Genetic Interactions between Brca1 and Gadd45a in Centrosome Duplication, Genetic Stability, and Neural Tube Closure

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GADD45a is a transcription target of the breast tumor suppressor gene BRCA. It was recently shown that mouse embryonic fibroblast cells carrying a targeted deletion of exon 11 of Brca1 (Brca11/11) or a Gadd45a-null mutation (Gadd45a−/−) suffer centrosome amplification. To study genetic interactions between these genes during centrosome duplication, we generated Brca11/11Gadd45a−/− mice by crossing each mutant. We found that all Brca11/11Gadd45a−/− embryos at embryonic days 9.5–10.5 were exencephalic and exhibited a high incidence of apoptosis accompanied by altered levels of BAX, BCL-2, and p53. The trigger for these events is likely the genetic instability arising from centrosome amplification that is associated, at least in part, with decreased expression of the NIMA-related kinase NEK2. We demonstrate that small interfering RNA-mediated suppression of Brca1 decreased Nek2 more dramatically in Gadd45a−/− cells than in wild-type cells and, conversely, that overexpression of Brca1 and/or Gadd45a up-regulated transcription of Nek2. Furthermore, we show that overexpression of Nek2 in Brca1-specific small interfering RNA-treated wild-type and Gadd45a−/− cells repressed abnormal centrosome amplification. These observations suggest that NEK2 plays a role in mediating the actions of BRCA1 and GADD45A in regulating centrosome duplication and in maintaining genetic stability.

Cells normally contain one or two centrosomes depending on their phases in the cell cycle. Centrosome duplication starts at later G2 phase and proceeds through S phase (1–4). Prior to mitosis, the duplicated centrosomes separate and move to the future poles of the spindle to initiate the bipolar spindle, which is required for equal segregation of chromosomes. Dysregulation of this process can cause centrosome malformation, chromosome unequal segregation, and, consequently, malignant transformation (5, 6). It has been shown that alterations of many factors, including Brca1 (the mouse homolog of human BRCA1), Gadd45a (growth arrest and DNA damage-inducible gene) and Nek2, affect centrosome duplication (7–10). However, the underlying mechanisms remain illusive.

The functions of BRCA1 have been the subject of extensive research since its cloning in 1994 (11). Mounting evidence reveals that BRCA1 plays essential roles in many biological processes, including transcription activation and repression, cell cycle regulation, chromatin remodeling, DNA damage repair, and centrosome duplication (reviewed in Refs. 12–14). It has been shown that mouse embryos carrying Brca1-null mutation have early embryonic death (15–19). Embryos carrying a homozygous deletion of Brca1 exon 11 (Brca11/11), which encodes 60% of the amino acids of the protein, die at later stages of gestation because of widespread apoptosis (20). We have found that haploinsufficiency of p53 can suppress apoptosis in Brca11/11 embryos and allow them to develop to adulthood; however, the survivors (Brca11/11; p53−/−) exhibit apoptosis in testes and thymus and eventually die of premature aging and tumorigenesis (20–23). In mutant mice in which the Brca1 exon 11 was specifically disrupted in mammary epithelium using a Cre/loxP approach, mammary tumors develop at low frequency after long latency (24). Further analysis revealed the involvement of multiple factors in the Brca1-associated tumorigenesis, including overexpression of the erbB2, c-myc, p27, and cyclin D1 genes as well as down-regulation or loss of p53 and p16 in the majority of tumors (25). As Brca1 is a transcription activator that regulates expression of a number of important genes, including p21, 14-3-3, Chk-1, and Gadd45a (26–29), part of the Brca1 mutant phenotypes could be due to changes in its downstream transcription targets.

GADD45A is one of several growth arrest- and DNA damage-inducible genes (30). Its expression can be regulated by a variety of genotoxic and non-genotoxic stresses, including UV radiation, methyl methanesulfonate, and ionizing radiation. GADD45A interacts with proliferating the cell nuclear antigen, p21, Cdc2, core histone, and MTK genes, indicating that GADD45A may be involved in multiple important cellular events (31, 32). GADD45A may also serve as a tumor suppressor, as its expression in multiple tumor lines suppresses their growth (33). It was shown that expression of BRCA1 induces expression of GADD45A, leading to JNK/SAPK-dependent apoptosis (34). Conversely, both the tumor-derived BRCA1 mutants and truncated BRCA1 mutants, which lack transactivation activity, are unable to activate the GADD45A promoter, indicating that BRCA1-mediated activation of the GADD45A promoter requires normal transcriptional properties of BRCA1 (34). Mouse Gadd45a−/− embryos exhibit a low frequency of exencephaly, but otherwise are developmentally normal. Mu-
BRCA1 Regulates Centrosome Duplication

Genotypes of animals derived from crosses between Brca1 and Gadd45a mutant mice

| Crosses | Total | No. of each genotype |
|---------|-------|----------------------|
|         |       | Brα<sup>−/−</sup> | Brα<sup>−/−</sup> | Brα<sup>−/−</sup> | Brα<sup>−/−</sup> | Brα<sup>−/−</sup> | Brα<sup>−/−</sup> | Brα<sup>−/−</sup> |
| Cross 1 (Brα<sup>−/−</sup> x Brα<sup>−/−</sup>) |       |                     |                     |                     |                     |                     |                     |
| At weaning | 145  | 1                    | 10                   | 0                    | 26                   | 54                   | 14                   | 38                   | 1                   |
| E12.5-15.5 | 26   | 1                    | 1                    | 1                    | 4                    | 7                    | 6                    | 2                    | 3                   |
| Cross 2 (Brα<sup>−/−</sup> x Brα<sup>−/−</sup>) |       |                     |                     |                     |                     |                     |                     |                     |
| At weaning | 42   | 5                    | 10                   | 0                    | 4                    | 23                   |                     |                     |
| E12.5-15.5 | 91   | 6                    | 14                   | 4                    | 13                   | 43                   | 13                   |                     |
| Cross 3 (Brα<sup>−/−</sup> x Brα<sup>−/−</sup>) |       |                     |                     |                     |                     |                     |                     |                     |
| At weaning | 341  | 121                  | 220                  | 0                    |                     |                     |                     |                     |
| E13.5-15.5 | 212  | 49                   | 111 (2)              | 52 (41)              | 23                   |                     |                     |                     |
| E11.5-12.5 | 190  | 60                   | 111 (4)              | 29 (14)              | 13                   |                     |                     |                     |
| E9.5-10.5  | 199  | 48 (4)               | 107 (13)             | 44 (44)              |                     |                     |                     |                     |
| Cross 4 (Brα<sup>−/−</sup> x Brα<sup>−/−</sup>) |       |                     |                     |                     |                     |                     |                     |
| At weaning | 182  | 75                   | 103                  | 4                    |                     |                     |                     |                     |
| E13.5-14.5 | 230  | 57                   | 121                  | 52 (3)               | 6                    |                     |                     |                     |
| E11.5-12.5 | 85   | 17                   | 42                   | 26 (1)               | 8                    |                     |                     |                     |
| E9.5-10.5  | 176  | 39                   | 80                   | 57 (2)               | 1                    |                     |                     |                     |

The phenotypic similarities between Brca1<sup>Δ111/Δ11</sup> and Gadd45a<sup>−/−</sup> cells prompted us to study the possible genetic interaction between Brca1 and Gadd45a in maintaining genome integrity. Using the existing Brca1<sup>Δ111/Δ11</sup> (20) and Gadd45a<sup>−/−</sup> (7) mice, we generated mice that are homozygous for mutations of both genes (Brca1<sup>Δ111/Δ11</sup>Gadd45a<sup>−/−</sup>). We found that virtually all Brca1<sup>Δ111/Δ11</sup>Gadd45a<sup>−/−</sup> embryos exhibited exencephaly, showing increased apoptosis in their neuroepithelium due to p53 activation, as haploid or complete loss of p53 repressed apoptosis and rescued embryonic lethality. Our further analysis uncovered a synergistic role of Brca1 and Gadd45a in regulating centrosome duplication and in maintaining genome integrity.

EXPERIMENTAL PROCEDURES

Mating and Genotyping of Mice—Genotyping of Brca1<sup>Δ111/Δ11</sup> and Gadd45a<sup>−/−</sup> mutant mice was as described (7, 20). Brca1<sup>Δ111/Δ11</sup> and Gadd45a<sup>−/−</sup> mice were crossed to generate Brca1 and Gadd45a double heterozygous (Brca1<sup>Δ111/Δ11</sup>Gadd45a<sup>−/+</sup>) mice, which were further crossed to generate Brca1<sup>Δ111/Δ11</sup>Gadd45a<sup>−/−</sup> mice. The genetic background is a mixture of 129, B6, and Black Swiss.

Apoptosis and Proliferation Analysis—Sections from E9.5 to E13.5 were analyzed for apoptosis using the ApopTag kit (Intergen Co., Purchase, NY) as recommended by the manufacturer. To evaluate cell proliferation rates, bromodeoxyuridine (BrdUrd) incorporation was measured using a cell proliferation kit (Amersham Biosciences) following the manufacturer’s directions.

Cell Cultures and Treatments—Primary MEFs were obtained from E14.5 embryos using a standard procedure. UBR-60 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and glutamine in the presence or absence of 1 μg/ml tetracycline for controlling BRCA1 expression as described (26). Plasmids bearing hemagglutinin (HA)-tagged GADD45a (36, 37) or pEGFP-N2K2A (38) were transfected into cultured cells using LipofectAMINE<sup>TM</sup> 2000 (Invitrogen). For small interfering RNA (siRNA) transfection, we plated 2.5 × 10<sup>5</sup> cells onto a 60-mm dish 1 day before transfection. siRNA specific for Brca1 (made by Dharmacon Research) and control siRNA (made by George Ploy) at a concentration of 0.36 μM were transfected into MEF cells at passage 2 using OligofectAMINE as described (39). After transfection, cells were harvested at 24, 48, 72, and 96 h and processed for immunofluorescence, Western blotting, and/or reverse transcription (RT)-PCR. The siRNA sequence for murine Brca1 was 5′-GACAGACAGAUCAAAGGCA-3′. The control siRNA was derived from human BRCA1 and differs from the corresponding region of mouse Brca1 by 2 bases. The siRNA sequences were also tagged with a fluoresecent group at the 3′-end. The transfection efficiencies for siRNA were ~70% as directly reviewed under a fluorescence microscope 48 h after transfection.

RT-PCR Analysis—Total RNAs were extracted from E10 embryos or MEFs. RT reactions were carried out using a first strand cDNA synthesis kit (Roche Applied Science). The cDNA samples were stored at −20 °C. One μg of RNA from each sample was used as template for each reaction, and 1 μl of cDNA from each sample was used for PCR. The optimal number of cycles for amplification was chosen according to the cycle number that yielded the strongest band while staying within the linear range. The ranges of cycles varied from 25 to 28 according to the specific target of RNA and primer set. The samples were heated to 94 °C for 2 min and then run through 25–28 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, followed by 72 °C for 10 min and then 4 °C. The primers used in this study were as follows: Nek2-1, 5′-GAA GAT TCG GAG GAA GAG CG-3′; Nek2-2, 5′-GAG GCA TTA GTC CAC ACA GC-3′; Gadd45a-1, 5′-GCA TTT GCA ATA TGA CTT TGC G-3′; Gadd45a-2, 5′-GTG CCA GGA GAT TAA TCA CG-3′; Brca1-1, 5′-CTT CAA GAA GAT GCA GAG AG-3′; Brca1-2, 5′-CAA TAA ACT GCT GGT CTC AGC-3′; glyceraldehyde-3-phosphate dehydrogenase-1, 5′-ACA GCC GCA TCT TCT TGT GC-3′; and glyceraldehyde-3-phosphate dehydrogenase-2, 5′-TTT GAT CTT AGA GGG GTC TGC-3′.

Centrosome Staining and Analysis—Cells grown on chamber slides (Falcon) were fixed in 2.5% paraformaldehyde, 25 mm MgCl<sub>2</sub> and phosphate-buffered saline (PBS) for 10 min at room temperature. The slides were then washed with 0.3 M glycine and PBS, permeabilized in 0.2% Triton X-100 and PBS, and incubated overnight with anti-GADPH (Sigma) diluted 1:1000 in 5% goat serum and PBS. The antibody complexes were detected with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Roche Applied Science) and stained with 4′,6-diamidino-2-phenylindole. For dual detection of centrosomes and microtubules, cells were fixed in ice-cold methanol for 10 min, and the permeabilization step was eliminated. Immuno-
staining was performed in four layers: anti-α-tubulin antibody (Sigma) followed by Texas Red-conjugated goat anti-mouse IgG (Vector Laboratories) and then anti–γ-tubulin antibody diluted 1:500 in 5% goat serum and PBS followed by fluorescein isothiocyanate-conjugated sheep anti-rabbit IgG (Roche Applied Science). Gray level images were acquired using a CCD camera (CH250, Photometries Ltd., Tucson, AZ) mounted on a Leica DMHRB epifluorescence microscope and pseudocolored using registration software. The centrosome/nucleus ratio is determined by counting centrosome numbers/cell, which contains only one nucleus.

Western Blot Analysis—Western blot analysis was accomplished according to standard procedures by ECL detection (Amersham Biosciences). The following primary antibodies were used: anti-p53 (Ab-7, Oncogene); anti-JNK1/JNK2 (BD Biosciences); anti-JNK3 (Upstate Biotechnology, Inc.); and anti-Bax, anti-Bcl-2, anti-α-Tub, and anti-NEK2 (Santa Cruz Biotechnology). Peroxidase-labeled goat anti-rabbit IgG (H + L) and goat anti-mouse IgG (H + L) antibodies (Kirkegaard & Perry Laboratories) were used as secondary antibodies.

Chromosome Preparation from E9.5 Embryos—E9 embryos were dissected and incubated at 37 °C for 2 h in medium containing 0.1 μg/ml Colcemid. The embryos were placed into 0.56% potassium chloride for 5 min and then fixed in a freshly prepared 3:1 mixture of methanol and glacial acetic acid at 4 °C overnight, followed by disaggregation in a 5-fold excess of aqueous 60% glacial acetic acid at room temperature for 5 min. The suspension was dropped across the surface of a slide, and the cells were spread and allowed to dry on the surface. The slides were stained with Giemsa.

RESULTS

Absence of Gadd45a Accelerates Embryonic Lethality of Brca1<sup>1/11/11</sup> Embryos—A previous investigation showed that the majority of Brca1<sup>1/11/11</sup> embryos die before birth, whereas Gadd45a<sup>+/−</sup> mice survive to adulthood (7). The animals double heterozygous for Brca1 and Gadd45a mutations were indistinguishable from wild-type controls (data not shown). However, no Brca1<sup>1/11/11</sup>Gadd45a<sup>−/−</sup> mice were found among 1246 pups generated from interbreeding with mice with several combinations of genotypes (Table I, Crosses 1–3), suggesting that the Brca1<sup>1/11/11</sup>Gadd45a<sup>−/−</sup> mutation is recessive lethal.

Next, we dissected pregnant females to study possible phenotypes of Brca1<sup>1/11/11</sup>Gadd45a<sup>−/−</sup> embryos during gestation. We found that Brca1<sup>1/11/11</sup>Gadd45a<sup>−/−</sup> embryos were presented at the expected ratios at E9.5–10.5 (Table I, Cross 3). However, all of the mutant embryos, especially those of older stages (from E11.5 to E15.5) were significantly smaller than the controls, and many of them were dying or dead (Fig. 1) (data not shown). We also analyzed Brca1<sup>1/11/11</sup> embryos that were in the same genetic background. We found that, although Brca1<sup>1/11/11</sup> embryos were distinguishably smaller than the wild-type controls at these stages of development, their development was relative normally and that they did not die until after E14.5 (Table I, Cross 4) (data not shown). Moreover, −1–2% of the Brca1<sup>1/11/11</sup> mice survived to adulthood (Table I, Cross 1, 1/145; and Cross 4, 4/182), whereas no Brca1<sup>1/11/11</sup>Gadd45a<sup>−/−</sup> mice were found in 1246 offspring generated from our crosses (Table I, Crosses 1–3). These observations indicate that the absence of Gadd45a accelerates embryonic lethality caused by the Brca1<sup>1/11/11</sup> mutation.

Brca1<sup>1/11/11</sup>Gadd45a<sup>−/−</sup> Embryos Display Exencephaly—One prominent feature of Brca1<sup>1/11/11</sup>Gadd45a<sup>−/−</sup> embryos was that the majority of them exhibited exencephaly because of failed closure of the anterior neural tube (Table I). In a normal...
embryo, the neural tube begins to close at E8.5 from multiple sites in the middle portion of the embryo and extends both anteriorly and posteriorly in a zipper-like fashion. By E9.5, most parts of the neural tube have already closed, and only small openings, called the neural pores, are left in both the anterior and posterior ends of the embryo. Our examination of E9.5–10.5 embryos indicated that all Brca1<sup>11/11</sup>Gadd45a <sup>−/−</sup> embryos failed to close their anterior neural tubes with varying severity (Fig. 1, A and B; and Table I, Cross 3). Whole-mount in situ hybridization using Otx2, which marks the neuroepithelium of the forebrain and midbrain, revealed that the unclosed region extended from Otx2-positive portions of the brain throughout the Otx2-negative hindbrain (Fig. 1E, arrow). We also analyzed Brca1<sup>11/11</sup>Gadd45a <sup>−/−</sup> embryos at E11.5–15.5 and found that ~80% of them exhibited exencephaly because of failure to close the anterior neural tube (Fig. 1, F and G; and Table I, Cross 3). Exencephaly also occurred in Brca1<sup>11/11</sup> and Gadd45a <sup>−/−</sup> embryos at much lower frequencies (Table I, Crosses 3 and 4). These observations indicate that the combined effect of the Brca1 exon 11 deletion and the Gadd45a-null mutation blocks anterior neural tube closure, leading to exencephaly.

Next, we performed histological analysis on the double mutant embryos. Fig. 2A shows that the mutant neural plate was elevated in E9.5 embryos, but its lateral edges did not bend toward each other. Although the thickness of the neuroepithelium was comparable with that of wild-type embryos, the lumina of the brain vesicles were not formed (Fig. 2, A–D). Examination of older embryos (E11.5–13.5) revealed that the mutant brains failed to develop further (Fig. 2E). Consequently, they contained only one layer of the neuroepithelium without any structures that were observed in normal brains. We also performed histological analysis on other parts of embryos and found that the mutant embryos contained all major internal organs despite being significantly smaller than the wild-type embryos (data not shown). This observation indicates that the combined mutations of Brca1 and Gadd45a do not affect organogenesis.

**Brca1<sup>11/11</sup>Gadd45a<sup>−/−</sup> Embryos Exhibit Increased Apoptosis**—Previous investigations revealed that altered cell death and proliferation are responsible for failure to close neural tubes in some mutant embryos (40, 41). To determine whether this is the case, we first analyzed E9.5 embryos for apoptosis. Our analysis revealed a significantly higher rate of apoptosis in the neuroepithelium of Brca1<sup>11/11</sup>Gadd45a <sup>−/−</sup> embryos compared with control embryos (Fig. 3, A–C and enlargements). In the midbrain and hindbrain, ~40% of the Brca1<sup>11/11</sup>Gadd45a <sup>−/−</sup> cells, 20% of the Brca1<sup>11/11</sup> cells, and ~5% of the wild-type cells were TUNEL-positive. In the forebrain, the apoptotic rates for all embryos were similar, except for Brca1<sup>11/11</sup> embryos, which contained more apoptotic cells in
the tip of the forebrain (Fig. 3B, arrow). Our examination of older embryos at E10.5–13.5 revealed that Brca1^+/Δ11, Gadd45a^−/− embryos continued to maintain higher apoptotic rates compared with Brca1^+/Δ11 embryos and other controls. These observations indicate that mutations of both Brca1 and Gadd45a synergistically increase cell death.

Next, we analyzed cell proliferation using BrdUrd incorporation in E9.5–13.5 embryos. Our analysis revealed no significant differences in proliferation in E9.5 embryos of all different genotypes (Fig. 3, D and E), although proliferation of Brca1^+/Δ11/Gadd45a^−/− and Brca1^Δ11/Δ11 embryos gradually decreased after E10.5 compared with Gadd45a^−/− and wild-type embryos (data not shown). A careful examination of E9.5 Brca1^+/Δ11/Gadd45a^−/− embryos revealed that many nuclei, including BrdUrd-positive ones, broke into pieces (Fig. 3F, arrowheads). This observation suggests that the primary effect of the doubly deficiency of Brca1 and Gadd45a at E9.5 is apoptosis, which occurs at different phases in the cell cycle, rather than limitation of cell proliferation. As the high rate of apoptosis at E9.5 correlated with exencephaly exhibited by all Brca1^+/Δ11/Gadd45a^−/− embryos, and the decreased proliferation occurred 1 or 2 days later, we believe that the alteration in cell death is responsible for the failure to close neural tubes.

Brca1 and Gadd45a Deficiency Synergistically Induces Centrosome Amplification—We have shown previously that Brca1^+/Δ11 and Gadd45a^−/− MEF cells exhibit centrosome amplification (7, 8). To determine whether this abnormality is enhanced by the combined deficiency of Brca1 and Gadd45a, we studied centrosomes in MEF cells. We found that ~45% of the Brca1^+/Δ11/Gadd45a^−/− MEF cells contained more than two centrosomes/nucleus, in contrast to ~24 and 15% of the Brca1^Δ11/Δ11 and Gadd45a^−/− MEF cells, respectively (Fig. 4, A and C–E). This observation indicates that deletion of Brca1 exon 11 and the Gadd45a-null mutation synergistically induces centrosome amplification. Faithful segregation of centrosomes is essential in maintaining genome integrity (5, 8). Consistent with this, we found that ~40% of the Brca1^+/Δ11/Gadd45a^−/− cells at passage 2 were aneuploid, containing.
41–60 (15%) and 61–80 (25%) chromosomes, respectively. Some of them also exhibited chromosome structural abnormalities (Fig. 4B) (data not shown). To determine whether centrosome amplification also occurs in vivo, we directly examined centrosomes in Brca1<sup>11/11Gadd45a<sup>−/−</sup></sup> embryos. Using antibodies to pericentrin, a major component of centrosomes, we performed immunofluorescent staining on tissue sections of E9.5 embryos. Our analysis revealed that ~20% of the Brca1<sup>11/11</sup> and Brca1<sup>11/11Gadd45a<sup>−/−</sup></sup> cells contained more than two centrosomes/cell, whereas no wild-type cells contained more than two centrosomes (Fig. 4, F–H).

Of note, the percentages of cells containing abnormally increased numbers of centrosomes were significantly lower in Brca1<sup>11/11Gadd45a<sup>−/−</sup></sup> embryos than in cultured Brca1<sup>11/11Gadd45a<sup>−/−</sup></sup> MEFs (45% versus 20%). We also failed to detect a significant difference in the percentages of cells with centrosome amplification between Brca1<sup>11/11</sup> and Brca1<sup>11/11Gadd45a<sup>−/−</sup></sup> embryos, whereas such a difference was significant in cultured Brca1<sup>11/11</sup> and Brca1<sup>11/11Gadd45a<sup>−/−</sup></sup> MEFs (24% versus 45%). These discrepancies could be due to the high rates of apoptosis in Brca1<sup>11/11Gadd45a<sup>−/−</sup></sup> embryos that prevented the accumulation of mutant cells. However, under the in vitro culture conditions, the effect of the absence of both genes was primarily manifested by significantly decreased proliferation, instead of cell death.

**Brca1 and Gadd45a Are Required for Maintaining JNK MAPK Activation in Vivo**—It was shown that overexpression of BRCA1 up-regulates GADD45A and triggers apoptosis through activation of JNK/SAPK in cultured cells (26). To determine whether this is the case in vivo, we checked the expression of these genes in E10.5 embryos and found that Gadd45a is expressed at a lower level in the Brca1 mutant embryos (Fig. 5A), and no changes in Brca1 were seen in Gadd45a mutant embryos (Fig. 5B). Next, we studied the expression levels of JNK1–3 by Western blot analysis. Antibodies to total proteins revealed no alterations in their expression levels (Fig. 5C), whereas anti-phospho-JNK antibodies, which detect activated forms of JNK1–3, detected significantly decreased levels in Brca1<sup>11/11Gadd45a<sup>−/−</sup></sup> embryos compared with embryos with other genotypes (Fig. 5D). This observation provides genetic evidence that supports the finding that ectopic expression of BRCA1 up-regulates GADD45A and activates JNK/SAPK (26). On the other hand, it rules out the possibility that the increased apoptosis in Brca1<sup>11/11Gadd45a<sup>−/−</sup></sup> embryos is due to JNK/SAPK activation.

**Brca1 and Gadd45a Double Deficiency Activates a p53-mediated Apoptotic Pathway**—In correlation with the dramatically increased apoptosis, we found that *bax*, a proapoptotic gene, was up-regulated, whereas *bcl-2*, a cell death inhibitor, was down-regulated (Fig. 5E). As both genes are involved in the p53-mediated cell death pathway, their alteration may suggest that activation of p53 is responsible for the cell death in Brca1 and Gadd45a double mutant embryos. Consistently, our Western blots revealed that p53 was slightly increased in mutant embryos (Fig. 5E). To provide further evidence of the involvement of p53 in the embryonic death of Brca1<sup>11/11Gadd45a<sup>−/−</sup></sup> embryos, we tested whether a p53-null mutation (42) could rescue the embryonic lethality of these embryos. Our analysis of 142 offspring from crosses between Brca1<sup>11/11Gadd45a<sup>−/−</sup>p53<sup>−/−</sup></sup> and Brca1<sup>11/11Gadd45a<sup>−/−</sup>p53<sup>−/−</sup></sup> mice detected three live Brca1<sup>11/11Gadd45a<sup>−/−</sup>p53<sup>−/−</sup></sup> mice and two Brca1<sup>11/11Gadd45a<sup>−/−</sup>p53<sup>−/−</sup></sup> mice at weaning, whereas no Brca1<sup>11/11Gadd45a<sup>−/−</sup>p53<sup>−/−</sup></sup> mice were found. All five of these mutant mice were significantly smaller than their littermate controls and died before 2 months.
of age without apparent causes (data not shown).

We also analyzed Brca1^Δ1/Δ11/Gadd45a+/−p53−/− embryos at various stages of embryonic development. Of eight mutant embryos studied between E16.5 and E18.5, one showed exencephaly (data not shown), and all others appeared normal, although they were smaller than the controls (Fig. 5F). TUNEL assay revealed no obvious apoptosis in Brca1^Δ1/Δ11/Gadd45a+/−-p53−/− embryos (Fig. 5, G and I), in contrast to widespread TUNEL-positive cells in Brca1^Δ1/Δ11/Gadd45a+/−p53+/− embryos (Fig. 5, H and J). These data provide direct evidence that the apoptosis and embryonic lethality of Brca1^Δ1/Δ11/Gadd45a+/−p53+/− embryos are caused by the activation of p53.

Brca1 and Gadd45a Double Deficiency Decreases Nek2 Levels, Accompanied by Centrosome Amplification and Genetic Instability—We next used a candidate approach to check expression of genes that are involved in centrosome duplication. Among a number of candidate genes examined by Western blot analysis, we found that Nek2, a NIMA-related Thr/Ser kinase, was present at lower levels in Brca1^Δ1/Δ11/Gadd45a+/− embryos (Fig. 6A). Because Nek2 is located in centrosomes and is known to play an important role in regulating centrosome duplication (43), we decided to investigate this further. RT-PCR analysis revealed that the decreased expression of Nek2 was more significant in Brca1^Δ1/Δ11/Gadd45a+/− embryos and MEFs than in cells carrying the mutation for each gene (Brca1^Δ1/Δ11 or Gadd45a+/−) (Fig. 6B). These data suggest that the down-regulation of Nek2 could be due to the synergistic action of the impaired function of both genes. Alternatively, the more significantly decreased Nek2 expression is secondary to severe abnormalities in the double mutant embryos. To distinguish between these possibilities, we performed siRNA-mediated acute suppression of Brca1 in wild-type cells (mimicking the Brca1 deficiency only) and Gadd45a+/− cells (mimicking the Brca1 and Gadd45a double deficiency) and compared Nek2 levels in these cells. Fig. 6 (C and D) shows that transfection with a Brca1-specific siRNA, but not a control siRNA, resulted in similar suppression of Brca1 after 72 h in both type of cells; however, Nek2 expression decreased more significantly in Gadd45a+/− cells than in wild-type MEFs. We also followed the expression patterns of Brca1 and Nek2 in Gadd45a+/− cells upon Brca1 suppression at different time points. Fig. 6E shows that transfection of the Brca1-specific siRNA effectively decreased Brca1 transcription starting from 24 h post-transfection, and the decreased expression of Nek2 occurred quickly as the Brca1 levels went down. These data indicate a synergistic action of both Brca1 and Gadd45a in maintaining Nek2 expression.

Next, we investigated whether the acute suppression of Brca1 in Gadd45a+/− cells could cause centrosome amplification and genetic instability as observed in Brca1^Δ1/Δ11, Gadd45a+/− cells. In the Brca1 siRNA-transfected Gadd45a+/− cells, we found that ~30% of the cells contained more than two centrosomes/cell 72 h after transfection compared with 12% of the cells in control siRNA-transfected cells (Fig. 6F). After normalizing the transfection efficiency (70%), the rate of centrosome amplification increased to 43%, which was similar to that of double mutant MEFs (45%). The increased number of centrosomes correlated with significantly increased percentages of aneuploid cells (Fig. 6G). These observations provide strong evidence that the Brca1 and Gadd45a double deficiency can cause significant down-regulation of Nek2, which is accompanied by centrosome amplification and genetic instability.

Expression of BRCA1 and GADD45A Up-regulates Nek2 Expression—Next, we determined whether overexpression of BRCA1 and/or GADD45A would increase Nek2 expression. We studied this using UBR-60 cells, which carry an inducible BRCA1 construct, and BRCA1 expression can be induced upon removal of tetracycline (26). Overexpression of GADD45A was achieved by transfecting GADD45A cDNA into these cells. The induction of BRCA1 by the removal of tetracycline and the expression of transfected GADD45A-HA were confirmed by Western blot analysis using antibodies to BRCA1 and HA, respectively (Fig. 7A). Fig. 7B shows that expression of BRCA1 or transfection of GADD45A alone up-regulated transcription of Nek2. Of note, transfection of GADD45A cDNA into UBR-60 cells in the absence of tetracycline (BRCA1 induction) did not cause an additional increase in Nek2 transcription (Fig. 7B). As BRCA1 positively regulates GADD45A expression in these cells (26), it is possible that endogenous GADD45A under BRCA1-inducing conditions is no longer a rate-limiting factor for Nek2 expression; therefore, transfection of GADD45A does not cause an obvious increase in Nek2 expression.

To provide additional evidence that the decreased Nek2 expression is responsible for the centrosome amplification associated with BRCA1 or BRCA1/GADD45A double deficiency, we investigated whether reconstitution of Nek2 could repress centrosome amplification in Brca1-specific siRNA-treated wild-type and Gadd45a−/− cells. We used these cells because the Brca1^Δ1/Δ11 and Brca1^Δ1/Δ11/Gadd45a+/− cells exhibited profound growth defects and were difficult for transfection (data not shown). Fig. 7 (C and D) shows that treatment of wild-type and Gadd45a−/− MEF cells with Brca1-specific siRNA resulted in 18 and 35% of the cells with more than two centrosomes, respectively. We then transfected these cells with pEFGP-NEK2A, which is the full-length isoform of human Nek2 (38). Fig. 7 (C and D) shows that expression of Nek2 overcame centrosome amplification in these cells. These data provide compelling evidence that Nek2 mediates the actions of BRCA1 and GADD45A in the regulation of centrosome duplication.
In this study, we have shown a role for genetic interactions between Brca1 and Gadd45a in maintaining normal development and neurogenesis during early post-gastrulation of mouse embryos. We demonstrated that the absence of Gadd45a accelerated embryonic lethality of Brca1<sup>Δ<sub>L1</sub>Δ<sub>L1</sub></sup> embryos and that all of the Brca1<sup>Δ<sub>L1</sub>Δ<sub>L1</sub>Δ<sub>L1</sub><sup>Δ<sub>L1</sub></sup></sup> embryos exhibited exencephaly and high rates of apoptosis due to the activation of p53 caused by genetic instability. We further demonstrated that Brca1 and Gadd45a play a synergistic role in maintaining expression of Nek2, which plays an essential role in regulating centrosome duplication.

The neural tube is first closed at the border between the spinal cord and hindbrain and in the forebrain, followed by a progressive apposition of the hindbrain neural plate in both the rostral and caudal directions. Neural tube closure defects (NTDs) are the most common congenital anomalies, with a frequency of 1/1000 live births in humans (44–46). Anencephaly (equivalent to exencephaly in mice) is a major type of NTD characterized by neural tube closure failure in cranial regions during embryonic development (47). It has been suggested that the combined genetic and environmental factors are major causes of NTDs (47). However, because of the polygenic nature of these diseases, the genes that cause the major types of human NTDs have not yet been identified. In mice, targeted mutations of >60 genes have been found to cause NTDs at varying penetrance (reviewed in Ref. 48). Analyses of these mutant mice revealed that abnormalities in cell migration, proliferation, and apoptosis could be major factors that prevent neural tube closure. Because Brca1<sup>Δ<sub>L1</sub>Δ<sub>L1</sub>Δ<sub>L1</sub><sup>Δ<sub>L1</sub></sup></sup> embryos exhibited significantly high rates of apoptosis, but no obvious alteration in proliferation, it is likely that the NTD is caused by the high rates of apoptosis, which prevents the normal process of neural tube closure.

We have previously shown that the absence of the full-length Brca1 isoform causes apoptosis in mutant embryos (20). Here, we have shown that Gadd45a deficiency enhances cell death in Brca1 mutant (i.e. Brca1<sup>Δ<sub>L1</sub>Δ<sub>L1</sub>Δ<sub>L1</sub><sup>Δ<sub>L1</sub></sup></sup>) embryos to a much higher extent compared with embryos carrying mutations of either gene. This observation suggests a synergistic action of these genes in apoptosis. A role for GADD45a in apoptosis is still contradictory. Microinjection of the exogenous GADD45a expression vector into human fibroblasts does not cause apoptosis, although it induces G<sub>s</sub> arrest (33). However, it has been shown that GADD45α may mediate BRCA1-induced apoptosis via activation of JNK and/or p38 MAPK (26, 49). Because this observation was made in cultured cells, we examined our Brca1<sup>Δ<sub>L1</sub>Δ<sub>L1</sub>Δ<sub>L1</sub><sup>Δ<sub>L1</sub></sup></sup>, Gadd45a<sup>−/−</sup>, and Brca1<sup>Δ<sub>L1</sub>Δ<sub>L1</sub>Δ<sub>L1</sub><sup>Δ<sub>L1</sub></sup></sup>, Gadd45a<sup>−/−</sup> embryos. Our data reveal significantly decreased JNK phosphorylation in Brca1<sup>Δ<sub>L1</sub>Δ<sub>L1</sub>Δ<sub>L1</sub><sup>Δ<sub>L1</sub></sup></sup>, Gadd45a<sup>−/−</sup> embryos. This observation suggests that, although JNK activation can induce apoptosis, the decrease in its activity does not prevent cell death. Of note, our data indicate that the increased apoptosis might be caused by the activation of the p53 gene caused by genetic instability, as haploid or complete loss of p53 repressed apoptosis and allowed some Brca1<sup>Δ<sub>L1</sub>Δ<sub>L1</sub>Δ<sub>L1</sub><sup>Δ<sub>L1</sub></sup></sup>, Gadd45a<sup>−/−</sup> mice to survive to adulthood. The increased levels of bax and the decreased expression of bcl-2 in Brca1<sup>Δ<sub>L1</sub>Δ<sub>L1</sub>Δ<sub>L1</sub><sup>Δ<sub>L1</sub></sup></sup>, Gadd45a<sup>−/−</sup> embryos are consistent with this observation.

A possible cause for genetic instability in Brca1<sup>Δ<sub>L1</sub>Δ<sub>L1</sub>Δ<sub>L1</sub><sup>Δ<sub>L1</sub></sup></sup>, Gadd45a<sup>−/−</sup> embryos is centosome amplification. Both Brca1 and Gadd45a have been implicated in centosome duplication (reviewed in Ref. 50). BRCA1 is located in centosomes and interacts with γ-tubulin, a major component of the centrosome (10, 51). Targeted mutation of Brca1 or Gadd45a results in centosome amplification in cultured MEF cells (7, 8). However, little is known about how Brca1 and Gadd45a control centosome duplication. Because BRCA1 positively regulates GADD45α expression (26, 34), a plausible explanation is that BRCA1 regulates centosome duplication through controlling GADD45α expression. However, we found that, in the MEF cells with loss of function of both Brca1 and Gadd45a, ~45% of the cells contained three or more centosomes. If Brca1 plays a function only through regulating Gadd45a, then the combined mutations of both genes would not be expected to dramatically increase centosome numbers. Thus, this observation suggests that the relationship between BRCA1 and GADD45α in centosome duplication is not just epistatic.

Of note, our data reveal the involvement of Nek2 in the centosome duplication process mediated by BRCA1 and GADD45α. Nek2 is located in centosomes, and transient overexpression of active NEK2 triggers centrosome splitting (the separation of parental centrioles), whereas prolonged expression of either active or inactive Nek2 leads to dispersal (severely diminished, fragmented, or undetectable) of centrioles (9). This observation indicates that the proper concentration of Nek2 is essential for centrome duplication. In our study, we found that Brca1<sup>Δ<sub>L1</sub>Δ<sub>L1</sub>Δ<sub>L1</sub><sup>Δ<sub>L1</sub></sup></sup>, Gadd45a<sup>−/−</sup> embryos and cultured MEF cells exhibited significantly lower levels of Nek2, suggesting that the centrosome amplification could be mediated by decreased expression of this gene. To provide further evidence for this hypothesis, we transfected an expression unit of Nek2 into wild-type and Gadd45a<sup>−/−</sup> cells that were treated with Brca1-specific siRNA. Our data indicate that reconstitution of Nek2 in these cells repressed centrosome amplification. Furthermore, we showed that overexpression of BRCA1 and/or GADD45α up-regulated transcripts of Nek2, establishing that both BRCA1 and GADD45α work upstream of Nek2. These studies provide compelling evidence that both BRCA1 and GADD45α are involved in maintaining Nek2 expression, whereas down-regulation of Nek2 in mutant cells could be a major cause for centrosome amplification. In turn, this causes genetic instability, increased apoptosis, and abnormal consequences.

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