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Mutant p53-dependent alterations of cancer metabolism and tumor microenvironment in pancreatic adenocarcinoma cells

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Coordinator: Prof.ssa Lucia De Franceschi

Tutor: Prof. Massimo Donadelli

Doctoral Student: Dott.ssa Giovanna Butera

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Giovanna Butera

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1. ABSTRACT

Pancreatic adenocarcinoma (PDAC) is one of the most aggressive and devastating human malignancies. Late diagnosis is due to an absence of specific symptoms at initial stages. In about 70% of PDACs, the tumor suppressor gene *TP53* is mutated generally resulting in conformational changes of mutant p53 (mutp53) proteins, making an important key in the carcinogenesis process not only through loss of wild type activity, but also through gain of specific mutant functions. In contrast to the tumor suppressive roles of wild-type p53, mutant p53 proteins support cancer progression by enhancing the ability of cancer cells to invade and metastasize, to confer chemoresistance, and to stimulate genomic instability. We focused our attention on novel molecular mechanisms by which gain of function (GOF) mutant p53 proteins play their oncogenic roles promoting cancer cell proliferation and chemoresistance. The main project is based on intracellular alterations induced by mutant p53 in cancer metabolism and reactive oxygen species (ROS) production, contributing to cancer development and aggressiveness. ROS are highly reactive byproducts of mitochondrial oxidative phosphorylation and are implicated in a plethora of biological events addressed to sustain each aspect of human cancer being able to act as second messengers in cellular signaling. In particular, we unveiled that mutp53 is able to inhibit SESN1 expression and consequently the amount of SESN1/AMPK complex, resulting in the downregulation of the AMPK/PGC-1α/UCP2 axis and ROS production. In this way GOF mutant p53 proteins, contrarily to its wild-type p53 counterpart, lead i) antiapoptotic effects, ii) proliferation and iii) chemoresistance in PDAC cells. These oncogenic roles given by GOF mutp53 are also detected through another mechanism that supports glycolytic metabolism in PDAC cells. Indeed, we demonstrated that mutant p53 prevents the nuclear translocation of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) stabilizing its cytoplasmic localization, thus supporting glycolysis of cancer cells and inhibiting cell death mechanisms mediated by nuclear GAPDH. We further show that the prevention of nuclear localization of GAPDH is mediated by both stimulation of AKT and repression of AMPK signaling, and is also associated with the formation of SIRT1:GAPDH complex. The blockage of GAPDH mutp53-dependent cytoplasmic stabilization is able to restore

the sensitivity of PDAC cells to the treatment with gemcitabine, permitting cancer cells to acquire sensitivity to anti-glycolytic drugs and suggesting a potential personalized therapeutic approach in human cancers carrying mutant *TP53* gene. In addition, we addressed our research on the extracellular roles of mutant p53 in the tumor microenvironment of PDAC cells. The cancer secretome is a rich repository to find useful information for both cancer biology and clinical oncology. A better understanding of biological features that are common or peculiar to different tumors could allow the identification of specific prognostic/predictive biomarkers for early diagnosis and tumor progression monitoring. This is particularly relevant for PDAC, which has extremely high mortality rate and is mainly due to lack of recognizable symptoms and exact assays for early detection. The objective of this study was to recognize a specific signature of biomarkers secreted by PDAC cells carrying GOF mutant p53. Comparing the secretome of p53-null PDAC cells before and after ectopic overexpression of R273H-mutp53 and R175H-mutp53, we found 23 differentially secreted proteins by both mutant p53 isoforms that might constitute a secreted signature driven by the hot-spot p53 mutants in PDAC. Furthermore, we also studied the functional effect of mutp53-driven secretome on cancer cells showing its influence on proliferation, chemoresistance, apoptosis, autophagy, and cell migration. These data constitute a prerequisite for the identification of a secreted biomarker signature for the early identification of mutant p53 PDAC patients. In conclusion, the discovery of novel mechanisms by which hot-spot mutant p53 isoforms induce pancreas cancer growth is crucial to identify specific and personalized therapies for PDAC patients bearing mutant *TP53* gene, representing a major therapeutic challenge for modern molecular oncology.

2. SOMMARIO

L’adenocarcinoma pancreatico duttale (PDAC) è una malattia letale e rappresenta una delle principali cause di morte per cancro. La diagnosi tardiva è dovuta ad un'assenza di screening efficaci che permettano di diagnosticarlo nei primi stadi della malattia. Ciò si traduce in un frequente ritardo nella diagnosi, che spesso viene diagnosticata solo quando il tumore è già in uno stadio avanzato e si è diffuso in altre parti del corpo. In più del 70% dei casi è presente un gene *TP53* mutato nelle cellule PDAC. P53 è un fattore di trascrizione che regola il ciclo cellulare e ricopre una importante funzione di soppressore tumorale. La maggior parte delle mutazioni presenti nel gene che codifica per p53 sono mutazioni missenso che causano l’espressione di isoforme di p53. Così, la proteina mutata non solo perde la sua funzione *wild type* ma può acquisire nuove proprietà biologiche chiamate *gain-of-function (GOF)* le quali contribuiscono allo sviluppo della patologia neoplastica. Lo scopo di questa tesi è stato quello di scoprire nuovi meccanismi molecolari attraverso i quali la proteina p53 mutata contribuisce alla progressione tumorale ed alla chemioresistenza al fine di identificare dei potenziali target terapeutici. Uno di questi meccanismi identificati è incentrato sullo studio delle specie reattive dell’ossigeno (ROS), che sono note indurre instabilità genomica ed altre alterazioni che favoriscono lo sviluppo di patologie quali il cancro. In questa tesi dimostriamo che le diverse isoforme di p53 mutata inducono alti livelli di ROS, attraverso l’inibizione di proteine antiossidanti, quali UCP2, ed identifichiamo una via di regolazione che coinvolge l’asse SESN1/AMPK/PGC-1α/UCP2. In questo modo, dimostriamo che p53 mutata favorisce la crescita delle cellule tumorali e chemioresistenza. Queste capacità oncogeniche date dalla proteina p53 mutata vengono riscontrate attraverso un secondo meccanismo incentrato sul metabolismo energetico delle cellule tumorali. Infatti, un altro obiettivo della tesi è incentrato sulla regolazione intracellulare dell’enzima glicolitico GAPDH (gliceraldeide 3-fosfato deidrogenasi) da parte della proteina p53 mutata con conseguente modulazione della proliferazione delle cellule tumorali. GAPDH è una proteina multifunzionale, capace di svolgere altri ruoli oltre al suo ruolo principale di enzima glicolitico. I risultati ottenuti dimostrano che p53 mutata stabilizza la proteina GAPDH nel citosol, stimolando l’effetto Warburg e impedendone

l’attivazione di meccanismi di morte cellulare indotti dal GAPDH nucleare. Investigando come questo avvenga, scopriamo che la stabilizzazione di GAPDH nel citosol data da p53 mutata, avviene atttraverso la stimolazione della proteina chinasi AKT e dalla repressione della chinasi AMPK ed è inoltre associata alla formazione del complesso GAPDH:SIRT1. Inoltre, il blocco della traslocazione nucleare del GAPDH da parte di p53 mutata rende più sensibili le cellule PDAC al trattamento di droghe antiglicolitiche; in questo modo GAPDH potrebbe essere un target terapeutico per questo tipo di cancro che possiede il gene *TP53* mutato. Il secondo progetto si basa sullo studio di biomarcatori secreti in cellule di PDAC con p53 mutato. Una migliore comprensione delle caratteristiche biologiche comuni e/o specifiche nei diversi tumori potrebbe consentire l'identificazione di specifici biomarcatori prognostici e/o predittivi per la diagnosi precoce e il monitoraggio della progressione tumorale. Questo è maggiormente richiesto in tumori come PDAC a causa della mancanza di sintomi e saggi riconoscibili per la diagnosi precoce. Il secretoma del cancro rappresenta il microambiente tumorale che svolge un ruolo chiave nei processi che promuovono la formazione dei tumori come l'angiogenesi e l'invasione. Confrontando il secretoma di cellule PDAC non esprimenti la proteina p53, con quelle in cui si ha una sovraespressione ectopica di R273H-mutp53 e R175H-mutp53, abbiamo trovato 23 proteine differenzialmente secrete da entrambe le isoforme mutanti di p53 che potrebbero costituire dei potenziali marcatori specifici per l’identificazione precoce di PDAC con p53 GOF. Inoltre, abbiamo anche studiato l'effetto funzionale del secretoma guidato da p53 GOF nelle cellule tumorali mostrando la sua influenza sulla proliferazione, la chemioresistenza, l'apoptosi e l'autofagia, così come sulla migrazione delle cellule. In conclusione, la scoperta di nuovi meccanismi mediante i quali p53 mutato stimola la progressione tumorale è importante per identificare terapie specifiche e mirate in tumori, esprimenti geni mutati per p53, che rappresentano una delle principali sfide terapeutiche per la moderna oncologia molecolare.

3. INTRODUCTION

Pancreatic cancer is one of the most frequent causes of tumor-associated deaths, and its incidence has recently increased in the western world [1]. There are different types of pancreatic cancer divided into two main groups; exocrine tumours that start in the exocrine cells where enzymes which help food digestion are made; endocrine tumours, also known as neuroendocrine tumours, that start in the endocrine cells which release insulin and other hormones. Most pancreatic cancers are exocrine and ductal adenocarcinomas [2]. Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic malignancy and has a poor prognosis, with a dismal overall 5-year survival rate of 5% [3]. Late diagnosis due to an absence of specific symptoms at initial stage, together with high metastatic potential, resistance to therapies, and a lack of biomarkers and screening methods, are the main causes of poor prognosis in PDAC. Standard treatments for advanced disease include therapy with gemcitabine (2′,2′-difluoro-2′-deoxycytidine; GEM) with a response rate of less than 20% [4]. Therefore, the identification of effective targets and novel therapeutic strategies to improve GEM effects in PDAC have been the topic of extensive investigation in the last few years [5]. PDAC presents genetic heterogeneity with a high number of mutations. Among the various important genes alterated in PDAC, *TP53* gene is the best-documented ones [6].

3.1 TUMOR SUPPRESSOR p53: THE GUARDIAN OF THE GENOME

P53 is one of the most important protein involved in signaling pathways that prevent tumour formation and progression. It is a transcription factor, with a modular structure formed by distinct functional domains: i) N-terminal transactivation domain (amino acid aa 1-73), which interacts with the transcriptional machinery [7]; ii) proline rich-regions (aa 63-97), which is required for p53 stabilization; iii) DNA binding domain (aa 93-312), that binds the responsive element on DNA and proteins that positively or negatively affect p53 activity, such as MDM2 or 53BP1 respectively

[8]; iv) oligomerization domain (aa 325-355),which is essential for tetramer formation and represents the active form of p53 [9] and v) C-terminal regulatory domain, containing residues post-translationally modified which are involved in modulation of its stability [10] (figure 1).

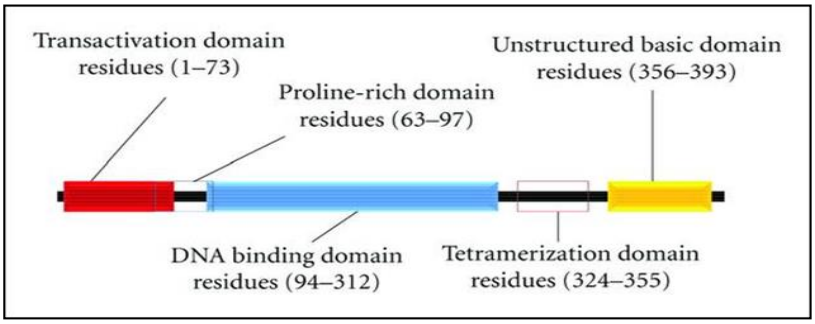


Figure 1. Multifunctional domains of p53. The p53 monomer consists of various multifunctional domains [11].

A lot of studies shows p53 at the centre of a molecular network that transduces signals deriving by stress conditions [12]. Under the non-stressed condition, the p53 protein is maintained at a low level in cells by the proteasome degradation pathway. MDM2, an E3 ubiquitin ligase, is the most critical negative regulator for p53 [13]. In response to a wide variety of stress signals, including DNA damage, nutritional starvation, hypoxia, the p53 protein is stabilized through post-translational modifications by a variety of enzymes. These enzymes include kinases, phosphatases, acetyltransferases, deacetylases, ubiquitin ligases, deubiquitinases, methylases, and sumoylases [8]. Once activated, p53 acts as a transcription factor and it becomes able to promote the coordinated expression of many target genes through the binding to specific DNA sequence in the regulatory regions of its target genes [14]. In this way, p53 regulates a wide range of cellular biological processes to maintain genomic integrity and prevent tumor formation, including cell cycle arrest, apoptosis, senescence, energy metabolism, anti-oxidant defense, autophagy, etc. (figure 2) [14], [15]. Because of its role as a key integrator in translating diverse stress signals into different cellular outcomes, p53 has been namely the “guardian of the genome”.

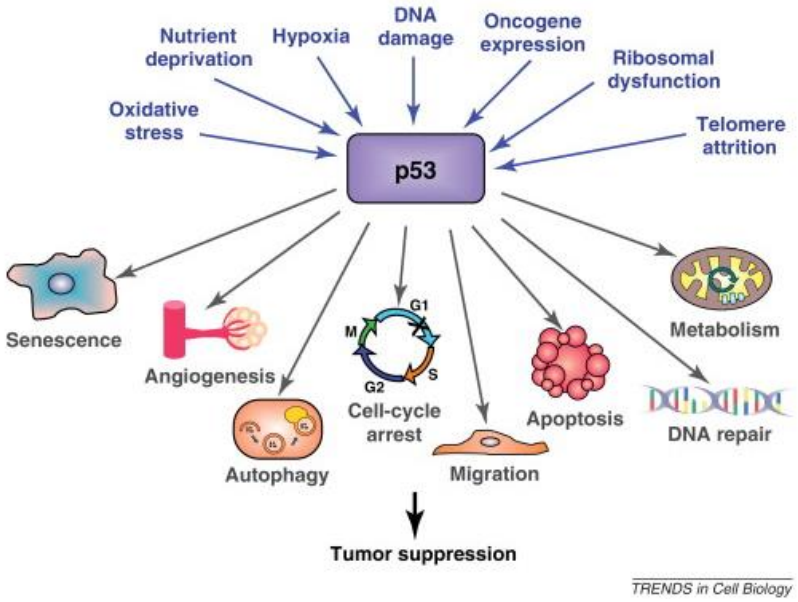


Figure 2: The p53 pathway in tumor suppression. Activation of the transcription factor p53 in response to different types of cellular stress can lead to cell survival as well as cell elimination. [15].

The importance of the p53 pathway in tumor suppression is strongly highlighted by the observation that mutations of the *TP53* gene are very frequent in human cancers. Indeed, whereas somatic *TP53* mutations contribute to sporadic cancer, germline *TP53* mutations cause a rare type of cancer predisposition known as Li-Fraumeni Syndrome (LFS) which is not associated with site-specific tumours, but rather with a variety of tumour types occurring at a relatively early age [16]. Accordingly, the absence of p53 predisposes to spontaneous development of neoplastic disease, as observed in p53 knockout mouse models [17]. Furthermore, somatic mutations in the *TP53* gene are one of the most common alterations in human cancers, occurring in more than 50% of cancer patients [18]. In patients with wild-type *TP53* gene , the p53 pathway is often compromised through the amplification of negative regulators, such as MDM2 [19] or the inactivation of upstream factors, as Chk2, ATM or p14ARF [20], [21].

3.2 MUTANT p53 AND ITS ONCOGENIC ROLE

Mutations in the *TP53* gene are among the most common gene-specific alterations in human cancers.The frequency of *TP53* gene mutations can vary considerably between cancer types, ranging from 10% in haematopoietic malignancies [22] to 50–70% in ovarian [23] and pancreas [24] cancers. Most of tumor suppressor genes, such as RB (retinoblastoma-associated protein), APC (adenomatous polyposis coli), and VHL (Von Hippel-Lindau tumor suppressor), are frequently inactivated by deletion or truncation mutations in tumors, resulting in the decreased or loss of expression of their proteins. Interestingly, the majority of p53 mutations in human cancer are missense mutations for more than 70% of them, which usually result in the expression of full-length mutant p53 proteins [25]. Although p53 mutations have been found in all coding exons of the *TP53* gene, the majority of mutations occur in the p53 DNA-binding domain, resulting in the loss of DNA-binding activity of mutant p53. In general, the p53 missense mutations can be classified into two main categories which are commonly referred to as "DNA-contact" and "conformational" mutations [26]. The first group includes mutations in residues directly involved in DNA binding, such as R248Q and R273H. The second group comprises mutations that cause local (such as R249S and G245S) or global (such as R175H and R282W) conformational distortions. The majority of p53 missense mutations occur at six 'mutational hot-spots' in the DNA-binding domain of p53, including residues R175, G245, R248, R249, R273, and R282 (figure 3)[26], [27].

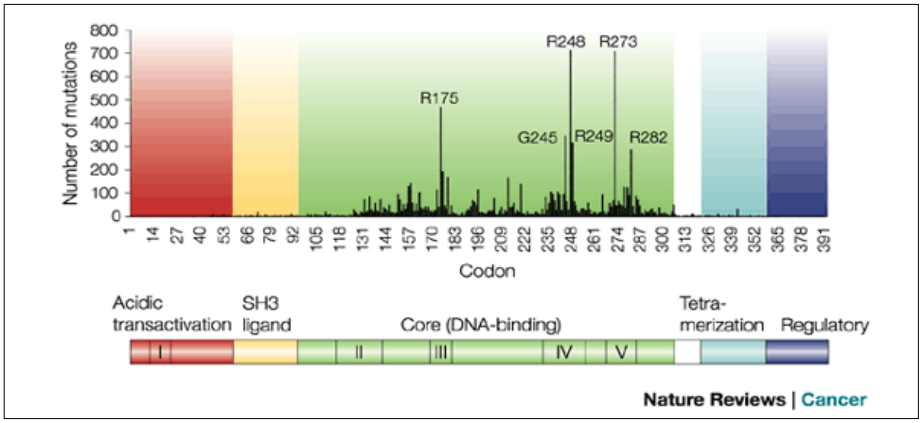


Figure 3. The distribution of hot-spot mutations along the p53 sequence [26].

While wild-type p53 protein is kept at a low level in cells by the proteasome degradation pathway under non-stressed conditions, mutant p53 protein usually accumulates to a high level in tumors, and the underlying mechanisms are not fully understood [27]. It has been well-documented that many tumor-associated mutant p53 proteins not only lose their tumor suppression functions, but also gain new oncogenic functions, totally independent on wtp53, which is termed the gain-of-function (GOF) of mutant p53, acquiring different GOF activities, including promoting cell proliferation, anti-apoptosis, metabolic changes, migration, invasion, angiogenesis, and metastasis (figure 4) [28], [29].

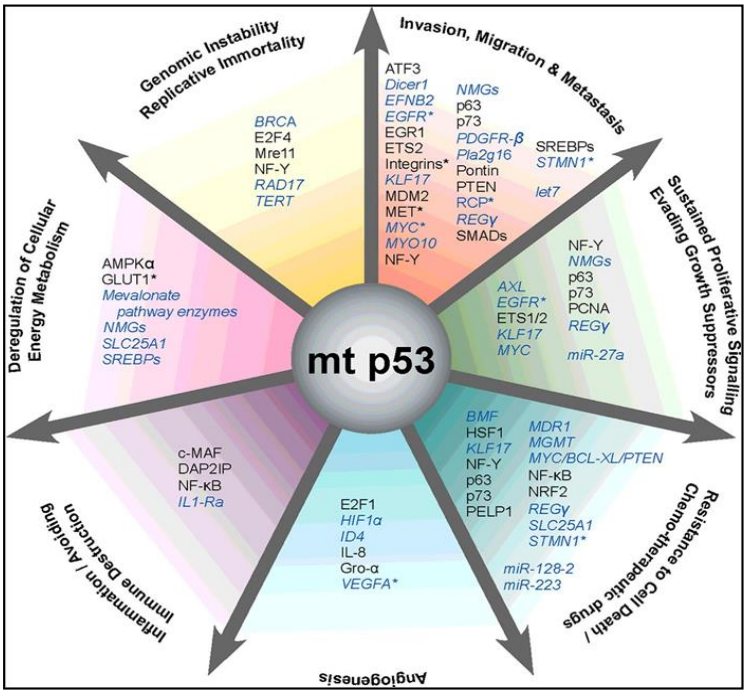


Figure 4: Selected oncogenic properties of mutant p53 proteins and their underlying mechanisms. Key cancer hallmarks and selected molecules that are associated with mutant p53 GOF [28].

Mutant p53 GOF contributes to cancer progression through direct interaction with proteins altering their function or through the transcriptional activation or repression of target genes and downstream molecules (figure 5) [29]. For example, mutant p53 has been shown to interact with the transcription factor NF-Y, and to up-regulate the expression of NF-Y target genes [30]. Furthermore, p53 mutants can bind and inactivate two homologues of the p53 family, p63 and p73.Given that they share amino ac-

id sequence identity in the DNA-binding domain, p53, p63 and p73 should have redundant functions in the regulation of gene expression [31].

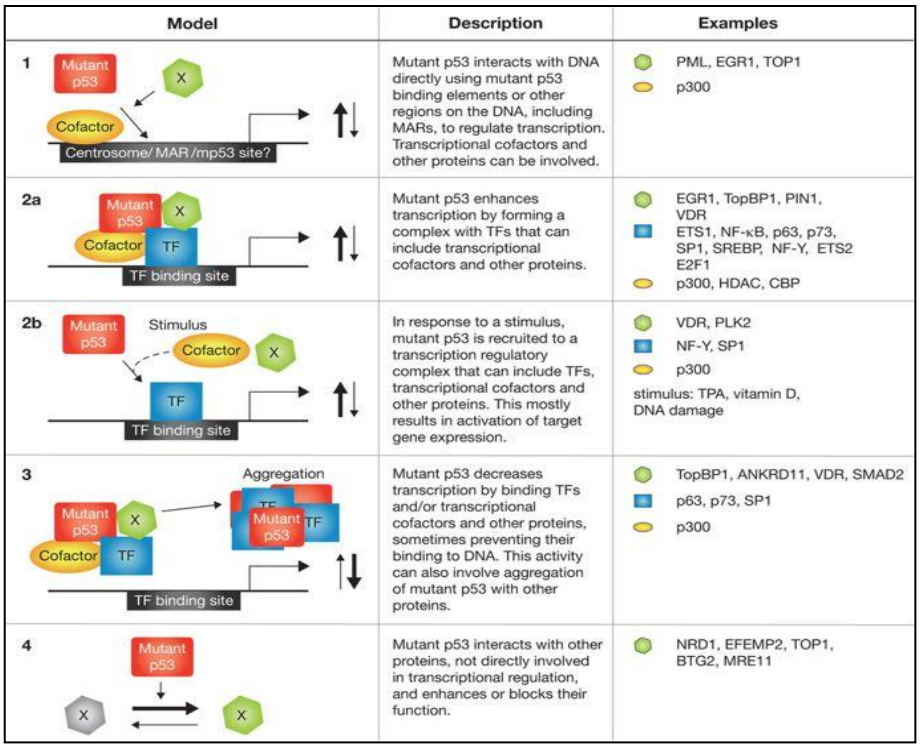


Figure 5: Mutant p53 and its interaction network. As part of its gain of function, mutant p53 interacts with different proteins to enhance or inhibit their activities. TF, transcription factor; X, any protein other than a transcription factor or transcriptional cofactor; MAR, matrix attachment region DNA element; mp53, mutant p53 [29].

The interaction between mutant p53 and p63/p73 are related with many aspects of the GOF of mutant p53, such as chemoresistance, migration, invasion, and metastasis [31]. In addition, most of mutant p53 isoforms are able to oligomerize with the wild-type protein encoded by the second allele, inhibiting its function, forming a hetero-tetramer unable to bind DNA, revealing a dominant negative function of mutant p53 [28]. An additional mechanism by which mutant p53 induces cancer progression is the up-regulation or down-regulation of a number of genes involved in different aspects of tumorigenesis, such as c-Myc, Fos, PCNA, IGF1R, EGR1, NF-κB, BCL-xL, IGF2, VEGFA, and others [28]. Furthermore, mutant p53 is also able to regulate non-coding RNA ,such as miRNAs, inducing or repressing the expression of certain

miRNAs to mediate new oncogenic activities [32]. Taken together, the modulation of gene transcription and the interference with pivotal signalling pathways are important mechanisms by which p53 mutants exert their oncogenic functions.

Chemoresistance

Chemoresistance causes reversion disease and metastasis, contrasts the development of the clinical outcome for the cancer patients, and remains the main obstacle to cancer therapy [33]. Several mechanisms are involved in chemoresistance, one of which is the involvement of oncogenes. One typical feature of mutant p53 proteins is their ability to confer an elevated drug resistance to cancer cells. The overexpression of various tumour-associated p53 mutants can render cancer cells more resistant to the effect of chemotherapeutic drugs [34], [35] whereas knockdown of endogenous mutant p53 sensitizes cancer cells to killing by such molecules [36]. The correlation between p53 mutation status and sensitivity to cytotoxic drugs has been confirmed by a large study conducted by the National Cancer Institute, USA, where 60 cell lines and more than 100 anticancer drugs were examined [37]. However, the way in which p53 influences drug resistance depends on different parameters including the way of action of the drug, genetic variations during carcinogenesis, and the kind of cancer [38]. For example, our group and others demonstrated that the treatment with the drug gemcitabine stabilizes mutant p53 in the nuclei of the cells and induces the expression of mutp53-target genes, as CdK1 (cyclin-dependent kinase 1) and CCNB1 (G2/mitotic-specific cyclin-B1), which are both involved in mitosis and cell proliferation, leading to gemcitabine resistance in pancreatic cancer cells [35], [39]. In temozolomide-resistant glioma cells, a correlation between mutant *TP53* gene and MGMT (O6-methylguanine DNA-methyl-transferase) expression was detected. While temozolomide kills cells by alkylating O6-guanine, MGMT in turn repairs alkylation. Therefore drug resistance may be caused by MGMT up-regulation [40]. In conclusion, mutant p53 is not only a crucial player in carcinogenesis, but it is also related with resistance to recognized cytotoxic anticancer drugs, such as gemcitabine, cisplatin, epirubicin, 5-fluorouracil, methotrexate and many other chemotherapeutics.

Therapeutic Strategies to Restore Wild-Type Activity to Mutant p53

Many different mutations and phenotypes allow a variety of strategies are being explored to target tumors expressing mutant p53s (Figure 6) [41]. Re-folding of this mutated and accumulated p53 leads to restoration and activation of defective proteins, resulting in high levels of active p53 with wild-type functionality and apoptotic cell death [42]. A variety of compounds that might restore wild-type p53 conformation and function have been characterized [42].

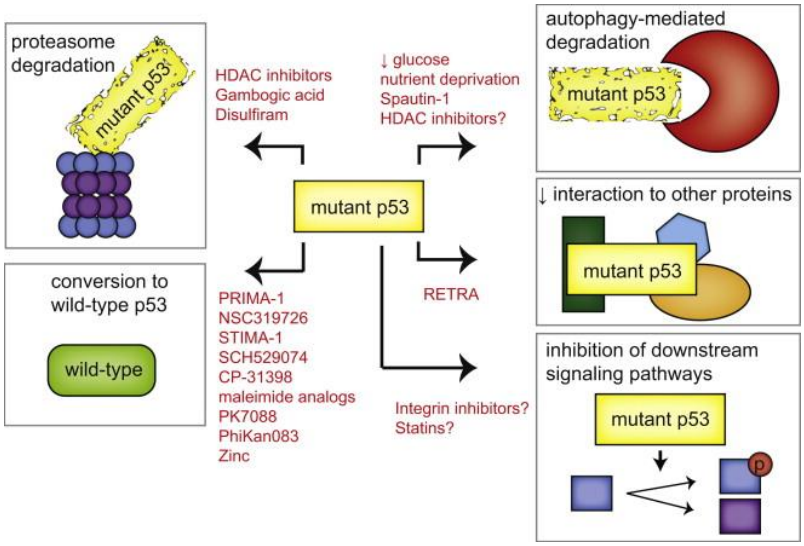


Figure 6: Strategies restoring p53 wild-type function. These strategies include promotion of mutant p53 degradation through the proteasome and autophagy pathways, restoration of wild-type p53 activity, interference with the interaction between mutant p53 and other proteins, and interference in signaling pathways downstream of mutant p53 [41].

The compound that represents an innovation in the reactivation of p53 is P53-Reactivation and Induction of Massive Apoptosis-1 (PRIMA-1). Treatment with this compound upregulated wtp53-target genes, such as BAX, PUMA and NOXA [43] and induced activation of caspases -2, -3 and -9 [44]. Under physiological conditions, PRIMA-1 and its methylated analogue PRIMA-1Met (also known as APR246) are converted into a reactive intermediate compound, MQ, which covalently binds to the core domain of p53 [45]. Due to their aberrant folding, mutant p53 proteins may expose cysteine residues, which are hidden in the core domain of wt-p53 [44]. This can lead to the formation of inter- and intramolecular disulfide bonds, locking mutant

p53 in an inactive conformation and causing protein aggregation. Thiol modification by reactive compounds, such as MQ, prevented the formation of such disulfide bonds and thus promoted correct folding and restoration of the wild-type function [45]. Although the fact that nuclear levels of both p53 and MDM2 are normally kept at low levels due to a regulatory circuit, a deregulated MDM2/p53 balance (e.g. by overexpression of MDM2) reduces the tumor suppressive functions of p53 [12]. Due to this antagonizing effect of MDM2 on p53, small molecules mimicing p53-binding residues can block the MDM2-p53 interaction. With this interaction blocked, p53 is no longer controlled by MDM2 and is reactivated in tumor cells harboring wt-p53 [46]. However, MDM2 is not the only a negative regulator of p53 and therefore other effects have to be considered. Again, p53 is involved in a complex network with several other actors. For example, it is also regulated by the proteins SirT1 (Sirtuin 1) [47] or Wip1 (wt-p53 induced phosphatase/PPM1D) [48]. Numerous MDM2 inhibitors have been developed during the past few years [49]. For example, RITA (Reactivation of p53 and Induction of Tumor cell Apoptosis) bound to the N-terminus of p53 (residues 1-63) and induced a conformational change, which transmitted from the N-terminus to the core and to the C-terminal domain, promoting the disruption of p53 and MDM2 complex [50]. This led to p53 accumulation and induction of p53-dependent apoptosis in a variety of tumor cell lines of different origin. Autophagy also plays a role in mutant p53 degradation. The degradation of mutant p53 was promoted by proteasomal inhibition and depended on functional autophagy machinery [51]. However, mutant p53 can inhibit autophagy [52], [53], indicating that the relationship between autophagy and mutant p53 is complex and the inhibition of autophagy by mutant p53 can further improve the overexpression of this oncognenic protein. Another small molecule named RETRA has been suggested to destabilize the interaction between p73 and mutant p53 [54]. Instead of targeting mutant p53 directly, another approach is to identify common pathways regulated by mutant p53 proteins in order to target these downstream pathways for therapeutic intervention [41].

3.3 CANCER METABOLISM: THE WARBURG EFFECT

Tumorigenesis is dependent on the reprogramming of cellular metabolism as both direct and indirect consequence of oncogenic mutations. The alterations in intracellular and extracellular metabolites that can accompany cancer-associated metabolic reprogramming have effects on gene expression, tumor microenvironment and the generation of signaling molecules such as reactive oxygen species (ROS) [55]. The common feature of this altered metabolism in cancer cells is increased glucose uptake and fermentation of glucose to lactate. Cellular metabolism of glucose to CO_2 and H_2O in normal adult cells is accomplished through the oxidative phosphorylation pathway that leads to the generation of ATP in the presence O_2. The Warburg effect, also called aerobic glycolysis, is the best known metabolic shift that occurs in cancer cells to support the biosynthetic requirements of uncontrolled proliferation [56]. During this phenomenon, even in the presence of oxygen and totally functioning mitochondria, tumour cells adopt glycolysis for their energy necessities and undergo both high rate glucose uptake and lactate production, as compared with normal cells (figure 7) [57], [58]. Per unit of glucose, aerobic glycolysis is an inefficient way of generating ATP compared to the amount obtained by mitochondrial respiration [59]. However, the rate of glucose metabolism through aerobic glycolysis is higher such that the production of lactate from glucose occurs 10-100 times faster than the complete oxidation of glucose in the mitochondria [60]. In this situation, the increased glucose consumption is used as a carbon source for anabolic processes needed to support cell proliferation [61]. In fact, this excessive organic substrate is also used for the *de novo* generation of nucleotides, lipids, and proteins. Furthermore, having a rate-limiting demand for ATP, proliferating cells are in an increased need of reducing equivalents in the form of NADPH obtained by the penthose phosphate pathway (PPP) [61]. A proposed mechanism for considering the biosynthetic function of the Warburg Effect is the regeneration of NAD+ from NADH in the pyruvate to lactate step that concludes aerobic glycolysis. This process may also influence the homeostasis of ROS generation by affecting the concentration of reducing equivalents in the mitochondria [62]. Finally, Warburg effect is also able to induce acidification of the microenvironment and other metabolic crosstalk, favoring cancer cell growth [62].

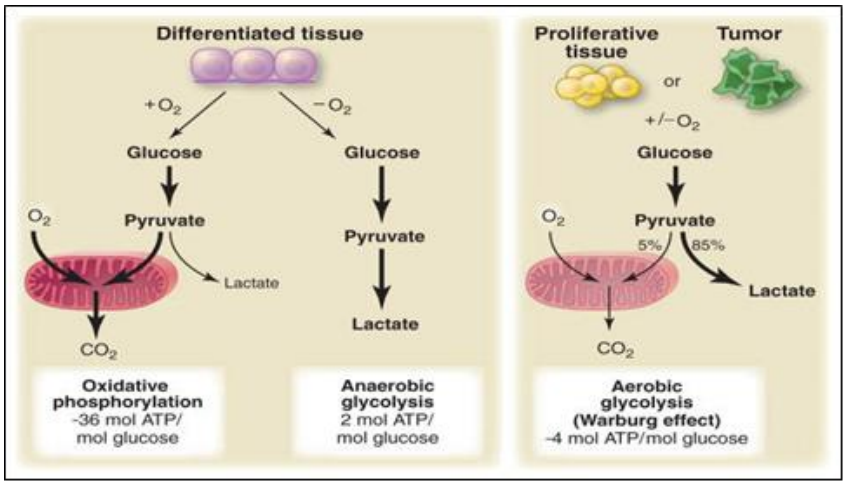


Figure 7: Warburg effect. Schematic representation of the differences between oxidative phosphorylation, anaerobic glycolysis, and aerobic glycolysis [61].

The opposite regulation on Warburg effect by Wild type and Mutant p53

Recent studies have shown that regulating energy metabolism is a critical role of wtp53 in tumor suppression [63]. Indeed, wt-p53 is described to regulate glycolysis, mitochondrial oxidative phosphorylation, pentose phosphate pathway (PPP), and lipid metabolism in cells. Functionally, wt-p53 represses glycolysis and the Warburg effect through multiple mechanisms as transcriptional regulation of genes involved in the glycolytic metabolism, including TIGAR (TP53-induced glycolysis and apoptosis regulator) and Parkin [64], [65]. For example, p53 transcriptionally blocks the expression of glucose transporters, as GLUT 1-4, and induces the expression of TIGAR which decreases the intracellular concentrations of fructose-2,6-bisphosphate, and thus reduces glycolysis and deflects glucose catabolism to the PPP [65]. On the contrary, tumor-associated mutant p53 was reported to promote tumor metabolic changes as a novel gain-of-function in promoting tumor development [66]. Mutant p53 encourages the Warburg effect both in cultured cells and mutp53 knock-in mice. This effect mainly occurs through promoting the translocation of GLUT1 (glucose transporter 1) to the plasma membrane, which is mediated by activated RhoA/ROCK signaling [66]. In addition, mutant p53 was reported to induce the expression of glycolytic enzyme hexokinase II, which could promote glycolysis [67].

3.4 NON-METABOLIC FUNCTIONS OF GLYCOLYTIC ENZYMES IN CANCER

In addition to their canonical roles in glycolysis, recent studies progressively uncovered some non-metabolic functions of glycolytic enzymes in tumorigenesis, becoming emerging targets for therapeutic intervention [68]. Emerging evidence showed that most glycolytic enzymes are deregulated in cancer cells and play important roles in tumorigenesis and all essential glycolytic enzymes can be translocated into nucleus where they participate in tumor progression independently of their canonical metabolic roles [69]. These non-canonical functions include anti-apoptosis, regulation of epigenetic modifications, modulation of transcription factors and co-factors, DNA repair activity, suggesting that these multifaceted glycolytic enzymes not only function in canonical glycolytic metabolism but also directly link metabolism to epigenetic and transcription programs implicated in tumorigenesis [70]. The trafficking of metabolic enzymes to the nucleus could be caused by covalent modifications or by forming new protein-protein interactions or protein complexes [71], [72]. However, the precise mechanisms at the basis of the regulation of these non-metabolic functions of the glycolytic enzymes are still largely unknown.

Multifaceted roles of GAPDH

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a glycolytic enzyme that catalyzes the reversible conversion of glyceraldehyde-3-phosphate (G-3-P) to 1,3-diphosphoglycerate in the cytosol of the cells. GAPDH acts as a homo tetramer containing four identical 37 kDa subunits [73]. It initially was identified as a glycolytic enzyme involved exclusively in cytosolic energy production [73]. However, emerging evidence indicates that GAPDH is a multifunctional protein displaying diverse activities distinct from its conventional metabolic role [74]. Its pleiotropic role is largely affected by ability of GAPDH to bind different macromolecules in the cell and by post-translational modifications in different amino acid residues such as phosphorylation, ADP ribosylation, and acetylation [75]. Specifically, several studies have demonstrated that the GAPDH has a variety of other

functions, including DNA repair, transcriptional regulation, membrane fusion and transport, autophagy, cell death and nuclear tRNA export [75], [76]. The multi-functional roles of GAPDH are strictly associated to its intracellular localization, which is not restricted to the cytosol for glycolytic energy production [76]. Indeed, after specific stimuli GAPDH can translocate in other subcellular compartments, such as nuclei in which it exerts a critical role in the regulation of cell death-related gene transcription, stimulation of apoptosis and modulation of cell fate. GAPDH has been also observed in the mitochondria, in which its binding to the voltage-dependent anion channel (VDAC) has been suggested to promote the release of proapoptotic proteins, such as cytochrome c (CytC) and apoptosis-inducing factor (AIF) [77]. On the other hand, GAPDH is involved in a series of age-related neurodegenerative disorders [78] and in tumor development and progression [79], promoting tumor-specific GAPDH transcriptional/post-transcriptional regulation and aging as prosurvival factor [80], [81]. For example, a study showed that GAPDH may translocate to the nucleus when cells encountered certain stress conditions such as oxidative stress. Specifically, oxidative stress-induced S-nitrosylation of GAPDH could promote translocation of GAPDH to the nucleus, where it could interact with Sp1 under oxidative stress conditions, and activate SNAIL transcription, promoting EMT and metastasis through its non-enzymatic function in the nucleus [82]. Our group demostrated that the inhibition of antioxidant uncoupling proteins UCP2 triggers PDAC cell death by ROS-dependent nuclear translocation of GAPDH [83] and later, we discovered that the synergistic PDAC cell growth inhibition given by everolimus and genipin treatment was due to a massive GAPDH nuclear translocation observed both *in vitro* and in mice xenograft [84]. Thus, understanding the biological functions of GAPDH beyond glycolysis will improve the ability to effectively target this enzyme in cancer therapy.

3.5 THE ENERGY SENSOR AMPK AND ITS REGULATION BY MUTANT p53.

AMP-activated kinase (AMPK) is a highly conserved serine/threonine protein kinase complex and a central metabolic sensor located at the crossroad between metabolic and signaling networks [85]. AMPK is a heterotrimeric complex composed of a catalytic α-subunit and two regulatory subunits, β and γ [86]. The γ-subunit enables AMPK to respond to changes in the ATP-to-AMP ratio as it contains domains that bind adenine nucleotides. Upon changes in the ATP-to-AMP or ATP-to-ADP ratio, AMPK is activated by an allosteric mechanism that stimulates its kinase activity phosphorylating downstream targets to redirect metabolism towards increased catabolism and decreased anabolism [86]. The two major upstream kinases responsible for AMPK activation are the tumor suppressor LKB1 and $Ca^{2+/}$calmodulin-dependent protein kinase kinase 2 (CaMKK2). LKB1 activates AMPK during energy stress, whereas CaMKK2 activity is induced by increased intracellular Ca^{2+} levels, regardless of the energy status of the cells [87]. AMPK restores ATP levels during metabolic stress by inhibiting ATP-consuming biosynthetic pathways while simultaneously activating pathways that regenerate ATP through the breakdown of macromolecules. In particular, AMPK phosphorylates several transcription factors (or co-factors) that are themselves master regulators of biosynthetic pathways (figure 8). In this way, AMPK can acutely restore energy balance but also reprogram cell metabolism transcriptionally in response to prolonged energetic decreases [87]. For example, AMPK promotes glucose uptake by phosphorylating TBC1D1 (TBC domain family, member 1) and TXNIP (thioredoxin-interacting protein), which control the translocation and cell-surface levels of glucose transporters GLUT4 and GLUT1, respectively [88]. It also promotes autophagy through several mechanisms like the activation of ULK1 (unc-51-like autophagy-activating kinase 1), which triggers the initiation of the autophagic cascade [88]. Regarding mutp53, it is able to inhibit AMPK signaling in head and neck cancer cells directly binding to the AMPKα subunit, thus gaining its oncogenic function and stimulating anabolic growth of cancer cells, in contrast to its wild type counterpart [89].

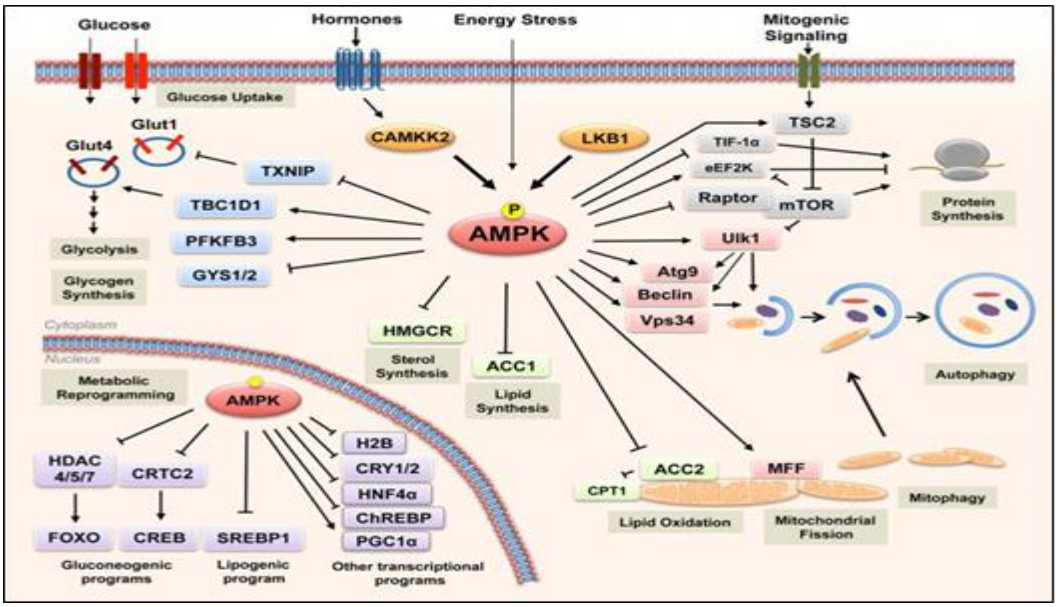


Figure 8: Substrates of AMPK regulate multiple metabolic processes in cells. AMPK is phosphorylated and activated by LKB1 and CAMKK2 in response to several stimuli. Its phosphorylation induces metabolic changes through the phosphorylation of substrates [87].

Mutp53s can directly interact with both AMPKα1 and AMPKα2 mainly through their DNA-binding domain (DBD), while the N-terminus of mutp53s is responsible for blocking the interaction between AMPKα and its upstream kinase LKB1 inhibiting its Thr172 phosphorylation and consequently preventing its activation [89]. Finally, our group revealed another mechanism by which mutp53 is able to inhibit AMPK-signaling [52]. In particular, p53 blocks AMPK by down-regulating Sestrin1/2 expression which are a class of proteins that can directly interact with AMPK subunits favoring their phosphorylation by upstream kinases and thereby resulting in AMPK signaling stimulation [52], [90]. Importantly, AMPK was recently identified as a negative regulator of the Warburg effect through inhibition of the hypoxia-induced factor 1 (HIF-1) pathway [91]. Therefore, the inhibition of AMPK by GOF mutp53s, which relieves the suppression of HIF-1 by AMPK, is expected to increase HIF-1 protein expression and thus lead to increased glucose influx and glycolysis. These phenomena could represent a further mechanism of GOF mutp53 to promote the Warburg effect and drastic metabolic changes in cancer cells.

3.6 THE ONCOGENE AKT AND ITS REGULATION BY MUTANT p53

Protein kinase B or Akt (PKB/Akt) is a serine/threonine kinase, which in mammals comprises three highly homologous members known as PKBalpha (Akt1), PKBbeta (Akt2), and PKBgamma (Akt3) [92]. PKB/Akt is activated in cells exposed to diverse stimuli such as hormones, growth factors, and extracellular matrix components. A well-known upstream target of Akt is phosphatidylinositol-3 kinases, PI3Ks, which is a lipid kinase family and a key enzyme in the generation of the second messenger phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P3) [93]. This allows the translocation of Akt from the cytoplasm to the plasma membrane, altering its conformation to allow subsequent phosphorylation by the phosphoinositide-dependent kinase-1 (PDK-1) [94]. PKB/Akt is then released from the membrane and translocates to other subcellular compartments. Phosphorylation of Akt results in full activation of Akt kinase activity and the subsequent regulation of multiple cellular processes, including the transmission of growth factor-dependent survival signal [95]. In particular, Akt inhibits apoptosis by phosphorylating and inactivating pro-apoptotic Bcl-2 family members, as Bad, and by inhibiting the release of cytochrome c. In addition, Akt changes mitochondrial membrane potential induced by multiple apoptotic stimuli, in a caspase-independent manner, and it acts maintaining mitochondrial integrity [96], [97]. In addition, Akt-regulated signalling plays a critical role in numerous processes which are known to be hallmark of cancer cells [98]. Akt regulates the transcription of death genes by phosphorylating forkhead family transcription factors or increasing the transcription of survival genes by activating NF-κB and CREB transcription factors [98]. Furthermore, it regulates energy metabolism by multiple mechanisms, including the expression and membrane translocation of glucose transporters. Akt may also indirectly activate the important rate-controlling enzyme phosphofructokinase-1 (PFK1) by directly phosphorylating and activating phosphofructokinase-2 (PFK2) [99] whose principal reaction product, fructose-2,6-bisphosphate (Fru-1,6-P2), is a potent allosteric activator of PFK1. Furthermore, Akt affects not only glycolysis, but is also able to improve mitochondrial respiration and oxidative phosphorylation [100], [101]. In fact, inhibiting Akt activation decreases ATP

production; activities of complexes I, II, and III; the mitochondrial membrane potential (ΔΨm); and F0F1-ATPase activity [100].

Intriguingly, there is a connection between p53 and Akt. Phosphorylation of MDM2 by Akt has been reported to result in the translocation of MDM2 to the nucleus, where it promotes the ubiquitination of p53, reducing wtp53 levels and promoting tumour growth [102]. Mutant p53-R273H is able to regulate PI3K/AKT signaling pathway, inducing Akt expression [103]. DAB2IP is a negative modulator of PI3K/AKT signaling, because it binds AKT limiting its activation in response to various stimuli [104]. It has been discovered that mutp53 binds and inhibits DAB2IP, favoring insulin-induced AKT activation in cancer cells [105]. *In vitro* studies have also shown increased transformation in cells having a combination of PI3K/Akt pathway activation and mutp53, as well as increased invasiveness of tumour cells [106]. Thus, Akt is an excellent candidate master regulator responsible for the classical biochemical features of cancer cells. Furthermore, it may constitute a "Warburg kinase" that can be specifically targeted to alter cancer cell energy metabolism for therapeutic benefit.

3.7 AUTOPHAGY: THE INTRACELLULAR DEGRADATION SYSTEM

Autophagy is a cellular catabolic degradation response to starvation or stress whereby cellular proteins, organelles and cytoplasm are digested and recycled [107]. Basal autophagy also has an important homeostatic function, maintaining protein and organelle quality control. Autophagy is controlled mainly by the kinase mammalian target of rapamycin (mTOR; also known as FRAP1), which is a downstream component of the PI3K pathway [107]. Autophagosomes are double-membrane vesicles that sequester cytoplasm and organelles. The autophagy-regulated or Atg proteins are required for the activation of autophagy, the formation of autophagosomes, the sequestration of intracellular constituents, and the targeting and fusion of autophagosomes to lysosomes [108]. One of the most common events in human cancer is the downstream kinase mTOR activation of the PI3K pathway. Autophagy was initially thought to be a tumor-suppression mechanism. Indeed, autophagy deficiency causes oxidative stress, activation of the DNA

damage response promoting cancer cell growth [109]. Nevertheless, cancer cells also rely on autophagy in many cases due to the increased metabolic and biosynthetic demands imposed by deregulated proliferation [110]. Wild type p53, in order to react to genotoxic or environmental stimuli, triggers autophagy in cancer cells through various mechanisms, as the stimulation of the nutrient energy sensor AMP-activated protein kinase (AMPK), the inhibition of the mammalian target of rapamycin (mTOR)[111]. On the contrary, mutant p53 proteins counteract autophagy through several mechanisms as the stimulation of Akt/mTOR pathway or through ATG12 repression [112]. Thus, the impact of autophagy regulation in the development of cancers and in response to therapies assumes increasing importance.

3.8 REACTIVE OXYGEN SPECIES IN CANCER

Reactive oxygen species (ROS) are highly reactive radicals, ions or molecules that can readily oxidize other molecules including lipids, amino acids, proteins, and nucleic acids [113]. ROS can appear from numerous intracellular sources; among them, the most important are mitochondria [114]. In mitochondria, ROS are produced as an inevitable byproduct of oxidative phosphorylation. The electron transport chain encompasses complexes I-IV and ATP synthase on the mitochondrial inner membrane. Superoxide is generated at complexes I and III and released in the intermembrane space or in the mitochondrial matrix [115]. Under normal physiological conditions, the intracellular levels of ROS are steadily maintained to prevent cells from damage. Detoxification from ROS is facilitated by non-enzymatic molecules (i.e. glutathione, flavenoids and vitamins A, C and E) or through antioxidant enzymes like superoxide dismutases (SODs), catalase or glutathione reductase, which specifically scavenge different kinds of ROS. Reactive oxygene species have been detected in almost all cancers where they promote many aspects of tumor development and progression [116]. In cancer cells high levels of reactive oxygen species can result from increased metabolic activity, oncogene activity, increased cellular receptor signaling and other events [117], [118]. Indeed, ROS in cancer are involved in in a plethora of biological events addressed

to sustain each aspect of cancer progression summarized in figure 9, like cell cycle progression and proliferation, cell survival and apoptosis, energy metabolism, cell motility, angiogenesis and maintenance of tumor stemness [116], [119]. Reactive oxygen species, particularly hydrogen peroxide, can act as second messengers in cellular signaling [120]. Indeed, ROS generation can activate several signaling pathway like the PI3K/Akt signaling, MAPK/Erk1/2pathway [116].The non-radical ROS hydrogen peroxide H_2O_2 regulates protein activity through reversible oxidation of its targets including protein tyrosine phosphatases or kinases, receptor tyrosine kinases and transcription factors [121]. Lipids are others cellular targets of ROS attacks. ROS react with polyunsaturated or polydesaturated fatty acids to initiate lipid peroxidation [122]. However, the role of ROS in cancer biology is ambiguous, indeed despite many studies attributed to ROS a pivotal role in promoting many events, many others have highlighted that a severe increase in ROS can induce cell death following a “non-specific” damage of macromolecules such as the irreversible oxidation of lipids, proteins or DNA [119]. Therefore, ROS represent an “Achilles heel” of cancer cells and new therapeutic improvement could be reached playing on this sophisticated redox cellular balance.

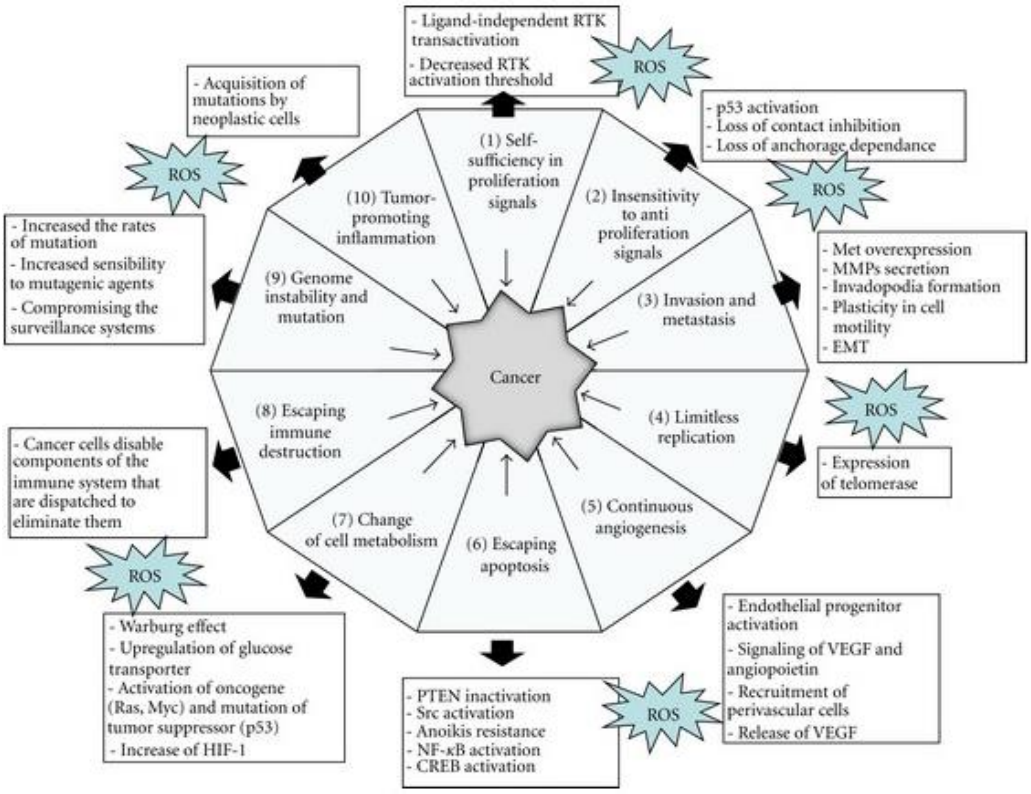


Figure 9: ROS play multiple roles in the hallmarks of cancers. Contribution of oxidants is indicated for each point [123]

UCP2: a key antioxidant player

The UCPs belong to the superfamily of anion transport carriers of the mitochondrial inner membrane [124] and some of them are involved in thermogenesis and regulation of mitochondrial ROS. UCP2 has been found in several tissues, including liver, brain, pancreas, adipose tissue, immune cells, spleen, kidney, and the central nervous system [125]. UCP2 acts as an important sensor of mitochondrial oxidative stress controlling the production of mitochondrial ROS. As revealed by studies with UCP2-null mice, its antioxidant function is generally implicated in cyto-protective activities [126]. The uncoupling of oxidative phosphorylation is a short circuit in which the transport of protons from the intermembrane space to the matrix bypasses ATP synthase resulting in a decrease of mitochondrial inner membrane potential; leakage of electrons from electron transport chain ETC and ROS generation (figure 10). Minor increases in the mitochondrial membrane potential induce ROS formation, whereas slight decreases can substantially diminish their production, without greatly lowering

the efficiency of oxidative phosphorylation [127]. Hence, the mild uncoupling of mitochondrial oxidative phosphorylation may represent the first line of defense against oxidative stress [127]. According to this pattern, UCP2 can dissipate the proton gradient to prevent the proton-motive force from becoming excessive, thus decreasing ROS produced by electron transport [128]. Mitochondrial superoxide ion is considered the initial and leading molecule of ROS signaling and is generally converted into hydrogen peroxide (H_2O_2) by superoxide dismutases. In addition, upon reaction with H_2O superoxide ion can generate hydroxyl radicals (•HO) implicated in lipid damage and protein oxidation [128]. Thus, UCP2 acts as a sensor of mitochondrial oxidative stress controlling the production of mitochondrial ROS and regulating redox-sensitive cytosolic signaling pathways. In addition to its antioxidant role, UCP2 may acts as a direct metabolic regulator contributing to the Warburg phenotype, promoting pyruvate efflux from mitochondria, restricts mitochondrial respiration, and increases the rate of glycolysis in cancer cells [129].

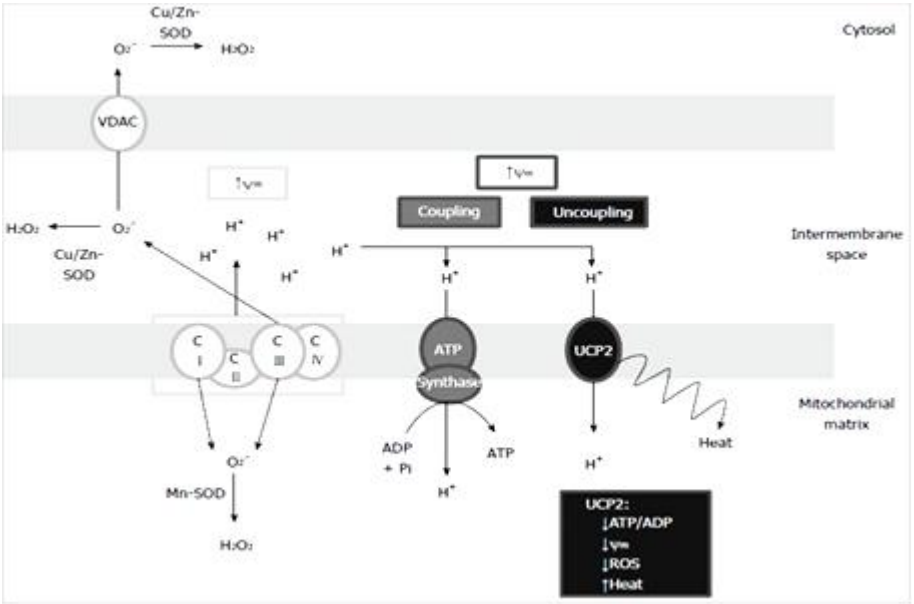


Figure 10. Uncoupling protein 2 uncoupling activity in oxidative phosphorylation. ROS: Reactive oxygen species; UCP2: Uncoupling protein 2; SOD: Superoxide dismutase; Mn-SOD: Manganese-superoxide dismutase [130].

The regulation of UCP2 can occur at various levels as transcriptional, translational and protein turn over regulation or post-transcriptional modifications [131]. One of

the most important mechanisms of the transcriptional regulation of UCP2 is mediated by the peroxisome proliferator-activated receptor gamma coactivator1-alpha (PGC-1α) [132], that has been shown to stimulate Ucp2 gene expression via two thyroid hormone response elements TREs located in the proximal Ucp2 promoter region [133]. PGC-1α can also indirectly induce Ucp2 gene expression by the link with sterol regulatory element-binding protein (SREBP) [134]. SREBP isoforms are known to regulate Ucp2 gene expression via either one of the two E-box motifs present on Ucp2 promoter [134].

Sestrins: crucial role in antioxidant defenses

The Sestrins constitute a family of stress-inducible proteins upregulated in cells exposed to a variety of environmental stresses including DNA damage, oxidative stress and energy deficiency. They contribute to redox homeostasis through the regulation of adenosine monophosphate-dependent protein kinase (AMPK)-mammalian target of rapamycin (mTOR) signaling, leading to inhibition of cellular anabolism and augmentation of catabolic processes such as beta-oxidation [135]. Sestrin-dependent activation of AMPK and suppression of mTORC1 activity are also critical for maintaining basal autophagy [136]. Thus, Sestrins can be important for autophagic elimination of dysfunctional mitochondria that leak electrons and produce pathogenic amounts of ROS. All members of Sestrin family are induced by oxidative stress, although they are subject to different induction mechanisms (figure 11) [137], [138]. Sesn1 is induced by hydrogen peroxide in a p53-dependent manner [139], whereas induction of Sesn2 by oxidative stress is only partially p53-dependent [140]. Silencing of either Sesn1 or Sesn2 in human fibroblasts significantly inhibit cell proliferation and accelerate cell senescence triggered by ROS accumulation [137]. The Sestrins were also shown to mediate the antioxidant activities associated with the p53 and FoxO transcription factors. While high levels of oxidative stress can lead to cell death through p53- and FoxO-dependent apoptotic gene transcription, low levels of oxidative stress cause moderate activation of p53 and FoxO that can induce Sestrins to reduce oxidative stress and prevent cell death [139], [141]. Despite their involvement in tumor suppression and genome protection, Sestrins are still expressed in

many cancers and might actually be required for maintaining the viability of cancer cells as part of the antioxidant defense system under certain conditions [142].

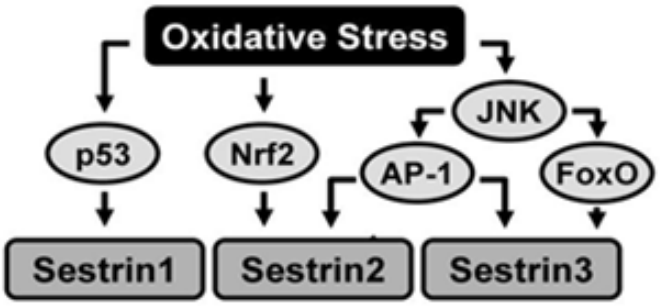


Figure 11: Regulation of Sestrin expression by oxidative stress. p53,Nrf2 and AP-1 are required for Sestrins induction upon oxidative stress [143]

3.9 THE IMPORTANCE OF SECRETOME AND TUMOR MICROENVIRONMENT

Secretome is referred to as the rich, complex set of molecules secreted from living cells, and is a vital aspect of cell–cell communication in eukaryotes. Proteins, lipids, micro-RNAs (miRNAs) and mRNAs are secreted into the extracellular space by a cell, tissue, organ, or organism at any given time and conditions [144]. Chronic perturbations in the secretome are often associated with altered cellular phenotypes indicative of pathological conditions [145], including obesity, diabetes, chronic inflammation and cancer. The widest application of secretome has been in the development of new diagnostic biomarkers for human disease classification like cancer [146]. Proteins of secretome play a key role in cell signaling, homeostasis, immune response, communication and migration [147]. Examples of secretory proteins include hormones, digestive enzymes, cytokines, chemokines, interferons (IFNs), colony-stimulating factors (CSFs), growth factors, and tumor necrosis factors (TNFs). The secretome of many cell types, including cancer cells, is released by distinct secretory processes. The best characterised of these pathways, the ‘classical’ or endo-

plasmic reticulum (ER)-Golgi pathway has traditionally been considered responsible for the majority of protein secretion. Proteins secreted through this pathway contain an N-terminal signal peptide that is crucial for recognition by the secretory apparatus. Classically secreted proteins are synthesised in the rough ER before transport to the Golgi apparatus in COPII-containing transport vesicles [148]. During passage through the Golgi, cargo proteins may be modified by processes such as proteolytic cleavage or glycosylation. Cargos are then sorted into secretory vesicles at the trans-golgi network (TGN), and secretion is achieved when Golgi-derived vesicles containing the secreted proteins are trafficked to and fuse with the plasma membrane, releasing their contents to the extracellular environment [149]. Whilst the majority of cytokines, growth factors and extracellular matrix components are believed to be classically secreted, several factors are known to be secreted via Golgi-independent, 'non-classical' pathways. These non-classical secretion pathways are, in general, less characterised than classical secretory events [150]. The need for developing more effective cancer biomarkers and therapeutic modalities has led to the study of cancer cell secretome as a means to identify and characterize diagnostic and prognostic markers and potential drug and therapeutic targets [151].

The role of secreted proteins in cancer

In addition to various pathological events, such as genomic instability and induction of oxidative stress, tumorigenesis and cancer progression may also strongly depend on extrinsic factors secreted by cancer cells. Secreted factors play a critical role in the tumour–microenvironment communications, representing a signal to cells at distant sites and affecting their phenotype (figure 12) [144]. The development of an adverse tumour microenvironment as the consequence of the crosstalk between cancer and stromal cells is one of the causes of low efficiency to cancer treatments. Indeed, it is reported that solid tumours take advantage of a co-evolution of neoplastic and stromal cells and that the extracellular matrix (ECM) plays a dynamic role in cancer invasion and migration. This complex cancer-microenvironment system is also strongly influenced by impaired vascularization and by interaction with the immune system cells [152]. Many biological pathways, such as NF-κB, MAPK, IL-1 are involved to orchestrate this complex crosstalk system [144], [153]. Those secreted fac-

tors, i.e. cytokines/chemokines, proteases, growth and angiogenic factors may regulate the crosstalk between stroma–cancer cells and tumour microenvironment. The amount of any constituent of the secretome can be regulated by alterations to *de novo* synthesis, to its half-life and to trafficking processes [154]. Furthermore, many of cancer cell-secreted proteins/enzymes/molecules can stimulate drug resistance through autocrine/paracrine mechanisms [155]. Thus, it's becoming fundamental to study specific secreted biomarkers which may clinically predict resistance mechanisms to specific drugs.

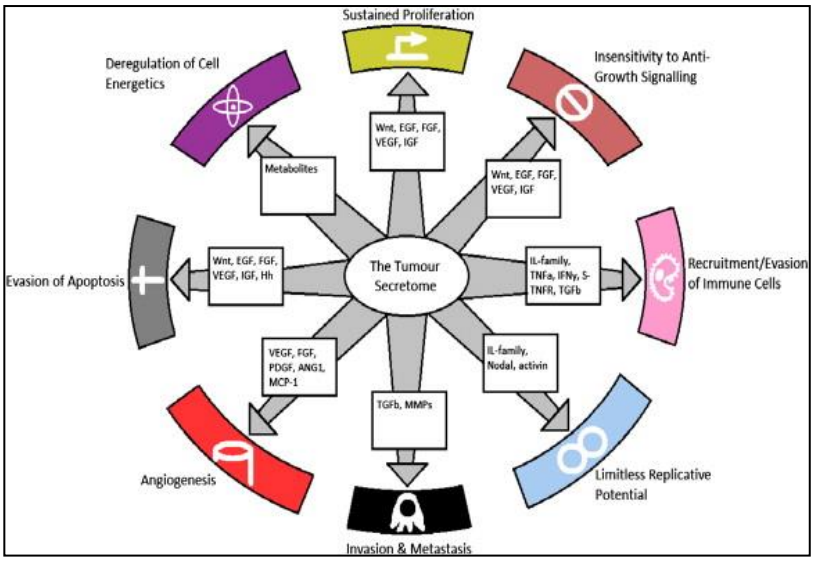


Figure 12: The tumour secretome and the hallmarks of cancer. The well-known secreted factors and the biological pathways involved. The secreted factors contribute to the hallmarks of cancer shown in the figure [144].

Mut p53 and tumour microenviroment

A novel mechanism to drive invasion by mutant p53 is the manipulation of tumour microenviroment [156]. Mutp53 proteins trigger the production and release of pro-inflammatory and immunomodulatory cytokines to stimulate an inflammatory cancer-associated microenvironment and to repress the immune system [156]. Indeed, mutp53 proteins, contrary to the wild-type counterpart, upregulate the expression of chemokines like CXCL5, CXCL8 and CXCL12 through the NF-κB-dependent pathway, highlighting a further molecular mechanism by which mutp53 proteins exert

their oncogenic activity [157]. Loging and Reisman showed that mutp53 proteins are able to repress the transcription of TIMP-3, which is crucial in the extracellular matrix (ECM) turnover and construction, contributing to increased activity of secreted MMPs in the ECM and, subsequently, to tumour invasion and metastasis [158]. Toschi *et al.* showed that, in human melanoma cells carrying mutp53 proteins, the reintroduction of wtp53 overcomes the mutp53 GOF and reduces cancer cell invasion into the ECM due to inhibition of secreted MMP-2 [159]. Another crucial element supporting the motility of tumour cells and their metastatic potential is covered by extracellular pH decrease [160]. This phenomenon is mainly related to the stimulation by mutp53 proteins of secreted lactate and implies an alteration of metabolism of cancer cells carrying mutant *TP53* gene. Tumour-derived lactate is able to induce inflammation and immune deficiency [160]. Low extracellular pH can induce expression and activity of many proteases, including MMP-2, MMP-9, cathepsin-B and cathepsin-L, enabling cancer cells to invade the surrounding tissue and generate metastasis. Lactate secretion can increase IL-17A production by both T-cells and macrophages, promoting chronic inflammation in tumour microenvironment [161], [162] Several studies attribute to mutp53 proteins also a critical role in tumour–stroma interaction [163] and the role of mutp53 proteins in the induction of the pro-angiogenic extracellular mediator VEGF [164]. Thus, mutp53 proteins play a crucial role in the promotion of Warburg effect in cancer cells that, through both stimulation of lactate production and reduction of extracellular pH, makes the tumour microenvironment suitable to cancer cell invasion and tumour dissemination.

4. AIMS OF THE STUDY

PDAC is the fourth leading cause of cancer-related deaths due to disease presentation at an advanced stage, early metastasis and generally a very limited response to radio and chemotherapy [1]. Mutations in the *TP53* gene occur in over 50% of human cancers, where most of them are missense mutations resulting in the expression of mutant forms of p53 [2]. In addition, p53 mutated proteins acquire new biological properties referred to as gain-of function (GOF) that contribute to the induction and maintenance of cancer [14]. Despite different models have been proposed to explain the GOF activities of mutant p53 in cancer, the detailed mechanisms remain largely unknown. The main aim of my PhD research was to discover novel molecular mechanisms by which GOF mutant p53 proteins promote PDAC cell proliferation and chemoresistance to identify specific and personalized therapies in tumors bearing mutant *TP53* gene. Thus, the aims of this thesis can be summarized as follows:

- to investigate novel mechanism by which mutant p53 oncogenic proteins regulate cancer metabolism. In particular, we focused on the ROS production in cancer cell mitochondria, identifying a signaling pathway involved, and on the regulation of the subcellular localization of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in pancreatic cancer cells bearing mutant p53 gene. We studied if mutp53s can support cell proliferation and chemoresistance, by stabilizing the cytoplasmic localization of GAPDH or by ROS production though SESN1/AMPK/PGC-1α/UCP2 axis.

-to recognize a specific signature of biomarkers secreted by PDAC cells carrying GOF mutant p53. We analyzed PDAC secretome by untargeted MS-analysis and compared secretome of p53-null PDAC cells before and after ectopic overexpression of R273H-mutp53 and R175H-mutp53, as compared to the mock vector used as control. We also investigated the functional role of mutp53-driven secretome, studying its influence on proliferation, chemoresistance, apoptosis and autophagy, as well as cell migration. These data constitute a prerequisite for the identification of a secreted biomarker signature for the early identification of mutant p53 PDAC patients.

5. MATERIAL AND METHODS

5.1 Cell culture

Pancreatic adenocarcinoma PaCa3 (WTp53), Panc1 (mutant p53-R273H), and AsPC1 (p53-null) cell lines were grown in in RPMI 1640 medium (Thermo Fisher, Milan, Italy), supplemented with 10% FBS and 50 µg/ml gentamicin sulfate (BioWhittaker, Lonza, Bergamo, Italy), and incubated at 37 °C with 5% CO_2. These cell lines were kindly provided by Dr. Aldo Scarpa (University of Verona, Italy). The clones C9 (mock) and H1 (stably expressing mutant p53-R273H) of the p53-null H1299 cells were kindly provided by Dr. Riccardo Spizzo (Centro di Riferimento Oncologico, National Cancer Institute, Aviano, Italy). All the cell lines were routinely tested to confirm lack of mycoplasma infection.

5.2 Drugs and chemicals

Gemcitabine (2',2'-difluoro-2'-deoxycytidine; GEM) was provided by Accord Healthcare (Milan, Italy) and solubilized in sterile bi-distilled water. 2-deoxyglucose (2-DG) was obtained from Sigma (Milan, Italy), solubilized bi-distillated sterile water and stored at −80 °C until use. The GAPDH inhibitor (S)-benzyl-2-amino-2-(S)-3-bromo-4,5-dihydroisoxazol-5-yl-acetate (AXP3009) has been designed and synthetized in the laboratory of Dr. Paola Conti at the Department of Pharmaceutical Sciences (University of Milan, Italy). AXP3009 was solubilized in methanol and stored at -80 ºC. Bruno *et al.* previously reported the chemical structure and the synthesis of AXP3009 compound [165]. The AKT inhibitor (SH-5) and the AMPK activator (AICAr) were obtained from Sigma, solubilized in DMSO and bi-distillated sterile water, respectively, and stored at -20 °C until use. N-acetyl-L-cysteine (NAC) and CP-31398 dihydrochloride hydrate were obtained from Sigma-Aldrich (Milan, Italy) and solubilized in bi-distilled sterile water. RITA [5,5'-(2,5-furandiyl)bis-2-thiophenemethanol; reactivation of p53 and induction of tumor cell apoptosis] was obtained from Sigma-Aldrich and solubilized in DMSO.

5.3 Liposome-mediated transient cell transfection

Exponentially growing cells were seeded in 96-well plates or in 60 mm cell culture plates. The ectopic expression of mutant p53 isoforms in AsPC1 p53-null cells was carried out transfecting pcDNA3-mutp53R273H or pcDNA3-mutp53R175H expression vectors, or their relative mock vector (pcDNA3). Wild-type and mutant p53 protein expression was transiently knocked-down by transfection with pRSUPER-p53 vector or its negative control (pRSUPER), kindly provided by Dr. Agami (The Netherlands Cancer Institute, Amsterdam). The silencing transfections were carried out for 48 h using Lipofectamine 3000 (Thermo Fisher), according to the manufacturer's instructions. Knock-down of GAPDH expression was obtained by transfecting cells with specific GAPDH small interfering siRNA or with a siRNA-CTRL (negative control) purchased from Life Technologies. Cells were transfected by siRNA at a final concentration of 50 nM using Lipofectamine 3000. The ectopic expression of wild-type or dominant negative (DN)-AMPK subunit γ2 was previously described [166].

5.4 Lentiviral cell transduction

To silence R273H mutp53 expression in Panc1 cells, we used plasmid pLKO.1 puro-vector encoding *TP53*-shRNA (TRCN0000003756 Sigma-Aldrich) indicated as p53-SH1. As negative control we used a non-target shRNA control (SHC016; Sigma-Aldrich) indicated as p53-NT. To generate viral particles, 293FT cells (Thermo Fisher) were transfected using pLKO.1 shRNA DNA vector together with ViraPower Lentiviral Packaging Mix (pLP1, pLP2 and pLP/VSV-G) (Thermo Fisher). Seventy-two hours later, viral supernatant was collected and transducing units per ml of supernatant were determined by limiting dilution titration in cells. A MOI (multiplicity of infection) of 5 to 1 (5 transducing viral particles to 1 cell) was used for transducing cells using Polybrene (Sigma-Aldrich) at a final concentration of 8 μg/ml to increase transduction efficiency. Twenty-four hours after transduction, puromycin selection (2 μg/ml) was performed for 48 h and mutant *TP53*-silenced cells were used for experiments.

5.5 Cell proliferation assay

Cells were seeded in 96-well plates (5×10^3 cells/well) and the day after transfected with the indicated constructs (see figure legends) and incubated with various compounds at the indicated conditions. At the end of the treatments, cell growth was measured by Crystal Violet assay (Sigma) according to the manufacturer's protocol, and absorbance was measured by spectrophotometric analysis (A_{595nm}).

5.6 Soft agar colony formation assay

Anchorage-independent growth was performed in soft agar. Briefly, 5 x 10^4 H1299 cells, mock and R273H clones, were resuspended in complete DMEM medium containing 0.6% agarose low-gelling temperature (A9045 - Sigma Aldrich, Milan, Italy) and seeded into 6-well plates containing 1.5 ml layer of solidified 1% agarose low-gelling temperature. After seeding the cells were untreated or treated with 50 µM AXP3009. Culture medium (100 µl each well) was added twice weekly. After 21 days, cells were fixed using a solution of 4% of Crystal Violet containing 1% ethanol. At the end of the growth period, colonies were photographed with 10X objective under an automated microscope (EVOS FL Auto, Thermo Fisher Scientific, Waltham, MA, USA Italy).

5.7 Apoptosis assay

Cells were seeded in 96-well plates (5×10^3 cells/well) and, the day after, were transfected with the indicated constructs (see figure legends) and incubated with various compounds at the indicated conditions. At the end of the treatments, cells were fixed with 2% paraformaldehyde in PBS at RT for 30 min, then washed twice with PBS and stained with annexinV/FITC (Bender MedSystem, Milan, Italy) in binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$) for 10 min at RT in the dark. Finally, cells were washed with binding buffer solution and fluorescence was measured by using a multimode plate reader (Ex_{485nm} and Em_{535nm}) (GENios Pro, Tecan, Milan, Italy). The values were normalized on cell proliferation by Crystal Violet assay.

5.8 Monodansylcadaverine staining and autophagosome formation assay

To quantify the induction of autophagy, cells were incubated with the fluorescent probe monodansylcadaverine (MDC) (Sigma), accordingly with the guidelines for studying autophagy [167]. MDC is a selective marker for acidic vesicular organelles, such as autophagic vacuoles. Briefly, cells were seeded in 96-well plates (5×10^3 cells/well) and treated with various compounds as indicated in the figure legend. At the end of the treatments, cells were incubated in culture medium containing 50 μM MDC at 37 °C for 15 min. Cells were then washed with Hanks buffer (20 mM Hepes pH 7.2, 10 mM glucose, 118 mM NaCl, 4.6 mM KCl, and 1 mM $CaCl_2$) and fluorescence was measured using a multimode plate reader (Ex_{340nm} and Em_{535nm}) (GENios Pro, Tecan). The values were normalized on cell proliferation by Crystal Violet assay.

5.9 Analysis of intracellular ROS

The non-fluorescent diacetylated 2′,7′-dichlorofluorescein (DCF-DA) probe (Sigma-Aldrich), which becomes highly fluorescent upon oxidation, was used to evaluate intracellular ROS production. Briefly, cells were plated in 96-well plates (5×10^3 cells/well) and, the day after, were treated with the various compounds. At the end of the various treatments, the cells were incubated in culture medium with 10 μM DCF-DA for 15 min at 37 °C. The cells were washed with Hanks' buffer (20 mM Hepes, pH 7.2, 10 mM glucose, 118 mM NaCl, 4.6 mM KCl, and 1 mM $CaCl_2$) and the DCF fluorescence was measured by using a multimode plate reader (Ex485 nm and Em535 nm) (GENios Pro, Tecan). The values were normalized on cell proliferation by crystal violet assay.

To evaluate mitochondrial superoxide ion ($O2^{-}\cdot$) production we used the nonfluorescent MitoSOX red probe (Molecular Probes). The probe is live-cell permeative and is rapidly and selectively targeted to the mitochondria where it becomes fluorescent after oxidation by O2•−, but not by other ROS or reactive nitrogen species. Briefly, cells were plated in 96-well plates (5 × 103 cells/well) and incubated in culture medium with 0.5 μM MitoSOX at 37 °C for 15 min. The cells were then washed with Hanks' buffer and fluorescence was measured by using a multimode plate reader (Ex535 nm and Em590 nm) (GENios Pro, Tecan). The values were normalized on

cell proliferation by crystal violet assay. Three independent experiments were performed for each assay condition.

5.10 RNA isolation and quantitative real-time PCR analysis

Total RNA was extracted from 10^6 cells using TRIzol Reagent (Thermo Fisher) and 1 µg of RNA was reverse transcribed using first-strand cDNA synthesis. Real-time quantification was performed in triplicate samples by SYBR Green detection chemistry with Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) on a 7900 HT Fast Real-Time PCR System (Thermo Fisher). Normalization was performed analyzing the ribosomal protein large P0 (RPLP0) mRNA expression level. The primers used were: *GAPDH* For, 5′-ATCAGCAATGCCTCCTGCAC-3′;Rev, 5′-TGGTCATGAGTCCTTCCACG-3′; RPLP0 For, 5′-ACATGTTGCTGGCCAATAAGGT-3′ and Rev, 5′-CCTAAAGCCTGGAAAAAGGAGG-3′. *PGC1α* for: 5' tgactggcgtcattcaggag 3' ;rev: 5' ccagagcagcacactcgat 3'; *UCP2* for: 5' ctcctgaaagccaacctcat 3';rev: 5' cccaaaggcagaagtgaagt 3'; *TP53* for: 5' ggcccacttcaccgtactaa 3';rev: 5' gtggtttcaaggccagatgt 3'; *SESN1* for: 5' ggacgaggaacttggcatta; 3'rev: 5' atgcatctgtgcgtcttcac 3'; *SESN2* for: 5' gcctgctacccagagaagac 3';rev: 5' cctccaggagcagcaagtt 3'. The thermal cycle reaction was performed as follows: 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The average of cycle threshold of each triplicate was analyzed according to the $2^{(-\Delta\Delta Ct)}$ method. Three independent experiments were performed for each assay condition.

5.11 Subcellular fractionation

Cells were washed with PBS and scraped into hypotonic buffer (10 mM HEPES pH 8.0, 10 mM KCl, 0.1% Igepal CA-630, 1.5 mM $MgCl_2$, 1 mM NaF, 0.5 mM Na_3VO_4, 0.5 mM DTT, 1 mM PMSF and 1 × protease inhibitor cocktail). The suspension was incubated on ice for 10 minutes and after centrifugation at 300 x *g* for 10 min at 4 °C, the supernatant was used as the cytoplasmic fraction. The pellet was washed twice with PBS and reconstituted in RIPA buffer (100 mM Tris HCl pH 8.0, 1% Triton X-100,100 mM NaCl, 0.5 mM EDTA and 1 × protease inhibitor cocktail). The suspension was incubated on ice for 15 minutes. After centrifugation at 15,000 x *g* for 10

min, the resultant supernatant was used as the nuclear fraction. To obtain whole cell lysates, cells were harvested, washed in PBS, and re-suspended in lysis buffer in the presence of phosphatase and protease inhibitors (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Igepal CA-630, 0.5% Na-Doc, 0.1% SDS, 1 mM Na_3VO_4, 1 mM NaF, 2.5 mM EDTA, 1 mM PMSF, and 1 × protease inhibitor cocktail). After incubation on ice for 30 minutes, the lysates were centrifuged at 5000 x *g* for 10 min at 4 °C and the supernatant fractions were used for Western blot analysis.

5.12 Immunoblot analysis

Cells were lysed in the presence of phosphatase and protease inhibitors (50 mM Tris HCl pH 8, 150 mM NaCl, 1% Igepal CA-630, 0.5% Na-Doc, 0.1% sodium dodecyl sulphate (SDS), 1 mM Na_3VO_4, 1 mM NaF, 2.5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluorid, and 1 × protease inhibitor cocktail). Whole protein extracts (50 μg/lane for whole cell lysate immunoblots and 5 μg/lane for cytoplasmic and nuclear cell lysate immunoblots) were resolved on a 12% SDS-polyacrylamide gel and electro-blotted onto PVDF membranes (Millipore, Milan, Italy). Membranes were blocked in 5% low-fat milk in TBST (50 mM Tris pH 7.5, 0.9% NaCl, 0.1% Tween 20) for 1 h at room temperature (RT) and probed overnight at 4 °C with anti-phospho(Ser473)-AKT (1:2000; Cell Signaling Technology, Danvers, MA, USA, #4060), anti-AKT (1:1000; Cell Signaling #9272), anti-α-tubulin (1:2500; Oncogene, La Jolla, CA, USA, #CP06-100UG), anti-phospho(Thr172)AMPKα (1:1000) (Cell Signaling, #2535), anti-AMPKα (1:1000) (Cell Signaling, #2603), anti-glyceraldehyde 3-phosphate dehydrogenase GAPDH (G-9) (1:1000; Santa Cruz #sc-365062), anti-LaminB1 (A-11) (1:1000; Santa Cruz #sc-377000), anti-p53 (BP 53.12) (1:2000; Santa Cruz #sc-81168), anti-Sirt1(D739) (1:1000, Cell Signaling #2493), anti-SESN1 (1:1000 GeneTex, Irvine, CA, USA; #GTX118141), anti-SESN2 (1:1000 Santa Cruz Biotech, #sc-393195), PGC-1α (1:1000 Calbiochem, #ST1202), anti-UCP2 (1:1000 Abnova, Taipei City, Taiwan; #PAB7242), The immunocomplexes were visualized by chemiluminescent substrates (Amersham Pharmacia Biotech, Milan, Italy) using ChemidocMP imaging system (Bio-Rad Laboratories, Milan, Italy) and the intensity of the chemilumines-

cence signal was quantified using NIH Image J software (http://rsb.info.nih.gov/nih-image/).

5.13 Immunoprecipitation assay

Cell extracts were solubilized in lysis buffer with 150 mM Hepes pH 7.5, 300 mM NaCl, 1% Triton-X100, phosphatase and protease inhibitors. Cells were harvested and lysed in lysis buffer and cleared by centrifugation. For each immunoprecipitation, 1 µg of antibody and 1 µg of rabbit or sheep IgG (Santa Cruz Biotech) as control were used. To immunoprecipitate we used an anti-GAPDH antibody from Santa Cruz (sc-365062). 1 mg of pre-cleared protein extracts were diluted in lysis buffer containing 0.05% BSA and incubated with Dynabeads® Protein G (Thermo Fisher) and antibodies, according to the manufacturer's instructions. Bead-bound immunocomplexes were rinsed with lysis buffer and eluted in 50 µl of SDS sample buffer for Western blotting. Immunoblotting was performed using the following primary antibodies: anti-Sirt1 (D739) (1:1000, Cell Signaling #2493), anti-GAPDH (G-9) (1:1000; Santa Cruz #sc-365062), anti-α-tubulin (1:2500; Oncogene #CP06-100UG). To identify AMPK-SESN1 complex, we used 2 µg of anti-mouse AMPKα antibody (Santa Cruz Biotech, #sc-74461), anti-mouse SESN1 (PA26) antibody (Santa Cruz Biotech, #sc-376170), or mouse IgG (Santa Cruz Biotech) as control were used. The immune complexes were collected by addition of protein A sepharose (Millipore), rinsed extensively with RIPA buffer and eluted in a non-reducing sample buffer for Western Blotting.

5.14 Immunofluorescence imaging of GAPDH subcellular localization

After lentiviral transduction, Panc1 cells were fixed in 4% paraformaldehyde for 15 min and, after 4 changes (10 min each) of PBS, were permeabilized with 0.1% Triton X–100 for 5 min in PBS. To saturate unspecific binding sites, the cells were incubated for 45 min at RT with a blocking solution containing 5% BSA and 0.05% Triton X-100 in PBS. Samples were then incubated overnight at 4 °C with anti-GAPDH (1:250; Santa Cruz #sc-365062) primary antibody diluted in blocking solution. After 3 washes with PBS (10 min each), cells were incubated for 1 hour at RT in the dark with specific secondary antibodies (1 µg/ml) conjugated with Alexa Fluor-488 (Mo-

lecular Probes, Eugene, OR, USA). The incubation with secondary antibody was followed by 10 min incubation at RT with 1 μg/ml of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich). Samples were mounted in anti-bleaching medium (Dako Fluorescent Mounting Medium). The negative control procedure omitted the primary antibody. Pictures were acquired under a Leica TCS SP5 AOBS laser confocal microscope (Leica-Microsystems, Wezlar, Germany). A 40X/1.25 NA oil-immersion objective (HCX PL APO 40x 1.25 OIL UV, Leica-Microsystem) was used.

5.15 MitoTracker and MitoSox colocalization analysis

For live cell imaging measurements, AsPC-1 cells/chamber were seeded on a four-chamber μslide, with 13 mm glass bottom (ibidi GmbH, Germany). After 24 h cells were transfected with pcDNA3-mutp53R175H, pcDNA3-mutp53R273H or the pcDNA3 empty vector (mock) by using Lipofectamine^{TM} 2000 according to the manufacturer's instructions. Forty-eight hours after transfection cells were incubated for 30 minutes with a staining solution made of MitoSox Red 1:1000 (Life Technologies) and Mitotracker Green 1:5000 (Life Technologies) in medium without FBS. Before the acquisition, the medium was replaced with a special medium without red phenol (DMEM/F12 NoPhenolRED, Life Technologies) to avoid any interference with the fluorescence signal. Cell images were captured using a confocal laser-scanning fluorescence microscope Leica SP5 (Leica Microsystem, Manheim, Germany) at × 63 magnification and processed using Adobe Photoshop and ImageJ softwares (Rasband, W.S., ImageJ, U. S. National Institute of Health, Bethesda, Maryland, USA (http://rsb.info.nih.gov/ij/, 1997–2008).

5.16 L-lactic acid quantification assay

AsPC1 cells were seeded in 96-well plates (5×10^3 cells/well) and transfected for 48 h. At the end of the treatments, culture medium has been harvested, centrifuged at 1500 x *g* for 10 min and diluted six-fold in H_2O. For each sample, 25 μL has been analyzed in a final reaction volume of 500 μL (Megazyme, #K-LATE 07/14). Absorbance at 340 nm has been read after 10 min. The activation of the reaction and L-

lactic acid concentration (g/L) has been calculated according to the manufacturer's instructions. The amount of L-lactic acid secreted by the cells in each sample was calculated by subtracting the amount of L-lactic acid in the medium (without cells) from the amount of L-lactic acid in the medium from each sample. The values obtained were normalized to the number of cells in each well.

5.17 GAPDH activity assay

Cells were resuspended in 0.2 ml of an ice-cold buffered solution containing 200 mM NaCl, 1 mM EDTA, 20 mM CHAPS and 10% sucrose at pH=7 and disrupted with three freeze-thaw cycles. The total soluble protein content of the cell lysates was assessed by measuring the absorbance at 280 nm using a Cary4000 spectrophotometer (Agilent Technologies). Aliquots of 10 µl were assayed for GAPDH activity using a modified version of the Ferdinand assay [168] in a buffered solution containing 10 mM triethanolamine, 10 mM sodium arseniate, 5 mM EDTA, 1.5 mM NAD^+ and 2.2 mM DL-glyceraldehyde 3-phosphate. NADH formation at 25°C was monitored at 340 nm. Each assay was carried out at least in duplicate. The initial velocity was determined by linear fitting of the initial phase of the kinetics. The ratio between GAPDH activity and total soluble protein content was calculated for each cell lysate.

5.18 Wound-closure cell migration assay

AsPC1 were seeded in 6-well plate and, the day after, were transfected with the indicated constructs. After 48h, AsPC-1 cells were washed six times in PBS (phosphate-buffered saline) and then incubated in serum-free RPMI for 22 h. Then, the medium of AsPC1 transfected cells was collected and transferred in untransfected AsPC1 p53 null cells, that were seeded in 6-well plate. The confluent AsPC1 p53-null monolayer was denuded of cells by scraping it with a sterile 200 µl pipette tip to create a wound through the center of the confluent cell layer. Cells were incubated for 48h and monitored with a microscope equipped with a camera. Images of cells movement were captured every 30 min for 48 h. A time-lapse video was created with the acquired images and then, the images were further analyzed quantitatively by using ImageJ computing software.

5.19 FACS Analysis

Cells were trypsinized and washed with PBS. Then, cells were incubated with anti-human CD325 (N-Cadherin) antibody conjugated with PE (Biolegend, Cat. No. CD325) or anti-human CD324 (E-Cadherin) antibody conjugated with PE (Bio-Legend Cat. No. 324106). Cells were immediately analyzed by flow cytometry. All fluorescences were analyzed with a FACScalibur flow cytometer (Becton Dickinson) using Blue Laser (488 nm). Unstained cells were used to set fluorescent negative and positive threshold.

5.20 Protein extraction from conditioned medium

The day after transient transfection, AsPC-1 cells were washed six times in PBS (phosphate-buffered saline) and then incubated in serum-free RPMI for 22 h. This serum-free time period of incubation has been chosen on the basis of our previous investigations in order to avoid cell injury. Cell viability, determined with 0.4% trypan blue solution (Thermo Fischer Scientific), was higher than 95%. The media containing secreted proteins were collected by centrifugation at 1,000 x g for 10 min to pellet floating cells and were defined as conditioned media (CM). After the addition of 1X protease inhibitor cocktail (Roche), CM were centrifuged again at 17,000 x *g* for 20 min at 4°C to pellet the remaining cell debris. Proteins in the CM were precipitated overnight at -20°C with 4 volumes of ice-cold acetone. The pellets were then collected by centrifugation at 17,000 x g for 20 min at 4°C and resuspended in 100 mM ammonium bicarbonate (NH_4HCO_3). Protein concentrations were determined using BCA protein assay (Sigma).

5.21 In-solution digestion

Before SWATH-MS analysis, CM proteins were digested following the protocol provided by the manufacture (Applied Biosystem). Briefly, samples were prepared to have 100 µg of protein in a final volume of 25 µl of 100 mM NH_4HCO_3. The proteins were reduced using 2.5 µl of dithiothreitol (200 mM DTT stock solution) (Sigma) at 90° for 20 min, and alkylated with 10 µl of Cysteine Blocking Reagent (Iodoacetamide, IAM, 200 mM Sigma) for 1 hour at room temperature in the dark. DTT stock solution was then added in order to destroy the excess of IAM. After dilution

with 300 µl of water and 100 µl of NH4HCO3 to raise pH, 5 µg of tryspin (Promega, Sequence Grade) was added and digestion was performed overnight at 37 °C. Trypsin activity was stopped by adding 2 µl of neat formic acid and digests were dried by Speed Vacuum.

5.22 Data acquisition

The digested samples were analyzed on a micro-LC Eksigent Technologies (Dublin, USA) interfaced to a 5600+ TripleTOF mass spectrometer system (AB Sciex, Concord, Canada) equipped with a DuoSpray Ion Source and a CDS (Calibrant Delivery System). The LC column was a Halo Fused C18 (AB Sciex, Concord, Canada). The mobile phase was a mixture of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B), eluting at a flow-rate of 15.0 µL min^{-1} at an increasing concentration of solvent B from 2% to 40% in 30 min. The injection volume was 4.0 µL and the oven temperature was set at 40 °C. For identification purposes the samples were subjected to data dependent analysis (DDA): the mass spectrometer operated using a mass range of 100–1500 Da (TOF scan with an accumulation time of 0.25 s), followed by a MS/MS product ion scan from 200 to 1250 Da (accumulation time of 5.0 ms) with the abundance threshold set at 30 cps (35 candidate ions can be monitored during every cycle). The ion source parameters in electrospray positive mode were set as follows: curtain gas (N2) at 25 psig, nebulizer gas GAS1 at 25 psig, and GAS2 at 20 psig, ionspray floating voltage (ISFV) at 5000 V, source temperature at 450 °C and declustering potential at 25 V. For the quantification the samples were subjected to cyclic data independent analysis (DIA) of the mass spectra, using a 25-Da window: the mass spectrometer was operated such that a 50-ms survey scan (TOF-MS) was performed and subsequent MS/MS experiments were performed on all precursors. These MS/MS experiments were performed in a cyclic manner using an accumulation time of 40 ms per 25-Da swath (36 swaths in total) for a total cycle time of 1.5408 s. The ions were fragmented for each MS/MS experiment in the collision cell using the rolling collision energy. The MS data were acquired with Analyst TF 1.7 (AB SCIEX, Concord, Canada). Two DDA and four DIA acquisitions were performed.

5.23 Protein database search

The DDA files were searched using Protein Pilot software v. 4.2 (AB SCIEX, Concord, Canada) and Mascot v. 2.4 (Matrix Science Inc., Boston, USA). The DIA files were converted to pseudo-MS/MS spectra with DIA-Umpire software and were searched as DDA files [169][170]. Chymotrypsin as digestion enzyme was specified for both the software. For Mascot we used 2 missed cleavages, the instrument was set to ESI-QUAD-TOF and the following modifications were specified for the search: carbamidomethyl cysteins as fixed modification and oxidized methionine as variable modification. A search tolerance of 0.08 Da was specified for the peptide mass tolerance, and 10 ppm for the MS/MS tolerance. The charges of the peptides to search for were set to 2 +, 3 + and 4 +, and the search was set on monoisotopic mass. The UniProt Swiss-Prot reviewed database containing human proteins (version 2015.07.07, containing 42131 sequence entries) was used and a target-decoy database search was performed. False Discovery Rate was fixed at 1%.

5.24 Bioinformatics and Statistics Software

The potential secretion pathways of regulated proteins were predicted with the SecretomeP 2.0 server [171] (http://www.cbs.dtu.dk/services/SecretomeP/) for classical and non-classical secretion, while the localization of signal peptide cleavage sites were predicted with SignalP version 3.0 (http://www.cbs.dtu.dk/services/SignalP/)[172].

The regulated proteins were analyzed by using STRING software (http://string-db.org), which is a database of known and predicted protein-protein interactions[173]. Differentially abundant proteins were subjected to GO classification via the Panther Classification System database [174][175] to investigate biological processes, molecular function, cellular compartment and protein class.

5.25 Protein quantification

The quantification was performed by integrating the extracted ion chromatogram of all the unique ions for a given peptide. The quantification was carried out with PeakView 2.0 and MarkerView 1.2. (ABSCIEX). The result file from the DDA acquisitions were used for the library generation using a protein FDR threshold of 1%

[176]. Six peptides per protein and six transitions per peptide were extracted from the SWATH files. Shared peptides were excluded as well as peptides with modifications. Peptides with FDR lower than 1.0% were exported in MarkerView for the t-test.

5.26 Statistical analysis

ANOVA analysis with GraphPad Prism 5 software or two-tailed t-test were used to calculate P values. Statistically significant results were referred with a P-value < 0.05. Values are the means of three independent experiments (± SD).

6. RESULTS

6.1 Mutant p53 and ROS metabolism

6.1.1 Mutant p53 proteins stimulate the production of ROS

To study the functional role of GOF mutant p53 proteins in the regulation of ROS production, we first analyzed the endogenous level of ROS by staining pancreatic cancer cell lines with the DCF probe. When PaCa3, expressing wild-type p53 protein, was knocked down for p53 expression the ROS level increased, accordingly with the antioxidant role of wild-type p53 (figure 13). On the contrary, the ROS level was decreased after knockdown of GOF mutant p53 in Panc1 cells. Consistent with this, exogenous overexpression of R175H or R273H mutant p53 proteins in AsPC1 cells (null for p53 expression) produced a drastic increase of ROS level, confirming the pro-oxidant role of mutant p53 isoforms in pancreatic cancer cells.

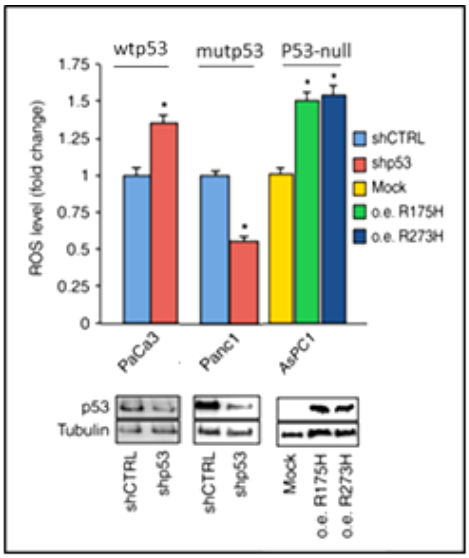


Figure 13: Mutant p53 proteins enhance ROS production in cancer cells. The indicated cell lines were transfected with the pRSuper-p53 vector and with plasmids for overexpression of mutant p53 (o.e. R175H; o.e. R273H), or their relative negative mock control (CTRL). DCF fluorescence intensity was analyzed by a multimode plate reader. Western blot of p53 was performed to test the effective knockdown of WT or mutant p53 and the overexpression of mutant p53 in the various cell lines indicated.

Then, we used small molecules, such as CP-31398 and RITA, which restore the wild-type transcriptionally competent conformation of mutant p53 proteins, as described in the introduction of this thesis. To activate the wild-type-like p53 function, we treated the cells with the p53-reactivators CP-31398 or RITA and we observed a decresead ROS level in both cancer cells having wild-type p53 (PaCa3) and mutant p53 (Panc1). As negative control, we also used p53-null AsPC1 cells to confirm that the effect of these compounds is mediated by p53 (figure 14).

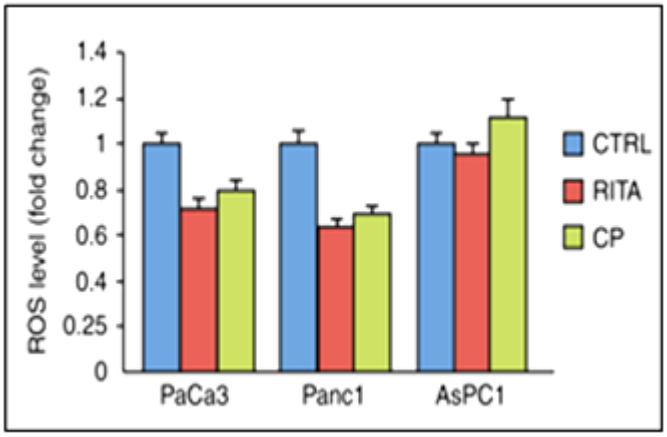


Figure 14: Reactivators of wtp53 decrease ROS production in both wtp53 (PaCa3) and mutp53 (Panc1) PDAC cells. The indicated cell lines were seeded in 96-well plates, incubated overnight, and treated with 20 μM CP-31398 or 40 μM RITA for 48 h. DCF fluorescence intensity was measured by a multimode plate reader.

To support these data and to investigate the subcellular source of ROS production by mutant p53 we analyzed mitochondrial superoxide ions ($O_2^{-}\cdot$) through MitoSox Red probe. We observed fluorescence emitted by MitoSox Red probe after exogenous overexpression of R175H or R273H mutant p53 isoforms in p53-null AsPC1 cells (figure 15 a) and also a colocalization of the fluorescence signals by MitoSox Red probe (revealing mitochondrial superoxide ions) and by MitoTracker Green (staining mitochondria) (figure 15b) indicating that mitochondria are a crucial source of ROS production induced by mutant p53 isoforms.

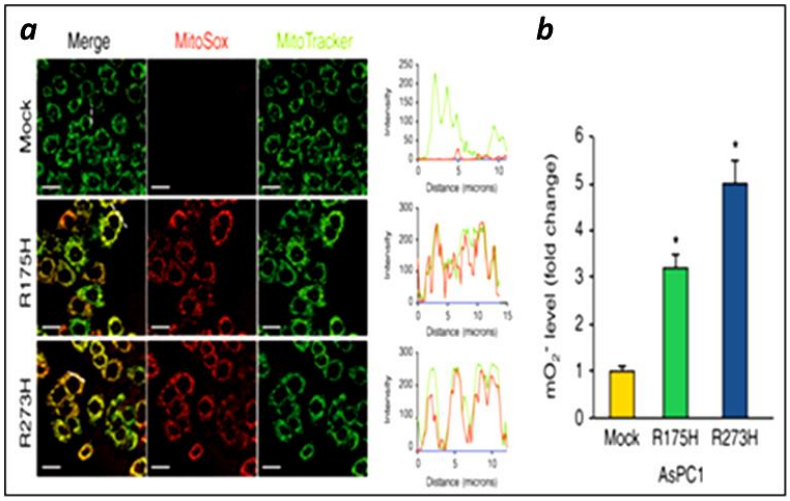


Figure 15: Mutant p53 proteins enhance mitochondrial superoxide ions production in cancer cells. *a)* Live cell imaging: 48 h after transfection with plasmid coding for R175H or R273H mutant p53, or with the mock vector (CTRL), cells were incubated for 30 min with MitoSox probe (red) and Mitotracker Green (green). The RGB profile plotted along the dashed line drawn in the merge image is also shown. Merge and single channel images come from a single z-plane. Scale bar 10 μm. *b)* Mitochondrial superoxide evaluation by MitoSox Red probe was analyzed with a multimode plate reader. Cells were seeded in 96-well plates, incubated overnight, and transfected with plasmid coding for R175H or R273H mutant p53, or the mock vector. All the experiments presented in this figure are representative of three biological replicates. P values were calculated with two-tailed t test. Statistical analysis: *$p < 0.05$ R175H or R273H vs Mock.

6.1.2 The oncogenic effects of mutant p53 are mediated by ROS induction

To explore the role of ROS stimulation on the oncogenic effects of mutant p53 proteins in PDAC cells we analyzed cell proliferation, apoptosis, and response to the drug gemcitabine (GEM) after addition of the radical scavenger NAC. First, we demonstrated that this antioxidant molecule was able to counteract ROS production by overexpression of R175H or R273H mutant p53 isoforms in AsPC1 p53-null cells (Fig. 16a). The hyper-proliferative effect and the anti-apoptotic effect induced by mutant p53 was counteracted by NAC treatment (Fig. 16b,c), demonstrating the functional involvement of ROS on these oncogenic events. Since my research team previously published that mutant p53 conferred chemoresistence to GEM treatment in pancreatic cancer cells [35], we investigated whether this function may be mediat-

ed by ROS. Figure 16d shows that mutant p53 expression reduced the sensitivity to GEM and that it was restored by the addition of the antioxidant compound NAC. Figure 16e shows an interesting result that may have therapeutic applications. Since is known that increasing ROS beyond a threshold level can inhibit cell proliferation inducing cell death, we investigated whether cancer cells expressing mutant p53 may acquire sensitivity to pro-oxidant agents. Thus, we discovered that R273H mutant p53 enhances cancer cell sensitivity to hydrogen peroxide, reversing the hyper-proliferative effect induced by mutant p53 and suggesting oxidant therapeutics in the treatment of cancer cells bearing mutant *TP53* gene.

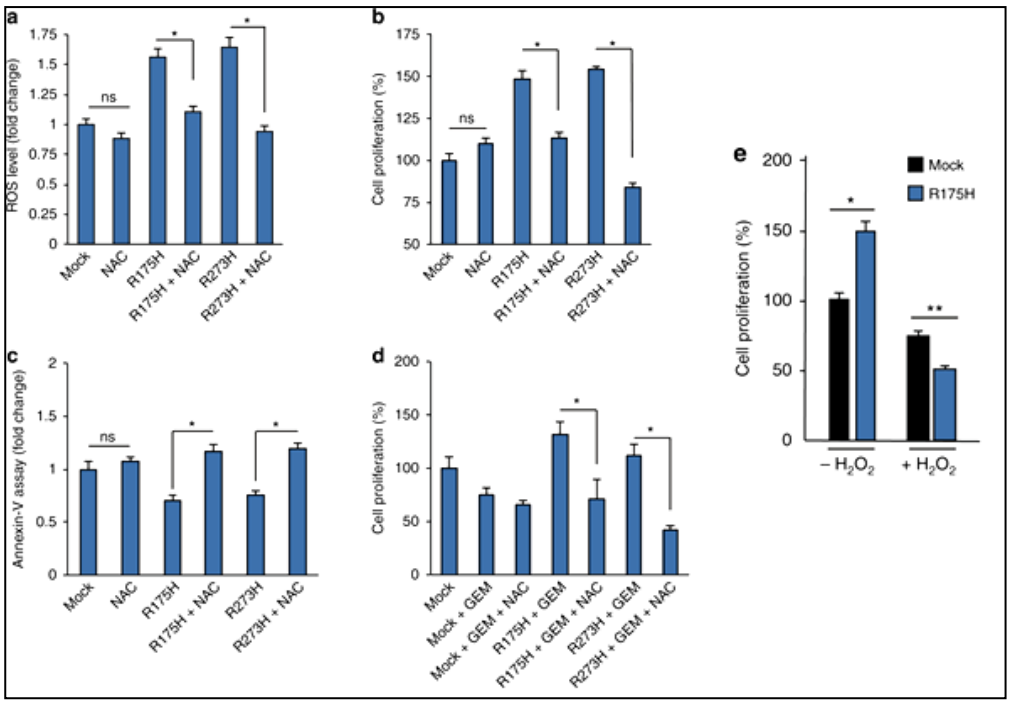


Figure 16: ROS induced by mutant p53 proteins are critical to mediate their oncogenic proprieties. a, b, c) AsPC1-p53 null cells were transfected with R175H or R273H vector, or mock control, and concomitantly treated with 7 mM NAC for 24 h. a) Intracellular ROS level was evaluated analyzing DCF fluorescence intensity using a multimode plate reader. b) Cell proliferation was measured by Crystal Violet assay and c) apoptosis was determined by the annexin V/FITC binding assay. Statistical analysis: *p < 0.05; R175H vs R175H + NAC and R273H vs R273H + NAC. d) AsPC1-p53 null cells were transfected and treated with 7 mM NAC and 1 μM GEM for 24 h. Cell proliferation was measured by Crystal Violet assay. Statistical analysis: *p < 0.05; R175H + GEM vs R175H + NAC + GEM and R273H + GEM vs R273H + NAC + GEM. e) AsPC1-p53 null cells were transfected with mock vector or with vector to express R175H mutant p53. After 24 h cells were treated with 100 μM H2O2 for further 24 h. Cell proliferation was measured by Crystal Violet assay. Statistical analysis: *p < 0.05 or ** p < 0.01 P R175H vs mock.

6.1.3 Mutant p53 downregulates UCP2 expression through the inhibition of PGC-1α

The mitochondrial uncoupling prototein UCP2 and its transcriptional activator PGC-1α are key factors in the maintenance of the mitochondrial redox balance [125][177]. Indeed, UCP2 is able to prevent the electron leakage from the respiratory chain, decreasing the superoxide ions production in mitochondria. Thus, we investigated wether mutant p53 induces ROS through the inhibition of the PGC-1α/UCP2 axis. The knock-down of endogenous mutant p53 in Panc1 cells determined the induction of both PGC-1α and UCP2 mRNAs, whereas the exogenous expression of the R273H mutp53 isoform inhibited their expression in p53-null AsPC1 (figure 17a). After treatment with the p53-reactivators CP-31398 or RITA we observed the increase of the expression level of UCP2 mRNA in mutant p53 Panc1 cells, whereas these compunds failed to modulate UCP2 expression in p53-null AsPC1 cells, further suggesting the involvement of p53 in their mechanism of action (figure 17b).

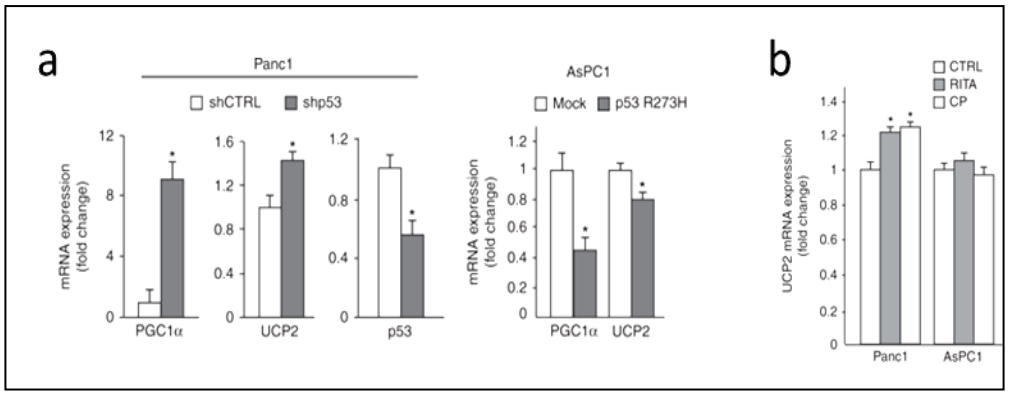


Figure 17: Mutant p53 downregulates UCP2 and PGC-1-α mRNAs levels. *a)* Panc1 mutR273H-p53 and AsPC1-p53 null cells were transfected with pRSuper-p53 vector and with plasmids for the ectopic expression of mutant p53-R273H or its relative negative control (CTRL). Gene expression analysis of the p53, UCP2, and PGC-1α was performed by RT-qPCR and was normalized to GAPDH mRNA. *$p < 0.05$. *b)* Panc1 mutR273H-p53 and AsPC1-p53 null cells were treated with 40 μM RITA and 20 μM CP-31398 for 48 h and gene expression analysis of UCP2 was performed by RT-qPCR and normalized to GAPDH mRNA. *$p < 0.05$.

We also demonstrated the inhibitory role of mutant p53 on the expression of PGC-1α and UCP2 proteins by Western blotting in Panc1 and AsPC1 cells (fig 18a). Fur-

thermore we analyzed the stimulation of UCP2 mRNA after mutp53-knockdown that was strongly inhibited by co-transfecting Panc1 cells with siRNA-PGC-1α (figure 18b), demonstrating the functional involvement of PGC-1α inhibition in the repression of UCP2 mRNA by mutant p53.

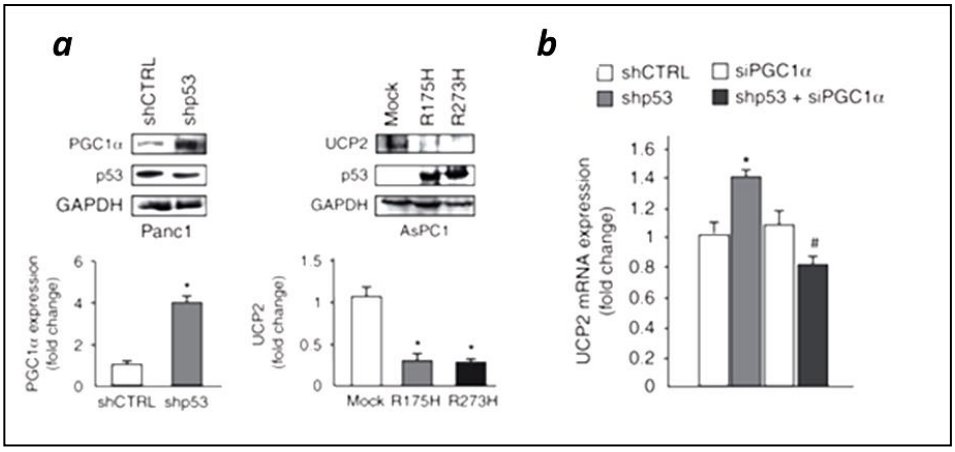


Figure 18: Mutant p53 downregulates UCP2 and PGC-1-α proteins levels. *a)* Western blotting analysis of Panc1 mutR273H-p53 and AsPC1-p53 null cells transfected with the indicate plasmids. Western blotting was performed using 50 μg of whole-cell extracts, probed with the indicated antibodies and quantified with ImageJ software. *b)* Panc1 mutR273H-p53 cells were transfected with pRSuper-p53 vector and siRNA-PGC-1α and relative controls and gene expression analysis of UCP2 was performed by RT-qPCR and normalized to GAPDH mRNA. *$p < 0.05$ shCTRL vs shp53; #$p < 0.05$ shp53 vs shp53 + siPGC-1α. The experiments are representative of three biological replicates

To confirm that PGC-1α/UCP2 axis inhibition is specifically due to a mutant p53-related GOF mechanism, we examined whether wild-type p53 is able to modulate this axis. The knock-down of wild-type p53 in PaCa3 cells failed to modulate mRNA levels of PGC-1α and also UCP2 (figure 19), in accordance with the lack of PGC-1α regulation and the absence of p53 binding sequences in the regulatory regions of the UCP2 gene.

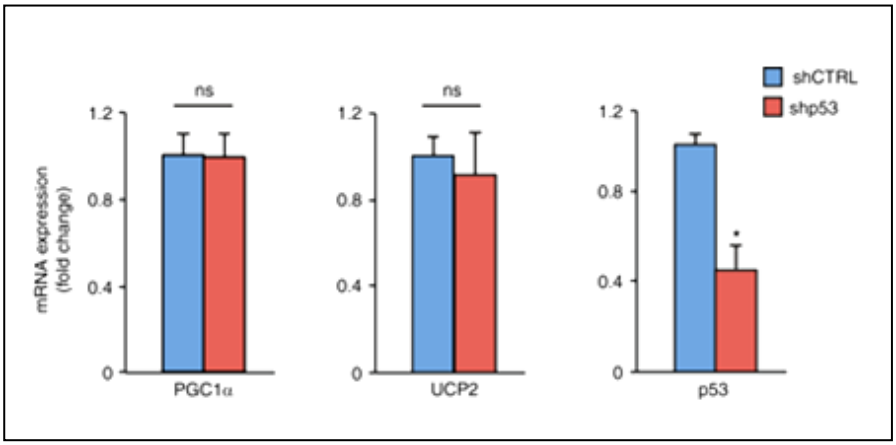


Figure 19: PGC-1α/UCP2 axis is not regulated by the endogenous basal level of wild-type p53. PaCa3 cells (WTp53) were transfected for 48 h with the pRSuper-p53 vector or its relative negative control (shCTRL). Gene expression analysis of p53, UCP2, and PGC-1α was performed by RT-qPCR and normalized to GAPDH mRNA. *p < 0.05 shp53 vs shCTRL

6.1.4 Mutant p53-dependent downregulation of the PGC-1α/UCP2 axis is mediated by the blockage of SESN1/AMPK signaling

Since AMPK signaling pathway has a crucial role in many biological functions and requires PGC-1α activity to modulate the expression of several key players in mitochondrial and glucose metabolism [178], we examined whether mutant p53 might inhibit PGC-1α/UCP2 axis through the upstream blockage of AMPK. We demonstrate that R273H mutant p53 inhibited the level of SESN1 and SESN2 mRNAs (figure 20a) and the protein expression of SESN1 and SESN2, as well as the P-AMPK level without affecting the total amount of AMPK (figure 20b). Importantly, we demonstrated by immunoprecipitation assay that mutant p53 decreased SESN1 binding to AMPK, likely as a consequence of SESN1 inhibition (figure 20b). In order to investigate the functional role of AMPK signaling in the inhibition of PGC-1α/UCP2 axis by mutant p53, we treated cells with AICA-R, a chemical activator of AMPK.

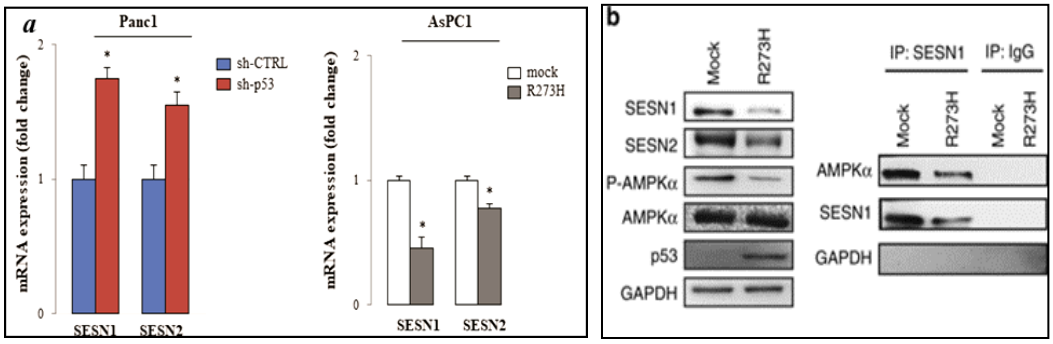


Figure 20: Mutant p53 inhibits UCP2 and PGC-1α through the inhibition of SESN1/AMPK signaling. *a)* Panc1 and AsPC1 were transfected for 48 h. Gene expression analysis of SESN1 and SESN2 was performed by RT-qPCR and normalized to GAPDH mRNA. *p < 0.05shp53 vs CTRL, R273Hvs Mock. *b)* H1299 p53-null cells stably expressing R273H mutant p53 (clone H1) and its respective mock control (clone C9) were used to identify the regulation of SESN1:AMPK complex by mutant p53. Left panel: western blotting was performed using 50 µg of whole-protein extracts and probed with the indicated antibodies. Right panel: SESN1 was immunoprecipitated from protein extracts using anti-SESN1 antibody (IP: SESN1) and western blot analysis was performed using indicated antibodies. Protein extracts were also immunoprecipitated with IgG as control.

Figure 21a shows that the repression of both PGC-1α and UCP2 mRNAs by overexpression of mutant p53 was reverted by AICA-R. In addition, we further demonstrated that transfection with a dominant negative isoform of AMPK γ (DN-AMPK), as compared with transfection with wild-type AMPK isoform γ (WT-AMPK), was able to inhibit the induction of both PGC-1α and UCP2 mRNAs after knock-down of endogenous mutant p53 (Fig. 21b). Altogether these data indicate that mutp53-dependent downregulation of the PGC-1α/UCP2 axis is mediated by the blockage of AMPK signaling. Finally, in order to investigate whether AMPK also plays a role in the final pro-oxidant effect of mutant p53, we analyzed ROS level after silencing endogenous mutant p53 with the concomitant transfection of DN-AMPK or WT-AMPK and we discovered that ROS level decreased in mutp53-knockdown conditions and it was recovered by DN-AMPK (figure 21c). Thus, all toghether these data show that the pro-oxidant inhibition of the PGC-1α/UCP2 axis by mutant p53 is due to AMPK signaling inhibition, which might be influenced by inhibition of SESN1 expression.

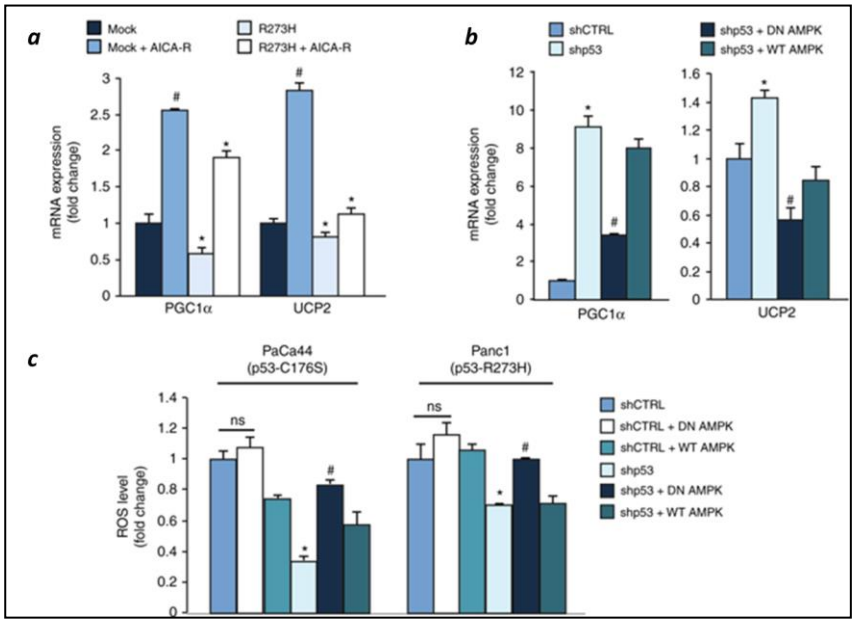


Figure 21: The mutp53-dependent downregulation of the PGC-1α/UCP2 axis is mediated by the blockage of AMPK signaling. *a)* AsPC1-p53 null cells were transfected with the vectors for the ectopic expression of p53-R273H and its mock control and treated with 1 mM AICA-R for 48 h. Gene expression analysis of the UCP2 and PGC-1α was performed by RT-qPCR and normalized to GAPDH mRNA. #p < 0.05 mock vs mock + AICA-R; *p < 0.05 mock vs R273H and R273H vs R273H + AICA-R. *b)* Panc1 mutR273H-p53 cells were transfected for 48 h with the indicated vectors and their relative negative controls. Gene expression analysis of UCP2 and PGC-1α was performed by RT-qPCR and normalized to GAPDH mRNA. *p < 0.05 shp53 vs CTRL; #p < 0.05 shp53 + DN-AMPK vs shp53 or shp53 + WT-AMPK. *c)* The indicated cell lines were transfected with pRSuper-p53, DN-AMPK, WT-AMPK vectors, or negative controls. ROS levels were analyzed using DCF probe by a multimode plate reader. *p < 0.05 shp53 vs CTRL; #p < 0.05 shp53 + DN-AMPK vs shp53 or shp53 + WT-AMPK.

6.1.5 The pro-oxidant and oncogenic effect of mutant p53 by UCP2 inhibition

To functionally demonstrate that the UCP2 blockage has a role on the pro-oxidant effect of mutant p53, we discovered that ROS level decreased after mutant p53 knockdown and it was recovered by siRNA-UCP2 or by the UCP2 innhibitor genipin in Panc1 cells (fig 22a). Furthermore, ROS level increase by R273H or R175H mutant p53 overexpression in p53-null AsPC1 cells was reduced by UCP2 overexpression (fig 22a). Figure 22b shows that UCP2 inhibition reduced the oncogenic hyper-

proliferative effect of mutant p53 in PDAC cells. Finally, we can conclude that UCP2 inhibition is a mechanism by which mutant p53 plays its oncogenic and pro-oxidant functions.

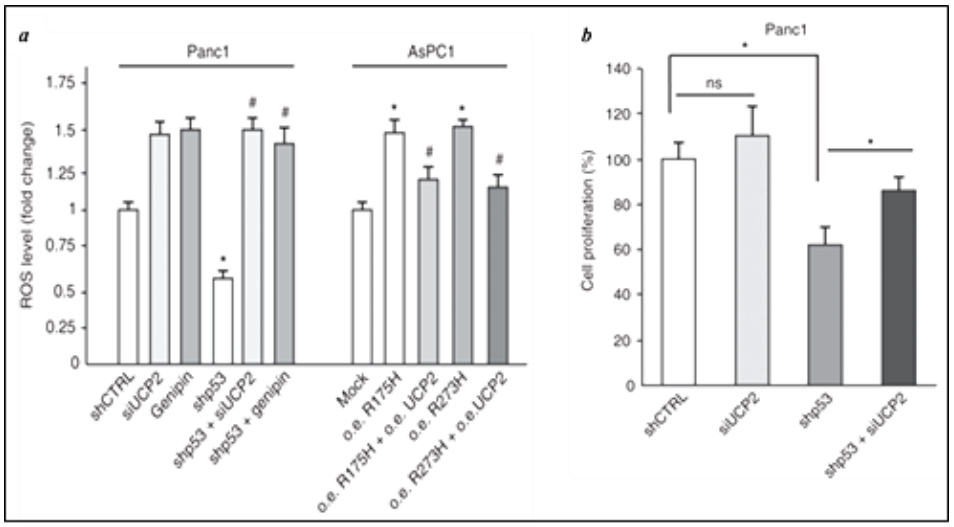


Figure 22: Mitochondrial superoxide production is due to mutp53-dependent UCP2 inhibition. *a)* Panc1 mutR273H-p53 and AsPC1-p53 null cells were transfected with pRSuper-p53 and vector for mutant p53 ectopic expression, respectively, and their relative controls. In addition, Panc1 cells were co-transfected with siRNA-UCP2, or treated with 150 μM genipin for 24 h; while AsPC1 cells were co-transfected with the UCP2 vector. ROS production, corresponding to DCF fluorescence intensity, was analyzed by a multimode plate reader. *$p < 0.05$ shCTRL vs shp53 or mock vs R175H or R273H; #$p < 0.05$ shp53 vs shp53 + siUCP2 or shp53 + genipin (Panc1 cells); R175H or R273H vs R175H + UCP2 or R273H + UCP2 (AsPC1 cells). *b)* Panc1 cells were transfected for 48 h with shp53 and/or siUCP2 and their relative negative controls. Cell proliferation was measured by Crystal Violet assay. *$p < 0.05$ shCTRL vs shp53; shp53 vs shp53 + siUCP2.

6.2 Mutant p53 and energy metabolism: the prevention of GAPDH nuclear translocation in PDAC cells

6.2.1 Mutant p53 prevents the nuclear translocation of GAPDH

To study another oncogenic role of mutp53 proteins in the metabolic regulation of cancer cells, we focused our attention on the regulation of the intracellular distribution of GAPDH. We modulated p53 expression in PDAC Panc1 and PaCa3 cell lines having mutant or wild-type *TP53* gene, respectively, by using liposome-mediated transient transfection assay and we analyzed the abundance of GAPDH in the cytosolic and nuclear fractions of the cells. When Panc1 cells were knocked-down for mutant p53 expression (left panel), the GAPDH expression level in the nuclear fraction increased revealing a role for mutant p53 in the prevention of the nuclear translocation of the enzyme (Figure 23A). Consistent with this, the exogenous expression of R273H mutant p53 in AsPC1 cells (null for p53 expression) (left panel) produced a drastic decrease of the GAPDH expression in nuclei (Figure 23B). To investigate whether this is a phenomenon specifically acquired by mutant proteins we knocked-down WTp53 (left panel) and analyzed cytosolic and nuclear GAPDH distribution, revealing that the WTp53 counterpart was not able to regulate the enzymatic nuclear translocation (Figure 23C). To check the purity of cytosolic and nuclear subcellular fractions we tested the abundance of α-tubulin and of Lamin B1, which are specifically expressed in cytosol and nucleus of the cells, respectively (Figure 23A-23C). We further strengthened these data through lentivirus-mediated transduction and immunofluorescence analysis by confocal microscopy using a different sequence to knock-down mutant p53 expression (p53-SH1) or its negative non-targeted control (p53-NT) in PDAC Panc1 cells. Figure 23D shows that mutp53 silencing unchanged the expression level of GAPDH (right panel), but it prompted GAPDH nuclear positivity as revealed also by XY, XZ and YZ orthogonal projections of confocal images.

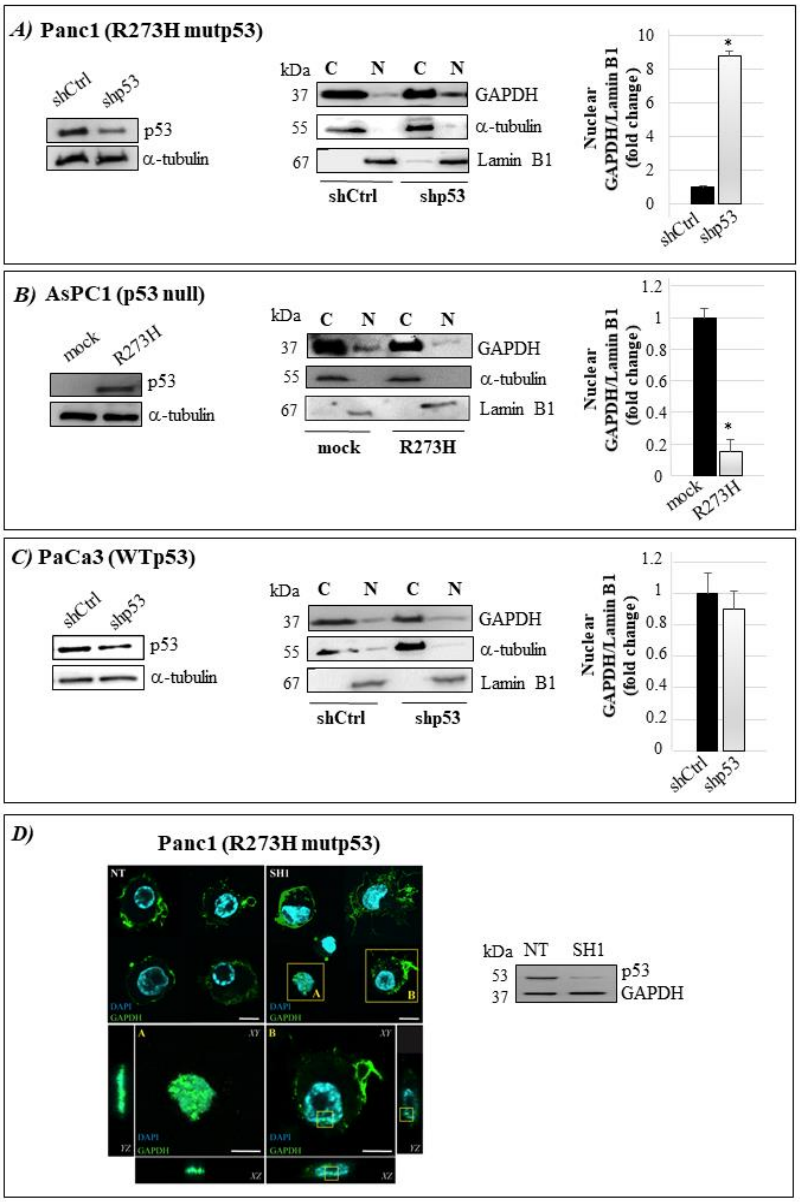


Figure 23. Mutant p53 prevents GAPDH nuclear translocation. *A-C)* Cytosolic and nuclear extracts were used for Western blotting of GAPDH, α-tubulin and Lamin B1 in Panc1 and PaCa3 cell lines transfected with pRSuper-p53 or mock vector, or in AsPC1 cells transfected with plasmids for R273H mutant p53 expression or its negative control. α-tubulin and Lamin B1 have been used as controls of the quality of the cytoplasmic and nuclear protein fractions, respectively. The amount of nuclear GAPDH in each extract was quantified using NIH Image J software and normalized to the amount of Lamin B1. Statistical analysis *p<0.05 Shp53 vs ShCtrl in Panc1 cells and *p<0.05 R273H vs mock in AsPC1 cells. *D)* Transduced Panc1 cells with vector encoding TP53-shRNA indicated as SH1 or its non-target shRNA control indicated as non-target (NT). In NT image, the GAPDH (green signal) is mainly localized into the cytoplasm and on the cell membrane. 48 hours after transduction, the enzyme is visible also into the nuclei (blue signal). *Inset A:* the cell is shown with a higher magnification. The enzyme is clearly visible inside the nucleus (as shown by the orthogonal projection, XZ

and YZ). *Inset B*: GAPDH (green spots) begins to localize in the nucleus as it can be appreciated from the orthogonal projection XZ and YZ (yellow squares). Scale bar: 10 µm.

6.2.2 Mutant p53 enhances the glycolytic activity of GAPDH and stimulates the L-lactate secretion

We further explored whether the stabilization of cytosolic GAPDH by mutp53 resulted in an enhanced glycolytic activity of the enzyme. Consistent with results reported in figure 23, figure 24A shows that mutp53 knock-down in Panc1 cells decreased GAPDH activity, which remained unchanged after WTp53 silencing in PaCa3 cells. Overall, in our experimental model we observed that WTp53 and mutp53 have dual opposite effects on the secretion of L-lactate, the metabolic compound generated by the glycolytic pathway. Figure 24B shows that L-lactate secretion was enhanced by mutp53, consistent with the cytosolic stabilization of GAPDH, while the WTp53 counterpart produced an inhibitory effect on L-lactate secretion, consistent with its overall negative effects on glycolysis [179].

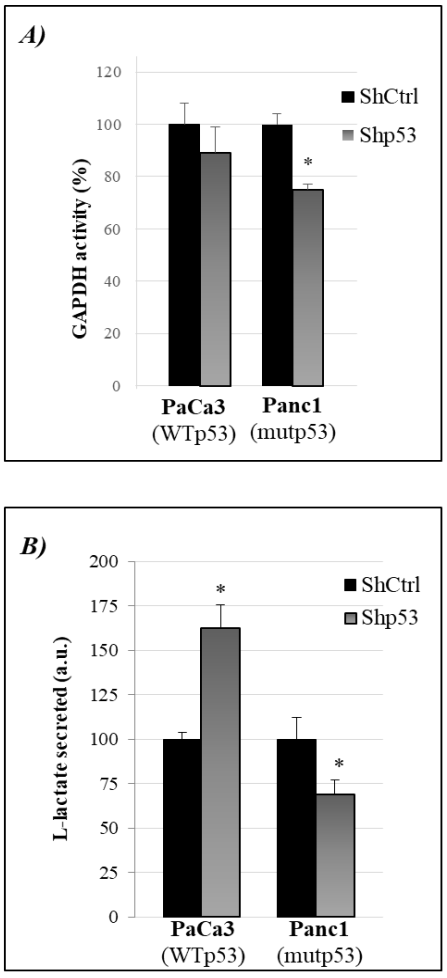


Figure 24. Mutant p53 enhances GAPDH glycolytic activity and L-lactate secretion. *A)* GAPDH activity was quantified in Panc1 and PaCa3 cells transfected with pRSuper-p53 vector or its negative control for 48 h. Statistical analysis *p<0.05 Shp53 vs ShCtrl. *B)* Panc1 and PaCa3 cells were transfected with pRSuper-p53 vector or its negative control for 48 h. L-lactic acid level in the culture medium has been analyzed by measuring the absorbance at 340 nm and the L-lactic acid concentration has been calculated as detailed in Material and Methods. Statistical analysis *p<0.05 Shp53 vs ShCtrl.

6.2.3 Prevention of nuclear localization of GAPDH by mutant p53 is mediated by regulation of SIRT1:GAPDH complex and of AMPK and AKT pathways.

Since it has been described that the binding of SIRT1 to GAPDH retains the latter protein in the cytosol as a mechanism to protect cytosolic GAPDH from nuclear translocation [180], we investigated whether mutp53 might stimulate SIRT1:GAPDH interaction. The large amount of protein extract used for the immunoprecipitation assay was obtained by a clone of H1299 cancer cells stably expressing R273H mutp53, which we previously used to study the oncogenic effects of mutp53s [52]. Figure 25 shows that H1299 cancer cells stably expressing R273H mutp53 presented enhanced levels of SIRT1 as compared to a mock clone of the same cells (left panel). Furthermore, immunoprecipitation assay revealed that SIRT1:GAPDH interaction was increased in R273H mutp53 expressing cells, as compared to mock (right panel), suggesting an involvement for this complex in the prevention of nuclear localization of GAPDH driven by mutp53 in cancer cells.

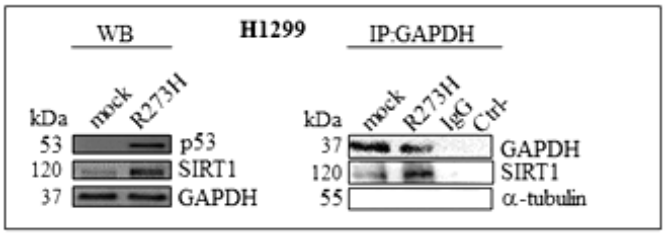


Figure 25. Prevention of nuclear localization of GAPDH by mutant p53 is mediated by regulation of SIRT1:GAPDH complex. Cell lysates from p53-null H1299 cancer cells (C9 clone) and H1299 stably expressing R273H mutp53 (H1 clone) were used to perform Western blotting by loading 30 μg of protein extracts and probed with the indicated antibodies. Left panel: GAPDH was used as control of equal protein loading. Right panel: GAPDH was immunoprecipitated from protein extracts of H1299 cell C9 clone (mock) or H1 clone stably expressing R273H mutp53 using anti-mouse GAPDH antibody (IP: GAPDH) and Western blotting was performed using anti-SIRT1 antibody. Negative control (Ctrl-) corresponds to lysis buffer without protein extracts immunoprecipitated with anti-GAPDH as described for mock or R273H samples. Protein extracts from H1299 cells (C9 mock clone) were also immunoprecipitated with an equal amount of mouse IgG as control. The blot exhibits equivalent GAPDH levels and the absence of α-tubulin expression in C9 and H1 clone samples as control of the quality of the immunoprecipitation.

Moreover, since GAPDH cellular distribution can also be regulated by post-translational modifications of the enzyme, we tested whether mutp53 can modulate AMPK and AKT signaling pathways, which are described to be involved in GAPDH phosphorylation in different amino acidic residues of the enzyme resulting in the inhibition or in the stimulation of GAPDH nuclear translocation, respectively [181], [182]. Figure 26 shows that mutp53 overexpression in p53-null PDAC AsPC1 cells strongly stimulated AKT signaling (P-AKT/AKT ratio) and inhibited AMPK signaling (P-AMPK/AMPK ratio).

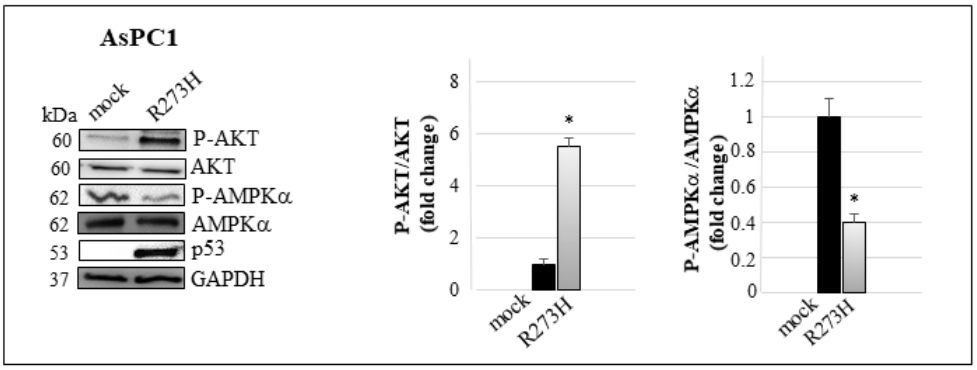


Figure 26. Mutant p53 regulates AMPK and AKT signaling pathways. Western blotting was performed using 40 μg of whole cell protein extracts from AsPC1 transfected with plasmids for R273H mutant p53 expression or its negative control (mock) and probed with the indicated antibodies. For quantitative analysis, bands were quantified using NIH Image J software and normalized to the amount of GAPDH. Statistical analysis * $p<0.05$ R273H vs mock.

To investigate the role of AMPK and AKT regulation on the prevention of nuclear localization of GAPDH by mutp53 we treated cells with the AMPK activator AICAr or with the AKT inhibitor SH-5. Figure 27A reports that the decrease of nuclear GAPDH by mutp53 was recovered by AICAr or SH-5 treatment. Accordingly, the increased level of secreted L-lactate by mutp53 was reversed after GAPDH silencing or cell treatment with AICAr or SH-5 (figure 27B). Altogether these results demonstrated that mutp53 adopted different mechanisms to prevent GAPDH nuclear translocation sustaining cytosolic glycolysis.

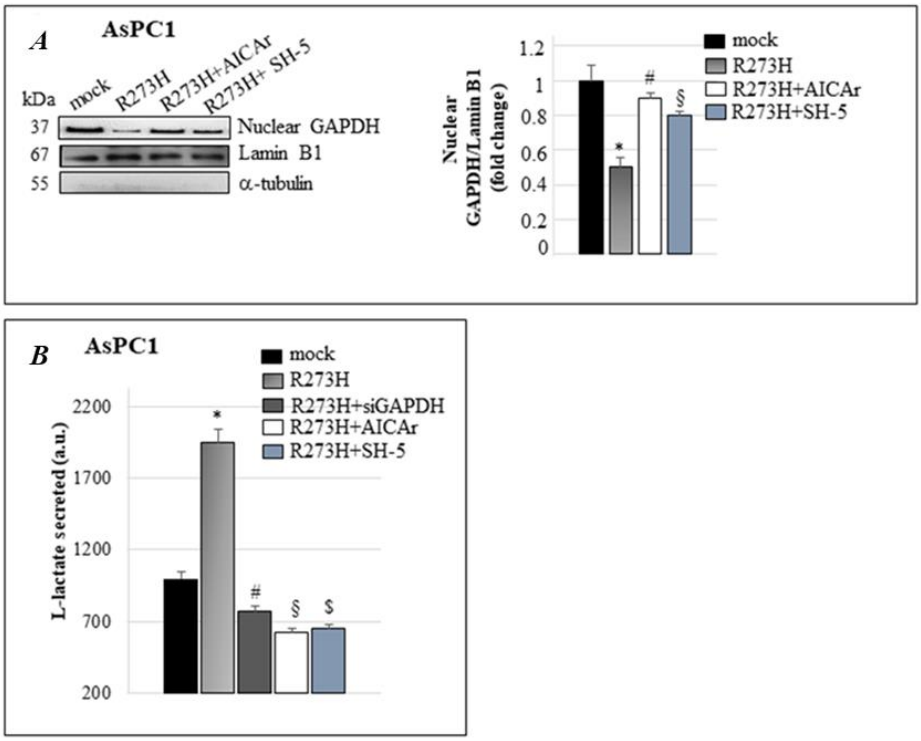


Figure 27: Prevention of nuclear localization of GAPDH by mutant p53 is mediated by regulation of AMPK and AKT pathways. *A)* AsPC1 cells were transfected with the vectors for the ectopic expression of p53-R273H or its mock control for 48 h and treated with 2 mM AICAr for 72 h or 15 μM SH-5 for 48 h. Western Blot analysis was performed using whole cell extracts of AsPC1 and probed with the indicated antibodies. α-tubulin and Lamin B1 have been used as controls of the quality of the nuclear protein fraction. Statistical analysis *p<0.05 R273H vs mock; #p<0.05 R273H + AICAr vs R273H; §p<0.05 R273H + SH-5 vs R273H. *B)* AsPC1 cells were transfected with the vectors for the ectopic expression of p53-R273H or its mock control for 48 h and for the knock-down of GAPDH using 50 nM siRNA-GAPDH for 48 h. AsPC1 cells were treated 2 mM AICAr for 72 h or 15 μM SH-5 for 48 h. L-lactic acid level in the culture medium has been analyzed as detailed in Material and Methods. Statistical analysis *p<0.05 R273H vs mock; #p<0.05 R273H + siGAPDH vs R273H; §p<0.05 R273H + AICAr vs R273H; $p<0.05 R273H + SH-5 vs R273H.

6.2.4 *GAPDH cytosolic stabilization contributes to the oncogenic effects of mutant p53*

To investigate the functional role of GAPDH cytosolic stabilization on the oncogenic effects of mutp53 we tested PDAC cell proliferation and apoptosis after R273H mutp53 overexpression in AsPC1 cells with the concomitant inhibition of the cytosolic glycolytic activity of GAPDH by the AXP3009 compound or after GAPDH siRNA transfection for gene silencing. Figures 28A and 28B show that a non-toxic

concentration of AXP3009 or cell transfection with GAPDH siRNA reversed R273H mutp53-dependent PDAC cell hyperproliferation and apoptosis inhibition, respectively.

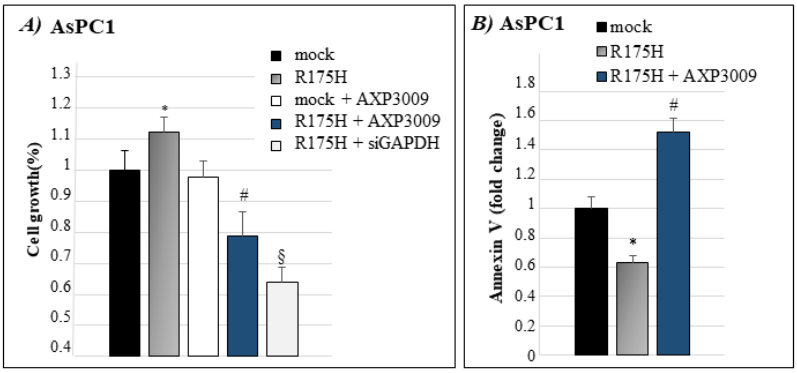


Figure 28. GAPDH cytosolic stabilization contributes to the oncogenic effects of mutant p53. *A)* Cell proliferation was measured by Cristal Violet assay and determined by the annexinV binding assay. *B)* apoptosis was AsPC1 cells were transfected with plasmids for R273H mutant p53 overexpression or its mock vector and for the knock-down of GAPDH using 50 nM siRNA-GAPDH for 48 h. The cells were also treated with 100 µM AXP3009 compound for 48 h. Statistical analysis *p<0.05 R273H vs mock; #p<0.05 R273H + AXP3009 vs R273H; §p<0.05 R273H + siGAPDH vs R273H.

To confirm the role of GAPDH cytosolic stabilization on the oncogenic effects of mutp53 we performed anchorage-independent soft agar colony assay. Figure 29 shows that R273H mutp53 favors the formations of the colonies (as compared to mock cells) and that AXP3009 treatment strongly reversed this phenomenon.

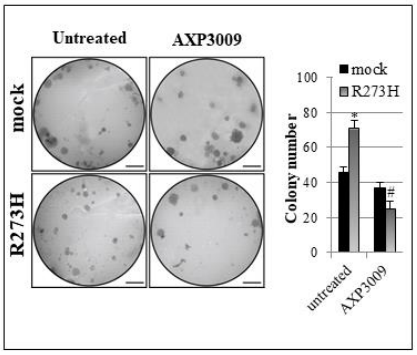


Figure 29: The GAPDH cytosolic stabilization by mutant p53 induces anchorage-independent cell growth. Representative images of soft agar colony assay. Upon 21 days of H1299 cell culture, the number of colonies in mock cells and mutp53 R273H cells, untreated or treated with 50 µM

AXP3009, was quantified by ImageJ software after staining with Crystal Violet. Results were shown as mean ± SEM of three independent experiment (right panel). Scale bar: 200 µm. Statistical analysis *p<0.05 R273H vs mock; #p<0.05 R273H + AXP3009 vs R273H.

Similar results have been observed also for R175H mutp53-dependent modulation of cell growth and apoptosis (figure 30A and 30B), extending the concept that GAPDH cytosolic stabilization may contribute to the oncogenic effects also of other GOF mutp53 isoforms.

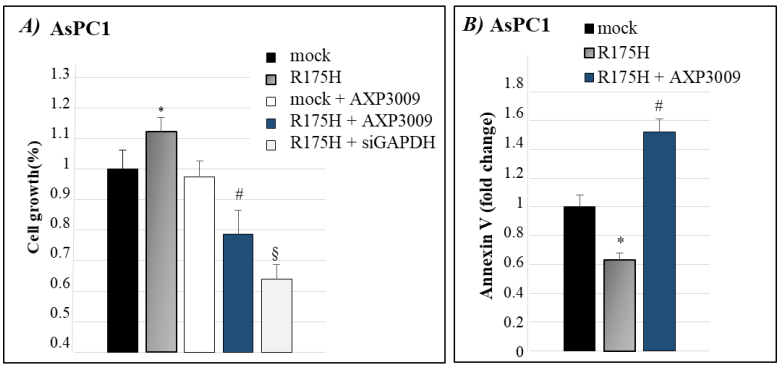


Figure 30: GAPDH cytosolic stabilization contributes to the oncogenic effects of mutant p53 isoforms. *A)* Cell proliferation was measured by Cristal Violet assay and *B)* apoptosis was determined by the annexin V-FITC binding assay. AsPC1 cells were transfected with plasmids for R175H mutant p53 expression or its mock vector and for the knock-down of GAPDH using 50 nM siRNA-GAPDH for 48 h. The cells were treated with 100 µM AXP3009 for 48 h. Statistical analysis *p<0.05 R175H vs mock; $^{\#}$p<0.05 R175H + AXP3009 vs R175H; §p<0.05 R175H + siGAPDH vs R175H.

Moreover, since we previously demonstrated that oncogenic effects of mutp53 in PDAC cells are mediated also by the counteraction of autophagy [52] and that mutp53s stimulate resistance to the drug gemcitabine in PDAC cells [52], we investigated whether GAPDH activity might contribute to regulate also these phenomena. In figure 31A we demonstrated that mutp53 decreased the amount of intracellular autophagic vesicles and this event was completely reversed by the addition of the AMPK activator AICAr or the AKT inhibitor SH-5, which also restored GAPDH nuclear prevention by mutp53 (figure 27A) and reduced L-lactate secretion prompted by mutp53 (figure 27B). In accordance with these observations, the shp53-induced increase of autophagy was counteracted in GAPDH silencing conditions in Panc1

cells, confirming the involvement of GAPDH in the inhibition of autophagy by mutp53 (figure 31B). Altogether, these data indicate that GAPDH expression is required to mediate several oncogenic effects of mutp53.

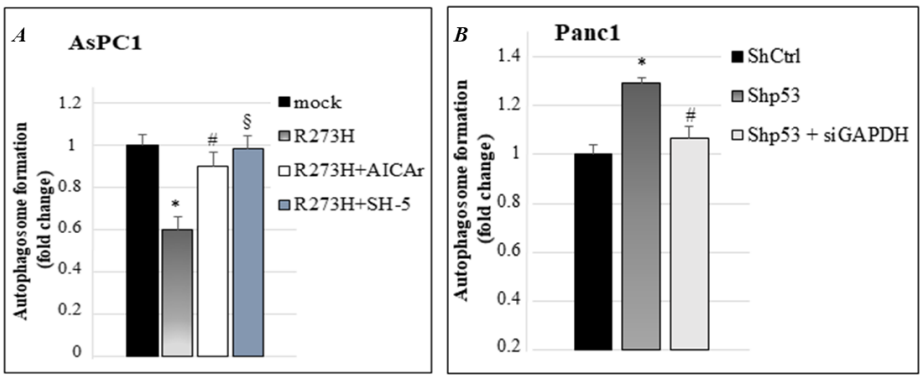


Figure 31: The GAPDH cytosolic stabilization by mutant p53 counteracts autophagosome formation. *A)* Autophagosome formation assay by MDC probe in AsPC1 cells transfected and treated with 2 mM AICA-R for 72 h or 15 μM SH-5 for 48 h. Statistical analysis *p<0.05 R273H vs mock; #p<0.05 R273H + AICAr vs R273H; §p<0.05 R273H + SH-5 vs R273H. *B)* Autophagosome formation assay in Panc1 cells transfected with pRSuper-p53 vector (Shp53) or its negative control (ShCtrl), in the absence or presence of siRNA-GAPDH for 48 h. Statistical analysis *p<0.05 shp53 vs shCtrl; #p<0.05 shp53 + siGAPDH vs shp53.

6.2.5 GAPDH cytosolic stabilization confers chemoresistance to gemcitabine and sensitizes cells to 2-deoxyglucose

Concerning drug chemoresistance, we demonstrated that GAPDH knock-down by siRNA restored PDAC cell sensitivity to gemcitabine even in mutp53-overexpressing conditions (figure 32A). Furthermore, we tested the role of GAPDH in PDAC cells endogenously expressing wild-type p53 or R273H mutp53. Figure 32B shows that GAPDH silencing had an undetectable effect on gemcitabine sensitivity of wt-p53 cells PaCa3 cells, while it enhanced the response to gemcitabine of mutp53 Panc1 cells.

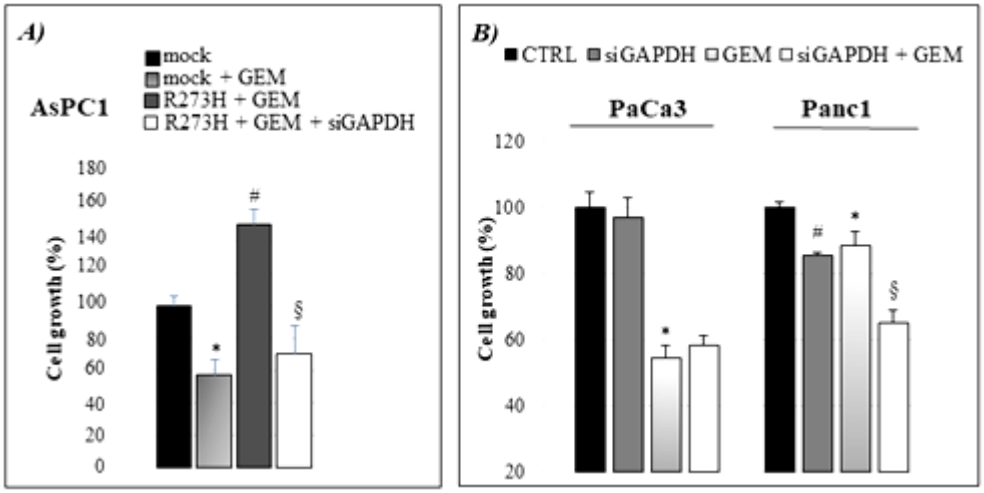


Figure 32: GAPDH cytosolic stabilization confers chemoresistance to gemcitabine. *A)* Cell proliferation was measured by Cristal Violet assay in AsPC1 transfected cells and treated with 1 μM gemcitabine (GEM) for 48 h. Statistical analysis *p<0.05 mock + GEM vs mock; #p<0.05 R273H + GEM vs mock + GEM; §p<0.05 R273H + GEM + siGAPDH vs R273H + GEM. *B)* Cell proliferation was measured by Cristal Violet assay in wt-p53 PaCa3 cells and mutp53-Panc1 cells transfected with siRNA GAPDH and/or treated with 1 μM gemcitabine (GEM) for 48 h. #p<0.05 siGAPDH vs CTRL; *p<0.05 GEM vs CTRL; §p<0.05 siGAPDH + GEM vs GEM.

Furthermore, we aimed to investigate whether PDAC cells bearing mutp53 might be more sensitive to the glycolytic standard inhibitor 2-DG, accordingly with the stimulatory role of mutp53 on glycolysis via GAPDH cytosolic stabilization. To functionally demonstrate the involvement of mutp53 on PDAC cell sensitivity to 2-DG, we evaluated the response of the cells after overexpression of R273H mutp53. Our data reported in figure 33 show that mutp53 overexpression conferred to p53-null AsPC1 cells a strong sensitization to 2-DG incubation, as compared to its negative mock control. These results demonstrated that targeting the glycolytic pathway may represent a potential therapeutic opportunity for cancer patients bearing mutations in the *TP53* gene.

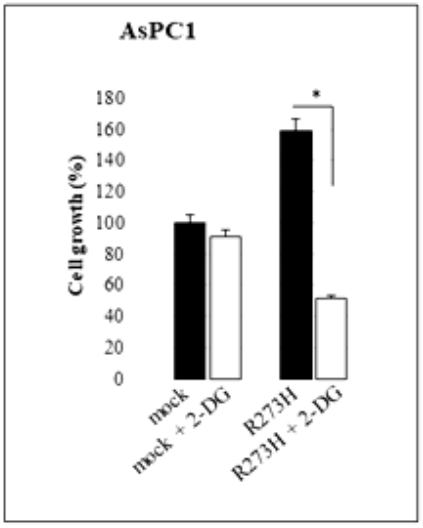


Figure 33: GAPDH cytosolic stabilization sensitizes cells to 2-deoxyglucose. Cell proliferation was measured by Cristal Violet assay in AsPC1 cells transfected as indicated and treated with 5 mM 2-DG for 48 h. Statistical analysis *p<0.05 R273H + 2-DG vs R273H.

6.3 Mutant p53 and tumor microenvironment

6.3.1 The oncogenic effects of mutant p53 are also mediated by alterations of the cancer cell secretome

To identify a specific signature of biomarkers secreted by PDAC cells carrying GOF mutant p53, primarily we want to find out whether mutant p53 may influence the secretome of PDAC cells in order to elucidate the functional role of mutp53-driven secretome. Thus, as summarized in figure 34, we induced the exogenous expression of R273H and R175H mutp53 in PDAC AsPC1 cell line (null for p53 expression) by using liposome-mediated transient transfection assay. After checking the transfection efficiency by Crystal Violet assay and Western Blotting, we collected and transferred the conditioned medium (CM) released by AsPC1 transfected cells to p53-null cancer cells. Cells cultivated in mutp53-driven secretome were collected to study the different functional effects of secretome driven by GOF mutp53.

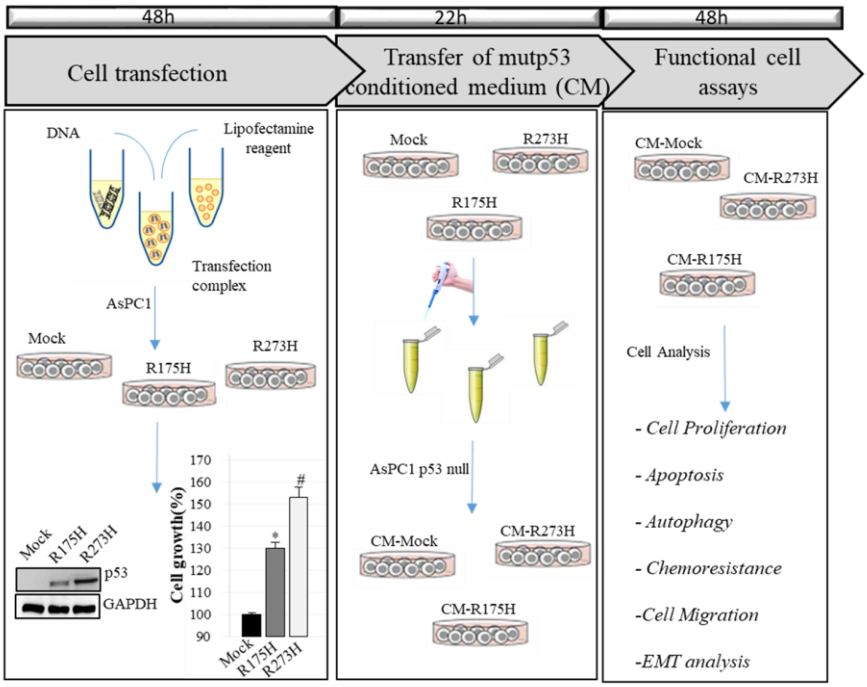


Figure 34: Model that summarize the approach used in this study. AsPC1 cells were transfected with plasmids for R273H or R175H mutant p53 over-expression or its mock vector for 48h. The quality of the transfection was verified by Western Blot, loading 30 µg of whole cell protein extracts and probed with the indicated antibodies, and by Crystal Violet assay. *$p < 0.05$ R175H vs Mock, #$p < 0.05$ R273H. After 48h, AsPC1 transfected cells were washed and then incubated in serum-free RPMI for 22h. Then, the conditioned medium (CM) of AsPC1 transfected cells was collected and transferred to untransfected AsPC1 p53 null cells. After 48h, several function assay listed in the figure was performed.

Figure 35A shows that both of the hot-spot mutations in the *TP53* gene, R273H and R175H, are able to induce PDAC cell proliferation through its driven conditioned medium. Thus, mutp53-driven secretome induces cell growth and inhibits apoptosis, as compared to its negative mock control p53-null cells (figure 35B).

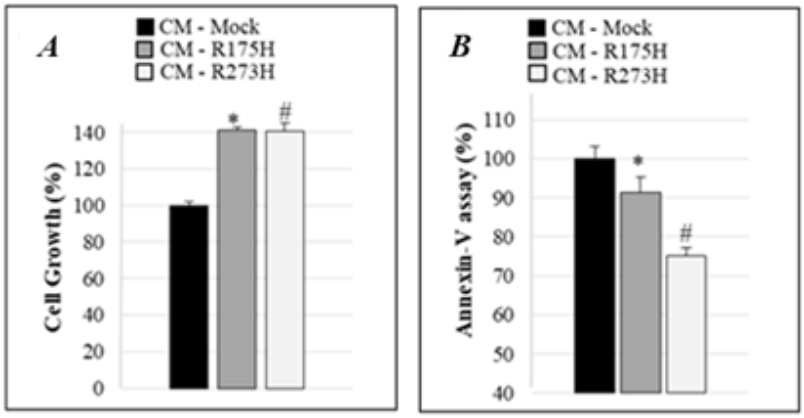


Figure 35: Mutant p53 influences cancer secretome, inducing cell growth and inhibiting apoptosis. *A)* Cell proliferation was measured by Cristal Violet assay and *B)* apoptosis was determined by the annexinV binding assay. Statistical analysis *p<0.05 R175H vs Mock, #p<0.05 R273H vs Mock.

Furthermore, since we had already demonstrated the oncogenic effects of mutp53 in PDAC cells which are also mediated by the counteraction of autophagy [52] and that mutp53 stimulates resistance to the drug gemcitabine in PDAC cells [35], we investigated whether mutp53-driven secretome can influence these mechanisms. Thus, we observed that secretome released by mutp53 cells having R275H or R175H mutations showed decreased amount of intracellular autophagic vesicles (figure 36A). Concerning drug chemoresistance, we demonstrated that R273H mutp53-driven secretome suppresses gemcitabine sensitivity (figure 36B), as compared to its mock control representing an important aspect to be further considered for clinical studies. In summary, the results obtained show that mutant p53 proteins are able to influence the secretion of components which contribute to hyperproliferation, blockage of apoptosis and autophagy and chemoresistance in PDAC cells showing a novel gain-of-function property that promote oncogenesis.

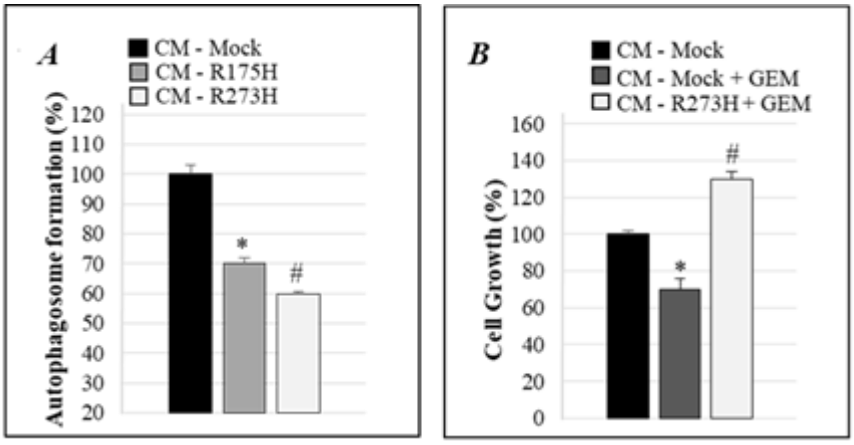


Figure 36: Mutp53-driven secretome inhibits autophagosome formation and induces chemo-resistance. *A)* Autophagosome formation assay through the incorporation of the MDC probe in AsPC1 cells. *p<0.05 R175H vs mock, #p<0.05 R273H vs Mock. *B)* Cell proliferation was measured by Cristal Violet assay in AsPC1 cells and treated with 1 μM gemcitabine (GEM) for 48 h. Statistical analysis *p<0.05 mock + GEM vs mock, #p<0.05 R273H+GEM vs Mock.

6.3.2 Mutp53-driven secretome stimulates cancer cell migration and epithelial-to-mesenchymal transition (EMT).

Since mutp53 proteins alter cancer cell secretome and tumour microenvironment through several mechanisms that we previously discussed in Cordani *et al.* [156], we wanted to further investigate if mutp53-induced modulation of secretome has a role in the stimulation of cancer cell migration. Using the same approach described in figure 34, we discovered that the conditioned medium of cells bearing R273H or R175H mutp53 are able to stimulate cell migration as compared to conditioned medium deriving from its mock control (figure 37A). For the betterment of this data, we used a clone of H1299 cancer cells stably expressing R273H mutp53, improving the exogenous expression of mutant p53 which has been used to study the oncogenic effects of mutp53s [52]. The quality and efficiency of transduction was verified in figure 38. Even the secretome released by H1299 cancer cells stably expressing R273H mutp53, induced cell migration in p53-null expressing cells (figure 37B).

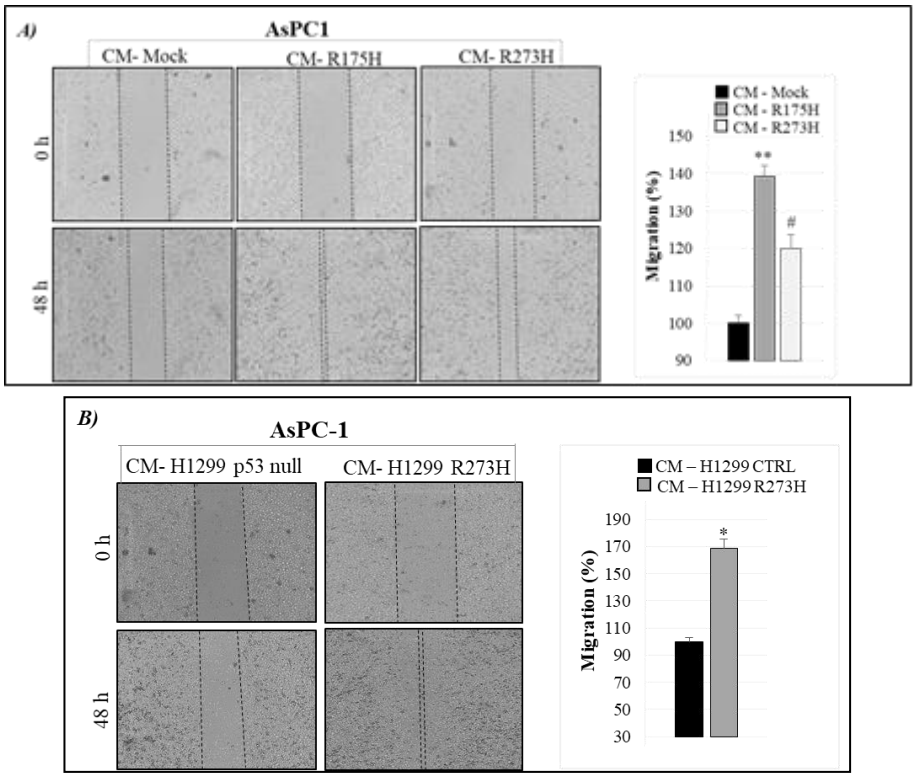


Figure 37. Mutp53-driven secretome induces cell migration. *A)* We performed wound closure cells assay on the confluent AsPC1 p53-null monolayer that received mutp53-driven secretome from AsPC1 transfected cells transduced cells. We have done a scratch in the cells monolayer at time zero, after that we have monitored cells for 48h and the images were analyzed quantitatively by using ImageJ computing software. **$p < 0.01$ R175H vs Mock; #$p < 0.05$ R273H vs Mock. *B)* The conditioned medium of H1299 clones C9 (mock) and H1 (stably expressing mutant p53-R273H) was transfered to AsPC1 p53-null cells and we performed wound clousure cells assay. We have done a scratch in the cells monolayer at time zero and we have monitored the cells for 48h. The images were analyzed quantitatively by using ImageJ computing software. *$p < 0.05$ H1299 R273H vs H1299 CTRL.

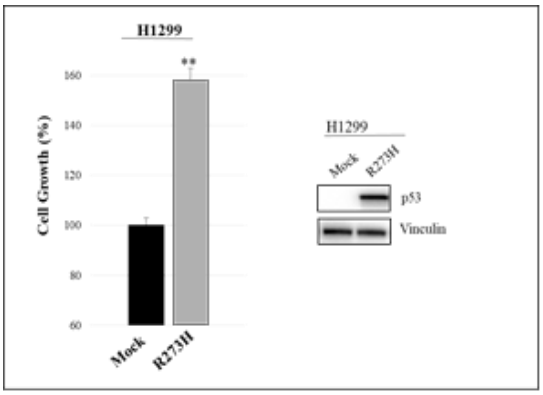


Figure 38: **Quality and efficiency of transduction.** H1299 p53 null were transducted with the clones C9 (mock) and H1 (stably expressing mutant p53-R273H) and then cell proliferation was measured by Cristal Violet assay. Western blotting was also performed using 40 µg of whole cell protein extracts from H1299 cells and probed with the indicated antibodies.

Furthermore, we found that R175H mutp53-driven secretome contributes to epithelial-mesenchymal transition (EMT) (figure 39). Mock and R175H transfected cells of AsPC1 was used as a control for the conditioned medium collected for further experiment. The existing data were confirmed also using conditioned medium, revealing an increase of N-cadherin and decrease of E- cadherin protein levels by using FACS analysis. These alterations are at the basis of the epithelial-to-mesenchymal transition [151] [152]. Indeed, the ratio of N-cadherin to E-cadherin in mutant p53 cells was higher when compared to the control (mock). Since N-cadherin promotes motility, invasion and produces a scattered phenotype with EMT in association with a reduction in the expression of E-cadherin [183], our results suggest that secretome released by mutant p53-expressing cells induces EMT transition. In conclusion, we discovered that mutp53-driven secretome alters tumour microenvironment, stimulating cell migration and EMT. The data obtained can have a relevance in the clinical practice and encourage to identify secreted biomarkers associated with the mutant *TP53* gene in tumour cells.

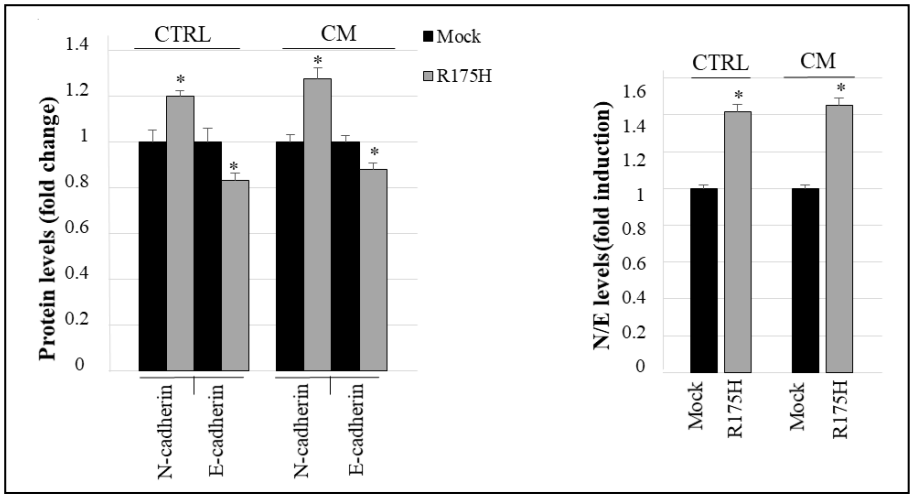
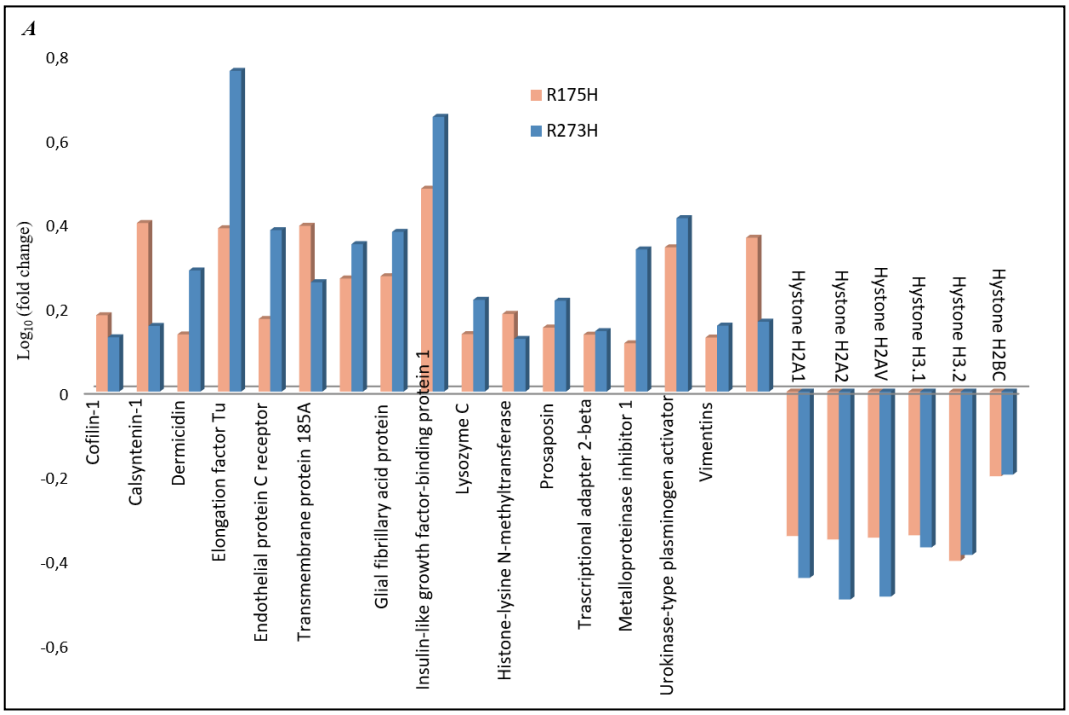


Figure 39. Mutp53-driven secretome induces EMT. We quantified N-cadherin and E-cadherin levels by FACS analysis in AsPC1 control cells transfected with plasmid for R175H over-expression and in the conditioned medium from transfected cells. We also calculated the ratio N to E ratio of cadherin levels. *$p < 0.05$ R175H vs Mock.

6.3.3 Biomarkers secreted from mutp53-driven secretome.

After verifying the oncogenic function of mutp53-driven secretome, we analyzed the composition of the conditioned medium released by AsPC1 expressing GOF mutp53 cells compared to AsPC-1 p53-null cells. After the transfection period (48 h), cells were washed and cultured in FBS-free medium for further 22 h. This FBS-free culture period has been defined on the basis of the absence of stess signals of cells. The secretomes were collected and proteins were precipitated for proteomics studies. The proteins were subjected to reduction, alkylation, and trypsin digestion followed by the mass spectrometry analysis (SWATH MS). We compared the secreted proteins of p53 mutants with the mock control as well as between them. We found 23 differentially secreted proteins by both R273H and R175H mutant p53 isoforms (figure 40A). Specifically, we identified 17 secreted proteins upregulated and 6 secreted proteins downregulated by mutp53 expression (fig 40 B). These proteins are involved in cancer progression and EMT and thus might constitute a secreted signature driven by the hot-spot p53 mutants in PDAC. We verified that the identified proteins are properly secreted proteins through bioinformatics analysis. These secreted proteins modulated by mutp53 isoforms may constitute a prerequisite for the identification of a secreted biomarker signature for the early identification of mutant p53 PDAC patient.



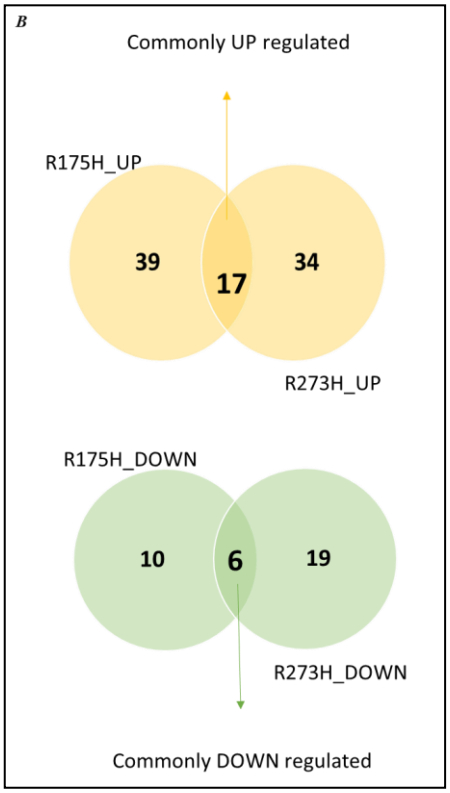


Figure 40: Biomarkers secreted from mut p53-driven secretome. *A* and *B)* AsPC1 cells were transfected with plasmids for R273H or R175H mutant p53 over-expression or its mock vector for 48 h. Then, AsPC1 transfected cells were washed and incubated in FBS-free RPMI for further 22 h. Then, the conditioned medium (CM) of AsPC1 transfected cells was collected and transferred to AsPC1 p53-null cells, not transfected. After 48 h, the proteins were analyzed by SWATH analysis followed by bioinformatics analysis.

6. DISCUSSION AND CONCLUSIONS

TP53 is one of the most frequently mutated genes in PDAC (~70%) [24] and in the other human cancers (~50%) [184]. Most of its mutations are missense mutations that result in the expression of mutant isoforms of p53 which acquire new biological properties referred as gain-of-function (GOF) [25]. These novel functions are involved in a plethora of different cellular pathways focused on cancer progression and aggressiveness, promoting chemoresistance, invasion, metastasis and the counteraction of apoptosis and cellular senescence [28] [29]. Mutant p53 GOF contributes to cancer progression through direct interaction with proteins altering their function or through the transcriptional activation/repression of mutp53-target genes and downstream molecules [29]. In many cancer types, p53 mutations are associated with high genomic instability, poor prognosis, poor response to chemotherapy. Indeed, our research group discovered that mutp53 confers resistance to the treatment with the DNA damaging drug gemcitabine in PDAC cells. Furthermore we observed that chemotherapy itself aberrantly stimulates mutant p53 activity in PDAC cells identifying a key process with potential for therapeutic targeting [35]. The influence of mutp53 in the clinical outcome of cancer patients, the high frequency of GOF mutations in the *TP53* gene and the involvment of mutp53 in a number of different cellular pathways have stressed the need to deeply investigate the events associated to cancer progression driven by mutp53 proteins in molecular oncology. Thus, I believe that a comprehensive discovery of the mechanisms by which mutp53 stimulates cancer aggressiveness is fundamental for its clinical implications and to counteract its oncogenic functions. Regarding the catabolic process of cellular self-digestion named autophagy, GOF mutant p53 proteins are able to orchestrate a plethora of events addressed to counteract autophagy in the various phases of the process, thus contributing to inhibit cell death and to sustain oncogenic activity. Autophagy deficiency causes oxidative stress, activation of the DNA damage response, and genome instability, a known cause of cancer initiation and progression [109], [185]. My research team discovered that mutant p53, in contrast to the autophagic function of wild-type p53, counteracts the formation of autophagic vesicles and their fusion with lysosomes through the repression of some key autophagy-related proteins [52]. Fur-

thermore, we previously reported the molecular interplay between the expression of mutant p53 proteins and autophagy regulation in cancer progression [112]. We further discovered novel molecular mechanisms by which mutp53 promotes cancer progression, one of these occurs through the ROS prodution. ROS are persistently elevated in cancer cells as a consequence of increased metabolic activity, mitochondrial dysfunction, and activation of oncogenes and are involved in the main features of aggressive cancer cell behavior, including genome instability, cellular hyperproliferation [116]. In the present study we discovered that mutant p53 proteins, contrarily to their wild-type p53 counterpart, can stimulate their oncogenic pro-oxidant conditions in PDAC cells. GOF mutant p53 isoforms contribute to enhance ROS levels in these cancer cells through a coordinated regulation of redox-related enzymes and signaling pathways, including SESN1/AMPK/PGC-1α/UCP2 axis. The proteins involved in this axis have various roles, including the production of ROS and the regulation of autophagy and mitochondrial metabolism. In particular, AMPK is a master regulator of cellular metabolism and it has been shown to control the expression of several mitochondrial enzymes and proteins [186], including PGC-1α which stimulates the expression of mitochondrial uncoupling proteins, as UCP2 [187]. Furthermore, AMPK triggers autophagy mainly through phosphorylation of several autophagy-related genes. The AMPK activators sestrins are not only negative regulators of ROS but also they can stimulate AMPK signaling and inhibit mTORC [188], which in turn is a central regulator of cell growth and plays a key role at the interface of the pathways that coordinately regulate the balance between cell growth and autophagy [189]. Thus, the inhibition of AMPK by repression of sestrins' expression results in ROS prodution and also in autophagy blockage by several ways, including the stimulation of mTOR complex. Regarding UCP2, we previously discovered that UCP2 has a central role in regulating the energetic metabolism of the cells, in addition to its well described antioxidant role [190]. This concept is supported by the demonstration that the channel formed by uncoupling proteins (UCPs) can also promote the mitochondrial efflux toward the cytosol of pyruvate and of Krebs cycle intermediates, regulating glucose and glutamine oxidation [191]. Indeed, we found that UCP2 also induces the expression of GLUT1 and pyruvate kinase isoform M2 [192] and GAPDH cytosolic stabilization [84], overall sustaining the glycolytic phenotype

of PDAC cells. Regarding glycolitic metabolism, we discovered another mechanism by which mutp53 induces the Warburg effect, supporting cell growth and chemioresistance. Indeed, apart from glycolysis, GAPDH participates in apoptosis, autophagy, iron metabolism, membrane trafficking, and other roles that depend on its subcellular localization [74]. In the present study, we demonstrate that mutant p53 stimulates glycolysis and lactate secretion through the cytosolic stabilization of GAPDH. In particular, we show that mutant p53 can induce the expression of SIRT1, a deacetylase enzyme which stimulates the proliferation and the expression of glycolytic genes in pancreatic neoplastic lesions [193]. In accordance with these observations, our data show that mutp53 stimulates lactate secretion with the concomitant upregulation of SIRT1 expression and the formation of SIRT1:GAPDH complex, which is reported to protect cytosolic GAPDH from nuclear translocation after various stimuli, retaining the glycolytic enzyme in the cytosol and promoting cell survival. Furthermore, we reveal that another mechanism by which mutant p53 stabilizes GAPDH cytosolic localization is due to the stimulation of AKT and inhibition of AMPK signaling pathways, which are reported to directly phosphorylate GAPDH in different amino acidic residues with opposite effects on the nuclear translocation of the enzyme. Finally, in this study we investigated the extracellular oncogenic function of mutp53 in tumour microenviroment in cells of PDAC, which has extremely high mortality rate mainly due to lack of biomarkers for early detection. An ever-increasing number of studies highlight the role of mutant p53 proteins in the alteration of cancer cell secretome that we previously summarized [156]. Thus, in this study we showed the functional effects of mutp53-driven secretome on cancer cells showing its influence on proliferation, chemoresistance, apoptosis and autophagy, as well as cell migration. Our data identified 23 differentially secreted proteins by both R273H and R175H mutant p53 isoforms. These proteins are involved in cancer progression and epithelial-to-mesenchymal transition and might constitute a secreted signature driven by the hot-spot p53 mutants in PDAC. This study is in progress and we are validating the identified secreted proteins in serum samples of a cohort of about 100 PDAC patients having WT or mutant *TP53* gene. These data might suggest the identification of targeted therapy specifically addressed to inhibit growth of PDACs carrying oncogenic mutant p53, which are strongly resistant to traditional chemotherapies.

In conclusion, this thesis shows several innovative mechanisms, summarized in figure 41, by which mutant p53 plays its oncogenic roles. The stimulation of glycolysis, the ROS production, and the alteration of tumour microenviroment might provide new therapeutic opportunities to be further considered for clinical studies in order to counteract chemoresistance in PDAC patients bearing mutant *TP53* gene.

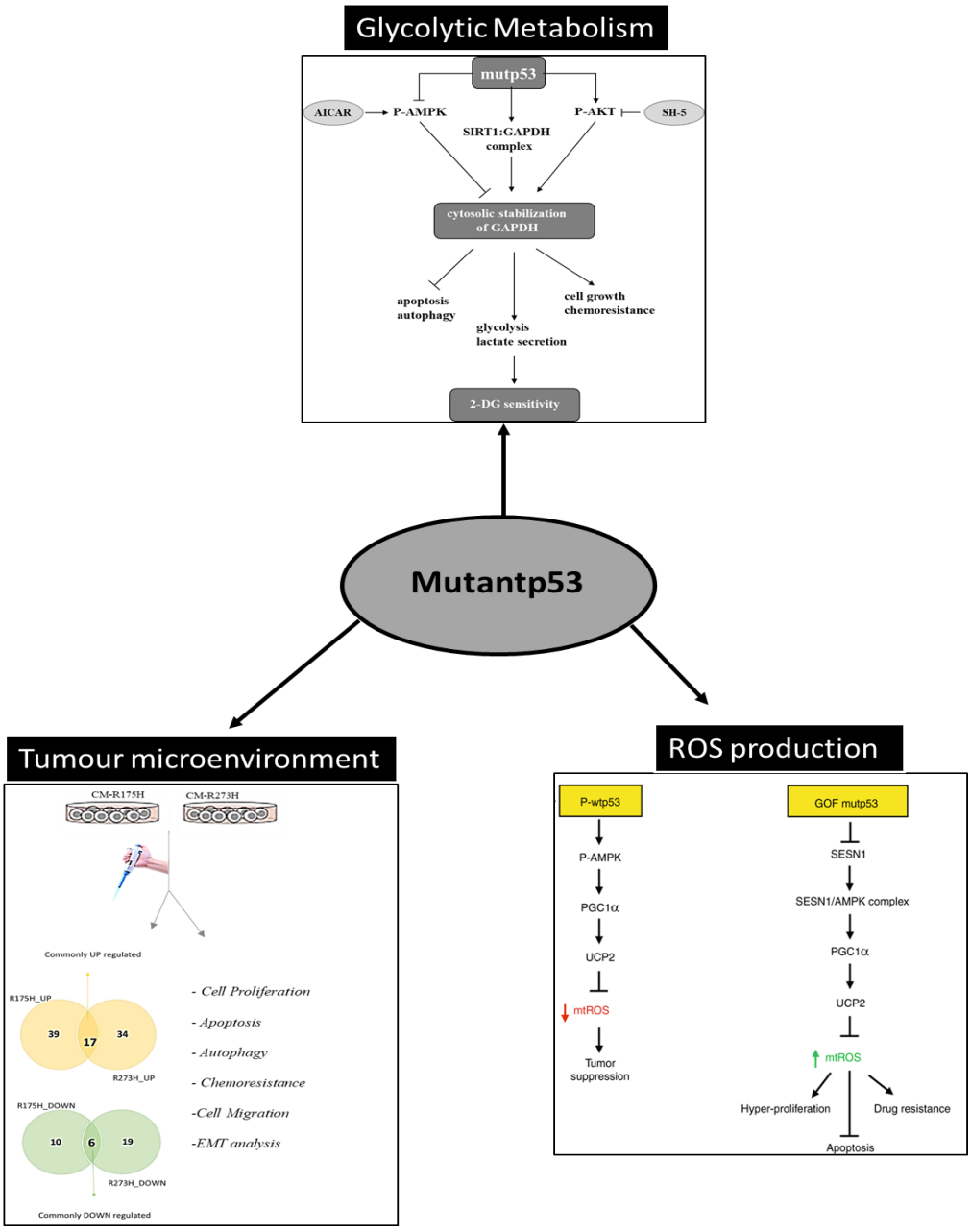


Figure 41: Representative summary of the data described in the thesis.

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9. ANNEXES

During the period of my PhD I studied the molecular mechanisms involved in chemoresistance of cancer cells, particularly in PDAC, bearing mutant p53 gene. I focused my attention on the molecular mechanism where mutp53 is involved. Specifically, I studied the regulation of nuclear translocation of the enzyme GAPDH by mutp53 with consequent regulation of the proliferation of tumour cells. A subsequent phase of my research also includes the study of biomarkers in PDAC cells and patients serum having the TP53 wild-type or mutated gene. A part of the project was carried out for three months in the Prof. Melo's lab at the University of Porto and I had won travel grants for the same. During the course of PhD, I presented my projects in various international and national conferences and I collaborated in the following projects:

1)"Mutant p53 prevents GAPDH nuclear translocation in pancreatic cancer cells favoring glycolysis and 2-deoxyglucose sensitivity". Giovanna Butera, Raffaella Pacchiana, Nidula Mullappilly, Marilena Margiotta, Stefano Bruno, Paola Conti, Chiara Riganti, Massimo Donadelli. BBA- Molecular Cell Research 2018.

2)"Mutant p53 blocks SESN1/AMPK/PGC-1α/UCP2 axis increasing mitochondrial $O2^{-\cdot}$ production in cancer cells" Marco Cordani, Giovanna Butera et al. British Journal of Cancer, 2018.

3)"Oncometabolites in cancer aggressiveness and tumor repopulation". Ilaria Dando,Elisa Dalla Pozza, Giulia Ambrosini,Margalida Torrens-Mas, Giovanna Butera, Nidula Mullappilly,Raffaella Pacchiana, Marta Palmieri, Massimo Donadelli. Journal of the National Cancer Institute. Submitted

4)"Autocrine mechanisms of cancer chemoresistance". Giovanna Butera, Raffaella Pacchiana, Massimo Donadelli . Semin Cell Dev Biol. 2018 Jun; 78:3-12 .

5)"UCP2 inhibition induces ROS/Akt/mTOR axis: Role of GAPDH nuclear translocation in genipin/everolimus . Dando I, Pacchiana R, Pozza ED, Cataldo I, Bruno S, Conti P, Cordani M, Grimaldi A, Butera G, Caraglia M, Scarpa A, Palmieri M, Donadelli M. Scarpa A, Palmieri M, Donadelli M. Free Radical. Biol. Med. 2017 Dec; 113:176-189 .

6) "A comparison study on RNase A oligomerization induced by cisplatin, carboplatin and oxaliplatin" Picone D, Donnarumma F, Ferraro G, Gotte G, Fagagnini A, Butera G, Donadelli M, Merlino A. J Inorg Biochem. 2017 Aug;173:105-112 .

7) “The antioxidant mitochondrial protein UCP2 promotes cancer development connecting the Warburg effect and autophagy” Marco Cordani, Giovanna Butera, Raffaella Pacchiana, Massimo Donadelli Translational Medicine Reports 2017; volume 1:6451.

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