Review

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The role of mitochondrial ATP synthase in cancer

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Abstract: The mitochondrial ATP synthase is a multi-subunit enzyme complex located in the inner mitochondrial membrane which is essential for oxidative phosphorylation under physiological conditions. In this review, we analyse the enzyme functions involved in cancer progression by dissecting specific conditions in which ATP synthase contributes to cancer development or metastasis. Moreover, we propose the role of ATP synthase in the formation of the permeability transition pore (PTP) as an additional mechanism which controls tumour cell death. We further describe transcriptional and translational modifications of the enzyme subunits and of the inhibitor protein IF1 that may promote adaptations leading to cancer metabolism. Finally, we outline ATP synthase gene mutations and epigenetic modifications associated with cancer development or drug resistance, with the aim of highlighting this enzyme complex as a potential novel target for future anti-cancer therapy.

Keywords: ATP synthase; cancer; mitochondria; permeability transition.

Introduction

Cancer cells undergo alterations in metabolic pathways that enable a number of regulatory processes, such as the enlargement of biosynthetic precursor pools, the production of signalling molecules or the generation of metabolites for post-translational or epigenetic modifications (Cairns et al. 2011; Wallace 2012). In many cancers, metabolic reprogramming mainly involves enhanced glucose uptake and glycolysis, resulting in the generation of ATP and lactic acid in the cytosol, even though mitochondria are functional and under normoxia, a metabolic condition that is termed *Warburg effect* (Warburg 1956). In rapidly growing tumours where hypoxia occurs instead, increased glycolysis can fuel sufficient ATP production and support bioenergetic homeostasis (Seyfried and Shelton 2010). Beyond the increase in glycolysis, many cancers additionally or alternatively display glutaminolysis and fatty acid oxidation in the mitochondria, generating tricarboxylic acid (TCA) cycle intermediates which are often used for anabolic and bio-energetic purposes (Lunt and Vander Heiden 2011). The requirement of functional mitochondria in cancer cells has been confirmed by mitochondrial (mt) DNA depletion (ρ0) studies. Cancer ρ0 cells show a decreased growth rate, reduced colony formation *in vitro*, and delayed tumour development in nude mice (Cavalli et al. 1997; Desjardins et al. 1985, 1986; King and Attardi 1989; Magda et al. 2008; Morais et al. 1994; Weinberg et al. 2010). Besides energy production by oxidative phosphorylation, functional mitochondria control many vital parameters in cancer cells, such as the redox status, the formation of reactive oxygen species (ROS) and the cytosolic Ca^{2+} levels. Importantly, changes in these parameters can impinge on biosynthetic pathways, cellular signal transduction, and apoptosis, through opening of the permeability transition (PT) pore (PTP) (Wallace 2012), the mitochondrial mega-channel activated by Ca^{2+} and oxidative stress (Bernardi et al. 2015; Giorgio et al. 2018; Kaludercic and Giorgio 2016).

The rate of oxidative phosphorylation is regulated by the activity of the mitochondrial ATP synthase (Boyer 1997). This enzyme is a large multi-subunit complex of 600 kDa. Electron microscopy of native mitochondrial membranes demonstrated that ATP synthase is associated in dimers that form long rows of oligomers in the inner membrane (Blum et al. 2019; Davies et al. 2011; Strauss et al. 2008). The monomeric enzyme (Figure 1A) is organized into a catalytic (F1) and a membranous (Fo) part linked by central and peripheral stalks. This large complex is a rotary motor. In tissues and cells, it catalyses the

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synthesis of ATP at the expense of the proton motive force generated by the respiratory chain in the presence of oxygen (Senior 2007), whereas it works in the direction of ATP hydrolysis when mitochondria are sufficiently deprived of oxygen and the membrane potential decreases (Chinopoulos and Adam-Vizi 2010; Di Pancrazio et al. 2004; Rouslin and Broge 1989). The core mammalian enzyme consists of 15 conserved subunits, the F1 subunits α–ε and the Fo subunits a–g, OSCP, A6L, and F6 (Wittig and Schägger 2008). In the Fo sector, single copies of subunits b, d, F6, and OSCP form the peripheral stalk, which extends from the top of the catalytic α3β3 sub-complex along its external surface down into the membrane domain (Dickson et al. 2006). The so-called minor subunits e, f, g, and A6L all span the membrane (Collinson et al. 1994); their role in maintaining dimer/tetramer stability has been established in mammals (Habersetzer et al. 2013; He et al. 2018) and in yeast (Arselin et al. 2004; Hahn et al. 2016). Recently, the structural organization of these minor subunits and the two hydrophobic proteins DAPIT and 6.8PL has been determined by Cryo-EM in a mammalian tetrameric enzyme (Gu et al. 2019). Furthermore, the mitochondrial enzyme complex can associate with the inhibitor protein IF1 (Gledhill et al. 2007). It has been demonstrated that IF1 binding to the catalytic sector of F1 in a 1:1 stoichiometry fully inhibits the ATPase activity and that such inhibition is optimal at low pH and low mitochondrial membrane potential, conditions occurring under ischaemia (Di Pancrazio et al. 2004). Finally, a novel function of ATP synthase in the formation of the mitochondrial mega-channel, which causes cell death through the release of pro-apoptotic factors, has been proposed. The finding that ATP synthase can reversibly undergo a Ca^{2+}-dependent transition to form the PTP (Giorgio et al. 2013; Urbani et al. 2019), opens new perspectives on the role of ATP synthase in the cancer field.

Here, we describe the involvement of ATP synthase in metabolism and progression of different cancer models by highlighting its functional roles as an enzyme and a PTP forming site. Moreover, we discuss the relevance of the inhibitor protein IF1 in cancer development. Finally, we list several mutations, transcriptional changes or epigenetic modifications at the ATP synthase gene level, that are responsible for the metabolic rewiring during cancer development or are associated to drug resistance.

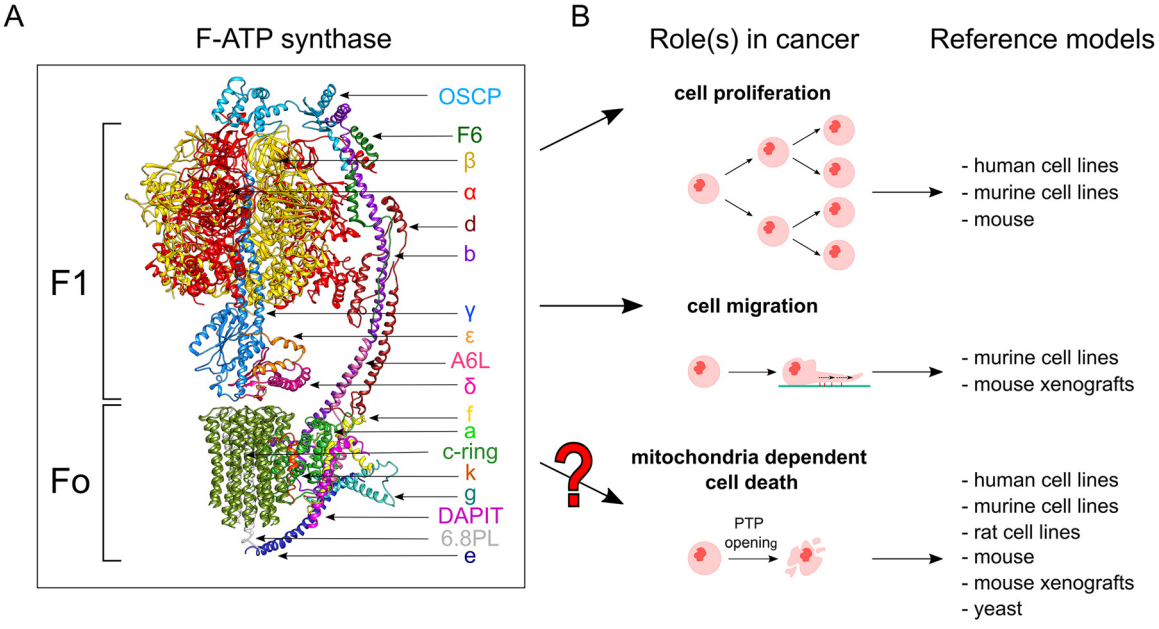

Figure 1: Mitochondrial ATP synthase monomer and its roles in cancer. (A) The subunits of the ATP synthase are mapped onto the monomeric structure of the mammalian ATP synthase from (Gu et al. 2019) (PDBid: 6J5K). The upper part contains the subunits of the F1 catalytic domain: the catalytic subunits α(3) and β(3), and the three central stalk subunits γ, δ and ε. The Fo membrane domain contains: the c-ring, the a subunit, and a number of supernumerary subunits: e, f, g, A6L, DAPIT, 6.8PL and k. The peripheral stalk extends from the top of the catalytic α3β3 subcomplex along its external surface down into the membrane domain (on the right of the model) and contains the OSCP, b, d and F6 subunits. Subunits are indicated with arrows and presented in different colours. (B) Representation of possible roles of ATP synthase in cancer and the reference models in which the enzyme involvement has been studied are shown.

ATP synthase functions in cancer

In this paragraph, we aim at presenting ATP synthase functions that have been associated in the literature with proliferation, invasive behaviour and cell death evasion of cancer models, as summarized in Figure 1B. The different aspects of functional involvement of ATP synthase might help to understand the relevance of the modifications at the level of the inhibitor protein IF1 or specific enzyme subunits and the epigenetic adaptations described in cancer and discussed in more detail in the following paragraphs.

Most cancer cells rely on aerobic glycolysis, a phenomenon termed *Warburg effect*. In this condition mitochondria are functional, ATP synthesis and respiration are essential to ensure rapid cell proliferation. Inhibition of ATP synthesis, either directly with oligomycin, or indirectly with rotenone or antimycin A (targeting respiratory complex I or III, respectively), causes a block of proliferation rate in different cell lines derived from murine leukaemia or human glioblastoma, cervix adenocarcinoma, non-small-cell lung cancer or osteosarcoma-derived cybrid 143B models (Sullivan et al. 2015). In osteosarcoma 143B cells, the inhibitory effect of oligomycin on ATP synthesis, which in turn causes a block of proliferation, is reverted by treatment with the uncoupler FCCP. Based on this finding the authors suggested that the respiratory chain uncoupled from ATP synthesis is sufficient to sustain proliferation, although it is possible that in a real tumour, in the absence of an efficient uncoupling system, the activity of the entire OXPHOS system, including ATP synthesis, is necessary.

Another evidence suggests that the electron transport chain has an essential role in cell proliferation by enabling the biosynthesis of aspartate (Birsoy et al. 2015). This result was achieved by a CRISPR-based negative selection screen for genes in human Jurkat leukaemic T-cells. Genes whose loss potentiates the anti-proliferative effects, due to mild inhibition of mitochondrial respiration, were identified. Even though respiratory chain inhibition impacts many processes, the supplementation of media with aspartate alone, or the expression of an aspartate transporter in cells, was sufficient to allow respiration-defective cells to proliferate in culture. These findings indicate the relevance of mitochondrial respiration in maintaining the aspartate levels during proliferation.

Although demonstrations *in vivo* are not available on the role of ATP hydrolysis in sustaining tumour growth, in a osteosarcoma cell model, in which anoxia-mimicking condition is induced, IF1-silenced clones (but not their control counterpart) hydrolyse ATP (Sgarbi et al. 2018). The measurement of glucose consumption and lactate production in osteosarcoma 143B and liver carcinoma HepG2 cell models showed that both cell lines exhibited increased glycolytic metabolism when subjected to hypoxia (Chevrollier et al. 2005). The Authors suggest that in the latter condition the adenine nucleotide transporter (ANT) isoform 2 (ANT2) allows glycolytic ATP uptake into mitochondria, and consequent ATP hydrolysis by the F1 component of ATP synthase. HepG2 cells, whose ANT2 expression level is much lower than that of 143B cells are more sensitive to hypoxia, which causes a block in the G1 phase of the cell cycle (Chevrollier et al. 2005).This suggests a possible role of ATP hydrolysis in cell proliferation, although other studies show that ATP synthesis is involved in cancer cell growth (García-Aguilar and Cuezva 2018). Concerning the role of ANT in the transport of glycolytic ATP to the matrix, Maldonado and coworkers made the unexpected observation that in four cell lines derived from different cancer tissues (liver, lung, pancreatic islets and oral epithelium) the mitochondrial transporter ANT is not involved in ATP translocation, suggesting an alternative transporter in cancer cells (Maldonado et al. 2016).

A different role of ATP synthase in cancer progression is the control of cell migration and metastasis. Primary tumours and lung metastases, derived from mouse adenocarcinoma 4T1 cells implanted into the mammary fat pads of female mice, were compared by gene expression microarrays, mtDNA content and respiration. The oxidative phosphorylation increases in circulating cancer cells, compared to primary tumour-derived cells, and correlates with a migratory behaviour (LeBleu et al. 2014). In invasive cancer cells, the transcription coactivator PGC-1α is responsible for enhanced mitochondrial biogenesis and increased oxidative phosphorylation. Transcripts for the catalytic α subunit of ATP synthase are upregulated according to the increased levels of PGC-1α in 4T1-derived circulating cells. This result is supported by the effects revealed in 4T1 cells overexpressing PGC-1α that display a higher oligomycin-sensitive respiration. Interestingly, the downregulation of PGC-1α in this breast cancer cellular model not only decreases the transcript levels of the genes for the respiratory chain complexes, but also modulates cell migration negatively (LeBleu et al. 2014).

The hypothesis of ATP synthase involvement in the permeability transition pore

Since its discovery in the 1990s, the PTP has been proposed as a key effector in the mitochondrial pathways leading to cell death. PTP activation is stimulated by Ca^{2+}, P_i, free fatty

acids and ROS, while it is inhibited by Mg^{2+} and acidic pH (Bernardi et al. 2015). A mitochondrial chaperone, the peptidyl-prolyl cis-trans isomerase cyclophilin D (CyPD) sensitizes the channel to Ca^{2+}-dependent permeability transition (Giorgio et al. 2010; Nicolli et al. 1996).

PTP inhibition is an important mechanism for cancer cells to escape from apoptosis, a fact which is confirmed by different studies in human models such as prostate and osteosarcoma cancer cells (Rasola et al. 2010); lung cancer cells (Jiang et al. 2019; Zhang et al. 2018); gastric cancer cells (Pan et al. 2015); pancreatic cancer cells (Chen et al. 2014); breast cancer MDA-MB-231 cells *in vitro* and in xenograft (Tang et al. 2018).

The adaptive responses of tumours, in which Ca^{2+} and ROS are under tight control, and matrix pH frequently decreases, may desensitize the PTP opening, thereby playing a role in resistance to cell death.

The involvement of ATP synthase in the events leading to cell death was firstly described in 1998 when two yeast strains lacking subunits b or δ of ATP synthase (or treated with oligomycin) and expressing the pro-apoptotic protein BAX, showed resistance to BAX- induced mortality (Matsuyama et al. 1998). A BAX- mediated effect was also blocked in yeast strains lacking subunits β or b of ATP synthase, thus confirming the role of this enzyme in cell mortality (Gross et al. 2000). In cancer cells, inhibition of ATP synthase has been shown to delay cytochrome *c* release from mitochondria by preventing ROS production, a cell death cascade which is induced by staurosporine treatment (Santamaría et al. 2006).

Only more recently, ATP synthase has been proposed as the major component of the PTP (Antoniel et al. 2017; Carraro et al. 2018; Giorgio et al. 2017, 2013; Guo et al. 2019, 2018; Lee et al. 2016; Neginskaya et al. 2019; Urbani et al. 2019), although these finding are debated, due to persistence of inner membrane permeability in human haploid HAP-1 cells lacking different ATP synthase subunits belonging to the Fo sector (Carroll et al. 2019; He et al. 2017a,b). The controversy might arise from the involvement of an alternative Ca^{2+}-activated channel formed by ANT, which may persist in the aforementioned haploid KO cells, as shown for the *c* subunit KO HAP-1 clones in electrophysiological studies (Neginskaya et al. 2019). These clones only revealed currents with smaller conductance than that of the PTP channel, which are sensitive to the selective ANT inhibitor, bongkrekic acid (Neginskaya et al. 2019). However, further studies aimed at characterizing the effects of point mutations on ATP synthase residues and affecting PT are required to clarify this debate.

So far, the definition of ATP synthase residues that are involved in PTP modulation through Ca^{2+}-, ROS- and pH-dependent mechanisms (Antoniel et al. 2017; Giorgio et al. 2018, 2017; Kaludercic and Giorgio 2016), might open new perspectives for therapeutic intervention in cancer.

High ROS have been linked to cancer incidence in numerous studies (Liou and Storz 2014) and are kept under control in tumours by enhancing anti-oxidant defences (DeNicola et al. 2012). This is particularly important in early tumorigenic phases, whereas at later neoplastic stages high ROS levels could be tolerated, as they increase cell motility and malignancy of the growing neoplasm (Trachootham et al. 2009). In the early tumour formation, PTP induction can be a key effector of oxidative stress-induced death, that may counteract tumour progression (Zou et al. 2017). Mitochondrial ROS/RNS can induce post-translational modifications at the level of ATP synthase subunits α, β, γ, OSCP and d, as previously described (Kaludercic and Giorgio 2016). Modifications of critical residues on these subunits may play a crucial role in favouring PTP opening (which is activated by ROS) in a pro-oxidative therapy. However, some of these modifications are known to inhibit the catalytic activity of the enzyme (Kaludercic and Giorgio 2016), causing toxicity for non-cancer cells. Although effective chemo- and radio-therapies are based on ROS production (Zou et al. 2017), the identification of specific residues involved exclusively in PTP activation by ROS, might be useful to develop more selective therapeutic strategies.

Mitochondrial Ca^{2+} is also important in tumour signalling, as shown by treating C2C12 myoblasts with either ethidium bromide (to reduce the mtDNA content by up to 80%) or with the uncoupler CCCP (Amuthan et al. 2001). Both treatments cause a drop in mitochondrial membrane potential which decreases the energetic drive for mitochondrial Ca^{2+} import. Such conditions convert the C2C12 cells from being non-tumorigenic to having an invasive phenotype (Amuthan et al. 2001). A possible explanation for the above finding might reside in the fact that matrix Ca^{2+} is the key inducer of PTP opening. Low Ca^{2+} levels in mitochondria might promote a pro-survival phenotype by preventing apoptosis. The site at which Ca^{2+} binds and induces conformational changes on ATP synthase leading to PTP opening, has been characterized on the catalytic β subunit. This demonstration was achieved by introducing the T163S point mutation on this subunit, which desensitizes HeLa cells from Ca^{2+}-mediated openings of the channel and decreases apoptosis *in vivo* (Giorgio et al. 2017). Small pharmacological molecules able to promote conformational changes on ATP synthase and favouring

Ca^{2+} binding and PTP opening, might be designed to develop a novel anti-cancer strategy.

Matrix acidification is another condition that can occur during hypoxia in cancer cells, depending on mitochondrial membrane depolarization (Poburko et al. 2011). Antoniel and coauthors characterized a key role of ATP synthase OSCP subunit in the pH-dependent modulation of the PTP (Antoniel et al. 2017), which is inhibited by acidic pH. The protonation of the unique, highly conserved histidyl residue H112 of the human protein causes inhibition of the channel, and protection from cell mortality. Moreover, the same protonation of the OSCP residue causes the pH-dependent release of the mitochondrial deacetylase SIRT3 from this subunit (Yang et al. 2016). Remarkably, in the absence of bound SIRT3, the increased level of acetylation of the OSCP K47 residue favours CyPD binding (Lee et al. 2016), which is sufficient to sensitize PTP opening in heart mitochondria (Karamanlidis et al. 2013). However, the effects of CyPD binding might be secondary to the pH-dependent desensitization of the PTP in cells where the CyPD expression level is low, which is an interesting hypothesis to test in future cancer studies. Another OSCP interactor, p53, is known to modulate PTP opening in cancer, which is described together with different OSCP-targeting molecules in (Giorgio et al. 2019). In conclusion, further mutagenesis studies are needed to identify molecules that are able to activate PTP opening in cancer cells by targeting specific sites that can be modified in the tumour environment in which ROS, Ca^{2+} and pH adaptations occur.

The role of the inhibitor protein IF1 in cancer

IF1 is a physiological inhibitor of the ATP synthase and was originally thought to only inhibit mitochondrial ATP consumption by preventing ATP hydrolysis under hypoxic conditions. The N-terminal part of the inhibitor protein contains the so called minimal inhibitory region (Bason et al. 2011; Cabezón et al. 2001), that interacts with the catalytic subunits α, β and γ, in a mechanism which has been precisely characterized (Gledhill et al. 2007). The C-terminal part of IF1 is known to be involved in IF1 dimer formation through its coiled-coil structure (Cabezón et al. 2001). Dimers self-associate in tetramers in a mechanism which is mediated by critical amino acid residues, including a conserved histidine at position 49 of the bovine IF1 sequence, in an equilibrium that has been described to be dependent on pH and ionic strength (Boreikaite et al. 2019).

More recent findings indicate that IF1 can also bind to ATP synthase under normal phosphorylating conditions (Barbato et al. 2015; García-Bermúdez et al. 2015; Kahancová et al. 2018) and that its role in preventing ATP hydrolysis is observed only under near anoxia both in cardiomyocytes (Ganitkevich et al. 2010) and osteosarcoma cancer cells (Sgarbi et al. 2018). The possibility of a different mechanism of binding during ATP synthesis remains to be elucidated, since the interactions by which the IF1 N-terminal domain binds the catalytic subunits of the enzyme have only been demonstrated in the reverse direction of catalysis and require the sequential hydrolysis of two ATP molecules (Bason et al. 2014). The Authors that firstly demonstrated a role of IF1 in inhibiting ATP synthesis (García-Bermúdez et al. 2015), also showed that IF1 binding to the enzyme is subjected to a stringent posttranslational regulation of the inhibitor protein which depends on the cellular metabolic state. Human IF1 is phosphorylated at S39 by a mitochondrial cAMP-dependent protein kinase that renders the inhibitor protein unable to bind the enzyme (García-Bermúdez et al. 2015). The adaptation of the IF1 inhibitory activity to metabolic requirements seems to be particularly important in some cancers in which IF1 is sharply upregulated. Colon, lung, breast and ovarian carcinomas overexpress IF1, which became an independent prognostic marker of disease progression for patients, given that the corresponding healthy tissues are essentially devoid of the inhibitory protein under physiological conditions (Esparza-Moltó et al. 2017; Formentini et al. 2012; Sánchez-Aragó et al. 2013).

In non-small-cell lung cancer (Gao et al. 2016), bladder carcinomas (Wei et al. 2015), and gliomas (Wu et al. 2015), a high expression level of IF1 in tumours predicts a worse patient prognosis. On the contrary, in colon and breast cancer patients, a high level of IF1 expression predicts a better outcome (Sánchez-Aragó et al. 2013; Zhang et al. 2016), especially in the group of triple-negative breast cancer patients (García-Ledo et al. 2017). In the case of breast cancer, lymph node metastases show a lower expression level of IF1 when compared with the primary tumours (Kurbasic et al. 2015). This finding suggests that breast cancer cells expressing low levels of IF1 may have a higher metastatic potential, which is in full agreement with the recent finding that low IF1 expression in triple-negative breast cancer cells confers a more invasive phenotype (García-Ledo et al. 2017) and with the finding that metastatic cells derived from mouse adenocarcinoma 4T1 cells implanted *in vivo* exhibit higher oxidative phosphorylation than their primary tumours, as described above (LeBleu et al. 2014). By contrast, human tissues that express high levels of IF1 under basal physiological conditions such as endometrium, kidney, liver and stomach do not show a

relevant increase in IF1 expression during oncogenesis (Esparza-Moltó et al. 2017; Sánchez-Aragó et al. 2013).

Some of the potential roles of IF1 in cancer have been explored by generating genetically modified mouse models in which the active mutant H49K of the inhibitor protein has been selectively overexpressed in neurons, hepatocytes or colonocytes (García-Aguilar and Cuezva 2018). The overexpression of IF1 in these tissues *in vivo* is sufficient to promote metabolic reprogramming into enhanced aerobic glycolysis. These Authors suggest that the activated glycolysis results from the inhibition of ATP synthase by IF1 and a consequent activation of AMPK (Esparza-Moltó and Cuezva 2018). Moreover, the same Authors have suggested that inhibition of ATP synthase by IF1 causes a backflow of electrons through the respiratory chain enhancing the production of mitochondrial ROS, which in turn activate the canonical NFκB pathway and favour proliferation (García-Aguilar and Cuezva 2018).

These results are in line with the effects of IF1 overexpression or downregulation in cultured cells (Formentini et al. 2012; Sánchez-Aragó et al. 2013; Sánchez-Cenizo et al. 2010). On the same line, the overexpression of IF1 in pancreatic β cells leads to a substantial decrease in ATP levels and in glucose-stimulated insulin secretion (Kahancová et al. 2020), while the downregulation of the inhibitor protein in the same model causes an increase in mitochondrial respiration and in ATP levels leading to insulin secretion (Kahancová et al. 2018).

On the contrary, in osteosarcoma cells in which IF1 is stably silenced, there is a decrease in ATP synthesis and ROS formation (Barbato et al. 2015; Sgarbi et al. 2018). These Authors demonstrated that in osteosarcoma parental cells IF1 is associated to ATP synthase and its stable downregulation causes a decreased state 3 respiration (Barbato et al. 2015) together with a decreased ROS production (Sgarbi et al. 2018). This effect does not seem to be caused by a different oligomerization of ATP synthase nor by differences in the level of the respiratory chain subunits, as revealed by BN-PAGE and SDS-PAGE followed by Western blotting, respectively (Barbato et al. 2015). However, the effect of IF1 described in parental osteosarcoma cells might depend on the stabilization of mitochondrial cristae, as previously observed in a HeLa cellular model (Campanella et al. 2008), which might in turn promote respiratory chain supercomplex formation, thus favouring state 3 respiration.

Another possible role of IF1 has been associated with the resistance from apoptosis in cells overexpressing this protein. It has been suggested that the preservation of mitochondrial morphology protects cells from death, in a process which involves the pro-fusion dynamin-related protein optic atrophy 1 (OPA1) (Faccenda et al. 2017) and the Ca^{2+}-dependent recruitment of dynamin-related protein 1(Drp1) (Faccenda et al. 2013), thus limiting cristae remodelling during apoptosis. These findings are in line with the evidence that HeLa cells, in which IF1 is downregulated, are more sensitive to cell death under stress conditions (Fujikawa et al. 2012). Moreover, the employment of a zebra fish model, lacking one of the two IF1 paralogs, showed visual impairment alongside increased apoptotic bodies and neuroinflammation both in brain and retina, in a process which involves a decline in OPA1 levels (Martín-Jiménez et al. 2018). These findings are in contrast with the lack of effect in IF1 knockout mice (Nakamura et al. 2013), and with the protective effect observed in IF1 KO mice against cardiac dysfunction in overload -induced hypertrophy (Yang et al. 2017). However, the role of IF1 as an anti-apoptotic factor might be part of the mechanisms occurring during tumorigenesis and further studies on cancer cell death *in vitro* might better clarify its role.

Expression of ATP synthase in cancer

In a recent work aimed at clarifying the functional consequences of ATP synthase deficiency, human HEK293 cells with a varying content of fully assembled ATP synthase were studied (Nuskova et al. 2020). Downregulation of the F1 subunits γ, δ and ε to a 15–80% of controls revealed that glycolysis compensates for insufficient mitochondrial ATP production, while reduced dissipation of mitochondrial membrane potential leads to elevated ROS production. Both insufficient energy supply and increased oxidative stress contribute to the resulting pathological phenotype. The threshold for manifestation of the ATP synthase defect and subsequent metabolic remodelling is 30% of residual ATP synthase activity. When the content of ATP synthase drops below 30%, metabolic adaptations are sufficient to sustain proliferation under glucose-rich conditions.

Besides the modulatory effect of the inhibitor IF1 on ATP synthase catalysis (García-Bermúdez and Cuezva 2016), the main other mechanisms described in the literature, promoting the rewiring of metabolism in human carcinomas, are the partial down- or up-regulation of the expression of ATP synthase subunits (Figure 2) and/or the occurrence of specific mutations at the level of the subunit genes (Table 1).

A decreased expression of the catalytic β subunit of the ATP synthase has been described in liver, kidney, colon

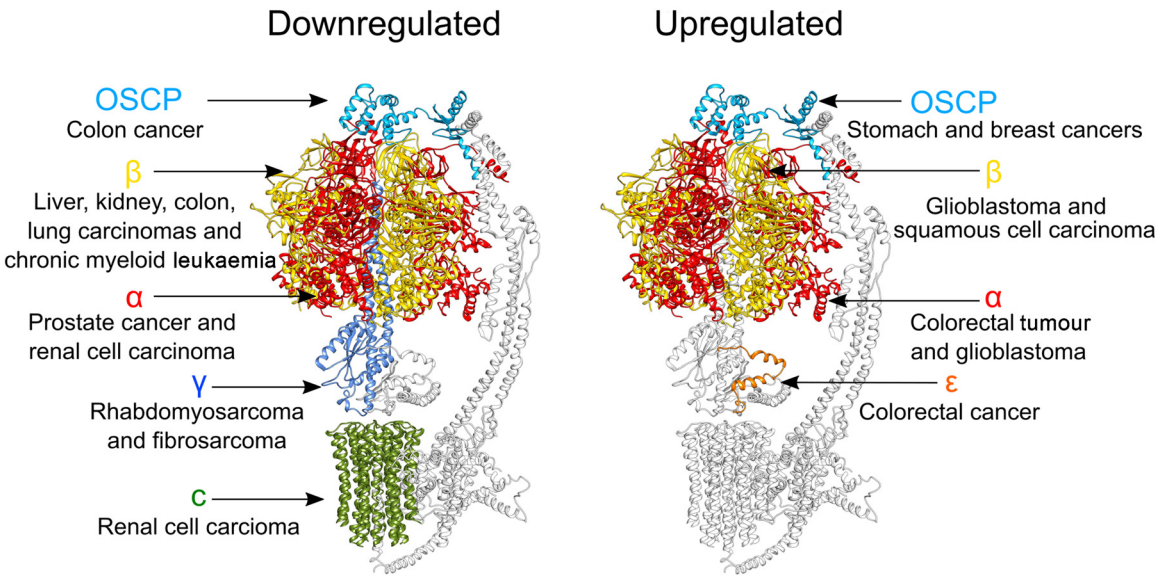

Figure 2: Altered expression of ATP synthase subunits in cancer. The downregulation (left) or upregulation (right) of ATP synthase subunits in cancer is mapped onto the monomeric structure of the mammalian ATP synthase from (Gu et al. 2019) (PDBid: 6J5K). The published Cryo-EM structure is presented in grey. The subunits with an altered expression level in the indicated cancer models are presented in colours. The subunits α, β, OSCP, ε, γ, c are shown in red, yellow, cyan, orange, blue and green, respectively.

human carcinomas (Cuezva et al. 2002) and in lung adenocarcinoma (Cuezva et al. 2004). In solid carcinomas, the β subunit downregulation is dependent on the specific repression of its mRNA translation (Ortega et al. 2010; Willers et al. 2010; Willers and Cuezva 2011), while, in chronic myeloid leukaemia, it is dependent on hypermethylation and silencing of the promoter of the *ATP5B* gene (Li et al. 2010). The decreased expression of this subunit might result in changes at the level of mitochondrial ATP synthesis and/or PTP desensitization to matrix Ca^{2+}. Interestingly, an acidic pH-dependent alternative splicing for the catalytic γ subunit has been reported for cancer cells (Endo et al. 1994), which in rhabdomyosarcoma and fibrosarcoma is regulated by a negative regulatory factor that excludes the exon 9 in acidic conditions (Hayakawa et al. 1998). Reduced levels of α subunit have been detected in prostate cancer samples and correlate with an earlier onset of the disease in patients (Feichtinger et al. 2018). Furthermore, in a cohort of clear cell renal cell carcinoma (ccRCC) the downregulation of the expression of *ATP5A1, ATPAF1, ATP5G1/G2/G3* genes was validated at the protein level using Western Blot and immunohistochemistry (Brüggemann et al. 2017). The down-regulation of the derived ATP synthase α and c subunits and an altered expression of the assembly factor ATPAF1, which is homologous of the yeast protein ATP11, in ccRCC is the basis for a reduced mitochondrial function and might alter cellular metabolism towards glycolysis. On the contrary, the α subunit expression facilitated the development of colorectal tumours with microsatellite instability (Seth et al. 2009), and its expression, together with that of the β subunit, was found to be up-regulated in glioblastoma and endothelial cells in tumour microenvironment (Xu and Li 2016) (Figure 2). The expression of the ATP synthase β subunit also increases with keratinocyte and HaCaT cell differentiation in normal skin, but also in some epidermis hyper-proliferative diseases, and squamous cell carcinoma (Xiaoyun et al. 2017). Oligomycin treatment prevents differentiation in the HaCaT cell model, which correlates with a decrease in mitochondrial ATP content, supporting the idea that the observed increase in the ATP synthase catalytic subunit has a role in promoting oxidative phosphorylation during HaCaT differentiation. The overexpression of ε subunit encoded by the *ATP5E* gene was detected in colorectal cancer cells from patients, compared to their normal counterparts (Figure 2) (Huang et al. 2019). Colon cancer cells in which the ε subunit was silenced showed markedly reduced invasive and migratory abilities and *ATP5E* gene downregulation significantly reduced the incidence of distant metastasis in a mouse xenograft model, suggesting an additional role of the upregulation of some ATP synthase subunits in a more invasive phenotype. In these colon cancer samples the ε subunit level was associated with the AMPK-AKT-HIF1 signalling axis, while its silencing led to the degradation of HIF1α under hypoxia through AMPK-AKT signalling (Huang et al. 2019). On the

same line, much higher mRNA levels of the OSCP subunit of ATP synthase were detected in a metastatic stomach cancer cell line than in a non-metastatic cancer cell line, that were obtained from the same patient (Salesiotis et al. 1995). High expression levels of the same gene were also observed in two breast cancer cell lines, BT20 and T47D. However, a correlation of the OSCP subunit level and the ATP synthase catalytic function or PTP modulation, a possible mechanism which has been previously proposed (Giorgio et al. 2019), was not described in this study and might be further investigated by monitoring respiration and swelling in cancer cells overexpressing the OSCP subunit. Overall the down- or up-regulation of critical ATP synthase subunits seems to be part of the cancer adaptations, on the one hand by desensitizing cells to permeability transition and cell death, and on the other hand by promoting mitochondrial respiration and an invasive phenotype.

Mutations of ATP synthase in cancer

Somatic mtDNA mutations have been increasingly observed in primary human cancers (Chatterjee et al. 2006) (Table 1). Amino acid changes have been detected at the level of the a subunit of ATP synthase, which is encoded by the *ATP6* gene, in pancreatic cancer cell lines (Jones et al. 2001) and in benign or malignant thyroid tumours (Maximo et al. 2002), in cervical cancer, osteosarcoma, bladder cancer, head and neck cancer, oesophageal cancer, leukaemia (Jiménez-Morales et al. 2018) and in acute myeloid leukaemia (Wu et al. 2018). The other mtDNA-encoded subunit of the enzyme in humans, A6L, derived from the *ATP8* gene, is mutated in breast, ovarian, cervical and thyroid cancers (Jiménez-Morales et al. 2018). Homoplasmic mutations in the *ATP6* and *ATP8* genes were found in patients with breast cancer (Grzybowska-szatkowska et al. 2014).

Yet, the question of whether such mutations contribute to the promotion of carcinomas remains to be clarified. With the aim of understanding the contribution of mtDNA mutations, cybrids containing a common HeLa nucleus and mtDNA with or without a homoplasmic pathogenic point mutation at nucleotide position 8993 or 9176 in the *ATP6* gene derived from patients with mitochondrial encephalomyopathy were studied.

When cybrids were transplanted into nude mice, the *ATP6* mutations conferred an advantage in the early stage of tumour growth. The restoration of mutations with a wild-type nuclear version of *ATP6* gene slowed down the tumour growth in transplantation. Conversely, expression of a mutant nuclear version of *ATP6* in the wild-type cybrids declined respiration and accelerated the tumour growth. Finally, these pathogenic mtDNA mutations seem to promote tumour formation by preventing apoptosis (Shidara et al. 2005). In line with these findings, Petros and coworkers introduced the pathogenic mtDNA ATP6 T8993G mutation into the PC3 prostate cancer cell line through cybrid transfer and tested these cells for tumour growth in nude mice. The resulting mutant (T8993G) cybrids were found to generate tumours that were seven times larger than the wild-type cybrids. The mutant tumours also generated significantly more ROS (Petros et al. 2005). The same mutation was introduced into PC3 prostate cancer cybrids to test their growth advantage in the bone

Table 1: Common DNA mutations on ATP synthase subunits in cancer.

Gene	cDNA variant	Predicted protein	PubMed ID
MT-ATP6	m.8557G > A	p.Ala11Thr	25110199
	m.8696T > C	p.Met57Thr	11245424; 16892080
	m.8701A > G	p.Thr59Ala	11861366; 12000737; 16892080; 30015870
	m.8705T > C	p.Met60Thr	30185817
	m.8716A > G	p.Lys64Glu	12000737; 16892080; 28986220
	m.8860A > G	p.Thr112Ala	11861366; 25110199; 30015870
	m.8854G > A	p.Ala110Thr	25110199
	m.8858G > C	p.Gly111Ala	25110199
	m.8914C > A	p.Pro130Thr	16205644; 27083309
	m.8932C > T	p.Pro136Ser	15647368; 28986220; 27083309;
	m.8953A > G	p.Ile143Val	17916247; 27083309
	m.8993T > G	p.Leu156Arg	15753359; 15647368; 18850577; 22778551
	m.9055G > A	p.Ala177Thr	11861366; 30015870
	m.9070T > G	p.Ser182Ala	11245424; 16892080
	m.9119T > G	p.Leu198Arg	25110199
	m.9131T > C	p.Leu202Pro	11861366; 27083309
	m.9130C > G	p.Leu202Val	25110199
	m.9137T > C	p.Ile204Thr	12000737; 16892080
	m.9176T > C	p.Leu217Pro	15753359
MT-ATP8	m.7778G > T(*)	p.Asp5Tyr	21167184
	m.8429C > A	p.Leu22Ile	25110199
	m.8439A > C	p.Gln25Pro	25110199
	m.8448T > C	p.Met28Thr	25110199
	m.8472C > T	p.Pro36Leu	30015870
	m.8472C > A	p.Pro36His	30015870
	m.8519G > A	p.Glu53Lys	25110199
ATP5PO	n.218A > G	p.Lys73Arg	18389087

The mutated subunits of ATP synthase found in cancer models, their nucleotide position in humans and the consequent amino acidic substitution are indicated; (*) refers to murine sequence.

microenviroment in xenografts, an effect that was shown to be mediated by upregulation of FGF-1 and FAK (Arnold et al. 2009), proteins that are expressed in prostate cancer cells when exposed to bone stromal cells.

Another study on the role of ATP synthase gene mutations in cancer was provided in yeast. *Saccharomyces cerevisiae* has been used as a model to investigate the functional consequences of cancer-associated missense mutations (8914C > A, 8932C > T, 8953A > G, 9131T > C) found in the mitochondrial *ATP6* gene of thyroid, parathyroid, prostate and breast cancer cells (Niedzwiecka et al. 2016). Although the four studied mutations affected conserved residues of the a subunit, only one of them (8932C > T) had a significant impact on mitochondrial function, due to the amino acid change P163S in the yeast a subunit, leading to a less efficient incorporation of this subunit into the ATP synthase complex (Niedzwiecka et al. 2016). Moreover, two mutations, corresponding to the human *ATP6*, P136S and K64E, found in prostate and thyroid cancer samples, respectively, were introduced in the yeast a subunit in an *OM45*-GFP background in which Om45p, the component of the porin complex in the outer mitochondrial membrane, was fused to GFP (Niedzwiecka et al. 2018). Both mutations increased sensitivity of yeast cells to compounds inducing oxidative stress, reduced ATP synthase catalytic activity in both directions, affected calcium homeostasis and delayed the activation of the yeast PTP upon calcium induction at 36 °C. This latest finding suggests that mitochondrial ATP synthase mutations, which accumulate during the carcinogenesis process, may also inhibit the propensity of cancer cells to undergo apoptosis.

In principle, mutations at the level of *ATP6* and *ATP8* genes could contribute to neoplastic transformation by changing cellular energy capacity, increasing mitochondrial oxidative stress and/or modulating apoptosis. However, in patients mutations or deletions of these genes are often responsible for neuromuscular disorders, cardiomyopathy, encephalopathy, NARP, MILS and LHON associated phenotypes (Kucharczyk et al. 2009), more than a neoplastic growth. The nuclear expression of the T8993G mutant *ATP6* in transgenic mice show functional motor deficiencies similar to those seen in human NARP patients, while enhanced performance was observed in others (Dunn and Pinkert 2012). On the other hand, mice carrying the G7778T mutation on the *ATP8* mitochondrial gene show increased ROS production but normal ATP levels under normal metabolic conditions. Interestingly, these mutant mice treated with drugs, causing endotoxemic acute liver failure and caspase 3 dependent apoptosis, exhibited liver protection and overall survival, most probably due to better hepatic energy status and decreased apoptosis than in their control counterpart (Eipel et al. 2011). However, none of the aforementioned *in vivo* models develop cancers in the presence of ATP synthase gene mutations, suggesting that other nuclear genes are required for activation of tumour growth in the initial phases.

Epigenetic modulation of ATP synthase induces drug resistance

Metabolic rewiring in cancer is tightly connected to changes at the epigenetic level. Enzymes that mediate the epigenetic status of cells catalyse posttranslational modifications of DNA and histones and influence metabolic gene expression (Johnson et al. 2015). Epigenetic characteristics reside also in mtDNA, which is modified in copy number per cell (Naviaux 2008; Smiraglia et al. 2008). Nuclear DNA methylation and mtDNA copy number regulation produce tissue-specific differences in gene expression (Delsite et al. 2002; Kitamura et al. 2007). Both help to set the metabolic phenotype of the cell and are clinically relevant for cancer survival (Naviaux 2008). This interplay between epigenetics and metabolism constitutes a new aspect of cancer biology and could lead to new insights for the development of anti-cancer therapeutics. Novel approaches are required especially for the transformed phenotypes that become resistant to chemo- or radio-therapy treatments.

A few examples in which modifications at the epigenetic level of ATP synthase genes (or promoters) are involved in cancer cell resistance to treatment are reported below. In different cell models of chronic myeloid leukaemia, the hypermethylation of the *ATP5B* nuclear gene, encoding for the catalytic β subunit of ATP synthase, causes a protein downregulation which is associated with disease progression and chemo-resistance (Koschmieder and Vetrie 2018). This evidence might be hypothesized as a cancer strategy to decrease the amount of the β subunit which represents the Ca^{2+} binding site that promotes PTP opening and apoptosis.

A study, in which novel mechanisms involved in multidrug resistance were explored with comparative proteomics, was focused on adriamycin resistance in leukaemia. Differences were analysed in global protein expression in the leukaemia cell line K562 and its multidrug resistance counterpart, K562/A02 (Li et al. 2010). Results indicated that mitochondrial ATP synthase β subunit down-regulation plays an important role in adriamycin

resistance in leukaemia cells, possibly through DNA methylation of the *ATP5B* promoter. Moreover, silencing of this catalytic subunit of ATP synthase in K562—the non-resistant parental cell line—was able to decrease sensitivity to adriamycin in these cells. The authors found a down-regulation of β subunit not only in K562/A02, but also in primary cells from patients with chronic myeloid leukaemia blast crisis, a progression of the disease in which conventional treatments are notoriously unsatisfactory. The hypermethylation status of the *ATP5B* gene promoter correlated with chemo-resistance, in primary cells from 22 chronic myeloid leukaemia patients of different stages.

Another important evidence is found in a missense mutation at the level of the OSCP subunit gene which has been detected in patients affected by sporadic colon adenocarcinoma (Table 1), suggesting a tumour-specific expression of an altered allele for this subunit (Kan et al. 2007). The downregulation of the OSCP subunit in chemo-resistant cancer cells remains to be elucidated. However, it seems plausible that, given the importance of this subunit in the modulation of PTP (Giorgio et al. 2019, 2018, 2013), the decreased level of this protein might be effective in tumours to inhibit the PTP opening and therefore to escape cell death. In line with this hypothesis, the downregulation of OSCP subunit has been observed in the colon cancer cell model COLO 205. Interestingly, Hsp90 inhibitors that stabilized OSCP levels elicited reversal effects on transformation and differentiation of these colon cancer cells. Moreover, in the same cells, Hsp90 inhibition increased OSCP levels more markedly than those of other ATP synthase subunits and promoted apoptosis via the mitochondrial pathway (Margineantu et al. 2007), a possible PTP-dependent event that awaits to be explored and hopefully exploited therapeutically.

ATP synthase catalytic activity was also hypothesized to be relevant in chemo-resistance. A comparative proteomics study aimed at the identification of mechanisms that cause chemo-resistance to 5-Fluorouracil (5-FU), a critical problem for chemotherapy of advanced colorectal cancer. Data showed a lower expression of the α subunit of ATP synthase together with a decrease level of the OSCP and d subunits in 5-FU–resistant cells compared with parent cells. 5-FU–resistant cell lines also showed decreased ATP synthase activity and reduced intracellular ATP content. The ATP synthase inhibitor, oligomycin, strongly antagonized the 5-FU inhibition of cell proliferation. Down-regulation of the subunit d by siRNA transfection of non-resistant cells increased cell viability in the presence of 5-FU (Shin et al. 2005).

On the same line, metabolic reprogramming caused by epigenetic loss of the ATP synthase subunit d, which is encoded by *ATP5H* gene, leads to ROS accumulation and HIF-1α stabilization under normoxia. Such metabolic changes, were generated in tumour cells subjected to sequential rounds of *in vitro* or *in vivo* immune selection via cognate cytotoxic T lymphocytes and correlated with multimodality resistance to immunotherapy, chemotherapy, and radiotherapy (Song et al. 2018). The subunit d loss in the tumour is strongly linked to failure of therapy, enhanced disease progression, and poor survival in patients with cancer. These results revealed the ATP synthase involvement in an immune-driven multimodality resistance to cancer therapy.

In HER2+ breast tumours the modulation of different ATP synthase subunits in the opposite direction (i.e. increased expression) was detected in cells with acquired resistance to HER2-targeted therapies. RNA-sequencing analysis showed that in cells resistant to trastuzumab, or trastuzumab and pertuzumab in combination, expression increased for β, γ and F6 subunits. Despite minimal changes in mitochondrial respiration, these cells exhibited selective dependency on ATP synthase function. Resistant cells were sensitive to inhibition of ATP synthase by oligomycin, and knockdown of F6 or β subunits rendered resistant cells responsive to a low dose of trastuzumab. Furthermore, combining ATP synthase inhibitor oligomycin with trastuzumab led to regression of trastuzumab-resistant tumours *in vivo* (Gale et al. 2020). A recent work on non-small-cell lung cancer cells subjected to X-ray irradiation showed that the expression level of the α subunit of ATP synthase is increased 24–96 h post-irradiation and that this is in line with an increased ATP hydrolysis in irradiated cells. It is reasonable to speculate that ATPase activity plays a role in survival of non-small-cell lung cancer cells after X-ray radiation. Interestingly, the Authors have shown that the inhibition of the ATPase activity enhances radio-sensitivity in these cancer cells and that the inhibition correlates with a higher mortality associated with the caspase 3 apoptotic pathway (Wang et al. 2017). A possible explanation of the role of ATP synthesis or hydrolysis, in the described breast or lung cancers, might be related to the requirement to support energy production in a metastatic phenotype, or the maintenance of mitochondrial membrane potential for cell survival, respectively. On the contrary, in myeloid leukaemia and colon cancer models described above, the downregulation of the ATP synthase subunits might be a strategy to desensitize PTP opening and avoid apoptosis. In conclusion, ATP synthase subunits are a novel critical target in cancer cells with

acquired resistance. Their expression levels are epigenetically modified by up- or down-regulation as a cancer-specific mechanism of therapy resistance.

Summary

Mitochondria participate to the metabolic and biosynthetic pathways that drive cancer development and progression. Besides energy production, they control many vital parameters in cancer cells, such as the formation of reactive oxygen species (ROS), the cytosolic Ca^{2+} levels and finally the switch leading to apoptosis. ATP synthase plays a critical role in these mitochondrial events and their adaptations in cancer, by affecting cancer metabolism, Ca^{2+} homeostasis and ROS formation. Moreover, this enzyme participates through its functions to different aspects of tumour growth. ATP synthase activity correlates in different cancer models with tumour progression, proliferation and metastatic behaviour. On the other hand, the desensitization of the PTP, a mitochondrial mega-channel which promotes apoptosis and has been proposed to be formed by ATP synthase, seems to be a key event in the primary phases of cancer development.

ATP synthase mutations or transcriptional/translational modifications at the level of enzyme components were found in different cancer models, a fact that suggests an early participation of this enzyme in the primary metabolic changes leading to cancer formation. Furthermore, the down- and up-regulation of ATP synthase subunits in treatment-resistant samples, highlight this enzyme as a novel target that needs to be further characterized for anti-cancer strategies.

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