Carlos Eduardo Cardanho dos Ramos

The long and winding road of STAT3 in the mitochondria

Monografia realizada no âmbito da unidade de Estágio Curricular do Mestrado Integrado em Ciências Farmacêuticas, orientada pelo Professor Doutor João António Nave Laranjinha e apresentada à Faculdade de Farmácia da Universidade de Coimbra

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(Carlos Eduardo Cardanho dos Ramos)

Agradecimentos…

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À Sara, por ser o meu porto seguro.

Ao A.S.J., porque *"sozinhos vamos onde pudermos, mas juntos vamos onde quisermos"*.

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Contents

Abbreviations

ATP – Adenosine TriPhosphate

Bcl-2 – B-cell lymphoma 2

Bcl-xL – B-cell lymphoma xtra-Large

CC – Coiled Coil domain

CL – Cardiolipin

CL^{ox} – oxidized Cardiolipin

Cyt *c* – Cytochrome *c*

DHQ – DiHydroQuercetin

ETC – Electron Transport Chain

$FADH_2$ – Flavin Adenine Dinucleotide

Fe-S – Iron-Sulphur cluster

FMN – Flavin MonoNucleotide

GRIM-19 – Gene associated with Retinoic-Interferon-induced Mortality 19

H_2O_2 – Hydrogen peroxide

Hsp90 – heat shock protein 90

ISCH – Ischemia

JAK2 – Jannus Kinase 2

LDH – Lactate DeHydrogenase

LK – LinKer domain

Mcl-1 – Myeloid cell leukaemia 1

MLS-SAT3E – mitochondria-targeted STAT3 with a mutation in the DNA-binding domain; overexpressed

MLS-STAT3 – mitochondria-targeted STAT3

MLS-STAT3 E434A/E435A – mitochondria-targeted STAT3 with the Glutamate residues at positions 434 and 435 mutated to Alanines (DNA binding domain)

MLS-STAT3 Y705F – mitochondria-targeted STAT3 with the Tyrosine residue at position 705 mutated to a Phenylalanine

MLS-STAT3 Y705F/S277A – mitochondria-targeted STAT3 with the Tyrosine residue at position 705 mutated to a Phenylalanine and the Serine residue at position 727 mutated to an Alanine

MLS-STAT3 Y705F/S727D – mitochondria-targeted STAT3 with the Tyrosine residue at position 705 mutated to a Phenylalanine and the Serine residue at position 727 mutated to an Aspartic acid

MnSOD – mitochondrial Manganese SuperOxideDismutase

MOMP – Mitochondrial Outer Membrane Permeabilization

MPTP – Mitochondrial Permeability Transition Pore

NADH – Nicotinamide Adenine Dinucleotide

p-Tyr705 – Phosphorylated Tyrosine at position 705

P-V – Phospho-valproic acid

ROS – Reactive Oxygen Species
Ser727 – Serine at position 727
SH2 domain – Src Homology domain
shRNA – short hairpin RNA (used to silence a target gene via RNA interference)
SOCS3 – Suppressor Of Cytokine Signalling 3
SOD – SuperOxide Dismutase
Src – family of tyrosine kinases
STAT3 – Signal Transducer and Activator of Transcription 3
STAT3 C3S – STAT3 with the Cysteine residues 418,426 and 468 mutated to Serines
STAT3 CA – Constitutively Active STAT3
STAT3-/- - STAT3 – null cells
STAT3+/+ – Cells with STAT3, same as Wild Type
TAD – TransActivation Domain
TMPD – TetraMethyl-*p*-PhenyleneDiamine
TOM20 – Translocase of Outer Membrane 20
Tyr705 – Tyrosine at position 705
WT – Wild Type
ΔH^+ – Mitochondrial membrane proton gradient
$\Delta\Psi$ – Mitochondrial membrane potential

Resumo

O STAT3, um fator de transcrição responsável pela ativação de vários oncogenes, tem sido estudado extensivamente dado o interesse biomédico da modulação da sua atividade. No entanto, esta proteína também exerce as suas ações, de uma forma não-transcricional, na mitocôndria. Em particular, tem sido proposto que o STAT3 regula a cadeia transportadora de eletrões (ETC), através da interação com os Complexos I e II, e medeia a abertura do poro transitório da membrana mitocondrial (MPTP). Este mecanismo revela um novo papel do STAT3 na proteção celular.

Neste trabalho foi abordada a atividade controversa do STAT3 na mitocôndria em células cancerígenas sujeitas a hipóxia, tentando formular uma hipótese inovadora, nomeadamente, que o STAT3 mitocondrial controla a produção de espécies reativas de oxigénio (ROS) favorecendo a proliferação e não a morte celular. Neste contexto, novas moléculas, como o ácido fosfo-valpróico (P-V) que bloqueia as ações nucleares e mitocondriais do STAT3, parecem ser candidatos promissores a fármacos, através da modulação da atividade do STAT3 no cancro.

Abstract

STAT3 has been extensively studied as a nuclear transcription factor and is responsible for the activation of many oncogenes. However, this protein also exerts its actions in a non-transcriptional way in the mitochondria. It has been proposed that STAT3 regulates the ETC, through interaction with Complexes I and II, and mediates MPTP opening. This mechanism unveils a new cytoprotective role for STAT3.

Here it is discussed how mitochondrial STAT3, in cancer cells under hypoxia, controls ROS production to favour cell proliferation rather than cell death, thus providing a original hypothesis for its action. Following this rationale, molecules, such as P-V, which targets STAT3 nuclear and mitochondrial actions, seem promising candidate drugs against cancer.

1. Introduction

Signal Transducer and Activator of Transcription 3 (STAT3) is a well-known transcription factor (Fig.1) that is expressed constitutively activated in various tumour cell lines including breast, colon, gastric, lung, head and neck, skin and prostate. It can be activated by the entire IL-6 family of cytokines and growth factors, such as epidermal growth factor, that results in phosphorylation of STAT3 in a single tyrosine residue, located at position 705 (p-Tyr705). This leads to an interaction between p-Tyr705 and the SH2 domain of two different STAT3 monomers to form dimers which translocate into the nucleus to induce transcription of genes involved in proliferation, cell survival, inflammation, metastasis and angiogenesis (Sivaraman *et al.*, 2014). Despite this, molecules that block SH2 and DNA-binding domains and p-Tyr705 have had little therapeutic value so far.

It was thought that STAT3 exerted its function solely as a nuclear transcription factor, but recent studies showed that it was also present in the mitochondria(Wegrzyn *et al.*, 2009)(Szczepanek *et al.*, 2012)(Gough *et al.*, 2009). There are a number of reports suggesting that mitochondrial-STAT3 can contribute to, or be sufficient for the growth and transformation of many malignancies (Zhang *et al.*, 2013). Coordinate regulation at the nucleus and mitochondria by this protein places it in a unique position to regulate cellular processes (Meier e Larner, 2014). Here it will be summarized how STAT3 influence mitochondrial function in health and disease, especially in cancer.

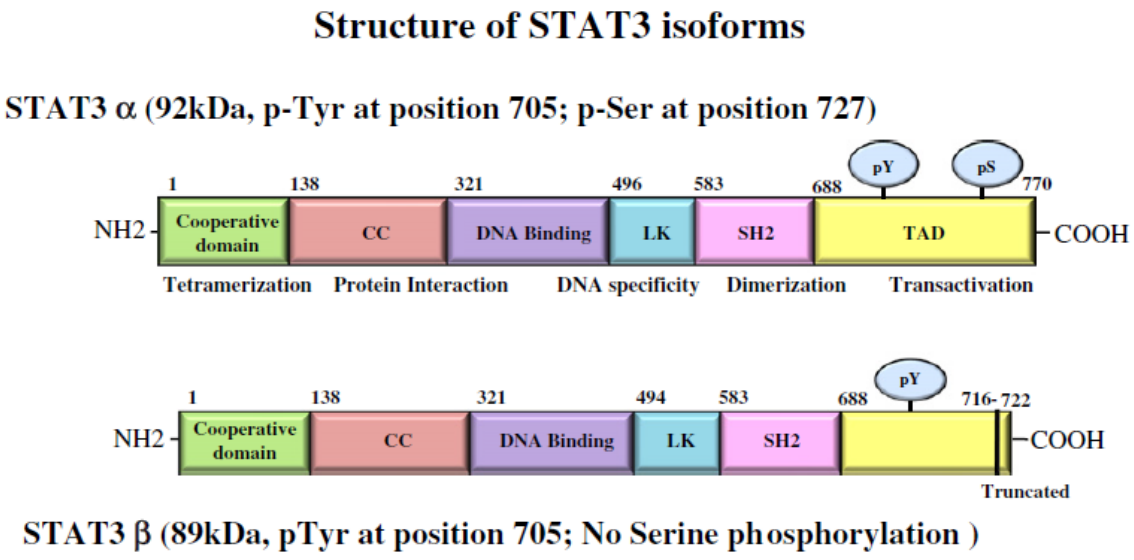

Fig.1 – STAT3 protein domains. Amino acid numbers indicate boundaries between each domain. The phosphorylation sites Tyr705 and Ser727 are also indicated. *Adapted from (Subramaniam et al., 2013).*

2. The role of STAT3 in the mitochondria

The function of mitochondrial STAT3 has been extensively studied and it is known that a small pool of STAT3 (5-10% of total) is located inside this organelle, in many tissues and cultured cells. However, the mitochondrial targeting sequence for STAT3 is likely cryptic and as such, is yet to be determined (Meier e Larner, 2014). Nonetheless, GRIM-19, a complex I component, acts as a chaperone to recruit STAT3 into the mitochondria (Tammineni *et al.*, 2013), but it is probable that other proteins are involved, as STAT3 is also known to interact with TOM20 (Boengler *et al.*, 2010).

Within the mitochondria, STAT3 modulates two major players in mitochondrial physiology: the Electron Transport Chain (ETC) (Wegrzyn *et al.*, 2009) and the Mitochondrial Permeability Transition Pore (MPTP) (Boengler *et al.*, 2010); influencing the mitochondrial membrane potential ($\Delta\Psi$) and proton gradient (ΔH^+), ATP production, reactive oxygen species (ROS) levels and cell death.

2.1. Mitochondrial STAT3 and the Electron transport chain

The ETC in the inner mitochondrial membrane consists of four protein complexes (I-IV). These complexes are responsible for driving the transport of electrons down the chain, by pairing electron donors (NADH and $FADH_2$) with specific electron acceptors and ultimately leading to the reduction of O_2 to H_2O. As a result of this energy transfer, protons (H^+ ions) are pumped into the inter-membrane space, generating an electrochemical gradient across the inner mitochondrial membrane. Complex V is then able to dissipate this proton gradient and couple the resulting energy release with ATP production. However, there is also an alternative reaction of Complexes I and III directly with O_2 that can lead to the formation of ROS, in particular superoxide radical (Meier e Larner, 2014). Under normal conditions, there are enzymatic antioxidant defence mechanisms (SOD, Catalase, Glutathione Peroxidase system, among others) that quickly reduce ROS to H_2O (Raedschelders, Ansley e Chen, 2012). Thereby, an impairment on ETC might result in reduced ATP production and increased ROS levels above the steady state in which bypass the reduction by enzymatic systems, which can ultimately lead to mitochondrial dysfunction and cell death.

STAT3 is thought to be an important regulator of the ETC as the absence of this protein in astrocytes leads to increased production of superoxide anion and other ROS, decreased $\Delta\Psi$ and decreased ATP production (Sarafian *et al.*, 2010). Additionally, ventricles from STAT3-null hearts have also showed elevated ROS levels(Hilfiker-Kleiner *et al.*, 2007), and in

Ras-transformed cells, loss of STAT3 led to a 50% reduction in ATP levels, mainly due to decreased activity of Complex V (Gough *et al.*, 2009).

To better understand how STAT3 might influence the ETC, Wegrzyn et al. compared STAT3+/+ with STAT3-/- pro-B cells and discovered that respiration was reduced in STAT3-/- cells when glutamate and succinate were used as substrates (Complex I and Complex II substrates, respectively).They also measured NADH and DHQ oxidase activities. NADH oxidase activity, which gives us information about Complexes I, III and IV, was reduced by 65% in STAT3-/- cells; while DHQ oxidase activity, which requires Complex III and IV, was not affected (Wegrzyn *et al.*, 2009).

To confirm the previous results, the same group assayed enzyme activities of the ETC and discovered a reduction by 40% and 85% in complex I and complex II, respectively, in Stat3−/− pro-B cells as compared with WT pro-B cells. Complex III and IV activities for both types of cells were not affected (Wegrzyn *et al.*, 2009).

These results suggest that STAT3 is essential for maximal activity of complexes I and II of the ETC (Fig. 2). This is not a surprise, as STAT3 is known to interact with the complex I subunit, GRIM-19 (Tammineni *et al.*, 2013), and it was found in Complex I and II immunoprecipitates (Wegrzyn *et al.*, 2009).

Expression of STAT3 in the heart is cardioprotective and reduces ROS levels, probably by preservation of mitochondria function during ischemia (Szczepanek *et al.*, 2012). To address this, Szczepanek et al. generated transgenic mice with cardiomyocyte-specific overexpression of mitochondria-targeted STAT3 with a mutation in the DNA-binding domain (MLS-SAT3E). Opposite to Wegrzyn et al., they discovered that, in transgenic mice, the maximal rate of respiration was modestly decreased by 20%, when glutamate + malate (Complex I) or succinate (Complex II) were used as substrates. They also observed a reduction in Complex I and II activities, but Complex III and IV activities were not affected (Szczepanek *et al.*, 2011). These different results might indicate that over-expression of mitochondrial STAT3 alters its protein–protein interactions such that its actions on the ETC become more protective under conditions of stress and less effective in regulating the activity of the ETC under basal conditions (Meier e Larner, 2014).

Interestingly, under basal conditions, MLS-STAT3E mice do not have increased ROS production nor have decreased $\Delta\Psi$ (Szczepanek *et al.*, 2011). However, ischemia induced a moderate decrease of state 3 respiration using glutamate plus malate (Complex I) as substrate

in WT hearts, while in MLS-STAT3E mitochondria there was only a minimal decrease of glutamate plus malate rates. Ischemia also increased H_2O_2 release from WT mitochondria (Fig. 2A) respiring on glutamate plus malate, whereas in transgenic mice mitochondria no such effect was observed (Fig. 2B). Once high levels of ROS induce cytochrome *c* release and consequently cell death, it's not surprising that cytochrome *c* release in WT was much higher when compared to MLS-STAT3E hearts under ischemia (Szczepanek *et al.*, 2011). Curiously, in MLS-STAT3E mice, the defect was localized to the distal portion of Complex I, *i.e.* the chain of iron-sulphur clusters or the quinone-binding site (Szczepanek *et al.*, 2011), which are the locus for production of ROS from ischemia-damaged Complex I (Chen *et al.*, 2008). All of these results indicate that the STAT3-dependent partial blockade of electron flow through Complex I decreases ROS production and blocks cytochrome *c* release into the cytosol, thus explaining the cytoprotective role of STAT3 in the heart (Fig. 2). These results are supported by and might explain why STAT3 import into the mitochondria is increased during ischemia (Szczepanek *et al.*, 2011).

2.2. Mitochondrial STAT3 and the Mitochondrial Permeability Transition Pore

The MPTP is a non-specific pore localized in the inner mitochondrial membrane but with contact sites in the outer membrane (Halestrap, 2009). It has a molecular cut-off of 1.5 kDa and under non-stressed conditions transiently opens and closes to maintain Ca^{2+} concentration gradients and $\Delta\Psi/\Delta H^+$ (Meier e Larner, 2014).

The primary trigger for opening the MPTP is Ca^{2+} overload, which is consistent with the fact that this pore is used for release of accumulated Ca^{2+}. However, MPTP opening enables free passage of protons into the mitochondria, thus dissipating the $\Delta\Psi/\Delta H^+$ and preventing Complex V from making ATP (Halestrap, 2009). Oxidative stress can also open MPTP. Indeed, during ischemia and reperfusion, pore opening is largely mediated by oxidative stress rather than an increase in Ca^{2+} concentration (Kim, Jin e Lemasters, 2006).

Ca^{2+} overload, ROS, but also other stimuli such as misfolded mitochondrial proteins, can lead to sustained MPTP opening (Meier e Larner, 2014). Since the matrix protein concentration is higher than that in the cytosol and intermembrane space, it exerts a colloidal osmotic pressure leading to swelling of the matrix compartment. As the matrix expands, it exerts pressure on the outer membrane which eventually ruptures. This event releases cytochrome *c* and other pro-apoptotic proteins which have the potential to initiate apoptotic cell death (Halestrap, 2009). Also, if MPTP is opened for a long time there is no $\Delta\Psi/\Delta H^+$ and no ATP production, thus, enhancing mitochondrial dysfunction.

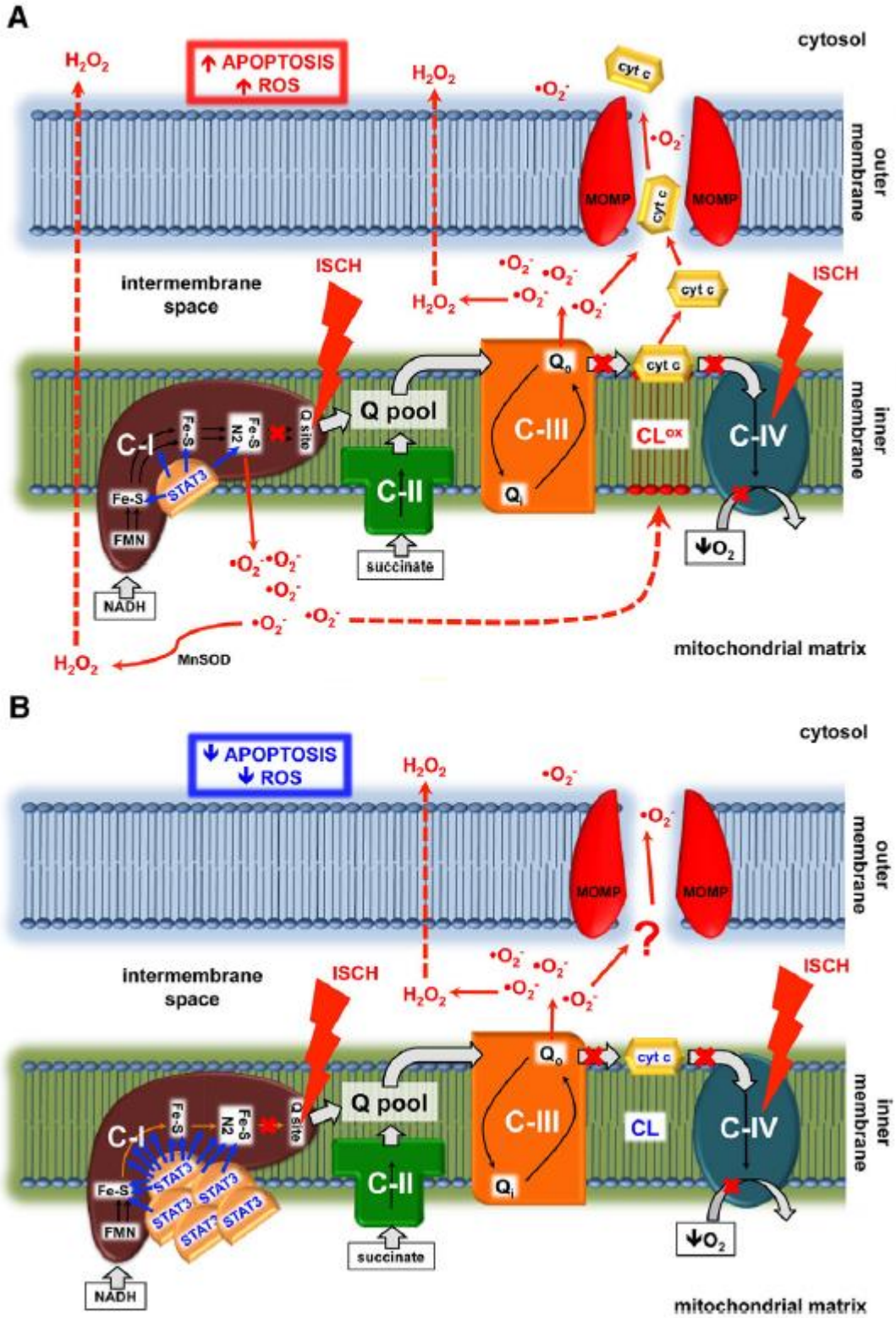

Fig. 2 – Postulated mechanism of the protective role of STAT3 in heart mitochondria during ischemia. (A) In wild type mitochondria ischemia increases superoxide production from complex I that is directed toward the matrix. This results in cardiolipin oxidation and cytochrome c delocalization from the inner membrane. Further damage to the mitochondria leads to outer membrane permeabilization, which allows the release of cytochrome c from mitochondria and the subsequent induction of apoptosis. **(B)** Overexpression of MLSSTAT3E in the mitochondria partially blocks electron flow through iron–sulfur clusters within complex I, resulting in blockade of superoxide generation from complex I during ischemia. This in turn decreases cardiolipin oxidation and preserves cytochrome c retention in the inner membrane. Lower levels of ROS and the lack of cytochrome c translocation into the cytosol attenuates apoptosis and increases cell viability during oxidative stress. *Adapted from (Szczepanek et al., 2012).*

In STAT3-/- mitochondria, MPTP opening occurred with lower concentrations of Ca^{2+} (Boengler *et al.*, 2010). Furthermore, MPTP more readily opens in WT mitochondria when treated with Stattic, a STAT3 inhibitor (Boengler *et al.*, 2013). This can be explained because STAT3 co-immunoprecipitates with Cyclophilin D, the only protein known to be absolutely required for pore functioning and which is thought to be activated in the mitochondrial matrix and translocate to the inner mitochondrial membrane to facilitate pore opening (Baines *et al.*, 2005). STAT3 inhibits MPTP opening, preventing mitochondria swelling and reducing cytochrome *c* release, mitigating cell death (Fig. 3)

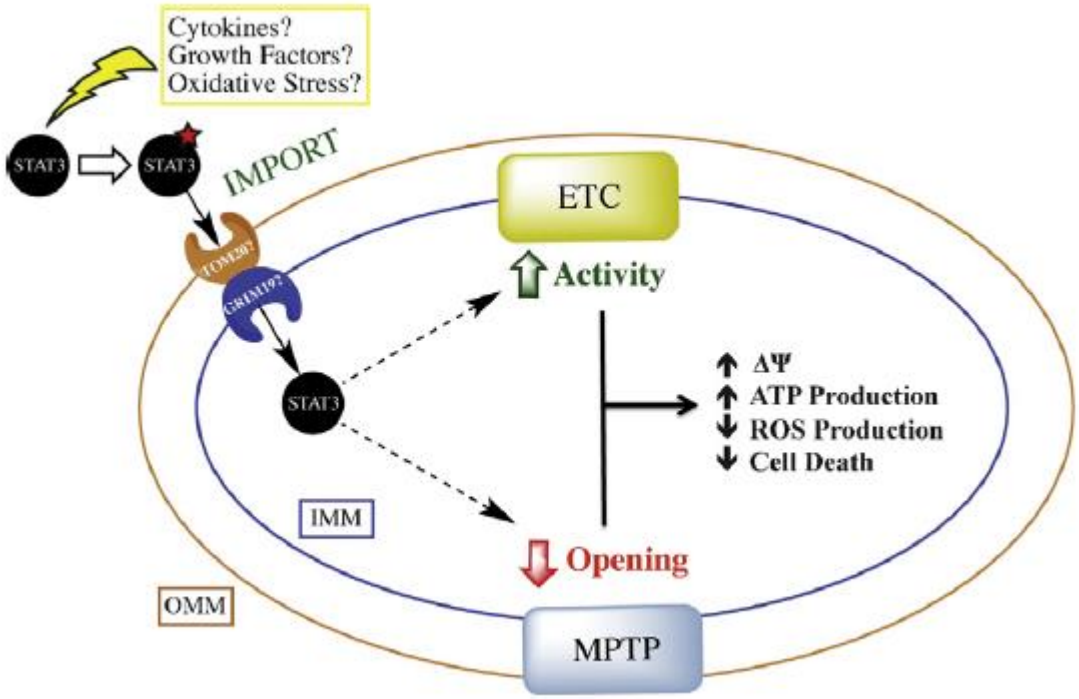

Fig. 3 – Model of STAT3's mitochondrial action. Upstream activation by cytokines, growth factors, or oxidative stress likely phosphorylated STAT3 in Ser727 is targeted to the mitochondria. Though still unknown, mitochondrial import of STAT3 may rely on translocases of the outer membrane (TOM20 potentially) and GRIM-19 in the inner mitochondrial membrane. Once imported, STAT3 modulates both the ETC and the MPTP to regulate ΔΨ, ATP production, ROS production and, ultimately, cell death. *Adapted from* (*Meier e Larner, 2014).*

2.3. How does STAT3 act in mitochondria?

In mitochondria, STAT3 doesn´t exert its actions as a transcription factor since mitochondrial proteins were present in similar levels between WT and STAT3-/- pro-B cells. Also, the amount of mitochondrial DNA–encoded genes was similar between WT and STAT3−/− pro-B cells, as were the concentrations of mitochondrial-encoded RNAs.

Moreover, MLS-STAT3 (STAT3 with a mitochondrial targeting sequence) showed no increase in SOCS3 expression, a STAT3-dependent gene (Wegrzyn *et al.*, 2009).

To determine which protein domains are required for ETC regulation, Wegrzyn *et al.* expressed, in STAT3−/− cells, different MLS-STAT3 that contained mutations in its DNA binding domain, Tyr705, or Ser727 and Tyr705 (Table 1). Mutation in either Tyr705 (MLS-STAT3 Y705F) or the DNA binding domain (MLS-STAT3 E434A/E435A) restored activity in Complex I and II. Interestingly, in Stat3−/− cells expressing a constitutively active STAT3 (STAT3 CA) that forms a dimer without being tyrosine phosphorylated, did not restore mitochondrial respiration. Ser727 mutated to an alanine (A) functioned as a dominant negative, but when mutated to an aspartic acid (D) functioned as a mimetic of a constitutively serine-phosphorylated. Expression of MLS-STAT3 Y705F/S727D restored Complex I and II activities, whereas, MLS-STAT3 Y705F/S277A was ineffective (Table 1) (Wegrzyn *et al.*, 2009).

With these results, we can conclude that known domains required for STAT3 nuclear action (Tyr705 and DNA binding domain) are not needed to drive its mitochondrial action. Interestingly, phosphorylation at Ser727 is crucial for STAT3 mitochondrial function and may be required for its import into this organelle (Zhang *et al.*, 2013). This is consistent with the fact that the relative concentration of serine-phosphorylated STAT3 is highly enriched in mouse mitochondria, when compared to the concentration present in the cytoplasm (Wegrzyn *et al.*, 2009).

	MLS-STAT3 Y705P	MLS-STAT3 E434A/E435A	MLS-STAT3 Y705P/S727D	MLS-STAT3 Y705P/S727A	STAT3 CA	MLS-STAT3E
Complex I	✓	✓	✓	×	×	↓
Complex II	✓	✓	✓	×	×	↓

Table 1 – How mutations in STAT3 influence Complex I and Complex II activities. This table resumes on how different mutations in STAT3 affect the activities of ETC complexes I and II. ✓**:** restored activity in STAT3-/- cells. ×: did not restore activity in STAT3 -/- cells. ↓: decreased activity.

3. Mitochondrial STAT3 and cancer

STAT3 mediates cellular differentiation, proliferation and survival functions through its nuclear actions, acting as a canonical transcription factor. However, mitochondrial localization of STAT3 and its actions regulating ATP production, ROS levels and cell death makes it ideally situated to influence mitochondrial death and survival pathways. This becomes apparent when considering the central role in the regulation of cell demise, either life or death.

The following sections will address the role mitochondrial STAT3 might have in cancer biology.

Transformation by activated Ras should be independent of STAT3, since the Ras family of oncogenes lacks tyrosine kinase activity. However, the ability of H-Ras to cause cell growth in soft agar was impaired in the absence of STAT3. Furthermore, growth of Ras-transformed tumours in mice was abrogated without STAT3. Accordingly, in a T24 cell line derived from a spontaneous H-Ras-transformed cell carcinoma, the average colony formation was the same when STAT3 or H-Ras were shRNA-ablated (Gough *et al.*, 2009).

Interestingly, reconstitution of STAT3-null cells with different STAT3 mutants showed that phosphorylation at Ser727 is essential to restore Ras-transformation, but domains associated with transcriptional activity were not required (Table 2). This is consistent with the fact that no detectable p-Tyr705 STAT3 was found in this type of cells. This suggests that Ras requires STAT3 to exert its mitochondrial function rather than its nuclear one. To confirm this, MLS-STAT3 (restricted to mitochondria) was expressed in STAT3-null cells and it favoured Ras-transformation (Gough *et al.*, 2009).

	STAT3 Y705P	STAT3 N-terminal	STAT3 DBD	STAT3 SH2	STAT3 NLS	STAT3 S727A	STAT3 S727D	STAT3 β
Ras-transformation	✓	✓	✓	✓	✓	✗	✓	✗

Table 2 – How mutations in STAT3 influence Ras-transformation. This table resumes on how different mutations in STAT3 affect Ras-transformation. ✓**:** restored activity in STAT3-/- cells. ✗: did not restore activity in STAT3 -/- cells.

Mitochondrial STAT3 not only contributes to Ras-mediated transformation, but it can also influence growth and metastasis of established tumour cells. Zhang *et al.* compared 4T1 murine breast cancer cells expressing different STAT3 mutants. They found that in MLS-

STAT3 S727A cells there were fewer colonies than in vector controls. On the other hand, MLS-STAT3 S727D cells showed increased number of colonies. Accordingly, cells expressing MLS-STAT3 S727A formed smaller tumours and invasion was less effectively when compared to cells expressing MLS-STAT3 S727D. The same group also observed that essential domains for STAT3 nuclear actions (Tyr705, DNA binding and SH2 domains) did not influence tumour size or invasion (Zhang *et al.*, 2013), supporting once more the dependence on mitochondrial STAT3.

These data is supported by many reports showing that phosphorylation at Ser727 is crucial for growth and transformation of many malignancies, including prostate and breast cancer (Yeh *et al.*, 2006)(Qin *et al.*, 2008).

We could now speculate that targeting STAT3 mitochondrial function might be as important as targeting its transcriptional actions, but first we need to understand exactly how mitochondrial STAT3 influences tumorogenesis.

3.1. Mitochondrial STAT3 sustains altered glycolytic and oxidative phosphorylation activities characteristic of cancer cells

Even in the presence of physiologic oxygen tension, cancer cells reprogram their energy metabolism limiting it largely to glycolysis. This phenomenon, known as the "Warburg effect" or "aerobic glycolysis", is characterized by an increase in glucose uptake and lactate production, and a decrease in oxidative phosphorylation activity (Hanahan e Weinberg, 2011). At first sight, STAT3 shouldn't have any influence in transformation and growth of tumour cells, since it exerts its mitochondrial actions primarily through regulation of the ETC. However, as mentioned before, STAT3 is needed for Ras full transforming potential.

Glycolytic fuelling in cancer cells has been associated with activated oncogenes, such as Ras (Hanahan e Weinberg, 2011). This is consistent with the fact that, in high glucose medium, Ras-expressing cells were viable, even in the absence of STAT3. On the other hand, cells lacking STAT3 were more sensitive when glucose was restricted (Gough *et al.*, 2009). This might indicate that most tumour cells still maintain, at least some, mitochondrial function and the necessity of ATP might lead cancer cells to derive it from oxidative phosphorylation in low glucose mediums, thus, evidencing the importance of STAT3 in these conditions. Still, this explanation seems insufficient to justify the 50% loss in ATP levels observed in STAT3-null cells (Gough *et al.*, 2009).

LDH converts pyruvate to lactate to complete the glycolysis process. Since cancer cells rely on glycolysis for energy production, the fact that LDH activity is higher in Ras-

transformed cells comes with no surprise. It is however unexpected that this increment requires mitochondrial STAT3 and phosphorylation at Ser727 (Gough *et al.*, 2009).

These findings suggest that mitochondrial STAT3 contribute to Ras-transformation not only through maximisation of the ETC, but mainly by supporting the metabolic shift favouring the glycolytic pathway.

3.2. Mitochondrial STAT3 regulates ROS levels preventing cell death and favouring cell proliferation

Overproduction of ROS results in oxidative stress, a cytotoxic process that can be an important mediator of damage to cell structures, including proteins and DNA, and trigger apoptosis. In contrast, at low concentrations, ROS, such as H_2O_2 and superoxide anion, can be beneficial in the redox regulation of cellular functions, acting on a number of cellular signalling pathways, thus promoting cell survival and proliferation (Valko *et al.*, 2007). This way, the intracellular oxidative potential must lie in a fairly narrow window: too low or too high and cells cease to grow, or die by apoptosis. Whether or not this window is shifted or expanded in cancer cells is yet to be proven (Li *et al.*, 2010).

Despite the mitochondrial STAT3 function discussed above, it has been proposed that the principal mechanism by which mitochondrial STAT3 favours cancer growth and survival is through regulation of oxidative stress.

Once oxygenation in tumours is not static but instead fluctuates, ranging from normoxia to hypoxia, mitochondrial STAT3 might have an important role preventing elevated ROS levels in cancer cells under ischemic conditions. This theory is supported by Gough et al. observation that Ras-expressing cells lacking STAT3 were much more sensitive to glucose restriction, when cultivated in reduced oxygen concentrations (Gough *et al.*, 2009). To confirm this, Zhang et al. measured H_2O_2 and superoxide anion levels through an indirect assay (tyrosine-nitrated proteins) in 4T1 cells, expressing various MLS-STAT3 mutants, subjected to hypoxia. They observed that cells expressing MLS-STAT3 and MLS-STAT3 S727D had significantly lower ratios of tyrosine-nitrated proteins when compared to WT and vector controls. Similarly, MLS-STAT3 S727A expressing cells showed higher levels of superoxide anion, when they assayed it using mitoSOX dye. Interestingly, MLS-STAT3 S727D cells also showed a significant increase in Complex I activity when cultivated in low oxygen concentrations (Zhang *et al.*, 2013).

ROS, especially H_2O_2 and superoxide anion, regulate signal transduction pathways via oxidation/reduction of critical cysteine residues in kinases, phosphatases and other regulatory proteins (Burhans e Heintz, 2009). Curiously, STAT3 can also be directly oxidized by H_2O_2 in cysteine residues, affecting its nuclear and mitochondrial actions (Li *et al.*, 2010). By analysing a number of cysteine substitution mutants, it was found that only oxidation at cysteine residues 418, 426, 468 (DNA binding domain) and 768 (TAD) resulted in altered STAT3 activity, probably because oxidation of these residues led to the formation of inter-chain disulphides and formation of trimers and tetramers (Li *et al.*, 2010)(Shaw, 2010). Curiously, these multimers inhibited DNA binding and reduced expression of STAT3-dependent genes.

In order to seek an effect of STAT3 oxidation in cell growth, Li *et al.* compared breast cancer cells expressing STAT3 with STAT3 C3S (redox insensitive, unable to form multimers). Under normoxic conditions, expression of STAT3 C3S increased cell proliferation, possibly by reduction of the cell cycle, as observations after 16 hours showed more cells expressing STAT3 C3S had reached G_2-M. However, under mild oxidative stress, STAT3 C3S cells were less resistant and, after 16 hours, fewer cells had reached G_2-M. Once STAT3 C3S is not able to form mutlimers, its DNA binding capacity is not affected by oxidative stress. This might lead us to believe that oxidation of STAT3 also alters its mitochondrial function to favour cell growth.

Peter Shaw proposed that oxidized STAT3 may be selectively taken up into mitochondria to stimulate Complex I and II, increasing ATP synthesis and limiting ROS generation (Shaw, 2010). This theory explains the observations of Li *et al.* On one hand, under normoxic conditions, STAT3 C3S inability to enter the mitochondria led to sufficiently elevated levels of ROS to accelerate the G_1 transition and thus, enhancing cell proliferation. On the other hand, under oxidative stress, STAT3 C3S was unable to cut ROS output from mitochondria, resulting in extremely high levels of ROS and induced senescence or apoptosis. Once ischemia is connected to high levels of ROS, this hypothesis supports the observation of Szczepanek *et al.* that ischemia increases mitochondrial levels of STAT3 and, therefore, explains the cytoprotective role of STAT3 in cancer cells under hypoxia.

Supporting its regulation of the ETC, STAT3 can also reduce cellular oxidative stress by acting as an important electron sink, through its oxidation by H_2O_2. Another relevant aspect that we must take into account is the STAT3 ability to mediate MPTP opening; preventing sustained opening of the pore might be an advantageous mechanism for cancer cells.

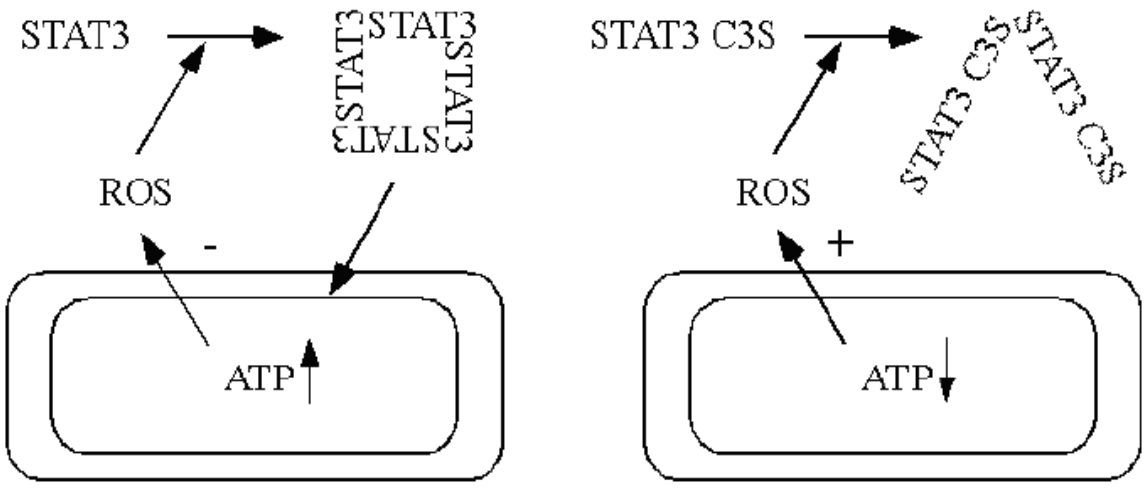

Fig.4 – Peter Shaw theory for STAT3 import to and actions on mitochondria. On the left, STAT3 can be oxidized to form tetramers that are selectively taken up into the mitochondria, increasing ATP production and reducing ROS levels. On the right, the redox insensitive STAT3 C3S mutant is unable to form tetramers and doesn't enter the mitochondria, resulting in reduced Complex I activity. *Adapted from* (Shaw, 2010).

4. Targeting mitochondrial STAT3

For the last decade, many STAT3 inhibitors have been studied, and although blockade of STAT3 in cultured tumour cells was found to induce apoptosis and inhibit cell proliferation, these compounds have had little therapeutic value so far. This might be because, until recently, only the nuclear actions of STAT3 were being targeted (Sivaraman *et al.*, 2014)(Meier e Larner, 2014). A recent study demonstrated the importance of targeting both mitochondrial and nuclear function of STAT3, in pancreatic cancer in mice (Mackenzie *et al.*, 2013).

Mackenzie *et al.* have identified phospho-valproic acid (P-V) (Fig. 5), a novel valproic acid derivative, as potent STAT3 inhibitor. Once STAT3 plays an essential role in the transformation and progression of pancreatic cancer, P-V was evaluated in different human pancreatic cancer xenografts in mice. It inhibited the growth by 60% to 97%, without any genotoxicity on Ames test and no acute toxicity. Suggesting that P-V is safe molecule that strongly supresses pancreatic cancer.

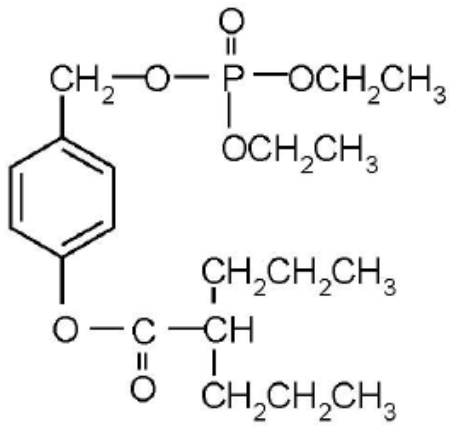

Fig.5 – The chemical structure of phospho-valproic acid (P-V).

P-V inhibits STAT3 phosphorylation through inhibition of JAK2 phosphorylation and Src activation (Mackenzie *et al.*, 2013). JAK2 and Src are overexpressed in human pancreatic cancer cells and have been connected to STAT3 phosphorylation, especially in the Tyr705 (Sivaraman *et al.*, 2014). P-V also disrupted STAT3-Hsp90 interaction, which optimizes the conformation of STAT3 for phosphorylation. This triple effect reduced STAT3 activation and decreased expression of STAT3-dependent antiapoptotic proteins, such as Mcl-1, survivin, Bcl-2 and Bcl-xL (Fig.6) (Mackenzie *et al.*, 2013).

Mitochondrial STAT3 proved to be an important molecular target of P-V, by preventing its import into this organelle. Although the mechanism is not entirely clear, P-V impaired the association STAT3-TOM20 and also led to decreased phosphorylation at Ser727 (Mackenzie *et al.*, 2013), both of which facilitate STAT3 entry into the mitochondria (Boengler *et al.*, 2010)(Zhang *et al.*, 2013). Reduced levels of mitochondrial STAT3 resulted in a decreased activity of Complex I by 29%, which in turn led to an increased superoxide anion generation. This increment in mitochondrial ROS levels collapsed the $\Delta\Psi$, activating the intrinsic apoptotic pathway (Fig.6). In addition, the lack of STAT3 could have a direct effect on $\Delta\Psi$, as STAT3 is known to inhibit the MPTP opening. Interestingly, P-V may also covalently modify STAT3 at its cysteine residues, which, according to Peter Shaw theory, may also reduce STAT3 mitochondrial levels.

This study demonstrates that targeting STAT3 nuclear actions is insufficient to reduce cell growth, what probably explains lack of clinical value of previous STAT3 inhibitors. Acting not only in the nucleus, but also in the mitochondria, makes P-V a promising candidate drug for pancreatic cancer.

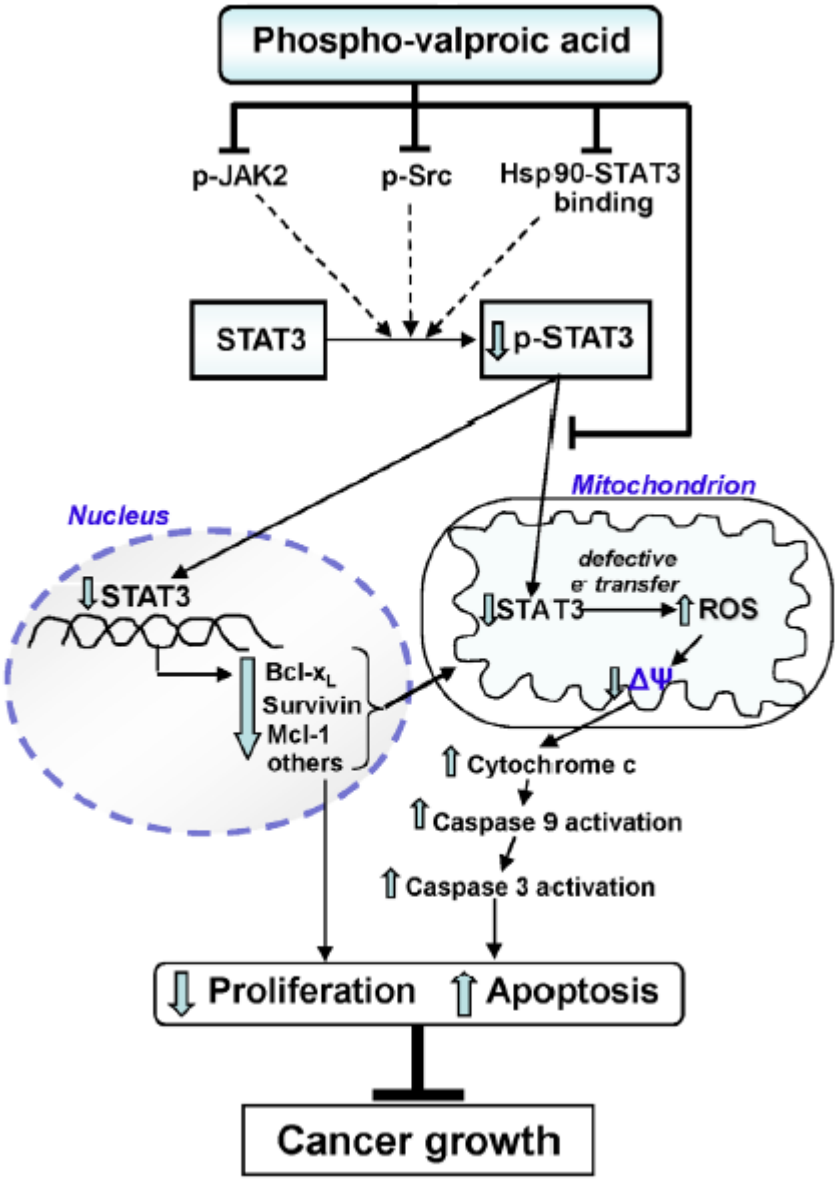

Fig. 6 – Proposed mechanism of action for P-V. Inhibition of phosphorylation at Tyr705, reduces dimerization of STAT3 and its translocation into the nucleus, resulting in decreased expression of antiapoptotic proteins. Inhibition of phosphorylation at Ser727, reduces STAT3-TOM20 interaction and its translocation into the mitochondria, resulting in decreased activity of Complex I and increased ROS levels. Mitochondrial STAT3 also inhibits MPTP opening which, coupled with elevated ROS levels, leads to the collapse of ΔΨ and activation of the intrinsic apoptotic pathway. *Adapted from* (Mackenzie *et al.*, 2013).

5. Conclusion and future perspectives

The road of STAT3 in the mitochondria is indeed a long and winding one, involving many different critical players for optimal function of this organelle. The ability of this protein to regulate ATP production and mitochondrial ROS levels, combined with its already known nuclear actions, lead us to speculate that STAT3 is associated with a multitude of biochemical pathways, thus affecting cell and organ homeostasis.

Mitochondria play a central role in the regulation of cell life and death. While this notion is important to induce apoptosis in cancer cells, it also takes us to a whole new level when it comes to cytoprotection. STAT3 might have a crucial role in preventing cell death in hypoxic conditions, especially in tissues with high levels of mitochondria, like the heart. Once overexpression of mitochondrial STAT3 enhanced cell survival, future reports should address this issue, maybe through a genetic approach, in order to increase expression of STAT3 during ischemia.

Although we have a general idea of how STAT3 acts in the mitochondria, future studies should elucidate how it regulates the ETC, especially Complex I. Once blocking STAT3 entry into the mitochondria seems a promising strategy to inhibit cancer development, we need to know exactly which proteins are involved in this import and how they co-relate with Peter Shaw Theory.

As more information about the role of mitochondrial STAT3 becomes available, we will be better guided to come up with the best strategy to involve this transcription factor in cancer cure.

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