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# Increased genomic instability is not a prerequisite for shortened lifespan in DNA repair deficient mice

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**Abstract**

Genetic defects in nucleotide excision repair (NER) are associated with premature aging, including cancer, in both humans and mice. To investigate the possible role of increased somatic mutation accumulation in the accelerated appearance of symptoms of aging as a consequence of NER deficiency, we crossed four different mouse mutants, $Xpa^{-/-}$, $Ercc6(Csb)^{-/-}$, $Ercc2(Xpd)^{m/m}$ and $Ercc1^{-/m}$, with mice harboring *lacZ*-reporter genes to assess mutant frequencies and spectra in different organs during aging. The results indicate an accelerated accumulation of mutations in both liver and kidney of Xpa defective mice, which correlated with a trend towards a decreased lifespan. Until 52 weeks, Xpa deficiency resulted mainly in 1-bp deletions. At old age (104 weeks), the spectrum had undergone a shift, in both organs, to G:C → T:A transversions, a signature mutation of oxidative DNA damage. $Ercc1^{-/m}$ mice, with their short lifespan of 6 months and severe symptoms of premature aging, especially in liver and kidney, displayed an even faster *lacZ*-mutant accumulation in liver. In this case, the excess mutations were mostly genome rearrangements. $Csb^{-/-}$ mice, with mild premature aging features and no reduction in lifespan, and $Xpd^{m/m}$ mice, exhibiting prominent premature aging features and about 20% reduction in lifespan, did not have elevated *lacZ*-mutant frequencies. It is concluded that while increased genomic instability could play a causal role in the mildly accelerated aging phenotype in the Xpa-null mice or in the severe progeroid symptoms of the Ercc1-mutant mice, shortened lifespan in mice with defects in transcription-related repair do not depend upon increased mutation accumulation.


*Keywords:* DNA-repair; Aging; Mutation; Mice

*Abbreviations:* NER, nucleotide excision repair; GG-NER, global genome NER; TC-NER, transcription coupled NER; XP, xeroderma pigmentosum; CS, cockayne syndrome; TTD, trichothiodystrophy; ICLR, interstrand crosslink repair; ERCC1, excision repair cross complementing-group 1

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## 1. Introduction

Genomic instability is thought to play a major role in cancer and, possibly, aging [1,2]. To monitor tissue-specific patterns of somatic mutation accumulation during aging, we have developed a transgenic mouse model harboring chromosomally integrated *lacZ*-plasmids [3]. After recovery of the plasmids from genomic DNA and their transfer into *Escherichia coli*, the mutations in the *lacZ* gene that have arisen in the mouse can be quantified and characterized. Using this model, which is sensitive to a broad range of mutational events, including genome rearrangements, we previously demonstrated organ-specific differences in mutation accumulation with age [4–6]. Presumably, differences in organ function, including metabolism and genome maintenance, in association with replicative history, underlie the divergence in mutation spectra as an endpoint of endogenously and environmentally inflicted DNA damage.

A major factor determining patterns of mutation accumulation in different tissues of mammals is likely to be the network of DNA repair pathways evolved to cope with destructive effects of DNA damage [7]. We previously demonstrated that in mice, completely defective for the multi-step 'cut and patch' pathway of nucleotide excision repair (NER) due to the ablation of the gene *Xpa*, mutations at the *lacZ* locus accumulate significantly faster with age than in control animals [8]. However, NER is an intricate mechanism and removes a broad range of helix-distorting lesions, from UV-induced DNA damage and numerous chemical adducts to oxidative damage produced by endogenous metabolism [9]. Within NER two subpathways are recognized, differing in damage recognition but sharing the same repair machinery: global genome NER (GG-NER) for the removal of distorting lesions anywhere in the genome and transcription-coupled NER (TC-NER) for the elimination of distorting DNA damage that blocks transcription.

At least three human syndromes, xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD) are associated with heritable defects in NER and are characterized by UV-sensitivity [10]. XP-patients display a high (>2000-fold increased) incidence of sun (UV)-induced skin cancer, the frequency of internal tumors is about 10-fold elevated and accelerated neurodegeneration is frequently observed. The disorder arises from mutations in one of the seven GG-NER genes (*XPA–XPG*). CS shows no predisposition to cancer, but leads to severely impaired physiological and neurological development, including retarded growth, cachexia, sensorineural hearing loss, retinal degeneration and strongly reduced lifespan. CS is caused by mutations in the CSA or CSB genes, which are specifically involved in TC-NER, as well as transcription-coupled repair of non-NER transcription-blocking lesions, probably including several forms of oxidative damage. GG-NER is unaffected in CS. The lack of cancer predisposition in this disease is explained by increased sensitivity to DNA damage-induced apoptosis, thereby protecting against tumorigenesis. TTD can be caused by mutations in the XPD gene and adds the hallmark features of brittle hair, nails and scaly skin to the symptoms of CS. The helicase encoded by the XPD gene is one of the 10 subunits of basal transcription factor IIH (TFIIH), which is required for multiple processes: GG-NER, TC-NER of NER and non-NER lesions, as well as transcription initiation by RNA polymerase I and II.

Many NER genes have been knocked-out and/or subtly mutated in mice to create models for the aforementioned human disorders [11]. The *Xpa* gene product is involved in damage verification and guiding cleavage of the damaged strand of DNA. *Xpa* knockout ($Xpa^{-/-}$) mice are completely deficient for both GG-NER and TC-NER, but can repair transcription-blocking non-NER lesions, including many oxidative DNA damages, in contrast to CS cells. As a consequence, $Xpa^{-/-}$ cells, mice and patients, are hyper-sensitive to UV-irradiation and numerous genotoxic agents that distort the DNA helical structure [12]. The Csb protein is thought to be involved in displacing RNA polymerase stalled by a DNA lesion and recruiting the NER (and perhaps base excision repair) machinery to the site of the lesion. $Csb^{-/-}$ mice are deficient in transcription-coupled repair of NER and non-NER types of damage, while their GG-NER capacity remains intact [13]. They show mild aging features including reduced growth and neurologic dysfunction. The Csb-null mutation reduces spontaneous tumorigenesis [14]. Complete inactivation of the Xpd helicase is not viable in the mouse or in cells, due to the essential transcription initiation function of the TFIIH complex. By mimicking a point mutation found in a TTD-patient, de Boer et al. created viable Xpd-mutant mice, which show many hallmarks of TTD, including premature aging features [15,16]. At the level of DNA repair the $Xpd^{m/m}$ mutation causes a partial defect in both GG-NER and TC-NER of NER and presumably also non-NER lesions. The ERCC1-XPF complex forms an endonuclease, required for the 5′-incision to remove the damage-containing oligonucleotide during NER, but also essential for interstrand crosslink repair (ICLR) [17]. Hence, Ercc1 knockout ($Ercc1^{-/-}$) mice are deficient in GG-NER, TC-NER and ICLR. These

mice show a severe phenotype including runted growth, progressive neurological abnormalities, kyphosis, a short lifespan of about 3 weeks and liver and kidney dysfunction [18]. At the cellular level the Ercc1 defect leads to accelerated nuclear polyploidization. The combination of a knockout allele with a truncated Ercc1 allele (*Ercc1*$^{-/m}$), resulting in a protein lacking the last seven amino acids [18], delays the onset of the premature aging phenotype and extends the maximal lifespan to about 6 months.

To further investigate the role of the different components of NER in premature aging, including cancer, it is important to assess integrity of the somatic genome during the entire lifetime of the DNA repair defective mice. Here we present, for the first time, complete lifespan studies of the four aforementioned NER mouse mutants with a side-by-side comparison of accumulated somatic mutations at a *lacZ*-reporter locus crossed into each NER-deficient background. The results indicate accelerated mutation accumulation in the *Xpa*$^{-/-}$ and *Ercc1*$^{-/m}$ mice, defective in DNA repair per se, but not in the *Csb*$^{-/-}$ or *Xpd*$^{m/m}$, with defects in both repair and transcription.

## 2. Materials and methods

### 2.1. Animal breeding and maintenance

*Xpa*$^{-/-}$ [12], *Csb*$^{-/-}$ [13] and *Xpd*$^{m/m}$ [15] mice were backcrossed at least 10 generations to C57BL6/JIco mice (Charles River, France). Each NER-mutant mouse model was crossed with C57BL/6J pUR288(lacZ)-transgenic mice, line 30 [4], to breed homozygous NER-mutant mice that were hemizygous for the pUR288(lacZ) concatemer. The homozygous NER-mutant mice were compared to a common C57BL/6 wild-type cohort, hemizygous for the pUR288(lacZ) transgene. *Ercc1*$^{+/-}$ or *Ercc1*$^{+/m}$ FVB mice [18] were crossed with *Ercc1*$^{+/-}$ C57Bl/6 mice, homozygous for pUR288(lacZ), to generate either *Ercc1*$^{-/-}$ or *Ercc1*$^{-/m}$ mice, hemizygous for the pUR288 concatemer in a C57BL6FVB/n background. Homozygous and heterozygous Wt siblings served as controls. The Ercc1-mutant and control mice were maintained in the animal facilities of the Erasmus University (Rotterdam, The Netherlands). All other mouse cohorts were maintained in the animal facilities of the RIVM (Bilthoven, The Netherlands) under specific pathogen-free (SPF) conditions. The room temperature was 20 °C and the light/dark cycle was 12 h/12 h. Standard lab chow (Hope Farms, The Netherlands) and water were supplied ad libitum. The animals were kept in groups of four or less per cage after weaning. Animals in longevity cohorts were removed from the study only when found dead or moribund. Asphyxiation by $CO_2$ was used for scheduled sacrificing. Liver and kidney were dissected and snap frozen in liquid nitrogen for DNA isolation. All research was in accordance with all applicable federal guidelines and institutional policies.

### 2.2. Plasmid rescue and mutant frequency determination

DNA was extracted by routine phenol/chloroform extractions. Complete protocols for plasmid rescue and mutant frequency determinations with this model have been provided elsewhere [19]. Briefly, between 10 and 20 μg genomic DNA was digested with HindIII for 1 h in the presence of magnetic beads (Dynal) precoated with lacI–lacZ fusion protein. The beads were washed three times to remove the unbound mouse genomic DNA. Plasmids were subsequently eluted from the beads by IPTG. After circularization of the plasmids with T4 DNA ligase they were ethanol-precipitated and used to electrotransform *E. coli C (ΔlacZ, galE*$^{-}$*)* cells. One thousandth of the transformed cells was plated on the titer plate (with X-gal) and the remainder on the selective plate (with p-gal). The plates were incubated for 15 h at 37 °C. Mutant frequencies were determined as the number of colonies on the selective plates versus the number of colonies on the titer plate (times the dilution factor of 1000). Each mutant frequency is based on at least 300,000 recovered plasmids. The background mutant frequency of this system is about $1 \times 10^{-5}$, as determined in *E. coli*, and consists mostly of false positive size-change mutants at HindIII star-activity sites [20].

### 2.3. Mutant characterization

Mutant colonies were taken from the selective plates and grown at 37 °C overnight in 96-well round-bottomed plates containing 150 μl LB medium per well, supplemented with 25 μg/ml kanamycin and 150 μg/ml ampicillin. A plate replicator was used to spot about 1 μl of each overnight culture on standard LB-agar media containing 75 μg/ml X-gal to screen for galactose insensitive host cells containing wild-type plasmids [21] and subtract their background. The plate replicator was also used to transfer about 1 μl of each overnight cell culture to 96-well PCR plates containing 25 μl total volume HotStarTaq Master Mix (Qiagen) and 250 nM of each primer, pUR4923-F (5′ TGG AGC GAA CGA CCT ACA CCG AAC TGA GAT 3′) and pUR3829-R (5′ ATA GTG TAT GCG GCG ACC GAG TTG CTC TTG 3′). After 35 amplification cycles, 5 μl of each PCR product was AvaI-digested and size-separated on 1% agarose gels. Mutant plasmids with restriction patterns resembling or deviating from the wild-type restriction pattern were classified as “no-change” and “size-change” mutants, respectively. Fifty microliter 50% glycerol per well was added to the overnight cultures for long-term storage at −80 °C.

One microliter of selected mutant glycerol stocks were grown overnight at 37 °C in 6 ml LB medium and subsequently used for plasmid mini preparation (Spin Miniprep Kit, Qiagen). Sequence reactions of purified mutant plasmids were outsourced to Davis Sequencing (Davis, CA, USA). The returned chromatograms were analyzed with Sequencher (Gene Codes,

Ann Arbor, MI, USA). The primers used for the sequence reactions were the same as described earlier [21].

### 2.4. Statistical analysis

Survival curves were compared in pairs using the logrank test. Two-way analysis of variance (ANOVA) was used per organ to test statistical differences between mutant frequencies of three or more age groups, using age and genotype as a factor. If this overall test was significant, several Student's *t*-tests were performed to compare two groups at the time. For differences between two groups only, Student's *t*-tests were applied directly. The total mutant frequencies between organs were tested with a three-way ANOVA, using the factors genotype, organ and age. The point mutation spectra were analyzed using a modified Bayesian approach, as described previously [6]. The modifications entailed: marking the number of cells as stochastic, rather than the number of mutants; the table of independent mutations of site by treatment group was viewed as a product of multinomials, and we eliminated the use of frequentist tests in the Bayesian analysis.

## 3. Results

### 3.1. Lifespan of DNA repair-deficient mice

Lifespan studies were performed with 45 ad libitum fed male mice per aging cohort of Wt, $Csb^{-/-}$, $Xpd^{m/m}$ and $Xpa^{-/-}$ mice. All these strains were bred to a C57BL/6J background and were hemizygous for the pUR288(lacZ) transgene on Chromosome 11. The resulting survival curves (Fig. 1) show a 50% survival and maximal lifespan in weeks of 110 and 134 for $Xpd^{m/m}$, 118 and 162 for $Xpa^{-/-}$, 123 and 160 for $Csb^{-/-}$, and 125 and 168 for Wt mice, respectively. The Logrank test revealed a significant difference between the survival curves of $Xpd^{m/m}$ and Wt mice ($p < 0.0001$). The differences between $Xpa^{-/-}$ and Wt ($p = 0.0633$) and between $Csb^{-/-}$ and Wt ($p = 0.9482$) were not statistically significant. The hazard ratio of $Xpd^{m/m}$ versus Wt was 2.4 (95% CI: 2.1–5.6), indicating a higher mortality rate of these mutants. Similar results were obtained with female cohorts with all curves shifted to the left, due to the shorter lifespan of female C57Bl/6 mice (Wijnhoven et al., in preparation). The $Ercc1^{-/-}$ mice, in a mixed C57Bl/6:FVB/n genetic background, have a mean survival of 3 weeks and maximal lifespan of 4 weeks, while the $Ercc1^{-/m}$ mice have a 50% survival of 16 weeks and a maximal lifespan of 28 weeks (Niedernhofer, unpublished data).

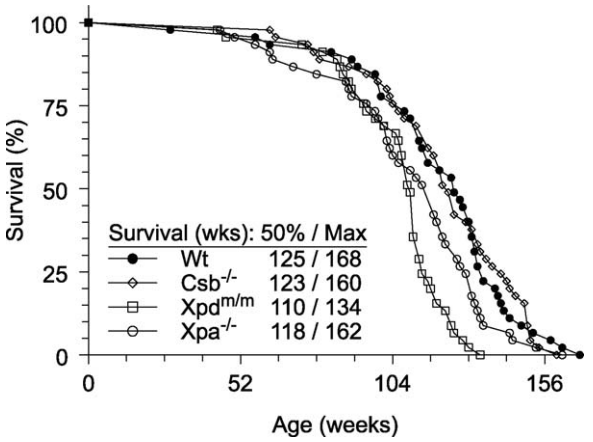


Fig. 1. Survival curves of male, NER-compromised mice.

### 3.2. Mutation accumulation in liver and kidney

Spontaneous mutant frequencies at the *lacZ* transgene locus were analyzed in liver and kidney of male Wt, $Csb^{-/-}$, $Xpd^{m/m}$ and $Xpa^{-/-}$ mice, 13, 52 and 104 weeks of age (Fig. 2). Four to 12 individual animals were analyzed per organ/genotype/age group. A significant age-related increase was found in all genotypes for both organs. The mean mutant frequency in liver of Wt

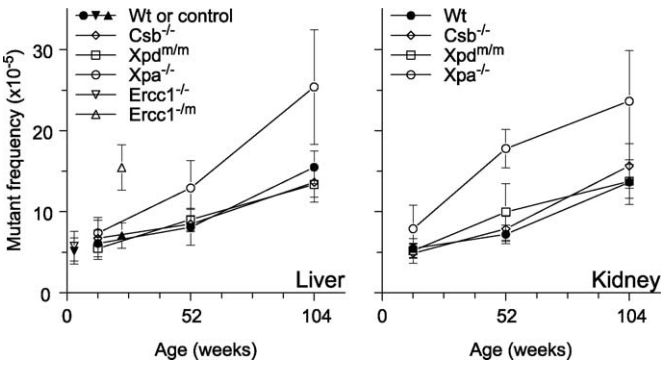


Fig. 2. Spontaneous *lacZ*-mutant frequencies (±S.D.) with age in liver and kidney of male NER-compromised mice. For numerical data, see Table 1.

Table 1
*LacZ*-mutant frequencies with age in liver and kidney of NER-compromised mice

| Organ | Genotype | Mean mutant frequency ± S.D. ($\times 10^{-5}$) | | | | |
|---|---|---|---|---|---|---|
| | | 3 weeks | 13 weeks | 23 weeks | 52 weeks | 104 weeks |
| Liver | Wt or control | 5.2 ± 1.6 | 6.1 ± 1.6 | 7.1 ± 1.6[a] | 8.1 ± 2.2[b] | 15.5 ± 2.0[c] |
| | *Csb*$^{-/-}$ | | 5.5 ± 1.4 | | 9.0 ± 1.4 | 13.4 ± 2.2 |
| | *Xpd*$^{m/m}$ | | 6.7 ± 2.6 | | 8.5 ± 0.9 | 13.6 ± 1.8 |
| | *Xpa*$^{-/-}$ | | 7.4 ± 1.6 | | 12.9 ± 3.4[b] | 25.4 ± 7.1[c] |
| | *Ercc1*$^{-/-}$ | 5.8 ± 1.8 | | | | |
| | *Ercc1*$^{-/m}$ | | | 15.5 ± 2.8[a] | | |
| Kidney | Wt | | 5.5 ± 1.2 | | 7.2 ± 1.1[d] | 13.6 ± 2.8[e] |
| | *Csb*$^{-/-}$ | | 5.2 ± 0.8 | | 10.0 ± 3.5 | 13.7 ± 2.1 |
| | *Xpd*$^{m/m}$ | | 4.9 ± 1.2 | | 7.9 ± 1.5 | 15.6 ± 2.7 |
| | *Xpa*$^{-/-}$ | | 7.9 ± 2.9 | | 17.8 ± 2.4[d] | 23.6 ± 6.2[e] |

Identical footnotes indicate the means compared; the resulting p-values are [a]$p = 0.0020$; [b]$p = 0.0045$; [c]$p = 0.0007$; [d]$p = 0.0002$;and [e]$p = 0.0085$.

mice was $6.1 \times 10^{-5}$ at 13 weeks and increased 2.5-fold to $15.5 \times 10^{-5}$ at 104 weeks of age (Table 1). During these 91 weeks of adult life, mutant frequencies in liver of *Csb*$^{-/-}$ and *Xpd*$^{m/m}$ mice were similar to Wt animals (Fig. 2). In contrast, the mean mutant frequencies at the two latest time points ($12.9 \times 10^{-5}$ and $25.4 \times 10^{-5}$ at 52 and 104 weeks, respectively), in livers of *Xpa*$^{-/-}$ mice were 1.6-fold increased over Wt (Table 1). An accelerated *lacZ*-mutant accumulation was not yet apparent in the liver of young, 13-week-old *Xpa*$^{-/-}$ mice, showing a mean mutant frequency of $7.4 \times 10^{-5}$. For the *Xpa*$^{-/-}$ genotype, this represented a 3.4-fold age-related increase in *lacZ*-mutant frequency between 13 and 104 weeks.

The results in the kidney were very similar to the situation in the liver (Fig. 2). The 2.5-fold mutant frequency increase from 13 to 104 weeks of age ($5.5 \times 10^{-5}$–$13.6 \times 10^{-5}$) in Wt mice was closely mimicked by *Csb*$^{-/-}$ and *Xpd*$^{m/m}$ mice, whereas the mean mutant frequencies in the kidney of *Xpa*$^{-/-}$ mice at 52 and 104 weeks ($17.8 \times 10^{-5}$ and $23.6 \times 10^{-5}$, respectively), corresponded to a 1.7-fold increase over Wt. As in liver, a significantly increased *lacZ*-mutant accumulation was not yet evident in the young, 13-week-old *Xpa*$^{-/-}$ mice, averaging $7.9 \times 10^{-5}$. In kidney of *Xpa*$^{-/-}$ mice, the age-related increase in mutant frequency at the *lacZ* locus was 3-fold. No significant differences were found between liver and kidney within genotypes.

In addition to the three relatively long-lived NER-deficient mice, spontaneous mutant frequencies were analyzed in liver of *Ercc1*$^{-/-}$ and *Ercc1*$^{-/m}$ mice, at 3 and 23 weeks of age, respectively. Although 3 weeks of age is close to the maximum lifespan of *Ercc1*$^{-/-}$ mice, there was no increased mutant frequency in liver compared to sibling controls (Fig. 2). In contrast, 23-week-old *Ercc1*$^{-/m}$ mice, also close to their maximum lifespan, showed a significant two-fold increased *lacZ*-mutant frequency compared to their sibling control mice (Fig. 2 and Table 1; $15.5 \times 10^{-5}$ and $7.1 \times 10^{-5}$, respectively).

### 3.3. Different types of mutations in *Xpa*$^{-/-}$ and *Ercc1*$^{-/m}$ mice

Next, mutant *lacZ*-plasmids recovered from the two mutants exhibiting increased spontaneous mutagenesis (Xpa and Ercc1) were characterized at the molecular level to determine whether the different repair defects resulted in distinct mutation spectra. A subclassification was made between "no-change" and "size-change" mutations, based on restriction fragment length variation compared to the Wt *lacZ*-plasmid. This analysis detects alterations of ≥50 bp as size-change mutants. About 48 mutants per mouse of four mice per organ/genotype/age group were categorized for all groups that showed a significant increase in total mutant frequency (Fig. 2) over the corresponding control group: liver and kidney of 1- and 2-year-old *Xpa*$^{-/-}$ mice, and liver of 23-week-old *Ercc1*$^{-/m}$ mice. The no-change mutant frequencies in liver were increased 1.5-fold both at 1 and 2 years of age ($p < 0.02$) in *Xpa*$^{-/-}$ compared to Wt mice (Fig. 3). In kidney a significant ($p < 0.002$) four-fold increase in no-change mutant frequencies was found in 1-year-old *Xpa*$^{-/-}$ over Wt animals. At 2 years of age, both no-change and size-change mutations were elevated in this organ (1.4- and 1.9-fold, respectively), albeit not significantly. In liver of 23-week-old *Ercc1*$^{-/m}$ mice, both no-change and size-change mutant frequencies were significantly ($p < 0.007$) increased, two- and three-fold, respectively, compared to sibling controls (Fig. 3). These

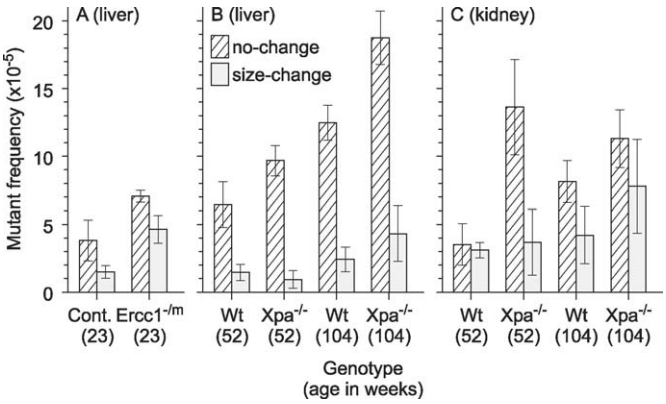


Fig. 3. Mean frequencies (±S.D.) of no-change and size-change mutants in (A) liver of 23-week-old sibling control and *Ercc1*$^{-/m}$ mice; (B) liver of 52- and 104-week-old Wt and *Xpa*$^{-/-}$ mice; and (C) kidney of 52- and 104-week-old Wt and *Xpa*$^{-/-}$ mice.

results indicate that a pure NER defect (as in *Xpa*$^{-/-}$ mice) mainly results in an induction of no-change mutations, whereas size-change mutations were also elevated in the liver of *Ercc1*$^{-/m}$ mice, likely reflecting their additional defect in ICLR.

### 3.4. Genome rearrangements in *Ercc1*$^{-/m}$ mice

Size-change mutants recovered from mutant and their sibling control mice were sequenced to investigate whether the ICLR defect of *Ercc1*$^{-/m}$ mice resulted in particular types of rearrangements (Tables 2 and 3). One of the 20 sequenced size-change mutants from *Ercc1*$^{-/m}$ livers was a *lacZ*-internal deletion of 2196 bp; the remaining 19 were genome rearrangements with one breakpoint in the *lacZ*-reporter gene and one elsewhere in the mouse genome, recognized as a plasmid carrying a truncated *lacZ* gene fused with a piece of the mouse genome [22]. Five of 19 sequenced size-change mutants from control livers were *lacZ*-internal deletions ranging from 536 to 3000 bp in size; the remaining 14 mutants were genome rearrangements. This difference in internal deletions between mutant and Wt was not statistically significant. The recovered fragments of the mouse genome in the genome rearrangement-derived mutant plasmids were blasted against the public mouse genome sequence to identify the location of the breakpoints and the surrounding sequences. The sequences at the breakpoints of both *lacZ*-internal size-change mutants and genome rearrangements, whether derived from *Ercc1*$^{-/m}$ or control mice, did not yield extended regions of homology (Table 2). Occasionally 1–8 bp stretches of micro-homology were found at the breakpoints, as reported for another *lacZ*-plasmid transgenic founder line, line 60, with integration sites on Chromosomes 3 and 4 [22]. Two breakpoints, one in each group, could not be located, since one was in a repetitive mouse sequence and the other in a fragment that matched an unassigned contig in the database. Of the remaining 18 and 13 genome rearrangements from *Ercc1*$^{-/m}$ and control mice, respectively, two breakpoints in each group (15 and 11%, respectively) were located on Chromosome 11 (the chromosome carrying the lacZ-reporter plasmids), representing intra-chromosomal events, such as deletions or inversions. The breakpoints of the other 16 (89%) and 11 (85%) genome rearrangements were located on other chromosomes, representing inter-chromosomal recombinations (Table 3). Hence, while the frequency of rearrangements in liver of *Ercc1*$^{-/m}$ mice was elevated (Fig. 3), they were similar in character to those occurring in the liver of control mice.

### 3.5. Point mutations in *Ercc1*$^{-/m}$ and *Xpa*$^{-/-}$ mice

In addition to size-change mutations, *Ercc1*$^{-/m}$ mice displayed a significant induction of no-change mutations in liver (Fig. 3). We sequenced a total of 80 no-change mutant plasmids recovered from *Ercc1*$^{-/m}$ and control mice, to examine whether the Ercc1 defect led to a specific point mutation fingerprint (Table 4). As a summary, the frequencies of the predominant base changes are plotted in Fig. 4. Compared to the spectrum of the control mice, Ercc1 deficiency caused a three-fold induction of G:C → T:A transversions (95%CI: 1.1–6.1) and a 10-fold induction of base changes at A:T base pairs (95%CI: 2.5–49). The 1.6-fold induction of 1 bp deletions was not statistically significant (95%CI: 0.54–5.2).

To investigate whether Xpa-deficiency induced a similar point mutational fingerprint, we analyzed 160 no-change mutant plasmids from liver and kidney of 52- and 104-week-old *Xpa*$^{-/-}$ and Wt mice (Table 4). The eight mutation spectra are summarized in Fig. 5. At

Table 2
Breakpoints in the *lacZ* transgene and the mouse genome of genome rearrangements in liver of 23 week old *Ercc1*$^{-/m}$ and control mice

| Mutant ID | Sequence (5'→3')[a] | Origin |
|---|---|---|
| Ercc1_017 | GTGGAGCGCCGAAATCCCGAATCTCTATCG^tgcggtggttgaactgcacaccgccgacgg | pUR288 |
| | aataaagtaaatatctaattaaaaaaaaaa^AGAGGACTGACTCAAACTTCCACCTGTGTT | Chromosome 12 |
| Ercc1_026 | TGGTCTGCTGCTGCTGAACGGCAAGCC**GTT**^gctgattcgaggcgttaaccgtcacgagca | pUR288 |
| | tcttatcaaatggtgcatttaaaatgg**gtt**^TTATTCCCAGGATGGATGTAATAGCTGTGA | Chromosome 13 |
| Ercc1_039 | GACTGGGTGGATCAGTCGCTGATTAAATA**T**^gatgaaaacggcaacccgtggtcggcttac | pUR288 |
| | tatttttattgtatttttcacatttgcat**t**^AGTTTATTTTAGAGTTCCTACAATGATTGT | Chromosome 12 |
| Ercc1_052 | CAGAAGCGGTGCCGGAAAGCTGGCTGGAGT^gcgatcttcctgaggccgatactgtcgtcg | pUR288 |
| | catgtggttggtgggaattgaactcaggac^CTCTGGAAGAGCATCCAGTGCTCTTAACCT | Chromosome 12 |
| Ercc1_065 | CAGGTTTCCCGACTGGAAAGCGGGCAGT**GA**^gcgcaacgcaattaatgtgagttagctcac | pUR288 |
| | aggaaggggcttctaaagcctcagccag**ga**^CCTCAGAGCCACCCCACCCCACTGCACCCC | Chromosome 11 |
| Ercc1_066 | GAAGGCCAGACGCGAATTATTTTTGATGG**C**^gttaactcggcgtttcatctgtggtgcaac | pUR288 |
| | ataagctaaacacacaactgggagagaca**c**^ACAAGGCATTTCCTTTGGAGCACATGATGA | Chromosome 14 |
| Ercc1_075 | GCTGTTCGCATTATCCGAACCATCCGCTGT^ggtacacgctgtgcgaccgctacggcctgt | pUR288 |
| | atttctactttgtttaacattgtctaatgc^CAGGAGTTATCAAAGGATAAACTGAGATAA | Chromosome 3 |
| Ercc1_085 | CTACCGGCGATGAGCGAACGCGTAACGCGA^atggtgcagcgcgatcgtaatcacccgagt | pUR288 |
| | gtgaaagtcgtgcatgcttataatgccatc^CCTGTCCAAGTCTATTCTAAAGAAGGCGGA | Chromosome 15 |
| Ercc1_103 | ATGGCGTTAACTCGGCGTTTCATCTGTGGT^gcaacgggcgctgggtcggttacggccagg | pUR288 |
| | ggccagcctggtctacaaagtgagttccag^CACAGCCAGGGCTATACAGAGAAACCCTGT | Chromosome 4 |
| Ercc1_107 | CCGCCAGTCAGGCTTTCTTTCACAGATG**TG**^gattggcgataaaaaacaactgctgacgcc | pUR288 |
| | agctctacagctctgcgagtcagtgtga**tg**^TGAGGTCCAAAGGCTCAGCAGTGGACGGAA | Chromosome 1 |
| Ercc1_121 | CGCAGCCGAACGACCGAGCGCAGCGAGTC**A**^gtgagcgaggaagcggaagagcgcccaata | pUR288 |
| | atctgaattagaactaaatacttaacagt**a**^CTTAGACTGTGGCTGGAAAGCTTCCATTTC | Chromosome 4 |
| Ercc1_125 | GCAAGCCGTTGCTGATTCGAGGCGTTAACC^gtcacgagcatcatcctctgcatggtcagg | pUR288 |
| | tagcattaaatatgagtctagggttgttct^CATGTGTGAATACTCGCAGTAAGTGAGGTG | Chromosome 6 |
| Ercc1_142 | TTTTTACGCGCCGGAGAAAACCGCCTCGC**G**^gtgatggtgctgcgctggagtgacggcagt | pUR288 |
| | ttgttcatttccatcacctgtttggatgt**g**^TTTTCCTGTTTTTCTGTAAGGACTTCTACC | Chromosome 12 |
| Ercc1_154 | GCATCCAGCGCTGACGGAAGCAAA**ACACCA**^gcagcagtttttccagttccgtttatccgg | pUR288 |
| | aggatctccttcataaagggatgc**acacca**^TTCCTGATGCTCAGGAGGTGACACAACAGC | Chromosome 14 |
| Ercc1_160 | AAGGCCAGACGCGAATTATTTTTGATGG**CG**^ttaactcggcgtttcatctgtggtgcaacg | pUR288 |
| | ggggagaggagaaaggggcaactgtggt**cg**^AGATGCAAAAAAGAAAGAAAGAAAAGGAAT | Chromosome 9 |
| Ercc1_169 | CAACGGGCGCTGGGTCGGTTACGGCCAGG**A**^cagtcgtttgccgtctgaatttgacctgag | pUR288 |
| | gccagctggcgcaggtagcagagcgggta**a**^ACTGGCTCGGATTAGGGCCGCAAGAAAACT | pUR288 |
| Ercc1_173 | GGTAGCAGAGCGGGTAAACTGGCTCGGAT**T**^agggccgcaagaaaactatcccgaccgcct | pUR288 |
| | caataatttgctctgacacggaagcttag**t**^CCTGAAATAGCATTATATATCTTAATGAAT | Chromosome 11 |
| Ercc1_174 | ATCCTGCTGATGAAGCAGAACAACTTTAAC^gccgtgcgctgttcgcattatccgaaccat | pUR288 |
| | tatggttgctttaaatgtccaataaagagt^CTGATGGAATTTTAAAAGCAAAAAATTGTT | Chromosome 2 |
| Ercc1_175 | CTGGCAAGCGGTGAAGTGCCTC**TGGATGTC**^gctccacaaggtaaacagttgattgaactg | pUR288 |
| | taaatatctcacaacactgtct**tggatgtc**^CGAGGGTTCTGCCCATGGCACCATTAGCTC | Chromosome 2 |
| Cont_193 | CCCAATACGCAAACCGCCTCTCCCCGCGC**G**^ttggccgattcattaatgcagctggcacga | pUR288 |
| | tctgtacagttccttgtaaagaggcaaaa**g**^AAGCTGGAAACATCAGCAGCTGTAGCAACA | Chromosome 8 |
| Cont_198 | ATCCCCGTTTACAGGGCGGCTTCGTCTGG**G**^actgggtggatcagtcgctgattaaatatg | pUR288 |
| | gccggaaaacctaccggattgatggtagt**g**^GTCAAATGGCGATTACCGTTGATGTTGAAG | pUR288 |
| Cont_206 | CAAATGGCGATTACCGTTGATGTTGAAGTG^gcgagcgatacaccgcatccggcgcggatt | pUR288 |
| | ccgagttgacgtcacgrggaaggcagagca^CATGGAGTGAAGAACCACCCTCGGCACATG | Chromosome ? |
| Cont_210 | ACCGAGCGCAGCGAGTCAGTGAGCGAGGA**A**^gcggaagagcgcccaatacgcaaaccgcct | pUR288 |
| | ttgaccgctgggatctgccattgtcagac**a**^TGTATACCCCGTACGTCTTCCCGAGCGAAA | pUR288 |
| Cont_234 | TTCGGCGGTGAAATTATCGATGAGCGTGG**T**^ggttatgccgatcgcgtcacactacgtctg | pUR288 |
| | gaaggtggtacatctcagctggaatgcat**t**^TGGTACAGAGTACAACTAGACTTCTCATCA | Chromosome 18 |
| Cont_237 | TACAACGTCGTGACTGGTACAACCCTGGCG^ttacccaacttaatcgccttgcagcacatc | pUR288 |
| | cctggtgattagaacaaggatcatatcata^CCCTCTTCTTGGTCCTCCCTTCTGATTCTC | Chromosome 11 |
| Cont_240 | AACGCCGTGCGCTGTTCGCATTATCCGAAC^catccgctgtggtacacgctgtgcgaccgc | pUR288 |
| | catcaagtgaaggatggagttgccatccca^GAGTCACATATCTGACCCATAAATGTTACT | Chromosome 5 |
| Cont_253 | AACCGACTACACAAATCAGCGATTTCC**ATG**^ttgccactcgctttaatgatgatttcagcc | pUR288 |
| | aaggcccattccatagatagtgtaaat**atg**^GCCAAGAACCCTAgCTTGGGAAGTTCACAA | Chromosome 11 |
| Cont_265 | TGCGTGACTACCTACGGGTAACAGTTTCTT^tatggcagggtgaaacgcaggtcgccagcg | pUR288 |
| | acagctacagtgtagttatatataataaa**t**^AAATCTTAAAAAAAAAAAGGAAAGAAAGAA | Chromosome X |
| Cont_267 | CGCCAGCTGGCGTAATAGCGAAGAGGCCCG^caccgatcgcccttcccaacagttgcgcag | pUR288 |
| | ctaccattaccagttggtctggtgtcgggg^ATCCGTCGACTCTAGAAAGCTTATCGATGA | pUR288 |
| Cont_268 | CGGCCAGGACAGTCGTTTGCCGTCTGAATT^tgacctgagcgcatttttacgcgccggaga | pUR288 |
| | gctcgggttagtctgatgactcgtggctg**t**^AATCCCAGCATTTGGGAGGCTGAGTTAGGA | Chromosome 10 |
| Cont_274 | GGCGGAAAACCTCAGTGTGACGCTCCCCGC^cgcgtcccacgccatcccgcatctgaccac | pUR288 |
| | gggccgcaagaaaactatcccgaccgcctt^ACTGCCGCCTGTTTTGACCGCTGGGATCTG | pUR288 |

Table 2 (*Continued*)

| | | |
|---|---|---|
| Cont_301 | GAATTTGACCTGAGCGCATTTTTACGCGCC^ggagaaaaccgcctcgcggtgatggtgctg | pUR288 |
| | ccctggctgtcctacaatttgctctccaga^CCAGAGGTGATCTCAAACTCACAGAGATCT | Chromosome 7 |
| Cont_307 | AACCATCCGCTGTGGTACACGCTGTGCG**AC**^cgctacggcctgtatgtggtggatgaagcc | pUR288 |
| | aaaattgccatagtcatagaaatttgtc**ac**^AGCAACAGAAAATAACTAATGTAGGACCTG | Chromosome 4 |
| Cont_308 | CAACGTGACCTATCCCATTACGGTCAATC**C**^gccgtttgttcccacggagaatccgacggg | pUR288 |
| | ccccgtttacagggcggcttcgtctgggga**c**^TGGGTGGATCAGTCGCTGATTAAATATGAT | pUR288 |
| Cont_321 | CGCGCCGGAGAAAACCGCCTCGCGGTGATG^gtgctgcgctggagtgacggcagttatctg | pUR288 |
| | cagatgcaggtagagattaacccgaaggcc^CACTTATCTTTGTAGAGATGGATCTAGTTT | Chromosome 7 |
| Cont_329 | AGGCCAGACGCGAATTATTTTTGATGGCG**T**^taactcggcgtttcatctgtggtgcaacgg | pUR288 |
| | tctttggctttcatgtcctgagtcacagt**t**^CCTTCAGAGTTCCTTCAATGCAGGAACTCA | Chromosome 7 |
| Cont_331 | ATGTGGTGGATGAAGCCAATATTGAAACC**C**^acggcatggtgccaatgaatcgtctgaccg | pUR288 |
| | taggggatagccactatgccatgcattgt**c**^CTTGATtCTCACCCCCTTACCTCATGGCCA | Chromosome 7 |
| Cont_332 | ACCCGAGTGTGATCATCTGGTCGCTGGGG**A**^atgaatcaggccacggcgctaatcacgacg | pUR288 |
| | cctagcagacaagaagagatgctggttaa**a**^GCCCCTTACCTAGCAGGCACGGAGAGACGC | Chromosome 9 |

[a] The capitalized nucleotides represent the recovered mutant sequence. Circumflexes and bold letters indicate breakpoints and direct homology between the breakpoint in the transgene and the mouse genome, respectively.

Table 3
Intra- and inter-chromosomal genome rearrangements in liver of 23-week-old *Ercc1*$^{-/m}$ and control mice

| Rearrangements | *Ercc1*$^{-/m}$ | Control | Line 60[a] |
|---|---|---|---|
| Intra-chromosomal | 2 (11%) | 2 (15%) | 20 (53%) |
| Inter-chromosomal | 16 (89%) | 11 (85%) | 18 (47%) |

[a] Data taken from [22].

52 weeks of age a dominant 13- and 23-fold induction of 1 bp deletions was observed in liver (95% CI: 2.9–54) and kidney (95% CI: 3.7–145), respectively. No hotspots, or sequence commonalities were detected at the sites of the deletions. The frame shifts occurred both within non-repetitive and reiterated sequences. In addition, G:C → T:A transversions were significantly induced 6.2-fold (95% CI: 1.2–28) in the kidney of *Xpa*$^{-/-}$ mice at 52 weeks of age. Remarkably, at 2 years of age the over-representation of 1 bp deletions was decreased in liver and disappeared in kidney in the *Xpa*$^{-/-}$ mice compared to Wt. In the 104 week age group mainly changes at G:C base pairs were increased over Wt, in particular, the 5.4- and 5.6-fold induction of G:C → T:A transversions in liver (95% CI: 0.8–34) and kidney (95% CI: 1.7–30), respectively.

The sequencing data also indicated the presence of three single nucleotide variations among the integrated copies of the transgene cluster in *lacZ*-plasmid transgenic founder line 30 (Table 5), similar to those reported earlier for founder line 60 [20]. Although these line 30 polymorphisms formally remain to be verified as we did for those in line 60 by denaturing gradient gel electrophoresis [20], they behaved as polymorphic variations, i.e., occurred at relative high frequencies, were present in addition to unique mutations in mutant plasmids and were found in Wt plasmids. In addition, two

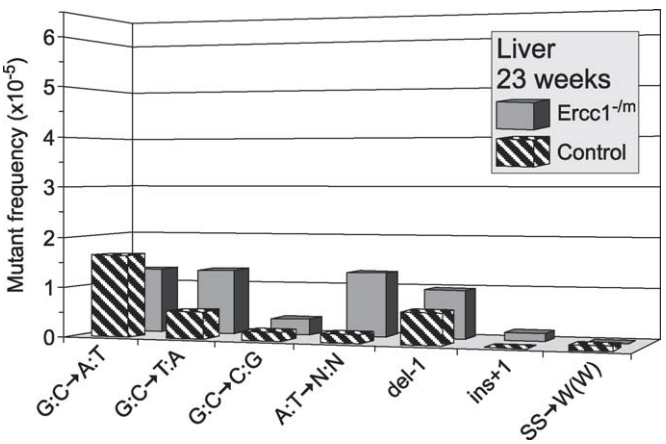


Fig. 4. Point mutational spectra of the most frequent changes observed in liver of 23-week-old *Ercc1*$^{-/m}$ and sibling control mice. Due to their infrequent occurrence all possible base changes affecting A:T base pairs were grouped together (A:T → N:N). Two neighboring strong (S = G or C) bases were also frequently replaced by one or two weak bases (W = A or T): SS → W(W). For a complete list of the no-change mutants, see Table 4.

Table 4
Sequenced *lacZ* no-change mutations recovered from liver and kidney of *Ercc1*$^{-/m}$, *Xpa*$^{-/-}$, Wt and control mice at specific ages

| Position[a] | pUR288 sequence (5'→3') | Alteration |
|---|---|---|
| Control liver, 23 weeks | | |
| 139 | ACCCCA **GGCTTT...GCTCGT** ATGTTG | 28bp del |
| 269 | CTGGTA **C** AACCCT | C→A |
| 274 | ACAACC **C** TGGCGT | delC |
| 630 | GAAGGC **C** AGACGC | C→T |
| 661 | TTAACT **C** GGCGTT | C→G |
| 691 | GGCGCT **G** GGTCGG | G→A |
| 837 | ATGAGC **G** GCATTT | G→A[c] |
| 1020 | CAGGTC **G** CCAGCG | delG |
| 1188 | CTGATT **G** AAGCAG | G→T |
| 1194 | GAAGCA **G** AAGCCT | G→T |
| 1290 [b] | CGTCAC **G** AGCATC | G→A[c] |
| 1338 | ATGGTG **C** AGGATA | C→T |
| 1341 | GTGCAG **G** ATATCC | G→A |
| 1387 | GCTGTT **C** GCATTA | C→T[c] |
| 1390 | GTTCGC **A** TTATCC | A→G |
| 1396 | ATTATC **C** GAACCA | C→T[c] |
| 1411 | CGCTGT **G** GTACAC | G→A |
| 1464 | AATATT **G** AAACCC | G→T |
| 1480 | GCATGG **T** GCCAAT | delT |
| 1588 | TCTGGT **C** GCTGGG | C→A |
| 1639 | ATCGCT **G** GATCAA | G→A |
| 1677 | CAGTAT **G** AAGGCG | G→A |
| 1772 | GAAATG **G** TCCATC | G→A |
| 1836 | TACGCC **C** ACGCGA | C→T |
| 1939 | ATCAGT **C** GCTGAT | C→A |
| 1972 | ACCCGT **G** GTCGGC | G→A |
| 1973 | CCCGTG **G** TCGGCT | G→A |
| 2054 | CCGCAC **G** CCGCAT | delG |
| 2085 | AAACAC **C** AGCAGC | C→T |
| 2612 | TGAAGC **G** ACCCGC | delG |
| 2745 [b] | GCGTGG **C** AGCATC | C→T |
| 2786 | AACCTA **CC** GGATTG | CC→AA |
| 2921 | ATTAGG **G** CCGCAA | delG |
| 2960 | CGCCTG **T** TTTGAC | T→A |
| 3002 | TACCCC **G** TACGTC | delG |
| 3024 | GAAAAC **G** GTCTGC | G→C |
| 3070 | ACCAGT **G** GCGCGG | G→A |
| Wt liver, 52 weeks | | |
| 440 | CTGGCT **GG** AGTGCG | GG→TT |
| 651 | TTTGAT **GG** CGTTAA | GG→TT |
| 692 | GCGCTG **G** GTCGGT | G→A |
| 726 | CCGTCT **G** AATTTG | G→T |
| 838 | TGAGCG **G** CATTTT | G→T |
| 851 | CCGTGA **C** GTCTCG | delC |
| 1218 [d] | TTCCGC **G** AGGTGC | G→T |
| 1555 [d] | TGCAGC **G** CGATCG | G→A[c] |
| 1272 | CTGATT **C** GAGGCG | C→T[c] |
| 1275 | GATTCGA **G** GCGTTA | G→T |
| 1290 | CGTCAC **G** AGCATC | G→A[c] |
| 1370 | CAACTT **T** AACGCC | delT |
| 1387 | GCTGTT **C** GCATTA | C→T[c] |
| 1433 | CCGCTA **C** GGCCTG | C→A |
| 1605 | GAATCA **GG** CCACGG | GG→AT |
| 1718 | TATTTG **C** CCGATG | C→A |
| 1827 | CTTTGC **G** AATACG | G→A[c] |
| 1827 | CTTTGC **G** AATACG | G→A[c] |
| 1874 | TAAATA **C** TGGCAG | C→A |
| 1982 | GGCTTA **C** GGCGGT | C→G |
| 1982 | GGCTTA **C** GGCGGT | C→G |
| 2244 | CCACAA **GG** TAAACA | GG→T |
| 2607 | GTAAGT **G** AAGCGA | G→T |

| Position[a] | pUR288 sequence (5'→3') | Alteration |
|---|---|---|
| *Ercc1*$^{-/m}$ liver, 23 weeks | | |
| 266 | TGACTG **G** TACAAC | G→A |
| 269 | CTGGTA **C** AACCCT | C→A |
| 363 | CCTTCC **C** AACAGT | delC |
| 489 | AACTGG **C** AGATGC | C→T |
| 601 | CATTTA **CTCGCCCACAGTTA** ATGTTG | 14bp ins |
| 838 | TGAGCG **G** CATTTT | G→T |
| 1027 | CCAGCG **G** CACCGC | delG |
| 1387 | GCTGTT **C** GCATTA | C→T[c] |
| 1442 | CCTGTA **T** GTGGTG | T→A |
| 1449 | GTGGTG **GAT** GAAGCC | GAT→CGA |
| 1782 | ATCAAA **A** AATGGC | A→T |
| 1826 | CCTTTG **C** GAATAC | C→A |
| 1827 [b] | CTTTGC **G** AATACG | G→A[c] |
| 1846 | CGATGG **G** TAACAG | delG |
| 1851 | GGTAAC **A** GTCTTG | A→C |
| 1874 | TAAATA **C** TGGCAG | C→A |
| 1910 | ACAGGG **CGGCTTCGTCTGGG** ACTGGT | 14bp del |
| 1922 | CGTCTG **G** GACTGG | G→T |
| 1926 | TGGGAC **T** GGGTGG | T→C |
| 1928 | GGACTG **G** GTGGAT | G→T |
| 2024 | GTTCTG **T** ATGAAC | T→A |
| 2024 | GTTCTG **T** ATGAAC | T→A |
| 2031 | ATGAAC **G** GTCTGG | G→C |
| 2032 | TGAACG **G** TCTGGT | delG |
| 2466 | TGCATC **T** AGCTGG | G→T |
| 2499 | AACCGC **C** AGTCAG | C→T |
| 2540 | TAAAAA **A** CAACTG | delA |
| 2607 | GTAAGT **G** AAGCGA | G→T |
| 2781 [d] | CGGAAA **A** CCTACC | delA |
| 2794 [d] | GGATTG **A** TGGTAG | A→T |
| 2913 | TGGCTC **G** GATTAG | G→A[c] |
| 2914 | GGCTCG **G** ATTAGG | G→C |
| 2971 | ACCGCT **G** GGATCT | G→A |
| 3121 | AACTGA **TGG** AAACCA | TGG→ATGA |
| 3175 | TGAATA **T** CGACGG | T→A |
| *Xpa*$^{-/-}$ liver, 52 weeks | | |
| 269 | CTGGTA **C** AACCCT | C→A |
| 285 | GTTACC **C** AACTTA | delC |
| 345 | GAGGCC **C** GCACCG | delC |
| 488 | AAACTG **G** CAGATG | delG |
| 630 | GAAGGC **C** AGACGC | C→T |
| 1145 [d] | TCTCTA **T** CGTGCG | T→G |
| 2314 [d] | CAGTAC **G** CGTAGT | G→A[c] |
| 1449 | GTGGTG **G** ATGAAG | delG |
| 1595 | GCTGGG **G** AATGAA | delG |
| 1814 | GCGCCC **G** CTGATC | delG |
| 1845 | GCGATG **GGTAACAG** TCTTGG | 8bp del |
| 1857 | AGTCTT **G** GCGGTT | G→C |
| 1881 | TGGCAG **G** CGTTTC | delG |
| 1895 | TCAGTA **T** CCCCGT | T→G |
| 1905 | CGTTTA **C** AGGGCG | C→T |
| 2032 | TGAACG **G** TCTGGT | delG |
| 2241 | GCTCCA **C** AAGGTA | C→T |
| 2492 | GCAATT **T** AACCGC | delT |
| 2540 | TAAAAA **A** CAACTG | delA |
| 2783 | GAAAAC **C** TACCGG | delC |
| 3104 | CCGCTA **C** AGTCAA | C→G |

Table 4 (*Continued*)

| Wt liver, 104 weeks | | | *Xpa*$^{-/-}$ liver, 104 weeks | | |
|---|---|---|---|---|---|
| 488 | AAACTG **G** CAGATG | G→A | 350 | CCGCAC **C** GATCGC | delC |
| 901 | ATGTTG **CC** ACTCGC | CC→A | 366 | TCCCAA **C** AGTTGC | C→T |
| 967 | GCGAGT **T** GCGTGA | T→A | 391 | GCGAAT **G** GCGCTT | G→A |
| 977 | TGACTA **C** CTACGG | C→A | 456 | CTTCCT **G** AGGCCG | G→T |
| 1396 | ATTATC **C** GAACCA | C→T[c] | 503 | CGGTTA **C** GATGCG | C→G |
| 1460 | AGCCAA **T** ATTGAA | T→G | 543 | ATTACG **G** TCAATC | delG |
| 1599 | GGGAAT **G** AATCAG | G→T | 589 | GTTACT **C** GCTCAC | C→A |
| 1792 | GGCTTT **CG** CTACCT | CG→AA | 623[b] | GCTACA **GG** AAGGCC | GG→AT |
| 1827 | CTTTGC **G** AATACG | G→A[c] | 701 | CGGTTA **C** GGCCAG | C→A |
| 1850 | GGGTAA **CA** GTCTTG | 2bp del | 819 | GATCAG **G** ATATGT | G→T |
| 1902 | CCCCGT **TTACAGGGCGGCTT** CGTCTG | 14bp del | 879 | ACTACA **C** AAATCA | C→T |
| 1994 | TGATTT **T** GGCGAT | delT | 1218 | TTCCGC **G** AGGTGC | G→T |
| 2049 | GCCGAC **C** GCACGC | C→T[c] | 1272 | CTGATT **C** GAGGCG | C→T[c] |
| 2664 | GGCCAT **T** ACCAGG | delT | 1550 | AATGGT **GC** AGCGCG | GC→TT |
| 2743 | ACGCGT **G** GCAGCA | G→A | 1746 | GAAGAC **C** AGCCCT | C→T |
| 3266 | CCATTA **CC** AGTTGG | CC→AA | 1784 | CAAAAA **A** TGGCTT | delA |
| | | | 2031 | ATGAAC **G** GTCTGG | G→T |
| Wt kidney, 52 weeks | | | *Xpa*$^{-/-}$ kidney, 52 weeks | | |
| 336 | AATAGC **G** AAGAGG | G→T | 235 | CGGATT **C** ACTGGC | C→A |
| 404 | TGCCTG **G** TTTCCG | G→A | 326 | CAGCTG **G** CGTAAT | G→A |
| 616 | AAAGCT **G** GCTACA | G→A | 467 | CGATAC **TGTC** GTCGTC | 4bp del |
| 616 | AAAGCT **G** GCTACA | G→A | 479 | CGTCCC **C** TCAAAC | delC |
| 1008 | CAGGGT **G** AAACGC | G→T | 503 | CGGTTA **C** GATGCG | C→G |
| 1128 | AGCGCC **G** AAATCC | G→A[c] | 677 | TCTGTG **G** TGCAAC | G→A |
| 1272 | CTGATT **C** GAGGCG | C→T[c] | 726 | CCGTCT **G** AATTTG | G→T |
| 1311 | CATGGT **C** AGGTCA | C→T | 748 | CATTTT **T** ACGCGC | delT |
| 1387 | GCTGTT **C** GCATTA | C→del | 755 | ACGCGC **C** GGAGAA | delC |
| 1387 | GCTGTT **C** GCATTA | C→T[c] | 869 | GCATAA **A** CCGACT | delA |
| 1671 | CCGGTG **C** AGTATG | C→T | 1056 | ATTATC **G** ATGAGC | G→T |
| 1733 | CGCGCG **CG** TGGATG | 2bp del | 1272 | CTGATT **C** GAGGCG | C→T[c] |
| 1827 | CTTTGC **G** AATACG | G→A[c] | 1722 | TGCCCG **A** TGTACG | delA |
| 2180 | GCACTG **G** ATGGTG | G→A | 1746 | GAAGAC **C** AGCCCT | C→T |
| 2344 | CATGGT **C** AGAAGC | C→A | 1927 | GGGACT **G** GGTGGA | G→A |
| 2487 | CGTTGG **C** AATTTA | C→T | 2098 | AGTTTT **T** CCAGTT | delT |
| 2689 | TGTTGC **A** GTGCAC | A→del | 2466 | TGCATC **G** AGCTGG | G→T |
| 3195 | ATGGGG **A** TTGGTG | A→T | 2515 | TTCTTT **C** ACAGAT | C→G |
| 3915 | ATGGGG **A** TTGGTG | A→T | 2567 | CGATCA **G** TTCACC | delG |
| Wt kidney, 104 weeks | | | *Xpa*$^{-/-}$ kidney, 104 weeks | | |
| 27[d] | CTCTCC **CC** GCGCGT | CC→AA | 440 | CTGGCT **GG** AGTGCG | GG→AT |
| 567[d] | CCCACG **G** AGAATC | G→T | 474[d] | GTCGTC **G** TCCCCT | G→T |
| 2651[d] | CTGGAA **G** GCGGCG | G→A | 479[d] | CGTCCC **C** TCAAAC | C→T |
| 252 | GTTTTA **C** AACGTC | C→T | 503[d] | CGGTTA **C** GATGCG | C→G |
| 313 | ATCCCC **C** TTTCGC | delC | 3059[d] | TTATGG **C** CCACAC | C→G |
| 487 | CAAACT **G** GCAGAT | G→A | 503 | CGGTTA **C** GATGCG | C→A |
| 1040 | GCCTTT **C** GGCGGT | delC | 503[d] | CGGTTA **C** GATGCG | delC |
| 1040 | GCCTTT **C** GGCGGT | delC | 518[d] | CATCTA **C** ACCAAC | C→A |
| 1120 | AACTGT **G** GAGCGC | G→A | 518 | CATCTA **C** ACCAAC | C→A |
| 1145 | TCTCTA **T** CGTGCG | T→G | 816 | GAAGAT **C** AGGATA | C→T |
| 1264 | AGCCGT **T** GCTGAT | T→TT | 963 | TGCGGC **G** AGTTGC | G→T |
| 1593 | TCGCTG **G** GGAATG | G→A | 1001 | TTTATG **G** CAGGGT | delG |
| 1827 | CTTTGC **G** AATACG | G→A[c] | 1272 | CTGATT **C** GAGGCG | C→T[c] |
| 1829 | TTGCGA **A** TACGCC | delA | 1290 | CGTCAC **G** AGCATC | G→T |
| 1905 | CGTTTA **C** AGGGCG | C→T | 1482 | ATGGTG **C** CAATGA | C→A |
| 2118 | TCCGGG **C** AAACCA | C→T | 1718 | TATTTG **C** CCGATG | C→A |
| 2487 | CGTTGG **C** AATTTA | C→T | 1774 | AATGGT **CC** ATCAAA | CC→AGA |
| 2499 | AACCGC **C** AGTCAG | C→T | 1784 | CAAAAA **A** TGGCTT | delA |
| 2575 | TCACCC **G** TGCACC | G→T | 2031 | ATGAAC **G** GTCTGG | G→T |
| 2616[d] | GCGACC **C** GCATTG | C→A | 2144 | CGAATA **C** CTGTTC | C→A |
| 2668[d] | ATTACC **A** GGCCGA | A→AT | 2534 | TGGCGA **T** AAAAAA | T→TT |
| 2700 | ACGGCA **G** ATACAC | delG | 2565 | CGCGAT **C** AGTTCA | C→T |
| 2920 | GATTAG **G** GCCGCA | G→A | 2635 | ACGCCT **GG** GTCGAA | GG→AA |

[a] Nucleotide numbering according to SYNPUR288V (GenBank, L09147).

[b] Recurrent mutations, i.e., identical mutants recovered from the same tissue sample; the frequency was 2 in all cases.

[c] G:C→A:T base change at CpG site.

[d] The gray blocks indicate compound mutants, all other mutants consisted of single mutations.

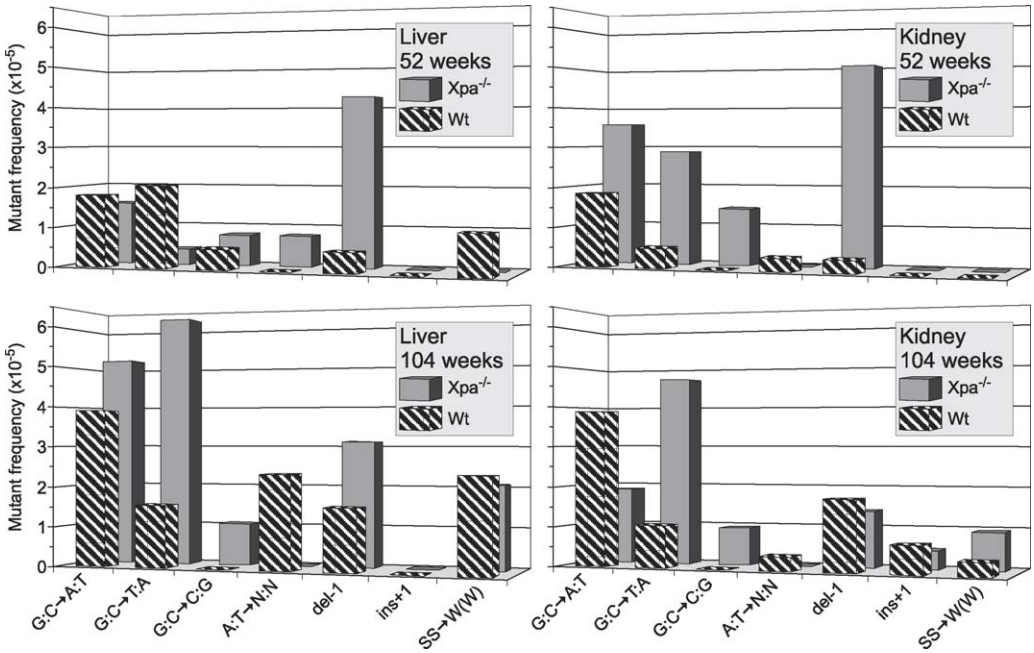


Fig. 5. Point mutational spectra of the most frequent changes observed in liver and kidney of 52- and 104-week-old Xpa$^{-/-}$ and Wt mice. See the legend of Fig. 4 for more details.

Table 5
Polymorphic sites in *lacZ* of pUR288 transgenic mice line 30

| Position[a] | pUR288 sequence (5'→3') | Variation | Frequency[b] |
|---|---|---|---|
| 106 [c] | CGCAAT **T** AATGTG | T→C | 25 |
| 227 [c] | CATGAT **T** ACGGAT | T→A | 25 |
| 1980 | TCGGCT **T** ACGGCG | T→A | 11 |

[a] Nucleotide numbering according to SYNPUR288V (GenBank, L09147).
[b] Occurrence of polymorphic sites on a total of 246 sequenced plasmids.
[c] Polymorphic sites at position 106 and 227 were linked in all cases.

of the three variations (106T → C and 227T → A) were linked in all 25 cases among the 246 plasmids analyzed. The 1980T → A variation was found 11 times (Table 5). The 2:1 ratio between the frequencies of these variations suggests that the linked polymorphisms occur twice per haploid genome and the unique polymorphism only once. Based on this assumption, the total integrated plasmid copy number can be deduced by dividing the total number of plasmids analyzed by the frequency of the polymorphic sites and multiplying by their assumed occurrence per haploid genome. Accordingly, we now estimate the copy number per haploid genome at $21 \pm 1$.

## 4. Discussion

Ample studies have been devoted to the role of DNA repair in removing damage to promote survival [23]. However, especially in mammals, much less effort has gone into analysis of the main molecular endpoint of DNA damage, i.e., gene mutations. Mutations are mainly the consequence of the erroneous processing of DNA lesions introduced by environmental or endogenous genotoxins during DNA replication, repair or recombination. With the generation of transgenic mouse models harboring mutational reporter genes that can be crossed into the various DNA repair-deficient mouse models, it is possible to determine the contribution of various DNA repair mechanisms to attenuating mutation accumulation over the lifespan of an organism [24]. This is especially interesting in view of the possible role of DNA repair systems in longevity assurance, as exemplified by the strong association of premature aging with defects in DNA repair [25]. In the present paper we have monitored a broad range of somatic mutations over the entire lifespan of four mouse models, harboring heritable mutations affecting different repair systems. The results indicate that only the Xpa and Ercc1 deficiency (defective in NER alone or in combination with ICLR, respectively) leads to increased genomic instability during aging, whereas the Xpd and Csb deficiency, which affect transcription and/or transcription-coupled repair, do not. We interpret these results as follows.

The mutation accumulation in the Xpa-null mice was not significantly elevated above littermate controls at 13 weeks of age, but was significant by 52 weeks. This rela-

tively late effect of the Xpa-null mutation on age-related mutation accumulation confirms our previous results, indicating that at young age, i.e., 2 months of age in that study, mutation frequencies were not different from the littermate controls, with significantly increased levels observed only after 4 months of age [8]. A reasonable conclusion would be that in liver, NER is not required to suppress mutations during development, but becomes important after pubescence, at least in rodents. It is possible that the increased post-pubescent mutagenesis is due to a change in exposure to environmental mutagens, e.g., dietary switch after weaning. Alternatively, it is possible that a higher rate of apoptosis in the developing organism effectively prevents mutation accumulation early in life. While histopathological examination of the $Xpa^{-/-}$ mouse cohort in this study has not been completed yet, preliminary results suggest a decreased latency and a somewhat increased incidence of tumors, many of hepatocellular origin, in these mice (Dr. Dolf Beems, personal communication). A slightly higher incidence of liver tumors has been reported previously for Xpa-null mice at about 20 months of age [26]. We also observed a reduced average lifespan of the Xpa-mutant mice as compared to Wt, but a similar maximum lifespan. Such survival curves typically reflect the stochastic effects of susceptibility to a disease [27]. It is possible that the observed accelerated mutation accumulation in the $Xpa^{-/-}$ mice is responsible for the increased cancer and shortened lifespan in these animals, indicating a role for NER in attenuating both cancer and segmental aging in mammals.

Analysis of the types of *lacZ*-mutants accumulating with aging in the Xpa-null mice (which were mainly point mutations), revealed a much higher frequency of 1 bp deletions, in both liver and kidney at 52 weeks of age compared to littermate controls. At old age (104 weeks), the spectrum shifted, in both organs, to G:C → T:A transversions, a signature mutation of oxidative damage. It is conceivable that the 1-bp deletions are caused by replication errors during polyploidization – an age-related physiological process in many tissues, that begins after weaning [28] – as a consequence of unrepaired spontaneous lesions that normally are substrates for NER, i.e., bulky adducts or other large distortions of DNA. It has been demonstrated that −1 frameshift mutations occur after translesion synthesis past platinum-GG adducts in vitro [29]. The change in the spectrum towards base substitutions at G:C's at older ages could be due to a much higher representation of relatively small oxidative DNA lesions, which are also subject to NER [9] and would be expected to lead predominantly to G:C to T:A transversions. At such late age, replication errors can still occur, e.g., associated with tissue regeneration, or with hyperplastic or neoplastic cell growth. We cannot exclude the possibility that alterations in cell composition in the organs, due to, e.g., lymphocyte infiltration, contributed to the shift in mutant spectra. Cell turnover could also be responsible, in part, for the disappearance of cells that underwent 1-bp deletion mutagenesis at young age.

The Ercc1-mutant mice differ from the Xpa-null mice in the sense that they are deficient for both NER and ICLR. Their severe phenotype and short lifespan is explained not by the NER defect (since NER-impaired Xpa-null mice fail to display these features), but the ICLR defect. Interestingly, mutations were only found to accumulate in the liver of those Ercc1-mutant mice harboring one knockout allele and one truncated Ercc1 allele, which extends the lifespan of these mice to about 6 months. Possibly, like in the Xpa-null mice, mutation accumulation in the Ercc1 hypomorph begins after development, or the rapid rate of liver cell degeneration and death in the Ercc1-null mutant precludes the fixation of mutations. Most of the Ercc1-specific mutations were size-change mutations, i.e., mutations inactivating a *lacZ* gene by a genome rearrangement event involving a breakpoint in a *lacZ* gene and a second breakpoint elsewhere in the mouse genome. This is consistent with their defect in ICLR, resulting in the accumulation of replication-induced double-strand break repair intermediates [17].

In a previous study on the accumulation of spontaneous genome rearrangements in normal mice with aging, we discovered that 50% of the events were intra-chromosomal, i.e., large deletions or inversions [22]. In contrast, in this present study most of the rearrangements resulted from inter-chromosomal recombination, in both the Ercc1-mutant and control animals (Table 3). Previously, we used *lacZ*-plasmid line 60 mice with integration sites on Chromosomes 3 and 4, while in the present study line 30 mice were used with a single integration site on Chromosome 11. This indicates that the relative frequency of translocations is founder line specific and could be due to the position of the *lacZ*-plasmid cluster on the chromosome. Indeed, the chromosomal integration sites in line 60 mice are in the E1 region of Chromosome 3 (half way along the chromosome) and the C5 region of Chromosome 4 (two-thirds of the way along the chromosome) [22], while the integration site of founder line 30 (used in this study) is on the centromeric tip of Chromosome 11 (region A1–A2; not shown). The proximal location on Chromosome 11 prevents the detection of all but relatively small intra-chromosomal recombinations; larger events would lead to loss of the centromere

and, therefore, the entire chromosome. If the orientation of the integration site in line 30, which is currently unknown, is towards the centromere, transpositions and inversions towards the distal end are the only detectable large intra-chromosomal rearrangements (for a detailed explanation of the different chromosomal events that can occur at the *lacZ* locus, see [22]).

Thus, in both the Xpa- and Ercc1-defective mice an accelerated increase of *lacZ* mutants with age was readily detected. In contrast, in the Xpd and Csb defective mice the age-related accumulation of *lacZ* mutants in liver and kidney was not significantly elevated compared to Wt. For the Csb-null mice this may be due to the presumed non-transcribed *lacZ*-reporter gene, rendering the mutation detection assay insensitive to TC-NER-related mutagenesis. Hence, Csb deficiency could potentially result in increased mutagenesis in transcriptionally active sequences, resulting in some age-related symptoms, including a significant growth reduction starting at maturity [13], but not substantial enough to cause a reduction in lifespan (Fig. 1). In the Xpd mice, the mutation causes hypomorphism, with about 30% residual NER activity, both global and transcription coupled [15]. In these mice we observed a 12 and 20% reduction in mean and maximum lifespan, respectively (Fig. 1) and a host of premature aging features [16,30]. It is possible that accelerated aging in Xpd mutants is caused by increased apoptosis as a consequence of RNA polymerase II stalling at the site of a lesion resulting from the helicase defect in TFIIH. Indeed, increased spontaneous apoptosis in the liver of these mice has been observed (Dr. Yousin Suh, personal communication). This high level of spontaneous apoptosis would be in keeping with the reduced incidence of cancer in these mice, and confirm the antagonistic relationship between cancer and aging with respect to apoptosis. Moreover, the high mortality rate towards the end of life suggests a rapid depletion of a vital compartment (e.g., stem cells), in contrast to a stochastic effect such as cancer (Fig. 1). While increased apoptosis can occur in combination with increased genomic instability [31], it is clear that the residual (GG)-NER activity in these mice is sufficient to prevent accelerated mutation accumulation. Due to the presumed non-transcribed nature of the *lacZ*-reporter gene, the results do not exclude a potential mutagenic effect of the partial TC-NER defect in Xpd-mutant mice.

In summary, complete lifespan studies of somatic mutation accumulation in various DNA repair deficient mice indicate a dichotomy between defects in global genome repair and transcription-related events. Only repair defects per se, i.e., Xpa or Ercc1 defects, were associated with accelerated mutation accumulation. This is in keeping with results obtained by Wijnhoven et al., who observed an accelerated increase of mutations at the *Hprt* locus in splenocytes of Xpc-null mice (only defective in GG-NER) [32]. Symptoms of premature aging were found in Xpd and Ercc1-mutant mice, but were milder in the Csb and not as obvious in the Xpa or Xpc-null mice, demonstrating that increased mutation frequency does not correlate directly with accelerated aging. How can this differential impact of DNA repair gene defects on genomic instability and aging be explained? First of all, it should be realized that DNA repair as a longevity assurance system is extremely broad, with hundreds if not thousands of genes playing, often complementary, roles. Hence, it is possible that some or even most heritable mutations in genes that impact DNA repair pathways show no effect on aging at all. Those gene defects that do impact aging can do this by accelerating tumor formation, a major hallmark of aging, by reducing organ and tissue function or by a combination of the two (which best resembles natural aging). While cancer requires somatic mutations, organ dysfunction in the transcription-related repair mutants or in the Ercc1-null mice may primarily be due to increased rates of DNA damage-induced apoptosis. While normal aging could, at least in part, be caused by increased cell death, mutation accumulation, especially large rearrangements, may impact normal patterns of gene expression in the cell, which could significantly contribute to age-related cellular degeneration [33]. This may explain why increased genome rearrangements are associated with segmental progeria, such as Werner syndrome [34]. It is possible that such mutational events also contribute to accelerated aging in the Ercc1-mutant mice.

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