Targeted disruption of the Nijmegen breakage syndrome gene *NBS1* leads to early embryonic lethality in mice

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Nijmegen breakage syndrome (NBS) is a rare autosomal recessive human disease whose clinical features include growth retardation, immunodeficiency, and increased susceptibility to lymphoid malignancies. Cells from NBS patients exhibit γ-irradiation sensitivity, S-phase checkpoint defects, and genomic instability. Recently, it was demonstrated that this chromosomal breakage syndrome is caused by mutations in the *NBS1* gene that result in a total loss of full-length NBS1 expression [1, 2]. Here we report that in contrast to the viability of NBS patients, targeted inactivation of *NBS1* in mice leads to early embryonic lethality in utero and is associated with poorly developed embryonic and extraembryonic tissues. Mutant blastocysts showed greatly diminished expansion of the inner cell mass in culture, and this finding suggests that NBS1 mediates essential functions during proliferation in the absence of externally induced damage. Together, our results indicate that the complex phenotypes observed in NBS patients and cell lines may not result from a complete inactivation of *NBS1* but may instead result from hypomorphic truncation mutations compatible with cell viability.

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Results and discussion

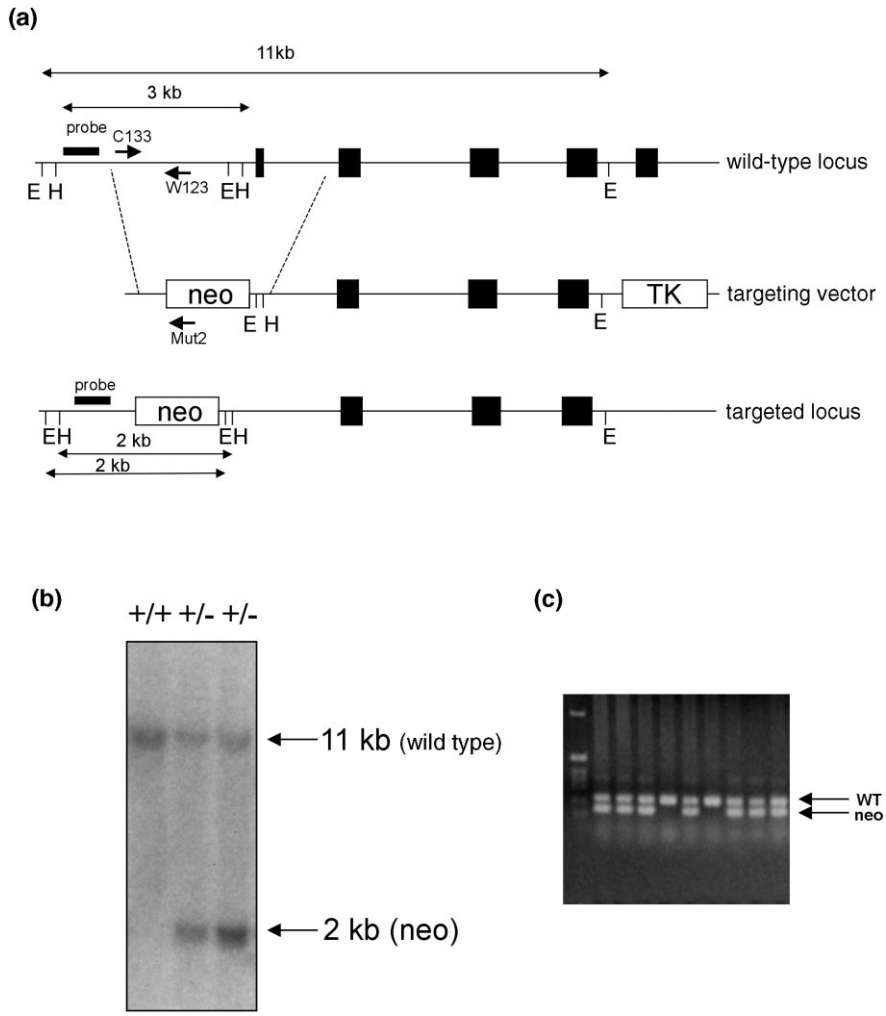
Since it is unknown whether defects in NBS patients are manifestations of hypomorphic or null mutations, it is possible that complete inactivation of NBS1 might reveal novel functions for this protein. We therefore decided to target the 5′ region of *NBS1* to produce a null allele. Ablation of *NBS1* was achieved by the deletion of 2 kb of DNA spanning the *NBS1* promoter, exon 1, and 700 bp of intron 1. Homologous recombination of the targeting vector into the *NBS1* gene results in the introduction of EcoRI and HincII sites adjacent to the neomycin resistance cassette (Figure 1a). These restriction sites were used for screening embryonic stem (ES) cells by southern blotting (Figure 1b), and the genotypes of the neomycin-resistant transformants were subsequently confirmed by PCR analysis (Figure 1c). From two out of four independently targeted $NBS1^{+/-}$ ES cell clones, chimeric mice were generated that transmitted the germ line *NBS1* mutation to offspring. $NBS1^{+/-}$ mice were viable and healthy, and they grew normally. Although the cancer risk in human NBS heterozygotes is high [3], 100 $NBS^{+/-}$ mice did not develop cancer spontaneously within 14 months, and tumors were not apparent in $NBS1^{+/-}$ mice 7 months after treatment with 4 Gy γ-irradiation (n = 32). However, it remains possible that $NBS1^{+/-}$ mice may develop cancer at a more advanced age.

$NBS1^{+/-}$ mice were fertile, but when they were interbred, homozygous mutants for the targeted *NBS1* allele were not found among the 312 offspring analyzed from one of the founder lines. These results indicate the embryonic lethality of the NBS1-deficient phenotype (Table 1). To determine the onset of lethality, we isolated embryos at various stages of gestation. At e7.5–e10.5 (where e = embryonic day), no $NBS1^{-/-}$ embryos were found among the 66 analyzed. However, several decidua containing resorbed embryos were found at e7.5–e10.5 (Table 1). This suggests that the death of $NBS1^{-/-}$ embryos occurred before e7.5.

To further investigate the nature of the lethality, we examined serial sections of embryos between e5.5 and e7.5 (Figure 2). At e7.5, 5 out of 16 embryos were found to be in an advanced stage of resorption. At e6.5, 3 out of 10 embryos were grossly smaller than their littermates, and their cells appeared morphologically disorganized and degenerated. These smaller embryos also lacked any detectable structures, such as embryonic or extraembryonic ectoderm or a preamniotic cavity (Figure 2). Even at e5.5, there was an overall reduction in size in 7 out of 21 embryonic sections examined (not shown). Both the timing of this apparent developmental block and the morphology of the $NBS1^{-/-}$ embryos are similar to the homozygous mutant phenotypes of *RAD50* [4], *RAD51* [5], *BRCA1* [6, 7], and *BRCA2* [8], which may function in similar DNA double-strand break (DSB) repair pathways [9].

To test whether $NBS1^{-/-}$ embryos survive until the blastocyst stage, we isolated preimplantation embryos at e3.5

Figure 1



Targeting of mouse *NBS1* **(a)** Genomic structure of the wild-type *NBS1* locus and targeting vector. A genomic clone was isolated from a mouse BAC library (Genome Systems) with a 700 bp *Bgl*II/*Pst*I probe derived from the 5′ end of mouse *NBS1* cDNA. In the targeting vector and targeted allele, the neomycin selection cassette replaces the first *NBS1* exon, including the translation initiation codon. Homologous recombination introduces EcoRI (E) and HincII (H) sites, which reduces the 11 kb wild-type EcoRI fragment and the 3.0 kb wild-type HincII fragment to 2 kb. Four successful targeting events were identified in 250 clones screened. **(b)** Identification of *NBS1* targeting in ES cells by southern blotting. EcoRI-digested genomic DNA from *NBS1*$^{+/+}$ and *NBS1*$^{+/-}$ ES cells were hybridized with a 300 bp *Pst*I probe upstream of exon 1; results were confirmed with HincII digestion of genomic DNA (not shown). **(c)** PCR screening of DNA from ES cells and mouse tails by the use of the following primers: C133 (5′-GCGTAAA TGGTTGATTGTCC-3′), W123 (5′-GATTG TCAGCACAGAAATCTTCCC-3′), and Mut2 (5′-GCGCTCCCCTACCCGGTAGATT-3′). PCR was performed with a mixture of all three primers. The C133/W123 combination generates a 256 bp wild-type band, and the C133/Mut2 combination generates a 150 bp mutant band. Location of PCR primers is indicated schematically in (a).

and observed them on a daily basis as they grew in culture for 4 days (Figure 3a). Out of 97 embryos genotyped on day 4, 12 were found to be *NBS1*$^{-/-}$ (Figure 3b; Table 1), and this finding indicates that at least some of the homozygous mutant embryos developed to the blastocyst stage. When *NBS1*$^{+/+}$ or *NBS1*$^{+/-}$ blastocysts were cultured in vitro, cells from the inner cell mass (ICM) proliferated rapidly and formed a colony on top of the trophoblast giant cells (Figure 3a, upper panels). In contrast, abnormal ICM development became evident in *NBS1*$^{-/-}$

Table 1

Genotypes of live births and embryos from NBS$^{+/-}$ intercrosses*

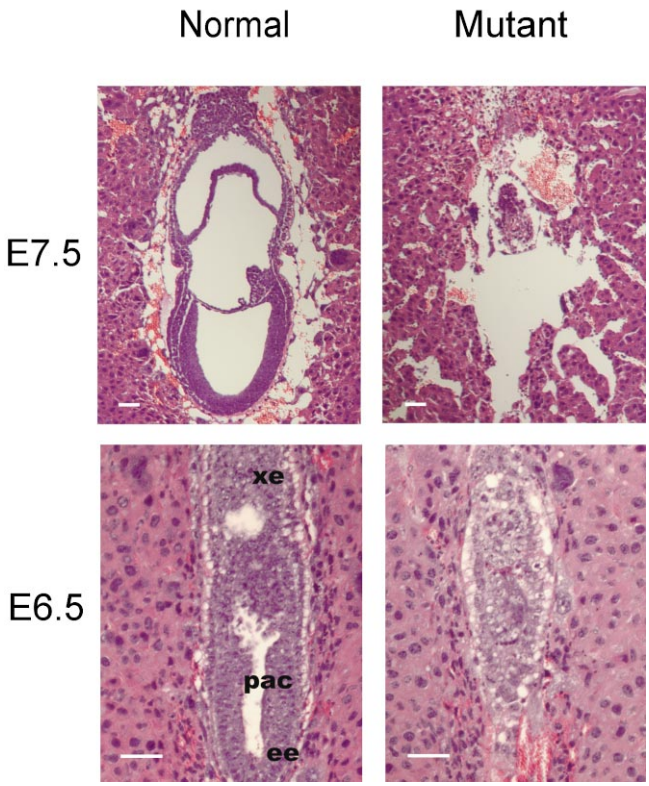
	NBS$^{+/+}$	NBS$^{+/-}$	NBS$^{-/-}$	Resorted or empty decidua	Total
Live births	116	196	0		312
Embryos, e8.5–10.5	8	22	0	7	37
Embryos, e7.5	4	20	0	5	29
In vitro growth of e3.5 blastocysts after 4 days					
Normal ICM	31	53	0		84
No ICM	0	1	12		18†

*The genetic background of the NBS1$^{+/-}$ mice is 129 × C57BL/6, and all the progeny and embryos are derived from one founder line. †PCR genotyping failed for five embryos that lacked ICM at day 4 (e7.5).

embryos after 2 days in culture, when the ICM appeared smaller than the controls. These ICM from *NBS1*$^{-/-}$ embryos continued to diminish in size until the majority of these cells died by day 4 in culture and left behind a monolayer of trophoblast giant cells (Figure 3a; lower panels). Nine out of twelve *NBS1*$^{-/-}$ embryos lacked any detectable ICM by day 4, and the remaining three *NBS1*$^{-/-}$ outgrowths were much smaller than those of the controls. Efforts to generate *NBS1*$^{-/-}$ ES cell lines by either culturing *NBS1*$^{+/-}$ ES cells in high concentrations of G418 or by retargeting *NBS1*$^{+/-}$ ES cells by using an independently derived construct conferring hygromycin resistance also failed to generate any *NBS1*$^{-/-}$ ES cells. Thus, it appears that NBS1 is essential for growth of early embryonic cells.

NBS1 plays a central role in detecting and signaling the presence of DSBs, which may arise either as a result of external damage or during normal metabolic processes [10]. NBS1, together with the Mre11/Rad50 complex, forms irradiation-induced foci that associate with DSBs until the damage is repaired [11]. Similarly, DSBs generated during physiological V(D)J (variable, diversity, joining) recombination cause the redistribution of NBS1 to sites of RAG-mediated V(D)J cleavage [12]. In NBS patient cells, which contain truncating or nonsense mutations in *NBS1*, the formation of ionizing radiation-induced Mre11/Rad50 foci and the inhibition of DNA synthesis in response to γ-irradiation are abrogated [2, 13]. It has been speculated that these mutations in NBS result in null alleles [1, 14]. However, the data presented here demonstrate that in contrast to the viability of NBS patients, complete loss of NBS1 in mice leads to embryonic lethality associated with defective cellular proliferation. Thus, we hypothesize that NBS patients may not carry null *NBS1* alleles. Rather, they may express a truncated NBS1 protein that abrogates DSB recognition/signaling functions but that still supports viability. Consistent with this prediction, a 70 kDa protein, which may be an N terminally truncated form of NBS1, has recently been identified in NBS patient cells (J. H. Petrini, personal communication).

Figure 2



Histological sections of embryos grown in utero to e7.5. E6.5 and e7.5 decidua from *NBS1*$^{+/-}$ intercrosses were sectioned and stained with hematoxylin and eosin. A midsagital section is shown for normal and presumed mutant embryos. Abbreviations are as follows: ee, embryonic ectoderm; xe, extraembryonic ectoderm; pac, preamniotic cavity. Scale bars represent 100 μm.

A very similar scenario is observed in cells carrying mutations in *MRE11*. Hypomorphic alleles of human *MRE11* have been discovered in an ataxia telangiectasia–like disorder, which, like NBS, is characterized by γ-radiation sensitivity, defective cell cycle checkpoints, and increased genomic instability [15]. However, like *NBS1* and *RAD50* null mutations, homozygous disruption of *MRE11* results in early embryonic lethality [16]. Recent studies in *S. cerevisiae* demonstrate that *MRE11* is required for sister chromatid recombinational repair [17]. Likewise, *MRE11* null chicken cells exhibit a reduced capacity for homologous recombination and undergo proliferative arrest associated with high levels of chromosome aberrations [18]. Additionally, *MRE11* complexes have been found in association with blocked DNA replication forks [19]. Thus, it is possible that the failure to repair DSBs that arise spontaneously during DNA replication in *MRE11*$^{-/-}$, *RAD50*$^{-/-}$, or *NBS1*$^{-/-}$ embryos might lead to massive cell death during embryogenesis. This embryonic lethality appears not to be rescued by the loss of p53, as we have been unable to obtain live *NBS1*$^{-/-}$*p53*$^{-/-}$ progeny (n = 100) by crossing the *NBS1* defect into the *p53*$^{-/-}$ background. Similarly, the embryonic lethality phenotype caused by deficiencies in *RAD51*, *BRCA1*, and *BRCA2* could not be rescued in a *p53* null background [5, 20, 21]. These data raise the possibility that NBS1 may function in similar recombinational repair pathways that are required for maintaining genomic stability in the absence of exogenous damage.

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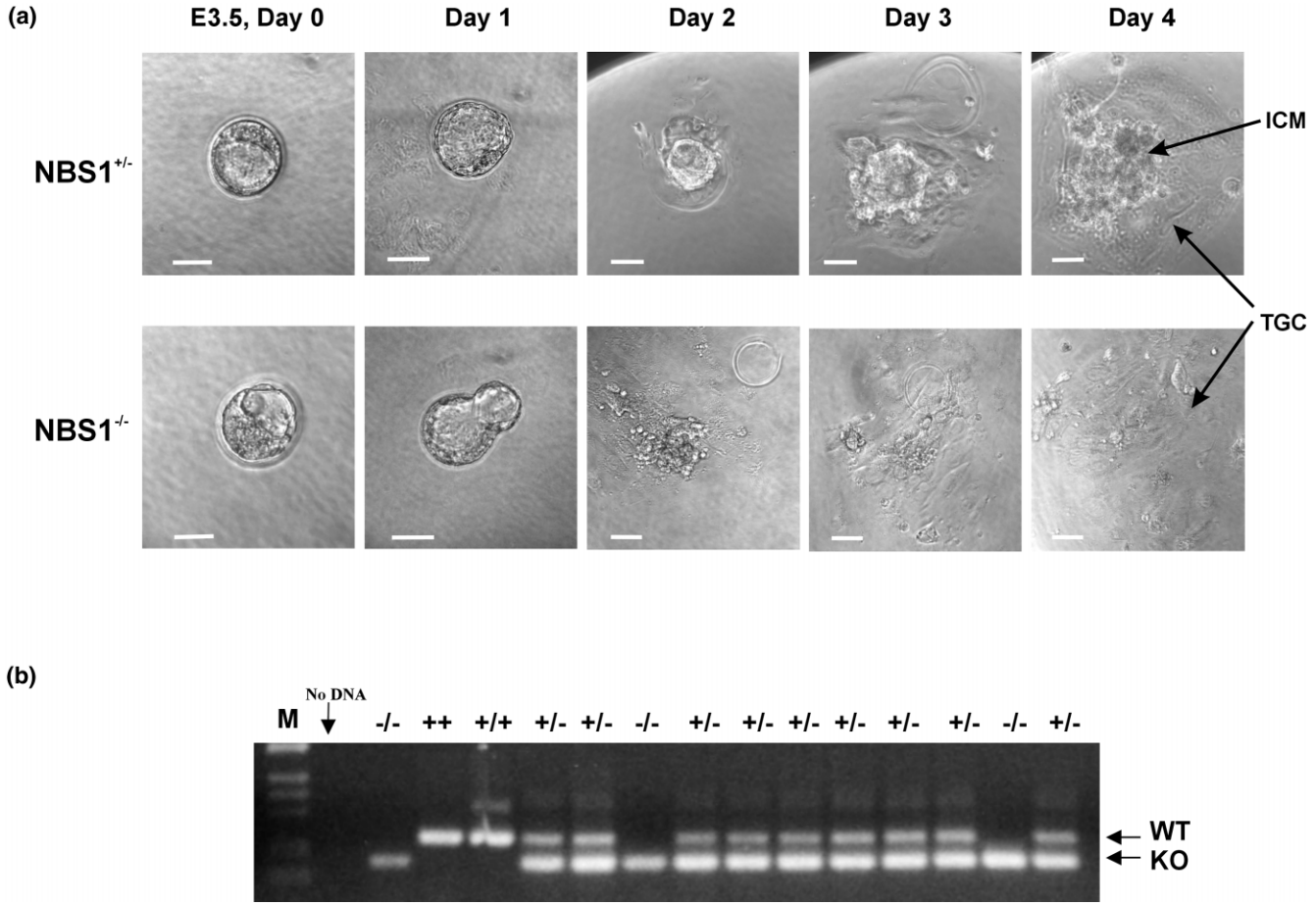
Figure 3



Defective growth of *NBS1*$^{-/-}$ blastocysts in vitro. **(a)** E3.5 blastocysts were isolated from *NBS1*$^{+/-}$ intercrosses and cultured in 96 well plates. Pictures were taken on a daily basis with a phase contrast microscope (Zeiss Axiovert 100) prior to genotyping by PCR. The upper panel shows growth of *NBS1*$^{+/-}$ blastocysts, and the lower panel is an example of *NBS1*$^{-/-}$ blastocyst outgrowth. Inner cell mass (ICM) and trophoblast giant cells (TGC) are indicated. The scale bar represents 100 μm. **(b)** Examples of PCR genotyping of blastocysts after 4 days in vitro culture. A nested PCR strategy was used. DNA preparation was by the incubation of individual blastocysts with 20 μl lysis buffer (100 mM KCl, 10 mM Tris HCl, 2.5 mM $MgCl_2$, 0.45% Tween 20, 0.45% NP40, and 1 mg/ml proteinase K) for 4–5 hr at 55°C. DNA samples (3–5 μl) were used for genotyping. In the first round of PCR, a common primer, C1400 (5′-GGCTAAGA TGTATTGCTCCG-3′), a wild-type specific primer, W210 (5′-GGATCTGGGAAGCTAAAGTATG-3′), and a mutant primer, 3PNT (5′- TAAAGCGCATG CTCCAGACT-3′) were used. For the second round of PCR, primers Com133, W123, and Mut2 (indicated in Figure 1) were used to amplify both wild-type and mutant fragments.

References

1. Varon R, Vissinga C, Platzer M, Cerosaletti KM, Chrzanowska KH, Saar K, *et al.*: **Nibrin, a novel DNA double-strand break repair protein, is mutated in Nijmegen breakage syndrome.** *Cell* 1998, **93:**467-476.
2. Carney JP, Maser RS, Olivares H, Davis EM, Le Beau M, Yates JR iii, *et al.*: **The hMre11/hRad50 protein complex and Nijmegen breakage syndrome: linkage of double-strand break repair to the cellular DNA damage response.** *Cell* 1998, **93:**477-486.
3. Seemanova E: **An increased risk for malignant neoplasms in heterozygotes for a syndrome of microcephaly, normal intelligence, growth retardation, remarkable facies, immunodeficiency and chromosomal instability.** *Mutat Res* 1990, **238:**321-324.
4. Luo G, Yao MS, Bender CF, Mills M, Bladl AR, Bradley A, *et al.*: **Disruption of mRad50 causes embryonic stem cell lethality, abnormal embryonic development, and sensitivity to ionizing radiation.** *Proc Natl Acad Sci USA* 1999, **96:**7376-7381.
5. Lim DS, Hasty P: **A mutation in mouse rad51 results in an early embryonic lethal that is suppressed by a mutation in p53.** *Mol Cell Biol* 1996, **16:**7133-7143.
6. Hakem R, de la Pompa JL, Sirard C, Mo R, Woo M, Hakem A, *et al.*: **The tumor suppressor gene Brca1 is required for embryonic cellular proliferation in the mouse.** *Cell* 1996, **85:**1009-1023.
7. Liu CY, Flesken-Nikitin A, Li S, Zeng Y, Lee WH: **Inactivation of the mouse Brca1 gene leads to failure in the morphogenesis of the egg cylinder in early postimplantation development.** *Genes Dev* 1996, **10:**1835-1843.
8. Sharan SK, Morimatsu M, Albrecht U, Lim DS, Regel E, Dinh C, *et al.*: **Embryonic lethality and radiation hypersensitivity mediated by Rad51 in mice lacking Brca2.** *Nature* 1997, **386:**804-810.
9. Karran P: **DNA double strand break repair in mammalian cells.** *Curr Opin Genet Dev* 2000, **10:**144-150.
10. Featherstone C, Jackson SP: **DNA repair: the Nijmegen breakage syndrome protein.** *Curr Biol* 1998, **8:**R622-R625.
11. Nelms BE, Maser RS, MacKay JF, Lagally MG, Petrini JH: **In situ**

visualization of DNA double-strand break repair in human fibroblasts. *Science* 1998, **280:**590-592.
12. Chen HT, Bhandoola A, Difilippantonio MJ, Zhu J, Bonner MJ, Tai X, *et al.*: **Response to RAG-mediated V(D)J cleavage by NBS1 and γ-H2AX.** *Science* 2000, **290:**1962-1964.
13. Young BR, Painter RB: **Radioresistant DNA synthesis and human genetic diseases.** *Hum Genet* 1989, **82:**113-117.
14. Cerosaletti KM, Desai-Mehta A, Yeo TC, Kraakman-Van Der Zwet M, Zdzienicka MZ, Concannon P: **Retroviral expression of the NBS1 gene in cultured Nijmegen breakage syndrome cells restores normal radiation sensitivity and nuclear focus formation.** *Mutagenesis* 2000, **15:**281-286.
15. Stewart GS, Maser RS, Stankovic T, Bressan DA, Kaplan MI, Jaspers NG, *et al.*: **The DNA double-strand break repair gene hMRE11 is mutated in individuals with an ataxia-telangiectasia-like disorder.** *Cell* 1999, **99:**577-587.
16. Xiao Y, Weaver DT: **Conditional gene targeted deletion by Cre recombinase demonstrates the requirement for the double-strand break repair Mre11 protein in murine embryonic stem cells.** *Nucleic Acids Res* 1997, **25:**2985-2991.
17. Bressan DA, Baxter BK, Petrini JH: **The Mre11-Rad50-Xrs2 protein complex facilitates homologous recombination-based double-strand break repair in *Saccharomyces cerevisiae*.** *MCB* 1999, **19:**7681-7687.
18. Yamaguchi-Iwai Y, Sonoda E, Sasaki MS, Morrison C, Haraguchi T, Hiraoka Y, *et al.*: **Mre11 is essential for the maintenance of chromosomal DNA in vertebrate cells.** *EMBO J* 1999, **18:**6619-6629.
19. Limoli CL, Giedzinski E, Morgan WF, Cleaver JE: **Inaugural article: polymerase eta deficiency in the xeroderma pigmentosum variant uncovers an overlap between the S phase checkpoint and double-strand break repair.** *Proc Natl Acad Sci USA* 2000, **97:**7939-7946.
20. Ludwig T, Chapman DL, Papaioannou VE, Efstratiadis A: **Targeted mutations of breast cancer susceptibility gene homologs in mice: lethal phenotypes of Brca1, Brca2, Brca1/Brca2, Brca1/p53, and Brca2/p53 nullizygous embryos.** *Genes Dev* 1997, **11:**1226-1241.
21. Shen SX, Weaver Z, Xu X, Li C, Weinstein M, Chen L, *et al.*: **A targeted disruption of the murine *Brca1* gene causes gamma-irradiation hypersensitivity and genetic instability.** *Oncogene* 1998, **17:**3115-3124.