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The pro-oncogenic protein IF_1 does not contribute to the Warburg effect and is not regulated by PKA in cancer cells

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ABSTRACT

The endogenous inhibitor of mitochondrial F_1F_o-ATPase (ATP synthase), IF_1, has been shown to exert pro-oncogenic actions, including reprogramming of cellular energy metabolism (Warburg effect). The latter action of IF_1 has been reported to be hampered by its PKA-dependent phosphorylation, but both reprogramming of metabolism and PKA-dependent phosphorylation are intensely debated. To clarify these critical issues, we prepared stably IF_1-silenced clones and compared their bioenergetics with that of the three parental IF_1-expressing cancer cell lines. All functional parameters: respiration rate, ATP synthesis rate (OXPHOS), and mitochondrial membrane potential were similar in IF_1-silenced and control cells, clearly indicating that IF_1 cannot inhibit the ATP synthase in cancer cells when the enzyme works physiologically. Furthermore, all cell types exposed to PKA modulators and energized with NAD^+-dependent substrates or succinate showed similar OXPHOS rate regardless of the presence or absence of IF_1. Therefore, our results rule out that IF_1 action is modulated by its PKA-dependent phosphorylated/dephosphorylated state. Notably, cells exposed to a negative PKA modulator and energized with NAD^+-dependent substrates showed a significant decrease of the OXPHOS rate matching previously reported inactivation of complex I. Overall, this study definitively demonstrates that IF_1 inhibits neither mitochondrial ATP synthase nor OXPHOS in normoxic cancer cells and does not contribute to the Warburg effect. Thus, currently the protection of cancer cells from severe hypoxia/anoxia and apoptosis remain the only unquestionable actions of IF_1 as pro-oncogenic factor that may be exploited to develop therapeutic approaches.

1. Introduction

Mitochondria metabolism and oxidative phosphorylation (OXPHOS) play central roles in cancer development, growth and metastasis [1–6]. Therefore, both the F_1F_o-ATPase complex (ATP synthase or H^+-ATPase) and its regulation is critical to cancer cells. The main endogenous regulator of the enzyme is the inhibitor protein IF_1, also called "ATPase inhibitory factor 1", firstly isolated from bovine heart mitochondria by Pullman and Monroy in 1963 [7]. IF_1 was found in all eukaryotic cells and it is characterized by high homology among species, particularly in mammals (see Fig. S1). This 84-amino acids bovine protein (81 in humans) was structurally and functionally characterized in mammalian cells over the following 50 years [8–13] and it was shown to be over-expressed in many cancers [14–17]. In aqueous solutions and in cells under physiological conditions it is present in the form of monomers, dimers and tetramers, in equilibrium with each other and the level of

Abbreviations: 143B, human 143B osteosarcoma cells; ATP synthase, mitochondrial F_1F_o-ATPase (complex V, H^+-ATPase); complex I, NADH:ubiquinone oxidoreductase (NADH dehydrogenase); complex II, succinate-ubiquinone oxidoreductase (succinate dehydrogenase); CS, cytrate synthase; db-cAMP, dibutyryl cyclic-AMP sodium salt; DMEM, Dulbecco's Modified Eagle Medium; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; FCCP, trifluoromethoxy carbonylcyanide phenylhydrazone; H89, H-89 dihydrochloride hydrate; HBSS, Hank's Balanced Salt Solution; HCT116, human colorectal carcinoma cells; HeLa, human cervix adenocarcinoma cells; IF_1, inhibitor factor 1, the endogenous inhibitor of the F_1F_o-ATPase complex; OCR, oxygen consumption rate; OSR, oligomycin-sensitive respiration; OXPHOS, oxidative phosphorylation; PKA, cAMP-dependent protein kinase; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TMRM, tetramethylrhodamine methyl ester; TOM20, translocase of outer mitochondrial membrane 20; $\Delta\mu_{H^+}$, mitochondrial electrochemical gradient; $\Delta\psi_m$, mitochondrial membrane potential..

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each form depends on both the pH and the ionic composition of the solution [18,19]. When cells experience anoxic or near-anoxic conditions as occurs in cardiomyocytes during ischemia, F_1F_o-ATPase works in reverse, as the mitochondrial electrochemical gradient ($\Delta\mu_{H+}$) is greatly decreased. The level of dimeric IF_1 (i.e. active form of IF_1, see Fig. 1) increases, and it binds to the catalytic domain of the complex, F_1, inhibiting its hydrolytic activity [see [20] for an extensive review]. The ATP produced by glycolysis in the cytosol is partially saved and mitochondria depolarization is avoided, preventing rapid cell death. In cancer cells IF_1 exerts an action qualitatively similar to that in cardiomyocytes, prolonging the survival period of poorly oxygenated or anoxic cells within the solid tumors [21], possibly allowing the development of the vascular system within the tumor mass [22]. This might provide a critical advantage to the cancer survival and proliferation. Furthermore, under conditions of severe hypoxia or anoxia the presence of IF_1 in cancer cells, where IF_1 can completely inhibit the hydrolytic activity of ATP synthase, further decreases the level of reactive oxygen species (ROS) compared to normoxia [23]. Indeed, as we have previously observed, hypoxia significantly decreases the level of ROS in both normal and transformed cells [23,24].

Besides these protective actions, IF_1 has also been shown to be important in the prevention of cristae remodeling and completion of apoptosis, by stabilizing the dynamin-related optic atrophy 1 (OPA1) pro-fusion protein [25]. Moreover, a very recent work proposes IF_1 as a modulator of the mitochondrial Ca^{2+} uniporter expression, contributing to preserve the intracellular Ca^{2+} homeostasis [26], which in turn allows to exert a good control of PTP opening and induction of apoptosis. Other investigations have identified a slight enhancement of ATP synthase activity in some cancer cell lines [27,28]. This effect has been attributed to the improvement of the inner membrane structure [27] or to a binding of IF_1 to the enzyme at a site other than that involved in the inhibition of the F_1F_o-ATPase complex working in reverse, which has been clearly identified [29,30]. In addition to the above actions, ATPase inhibitor has been proposed to promote the metabolic shift of cancer cells towards a Warburg phenotype by inhibiting the ATP synthesis of the F_1F_o-ATPase complex [15]. This appeared to be an interesting point even if the experiments found no support in other studies on the ATP synthase and its modulators [27–29,31]. A few years later, the same authors reported that IF_1 is subjected to a post-translational regulation by phosphorylation and that only the dephosphorylated form of IF_1 could inhibit the ATP synthase activity [32]. They showed that IF_1 was phosphorylated in Ser-39 (i.e. Ser-14 in the mature protein, Fig. S1) and that it is phosphorylated by a mitochondrial cAMP-dependent protein kinase (PKA). The authors suggested that phosphorylation rendered IF_1 unable to bind to the ATP synthase, thus inactivating IF_1, while dephosphorylation of IF_1 allowed it to bind to the ATP synthase. The covalent regulation of IF_1 seems interesting, but neither the site of IF_1 binding to the enzyme nor its mechanism have been proposed, so the issues are still deeply debated [33,34].

The wide interest of our laboratory on the role of IF_1 both in biology

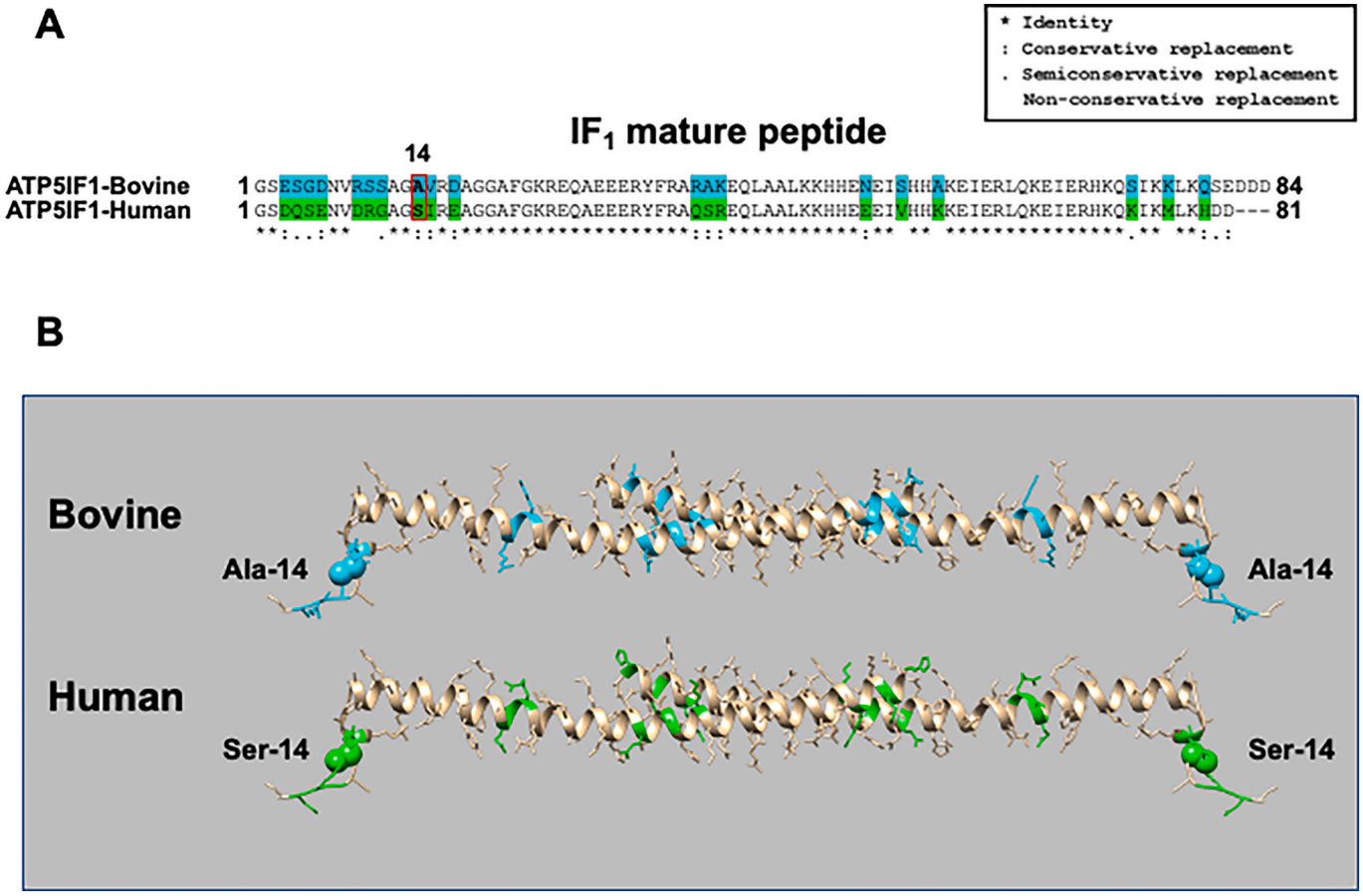

Fig. 1. Protein sequence alignment of bovine and human IF_1.
(A) Pairwise alignment of the complete sequence of bovine (UniprotKB - P01096) and human (UniprotKB - Q9UII2) IF_1 protein, performed by the Clustal omega program. Amino acid substitutions between bovine and human sequences are highlighted in cyan and green, respectively. A red rectangle identifies the amino acids semiconservative replacement at position 14: bovine alanine/human serine. (B) Depiction of the bovine IF_1 3D structures based on x-ray diffraction models deposited in the RCSB Protein Data Bank (PDB codes: 1GMJ and 1OHH). IF_1 structure of each model is truncated but partially overlapping, allowing the superposition of the two structures by the match-maker command in CHIMERA software. Human 3D structure was obtained at the Swiss-model server by protein 3D homology-modelling, using the two bovine IF_1 structures as templates, following superposition of the overlapping regions as described above.

and in tumorigenesis, has prompted us to evaluate the conditions and possibly the mechanism by which IF_1 regulates the mitochondrial bioenergetics in cancer cells, in particular under limited oxygen availability [21,23,28]. In the course of these studies, we found a series of inconsistencies that persuaded us to pursue a more in-depth investigation on the possible modulation of the IF_1 regulatory action by PKA-dependent phosphorylation. We exploited cell models expressing different levels of IF_1 and their recently developed stable IF_1-silenced clones, to characterize their bioenergetics. Here, we provide further experimental data to answer the two main open questions: first, does IF_1 contribute to the metabolic reprogramming of cells to the aerobic glycolysis (i.e. Warburg effect) and second, does PKA affect the action of the ATP synthase inhibitor protein?

Current work, carried out on cell lines derived from three different types of cancer, has allowed us to rule out that IF_1 inhibits the physiological function of ATP synthase and that the regulatory action of IF_1 is not affected by PKA-dependent covalent modification.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (A7030), digitonin (D5628), dibutyryl cyclic-AMP sodium salt (db-cAMP) (D0627), Dulbecco's Modified Eagle Medium (DMEM) (D5030), Glucose (G7021), Glutamine (G3126), H-89 dihydrochloride hydrate (H89) (B1427), phenylmethylsulfonyl fluoride (PMSF) (P7626), Protease inhibitors (P8340), Pyruvate (P2256), sodium deoxycholate (D6750), Triton X-100 (X-100), Malonic acid (M4795), L-Malic acid (M1000), L-Glutamic acid (G1251), Sodium succinate dibasic hexahydrate (S5047), Dimethyl sulfoxide (D4540), Antimycin A from Streptomyces sp. (A8674), Rotenone (R8875), FCCP (C2920), Oligomycin A (75351), Adenosine 5′-diphosphate sodium salt (A2754), β-actin (A5441) and goat anti-rabbit IgG (1706515) antibodies were all purchased from Sigma-Aldrich (Merk) (St. Luis, MO, USA). ECL PRIME (RPN2232) and SELECT (RPN2235) were from Amersham (Merk). MUSE Count and Viability Kit (MCH100102) is from Luminex (Austin, TX, USA). Tetramethylrhodamine methyl ester (TMRM) (T668) and Horseradish peroxidase-conjugated secondary antibody goat anti-mouse mouse IgG H + L (G21040) were from Thermo Fisher Scientific (Waltham, MA, USA). ATP Bioluminescence Assay Kit LCS II (ref 1169969591) was from Roche (Basel, Switzerland). IF_1 (AB110277), TOM20 (AB186735) and ATP synthase d subunit (AB110275) antibodies were from Abcam (Cambridge, UK). Citrate synthase (16131-1-AP) antibody was from Proteintech (Rosemont, IL, USA).

2.2. Cell culture and treatment

Controls and IF_1-silenced clones derived from 143B osteosarcoma, HCT116 colon carcinoma and HeLa cervix adenocarcinoma human cell lines were grown in DMEM high glucose supplemented with 10 % FBS (Thermo Fisher Scientific, Waltham, MA, USA). Suppression of IF_1 expression in HCT116 and HeLa cells was obtained as previously reported for the 143B cells [28]. The screening of IF_1 silencing level in the clones derived from HCT116 and HeLa cells is shown in Fig. S2, whilst osteosarcoma derived clones were reported in Sgarbi et al. [21]. Parental cell lines and clones were seeded at 10×10^4 cells/cm^2 and, when required, 48 h later cells were treated with the PKA agonist: 100 μM db-cAMP or 10 μM competitive inhibitor H89 for 12 h before processing. All the cell types were incubated in a humidified atmosphere at 37 °C containing 5 % CO_2 in the presence or absence of PKA modulators.

2.3. Cell viability assay

Viability assessment of cancer cells, treated or not with FCCP, was performed by using the MUSE Count and Viability Kit (Thermo Fisher Scientific). Parental and IF_1-silenced cells were exposed to 1 μM FCCP in a medium devoid of FBS, up to 24 h. Periodically, the medium was collected together with the adherent cells and an aliquot was assayed to measure the percentage of dead cells. The dot plot post-analysis of dead cells was performed by using the Flowing software.

2.4. Immunoblot analysis

Cells were lysed and proteins, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), were blotted onto nitrocellulose membranes to perform a semiquantitative analysis according to Barbato et al. [28]. Blots of resolved proteins were incubated with both mouse monoclonal anti IF_1 (12 kDa), anti F_1F_o-ATPase d subunit (19 kDa), anti TOM20 (15 kDa), anti β-actin (42 kDa), and rabbit polyclonal anti CS (52 kDa) primary antibodies. β-actin was used as loading control. Immunodetection of primary antibody was carried out with secondary goat anti-mouse and anti-rabbit IgGH + L antibody labelled with horseradish peroxidase. Chemiluminescent detection of the specific proteins was performed with the ECL Western Blotting Detection Reagent Kit by using the ChemiDoc MP system equipped with ImageLab software (BioRad, Hercules, CA, USA) to perform the densitometric scanning of the protein band intensity.

2.5. Protein determination

Protein concentration was measured by the method of Lowry et al. [35] in the presence of 0.3 % (weight to volume ratio) sodium deoxycholate. Bovine serum albumin was used as standard.

2.6. Mitochondrial ATP synthesis assay and cellular ATP hydrolysis assessment

The oligomycin-sensitive ATP synthase activity was determined in digitonin-permeabilized cells according to the method described by Baracca et al. [36]. To identify the optimal amount of digitonin, we performed a titration of the detergent concentration (ranging from 30 to 60 μg/ml) for each cell type (2 mg/ml), and we also tested different times of incubation. The percentage of permeabilized cells was assessed at the microscope using trypan blue dye. We chose 45 μg/ml, the lowest digitonin concentration that allows to observe the maximal value of the oligomycin-sensitive ATP synthesis rate after 10 min of incubation with cells. The synthesis of ATP driven by complex I or complex II was induced by adding 10 mM glutamate/10 mM malate (+ malonate) and 20 mM succinate (+ rotenone), respectively. The reaction was started with 0.5 mM ADP and 3 min later it was stopped by adding dimethyl sulfoxide (80 %) to the reaction mixture. Newly synthesized ATP was measured under basal condition by a Bioluminescence assay kit based on the use of the luciferin/luciferase system, according with the manufacturer's instructions. The amount of ATP measured was normalized to the protein content [37].

ATP hydrolysis by ATP synthase of intact cells was evaluated by comparing the ATP content of FCCP-exposed cells with their controls using the above-mentioned bioluminescence assay kit.

2.7. Oxygen consumption rate

First, we performed a titration of the digitonin concentration (ranging from 20 to 60 μg/ml) for each cell type (1 mg/ml) and tested different incubation times to choose the optimal cell permeabilization conditions. The respiratory rate of the cells was measured by a polarographic assay using a Clark-type oxygen electrode in a buffer containing 250 mM sucrose, 20 mM Tris/Cl, 4 mM $MgSO_4$, 0.5 mM EDTA, and 10 mM KH_2PO_4, pH 7.4. Oxygen consumption rate (OCR) was measured under both basal (intact cells: 1.5 mg/ml cellular protein) and state 3 condition (exogenous 0.5 mM ADP and permeabilized cells: 1 mg/ml cellular protein; 30 μg/ml digitonin; detergent incubation time 1 and 2 min for HCT116/HeLa and 143B cells, respectively). Under state 3

condition, the complex I- and complex II-dependent oxygen consumption was measured at 30 °C (saturating oxygen concentration is 204.1 μM) by adding 10 mM glutamate/10 mM malate (plus 1.8 mM malonate) or 20 mM succinate (plus 1 μM rotenone), respectively, and 0.5 mM ADP as substrates [38]. We calculated the initial rate of respiration under both basal and state 3 condition by evaluating the oxygen concentration decline during the first 2 min of reaction.

2.8. Fluorescence microscopy

Cells were plated in 35-mm Petri dishes coated with poly-D-lysine at a density of 20×10^4 cells/cm^2 and were grown in basal conditions for 48 h. Mitochondrial membrane potential was evaluated by incubating intact cells with 20 nM tetramethylrhodamine methyl ester (TMRM, Molecular Probes, Eugene, OR, USA) for 30 min, essentially as reported by Baracca et al. [36]. The dish was washed once with HBSS and immediately images were acquired on an inverted fluorescence microscope Olympus IX50 (Olympus, Center Valley, Pennsylvania) equipped with a monochrome CCD camera. Multiple fluorescence high-power images (magnification 40×) were acquired by the IAS2000 software (Delta Sistemi, Italy) and deconvolved by using the AutoDeblur/AutoVisualize software (AutoQuant Imaging, NY, USA).

2.9. Flow cytometry analysis

Flow cytometry evaluation of mitochondrial membrane potential under basal conditions was performed using a MUSE cytometer (Merk Millipore, Darmstadt, Germany) after loading the cells with 20 nM TMRM, according to Sgarbi et al. [24]. Essentially, the cells were incubated with the dye for 30 min at 37 °C and wells were then washed with HBSS to remove any remaining unincorporated dye. The cells were rapidly trypsinized, diluted to the optimal density with HBSS supplemented with 10 % FBS and immediately analyzed. The cell fluorescence intensity was measured using a 532 nm excitation laser and a 576/28 nm emission filter; a total of 10.000 events were acquired for each analysis. Cytometry histogram post-analysis of the mean fluorescence distribution of TMRM-loaded cells was performed by the Flowing software (Cell Imaging Core, Turku Centre for Biotechnology, University of Turku).

2.10. Data analysis

Results were analyzed by means of the one-way analysis of variance (ANOVA) with Bonferroni's post-hoc test, unless otherwise specified in the figure legends. Statistical analysis was performed by running GraphPad Prisme for Windows (GraphPad Software). Data are reported as mean ± SD of at least three independent experiments. A level of $P < 0.05$ was selected to indicate statistical significance.

3. Results

3.1. IF_1 is expressed differently in cancer cells and IF_1 knockdown does not affect the mitochondrial mass

It is well documented that IF_1 is overexpressed in many human tumors, but the molecular mechanisms at the basis of the pro-oncogenic actions of IF_1 are still a matter of discussion. Herein, we ought to clarify some aspects of the mechanism elicited by IF_1 in three different tumor cell lines. As a first step we evaluated the expression of IF_1 in each parental cell types. Fig. 2A shows the immunoblot of both IF_1 and ATP synthase d subunit of each cancer cell line. Analysis of the chemiluminescent bands provides an empirical parameter related to the extent of the IF_1/d subunit mass ratio (i.e. it is not the stoichiometric ratio). This ratio varies significantly from one cancer cell line to another as the IF_1/d subunit ratio increases significantly from osteosarcoma (143B) to colon carcinoma (HCT116), to cervix adenocarcinoma (HeLa) human derived cells (Fig. 2B). All the stably IF_1-silenced clones were virtually devoid of the inhibitor, whilst the scrambled cells expressed a content of IF_1 as the corresponding parental cells (Fig. 2C).

The energetic metabolism of eukaryotic cells mainly occurs in mitochondria, therefore it is critical to consider their content when analyzing and comparing different types of cells [39]. Therefore, we quantified specific mitochondrial proteins recognized as an index of their mass: citrate synthase (CS), a specific Krebs cycle enzyme, and TOM20, a receptor subunit of the translocase of the outer mitochondrial membrane (TOM complex). Fig. 3A and B show that mitochondrial mass is higher in both HCT116 and HeLa cells than in 143B cells, although statistical significance for HCT116 is only achieved by quantifying the TOM20 level. In addition, we evaluated and compared the CS level of each cancer cell type with that of derived clones (Fig. 3C and D). Indeed, no significant difference was observed between control cells and IF_1-depleted clones. Cytofluorometric analysis performed by using the nonyl acridine orange (NAO), a non membrane potential-dependent fluorescent probe, confirmed that IF_1-silencing does not affect the mitochondrial content of any cancer cell line tested (Fig. S3). Furthermore,

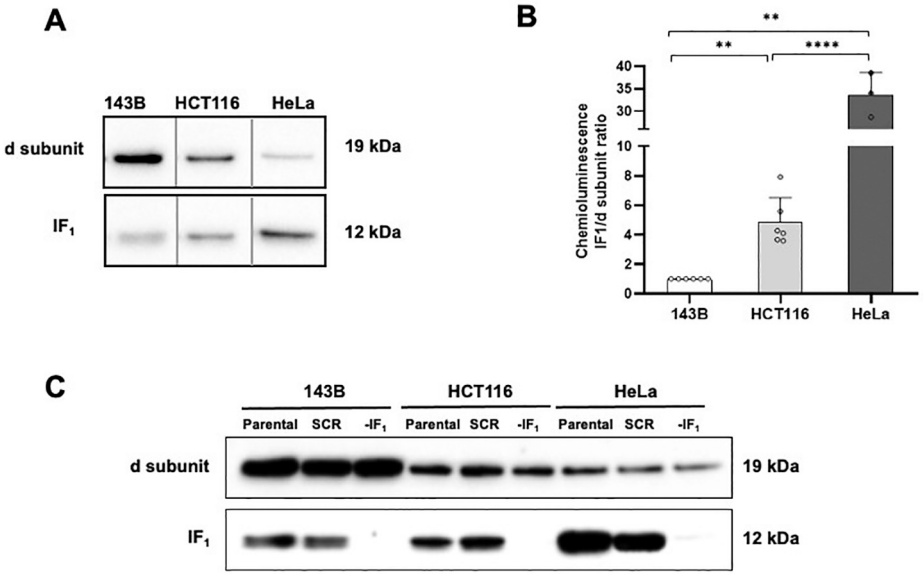

Fig. 2. Expression level of IF_1 in three different types of cancer cell lines.
(A) Typical electrophoretic separation and immunodetection of both IF_1 and F_1F_o-ATPase d subunit in lysates of 143B, HCT116 and HeLa cancer cell lines. Bars indicate that one lane was removed from the blot. (B) The scanned images were quantified by ImageLab software; protein levels of IF_1 were normalized to d subunit, taken as the internal standard of ATP synthase. Data are means ± SD of at least three independent experiments and are reported as a percentage of parental 143B cells. $^{**}P < 0.01$ and $^{****}P < 0.0001$, indicate the statistical significance using the One-sample *t*-test to compare 143B with the other two cell lines, and the two-tailed t-test to compare HCT116 and HeLa. (C) Representative IF_1 expression level in parental cells (143B, HCT116, HeLa) and related scrambled (SCR: C4, A6, B1) and IF_1-silenced (-IF_1: E9, C3, F3) clones. The wells were overloaded to better evidence the lack of IF_1 in the stably silenced cells.

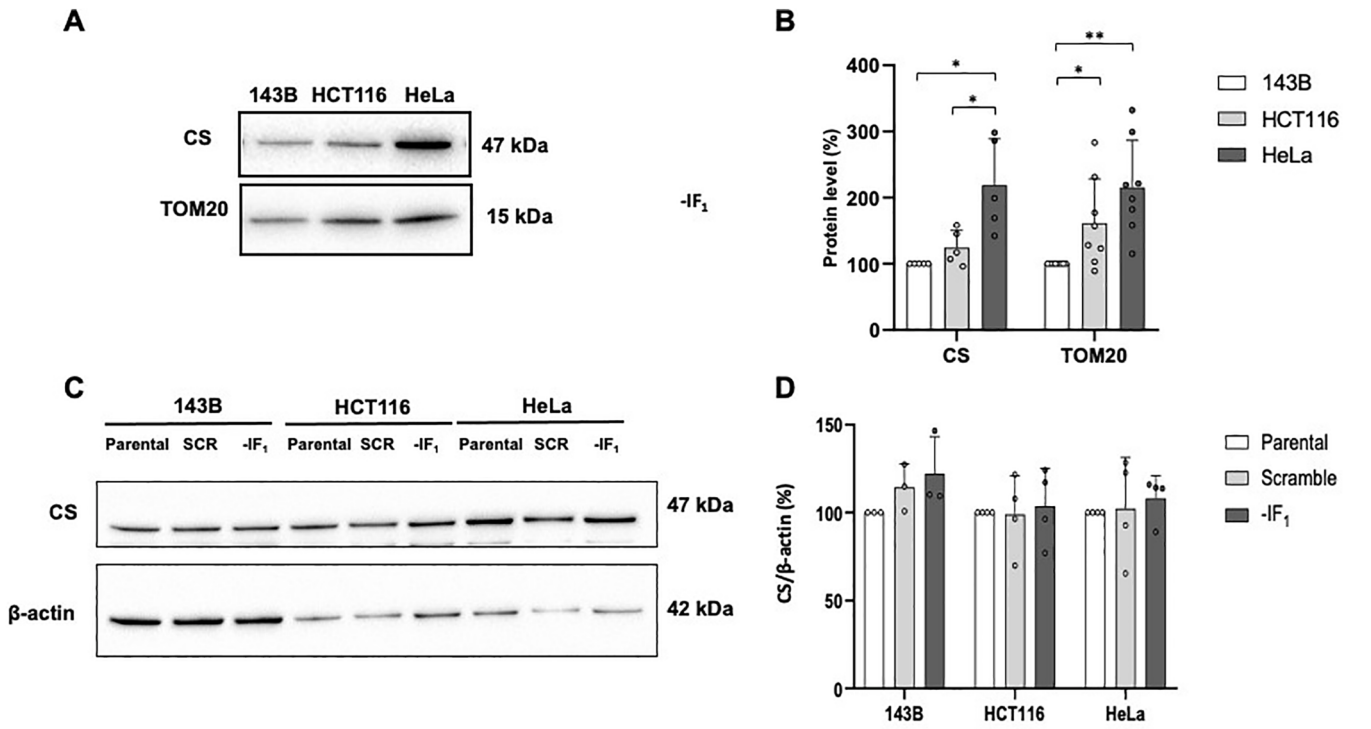

Fig. 3. IF_1 silencing does not change the mitochondrial mass in cancer cell lines.
A) Representative electrophoretic separation and immunodetection of citrate synthase (CS) and TOM20 levels in parental cells. (B) Scanned images were quantified by ImageLab software and the histogram shows the densitometric analysis. Data are means ± SD of at least five independent experiments and are reported as a percentage of 143B cells. $^{*}P < 0.05$, $^{**}P < 0.01$, indicate the statistical significance using the One-sample *t*-test to compare 143B with the other two cell lines, and the two-tailed t-test to compare HCT116 and HeLa. (C) Representative electrophoretic separation and immunodetection of citrate synthase (CS) in each parental cell type and its derived scrambled (SCR) and IF_1-silenced (-IF_1) clones. (D) Scanned images were quantified by ImageLab software; protein levels of CS were normalized to β-actin, taken as loading control. The histogram shows the relative CS levels of the SCR (C4, A6, B1) and -IF_1 (E9, C3, F3) clones expressed as a percentage of the respective parental cells (143B, HCT116, HeLa). Data are means ± SD of at least three independent experiments.

measurements of citrate synthase activity in parental and IF_1-silenced cells matched the assessment of mitochondrial mass performed by western blot analyses quantifying CS protein (Fig. S4A). Overall, normalization of enzyme activities to the mitochondrial mass can be avoided when parental cells are compared to corresponding IF_1-silenced clones. Incidentally, as shown in Fig. S5, we analyzed the levels of OXPHOS complexes in all parental and IF_1-silenced cells; however, no significant difference was found between IF_1-expressing and IF_1-silenced cells, as previously reported for osteosarcoma 143B cells [28].

3.2. *IF_1 expression does not inhibit ATP synthase and OXPHOS in cancer cells*

A number of papers, mostly from the same research group, report an inhibiting effect of IF_1 on the ATP synthase activity of cancer cells [see for reviews [33,40]. To clarify the role of IF_1 on the ATP synthase activity, we extended the study earlier performed on the 143B osteosarcoma cell line [21,28] to other tumor cell types, namely HeLa and HCT116. Indeed, the ATP synthase of the latter cancer cell lines was reported to be significantly inhibited by IF_1 [15] and this was associated with an increase of the aerobic glycolysis (Warburg effect) [16]. Here, we demonstrate by means of three independent experimental approaches that the silencing of IF_1 in cancer cells never determines significant increase of the ATP synthesis rate (Figs. 4-6). Fig. 4 shows data from the direct assay of the maximal ATP synthesis rate by a well-known luminometric method routinely used in our laboratory. Clearly, the rate of ATP synthesis by OXPHOS of the parental cell lines and their IF_1-silenced clones was similar, whatever NAD-dependent substrate or succinate was used to energize the mitochondria.

A second approach to evaluate the possible modulation of the ATP synthesis rate in cancer cells by IF_1 was based on the polarographic measurement of the endogenous respiration rate (i.e. oxygen consumption rate, OCR). The histograms reported in Fig. 5 show that there is no difference between the endogenous respiration of IF_1 expressing and IF_1-silenced cancer cells, excluding that IF_1 can inhibit the ATP synthase activity under basal conditions.

To further support the lack of control of the ATP synthase by IF_1 under basal conditions, we assayed the endogenous mitochondrial membrane potential ($\Delta\psi_m$) of the different cancer cells containing or lacking the inhibitor protein. We loaded the cells with 20 nM TMRM, a recognized potentiometric probe of $\Delta\psi_m$, and cells were analyzed by flow cytometry. Fig. 6 shows the distribution of the fluorescent cell populations (A) and the related fluorescent mean values (B). $\Delta\psi_m$ was slightly higher in IF_1-silenced 143B cells, and unchanged in both IF_1-silenced HCT116 and HeLa cells compared to controls. Fluorescence microscopy images of TMRM-loaded cancer cell lines matched the flow cytometry results (Fig. 6C). Since the ATP synthesis rate is inversely related to $\Delta\psi_m$, it is clear that the presence of IF_1 did not induce ATP synthesis decrease: eventually it could improve the rate as in 143B cells, according with previous observations [28].

The conclusion of this set of experiments is that the ATP synthesis rate by OXPHOS was not affected whether IF_1 was present or absent in the three cancer lines tested. This experimental work was necessary to extend and support our previous observations on the regulation of OXPHOS by IF_1 in osteosarcoma cells [28] and it pushed us to investigate the proposed PKA dependent regulatory mechanism of the IF_1 action.

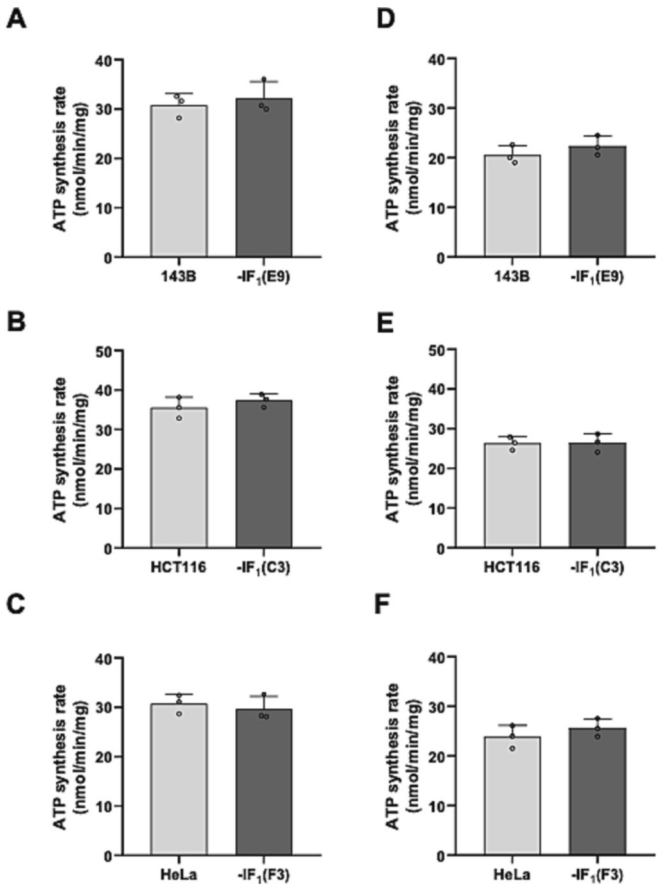

Fig. 4. Silencing of IF_1 does not increase the OXPHOS rate in cancer cell lines. Parental and IF_1-silenced cells were cultured in high glucose complete medium for 48 h. The rate of ATP synthesis (i.e. OXPHOS) was measured in digitonin-permeabilized cells energized with either 10 mM glutamate/10 mM malate plus 1.8 mM malonate (A-C) or 20 mM succinate plus 1 μM rotenone (D-F) by adding 0.5 mM ADP. No significant changes were observed in the IF_1-silenced clones compared to the respective parental cell lines: 143B (A and D), HCT116 (B and E) and HeLa (C and F). Data are means ± SD of three independent experiments.

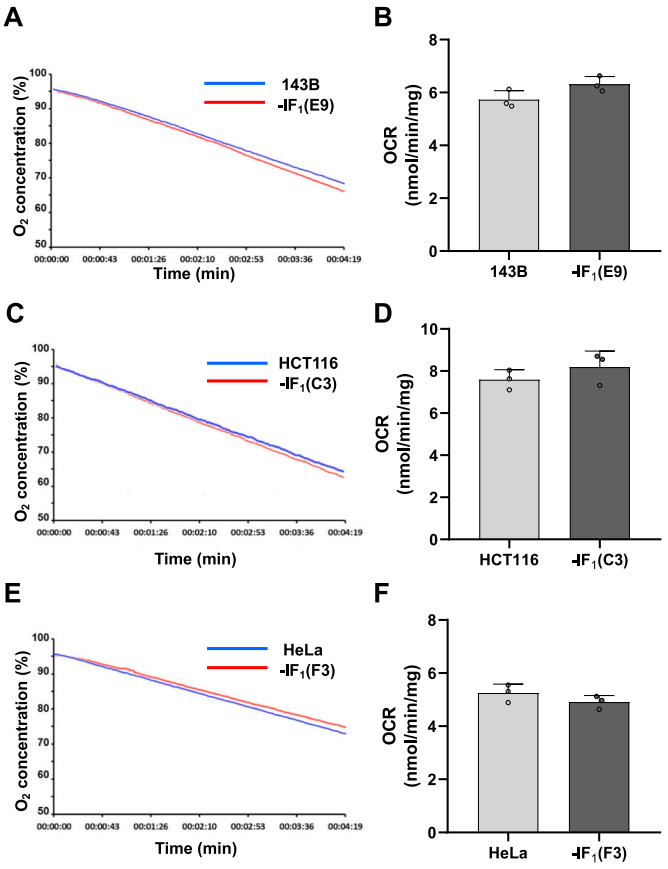

Fig. 5. Mitochondrial respiration is similar in IF_1-silenced clones and related parental cancer cell lines. Representative respiration traces (A, C, E) and histograms showing the endogenous OCR (B, D, F) of each parental cell type and its derived silenced clone. The histograms report means ± SD of three independent experiments.

3.3. PKA activity does not affect the action of IF_1 on ATP synthase working physiologically

To answer the question whether the PKA dependent phosphorylation/dephosphorylation state of IF_1 affects its inhibitory action on the ATP synthase complex, we set experiments in which the PKA activity was either stimulated or inhibited by db-cAMP and H89, respectively [32,41]. All the cancer cell types expressing or lacking IF_1 were pre-incubated with or without the PKA effectors for 12 h, then were harvested, softly permeabilized with digitonin as previously reported [21], and assayed for both ATP synthesis and OCR (state 3 respiratory condition) driven by the NAD^+-dependent substrates glutamate/malate (Fig. 7). Surprisingly, the effects of the PKA modulators on both ATP synthesis rate and OCR were similar in controls and corresponding IF_1 lacking cells. Notably, the stimulation of PKA by db-cAMP had no effects on either the ATP synthesis rate or OCR independently of the presence or absence of IF_1. On the contrary, the inhibition of PKA activity by H89 resulted in severe decrease of both the ATP synthesis rate (40–70 %) and OCR (40–60 %) independently of the presence or absence of IF_1.

We were aware of earlier studies where subunits of complex I, the first entry point of the electron transport chain, were found to be phosphorylated and capable of improving mitochondrial respiration rate [42–44]. Therefore, we repeated the experiments by fueling respiration with succinate, a FAD-dependent substrate that bypasses the action of complex I. These experiments clearly show that neither the PKA inhibitor, H89, or the activator, db-cAMP, affected the respiration rate and the ATP synthesis rate in all the cell types tested whether IF_1 was present or absent (Fig. 8). No changes of the mitochondrial mass were observed in all the cells and clones upon exposure to the modulators of PKA, as observed by the measurements of citrate synthase activity (Fig. S4B-D). Therefore, the data exclude any effect of PKA dependent phosphorylation (and dephosphorylation) of IF_1 on the ATP synthesis activity of the F_1F_o-ATPase complex. These conclusions were confirmed by OCR measurements under basal conditions (Fig. 9).

3.4. PKA does not affect the inhibition of ATP synthase hydrolytic activity by IF_1 in cancer cells

To complete the study on the possible effect of phosphorylation/dephosphorylation of IF_1 on the ATP synthase complex activities, we also assessed the ATP hydrolytic activity of the enzyme in presence and absence of PKA effectors (Fig. 10). We assessed the hydrolytic activity of the F_1F_o-ATPase complex adopting an indirect method since the more direct coupled enzyme assay, based on the spectrophotometric or fluorometric evaluation of NADH decay [45], could not be exploited due to interference from many cell dehydrogenases which could affect and confuse the results. The enzyme complex was then induced to hydrolyze ATP exposing the cells to the uncoupler FCCP for 12 h [21] and the cell steady state ATP content was finally measured. As expected, under uncoupling condition, the ATP level dropped down in all cells. In IF_1-silenced clones ATP level decreased more than in parental cells due to uncontrolled ATP hydrolytic activity of F_1F_o-ATPase which works in

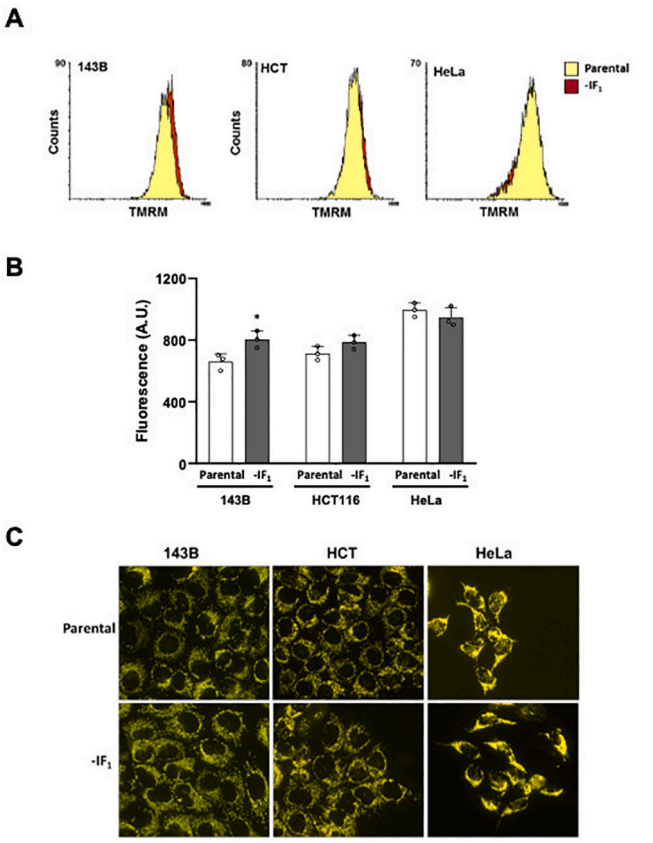

Fig. 6. Mitochondrial membrane potential of IF_1-silenced cells. (A) Typical flow cytometry analysis of TMRM-loaded cells under basal condition. (B) Quantification of mitochondrial membrane potential based on the cell fluorescence mean values. Bars show the mean ± S.D. of four independent experiments. (C) Representative fluorescence images (magnification 40×) of TMRM-loaded cells. *$P < 0.05$, indicate statistical significance of data using two-tailed *t*-test.

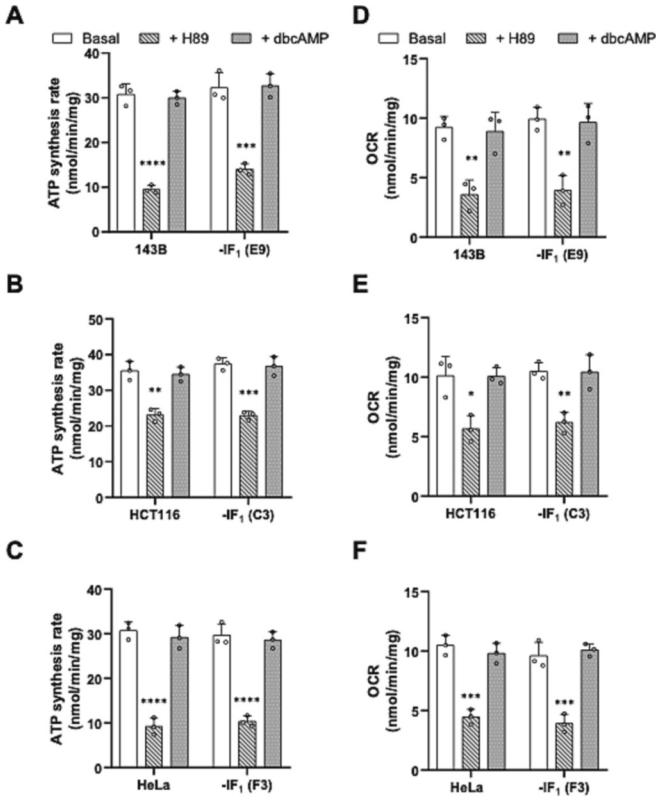

Fig. 7. PKA effectors modulate OXPHOS driven by NAD^+-dependent substrates in cancer cells.
Parental and IF_1-silenced cells were exposed to both H89 and db-cAMP for 12 h before processing. Both the ATP synthesis rate (A-C) and the oxygen consumption rate (state 3 respiration) (D-F) were maximized by adding glutamate/malate and ADP in digitonin-permeabilized cells. Treatment with H89 caused a significant decrease in both OXPHOS and state 3 respiratory rate in all IF_1-silenced and parental cells: 143B (A and D), HCT116 (B and E), and HeLa (C and F). Data are means ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$ indicate the statistical significance compared to basal condition.

reverse [21]. The extent of the ATP drop was slightly different in the three cancer cell types, possibly due to the different production of ATP by glycolysis and to the different content of both the ATP synthase complex and its endogenous regulator, IF_1. Notably, under uncoupling conditions PKA modulators similarly affected the ATP level in each cancer cell type independently of the IF_1 presence. Moreover, the presence of the PKA inhibitor H89 does not positively modulate the ATP level of FCCP-exposed parental cells. Of note, even the lowest ATP level found in parental and IF_1-silenced HCT116 cells upon the exposure to the uncoupler did not affect viability during the experiment (Fig. S6).

These results clearly demonstrate that PKA-mediated phosphorylation/dephosphorylation does not affect the inhibitory action of IF_1 on ATP synthase working in reverse.

4. Discussion

We studied different cancer cell lines to extend the knowledge of cancer bioenergetics and definitely establish whether the main mechanisms of action of IF_1 as a pro-oncogenic protein include inhibition of ATP synthase working physiologically, thus contributing to the onset of the Warburg effect. To achieve this goal, it was also necessary to consider and ascertain whether PKA modulates the action of IF_1 on mitochondrial ATP synthase by influencing its pathophysiological activities. To these end, experimental work was performed on cell lines from three different types of cancer characterized by a significantly different IF_1/ATP synthase ratio, including cancer cells in which the synthesis of ATP by ATP synthase was found to be inhibited by IF_1 [15].

The main findings of the present study are that (i) activation or inhibition of PKA cannot affect the action of IF_1 and its modulation of ATP synthase activities (i.e. ATP synthesis and ATP hydrolysis) and (ii) IF_1 does not inhibit the ATP synthesis rate via OXPHOS in cancer cells.

Consequently, the results of the present study clearly indicate that inhibition of the ATP synthase complex by IF_1 when the enzyme is functioning physiologically (i.e. normoxia and ATP synthesizing enzyme) does not occur. Interestingly, this unambiguous conclusion based on cell model experiments finds a strong match in the in vitro experiments conducted by John Walker and coworkers on the isolated sector of ATP synthase, F_1, which was analyzed with the purified inhibitory protein IF_1 in different proportions and environmental conditions [18,19]. Furthermore, very recent experiments by Noji et al. [46] elucidated how IF_1 achieves the unidirectional inhibition of the enzyme when it hydrolyzes ATP. They conducted single molecule manipulation of IF_1-inhibited *bovine* F_1 with magnetic tweezers and showed that the inhibited F_1 complex was activated by releasing IF_1 when its rotor domain was forcibly rotated in clockwise direction (i. e. synthesis direction) in the presence of ADP and inorganic phosphate [46,47].

As a corollary of the current study, we highlight that inhibition of PKA modulates OXPHOS (Figs. 7 and 9), although the proposed PKA-dependent phosphorylated (and dephosphorylated) state of IF_1 cannot affect the rate of ATP synthesis by the ATP synthase complex. Indeed, our experiments provided these results regardless of whether IF_1 was present or absent in the cells and the modulation of OXPHOS only

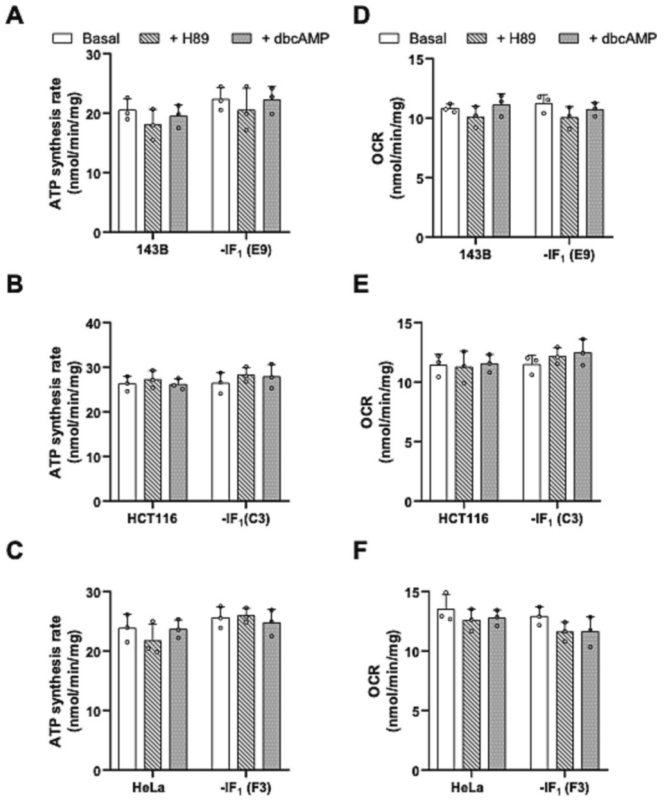

Fig. 8. PKA effectors do not regulate OXPHOS driven by succinate in cancer cells.

Parental and IF_1-silenced cells were exposed to both H89 and db-cAMP for 12 h before processing. Both the ATP synthesis rate (A-C) and the oxygen consumption rate (D-F) were evaluated in digitonin-permeabilized cells in presence of ADP and by adding succinate to energize mitochondria. Treatment with either H89 or db-cAMP did not significantly change OXPHOS or state 3 respiration rate in any of the IF_1-silenced or parental cells: 143B (A and D), HCT116 (B and E) and HeLa (C and F). Data are means ± SD of three independent experiments.

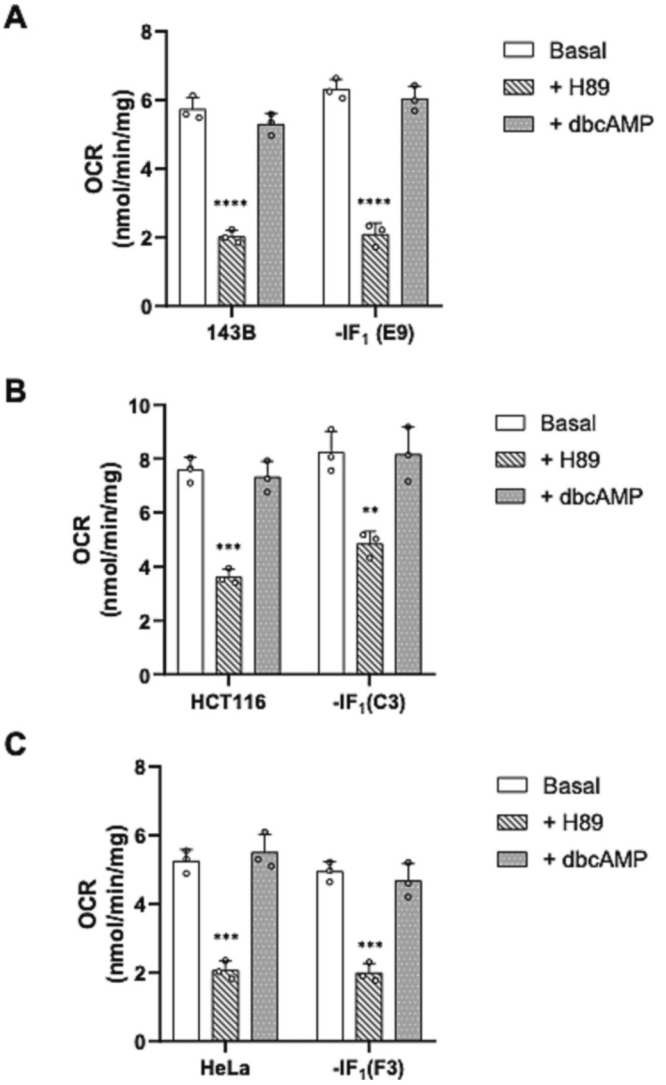

Fig. 9. PKA inhibition by H89 decreases the endogenous respiration of cancer cells.

Parental and IF_1-silenced cells were exposed to both H89 and db-cAMP for 12 h before processing. The basal OCR was evaluated in both IF_1-silenced and parental 143B (A), HCT116 (B) and Hela (C) intact cells. Upon H89 treatment, a significant decrease of the basal respiration rate in both parental and IF_1-silenced cells was observed. The PKA activator db-cAMP did not affect the basal OCR in any of the cells examined. Data are means ± SD of three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ indicate the statistical significance compared to basal condition.

occurred when we used NAD^+-dependent substrates as fuel. In fact, the use of succinate as substrate did not show any PKA-dependent modulation of the OXPHOS rate (Fig. 8). Thus, the modulation of OXPHOS by PKA-dependent phosphorylation must be ascribed to NADH-ubiquinone oxidoreductase (complex I), the first entry point of the electron transport chain, which oxidizes NADH to fuel the respiratory chain allowing for the generation of ATP by OXPHOS. Indeed, several complex I subunits can be phosphorylated [43,48] and pioneer work by Papa et al. [42] proposed that the enzyme was phosphorylated through the cAMP/PKA pathway in cells. Subsequent studies on both isolated complex I and submitochondrial particles from bovine heart indicated that the ESSS subunit (NDUFB11) of complex I was phosphorylated at serine 20 [43]. The demonstration that IF_1 does not inhibit the ATP synthase under physiological conditions contradicts published work reporting that IF_1 inhibits ATP synthesis in cancer cells (HCT116 and HeLa), where it is overexpressed [15,49]. The conclusions of the latter authors were mostly based upon indirect assays, evaluating the rate of oligomycin-sensitive respiration (OSR) under basal cell culture conditions. Moreover, the model used were cells transfected with a plasmid encoding IF_1, a condition favoring the formation of protein aggregates (amyloids) that can affect the function of mitochondria, inhibiting OXPHOS [50–54]. In addition, most experiments were performed in transiently transfected cells, which are heterogeneous and dynamic models, where assessment of cellular bioenergetics is difficult to define. Finally, the evidence that IF_1 activity is insensitive to PKA action also disagrees with previous work stating that IF_1 does not bind to and inhibit ATP synthase when the cAMP/PKA pathway is activated [32]. Indeed, the authors assayed the OSR rate only under basal cell culture conditions and concluded that IF_1 inhibits the rate of ATP synthesis by the F_1F_o-ATPase complex, the results and conclusions here reported are based on three different and independent experimental approaches: direct luminometric assay of oligomycin-sensitive ATP synthesis rate, oxygen consumption rate in both basal and state 3 respiratory conditions using both NAD^+- and FAD-dependent substrates, and evaluation of the mitochondrial membrane potential of the cells by flow cytometry. Moreover, the investigation was performed analyzing both parental cell types and their derived stably IF_1-silenced clones. Notably, under uncoupling conditions, the ATP level in each cancer cell type was similarly affected by PKA modulators regardless of the presence of IF_1. In particular, the PKA inhibitor H89 significantly decreased the cellular ATP level compared to the basal value of the corresponding uncoupled cell type, clearly suggesting that other targets than IF_1 are modulated by PKA and affect cellular ATP.

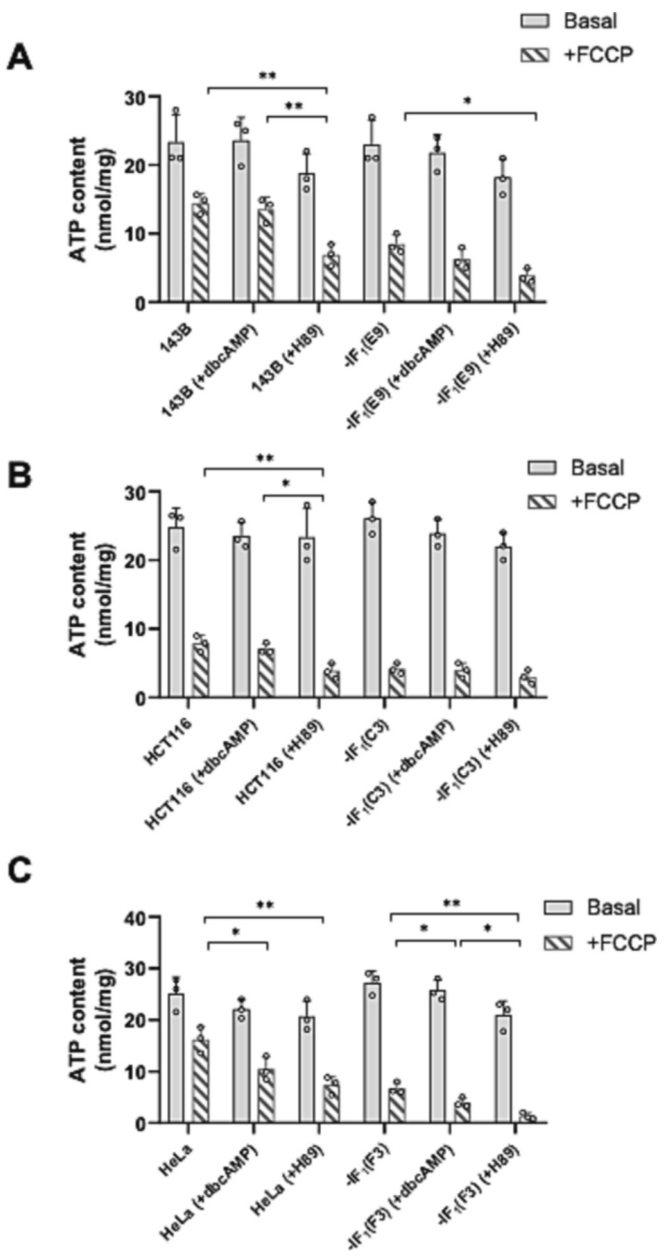

Fig. 10. PKA modulation influences the ATP content of cancer cells independently of IF_1.
(A-C) Parental cells and their derived IF_1-silenced clones were exposed or not to both PKA inhibitor H89 and PKA activator db-cAMP for 12 h to achieve a steady-state metabolic condition. All cell types were then exposed to the FCCP uncoupler (1 μM) to induce ATP synthase to work in reverse. After 20 min of treatment, the ATP content of the cells was analyzed by bioluminescence assay kit, according to the manufacturer's instructions. Data are means ± SD of three independent experiments. $^*P < 0.05$ and $^{**}P < 0.01$ indicate statistical significance.

Overall, our experimental work states that IF_1 does not inhibit the ATP synthesis activity of the mitochondrial F_1F_o-ATPase complex and that the action of IF_1 is not regulated by a cAMP/PKA-dependent mechanism in cancer cells.

5. Conclusions

IF_1 in cancer cells does not trigger inhibition of ATP synthesis and consequently OXPHOS. At present, the pro-oncogenic action of IF_1 cannot include inhibition of physiologically working ATP synthase, thus IF_1 does not contribute to metabolic reprogramming by shifting energy metabolism towards aerobic glycolysis in cancer cells (Warburg effect), as previously proposed by Cuezva et al. [15]. Hence, two main mechanisms underlie the pro-oncogenic role of IF_1: first, the resistance of cancer cells to death by apoptosis which IF_1 sustains by both the preservation of mitochondrial cristae ultrastructure [55] and by increasing the concentration of Ca^{2+} necessary for the opening of PTP [56]. Second, the prolonged survival of cancer cells under conditions of severe hypoxia/anoxia due to the energy conservation enabled by IF_1 which limits the hydrolase activity of ATP synthase [21].

The present study carried out in human osteosarcoma, cervical, and colon cancer cells provides a compelling example of how incomplete and/or superficial analysis of a complex biochemical process as OXPHOS can lead to misleading conclusions and negatively affect the development of bioenergetic knowledge in pathophysiology. Indeed, appropriate controls such as the use of different substrates to assay OCR and/or OXPHOS and comparing stable IF_1-silenced clones with the corresponding parental controls, are essential to provide reliable information for understanding biological processes. Consequently, the search for potential new therapeutic approaches and/or molecules would develop from an appropriate hypothesis.

CRediT authorship contribution statement

Conceptualization, GS, AB; Investigation, RR, GSg, VDD, SG; Methodology, GSg, RR; Supervision, AB, GS; Writing - original draft, GS, AB; Writing - review & editing, GS, AB, VG, VDD; Funding acquisition, GS, AB, VG; Project administration GS, AB, GSg.

Declaration of competing interest

The authors declare no financial conflict of interest that might be construed to influence the results or interpretation of the manuscript.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2023.166879.

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