The F1Fo-ATPase inhibitor, IF1, is a critical regulator of energy metabolism in cancer cells

The F_1F_o-ATPase inhibitor, IF_1, is a critical regulator of energy metabolism in cancer cells

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Abstract

In the last two decades, IF_1, the endogenous inhibitor of the mitochondrial F_1F_o-ATPase (ATP synthase) has assumed greater and ever greater interest since it has been found to be overexpressed in many cancers. At present, several findings indicate that IF_1 is capable of playing a central role in cancer cells by promoting metabolic reprogramming, proliferation and resistance to cell death. However, the mechanism(s) at the basis of this pro-oncogenic action of IF_1 remains elusive. Here, we recall the main features of the mechanism of the action of IF_1 when the ATP synthase works in reverse, and discuss the experimental evidence that support its relevance in cancer cells. In particular, a clear pro-oncogenic action of IF_1 is to avoid wasting of ATP when cancer cells are exposed to anoxia or near anoxia conditions, therefore favoring cell survival and tumor growth. However, more recently, various papers have described IF_1 as an inhibitor of the ATP synthase when it is working physiologically (i.e. synthethizing ATP), and therefore reprogramming cell metabolism to aerobic glycolysis. In contrast, other studies excluded IF_1 as an inhibitor of ATP synthase under normoxia, providing the basis for a hot debate. This review focuses on the role of IF_1 as a modulator of the ATP synthase in normoxic cancer cells with the awareness that the knowledge of the molecular action of IF_1 on the ATP synthase is crucial in unravelling the molecular mechanism(s) responsible for the pro-oncogenic role of IF_1 in cancer and in developing related anticancer strategies.

Introduction

During the last decades, numerous proteins have been shown to express pro-oncogenic action in different cancers. Among these proteins a peculiar and interesting role is played by the endogenous inhibitor of the mitochondrial ATP synthase (F_1F_o-ATPase), IF_1, also called “Inhibitor Factor 1”. It is a low molecular mass protein first identified in bovine heart mitochondria by Pullman & Monroy [1] and subsequently it was found ubiquitously expressed in eukaryotes. It has been described in monocellular organisms [2, 3] and more extensively in mammalian tissues, where it was found present at different levels [4-6], and in particular in slow and fast beating hearts animals [7]. The presence of the intrinsic inhibitor and its interaction with the ATP synthase appear of critical importance in eukaryotic cellular life, therefore it is worth clarifying its role(s) also in cancer cells.

The human mature protein, encoded by the nuclear gene ATP5IF1 in chromosome 1, is composed of 81 amino acids and presents a high sequence homology of about 75% with the bovine IF_1 (Figure 1A). In aqueous solution, IF_1 exists as monomers, dimers, and oligomers depending on the pH. At slightly acidic pH the equilibrium is forced toward the dimeric state (Figure 1B) and the ATP hydrolytic activity of the enzyme is fully inhibited, whereas at alkaline pH IF_1 is predominantly oligomeric and inactive [8, 9]. In addition, the oligomeric state and consequently the inhibitory potency of IF_1 is sensitive to both the ionic strength and the nature of cationic species [8].

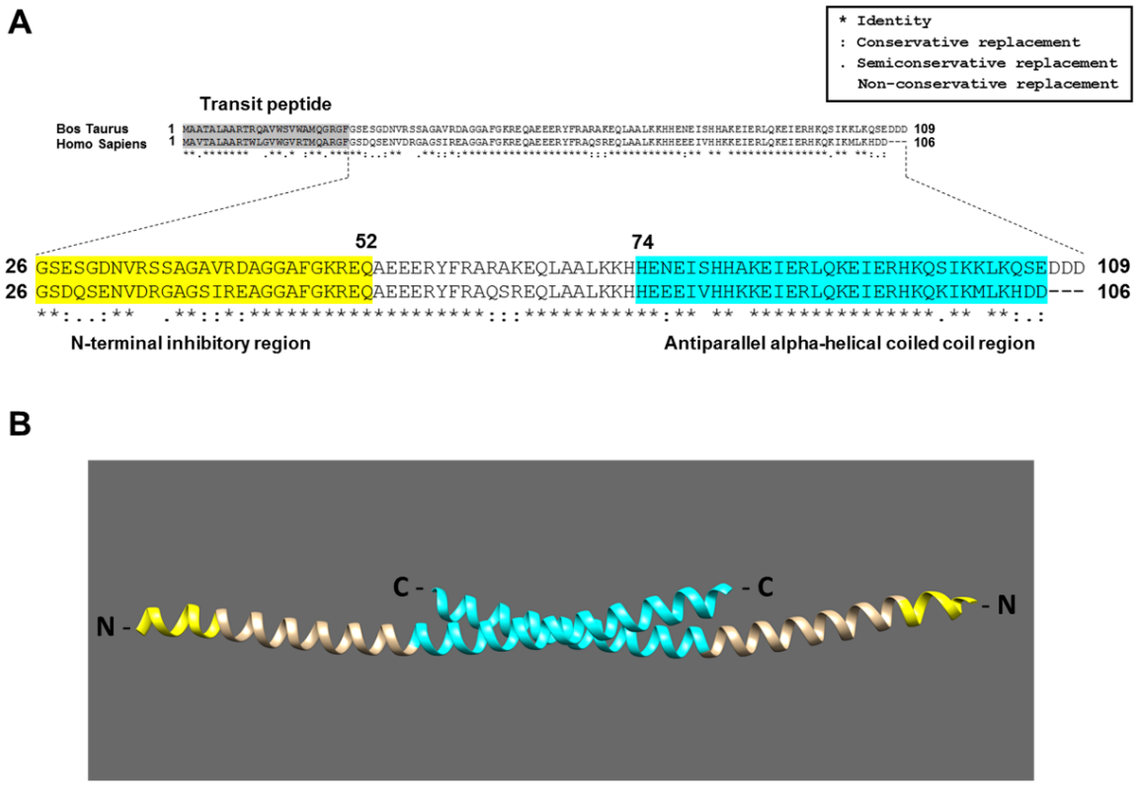

Figure 1. Protein sequence alignment of bovine and human IF_1 and its dimeric structure. (A) Pairwise alignment of the complete sequence of bovine (UniprotKB - P01096) and human (UniprotKB - Q9UII2) IF_1 protein, performed by Clustal omega program. The N-terminal inhibitory and the antiparallel α-helical coiled coil region are dyed in yellow and cyan, respectively. (B) 3D depiction of the bovine dimeric IF_1 (PDB code: 1GMJ). The representation shows the monomer association via an anti-parallel α-helical coiled-coil in their C-terminal regions (cyan) and the N-terminal inhibitory regions extending in opposite regions (yellow). The N-terminal domain of both monomers are truncated because only few residues of these domains were resolved in the structure.

In eukaryotes, IF_1 is found in mitochondrial matrix, but it is still unclear whether under physiological conditions it is free or bound to the F_1F_o-ATPase. Interestingly, a very recent paper challenged the hypothesized contribution of IF_1 to the formation of inactive ATP synthase tetramers [10]. Nonetheless, the coexistence of active ATP synthase dimers and inactive tetramers, resulting from the binding of IF_1 to ATP synthase dimers in the hydrolytic mode, may be hypothesized considering that individual cristae within the same mitochondrion can have different membrane potentials [11]. Alternatively, under conditions leading to ATP synthase dimers in the hydrolytic mode, tetramers could result from insufficient IF_1 to inhibit all the ATP synthase dimers [10].

At present, IF_1 is considered to be a reversible non-competitive inhibitor of ATP hydrolysis catalyzed by isolated F_1-ATPase and *in situ* F_1F_o-ATPase under conditions of collapsed proton motive force [8] (see scheme, Figure 2). This occurs in the ischemic heart (for review see [12, 13]) and in cancer cells exposed to anoxia or anoxia mimicking uncoupling conditions [14].

The F_1F_o-ATPase is a multimeric complex of about 600 kDa mass, located in the inner mitochondrial membrane where it phosphorylates ADP with inorganic phosphate at the expenses of the proton motive force ($\Delta\mu_{H^+}$) produced by the respiratory chain. Its catalytic moiety, F_1, constituted by 9 subunits, $\alpha_3\beta_3\gamma\delta\varepsilon$ in order of decreased molecular weight, is bound to the membrane sector, F_o, and protrudes within the matrix. The enzyme complex contains three equivalent catalytic sites located at the α–β interfaces that in situ differ one another for the nucleotide affinity. This affinity depends on the interaction of each αβ dimer with the γ-subunit. This subunit rotates during the catalytic turnover, therefore on a 360 degrees round, all the three catalytic sites experience the three different affinity states [15]. Notably, the ATP synthase is a reversible enzyme that under collapsed $\Delta\mu_{H^+}$ hydrolyzes ATP, produced by glycolysis/glycogenolysis in the cytosol of the cells, to contribute re-establishing and maintaining $\Delta\mu_{H^+}$. Incidentally, a similar condition occurs in vivo on an ischemic tissue. Indeed, the cellular energy metabolism generates acidification of both cytosolic and mitochondrial matrix compartments promoting the formation of the active dimeric state of IF_1 that binds two F_1 molecules, as clearly established by Cabezon et al. [16]. Under collapsed $\Delta\mu_{H^+}$, cytosolic ATP can cross the inner mitochondrial membrane in exchange for ADP [17] and the enzyme pumps H^+ from the matrix to the intermembrane space exploiting the energy released by ATP hydrolysis (Figure 2).

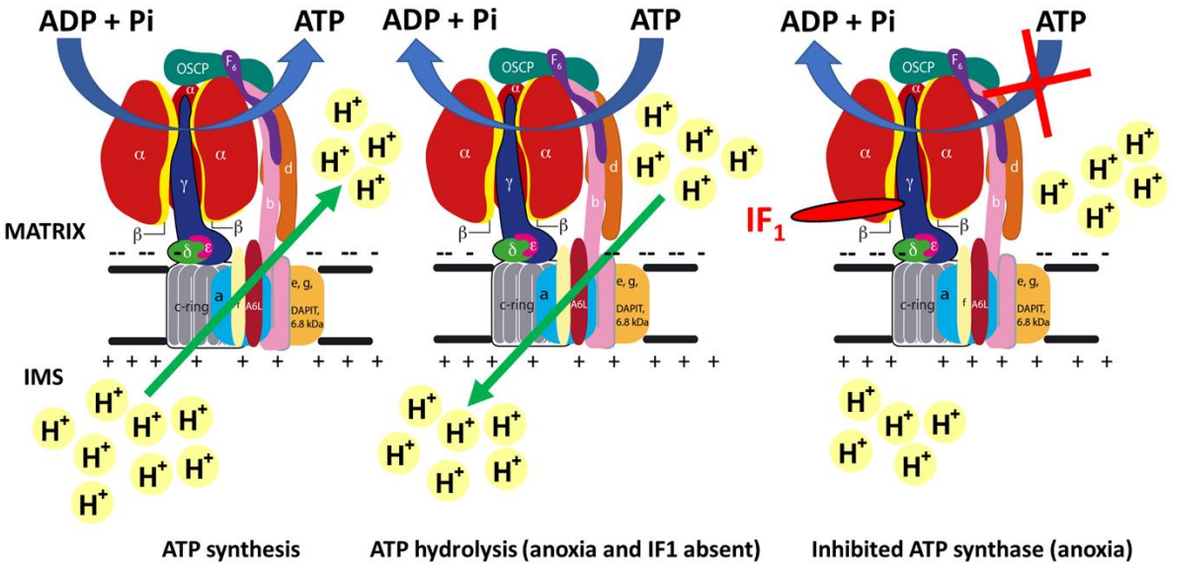

Figure 2. Schematic presentation of the mitochondrial F_1F_o-ATPase catalytic activities. The Schematic subunit composition of mitochondrial ATP synthase is from Prof. J.E. Walker Medical Research Council, Mitochondrial Biology Unit, University of Cambridge (http://www.mrc-mbu.cam.ac.uk/projects/2679/subunit-composition**.** Left panel: proton transport drives ATP synthesis in cells under physiological condition (normoxia). Central panel refers to IF_1-silenced cancer cells exposed to anoxia or anoxia-mimicking (uncoupling) conditions. The enzyme pumps protons from the mitochondrial matrix to the IMS (intermembrane space; i.e. intracristae space). The right panel shows the block of the ATPase activity in cancer cells due to the binding of the inhibitor, as it occurs in vivo under anoxia. Green and red arrows indicate allowed and forbidden proton translocation through the ATP synthase, respectively.

The reversal activity of the ATP synthase and the contribution of the ATP/ADP exchange to the transmembrane potential recovery allow mitochondria to save many of their functions, promoting the preservation of cellular homeostasis, and impede the cell death. To this aim, the inhibitory action of IF_1 is fundamental since it allows to maintain the correct balancing between the $\Delta\mu_{H^+}$ and the ATP levels, avoiding an excessive ATP consumption by the F_1F_o-ATPase complex [13].

The molecular mechanism of inhibition has been described in detail by Walker and coworkers [18-20]. In synthesis, the same Authors proposed that the inhibitor binds F_1 when it hydrolyzes ATP: IF_1 interacts with the αβ dimer at the lowest affinity site for nucleotides (i.e. the empty catalytic site) and following the hydrolysis of two molecules of ATP, it moves into the α/β hexamer preventing the rotor from turning further (Figure 3).

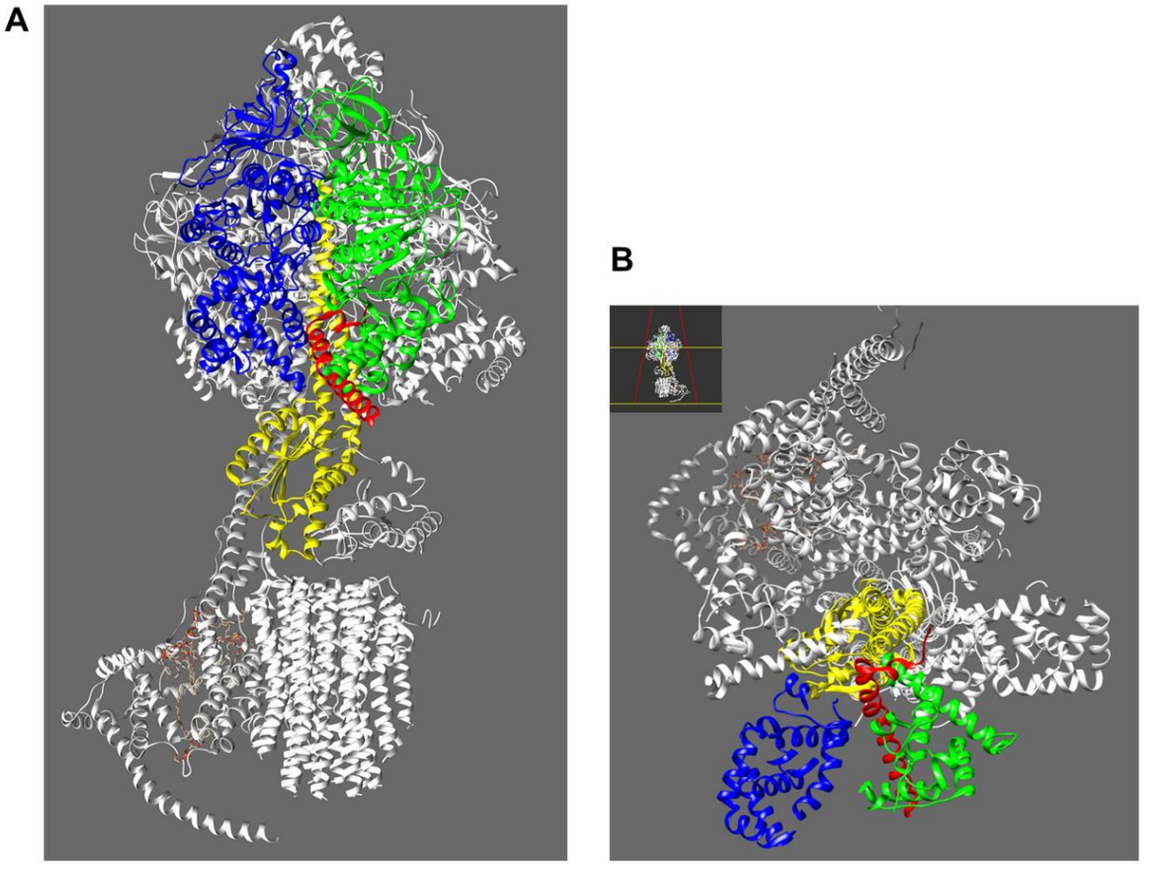

Figure 3. Binding of IF_1 to the F_1F_o-ATPase. (A) The 3D depiction shows the binding of the N-terminal inhibitory region of IF_1 (red) to the F_1F_o-ATPase. The N-terminal region of IF_1 occupies an aqueous cleft between the C-terminal domain of the β_{DP} (green) and α_{DP} (blue) subunits of the F_1 domain and interacts with the γ subunit (yellow) (PDB code: 7AJF). (B) Upper view of the transversal section of the F_1 domain in close proximity to IF_1 and γ-subunit interaction is shown.

The presence of an endogenous inhibitor of the ATP synthase appears to be of great importance in all the biological systems containing ATP synthases, although its mechanisms of action are still debated, particularly in cancer cells. Several authors from different laboratories reported that IF_1 is overexpressed in human cancer and this has led to the hypothesis about its pro-oncogenic role [see below]. However, in the last two decades, besides the originally described action as an inhibitor of the ATP hydrolytic activity of the enzyme driven by a severe drop of the inner mitochondrial membrane potential ($\Delta\Psi_m$) (i.e. the main component of $\Delta\mu_{H^+}$), several other functions have been ascribed to IF_1 (Table 1).

The present review will describe and discuss two main aspects of proposed roles for IF_1 in cancer. First, the controversial modulation of the ATP synthase activity in cancer cells [5, 21-23] and second, the contribution of IF_1 to modulate reactive oxygen species (ROS) levels and associated protection of cancer cells from apoptosis [23-27].

This will contribute the understanding of the IF_1 functions and the clarification of the molecular mechanisms through which the F_1F_o-ATPase is controlled by its endogenous inhibitor. Considering the importance of the ATP synthase, the main producer of energy in human cells to which the whole cell metabolism is strictly associated, understanding the function(s) and defining the molecular mechanism(s) of IF_1 action in cancer cells is essential to open new effective approaches to fight this disease.

Table 1 Proposed roles for IF_1

Action	Mechanism of action	Citation
IF_1 regulates the ATP hydrolysis in hepatoma 22a mitochondria	Almost all F_1F_o-ATPase in hepatoma mitochondria have IF_1 bound in a not inhibitory manner, but mitochondria preincubated with an uncoupler decreased the rate of ATP hydrolysis, indicating activation of IF_1	Chernyak et al. 1991 [28]
IF_1 contributes to the myocardial ischemic preconditioning	IF_1 contributes to the protective mechanism of myocardial preconditioning during the critical phase of the very early reperfusion of the ischemic heart by slowing down the reactivation of the ATP synthase	Bosetti et al. 2000 [29]
IF_1 modulates the activity of angiostatin on the endothelial cell surface	IF_1 binding to surface F_1F_o-ATPase conserves ATP particularly at low extracellular pH and it could contribute to modulate angiogenesis	Burwick et al. 2005 [30]
IF_1 promotes dimerization of the mitochondrial F_1F_o-ATP synthase	Overexpression of IF_1 in AS-30D hepatoma mitochondria correlated with an increase in the Dimer/Monomer (D/M) ratio of the ATP synthase. Removal of IF_1 increased the F_1F_o-ATPase activity and decreased the D/M ratio of the ATP synthase	García et al. 2006 [31]
IF_1 protects the liver from sepsis in rats	It was observed that suppression of IF_1 expression caused elevated mitochondrial F_1F_o-ATPase activity, contributing to the bioenergetic failure in the liver during late sepsis	Huang et al. 2007 [32]
IF_1 could play a role in regulating different mitochondrial functions in proximal and distal tubule of nephrons	By measuring the mitochondrial membrane potential and the respiration both in normal conditions and in presence of a specific inhibitor, the Authors hypothesized the role of IF_1 in the regulation of the mitochondrial function of different parts of the kidney tubules	Hall et al. 2009 [33]
Atpif1 deficiency reduces the synthesis of haem	Atpif1 regulates the catalytic efficiency of vertebrate ferrochelatase to synthesize haem and it loss may result in congenital sideroblastic anaemias and mitochondriopathies	Shah et al. 2012 [34]
IF_1 limits the apoptotic signalling cascade by preventing mitochondrial remodelling	IF_1 contributes to the structural re-arrangement of mitochondria during apoptosis, modifying the mobilization of Cytochrome c and so altering the downstream cascade of events	Faccenda et al. 2013 [35]
IF_1 as an essential factor for PARK2 recruitment and mitophagy	In uncoupled mitochondria IF_1 promotes collapse of $\Delta\Psi m$ and activation of the PINK-PARK2 mitophagy pathway by blocking the ATPase activity of the F_1F_o-ATP synthase	Lefebvre et al. 2013 [36]
IF_1 plays a role in the glucocorticoids cellular stress-induced program	IF_1 coimmunoprecipitate with the glucocorticoid-induced protein kinase, Sgk-1, at neutral pH, allowing mitochondria to contribute the cellular stress-induced program of the hormones	O'Keeffe et al. 2013 [37]
IF_1 is suggested to promote metabolic preconditioning in neurons	An IF_1 mutant form inhibits the F_1F_o-ATPase increasing $\Delta\Psi m$ and ROS that promotes Akt/p70S6K and PARP repair pathways and Bcl-xL protection from cell death	Formentini et al. 2014 [38]

Reciprocal activation of IF_1 and NF-kB drive Hepatocellular Carcinoma Angiogenesis and Metastasis	IF_1 promoted Snai1 and vascular endothelial growth factor (VEGF) expression by way of activating nuclear factor kappa B (NF-kB) signaling and $IF_1$was directly transcribed by NF-kB, thus forming a positive feedback signaling loop	Song et al. 2014 [39]
Inhibition of $ATPIF_1$ Ameliorates Severe Mitochondrial Respiratory Chain Dysfunction in Mammalian Cells	A genome-wide genetic screen in haploid human cells revealed that loss of $ATPIF_1$ strongly protects against antimycin-induced ETC dysfunction and cell death by allowing for the maintenance of mitochondrial membrane potential. The Authors suggested that inhibition of $ATPIF_1$ can ameliorate severe ETC dysfunction in mitochondrial pathology	Chen et al 2014 [40]
IF_1 is a potential prognostic marker for the migration and invasion of glioma	IF_1 may promote glioma cell migration and invasion through the nuclear factor-κB (NF-κB)/Snai1 axis since IF_1 knockdown inhibited the expression of NF-κB and Snai1, and led to increased E-cadherin expression and reduced vimentin expression.	Wu et al. 2015 [41]
IF_1 mediates the tumor cell cycle	IF_1 knockdown on breast carcinoma cell proliferation suppresses cyclins and cyclin-dependent kinases related to G1/S transition and then induction of G0/G1 arrest	Wei et al. 2015 [42]
IF_1 is a prognostic marker and contributes to proliferation and invasion of human gastric cancer	IF_1 knocked down in gastric cancer cell line SGC-7901 led to a significant reduction of cell proliferation and to the increase of cell death. IF_1-expressing and -silenced SGC-7901 cells implanted in nude mice confirmed the in vitro results	Yin et al. 2015 [43]
ATPase IF_1 expression is a prognostic factor in non-small cell lung cancer	Elevated expression of IF_1 may be associated with lymph node metastasis of non-small cell lung cancer and serves as an independent prognostic and recurrent indicator for the patients	Gao et al. 2016 [44]
IF_1 contributes the control of mitochondrial remodelling and apoptosis	IF_1 activates a pro-oncogenic mechanism of evasion of apoptosis occurring through optic atrophy 1 (OPA1)-dependent maintenance of cristae shape and preservation of redox balance	Faccenda et al. 2017 [45]
IF_1 seems to contribute the benzopyrene-induced reprogramming of cancer cells to the Warburg effect	The molecular mechanism reported is mainly based on the IF_1 expression increase induced by benzopyrene	Hardonnière et al. 2017 [46]
Role of IF_1 in mitohormesis	The authors hypothesize that the actions of IF_1 on the F_1F_o-ATPase are the bases through which the enzyme modulates signaling pathways that allow mitohormesis response (i.e. on ATP, ROS and target of rapamycin)	Esparza-Moltó et al. 2017 [47]
IF_1 sustains migration, invasion and proliferation pancreatic ductal adenocarcinoma cells	IF_1 in both pancreatic acinar cells and pancreatic ductal adenocarcinoma allow to maintain ΔΨm and ATP levels in conditions of chemical hypoxia	Tanton et al. 2018 [48]
Reduction of IF_1 leads to visual impairment in vertebrates	Alterations in IF_1 expression lead to visual impairments in both zebrafish larvae and mice that are associated with an observed interlink between IF_1 level and OPA1 processing	Martín-Jiménez et al. 2018 [49]
IF_1 regulates glucose-stimulated insulin secretion	IF_1 in pancreatic β-cells is bound to the ATP synthase also under normal physiological conditions and its silencing increases insulin secretion over a range of glucose concentrations	Kahancová et al. 2018 [50]
IF_1 inhibition improves the antitumor effect of YC-1 against hepatocellular carcinoma	Limiting the action of IF_1 the p-STAT3 level decreased, determining increased expression of the tumor suppressor gene E-cadherin	Ding et al. 2018 [51]
Overexpression of IF_1 acts as a tumor suppressor in colorectal carcinomas	Overexpression of mitochondrial IF_1 prevents metastatic disease of colorectal cancer by enhancing anoikis and tumor infiltration of natural killer cells	González-Llorente et al. 2019 [52]
IF_1 contributes the control of Ca^{2+} homeostasis	IF_1 is required for maintaining mitochondrial Ca^{2+} homeostasis by regulating the expression of the Ca^{2+} uniporter (MCU) via the AMPK/CREB pathway	Faccenda et al. 2021 [53]

IF_1 is overexpressed in human cancer

The first evidence of overexpressed IF_1 in mammalian cancer was provided by Luciakova and Kuzela [54] who observed that the content of F_1-ATPase in mitochondria of Zajdela hepatoma and Yoshida sarcoma did not differ significantly from that measured in mitochondria of rat liver and heart, whereas the content of IF_1 revealed that the tumor mitochondria contained 2-3-times more ATPase inhibitor than rat liver and heart. Subsequently, Capuano et al. [55] analyzing the oxidative phosphorylation enzymes in human normal and neoplastic cells confirmed the higher level of IF_1 compared to that of the F_1F_o-ATPase. In a more recent paper, Bravo et al. [56] agreed with previous studies and reported a 2-fold enhancement of IF_1 content in hepatoma AS-30D submitochondrial particles compared to normal rat liver controls. Finally, Cuezva et al. [5, 25, 26] reported IF_1 to F_1F_o-ATPase ratios greater than two in different types of human and cancer cells. High levels of IF_1 in a number of cancers have been linked to increased glycolysis, resistance to cell death, increased migration, and proliferation [39]. These connections and the need to define the role of this overexpressed protein in many cancers pushed a number of researchers to deeper investigate the function(s) and the mechanism(s) of action of IF_1 in cancer cells. Only with a clear-cut identification of the molecular mechanisms of action of a pro-oncogenic factor as IF_1, will the scientific basis be established for drug development to oppose proliferation and diffusion of cancer cells, and resistance to the action of drugs.

IF_1 and metabolic reprogramming in cancer cells

Cancer cell metabolism is extremely heterogeneous and it might promptly change to adapt and suitably interact with the microenvironment. The heterogeneity of the metabolism, the capability to interact with different microenvironments, and the great proliferation rate characterizing cancer cells require reprogramming their metabolism compared to parental, non-transformed cells. Alterations of cellular metabolism are considered crucial hallmarks of cancer and are essentially determined by mutational events that combine altering multiple and fundamental signalling pathways [57-60]. Here, the energetic metabolism of cancer cells, in particular the modulation of the activity of mitochondrial F_1F_o-ATPase, that can be affected by IF_1, will be addressed.

It is well known that many cancer cells adopt the so-called aerobic glycolysis, “Warburg effect” [61], consisting in a higher glycolytic flux (2-17 fold) compared to normal cells in the presence of physiological concentrations of oxygen [62]. Aerobic glycolysis is certainly induced by oncogenes such as MYC and the hypoxia inducible factor HIF-1 that increase the level of glycolytic enzymes and inhibit pyruvate oxidation, therefore limiting the rate of the tricarboxylic acid cycle and the coupled rate of oxidative phosphorylation

(OXPHOS) [63, 64]. This allows cancer cells to meet both the great requirement of glycolytic intermediates to produce mass, reducing equivalents (NADPH), and supply ATP to support the high anabolism necessary for the great proliferation rate of cancer cells [65, 66]. According to the above evidence and considering the tight control of ADP phosphorylation by the mitochondrial membrane potential, $\Delta\Psi_m$, one might assume that IF_1 does not play any significant role in mediating the shift of cancer cells to an enhanced aerobic glycolysis. Nonetheless, in several papers, proposing the involvement of IF_1 in the modulation of ATP synthesis is proposed. Indeed, over the past decade Cuezva and coworkers reported that IF_1 is as an important player in cancer cell acquisition of the Warburg metabolic phenotype [5, 26, 67]. The first of these studies was based on assays carried out in mouse hepatoma (Hepa 1–6), human hepatocarcinoma (HepG2), and cervix carcinoma (HeLa) cells in which IF_1 was transiently overexpressed or silenced [5]. The authors observed that overexpression of IF_1 in cells expressing low levels of IF_1 triggered the upregulation of aerobic glycolysis and the inhibition of state 3 respiration rate (ADP-stimulated respiration rate) with concurrent mitochondrial hyperpolarization. In addition, the authors observed that IF_1 overexpression mimicked the effects of cell treatment with the ATP synthase inhibitor oligomycin, and from this observation drew the conclusion that IF_1 controls the activity of oxidative phosphorylation by inhibiting the F_1F_o-ATPase under the physiological conditions of ATP synthesis. Subsequently, the same authors reported similar findings with human colon tumor cells (HCT116) [25]. Recently, Kahancova et al. [50] inferred that IF_1 inhibits the ATP synthesis rate in a model of pancreatic β-cells. Their hypothesis was based on the observation that both ATP concentration and state 3 respiration decreased in cells overexpressing IF_1, and that IF_1 silencing resulted in the opposite effect. However, in neither set of experiment was the synthetic or hydrolytic activity of the F_1F_o-ATP synthase determined directly *in situ*, although it would have been crucial to do so. In addition, both overexpression and silencing of IF_1 were transient, and therefore the cellular content of IF_1 cell content was heterogeneous and cells experienced an unstable condition that is far from an unperturbed steady-state.

Conversely, other researchers, working independently, reported that IF_1 inhibited the ATP synthase complex only when it works in reverse, hydrolyzing ATP, a condition widely described in the earliest literature as above mentioned. Indeed, Campanella et al. [22] in HL-1, C2C12, and HeLa cells observed that IF_1 overexpression increased the formation of dimeric ATP synthase complexes that was associated with an increased F_1F_o-ATP synthase activity. Although the authors approached the study by means of an indirect method based on the assay of $\Delta\Psi_m$ in cells containing or lacking IF_1, under conditions of fully active or oligomycin-inhibited oxidative phosphorylation, the decrease in $\Delta\Psi_m$ induced by IF_1 was confirmed subsequently by others [14, 21, 23, 48, 68] directly measuring the ATP synthesis rate using reliable luminometric assays. The presence of IF_1 never caused an inhibition of the ATP synthesis rate by OXPHOS in different cancer cell lines. In addition, Sgarbi et al. [14] showed that

also in severe hypoxia (up to 0.1% O_2) osteosarcoma 143B cells synthesized ATP, although at a lower rate compared to normoxic conditions, but the rate was unchanged whether IF_1 was expressed or silenced. These results were in line with the $\Delta\Psi_m$ measurements carried out in IF_1-expressing and -silenced cells and crucially, $\Delta\Psi_m$ values were unaffected by the addition of oligomycin, thus excluding any contribution to $\Delta\Psi_m$ from ATP hydrolysis. Under anoxia-mimicking conditions (uncoupling conditions) the presence of IF_1 allowed the $\Delta\Psi_m$ collapse, whilst in IF_1-silenced cells it was significantly preserved at the expenses of glycolytic ATP [27]. It should be stressed, that in osteosarcoma cells the expression of IF_1 was sufficient to inhibit the entire ATPase activity, as shown by measuring the ATP level of oligomycin-inhibited and control cells exposed to an uncoupler [14]. Similar experiments were performed also using a stably IF_1-silenced human embryonic kidney cell lines (HEK-293), and they fully confirmed the results obtained in the osteosarcoma experiments. IF_1-induced $\Delta\Psi_m$ collapse matched results obtained under uncoupling conditions in HeLa cells [36]. Finally, ATP hydrolysis by ATP synthase reconstituted into phospholipid vesicles was, as expected, inhibited by IF_1, and ATP synthesis could only be observed in the presence of IF_1 to inhibit low levels of uncoupled enzyme in the vesicles, which otherwise would destroy any newly synthesized ATP [68]. The clear conclusion from the above investigations is that IF_1 is excluded from contributing to the metabolic reprogramming of cancer cells towards a Warburg effect under normal and even in hypoxic conditions.

Besides the above experimental results, the inhibition of the ATP synthase by IF_1 under physiological conditions could be excluded in principles. As described briefly in the Introduction section, the molecular mechanism of the IF_1 mediated-ATP hydrolytic inhibition has been exhaustively clarified [18]. As all recognize, the ATP synthase complex is a reversible enzyme: it can catalyze both the synthesis of ATP driven by proton translocation from the IMS to the matrix and the reverse reaction pumping protons towards the IMS driven by ATP hydrolysis. Clearly, the direct and the reverse reaction proceed through mirror mechanisms. IF_1 cannot inhibit the synthesis of ATP by the same mechanism adopted to block ATP hydrolysis. Indeed, as widely demonstrated, conditions leading to ATP synthesis (i.e. proton motive force onset and returning to matrix pH physiological value) promoted IF_1 release from the enzyme, as occurs in reperfusion following an ischemic insult [4, 29, 69, 70]. Therefore, the proposed role of IF_1 as inhibitor of ATP synthesis by OXPHOS lacks a clear-cut molecular mechanism.

Finally, some years ago, it was reported that IF_1 in mitochondria can be phosphorylated and dephosphorylated at residue Ser-14 and that the only dephosphorylated protein binds to and inhibits the mitochondrial ATP synthase [67]. This observation could be of considerable scientific and translational interest. However, it is noteworthy that Ser-14 is not an absolutely conserved residue in mammalian IF_1 proteins, and therefore this proposed mechanism of regulation requires further investigation.

IF_1 modulates reactive oxygen species in cancer cells

Another important and undefined aspect of the overexpression of IF_1 in cancer cells concerns its possible role in homeostasis of reactive oxygen species (ROS) homeostasis, given the roles of ROS in cancer pathogenesis and expansion. Low levels of ROS are modulators at transcriptional sites and support cancer development and progression [71, 72]. Elevated levels of ROS have been observed generally in cancer cells, and increased levels of mitochondrial ROS were shown to promote cell proliferation, survival, and migration [73-75]. For these reasons the targeting of mitochondria to the disruption of cell redox communication might be considered to be promising for the development of an anticancer strategy [76]. Indeed, high levels of ROS are toxic to cells [75] and the induction of their elevation by therapeutic intervention could represent a valid approach to cancer therapy, as supported by various studies [77-79]. Therefore, ROS might be considered to be a double-hedged swords in cancer. This section will be focused mainly on the association between IF_1, the levels of ROS, and resistance to cell death in cancer cells.

Campanella et al. [24] analyzed and compared the rate of oxidation of dihydroethidium (DHE) in HeLa cells with IF_1 either transiently overexpressed ($+IF_1$) or silenced ($-IF_1$) in order to evaluate the respective ROS level and to assess the possible role of IF_1 in regulating autophagy. The experiments showed that the expression of IF_1 played a significant role in defining both resting rates of ROS generation and cellular content of mitochondria, allowing the conclusion to be drown that IF_1 diminished mitochondrial ROS generation, limiting autophagy which was significantly increased by knockdown of IF_1. The action of IF_1 as a decreasing factor of ROS level in cells found a strong corroboration by the work of Fujikawa et al. [23] who showed that the ROS level Hela cells where IF_1 had been knocked down stably was double the level in controls, and associated this to the higher $\Delta\Psi_m$ measured in IF_1 knockdown cells. However, it is noteworthy that although ROS levels increased in IF_1 knockdown HeLa cells, their growth was unaffected. Similar results were obtained in osteosarcoma 143B cells in our laboratory [21]. Further support to the assertion that IF_1 is capable to limiting ROS levels in cancer cells came from a comparison of ROS levels in osteosarcoma cells grown under normoxic and severe hypoxic conditions (0.5% O_2) [27]. By means of the CellROX fluorescent probe which senses peroxides and other oxidants, total ROS were assayed in osteosarcoma 143B cells containing IF_1 and in the same cells where IF_1 had been stably silenced. Under both normoxic and hypoxic conditions the presence of IF_1 clearly correlated with a significantly lower level of ROS than in its absence. Concurrently, this work also showed that in osteosarcoma IF_1-silenced cells, the superoxide level in mitochondria as measured with the MitoSOX Red probe, was higher than that in cells where IF_1 was being expressed. Interestingly, the level of ROS in hypoxic osteosarcoma cells has decreased significantly compared to the normoxic conditions whether or

not IF_1 was expressed. These findings are consistent with previously reported results obtained by analyzing the O_2 tension-dependence of ROS levels in human fibroblasts [80].

However, a number of papers reported dissimilar results, where in contrast, the levels of ROS were enhancement by increases in the levels of IF_1. Thus, Formentini et al. [25] observed that upregulation of IF_1 in HCT116 cells triggered a significant increase in the production of superoxide, as evaluated by the MitoSOX probe and that a pretreatment with the mitochondrial antioxidant MitoQ [81] quenched superoxide production in IF_1 overexpressing cells, and also ameliorated the basal level of mitochondrial ROS in control cells. The authors also reported that overexpression of IF_1 did not affect the levels of cellular hydrogen peroxide or the GSH/GSSG ratio. The latter observations seem quite in contrast due to the well-known action of the superoxide dismutases, which are ubiquitously present in human cells, to convert the superoxide radicals into hydrogen peroxide with high rate and efficiency [82]. On the basis of their findings, the authors proposed the following sequence of events: IF_1 overexpression → upregulation of the level of ROS (o superoxide) → promotion of NF-kB transcriptional activation via IkBα downregulation and phosphorylation → increase rate of proliferation, invasiveness and Bcl-xL-mediated resistance to drug-induced apoptosis. The role of IF_1 in conferring resistance to staurosporin-induced apoptosis was supported by experimental evidence carried out in different types of cancer cells [25, 26, 45]. However, according to Faccenda et al. [45] it occurred independently from activation of the NF-kB pathway. Indeed, dimeric IF_1 could stabilize the oligomerization of the ATP synthase binding between two monomeric ATP synthases in two adjacent dimers [10]. This might prevent OMA1-induced OPA1 processing that impedes mitochondrial cristae re-modelling [45, 53], thus inhibiting cytochrome c release and the activation of the intrinsic apoptotic pathway. OMA1 is a mitochondrial inner membrane metallopeptidase and its activation may be promoted by various stress cellular signals as low cellular ATP level, altered $\Delta\Psi_m$ and increased oxidative stress [83]. As supported by experimental data, in addition to its direct role in mitochondria morphology and cristae shaping, IF_1 could counteract apoptosis and confer chemoresistance to cancer cells by preserving ATP level that in turn contributes to the maintenance of an efficient antioxidant defense system (i.e. GSH and peroxiredoxins) and low cellular level of ROS [45]. To conclude, we should point out that in cancer cells the link between ROS and IF_1 will be revealed only when the action of IF_1 on the ATP synthase has been established definitively.

Concluding remarks

Three main points are clear-cut and generally agreed upon by the scientific community concerning the role of IF_1 in cancer cells: first, IF_1 is overexpressed in many cancer cells; second, IF_1 displays a pro-oncogenic potential; and third, IF_1 inhibits the ATP hydrolytic activity of the mitochondrial F_1F_o-ATPase

under conditions of collapsed $\Delta\mu_{H^+}$ in cells, such as occurs under conditions of severe O_2 deficiency such as is found in solid tumors, where oxygen deficiency occurs. In addition, other molecular mechanisms have been advanced to explain the pro-oncogenic activity of IF_1, but until now, the experimental findings are contradictory and certainly insufficient. In particular, the proposed role of IF_1 in metabolic reprogramming of cancer cells under the condition of F_1F_o-ATPase synthetizing ATP requires further experimental validation, as this facet is advanced as a molecular explanation for the role of IF_1 in tumorigenesis. An understanding of the biological roles and mechanism(s) of the interaction of IF_1 with the ATP synthase is crucial for developing valuable diagnostic and therapeutic targets for cancer and possibly other human diseases.

Perspectives

(i) Defining the molecular mechanism(s) of the pro-oncogenic action of IF_1, the main regulator of mitochondrial ATP synthase, is essential if the protein is to be developed as a target for cancer therapy.

(ii) Our current knowledge of the molecular action of IF_1 is that it acts to prevent ATP hydrolysis by the ATP synthase working in reverse (for example during anoxia). In dispute is the role of IF_1 in normoxic cancer cells since it has been proposed that IF_1 also inhibits the ATP synthase activity. Accordingly, divergent modulation of intracellular ROS homeostasis has been reported.

(iii) Future reproducible experiments are required to resolve the currently opposed views concerning the action of IF_1 in normoxic cancer cells. These experiments should also identify both the site of binding of IF_1 to the enzyme and its molecular mechanism of action.

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G.S. (Giancarlo Solaini) and A.B. (Alessandra Baracca) wrote the manuscript; All authors (G.S. A.B. and G.Sg., Gianluca Sgarbi) contributed to the discussion, writing, review and editing the manuscript.

Declaration of Interests:
The authors declare no conflict of interest.

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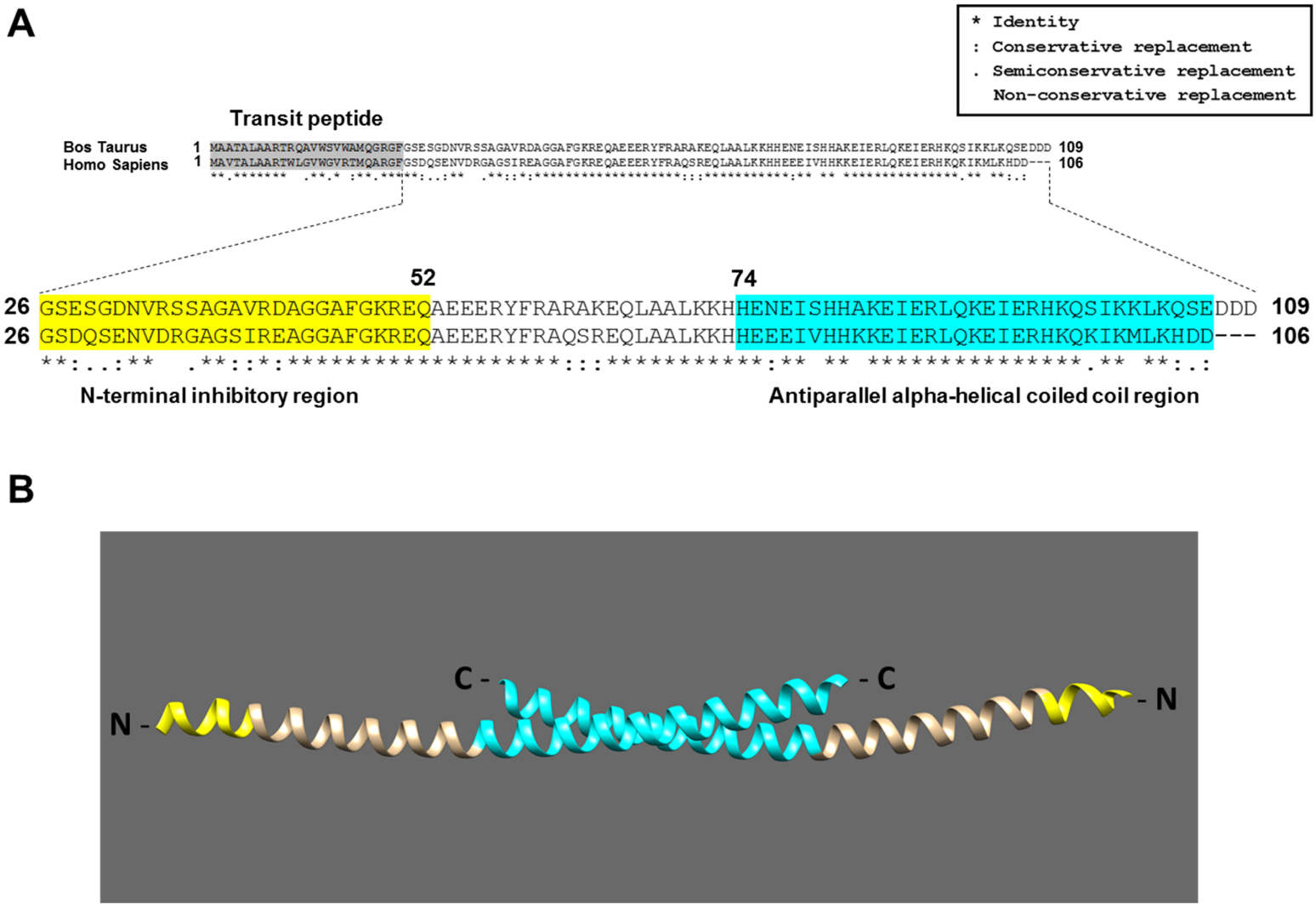
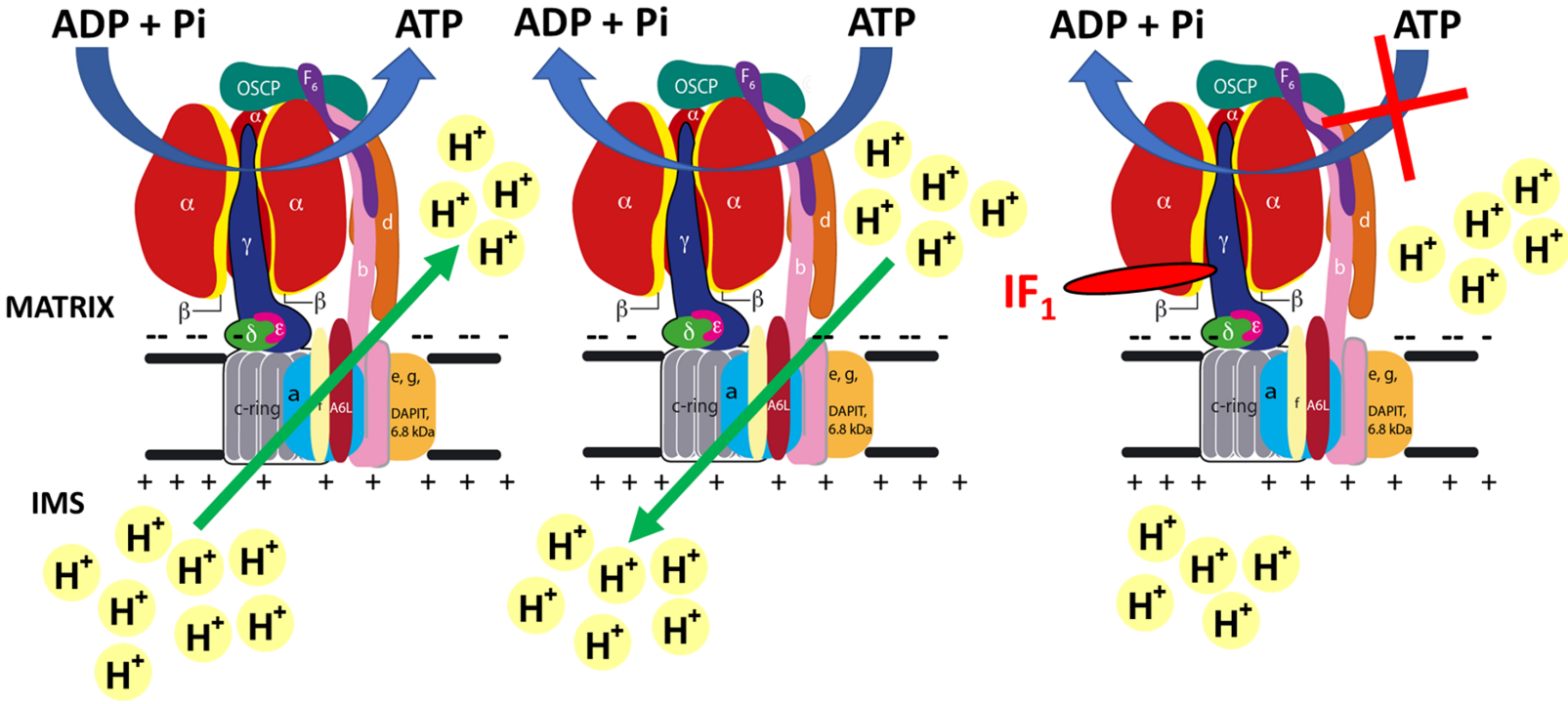

A
* Identity
: Conservative replacement
. Semiconservative replacement
Non-conservative replacement
Transit peptide
Bos Taurus 1 MAATALAARTRQAVWSVWAMQGRGFGSESGDNVRSSAGAVRDAGGAFGKREQAEEERYFRARAKEQLAALKKHHENEISHHAKEIERLQKEIERHKQSIKKLKQSEDDD 109
Homo Sapiens 1 MAVTALAARTWLGVWGVRTMQARGFGSDQSENVDRGAGSIREAGGAFGKREQAEEERYFRAQSREQLAALKKHHEEEIVHHKKEIERLQKEIERHKQKIKMLKHDD--- 106
52
74
26 GSESGDNVRSSAGAVRDAGGAFGKREQAEEERYFRARAKEQLAALKKHHENEISHHAKEIERLQKEIERHKQSIKKLKQSEDDD 109
26 GSDQSENVDRGAGSIREAGGAFGKREQAEEERYFRAQSREQLAALKKHHEEEIVHHKKEIERLQKEIERHKQKIKMLKHDD--- 106
N-terminal inhibitory region
Antiparallel alpha-helical coiled coil region
B
C -
- C
N -
- N

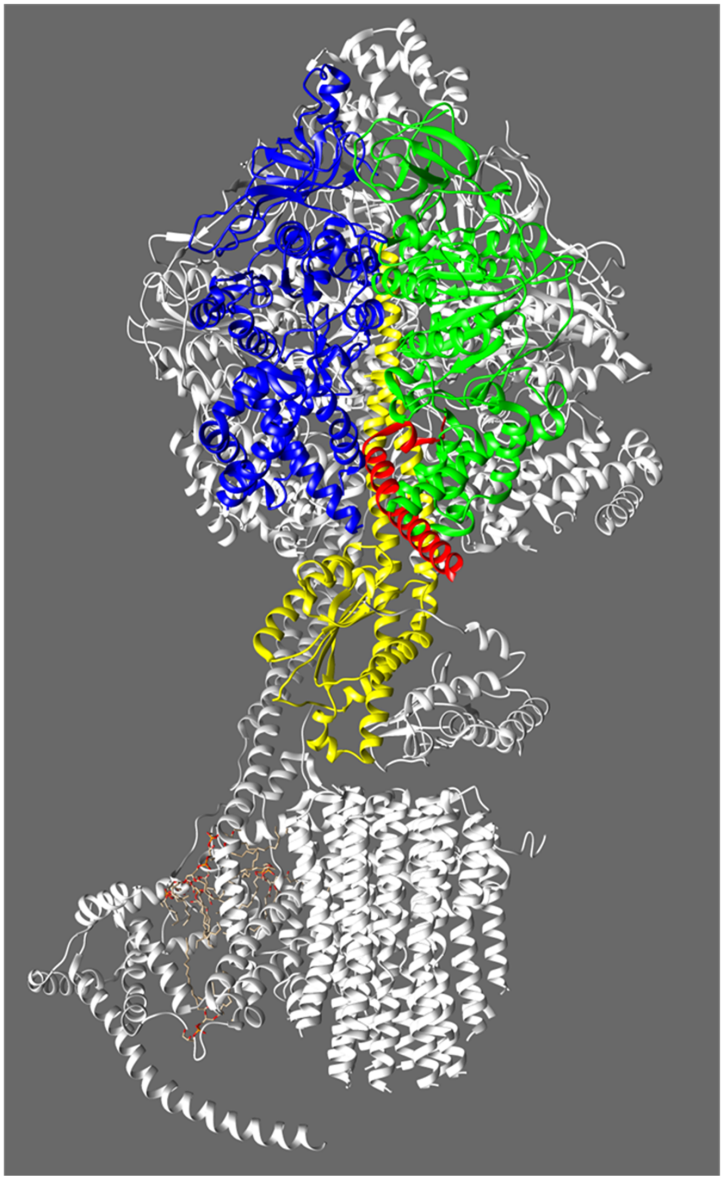


ATP synthesis

ATP hydrolysis (anoxia and IF1 absent)

Inhibited ATP synthase (anoxia)

A



B