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Role of the mismatch repair gene, *Msh6*, in suppressing genome instability and radiation-induced mutations

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Abstract

Mismatch repair (MMR) is critical for preserving genomic integrity. Failure of this system can accelerate somatic mutation and increase the risk of developing cancer. MSH6, in complex with MSH2, is the MMR protein that mediates DNA repair through the recognition of 1- and 2-bp mismatches. To evaluate the effects of MSH6 deficiency on genomic stability we compared the frequency of *in vivo* loss of heterozygosity (LOH) between MSH6-proficient and deficient, 129S2 x C57BL/6 F1 hybrid mice that were heterozygous for our reporter gene *Aprt*. We recovered mutant cells that had functionally lost APRT protein activity and categorized the spectrum of mutations responsible for the LOH events. We also measured the mutant frequency at the X-linked gene, *Hprt*, as a second reporter for point mutation. In *Msh6*−/−*Aprt*+/− mice, mutation frequency at *Aprt* was elevated in both T cells and fibroblasts by 2.5-fold and 5.7-fold, respectively, over *Msh6* +/+*Aprt*+/− littermate controls. While a modest increase in mitotic recombination (MR) was observed in MSH6-deficient fibroblasts compared to wild type controls, point mutation was the predominant mechanism leading to APRT deficiency in both cell types. Base substitution, consisting of multiple types of transitions, accounted for all of the point mutations identified within the *Aprt* coding region. We also assessed the role of MSH6 in preventing mutations caused by a common environmental mutagen, ionizing radiation (IR). In *Msh6*−/−*Aprt*+/− mice, 4 Gy of X-irradiation induced a significant increase in point mutations at both *Aprt* and *Hprt* in T cells, but not in fibroblasts. These findings indicate that MutSα reduces spontaneous and IR-induced mutation in a cell-type dependant manner.

Keywords

loss of heterozygosity; mismatch repair; MSH6; MutSα; mitotic recombination; *Aprt*

1. Introduction

To maintain genomic integrity, DNA must be replicated with high fidelity. It is crucial to correct any errors made during DNA replication before these errors are converted into permanent

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mutations in progeny cells. Mutations introduced into critical genes such as those involved in DNA repair, apoptosis, or growth control could ultimately lead to tumorigenesis.

One set of genes critical to maintaining genomic stability and preventing tumorigenesis are those involved in the mismatch repair (MMR) pathway. MMR has been shown to correct errors made by DNA replication complexes by recognizing and repairing mispaired or extrahelical nucleotides. MutS complexes, either *Msh2-Msh6* (MutSα) or *Msh2-Msh3* (MutSβ), recognize mismatches consisting of either 1–2 bps or 2–5 bps, respectively [1]. MutS complexes are vital to initiating repair through recruitment of MutL complexes that conduct the excision of the unpaired nucleotide(s), resynthesis of the nascent strand, and ligation of the single-stranded gap. Therefore, lack of functional MutS subunit proteins, particularly MSH2 and to a lesser extent MSH6, results in a mutator phenotype. The mutator phenotype is associated with the etiology of certain hereditary diseases, such as familial pancreatic cancer, endometrial adenocarcinomas, and in particular, hereditary non-polyposis colorectal cancer (HNPCC) [2–5].

MutSα has also been implicated in the suppression of recombination between divergent DNA sequences, as first demonstrated in bacterial conjugant crosses between *E. Coli* and *Salmonella typhimurium*. The interspecific recombination was elevated a 1000-fold in a *mutS*-deficient background [6]. In mammalian cells, MMR is potentially involved in heteroduplex rejection during recombination. The RecQ helicase, BLM, was observed to co-localize with MLH1 and MSH6 at DNA double-strand breaks [7,8]. The hMSH2/6 complex was later demonstrated to stimulate BLM-mediated disruption of Holliday junction intermediates by seven-fold [9]. This interaction suggested that MutSα may regulate the activity of RecQ helicases to prevent mitotic recombination between “homeologous” chromosomes *in vivo*. We previously showed that mitotic recombination *in vivo* between homeologous chromosomes is increased in 129XC57F1 *Mlh1* null mice [10].

MutSα has also been shown to be involved in the repair of DNA damage caused by reactive oxygen species (ROS) [11]. One of the most common consequences of ROS exposure is the incorporation of oxidized nucleotides such as 8-oxoguanine (8-oxoG or °G) into the genome. Mismatch repair has been shown to repair oxidative damage by lowering the level of 8-oxoGs that are incorporated into the genome, presumably by recognizing mismatches different from those recognized by BER [11]. While BER can initiate the repair of °G:A and °G:C mispairs, no such role has been demonstrated for processing other mispairs such as °G:T or °G:G. Perhaps MMR is responsible for correcting these single nucleotide mismatches through MutSα-mediated repair.

Ionizing radiation (IR) can cause base damage, such as 8-oxoG, single- or double-strand breaks [12,13]. Double-strand breaks in S phase are primarily repaired by homologous recombination [14]. If MutSα regulates mitotic recombination (MR), then it could play an important role in preventing hyperrecombination in IR-exposed cells. MLH1 has already been demonstrated to suppress MR at the *Aprt* reporter gene in kidney cell lines exposed to 5.5 Gy 137 Cs-γ radiation [15].

To evaluate the effects of MutSα deficiency on genome stability under both spontaneous and stressed conditions, we utilized a murine assay to measure *in vivo* mutant frequency at an endogenous gene in *Msh6*−/− 129XC57F1 hybrid mice. T-lymphocytes and fibroblasts that had undergone *in vivo* LOH at *Aprt* in *Msh6*−/−*Aprt*+/−mice were recovered by their resistance to adenine analog 2, 6-diaminopurine (DAP). We then categorized the mutation spectra and calculated the frequency of different mutational mechanisms such as point mutation and mitotic recombination. Under normal conditions, we observed that MSH6 plays a strong role in the repair of base substitutions and, to a lesser extent, in the tissue-specific suppression of

recombination between homeologous chromosomes. We also characterized the mutations arising in *Msh6*−/−*Aprt*+/− mice that were exposed to 4 Gy of X-irradiation. We observed that MSH6 prevented a wide spectrum of IR-induced base substitution again in a tissue-specific manner. Interestingly, cell survival in MSH6-deficient backgrounds was not increasing. These results suggest that MutSα is only critical for preventing IR-induced mutation in cell types that are more susceptible to this type of damage, but not critical for triggering apoptosis in response to this type of damage.

2. Materials and Methods

2.1. Mice

Aprt heterozygous mice, originally generated by gene targeting, were maintained in a 129S2 background [16]. The mutant (targeted) *Aprt* allele has a neo insertion in the third exon, making it distinguishable from the wild-type (non-targeted) *Aprt* allele. MSH6-deficient mice were kept in a C57BL/6 background [17]. A subset of these *Msh6* mutant mice were backcrossed for four generations to 129S2 strain mice to generate N4129S2 *Msh6*+/−*Aprt*+/−mice. We analyzed three polymorphic SSR markers, D8mit155, D8mit190, D8mit75, located 1.0, 21.0, and 37.3 cM from the centromere of mouse chromosome 8 to ensure the $Aprt^{Neo}$ -containing chromosome 8 was mostly, if not entirely, derived from the 129S2 background in N4129S2 *Msh6*+/−*Aprt*+/− mice. The N4129S2 *Msh6*+/−*Aprt*+/− mice were crossed with C57BL/6 *Msh6*+/− mice to produce 129S2 x C57BL/6 F1 hybrid mice.

For X-irradiation experiments, 129 x C57 F1 hybrid mice were exposed to 4 Gy ionizing radiation using a Torrex cabinet X-ray system (Faxitron, Wheeling, IL). Mice were whole-body irradiated at 7 weeks and sacrificed at 15 weeks.

2.2. Isolation and molecular characterization of mutant clones

The isolation and characterization of DAP-resistant (DAP^r) cell colonies have been previously reported [18]. Briefly, ear fibroblasts and splenic T-lymphocytes from *Msh6*−/−*Aprt*+/− mice and *Msh6*+/+*Aprt*+/− littermate controls were plated into media containing 50μl 2, 6-diaminopurine (DAP). DAP^r colonies were harvested and analyzed by allele-specific PCR to determine whether or not they lost (class I) or still retained (class II) the non-targeted *Aprt* allele. Class I clones were further assayed for genotypes at microsatellite marker loci on chromosome 8. This assay distinguished between mitotic recombination, gene conversion/deletion and loss of the C57 homolog. Class II clones were subjected to DNA sequencing of all five exons of the non-targeted *Aprt* allele to detect point mutations. Only mutations with clear consequences to the amino acid sequence where included in the spectrum. Mutation at the *Hprt* locus in *Aprt* wild-type mice was evaluated by clonal recovery of 6-thiogaunine (6-TG) resistant variants, as has been described [19].

3. Results

3.1. Elevation of spontaneously occurring mutations in T cells of *Msh6*−/− mice

To evaluate the importance of MSH6 in maintaining genomic stability, we measured the *in vivo* frequency of loss of *Aprt* heterozygosity that produced APRT-deficient cells. We found that the median frequency of DAP^r clones in T-lymphocytes of *Msh6*−/− mice was 2.5-fold higher than that of *Msh6* wild type control mice, 25.0×10^{-6} vs. 10.0×10^{-6} ($P = 0.0132$, Mann-Whitney U-test, Table 1).

To identify the types of mutational events that arise in the absence of MSH6, we divided DAP^r clones into two groups based on the physical loss (Class I) or retention (Class II) of the untargeted *Aprt* allele. The physical loss of *Aprt* may be attributed to multi-locus events such

as mitotic recombination, gene conversion or chromosome loss. The physical retention of the wild type *Aprt*, as determined by allele-specific PCR, indicates either point mutation or epigenetic events restricted to *Aprt* [18,19]. Previous studies in our laboratory have consistently shown that the majority of the DAP^r T cell clones in both inbred and F1 hybrid mice were class I [20,21]. Analysis of polymorphic simple sequence repeat (SSR) markers along the length of chromosome 8 revealed that over 95% of class I clones arise by mitotic recombination (MR) in each study. In this study, we found that about half of the DAP^r T cell clones isolated from *Msh6*+/+ mice were class I (Table 2). Consistent with previous findings, all class I clones analyzed from both control and *Msh6*−/− groups were due to mitotic recombination (data not shown). However, the vast majority of the DAP^r T cell clones obtained from *Msh6*−/− mice were class II (70/84, 83%). This suggests that the overall increase in mutant frequency of DAP^r T cells in MSH6 deficient mice was primarily due to either point mutation or epigenetic silencing at *Aprt*, not mitotic recombination.

To test if MSH6 suppresses point mutation globally, we also estimated the mutant frequency at the X-linked *Hprt* locus. This gene serves as a reporter for point mutation and small deletions given that it is functionally hemizygous. In our study, T cell colonies resistant to 6-thiogaunine (6-TG) were isolated from *Msh6*−/−*Aprt*+/+ mice or wild type littermates. The median frequency of 6-TG^r T cells was 11.7-fold higher in the MSH6-deficient vs. proficient group, 21.0×10^{-6} vs. 1.8×10^{-6} ($P = 0.0078$, Mann-Whitney U-test), suggesting that point mutation is also increased at this locus (Table 3).

3.2. IR induces more mutations in T cells of *Msh6−/−* mice

To evaluate the role of MSH6 in correcting DNA damage from ionizing radiation, we measured the frequency of 6-TG^r T cells from *Msh6*−/− and *Msh6*+/+ mice. In wild type T cells, IR induced a 20.1-fold higher 6-TG^r frequency compared to untreated cells, 36.2×10^{-6} vs. 1.8×10^{-6} respectively (Table 3). This indicates that *Hprt* is a sensitive reporter for IR-induced DNA damage. In *Msh6*−/− T cells, IR induced an 8-fold higher 6-TG^r frequency compared to untreated *Msh6*−/− T cells, 169.0×10^{-6} vs. 21.0×10^{-6} (Table 3). The induced mutant frequency in IR-treated *Msh6*−/− T cells was elevated 4.7-fold compared to IR-treated wild type controls, 169.0×10^{-6} vs. 36.2×10^{-6} respectively when subtracting out corresponding spontaneous frequencies (Table 3). This difference was statistically significant ($P = 0.0004$, Mann-Whitney *U*-test). This indicates that MSH6 is critical in reducing IR-induced mutations in this cell type at *Hprt*.

In order to determine if the observations in MSH6-deficient cells were unique to *Hprt*, we measured the mutation frequency at *Aprt*. In T cells, IR-treatment induced a 9.3-fold higher DAP^r frequency in MSH6-deficient vs. MSH6-proficient cells, 84.0×10^{-6} vs. 9.0×10^{-6} respectively when correcting for spontaneous mutation (Table 1). IR-treated wild type T cells had a DAP^r frequency that was slightly higher than in untreated wild type cells, 19.0×10^{-6} vs. 10.0×10^{-6}. However, this difference was statistically significant ($P = 0.0184$, Mann-Whitney U-test). In IR-treated *Msh6*−/− T cells, we observed an induction of DAP^r frequency that was 3.4-fold over the untreated *Msh6*−/− T cells, 84.0×10^{-6} vs. 25.0×10^{-6} ($P = .0262$, Mann-Whitney *U*-test). Thus, MSH6 plays a critical role in suppressing IR-induced mutation in T cells.

3.3 Most IR-induced mutations in T cells of *Msh6−/−* mice are base substitutions

In IR-treated wild type T cells, class I colonies made up 60% (12/20) of the total DAP^r clones we isolated (Table 2). In IR-treated *Msh6*−/− T cells, Class I mutations made up only 15.5% (13/84) of the total DAP^r colonies isolated. Subtracting the spontaneous class II mutant frequency 20.8×10^{-6}, the absolute class II mutant frequency induced by irradiation in MSH6-deficient T cells was 71.3×10^{-6}. This induced frequency was 29.7-fold higher than the induced

mutant frequency in MSH6-proficient T cells, 71.3 $\times 10^{-6}$ vs. 2.4 $\times 10^{-6}$ (Table 2), indicating that MSH6 primarily suppresses IR-induced class II mutations.

Class II mutations were predominantly responsible for the elevated mutant frequency in *Msh6* −/− T cells (Table 2). Therefore, we further classified DAP-resistant class II colonies by sequencing all five exons of *Aprt*. Of the 32 clones analyzed, 23 had sequence alterations within a coding region. These alterations consisted of 14 transitions, 7 transversions, 1 single base-pair insertion, and 1 single base-pair deletion (Table 4). All the mutations reported here are unique and alter the amino acid sequence of APRT. Transitions accounted for 61% and transversions for 30% of DNA alterations (Table 4). Frameshift mutations accounted for the remaining 9%. The distribution of mutations in IR-treated mice was similar to what was observed in untreated mice. We then calculated the frequency of each type of mutation in treated and untreated spleens for a side-by-side comparison. We observed a proportional increase of each type of base substitution in treated group ranging from 3.4-fold to 5.6-fold (data not shown). Therefore, MSH6 prevents a wide range of IR-induced base substitutions in T cells.

3.4 Spontaneous base substitutions and, to a lesser extent, mitotic recombination are increased in fibroblasts of *Msh6−/−* mice

As mentioned earlier, the role of mismatch repair in reducing point mutation has been demonstrated in a wide variety of experimental assays and animal models. However, the lack of functional MMR has only been implicated in causing cancer in certain tissue types, particularly colonic epithelium [24]. In order to identify possible tissue-specific differences in the types of mutations suppressed by MSH6, we compared the mutant frequencies of 6-TG^r and DAP^r clones in ear fibroblasts of MSH6-deficient and proficient mice. MSH6-deficient fibroblasts had a 6.8-fold higher 6-TG^r frequency compared to wild type controls, 21.0 $\times 10^{-6}$ vs. 3.1 $\times 10^{-6}$ respectively (Table 3). This difference was statistically significant by a chi-square test of the number of 6-TG^r clones isolated divided by the total number of viable cells in each group (*Msh6*+/+: 2/646,184; *Msh6*−/−: 18/854,481; P =.0058).

We also observed a significant 5.7-fold elevation in DAP^r mutant frequency in *Msh6*−/−*Aprt* +/− mice vs. *Msh6*+/+*Aprt*+/− mice (130.0 $\times 10^{-6}$ vs. 23.0 $\times 10^{-6}$ respectively, P =.0002, Mann-Whitney U-test, Table 1). Class II variants again accounted for the majority of clones isolated from *Msh6*−/−*Aprt*+/− fibroblasts (47/77, 61%, Table 2). Class I events accounted for the majority of the total in the control group (88%, Table 2). This is comparable to our previous studies in *Mlh1*+/+ in which class I clones accounted for 67% of DAP^r fibroblasts (10). Also consistent with our previous studies in MMR-proficient fibroblasts, all class I fibroblasts arose by mitotic recombination based on the observed pattern of loss of microsatellite markers on chromosome 8 (data not shown).

We sequenced all five exons of the non-targeted *Aprt* allele in class II fibroblast variants. As mentioned earlier, class II variants can arise either by intragenic mutation or epigenetic inactivation. Past studies using our assay indicated that epigenetic silencing accounts for a majority of class II events fibroblasts in wild type mice, with only 9 DNA sequence alterations identified out of 42 total clones (data not published). In *Msh6*−/−mice, however, intragenic alterations in coding sequences were identified in 18 of 21 fibroblast clones analyzed (84%). Base substitutions accounted for all 18 of those mutations. Of the 18 identified mutations, 10 were unique and consisted of three of the four possible types of transitions (Table 4).

3.5 Fibroblasts are less sensitive to IR-induced mutagenesis

We next assessed the effects on 4 Gy ionizing radiation on the fibroblasts of *Msh6*+/+*Aprt*+/− and *Msh6*+/+*Aprt*+/− using the same approach carried out for T cells. IR-treatment induced

a 1.8-fold higher mutant frequency in wild type fibroblasts compared to untreated fibroblasts, 41.0×10^{-6} vs. 23.0×10^{-6} respectively (Table 1). However, this increase was not statistically significant ($P = 0.0559$, Mann-Whitney *U*-test). Ionizing radiation also had no apparent effect on fibroblasts in *Msh6*−/−*Aprt*+/−mice. DAP^r frequencies in treated and untreated MSH6-deficienct fibroblasts were 124.0×10^{-6} and 130×10^{-6}, respectively (Table 1). This suggests that fibroblasts are relatively insensitive to IR-induced mutagenesis at *Aprt*, even when MSH6 is absent.

In order to determine if our observations with IR-treated, MSH6-deficient mice were unique to *Aprt*, we also measured the mutation frequency at *Hprt*. IR induced a 2.4-fold higher mutant frequency in *Msh6*−/− fibroblasts compared to wild type controls, 31.0×10^{-6} vs. 12.9×10^{-6} when correcting for spontaneous mutation (Table 3). Ionizing radiation increased the mutant frequency at *Hprt* 5-fold in wild type fibroblasts (Table 3). In IR-treated *Msh6*−/− fibroblasts, the mutant frequency was 2.5-fold higher than that of untreated *Msh6*−/− fibroblasts, 52.0×10^{-6} vs. 21.0×10^{-6} (Table 3). This 2.5-fold increase in fibroblasts is modest when considering that IR treatment increased *Hprt* mutant frequency 9-fold in *Msh6*−/− T cells compared to untreated *Msh6*−/− T cells (Table 3). This was also the case at *Aprt* (see above), indicating that MSH6 is more important in preventing mutations in T cells than in fibroblasts.

4. Discussion

4.1 Evidence of functional redundancy for MutS complexes

Bacteria have one protein, mutS, which recognizes mismatches of one or multiple nucleotides. This protein functionally suppresses both base substitutions and frameshifts of one or more base pairs [1]. Eukaryotic cells have evolved two complexes to presumably split these duties. MutSα has been proposed to prevent base substitutions and MutSβ to prevent frameshifts of two or more base pairs. However, both complexes have been implicated in preventing 1-bp frameshifts, but few studies have addressed whether or not this function is completely redundant. One study showed that MutSα plays a stronger role than MutSβ in correcting 1-bp IDL's within the transgenic reporter, supFG1 [25]. Our data obtained from an endogenous reporter gene showed that loss of MutSα, via MSH6-deficiency, does not result in a relatively large increase in 1-bp IDL's compared to wild type mice, even after exposure to 4 Gy of IR. This is consistent with studies measuring spontaneous mutant frequencies within the transgenic reporters cII and lacI in MSH6-deficient mice, as well as in results in vitro [17,25,26]. This suggests that MutSα and MutSβ complexes can prevent 1-bp frameshifts independently in mammalian cells. Perhaps mammalian cells evolved this redundancy to combat an increased rate of these types of lesions under certain environmental conditions.

4.2 MMR is more important in certain cell types

The role of MutSα in recognizing certain types of mismatched nucleotides is well conserved between yeast, mice, and human cell lines. Our work indicates that MutSα at least plays a role in preventing base substitutions. However, the role of MutS complexes in suppressing recombination has been previously studied without taking tissue-specificity into account. We found that MSH6 suppressed spontaneous mitotic recombination in fibroblasts (Table 2). We, however, did not observe such a role for MSH6 in T cells (Table 2). Tissue-specific suppression of spontaneous mitotic recombination by MMR has previously been reported in *Mlh1*−/− mice (10), we observed that that MSH6 suppresses mitotic recombination in a similar manner. These findings raise an issue not testable by studies in lower organisms or cell lines. What are the tissue-dependant factors that make MMR necessary for preventing mutation in some cell types but not others?

One recent review article highlighted several tissue-specific factors that could explain why MMR-deficient tumors only arise in certain cell types. Such factors included high cell turnover, higher exposure to environmental stress, or hormonally-triggered cell growth [24]. Our work showed that MSH6 prevented IR-induced mutations in T cells, but not in fibroblasts. T cells are known to undergo apoptosis and repopulation (presumably from hematopoietic stem cells) after IR-exposure [27]. Fibroblasts, by contrast, arrest in G_o 24 hours after IR-treatment [28]. It is likely that MutSα is not important for repairing IR-induced DNA damage in cell types that arrest after damage since DNA is not actively replicating. This could explain why we observed no increase in mutant frequency at *Aprt* in fibroblasts of *Msh6*−/− mice after their exposure to IR (Table 1), and only a relatively small increase at *Hprt* (Table 3).

4.3 MutSα does not trigger IR-induced apoptosis

Another possible tissue-dependant factor could be that MutSα plays a stronger role in triggering apoptosis in T cells in response to environmental DNA damage. Both MLH1 and MSH2-dependant apoptosis in response to mutagenic agents has been demonstrated in both cell lines and mouse tissues [29–31]. Perhaps the absence of MutSα allowed mutated T cells to propagate due to increased survivability. This could explain the wide range of base substitutions observed in class II DAP^r clones isolated from IR-treated *Msh6*−/− T cells (table 4). However, the mean cloning efficiencies of IR-treated MSH6-proficient and deficient T cells in both the *Aprt* and *Hprt* studies were very similar (Tables 1 and 3). This is consistent with cell survival studies in *Msh6*−/− mouse ES cells exposed to UVC light [32]. MSH6 has been implicated in triggering apoptosis in mouse embryonic fibroblasts exposed to UVB light [33]. However, as in T cells, there was no increase in ear fibroblast survival in IR-treated *Msh6*−/− mice (Tables 1 and 3). This suggests that MutSα is not important for triggering apoptosis in response to certain environmental agents such as ionizing radiation.

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Abbreviations

Aprt
: adenine phosphoribosyltransferase

DSB
: double-strand break

Hprt
: hypoxanthineguanine phosphoribosyltransferase

IR
: ionizing radiation

MF
: mutant frequency

MMR
: mismatch repair

MR
: mitotic recombination

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Table 1

Frequencies of DAP^r-variants

		T cells			Fibroblasts		
IR (Gy)	**Genotype**	**# spleens**	**C.E. (mean ± s.e.)**	**Median freq.**	**# ears**	**C. E. (mean ± s.e.)**	**Median freq.**
0	*Msh6*+/+	14	4.55 ± 0.35	10.0×10^{-6} [a]	33	1.14 +/− 0.05	23.0×10^{-6} [c]
0	*Msh6*−/−	11	4.24 ± 0.83	25.0×10^{-6} [a]	22	1.06 +/− 0.04	130.0×10^{-6} [c]
4	*Msh6*+/+	7	1.33 ± 0.12	19.0×10^{-6} [b]	14	0.61 +/− 0.05	41.2×10^{-6} [d]
4	*Msh6*−/−	10	1.04 ± 0.15	109.0×10^{-6} [b]	20	0.54 +/− 0.04	124.0×10^{-6} [d]

[a] *P*-value = 0.0132;

[b] *P*-value = 0.0037;

[c] *P*-value=0.0002;

[d] *P*-value=0.0023. Mann-Whitney *U*-test

C.E.: cloning efficiency, s.e. standard error.

Table 2

Classification of DAP^r-variants

		T cells					Fibroblasts				
IR (Gy)	**Genotype**	**Class II/ total**	**% Class II**	**Total mutant frequency** [a]	**Class I frequency**	**Class II frequency**	**Class II/ total**	**% Class II**	**Total mutant frequency** [a]	**Class I frequency**	**Class II frequency**
0	*Msh6*+/+	17/33	52	10.0×10^{-6}	4.8×10^{-6}	5.2×10^{-6}	8/67	12	23.0×10^{-6}	20.2×10^{-6}	2.76×10^{-6}
0	*Msh6*−/−	70/84	83	25.0×10^{-6}	4.3×10^{-6}	20.8×10^{-6}	47/77	61	130.0×10^{-6}	50.7×10^{-6}	79.3×10^{-6}
4	*Msh6*+/+	8/20	40	19.0×10^{-6}	11.4×10^{-6}	7.6×10^{-6}	n/a	n/a	41.2×10^{-6}	n/a	n/a
4	*Msh6*−/−	71/84	85	109×10^{-6}	16.9×10^{-6}	92.1×10^{-6}	n/a	n/a	124.0×10^{-6}	n/a	n/a

Class I: physical loss of untargeted *Aprt* allele; *Class II*: physical retention of untargeted *Aprt* allele

[a] from Table 1. n/a, not analyzed

Table 3

Frequencies of *Hprt* mutants

		T cells			Fibroblasts		
IR (Gy)	**Genotype**	**# spleens**	**C.E. (mean ± s.e.)**	**Median freq.**	**# ears**	**C.E. (mean ± s.e.)**	**Median freq.**
0	*Msh6*+/+	8	5.2 ±.04	1.8×10^{-6} [a]	16	1.20 ±.05	3.1×10^{-6}
0	*Msh6*−/−	9	5.8 ±.09	21.0×10^{-6} [a]	22	1.20 ±.04	21.0×10^{-6}
4	*Msh6*+/+	10	1.24 ±.14	38.0×10^{-6} [b]	12	0.55 ±.04	16.0×10^{-6} [c]
4	*Msh6*−/−	7	1.34 ±.24	190.0×10^{-6} [b]	14	0.63 ±.04	52.0×10^{-6} [c]

[a] *P*-value = 0.0078;

[b] *P*-value = 0.0004;

[c] *P*-value=0.0166. Mann-Whitney *U*-test

C.E.: cloning efficiency

Table 4

Spectrum of DAP^r class II mutants

	T cells		Fibroblasts
	IR-treated	**Untreated**	**Untreated**
Alterations detected	23	16	18
Clones analyzed	32	25	21
%	72	64	86
Transitions (%)	14 (61)	10 (63)	16 (89)
T>C	5 (22)	3 (19)	5 (28)
C>T	4 (17)	3 (19)	5 (28)
A>G	1 (4)	1 (6)	0
G>A	4 (17)	3 (19)	6 (33)
Transversions (%)	7 (30)	6 (38)	2 (11)
T>A	1 (4)	1 (6)	0
G>C	1 (4)	1 (6)	0
C>A	1 (4)	1 (6)	0
G>T	4 (17)	3 (19)	2 (11)
Frameshifts (%)	2 (9)	0 (0)	0 (0)
(+1) IDL's	2	0	0