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EFFECTS OF p66Shc EXPRESSION ON BIOENERGETICS AND CELL VIABILITY OF B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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Summary

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During tumorigenesis many oncogenic pathways are reprogrammed and modulate mitochondrial metabolism, decreasing oxidative phosphorylation (OXPHOS) activity. They inhibit ATP synthesis and ROS production from OXPHOS complexes favoring metabolic switching toward a more glycolytic metabolism and tumor growth. This metabolic rewiring is well established in solid tumor but very little is known about this event in non-solid tumors such as leukemias. Indeed, it has been demonstrated that in chronic lymphocytic leukemia (B-CLL), cells show a high OXPHOS activity and elevated ROS levels. Notably, expression of the p66Shc protein, whose mitochondrial fraction modulates cell bioenergetics and increases ROS generation, is lost during B-CLL tumorigenesis.

In this work, I have investigated the effects of the expression of p66Shc on bioenergetics and viability of B-CLL cells. I have used MEC-1 cells, a B-CLL cell model that, at variance form all solid tumor cell models that I have tested, does not express p66shc. In collaboration with Prof. Baldari's group at the University of Siena, p66Shc was expressed in MEC-1 cells and I found that a fraction of it is located in the intermembrane space of mitochondria. I checked the effect of p66Shc expression on mitochondrial respiration through extracellular flux analyzer (Seahorse XF24), which allows measuring the oxygen consumption rate (OCR) of a monolayer of adherent cells. I adapted the protocol to non-adherent cells by coating experimental wells with poly-L-lysine before plating cells and performing the analysis. I found that p66Shc expression decreases both basal and maximal respiration. These differences were maximized when cells were kept in low serum and in the absence of glucose, a condition that increases OXPHOS, blunting glycolysis and the pentose phosphate pathway, which leads to oxidative stress and mimics the microenvironment of solid tumors (which usually express p66Sshc and undergo the Warburg metabolic phenotype). p66Shc expression also decreases mitochondrial membrane potential, total ATP levels and renders cells less dependent from OXPHOS to ATP supply without changes mitochondrial mass.

In order to understand whether a modulation of respiratory chain activity could account for the observed OCR differences, I analysed the respiratory chain complex assembly

and stability. I did not observe any variation in total level of RC complexes. Thus, I performed the blue native polyacrylamide gel electrophoreses (BN-PAGE) in order to separate each OXPHOS complex in native conditions and then to perform SDS-PAGE to assess the expression level of each complex subunits. I could find that complex I is less assembled in p66shc expressing cells, whereas the assembly of other respiratory complexes and of ATP synthase were not affected by p66shc expression. Consistent with this data, p66shc-expressing cells show lower complex I activity.

I also analyzed the possibility that RC complex can be regulated by post-translational modifications and by interaction with other mitochondrial proteins. Low concentration of 3-nitropropionic acid deletes the differences in OCR indicating that complex II is involved in the definition of OCR differences observed after p66Shc expression. By using BN-PAGE I found that the mitochondrial chaperones TRAP1 and cyclophilin D (CyP-D) and the kinase ERK bind succinate dehydrogenase (SDH), *i.e.* RC complex II. Remarkably, the quantity of ERK that interacts with SDH was higher in p66Shc-expressing cells than in wild-type MEC-1 cells; moreover, p66shc expression increased phosphorylation of the fraction of ERK associated to SDH. The activity of SDH is reduced in p66Shc expressing cells and its function is modulated by ERK and TRAP1 activity. p66Shc expression increases mitochondrial ERK activation without any effects on TRAP1 protein levels, meaning that differences in SDH activity are caused by ERK activation that induces post-transcriptional modification on SDH or TRAP1 that interact with SDH. This could be particularly relevant because during tumorigenesis SDH is inhibited by its interaction with TRAP1, leading to accumulation of succinate and the ensuing stabilization of the HIF transcription factor, and to a decrease in ROS levels; both these events favour tumor growth.

Moreover, both a low level of oxidative stress and the activity of the mitochondrial fraction of ERK contribute to tumor cell survival by inhibiting opening of the permeability transition pore (PTP), a mitochondrial channel whose opening irreversibly commits cells to death. I check the effect of p66Shc on cell viability. I cultured cells in depletion conditions, low serum and absence of glucose, (a condition that mimics the microenvironment of p66shc-expressing solid tumors), that stimulates mitochondrial respiration. I found that p66shc expression protected cells from death. Even if this result

is in accord with an increased phosphorylation of mitochondrial ERK, which has a survival role and whose activation is strongly increased in starvation conditions, it is extremely surprising, because these depletion conditions kill cells following oxidative stress induction, and mitochondrial p66Shc is known to have a powerful pro-oxidant activity. In depletion conditions, I found that p66Shc expression correlates with more active mitochondrial ERK and that inhibition of ERK decreases cell viability. I also found that inhibition of mitochondrial chaperones activity with Cyclosporine A, an inhibitor of CyP-D, or 17AAG, an inhibitor of TRAP1, also affects cell viability under depletion condition, thus supporting the hypothesis that mitochondrial ERK is involved in survival mechanism induced by p66Shc expression. I also found that p66Shc expression protected cells from death induced by mitochondrial-derived oxidative stress such as EM-20-25, which is also mPTP inducer, and the chemotherapeutic cisplatin that induces ROS from RC complex I. add hydrogen peroxide

Taken together these data indicate that regulation of RC complex activity can be linked to regulation of cell survival. Indeed, in B-CLL cells a high level of mitochondrial respiration is essential to support survival of cells. We hypothesize that the mitochondrial branch of the Ras-ERK signaling pathway regulates mitochondrial bioenergetics by affecting SDH activity and resistance to apoptosis of B-CLL cells.

Moreover, these data highlight a possible new function of p66Shc. Indeed, p66Shc through the activation of mitochondrial ERK and inhibition of SDH activity can decrease OXPHOS activity and ROS production, a condition that is necessary in the early stages of tumorigenesis. It can also support tumour cell growth making mPTP less sensitive to opening by activating mitochondrial ERK. Indeed, many cancer types show high levels of p66Shc expression although its pro-apoptotic function.

Sommario

Durante il processo di tumorigenesi molte vie di segnale che regolano il metabolismo cellulare cambiano a favore di un metabolismo glicolitico. Molte di esse convergono sul mitocondrio per diminuire l'attività della fosforilazione ossidativa (OXPHOS). In particolare, queste vie diminuiscono l'attività dei complessi respiratori, la produzione di ATP e ROS da essi favorendo un metabolismo glicolitico necessario per la crescita tumorale. Questa modifica del metabolismo cellulare è tipica e ben decritta per i tumori solidi ma poco si sa sulle caratteristiche metaboliche e l'adattamento metabolico che avviene nelle leucemie. Nelle cellule di leucemia linfocitica cronica (B-CLL) è stato dimostrato che, a differenza dei tumori solidi, la fosforilazione ossidative è molto attiva e il livello di stress ossidativo nelle cellule è molto elevato. Queste cellule sono resistenti alla morte cellulare e mancano dell'espressione della proteina p66Shc. La frazione mitocondriale di questa proteina è noto influenzare la bioenergetica la produzione di ROS delle cellule.

Durante il lavoro di tesi mi sono concentrata sul definire gli effetti dell'espressione della proteina p66Shc sulla bioenergetica e la vitalità delle cellule di B-CLL.

Ho impostato il mio lavoro partendo dallo studiare l'effetto della proteina sul modello cellulare di leucemia linfocitica cronica MEC-1. A differenza dei modelli cellulari di tumori solidi le MEC-1 non esprimono p66Shc, come le cellule di pazienti B-CLL. In collaborazione con il gruppo di Tatiana Baldari dell'università di Siena, ho espresso p66Shc nelle cellule MEC-1. Una frazione della proteina è stata trovata nello spazio intermembrana dei mitocondri. Quindi sono andata ad analizzare il suo effetto sulla respirazione mitocondriale delle cellule MEC-1. Per fare questo ho utilizzato l'analizzatore del flusso extracellulare Seahorse XF24 che consente di misurare la respirazione mitocondriale di un mono strato di cellule adese. Dopo aver settato il protocollo anche per le cellule in sospensione, facendole aderire su un film di poly-L-lisina, ho osservato che l'espressione di p66Shc nelle MEC-1 diminuiva la respirazione mitocondriale sia massima che basale. Queste differenze sono massimizzate in assenza glucosio. L'assenza di glucosio è molto importante perché bloccando la glicolisi e la via

dei pentoso fosfati aumenta l'attività della catena respiratoria e lo stress ossidativo. Questa condizione mima l'ambiente in cui vivono le cellule dei tumori solidi le quali solitamente esprimono p66Shc e presentano un metabolismo glicolitico. L'espressione di p66Shc nelle cellule MEC-1 diminuisce anche il potenziale di membrana, i livelli totali di ATP e rende le cellule meno dipendenti dall'ATP prodotto dalla respirazione mitocondriale senza però modificare la massa mitocondriale.

Per poter spiegare come l'espressione di p66Shc può modificare la respirazione mitocondriale. Ho analizzato la struttura dei complessi della catena respiratoria. Se da una parte i livelli proteici totali dei complessi respiratori non cambiano dopo l'espressione di p66Shc, i livelli di complesso I assemblato diminuiscono mentre gli altri complessi rimangono invariati. A sostegno di questa osservazione, l'attività enzimatica del complesso I è minore nelle cellule che esprimono p66Shc.

Ho analizzato anche la possibilità in cui i complessi della catena respiratoria possano essere modulati da modifiche post-traduzionali o dall'interazione con altre proteine. Ho osservato che l'inibizione parziale del complesso II con basse concentrazioni di acido 3-nitropropionico diminuisce le differenze che si osservano nella respirazione basale dopo l'espressione di p66Shc nelle cellule MEC-1. Il livello proteico totale e l'assemblaggio del complesso II non variano. Sul complesso II pero, è presente un complesso multi proteico in cui sono coinvolte la frazione mitocondriale di ERK e GSK3 e gli scaperoni mitocondriali TRAP1 e CyP-D. L'espressione di p66Shc aumenta i livelli proteici di GSK3 ed ERK sul complesso II, ma non varia i livelli proteici di TRAP1 e CyP-D. p66Shc aumenta anche l'attività di ERK nel mitocondrio. A questa condizione è associata una minor attività del complesso II. L'inibizione della frazione mitocondriale di ERK e dello scaperone TRAP1 aumentano l'attività enzimatica del complesso II. L'espressione di p66Shc non influenza i livelli proteici totali di TRAP1 ma aumenta l'attivazione di ERK nel mitocondrio ed inibisce l'attività di GSK3. Questo significa che l'attività del complesso II è modulata da ERK, che come chinasi può fosforilare direttamente il complesso II o le proteine coinvolte nell'interazione con esso. Questo effetto di p66Shc può essere molto importante nella tumorigenesi. Infatti, il complesso II viene inibito da TRAP1 portando alla stabilizzazione del fattore pro-oncogenico HIF e alla diminuzione della produzione di ROS. Questi due eventi sono fondamentali per la crescita tumorale. Quindi p66Shc

diminuendo l'attività del complesso II attraverso l'attivazione di ERK potrebbe stimolare la glicolisi e indurre l'adattamento metabolico alla base dello sviluppo tumorale.

La diminuzione dei livelli di stress ossidativo e l'attività della frazione mitocondriale di ERK contribuiscono alla sopravvivenza delle cellule tumorali attraverso l'inibizione dell'apertura del poro di transizione di permeabilità (mPTP), un canale mitocondriale la cui apertura porta alla morte delle cellule. Ho analizzato gli effetti dell'espressione di p66Shc sulla vitalità delle cellule MEC-1. In condizioni basali (10% FBS) non si osserva nessuna differenza nella vitalità. Coltivando le cellule in assenza di siero e glucosio, l'espressione di p66Shc protegge le cellule dalla morte indotta dallo stress ossidativo. Questa condizione di deplezione infatti, mima l'ambiente dei tumori solidi che normalmente esprimono p66Shc, stimola l'attività della catena respiratoria e i ROS prodotti da essa. In condizione di deplezione ERK è attivo e l'espressione di p66Shc ne induce ulteriormente la fosforilazione. Questi dati sono in accordo con l'aumento dell'attività della frazione mitocondriale di ERK che normalmente è associata con la maggior sopravvivenza delle cellule. D'altro canto però essi sono in contrasto con la funzione pro-ossidante di p66Shc. Infatti, la deplezione di siero e glucosio, seguita dall'aumento dello stress ossidativo dovrebbe indurre l'attività pro-ossidante di p66Shc inducendo la morte nelle cellule. L'inibizione dell'attivazione di ERK diminuisce la vitalità delle cellule in condizione di deplezione come pure l'inibizione degli scaperoni mitocondriali TRAP1 e CyP-D. Questo indica che la frazione mitocondriale di ERK attiva è coinvolta nella regolazione della vitalità cellulare. L'espressione di p66Shc protegge le cellule dalla morte indotta da altri stimoli che inducono stress ossidativo a livello dei mitocondri come l'EM20-25, che è anche un induttore del poro, ed il Cisplatino, un chemioterapico che induce la produzione di ROS a livello del complesso I.

Questi dati indicano che la regolazione dei complessi della catena respiratoria può influenzare la vitalità cellulare. Infatti, nelle cellule di B-CLL la respirazione mitocondriale e lo stress ossidativo elevati sono essenziali per rendere le cellule resistenti alla morte cellulare. Noi ipotizziamo che la frazione mitocondriale della via di segnale RAS-ERK possa regolare la bioenergetica delle cellule influenzando l'attività del complesso II e anche la loro vitalità. Inoltre, questi dati definiscono una nuova funzione per p66Shc.

Infatti, p66Shc attraverso l'attivazione della frazione mitocondriale di ERK e l'inibizione del complesso II può diminuire l'attività OXPHOS e la produzione di ROS. Questi eventi sono essenziali nelle prime fasi di sviluppo dei tumori. Inoltre, p66Shc può supportare la crescita tumorale rendendo il poro meno sensibile all'apertura attivando la frazione mitocondriale di ERK. Questo spiegherebbe perché molti tumori solidi esprimono elevate livelli di p66Shc sebbene questa proteina presenti funzioni pro-apoptotiche.

1. Introduction

1.1   Cancer

Cancers are a group of diseases characterized by abnormal cell growth with the potential to invade and spread in the body. The pathophysiological processes underlying neoplastic transformation are extremely complex, with several intermediate steps in the stepwise progression from normalcy to malignancy via a series of premalignant cell stages. Genetic alterations such as point mutations and chromosome instability characterize each of these steps, favouring cell survival, cell proliferation and dissemination in the body. Despite this complexity, and the definition of cancer as a real, albeit abnormal, tissue composed by different cell types involved in heterotypic interactions with each other, all cancer types can be defined by a small number of underlying features (Hanahan & Weinberg 2011) (**Figure 1.1**):

- Self-sufficiency in growth signals
- Insensitivity to growth-inhibitory (anti-growth) signals
- Evasion of programmed cell death (apoptosis)
- Limitless replicative potential
- Sustained angiogenesis
- Tissue invasion and metastasis
- Genome instability and mutations
- Tumour-promoting inflammation
- Avoidance of immune destruction
- **Deregulation of cellular energetics**.
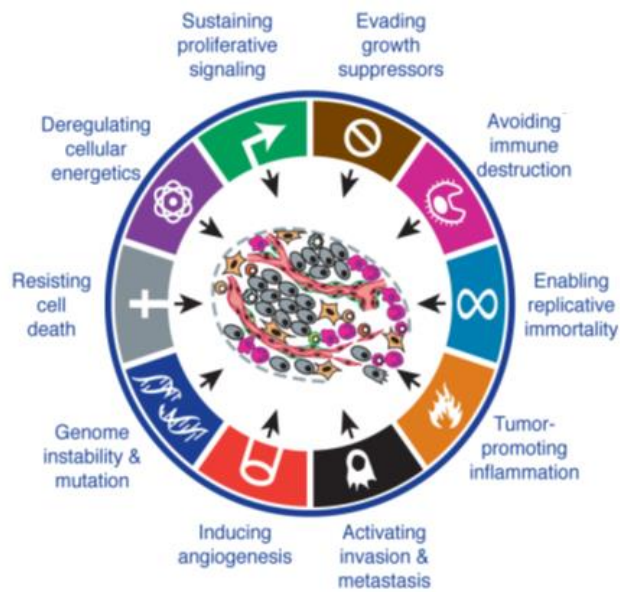
Figure 1.1 **Hallmarks of cancer cells** (Hanahan & Weinberg 2011)

Each of the hallmarks listed above is regulated by one or more molecular circuits that control a biological routine in normal cells, but are dysregulated in cancer cells, providing them with selective advantages. These circuitries are strictly interconnected and cross talk to each other (**Figure 1.2**) (Hanahan & Weinberg 2011).

A detailed comprehension of the oncogenic mutations and of the biochemical alterations that characterize each cancer type is mandatory for the development of effective antineoplastic therapies.
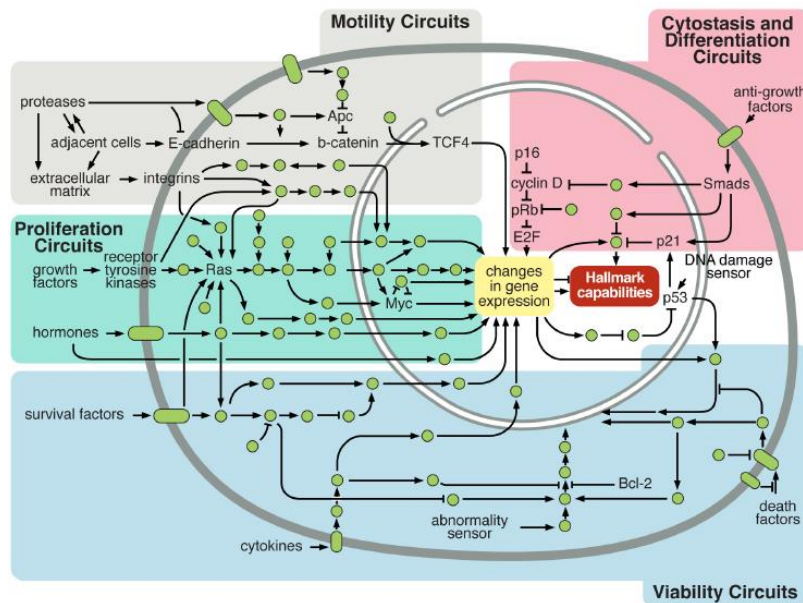
Figure 1.2 **Intracellular Signalling Networks Regulate the Operations of the Cancer Cell** (Hanahan & Weinberg 2011)

1.2   Cancer metabolism

Cancer metabolism has received a substantial amount of interest over the last decade. There are many evidences by which oncogenic signal pathways are strictly connected with metabolic activities. Moreover, the accumulation of oncogenic metabolites (oncometabolites) produced by reprogrammed cell metabolism is involved in the formation and malignant progression of cancers. Thus, metabolic reprogramming is being increasingly recognised as a key feature of cancer cells. The identification of metabolic changes of cancer cells can be instrumental to set up anti-neoplastic strategies (Schulze & Harris 2012).

In multicellular organisms, growth factors stimulate cells to take up nutrients from the environment only when it is necessary, and when nutrient availability exceeds cell needs, homeostasis is reached through inhibitory or catabolic signals. Cancer cells overcome these controls by acquiring genetic mutations that alter signalling pathways. Many studies have demonstrated that many oncogenic mutations constitutively activate the uptake and metabolism of nutrients that fuel cell growth and proliferation (Vander Heiden et al. 2009). Energy metabolism is essential to support cell growth, proliferation and survival because it provides energy, lipids, nucleotides and amino acids. Cancer cells

change the regulation of energy metabolism to support their abnormal growth and proliferation (Hanahan & Weinberg 2011; Cairns et al. 2011; Tennant et al. 2009).

In the presence of oxygen, normal cells metabolize glucose to carbon dioxide through glycolysis, followed by the oxidation of glycolytic pyruvate in the mitochondrial tricarboxylic acid (TCA) cycle. This produces NADH (reduced nicotinamide adenine dinucleotide) that fuels mitochondrial respiration to generate adenosine 5'-thriphosphate (ATP), with a minimal production of lactate. Under anaerobic conditions, glycolysis is favoured and normal cells divert glycolytic pyruvate into lactate production, whereas only a very low amount of pyruvate is utilized in the TCA cycle. By contrast, cancer cells produce large amounts of lactate even in the presence of oxygen sustaining ATP production by glycolysis and decreasing mitochondrial metabolism. This kind of metabolism is termed "aerobic glycolysis" (Figure 1.3) (Vander Heiden et al. 2009).

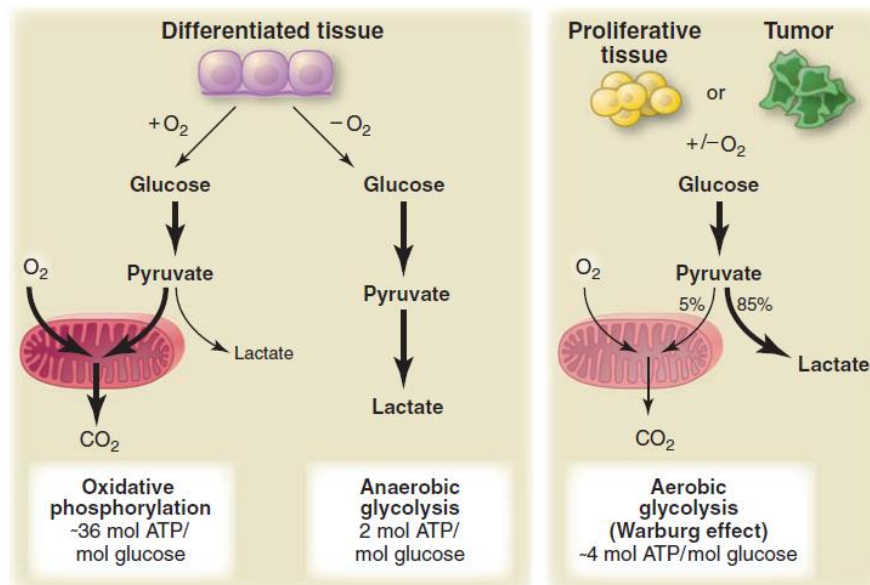Figure 1.3 **ATP yield of OXPHOS, anaerobic glycolysis and aerobic glycolysis (Warburg Effect)** (Vander Heiden et al. 2009)

1.2.1 The Warburg effect

In 1897, Louis Pasteur postulated the "Pasteur effect" in which cells stop to produce lactic acid in the presence of oxygen (Koppenol et al. 2011). In 1924, Otto Warburg observed that rapidly growing tumour cells consumed glucose at high rate compared

with normal cells in the presence of oxygen. Most of the glucose derived-carbon was secreted by neoplasm in the form of lactate (Warburg 1924; Frezza & Gottlieb 2009). He postulated that this phenomenon, termed "aerobic glycolysis", was provoked by mitochondrial respiration impairment and was the cause of neoplastic transformation of cells (Warburg 1956a; Warburg 1956b). In the same period, Herbert Grace Crabtree observed that increased glycolysis in cancer and normal proliferating cells inhibited respiration. This observation is now known as the "Crabtree effect" (Crabtree 1929; Frezza & Gottlieb 2009; Rasola et al. 2014).

The oncogene revolution, *i.e.* the discovery of genes whose mutations could prompt tumorigenesis (Hunter 1997; Knudson 2002), pushed tumour metabolism to the margins of cancer research. Warburg's observations were forgotten for a long period, and the metabolic alterations of tumours were simply considered as an epiphenomenon of changes elicited in neoplastic cells by mutations in oncogenes and tumour suppressor genes. Despite this, several further metabolic alterations and adaptations of cancer cells have been discovered in the following decades that refine the Warburg theory (Figure 1.4).

Thanks to these studies, we now appreciate that the upregulation of glycolysis first observed in 1920s is only one face of a dramatic metabolic reprograming that induces alterations in all major anabolic pathways and confers essential bioenergetic and biosynthetic advantages to the rapid proliferating tumour cells (Figure 1.4) (Cairns et al. 2011; Tennant et al. 2010; Koppenol et al. 2011).
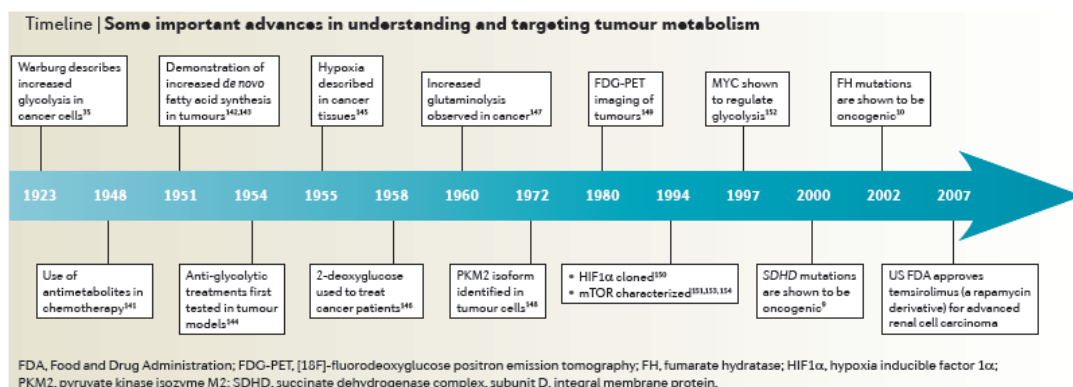

Figure 1.4 **Timeline indicating some important advances in understanding and targeting tumour metabolism** (Tennant et al. 2010)

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Today, it has been well characterized that cells of many solid tumours develop aerobic glycolysis (Vander Heiden et al. 2009) (Figure 1.3) in order to adapt to the stressful and dynamic microenvironment typical of cancer, where the availability of nutrient such as glucose, glutamine and oxygen are spatially and temporary heterogeneous (Cairns et al. 2011). Glycolysis is less efficient to produce ATP than OXPHOS but the enzymes of the glycolytic pathway have a high V_{MAX}. Thus, glycolytic ATP can exceed the ATP produced by OXPHOS, and glycolysis can generate ATP faster than OXPHOS, providing sufficient energy to sustain a high rate cell proliferation in conditions of high glucose availability. Furthermore, the high rate of glycolysis allows cells to maintain biosynthetic fluxes during rapid proliferation. Indeed, glucose degradation is used to generate building blocks essential for cell proliferation. Intermediates of glycolysis such as glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) feed the pentose phosphate pathway (PPP) to produce ribose for nucleotides. The 3-phosphoglycerate can be converted to glycine for purine and non-essential amino acid synthesis and NADPH that is important for ROS scavenging systems (glutathione etc). To fuel the TCA cycle, pyruvate derived from glycolysis needs to be imported into mitochondrial matrix and phosphorylated by pyruvate dehydrogenase (PDH). In tumour cells, the rate of glycolysis can overpace PDH activity. In these conditions, pyruvate accumulates in cytosol and lactate dehydrogenase A (LDH-A) rapidly consumes it (Ralph J. DeBerardinis et al. 2008). Thus, the functions of the TCA cycle shift from the production of NADH for OXPHOS to the production of biosynthetic intermediates, *i.e.* citrate for fatty acid synthesis, aspartate for nucleotide synthesis, oxaloacetate and α-ketoglutarate for non-essential amino acids.

In order to maintain the TCA cycle despite low pyruvate uptake and less carbon intermediates to other pathways, the metabolism of glutamine (glutaminolysis) is increased to produce α-ketoglutarate to feed the TCA cycle, producing ATP and biosynthetic intermediates (Figure 1.5) (Cairns et al. 2011; Ralph J DeBerardinis et al. 2008; Deberardinis et al. 2008).
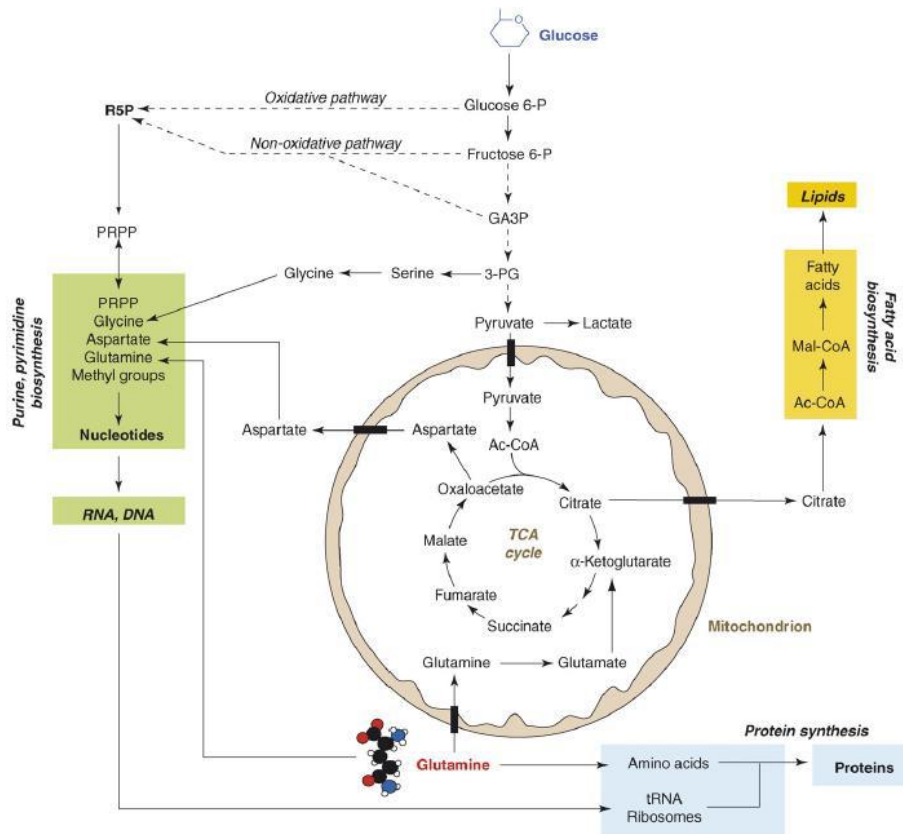
Figure 1.5 **Tumour cells obtain biosynthetic precursors from glucose and glutamine metabolism** (Deberardinis et al. 2008).

1.2.2 Changes in signal transduction that affect tumor cell metabolism

Cancer cells acquire mutations that chronically activate several pathways involved in the control of cell growth and proliferation, losing the capability of regulating these biological processes (Ralph J DeBerardinis et al. 2008).

Several crucial components of these pathways that are dysregulated in tumor cells have now been implicated in the control of metabolism. These include the growth-factor activated PI3K pathway; the transcription factors HIF-1α and MYC; the metabolic master switch AMPK, the tumour suppressor p53, and the RAS oncogenic pathway (Cairns et al. 2011).

1.2.2.1 The PI3K/AKT pathway

 PI3K signalling is one of the most commonly activated pathways in human cancers by mutations in tumour suppressor genes such as PTEN, in PI3K itself, and by aberrant

signalling from receptor tyrosine kinases (Tennant et al. 2010; Ralph J DeBerardinis et al. 2008).

This pathway is not only important for growth and survival signals but also has profound effect on cellular metabolism. Through the activation of its effector protein kinase AKT, PI3K modulates both expression and activity of key glycolytic enzymes, such as hexokinase 2 (HK II), the enzyme that converts glucose to glucose-6-phosphate (G6P), and phosphofructokinase 2 (Vander Heiden et al. 2009; Cairns et al. 2011; Ralph J DeBerardinis et al. 2008). By activating the mTOR complex, a branch point for growth signals and nutrient availability, the PI3K/AKT pathway stimulates protein and fatty acids biosynthesis. In addition, mTOR indirectly induces other metabolic changes by activation of hypoxia inducible factor 1 (HIF-1), resulting in a shift toward a more glycolytic metabolism (Cairns et al. 2011).

1.2.2.2 Hypoxia-induced factor 1 (HIF-1) signalling

HIF-1 is the major transcription factor involved in cellular response to low oxygen conditions (hypoxia). It regulates genes encoding glucose transporters, glycolytic enzymes and lactate dehydrogenase (LDH-A), stimulating cells to consume glucose and produce lactate. HIF-1 activity requires the HIF-1α subunit, whose expressions is related to activation of PI3K/AKT/mTOR pathway. During normoxia, HIF-1α is degraded by the ubiquitin-proteasome system following prolyl-hydroxylation induced by the cytosolic prolyl-hydroxilase (PHD) enzyme. During hypoxia, HIF-1α is stabilised and activates a genetic program that supports cell viability in low oxygen conditions, including motility, angiogenesis and metabolic rewiring (Semenza 2010; Cairns et al. 2011).

In cancer cells, HIF-1α can be stabilised also under normoxic conditions favouring the Warburg metabolic phenotype; this condition is called "pseudo-hypoxia". Here, the stabilization of HIF-1α occurs as a result of mutations in oncogenic signalling pathways and, interestingly, in the mitochondrial TCA enzymes succinate dehydrogenase (SDH) and fumarate hydratase (FH). Mutations in SDH and FH lead to a cytosolic accumulation of succinate and fumarate, which act as oncometabolites by inhibiting PHD and the ensuing degradation of HIF-1α. HIF-1α associates into the nucleus with HIF-1β to form the HIF1 transcription complex that activates the transcription of (i) glucose transporters

and glycolytic enzymes; (ii) isoform 2 of pyruvate kinase (PKM2), the enzyme that converts phosphoenolpyruvate (PEP) into pyruvate; PKM2 shows a lower activity compared with the isoform usually expressed in normal cells (PKM1); (iii) pyruvate dehydrogenase kinase (PDK), which inhibits the mitochondrial pyruvate dehydrogenase complex. The consequent reduction of pyruvate flux into mitochondria results in a low rate of OXPHOS and oxygen consumption, reinforcing the glycolytic phenotype (Cairns et al. 2011; Frezza & Gottlieb 2009; Ralph J DeBerardinis et al. 2008). Moreover, slowing the final glycolytic steps by PKM2 expression allows glycolytic intermediates to enter other pathways such as uridine diphosphate-glucose synthesis, glycerol synthesis and the PPP (see Figure 1.4) (Cairns et al. 2011). These pathways generate macromolecule precursors that are necessary to support cell proliferation, and reducing equivalents such as NADPH to be used by antioxidant systems (Cairns et al. 2011). Furthermore, PKM2 functions also as a transcriptional coactivator of HIF-1α by directly interacting with it, taking part in a positive feedback loop that enhances HIF-1 activity, leading to metabolism reprogramming in cancer cells (Besinger Steven J. 2012).

1.2.2.3 MYC

The transcription factor MYC regulates cell growth, proliferation and metabolism. In cancer cells, its upregulation supports the development of a Warburg-like phenotype by inducing glucose transporters and glycolytic enzymes as well as PDK and LDH-A (Cairns et al. 2011). Furthermore, MYC also provides macromolecules and antioxidants that are required for tumour cell growth through the induction of glutaminolysis by directly activating genes involved in glutamine metabolism such as glutamine transporters and glutaminase-1 (GLS-1). GLS-1 converts glutamine into glutamate which, transformed into α-ketoglutarate, feeds the TCA cycle providing carbon for fatty acid, amino acid, purine and pyrimidines synthesis. Glutamate could also be converted into the antioxidant molecule glutathione for contributing to redox homeostasis (Cairns et al. 2011). Furthermore, like HIF-1α, MYC increases the expression of PKM2 thereby controlling the rate of glycolysis and the generation macromolecules precursors and reducing equivalent such as NADPH (Cairns et al. 2011).

1.2.2.4 AMP-activated protein kinase

AMP-activated protein kinase (AMPK) is the crucial sensor of the energy status of cells and plays an important role in the cellular response to metabolic stress. The AMPK complex is a potent inhibitor of mTOR and functions as a metabolic checkpoint, regulating cellular response to energy availability. During periods of energetic stress, AMPK becomes activated in response to an increased AMP/ATP ratio. Accumulated AMP activates AMPK shifting cells to oxidative metabolism and inhibiting cell proliferation (Cairns et al. 2011). Suppression of AMPK signalling in cancer cells uncouples fuel signals from growth signals, e.g. by activating mTOR and HIF, allowing cells to proliferate under abnormal nutrient conditions and supporting the shift towards glycolytic metabolism (Cairns et al. 2011; Vander Heiden et al. 2009).

1.2.2.5 p53 and metabolism

p53 is an important transcription factor and a tumour suppressor involved in the DNA damage response (DDR), apoptosis and regulation of metabolism (Vousden & Ryan 2009). It activates the expression of HK II, but it also inhibits further glycolytic steps by upregulating the expression of TIGAR (TP-53 induced glycolysis and regulator of apoptosis), an enzyme that decreases the levels of the glycolytic activator fructose-2,6-bisphosphate. By redirecting G6P to the PPP pathway, p53 provides NADPH to generate a reduced form of glutathione that protect cells from oxidative stress (ROS). p53 also promotes OXPHOS through the expression of SCO2, which is required for the assembly of complex IV of the electron transport chain. Thus, the loss of p53 that occurs in most tumors favours the Warburg phenotype, activating glycolysis and inhibiting OXPHOS activity (Cairns et al. 2011).
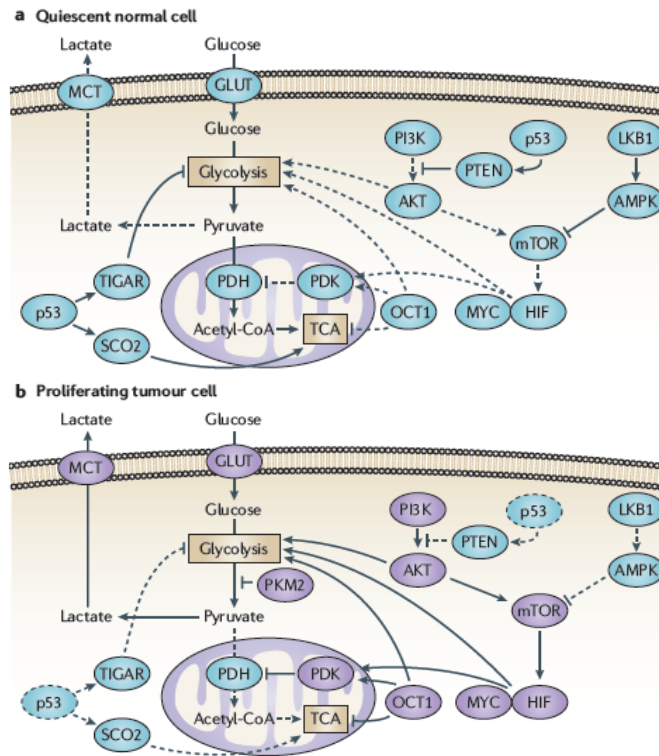
Figure 1.6 **Molecular mechanisms driving the Warburg phenotype from quiescent normal cells (a) to proliferating tumor cells (b)** (Cairns et al. 2011).

1.2.2.6 RAS

Ras proteins H-Ras, N-Ras, K-Ras4A and K-Ras4B, encoded by the RAS gene, are small GTPase that cycle between inactive guanosine diphosphate (GDP)-bound and active guanosine triphosphate (GTP)-bound conformations (Ras-GDP and Ras-GTP, respectively). Ras proteins are activated by soluble growth factors following their binding to membrane cognate receptors (Shubbert et al. 2007). Active Ras-GTP can interact with many effector proteins, controlling a variety of transduction pathways (Figure 1.7) (Karnoub & Weinberg 2008).

Ras-GTP can directly interact with the serine/threonine kinase Raf-1, which in turn activates protein kinases MEK and then ERK1/2 (Steelman et al. 2011; McCubrey et al. 2007; Karnoub & Weinberg 2008). ERK1/2, a serine/threonine protein kinase, can phosphorylate cytosolic and nuclear substrates, including transcription factors such as JUN, and ElK-1 that control cell proliferation and resistance to apoptosis.

Activating Ras mutations occur in many types of cancers: KRAS mutations are prevalent in pancreatic, colorectal, endometrial, biliary track, lung and cervical cancers; KRAS and

HRAS mutations are found in myeloid malignancies; and NRAS and HRAS mutations predominate in melanoma and bladder cancers, respectively (Karnoub & Weinberg 2008; Shubbert et al. 2007).
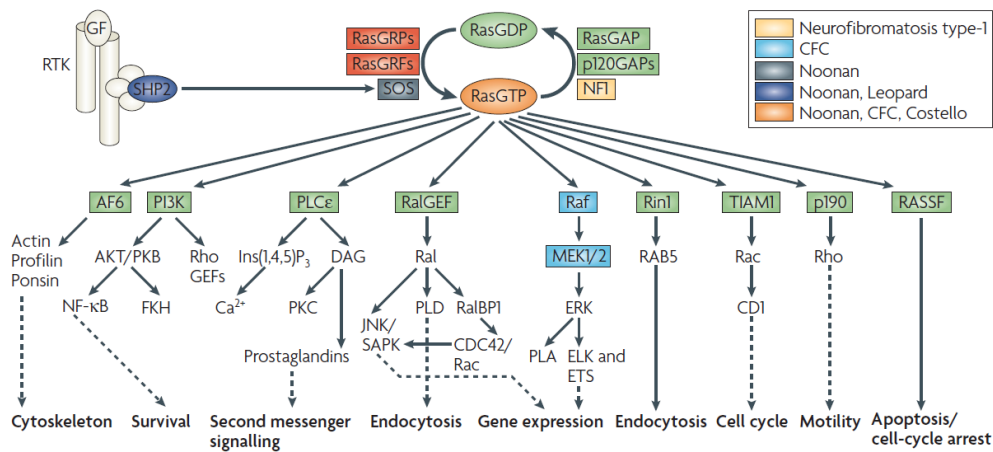
Figure 1.7 **Ras signalling network** (Karnoub & Weinberg 2008)

In the last few years increasing evidences indicate that hyperactive Ras stimulates glutamine uptake and regulates the metabolic switch toward the Warburg phenotype (Figure 1.8) (Ramanathan et al. 2005; Yun 2009; Weinberg 2010; Yang et al. 2012). Multiple molecular mechanisms underpin the metabolic role of Ras signalling: direct activation of both ERK and PI3K/AKT transduction axes, both of which are directly involved in metabolic control (see above); and activation of the transcription factors MYC and HIF-1 (Kikuchi et al. 2009; Sears et al. 1999).
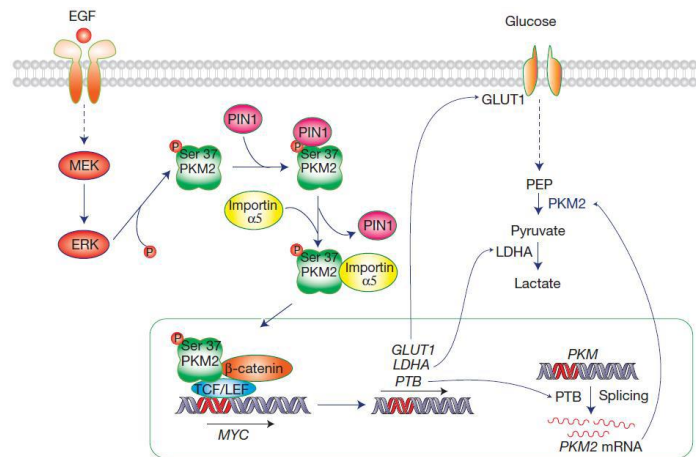
Figure 1.8 **ERK1/2-dependent phosphorylation and nuclear translocation of PKM2 promotes the Warburg effect** (Yang et al. 2012)

1.2.3 Redox status

Reactive oxygen spices (ROS) are highly reactive molecules that are produced by metabolism of normal cells as normal byproducts. They behave as oxidants, which can extract electrons from DNA, proteins, lipids and other molecules. Superoxide anion ($O_2^{.-}$), generated as an OXPHOS byproduct, is the precursor of all major forms of ROS, including hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-).

ROS can act as signalling molecules, in redox signalling systems that usually involve protein kinase (PK) activation, mainly through inhibition of protein phosphatases (PP) that have reactive cysteine residues in their catalytic site, whose oxidation leads to enzyme inactivation. The phosphorylation enhancement that characterizes conditions of increased intracellular ROS levels can promote several cellular events such as proliferation, differentiation and cell migration. It involves all major classes of tyrosine and serine/threonine kinases (Ralph et al. 2010; Fogg et al. 2011; Kamata & Hirata 1999).

A crucial component of the biological effects of ROS is the intensity of the signal. Indeed, an increase in intracellular ROS levels under a certain threshold can prompt cell proliferation and survival through the post-translational modifications of kinases and phosphatases, and ROS can also induce the expression of stress-responsive genes such as HIF-1α (Giannoni et al. 2005; Cao et Al. 2009). Instead, high levels of ROS can overload oxidant buffering systems, damaging all major classes of cell macromolecules

and leading to protein inactivation, DNA mutagenesis, membrane permeabilization and eventually cell death (Cairns et al. 2011).

Cells counteract the detrimental effects of ROS by activating anti-oxidant systems such as superoxide dismutase (SOD), which catalyses the dismutation of superoxide into H_2O_2; catalase, which converts peroxide into H_2O and O_2, and GSH peroxidase/GSSG reductase and TRX/thioredoxin reductase (TRXR) systems. GSH peroxidase transfers electrons from reduced glutathione (GSH) to reduce H_2O_2 to water. The oxidised glutathione produced (GSSG) is reduced back to GSH by glutathione reductase utilizing NADPH. The TRX redox system catalyses the reduction of disulphides (S-S) within oxidised proteins, such as TRX. TRXR and NADPH reduce oxidised TRX. Importantly, several of these detoxification systems rely on the reducing power of NADPH to maintain their activities (Ralph et al. 2010; Cairns et al. 2011).

Cancer cells exhibit changes in their redox status, with elevated ROS levels caused both by changes in signalling pathways, metabolic networks and mitochondrial function, and by exposure to fluctuating conditions of oxygen and nutrient supply in the surrounding environment characterised by prolonged hypoxia and nutrient deprivation. Aberrant signalling, such as upregulation of translation mediated by hyperactivation of mTOR, or PI3K/AKT signalling induction, can increase ROS levels (Li et al. 2010; Nogueira et al. 2008; Ozcan et al. 2008). The loss of p53 increases ROS levels as p53 drives GSH *de novo* biosynthesis by upregulating the expression of glutaminase 2 (GLS2) (Suzuki et al. 2010), the master antioxidant transcription factor NRF2 (Chen et al. 2009). Normally, it is maintained at low levels through proteasomal degradation mediated by KEAP1 protein (kelch-like ECH-associated protein 1). Under oxidative stress, NRF2 binds onto antioxidant response element (AREs) and turns on DNA stress response genes, such as Heam oxygenase 1 (OH-1), activating the cellular antioxidant response. In cancer cells, accumulation of NRF2 creates an environment conducive for cell growth and protects against oxidative stress, chemotherapeutic agents, and radiotherapy (Jaramillo & Zhang 2013). It has been demonstrated that loss of p53 disrupts the NRF2-KEAP1 interaction that induces constitutive transcription of NRF2 target genes protecting cancer cells from increased oxidative stress (Cairns et al. 2011; DeNicola & Al. 2011). Moreover, excess of fumarate due to mutations in fumarate hydratase can inactivate KEAP1 protein through

its succination (see after) thereby stabilizing NRF2 (Frezza et al. 2011). Thus, enhanced ROS detoxification and additional NRF2 functions are pro-tumorigenic. Increased ROS levels lead to HIF family protein stabilisation, which in turn induces RAS, MYC and p53, supporting cell transformation (Gordan et al. 2007). ROS can also directly act on receptor tyrosine kinases inducing their ligand-independent activation or inhibiting the activity of phosphatases (McCubrey et al. 2007; Weinberg & Chandel 2009). At later neoplastic stages, high ROS levels damage nucleic acids resulting in genomic instability that can induce further oncogenic lesions resulting in a positive loop that boosts the rate of ROS production and proliferation, resistance to stress conditions, invasiveness and metastatic properties (Ralph et al. 2010; Guzzo et al. 2014).

These increased levels of ROS must be kept under tight control by enhancing anti-oxidant defences, in order to avoid lethal oxidative stress. This is particularly important in the early tumorigenic phases in which cells are more sensitive to ROS-induced cell death because they have not yet adapted the redox control systems to metabolic and signalling changes (Andrea Rasola & Bernardi 2014). In accord with this model, several oncogenes induce a ROS scavenging program in early phases of tumour growth, based on upregulation of MYC, which supports antioxidant capacity by driving PPP-based NADPH production, and of Ras, which induces stable flow of glutamine and GSH generation, thus supporting antioxidant defence (Cairns et al. 2011; Guzzo et al. 2014; Denicola et al. 2012).

Importantly, in all stages of tumour growth malignant cells are endowed with an extremely fragile redox balance, which renders them vulnerable to oxidants (Rasola et al. 2014).

The control of ROS and the mechanisms designed to counter them allow cancer cells to avoid the detrimental effects of oxidative stress, but also increase their vulnerability to additional increases of ROS that could be used as anti-neoplastic strategies (Figure 1.9) (Cairns et al. 2011).
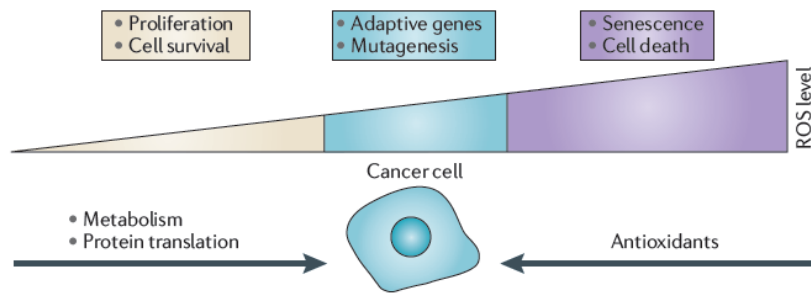
Figure 1.9 **Relationship between the levels of ROS and cancer** (Cairns et al. 2011).

1.3 Mitochondria and cancer

Many of the oncogenic pathways and tumour suppressor genes that have been mentioned in the section 1.2.2 change mitochondrial metabolism decreasing mitochondrial respiration and driving the metabolic switching toward a Warburg phenotype (Figure 1.10).

These organelles are characterised by a double membrane structure that creates two mitochondrial compartments: an internal matrix surrounded by the inner mitochondrial membrane (IMM) and a narrower intermembrane space surrounded by the outer mitochondrial membrane (OMM). The mitochondrial matrix and the inner membrane are the sites of numerous enzymes, including those involved in citric acid cycle and oxidative phosphorylation (OXPHOS).

Mitochondria support energy demand of cells synthesizing ATP through the process of OXPHOS, but they also control cell death programs. Among mitochondrial death effectors, opening of the mitochondrial channel called permeability transition pore (mPTP) has a crucial role in committing cells to death (Figure 1.10). ROS, which mainly derive from OXPHOS, are strong mPTP inducers. Thus, cancer cells, which show a decrease in OXPHOS activity, can reduce ROS levels, thus inhibiting the mPTP and supporting cell viability (Wallace 2012; Fogg et al. 2011).
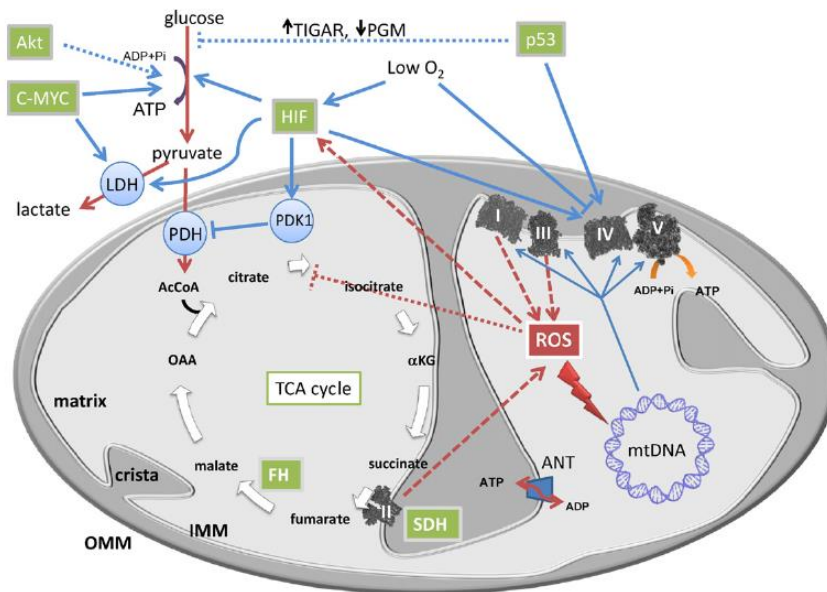
Figure 1.10 **Mitochondria as targets for multiple metabolic transformation events**. Genetic reprogramming and environmental changes induce metabolic perturbations in cancer cells. The activation of Akt and MYC oncogenes and the loss of p53 tumour suppressor gene are among the most frequent events in cancer. Furthermore, all solid tumours are exposed to oxidative stress and hypoxia, hence to HIF activation. These frequent changes in cancer cells trigger a dramatic metabolic shift from OXPHOS to glycolysis. In addition, direct genetic lesions of mtDNA or of nuclear encoded mitochondrial enzyme (SDH or FH) can directly down-modulate OXPHOS in cancer (Frezza & Gottlieb 2009).

1.3.1 Mitochondrial bioenergetics

The reducing equivalents derived from carbohydrates and fats are completely oxidised by oxygen via electron transport chain (ETC). The ETC collects electrons from NADH and H^+ via complex I (NADH oxidoreductase) and from succinate by complex II (succinate dehydrogenase SDH) and transfers each pair of electrons to reduce ubiquinone to ubiquinol. Ubiquinol then transfers electrons to complex III (the bc_1 complex or ubiquinone: cytochrome *c* oxidoreductase) which in turn donates electrons to cytochrome *c*, which finally passes them to complex IV (cytochrome *c*, COX or reduced cytochrome *c*: oxygen oxidoreducatase). Here two electrons are passed to an atom of oxygen to create molecules of water. The energy that is released by the ETC is used to pump protons out across the inner mitochondrial membrane through complexes I, III and IV. This creates an electron gradient or capacitance ($\Delta p_m = \Delta \mu_{\boxed{}} + \Delta pH$) that is positive, acid on the outside, negative, and alkaline on the inside. This proton gradient is utilised by complex V (ATP synthase) to generate ATP via the flow of protons back into the

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matrix through a proton channel in the ATP synthase enzyme. Δp_m is directly linked to the activity of ETC complexes. Indeed, it regulates the ETC activity: at high potentials further proton pumping is inhibited, whereas a decrease of Δp_m through proton utilisation allow the ETC to rebuild Δp_m (Hüttemann et al. 2008; Wallace 2005).

Usually, the major ROS production occurs at the level of complex I and III, as these respiratory complexes can donate an electron directly to O_2 to generate superoxide. Superoxide anion ($O_2^{.-}$), which is a strong oxidizing agent, is rapidly dismutated by mitochondrial manganese superoxide dismutase (MnSOD) into hydrogen peroxide (H_2O_2), which can diffuse out into the cytosol and the nucleus.

Mitochondrial ROS generation can be enhanced by partially inhibiting electron flux through the ETC, either by diminishing the efficiency of one of the ETC complexes or by inhibiting ATP synthase. This increases the proton gradient, complex I, III and IV cannot further pump protons, therefore the ETC stalls and in the end this results in $O_2^{.-}$ accumulation (Wallace 2005).

In glycolytic cancer cells, the down-modulation of mitochondrial metabolism can strongly impact on mitochondrial superoxide levels (Murphy 2009; Holmstrom & Finkel 2014). This inhibition of mitochondrial bioenergetics can be ascribable to mutations in mitochondrial and nuclear genes encoding mitochondrial TCA cycle enzyme and ETC complexes (Wallace 2012), to changes in the content and assembly of ETC complexes (Solaini et al. 2011) or to altered modulation of ETC complex activities induced by mitochondrial proteins that become deregulated during tumorigenesis (Sciacovelli et al. 2013).

1.3.1.1 Mutations that alter mitochondrial metabolism in cancer

Mitochondrial proteins are encoded both by nuclear genes and by mitochondrial DNA (mtDNA), a circular molecule of about 16.5Kb where 13 genes for subunits of complex I, complex III, complex IV and ATP synthase are located. Somatic and germline mutations in genes for mitochondrial proteins have been reported in a wide variety of neoplasms (Frezza & Gottlieb 2009; Wallace 2012).

Many of these mutations affect genes encoding subunits of respiratory complex I, the first site of electron transport chain and an active site of ROS generation. Mutations in

complex I are thought to promote tumorigenesis by unbalancing cell redox equilibrium. For instance, mutations in oncocytic thyroid carcinomas and renal oncocytomas correlate with low respiration rate, decreased complex I and III activities, reduced ATP content and high ROS production (Bonora et al. 2006; Simonnet et al. 2003). Tumour cells bearing mtDNA mutations on genes encoding the complex I subunit NADH dehydrogenase subunit 6 (ND6), have a defective respiration and subsequently overproduce ROS, increasing their metastatic potential (Ishikawa et al. 2008). However, complex I deficiency renders tumour cells prone to cell death induction through opening of the mPTP (Porcelli et al. 2009).

Mutations in subunits of other respiratory chain complexes have been found in many cancers. In colorectal, ovarian, thyroid, and bladder cancers, complex III mtDNA mutations are found (Gaude & Frezza 2014; Fliss et al. 2000). Complex IV is the rate limiting step of ETC and a well characterised site of ROS production. The link between complex IV and cancer is controversial (Permut-Wey 2011; Petros 2005). Mutations in mtDNA genes for complex IV subunitsare generally tumour-suppressing, whereas nuclear DNA mutations are tumour-promoting (Gaude & Frezza 2014). mtDNA mutations in ATP synthase (complex V) subunits have been found in thyroid, pancreatic and prostate cancers, where they would protect from apoptosis (Gaude & Frezza 2014; Maximo et al. 2002; Petros et al. 2005).

Oncogenic mutations in mitochondrial proteins encoded by nuclear genes have been linked to both sporadic and hereditary forms of cancers, and usually affect genes encoding TCA cycle enzymes.

Isocytrate dehydrogenase (IDH1-2) catalyses the reversible conversion of isocitrate into 2-oxoglutarate. Mutations in the cytosolic and mitochondrial isoforms are associated with glioma, glioblastoma, colon and prostate cancers, acute myeloid leukaemia and B-acute lymphoblastic leukaemia. Mutated IDH generates the oncometabolite 2-hydroxyglutarate (2-HG). Normally, this molecule is found at trace level in cells. High levels of 2-HG are associated with alterations in cellular genomic methylation and transcription patterns. It seems to be involved in the epigenetic modifications of glioblastoma cells (Gaude & Frezza 2014; Wallace 2012).

Two other nuclear-encoded TCA cycle enzymes highlight the important link between mitochondrial metabolism and cancer. Less than a decade ago it has been demonstrated that mutations in SDH and fumarate hydratase (FH), both enzymes of the TCA cycle, were found to be the initiating events of familiar paraganglioma and leiomyoma, and papillary renal carcinoma, respectively (Baysal et al. 2000; Tomlinson et al. 2002; Frezza & Gottlieb 2009; Bardella et al. 2011).

Fumarate hydratase catalyses the reversible conversion of fumarate into malate, which is found to be downregulated in sporadic clear cell carcinomas, glioblastoma and deleted in Myc-amplified neuroblastoma (Gaude & Frezza 2014). Its tumorigenic activity has been attributed to the abnormal accumulation of fumarate, which acts as an oncometabolite by inhibiting PHDs, histone and DNA demethylases leading to tumorigenesis. Moreover, fumarate takes part in the process of protein succination, in which it is covalently bound to cysteine residues. Succination of KEAP1, the negative regulator of the transcription factor Nrf2, leads to its inactivation and to the ensuing establishment of the anti-oxidant program controlled by Nrf2, which supports tumour formation. Interestingly, thiol residues of GSH are also succinated, and this event is linked to increased oxidative stress (Gaude & Frezza 2014).

Succinate dehydrogenase (SDH) is an integral mitochondrial inner membrane protein complex that oxidises succinate to fumarate and transfers two electrons to Coenzyme Q10 (CoQ). The reduced ubiquinol transfers electrons from complex II to complex III for further oxidation. Thus, SDH is a component of both the TCA cycle and of the ETC. It is composed by four subunits: SDHA-B-C-D. SDHA binds the substrate succinate; SDHB contains the iron-sulfur centres for electron transfer; SDHC and SDHD anchor SDH into the mitochondrial membrane and form the CoQ binding site. Moreover, the SDH holoenzyme is assembled by SDH assembly factor 1 and 2 (SDHF1-2) (Wallace 2012).
SDH mutations inducing paraganglioma and pheochromocytoma are mainly found in genes encoding SDHC, SDHD and SDHB subunits and generally result in truncated proteins that impair the assembly of the SDH holoenzyme and therefore prevent its succinate-ubiquinone oxidoreductase (SQR) enzymatic activity (Douwes Dekker et al.

2003; Yankovskaya et al. 2003). In addition, mutations in SDH subunits B, C and D that allow enzyme assembly could be associated with mitochondrial electron transport function, and therefore increase ROS generation following electron reaction with O_2 to generate O_2^-. Depending on the mutation sites, the decreased activity of SDH can produce high or low levels of ROS (Figure 1.11). We can mimic the effects of these mutations by using different SDH inhibitors. Thenoyltrifluoroacetate (TTFA) targets SQR activity of complex II, hence it increases ROS levels and induces apoptosis, whereas 3-nitropropionic acid (3NP) inhibits SDH activity in the A subunit, thus protecting cells from ROS overload and death (Grimm 2013).
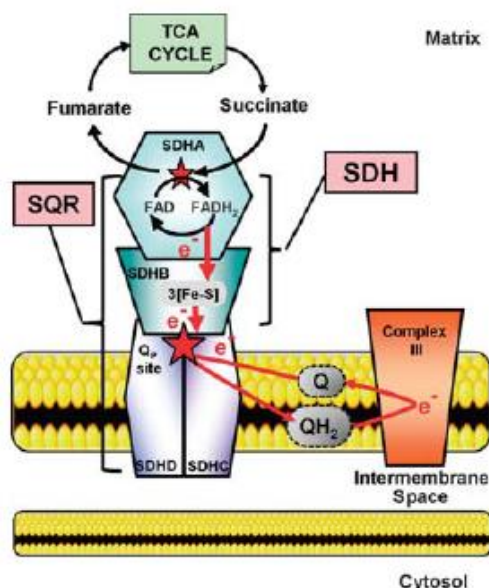
Figure 1.11 **Structure of complex II.** Red stars define mutation sites (Lemarie & Grimm 2011)

It has been demonstrated in solid tumours that inhibition of SDH increases mitochondrial and cytosolic succinate levels, which in turn inhibits PHDs, thus causing stabilisation of hypoxia-inducible factor 1 α (HIF-1α). Stabilised HIF-1α translocates into the nucleus, where it activates genes involved in the metabolic shift toward aerobic glycolysis (Figure 1.12; for the oncogenic effects of HIF1, see above) (Wallace 2012; Frezza & Gottlieb 2009).

SDH deficient cells have also an increased generation of mitochondrial ROS, which can contribute to HIF-1α stabilisation by inactivating PHDs (Bardella et al. 2011; Guzy et al.

2008; Frezza & Gottlieb 2009). Moreover, the long term generation of ROS by SDH can contribute to genomic instability and further tumour development (Lemarie & Grimm 2011; Grimm 2013).
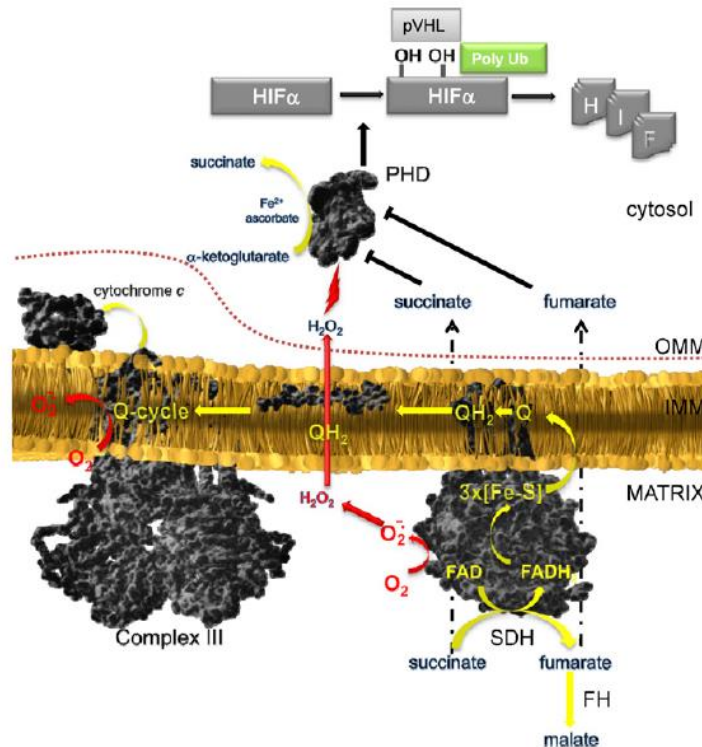
Figure 1.12 **The physiological roles of SDH in the TCA cycle and the ETC and its potential roles in cancer** (Frezza & Gottlieb 2009)**.**

ROS generated by SDH can also contribute to the regulation of apoptosis . It has been proposed that apoptosis induction can be correlated with the release of SDHA and SDHB from the inner mitochondrial membrane into the matrix. The detached SDHA/SDHB complex remains enzymatically active and can oxidize succinate, but electrons are transferred to oxygen as the downstream physiological acceptor SDHC/SDHD is missing in these conditions. The consequent generation of ROS would cause apoptosis induction (Grimm 2013). These data are in accord with the down-modulation of SDH assembly or activity observed in many cancer cell types that must escape cell death (Lemarie & Grimm 2011).

1.3.1.2 Alterations in ETC complex assembly and supercomplex formation

In mitochondria, ETC complexes are organised in supramolecular structures called supercomplexes in order to maximize respiratory efficiency (Wittig et al. 2007; Schägger & Pfeiffer 2001). The arrangement of ETC complexes into assembly lines increases electron transfer rates due to substrate channelling (Schägger & Pfeiffer 2001). Alterations in ETC complex assembly and in the stoichiometry of supercomplexes can change the complex activity by blocking electron flow through the ETC, thus inducing ROS formation. For example, it is possible that an inhibition of complex I assembly, as observed in renal oncocytomas and in lung epidermoid carcinoma (Ishikawa et al. 2008; Lenaz et al. 2010), causes a defect in its association with complex III in supercomplexes; consequently, electrons cannot be correctly channelled from NADH to the complex III redox centre, leading to a ROS overproduction that can favour tumorigenesis.

Low levels of ATP synthase are often observed in clear cell-type renal cell carcinoma, favour glycolytic ATP synthesis and probably indicate that mitochondria are in an inefficient structural and functional state (Simonnet et al. 2003; Bonora et al. 2006; Solaini et al. 2011).

1.3.1.3 Modulation of respiration by post translational modification

OXPHOS can be regulated by post-translational modifications (PTMs), including nitrosylations, glutathionylations, acetylations and phosphorylations, which provide a very adaptable and dynamic system of respiration control.

Sirtuins carry out deacetylation reactions; among these, SIRT3 deacetylates many mitochondrial proteins such as SDHB, subunits of complex I and V but also cyclophilin D (CyP-D) and UCP2 that modulate mitochondrial permeability and electron flow respectively (Boland et al. 2013).

Numerous kinases and phosphatases appear to be located into mitochondria where they can modulate a variety of mitochondrial processes (Hebert-Chatelain 2013). There are many evidences that OXPHOS complexes use reversible phosphorylation to modulate their activity. It has been proposed that complex I, III, and IV, which pump protons, can be phosphorylated when the proton gradient is high ($\Delta\mu_m$ >150mV) in order to prevent further proton pumping. In contrast, the dephosphorylated complexes would be maximally active (Hüttemann et al. 2008). Moreover, phosphorylation of specific

OXPHOS complexes can lead to supercomplex formation (Hebert-Chatelain 2013) and can also control apoptosis induction (Kadenbach et al. 2004). Thus, alterations in the phosphorylation pattern of OXPHOS complexes can change their activity and can modulate bioenergetic changes and resistance to apoptosis typical of cancer cells. However, it must be highlighted that the physiological meaning of most PTMs is generally unknown, and functional correlation are in general hypothetic.

The mitochondrial fraction of the serine/threonine kinase PKA phosphorylates complex I subunit NDUFS4, stimulating the activity of complex I (Papa et al. 1996; Papa et al. 2012). PKA-dependent phosphorylation also regulates the allosteric inhibition by ATP of complex IV, as well as its enzymatic activity (Acin-Perez et al. 2009). In human tumours, K-Ras-dependent dysregulation of the cAMP/PKA pathway results in fragmentation of mitochondria and inhibition of complex I activity (Papa et al. 2012).

Mitochondrial Src family tyrosine kinases can increase complex IV activity and decrease the activities of complex I, III and V (Tibaldi et al. 2008; Hebert Chatelain et al. 2011). In osteoclasts, c-Src increases the enzymatic activity of cytochrome *c* oxidase (Miyazaki et al. 2003; Hebert-Chatelain 2013). c-Src can translocate into mitochondria after EGFR stimulation decreasing complex IV activity and ATP production. In osteosarcoma and prostate carcinoma cells c-Src increases cellular respiration, but its regulatory effects on respiration probably depend on the bioenergetic features of cancer cells (Hebert-Chatelain 2013). Finally, it has been demonstrated that mitochondrial PKCε phosphorylates complex IV increasing its activity during ischemia (Guo et al. 2007).

The serine/threonine kinase ERK, is not only located in the cytosol and in the nucleus but also in mitochondria (A. Rasola et al. 2010). Mitochondrial ERK appears to promote ATP synthesis, to maintain mitochondrial membrane potential and prevent cytochrome *c* release (Arciuch et al. 2009) and opening of the permeability transition pore (A. Rasola et al. 2010). Thus, it is possible that mitochondrial ERK will phosphorylate OXPHOS complexes and other mitochondrial proteins in order to regulate bioenergetics and apoptosis of cells.

1.3.1.4 Modulation of OXPHOS activity by protein interactions

Activity of OXPHOS complexes can be regulated by the interaction with other mitochondrial proteins. These interactions can induce temporary and dynamic changes in the structure of OXPHOS complexes that can affect mitochondrial metabolism. Aberrant interactions between regulatory proteins and ETC complexes induce bioenergetic deregulation that can contribute to tumorigenic transformation. For instance, the mitochondrial fraction of the transcription factor STAT3 can modulate respiration by regulating the activity of complex I and II (Wegrzyn et al. 2009). In liver cancer cells, a mitochondrial fraction of the serine protease inhibitor SERPIN B3 interacts with respiratory complex I, inhibiting ROS production and protecting cancer cells from death (Ciscato et al. 2013).

Chaperones control the correct folding of nascent polypeptides and the productive assembly of multimeric complexes, while minimizing dangerous misaggregations in the protein-rich intracellular environment. These proteins also control the conformational changes associated to molecular dynamics, as in the case of propagation of signals through reversible phosphorylations, and in the regulation of protein degradation and turn-over. Through the interaction with their targets, chaperones maintain the correct folding of proteins and regulate protein activities (Akerfelt et al. 2010). It is therefore conceivable that the activity of OXPHOS complexes can be finely tuned by mitochondrial chaperones; examples include CyP-D and Hsp90-family chaperones.

Cyclophylin D (CyP-D) is a mitochondrial peptidyl-prolyl *cis-trans* isomerases located in the matrix compartment, where it is involved in the regulation of the mPTP. CyP-D binding on mPTP favours mPTP opening and commits cells to death.

The chaperone function of CyP-D is regulated by PTMs. Indeed, CyP-D undergoes phosphorylations, acetylations and nitrosylations, and these events lead to CyP-D dependent activation of mPTP opening (Andrea Rasola et al. 2010; Shulga et al. 2010; Nguyen et al. 2011). Furthermore, CyP-D can interact with several proteins, including the chaperones Hsp-90 and TRAP1and the tumor suppressor p53 (Bernardi 2013). It has been proposed that CyP-D binds to TRAP1 and Hsp90 creating a complex that renders CyP-D no longer active as a mPTP inducer (Kang et al. 2007;). Moreover, in several

tumour cell models it has been demonstrated that the mitochondrial fraction of GSK3 phosphorylates CyP-D facilitating mPTP opening. An active mitochondrial fraction of ERK inhibits GSK3 activity blocking the phosphorylation of CyP-D and mPTP opening (Andrea Rasola et al. 2010; A. Rasola & Bernardi 2014)

CyP-D is upregulated in hormone dependent human cancers such as cervix human cancers protecting cancer cells from death (Schubert & Grimm 2004).

CyP-D can associate with the with the OSCP subunit in the lateral stalk of the F1FO ATP synthase, , decreasing its enzymatic activity (Giorgio et al. 2013).

Thus, it is possible that PTM-mediated alterations of CyP-D functions and interactions could affect the state of the mPTP.

Heat shock family protein (HSPs) are chaperones expressed after proteotoxic insults such as heat or oxidative stress. In these conditions, HSPs maintain the correct protein folding, prevent protein aggregation, transport proteins across cell membranes, and are involved in protein degradation and in the control of signal transduction. Hsp90, the most abundant HSP protein in cells, is critical for tumour cells to tolerate stressful environments which induce protein unfolding, and to maintain the function of mutant proteins produced during tumorigenesis (Kang 2012).

TRAP1 is a homolog of Hsp90 and resides in the mitochondrial matrix. It shares with Hsp90 the domain architecture, the function of molecular chaperone, and the homo-dimeric quaternary structure. The ATP binding site is also conserved between TRAP1 and Hsp90 and is inhibited by geldanamycin. TRAP1 is also dynamically regulated by PTMs such as phosphorylation by the serine/threonine kinase PINK1 and acetylations (Kang 2012; Lavery et al. 2014).

High levels of TRAP1 expression have been found in many tumour types, where TRAP1 is involved in critical mitochondrial pathways such as regulation of mitochondrial bioenergetics, protection from oxidative stress, and cell death (Rasola et al. 2014). It has been recently demonstrated that TRAP1 is involved in the induction of the pseudo-hypoxic phenotype in the early stages of tumorigenesis. It interacts with subunit A of SDH, inhibiting its enzymatic activity. This leads to succinate accumulation in the cytosol and to the consequent inhibition of prolyl hydroxylase enzyme (PHD) that stabilizes HIF-

1α. HIF-1α translocates into the nucleus, where it activates genes that inhibit mitochondrial metabolism and activate glycolysis. Moreover, TRAP1 interacts with complex IV decreasing its activity. Thus, TRAP1 expression decreases respiration, oxygen-coupled ATP synthesis, induces accumulation of TCA intermediates and shifts the ATP production to glycolysis while inhibiting fatty acid oxidation. Together these data sustain a TRAP1-dependent switch toward a Warburg phenotype (Sciacovelli et al. 2013; Yoshida & et al. 2013).

The interaction of TRAP1 with SDH and complex IV slows down mitochondrial respiration, and keeps the synthesis of ROS low abrogating ROS-dependent opening of the mitochondrial PTP and promoting *in vitro* tumorigenesis. This defines a mechanism through which TRAP1 develops its anti-oxidant activity (Guzzo et al. 2014; Kang 2012).

1.3.2 Mitochondrial pathways of cell death

Mitochondria orchestrate a wide number of signals to determine cell commitment to death or survival, as "pro-life" and "pro-death" signals converge at the level of the mitochondrial outer membrane (OMM). OMM permeabilization results in release of cytochrome *c* and other apoptogenic proteins (e.g. AIF, SMAC/Diablo, Omi/HtrA2) into the cytosol. Cytochrome *c* interacts with APAF1 to form a complex known as apoptosome, which triggers the activation of caspase-9 and a resulting cascade of caspase activation, leading to cell destruction (Tait et al. 2010).

The Bcl-2 family proteins play a critical role in the induction of OMM permeabilization. Both pro- and anti- apoptotic members, compose this family. The pro-apoptotic Bcl-2 proteins BAX, BAK, BAD, BIM, and BID; and the anti-apoptotic Bcl-2 proteins such as Bcl-2, Bcl-X$_L$, MCL-1, BAK and BAX, regulate OMM permeability in responses to apoptogenic signals, and are in turn regulated either at the transcriptional levels by several factors, among which the p53 tumour suppressor (Coultas et al. 2003; Levine 1997). Overexpression of Bcl-2 is oncogenic in a number of haematological malignancies as well as in solid tumours such as prostate, colorectal, lung and gastric cancers. Other cancers show either overexpression of other Bcl-2 anti-apoptotic proteins, Bcl-X$_L$ and MCL-1, or loss of the pro-apoptotic ones (Kirkin et al. 2004). Inactivating mutations and impaired

expression of BAK and BAX have been reported in gastric and colorectal cancers (Fogg et al. 2011; Kirkin et al. 2004).

An important mitochondrial structure involved in the mitochondrial apoptosis is the mPTP, a channel that resides in the inner mitochondrial membrane (IMM). The PTP is a voltage and Ca^{2+}- dependent, high conductance channel, whose prolonged openings prompt cell death inducing a bioenergetic catastrophe: equilibration of the proton gradient that causes mitochondrial depolarisation, respiration inhibition and generation of ROS. Furthermore, it induces massive release of Ca^{2+}, swelling of mitochondria leading to breaches in the OMM that induce the release of intermembrane proteins (A. Rasola & Bernardi 2014). Prolonged mPTP openings are induced by Ca^{2+} and ROS and constitute the point of no return toward the demise of the cell, irreversibly committing it either to apoptosis or to necrosis.

The mitochondrial chaperone CyP-D is a crucial regulator of the mPTP, favouring its opening; the CyP-D inhibitor cyclosporine A (CsA) desensitizes mPTP opening. Dynamic changes of CyP-D function affect the mPTP state, providing flexibility in its regulation (Andrea Rasola & Bernardi 2014; Bernardi 2013). Recently, it has been proposed that the mPTP is formed by dimers of the F1FO ATP synthase (Figure 1.13), the enzymes that catalyse ATP production, and that CyP-D interacts with ATP synthase subunit b, d and OSCP (Giorgio et al. 2013; Giorgio et al. 2009). Thus, this discovery provides a link between the bioenergetics of cells and apoptosis, defining CyP-D as common regulator of these two important mitochondrial functions.
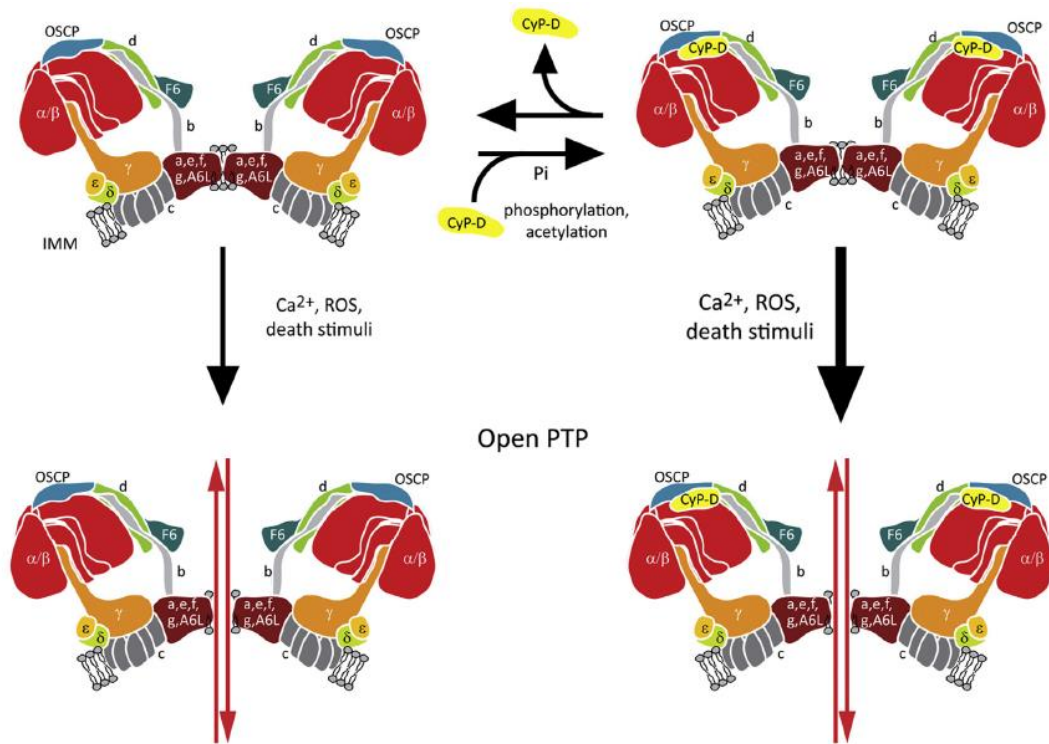
Figure 1.13 **Model of PTP structure.** The PTP is formed at the interface between two ATP synthase monomers. Phosphorylation and acetylation of CyP-D favor and pore opening following increases in Ca^{2+}, ROS or a variety of death stimuli. IMM, inner mitochondrial membrane. (metti REF)

mPTP triggers cell death following oxidative stress. Indeed, oxidation of key cysteine residues induces mPTP opening (A. Rasola & Bernardi 2014). The capability to escape cell death induction following exposure to stress conditions is a mainstay in cell progression towards malignancy (Hanahan & Weinberg 2011). Therefore, cancer cells adapt their intracellular circuits to make mPTP less sensitive to opening. Cancer cells desensitize mPTP by different ways in which protein kinases, mitochondrial hexokinase II and mitochondrial chaperons are involved. As respiratory chain complexes are one of the primary source of ROS, and ROS elicit mPTP opening, regulation of OXPHOS complex activity is crucial to control mPTP state and cell death. It has been recently demonstrated that overexpression of SERPINB3 (SB3), a serine protease inhibitor, installs an anti-oxidant defence mechanism in the neoplastic cells: SB3 located inside mitochondria, mainly during oxidative stress, inhibits respiratory complex I blocking ROS generation and protecting cells from mPTP opening (Figure 1.15) (Ciscato et al. 2013). (A. Rasola & Bernardi 2014).

1.3.2.1. Kinase signalling to PTP in cancer

mPTP regulation by kinase signalling has been investigated mainly in cardiac models.

A fraction of GSK3, a serine/threonine kinase crucially involved in a variety of biological processes, is located inside mitochondria (mGSK3), where it constitutes the integration point to funnel a multiplicity of signals to target at or in close proximity to the mPTP (Chiara & Rasola 2013; Andrea Rasola & Bernardi 2014; Boland et al. 2013). It has been demonstrated that mGSK3 is induced by ROS produced by respiratory complex I (King et al. 2008). Active GSK3 has a double effect on mPTP. In the cytosol, activated GSK3 interacts with Bax and prompts its mitochondrial translocation, thus inducing mPTP and tumour cell death (Chiara et al. 2012). These mechanisms are supported by studies carried out on the Gold(III)-ditiocarbamato complex AUL12, a gold-based chemotherapeutic of new generation (Figure 1.15) (Chiara & Rasola 2013; Chiara et al. 2012). At the level of mitochondria, it facilitates mPTP opening through the phosphorylation of CyP-D (A. Rasola et al. 2010). mGSK3 is inhibited by kinase pathways activated during the process of neoplastic transformation (Figure 1.14), such as Ras/ERK signalling. A fraction of active ERK locates in mitochondria, where it inhibits mGSK3 by serine phosphorylation; this results in ablation of CyP-D phosphorylation, making more difficult the opening of the mPTP (A. Rasola et al. 2010; Masgras et al. 2012; Traba et al. 2012). Therefore, this mitochondrial kinase pathway contributes to the resilience to undergo death that characterise neoplastic cells and confers them key advantages during neoplastic transformation (A. Rasola et al. 2010; Andrea Rasola et al. 2010). It is possible that ROS generated by OXPHOS complexes, through the inhibition of phosphatases such as PP2A that target ERK, lead to activation of the mitochondrial fraction of ERK, contributing to protection from cell death. Indeed, in the heart it has been shown that PP2A inhibits mPTP opening and is crucial for cell survival (Lu et al. 2007).
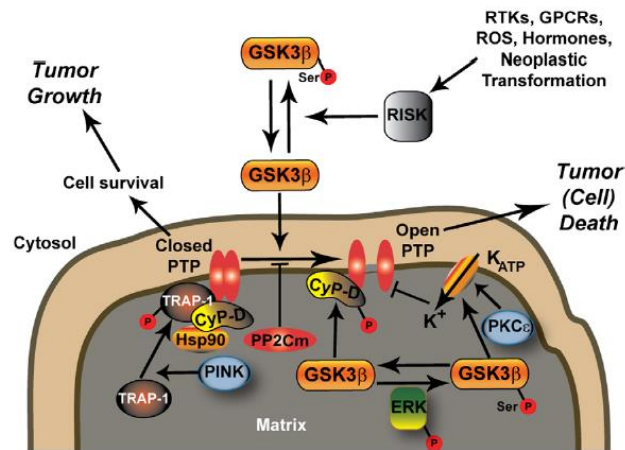
Figure 1.14 **Kinase signalling to the mPTP** (Andrea Rasola et al. 2010)

1.3.2.2. Mitochondrial hexokinase II and mPTP

Hexokinase is the first enzyme of glucose metabolism, which converts glucose to glucose-6-phosphate. The isoform II of the enzyme (HK II) is overexpressed during neoplastic transformation, and it plays a central role in the metabolic rewiring of neoplastic cells toward efficient glucose utilisation. It displays a N-terminal mitochondrial anchoring domain, which allows HK II binding to the OMM in order to maximally exploit mitochondrial ATP for glucose phosphorylation (Andrea Rasola & Bernardi 2014).

The fraction of HK II bound onto mitochondria delivers survival signals that stabilise mPTP in the closed conformation, whereas HK II detachment from mitochondria propagates a conformational change to molecules of the IMM leading to mPTP opening. It has been demonstrated that in many tumour cell models HK II release from mitochondria prompts mPTP opening and apoptosis (Machida et al. 2006; Chiara et al. 2008). HK II integrates a variety of signals that determine its association/dissociation with mitochondria and therefore survival or death, respectively, of tumour cells. HK II binding to mitochondria is under the control of the cytosolic pool of GSK3 and of the survival kinase AKT. AKT-dependent phosphorylation of HK II favours its binding to the OMM and inhibits cytochrome *c* release, and association of HK II to the OMM is favoured when cytosolic GSK3 is inhibited by AKT phosphorylation (Figure 1.15) (Mathupala et al. 2006; Robey et al. 2006; Andrea Rasola et al. 2010; A. Rasola & Bernardi 2014). Mitochondrial HK II also displays an anti-oxidant function that inhibits

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mPTP opening and is independent of HK enzymatic activity. It has been demonstrated in tumour cells that HK II interacts with TIGAR under hypoxia and limits ROS levels, protecting them from death (Cheung et al. 2012).

1.3.2.3 Mitochondrial chaperones and mPTP

Multichaperone platforms might maintain the appropriate folding of pore components and regulators. Chaperones are targets of protein kinases, and a network of phosphorylation events activated by oxidative stress might control mPTP opening by modulation of mitochondrial chaperone activity. For instance, TRAP1 is substrate of the serine/threonine kinase PINK1, and this phosphorylation is required to prevent apoptosis induced by oxidative stress (Pridgeon et al. 2007). In cancer cells, it has been demonstrated that TRAP1 regulates indirectly oxidative-dependent mPTP opening interacting with SDH. SDH is an important site of ROS generation, and TRAP1 markedly inhibits ROS generation by interacting with the catalytic site of the SDHA subunit. Thus, the inhibition of SDH by TRAP1 protects neoplastic cells from oxidative insults and from the lethal opening of the mPTP (Figure 1.15) (Guzzo et al. 2014; Andrea Rasola & Bernardi 2014). Moreover, both TRAP1 and the mitochondrial fraction of Hsp90 interact with CyP-D and antagonise its function of mPTP sensitisation, thus rendering mPTP less prone to opening (Kang et al. 2007).

It is possible that other mitochondrial protein kinases phosphorylate TRAP1 regulating the inhibitory effect of TRAP1 on SDH, ROS induction and mPTP regulation.
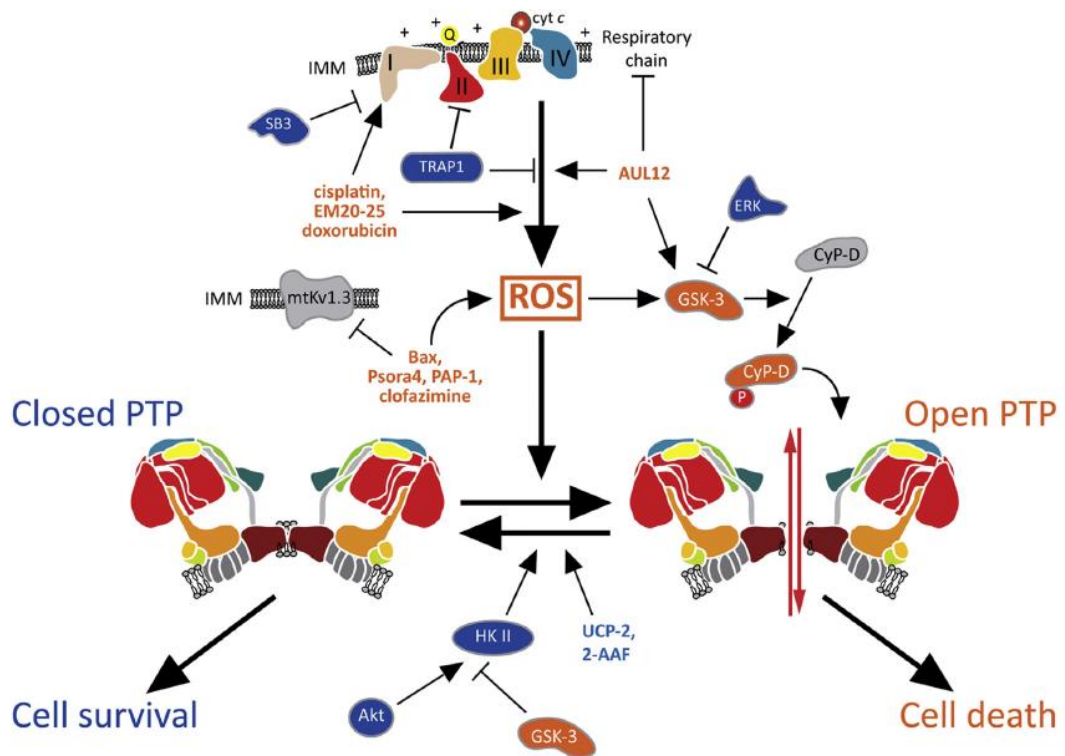
Figure 1.15 **Mechanisms of PTP regulation in tumour cells** (Andrea Rasola & Bernardi 2014)

1.4　　B-cell chronic lymphocytic leukaemia (B-CLL)

B-CLL is the most common form of adult leukaemia in Western countries. It has a variable course: some patients die within 1 year from diagnosis and others have a life longer than 10 years. The disease mainly affects elderly individuals. In the 1967 Dameshek suggested that B-CLL was an accumulative disease of a functionally inactive population of lymphocytes (Dameshek 967). Clinical features of B-CLL include immunodeficiency and accumulation of monoclonal B-cells in peripheral blood, bone marrow (BM) and lymph nodes (LN). B-CLL cells are mature B-cells, established from a CD5$^+$ B-cell subset with preferential expression of surface membrane immunoglobulin with low affinity for autoantigen (Jurlander 1998). Some studies have identified B-CLL cells derived from mantle-zone follicle-centre B cells, others consider B-CLL cells as related to memory B cells (Guipaud et al. 2003).

1.4.1 Apoptotic resistance in B-CLL

B-CLL cells have an altered ability to undergo apoptosis *in vivo*. Indeed, it was hypothesised that B-cell accumulation at an early disease stage is not due to accelerate proliferation of malignant clones, but rather to an undefined defect in apoptosis resulting in their increased lifespan. The mechanisms that lead to apoptotic resistance in B-CLL cells are not yet understood. All B-CLL cells express high levels of Bcl-2 and considerable levels of BCL-2-related myeloid cell leukemia-1 (MCL-1)(Longo et al. 2008). Chromosomal alterations, but also the aberrant activation of B cell receptor (BCR) and kinase signalling and microenvironment are involved in anti-apoptotic phenotype of B-CLL clones (Scupoli & Pizzolo 2012).

1.4.1.1 Chromosomal alteration in B-CLL

Many B-CLL patients have karyotypic abnormalities usually associated with advanced stage of the disease and mainly involve genes encoding proteins that regulate the G1 phase of cell cycle.

17q deletion is a very important chromosomal aberration associated with resistance to treatment and poor prognosis because it causes the deletion of p53 tumour suppressor (Fabris et al. 2008). Deletions of the p53 protein induce DNA instability, alteration in Bcl-2 family protein expression and *in vitro* apoptotic resistance following treatment with chemotherapeutics (Turgut et al. 2007) (Jurlander 1998).

Aberrations in chromosome *14q* consist in translocations that create chimeric genes or hypo-methylation of cytosine in the gene promoter result in high expression of the *bcl-2* gene (Jurlander 1998), contributing to apoptosis resistance of leukemic cells (Bouley et al. 2006).

It has been argued that these molecular aberrations are not responsible for initiation of B-CLL, but are secondary events arising in the course of disease, as they are not present in all patients (Jurlander 1998).

1.4.1.2 BCR signalling in B-CLL

BCR is an antigen-binding transmembrane immunoglobulin complex, which enables the transmission of signals inside the cell. Oligomeric or multimeric antigen engagement

leads to BCR cross-linking and to the phosphorylation of its intracellular region by the Src family kinase Lyn. This phosphorylation recruits spleen tyrosine kinase (SYK) to the receptor, where it becomes active by phosphorylation. Activation of SYK propagates the signal activation to downstream signalling proteins through the formation of a plasma membrane-associated signalling complex, named signalosome, which assembles signal molecules, such as SYK itself, phospholypase-CƳ2 (PLCγ2), PI3K, Bruton's tyrosine kinase (BTK). The signalosome coordinates and regulates downstream cellular events, including signalling mediated by increased Ca^{2+} concentration and induction of gene expression (Figure 1.16). These signalling events regulate proliferation, survival, differentiation and death of B-CLL cells (Figure1.16) (Scupoli & Pizzolo 2012).
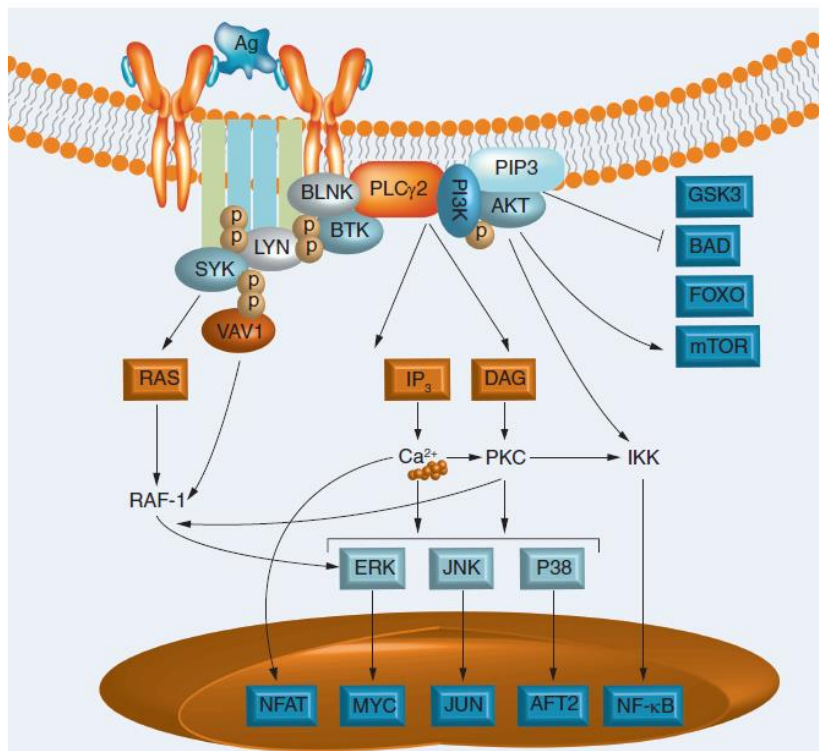
Figure 1.16 **B-cell receptor-activation pathways following antigenic stimulation**
(Scupoli & Pizzolo 2012)

In B-CLL, BCR exhibits high intrinsic activity. Its interactions with presently unknown self-antigens or environmental antigens trigger anti-apoptotic signals, thereby contributing to the accumulation of leukemic cells and favouring secondary genetic mutations. Indeed, *in vitro* experiments have shown that BCR stimulation with anti-IgM is able to

transmit survival signals inside B-CLL cells, and that these signals are associated with enhanced activation of the NF-κB, ERK and AKT pathways (Scupoli & Pizzolo 2012).

Key signalling molecules downstream to BCR engagement are constitutively active in B-CLL cells resulting in a ligand independent, tonic BCR signal (Figure 1.16). Lyn tyrosine kinase is upregulated in B-CLL cells, and it is found both on the cell surface and in the cytosol, where it plays an anti-apoptotic role; accordingly, high levels of tyrosine phosphorylation have been observed in B-CLL cells (Contri et al. 2005; Trentin et al. 2008; Zonta et al. 2014).

A key regulator of cell survival in B-CLL is PI3K/AKT signalling, which is induced in B-CLL by several cytokines, including IL-4 and IL-2 (Longo et al. 2008). GSK3 is another downstream effector of PI3K which takes part in the survival of B-CLL cells (Gold et al. 1999).

PKC is constitutively activated in B-CLL and contributes to B-CLL cell survival by activating the ERK pathway, the transcription factor NF-κB and increasing the expression of the anti-apoptotic genes *Bcl-XL*, *Mcl-1* and XIAP (Barragan et al. 2002).

The Raf/MEK/ERK pathway is constitutively active in half of B-CLL cases and regulates the expression and functions of several proteins that promote cell survival in B-CLL cells. Increased ERK phosphorylation in response to BCR stimulation is associated with poor prognosis and with activation of the transcription factor NF-ATc1, inhibiting BCR-induced anergy, one of the apoptotic mechanisms utilized to get rid of auto reactive B-lymphocytes. Pro-apoptotic BH3-only proteins BAD and BIM are also inhibited by the ERK signalling (Scupoli & Pizzolo 2012).

1.4.1.3 Microenvironment

The term "microenvironment" collectively describes cellular, structural, and soluble components of the anatomic compartment in which B-CLL cells reside. Stimuli from microenvironment have a key role in the onset and progression of B-CLL. Indeed, when placed in culture, B-CLL cells spontaneously undergo apoptosis unless appropriate microenvironmental factors are provided. Co-culture with different types of stromal and monocyte-derived cells, designated "nurse-like cells," promote B-CLL cell survival by providing many factors such as cytokines, chemokines, CD40, BAFF, integrins, and

components of the extracellular matrix (Figure 1.17). These extrinsic factors activate several intracellular pathway such as PI3K/AKT, NF-κB and MAPK pathways as well as the kinases SYK and LYN. In tissues, B-CLL cells can interact with cells such as T-lymphocyte and stromal cells that provide all factors necessary for apoptosis resistance, survival and proliferation (Wiestner 2012).
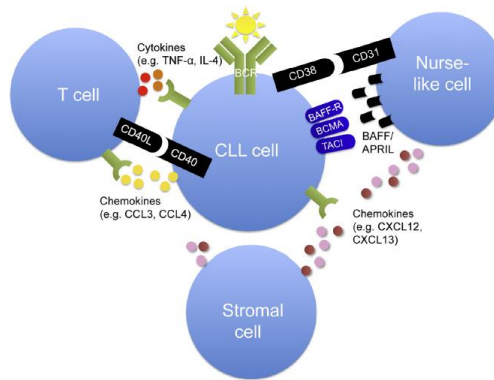
Figure 1.17 **Microenvironmental interactions of CLL** (de Weerdt et al. 2013)**.**

Reactivation of apoptosis is the therapeutic approach for B-CLL. Apoptosis can be induced by inhibiting NF-κB or the PI3K/AKT pathway, by enhancing p53-dependent apoptosis, by stimulation of the extrinsic pathway through death receptors, or with chemotherapeutics and monoclonal antibodies (Billard 2013).

However, up to now there are no effective chemotherapeutics against B-CLL. Thus, finding novel mechanisms of apoptosis resistance in B-CLL cells may define novel drug targets.

1.4.2 B-CLL metabolism

All metabolic features of cancer cells, mentioned before, have been extensively studied in solid tumours. Very little is known on metabolic and mitochondrial changes that characterise blood cell tumours, such as leukaemias.

It is known that T lymphocytes maintain low rate of glycolysis and predominantly oxidize glucose-derived pyruvate via OXPHOS or engage fatty acid oxidation to synthesize ATP. However, upon activation T cells become highly proliferating and shift their metabolism towards aerobic glycolysis, which is reminiscent of the Warburg phenotype of proliferating tumour cells (Pearce et al. 2013; Ralph J DeBerardinis et al. 2008). B lymphocytes do share certain fundamental metabolic characteristics with T cells, including their induction of glycolysis after activation. We might speculate that short-lived plasma cells and memory B cells have a metabolic profile similar to that of resting and memory T cells, *i.e.* mainly relying on OXPHOS for their metabolic needs (Pearce & Pearce 2013).

Leukemic cells usually face normoxic conditions, which is different from solid tumour cells, often growing in hypoxic conditions. It was therefore proposed that B leukemic cells maintain a high OXPHOS activity, without shifting toward an aerobic glycolysis phenotype (Jia & Gribben 2014).

Metabolism of B-CLL cells remains a relatively unexplored area. Compared with normal lymphocytes, B-CLL cells were shown to display a substantial increase in ROS levels, causing oxidative DNA damage and mtDNA mutations. Intracellular ROS levels directly correlate with B-CLL aggressiveness. It has been demonstrated that B-CLL cells have increased mitochondrial biogenesis and elevated mitochondrial OXPHOS but do not have increased aerobic glycolysis (Jitschin et al. 2014). This higher mitochondrial respiration leads to an increased ROS production and favours oxidative stress. In accord with these observations, mitochondrial mass and membrane potential, ATP production, mitochondrial DNA copy number, oxygen consumption and ETC activity are all increased in B-CLL cells with respect to non-malignant B lymphocytes (Jitschin et al. 2014). Moreover, manganese superoxide dismutase (MnSOD2) expression is significantly reduced in B-CLL and mitochondrial superoxide levels are increased. Finally, inhibition of F1FO ATP synthase with the benzodiazepine derivate PK11195 blocks oxidative phosphorylation, induces overproduction of superoxide and apoptosis only in B-CLL cells (Jitschin et al. 2014). In turn, gene expression of antioxidant enzymes such as catalase

and the stress-responsive molecule haem oxygenase 1 (OH-1) were upregulated in B-CLL with respect to normal B cells to counteract the intrinsic increase of oxidative stress. Therefore, it is proposed that increased mitochondrial ROS production leads to an intrinsic compensatory upregulation of cellular anti-oxidant defences, including OH-1, a positive regulatory signal of TFAM that drives mitochondrial biogenesis, leading to more ROS-generating mitochondria (Figure 1.18)
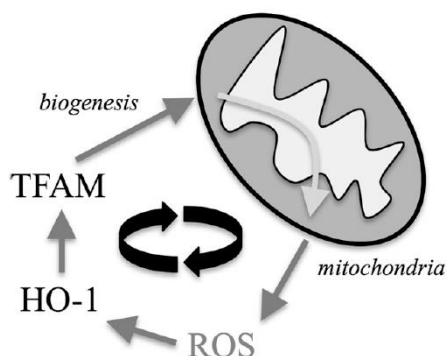
Figure 1.18 **The proposed mechanism by which mitochondrial ROS and mitochondrial biogenesis form a self-amplifying feedback loop in CLL cells** (Jitschin et al. 2014)

This model is in accord with the observations by which leukemic cells meet their high energy demand by increasing mitochondrial ETC activity, rather that aerobic glycolysis (Jia et al. 1996).

The main sources of superoxide radicals in mitochondria are respiratory complex I and complex III. Recently, however, it became clear that also complex II is a source of superoxide in mitochondria. Thus, it is possible that alterations in the regulation of complex II activity can affect superoxide production in B-CLL cells.

Manipulating the redox status of B-CLL cells it is possible to build up therapeutic strategies. Antioxidant treatment of B-CLL cells can neutralise endogenous ROS overproduction protecting the immune system. On the other hand, induction of ROS overproduction by targeting mitochondrial ETC could selectively kill B-CLL cells (Jia & Gribben 2014; Lilienthal et al. 2011).

1.5   p66Shc

p66Shc belongs to the ShcA family of adaptor proteins that consists of three members: p46Shc, p52Shc, and p66Shc, which derive from alternative splicing of the same SHC1 locus.

p66Shc isoform differs from p52shc and p46shc for the presence of an additional N-terminal proline-rich collagen homology domain (CH2), which contains a serine phosphorylation site (Ser36) that is critical for its pro-oxidant properties and by a functional region that is responsible for the interaction of p66shc with cytochrome *c* (Figure 1.19).
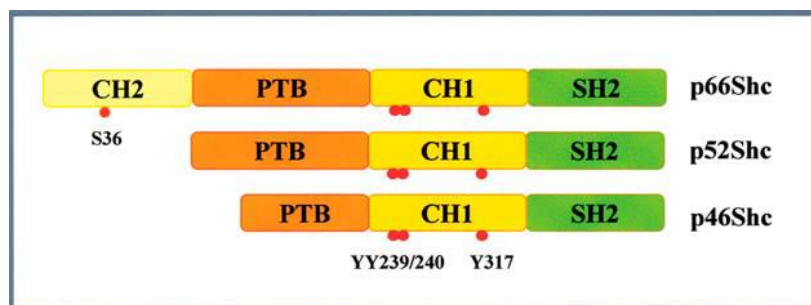
Figure 1.19 **Schematic structure of ShcA proteins**

1.5.1 p66Shc localisation and oxidative stress

The cytosolic fraction of p66Shc is associated to the plasma membrane, where it negatively regulates signalling triggered by the EGF and IGF receptors, as well as T-cell antigen receptor that mediates receptor tyrosine kinase signalling (RAS/ MAPK), promotes Rac1 activation and triggers NADPH membrane oxidase ROS production (Pacini et al. 2004). The negative effect of p66shc on mitogenic signalling involves competitive inhibition of p52Shc binding to its upstream partners (Pellegrini et al. 2005). It is also possible that increased expression of p66Shc results in an elevated basal activity of ERK/MEK in the absence of stimuli, thus minimizing the extent of further activation by growth factors such as EGF (Veeramani et al. 2005).

In the nucleus, p66shc can inhibit Forkhead family transcription factors, such as FKHRL1 that positively regulates genes encoding proteins involved in ROS scavenging. A fraction

of p66Shc has been found constitutively associated with mitochondria in the mitochondrial intermembrane space (IMS) where it interacts with cytochrome c and takes part in ROS production. Oxidative stress induces an increase in mitochondrial p66Shc. Mitochondrial p66shc forms a constitutive complex with the Hsp70 chaperone (Pellegrini et al. 2005). p66Shc is phosphorylated at Ser36 by oxidative stress-induced stimuli such as UV radiation or H_2O_2 treatment. Stress-activated kinase can lead to the Ser36 phosphorylation of p66shc, which leads to p66Shc prolyl isomerization by Pin-1 and dephosphorylation by PP2A. In the model proposed by Giorgio et al. in 2005, which is based on experiments performed on mouse embryonic fibroblasts and on isolated mitochondria stimulated with H_2O_2, p66Shc can also translocate to the IMS through the TIM/TOM mitochondrial import machinery. Here, its redox-active region (present at the N-terminal domain) is involved in the transfer of electrons from reduced cytochrome c to molecular oxygen and, finally, in mitochondrial H_2O_2 generation (Figure 1.20) (Giorgio et al. 2005; Migliaccio et al. 1999). Increased intracellular H_2O_2 levels activate PKCβ and lead to the activation of a self-triggered loop (Lebiednska-Arciszewska et al. 2014) resulting in a rise of intracellular ROS levels that leads to mitochondrial permeability transition and cytochrome c release (Pellegrini et al. 2005). p66Shc is also a downstream effector of p53 in stress-induced apoptosis. Activated p53 induces and is required for upregulation of p66Shc (Trinei et al. 2002). p66shc is therefore an important player in stress-induced apoptosis and ROS-induced ROS production. It plays an important role in the elimination of damaged cells via ROS-induced apoptosis (Figure 1.20) (Giorgio et al. 2005).
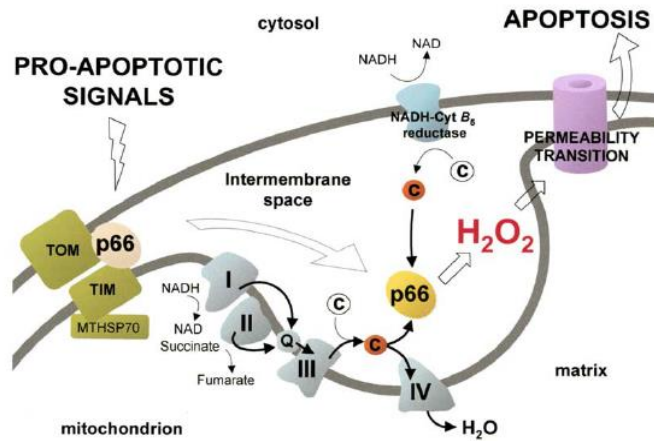
Figure 1.20 **model of p66Shc redox activity**

1.5.2 p66Shc and cancer

The ablation of p66Shc in mice has no negative impact on the health of animals.

A positive correlation has been demonstrated between p66Shc protein levels and the proliferation rate of cells from several tumour types, including human breast, ovarian, thyroid, colorectal and prostate cancer cell (Lebiednska-Arciszewska et al. 2014). Veeramani et al. correlates the requirement for ROS production in the proliferation of tumours stimulated by p66Shc . Indeed, ROS generated by p66Shc activation inhibit protein tyrosine phosphatases, leading to the activation of several kinases, including ERK/MAPK and to the ensuing induction of cell proliferation and survival (Figure 1.21) (Veeramani et al. 2012; Rajendran et al. 2010; Veeramani et al. 2005).
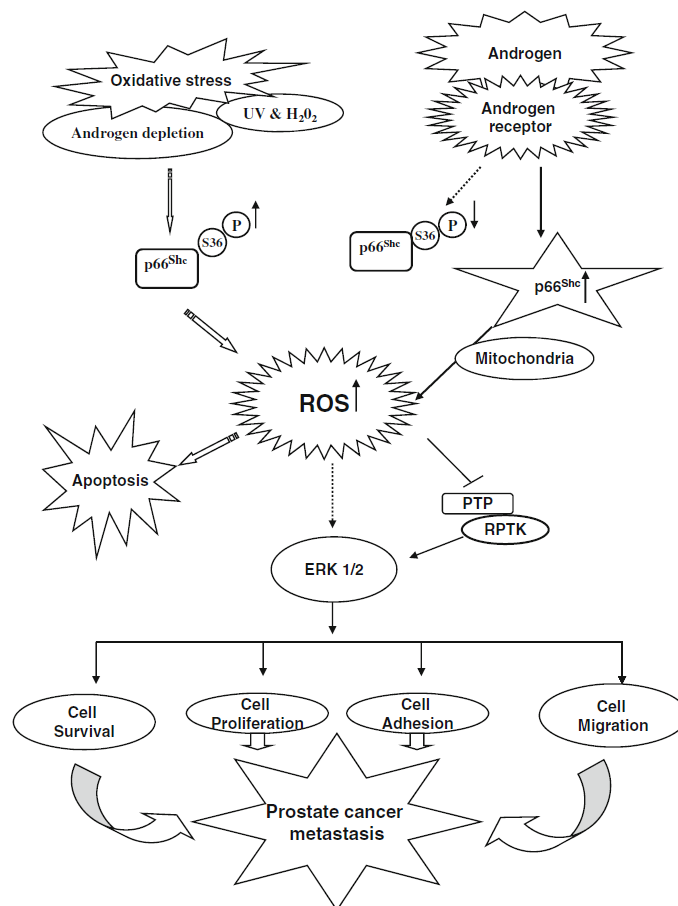
Figure 1.21 **p66Shc signalling and its novel mechanism regulated by androgens in prostate cancer cells** (Rajendran et al. 2010)**.**

Conversely, many cancer types, including lung cancer, malignant ovarian epithelial cancer and melanoma show low levels of p66shc protein. They include human (Lebiednska-Arciszewska et al. 2014). Loss of p66Shc expression leads to constant Ras activation and unregulated cellular proliferation, which is responsible for lung cancer development (Ma et al. 2010; Lebiednska-Arciszewska et al. 2014). In lung cancers, lower levels of p66Shc correlate with high expression of Nrf2 and may be responsible not only for metastasis (due to inhibition of anoikis) but also for drug resistance (due to higher NRF2 levels) (Du et al. 2013; Lebiednska-Arciszewska et al. 2014).

These data suggest that p66Shc expression is differently regulated in diverse tumor types. To understand the meaning of these regulatory events for neoplastic progression it is crucial to better elucidate the biochemical functions of p66Shc.

1.5.3 p66shc and B-CLL

In murine B-cells, p66Shc deficiency results in enhanced proliferative response of mouse B-cells to BCR engagement (Finetti et al. 2008). In B-CLL cells it has been proposed that p66Shc acts as a negative regulator of B-cell survival due to its dual activity as attenuator of BCR signalling and promoter of apoptosis (Capitani et al. 2010). Indeed, the expression of p66shc enhances B-cell apoptosis in cells from B-CLL patients, in the Ramos Burkitt's lymphoma cell line, in B-cells from p66shc$^{-/-}$ mice and B-CLL cells where p66shc expression is abrogated. This event correlates with decreased BCR-dependent activation of SYK, AKT and ERK protein kinases which causes a profound imbalance between pro-apoptotic (BAX, BAK) and anti-apoptotic (Bcl-2, Bcl-X$_L$) Bcl-2 family members compared with normal controls.

A potential mechanism through which p66Shc could induce apoptosis involves its capacity to uncouple BCR from PI3K/AKT and Ras /ERK activation, as it competes with the mitogenic isoform of p66Shc, p52Shc, for recruitment on activated BCR. Indeed, both PI3K/AKT and Ras /ERK pathways increase the expression of anti-apoptotic Bcl-2 family proteins. Notably, p66Shc expression is lower in B-CLL patients with poor prognosis which also harbour non-mutated IGHV genes (U-CLL) than in CLL patient with good prognosis, characterized by somatic mutation in IGHV genes (M-CLL) (Capitani et al. 2010).

In B-CLL, the impaired expression of p66Shc is also correlated with the homing of B-CLL cells in lymph nodes. The impaired expression of p66Shc in B-CLL cells is associated with S1P1 receptor defect, which usually favour the egress of B cell from lymphoid tissues and an upregulation of CCR7 that leads to the accumulation of B-CLL in bone marrow and lymph nodes (Capitani et al. 2012).

1.5.4 p66shc and cancer metabolism

Several studies suggest that p66Shc plays a role in the metabolic changes of tumour cells. It was demonstrated that knocking-down p66shc expression inhibits mitochondrial OXPHOS, whereas it increases glycolysis in several cell lines obtained from solid tumors

(Nemoto & Finkel 2002). More recently it was shown that p66Shc silencing in solid tumours enhances glycolytic metabolism by improving glucose uptake and redirect glucose-derived carbon into anabolic pathways via the activity of mTOR and of one of its major downstream effectors, S6 Kinase (Soliman et al. 2014; Lebiednska-Arciszewska et al. 2014). Thus, p66Shc could switch the energy balance depending on the specific bioenergetics context to maintain proper ATP levels.

The hypothesis that p66Shc expression translates nutrient availability into mitochondrial ROS production has important implication in cancer biology. In highly proliferative cells, increased oxidative stress might contribute to the elevate p66Shc levels, which could be involved in carcinogenesis. Although p66shc was initially reported as a pro-apoptotic molecule, cancer cells that are adapted to survive in metabolically stressed, nutrient deficient microenvironment, often prefer glycolysis. These cells are also correlates with cell proliferation high metastatic potential and poor prognosis (Lebiednska-Arciszewska et al. 2014).

2. Aim of the project

In tumour cells, mitochondria play a pivotal role in the regulation of bioenergetics and viability of cells. For these reasons, oncogenic signalling pathways converge onto mitochondria and readjust mitochondrial physiology. They decrease OXPHOS activity, change redox state of cells, make mPTP less sensitive to opening and induce a more glycolytic phenotype in order to support the oncogenic process. In B-CLL, little is known on how oncogenic signalling pathways that converge onto mitochondria readjust mitochondrial physiology, even if some reports suggest that B-CLL cells undergo a metabolic shift towards a more oxidative metabolism.

The aim of this thesis is the investigation of the role of p66Shc in the regulation of OXPHOS activity and cell survival in B-CLL cells. I have focused on the interplay between p66Shc and the Ras/MEK/ERK pathway in mitochondria. In particular, I have tried to define the functional relationship among mitochondrial kinases ERK and GSK3, and chaperones CyP-D and TRAP1 after p66Shc expression in B-CLL cells, and how these proteins impact on mitochondrial physiology in terms of regulation of mitochondrial respiration and cell survival of B-CLL.

3. Materials and Methods

3.1   Chemicals and Antibodies

Tetramethylrhodamine methyl ester (TMRM); FITC-conjugated Annexin-V was from Boehringer Mannheim (Indianapolis, IN); indirubin-3'-oxime and CsA were from Calbiochem (San Diego, CA); cyclosporin H (CsH) was a generous gift of Dr. Urs Ruegg, Geneva; all other chemicals were from Sigma (Milan, Italy).

Mouse monoclonal anti GSK-3α/β, Grim19 and SDHA, goat polyclonal anti Calnexin and HK II, and rabbit polyclonal anti ERK2, PARP and TOM20 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit polyclonal anti phospho-ERK1/2 (Thr202/Tyr204) and anti phospho GSK-3α/β (Ser21/9) and rabbit monoclonal anti caspase-3 antibodies were from Cell Signaling (Beverly, MA); GAPDH, CyP-D and anti prohibitin antibodies were from Qiagen, Millipore, Calbiochem and Lab Vision (Fremont, CA), respectively; mouse monoclonal anti HSP90 and TRAP1 antibodies were from BD Biosciences; rabbit polyclonal anti AIF antibody were from Exalpha Biologicals (Shirley, MA); the mouse monoclonal OXPHOS antibody cocktail recognizing CI (NDUFB8), CII (SDHB), CIII (core2), CIV (COXII) and CV (α), and the mouse monoclonal anti NDUFS3 antibody were from Mito-Sciences (Eugene, OR); rabbit monoclonal anti NDUFS1 was from Abcam; rabbit polyclonal anti SHC was from upstate.

3.2   Cell cultures

MEC p66shc expressing cells and MEC pc control cells were kindly provided by Nagaja Capitani (Department of Life Sciences, University of Siena, Siena, Italy). The transfection of pc3.1DNA (+) empty vector or containing p66shc cDNA sequence was performed by electroporation as described by Pacini et al. 2004 (Pacini et al. 2004). Cells were grown in high L-glutamine (300mg/ml) RPMI 1640 medium (Invitrogen) supplemented with 0.1 or 10% fetal bovine serum (FBS).The FBS and glucose starvation experiments were done culturing cells in no glucose RPMI 1640 medium (Invitrogen) supplemented with 0.1% fetal bovine serum (FBS). All culture media were supplemented by 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen) as antibiotics and cells were cultured in a humidified atmosphere of 5% CO_2/95% air at 37°C.

3.3   Cell lysis and mitochondrial fractionation

Total cell extracts were prepared lysing cells in a buffer composed by 150 mM NaCl, 20 mM Tris-HCl pH 7.4, 5 mM EDTA, 10% glycerol, 1% Triton X-100 (Lysis Buffer), in the presence of phosphatase and protease inhibitors (Sigma). Lysates were then cleared with a centrifugation at 18,000 x g for 30 min. All procedures were carried out at 4°C. The proteins were quantified using a BCA Protein Assay Kit (Thermo Scientific-Pierce) incubating samples at 37°C for 30 minutes.

Mitochondria were isolated after cell disruption with electrical potter (Sigma) in a buffer composed by 250 mM sucrose, 10 mM Tris-HCl, 0.1 mM EGTA-Tris, pH 7.4. Nuclei and plasma membrane fractions were separated by a first mild centrifugation (700 x g, 10 min); mitochondria were then spinned down at 7,000 x g, 10 min, and washed twice (7,000 x g, 10 min each). All procedures were carried out at 4°C.

In order to define sub-mitochondrial protein localization, isolated mitochondria were digested with trypsin at different concentrations at 4°C for 1 hour. Where indicated, 0.1% SDS was added before trypsin. Trypsin was then inactivated with a protease inhibitor cocktail (Sigma), and mitochondria were lysed and spun at 18,000 x g at 4°C for 10 min.

3.4   Immunoprecipitations and western blot

Protein immunoprecipitations were carried out on 3 mg of total cellular extracts. Lysates were pre-cleared by incubating them with protein A-Sepharose beads (Sigma) for 1 hour at 4°C; they were then incubated in agitation for 18 hours at 4°C with the antibody conjugated to fresh protein A-Sepharose beads depending on the antibody isotype. Where indicated, an anti mouse IgG antibody was added as a negative isotype control. Beads were then washed several times in Lysis buffer.

Proteins extracted from total or mitochondrial cell lysates or from immunoprecipitations were then boiled for 5 min in Laemmli sample buffer, separated in reducing conditions on SDS-polyacrylamide gels and transferred onto Hybond-C Extra membranes (Amersham) following standard methods. Primary antibodies were incubated for 16

hours at 4°C, a horseradish peroxidase-conjugated secondary antibodies was added for 1 hour at room temperature. Proteins were then visualized by enhanced chemiluminescence (Millipore).

3.5　Blue native polyacrylamide gel electrophoresis (BN-PAGE)

BN-PAGE experiments were performed on pellets of mitochondria isolated as described. ETC complexes were extracted from 250 µg of mitochondria by resuspending them at 10 mg/ml in a buffer composed by 1M aminocaproic acid, 50 mM Bis Tris pH 7, in the presence of 2% n-dodecyl-β-D-maltoside (DDM) at 4°C. Mitochondria were then spinned at 100,000 x g for 30 min and supernatants were loaded on 1D-Native-PAGE 3-12% Bis-Tris pre-cast gradient gels (Invitrogen) after addition of Coomassie Blue G250 (Invitrogen). Samples were run for 20 min at 150V and then for 2 hours and 10 minutes at 250V. Bands were visualized after 18 hours of Coomassie Blue G-250 staining. Bands corresponding to different ETC complexes were cut and subjected to 2D-SDS-PAGE, in order to separate single protein components that were identified by Western immunoblotting.

3.6　Cytofluorimetric analyses

The flow cytometry was used to assess cell death, mitochondrial membrane potential ($\Delta\mu_m$) and mitochondrial mass.

Cell death analysis was carried out by incubating cells for 30 minutes at 37°C in a buffer characterised by 135 mM NaCl, 10 mM HEPES, 5 mM $CaCl_2$ (FACS mix solution). FITC-conjugated Annexin-V (Boehringer Mannheim) and propidium iodide (PI, 1 µg/ml; Sigma) were added to detect phosphatidyl-serine exposure on the cell surface (increased FITC conjugated Annexin-V staining) and loss of plasma membrane integrity (PI permeability and staining); Annexin-V and PI negative cells were considered viable.

For mitochondrial membrane potential analysis cells were incubated for 20 min at 37°C in FACS mix solution with TMRM (20 nM) a potentiometric dye and CsH an inhibitor of the mitochondrial multidrug resistant pump (1.6 μM).

For mitochondrial mass analysis cells were incubated for 15 min at 37°C in FACS mix solution with 10-N-nonyl acrydine orange (NAO, 20 nM; Invitrogen), which binds to cardiolipin in mitochondrial membranes. Changes in forward and side light scatter were assessed at the same time to measure alterations in cell dimension and granularity, respectively. Samples were analysed on a FACSCanto II flow cytometer (Becton Dickinson). Data acquisition and analysis were performed using FACSDiva software.

3.7 Measurements of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR)

The rate of oxygen consumption was assessed in real-time with the XF24 Extracellular Flux Analyzer (Seahorse Biosciences), which allows to measure OCR changes after up to four sequential additions of compounds on adherent cells. This protocol was adapted to non-adherent cells by coating multiwell plates was with poly-L-lysine solution (0.01%). Cells were incubated in a humidified atmosphere of 5% CO2/95% air at 37°C for 1h, collected and plated (2.5×10^5 cells/well) in a running DMEM medium (supplemented with 25 mM glucose, 2 mM glutamine, 1 mM sodium pyruvate, and without serum and sodium bicarbonate); cells were then centrifuged twice at 400g for 10 minutes and then the experiment was started. OCR values were normalized for both the cell number and the protein content of each sample. An accurate titration with the uncoupler FCCP was performed for each cell type, in order to utilize the FCCP concentration that maximally increases OCR.

Together with OCR measurements, values of ECAR were also recorded as the absence of sodium bicarbonate allows changes in the pH of the medium.

3.8   Measurements of ETC complex I and II activity

To measure the enzymatic activity of respiratory chain complexes, cells were homogenized with an electric potter (Sigma) in a buffer composed by 250 mM sucrose, 10mM Tris-HCl, 0.1 mM EGTA-Tris, pH 7.4, protease and phosphatase inhibitors, and mitochondria were isolated as described above. Mitochondrial enriched fractions (40 µg per trace) were used for spectrophotometric recordings.

For complex I activity, the rotenone-sensitive NADH-CoQ oxidoreductase activity was detected following the decrease in absorbance caused by NADH oxidation at 340 nm (ε=6.2 mM-1 cm-1). Reactions were performed at 30°C in 10 mM Tris-HCl pH 8 buffer containing 5 µM alamethicin, 3 mg/ml BSA, 5 µM (per favore usa il symbol per ogni lettera greca) sodium azide, 2 µM antimycin A, 65 µM Coenzyme Q1, and 100 µM NADH. The NADH-ubiquinone oxidoreductase activity was measured for 3-5 min before the addition of rotenone (10 µM), and for an additional 3-5 min after rotenone.

Complex II activity was measured by following the reduction of 2-6 dichlorophenolindophenol (DCPIP) at 600 nM (ε=19.1 mM-1 cm-1). Mitochondria were pre-incubated for 10 min at 30°C in a buffer composed of 25 mM potassium phosphate pH 7.2, 20 mM sodium succinate, and 6 µM alamethicin. After the pre-incubation time, sodium azide (5 µM), antimycin A (2 µM), rotenone (2 µM) and DCPIP (50 µM) were added for 1 min to the medium. Reaction was performed at 30°C and started after the addition of an intermediate electron acceptor (Coenzyme Q1, 65 µM). The pharmacological inhibition of TRAP1 was performed adding 5 µM of 17AAG, directly in the buffer during the analysis. Instead, the inhibition of ERK was performed pre-incubating mitochondria with 50µM of PD98059 for 10 min at 30°C in the buffer without RC complex inhibitors. Each measurement of ETC complex activity was normalized for protein content.

3.9   Intracellular ATP determination

Intracellular ATP was quantified by an Adenosine TriPhosphate (ATP) monitoring system based on firefly luciferase, the ATP determination kit ATPlite[TM] from PelkinElmer following manufacturer's instructions. Cells were kept for two hours in the different

experimental conditions. 50.000 cells/wells were suspended in 100 µl of medium and then were analysed in a 200 µl final volume 96-well plates adding 100 µl of reconstituted reagent solution. The luciferase signal was read using a Fluoroskan Ascent FL microplate reader. A standard curve was generated using solutions of known ATP concentrations. ATP levels were calculated as nanomoles of ATP and normalized for protein content and number of cells.

3.10   Statistics

Unless otherwise stated each experiment was repeated at least three times. Data are presented as average ± S.D. or, for clarity, as representative experiments (see figure legends for details).

4. Results

4.1   A B-CLL cell model: MEC-1 cells

B-CLL cells spontaneously undergo apoptosis when placed in culture unless appropriate microenvironmental factors are provided. For this reason, I decided to begin my study with an established B-CLL cell model. As B-CLL cells display a characteristic resistance to immortalization, very few B-CLL cell lines have been described. Stacchini and co-workers (Stacchini et al. 1999) established and characterised a cell line that was obtained from the peripheral blood (PB) of a patient with B-CLL. They called this cell line MEC-1. These cells are positive to Epstein bar virus (EBNV[+]) and are characterised by chromosomal abnormalities typical of B-CLL: deletion of the short arm of chromosome 17, which implies the loss of p53 that is typical of the more aggressive form of B-CLL, and abnormalities of chromosome 12. They also overexpress Bcl-2 protein and express fairly large amounts of Bcl-xL indicating that the pattern of Bcl-2 family protein of gene expression is shifted toward prevention of apoptosis (Stacchini et al. 1999).

4.2   p66Shc involvement in the regulation of mitochondrial physiology of B-CLL cells

It has been demonstrated that MEC-1 cells do not express p66shc (Capitani et al. 2012). We hypothesise that p66Shc expression might affect bioenergetics and survival features of B-CLL cells by interacting with the mitochondrial branches of oncogenic signalling pathways. Therefore, we have investigated the role of p66Shc expression on the mitochondrial physiology of B-CLL cells, by comparing MEC-1 cells with or without p66Shc expression.

4.2.1 A fraction ofp66Shc is in mitochondria of MEC-1 cells

In a variety of cell lines derived from solid tumors, including osteosarcoma (SAOS cells), hepatocellular carcinoma (HUH-7 cells) and cervical cancer (HeLa cells), p66Shc is highly expressed, whereas MEC-1 cells do not express p66Shc (Figure 4.1A). Thus, to study the effect of p66Shc expression in B-CLL cells, the protein was stably transfected in MEC-1 in collaboration with the group of Prof. Baldari from the University of Siena, and p66Shc

expression levels were analysed by Western blot (Figure 4.1B). It has been demonstrated that a fraction of p66Shc is into mitochondrial intermembrane space of MEF cells where it affects bioenergetics by interacting with cytochrome *c* (Giorgio et al. 2005).
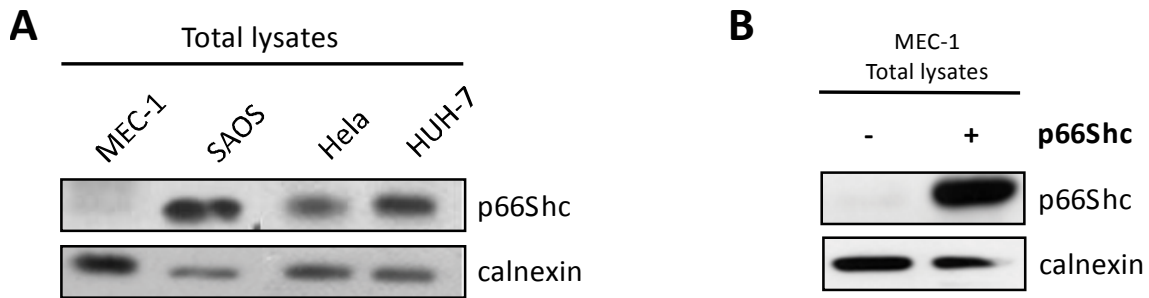

Figure 4.1 **p66Shc expression in diverse tumor cell types** (A) p66Shc levels in MEC-1B-CLL cells, SAOS-2 osteosarcoma cells, HeLa cervix carcinoma cells and HUH-7 hepatoma cells. (B) p66Shc levels in MEC-1 after stable transfection. In (A) and (B) calnexin is used as a loading control.

To check the subcellular localization of p66Shc in MEC-1 cells, a cellular fractionation was performed. A fraction of p66Shc was found into crude mitochondria of p66Shc expressing MEC-1 cells (Figure 4.2A). The sub-mitochondrial localisation of p66Shc protein in MEC-1 cells was defined treating mitochondria with increased concentrations of trypsin to degrade mitochondrial proteins from external to more internal compartments and to get rid of non-mitochondrial contaminants. The outer mitochondrial membrane (OMM) marker TOM20 was degraded by trypsin whereas prohibitin, the matrix marker, was not. The p66Shc protein levels decrease in a way similar to those of the protein AIF, an intermembrane space marker. This means that the mitochondrial fraction of p66Shc is principally localised in the intermembrane space (Figure 4.2A).

A

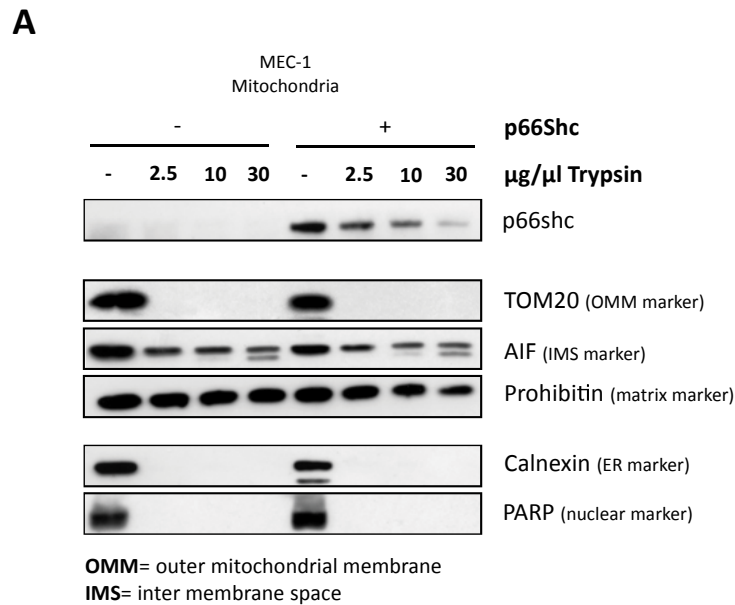OMM= outer mitochondrial membrane
IMS= inter membrane space

Figure 4.2 **After expression in MEC-1 cells, p66Shc is in the inter membrane space fraction of mitochondria.** (A) Mitochondrial sub fractionation of MEC-1 cells by 1h of trypsin at 2.5-10-30 µg/µl. In the Western blot, calnexin is used as an endoplasmic reticulum marker, PARP as a nuclear marker, TOM 20 as an OMM marker, AIF as an IMS marker and prohibitin as an IMM marker.

It has been demonstrated that p66Shc is a positive regulator of oxidative stress, and that it induces apoptosis after its activation by specific stimuli such as H_2O_2 and UV light (Migliaccio et al. 1999). Consistent with these data, flow cytometry analysis by annexin/PI staining revealed that treatment with H_2O_2 is more toxic on p66Shc expressing cells than on control cells (Figure 4.3A). As expected, treatment with N-acetyl cysteine, an anti-oxidant molecule that prevents oxidative stress providing substrates for glutathione anti-oxidant system, rescued cell from oxidative stress damage and subsequent cell death.
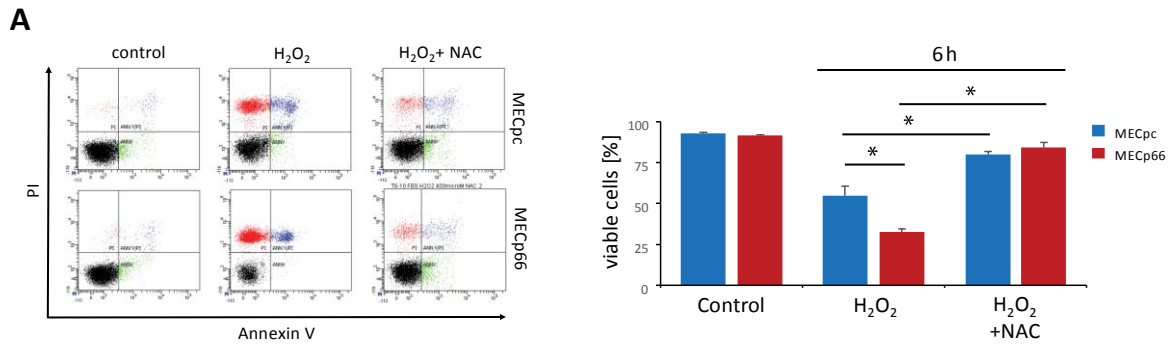
A

Figure 4.3 **After expression in MEC-1 cells, p66Shc increases cell death after H₂O₂ treatment.** (A) Analysis of viable cells with AnnexinV/PI staining by flow cytometry and quantification of viable cells (MECpc, blue squares; MECp66, red square) after 6h of H₂O₂ (1mM) treatment. The bar graph was derived from the elaboration of flow cytometry data. N-acetyl cysteine (NAC) at 1mM concentration was used as an anti-oxidant molecule to confirm that cell death are induced by oxidative stress. Control cells were kept in complete medium. Values are expressed as the mean number of viable cells as a percentage of total cells ± SD.

4.2.2 p66Shc expression decreases mitochondrial respirationin MEC-1 cells

It was demonstrated that B-CLL cells that do not express p66Shc (Capitani et al. 2010) have high mitochondrial respiration but glycolysis does not change (Jitschin et al. 2014). I have investigated the effects of p66Sh expression on bioenergetic parameters of MEC-1 cells.

I have analysed mitochondrial respiration with a XF24 extracellularflux analyser. This instrument allows the measurement of the oxygen consumption rate (OCR) on a monolayer of adherent cells. To measure OCR of MEC-1 cells, which are non-adherent cells, I coated wells with poly-L-lysine. This allowed MEC-1 cells to form a monolayer which could undergo analysis (Figure 4.4A). I found that p66Shc-expressing MEC-1 cells (light blue trace) slow down basal respiration in comparison with control cells (orange trace) (Figure 4.4B). Coupled respiration, *i.e.* the fraction of respiration utilized to synthesize ATP, was defined by using the ATP synthase inhibitor Oligomycin, and did not show any p66Shc-dependent change. Subsequent addition of a low concentration of the proton uncoupler FCCP (150μM) stimulates maximal respiration, *i.e.* the maximal activity of respiratory chain complexes measured as an OCR increase. This parameter can be used also to uncover defects or modulations of the respiratory function. The maximal respiration was lower in p66Shc expressing cells than in control cells (Figure 4.4B). Thus,

p66Shc can affect mitochondrial respiration decreasing ETC complex activity. We also compared the mitochondrial respiration of cells in basal condition (red and blue traces) and after 1h of glucose starvation (light blue and orange traces) (Figure 4.4C), as this condition switched off glycolysis and further increased mitochondrial respiration. We found that basal respiration of p66shc expressing cells did not change following starvation, whereas basal respiration of control cells increased. Thus, p66Shc expressing cells are using almost their maximal respiration as basal respiration and cannot further stimulate mitochondrial respiration. The addition of the ETC inhibitors rotenone and antimycin A fully abolished the OCR, indicating that it derives from respiration (Figure 4.4B and C). Together these data indicate that p66Shc expression decreases mitochondrial respiration of MEC-1 cells.

The differences in mitochondrial respiration that we have observed could be due to differences in cell numbers. To exclude this possibility the data were normalised for cell number and protein content.

A

before analysis   after analysis

MEC pc

MEC p66

B

Oligomycin 1γ   FCCP 150µM   Rotenone 1µM   Antymicin A 1µM

MECpc
MECp66

OCR (pMoles/min)
TIME (min)

C

Oligomycin 1γ   FCCP 150µM   Rotenone 1µM   Antymicin A 1µM

MECpc
MECpc 1h no glu
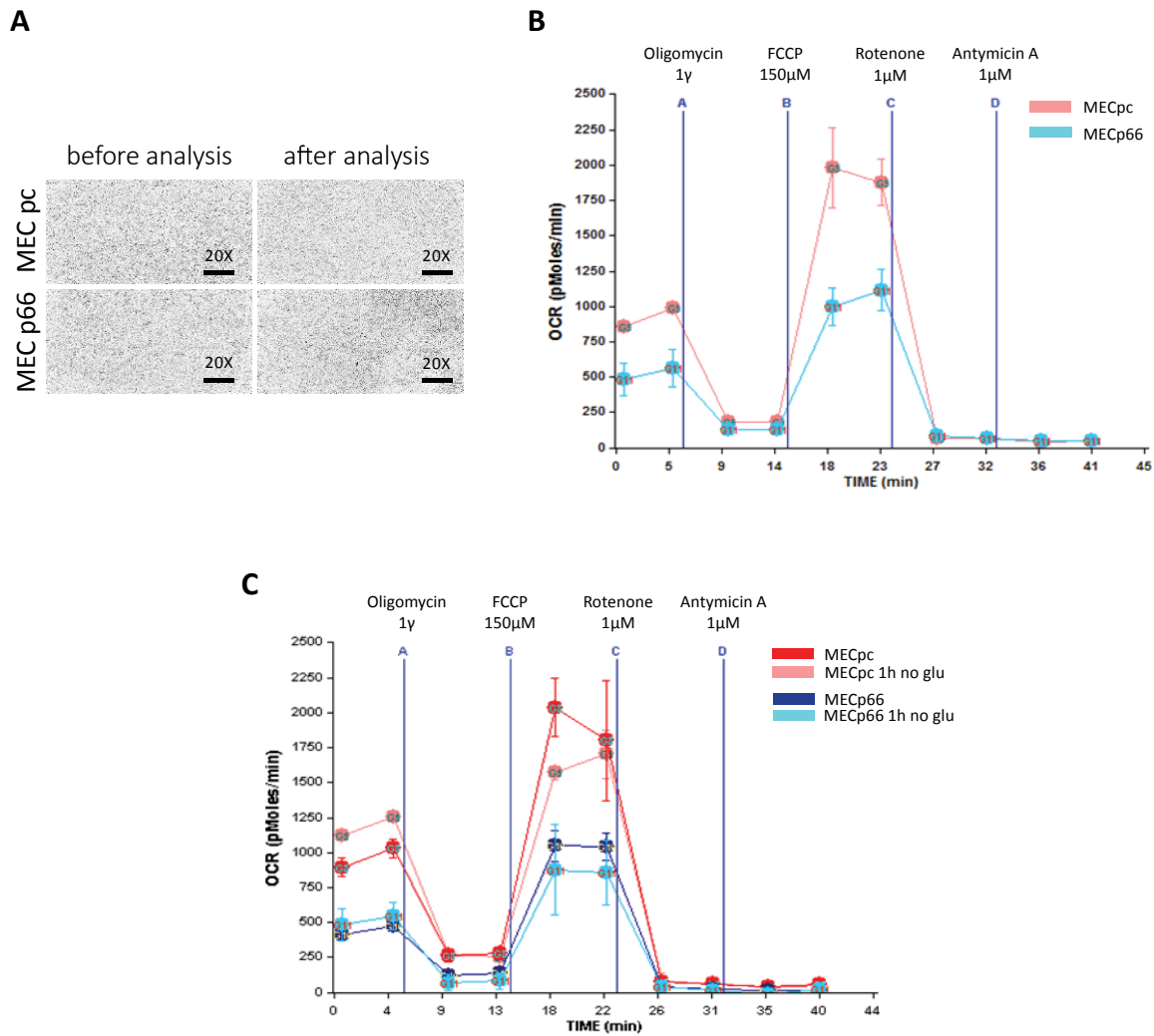MECp66
MECp66 1h no glu

OCR (pMoles/min)
TIME (min)

Figure 4.4 **p66shc expression decreases mitochondrial respiration** (A) Monolayer of non-adherent cells placed on poly-L-Lysine before and after oxygen consumption (OCR) rate analysis. (B) Representative OCR traces performed onMEC-1 cells transfected with empty pcDNA3.1 (MECpc) and MEC-1 cells transfected withpcDNA3.1 with p66Shc sequence (MECp66) (orange trace and light blue trace, respectively). Subsequent addition of Oligomycin, FCCP, the complex I inhibitor Rotenone, and complex III inhibitor Antimycin A are reported. (C) Representative traces of OCR measurement performed in basal conditions on MECpc and MECp66 (red and blue traces respectively) or after 1h of absence of glucose (orange for MECpc and light blue MECp66 respectively).

We also checked the effects of p66Shc expression on glycolysis in MEC-1 cells using the extracellular flux analyser that besides OCR allows monitoring the extracellular acidification rate (ECAR), which is caused by lactate efflux from cells and is proportional to the rate of conversion of pyruvate to lactic acid. Hence, the ECAR increases when the glycolytic flux is diverted from entering the TCA cycle, which can be due to an increase in glycolytic activity, a decrease in the rate of TCA, or both. We observed that the ECAR did not change between the two cell lines (Figure 4.5A).
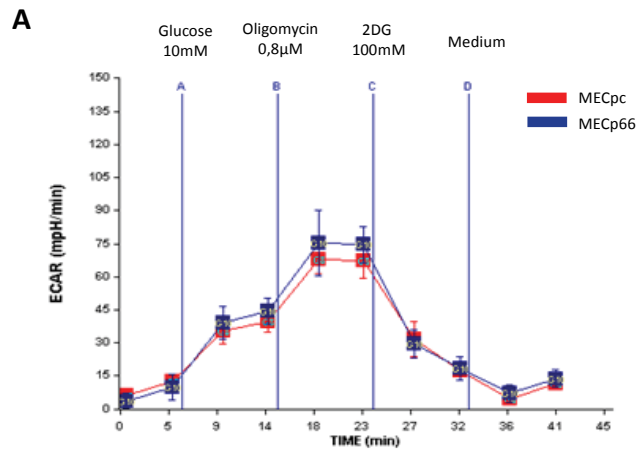
A

Figure 4.5 **p66shc expression does not affect ECAR** (A) Representative traces of the extracellular acidification rate (ECAR) performed on MECpc (red trace) and MECp66 (blue trace); subsequent addition of glucose, the ATP synthase inhibitor oligomycin, the glycolysis inhibitor 2-deoxy-glucose and medium are performed.

I also checked the mitochondrial membrane potential ($\Delta\psi_m$) of cells with a potentiometric dye, tetramethylrodamine (TMRM). I observed that p66Shc expressing cells had a lower $\Delta\psi_m$ than control cells (Figure 4.6A), in accord with an inhibitory role played by p66Shc on ETC activity.

A
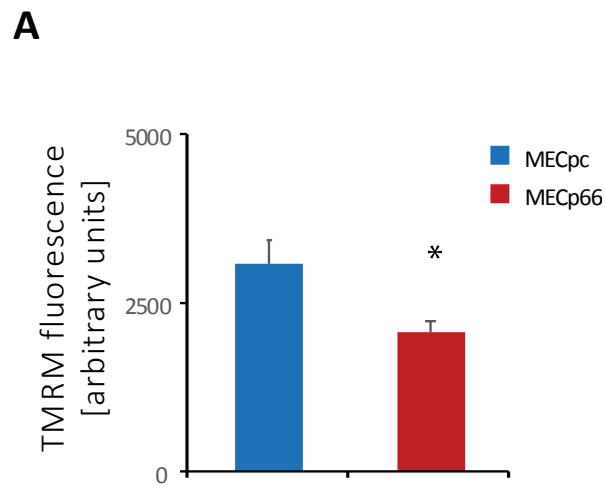
Figure 4.6 p66Shc expression decreases mitochondrial membrane potential ($\Delta\psi_m$) (A) Measurement of mitochondrial membrane potential of MECpc and MECp66 cells stained with TMRM. Mean fluorescence intensity as assessed by FACS analysis is shown. Histograms represent mean ± SD.

Changes in mitochondrial content can affect measurements of OCR and $\Delta\psi_m$. To exclude this possibility we evaluated the mitochondrial mass by staining cells with NAO, a

fluorescent probe that binds the mitochondrial lipid cardiolipin, and the protein levels of mitochondrial house-keeping proteins such as TOM20 and prohibitin. We detected comparable levels of mitochondrial mass and mitochondrial protein levels in the two cell types (Figure 4.7 A-B).
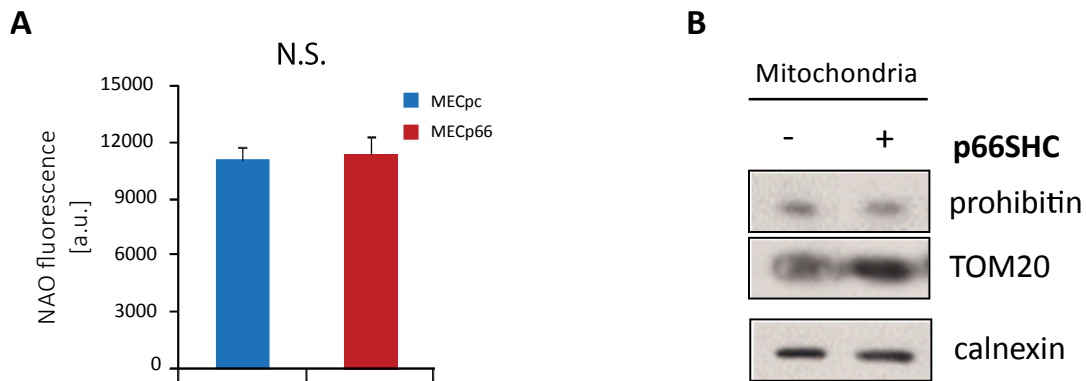
A

B

Figure 4.7 **p66Shc expression does not change mitochondrial content in cells**(A) Measurement of mitochondrial mass by mean florescence intensity of NAO. (B) Protein levels of mitochondrial house-keeping proteins. Calnexin was used as loading control. Histograms represent mean ± SD.

Mitochondrial respiration is the main source of ATP. When I checked the total amount of ATP of cells, I found that p66shc expressing cells display lower ATP levels than control cells (Figure 4.8A). Addition of the ATP synthase inhibitor oligomycin decreased the ATP content of cells, as expected, but it was less effective in p66Shc expressing cells, indicating that these cells have a lower OXPHOS activity than their wild-type counterparts.
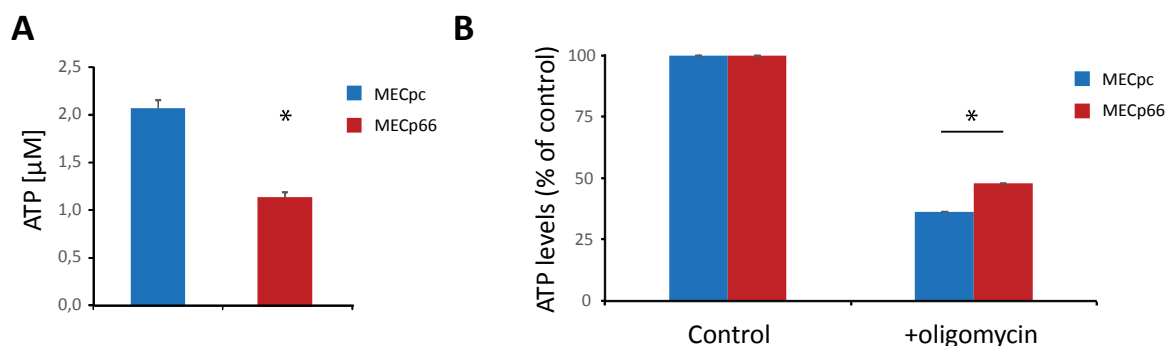
A

B

Figure 4.8 **p66Shc expression decreases mitochondrial ATP levels**. (A) ATP levels measured in standard conditions. (B) ATP levels expressed as percentage of control of MECpc and MECp66 cells treated with 0.8µl of oligimycin for 2h. Values are normalised for cell number and protein content. Histograms represent mean ± SD.

These data demonstrate that p66shc expression decreases mitochondrial OXPHOS in B-CLL MEC-1 cells with respect to wild-type cells (MECpc).

4.2.3 p66Shc expression decreases the assembly and activity of mitochondrial complex I (NADH dehydrogenase)

Variation in mitochondrial respiration can result from changes in synthesis/degradation rate of RC protein, assembly/stability of RC complexes or supercomplexes and post-translational modifications.

I started to investigate the assembly/stability of RC complexes by extracting each ETC complexes from isolated mitochondria with dodecyl maltoside, a detergent that allows to extract mitochondrial complexes as monomers and dimers, and subsequently performing an electrophoresis under native conditions (blue native polyacrylamide gel electrophoresis, BN-PAGE) (Wittig et al. 2007). This technique is used for isolation of protein complexes from biological membranes maintaining their native structure and protein mass; furthermore, it can be applied to identify physiological protein-protein interactions. Separated complexes were stained with Coomassie Blue G-250. This staining revealed a decrease in a band corresponding to complex I in p66Shc expressing cells compare with the control one (Figure 4.9A). Native bands corresponding to each ETC complex were cut and then loaded onto SDS-PAGE gels in order to perform a second dimension electrophoresis in denaturant conditions; this allowed separation of complexes into single protein components, which were therefore identified by Western immunoblotting (Figure 4.9A). We found a decrease in Complex I catalytic subunits, such as NDUFS1, NDUFS8 and accessory subunit GRIM19 whereas the amount of other respiratory chain complexes as complex III and V was not affected by p66Shc expression. Thus, p66Shc decreases the protein levels of assembled complex I. An imbalance between synthesis and degradation rate were excluded because the mitochondrial protein levels of complex I analysed by SDS-PAGE revealed no differences between the two cell lines (Figure 4.9B).
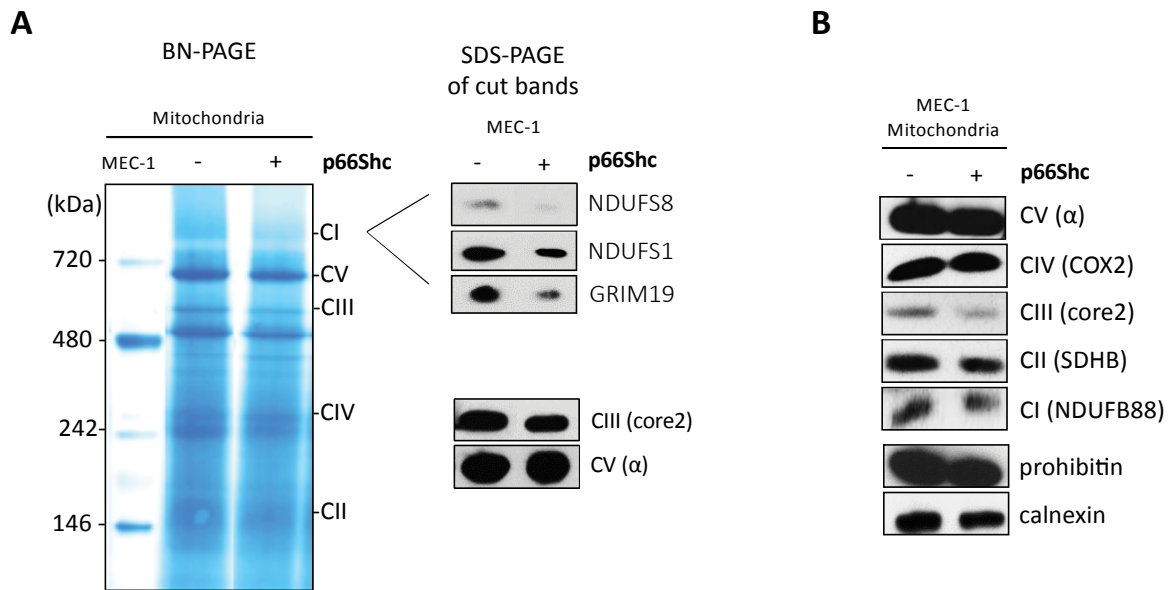
Figure 4.9 **p66shc decreases complex I assembly and activity (NADH dehydrogenase).** (A) Mitochondria are solubilised in 2% of dodecyl maltoside and ETC complexes were separated through a blue native gel electrophoresis (BN-PAGE). Bands corresponding to complex I, II, III and V were excised, run on SDS-PAGE and probed with the following antibodies: NDUFS8, NDUFS1, and Grim19 for complex I, core 2 for complex III and α for complex V. (B) Protein levels of respiratory chain complexes (ETC). Prohibitin was used as mitochondrial marker and calnexin as loading control.

The alteration of native structure of RC complexes can affect their activity. In accordance with the data of a less assembled complex I, the rotenone-sensitive NADH-CoQ oxidoreductase activity measured in isolated mitochondria was remarkably lower in p66Shc expressing cells compared with control ones (Figure 4.10A).
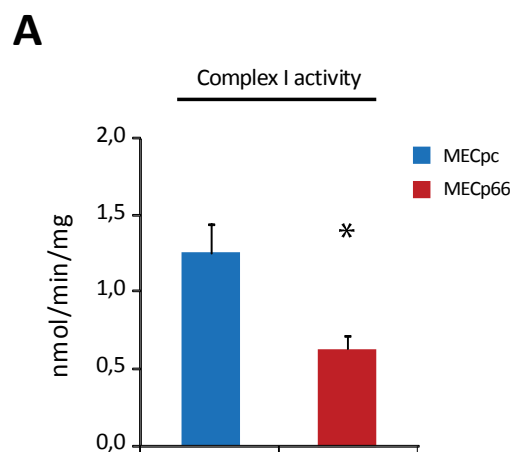

Figure 4.10 **p66shc decreases complex I activity (NADH dehydrogenase).** (A) Complex I enzymatic activity measured in isolated mitochondria. Values were normalized for mitochondrial protein content and expressed as nmol/min/mg. Histograms represent mean ± SD.

These data demonstrated that p66Shc expression in MEC-1 cells modulates mitochondrial respiration decreasing complex I assembly and activity. Complex I is usually organised in supercomplexes together complex III and IV, and supercomplex formation depends on the amount of each RC complex involved. Thus, to complete these observations it would be necessary to analyse if changes in RC complex I assembly also modulate supercomplex formation and activity.

4.2.4 p66Shc expression results in modulation of complex II activity (succinate dehydrogenase)

In our laboratory, it has been recently demonstrated that the down-modulation of succinate dehydrogenase (SDH) activity by interaction with the mitochondrial chaperone TRAP-1 can contribute to the tumorigenic process by modulating HIF1 transcriptional activity and the redox equilibrium of the cells (Sciacovelli et al. 2013). We therefore measured the OCR of cells after treatment with the SDH activity inhibitor 3-nitropropionic acid (3NP, 20µM); this concentration was enough to decrease SDH activity without completely inactivate it. We observed that basal respiration of p66Shc expressing cells treated with 3NP (light blue trace) did not change whereas in control cells (orange trace) decreased up to the values of p66Shc expressing cells (Figure 4.11A). These results demonstrate that SDH modulation is involved in maintaining the respiration differences induced by p66Shc expression in MEC-1 cells. We confirmed this observation measuring the succinate-CoQ reductase (SQR) enzymatic activity of complex II (succinate dehydrogenase) spectrophotometrically. We found that SQR activity was decreased in p66Shc expressing cells compared with control ones (Figure 4.11B), even if the mitochondrial protein levels did not change after the expression of p66Shc as described before (Figure 4.9B). Therefore, modulation of SDH activity by p66Shc contributes to decrease mitochondrial respiration.
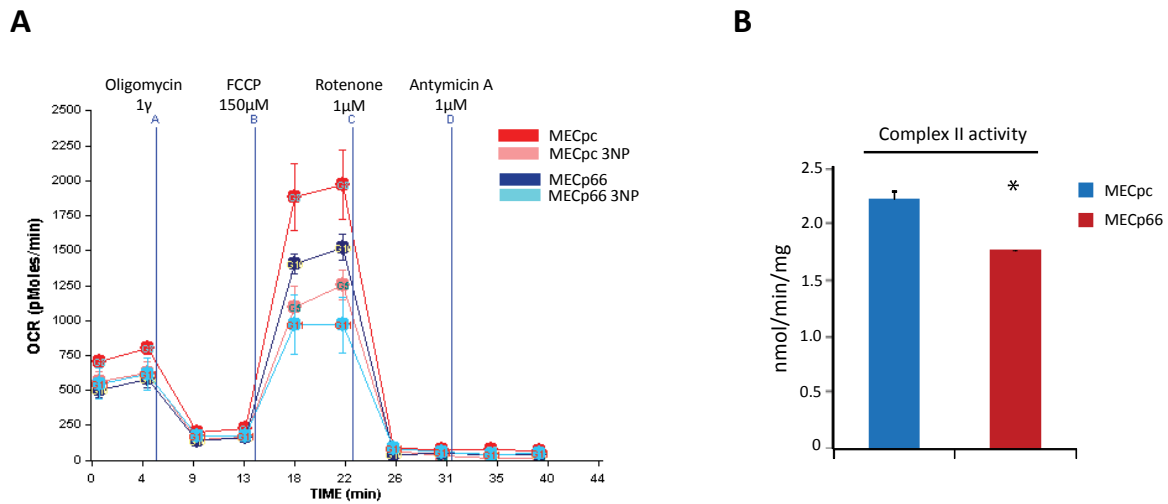
Figure 4.11 **Modulation of complex II activity is involved in changes of mitochondrial respiration induced by expression p66Shc** (A) Measurement of succinate-CoQ reductase (SQR) enzymatic activity of complex II. Values are normalised for mitochondrial protein content and are expressed as nmol/min/mg. (B) Representative OCR traces recorded in basal conditions on MECpc and MECp66 (red and blue traces respectively) or after 1h of treatment with 50µM 3-nitropropionic acid the inhibitor of SDH activity, (orange for MECpc and light blue MECp66 respectively). Histograms represent mean ± SD.

The activity of RC complexes can be modulated by post-translational modifications such as phosphorylations or by interaction with other proteins, resulting in alteration in mitochondrial respiration (see section 1.3.1.3-1.3.1.4 of introduction). As described before the native protein levels (Figure 4.9A BN-PAGE) and the total mitochondrial levels (Figure 4.9B) of SDH do not change, indicating that SDH is stable and assembled even after p66Shc expression. Therefore, to explore the possibility that p66Shc down-modulates SDH activity inducing changes in interactions or PTMs of SDH, we loaded the SDH band cut by BN-PAGE onto SDS-PAGE. In these conditions, I detected a multiprotein complex that co-migrates with SDH in both cell types (Figure 4.12A). Proteins associated with SDH included the mitochondrial chaperones CyP-D and TRAP1 and a mitochondrial fraction of protein kinases ERK and GSK3. Notably, the fraction of ERK that co-migrates with SDH is in its active form (Figure 4.12A). I also observed that the protein levels of TRAP1 and CyP-D that co-migrate with SDH did not change between the two cell lines, whereas the levels of ERK and GSK3 were higher and ERK was more phosphorylated in p66Shc expressing cells compared with control cells. SDH protein levels were the same (Figure 4.12A) indicating that increasing ERK and GSK3 levels were not due to differences in the protein content of SDH. Thus, SDH interacts with a multiprotein complex,

composed by mitochondrial chaperones and kinases, and p66Shc can affect the composition of the multiprotein complex on SDH.
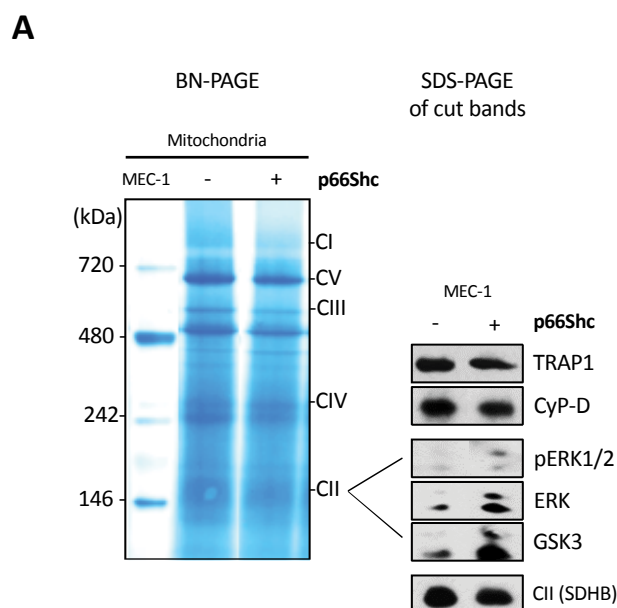

Figure 4.12 **The multiprotein complex on complex II upon expression p66Shc by.** (A) Mitochondria are solubilised in 2% of dodecyl maltoside and ETC complexes were separated through a blue native gel electrophoresis (BN-PAGE). Bands corresponding to complex II were excised, run on SDS-PAGE and stained with SDHB, TRAP1, CyP-D, pERK1/2, ERK and GSK3α/β antibodies.

4.2.4.1 TRAP1 and the mitochondrial fraction of ERK modulate SDH enzymatic activity in a p66Shc-dependent way

It has been demonstrated that the mitochondrial chaperone TRAP1 interacts with SDH and down-modulates complex II activity, leading to accumulation of the oncometabolite succinate in the cytosol and to stabilisation of the pro-neoplastic transcription factor HIF1-α (Sciacovelli et al. 2013). Therefore, I analysed the effect of TRAP1 inhibition on the succinate-CoQ reductase (SQR) enzymatic activity of complex II (succinate dehydrogenase) in our model. I observed that the pharmacological inhibition of the mitochondrial chaperone TRAP1 by 17AAG significantly increased complex II activity in p66Shc expressing cells but not in control cells (Figure 4.13A) indicating that the expression of p66Shc in MEC-1 cells down-modulates complex II activity through its interaction with TRAP1. I performed an immunoprecipitation of the SDHA subunit, without observing any difference in the interaction between SDHA and TRAP1 between the two cell lines (Figure 4.13B); also, TRAP1 mitochondrial protein levels were

unchanged (Figure 4.13C). Thus, the down-modulation of complex II activity by TRAP1 is not caused by differences in TRAP1 expression levels but probably by changes in post-transcriptional modification of TRAP1 induced after p66Shc expression.
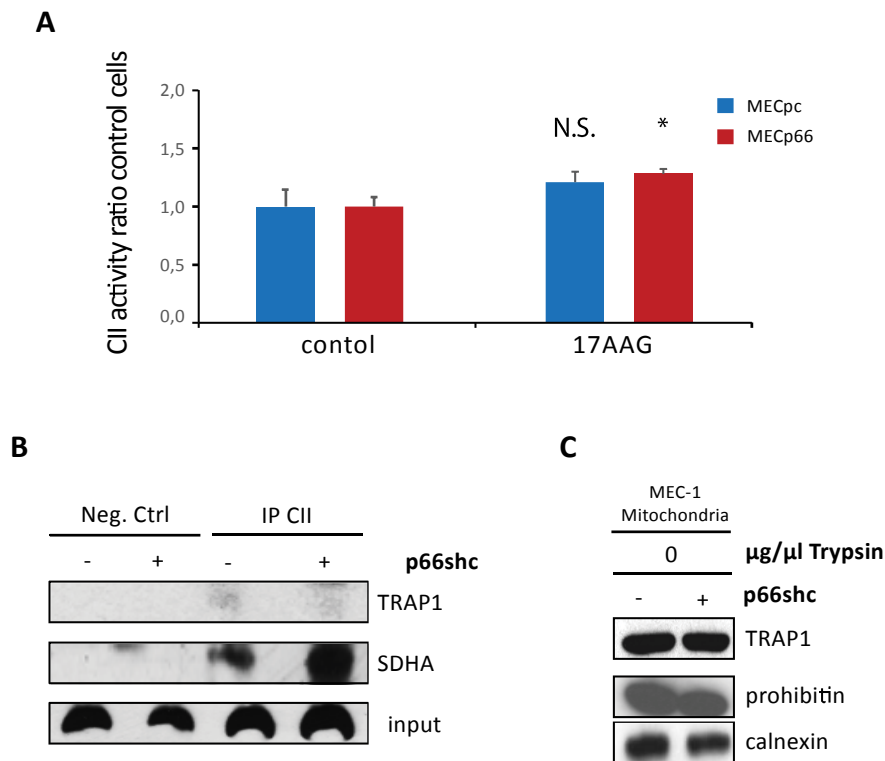

Figure 4.13 **TRAP1 modulates RC complex II activity upon expression p66Shc** (A) Succinate-CoQ reductase (SQR) enzymatic activity of complex II in isolated mitochondria treated or not with 5µM of TRAP1 inhibitor 17AAG. Values are normalised for mitochondrial protein content and expressed as a ratio of not treated (control) cells. Histograms represent mean of ratios ± SD. (B) Immunoprecipitation of SDH subunit A carried out on total lysate. As a negative control for immunoprecipitation an anti-mouse IgG was used. (C) Protein levels of mitochondrial chaperones CyP-D and TRAP1. Prohibitin was used as a mitochondrial marker and calnexin as a loading control.

The presence of a mitochondrial fraction of ERK and GSK3 is consistent with data obtained in our laboratory on several cells obtained from solid tumours, in which a branch of the RAS-ERK pathway was found in mitochondria, and it was demonstrated that an active mitochondrial ERK phosphorylated on Thr202/Tyr204 correlates with an inactive GSK3α/βphosphorylated onSer21/9 (Andrea Rasola et al. 2010). The RAS-ERK pathway is one of the pathways constitutively activated in B-CLL cells (Scupoli & Pizzolo 2012). Accordingly, I confirmed that in MEC-1 cells, ERK and GSK3 were in mitochondria and their activity were inversely correlated. Phosphorylated ERK and GSK3 were found in

the matrix fraction of mitochondria treated with high concentration of trypsin, ERK was active and GSK3 was inhibited respectively (Figure 4.14A).

In MEC-1 cells, p66Shc expression modulated the activity of mitochondrial fraction of ERK and GSK3 by increasing the phosphorylation of ERK, which correlated with a strong inhibition of GSK3. Indeed, even if the total mitochondrial protein levels of ERK were the same between the two cell lines, p66Shc expressing cells showed higher phosphorylation of ERK. In these cells the mitochondrial and phosphorylation levels of GSK3 were also increased (Figure 4.14A). We cannot exclude that the GSK3 increased phosphorylation observed between the two cell lines is due to increased total mitochondrial GSK3 levels. Thus, p66Shc expression leads to activation of mitochondrial fraction of ERK but not the cytosolic ones (Figure 4.14A).

Therefore, I explored the possibility that SDH activity was regulated at the post-transcriptional level by the RAS-ERK pathway. Indeed, treating mitochondria with PD98059, a MEK-ERK protein kinase inhibitor I observed an increase in SDH activity in both cell lines, which was more pronounced in p66Shc expressing cells (Figure 4.14B). Thus, active ERK can modulate SDH activity through post-transcriptional modifications.
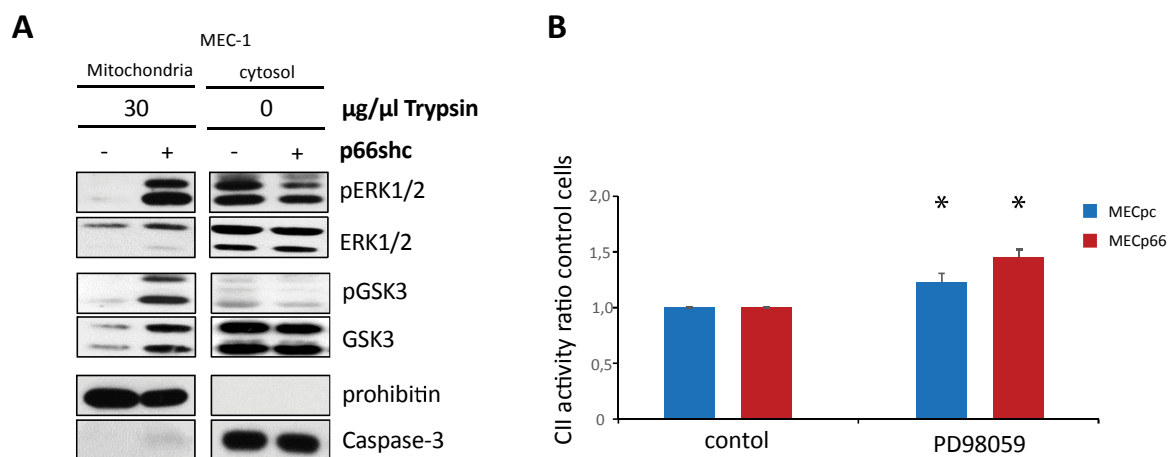
Figure 4.14 **Phosphorylation of mitochondrial ERK is increased upon p66shc expression and modulates complex II activity.** (A) Phosphorylation status of ERK1/2 (Thr202/Tyr204) and GSK3α/β (Ser21/9) in cytosolic and mitochondrial fraction. Mitochondria are treated 1h with 30 μg/μl of trypsin. In the blot prohibitin is used as mitochondrial marker and caspase-3 as cytosolic marker. (B) Succinate-CoQ reductase (SQR) enzymatic activity of complex II in isolated mitochondria treated or not with 50μM of MEK-ERK inhibitor PD98059. Values are normalised for mitochondrial protein content and expressed as a ratio of not treated control cells. Histograms represent mean of ratios ± SD.

Thus, SDH modulation by p66Shc expression is not caused by differences in protein levels of TRAP1 and ERK in mitochondria but may be due to increased levels of active ERK in the multimolecular complex associated with SDH. Active ERK on SDH can affect its activity through post-transcriptional modifications that may occur directly on SDH or on other proteins of the multimeric complex such as TRAP1.

4.2.5 p66Sch expression protects cells from death induced by mitochondrial oxidative stress

A key component of mitochondrial apoptotic machinery is the mPTP. The inhibition of mPTP opening might be used by tumour cells to avoid cell death. mPTP can be induced by oxidative stress. Thus, neoplastic cells maintain oxidative stress under strict control boosting anti-oxidant defences (A. Rasola & Bernardi 2014). B-CLL cells, that show high mitochondrial respiration, which probably contributes to rising their intracellular ROS levels, counteracted by increased anti-oxidant defences (Jitschin et al. 2014). The mitochondrial respiratory chain is one of the main sites of ROS and oxidative stress production in cells. Thus, we would like to understand the effect of p66Shc expression on cell viability of MEC-1 cells.

In basal conditions, we did not observe any difference in the number of viable cells between the two cell lines. Thus, we decided to induce oxidative stress in cells, culturing them in 0,1% FBS and in the absence of glucose. This condition mimics the environment found by many tumor types (low availability of nutrients and growth factors) and induces oxidative stress in cells by stimulating mitochondrial respiration and inhibiting anti-oxidant defences (Figure 4.4C). After 48h of culture, we observed that the number of viable p66Shc expressing cells was higher compared with control ones (Figure 4.15A). The toxicity of starvation was prevented by anti-oxidant molecule N-acetyl cysteine (NAC) that rescues cell from oxidative stress damage and subsequent cell death. This observation gave evidences that p66Shc expression in MEC-1 cells reduces oxidative stress-dependent cell death, probably through its inhibitory effects on mitochondrial respiration.
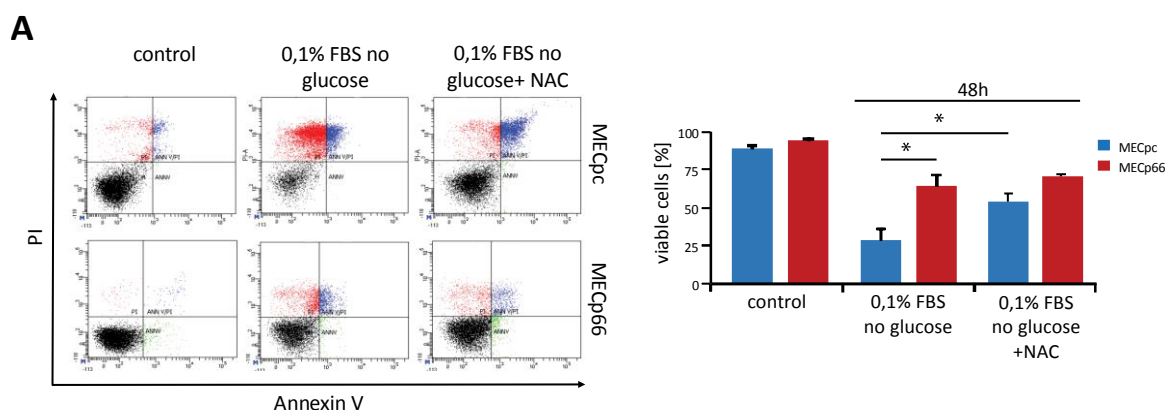
A

Figure 4.15 **Induction of cell death by mitochondrial oxidative stress: starvation conditions.** (A) Quantification of viable or MECpc (blue square) and MECp66 (red square) cells after 48h of 0.1% FBS and absence of glucose culture. Control cells were cultured in 10% of FBS. N-acetyl cysteine (NAC) at 1mM concentration was used as anti-oxidant molecule to confirm that cell death is induced by oxidative stress. A representative flow cytometry analysis after AnnexinV/PI staining is reported. Bar graph derived from the elaboration of flow cytometry-derive data of viable cell number. Values are expressed as the mean of percentage of viable cells ± SD.

I treated cells with EM20-25, a PTP inducer and BH3 mimetic compound that induces mitochondrial ROS production and massive cell death in several tumour cell models (A. Rasola et al. 2010; Ciscato et al. 2013) (Figure 4.16A). I observed that p66Shc expressing cells had higher viable cell number compared with control ones after EM20-25 treatment, and NAC protected cells from death (Figure 4.16A) indicating that the treatment exerts a pro-oxidant action which could be of different entity in the two cell lines. Thus, p66Shc expression renders cells less sensitive to this type of cell death, elicited by mitochondrial oxidative stress.

Another compound that causes oxidative stress by acting on the respiratory chain is the chemotherapeutic cisplatin, as it has been recently shown by our group that the rapid rise in ROS levels prompted by cisplatin treatment is strictly regulated by complex I, independently of its effects as DNA damaging agent (Ciscato et al. 2013). The treatment of MEC-1 cells with cisplatin showed that p66Shc expressing cells had more viable cells compare with control ones and that NAC protected cells from death (Figure 4.16B).
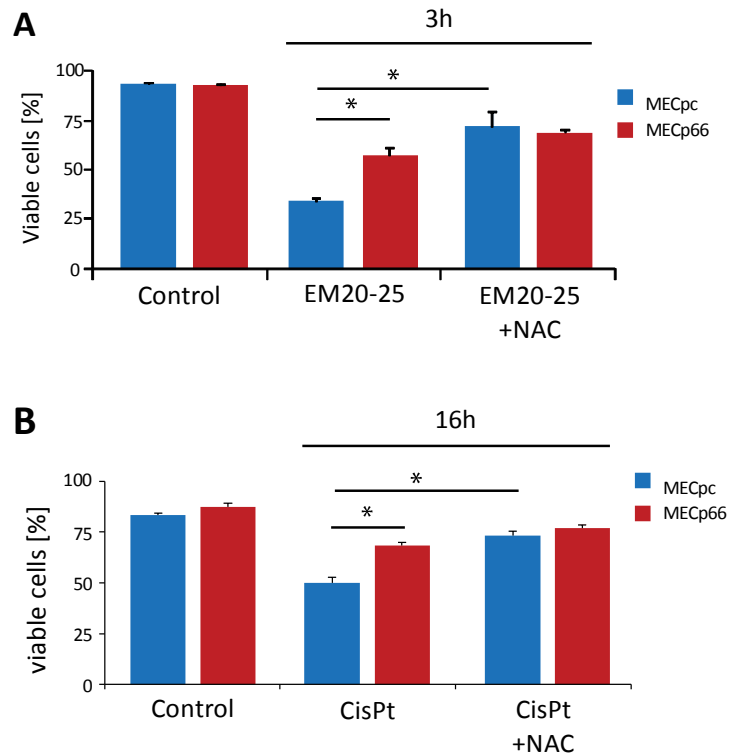
Figure 4.16 **Induction of cell death by mitochondrial oxidative stress.** (A) Number of viable MECpc (blue square) and MECp66 (red square) cells after 3h of EM20-25 (500μM) treatment. (B) Number of viable MECpc (blue square) and MECp66 (red square) cells after 16h of cisplatin (30μM) treatment. N-acetyl cysteine (NAC) at 1mM concentration was used as anti-oxidant molecule to confirm that cell death is induced by oxidative stress. In (A) and (B) control cells were cultured in 0,1% FBS. Bar graph derived from the elaboration of flow cytometry-derive data of viable cell number. Values are expressed as the mean of percentage of viable cells ± SD.

Together these data demonstrated that p66Shc expression in MEC-1 cells induces RC regulation changes that protect cells from death induced by mitochondrial-derived oxidative stress. These observations are consistent with the lower mitochondrial respiration observed in MEC-1 p66Shc expressing cells due to down-modulation of RC complex I and II activity.

The higher viability of p66Shc expressing cells can be due to a lower mitochondrial ROS production linked to a lower mitochondrial respiration. Importantly, these data are in contrast with the pro-apoptotic and pro-oxidant activity of p66Shc expression (Giorgio et al. 2005). Notably, p66Shc expression induced an unbalance of Bcl-2 family proteins toward a pro-apoptotic phenotype (Capitani et al. 2010), whereas we did not observe

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any differences in the protein levels of Bcl-2 family proteins in MEC-1 cells after p66Shc expression (Figure 4.17A)
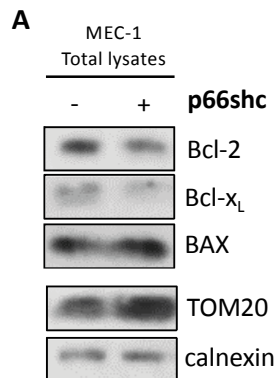

Figure 4.17A **p66Shc does not affect bcl-2 family proteins** (A) Protein levels of Bcl-2, Bcl-x$_L$ and BAX. TOM20 was used as a mitochondrial marker and calnexin as a loading control.

4.2.6 Mitochondrial ERK1/2 can modulate cell viability under stress conditions.

In several tumor cell types it has been demonstrated that high phosphorylation of ERK protects cell from death through inhibition of GSK3 that blocks CyP-D phosphorylation and makes mPTP less sensitive to opening (A. Rasola et al. 2010). In accord with reports indicating that p66Shc increases phosphorylation and activation of ERK, thus supporting proliferation of solid tumour cells (Veeramani et al. 2005), I found that p66Shc expression in B-CLL MEC-1 cells increased activation of the mitochondrial fraction of ERK (Figure 4.14 A).

I asked whether mitochondrial ERK has a role in cell death induced by oxidative stress. Starvation conditions, inhibits growth factor dependent pathways, activates oxidative dependent pathways, and highlights mitochondrial survival pathways under stress conditions. These conditions decreased mitochondrial ERK activation in both cell lines (Figure 4.18A). This event was linked with a reduction of cell viability (Figure 4.18B). The inhibition of active ERK with PD98059 in starvation conditions induced cell death in both cell types (Figure 4.18B). These data indicate a link between decreased mitochondrial ERK activation and cell death. p66Shc expressing cells showed high mitochondrial active ERK in basal and also under starvation conditions (Figure 4.18A). The number of viable p66Shc expressing cells was always higher compared with the control ones (Figure

4.18B). Thus, activation of ERK in mitochondria is associated with high number of viable cells.
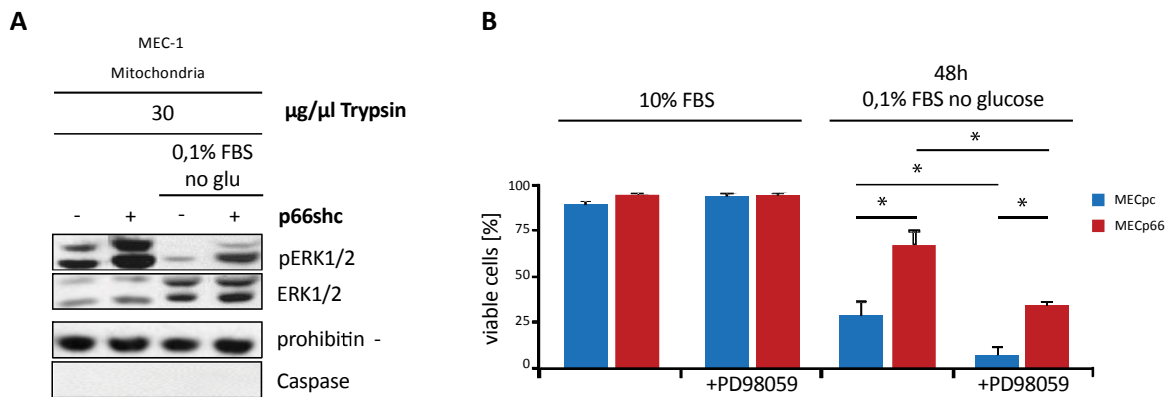
Figure 4.18 **inhibition of ERK1/2 decreases cell viability** (A) Phosphorylation status of ERK1/2 (Thr202/Tyr204)in mitochondrial fraction of cells cultured in 10% FBS and in 0,1%FBS and absence of glucose for 16h. prohibitin was used as mitochondrial marker and calnexin as loading control. (B) Number of viable cells for MECpc (blue square) and MECp66 (red square) cells after 48h of culture with 10% or0,1% FBS and absence of glucose culture treated or not with the MEK-ERK inhibitor PD98059 (50µM).The bar graph is obtained from the elaboration of flow cytometry data and indicates the mean percentage of viable cells.

In addition, in both cell types also the inhibition of the mitochondrial chaperone CyP-D with cyclosporine A (CsA) protected cells from death (Figure 4.19A), whereas inhibition of mitochondrial chaperone TRAP1, with 17AAG, decreased cell viability (Figure 4.19A).

The results on cell viability under starvation conditions could not be directly linked with mitochondrial ERK activity because they derive from flow cytometry analysis performed on whole cells. However, the effects of mitochondrial chaperones CyP-D and TRAP1 inhibition on cell viability indicates that mitochondria and mitochondrial ERK are involved in the regulation of cell death in MEC-1 cells. Thus, mitochondrial ERK, CyP-D and TRAP1 can take part in the survival pathways that protect cells from death induced by oxidative stress.

5. Discussion

The metabolism of healthy cell is based on mitochondrial oxidative phosphorylation to supply the energy needed for biochemical processes. During the growth of solid tumours cancer cells proliferate in an environment characterized by low oxygen and nutrient availability, and therefore rely on aerobic glycolysis to support their energy demand; this metabolic adaptation is termed "the Warburg effect" (Vander Heiden et al. 2009). The attention toward rewiring of metabolism has progressively risen, even though it remains unclear whether metabolic changes drive the process of neoplastic transformation or simply take part in it (Hanahan & Weinberg 2011).

Mitochondria are at the heart of bioenergetic processes, and they also control many survival pathways whose deregulation allows neoplastic cells to escape apoptotic stimuli. Therefore, it is possible that connections exist in the biochemical mechanisms that control bioenergetic and survival routines in mitochondria. For instance, in the inner mitochondrial membrane resides the permeability transition pore (mPTP), whose opening induced by specific pro-apoptotic stimuli commits cells to death, and mitochondrial respiration is strictly connected with the mPTP. Indeed, respiratory chain is the major source of ROS production which induce mPTP opening and play an important role in determining cell fate; moreover, the functionality of OXPHOS contribute to the membrane potential across the inner mitochondrial membrane, which defines the threshold of mPTP opening.

Signalling pathways that are usually found altered in tumours control many mitochondrial functions. One example is provided by the RAS-ERK axis, which has a mitochondrial branch that promotes the viability of solid tumour cells (A. Rasola et al. 2010). The aberrant activation of these kinases can be due to constitutive activation of upstream receptors, to oncogenic mutations or to changes in the redox equilibrium of the neoplastic cell. In this regard, an important role can be played by p66Shc, whose expression increases the phosphorylation of ERK in prostate and breast cancer models (Veeramani et al. 2005; Veeramani et al. 2012). A fraction of this protein was found in the intermembrane space of mitochondria, where it produces ROS by subtracting electrons from OXPHOS at the level of cytochrome *c* (Giorgio et al. 2005). Thus, p66Shc can affect OXPHOS activity of tumour cells, and it has been recently demonstrated that p66Shc in solid tumours inhibits glycolytic metabolism in favour of oxidative metabolism

(Soliman et al. 2014). Together these data suggest that deregulated kinase signalling in mitochondria can aberrantly modulate OXPHOS complexes, possibly through the involvement of proteins such as p66Shc that link redox changes to kinase signalling. Moreover, also mitochondrial chaperones such as TRAP1 and CyP-D can control both survival pathways and bioenergetic functions of mitochondria, and can be targeted by kinase signalling.

Most of these data on mitochondrial features that characterize malignancies were obtained on cells obtained from solid tumors. Very little is known on the metabolism of blood tumor cells. Among these, B-cell chronic lymphocytic leukaemia (B-CLL) seems particularly interesting, because a distinctive feature of this neoplasm is its high resistance to apoptotic stimuli, which implies an important role played by mitochondria. For B-CLL no effective chemotherapy exists, and usually patients develop drug resistance, which highlights the importance of finding novel anti-neoplastic strategies, and targeting metabolic features of these malignant cells could be an interesting therapeutic option. In B-CLL cells circulate in blood or are found in lymph nodes, where oxygen and nutrient availability is high. It is therefore possible that B-CLL are endowed with an efficient mitochondrial oxidative metabolism, in contrast with the Warburg phenotype that characterizes most solid tumors. A high OXPHOS activity can induce oxidative stress in B-CLL cells, and this must be counteracted by increased anti-oxidant systems (Jitschin et al. 2014).

Nothing is known on the molecular circuitry that regulate OXPHOS activity and viability in B-CLL cells. A recent study shows that B-CLL cells lose p66Shc expression (Capitani et al. 2010), which could make these cells more resistant to cell death elicited by oxidative stress. In this scenario I have investigated the effect of p66Shc expression, which increases the activation of ERK in solid tumour cells, on mitochondrial respiration and cell viability of B-CLL cells.

I have observed that p66Shc expression decreases mitochondrial respiration without any change in glycolysis. This means that B-CLL cells depend on L-glutamine metabolism as carbon source and that the loss of p66Shc dramatically increases mitochondrial oxidative metabolism that is fundamental to support viability of this kind of cells. I propose that decreased mitochondrial respiration is due to decreased activity of

respiratory complex I and II. In more detail, there is a decrease in complex I assembly with a consequent inhibition of its activity, and a decrease in complex II activity caused by a multi-protein complex in which TRAP1 and active ERK are involved.

We found that also in B-CLL cells, as in cells derived from solid tumors, TRAP1 decreases SDH activity. Indeed, TRAP1 inhibition increases SDH activity in B-CLL cells and this effect is more pronounced in p66Shc expressing cells. Thus, p66Shc inhibits mitochondrial respiratory chain by affecting TRAP1 activity. It remains to elucidate the mechanism by which p66Shc modulates TRAP1 inhibitory effect on SDH. It has been demonstrated that TRAP1 can promote neoplastic transformation through succinate accumulation and stabilisation of HIF1-α. Moreover, it has anti-oxidant effects and protects cells from mPTP opening (Sciacovelli et al. 2013; Guzzo et al. 2014). It is possible that TRAP1 inhibition of SDH affects B-CLL resistance to cell death through these mechanisms. However, further studies must be carried out in order to elucidate the crucial role of TRAP1 in the regulation of mitochondrial respiration in B-CLL cells and in p66Shc expressing cells to define a connection between SDH inhibition by TRAP1 and tumorigenesis.

Several studies have shown important metabolic alterations in cells with oncogenic RAS (see section 1.2.2.6 of introduction). A fraction of active ERK was found in mitochondria of solid tumour cell models, but its possible effects on mitochondrial OXPHOS were not evaluated (Andrea Rasola et al. 2010). The RAS-ERK pathway is hyperactivated in half of B-CLL patients (Scupoli & Pizzolo 2012). We found that a fraction of ERK is in mitochondria of B-CLL cells and that its inhibition increases SDH activity. Thus, mitochondrial ERK takes part in the regulation of mitochondrial respiration on B-CLL cells. p66Shc expression increases activation of ERK in mitochondria of B-CLL cells, in accord with what was found in solid tumours, where p66Shc expression increases ERK activation, even if these experiments were not carried out on the mitochondrial fraction of these molecules (Veeramani et al. 2005). I have also found that inhibition of ERK increases SDH activity, suggesting that p66Shc inhibits SDH activity by increasing the activation of ERK. ERK can decrease SDH activity by post-transcriptional modifications on SDH subunits: it can directly phosphorylate SDH or proteins involved in the multiprotein complex bound to SDH, such as TRAP1. Further studies are necessary to elucidate the

mechanisms by which ERK regulates SDH activity. Most solid tumours and leukaemias show activation of the RAS-ERK pathway.

How p66Shc could affect ERK activation in mitochondria remains unclear. It is possible that high activation of mitochondrial ERK is due to an adaptation of cells to the stable expression of p66Shc. Indeed, p66Shc increases H_2O_2 levels. Interacting with cytochrome *c*, p66Shc can render less efficient the respiratory chain at the level of complex III, thus generating ROS in the matrix. These ROS may inhibit mitochondrial phosphatases, therefore increasing mitochondrial ERK phosphorylation. Cells counteract this increase in ROS levels by inhibiting mitochondrial respiration, the most important site of ROS production in cells, or increasing anti-oxidant systems, thus allowing cells to survive.

Our data indicate that SDH activity, TRAP1 and ERK are involved in the control of mitochondrial respiration activity of B-CLL cells (Jitschin et al. 2014). The expression of p66Shc in B-CLL modulates the interplay among these proteins and is probably involved in the establishment of a glycolytic phenotype. Hence, p66Shc could contribute to the neoplastic process in solid tumors through the inhibition of SDH and the ensuing stabilization HIF1-α upstream to TRAP1 activity, and it could also decrease ROS production from mitochondrial respiration.

In highly proliferating solid tumour cells, increased oxidative stress can contribute to elevate p66Shc expression. Although p66Shc was initially reported as a pro-apoptotic molecules, several solid tumour cells are glycolytic and express p66Shc (Lebiednska-Arciszewska et al. 2014). B-CLL cells show high oxidative stress counteract by increased anti-oxidant systems. We checked the effect of p66Shc expression on viability of B-CLL cells. p66Shc expressing cells die when p66Shc is stimulated by H_2O_2. These data are in accordance with pro-apoptotic effect previously described for p66Shc protein in B-CLL (Capitani et al. 2010; Migliaccio et al. 1999; Pacini et al. 2004). In contrast, I observed that p66Shc expression in B-CLL cells protected from death induced by mitochondrial-derived oxidative stress. Thus, we confirm that B-CLL cells, which do not express p66Shc, have a high oxidative stress due to high mitochondrial respiration, whereas p66Shc expression decreases mitochondrial respiration. This differential response to cell death underlines a differential involvement of p66Shc in different mechanism of cell death induction. It is possible that H_2O_2 can stimulate p66Shc to induce an unbalance in Bcl-2

family proteins toward a pro-apoptotic phenotype (Capitani et al. 2010). On the other hand, p66Shc expression can protect cells from death induced by mitochondrial-dependent oxidative stress because it decreases mitochondrial respiration. Accordingly, we found that active ERK is in mitochondria of B-CLL cells and its inhibition increases cell death induced by mitochondrial-dependent oxidative stress. In these conditions, p66Shc expressing cells are more viable. It is possible that low complex II activity inhibited by active ERK decreases ROS production but also decreases mitochondrial membrane potential and makes mPTP less sensitive to opening (A. Rasola & Bernardi 2014) protecting cells from death. In p66Shc expressing cells, this effect is more pronounced because mitochondrial ERK is more active and SDH is more inhibited. Further investigation are necessary to elucidate how mitochondrial active ERK protect cell from death and how p66Shc is involved in these mechanisms. Thus, the RAS-ERK pathway plays a key role also in mitochondrial mechanisms that regulate cell viability in B-CLL cells probably by regulating SDH activity and mitochondrial respiration. In this model, p66Shc would protect cells from death decreasing mitochondrial respiration via ERK modulation, therefore leading to mPTP modulation.

Further studies must be carried out in order to highlight the molecular circuitry of regulation of complex II activity and viability of cells by ERK. p66Shc expression affecting ERK activity, decreases mitochondrial respiration and protect B-CLL from death induced by mitochondrial-derived oxidative stress. Thus, p66Shc play a pivotal role in regulation of mitochondrial respiration and metabolism of cancer cells. Further studies are necessary to define the role of p66Shc on SDH regulation because the stabilisation of HIF1-α leaded to SDH inhibition and succinate accumulation could explain tumorigenesis of p66Shc expressing tumour cells. Finally, highlight the molecular circuitry through which p66Shc can induce changes in mitochondrial respiration is very important to find new target for drug design.

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