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ORIGINAL ARTICLE

Homologous recombination-mediated repair of DNA double-strand breaks operates in mammalian mitochondria

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Abstract Mitochondrial DNA is frequently exposed to oxidative damage, as compared to nuclear DNA. Previously, we have shown that while microhomology-mediated end joining can account for DNA deletions in mitochondria, classical nonhomologous DNA end joining, the predominant double-strand break (DSB) repair pathway in nucleus, is undetectable. In the present study, we investigated the presence of homologous recombination (HR) in mitochondria to maintain its genomic integrity. Biochemical studies revealed that HR-mediated repair of DSBs is more efficient in the mitochondria of testes as compared to that of brain, kidney and spleen. Interestingly, a significant increase in the efficiency of HR was observed when a DSB was introduced. Analyses of the clones suggest that most of the recombinants were generated through reciprocal exchange, while ~ 30% of recombinants were due to gene conversion in testicular extracts. Colocalization and immunoblotting studies showed the presence of RAD51 and MRN complex proteins in the mitochondria and immunodepletion of MRE11, RAD51 or NIBRIN suppressed the HR-mediated repair. Thus, our results reveal importance of homologous recombination in the maintenance of mitochondrial genome stability.

Keywords Mitochondrial genome stability · Genomic instability · Double-strand break repair · DNA damage · NHEJ · MMEJ · Alternative NHEJ · Gene conversion · Reciprocal exchange

Introduction

Maintaining stability of the genome is one of the major challenges for a cell [1, 2]. The genome of an organism is under constant threat from several exogenous (ionizing radiation, chemotherapeutic agents, etc.) and endogenous factors (free radicals, enzymatic actions, etc.) that can lead to various DNA damages and alterations in the genetic material, if not repaired [3–6]. Among the thousands of DNA lesions that occur within a cell per day, DNA breaks, particularly double-strand breaks (DSBs) are considered as the most detrimental [7, 8]. Failure in DSB repair could lead to chromosomal rearrangements, carcinogenesis, premature ageing and several diseases [9–12].

Repair of DSBs occurs through two major pathways, namely homologous recombination (HR) and nonhomologous end joining (NHEJ) [13–19]. HR operates generally in late S and G2 phases of the cell cycle, utilising homology sequence from the sister chromatid and is thus accurate. In contrast, NHEJ is less accurate and occurs by end joining of broken DNA. Since NHEJ operates throughout the cell cycle, despite being imprecise, it helps in maintaining integrity of the genome within the cell [11, 20–23]. In addition to these, recently, an alternative pathway of NHEJ, known as microhomology-mediated end joining (MMEJ) has been elucidated, which utilises microhomology regions and is known to result in larger deletions [5, 19, 24–26]. Owing to its erroneous nature, this pathway is less faithful than

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classical NHEJ (c-NHEJ) and can result in genomic instability and chromosomal translocations [25].

Mitochondria, also known as the 'powerhouse of the cell', are unique, as they have their own genome, unlike other organelles and are semiautonomous. Mammalian mitochondrial DNA (mtDNA) is about 16 Kb in size and is a closed circular double-stranded DNA. The human mtDNA contain 37 genes, of which 2 code for rRNAs, 22 for tRNAs and 13 for polypeptides that are part of the enzyme complex of oxidative phosphorylation [27]. Since the mitochondrion is involved in ATP generation and oxidative phosphorylation, it is exposed to reactive oxygen species (ROS) continuously, explaining the elevated levels of oxidative damage in the mitochondrial DNA compared to nuclear DNA [28, 29]. Therefore, accumulation of mutations and increased frequency of deletions are reported in mtDNA in comparison to nuclear DNA [30].

Although > 50-fold increase in mutations has been reported, the repair mechanism associated with it is not well studied in mitochondria [31–35]. It was previously thought that mitochondria are devoid of efficient repair, as DNA repair genes are absent. However, studies have revealed the presence of base excision repair (BER) in mitochondria and suggested that most of the proteins involved in mtDNA repair are translocated into mitochondria either from the cytoplasm or nucleus [36–42]. Previously, two models were proposed to explain how mitochondrial nucleoids mediate mtDNA inheritance. One of the studies proposed that mtDNA is replicated within mitochondrial nucleoids with little or no exchange of genetic information between nucleoids [43], while the other study suggested remodelling of nucleoids with exchange of mtDNA [44]. In a recent study, functional complementation of two heterologous mtDNA nucleoids was shown in wild type cells, suggesting consistent genetic background of nucleoids. However, exchange among mtDNA within the nucleoid is controversial [45, 46].

Unlike nucleus, the mechanism for double-strand break repair is less studied in mitochondria. In a recent study, it was reported that while MMEJ is active in mammalian mitochondria, NHEJ was undetectable [26]. Although the existence of MMEJ could explain the presence of mitochondrial deletions and consequent genetic disorders, the mechanism responsible for stability of mitochondrial genome is largely unclear [47]. Therefore, in the present study, we investigate the DSB repair mechanism that is responsible for mitochondrial genomic stability. Using various biochemical assays, we report that homologous recombination is the major DSB repair pathway in mammalian mitochondria. We observed that mitochondrial extracts from testes, brain, kidney and spleen possess proficient HR. Induction of DSBs, in one of the two plasmid DNA substrates used for the study significantly enhanced HR in mitochondria. Evaluation of the molecular mechanism revealed that recombination mostly occurred through reciprocal exchange, rather than gene conversion. We also provide evidence for presence of HR proteins such as RAD51, MRE11 and NIBRIN in mitochondrial extracts and their involvement in HR-mediated repair.

Materials and methods

Plasmid constructs

Plasmids pTO223 and pTO231 were a kind gift from Dr. Christian Sengstag, Switzerland. The construction of plasmids pTO223 and pTO231 was described previously [48]. The supercoiled plasmids were purified and used for the study as described earlier [49, 50].

The plasmid DNA substrates were used for HR assay either as circular DNA (circular- circular; pTO223 + pTO231) or circular–linear (pTO223 + pTO231/SalI) along with mitochondrial proteins, heat-inactivated protein, or no protein (negative control) as described below.

Preparation of mitochondrial protein extracts

Mitochondrial protein extracts were prepared following isolation of mitochondria based on differential centrifugation as described [26, 51]. Brain, testes, spleen and kidney were dissected out from 4- to 6-week-old male Wistar rats. Each tissue was minced on ice and washed multiple times using ice-cold PBS. Following homogenization in the buffer [70 mM of sucrose, 200 mM of mannitol, 1 mM of EDTA, and 10 mM of HEPES (pH 7.4), 0.5% BSA per g tissue] in a Dounce-type homogenizer (20-30 strokes), homogenate was centrifuged (3000 rpm, 10 min at 4 °C) to remove nuclei and cellular debris from the supernatant that contains the cytosol and mitochondria. This step was repeated at least three times to avoid nuclear contamination in the cytosolic fraction. The cytosolic fraction was centrifuged at 12,000 rpm (30 min at 4 °C) to pellet the mitochondria. The mitochondrial pellet was washed in suspension buffer [10 mM of Tris–HCl (pH 6.7), 0.15 mM of $MgCl_2$, 0.25 mM of sucrose, 1 mM PMSF and 1 mM DTT] twice and lysed in mitochondrial lysis buffer [50 mM of Tris–HCl (pH 7.5), 100 mM of NaCl, 10 mM of $MgCl_2$, 0.2% Triton X-100, 2 mM of EGTA, 2 mM of EDTA, 1 mM of DTT and 10% glycerol] by mixing (30 min at 4 °C) along with protease inhibitors (PMSF (1 mM), aprotinin (1 µg/ml), pepstatin (1 µg/ml) and leupeptin (1 µg/ml). Mitochondrial extract was centrifuged at 12,000 rpm (5 min) and the supernatant (mitochondrial fraction) was aliquoted, snap-frozen and stored at − 80 °C till use.

Mitochondrial protein extracts were also prepared from rat tissues as per manufacturer's instructions using mitochondrial extraction kit (Imgenex, USA).

Proteinase K treatment to mitochondria

Mitochondrial pellet was incubated on ice for 3 h with increasing concentrations of Proteinase K (1, 3, 5, 10, 15 µg/ml). Following incubation, the pellet was centrifuged and supernatant was discarded. The mitochondrial pellet was then lysed, extract was prepared as described above, aliquoted and stored.

Preparation of whole cell extracts

Cell-free extracts were prepared in ice-cold conditions as described previously [26, 52, 53]. In brief, cells were washed in ice-cold phosphate-buffered saline (PBS) and in 200 µl of HLB/ml cell pellet [Hypotonic Lysis Buffer: 10 mM Tri-HCl (pH 8.0), 1 mM of EDTA and 5 mM of DTT]. Pellet was resuspended in hypotonic buffer and incubated for 20 min at 4 °C. Cells were homogenized in the presence of protease inhibitors consisting of PMSF (1 mM), aprotinin, pepstatin and leupeptin (1 µg/ml each) and incubated in 0.5 volumes of HSB [High Salt Buffer: 50 mM of Tris–HCl (pH 7.5), 1 M of KCl, 2 mM of EDTA and 2 mM of DTT] on ice (20 min). The extract was centrifuged (3 h at 42,000 rpm, 4 °C; SW41 rotor, Beckman ultracentrifuge, Model L8-70M), supernatant was dialysed against DB [Dialysis Buffer: 20 mM Tris–HCl (pH 8.0), 100 mM of potassium acetate, 20% glycerol, 0.5 mM of EDTA and 1 mM of DTT] for 16 h. The clarified extract was aliquoted, quick-frozen in liquid nitrogen and stored at − 80 °C.

In vitro HR assay

HR reactions were performed as described earlier with slight modifications [48, 49, 54]. Reactions were carried out in a volume of 25 µl by incubating 500 ng each of plasmids pTO223 and pTO231 in a buffer containing 35 mM of HEPES (pH 7.9), 10 mM of $MgCl_2$, 1 mM of DTT, 1 mM of ATP, 50 µM of dNTPs, 1 mM of NAD and 100 µg/ml of BSA at 30 °C (for testes) and 37 °C (for other tissues) for 30 min. 2 µg of mitochondrial extracts (increasing concentrations of proteins as specified or heat-inactivated extracts, or immunoprecipitated extracts) were used for the HR reaction. The HR reactions were terminated by EDTA (20 mM), proteinase K (200 µg/ml) and SDS (0.5%). The products were purified by phenol/chloroform extraction, followed by precipitation with ethanol and glycogen, and pellet was dissolved in TE buffer (20 µl). 2 µl of the purified DNA was used for transformation into electro-competent *Escherichia coli* DH5αF^{-} and Tg1. The bacterial cells were then plated on ampicillin and kanamycin plates to score the transformants and recombinants, respectively. The recombination frequency was calculated as the ratio of the number of recombinants to the number of transformants obtained per microgram of DNA. For all the experimental conditions, a control reaction without extract was used to measure the recombination catalysed by *E. coli*. Fold change was measured by determining the increase in recombination frequency with respect to no protein control.

Restriction digestion analysis of recombinants

The colonies were grown individually in 2 ml of LB broth for 12 h at 37 °C. Plasmid DNA was isolated as per standard protocol [55]. Recombinant plasmid DNA was subjected to restriction enzyme digestion using EcoRI/SalI (for reciprocal exchange) and SalI/HindIII (for gene conversion). After the plasmid isolation, ~ 1 µg of DNA was incubated in NEB buffer 3 and BSA along with EcoRI and SalI (4 units each) at 37 °C for 4 h. The digested products were resolved on 1% agarose gel to confirm recombination (1.5 Kb fragment release). The positive clones were then digested with HindIII (4 units) at 37 °C in NEB buffer 2 for 2 h followed by SalI (4 units) digestion in NEB buffer 3 for 2 h at 37 °C. The digested products were resolved on 1% agarose gel to test the 1.5 Kb fragment release. For each set of digestion, both plasmids with nonfunctional neomycin gene were also restriction digested as controls. These plasmids give the fragment size of 1.2 Kb (pTO223) and 1.25 Kb (pTO231) upon EcoRI/SalI digestion, whereas a 1.2 Kb (pTO223) and 4.1 Kb (pTO231) upon HindIII/SalI digestion.

Immunoprecipitation (IP)

IP experiments were performed as described previously with modifications [52, 56, 57]. Protein A agarose beads (Sigma) were activated by immersing in water and incubated in IP buffer [300 mM of NaCl, 20 mM of Tris–HCl (pH 8.0), 0.1% of NP40, 2 mM of EDTA, 2 mM of EGTA, and 10% glycerol] for 30 min on ice following which the beads were conjugated with appropriate antibody at 4 °C for overnight to generate antibody-bead conjugate. The antibody-bead conjugates were then separated by centrifugation and incubated with mitochondrial extracts at 4 °C overnight. The conjugate bound to target proteins was separated and washed. The protein depletion was confirmed in the resulting supernatant by immunoblot analysis and quantified using Multi Gauge (V3.0). The immunodepleted extracts were then used for HR assays.

Immunocolocalization

Approximately 50,000 HeLa cells were grown in chamber slides in MEM medium supplemented with 10% FBS and 1% penicillin–streptomycin (Sigma) for 24 h. The cells were stained with either 100 ng/ml of Mitotracker Red 580 (Invitrogen) or 100 ng/ml Mitotracker Green FM

(Invitrogen) at 37 °C in a CO_2 incubator for 30 min. Cells were washed twice with 1 × PBS, fixed in 2% paraformaldehyde (20 min) and permeabilized with 0.1% Triton X-100 (5 min) at room temperature. BSA (0.1%) was used for blocking (30 min) and subsequently the cells were incubated with appropriate primary antibody at room temperature (4 h). FITC- or Alexa Fluor-conjugated secondary antibodies were used for detection of the signal. After washing, the cells were stained with DAPI, mounted with DABCO (Sigma) and observed under confocal laser scanning microscope (Zeiss) at 63× magnification. The images were processed using Zen Lite software.

Immunoblotting

Immunoblotting analysis was performed as described before [5]. ~ 15 µg of protein was resolved on 8–10% SDS-PAGE, proteins were transferred to PVDF membrane (Millipore, USA), blocked with 2% BSA for 1 h at 4 °C, probed with appropriate primary antibodies against PCNA (1:750), RAD51 (1:750), MRE11 (1:750), NIBRIN (1:750), VDAC (1:750), ACTIN (1:5000), SMAC/DIABLO (1:5000), CYTOCHROME C (1:5000) and TFAM (1:250). The numbers in the parenthesis indicate the dilution. Blots were washed in PBST (1× PBS and 0.1% Tween 20) several times and incubated with biotinylated secondary antibodies (Santa Cruz; 1:10,000) at 4 °C for 4 h. The blots were rinsed, incubated with 250 ng/ml streptavidin-HRP (Sigma, USA) for 30 min and washed. The blots were developed using chemiluminescent solution (Immobilon TM western; Millipore, USA) and scanned by gel documentation system (LAS 3000; Fuji, Japan).

Statistical analysis

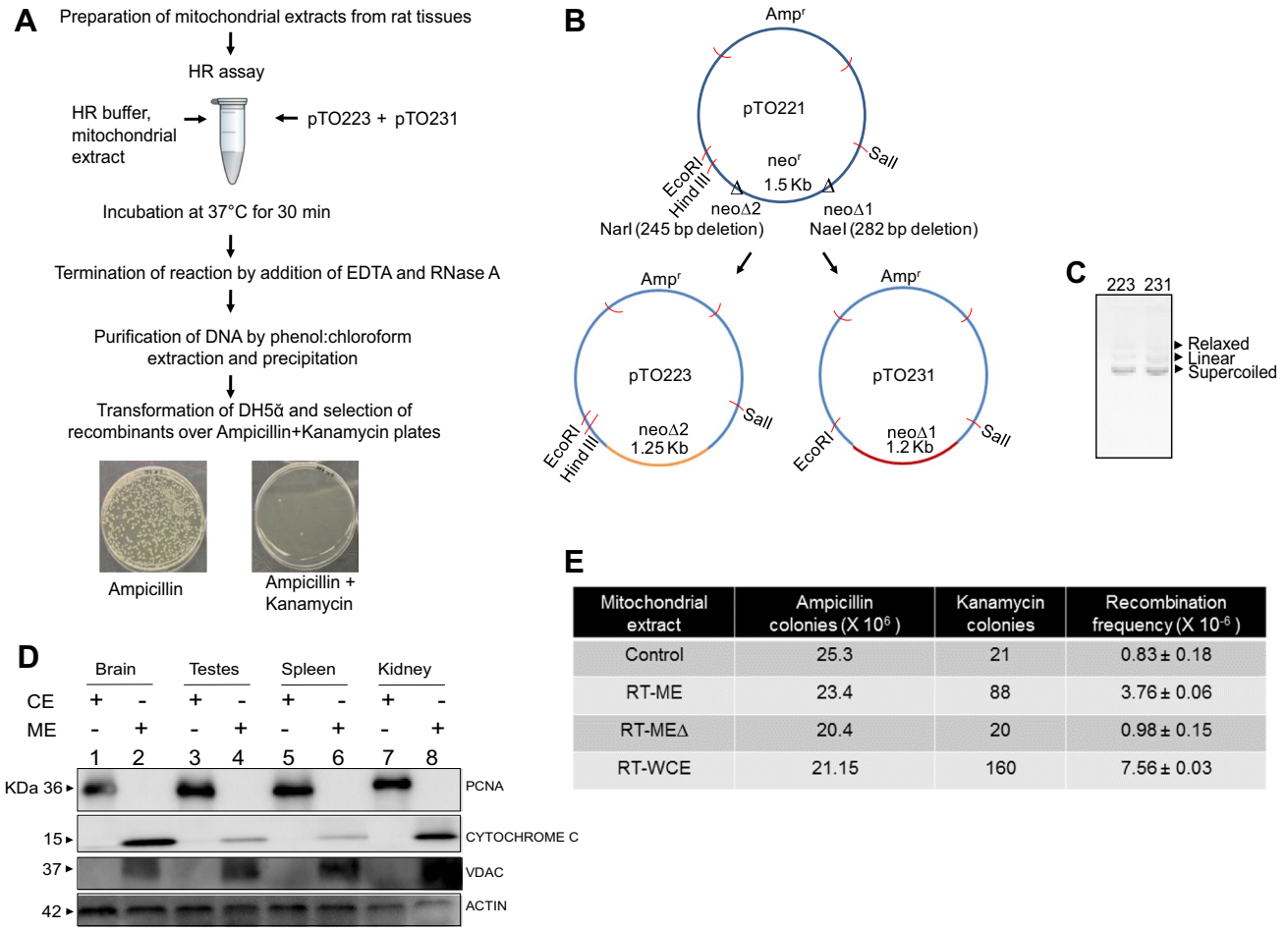
Every experiment presented in the current study was repeated a minimum of three times (biological repeats). Transformation efficiency and recombination frequency were determined for every experiment. The values obtained were then used for calculating the standard deviation using Microsoft Excel 2007 and represented as mean ± SD in the tables. The error bars for the bar diagrams representing the fold change values were obtained using GraphPad Prism ver 5.0 based on data obtained from three independent repeats by performing Student's *t* test (two tailed). Error bars have been shown depicting mean ± SEM (ns: not significant, $*p < 0.05$, $**p < 0.005$, $***p < 0.0001$). Colocalization analysis was done using JaCoP in ImageJ software. The values were plotted in GraphPad Prism 5.0 and the significance was calculated using the same. The values were obtained for at least 50 cells per sample. One-way ANOVA was performed using Tukey's multiple comparison test in GraphPad Prism ver 5.0 and significance was calculated and presented as table ($p < 0.05$).

Results

Mitochondrial protein extracts support DSB repair by homologous recombination

In a previous study, we reported that mitochondrial extracts are proficient in MMEJ, the backup DSB repair pathway, although classical NHEJ was undetectable [26]. The use of microhomology regions during MMEJ results in mitochondrial DNA deletion; hence presence of this pathway alone, fails to explain maintenance of mitochondrial genome stability when DSBs are generated upon DNA damage. Therefore, we investigated whether homologous recombination-mediated DSB repair is proficient in mitochondria isolated from various tissues of rat. To study this, recombination assay was performed as outlined in the schematic presentation, using plasmids (~ 80% supercoiled form) with two independent mutations at different regions of neomycin gene (Fig. 1a–c). The mitochondrial extracts (ME) were prepared from brain, testes, spleen and kidney of rats and the purity was confirmed by immunoblotting using specific mitochondrial markers, VDAC and Cytochrome C, which were undetectable in cytoplasmic extracts (CE) (Fig. 1d). The nuclear contamination in mitochondrial extracts was probed using PCNA and results showed that while its presence was observed in cytosolic extracts, it was undetectable in mitochondrial extracts (Fig. 1d). β-ACTIN served as the loading control (Fig. 1d). Hence, our results revealed that the mitochondrial extracts used for the study were pure and there were no contaminating cytoplasmic and nuclear proteins.

The ability of mitochondrial proteins to catalyse HR-mediated repair was tested by incubating mitochondrial extracts from rat testes with plasmids bearing two independent deletions in neomycin gene. Upon HR-mediated DSB repair, a functional neomycin gene will be restored and can be scored by transforming into *E. coli* DH5α (Fig. 1a, b). Background recombination catalysed by *E. coli* was assessed by performing the reaction in the absence of proteins. Results showed that mitochondrial extracts (2 µg) from rat testes are proficient in homologous recombination, with an efficiency of 3.76×10^{-6} when incubated with plasmid DNA substrates at 30 °C, while it was only 0.98×10^{-6}, when heat-inactivated extracts were used, which was comparable to the efficiency seen when reactions were performed in the absence of extracts (0.83×10^{-6}) (Fig. 1e). Recombination catalysed by whole cell extracts from rat testes served as positive control, with a recombination frequency of 7.56×10^{-6}, which was higher than that of the mitochondrial extracts (Fig. 1e).



Mitochondrial extract	Ampicillin colonies (X 10^6)	Kanamycin colonies	Recombination frequency (X 10^{-6})
Control	25.3	21	0.83 ± 0.18
RT-ME	23.4	88	3.76 ± 0.06
RT-MEΔ	20.4	20	0.98 ± 0.15
RT-WCE	21.15	160	7.56 ± 0.03

Fig. 1 Homologous recombination assay to evaluate efficiency of HR-mediated DSB repair in mitochondrial extracts from rat testes. **a** Flowchart representing steps involved in HR assay. Two plasmid DNA substrates (500 ng each) bearing independent neomycin mutations were incubated with mitochondrial extracts in HR buffer as indicated. The products were purified, transformed into *E. coli* and selected on ampicillin/kanamycin plates to evaluate recombinants. Recombination frequency was calculated as the ratio of the number of recombinants to the number of transformants obtained per microgram of DNA. **b** Schematic showing parental plasmids (pTO221) and two of its variants, wherein two different regions of neomycin gene were mutated (pTO223 and pTO231). Both the plasmids have similar backbone/sequence except the mutations in the neomycin gene. **c** Agarose gel electrophoresis image showing the supercoiled form of pTO223 and pTO231 DNA. **d** Evaluation of purity of mitochondrial extracts. Mitochondrial extracts (ME) and cytosolic extracts (CE) were prepared from brain, testes, spleen, and kidney of rat and western blotting was performed to evaluate the purity using specific markers. PCNA was used as nuclear and cytosolic marker, while VDAC and CYTOCHROME C were used as mitochondrial markers. ACTIN served as loading control. **e** Comparison of HR efficiency between mitochondrial extracts (ME) and whole cell extracts (WCE) of rat testes. Either mitochondrial extract or whole cell extract (2 µg) was incubated with plasmid DNA substrates (500 ng) in HR buffer, products were purified, transformed in *E. coli* to select recombinants. Recombination frequency was calculated and presented

Further, we observed a concentration-dependent increase in the recombination frequency when protein titration was performed using mitochondrial extracts prepared from testes (1, 2 and 3 µg, 30 °C for 30 min). While 1 µg of ME exhibited a 1.16-fold increase in recombination frequency, compared to no protein control, an increase of 3.3- and 5.8-fold was observed when 2 and 3 µg of protein, respectively were used (Table 1A, Suppl. Figure 1A). Time course studies revealed that an incubation time of 30 min resulted in maximum recombination frequency, which was ~ 4-fold higher than no protein control (Table 1B, Suppl. Figure 1B). Incubation for longer time periods resulted in decreased recombination frequency, which could be due to nuclease activity present in the extracts. Depletion of buffer components such as ATP and dNTPs resulted in significant decrease in recombination frequency as compared to the controls. However, depletion of NAD had no significant effect on recombination frequency (Suppl. Figure 1C). Thus, our results suggest that the mitochondria in rat testes are proficient in HR.

Table 1 HR efficiency of mitochondrial extract in the presence of increasing concentrations of protein and at different incubation times

Mitochondrial extract	Recombination frequency ($\times 10^{-6}$)	Fold change
A		
Control	1.12 ± 0.66	–
Testes-ME (1 µg)	1.25 ± 0.98	1.16
Testes-ME (2 µg)	3.7 ± 1.45	3.3
Testes-ME (3 µg)	6.5 ± 0.04	5.8
B		
Control	0.53 ± 0.38	–
0 min	0.56 ± 0.41	1.05
15 min	0.6 ± 0.4	1.13
30 min	2.19 ± 0.49	4.13
60 min	1.5 ± 0.05	2.83
120 min	0.96 ± 0.34	1.81

A: Comparison of HR at different concentrations (1, 2 and 3 µg) of mitochondrial extracts (ME) from rat testes when incubated along with HR plasmids. Data shown is derived from three independent repeats

B: The mean fold change in recombination efficiency when mitochondrial extract from rat testes (2 µg) and HR plasmids were incubated for different time points (0, 15, 30 min, 1, 2 h). Data shown is based on three independent repeats. In both panels, control indicates HR assay in the absence of mitochondrial proteins

Mitochondrial extracts from various tissues possess HR activity

Since ME from testes showed HR activity, we were interested in testing whether mitochondrial extracts from tissues such as brain, spleen and kidney also possess recombination-mediated repair. To test this, an HR assay was performed using protein extracts prepared from purified mitochondria from each tissue after confirming the purity (Fig. 1a–d). Results showed HR activity in all mitochondrial extracts tested, although the recombination frequency was highest in testes (~ 3.98×10^{-6}) (Fig. 2a). Compared to no protein controls, mitochondrial extracts from tissues showed three to fourfold increase in recombination frequency, when analysed after equalization for TFAM, a mitochondrial transcription factor (Fig. 2a). An elevated recombination frequency of 15.26×10^{-6} was observed when bacterial cells with $RecA^+$ background (Tg1 cells) were used and thus impact of incubation with mammalian tissue extracts could not be judged (Suppl. Figure 2). The observed higher recombination rate in $RecA^+$ bacteria as compared to mammalian cells is consistent with previous reports [49, 54, 58].

To compare the level of homologous recombination in whole cell extracts prepared from different tissues, HR assay was performed. Testicular extracts showed the highest recombination efficiency (8.08×10^{-6}). Besides, extracts prepared from brain (5.47×10^{-6}), spleen (5.87×10^{-6}) and kidney (6.23×10^{-6}) exhibited remarkable HR efficiency (Fig. 2b, c; Suppl. Figure 3). Comparison of HR efficiency between mitochondrial extracts and whole cell extracts revealed 2–3-fold difference in recombination (Fig. 2b, c). To rule out the possible contamination of cytoplasmic proteins bound to mitochondrial membrane, we performed digestion with increasing concentrations of proteinase K, prior to preparation of mitochondrial extracts (Fig. 2d). Immunoblotting studies were performed to check the digestion of outer membrane by probing with antiVDAC and the intact inner membrane with antiCYTOCHROME C. Results revealed equal levels of mitochondrial proteins even after proteinase K digestion. Further, recombination assay using mitochondrial extracts revealed no significant difference in recombination efficiency (Fig. 2d, e). Importantly, immunoblotting of RAD51, MRE11 and NIBRIN revealed their presence in the mitochondrial extracts following digestion with proteinase K (Fig. 2e).

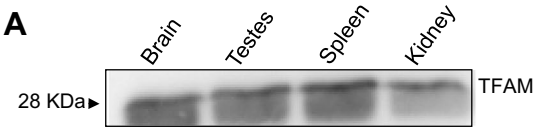
HR assay using HeLa cells showed that mitochondrial recombination occurred even in cancer cells and the efficiency of recombination was higher than that of normal tissue extracts (Suppl. Figure 4A). The purity of extracts was confirmed by probing with PCNA and CYTOCHROME C as described above (Suppl. Figure 4B).

Reciprocal exchange, but not gene conversion, is the predominant recombination activity in mitochondria

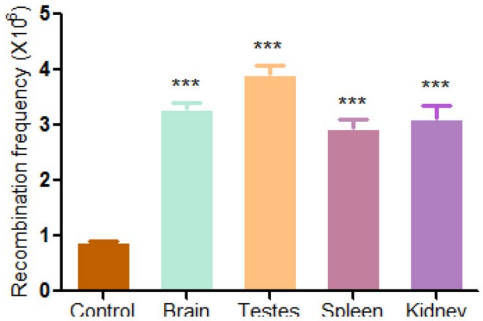
Homologous recombination-mediated DSB repair can occur either through reciprocal exchange or gene conversion. If the recombination is mediated through reciprocal exchange, functional neomycin (neo) gene would be restored by crossing over mechanism, whereas in case of gene conversion it can be with or without crossing over (Fig. 3a, b). Restriction digestion of the recombinant clones (positive for neo-selection) obtained after transformation of HR assay products would allow distinguishing between the two events due to difference in restriction sites. In the upstream position, neoΔ1 has only EcoRI site, while neoΔ2 has an additional site of HindIII, in contrast to a single downstream SalI site (Fig. 3a, b). When the clones are digested with EcoRI/SalI, a fragment of 1.5 Kb is released if the recombinant clones are due to reciprocal exchange, whereas the plasmid carrying the deleted neomycin gene releases fragments of 1.25 Kb (pTO223) or 1.20 Kb (pTO231) (Fig. 3a, d). In contrast, if neomycin gene is restored by virtue of gene conversion, HindIII/SalI digestion would release a fragment of 1.5 Kb, while the plasmids with nonfunctional neomycin gene releases either a fragment of 1.25 Kb (pTO223) or a linearized plasmid (pTO231, 4.1 Kb) as there is no HindIII site (Fig. 3b, e).

Restriction enzyme digestion analysis and sequencing of recombinant clones (resistant to kanamycin) showed

A



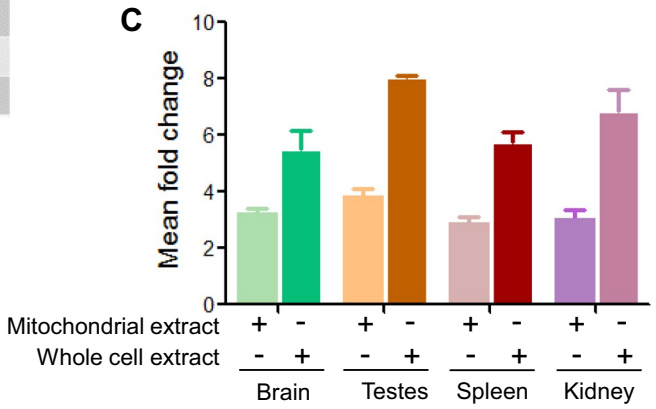
Mitochondrial extract	Ampicillin colonies ($X10^6$)	Kanamycin colonies	Recombination frequency ($X10^{-6}$)
Control	9.04	8	0.88 ± 0.039
Brain	7.83	22	2.86 ± 0.12
Testes	8.27	33	3.98 ± 0.08
Spleen	7.63	20	2.58 ± 0.15
Kidney	7.99	22	2.76 ± 0.016



B

Extract	Recombination frequency ($X\ 10^{-6}$)	
	Whole cell extract	Mitochondrial extract
Brain	5.47	2.52
Testes	8.08	3.70
Spleen	5.87	2.58
Kidney	6.23	2.46

C



D

Mitochondrial extract (Proteinase K treated)	Ampicillin colonies ($X10^6$)	Kanamycin colonies	Recombination frequency ($X10^{-6}$)
Control	12.04	10	0.83 ± 0.19
0 µg/ml	10.08	36	3.57 ± 0.027
1 µg/ml	9.78	36	3.68 ± 0.22
3 µg/ml	10.36	34	3.28 ± 0.038
5 µg/ml	8.99	35	3.89 ± 0.013
10 µg/ml	10.17	34	3.34 ± 0.08
15 µg/ml	10.55	36	3.45 ± 0.025

E

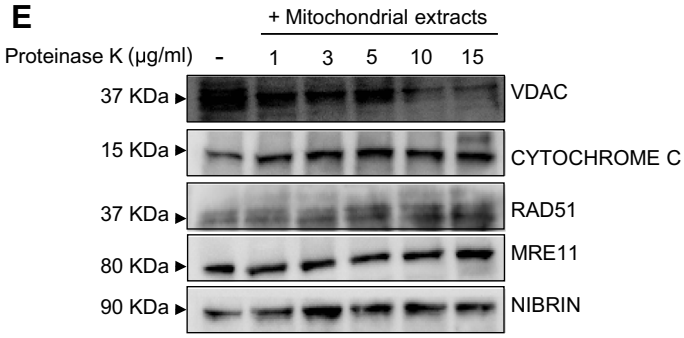


Fig. 2 Comparison of HR efficiency in mitochondrial extracts prepared from various rat tissues. **a** Comparison of HR efficiency in mitochondrial extracts prepared from brain, testes, spleen and kidney. In all cases, ~ 2 µg of mitochondrial extracts equalized for TFAM (Mitochondrial transcription factor A), were incubated with plasmid DNA substrates, recombinants screened and recombination frequency was determined. Bar diagram showing the mean fold change for recombination frequency of mitochondrial extracts from different tissues, with respect to no protein control is shown. Error bar was calculated as mean ± SEM and shows cumulative of nine repeats, including three biological repeats (*ns* not significant, $*p < 0.05$, $**p < 0.005$, $***p < 0.0001$). **b** Table showing comparison of recombination frequency between the mitochondrial extracts and the whole cell extracts prepared from brain, testes, spleen and kidney. **c** Bar graph showing the difference in fold change in recombination frequency between the mitochondrial and whole cell extracts from different tissues. **d** Table showing the recombination frequency of mitochondrial extracts when prepared following proteinase K treatment of purified mitochondria to remove proteins that stick to mitochondrial outer membrane. Mitochondria were isolated and treated with increasing concentrations of proteinase K (0, 1, 3, 5, 10, 15 µg/ml) and used for preparation of extracts. Standard deviation was calculated by comparing HR activity of proteinase K treated samples to that of untreated control as mean ± SEM and is based on three biological repeats with $p > 0.05$. **e** Immunoblot showing the expression of VDAC, CYTOCHROME C, RAD51, MRE11 and NIBRIN in mitochondrial extracts following digestion with proteinase K as described above

that 49 and 48 clones out of 50 were due to reciprocal exchange in case of brain and spleen mitochondria, respectively. 41 out of 48 recombinants were due to reciprocal exchange in case of kidney, while it was 29 out of 45 in testes mitochondrial extracts (Fig. 3c–f). The results were further confirmed by sequencing the recombinant plasmids (Suppl. Figure 5). Hence our results suggest that neomycin gene in > 90% recombinants were restored by

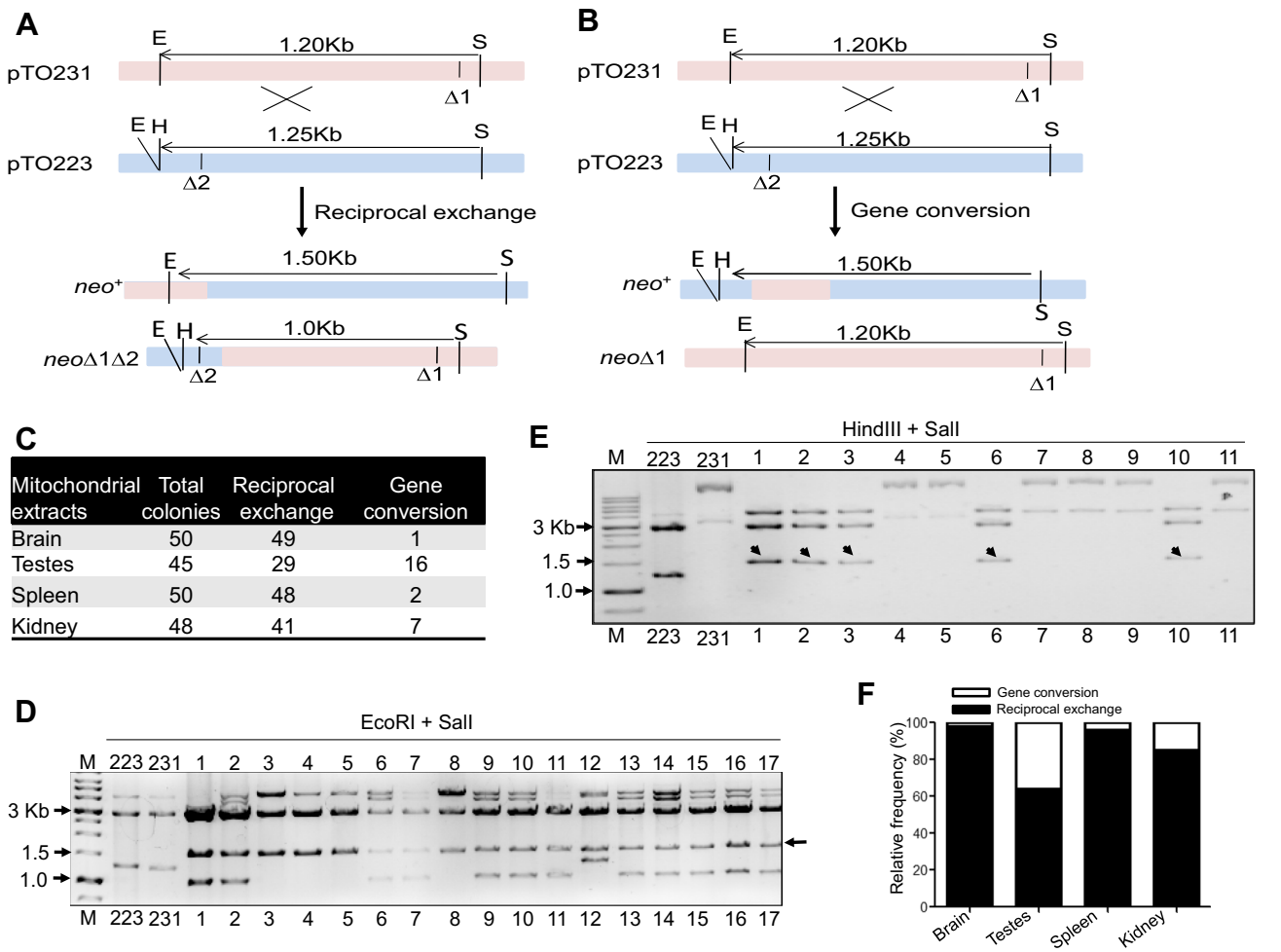
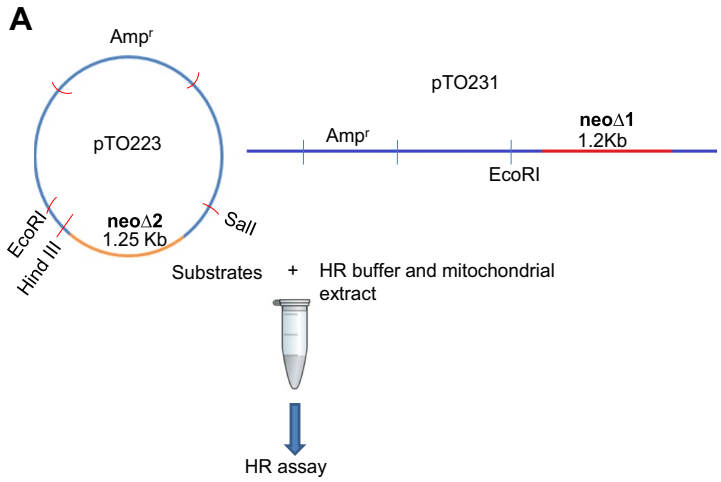


Fig. 3 Assessment of frequency of reciprocal exchange and gene conversion in mitochondrial extracts of different rat tissues. **a** Schematic presentation depicting recreation of restriction enzyme sites in neomycin gene, when recombinants are generated via reciprocal exchange. In this case, the clones release a 1.5 Kb fragment upon EcoRI/SalI digestion, whereas it will be only linearized following HindIII/SalI digestion. **b** Schematic showing the restoration of functional neomycin gene in recombinants by gene conversion. Gene conversion will yield a fragment of 1.5 Kb size upon HindIII/SalI digestion, as depicted. **c** Summary of number of clones evaluated to determine generation of recombinants either by reciprocal exchange or gene conversion in different mitochondrial extracts. **d** Representative agarose gel profile showing the EcoRI + SalI digestion products. The fragment release is indicated using an arrow. **e** Representative agarose gel profile showing HindIII + SalI digestion. The yield of a 1.5 Kb fragment indicates gene conversion and is marked using an arrow. **f** Bar graph showing the distribution of recombinants that had undergone either reciprocal exchange or gene conversion

reciprocal exchange, rather than gene conversion, particularly in mitochondria of somatic tissues (Fig. 3f). Interestingly, ~ 40% of the recombinants were due to gene conversion, in the case of mitochondria from testes (Fig. 3f). It is also important to point out that gene conversion observed here is the minimum, due to limitation of the assay.

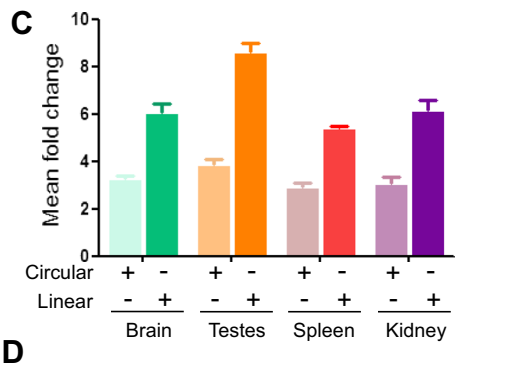
Introduction of a double-strand break improves the efficacy of HR significantly

The Szostak model for DSB repair suggests that HR-mediated repair is improved significantly when a DSB is introduced [59]. Since we observed efficient HR in the mitochondrial extracts, we wondered whether generation of a DSB by linearizing one of the two HR plasmids could improve the efficacy of recombination frequency. To test this, one of the plasmids, pTO231 was linearized using SalI (Fig. 4a) and subjected to HR assay as described (Figs. 1a, 4a). Results showed a significant increase in recombination frequency when one of the plasmid DNA substrates was linearized; mitochondria from brain showing a recombination frequency of 5.88×10^{-6}, testes with 6.95×10^{-6}, spleen with 4.53×10^{-6} and kidney with 5.38×10^{-6} (Fig. 4b). This recombination frequency was 2.2–3-fold higher than that of intact plasmid substrates (Fig. 4c, d). A similar increase in the efficiency of HR was also reported previously, when



B

Mitochondrial extract	Ampicillin colonies ($X10^6$)	Kanamycin colonies	Recombination frequency ($X10^{-6}$)
Control	30.43	28	0.92 ± 0.13
Brain	17.51	103	5.88 ± 0.06
Testes	21.01	146	6.95 ± 0.11
Spleen	25.38	115	4.53 ± 0.02
Kidney	20.07	108	5.38 ± 0.22



D

Mitochondrial extract	Circular	Linear	Fold difference
Brain	2.86	6.05	2.11
Testes	3.98	8.25	2.1
Spleen	2.58	5.97	2.31
Kidney	2.76	6.18	2.24

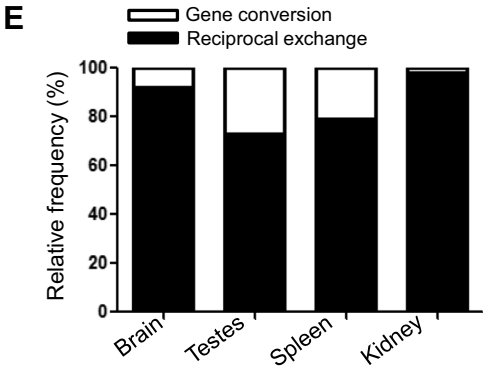


Fig. 4 Evaluation of HR efficiency following induction of a double-strand break in one of the plasmid DNA substrates. **a** Schematic presentation showing HR assay after introducing a DSB on plasmid pTO231 (linearization) by SalI digestion. For other details, refer Fig. 1a. **b** Table showing comparison of recombination frequency of the mitochondrial extracts (2 µg) from various tissues of rats, when incubated with circular–linear HR substrates (500 ng each). Recombination frequency represents the mean of three independent repeats. Standard deviation was calculated as mean ± SEM with $p < 0.005$. **c** Bar graph showing the difference in recombination frequency (fold change) between circular–circular and circular–linear DNA substrates. **d** Comparison of recombination frequency of mitochondrial extracts when circular–circular and circular–linear plasmid DNA substrates were used. The fold change represents the increase in the recombination frequency between the two types of DNA substrates. **e** Bar diagram showing the relative frequency of reciprocal exchange and gene conversion

mouse testicular cell-free extracts were used for the study [49, 54].

Restriction enzyme digestion of the recombinant clones revealed that reciprocal exchange was predominant particularly in case of mitochondrial extracts from brain and kidney. However, both testes and spleen mitochondrial extracts exhibited increased levels (30–35%) of gene conversion (Fig. 4e). Hence, our results demonstrate that introduction of a DSB can improve the HR efficacy significantly, in mitochondrial extracts.

HR proteins are localized to mitochondria

To examine whether the HR proteins are present in mitochondria within cells, immunofluorescence analyses of various HR proteins were performed in HeLa cells. The cells were preincubated with either Mito Tracker Deep Red or MitoTracker Green FM, dyes that stain mitochondria, followed by incubation with appropriate primary antibodies and labeled secondary antibodies. Confocal microscopy studies showed mitochondrial localization

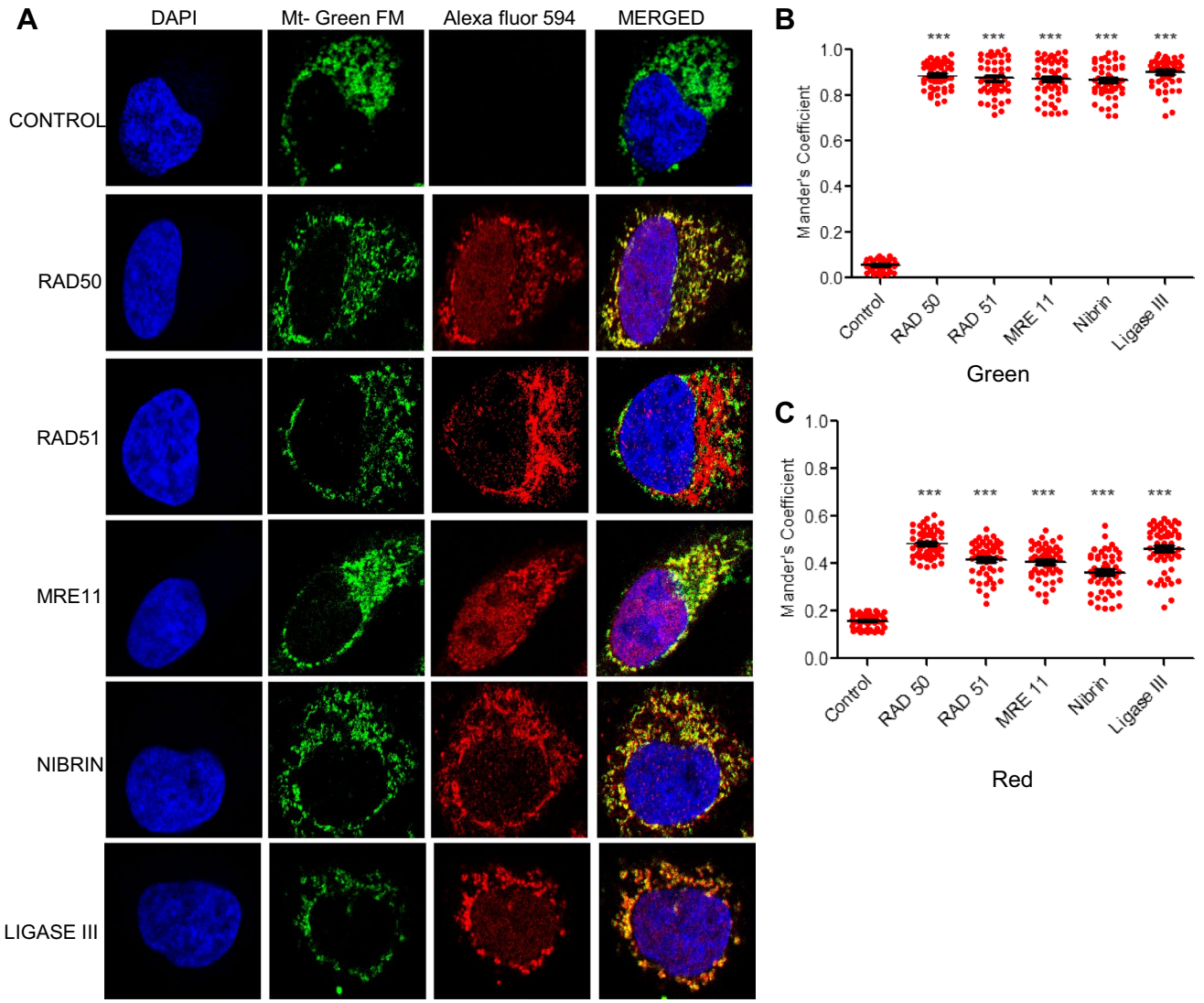


Fig. 5 Immunofluorescence studies showing localization of RAD50, RAD51, MRE11, NIBRIN and LIGASE III in mitochondria of HeLa cells. **a** Representative images of colocalization of RAD50, RAD51, MRE11, NIBRIN and LIGASE III with mitochondrial signal in HeLa cells. Alexa Fluor-conjugated secondary antibodies were used for detecting proteins. Mt-Green FM is Mitotracker Green, a mitochondrial marker. DAPI is used as nuclear stain. **b** Colocalization analyses using JaCoP in Image J software. Minimum of 50 cells were used for analysis of colocalization of red and green signals, and plotted as the colocalization value of green overlapping red (dot plot marked as green). *Y*-axis depicts the Mander's colocalization coefficient value calculated for green over red and plotted in the form of dot plot. **c** Colocalization analyses values plotted as red overlapping green (dot plot marked as red). *Y*-axis depicts the Mander's colocalization coefficient value calculated for red over green and plotted in the form of dot plot. The significance for both the plots was calculated using GraphPad Prism 5.0 with respect to secondary control and shown as mean ± SEM (*ns* not significant, $*p < 0.05$, $**p < 0.005$, $***p < 0.0001$)

of RAD51, RAD50, MRE11, LIGASE I and NIBRIN in HeLa cells (Fig. 5, Suppl. Figure 6). As expected, expression of these proteins was also seen in the nucleus, which was counter stained by DAPI. Thus, our results reveal presence of specific HR-associated DSB repair proteins in mitochondria.

HR in mitochondria is dependent on RAD51, MRE11 and NIBRIN

Presence of well-known HR proteins such as RAD51, MRE11 and NIBRIN was tested in mitochondrial extracts by western blotting. Both mitochondrial and cytosolic extracts

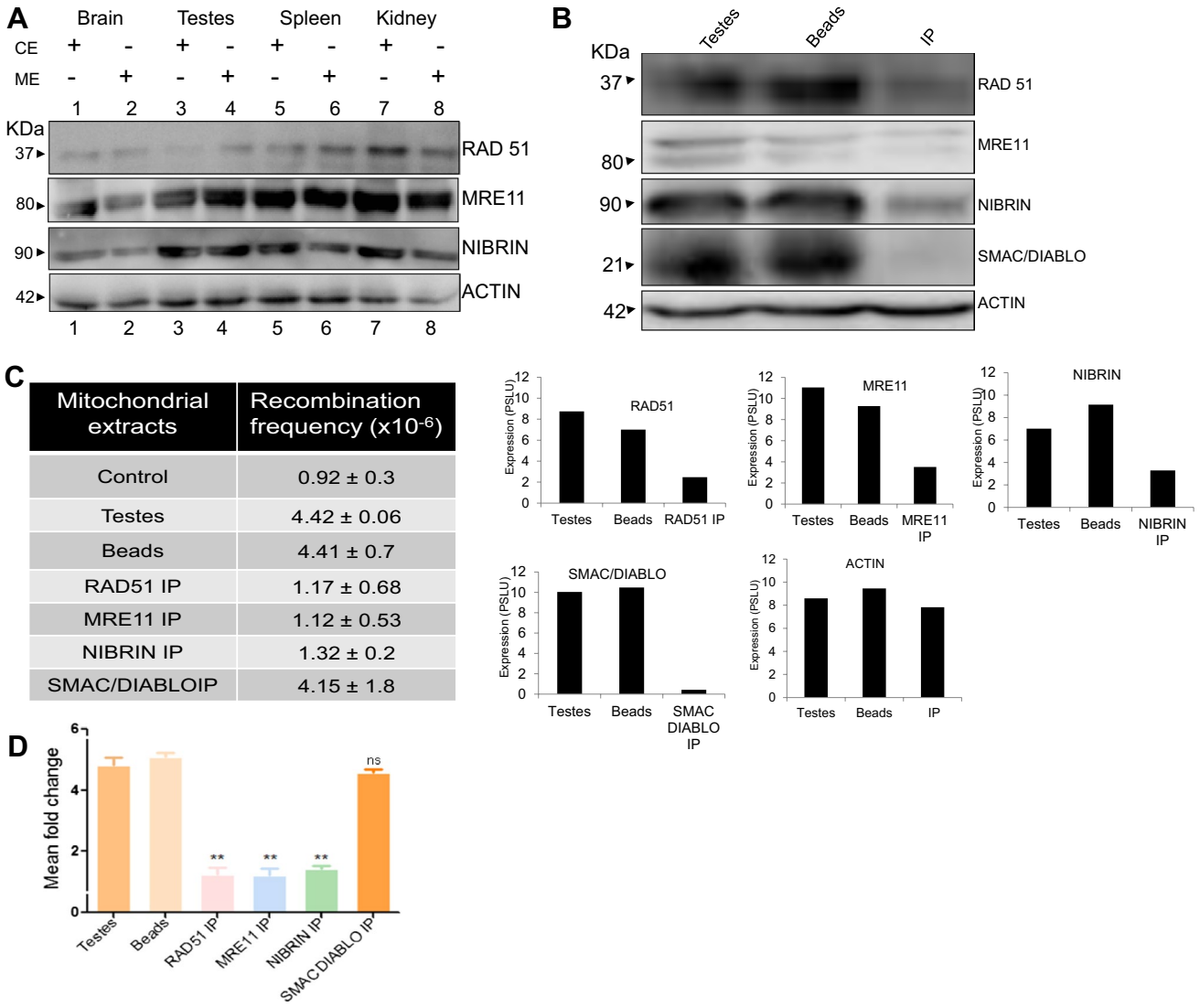


Fig. 6 Evaluation of involvement of DSB repair proteins in HR-mediated repair in mitochondria. **a** Immunoblot analyses showing expression levels of MRE11, RAD51 and NIBRIN in mitochondrial extracts of brain, testes, spleen and kidney. Cytosolic extracts prepared from same tissues served as the control. **b** Immunoblotting showing efficacy of immunoprecipitation of RAD51, MRE11, NIBRIN and SMAC/DIABLO in mitochondrial extracts of rat testes and its quantification. The bar graphs corresponding to the immunodepletion are expressed in PSL units. β-ACTIN served as the loading control. **c** Evaluation of recombination frequency in immunodepleted mitochondrial extracts of rat testes in comparison to mitochondrial extracts. Mean recombination frequency represents the mean of two independent experiments using immunodepleted extracts. **d** Bar diagram showing the mean fold change in recombination frequency of the IP extracts with respect to that of no protein control. Error bars were calculated as SEM and shows a cumulative of six repeats, which includes three biological repeats (*ns* not significant, $*p < 0.05$, $**p < 0.005$, $***p < 0.0001$)

of respective tissues were used for analysis after equalizing with the loading control β-ACTIN. Results showed robust expression of all three proteins investigated in both cytosolic and mitochondrial extracts, particularly in testes, spleen and kidney (Fig. 6a).

To investigate the role of RAD51, MRE11 and NIBRIN in mitochondrial extract-mediated HR, proteins of interest were immunodepleted using respective antibodies from the mitochondrial extracts of rat testes. SMAC/DIABLO, a protein not involved in HR served as a negative control. To determine the extent of immunodepletion, western blotting was performed using antibody against respective proteins (Fig. 6b). Results showed significant reduction upon IP in case of all four proteins, while the loading control β-ACTIN levels were comparable (Fig. 6b). The HR assay using RAD51-, MRE11- and NIBRIN-immunodepleted extracts revealed significant reduction in recombination frequency, when compared to controls (Fig. 6c, d). Interestingly, there was no significant change in recombination frequency when SMAC/DIABLO depleted extract or beads alone

(supernatant) were used for the study (Fig. 6c, d). Thus, immunoprecipitation studies suggest that HR in mitochondria is mediated by RAD51 and MRN complex proteins such as MRE11 and NIBRIN.

Discussion

Various mitochondrial genomic alterations leading to several disorders, myopathy, ageing, etc., have been suggested by previous studies [26, 47, 60, 61]. However, the mechanism by which mitochondrial DNA integrity is maintained when DSBs are generated is not well understood [29, 62]. The importance of mitochondria in maintaining the cellular homeostasis explains the requirement of precise DNA repair mechanisms [63].

Mitochondria possess proficient HR-mediated DSB repair mechanism

In the current study, we show that the mitochondrial extracts prepared from various tissues of rat possess efficient HR activity. Introduction of a DSB resulted in significant increase in the recombination frequency, irrespective of cell type. These results suggest that mitochondria possess HR protein machinery to recognize breaks and repair through recombination. Preliminary studies suggested potential HR-mediated DSB repair in mitochondria [29, 64]. Efficient HR in mice testicular cell-free extracts was also reported previously [49, 54].

The mitochondrial extracts used in this study were carefully prepared as described [26] and purity of the extracts was confirmed by immunoblotting using specific mitochondrial markers, VDAC and CYTOCHROME C and nuclear marker PCNA, to rule out nuclear and cytosolic contamination. Besides, we also prepared extracts using mitochondrial purification kit and observed that the results were consistent, irrespective of the method used. These ruled out the possibility that the observed HR in our study is due to nuclear contamination. Apart from this, we also observed mitochondrial localization of HR proteins such as MRE11, RAD51 and NIBRIN through immunofluorescence studies in HeLa cells.

The possibility of recombination activity contributed by the bacterial strain used for transformation was ruled out using both, no protein control and heat-inactivated mitochondrial extracts. Thus, our study demonstrates that mitochondria purified from cells of four different organs from rat, and cancer cells possess efficient homologous recombination.

HR in mitochondria is mediated by RAD51 and MRN complex

Western blotting studies revealed the presence of critical HR proteins such as RAD51, MRE11 and NIBRIN in mitochondrial extracts prepared from different rat tissues. The results were also consistent with colocalization studies in HeLa cells, where these proteins were present in mitochondria as well as in the nucleus. Our results are also consistent with the previous reports showing the presence of RAD51 and MRE11 in mitochondria [29, 65, 66]. Most importantly, we observed that immunodepletion of RAD51, MRE11 and NIBRIN led to significant decrease in HR efficiency, suggesting their involvement in maintenance of mitochondrial genome stability.

Although we and others were able to detect the HR proteins in mitochondria, the mechanism of translocation of these proteins to mitochondria is yet to be understood. Most of the proteins of interest lack a mitochondrial localization signal (MLS). However, an alternative translation initiation mechanism is reported for Ligase III [67]. Interestingly, this mechanism is known to produce proteins with or without MLS from the same mRNA [67]. The other mechanism utilises Mia40 transport machinery, which is known for import of proteins such as Tim and APE1 to mitochondrial intermembrane space (IMS). These small proteins bind to Mia40 and get translocated to IMS [68, 69]. It is also possible that there are other unknown mechanisms in mitochondria responsible for transport of such proteins that need to be investigated.

HR-mediated DSB repair in mitochondria is predominantly dependent on reciprocal exchange

The analysis of the recombinant clones in our assays revealed that HR in mitochondria was primarily through reciprocal exchange particularly in somatic cells. Interestingly, mitochondria from testicular cells showed significant frequency of gene conversion, besides reciprocal exchange. The result was consistent, irrespective of whether circular–circular or circular–linear DNA substrates were used. It is also expected that upon incubation in mitochondrial extracts, one of the plasmids gets linearized due to nucleases present in the extract. Thus, it is understandable that, although, introduction of a DSB externally improved the efficacy of HR significantly, it did not significantly affect the occurrence of gene conversion vs. reciprocal exchange. This observation was consistent with one of the previous studies, where HR-mediated DSB repair through gene conversion was maximal in spermatocytes (meiotic in nature) compared to spermatogonia or spermatids (somatic in nature) [54].

DSBs are generally formed during leptotene in germ cells and repaired during zygotene and the number of

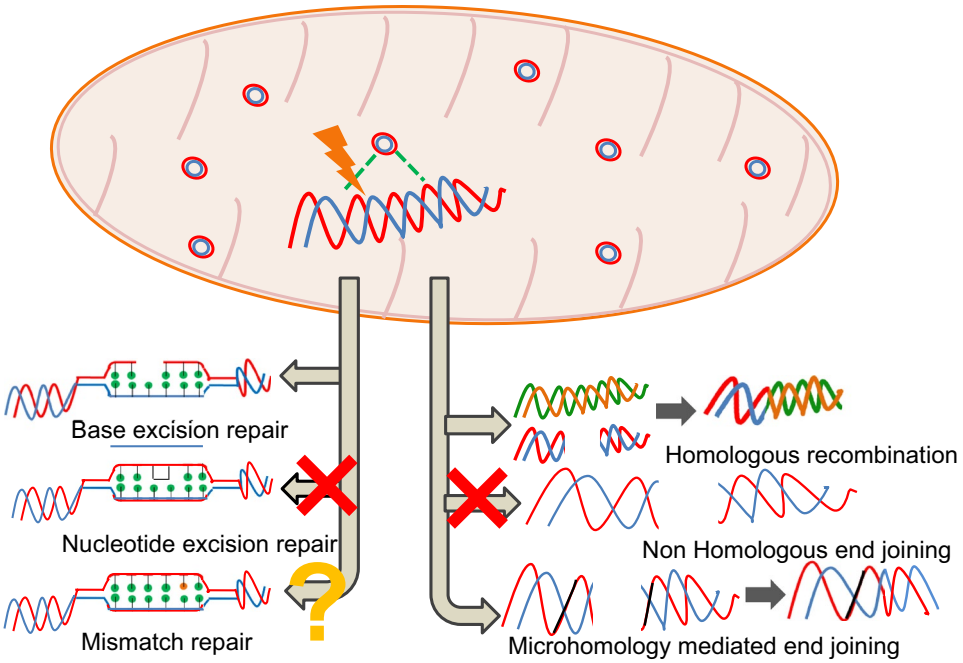


Fig. 7 Cartoon depicting various DNA repair pathways in mitochondria. Among the three major repair pathways of excision repair in nucleus, mitochondria are proficient only with base excision repair, while the existence of mismatch repair is not clear. Our study shows that homologous recombination and microhomology-mediated end joining are two major double-strand break repair pathways in mitochondria, whereas nonhomologous end joining is undetectable

crossovers is less as compared to the DSBs generated [70]. The DSBs not undergoing repair through crossovers require fast repair, which are thus mediated through gene conversion [71]. In somatic tissues, the breaks induced in nuclear genome are less, as compared to germline tissues, which may afford delayed repair by reciprocal exchange [71]. Thus, it is possible that mitochondria present in somatic tissues would have evolved a machinery favoring reciprocal exchange rather than gene conversion, unlike mitochondria from germ cells, which prefer fast and accurate repair [54, 72].

DSB repair pathways in mitochondria

Based on the current study, we propose HR-mediated DSB repair for maintaining stability of mitochondrial genome. Previous studies also support such a conclusion [29, 64]. Since there are multiple copies of mitochondrial DNA present, any damage to mitochondrial DNA facilitates the use of the homologous region of the other copy of DNA and repair is achieved by homologous recombination. This could be mediated by reciprocal exchange or gene conversion depending on the cell type. HR is error-free repair and thus repairs the DNA without any loss of sequences (Suppl. Figure 7, Fig. 7). However, if the break is proximal to the direct repeat regions of the mitochondrial genome, repair is done by microhomology-mediated end joining. MMEJ is an error-prone repair and results in deletions of varying lengths. Our previous study reports the existence of such an error-prone pathway and the absence of c-NHEJ [26] (Fig. 7). The occurrence of MMEJ can explain the prevalence of large mitochondrial deletions and human disorders associated with it.

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Compliance with ethical standards

Conflict of interest The authors disclose that there is no potential conflict of interest.

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