Lack of PTEN sequesters CHK1 and initiates genetic instability

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Summary

***Pten*$^{-/-}$ cells display a partially defective checkpoint in response to ionizing radiation (IR). The checkpoint defect was traced to the ability of AKT to phosphorylate CHK1 at serine 280, since a nonphosphorylated mutant of CHK1 (S280A) complemented the checkpoint defect and restored CDC25A degradation. CHK1 phosphorylation at serine 280 led to covalent binding of 1 to 2 molecules of ubiquitin and cytoplasmic CHK1 localization. Primary breast carcinomas lacking PTEN expression and having elevated AKT phosphorylation had increased cytoplasmic CHK1 and displayed aneuploidy ($p < 0.005$). We conclude that loss of PTEN and subsequent activation of AKT impair CHK1 through phosphorylation, ubiquitination, and reduced nuclear localization to promote genomic instability in tumor cells.**

Introduction

Impairment of DNA damage checkpoint pathways is closely linked to cellular transformation (Elledge, 1996). Inherited mutations in the DNA damage checkpoint genes *ATM*, *p53*, *CHK2*, and *BRCA1* are associated with a predisposition to malignancy in humans (Bell et al., 1999; Cortez et al., 1999; Kastan et al., 1992; Malkin et al., 1990; Savitsky et al., 1995; Tibbetts et al., 2000). These genes protect the body from neoplasia by preventing genetic damage from being passed onto daughter cells. Depending upon the type of DNA damage, ATM or ATR kinase is required for initiation of the checkpoint response (Canman et al., 1998; Wright et al., 1998). ATM and ATR kinase phosphorylate a variety of substrates, including BRCA1, p53, CHK1, and CHK2 (Guo et al., 2000; Siliciano et al., 1997; Tibbetts et al., 2000). DNA damage activates the CHK1 protein and leads to a marked increase in the phosphorylation and destruction of CDC25A. As a result, cyclin/cdk complexes remain phosphorylated in an inactive state, and cells undergo a transient arrest in the G1, S, and G2 phases of the cell cycle (Mailand et al., 2000; Sorensen et al., 2003; Zhao et al., 2002).

In addition to the role of CHK1 in checkpoint activation, it participates in the control of the normal cell cycle, in part through the regulation of Cdc25A (Chen et al., 2003; Sorensen et al., 2003; Zhao et al., 2002). Reduction of CHK1 with RNAi in human cells results in increased CDC25A (Sorensen et al., 2003; Zhao et al., 2002). In some cells, CHK1 protein and its kinase levels are low in resting cells, increase in G1 and S phase, and fall in late G2/M (Kaneko et al., 1999). Conditional biallelic inactivation of *chk1* in mouse embryonic stem cells leads to numerical and structural chromosomal aberrations and apoptosis after only a few cell divisions (Liu et al., 2000; Takai et al., 2000). Therefore, CHK1 has a critical role in maintaining genomic stability in a normal cell cycle.

PTEN, a potent tumor suppressor, is inactivated in a large proportion of human tumors (Li et al., 1997; Steck et al., 1997). Inactivation of PTEN leads to increased levels of the phospholipid phosphatidylinositol-3,4,5-trisphosphate, which activates the PI-3 kinase signaling cascade (Maehama and Dixon, 1998;

SIGNIFICANCE

Upregulation of the PI-3 kinase pathway—whether through inactivation of PTEN or activation of PI-3 kinase and AKT—is frequently observed in human solid tumors. In mice, mutation of PTEN stimulates AKT kinase in normal cells but is not sufficient to induce tumorigenesis. Nevertheless, cells lacking PTEN have an enhanced capacity to evolve into tumors. Here we demonstrate that CHK1, a critical regulator of chromosomal stability, is impaired in cells lacking PTEN due to AKT-mediated phosphorylation at serine 280, which triggers ubiquitination and cytoplasmic sequestration. ATM kinase therefore has access to a smaller pool of nuclear CHK1 to initiate the DNA damage checkpoint. This impairment of CHK1 function contributes to the rapid rate at which PTEN mutant cells evolve into tumors.

Stambolic et al., 1998). Examination of the consequences of inactivation of PTEN in normal cells links it to the regulation of cell characteristics such as size, apoptosis, and proliferation status (Kishimoto et al., 2003). Aberrations in enzymatic signaling due to PTEN loss include activation of AKT kinase (protein kinase B) and p70 S6 kinase (Cantley and Neel, 1999; Lu et al., 1999). Cells lacking PTEN therefore have persistent phosphorylation of AKT substrates such as GSK3β, FOXO1, p27, MDM2, TSC2, and BAD, and S6K substrates such as ribosomal subunit S6 (Wishart and Dixon, 2002). Inactivation of PTEN is by itself insufficient to cause cellular transformation; however, over a brief period of time, a subset of cells lacking PTEN evolve into tumors (Sulis and Parsons, 2003). It has been observed in a small cohort of breast carcinomas that PTEN-deficient tumors tend to be aneuploid (Bose et al., 2002); therefore, it is possible that lack of PTEN stimulates genetic instability. This idea is supported by the observation that PTEN-deficient cells have attenuated function of p53, a critical checkpoint protein (Mayo et al., 2001; Zhou et al., 2001).

Several lines of evidence suggest a role for PTEN and the PI-3 kinase pathway in the regulation of CHK1. During the cell cycle, AKT kinase activity rises in G2/M, which is coincidental with the fall in CHK1 kinase activity (Shtivelman et al., 2002). Inhibition of the PI-3 kinase pathway with PTEN or a PI-3 kinase inhibitor leads to cell cycle arrest in G2 phase (Kandel et al., 2002; Saito et al., 2003; Shtivelman et al., 2002). Such inhibition is relieved by overexpression of constitutively active AKT kinase. Importantly, AKT inhibits CHK1 kinase activity, whereas PI-3 kinase inhibitor LY294002 enhances its kinase activity (Shtivelman et al., 2002). Review of the literature also supports the notion that the PI-3 kinase-PTEN-AKT pathway regulates CHK1-dependent checkpoint activation. A PI-3 kinase inhibitor shortens the IR-induced G_2 arrest (Blasina et al., 1999), whereas constitutively active AKT blocks hydroxyurea-induced activation of chk1 and DNA damage-induced arrest in G2 (Shtivelman et al., 2002). The ability of AKT to regulate CHK1 potentially occurs through a consensus AKT phosphorylation site at amino acid 280 of CHK1, which AKT is capable of phosphorylating in vitro and in vivo (King et al., 2004). Thus, the existing literature argues in favor of AKT regulating CHK1 function. In this report, we demonstrate that CHK1 nuclear localization is attenuated through phosphorylation and ubiquitination in PTEN-deficient cells, which leads to abnormal checkpoint control and genetic instability.

Results

Generation of Pten$^{-/-}$ embryonic stem cells

Mouse embryonic stem (MES) cells rely upon chk1 and chk2 to mediate the G_2 checkpoint activated by IR (Hirao et al., 2000; Liu et al., 2000; Takai et al., 2000). To investigate the role of the *PTEN* tumor suppressor in checkpoint regulation, we generated a series of *Pten* $^{-/-}$ (null) MES cell clones by mutating both *Pten* alleles with neomycin and hygromycin targeting constructs (Figure 1A) (Podsypanina et al., 1999). The mutations were designed to inactivate *Pten* by deleting the phosphatase domain. Multiple *Pten*$^{-/-}$ clones were identified (Figure 1B) and confirmed for their diploid karyotypic status (data not shown). *Pten*$^{-/-}$ clones expressed no detectable Pten protein (Figures 1C and 1D). Such cells had enhanced Akt phosphorylation and kinase activity as measured by immunoblot with antibodies that detected phospho-serine-473 of Akt and its phosphorylated substrates Gsk3-α and -β when compared to *Pten*$^{+/+}$ MES cell (Figures 1C and 1D). Clones of all three genotypes (*Pten*$^{+/+}$, $^{+/-}$, and $^{-/-}$) proliferated at similar rates when grown in the presence of 15% fetal bovine serum and leukemia inhibitory factor on feeder cells (data not shown).

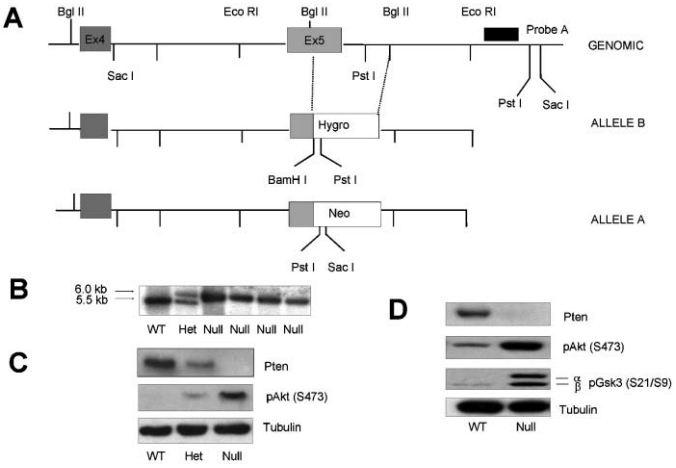

Figure 1. Generation of murine Pten knockout embryonic stem cells

A: A map of the genomic Pten locus and the two constructs used to disrupt it. Correct targeting results in the deletion of the indicated fragment located in between two Bgl II restriction sites by a hygromycin resistance cassette in one allele and a neomycin in the other.

B: Southern blot analysis of the isolated clones using Probe A. Genomic DNA was digested with PstI. *Pten*$^{+/-}$ wild-type (WT), +/− (Het), and −/− (Null) clones are shown.

C: Western blotting of a wild-type, a heterozygous, and a representative nullizygous ES clone. The heterozygous clone expresses a lower amount of the Pten protein than does the wild-type clone.

D: Analysis of phospho-Akt (S473) and phospho-Gsk3 (serines 21 and 9) in the wild-type and the representative nullizygous clone. Pten null cells have a high basal level of phosphorylated Akt and glycogen synthase kinase 3.

Checkpoint defects in Pten$^{-/-}$ cells

To examine the ability of the cells to respond to IR exposure, cells were treated with 10 Gy. We consistently noticed that *Pten*$^{-/-}$ cells did not arrest in G_2 as efficiently as *Pten*$^{+/+}$ cells (Figure 2A). Moreover, 24 hr after radiation exposure, approximately half of the bromodeoxyuridine-labeled Pten$^{-/-}$ cells had entered G1 phase, while the labeled *Pten*$^{+/+}$ cells were predominantly arrested in G_2. To determine whether the early response to IR was affected as well, MES cells were irradiated and examined for their mitotic indices 1 and 2 hr later. *Pten*$^{-/-}$ cells failed to efficiently induce the early checkpoint at both time points (Figure 2B). This result was verified when metaphases were counted 30, 60, and 90 min post-IR exposure of 1 Gy (Figure 2C). Chromosome examination revealed that *Pten*$^{-/-}$ cells prematurely exited from G2 phase after IR treatment, as is evident from mitotic index as well as higher frequency of chromatid gaps and breaks observed in Pten$^{-/-}$ cells (Figure 2D).

Phosphorylation of chk1 serine 280 is regulated by Akt and Pten

To determine whether phosphorylation of chk1 occurs in vivo, an affinity purified anti-chk1 phosphoserine 280 antibody was

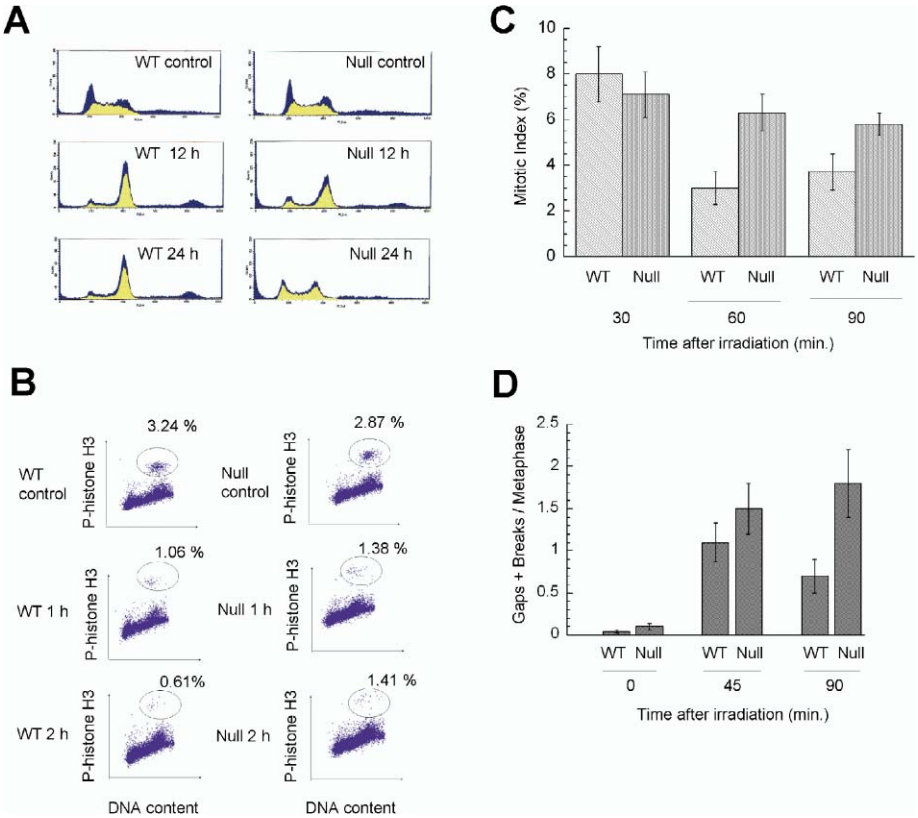

Figure 2. Defect in checkpoint response of Pten$^{-/-}$ cells

A: Defect of cell cycle response to γ radiation in *Pten*$^{-/-}$ cells. S phase cells were pulsed with bromodeoxyuridine (BrdU, marked yellow) and then exposed to 10 Gy of ionizing radiation. Total DNA was stained with propidium iodide (marked blue). Histograms show superimposed images.

B and C: Rapid G_2 checkpoint is defective in *Pten*$^{-/-}$ cells. **B:** Cells were irradiated with 10 Gy of γ radiation and costained with phospho-histone H3 (S10) antibody and propidium iodide. **C:** ES cells were treated with 1 Gy of γ rays. Cells were harvested 30, 60, and 90 min later and examined for mitoses. $p < 0.01$ at 60 min.

D: *Pten* null cells enter mitosis with unrepaired chromosomes. Giemsa-stained chromosomes from metaphases were analyzed for double-stranded gaps and breaks 0, 45, and 90 min after treatment with 1 Gy of ionizing radiation. Four different *Pten*$^{-/-}$ clones have been determined to have checkpoint defects in response to ionizing radiation. Error bars represent the standard deviation of the mean of three independent experiments in which 100 cells were counted.

generated. To test whether the antibody was able to specifically detect phosphorylation of S280 by immunoblot, Chk1 was knocked down in mouse fibroblasts with a short hairpin RNAi retroviral construct that targeted Chk1. Downregulation of total protein expression led to diminution of the phospho-specific band as well (Figure 3A, upper panel). In addition, treatment of cell extracts from Pten$^{-/-}$ cells with calf intestinal alkaline phosphatase led to a considerable reduction in detectable phosphoprotein in the phosphatase-treated lane (Figure 3A, lower panel). Analysis of the same immunoblot with a monoclonal antibody to chk1 demonstrated that chk1 protein was present in the same lane. In order to examine the ability of myr-AKT to phosphorylate chk1, we cotransfected HEK293 cells with either wild-type chk1 or chk1^{S280A} (a mutant of chk1 in which the serine at 280 has been substituted with an alanine). AKT stimulated chk1 serine 280 phosphorylation of wild-type chk1 but not of the chk1^{S280A} mutant (Figure 3B).

To test whether growth factors stimulate phosphorylation of chk1 on serine 280 in a PI-3 kinase-dependent manner, HEK293 cells were transfected with wild-type mouse chk1 and serum starved for 24 hr prior to stimulation with IGF1 in the presence or absence of inhibitors. As expected, IGF1 stimulated AKT and ERK phosphorylation, and PI-3 kinase inhibitors blocked AKT activation and partially impaired ERK phosphorylation. IGF1 stimulated chk1 phosphorylation on serine 280 (Figure 3C). Inhibition of PI-3 kinase with either wortmannin or LY294002 blocked induction of phosphorylation at the serine 280, but inhibition with rapamycin did not. The MEK inhibitor PD98059 inhibited IGF1-induced activation of the pathway but not to the same extent as the PI-3 kinase inhibitors in a setting of complete inactivation of ERK phosphorylation. Examination of NIH/3T3 cells for the ability to phosphorylate endogenous chk1 in response to growth factor stimulation showed that stimulation with either IGF1 or insulin led to induction of a band with the phospho-serine 280 antibody (Figure 3D). This signal was repressed when either ligand was added to cells in the presence of wortmannin. The aforementioned data demonstrate that the PI-3 kinase is able to stimulate phosphorylation of chk1 serine 280. To determine whether Pten regulates phosphorylatation of chk1 serine 280, the phosphospecific antibody to chk1 serine 280 was used to immunoprecipitate chk1 in *Pten*$^{+/+}$ and *Pten*$^{-/-}$ MES cells. Unstimulated *Pten*$^{-/-}$ MES cells contained detectable phosphoserine-280 chk1, while *Pten*$^{+/+}$ cells did not (Figure 3E).

Serine 280 is an important regulator of CHK1 cellular localization

As demonstrated above, AKT kinase effectively phosphorylates chk1-S280 in vivo. To determine whether chk1-S280 is responsible for the checkpoint defect seen in *Pten*$^{-/-}$ MES cells, wild-type, S280A, and S280E chk1 were expressed in Pten$^{-/-}$ ES cells, irradiated, and treated with nocodazole. At 24 hr post-treatment, both wild-type and chk1^{S280A} rescued the phenotype. No rescue was seen with the phosphomimetic chk1^{S280E} construct. Surprisingly, chk1^{S280A} decreased the number of mitotic cells even below the level seen in the wild-type counterparts (Figure 4A). This would argue that the chk1^{S280A} mutant protein is more potent in activating the checkpoint than the

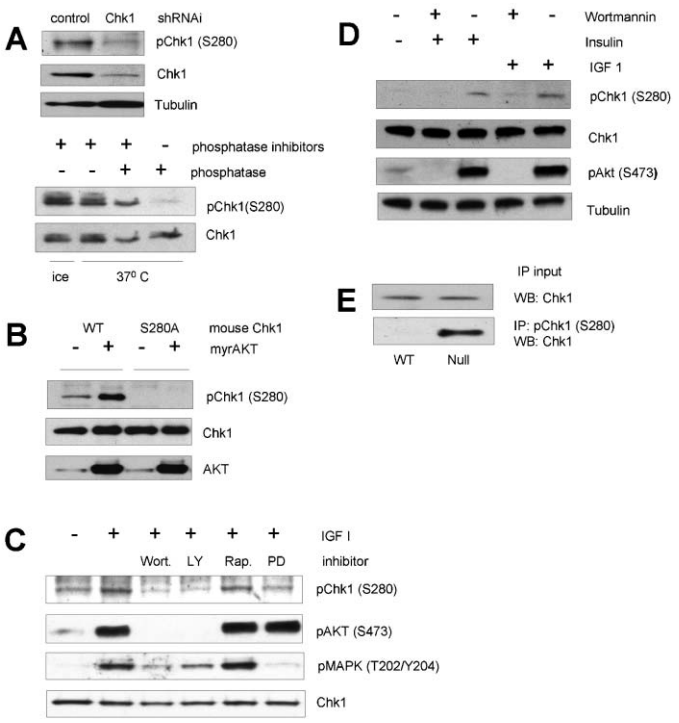

Figure 3. Phosphorylation of chk1 at serine 280

A: Reactivity of Chk1 phospho-specific antibody (S280). Upper panel: chk1 was knocked down by shRNAi in MEFs. Cell lysates were resolved by SDS-PAGE and the membrane was probed for pS280-Chk1. The same membrane was stripped and reprobed for total Chk1. Lower panel: protein extracts from Pten nullizygous MES cells were treated with calf intestinal phosphatase in the presence or absence of phosphatase inhibitors and analyzed by Western blotting. Phosphatase treatment of protein extracts affects the ability of the antibody to recognize the phosphorylated epitope.

B: AKT phosphorylates chk1 at serine 280. pChk1(S280) antibody recognizes the wild-type but not mutant (S280A) mouse protein in a cotransfection experiment with myristoylated AKT in HEK293 cells. Cells were cotransfected with either wild-type or S280A chk1 constructs and myrAKT. Lysates were resolved by SDS-PAGE and transferred to PVDF membranes prior to incubation with the indicated antibodies.

C: Phosphorylation of S280 is dependent on IGF I-induced activation of PI-3K/AKT signaling. HEK293 cells were transfected with mouse Chk1 and starved for 24 hr in serum-free media. Wortmannin (100 nM) was added 15 min prior to mitogen addition. LY 294002 (10 μM), rapamycin (200 nM), and PD980059 (50 μM) were added 2 hr prior to IGF I addition. Cells were stimulated with IGF I (100 ng/ml) for 15 min, lysed, and analyzed by Western blot with the indicated antibodies.

D: Phosphorylation of endogenous chk1 in NIH/3T3 cells is growth factor-dependent and requires PI-3 kinase. Cells were starved for 24 in serum-free DMEM, preincubated with 100 nM wortmannin for 15 min, and treated with insulin (100 ng/ml) or IGF I (100 ng/ml) for 15 min.

E: Increased levels of pS280-chk1 levels are present in PTEN$^{-/-}$ cells. Endogenous pChk1 was precipitated from MES cell lysates using phospho-specific anti-phosphoserine 280 antibody. Proteins were resolved by SDS-PAGE and detected with Chk1 antibody.

wild-type protein. To further investigate the effect of the mutation, we considered the possibility that stability of the protein might be affected. Therefore, U20S cells were transfected with wild-type, S280A, and S280E CHK1 constructs. Protein synthesis inhibitor cycloheximide was added to the media 24 hr posttransfection. No difference in CHK1 protein levels was observed up to 6 hr after addition of the inhibitor, arguing that CHK1 activity was regulated by a mechanism other than half-life. Additionally, there was no difference in in vitro kinase activity after immunoprecipitation of transfected untreated cell lysates (data not shown).

Upon DNA damage, CHK1 protein has been shown to localize to the nucleus, where it could be activated by ATM and ATR kinases and initiate the checkpoint response (Jiang et al., 2003). To determine whether subcellular localization of CHK1 could be altered due to activation of the PI-3K-AKT pathway, CHK1 was expressed in U2OS cells and examined by indirect immunofluorescence. Although primarily nuclear, a significant amount of CHK1 was also detected in the cytoplasm of cells. The nuclear-to-cytoplasmic ratio for wild-type CHK1 and the S280A mutant was greater than for the S280E mutant (Figures 4B–4D). The S280E mutant exhibited a pancellular localization, being present in both the nucleus and cytoplasm regardless of radiation treatment. Thus, it appears likely that the defect of chk1 seen in *Pten*$^{-/-}$ cells may be due to altered intracellular localization.

Altered signaling of chk1 due to S280 phosphorylation

Cytoplasmic localization of CHK1 would render it inaccessible to activation by ATM/ATR in response to DNA damage. To test the fidelity of the chk1 signal in Pten$^{-/-}$ cells, wild-type and nullizygous ES cells were irradiated with 10 Gy and examined for the induction of chk1 serine 345 phosphorylation. *Pten*$^{-/-}$ cells failed to efficiently activate S345 phosphorylation as well as wild-type, although the total levels of chk1 were similar between wild-type and *Pten*$^{-/-}$ (Figure 4E, upper left panel). One of the major substrates of chk1 is cdc25A, which is rapidly degraded upon phosphorylation (Sorensen et al., 2003; Zhao et al., 2002). As expected in a setting of reduced chk1 function, increased levels of cdc25A were observed in *Pten*$^{-/-}$ cells after IR treatment at all time points (Figure 4E, upper left panel) (Mailand et al., 2000). Interestingly, expression of the S280A, but not the S280E, Chk1 mutant in Pten nullizygous MES caused reduction in the expression of cdc25A protein levels after radiation treatment (Figure 4F), arguing that phosphorylation of Chk1 on serine 280 contributes to the regulation of cdc25A protein levels. In addition, these data are consistent with the mitotic rescue seen with the S280A mutant-transfected PTEN nulllizygous MES (Figure 4A). The CHK1 S280E mutation also affected serine 345 phosphorylation after hydroxyurea treatment of U2OS cells (Figure 4E, lower panel). Therefore, present data suggest that altered cellular localization of chk1 reduces its capacity to be phosphorylated at serine 345 and regulate the cell cycle.

Endogenous AKT regulates the localization of CHK1

One question that is raised by the above results is, how does AKT kinase access CHK1 in the nucleus? A recent study of AKT function has determined that a subset of phosphorylated AKT at serine 473 resides in the nucleus of endothelial cells (Adini et al., 2003). To test the specificity of the phospho-serine 473 AKT antibody by immuofluorescence, wild-type and AKT1$^{-/-}$ fibroblasts were stimulated with IGF I to activate the PI3K/AKT axis. Clearly, staining was much brighter in the wild-type cells; in particular, staining of the nucleus and membrane was present in wild-type but not AKT1$^{-/-}$ fibroblasts (Supplemental Figure S1). Dim cytoplasmic staining was seen in the mutant cells, which may reflect AKT2 present in the cells. To validate

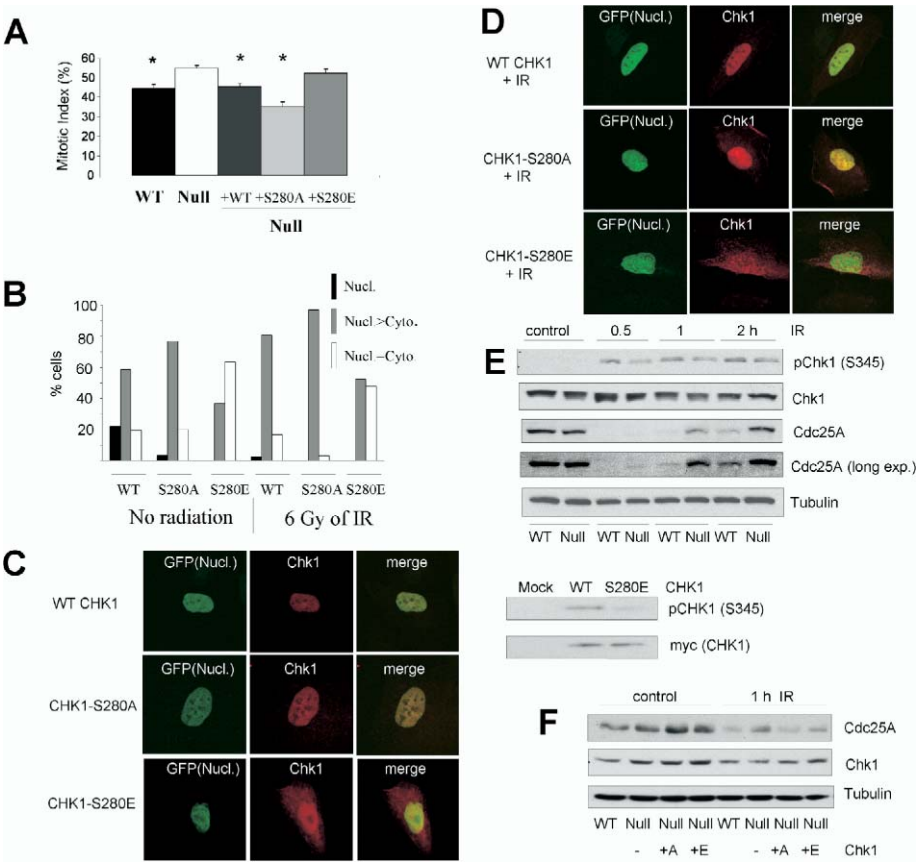

Figure 4. Defective Chk1 signaling is due to serine 280 modification

A: Wild-type and S280A chk1 rescue IR-induced phenotype of PTEN$^{-/-}$ cells. *Pten*$^{-/-}$ cells were transfected with wild-type, S280A, and S280E mouse Chk1, irradiated with 10 Gy of γ rays, treated with nocodazole, and harvested at 24 hr. Cells were stained with phospho S10-Histone H3 antibody and examined under fluorescent light. * indicates $p < 0.003$ using student's two-tailed t test when compared to null.

B: Graph depicting the distribution of exogenous CHK1 in U2OS cells. Here, at least 60 cells were counted and evaluated by eye.

C and D: Expression of exogenous CHK1 in U2OS cells showing nuclear localization of the S280A mutant and cytoplasmic staining of the S280E protein. Cells were cotransfected with myc-tagged CHK1 and histone H2B-EGFP constructs. 18 hr after transfection, cells were irradiated with 6 Gy of IR. 20 hr posttransfection, unirradiated (**C**) and irradiated (**D**) cells were fixed and stained.

E: Upper panel: Pten null MES cells are defective in Chk1-responsive signaling. Cells were treated with 10 Gy of ionizing radiation and harvested at indicated times. Null cells display decreased phosphorylation of S345-chk1 and increased expression of Cdc25A. Longer exposure of Cdc25A blot shows that Cdc25A is not fully degraded at 30 min after radiation in Pten$^{-/-}$ cells. Lower panel: CHK1 S280E is not phosphorylated as well as wild-type on serine 345 after hydroxurea treatment. U2OS cells were transfected with WT, S280E myc-tagged, and empty pCDNA3 expression vectors. After 24 hr, 3 mM hydroxyurea was added to cells for 17 hr. Lysates were immunoblotted and probed with the indicated antibodies.

F: Pten nullizygous cells were transfected with wild-type, S280A, or S280E mChk1 and assayed by Western for Cdc25A expression 1 hr after IR treatment. S280A (denoted A) but not S280E (denoted E) mutant affects CDC25A protein levels in response to ionizing radiation.
Error bars represent the standard deviation of three independent experiments in which at least 400 cells were counted.

the specificity of CHK1 monoclonal antibodies, siRNA for CHK1 was transfected into U2OS cells. Reduced CHK1 signal was evident in the cells transfected with siRNA specific to CHK1, but not in cells transfected with control siRNA (Supplemental Figure S3).

Since it has been shown that during G2/M, AKT kinase levels are highest when CHK1 kinase levels are lowest, we explored the localization of endogenous chk1 and phosphoserine 473 AKT in synchronized U2-OS cells (Shtivelman et al., 2002) (Figure 5A). Cells were synchronized at the G1/S boundary with a double thymidine block and released into fresh media. At 0 hr, before the addition of fresh media, threonine 308 was phosphorylated, but serine 473 was barely detectable on AKT. At 1 hr after the addition of fresh media, phosphorylation at serine 473 was increased, which began to subside at 2 hr and was followed by a wave of increased phosphorylation at 7 hr in late G2 (Figure 5B) Using immunofluorescence of synchronized cells, we determined that endogenous nuclear phosphoserine 473 of AKT could be detected in the nucleus throughout the cell cycle, and that in late G2, an increase in both nuclear and cytoplasmic AKT could be seen. The change in AKT intensity at 7 hr and 8 hr correlated with a reduction in nuclear CHK1 staining that was apparent at 7 hr and 8 hr. Representative images from 2 hr and 7 hr are shown (Figure 5C). These data demonstrate that AKT has access to the CHK1 substrate in the nucleus and is most active when nuclear CHK1 levels are decreasing. Loss of CHK1 from the nucleus in G2 may explain why Cdc25A levels rise in G2/M of a cell cycle (Bernardi et al., 2000).

To determine whether loss of PTEN and activation of AKT resulted in exclusion of endogenous CHK1 protein from the nuclear compartment, expression of PTEN in U2OS cells was reduced by means of RNA interference with a retroviral shRNA vector for PTEN. Pooled infected cells were larger and showed reduced expression of PTEN and increased reactivity with phospho S473-AKT antibody when compared to hairpin control cells (Figure 5D). When examined by indirect immunocytofluorescence, many of the cells infected with PTEN shRNA displayed increased staining for phosphorylated AKT with numerous nuclear foci (>20). Simultaneous examination of CHK1 showed that cells with nuclear phosphoS473-AKT had cytosolic CHK1 (Figure 5D). This phenomenon was observed only rarely in the control hairpin cells.

To test the hypothesis that inhibition of AKT could result in retention of CHK1 in the nucleus, cells were transfected with short interfering RNAs to AKT and confirmed by immunoblot (Supplemental Figure S2). As expected, the CHK1 signal was exclusively nuclear, demonstrating that downregulation of the AKT kinase levels resulted in nuclear compartmentalization of

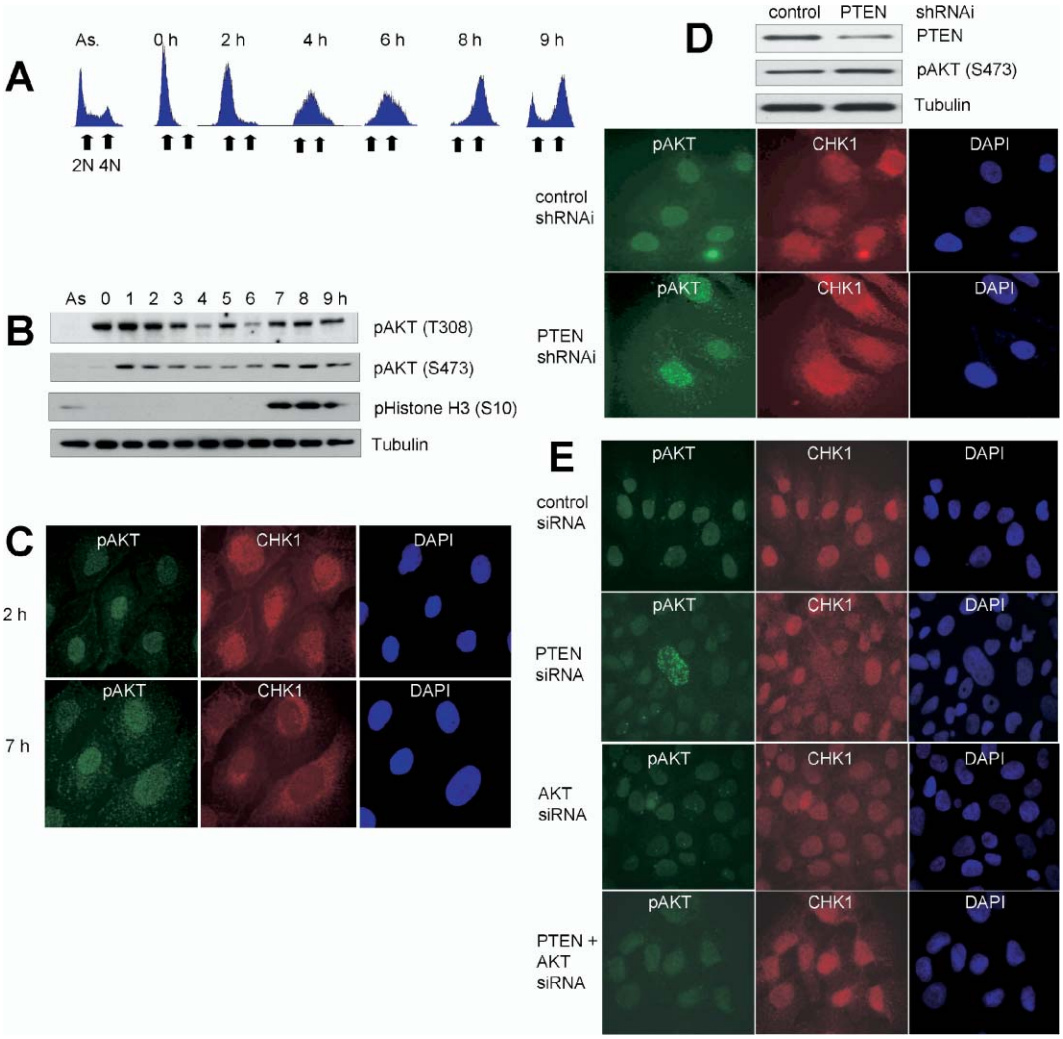

Figure 5. PI3-K/AKT pathway regulates localization of endogenous CHK1

A–C: Analysis of endogenous pAKT and endogenous CHK1 in synchronized U2OS cells. **A:** Flow cytometry profile of synchronized cells at indicated times after release from thymidine block. **B:** Biphasic increased AKT kinase activity as demonstrated by phosphorylated Thr 308 and Ser 473. **C:** Nuclear AKT phosphoserine 473 is declining in early S phase cells (2 hr time point) and is coincidental with nuclear CHK1 staining pattern, whereas it is increasing in premitotic G2 cells (7 hr time point) in the nucleus and cytoplasm when CHK1 is largely perinuclear and cytoplasmic. G-4 antibody was used to detect CHK1.

D: Activation of nuclear AKT results in shift of CHK1 from the nuclear compartment. PTEN was knocked down in U2OS cells by shRNA. Upper panel shows immunoblot analysis of cells with control and PTEN hairpin. Lower panel shows indirect immunofluorescence of cells with activated nuclear AKT with concomitant cytosolic CHK1 staining. For comparison, cells infected with the control hairpin RNAi vector are shown. Here, CHK1 is mainly in the nucleus. G-4 monoclonal antibody was used to detect CHK1.

E: Activation or inhibition of endogenous AKT affects CHK1 localization. U2OS cells were transfected with either control, PTEN, AKT, or PTEN + AKT siRNA. PTEN downregulation results in activation of nuclear AKT and causes CHK1 to localize to the cytosol (as also shown in **D**), whereas AKT downregulation causes CHK1 to remain in the nuclear compartment. After double knockdown of PTEN and AKT, CHK1 can be observed in the nuclei of cells. Here, CHK1 was detected with DCS-310 monoclonal antibody. Nuclei were stained with DAPI. Images were photographed with a 100× oil objective with a SPOT camera.

CHK1 (Figure 5E). In addition, in the PTEN and AKT double knockdown, CHK1 was localized to the nucleus. PTEN alone was knocked down as well to test the reproducibility of CHK1 shift to the cytosol. Again, a striking increase in nuclear phosphoS473-AKT was detected that was associated with a shift of CHK1 out of the nucleus.

Serine 280-phosphorylated Chk1 covalently associates with ubiquitin

In response to DNA damage, ATR and ATM kinases phosphorylate CHK1 (Liu et al., 2000; Sorensen et al., 2003; Zhao and Piwnica-Worms, 2001). Recently, Jiang et al. demonstrated that following DNA damage, S345-phosphorylated forms of CHK1 are predominantly associated with chromatin, and that this generated a consensus site for 14-3-3 protein binding to CHK1 that played a role in CHK1 nuclear retention (Jiang et al., 2003). We were interested in determining whether association of CHK1 with 14-3-3 proteins may help to explain the compartmentalization of CHK1 S280 mutants. However, no binding of 14-3-3σ or ζ proteins to CHK1 could be observed in presence or absence of DNA damage either by far Western or coimmu-

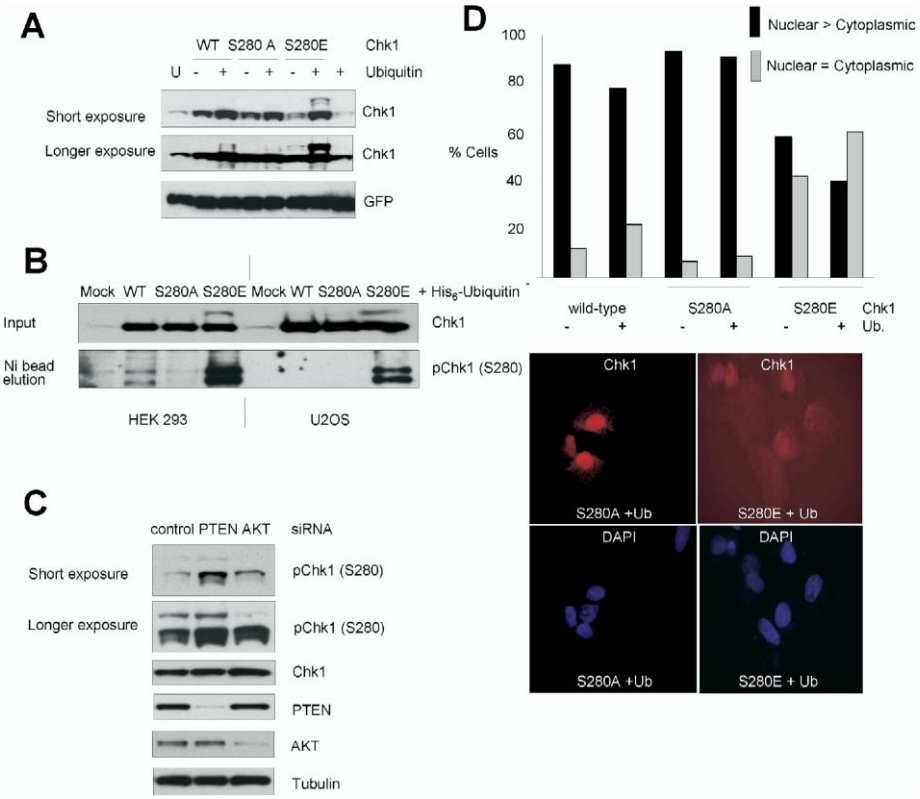

Figure 6. Association of phosphomimetic S280E Chk1 mutant with ubiquitin

A: S280E but not S280A mutant of mouse chk1 is ubiquitinated when transiently expressed in HEK 293 cells. HEK 293 cells were cotransfected with mChk1 constructs (WT, S280A, S280E), ubiquitin (+), empty vector (−), and GFP (as a transfection control). U indicates transfection with only GFP. 48 hr posttransfection, cells were lysed and lysates were analyzed by Western blotting.

B: Covalent binding of Chk1 and ubiquitin. HEK 293 and U2OS cells were transfected with His_6-Ub and wild-type, S280A, or S280E Chk1 mutants. 24 hr later, the cells were lysed under denaturing conditions. Ubiquitin-associated proteins were purified on Ni-NTA agarose beads and eluted. The eluates were fractionated by SDS-PAGE and analyzed by Western blotting with pChk1 (S280) antibodies. Input is shown above.

C: Ubiquitination of Chk1 is PI3K/AKT-dependent. PTEN or AKT was knocked down in U2OS cells by siRNA technology. The knockdown cells were transfected with wild-type Chk1 and His_6-Ub. Cellular lysates were analyzed by Western blotting with indicated antibodies. Downregulation of endogenous PTEN increases Chk1 ubiquitination, whereas downregulation of AKT decreases it.

D: Association of S280E but not S280A mutant with ubiquitin affects cytosolic localization of Chk1. Wild-type, S280A, and S280E Chk1 were transfected into U2OS cells either alone or with ubiquitin in the presence of H2B-EGFP plasmid to monitor transfection. 18 hr later, the cells were fixed and stained with Chk1 (DCS-310) antibody. Only cells expressing weak and moderate levels of GFP were evaluated. Here, at least 50 cells were counted for each group. Lower panel shows S280A and S280E transfected cells stained with DCS-310 antibody or DAPI. Note that the dilution of the antibody was such that endogenous CHK1 was not detected.

noprecipitation techniques (data not shown). Thus, regulation of molecular transport of CHK1 by 14-3-3 proteins does not seem to be a likely scenario in this setting.

We next explored the possibility that monoubiquitination could regulate the localization of CHK1. Transfection of the S280E mutant into HEK 293 cells generated a slower migrating band that was consistent with monoubiquitination (Figure 6A). To see if ubiquitination was indeed involved in regulation of Chk1, we cotransfected ubiquitin with chk1, chk1S280A, and chk1 S280E. Interestingly, more of the same species of slower migrating chk1 was detected in the S280E lane. In addition, potentially covalent ubiquitination of wild-type chk1 was evident in a longer exposure of the immunoblot, but such modification of the S280A mutant was barely detectable. This result was only seen when untagged Chk1 was used in the experiment. N- and C-terminus-tagged Chk1 constructs (both human and mouse) did not display this property. We wished to confirm that the shifted product was indeed Chk1 covalently attached to the ubiquitin polypeptide. For this purpose, HEK 293 and U2OS cells were cotransfected with pCIN4-mChk1 (WT, S280A, S280E) and pCMV-His_6-ubiquitin and lysed under denaturing conditions. Ubiquitin-associated proteins were isolated by incubating the lysates with Ni agarose beads. Analysis of the SDS-PAGE-resolved eluates showed that Chk1 S280E was covalently attached to one or two molecules of ubiquitin (Figure 6B). We wished to investigate whether modification of the PI3K/AKT pathway might have an effect on ubiquitination of Chk1. To this end, endogenous PTEN or AKT were knocked down by siRNA in U2OS cells. Wild-type Chk1 and ubiquitin were then coexpressed. In the setting of reduced PTEN, the signal of the upper shifted band was increased when compared to the control. Conversely, downregulation of cellular AKT resulted in decrease of the signal (Figure 6C).

If ubiquitination were involved in the regulation of Chk1 compartmentalization, one would expect to see a difference in subcellular localization of Chk1 mutants in the presence of excess ubiquitin. For this purpose, Chk1 constructs were expressed in U2OS cells in the absence or presence of exogenous ubiquitin. Coexpression of S280A Chk1 with ubiquitin had no effect on localization of the protein. Here, most transfected Chk1 could be seen in the nuclear compartment. However, transfection of ubiquitin shifted the phosphomimetic S280E mutant into the cytoplasm. Now, 60% of cells display an equal distribution of Chk1 between nucleus and cytosol, versus 40% in the absence of excess ubiquitin (Figure 6D).

Alteration of chk1 localization in breast carcinomas and genetic instability

The results described above demonstrate that activation of the PI3K-AKT pathway affects CHK1 directly. To test whether loss of PTEN might influence CHK1 in primary human carcinomas, immunohistochemical analysis was performed on a panel of breast carcinomas with antibodies to PTEN, phosphoS473-AKT, and CHK1. Normal epithelial ducts express more PTEN than the surrounding stroma and stain weakly in the cytoplasm with the phospho-AKT (473) antibody (Figures 7A and 7B). Nor-

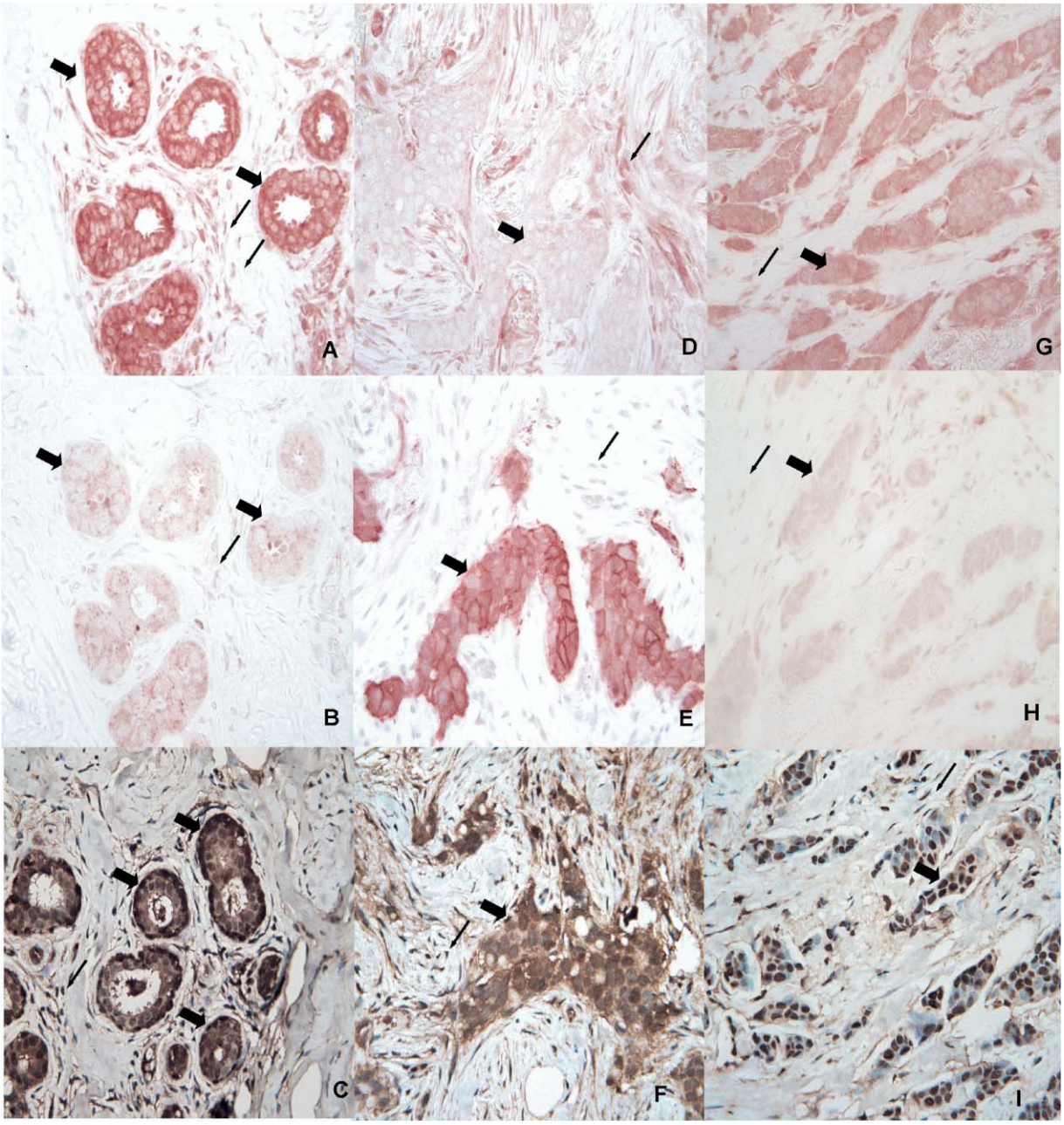

Figure 7. Breast carcinomas with elevated AKT phosphorylation and reduced PTEN protein have altered distribution of CHK1

Staining with antibodies to PTEN, phosphoAKT (S473), and CHK1 were performed in breast carcinomas. Twelve tumor biopsies were examined for CHK1 localization, six with low PTEN and elevated phospho-AKT (S473), and six with normal levels of PTEN and phospho-AKT (S473). Representative examples of PTEN (**A, D,** and **G**), pAKT-473 (**B, E,** and **H**), and CHK1 (**C, F,** and **I**) staining are shown. Adjacent normal ducts had high PTEN (**A**), weak phospho-AKT (S473) (**B**), and strong nuclear (**C**) CHK1 staining. Tumor cells with reduced PTEN (**D**) and increased phospho-AKT (S473) (**E**) had increased cytoplasmic and reduced nuclear CHK1 staining (**F**). Tumor cells with normal levels of PTEN (**G**) and weak phospho-AKT (**H**) staining had primarily nuclear CHK1 (**I**) staining. Shifted CHK1 staining into the cytoplasm was seen in all six cases with altered PI-3 kinase signaling. Nuclear CHK1 staining was seen in all six cases with normal PI-3 kinase signaling. $\chi^2 < 0.005$. Wide arrows indicate epithelial cells, while narrow arrows indicate stromal cells.

mal duct cells had primarily nuclear CHK1, as did the surrounding stroma (Figure 7C). Tumor cells with downregulated PTEN (Figure 7D) and increased staining for phosphoS473-AKT (Figure 7E) at the membrane and throughout the cell had cytoplasmic and nuclear CHK1 at roughly equivalent levels (Figure 7F). On the other hand, tumors with high PTEN and low phospho-AKT (S473) expression that was consistent with normal epithelial cells (Figures 7G and 7H) had largely nuclear CHK1 (Figure 7I). These data are consistent with AKT kinase regulating CHK1 localization in human cells lacking PTEN.

CHK1 is a candidate tumor suppressor that preserves genomic integrity during each cell cycle in response to endoge-

nously and exogenously generated DNA lesions (Bartek and Lukas, 2003). To see whether loss of PTEN affects genetic stability, we analyzed a set of primary invasive stage II and stage III breast carcinomas with normal and low PTEN expression. In a panel of 109 tumors, lack of PTEN expression was associated with an increased incidence of aneuploidy in breast carcinoma ($\chi^2 = 8.34$; $p < 0.005$). While 25 of 77 (32%) tumors with normal PTEN were diploid, only 2 of 32 (6%) tumors lacking PTEN had normal ploidy. These results confirm the initial correlation of aneuploidy and loss of PTEN expression that we observed previously in a smaller group of breast tumors (Bose et al., 2002). Moreover, they suggest that the cytoplasmic localization of CHK1 seen in PTEN-deficient cells is contributing to their aneuploidy. To confirm the link between PTEN inactivation and aneuploidy, we examined prostatic intraepithelial neoplasia (PIN) from eleven *Pten*$^{+/-}$ mice for aneuploidy (Podsypanina et al., 1999). We found that 6 of 11 PIN lesions (55%) were aneuploid. Analysis of MES cells after over 30 passages or after recovery and expansion from 3 Gy of ionizing radiation demonstrated no evidence of aneuploidy or chromosomal rearrangement due to lack of *Pten*. These data suggest that the deficit in checkpoint function is insufficient to cause frank aneuploidy in the absence of phenotypic selection.

Discussion

CHK1 is in the first line of defense against DNA damage, and can be activated by a wide variety of stimuli that include ultraviolet light, ionizing radiation, and hydroxyurea. By rapidly modulating the level of CDC25A, CHK1 is able to arrest the cell cycle at the G1/S boundary, S, and G2 phases by altering the kinase activity of cyclin/cdk complexes E, A, and B, respectively (Figure 8). In this study, we have shown that PTEN tumor suppressor is an important modulator of CHK1 function. In response to ionizing radiation, *Pten*$^{-/-}$ cells had diminished checkpoint control as early as 1 and 2 hr after treatment (Figure 2), a phenotype also observed in MES cells lacking chk1 and RNAi-mediated knockdown of CHK1 in human cells (Takai et al., 2000; Zhao et al., 2002). Because of their checkpoint defect, *Pten*$^{-/-}$ cells displayed more than a 2-fold increase in the number of DNA double-strand gaps and breaks 90 min after treatment with a low dose of ionizing radiation (Figure 2D). We also have demonstrated that the PI-3 kinase pathway and AKT were able to stimulate phosphorylation of chk1 serine 280 in vivo, and that cells lacking Pten had elevated phosphorylated chk1 at serine 280 (Figure 3). Serine 280 phosphorylation reduced the protein's ability to be phosphorylated at another residue, serine 345, in response to DNA damage (Figure 4E). As a consequence of reduced Chk1 activity, *Pten*$^{-/-}$ cells were unable to properly regulate cdc25A and the cell cycle (Figures 2 and 4). The influence of the PI-3 kinase pathway on chk1 signaling is demonstrated by the ability of chk1^{S280A}, a mutant that AKT cannot phosphorylate, to suppress the checkpoint defect seen in Pten$^{-/-}$ cells and to restore the reduction of CDC25A after radiation (Figure 4). Since S280A chk1 rescued the IR-induced phenotype of *Pten*$^{-/-}$ cells, we looked at subcellular distribution of CHK1 in U2OS cells. We discovered that CHK1^{S280A} localized mostly to the nuclear compartment, whereas the phosphomimetic (S280E) mutant could be predominantly seen in the cytoplasm. In addition, at the G2 phase of the cell cycle, endogenous CHK1 could be seen departing from the nucleus at the time of peak AKT kinase phosphorylation at threonine 308 and serine 473 and nuclear localization (Figure 5). Such reduction in nuclear CHK1 may explain the increase in Cdc25A protein seen in G2/M phases of the cell cycle (Bernardi et al., 2000). Reduction of PTEN in U2OS cells with RNAi led to a marked increase in nuclear foci of phospho-AKT (S473) and increased cytoplasmic CHK1. Serine 280 phosphorylation appears to be a trigger for monubiquitinatiton of CHK1, which we have shown to influence cellular localization of CHK1 (Figure 6). Overall, this suggests that increased serine 280 phosphorylation and ubiquitination of CHK1 in PTEN$^{-/-}$ cells promotes enhanced cytoplasmic localization, thereby inhibiting its checkpoint function.

Reduced CHK1 function is associated with genomic instability and cancer. Mutation of one allele of chk1 stimulates tumor development in the WNT mouse mammary tumor model (Liu et al., 2000). In these tumors, no loss of heterozygosity of the wild-type chk1 allele occurs. Moreover, normal mouse *chk1*$^{+/-}$ cells have partial deficits in checkpoint function (Lam et al., 2004). Since *Pten*$^{-/-}$ cells also have a reduced amount of chk1 function, we surmise that the chk1 defect seen in Pten$^{-/-}$ cells contributes to tumor development. In addition, activation of AKT inhibits p21, p27, and CDC25B, key inhibitors of cyclin/cdk complexes that facilitate cell cycle arrest in response to checkpoint activation; moreover, AKT phosphorylates and activates MDM2 to stimulate p53 destruction (Baldin et al., 2003; Fujita et al., 2002; Liang et al., 2002; Mayo and Donner, 2001; Rossig et al., 2001; Shin et al., 2002; Zhou et al., 2001).

The major substrate of CHK1 is CDC25A, which is often overexpressed in human breast cancer and is able to cooperate with mutant *h-ras* to transform mouse embryo fibroblasts (Cangi et al., 2000; Galaktionov et al., 1995). Overexpression of CDC25A activates cyclin E/Cdk2 complexes, a complex shown to induce unscheduled firing of origins of replication and induction of chromosome instability (Blomberg and Hoffmann, 1999; Spruck et al., 1999). Therefore, it seems reasonable to conclude that the aneuploidy seen in PTEN-deficient tumors is due at least in part to reduced CHK1 function. Our finding that CHK1 is shifted into the cytoplasm of primary breast tumor cells with phosphorylated AKT and reduced PTEN protein expression supports this hypothesis (Figure 7).

Loss of PTEN leads to increased proliferation and reduced apoptosis in nontumor cells (Kishimoto et al., 2003). Although altered proliferation and apoptosis are potent stimuli for tumor formation, the pace at which spontaneous *Pten*$^{-/-}$ tumors arise from *Pten*$^{-/-}$ normal cells suggests that they have acquired a new capacity to evolve into tumors (Backman et al., 2004; Kimura et al., 2003; Suzuki et al., 2001; Trotman et al., 2003; Wang et al., 2003). One explanation for this increased evolutionary capacity is that *Pten*$^{-/-}$ cells have enhanced genetic plasticity that allows for the selection of tumor traits. We liken the modest defect in checkpoint control seen in cells lacking PTEN to a chink in the armor or a pull in a garment. Such a weakness is significant, since over many generations, a cell lacking *PTEN* has the opportunity to select for further genetic instability and cooperating mutations that facilitate tumor growth.

Experimental procedures

Plasmid constructs and gene targeting

The *Pten* targeting construct containing a resistance cassette was electroporated into murine W9.5 embryonic stem cells (Podsypanina et al., 1999).

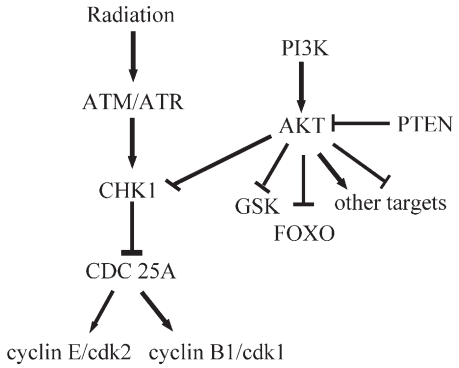

Figure 8. Model of PTEN regulation of DNA damage checkpoints

AKT activation due to the loss of PTEN phosphorylates CHK1 at serine 280 and leads to its monoubiquitination and sequestration from the nucleus. Outside of the nucleus, CHK1 is not able to respond to its activating kinases ATM and ATR, nor can it phosphorylate substrates such as CDC25A.

Recombinants were selected on mitomycin C-treated feeder cells in ES media (DMEM supplemented with nonessential amino acids, L-Glutamine, nucleosides, 2-mercaptoethanol, 15% defined fetal bovine serum, and 1000 U/ml of leukemia inhibitory factor [LIF]) and 2 mg/ml of G418 or 0.2 mg/ml hygromycin B for a period of 10 days. Heterozygous mutant cells were retargeted with a construct bearing the hygromycin-bearing cassette. Multiple aliquots of cells were frozen and used during the course of the study to limit variability caused by passage number. For the generation of mouse chk1 expression vector, mchk1 cDNA (gift of Stephen J. Elledge, Harvard Medical School) was subcloned into the Not I site of pCIN4 (gift of Wei Gu, Columbia University). Phosphorylation site mutants were generated with the Quick Change XL Mutagenesis Kit (Stratagene) using pCIN4-mChk1 as a template. pCMV6 and pCMV6-myrAKT-HA were provided by Thomas Franke (Columbia University). pCMV-His_6-Ubiquitin vector was a gift of Richard Baer (Columbia University). The human CHK1 expression vector was described previously and was the template for the human CHK1 codon 280 mutants (Zhao and Piwnica-Worms, 2001).

Radiation treatment and immunoblotting

Embryonic stem cells were maintained on gelatinized dishes without feeder cells in ES media supplemented with 1000 U/ml of LIF. 70% confluent ES cells seeded on 6-well plates were treated with IR from a ^{137}Cs source and harvested in Laemmli sample buffer at different time points. HEK 293 cells were transfected by the calcium phosphate method. When pCIN4-mChk1/pCMV6-myrAKT were used, cells were harvested 24 hr later in Laemmli sample buffer. Protein extracts were boiled for 5 min and precleared by centrifugation. Twenty-five micrograms of protein were resolved by SDS-PAGE on 4%–20% or 8% Tris-Glycine gels. Proteins were transferred to Immobilon-P membrane (Millipore) and visualized by enhanced chemiluminescence (Pierce). PTEN was detected with polyclonal antibody purchased from Cascade, and pAKT (S473), pAKT (T308), AKT, pChk1 (S345), pGSK3 (S21/9), pMAPK (T202/Y204) were from Cell Signaling. For determination of Cdc25A expression, cells were lysed in mammalian cell lysis buffer supplemented with 200 nM okadaic acid in place of 1 μM microcystin (Zhao and Piwnica-Worms, 2001). Monoclonal antibodies to CHK1 (G-4) and CDC25A (F6) were obtained from Santa Cruz Biotechnology. Polyclonal phosphohistone H3 (S10) antibody was from Upstate Biotechnology. Phospho-Chk1 (S280) antibody was raised in rabbits immunized with the mouse peptide CRPRAT-pS-GGMS-NH2 covalently attached with cysteine to KLH and affinity purified. This antibody does not crossreact with human CHK1.

Growth factor stimulation

For determination of the effect of growth factor stimulation on phosphorylation of Chk1 protein, mChk1-transfected HEK 293 cells or NIH/3T3 cells were starved for 24 hr in serum-free DMEM. 100 nM Wortmannin was added 15 min prior to growth factor addition to inhibit PI3-K. Cells were pretreated with 10 μM LY294002, 50 μM PD980059, and 200 nM Rapamycin for 2 hr prior to mitogen addition. Insulin and IGF I (Sigma) were added to concentration of 100 ng/ml for 15 min.

Phosphatase treatment

Pten nullizygous ES cells were lysed in buffer containing 50 mM Tris-Cl (pH 8.0), 100 mM NaCl, 10 mM $MgCl_2$, 0.5 % Nonidet P-40, 2 mM DTT, 2 mM PMSF, 10 μg of aprotinin per ml, and 5 μg of leupeptin per ml. Clarified lysates containing 150 μg of total protein were incubated with 5 U of calf intestinal phosphatase (NEB) in the absence or presence of 10 mM β-glycerol phosphate and 10 mM NaF at 37°C for 1 hr.

Isolation of ubiquitin-conjugated proteins

60% confluent HEK 293 or U2OS cells in 100 mm dishes were transfected with 5 μg pCIN4-mChk1 (WT, S280A, S280E) and 5 μg pCMV-His_6-Ub. 24 hr after transfection, cells were lysed under denaturing conditions according to procedure described by Choudhury et al. (2004).

Checkpoint rescue

60% confluent *Pten*$^{-/-}$ MES cells growing in gelatinized T75 cm^2 flasks were transfected with 20 μg of pCIN4-mChk1 (WT, S280A, S280E) by the Lifofectamine 2000 method. 48 hr posttransfection, cells were reseeded on gelatinized T25 cm^2 flasks and allowed to attach overnight. Cells were then irradiated with 10 Gy of ionizing radiation from a ^{137}Cs source at a 1 Gy/min rate. Nocodazole was added to final concentration of 0.2 μg/ ml. Cells were harvested 24 hr later and were fixed in 2% paraformaldehyde for 10 min and resuspended in 70% ethanol/PBS. Fixed cells were stained with rabbit phospho histone H3 (S10) antibody (Upstate Biotechnology), washed, and incubated with Alexa Fluor 488 anti-rabbit IgG (Molecular Probes). After washing, the positive cells were scored under fluorescent light. At least 400 nuclei were measured for each time point in triplicate. For the CDC25A rescue, PTEN wild-type and nullizygous MES were seeded at 60% confluency on gelatinized 6-well plates. Nullizygous cells were transfected with 0.6 μg of either wild-type, S280A, or S280E mChk1 expression vector. 18 hr posttransfection, the cells were exposed to 10 Gy of IR from a ^{137}Cs source. After 1 hr, both unirradiated and irradiated cells were lysed in buffer MCLB. Cell lysates were resolved by SDS-PAGE and analyzed by WB for CDC25A expression levels.

Supplemental data

Supplemental data for this article can be found at http://www.cancercell.org/cgi/content/full/7/2/193/DC1/.

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