Targeted Disruption of Hepatic Frataxin Expression Causes Impaired Mitochondrial Function, Decreased Life Span, and Tumor Growth in Mice

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Abstract:

We have disrupted expression of the mitochondrial Friedreich ataxia protein frataxin specifically in murine hepatocytes in order to generate mice with impaired mitochondrial function and decreased oxidative phosphorylation (OXPHOS). These animals have a reduced life span and develop multiple hepatic tumors. Livers also show increased oxidative stress, impaired respiration and reduced ATP levels paralleled by reduced activity of iron-sulfur cluster (Fe/S) containing proteins (ISP), which all leads to increased hepatocyte turnover by promoting both apoptosis and proliferation. Accordingly, phosphorylation of the stress-inducible p38 MAP kinase was found to be specifically impaired following disruption of frataxin. Taken together, these findings indicate that frataxin may act as a mitochondrial tumor suppressor protein in mammals.

Introduction:

Mitochondria generate readily available energy equivalents by conversion of macronutrient intermediates into ATP by oxidative phosphorylation (OXPHOS). This process is dependent on iron-sulfur clusters (Fe/S), which are essential parts of mitochondrial enzymes, including aconitase, and complexes I, II and III of the respiratory chain (1).

Friedreich's Ataxia is an inherited neurodegenerative disorder (2) caused by reduced expression of the mitochondrial protein frataxin (3) leading to premature death due to cardiac failure (2), diabetes mellitus and insulin resistance (4), as well as impaired ATP synthesis in muscle of humans (5, 6). Concurrently it was shown that frataxin overexpression promotes ATP synthesis and interacts with the respiratory chain (7, 8). While the primary function of frataxin is still a matter of debate (9), increasing evidence suggests that this protein directs the intramitochondrial synthesis of Fe/S clusters (1, 10-12). Individuals suffering from Friedreich ataxia have a reduced life expectancy of 38 years in average (2), and show indications for increased oxidative stress (13-15). Overexpression of frataxin has been shown to reduce intracellular accumulation of reactive oxygen species (ROS) and to prevent menadione-induced malignant transformation of fibroblasts (16). Furthermore, disruption of
the frataxin homologue in yeast has been shown to cause increased sensitivity against oxidants and promote oxidative damage to both nuclear (17) as well as mitochondrial DNA (18). In addition, fibroblasts from Friedreich patients exhibit increased sensitivity against ionizing radiation and show an increased frequency of transforming events (19). Although cancer is not considered a typical feature of the disorder, Friedreich ataxia patients exhibit various types of malignant or proliferative disorders (20-28).

To evaluate the role of frataxin in liver tissues, we have now disrupted expression of this protein specifically in hepatocytes of C57b/6 mice. We here demonstrate the presence and efficacy of the disruption, Fe/S dependent alterations of enzyme activities, decreased OXPHOS and increased reactive oxygen species (ROS) formation, decrease of life span, and show formation of multiple liver tumors, paralleled by impaired p38 MAP kinase phosphorylation cumulating in induction of both apoptosis and proliferation. Taken together, the findings indicate that lack of frataxin expression may promote tumor growth in mammals, and that frataxin may thus be considered a mitochondrial tumor suppressor protein located upstream of p38 MAP kinase.

Results:

Hepatocyte-specific disruption of frataxin expression

Frataxin is a nuclear encoded protein with an N-terminal signal targeting the protein to the mitochondrial compartment (2). We have used the cre/loxP system to remove exon 4 of the frataxin gene in a tissue-specific manner as previously described (11, 29) except for hepatocyte-specific expression of Cre recombinase was obtained by using mice carrying an albumin promoter-driven Cre transgene (30). Presence, efficiency and specificity of disruption were shown at genomic, transcriptional and translational levels by using genomic PCR by primers flanking the loxP sites of the targeting allele (11) (Fig. 1a), reversely transcribed PCR with primers located in exons 3 and 5 of the frataxin gene (Fig. 1b), and immunoblotting against murine frataxin protein (Fig. 1c). The findings so far indicate that disruption of frataxin expression is restricted to liver specimen from knockout mice (Fig. 1a-
c), and that disruption efficacy is almost complete (Fig. 1c), since faint remnant signals (Fig. 1c, left lane) are likely due to non-hepatocyte cells contained in the liver samples.

**Decreased life span and liver tumor formation in frataxin knockout mice**

Although knockout mice were born in the expected Mendelian frequency (data not shown, P=0.7351) and had normal body weight (Fig. 2a), they subsequently failed to thrive, as indicated by a lack of weight gain (Fig. 2a) and a specific reduction of body fat as determined by nuclear magnetic resonance (data not shown). To investigate whether liver function might be impaired in knockout mice, serum levels for albumin (Alb), cholinesterase (ChE), L-alanine transferase (ALAT), and lactate dehydrogenase (LDH) were determined (Figs. 2b – 2e). The first two parameters (Alb, ChE) are commonly used to quantify synthesis capacity of hepatocytes, and were found to be moderately but significantly reduced (Fig. 2b, 2c). Two other parameters to quantify putative hepatocyte damage were determined (ALAT, LDH), and were found to be mildly increased (Fig. 2d, 2e), suggesting continuous damage to hepatocytes. Nevertheless, changes were found to be comparably moderate, but may be sufficient to contribute to the failure to thrive leading to hepatic cachexia (31).

Furthermore, knockout mice exhibited a significantly decreased life expectancy (Fig. 2f) leading to reduction of the number of knockout animals by almost 50% at an age of 30 weeks after birth. While wild-type C57bl6 mice are known to be susceptible to liver tumor development at senescence only, anatomical evaluation of young knockout mice revealed the presence of multiple liver tumors (Fig. 2g), which were not observed in age-matched control animals. The average number of macroscopically visible tumors per animal was 32.4 (+ 15.7, P = not applicable, since number of tumors in control group equalled zero). In these tumors, a highly increased number of mitotic and apoptotic hepatocytes as well as numerous polyploid and poorly differentiated cells were observed (Figs. 2h and 2i).

**Increased oxidative stress in frataxin knockout mice**


Previously published evidence suggests an increase of oxidative stress or ROS formation in fibroblasts or blood samples from Friedreich ataxia patients (13-15). Therefore, we questioned whether oxidative stress might be elevated in livers of frataxin knockout mice. First, we quantified a marker for lipid oxidation, so-called thiobarbituric-acid reactive substances (TBARS), which we found to be significantly elevated in liver specimen from frataxin knockout mice (Fig. 3a). Subsequently, levels of reduced and oxidized glutathione in such specimen were determined. Glutathione in its reduced state (GSH) confers to the quantitatively most important buffering system against oxidative stress in mammals. Consistently, reduced glutathione levels have been found diminished in blood samples of Friedreich ataxia patients (14), and have been described to be elevated in murine fibroblasts overexpressing human frataxin (16). In liver specimen of our knockout mice, levels of oxidized glutathione (GSSG) were found to be significantly elevated (Fig. 3b) while levels of reduced glutathione were not found to be affected by disruption of frataxin expression (Fig. 3c). Taken together, these findings suggest that a detectable increase in oxidative stress occurs in liver specimen of knockout mice, although the overall buffering capacity against ROS remains unaffected as reflected by unaltered levels of reduced glutathione.

Reduction of hepatic mitochondrial function in frataxin knockout mice

Disruption of mitochondrial proteins may reduce the number of mitochondria per cell. We therefore quantified mitochondrial marker proteins including cytochrome C (see below, Fig. 5e) as well as mitochondrial DNA (mtDNA) content by Southern blotting using the mitochondrially encoded subunit III of cytochrome oxidase as a probe (mtCOXIII, Fig. 4a, upper panel). After normalization of the mtCOXIII signal to 18S rDNA (Fig. 4a, lower panel), no significant difference in mtDNA content was observed when knockout and control animals were compared (P=0.501). Subsequent quantification of enzymes within the Krebs cycle as well as the respiratory chain indicated that lack of frataxin indeed selectively affects activities of those proteins containing Fe/S clusters, including aconitase, and complexes I, II and III of the respiratory chain (Fig. 4b). Concurrently, activity of fumarate hydratase, an enzyme
functioning independently of Fe/S clusters, was found to be unaffected (Fig. 4b). Taken together, these findings suggest an impairment of Krebs cycle flux, which should lead to decreased oxidative capacity and ultimately an energy deficit within affected cells. Hence, we quantified oxygen consumption, which was found to be reduced in liver specimen of frataxin knockout mice when compared to control animals (Fig. 4c), while other tissues of knockout animals showed a respiratory activity comparable to that of controls (data not shown). Subsequent quantification of ATP levels revealed a remarkable reduction in liver tissues of knockout animals (Fig. 4d), while other tissues of knockout animals contained normal amounts of ATP in comparison to control animals (not shown). Taken together, these findings suggest that disruption of frataxin causes a specific impairment of Fe/S cluster containing mitochondrial enzymes leading to an impairment of respiration and ATP synthesis, consistent with previously published findings regarding Fe/S enzyme activity (10-12) and OXPHOS (5, 7, 8) in states of altered frataxin expression.

Increased apoptosis in hepatocytes of frataxin knockout mice

Impaired OXPHOS, due to disruption of frataxin as well as due to other reasons, causes depletion of intracellular ATP (Fig. 4d). Chronic depletion of ATP as well as increased ROS (see above) may cause programmed cell death by activation of previously established molecular pathways. Translocation of the pro-apoptotic protein Bax to the mitochondria is an early event during apoptosis in eukaryotic cells. Accordingly, in hepatic tissue lysates lacking frataxin (Fig. 5a), we observed an increase in expression of Bax (Fig. 5b) as well as a translocation of Bax to the mitochondrial fractions of tissue lysates (Fig. 5c). A subsequent release of cytochrome C from the mitochondria to the cytosol was consistently observed (Fig. 5d) while the content of membrane associated cytochrome C in the mitochondrial fractions was found unaltered (Fig. 5e), again suggesting a normal amount of mitochondria in frataxin knockout hepatocytes (see also Fig. 4a). Cleavage of caspase 3 into its active form reflects a terminal stage of the pro-apoptotic program. Accordingly, only hepatocytes of knockout animals showed detectable level of activated caspase 3 protein (Fig. 5f). To validate these
findings by an independent method, TUNEL stains, reflecting apoptotic cells, were performed. This assay also showed an increased number of apoptotic events in sections of *frataxin* knockout livers (P<0.01, Fig. 5g). Of note, no activation of pro-apoptotic p53 was observed (data not shown), consistent with a previously described activation of Bax independent of p53, e.g. by arsenic trioxide (32), a substance that interestingly functions as an inhibitor of mitochondrial Krebs cycle activity. Taken together, these findings demonstrate an induction of apoptotic pathways in frataxin-deficient hepatocytes.

*Increased proliferation in hepatocytes of frataxin knockout mice*

Since an increased frequency of apoptotic events alone is not sufficient to explain neither tumor formation nor tumor growth, and since increased levels of ROS may cause tumor formation and/or growth, we asked whether increased apoptotic events were paralleled by an induction of pro-proliferative molecular pathways in livers of *frataxin* knockout mice, together potentially causing an increased hepatocyte turnover. Therefore, we first quantified two potentially oncogenic members of the family of heat shock proteins, HSP70 and HSP25, the latter being the murine homologue of human HSP27. While expression of HSP70 was found to be decreased in liver specimen from *frataxin* knockout mice (Fig. 5h), protein levels of HSP25 were increased (Fig. 5i). Regarding impaired OXPHOS activity (Fig. 4c and 4d), it should be noted that activation of HSP70 is known to be an ATP-dependent process, while induction of HSP27, and hence presumably also HSP25, occurs independently of ATP (33).

Next, we quantified expression levels and phosphorylation status of the three major members of the mitogen-activated protein kinase family, p44/42, SAPK/JNK, and p38 MAP kinase. While no change in expression or phosphorylation of p44/42 or SAPK/JNK was detected (data not shown), phosphorylation of p38 MAP kinase was found to be impaired in knockout liver specimen (Fig. 5j), while basal p38 expression remained unaltered (Fig. 5k). The MAP kinase p38 in its phosphorylated state functions as a tumor suppressor protein (34-36), specifically in liver (37, 38), and has been shown to suppress growth by inhibition of
cyclin D1/cyclin-dependent kinase 4 (cdk4) complexes (34). We subsequently quantified cdk4 expression, which we found to be increased in knockout specimen (Fig. 5l) suggesting promotion of G1 to S transition of the cell cycle, consistent with a persistent pro-proliferative stimulus. Concurrently, immunostaining with an antibody against Ki-67 protein, a marker for proliferating cells, revealed a significant increase in the number of cells about to divide in knockout animals when compared to liver sections from control genotypes (Fig. 5m, P<0.00001). Taken together, these findings suggest that disruption of frataxin causes a reduction in OXPHOS and an increase in ROS formation, impaired phosphorylation of p38, and increased expression of cdk4, leading to increased proliferation of knockout hepatocytes.

**Discussion:**

By disruption of frataxin in murine hepatocytes, we here show impaired mitochondrial function, decreased life span, and, unexpectedly, formation of tumors in knock-out mice. Consistent with previously published data, we and others have shown that frataxin controls mitochondrial function and ATP synthesis (5-8, 10), as suggested by one of its proposed primary functions, the control of Fe/S cluster synthesis (1, 12). Secondly, disruption of frataxin causes increased formation of ROS, as indicated by elevated levels of TBARS and oxidized glutathione (GSSG) in liver specimen from knock-out mice, and consistent with previously published findings (13-16). Nevertheless, buffering capacity against ROS remained unaffected as indicated by unaltered levels of reduced glutathione (GSH), owing some support to recent data suggesting a rather limited role of oxidative stress in the development of the Friedreich ataxia phenotype (39). Thirdly, we observed reduction of life span in affected mice consistent with previously published data on extension of life span in eukaryotes with an increase in mitochondrial respiration (40) as well as reduced life expectancy due to increased ROS formation in mice (41). Lastly, we found that frataxin deficiency promotes tumor formation in mice by impairing activation of the tumor suppressor p38 MAP kinase (34-36), which has been found to be important for growth and tumorigenesis...
especially in liver (37, 38). Specifically, deficiency of frataxin leads to an enhanced hepatocyte turnover by simultaneous induction of both apoptosis, which is typically observed following depletion of ATP and occasionally observed following induction of ROS, as well as proliferation, which may be induced by impaired phosphorylation of p38 MAP kinase and possibly increased formation of ROS. Further experiments employing liver-specific frataxin knock-outs as well as inbred, genetically unmodified mice will have to show whether reduction of Fe/S-dependent enzymes and OXPHOS is sufficient to impair phosphorylation of p38 MAP kinase, especially since ROS are known to typically induce rather than impair activity of stress kinases (42), and specifically p38 MAP kinase (42).

Numerous cancer specimen exhibit mtDNA deletions, reduced mitochondrial content, altered mitochondrial morphology and impaired oxidative capacity (43-45) as well as an increase in glycolytic rate and lactate production (46, 47). Consistently, disorders of the respiratory chain predispose to hepatocellular carcinoma in humans (48), and rare inherited deficiencies of mitochondrial succinate dehydrogenase subunits or mitochondrial fumarate hydratase can cause tumors in humans (49). In this regard, it has been predicted that these inherited deficiencies should cause increased formation of ROS in parallel with impaired OXPHOS, hypothetically culminating in both increased apoptosis as well as proliferation (49). By using our mouse model of impaired OXPHOS due to depletion of frataxin, we here confirm this hypothesis. However, while oxidative stress is observed in frataxin-deficient liver specimen, a primary role of ROS in tumor formation remains a matter of debate since (i) the buffering capacity of knockout hepatocytes against ROS was found to be unaltered, and (ii) the tumor suppressor p38 was found less phosphorylated in frataxin knockout animals than in control mice, while significant levels of ROS typically induce p38 activity (42). Further experiments employing transformed cell lines overexpressing frataxin may be both useful and required to dissect the concurrent roles of impaired OXPHOS and increased ROS formation in frataxin-dependent induction of tumor growth, and are currently underway.

Friedreich ataxia is a disease predisposing to occasional tumors at young age (20-28); nevertheless, malignant disorders are not considered a mandatory complication of the
disease. As previously discussed for a similar apparent inconsistency in fumarate hydratase deficient individuals (50), Friedreich ataxia patients typically exhibit a decreased life expectancy of 38 years on average, which may prevent tumors to evolve into a clinically visible state. Furthermore, while Friedreich ataxia patients exhibit reduced, albeit detectable, levels of frataxin protein in their tissues (2), knock-out mice including our liver-specific animals have undetectable expression levels, potentially accelerating the phenotype, as previously discussed for disruption of frataxin expression in tissues other than liver (29). Hence the phenotype observed here is probably induced by complete disruption of frataxin expression, while Friedreich ataxia patients (as well as their heterozygous relatives) may exhibit an increased risk for malignancies only if they obtain a normal life span, which should be taken into account as soon as successful treatments for the disease have been established. Furthermore, prospective studies to determine the cancer risk in first degree relatives of Friedreich ataxia patients might be useful to further test this hypothesis.

In summary, we here have shown that lack of hepatic frataxin expression causes liver tumor growth in mice following impaired mitochondrial function and increased ROS formation, and that this unprecedented effect of a mitochondrial protein may be mediated by modulating the activity of p38 MAP kinase. Hence, frataxin might be considered a metabolic tumor suppressor protein located upstream of established stress kinases in mammals.

Methods:

**Generation of knockout mice:** Animals were generated (11), bred (29) and maintained (29) as described before except for beta-cell specific *Ins2-cre* mice were replaced by hepatocyte-specific *Alb-cre* (30) animals, which were 67% C57bl6 and 33 % FVB of origin, while *frataxin loxP* animals were at least 90% of C57bl6 origin. Genotyping and detection of knockout animals at genomic and transcriptional levels were previously described (29). Detection of knockout at translational levels was performed with a polyclonal antibody against mouse frataxin (11) by immunoblotting as described (16).
Histology and immunohistochemistry: Methods have been described before (29), except for TUNEL assays were performed by using a TACS XL Apoptosis Detection Kit (Trevigen, Gaithersburg, MD).

Southern blotting: Methods have been described before (51) except for Nhe I (Roche, Basel, Switzerland) was used for enzymatic restriction of murine DNA prior to gel electrophoresis.

Metabolic and enzymatic assays: Mitochondrial enzyme activities were determined as described before (52). Oxygen consumptions, ATP contents and mitochondrial membrane potentials were measured as previously described (7). Measurements of TBARS were performed as previously described (53). Quantification of oxidized and reduced glutathione was performed as previously described (54). Serum levels for albumin, L-alanine transferase (ALAT), cholinesterase (ChE) and lactate dehydrogenase (LDH) were thankfully determined by the clinical laboratory of the German Institute for Human Nutrition employing standard assays. All assays were performed in samples derived from at least 4 animals per genotype.

Signal transduction: Immunoblots were performed as described before (16) except for additional polyclonal antibodies against Bax, basal p38, Thr180/Tyr182 phosphorylated p38, basal p44/42, Thr202/Tyr204 phosphorylated p44/42, basal SAPK/JNK, and Thr183/Tyr185 phosphorylated SAPK/JNK (all from Cell Signalling, Beverly, MA), basal p53 (Novo-Castra Laboratories, Newcastle upon Tyne, UK), basal p53 (Exalpha Biologicals, Boston, MA), HSP25 (Stressgen, Victoria, BC), and additional monoclonal antibodies against cdk4, Ser15 phosphorylated p53, and basal p53 (all from Cell Signalling), cytochrome C (BD Biosciences, Franklin Lakes, NJ), HSP70 (Stressgen), and α-tubulin (Sigma-Aldrich) were used, and phosphatase inhibitors (Complete, Roche) were added whenever applicable.

Statistical analyses: Methods have been described before (29).

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Note added in proof:
A corresponding manuscript describing inhibitory effects of frataxin on cancer growth was accepted for publication at the Journal of Biological Chemistry (digital object identifier: 10.1074/jbc.M511064200, http://www.jbc.org/cgi/doi/10.1074/jbc.M511064200)

Legends to figures:

Figure 1: Efficacy and specificity of hepatocyte-specific frataxin knockout in mice.
Panel a depicts detection of the disrupted frataxin allele by PCR amplification of genomic DNA. Panel b shows detection of a missing exon 4 within frataxin cDNA by reversely transcribed PCR in liver RNA samples of knockout mice. In Panel c immunoblots against murine frataxin protein (18 kD) and a Ponceau red stain of the corresponding blot (loading control, also applies to Figs. 5c and 5e) are displayed.

Figure 2: Decreased life span and liver tumor formation due to frataxin disruption.
Panel a shows body weight gain in offspring starting at 3 weeks of age (squares indicate control animals, triangles indicate knockout animals). Panels b to e depict serum levels for albumin (b), cholinesterase (c), L-alanine transferase (d), and lactate dehydrogenase (e) in frataxin knockout mice (black) and control genotypes (grey) (both apply to all subsequent figures) Panel f shows a Kaplan-Meier survival graph of knockout mice (black line) versus control genotypes (grey line). Panels g, h and i displays a typical liver specimen of hepatocyte-specific frataxin knockout mice; original enlargement: b = none, c = 40fold, and d = 200fold.
Figure 3: Increased markers of oxidative stress following frataxin disruption. Panel a depicts amounts of thiobarbituric reactive substances (TBARS) in liver specimen of frataxin knockout mice and control genotypes. Error bars indicate standard deviations (applies to all subsequent figures). Panel b depicts levels of oxidized glutathione, and Panel c depicts levels of reduced glutathione in liver specimen.

Figure 4: Impaired mitochondrial function due to frataxin disruption. Panel a depicts Southern blotting of mtDNA of liver samples from frataxin knockout mice and control genotypes employing a mtCOXIII probe; below the corresponding loading control (18S rDNA). Panel b depicts specific activities of mitochondrial enzymes in liver samples from frataxin knockout mice and control genotypes. Abbreviations are Aco = aconitase, C I = complex I, C II = complex II, C III = complex III, and Fum = fumarate hydratase. Panel c depicts oxygen consumption of liver specimen from frataxin knockout mice and control genotypes. Panel d depicts ATP content of liver specimen from frataxin knockout mice and control genotypes.

Figure 5: Frataxin disruption promotes both apoptosis and proliferation. Panel a depicts immunoblots against murine frataxin (upper) and a subsequent re-blot against α-tubulin (lower, loading control) (the latter also applies to panels 5b, 5d, 5h, 5i, 5j, 5k, and 5l) in whole cell lysates. Panel b depicts immunoblot against Bax in whole cell lysates and Panel c immunoblot against Bax in mitochondrial fractions of whole cell lysates. Panel d depicts immunoblot against cytochrome C in cytosolic fractions of whole cell lysates of liver samples. Panel e depicts immunoblot against cytochrome C in mitochondrial fractions of whole cell lysates. Panel f depicts a typical stain against activated/cleaved caspase 3, and Panel g depicts a typical TUNEL stain, both on sections from frataxin knockout mice (left) and control genotypes (right); original enlargement 200fold, except otherwise indicated.
Panel h depicts an immunoblot in whole cell lysates against heat shock protein 70 (HSP70), and Panel i against HSP25. Panel j depicts immunoblot against phosphorylated p38 MAP kinase in liver samples from frataxin knockout mice, panel k depicts a blot against basal p38 MAP kinase in these samples, and Panel l depicts immunoblot against cyclin-dependent kinase 4. Panel m depicts a typical immunostaining against Ki-67 on sections from frataxin knockout mice (left) and control genotypes (right); original enlargement 200fold.
References:


Comparison of TBARS, GSSG, and GSH levels between KO and lox genotypes.