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Genetic Evidence for the Involvement of Mismatch Repair Proteins, PMS2 and MLH3, in a Late Step of Homologous Recombination

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Running title: **Role of PMS2 and MLH3 in Homologous Recombination**

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Abstract

Homologous recombination (HR) repairs DNA double-strand breaks (DSBs) using intact homologous sequences as template DNA. Broken DNA and intact homologous sequences form joint molecules (JMs), including Holliday junctions (HJs), as HR intermediates. HJs are resolved to form crossover and noncrossover products. A mismatch repair factor, MLH3 endonuclease produces the majority of crossovers during meiotic HR. It remains elusive whether mismatch repair factors promote HR in non meiotic cells. We disrupted genes encoding the MLH3 and PMS2 endonucleases in the human B cell line, TK6, generating null *MLH3*^{-/-} and *PMS2*^{-/-} mutant cells. We also inserted point mutations into the endonuclease motif of *MLH3* and *PMS2* genes, generating *MLH3*^{DN/DN} and *PMS2*^{EK/EK} cells. *MLH3*^{-/-} and *MLH3*^{DN/DN} cells showed a very similar phenotype, 2.5 times decrease in the frequency of heteroallelic HR-dependent repair of a restriction-enzyme-induced DSBs. *PMS2*^{-/-} and *PMS2*^{EK/EK} cells showed a phenotype very similar to that of the MLH3 mutants. These data indicate that MLH3 and PMS2 promote HR as an endonuclease. The *MLH3*^{DN/DN} and *PMS2*^{EK/EK} mutations had an additive effect on the heteroallelic HR. *MLH3*^{DN/DN}/*PMS2*^{EK/EK} cells showed normal kinetics of γ -irradiation-induced Rad51 foci but a significant delay in the resolution of Rad51 foci and three times decrease in the number of cisplatin-induced sister chromatid exchange (SCE). The ectopic expression of the Gen1 HJ resolvase partially reversed the defective heteroallelic HR of *MLH3*^{DN/DN}/*PMS2*^{EK/EK} cells. We propose that MLH3 and PMS2 promote HR as endonucleases, most likely by processing JMs in mammalian somatic cells.

Introduction

The mismatch repair (MMR) pathway corrects the mismatch formed during DNA replication (1–5). MMR is initiated by the recognition of mismatches by the heterodimers, MSH2-MSH6 (MutS α), and MSH2-MSH3 (MutS β) (5–10). Upon recognition, the MutS heterodimers interact with one of the three MutL heterodimers, MLH1-PMS2 (MutL α), MLH1-PMS1 (MutL β), and MLH1-MLH3 (MutL γ) (11–13). A single-strand break formed by the MLH1-PMS2 endonuclease serves as entry points for the exonuclease activity that removes mismatched DNA. The endonuclease activity of MLH1-PMS2 depends on the metal-binding motif DQHA(X)₂E(X)₄E present on PMS2 and the last ten residues of MLH1 (14). This nuclease-active site is conserved in MLH3 but not in PMS1 (15).

A subclass of the MMR proteins is involved in double-strand break (DSB) repair. Firstly, MutS complexes play a role in the rejection of heteroduplex DNA containing insertion/deletion mismatches when the nucleotide sequences of two partner DNAs are not identical (16, 17). Second, MutS α may recognize mismatches within the heteroduplex region of the JMs and avoid recombination, collaborating with RecQ helicases (18). Third, MLH1 can affect nonhomologous end-joining (NHEJ) (19), which repairs 80% of the ionizing-radiation-induced DSB in the G2 phase (20). Fourth, a subset of MSH and MLH proteins promote meiotic HR, which function is distinct from their MMR functions. MLH1-MLH3, which has a minor role in MMR, is critical for producing meiotic crossover products in mice and *Saccharomyces cerevisiae* (*S. cerevisiae*) (21, 22). MLH1, MLH3, and PMS2 are essential for the progression of meiotic HR in mice (23–28). The role played by the putative endonuclease activity of PMS2 in the resolution of meiotic HR intermediates has not yet been clarified in the mouse or human but recent studies have unveiled new insights into the molecular mechanisms of MLH1-MLH3 and the role of its endonuclease activity (29, 30). Another unsolved question is whether MLH3 and PMS2 promote HR in mammalian somatic cells.

HR initiates DSB repair by resecting DSBs leading to the formation of 3' single-strand overhangs, followed by polymerization of Rad51 on the single-strand DNA (31–33). The resulting Rad51 nucleoprotein filaments undergo homology search and pairing with the intact duplex DNA donor to form joint molecules (JMs) such as double Holliday junctions (dHJs) with the help of Rad54 (33–35). JMs are resolved into individual DNA duplexes to allow chromosomes to separate in the anaphase. The separation is performed by two alternative processes, the dissolution and resolution

pathways. The phenotypic analysis of meiotic HR indicates that only 10% of the DSBs (*M. musculus*) form dHJs, and these are almost exclusively processed by the resolution pathway, involving the activity of MLH1-MLH3 (22). In somatic cells, the resolution of HJs is done by a number of structure-specific endonucleases, MUS81-EME1, SLX1-SLX4, XPF-ERCC1, and GEN1 (36–39). Mice deficient in either MUS81-EME1 or SLX1-SLX4 or GEN1 are all viable, whereas mice deficient in both MUS81-EME1 and GEN1 are synthetic lethal (40–43), suggesting a substantial functional overlap between the two nucleases. Although yeast genetic studies have precisely monitored the formation of HR intermediate molecules such as HJs over time upon DSB formation during both meiosis and mitosis (21, 34, 44, 45), no equivalent phenotypic assays are available in the phenotypic analysis of HR in mammalian somatic cells.

There are two major DSB repair pathways in mammalian cells, HR and nonhomologous end-joining (NHEJ). The two pathways differentially contribute to cellular tolerance to anti-malignant therapies. These pathways contribute to tolerance to radiotherapy with HR functioning in the S to G2 phases and NHEJ functioning in the whole cell cycle (46). HR, but not NHEJ, repairs DSBs induced by camptothecin (Top1 poison) and olaparib (poly[ADP ribose]polymerase poison). NHEJ plays the dominant role in repairing DSBs induced by ICRF-193 (catalytic inhibitor of Top2) (47, 48). Thus, the sensitivity profile of DSB-repair mutants to these chemotherapeutic agents help to discriminate which repair pathway is compromised in the mutants.

To investigate the role for MLH3 and PMS2 as nucleases in DSB repair of somatic mammalian cells, we inserted point mutation into the DQHA(X)₂E(X)₄E motif of the endogenous *MLH3* and *PMS2* genes of the human TK6 B cell line (49) and generated *MLH3*^{D1223N/D1223N} and *PMS2*^{E705K/E705K} cells. These mutants exhibited increased sensitivities to camptothecin and olaparib, a few times decrease in the frequency of both SCE and the heteroallelic HR, and delayed resolution of γ -ray-induced Rad51 foci, indicating a defect in HR in later steps. Surprisingly, their role seems to be mostly independent of MLH1. We conclude that the MLH3 and PMS2 proteins promote DSB repair by HR presumably by processing JMs in human cells.

Results

MLH3 and PMS2 mutants, but not MSH2 and MLH1 mutants, are sensitive to both camptothecin and olaparib

We disrupted the *PMS2* and *MLH3* genes in TSCER2 cells (50, 51), a TK6 sub-line for measuring heteroallelic HR, generating *PMS2*^{-/-} and *MLH3*^{-/-} cells (Figures S1 and S2). We also generated *MSH2*^{-/-} cells (Figures S3A and B), as MSH2 plays a major role in MMR but is not involved in the resolution of HJs in *S. cerevisiae* (21). *MSH2*^{-/-} cells were tolerant to an alkylating agent, temozolomide (Figure S3C), as expected from a defect in MMR (52). *PMS2*^{-/-} and *MLH3*^{-/-} cells were sensitive to camptothecin, γ -irradiation, and olaparib (Figures 1A and B), whereas *MSH2*^{-/-} cells were tolerant to these damaging agents (Figures S3D, E, and F). These data suggest the involvement of PMS2 and MLH3 in HR-dependent DSB repair independently of their functioning in MMR or independently of their interaction with Mut α (MSH2-MSH6) or Mut β (MSH2-MSH3) heterodimers. We generated *MLH1*^{-/-} cells (Figure S4) and verified a defect in MMR by confirming the marked tolerance to temozolomide (53) (Figure S4F). We found no noticeable sensitivity of *MLH1*^{-/-} cells to γ -irradiation (Figure 1A). We disrupted the *MUS81* gene in *wild-type* and *PMS2*^{-/-} TK6 clones (Figure S5). The resulting *MUS81*^{-/-} cells showed a phenotype very similar to that of *PMS2*^{-/-} cells. *MUS81*^{-/-}/*PMS2*^{-/-} cells showed higher sensitivity to olaparib than *MUS81*^{-/-} and *PMS2*^{-/-} cells (Figure 1C). These observations support the notion that PMS2 and MUS81 act independently of each other in HR-mediated DSB repair.

To investigate the catalytic role of MLH3 and PMS2, we inserted point mutations into the endogenous *MLH3* and *PMS2* genes at the highly conserved DQHA(X)₂E(X)₄E metal-binding motif. The replacement of the glutamic acid residue in position 705 by lysine (E705K) in human PMS2 completely inactivates its endonuclease activity (15, 54, 55). Likewise, the D523N and E529K mutations in *S. cerevisiae* MLH3, which correspond to the D1223N and E1229K mutations in human MLH3, impair both MMR and the resolution of JMs in meiotic HR in mice and *Saccharomyces cerevisiae* (21, 22, 56–59). We thus generated *PMS2*^{E705K/E705K} cells (Figure S1), and *MLH3*^{D1223N/D1223N} and *MLH3*^{E1229K/E1229K} cells (Figure S6). These mutants are hereafter written as *PMS2*^{EK/EK}, *MLH3*^{DN/DN}, and *MLH3*^{EK/EK} cells. The sensitivity profile of *PMS2*^{EK/EK} cells was the same as that of *PMS2*^{-/-} cells (Figure 1A). Likewise, *MLH3*^{-/-}, *MLH3*^{DN/DN}, and *MLH3*^{EK/EK} cells showed the same phenotype (Figure 1B). These observations suggest that PMS2 and MLH3 contribute to HR mediated DSB repair as the endonuclease.

The repair of γ -ray-induced DSBs during G2 phase is severely compromised in the PMS2 and MLH3 mutant cells

To monitor DSB repair selectively during the G2 phase when HR is active, we exposed cells to ionizing-radiation and measured the number of chromosomal aberrations in mitotic chromosome spreads at three hours after ionizing-radiation. Only cells that were γ -irradiated at the G2 phase, but not S phase, can enter the M phase within three hours (60). This method allows for evaluating the capability of HR to repair DSBs with several times higher sensitivity than the analysis of the γ -irradiation-sensitivity of asynchronous cell populations (Figures 1A and B). Indeed, γ -irradiation increased the number of chromosomal breaks by 1.0 per *MUS81*^{-/-} cell and only 0.2 per *wild-type* cell (Figure 2B). Remarkably, the total numbers of chromosome aberrations induced by γ -rays were around ten times higher in the PMS2 and MLH3 mutant cells in comparison with *wild-type* cells (Figure 2B). The total number of mitotic chromosome aberrations was significantly higher in *MLH1*^{-/-} cells, but not in *MSH2*^{-/-} cells, in comparison with *wild-type* cells (Figure 2B). We conclude no significant contribution of canonical MMR involving MSH2 to DSB repair during the G2 phase. The total numbers of γ -ray-induced chromosome aberrations increased to very similar extents in the five mutants, *PMS2*^{-/-}, *PMS2*^{EK/EK}, *MLH3*^{-/-}, *MLH3*^{DN/DN}, and *MLH3*^{EK/EK} cells (Figure 2B). These data suggest that PMS2 and MLH3 significantly contribute to DSB repair as endonuclease.

We counted the number of chromosome aberrations distinguishing chromatid-type breaks (one of the two sister chromatids is broken), isochromatid-type breaks (two sister chromatids are broken at the same sites) and radial chromosomes (comprise the association of two or more chromatids) (Figure 2A). Ionizing irradiation of *RAD54*^{-/-} cells caused a more significant increase in the number of chromatid-type breaks than that of isochromatid-type breaks (Figure 2B). This observation agrees with the role of RAD54 in promoting strand exchange and JM formation. In contrast, *MUS81*^{-/-} cells showed marked increases in the numbers of isochromatid-type breaks. Isochromatid-type breaks result from abnormal processing of JMs between broken and intact sister chromatids, as the persistent presence of JMs interferes with local chromosome condensation of both sister chromatids, leading to microscopically visible breakage of the two chromosomes at the same sites (39, 61, 62). Radial chromosomes may be caused by the abnormal separation of JMs containing two sisters, leading to inverted chromosome fusions. Like *MUS81*^{-/-} cells, the PMS2 and MLH3 mutants showed significant increases in the numbers of both isochromatid-type breaks and radial chromosomes (Figure 2B). These data suggest that PMS2 and MLH3 promote HR-dependent DSB repair after formation of JMs as does MUS81.

Surprisingly, the *MLH1*^{-/-} phenotype is not as severe as expected from the phenotypes of the PMS2 and MLH3 mutants, particularly no significant alteration regarding isochromatid-type breaks. A moderate increase in the number of chromatid-type breaks in *MLH1*^{-/-} cells suggests that the MLH1-MLH3 and MLH1-PMS2 heterodimers may play a minor role in NHEJ-mediated DSB repair, as suggested previously (19). One possible scenario is that an MLH1-independent alternative mechanism of PMS2 and MLH3 might be present in the process observed in this study. These data suggest that PMS2 and MLH3 promote HR-dependent DSB repair after the formation of JMs, as does MUS81.

PMS2^{EK/EK}/MLH3^{DN/DN} double mutant cells display stronger HR defects than PMS2^{EK/EK} and MLH3^{DN/DN} cells

We chose *MLH3*^{DN/DN} cells as a representative MLH3 mutant due to the phenotypic similarity among *MLH3*^{-/-}, *MLH3*^{DN/DN}, and *MLH3*^{EK/EK} cells. Likewise, we chose *PMS2*^{EK/EK} cells for the subsequent analyses. To investigate the functional relationship between the PMS2 and MLH3 endonucleases, we generated *PMS2*^{EK/EK}/*MLH3*^{DN/DN} double mutant cells. The doubling time was 12.5 hours for *wild-type*, 12.7 hours for *PMS2*^{EK/EK}, 12.7 hours for *MLH3*^{DN/DN}, and 14.3 hours for *PMS2*^{EK/EK}/*MLH3*^{DN/DN} cells. The plating efficiency of these cells was 50 to 60% for all genotypes. *PMS2*^{EK/EK}/*MLH3*^{DN/DN} cells showed high sensitivity to camptothecin and γ -rays, higher than *MLH3*^{DN/DN}, and slightly higher than *PMS2*^{EK/EK}, suggesting a prominent role of PMS2 in these assays (Figure 1D). *PMS2*^{EK/EK}/*MLH3*^{DN/DN} cells also showed a higher sensitivity to olaparib than did *PMS2*^{EK/EK} and *MLH3*^{DN/DN} cells (Figure 1D). The number of γ -ray-induced chromosomal breaks was more than 50% higher in *PMS2*^{EK/EK}/*MLH3*^{DN/DN} cells than in *PMS2*^{EK/EK} and in *MLH3*^{DN/DN} cells (Figure 2). We, therefore, conclude that PMS2 and MLH3 contribute to HR as the endonuclease independently of each other.

We monitored DSB repair kinetics by measuring the number of γ H2AX foci with time after γ -irradiation (Figure 3). The numbers of γ H2AX foci were very similar among *MLH3*^{DN/DN}, *PMS2*^{EK/EK}, and *wild-type* cells at two hours after ionizing irradiation. The numbers of γ H2AX foci reduced more slowly in *MLH3*^{DN/DN} and *PMS2*^{EK/EK} cells in comparison with *wild-type* and *MLH1*^{-/-} cells (Figure 3). The delayed DSB repair kinetics observed more than two hours after ionizing irradiation is consistent with the fact that HR needs a longer time to complete DSB repair than does NHEJ (20). *PMS2*^{EK/EK}/*MLH3*^{DN/DN} cells showed a more prominent delay in DSB repair at 8 hours

in comparison with $MLH3^{DN/DN}$ and $PMS2^{EK/EK}$ cells (Figure 3). We conclude that PMS2 and MLH3 promote DSB repair independently of each other in an MLH1-independent manner.

Resolution of γ -ray-induced RAD51 foci is delayed in the PMS2 and MLH3 mutant cells

To evaluate if PMS2 and MLH3 act in the early and late steps of HR, we analyzed the formation of Rad51 foci over time after γ -irradiation (Figure 4). The number of Rad51 foci peaked at two hours after γ -irradiation in *wild-type* TK6 cells (60). $MLH3^{DN/DN}$, $PMS2^{EK/EK}$, and $PMS2^{EK/EK}/MLH3^{DN/DN}$ cells showed the same extent of Rad51 foci at two hours as *wild-type* cells. Thus, PMS2 and MLH3 are dispensable for DSB resection and the polymerization of Rad51 on resected DSBs. Remarkably, $MLH3^{DN/DN}$, $PMS2^{EK/EK}$, and $PMS2^{EK/EK}/MLH3^{DN/DN}$ cells showed a significant delay in the resolution of Rad51 foci in comparison with *wild-type* and $MLH1^{-/-}$ cells (Figure 4). Both $MLH3^{DN/DN}$ and $PMS2^{EK/EK}$ single mutants showed a similar delay in the resolution of Rad51 foci in comparison with $MUS81^{-/-}$ cells, but this effect was more prominent in the $PMS2^{EK/EK}/MLH3^{DN/DN}$ double mutant cells (Figure 4B). All mutants were less sensitive than $RAD54^{-/-}$ cells. We, therefore, conclude that the PMS2 and MLH3 endonucleases promote HR-dependent DSB repair after the polymerization of Rad51 at DSBs.

$MLH3^{DN/DN}$ and $PMS2^{EK/EK}$ cells are deficient in heteroallelic HR

To assess the involvement of PMS2 and MLH3 in the resolution of HJs, we measured the frequency of heteroallelic recombination between the allelic thymidine kinase (*TK*) genes carrying compound heterozygous mutations (47, 50, 60, 63) (Figure 5A). One of the two allelic *TK* genes carries an *I-Sce1* site, and a mutation in the exon 5 localizes 108 nucleotides downstream of the *I-Sce1* site. When *I-Sce1*-induced DSBs are repaired by either the gene conversion (HR) that associates with crossover or long-tract gene conversion, it can restore an intact *TK* gene. These restoration events are detectable by counting the frequency of drug-resistant colonies (50). The HR frequency was 60% smaller in $MUS81^{-/-}$ cells compared with *wild-type* cells (Figure 5B), suggesting that a majority of the heteroallelic recombination events involve the formation of HJs. The PMS2 and MLH3 mutants, including $MLH3^{DN/DN}$ and $PMS2^{EK/EK}$ cells, showed 60-70% decreases, and $PMS2^{EK/EK}/MLH3^{DN/DN}$ and $MUS81^{-/-}/PMS2^{-/-}$ cells showed further declines in the frequency of crossover events when compared with *wild-type* cells (Figure 5B). These observations suggest that the endonuclease-activity of PMS2 and MLH3 may be involved in the resolution of HJs. This function of PMS2 and MLH3 is also independent of MLH1 and MSH2.

We further assessed the involvement of PMS2 and MLH3 in the resolution of HJs by measuring SCE events, crossover-type HR (62, 64, 65). To induce SCE, we treated cells with cisplatin, an interstrand crosslinking agent. The number of cisplatin-induced SCE events was measured by subtracting the number of SCE before cisplatin-treatment from the number of SCE post-treatment (Figure 5C and 5D). The treatment increased the SCE frequency by 11 events per 100 mitotic *wild-type* cells (Figure 5D). The number of induced SCE was 50% smaller in *MUS81*^{-/-} cells when compared with *wild-type* cells. *MLH3*^{DN/DN} and *PMS2*^{EK/EK} cells also showed 50% decreases, and *PMS2*^{EK/EK}/*MLH3*^{DN/DN} cells showed an 80% decrease in the SCE in comparison with *wild-type* cells (Figure 5C and 5D). In summary, PMS2 and MLH3 contribute to crossover formation most likely by promoting the resolution of HJs, as does MUS81, but MLH1 is not involved in this process.

The loss of MLH1 does not impair HR-dependent DSB repair

MLH1 physically interacts with PMS2 and MLH3 as heterodimers and thereby stabilizes the two endonucleases (66). Here to evaluate the role of MLH1 in the mitotic HR we have employed five phenotypic assays, (i) sensitivity to camptothecin, γ -irradiation and olaparib (Figure 1A), (ii) γ -ray-induced chromosome aberrations (Figure 2B), (iii) measuring the number of γ H2AX and Rad51 foci over time after γ -irradiation (Figure 3 and 4), (iv) measuring the frequency of the heteroallelic recombination (Figure 5A and 5B), and (v) SCE induced by cisplatin (Figure 5C and 5D). Unexpectedly, as mentioned above, all of these phenotypic assays consistently showed that *MLH1*^{-/-} cells were proficient in HR-mediated DSB repair. We, therefore, conclude that MLH1 is dispensable for the functioning of human MLH3 and PMS2 in mitotic HR. It represents to our knowledge the first example, where MLH1 is not required for the functioning of MLH3 and PMS2. Indeed, MLH1 has been shown to be required for the functioning of MLH3 and PMS2 in MMR and in meiotic HR in mice (24, 25).

We speculated that the MLH1-independent function of PMS2 and MLH3 in mitotic HR can be achieved through I) homodimer formation, II) forming heterodimer with unknown partner protein which stabilize the PMS2 and MLH3 proteins, and III) MLH3-PMS2 heterodimer. We could not examine these possibilities due to the lack of specific antibodies and no appropriate method of inserting functional tag sequences into PMS2 and MLH3. We therefore investigated PMS2 and MLH3 homodimer and heterodimer formation through 3D-structure modeling using a standard

homology modeling pipeline based on the HHpred and RosettaCM methods (67–69). Structural analysis of the resulting models supports the potential homodimer and heterodimer formation (Figure S8A, S8B, and S8C).

Significant rescue of the defective HR of the MLH3 and PMS2 mutants by ectopic expression of GEN1.

We reason that if the PMS2 and MLH3 endonuclease activities promote HR by processing HJs, the mutant phenotype of $MLH3^{DN/DN}$ and $PMS2^{EK/EK}$ cells could be suppressed by ectopic expression of one of the resolvases described for HJs. We chose GEN1 as the HJ resolvase (70), and used the *GEN1* transgene carrying mutations in its nuclear export signal (NES) and fused with the nuclear localization signal (NLS) (71) (Figure 6A). We added the FLAG tag to this *GEN1* transgene and inserted it into the pMSCV retroviral expression vector, which allows for the bi-cistronic expression of the *GFP* and *GEN1* transgenes (72) (Figure S7). We produced recombinant retrovirus and infected them into TK6 clones. To confirm the expression of the transgene, we performed western blot analyses using an anti-flag antibody (Figure S7D). We measured the ionizing-radiation sensitivity and calculated LD50, the dose of γ -rays that reduced the survival of cells to 50% relative to non-irradiated cells (Figure 6B). The expression of the *GEN1* transgene reversed the ionizing-radiation sensitivity of $MUS81^{-/-}$ cells, but not *wild-type* or $RAD54^{-/-}$ cells. Thus, the *GEN1* transgene is able to selectively normalize the defective processing of JMs during HR mediated DSB repair.

The *GEN1* transgene restored the tolerance of $MLH3^{DN/DN}$, $PMS2^{EK/EK}$, and $PMS2^{EK/EK}/MLH3^{DN/DN}$ cells to γ -rays at least partially (Figure 6B). The rescue effect of *GEN1* transgene was more efficient in $PMS2^{EK/EK}/MLH3^{DN/DN}$ double mutant cells compare to $MLH3^{DN/DN}$ and $PMS2^{EK/EK}$ cells. In agreement with this finding, the *GEN1* transgene significantly reduced the total number of chromosomal aberrations in these mutants as well as $MUS81^{-/-}$ cells (Figure 6C). Importantly, the *GEN1* transgene expression reduced the number of isochromatid-type breaks to a considerably greater extent than that of chromatid breaks (Figure 6C). The *GEN1* transgene increased the frequency of heteroallelic HR in $MUS81^{-/-}$ cells by 60% but had no effect on that in *wild-type* or $RAD54^{-/-}$ cells (Figure 6D), suggesting that a substantial fraction of heteroallelic HR involves HJ formation as HR intermediates. The *GEN1* transgene restored heteroallelic HR in $MLH3^{DN/DN}$, $PMS2^{EK/EK}$, $PMS2^{EK/EK}/MLH3^{DN/DN}$, as well as $MUS81^{-/-}$ cells but not *wild-type* or $RAD54^{-/-}$ cells

(Figure 6D). In summary, the PMS2 and MLH3 endonuclease activities facilitate the separation of HJs.

Discussion

We demonstrate that human PMS2 and MLH3 promote DSB repair by HR in human somatic cells. Previous studies failed to uncover their role in the repair of X-ray-induced DSBs, presumably because murine primary cells deficient in PMS2 are slightly resistant to ionizing radiation due to defective MMR of damaged nucleotides (73). Strikingly, the defective HR phenotype of the PMS2 and MLH3 mutants derived from the TK6 cell line was as prominent as that of TK6 cells deficient in MUS81, an important endonuclease involved in the resolution of HJs (36, 37) (Figures 2B, 5B, 5C, and 5D). Furthermore, *PMS2^{EK/EK}/MLH3^{DN/DN}* cells displayed a significantly stronger phenotype than did *MUS81^{-/-}* cells, including 15 times more mitotic chromosome breaks induced by γ -irradiation at the G2 phase (Figure 2B) and ~80% decrease in the number of cisplatin-induced sister chromatic exchanges (Figure 5C and 5D) in comparison with *wild-type* cells. The contribution of PMS2 and MLH3 to HR is totally independent of their functioning in MMR since MSH2 and MLH1 are required for MMR but dispensable for HR (Figure 2). In summary, human PMS2 and MLH3 significantly contribute to the genome stability of somatic cells through at least two distinct mechanisms; MMR and DSB repair by HR.

The present study shows compelling genetic evidence for the requirement of the PMS2 and MLH3 endonuclease activity for the efficient resolution of HJs. *MLH3^{DN/DN}* and *MLH3^{-/-}* cells showed the same phenotype in the defective HR (Figure 2). Likewise, the phenotype of *PMS2^{EK/EK}* cells was very similar to that of *PMS2^{-/-}* cells (Figure 2). These data indicate PMS2 and MLH3 promote HR as the endonuclease. In the *MLH3^{DN/DN}* and *PMS2^{EK/EK}* mutants, the initial kinetics of γ -ray-induced RAD51 focus formation was normal, whereas its resolution was significantly delayed (Figure 4). We, therefore, conclude that the PMS2 and MLH3 endonuclease activities promote a late step of HR, most likely after the formation of JMs. The *MUS81^{-/-}*, *MLH3^{DN/DN}*, and *PMS2^{EK/EK}* mutants all showed a ~40% decrease in the frequency of cisplatin-induced SCEs (Figure 5D). Furthermore, ectopic expression of GEN1, a typical HJ resolvase, reversed the defective heteroallelic HR of *MUS81^{-/-}*, *MLH3^{DN/DN}*, and *PMS2^{EK/EK}* cells by 30% - 50% (Figure 6). In conclusion, the endonuclease activity of PMS2 and MLH3 process HJs generating both crossover and noncrossover products.

The PMS2-MLH1 and MLH3-MLH1 heterodimers are involved in both MMR and meiotic HR in *S. cerevisiae* and mice (21, 24, 25, 66). Unexpectedly, we observed that only PMS2 and MLH3, but not MLH1, are involved in HR in human somatic cells. The PMS2 and MLH3 proteins may form homodimers and heterodimers when they are involved in HR in the same manner as the MutL homologs form heterodimer mediated by their C-terminal region (14). Indeed, homodimers of yeast Mlh1 have been reported, and an increase of their formation can inhibit MMR (74). In addition, in support of a possible heterodimer formation, a recent study in budding yeast found co-immunoprecipitation of Mlh3 with Pms1 (the equivalent of PMS2) (ref59 BioRxiv). The crystal structure of the C-terminal region of human MLH1 (pdb code 3RBN) showed that human MLH1 could form homodimers with the same residues involved in the heterodimer formation (14). The HHpred and RosettaCM analysis also suggested that the homodimer as well as heterodimer formation is possible (Figure S8). Future studies will demonstrate the homodimer and heterodimer formation as well as the interaction with an unidentified partner protein.

MLH3 and PMS2 have strong tumor suppressor activities, and it is believed that these activities are attributable exclusively to their functioning of MMR (75). The current study suggests that these endonucleases may contribute to tumor suppression also by promoting the resolution of HJs. The MUS81-EME1, SLX1-SLX4, and GEN1 endonucleases all play the critical role in genome maintenance particular when Bloom helicase is attenuated (62, 76, 77). Nonetheless, it remains unclear how much these endonucleases contribute to tumor suppression in the human. A defect in the resolution of HJs can pose a more serious threat to genome stability in comparison with the initial step of HR because the former deficiency not only leaves DSBs unrepaired but also can cleave intact sister chromatids (78). The following two mouse experiments suggest the critical role played by HJ resolvases in tumor suppression. SLX4 serves as docking sites for the MSH2, MUS81-EME1, SLX1-SLX4, and XPF-ERCC1 endonucleases (78). SLX4 plays a dominant role in preventing carcinogenesis, as evidenced by the data that the loss of SLX4 decreases a median survival time of mice to ~90 days due to enhanced tumorigenesis (79). The *MUS81* null mutation reduces the life expectancy of p53 null deficient mice by about 30% due to an increase in carcinogenesis (80). The critical role of MLH3 and PMS2 in the resolution of HJs emphasizes their strong tumor suppressor activities in addition to their function in MMR (21, 22, 81).

In this study, we characterized a major role of MLH3 and PMS2 in the DSB repair that is independent of MSH2 and MLH1. These results highlight an additional layer of the multifunctional role played by the MMR proteins (66). Studies on the molecular mechanisms of the process identified here will allow deciphering if this process is mediated by the homodimeric form of PMS2 and MLH3, complex with not yet characterized partners or a new pathway of DSB repair.

Experimental procedures

Cell clones

All the clones used in this study are summarized in Table 1.

Cell culture

Cell culture conditions for human TK6 cells was as described previously (82). Briefly, TK6 cells were grown in RPMI 1640 medium (Nacalai Tesque Inc., Kyoto, Japan) supplemented with 10% heat-inactivated horse serum (Gibco, Life technologies, New Zealand), 200 µg/ml sodium pyruvate and 100 U/ml penicillin -100 µg/ml streptomycin at 37⁰ C in 5% CO₂ atmosphere.

Generation of human PMS2^{-/-} TK6 B cells

To generate a pair of TALEN expression plasmids against the *PMS2* gene, we used a Golden Gate TALEN kit and a TAL effector kit (Addgene, US) (83, 84). The TALEN target sites are shown in Figure S1A. The gene-targeting constructs were generated from the genomic DNA of TK6 cells by amplifying with primers *Hind*III-flanked F1 and *Hind*III -flanked R1 for the 5'-arm and *Xba*I-flanked F2 and *Xba*I-flanked R2 for the 3'-arm. The 5'-arm and 3'-arm PCR products were cloned into the corresponding sites of the DT-ApA/puro or DT-ApA/hygro vectors. 10 µg TALEN-expression plasmids and 10 µg linearized gene-targeting vectors were transfected into 10×10⁶ TK6 cells using the Bio-Rad Gene Pulser II Transfection System at 250 V and 950 µF. After electroporation, cells were released into 20 ml drug-free medium containing 10% horse serum. 48 hours later, cells were seeded into 96-well plates with both hygromycin and puromycin antibiotics for two weeks. The gene disruption was confirmed by genomic PCR using primers P1, P2, P3, P4 (Figure S1B) and RT-qPCR using primers P5 and P6 (Figure S1C). All primers used in this study are shown in Table S1.

Generation of nuclease dead human PMS2^{E705K/E705K} TK6 B cells

To generate nuclease dead human *PMS2*^{E705K/E705K} TK6 B cells, we designed a guide RNA targeting intron sequence upstream of 12th exon using the Zhang CRISPR tool (85) and gene-targeting constructs. The CRISPR-target site is depicted in Figure S1C. The gene-targeting constructs were generated using SLiCE (Seamless Ligation Cloning Extract). The genomic DNA was amplified with primers F3 and R3 from the *PMS2*-gene locus and the PCR product was used as template DNA for amplifying the 5' arm. The 5'-arm was amplified using primers F4 and R4, where each primer shared 20-base pair-end homology with the insertion site of the vector. The sequence intended as the 3' arm of the *PMS2* targeting construct was amplified by PCR as two fragments using overlapping primers (F5 and R5) and that included a point mutation to change codon 705 from glutamic acid to lysine. The two fragments were then combined by chimeric PCR to yield the 3' targeting arm including the codon 705 mutation. The 3'-arm was amplified using primers F6 and R6, where each primer shared 20-base pair-end homology with the insertion site of the vector. Both vectors, DT-ApA/neo and DT-ApA/hygro, were linearized with *NotI* and *XbaI*. All the fragments of the vectors and inserts were purified using a QIAquick gel extraction kit (QIAGEN, Netherlands). The gene-targeting constructs were generated in a single reaction mixture containing DT-ApA/neo or DT-ApA/hygro vectors, 5'- and 3'-arms, and 2×SLiCE buffer (Invitrogen, US) and incubated for 30 min at room temperature. 6 µg of CRISPR and 2 µg of each gene-targeting vector were transfected into 4×10⁶ TK6 cells using the Neon Transfection System (Life Technologies, US). After electroporation, cells were released into 20 ml drug-free medium containing 10% horse serum. Forty-eight hours later, cells were seeded into 96-well plates for selection with both neomycin and hygromycin antibiotics for two weeks. The gene disruption was confirmed by RT-PCR using primers F7 and R7 followed by direct sequencing (Figures S1D and S1E). The drug resistance markers are flanked by *loxP* sites, and were thus excised from *PMS2*^{E705K/E705K} cells by transient expression of cre-recombinase, leading to the generation of *PMS2*^{E705K/E705K} cells.

Generation of human *MLH3*^{-/-} TK6 B cells

To disrupt the *MLH3* gene, we designed a guide RNA targeting the sixth exon using the Zhang CRISPR tool (85) and gene-targeting constructs. The CRISPR-target site is depicted in Figure S2A. The gene-targeting constructs were generated using SLiCE (Seamless Ligation Cloning Extract). The genomic DNA was amplified with primers F8 and R8 from the *MLH3*-gene locus and the PCR product was used as template DNA for amplifying the 5'- and 3'-arms. The 5'-arm was amplified using primers F9 and R9 and the 3'-arm was amplified using primers F10 and R10, where each primer shared 20-base pair-end homology with the insertion site of the vector. Both vectors, DT-

ApA/neo and DT-ApA/hygro, were linearized with *Afl*III and *Apa*I. All the fragments of the vectors and inserts were purified using a QIAquick gel extraction kit (QIAGEN, Netherlands). The gene-targeting constructs were generated in a single reaction mixture containing DT-ApA/neo or DT-ApA/hygro vectors, 5'- and 3'-arms, and 2×SLiCE buffer (Invitrogen, US) and incubated for 30 min at room temperature. 6 µg of CRISPR and 2 µg of each gene-targeting vector were transfected into 4×10⁶ TK6 cells using the Neon Transfection System (Life Technologies, US). After electroporation, cells were released into 20 ml drug-free medium containing 10% horse serum. Forty-eight hours later, cells were seeded into 96-well plates for selection with both neomycin and hygromycin antibiotics for two weeks. The gene disruption was confirmed by RT-PCR using primers F11 and R11 (Figure S2B), and by Southern blot analysis with a 0.6 kb probe amplified by PCR from genomic DNA using F12 and R12 (Figure S2C). The genomic DNA of the candidate clones was digested with *Eco*RI for Southern blot analysis.

Generation of human MSH2^{-/-} TK6 B cells

To disrupt the *MSH2* gene, we designed a guide RNA targeting the 4th exon using the Zhang CRISPR tool (85) and gene-targeting constructs. The CRISPR-target site is depicted in Figure S3A. The gene-targeting constructs were generated using SLiCE (Seamless Ligation Cloning Extract). The genomic DNA was amplified with primers F22 and R22 from the *MSH2*-gene locus and the PCR product was used as template DNA for amplifying the 5'- and 3'-arms. The 5'-arm was amplified using primers F23 and R23 and the 3'-arm was amplified using primers F24 and R24, where each primer shared 20-base pair-end homology with the insertion site of the vector. Both vectors, DT-ApA/neo and DT-ApA/puro, were linearized with *Afl*III and *Apa*I. All the fragments of the vectors and inserts were purified using a QIAquick gel extraction kit (QIAGEN, Netherlands). The gene-targeting constructs were generated in a single reaction mixture containing DT-ApA/neo or DT-ApA/puro vectors, 5'- and 3'-arms, and 2×SLiCE buffer (Invitrogen, US) and incubated for 30 min at room temperature. 6 µg of CRISPR and 2 µg of each gene-targeting vector were transfected into 4×10⁶ TK6 cells using the Neon Transfection System (Life Technologies, US). After electroporation, cells were released into 20 ml drug-free medium containing 10% horse serum. Forty-eight hours later, cells were seeded into 96-well plates for selection with both neomycin and puromycin antibiotics for two weeks. The gene disruption was confirmed by genomic PCR using primers F25, F26 and R25 (Figure S3).

Generation of human MLH1^{-/-} TK6 B cells

To disrupt the *MLH1* gene, we designed a guide RNA targeting the 8th exon using the Zhang CRISPR tool (85) and gene-targeting constructs. The CRISPR-target site is depicted in Figure S4A. The gene-targeting constructs were generated using SLiCE (Seamless Ligation Cloning Extract). The genomic DNA was amplified with primers F27 and R27 from the *MLH1*-gene locus and the PCR product was used as template DNA for amplifying the 5'- and 3'-arms. The 5'-arm was amplified using primers F28 and R28 and the 3'-arm was amplified using primers F29 and R29, where each primer shared 20-base pair-end homology with the insertion site of the vector. Both vectors, DT-ApA/neo and DT-ApA/puro, were linearized with *Afl*III and *Apa*I. All the fragments of the vectors and inserts were purified using a QIAquick gel extraction kit (QIAGEN, Netherlands). The gene-targeting constructs were generated in a single reaction mixture containing DT-ApA/neo or DT-ApA/puro vectors, 5'- and 3'-arms, and 2×SLiCE buffer (Invitrogen, US) and incubated for 30 min at room temperature. 6 µg of CRISPR and 2 µg of each gene-targeting vector were transfected into 4×10⁶ TK6 cells using the Neon Transfection System (Life Technologies, US). After electroporation, cells were released into 20 ml drug-free medium containing 10% horse serum. Forty-eight hours later, cells were seeded into 96-well plates for selection with both neomycin and puromycin antibiotics for two weeks. The gene disruption was confirmed by Southern blot analysis (genomic DNA was digested with *Sph*I) with a 0.52 kb probe amplified by PCR from genomic DNA using F30 and R30 (Figure S4B and S4C). The candidate clones were further confirmed by RT-PCR using primers F31 and R31 (Figure S4D) and Western blot analysis (Figure S4E).

Generation of human MUS81^{-/-} TK6 B cells

To generate a pair of TALEN expression plasmids against the *MUS81* gene, we used a Golden Gate TALEN kit and a TAL effector kit (Addgene, US) (83, 84). The TALEN target sites are shown in Figure S5A. The gene-targeting constructs were generated from the genomic DNA of TK6 cells by amplifying with primers *Sac*I-flanked F19 and *Bam*HI-flanked R19 for the 5'-arm and *Bam*HI-flanked F20 and R20 for the 3'-arm. The 3'-arm PCR products were cloned into pCR-Blunt II-TOPO vector. The 5'-arm PCR products were cloned into the *Sac*I site of the pCR-Blunt II-TOPO vector containing 3'-arm. The *Bam*HI fragment containing either the *bsr^R* or *puro^R* gene was cloned into the *Bam*HI site between the 3'-arm and the 5'-arm in the pCR-Blunt II-TOPO vector. 10 µg TALEN-expression plasmids and 10 µg linearized gene-targeting vectors were transfected into 10×10⁶ TK6 cells using the Bio-Rad Gene Pulser II Transfection System at 250 V and 950 µF. After electroporation, cells were released into 20 ml drug-free medium containing 10% horse

serum. 48 hours later, cells were seeded into 96-well plates with both blasticidin and puromycin antibiotics for two weeks. The genomic DNAs of the isolated clones resistant to both hygromycin and puromycin were digested with *DraI* for Southern blot analysis. A 0.6 kb probe was generated by PCR of genomic DNA using primers F21 and R21 (Figure S5B).

Generation of nuclease dead human $MLH3^{D1223N/D1223N}$ and $MLH3^{E1229K/E1229K}$ TK6 B cells

To generate nuclease dead human $MLH3^{EK/EK}$ and $MLH3^{DN/DN}$ TK6 B cells, we designed a guide RNA targeting intron sequence upstream of seventh exon using the Zhang CRISPR tool (85) and gene-targeting constructs. The CRISPR-target site is depicted in Figure S6. The gene-targeting constructs were generated using SLiCE (Seamless Ligation Cloning Extract). The genomic DNA was amplified with primers F13 and R13 from the *MLH3*-gene locus and the PCR product was used as template DNA for amplifying the 5' arm. The 5'-arm was amplified using primers F14 and R14, where each primer shared 20-base pair-end homology with the insertion site of the vector. The sequence intended as the 3' arm of the *MLH3* targeting construct was amplified by PCR as two fragments using overlapping primers (F15 & R15 for $MLH3^{DN/DN}$ and F16 & R16 for $MLH3^{EK/EK}$ cells) and that included a point mutation to change codon from aspartic acid to asparagine ($MLH3^{DN/DN}$) and glutamic acid to lysine ($MLH3^{EK/EK}$) subsequently. The two fragments were then combined by chimeric PCR to yield the 3' targeting arm including the mutation. The 3'-arm was amplified using primers F17 and R17, where each primer shared 20-base pair-end homology with the insertion site of the vector. Both vectors, DT-ApA/neo and DT-ApA/hygro, were linearized with *NotI* and *XbaI*. All the fragments of the vectors and inserts were purified using a qiaquick gel extraction kit (QIAGEN, Netherlands). The gene-targeting constructs were generated in a single reaction mixture containing DT-ApA/neo or DT-ApA/hygro vectors, 5'- and 3'-arms, and 2×SLiCE buffer (Invitrogen, US) and incubated for 30 min at room temperature. 6 µg of CRISPR and 2 µg of each gene-targeting vector were transfected into 4×10^6 TK6 cells using the Neon Transfection System (Life Technologies, US). After electroporation, cells were released into 20 ml drug-free medium containing 10% horse serum. Forty-eight hours later, cells were seeded into 96-well plates for selection with both neomycin and hygromycin antibiotics for two weeks. The site directed mutagenesis was confirmed by genomic PCR using primers F18 and R18 followed by direct sequencing (Figures S6C and D). The drug resistance markers are flanked by *loxP* sites, and were thus excised from $MLH3^{DN/DN}$ and $MLH3^{EK/EK}$ cells by transient expression of cre-recombinase, leading to the generation of $MLH3^{DN/DN}$ and $MLH3^{EK/EK}$ cells.

Colony-survival assay

To measure sensitivity, cells were treated with camptothecin (Topogen, Inc, US), olaparib (Funakoshi, Japan) and irradiated with ionizing radiation (^{137}Cs). Cell sensitivity to these DNA-damaging agents was evaluated by counting colony formation in methylcellulose plates as described previously (86).

Heteroallelic crossover analysis

The human lymphoblastoid cell line TSCER2 is a TK6 derivative with an I-*Sce1* site inserted into the TK locus (50, 51). TSCER2 cells are compound heterozygous ($TK^{-/-}$) for a point mutation in exons 4 and 5. DSB occurring at the I-*Sce1* site results in homologous recombination between the alleles and produces TK-proficient revertants ($TK^{+/-}$). 4×10^6 TK6 cells were transfected with 6 μg I-*Sce1* expression vector using the Neon Transfection System (Life Technologies, US) with 3X pulse at 1350 V and with 10 msec pulse width and released into 20 ml drug-free medium containing 10% horse serum. After 48 hours, cells were seeded as 1×10^6 cells per 96-well plates, with 2'-deoxycytidine (Sigma D0776), hypoxanthine (Sigma H9377), aminopterin (Sigma A3411) and thymidine (Sigma T9250) (CHAT for TK-revertants) medium. Drug resistant colonies were counted 2 weeks later.

Chromosomal aberrations analysis

TK6 cells were irradiated with 1 Gy of IR. The cells were then treated with 0.1 $\mu\text{g}/\text{ml}$ colcemid (GIBCO-BRL, Grand Island, NY) and incubated at 37°C for 3 hours. Experimental conditions for chromosomal aberration analysis were as described previously (72). Briefly, harvested cells were treated with 1 ml of 75 mM KCl for 15 min at room temperature and fixed in 5 ml of a freshly prepared 3:1 mixture of methanol/acetic acid. The cell suspension was dropped onto a glass slide and air-dried. The slides were stained with 5% Giemsa solution (NacalaiTesque) for 10 min and air-dried after being rinsed carefully with water. All chromosomes in each mitotic cell were scored at $1,000\times$ magnifications. A total of 50 mitotic cells were scored for each group using a microscope.

Sister chromatid exchange (SCE) analysis

TK6 cells were incubated with or without cisplatin (2 μM). After one hour, cells were washed and released into BrdU (100 mM) containing media. Cells were incubated for 2 more cell cycle and

treated with Colcemid (0.1 mg/ml) for 3 hours before being harvested. Metaphase chromosomes were prepared and assayed for SCEs as previously described (64).

Immunostaining and microscopic analysis

Cells were fixed with 4% paraformaldehyde (Nacalai Tesque, Japan) for 10 min at room temperature and permeabilized with 0.5% TritonX-100 (Sigma, St. Louis, US) for 30 min. Images were taken with a confocal microscope (TCS SP8, Leica Microsystems, Germany).

Antibodies

Anti- γ H2AX mouse monoclonal (1:1000, Millipore, US); anti-Rad51 rabbit polyclonal (1:500, Sigma, US); anti-MLH1: (1:1000, ab92312, abcam); anti-FLAG: (1:500, F1804, Sigma); Mouse monoclonal α - β -Tubulin (Sigma,US); alexa fluor 488-conjugated anti-mouse IgG (1:1000, Molecular Probes); alexa fluor 488-conjugated anti-rabbit IgG (1:1000, Molecular Probes); Goat monoclonal a-mouse HRP(Invitrogen, US)

Construction of Flag-tagged hGen1 with nuclear localization signal expressing TK6 cell lines

Flag-tagged hGen1-NES (4A)-NLS⁺ expressing TK6 cells were generated using a genetically modified retroviral vector as described (Figure S7) (72). Briefly, the coding sequence for hGen1-NES (4A)-3xNLS⁺-3xFlag was cloned into the pMSCV retroviral expression vector (Clontech) (Figure S7A). The newly engineered retroviral expression vector was co-transfected into human 293T cells with a helper plasmid (pClampho) expressing the viral gag, pol and env proteins to produce viral supernatant. The viral supernatant was collected after 48h and used to transduce into *wild-type*, *PMS2^{EK/EK}*, *MLH3^{DN/DN}*, *PMS2^{EK/EK}/MLH3^{DN/DN}*, *MUS81^{-/-}*, and *RAD54^{-/-}* TK6 mutant strains (Figure S7B). The efficiency of each step was assessed by quantifying the number of cells expressing GFP (Figure S7C). The expression of hGen1-NES (4A)-3xNLS⁺-3xFlag was further confirmed by western blot (WB). Experimental conditions for WB analysis were as described previously (87). The following antibodies were used: anti-Flag antibody for overnight at 4°C and anti-mouse IgG HRP-linked antibody for 1h at room temperature was used as the primary and secondary antibodies respectively (Figure S7D).

Quantification and Statistical Analysis

For all statistical analyses with a p-value, unpaired Student's t-test was used. Error bars represent standard deviation (SD), as indicated in the legends. We calculated the propagation of errors using the following formula: $\sqrt{((SD \text{ with IR treatment})^2 + (SD \text{ without IR treatment})^2)}$

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Conflict of interest

None declared.

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Table 1. Panel of cell line used in this study

| Genotype | Parental Cell line | Marker genes | References |
|---|--------------------|--|------------|
| <i>PMS2</i> ^{-/-} | TK6 | hygro ^R , puro ^R | # T |
| <i>PMS2</i> ^{EK/EK} | TK6 | - | # C |
| <i>MLH3</i> ^{-/-} | TK6 | hygro ^R , neo ^R | # C |
| <i>MLH3</i> ^{DN/DN} | TK6 | - | # C |
| <i>MLH3</i> ^{EK/EK} | TK6 | - | # C |
| <i>PMS2</i> ^{EK/EK} <i>MLH3</i> ^{DN/DN} | TK6 | - | # C |
| <i>PMS2</i> ^{EK/EK} <i>MLH3</i> ^{EK/EK} | TK6 | - | # C |
| <i>MUS81</i> ^{-/-} | TK6 | bsr ^R , puro ^R | # T |
| <i>RAD54</i> ^{-/-} | TK6 | neo ^R , puro ^R | T (47) |
| <i>MLH1</i> ^{-/-} | TK6 | hygro ^R , puro ^R | # C |
| <i>MSH2</i> ^{-/-} | TK6 | neo ^R , puro ^R | # C |

= This study; T = TALEN; C = CRISPR

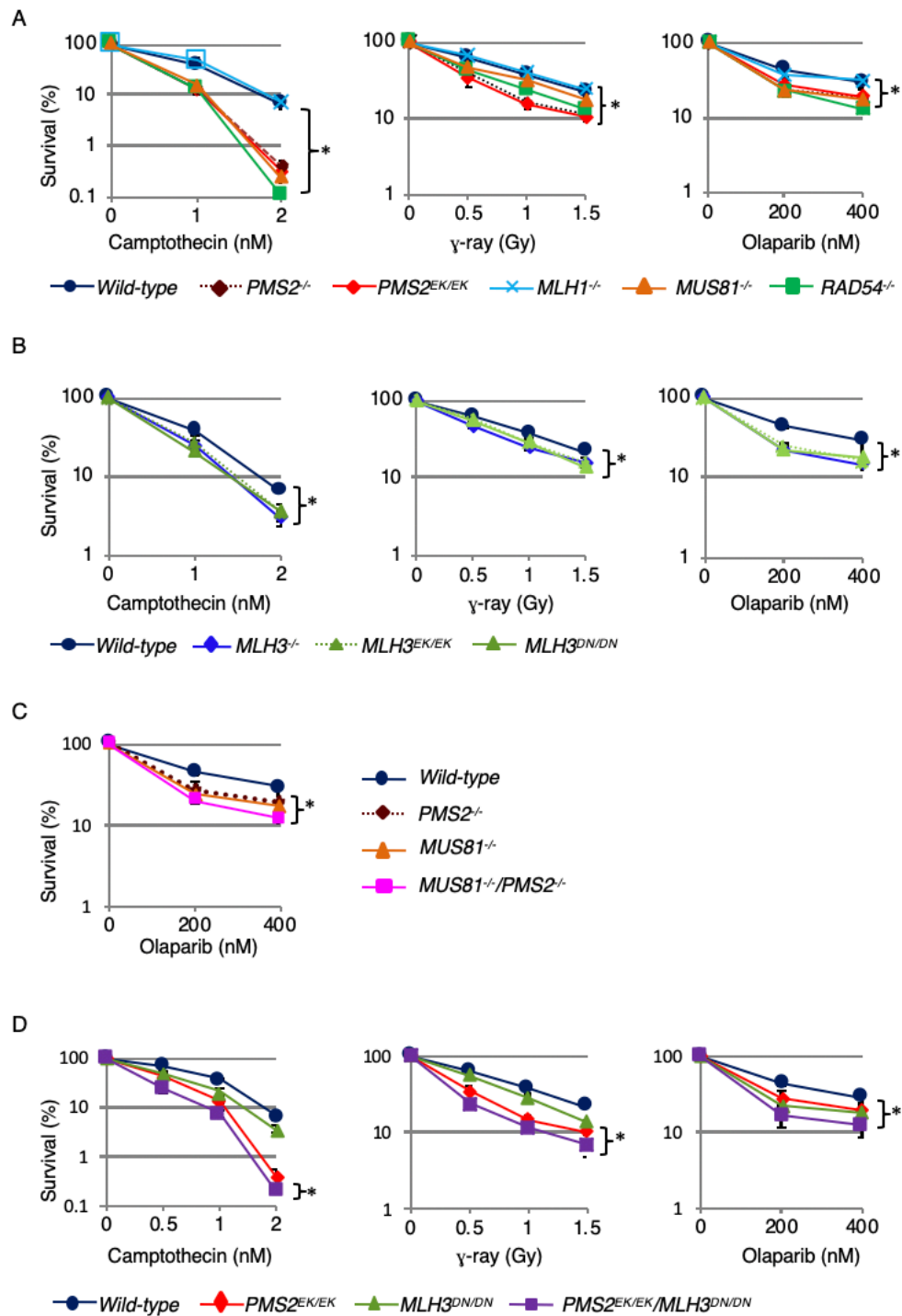


Figure 1. *MLH3* and *PMS2* mutants are sensitive to camptothecin, γ -rays and olaparib. (A) Clonogenic cell survival assay following exposure of *PMS2* mutants to Camptothecin, γ -rays and olaparib (PARP inhibitor). The *x*-axis represents the dose of the indicated DNA-damaging agent on a linear scale; the *y*-axis represents the survival fraction on a logarithmic scale. Error bars show the SD of the mean for three independent assays. Statistical analyses were performed by student's t-test (**P*<0.01). (B) Clonogenic cell survival assay following exposure of *MLH3* mutants to Camptothecin, γ -rays and olaparib (PARP inhibitor). Cellular sensitivity is shown as in figure A. Statistical analyses were performed by student's t-test (**P*<0.01). (C) Clonogenic cell survival assay following exposure of *MUS81*^{-/-}*PMS2*^{-/-} mutants to olaparib (PARP inhibitor). Cellular sensitivity is shown as in figure A. (D) *PMS2*^{EK/EK}/*MLH3*^{DN/DN} double mutant cells show stronger HR defects than *PMS2*^{EK/EK} and *MLH3*^{DN/DN} cells. Clonogenic cell survival assay following exposure of *PMS2*^{EK/EK}, *MLH3*^{DN/DN}, *PMS2*^{EK/EK}*MLH3*^{DN/DN} mutants to Camptothecin, γ -rays and olaparib (PARP inhibitor). Cellular sensitivity is shown as in figure 1A. Statistical analyses were performed by student's t-test (**P*<0.05).

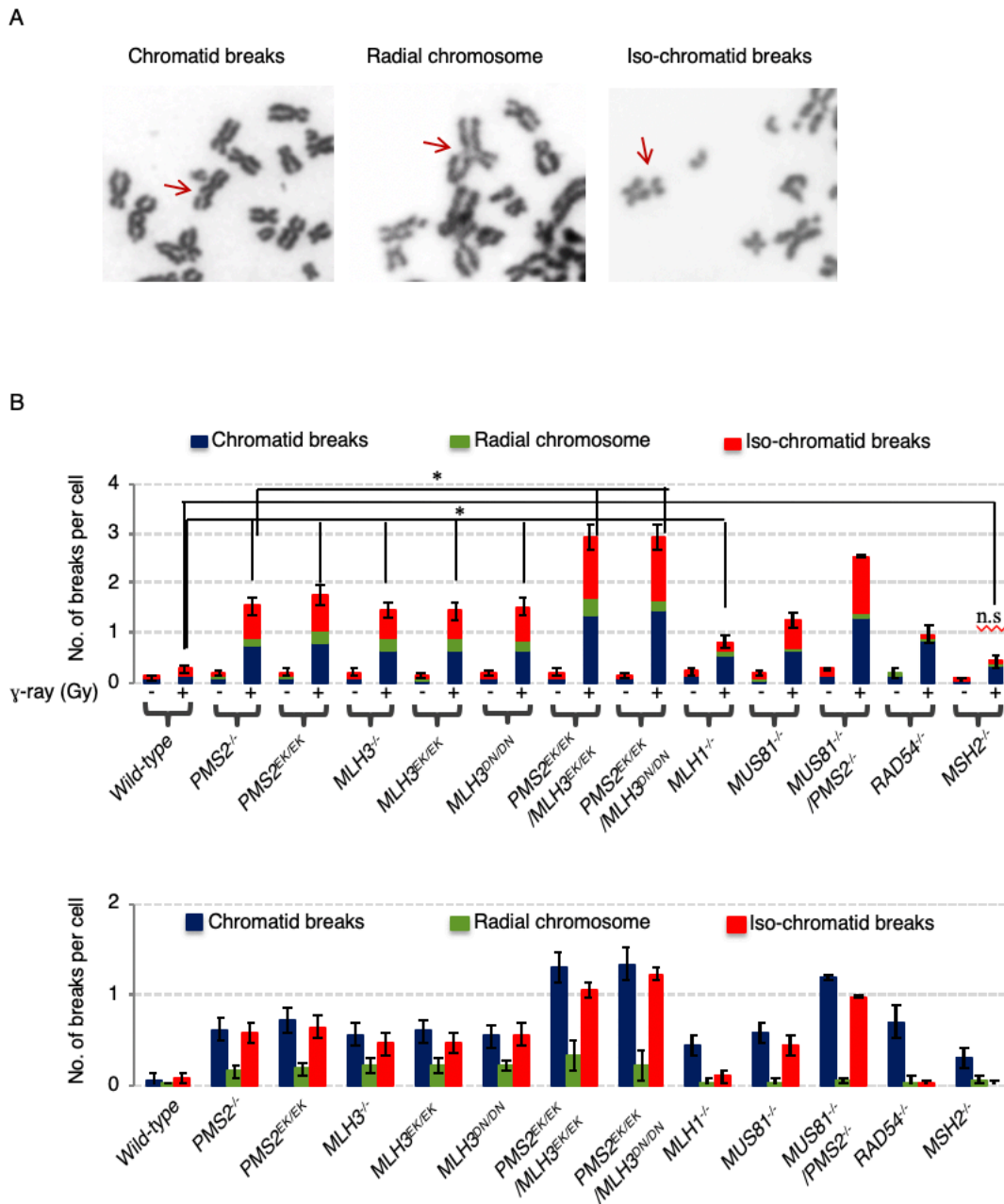


Figure 2. HR-mediated repair of γ -ray-induced DSBs is severely compromised in the PMS2 and MLH3 mutant cells. (A) Representative images of chromatid breaks, isochromatid breaks and radial chromosome after irradiation of 1 Gy IR. (B) Number of breaks per mitotic cells in the indicated genotypes (Upper panel). Error bars represent standard deviation. The asterisks indicate $P < 0.001$, calculated by Student's t test. At least 50 mitotic cells were counted for each cell line.

The subtracted numbers of breaks before the exposure from breaks after the exposure (Lower panel). Error bars represent propagation of error.

Maminur, et al., Figure 3

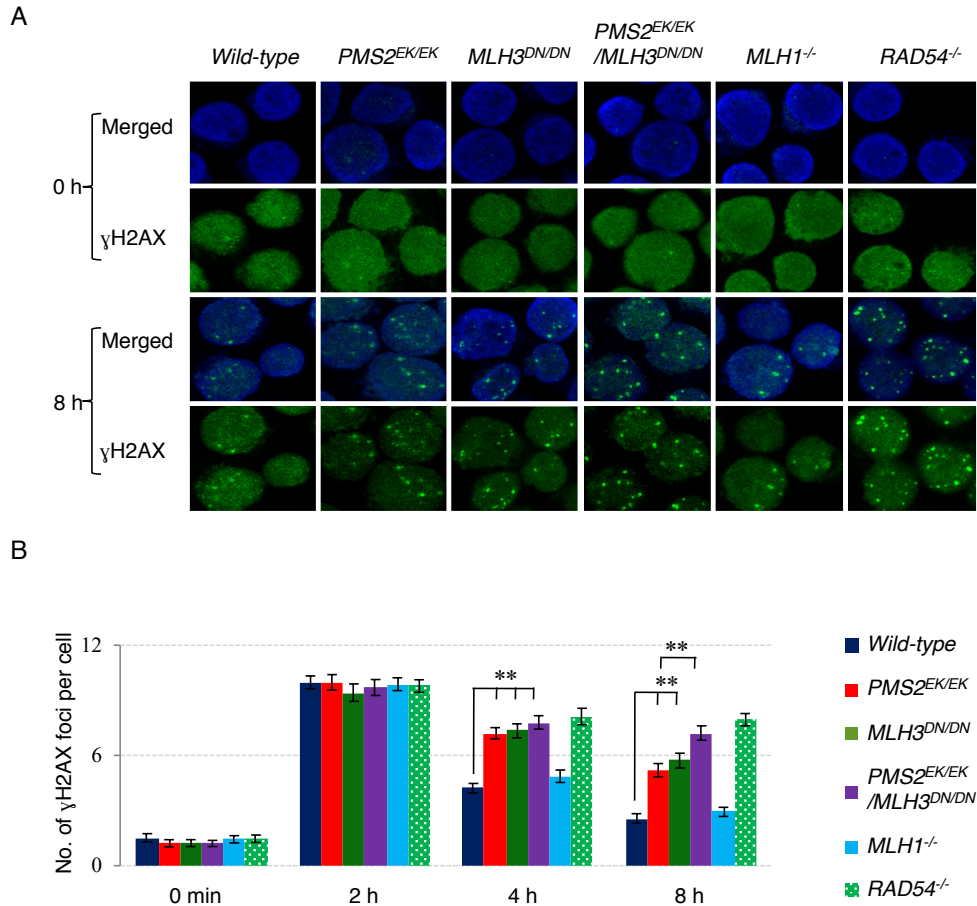
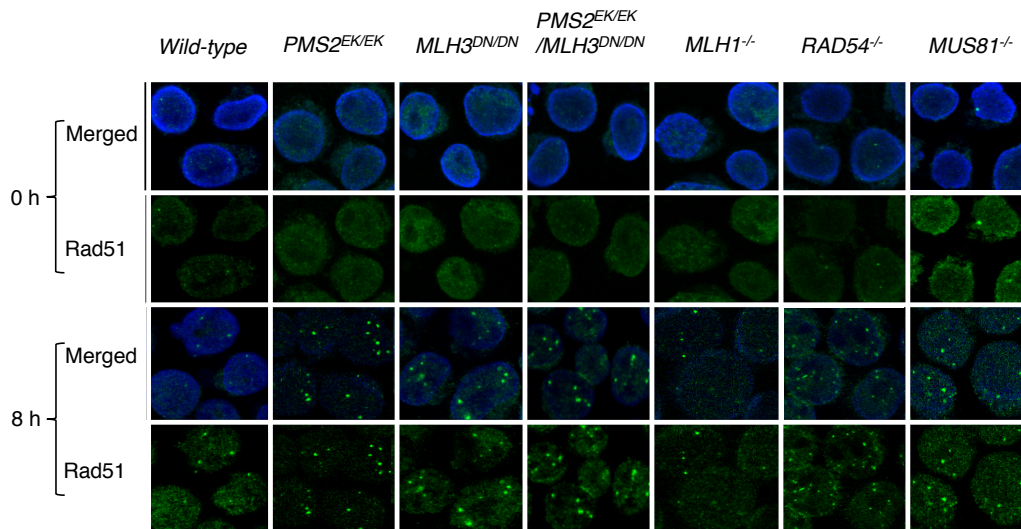


Figure. 3 Following ionizing radiation, γ H2AX foci appear with normal kinetics but persist for longer time in the *PMS2* and *MLH3* mutants in comparison with wild-type cells. (A) Representative fluorescence microscopic images of γ H2AX foci in the indicated cell lines before and 8 h after irradiation of 1 Gy IR. **(B)** Quantification of γ H2AX foci number per cell at indicated time points. At least 100 cells were counted per condition in each experiment. Statistical analyses were performed by student's t-test (** $P < 0.001$).

A



B

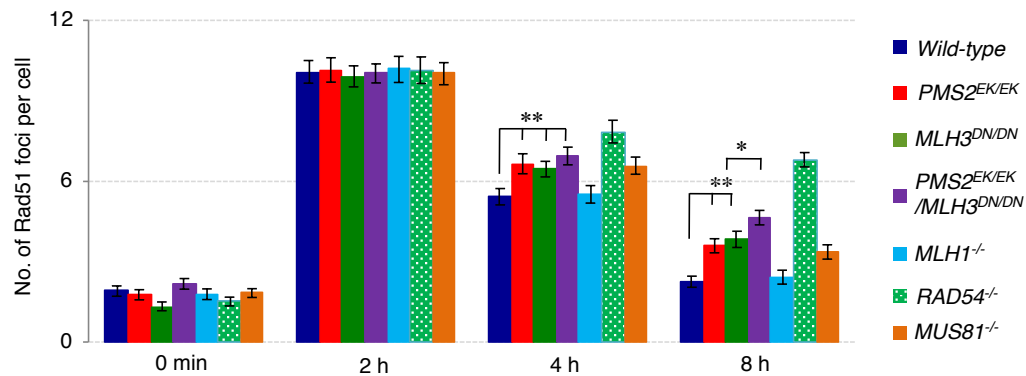
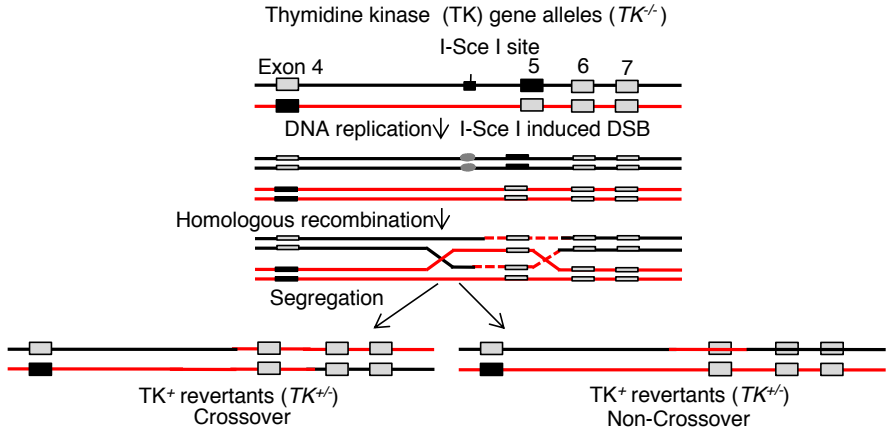
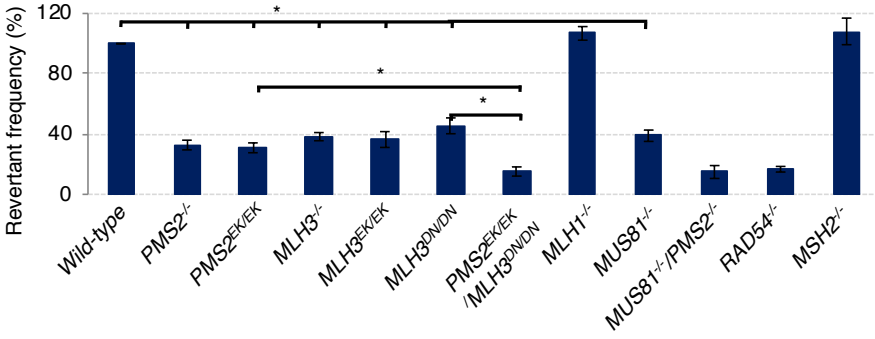


Figure. 4 Following ionizing radiation, Rad51 foci appear with normal kinetics but persist for longer time in the *PMS2* and *MLH3* mutants in comparison with wild-type cells. (A) Representative fluorescence microscopic images of Rad51 foci in the indicated cell lines before and 8 h after irradiation of 1 Gy IR. (B) Quantification of Rad51 foci number per cell at indicated time points. At least 100 cells were counted per condition in each experiment. Statistical analyses were performed by student's t-test (* $P < 0.01$ and (** $P < 0.001$).

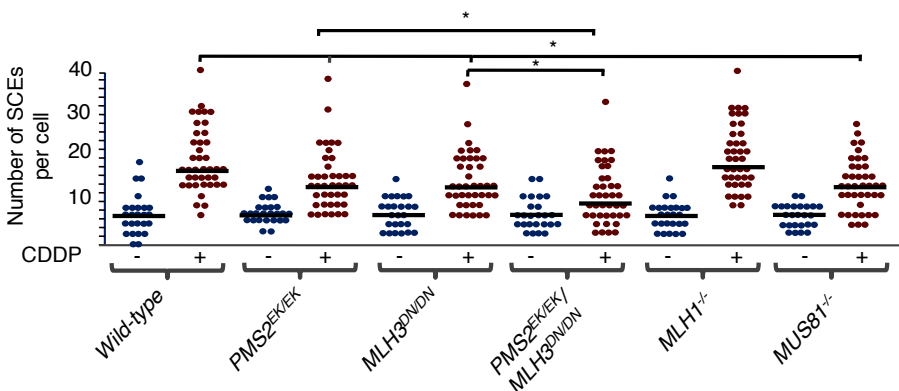
A



B



C



D

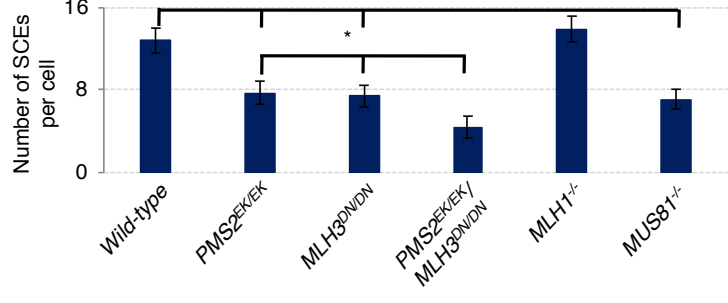


Figure 5. *MLH3*^{DN/DN} and *PMS2*^{EK/EK} cells are deficient in HR associated with crossover. (A) Schematic diagram showing DSB-repair events that repair I-*Sce1*-induced DSBs in the endogenous thymidine kinase (*TK*) locus. *TK*^{-/-} cells carry an I-*Sce1* site in intronic sequences of the *TK* allelic gene. The site of mutations in the *TK* allelic genes are marked as closed rectangles at exon 4 and exon 5. When a DSB at the I-*Sce1* site is repaired by HR, TK-proficient revertants (TK^{+/-}) are generated by crossover resolution from TSCER2 cells under CHAT selection. (B) Histogram representing the frequency of DSB-repair events (*y*-axis) in the indicated genotypes (*x*-axis). Error bars indicate SD of more than three independent experiments. Statistical analyses were performed by student's t-test (*P*<0.01). (C) Nuclease dead *PMS2* and *MLH3* mutants displayed reduced number of sister chromatid exchange (SCE) events induced by cisplatin (CDDP). The distribution of SCE events per 100 chromosomes is shown for the indicated cell types. Mean values for SCE before and after exposure to the DNA damaging agents are indicated. Statistical analyses were performed by student's t-test (*P*<0.01). (D) The subtracted numbers of SCEs before the exposure from SCEs after the exposure. Error bars indicate SEM. Statistical analyses were performed by student's t-test (*P*<0.01).

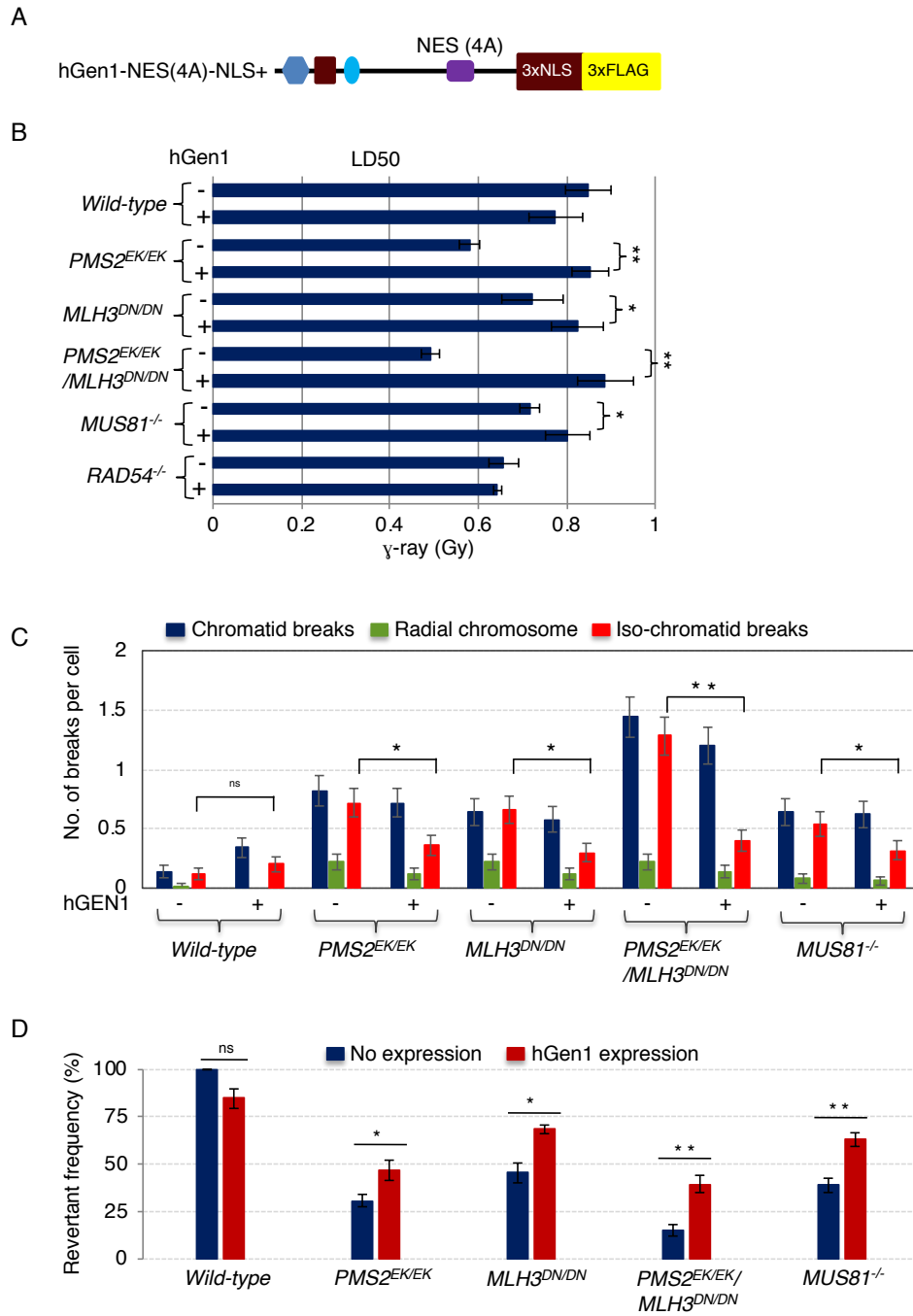


Figure 6. Significant rescue of the defective HR of the *MLH3* and *PMS2* mutants by ectopic expression of GEN1. (A) Schematic representation of hGEN1 with a mutated NES and 3xNLS sequences. (B) Clonogenic cell survival to the indicated DNA-damaging agents was analyzed as described in Figure 1A. Lethal dose 50% (LD50) is the concentration of DNA damaging agents that reduces cellular survival to 50% relative to cells non-treated with DNA damaging agents. Error bars show the standard deviation of the mean of at least three independent experiments. Statistical analyses were performed by student's t-test (* $P < 0.05$ and (** $P < 0.01$)). (C) Number of IR-induced chromosomal aberrations per mitotic cells in the indicated genotypes. Error bars represent standard deviation. At least 50 mitotic cells were counted for each cell line. Statistical analyses were performed by student's t-test (* $P < 0.05$ and (** $P < 0.01$)). (D) Frequency of heteroallelic HR, associated with crossover was measured and calculated as described in Figure 5B. Statistical analyses were performed by student's t-test (* $P < 0.05$ and (** $P < 0.01$)).