RADIATION-INDUCED SPERM DNA LESIONS ARE REPAIRED IN THE EGG BY MATERNAL NHEJ AND HR PATHWAYS

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ONE-SENTENCE SUMMARY

Genetic disruption of DNA double-strand break repair pathways in female mice significantly increased the frequency of zygotes with chromosomal structural aberrations after paternal exposure to ionizing radiation.

ABSTRACT

The final weeks of male germ cell differentiation occur in a DNA repair-deficient environment and normal development depends on the ability of the egg to repair DNA damage in the fertilizing sperm. Genetic disruption of maternal DNA double-strand break repair pathways in mice significantly increased the frequency of zygotes with chromosomal structural aberrations after paternal exposure to ionizing radiation. These findings demonstrate that radiation-induced DNA sperm lesions are repaired after fertilization by maternal factors and suggest that genetic variation in maternal DNA repair can modulate the risk of early pregnancy losses and of children with chromosomal aberrations of paternal origin.

TEXT

Chromosomal defects transmitted through male and female germ lines are associated with pregnancy loss, developmental defects, infant mortality, infertility and genetic diseases in the offspring (*1*). The parental origins of *de novo* genetic and chromosomal defects are not random: e.g., autosomal aneuploidy has a preferential maternal origin (*2*), while point mutations and structural chromosomal rearrangements have a preferential paternal origin (*3*). Male germ cells are susceptible to the accumulation of DNA lesions in fertilizing sperm because their DNA repair capacity declines during the latter part of spermatogenesis (*4*). In contrast, the mammalian oocyte is capable of repairing DNA damage throughout oogenesis and provides gene products that are responsible for repairing DNA damage in both paternal genomes after fertilization (*5, 6*). These maternal gene products persist to sustain the zygote until its genome is fully activated, which occurs at the 2-cell stage in the mouse (*7*) and even later in the human embryo (*8*).

Mouse strains are known to vary substantially in the efficiency of oocytes to repair DNA lesions in the fertilizing sperm (*9*) but the molecular mechanisms responsible for these differences are not understood. Chromosome-type aberrations, i.e. affecting both sister chromatids, are the main type of aberration found in zygotes after paternal exposure to ionizing radiation and chemical mutagens (*10*). These findings indicate that double strand breaks (DSBs) are obligatory intermediates and that their repair occurs during the G1 phase of the zygotic cell cycle. DSB repair can occur via two mechanistically distinct pathways: non-homologous end joining (NHEJ), i.e., direct rejoining of broken ends with minimal requirement for homology (*11, 12*), and homologous recombination (HR), in which a sister duplex provides information to repair the damaged duplex (*13, 14*). The NHEJ pathway involves the DNA-PK (DNA protein kinase) complex which is composed of the Ku70/Ku86 DNA end-binding heterodimer and the

catalytic subunit DNA-Pkcs (*15*), the latter is defective in the immunodeficient Scid mouse strain (*16*). The HR pathway involves the RAD52 epistasis group including RAD54 (*12*), which interacts with RAD51 and is required for DSB repair and sister chromatid exchange (*17, 18*). Disruption of either DSB repair pathway in somatic cells confers increased radiation sensitivity (*19*).

We tested the hypothesis that sperm DNA lesions induced by ionizing radiation a week prior to fertilization persist unrepaired in sperm and that after fertilization maternal DSB repair pathways are involved in the repair of these lesions. We used NHEJ-defective Scid and HR-defective Rad54 null female mice to investigate how disruption of the NHEJ and HR pathways in the fertilized egg altered the repair of radiation-induced sperm DNA lesions. B6C3F1 males were treated with 4 Gy gamma rays 7 days prior mating with normal (C57BL/6J), Scid or Rad54 null females. First-cleavage zygote metaphases were collected for each maternal genotype and analyzed by the PAINT/DAPI chromosome painting procedure (Figure 1) to determine the frequencies and types of zygotes with chromosomal structural aberrations and the types of aberrations, i.e., chromosome- vs. chromatid-type, and rejoined vs. unrejoined.

Disruption of the NHEJ pathway in female mice did not change the baseline frequencies of chromosomal aberrations with respect to C57BL/6J females in matings with unirradiated males (Table 1). After paternal exposure to ionizing radiation, the frequency of zygotic metaphases with chromosomal aberrations was significantly higher in NHEJ-defective females than in control females ($P<0.001$; Table 1). Analysis of the types of chromosomal aberrations showed that there was a 2.4-fold increase ($P<0.001$, Figure 2A) in the number of chromosome-type aberrations in NHEJ-defective females, while chromatid-type aberrations were unchanged (Figure 2B). Both unrejoined (i.e., acentric fragments and breaks) and rejoined (i.e., dicentrics,

translocations, etc.) chromosomal aberrations were significantly higher in NHEJ-defective females with respect to C57BL/6J females (P<0.001; Figure 2C-D). These results indicate that radiation-induced sperm DNA lesions persisted in the maturing sperm for at least 7 days prior to fertilization before they were repaired in the fertilized egg by the NHEJ pathway and that this repair occurred during the G1 phase of the first cell cycle of development (Figure 2E). Consistent with observations in somatic Scid cells (*20, 21*), disruption of the NHEJ pathway in the fertilized egg through abrogation of DNA-PKcs function resulted in both a reduction in repair, as indicated by the increase in unrejoined aberrations, and an increase in misrepair of DSBs, as indicated by the increase in rejoined aberrations. This supports the existence of a NHEJ repair mechanism that is DNA-PK-independent and prone to misrejoining (*22, 23*).

Disruption of the HR pathway in female mice also did not change the baseline frequencies of chromosomal aberrations with respect to C57BL/6J females in matings with unirradiated males (Table 1). After paternal exposure to ionizing radiation, HR-defective females had 1.4-fold higher frequency of zygotic metaphases with chromosomal aberrations (P=0.008); most notably, they had a 4.2-fold increase in the frequency of zygotes with chromatid-type aberrations (Table 1). Analysis of the types of chromosomal aberrations showed that chromosome-type aberrations were unaffected (Figure 2A), while HR-defective females had a 5.5-fold increase in chromatid-type aberrations (P<0.001, Figure 2B). Thus, HR deficiency and NEHJ deficiency showed an opposite effect on the production of chromosome- and chromatid-type aberrations. HR-defective females had also a minor increase in zygotes with unrejoined aberrations (Figure 2D). The findings demonstrate that the HR pathway also participate in the repair of radiation-induced sperm DNA lesions and postulate a class of DSBs that persisted unrepaired beyond the G1 phase and, in the absence of a functional HR pathway, produced

chromatid-type aberrations during the S/G2 phase. This class of DSBs may represent lesions that: (a) escaped repair by the functional NEHJ pathway during G1; or (b) were generated by misrepair of other types of DNA lesions by other DNA repair pathways during the G1 phase of the cell cycle.

We next examined how disruption of the maternal NHEJ and HR pathways affected the progression through the first cell cycle of development (Table 2). Eggs were classified as unfertilized or fertilized (*24*), and fertilized eggs were further categorized as: (a) arrested before S-phase (based on the absence of pronuclei or mitotic chromosomes); (b) in S-phase (based on the presence of the two parental pronuclei); and (c) beyond S-phase (based on the presence of mitotic chromosomes). Paternal exposure to radiation slightly reduced the number of eggs that were fertilized, across all three maternal genotypes ($P=0.09$), but did not significantly affect the progression through the first cell cycle in NHEJ-defective females. However, when HR-defective females were mated with irradiated males there was a significant reduction in the number of fertilized eggs that reached the zygotic metaphase stage when compared with control mice mated to irradiated males ($P<0.001$) and with HR-defective females mated to unirradiated males ($P<0.05$). The data in Table 2 indicates that this was due to a failure of some fertilized eggs to form pronuclei rather than to a general delay in cell cycle progression. Formation of the male pronucleus requires dramatic chromatin remodeling of the fertilizing sperm DNA as protamines are removed and oocyte-supplied histones are assembled onto the sperm DNA (*25*). Our results suggest the involvement of Rad54 in the remodeling of the sperm nucleus into the male pronucleus. Indeed, Rad54 can catalyze nucleosome redistribution along the DNA independently of its association with Rad51 (*26, 27*).

Our findings demonstrate that exposure of male germ cells to ionizing radiation induces DNA lesions that persist in sperm for at least 7 days prior to fertilization and that these lesions are then repaired in the fertilized egg by the maternal DSB repair pathways. Both NHEJ and HR pathways are involved in the repair of radiation-induced sperm DNA lesions during the first cell cycle after fertilization, with the NHEJ pathway playing a greater role than the HR pathway. Also, because both chromosome- and chromatid-type aberrations were affected, our results suggest that maternal DSB repair mechanisms are operating in G1 as well as S/G2 phases of the zygotic cell cycle. Our findings also suggest that maternal Rad54 may be involved in male pronuclear formation during the early phases after fertilization.

The results of the present study highlight the importance of maternal DNA repair during the early phases of mammalian development in assuring the genomic integrity of the conceptus. As chromosomal aberrations in zygotes are quantitatively associated with various types of abnormal reproductive outcomes (*28*), our results show that quantitative and qualitative differences in the efficiency of maternal DNA repair genes can directly alter the risk of early pregnancy losses and of children with chromosomal aberrations of paternal origin. Thus, a fully DNA-proficient egg that is capable of repairing DNA lesions carried by the fertilizing sperm is essential for proper embryonic development and birth of a healthy baby.

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MATERIALS AND METHODS

Studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals and were approved by the LLNL Institutional Animal Care and Use Committee. B6C3F1 males were purchased from Harlan Sprague-Dawley Inc. (Indianapolis, IN, USA); C57BL/6J and Scid females were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). A colony of Rad54 deficient mice was established and maintained at LLNL from two Rad54 null breeding pairs (*18*). Both DSB repair mutations were on a C57BL/6J background.

Mice were 6-8 weeks of age at the beginning of the experiments and were maintained under a 12 hr light/dark photoperiod at room temperature of 21-23° C and relative humidity of 50 ± 5%. Pelleted food and sterilized tap water were provided *ad libitum*. Male mice received a whole body irradiation dose of 4 Gy with a delivery rate of 0.61 Gy/min using a ^{137}Cesium Mark 1 Irradiator (J. L. Shepherd and Assoc., Glendale, CA) and were mated with unirradiated females 7 days later. Control males were sham irradiated. Female mice were superovulated by an i.p. injection of 7.5 I.U. of pregnant mare's serum (PMS, Sigma Co.) followed 48h later by an i.p. injection of 5.0 I.U. of human chorionic gonadotrophin (hCG, Sigma Co.). Scid females did not respond as well as the other maternal genotypes to superovulation. The number of ovulated eggs were 20.3 ± 4.6, 21.1 ± 3.9 and 13.2 ± 2.5 in C57BL/6J, Rad54 null and Scid females, respectively.

For each data point, a minimum of three repetitions, each using 12 mating pairs, were conducted. In each repetition, zygotes from all mated females were pooled and processed simultaneously. Animal mating, zygote collection and processing, chromosome painting and metaphase analyses were conducted as previously described (*28*).

The ANOVA test was used for comparing the progression during the first cell cycle and the Chi-square test was used for comparing the frequencies of zygotes with chromosomal aberrations and types of chromosomal aberrations among the three maternal genotypes.

Table 1 – Chromosomal aberrations in zygotes of three maternal genotypes mated to unirradiated (0 Gy) or irradiated (4 Gy) males.

Maternal genotype	Paternal exposure (Gy)[a]	Zygotes analyzed	Zygotes with aberrations		Zygotes with chromosome-type aberrations		Zygotes with chromatid-type aberrations	
			Number[b]	% ± S.E.[c]	Number	% ± S.E.[c]	Number	% ± S.E.[c]
C57BL/6J	0	117	2	1.7 ± 0.7	2	1.7 ± 0.7	0	0
C57BL/6J	4	261	56 (3)	21.5 ± 1.6	55	21.1 ± 1.7	4	1.5 ± 0.9
Scid	0	61	1	1.6 ± 1.1	1	1.6 ± 1.1	0	0
Scid	4	71	34 (1)	47.9 ± 6.6[f,g]	34	47.9 ± 6.6[f,g]	1	1.4 ± 1.9
Rad54 null	0	175	4[d] (1)	2.3 ± 0.9	2	1.1 ± 1.0	3[d]	1.7 ± 1.1
Rad54 null	4	155	45 (4)	29.0 ± 2.4[e]	38	24.5 ± 2.2	11	7.1 ± 1.8[h]

[a]Seven days prior to mating; males were B6C3F1 for all the experiments.

[b]Zygotes with both chromosome-type and chromatid-type aberrations are reported in parenthesis.

[c]Standard error across the minimum of three pools of zygotes.

[d]One zygote with a chromatid exchange in the maternal chromosomes.

[e]P=0.08 vs C57BL/6J; 4Gy.

[f]P<0.01 vs C57BJ/6J; 4 Gy.

[g]P<0.05 vs Rad54 null; 4 Gy.

[h]P<0.05 vs C57BL/6J; 4 Gy and Rad54 null; 0 Gy.

Table 2 – Frequencies and types of eggs recovered from females with three maternal genotypes mated to unirradiated (0 Gy) or irradiated (4 Gy) males.

Maternal genotype	Paternal exposure (Gy)[a]	Total eggs	Unfertilized eggs		Fertilized eggs					
					Arrested before S-phase		In S-phase		Beyond S-phase	
			Total	% ± S.E	Total	%[b] ± S.E	Total	%[b] ± S.E	Total	%[b] ± S.E
C57BL/6J	0	246	59	24.0 ± 2.7	29	15.5 ± 1.2	5	2.7 ± 1.2	153	81.8 ± 2.5
C57BL/6J	4	660	191	28.9 ± 2.3	87	18.6 ± 1.9	16	3.4 ± 1.6	366	78.0 ± 3.0
Scid	0	160	43	26.9 ± 1.9	19	16.2 ± 4.7	3	2.6 ± 1.5	95	81.2 ± 6.2
Scid	4	283	98	34.6 ± 2.8	47	25.4 ± 3.3	5	2.7 ± 1.2	133	71.9 ± 2.6
Rad54 null	0	466	135	29.0 ± 3.9	84	25.4 ± 6.2	7	2.1 ± 0.9	240	72.5 ± 6.7
Rad54 null	4	607	219	36.1 ± 3.1	155	39.9 ± 2.4[c,d]	18	4.6 ± 1.7	215	55.4 ± 1. 4[c,d]

[a]Seven days prior to mating; males were B6C3F1 for all the experiments.

[b]Frequencies among fertilized eggs.

[c]P<0.05 vs Rad 54 null; 0 Gy.

[d]P<0.001 vs C57BL/6J; 4 Gy.

FIGURES

Figure 1. Photomicrographs of first-cleavage (1-Cl) zygote metaphases using the PAINT/DAPI procedure. Metaphases were hybridized with chromosome-specific painting probes for chromosomes 1, 3, 5, X and Y labeled with FITC and chromosomes 2, 4, 6, X and Y labeled with biotin and signaled with Texas Red™. With this probe combination, chromosomes 1, 3 and 5 appear green, chromosomes 2, 4 and 6 appear red and the sex chromosomes appear yellow. Paternal chromosomes can be identified because they contain the Y chromosome or they are less condensed than maternal chromosomes. Bars represent 10 μm. (**A**) Normal 1-Cl zygote metaphase from a C57BL/6J female with the Y-bearing sperm-derived chromosomes on the left. (**B**) 1-Cl zygote with Y-bearing sperm-derived chromosomes (on the right) obtained from a Scid female mated to an irradiated male showing a ring aberration involving one paternal chromosome painted in green. (**C** to **E)** Examples of chromatid-type aberrations in paternal chromosomes observed in 1-Cl zygotes from Rad54 null females mated with irradiated males: (**C**) incomplete interchromatid exchange; (**D**) triradial; (**E**) chromatid break.

Figure 2. Relative induction of chromosome-type (**A**) and chromatid-type (**B**), rejoined (**C**) and unrejoined (**D**) aberrations in C57BL/6J, Rad54 null and Scid females mated to unirradiated (0 Gy) or irradiated (4 Gy) males. The number of aberrations per metaphase analyzed is reported on the Y axis, while the bars represent the standard error. For Rad54 null females, the chromatid-type data includes a single chromatid-exchange involving at least 6 chromosomes that was found in the maternal chromosomes of a zygote fertilized by an untreated sperm. This was the only zygote in the entire study with a chromosomal aberration of maternal origin. (**E**) Model for the

repair of ionizing radiation-induced sperm DNA lesions by maternal DSB repair pathways after fertilization. The results with NHEJ-defective females show that DSBs induced by irradiation of sperm before fertilization are repaired by the NHEJ pathway during the G1 phase of the first cell cycle. In the absence of a functional NHEJ pathway, unrepaired DSBs produce chromosome-type aberrations and increased unrejoined chromosomal breaks. The results with HR-defective females show that some radiation-induced lesions are repaired during the S/G2 phase of the cell cycle. In the absence of a functional HR pathway, there is an increase in chromatid-type aberrations. These may be a class of DSBs that escape repair by the functional NHEJ pathway during G1, or DSBs generated by misrepair of other sperm DNA lesions by other DNA repair pathway during G1.

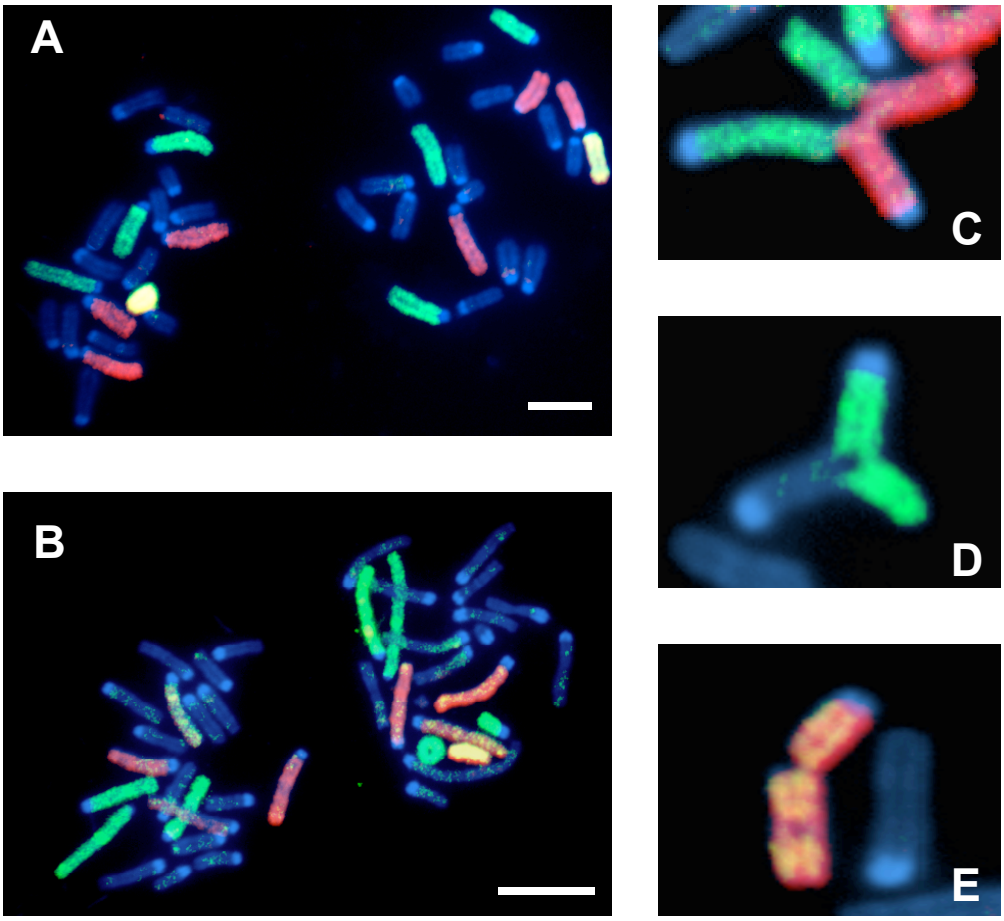

Figure 1

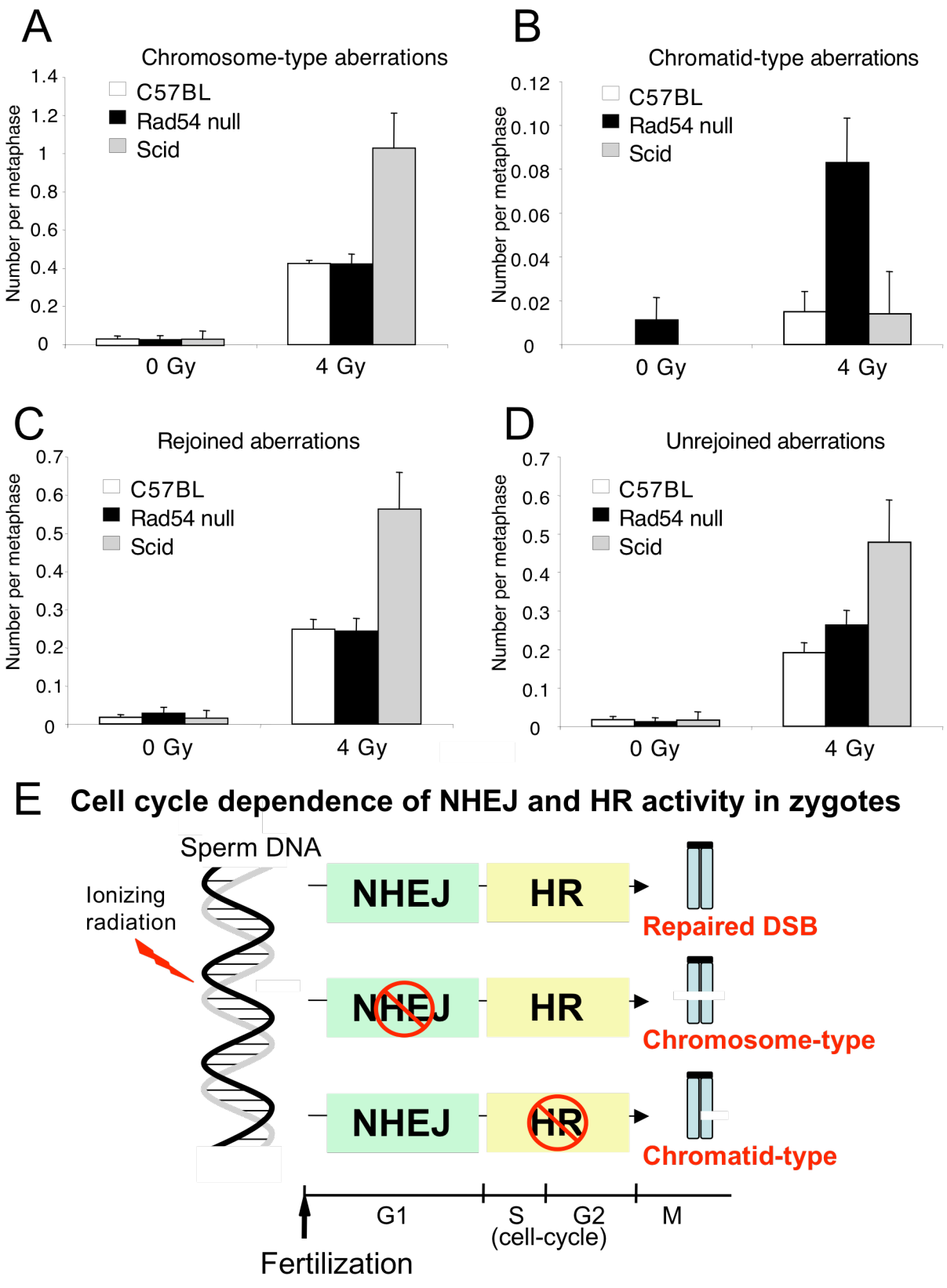
A
Chromosome-type aberrations
Number per metaphase
C57BL
Rad54 null
Scid
0 Gy
4 Gy
B
Chromatid-type aberrations
C
Rejoined aberrations
D
Unrejoined aberrations
E
Cell cycle dependence of NHEJ and HR activity in zygotes
Sperm DNA
Ionizing radiation
NHEJ
HR
Repaired DSB
Chromosome-type
Chromatid-type
G1
S
G2
M
(cell-cycle)
Fertilization

Figure 2

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