Cyclin-dependent kinase subunit (Cks) 1 or Cks2 overexpression overrides the DNA damage response barrier triggered by activated oncoproteins

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Cyclin-dependent kinase subunit (Cks) proteins are small cyclin-dependent kinase-interacting proteins that are frequently overexpressed in breast cancer, as well as in a broad spectrum of other human malignancies. However, the mechanistic link between Cks protein overexpression and oncogenesis is still unknown. In this work, we show that overexpression of Cks1 or Cks2 in human mammary epithelial and breast cancer-derived cells, as well as in other cell types, leads to override of the intra–S-phase checkpoint that blocks DNA replication in response to replication stress. Specifically, binding of Cks1 or Cks2 to cyclin-dependent kinase 2 confers partial resistance to the effects of inhibitory tyrosine phosphorylation mediated by the intra–S-phase checkpoint, allowing cells to continue replicating DNA even under conditions of replicative stress. Because many activated oncoproteins trigger a DNA damage checkpoint response, which serves as a barrier to proliferation and clonal expansion, Cks protein overexpression likely constitutes one mechanism whereby premalignant cells can circumvent this DNA damage response barrier, conferring a proliferative advantage under stress conditions, and therefore contributing to tumor development.

Cyclin-dependent kinase subunit (Cks) proteins are small (9 kDa) cyclin-dependent kinase (Cdk)-interacting proteins that have been found frequently overexpressed in breast cancer (1, 2), as well as in a broad spectrum of other human malignancies (1, 3–6). Originally discovered in fission (7) and budding yeast (8), Cks proteins are expressed in all eukaryotic lineages, including the highly conserved paralogs Cks1 and Cks2 in mammals (9). The structural basis for the Cks-CDK interaction is well understood, because the heterodimeric complex has been determined by X-ray diffraction crystallography (10). In addition, the genetic analysis of Cks protein function in mammals is quite developed. KO mouse models have been generated for both Cks1 (11) and Cks2 (12), revealing individual specialized biological roles for the two paralogs. Cks1 has a specific function not shared by Cks2, by acting as an essential cofactor for the Skp1-Cul1-F-box protein (SCF)–Skp2 complex in targeting, among others, the Cdk inhibitors p27, p21, and p130 for proteolytic degradation by the 26S proteasome (11, 13). Cks2, on the other hand, is essential for meiosis, a phenotype resulting from the absence of both Cks proteins in germ cells, because Cks1 is not expressed in these cells, which are therefore completely deficient for Cks protein expression (12). The absence of both Cks1 and Cks2 results in very early embryonic lethality in mice, with the embryos dying before reaching the blastocyst stage (14). The shared essential redundant function of Cks proteins resides in their requirement for the G2- to M-phase cell cycle transition because they are vital for efficient transcription of a number of crucial cell cycle control genes (14). However, the precise molecular functions of Cks proteins remain mainly unknown. In addition, the mechanistic link between Cks protein overexpression and oncogenesis remains unknown. To elucidate the role(s) of Cks proteins in breast cancer development, we characterized the cellular phenotype of Cks protein overexpression.

Results and Discussion

In the course of a systematic analysis of the cellular phenotype conferred by Cks protein overexpression, we noticed that whereas HEK293A and hTERT-immortalized human mammary epithelial (IME) cell lines overexpressing either Cks1 or Cks2 exhibited similar cell cycle distributions to control cells (Fig. 1*A*, *Upper* and Fig. S1*A*), they were resistant to cell cycle arrest in the presence of high levels of thymidine (Fig. 1*A*, *Lower* and Fig. S1*A*). Thymidine, via allosteric inhibition of ribonucleotide reductase and concomitant depletion of deoxyribonucleotide triphosphate pools, causes replication stress and triggers the intra–S-phase checkpoint. Whereas control cells incubated in thymidine overnight arrested as a peak in early S phase, Cks1- and Cks2-overexpressing cells continued to progress through S phase, albeit slowly, presumably attributable to limiting deoxynucleotide triphosphate levels.

Cell cycle arrest in S phase in response to low deoxynucleotide triphosphate levels is mediated by the intra–S-phase checkpoint, which senses replication stress and prevents firing of new replication origins (reviewed in 15). To determine if Cks protein overexpression overrides the intra–S-phase checkpoint, we used DNA combing technology, outlined in Fig. 1*B* (16, 17), to monitor new DNA replication origin firing. New origin firing in the presence of the ribonucleotide reductase inhibitor hydroxyurea (HU) was compared in cells overexpressing Cks1 or Cks2 vs. controls (Fig. 1*C*). Whereas HU treatment reduced new origin firing in the control cell population, new origin firing was actually increased in both the Cks1- and Cks2-overexpressing populations, indicating a failure of the intra–S-phase checkpoint. To determine whether this phenomenon is physiologically relevant, particularly in the context of breast tumorigenesis, we compared the level of Cks2 mRNA overexpression in these retrovirally transduced cell lines with those in breast cancer-derived cell lines that overexpress Cks2. We decided to focus our studies mainly on Cks2 because, unlike Cks1, it is not involved in SCF-dependent degradation of Cdk inhibitors (11); therefore, any observed phenotype attribut-

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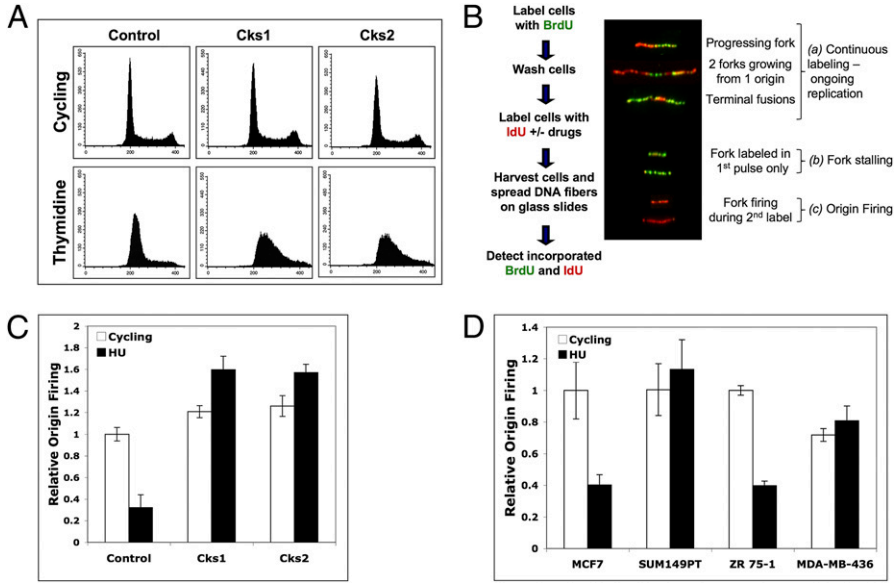

Fig. 1. Cells overexpressing Cks proteins are resistant to thymidine or HU arrest because of increased origin firing. (*A*) FACS profile of control and Cks1- and Cks2-overexpressing HEK293A cells (293A) either cycling (*Upper*) or arrested overnight in 2 mM thymidine (*Lower*). Stable cell lines were obtained via retroviral transduction and selection. (*B*) Outline of DNA combing assay method. Relative origin firing in control and Cks1- and Cks2-overexpressing 293A (*C*) and breast cancer-derived (*D*) cell lines when cycling or treated with HU as determined by DNA combing. Error bars shown in *C* and *D* represent SEM.

able to Cks2 overexpression is unlikely to be biased as a consequence of possible effects on the levels of Cdk inhibitors. Using published transcriptional array data (18), we selected several breast cancer-derived cell lines that overexpress Cks2 and compared the levels with those of HEK293A and IME cells, as well as with the retrovirally transduced populations used in Fig. 1 *A* and *C*. The breast cancer-derived cell lines expressed Cks2 mRNA at levels two- to fourfold higher than HEK293A and IME cells (Fig. S1*B*), whereas the retrovirally transduced HEK293A and IME cells expressed Cks2 mRNA at levels 15- to 20-fold higher than the control cells (Fig. S1*C*). Therefore, to determine whether intra–S-phase checkpoint override occurs at levels of Cks2 expression characteristic of breast cancer-derived cell lines that overexpress Cks2, we carried out a titration experiment using a recombinant Cks2 adenovirus in IME cells. With increasing Cks2 expression, there was progressive override of the intra–S-phase checkpoint, as determined by new origin firing in the presence of HU (Fig. S1*D*). Interestingly, saturation occurred at an expression level of three- to fourfold over that of the controls, which is typical of breast cancer-derived cell lines that overexpress Cks2 (Fig. S1*B*). Therefore, overexpression of Cks proteins overrides the intra–S-phase checkpoint at levels that are relevant to breast cancer development. To confirm that overexpression of Cks2 in breast cancer-derived cell lines correlates with override of the intra–S-phase checkpoint, we analyzed new origin firing in the cell lines SUM149-PT and MDA-MB-436, which express Cks2 mRNA at approximately fourfold the level in IME cells, and compared it with that in the MCF7 and ZR 75-1 lines, which do not overexpress Cks2 (18). We confirmed that the aggregate level of Cks1 and Cks2 protein was higher in SUM149-PT and MDA-MB-436 cells compared with MCF7 and ZR75-1 cells by carrying out Western blots (Fig. S1*E*). Whereas HU treatment of MCF7 and ZR 75-1 cells reduced new origin firing, similar treatment of SUM149-PT or MDA-MB-436 cells did not lead to a reduction of new origin firing (Fig. 1*D*), indicating checkpoint override when Cks2 is overexpressed in breast cancer-derived cell lines.

The intra–S-phase checkpoint in response to replication fork stress depends on a signaling pathway involving the kinases ataxia telangectasia-related (ATR) and Chk1. After replication fork stress is sensed, ATR activates Chk1 by phosphorylation at Ser345 (19–21). To determine whether this part of the signaling pathway is disrupted in cells overexpressing Cks proteins, we treated Cks1- and Cks2-overexpressing cells, as well as controls, with thymidine overnight and analyzed Chk1 phosphorylation status. As can be seen in Fig. 2*A*, thymidine treatment induced similar increases in Chk1 phosphorylation in all cell populations regardless of Cks protein overexpression. Activation of Chk1 leads to inactivation of Cdk2 by promoting phosphorylation-dependent turnover of the Cdk-activating phosphatase Cdc25A (reviewed in 22), thereby causing the accumulation of inactive Cdk2 that is phosphorylated on Tyr15 (23). We therefore compared the levels of Cdc25A in Cks-overexpressing and control cells treated with thymidine. As can be seen in Fig. 2B, overexpression of either Cks1 or Cks2 had no impact on the checkpoint-mediated reduction in Cdc25A levels caused by thymidine treatment. We then determined whether Cdk2 in cells overexpressing Cks proteins is equivalently phosphorylated on Tyr15 compared with controls. Cdk2 was immunoprecipitated from extracts of Cks-overexpressing and control cells, either untreated or treated with thymidine. Immunoprecipitates were then separated by SDS/PAGE and blotted with antibodies that detect phosphorylated Tyr15, followed by stripping and reprobing with Cdk2 antibodies. As shown in Fig. *C*, Cdk2 was equivalently phosphorylated on Tyr15 in Cks-overexpressing populations and controls. Therefore, Cks protein overexpression does not affect checkpoint signaling through modulation of Cdk2 inhibitory phosphorylation.

Because Cdk2 is phosphorylated on Tyr15 to equivalent levels in control and Cks-overexpressing cells, Cks proteins could override the intra–S-phase checkpoint by acting downstream of Cdk2 or by maintaining Tyr15-phosphorylated Cdk2 in an active state. To distinguish between these possibilities, we carried out DNA combing experiments to compare the effects of HU and roscovitine, a direct Cdk2 inhibitor, on control and Cks2-overexpressing cells. In control cells treated with roscovitine, replication origin firing was reduced to levels comparable to those of cells treated with HU (Fig. 3*A*), and the effects of roscovitine and HU were not additive, consistent with the observed checkpoint-mediated decrease in origin firing being a result of the inhibition of Cdk2. However, when Cks2-overexpressing cells were treated with roscovitine, origin firing was decreased in the presence of HU (Fig. 3*A*), arguing against the idea that Cks proteins act downstream of Cdk2 and supporting the hypothesis that Cks protein

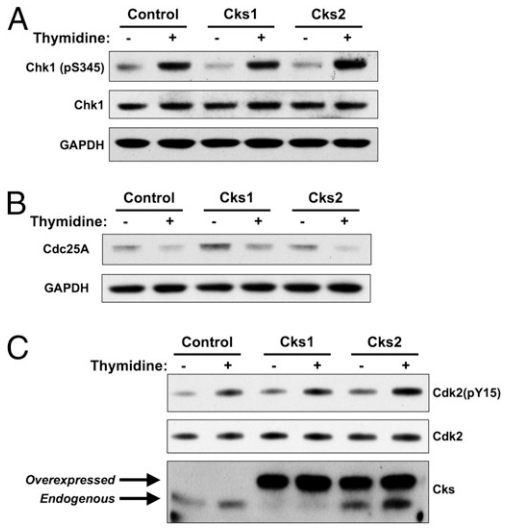

Fig. 2. The intra–S-phase checkpoint signaling pathway is intact in cells overexpressing Cks proteins. Western blot analysis of cycling or thymidine-treated HEK293A cell extracts for Chk1 phosphorylation (*A*) and Cdc25A degradation (*B*). (*C*) Western blot analysis of Tyr15 phosphorylation levels of Cdk2 protein immunoprecipitated from cycling or thymidine-treated HEK293A cells. (*Bottom*) Same blot probed with antibody that recognizes both Cks1 and Cks2.

binding maintains Tyr15-phosphorylated Cdk2 in an active state. To address this question further, we then immunoprecipitated Cdk2 from thymidine-arrested, control or Cks2-overexpressing cells, and analyzed Cdk2 kinase activity in vitro using recombinant retinoblastoma (Rb) protein as a substrate. Cdk2 from Cks2-overexpressing cells had increased kinase activity toward Rb, even though the Tyr15 phosphorylation levels are comparable to controls (Fig. 3*B*). The graph shows quantification of five different Cdk2 kinase experiments, demonstrating that the result is statistically significant ($P = 0.00055$, Student t test). Consistent with these results, in vitro phosphorylation of Rb at the Cdk2-specific residue Thr821 was increased when Cdk2 immunoprecipitates were prepared from thymidine-arrested cells that overexpress Cks2 compared with immunoprecipitates prepared from thymidine-arrested control cells (Fig. 3*C*). We directly confirmed this hypothesis by performing a reconstituted in vitro cyclin E/Cdk2 kinase assay using purified recombinant proteins. As can be observed in Fig. 3*D*, addition of Cks1 significantly increased the activity of fully Tyr15-phosphorylated Cdk2, previously incubated with Wee1 for 1 h (Fig. S2*B*). A Cks1 mutant defective in Cdk binding (Cks1 E63Q) (10) was inactive in this assay, indicating that Cdk2 activation depends on direct interaction. On the other hand, a mutant defective in phosphoprotein binding (Cks1 A.M.) activated Cdk2 similar to WT Cks1 (Fig. 3*D*). This triple mutant (K11E, S51E, and R71A) eliminates all anion contact residues required for phosphoprotein binding based on the crystal structure (10, 24), and the result argues against Cks1 in this context serving as a docking factor for partially phosphorylated Cdk2 substrates. The same activation profile was observed for non-phosphorylated Cdk2 (Fig. S2*A*), indicating that Cks-dependent activation of Cdk2 does not depend on Y15 phosphorylation. These results show that Cdk binding but not phosphoprotein binding is critical for Cks-dependent activation of Cdk2 kinase. Note that in these in vitro experiments, Cdk2 kinase activity is maximally inhibited by saturating Wee1 phosphorylation or not inhibited at all (Fig. S2*B*). However, checkpoint-associated Tyr15 phosphorylation in vivo occurs in a much narrower dynamic range, as is evident when comparing Cdk2 from cycling and checkpoint-arrested cells (Fig. 2*C*). Therefore, Cks1 binding in vivo is likely to restore checkpoint-inhibited Cdk2 activity to a level more similar to that observed in nonperturbed cells. Finally, because Cdk1 (like Cdk2) is inhibited by Tyr15 phosphorylation under conditions of replicative stress, we carried out a preliminary analysis of Cdk1 inhibition after treatment with thymidine and observed similar Cks-dependent reactivation (Fig. S2 *C* and *D*).

To confirm our in vitro results, we carried out DNA combing assays using WT and mutant Cks2 proteins. IME cells overexpresssing Cks2-E63Q, defective in Cdk binding, showed a reduction in origin firing similar to control cells when treated with HU (Fig. 3*E*), indicating that the Cks-dependent checkpoint override depends on Cdk2 binding. In contrast, a Cks2 allele defective in phosphoprotein binding (25) behaved similarly to WT Cks2 (Fig. 3*F*), confirming that checkpoint override does not depend on phosphoprotein binding.

Activation of oncogenes triggers DNA damage checkpoint responses, including the intra–S-phase checkpoint, which function as protective mechanisms against the generation of genomic instability and tumor development (26, 27). In fact, during malignant transformation, the DNA damage response precedes mutations in tumor suppressors, such as p53, thus functioning as an anticancer barrier specifically during early stages of tumorigenesis (26, 27). Cyclin E is an oncoprotein implicated in breast cancer etiology that has been shown to trigger the DNA damage response (28). To test the hypothesis that Cks protein up-regulation is an adaptive response that allows cells to evade the oncoprotein-induced replicative barrier, we overexpressed cyclin E in IME cells via adenoviral transduction and monitored both DNA replication and origin firing in the presence of normal or elevated levels of Cks2. As has been shown previously (29), overexpression of cyclin E decreases the rate of DNA replication, as measured by the rate of BrdU incorporation (Fig. 4*A*, with quantification in Fig. 4*B*). However, overexpression of Cks2 restores the normal rate of DNA replication (Figs. 4 *A* and *B*). To show that these effects are mediated by checkpoint activation and subsequent override, we carried out parallel DNA combing experiments. As shown in Fig. 4*C*, overexpression of cyclin E caused a decrease in origin firing in control cells, consistent with triggering of the intra–S-phase checkpoint. In contrast, cells overexpressing Cks2 were able to overcome the replicative barrier imposed by high levels of cyclin E and continued to fire replication origins, indeed at higher than normal levels (Fig. 4*C*). These data confirm that overexpression of Cks proteins may constitute a mechanism of escape from the oncoprotein-induced DNA damage response barrier during the course of breast cancer development. To test this hypothesis further, we screened a set of protein samples obtained from human breast carcinoma biopsies for levels of cyclin E by Western blotting and stratified them into three cohorts based on normalized levels of cyclin E (Table S1). The highest expressing ($n = 8$) and lowest expressing ($n = 8$) specimens were then screened for expression of Cks2 by quantitative real-time PCR. Even though overexpression of Cks2 does not have a direct effect on the levels of cyclin E (Fig. S3*A*) and overexpression of cyclin E does not affect the levels of Cks2 (Fig. S3*B*), the results in Fig. 4*D* show a very strong correlation between high cyclin E levels and high Cks2 levels in human breast tumor samples ($P = 0.0003$, Student's t test), consistent with a role for Cks2 in escape from oncogene-induced checkpoint barriers in breast tumor development. A correlation between high cyclin E levels and high Cks1 levels was also observed in these breast tumor samples (Fig. S3*C*), although it was not as strong as the correlation observed for Cks2. Finally, to investigate the generality of this mechanism, we determined the effect of Cks2 overexpression on the antiproliferative response of IME cells to expression of activated h-Ras. As can be seen in Fig. 4*E*, overexpression of activated h-Ras via retroviral transduction led to a significant proliferative defect resulting from oncogene stress. However, co-overexpression of Cks2 restored proliferation to an intermediate level relative to controls, suggesting that overexpression of Cks proteins constitutes a general mechanism for evading at least some oncoprotein-induced growth barriers. Indeed, it has been shown that down-regulation of checkpoint genes can override the antiproliferative effect caused by activated

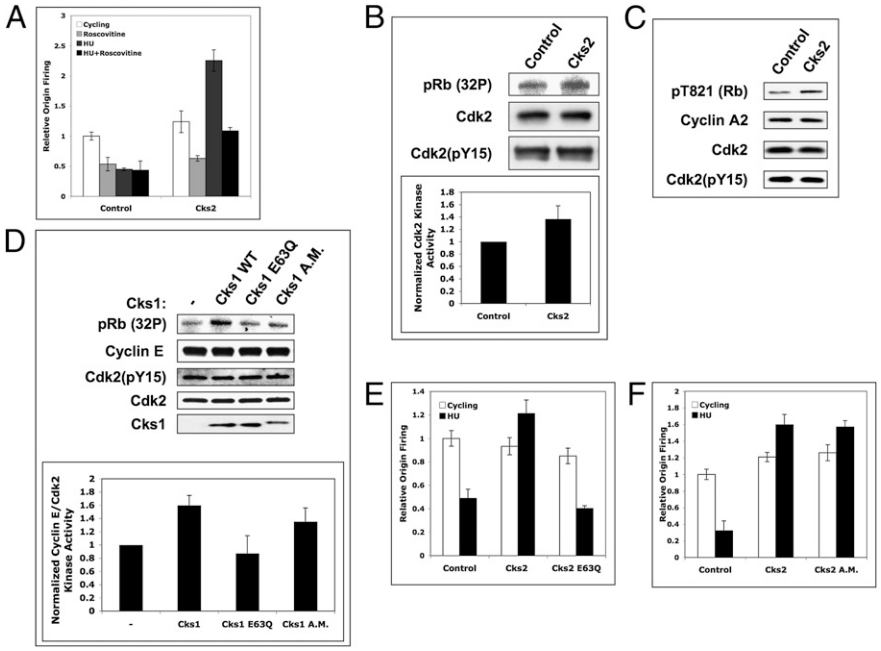

Fig. 3. Cks proteins override the intra–S-phase checkpoint by partially restoring the kinase activity of Tyr15-phosphorylated Cdk2. (*A*) Effect on origin firing of inhibition of Cdk2 by roscovitine (100 μM) in control and Cks-overexpressing HEK293A cells cycling or treated with HU. (*B*) In vitro kinase activity of Cdk2 immunoprecipitates from thymidine-arrested control and Cks2-overexpressing HEK293A cells, with the graph representing quantification of five different experiments. The substrate is recombinant Rb. (*C*) In vitro phosphorylation of the Cdk-specific T821 residue of Rb by Cdk2 immunoprecipitates from thymidine-arrested control and Cks2-overexpressing HEK293A cells. (*D*) In vitro kinase activity of recombinant CycE/Cdk2 purified from insect cells in the presence of either WT or mutant Cks1 proteins, with the graph representing quantification of three different experiments. Cks1 A.M. is a triple mutant (K11E, S51E, and R71A) incapable of anion or phosphoprotein binding. Relative origin firing of cycling and HU-treated IME (*E*) or HEK293A (*F*) control and indicated Cks2 mutant-overexpressing cells. Error bars represent SE (*A*, *E*, and *F*) or SD (*B* and *D*). (*E*) Adenoviral transduction of IME cells was used to analyze the Cks2 E63Q mutant, because this protein did not accumulate sufficiently using retroviral transduction.

h-Ras (30, 31). These results suggest that anticancer therapies that depend on robust S-phase checkpoint function might not be effective in cells overexpressing Cks proteins. Additionally, targeting Cks protein function might be a successful prophylactic strategy for individuals with a genetic predisposition to breast cancer and other malignancies. In fact, Cks1 deficiency is protective against tumor development in genetically engineered mouse models of lymphoma (32) and mammary tumorigenesis (Table S2).

If the role of Cks protein overexpression in tumorigenesis is to override the intra–S-phase checkpoint, it is likely that other elements in the signaling pathway would be found mutated or deregulated in tumors. This is indeed true, because CDC25A is frequently overexpressed (33, 34), whereas ATR and CHK1 are mutated (35–37). Interestingly, CDC25A overexpression synergizes with expression of activated oncoproteins, whereas hemizygous mutation of CDC25A is protective in a mouse mammary tumorigenesis model (38, 39), consistent with the idea that attenuation of the intra–S-phase checkpoint potentiates the efficacy of oncoproteins by removing replicative barriers.

The mechanism whereby Cks proteins activate Cdk2 remains to be elucidated. In this regard, the crystal structure of the Cks1-Cdk2 heterodimer is not informative, because Cks1 binding appears to confer minimal structural alteration on Cdk2 (10). However, that structural analysis was carried out on Cdk2 that was catalytically inactive because of a lack of both T-loop phosphorylation and cyclin binding. Cks protein binding might therefore have a greater structural impact on Cdk2 that is in an activated state. Alternatively, it is possible that the role of Cks proteins in the context described above is not allosteric but as a substrate-binding adapter, albeit one not dependent on phosphoprotein binding. Further experimentation will be required to distinguish between these models.

Experimental Procedures

Cell Culture. HEK293A and MCF7 cells were cultured in DMEM (Gibco) supplemented with 10% newborn calf serum (NCS; Gemini Bioproducts), 1% PSQ (2 mM L-Glutamine, 100 U/mL penicillin, 100 U/mL streptomycin; Invitrogen). IME cells were cultured in MCDB 131 (Gibco), with 1% NCS (Gemini Bioproducts), 1% PSQ, 70 μg/mL bovine pituitary extract (Hammond Cell Tech), 5 μg/mL human holotransferrin (Sigma), 0.5 μg/mL hydrocortisone (Sigma), 10 ng/mL human EGF (Invitrogen), and 5 μg/mL insulin (Sigma). MDA-MB-436 cells were cultured in DMEM/Ham's F-12 (Gibco), with 10% (vol/vol) NCS and 1% PSQ. SUM149PT cells were cultured in Ham's F-12, with 5% (vol/vol) NCS, 5 μg/mL insulin, and 1 μg/mL hydrocortisone. ZR 75-1 cells were grown in RPMI-1640, with 10% (vol/vol) NCS and 1% PSQ. Cks1 and Cks2 were stably introduced into cells via retroviral gene transfer. Briefly, empty, Cks1-expressing, or Cks2-expressing retroviral vectors were transfected by calcium phosphate into Phoenix cells, and supernatant was collected 48 h after transfection, filtered, and added to freshly seeded cells. After 48 h, cells were selected in 4 μg/mL puromycin (EMD). Mixed populations or individual clones were isolated and expanded, and expression levels were determined by real-time PCR and Western blotting.

Flow Cytometry. Cells were grown in monolayers for 18 h, with or without 2 mM thymidine (Sigma) and with or without 30 min of labeling with 20 μM BrdU (Sigma), and were then harvested by trypsinization, washed in PBS (Invitrogen), fixed in 70% (vol/vol) ethanol overnight, washed in washing buffer (PBS, 1% BSA, 0.5% Tween 20), and, for BrdU staining, treated with 0.1 mM borax (Sigma) for 1 h and then with 2 mM HCl before staining with fluorescin-labeled anti-BrdU antibodies (Phoenix Flow Systems). Cells were washed with washing buffer, stained using propidium iodide overnight at 4 °C, run on a FACSCalibur flow cytometer (Becton–Dickinson), and analyzed using CellQuest (Becton Dickinson).

Fluorescence Microscopy. Fluorescently labeled cells or DNA was examined on an Axioscope 2 microscope mounted with an AxioCam MRm monochrome charge-coupled device camera (both from Zeiss). A mercury lamp, conventional microscope optics, and selective wavelength filters were used. A Zeiss Plan-Neofluar 63X/1.25 oil immersion lens was used to visualize

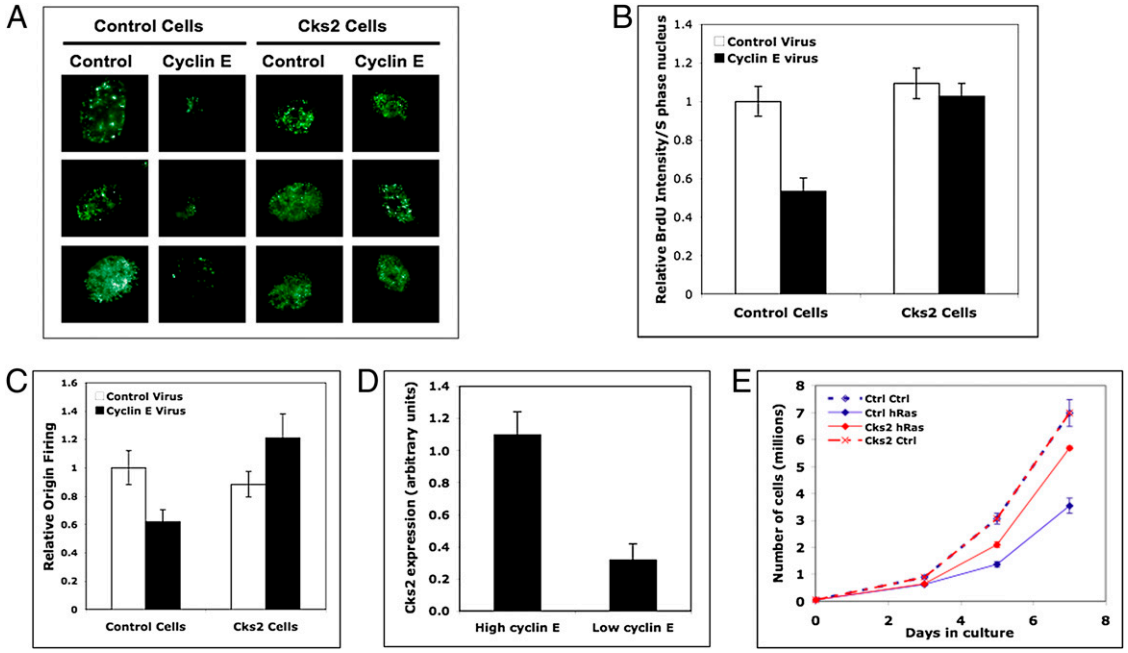

Fig. 4. Overexpression of Cks proteins enables cells to overcome the replicative barrier imposed by activated oncoproteins. (*A* and *B*) DNA replication rate measured by the rate of BrdU incorporation in control and Cks2-overexpressing IME cells transduced with either control or cyclin E-expressing adenovirus. (*C*) Relative origin firing of control or Cks2-overexpressing IME cells transduced with control or cyclin E adenovirus. (*D*) Relative levels of Cks2 mRNA measured by real-time PCR in breast cancer samples expressing low or high levels of cyclin E. (*E*) Growth analysis of control and Cks2-overexpressing IME cells expressing activated h-Ras. Error bars represent SE.

stained DNA. Images at appropriate wavelengths were captured using AxioVision software (Zeiss).

Immunofluorescence. Cells were grown in monolayers on coverslips. To incorporate BrdU, cells were incubated with 20 μM BrdU for 10 min, washed in PBS, and fixed for 1 h in MeOH, followed by 70% (vol/vol) EtOH at 4 °C overnight. Cells were permeabilized in 15% acetone and 15% (vol/vol) MetOH in 70 mM NaOH and were rinsed in 0.07 M HCl. Cells were rinsed twice in PBS; incubated for 15 min in washing buffer [0.05 M Tris-HCl (pH 7.6), 0.3 M NaCl, and 0.02% Tween-20] and for 15 min in blocking buffer (1% BSA and 0.5% Tween-20 in PBS); and then incubated for 1 h with sheep–anti-BrdU antibody (M20105S; Biosite), washed three times for 20 min in washing buffer, and incubated for 1 h with FITC-conjugated donkey–anti-sheep secondary antibody (Jackson ImmunoResearch) diluted in blocking buffer. Cells were washed in washing buffer three times for 20 min, mounted in ProLong Gold antifade mounting medium containing DAPI (Invitrogen), and analyzed by microscopy. Images of 10 cells were analyzed for each sample. Cell nuclei were defined based on DAPI staining, and fluorescence intensity in the FITC channel was calculated as a measure of BrdU incorporation.

DNA Combing. Cells were grown in monolayers, labeled for 15 min with 15 μM BrdU (Sigma), washed in PBS, and labeled for 45 min with 150 μM iododeoxyuridine (IdU; Sigma) in the presence or absence of 2 mM HU (Sigma), with or without 100 mM roscovitine (LC Laboratories). Cells were harvested and washed in PBS, and DNA spreads were prepared as previously described (17). BrdU-labeled tracks were detected with rat anti-BrdU (Accurate) and Alexa-fluor488–conjugated chicken anti-rat secondary antibody (Invitrogen Molecular Probes), followed by IdU detection using mouse anti-BrdU/IdU (Becton–Dickinson) and Cy3-conjugated goat anti-mouse antibody (Jackson ImmunoResearch). Fibers were examined by microscopy. At least 100 replication tracks were analyzed for each experiment.

Immunological Procedures and Reagents. Cell lysates from cycling or thymidine-arrested cells (18 h in 2 mM thymidine) were prepared in Tris-HCl lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 50 mM Na_3VO_4, 1 mg/mL leupeptin, 1 mg/mL pepstatin, 2 mg/mL aprotinin], and protein concentrations were determined by Bradford protein assay (Bio-Rad). Protein extract was loaded onto SDS/PAGE gels, run for 4 h, transferred onto a PVDF membrane (Immobilon P or PS; Millipore), and immunoblotted with the following antibodies: Chk1 (Santa Cruz Biotechnology), Chk1-P (Cell Signaling), Cdc25A (Abcam), Cdk1 (Santa Cruz Biotechnology), Cdk2 (Santa Cruz Biotechnology), Cdk1/2(pY15) (Cell Signaling), Rb(pT821) (Epitomics), Cks1/2 (Santa Cruz Biotechnology), cyclin A (Santa Cruz Biotechnology), cyclin E (Santa Cruz Biotechnology), GAPDH (Abcam), and Ku86 (Santa Cruz Biotechnology). For immunoprecipitation, anti-Cdk1 (Santa Cruz Biotechnology) or anti-Cdk2 (Santa Cruz Biotechnology) beads were incubated for 3 h at 4 °C with 3 mg of total protein, washed three times with lysis buffer, boiled with SDS/PAGE loading buffer, and processed for immunoblotting. For in vitro kinase assay, Cdk1 or Cdk2 immunoprecipitates were washed three times with lysis buffer and one time with kinase buffer [20 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$] and were incubated in kinase buffer with 250 μg of recombinant Rb protein (Prolias), with or without ^{32}P γ-ATP (MP Biomedicals), for 1 h at 37 °C. Reactions were stopped with SDS/PAGE loading buffer, and samples were processed for immunoblotting. For reactions with ^{32}P γ-ATP, immunoblots were analyzed by autoradiography using X-ray film with intensifying screens (Cdk2 experiments) or a phosphorimager (Cdk1 experiment).

Reconstituted Cdk2 Kinase Assay on pRb. Five micrograms of His-Cdk2/CycE purified from insect cells was phosphorylated on Y15 by incubation with 0.5 μg of Gst-Wee1 (Abcam) for 60 min at 37 °C, in kinase buffer [20 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, 1 mM DTT, 250 μM ATP]. The same incubation of His-Cdk2/CycE without adding Wee1 was performed as a nonphosphorylated Cdk2 control. Wee1 was depleted from the reaction by incubation with 20 μL of glutathione beads for 2 h at 4 °C, and supernatant aliquots containing His-Cdk2/CycE were stored at −80 °C in kinase buffer with 30% (vol/vol) glycerol. For each His-Cdk2/CycE kinase reaction, 40 ng of Cdk2/CycE was incubated overnight at 4 °C with 15 ng of Cks1 in kinase buffer supplemented with 100 nmol of Hsp90 and 50 nmol of Cdc37, and kinase activity was assayed by incubation with 200 ng of recombinant C-terminal Rb peptide (Abcam), with ^{32}P γ-ATP (MP Biomedicals), for 1 h at 30 °C. Reactions were stopped and processed as described above.

Real-Time PCR. Total RNA was extracted from cells using RNeasy columns (Qiagen) according to the manufacturer's instructions. RNA (5 μg/mL) was reverse-transcribed, amplified, and quantified using iQ Sybr green Supermix (Bio-Rad) and a Chromo4 real-time PCR detector (Bio-Rad). Each sample was run at least in triplicate. Primers can be obtained on request. Expression levels were determined relative to a control mRNA, using the formula: $2^{CT(\text{control mRNA}) - CT(\text{mRNA of interest})} \times 10{,}000$, where CT is the threshold cycle. β-Actin, γ-actin, or GAPDH was used as

a control mRNA. SDs of the relative expression values were calculated, taking error propagation into account with the formula: $\{[2^{-CT(\text{mRNA of interest})} \times \ln 2 \times 2^{CT(\text{control mRNA})}]^2 \times SD_{CT(\text{control mRNA})}{}^2 + [2^{CT(\text{control mRNA})} \times \ln 2 \times 2^{-CT(\text{mRNA of interest})}]^2 \times SD_{CT(\text{mRNA of interest})}{}^2\}^{1/2} \times 10{,}000$.

Breast Tumor Samples. Breast cancer specimens from 22 patients treated at Innsbruck Medical University were analyzed. Tumor specimens were obtained immediately after surgery and brought to the pathologist, and part of the tissue was pulverized with liquid nitrogen and stored at −70 °C. The study was approved by the Institutional Review Board. Clinical, pathological and follow-up data were stored in a database in accordance with hospital privacy rules. Estrogen receptor status and progesterone receptor status were identified immunohistochemically.

Mouse Aging Study, Necropsies, and Histopathology. Animals either WT ($^{+/+}$), heterozygous ($^{+/-}$), or homozygous ($^{-/-}$) for Cks1 deletion and also carrying both a p53 heterozygous deletion and mammary tissue-specific expression of a stabilized cyclin E mutant were generated, and female mice were aged as described (40). Only female mice that reached the 18-mo end point or had to be euthanized because of the development of mammary tumors were used in this study. Necropsies were performed on these animals, and mammary tissues were dissected and fixed overnight in 10% buffered formalin solution (Sigma–Aldrich). Tissues were then washed in 70% (vol/vol) ethanol and routinely processed; they were then embedded in paraffin, sectioned to 6 μm, stained with H&E, and analyzed by light microscopy. For immunohistochemistry, paraffin-embedded sections were deparaffinized and rehydrated by xylene and a graded alcohol series. Antigen retrieval was performed by microwave in Vector Labs retrieval solution (pH 6.0; BD Pharmingen). Antibody staining and counterstaining with hematoxylin were performed as described (40), and primary antibodies for human cyclin E (Santa Cruz Biotechnology) and PCNA (Santa Cruz Biotechnology) were used.

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