Chk1 is haploinsufficient for multiple functions critical to tumor suppression

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Summary

The haploinsufficient tumor suppressor *Chk1* is essential for embryonic cells, but the consequences of *Chk1* loss in adult tissues are unknown. Using conditional *Chk1* mice, we find that proliferating mammary cells lacking Chk1 undergo apoptosis leading to developmental defects. Conditional Chk1 heterozygosity increased the number of S phase cells and caused spontaneous DNA damage. *Chk1*+/− epithelia also exhibit a miscoordinated cell cycle in which S phase cells display an early mitotic phenotype. These cells maintain high levels of Cdc25A, which can promote inappropriate cell cycle transitions. Thus, *Chk1* heterozygosity results in three distinct haploinsufficient phenotypes that can contribute to tumorigenesis: inappropriate S phase entry, accumulation of DNA damage during replication, and failure to restrain mitotic entry.

Introduction

Genomic instability is a hallmark of tumorigenesis. Checkpoint pathways help maintain genomic stability, and many components of these pathways are tumor suppressors. The majority of tumor suppressors require that both alleles are lost in tumors. Chk1 is a tumor suppressor that does not conform to this model. Chk1 is a protein kinase involved in transducing DNA damage signals and is required for both the intra-S phase and G2/M checkpoints. Tumors observed in germline *Chk1*+/− mice do not appear to lose the second allele of *Chk1* (Liu et al., 2000), suggesting that Chk1 is a haploinsufficient tumor suppressor. However, the molecular mechanisms by which Chk1 heterozygosity may regulate tumor progression are not well understood.

In response to replicative stress, ATR phosphorylates Chk1 at Ser317 and Ser345, which upregulates Chk1 kinase activity (Liu et al., 2000; Zhao et al., 2002). Activated Chk1 prevents cell cycle progression by inhibiting the cell cycle phosphatases Cdc25A and Cdc25C. Chk1-mediated phosphorylation of Cdc25A results in its degradation, thereby preventing DNA replication during exposure to genotoxic agents (Sanchez et al., 1997; Zhao et al., 2002). These events facilitate the prevention of mitotic entry at the G2 boundary, since Tyr15 of Cdc2 remains phosphorylated.

Chk1 is indispensable in mammals for proper embryogenesis (Liu et al., 2000; Takai et al., 2000; Zachos et al., 2003). *Chk1* null mice exhibit embryonic lethality (E6.5) due to a periimplantation defect, and this lethality was not rescued in a p53 null background (Liu et al., 2000; Takai et al., 2000). However, the role of Chk1 in adult tissues has not been investigated. This is particularly important because Chk1 has been proposed to be a potential drug target for anticancer therapies (Hapke et al., 2001; Li and Zhu, 2002; McKinstry et al., 2002; Zhou et al., 2003; Zhou and Sausville, 2003). In order to know whether Chk1 has potential as an antitumor drug target, it is critical that we understand the consequences of eliminating Chk1 in adult tissues.

Alterations in Chk1 may play a critical role in the etiology of human cancer. For example, genetic mutations for *Chk1* have been observed in sporadic, endometrial, and gastrointestinal tumors displaying microsatellite instability (Bertoni et al., 1999; Menoyo et al., 2001). Furthermore, LOH at chromosome 11q21-24, a region containing several tumor suppressors, including *Chk1*, has been observed in a variety of human malignancies (Gentile et al., 1999; Gudmundsson et al., 1995; Launonen et al., 1998). Interestingly, the Chk1-regulated oncoprotein Cdc25A is highly expressed in a multitude of human cancers and is associated with poor prognosis (Cangi et al., 2000; Dixon et al., 1998; Gasparotto et al., 1997; Wu et al., 1998). These results

SIGNIFICANCE

DNA replication and damage checkpoints coordinate cellular responses to genotoxic stress. The Chk1 kinase is a key regulator of both S phase progression and mitotic entry, and has been proposed as a potential antitumor drug target. Here we find that Chk1 is essential for proliferating cell viability in the breast. We describe a novel haploinsufficiency phenotype in Chk1 conditional heterozygous tissues, which exhibit cells accumulating in an erroneous S phase state with premature localized chromatin condensation, characteristics commonly found in malignant cells. Accordingly, an increase in LOH at 11q24-25, a region containing the Chk1 locus, has been reported in human tumors. The fact that Chk1 levels are critical for genomic stability and survival of proliferating cells may limit the usefulness of drugs that inhibit Chk1 as anticancer agents.

suggest that the loss of Chk1 function may have deleterious consequences in mammalian cells, possibly due to Cdc25A deregulation, but this has yet to be demonstrated in an in vivo animal model.

To investigate the role of Chk1 in adult tissues and to explore its role in tumorigenesis, we generated a mouse that was conditional for *Chk1* in the mammary gland. We found that Chk1 was essential for mammary epithelial cell growth. However, during the analysis of these animals, we also observed that Chk1 mice showed strong phenotypes in a heterozygous background. These studies demonstrate that Chk1 is haploinsufficient for control of cell proliferation, genomic stability, and cell cycle coordination, and explain its role as a haploinsufficient tumor suppressor.

Results

Homozygous deletion of *Chk1* results in impaired development

To study the role of Chk1 during somatic cell development, we targeted mice for homozygous disruption of *Chk1* in the mouse mammary gland using a conditional floxed allele. The mammary gland provides an ideal model in which to study the role of developmentally essential genes in somatic cells in vivo. The majority of mammary gland development occurs postnatally; thus, defects arising from the genetic disruption of *Chk1* in mammary epithelia may affect the ability to nurse young, yet deletion should not impair the general viability of the animal.

As shown previously, conditional deletion of exon 2 of the *Chk1* gene by Cre recombinase resulted in a null phenotype in mouse *Chk1 FLOX/−* embryonic stem cells (Liu et al., 2000). Three independent ES cell clones that contained the *Chk1+/Flox* genotype were injected into blastocysts and implanted into C57BL/6 female mice. In total, 15 chimeric males were obtained. When backcrossed to C57BL/6 female mice, four of these chimeras transmitted the targeted allele to their offspring to generate the *CHK1+/FLOX* mice (data not shown).

The availability of the mammary-specific whey acidic protein-Cre recombinase (*WAP-Cre*) transgenic mice facilitated the deletion of the conditional *Chk1* allele from somatic lobuloalveolar precursor cells in the mammary gland (Wagner et al., 1997a). Cre expression is increased in these transgenic mice at day 10 of pregnancy, when mammary epithelial cells (MECs) are highly proliferative and subsequently undergo differentiation into milk-producing lobuloalveolar mammary epithelial cells (LMECs) (Figure 1). However, only approximately 50%–60% of the *partially differentiating* LMEC precursors, during mid-pregnancy (P10–14), actually express *WAP-Cre*. In the remaining mammary lobuloalveolar and ductal cells, transgene expression was not observed (Wagner et al., 2002). Furthermore, during early lactation, e.g., at day 1 of lactation (L1), an additional 10%–15% of *fully differentiated* LMECs will express *WAP-Cre*, resulting in the generation of a second population of cells activating *WAP-Cre* (Figure 1). In combination with the Flox-Stop-Flox R26R-LacZ (*R26R-LacZ*) reporter line, Cre expression and recombination in mammary epithelial cells can be readily visualized during pregnancy and lactation (Soriano, 1999; Wagner et al., 2002). This not only provides a convenient assay for recombination in the mammary gland, but also facilitates the analysis of tissue morphology after deleting *Chk1* from epithelial cell precursors.

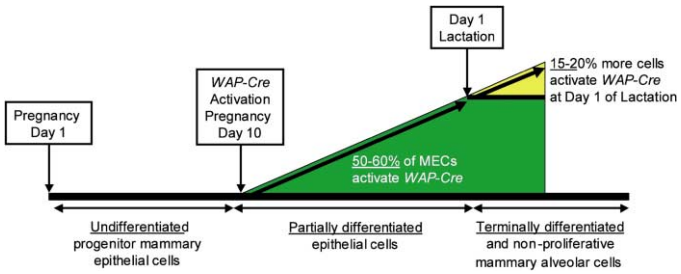

Figure 1. *WAP-Cre*: A developmentally timed mouse mammary gland model for the conditional deletion of *Chk1* kinase

Mice carrying the conditional floxed allele for *Chk1* were bred with mammary-specific *WAP-Cre* mice, generating bigenic animals (*WAP-Cre // Chk1 +/F* or *F/F*). WAP-Cre is activated in two waves: first, beginning at day 10 of pregnancy (green), Cre will be expressed in 50%–60% of the progenitor mammary epithelial cells (MECs), resulting in the removal of either one (*+/F*) or both (*F/F*) floxed alleles for *Chk1*, respectively, in these cells. At day 1 of lactation (yellow), an additional population of 15%–20% of the *terminally differentiated* mammary lobuloalveolar cells in these glands will activate *WAP-Cre* and delete any conditional alleles for *Chk1* in this population. This results in the deletion of *Chk1* in 65%–80% of the mammary epithelial cells in this bigenic gland.

Mammary glands from either *WAP-Cre/+ // R26R-LacZ/+ // Chk1 Flox/Flox*, *+/Flox*, or wild-type (herein referred to as *Chk1 F/F*, *+/F* or *+/+*) females were removed at day 1 of lactation, approximately 11 days after *WAP-Cre* expression. Detection of Cre-mediated recombination was revealed by β-galactosidase staining of L1 trigenic mammary gland wholemounts (Figures 2A and 2B). *Chk1 F/F* mammary glands exhibited a dramatic reduction in the number of LMECs as indicated by the decreased numbers of β-galactosidase-stained cells (Figure 2A). Importantly, this decrease in LMECs was proportional to the percentage of cells expressing *WAP-Cre* (50%–60%) during pregnancy, as previously described. Conversely, trigenic heterozygous (*+/F*) tissues for Chk1 showed only slightly reduced levels of alveolar development, as compared to the wild-type mammary gland (Figure 2A). Therefore, the *Chk1 F/F* glands exhibited a substantial decrease in the number of LMECs following Cre-mediated recombination.

Histological analysis of conditional homozygous *Chk1* tissue confirmed the marked decrease in the number of Chk1 F/F LMECs, as compared to wild-type (Figure 2B). Quantitation of DAPI-stained nuclei in *Chk1 +/F* and *F/F* tissue sections indicated a 15%–18% and 55% reduction, respectively, in the number of LMECs present at day 1 of lactation, as compared to wild-type. As a result of the substantial loss of milk-producing LMEC precursors, conditional *Chk1 F/F* mothers failed to nurse their young. Subsequently, the pups of these animals had to be foster nursed in order for litter survival. In contrast, Chk1 heterozygous animals were able to support their offspring.

To examine recombination in the *Chk1 F/F* glands, PCR-mediated analysis of recombination at the conditional Chk1 locus was conducted at various intervals during mammary gland development. DNA was extracted from *Chk1 +/+*, *+/F*, and *F/F* mice at days 12 and 16 of pregnancy and day 1 of lactation (2, 4, and 11 days post-Cre activation, respectively). The recombined *Chk1 F/F* alleles were detected, at P12, but these LMECs were lost by day 16 (P16, Figure 2C), indicating a lack of recom-

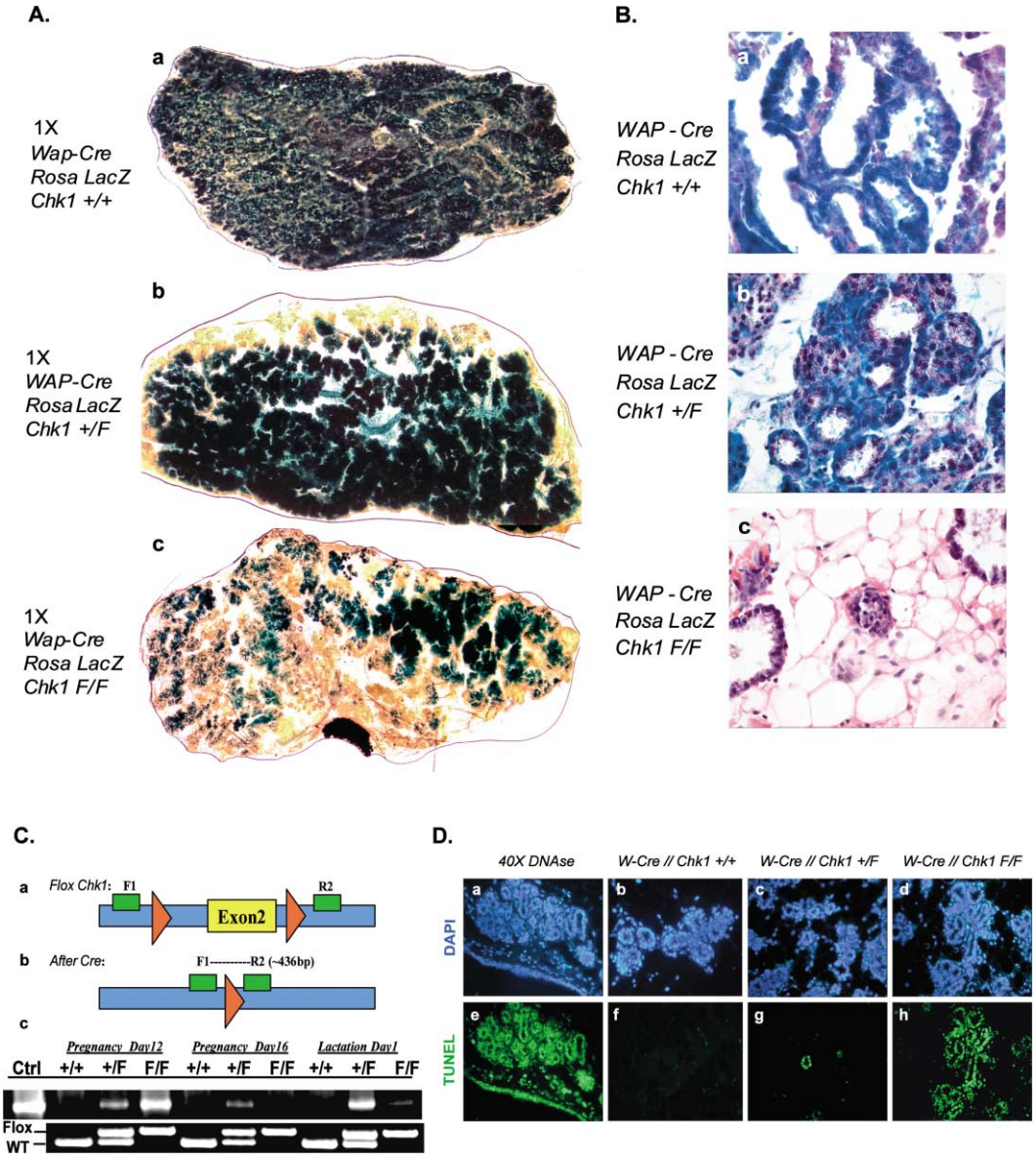

Figure 2. Conditional deletion of *Chk1* from the mammary gland by WAP-Cre results in massive apoptosis and impaired development

A: X-gal stained wholemounts of recombinant bigenic *WAP-Cre // Flox-Stop-Flox ROSA lacZ // Chk1 +/+* (**a**) or recombinant tri-genic *WAP-Cre // Flox-Stop-Flox Rosa LacZ //* and either *Chk1 +/Flox* (**b**) or *Chk1 Flox/Flox* (**c**) mammary glands.

B: Hematoxylin and eosin stained sections of X-gal stained *WAP-Cre // Chk1 +/+* (**a**) or trigenic glands for either *WAP-Cre // Rosa LacZ // Chk1 +/Flox* (**b**) or *Chk1 Flox/Flox* alleles (**c**). Shown at 40× magnification.

C: Schematic for the conditional allele for *Chk1* (**a**); red triangles indicate the two *loxP* sites flanking Exon 2 of the *Chk1* gene; green boxes indicated by F1 and R2 represent primers designed to detect recombination. **b:** Upon recombination, exon 2 is deleted, allowing for the detection of recombinant DNA by PCR using the F1/R2 primer set. **c:** PCR-mediated recombination yielding a 436 bp product from DNA harvested from *WAP-Cre // Chk1 +/+, +/F, or F/F* tissue during days 12 and 16 of pregnancy and day 1 of lactation (approximately 2, 6, and 11 days post-Cre activation, respectively). Genotyping primers, flanking the 5′ loxP site, were used to identify the genotype of the tissue.

D: Terminal transferase TUNEL assays on sections of bigenic day 12 of pregnancy mammary glands from *WAP-Cre // Chk1 +/+, +/F, or F/F* animals. DAPI staining (**a**–**d**) and TUNEL-positive nuclei (green) are shown for the Dnase-treated control (**e**), wild-type (**f**), *Chk1 +/F* tissues (**g**), and *Chk1 F/F* (**h**) samples.

bined cells. Interestingly, the recombinant Chk1 double-floxed allele was again detected at day 1 of lactation, coincident with an additional 15%–20% of the LMECs activating *WAP-Cre* (Figures 1 and 2C). In contrast, the levels of recombination in the *Chk1 +/F* LMECs remained constant during pregnancy and lactation (Figure 2C). As a control, recombination was never detected in *WAP-Cre // Chk1 +/+* tissues due to the lack of a floxed Chk1 allele (Figure 2C). Thus, these results suggested that by day 1 of lactation, homozygous floxed LMECs undergo apoptosis, and these mice fail to lactate due to the loss of these LMEC precursors.

To investigate the possibility of apoptosis during develop-

ment, female conditional Chk1 mice were mated and glands were harvested at pregnancy day 12 (P12), approximately 2 days after WAP-driven Cre expression begins to increase in these tissues. Sections from these conditional glands were screened for the presence of apoptotic LMEC progenitors using a terminal transferase-based TUNEL assay. P12 *Chk1 F/F* mammary glands exhibited substantial apoptosis in approximately 10%–12% of differentiating LMECs, while *Chk1* conditional heterozygotes and wild-type glands demonstrated only 3% and >1%, respectively (Figure 2D). This level of progenitor cell apoptosis in the *Chk1 F/F* mammary gland is sufficient to impair the normal development of this tissue. Therefore, the deletion of Chk1 from proliferating somatic cells in vivo results in cell lethality mediated by a currently unknown apoptotic mechanism.

Chk1 is a haploinsufficient tumor suppressor

Since the role of Chk1 is thought to maintain functionally intact DNA replication and G2/M checkpoints, we next examined cell cycle progression in the mammary glands harvested from day 1 lactating *WAP-Cre // Chk1* +/+, +/*F*, or *F/F* mice. Females were injected intraperitoneally with BrdU and glands were harvested after 2 hr, approximately equal to a 30 min label time in vivo, to measure the levels of DNA synthesis within these tissues (Kriss and Revesz, 1962; Rocha et al., 1990). Wild-type mammary glands displayed approximately 6.5% BrdU positive cells, consistent with previously published reports (Figures 3A and 3B) (Wagner et al., 1997b). *Chk1 F/F* samples showed even lower levels of proliferation at approximately 4%, presumably due to the apoptosis of previously replicating cells (Figures 3A and 3B). Strikingly, in *Chk1* heterozygous tissues, a 6-fold increase (~35%) in the number of BrdU-stained cells was detected, as compared to wild-type mammary glands (Figures 3A and 3B). This haploinsufficiency defect has not been previously reported in mammalian cell culture using Chk1-specific siRNAs.

To explore this phenomenon, sections were costained for BrdU incorporation and an antibody that recognizes phosphorylated Thr187 on the Cdk inhibitor, p27. The phosphorylation of p27 at this particular threonine marks the G1/S transition in mammalian cells. A feedback loop is created since Cdk2/cycE phosphorylates p27 at T187, targeting it for destruction. Upon staining, Chk1 wild-type samples could be divided into three populations: those that are in late G1 represented by only phospho-p27, those nuclei in mid-late S phase, marked by only BrdU, or those proliferating cells which have exited late G1 and have entered early S signified by the staining of both markers (Figures 3A and 3C). The specificity of this antibody was validated by the absence of detectable staining in p27−/− mammary epithelium (Figure 3A). *Chk1* heterozygous LMECs prominently displayed a 10-fold increase in the number of nuclei with phospho-p27 (late G1) staining as compared to wild-type (Figures 3A and 3C). Approximately 85% of the nuclei exhibiting BrdU incorporation also costained for phospho-p27 Thr187, indicating that these cells are in a stage with active Cdks. It is possible that phosphor-p27 T187 causes conditionally *Chk1* +/*F* cells to exit G1, resulting in substantially increased rates of DNA synthesis.

Haploinsufficiency causes partial chromatin condensation during S phase

To ascertain whether *Chk1* +/*F* cells are cycling through to mitosis, tissue sections were stained for phosphorylated serine 10 of histone H3, a commonly used mitotic marker. Phospho-H3 staining was detected in over 30% of the *Chk1* +/*F* nuclei, as large punctate foci at the periphery of the nucleus (Figures 4A and 4C). This level of minor H3 phosphorylation is usually indicative of cells in late G2 phase of the cell cycle (Crosio et al., 2002). Unexpectedly, a large majority of the *Chk1* +/*F* cells, which contained phosphorylated H3 staining, also prominently displayed BrdU incorporation *within the same nucleus* (Figure 4A). It is highly unlikely that these *Chk1* +/*F* cells could have entered into late G2/M within the in vivo time frame of BrdU labeling (Christov et al., 1993; Goepfert et al., 2000; Rajan et al., 1996). In contrast, wild-type glands rarely exhibited both BrdU staining and phospho-H3 accumulation, since these processes are normally restricted to S phase and mitosis, respectively (Figure 4A). Thus, in vivo analysis of Chk1 heterozygous tissues revealed not only inappropriate DNA replication, but also spontaneous partial chromatin condensation prior to the completion of S phase, which may lead to genomic instability due to the lack of apoptosis observed.

To examine this pattern seen for phosphorylated histone H3 in *Chk1* +/*F* glands, tissues were stained for the suspected histone H3 kinase, Aurora B. A marker for mitotic progression, Aurora B kinase was readily observed in wild-type cells progressing through mitosis (data not shown) (Crosio et al., 2002; Terada et al., 1998). Conversely, Aurora B colocalized exclusively with phospho-H3 to the same large condensed nuclear foci at the nucleoplasmic side of the nuclear membrane in *Chk1* +/*F* LMECs (Figure 4B). Quantitative analysis of the number of cells demonstrating Aurora B or phospho-H3 costaining revealed an approximately 1:1 ratio (Figures 4B and 4C), consistent with the >90% colocalization observed. INCENP, the chromosomal passenger protein necessary for Aurora B activity during mitosis, also colocalized to these large discrete foci (data not shown). Although Aurora B and INCENP have a conserved role for inducing chromatin condensation during mitosis, these cells still fail to fully condense their DNA, as only 6% of these *Chk1* +/*F* nuclei proceeded to accumulate phospho-histone H3 staining characteristic of a mitotic cell. These data suggest that due to *Chk1* haploinsufficiency, these cells display a miscoordinated cell cycle marked by premature chromatin condensation prior to completion of DNA synthesis.

Further evidence of miscoordination comes from costaining of phospho-p27 (late G1) and Aurora B (mitosis) in Chk1 heterozygotes. We observed coexpression of these two markers in approximately 25%–30% of 1000 epithelia quantitated (Supplemental Figure S1A at http://www.cancercell.org/cgi/content/full/6/1/45/DC1). This is in contrast to wild-type tissues, which showed <1% coexpression (Supplemental Figure S1A). In addition to the BrdU/p-H3 phenotype above, these data further support a state of cell cycle miscoordination in Chk1 conditional heterozygotes.

Cdc25A accumulates in *Chk1* heterozygous tissues

The oncoprotein Cdc25A phosphatase is involved in the activation of Cdk2/CycE at the G1/S transition and facilitates activation of DNA replication. Since Chk1 has been implicated in the negative regulation of Cdc25A (Sorensen et al., 2003), we examined Cdc25A protein levels in whole tissue lysates from day 1 lactating heterozygous Chk1 glands by Western blotting. A marked increase in the total amount of Cdc25A protein was observed in heterozygous *Chk1* lysates compared to wild-type

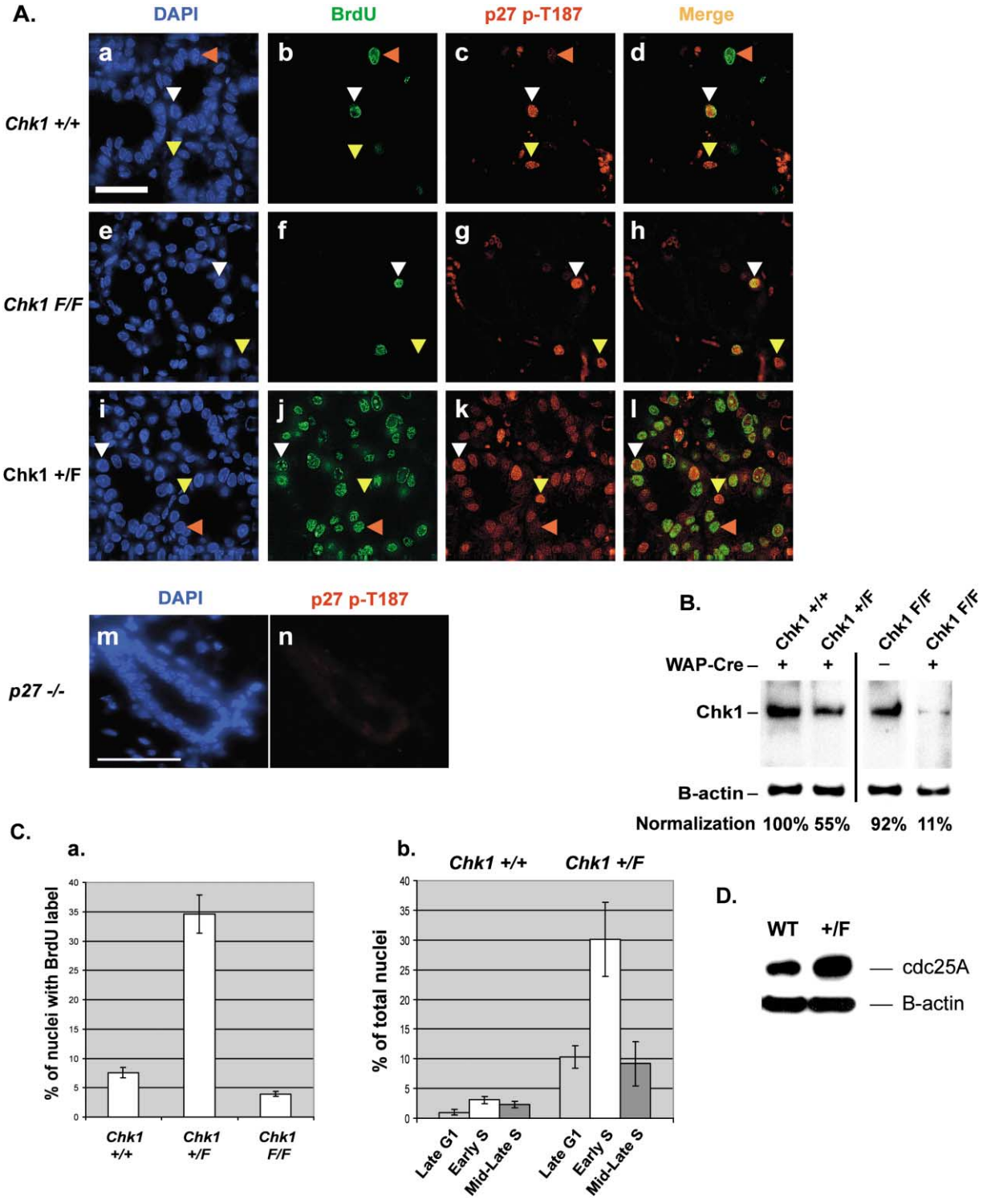

Figure 3. *Chk1* heterozygosity in somatic LMECs results in increased numbers of cells in S phase

A: BrdU was injected 2 hr prior to biopsy from *Chk1 +/+* (**a–d**), *Chk1 F/F* (**e–h**), or *Chk1 +/F* (**i–l**) mammary glands at day 1 of lactation (all tissues carried the *WAP-Cre* transgene). Paraffin-embedded glands were stained with DAPI (**a**, **e**, and **i**), anti-BrdU-FITC (**b**, **f**, and **j**), or anti-phospho-p27 T187 (Texas Red, **c**, **g**, and **k**). Merged images are shown (**d**, **h**, and **l**). Scale bars represent 50 μm. Arrows indicate BrdU only (red), phospho-p27 only (yellow), or both phospho-p27 and BrdU (white) stained nuclei. **m** and **n** represent the negative control for the phospho-p27 Ab by staining *p27−/−* tissues.
B: Lysates generated from day 1 lactating *WAP-Cre // Chk1 +/+*, *WAP-Cre // Chk1 +/F*, *WAP-Cre // Chk1 F/F*, or Chk1 F/F mammary glands were electrophoresed on SDS-PAGE gels, and Western analysis was carried out using a monoclonal antibody against Chk1. β actin blotting is shown as a loading control. Bands were quantitated using Image QuaNT software. Normalization is shown in terms of percentages of the CHK1/β actin ratio compared to *WAP-Cre // Chk1 +/+* (100%).
C: Epithelial cells from tissue sections were counted and the percentage of nuclei with BrdU incorporation are shown for the labeled *WAP-Cre // Chk1* genotypes (**a**). Graph representing the total percentage of cells marked by either phospho-p27 only (late G1), phospho-p27 + BrdU (early S phase), or BrdU only (mid-late S phase) in *WAP-Cre // Chk1 +/+* or *+/F* tissue sections (**b**).
D: Lysates generated from day 1 lactating *WAP-Cre // Chk1 +/+* or *+/F* mammary glands were electrophoresed on SDS-PAGE gels and Western analysis was carried out using a monoclonal anti-Cdc25A antibody. β actin blotting is shown as a loading control.

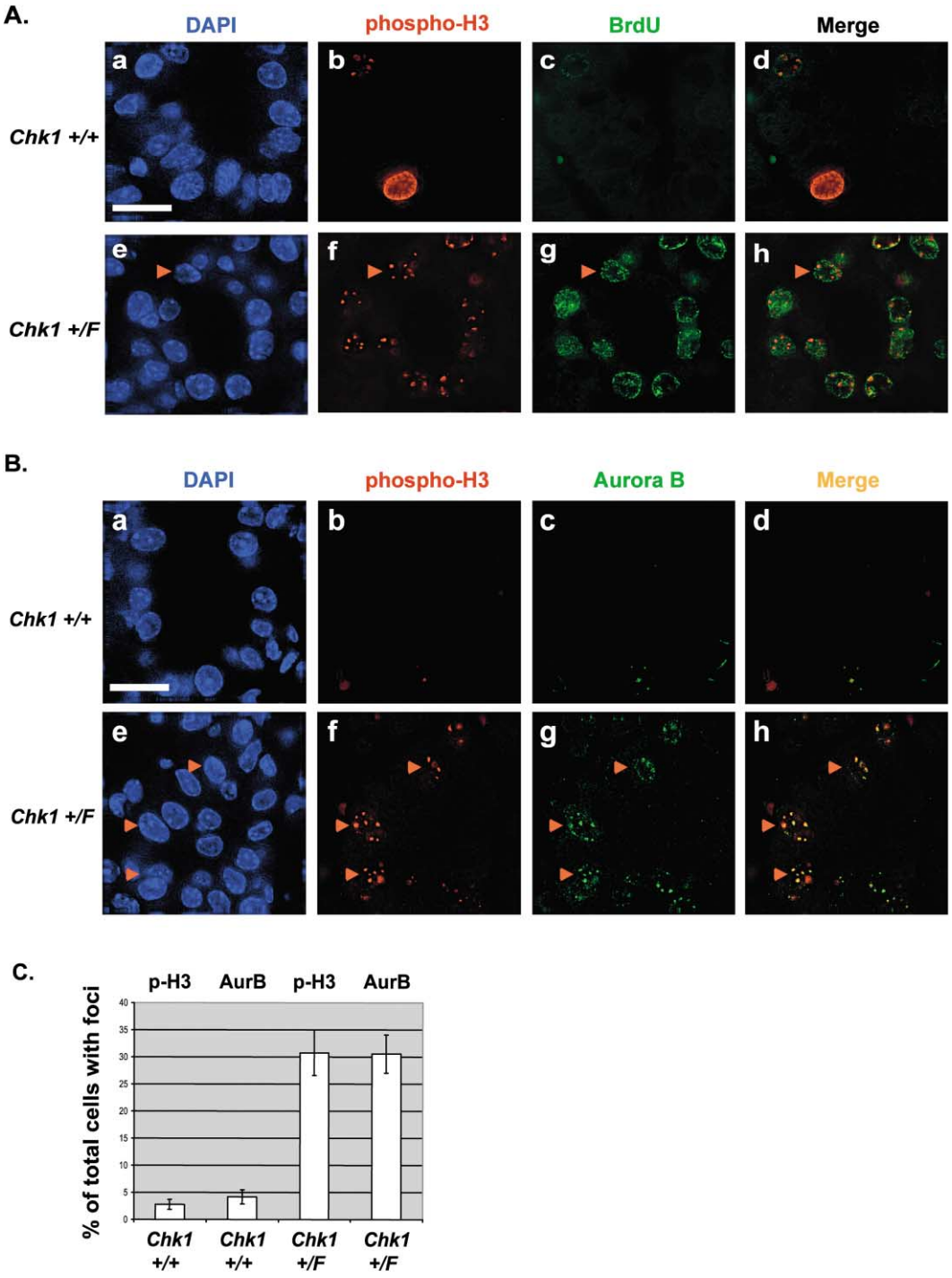

Figure 4. *Chk1* heterozygosity results in cell cycle miscoordination

A: Sections of either *WAP-Cre // Chk1 +/+* (**a**–**d**) or *WAP-Cre // Chk1 +/F* (**e**–**h**) were stained with anti-phosphorylated histone-H3 Ser10 (Texas Red, **b** and **f**) or anti-BrdU-FITC (**c** and **g**). Representative merged images are shown in **d** and **h**. Scale bars represent 10 μm. Red arrows indicate representative nuclei.
B: Sections of either *WAP-Cre // Chk1 +/+* (**a**–**d**) or *WAP-Cre // Chk1 +/F* (**e**–**h**) were stained with anti-phosphorylated histone-H3 Ser10 (Texas Red, **b** and **f**) and anti-Aurora B antibodies (**c** and **g**). Merged images are shown (**d** and **h**). Scale bars represent 10 μm.
C: Epithelial cells from tissue sections were counted each for *WAP-Cre // Chk1 +/+* or *Chk1 +/F* samples, and the percentage of nuclei demonstrating >3 large nuclear foci for phospho-H3 or Aurora B is displayed.

extracts (Figure 3D). This suggests that an inadequate level of Chk1 kinase activity exists in vivo to properly regulate Cdc25A protein levels. To directly examine Chk1 deficiency, we measured Chk1 protein levels in tissue lysates from Chk1 F/F (with and without Cre), +/F, and +/+ glands. We observed a 45% reduction in Chk1 protein in heterozygotes in Western blots, as compared to wild-type lysates (Figure 3B). Thus, Chk1 levels appear to be reduced by approximately one half in conditional

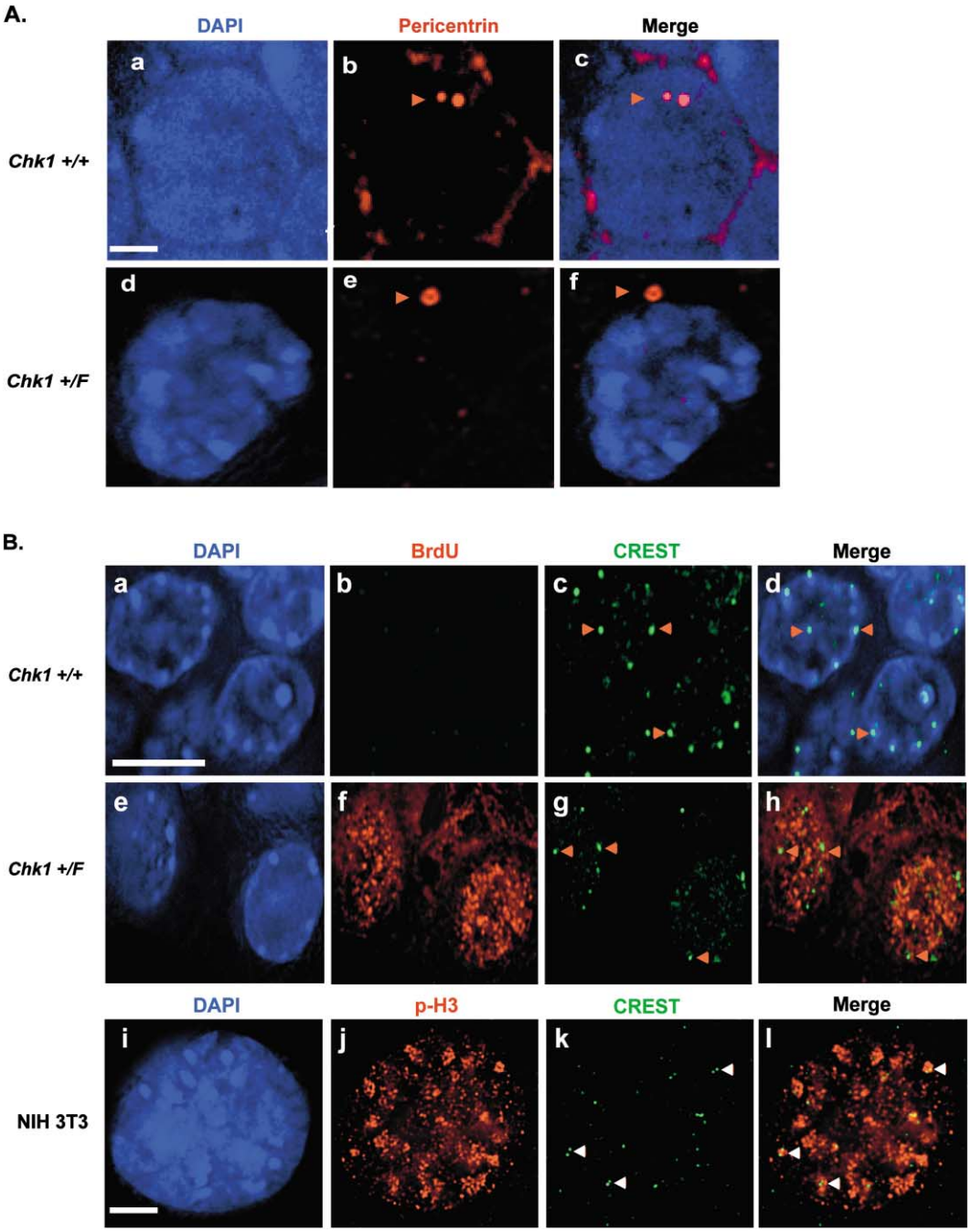

Figure 5. Heterozygous *Chk1* cells are in early S phase

A: *WAP-Cre // Chk1 +/+* and *WAP-Cre // Chk1 +/F* tissues were stained with anti-pericentrin (**b** and **e**). Blue represents DAPI-stained DNA (**a** and **d**). Red arrows indicate either 1 or 2 centrosomes (red). Merged images are shown in **c** and **f**). Scale bar indicates 1 μm.

B: Tissues from **A** were stained with CREST autoimmune sera specific for centromeres (green, **c**, **g**, and **k**). BrdU incorporation is indicated in red (**b** and **f**). Merged images are shown in **d**, **h**, and **l**. DAPI-stained DNA is shown in **a**, **e**, and **i**. Red arrows indicate unduplicated centromeres. A representative G2/M NIH 3T3 cell stained for phospho-H3 (red, **j**) and CREST is shown. White arrows indicate duplicated centromeres. Scale bar indicates 5 (**a**–**h**) and 1 (**i**–**l**) μm.

heterozygotes, which explains the haploinsufficient phenotype. The high levels of Cdc25A and the reduced level of Chk1 in Chk1 +/F tissues, in conjunction with the increased percentages of cells undergoing DNA replication and premature chromatin condensation, help delineate a critical interaction between Chk1 and Cdc25A in regulating proper G1 exit and S phase entry.

DNA replication markers indicate early-mid S phase

Phospho-p27/BrdU and phospho-H3/Aurora B staining should reflect two mutually exclusive stages of the cell cycle, early S and G2/M, respectively. Yet these markers appeared to be coexpressed in LMECs heterozygous for *Chk1*. Thus, it was important to utilize other independent markers to ascertain the

correct stage of cell cycle in these LMECs. Accordingly, we stained *Chk1 +/F* tissues for pericentrin, a peripheral centrosomal marker, which should indicate whether these cells were in early S or G2 depending upon the number of centrosomes displayed. In wild-type tissues, we detected some cells in G2 represented by the presence of two centrosomes (Figure 5A). In contrast, *Chk1 +/F* LMECs displayed only a single centrosome along with the characteristic peripheral chromatin condensation mentioned previously (Figure 5A, for quantitation see Supplemental Data). These data indicate that these *Chk1* heterozygous cells have not yet replicated their centrosomes, suggesting a cell cycle period prior to G2.

As centromeres are the last segment of chromosomes to duplicate during DNA replication, we examined them to confirm that these cells were in early S phase, as suggested by the results of the phosho-p27 staining. The autoimmune sera from scleroderma CREST patients has widely been used to stain for centromeres in mammalian cells (Brenner et al., 1981; Moroi et al., 1980). If cells in S phase have yet to duplicate their centromeres during DNA replication, then early S phase mouse nuclei with 20 chromosomes should only exhibit twenty individual centromeric foci (Figure 5B and Supplemental Figure S1). In contrast, the centromere number will double to 40 centromeres (indicated by 20 paired sister chromatid doublets) if such cells progress through late S phase and into G2 (NIH3T3, Figure 5B). *Chk1* heterozygous tissues costained for both BrdU incorporation and centromeres (CREST) displayed only single centromeres while exhibiting DNA synthesis, confirming that they exist in a stage before late S phase (Supplemental Figure S1). These results indicate that these *Chk1* heterozygous cells are in early-mid S phase and display a miscoordinated cell cycle by concurrently exhibiting mitotic markers.

Chk1 haploinsufficiency causes DNA damage

Due to the large number of cells undergoing inappropriate DNA replication while prematurely condensing their chromatin, we sought to ascertain whether these *Chk1 +/F* LMECs were experiencing spontaneous *endogenous* DNA damage. To address this, tissue sections were stained for DNA damage response proteins using indirect immunofluorescence with antibodies against mouse 53BP1 (Figure 6A), MDC1 (Figure 6B), and phospho-H2A.X (Figure 6C). A hallmark of many proteins involved in DNA repair is their ability to form nuclear foci in response to DNA damage. The appearance of punctate nuclear foci, without the addition of any *exogenous* DNA damaging agents (note: the *Chk1 +/+* and *+/F* mice were all expressing Cre recombinase—see Discussion) were quantitatively scored for each individually stained DNA damage protein.

This analysis revealed that a significant number of heterozygous LMECs were undergoing a DNA damage response in *Chk1* conditional heterozygotes. The occurrence of multiple ($>$6) DNA damage foci per nuclei were seen in 36%–38% of the cells across the mammary glands of *Chk1 +/F* mice (Figure 6D). All the checkpoint markers used (MDC1, 53BP1, p-H2A.X) closely correlated with one another ($\pm$2%) in terms of the total number of cells forming DNA damage foci (Figure 6D). In contrast, wild-type LMECs displayed foci in less than 6.5% of those cells stained for damage response proteins (Figure 6D). The recently published colocalization of MDC1 with phosphorylated H2AX, a variant of histone H2A, was also observed in these heterozygous Chk1 LMECs (Figure 6C) (Schultz et al., 2000; Stewart et al., 2003). Since the phosphorylation of H2A.X is thought to only occur during a DNA damage response, these heterozygous *Chk1* LMECs appear to accumulate *endogenous* DNA damage. The foci formation observed by DNA repair proteins suggest that these *Chk1 +/F* LMECs are undergoing an aberrant S phase resulting in spontaneous DNA damage, in vivo, at day 1 of lactation.

To confirm that heterozygosity for *Chk1* is inducing the DNA damage observed in the conditional heterozygotes, germline *Chk1+/−* mammary glands without *WAP-Cre* were stained for these same checkpoint related proteins. As expected, 53BP1 (Figure 6A) and MDC1 (Figure 6B) each exhibited increased levels of DNA damage foci formation relative to wild-type tissues (Figure 6D). Interestingly, germline heterozygotes (*+/null*) displayed fewer total nuclei, which stained for these particular markers, than their *Chk1* conditional heterozygous counterparts, suggesting the possibility that some precursors for these cells were selected against earlier in development in the absence of wild-type levels of Chk1 (Figure 6D).

Homozygous Chk1 progenitor cells exhibit the haploinsufficient phenotype

To ascertain whether conditional *Chk1 F/F* homozygous LMEC progenitors also displayed a similar phenotype, mammary glands from day 12–12.5 of pregnancy, 2–2.5 days post Cre activation, were harvested from Chk1 +/+ and Chk1 F/F females and stained for BrdU and phospho-H3. Indeed, Chk1 F/F cells displayed cell cycle anomalies (Figure 7A) very similar to those of Chk1 +/F LMECs at lactation (Figure 4A). In contrast, wild-type tissues properly displayed S phase staining separately from those nuclei undergoing mitosis (Figure 7A).

Additionally, Chk1 F/F tissues at P12–P12.5 were stained for DNA damage markers to provide an explanation for the apoptosis observed in Figure 2D and the subsequent failure in development. In correlation with the cell cycle miscoordination, MDC1 and phospho-H2A.X DNA damage foci were observed in approximately 10% of Chk1 F/F nuclei (Figure 7B). Importantly, nuclei with highly saturated phospho-H2A.X staining patterns were particularly striking (Figure 7B, top arrow). Strong H2A.X phosphorylation following apoptotic DNA fragmentation has been previously reported (Rogakou et al., 2000). As such, the elevated levels of phosphorylated H2A.X in homozygous Chk1 cells may be a result of the DNA fragmentation seen during programmed cell death.

Aurora B is temporally mislocalized

To verify whether Aurora B sequestration only occurred in those cells that were experiencing a DNA damage response in vivo, tissue sections were costained with antibodies against Aurora B and MDC1. The pattern of immunofluorescence demonstrated that the large distinct foci for Aurora B only localized to condensed chromatin in those *Chk1* heterozygous cells undergoing a DNA damage response. These nuclei were marked by punctate foci formed by both MDC1 and Aurora B (Figure 8A). It is important to note that Aurora B did not colocalize with MDC1. Note that in wild-type tissues, Aurora B was also visualized at the periphery of nuclei undergoing a DNA damage response, albeit at levels far lower than heterozygous cells (Figure 8A).

Closer examination of the large Aurora B foci revealed that the heterochromatin in these areas appeared to be condensed into large discrete foci represented by increased DAPI absorption (Figure 8A). Aurora B colocalized with these large peripheral condensed DAPI foci (Figure 8A). This is reminiscent of nuclei

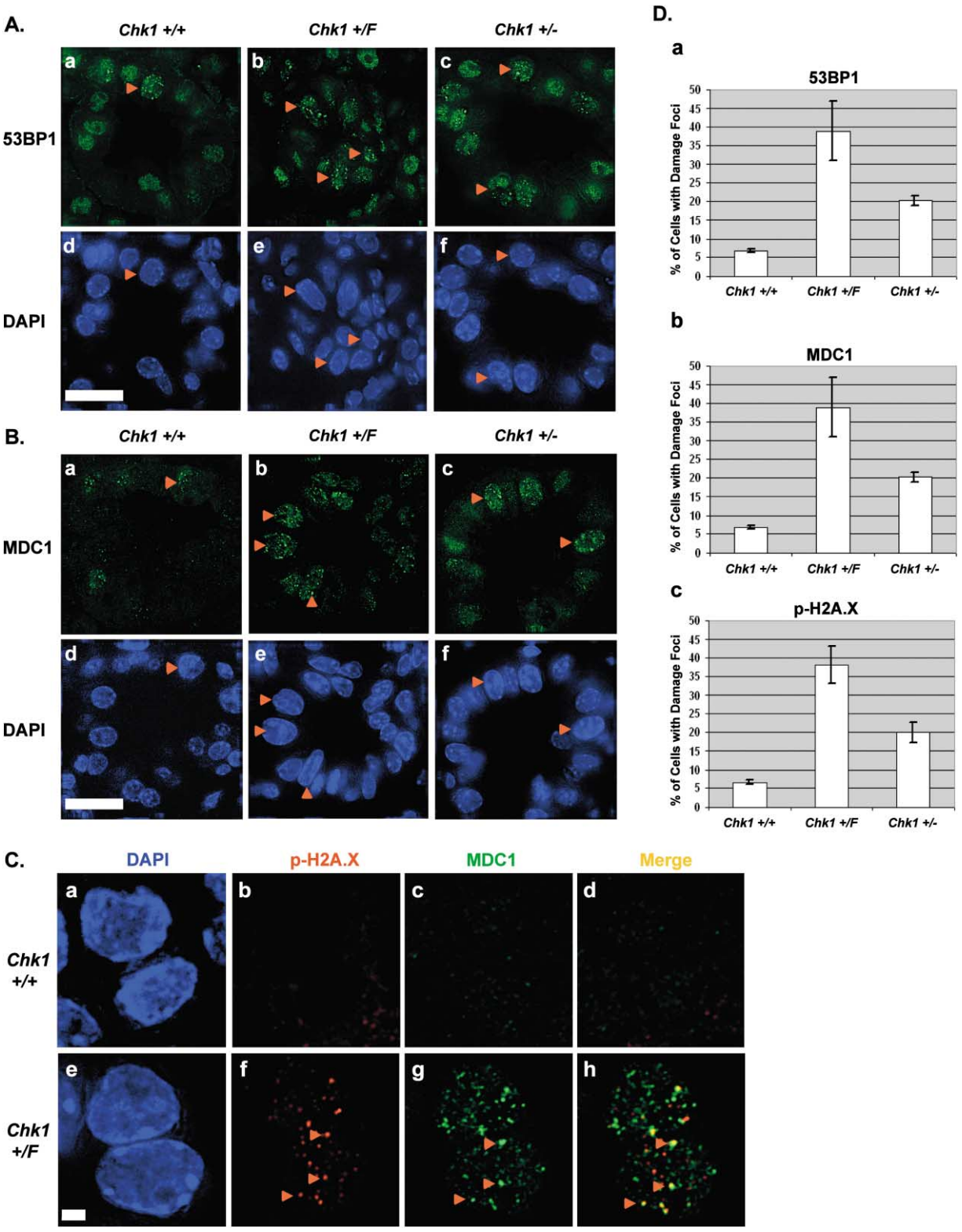

Figure 6. *Chk1* heterozygous LMECS exhibit endogenous DNA damage

A and B: *WAP-Cre // Chk1 +/+* (**a** and **d**), *WAP-Cre // Chk1 +/F* (**b** and **e**), or germline *Chk1+/−* lacking WAP-Cre (**c** and **f**) mammary glands were sectioned and stained (green) against either anti-53BP1 (**A**) or anti-MDC1 (**B**). Arrows indicate representative nuclei with endogenous DNA damage foci. Scale bars represent 10 μm.

C: WAP-Cre // Chk1 +/+ (**a**–**d**) or WAP-Cre // Chk1 +/F (**e**–**h**) nuclei were costained using antibodies against phospho-H2A.X (**b** and **f**) and MDC1 (**c** and **g**). Merged images for the two panels are shown in **d** and **h**. Red arrows indicate representative endogenous nuclear DNA damage foci. Scale bars represent 1 μm.

D: Quantitation of the total percentage of mammary lobuloalveolar epithelium displaying >6 foci/nuclei when staining for 53BP1 (**a**), MDC1 (**b**), or phospho-H2A.X (**c**) in tissue sections presented in **A**–**C**.

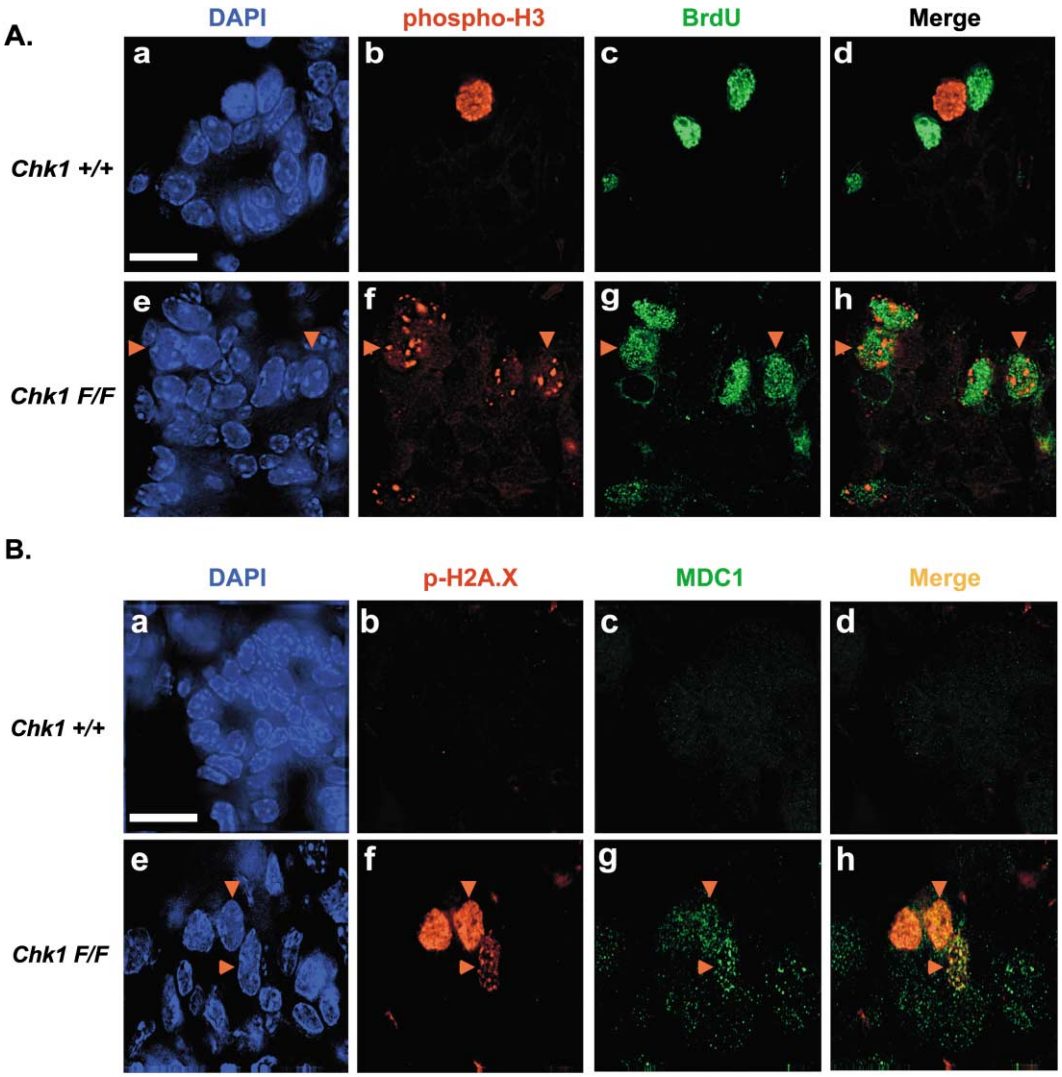

Figure 7. Following recombination, Chk1 F/F tissues reveal cell cycle miscoordination

A: Sections of either WAP-Cre // Chk1 +/+ (**a**–**d**) or WAP-Cre // Chk1 F/F (**e**–**h**) were stained with anti-phosphorylated histone-H3 Ser10 (Texas Red, **b** and **f**) or anti-BrdU-FITC (**c** and **g**). Representative merged images are shown in **d** and **h**. Scale bars represent 10 μm. Red arrows indicate representative nuclei. **B:** WAP-Cre // Chk1 +/+ (**a**–**d**) or WAP-Cre // Chk1 F/F (**e**–**h**) nuclei were costained using antibodies against phospho-H2A.X (**b** and **f**) and MDC1 (**c** and **g**). Merged images for the two panels are shown in **d** and **h**. Red arrows indicate representative nuclei. Scale bars represent 10 μm.

which undergo premature chromatin condensation (PCC) in response to hydroxyurea or other agents, which cause DNA damage, leading to an activated DNA damage checkpoint response (Nghiem et al., 2001). The DAPI staining of *Chk1* heterozygous cells appeared to be concentrated in large discrete darkly stained regions analogous to previous descriptions of cells undergoing premature chromatin condensation. These results suggest that during a mammalian DNA damage checkpoint response, Aurora B kinase may be sequestered to partially condensed regions of chromatin.

Discussion

In contrast to previous studies that focused primarily on cell culture or early embryogenesis experiments, in this study, we define the in vivo phenotype of conditional *Chk1* heterozygous and homozygous somatic cells in breast tissue. We present evidence that the Chk1 regulated oncoprotein Cdc25A accumulates in *Chk1* heterozygous tissues. The lobuloalveolar mammary epithelial cells (LMECs) from conditional *Chk1* mice demonstrated distinct but striking phenotypes depending on the disruption of one or both alleles for *Chk1* and demonstrate an important role for Chk1 in maintaining the genomic integrity of developing somatic tissues.

Chk1 deletion results in cell lethality

The initial phenotype observed in *Chk1* homozygous floxed mice was a failure of mothers to nurse their young. This lactogenic defect was caused by a rapid wave of apoptosis in the LMEC precursor population during mid-pregnancy (P10–P14), once *Chk1* was deleted. These results correlate with previous studies using conditional *Chk1* ES cells treated with Adeno-Cre recom-

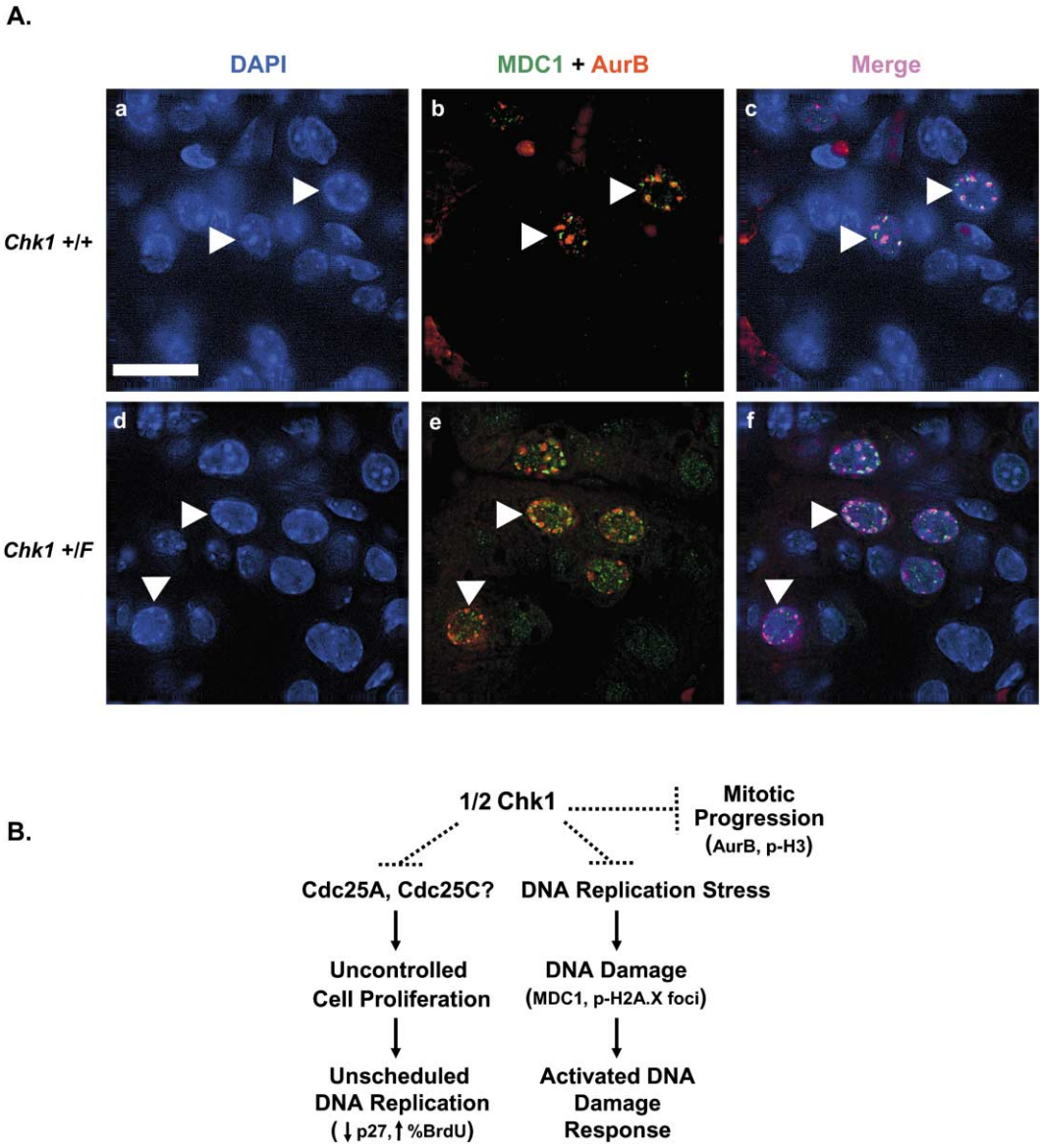

Figure 8. Aurora B is localized to foci of condensed chromatin in cells experiencing DNA damage

A: Sections of *WAP-Cre // Chk1 +/+* (**a**–**c**) or *WAP-Cre // Chk1 +/F* (**d**–**f**) mammary glands were costained with anti-MDC1 (green) and anti-Aurora B (Texas Red). Merged images for DAPI (blue) with MDC1 and Aurora B are shown (**c** and **f**). White arrows represent nuclei containing DAPI and Aurora B foci indicative of condensed chromatin (pink). Scale bars represent 10 μm.

B: A model for the consequences of *Chk1* heterozygosity. Deletion of a single *Chk1* allele results in the disruption of ordered cell cycle events. These cells exhibit an increase in p27 phosphorylation facilitating entry into S phase. As a result of unscheduled DNA synthesis and *Chk1* haploinsufficiency, DNA damage accumulates, probably due to an inability to detect and repair replicative lesions. Cell cycle miscoordination occurs in part due to inappropriate Cdc25 activity leading to activation of Cdks at the G1/S and G2/M boundaries. In addition, compromised G2/M checkpoint activation led to partial chromatin condensation and Aurora B kinase sequestration. Dotted lines indicate partially defective regulation.

binase, which also exhibited high levels of apoptosis within 36–48 hr postinfection (Liu et al., 2000). However, the 10%–20% of LMECs that lost *Chk1* during Cre activation, at day 1 of lactation, do not undergo apoptosis, suggesting it is not completely essential. How can this be explained? These lactating LMECs are terminally differentiated cells, and as such they do not undergo cellular proliferation (Wagner et al., 2002). Due to this nonproliferative state, they do not exhibit the apoptotic cell death observed in the mid-pregnant LMEC precursors, since they do not incur replicative stresses from undergoing DNA synthesis, which we feel are likely to require Chk1 function for survival. This explains the appearance of recombinant β-galactosidase-stained cells at day 1 of lactation in *Chk1* F/F tissues. In support of this hypothesis, TUNEL analysis in *Chk1* F/F tissues did not reveal any increased levels of apoptosis over wild-type tissues at day 1 of lactation (data not shown).

Chk1 heterozygosity causes an abnormal cell cycle

In contrast to *Chk1* null mammary glands, *Chk1 +/F* glands after Cre expression were apparently functional with respect to

tissue function. However, these heterozygous LMECs exhibited an enhanced propensity to undergo DNA synthesis, while simultaneously undergoing other cell cycle events that normally do not coincide with S phase. For example, *Chk1 +/F* LMECs immunostained for both p27 T187 phosphorylation and BrdU incorporation revealed that greater than 30% of these cells had exited late G1 and subsequently entered an S phase-like state, distinct from the *Chk1 +/+* controls. In addition, *Chk1 +/F* LMECs appear to experience DNA damage during this S phase state as indicated by the number of damage foci detected. Interestingly, these cells accumulate Aurora B at large foci localized to the nucleoplasmic side of the nucleus, and these foci colocalized with phosphorylated histone H3 at Ser10, indicative of DNA condensation at these areas typical of late G2. Although the expression of these two markers suggests that the *Chk1* heterozygous cells are in G2/M, the analysis of centrosomal (pericentrin) and centromeric (CREST) markers indicates a time frame during early S phase, since these cells have not undergone centrosome duplication, nor have they fully completed one full round of DNA synthesis by replicating their centromeres.

Our findings suggest a possible role for Chk1 in control of the G1/S transition. *Chk1* heterozygous cells show an inappropriately high number of LMECs incorporating BrdU, suggesting an inappropriate number of these cells traversing S phase (Figure 8B). Formally, in an animal model such as this, we cannot distinguish between inappropriate G1 exit versus a slowing down of cells during S phase as an explanation for this increase. However, *Chk1* heterozygous cells have increased levels of Cdc25A, and it has been previously shown that overexpression of Cdc25A can accelerate the G1/S transition via the premature activation of cyclin E- and cyclin A-dependent kinases (Blomberg and Hoffmann, 1999; Molinari et al., 2000; Vigo et al., 1999). Thus, we favor the explanation that cells are inappropriately exiting G1. This is consistent with the observation that these cells also show increased levels of phospho-T187 p27, which is typically observed in late G1-early S phase nuclei committed to DNA synthesis. Cdk2/cycE activity has been proposed to regulate this phosphorylation of p27 (Sheaff et al., 1997), which could be caused by Cdc25A overproduction. However, once S phase entry has occurred, a prolonged S phase may also result as a consequence to unresolved replication stress. As such, these results suggest a possibly indirect role for Chk1 in regulating the G1/S transition through its direct control of Cdc25A protein levels.

Chk1 +/F tissues experience a damaged S phase

Previous studies have demonstrated that Chk1 is essential for halting replication origin firing in response to DNA damage (Feijoo et al., 2001). In addition, chicken tumor cells engineered to lack Chk1 fail to maintain viable replication forks or suppress futile origin firing when DNA polymerase is inhibited, leading to incomplete genome duplication and diminished cell survival after release from replication arrest (Zachos et al., 2003). Chk1 is therefore a critical responder to DNA replicational stress. Haploinsufficiency for this function would be expected to lead to an inability to properly repair replication problems, resulting in the accumulation of damage at replication structures. Hence, the appearance of DNA damage foci containing MDC1, 53BP1, and phospho-H2A.X proteins in *Chk1 +/F* LMECs supports the presence of spontaneous DNA damage in these cells. This damage is not due to Cre expression, because it is also observed in nonconditional heterozygous *Chk1* mice, which do not express Cre. Thus, the haploinsufficiency observed here suggests that physiological Chk1 protein levels play a critical role in the regulation of DNA replication (Feijoo et al., 2001; Lee et al., 2003).

Chk1 shows haploinsufficiency for prevention of premature mitosis

Previous studies revealed that increased levels of Cdc25A are sufficient to override the restraint of mitotic entry during S phase, resulting in premature chromatin condensation (Molinari et al., 2000). Moreover, Cdc25A has been shown to play a significant role in activating Cdc2/cycB during mitosis (Mailand et al., 2000). Due to the increased levels of Cdc25A observed in *Chk1* heterozygotes, the localized chromatin condensation observed may be a result of premature activation of Cdc2/cycB complexes during S phase. Active Cdc2/cycB complexes inhibit protein phosphatase 1, which is an event necessary for the activation of Aurora B during mitosis (Goto et al., 2002; Murnion et al., 2001; Sugiyama et al., 2002). Since the expression of a nonphosphorylatable Cdc2 Y15A mutant in human cells induced premature chromatin condensation (Jin et al., 1996), it appears plausible that the chromatin condensation observed in Chk1 heterozygotes and the localization of Aurora B to these structures is a result of at least partial activation of Cdc2, possibly through inappropriate Cdc25A activation (Figure 8B).

All of the phenotypes observed in the heterozygous animals are also observed in the homozygous nulls. The difference is that the homozygous null cells all appear to undergo apoptosis, indicating that their cellular defects are significantly more severe. Mammalian Chk1 appears to perform a similar function to the budding yeast Chk2 homolog, Rad53, which also has an important role in stabilizing stalled replication forks and recovering from replicative stress (Sogo et al., 2002; Desany et al., 1998). It is likely that in Chk1 F/F cells, the inability to resolve defective replication structures coupled with miscoordination of cell cycle and inappropriate mitotic entry is responsible for the induced apoptosis.

Our current data support a model in which haploinsufficiency in *Chk1* heterozygotes can result in three distinct haploinsufficient phenotypes that each can contribute to tumorigenesis: inappropriate entry into S phase, accumulation of DNA damage during replication, and a failure to restrain mitotic entry in the presence of a damaged S phase. These results explain Chk1's role as a haploinsufficient tumor suppressor and suggest that inactivation of a single allele of *Chk1* in human tumors by mutation, chromosomal deletion, or epigenetic silencing may be a common source of genomic instability driving tumorigenesis.

Chk1 has been proposed to be a potential drug target for anticancer therapies (Hapke et al., 2001; Li and Zhu, 2002; McKinstry et al., 2002; Zhou and Sausville, 2003; Zhou et al., 2003). However, our studies suggest that Chk1 is essential for adult proliferating cells. As death of nontumor proliferating cells limits the usefulness of many current chemotherapeutic treatments, it is likely that Chk1 inhibition might also have pronounced toxicity that may limit its effectiveness in this respect. Whether tumor cells will prove more or less resistant to loss of Chk1 function than wild-type cells remains to be determined and will be critical for the success of checkpoint pathway intervention as an anticancer strategy.

Experimental procedures

Generation of conditional Chk1 mice

The Flox targeting vector (pQL456) was constructed as follows: the *lox*P-neo-*lox*P cassette was ligated to a 2.2 kb exon 2-containing genomic fragment, of which the last 59 bp was replaced by an EcoRV and a *lox*P site. The *lox*P-neo-*lox*P-E2-*Rv*-*lox*P centerpiece was flanked by 1.9 kb of 5′ untranslated region and 4.3 kb of 3′ genomic sequence carrying exons 3–5 (Liu et al., 2000). The *Chk1* +/*Flox* ES cells was created by targeting one *Chk1* allele with linearized pQL456 vector followed by the excision of the neo marker by transient expression of Cre recombinase. Three *Chk1* +/*Flox* ES clones were injected into blastocysts to generate chimera, of which two produced germline transmission.

Trigenic mice were generated by mating the mammary-specific WAP-Cre line (Henninghausen through NCI Frederick) with conditional floxed allele *Chk1* animals. Concurrent mating of Flox Stop Flox Rosa LacZ mice (*R26R*) with either the conditional *Chk1* or the *WAP-Cre* animals, was done to efficiently breed trigenic litters. Genotyping, by PCR, was conducted on mouse tail DNA using specific primers designed against *WAP-Cre* (Wagner et al., 1997a) or *LacZ* (Rijnkels and Rosen, 2001). We designed the Chk1 F1 (5′-ACC TGC CCG CAA CTC CCT TTC-3′) and R1 (5′-CCA TGA CTC CAA GCA CAG CGA-3′) genotyping primers to flank the 5′ LoxP site of the conditional mouse *Chk1* allele. This yielded a 380 bp PCR product for the conditional allele in comparison to a 318 bp product for the wild-type allele (54°C for 35 cycles). The presence of the *Chk1* null allele was confirmed by using a primer set against the neomycin gene of the following sequence, NeoF (5′-GAT CGG CCA TTG AAC AAG ATG G-3′) and NeoR (5′-CCT GAT GCT CTT CGT CCA GAT C-3′), respectively.

Mammary gland biopsy and histological analysis

Inguinal mammary glands were harvested at day 1 of lactation from mice who had given birth within the last 4–8 hr. Glands were fixed in 4% paraformaldehyde for either a 2 hr fixation period for immunocytochemistry and TUNEL analysis or 4 hr for histology. Tissues were then paraffin embedded and 5 μm sections were then cut and placed on glass slides. X-Gal staining of mammary gland tissue was incubated O/N at 37°C in X-gal staining buffer following 1.5–2 hr fixation in 4% paraformaldehyde as previously described (Rijnkels and Rosen, 2001). H&E staining of sections from whole-mounted, paraffin embedded tissues followed standard histological protocols. Whole mounts of day 1 of lactation mammary glands stained with X-Gal were taken on a Leica MZ FLUOIII stereomicroscope. Images for the X-gal and H&E staining on mammary gland sections were taken on an Olympus BX41 system microscope and processed with Adobe Photoshop.

TUNEL and PCR recombination analyses

Inguinal mammary glands from day 12 of pregnancy were harvested and fixed, as described above. Sections were then deparaffinized and rehydrated. Following 15 min of antigen retrieval in 10 mM sodium citrate, the slides were then cooled and washed 4 × 3 min in ddH2O and then once for 3 min in PBS. The protocol for the TdT terminal transferase kit was then followed (Roche and ChromaTide dUTP, Molecular Probes). Sections were then mounted with Vectashield with DAPI (Vector Labs). For PCR-based analysis of recombination in *Chk1* +/*F* and *F*/*F* tissues, DNA was extracted from 5 μM sections from 3–5 separate single parous mouse mammary glands of such genotype. Recombinant DNA was detected using the forward genotyping primer Chk1 F1 and a reverse primer Chk1 R2 (5′-TGC AAC AGC TTC TTC AGT TAT TC-3′). Using 200 ng of genomic DNA, a recombinant 436 bp band representing the recombined Chk1 allele could be detected using the Expand High Fidelity PCR system (Roche) at 50°C for 40 cycles in a Perkin Elmer thermocycler.

Immunofluorescence microscopy and antibodies

Immunofluorescent staining of mammary gland tissue was as follows: sections were prepared as described for TUNEL analysis until the PBS step. Sections were then blocked with goat serum (Sigma) 1:100 in blocking solution (3% BSA and 0.1% Tween 20 in PBS [pH 7.4]) for 1 hr. Specific primary antibodies were then added at the appropriate dilution in blocking buffer and then incubated overnight at RT in a humidified chamber. BrdU analyses were conducted using a mouse monoclonal anti-BrdU antibody conjugated to FITC (Becton Dickinson) (1:5). Mouse Aurora B was detected in sections using a mouse monoclonal anti-Aurora B/AIM-1 antibody, 1:200 (Transduction Labs). Mouse INCENP was detected in sections using a rabbit polyclonal antibody, 1:200 (a generous gift from Dr. William Earnshaw). Visualization of mitosis was done with a rabbit anti-phospho-S10 Histone H3 antibody, 1:200 (Upstate). DNA damage foci were detected using rabbit polyclonal antibodies against 53BP1, 1:200 (a gift from Dr. Phil Carpenter), MDC1 1:200 (Elledge Lab), and a mouse monoclonal phospho-Ser139 H2A.X antibody, 1:200 (Upstate). Mouse monoclonal pericentrin antibodies were obtained from Pharmingen (1:500). The CREST auto-immune serum was a kind gift from Dr. William Brinkley (1:500). Indirect immunofluorescent detection was conducted using secondary antibodies obtained from Molecular Probes at 1:200 dilution. Images were captured on a Zeiss/Applied Precision SoftWoRx Image Restoration Microscope (deconvolution) and processed using Adobe Photoshop or SoftWoRx proprietary software, respectively.

Tissue homogenization and Western blotting

As described previously (Said and Medina, 1995), tissue biopsies (approximately 0.5 grams) of lactating mammary glands from *Chk1* +/+ and +/*F* animals were directly homogenized in lysis buffer (Said and Medina, 1995) with the appropriate protease inhibitor cocktail (Roche). Protein analysis, electrophoresis, and blotting followed standard protocols. Mouse monoclonal Cdc25A (Santa Cruz) and rabbit polyclonal β actin antibody (Cell Signal) antibodies were used at 1:1000 in PBST.

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