Homologous recombination (HR) repairs DNA double-strand breaks 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, but it remains elusive whether mismatch repair factors promote HR in nonmeiotic 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 endonuclease death MLH3DN/DN and PMS2EK/EK cells. MLH3−/− and MLH3DN/DN cells showed a very similar phenotype, a 2.5-fold decrease in the frequency of homologous recombination (HR) and the role of its endonuclease activity (22, 23). MLH1, MLH3, and PMS2 are essential for the progression of meiotic HR in mice (24–26). MLH1-PMS2 (MutLβ), or MLH1-MLH3 (MutLγ) (11–13). A single-strand break formed by the MLH1-PMS2 endonuclease serves as an entry point for the exonuclease activity that removes mismatched DNA. The endonuclease activity of MLH1-PMS2 depends on the metal-binding motif DQHa2ExE present on PMS2 and the last 10 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. First, 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, MutSα 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). The resulting HR intermediates has not yet been clarified in mice or humans, but recent studies have unveiled new insights into the meiotic crossovers in mice and Saccharomyces 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 mice or humans, but recent studies have unveiled new insights into the molecular mechanisms of MLH1-MLH3 and the role of its endonuclease activity (22, 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 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 (MutSα) and MSH2-MSH3 (MutSβ) (5–10). Upon recognition, the MutS heterodimers interact with one of the three MutL heterodimers MLH1-PMS2 (MutLα), MLH1-PMS1 (MutLβ), or MLH1-MLH3 (MutLγ) (11–13). A single-strand break formed by the MLH1-PMS2 endonuclease serves as an entry point for the exonuclease activity that removes mismatched DNA. The endonuclease activity of MLH1-PMS2 depends on the metal-binding motif DQHa2ExE present on PMS2 and the last 10 residues of MLH1 (14). This nuclease-active site is conserved in MLH3 but not in PMS1 (15).
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 (Mus 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 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 helps 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 a point mutation into the DQHA2XExE motif of the endogenous MLH3 and PMS2 genes of the human TK6 B cell line (49) and generated MLH3D1223/MLH3D1223 and PMS2E705K/E705K cells. These mutants exhibited increased sensitivities to camptothecin and olaparib, a few-fold decrease in the frequency of both sister chromatid exchange (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 subline for measuring heteroallelic HR, generating PMS2−/− and MLH3−/− cells (Figs. S1 and S2). We also generated MSH2−/− cells (Figs. S3, A 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 (Fig. S3C), as expected from a defect in MMR (52). PMS2−/− and MLH3−/− cells were sensitive to camptothecin, γ-irradiation, and olaparib (Fig. 1, A and B), whereas MSH2−/− cells were tolerant to these damaging agents (Figs. S3, D–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 MutSα (MSH2-MSH6) or MutSβ (MSH2-MLH3) heterodimers. We generated MLH1−/− cells (Fig. S4, A–E) and verified a defect in MMR by confirming the marked tolerance to temozolomide (53) (Fig. S4F). We found no noticeable sensitivity of MLH1−/− cells to camptothecin, γ-irradiation, and olaparib (Fig. 1A). In addition, we completely deleted the whole MLH1 locus (57 kb) from TK6 cells (Fig. S4, G–J) and confirmed that the resulting MLH1 null cells were tolerant to camptothecin (Fig. S4J). We disrupted the MUS81 gene in WT and PMS2−/−/− TK6 clones (Fig. 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 (Fig. 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 DQHA2XExE 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 S. cerevisiae (21, 22, 29, 56–58). We thus generated PMS2E705K/E705K cells (Fig. S1) and MLH3D1223/MLH3D1223 and MLH3E1229K/E1229K cells (Fig. S6). These mutants are hereafter written as PMS2E/E, MLH3D/DD, and MLH3E/E. The sensitivity profile of PMS2E/E cells was the same as that of PMS2−/− cells (Fig. 1A). Likewise, MLH3−/−, MLH3D/DD, and MLH3E/E cells showed the same phenotype (Fig. 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 3 h after ionizing radiation (59). Only cells that were γ-irradiated at the G2 phase, but not at the S phase, can enter the M phase within 3 h (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 (Fig. 1, A and B). Indeed, γ-irradiation increased the number of chromosomal breaks by 1.0 per MUS81−/− cell and only 0.2 per WT cell (Fig. 2B). Remarkably, the total numbers of chromosome aberrations induced by γ-rays were around 10 times higher in the PMS2 and MLH3 mutant cells compared with WT cells (Fig. 2B). The total number of mitotic chromosome aberrations was significantly higher in MLH1−/− cells, but not in MSH2−/− cells, compared with WT cells (Fig. 2B and Fig. S4K). We conclude that there is 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<sup>−/−</sup>, PMS2<sup>EK/EK</sup>, MLH3<sup>−/−</sup>, MLH3<sup>DN/DN</sup>, and MLH3<sup>Ek/EK</sup> cells (Fig. 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 (where one of the two sister chromatids is broken), isochromatid-type breaks (where two sister chromatids are broken at the same sites), and radial chromosomes (which comprise the association of two or more chromatids) (Fig. 2A). Ionizing irradiation of RAD54<sup>−/−</sup> cells caused a more significant increase in the number of chromatid-type breaks than that of isochromatid-type breaks (Fig. 2B). This observation agrees with the role of Rad54 in promoting strand exchange and JM formation. In contrast, MUS81<sup>−/−</sup> cells showed marked increases in the numbers of isochromatid-type

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**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, S.D. 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 A. Statistical analyses were performed by Student’s t test (*, p < 0.01). C, clonogenic cell survival assay following exposure of MUS81<sup>−/−</sup> and PMS2<sup>−/−</sup> mutants to olaparib (PARP inhibitor). Cellular sensitivity is shown as in A. The x axis represents the dose of olaparib (nM). Statistical analyses were performed by Student’s t test (*, p < 0.01). D, PMS2<sup>−/−</sup>, MLH3<sup>−/−</sup> double mutant cells show stronger HR defects than PMS2<sup>EK/EK</sup> and MLH3<sup>DN/DN</sup> cells. Shown is a clonogenic cell survival assay following exposure of PMS2<sup>EK/EK</sup>, MLH3<sup>DN/DN</sup>, and PMS2<sup>EK/EK</sup> MLH3<sup>DN/DN</sup> mutants to camptothecin, γ-rays, and olaparib (PARP inhibitor). Cellular sensitivity is shown as in A. Statistical analyses were performed by Student’s t test (*, p < 0.05).
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 MLH1 \(^{-/-}\) cells, the PMS2 and MLH3 mutants showed significant increases in the numbers of both isochromatid-type breaks and radial chromosomes (Fig. 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\(^{EKEK}\)/MLH3\(^{DN/DN}\) double mutant cells display stronger HR defects than PMS2\(^{EKEK}\) 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\(^{EKEK}\) cells. Likewise, we chose PMS2\(^{EKEK}\) cells for the subsequent analyses. To investigate the functional relationship between the PMS2 and MLH3 endonucleases, we generated PMS2\(^{EKEK}/MLH3\(^{DN/DN}\) double mutant cells. The doubling time was 12.5 h for WT, 12.7 h for PMS2\(^{EKEK}\), 12.7 h

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**Figure 2.** HR-mediated repair of \(\gamma\)-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 cell in the indicated genotypes (top). Error bars, S.D. The asterisks indicate \(p < 0.001\), calculated by Student’s \(t\) test. At least 50 mitotic cells were counted for each cell line. The number of breaks before the exposure was subtracted from breaks after the exposure (bottom). Error bars, propagation of error.
for MLH3<sup>DN/DN</sup>, and 14.3 h for PMS2<sup>EK/EK</sup>/MLH3<sup>DN/DN</sup> cells. The plating efficiency of these cells was 50–60% for all genotypes. PMS2<sup>EK/EK</sup>/MLH3<sup>DN/DN</sup> cells showed high sensitivity to camptothecin and γ-rays, higher than MLH3<sup>DN/DN</sup> and slightly higher than PMS2<sup>EK/EK</sup>, suggesting a prominent role of PMS2 in these assays (Fig. 1D). PMS2<sup>EK/EK</sup>/MLH3<sup>DN/DN</sup> cells also showed a higher sensitivity to olaparib than did PMS2<sup>EK/EK</sup> and MLH3<sup>DN/DN</sup> cells (Fig. 1D). The number of γ-ray–induced chromosomal breaks was more than 50% higher in PMS2<sup>EK/EK</sup>/MLH3<sup>DN/DN</sup> cells than in PMS2<sup>EK/EK</sup> and in MLH3<sup>DN/DN</sup> cells (Fig. 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 γH2AX foci with time after γ-irradiation (Fig. 3). The numbers of γH2AX foci were very similar among MLH3<sup>DN/DN</sup>, PMS2<sup>EK/EK</sup>, and WT cells at 2 h after ionizing irradiation. The numbers of γH2AX foci reduced more slowly in MLH3<sup>DN/DN</sup> and PMS2<sup>EK/EK</sup> cells compared with WT and MLH1<sup>−/−</sup> cells (Fig. 3). The delayed DSB repair kinetics observed more than 2 h after ionizing irradiation is consistent with the fact that HR needs a longer time to complete DSB repair than does NHEJ (20). PMS2<sup>EK/EK</sup>/MLH3<sup>DN/DN</sup> cells showed a more prominent delay in DSB repair at 8 h compared with MLH3<sup>DN/DN</sup> and PMS2<sup>EK/EK</sup> cells (Fig. 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 whether PMS2 and MLH3 act in the early and late steps of HR, we analyzed the formation of Rad51 foci over time after γ-irradiation (Fig. 4). The number of Rad51 foci peaked at 2 h after γ-irradiation in WT TK6 cells (59, 60). MLH3<sup>DN/DN</sup>, PMS2<sup>EK/EK</sup>, and PMS2<sup>EK/EK</sup>/MLH3<sup>DN/DN</sup> cells showed the same extent of Rad51 foci at 2 h as WT cells. Thus, PMS2 and MLH3 are dispensable for DSB resection and the polymerization of Rad51 on resected DSBs. Remarkably, MLH3<sup>DN/DN</sup>, PMS2<sup>EK/EK</sup>, and PMS2<sup>EK/EK</sup>/MLH3<sup>DN/DN</sup> cells showed a significant delay in the resolution of Rad51 foci compared with WT and MLH1<sup>−/−</sup> cells (Fig. 4). Both MLH3<sup>DN/DN</sup> and PMS2<sup>EK/EK</sup> single mutants showed a similar delay in the resolution of Rad51 foci compared with MLH3<sup>DN/DN</sup>, but this effect was more prominent in the PMS2<sup>EK/EK</sup>/MLH3<sup>DN/DN</sup> double mutant cells (Fig. 4B). All mutants were less sensitive than RAD54<sup>−/−</sup> cells. We therefore conclude that the PMS2 and MLH3 endonucleases promote HR-dependent DSB repair after the polymerization of Rad51 at DSBs.

**MLH3<sup>DN/DN</sup> and PMS2<sup>EK/EK</sup> 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 (<em>TK</em>) genes
carrying compound heterozygous mutations \((47, 50, 60, 63)\) (Fig. 5A). One of the two allelic \(TK\) genes carries an I-SceI site, and a mutation in the exon 5 localizes 108 nucleotides down-stream of the I-SceI site. When I-SceI–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% lower in \(MUS81^{2/2}\) cells compared with WT cells \((Fig. 5B)\), 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 \(PMS2^{EK/EK}/MLH3^{DN/DN}\) cells showed further declines in the frequency of crossover events when compared with WT cells \((Fig. 5B)\). 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 cross-linking agent. The number of cisplatin-induced SCE events was measured by subtracting the number of SCEs before cisplatin treatment from the number of SCEs post-treatment \((Fig. 5, C and D)\). The treatment increased the SCE frequency by 11 events per 100 mitotic WT cells \((Fig. 5D)\). The number of induced SCEs was 50% smaller in \(MUS81^{-/-}\) cells compared with WT 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 compared with WT cells \((Fig. 5, C and D)\). 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 hetero-dimers 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 camptotheacin, \(\gamma\)-irradiation, and olaparib \((Fig. 1A and Fig. S4J)\), (ii) \(\gamma\)-ray–induced chromosome aberrations \((Fig. 2B and Fig. S4K)\), (iii) measuring the number of \(\gamma\)H2AX and Rad51 foci over time after \(\gamma\)-irradiation \((Figs. 3 and 4)\), (iv) measuring the frequency of the heteroallelic recombination \((Fig. 5, A and B)\), and (v) SCE induced by cisplatin \((Fig. 5, C and D)\). Unexpectedly, as mentioned above, all of these phenotypic assays consistently showed that \(MLH1^{-/-}\) cells were proficient in HR-mediated

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**Figure 4.** Following ionizing radiation, Rad51 foci appear with normal kinetics but persist for a longer time in the \(PMS2\) and \(MLH3\) mutants compared with WT cells. A, representative fluorescence microscopic images of Rad51 foci in the indicated cell lines before and 8 h after irradiation of 1-Gy IR. Green specks, Rad51 signal; blue, nuclei. DAPI, 4',6-diamidino-2-phenylindole. B, quantification of the number of Rad51 foci/cell at the indicated time points. At least 100 cells were counted per condition in each experiment. Statistical analyses were performed by Student’s t test \((*) p < 0.01; ** p < 0.001)\).
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).

Figure 5. MLH3<sub>DN/DN</sub> and PMS2<sub>EK/EK</sub> cells are deficient in HR associated with crossover. A, schematic diagram showing DSB repair events that repair I-SceI-induced DSBs in the endogenous TK locus. TK<sup>−/−</sup> cells carry an I-SceI site in intronic sequences of the TK allelic gene. The sites of mutations in the TK allelic genes are marked as closed rectangles at exon 4 and exon 5. When a DSB at the I-SceI site is repaired by HR, TK-proficient revertants (TK<sup>+/−</sup>) 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, S.D. 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 a reduced number of 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 number of SCEs before the exposure was subtracted from SCEs after the exposure. Error bars, S.E. Statistical analyses were performed by Student’s t-test (p < 0.01).
We speculated that the MLH1-independent function of PMS2 and MLH3 in mitotic HR can be achieved through (i) homodimer formation, (ii) formation of a heterodimer with an unknown partner protein, which would stabilize the PMS2 and MLH3 proteins, and (iii) formation of an 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 (Fig. S8, A–C).

**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) (Fig. 6A). We added the FLAG tag to this GEN1 transgene and inserted it into the pMSCV retroviral expression vector, which allows for bicistronic expression of the GFP and GEN1 transgenes (72) (Fig. S7). We produced recombinant retrovirus and infected them into TK6 clones. To confirm the expression of the transgene, we performed Western blotting analyses using an anti-FLAG antibody (Fig. 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 nonirradiated cells (Fig. 6B). The expression of the GEN1 transgene reversed the ionizing radiation sensitivity of MLH3^−/−^ cells, but not WT or RAD54^−/−^ cells. Thus, the GEN1 transgene is able to selectively normalize the defective processing of IMs 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 (Fig. 6B). The rescue effect of GEN1 transgene was more efficient in PMS2^EK/EK^/MLH3^DN/DN^ double mutant cells compared with 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 MLH3^−/−^ cells (Fig. 6C). Importantly, the GEN1 transgene expression reduced the number of isochromatid-type breaks to a considerably greater extent than that of chromatid breaks (Fig. 6C). The GEN1 transgene increased the frequency of heteroallelic HR in MLH3^−/−^ cells by 60% but had no effect on that in WT or RAD54^−/−^ cells (Fig. 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^, and MLH3^−/−^ cells but not WT or RAD54^−/−^ cells (Fig. 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) (Figs. 2B and 5B, C, and D). Furthermore, PMS2^EK/EK^/MLH3^DN/DN^ cells displayed a significantly stronger phenotype than did MLH3^−/−^ cells, including 15 times more mitotic chromosome breaks induced by γ-irradiation at the G2 phase (Fig. 2B) and an ~80% decrease in the number of cisplatin-induced sister chromatid exchanges (Fig. 5, C and D) compared with WT cells. The contribution of PMS2 and MLH3 to HR is totally independent of their functioning in MMR because MSH2 and MLH1 are required for MMR but dispensable for HR (Fig. 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 (Fig. 2). Likewise, the phenotype of PMS2^EK/EK^ cells was very similar to that of PMS2^−/−^ cells (Fig. 2). These data indicate that 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 (Fig. 4). We therefore conclude that the PMS2 and MLH3 endonuclease activities promote a late step of HR, most likely after the formation of IMs. The MLH3^−/−^, MLH3^DN/DN^, and PMS2^EK/EK^ mutants all showed a ~40% decrease in the frequency of cisplatin-induced SCEs (Fig. 5D).

Furthermore, ectopic expression of GEN1, a typical HJ resolvase, reversed the defective heteroallelic HR of MLH3^−/−^, MLH3^DN/DN^, and PMS2^EK/EK^ cells by 30–50% (Fig. 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, and 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 (75). The crystal structure of the C-terminal region of human MLH1 (Protein Data Bank code 3RBH) 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 both the homodimer and heterodimer formation are possible (Fig. 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 function of MMR (76). 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 a critical role in genome maintenance, particularly when Bloom helicase is attenuated (62, 77, 78). Nonetheless, it remains unclear how much these endonucleases contribute to tumor suppression in humans. A defect in the resolution of HJs can pose a more serious threat to genome stability compared with the initial step of HR because the former deficiency not only leaves DSBs unrepai red but also can cleave intact sister chromatids (79). The following two mouse experiments suggest the critical role played...
Role of PMS2 and MLH3 in homologous recombination

Table 1
Panel of cell lines used in this study

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Parental cell line</th>
<th>Marker genes</th>
<th>Sources</th>
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<tr>
<td>PMS2</td>
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by HJ resolvases in tumor suppression. SLX4 serves as a docking site for MUS81-EME1, SLX1-SLX4, and XPF-ERCC1 endonucleases and MSH2-MSH3 mismatch repair factor (79). SLX4 plays a dominant role in preventing carcinogenesis, as evidenced by the data indicating that the loss of SLX4 decreases the median survival time of mice to ~90 days due to enhanced tumorigenesis (80). The MUS81 null mutation reduces the life expectancy of p53 null mice by about 30% due to an increase in carcinogenesis (81). 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, 82).

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 determination of whether this process is mediated by the homodimeric form of PMS2 and MLH3, a complex with not yet characterized partners, or a new pathway of DSB repair.

Experimental procedures

Cell clones

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

Cell culture

Cell culture conditions for human TK6 cells were as described previously (83). 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, Ltd., Auckland, New Zealand), 200 μg/ml sodium pyruvate, and 100 units/ml penicillin plus 100 μg/ml streptomycin at 37 °C in 5% CO₂ atmosphere.

Generation of human PMS2<sup>-/-</sup> 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) (84, 85). The TALEN target sites are shown in Fig. S1A. The gene-targeting constructs were generated from the genomic DNA of TK6 cells by amplifying with primers HindIII-flanked F1 and HindIII-flanked R1 for the 5’-arm and XbaI-flanked F2 and XbaI-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 of TALEN expression plasmids and 10 μg of 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 microfarads. After electroporation, cells were released into 20 ml of drug-free medium containing 10% horse serum. 48 h later, cells were seeded into 96-well plates with both hygromycin and puromycin antibiotics for 2 weeks. The gene disruption was confirmed by genomic PCR using primers P1, P2, P3, of P4 (Fig. S1B) and RT-quantitative PCR using primers P5 and P6 (Fig. S1C). All primers used in this study are shown in Table S1.

Generation of nuclease-dead human PMS2<sup>E705K/E705K</sup> TK6 B cells

To generate nuclease-dead human PMS2<sup>E705K/E705K</sup> TK6 B cells, we designed a guide RNA targeting intron sequence upstream of the 12th exon using the Zhang CRISPR tool (86) and gene-targeting constructs. The CRISPR target site is depicted in Fig. 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-bp 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 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-bp 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 of the fragments of the vectors and inserts were purified using a QIAquick gel extraction kit (Qiagen, Venlo, 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 2X SLiCE buffer (Invitrogen) 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, Inc.). After electroporation, cells were released into 20 ml of drug-free medium containing 10% horse serum. 48 h later, cells were seeded into 96-well plates for selection with both neomycin and hygromycin antibiotics for 2 weeks. The gene disruption was confirmed by RT-PCR using primers F7 and R7 followed by direct sequencing (Fig. S1, D and E). The drug resistance markers are flanked by loxP sites and were thus excised from PMS2<sup>E705K/E705K</sup> TK6 cells by transient expression of Cre recombinase, leading to the generation of PMS2<sup>E705K/E705K</sup> TK6 cells.
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targeting constructs. The CRISPR target site is depicted in Fig. S2A. The gene-targeting constructs were generated using SLiCE. 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-bp end homology with the insertion site of the vector. Both vectors, DT-ApA/neo and DT-ApA/hygro, were linearized with AflII and ApaI. All of the fragments of the vectors and inserts were purified using a QIAquick gel extraction kit (Qiagen). 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) 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). After electroporation, cells were released into 20 ml of drug-free medium containing 10% horse serum. 48 h later, cells were seeded into 96-well plates for selection with both neomycin and hygromycin antibiotics for 2 weeks. The gene disruption was confirmed by RT-PCR using primers F11 and R11 (Fig. S2B) and by Southern blotting analysis with a 0.6-kb probe amplified by PCR from genomic DNA using F12 and R12 (Fig. S2C). The genomic DNA of the candidate clones was digested with EcoRI for Southern blotting analysis.

**Generation of human MSH2^+/− TK6 B cells**

To disrupt the MSH2 gene, we designed a guide RNA targeting the fourth exon using the Zhang CRISPR tool (86) and gene-targeting constructs. The CRISPR target site is depicted in Fig. S3A. The gene-targeting constructs were generated using SLiCE. 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-bp end homology with the insertion site of the vector. Both vectors, DT-ApA/neo and DT-ApA/puro, were linearized with AflII and ApaI. All of the fragments of the vectors and inserts were purified using a QIAquick gel extraction kit (Qiagen). 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) 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). After electroporation, cells were released into 20 ml of drug-free medium containing 10% horse serum. 48 h later, cells were seeded into 96-well plates for selection with both neomycin and puromycin antibiotics for 2 weeks. The gene disruption was confirmed by Southern blotting analysis (genomic DNA was digested with SphI) with a 0.52-kb probe amplified by PCR from genomic DNA using F30 and R30 (Fig. S4, B and C). The candidate clones were further confirmed by RT-PCR using primers F31 and R31 (Fig. S4D) and Western blotting analysis (Fig. S4E).

**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 (86) and gene-targeting constructs. The CRISPR target site is depicted in Fig. S4A. The gene-targeting constructs were generated using SLiCE. 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-bp end homology with the insertion site of the vector. Both vectors, DT-ApA/neo and DT-ApA/puro, were linearized with AflII and ApaI. All of the fragments of the vectors and inserts were purified using a QIAquick gel extraction kit (Qiagen). 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) 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). After electroporation, cells were released into 20 ml of drug-free medium containing 10% horse serum. 48 h later, cells were seeded into 96-well plates for selection with both neomycin and puromycin antibiotics for 2 weeks. The gene disruption was confirmed by Southern blotting analysis (genomic DNA was digested with SphI) with a 0.52-kb probe amplified by PCR from genomic DNA using F30 and R30 (Fig. S4, B and C). The candidate clones were further confirmed by RT-PCR using primers F31 and R31 (Fig. S4D) and Western blotting analysis (Fig. 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) (84, 85). The TALEN target sites are shown in Fig. S5A. The gene-targeting constructs were generated from the genomic DNA of TK6 cells by amplifying with primers SacI-flanked F19 and BamHI-flanked R19 for the 5′-arm and the 3′-arm PCR products were cloned into the SacI site of the pCR-Blunt II-TOPO vector. The 5′-arm PCR products were cloned into the SacI site of the pCR-Blunt II-TOPO vector containing the 3′-arm. The BamHI fragment containing either the bsR or puroR gene was cloned into the BamHI site between the 3′-arm and the 5′-arm in the pCR-Blunt II-TOPO vector. 10 μg of TALEN expression plasmids and 10 μg of linearized gene-targeting vectors were transfected into 10 × 10^6 TK6 cells using the Bio-Rad Gene Pulser II Transfection System at 250 V and 950 microfarads. After electroporation, cells were released into 20 ml of drug-free medium containing 10% horse serum. 48 h later, cells were seeded into 96-well plates with both blasticidin and puromycin antibiotics for 2 weeks. The genomic DNAs of the isolated clones resistant to both hygromycin and puromycin were digested with Dral for Southern blotting analysis. A 0.6-kb probe was generated by PCR of genomic DNA using primers F21 and R21 (Fig. S5B).
Generation of nuclease-dead human MLH3<sub>D1223N/D1223N</sub> and MLH3<sub>E1229K/E1229K</sub>-expressing TK6 B cells

To generate nuclease-dead human MLH3<sup>EK/EK</sup> and MLH3<sup>DN/DN</sup>-expressing TK6 B cells, we designed a guide RNA targeting intron sequence upstream of seventh exon using the Zhang CRISPR tool (86) and gene-targeting constructs. The CRISPR target site is depicted in Fig. S6. The gene-targeting constructs were generated using SLiCE. 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-bp 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 and R15 for MLH3<sup>DN/DN</sup> and F16 and R16 for MLH3<sup>EK/EK</sup>) cells that included a point mutation to change codon from aspartic acid to asparagine (MLH3<sup>DN/DN</sup>) and glutamic acid to lysine (MLH3<sup>EK/EK</sup>) 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-bp 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 of the fragments of the vectors and inserts were purified using a QIAquick gel extraction kit (Qiagen). 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) 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<sup>6</sup> TK6 cells using the Neon Transfection System (Life Technologies). After electroporation, cells were released into 20 ml of drug-free medium containing 10% horse serum. 48 h later, cells were seeded into 96-well plates for selection with both neomycin and hygromycin antibiotics for 2 weeks. The site-directed mutagenesis was confirmed by genomic PCR using primers F18 and R18 followed by direct sequencing (Figs. S6, C and D). The drug resistance markers are flanked by loxp sites and were thus excised from MLH3<sup>DN/DN</sup> and MLH3<sup>EK/EK</sup> cells by transient expression of Cre recombinase, leading to the generation of MLH3<sup>DN/DN</sup> and MLH3<sup>EK/EK</sup> cells.

Colony survival assay

To measure sensitivity, cells were treated with 0.1 μg/ml colcemid (GIBCO-BRL) and incubated at 37 °C for 3 h. 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 (Nacalai Tesque) for 10 min and air-dried after being rinsed carefully with water. All chromosomes in each mitotic cell were scored at ×1000 magnification. A total of 50 mitotic cells were scored for each group using a microscope.

SCE analysis

TK6 cells were incubated with or without cisplatin (2 μM). After 1 h, cells were washed and released into bromodeoxyuridine (100 mM)-containing media. Cells were incubated for two more cell cycles and treated with colcemid (0.1 mg/ml) for 3 h before being harvested. Metaphase chromosomes were prepared and assayed for SCEs as described previously (64).

Immunostaining and microscopic analysis

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

Antibodies

The following antibodies were used: anti-γH2AX mouse monoclonal (1:1000; Millipore); anti-Rad51 rabbit polyclonal (1:500; Sigma); anti-MLH1 (1:1000; ab92312, Abcam); anti-FLAG (1:500; F1804, Sigma); mouse monoclonal α-β-tubulin (Sigma); Alexa Fluor 488-conjugated anti-mouse IgG (1:1000; Molecular Probes); Alexa Fluor 488-conjugated anti-rabbit IgG (1:1000; Molecular Probes); and goat monoclonal α-mouse horseradish peroxidase (Invitrogen).

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

FLAG-tagged hGen1-NES (4A)-NLS<sup>+</sup>-expressing TK6 cells were generated using a genetically modified retroviral vector as

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The human lymphoblastoid cell line TSCER2 is a TK6 derivative with an I-SceI site inserted into the TK locus (50, 51). TSCER2 cells are compound heterozygous (TK<sup>−/−</sup>) for a point mutation in exons 4 and 5. A DSB occurring at the I-SceI site results in homologous recombination between the alleles and produces TK-proficient revertants (TK<sup>+/−</sup>). 4 × 10<sup>6</sup> TK6 cells were transfected with 6 μg of I-SceI expression vector using the Neon Transfection System (Life Technologies) with 3× pulse at 1350 V and with 10-ms pulse width and released into 20 ml of drug-free medium containing 10% horse serum. After 48 h, cells were seeded as 1 × 10<sup>5</sup> cells/96-well plate, with 2′-deoxyuridine (Sigma, D0776), hypoxanthine (Sigma, H9377), amniopterin (Sigma, A3411), and thymidine (Sigma, T9250) (CHAT for TK-revertants) medium. Drug-resistant colonies were counted 2 weeks later.

Chromosomal aberration analysis

TK6 cells were irradiated with 1-Gy IR. The cells were then treated with 0.1 μg/ml colcemid (GIBCO-BRL) and incubated at 37 °C for 3 h. 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 (Nacalai Tesque) for 10 min and air-dried after being rinsed carefully with water. All chromosomes in each mitotic cell were scored at ×1000 magnification. A total of 50 mitotic cells were scored for each group using a microscope.

Heterallelic crossover analysis

The human lymphoblastoid cell line TSCER2 is a TK6 derivative with an I-SceI site inserted into the TK locus (50, 51). TSCER2 cells are compound heterozygous (TK<sup>−/−</sup>) for a point mutation in exons 4 and 5. A DSB occurring at the I-SceI site

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described (Fig. S7) (72). Briefly, the coding sequence for hGen1-NES (4A)-3xNLS+3xFLAG was cloned into the pMSCV retroviral expression vector (Clontech) (Fig. 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 48 h and used to transduce into WT, PMS2\(^ {2E/K}\), MLH3\(^ {DN/DN}\), PMS2\(^ {2E/K}\)/MLH3\(^ {DN/DN}\), MUS81\(^ {-/-}\), and RAD54\(^ {-/-}\) TK6 mutant strains (Fig. S7B). The efficiency of each step was assessed by quantifying the number of cells expressing GFP (Fig. S7C). The expression of hGen1-NES (4A)-3xNLS+3xFLAG was further confirmed by Western blotting. Experimental conditions for Western blotting analysis were as described previously (88). Anti-FLAG antibody overnight at 4 °C and anti-mouse IgG horseradish peroxidase–linked antibody for 1 h at room temperature were used as the primary and secondary antibodies, respectively (Fig. S7D).

Quantification and statistical analysis

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

Data availability

All of the data described are contained within the article.

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: MMR, mismatch repair; DSB, double-strand break; NHEJ, nonhomologous end-joining; HR, homologous recombination; JM, joint molecule; HJ, Holliday junction; dHJ, double Holliday junction; SCE, sister chromatid exchange; NES, nuclear export signal; NLS, nuclear localization signal; SLICE, seamless ligation cloning extract; TK, thymidine kinase; Gy, gray; PARP, poly(ADP-ribose) polymerase.

References


Role of PMS2 and MLH3 in homologous recombination


Genetic evidence for the involvement of mismatch repair proteins, PMS2 and MLH3, in a late step of homologous recombination
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