

Induction of Oxidative Metabolism by Mitochondrial Frataxin Inhibits Cancer Growth

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More than 80 years ago Otto Warburg suggested that cancer might be caused by a decrease in mitochondrial energy metabolism paralleled by an increase in glycolytic flux. In later years, it was shown that cancer cells exhibit multiple alterations in mitochondrial content, structure, function, and activity. We have stably overexpressed the Friedreich ataxia-associated protein frataxin in several colon cancer cell lines. These cells have increased oxidative metabolism, as shown by concurrent increases in aconitase activity, mitochondrial membrane potential, cellular respiration, and ATP content. Consistent with Warburg's hypothesis, we found that frataxin-overexpressing cells also have decreased growth rates and increased population doubling times, show inhibited colony formation capacity in soft agar assays, and exhibit a reduced capacity for tumor formation when injected into nude mice. Furthermore, overexpression of frataxin leads to an increased phosphorylation of the tumor suppressor p38 mitogen-activated protein kinase, as well as decreased phosphorylation of extracellular signal-regulated kinase. Taken together, these results support the view that an increase in oxidative metabolism induced by mitochondrial frataxin may inhibit cancer growth in mammals.

Friedreich ataxia is an inherited neurodegenerative disorder (1) caused by the reduced expression of mitochondrial frataxin protein (2) leading to premature death due to cardiac failure (1), diabetes mellitus and insulin resistance (3), as well as impaired ATP synthesis in humans (4, 5). Concurrently it was shown that frataxin promotes oxidative metabolism and ATP synthesis when overexpressed in fibroblasts (6), possibly by a direct interaction with the respiratory chain (7). Although the primary function of frataxin is still a matter of debate (8), some evidence suggests that this protein directs the intramitochondrial synthesis of Fe/S clusters (9–12). Individuals suffering from Friedreich ataxia have a reduced life expectancy of 38 years on average (1) and show increased oxidative stress (13–15). Overexpression of frataxin has been shown to reduce intracellular accumulation of reactive oxygen species (ROS)³ 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 to oxidants and promote oxidative damage to both nuclear (17), as well as mitochondrial, DNA (18). In addition, fibroblasts from Friedreich ataxia patients exhibit increased sensitivity against ionizing radiation and show an increased frequency of transforming events (19). Although malignant disease is not considered a typical feature of the disorder, Friedreich ataxia patients exhibit various types of cancer atypical for their young age (reviewed in Refs. 3 and 16). Lastly, targeted disruption of frataxin in murine hepatocytes causes decreased life span and liver tumor formation in mice.⁴

In parallel to these latter findings in rodents, we questioned whether frataxin might conversely suppress cancer growth when stably overexpressed in previously transformed cells. Here we show that cancer cells overexpressing frataxin have increased oxidative metabolism, decreased growth rates and population doubling times, show inhibited colony formation capacity in soft agar assays, and exhibit a reduced capacity for tumor formation when injected into nude mice, paralleled by increased phosphorylation of the tumor suppressor p38 MAP kinase and decreased phosphorylation of ERK. Hence, increased oxidative metabolism may suppress malignant growth in mammals, and frataxin may function as a metabolically active mitochondrial tumor suppressor protein.

EXPERIMENTAL PROCEDURES

Cell Culture and Stable Transfection of Colon Cancer Cells—Transfection experiments were performed as described previously (6, 16), except that the retroviral expression backbone *pBabePuro* was replaced with *pCiNeo* (Promega, Madison, WI), which was subjected to calcium phosphate transfection (20). Overexpression and detection of hemagglutinin-tagged human frataxin was performed as described previously (16). Cells were selected and maintained in Dulbecco's modified Eagle's medium containing 4.5 g/liter D-glucose (Sigma-Aldrich), 10% fetal bovine serum (Biochrom KG, Berlin, Germany), and neomycin (Invitrogen) (700 mg/liter for MIP101, 500 mg/liter for DLD2, and 800 mg/liter for HT-29 cells).

Metabolic and Enzymatic Assays—Aconitase activity was determined spectrometrically in mitochondria-enriched fractions as described previously (21) by monitoring the formation of NADPH at 340 nm. The assay mixture contained 50 mM Tris-HCl, pH 7.5, 60 mM sodium citrate, 1 mM MnCl₂, 0.4 mM NADP⁺, and 4 units of NADP⁺ isocitrate dehydrogenase for a final volume of 1 ml. An appropriate volume of cell extract was brought up to 150 μl with 50 mM Tris-HCl

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³ The abbreviations used are: ROS, reactive oxygen species; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; SAPK, stress-activated protein kinase.

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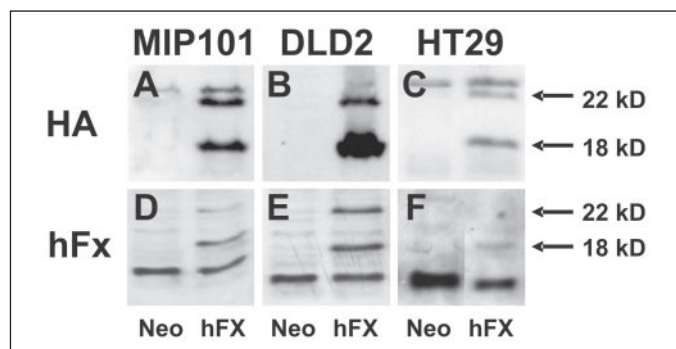


FIGURE 1. Stable overexpression of human frataxin in colon carcinoma cells. *A*, depicts immunoblot against hemagglutinin (HA) detecting the fully processed (18 kDa) and precursor (22 kDa) isoforms of stably overexpressed, hemagglutinin-tagged human frataxin in MIP101 cells (hFX). Neo, mock-transfected control cells. *B*, depicts immunoblots as in *A* using lysates from DLD2 cells. *C*, depicts immunoblots as in *A* using lysates from HT29 cells. *D*, depicts immunoblot against human frataxin (hFX) detecting the fully processed (18 kDa) and precursor (22 kDa) isoforms of both endogenous and stably overexpressed human frataxin in MIP101 cells. Neo, mock-transfected control cells. *E*, depicts immunoblots as in *D* using lysates from DLD2 cells. *F*, depicts immunoblots as in *D* using lysates from HT29 cells.

and loaded into one well of a 96-well plate. To start the reaction, 150 μ l of assay mixture were added, and the absorbance change at 340 nm was measured for 60 min at 37 $^{\circ}$ C. The aconitase activity was calculated from the slope of the linear portion.

For subcellular fractionation, $\sim 70 \times 10^6$ cells were scraped from the plates, suspended in 25 ml of phosphate-buffered saline, and centrifuged at $800 \times g$ for 10 min at room temperature. The resulting cell pellet was resuspended in 0.8 ml of MSH buffer (210 mM mannitol, 70 mM sucrose, 5 mM K^+ -Hepes, pH 7.5), homogenized on ice in a Teflon homogenizer, and centrifuged at $800 \times g$ for 5 min at 4 $^{\circ}$ C. The pellet was resuspended in 50 mM Tris-HCl, sonicated, and treated with 0.1% Triton X-100 for 10 min. After a further centrifugation at $10,000 \times g$ for 10 min at 4 $^{\circ}$ C, the mitochondria-enriched fraction (supernatant) was achieved, and aconitase activity was immediately measured.

Oxygen consumption, ATP content, and mitochondrial membrane potential were measured as previously described (6) using a Clark-type electrode (Hansatech; Norfolk, England, UK), a luciferase-based commercial kit (Sigma-Aldrich), and the fluorescent dye rhodamine 123 (Sigma-Aldrich), respectively. Intracellular quantification of ROS accumulation in cultured cells was performed as previously described (16, 22) by fluorescence-based quantification of the oxidation of the intracellular dye dihydrodichloro-fluorescein (Molecular Probes/Invitrogen).

Signal Transduction—Immunoblots were performed as described previously (16), except for the additional polyclonal antibodies 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 Signaling, Beverly, MA), and an additional monoclonal antibody against α -tubulin (Sigma-Aldrich) was used, and phosphatase inhibitors (CompleteTM, Roche Applied Science) were added whenever applicable.

Proliferation Assays—Population doubling time determinations, soft agar assays, and nude mice experiments were performed as described previously (16).

Statistical Analyses—The statistical analysis methods used have been described previously (23), except the *U* test was used for soft agar assays, and the Wilcoxon test was used for nude mice experiments.

RESULTS

Using standard calcium phosphate transfection techniques, we have stably overexpressed human frataxin carrying a hemagglutinin tag at the

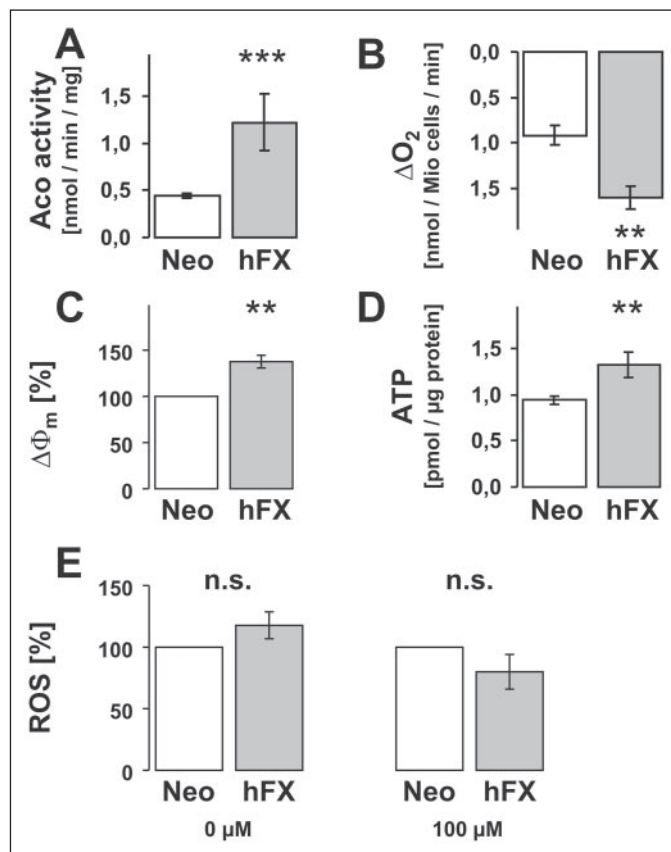


FIGURE 2. Increased oxidative metabolism in carcinoma cells overexpressing frataxin. *A*, depicts aconitase activities in native mitochondrial fractions. Open bars, (Neo) mock-transfected MIP101 cells; filled bars, (hFX) overexpressing MIP101 cells; means \pm S.D. (also applies to *B*–*E*). *B*, oxygen consumption of whole cells. *C*, the mitochondrial membrane potential. *D*, ATP content in whole cell lysates. *E*, dichloro-fluorescein fluorescence in untreated (left pair) and hydrogen peroxide-treated (right pair) cells. **, $p < 0.005$; ***, $p < 0.0005$; n.s., not significant.

C terminus in different human colon carcinoma cell lines, including MIP101, DLD2, and HT29, as shown by immunoblotting against the hemagglutinin tag solely detecting the overexpressed protein (Fig. 1, *A*–*C*), as well as against amino acids corresponding to exon 4 of the frataxin gene, detecting both endogenously expressed as well as overexpressed human frataxin protein (Fig. 1, *D*–*F*). Although colon carcinoma cells completely lacked endogenous expression of frataxin (Fig. 1, *D*–*F*), it should be noted that non-transformed cell lines, including primary human fibroblasts, expressed detectable levels of endogenous frataxin (data not shown).

To investigate whether overexpression of frataxin in cancer cells leads to metabolic alterations similar to those found in non-transformed fibroblasts subsequent to frataxin overexpression (6), we quantified aconitase activity, oxygen consumption, mitochondrial membrane potential, and lastly ATP levels. Consistent with previously published findings in frataxin-deficient states (9), overexpression of frataxin in colon carcinoma cells led to a significant increase in activity aconitase (Fig. 2*A*, $p < 0.0000001$) (DLD2, $p < 0.005$; HT29, $p < 0.00005$; data not shown), an enzyme that is dependent on Fe/S clusters (24). In accordance with our previous findings in non-transformed fibroblasts (6), overexpression of frataxin in colon carcinoma cells similarly increased cellular respiration ($p < 0.01$) (Fig. 2*B*) (DLD2, $p < 0.05$, data not shown), as well as the mitochondrial membrane potential ($p < 0.01$) (Fig. 2*C*), which is known to be the driving force for mitochondrial ATP synthesis. Accordingly and consistent with previously published find-

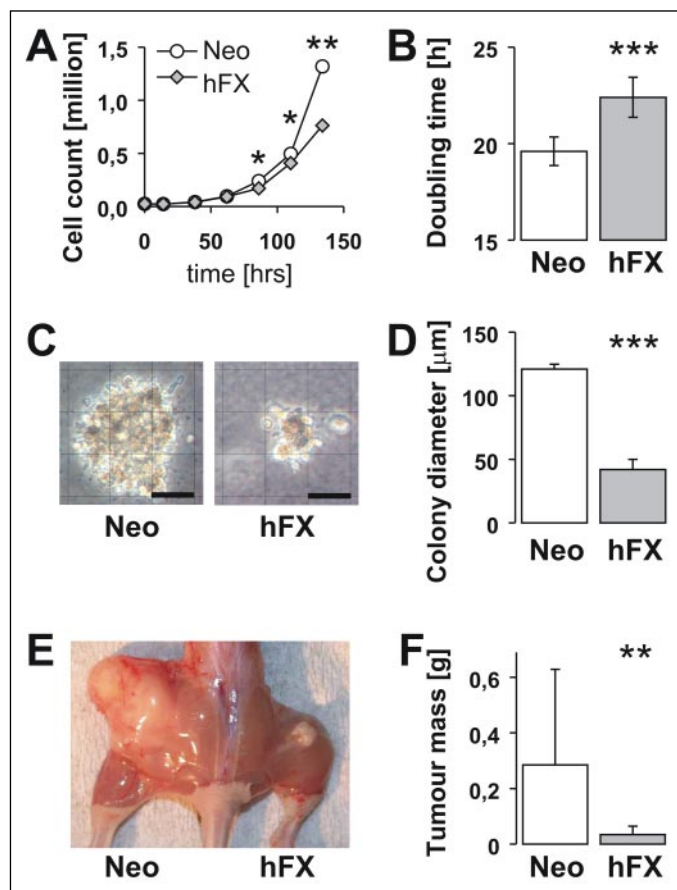


FIGURE 3. Decreased growth of carcinoma cells overexpressing frataxin. *A*, depicts growth characteristics of overexpressing hFx MIP101 cells (filled squares) and mock-transfected control cells (open circles) by absolute numbers. *B*, depicts growth characteristics of overexpressing hFx MIP101 cells and mock-transfected control cells by population doubling times. Open bars, (Neo) mock-transfected MIP101 cells; filled bars, (hFx) overexpressing MIP101 cells; means \pm S.D. (also applies to *D* and *F*). *C* and *D*, depict anchorage-independent growth in soft agar of overexpressing hFx MIP101 cells and mock-transfected control cells. Scale bars, 50 μ m (median + semiquartile differences). *E*, depicts typical tumors in nude mice derived from previously injected hFx MIP101 cells (right hind, top angle) and Neo control cells (left hind, top angle). *F*, the corresponding relative tumor masses derived from overexpressing hFx MIP101 cells and mock-transfected control cells (median + semiquartile differences). *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$.

ings in frataxin-overexpressing (6) as well as frataxin-deficient states (4, 5), we observed significantly increased levels of ATP in colon carcinoma cells overexpressing frataxin ($p < 0.05$) (Fig. 2*D*) (DLD2, $p < 0.05$, data not shown).

Frataxin has been described to reduce accumulation of ROS when overexpressed in non-transformed fibroblasts (16), and its yeast homologue Yfh1 has been shown to prevent damage to nuclear as well as mitochondrial DNA in *Saccharomyces cerevisiae* (17, 18). Furthermore, mice lacking frataxin in pancreatic β cells (23) or hepatocytes⁴ exhibit an increased expression of oxidative stress markers. We therefore questioned whether overexpression of frataxin in colon carcinoma cells might alter ROS accumulation, which was not the case. Neither in the basal state ($p = 0.423$) (Fig. 2*E*, left pair of bars) nor after exposure to hydrogen peroxide ($p = 0.150$) (Figs. 2*E*, right pair of bars) were significant differences in intracellular ROS accumulation observed.

Taken together, the findings so far suggest that colon carcinoma cell lines lack endogenous frataxin protein, and that overexpression of frataxin increases oxidative metabolism, whereas ROS accumulation remains unaffected by frataxin. These findings lend some support to

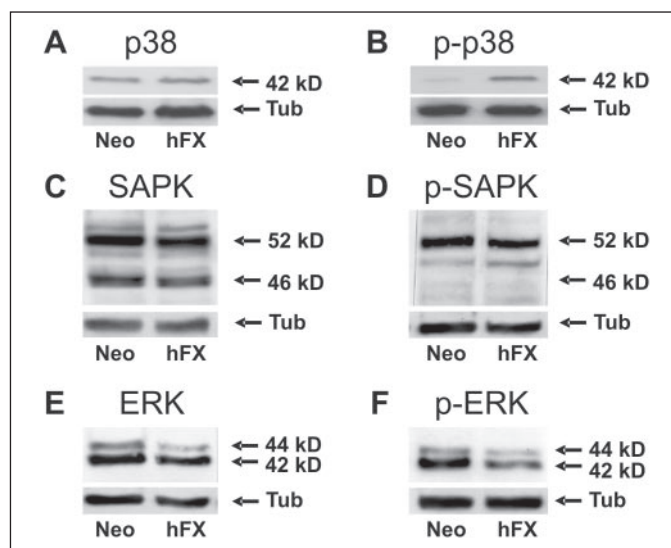


FIGURE 4. Expression and phosphorylation patterns of MAP kinases in carcinoma cells overexpressing frataxin. *A*, depicts immunoblots against total p38 MAP kinase (upper pair) in mock-transfected Neo control cells and overexpressing hFx MIP101 cells (applies to all subsequent panels). Lower pair, depicts corresponding blot against α -tubulin (Tub) (stripped and reincubated with additional primary antibody (applies to all subsequent panels)). *B*, depicts immunoblots against phosphorylated p38 MAP kinase. *C*, depicts immunoblots against total SAPK. *D*, depicts immunoblots against phosphorylated SAPK. *E*, depicts immunoblots against total ERK. *F*, depicts immunoblots against phosphorylated ERK.

recently published findings in frataxin knock-out mice questioning the link between frataxin deficiency and ROS accumulation (25).

Following stable transfection with frataxin, colon carcinoma cells showed significantly reduced growth rates when compared with mock-transfected cells ($p < 0.01$ after 134 h) (Fig. 3*A*) (DLD2, $p < 0.001$ after 134 h, data not shown). Accordingly, population doubling times in cancer cells overexpressing frataxin were significantly increased (Fig. 3*B*, $p < 0.0001$) (DLD2, $p < 0.0001$; data not shown). To further test whether frataxin overexpression also affected anchorage-independent growth, soft agar assays were performed; a pronounced reduction in colony-forming capacity and colony size was observed in the case of frataxin-overexpressing cells ($p < 0.000001$) (Fig. 3, *C* and *D*) (DLD2, $p < 0.000001$, data not shown). Lastly, we tested whether tumor formation in nude mice would be similarly affected. Indeed, tumors derived from colon cancer cells with an enhanced oxidative metabolism due to overexpression of frataxin were significantly smaller than those derived from mock-transfected control cells ($p < 0.01$) (Fig. 3, *E* and *F*) (DLD2, $p < 0.05$; HT29, $p = 0.066$, data not shown), thus suggesting that increased oxidative metabolism following overexpression of frataxin efficiently inhibits expansion of cancer cells *in vivo*.

Hepatic disruption of *frataxin* in mice causes liver tumors and impairs phosphorylation of the tumor suppressor p38 MAP kinase, whereas other MAP kinases, including ERK and SAPK, remain unaffected.⁴ Hence we questioned whether overexpression of frataxin would conversely promote activation of p38 MAP kinase. Indeed, although basal expression of p38 was unaffected in frataxin-overexpressing cells (Fig. 4*A*), phosphorylation of p38 was found to be increased in cells overexpressing frataxin (Fig. 4*B*), thereby potentially explaining reduced growth rates and diminished tumorigenesis of these cells (26–30). Although expression levels and phosphorylation of SAPK remained unaffected (Fig. 4, *C* and *D*), we observed reduced phosphorylation of ERK in frataxin-overexpressing cells (Fig. 4, *E* and *F*), also consistent with reduced growth rates and diminished tumorigenesis of these cells (31). Taken together, overexpression of frataxin significantly

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impairs growth and tumorigenesis of various colon cancer cell lines by induction of oxidative metabolism paralleled by altered expression and phosphorylation patterns of MAP kinases, p38, and ERK.

DISCUSSION

More than 80 years ago, Otto Warburg suggested that malignant growth might be caused by a decrease in mitochondrial energy metabolism paralleled by increased glycolytic flux (32, 33). This hypothesis has never been unambiguously confirmed, although increasing evidence suggests a close link between metabolic and genetic changes observed during malignant growth (reviewed in Ref. 34). We here have shown that overexpression of mitochondrial frataxin in transformed cells reduces malignant growth and inhibits their tumorigenic capacity by induction of oxidative metabolism. Although Warburg hypothesized that an increase in glycolysis might be the primary cause of malignant growth (32, 33), our current findings rather suggest that the efficiency of mitochondrial energy conversion might be the key metabolic factor. Whether the availability of oxygen is correlated with growth of such cells (as tentatively suggested by the findings depicted in Fig. 3) remains to be investigated.

Frataxin is a mitochondrial protein found to be reduced in Friedreich ataxia. Although the primary function of frataxin is still a matter of debate (8), there is no doubt as to its involvement in the control of oxidative metabolism. Although reduced expression of frataxin causes impaired oxidative phosphorylation in rodents and humans (4, 5), overexpression of frataxin in non-transformed fibroblasts has been previously linked to increased oxidative metabolism (6). We here provide evidence that three different colon cancer cell lines (MIP101, DLD2, and HT29) lack endogenous expression of frataxin, whereas non-transformed cell lines, including primary human fibroblasts, express detectable levels of endogenous frataxin.⁵ Although it is known that most metabolically active non-transformed cells express significant levels of frataxin (35), further studies are required to test whether lack of frataxin is a common trait of transformed cell lines and/or cancer tissues in general.

Because overexpression of frataxin in non-transformed fibroblasts was shown to increase oxidative phosphorylation previously (6, 7), it is not surprising that overexpression of this protein in cancer cells induces oxidative metabolism as well, as shown in the present study. Hence, frataxin can be used as a molecular tool to specifically enhance mitochondrial metabolism in cancer cells. Such enhancement efficiently impairs malignant growth and reduces the tumorigenic capacity of previously transformed cells, providing evidence for a close link between oxidative metabolism and cancer growth.

Several groups have previously shown that frataxin may influence the formation and accumulation of ROS in yeast, cultured mammalian cells, rodents, and finally humans (13–18, 23). Nevertheless, recent findings question the primary relevance of ROS formation in frataxin deficiency (25). To evaluate whether overexpression of frataxin in colon cancer cells might influence the endogenous production of ROS, intracellular oxidation of dihydro-dichloro-fluorescein was quantified and found unaltered. The subsequent addition of hydrogen peroxide did not reveal any differences in the accumulation of exogenous ROS. Hence, a putative effect of ROS on cell division or anchorage-independent growth appears unlikely in the specific experimental setting employed.

Targeted disruption of *frataxin* in murine hepatocytes causes multiple liver tumors and specifically impairs phosphorylation of the tumor suppressor p38 MAP kinase, whereas other members of the MAP kinase

family remain unaffected.⁴ Based on these findings, we quantified the basal expression and phosphorylation status of three main MAP kinases. Phosphorylation of p38 was found to be increased following the overexpression of frataxin, consistent with findings in frataxin knock-out animals. Although ERK and SAPK were found unaltered in frataxin knock-out animals, we here observed reduced activation of ERK following overexpression of frataxin. Because ERK is known to be activated by oncogenic *ras* and *raf*, impaired activation might be considered consistent with impaired malignant growth (31). Taken together, overexpression of frataxin may exert its anti-proliferative activity by induction of oxidative metabolism leading to increased phosphorylation of the tumor suppressor p38 and impaired phosphorylation of pro-proliferative MAP kinases, namely ERK.

Numerous cancer specimens exhibit mtDNA deletions, reduced mitochondrial content, altered mitochondrial morphology, and impaired oxidative capacity (36–38), as well as an increase in glycolytic rate and lactate production (32, 33). These observations have been corroborated in numerous subsequent studies during the past decades (reviewed in Ref. 34). Additionally, a recent publication on clonal cell lines expressing an increasing number of oncogenes suggested that transforming events may lead to a metabolic state similar to that proposed by Warburg in 1924 (34). It should be noted, however, that Ramanathan *et al.* (34) found specific metabolites, including ATP, regulated differently from our current findings for reasons that remain to be evaluated. Nevertheless, both studies come to the conclusion that impaired mitochondrial metabolism, and specifically reduced Krebs cycle activity, may promote malignant growth. Consistently, disorders of the respiratory chain predispose to hepatocellular carcinoma in humans (39), and rare inherited deficiencies of mitochondrial succinate dehydrogenase subunits or mitochondrial fumarate hydratase can cause tumors in humans (40).

Ingestion of fructose causes depletion of ATP in rodents (41) and humans (42). If ATP depletion is a relevant factor in the promotion of tumor growth, as suggested by our current findings, increased consumption of fructose should be associated with tumor formation in rodents and humans. Indeed, chronic ingestion of fructose has been shown to promote growth of chemically induced liver tumors in rats (43) and has been found to be associated with several cancers in humans in a dose-dependent manner (44, 45). Furthermore, type 2 diabetes mellitus has been associated with impaired expression of oxidative phosphorylation-related genes (46, 47) and was associated with altered phosphorylation patterns of p38 MAP kinase (48). Consistently, type 2 diabetes has been associated with the increased prevalence of numerous types of cancers in humans (49).

In summary, we have shown that induction of oxidative metabolism efficiently suppresses malignant growth *in vitro* and *in vivo*, as previously hypothesized (32, 33). Although further studies are required and warranted, induction of mitochondrial energy conversion, rather than suppression of glycolysis (32, 33), might be worth considering in efficiently preventing cancer growth in mammals.

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