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Cell death regulation by mitochondrial chaperones in cancer cell models

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Summary

Cancer cells are endowed with the capability to evade normal apoptotic signaling, as they display a constitutive hyperactivation of kinase signaling pathways. Integration of survival and death stimuli occurs on mitochondria, where many of these signals converge in the regulation of a channel termed permeability transition pore (PTP). PTP opening commits cells to death, and it is regulated by a variety of factors, among which molecular chaperones play a pivotal role. Here I have studied how mitochondrial chaperones interact with signal transduction pathways, modulate the PTP and more in general mitochondrial bioenergetics, and how these regulatory networks control tumor cell viability. In a first part of my work, I have explored a functional connection between the Ras/ERK signaling axis, whose constitutive activation characterizes most tumors and prompts their growth and survival, and cyclophilin D (CyP-D), a mitochondrial chaperone that regulates the PTP. A fraction of active ERK was found to be located in mitochondria in RWPE-2 cells, obtained by *v*-Ki-Ras transformation of the epithelial prostate RWPE-1 cell line; in metastatic prostate cancer DU145 cells; and in osteosarcoma SAOS-2 cells. All these tumor cells displayed marked resistance to death caused by apoptotic stimuli like arachidonic acid and the BH3 mimetic EM20-25, which cause cell death through the mitochondrial PTP. PTP inhibition and the ensuing resistance to cell death induced by arachidonic acid or EM20-25 could be ablated by inhibiting ERK with the drug PD98059 or with a selective ERK activation inhibitor peptide. ERK inhibition enhanced GSK-3-dependent phosphorylation of CyP-D), whereas GSK-3 inhibition protected from PTP opening. Neither active ERK in mitochondria nor pore desensitization were observed in non-transformed RWPE-1 cells. Thus, in tumor cells mitochondrial ERK activation desensitizes the PTP through a signaling axis that involves GSK-3 and CyP-D. In a second part of my thesis work, I have investigated the activity of a second mitochondrial chaperone, TRAP1/HSP75, overexpressed in tumor cells and proposed to be involved in regulation of the pore. I have determined that

TRAP1 interacts with CyP-D and characterized its survival function against a wide spectrum of death stimuli inducing oxidative stress, including diamide, exposure to TNFα, and glucose deprivation. Moreover, I have found that knocking-down TRAP1 expression level through RNA interference in SAOS-2 osteosarcoma cells facilitates PTP opening, thus lowering the threshold for committing cells to death. TRAP1 modulates cell metabolism and possibly the response to oxidative stress by reducing mitochondrial respiration and the activity of respiratory chain complex I, with which TRAP1 directly interacts, both in cells and in tumor samples. Notably, down-modulation of TRAP1 ablates the tumorigenic potential of SAOS-2 cells both *in vitro* and *in-vivo*. Altogether, these data indicate that mitochondrial chaperones such as CyP-D and TRAP1 play an important role in tumor progression and constitute a possible target for anti-neoplastic intervention.

Riassunto

Le cellule tumorali sono caratterizzate dalla capacità di evadere il normale signale apoptotico, così come mostrano una iper-attivazione costitutiva delle vie di signale kinasico. L'integrazione degli stimoli di sopravvivenza e morte si concentra nei mitocondri, dove molti di questi segnali convergono nella regolazione di un canale chiamato poro della transizione di permeabilità (PTP). L'apertura del PTP porta le cellule alla morte ed è regolata da una varietà di fattori e fra questi gli chaperoni giocano un ruolo fondamentale. Nel mio lavoro di tesi ho studiato come gli chaperoni mitochondriali si integrano nelle vie di trasduzione del segnale , modulando il PTP e più in generale la bioenergetica mitocondriale e come questi network regolatori controllano la vitalità cellualre. Nella prima parte del mio lavoro ho studiato una possibile connessione fra la via del segnale Ras/ERK, la cui attivazione costitutiva caratterizza molti tumori favorendo la loro crescita e sopravvivenza, e la ciclofilina D (CyP-D), uno chaperone mitocondriale che regola il PTP. Una frazione di ERK attivo è stato trovato nei mitocondri delle cellule RWPE-2, ottenute tramite trasformazione con *v*-ki-Ras a partire da cellule dell'epitelio prostatico RWPE-1; in cellule metastatiche di tumore prostatico DU145; e in cellule di osteosarcoma SAOS-2. Tutte queste cellule tumorali mostrano una marcata resistenza alla morte indotta da stimoli pro-apoptotici come l'acido arachidonico e il BH3 mimetico EM20-25, i quali inducono la morte cellulare attraverso il PTP mitocondriale. L'inibizione del PTP e la conseguente resistenza alla morte cellulare indotta da acido arachidonico o EM-20-25 può essere abolita dall'inibizione di ERK con il farmaco PD98059 o con un peptide selettivo inibitorio di ERK. L'inibizione di ERK aumenta la fosforilazione GSK-3 dipendente della CyP-D, mentre l'inibizione di GSK3 protegge dall'apertura del poro. Ne ERK attivo nei mitocondri, ne desensibilizzazione del poro è stata osservata in cellule non trasformate RWPE-1. In conclusione, nelle cellule tumorali l'attivazione dell'ERK mitocondriale desensibilizza il PTP attraverso un asse di segnale che coinvolge GSK3 e Cyp-D. Nella seconda parte del mio lavoro

di tesi, ho studiato l'attività di un secondo chaperone mitocondriale, TRAP1/HSP75, fortemente espresso nelle cellule tumorali e che è stato proposto essere coinvolto nella regolazione del poro. Ho dimostrato che TRAP1 interagisce con la CyP-D ed ho caratterizzato la sua funzione di pro-sopravvivenza nei confronti di un vasto spettro di stimoli di morte, incluso lo stress ossidativo, la diamide, il TNFα, e la deplezione di glucosio. Inoltre ho trovato che il knocking-down dei livelli di espressione di TRAP1 attraverso la tecnica dell'RNA interference in cellule di osteosarcoma SAOS-2 facilita l'apertura del PTP, abbassando la soglia per portare le cellule alla morte. TRAP1 modula inoltre il metabolismo cellulare probabilmente la risposta allo stress ossidativo e l'attività della del complesso I della catena respiratoria, con il quale TRAP1 interagisce direttamente sia in cellule che campioni tumorali. La down- regolazione di TRAP1 abolisce il potere tumori genico delle cellule SAOS-2 sia *in vitro* che *in vivo.* Tutti insieme questi dati indicano che gli chaperoni mitocondriali come CyP-D e TRAP! Giocano un ruolo importante nella progressione tumorale e costituiscono un possibile target di nuove terapie antineoplastiche.

Cell death regulation by mitochondrial chaperones in tumor cell models

1. Introduction

Cancer is a complex and heterogeneous set of diseases, in which a group of cells loses the homeostatic control of basic biological processes such as environmental interactions, metabolism, proliferation and death, resulting in an unrestrained growth that intrudes upon and destroys adjacent tissues, and sometimes induces cell spreading to distant locations in the body. Given the growing medical importance of cancer, which is one of the leading causes of death, during the last century research has intensively studied the etiology, pathogenesis, epidemiology, the clinical features of cancer, generating a complex body of knowledge and a wide array of therapeutic approaches. It is now clear that tumorigenesis is caused by inherited or, more often, genetic alterations, which induce a multistep process endowed with a group of stereotypical features.

1.1 Hallmarks of cancer

Cancer cells are characterized by changes in a variety of intrinsic biological processes and in environmental interactions that lead to loss of tissue homeostasis. Importantly, cancer development requires complex heterotypic interactions among neoplastic cells and several other cell types (Hanahan and Weinberg 2000).

There are eight essential alterations that hallmark cancer and regulate malignant growth (Hanahan and Weinberg 2000; Klein et al. 2008; Mantovani et al.2008):

1. *Self sufficiency in growth signals*
2. *Insensitivity to growth inhibitory signals*
3. *Evasion of programmed cell death (Apoptosis)*
4. *Limitless replicative potential*
5. *Sustained angiogenesis*
6. *Tissue invasion and metastasis*
7. *Induction of an inflammatory microenvironment*
8. *Metabolic changes*

Each of these changes is the result of the successful breaching of anticancer defenses present in cell and tissues.

Several genetic alterations can drive the stepwise transformation of normal cells into malignant derivatives. Thus, tumor development can be viewed as a phenomenon that proceeds in a sort of Darwinian evolution, in which subsequent genetic changes confer advantages to tumor cells, allowing them to face the hostile surrounding environment.

Genetic mutations leading to neoplastic transformation can occur either in *oncogenes (*dominant gain of function mutations) or in *tumor suppressor genes* (recessive loss of function mutations); products of these genes include protein involved in the regulation of diverse biological processes, such as growth, proliferation, apoptosis, metabolism, and DNA repair.

1.1.1 Self sufficiency in growth signals

Normal cells require mitogenic growth factors (GF) in order to move from a quiescent state into a proliferative one. These signals are transmitted into the internal space of cells by receptors that bind diffusible growth factors. As a consequence, through activation of intra-cellular cascades, this signals control anabolism, catabolism, the entry into the cell cycle and mainly regulation of gene expression. Conversely, cancer cells acquire autonomy from exogenous GFs in several ways: they can become capable to synthesize the GFs they need (e.g.,

sarcomas produce TGFα; Fedi et al. 1997), to overexpress GF-binding receptors (e.g. EGF-R /erbB in breast cancer; Slamon et al.1987) or to structurally change GF receptors in order to obtain constitutively active signals (for example truncated versions of EGF receptor lacking much of its cytoplasmic domain; Fedi et al.1997). Mutations can also occur on downstream, cytosolic components of GS signaling: a classical example is hyper activation of the MAP kinase cascade, observed in the majority of cancers and caused by mutations in several components of the signaling pathway (Dhillon et al. 2007).

1.1.2 Insensitivity to growth inhibitory signals

Multiple anti-proliferative stimuli operate to counteract growth signals and maintain tissue homeostasis . These type of signals include both soluble factors and immobilized inhibitors embedded in the extracellular matrix. Antigrowth signals block proliferation prompting cell exit from the cycle, in the G_0 quiescent state, which frequently preludes to the acquisition of specific differentiation traits. Cancer cells evade these stimuli by modulating components that govern the transit of cells through the G1 phase of the cell cycle. A classical pathway altered in cancer cells is the one controlled by the protein *pRb* encoded by the retinoblastoma tumor suppressor gene Rb1.pRB blocks proliferation sequestering the *E2F1* transcription factor, a master switch that controls the transition from G1 into S phase. Disruption of this cascade leads to forced proliferation (Goodrich et al. 2006), and mutational inactivation of the two Rb1 alleles causes pediatric retinoblastoma and is involved in many tumor types.

Another important player in avoiding post mitotic differentiation is activation of the proto-oncogene c-MYC, a transcription factor whose deregulated expression is observed in a wide spectrum of cancers and is associated to aggressive, poor differentiated neoplasias. c-MYC, in association with the transcription factor MAX, activates the expression of genes that directly induce proliferation (e.g. those encoding Cyclin D2 or CDK4; Pelengaris et al.2002). Cell differentiation is controlled by MAD-MAX transcription factor complexes: c-MYC over-

expression disrupt MAD-MAX interaction in several tumors favoring formation of c-MYC-MAX complexes (Pelengaris et al.2002)

1.1.3 Evasion of programmed cell death (Apoptosis)

Tumors are characterized by an high proliferation rate that is sustained also by the development of a robust anti-apoptotic platform. Signals that elicits apoptosis converge on the mitochondria, which respond to stimuli by realizing cytochrome *c* that activates intracellular protease called *caspases* (this feature will be discussed in detail in paragraph 1.4.).

1.1.4 Limitless replicative potential

Mammalian cells carry an intrinsic cell autonomous program that limits their multiplication, independently of cell-to-cell signaling. Once cells have progressed through a certain number of doublings, they stop growing and enter a state called senescence. Cellular senescence is characterized by a largely irreversible cell cycle arrest that can be triggered by many types of intrinsic and extrinsic stress. These include telomere malfunction, oncogene activation and tumor suppressor gene inactivation (Prieur and Peeper, 2008; Kuilman et al.2010). Ultimately, such events culminate in the activation of a tumor suppressor gene network. Many signals that induce senescence, including those resulting from telomeric malfunctioning, converge on p53 and pRB, which have been proposed to act as gate keeper tumor suppressors (Prieur and Peeper, 2008). Since the first description of Oncogene-Induced cellular Senescence (OIS) little over a decade ago, many subsequent studies have confirmed that OIS prevents cells from undergoing oncogenic transformation in vitro. However, it has long been debated whether any in vivo correlates exist. It is only since recent years that evidence has been accumulating indicating that OIS in vivo does correspond to a major protective mechanism against cancer (Prieur and Peeper, 2008). One strategy used by malignancies to replicate in a limitless way is the maintenance of *telomeres*. The ends of chromosomes, called telomeres, are characterized by repeats of 6 bp

sequence elements that control the number of duplications in a cell life. Each passage of doublings lead to a loss of 50-100 bp of telomeric DNA. Malignancy are able to avoid also this control: a common strategy used is the up-regulation of telomerase enzyme which adds hexanucleotide repeats onto the end of telomeric DNA, regenerating them.(Hanahan and Weinberg, 2000).

1.1.5 Sustained angiogenesis

Physiological angiogenesis is a process initially defined as the spreading of capillary venules from pre-existing vessels. Ischemia and oxygen decrease initiate a cascade of events that stimulates new vessels formation in order to increase nutrient and oxygen supply. This complex process requires interactions among different cell types, extracellular matrix, growth factors, several cytokines. The process of cancer angiogenesis relies on many of the same processes involved in the physiological angiogenesis that in cancer are constitutively active. When the tumor demand for oxygen and nutrients surpasses the local supply (which typically occurs before the tumor reaches 1-2 mm of diameter) there are some internal regions in primary tumor mass that are not supply by nutrient and oxygen: this hypoxic micro-environment induces angiogenesis through the expression of HIFs proteins. In addition to hypoxia, other factors can aberrantly up-regulate HIFs expression under normoxic conditions in tumors, for example mutations in the tumor suppressor gene von Hippel Lindau (VHL) that enhance HIF1α activity preventing its degradation in renal carcinomas.

Cancer ability to stimulate new angiogenesis is a pre requisite for the rapid expansion that underpins the formation of macroscopic solid tumors (Hanahan and Weinberg 2000; Ellis et al. 2008; Chung et al. 2010). There are many evidences in which cancer cells up-regulates angiogenesis initiating signals (for example vascular endothelial growth factors ,VEGF, and fibroblast growth factors FGF 1/2) and down regulate the endogenous inhibitors (for example β-interferon or thrombospondin-1). New vessels induced by cancer cells are quite different from normal one because they are tortuous and disorganized. Moreover they are leakier than normal: this contribute to the generation of an interstitial

hypertension that limits delivery of anti- neoplastic drugs and to the spreading of metastases.

1.1.6 Tissue invasion and metastasis

Hallmarks of cancer malignancy are the capability of neoplastic cells to trespass tissue boundaries, to colonize both adjacent districts (invasion) and distant areas, utilizing in this case the blood or the lymph flux (metastasis). The pathological importance of these processes is highlighted by the observation that cancer lethality is caused in the vast majority of cases by invasive or metastatic tumor masses. Invasion and metastasis are complex events and their genetic and biochemical determinants remain not completely understood.

Epithelial tissues, representing the origins of most solid tumors, form relatively rigid sheets of cells. They are separated from the stroma by a basement membrane and are highly organized by lateral belts of cell–cell adhesion complexes. During the progression from a tumor in situ to an invasive carcinoma, epithelial tumor cells are released from their neighbors and breach the basement membrane barrier. The process underlying this phenomenon has often been suggested to involve EMT (Epithelial to Mesenchymal transition; Geiger et al.2009). EMT is an evolutionarily conserved developmental process. Conventionally, epithelial cells are defined as surface barrier cells with secretory functions that show distinct apical versus basolateral polarity established by adherens and tight junctions. Mesenchymal cells serve scaffolding or anchoring functions and have multifunctional roles in tissue repair and wound healing. During embryonic development, certain differentiated polarized epithelial cells, on extracellular cues, undergo profound morphogenetic changes, collectively referred to as EMT. This is typified by the dissolution of cell–cell junctions and loss of apico–basolateral polarity, resulting in the formation of migratory mesenchymal cells with invasive properties (Singh and Settleman 2010). Although the mechanistic details behind the metastatic spread of cancer remain obscure, it has been reported that transforming growth factor β (TGFβ) acts as major player in the metastatic process. Ordinarily tumor suppressor TGFβ mediates growth arrest and apoptosis

but recently Adorno et al. (2009) identify a mechanism in cells expressing mutant p53, which enables TGFβ to switch to an oncogenic role by promoting mutant p53-mediated suppression of the related p63 protein, thus uncovering a new role for p63 in the negative regulation of metastasis. (Clohessy and Pandolfi 2009).

As soon as tumor cells lose contact with the basement membrane during invasion they hit another barrier against metastasis: *anoikis* (cell death induced by inappropriate or loss of cell adhesion).Anoikis could hamper metastasis by inducing apoptosis when tumor cells enter "foreign" environments and so its suppression, therefore, is likely to be a prerequisite for tumor cells to successfully metastasize to distant sites .Consistent with this, most cell lines established from human tumors contain populations of cells that survive when confronted with lack of adhesion to culture plates (Geiger et al. 2009).This is not the only defensive mechanism against tumors. Oncogenic protein mutations can be recognized by the immune system. In particular Cytotoxic T Lymphocyte (CTL) can efficiently discover and destroyed cells the express antigens associated to MHC class I proteins with the help of Natural Killer (NK) cells that can lysate transformed cells that are not expressing MHC class I proteins (Kumar et al.2010).

Several classes of proteins are involved in tethering cells to their surroundings in a tissue, and many of these are altered when cells acquire metastatic capacity. Proteins affected include cell-cell adhesion molecules (CAMs, for instance E-cadherin) but also integrins that regulate interactions between cells and the extracellular matrix and extracellular proteases (Geiger et al.2009). Recently it was proposed that metastasis can evolve following two different models and this is crucial from the therapeutic point of view (fig.1).

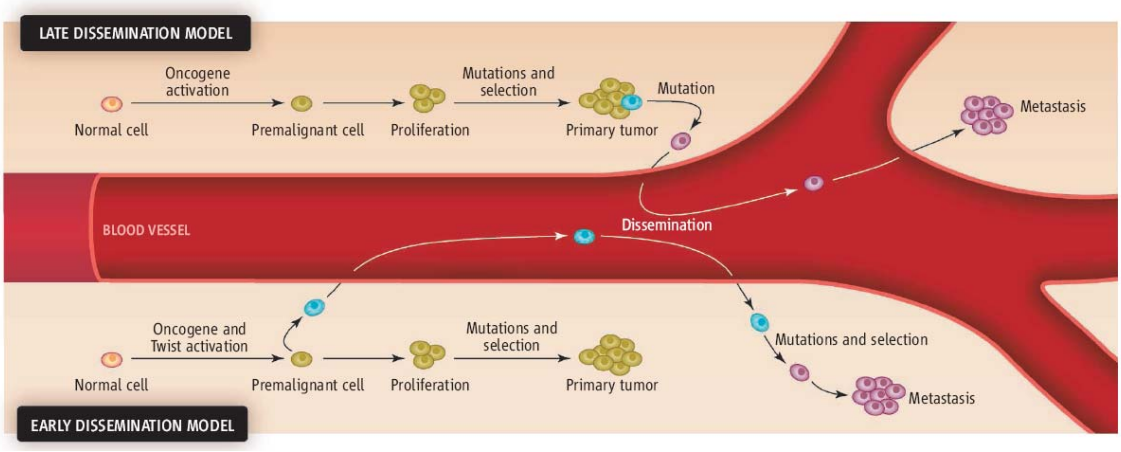


Figure 1. Late and early metastatic cascade model. The late dissemination model postulates that the expansion of metastatic clones occurs into the primary tumor, these aggressive cells would have a genetic profile similar to that of primary tumor cells. The early dissemination model proposes that mutations accumulate at distant sites, where metastatic cells genetically diverge from primary tumor cells (From Klein CA., Science 2008)

The first one is the *linear progression model*, also called the *late dissemination model* . In this model, cancer cells undergo genetic and epigenetic alterations, multiple successive rounds of mutations and selections for competitive fitness in the context of primary tumors. After a number of such rounds, cells that can proliferate leave the primary accrual site to seed and grow in secondary loci. Therefore, this clonal expansion is strictly related to tumor size and, more importantly, metastases are genetically similar to primary tumors.

The second model is the *parallel or early dissemination model*. This type of progression predicts an early dissemination of metastatic founder cells, with no need of accumulating mutations in the primary tumor mass. So, a great genetic disparity exists between cells derived from the metastatic founder and those grown in the primary tumor, as microenvironment becomes the limiting factor in the gathering of site-specific genetic and epigenetic mutations (Klein 2008;Klein 2009).

1.1.7 Cancer related inflammation

Links between cancer and inflammation were first made in the 19th century, on the basis of observation that tumors often arose at sites of chronic inflammation and that inflammatory cells were present in tumor biopsies. The role of inflammation in cancer is controversial: cells involved in the inflammatory response such as CTL or NK are crucial for the control and elimination of nascent tumors (Mantovani et al.2008; Mantovani 2009; Steer et al. 2010) fig. 2), and tumor cells must develop a variety of strategies to escape this kind of surveillance.

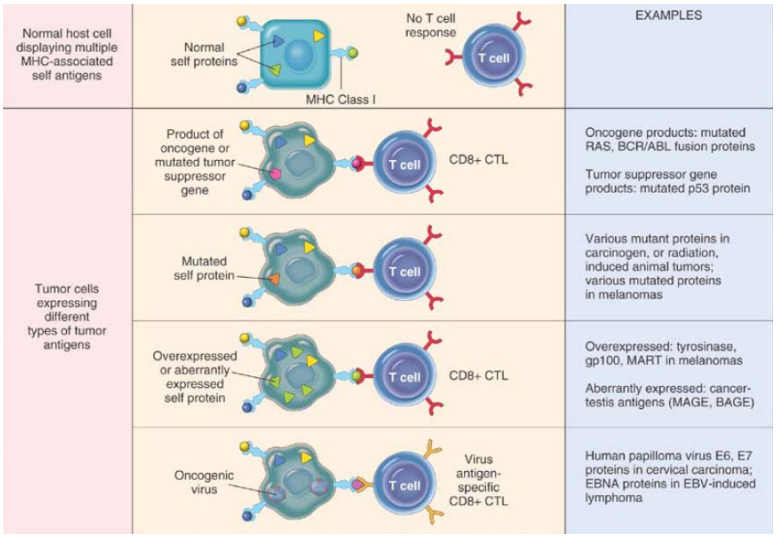


Figure 2. Principal classis of tumor antigens recognized by CTL CD8+ cells. Some proteins expressed or mutated in cancer (oncoproteins, mutated self proteins or oncogenic viruses) can be recognized by the immune system if associated to MHC I and destroyed by CTL cells (from Kumar et al.Elsvier 2010)

Conversely, epidemiological studies have shown that chronic inflammation predisposes individual to various types of cancer, and that infections and inflammatory responses are connected to cancer in 15-20% of total cases (Balkwill and Mantovani 2001). There are many triggers of chronic inflammation that increase the risk of developing cancer; these include microbial infections (for example *H.Pylori* is associated with gastric cancer and gastric mucosal lymphoma; Mantovani et al. 2008), autoimmune diseases (for example the association between inflammatory bowel disease and colon cancer, Mantovani et al.2008). Hallmarks of cancer-related inflammation include the presence of inflammatory cells and mediators in tumor tissues, tissue remodeling and

angiogenesis similar to those observed in chronic inflammatory responses. Cancer and inflammation are functionally connected in two different ways (fig.3):

- inflammatory conditions activate a genetic program in cancer cells that increases the rate of neoplastic transformation (extrinsic pathway)
- genetic alterations of cancer cells (e.g. oncogene activation) drive the production of inflammatory cytokines (intrinsic pathway). Typical examples in this context are activation of the receptor tyrosine kinase *RET*, which drives the development of papillary thyroid carcinoma (Borrello et al.2005), induction of the RAS-RAF signaling pathway or of the transcription factor MYC . In all these cases, a transcriptional program similar to the one occurring during inflammation is induced, with the production of tumor-promoting inflammatory chemokines and cytokines (Sparmann et al.2004 ; Sumimoto et al. 2006; Shchors et al.2006).

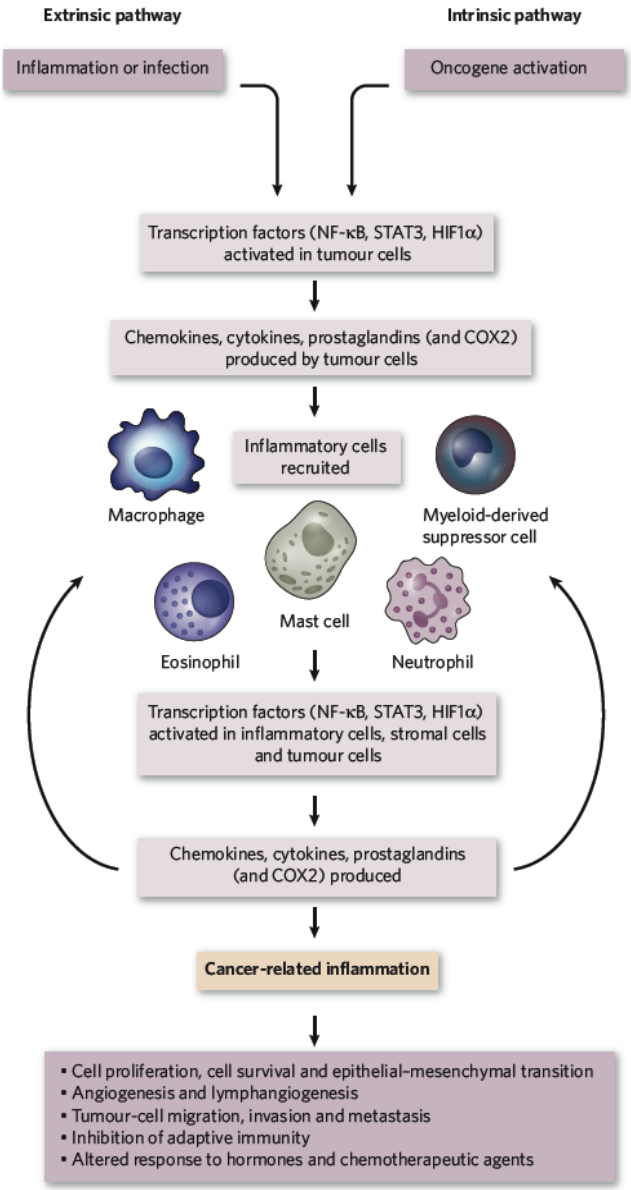


Figure 3. Pathways that connect inflammation and cancer

Cancer and inflammation are connected by two pathways: the intrinsic one activated by genetic events and the extrinsic one due to infectious conditions and inflammation. Both pathways converge in the activation of transcription factors (NF-kB, STAT3, HIF1α) that coordinate the production of chemokines and cytokines. (from Mantovani et al. Nat Rev Canc. 2008)

Tumor suppressor proteins (e.g. VHL, TGFβ and the phosphatase PTEN) can also regulate the production of inflammatory mediators .

Both intrinsic and extrinsic pathways converge on the activation of endogenous transcription factors, which elicit cytokine synthesis. Inflammatory pathways are also involved in migration, invasiveness and ectopic survival. As an example, the

chemokine receptor CXCR4 is expressed in tumors, and its expression correlates with the metastasis of colorectal, breast, liver and oesophageal cancer.

1.1.8 Metabolic changes

The link between cancer and altered metabolism is not new, as many observations made during the early period of cancer biology research identified metabolic changes as a common feature of cancerous tissues. One of these features is the prevalent use of glycolysis as source of energy also in presence of abundant oxygen. This phenomenon was discovered by Otto Warburg at the beginning of 20^{th} century (Warburg ,1956) and cancer glucose avidity is currently used in clinical practice to diagnostic tumor presence through PET technique (positron electron tomography). In cancer cells were also present other metabolic alterations, and all will be discussed in paragraph 1.3.

1.2 Oncogenes, Tumor suppressor genes and signaling

All eukaryotic cells coordinate their growth with the availability of nutrients in their environment. Signaling transduction is the process by which an extracellular signaling molecule activates a membrane receptor that in turn alters intracellular molecules to create a response. Signaling cascades are involved in tumorigenesis because through coordinated phosphorylation of proteins they directly regulate proteins synthesis, cell cycle, metabolism, transcription factors (Hunter, 2000). Three decades of basic cancer research have revealed that virtually every type of cancer cell display mutations in components molecular pathways that transduce growth factor-derived signals. Most human tumors harbour activating oncogenic mutations on master regulators of these cascades(for example several receptor tyrosine kinases, the Ras proteins, P(I)3K, Raf, etc.) or inactivating mutations in tumor suppressor genes that act as negative regulators in these pathways (for example phosphatase and tensin homolog PTEN and neurofibromin; Beder et al.2005; Shaw and Cantley,2006). Notably, these biochemical axes form an

intersecting biochemical network; as a consequence, each mutation has multiple consequences that affect cell growth, proliferation, motility and survival, rendering each of these processes unrestricted by environmental cues (Shaw and Cantley, 2006). These evidences have made components of these signaling cascades attractive targets for selective therapeutic interventions (Knight and Shokat 2010; Zhang et al. 2009). The main classes of signaling molecules are:

- *Growth Factor receptors* ;
- *Cytosolic Serine/Threonine or Tyrosine Kinases*;
- *GTPases;*
- *Phosphatases;*
- *Adaptor, chaperone and scaffold proteins;*

1.2.1. RTKs

RTKs are a class of approximately 60 molecules divided into 20 subfamilies, able to transfer phosphate group from ATP to the hydroxyl group of tyrosine residues on signal transduction molecules (fig.4).

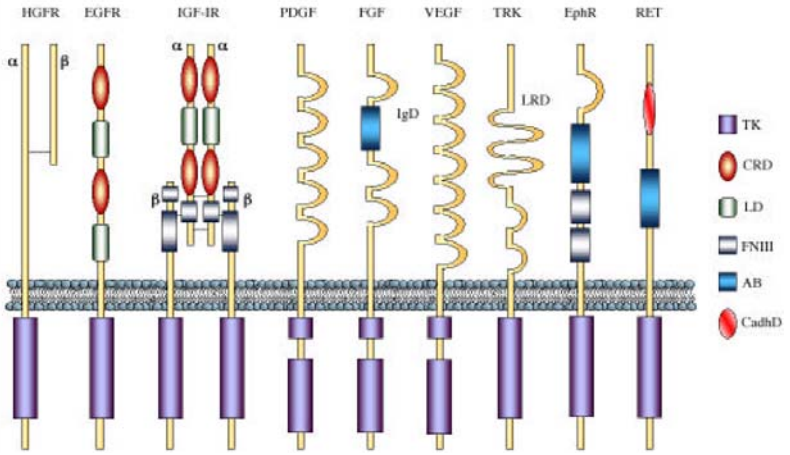


Figure 4. Schematic structure of the main human RTK families. Abbreviations are: TK, tyrosine kinase domain; CRD, cystein-richdomain; LD, leucine domain; FNIII, fibronectin type III-like domain; AB, acidic box; CadhD; cadherin-like domain; LRD, leucine-richdomain; IgD, immunoglubulin-like domain (from Fassetta et al.2004)

They are trans-membrane proteins characterized by a common structure: all of them display an extracellular domain (usually glycosilated) connected through a

single trans-membrane alpha helix to the cytoplasmatic portion. The extracellular domain contains the binding sites for growth factors, while in the cytoplasmic region are located the tyrosine kinase core and the regulatory sequences and the interaction domains. RTKs transduce pro survival signals from the extracellular environment to cytoplasm, modulating gene expression. Binding to GFs induces receptor dimerization and leads to trans-phosphorylation of receptor on tyrosine residues: this autophosphorylation is a key event because it completely activates the kinase domain and favor recruitment of scaffold proteins and downstream enzymes that express interacting SH2 or PTB domains . Among the main downstream targets activated by RTKs are Ras, MAPK, P(I)3K and STATs which orchestrate anabolic responses.. As a consequence, deregulated activation of RTKs transform these receptors in potent *oncoproteins*. Deregulations of RTKs can be caused by different modifications, for example receptor over-expression or activating mutation of either ligand or receptor (fig.5) (Blume-Jensen et al. 2001; De bacco et al.2004; McKay et al.2007).

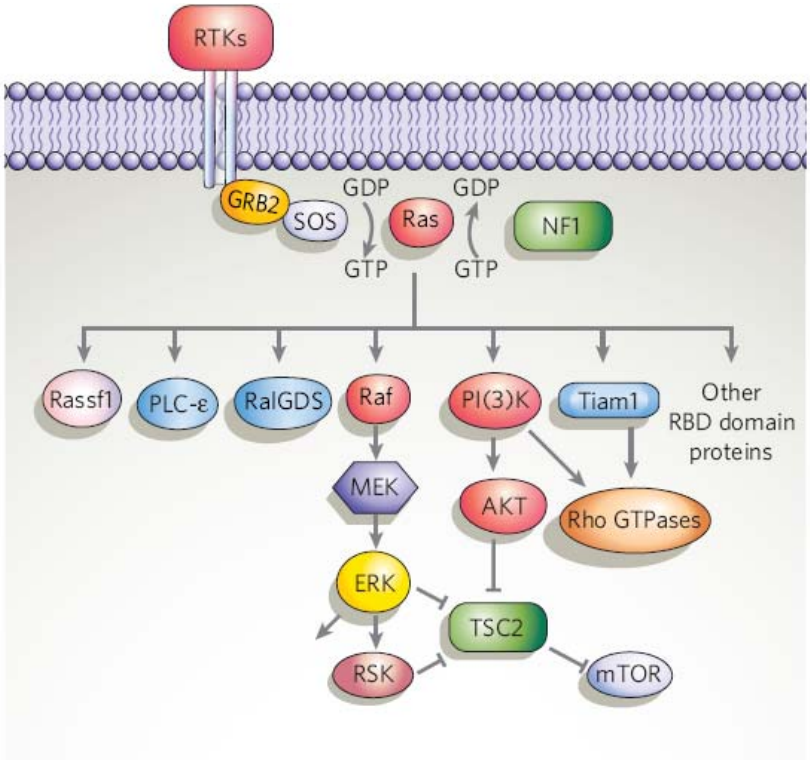


Figure 4. RTKs and downstream signals

Activation of RTKs on plasma membrane switches on several signaling pathways (from Shaw et al. Nature 2006).

1.2.2 Ras

Ras GTPases control different cascades. Although initially thought to occur mainly on the plasma membrane, there is increasing evidence that isoform specific RAS take place at different cellular compartments and within different region of plasma membrane (Hancock et al.2003; Buday and Downward 2008; Schubbert et al. 2007; Karnoub and Weinberg 2008). Activating mutations in *K-Ras* and *N-Ras* occur in varying frequencies in different types of cancer. These mutations, found at codon 12,13,or 61, prevent efficient GTP hydrolysis, rendering Ras in a active GTP-bound state (Dhillon et al.2007).

1.2.3 MAPK (Mitogen-activated protein kinase pathway in cancer

MAPKs are evolutionary conserved kinases that link extracellular signals to the biochemical machinery that controls cell proliferation and death.
These evidences validate the importance of this signaling cascade in neoplastic transformation. Signaling to MAPKs is composed of three tier kinase modules: a MAPK is activated following sequential activating phosphorylations: a G protein-regulated MAPKKK (mitogen activated protein kinase kinase kinase) phosphorylates a MAPKK, which in turn activates a MAPK (Dhillon et al.2007) (fig.5).

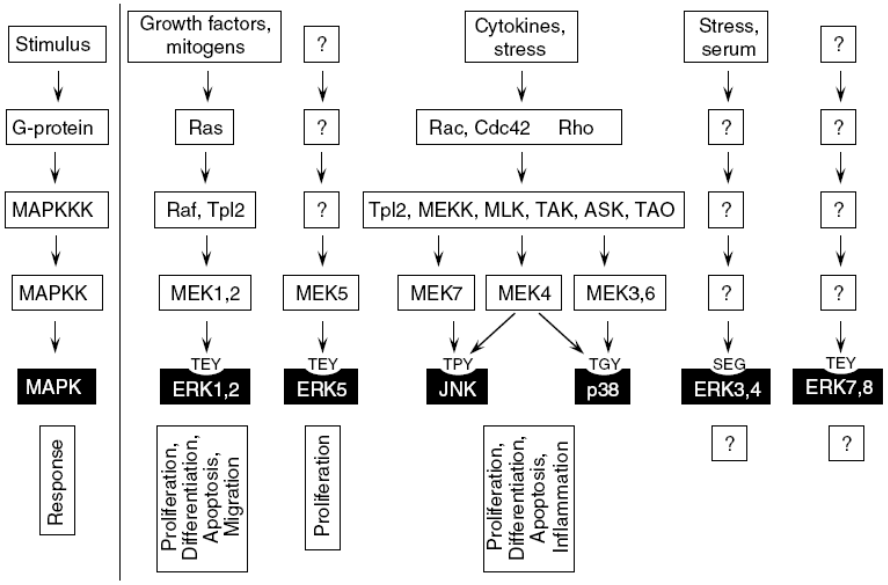


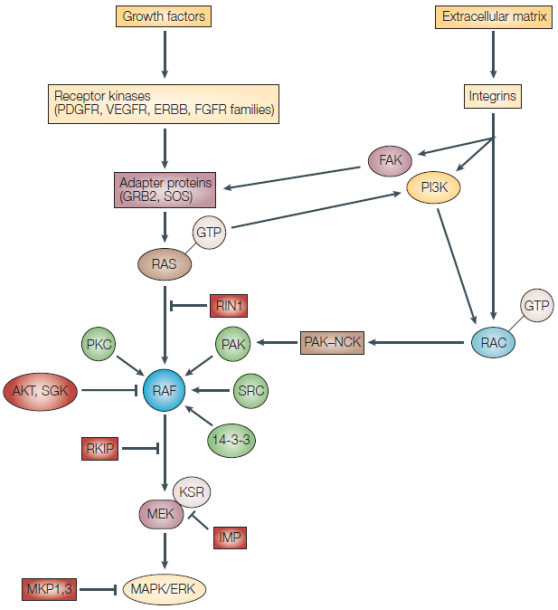
Figure 5. **Schematic overview of MAPK pathways** (from Dhillon et al. Oncogene 2007).

These cascades are activated not only by RTKs receptors but also by stress conditions and cytokines. We can identify two main modules:

1) *ERK pathway*

ERK signaling is a fundamental inducer of cell proliferation, motility and survival, but, as for most transduction cascades, it is involved in a variety of signals, including response to stresses, depending on the cellular and environmental context (Dhillon et al.2007). In the ERK cascade, ligand-mediated activation of RTKs triggers guanosine triphosphate (GTP) loading of the RAS GTPase which can then recruit Raf kinases to the plasma membrane. The final effectors are *ERK1/2* that are activated upon phosphorylations by MEK (*MEK1/2*) which is itself activated when phosphorylated by *Raf-1, Raf-B and Raf-A*. (fig 6.)

A.



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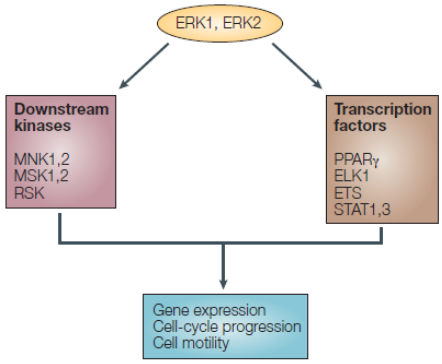


Figure 6. **Schematic overview of ERK pathways (A) and targets of activated ERK1/2(B)** (from Sebolt-Leopold JS et al. Nat Rev Cancer 2004).

ERK signaling is switched on by numerous extracellular signals ,mainly mitogens that are essential for neoplastic progression. ERK signaling deregulation is observed in the majority (one third are Ras dependent) of human cancers. Most cancer associated lesions that constitutively activate this module occur at the level of RTKs, RAS or RAF, whose activating mutations are oncogenic. However, there is also the possibility of amplifications or deregulations of ERK targets, mainly transcription factors, or of inactivation of negative regulators, such as

neurofibromin (Larizza et al.2009), a Ras-GAP (GTPase activating protein) that inactivates Ras (Dhillon et al.2007).

Recently, some articles were published in which an extra-nuclear activity of ERK that can be crucial for malignancy was identified. Interstingly, ERK was found in mitochondria, the organelles which the apoptotic machinery is regulated in (Galli et al.2009). The Raf kinase is direct effector of Ras that is present at the apex of ERK pathway kinase module. The three Raf proteins (Raf-A, Raf-1 and Raf-B) are similar from the structural point of view but they differ considerably in their modes of regulations, tissue distribution and abilities to activate MEK. Once bound to to Ras, Raf kinases are activated by a complex sequence of events involving not only phosphorylations but also protein- protein and protein-lipid interactions that neutralize auto-inhibition and favor the activation of kinase domain. Several mutations were found in the Raf gene: one of these is an activating mutation in one of the Raf isoform, B-Raf, have been found in a large (more than 60%) fraction of human malignant melanomas, and in some colon, lung and thyroid tumors. (Shaw and Cantley, 2006; Wan et al.2004).

2) *Stress activated MAPK pathways*

Many MAP kinases also participate in stress signaling, with a different relative importance depending on the context. Indeed, in the majority of cases environmental stimuli induce a wide array of MAPK responses, and the integration among all signals determine the biological response (Fassetta et al, 2006). Stress conditions are important for tumor growth, and cancer cells are always exposed to various stress conditions including hypoxia, matrix detachment, inflammation, metabolite deprivation, reactive oxygen and nitrogen species (ROS and NOS), dysregulations of energy production and moreover genotoxic and pharmacological stress caused by chemotherapy and radiotherapy. Stress MAPK kinases are anti-proliferative and pro-apoptotic proteins, and therefore they display an anti-neoplastic function; however, in some case they can contribute to tumorigenesis (Dhillon et al.2007).

The most important stress kinases are *JNK and p38.*

The *JNK* family of proteins is predominantly activated by cytokines, UV radiations, growth factor deprivation, DNA-damaging agents, some G-protein coupled receptors and serum (Weston and Devis 2002). Three genes, Jnk1, JnK2 and Jnk3, encode ten JNK proteins obtained through alternative splicing; however, only Jnk1 and jnk2 are ubiquitously expressed. When phosphorylated, JNK kinases activate transcription factor *c-Jun* through phosphorylation in Serine 63 and 73, leading to actvation of a wide sprectrum of transcription factors such as AP-1, ATF-2, HSF-1 and STAT3 (Ip and Davis 1998). JNK is also able to bind and phosphorylate *p53*, which is one of the main tumor suppressor proteins, acting as a molecular guardian of genome integrity (Vousden and Lane 2007). JNK increases p53 transcriptional activity and stability. Nevertheless, with respect to cancer JNK is a double face kinase: it has been reported that, in Ras-induced tumorigenesis, its main target c-Jun can cooperate in cellular transformation (Kennedy et al.2003): leading to a potent transcriptional repression of the p53 gene (Eferl et al.2003). For all these reasons JNK inhibitors for cancer treatment has an unclear usefulness and are not very common in therapy.

In mammals *p38* isoforms are strongly activated by environmental stresses and inflammatory cytokines, and are required for TNFα and interleukin-1 expression during inflammatory responses. The four isoforms of p38, α,β,γ and δ, are both characterized by the presence of conserved *Thr-Gly-Tyr* phosphorylation motif in their activation loop, that are necessary for activation sustained by MEK3 and MEK6, which are themselves activated by stresses. Effectors of p38 tumor suppressor activity include p53 and a more general role of apoptosis induction and cell cycle inhibition. in several tumors, e.g. hepatocellular carcinomas, p38 expression level are reduced in comparison to adjacent normal tissue (Dhillon et al.2007; Iyoda et al.2003).

1.2.4 PI3K/Akt pathway

The *P(I)3K* pathway has been implicated in cancer since its discovery associated with viral oncoproteins 20 years ago (Shaw and Cantley 2006), but it has become apparent later that is one of the most frequently targeted pathways in all sporadic

human tumors, with mutations in one or in another cascade component that accounts for up to 30% of all human cancers. P(I)3K can be activated both by RTKs and by Ras (fig.7); it follows that a strict cross-talk exists between the PI3K and the ERK pathways. PI3K in turn activates several downstream signaling molecules. PI3K enzymatic activity is through the generation of the lipid phosphatydil-inositol-3-4-5-triphosphate (PtdIns(3,4,5)P_3), which acts as a second messenger. P(I)3K is composed by a 85 kDa regulatory (p85α) and a 110 kDa (p110α) catalytic subunit. There are three classes of P(I)3Ks: *Class* I_A P is activated by RTKs on the plasma membrane through the interaction between phospgo-tyrosine residues on the receptor and Src homology domains (SH2) of the p85α subunit. In *Class* I_B the kinase is primarily activated by small G-proteins such as Ras and by β/γ subunits of G-protein coupled receptors. In both cases kinase activation leads to generation of D3 phosphorylated- lipid products. The third class , *Class I,* owns not only lipid kinase activity but also exerts serine/threonine kinase activity on protein substrates (Franke 2008).

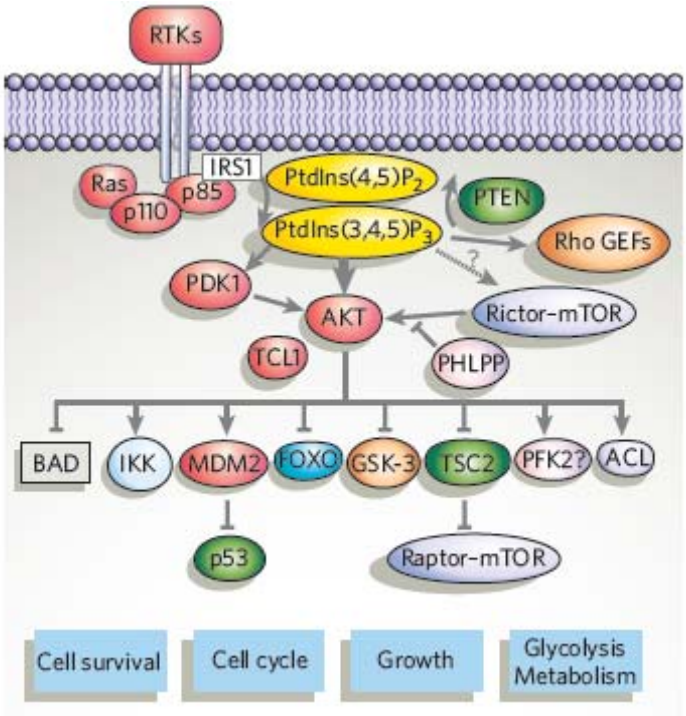


Figure 7. P(I)3K/Akt network: Akt controls cell survival, cycle, growth and metabolism (from Shaw et al. Nature 2006).

The main cascade activated by P(I)3K is the *AKT pathway*, which displays a pivotal role in the development of malignancies (fig.7). The *Akt* gene is the cellular homolog of the v-akt oncogene transduced by AKT8,an acute transforming retrovirus in mice that was originally described in 1977 and cloned in 1991 (Franke 2008). Structurally, all Akt isoforms share N-terminal regulatory domain including a pleckstrin homology domain (PH), a region connecting the PH domain with serine/threonine specificity and a C-terminal region necessary for induction and maintenance of its kinase activity. All isoforms are activated after binding between D3 phosphorylated phosphoinositide products of P(I)3K and PH domain (Franke 2008). In mammalian cells we can found three isoforms: *Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBγ)* : Akt1 is ubiquitously expressed, while Akt2 is highly expressed in insulin sensitive tissues (muscle, liver and adipose tissue) and Akt3 in brain and testis . Only Akt2 and Akt3 seemto be highly expressed in cancers, where they control at least four different intersecting biological processes, cell survival, cell-cycle progression, growth and metabolism, through phosphorylation of several substrates (fig. 7).

Amplifications of genes encoding subunits of P(I)3K and AKT2 have been observed in a variety of tumor types, for instance in ovarian, breast and pancreatic cancers but also point mutations on other components of the pathway, for example in 3-phosphoinositide-dependent protein kinase 1 (PDK1), in PTEN phosphatase and TCL1 (oncogene from T cells leukaemias), frequent in colon carcinomas (Shaw and Cantley 2006).

-*Glycogen synthase kinase-3 (GSK3)* is a highly conserved protein kinase that controls a number of critical cell events. This enzyme has several intriguing regulatory characteristics, it is centrally involved in regulating cellular structure, function and survival, and it is linked to several prevalent diseases, such as diabetes and Alzheimer's disease. Akt is able to phosphorylate GSK3 inhibiting it's activity (Jope and Johnson 2004).

- *Chk1 protein kinase*, that is implicated in a radiation damage check point, and *MDM2* that is a human oncogene product that degrades p53 tumor suppressor are activated by this kinase;

-*BAD* pro apoptotic protein that is inactivated by Akt;

-*FOXO* family of forkhead transcription factors that are inactivated and sequestrated in cytoplasm after Akt phosphorylation. (FOXO proteins control pro-apoptotic and stress-response genes expression);
-*TSC2* tumor suppression protein, tuberin, that blocks mTOR activity is inactivated by Akt;
-*Phosphofructokinase 2 (PFK2)* a glycolitic enzyme is activated by Akt, stimulating glycolysis;
-*ATP citrate lyase* (ACL), activated by Akt, impinge upon fatty acid synthesis ;
-*HIF-1*α (Hypoxia inducible factor 1 α), is activated by this kinase, stimulating glucose metabolism.
All these pathway controlled by Akt make this kinase one of the first target for anti-cancer therapy.

1.2.5 mTOR signaling

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that forms two distinct multimeric complexes, *mTORC1* and *mTORC2*. mTORC1 controls cell growth in response to nutrient and growth factor availability, while mTORC2 is thought to mediate cell proliferation and cell survival. AKT is able to activate mTORC1, phosphorylating and inhibiting the TSC1/TSC2 negative regulatory complex (fig.8). Once active, mTORC1 promotes cell growth by directly phosphorylating the regulators of translation *S6K1* and *4E-BP1* and also by inhibiting autophagy and facilitating lipid and mitochondrial biogenesis. Linking m-TORC1 regulation to oncogenic PI3K/AKT activity provides a strong rationale for targeting mTORC1 in cancer and for using rapamycin as a selective chemotherapeutic in clinical trials. Unfortunately, the effectiveness of rapamycin as a single anticancer agent is stifled in part by strong mTORC1 dependent negative feedback loops that become inactive during mTORC1 inhibition by rapamycin (Franke 2008). The importance of this pathway is evident looking at the different signaling cascades that intersect it: in fact, mTORC1 can crosstalk

with ERK1/2, that increase its activity through inhibitory phosphorylation of the negative regulator TSC2; moreover, mTORC1 activity is strictly controlled by availability of nutrients (such as glucose, oxygen and aminoacids). In this context, an important mTORC1 inhibitor is the Ser/Thr kinase AMP-activated protein kinase (*AMPK)*. When intracellular ATP levels drop and consequently AMP levels rise, as in the conditions of hypoxia or glucose deprivation encountered by the growing primary tumor mass, AMP directly binds to AMPK, causing the exposure of a threonine residue in the activation loop that is activated by phosphorylation mediated by the LKB1 kinase. ActiveAMPK inhibits mTORC1 both by direct phosphorylation and by inhibition of the TSC2 protein (Shaw and Cantley 2006).Unlike mTORC1, mTORC2 is not effectively inhibited of rapamycin .The main target identified for mTORC2 is AKT, that can be activated by phosphorylation on a critical regulatory site (fig.8). In this context, anti-cancer research is developing new tailored inhibitors, and a class of them, prototype mTOR kinase inhibitors (TKIs) are just now becoming available for research (Sparks and Guertin 2010).

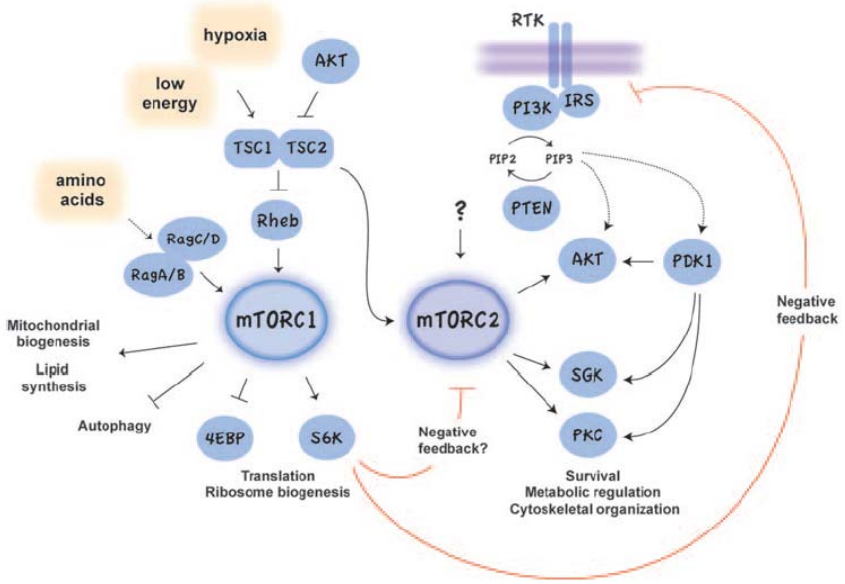


Figure 8. mTORC1 and mTORC2 signaling branches. mTOR is the catalytic core of two complexes called mTORC1 and mTORC2. mTORC1 is sensitive to growth factors, hypoxia, low energy and aminoacids. mTORC1 control protein synthesis through 4E-BPI and S6K1, mitochondrial and lipid

biogenesis, blocks autophagy. mTORC2 phosphorylates some important kinases, PKC,SGK and AKT (from Sparks et al. Oncogene 2010) .

1.3 Cancer cell metabolism

Cancer development involves major alterations in cell metabolism. Over the past decades, accumulating evidence of a metabolic reorganization during cancer progression has emerged from studies on various tumor types. Enhanced *glycolysis*, increased *de novo fatty acid synthesis* and *glutamine* dependence are now considered three metabolic traits shared by malignancies (REF). Non-proliferating cells from differentiated tissue predominantly use catabolic metabolism and mitochondrial oxidative phosphorylation (OXPHOS)to fuel their energetic needs . In sharp contrast, in tissue with high proliferative rate and in cancer, metabolicpathways are reorganized to allow cell-cycle progression, leading to a shift toward an anabolic metabolism, endowed with a high glucose utilization, lactate production and biosynthesis of macromolecules (fig. 9)(Frits and Fajas 2010).

1.3.1 Glycolysis in cancer

Early in the last century (Warburg et al.1927), Warburg observed that tumor cells have a higher rate of glucose metabolism than their normal counterparts and boost glycolysis even under normoxic conditions (Vander Heiden et al.2009;Warburg 1956). Today, induction of aerobic glycolysis in tumor cells is called the Warburg effect. Warburg hypothesized that tumor cells develop a defect in mitochondria that leads to impaired aerobic respiration and a subsequent reliance on glycolytic metabolism (Warburg, 1956). However, subsequent works showed that mitochondrial function is not impaired in most cancer cells, suggesting that the shift towards glycolysis during neoplastic transformation occurs as a consequence of regulatory mechanisms.

We still do not understand exactly what is the advantage obtained by tumor cells in moving their metabolic demand towards glucose usage. The current model postulates that the preferential use of aerobic glycolysis offers the following advantages to highly proliferative cells. First, it focuses cells on the use of glucose, which is the most abundant extracellular nutrient.; second, the flux of ATP derived from glycolysis can exceed the one produced during OXPHOS, despite the low efficiency of glycolysis, when glucose is provided at a sufficiently high concentration; third, glucose utilization provides essential metabolic intermediates for the biosynthesis of diverse macromolecules (lipids, proteins and nucleic acids) and for anti-oxidative defenses (Vander Heiden et al.2009).

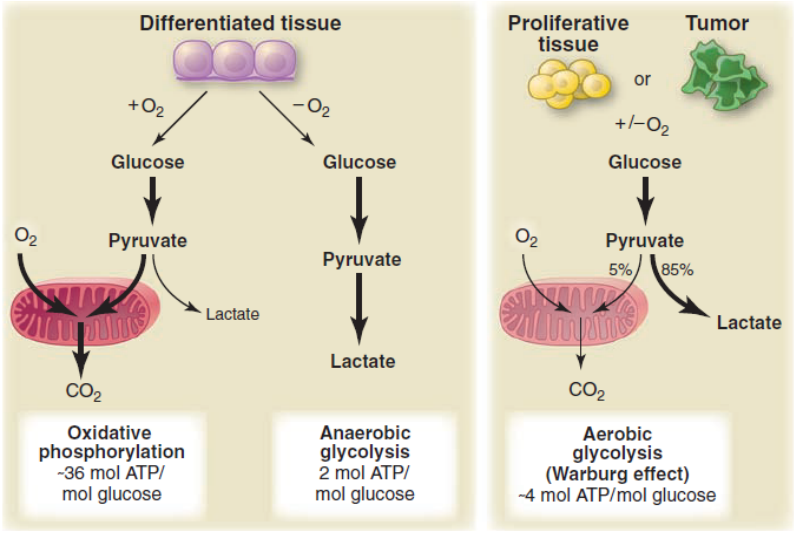


Figure 9. Schematic difference between oxidative phosphorylation, anaerobic glycolysis and aerobic glycolysis (Warburg Effect). (from Vander Heiden et al. Science 2009)

The molecular mechanisms responsible for this shift are under intense scrutiny, and their unraveling will allow to understand how changes in the metabolic profile can provide tumors with a growth advantage (Denko 2008). There are two main mechanisms explored by researchers:

1) *Mutations that alter metabolism.*Oncogenic mutations, both in oncogenes and in tumor suppressor genes can directly influence metabolism. For example, Ras and Akt mutations increase glycolytic flux at different levels (Hsu and Sabatini

2008), whereas p53 inhibits glycolysis by inducing the expression of TIGAR, which decreases fructose 2-6-biphosphate levels (Hsu and Sabatini 2008; anche Cairns et al. 2011).

2) *Environmental stresses. A variety of signals reaching the tumor from the surrounding environment can modulate the metabolism of the neoplastic cell; these signals include hypoxia, acidosis and interstitial pressure.* Hypoxia is perhaps the most pervasive between cellular stresses, and occurs when O_2 delivery does not meet the demand of a certain tissue. Tumour cells respond to hypoxic conditions mainly by inducing the *hypoxia-inducible factor 1 (HIF1) transcription factor*. HIF1 was initially identified because of its response to low O_2 concentration. It is now apparent that HIF1 activation is one of the main switches that prompt tumor progression, as it induces epithelial mesenchymal transition and angiogenesis in addition to metabolic changes (Denko 2008), and can be regulated even under normoxic conditions by a variety of other factors such as oncogenes such as Ras, SRC and PI3K, or loss of tumour suppressors such as VHL (von Hippel–Lindau) or PTEN phosphatase. Furthermore, increased levels of metabolites such as succinate and fumarate (King et al.2006), or O_2 by-products such as free radicals, can also stabilize HIF1α. Activation of the HIF1 transcriptional program has two major effects on metabolism: first it stimulates glycolysis, promoting glucose uptake by induction of the glucose transporters GLUTs and the expression of several enzymes of the glycolytic chain (for example hexokinase, phosphofructokinase 1 and aldolase); second, HIF1 down-regulates oxidative phosphorylation, transactivating pyruvate dehydrogenase kinase 1 (PDK1) that inhibits the entry of pyruvate into the Krebs cycle through phosphorylation of pyruvate dehydrogenase (fig 10) (Denko 2008).

Product(s) of HIF1 target gene(s)	Metabolic function
Glucose transporters GLUT1 and GLUT3	Glucose entry into the cell
HK2	Phosphorylation of glucose
PGI, PFK1, aldolase, TPI, GAPDH, PGK, PGM, enolase, PK, PFKFB1–4	Glycolysis
LDHA	Conversion of pyruvate to lactate
MCT4	Removal of lactate from the cell
PDK1, MXI1	Decreased mitochondrial activity
COX4I2, LON protease	Increased O_2 consumption in hypoxia

Figure 10. HIF targets that regulate glucose metabolism

COX4I2, cytochrome oxidase isoform 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT,glucose transporter; HIF, hypoxia-inducible factor; HK, hexokinase; LDHA, lactate dehydrogenase A; McT, monocarboxylate trasporter; MXI, max interactor; PDK, pyruvate dehydrogenase kinase; PFK, phosphofructokinase; PFKFB, 6-phospho-2-kinase/fructose 2,6 bisphosphatase; PGK, phosphoglycerate kinase; PGI, phosphoglucose isomerase; PGM, phosphoglycerate mutase; PK, pyruvate kinase; TPI, triosephosphate isomerase. (from Denko et al. Nat Rev Cancer 2008).

Glycolysis can also be enhanced through inhibition of the functional assembly of respiratory chain complexes. A recent model proposes that respiratory complexes are organized in functional super-complexes, for example aggregates of Complex I-III-IV, which form the so-called "*respirasome*" (Acin-Perez et al.2008). The correct supra-complex assembly would be a crucial regulatory event in obtaining a fully functional respiratory chain. Recently it was proposed that OXPHOS impairment in cancer cells can be due to a defective assembly of these super-complexes, whose disassembly could favor the switch towards glucose metabolism (Baracca et al.2010).

1.3.2 De-novo fatty acid synthesis

One of the main metabolic characteristics of neoplastic cells is the increase in *de-novo fatty acid* (FA) *synthesis*. Although this process is very active during embryogenesis, most adult normal cells and tissues, even those with high cellular turnover, preferentially use circulating FA for the synthesis of new structural lipids. In contrast to this, various tumors undergo exacerbated endogenous fatty acid synthesis, irrespective of the levels of extracellular lipids. De novo FA

biosynthesis is required for cancer cells to synthesize new membranes and lipid rafts for increased signaling, and lipid synthesis intermediates, such as malonyl-CoA, participate in the transcriptional regulation of growth factor receptors. De novo FA synthesis also facilitates the production of lipids that regulate the activity of various oncogenes. Indeed phosphatidylinositol, phosphatidylserine or phosphatidylcholine contribute in the activation of proliferative and survival pathways, notably PI3K/AKT, Ras or Wnt cascades (Fritz and Fajas 2010). The increased lipogenesis in cancer is caused by over-expression and/or hyperactivity of lipogenic enzymes such as ATP citrate lyase (ACL),acetyl-CoA carboxylase (ACC) or the fatty acid synthase (FAS). ACC carboxylates acetyl-CoA to form malonyl-CoA, which is further converted to long-chain FA by FAS. Interestingly, studies with chemical inhibitors have revealed that inhibition of FAS activity results in decreased proliferation and increased apoptosis of cancer cells (Fritz and Fajas 2010).

1.3.3. Glutamine metabolism

The third main feature of tumor metabolism is the increased dependence on *glutamine* for growth and proliferation. Essential functions of glutamine include its conversion to glutamate as a metabolic intermediate to be channeled into TCA cycle and its usage as a precursor in the biosynthesis of nucleic acid, certain amino-acids and gluthatione.(Lu et al.2010).

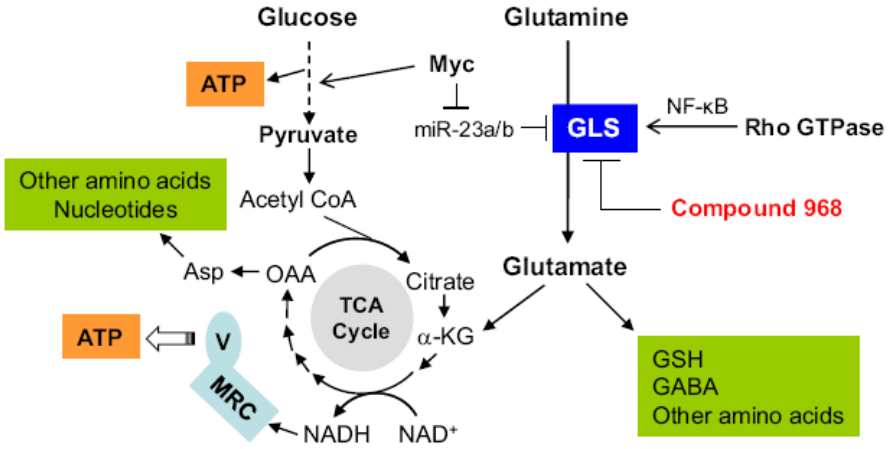


Figure 11. Pathways of glutamine metabolism. The interconnection between glutamine metabolism and glucose metabolism is highlighted. Inhibition of glutaminase by compound 968 suppresses oncogenic transformation induced by Rho GTPases. GLS, glutaminase; TCA cycle, tricarboxylic acid cycle; MRC, mitochondrial respiratory chain; V, mitochondrial respiratory complex V; OAA, oxaloacetate; Asp, aspartate; a-KG, a-ketoglutarate, miR=micro RNA (from Lu et al. Cancer cell preview 2010)

The mitochondrial enzyme glutaminase (*GLS*) catalyzes the conversion of glutamine to glutamate. Increased expression of GLS is often observed in tumor and in rapidly dividing cells: a recent study demonstrates that GLS expression is enhanced both by Rho GTPases in a NF-κB-dependent manner (Wang et al.2010) and by c-Myc (Gao et al.),thus providing molecular links between glutamine metabolism and oncogenic signals (fig.11). .

1.4 Tumors and apoptosis

1.4.1 Cell death machinery

Cancer cells are characterized by the development of a roboust anti-apoptotic platform. Apoptosis is the most common and well-defined form of programmed cell death (PCD), which is a physiological 'cell-suicide' program essential for

embryonic development, immune-system function and the maintenance of tissue homeostasis in multicellular organisms (Chiara and Rasola 2007).

Apoptosis is a tightly regulated multi-step process: it is characterized by cell shrinkage, loss of membrane lipid asymmetry, chromatin condensation, and nuclear and cell fragmentation. These features result in the formation of apoptotic bodies that are then engulfed by neighboring phagocytic cells, which bind to the membrane lipid phosphatidylserine on the cell surface.

Dysregulation of apoptosis has been implicated in numerous pathological conditions, including neurodegenerative diseases, autoimmunity and cancer. In mammalian cells apoptosis effectors are a family of cysteine proteases known as *caspases*. To keep the apoptotic program under control, caspases are initially expressed in cells as inactive pro-caspase precursors. When *initiator caspases* (such as caspase-8 and caspase-9) are activated by oligomerization, they cleave the precursor forms of *effector caspases*, such as caspase-3, caspase-6 and caspase-7 . Once activated, effector caspases in turn cleave a specific set of cellular substrates, resulting in a constellation of biochemical and morphological changes that are associated with the apoptotic phenotype. There are two pathways by which caspase activation is triggered :

1) *The extrinsic apoptotic pathway*
2) *The intrinsic apoptotic pathway.*

The *extrinsic pathway* is switched on by the engagement of death receptors on the cell surface. Binding of ligands such as FASL and tumour necrosis factor (TNF) to FAS and the TNF receptor (TNFR), respectively, induces the formation of the death-induced signaling complex (DISC).This complex recruits caspase-8 and promotes the activation of pro-caspase that follows (caspases 3-6-7)

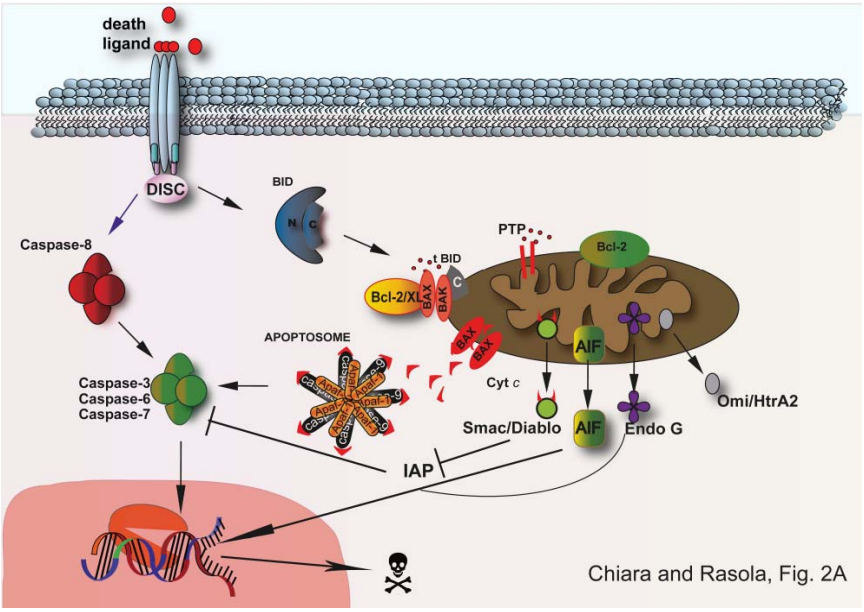


Figure 12. The extrinsic apoptosis pathway (from Chiara and Rasola, 2007)

This pathway can also converge on mitochondria through activation of a protein of BCL-2 family (see below), BID, that once cleaved, induces mitochondrial outer membrane (OMM) permeabilization with subsequent release of pro-apoptotic proteins (fig.12).

The *intrinsic pathway* is triggered by various extracellular and intracellular stresses, such as growth-factor withdrawal, hypoxia, DNA damage and oncogene induction. Signals that are transduced in response to these stresses converge on mitochondria, inducingpermeabilization of the OMM, release of cytochrome *c* and other pro-apoptotic molecules, and the ensuing formation of the *apoptosome*. Apoptosome isa large protein complex that contains cytochrome *c,* apoptotic protease activating factor 1 , *APAF1*, and the initiator *caspase-9* and that, similarly to the DISC,leads to a full-blown caspase activation. Cell death is regulated at various levels by several proteins. Among proteins released by mitochondria, endonuclease G and apoptosis-inducing factor (AIF) and might induce cell death independently of caspase activation; SMAC/DIABLO and OMI/HtrA2 promote caspase activation by counteracting inhibitor of apoptosis (IAP)-mediated caspase inhibition (Okada and Mak, 2004) (fig.12).

Mitochondrial membrane permeabilization is regulated by members of the *BCL2* family of proteins.

These are the master switches that decide whether a cell is committed to survive or must be primed for apoptosis.. Functionally, they can be subdivided in three main classes: a subset of these molecules (Bcl-2, Bcl-X_L, Bcl-w, A1 and Mcl-1) is characterized by four BH domains (BH1/4) and by a hydrophobic tail that allows membrane insertion. These proteins are localized on the OMM and on other intracellular membranes and display a central anti-apoptotic function by contributing to the mitochondrial electrochemical homeostasis. Conversely, other Bcl-2 family proteins are pro-apoptotic. They can be subdivided in "multi-BH domain" molecules (Bax, Bak, Bok), which share three BH domains (BH1/3) and a hydrophobic tail, and in "BH3-only" protiens, whose sequence homology is restricted to the short BH3 region. Bax/Bak are the central effectors of apoptosis induction. Once activated, they form oligomers on the OMM and cause its permeabilization .As a result, mitochondria depolarize and release apoptogenic proteins. BH3-only proteins are sentinels of stress insults in the cytosol and in other subcellular districts and are recruited by specific cytotoxic signals onto mitochondria, where they act as death inducers engaging Bax/Bak and/or inhibiting anti-apoptotic Bcl-2 family proteins (Chiara and Rasola 2007). In the last years there were developed several anti neoplastic drugs that act on Bcl-2 proteins. This class of compounds are called BH3-mimetics and promote cell death blocking pro-survival Bcl-2 proteins (Milanesi et al.2006). Therefore, mitochondria are obligate participants in both intrinsic- and extrinsic-mediated apoptosis. When a stress stimulus tips the death/survival balance towards a lethal outcome, this leads to mitochondrial depolarization, swelling and release of apoptogenic proteins. The mechanism is not so clear, one possibility is the opening of mitochondrial Permeability Transition Pore (PTP).

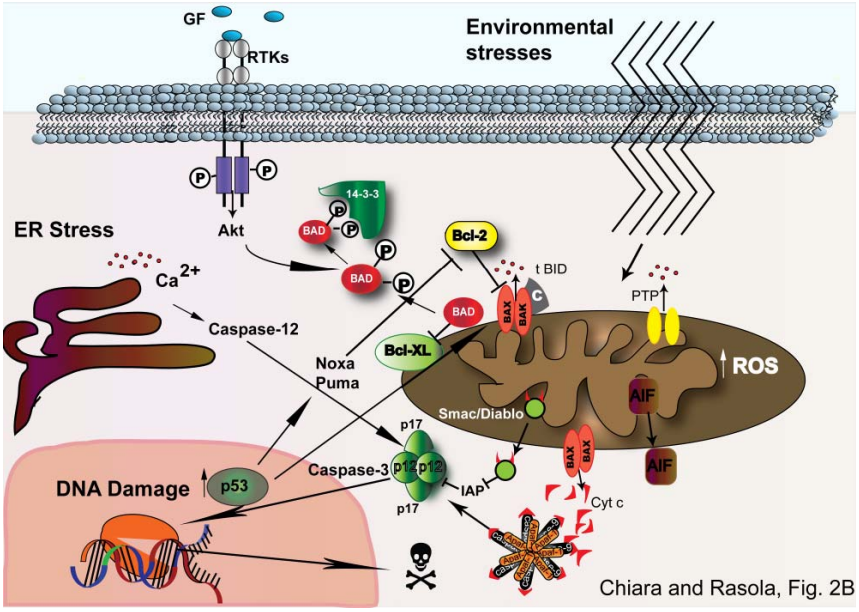


Figure 12. The intrinsic apoptosis pathway (from Chiara and Rasola, 2007)

1.4.2 The mitochondrial Permeability Transition Pore (PTP)

The *Permeability Transition Pore* (PTP) is a voltage- and Ca^{2+}-dependent, cyclosporine A (CsA) sensitive, high conductance channel , located in the inner mitochondrial membrane (IMM). The Permeability Transition is a sudden increase of IMM permeability to solutes with a molecular mass up to 1500 Da, due to the opening of this channel. The PTP in its fully open state reaches 3nm of diameter, and it is strictly regulated by a number of effectors. The primary consequence of a prolonged PTP opening is mitochondrial depolarization due to equilibration of the proton gradient, followed by respiratory inhibition, as matrix pyridine nucleotides (PN) are lost. Equilibration across the IMM of ions and of solutes with molecular masses below the pore size induces massive release of the Ca^{2+} stored in the matrix and extensive swelling of mitochondria, caused by the colloidal osmotic pressure exerted by the high concentration of mitochondrial matrix proteins. As a consequence, the unrestricted cristae unfolding causes breaches in the OMM

and release of inter-membrane proteins (Rasola and Bernardi 2007).To summarize, the PTP opening prompts the demise of the cell, either through apoptosis (if enough ATP is present to fuel caspase activity) or through necrosis, which follows loss of Ca^{2+} homeostasis and mitochondrial depolarization, but is not regulated.

Molecular structure of the PTP

The molecular structure of the pore remains unsolved. The present consensus model postulates that the channel is originated by a supra-molecular complex. Several layers of complexity render dissection of single components a difficult task. Assemblage of PTP components might be a rare event, to avoid unwanted mitochondrial depolarization and damage. It is also possible that, in particular cell types or following specific triggering conditions, different protein multimers assemble to form the PTP, possibly resulting in diverse sensitivities to modulators or in changes of conductance properties. A subset of proteins was proposed to constitute core components of the channel, but rigorous genetic testing in the last few years has excluded this possibility for most of them, while for one of this, mitochondrial phosphate carrier (PiC) there is no rigorous genetic testing done yet. The PTP is clearly sensitized by the matrix peptidyl-prolyl cis–trans isomerase CyP-D (see also below, paragraph 1.5.3). However, the pore is still present in mitochondria and cells obtained from mice where the gene encoding CyP-D was knocked-out. CyP-D ablation increases the Ca^{2+} load required to open the PTP, unmasking inhibition by Pi, and abolishes sensitivity to the CyP-D ligand CsA, conclusively demonstrating that CyP-D is an important regulator of the PTP, but not a component of the channel.

1.4.3 PTP dysregulation in tumors

The hypothesis that PTP could have a role in cell death was already proposed nearly 20 years ago (Crompton and Costi 1988). Several PTP inhibitors (bongkrekate, CsA and its derivatives) were reported to protect from cell death both *in vitro* and *in vivo*. *In vitro*, these inhibitors abolish cytochrome *c* release and protect different cell types from apoptosis induced by glucocorticoids, neurotoxins and tumor necrosis factor α (TNFα) (Rasola and Bernardi 2007; Soriano et al.2004); *in vivo*, CsA is effective in a variety of conditions such as muscular dystrophy caused by collagen VI deficiency (Rasola and Bernardi 2007; Angelin et.al 2007).

Mitochondria are involved in more than 40 known human diseases. The effects of CsA in treatment have implicated a PTP-dependent mitochondrial dysfunction and Ca^{2+} deregulation in many of these conditions, including ischemia-reperfusion (I/R) injury of the heart (Griffith and Halestrap 1993), ischemic and traumatic brain damage, muscular dystrophy caused by collagen VI deficiency (Angelin et al.2007), amyotrophic lateral sclerosis (Keep et al. 2001), acetaminophen hepatotoxicity (Haouzi et al. 2002), hepatocarcinogenesis by 2-acetyl-aminofluorene (Klohn et al.2003), and fulminant, death receptor-induced hepatitis (Soriano et al.2004). Tumor cells are more resistant to the breakdown of the OMM following treatment with a variety of stress factors (Rasola and Bernardi 2007). Indeed, an involvement of an altered PTP opening in cancer is suggested by several pieces of evidence:

1) PTP opening can be both the cause and the consequence of increased ROS unbalance, and part of an amplification loop during apoptosis induction. Notably, inhibitors of chemotherapy-induced apoptosis include several antioxidant agents;

2) resistance to chemotherapeutics is related to a reduced release of Ca^{2+} from intracellular stores upon apoptosis induction (Chandra et al.2002);

3) cell treatment with chemotherapeutic agents reduces the interaction of the anti-apoptotic Bcl-2 proteins with mitochondria (Verrier et al.2004);

4) tumor cells escape apoptosis elicited by hypoxia and matrix-detachment (anoikis), both of which activate the PTP (Brenner and Grimm 2006);

5) the plant lipids jasmonates, which target the PTP, selectively induce apoptosis in transformed tumor cells , making the PTP itself a potential target in cancer therapy (Rotem et al.2005);

Moreover, in a model of hepatocarcinogenesis induced bythe arylamine 2-acetylaminofluorene (AAF), our group found that PTP desensitization induces a tumor-promoting adaptive response that selects apoptosis resistant hepatocytes (REF). Thus, inhibition of liver apoptosis through PTP adaptation might play a role in the onset of liver cancer (Rasola and Bernardi 2007).

1.4.4 Signaling transduction to PTP

The continuous crosstalk between mitochondria and other cell compartments suggests that several proteins can play regulatory roles on the pore, connecting signaling cascades known to control the apoptotic process with the PTP, as this is a key final point in cell commitment to death. These complex regulatory mechanisms could play a pivotal role in neoplastic transformation, where signal transduction cascades are deregulated.

An example of this interplay between mitochondria and cytosolic signal transduciton is *hexokinase* (HK). HK initiates all major pathways of intracellular glucose utilization, and type II hexokinase (*HK II*) is highly expressed in most cancer cells, where it mainly localizes on the OMM (fig.13).

Mitochondrial HK II is extremely important in neoplastic transformation. In highly glycolytic, aggressive tumors, it fosters cell growth in the hypoxic conditions of primary tumor mass accrual. This would contribute to the Warburg effect, *i.e.* to the uncoupling between glycolysis enhancement and oxygen availability, supporting cell proliferation when the cancer mass outgrows the surrounding blood vessels. Notably, signal transduction pathways converge on mitochondrial HK II (fig 13). This is of pivotal importance in cancer, because tumor cells are endowed with hyper-activation of these same anabolic transduction pathways. Accordingly, in hepatocellular carcinoma cells hypoxia stimulates growth via HK II induction, whereas HK II inhibition induces

apoptosis (Kim et al.2007), and release of HK II from mitochondria prompts apoptosis in glioma cells (Machida et al.2006). Detachment of HK II from mitochondria by a selective peptide induces PTP opening and cell death in tumor cell models and in cardiomyocytes (Jahaszova et al.2009). In experiments with CsA and its derivatives, and with cells and mitochondria derived from CyP-D knock-out mice, our group has shown that CyP-D enhances PTP opening and the ensuing apoptosis triggered by detachment of HK II from mitochondria (Chiara et al.2008) . These observations suggest that mitochondrial HK II delivers a survival signal that stabilizes the PTP in the closed conformation, whereas HK II detachment from mitochondria would propagate a conformational change to molecules of the IMM, eventually leading to pore opening (fig 13). Moreover, in this model the knockout of CyP-D inhibits apoptosis, at variance from what has been reported in other experimental setting.

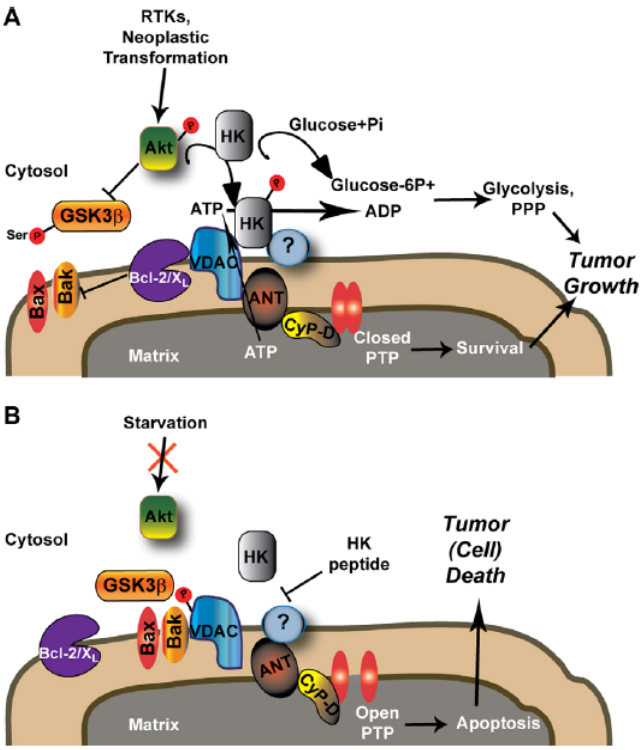


Figure 13. PTP regulation by hexokinase. (A) Akt activation by receptor tyrosine kinases (RTKs) or during neoplastic transformation keeps HK bound to the mitochondrial surface, both through a direct HK phosphorylation and through an inactivating serine phosphorylation of GSK3β. Inactive GSK3β cannot phosphorylate VDAC, favoring its binding to HK and to anti-apoptotic Bcl-2 family proteins, and displacing

the binding of pro-apoptotic Bax/Bak. However, VDAC is dispensable for HK interaction with mitochondria, suggesting that (an) unknown partner(s) is/are involved in this process. HK utilizes the ATP synthesized by mitochondria to start glucose metabolism, and stabilizes the PTP in a closed state. (B) Either Akt inactivation or treatment with a HK peptide induce HK detachment from the outer mitochondrial membrane, possibly leading to conformational changes of ANT and CyP-D and eventually to PTP opening and cell death. GSK3β is activated by Akt inhibition. The ensuing VDAC phosphorylation leads to Bcl-2/X_L displacement and favors Bax/Bak activation, but PTP opening induced by HK detachment from mitochondria does not require VDAC.

Thus, by integrating information from growth factor signaling, mitochondrial HK II could act as a potent anti-apoptotic "porekeeper" to suppress cell death either in conditions of adequate nutrition or when kinase pathways are aberrantly boosted by malignant transformation. The survival kinase Akt, which is activated by growth factors and in most tumor cells, promotes HK II binding to mitochondria. Indeed, Akt phosphorylates both HK II itself or GSK3β. Akt-dependent phosphorylation of mitochondrial HK II inhibits Ca^{2+}-induced cytochrome *c* release (Miyamoto et al.2008), and association of HK II to the OMM is favoured when GSK3β is inactivated by Akt phosphorylation (Mathupala et al.2006; Robey et al.2006). Accordingly, activation of GSK3β was shown to induce release of HK II, enhancing susceptibility to cell death (Mathupala et al.2006; Robey et al.2006). This was proposed to require GSK3β phosphorylation of VDAC, which would displace Bcl-2 from its interaction with VDAC, favoring the binding between VDAC and the pro-apoptotic Bax/Bak proteins in conditions of growth factor deprivation (Mathupala et al.2006; Robey et al.2006). This would result in increased sensitivity of mitochondria to PTP induction. However, mitochondrial displacement of HK II induced cell death also after insulin-dependent Akt activation or in cells lacking VDAC1 and 3 (and without detectable binding between HK II and residual VDAC2), and even in the absence of Bax and Bak (Majewski et al.2004). Finally, it remains unclear what mediates the interaction between HK II, which is located on the mitochondrial surface, and PTP regulators or components in the inner mitochondrial compartments.

PTP regulation by kinase signaling has been widely investigated in cardiac models, but it is reasonable to envisage that tumor cells could display a similar biochemical machinery to keep PTP under control. A group of kinases, termed

RISK (Reperfusion Injury Salvage Kinase), confers cardioprotection when activated during post-ischemic myocardial reperfusion or ischemic pre- and post-conditioning.(Hausenloy et al.2007). Several agents, including growth factors and hormones, reduce myocardial infarct size by activation of the RISK pathway (fig.14). RISKs include the survival kinases Akt and ERK1/2, PKCε, PKG and p70s6K, and protect from cell death by phosphorylation of Bcl-2 family proteins and inhibition of the mitochondrial PTP, with a possible interplay between these events (Majewski et al.2004; Baines et al.2007). A substrate of the RISK pathway is GSK3β which, unlike most kinases, is constitutively active in unstimulated cells, exerting a tonic inhibitory effect on its downstream targets. GSK3β is inactivated by serine phosphorylation induced by a variety of signaling pathways, whereas autophosphorylation at a specific tyrosine increases its overall catalytic efficiency.

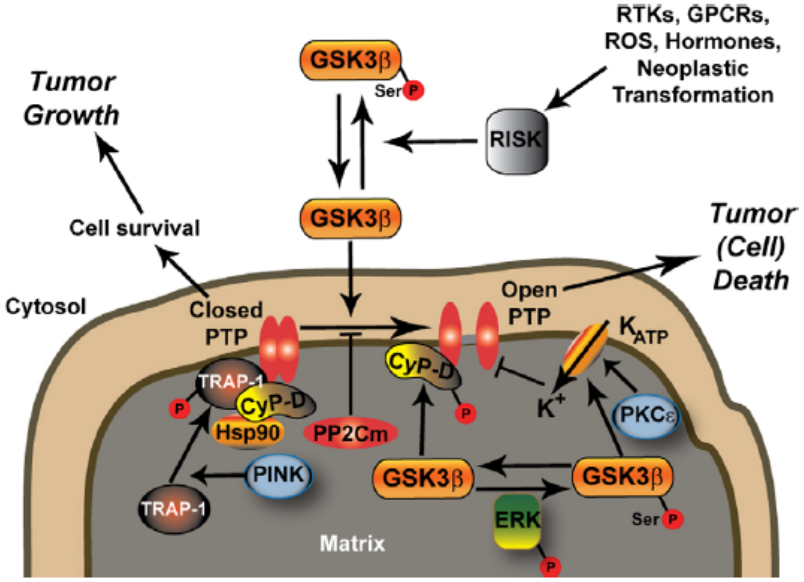


Figure 14. Kinase signaling to the PTP. Several kinase pathways converge on PTP regulation. A common target, at least during post-ischemic reperfusion, is GSK3β, which isinactivated by a group of survival kinases termed RISK (reperfusion injury salvage kinases). A small pool of GSK3β is in the mitochondrial matrix, where it favors PTP opening,possibly through CyP-D phosphorylation. In tumors, mitochondrial GSK3β is inactivated by ERK-dependent phosphorylation, raising the threshold for pore opening and therefore enhancing cell survival. GSK3β inactivation, and PKCe activity, could also contribute in keeping closed the

PTP by opening mitochondrial KATP channels. CyP-D regulation of the pore is also influenced by the chaperones Hsp90 and TRAP-1; TRAP-1 is activated by the Ser/Thr kinase PINK1. Finally, PTP opening is inhibited by the phosphatase PP2Cm through unknown mechanisms. RTKs: receptor tyrosine kinases; GPCRs: G protein coupled receptors.

Deregulation of this GSK3β fine tuning has been associated with a variety of diseases, such as neurodegenerative diseases, cancer and ischemia/reperfusion injury, which involve disturbances in apoptosis regulation . In cardiomyocytes, receptor tyrosine kinase or G protein-coupled receptor activation, or ROS production induced by reoxygenation after prolonged hypoxia lead to activation of diverse RISK components, and ultimately converge on PTP desensitization downstream to inactivating GSK3β phosphorylation. Accordingly, expression of a constitutively active GSK-3β failed to protect myocytes against oxidative stress, whereas transfection with a kinase-dead enzyme was protective. Moreover, infarct size in mice was reduced by CsA and by a GSK3β inhibitor, whereas in mice expressing a constitutive active GSK3β only CsA was effective. As in mitochondria from wild-type animals the PTP inhibitory effect of CsA and of the GSK3β inhibitor were additive, it was proposed that GSK3β facilitates pore opening independently of CyP-D.

This PTP regulation is operated by changing the ROS and/or Ca^{2+} threshold of PTP opening, and should be elicited by a mitochondrial pool of GSK3β acting as an integration point to funnel a multiplicity of survival pathways to target(s) at or in close proximity to the PTP. It must be stressed that GSK3β has further regulatory mechanisms, including phosphorylation at additional Ser residues, complex formation with scaffold proteins, priming of substrates and of the enzyme itself and intracellular translocation, as a consequence, GSK3β regulation of the pore could be finely tuned in several ways. Moreover, GSK3β could indirectly regulate the PTP. These include the already discussed modulation of HK II binding to mitochondria, regulation of the interaction between ANT and CyP-D, mitochondrial translocation of pro-apoptotic Bcl-2 family members, degradation of anti-apoptotic Bcl-2-like proteins. GSK3β inhibition also reduces infarct size via the opening of ATP-dependent K^+ channels and the ensuing PTP

closure, and phosphorylation of mitochondrial serine/threonine protein phosphatases (e.g. the subunit B of PP2A that possesses a consensus site for GSK3β) could directly affect the pore. Indeed, a mitochondrial matrix-targeted protein phosphatase 2C family member (PP2Cm) was shown to inhibit PTP opening and to be essential for cell survival , whereas PTP-mediated apoptosis was shown to be inducible by the protein phosphatases PP2B and SHP-1 in different cell models.

It is possible that other kinase/phosphatase signaling pathways can converge on the PTP through GSK3β regulation, or alternatively bypass it to directly impinge on the pore. PKCε plays a critical role in cardioprotective signaling pathways, targeting mitochondrial ATP-sensitive K^+ channels, respiratory chain components, ROS production and both ANT and VDAC. PKCε can inhibit PTP opening in a phosphorylation-dependent reaction and also in isolated mitochondria, conferring protection from reperfusion-induced cell death. The master survival kinase Akt protects the ischemic heart through inhibition of the PTP, and growth factors provided before ischemia reduce infarct size and increase the fraction of activated Akt in mitochondria, where it complexes with ANT upon reperfusion. As in the case of GSK3β, Akt could also indirectly modulate pore opening through the targeting of Bcl-2 family proteins, either affecting their subcellular localization or their degradation or regulating them at the transcriptional level. In hepatoma cells Akt inactivation by oxidative stress induces mitochondrial injury associated with opening of the PTP but independently of both GSK3β and Bcl-2 family proteins. In pheochromocytoma cells, GSK3β causes mitochondrial pore opening and the subsequent cell death via upregulation of the stress kinase JNK.

The limited knowledge of proteins that regulate the pore, and the lack of information on PTP components, make an arduous task the subtle dissection of the interplay between kinase pathway and the pore, and of its functional meaning. Furthermore, as it is always the case for (de)phosphorylation events, critical components are the timing, duration and intensity of the signal, which are in turn dependent on the cell type, the subcellular localization of scaffolding, adapter and regulatory proteins, on the assembly of multimeric complexes and even on specific non-proteinaceous components (e.g. lipid species). In addition, the

absence of recognized mitochondrial import sequences in most known kinases ask for laborious investigations to assess the direct interaction between these enzymes and molecules located in inner mitochondrial compartments. In this framework, an important role can be played by molecular chaperones (fig.14). A chaperone homologous to heat shock protein 90 (Hsp90), termed tumor necrosis factor receptor-associated protein-1 (TRAP-1), is localized in mitochondria of tumor cells and of nervous tissue, but not in most normal tissues . Both TRAP-1 and the mitochondrial fraction of Hsp90 interact with CyP-D and antagonize its function of PTP sensitization.

1.5 Mitochondrial chaperones in tumorigenesis

1.5.1 Chaperones activity

Molecular chaperones are involved in the correct folding of nascent polypeptides and in the productive assembly of multimeric protein complexes , while minimizing the danger of aggregation in the protein-rich intracellular environment. These proteins are also involved in 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 (Akerfelt et al.2010). The mechanism that cells use to ensure the quality of intracellular proteins is the selective destruction of misfolded or damaged polypeptides. In eukaryotic cells, a large ATP-dependent proteolytic machine, the 26S proteasome, prevents the accumulation of non-functional, potentially toxic proteins. This process is of particular importance in protecting cells against harsh conditions (for example, heat shock or oxidative stress) and in a variety of diseases (for example, cystic fibrosis and the major neurodegenerative diseases). Therefore, defects in chaperone activity could play a central role in different types of disease.

1.5.2 Cyclophilin D

Cyclophilin D (CyP-D) is a matrix mitochondrial chaperone endowed with a peptidyl-prolyl *cis-trans* isomerase activity. CyP-D is encoded by the *Ppif* gene, and belongs to the family of immunophilins . The catalytic function of cyclophilins requires a CLD (cyclophilin-like domain) and leads to conformational modification of the peptidic-binding of proline residues (from *trans* to *cis* and conversely). CyP-D is targeted by CsA, and this interaction inhibits PTP opening. As CsA delays PTP opening by binding to CyP-D, one would expect only that CyP-D favors PT and cell death. However, CyP-D overexpression reportedly *desensitizes* cells from apoptosis induced by the overexpression of caspase-8 (but not Bax) or by exposure to arsenic trioxide (Green and Kroemer 2004). Thus, CyP-D may play a role as a survival molecule, possibly acting on target(s) other than the PTP. Generation of $Ppif^{-/-}$ mice clearly demonstrates that CyP-D is dispensable for embryonic development and viability of adult animals. Various cell types isolated from CyP-D-deficient mice normally undergo apoptosis in response to various stimuli, including etoposide, staurosporine, and TNFα, and tBID or Bax caused cyt *c* release from isolated mitochondria (Baines et al.2005). Instead, CyP-D- deficient cells are significantly more resistant to necrosis induced by a Ca^{2+} ionophore (A23187) or by H_2O_2, and cardiac ischemia/reperfusion injury causes less damage in $Ppif^{-/-}$ animals, similarly to what is observed following treatment of wild-type animals with CsA. However, it must be highlighted that results obtained on $Ppif^{-/-}$ mice or cells can only be interpreted in terms of the role of CyP-D, not of the PTP, in cell death.(Rasola and Bernardi 2007). Its role in PTP regulation renders CyP-D an interesting target for anti-neoplastic therapies but also for diseases in which PTP is responsible for enhanced cell death rate. Recently Pastorino and co-workers have demonstrated that demonstrating that CyP-D is regulated by post-traslational modifications.They found that ethanol sensitizes PTP opening in part by increasing the acetylation and activity of CyP-D through a inhibition of sirtuin-3,

an NAD^+ deacetylase that is localized in the mitochondrial matrix (Shulga and Pastorino, 2010). This article is important because suggests that CyP-D could be maybe regulated also by other post-traslational modifications.

1.5.3 Heat shock proteins in tumors

The heat shock response is a highly conserved mechanism in all organisms from yeast to humans that is induced by extreme proteotoxic insults such as heat, oxidative stress, heavy metals, toxins and bacterial infections.

The heat shock response is mediated by *Heat shock proteins (HSP)*. These proteins ,named accordingly with their molecular weight (for example HSP-70 is a 70 KDa protein), function as intra-cellular chaperones for other proteins. They play an important role in protein-protein interactions such as folding and assisting in the establishment of proper protein conformation and prevention of unwanted protein aggregation. HSPs also contribute to the stabilization of partially unfolded proteins, and to the transport of proteins across cell membranes, and are also involved in protein degradation and in the control of signaling activation. Their conservation among different eukaryotes suggests that heat shock proteins are essential for survival in a stressful environment.

Some HSPs are also involved in tumorigenesis. One of the best studied HSPs in tumors is HSP90. *Heat shock protein 90 (HSP90)* is an evolutionarily conserved molecular chaperone that participates in stabilizing and activating more than 200 proteins —referred to as HSP90 'clients'- many of which are essential for constitutive cell signaling and adaptive responses to stress. To accomplish this task, HSP90, the chaperone HSP70, and additional proteins termed *co-chaperones* form a dynamic complex known as the HSP90 chaperone machine. Cancer cells use the HSP90 chaperone machinery to protect an array of mutated and over expressed oncoproteins from misfolding and degradation. Therefore, HSP90 is recognized as a crucial facilitator of oncogene addiction and cancer cell survival. For all these features HSP-90 is an important therapeutic target in cancer.

The first HSP90 inhibitor, 17-AAG (tanespimycin), entered clinical trials in 1999. In 2004, a second HSP90 inhibitor, 17-DMAG (alvespimycin), entered in clinical

experimentation .Owing to extensive efforts in rational drug design and discovery, HSP90 inhibitors are currently undergoing clinical evaluation in cancer patients (Trapel et al.2010).

HSP90 is a member of a small superfamily of functionally unrelated proteins (that also comprises DNA gyrase, histidine kinase and the DNA mismatch repair protein MutL) characterized by a unique ATP-binding pocket. The conserved chaperone structure consists of three domains: an amino terminal region (N domain) that contains an ATP and drug-binding site and co-chaperone-interacting motifs; a middle (M) domain that provides docking sites for client proteins and co-chaperones, and that participates in forming the active ATPase; and a carboxy-terminal (C) domain that contains a dimerization motif, a second drug-binding region and interaction sites for other co-chaperones. Dimerization of two HSP90 protomers through their C domains is necessary for chaperone function. Although HSP90 is primarily a cytoplasmic protein, it was also found in organelles such as mitochondria (REF); in addition, mammalian cells also express two compartmentally restricted HSP90 homologues (Trapel et al.2010):

- *Glucose-regulated protein 94 (GRP94)* found in the endoplasmic reticulum (ER);
- *Tumour necrosis factor receptor-associated protein 1 (TRAP1)* localized into mitochondria.

Like other HSP90 proteins, both GRP94 and TRAP1 possess ATPase activity but both lack knownco-chaperones. Recent studies suggest that GRP94 is essential for the maturation and secretion of insulin-like growth factors, which are autocrine mitogens that have a key role in transformation. ATP binding and hydrolysis are essential for the chaperone activity of GRP94, and a comparison of the nucleotide-binding pocket of GRP94 with that of HSP90 suggests that GRP94-specific inhibitors can be designed. In light of these recent findings, GRP94 should be evaluated as a bona fide anticancer target. (Trapel et al.2010).

1.5.4 TRAP1 mitochondrial chaperone

TRAP1 protects mitochondria from oxidative stress. Intriguingly, TRAP1 expression is low in the mitochondria of normal tissues (with the exception of the brain and testis) but is markedly increased in tumour mitochondria (Kang et al.2007). Like HSP90, TRAP1 is a phosphoprotein the phosphorylation of which by PTEN-induced putative kinase 1 (PINK1) is necessary to prevent oxidative stress-induced apoptosis(Pridgeon et al.2007).

TRAP1 (also known as HSP75), is a molecular chaperone homologous to HSP90 (Chen b. et al.2005). However, TRAP1 does not bind the classical HSP90 co-chaperones , it displays a higher ATP binding affinity than HSP90, possesses a typical ATPase cycle and it is almost exclusively restricted to mitochondria (Felts et al.2000). Many chaperones are endowed with a protective role against acute and chronic stress, and an anti-apoptotic function is emerging for TRAP1 as well. Indeed, in natural killer cells TRAP1 protects from granzyme M-mediated generation of reactive oxygen species and apoptosis (Hua et al.2007), and in human osteosarcoma SAOS-2 cells and colon carcinoma cells high TRAP1 levels elicit resistance to oxidants and chemotherapeutics (Costantino et al.2009). TRAP1 levels increase as an adaptation to oxidant exposure, and cells become more resistant to chemotherapeutics, suggesting a role for the antiapoptotic effect of TRAP1 in tumorigenesis. Consistently, Fluorouracil (FU) resistant colon carcinoma cells and other tumor cells upregulate TRAP1 protein levels (Montesano Gesualdi et al.2007). The importance of TRAP1 in neoplastic transformation is further supported by the recent finding that mitochondria from tumor cells contain TRAP1 in a complex with HSP90, and that disabling the ATPase activity of these chaperones leads to the selective death of neoplastic cells (Kang et al.2007). Moreover TRAP1 silencing in prostate cancer cell lines caused apoptosis, as did its targeting with mitochondria-specific HSP90 inhibitors (Leav et al.2009), and TRAP1 inhibition leads to the collapse of mitochondrial integrity, cytochrome *c* release, and caspase activation in several tumour cell lines and in several murine tumour models with little effect on non-transformed cells and minimal *in vivo* toxicity (Kang et al.2009;Kang et al.2007). Another important work raised the attention on TRAP1: the chaperone was found to be a substrate of the serine/threonine kinase PINK-1, whose mutations cause an autosomal

recessive form of Parkinson's disease. TRAP1 phosphorylation by PINK1 protects cells from apoptosis (Pridgeon et al.2007). This finding supports the notion that TRAP1 plays a role in Parkinson's disease, but also suggests that Ser phosphorylation may be an important regulatory mechanism in TRAP1 function. Taken together, these observations indicate that TRAP1 may inhibit some key apoptogenic events in mitochondria, whose loss of regulation could contribute to different pathological conditions.

2. Aim of the work

The aim of my work of thesis is the investigation of the molecular mechanisms by which mitochondrial chaperones regulate the response of tumor cells to death stimuli. In particular, I have analyzed a possible regulation of the mitochondrial Permeability Transition Pore (PTP) by signaling pathways, focusing mainly on MAPK cascade, which is hyper-activated in the majority of cancers. As the mitochondrial chaperone Cyclophilin D (CyP-D) is an important regulator of PTP, I have studied whether and how CyP-D activity on the pore is influenced by MAPK activation in tumor cells. Another mitochondrial chaperone, called TRAP1, is overexpressed during neoplastic transformation, and it was reported to interact with CyP-D, inhibiting PTP opening. My work concentrated in trying to better dissect what is the role of TRAP1 in PTP regulation and in tumorigenesis, with a particular attention on the bioenergetic features that characterize tumor cells.

3. Materials and Methods

3.1 Cellular models

Human SAOS-2 osteosarcoma cells , human cervical cancer cells Hela and human colon-rectal carcinoma cells HCT-116 were purchased from ATCC and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin and 100 μg/ml streptomycin, in a humidified atmosphere of 5% CO_2/95% air at 37°C. TRAP1 stable interference was achieved by transfecting SAOS-2 cells with TRAP1 shRNA (sequence TGCTGTTGACAGTGAGCGACCCGGTCCCTGTACTCAGAAATAGTGAAG CCACAGATGTATTTCTGAGTACAGGGACCGGGCTGCCTACTGCCTCGG A) or scrambled shRNA both contained in a pSM2 vector (by Open Biosystem). Cells were selected with the use of 0.8 μg/ml of puromycin (Sigma) to obtain stable transfected clones.

Diaphragm adult fibroblasts were obtained by SV40 immortalization of primary cells from wild-type and *Ppif*$^{-/-}$ mice (Chiara et al.2008) and grown in DMEM as above.

RWPE-1 are human epithelial prostate cells immortalized with HPV virus, whereas RWPE-2 cells are obtained from RWPE-1 through transformation after stable expression of v-ki-RAS . Prostate cells are grown in a humidified atmosphere of 5% CO_2/95% air at 37°C using Keratinocyte medium (Invitrogen) supplemented with 25 mg of bovine pituitary extract, 100 μg of recombinant human EGF, 100 units/ml penicillin, 100 μg/ml streptomycin (Bello et al, 1997; Webber et al, 1997).

For the expression of all vectors, cells were stably transfected with Lipofectamine 2000 (Invitrogen). To this aim, cells were grown in standard medium in 25 cm^2 flasks (BD Falcon). When sub-confluence was reached, cells were treated with

Lipofectamine following manufacturer's instructions. After 72 h, antibiotics for selection were added to the medium to obtain stable transfected cells.

3.2 Biochemical analyses

3.2.1 Protein preparation

Cells were washed in PBS solution ((140 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2PO_4, 1.8 mM KH_2PO_4) and detached from flasks with the use of trypsin 0.25% (Invitrogen) at 37°C. After centrifugation at 900 *g* for 5 minutes cells were resuspended at 4°C in a lysis buffer composed by 140 mM NaCl, 20 mM Tris-HCl pH 7.4, 5 mM EDTA, 10% glycerol, 1% Triton X-100, in the presence of phosphatase and protease inhibitors, Sigma. After few minutes on ice, lysates were cleared through a centrifugation at 13000 *g* for 30' minutes at 4°C, and supernatant were collected. In other cases cells were washed in PBS at 4°C and detached with the use of a cell scraper (BD Falcon) in the presence of lysis buffer and after few minutes on ice they were centrifugated at 13000 *g* at 4°C and supernatants were collected. When not used immediately, proteins were stored at -80°C.

Proteins were quantified using a BCA Protein Assay Kit (Thermo Scientific-Pierce).The BCA Protein Assay combines the well-known reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) by bicinchoninic acid. The first step is the chelation of copper with protein in an alkaline environment to form a light blue complex. In the second step of the color development reaction, bicinchoninic acid (BCA) reacts with the reduced (cuprous) cation that was formed in step one. The intense purple-colored reaction product results from the chelation of two molecules of BCA with one cuprous ion. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations. BSA (Sigma) was used a standard.

3.2.2 Isolation of mitochondria

Mitochondria were isolated either from cells, or from solid tumors or from livers of C57BL/6 mice through sequential centrifugations. To obtain mitochondria, cells were disrupted with a glass-Teflon or 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 *g,* 10 min), and mitochondria were then spinned down at a higher speed (7000 *g,* 10 min) and washed twice (7000 *g*, 10 min each one). All procedures were carried out at 0–4°C.

In order to define submitochondrial localization of proteins, a trypsin assay was carried out. Briefly, 70 μg of isolated mitochondria were treated with trypsin at different concentrations at 4°C for 1h. Where indicated, 0.1% SDS was added before trypsin. After inactivating trypsin with protease inhibitor cocktail (Sigma), mitochondria were spinned (18000 *g,* 10 min, 4°C) and loaded on a SDS-PAGE or used for CRC assays.

3.2.3 SDS PAGE and Western immunoblotting

In order to perform SDS-polyacrylamide gel electrophoreses (SDS-PAGE), cell extracts prepared as in 3.3.1. Samples were then reduced with the addition of a loading buffer composed by SDS 10%, TRIS 250mM pH 6.8, Glycerol 50%, β-mercaptoethanol 12,5%, Brome Phenol Blue 0.02% and denatured at 95°C for 5 minutes, run and separated on SDS-PAGE (10-12 % acrylamide for running gels, 4% acrylamide for stacking gels) and transferred onto nitrocellulose Hybond-C Extra membranes (Amersham, Little Chalfont, UK). Molecular standards used for gels were SeeBlue® Plus2 Pre-Stained Standard (Invitrogen).

Primary antibodies were solubilized in TBS TWEEN 0.1%, alone or with BSA 10% or milk 5%, incubated 16 hours at 4°C in , washed three times in TBS TWEEN 0.1%, incubated for 1 hour with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare), solubilized in the same buffers at dilutions

ranging from 1:5000 to 1:10000 . Proteins were visualized by enhanced chemiluminescence (Millipore, Billerica, MA) and using high performance chemiluminescence films (GE Healthcare). When required, densitometric analyses of the observed bands were performed with Quantity One software. When required, densitometric analyses of the observed bands were performed with Quantity One software.

3.2.4 Protein immuneprecipitation

Protein immuneprecipitations were carried out on 3-5 mg of total cellular extracts or on 300-400 μg of isolated mitochondria extracts. Lysates were pre-cleared with an incubation with Sepharose A or G (by Sigma) for 1 h at 4°C in agitation centrifuged at 13000 *g* for 2 minutes and supplemented with fresh Sepharose conjugated with the antibody against the protein to immuneprecipitate and incubated in agitation for 18 h at 4°C. Antibody conjugation was performed at room temperature in agitation using 30-50 μl of packed Sepharose and 1μg of antibody /mg of total protein. Lysates were then centrifuged at 13000 *g* for 2 minutes, the sepharose pellet was washed several times in lysis buffer , denatured at 95°C for 5 minutes with Loading Buffer and loaded on a SDS PAGE.

3.2.5 In vitro phosphorylation assay

In order to measure *in vitro* phosphorylation of the presumed kinase target CyP-D, a kinase assay was performed by incubating FLAG-CyP-D (the CyP-D cDNA was cloned in a pcDNA3 vector by Invitrogen with a FLAG tag added at its 3'end) obtained from 10^7 cells with the recombinant kinase GST-GSK-3β (200 ng) for 30 minutes at 30°C in a buffer containing 25 mM Tris-HCl (pH 7.5), 5 mM β-glycerophosphate, 2mM dithiothreitol (DTT), 0.1 mM Na_3VO_4, 10 mM $MgCl_2$, supplemented with 50 μM ATP. The GSK-3β inhibitor indirubin-3'-oxime was used as a selectivity control and preincubated 30 minutes with the

enzyme. Samples were then run on SDS-PAGE and visualized as above (paragraph 3.3.3), and phosphorylation determind with the use of anti phospho-Ser/Thr antibodies.

3.2.6 In vitro isomerase assay

The peptidyl-prolyl isomerase (PPIase) activity of the mitochondrial chaperone CyP-D was assessed on the recombinant protein (10 ng per experimental point). The FLAG-CyP-D (obtained as described above in paragraph 3.3.4) was purified by immunoprecipitation with a FLAG displacing peptide (Sigma). The enzymatic assay was a spectrophotometric analysis of the rate of hydrolysis of the peptide N-succinyl-Ala-Ala-*cis*-*trans*-Pro-Phe-p-nitroanilide by chymotrypsin as previously reported (Clarke et al. 2002). Chymotrypsin hydrolyzes only the *trans* form of the peptide, and this is measured as a rise in absorbance at 410 nM. The concentration of the *cis* form is maximized by using a peptide stock dissolved in trifluoroethanol containing 470 mm LiCl, and its hydrolysis is limited by the rate of *cis*-*trans* isomerization.

3.2.7 Hydrogen peroxide detection with Amplex Red

The enzymatic determination of hydrogen peroxide can be accomplished with high sensitivity and specificity using N-acetyl-3,7-dihydroxyphenoxazine (Amplex Red), a highly sensitive and chemically stable fluorogenic probe for the enzymatic determination of H_2O_2. Enzyme-catalyzed oxidation of Amplex Red, which is a colorless and nonfluorescent derivative of dihydroresorufin, produces highly fluorescent resorufin, which has an excitation maximum at 563 nm and emission maximum at 587 nm. Amplex Red was purchased by Invitrogen and used following manifacture's protocols. We detected hydrogen peroxide in 50-100 μg of fresh total lysates from SAOS-2 cells.

3.3 Physiological analyses

3.3.1 Measurement of mitochondrial Ca^{2+} retention capacity

The Ca^{2+} retention capacity (CRC) assay was used to assess PTP opening following trains of Ca^{2+} pulses and measured fluorimetrically at 25°C in the presence of the Ca^{2+} indicator Calcium Green-5N (1 μM; λ_{exc}: 505 nm; λ_{em}: 535 nm; Molecular Probes). Experiments were performed either on isolated mitochondria or on whole cells (Chiara et al 2008). Cells were washed in an isotonic buffer (130 mM KCl, 1 mM Pi-Tris, 10 mM Tris/Mops, and 0.1 mM EGTA/Tris, pH 7.4), and then permeabilized with 150 μM digitonin (20 min, 4°C), increasing EGTA to 1 mM. Digitonin was then eliminated and the number of cells carefully assessed before starting each experiment. Permeabilized cells or isolated mitochondria were placed in low (10 μM) EGTA in the presence of 2 μM rotenone/5 mM succinate, 10 μM cytochrome *c*, and Calcium Green-5N, which does not permeate mitochondria. Cells or mitochondria were then exposed to Ca^{2+} spikes, and fluorescence drops were used to assess mitochondrial Ca^{2+} uptake. PTP opening was detected as a fluorescence increase. Calcium Green-5N fluorescence was measured either with a fluorescence spectrometer LS50B (Perkin Elmer, Waltham, MA) or with a Fluoroskan Ascent FL fluorimeter (Thermo Electron Corporation, Waltham, MA).

3.3.2 Measurements of oxygen consumption

Mitochondrial oxygen consumption was measured polarographically at 25°C with a Clark oxygen electrode (Yellow Springs Instruments, OH, USA). Mitochondria (0.6 mg per experimental point) or cells (3 million/points) were incubated in a solution of 130 mM KCl, 10 mM Tris-Mops, 1 mM Pi-Tris, 20 mM EGTA-Tris and 2 μM rotenone/5 mM succinate to assay basal respiration (state 4). ADP (200 μM) was subsequently added to measure state 3 respiration, followed by the uncoupling agent dinitrophenol (100 μM) to assess the maximal respiration rate.

The rate of oxygen consumption was also measured *in vivo* on cell monolayers with the XF24 Extracellular Flux Analyzer (Sea Horse Biosciences), which measures both the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) using fluorophores contained in a sensor cartridge that is hydrated for 24h at 37°C without CO_2. The XF24 instrument is able to add compounds to each well through four injection, allowing a real-time evaluation of their metabolic effects. We plated cells (50000/well) the day before the experiment in a 24 multiwell plate in a DMEM/10% serum medium. Before starting measurements, cells were placed in a running DMEM (50 mM glucose, 2mM glutamine, 1mM sodium Pyruvate, no serum and NaCl x mM pH 7.4) and pre-incubated for 1h at 37°C without CO_2.

3.3.3 Determination of mitochondrial respiratory chain enzyme activities

To measure the enzymatic activity of respiratory chain complexes, cells or biopsies were homogenized with an electric potter (Sigma) in a buffer composed by 250 mM sucrose, 10 mM Tris-HCl, 0.1 mM EGTA-Tris, pH 7.4, Percoll 10% , protease and phosphatase inhibitors. 200-400 μg of total homogenate was used for a single trace. For Complex I activity we evaluated exogenous NADH (100 μM) oxidation as decrease in absorbance at 340 nm at 30°C with a spectrophotometer . Reaction started in a buffer with 10 mM Tris, 3 mg/ml BSA, 300 μM sodium Azide and 1μM Antimycin A (in order to block Complex IV and Complex III respectively), Alamethycin 5μM (to disrupt mitochondria). Coenzyme Q1 (20 μM) was added with NADH as electron acceptor to start reaction. After few minutes we added also rotenone (20 μM) to evaluate NADH consumption not due to Complex I activity. All complexes activity were normalized for Citrate Synthase activity. This assay follows citrate formation as increase in absorbance at 420 nm at 37°C with a spectrophotometer. Reaction buffer was composed by 100mM Tris-HCl pH 8, 100 μM DTNB, 300 μM Acetyl -CoA, 500 μM Oxaloacetate. For Complexes II-III activity we evaluated cytochrome C reduction as decrease in absorbance at 550 nm at 37°C with a

spectrophotometer . The buffer used contains 20 mM K_2HPO_4 pH7.5, 1 mg/ml BSA, 500 μM sodium Azide, 50 μM ATP, 10 mM Succinate and 50 μM Cytochrome C oxidized.

3.4 Flow cytometry analysis of mitochondrial depolarization and cell death

Flow cytometry is a technique used to separated population of cells in suspension on the basis of light scattering and fluorescence. It can be successfully used for the evaluation of cell death and mitochondrial depolarization through the fluorescence of specific molecular probes.

Flow cytometry recordings were performed as described (Gramaglia et al. 2004; Rasola et al.2001;Fassetta et al.2006). Briefly, at the end of the incubation, cells were resuspended in 135 mM NaCl, 10 mM HEPES, 5 mM $CaCl_2$ and incubated at 37°C in: a) Tetramethyl Rhodamine Methyl Ester (TMRM,10 nM), to detect mitochondrial depolarization (reduced TMRM staining).When TMRM was used to detect mitochondrial membrane potential, in each experiment one sample was treated with the protonophore FCCP (4μM) as a positive control (full mitochondrial depolarization). Moreover, as TMRM can be pumped out of cells by multidrug resistance systems, and CsA is a multidrug resistance inhibitor, the CsA analogue cyclosporin H, which is inactive on the PTP, was used to block multidrug resistance in the absence of CsA. ; b) FITC-conjugated Annexin-V and propidium iodide (PI, 1 μg/ml), 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); c) Mitosox (1μM) to detect mitochondrial superoxide production; d) N-acrydine orange (NAO 20μM, Invitrogen) to evaluate mitochondrial mass (to detect cardiolipin present in mitochondrial membranes). Samples were analyzed on a FACS Canto II flow cytometer (Becton Dickinson, San Diego, CA,USA). Data acquisition and analysis were performed using FACSDiva software and with WinMDI software.

3.5 Tumorigenesis assays

3.5.1 In vitro tumorigenesis: Focus Forming and Soft Agar assays

These assays evaluate in vitro tumorigenic features of cells. The Focus Forming experiment measures the capacity of cells to grow escaping contact inhibition and forming aggregates celled foci,and it is considered of the standard transformation assay.Cells (1 million) were plated in 10 cm Petri dishes (BD Falcon) in the presence of DMEM supplemented with 10% serum. When cells reached sub-confluence, medium was changed and serum decreased to 2%, 0.5% or 0% and changed every 4th day. At the 15^{th} day from serum decrease, proteins were collected from some dishes after washing cells in PBS at 4°C and detaching them with a scraper in the presence of lysis buffer. After 25 days from serum starvation cells were washed in PBS, fixed in methanol for 30 minutes and foci colored with GIEMSA solution for 1 h. After washing in deionized water, size and number of foci was analyzed with Image Analyzer software.

The soft agar assay evaluates the capability of cells to grow in anchorage-independent conditions, avoiding anoikis-induced cell death and forming colonies. The assay was performed in 6 cm Petri dishes or in x cm2 multiwells ostar (BD Falcon). Petri dishes or wells were covered by a first layer of DMEM without serum supplemented with low melting point Agarose (Promega) at a final concentration of 0.8%. This layer solidifies at 4°C in 20 minutes. On top of this, another layer of DMEM with 0.5% serum, and agarose at final concentration 0.7% was plated on it. Cells ($2.5x10^4$) had been added in this mixture, and remain embedded in the upper agarose layer. Dishes or wells were again placed at 4°C (20 minutes) and then for 10 minutes at room temperature, and finally were maintained in a humidified atmosphere of 5% CO_2/95% air at 37°C. Medium (DMEM 0.5% serum) was added on the top of the two layer and changed once a week. At the 21^{th} day dishes or wells were washed in PBS and fixed in methanol

for 30 minutes. Colonies were stained with Cristal Violet 0.005% and analyzed with Image Analyzer software.

3.5.2 In vivo tumorigenesis assay: inoculation of tumor cells in SCID mice

This assay evaluates in vivo capacity of cells to develop a solid tumor after injection in animals called SCID mice that are not able to kill injected cancer cells. SCID or nude mice are transgenic animals characterized by a severe immune-deficiency caused by an insufficient production of lymphocyte T, caused by lack of thymus development, which in turn depends on a deletion of the FOXN1 gene. This experiment was done in collaboration with Prof. DeFilippi's group (University of Torino). Twelve female mice, 3 months old, were injected on flanks with 100 μl of a suspension of 5 million of cancer cells in sterile PBS with or without Matrigel (basement membrane matrix, which addiction tothe suspension is used to favor tumor solid growth).The operator followed solid tumor growth for three weeks. At this point mice were sacrificed and tumors stored at -80°C.

4. Results

4.1 ERK activation and localization in cancer cell models

In the first part of my work I focused my attention on a possible functional circuit that connects the Ras/ERK transduction pathway to the mitochondrial PTP. The rationale is that: a) chronic activation of the Ras/ERK signaling pathway is frequently induced in neoplastic transformation, where it correlates with worsening of tumor stage and grade and favors metastasis formation; b) PTP inhibition is observed in several type of cancers. Moreover, activation of Ras/ERK signaling contributes to the development of the robust anti-apoptotic platform that characterizes cancer cells, and PTP inhibition has a central role in cell death inhibition. As a first model to study ERK activation and the pore, we utilized cells called RWPE-1 and RWPE-2. RWPE-1 cells are immortalized epithelial prostate cells that lack any tumorigenic potential, while RWPE-2 are made tumorigenic by expression of *v*-Ki-Ras in RWPE-1 cells (Webber et al.1997). As expected, ERK is constitutively active only in RWPE-2 cells (fig. 15).

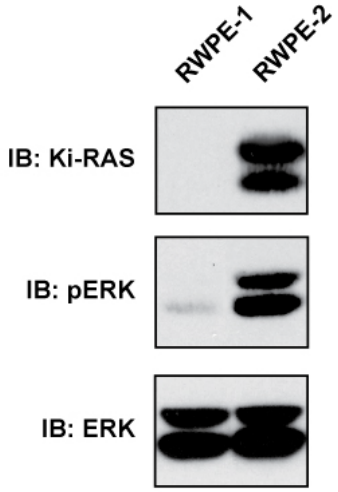
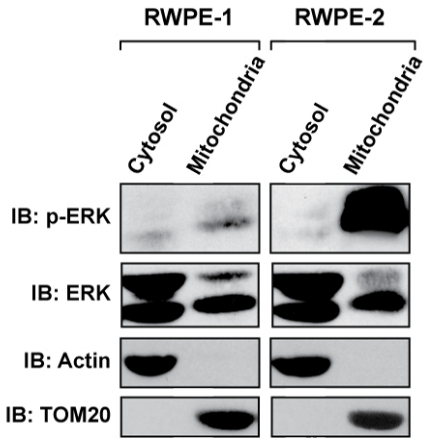


Figure 15. ERK is constitutively active in cells transformed with *v*-Ki-Ras. Western immunoblot from total lysates of RWPE-1 and RWPE-2. ERK activation is displayed as protein phosphorylation (pERK).

As the PTP is located across the inner mitochondrial membrane (IMM), its direct regulation by ERK requires that also the kinase is in mitochondria. I therefore explored ERK subcellular localization. As expected, most of the enzyme is in the cytosol but I found a fraction of the p42ERK2 isoform in mitochondria both in RWPE-1 and RWPE-2 cells; notably, mitochondrial ERK2 is active only in transformed cells (fig.16A). The upstream component of the ERK cascade, *K*-Ras, was not located in mitochondria (fig.16B).

A.



B.

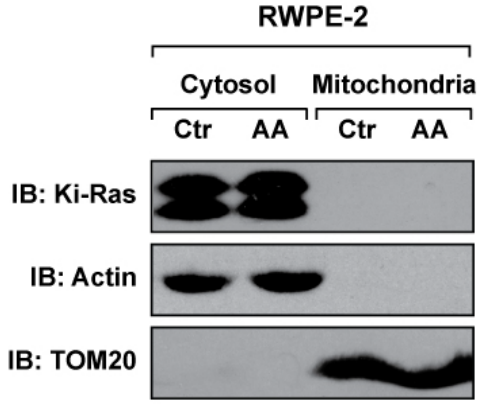


Figure 16. A fraction of active ERK is localized in mitochondria of RWPE2 cells, while K-Ras is in the cytosolic fraction.

A. Subcellular distribution of phospho- and total ERK was assessed by Western immunoblot in the mitochondrial and cytosolic fractions of RWPE-1/-2 cells. Blots were probed with an anti TOM20 as a

mitochondrial marker and with an anti actin as a cytosolic marker. **B.** Subcellular distribution of K-Ras was assessed in the mitochondrial and cytosolic fractions of RWPE-1/-2 cells. Cells were also treated with arachidonic acid (AA, 50 μM) for 1 hour. Blots were probed with an anti TOM20 as a mitochondrial marker and with an anti actin as a cytosolic marker.

As the mitochondrial localization of ERK is a novel finding, with few similar observations in the literature (REFs), I studied whether this result was peculiar for this *in- vitro* modelof cell transformation, or if it was a more general feature of tumor cells. To answer this question, I analyzed several human cancer cell models, and I found that a fraction of active ERK is always found in mitochondria (in fig. 17 the osteosarcoma SAOS-2 cells and the prostate cancer DU145 cells are shown).

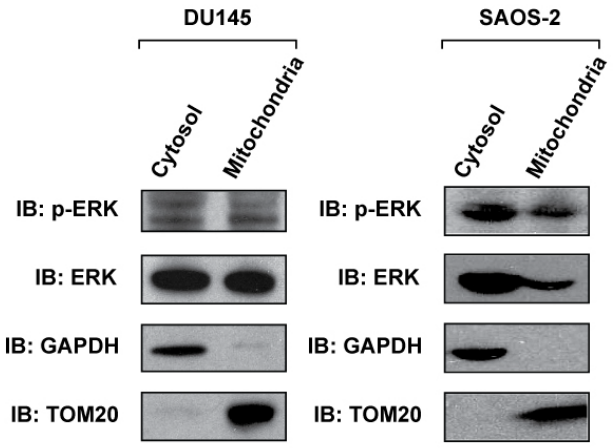


Figure 17. Active ERK is found in mitochondria of diverse tumor cell types. Subcellular distribution of phospho-ERK was assessed in the cytosolic and mitochondrial fractions of DU145 and SAOS-2 cells. Blots were probed with an anti total ERK to ascertain protein levels, with an anti GAPDH as a cytosolic marker and with an anti TOM20 as a mitochondrial marker.

Given the complexity of mitochondrial ultrastructure, which is composed by two membranes, an intermemebrane space and an internal matrix, a direct interaction with the PTP requires that ERK is found in proximity of the IMM. I therefore investigated the submitochondrial localization of ERK. To do this, I used a trypsin digestion assay on isolated mitochondria from SAOS-2 cells, exploiting the principle that progressively higher protease concentrations are needed to access internal organelle compartments and to cleave proteins found therein. I

found that ERK2 was not digested under conditions that disrupted external mitochondrial markers (fig. 18A).

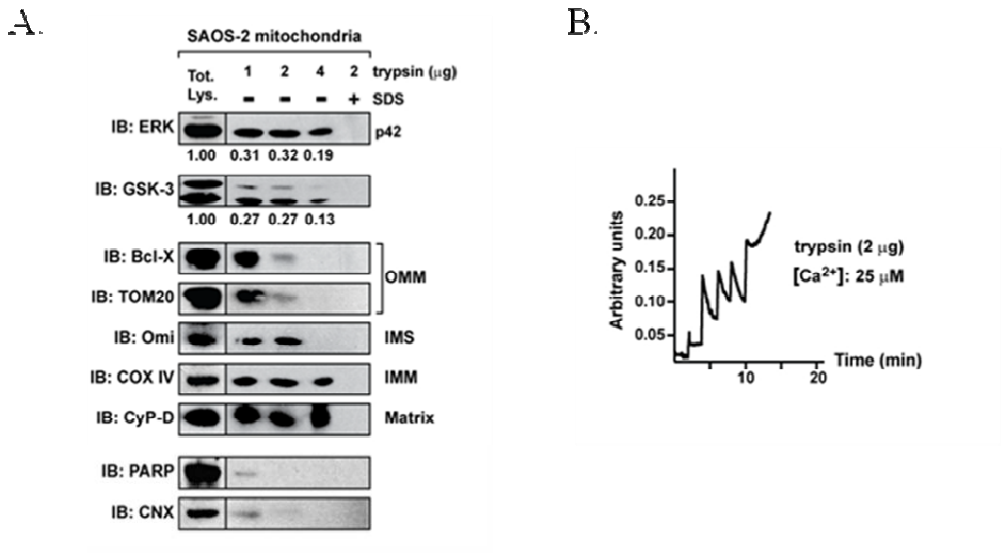


Figure 18. A fraction of ERK and of GSK3 is located in mitochondria.
A. Isolated mitochondria (70μg/point) were treated for 1 hour with the reported quantities of trypsin and analyzed by Western immunoblot (upper part) or by CRC (lower part). Where indicated, 0.1% SDS was added before trypsin. Blots were probed for ERK and GSK3, and a densitometric analysis of the bands is reported and compared to the enzyme level in the total lysate; and for a panel of mitochondrial markers of the outer and inner membrane (OMM and IMM, respectively), of the intermembrane space (IMS) and of the matrix. COX IV is a cytochrome oxidase subunit. PARP was used as an example of a non-mitochondrial soluble protein; calnexin (CNX) as an endoplasmic reticulum marker. **B.** PTP opening of mitochondria SAOS-2 cells treated with trypsin (2μg) with Ca2+ retention capacity assay (CRC). Calcium Green-5N fluorescence is reported as arbitrary units on the *y* axis. As the probe does not permeate mitochondria, Ca2+ uptake into the organelles is displayed by a rapid decrease of the fluorescence spike following administration to the cells of subsequent Ca2+ pulses (5 μM each).

Increasing trypsin concentrations fully digested intermembrane space (IMS) proteins, but not inner membrane or matrix components, and only partially affected ERK2 (fig. 18A). ERK2 was eliminated only when mitochondrial membranes were disrupted with SDS before trypsin treatment (fig. 18A). Therefore, the protease-resistant fraction of ERK resides in the matrix, whereas the minor portion of the enzyme digested by trypsin is in external mitochondrial compartments . A densitometric analysis on the trypsin-treated mitochondria *vs*. the cytosol showed that about 1.5% of total ERK is in matrix. Notably,

mitochondria treated with low trypsin concentration maintain the ability to take up Ca^{2+} and to open the PTP, as evaluated by the CRC assay in fig. 18B.

4.2 Effect of Ras transformation on cell death and PTP opening

in a first attempt to functionally connect mitochondrial ERK activity and pore regulation, I assessed cell sensitivity to PTP inducers in the human prostate epithelial cell lines RWPE-1 and RWPE-2 . Cells were treated with arachidonic acid (AA, 50μM for 1h), a fatty acid that acts as a potent PTP inducer (Penzo et al. 2004), and with the BH3 mimetic EM-20-25 (500μM, 1h), characterized in our laboratory as a pore inducer (Milanesi et al. 2006). After incubation with the two compounds, cell death was evaluated by cytofluorimetric inspection, staining cells with Annexin V and Propidium iodide (PI; see Methods).

AA caused a marked cell death in RWPE-1 cells, whereas RWPE-2 cells were less sensitive (fig. 19A-B). Pre-incubation with the pore inhibitor CsA (4μM for 30') inhibited cell death, confirming that AA acts through pore opening.

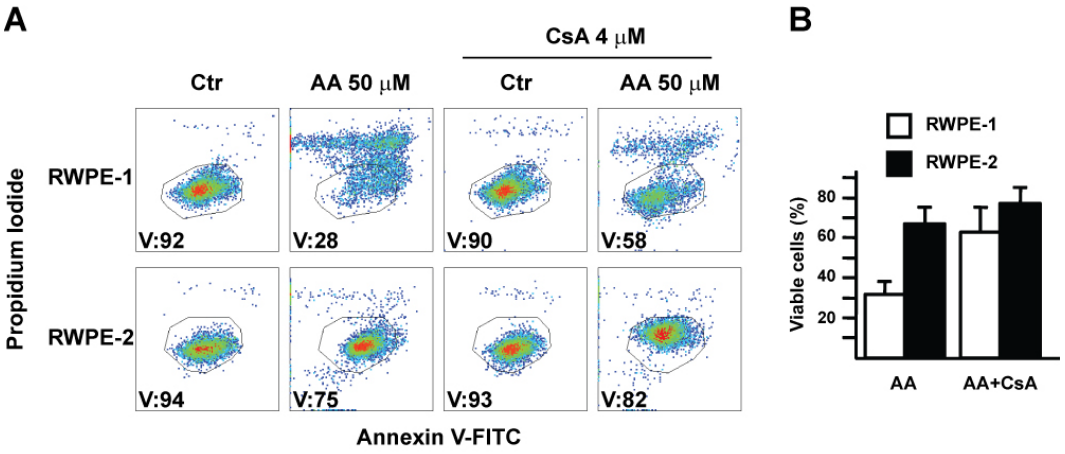


Figure 19. Ki-Ras-transformed RWPE-2 prostate cells are more resistant than non-transformed RWPE-1 cells to apoptosis and mitochondrial depolarization induced by PTP agonists. A. Outputs of multiparametric FACS analyses (propidium iodide *vs* Annexin V-FITC) show cell death induction of RWPE-1/2 cells exposed for 1 hour to arachidonic acid (AA).Viable cells (V, double negative for propidium iodide

and Annexin V-FITC) are delimited by the quadrant and their percentages (±SD) are shown on histograms (**B**).

Since AA is a direct PTP inducer, Ki-Ras-mediated transformation might act on the pore itself. In this case, RWPE-2 cells should be similarly protected from the effects of a second PTP opener, unrelated to AA. For these reasons we tested the BH3 mimetic EM20-25, which, unlike its parent compound HA14-(Wang et al. 2000), does not cause inhibition of respiration or uncoupling, and specifically kills cells by opening the PTP (Milanesi et al. 2006). As for AA, RWPE-1 cells treated with EM20-25 underwent death to a significantly higher degree than RWPE-2, and cell death was inhibited once again by CsA, as indicated in figure 20. Altogether these experiments show that cells transformed with *v*-Ki-Ras have a higher threshold for PTP opening.

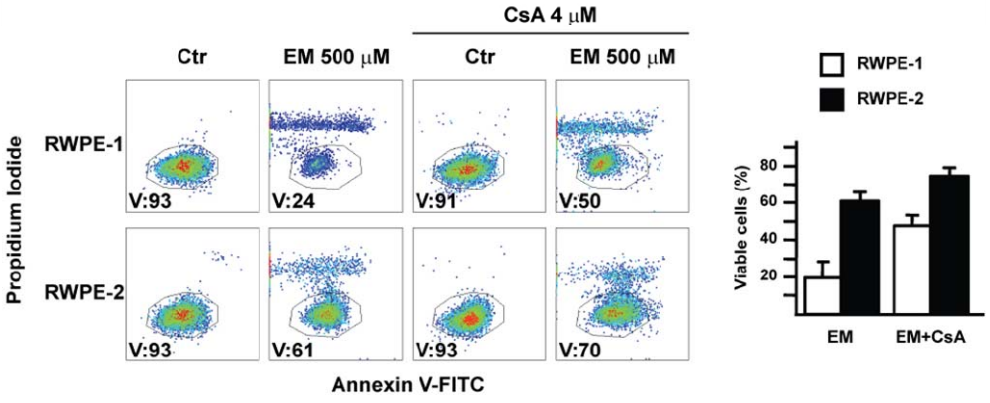


Figure 20. . Ki-Ras-transformed RWPE-2 prostate cells are more resistant than non-transformed RWPE-1 cells to apoptosis and mitochondrial depolarization induced by PTP agonists. A. Outputs of multiparametric FACS analyses (propidium iodide *vs* Annexin V-FITC) show cell death induction of RWPE-1/2 cells exposed for 1 hour to EM 20-25. Viable cells (V, double negative for propidium iodide and Annexin V-FITC) are delimited by the quadrant and their percentages (±SD) are shown on histograms (**B**).

These assays constitute an indirect evidence of a functional link between ERK activation and PTP inhibition. I therefore investigated more closely pore opening, with two distinct approaches: the study of mitochondrial depolarization, a direct consequence of PTP opening, and the calcium retention capacity assay (CRC), which directly measures PTP (see Methods). As shown in fig. 21, both EM20-25 and AA are able to induce mitochondrial depolarization, and their effect is higher

in RWPE-1 than in RWPE-2 cells. As expected, mitochondrial depolarization is caused by PTP opening,, as it is reverted by the use of CsA (figure 21).

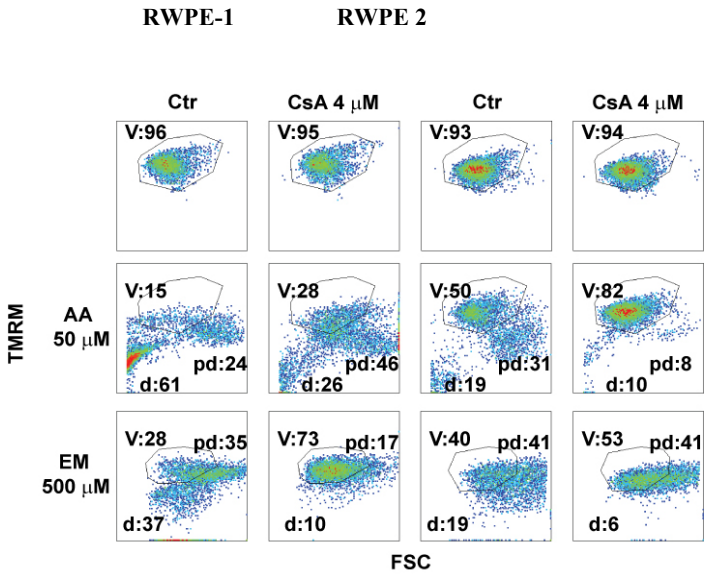


Figure 21. Mitochondrial depolarization in RWPE cells. FACS analysis (Forward Scatter, FSC, *vs* TMRM) showing mitochondria depolarization in RWPE-1/2 cells exposed for 1 hour to AA or EM20-25. Viable cells (V, TMRM positive) are delimited by the quadrant. Cells displaying partial mitochondrial depolarization (reduced TMRM staining) are indicated as pd (partially depolarized), whereas cells with fully depolarized mitochondria are shown as d (depolarized). Throughout the Figure, numbers are percentages. Where indicated, CsA was preincubated for 30 minutes.

Interaction between ERK and the PTP was further evaluated with the CRC assay (see Methods). With this approach pore opening is induced by a Ca^{2+} overload. The assay is performed by adding pulses of Ca^{2+} to mitochondria or permeabilized cells kept in a Ca^{2+}–free medium, in the presence of the Ca^{2+}-sensitive fluorescent probe Calcium Green 5N. Pulses are visualized as probe fluorescence increase; as Ca^{2+} is rapidly taken up by mitochondria, a fluorescence decrease follows, until Ca^{2+} induces PTP opening and is massively released by mitochondria, inducing a dramatic increase in probe fluorescence. The quantity of Ca^{2+} accumulated by mitochondria before PTP opening gives a semi-quantitative information on the effects of inducers or inhibitors, which decrease or increase, respectively, the number of pulses required to open the pore.

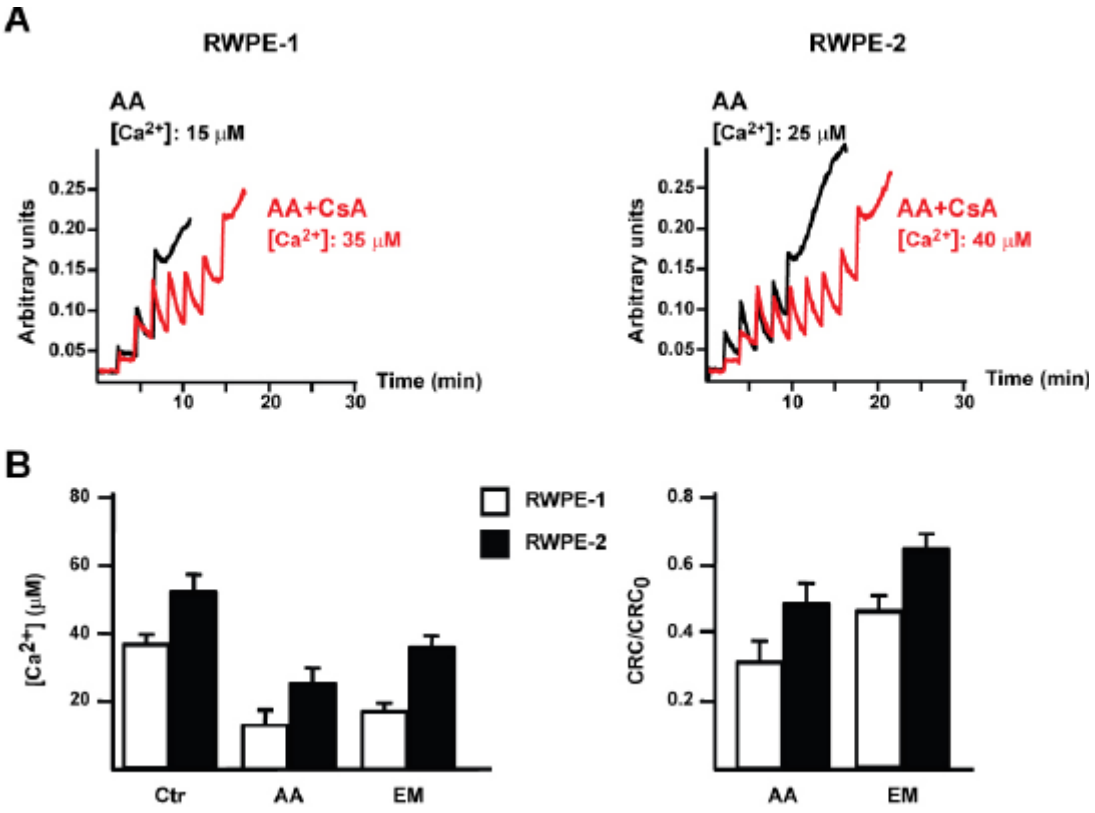


Figure 22. RWPE-2 cells are less sensitive than RWPE-1 cells to pore opening. A. PTP opening of RWPE-1/2 cells treated with arachidonic acid (AA) is measured with the whole-cell Ca^{2+} retention capacity assay (CRC). Calcium Green-5N fluorescence is reported as arbitrary units on the *y* axis. As the probe does not permeate mitochondria, Ca2+ uptake into the organelles is displayed by a rapid decrease of the fluorescence spike following administration to the cells of subsequent Ca2+ pulses (5 μM each). CsA (in red)increases the number of spikes before the permeability transition, recorded as a marked fluorescence increase, occurs, thus establishing the PTP-dependence of Ca2+ release. In the histograms of (**B**.), both the Ca^{2+} concentration required to open the pore (on the left) and the ratio between the CRC detected in the presence (CRC) and absence (CRC0) of the agonist (on the right) are reported. Agonist concentrations were as follows: AA, 1 μM; EM20-25 (EM), 50 μM. Numbers are mean values of percentages(±SD).

With this approach, and in accord with my previous data, I have shown that RWPE-2 cells have a higher threshold for PTP opening than RWPE-1 cells, both in unstimulate conditions and after treatment with either AA or EM20-25 (fig. 22).

4.3 Inhibiting ERK favors PTP opening

Having established that Ras transformation protects RWPE cells from death stimuli through PTP inhibition, I studied whether ERK directly modulates the PTP. To this aim, I blocked ERK activation using two different approaches: a drug called PD98059, which is a potent inhibitor of MEK (the kinase responsible for ERK phosphorylation and activation), and a soluble and cell-permeable peptide called EIP (ERK1/2 -inhibiting- peptide) which corresponds to the N-terminus of MEK and selectively inactivates ERK by direct binding . First I assessed the effectiveness of these two compounds, and I found that both are efficient in blocking ERK phosphorylation, also in the mitochondrial fraction (fig. 23).

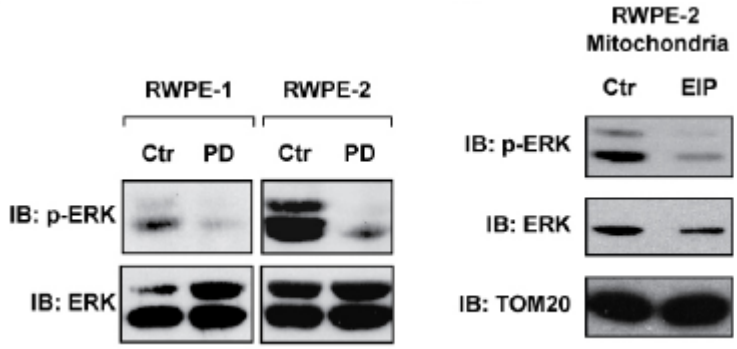
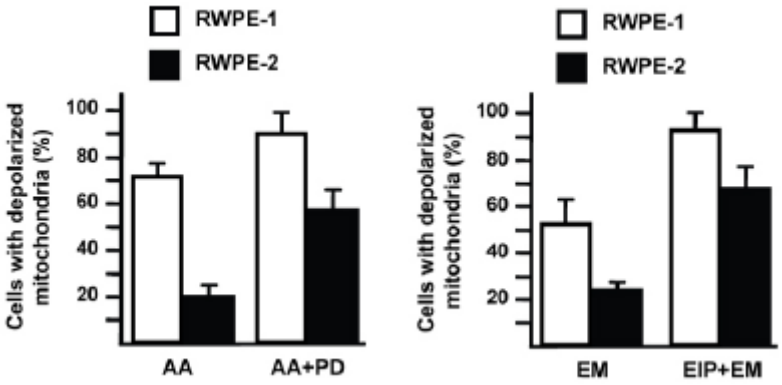


Figure 23. PD98059 and EIP decrease ERK activation in both RWPE-1 and RWPE-2 cells. On the left, inhibition of ERK activation by the drug PD98059 was assessed by Western immunoblot on RWPE-1 and RWPE-2 cell lysates. Blots were probed with an anti total ERK to ascertain protein levels. On the right inhibition of ERK activation by an ERK inhibition peptide (EIP, 50 μM) was assessed on lysates of RWPE-2 mitochondria. The blot was probed with an anti TOM20 as a mitochondrial marker and with an anti GAPDH (not shown) as a cytosolic marker.

Then, I looked for the biological consequences of ERK inhibition, and I found that pre-treatment with either PD or EIP increases the effect of the pore inducers. Indeed, ERK inhibitors enhanced both mitochondrial depolarization and PTP opening caused by AA or EM20-25 (fig. 24A), and also when used by themselves sensitized cells to pore opening (fig. 24B).

A.



B.

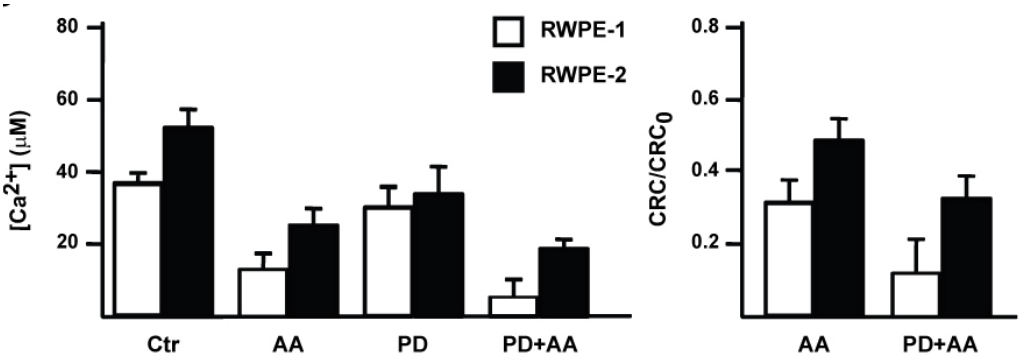


Figure 24. Mitochondrial deopolarization induced by pore openers is enhanced by ERK inhibitors. A. Mitochondria depolarization of RWPE-1/2 cells exposed to 50 μM AA or to 500 μM EM20-25 (EM) for 1 hourwith or without a 30 minute pre-incubation with an ERK inhibitors peptide (PD 98059 40μM ;EIP 50 μM; Numbers are mean values of percentages (±SD). In the histograms of (B.), both the Ca^{2+} concentration required to open the pore (on the left) and the ratio between the CRC detected in the presence (CRC) and absence (CRC0) of the agonist (on the right) are reported. Agonist concentrations were as follows: AA, 1 μM; PD98059 (PD), 40 μM;EM20-25 (EM), 50 μM. Numbers are mean values of percentages (±SD).

4.4 Kinase regulation of the PTP

despite all these evidences provide a correlation between ERK activation and PTP inhibition, the identification of an ERK substrate

on the pore is made impossible by the lack of information on the molecular composition of the PTP. So I focused my attention on CyP-D, the best-characterized PTP regulator. To be an ERK substrate, CyP-D must be phosphorylated on serine or threonine residues, as ERK is a Ser/Thr kinase, but no CyP-D phosphorylation had ever been reported. So, I performed an immunoprecipitation with an anti-phospho-Ser/Thr residues, in conditions of modulation of ERK activity: cells depleted of serum or treated with the EIP (ERK inhibition in both conditions), or exposed to serum (ERK activation). As reported in fig. 25A, CyP-D turned out to be phosphorylated on Ser/Thr residues, and its phosphorylation inversely correlated with ERK activity. Moreover, I also found that CyP-D directly interacts with ERK . This interaction is not modulated by the degree of kinase activity, as shown by the immunoprecipitation of a CyP-D tagged with a FLAG peptide (fig. 25B); . this construct was used as there is no available anti CyP-D antibody to be used in immunoprecipitations. Remarkably, this interaction was observed in purified mitochondria(fig. 25B)

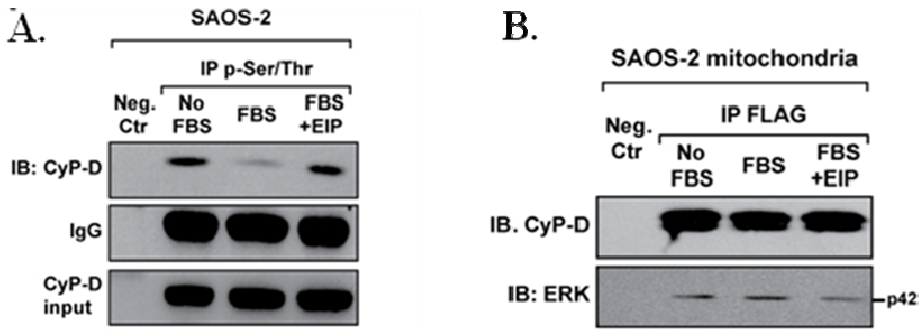


Figure 25. ERK activity modulates mitochondrial depolarization and CyP-D phosphorylation through GSK3. A. Immunoprecipitations on lysates of SAOS-2 cells starved overnight (NO FBS) and then stimulated for 15 minutes with serum with or without a 30 minute preincubation with an ERK inhibition peptide (EIP 50 μM; lanes FBS and FBS+EIP). Upper part: P-Ser/Thr were immunoprecipitated from total cell lysates; co-immunoprecipitation of CyP-D is shown, and IgGand input of CyP-D are reported as loading control. **B**. The FLAG peptide was immunoprecipitated from mitochondria of SAOS-2 cells expressing FLAG-CyP-D. Immunoprecipitation of CyP-D and co-immunoprecipitation of ERK2 (p42) are shown. The negative control was a SAOS-2 lysate incubated with the non-conjugated resin (A) or a lysate of SAOS-2 wild-type mitochondria incubated with the FLAG-conjugated resin (B).

Thus, ERK activity could impinge upon pore opening through the negative regulation of CyP-D phosphorylation, possibly by inhibiting an intermediate kinase. A candidate substrate of ERK as a PTP regulator was the GSK-3 kinase. In fact: a) GSK-3 inhibition by Ser-phosphorylation was reported to desensitize PTP opening (Juhaszova et al.2009); b) ERK can phosphorylate GSK-3 on a serine residue, inhibiting its activity (Ding et al.2005); c) I found that a fraction of GSK-3 is located in the mitochondrial matrix (fig.18), corresponding to about 1% of the total cell enzyme. To explore the possible involvement of GSK-3 in PTP regulation, I immunoprecipitated GSK-3 from mitochondria in the same conditions of ERK modulation as above. As shown in fig. 26, mitochondrial GSK-3 turned out to be associated to CyP-D in the same conditions in which CyP-D is phosphorylated, i.e. when ERK is inhibited (compare fig. 25 and fig. 26) and GSK-3 is active (as shown by inhibition of its serine phosphorylation in fig. 26). GSK-3GSK-3GSK-3GSK-3

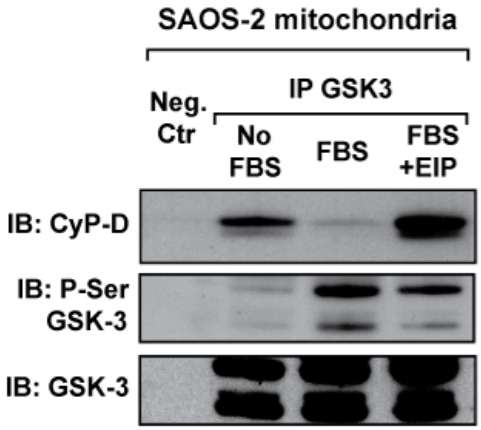


Figure 26. CyP-D interacts directly with GSK3 in mitochondria of SAOS-2 cells.
GSK-3 was immunoprecipitated from SAOS-2 mitochondria. Co-immunoprecipitation of CyP-D and Ser-phosphorylation of GSK-3 are shown. The negative control was a SAOS-2 lysate incubated with the non-conjugated resin.

To confirm that GSK-3 phosphorylates CyP-D I performed a phosphorylation *in vitro* assay, and I found that a purified GSK-3 fusion protein was able to phosphorylate CyP-D, and this was inhibited by the GSK-3 inhibitor indirubin-3'-oxime (fig. 27). An *in silico* analysis was performed with Scansite and NetPhosK

software, and residues S38, S39 and S123 on human CyP-D were identified as possible GSK-3 targets.

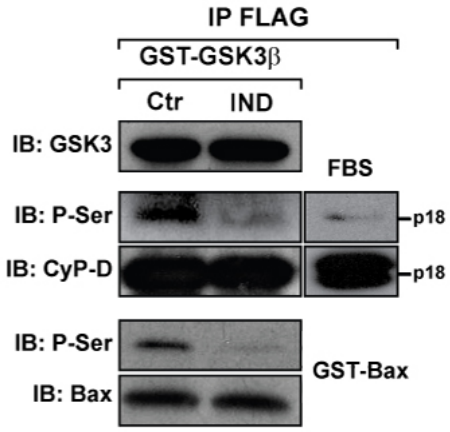


Figure 27. ***In vitro*** **CyP-D phosphorylation by recombinant GST-GSK3β.** The FLAG peptide was immunoprecipitated from lysates of SAOS-2 cells expressing FLAG-CyP-D. GST-Bax was used as a positive control for enzyme activity. Ser-phosphorylation was analyzed on the same quantity of FLAG-CyP-D immunoprecipitated from SAOS-2 cells kept with serum. Indirubin-3'-oxime addiction to the assay buffer (3μM), a well know inhibitor of GSK3, completely abolishes CyP-D Ser-phosphorylation.

Finally, to evaluate the importance of CyP-D phosphorylation on mitochondrial pore regulation, I performed a set of physiological experiments. First, I over-expressed CyP-D; in these conditions, the slight PTP induction in CRC caused by ERK inhibition was enhanced (fig. 28A); second, I used the EIP inhibitor in fibroblasts derived from CyP-D knock-out mice, and found that it was ineffective in triggering any pore opening (fig. 28B); third, I found that mitochondrial depolarization induced by EM20-25, and the enhancing effect of EIP, were abolished by the GSK-3 inhibitor indirubin-3'-oxime (fig. 28C), further confirming that GSK-3 is a downstream target of ERK in PTP regulation.

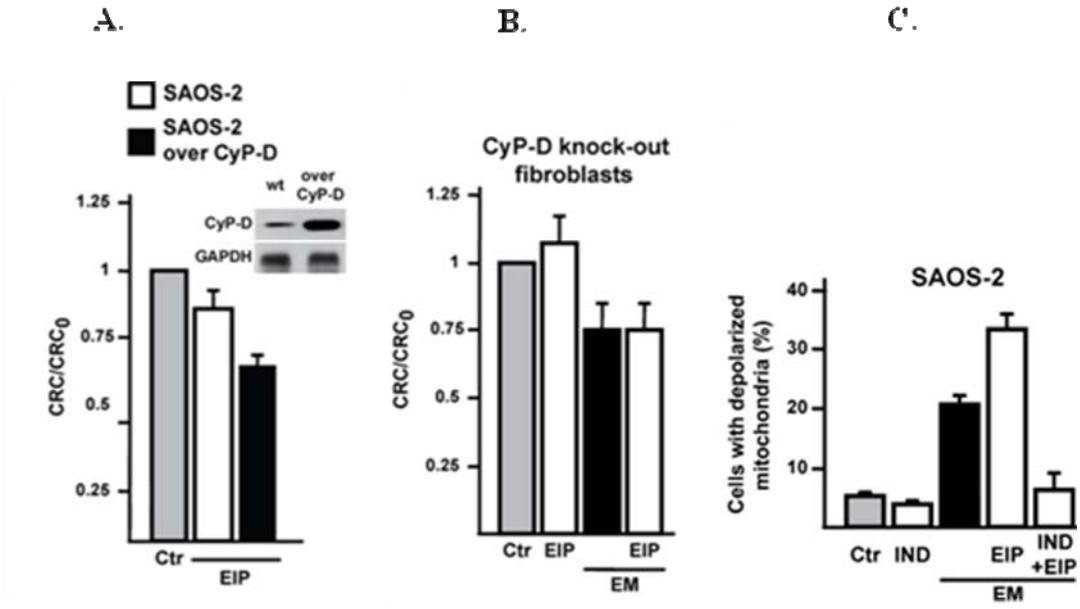


Figure 28. CyP-D levels and GSK3 inhibition modulate ERK inhibitors toxicity.

Ratio between CRC detected in the presence (CRC) and absence(CRC_0) of EIP are reported for wild-type or CyP-D-overexpressing SAOS-2 cells (**A**), or for CyP-D knock-out fibroblasts (**B**.),which were also treated with EM20-25. Numbers are mean values of four experiments (±SD). **C**. Mitochondria depolarization of SAOS-2 cells exposed to 500 μM EM20-25 (EM) for 1 hour with or without a 3 hour pre-incubation with IND (3 μM) and/or a 30 minute pre-incubation with EIP 50μM. Numbers are mean values of percentages (±SD) of four FACS analyses.

4.5 Characterization of a novel CyP-D inhibitor: antamanide

The characterization of the ERK-GSK3-CyP-D signaling axis in tumor cells and, more in general, the involvement of CyP-D in PTP regulation in several diseases has increased the interest in the finding of novel and more selective CyP-D inhibitors, devoid of toxic effects . In this framework, I have participated in the characterization of one of these candidate molecules, antamanide, a monocyclic homodetic decapeptide isolated from the poisonous mushroom *Amanita Phalloides* (fig. 29A). Antamanide was investigated because it displays some biological activities, e.g. its action as an immunosuppressant, that remind those of the CyP-D inhibitor CsA (Siemion et al.1992). By performing a CRC assay on

isolated mitochondria from mouse liver, we found that antamanide inhibited PTP opening in a way similar to CsA, but it is less effective than Ub0, which acts in a CyP-D independent fashion (fig. 29B).

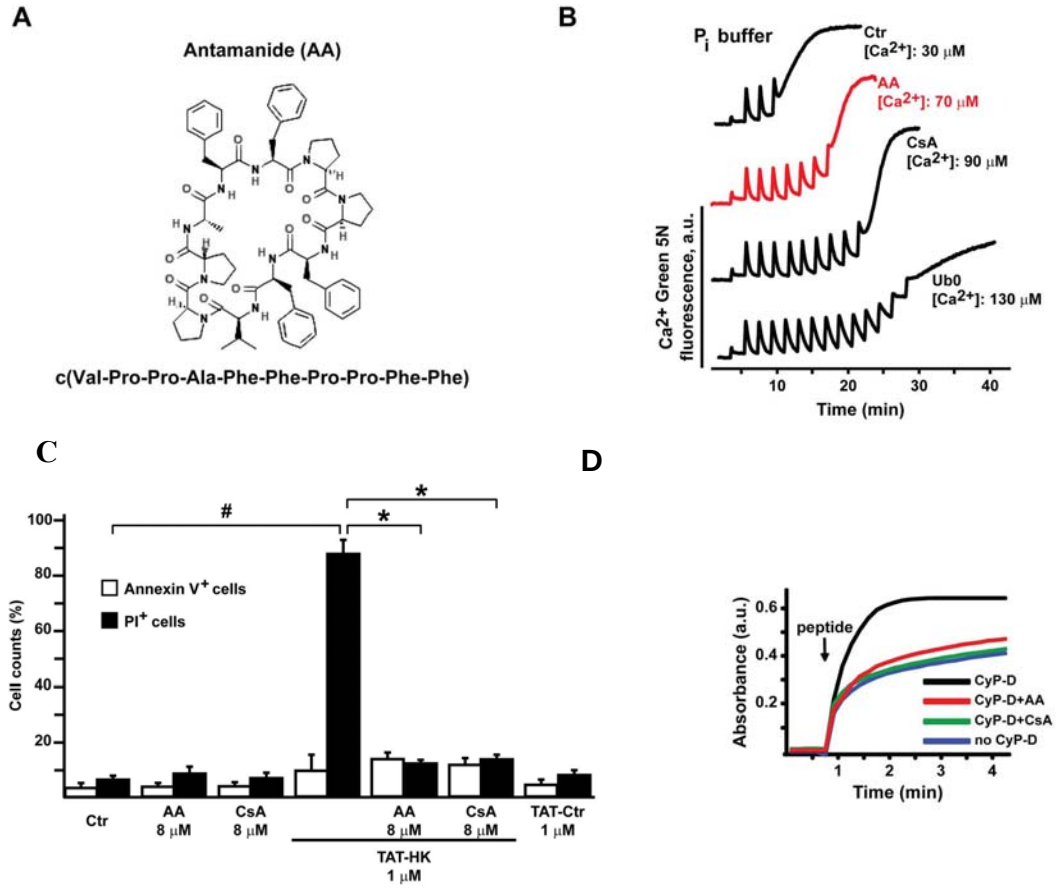


Figure 29. Antamanide structure and effect on the PTP, on cell viability and on the CyP-D catalytic activity. A, Chemical structure of antamanide. B, CRC experiment; traces are reported as in fig.22 Antamanide (AA; red trace, 8 μM) or CsA (0.8 μM) act as pore inhibitors, as they increase the threshold Ca^{2+} concentration required to trigger permeability transition. Ub0 was used at 25 μM concentration. C, cytofluorimetric analysis of cell death induction in HeLa cells treated for 1 hour with TAT-HK. Where indicated, cells were preincubated or 30 minutes with CsA or AA. TAT-Ctr was an unrelated peptide used as a negative control. Cells were double stained with Annexin V-FITC and PI. D, the isomerase activity assay (see Materials and Methods) shows that AA acts as an enzymatic inhibitor of CyP-D. After addition of the substrate peptide N-succinyl-Ala-Ala-*cis*-*trans*-Pro-Phe-p-nitroanilide (arrow), chymotrypsin cleaves the trans-isomeric form, causing a rise in absorbance. CsA: 8 μM; AA: 8 μM.

We also found that antamanide is effective in cells, inhibiting cell death caused by the PTP inducer TAT-HK, a peptide (sequence: MIASHLLAYFFTELNbA-

GYGRKKRRQRRRG) that opens the PTP by detaching hexokinase II from mitochondria (fig. 29C; Chiara et al, 2008). Then we evaluated with a in vitro-isomerasic assay if The effect of antamanide is on CyP-D, as it does not affect the PTP in cells derived from CyP-D knock-out mice (fig.30). Moreover, antamanide directly blocks the isomerase activity of CyP-D (fig.29D).

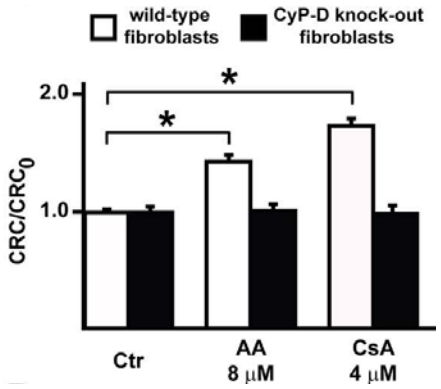


Figure 30. Effect of AA on PTP opening in wild-type or CyP-D knock-out mouse fibroblasts.
The CRC/CRC_0 ratio calculated indicates that both AA and CsA display an inhibitory effect on the PTP in wild-type, but not in CyP-D knock-out fibroblasts. Results are mean±SD of at least 4 experiments. Significant differences were observed between ratios in control conditions and after treatment with either AA or CsA (Student's *t* test analysis; *: $p<0.01$)

4.6 The mitochondrial chaperone TRAP1 interacts with CyP-D and inhibits cell death in tumor cells

The first part of my work has shown that cell death is inhibited in tumor cells through the regulation of the mitochondrial chaperone CyP-D, and that this regulation relies on an ERK-GSK-3 transduction axis that impinges on the mitochondrial PTP. I therefore asked if another mitochondrial chaperone, TRAP1/HSP-75, which was shown upregulated in tumor cells, where it interacts with CyP-D (Kang et al.2007), could be involved in pore inhibition and in cancer cell survival. In a first experiment I confirmed the interaction between TRAP1 and CyP-D. In fact, immunoprecipitating FLAG-Cyp-D in SAOS-2 osteosarcoma cells I found co-immunoprecipitation of TRAP1 (fig. 31A). The CyP-D/TRAP1 interaction was not modulated when cells were pretreated with the CyP-D

inhibitor CsA (fig. 31B). The presence of ERK in association with CyP-D (fig. 25B) suggests that, similarly to CyP-D, also TRAP1 could be targeted by the ERK signaling pathway, in accord with data showing that TRAP1 can be phosphorylated, albeit by another kinase, PINK1 (Pridgeon et al, 2007).

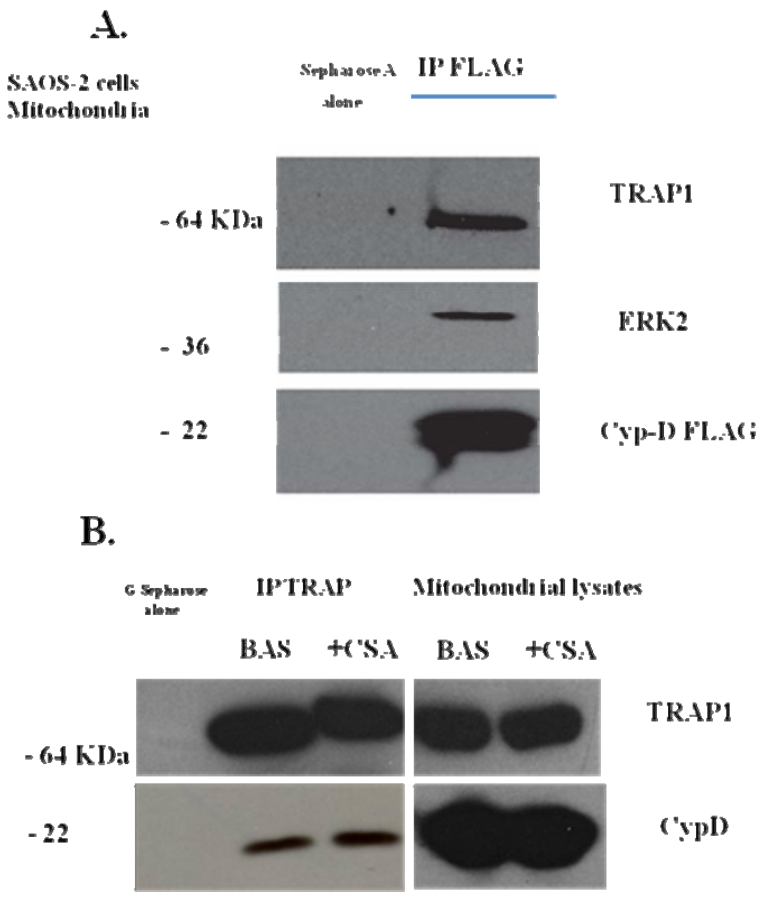


Figure 31. Co-mmunoprecipitation of TRAP1 and ERK2 with CyP-D.

A. Anti FLAG peptide immunoprecipitations (IP) on mitochondria of SAOS-2 cells expressing FLAG-CyP-D. Immunoblot results for CyP-D, ERK2 (p42) and TRAP1 are shown. **B.** Anti TRAP1 IP on mitochondria of SAOS-2 cells treated or not with CsA 4μM for 30'. Immunoblot results for CyP-D, ERK2 (p42) and TRAP1 are shown. Both in A and in B, as a negative control, protein Sepharose A was loaded after incubation with mitochondria lysates in the absence f antibody. In **B**, mitochondrial total lysates were loaded as control of protein expression.

In order to better characterize TRAP1 biological functions its expression was down-regulated in osteosarcoma cells using a RNA interference technique with stable transfection of short hairpin RNA (shTRAP1) against the chaperone (this

was done by the Prof. Esposito group at the University of Napoli). TRAP1 protein levels were decreased of more than 80% with respect to SAOS-2 cells transfected with a scrambled shRNA (fig.32).The two cell types were called SAOS-2 shTRAP1 and SAOS-2 shSCR, respectively.

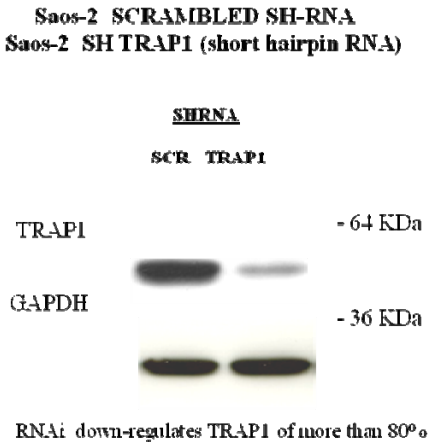


Figure 32. Analysis of TRAP1 expression level in SAOS-2 shSCR and SAOS-2 shTRAP1 cells.
GAPDH was used as a protein loading control.

I exploited these cell models to evaluate the role of TRAP1 in the response to stress stimuli, to explore the hypothesis of a role of this chaperone in the regulation of PTP opening and, more in general, in the survival of tumor cells exposed to adverse environmental conditions.

I performed experiments of cell death induction using a panel of diverse stimuli: diamide, TNFα and glucose deprivation. All these conditions induce oxidative stress, which in turn can prompt pore opening (this was already shown for diamide; Petronilli et al.1994); and TRAP1 was reported to act as anti-oxidant molecule (Montesano Gesualdi et al.2007). Cell death analysis was performed with a cytofluorimetric inspection (see Methods),and SAOS-2 shTRAP1 cells turned out to be sensitized to all these death stimuli (fig. 33). The involvement of reactive oxygen species (ROS) as cell death inducers was confirmed in the case of glucose starvation by the use of an anti-oxidant, NAC (N-acetyl Cysteine), which abrogated toxicity (fig. 33).

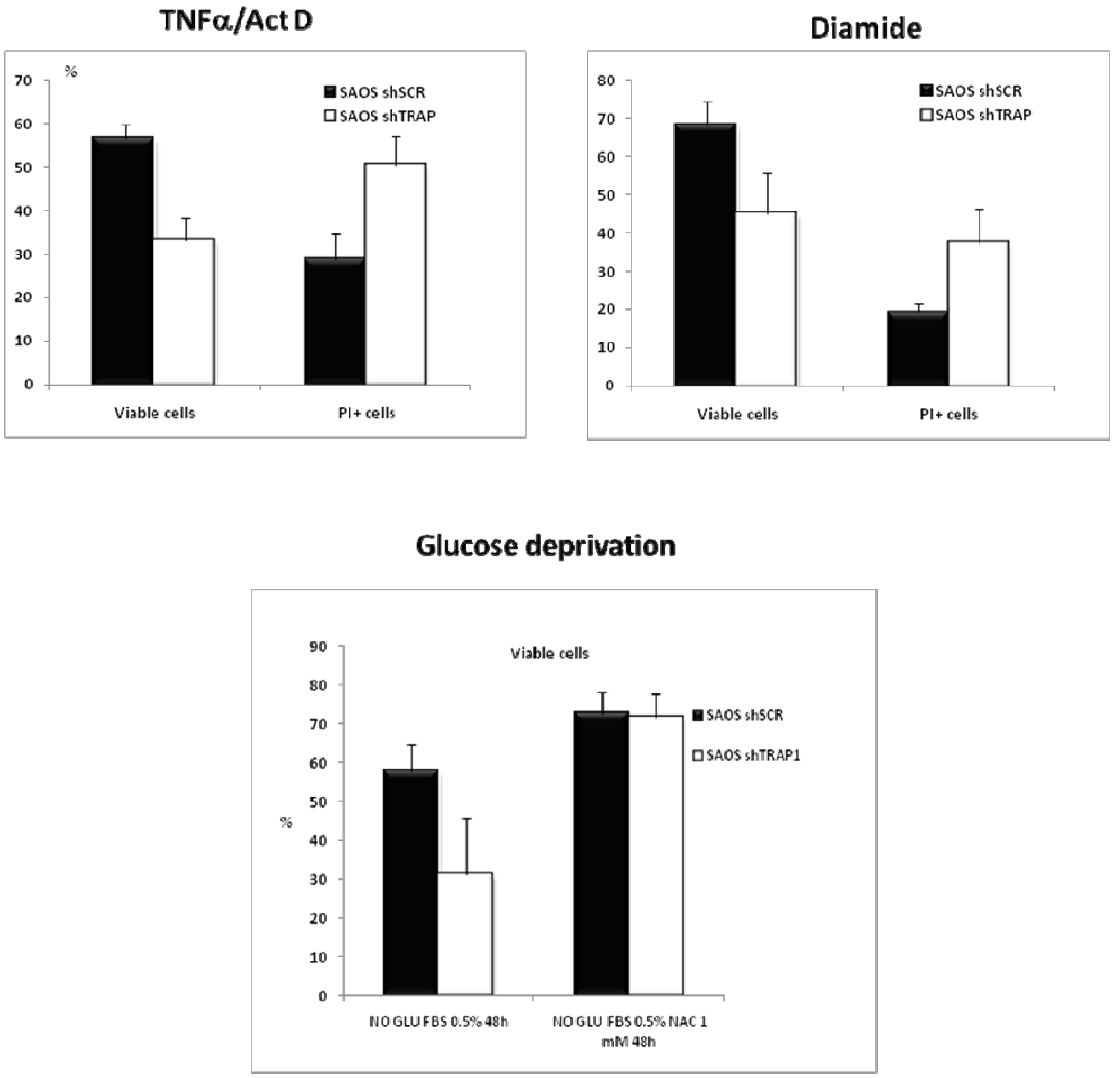


Figure 33. Knocking-downTRAP1 expression sensitizes SAOS-2 cells to several death stimuli.

SAOS-2 shSCR and SAOS-2 shTRAP1 cells were treated with TNFα (50μg/ml) plus Actinomycin D (0,25 μg/ml) for 24h; with diamide (500μM); or plated in a no glucose, low serum (0.5%) medium for 48h, with or without N-acetyl Cysteine (1mM). Cell death was measured cytofluorimetrically after staining with Annexin V-FITC and PI. Bar graphs show the percentage of both viable (double negative) and dead (PI positive) cells.

ROS are important second messenger, and cells can utilize ROS for transmitting a variety of signals or, above a certain concentration threshold, as death inducers. In particular their role in cancer progression is controversial because they have been described to favor both cancer progression (for example by increasing DNA mutations) and tumor cell death (Trachootham et al. 2009; D'Autréaux and Toledano 2007; Grek and Tew 2010). The PTP can be activated by ROS (Rasola and Bernardi 2007). To investigate any PTP modulation by TRAP1, I performed a

CRC assay comparing the responses of SAOS-2 shSCR and SAOS-2 shTRAP1 to the pore inducer diamide (fig.34). Diamide was more effective as a pore inducer, in SAOS-2 shTRAP1 cells than in SAOS-2 shSCR cells and this difference was statistically significant ($p< 0.001$ with a Student's *t* test).

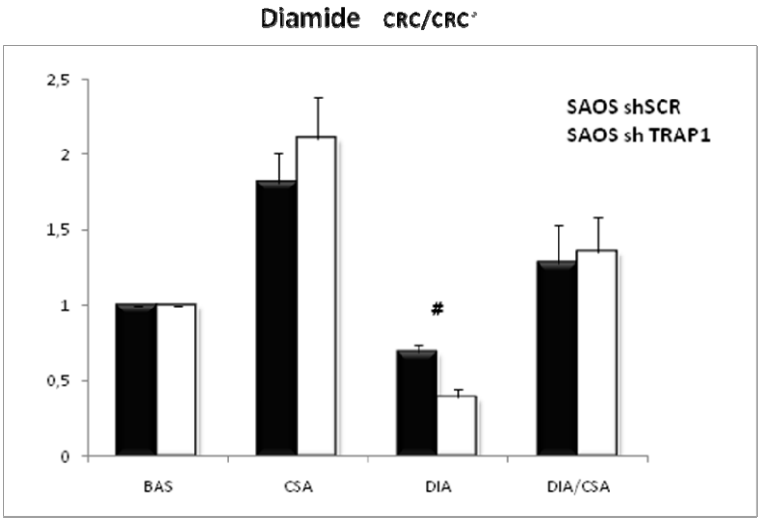


Figure 34. Knocking-down TRAP1 expression sensitizes SAOS-2 cells to PTP opening.
In this Ca2+ retention capacity (CRC) experiment, SAOS-2 shSCR and SAOS-2 shTRAP1 cells were treated with Diamide (50μM) with or without CsA (4μM) for 3' before starting recordings. Bar graphs display the ratio between the CRC detected in the presence (CRC) and absence (CRC0) of each treatment. Results are mean±SD of at least 4 experiments. PTP sensitization to diamide by knocking-down TRAP1 is statistically significant (Student's *t* test analysis; #: $p<0.01$).

After showing that TRAP1 protects from oxidative stress by inhibiting PTP opening, I investigated whether it displays a constitutive activity in diminishing cellular ROS levels. I quantified hydrogen peroxide production in total lysates with a spectrophotometer assay based on the substrate Amplex-Red (see Methods), and I found a statistically significant increase of about 35% in cellular hydrogen peroxide levels in cells with low levels of TRAP1 with respect to their wild-type counterparts (fig. 35). In particular cells SAOS-2SH TRAP1produces more in hydrogen peroxide and this connected to TRAP1 absence.

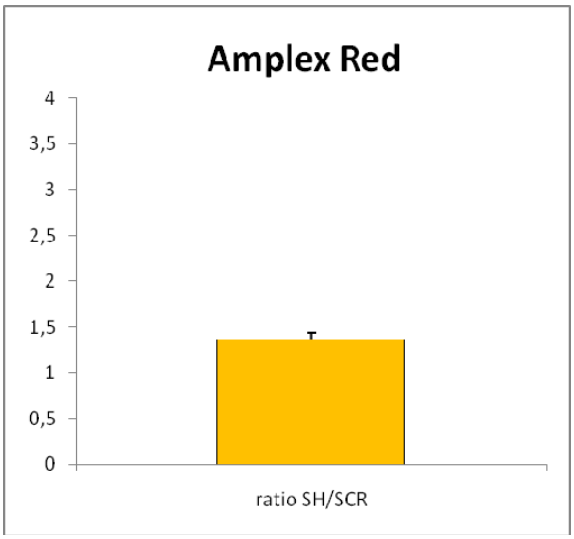


Figure 35. Hydrogen peroxide quantification with Amplex-Red. In the graph, the ratio between SAOS-2 shTRAP1and SAOS-2 shSCR cells is reported. The assay was performed as described in Materials and Methods.

4.7 Regulation of cellular respiration by TRAP1

As the mitochondrial respiratory chain is the principal source of ROS in the cell, I investigated whether cellular respiration was affected by TRAP1expression. This could have a more general relevance in tumorigenesis, as cancer cells are characterized by a decrease in oxidative phosphorylation rate as part of the Warburg effect. I measured in vivo oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in SAOS-2 cell monolayers, using the extracellular flux analyzer XF-24 (see Methods). After titrating cells with the uncoupler FCCP to measure the maximal respiration (data not shown), I treated cells with a sequence of four stressors of the respiratory chain: oligomycin, an ATP synthase blocker, to understand what fraction of the respiratory rate was devoted to ATP synthesis; FCCP itself, to reach the maximal respiration;rotenone, in order to block respiratory chain complex I, and antimycin A,, an inhibitor of complex III activity.

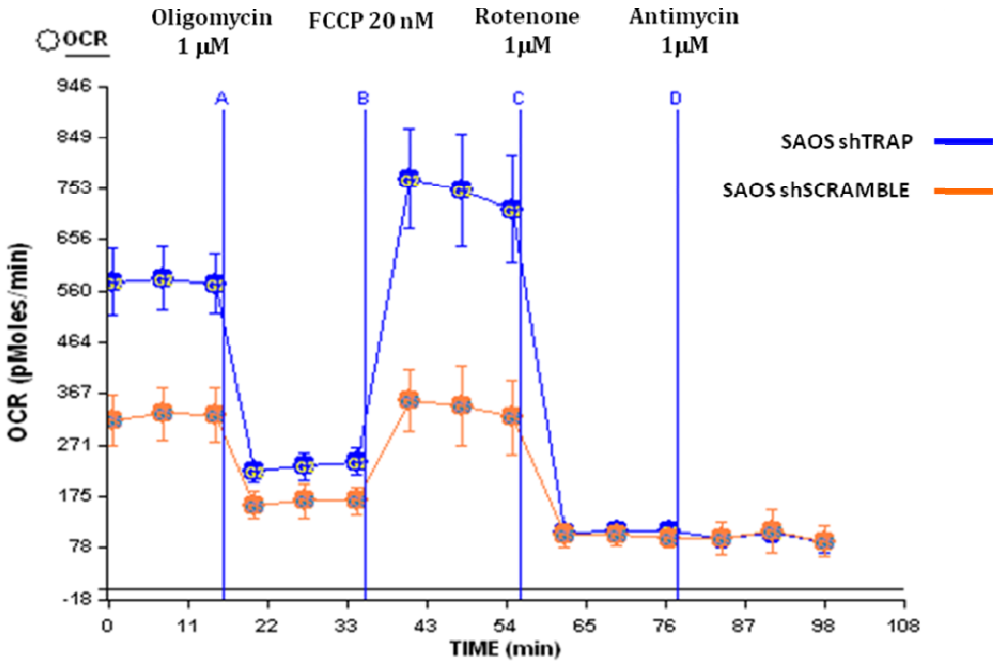


Figure 36. TRAP1 silencing increases cellular respiration in SAOS-2 cells. In the graph, kinetic changes of oxygen consumption rate (OCR) are reported; cells were sequentially treated with oligomycin (1 µM), FCCP (20 nM), rotenone (1 µM), and antimycin (1 µM). Traces are orange and blue for SAOS-2 shSCR and SAOS-2 shTRAP1cells, respectively, r

With this approach I obtained three different pieces of information (fig. 36). First: SAOS-2 shTRAP1 cells have a basal OCR that is almost the of SAOS-2 shSCR cells; second: SAOS-2 shSCR cells are respiring at the maximal velocity in basal conditions, as shown by the lack of OCR increase following FCCP treatment; third, the OCR difference between SAOS-2 shSCR and SAOS-2 shTRAP1 could depend on a Complex I inhibition, because rotenone virtually abrogates OCR, which reach the same baseline in the two cell types. To avoid any artifact, these parameters were I carefully normalized for the number of cells and for the mitochondrial mass, assessed respectively with protein determination and with 10-N Nonyl-Acridine-Orange (NAO) staining (NAO is a fluorescent probe that binds cardiolipin in the mitochondrial membrane; data not shown). Therefore, I considered the possibility that there is a different activity of Complex I in SAOS-2 cells with high or low SH TRAP1 protein level, and that this difference is responsible for OCR changes. To clarify this point I performed Complex I *in vitro* activity assay.

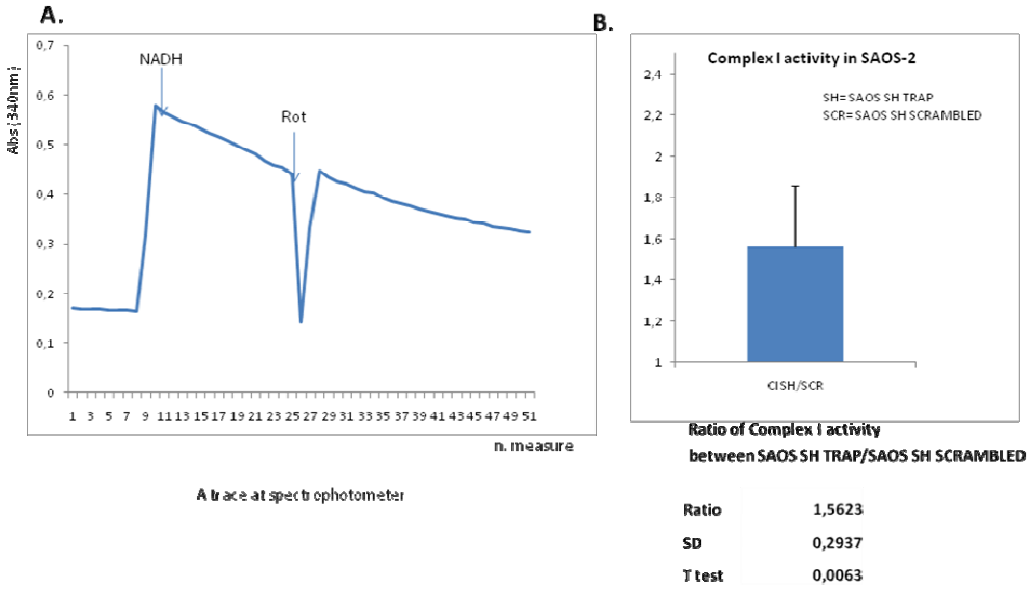


Figure 37. Knocking-down TRAP1 protein expression increases activity of respiratory chain Complex I in SAOS-2 cells. A. typical result of a spectrophotometric measurement at 340 nm; the decrease in absorbance indicates oxidation of exogenous NADH. Rot, addition of rotenone (20 μM). **B.** quantification of the ratio of Complex I activity between SAOS-2 shTRAP1 and SAOS-2 shSCR cells. Data were normalized for the protein content and for citrate synthase activity. The increase in Complex I activity caused by RNA interference on TRAP1 is statistically significant following a Student's *t* test analysis ($p<0.01$).

As shown in fig. 37B, silencing TRAP1 leads to a significant increase in Complex I activity of about 1,5 times. To understand if this result has a general meaning I moved to other tumor models. TRAP1 was silenced in another tumor cell line (by the Prof. Esposito group at the University of Napoli), HCT-116 colon carcinoma cells (fig. 38A). in accord with results obtained in SAOS-2 cells, also in HCT-116 cells I observed an increase of about two times in Complex I activity when TRAP1 was knocked-down (fig. 38B).

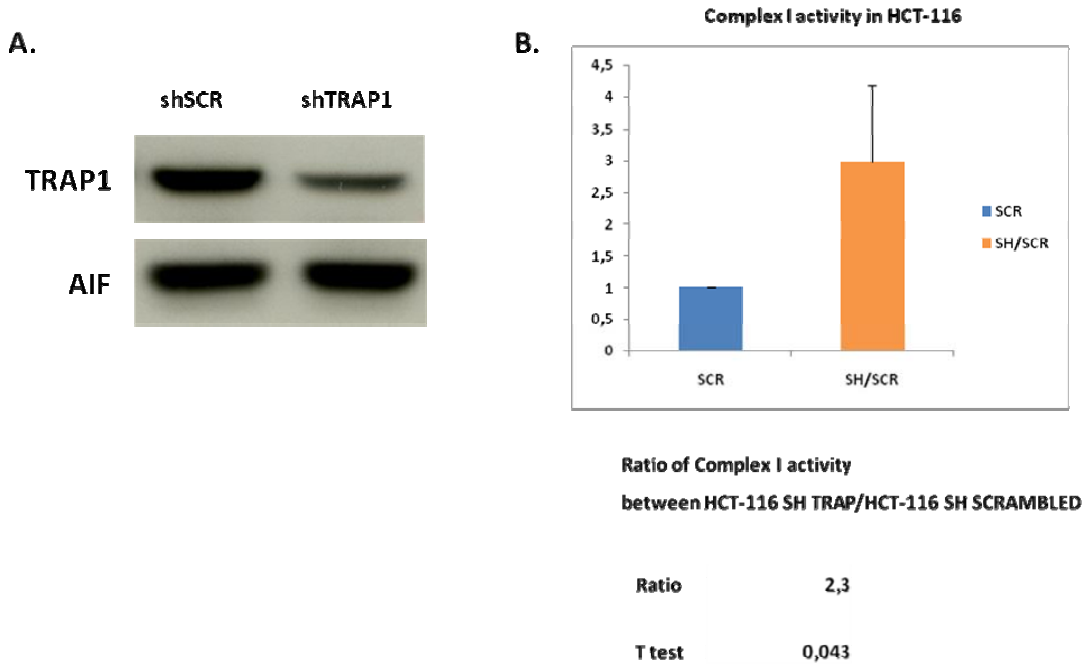
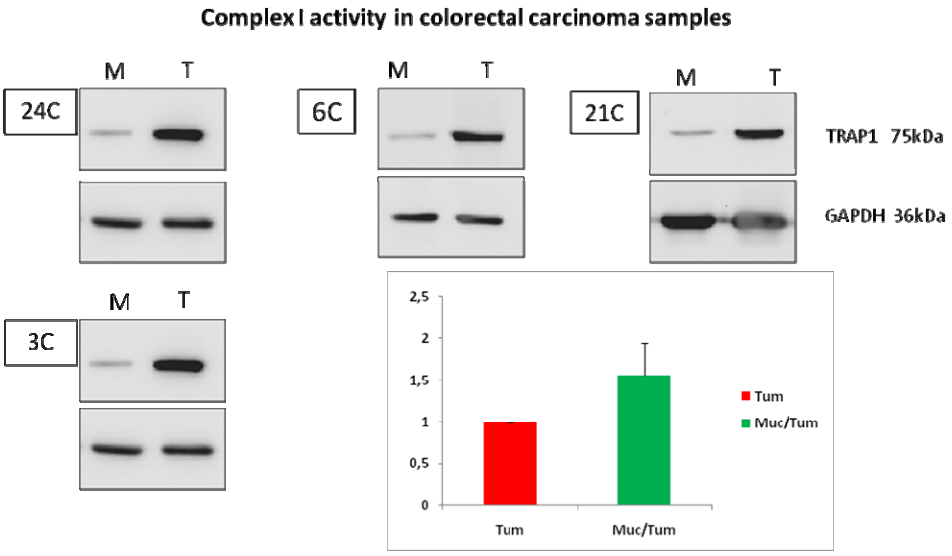


Figure 38. TRAP1 interference increases Complex I activity also in HCT-116 colon carcinoma cells.
A, Western immunoblot showing TRAP1 protein downregulation following stable transfection of HCT-116 cells with either a shRNA SCR or a shRNA for TRAP1. AIF was used as a protein loading control. B, Analysis of Complex I activity in HCT-116 shSCR or in HCT-116 shTRAP1 cells is reported as in fig.37.

I extended my analysis towards human cancer biopsies (collected by dr. Landriscina, University of Foggia). I measured Complex I activity in a panel of colon carcinoma samples, and I compared the result to healthy mucosae derived from the adjacent, non-transformed tissue of every patient. TRAP1 expression is increased in tumor samples compared to mucosae (fig. 39). In accord with the previous analyses, I found that increased TRAP1expression decreases Complex I activity in tumor homogenates. (fig. 39A)

A.

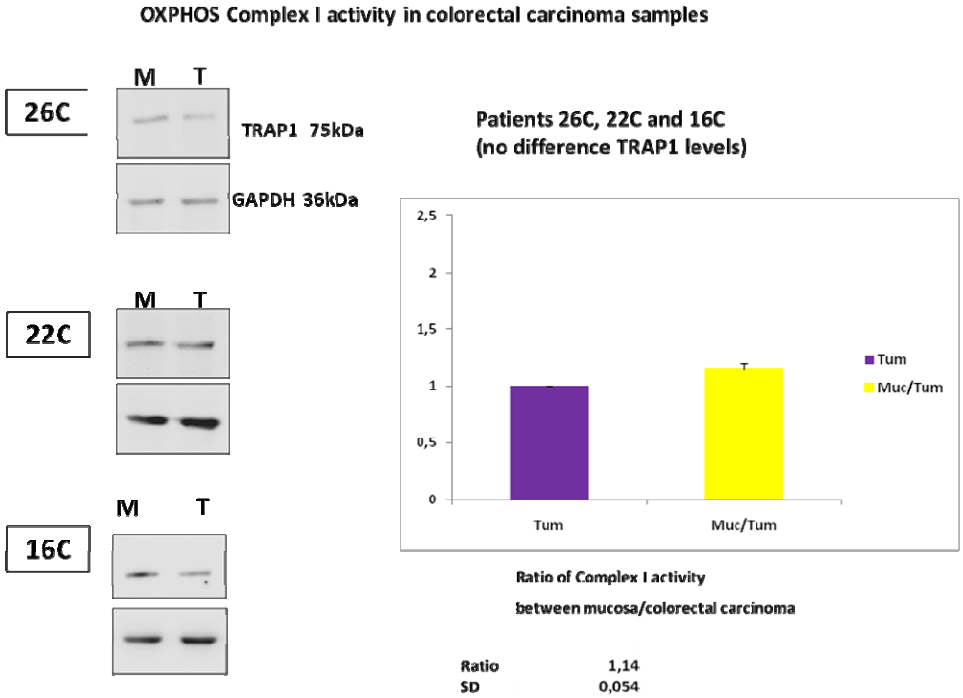


Ratio of Complex I activity

between mucosa/colorectal carcinoma

Ratio	1,55
SD	0,38
T test	0,05

B.



Ratio of Complex I activity

between mucosa/colorectal carcinoma

Ratio	1,14
SD	0,054

Figure 39. TRAP1 expression levels and activity of respiratory chain Complex I in colorectal cancer samples from human patients. Western immunoblot show TRAP1 expression levels in mucosae (M) and tumors (T) samples. GAPDH was used as a protein loading control. Complex I activity was assayed in homogenates derived from each biopsy; the ratio between Complex I activity in mucosa and in tumor samples of each patient is calculated, and the mean value reported in the bar graphs as in fig.37. In **A,** results of a group of patients showing increased TRAP1 levels in tumors *vs.* mucosa; in **B**, results of a group of patients showing no TRAP1 variation.

I performed the same analysis on samples in which there is no difference in TRAP1 expression level between colon carcinoma and healthy mucosa, and I could not detect any variation in Complex I activity between tumors and mucosae (fig. 39B).

Altogether, these data suggest that TRAP1 is a regulator of Complex I activity in tumors. To support this hypothesis, I looked for a direct interaction between Complex I and TRAP1 in cancer cells. To this aim, I immunoprecipitated total Complex I, both in SAOS-2 cells and in tumors obtained from *in vivo* tumorigenesis assays (see below), and I found that TRAP1 associates to it (fig.40). Interestingly, also ERK co-immunoprecipitated with TRAP1/Complex I both in vitro and in vivo (fig. 40), suggesting an important regulatory role of this kinase on respiratory activity during tumorigenesis.

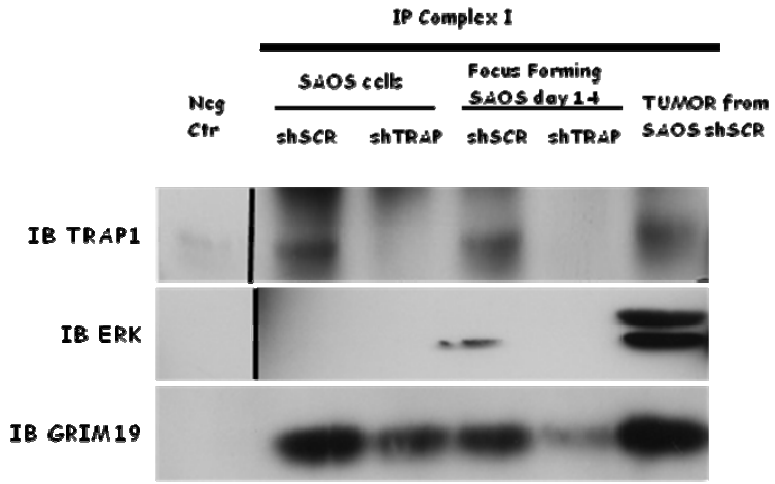


Figure 40. Immunoprecipitation against Complex I. The Western blot was incubated with antibodies against TRAP1 to see if there is a modulation in its levels; a subunit of Complex I (GRIM-19) as control of immunoprecipitation, and ERK kinase. In the first lane from left was loaded as negative control a sample from SAOS-2 shSCR cells with Sepharose alone without antibodies. In the second lane was loaded immunoprecipitated from SAOS-2 shSCR or shTRAP1cells that were in normal growth condition then in the next lanes immunoprecipitated from lyastes of osteosarcoma cells at the 15th day of focus forming.

4.8 TRAP1 is necessary for tumorigenesis

Overexpression of TRAP1 in tumor samples and its role as a PTP- and cell death inhibitor suggested that it could play an important role during neoplastic transformation. To study TRAP1 involvement in tumorigenesis I used both *in-vitro* and *in-vivo* approaches . A first *in vitro* experiment was the focus forming assay. After several days in culture, transformed cells are characterized by the capability to escape contact inhibition, forming aggregates called foci. I measured number and size of foci formed by SAOS-2 shSCR and SAOS-2 shTRAP1 cells kept in culture for three weeks at different serum concentrations, and found that knocking-down TRAP1 almost abolishes the transforming capacity of SAOS-2 cells (upper graph of fig. 41).

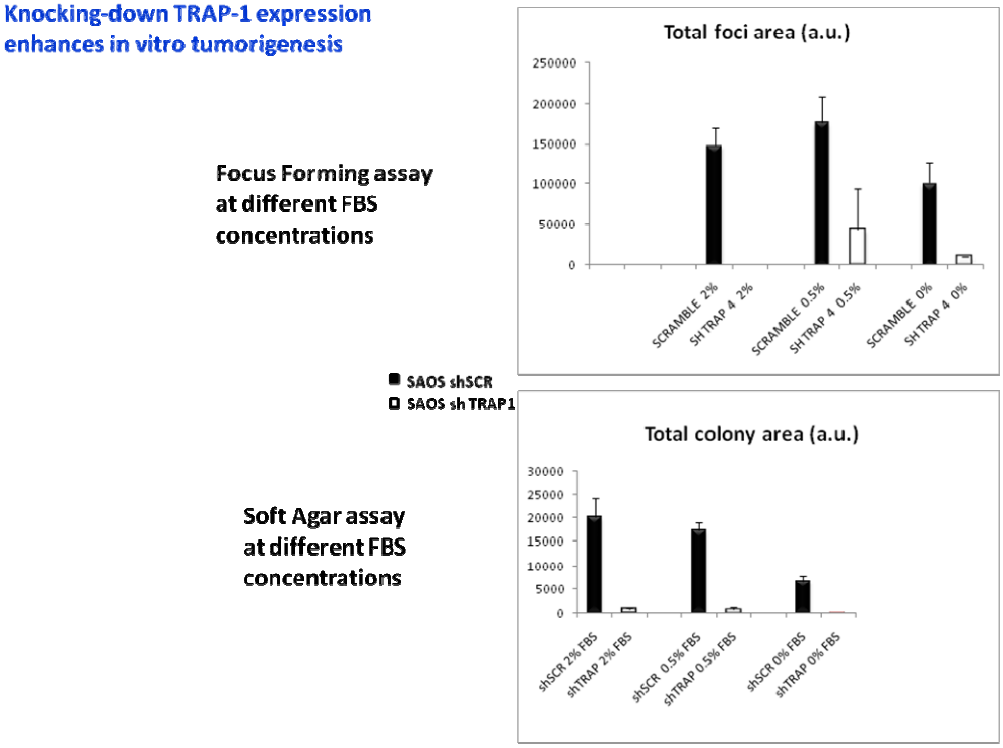


Figure 41. *In vitro* tumorigenesis assays on SAOS-2 shSCR and SAOS-2 shTRAP1 cells. In the upper part of the figure, the result of a focus forming assay is reported, whereas the outcome of a soft agar assay is in the lower graph. Bars indicate the total area of foci or of colonies (upper and lower graph, respectively), in arbitrary units. Data were obtained in the presence of different serum concentrations (2%,0,5% and 0%). Values are the mean±SD of at least three experiments.

A second experiment of *in vitro* tumorigenesis, the soft agar assay, determines the capacity of cells to grow in conditions of anchorage independence, forming tridimensional aggregates called colonies; this is another typical feature of cancer cells. SAOS-2 shSCR cells were able to form colonies at all serum concentrations, and again RNA interference on TRAP1 virtually abrogates colony formation (fig. 41, bottom graph). Altogether these assays indicate that TRAP1 is essential for tumorigenesis, at least in *in vitro* conditions, but the molecular mechanism(s) by which TRAP1 influences tumor growth remain poorly understood. I have found that TRAP1 protects cells from insults that induce oxidative stress (fig.33). Therefore, I carried out a soft agar assay in the presence of antioxidants, the vitamin E analogue Trolox. In these conditions I could measure a dramatic increase in colony formation (fig. 42), suggesting that an efficient anti-oxidant machinery is crucial for tumor development.

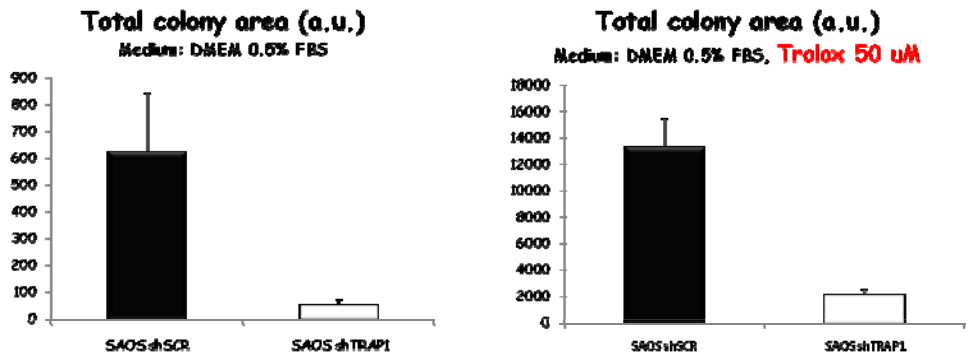


Figure 42. Soft agar assay on SAOS-2 shSCR and SAOS-2 shTRAP1 cells.. Data are reported as in fig.41. In the graph on the right, bars report colony growth when cells were kept in the presence of the anti-oxidant Trolox (50 μM). Note the different scales between graphs on the left and on the right.

Analysis of in vitro tumorigenesis properties is an interesting approach, but it does not tell anything about the role of TRAP1 in a real process of neoplastic development. Hence, I performed an in vivo tumorigenesis assay, in collaboration with the group of Prof. Deilippi, University of Torino. SAOS-2 shSCR and SAOS-2 shTRAP1 cells were inoculated in SCID mice and tumor growth was followed for 21 days. The experiment was carried out in two different conditions, with or without Matrigel, a protein mixture that mimics extracellular matrix and favours cancer cell aggregation and growth of a tumor mass. Remarkably, SAOS-2 shSCR formed solid growing tumors, whose growth was enhanced by Matrigel, whereas SAOS-2 shTRAP1 were totaaly unable to form tumors in SCID mice (fig.43)

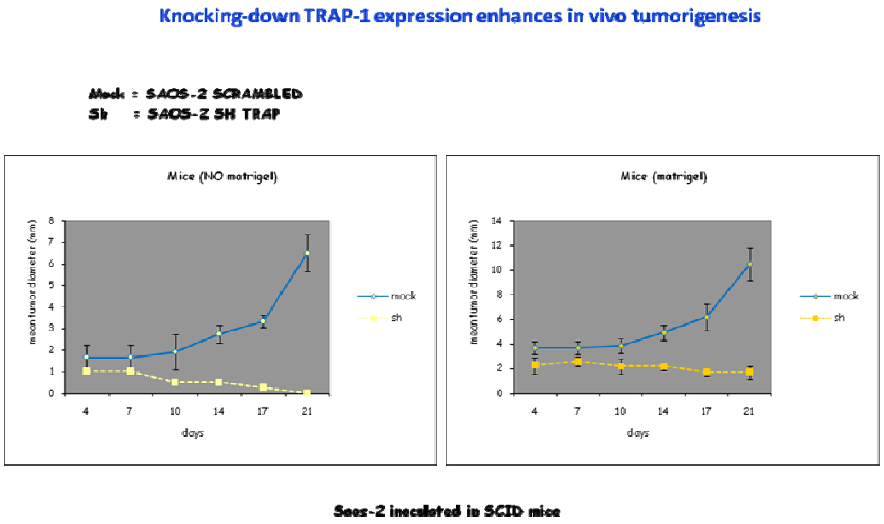


Figure 43. Tumor growth in SCID mice injected with SAOS-2 shSCR or SAOS-2 shTRAP1 cells. Tumor growth is reported as increase in the diameter of the tumor mass (in mm, on the *y* axis) during three weeks (*x* axis). Tumors derived from SAOS-2 shSCR or SAOS-2 shTRAP1 cells are indicated in blue and yellow, respectively. In the experiment reported on the right, cells were injected within a Matrigel suspension.Values are the averages of 8 different tumors.

5. Discussion

Cancer cells are characterized by the capability to escape cell death avoiding normal apoptotic signals. Integration of pro-survival and death stimuli converge on mitochondria where the channel called permeability transition pore (PTP) displays a key role in the commitment of cells to death. PTP has a pivotal role in several diseases and its opening is regulated by mitochondrial chaperones CyP-D and TRAP1. Chronic activation of the Ras/ERK signaling pathway is frequently induced in neoplastic transformation and it correlates with worsening of tumor stage and grade. I have found evidence that a functional circuit connects the Ras/ERK transduction pathway and the mitochondrial PTP and that in cancer cell models mitochondrial activation of ERK results in desensitization of pore opening and increased resistance to death stimuli, a finding that has major implications for tumorigenesis. I found that ERK is constitutively activated in mitochondria of cancer cells, but not in immortalized, non-transformed prostate cells. Dysregulation of pore opening has been implicated in several diseases and several observations link the PTP to tumor progression. The finding that CyP-D it a downstream target of ERK through GSK3 activity modulation opens to new targets and strategies for therapeutic intervention. ERK acts as a priming kinase on GSK-3 (Ding et al.2005), therefore complete GSK-3 inhibition requires the activity of a second kinase; in addition, also GSK-3 itself needs a priming kinase to phosphorylate its target. For these reasons, the network of kinase regulatory interactions on CyP-D and on the PTP must be more complex than what we have shown, and further investigations to dissect these molecular mechanisms are therefore necessary. Regulation of CyP-D by post-traslational modifications is a starting point for the study of pore opening regulation in different diseases and opens to the possibility of study other signaling pathway possibly involved mitochondrial functionality and in cell death. For these reasons I focused my attention also on the study of novel inhibitors of CyP-D that could be useful in other therapies. We found that Antamanide, a cyclic peptide from *Amanita*

phalloides inhibits CyP-D and pore opening in CRC and cell death experiments. In tumor cells another chaperone is known to modulate PTP opening (Kang et al.2007) the heat shock protein TRAP1. We found TRAP1 associated with CyP-D and ERK in mitochondria of SAOS-2 human osteosarcoma cells and we characterized some of its activities in this model. First we have found that TRAP1 regulates PTP, in fact its silencing is linked to decreased threshold for pore opening in presence of several pro-apoptotic stimuli. TRAP1 is also well studied as an anti-oxidant protein. In this context I found that cells silenced for TRAP1 produces more hydrogen peroxide and that are more sensitive to stimuli connected to ROS production that are important factors for control of tumor progression. I also found that TRAP1 is essential for both *in vitro* and *in vivo* tumorigenesis: without high expression of TRAP1 aggressive tumorigenic cell SAOS-2 are not able to grow in immune-compromised SCID mice neither form colonies in softagar or foci in focus forming assay. We have shown that ROS increase due to silencing of TRAP1 correlated with an increase in respiration rate, and in particular in Complex I increased activity. TRAP1 interacts directly with Complex I in immunoprecipitations from SAOS-2 cells lysates and up-regulation of TRAP1 levels correspond to decreased Complex I activity in vitro experiments not only in cell lines but also samples from human patients. For all these evidences, TRAP1 is an interesting target for therapeutic intervention in cancer. The involvement of TRAP1 in Complex I regulation could be important not only for cancer progression but also for Parkinson disease. As described in the introduction TRAP1 is the target of PINK1, a kinase whose inactivating mutations lead to some some genetic forms of Parkinson disease. In this context regulation of complex I through TRAP1 could be a new target of therapy.

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