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► To cite this version:

René Thierbach, Gunnar Drewes, Markus Fusser, Anja Voigt, Doreen Kuhlow, et al.. The Friedreich's Ataxia protein frataxin modulates DNA base excision repair in prokaryotes and mammals. *Biochemical Journal*, 2010, 432 (1), pp.165-172. 10.1042/BJ20101116 . hal-00529109

HAL Id: hal-00529109

<https://hal.science/hal-00529109>

Submitted on 25 Oct 2010

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

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Abbreviations used: BER, base excision repair; Fpg, formamido-pyrimidine DNA glycosylase; FRDA, Friedreich's ataxia; ISCs, iron-sulphur-clusters; MUTYH, human mutY homolog (*E. coli*); NER, nucleotide excision repair; 8-oxoG, 7,8-dihydro-8-oxoguanine; ROS, reactive oxygen species; SD, standard deviation; SSB, DNA single strand break;

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Short title: **Frataxin controls DNA repair**

ABSTRACT

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 iron-sulphur-clusters (ISCs). 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. Here 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 *S. 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 unaltered by frataxin overexpression. These findings indicate that frataxin modulates DNA repair mechanisms likely due to its impact on ISC-dependent repair proteins, linking mitochondrial dysfunction to DNA repair and tumour initiation.

Key words:

oxidative stress, lifespan, DNA repair, iron-sulphur cluster, mutagenicity, tumourigenesis

INTRODUCTION

Iron-sulphur clusters (ISCs) 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, iron uptake regulation, and others. In the past ten years, significant knowledge about ISC biogenesis and the assembly of ISC proteins was 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 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 have the frataxin protein have been experimentally shown [6, 7].

Defects in the frataxin gene and thereby a reduced level of functional frataxin protein leads to a human disease named Friedreich's ataxia (FRDA) [8] with serious clinical features including abnormalities in glucose metabolism, limb and gait ataxia, hypertrophic cardiomyopathy and reduced expectancy of life. While unconditional deletion of frataxin in mice causes early embryonic lethality [9], Cre/loxP conditional knock-out mice [10-12] and GAA repeat knock-in 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^{-/-} [12]. These mice exhibit impaired mitochondrial energy metabolism, increased oxidative stress and severely reduced lifespan [12]. Unexpectedly, we additionally observed liver tumour formation in almost all animals within the first twelve months of life, whereas control animals rarely exhibit such tumours during this time period. This finding is consistent with several reports about malignant disorders in FRDA patients [14-17]. Moreover we have described reduced malignancy in cancer cells which stably over-express frataxin [18, 19] in a potentially p38 MAP kinase dependent manner [19]. Consistently, in AlbFxn^{-/-} mice, we have shown impaired activation of the p38 MAP kinase 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 ISC [20, 21] has been proposed and studied in more detail: The ISC containing DNA glycosylase MUTYH (human mutY homolog [*E. coli*]) has been shown to be involved into base excision repair (BER), while alterations in the human *MUTYH* 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. Furthermore the DNA repair helicases XPD and FancJ, which are involved in nucleotide excision repair (NER) and which are associated with the human diseases *Xeroderma pigmentosum* and Fanconi 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 ISC in meanwhile 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 analyzing 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 (Munich, Germany).

Mutagenicity in *Salmonella typhimurium*

S. typhimurium strains TA104 and TA1538 were kindly provided by Dr. Bruce N. Ames (University of California, Berkeley, CA, USA). Human frataxin cDNA was recovered by amplification from previously described plasmids harbouring hemagglutinin-tagged frataxin [19] with primers including suitable restriction sites for the target vectors, AmesBac_FX_FWD 5'-CGCGGATCCCCATGGGAACCTTTGGGCCACCCAGGCTCT-3' and AmesBac_FX_REV 5'-TGGGAATTCAAGCTTCTAGATGTATCGGTACGTGTCTGC-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 localisation signal peptide [25]. The PCR product was cloned into pCR2.1 (Invitrogen) and confirmed by sequencing. The frataxin cDNA was then sub-cloned into the ampicillin-resistant target vector pKK223-3 by digestion with NcoI and HindIII. Orientation was tested by digestion with BamH1/HindIII and BamH1/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-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 *S. typhimurium* LT2 by passaging them through the restriction-deficient, but methylation-proficient *S. typhimurium* strain LB5000. Plasmids were recovered and used for the transformation of the strains *S. 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]. Shortly, nutrient broth (25 g Oxoid nutrient broth No. 2/litre) was inoculated with bacteria and grown overnight. Before the experiment bacteria were centrifuged, resuspended in medium B (1.6 g Bacto nutrient broth and 5 g NaCl/litre) and adjusted nephelometrically to a titre of 1.1 to 1.8 x 10⁹ 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 : 25 mM sodium phosphate buffer, pH 7.4) was added, and the mixture was poured onto a Petri dish containing 22 ml of minimal agar (1.5 % agar in Vogel-Bonner E medium with 2 % glucose). After incubation for 3 days in the dark, the colonies (his⁺ revertants) were counted. Initially, a dose-finding experiment was carried out. The compounds were then tested using 3 plates

for each condition. In virtually all cases, the individual values deviated from the mean by less than 10 % or by less than 10 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) on Nov. 2, 2005. Cells were thawed and propagated in Dulbecco's Modified Eagle Medium containing 4,5 g/l D-glucose and 10 % foetal bovine serum, and three passages later frozen in aliquots.

Transfection experiments were performed as described previously using pCINeo (Promega, Madison WI) (control) and pCINeo-hFX [19] the latter containing a full-length frataxin, *i.e.* including the N-terminal mitochondrial localisation signal and a C-terminal hemagglutinin tag. V79 cells were selected and maintained in Dulbecco's Modified Eagle Medium containing 4,5 g/l D-glucose, 10 % foetal bovine serum and 700 mg/l neomycin. Transfected cells were propagated in as above, and three passages later frozen in aliquots. 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 protein by polyacrylamide gel electrophoresis and Western blotting using an anti-hemagglutinin antibody (Roche Diagnostics Corp., Basel, Switzerland) followed by a horseradish peroxidase labelled secondary antibody and standard chemoluminescent detection.

To determine the growth curve, we seeded 2.5×10^4 cells into 72 replica 6-cm plastic dishes. The cells in eight dishes were counted every twelve hours, and growth curves were drawn. The population doubling time was determined from the growth curve. Oxygen consumption was measured by using a Clark-type electrode (Hansatech; Norfolk, England, UK) as previously described [28]. For each measurement two million cells suspended in Hanks Balanced Salt Solution were used.

HPRT 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 potassium bromate (KBrO₃) for 1 h in 100-mm Petri dishes before sub-cultivation. Treated cultures as well as control cells were transferred as needed (minimum 2×10^6 cells). Following the 3rd treatment from each culture 1×10^6 cells were plated into five new 100-mm dishes with selective medium containing 7 µg/µl 6-thioguanine to determine the number of mutants. Plating efficiency was determined in non-selective medium plating 200 cells in 60-mm dishes. After one week, colonies were fixed with methanol, stained with sulforhodamine-B and counted.

Isolation of hepatocytes

AlbFxn^{-/-} mice were generated, bred and verified as described before [12]. For determination of oxidative DNA modifications, hepatocytes from three-week old AlbFxn^{-/-} 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 µm-gauze. Hepatocytes were centrifuged at 15 x 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, Basel, Switzerland). Cells were irradiated for 10 min on ice from a distance of 0.38 meters with a 1000 W halogen lamp. This treatment oxidizes bases in DNA, but only causes few direct strand breaks [30]. Subsequently cells were either directly analyzed or maintained under standard culture conditions for four hours.

Quantification of DNA modifications

An alkaline elution assay [31] in combination with 1 µg/ml Fpg protein (formamido-pyrimidine DNA glycosylase), the bacterial functional analogue of 8-oxoguanine glycosylase (OGG1) protein, was used to quantify various types of DNA modifications in V79 cells [30] and in hepatocytes [32]. In this manner DNA single-strand breaks (SSB) were determined by incubation of the cellular DNA without Fpg protein immediately after cell lysis. The number of modifications sensitive to Fpg protein was determined using parallel filters with the repair enzyme. To quantify the Fpg-sensitive modifications the number of SSB was subtracted, whereas the background levels observed with untreated controls were subtracted in all cases. γ -Irradiated cells were used for calibration, assuming that 6 Gy induce 1 SSB /10⁶ base pairs [31].

Cleavage activity of OGG1 in cell extracts

About 12 million V79 Chinese hamster fibroblasts or hepatocytes were washed with phosphate buffer and the cell pellet was resuspended in 200 µl lysis buffer (200 mM Tris HCl pH 8, 10 mM EDTA pH 8, 2.5 M NaCl) containing protease inhibitors (Complete Mini from Roche Applied Science). After cells were sonicated carefully the suspension was centrifuged at 85 000 x g and 4 °C for 45 min. The resulting supernatant was used to determine the protein concentration according to the method of Bradford. Cleavage activities at 7,8-dihydro-8-oxoguanine (8-oxoG) sites in these cell extracts were measured by incubating 5 µg protein in a total volume of 32 µl for 1 h at 37 °C with a 34mer 5' [³²P]-labelled double-stranded oligodesoxy-ribonucleotide containing an 8-oxoG residue in the labelled strand at position 16 opposite a cytosine. After performing a denaturing polyacrylamide gel electrophoresis the extent of DNA cleavage was quantified by means of a phosphor-imager (Storm, Molecular Dynamics) [33].

Statistical analyses

Kolmogorov-Smirnov-Test was used for normality tests. We found normality in all experiments. The average and SD are shown; if not noted otherwise unpaired Student's t-test was used. A p-value below 0.05 was considered statistically significant.

RESULTS

Tumorigenicity in frataxin-deficient AlbFxn^{-/-} 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 absence of detectable frataxin protein following disruption is shown by immunoblotting in Fig. 1A. As previously published, hepatocyte-specific frataxin knock-out mice develop liver tumours early in life (Fig. 1B) [12].

The previously described increase in oxidative stress in liver specimen of AlbFxn^{-/-} 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 three-week old AlbFxn^{-/-} mice and quantified basal levels of oxidative DNA modifications in terms of single strand breaks (SSB) and Fpg-sensitive modifications (Fpg). The latter include oxidative purine modifications such as 8-oxoG and sites of base loss [34]. As depicted in Fig. 1C, we observed that the deficiency of frataxin was associated with significantly increased levels of Fpg-sensitive lesions, but not of SSB. 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 further elucidate 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 before-mentioned findings in frataxin-deficient mice, we assumed that overexpression of frataxin would conversely increase 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 firstly generated two prokaryotic systems stably overexpressing human frataxin (Fig. 2A). As host cells, we used the *Salmonella typhimurium* strain TA1538 which preferentially detects frame-shift mutations [37], and the strain TA104 which preferentially detects base substitutions [38]. Accordingly, the latter strain is highly sensitive to oxidative damage following exposure to reactive oxygen species (ROS) [38]. Consistent with our hypothesis, we observed a significant reduction in the number of spontaneous revertants only in TA104 *Salmonella* overexpressing frataxin, while the spontaneous mutation frequency in TA1538 bacteria remained unaffected (Fig. 2B). While ISC formation has not been directly quantified in these *Salmonella* strains, these findings insinuate 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 overexpression of frataxin caused a significant reduction of mutation frequency (Fig. 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 before-mentioned effects of frataxin on prokaryotic DNA mutations to eukaryotes, we used the previously established HPRT

mutagenicity test. We generated a V79 Chinese hamster fibroblast cell line overexpressing hemagglutinin-tagged human frataxin protein (V79-hFX) (Fig. 3A) and characterized these cells in comparison to mock-transfected cells (V79-Neo). As previously shown for frataxin-overexpressing murine fibroblasts [28] and colon cancer cells [19] we observed increased respiration rates in V79-hFX cells (Fig. 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 5 mutations per 1×10^6 cells (data not shown). In order to test ROS-induced mutagenicity we used KBrO_3 to induce mainly Fpg-sensitive oxidative DNA damage [40]. We tested KBrO_3 -dependent mutagenicity in the V79 cells and found diminished mutation frequencies in V79-hFX cells ($p=0.06$) (Fig. 3C). These findings suggest that ISC synthesis-promoting frataxin may reduce mutagenicity of KBrO_3 and consequently oxidative damage.

The 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 Fig. 4A, V79-hFX cells show a significant reduction of the basal levels of SSB. Moreover, we observed a trend ($p=0.096$) towards reduced Fpg-sensitive DNA modifications (Fig. 4A).

Subsequently, we tested whether frataxin-overexpressing cells differ from control cells in regards to the inducibility of DNA damage by exposing them to the photosensitizer Ro19-8022 plus visible light. In agreement with our previously published data [41], the treatment generates predominantly oxidative purine modifications (8-oxoG) and only few SSB and other types of lesions (Fig. 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 (Fig. 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 (Fig. 1B) and have increased basal levels of Fpg-sensitive base modifications (Fig. 1C) gave rise to 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 hours after damage induction by Ro19-8022 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 (Fig. 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 therefore is 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 (Fig. 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 (Fig. 5B). Taken together, these findings suggest that increased DNA repair capacity in frataxin-overexpressing cells (Fig. 4C) is unrelated to ISC-independent repair mechanisms (Fig. 5B) but rather depends on ISC-containing DNA repair enzymes.

DISCUSSION

The findings summarized in this manuscript suggest an important role of 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.

Due 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]. While 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 knock-out 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 current study, we show 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^{-/-}-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 here 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, the 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 trans-lesion 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 indicate that frataxin affects base-substitution-dependent mutagenesis only, which is also known to occur following exposure to oxidative stressors. Hence, our current findings are in accordance with previous publications 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 transformation in our prokaryotic model: This effect can be either 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 previously suggested [6, 7].

To extend the effect of frataxin on mutagenicity to eukaryotic systems we established V79 Chinese hamster fibroblasts stably overexpressing the 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_3 generates oxidative DNA modifications, in particular 8-oxoG [49], we used KBrO_3 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_3 in our hands is rather weak, which is consistent with long-standing findings [50]. Given the low basal mutagenic efficiency of KBrO_3 , the comparably weak protective effect of frataxin in this particular case is not really surprising.

To further elucidate 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 SSB 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 in regards 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 the iron-detoxification. In contrast, four hours 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 to control cells, thus indicating that DNA repair capacity is in fact modulated by frataxin (Fig. 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 $\text{AlbFxn}^{-/-}$ mice was increased, notably in the absence of any external ROS-inducing or mutagenic stimulus (Fig. 1).

Frataxin is a mitochondrial protein that is crucial for the mitochondrial biosynthesis of ISC. However, DNA repair and specifically base excision repair is mediated by several enzymes which partly depend on ISCs, while 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, and 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 strongly suggest that frataxin exerts its effects via modulation of ISC-dependent DNA repair enzymes.

Moreover, the findings described in this 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 evolutionary conserved throughout most species [25].

Taken together, the present results suggest that frataxin and apparently ISC availability modulate DNA repair capacity in evolutionary distinct *phylae* and protect mammals from spontaneous liver tumour formation.

AUTHOR CONTRIBUTION

René Thierbach designed and conducted experiments, evaluated the results and drafted the manuscript. 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.

FUNDING

Grant support was received from the German Research Foundation (DFG) grants [Th1392/1-1, Ri1076/1-3 and Ep11/5-3]. This work is part of the research program of the Jena Centre for Systems Biology of Ageing – JenAge funded by the German Ministry for Education and Research (Bundesministerium für Bildung und Forschung – BMBF; support code: 0315581[A-D]).

FIGURE LEGENDS**Figure 1 Liver tumor formation and basal levels of oxidative DNA modifications in frataxin-deficient AlbFxn^{-/-} knock-out mice**

(A) Hepatocyte-specific disruption of frataxin expression in the liver and loading control are displayed by immunoblot against murine frataxin protein and alpha-tubulin, respectively. (B) Typical liver specimen of control-mice and AlbFxn^{-/-}-mice. (C) Numbers of DNA single strand breaks (SSB) and Fpg-sensitive modifications (Fpg) in untreated hepatocytes as quantified by alkaline elution technique. The columns indicate means of 11 mice in each group + SD, the significance of the difference between the two groups according to a paired t-test is indicated.

Figure 2 Human frataxin protein protects from specific mutations in *S. typhimurium*

(A) Immunoblot analysis of recombinant frataxin expression in cytosolic preparations (20 µg protein per lane) of *S. typhimurium* strains TA104-hFX and TA1538-hFX against murine monoclonal HA clone 12CA5 (Roche) (18 kD). Neo, mock-transfected control strains. (B) Number of spontaneous revertant colonies per plate. Means of three (TA104) and four (TA1538) independent experiments + SD are shown. (C) Revertants per plate in the presence of varying concentrations of benzo[a]pyrene 4,5-oxide. *white*=Neo, *black*=hFX.

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

(A) Immunoblot against hemagglutinin detecting the fully processed (18 kD) and precursor (21 kD) isoforms of stably overexpressed, hemagglutinin-tagged human frataxin in V79 cells (hFX) and mock-transfected control cells (Neo). (B) Oxygen consumption, measured by a Clark-type electrode, as indicator for frataxin-dependent increase of ISC protein activity. Columns indicate means of three independent experiments + SD. (C) KBrO₃ dependent mutagenicity in V79 cells. Columns indicate means of eight independent experiments + SD.

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

(A) Steady state (basal) levels of DNA single-strand breaks (SSB) and Fpg-sensitive modifications (Fpg) in V79-hFX and control cells ($p_{\text{SSB}}=0.029$, $p_{\text{Fpg}}=0.096$). (B) Numbers of SSB and Fpg-sensitive modifications induced by exposure of the cells to the photosensitizer Ro19-8022 plus visible light. (C) Residual numbers of Fpg-sensitive modifications after DNA damage induction as in B, followed by a repair incubation of 4 hours in complete medium ($p=0.004$). Columns indicate means of 7 independent experiments + SD, the significance of the difference between the two cell lines according to a paired t-test is indicated.

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) Means of cleavage activity (% cleavage of the 34-mer) obtained from six independent experiments.

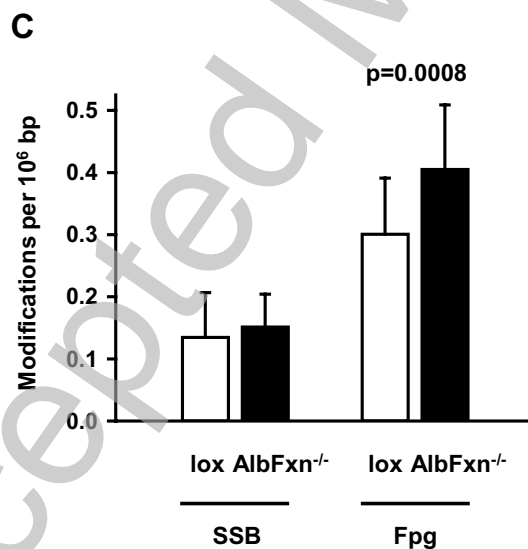
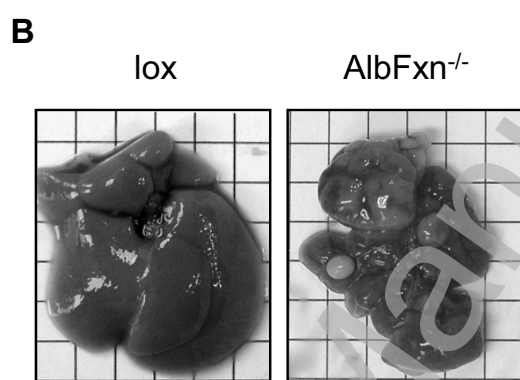
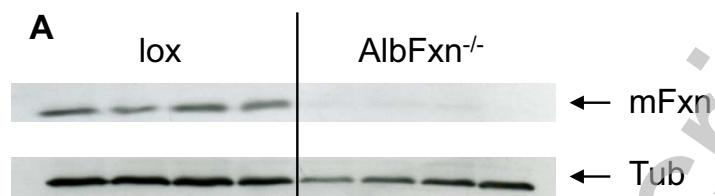
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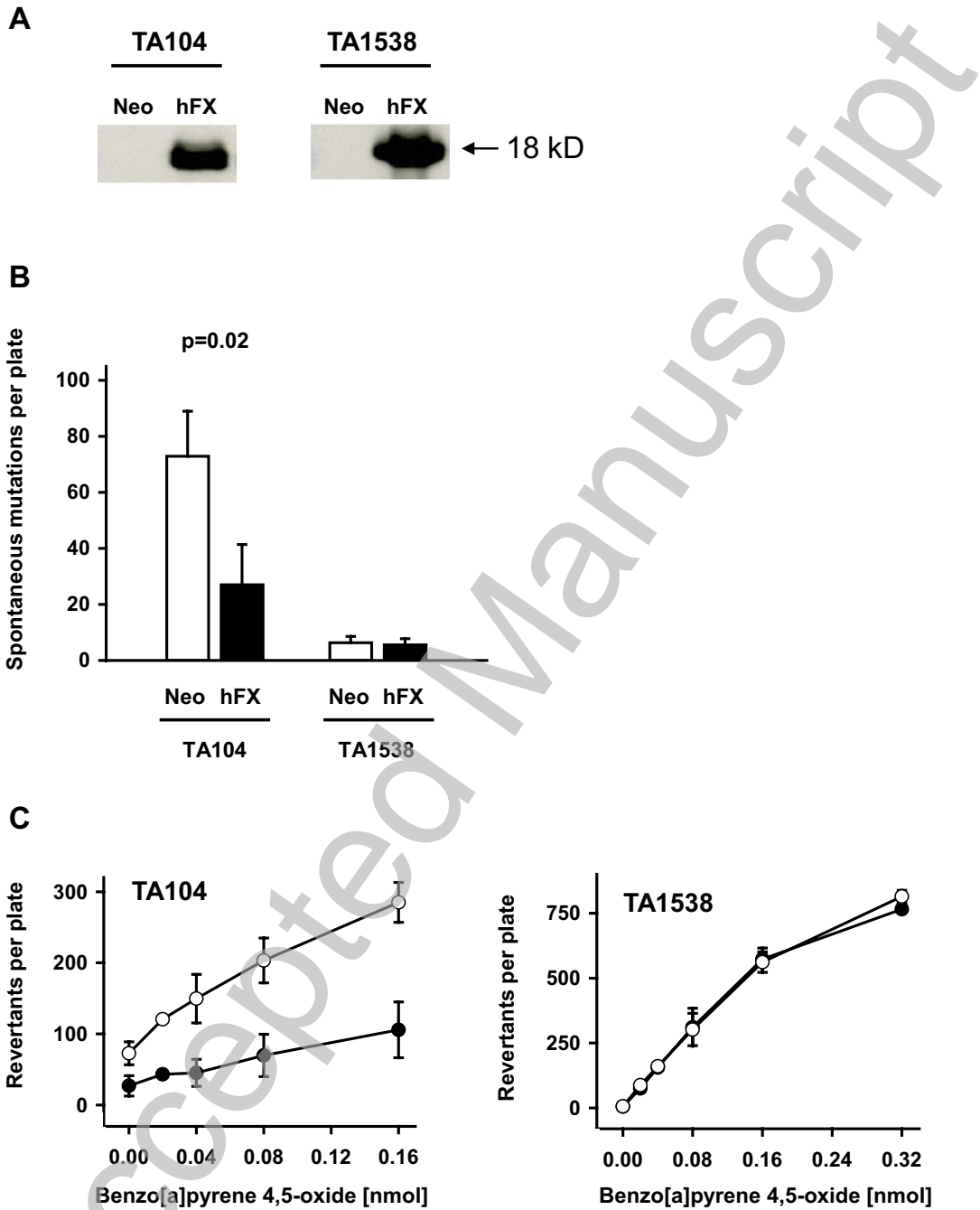
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Figure 1



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Figure 2



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Figure 3

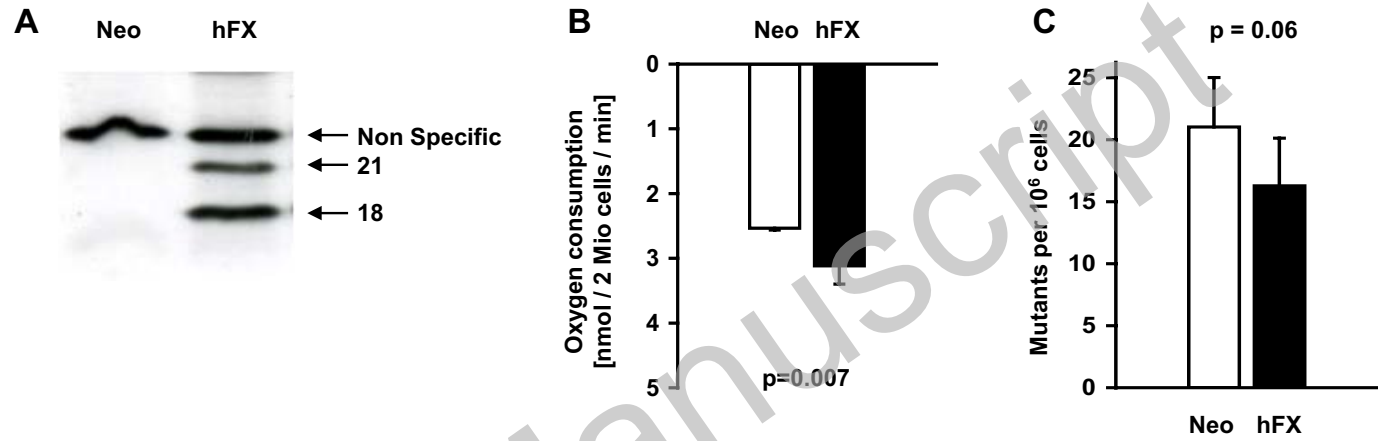


Figure 4

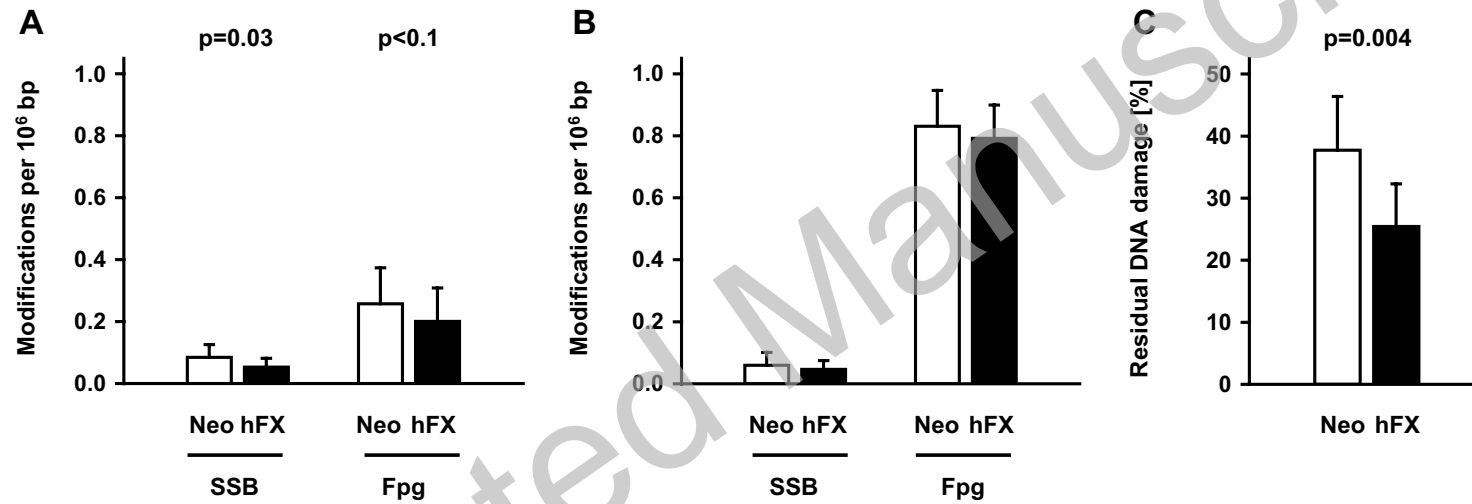


Figure 5

