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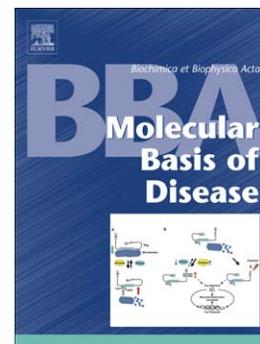
Differential metabolic consequences of fumarate hydratase and respiratory chain defects

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**Differential metabolic consequences  
of Fumarate Hydratase and respiratory chain defects**

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**Running title:** FH deficiency in primary cells

## Abstract

Defects of the oxidative energy production pathway lead to an amazing variety of disease phenotypes, ranging from childhood encephalomyopathies to hereditary tumor formation. A key enzyme of tricarboxylic cycle, fumarate hydratase (FH), is involved in encephalopathies, but also in leiomyoma formation, and occasionally also in various types of cancer. MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes) and NARP (neuropathy ataxia retinitis pigmentosa) are progressive neurological disorders, caused by mitochondrial DNA mutations and respiratory chain (RC) deficiency. The diseases lead to disability and premature death, but not to tumorigenesis. We studied the cellular consequences of FH and RC deficiencies, aiming to identify general responses to energy metabolism defect and those specific for FH deficiency, suggestively connected to tumorigenesis.

Unlike in RC deficiency, the FH-deficient diploid human fibroblasts showed no signs of oxidative stress, but had a reduced redox state with high glutathione levels. The cytoplasmic FH isoform, previously described but with an unknown function, was completely lacking in all FH-deficient lines. Fumarate was increased in two of our FH lines, but accumulation of HIF-1a was not detected. Glycolysis was induced in both MELAS and in FH deficiency.

Accumulation of fumarate in primary fibroblasts did not activate a hypoxia response, suggesting that hypoxia activation due to fumarate accumulation may be a tissue-specific response. The lack of cytoplasmic form of FH and the reduced redox

environment were typical for all FH-mutant lines, and their role in FH-related tumorigenesis requires further attention.

ACCEPTED MANUSCRIPT

## Introduction

The mitochondrial tricarboxylic acid cycle (TCA, also known as Krebs cycle or citrate cycle) has recently been shown to have a role in growth signalling, since two of its enzymes, succinate dehydrogenase (SDH) and fumarate hydratase (FH) have been identified as tumor suppressors [1-5]. Dominant mutations of succinate dehydrogenase subunits B, C and D are associated with development of pheochromocytomas and paragangliomas, and FH defects predispose to hereditary leiomyomatosis and renal cell cancer syndrome (HLRCC, OMIM #605839), as well are occasionally associated with ovary adenocarcinomas, Leydig cell tumors and cerebral cavernomas [5-8]. Autosomal recessive defects of FH and SDH result in devastating infantile encephalopathies [9], similar to those caused by recessive mutations of the mitochondrial respiratory chain (RC) and by some mitochondrial DNA (mtDNA) mutations. As TCA and RC generate most of the cellular ATP, it is likely that the energetic defect underlies the severe childhood encephalopathies. However, tumor predisposition is specific to FH and SDH mutations, not a feature of relative common RC defects.

Germ line heterozygosity for FH mutations underlies HLRCC, and the tumors are generated after somatic loss of the normal allele [10]. The underlying primary events of tumorigenesis have remained elusive. Both FH and SDH deficient tumors show induction of hypoxia pathway [11-13], which has been suggested to drive tumor

formation [12, 14]. Hypoxic response contributes to growth of several tumor types [15]. However, its role in primary tumorigenesis in FH-deficiency remains still suggestive.

We studied the effects of FH and RC deficiencies on cellular metabolism, hypoxia response, and redox metabolism. Both conditions result in reduced ATP production, but only FH-deficiency is associated with tumorigenesis. Both common energy-deficiency responses and FH-specific changes were identified, and their putative roles in FH-related tumor formation are discussed.

## Results

### **Localization of mutant FH protein: lack of cytoplasmic form in FH-deficiency.**

For the activities of mutant FH forms, please see methods. First we established whether the FH mutations affected the protein amounts or the localization, by cellular fractionation and Western blot analysis. Mitochondrial FH is translated as a ~54 kDa precursor, which is processed upon mitochondrial transport by cleavage of the N-terminal targeting signal, resulting in a mature form of 48 kDa. In yeast, the mature form is distributed to both cytoplasm and mitochondria after presequence cleavage [16]. In our cells, the mature 48 kDa form of FH was present in the mitochondria of all the FH-deficiency (FH<sup>-</sup>), RC-deficient and control lines (Figure 1A), albeit in three out of four FH lines the amount was slightly reduced. In MELAS and NARP, mitochondrial FH was clearly increased, which may be compensatory for RC defect. However, the cytoplasmic FH isoform was present in low amounts, ~10% of controls' level, in the cytoplasm (Figure 1B), whereas it was readily detected in the cytoplasmic fraction of RC-deficient lines and controls (Figure 1B).

### **Accumulation of fumarate in cells with homozygous FH mutations**

The mass spectrometric analysis of whole cell lysates showed that only the FH-1 and FH-2 lines, carrying homozygote mutations of FH and residual FH activities of <1% and <0.5%, had significantly increased fumarate concentration (Figure 1C). The lines FH-3 and FH-4 with compound heterozygote FH-mutations (activities ~20% and

~5%, respectively), as well as the MELAS and NARP lines, had fumarate levels similar to controls. All other metabolite levels analyzed (malate,  $\alpha$ -ketoglutarate, succinate, citrate/isocitrate, pyruvate, 2-phospho-glycerate/3-phospho-glycerate, glucose-1-phosphate, phosphoenolpyruvate and glyceraldehyde-3-phosphate), were similar in all lines (data not shown).

### **Metabolic and redox state of FH and RC lines**

Since the cellular metabolic and redox states have been associated to growth control [10, 14, 17], we examined the metabolic status and redox state of the cells.

Hexokinase (HK) is the first regulatory point of glycolysis. HK1 and HK2 are the two HK isoforms expressed ubiquitously or in proliferative states, respectively. HK1 mRNA level was unchanged in FH- and RC-deficient cells compared to controls, whereas the that of of HK2 was increased 1.5 – 3.5-fold, in FH-deficiency, MELAS and NARP (Figure 2A). These results suggest similar up-regulation of glycolysis in all these cell lines – a logical consequence of dysfunctional oxidative energy production pathway.

We then searched for evidence for reactive oxygen species in the cell lines. The activity of catalase was ~30% decreased in FH-deficient cells compared to controls, while two times higher in MELAS (Figure 2B). The cellular levels of  $H_2O_2$ , the long-lived, membrane-crossing source of hydroxyl radical, were lower in FH-deficiency than in controls, and in control level also in the RC-deficient lines (Figure 2C).

Next, we focused on the major intracellular redox buffer, glutathione (GSH).

GSH concentration was significantly higher in FH-deficient cells than in controls, but not in the RC-deficient lines MELAS or NARP (Figure 2D). The mRNA levels of both the catalytic and the modifier subunits of glutamate:cysteine ligase, catalyzing the rate-limiting step of GSH synthesis, were increased in FH-deficiency (1.5 to 4.5-fold for catalytic,  $p < 0,05$ , and 1.2-1.9-fold for modifier subunit,  $p < 0,05$ ). The concentration of cysteine, the limiting metabolite for GSH synthesis was increased in FH-lines, but not in RC-deficient lines (Figure 2E). All these data suggest increased GSH synthesis in FH-. The ratio of the activities of glutathione peroxidase 1 and glutathione reductase tended to be lower in FH-deficiency than in controls, and especially lower than in MELAS; FH-1 and FH-3 showed a significantly low ratio (2F). All these data indicate a reduced redox status and no oxidative stress in FH-deficiency, whereas some indications for ROS in RC-lines were detected.

### **No hypoxia response in FH- cells**

HIF-1a activation and nuclear localization has previously been associated with fumarate accumulation and tumorigenesis. We found no activation of the major regulator of hypoxia signalling in four FH-lines or in the RC- cells (Figure 3).

### **Growth rates unaffected by FH-deficiency**

No significant difference was observed in the growth rates of FH deficient fibroblasts versus controls in standard culture conditions (Figure 4), indicating that FH deficiency or fumarate accumulation do not provide a growth advantage in diploid primary cells.

## Discussion

Knowledge on the roles of energy metabolism enzymes in human disease has expanded during recent years from infantile encephalopathies to neurodegeneration and tumor predisposition. Familial predisposition to tumors has so far been reported only in association with dysfunction of two Krebs cycle enzymes, fumarate hydratase and succinate dehydrogenase. This suggests a role for these enzymes in growth regulation, not directly involved in oxidative ATP production. The primary mechanism, how a metabolic enzyme works as tumor suppressor, is still unresolved. Suggested mechanisms include fumarate accumulation and consequent HIF-1 $\alpha$  activation, as well as oxidative stress [10-12, 14, 17-19]. We studied here the metabolic consequences of FH deficiency and RC defects, both involved in energy production pathway. RC deficiencies have not been found to be associated with tumor predisposition, although they are common causes of metabolic disease.

FH has been shown to exist in two cellular compartments, the mitochondria and the cytoplasm, in the yeast *S. cerevisiae* [16], but the function of the cytoplasmic isoform is unknown in all organisms. In yeast, one FH precursor, common to both isoforms, is targeted to mitochondria, and the translocation is tightly coupled with translation [16, 20]. The targeting peptide is cleaved by the mitochondrial processing peptidase, and the mature form released partially to mitochondrial matrix, and partially to the

cytoplasm [21]. The mammalian cytoplasmic FH has been shown to exist in a number of previous studies [22-24], but its presence was recently questioned [25, 26]. We found clear evidence for the presence of both the mitochondrial and the cytoplasmic FH isoforms in primary healthy human fibroblasts as well as in those with RC deficiencies. However, the cytoplasmic FH was almost completely lacking in all our FH-deficient lines, irrespective of the underlying mutation. Intriguingly, mutations in any domain of the yeast FH abolish export of the mature cytoplasmic FH [27]. RC deficiency did not affect the FH isoform localization suggesting that the mislocalization is not energy dependent. Our finding can be explained either by translation-coupled translocation as in yeast, or decreased stability of the cytoplasmic defective isoform. Therefore, all our FH-deficient cells lack the enzyme activity almost completely, although residual activity remains in the mitochondria. Our finding emphasizes the need for clarification of the function of the cytoplasmic isoform and its relevance for FH-related pathogenesis.

FH and RC deficiencies shared some metabolic consequences, such as upregulated glycolysis, but also had marked differences. FH defect did not cause oxidative stress and the redox environment was reduced, whereas RC defects resulted in activation of oxidative stress defence. Fumarate increase has previously been described to affect redox status, favouring GSH production [28], but in our compound heterozygote cell lines, which also showed high GSH, total fumarate concentration was similar to controls. This suggested that fumarate alone was not responsible of the observed

redox change. However, by measuring total cellular fumarate, we cannot exclude local increases, or uneven distribution of the metabolite between cellular compartments.

High cellular GSH is typical for undifferentiated cell populations, such as fetal tissues, stem cells and tumors [29, 30], and has potential to directly inhibit cellular differentiation (reviewed in [30, 31]). Smooth muscle homeostasis is maintained by stem cell differentiation [32, 33], and it is tempting to hypothesize that FH-deficient smooth muscle progenitor cells with reduced redox status could be less prone to differentiate, increasing the likelihood of leiomyomas in FH-deficient patients. Our results do not, however, explain the tissue specificity. Our results do not support oxidative stress as a prime inducer of FH-deficiency associated phenotypes.

Hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) was not induced in any of our FH- and RC-deficient fibroblasts. FH-deficient myomas have been shown to have increased fumarate levels and HIF-1 $\alpha$  protein amounts, and therefore HIF-1 $\alpha$  activation has been proposed to underlie primary events of tumorigenesis in FH deficiency [10-12, 14, 17, 19]. Fumarate, succinate, pyruvate, oxalacetate and other 2-oxoacids were also shown to promote HIF-1a stabilization and accumulation [13, 34-37], through inhibition of prolyl hydroxylase 2 [11, 37, 38]. Several studies using cancer cells have shown accumulation of HIF-1 $\alpha$  in response to increased fumarate levels [34, 36]. In our study, only those two FH-deficient lines, which were homozygous for an active site mutation, showed accumulation of fumarate, but even they did not show HIF-1 $\alpha$  activation. These data suggest that fumarate accumulation does not always lead to

HIF-1 $\alpha$  activation but the effect may be tissue specific. The consequences of fumarate in smooth muscle cell metabolism warrants further study.

In conclusion, we show here that fumarate hydratase deficiency results in a loss of cytoplasmic FH isoform and reduced cellular redox state. Signalling functions of metabolites are only starting to be revealed. Fumarate is a by-product of purine biosynthesis [39], and therefore also formed in the cytoplasm. Lack of cytoplasmic FH could lead to local increase of cytoplasmic fumarate levels, which could affect purine metabolism, genome stability and tumor formation in a redox environment favoring undifferentiated state. Our data shows that detailed studies on the role of cytoplasmic FH and tissue-specific consequences of fumarate increase are warranted to understand FH-associated tumorigenesis.

## Materials and Methods

### Chemicals

Digitonin, GSSG, GSSG reductase, NADPH,  $\text{NaN}_3$ , and N-dodecyl-maltoside were from Sigma-Aldrich, Co. (St. Louis, Missouri, U.S.A.). GSH and NP-40 from Fluka (Sigma-Aldrich).  $\text{H}_2\text{O}_2$  (Perdrogen) from Riedel-de-Haën (Sigma-Aldrich). All chemicals were of the highest purity available.

### Antibodies

Mouse monoclonal antibodies were used against HIF-1 $\alpha$  (BD Biosciences, San José, California, U.S.A.),  $\beta$ -actin (Sigma-Aldrich, Co.) and SDHA (Molecular Probes, Invitrogen, Carlsbad, California, U.S.A.). Rabbit polyclonal antibodies were used against MAT-1 (Santa Cruz Biotech, Santa Cruz, California, U.S.A.) and FH (Nordic Immunology, Tillburg, The Neatherlands). Secondary antibodies horseradish-peroxidase-conjugated goat anti-mouse and goat anti-rabbit were purchased from Molecular Probes.

### Cell lines

Primary human fibroblasts were obtained by informed consent for research purposes with approval from the Ethical Review Boards of the involved Universities. Four fumarate hydratase deficient fibroblast lines were used: FH-1, homozygous for the E362Q mutation, with <1% remnant FH activity [40]; FH-2, homozygous for the and

Q376P mutation, with <0.5% remnant FH activity [41]; FH-3, is a compound heterozygote with InsK477/R233H with ~20% remnant FH activity [42]; and FH-4, is a compound heterozygote with delV387 and Q386H changes in one allele, and P369S in the other, with ~5% remnant FH activity [43]. Both mother and grand-mother of this child had uterine leiomyomas, and paternal relatives had lung and laryngeal cancers [43]. The family members of FH1-3 were not reported to have tumors at the time of examination, but follow-up information was not available. The respiratory chain deficient lines were from a 18 year-old female patient with mitochondrial encephalomyopathy, lactic acidosis and stroke-like-episodes syndrome (MELAS) due to a T3271C mutation in tRNA-leucine of mitochondrial DNA (mtDNA), with ~80% of mutant mtDNA, and from a 3 year-old patient with neurogenic muscle-weakness ataxia and retinitis pigmentosa syndrome (NARP), due to the T8993C mutation in mtDNA with ~90% mutant mtDNA. Control fibroblasts were from an adult, a child of 5 months and another of one year of age. None of the controls had an inherited disorder. FH-4 line was available only for part of the studies due to technical reasons. Typical passage numbers for the cells used in the studies were between ~10-25.

### **Cell culture**

Cells were cultured in Dulbecco's modified Eagle's medium with 4.5 g/l glucose and 2 mM L-glutamine, supplemented with 10% fetal bovine serum, uridine 50 µg/ml, penicillin 100 U/ml and streptomycin 100 µg/ml, at 37 °C in the presence of 5% CO<sub>2</sub>. All cell culture reagents were from Gibco-Invitrogen (Carlsbad, California).

### **Preparation of nuclear, mitochondrial, post-nuclear, post-mitochondrial and whole cell extracts**

Whole cell extracts: Primary fibroblast lines were trypsinized and pelleted at 800 x g, 5 min. The cell pellets were suspended and washed once in phosphate-buffered saline solution (PBS). For enzyme activity determination and mitochondrial protein detection, the cell pellet was resuspended in n-dodecyl-maltoside 1.5% (w/v) in PBS, supplemented with protease inhibitors (Complete Protease inhibitor, Roche, Basel, Switzerland), incubated at 4 °C for 30 min with rock-agitation, and centrifuged at 16,600 x g for 20 min at 4 °C. The supernatant ("whole cell extract") was used immediately for protein quantification and enzyme activity determination, or stored at -80 °C.

Mitochondrial extracts were prepared as described in [44], with slight modifications. Briefly, cells were trypsinized, pelleted (800 x g, 5 min) and washed once in PBS. Cell pellet was resuspended in digitonin (0.8 mg digitonin in PBS/mg protein), supplemented with protease inhibitors (Complete Protease inhibitor, Roche) and incubated for 5 min on ice. After dilution of samples with PBS, suspensions were centrifuged at 10,000 g for 10 min at 4 °C. This supernatant was the post-mitochondrial fraction, and the pellet contained enriched fraction of mitochondria. The pellet was resuspended in 1% n-dodecyl-maltoside in PBS, supplemented with protease inhibitors, incubated 15 min on ice, and centrifuged at 20,000 x g for 20 min

at 4 °C. The resulting supernatant was referred to as mitochondrial extract. It was used immediately for protein quantification and stored at -80 °C for protein detection.

Protein concentrations were measured at 595 nm, using BioRad Protein Assay (BioRad, Hercules, California, U.S.A.), according to Bradford method.

### **Gel electrophoresis and western blotting**

Whole cell extracts, mitochondrial extracts or nuclear extracts were resolved by SDS-PAGE on 7.5% (HIF-1a) or 10% (FH) Tris-Glycine gels. Proteins were blotted to PVDF (Immobilon-FL, Millipore Corporation, Bedford, MA, U.S.A.) with a semi-dry transfer apparatus (Amersham Biosciences, GE Healthcare) with 25mM Tris, 190mM glycine and 20% methanol. Membranes were blocked for 1h at room temperature in 5% milk in TBST (20mM Tris, 140mM NaCl, 0,1% Tween), and incubated with primary antibody (1:200, anti-HIF-1a; 1:200 anti-FH; 1:5000, anti- $\beta$ -actin; 1:10000 anti-SDHA) overnight at 4 °C. After washing in TBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Invitrogen; diluted in 5% milk in TBST; 1:4,000 anti-mouse for HIF-1a; 1:2,000 anti-rabbit for FH; 1:5000 anti-mouse for  $\beta$ -actin; 1:5000 anti-mouse for SDHA) for 1 h at room temperature. As a positive control for HIF-1 $\alpha$  activation we utilized fibroblasts treated with 100 $\mu$ M cobalt chloride for 4h in regular cell culture conditions. Chemiluminescent detection was performed using ECL Plus (Amersham Biosciences) or SuperSignal (Pierce, Rockford, Illinois, U.S.A.) on X-Omat AR and X-Omat LS films (Kodak, Hemel,

United Kingdom), or detected with Typhoon fluorescence scanner (GE Biosciences), and the signal was quantified utilizing Image-Quant software (GE Biosciences).

### **Metabolite extraction and analysis**

The cells were collected in equal confluency, by trypsinization, pelleted (800 x g, 5 min), washed once in PBS and the pellet (800 x g, 5 min) frozen at -80 °C until extraction. The cell pellet was suspended in 2.5 ml of boiling 80 % (w/w) ethanol and heated for 3 min in a boiling water bath. The samples were then centrifuged and the supernatant recovered. Pellets contained the macromolecules of the cells and were discarded. The supernatants were evaporated in a vacuum drier (SpeedVac). The dry sample was dissolved in 200 µL of deionized water. The solution was filtered through a 0.45 µm-membrane centrifugal filter at 12,000 g for 5 min. The filtrate with the metabolites was then analysed by anion exchange liquid chromatography with tandem mass spectrometric detection. The LC-MS analysis method was a modification of the method described in [45]. Metabolites analyzed were malate, fumarate, α-ketoglutarate, succinate, citrate/isocitrate, pyruvate, 2-phospho-glycerate/3-phospho-glycerate, glucose-1-phosphate, phosphoenolpyruvate and glyceraldehyde-3-phosphate.

### **Quantitative PCR**

Total RNA was extracted from fresh cell pellets using RNeasy mini RNA extraction kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer instructions.

cDNA was synthesised using M-MLV polymerase according to the manufacturer instructions, with random RNA hexamers primers (Roche). Real-time quantitative amplification was carried out using TaqMan system according to the manufacturer's protocol, with standardized Assays-on-Demand probes (Applied Biosystems, Foster City, California, U.S.A.). Fluorescence was detected with AbiPrism (Applied Biosystems) and the cDNA levels were determined at the exponential phase of PCR reaction, with the ABI7000 software (Applied Biosystems).

### **Glutathione and cysteine concentration**

The thiol metabolism was evaluated by measuring the total and free glutathione levels as previously described [46]. In brief, free sulphhydryls of thiols were derivatized with monobromobimane to form fluorescent complexes. The derivatized thiols were then separated by high-performance liquid chromatography (HPLC) and quantified by fluorimetry. In order to measure the total (reduced + oxidized) concentration of glutathione or cysteine, aliquots of the samples were first treated with 10 $\mu$ M dithiothreitol to reduce disulphides. Another aliquot was immediately treated with 15% (w/v) sulfo-salicylic acid to precipitate proteins. This allows separation of small-molecule mixed disulphides from larger complexes, such as protein-glutathione.

### **Enzyme activity measurements**

Enzyme activities were determined in whole cell extracts.

Glutathione peroxidase (GPx) assay followed the consumption of NADPH by the coupled reaction of glutathione reductase at 340 nm, using H<sub>2</sub>O<sub>2</sub> as substrate [47]. GPx activity unit corresponded to the formation of 1 μmol GSSG per min per milligram of protein. Glutathione reductase (GR) assay followed the consumption of NADPH at 340 nm [28]. GR activity unit corresponded to the consumption of 1 μmol GSSG/min/mg protein. Catalase activity assay followed the consumption of H<sub>2</sub>O<sub>2</sub> at 240 nm, at pH 7.0. Catalase activity unit corresponded to 1 μmol H<sub>2</sub>O<sub>2</sub> consumed/min/mg protein.

#### **Analysis of H<sub>2</sub>O<sub>2</sub> concentration**

Cells were seeded at the density of 5000 cells per well in 96-well plates and allowed to attach overnight. The wells were washed with PBS and replaced with normal culture medium. Background fluorescence (excitation 485 nm, emission 530 nm) was measured using a microplate fluorescence reader (Plate Chameleon, Hidex, Turku, Finland). Normal medium was replaced with medium containing 50 μM dichlorofluorescein diacetate (DCFH-DA, Molecular Probes) and 0.1 % dimethylsulphoxide (DMSO) or, as control, normal medium containing 0.1% DMSO, and incubated at 37 °C, for 30 min in the cell incubator. Cells were then washed once with PBS, and normal medium was added. DCFH fluorescence was measured. Background and DCFH fluorescence were normalized against control wells. As a positive control, some cells were treated with 10mM H<sub>2</sub>O<sub>2</sub> for 5 min at 37 °C after DCFH was removed, and then DCFH fluorescence was measured. DCFH oxidation is

driven mostly by H<sub>2</sub>O<sub>2</sub> in the cellular context, although reaction with other oxidants, such as peroxynitrite, can contribute in a low level to the signal [48].

### **Cell proliferation assay**

Primary fibroblasts were plated in luminescence-compatible 96-well plates (500 cells/well). The cell number was estimated using ViaLight HS proliferation assay (Cambrex BioScience Rockland, Charles City, Iowa, U.S.A.). The cells were allowed to attach, after which the first measurement was performed (point zero). Further measurements were made 1, 3, 5 and 9 days after plating. Each cell line in each time point was normalized to its own point zero, since somewhat decreased ATP production could be expected in the mitochondrial disease lines. The growth rates of the different patient lines were compared to the controls. The cells used for the proliferation assay were in passage numbers (p): control 1 (p=18), control 2 (p=34), control 3 (p=21), FH-1 (p=23), FH-2 (p=23), FH-3 (p=20), MELAS (p=18) and NARP (p=10).

### **Statistical analysis**

The number of independent replicates per experiment was typically five. Each patient cell line was compared to the pooled controls using student's t-test for two-tailed distributions with unequal variance. P-values under 0.05 were considered significant.

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## Figure Legends

### Figure 1. Fumarate hydratase protein levels and localization in fibroblast lines.

(A) Mitochondrial extracts (10  $\mu$ g protein / well) of FH patient fibroblast samples and controls were analyzed by Western blot, with FH-specific antibody. All cell lines show almost completely the mature processed FH form of ~48 kDa. SDHA as a mitochondrial marker shows equal loading. The bands of FH and SDH were quantified against the background using Image J software. (B) Same analysis as in A, with cytoplasmic extracts (10  $\mu$ g protein / well), also showing  $\beta$ -actin as a loading control. FH1-4 lines lack the mature 48 kDa isoform in the cytoplasm. SDHA shows the lack of mitochondria in the extracts. The bands of FH and  $\beta$ -actin were quantified against the background using Image J software. (C) Fumarate concentration ( $\mu$ M / mg protein) in whole cells. Significant changes are marked: \*\*( $p < 0.01$ ) or \*\*\*( $p < 0.001$ ). The controls represent the average  $\pm$ sd of the values for three individual fibroblast lines controls 1-3; all other values represent the average  $\pm$ sd for each cell line. Abbreviations: FH-1, fumarate dehydratase-deficient patient sample, E362Q homozygote; FH-2, Q376P homozygote; FH-3, compound heterozygote InsK477/R233H; FH-4, compound heterozygote with delV387+Q386H/P369S; SDHA, succinate dehydrogenase subunit A; MELAS, fibroblasts from a patient with mutation T3271C (~80% mutated mtDNA); NARP, sample with T8993C (~90% mutated mtDNA).

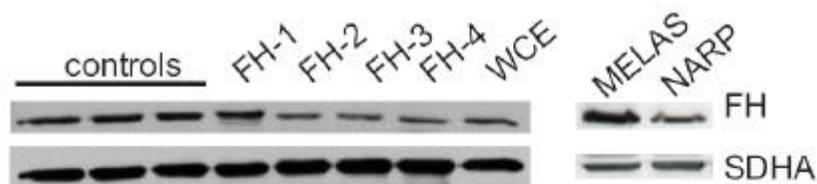
**Figure 2. Energy and redox metabolism.** (A) HK2 transcriptional levels were determined by quantitative-PCR. (B) Catalase activity in whole cell extracts. (C) H<sub>2</sub>O<sub>2</sub> levels in whole cells, measuring fluorescence intensity of dichlorofluorescein-H<sub>2</sub>O<sub>2</sub> reaction product, normalized against control wells after subtraction of background fluorescence. (D) GSH concentration, determined by HPLC of a fluorescently-derivatized compound, in the absence of DTT, on whole cell extracts. (E) Cysteine concentration determined by HPLC of fluorescently-derivatized compound on whole cell extracts. (F) The ratio of enzyme activities of glutathione reductase (GR) and glutathione peroxidase 1 (GPx1) in whole cell extracts. Significant changes determined by the two-tailed t-test are denoted as \*(p<0.05), \*\*(p<0.01) or \*\*\*(p<0.001). The controls represent the average±sd of the values for three individual fibroblast lines controls 1-3; all other values represent the average±sd for each cell line. Samples as in Figure 1.

**Figure 3. Accumulation of HIF-1 $\alpha$  protein in whole cell extracts.** Western blot for HIF-1 $\alpha$  (A) was performed with whole cell extracts (10  $\mu$ g / well, except for CoCl<sub>2</sub>-treated control, 5  $\mu$ g / well).  $\beta$ -actin was used as loading control. Abbreviations as in Fig 1.

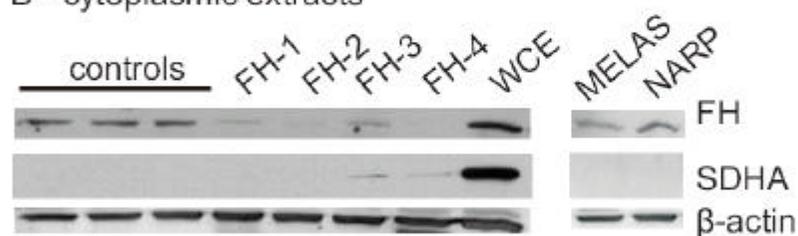
**Figure 4. Growth curves of the cell lines.** Cells were seeded in 96-well plates, and the number of cells was estimated from the amount of ATP detected in each well, normalized for each sample for its own point-zero value. Samples are as follows:

control-1-3 control fibroblasts; FH-1, FH-deficient fibroblasts with homozygous E362Q mutation; FH-2 with homozygous Q376P mutation; FH-3 with compound heterozygosity for InsK477/R233H; FH-4, compound heterozygosity for delV387+Q386H/P369S; MELAS, cells with MELAS mutation T3271C (~80% mutated mtDNA); NARP, cells with NARP mutation T8993 (~90% mutated mtDNA).

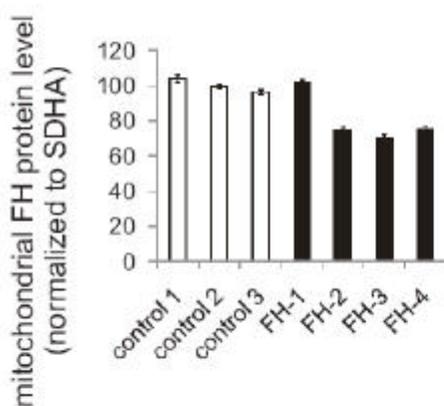
## A - mitochondrial extracts



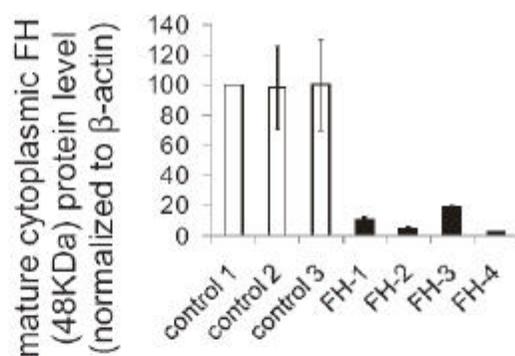
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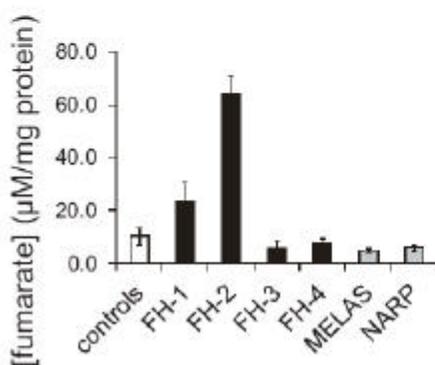
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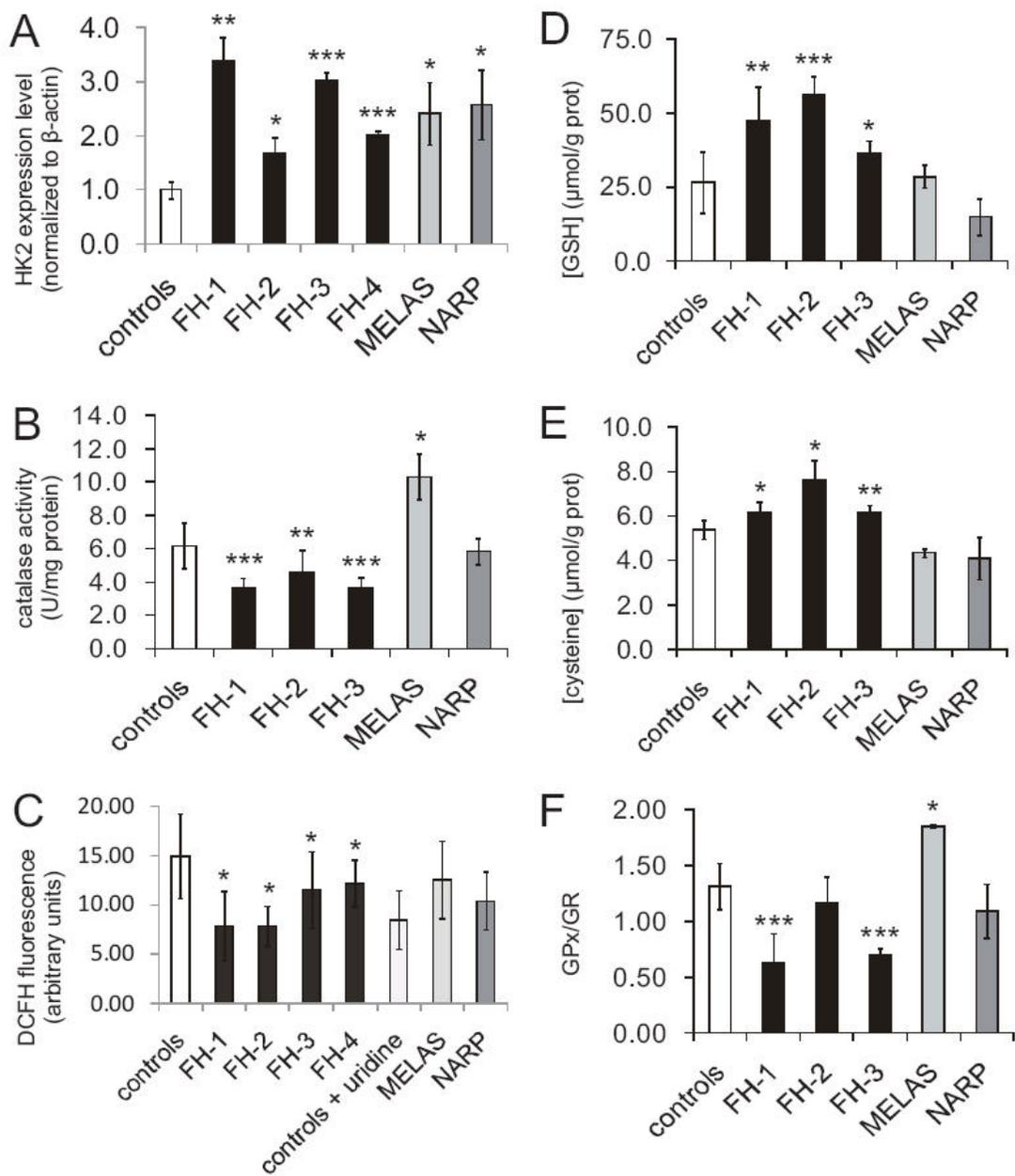


## D

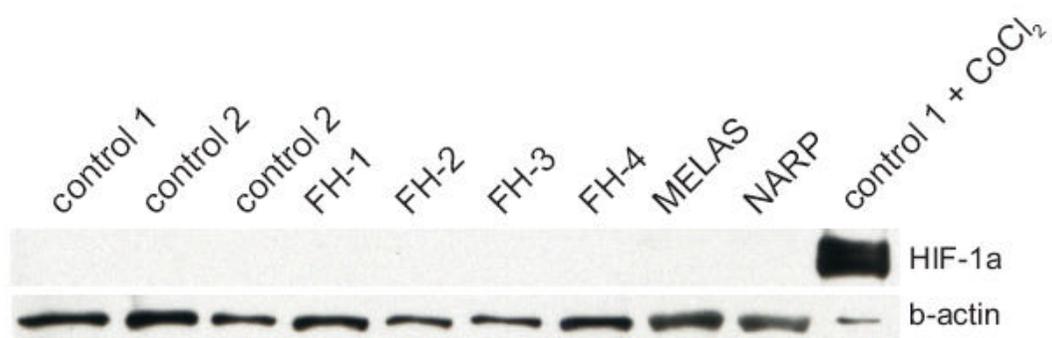


## E





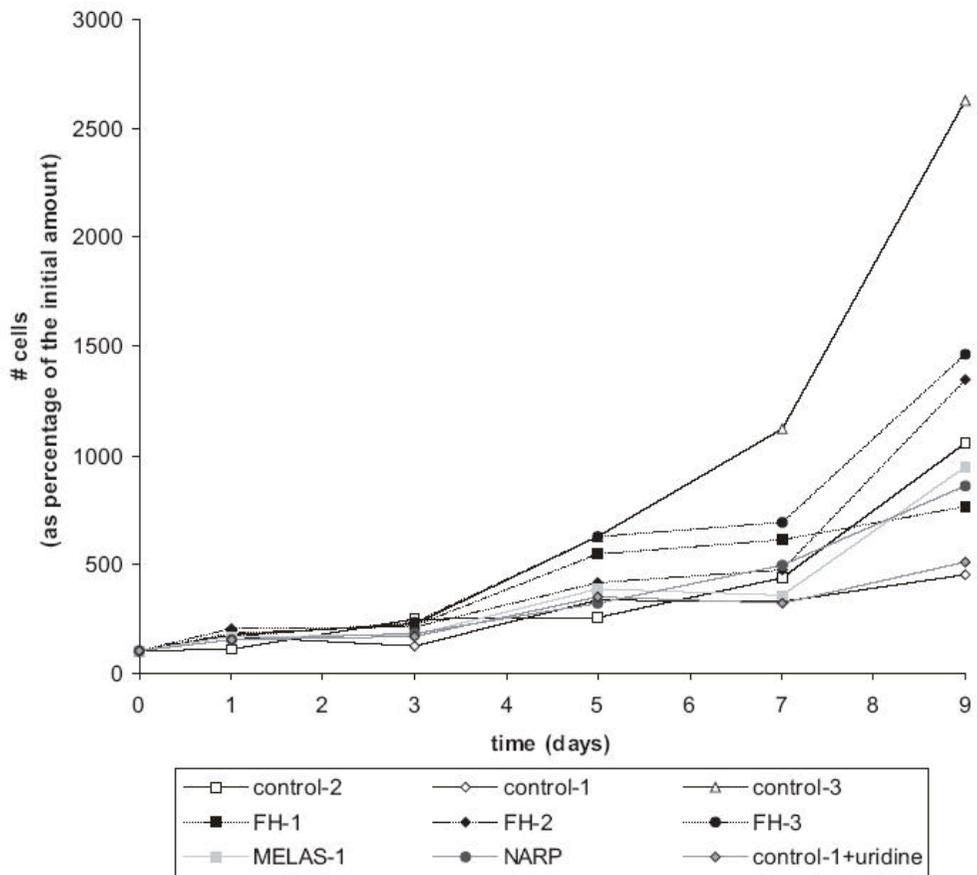
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Figure 2



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Figure 3

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Figure 4