 segregate their chromosomes aberrantly (57, 59). At the nonpermissive temperature, significant mitotic delay, unequal nuclear division, and decreased viability were observed in yeast cells carrying *hshec1-113*, a temperature-sensitive mutant allele of human HEC1 (59). Increased frequencies of chromosome segregation errors were also detected in the *hshec1-113* mutant at permissive temperatures. Hec1p has been found to interact physically or genetically with a number of proteins important for G<sub>2</sub>/M progression and chromosome segregation, including SMC (structural maintenance of chromosomes) proteins and yeast centromere protein Ctf19p (10, 26, 59). A potential role for Hec1p in modulating chromosome segregation in part through interactions with SMC proteins has been suggested (59). There is no protein with sequence similarity to Rb in the entire *S. cerevisiae* genome. Without interference from endogenous Rb, yeast strains in which the null mutation of scHEC1 has been complemented by hsHEC1 (59) therefore provide useful tools to address the consequence of the interaction between Rb and hsHec1p for chromosome segregation.

The biological significance of the interaction between Rb and hsHec1p is demonstrated here by reconstitution of these proteins in a heterologous *in vivo* yeast system. Expression of Rb resulted in a decrease in the rate of chromosome segregation errors in cells carrying a mutant form of hsHec1p and an increase in the survival rate of *smc1* mutant cells with defects in chromosome segregation. These results suggest that Rb plays a positive regulatory role in chromosome segregation.

## MATERIALS AND METHODS

**Strains and plasmids.** *S. cerevisiae* haploid and diploid strains carrying the *hshec1-113* mutant have been described previously (59). A new yeast strain, 4bWHL273 (*matx ade2 lys2 ura3 trp1 smc1-2::LEU2*), is one of the meiotic segregates of the diploid strain from the mating between 3bAS273 (a gift from D. Koshland) and YPH1015 (a gift from P. Hieter). The full-length 2.8-kb *RB* cDNA, or the cDNA for the H209 mutant *RB* derivative (*Cys706* changed to Phe), was inserted in two sets of plasmids, p415GAL1 (41) and pESC::TRP1 (Stratagene), by use of *Bam*HI and *Sal*I. The resultant plasmids were used to transform the above-mentioned strains. By a procedure described previously (21), cells were cultured in 2% raffinose overnight at 25°C before Rb expression was induced in medium containing additional 2% galactose for the indicated number of hours. The YEp195-GC15C plasmid was generated by inserting the *GAL1* promoter, hsHEC1 cDNA (59), and *CYC1* terminator (41) into the YEplac195 vector (16) for the expression of hsHec1p. The YEp195-GEKC plasmid was generated by site-directed mutagenesis for the expression of the *hshec1-EK* mutant (Glu234 changed to Lys). To express myc-tagged Smc1p, the full-length *SMC1* was generated by PCR, sequenced, and fused with the c-myc tag in the pESC plasmid.

**Immunoprecipitation and immunoblotting.** The preparation of yeast cell lysates, immunoprecipitation, and immunoblotting have been described previously (59). Human hsHec1p, *S. cerevisiae* Smc1p, and human Rb were precipitated or immunoblotted with anti-hsHec1p monoclonal antibody (MAb) 9G3, mouse anti-Smc1p antiserum (59), and anti-Rb MAb 11D7, respectively.

Human bladder carcinoma T24 cells were cultured and synchronized at different stages of the cell cycle as described previously (8, 10). Cells were lysed and immunoprecipitated by procedures described previously (8, 10).

For immunoprecipitation and immunoblotting of human SMC1 (hSMC1) from T24 cells, mouse anti-hSMC1 antiserum was obtained from mice immu-

nized with glutathione *S*-transferase (GST) protein fused with the peptide region of hSMC1 isolated from a yeast two-hybrid screen (10, 11, 59).

**Colony sectoring assays.** Colony sectoring assays were used to measure the frequencies of chromosome missegregation, as described previously (33, 59). Five single pink colonies of each diploid strain that contains a homozygous *ade2-101* ochre color mutation and a dispensable chromosome fragment carrying a copy of *SUP11* were picked and cultured to log phase in histidine-free supplemented minimal medium at 25°C for 3 days. Cells were diluted and incubated at 30°C for 4 h (one generation) in fresh medium supplied with histidine and containing 2% galactose and 2% raffinose to induce the expression of Rb or the H209 mutant. An aliquot of culture was then removed and plated on medium containing 6 mg of adenine per liter. The plates were incubated at 30°C for 6 days and at 4°C overnight before observation. The remaining cultures were used for detecting the expression of Rb or for examining the interaction between Rb and hsHec1p as described above.

**Immunoaffinity purification.** Yeast cell lysate was prepared as described previously (59). Smc1p was partially purified from this lysate with mouse anti-Smc1p polyclonal antibodies by immunoaffinity chromatography, according to modification of a procedure described previously (25, 29). Antibodies were incubated with 50  $\mu$ l of protein A-Sepharose beads for 2 h at 4°C and washed twice with 1 ml of Tris-buffered saline (50 mM Tris [pH 8.0], 125 mM NaCl). A 1.5-ml portion of cell lysate (about 50 mg of total protein) was added to the antibody-protein A-Sepharose beads and incubated for another 1 h at 4°C. The mixture was then loaded on a minicolumn and washed sequentially with 4 ml of XBE2 buffer (20 mM potassium HEPES [pH 7.7], 0.1 M KCl, 10% glycerol, 2 mM MgCl<sub>2</sub>, 5 mM EGTA), 0.5 ml of XBE2 with 0.4 M KCl, and 0.5 ml of XBE2. For elution, 150  $\mu$ l of XBE2 containing a 4- $\mu$ g/ $\mu$ l concentration of a GST fusion with the C-terminal region of Smc1p (59) was used. Fifty microliters of elution buffer was first allowed to flow in, and then the other 100  $\mu$ l was loaded. After incubation at 4°C for 4 h, the elution buffer was allowed to flow through and collected. The elution product was incubated with 100  $\mu$ l of glutathione-Sepharose that was prewashed with XBE2 for 1 h at 4°C three times to completely remove the GST fusion protein.

For multiple samples in the same experiment, equal numbers of yeast cells that contained comparable amounts of total proteins were lysed. The cell lysates were added to the antibody-protein A-Sepharose beads for immunoaffinity purification as described above. The eluted products from each sample were calibrated with comparable protein concentrations for use in gel shift assays. To minimize the effect of any quantitative variations, the amount of Smc1p in each purified product was adjusted according to the immunoblotting results.

**Gel mobility shift assay.** Approximately 2  $\mu$ l of the purified product described above was incubated with the 230-bp M13 replicative-form (RF) DNA fragment in 20  $\mu$ l of XBE2 buffer with 0.5 mg of bovine serum albumin per ml. The 230-bp M13 RF DNA fragment was digested from the same region of M13 genomic DNA described previously (1), although *Hind*III was used instead of *Eco*RI. The DNA fragment was end labeled with [ $\alpha$ -<sup>32</sup>P]dCTP by the fill-in reaction with Klenow enzymes. DNA fragments labeled with 10,000 to 20,000 cpm were used as substrates. For competition, unlabeled 230-bp M13 fragment, a 220-bp pUC19 fragment digested with *Ava*II from a pUC19-derived vector (1), and a 240-bp CEN3 fragment (bp, 113925 to 114168) generated by PCR amplification from yeast genomic DNA with the primers described previously (40) were used. The DNA-protein reaction mixtures were loaded on a 5.5% acrylamide gel and run at 4°C in a buffer containing 20 mM HEPES [pH 7.5] and 0.1 mM EDTA. The results were quantified using a densitometer and ImageQuant v1.1 (Molecular Dynamics).

## RESULTS

**hsHec1p specifically interacts with Rb through the IxCxE motif.** hsHec1p was originally identified using Rb as the bait in a yeast two-hybrid screen (10, 13). In order to determine the specific region of Rb required for binding hsHec1p, a deletion set that had previously been used to delineate the binding domain for protein phosphatase 1 $\alpha$  was employed (13). Amino acids 301 to 928 of the Rb protein and several carboxy-terminal deletion mutants, as well as the H209 point mutant with residue 706 changed from cysteine to phenylalanine, were fused with the yeast Gal4 DNA-binding domain. Full-length hsHec1p protein was fused with the Gal4 transactivation domain. The results showed that Rb uses the same T-antigen-binding domain to interact with hsHec1p, and the H209 point mutation abolished this binding (Fig. 1A). hsHec1p sequences required for binding Rb were also determined in a reciprocal manner, using a series of hsHec1p deletion mutants. These mutants showed that the central region of hsHec1p, from amino acids 128 to 251, binds to Rb (Fig. 1B). Two Rb-related

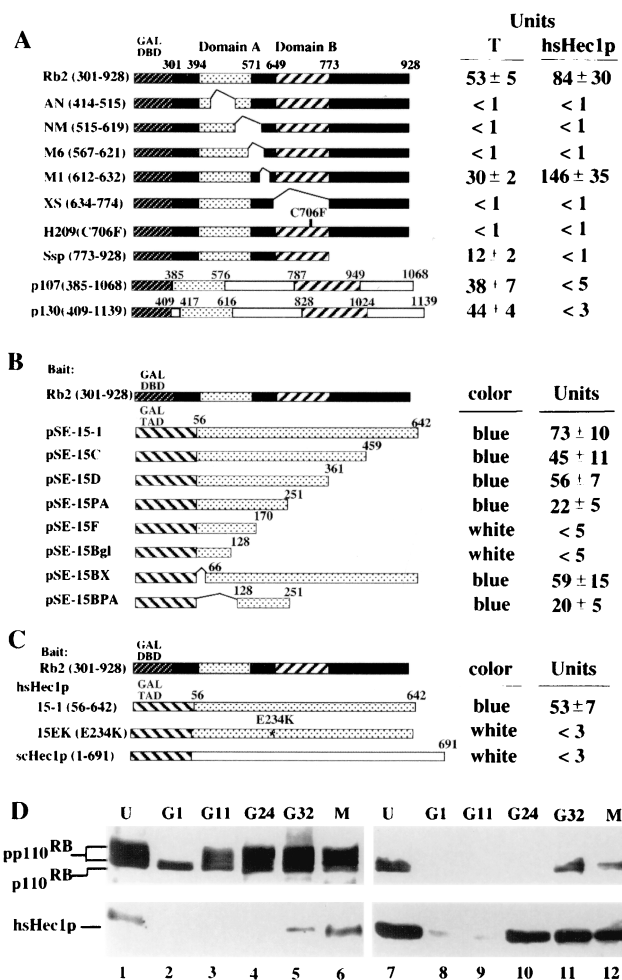


FIG. 1. Specific interaction between Rb and hsHec1p. (A) hsHec1p and T antigen bind to similar regions of the Rb protein. The Gal4 DNA-binding domain (DBD) (amino acids 1 to 147; stippled box) was fused to various Rb mutants, p107 (amino acids 385 to 1068), or p130 (amino acids 409 to 1139). The simian virus 40 T-antigen-binding domains A and B are shown as shaded and hatched boxes, respectively. hsHec1p or T antigen (13) was expressed as a Gal4 transactivation domain fusion protein and used to test for interaction with Rb fusion proteins in yeast two-hybrid assays. Transformants were grown in liquid cultures and used for *o*-nitrophenyl- $\beta$ -D-galactopyranoside quantitation of  $\beta$ -galactosidase activity as described previously (13). (B) Various hsHec1p mutants were fused with the Gal4 transactivation domain (TAD) (hatched box). Rb (amino acids 301 to 928) was expressed as the fusion with the Gal4 DNA-binding domain used for panel A. (C) Rb was expressed as the same fusion used for panel B. Wild-type hsHec1p (15-1), an hsHec1p mutant with amino acid 234 changed from E to K (15EK), and scHec1p were fused with the Gal4 transactivation domain. (D) Cell cycle-dependent interaction between Rb and hsHec1p. T24 cells were density arrested at G<sub>1</sub> (lanes 2 and 8) and then released for reentry into the cell cycle. At different time points after release as indicated above the lanes, 5 × 10<sup>6</sup> cells were collected, lysed, and immunoprecipitated with anti-Rb MAb 11D7 (lanes 1 to 6) or with anti-hsHec1p MAb 9G3 (lanes 7 to 12). The immune complexes were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting with MAb 11D7 (upper panel) or with 9G3 (lower panel). G<sub>1</sub> represents 11 h after release and corresponds to G<sub>1</sub>, G<sub>24</sub> marks 24 h after release and corresponds to S, and G<sub>32</sub> marks 32 h after release and corresponds to G<sub>2</sub>. M phase lysates (lanes 6 and 12) were obtained from cells treated with nocodazole (0.4  $\mu$ g/ml). Lanes 1 and 7, unsynchronized cells.

proteins, p107 and p130 (14, 20, 36, 39), did not interact with hsHec1p (Fig. 1A).

The region of hsHec1p that binds Rb was not conserved in yeast scHec1p. Thus, it is likely that the interaction between Hec1p and Rb is not conserved in yeast. To test this notion, a

yeast two-hybrid assay was performed using the above-described construct, with the Rb sequence fused with the Gal4 DNA-binding domain and a plasmid for the expression of yeast scHec1p sequence fused with the Gal4 transactivation domain. As predicted, yeast scHec1p failed to bind Rb (Fig. 1C).

An examination of the hsHec1p sequence showed that it contains an IxCxE motif, which has been implicated as the specific Rb-binding site in many proteins (reviewed in reference 5). This motif is not found in yeast scHec1p, suggesting that the inability of Rb to bind to yeast scHec1p may be due to the lack of the IxCxE sequence. To verify this possibility, a point mutant with residue 234 changed from glutamic acid to lysine in this motif was tested in a yeast two-hybrid assay. This mutation abolished the ability of hsHec1p to bind to Rb (Fig. 1C). However, *hshec1-EK*, with this mutation, was able to rescue the yeast *schec1* null mutant (data not shown) and generate the strain WHL101EK. This indicated that hsHec1p proteins, with or without an Rb-binding site, are able to perform their essential cellular function in yeast.

**Rb and hsHec1p interact at G<sub>2</sub>/M phase in mammalian cells.** The interaction between Rb and hsHec1p was also examined by coimmunoprecipitation following cell cycle progression. As shown in Fig. 1D, hsHec1p binds to Rb specifically at G<sub>2</sub>/M phase in human bladder carcinoma T24 cells, which were synchronized as described previously (8). Similar to most of other Rb-associated proteins, hsHec1p binds specifically to the hypophosphorylated form of Rb that reappears during M phase.

**The specific interaction between Rb and hsHec1p is reconstituted in yeast.** Wild-type Rb and the H209 mutant Rb were expressed under control of the *GAL1* promoter through *LEU2*-selectable plasmids (p415GAL1) in the same yeast strain that carries an *hshec1-113* mutant allele (59). As a negative control, wild-type Rb was also expressed in a yeast strain carrying the *hshec1-113EK* allele, which encodes a hshec1-113p without Rb-binding activity. The *hshec1-113EK* cells demonstrated no apparent difference in the temperature-sensitive (*ts*) phenotype compared with the *hshec1-113* cells (59).

Wild-type Rb coimmunoprecipitated with hshec1-113p but not with hshec1-113EK. The H209 mutant of Rb failed to form a complex with hshec1-113p (Fig. 2A, panel b). Consistent with a previous report (21), both hypophosphorylated and hyperphosphorylated forms of Rb were detected in these unsynchronized cells, but the H209 mutant was deficient in hyperphosphorylated forms. The abundance of hshec1-113p protein did not vary significantly when either Rb or the H209 mutant was expressed (Fig. 2A, panel c). These results suggested that the specific interaction between hsHec1p and Rb could be reconstituted in yeast cells.

To explore whether M phase-specific binding exists in yeast cells expressing Rb, the interaction was examined during cell cycle progression. Cells from the strain carrying the *hshec1-113* allele were induced to express Rb and then synchronized in early S phase by treatment with hydroxyurea. After release from treatment, an equal aliquot of cells was taken out every 20 min (Fig. 2B; lanes 1 to 10). hshec1-113p was coimmunoprecipitated by anti-Rb MAb in cells that entered M phase, according to DNA content analysis (Fig. 2C and D), and morphology was observed under the microscope. Similarly, hshec1-113p and Rb were coimmunoprecipitated in cells synchronized at metaphase with nocodazole (Fig. 2B, lanes 11 and 12) but not in cells released from nocodazole treatment for 1 h. These results indicated that the M phase-specific interaction between Rb and hsHec1p can also be reconstituted in yeast cells.

**Rb specifically enhances the fidelity of chromosome segregation.** The reconstitution of the specific interaction between Rb and hsHec1p in yeast cells provided an *in vivo* system for

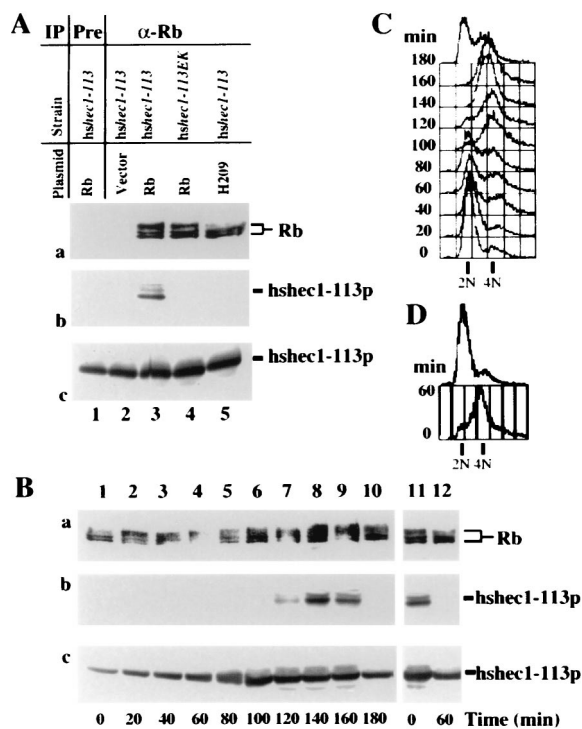


FIG. 2. Reconstitution of the interaction between Rb and hsHec1p in yeast. (A) Specific interaction between hsHec1p and Rb. Yeast cells were diluted to an optical density at 600 nm of 0.75 in fresh medium with 2% galactose and 2% raffinose and then cultured at 30°C for 4 h. Aliquots of cell lysate were immunoprecipitated (IP) with nonspecific IgG (lane 1) or with anti-Rb ( $\alpha$ -Rb) MAb 11D7 (lanes 2 to 5) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The same blot was probed with MAb 11D7 for Rb (a) or MAb 9G3 for hshec1-113p (b). Panel c shows the endogenous level of hshec1-113p in the cells used in panels a and b. For each lane in panel c, aliquots of the same lysates used in panel a were immunoprecipitated and immunoblotted with 9G3. The yeast strains and plasmids used to express Rb are indicated for each lane. (B) The yeast cells carrying the *hshec1-113* allele and an Rb expression vector were treated for 5 h with 0.1 M hydroxyurea or 20  $\mu$ g of nocodazole per ml in medium containing 2% galactose and 2% raffinose. At different time points after release (indicated under each lane [lanes 1 to 10, release from hydroxyurea; lanes 11 and 12, release from nocodazole]), cells were collected, lysed, and immunoprecipitated with  $\alpha$ -Rb MAb 11D7. The immunoprecipitates were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by immunoblotting with 11D7 for Rb (a) or with 9G3 for hshec1-113p (b). Aliquots of the same lysates were immunoprecipitated and immunoblotted with 9G3 (c). hshec1-113p was co-precipitated by Rb specifically at 120 to 160 min after release from hydroxyurea treatment, corresponding to G<sub>2</sub>/M phase, or metaphase arrest by nocodazole (time zero, lane 11). (C and D) The DNA content of the same cells used for panel B was analyzed by fluorescence-activated cell sorting as described previously (59).

investigation of the consequence of this interaction. If hsHec1p plays a crucial role in maintaining the fidelity of chromosome segregation as described previously (59), we surmised that Rb modulates hsHec1p and enhances this activity. To test this hypothesis, we examined the rate of chromosome missegregation by using the colony sectoring assay (33) after induction of Rb expression in the *hshec1-113* diploid strain. This strain was chosen because of its higher rate of chromosome segregation errors during mitosis (59). The total numbers of pink colonies (representing 1:1 segregation of a single dispensable chromosome fragment carried by this yeast strain), half-pink, half-red sectored colonies (representing 1:0 segregation), and half-white, half-red sectored colonies (representing 2:0 segregation) were counted. The rates of chromosome loss and nondisjunction in the first division were determined by the

TABLE 1. Rb reduces chromosome segregation errors

Relevant genotype	Plasmid	No. of colonies <sup>a</sup>	Missegregation rate (%)	
			1:0 events <sup>b</sup>	2:0 events <sup>c</sup>
hsHEC1		6,096	0.02	0.01
hshec1-113	Vector	6,687	1.47	0.56
hshec1-113	Rb	12,641	0.31	0.10
hshec1-113	H209	4,299	1.54	0.60
hshec1-113EK	Vector	5,392	1.48	0.46
hshec1-113EK	Rb	6,554	1.51	0.50

<sup>a</sup> Total number of pink colonies.

<sup>b</sup> Number of half-red, half-pink colonies divided by total number of pink colonies.

<sup>c</sup> Number of half-red, half-white colonies divided by total number of pink colonies.

frequencies of half-pink, half-red colonies and half-white, half-red colonies, respectively. As shown in Table 1, Rb expression decreased the frequency of chromosome segregation errors due to chromosome loss or nondisjunction by approximately fivefold. In contrast, expression of the H209 mutant of Rb or the vector alone had no effect. As another control, we examined the influence of Rb expression on the strain carrying the *hshec1-113EK* mutant; it had no significant effect.

The observed difference in the frequencies of chromosome missegregation is not due to the variable cell growth or cell cycle status, because expression of Rb or H209 has no significant effects on these processes in yeast cells carrying either wild-type *HEC1* alleles (21) or the mutant *hshec1-113* allele (data not shown). Therefore, the fidelity of chromosome segregation is enhanced specifically by interaction between Rb and hsHec1p.

**Rb enhances the ability of hsHec1p to suppress lethality caused by an *smc1* mutation.** hsHec1p plays an essential role in chromosome segregation in part through interacting with SMC1 protein, which, in a complex with SMC3, is involved in sister chromatid cohesion (24, 37, 54). The mutated *hec1p* fails to interact with Smc1p physically in the *hshec1-113* mutant cells at the nonpermissive temperature (59). Overexpression of Hec1p suppresses the lethal phenotype of the *smc1-2* mutant strain (1-1bAS172) at 37°C (55, 59). If Rb enhances the activity of mutated hshec1p in the maintenance of proper chromosome segregation, it is likely that Rb also enhances the activity of wild-type hsHec1p in suppression of defective chromosome segregation due to the *smc1* mutation. To test this hypothesis, we employed the yeast strain 2bAS273, which also carries the *smc1-2* mutant allele and has a lethal phenotype at temperatures above 33°C (D. Koshland, personal communication). The isogenic strain 4bWHL273, carrying the same *smc1-2* allele, was generated from 2bAS273 and transformed by plasmids expressing hsHec1p and Rb under control of the *GAL1* promoter. Cells overexpressing hsHec1p grew at 34°C, a nonpermissive temperature for this *smc1* mutant strain, while cells not overexpressing hsHec1p failed to grow, whether Rb was expressed or not (Fig. 3A). Interestingly, if the temperature was raised further to 35 to 36°C, cells overexpressing hsHec1p also failed to grow. Cells overexpressing both hsHec1p and Rb, however, continued to grow, whereas cells expressing the H209 mutant hardly survived at this higher temperature. Overexpression of hshec1-EK suppressed the *smc1-2* mutant at 34°C, but coexpression of Rb and hshec1-EK did not suppress it if the temperature was raised further (Fig. 3B). These results suggested that the specific interaction between Rb and wild-type hsHec1p results in the enhancement of the fidelity of

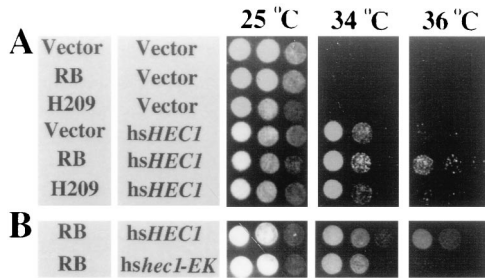


FIG. 3. Rb suppresses the ts phenotype of the *smc1-2* mutant through HsHec1p. (A) 4bWHL273 (*smc1-2*) cells were double transformed by *hsHEC1* in a *GAL1*-inducible and *URA3*-selectable vector (a YEplac195-based vector), by *RB* or the H209 *RB* mutant cDNA in a *GAL1*-inducible and *TRP1*-selectable vector (pESC::*TRP1*), or by the vectors alone. (B) 4bWHL273 cells were double transformed by *hsHEC1* or *hshec1-EK* in the *GAL1*-inducible and *URA3*-selectable vector and by Rb in the *GAL1*-inducible and *TRP1*-selectable vector. Different dilutions of log-phase cells grown at 25°C were inoculated on three plates with 2% galactose in the same manner and incubated at 25, 34, or 36°C.

chromosome segregation, probably mediated by the interaction between Hec1p and Smc1p.

**Specific binding of Smc1p to highly structured DNA.** SMC1 protein has been suggested to associate preferentially with highly structured DNA regions of chromatin, such as AT-rich DNA, bent DNA, and scaffold-associated regions (1, 22, 24, 29), and to mediate intermolecular cross-linking in sister chromatid cohesion (24). An in vitro binding assay for investigation of SMC1 DNA-binding activity has been established by using a 230-bp M13 RF DNA fragment (bp 6001 to 6231), which has a very high potential to form secondary structures, e.g., stem-loops, and therefore mimics highly structured DNA regions (1). The carboxyl-terminal region of SMC1 protein had been shown to mediate this specific DNA-binding activity (1).

To partially purify the Smc1p protein complex from yeast cell lysates, anti-Smc1p polyclonal antibodies (59) and a single-step immunoaffinity approach (25, 29) were employed. The affinity-bound proteins were eluted by the use of a highly concentrated GST fusion protein that had been used as the antigen to raise the antibodies (59). Excessive GST fusion protein was subsequently removed with glutathione-Sepharose. The affinity-purified fraction (APF) was incubated with the 230-bp M13 RF DNA fragment. Specific DNA-binding activity for the APF was detected by gel mobility shift assays (Fig. 4A, lanes 1 to 3). The abundance of the specific DNA-protein complex increased when more APF was added. Meanwhile, the mobility of the DNA-protein complex decreased and formed a more slowly migrating band (Fig. 4A, lane 3, bar). This stoichiometric effect is consistent with previous observations for the DNA-binding activity of another SMC-containing complex, the 13S condensin in *Xenopus* (29). This DNA-protein complex is not likely to be contaminated by the eluting antigen, which, encompassing the C-terminal DNA-binding region of Smc1p (1), formed a faster-migrating complex with the same DNA substrate (data not shown).

In order to determine the specificity of this DNA-binding activity, we also tested the APF from the *smc1-2* mutant cells cultured at 37°C, with *smc1p* inactivated (55). Our observation suggested that this mutated protein is unstable and barely detectable in these mutant cells cultured for 6 h at 37°C (Fig. 4B). No Smc1p-containing complex was obtained from these cells using the same purification procedure (Fig. 4C), and therefore, no DNA-binding activity was detected (Fig. 4A, lanes 4 to 6).

To determine whether this Smc1p-associated activity is spe-

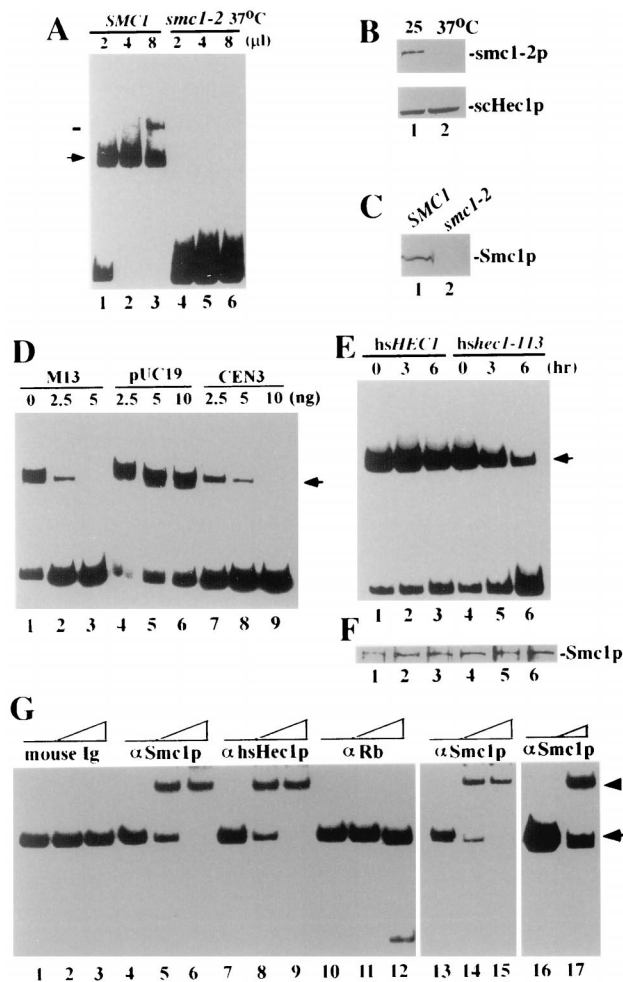


FIG. 4. DNA-binding activity of Smc1p and Hec1p complexes. (A) DNA-binding activity of Smc1p purified from equal numbers of wild-type cells (lanes 1 to 3) and *smc1-2* ts mutant cells (lanes 4 to 6) with a 230-bp M13 RF DNA fragment. The amount of concentrated protein in each lane is shown, and the position of the DNA-protein complex is indicated (arrow). Note that a slower-mobility complex (bar) appeared when more protein was added. (B) Immunoblotting by mouse anti-Smc1p polyclonal antibodies (upper panel) or by anti-scHec1p polyclonal antibodies (lower panel) of lysates from *smc1-2* ts mutant cells cultured at 25°C (lane 1) or 37°C (lane 2) for 6 h. (C) Immunoblotting of Smc1p purified from wild-type (lane 1) or *smc1-2* mutant (lane 2) cells cultured at 37°C for 6 h. (D) Competition of DNA-binding activity by unlabeled DNA fragments. The amount of competitor DNA added in each reaction is indicated above each lane. (E) DNA-binding activity of Smc1p purified from wild-type *hsHEC1* cells (lanes 1 to 3) or from the *hshec1-113* mutant cells (lanes 4 to 6) with the 230-bp M13 fragment. Cells were cultured at 25°C until log-phase growth and then shifted to 37°C for 0, 3, and 6 h before harvest. (F) Comparable amounts of Smc1p in each of the APFs were measured by immunoblotting with anti-Smc1p antibodies and were used for panel E. (G) Antibody supershift assay. Anti-Smc1p ( $\alpha$ Smc1p) antibodies and anti-hsHec1p MAb 9G3 supershifted the DNA-protein complex formed by APF from *hshec1-113* cells expressing Rb (lanes 1 to 12), but mouse IgG or anti-Rb MAb 11D7 did not. Anti-Smc1p also supershifted the DNA-binding complex formed by APF from *hshec1-113* cells not expressing Rb (lanes 13 to 15) and by APF from the wild-type *hsHEC1* cells (lanes 16 and 17). Lanes 1, 4, 7, 10, 13, and 16, no antibodies; lanes 2 and 3, 0.5 and 1  $\mu$ g of mouse IgG, respectively; lanes 5 and 6, 0.5 and 1  $\mu$ g anti-Smc1p antibody, respectively; lanes 8 and 9, 0.5 and 1  $\mu$ g of 9G3, respectively; lanes 11 and 12, 0.5 and 1  $\mu$ g of 11D7, respectively; lanes 14 and 15, 0.5 and 1  $\mu$ g of anti-Smc1p antibody, respectively; lane 17, 0.5  $\mu$ g of anti-Smc1p antibody. The original shift is indicated by an arrow, and the antibody supershift is indicated by an arrowhead.

cific to the highly structured DNA, the DNA-binding activity was competed by unlabeled DNA fragments containing the scaffold-associated region of *S. cerevisiae* CEN3. This centromere region was suggested to be a preferential binding site of SMC proteins and was able to compete with the M13 fragment in the *in vitro* DNA-binding assay of recombinant SMC1 (1). As shown in Fig. 4D, the Smc1p-associated DNA-binding activity that we detected in the yeast cells can also be competed by unlabeled CEN3 DNA and M13 DNA fragment but not by the region on pUC19 DNA with the least potential to form the secondary structures (1).

We also used a similar procedure to partially purify myc-tagged Smc1p from yeast cells overexpressing myc-Smc1p by use of anti-c-myc MAb and elution with the corresponding peptide. myc-Smc1p has the same DNA-binding activity (data not shown).

#### Hec1p modulates specific DNA-binding activity of Smc1p.

To test whether a deficiency in Hec1p activity affects the function of Smc1p, we examined the activity of Smc1p in the *hshec1-113* mutant yeast cells and compared it with that in cells expressing wild-type *hsHec1p*. Cells expressing wild-type *hsHec1p* or mutant *hshec1-113p* were cultured at the permissive temperature (25°C) and then shifted to 37°C for different periods of time. Equal numbers of cells were harvested and lysed. The resultant cell lysates from different samples contained comparable amounts of total proteins and were subjected to affinity purification of Smc1p. The DNA-binding activity of Smc1p in the wild-type cells did not change significantly after the cells were shifted to 37°C. In the *hshec1-113* mutant cells, however, this Smc1p activity dramatically decreased, and only less than 20% remained after 6 h at 37°C (Fig. 4E). The amount of Smc1p expressed in the *hshec1-113* cells was comparable to that in the wild-type cells (Fig. 4F), suggesting that the functional defect resulted specifically because of the mutated *hec1p*. The mobilities of the DNA-binding complexes from the wild-type cells and the mutant *hshec1-113* cells were very similar; only if gel electrophoresis was prolonged more than usual could they be distinguished (data not shown). It is therefore likely that Hec1p is present in the DNA-binding complex. As shown in Fig. 4G, anti-Hec1p antibodies and anti-Smc1p antibodies, but not anti-Rb antibodies or mouse immunoglobulin G (IgG), were able to supershift the DNA-binding complex. These results suggest that Hec1p is present in the complex with Smc1p to mediate the DNA-binding activity. Similar results were observed with APF from yeast cells expressing the wild-type Hec1p or cells not expressing Rb (Fig. 4G, lanes 13 to 17).

**Rb, through *hsHec1p*, enhances the DNA-binding activity of Smc1p.** If Rb enhances the fidelity of chromosome segregation, which may be mediated by the DNA-binding activity of Smc1p through *hsHec1p*, this Smc1p activity should increase in the cells expressing Rb. To test this hypothesis, we examined the DNA-binding activity of Smc1p in the same Rb-reconstituted yeast strains that had been tested for frequencies of chromosome missegregation. As in the colony sectoring assay, the cells were cultured at 30°C for 8 h while either Rb or the H209 mutant was induced. In the *hshec1-113* cells carrying only the empty vector, the DNA-binding activity of Smc1p decreased dramatically, to 30 to 40% of the wild-type level (Fig. 5A and C). These results are consistent with the abnormally high frequencies of chromosome missegregation in the same cells at the permissive temperature (Table 1). In cells expressing wild-type Rb, however, Smc1p DNA-binding activity was restored nearly to normal levels. Expression of the H209 mutant had no such effect, nor did wild-type Rb expression alone

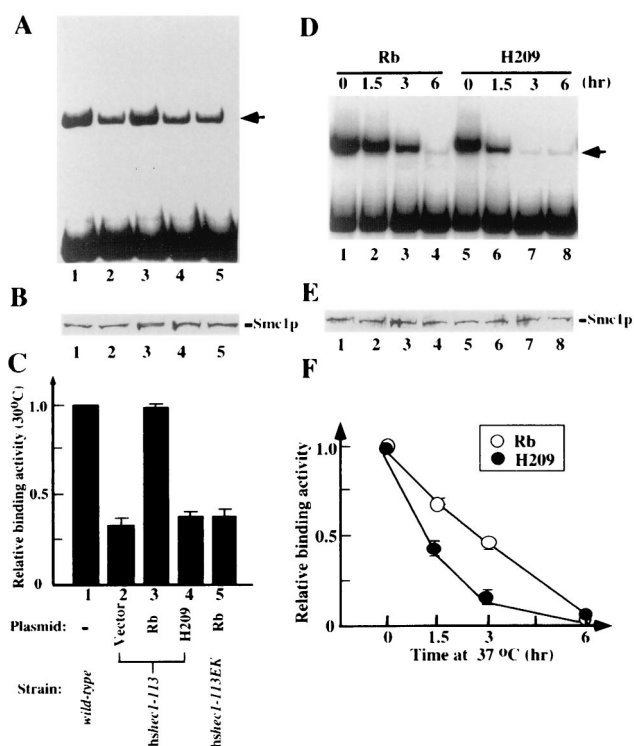


FIG. 5. Rb enhances the DNA-binding activity of Smc1p through *hsHec1p*. (A) DNA-binding ability of Smc1p purified from equal numbers of cells expressing various forms of Rb and *hsHec1p*. Expression of Rb and the H209 mutant was induced by addition of 2% galactose to the medium, and cells were cultured at 30°C in this medium for 8 h before harvest. (B) Immunoblot showing that comparable amounts of Smc1p were detected in each of the affinity-purified products used for panel A. (C) Histogram showing relative binding activity of Smc1p in each lane of panel A. (D) DNA-binding ability of Smc1p purified from *hshec1-113* cells expressing Rb (lanes 1 to 4) or the H209 mutant (lanes 5 to 8). Cells were cultured at 25°C in medium containing 2% galactose for 8 h to induce the expression of wild-type Rb or the H209 mutant and then shifted to 37°C for 0, 1.5, 3, or 6 h before harvest. (E) Immunoblot showing comparable amounts of Smc1p in each of the APFs used for panel D. (F) Effect of Rb on the DNA-binding activity of Smc1p in *hshec1-113* cells. The relative DNA-binding activity of Smc1p indicates the ratio between the quantified density result of each lane in panel D and that of lane 1 for Rb or lane 5 for H209. Bars represent standard errors from three separate experiments.

affect cells carrying the *hshec1-113EK* allele, which encodes a protein that cannot bind to Rb.

To further corroborate this finding, the dynamic effect of Rb on the activity of *hsHec1p* was examined. *hshec1-113* cells were cultured at 25°C in medium containing 2% galactose to induce the expression of Rb or the H209 mutant and then shifted to 37°C for different periods of time. As in the previous experiment (Fig. 4E), the DNA-binding activity of Smc1p began to decrease after cells were shifted to 37°C (Fig. 5D and F). This decrease of Smc1p activity, however, was significantly retarded during the first 3 h at 37°C in the cells expressing Rb compared with the cells expressing H209 mutant (Fig. 5F). By 6 h, Smc1p activity in both strains was very low. This result suggested that Rb can restore much of the activity impaired by mutation of *hshec1p* but cannot by itself complement the complete loss of *hshec1p*. Interestingly, Rb was not found in the Smc1p DNA-binding complex, since anti-Rb antibodies were not able to supershift the complex formed by the APF from *hshec1-113* cells expressing wild-type Rb (Fig. 4G). Rb thus appears to function like a chaperone, consistent with a previous proposal (6).

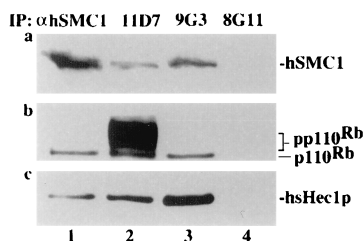


FIG. 6. Rb, hsHec1p, and hSMC1 protein form a complex in human cells. Asynchronous fast-growing human T24 cells ( $6 \times 10^6$ ) were lysed and immunoprecipitated (IP) by mouse anti-hSMC1 ( $\alpha$ hSMC1) antiserum (lane 1), anti-Rb MAb 11D7 (lane 2), anti-hsHec1p MAb 9G3 (lane 3), and anti-GST MAb 8G11 (lane 4). The immunocomplexes were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by immunoblotting with anti-hSMC1 antiserum to detect human SMC1 (a), with 11D7 to detect Rb (b), and with 9G3 to detect hsHec1p (c).

The above results suggest that a potential role of Rb in the modulation of SMC1 through hsHec1p exists and that both Hec1p and SMC1 proteins are functionally conserved from yeast to humans (24, 54, 59). It is therefore likely that Rb, Hec1p, and SMC1 may form a single complex in mammalian cells. To test this notion, hsHec1p and human SMC1 protein were coimmunoprecipitated with each other in human T24 cells (Fig. 6), consistent with our previous observation showing that the Hec1p-SMC1 interaction is conserved (59). Interestingly, the hypophosphorylated form of Rb was also coimmunoprecipitated. As a control, anti-GST MAb 8G11 did not coimmunoprecipitate any of these proteins (Fig. 6). These results suggest that Rb is present in a complex with Hec1p and SMC1 and support a potential role for Rb in modulating the activity of SMC1.

## DISCUSSION

In this study, we have employed a yeast system to address the function of Rb in chromosome segregation. Expression of Rb reduced chromosome segregation errors in cells carrying a mutant form of hsHec1p and enhanced the survival rate of *smc1* mutant cells with defects in chromosome segregation. Complexes of Hec1p and Smc1p play essential roles in chromosome segregation. Rb appears to chaperone Hec1p and indirectly to enhance the DNA-binding activity of Smc1p. These results reveal a novel biological activity of Rb intimately linked to its role in carcinogenesis and cancer progression.

The lack of an Rb homolog in yeast allowed us to address Rb function using yeast machinery as a powerful assay tool, without interference from endogenous Rb. Mechanisms similar to those governing Rb phosphorylation in mammalian cells have been demonstrated in yeast (21). However, no significant differences in cell morphology, growth rate, cell cycle progression, or mating pheromone response were observed in yeast cells expressing human wild-type or mutant Rb. These results suggest that Rb does not exert a function when yeast lacks specific cellular mediators of the antiproliferation and differentiation functions of Rb during  $G_1$  phase. Alternatively, potential mediators of such functions in yeast are unable to interact with Rb; such is the case with yeast Hec1p, which has no Rb-binding motif. In either case, the lack of both Rb and mediators of Rb function in yeast made it possible to exploit the yeast cell as an assay system for chromosome segregation and to reconstitute the interaction between hsHec1p and Rb in this system. This assay system ensures that the observed phenomena are direct and specific consequences of Rb expression and are specifically mediated by hsHec1p.

Expression of Rb decreased the frequency of chromosome segregation errors fivefold but was insufficient alone to rescue the yeast cells completely from aberrant mitosis. The fivefold enhancement is likely reminiscent of the physiological effect from a high-level regulator on the basic machinery for chromosome segregation. This improvement in the fidelity of chromosome segregation, however, would be quite significant in higher organisms, considering the millions of cells undergoing mitosis or meiosis daily. In the case of a lack of functional Rb, chromosome segregation errors in mammalian cells are expected to occur at a frequency similar to that for the wild-type yeast. Apparently, a higher fidelity of chromosome segregation is required for higher organisms to avoid errors in the more complicated chromosome segregation.

The biochemical mechanisms by which Rb modulates the activity of Hec1p and by which Hec1p modulates the activity of Smc1p remain to be elucidated. Our studies of DNA-binding activity of Smc1p from cells with different genetic backgrounds have provided some important clues leading to the understanding of these biochemical mechanisms. The DNA-binding activity of SMC1 is suggested to serve as a biochemical basis for its function in the chromatid assembly essential for sister chromatid cohesion and chromosome segregation (24). The modulation of this activity will undoubtedly affect the biological function of SMC1 in chromosome segregation, although other functions of Smc1p may also be influenced. It has been suggested that SMC1 forms complexes with various proteins, most of which, however, have not been revealed (24, 54). Our results indicate that Hec1p is present in the DNA-binding complex of Smc1p and also suggest that Hec1p is important for the biochemical activity of this complex. Consistently, the interaction between Hec1p and Smc1p is critical for proper chromosome segregation (59). Although purified recombinant protein containing the C-terminal region of SMC1 was shown to have the DNA-binding activity (1), it is likely that SMC1 requires other cofactors, such as Hec1p, to enhance its activity for a more stable binding of structured DNA. Rb appears to enhance the DNA-binding activity of Smc1p through Hec1p. This positive regulatory effect of Rb has also been observed in a number of transcription factors, such as MyoD (47), the glucocorticoid receptor (53), C/EBP $\beta$  (6), NF-IL6 (7), and c-jun (45). Our results showing that Rb is not a component of the DNA-binding complex formed by Smc1p and Hec1p suggest that Rb may serve as a chaperone for Hec1p, presumably by stabilizing its active conformation. Taken together, the results presented here suggest a potential role of Rb in regulation of SMC1 through hsHec1p.

The functional analysis of Rb in the heterologous yeast system is further supported by the *in vivo* interaction between Rb, Hec1p, and SMC1 in mammalian cells. The presence of Rb in a complex with Hec1p and Smc1p suggests the relevance of the novel Rb function revealed by the yeast study to mammalian cells where Rb exists. The complex formed between Rb and SMC1 indicates a biological role of Rb in the SMC1 activity, although Rb is not present in the DNA-binding complex of SMC1. Rb thus appears to modulate the activity of SMC1 before SMC1 binds to chromatin DNA. Unlike the activities of Hec1p and SMC1, this M phase activity of Rb does not appear to be required by either yeast or mammalian cells for their basic machinery of chromosome segregation or for cell survival. Nevertheless, such an activity of Rb in regulating SMC1 could be important for higher fidelity of chromosome segregation and higher integrity of mitotic chromosome structures in mammalian cells.

Whether loss of Rb function leads to a decrease in the fidelity of chromosome segregation in mammalian cells re-

mains to be explored. Nonetheless, such an activity for Rb may explain in part the abnormal process of mitosis observed in Rb-deficient fibroblasts and the chromosome abnormalities observed in Rb-deficient tumor cells (12, 28). It may also provide some clues for explaining observations that the majority of human retinoblastomas losing the wild-type allele and reduplicating the mutant allele early in the course of carcinogenesis result from nondisjunction and misproportioning of sister chromatids (4, 19). Interestingly, yeast or human cells lacking functional Hec1p complete mitosis with unseparated or unequally separated chromosomes and enter a new cell cycle, leading to hyperploidy and aneuploidy (10, 57, 59). This is similar to the phenomenon observed in Rb-deficient fibroblasts treated with nocodazole (12, 28). Since neither loss of Rb function nor loss of Hec1p function appears to affect the mitotic checkpoint control (28, 59), microtubule-destabilizing agents probably challenge the chromosome segregation process in Rb-deficient cells and thereby induce a high frequency of aberrant mitosis. These results indirectly support the role of Rb in chromosome segregation.

Taken together, the results of this study, using a heterologous yeast system, provide a useful assay and more direct evidence for revealing the mechanistic process of a novel function of Rb in chromosome segregation. The study thereby contributes to explaining a new critical role of Rb in human carcinogenesis.

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The first two authors contributed equally to this report.

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