The Inhibitor Protein (IF₁) of the F₁F₀-ATPase Modulates Human Osteosarcoma Cell Bioenergetics*

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Background: The role of the inhibitor factor, IF₁, of the F₁F₀-ATPase complex has not been fully defined.

Results: IF₁-silenced osteosarcoma cancer cells show increased mitochondrial membrane potential (Δψₘ) and decreased ADP-induced respiration rate.

Conclusion: IF₁ plays a role in the modulation of Δψₘ and oxidative phosphorylation rate in osteosarcoma cells.

Significance: These data provide novel insights into the role of IF₁ in tumor cells metabolism.

The bioenergetics of IF₁ transiently silenced cancer cells has been extensively investigated, but the role of IF₁ (the natural inhibitor protein of F₁F₀-ATPase) in cancer cell metabolism is still uncertain. To shed light on this issue, we established a method to prepare stably IF₁-silenced human osteosarcoma clones and explored the bioenergetics of IF₁ null cancer cells. We showed that IF₁-silenced cells proliferate normally, consume glucose, and release lactate as controls do, and contain a normal steady-state ATP level. However, IF₁-silenced cells displayed an enhanced steady-state mitochondrial membrane potential and consistently showed a reduced ADP-stimulated respiration rate. In the parental cells (i.e. control cells containing IF₁) the inhibitor protein was found to be associated with the dimeric form of the ATP synthase complex, therefore we propose that the interaction of IF₁ with the complex either directly, by increasing the catalytic activity of the enzyme, or indirectly, by improving the structure of mitochondrial cristae, can increase the oxidative phosphorylation rate in osteosarcoma cells grown under normoxic conditions.

Tumor cells exhibit profound genetic, biochemical, and histological differences with respect to the original non-transformed cellular types (1). The biochemical features of most fast-growing tumor cell types show a significantly modified energy metabolism that is characterized by a higher glycolytic flux (aerobic glycolysis) compared with normal cells: the so-called Warburg effect (2, 3). Indeed, different biochemical mechanisms may contribute to increasing the glycolytic rate of tumor cells, one which is the inhibition of mitochondrial ATP synthase (F₁F₀-ATPase) by the natural inhibitor protein, IF₁ (4).

The inhibitor protein, a basic, heat-stable, and highly evolutionarily conserved protein, is constituted of 106 amino acids in humans (5), and is the master regulator of the ATP hydrolytic activity of the ATP synthase complex. It is well established that when the electrochemical proton gradient across the mitochondrial inner membrane collapses becoming insufficient to drive ATP synthesis, as it occurs when mitochondrial respiration is dramatically impaired, ATP synthase reverses its function and hydrolyzes ATP to restore the membrane potential (6–8). Under these conditions, cell energy metabolism is shifted toward glycolysis resulting in a significant decrease in pH that induces the inhibition of ATP synthase by IF₁ (9). The pathways mediating this inhibition require the hydrolysis of two molecules of ATP by the IF₁-ATP synthase complex (10). The net effect is to prevent massive glycolytic ATP depletion to protect cells from death. Indeed, using oligomycin as a specific inhibitor of ATP synthase, it has been shown that, in ischemia, a considerable fraction (30–50%) of the decline in cytoplasmic high energy phosphates is due to hydrolysis by ATP synthase working in reverse (7, 11).

Besides the well established function described above, a number of other roles have been proposed for IF₁ in recent years, including involvement in ischemic preconditioning (12, 13), and in stabilizing both the dimeric form of ATP synthase and the structure of mitochondrial cristae (14, 15). A role in tumorigenesis is also suggested by the higher expression of IF₁ in a number of carcinomas than in normal tissues (4, 16–19). The latter point is of peculiar interest and is worth considering further for several reasons: the binding of IF₁ to ATP synthase is supposed to be strictly dependent on the environmental conditions of cells, and both acidic pH and mitochondrial membrane potential collapse seem to be required, as occurs during ischemia (6). These conditions, however, have only been marginally considered in recent studies. In addition, most of these studies were performed in cells with transient IF₁ overexpression or silencing. Transient transfection results in a heterogeneous population of cells due to the coexistence of transfected and control cells. Moreover, studies on transiently transfected cells do not represent a stable steady-state metabolic condition, but a dynamic situation in which the cells are adapting to the change in IF₁ content, producing potentially ambiguous bio-

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4 The abbreviations used are: F₁F₀-ATPase, ATP synthase complex; TMRM, tetramethylrhodamine methyl ester; IF₁, natural inhibitor protein of ATP synthase complex; OXPHOS, oxidative phosphorylation.
chemical data. Finally, transient transfection does not allow long-term changes to be studied. Given these restrictions, to dissipate ambiguities and clarify the contribution of the inhibitor protein to tumor viability and metabolism, we prepared IF₁-silenced clones from the human osteosarcoma 143B cell lines and assayed the main bioenergetic parameters. The purpose of this study was to examine in stable IF₁-silenced human osteosarcoma cells both the role played by IF₁ and the mechanism IF₁ adopts in tumor cells to regulate energy homeostasis and possibly to control mitochondrial mass, structure, and function.

EXPERIMENTAL PROCEDURES

Cell Culture, Transient Cotransfection, and Selection of Stable IF₁-silenced Clones—Human osteosarcoma 143B cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin, 0.25 μg/ml of amphotericin B, 4 mM glutamine, and 1 mM pyruvate at 37 °C in a humidified atmosphere (5% CO₂). For transient transfection we used a pCMV6-XL5-IF₁ expression plasmid transfected with an alternatively single shRNA vector or a scrambled negative control construct cloned in a pGFP-V-RS plasmid (#1 Gi325933, #2 Gi325934, #3 Gi325935, and #4 Gi325936 and TR30013, respectively). All plasmids used were from Origene (Rockville, MD). Equal amounts of the two vectors (4 μg total DNA) were transfected overnight using polyethylenimine (PEI). After 24 h the medium was replaced with complete DMEM and the cells were cultured for a further 24 h before analyzing the level of IF₁ silencing. To establish stable clones, parental 143B cells were seeded and transfected as described above with 2 μg of either Gi325936 or TR30013 plasmid DNA. 48 h later, cells were split and selected for stable transformation in the presence of 1 μg/ml of puromycin, changing the medium every day. Single colonies were then subcloned by limiting dilution and finally all the clones obtained were assayed for IF₁ expression.

Mitochondrial Isolation—Coupled mitochondria were isolated from cells according to the method by Kun et al. (20), modified to exclude digitonin treatment. Essentially, cell homogenates were obtained using a glass Potter-Helvehjem homogenizer with a motor-driven Teflon pestle in isolation buffer (0.22M mannitol, 0.07M sucrose, 0.02M HEPES, 1 mM phenylmethanesulfonyl fluoride). Crude extracts were centrifuged at 2000 × g for 10 min (Sorvall SS34 rotor) to remove nuclei and plasma membrane fragments, and then the supernatant was centrifuged at 10,000 [times] g for 10 min (Sorvall SS34 rotor) to obtain the mitochondrial pellet. Mitochondria were washed in 0.25 mM sucrose, 0.02 mM HEPES, 1 mM K-EDTA, and 0.1 mM K-EGTA, pH 7.4, and resuspended in the same buffer to a concentration of ~10 mg/ml of protein.

Cell Growth—Cell growth was assessed after seeding 2 × 10⁵ cells in complete DMEM and culturing the cells for up to 72 h. Adherent cells were trypsinized and collected, and the growth of cell lines was assayed using the trypan blue exclusion test. Cell count was performed every 24 h without changing the medium.

SDS-PAGE and Western Blot Analysis—Osteosarcoma cellular lysates were separated by SDS-PAGE and blotted onto nitrocellulose membranes to perform semiquantitative analysis of protein content according to Baracca et al. (21). Blots of resolved proteins were incubated either with primary mouse monoclonal antibodies specific for the d-subunit of F₁Fₒ-ATPase (19 kDa) and for IF₁ (12 kDa) or with a mixture of five primary mouse monoclonal antibodies specific for single subunits of each OXPHOS complex (MitoSciences Inc., Eugene, OR) as reported in Sgarbi et al. (22). Actin (42 kDa) and porin (35 kDa), used as loading controls for cells and mitochondria, respectively, were immunodetected with mouse monoclonal anti-actin (Sigma) and anti-porin (Mito-Sciences Inc., Eugene, OR) primary antibodies. Immunodetection of primary antibody was carried out with secondary goat anti-mouse IgG₄ antibody (Invitrogen) labeled with horseradish peroxidase. Chemiluminescent detection of the specific proteins was performed with the ECL Western blotting Detection Reagent Kit (GE Healthcare, Waukesha, WI) using the ChemiDoc MP system equipped with the ImageLab software (Bio-Rad) to perform the densitometric scanning of the relative protein intensity.

BN-PAGE Analysis and Western Blot Analysis—The organization of the ATP synthase complex and the binding of IF₁ to the monomeric and/or oligomeric form of the enzyme were analyzed in digitonin-treated mitochondria (2.5:1 (w/w) digitonin:protein ratio) by one-dimensional blue native-PAGE (23). Following electrophoresis under non-denaturating conditions, proteins were immediately electrophoresed onto nitrocellulose membranes under denaturing conditions. ATP synthase and IF₁ protein bands were detected using anti-α subunit and anti-IF₁ primary monoclonal antibodies (MitoSciences Inc.), respectively, and a secondary goat anti-mouse IgG₄ antibody labeled with horseradish peroxidase (Invitrogen). The immunoblots were detected and quantified by chemiluminescence using the ECL Western Blotting Detection Reagent Kit (Amer sham Biosciences).

In-gel ATPase Activity—Immediately after the electrophoretic run of the protein complexes extracted from digitonin-treated mitochondria, ATPase activity was assayed on the native gel using an enzymatic colorimetric method (24). White-stained ATP synthase bands were acquired using a GS-800 densitometer (Bio-Rad) with a blue filter to minimize the interference from the residual Coomassie Blue.

Flow Cytometric Assessment—Flow cytometry of GFP positive cells was performed using a FACSaria cytometer (BD Biosciences). Excitation was at 488 nm and fluorescence emission was measured at 530/30 nm. Data acquisition and analysis was performed with BD FACS Diva and Flowing Software, respectively.

The inner mitochondrial membrane potential was measured by staining cells with 20 nm tetramethylrhodamine methyl ester (TMRM) (Molecular Probes, Eugene, OR), a lipophilic probe that enters mitochondria in a Δψₒ⁻ dependent manner (25). The cells were incubated with the probe for 30 min at 37 °C in the absence or presence of 0.6 μM oligomycin and wells were then washed twice with Hanks’ balanced salt solution to remove any remaining unincorporated dye. The cells were rapidly
**RESULTS**

**shRNA-mediated Stable Silencing of the IF₁, Inhibitor Protein in Osteosarcoma 143B Cells**—To obtain stable suppression of the IF₁ inhibitor protein in human osteosarcoma 143B cells, which are cancer cells expressing IF₁, to a high extent (data not shown), we screened four IF₁ gene-specific shRNA expression plasmids (Fig. 1A) to identify the 29-mer shRNA sequence with the most powerful IF₁ silencing competence (Fig. 1, A and C). The transient cotransfection of the cells with the IF₁ expression plasmid together with each shRNA construct, resulted in different efficiency of silencing, and the maximal reduction of IF₁ expression (about ~70%) was observed using plasmid #4 (Fig. 1B). To set up the stable transfection, the latter vector (Fig. 1C) and a scrambled plasmid were used. After 24 h of culture, the morphology of cells transfected with either the scrambled or #4 plasmid was similar and the transfection efficiency was about 30% as estimated on the basis of the GFP positive cells, GFP being encoded by each shRNA plasmid (Fig. 1D). Following puromycin selection and subcloning, several clones were obtained for both scrambled and shRNA #4 plasmids. Immunodetection of IF₁ protein bands obtained by SDS-PAGE and Western blotting showed a remarkable suppression of IF₁ expression in all the silenced clones (Fig. 2A). Densitometric analysis of the bands revealed that clones A7 and D9 expressed the least IF₁ content (6 and 9% of the controls, respectively). The morphology of the IF₁-silenced and scrambled clones was similar to that of the parental cells (Fig. 2B, upper panels). Transfection stability and cell homogeneity of IF₁-silenced and scrambled clones were evaluated by both fluorescence microscopy (Fig. 2B, lower panels) and flow cytometry (Fig. 2C). Nearly all cells (99%) were found to be positive for GFP expression. Moreover, the morphology and adhesion capacity of IF₁-silenced and control cells appeared indistinguishable, as shown in the brightfield microscopy images (Fig. 2B, upper panels).

**IF₁ Silencing Does Not Affect the Viability and Proliferation Rate of Osteosarcoma Cells, nor the Main Metabolic Parameters**—We first analyzed and compared the growth rate of two IF₁-silenced clones with both parental and scrambled human osteosarcoma cells. The results shown in Fig. 3A indicate that under normal growth conditions the two IF₁-silenced clones grow and proliferate similarly to both the parental cell line and the scrambled clone. In addition, no difference was observed between IF₁ expressing or non-expressing cells in glucose consumption and lactate release after 24 h of cells growth. Incidentally, the lactate release to glucose consumption ratio was approximately two (Fig. 3, B and C). Finally, the steady-state level of cellular ATP was about 25 nmol/mg of protein in all cells analyzed, independently of IF₁ content (Fig. 3D). On the basis of earlier studies on both isolated mitochondria and sub-mitochondrial particles (30, 31), the above results were expected because cells were grown under conditions that did not favor binding and inhibition of IF₁ to the F₁F₀-ATPase complex. Indeed, most of the above data agree with those recently reported by Fujikawa et al. (32), who analyzed the only stable IF₁-depleted cell line (HeLa cells) as yet studied. However, to clarify the conflicting hypothesis on the role of IF₁ in cancer cell bioenergetics, the activity of both the respiratory particles (30, 31), the above results were expected because cells were grown under conditions that did not favor binding and inhibition of IF₁ to the F₁F₀-ATPase complex. Indeed, most of the above data agree with those recently reported by Fujikawa et al. (32), who analyzed the only stable IF₁-depleted cell line (HeLa cells) as yet studied. However, to clarify the conflicting hypothesis on the role of IF₁ in cancer cell bioenergetics, the activity of both the respiratory
chain and the F$_1$F$_0$-ATPase complex was analyzed in both parental and IF$_1$-silenced osteosarcoma cells.

**Bioenergetic Changes in IF$_1$-silenced Osteosarcoma Cells**—Some studies reported that overexpression of IF$_1$ in cells promotes tumor progression by either reducing mitochondrial ATP synthase activity and increasing aerobic glycolysis (4), or inducing a decrease that is associated with enhanced proneness to apoptotic cell death by cytochrome c release from mitochondria (19, 33). In addition, Fujikawa et al. (32) reported a nearly 40% increase in IF$_1$ null cells and proposed that it was the result of an increased ATP hydrolytic activity of the F$_1$F$_0$-ATPase complex under normoxic conditions. To address these conflicting issues, we first evaluated the respiratory chain activity by assaying the oxygen consumption rate in digitonin-permeabilized cells, and found that removal of IF$_1$ from cells caused a modest (about 20%) but reproducible decrease in the ADP-induced respiration rate (state 3) (Fig. 4, A and B). At variance, no difference was observed in both state 4 and dinitrophenol uncoupled respiration rate in IF$_1$-silenced and control cells (Fig. 4, C and D). Overall, these data indicate that the presence of the inhibitor protein in osteosarcoma 143B cells can enhance the rate of ATP synthesis via OXPHOS, and suggest that the absence of IF$_1$ has no effect on respiratory chain activity. In addition, no significant difference was observed in the level of OXPHOS complexes between IF$_1$-silenced and control cells (Fig. 5). On the basis of earlier studies on perfused hearts (11), this result was quite unexpected because the conditions to which the cells were exposed (normoxia and slightly basic pH) should not favor the binding of IF$_1$ to the ATP synthase complex (34, 35). On the other hand, IF$_1$ has been regarded as an unidirectional inhibitor of ATP hydrolysis without effects on ATP synthesis (36) at least under steady-state conditions (37). To verify whether the ATP hydrolysis rate of the ATP synthase complex showed similar behavior to those earlier reported, we tested the ATPase activity of our set of cells. Because in cells this assay cannot give reliable results due to the presence of many ATP hydrolyzing enzymes and technical restrictions, the ATP hydrolytic activity of the ATP synthase was assayed in isolated uncoupled mitochondria (Fig. 6). As expected, the oligomycin-sensitive ATPase activity of mitochondria was pH sensitive (31, 38), being at pH 6.7 about half than at pH 7.4. As expected and in accord with previous data obtained from organ extracts and the isolated enzyme, the oligomycin-sensitive ATPase activity of mitochondria from IF$_1$-silenced cells resulted in being 2–3– and 4–5-fold higher than in controls at pH 7.4 and 6.7, respectively. Incidentally, the
latter data match those reported by Cabezon et al. (35) analyzing the pH dependence of the isolated IF1-F1-ATPase complex activity.

Mitochondrial Membrane Potential of IF1-silenced Cells Is Higher Compared with Parental Cells—Given the different bioenergetic behavior of IF1-silenced cells compared with controls, we measured the Δψm of all cell types by examining the fluorescence of TMRM-loaded cells. Fig. 7A shows typical images of the four types of cells observed by fluorescence microscopy. The fluorescence intensity of IF1-silenced cells was higher than controls, indicating a higher endogenous Δψm. These results were assessed and quantified by flow cytometry.
and representative cell fluorescence distribution is reported in Fig. 7B. The mean fluorescence values of IF1-silenced cells was nearly 30% higher than in control cells, and no difference was observed between IF1-silenced clones and controls upon the addition of oligomycin (Fig. 7, B and C), indicating that the endogenous steady-state \( \Delta \psi_m \) of the IF1-silenced cells is significantly increased compared with controls, but is still far below its maximum (i.e. when cells are exposed to oligomycin). Interestingly, the mitochondrial mass of all the cell types was similar, as determined by the citrate synthase assay (Fig. 7D). These unexpected, but somehow intriguing results pushed us to analyze the ATP synthase oligomers directly from cells (data not shown). Noteworthy, IF1 was bound to the F\(_1\)F\(_0\)-ATPase complex and, eventually to which form of the enzyme (i.e. monomeric and/or oligomeric).

The Inhibitor Protein IF1 Binds to the ATP Synthase Complex in Its Dimeric Form—The ATP synthase complex has been shown to be present in dimers in mitochondria from several species (see Ref. 39 for a recent review). The choice and quality of the detergent and the conditions used to solubilize the ATP synthase complex is critical if oligomerization of the enzyme has to be studied (40). Indeed, on the basis of an empirical evaluation, to disaggregate the inner mitochondrial membrane, we chose a 2.5:1 (w/w) digitonin:protein ratio (Fig. 8A), and results were also confirmed when digitonin was used to extract the ATP synthase oligomers directly from cells (data not shown). Noteworthy, IF1 binds exclusively to the ATP synthase complexes even when a higher digitonin:protein ratio is used to extract proteins from mitochondria (Fig. 8B).

Fig. 9A shows the in-gel ATPase activity of the F\(_1\)F\(_0\)-ATPase complex following blue native-gel electrophoretic separation of the digitonin-extracted proteins. It is clearly seen that both monomers and dimers are ATP hydrolysis competent. The blotted ATP synthase protein bands were immunodetected and quantified using an \( \alpha \)-subunit monoclonal antibody (Fig. 9B): the ATP synthase complex from all different cells distribute similarly between the monomeric (53–58%) and dimeric (42–47%) forms, as quantitatively shown in Fig. 9D. This occurred independently of the presence of IF1, as confirmed in Fig. 9C. Indeed, immunodetection of IF1 clearly indicates that the main target of IF1 is the dimeric form of the ATP synthase in controls, and, as expected, no band was observed in IF1-silenced cells.

**DISCUSSION**

The main findings of the present study are: first, IF1 does not affect the mitochondrial volume nor the level of dimeric F\(_1\)F\(_0\)-ATPase in human osteosarcoma cells; second, IF1-silenced cells have a reproducibly higher \( \Delta \psi_m \) associated with a lower respiration rate than parental cells; and third, IF1 has a higher affinity for the dimeric form of ATP synthase than the monomeric form. Moreover, we report the method to prepare stable IF1-silenced clones from human osteosarcoma cells. Indeed, a brief comment on this point is that the transient transfection efficiency of osteosarcoma cells by using either the scrambled or the #4 plasmid was about 30% and upon puromycin selection and subcloning, stable transfected clones were obtained. The IF1 content of most of the silenced clones was found to be strongly reduced (90–95%) compared with parental cells and the silencing is still present after two years of cells cycling.

An intriguing paper (41) recently published reports that the mitochondrial volume of HeLa cells is regulated by IF1. This work was based on the analysis of cells in which IF1 was transiently either overexpressed or silenced: in the former case a higher mitochondrial volume was described and the lower mitochondrial volume found in the presence of reduced IF1 level was ascribed to enhanced autophagy compared with controls. However, Fujikawa et al. (32) did not observe any difference between IF1 knockdown and control HeLa cells. Accordingly, in the present study, the mitochondrial content of
osteosarcoma cells was found unchanged in IF₁-silenced clones and in controls, as assessed by measuring citrate synthase activity and by comparing the porin content of cells following electrophoretic separation of proteins by SDS-PAGE and immunoblotting (Figs. 2A and 5).

This investigation clarifies previous conflicting data concerning the difference between the main bioenergetic parameters of human cancer cells containing high levels of IF₁ protein and stable null IF₁ clones. Under normal conditions (i.e. normoxia) in which the inhibitor protein is not supposed to inhibit the ATP hydrolytic activity of the enzyme, the silenced IF₁ cells exhibited the same growth, glucose consumption, and steady-state ATP levels as control cells. These results were expected on the basis of earlier studies mostly obtained analyzing organs in perfusion (11) or isolated mitochondria from heart (7) or liver (42) exposed to ischemic conditions. However, the IF₁-silenced osteosarcoma cells also showed some intriguing features, including: reduced state 3 respiration rate, enhanced Δψᵢ, and similar presence of dimeric ATP synthase compared with controls, the latter implying that IF₁ does not contribute to inducing ATP synthase dimerization.

In the last decade, this issue has been addressed in a number of studies by means of different approaches and techniques. García et al. (14) studied different higher eukaryotic cells and proposed that IF₁ promotes dimerization of the ATP synthase. Accordingly, Campanella et al. (19) reported that IF₁ transiently overexpressed in HeLa cells enhanced the dimer:monomer ATP synthase ratio. However, the dimer:monomer ratio of the current study carried out in permanently IF₁-silenced clones definitely shows that it is independent of the IF₁ presence as previously proposed by Tomasetig et al. (43), in bovine heart mitochondria.

The increased Δψᵢ of IF₁-silenced osteosarcoma 143B cells studied in the present work matches the results recently...
reported analyzing stable IF₁-silenced HeLa cells (32), but our data are in contrast with those reported by Sanchez-Cenizo et al. (4), who found increased Δψₘ in different carcinoma cell lines overexpressing IF₁. We believe that the latter result may be only apparent due to the transient overexpression of IF₁ that results in heterogeneous population of cells carrying different IF₁ expression levels. However, we cannot exclude that different types of cancer cells as used in Ref. 4 behave differently from HeLa (19, 32) and osteosarcoma cells.

According to Fujikawa et al. (32), the significantly higher steady-state Δψₘ level found in IF₁-silenced cells than in controls was somewhat expected because it might be due to the presence of F₁,F₀-ATPase complexes that reverse the physiological activity and hydrolyze ATP in an uncontrolled manner. Therefore, more ATP hydrolysis occurs in the absence of IF₁ than in its presence, setting a higher steady-state Δψₘ. Never-
The inhibitor protein does not affect the dimerization of ATP synthase. A, representative in-gel activity staining of the monomeric and oligomeric ATP synthase forms extracted from digitonin-treated mitochondria of parental, scrambled, and IF1-depleted cells and separated by BN-PAGE. B, monomeric and oligomeric distribution analysis of the ATP synthase performed by immunodetection of the F1α-subunit after separation of the proteins by BN-PAGE, followed by blotting of the native complexes onto nitrocellulose membrane. C, immunoblot analysis of the IF1 binding to the ATP synthase complexes. The same amount of protein used in panels A and B were loaded. D, histograms represent the densitometric analysis of the monomer (dark bar) and dimer (gray bar) of the ATP synthase complex immunodetected and shown in panel B. The representative data were confirmed in three independent experiments.

Nevertheless, this hypothesis is not supported by some of our results, including the same glucose consumption and the same steady-state ATP level measured in IF1-silenced and control cells. Moreover, we observed that the Δψm of both IF1-silenced and control cells increased and reached the same level when exposed to 0.6 μM oligomycin (Fig. 7, B and C), a concentration capable of selectively inhibiting ATP synthase in cells (44). According to a recent report (45), when ATP hydrolysis significantly contributes to the maintenance of Δψm, the addition of oligomycin results in a Δψm collapse even in the presence of IF1. The observed higher Δψm shown by IF1-silenced cells compared with controls appears somehow intriguing because in normoxia IF1 should not affect F1F0-ATPase unless uncoupled conditions are considered (32, 46), therefore the enhanced Δψm seen in IF1-silenced cells deserves a different explanation.

First, it has been reported that besides binding the β subunit of F1, IF1 can bind another protein of low Mr of the inner mitochondrial membrane (47) and this might lead to Δψm enhancement in respiring mitochondria. This hypothesis does not seem acceptable because native PAGE analysis in our hands never showed the inhibitor protein in bands other than the IF1-ATP synthase complexes (Fig. 8B). A second possibility might originate from a recent study showing that dimers of the F1F0-ATPase incorporated into lipid bilayers form channels with the key features of the mitochondrial permeability transition pore (48). Indeed, one might speculate that the absence of IF1 could dysregulate the opening of the pore, resulting in a steady-state Δψm higher than in controls. Finally, it could be that the lack of IF1 decreases the dimeric form of the ATP synthase complex (19), and this might reduce the enzyme activity that in turn results in a higher Δψm in OXPHOS competent mitochondria. However, this is not the case because the distribution of ATP synthase between the dimeric and monomeric forms was found to be similar in IF1-silenced and control cells (Fig. 9B). If we now recall that the respiration rate under ADP phosphorylation conditions was higher in controls than in IF1-silenced cells, our data altogether suggest that IF1 can enhance the ATP synthesis rate via OXPHOS. This might occur through a direct interaction of IF1 with the ATP synthase at the αβ interface, as it has been proposed to occur as the first step of the inhibitory pathway described by Bason et al. (10), or IF1 might bind the ATP synthase complex at a different site that, however, should favor catalysis by enhancing the rate of ATP release once it has been formed (49). Finally, another possibility that we are at present exploring, is that the binding of IF1 to ATP synthase contributes to the modeling of mitochondrial cristae, as suggested by Campanella et al. (19), and this could in turn increase the OXPHOS rate.

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REFERENCES

1. Hanahan, D., and Weinberg, R. A. (2011) Hallmarks of cancer: the next generation. Cell 144, 646–674
2. Frezza, C., and Gottlieb, E. (2009) Mitochondria in cancer: not just innocent bystanders. Semin. Cancer Biol. 19, 4–11
3. Moreno-Sánchez, R., Rodríguez-Enríquez, S., Marín-Hernández, A., and Saavedra, E. (2007) Energy metabolism in tumor cells. FEBS J. 274, 1393–1418
4. Sánchez-Cenizo, L., Formentini, L., Aldea, M., Ortega, A. D., García-Huerta, P., Sánchez-Aragó, M., and Cuevas, J. M. (2010) Up-regulation of the ATPase inhibitory factor 1 (IF1) of the mitochondrial H+ -ATP synthase in human tumors mediates the metabolic shift of cancer cells to a Warburg phenotype. J. Biol. Chem. 285, 25308–25313
5. Ichikawa, N., Ushida, S., Kawabata, M., and Masazumi, Y. (1999) Nucleotide sequence of cDNA coding the mitochondrial precursor protein of
the ATPase inhibitor from humans. *Biosci. Biotechnol. Biochem.* **63**, 2225–2227

6. Harris, D. A., and Das, A. M. (1991) Control of mitochondrial ATP synthesis in the heart. *Biochem. J.* **280**, 561–573

7. Rouslin, W. (1991) Regulation of the mitochondrial ATPase in situ in cardiac muscle: role of the inhibitor subunit. *J. Bioenerg. Biomembr.* **23**, 873–888

8. Di Lisa, F., and Bernardi, P. (1998) Mitochondrial function as a determinant of recovery or death in cell response to injury. *Mol. Cell. Biochem.* **184**, 379–391

9. Solaini, G., and Harris, D. A. (2005) Biochemical dysfunction in heart mitochondria exposed to ischaemia and reperfusion. *Biochem. J.* **390**, 377–394

10. Bason, J. V., Montgomery, M. G., Leslie, A. G., and Walker, J. E. (2014) Pathway of binding of the intrinsically disordered mitochondrial inhibitor protein to F1-ATPase. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 11305–11310

11. Jennings, R. B., Reimer, K. A., and Steenbergen, C. (1991) Effect of inhibition of the mitochondrial ATPase on net myocardial ATP in total ischaemia. *J. Mol. Cell. Cardiol.* **23**, 1383–1395

12. Bosetti, F., Yu, G., Zucchi, R., Ronca-Testoni, S., and Solaini, G. (2000) Myocardial ischemic preconditioning and mitochondrial F1F0-ATPase activity. *Mol. Cell. Biochem.* **215**, 31–37

13. Formentini, L., Pereira, M. P., Sánchez-Cenizo, L., Santacatterina, F., Lucas, J. J., Navarro, C., Martínez-Serrano, A., and Acevea, J. M. (2014) In vivo inhibition of the mitochondrial H+/ATP synthase in neurons promotes metabolic preconditioning. *EMBO J.* **33**, 762–778

14. García, J. J., Morales-Ríos, E., Cortés-Hernandez, P., and Rodríguez-Zava, J. S. (2006) The inhibitor protein (IF1) promotes dimerization of the mitochondrial F1F0-ATP synthase. *Biochemistry* **45**, 12695–12703

15. Strauss, M., Hofhaus, G., Schröder, R. R., and Kühlbrandt, W. (2008) Dimer ribbons of ATP synthase shape the inner mitochondrial membrane. *EMBO J.* **27**, 1154–1160

16. Lucciaková, K., and Kuzela, S. (1984) Increased content of natural ATPase in liver mitochondria exposed to ischaemia and reperfusion. *Biochem. J.* **296–310

17. Borsi, E., Perrone, G., Terragna, C., Martello, M., Dico, A. F., Solaini, G., Baracca, A., Sgarbi, G., Pasquinelli, G., Valente, S., Zamagni, E., Tacchetti, P., Martinelli, G., and Cavo, M. (2014) Hypoxia inducible factor-1α as a therapeutic target in multiple myeloma. *Onco Targets Ther.* **5**, 1779–1792

26. Baracca, A., Sgarbi, G., Padula, A., and Solaini, G. (2013) Glucose plays a main role in human fibroblasts adaptation to hypoxia. *Int. J. Biochem. Cell Biol.* **45**, 1356–1365

27. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. I. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275

28. Sgarbi, G., Giannone, F., Casalena, G. A., Baracca, A., Baldassare, M., Longobardi, P., Caraceni, P., Derenzini, M., Lenaz, G., Trérè, D., and Solaini, G. (2011) Hypoxia fully protects mitochondria of explanted livers. *J. Bioenerg. Biomembr.* **43**, 673–682

29. Solaini, G., Baracca, A., Parenti Castelli, G., and Strambini, G. B. (1993) Tryptophan phosphorescence as a structural probe of mitochondrial F1-ATPase ε-subunit. *Eur. J. Biochem.* **214**, 729–734

30. Barogi, S., Baracca, A., Parenti Castelli, G., Bovina, C., Formiggini, G., Marchetti, M., Solaini, G., and Lenaz, G. (1995) Lack of major changes in ATPase activity in mitochondria from liver, heart, and skeletal muscle of rats upon ageing. *Mech. Ageing Dev.* **84**, 139–150

31. Solaini, G., Baracca, A., Gabellieri, E., and Lenaz, G. (1997) Modification of the mitochondrial F1F0-ATPase epsilon subunit, enhancement of the ATPase activity of the IFα-IFβ complex and IF1-binding dependence of the conformation of the ε subunit. *Biochem. J.* **327**, 443–448

32. Fujikawa, M., Ishihara, H., Nakamura, J., and Yoshida, M. (2012) Assessing actual contribution of IF1, inhibitor of mitochondrial F1F0, to ATP homeostasis, cell growth, mitochondrial morphology, and cell viability. *J. Biol. Chem.* **287**, 18781–18787

33. Faccenda, D., Tan, C. H., Duchen, M. R., and Campanella, M. (2013) Mitochondrial IF1 preserves cristae structure to limit apoptotic cell death signaling. *Cell Cycle* **12**, 2530–2532

34. Lippe, G., Sorgato, M. C., and Harris, D. A. (1988) The binding and release of the inhibitor protein are governed independently by ATP and membrane potential in ox-heart submitochondrial vesicles. *Biochim. Biophys. Acta* **933**, 1–11

35. Cabezón, E., Butler, P. J., Runswick, M. J., and Walker, J. E. (2000) Modulation of the oligomiserization state of the bovine F1-ATPase inhibitor protein, IF1, by pH. *J. Biol. Chem.* **275**, 25460–25464

36. Horstman, L. L., and Racker, E. (1970) Partial resolution of the enzyme catalyzing oxidative phosphorylation: XXII. interaction between mitochondrial adenosine triphosphatase inhibitor and mitochondrial adenosine triphosphatase. *J. Biol. Chem.* **245**, 1336–1344

37. Husain, I., Jackson, P. J., and Harris, D. A. (1985) Interaction between F1-ATPase and its naturally occurring inhibitor protein. Studies using a specific anti-inhibitor antibody. *Biochim. Biophys. Acta* **806**, 64–74

38. Baracca, A., Degli Esposti, M., Parenti Castelli, G., and Solaini, G. (1992) Purification and characterization of adenosine triphosphatase from eel liver mitochondria. *Comp. Biochem. Physiol.* **101B**, 421–426

39. Chaban, Y., Boekema, E. J., Dukindka, N. V. (2014) Structures of mitochondrial oxidative phosphorylation supercomplexes and mechanisms for their stabilisation. *Biochim. Biophys. Acta* **1837**, 418–426

40. Bisetto, E., Comelli, M., Salzano, A. M., Picotti, P., Scaloni, A., Lippe, G., and Mavelli, I. (2013) Proteomic analysis of F0F1-ATP synthase superassembly in mitochondria of cardiomyoblasts undergoing differentiation to the cardiac lineage. *Biochim. Biophys. Acta* **1827**, 807–816

41. Campanella, M., Seraphin, A., Abeti, R., Casswell, E., Echave, P., and Duchen, M. R. (2009) IF1, the endogenous regulator of the F1F0-ATP synthase, defines mitochondrial volume fraction in HeLa cells by regulating autophagy. *Biochim. Biophys. Acta* **1787**, 393–401

42. Schwerzmann, L. K., and Pedersen, P. L. (1981) Proton–adenosinetriphosphatase complex of rat liver mitochondria: effect of energy state on its interaction with the adenosinetriphosphatase inhibitory peptide. *Biochemistry* **20**, 6305–6311

43. Tomasetig, L., Di Pancrazio, F., Harris, D. A., Mavelli, I., and Lippe G. (2009) Tryptophan phosphorescence as a structural probe of mitochondrial F1-ATPase ε-subunit. *Eur. J. Biochem.* **214**, 729–734

44. Solaini, G., Harris, D. A., Lenaz, G., Sgarbi, G., and Baracca, A. (2008) The study of the pathogenic mechanism of mitochondrial diseases provides information on basic bioenergetics. *Biochim. Biophys. Acta* **1777**, 941–945
45. Porcelli, A. M., Angelin, A., Ghelli, A., Mariani, E., Martinuzzi, A., Carelli, V., Petronilli, V., Bernardi, P., and Rugolo, M. (2009) Respiratory complex I dysfunction due to mitochondrial DNA mutations shifts the voltage threshold for opening of the permeability transition pore toward resting levels. *J. Biol. Chem.* **284**, 2045–2052

46. Walker, J. E. (2013) The ATP synthase: the understood, the uncertain and the unknown. *Biochem. Soc. Trans.* **41**, 1–16

47. Lopez-Mediavilla, C., Vigny, H., and Godinot, C. (1993) Docking the mitochondrial inhibitor protein IF1 to a membrane receptor different from the F$_1$-ATPase β subunit. *Eur. J. Biochem.* **215**, 487–496

48. Giorgio, V., von Stockum, S., Antoniel, M., Fabbro, A., Fogolari, F., Forte, M., Glick, G. D., Petronilli, V., Zoratti, M., Szabó, I., Lippe, G., and Bernardi, P. (2013) Dimers of mitochondrial ATP synthase form the permeability transition pore. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 5887–5892

49. Boyer, P. D. (1997) The ATP synthase: a splendid molecular machine. *Annu. Rev. Biochem.* **66**, 717–749