

# The Friedreich's ataxia protein frataxin modulates DNA base excision repair in prokaryotes and mammals

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DNA-repair mechanisms enable cells to maintain their genetic information by protecting it from mutations that may cause malignant growth. Recent evidence suggests that specific DNA-repair enzymes contain ISCs (iron–sulfur clusters). The nuclear-encoded protein frataxin is essential for the mitochondrial biosynthesis of ISCs. Frataxin deficiency causes a neurodegenerative disorder named Friedreich's ataxia in humans. Various types of cancer occurring at young age are associated with this disease, and hence with frataxin deficiency. Mice carrying a hepatocyte-specific disruption of the frataxin gene develop multiple liver tumours for unresolved reasons. In the present study, we show that frataxin deficiency in murine liver is associated with increased basal levels of oxidative DNA base damage. Accordingly, eukaryotic V79 fibroblasts overexpressing human frataxin show

decreased basal levels of these modifications, while prokaryotic *Salmonella enterica* serotype Typhimurium TA104 strains transformed with human frataxin show decreased mutation rates. The repair rates of oxidative DNA base modifications in V79 cells overexpressing frataxin were significantly higher than in control cells. Lastly, cleavage activity related to the ISC-independent repair enzyme 8-oxoguanine glycosylase was found to be unaltered by frataxin overexpression. These findings indicate that frataxin modulates DNA-repair mechanisms probably due to its impact on ISC-dependent repair proteins, linking mitochondrial dysfunction to DNA repair and tumour initiation.

**Key words:** DNA base excision repair, frataxin, Friedreich's ataxia, iron–sulfur cluster, oxidative stress, tumorigenesis.

## INTRODUCTION

ISCs (iron–sulfur clusters) are small inorganic molecules that can be found in all kingdoms of life. Being integrated into a number of proteins, ISCs are involved in pivotal biochemical functions including the citric acid cycle, amino acid synthesis, respiration, nucleotide metabolism and iron-uptake regulation. In the last 10 years, significant knowledge about ISC biogenesis and the assembly of ISC proteins has been gained [1,2]. In eukaryotes, mitochondria harbour the ISC-assembly machinery. Although these pathways are still awaiting further elucidation, it appears generally accepted that the FRDA (Friedreich's ataxia)-associated protein frataxin is crucial for ISC biosynthesis [3] and plays a primary role in ISC protein maturation in human cells [4,5]. It should be noted, however, that additional functions for the frataxin protein have been shown experimentally [6,7].

Defects in the frataxin gene and thereby a reduced level of functional frataxin protein leads to a human disease named FRDA (OMIM 229300), with serious clinical features including abnormalities in glucose metabolism, limb and gait ataxia, hypertrophic cardiomyopathy and reduced expectancy of life. Although unconditional deletion of frataxin in mice causes early embryonic lethality [9], *Cre/loxP* conditional knockout mice [10–12] and GAA repeat knockin mice [13] unambiguously

reflect important physiological and biochemical features of the human disease.

We have previously generated and characterized mice with a *Cre/loxP*-mediated hepatocyte-specific disruption of frataxin named *AlbFxn<sup>-/-</sup>* [12]. These mice exhibit impaired mitochondrial energy metabolism, increased oxidative stress and severely reduced lifespan [12]. Unexpectedly, we also observed liver tumour formation in almost all animals within the first 12 months of life, whereas control animals rarely exhibit such tumours during this time period. This finding is consistent with several reports of malignant disorders in FRDA patients [14–17]. Moreover, we have described reduced malignancy in cancer cells which stably overexpress frataxin [18,19] in a potentially p38 MAPK (mitogen-activated protein kinase)-dependent manner [19]. Consistently, in *AlbFxn<sup>-/-</sup>* mice, we have shown impaired activation of the p38 MAPK pathway, as well as changes in the rates of proliferation suggesting a tumour-promoting effect of frataxin deficiency as one relevant cause for the tumour formation [11]. However, potential changes in tumour-initiation processes were not studied at that time.

Since then, dependency of DNA-repair enzymes on ISCs [20,21] has been proposed and studied in more detail: the ISC-containing DNA glycosylase MUTYH [human mutY homologue (*Escherichia coli*)] has been shown to be involved in BER (base excision repair), and alterations in the human *MUTYH*

Abbreviations used: BER, base excision repair; DMEM, Dulbecco's modified Eagle's medium; Fpg, formamido-pyrimidine DNA glycosylase; FRDA, Friedreich's ataxia; HPRT, hypoxanthine phosphoribosyltransferase; ISC, iron–sulfur cluster; LB, Luria–Bertani; MAPK, mitogen-activated protein kinase; MUTYH, human mutY homologue (*Escherichia coli*); OGG1, 8-oxoguanine DNA glycosylase 1; 8-oxoG, 7,8-dihydro-8-oxoguanine; ROS, reactive oxygen species; SSB, DNA single-strand break.

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gene are associated with cancer [22]. Boal et al. [23] have demonstrated a functional role of the ISC in mutY and two other BER enzymes, namely Nth and AfUDG (*Archeoglobus fulgidus* uracil DNA glycosylase). Furthermore, the DNA-repair helicases XPD (xeroderma pigmentosum complementation group D) and FancJ (Fanconi's anaemia complementation group J), which are involved in NER (nucleotide excision repair) and which are associated with the human diseases xeroderma pigmentosum and Fanconi's anaemia, frequently resulting in skin cancer, leukaemia and solid tumours, have been shown to contain ISCs that appear to be essential for their respective helicase activities [24].

This recently established and presumably essential role of ISCs in previously defined processes of DNA repair, together with the known relevance of DNA-repair enzymes on tumour initiation, suggests that ISCs may be relevant for tumour suppression. In the present study, we have tested this hypothesis by analysing a murine model of targeted disruption of the ISC synthesis-modulating frataxin gene in mammalian liver, as well as two model systems overexpressing frataxin, with respect to DNA repair and tumour initiation.

## MATERIALS AND METHODS

### Chemicals

Benzo[*a*]pyrene 4,5-oxide was kindly provided by Dr Albrecht Seidel (Biochemisches Institut für Umweltcarcinogene, Grosshansdorf, Germany). All other chemicals were purchased from Sigma–Aldrich.

### Animal experimentation

Mice were kept in accordance with the Federation of European Laboratory Animal Science Associations regulations and all experiments were approved by the corresponding institutional review board.

### Mutagenicity in *Salmonella enterica* serotype Typhimurium

*Salmonella* Typhimurium strains TA104 and TA1538 were kindly provided by Dr Bruce N. Ames (University of California, Berkeley, CA, U.S.A.). Human frataxin cDNA was recovered by amplification from previously described plasmids harbouring haemagglutinin-tagged frataxin [19] with primers including suitable restriction sites for the target vectors, AmesBac\_FX\_FWD (5'-CGCGGATCCCCATGGGAACCTTG GCCACCCAGGCTCT-3') and AmesBac\_FX\_REV (5'-TG-GGAATTCAAGCTTCTAGATGTATCGGTACGTGTCTGC-3') (Invitrogen). Primers were designed to generate a coding sequence beginning at amino acid 88, thereby leading to a cDNA lacking the N-terminal mitochondrial localization signal peptide [25]. The PCR product was cloned into pCR2.1 (Invitrogen) and confirmed by sequencing. The frataxin cDNA was then subcloned into the ampicillin-resistant target vector pKK223-3 by digestion with NcoI and HindIII. Orientation was tested by digestion with BamHI/HindIII and BamHI/SacI. The pKK223-3 plasmid containing the coding sequences for frataxin was digested with HindIII and SalI restriction enzymes and then cloned into the neomycin-resistant plasmid pKKneo [26]. The bacteria containing either pCR2.1 or pKK223-3 were grown on LB (Luria–Bertani) medium with ampicillin (100 µg/ml). For selection of pKKneo, LB medium was supplemented with neomycin (50 µg/ml). The pKKneo vector containing frataxin cDNA and the control vector pKKneo [26] were adapted to the restriction enzymes of *Salmonella* Typhimurium LT2 by passaging them through the restriction-deficient, but

methylation-proficient, *Salmonella* Typhimurium strain LB5000. Plasmids were recovered and used for the transformation of the strains *Salmonella* Typhimurium TA104 and TA1538 with frataxin and control vectors. The names of the resulting bacterial strains consisted of the short name of the recipient cells (TA104 and TA1538) and the expressed protein, namely TA104-hFX, TA104-Neo, TA1538-hFX and TA1538-Neo.

The so-called Ames test was performed as described previously [27]. Briefly, nutrient broth (25 g/l Oxoid nutrient broth No. 2) was inoculated with bacteria and grown overnight at 37°C. Before the experiment, bacteria were centrifuged at 9000 *g* for 10 min, resuspended in medium B (1.6 g/l Bacto nutrient broth and 5 g/l NaCl) and adjusted nephelometrically to a titre of  $(1.1-1.8) \times 10^9$  bacteria (colony-forming units)/ml. The bacterial suspension (100 µl) and benzo[*a*]pyrene 4,5-oxide (in 10 µl DMSO) were added sequentially to a glass tube containing 500 µl of 150 mM KCl at 37°C. After 60 min, 2 ml of 45°C warm top agar (0.55 % agar, 0.55 % NaCl, 50 µM histidine, 50 µM tryptophan, 50 µM biotin and 25 mM sodium phosphate buffer, pH 7.4) was added, and the mixture was poured into a Petri dish containing 22 ml of minimal agar (1.5 % agar in Vogel-Bonner E medium with 2 % glucose). After incubation at 37°C for 3 days in the dark, the colonies (his<sup>+</sup> revertants) were counted. Initially, a dose-finding experiment was carried out. The compounds were then tested using three plates for each condition. In virtually all cases, the individual values deviated from the mean by less than 10 % or by less than ten colonies.

### Origin, culture and stable transfection of V79 Chinese hamster cells

V79 Chinese hamster cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (German Collection of Microorganisms and Cell Lines, Braunschweig, Germany). Cells were thawed and propagated in DMEM (Dulbecco's modified Eagle's medium) containing 4.5 g/l D-glucose and 10 % fetal bovine serum, and three passages later frozen in aliquots of 1 ml.

Transfection experiments were performed as described previously using pCINeo (Promega) (control) and pCINeo-hFX [19]; the latter containing a full-length frataxin, i.e. including the N-terminal mitochondrial localization signal and a C-terminal haemagglutinin tag. V79 cells were selected and maintained in DMEM containing 4.5 g/l D-glucose, 10 % fetal bovine serum and 700 mg/l neomycin. Transfected cells were propagated as described above, and three passages later frozen in aliquots of 1 ml. The names of the resulting cell lines are V79-Neo and V79-hFX. Detection of human frataxin was performed on protein extracts containing 30 µg of protein by PAGE and Western blotting using an anti-haemagglutinin antibody (Roche Diagnostics) followed by a horseradish-peroxidase-labelled secondary antibody and standard chemoluminescence detection.

To determine the growth curve, we seeded  $2.5 \times 10^4$  cells into 72 replica 6-cm-diameter plastic dishes. The cells in eight dishes were counted every 12 h, and growth curves were created. The population doubling time was determined from the growth curve. Oxygen consumption was measured using a Clark-type electrode (Hansatech) as described previously [28]. For each measurement  $2 \times 10^6$  cells suspended in HBSS (Hanks balanced salt solution) were used.

### HPRT (hypoxanthine phosphoribosyltransferase) gene mutation test in Chinese hamster V79 cells

Six independent lines of frataxin-overexpressing and mock-transfected V79 cells were treated three times with 10 mM KBrO<sub>3</sub> for 1 h in 100-mm-diameter Petri dishes before subcultivation.

Treated cultures as well as control cells were transferred as needed (minimum  $2 \times 10^6$  cells). Following the third treatment,  $10^6$  cells from each culture were plated into five new 100-mm-diameter dishes with selective medium containing  $7 \mu\text{g}/\mu\text{l}$  6-thioguanine to determine the number of mutants. The plating efficiency was determined in non-selective medium plating 200 cells in 60-mm-diameter dishes. After 1 week, colonies were fixed with methanol, stained with sulforhodamine-B and counted.

### Isolation of hepatocytes

AlbFxn<sup>-/-</sup> mice were generated, bred and verified as described previously [12]. For determination of oxidative DNA modifications, hepatocytes from 3-week-old AlbFxn<sup>-/-</sup> mice and control genotypes (littermates) were isolated by a modified two-step collagenase perfusion technique [29]. Briefly, the liver was perfused *in situ* via the portal vein for 15 min with an EGTA-containing buffer at 37 °C. The perfusion was continued for another 30 min with a collagenase-containing buffer. The liver was removed; hepatocytes were carefully dissociated in a suspension buffer and filtered through a 100- $\mu\text{m}$  gauze. Hepatocytes were centrifuged at 15 g for 10 min and washed twice in suspension buffer, before viability was determined by Trypan Blue exclusion.

### DNA-damage induction by photosensitization

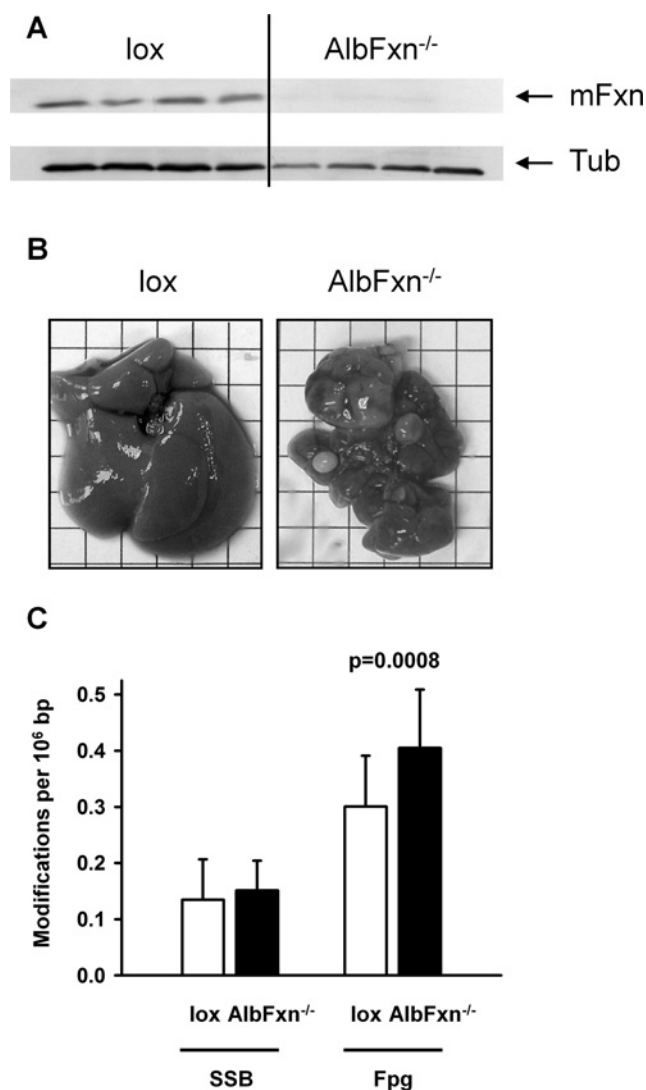
In order to induce DNA damage, exponentially growing V79-hFX cells and controls were irradiated with visible light in a phosphate buffer containing 50 nM of the photosensitizer Ro 19-8022 (Hoffmann La Roche). Cells were irradiated for 10 min on ice from a distance of 0.38 m with a 1000 W halogen lamp. This treatment oxidizes bases in DNA, but causes few direct strand breaks [30]. Subsequently, cells were either analysed directly or maintained under standard culture conditions for 4 h.

### Quantification of DNA modifications

An alkaline elution assay [31] in combination with 1  $\mu\text{g}/\text{ml}$  Fpg (formamido-pyrimidine DNA glycosylase), the bacterial functional analogue of OGG1 (8-oxoguanine DNA glycosylase 1), was used to quantify various types of DNA modification in V79 cells [30] and in hepatocytes [32]. In this manner, SSBs (DNA single-strand breaks) were determined by incubation of the cellular DNA without Fpg immediately after cell lysis. The number of modifications sensitive to Fpg was determined using parallel filters with the repair enzyme. To quantify the Fpg-sensitive modifications, the number of SSBs was subtracted, whereas the background levels observed with untreated controls were subtracted in all cases.  $\gamma$ -Irradiated cells were used for calibration, assuming that 6 Gy induces 1 SSB/ $10^6$  bp [31].

### Cleavage activity of OGG1 in cell extracts

Approx.  $1.2 \times 10^7$  V79 Chinese hamster fibroblasts or hepatocytes were washed with phosphate buffer, and the cell pellet was resuspended in 200  $\mu\text{l}$  of lysis buffer (200 mM Tris/HCl, pH 8, 10 mM EDTA and 2.5 M NaCl) containing protease inhibitors (Complete™ Mini from Roche Applied Science). After cells were sonicated carefully, the suspension was centrifuged in a 60Ti rotor at 45 000 rev./min for 45 min at 4 °C. The resulting supernatant was used to determine the protein concentration according to the method of Bradford. Cleavage activities at 8-oxoG (7,8-dihydro-8-oxoguanine) sites in these cell extracts were measured by incubating 5  $\mu\text{g}$  of protein in a total volume of 32  $\mu\text{l}$  for 1 h at 37 °C with a 34-mer 5' <sup>32</sup>P-labelled double-stranded oligodeoxyribonucleotide containing an 8-oxoG residue in the la-



**Figure 1** Liver tumour formation and basal levels of oxidative DNA modifications in frataxin-deficient AlbFxn<sup>-/-</sup> mice

(A) Hepatocyte-specific disruption of frataxin expression in the liver and loading controls are displayed by immunoblotting against murine frataxin protein (mFxn) and  $\alpha$ -tubulin (Tub) respectively. (B) Typical liver specimen of control and AlbFxn<sup>-/-</sup> mice. (C) Numbers of DNA SSBs and Fpg-sensitive modifications (Fpg) in untreated hepatocytes as quantified using the alkaline elution technique. Results are means + S.D. for 11 mice in each group. Significant difference between the groups is indicated according to a paired Student's *t* test.

belled strand at position 16 opposite a cytosine. After performing denaturing PAGE, the extent of DNA cleavage was quantified using a Storm Phosphorimager (Molecular Dynamics) [33].

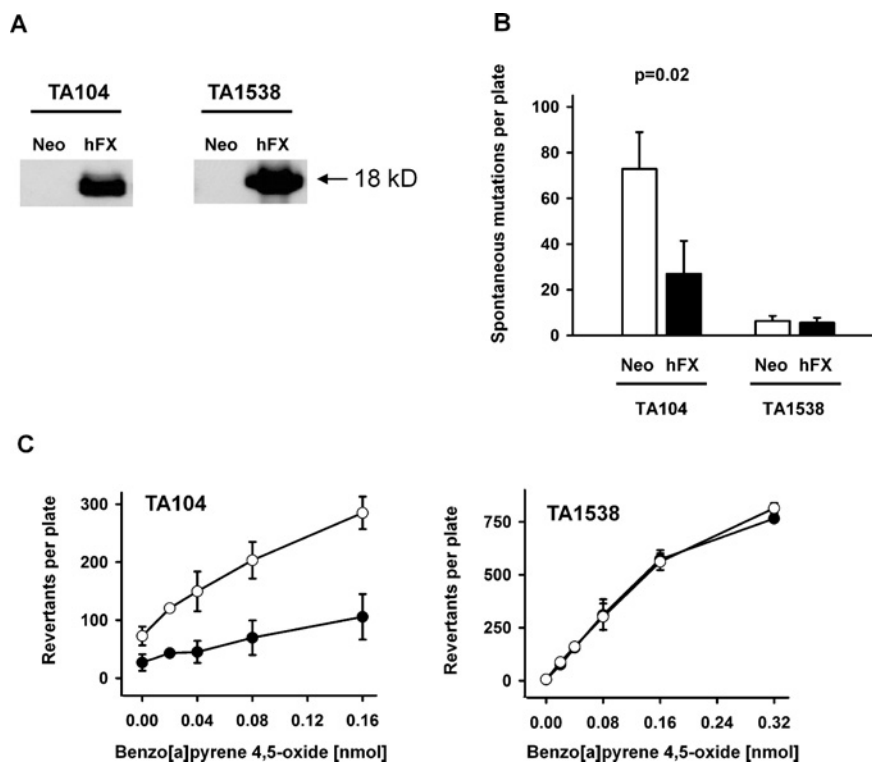
### Statistical analyses

A Kolmogorov–Smirnov test was used for normality tests. We found normality in all experiments. Unless noted otherwise, an unpaired Student's *t* test was used.  $P < 0.05$  was considered to be statistically significant.

## RESULTS

### Tumorigenicity in frataxin-deficient AlbFxn<sup>-/-</sup> mice is correlated with oxidative DNA base modifications

In the present study, we used the Cre/loxP system to remove exon 4 of the frataxin gene in a hepatocyte-specific manner. The



**Figure 2** Human frataxin protein protects from specific mutations in *Salmonella Typhimurium*

(A) Immunoblot analysis of recombinant frataxin expression in cytosolic preparations (20  $\mu$ g of protein/lane) of *Salmonella Typhimurium* strains TA104-hFX and TA1538-hFX against murine monoclonal HA clone 12CA5 (Roche) (18 kDa). Neo, mock-transfected control strains. (B) Number of spontaneous revertant colonies per plate. Results are means  $\pm$  S.D. for three (TA104) and four (TA1538) independent experiments. (C) Revertants per plate in the presence of various concentrations of benzo[a]pyrene 4,5-oxide. ○, Neo; ●, hFX.

absence of detectable frataxin protein following disruption was shown by immunoblotting (Figure 1A). As published previously, hepatocyte-specific frataxin-knockout mice develop liver tumours early in life (Figure 1B) [12].

The previously described increase in oxidative stress in liver specimens of AlbFxn<sup>-/-</sup> mice [12] led to the assumption that there may be an increased oxidative DNA damage in such liver cells. We tested this possibility in isolated hepatocytes from 3-week-old AlbFxn<sup>-/-</sup> mice and quantified basal levels of oxidative DNA modifications in terms of SSBs and Fpg-sensitive modifications. The latter include oxidative purine modifications such as 8-oxoG and sites of base loss [34]. As depicted in Figure 1(C), we observed that the deficiency of frataxin was associated with significantly increased levels of Fpg-sensitive lesions, but not of SSBs. This selectivity is an indication that the effect is more likely to be caused by a retardation of specific repair mechanisms rather than by an increased generation of the damage. To test this hypothesis and elucidate further the underlying mechanisms, we decided to overexpress human frataxin in both prokaryotic and eukaryotic model systems.

#### Overexpression of the human frataxin protein reduces DNA base substitution-mediated mutations in prokaryotes

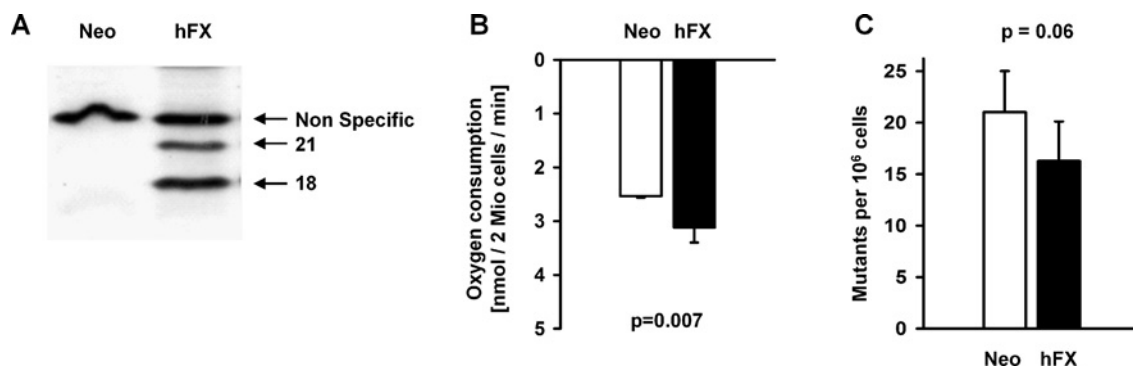
Impaired frataxin expression in humans reduces the activity of ISC-dependent enzymes [35], whereas overexpression of frataxin has been shown to increase the activity of ISC-containing enzymes [28]. Given the above-mentioned findings in frataxin-deficient mice, we assumed that overexpression of frataxin would conversely increase the ISC-mediated DNA-repair capacity and therefore ultimately reduce the frequency of mutations. To test this hypothesis, we decided to use the

Ames test [36], and first generated two prokaryotic systems stably overexpressing human frataxin (Figure 2A). As host cells, we used the *Salmonella Typhimurium* strain TA1538, which preferentially detects frameshift mutations [37], and strain TA104, which preferentially detects base substitutions [38]. Accordingly, the latter strain is highly sensitive to oxidative damage following exposure to ROS (reactive oxygen species) [38]. Consistent with our hypothesis, we observed a significant reduction in the number of spontaneous revertants only in TA104 *Salmonella* overexpressing frataxin, whereas the spontaneous mutation frequency in TA1538 bacteria remained unaffected (Figure 2B). Although ISC formation has not been quantified directly in these *Salmonella* strains, these findings indicate that ISC-synthesis-promoting frataxin reduces spontaneous mutation frequency in a *Salmonella* strain sensitive to oxidative stress.

Benzo[a]pyrene 4,5-oxide, a metabolite of benzo[a]pyrene, is a well-characterized mutagen [39] and was tested in both frataxin-overexpressing strains TA1538-hFX and TA104-hFX, as well as the corresponding control strains TA1538-Neo and TA104-Neo. In all strains, a dose-dependent increase in the number of revertants was detected. However, only in the TA104 strains did overexpression of frataxin cause a significant reduction of mutation frequency (Figure 2C). The ISC-synthesis-promoting frataxin therefore specifically reduces benzo[a]pyrene 4,5-oxide-induced base substitutions, but not frameshift mutations.

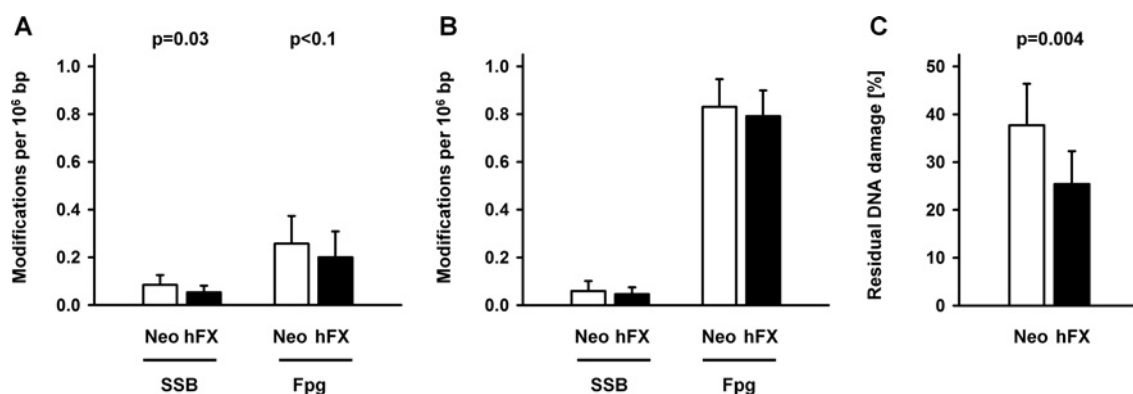
#### Overexpression of the human frataxin protein reduces DNA base substitution-mediated mutations in eukaryotes

To possibly extend the above-mentioned effects of frataxin on prokaryotic DNA mutations to eukaryotes, we used the previously established HPRT mutagenicity test. We generated a V79 Chinese



**Figure 3** Human frataxin protein diminishes the mutation rate in mammalian hamster fibroblasts

(A) Immunoblotting against haemagglutinin detecting the fully processed (18 kDa) and precursor (21 kDa) isoforms of stably overexpressed haemagglutinin-tagged human frataxin in V79 cells (hFX) and mock-transfected control cells (Neo). (B) Oxygen consumption, measured using a Clark-type electrode, as an indicator for frataxin-dependent increase in ISC protein activity. Results are means + S.D. for three independent experiments. (C)  $\text{KBrO}_3$ -dependent mutagenicity in V79 cells. Results are means + S.D. for eight independent experiments.



**Figure 4** Human frataxin protein promotes DNA repair in mammalian hamster fibroblasts

(A) Steady-state (basal) levels of SSBs and Fpg-sensitive modifications (Fpg) in V79-hFX and control cells. (B) Numbers of SSB and Fpg-sensitive modifications induced by exposure of the cells to the photosensitizer Ro 19-8022 plus visible light. (C) Residual numbers of Fpg-sensitive modifications after DNA damage induction as in (B), followed by a repair incubation of 4 h in complete medium. Results are means + S.D. for seven independent experiments. Significant difference between the groups is indicated according to a paired Student's *t* test.

hamster fibroblast cell line overexpressing haemagglutinin-tagged human frataxin protein (V79-hFX) (Figure 3A) and characterized these cells in comparison with mock-transfected cells (V79-Neo). As shown previously for frataxin-overexpressing murine fibroblasts [28] and colon cancer cells [19], we observed increased respiration rates in V79-hFX cells (Figure 3B), indicating that the protein is functional.

Next, we quantified mutation rates in these cells. Both overexpressing and control cells showed low spontaneous mutation frequencies, i.e. below five mutations per  $10^6$  cells (results not shown). In order to test ROS-induced mutagenicity, we used  $\text{KBrO}_3$  to induce mainly Fpg-sensitive oxidative DNA damage [40]. We tested  $\text{KBrO}_3$ -dependent mutagenicity in the V79 cells and found diminished mutation frequencies in V79-hFX cells ( $P=0.06$ ) (Figure 3C). These findings suggest that ISC-synthesis-promoting frataxin may reduce the mutagenicity of  $\text{KBrO}_3$  and consequently oxidative damage.

#### Steady-state levels of DNA damage in frataxin-overexpressing fibroblasts are decreased, but the extent of DNA damage induction remains unchanged

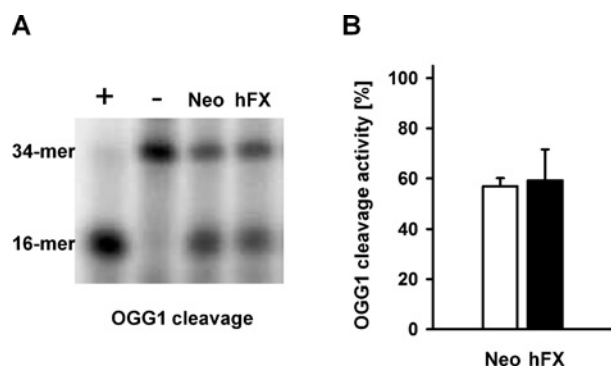
To analyse the impact of the frataxin overexpression on the steady-state levels of DNA damage, we quantified both SSB and Fpg-sensitive modifications in V79-hFX and control cells by alkaline

elution, as described in the Materials and methods section. As shown in Figure 4(A), V79-hFX cells show a significant reduction in the basal levels of SSBs. Moreover, we observed a trend ( $P=0.096$ ) towards reduced Fpg-sensitive DNA modifications (Figure 4A).

Subsequently, we tested whether frataxin-overexpressing cells differ from control cells with regard to the inducibility of DNA damage by exposing them to the photosensitizer Ro 19-8022 plus visible light. In agreement with our previously published results [41], the treatment generates predominantly oxidative purine modifications (8-oxoG) and only few SSBs and other types of lesion (Figure 4B). Notably, and of relevance for the findings summarized within the next paragraph, no differences between V79-hFX and control cells were detectable for either type of DNA damage directly after exposure to visible light (Figure 4B). This indicates that frataxin-overexpressing and control cells do not differ with respect to their susceptibility to the induction of oxidative DNA damage.

#### Frataxin-overexpressing fibroblasts have an increased DNA-repair capacity

Our initial observation that frataxin-deficient mice develop liver tumours (Figure 1B) and have increased basal levels of Fpg-sensitive base modifications (Figure 1C) gave rise to



**Figure 5 Frataxin has no influence on ISC-independent DNA-repair capacity**

Cleavage activity at 8-oxoG sites observed in total protein extracts of V79-hFX and control cells. (A) Representative gel showing the cleavage of a 34-mer oligonucleotide containing an 8-oxoG residue in a central position by incubation with Fpg (+; positive control), buffer only (-; negative control), extracts of frataxin-overexpressing cells (hFX) and control cells (Neo). (B) Results are mean + S.D. cleavage activities (percentage cleavage of the 34-mer) for six independent experiments.

the assumption that frataxin overexpression and subsequently increased ISC availability may stimulate the activity of ISC-dependent DNA repair. To test this hypothesis, we compared the residual, i.e. unrepaired number of Fpg-sensitive modifications in frataxin-overexpressing and control cells 4 h after damage induction by Ro 19-8022 plus light (see above). The results indicate that frataxin-overexpression is associated with a significantly lower number of unrepaired Fpg-sensitive oxidative purine modifications, i.e. more efficient repair (Figure 4C).

#### Increased DNA-repair capacity is unrelated to ISC-independent repair

It has been demonstrated previously that the repair of Fpg-sensitive oxidative purine modifications in mammalian cells depends largely on the activity of the repair glycosylase OGG1, the eukaryotic functional homologue of the bacterial Fpg protein [34]. OGG1, however, is not an ISC protein and its activity is therefore not expected to depend on frataxin. To determine the activity of OGG1 in V79-hFX and control cells, we used a cleavage assay, in which the incision capacity of cell extracts at single 8-oxoG residues in synthetic oligonucleotides is measured (Figure 5A). Very similar cleavage activity was observed in total protein extracts of frataxin-overexpressing and control cells, indicating that the ISC-independent enzyme OGG1 remains unaffected by altered ISC synthesis rates (Figure 5B). Taken together, these findings suggest that increased DNA-repair capacity in frataxin-overexpressing cells (Figure 4C) is unrelated to ISC-independent repair mechanisms (Figure 5B), but rather depends on ISC-containing DNA-repair enzymes.

#### DISCUSSION

The findings of the present study suggest an important role for a regulator of ISC synthesis, the frataxin protein, in DNA-damage repair in both prokaryotes and mammals. In particular, the removal of endogenously generated oxidative DNA base damage appears to strongly depend on frataxin expression.

Owing to environmental factors and normal metabolic processes within eukaryotic cells, a significant amount of DNA damage occurs on a regular basis. Two main types of DNA damage are oxidized bases (e.g. 8-oxoG) and DNA strand interruptions

due to biochemical by-products such as ROS. DNA-repair processes are constantly active as they respond to permanently occurring damages in the DNA structure. When normal repair processes fail and removal of damaged cells by apoptosis or senescence does not take place, damage may result in DNA mutations which subsequently can lead to the formation of malignantly transformed cells.

Reduced expression of the mitochondrial protein frataxin causes the neurodegenerative disorder FRDA which has been linked to various types of cancer on an anecdotal basis [14–17]. The essential role of frataxin as a mitochondrial iron chaperone for ISC biogenesis is widely accepted, and frataxin deficiency in humans, mice and yeast leads to severe alterations of mitochondrial and extra-mitochondrial ISC protein activity and function [10,35,42]. Although the malignant disorders observed in FRDA patients have been tentatively attributed to the formation of non-B DNA structures (triplexes or sticky DNA) based on intronic GAA triplet repeats [43], the effects observed in our model systems cannot be attributed to such triplet-repeat-dependent structures: neither the knockout model nor the overexpressing models carry such GAA repeats. Hence, the current observations should be considered a direct consequence of the altered frataxin protein levels rather than altered DNA structure and/or triplet repeat formation.

In the present study, we have shown that altered expression of the frataxin protein modulates activity of the DNA-repair system and is correlated with altered oxidative DNA modifications as well as altered mutagenicity, culminating in tumour formation in mice. In particular, frataxin deficiency leads to reduced activity of ISC proteins and tumour formation in the affected tissue, the liver, in AlbFxn<sup>-/-</sup>-mice [12] which is associated with an increase in Fpg-sensitive DNA modifications due to frataxin deficiency, as shown in the present study. In contrast, we additionally show that overexpression of human frataxin in the prokaryote *Salmonella* Typhimurium efficiently reduces mutagenicity of the well-characterized compound benzo[*a*]pyrene 4,5-oxide. Specifically, strain TA104, which is known to detect base substitutions and to be sensitive to oxidative mutagens [38], shows both reduced spontaneous as well as benzo[*a*]pyrene 4,5-oxide-induced mutation rates [44,45]. In contrast, in the frameshift-mutation-sensitive strain TA1538 [37], frataxin expression does not show any detectable effect. These differential effects argue against a toxicokinetic mechanism; a possible mechanism may be a frataxin-mediated change in the pattern of translesion synthesis DNA polymerases, which are important in the fixation of mutations induced by benzo[*a*]pyrene 4,5-oxide in *Salmonella* [46]. Taken together, these observations are consistent with the notion that frataxin affects base-substitution-dependent mutagenesis only, which is also known to occur following exposure to oxidative stressors. Hence, our findings are in accordance with previous studies that suggest increased sensitivity to oxidative stress in states of frataxin deficiency [12,47,48]. It remains to be elucidated, however, how frataxin protects from revertant mutations in our prokaryotic model. This effect can be explained by increased effectiveness of DNA damage repair due to increased ISC formation, but could also be interpreted to be caused by ferroxidase and/or iron-detoxification activity of frataxin which then would protect the DNA from oxidative damage, as suggested previously [6,7].

To extend the effect of frataxin on mutagenicity to eukaryotic systems, we established V79 Chinese hamster fibroblasts stably overexpressing human frataxin, and showed increased oxygen consumption as a prototypical marker for elevated ISC protein activity within the mitochondria [19,28,35]. Because an exposure of mammalian cells to KBrO<sub>3</sub> generates oxidative DNA

modifications, in particular 8-oxoG [49], we used KBrO<sub>3</sub> in the HPRT test and observed a trend towards a reduced mutagenicity in states of frataxin overexpression ( $P = 0.06$ ). It should be noted, however, that mutagenicity of KBrO<sub>3</sub> in our hands is rather weak, which is consistent with longstanding findings [50]. Given the low basal mutagenic efficiency of KBrO<sub>3</sub>, the comparably weak protective effect of frataxin in this particular case is not really surprising.

To elucidate further the mechanistic basis for ISC-mediated DNA damage protection, we used the highly sensitive alkaline elution technique [30]. We observed reduced steady-state levels of SSBs and Fpg-sensitive modifications, suggesting that frataxin either protects against oxidative DNA damage or promotes DNA repair. However, it should be emphasized that these findings need to be interpreted with caution since (i) SSB levels were near the detection limit of the technique, and (ii) findings on Fpg-sensitive modifications were not statistically significant.

To test whether frataxin overexpression directly protects from induction of DNA damage, or whether frataxin rather promotes DNA repair, we induced oxidative DNA damage by photosensitization. When quantifying the degree of damage immediately after irradiation, no difference with regard to DNA-damage frequency was observed. Thereby we aimed to exclude the possibility that frataxin affects the primary induction of damage in particular with regard to iron detoxification. In contrast, 4 h after induction of damage to a similar degree in frataxin-overexpressing and control cells, a significant reduction in the extent of DNA damage was observed in frataxin-overexpressing V79 cells when compared with control cells, thus indicating that DNA-repair capacity is, in fact, modulated by frataxin (Figure 4). Consistent with the findings in frataxin-overexpressing cells, we could inversely show that the steady-state level of Fpg-sensitive DNA modifications in hepatocytes isolated from young frataxin-deficient AlbFxn<sup>-/-</sup> mice was increased, notably in the absence of any external ROS-inducing or mutagenic stimulus (Figure 1).

Frataxin is a mitochondrial protein that is crucial for the mitochondrial biosynthesis of ISCs. However, DNA repair and specifically BER is mediated by several enzymes which partly depend on ISCs, whereas others do not require ISCs, the latter including OGG1. Accordingly, we here observed that OGG1 is not affected by expression levels of frataxin and hence ISC availability. Although experimentally highly challenging, future experiments will ideally have to show which ISC-dependent repair enzymes are affected by frataxin expression. However, given (i) the frataxin-mediated induction of repair capacity, as well as (ii) the fact that ISC-independent OGG1 activity does not contribute to this phenotype, it strongly suggests that frataxin exerts its effects via modulation of ISC-dependent DNA-repair enzymes.

Moreover, the findings described in the present study indicate that a human mitochondrial protein can be functional not only in mammalian cells, but also in prokaryotes, which lack mitochondria. This observation is consistent with the fact that frataxin is evolutionarily conserved throughout most species [25].

Taken together, the present study suggests that frataxin and apparently ISC availability modulate DNA-repair capacity in evolutionary distinct phyla and protect mammals from spontaneous liver tumour formation.

## AUTHOR CONTRIBUTION

René Thierbach designed and conducted experiments, evaluated the results and drafted the paper. Gunnar Drewes conducted the majority of the experiments and performed the data analysis. Markus Fusser, Anja Voigt, Doreen Kuhlow, Urte Blume, Tim J. Schulz and Carina Reiche conducted experiments. Hansruedi Glatt and Bernd Epe designed experiments and co-wrote the paper. Pablo Steinberg and Michael Ristow designed experiments, supervised the study and co-wrote the paper.

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