raphy screening for the early detection of breast cancer, a question to be resolved by epidemiological studies.

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37. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
38. Flag-tagged NLS-Brca1 1351 to 1552 was constructed with the univector plasmid fusion system (*29*). The host vector is based on pCMV2-Flag (Sigma). The SV40 large T antigen NLS was inserted between the Hind III and Not I sites with the following primers: AGCTTCCCAAGAAGAAGAGGAAGGC and GGCCGCCTTCCTCTTCTTCTTGGGA. pUNI15 Brca1 1351 to 1552 was made with the same primers as those for pDC115 described above. The serine to alanine mutations were made by single-stranded mutagenesis with the following primers: S1423A, ACAGCATGGGGCCCAGCCTTCTAACAG; and S1524A, AAACTACCCAGCTCAAGAGGAACTCATTAAGGTTGTT. All mutations and PCR products were sequenced. Transfections were performed by standard calcium phosphate technique (*30*).
39. pBABEpuro HABrca1 was made by digesting pCDNA3βHABrca1 (*16*) with Sal I and Xho I and inserting it into the Sal I site of pBABEpuro. The serine to alanine mutations were inserted into wild-type Brca1 in pBABEpuro by exchanging a Pfl MI to Apa I Brca1 fragment from the mutated gene into pBABEpuro HABrca wild type. This deletes a portion of Brca1 because an Apa I site had been created by the introduction of the S1423A mutation. This deleted Brca1 segment was reintroduced as an Apa I to Apa I fragment derived from the full-length S1423A/S1524A mutant in pBSKII(−) reconstituting full-length HABrca1 S1423A/S1524A. Retrovirus was made by cotransfection of the pBABEpuro vectors with amphotrophic packaging DNA into 293T cells essentially as described (*30*). Viral supernatants were used to infect the HCC1937 cells. Two days after infection, cells were selected for puromycin resistance with puromycin (1 μg/ml; Sigma).

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Control of the DNA Damage Checkpoint by Chk1 and Rad53 Protein Kinases Through Distinct Mechanisms

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In response to DNA damage, cells activate checkpoint pathways that prevent cell cycle progression. In fission yeast and mammals, mitotic arrest in response to DNA damage requires inhibitory Cdk phosphorylation regulated by Chk1. This study indicates that Chk1 is required for function of the DNA damage checkpoint in *Saccharomyces cerevisiae* but acts through a distinct mechanism maintaining the abundance of Pds1, an anaphase inhibitor. Unlike other checkpoint mutants, *chk1* mutants were only mildly sensitive to DNA damage, indicating that checkpoint functions besides cell cycle arrest influence damage sensitivity. Another kinase, Rad53, was required to both maintain active cyclin-dependent kinase 1, Cdk1(Cdc28), and prevent anaphase entry after checkpoint activation. Evidence suggests that Rad53 exerts its role in checkpoint control through regulation of the Polo kinase Cdc5. These results support a model in which Chk1 and Rad53 function in parallel through Pds1 and Cdc5, respectively, to prevent anaphase entry and mitotic exit after DNA damage. This model provides a possible explanation for the role of Cdc5 in DNA damage checkpoint adaptation.

Arrest of the cell cycle in response to the DNA damage checkpoint in *Saccharomyces cerevisiae* does not require inhibitory phosphorylation of Cdk1 (*1, 2*), and cells arrest in metaphase with active Clb/Cdk1 (*3*). Thus, given the role of Chk1 in Cdk phosphorylation in other systems, it seemed unlikely that *S. cerevisiae* Chk1 (YBR274w) would be required for checkpoint control. Disruption of *CHK1* revealed that it is not essential, and *chk1* mutants grown asynchronously were not sensitive to γ- or UV-radiation (*4*). How-

ever, *chk1* mutants were mildly sensitive to ionizing radiation when synchronized in G_2/M with the anti-microtubule drug nocodazole (*4*). The *chk1* mutants were not sensitive to hydroxyurea (HU) and had no defects in the S phase checkpoint or the transcription of genes induced by DNA damage (*4*).

The integrity of the DNA damage checkpoint in *chk1* mutants was examined in *cdc13chk1* mutant strains. The *cdc13-1* mutants accumulate single-stranded DNA at the non-permissive temperature and exhibit a checkpoint-dependent pre-anaphase arrest (*5, 6*). In contrast, *cdc13chk1* double mutants failed to arrest and progressed through multiple cell cycles (Fig. 1A). When cells were arrested with nocodazole, treated with UV-irradiation, and then released, *chk1* mutants also failed to delay the cell cycle as wild-type (WT) cells do (Fig. 1B). Thus, Chk1 is required to prevent mitosis in the presence of DNA damage. Passage through mitosis in the presence of DNA damage is thought to be a catastrophic event because *cdc13* mutants with mutations in checkpoint genes such as *rad9*, *mec1*, and *rad53* exhibit a greater loss of viability than do cells in which only *cdc13* is mutated (*5*). In contrast, *cdc13chk1* mutants did not show enhanced lethality (Fig. 1C). Furthermore, *rad9* was epistatic to *chk1*; *cdc13rad9chk1* and *cdc13rad9* mutants exhibited a similar viability loss. These results indicate that defects in *cdc13rad9* mutants other than the failure to arrest the cell cycle may account for enhanced lethality.

To explore Chk1 regulation, we examined HA-Chk1 in cells exposed to UV or γ-IR or treated with methyl methane sulphonate (MMS) and observed that Chk1, like that of *Schizosaccharomyces pombe* (*7*), underwent a shift in electrophoretic mobility that could be reversed by phosphatase treatment (Fig. 1D). Chk1 modification was dependent on *MEC1* and *RAD9* but independent of *RAD53* (Fig. 1E). Furthermore, activation of Rad53 was not dependent upon *CHK1* (*4*). Like Rad53 (*8, 9*), Rad9 interacted with Chk1 in two-hybrid analysis (*4*) [Web figure 1 (*10*)]. These results indicate that both Rad53 and Chk1 are regulated by Mec1 and Rad9.

The Dun1 protein kinase is regulated by Rad53 (*11*) and is also required for metaphase arrest in response to DNA damage (*12*). Chk1 appears to function in a different pathway from Rad53 and Dun1 because *chk1rad53* and *chk1dun1* double mutants were more sensitive to DNA damage than single mutants (Fig. 2A) (*4*). We examined the relative contribution of Chk1 and Rad53 to cell cycle arrest. We synchronized *cdc13* strains containing *mec1*, *rad53*, or *chk1* mutations in G_1 with α-factor and then incubated cells at 32°C to activate the DNA damage checkpoint pathway (Fig. 2B). The *cdc13mec1* mutants completed mitosis with a 15-min delay relative to that of WT cells, whereas *cdc13chk1* and *cdc13rad53* mutants showed an additional 20- to 60-min delay. This delay was abolished in a *cdc13chk1rad53* mutant, which completed mitosis with identical kinetics to those of the *cdc13mec1* mutant (Fig. 2B). These data indicate that Chk1 and Rad53 participate in two distinct pathways that together are responsible for *MEC1*-dependent cell cycle arrest.

Progression through mitosis is regulated

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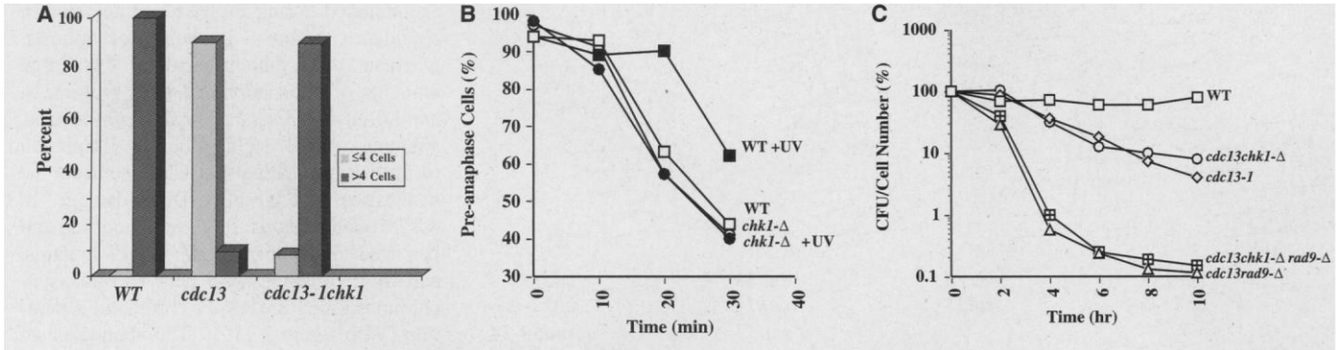

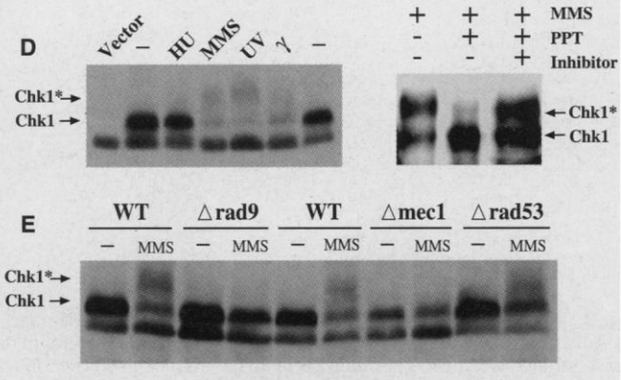

Fig. 1. Analysis of Chk1 and its regulation in response to DNA damage. (**A**) *chk1* mutants are defective in the DNA damage checkpoint. Y300 (WT), Y816 (*cdc13-1*), and Y836 (*cdc13-1chk1-Δ*) cells were grown at room temperature in YPD, then plated on prewarmed YPD plates (30°C) and incubated at 30°C. After 16 hours, cells were examined for microcolony formation. The light bars represent the percent of cells that exhibited a large-budded arrest, and the dark bars represent the percent of cells that formed a microcolony. (**B**) Failure of chk1 mutants to delay anaphase in response to DNA damage. Y300 (WT) and *chk1* (Y801) cells were synchronized in metaphase in YPD containing 10 μg/ml nocodazole, treated with 70 J/m^2 UV, and released into the cell cycle at 30°C. At indicated time points, samples were removed and processed for DNA staining by DAPI (4′,6′-diamidino-2-phenylindole) to evaluate nuclear morphology. (**C**) Failure of *chk1* mutations to enhance the lethality of a *cdc13-1* mutant. TWY397 (WT), TWY431 (*cdc13-1*), Y803 (*cdc13-1chk1-Δ*), TWY72 (*cdc13-1rad9-Δ*), and Y802 (*cdc13-1rad9-Δchk1-Δ*) cells (*28*) were grown to log phase in YPD medium at room temperature, then shifted to 37°C. Aliquots were taken every two hours, analyzed for total cell numbers and plated on YPD plates at room temperature to measure viability. Data points in (C) are expressed as the percentage of colony-forming units/cell number (normalized to the initial timepoint). (**D**) Modification of Chk1 in response to DNA damage. (Left) WT cells (Y300) containing either empty vector (pRS415) or pML107(pRS415-HA-Chk1) (*28*) were grown at 30°C in SC-Leu medium to log-phase, then DNA-damaging agents were introduced: HU (0.2 M); MMS (0.1% v/v); UV(50 J/m^2); and γ irradiation (15 krad). After1 hour, cells were harvested for protein immunoblotting (*29*). (Right) Cells containing pML107 were treated with MMS as above, and HA-Chk1 was immunoprecipitated and immunoblotted with anti-HA antibodies. Immunoprecipitates were analyzed alone or treated with alkaline phosphatase with or without phosphatase inhibitor as indicated. Asterisk denotes phosphorylated Chk1. (**E**) WT (Y300), *Δrad9* (Y438), WT (Y607), *Δmec1* (Y581), and *Δrad53* (Y608) cells containing pML107(pRS415-HA-Chk1) were treated with MMS (0.1% v/v) and analyzed as in (A).

by the anaphase-promoting complex (APC), which exists in two forms; APC^{CDC20} triggers chromosome segregation by degrading Pds1, whereas APC^{CDH1} triggers mitotic exit through the degradation of mitotic cyclins. Pds1 is required for cell cycle arrest after DNA damage (*13*) and is phosphorylated in response to DNA damage in a *MEC1*-dependent, *RAD53*-independent manner (*14*). To test whether Chk1 contributes to damage-induced phosphorylation of Pds1, WT, *cdc13*, and *cdc13chk1* mutants were released from α-factor at 32°C and examined for Pds1 modification. Pds1 exists in two forms and both exhibited a reduced electrophoretic mobility in *cdc13* mutants (Fig. 3A) (*15*). This reduced mobility was abolished in *cdc13chk1* mutants but was unaltered in *cdc13rad53* mutants (Fig. 3A). To determine if Chk1 could bind and phosphorylate Pds1 directly, we infected insect cells with baculoviruses encoding either Gst-Chk1, a catalytically inactive Chk1 mutant (Gst-$Chk1^{kd}$), or Gst-Rad53 along with a baculovirus encoding Myc-Pds1. The Gst-fusion proteins were affinity purified and immunoblotted for Pds1. Seven percent of the total Myc-Pds1 was bound by Gst-Chk1, but not by Gst-Rad53 (Fig. 3B). The Myc-Pds1 associated with Gst-Chk1 was of a slower electrophoretic mobility than that bound to Gst-$Chk1^{kd}$ and similar to that of endogenous Pds1 in the presence of DNA damage. The mobility shift was reversed by phosphatase treatment (*4*). Incubation of kinase-bound Myc-Pds1 with [γ-^{32}P]ATP showed phosphorylation of Myc-Pds1 in the Gst-Chk1 but not Gst-$Chk1^{kd}$ preparations (Fig. 3C), suggesting that Pds1 can be phosphorylated directly by Chk1.

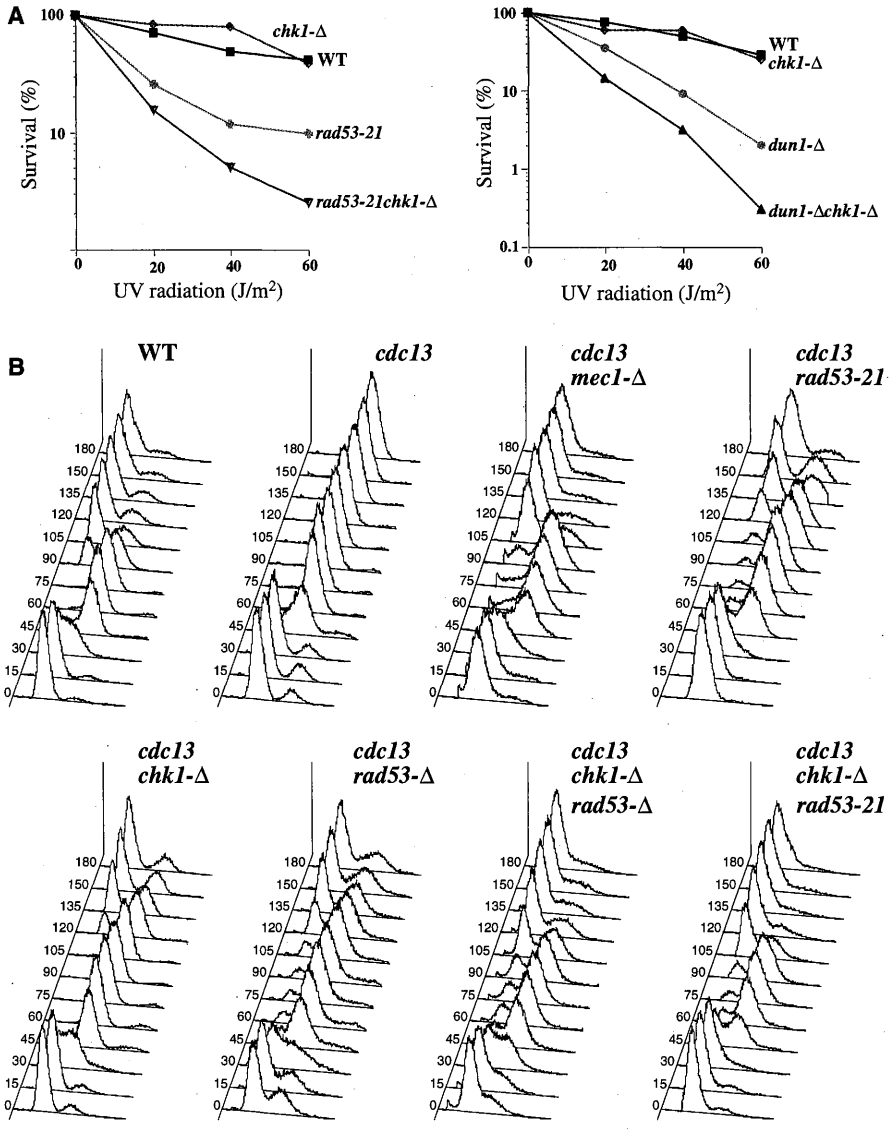

Fig. 2. Independent roles of CHK1 and RAD53 in the DNA damage checkpoint. (**A**) *CHK1* loss enhances the DNA damage sensitivity of *rad53* and *dun1* mutants. Logarithmic cultures of the yeast strains WT (Y300), *rad53-21* (Y301), *chk1*-Δ (Y801) *rad53-21chk1*-Δ (Y858), *dun1*-Δ (Y286), and *dun1*-Δ *chk1*-Δ (Y857) (*28*) were plated on YPD and treated with increasing doses of UV radiation. The viability was examined after 3 days at 30°C. (**B**) Requirement of Chk1 and Rad53 for delay of mitotic entry in response to DNA damage. Y808 (WT), Y809 (*cdc13-1*), Y812 (*cdc13-1mec1*-Δ), Y810 (*cdc13-1rad53-21*), Y811 (*cdc13-1chk1*-Δ), and Y814 (*cdc13-1rad53-21chk1*-Δ) (*28*) cells were synchronized in G_1 with α-factor at room temperature and released from the block at 32°C. The α-factor was added back to the culture after the majority of cells (≥80%) had budded to prevent a second cell cycle. Aliquots were withdrawn at timed intervals to examine DNA content by fluorescence-activated cell sorting (FACS) analysis.

If Chk1 functions primarily to control Pds1, then cells containing null mutations in both *chk1* and *pds1* should exhibit a checkpoint defect equivalent to that of *chk1* and *pds1* single mutants. The kinetics of anaphase entry were analyzed in strains synchronized by exposure to hydroxyurea (HU) and released into the cell cycle (*16*). The *cdc13pds1*, *cdc13chk1*, and *cdc13chk1pds1* mutants displayed quantitatively similar checkpoint defects (Fig. 3C), indicating that Pds1 is the principal checkpoint effector regulated by Chk1.

To determine whether Chk1 might function to maintain the abundance of Pds1, we examined the amount of Pds1 in cells exposed to DNA damage. In WT cells, Pds1 accumulated during S phase and declined in abundance before completion of mitosis, whereas *cdc13* mutants arrested with large amounts of phosphorylated Pds1 (Fig. 3, A and D). In *cdc13mec1* and *cdc13chk1* strains, amounts of Pds1 declined before completion of mitosis, indicating that Chk1 controls the abundance of Pds1 after DNA damage. In *cdc13rad53* mutants, Pds1 was modified and persisted longer than in *cdc13chk1* mutants, and this correlated with delayed and asynchronous kinetics of sister chromatid separation [Web figure 2 (*10*)]. The abundance of Pds1 in *cdc13rad53* mutants appears to depend upon *CHK1* because Pds1 destruction in the *cdc13rad53chk1* mutant resembled Pds1 degradation in *cdc13mec1* mutants. Rad53 also appears to contribute to the timing of Pds1 destruction because Pds1 degradation was 10 min faster in *cdc13rad53chk1* mutants than in *cdc13chk1* mutants.

If Chk1 functions to prevent Pds1 degradation, providing an indestructible Pds1 should restore cell cycle arrest in *chk1* mutants. We tested the effects of expressing a destruction box mutant Pds1 (dbmPds1) in synchronized WT, *cdc13*, *cdc13chk1* and *cdc13rad53* cells. In the absence of dbmPds1, WT cells reentered G_1 after 180 min; *cdc13* cells arrested in metaphase for the duration of the experiment, *cdc13chk1* cells underwent mitosis at 300 min, and *cdc13rad53* mutants underwent mitosis between 300 and 360 min (Fig. 4, A and B). Expression of dbmPds1 in WT cells

blocked anaphase (Fig. 4, A and B), but after a delay these cells exited mitosis, reinitiated DNA synthesis (Fig. 4A), and formed an additional bud on an already large budded cell in the absence of chromosome segregation (rebudding) (Fig. 4B), much like extra spindle pole body (*esp1*) mutants (*17*). Expression of dbmPds1 in *cdc13chk1* mutants blocked anaphase, and cells did not rereplicate or rebud, indicating a fully proficient checkpoint pathway. Although dbmPds1 expression blocked anaphase in *cdc13rad53*, these cells exhibited rebudding and re-replication similar to that of WT cells (Fig. 4, A and B), indicating a role for Rad53 in a pathway distinct from that which controls Pds1 degradation.

Rad53's checkpoint function has been elusive, and the observation that it functions in control of mitotic exit provides an important clue to unravel its function in anaphase, control. Exit from mitosis and rebudding requires inactivation of Clb-Cdk1. We therefore tested whether Rad53 might function to maintain high Clb2-Cdk1 activity. HA-Clb2-Cdk1 activity was measured in WT, *cdc13*, *cdc13rad53*, and *cdc13chk1* cells released from a G_1 block at 32°C (Fig. 4C). In WT cells, HAClb2-associated H1 kinase activity was absent in G_1, peaked before anaphase, and declined as cells reentered G_1. Kinase activity remained high during the *cdc13* induced checkpoint arrest. The *cdc13rad53* mutants showed a pattern of Clb2-Cdk1 activity similar to that of WT cells, although with a slightly delayed accumulation and decline. The amounts of kinase activity in *cdc13chk1* cells remained high throughout the experiment but in longer time courses began to decline just after completion of anaphase (165 min) (*4*). Thus, Rad53 is required to maintain Clb2-Cdk1 activity after damage.

Rad53 has a role in the prevention of both anaphase and mitotic exit in the presence of DNA damage. Inappropriate entry into anaphase apparently does not cause mitotic exit because preventing anaphase by expression of nondegradable Pds1 did not prevent mitotic exit. To determine whether activation of the mitotic exit pathway might cause inappropriate anaphase entry, we performed epistasis experiments using mutations in *CDC14*, a phosphatase, and *CDC5*, a kinase, that block mitotic exit by preventing activation of APC^{CDH1}. Cells with *cdc14* and *cdc5* mutations alone arrest with long spindles, degraded Pds1, and high Clb-Cdk1 activity (*18–20*) but have no change in the rate of anaphase entry in the absence of DNA damage (*4*). We examined the kinetics of anaphase entry of *cdc13rad53*, *cdc13rad53cdc14-1*, and *cdc13rad53cdc5-1* triple mutants released from a G_1 block at the nonpermissive temperature for *cdc13*, *cdc14*, and *cdc5*. The presence of *cdc14* blocked mitotic exit but did not slow the rate of anaphase in the *cdc13rad53* mutant (Fig. 5B), demonstrating that execution of mitotic exit does not affect anaphase entry. In contrast, *cdc13rad53cdc5-1* mutants displayed a delay in anaphase relative to that of *cdc13rad53* or *cdc5-1* strains (Fig. 5, A and B). This result suggested that Rad53 might inhibit Cdc5 function to control both anaphase entry and mitotic exit. Consistent with this idea, overproduction of *CDC5* forced *cdc13cdc14RAD53* cells through the metaphase-anaphase transition (Fig. 5C). Overproduction of Cdc5 can also force *cdc13* mutants through anaphase and mitotic exit at 34°C (*4*). Thus, Cdc5 activity is rate-limiting for both anaphase entry and mitotic exit in the presence of DNA damage.

The phenotype of *chk1* mutants is unique

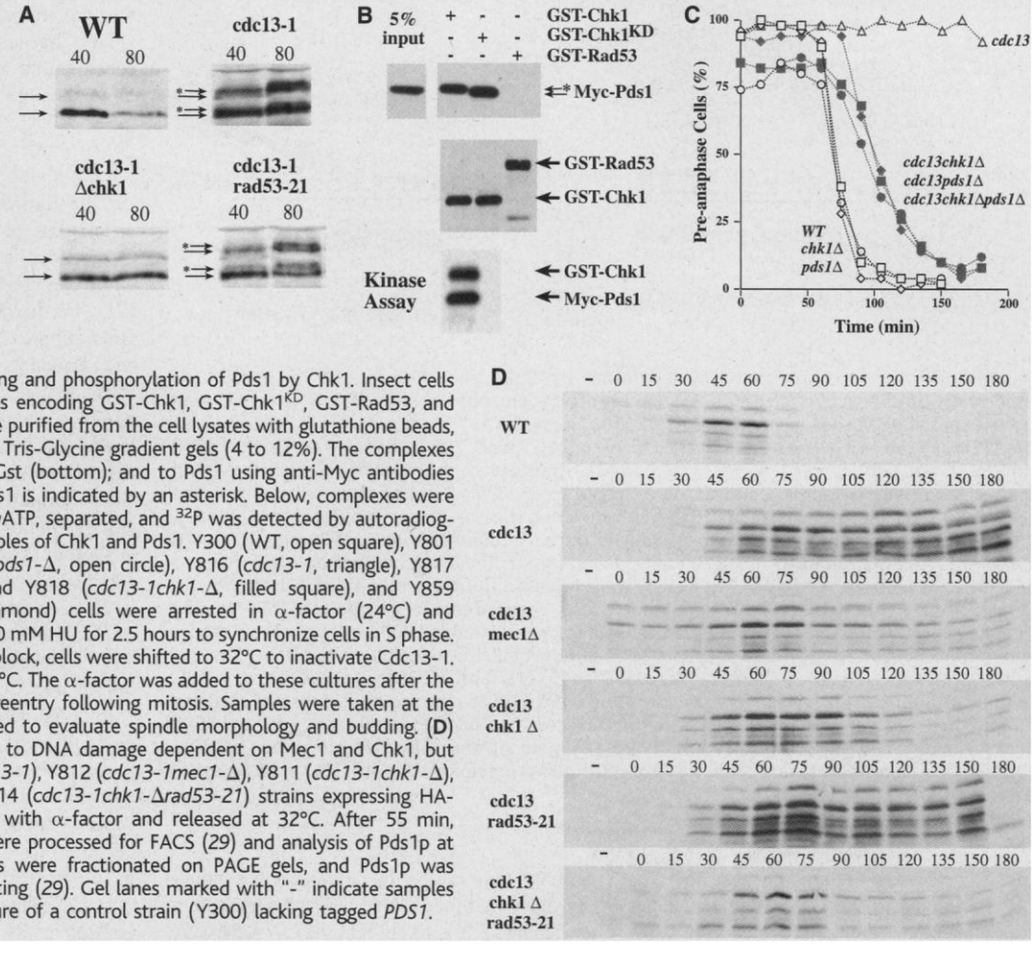

Fig. 3. Regulation of phosphorylation of Pds1 by Chk1 in vivo and in vitro. (**A**) Chk1-dependent phosphorylation of Pds1 in response to DNA damage. Y808 (WT), Y809 (*cdc13-1*), Y810 (*cdc13-1rad53-21*), and Y811 (*cdc13-1chk1-Δ*) strains were synchronized in G_1 and treated as in Fig. 2B. Aliquots were withdrawn at timed intervals to examine Pds1-HA protein levels (*29*) at 40 and 80 min following α-factor release. The arrows indicate different forms of Pds1-HA, and the shifted forms of Pds1 is indicated by an asterisk. (**B**) Binding and phosphorylation of Pds1 by Chk1. Insect cells (Hi5) were coinfected with viruses encoding GST-Chk1, GST-$Chk1^{KD}$, GST-Rad53, and Myc-Pds1. Gst-Chk1 proteins were purified from the cell lysates with glutathione beads, and complexes were separated on Tris-Glycine gradient gels (4 to 12%). The complexes were blotted with antibodies to Gst (bottom); and to Pds1 using anti-Myc antibodies (top) (*29*). The shifted form of Pds1 is indicated by an asterisk. Below, complexes were incubated in the presence of ^{32}P γATP, separated, and ^{32}P was detected by autoradiography. (**C**) Equivalent checkpoint roles of Chk1 and Pds1. Y300 (WT, open square), Y801 (*chk1-Δ*, open diamond), Y815 (*pds1-Δ*, open circle), Y816 (*cdc13-1*, triangle), Y817 (*cdc13-1pds1-Δ*, filled circle), and Y818 (*cdc13-1chk1-Δ*, filled square), and Y859 (*cdc13-1chk1-Δpds1-Δ*, filled diamond) cells were arrested in α-factor (24°C) and released into media containing 200 mM HU for 2.5 hours to synchronize cells in S phase. During the last 30 min of the HU block, cells were shifted to 32°C to inactivate Cdc13-1. Cells were released from HU at 32°C. The α-factor was added to these cultures after the HU release to prevent cell cycle reentry following mitosis. Samples were taken at the indicated timepoints and processed to evaluate spindle morphology and budding. (**D**) Accumulation of Pds1 in response to DNA damage dependent on Mec1 and Chk1, but not Rad53. Y808 (WT), Y809 (*cdc13-1*), Y812 (*cdc13-1mec1-Δ*), Y811 (*cdc13-1chk1-Δ*), Y810 (*cdc13-1rad53-21*), and Y814 (*cdc13-1chk1-Δrad53-21*) strains expressing HA-tagged Pds1p were synchronized with α-factor and released at 32°C. After 55 min, α-factor was added back. Cells were processed for FACS (*29*) and analysis of Pds1p at 15-min intervals. Protein extracts were fractionated on PAGE gels, and Pds1p was visualized by protein immunoblotting (*29*). Gel lanes marked with "-" indicate samples taken from an asynchronous culture of a control strain (Y300) lacking tagged *PDS1*.

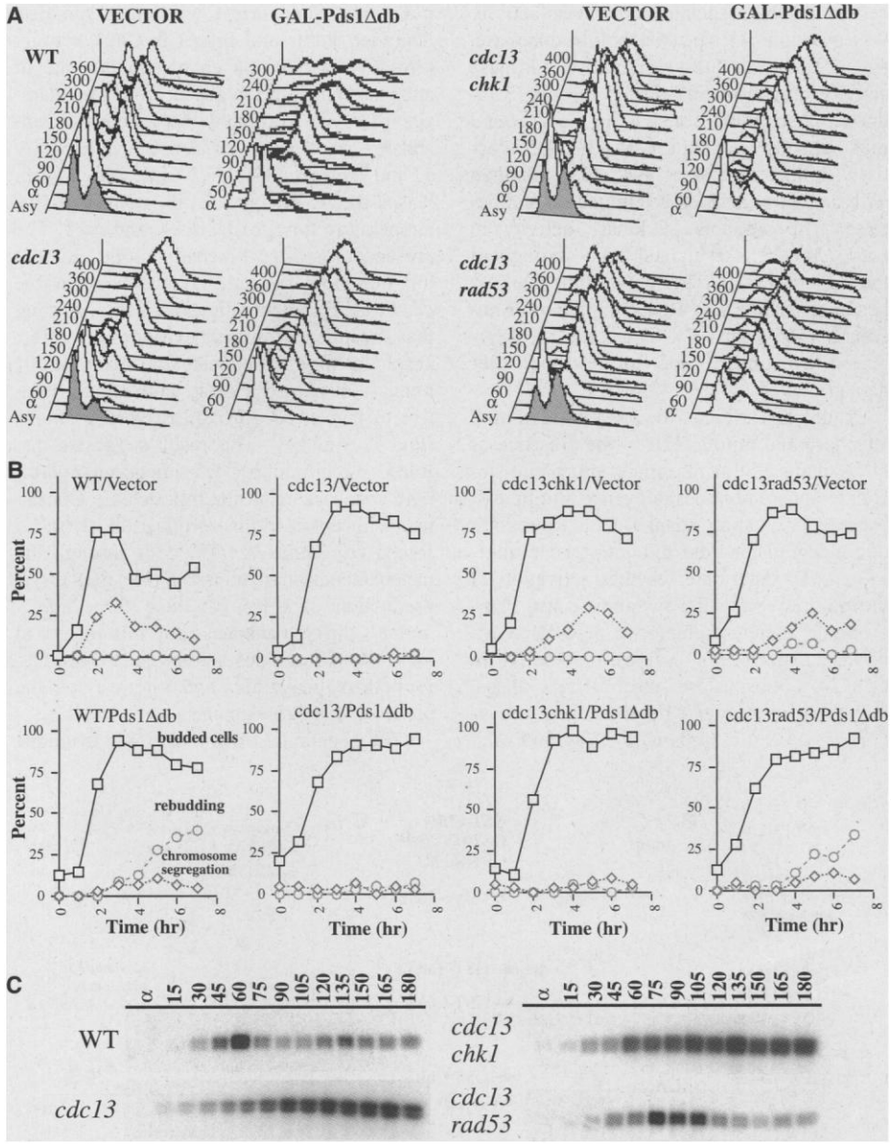

Fig. 4. Controls of sister chromatid separation by Chk1 and mitotic exit by Rad53. (**A**) Restoration of checkpoint arrest in *chk1* mutants by an indestructible Pds1. The following strains contained either vector alone (pRS416, *CEN URA3*) or pOC58 (*CEN URA3 GAL-dbmΔPDS1*) (*15*): WT (Y823 and Y826), *cdc13-1* (Y827 and Y830), *cdc13-1rad53-21* (Y832 and Y835), and *cdc13-1chk1-Δ* (Y837 and Y840). Strains were grown in SC-Ura raffinose and arrested in G_1 with α-factor. Cultures were shifted to 32°C for 1 hour before release from the block at 32°C. Galactose was added to the culture when most of the cells had entered S phase, as evidenced by budding, and galactose-induced transcription was shut down with glucose 70 min after induction. Aliquots of cells were removed to examine DNA content by FACS analysis (*26*). (**B**) Cells were treated as in (A), except were released from α-factor at 32°C into YP with 2% galactose. At the indicated times, aliquots were removed and processed for DAPI staining and cytological analysis. Squares, budded cells; diamonds, rebudded cells; circles, chromosome segregation. (**C**) Failure of *rad53* mutants to maintain Clb2/Cdk1 activity after DNA damage. Y300 (WT), Y816 (*cdc13-1*), Y831 (*cdc13-1rad53-21*), and Y836 (*cdc13-1chk1-Δ*) cells containing pMT812 (Clb2-HA) were synchronized in SC-Ura at room temperature and released at 37°C. The α-factor was added back after 80% of cells had budded to all strains except Y860 to prevent subsequent cycles. Portions were removed at the indicated times to examine Clb2/Cdk1 histone H1 kinase activity from Clb2-HA precipitated with HA antibodies as described (*30*).

among checkpoint mutants because they are only mildly sensitive to DNA damage. Thus, failure to arrest the cell cycle with DNA damage may not be the sole cause for lethality seen in other DNA damage checkpoint mutants (*5*). It is likely that the DNA damage response includes DNA repair processes that are not related to cell cycle arrest but are possibly more relevant to the survival of damaged cells. The *mec1*, *rad9*, and *rad53* mutants are also defective for these non-checkpoint pathways, whereas *chk1* mutants specifically influence cell cycle arrest.

This study reveals an additional function for Chk1 distinct from Cdk inhibition (*21*). Our results are consistent with a model (Fig. 5D) in which *MEC1* and *RAD9* cooperate to activate Rad53 and Chk1 after DNA damage. These kinases control distinct but mutually reinforcing pathways required to prevent cell cycle progression. The Chk1 branch controls phosphorylation and abundance of Pds1 to prevent anaphase entry. It is possible that phosphorylation of Pds1 prevents its degradation, but proof of this model will require identification and mutation of the sites phosphorylated in response to DNA damage. The Chk1 branch also helps prevent mitotic exit. This function probably also operates by preventing degradation of Pds1, since nondegradable Pds1 rescues both the anaphase entry and mitotic exit defect in *cdc13chk1* strains. The Rad53 branch also functions to prevent Pds1 degradation and to prevent activation of the mitotic exit pathway. However, control of the amount of Pds1 cannot be the only function of Rad53 in delaying anaphase because *pds1chk1* mutants show a considerable anaphase delay in response to DNA damage (Fig. 3C), whereas *rad53chk1* mutants have virtually no cell cycle delay. Other evidence also indicates Rad53 and Pds1 function in separate pathways (*22*).

The Rad53 branch of the DNA damage checkpoint pathway may function through Cdc5. Cdc5 is phosphorylated in response to DNA damage in a *RAD53*-dependent manner (*23*). Furthermore, we show that *cdc5* mutants suppress the checkpoint deficiency of *rad53* mutants and *CDC5* overproduction can override checkpoint arrest. The simplest model to explain our observations is that Rad53 inhibits the Cdc5 pathway, perhaps Cdc5 function itself. This model requires that Cdc5 normally has a role in promoting anaphase which must be inhibited in response to DNA damage. Although Cdc5 is not required for degradation of Pds1 (*19*), its homologs have been implicated in APC activation in other organisms (*24*) and could thus conceivably influence Pds1 stability. Cdc5 is required for cyclin degradation and mitotic exit, and a role for Rad53 in inhibiting Cdc5 could also explain the requirement for Rad53 in maintaining Clb2/Cdk1 activity. However, the mitotic exit aspect of Rad53 function is separable from regulation of anaphase entry because *cdc14* mutants block mitotic exit but have no effect on anaphase.

CDC5 has been implicated in checkpoint control in the adaptation pathway (*25*). The

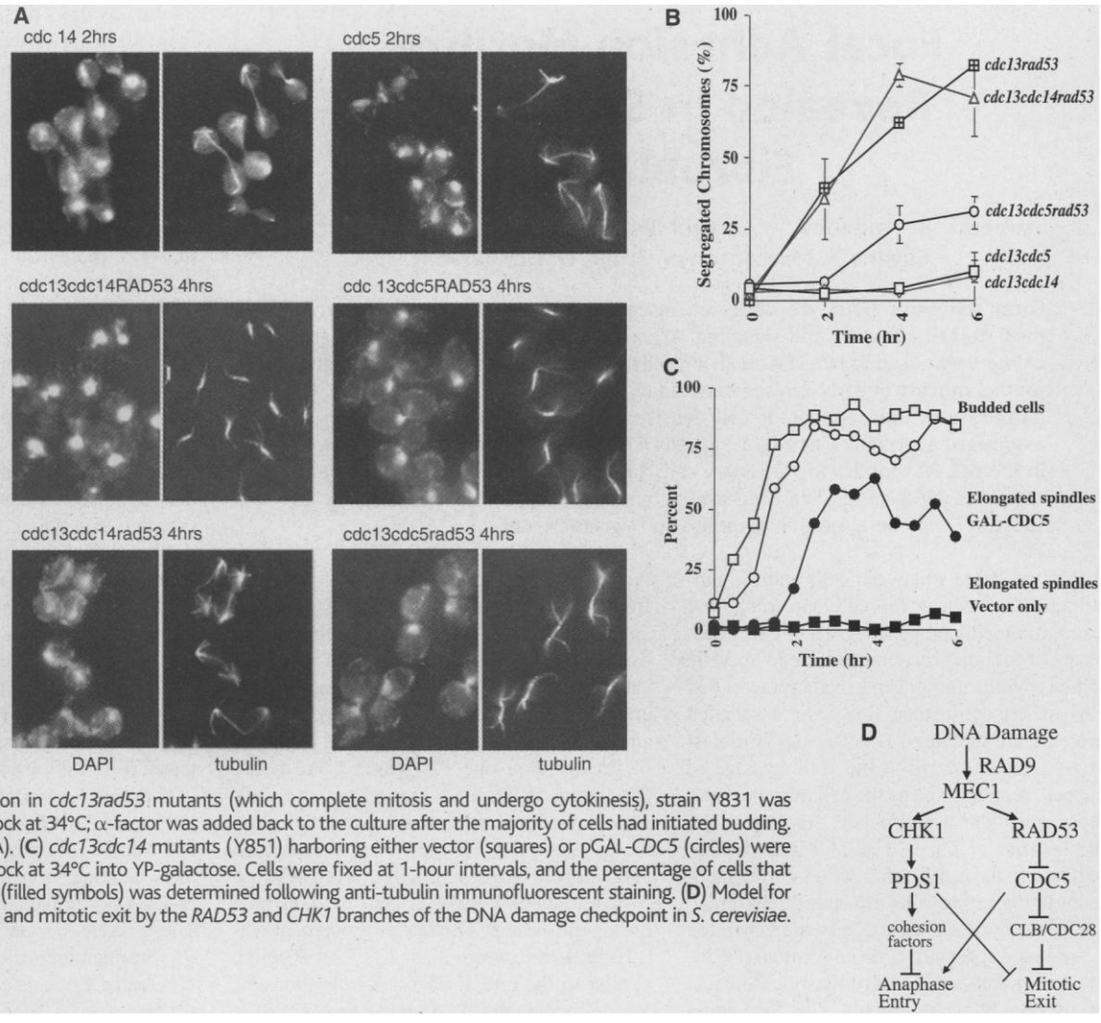

Fig. 5. Inactivation of Cdc5, but not Cdc14, can restore anaphase delay in *rad53* mutants. (**A**) Strains Y849 (*cdc14-1*), Y850 (*cdc5-1*), Y851 (*cdc13-1cdc14-1*), Y852 (*cdc13-1cdc5-1*), Y853 and Y854 (*cdc13-1cdc14-1rad53-21*), and Y855 and Y856 (*cdc13-1cdc5-1rad53-21*) were arrested in α-factor and released into fresh media at 34°C. At the indicated time points, samples were removed and processed for DNA staining by DAPI and anti-tubulin immunofluorescence to evaluate mitotic spindles. (**B**) The percentage of Y851 (*cdc13-1cdc14-1*), Y852 (*cdc13-1cdc5-1*), Y854 (*cdc13-1cdc14-1rad53-21*) and Y855 and Y856 (*cdc13-1cdc5-1rad53-21*) cells with segregated chromosomes was calculated from three separate experiments, as in (A). Error bars represent one standard deviation. To compare these results with chromosome segregation in *cdc13rad53* mutants (which complete mitosis and undergo cytokinesis), strain Y831 was released from an α-factor block at 34°C; α-factor was added back to the culture after the majority of cells had initiated budding. Cells were processed as in (A). (**C**) *cdc13cdc14* mutants (Y851) harboring either vector (squares) or pGAL-*CDC5* (circles) were released from an α-factor block at 34°C into YP-galactose. Cells were fixed at 1-hour intervals, and the percentage of cells that had elongated their spindles (filled symbols) was determined following anti-tubulin immunofluorescent staining. (**D**) Model for regulation of anaphase entry and mitotic exit by the *RAD53* and *CHK1* branches of the DNA damage checkpoint in *S. cerevisiae*. See text for details.

cdc13-1 mutants maintain cell cycle arrest for approximately 24 hours, then reenter the cell cycle with unrepaired DNA damage. An allele of *CDC5*, $cdc5^{AD}$, blocked this adaptation and allowed the *cdc13* mutant to remain arrested for more than 24 hours. One interpretation of our results is that the adaptation response may be due to a gradual weakening of the *RAD53*-branch of the checkpoint pathway. $cdc5^{AD}$ could represent a hypomorphic allele of *CDC5* capable of re-enforcing the *RAD53* branch of the checkpoint.

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28. Plasmid and strain construction: All strains were congenic with Y300 except those derived from the strains provided by T. Weinert (TW) as indicated and were derived with standard genetic techniques (*10*). *CHK1* (accession number AF117345) was disrupted by using the disruption cassette pYS51 (*chk1::HIS3*). *PDS1* was disrupted by using pAY55 (*13*). The *cdc14-1* allele was introduced into Y300 using the pSD231 cassette. The *cdc5-1* allele was backcrossed into the Y300 background five times from an initial cross with strain 11359-10-4A provided by D. Toczyski and L. Hartwell. Strains containing deletions of *MEC1* or *RAD53* are maintained by expression of *RNR1* under *GAP* control (*26*). The *RAD9* and *CHK1* ORFs were introduced into pACT-lox and pAS2-lox via the UPS reaction (*27*).

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Yolanda Sanchez; Brian A. Desany; William J. Jones; Qinghua Liu; Bin Wang; Stephen J. Elledge

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