Frataxin participates to the hypoxia-induced response in tumors

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Defective expression of frataxin is responsible for the degenerative disease Friedreich's ataxia. Frataxin is a protein required for cell survival since complete knockout is lethal. Frataxin protects tumor cells against oxidative stress and apoptosis but also acts as a tumor suppressor. The molecular bases of this apparent paradox are missing. We therefore sought to investigate the pathways through which frataxin enhances stress resistance in tumor cells. We found that frataxin expression is upregulated in several tumor cell lines in response to hypoxic stress, a condition often associated with tumor progression. Moreover, frataxin upregulation in response to hypoxia is dependent on hypoxia-inducible factors expression and modulates the activation of the tumor-suppressor p53. Importantly, we show for the first time that frataxin is in fact increased in human tumors in vivo. These results show that frataxin participates to the hypoxia-induced stress response in tumors, thus implying that modulation of its expression could have a critical role in tumor cell survival and/or progression.

Cell Death and Disease (2011) 2, e123; doi:10.1038/cddis.2011.5; published online 24 February 2011

Subject Category: Cancer

Friedreich's ataxia (FRDA) is a degenerative inherited disease because of deficient expression of the nuclear-encoded mitochondrial protein frataxin. Frataxin is required for organismal development, since its complete loss results in embryonic lethality in mouse,2 in plant Arabidopsis3 and for organismal development, since its complete loss results in embryonic lethality in mouse,2 in plant Arabidopsis3 and in developmental arrest in the nematode C. elegans.4 Partial expression of frataxin allows instead organismal development and survival, yet results in progressive degeneration of specific tissues. In fact, frataxin deficiency in humans critically affects survival of large primary neurons of the dorsal root ganglia, cardiomyocytes and pancreatic β-cells, accounting for FRDA syndromic features.1

Frataxin is therefore crucial for cell survival. Although the exact function of this protein is still unclear, it has been implicated in different biological functions, such as iron homeostasis,5 iron–sulfur cluster (ISC) biosynthesis,6 protection from oxidative stress and apoptosis.7 Conflicting results have been reported on the role of frataxin in cellular growth: both frataxin knockdown and frataxin overexpression were shown to impair cell growth.8–11 Very little is known regarding the molecular regulation of frataxin expression: histone deacetylase inhibitors, erythropoietin, cisplatin, 3-nitropropionic acid and hemin increase levels of frataxin2 in vitro (for review, see ref. 12). Moreover, transcription factors peroxisome proliferator-activated receptor gamma, 13 positively regulate frataxin expression. However, the molecular bases underlying the potential role of frataxin in cancer are unknown.

Hypoxia is a characteristic feature of most solid tumors that promotes the angiogenic switch necessary for tumor progression.15 The hypoxic environment found in tumors is not only dramatically associated with tumor progression but also confers tumor resistance to chemotherapy and radiotherapy. Cells under hypoxic stress can either develop an adaptive response, increasing the rate of glycolysis and angiogenesis, or undergo cell death through apoptosis and/or necrosis.16,17 The ability of tumor cells to maintain a balance between adaptation to hypoxia and cell death is mainly mediated by HIFs, which are essential for the regulation of the expression of a large number of hypoxia-responsive genes.16,17 HIFs are heterodimers consisting of either HIF-1α or HIF-2α bound to HIF-β/ARNT subunit. Although HIF-1α/β is constitutively expressed, both HIF-α subunits are degraded by oxygen availability. Under normoxia, HIF-α subunits are degraded by the proteasome through binding to the Von Hippel–Lindau E3 ligase. Low oxygen tension stabilizes the HIF-α subunits by inhibiting its degradation thus activating hypoxia adaptation responses. HIFs regulate the expression of genes containing conserved hypoxia-responsive element (HRE).18 Murine frataxin was shown to possess an HRE and its expression is indeed controlled by HIF-2α.14 In addition, a direct correlation between upregulation of HIF expression and tumor progression is well documented.16

Stabilization of the tumor-suppressor p53 is one of the many cellular responses triggered by hypoxic conditions,19,20 p53 itself is indeed modulated by HIF transcription factors21 and is involved in the metabolic alterations, which occur in FRDA, Friedreich's ataxia; HIF, hypoxia-inducible factor; HRE, hypoxia-responsive element; ISC, iron–sulfur cluster; ROS, reactive oxygen species

Received 27.8.10; revised 13.1.11; accepted 19.1.11; Edited by P Salomoni

Keywords: frataxin; hypoxia; p53; HIF; Friedreich's ataxia; tumor

Abbreviations: FRDA, Friedreich's ataxia; HIF, hypoxia-inducible factor; HRE, hypoxia-responsive element; ISC, iron–sulfur cluster; ROS, reactive oxygen species

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cancer cells. Hypoxia associated with tumor progression, not only results in enhanced stabilization of the master regulator HIF-1α but also in an increased leakage of reactive oxygen species (ROS) from the mitochondrial electron transport chain. Mitochondrial ROS production in turn activates p53 (ref. 24) while frataxin dampens oxidative stress. Moreover, a link between frataxin, oxidative stress and p53 has already been observed in C. elegans where the absence of cep-1, the C. elegans p53 homologue, significantly suppresses the increase in lifespan induced by reduced expression of the frataxin homologue frh-1 (ref. 26). cep-1 also regulates the induction of gst-4 (ref. 27), an antioxidant gene controlled by the redox-transcription factor skn-1, the C. elegans homolog of Nfr2, whose activation is impaired in human cells with defective frataxin expression.

Considering that hypoxia is a major stress signal for tumor cells, and that the murine frataxin is regulated by HIF, we hypothesized that frataxin could participate in tumor adaptation to hypoxia by regulating p53-dependent metabolic pathways. In this study, we found that hypoxia induces frataxin expression in different human tumor cell lines in a HIF-1α-dependent manner. In addition, frataxin silencing in tumor cells prevented hypoxia-induced p53 activation. Our data thus suggest that frataxin expression could have a critical role in tumor cell survival and/or progression. Importantly, in support of this idea, we now provide evidence for an in vivo increase in frataxin in human glioblastoma and colon cancer samples.

Results

Hypoxia induces frataxin expression in tumor cells. As tumor progression is associated with hypoxia, we analyzed frataxin expression on hypoxic stress in several tumor cell lines, such as two human glioblastoma cell lines U87 and U118, colon carcinoma HCT116 and human epithelial cervical carcinoma HeLa cells. The effect of hypoxia was also tested on immortalized B lymphoblasts derived from a FRDA patient and on control-matched immortalized B lymphoblasts derived from a healthy brother. Cells were subjected to hypoxia (1% O2) and collected at the time indicated. Longer exposures to hypoxia resulted in cell death (data not shown). As expected, HIF-1α protein expression increased on hypoxia compared with untreated cells (21% O2) (Figure 1). Importantly, both frataxin protein (Figure 1) and mRNA (Supplementary Figure 1) were significantly upregulated following severe hypoxic stress in all the cell types analyzed. Frataxin expression was induced in the different cell lines at different time points and with different intensity, probably reflecting differential cell-type sensitivity to low oxygen. Of note, transcriptional upregulation of frataxin appears to be modest compared with the effect on protein level. This observation suggests that, consistent with our recent report describing frataxin stability under proteasome control in normoxia, additional mechanisms for frataxin protein stabilization could also exist in hypoxic conditions.

Hypoxia-induced frataxin upregulation is mediated by HIF. HIFs are main mediators of hypoxia. Under low oxygen conditions, they translocate to the nucleus where they act as transcription factors for different HRE-containing genes. As murine frataxin was shown to possess an HRE for HIF-2α, we assessed whether frataxin upregulation in response to hypoxia in human cancer cells is mediated through the HIF pathway. We took advantage of glioblastoma cell lines TB10, U87 and U118, which stably express shRNA against HIF-1α, resulting in 70–80% knockdown of its expression. Wild-type cells and cells stably expressing shHIF-1α were subjected to hypoxia and frataxin expression was analyzed. As expected, on shRNA treatment, hypoxia-induced HIF-1α expression was significantly prevented in cells stably expressing shHIF-1α. Importantly, while HIF-2α expression was unchanged, the induction of frataxin on hypoxic stress was almost completely abolished (Figure 2). These results suggest that frataxin induction in response to hypoxia is controlled by HIF-1α in human cells.

Frataxin deficiency differently affects p53 activation in normoxia or hypoxia. Hypoxia modulates the activation of the tumor-suppressor p53, one of the most important defender against tumor development. We analyzed hypoxia-induced p53 activation in immortalized B lymphoblasts derived from a FRDA patient, compared with control-matched immortalized B lymphoblasts derived from a healthy brother. Cells undergoing hypoxia showed p53 phosphorylation at ser15, a classical marker of p53 activation. Interestingly, frataxin-defective cells had increased basal...
level of phosphorylated p53 but a less efficient activation of p53 on hypoxia (Figure 3a).

To further address the role of frataxin in modulating p53, we also interfered with frataxin expression using a shRNA-based approach in HeLa and U118 cell line, achieving a 60–70% frataxin knockdown. Cells were then placed into hypoxic conditions and p53 activation was analyzed by western blot. Similarly to FRDA cells, frataxin knockdown HeLa (Figure 3b) and U118 (Figure 3c) cells had increased basal level of phosphorylated p53 compared with control cells. On hypoxia exposure, however, both HeLa (Figure 3b) and U118 (Figure 3c) failed to upregulate frataxin and to activate p53. Thus, cells with slightly activated p53 might respond less efficiently to hypoxia. Importantly, normal frataxin levels appear to be required for optimal p53 activation following hypoxia.

**Figure 2** HIFs mediate hypoxia-induced frataxin upregulation. Left panels: human glioblastoma cells TB10 (a), U87 (b) and U118 (c) wild type or stably interfered for HIF-1α (shHIF-1α) were exposed to severe hypoxia for 18 h and frataxin (Fxn), tubulin (Tub), HIF-1α and HIF-2α expression analyzed. Data are representative of four, three and three independent experiments for TB10, U87 and U118 cells, respectively. Right panels: densitometric quantification of frataxin upregulation. Frataxin expression was normalized with tubulin and frataxin expression in normoxia set to one. Data represent the mean ± 1 S.E.M. from the different independent experiments performed for cell lines described in left panels. P-values were calculated with Student’s t-Test: *P < 0.05; **P < 0.01

**Figure 3** Frataxin deficiency prevents p53 activation in response to hypoxia. (a) FRDA patient-derived B cells (FRDA) and respective control cells (Healthy) were placed into hypoxic chamber for 18 h and frataxin (Fxn), actin (Act), total p53 (p53Tot) and phosphorylation at serine 15 of p53 (pS15-p53) expression analyzed. Data are representative of six independent experiments. (b) HeLa cells were transiently transfected with plasmid for frataxin gene interference (shFxn) each 2 days. Frataxin (Fxn), actin (Act), total p53 (p53Tot) and phosphorylation at serine 15 of p53 (pS15-p53) were analyzed after three rounds of transfection and exposure or not to hypoxic conditions for 18 h. Data are representative of three independent experiments. (c) hFxnRNAi and control U118 cells were treated for 5 days with doxycycline. Frataxin (Fxn), actin (Act), total p53 (p53) and phosphorylation at serine 15 of p53 (pS15-p53) expression were analyzed after exposure or not to hypoxia for 18 h. Data are representative of four independent experiments.

Frataxin is upregulated in human tumor samples *in vivo*. To assess whether frataxin levels are modulated in hypoxic tissues *in vivo*, frataxin was quantitated in human glioblastoma and colon carcinoma tumor samples. Analysis was performed comparing central tumor sample with the
adjacent healthy tissue of the same patient. Strikingly, frataxin resulted to be significantly upregulated in 485% of tumor samples analyzed (Figures 4a and b). Frataxin expression was upregulated in 12 out of 14 glioblastoma (Figure 4a), and in 17 out of 20 colorectal carcinoma tumor samples (Figure 4b), compared with the adjacent healthy tissue. The average frataxin upregulation resulted in 2.50- and 1.42-fold increase in glioblastoma and colorectal carcinomas, respectively, and was statistically significant both in glioblastomas and colorectal carcinomas ($P<0.01$).

In one case of glioblastoma, we first obtained surgical resections from the primary tumor and then from a relapse. In the relapse samples, the tumor core presented extended necrotic areas, a feature usually associated to severely hypoxic tissue and the adjacent peripheral tissue was infiltrated by neoplastic cells. Interestingly, frataxin expression was higher in the relapse sample, compared with the primary sample, both in the core and in the adjacent peripheral areas (Figure 5a).

In addition, for other six glioblastoma patients, two peripheral healthy samples adjacent to the tumor resection edge were collected. P1 is closer than P to the tumor central core. Interestingly, we could correlate frataxin expression level with the distance to the tumor core sample: the closer is the peripheral sample to the tumor core, the higher is the frataxin expression (Figure 5b).

Moreover, although the lack of information regarding p53 mutations in the samples analyzed does not allow to directly link frataxin expression to p53 activation, according to what we observed in human cell lines undergoing hypoxia, frataxin upregulation correlated with p53 expression and activation (Supplementary Figure 2).

**Discussion**

This study shows that frataxin is upregulated on hypoxia in tumor cell lines and that this event is dependent on HIF-1$\alpha$. Moreover, frataxin is required for optimal p53 activation following hypoxia in tumor cell lines. Importantly, here we report that frataxin is in fact upregulated *in vivo* in human glioblastoma and colon tumor samples.

Tumor proliferation depends on adequate nutrients and oxygen supply but tumor cells also need to survive under hostile conditions that include hypoxia and increased oxidative stress. The ability of tumor cells to maintain a balance

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**Figure 4** Frataxin is upregulated in human glioblastoma and colon cancer. Protein extracts (100 $\mu$g) were prepared from human glioblastoma and colorectal carcinoma (Tumor) and corresponding normal samples (Healthy). Frataxin expression was analyzed by western blot and normalized with tubulin and $\beta$-actin for glioblastoma (a) and colon carcinoma (b), respectively, and frataxin expression in healthy samples set to one. The densitometric quantification of normalized frataxin in the various samples as well as mean upregulation is indicated (mean ± 1 S.E.M.). Statistical analysis was performed by Student’s t-test; *$P<0.05$; **$P<0.01$
Frataxin binds iron and is involved in iron metabolism. Considering that iron controls HIFs degradation and that HIFs, in turn, can regulate iron metabolism modulating the levels of hepcidin, a functional interplay between HIFs and frataxin is expected. Although frataxin deficiency was reported to result in upregulation of HIF-1x proteins in cardiomyocytes of conditional frataxin knockout mutant mice with evident cardiomyopathy, we could not detect any significant differences of HIFs protein expression between healthy control and FRDA lymphoblasts or between wild-type and shFxn HeLa and U118 cells, both in normoxia and hypoxia conditions. Moreover, a role for HIF-2x but not HIF-1x in iron absorption of intestinal epithelium in mice has been reported: only HIF-2x can regulate the iron transporter DMT1 (ref. 35). It is noteworthy that contrasting effects between HIF-1x and HIF-2x have also been described on p53 activation in human cells. Although HIF-1x activates p53 (ref. 19), HIF-2x has been recently reported to inhibit p53 and ROS production. Considering that murine frataxin is controlled by HIF-2x but that in our cell system human frataxin upregulation in response to hypoxia depends primarily on HIF-1x, it would be interesting to further investigate whether such a discrepancy depends on cell and/or tissue type or on species differences.

Given the role of frataxin in ISC biogenesis, the HIF-1x-mediated frataxin upregulation is apparently in contrast with the pathways leading to mitochondrial dysfunctions in hypoxic cells. In this context, miR-210, which is a key microRNA upregulated by HIF-1x under hypoxic conditions, leads to downregulation of the scaffold protein ISCU in order to repress the mitochondrial electron transport and the Krebs’ cycle. However, the repression of ISCU by miR-210 concomitantly contributes to the generation of ROS during hypoxia, and frataxin, in addition to its function as iron chaperone, also has a key function in the adaptive response to oxidative stress. As oxidative stress correlates with aggressiveness of tumors and promotes tumor cell survival under hypoxic conditions, frataxin induction may contribute to tumor cells survival under hypoxia by regulating cells antioxidant response. A fine-tuned regulation already exists between hypoxia, HIF, p53 activation and ROS production: hypoxia stimulates mitochondrial ROS production, which modulates HIF-1x and HIF-1x, in turn, has a crucial homeostatic role in managing oxygen consumption to balance ATP and ROS production. Moreover, hypoxia activates p53, which has both pro-oxidant and anti-oxidant effects. We now add a new player to this complex loop by providing data showing that frataxin deficiency induces p53 activation in normoxia while preventing it on hypoxia. Frataxin deficiency in FRDA patients leads to increased ROS production, which could in turn activate p53 resembling hypoxic stress condition. This basal stress could then interfere with further adequate adaptive hypoxic responses preventing optimal p53 activation. In support of our findings, several evidences point to differential p53 biological responses depending on the degree of damage or stress as a conserved evolutionary mechanism from nematodes to mammals. Moreover, consistent with a role of frataxin in regulating p53 responses, we have previously shown that frataxin deficiency modulates longevity and antioxidant gst-4 activation in C. elegans,
in a p53-dependent manner and a recent report describes partners proteins of p53 and ATM significantly altered in FRDA patients. Overall, frataxin levels may contribute to the survival pathways of tumor cells via a fine-tuned regulation of the anti-oxidant response under hypoxic environment, likely influencing metabolic pathways by modulating the hypoxia-induced p53 stress response. The mechanisms underlying differential modulation of p53 activation by frataxin expression levels in normoxia and hypoxia with possible consequent recruitment of different p53 downstream targets will require further investigation.

Last but not least, considering that in FRDA, frataxin expression is inversely correlated with disease onset, progression and severity, it is noteworthy that frataxin protein level could be upregulated on hypoxia even in FRDA patient derived lymphoblastoid cells. Although hypoxia cannot be considered as a therapeutic strategy for this disease, further unraveling the mechanisms contributing to hypoxia-induced frataxin upregulation could be crucial to identify novel drugs capable of increasing frataxin level in FRDA patients.

In summary, frataxin is crucial for cell life since reduction of its expression leads to neurodegenerative disease FRDA and complete knockout is lethal in several organisms such as mice and plants. Frataxin overexpression can protect tumor cells from apoptosis but can also act as a tumor suppressor. We herein provide explanation for this apparent paradox showing that frataxin participates, by regulating p53 activation, in tumor adaptation to hypoxia, a critical feature associated with tumor growth and progression, thus suggesting that frataxin levels can influence tumor cell fate. We show indeed for the first time that frataxin is increased \textit{in vivo} in human tumor tissues.

Materials and Methods

\textbf{shRNA construct (shFxn).} Frataxin was depleted in human cells by RNAi using a \textit{pSUPER} vector-based approach. Self-complementary inverted repeat sequences of frataxin (19 nucleotide long frataxin gene-specific targeting sequence 5'GGTCCGAACCACTGAGGCT-3', position 167–185 of the coding sequence), spaced by 9 base loop region 5'TCCAAGAGA-3' were synthesized (Invitrogen, San Giuliano Milanese, Italy) as single-strand oligonucleotides. These 64 base long oligonucleotides containing the coding and complementary sequence of these components, together with BglII and HindIII restriction site sequences at the 5' and 3' ends respectively, were then annealed by heating to 89°C for 30 min, and then to 65°C for 10 min, in 50 mM Tris-HCl, 10 mM MgCl2, 10 mM DTT, 1 mM ATP, 25 mg/ml BSA, 10% DMSO. Successful annealing was checked by agarose gel electrophoresis, and the obtained double-stranded DNA was cloned into the \textit{pSUPER} vector via the BglII and HindIII restriction sites. All constructs were checked by DNA sequencing. The inducible \textit{hFxnRNAi} construct (\textit{pSUPERIOR.PURO} vector) and \textit{pcDNA6/TR} plasmid were a kind gift of Dr. Cortopassi.

\textbf{Cell culture, transfections and hypoxia.} Immortalized GM15850B lymphoblasts, from a clinically affected FRDA patient, and immortalized GM15851B lymphoblasts from a clinically unaffected brother of GM15850 were obtained from NIGMS Human Genetic Cell Repository, Coriell Institute for Medical Research (Camden, NJ, USA) and were cultured in RPMI 1640 supplemented with 15% fetal bovine serum. HeLa, U87, U118, HCT116 and TB10 cells were cultured in DMEM supplemented with 10% fetal bovine serum (HyClone, Rockford, IL, USA).

To create severe hypoxic environmental conditions, cells were placed into a modular incubator chamber (Billups-Rothenberg, Del Mar, CA, USA) and gas without oxygen (95%N2, 5% CO2) was flushed into the chamber for 10 min. Cells were then cultured into a normal CO2 incubator for different time periods.

\textbf{Generation of stably shHIF-1 \textit{a} cell lines.} Glioblastoma cell lines TB10, U87 and U118 with silencing of HIF-1\textit{a} were obtained, as previously described, by retroviral mediated expression of shRNA targeting nucleotide specific for HIF1-\textit{a} sequence resulting in 70–80% knockdown of its expression.

\textbf{Generation of stable doxycycline/tetracycline-inducible \textit{hFxnRNAi} cell line.} Human U118 glioblastoma cells stably transfected using Lipofectamine 2000 (Invitrogen) with \textit{pcDNA6/TR} plasmid, therefore expressing the tetracycline repressor, were first generated by 10 days selection with blasticidin hydrochloride (Invitrogen) 2 \mu g/ml. Cells were maintained in DMEM supplemented with 10% tetracycline-free fetal bovine serum (HyClone, Rockford, IL, USA).

Stably expressing \textit{pcDNA6/TR} U118 cells were then transfected with inducible \textit{hFxnRNAi} construct or the empty \textit{pSUPERIOR.PURO} vector as a control for the inducible system. At 24 h after transfection, the cells were split and grown in medium containing 1.5 \mu g/ml puromycin (Sigma, Milan, Italy). Single clones were isolated and expanded.

The efficiency of the inducible RNAi system was established analyzing frataxin mRNA levels by quantitative real-time PCR after 5 days of treatment with 8 \mu g/ml doxycycline (Sigma) of \textit{hFxnRNAi} and control clones. Three \textit{hFxnRNAi} clones showing >70–80% frataxin reduction were selected, as well as control clones in which frataxin level remained unchanged (data not shown).

\textbf{Quantitative RT-PCR analysis.} Total RNA (500 ng) was retrotranscribed using SuperScript Vilo \textit{cDNA} Synthesis Kit (Invitrogen) according to the manufacturer’s instructions. One-twentieth of the cDNA was used to quantify the transcripts by real-time RT-PCR employing the 7300HT Fast Real-Time PCR System Applied by Biosystem (Carlsbad, CA, USA). Gene specific primers were designed by the Probe Finder software (Roche Applied Science, Milan, Italy) and the cDNA was amplified and quantified using the SensiMix \textit{dT} kit by Quantace (Bioline, London, UK) according to the manufacturer’s instructions. The tata-binding protein mRNA was used as housekeeping invariant reference gene and the relative quantity was calculated accordingly. Specific forward primer for frataxin is situated in the third exon: 5'-CATCACGGTTTGGAGACTATGTCGT-3' and specific reverse primer situated in the fourth exon: 5'-TTTGGCCTCCTGTGTTATC-3'.

\textbf{Western blot.} Total cell extracts were prepared in ice-cold modified RIPA lysis buffer (50 mM sodium phosphate pH 7.2, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA) supplemented with complete protease inhibitor cocktail (Roche Diagnostics, Milan, Italy). Cell lysates (100 \mu g) were resolved by SDS-PAGE and analyzed by immunoblot with specific mAb anti-frataxin (Immunological Sciences, Rome, Italy), mAb anti-tubulin (Sigma), mAb anti-HIF-1\textit{a} (Becton Dickinson, Milan, Italy), mAb anti-HIF-2\textit{a} (Santa Cruz Biotechnology, Heidelberg, Germany), mAb anti-Jacltin (Sigma), mAb anti-p53 (Santa Cruz Biotechnology) mAb anti-phosphorylated p53 (ser 15) (Cell Signaling Technology, Danvers, MA, USA) using ECL system detection (GE Healthcare Europe GmbH, Milan, Italy). Densitometric analyses were performed using ImageJ software from the U.S. National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/.

\textbf{Total cell extracts from human tumor samples.} Colorectal adenocarcinoma and corresponding normal tissue from the same patients were obtained from 20 patients who underwent surgical resection. Glioblastoma tissue and corresponding normal counterpart adjacent to the tumor (resection edge) from the same patients were obtained from 14 patients who underwent surgical resection. The normal corresponding tissues were confirmed to be tumor-free by histological analysis. Each sample was frozen in liquid nitrogen, stored at –80°C, and subsequently homogenized on ice, in ice-cold modified RIPA lysis buffer supplemented with complete protease inhibitor cocktail. Frataxin, p53 and p53 phosphorylation were analyzed by western blot. Protein expression in human tumor samples was tested in duplicates or triplicates when possible.

\textbf{Statistical analysis.} The significance of differences between populations of data were assessed according to the Student’s \textit{t}-test with a level of significance of at least \(P < 0.05\) (alpha conventionally equal to 0.05).
Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. This work was funded in part by FIRC 2003 and Atena Outils Association for GM, FILAS grant from Region and Lazio for AL, National Ataxia Foundation, Ataxia UK, Friedrich's Ataxia Research Alliance, and Telethon-Italy (Grant GGGP06059) for RT. IG is a fellowship holder of the Association Francaise de l'Ataxie de Friedrich (AFAF).

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