Published in final edited form as:

Mol Immunol. 2010 October ; 47(16): 2611–2618. doi:10.1016/j.molimm.2010.05.291.

The pattern of somatic hypermutation of Ig genes is altered when *p53* is inactivated

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Abstract

Mice with a deletion of the *p53* gene have normal antibody titers against sheep red blood cells and normal switching to all Ig isotypes. In older mice (11 and 16 weeks old) the somatic hypermutation (SHM) frequencies are progressively reduced. In young mice (8 weeks old) with *p53* deletion, the SHM frequencies are normal. However, the mutation pattern is changed in all *p53*^{−/−} mice: mutations at A are increased. Surprisingly, deletion of the *Ung2* gene in addition to the deletion of *p53* corrected the A mutation frequencies to those of control mice. Known interactions of p53 protein with several proteins involved in error-prone BER during SHM may explain these findings. There is no indication that the absence of p53 affects the function of AID. Inactivation of *p21* does not alter SHM, supporting the idea that the p53 protein is involved in SHM by its direct association with the SHM process. There is no significant change of mutations at T. Thus, the hypermutability at A is strand-biased (transcription? replication?). The translesion polymerase pol eta has so far been found to be the sole mutator at A and T in mice. However, the pattern in *p53*^{−/−} mice is compatible with the possible inhibition by p53 of another translesion polymerase, pol iota, which in the absence of *p53* may be recruited to error-prone repair of abasic sites in SHM.

Keywords

Somatic hypermutation; p53; AID; Ig genes; knockout mice; pol eta; pol iota

1. Introduction

The activation-induced cytosine deaminase (AID) initiates the process of somatic hypermutation (SHM) of Ig genes by creating uracils in the variable (V) region and flanking DNA sequences (reviewed in (Longerich et al., 2006). Many of the uracils are repaired in an error-free mode, however, an as yet unknown proportion of uracils are treated by invoking error-prone mechanisms (Liu et al., 2008; Longerich et al., 2006; Storb et al., 2009). Lesion-

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The authors have no conflicting financial interests.

bypass polymerases introduce mutations at the uridine and sequences within a dozen or so nucleotides in the vicinity of the uridine (Longerich et al., 2006). Both mismatch repair (MMR) and base excision repair (BER) proteins are involved in the error-processes (Longerich et al., 2006). Nucleotide excision repair has not been found to play a role in SHM (Kim et al., 1997).

Since p53 is a major monitor of genome integrity, it can be expected that p53 is involved in the process of SHM. p53 is expressed in mouse germinal center B cells (Ranuncolo et al., 2007). p53 has been shown to be involved directly in BER (Seo and Jung, 2004). It has been proposed that p53 would facilitate DNA repair by allowing cells to remain in G1 before resuming cycling (Smith and Seo, 2002; Vousden and Prives, 2009). Mutations during SHM have been shown to occur in the G1 phase (Faili et al., 2002b) and Gasior, S. and U.S., unpublished). Would one expect then that the mutation frequency is increased when p53 is inactivated because cells with AID-induced uracils would have shorter times in G1 and therefore less time to repair the lesions? Certainly, general mutations have been found to increase in the absence of p53 (Zhou et al., 2001). However, since DNA repair by MMR and BER is co-opted in SHM into introducing mutations, the opposite may occur, namely that in the absence of p53 and due to the shorter time in G1 fewer mutations would occur. Furthermore, the pattern of mutations may partly depend on interactions of p53 with MMR and BER, as well as influencing the targeting of various lesion-bypass polymerases.

In order to obtain an insight into a possible role of p53 in SHM we have determined the effects of the inactivation of the *p53* gene on the frequency and pattern of SHM.

2. Materials and methods

2.1 Mice

The *p53* knockout mice (Donehower et al., 1992), the *p21* knockout mice (Brugarolas et al., 1995) and their age-matched wildtype littermates were obtained from The Jackson Laboratory and were further bred in our mouse facility. The *p53*^{-/-} mice were C57BL/6. *Ung*^{-/-} mice were a gift of D. Barnes and T. Lindahl (Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, U.K.), and were bred in our mouse facility with B6 mice. The *ung*^{-/-} mice in this study were obtained by crossing *p53*^{-/-}, *p53*^{+/-}, or *p53*^{+/+} mice on a B6 background with *ung*^{-/-} or *ung*^{+/-} mice on a mainly B6 background but some with a mixed (F3) B6 and 129/Sv (129) background. Three mice were a mix of B6 and 129 at the Ig heavy chain locus. Based on the IgH sequences we obtained (see below in section 3.3), 26, 26, and 35% of the mutated IgH genes were 129 in the *ung*^{-/-}, the *p53*^{+/-}*ung*^{-/-}, and the *p53*^{-/-}*ung*^{-/-} mouse 2 (mice 8, 9, and 11, Tables 2A, 2B), respectively. The 129 strain has an inactivated pol iota gene. We determined the proportion of the active and inactive 129 *iota* gene and found that all of the *ung*^{-/-} mice (mice #8–11 Tables 2A, 2B) had only the active pol iota gene.

A male *Msh6*^{+/-} mouse was obtained from the Mouse Repository at the National Cancer Institute (Rockville, MD) and bred with B6 in our facility. *p53*^{+/-} mice were mated either to *Msh6*^{+/-} mice or *Msh6*^{-/-} in an attempt to obtain *p53*^{-/-}*Msh6*^{-/-} mice. The primers used for genotyping the mice are shown in Supplementary Table 1a.

2.2 Immunization

The mice were immunized by an intra-peritoneal injection of sheep red blood cells (2×10^8 cells) followed by a secondary immunization after 21 days. The spleens and blood were collected after 21+ 4 days.

2.3 Analysis of SHM

PNA-high GL7+ B220+ (germinal center, mutating B cells) cells and an equivalent number of PNA-low GL7-low B220+ (non-germinal center B cells) cells were obtained from spleens of immunized mice as described (Longerich et al., 2005). DNA was extracted using DNeasy columns (QIAGEN). VHJ558-rearranged *IgH* genes were amplified using primers VHJ558BLF (GGAATTCGCCTGACATCTGAGGACTCTGC) and JCIIntronR (GACTAGTCCTCTCCAGTTTCGGCTGAATCC) as described (Longerich et al., 2005). Around 10,000 cell equivalents of template DNA were used for the PCR reaction at the following PCR cycling conditions: 1 cycle at 95 °C for 4 min, 95 °C for 40 s, and 64–58 °C for 40 s (touchdown annealing), 13 cycles at 72 °C for 4 min, followed by 27 cycles at 95 °C for 40 s, 57 °C for 40 s, 72 °C for 4 min, and a final extension at 72 °C for 7 min. The PCR products were resolved by agarose gel electrophoresis. The band encompassing the JH4 rearrangement of the VHJ558 *IgH* gene was excised, and DNA was purified using a gel extraction kit (Qiaquick; QIAGEN). PCR products were cloned using the Zero Blunt Topo PCR Cloning Kit (Invitrogen). Individual colonies were picked for automated DNA preparation and sequencing at the University of Chicago Cancer Research Center DNA Sequencing Facility.

2.4 Real-time RT-PCR

Total RNA was made from PNA-high and PNA-low B-cells with RNA STAT-60 (Tel-Test Inc). cDNA was made by the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) using oligo-dT. Real-time PCRs were run using gene specific primers and analyzed on a MYiQ system with SYBR Green SuperMix (Bio-Rad Laboratories). PCR conditions were 95°C for 30 s, 58°C for 45 s, and 72°C for 60 s for 40 cycles. Mouse beta actin was used as a reference for the relative quantification of *pol eta*, *pol iota* and *Msh2* using the Pfaffl method (Pfaffl, 2001). The analysis was done in duplicates. The primers used in this analysis are shown in Supplementary Table 1b.

2.5 ELISA assays

ELISA assays were performed using the SBA clonotyping System/AP kit according to the manufacturer's instructions (cat # 5300-04, Southern Biotechnology Associates) as described (Shen et al., 2006). The concentration of the immunoglobulins in the samples was calculated based on the standard readings. The analysis was done in duplicates.

2.6 Hemagglutinin assays

Serum was diluted 1:1 with 1× PBS and incubated for 30 minutes at 50° C. Serial dilutions of 1:2 were mixed with equal volumes of sheep red blood cells (2×10^8 cells/ml). The highest dilution at which agglutination was seen is noted in Table 1.

2.7 Statistical Analyses

The p-values were calculated using Fisher's exact test for 2×2 contingency tables. For example, for the WRC, GYW hotspot comparison, a 2×2 table of mutation counts was constructed, where the rows index mice (*p53*^{−/−} versus controls) and the columns index type of mutations (mutations in WRC, GYW hotspots versus other mutations at C and G).

3. Results

3.1 General immune status of *p53*^{−/−} mice

All mice in this study responded with essentially equal anti-sheep red blood cell (SRBC) titers four days after a secondary immunization with SRBC (Table 1). The serum levels of isotype switched Igs were the same in *p53*^{−/−} mice as in their wildtype littermates (Fig. 1).

Others also found no effect of *p53* inactivation on class switch recombination (Ramiro et al., 2006). As expected, in all *ung*^{-/-} mice IgM in the serum was raised considerably, and the levels of IgG1, IgG2a and IgG3 were at least two-fold lower than in *ung* wildtype littermates. These isotype changes are as in previous studies with *ung*^{-/-} mice (Rada et al., 2002; Shen et al., 2006). As before, we found no decrease in IgG2b in *ung*^{-/-} mice (Shen et al., 2006). Normal levels of IgA had been noticed before in young *ung*^{-/-} mice, but not in very old mice (Rada et al., 2002). The mice in our study were young. These findings suggest that the overall immune responsiveness was not dependent on *p53*. Furthermore, lack of *p53* did not ameliorate the inhibition of isotype switching in *ung*^{-/-} mice (Fig. 1). The total numbers of nucleated spleen cells were similar in all mice, except that they were drastically increased in the oldest, 16 week old *p53*^{-/-} mouse (Table 1). Surprisingly though, the numbers of total PNA-hi (= germinal center) B cells were similar in the oldest *p53*^{-/-} mouse and its wildtype littermate (Table 1).

3.2 Production of *p53*^{-/-} MMR or BER double-deficient mice

Since the major DNA repair systems involved in SHM are mismatch repair (MMR) and base excision repair (BER) (reviewed in (Longerich et al., 2006) we wanted to produce double-knockout mice of essential components of these systems and *p53* to help explain the mutation patterns described below. We were unable to obtain any double-knockout mice for Msh6, the partner of Msh2 in the repair of single base-mismatches. Among 115 offspring of multiple crosses, we would have expected to obtain ~17 *Msh6*^{-/-}*p53*^{-/-} pups. None were obtained. Thus, deletion of both genes may be embryonic lethal (Young et al., 2007). We obtained the expected ratios of *p53*^{-/-} offspring. Double-knockout offspring for *p53* and *ung* were at about 50% of the expected ratios. We did not find tumors in any of the *p53*^{-/-} mice, regardless of the *ung* genotype. Presumably they were too young. Tumors have been seen in *p53*^{-/-} mice before 10 weeks of age, but the average time to tumor appearance was 20 weeks (Donehower et al., 1992). We analyzed most of the mice at only 8 weeks of age since their SHM frequency was normal at that time (Table 2A). The oldest *p53*^{-/-} mouse in this study was sacrificed at 16 weeks. It had no obvious tumors, but an enlarged spleen.

3.3 Somatic hypermutation of Ig genes in *p53*^{-/-} mice

PNA-hi B cells were isolated from mice four days after secondary immunization with SRBCs and DNA sequences of cells with JH4 rearrangements of the VHJ558 gene were determined (Jolly et al., 1997). Strain 129 IgH sequences from the three mice with 129 and B6 IgH genes were compared to the 129 sequence, and B6 sequences were compared to B6, respectively. All mice had mutations in the 5' region of the JH4-Cmu intron. In wildtype controls 48 to 65% of the sequences had mutations (Table 2A (d), Mut/total clones). This measure of mutability was greatly reduced to 16 and 8% of the sequences in the older *p53*^{-/-} mice (Table 2A (d) #2 and 4 compared with #1 and 3), but not in the youngest mice, regardless of their *p53* and/or *ung* genotype (Table 2A (d) #6, 10 and 11 compared with #5, 8, and 9, respectively). Also, the mutation frequencies per 10³ nucleotides in sequences with mutations were reduced 2-fold or more in the older *p53*^{-/-} mice compared with their wt littermates, but not in the youngest mice (Table 2A (e) #2 and 4, compared with their wt littermates, #1 and 3). We assume that SHM in the young (8 week old) mice represents the most direct effect of the deletion of *p53* on SHM and have therefore analyzed the effect of additional *ung*-deletion only in 8 week old mice. The reduction of mutations in the older mice (Fig. 2) may be a signature of cumulative events in the B cells already before immunization. In addition, the drastically reduced mutability in the oldest *p53*^{-/-} mouse may be due to stress of SHM in the overall more severely compromised environment that then eliminated cells with a high mutation load. This may relate mainly to non-Ig genes. E.g. the tumor suppressor gene *Bcl6* is a relatively efficient target of AID (Pasqualucci et al.,

1998; Peng et al., 1999; Shen et al., 1998) and many other non-Ig genes are subject to SHM (Liu et al., 2008; Storb et al., 2009).

We have no evidence that p53 is involved in the targeting of AID. The mutation frequencies for C + G in the *p53*^{-/-}*ung*^{-/-} mice is 4.51×10^{-3} nts, compared with 4.64×10^{-3} for the *p53* wt, *ung*^{-/-} controls. Most of these are transition mutations, an even more directly correlated measure of AID targeting. The mutation frequencies for C+G transitions were 4.3×10^{-3} and 4.27×10^{-3} in the *p53*^{-/-}*ung*^{-/-} versus their *p53* wt, *ung*^{-/-} controls, respectively. The lack of an effect of *p53* inactivation on AID targeting is also supported by the finding that isotype switching is not altered (Fig. 1). Finally, there is no change in the targeting of AID to the known AID hotspots (Supplementary Table 2). The Ig class switch recombination (CSR) in *p53*^{-/-} mice is not altered (Fig.1) presumably because AID and ung are sufficient, and lesion bypass polymerases are not required for CSR.

However, there are significant changes in mutation patterns in the *p53*^{-/-} mice. A major change in the pattern of SHM was seen with mutations at A. The percentage of A mutations over total mutations in mutated sequences ranged narrowly between 30 and 36% in the *p53* wt mice, similar to what is generally found for SHM in normal mice (Table 2B (a), # 1, 3, 5, and 7; summarized in Table 2C). In the *p53*^{-/-} mice, however, mutations at A were at 45 to 72% of total mutations (Table 2B (a), # 2, 4, 6); summarized in Table 2C). The most significant change in mutations at A were A to G transitions (Table 2B (b); summarized in Table 2C). Surprisingly, when *ung* was deleted in addition to the deletion of *p53*, the A mutations over total mutations and the % mutations at A to G were normal: only 30 % of all mutations were at A and 13 or 15% of all mutations were A to G (Table 2B (b), #10–11; summarized in Table 2C). This suggests that when *p53* is deleted, the increased mutability at A depends on ung.

As observed previously (Rada et al., 2002; Shen et al., 2006), all *ung*^{-/-} mice have increased C to T and G to A transition mutations (Table 2B (b), #8–11; summarized in Table 2C). This is not affected by the *p53* status.

There is also a significant reduction of C to A transversion mutations in *p53*^{-/-} mice. Unlike the A-G transitions, this C-A transversion is independent of ung since the reduction of C -A is still prominent in *ung*^{-/-} mice (Table 2B (b)). The C-A changes may relate to a p53 effect on mismatch repair during transcription or while the leading strand DNA is replicated. The C-A change may be also strand-biased since the complementary reductions of G to T transversions are significant in *p53*^{-/-} mice only in the presence of ung. However, this interpretation is complicated by the very low G-T transversion mutation frequencies in *ung*^{-/-} mice. While G-A and C-T transitions are high in *ung*^{-/-} mice, as expected, it is not clear why there is an imbalance in C-A versus G-T transversions in *ung*^{-/-} mice with wt *p53* (Table 2B (b)).

Mice deficient in p21 did not show the SHM changes observed in the *p53*^{-/-} mice (Table 2D). This suggests that the pathway from p53 through p21 is not involved in SHM. Most likely, p53 affects SHM because p53 protein interacts with proteins involved in SHM (Table 3; see Discussion).

4) Discussion

4.1 Increased mutations at A

There are several known functions of p53 that may relate to factors known to be involved in SHM. p53 protein interacts directly or indirectly with a considerable number of factors, and p53 can act as an activator or suppressor of transcription by interacting with regulatory DNA

sequences, as summarized in Table 3. Deletion of *p53* decreases BER (Table 3 b.; (Offer et al., 2001; Offer et al., 1999; Smith and Seo, 2002; Zhou et al., 2001). Reduced general BER would be expected especially due to reduced activity of pol beta (Table 3 d., e.; (Seo et al., 2002; Zhou et al., 2001). However, SHM invoking error-prone polymerases may be more active in *p53*^{-/-} mice because the reduced activity of the relatively high fidelity pol beta may allow error-prone lesion bypass polymerases, such as pol eta or pol iota (see below), to be more efficiently recruited. In addition, ung activity would be increased, and the transcription of the *APE1* gene would be increased, thus helping the resolution of the AID-induced uracil by error-prone BER (Table 3 f., i.; (Lu et al., 2004; Zaky et al., 2008). Furthermore, it has been shown in cell lines that *p53* decreases the efficiency of translesion DNA synthesis, but increases its fidelity (Table 3 g.; (Avkin et al., 2006). Complementing this finding, deletion of *p53* increased the mutagenesis by translesion polymerases (Table 3 h.; (Avkin et al., 2006). Thus, error-prone repair during SHM would increase in the absence of *p53*. Apparently, the expected decreased transcription of the *Msh2* and *PCNA* genes in the absence of *p53* (Table 3 j., k.; (Scherer et al., 2000; Shan and Morris, 2005) is not sufficiently severe to reduce SHM below the levels of *p53* wild type mice, except perhaps in the older mice, but see above (first paragraph in section 3.3). In fact, we found a slight increase of stable *Msh2* mRNA in *p53*^{-/-} mice (Fig. 3).

These considerations lead to the conclusion that the increased mutation frequencies at A are mainly due to less restricted recruitment of translesion DNA polymerases in *p53*-deficient cells during SHM. Any changes of SHM in *p53*^{-/-} mice that are normalized in the absence of *ung* are likely due to BER that depends on the creation of an abasic site by ung.

The dependence of A mutations on ung is contrary to the current dogma that most A (and T) mutations are due to coupling of pol eta with mismatch repair (MMR), not with ung-initiated base excision repair (BER) (Delbos et al., 2005; Longerich et al., 2006). However, in the *p53*^{-/-} mice only the mutations at A, not those at T are increased. Thus, there is an A>T strand bias (Table 2B (i)) which is even higher than normal for SHM (Longerich et al., 2006; Storb et al., 1998). We assume that mutations at A indicate that a T in the transcribed (template) strand has been copied erroneously. Meaning that the non-transcribed (coding) strand had acquired an AID-induced uracil and that a "long" patch of nucleotides had been replaced by error-prone DNA synthesis, rather than only the uracil (Klungland and Lindahl, 1997). If the excess of mutations at A over T was created by the same mechanism that mutates C and G, one would expect a corresponding strand-bias for mutations at C and G, namely increased mutagenesis at C over G, representing error-prone copying of G on the transcribed strand. However, while in the *p53*^{-/-} mice the A/T ratio increases compared with wildtype, the C/G ratio decreases in the two oldest mice and remains about the same in the youngest mouse (Table 2B (j) #2, 4, 6 versus #1, 3, 5). This suggests that the effect of *p53* is strand-biased for A mutations, but a correlated strand bias for C/G mutations does not occur. The increase of mutations at A in *p53*^{-/-} mice depends on ung and appears to involve the recruitment of translesion polymerases preferably during the error-prone synthesis in the non-transcribed strand.

AID targeting of the non-transcribed strand would require that this strand be single-stranded at the time of AID action. During transcription of linear, double-stranded DNA in vitro, AID has been found to mainly target the non-transcribed strand, suggesting that it attacks DNA in the transcription bubble (Chaudhuri et al., 2003; Pham et al., 2003). However, in the case of AID action in double-stranded supercoiled DNA that was transcribed, a strand bias was not observed (Shen et al., 2005; Shen and Storb, 2004; Shen et al., 2009). This suggested that AID may interact on both DNA strands with flipped-out cytosines created in negative supercoils behind the transcribing RNA polymerase. Since there is no evidence that AID targeting is altered in *p53*^{-/-} mice, the strand bias is unlikely related to altered strand-

specific AID targeting. It may therefore relate to the recruitment of BER or MMR or lesion bypass DNA polymerases. Strand specificity in the treatment of AID created uracils could relate to transcription. Alternatively, it could relate to DNA replication. There is a strong origin of replication at the 3' end of the IgH locus in mature B cells (Zhou et al., 2002). Thus in the mutating B cells, the leading strand would be the non-transcribed strand. In summary, the reason for the observed strand bias of A mutations in *p53*^{-/-} mice currently has not been resolved.

4.2 Does pol iota play a role in SHM of *p53*^{-/-} mice?

Mutations at A and T during SHM in mice were found to be entirely due to pol eta (Delbos et al., 2007). Since the increase of mutations at A in *p53*^{-/-} mice could have been due to increased pol eta, we determined the levels of stable pol eta transcripts in PNAhi B cells of *p53*^{-/-} and wt control mice (Fig. 3). There was no difference. Pol iota, on the other hand, seemed unlikely to play a role in SHM of mice, since the pattern and frequencies of mutations during SHM were reported to be the same in the 129 strain mice that are deficient in pol iota as in pol iota wildtype mice (C57BL/6) (McDonald et al., 2003). However, in a human B cell line, pol iota-deficiency caused a major reduction in SHM that was remedied by the expression of a transfected *pol iota* gene (Faili et al., 2002a). Interestingly, the human cell line assayed in that study, BL2, is deficient in p53 (Gao et al., 1999). It is therefore possible, that pol iota was allowed to be active in this cell during SHM, whereas in the presence of p53 (in germinal center B cells) pol iota may be prohibited from access to post-C-deamination processes. Levels of pol iota mRNAs were not changed in *p53*^{-/-} mice. Comparing the error rates of pol iota with those of pol eta at different nucleotides (Table 4) shows that A to G mutations are a hallmark of the error rates of pol iota (Tissier et al., 2000). When pol iota copies undamaged DNA, it copies T with G three times more often than copying it with the correct A. Table 4 summarizes some of the mutation patterns in *p53*^{-/-} mice in parallel with the error rates of pol iota versus those of pol eta. Both pol iota and pol eta are candidates for causing the increase in A to G mutations in *p53*^{-/-} mice. However, pol iota is 52x more error prone for this process. On the other hand, pol iota is highly error-free when copying A, resulting in very few T to N mutations (Table 4 (c)), while pol eta has error rates for copying A that are only slightly lower for copying A with G or even higher for copying A with A or C than its error rates for copying T (Kunkel et al., 2003).

Clearly, there is no increase in mutations at T in *p53*^{-/-} mice (Table 2B (a), (b); Table 4) as would have been expected if the mutations were due to increased activity of pol eta. Thus it is possible that mutations are created at A and T by the action of pol eta in the presence of p53, but in the absence of p53, mutations are increased at A and decreased at T because pol iota partially replaces pol eta.

The effects of *p53* inactivation on SHM reveal another novel role added to the vast repertoire of p53 functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to B. Kee for technical advice and use of her instrument for Real Time PCR, W. Buikema and C. Hall for DNA sequencing, and R. Duggan for flow cytometric cell sorting. We also thank T.E Martin for many suggestions for the manuscript. The work was supported by National Institutes of Health grants AI047380 and AI053130. S.R. was supported by a postdoctoral fellowship from the Cancer Research Institute.

Appendix

There are three Supplementary Tables 1A, 1B, and 2.

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Fig.1. CSR activity in wildtype and *p53*^{-/-} mice

The data shown are an average of two independent ELISA experiments. The numbers on the x-axis refer to the mouse numbers in Table 2A. The y-axis shows the concentration of specific immunoglobulins.

Fig.2. Mutation frequencies in wildtype and *p53*^{-/-} mice

The pie charts depict the number of point mutations per DNA clone (1.24 kb). The numbers in the central circle are the total number of clones sequenced. The numbers in parentheses () refer to the mouse numbers in Table 2A.

Fig. 3. Relative mRNA expression in wildtype and *p53*^{-/-} mice

The data shown are an average of two independent real time RT-PCR assays. The *pol eta*, *pol iota*, *Msh2* and AID (*aicda*) mRNA levels were normalized to *beta actin* mRNA (shown as 1). The y-axis shows the mRNA levels of *pol eta*, *pol iota*, *Msh2* and *aicda* in *p53*^{-/-} mouse relative to levels in wildtype mouse.

Table 1General status of *p53*^{-/-}, *p21*^{-/-}, and wildtype mice

Mouse ^a	Age	Anti-SRBC ^b	No. of PNA-hi B cells	Total no. of nucleated spleen cells
(1) wildtype mouse 1	11 weeks	1/1024	195,428	72 × 10 ⁶
(2) <i>p53</i> ^{-/-} mouse 1	11 weeks	1/1024	205,616	74 × 10 ⁶
(3) wildtype mouse 2	16 weeks	1/1024	251,645	84 × 10 ⁶
(4) <i>p53</i> ^{-/-} mouse 2	16 weeks	1/1024	270,228	226 × 10 ⁶
(5) wildtype mouse 3	8 weeks	1/2048	229,312	98 × 10 ⁶
(6) <i>p53</i> ^{-/-} mouse 3	8 weeks	1/2048	240,041	104 × 10 ⁶
(7) <i>ung</i> control mouse	8 weeks	1/2048	265,482	84 × 10 ⁶
(8) <i>ung</i> ^{-/-} mouse	8 weeks	1/2048	227,856	80 × 10 ⁶
(9) <i>p53</i> ^{+/-} <i>ung</i> ^{-/-} mouse	8 weeks	1/1024	215,221	96 × 10 ⁶
(10) <i>p53</i> ^{-/-} <i>ung</i> ^{-/-} mouse 1	8 weeks	1/1024	220,662	89 × 10 ⁶
(11) <i>p53</i> ^{-/-} <i>ung</i> ^{-/-} mouse 2	8 weeks	1/1024	218,016	92 × 10 ⁶
<i>p21</i> wildtype mouse	8 weeks	1/1024	~185,000	90 × 10 ⁶
<i>p21</i> ^{-/-} mouse	8 weeks	1/1024	~180,000	83 × 10 ⁶

^aThe numbers in parenthesis refer to the mouse numbers in Table 2A.^bHemagglutinin assay

Table 2A

SHM is altered in *p53*^{-/-} mice

Mouse	1	2	3	4	5	6	7	8	9	10	11
	wt1	<i>p53</i> ^{-/-} 1	wt2	<i>p53</i> ^{-/-} 2	wt3	<i>p53</i> ^{-/-} 3	wt <i>ung</i> ⁺ (a)	<i>ung</i> ^{-/-}	<i>p53</i> ^{+/+} <i>ung</i> ^{-/-}	<i>p53</i> ^{-/-} <i>ung</i> ^{-/-} 1	<i>p53</i> ^{-/-} <i>ung</i> ^{-/-} 2
Tot cIs(b)	50	87	37	123	123	106	66	60	80	98	90
Mut cIs(c)	24	7	22	20	61	63	43	37	42	42	42
Mut/tot(d)	0.48	0.08	0.59	0.16	0.49	0.59	0.65	0.62	0.53	0.43	0.47
Tot muts(e)	153	18	189	91	219	270	285	302	216	370	196
Mut/10 ³ (f)	5.13	2.07	6.92	3.66	2.89	3.45	5.34	6.57	4.14	7.09	3.76
	2<1 p3×10⁻⁷ 4<3 p7×10⁻⁵										
Age	16 weeks	16 weeks	11 weeks	11 weeks	8 weeks	8 weeks	8 weeks	8 weeks	8 weeks	8 weeks	8 weeks

(a) wt littermate of *ung*^{-/-} mice

(b) Total clones sequenced

(c) Total clones with mutations

(d) Proportion of total clones that had mutations

(e) Total mutations in all sequences

(f) Mutations per 10³ nucleotides in mutated sequences. The decreased mutation frequencies in mice *p53*^{-/-} 2 and 4, compared with their wt littermates is highly significant.

Table 2B

The pattern of SHM is altered in *p53*^{-/-} mice

Mouse	1	2	3	4	5	6	7	8	9	10	11
	wt1	<i>p53</i> ^{-/-} 1	wt2	<i>p53</i> ^{-/-} 2	wt3	<i>p53</i> ^{-/-} 3	wt <i>ung</i> ⁺ (a)	<i>ung</i> ^{-/-}	<i>p53</i> ^{+/+} <i>ung</i> ^{-/-} 1	<i>p53</i> ^{-/-} <i>ung</i> ^{-/-} 1	<i>p53</i> ^{-/-} <i>ung</i> ^{-/-} 2
A mut % (a)	32	72	33	50	30	45	36	31	34	30	30
A-G % (b)	13	22	15	25	14	23	17	15	20	13	15
			2+4+6 > 1+3+5 p4×10⁻⁴(c)				10+11 = 8+9 p0.13				
A-C % (b)	11.8	22.2	9.5	13.2	7.3	11.1	7	7.9	7.4	6.8	8.7
A-T % (b)	6.5	28	8.5	11	8.7	11	11.6	7.6	6.9	10.3	6.1
			2+4+6 > 1+3+5 p0.06(d)				10+11 = 8+9 p0.38				
C-T % (b)	5	0	10	7	7	11	10	14	14	19	18
			2+4+6 > 1+3+5 p0.06(d)				8+9+10+11 > 1+3+5+7 p8.5×10 ⁻⁸ (g)				
C-A % (b)	4.6	0	2.6	0	5.5	1.9	1.1	2.6	3.2	0.2	0.5
			2+4+6 < 1+3+5 p0.01(e)				10+11 < 8 + 9 p8×10⁻⁴(e)				
C-G % (b)	1.3	0	2.6	3.3	4.6	1.1	3.9	0	0	0.3	0.5
G-A % (b)	9	6	15	10	15	7	14	30	26	29	20
			2+4+6 < 1+3+5 p0.01(f)				10+11 = 8+9 p0.41				
			2+4+6 < 1+3+5 p0.01(f)				8+9+10+11 > 1+3+5+7 p3.4×10 ⁻¹³ (g)				
G-C % (b)	13.7	11.1	7.9	7.7	6.4	7.4	6	0.7	0.5	0.5	1
G-T % (b)	9.8	0	3.7	5.5	4.1	1.9	4.9	0.3	0	1.1	0
			2+4+6 < 1+3+5 p0.03(h)				10+11 = 8+9 p0.37				
T mut % (a)	25.1	11.5	25.3	17.3	25	23.9	23.7	20.6	21.5	19.7	29.7
T-C % (b)	12	6	11	3	14	12	10	8	16	8	17
T-A % (b)	8.5	5.5	9	6.6	7.3	7.8	7.7	7.3	3.2	6.8	7.1
T-G % (b)	4.6	0	5.3	7.7	3.7	4.1	6	5.3	2.3	4.9	5.6
A/T(i)	1.26	6.5	1.29	2.81	1.25	1.85	1.52	1.48	1.82	1.49	1.02
C/G(j)	0.34	0	0.58	0.43	0.75	0.84	0.58	0.59	0.68	0.63	0.9

Mouse	1	2	3	4	5	6	7	8	9	10	11
	wt1	<i>p53</i> ^{-/-} 1	wt2	<i>p53</i> ^{-/-} 2	wt3	<i>p53</i> ^{-/-} 3	wt <i>ung</i> ⁺ (d)	<i>ung</i> ^{-/-}	<i>p53</i> ^{+/+} <i>ung</i> ^{-/-}	<i>p53</i> ^{-/-} <i>ung</i> ^{-/-} 1	<i>p53</i> ^{-/-} <i>ung</i> ^{-/-} 2
Age	16 weeks	16 weeks	11 weeks	11 weeks	8 weeks	8 weeks	8 weeks	8 weeks	8 weeks	8 weeks	8 weeks

- (a) Proportion of total mutations that were at A or at T
- (b) Proportion of total mutations that were the respective changes
- (c) The increase in A-G in the three *p53*^{-/-} mice (#2, 4, 6) compared with their wt littermates is highly significant (Fisher's exact test).
- (d) The increase in A-T in the three *p53*^{-/-} mice (#2, 4, 6) compared with their wt littermates is borderline significant. The increase in A-C mutations is not significant (p0.16)
- (e) The decrease in C-A in the three *p53*^{-/-} mice (#2, 4, 6) and two *p53*^{-/-}*ung*^{-/-} mice (#10, 11) compared with their *p53*⁻wt littermates is significant
- (f) The decrease in G-A in the three *p53*^{-/-} mice (#2, 4, 6) compared with their wt littermates is significant
- (g) C-T and G-A transitions are significantly different between all *ung*^{-/-} mice and all wt mice
- (h) The decrease in G-T in the three *p53*^{-/-} mice (#2, 4, 6) compared with their littermates is significant
- (i) % mutations at A/% mutations at T
- (j) % mutations at C/% mutations at G
- Bold: mutations in *p53*^{-/-} mice are significantly different (> or <) from *p53*⁻wt**

Table 2C

Summary of a subset of mutations

Mice	A mutations (%)	A to G (%)	T to C (%)	C to T (%)	G to A (%)
Wt	33	15	12	8	13
p53-/-	56	23	7	6	8
P-value*	4×10 ⁻⁴	4×10 ⁻⁴	0.25	0.34	0.01
p53-/- ung -/-	30	14	13	19	25
P-value**	0.6	0.13	1.0	0.10	0.41
Ung-/-	31	16	12	16	26
P-value*	0.38	0.42	0.31	9×10 ⁻⁸	3×10 ⁻¹³

P-value

* versus wt;

** versus ung-/-

Bold, increased mutations at A and A-to-G in p53-/- versus wt

Individual mouse data are in Table 2B: wt = #1, 3, 5, 7; p53-/- = #2, 4, 6; p53-/- ung-/- = #11, 12; ung-/- = #8-11

Table 2D

SHM in *p21*^{-/-} mice is normal

Mouse	wt	<i>p21</i> ^{-/-}
Tot clones ^(a)	24	22
Mut clones ^(b)	10	8
Mut/tot clones ^(c)	42	36
Tot muts ^(d)	45	51
Muts/10 ³ ^(e)	3.62	5.1
A mutations ^(f)	14	17
A muts % ^(g)	31	33
C mutations ^(f)	4	6
C muts % ^(g)	9	12
G mutations ^(f)	17	15
G muts % ^(g)	38	29
T mutations ^(f)	10	13
T muts % ^(g)	22	25
Age	8 weeks	8 weeks

- (a) Total clones sequenced
- (b) Total clones with mutations
- (c) Proportion of total clones that had mutations
- (d) Total mutations
- (e) Mutations per 10³ nucleotides in mutated sequences.
- (f) Number of mutations at the indicated nucleotide
- (g) Proportion of total mutations that were at the indicated nucleotide

There are no significant differences between the wt and *p21*^{-/-} littermates.

Table 3

List of proteins, genes and mechanisms that are likely involved in SHM and that are regulated by p53

p53	Reference
a.Expressed in germinal center B cells	{Ranuncolo, 2007 #143}
b.Enhances BER	{Offer, 2001 #163; Offer, 1999 #164} {Zhou, 2001 #141} {Smith, 2002 #142}
c.Decreases mutagenesis	{Zhou, 2001 #141}
d.Pol beta almost absent in p53 ^{-/-} mice	{Seo, 2002 #162}
e.Interaction with pol beta required for enhanced BER	{Zhou, 2001 #141}
f.Decreases Ung2 activity due to activation of phosphatase	{Lu, 2004 #77} }
g.Decreases efficiency of translesion DNA synthesis	{Avkin, 2006 #161}
h.Deletion of p53 increases mutagenesis by translesion pols.	{Avkin, 2006 #161}
i.Reduces transcription of APE1 gene	{Zaky, 2008 #56}
j.Increases transcription of Msh2 gene	{Scherer, 2000 #55}
k.Increases transcription of PCNA gene	{Shan, 2005 #54}

Table 4

Altered mutation pattern in *p53*^{-/-} mice may be due to activation of pol iota

Mutation	Observed mutations		Change ^(a)	P-value ^(b)	Reported error rates of translesion polymerases		
	% of total mutations	wt			pol iota error rate ^(c)	pol eta error rate ^(d)	error rate iota/eta ^(e)
A to G	15	23	UP	0.0004	3×10 ⁰	5.8×10 ⁻²	52× UP
A to T	8	17	UP	0.06	6.7×10 ⁻¹	6×10 ⁻³	112× UP
T to N	27.3	17.6	DOWN	0.25	1-2×10 ⁻⁴	8-14×10 ⁻³	40-141× DOWN

^(a) Increased or decreased mutations in *p53*^{-/-} versus wt mice

^(b) P-value of mutations in *p53*^{-/-} versus wt mice

^(c) Tissier et al., 2000

^(d) Kunkel et al., 2003

^(e) Increased or decreased error rate of pol iota compared with pol eta