

Gen1 and *Eme1* Play Redundant Roles in DNA Repair and Meiotic Recombination in Mice

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Resolution of the Holliday junction (HJ) is essential for homologous recombination and DNA repair. In *Saccharomyces cerevisiae*, HJ resolvase Yen1 and the Mus81-Mms4 complex are redundant in DNA damage repair. In cultured mammalian cells, such redundancy also exists between Yen1 ortholog GEN1 and the Mus81-Mms1 ortholog MUS81-EME1. In this report, we further tested if GEN1 and EME1 redundantly affect HJ-related physiological processes in mice. We found that combined homozygous mutations of *Gen1* and *Eme1* led to synthetic lethality during early embryonic stages. Homozygous *Gen1* mutations did not cause DNA repair deficiency in mouse embryonic fibroblast (MEF) cells, but made heterozygous *Eme1* mutant MEFs more sensitive to various DNA-damaging reagents. *Gen1* mutations also reduced the meiotic recombination efficiency in *Eme1* mutant mice. These results suggest that *Gen1* and *Eme1* play redundant roles in DNA repair and meiotic recombination *in vivo*.

Keywords: *Gen1*, *Eme1*, DNA repair, meiotic recombination

Introduction

A HOLLIDAY JUNCTION (HJ) is a branched intermediate structure that usually exists in meiosis and DNA recombination repair (Matos and West, 2014). HJs connect two double-stranded DNA molecules together so that they must be resolved to allow the segregation of both molecules (West, 2009). Defective HJ resolution may lead to sterility or increased susceptibility to cancer (Holloway *et al.*, 2008; Matos *et al.*, 2011). Several evolutionary conserved pathways are involved in HJ resolution in eukaryotic organisms.

In *Saccharomyces cerevisiae*, the Yen1 nuclease can symmetrically cut HJ in a manner analogous to the *Escherichia coli* HJ resolvase RuvC (Ip *et al.*, 2008; Rass *et al.*, 2010), whereas the XPF-family heterodimeric endonuclease complex Mus81-Mms4 can asymmetrically cleave HJ (Boddy *et al.*, 2001; Chen *et al.*, 2001). Slx1, another structure-selective endonuclease, is also capable of processing HJs (Fricke and Brill, 2003; Fekairi *et al.*, 2009; Munoz *et al.*, 2009). In addition to these structure-specific nucleases, HJs can be dissolved by the Sgs1-Top3-Rmi1 (STR) complex to produce noncrossover recombinants (Ellis *et al.*, 1995; Wu and Hickson, 2003; Cejka *et al.*, 2010).

In human cells, the Yen1 ortholog GEN1, Mus81-Mms4 orthologs MUS81-EME1, and the Slx1 ortholog SLX1 can process HJs in similar ways as they do in yeast cells (Ip *et al.*, 2008; Matos and West, 2014). It has been shown that GEN1 forms a dimer that juxtaposes two products in a substrate-like complex, and the chromodomain of GEN1 is indispensable for its DNA recognition and cleavage activities (Lee *et al.*, 2015; Liu *et al.*, 2015). Both MUS81 and SLX1 can be activated by interacting with SLX4, a scaffold protein that serves as an essential docking platform to cooperate with multiple structure-specific endonucleases (Fekairi *et al.*, 2009; Munoz *et al.*, 2009; Svendsen *et al.*, 2009). HJs could also be dissolved by the Bloom's syndrome complex (BLM helicase-topoisomerase III α -RMI1/2) to produce noncrossover recombinants (Ellis *et al.*, 1995; Wu and Hickson, 2003; Cejka *et al.*, 2010).

Yen1 and the Mus81-Mms4 complex play redundant roles in HJ resolution in yeasts. Deletion of *yen1* in *S. cerevisiae* does not affect DNA repair, whereas disruption of both *yen1* and *mus81* caused hypersensitivity to various DNA-damaging reagents (Blanco *et al.*, 2010; Ho *et al.*, 2010; Tay and Wu, 2010). During meiosis, deletion of both *yen1* and *mms4* also caused more severe decrease in chromosome crossover than

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the single mutations did (Zakharyevich *et al.*, 2012). In addition, ectopic expression of human *GEN1* could overcome HJ defects caused by *mus81* deletion in fission yeasts, resulting in the formation of chromosome crossover (Lorenz *et al.*, 2010). Recent studies in mitotic human cells have shown sequential activation of *GEN1* and *MUS81-EME1* to eliminate persistently jointed DNA molecules (Matos *et al.*, 2011; Matos and West, 2014). Depletion of both *GEN1* and *MUS81* in cultured cells from Bloom's syndrome patients resulted in elongated and segmented chromosomes, as well as high levels of cell mortality (Wechsler *et al.*, 2011; Wyatt *et al.*, 2013). These results indicate that the functional redundancy between *GEN1* and *MUS81-EME1* also exists in mammalian cells. However, little evidence has been provided for the functional redundancy between *GEN1* and *EME1*, making this conclusion remain to be confirmed in mammals.

We have generated *Gen1* and *Eme1* mutations in mice by insertional mutagenesis with the *piggyBac* (PB) transposon. We report synthetic lethality, reduced DNA repair, and decreased meiotic recombination efficiency in mice and cells carrying both mutations. These results suggest a redundant role between *Gen1* and *Eme1* in DNA recombination in mice.

Materials and Methods

Mice

All animal experiments were performed in accordance with protocols approved by the Animal Care and Use Committee of the Institute of Developmental Biology and Molecular Medicine (IDM), Fudan University. Both *Gen1* (081125049-HLA) and *Eme1* (081028120-HLA) mutants were generated by inserting a PB transposon in target genes during the process of a large-scale insertional mutagenesis project on the FVB/NJ background (Ding *et al.*, 2005; Sun *et al.*, 2008). In the *Gen1*^{PB} allele, the PB insertion was mapped in the second intron (Chr:12.11268138, Ensembl release 54). In the *Eme1*^{PB} allele, the PB inserts into the third intron (Chr: 11.94510958, Ensembl release 54).

Cell culture and DNA damage assay

Mouse embryonic fibroblasts (MEFs) were isolated from E14.5 embryos and cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin. The assay for sensitivity to DNA damage was performed as previously described (Lei *et al.*, 2012). In brief, MEFs of indicated genotype were plated in three wells of a six-well plate at a density of 5×10^4 per well for 12 h, then treated with CPT (264933; Jingke Chem), Etoposide (E1383; Sigma), MMC (M4287; Sigma), MMS (129925; Sigma), or HU (H8627; Sigma) at indicated doses for 24 h. Surviving cells in each well were counted by FACS after 1-week incubation. Cell survival rate was calculated by dividing survival cell number in each well by mean survival cell number in control group without drug treatment. Statistical analysis was performed by unpaired *t*-test.

Polymerase chain reaction

Genotyping polymerase chain reaction (PCR) was performed with a PB-specific primer (LB2) and two flanking

genomic primers (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/dna). PCR and reverse transcription polymerase chain reaction (RT-PCR) primers are listed in Supplementary Table S1.

Measurement of meiotic recombination efficiency

Male *Gen1* mutant mice with mixed FVB/NJ and C57BL/6J background were bred in two steps: *Gen1*^{PB/+} (FVB/NJ) mice were mated with C57BL/6J wild-type mice to generate *Gen1*^{PB/+} (FVB/C57); *Gen1*^{PB/+} (FVB/C57) mice were then mated with *Gen1*^{PB/+} (FVB/NJ) mice to generate wild-type and *Gen1*^{PB/PB} mice with mixed background. Combined *Gen1* and *Eme1* mutant mice with mixed background were generated by mating *Gen1*^{PB/+} mice (FVB/C57) with *Gen1*^{PB/+}; *Eme1*^{PB/PB} mice (FVB/NJ). Heterozygosity of the single nucleotide polymorphisms (SNPs) in these mice was confirmed using Sanger sequencing after genotyping PCR (Supplementary Table S1). The meiotic recombination efficiency was measured in the offspring of male mice with indicated genotypes and FVB/NJ females by genotyping PCR and Sanger sequencing. Meiotic recombination efficiency = [No. (rs32032816^{G/G}; rs32330931^{A/G}) + No. (rs32032816^{C/G}; rs32330931^{G/G})]/No. (all offspring).

Statistics

Statistical methods were indicated in the figure legend. The threshold for significance was set at $p < 0.05$.

Results

Disruption of *Gen1* and *Eme1* causes synthetic lethality

We have isolated *Gen1* and *Eme1* mutants from a PB insertional mutagenesis project in mice (Sun *et al.*, 2008). The PB transposon was inserted into the second and third intron of *Gen1* and *Eme1*, respectively. Both mutants were viable and fertile. RT-PCR analysis of individual mutant alleles showed that gene expression was reduced or extinguished in MEF cells isolated from heterozygous or homozygous mutants, respectively (Fig. 1A, B). To test if *Gen1* and *Eme1* play redundant roles in mice, we first tried to generate mice carrying both mutations. We have successfully obtained double heterozygous mice (*Gen1*^{PB/+}; *Eme1*^{PB/+}) and *Gen1*^{PB/+}; *Eme1*^{PB/PB} mutants. However, inconsistent with expected Mendelian distribution, double homozygotes (*Gen1*^{PB/PB}; *Eme1*^{PB/PB}) were observed neither among 65 neonates generated from inbreeding of *Gen1*^{PB/+}; *Eme1*^{PB/+} mice (Fig. 1C, $p = 0.015$), nor among 48 progenies born by *Gen1*^{PB/+}; *Eme1*^{PB/PB} inbreeding (Fig. 1D, $p = 1 \times 10^{-6}$), indicating prenatal lethality of double homozygotes. To precisely pinpoint the time of embryonic death, embryos from *Gen1*^{PB/+}; *Eme1*^{PB/PB} intercrosses were dissected at different stages and genotyped by PCR (Fig. 1D). We failed to identify double homozygotes at embryonic day 8.5 (E8.5) (0/12, $p = 0.032$), or even at E3.5 (0/19 blastocysts, $p = 0.004$), indicating that *Gen1*^{PB/PB}; *Eme1*^{PB/PB} embryos could not survive through early embryonic development. The synthetic lethality caused by *Gen1* and *Eme1* mutations indicates that both genes are required for embryonic development.

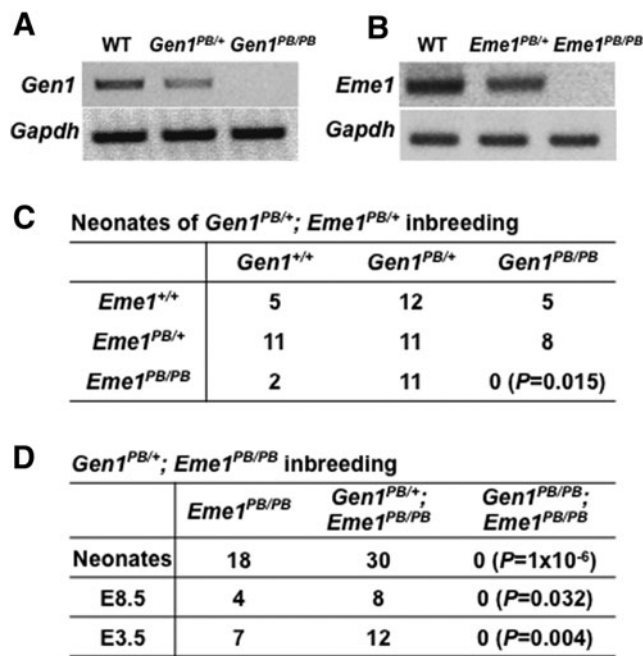


FIG. 1. Disruption of *Gen1* and *Eme1* caused synthetic lethality. RT-PCR showed disrupted expression of *Gen1* (A) and *Eme1* (B) in MEFs bearing homozygous mutations, respectively. Genotyping PCR of neonates (C, D) and embryonic day 8.5 (E8.5) offspring (D), and E3.5 blastocysts (D) from indicated mice inbreeding detected no double homozygous mutant (*Gen1^{PB/PB}; Eme1^{PB/PB}*), respectively. *p*-Value of Fisher's exact test was labeled in the figure. MEFs, mouse embryonic fibroblasts; PB, *piggyBac*; RT-PCR, reverse transcription polymerase chain reaction; WT, wild-type.

Gen1 and Eme1 play redundant roles in cellular resistance to DNA damage-induced cell death

We speculate that the lethality of *Gen1* and *Eme1* double mutants may be due to the redundant roles of GEN1 and the MUS81-EME1 complex in DNA repair in mice. To confirm this hypothesis, we prepared primary MEFs carrying different combinations of *Gen1* and *Eme1* mutations to test their sensitivities to various DNA damaging reagents, such as mitomycin C (MMC), methyl methanesulfonate (MMS), camptothecin (CPT), and Etoposide. We did not observe any significant differences in the survival rates between the wild-type and *Gen1^{PB/PB}* MEFs (Fig. 2A–D).

However, *Gen1^{PB/PB}* mutations significantly increased the sensitivity of *Eme1^{PB/+}* MEFs to DNA-damaging reagents. Compared with that of *Eme1^{PB/+}* MEFs, the survival rate of *Gen1^{PB/PB}; Eme1^{PB/+}* MEFs dropped by ~12% under the treatment with three different concentrations of CPT or Etoposide (Fig. 2A, B). Although low and high concentrations of MMC treatment did not cause any differences, medial level of MMC (0.67 μg/mL) killed slightly more *Gen1^{PB/PB}; Eme1^{PB/+}* MEFs than *Eme1^{PB/+}* MEFs (Fig. 2C). The survival rates of *Gen1^{PB/PB}; Eme1^{PB/+}* and *Eme1^{PB/+}* MEFs are comparable with each other after MMS treatment, both lower than those of the wild-type or *Gen1^{PB/PB}* MEFs (Fig. 2D). Meanwhile, DNA replication stress by hydroxyurea (HU) treatment did not bring any differences between the wild-type and *Gen1^{PB/PB}; Eme1^{PB/+}* MEFs (Fig. 2E).

Real-time RT PCR showed that *Gen1* mRNA was undetectable in homozygous mutant MEFs (Fig. 2F), whereas ~31% of *Eme1* mRNA still remained in *Gen1^{PB/PB}; Eme1^{PB/+}* MEFs (Fig. 2G).

These results indicate that *Gen1* and *Eme1* are functionally redundant in the resistance to DNA damage-induced cell death in mammalian cells.

Gen1 and Eme1 play redundant roles in meiotic recombination

As the intermediate structure of DNA recombination, HJs exist not only in DNA repair, but also in meiosis. We thus checked if *Gen1* and *Eme1* also play redundant roles in meiotic recombination in mice.

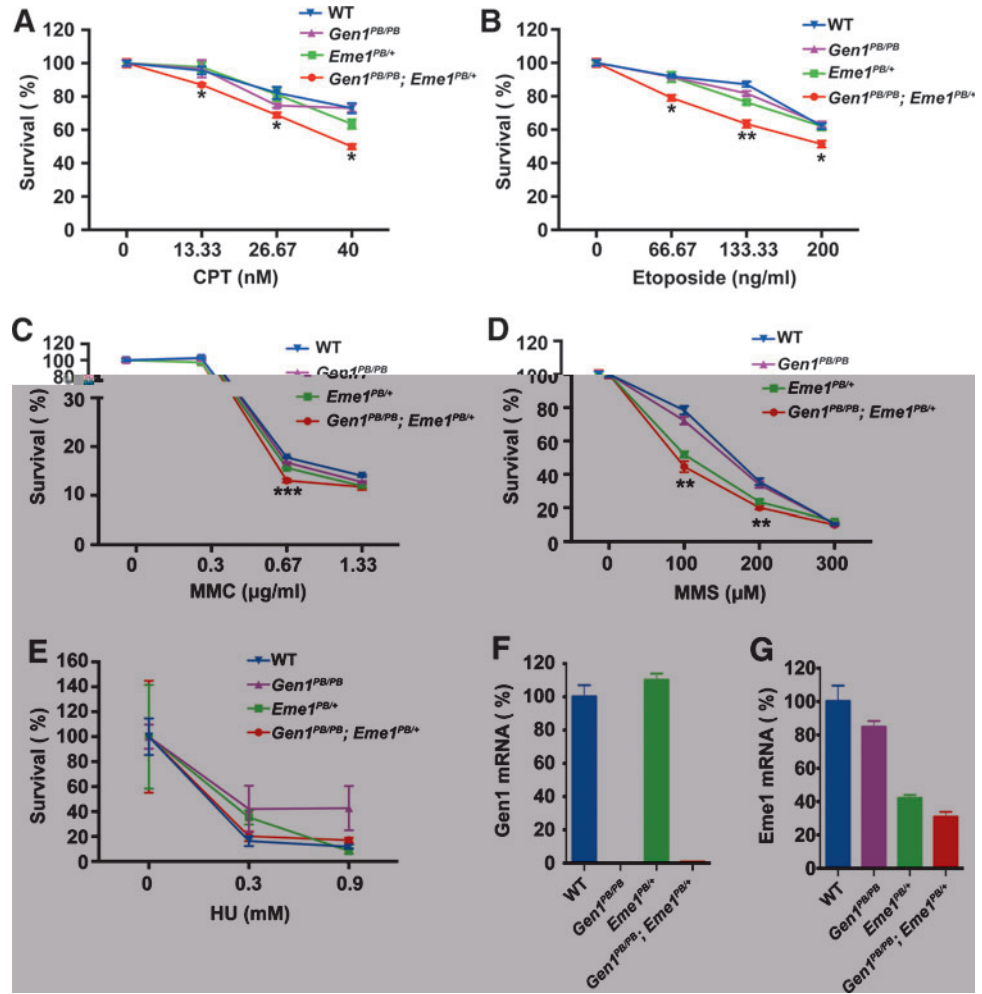
We first introduced *Gen1* and *Eme1* mutations into the mixed genetic background of FVB/NJ and C57BL/6J by breeding, then collected ~100 offspring of each of the four genotypes (WT, *Gen1^{PB/PB}*, *Eme1^{PB/+}*, and *Gen1^{PB/PB}; Eme1^{PB/+}*) to measure the meiotic recombination efficiency by scoring the segregation of SNPs from both ancestral genetic background. After screening >40 SNPs that are not only different in FVB/NJ and C57BL/6J, but also mapped ~25–50 Mbps apart from each other (Supplementary Table S2), we found a pair of Chr. Seven SNPs (rs32032816 and rs32330931) that were both heterozygous in all of the four genotypes so that they could be used to calculate the recombination efficiency (Fig. 3A).

The calculated meiotic recombination efficiency in the offspring of wild-type male mice was 32.22% (29/90). Similar results were obtained in the offspring of *Gen1^{PB/PB}* (33.96%, *n* = 106) and *Eme1^{PB/+}* (36.89%, *n* = 103) males. However, the rate was significantly reduced in the offspring of *Gen1^{PB/PB}; Eme1^{PB/+}* mice. Only 17.48% (18/103, *p* = 0.0193) of the progeny were identified as recombinants. Thus, disruption of *Gen1* affects meiotic recombination not on the wild type, but on the *Eme1* mutant background (Fig. 3B). Other than this, we did not find obvious differences in the size and gross morphology of the testis in *Gen1^{PB/PB}; Eme1^{PB/+}* and other mutants (Supplementary Fig. S1A–D). The mating between male mice of each genotype and WT FVB/NJ females resulted in similar litter sizes as well (Supplementary Fig. S1E). Taken together, these results suggest that *Gen1* and *Eme1* also play redundant roles in meiotic recombination.

Discussion

GEN1, MUS81-EME1, and SLX1 are three HJ resolvases that could process persistent HJs in mammalian cells (Boddy *et al.*, 2001; Chen *et al.*, 2001; Fricke and Brill, 2003; Ip *et al.*, 2008; Fekairi *et al.*, 2009; Munoz *et al.*, 2009; Rass *et al.*, 2010). However, their contributions to HJ-mediated processes *in vivo* have not been well defined. In this study, we found that a *Gen1* mutation alone did not affect DNA repair or meiotic recombination in mice. It caused synthetic lethality, nevertheless, when combined with *Eme1* mutations at an early embryonic stage. In addition, the combination of *Gen1^{PB/PB}; Eme1^{PB/+}* mutations makes MEFs more sensitive to DNA damage and mice less capable of meiotic recombination. These results indicate that *Gen1* and *Eme1* are functionally redundant in DNA repair and meiotic recombination in mice.

FIG. 2. *Gen1* mutation increased the sensitivity of *Eme1*^{PB/+} MEFs to DNA-damaging reagents. WT, *Gen1*^{PB/PB}, *Eme1*^{PB/+}, and *Gen1*^{PB/PB}; *Eme1*^{PB/+} MEF cells were subjected to doses of CPT (A), Etoposide (B), MMC (C), MMS (D), and HU (E) as indicated. The survival rate of the cells was quantified 1 week later. Compared with *Eme1*^{PB/+} MEF cells, *Gen1*^{PB/PB}; *Eme1*^{PB/+} MEF cells were more sensitive to CPT (A), Etoposide (B), and MMC (C). (F) Real-time RT-PCR results of *Gen1* in the four cell lines. (G) Real-time RT-PCR results of *Eme1* in the four cell lines. Data are presented as mean ± SEM. *, *p* < 0.05; **, *p* < 0.01, ***, *p* < 0.001; unpaired *t* test. CPT, camptothecin; HU, hydroxyurea; MMC, mitomycin C; MMS, methyl methanesulfonate.

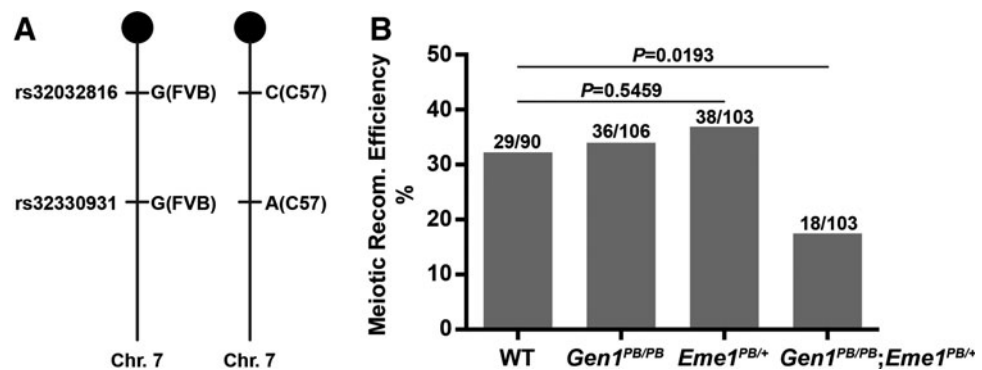


Compared with those of the *Mus81* and *Eme1* mutants, the phenotypes related to DNA recombination are milder in *Gen1* mutants. Homozygous deletion of *Eme1* caused hypersensitivity to MMC and other DNA-damaging reagents in embryonic stem cells and MEFs (Abraham *et al.*, 2003). *Mus81*^{-/-} mice exhibited significant meiotic defects such as the depletion of mature epididymal sperm and partial failure of DNA double-strand breaks repair (Holloway *et al.*, 2008). Thus, *GEN1* likely plays a supporting role in DNA repair and meiosis recombination in mice. This may also explain why *Gen1*^{PB/PB}; *Eme1*^{PB/+} MEFs showed only a

mild additional effect on drug sensitivity when compared with *Eme1*^{PB/+} MEFs, in which substantial amount of *Eme1* expression could still be detected (Fig. 2G).

Increased sensitivity of *Gen1* and *Eme1* double mutants to DNA damaging reagents may be a result of multiple cellular events. It has been shown that codepletion of *MUS81* and *GEN1* in human fibroblasts led to severe chromosome segregation defects (Wyatt *et al.*, 2013). *MUS81*- and *GEN1*-codepleted HeLa cells also showed genome instability, which is exemplified by impaired replication fork movement and S-phase progression, endogenous checkpoint

FIG. 3. Decreased meiotic recombination efficiency in *Gen1*^{PB/PB}; *Eme1*^{PB/+} mice. (A) Brief illustration shows the genotype of two SNPs on FVB/NJ and C57BL/6J background, respectively. (B) Meiotic recombination efficiency of WT, *Gen1*^{PB/PB}, *Eme1*^{PB/+}, and *Gen1*^{PB/PB}; *Eme1*^{PB/+} mice. Recombinant pups/total pups is labeled above each column. *p* Value was calculated by Fisher's exact test. SNPs, single nucleotide polymorphisms.



activation, chromosome segmentation, and multinucleation (Sarbjana *et al.*, 2014). These events may also contribute to the synthetic lethality observed in *Gen1^{PB/PB}; Eme1^{PB/PB}* embryos, which may experience DNA damages caused by environment during development.

Meanwhile, mutant embryos may die of HJ processing deficiency during mitosis. DNA replication is known to produce HJs (Petermann and Helleday, 2010). Failure of the resolution of replication-induced HJs could cause delayed or arrested mitosis in mammalian cells, leading to cell death in the absence of exogenous DNA damage (Garner *et al.*, 2013). Depletion of GEN1 in human cells expressing mutant SLX4 that no longer binds and activates MUS81 would result in dysfunctional mitosis and cell death under normal culturing conditions (Garner *et al.*, 2013; Sarbjana *et al.*, 2014). Double mutations of GEN1 and EME1 may cause cell death through similar mitotic defects.

Conclusion

In summary, GEN1 and MUS81-EME1 are functionally redundant in mice to ensure the elimination of HJs during both mitotic DNA damage repair and meiotic recombination. Given that both MUS81-EME1 and SLX1 interact with the scaffold protein SLX4 to resolve HJs together in mammalian cells, it would be reasonable to speculate that GEN1 and SLX1 are also functionally redundant in these processes *in vivo*.

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Disclosure Statement

No competing financial interests exist.

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