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Genetic instability and mammary tumor formation in mice carrying mammary-specific disruption of Chk1 and p53

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Abstract

Checkpoint kinase 1 (Chk1) is a key element in the DNA-damage response pathway that is required for maintaining genomic stability. To study the potential role of Chk1 in mammary tumorigenesis, we disrupted it using a Cre/loxP system. We showed that although Chk1 heterozygosity caused abnormal development of the mammary gland, it was not sufficient to induce tumorigenesis. Simultaneous deletion of one copy of p53 failed to rescue the developmental defects; however, it synergistically induced mammary tumor formation in *Chk1*$^{+/-}$; *MMTV-Cre* animals with a median time to tumor latency of about 10 months. Chk1 deficiency caused a preponderance of abnormalities, including prolongation, multipolarity, misalignment, mitotic catastrophe and loss of spindle checkpoint, that are accompanied by reduced expression of several cell cycle regulators, including Mad2. On the other hand, we also showed that Chk1 deficiency inhibited mammary tumor formation in mice carrying a homozygous deletion of p53, uncovering a complex relationship between Chk1 and p53. Furthermore, inhibition of Chk1 with a specific inhibitor, SB-218078, or acute deletion of Chk1 using small hairpin RNA killed mammary tumor cells effectively. These data show that Chk1 is critical for maintaining genome integrity and serves as a double-edged sword for cancer: although its inhibition kills cancer cells, it also triggers tumorigenesis when favorable mutations are accumulated for cell growth.

Keywords

Chk1; mitotic catastrophe; genome integrity; mammary cancer; SB-218078

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Conflict of interest
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Introduction

The majority of cancers are characterized by abnormalities of the cell cycle (Kastan and Bartek, 2004; Deng, 2006). Most commonly, G1/S checkpoint abrogation figures prominently in these abnormalities, primarily because of mutations in the broadly important tumor suppressor p53, which enforces the checkpoint through p21 (Harper *et al.*, 1993; el-Deiry *et al.*, 1993; Brugarolas *et al.*, 1995; Deng *et al.*, 1995). Mitotic catastrophe, however, can be avoided if these cells retain a functional G2/M checkpoint. Loss of this checkpoint can send cancer cells down an apoptotic pathway, which explains why abrogation of the G2/M checkpoint is an interest target for antitumor therapy, particularly when combined with traditional DNA-damaging agents. An especially attractive aspect of this approach is that cells with mutated p53 may be especially vulnerable to such treatment, whereas normal cells with functional p53 and an intact G1/S checkpoint should be relatively unaffected. If this is the case, drugs that abrogate the G2/M checkpoint represent a new class of anticancer agents that target p53-null tumors, which may otherwise be resistant to chemotherapy and radiation (Lowe *et al.*, 1994).

The regulation of cell cycle progression involves overlapping pathways, but it has become clear that the checkpoint kinase 1 (Chk1) is essential in regulating progression in multiple phases of the cell cycle, including the G1/S and G2/M transition, as well as cytokinesis (Liu *et al.*, 2000; Zhao *et al.*, 2002; Xiao *et al.*, 2003; Peddibhotla *et al.*, 2009). It has been shown that Chk1 mediates signals from the upstream DNA-damage response kinases ATR and ATM, and acts principally by phosphorylating Cdc25A at inhibitory sites, blocking cellular entry into mitosis (Bartek and Lukas, 2003; Zhang *et al.*, 2008). Chk1 also has a role in delaying the onset of anaphase in mitotic cells when there is DNA damage (Collura *et al.*, 2005). It has been shown that the hereditary breast cancer-associated gene-1 (BRCA1), which has a vital role in genome surveillance and represses breast cancer formation, is also essential in activating Chk1 in response to DNA damage (Miki *et al.*, 1994; Yarden *et al.*, 2002; Deng and Wang, 2003).

Studying functions of Chk1 in tumorigenesis and progression is made difficult by the fact that *Chk1*$^{-/-}$ embryos die at E6.5 (Liu *et al.*, 2000; Takai *et al.*, 2000). Using a Cre-LoxP-mediated approach, however, indicated that haploid loss or complete loss of Chk1 in the mouse mammary gland failed to cause tumor formation, and instead resulted in extensive underdevelopment (Lam *et al.*, 2004). It is noted that Chk1 heterozygous mammary epithelial cells exhibited three distinct haploinsufficient phenotypes, including inappropriate S-phase entry, accumulation of DNA damage during replication, and premature mitotic entry. It was therefore proposed that Chk1 had multiple functions critical to tumor suppression, and that Chk1 haploinsufficient phenotypes could cause mammary tumorigenesis. However, experimental evidence of tumor formation in mammary epithelial-specific Chk1 mutant mice has not yet been provided (Lam *et al.*, 2004).

To investigate whether long-term Chk1 deficiency could induce tumorigenesis and how Chk1 deficiency affects cells differently depending on their p53 status, we created an animal model carrying conditional knockout of both Chk1 and p53 in the mammary gland. Our analysis revealed that Chk1 is critical for viability of both normal mammary epithelial cells

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and cancer cells, providing a molecular basis for the observed dual function of Chk1 in tumor inhibition and promotion under different conditions.

Results

Targeted disruption of Chk1 and p53 in mouse mammary epithelium

Chk1 was disrupted in mammary tissue by crossing a mouse strain that carries a loxP-flanked exon 2 of Chk1 (Liu *et al.*, 2000) with *MMTV-Cre* transgenic mice (Wagner *et al.*, 1997). Analysis of the mammary glands of *Chk*$^{+/Co}$;*MMTV-Cre* and *Chk1*$^{Co/Co}$;*MMTV-Cre* mice at 2 months to 12 months of age detected abnormal mammary development (Figures 1a–c). The mutant glands appeared less dense than controls, and this abnormality was more impressive when the alveoli were visualized in high power images. Our previous studies indicated that mammary defects caused by Brca1 mutation could be rescued by p53 deficiency (Xu *et al.*, 2001; Li *et al.*, 2007). As Chk1 activity is regulated at least in part by Brca1 (Yarden and Brody, 2001), we were interested in investigating whether the developmental defect associated with Chk1 mutation could be rescued by simultaneous deletion of p53. Therefore, the *Chk1*$^{Co/+}$;*MMTV-Cre* mice were further crossed with *p53*$^{Co/+}$ mice (Jonkers *et al.*, 2001) to generate mice with various genotypes. Analysis of these mutant mice indicated that mammary tissues of *Chk1*$^{+/Co}$;*p53*$^{+/Co}$;*MMTV-Cre*, *Chk1*$^{+/Co}$;*p53*$^{Co/Co}$;*MMTV-Cre* and *Chk1*$^{Co/Co}$;*p53*$^{Co/Co}$;*MMTV-Cre* mice were also underdeveloped (Figures 1d, e, and data not shown). Quantitative measurement of mammary branches and alveoli of mutant and control mice within arbitrary areas of the same size revealed that haploid loss or complete loss of Chk1 significantly impaired mammary branch morphogenesis and alverolization, and that these defects could not be rescued by haploid or complete loss of p53 (Figure 1f).

Mammary hyperplasia and tumor formation in Chk1 and p53 double-mutant mice

Despite the observation that impaired p53 function did not rescue the growth defects, hyperplastic and noninvasive focal lesions were detected in the mammary glands of some *Chk1*$^{+/Co}$;*p53*$^{+/Co}$;*MMTV-Cre* mice starting at 12 months of age (Figures 1g–i). In *Chk1*$^{+/Co}$;*p53*$^{Co/Co}$;*MMTV-Cre* glands, the formation of multifocal hyperplasia was more uniform than that in *Chk1*$^{+/Co}$;*p53*$^{+/Co}$;*MMTV-Cre* glands, suggesting that deletion of the remaining wild-type p53 allele had a stronger impact in inducing this lesion. Deletion of the remaining wild-type Chk1 allele, however, had a negative impact, as no hyperplastic foci were observed in the mammary glands of *Chk1*$^{Co/Co}$;*p53*$^{Co/Co}$;*MMTV-Cre* mice of similar age.

To study the potential impact of mutations of Chk1 and p53 on mammary neoplasia, we followed cohorts of animals that had gone through multiple cycles of pregnancy for tumor formation. Twenty out of 33 (60%) *Chk1*$^{+/Co}$;*p53*$^{+/Co}$;*MMTV-Cre* mice developed tumors, with a median time to tumor latency of approximately 10.5 months, whereas none of the control mice (*p53*$^{+/Co}$;*MMTV-Cre* and *Chk1*$^{+/Co}$;*MMTV-Cre*, *Chk1*$^{Co/Co}$;*MMTV-Cre*, or *MMTV-Cre* mice, n412 of each) developed tumors during the same period of time (Figure 2a). These data suggest that haploid loss of Chk1 and p53 synergistically induces mammary tumor formation.

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We further showed that 7 out of 11 $Chk1^{+/Co}$;$p53^{Co/Co}$;*MMTV-Cre* mice (64%) developed mammary tumors with a median time to tumor latency of 9 months. Thus, heterozygous loss of p53 allowed tumor formation in $Chk1^{+/Co}$ mice, whereas the loss of the remaining wild-type p53 allele slightly increased tumorigenesis (Figure 2a). Next, we studied the effect of homozygous loss of Chk1 on tumorigenesis. We showed that 3 out of 21 $Chk1^{Co/Co}$;$p53^{+/Co}$;*MMTV-Cre* mice (14%) developed mammary tumors with a median time to tumor latency of 21 months. Two out of seven $Chk1^{Co/Co}$;$p53^{Co/Co}$;*MMTV-Cre* mice (29%) developed mammary tumors with a median time to tumor latency of 14 months (Figure 2a). This observation indicates that although Chk1 heterozygosity promotes tumorigenesis, loss of both copies of Chk1 attenuates the synergistic effect in tumor formation. This observation also suggests that a certain level of Chk1 function is needed to support tumor formation. Consistent with this, our study indicated that mammary tumors derived from the $Chk1^{+/Co}$;$p53^{+/Co}$;*MMTV-Cre* mice and cell lines isolated from these tumors still expressed Chk1, although at a lower level compared with Chk1 wild-type controls (Figures 2b and c), whereas all tumors analyzed from these mice lost p53 protein (Figure 2c, and data not shown). These data suggest that Chk1 behaves in a haploinsufficient manner for mammary tumor formation in a p53-deficient background.

An interesting finding came from analyzing $p53^{Co/Co}$;*MMTV-Cre* mice, which were initially set up as a control for tumorigenesis in $Chk1^{+/Co}$;$p53^{Co/Co}$;*MMTV-Cre* mice and $Chk1^{Co/Co}$;$p53^{Co/Co}$;*MMTV-Cre* mice. Among a studying population of 10 $p53^{Co/Co}$;*MMTV-Cre* mice, 4 developed lymphoma at 7–8 months of age, 5 developed mammary tumor at 8 months and the remaining 1 developed a mammary tumor at 13 months (Figure 2a). The higher tumor incidence of $p53^{Co/Co}$;*MMTV-Cre* mice than $Chk1^{+/Co}$;$p53^{Co/Co}$;*MMTV-Cre* mice and $Chk1^{Co/Co}$;$p53^{Co/Co}$;*MMTV-Cre* mice suggests that impaired Chk1 function inhibits tumorigenesis induced by p53 deficiency. Thus, the relationship between Chk1 and p53 in tumorigenesis is complex with the following features: (1) $Chk1^{+/Co}$, $Chk1^{Co/Co}$ or $p53^{+/Co}$ mutation alone in the mammary gland does not cause mammary tumor formation; (2) $Chk1^{+/Co}$;*MMTV-Cre* or $Chk1^{Co/Co}$;*MMTV-Cre* together with $p53^{+/Co}$ mutation synergistically induces mammary tumorigenesis; and (3) homozygous loss of p53 causes mammary tumor formation at a relatively high frequency, which is reduced by heterozygous loss of Chk1 ($Chk1^{+/Co}$;$p53^{Co/Co}$;*MMTV-Cre*), and is further inhibited by homozygous loss of Chk1 ($Chk1^{+/Co}$;$p53^{Co/Co}$;*MMTV-Cre*).

Mammary tumors exhibited diverse histopathological features

Mammary tumors developed in the $Chk1^{+/Co}$;$p53^{+/Co}$;*MMTV-Cre* mice were analyzed for their histopathological features. The majority of tumors were adenocarcinomas, but were highly diverse in histopathology. A characteristic feature of the tumors was the presence of giant cells that had nuclear diameters many times larger than those of surrounding cells (Figures 3a and b). The nucleus of the giant cells was usually misshapen, containing smaller projections seeming to be in a process of separation (Figure 3b). Most tumor cells exhibited nuclear polymorphism (Figure 3c). Sometimes, a distinct histological boundary was formed within tumors, where cells with a distinct nuclear histology were adjacent (Figure 3d, arrows). The diverse histological appearance suggests that the impaired function of Chk1 and p53 might lead to random genetic alterations that initiated the tumorigenesis. A majority

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of tumors exhibited extensive angiogenesis (Figure 3e), although small patches of tumor cells of uniform size and with less angiogenesis were also observed (Figure 3f). We had also analyzed three tumors of varying sizes (0.5, 1 and 2 cm) that were developed in a single mouse. The data indicated that all these tumors contained many necrotic cells, with a higher number of necrotic cells in larger tumors (Figure 3g), possibly due to an increasing difficulty in getting nutrition supply for these cells. We further showed that only about 1% of cancer cells were 5-bromodeoxyuridine labeled, suggesting that these tumors were not highly proliferative. It is noted that all giant cells were 5-bromodeoxyuridine negative (Figure 3h), and PCNA negative and phospho-p27 positive, suggesting that they were in G1 phase (Supplementary Figure 1).

It is also noted that tumors derived from *Chk1*$^{Co/Co}$;*p53*$^{Co/Co}$;*MMTV-Cre* mice exhibited similar diverse histopathological features similar to those derived from *Chk1*$^{+/Co}$;*p53*$^{+/Co}$;*MMTV*-Crie mice (Supplementary Figures 2a–c). In contrast, tumors derived from *p53*$^{Co/Co}$;*MMTV-Cre* mice displayed uniform histological features and were highly proliferative (Supplementary Figure 2d), which is consistent with the view that loss of Chk1 suppressed p53-null status-induced mammary tumorigenesis.

Mammary tumors exhibited marked aneuploidy and chromosomal aberrations

The nuclear polymorphism of these tumors prompted us to investigate the status of chromosomes derived from tumor cells. Chromosome spreads of primary tumor cells from *Chk1*$^{+/Co}$;*p53*$^{+/Co}$;*MMTV-Cre* mice cultured overnight showed that ~68% cells were aneuploid (Figure 4a). In nearly 40% of the spreads, distinct double minute chromosomes were detected that mapped to chromosome 9, as confirmed by fluorescence *in situ* hybridization (Figure 4b). To further characterize chromosomal aberrations, we performed spectral karyotyping on metaphase spreads derived from early passages of two primary tumors.

Twelve spreads were analyzed from the tumor, D644. These cells showed a wide variation in ploidy levels (68–86 chromosomes), with near tetraploid to hypertetraploid cells (Figure 4c). A variety of structural chromosomal aberrations were identified by spectral karyotyping analysis (Figure 4c). It is noted that a reciprocal translocation of T(1;5)(5;1) was detected clonally (>90%). In single spreads, chromosomal aberrations such as non-reciprocal and Robertsonian translocations were also identified that were non-clonal. Complex translocations occurred because of fusions from parts of different chromosomes (Chr2, 3, 4, 8, 16, 19 and X). These aberrant fusions resulted in elongated chromosomes and were seen clonally in two metaphase spreads. A striking structural aberration was a sister chromatid break independently detected on Chr3, Chr14 and ChrX. A variety of chromosomal deletions were also found, notable among which was a deletion of Chr11 in >80% of the spreads analyzed. The second tumor, D643, contained an average number of chromosomes (~80, near tetraploid) and also structural aberrations, including chromosome translocations, insertions, deletions and fusions (Supplementary Figure 3).

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Chk1 haploinsufficiency resulted in mitotic catastrophe

To further investigate the source of this chromosome damage, as well as the origin of the polynuclear features in Chk1-associated tumors, we examined mitoses in three cell lines (369, 459 and 644) derived from *Chk1*$^{+/Co}$;*p53*$^{+/Co}$;*MMTV-Cre* tumors using time-lapse photography. Although mitosis of most *Chk1*$^{+/+}$control tumor cells (MMTV-neu, a cell line that was derived from a mammary tumor of a MMTV-neu,p53$^{+/-}$ mouse; or MMTV-ras, a cell line that was derived from a mammary tumor of a MMTV-ras,p53$^{+/+}$ mouse) (Brodie *et al.*, 2001b) was completed within 60 min, mitosis in many Chk1 mutant cells could last more than 8 h, which often led to cell death (Figure 5a). Many mutant cells encountered difficulties in cytokinesis. This cell (Figure 5b) already had a cleavage furrow at the time we noticed it. The cell became slightly elongated with a clearer cleavage furrow at 2 h. However, the cleavage was aborted and the cell maintained single at 4 h. At 6 h, the cell began a new round of cell division and formed a new cleavage furrow at 7.5 h. This time the cleavage was effective, but it resulted in the death of one daughter cell (Figure 5b). We also found that many divisions yielded daughter cells of different sizes, and that the smaller cells always died shortly after the division (Figure 5c).

It is conceivable that smaller cells might obtain less amount of DNA, and could not therefore maintain their normal cell cycle progression. To investigate this, we transfected mutant cells with histone H2B-GFP expression vector so that their chromosomes could be monitored during cell division. We found that many cells entered mitosis, but they were arrested at metaphase and then directly returned to interphase (Figure 5d). In some cases, cells underwent unequal segregation of their DNA. Such segregations were usually not successful and resulted in mitotic abortion (Figure 5e). As summarized in Figure 5f, the time-lapse study revealed a range of mitotic catastrophe in Chk1 mutant cells. These include: (1) cell death during mitosis; (2) mitotic prolongation, defined as delayed cytokinesis beyond 1 h; (3) multipolarity, defined as the presence of more than two poles of cytokinesis; and (4) asymmetric cleavage furrow, leading to the formation of daughter cells of different size. About 95% of mitotic figures exhibited one or more of these abnormalities. We have also examined mitotic figures of tumor cells derived from *MMTV-neu* mice under the same condition and found that over 40% of cells were normal (Figure 5f). Although the above abnormalities could be detected in the *MMTV-neu* cells, they were much milder than those observed in Chk1 mutant cells.

To investigate whether the mitotic catastrophe phenotypes could also be recapitulated in the cells that carry acute knockdown of Chk1, we performed small hairpin RNA knockdown of Chk1 in both *MMTV-neu* and *MMTV-ras* cells. Our analysis indicated that suppression of Chk1 in both cell lines significantly reduced their proliferation (Supplementary Figure 4). Time lapse revealed similar mitotic catastrophe in these cells (Supplementary Figure 5 and Figure 6) compared with the Chk1+/− cells shown earlier (Figure 5). Altogether, these data revealed an essential role of Chk1 in maintaining genome integrity.

Haploid loss of Chk1 impaired the spindle checkpoint

It was shown previously that Chk1 deficiency in yeast and chicken DT40 cells caused defective spindle checkpoint (Collura *et al.*, 2005; Zachos *et al.*, 2007). As *Chk1*$^{+/-}$ cells

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exhibited mitotic catastrophe, we suspected that the spindle checkpoint might be impaired in these cells. To examine this, we treated *Chk1*$^{+/-}$ and control cells with nocodazole, which depolymerizes microtubules and activates the spindle checkpoint. We found that although *Chk1*$^{+/+}$ cells (*MMTV-neu*) were able to maintain a relatively high mitotic index 48 h after treatment (~40%) (Figure 6a), the mitotic index of *Chk1*$^{+/-}$ and *Brca1*$^{-/-}$ cells declined to ~20%, which is closer to Brca1 mutant cells (~10%) that are known to be defective in the spindle checkpoint (Wang *et al.*, 2004). These data suggest that the haploid loss of Chk1 impairs the spindle checkpoint, highlighting an essential role for Chk1 in this process.

Multiple factors have a role in the spindle checkpoint (Deng, 2006). We found that Chk1 mutant cells had reduced levels of Bub1, BubR1 and Mad2, with the latter reduced in all three *Chk1*$^{+/-}$ cell lines examined (Figure 6b). This observation suggests that Mad2 has an important role in mediating Chk1 function in the spindle checkpoint. To investigate whether reduced Chk1 activity is a direct cause for the reduced levels of Mad2, we performed small hairpin RNA-mediated knockdown of Chk1 in MMTV-neu cells. Our data indicated that as Chk1 levels gradually reduced after lentiviral-mediated infection of small hairpin RNA specific for Chk1, Mad2 expression also decreased (Figure 6c). Next, we treated these with SB-218078, a Chk1-specific inhibitor (Jackson *et al.*, 2000), and found that inhibition of Chk1 resulted in the downregulation of Mad2 (Figure 6d). Thus, it is conceivable that reduced expression of Mad2 triggered by Chk1 haplo-insufficiency cannot hold cells in the metaphase, thereby leading to premature entry into anaphase and mitotic abnormalities.

Inhibition of Chk1 with SB-218078 effectively killed mammary tumor cells

Corresponding to the defective spindle checkpoint and high rate of mitotic catastrophe, *Chk1*$^{+/-}$ mutant cancer cells grew much slower than did the cancer cell lines driven by MMTV-ras and MMTV-neu (Figure 6e, and data not shown). The reduced growth of the *Chk1*$^{+/-}$ mutant cancer cells was even more pronounced than the *Brca1*$^{-/-}$ cancer cells (Figure 6e), which also suffer mitotic catastrophe but to a lesser extent (Xu *et al.*, 1999; Wang *et al.*, 2004). Moreover, our earlier data indicated that Chk1 heterozygosity or Chk1 homozygosity (complete loss of Chk1) inhibited tumorigenesis in *p53*$^{Co/Co}$;*MMTV-Cre* mice (Figure 2a). This observation prompted us to investigate the effect of inhibition of Chk1 using a Chk1 inhibitor, SB-218078, on these tumor cell lines. We found that this drug, which is a more specific inhibitor for Chk1 than some other Chk1 inhibitors (Jackson *et al.*, 2000), effectively killed three mammary tumor cell lines, *MMTV-ras*, *Brca1*$^{-/-}$ (69) and *Chk1*$^{+/-}$ (459) (Figure 6f). It is noted that *Chk1*$^{+/-}$ cells were most sensitive to SB-218078 treatment, perhaps due to their lower levels of Chk1, compared with Ras and *Brca1*$^{-/-}$ cells. We had also compared the behavior of the SB-218078-treated cells and the Chk1-small hairpin RNA-treated cells during mitosis using time lapse, and found that these cells similarly exhibited high levels of mitotic catastrophe under both conditions (Supplementary Figures 7 and 8). These data suggest that inhibition of Chk1 with a specific inhibitor is effective to block growth of a variety of tumor cells.

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Discussion

Previous investigations indicated that Chk1 deficiency induced a profound growth defect and cell cycle abnormalities (Liu *et al.*, 2000; Takai *et al.*, 2000; Lam *et al.*, 2004; Peddibhotla *et al.*, 2009). However, whether Chk1 loss can cause tumorigenesis is unknown. Using conditional Chk1 mutant mice, we found that either complete or heterozygous loss of Chk1 in the mammary gland causes profound growth defect, similar to early $Chk1^{-/-}$ embryos that cannot be overcome by p53 deficiency. These data suggest that Chk1 is critical for the viability of mammary epithelial cells. However, we found that the loss of one or both p53 alleles in the $Chk1^{+/Co}$ and $Chk1^{Co/Co}$ mammary gland was eventually able to cause hyperplastic foci formation and resulted in tumor formation after a long latency. This indicates that some $Chk1^{+/-}$ and $Chk1^{-/-}$ cells are able to overcome the growth defect, perhaps after accumulating multiple changes.

Why does the absence of p53 not rescue the developmental defects, but allows tumor formation associated with Chk1 deficiency? Previous investigations showed that Chk1 is an essential kinase involved in the regulation of G1/S and G2/M cell cycle checkpoints and in cytokinesis (Liu *et al.*, 2000; Zhao *et al.*, 2002; Xiao *et al.*, 2003; Peddibhotla *et al.*, 2009). Reduced Chk1 function in Pten-deficient cells leads to the accumulation of double-stranded DNA breaks and genetic instability, particularly at fragile sites (Puc and Parsons, 2005; Puc *et al.*, 2005; Durkin *et al.*, 2006). It was also shown that Chk1 has a regulatory role in the spindle checkpoint in chicken DT40 cells (Zachos *et al.*, 2007) and in U2OS cells (Carrassa *et al.*, 2009). Here, our data provide *in vivo* evidence that Chk1 is essential for the spindle checkpoint in mouse mammary epithelial cells and mammary cancer cells. Thus, Chk1 deficiency yields profound abnormalities, as evidenced by mitotic catastrophe, which is much more severe than that caused by Brca1 mutation (Shen *et al.*, 1998; Xu *et al.*, 1999; Wang *et al.*, 2004). This may account for the reason why absence of p53 could not rescue the lethality caused by Chk1 deficiency, although the absence of p53 is sufficient to suppress the lethality and growth defects associated with Brca1 mutation (Xu *et al.*, 2001; Li *et al.*, 2007). However, the abnormalities in multiple cell cycle checkpoints and cytokinesis in Chk1 mutant cells could eventually result in accumulation of genetic alterations that may cooperate with p53 deficiency to induce tumor formation.

As evidence for this view, we found numerous chromosome structural aberrations in mammary cancer cells. We also showed that a majority of tumor cells contained 80 or more chromosomes, suggesting that tetraploidization may serve as a major mechanism that contributes to tumorigenesis, that is, increase gene dose to overcome profound growth defects associated with impaired Chk1 function. Chromosome tetraploidization frequently occurs before aneuploidy during tumorigenesis (Margolis, 2005). It also occurs in mammary cancers developed in transgenic mice with overexpression of oncogenes, such as Aurora-A (Wang *et al.*, 2006). We suspect that the failure of cytokinesis in the Chk1 mutant cells could be, in part, responsible for this phenotype.

Our data revealed that Chk1 mutant tumor cells lost the spindle checkpoint that is accompanied by reduced expression levels of several members in two evolutionarily conserved protein families: Bub1, BubR1 and, most pronounced, Mad2. It is known that

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Mad2 binds to unattached kinetochores and inhibits anaphase-promoting complex together with BubR1 (Sudakin *et al.*, 2001; Tang *et al.*, 2001). Mad2 deficiency results in premature anaphase onset, chromosome missegregation and apoptosis, leading to early lethality at embryonic day 5, whereas Mad2 haploinsufficiency causes lung tumors after a long latency (Dobles *et al.*, 2000; Michel *et al.*, 2001). We have also shown previously that Mad2 has an essential role in mediating functions of Brca1 in the spindle checkpoint (Wang *et al.*, 2004). On the basis of these findings, we postulated that the reduced levels of Mad2 might be responsible, at least in part, for the spindle checkpoint defect observed here. Consistently, our data revealed that inhibition of Chk1 reduces Mad2 expression and triggers cell death, although it remains unclear how Chk1 affects Mad2 expression. This is an interesting issue that will be investigated further in future studies.

Although our study reveals that absence of p53 enhances tumorigenesis in Chk1 mutant cells, it also indicates that loss of Chk1 inhibits p53 deficiency-associated tumor formation. Several lines of evidence indicate that Chk1 is critical for viability of cancer cells, which may provide a clue to understand this complex pattern of Chk1 and p53 interaction. First, in all mammary tumor formation in $Chk1^{+/Co}$;$p53^{+/Co}$;*MMTV-Cre* mice, there was no loss of heterozygosity for Chk1, although p53-heterozygous mice did lose their remaining copy of p53 wild-type allele in all tumors examined. Second, tumor incidence of $p53^{Co/Co}$;*MMTV-Cre* mice is much higher than that of $Chk1^{+/Co}$;$p53^{Co/Co}$;*MMTV-Cre* and $Chk1^{Co/Co}$;$p53^{Co/Co}$;*MMTV-Cre* mice. Finally, we found that acute knockdown of Chk1 in both Neu and Ras cells, which were derived from mammary cancers of *MMTV-neu* and *MMTV-ras* transgenic mice (Muller *et al.*, 1988; Brodie *et al.*, 2001a), resulted in profound growth defects and mitotic catastrophe. These observations prompted us to hypothesize that complete loss of Chk1 is harmful to cancer cell growth because of profound genetic instability and growth defects. This would also explain why treatment with a pharmaceutical antagonist to Chk1 effectively kills cancer cells, perhaps due to the fact that the treated cells do not have sufficient time to undergo profound genome alterations to overcome the growth defect triggered by Chk1 inhibition.

In summary, using the Cre/loxP system to mutate both Chk1 and p53 in mammary tissue, we showed that Chk1 acts as a haploinsufficient tumor suppressor and cooperates with p53 to inhibit mammary tumor formation. We also provide *in vivo* evidence that Chk1 has an essential role in the spindle checkpoint to maintain genome integrity. As Chk1 is critical for viability of cancer cells, we have tested the effect of SB-218078 on cell growth *in vitro*, and our data indicate that treatment of SB-218078 can efficiently inhibit growth of multiple tumor cells. Chk1 inhibition is being pursued as a promising target for the treatment of cancer (Blagden and de Bono, 2005). The oldest of such drugs, UCN-01, has been in development for the longest period of time and is now in Phase II clinical trials (Hotte *et al.*, 2006). However, phase I trials revealed certain disadvantages of this drug, including dose-limiting toxicities (Kortmansky *et al.*, 2005) and extremely long half-life due to binding to a1-acid glycoprotein (Fuse *et al.*, 1998). Thus, our data suggest that SB-218078, which is a more specific inhibitor for Chk1 than UCN01 (Jackson *et al.*, 2000), may also be tested in the therapeutic treatment of breast cancers.

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Materials and methods

Mice and mating

The *Chk1*$^{Co/+}$ (Liu *et al.*, 2000), *p53*$^{Co/+}$ (Jonkers *et al.*, 2001) and MMTV-Cre (Wagner *et al.*, 1997) mice were crossed to generate mice of different genotypes, including *Chk1*$^{+/Co}$*;MMTV-Cre*, *Chk1*$^{Co/Co}$*;MMTV-Cre*, *Chk1*$^{+/Co}$*;p53*$^{+/Co}$*;MMTV-Cre*, *Chk1*$^{+/Co}$*;p53*$^{Co/Co}$*;MMTV-Cre*, *Chk1*$^{Co/Co}$*;p53*$^{+/Co}$*;MMTV-Cre*, *p53*$^{+/Co}$*;MMTV-Cre*, *p53*$^{Co/Co}$*;MMTV-Cre* and *Chk1*$^{Co/Co}$*;p53*$^{Co/Co}$*;MMTV-Cre*. Genotyping of these mice was performed as described (Wagner *et al.*, 1997; Liu *et al.*, 2000; Jonkers *et al.*, 2001). The female mice were kept with males for continuous mating and the number of pregnancies was recorded. When killed, one of the fourth glands was used for whole-mount preparation and the others used for DNA, RNA and/or histological analysis (Deng and Xu, 2004). The protocol for animal studies was approved by the 'Animal Care and Use Committee' of the National Institute of Diabetes and Digestive and Kidney Diseases.

RNA isolation and reverse transcription–PCR

Total RNA was isolated from cells or tissues with STAT-60 following the manufacturer's protocol (TEL-TEST, Friendswood, TX, USA). Complementary DNA was synthesized with Cells-to-cDNAII (Ambion, Austin, TX, USA). Primer sequences are as follows:

Gapdh: forward 5′-ACAGCCGCATCTTCTTGTGC-3′, reverse 5′-CACTTTGCCACTGCAAATGG-3′; Chk1: forward 5′-TTTGGGAGAAGGTGCCTATG-3′, reverse 5′-TTC TGGACAGTCTATGGCCC-3′.

Cell culture, chromosome spread and spectral karyotyping analysis

Brca1 mutant cell lines 69 and 780, and MMTV-ras and MMTV-neu were as described (Brodie *et al.*, 2001b). Establishment of cell lines 399, 459 and 644, from primary mammary tumors in *Chk1*$^{+/Co}$*;p53*$^{+/Co}$*;MMTV-Cre* mice, and chromosomal spread were as described (Deng and Xu, 2004). Spectral karyotyping was performed as described (Padilla-Nash *et al.*, 2006). Fluorescence *in situ* hybridization was performed on metaphase spreads by hybridization with whole chromosome paints against target chromosomes.

Whole-mount staining of mammary glands, histology, immunohistochemical staining and western blotting

Whole-mount staining of mammary glands was carried out as described (Deng and Xu, 2004). For histology, tissues were fixed in 10% formalin, blocked in paraffin, sectioned, stained with hematoxylin and eosin, and examined by light microscopy. Detection of primary antibodies was performed using the Zymed Histomouse SP Kit (Zymed, South San Francisco, CA, USA) according to the manufacturer's instructions. Western analysis was performed using standard procedures. Antibodies for Chk1 and p53 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody for 5-bromodeoxyuridine was purchased from Covance (Princeton, NJ, USA).

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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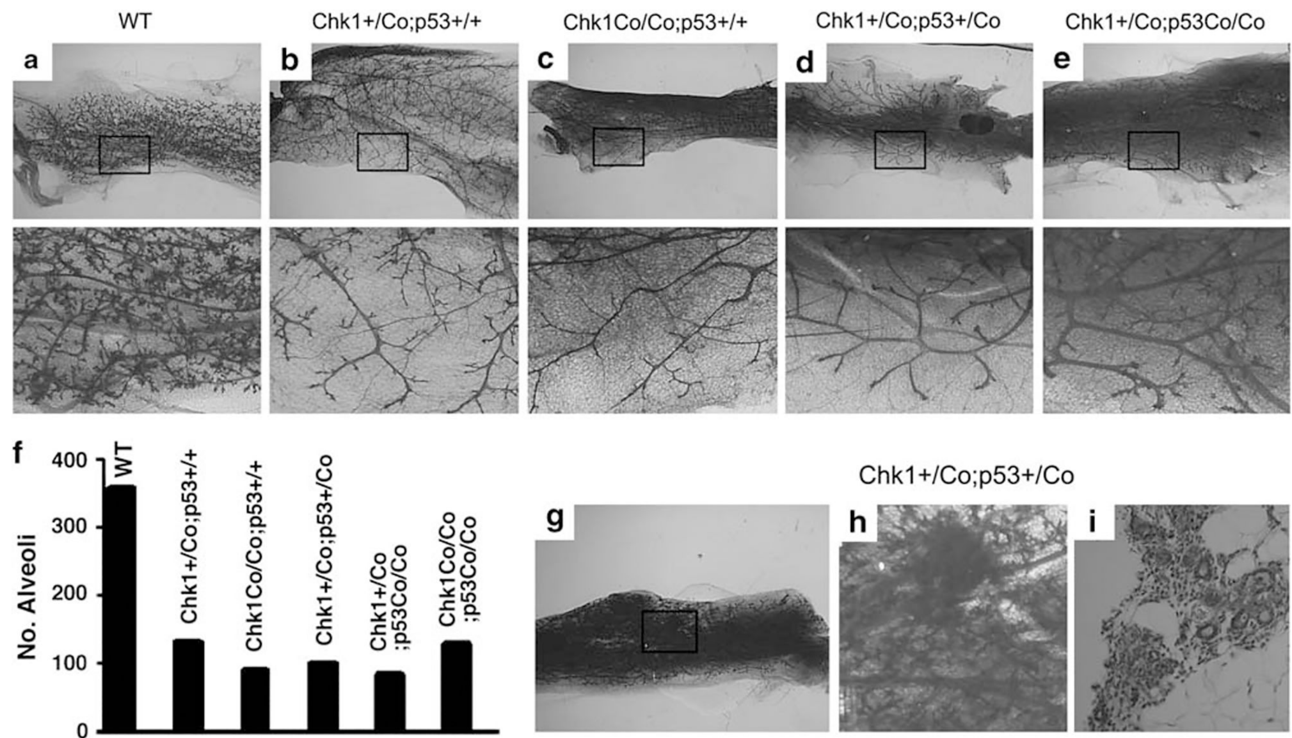

Figure 1.

Haploid or complete loss of Chk1 impaired mammary gland development independent of p53. (**a–e**) Whole-mount imaging of mammary glands from 12-month-old parous WT (**a**), *Chk1*$^{+/Co}$;*p53*$^{+/+}$;*MMTV-Cre* (**b**), *Chk1*$^{Co/Co}$;*p53*$^{+/+}$;*MMTV-Cre* (**c**), *Chk1*$^{+/Co}$;*p53*$^{+/Co}$;*MMTV-Cre* (**d**) and *Chk1*$^{+/Co}$;*p53*$^{Co/Co}$;*MMTV-Cre* (**e**) mice. Boxed areas are enlarged and placed underneath. (**f**) Quantitative measurement of mammary branches and alveoli of mutant and control mice within arbitrary areas of equal size, which were counted under high power magnification. (**g–i**) Whole-mount imaging of a mammary gland from a 12-month-old parous *Chk1*$^{+/Co}$;*p53*$^{+/Co}$;*MMTV-Cre* mouse showing increased branching morphogenesis and hyperplastic foci (**g**). The boxed area is enlarged (**h**) and sectioned (**i**).

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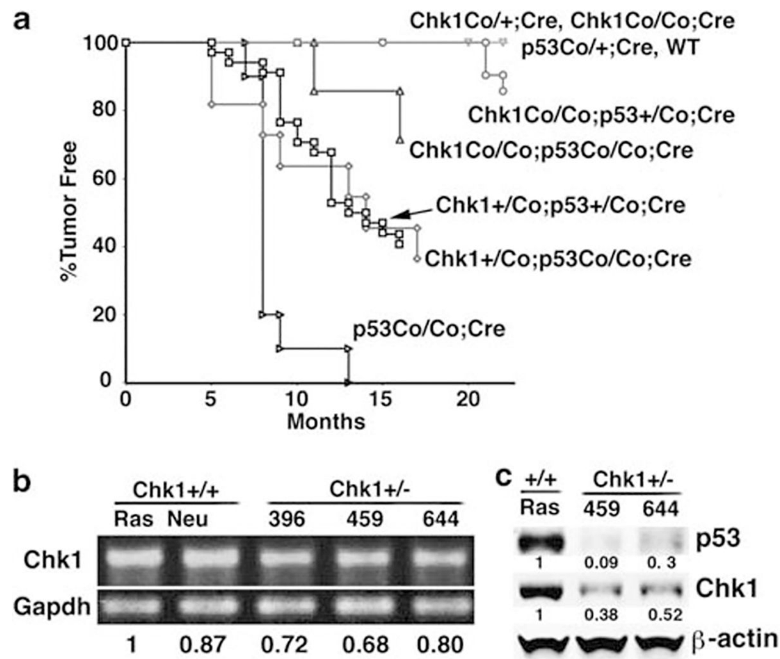

Figure 2.

Mammary tumor formation in mice carrying mammary-specific deletion of Chk1 and p53. (**a**) Kaplan–Meier survival curve of mice with different genotypes as indicated. (**b**) Transcriptional levels of Chk1 in three cell lines derived from three mammary tumors of *Chk1*$^{+/Co}$;*p53*$^{+/Co}$;*MMTV-Cre* mice (396 459, 644), and two cell lines derived from Chk1 wild-type MMTV-ras (Ras) and MMTV-neu (Neu) mice, as revealed by reverse transcription–PCR. (**c**) Protein levels of Chk1 and p53 in *Chk1*$^{+/-}$ (644 and 459) and Ras cell lines as revealed by western blot analysis. The intensity of bands was measured by Quantity One Software (Bio-Rad, Hercules, CA, USA) and normalized using intensity of Gapdh or β-actin. The quantified numbers are shown at the bottom of the pictures.

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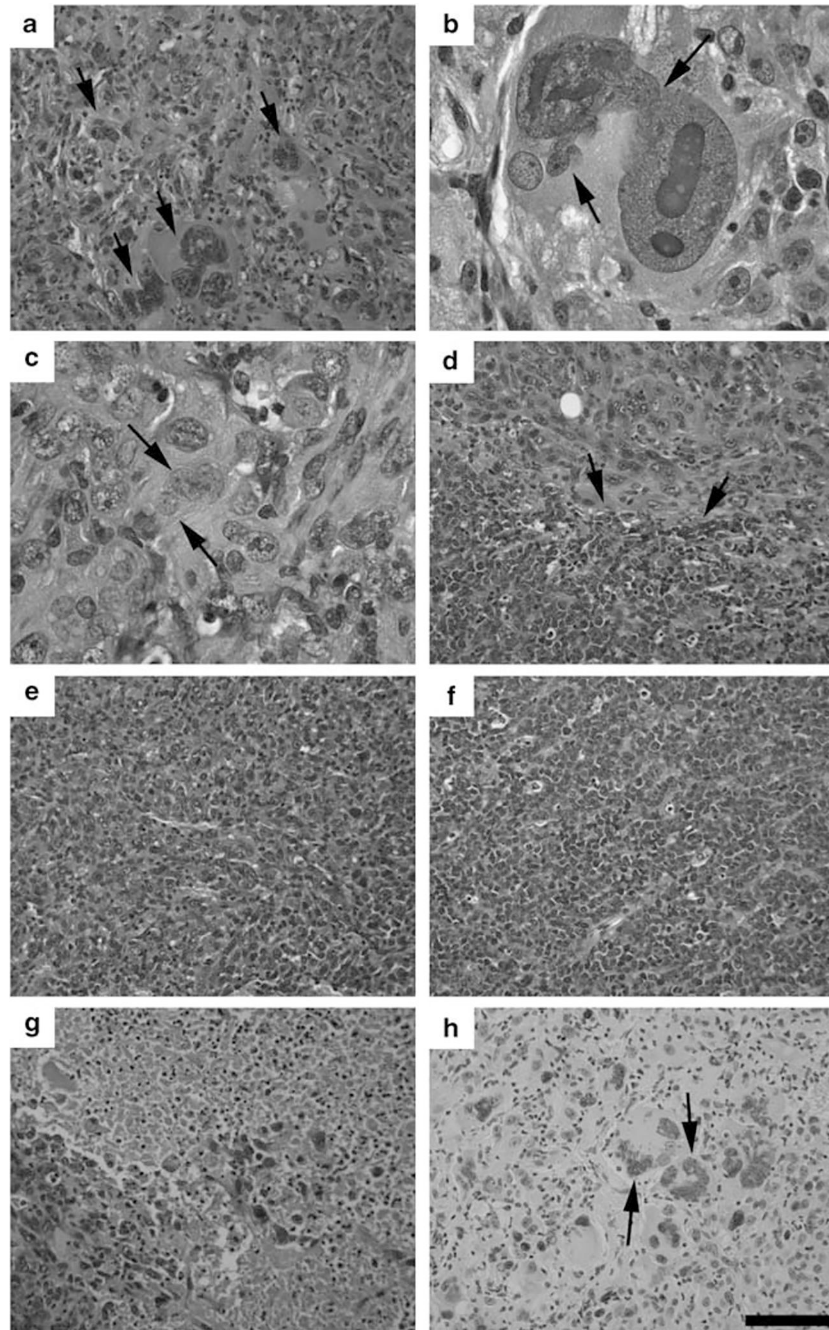

Figure 3.

Histologic diversity of mammary tumors found in *Chk1*$^{+/Co}$;*p53*$^{+/Co}$;*MMTV-Cre* mice as revealed in hematoxylin and eosinstained sections. (**a**) An adenocarcinoma-containing cell with a giant nucleus. (**b**) An enlarged image of the nucleus of a giant cell. (**c**) Nuclear polymorphism (arrows). (**d**) Distinct histological boundaries (arrows). (**e**, **f**) Most tumors exhibited extensive angiogenesis (**e**), although small patches of tumor cells of uniform size, and less angiogenesis were also observed (**f**). (**g**) Areas of necrotic cells. More necrotic areas

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can be found in tumors with larger sizes. (**h**) 5-Bromodeoxyuridine (BrdU) labeling showing that all giant cells are BrdU negative (arrows).

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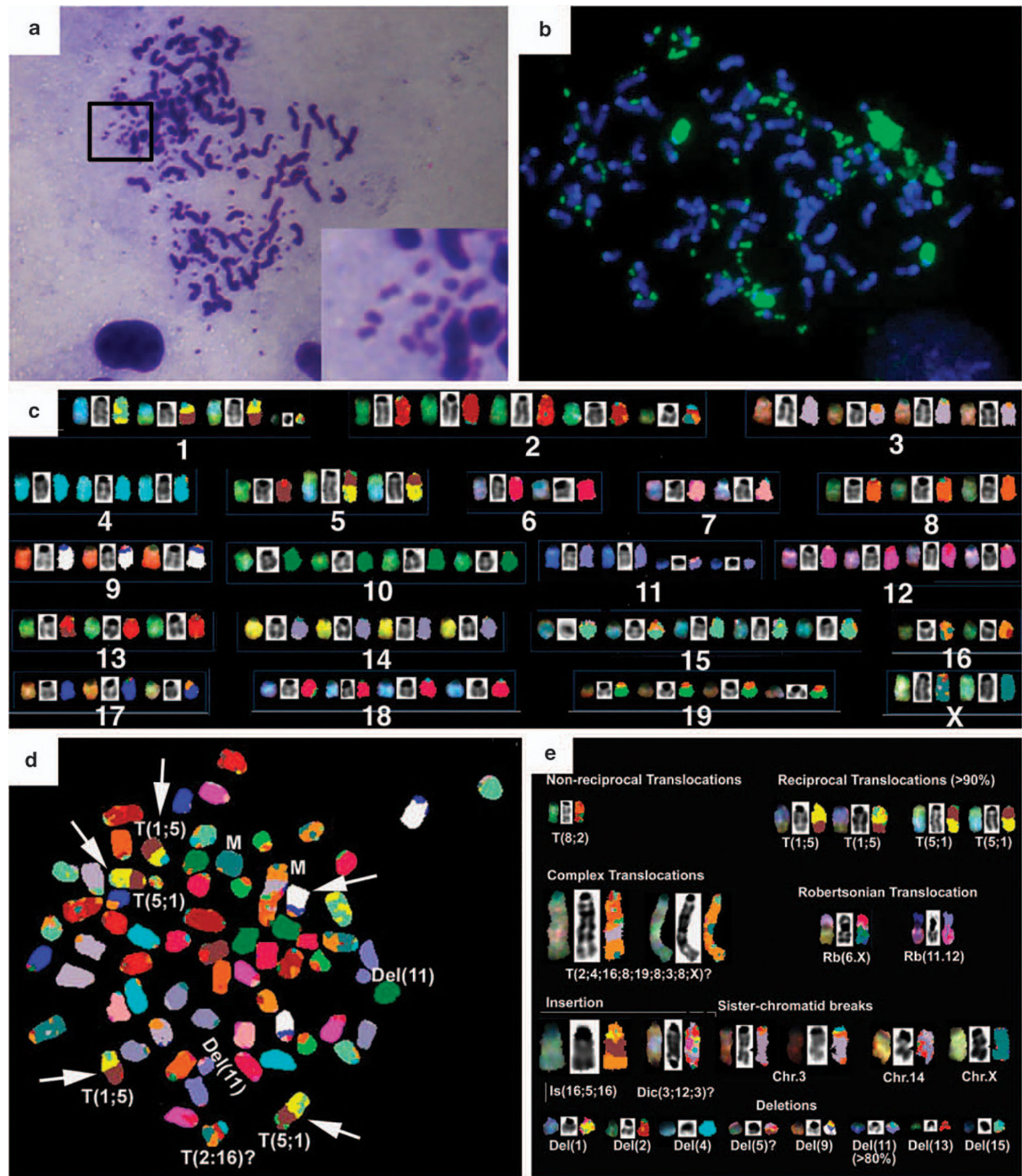

Figure 4.

Spectral karyotyping (SKY) analysis of chromosomes prepared from primary mammary tumors developed in *Chk1*$^{+/Co}$;*p53*$^{+/Co}$;*MMTV-Cre* mice and a cancer cell line, 644. (**a**) Metaphases of cells showing polypoidy and double minute chromosomes. (**b**) Fluorescence *in situ* hybridization on metaphase spreads from D644, showing double minutes that map to chromosome 9 (green) using a whole-chromosome probe for chromosome 9. (**c**) SKY classification of chromosomes. (**d**) A representative SKY image showing chromosomal aberrations as indicated. (**e**) A summary of all chromosomal aberrations from D644.

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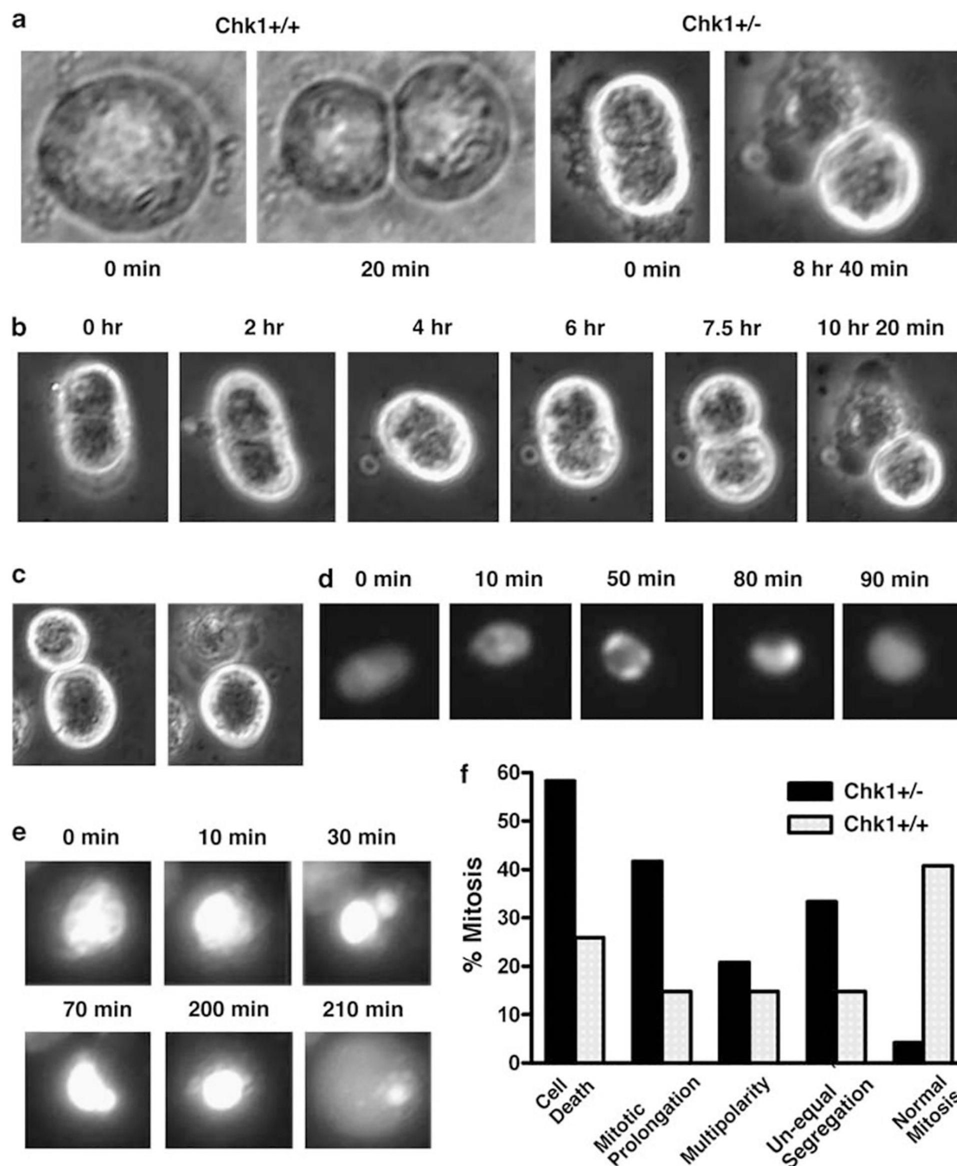

Figure 5.

Mitotic catastrophe and abortive chromosome segregation revealed by time-lapse experiment. (**a**) Images showing abnormal (from a *Chk1*$^{+/-}$ cell) and normal (from an MMTV-neu cell that is wild type for Chk1) mitoses. (**b**) Time-lapse images showing abnormal mitosis of one *Chk1*$^{+/-}$ cell. (**c**) Asymmetric division of *Chk1*$^{+/-}$ cells produces cells of different size and causes the subsequent death of a smaller cell. (**d**, **e**) Abnormal mitosis observed in cells carrying a stably expressed histone H2B-GFP expression vector, allowing their DNA to be visualized. (**f**) Summary of mitotic abnormalities in *Chk1*$^{+/-}$ mutant and Neu cells.

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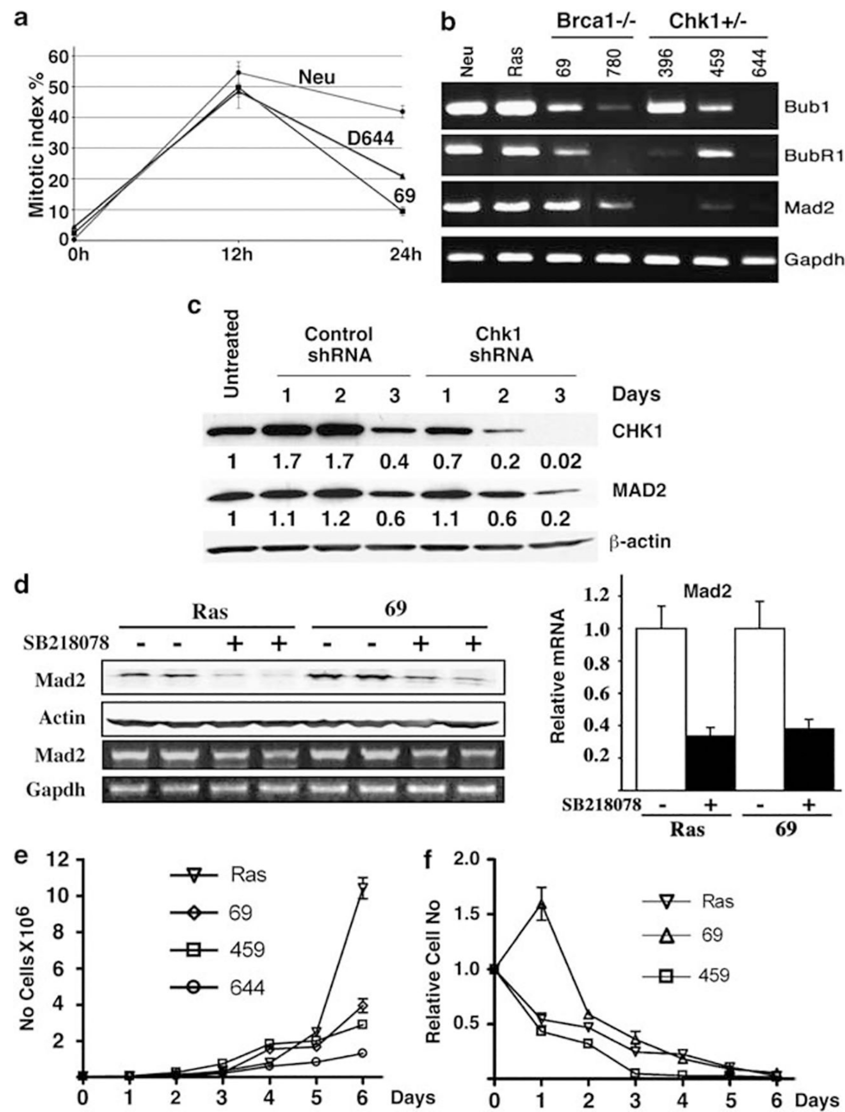

Figure 6.

Haploid loss of Chk1 impaired by the spindle checkpoint is partially mediated by Mad2. (**a**) Mitotic index of cells at 12 and 24 h after treatment with nocodazole. (**b**) Expression of several genes involved in the spindle checkpoint pathway as revealed by reverse transcription (RT)–PCR. (**c**) Small hairpin RNA-mediated knockdown of Chk1 in MMTV-neu cells decreases Mad2 as revealed by western blot analysis (left), which is quantified on the basis of untransfected cells (right). (**d**) Treatment of Chk1 wild-type cells with SB-218078 results in reduced levels of Mad2 as revealed by western blot analysis (left) and real-time RT–PCR (right). A quantitative measurement is provided (right). (**e**) Growth curve of cell lines without receiving SB-218078 (Cat. no. 559402, Calbiochem, La Jolla, CA, USA). (**f**) Relative response of cell lines to SB-218078 (1 μg/ml) treatment compared with untreated cells.