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GAPDH AND MITOCHONDRIAL DYNAMICS REGULATION: NOVEL INSIGHTS TO TREAT PDAC HAVING *TP53* MUTANT GENE

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GAPDH AND MITOCHONDRIAL DYNAMICS REGULATION:
NOVEL INSIGHTS TO TREAT PDAC HAVING TP53 MUTANT GENE

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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* has missense mutations generally resulting in conformational changes of mutantp53 (mutp53) proteins. This represents important event in the carcinogenesis process, not only through loss of p53 wild-type activity, but also through gain of specific mutp53 functions. In contrast to the tumor suppressive roles of wild-type p53, mutp53 proteins support cancer progression by enhancing the ability of cancer cells to invade and metastasize, to confer chemo-resistance, and to stimulate genomic instability. We focused our attention on novel molecular mechanisms by which mutp53 proteins play their oncogenic roles promoting cancer cell proliferation and chemoresistance. The need to find a better strategy by identifying novel mechanisms involving mutp53 in PDAC is thereby tremendously increasing. The main objective of the project is to find a better therapeutic strategy than existing drugs, as gemcitabine (GEM), which is not efficient in the treatment of chemoresistant PDAC cells.

In general, thesis is dealt with Pancreatic cancer (PDAC), p53, mitochondrial dynamics and cancer cell metabolism.

The first part of my project was based on testing a panel of novel inhibitors of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which is a key enzyme of the glycolytic pathway having moonlight properties. These novel inhibitors were synthesized in the lab of Prof. Paola Conti (University of Milan, Italy). Among the selected inhibitors, AXP-3019 was able to strongly reduce the PDAC cell proliferation carrying mutant *TP53* gene, both *in vitro* and *in vivo* mice models. Decreased glycolytic flux in PANC-1 cells has been confirmed by decrease in lactate secreted levels. Apoptosis and autophagy assays also confirmed the efficiency of the inhibitor. The role of autophagy was also then reconfirmed by administering the lysosomal activity inhibitor chloroquine (CQ) which reverts the autophagy. Finally, to investigate the synergistic effect between GAPDH inhibitors and the standard drug GEM, we performed a drug combination

studies by using CalcuSyn software, revealing that GAPDH inhibition synergistically enhanced PDAC cell death when cells were co-treated with GEM.

In the second part of my project, we aimed to understand whether mutp53 was able to regulate mitochondrial dynamics in PDAC cells, since mitochondrial dynamics plays a major role in maintaining the balance and equilibrium of the cells. We analyzed a panel of specific genes and respective proteins in PaCa3 (WTp53), PANC-1 (mutp53) and AsPC1 (p53 null) cell lines, in order to investigate their different involvement in mitochondrial dynamics. Further, dynamics of the mitochondria were observed and statistically quantified using confocal microscopy. The Optic atrophy 1 (OPA1), a master fusion regulator of mitochondrial dynamics is found to be inhibited by mutp53 and this inhibition has been involved in mutp53 dependent cell growth and anti-apoptotic events. Furthermore, the roles of OPA1 inhibition by mutp53 in other metabolic pathways were partially revealed. From this we revealed that, modulating the expression of OPA1 and understanding the role of mitochondrial dynamics pathway in cancer, might be useful to identify novel and more efficient strategies to treat PDAC disease.

From the above studies, we are able to confirm that the deep knowledge of the metabolic alterations and related pathways are necessary for the identification of efficient treatment strategies, for reducing the drug resistance, and finally improving the survival rate of PDAC patients having mutp53. Also, target therapy based on glycolytic pathway and mitochondrial dynamics should be considered for novel treatment strategies.

2. SUMMARY

L'adenocarcinoma pancreatico (PDAC) è uno dei tumori maligni umani più aggressivi e devastanti la cui diagnosi tardiva è causata dall'assenza di sintomi specifici nelle fasi iniziali. In circa il 70% dei PDAC, il gene soppressore del tumore TP53 ha mutazioni missenso che generalmente determinano cambiamenti conformazionali delle proteine p53 mutanti (mutp53). Questo rappresenta un evento importante nel processo di carcinogenesi, non solo per la perdita dell'attività wild-type, ma anche l'acquisizione di nuove funzioni di p53 mutata. In contrasto con il ruolo soppressivo della forma wild type, la proteina mutata favorisce la progressione del cancro aumentando la capacità delle cellule tumorali di invadere, metastatizzare, di conferire chemio-resistenza e di stimolare l'instabilità genomica. Nel presente lavoro di tesi abbiamo focalizzato l'attenzione sui nuovi meccanismi molecolari attraverso i quali p53 mutata svolge il proprio ruolo oncogenico, promuovendo la proliferazione delle cellule tumorali e la chemioresistenza. La necessità di trovare una strategia vincente, identificando nuovi meccanismi di mutp53 nel tumore del pancreas è particolarmente attuale. Lo scopo principale del progetto è quello di trovare una strategia terapeutica in alternativa ai farmaci esistenti, come la gemcitabina (GEM), scarsamente efficaci nel trattamento delle cellule PDAC resistenti alla chemio.

La prima parte del mio progetto è incentrata sull'analisi di un pannello di nuovi inibitori dell'enzima gliceraldeide 3-fosfato deidrogenasi (GAPDH), enzima chiave del percorso glicolitico. I nuovi inibitori sono stati sintetizzati nel laboratorio della Prof. Paola Conti (Università di Milano, Italia). Tra gli inibitori selezionati, AXP-3019 è stato in grado di ridurre in modo significativo la proliferazione delle cellule di PDAC che portano il gene TP53 mutante, sia *in vitro* che nei modelli di topi *in vivo*. La diminuzione del flusso glicolitico nelle cellule PANC-1 è stata confermata dalla diminuzione dei livelli di lattato secreto. Anche i saggi di apoptosi e autofagia hanno confermato l'efficienza dell'inibitore. Il ruolo dell'autofagia è stato inoltre confermato utilizzando un inibitore specifico, la clorochina (CQ).

Infine, allo scopo di indagare l'effetto sinergico tra gli inibitori di GAPDH e il farmaco di riferimento, abbiamo effettuato uno studio di *drug combination* il

cui risultato ha permesso di mettere in evidenza un aumento di morte cellulare dopo trattamento combinato dell'inibitore + la gemcitabina maggiore rispetto alla morte cellulare ottenuta dopo il trattamento con il singolo farmaco.

Nella seconda parte del mio progetto, abbiamo cercato di indagare il ruolo di mutp53 nella *mythocondrial dynamics*, la cui importanza nel mantenimento dell'equilibrio cellulare è di notevole importanza.

Abbiamo analizzato i livelli di espressione di mRNA e di specifiche proteine coinvolte nella dinamica mitocondriale nelle linee cellulari di PaCa3 (WTp53), PANC-1 (mutp53) e AsPC-1 (p53 null) utilizzando varie metodiche. OPA1, la principale proteina coinvolta nel processo di fusione mitocondriale, risulta inibita da mutp53 e tale inibizione è correlata alla crescita cellulare dipendente da mutp53 e agli eventi anti-apoptotici.

Inoltre, sono stati parzialmente rivelati i ruoli dell'inibizione dell'OPA1 da parte di mutp53 in altre vie metaboliche. Da ciò si evidenzia che, modulando l'espressione dell'OPA1 e comprendendo il ruolo della via mitocondriale nel cancro, potrebbe essere utile individuare nuove e più efficaci strategie per il trattamento della PDAC. Dagli studi fin qui condotti, possiamo confermare che la conoscenza approfondita delle alterazioni metaboliche e delle relative vie è necessaria per individuare strategie terapeutiche efficaci, ridurre la resistenza ai farmaci e infine migliorare la sopravvivenza dei pazienti affetti da PDAC con p53 mutata. Inoltre, è possibile prendere in considerazione una terapia mirata basata sulla via glicolitica e sulla dinamica mitocondriale al fine di identificare nuove strategie di trattamento.

3. INTRODUCTION

Cancer is one of the major public health problem worldwide and one of the major causes of death. According to statistics, cancer is estimated to be the second cause of death in United States and also in western countries [1]. For all the stages combined, the survival rate is highest for prostate cancer (98%), melanoma of the skin (92%), and female breast cancer (90%) and lowest for cancers of the pancreas (9%), liver (18%), oesophagus (19%), and lung (19%) [2]. Of this pancreatic cancer is the seventh cause of total cancer in the world and estimated to have a survival rate of 5% over five years. About 85% of pancreatic cancer is by ductal adenocarcinoma tumor type (PDAC) and 5% is by endocrine tumor type [3]. The overall decrease in survival rate of pancreatic cancer might be due to failure in early prognosis where patients rarely exhibit symptoms at early stage. The pancreatic cancer has some prevalent genetic mutations and most common ones are *KRAS*, *CDKN2A* (encoding p16), *TP53* and *SMAD4* [4]. This late diagnosis being asymptomatic in the early stage, 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% [5]. With all these, it's highly recommended to understand treatment methods and increase the efficiency of treatments to pancreatic cancer that can control the metastasis and detect at an early stage. In general, this thesis deals with pancreatic cancer (PDAC), p53, mitochondrial dynamics and cancer cell metabolism aspects combined together in two different projects.

3.1 *TP53* – VITAL PLAYER OF GENOME

TP53 is a vital tumor suppressor gene codifying for the protein 53 (p53), which exerts tumor suppressor functions and it is also called guardian of genome. *TP53* gene is the most frequently mutated gene in human cancers [6]. Human p53 is a nuclear phosphoprotein having molecular weight of 53 kDa encoded by a 20-Kb gene containing 11 exons and 10 introns, which is located on the small arm of chromosome 17 [7]. p53 belongs to a family of proteins including also p63 and p73, having similar structural and functional roles. In particular, p53 has evolved to have

crucial role in apoptosis and in preventing tumor development. The proteins p63 and p73 have defined roles in developmental biology as well [8]. p53 works as a transcription factor and it has been extensively studied from past 20 years due to its multifaceted roles especially in prevention of tumor progression. p53 has a modular protein domain structure: N terminus is comprised of transcriptional activating domains (TAD1 and TAD2) spanning the residues from 1-70, helping in the enhancement of transcription of p53 target genes by recruiting histone modifying enzymes co-activator complexes, and other molecules involved in the transcriptional machinery [9]; followed by this domain, there is the proline rich domain (PRD) from 60-95 residues, helping in protein interactions in the presence of PxxP motifs, also having a role in p53 stabilisation [10]. The central core of p53 having residues from 100 - 300, which is DNA binding domain (DBD), helps in sequence-specific binding of the protein to p53 response elements in the DNA [11]. The tetramerization domain (Tet) comprises residues 325 - 356 and is required for formation of the active tetrameric form of p53 [12]. Finally, at the C-terminus end, there is a basic lysine rich domain called Regulatory Domain (Reg) from 363 - 393 where DNA is bounded in a non-sequence specific manner and helping in undergoing post-translational modifications of p53, especially for protein stability [13] (figure1).

Figure 1: Modular protein domain structure of p53. The p53 monomer consists of various multifunctional domains (modified by [13]).

p53 pathway is activated in response to various stress stimulated pathways triggered by oncogene activation, DNA damage, telomere erosion, ribonucleotide depletion, altered mitochondrial and ribosomal biogenesis, nutrient deprivation, hypoxia and loss of cell contacts [14]. Various stress stimuli are integrated with the involvement of extensive covalent post-translational modifications of both p53 and its regulators. During stress, post-translational modifications occurs in p53

conserved residues, including phosphorylation, acetylation, methylation, mono- and poly-ubiquitination, sumoylation, neddylation, ADP-ribosylation and glycosylation. These modifications can regulate p53 stability, DNA binding, tri dimensional conformation, cellular localisation and protein-protein interactions [15]. Furthermore, the activation of p53 mediates senescence, apoptosis, tumor suppression, cellular homeostasis, cell growth arrest, protection of stemness, anti-angiogenesis and others. The main target genes of p53 belonging to all these cellular responses are *p21*, *Btg2* for cell cycle arrest, *P53r2*, *Ddb2*, *Mgmt*, *Gadd45α* for DNA repair, *Puma*, *Noxa*, *Bax*, *Pig3* for apoptosis, *Pai1*, *Pml*, *Cdkn1α* for senescence, *Tsp1*, *Bai1*, *Epha2*, *Col4a1* for anti-angiogenesis [16].

Similarly, somatic mutations, signalling defects, having ATM/Chk2 mutation, viral proteins, nuclear exclusion, misregulation of proteins like Mdm2/4, p14ARF, MAGE can lead to inactivation of p53. Tumorigenesis, gain of function properties, therapy resistance and poor prognosis are the result of inactivation of p53 [17] (figure 2). 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".

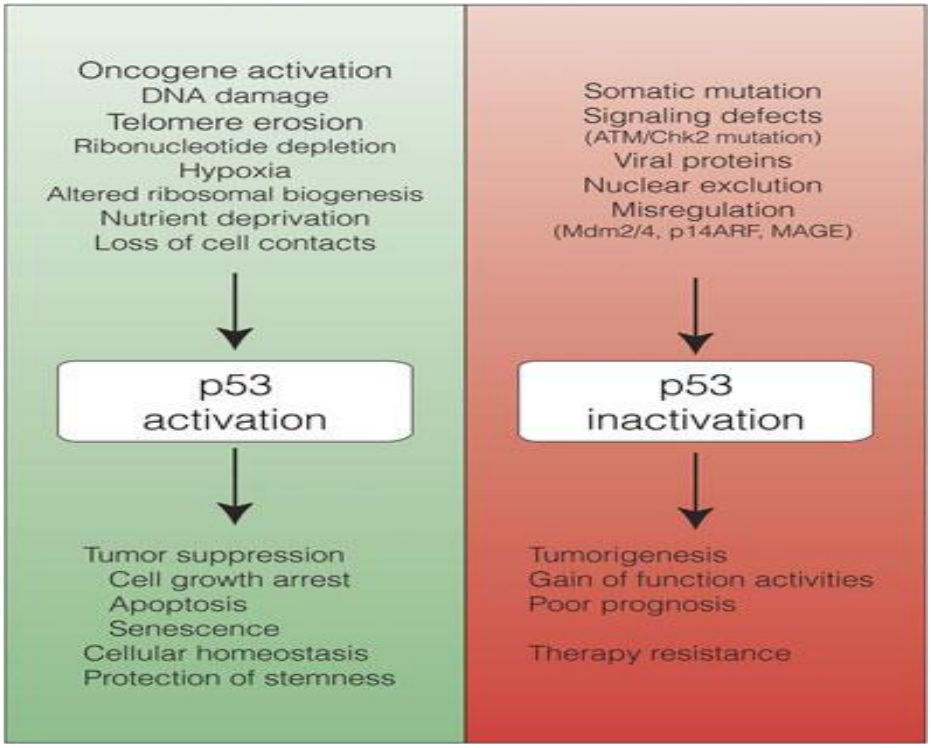

Figure 2: p53, called guardian of genome, shows different functions when activated and inactivated. Schematic representation of those functions [17].

In the absence of stress, p53 is maintained at very low level by its targeting with MDM2 E3 ligase, which determines the continuous degradation of p53 by the ubiquitin–dependent proteasome pathway. Mdm2 gene binds to transactivating domain of N-terminus and thus targeting C-terminal end having Lys residues of p53 for post-translational modifications, as ubiquitination, thus followed by proteosomal degradation, and thus preventing further gene transactivation [18] (figure 3). MDM2 is also a transcriptional target of p53 thereby creating an auto-regulatory loop. Here p53 controls the expression of its own negative regulator. MDMX, which is a MDM2 related protein also inhibits the activation of p53 [19] (figure 3).

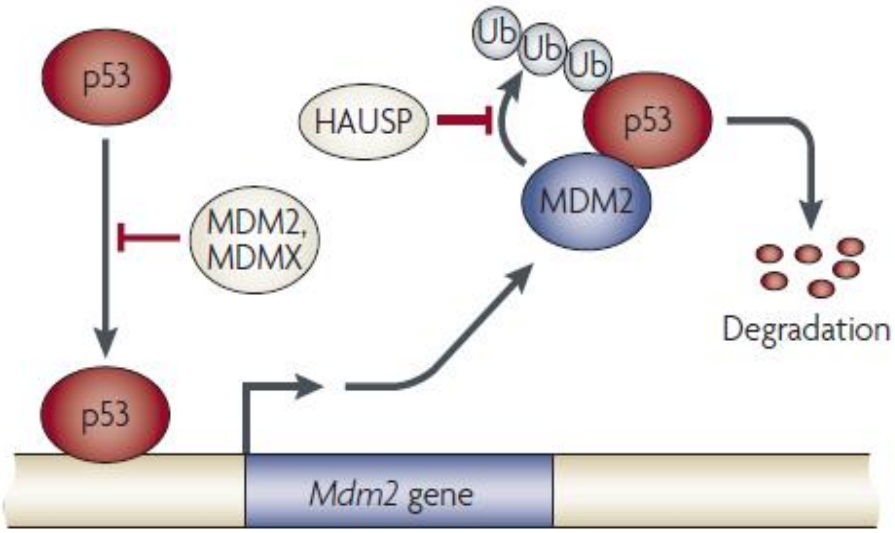

Figure 3: p53 and its upstream activation. p53 is targeted by MDM2 E3 ligase in absence of stress [19].

The p53 pathway which responds to different stress stimuli and thereby reducing tumor growth is strongly highlighted by the fact that mutations in the *TP53* gene are more frequent in most of the existing cancers. Recent studies showed that the restoration or re-expression of p53 in tumors, lacking p53, caused a fast and extensive tumor regression in established tumors, which is due to induction of p53-dependent apoptosis or senescence [20].

Recently, p53 is identified as an unsophisticated enhancer which is common among transcription factors. P53 identifies a set of core enhancers regardless of strong content, making variations in chromatin landscape and nucleosome positioning [21]. As previously discussed, p53 is involved in various canonical effector pathways employing certain genes in different cellular context, but it is also possible that p53 activates highly unuseful transcription network in which none of the target genes is involved in tumor suppressive activity. A clear-cut answer to this question might pave the way for better p53-related cancer therapies.

3.2 P53 MUTATIONS AND ONCOGENESIS

Abnormalities and mutations in the tumor suppressor gene p53 are among the most frequent molecular events in human neoplasia. Somatic p53 mutations are found in most of the cancer types and about half of all ovarian, colorectal, and oesophageal cancers. In the rest of tumors, p53 pathway is impaired or functionally restrained because of either the amplification of negative regulators like MDM2 [22] or inactivation of upstream factors, as ATM, Chk2 [23].

Germline p53 mutations are found in 660 families, which comprises mainly of missense mutations (75.2%). Of this 75% of mutations can be seen in the DNA binding domain and 19.6% in the tetramerization domain [24]. It is reported that germline mutations cause a rare type of cancer known as Li-Fraumeni Syndrome (LFS) or Li-Fraumeni-like syndrome (LFL), which are a familial clustering of early onset tumors, including sarcomas, breast cancers, brain tumors and adrenal cortical carcinomas [25]. In somatic mutations, most of them accounts to missense mutation which comprises 76.7%, leading to expression of full length mutp53 proteins by substituting a single amino acid [26]. Of this missense mutations, DNA binding domain has 93% of mutations followed by tetramerization domain having 1.6% [27]. Deletion or mutation in the tetramerization domain causes the lost or the decrease of the tetrameric structure of p53, resulting in reduced or eliminated transcriptional activity [28].

Most of the missense mutations in p53 have been identified in hotspot mutation sites, which have amino acid residues R175, G245, R248, R249, R273,

and R282 [29] (figure 4,5). These can be subdivided in DNA contact mutations, involving for instance R248 and R273, which make direct contact with target DNA sequence impairing the transcriptional activity of WTp53 or conformational mutations (R175, G245, R282 and R249), which determine more dramatic conformational changes of protein structure of p53 [30][31].

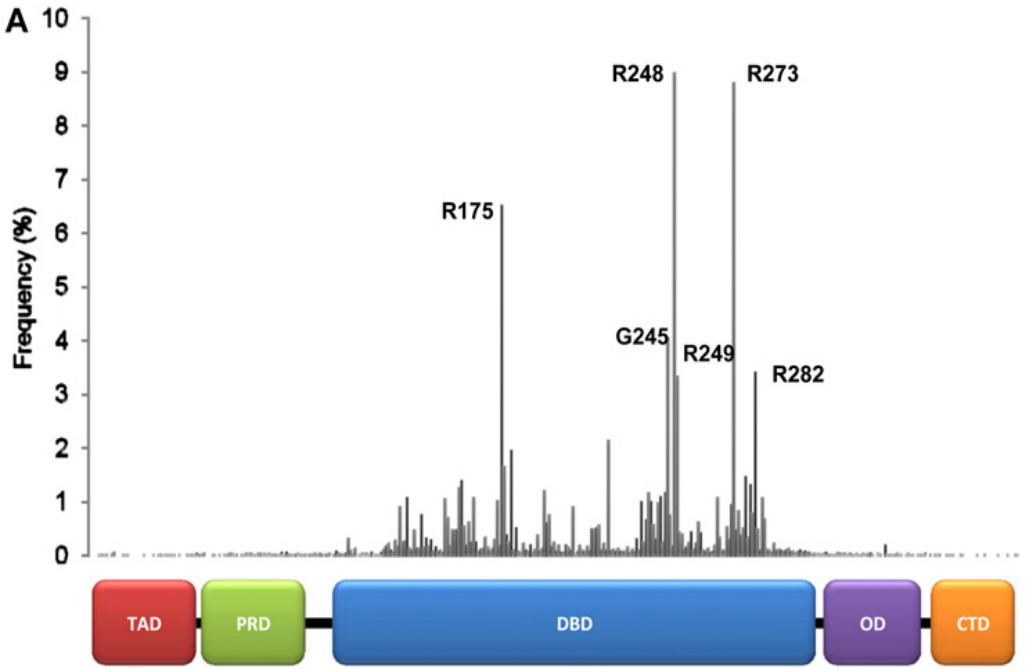

Figure 4: TP53 missense mutations data for human cancer patients[26]

Amino Acid Residue	Frequency of Alteration
248	7.0%
273	6.7%
175	5.1%
245	3.3%
249	2.9%
282	2.9%

Mutation	Overall Frequency	Wild-Type Codon	Mutant Codon	CpG	Class
R175H	4.6%	CGC	CAC	Yes	Conformation
R248Q	3.5%	CGG	CAG/CAA	Yes	DNA Contact
R273H	3.1%	CGT	CAT	Yes	DNA Contact
R248W	2.8%	CGG	TGG	Yes	DNA Contact
G245S	2.8%	GGC	AGC	Yes	Conformation
R273C	2.7%	CGT	TGT	Yes	DNA Contact
R282W	2.4%	CGG	TGG	Yes	DNA Contact
R249S	1.8%	AGG	AGT	No	Conformation
G245D	0.68%	GGC	GAC	No	Conformation

Figure 5: Hotspot residues of p53 and common missense mutations of p53 [26]

The wild type p53 gene undergoes proteasomal degradation to balance and keep the level low under unstressed conditions [32]. However, during tumorigenesis the expression level of mutp53 protein is maintained high because it is accumulated to a high level, stabilized and protected not to be degraded by proteasomal degradation by various oncogenic signalling pathways [33]. TP53 mutations are first considered to inactivate the onco-suppressive capacity of WTp53 gene by loss of function referred as LOF. Since p53 acts as a tetramer, p53 mutant can show a dominant negative (DN) effect over their wild-type counterpart, helping in gain of function (GOF). In most cases, *TP53* gene mutations are followed by the deletion of existing wild-type allele, which is known as loss of heterozygosity (LOH), suggesting the facts that complete loss of wild-type p53 provides cancer cells with a selective advantage, despite of their dominant negative effect exerted by mutp53. Indeed, from the recent studies, it's been clear that LOH favours mutp53 for stabilisation and attaining further oncogenic properties [34] (figure 6).

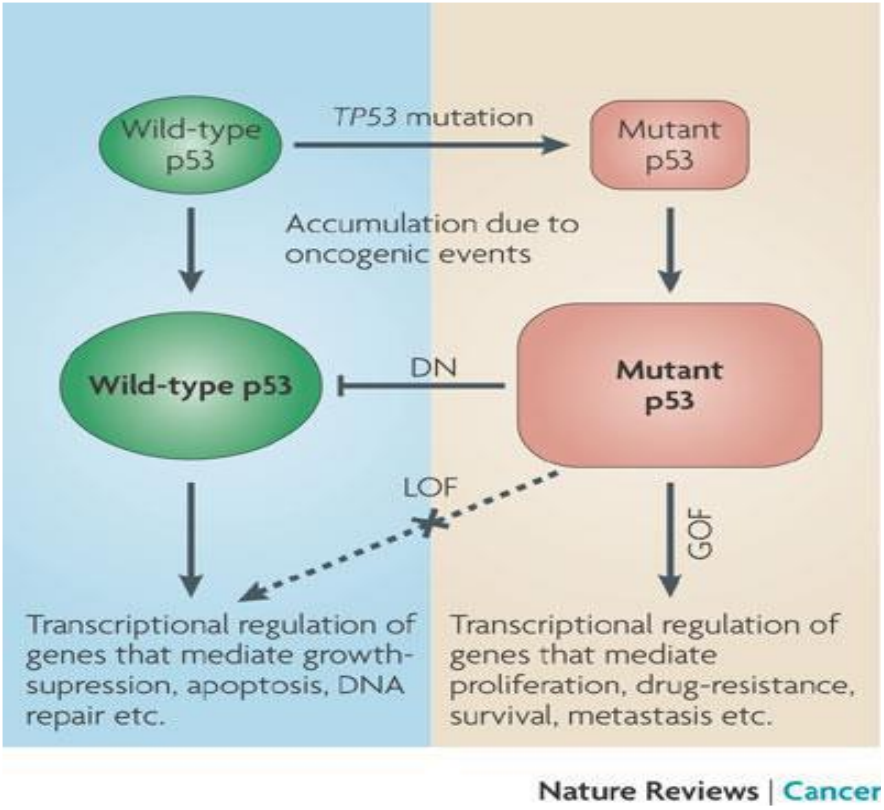

Figure 6: Functionality of p53. mutp53 shows Loss of Function (LOF) and Gain of Function (GOF) properties [35].

The main functions of GOF mutp53 is oncogenic property including invasion, migration, angiogenesis, increased proliferation, reduced apoptosis and autophagy, genomic instability, chemo resistance and so on (figure 6). The hotspot mutations mentioned in Figure 4 and 5 are examples of GOF. Along with this, one of the crucial consequences of GOF is to affect the metabolism and the response to oxidative stress of cancer cells [36].

Mutp53 generally executes its GOF activities via distinct molecular mechanistic pathways. Although p53 mutants generally lost their capability to bind DNA they can interact with other transcription factors or co-factors, increasing the gene transcription and expression. It is possible also to inhibit gene transcription by interacting with DNA binding activity of other transcription factors or repressors. In some other cases, they can directly bind to DNA with the help of special structured DNA such as matrix attachment regions (MARS) and then regulate the transcription. Also, they could bind to various proteins or enzymes, not related to transcription, but instead managing cellular functions [37].

Majority of GOF are related to oncogenesis. Also, all the mutations of p53 confer to different biological properties which seem to influence the prognostic significance in mutated tumors[38]. Mutp53 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 [39]. For example, mutp53 has been shown to interact with the transcription factor NF-Y, and to up-regulate the expression of NF-Y target genes [40]. It has been reported that mutp53 upregulates CDK4/Cyclin, C-myc or others for cellular proliferation, TGFβ and EGFR for metastasis, Mre-11-Rad50-NBS complex for genomic instability, RhoA/Glut1 cascade for metabolism, Tap63/73 and others for survival, downregulates AMPK pathway for autophagy and upregulates mTOR pathway for cell growth and protein synthesis (Figure 7).

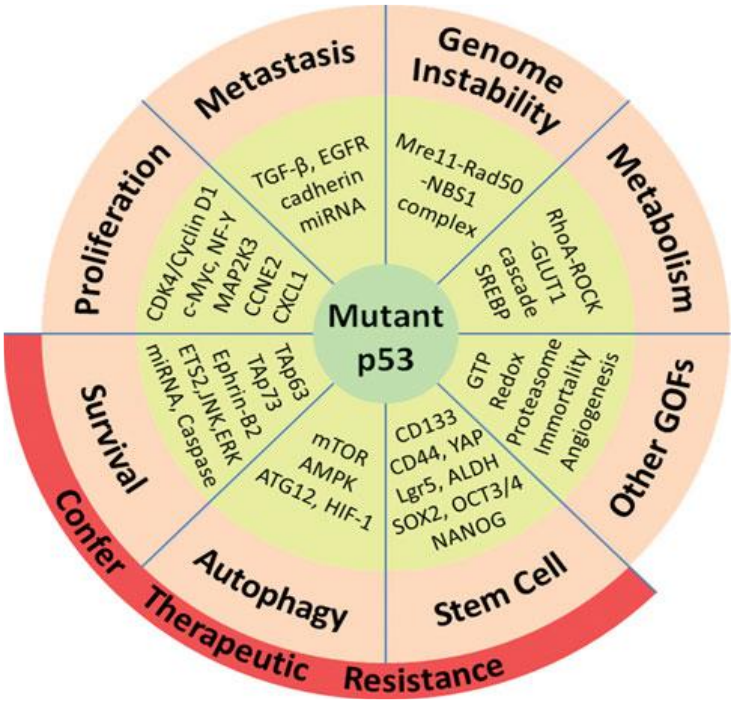

Figure 7: mutp53 and its interacting partners. mutp53 is involved in various functions, as autophagy, stem cell maintenance, metabolic functions, genomic instability, proliferation and invasion and others [37].

Furthermore, p53 mutants can bind and inactivate two homologues of the p53 family, p63 and p73. Given that they share amino acid sequence identity in the DNA-binding domain, p53, p63 and p73 should have redundant functions in the regulation of gene expression [41]. The interaction between mutp53 and p63/p73 are related to many aspects of the GOF of mutp53, such as chemoresistance, migration, invasion, and metastasis [41]. p63 and p73 are found to homo- and hetero-dimerize each other, but has not reported to bind to wild-type p53. On the contrary, the mutant isoform R175H of p53 has been reported to bind to family members, including p63 and p73 through an interaction that involves its DNA binding domain (DBD) and the C-terminal transactivation inhibitory (TI) domain of both p63 andp73 isoforms [42]. Through this aggregation process, mutp53 exerts a dominant negative effect on p63 and p73, thus inhibiting their functions [43]. An additional mechanism by which mutp53 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 [44]. Considering all these, modulation of gene transcription and interference with various signalling pathways are important mechanisms by which p53 mutants exert their oncogenic functions.

3.2.1 Chemoresistance

The major principle of cancer therapy is to decrease cancer cell proliferation and induce apoptosis. Cancer cells may become resistant to therapy for their survival. In some cases, they survive by adapting to therapeutic treatments by mutating the*TP53* gene in their later stages of oncogenesis. During recent years it has been shown that p53 mutants are key molecules endowing cancer cells with chemoresistance [37]. Chemoresistance causes reversion disease and metastasis, contrasts the clinical outcome of cancer patients, and a major keypoint in therapy [45]. There are many mechanisms involved in chemoresistance. One of them is the evidence that mutp53 can induce the expression of its target gene MDR1 (multi drug resistance gene 1), representing the first identification of mutp53 involvement in chemoresistance [46]. Indeed, MDR1 which is also known as ABCB1 (ATP Binding Cassette Subfamily B Member 1), an ATP binding cassette (ABC) transporter and mediating the efflux of drugs from cells in a ATP-dependent manner, conferring multi-drug resistance (MDR) is demonstrated to be stimulated by mutp53 [47]. Further, a different regulation has been reported by WTp53. Also, another mechanism has been shown, even due to low DNA damage, PLK2 and TopBP1 enhances the recruitment of nuclear factor Y(NF-Y), leading to increased expression of genes involved in chemoresistance and proliferation [39] (Figure 8). Overexpression of various tumor associated mutp53 can determine cancer cells more resistant to therapeutic drugs [48]. On the other side, the knockdown of endogenous mutant p53 sensitizes cancer cells to killing by such molecules [30]. 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, by which 60 cell lines and more than 100 anticancer drugs were examined [49]. 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 tissue type of cancers [50]. 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 [48][51].

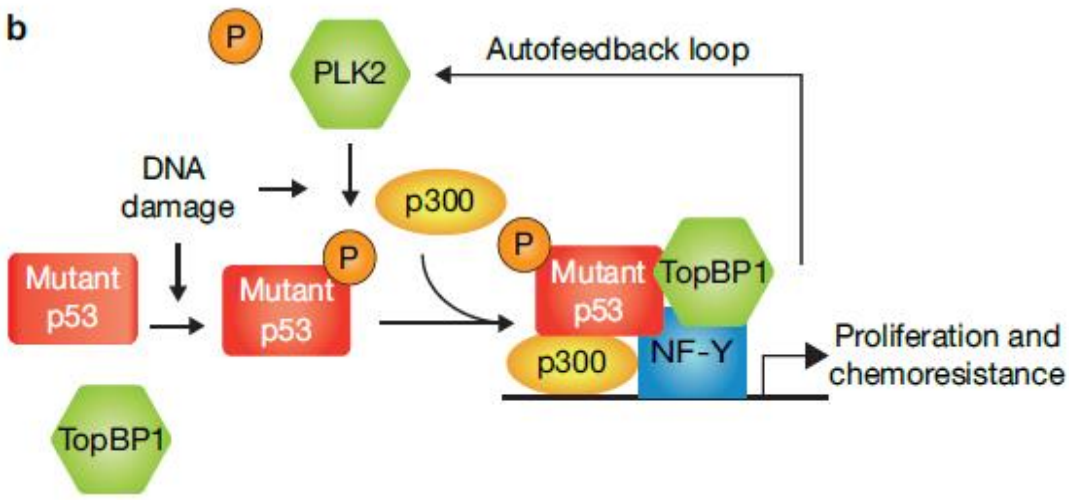

Figure 8: One of the mechanisms of mutp53 involved in chemoresistance [39]

3.2.2 Cellular Metabolism

Some of the missense mutation of p53 that are known to be gain of function mutation are involved in the deregulation of cellular metabolism, independent of the main role that WTp53 exerts. It has been reported that mutp53 stimulates mevalonate pathway, which is responsible for cholesterol production and further for membrane biogenesis and cell division. In this context, mutp53 acts as coactivator of SREBPs (sterol regulatory element binding transcriptional factors), which are transcriptional pathways capable of activating mevalonate pathway enzymes [52]. This stimulation by mutp53 is required for cells to maintain a malignant state. Similarly, under metabolic stresses, mutp53 increases lipid production and aerobic glycolysis (the Warburg Effect) by inhibiting AMP activated protein Kinase (AMPK) signalling [53] or by RhoA–ROCK–GLUT1 cascade[54]. Mutp53 inhibits AMPK activation by binding tightly to AMPK α subunit and blocking its phosphorylation at Thr172 residue by LKB1 (liver kinase B1) or blocking AMPK-LKB1 interaction [55]. Taken together, these studies reveal

that p53 mutants sustain cancer cell survival through metabolic regulation (figure 9).

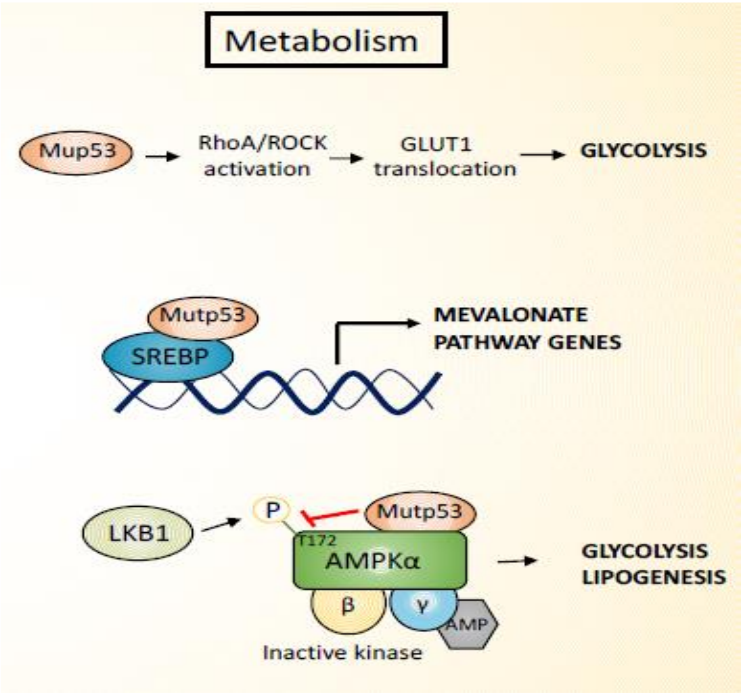

Figure 9*:* p53 mutants sustain cancer cell survival through metabolic regulation [34]

3.2.3 Cell Proliferation

One of the major outcome of GOF of mutp53 is uncontrolled cellular growth. Some of the mutant variants as R175H, R273H, D281G are able to form ternary complex with NF-Y and p300 as co-factor. This binding mediates histone acetylation and further transcription of NF-Y, activating target genes responsible for cell cycle progression and DNA synthesis (figure 8). These target genes include CCNA2, CCNB1, CCNB2,CDK1, and CDC25C [40]. Furthermore, the genes as C-Myc, MAP2K3, CXCL1 and CCNE2 are also induced by mutp53 following various mechanisms and which are responsible for cell proliferation [56]. Recently, a novel mechanism has been elucidated in hepatocellular carcinoma (HCC) having mutp53 (R249S), where oncoproteins as CDK4/Cyclin D1, PIN1 and c-MYC are involved in the hyper-proliferative mechanism [57] (figure 10). Initially, p53-R249S is phosphorylated by CDK4/Cyclin D1, interacts with PIN1, which helps the complex

to be transported to the nucleus. Later, in the nucleus, the complex binds and stabilises c-MYC, becoming more active and facilitating the transcription of target genes including ribosomal biogenesis genes, consequently increasing HCC cell proliferation and survival [58].

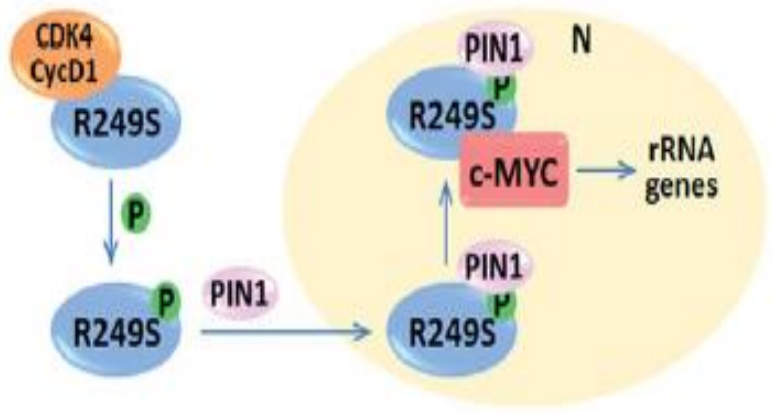

Figure 10: Cellular proliferation, outcome of GOF of mutp53 and its mechanism [57]

3.2.4 Cell death related mechanism: apoptosis and autophagy

To increase the apoptosis, autophagy or any kind of cell death mechanism is one of the main strategies of any cancer therapeutic drugs. In response to external stress, WTp53 can undergo post-translational modification including acetylation and phosphorylation leading to robust apoptosis, showing an increased expression of apoptosis-related genes, as PUMA, BID, BAX, NOXA, initiating MOMP and driving caspase pathway, followed by apoptotic cell death [59]. Mutation in p53 impairs transcriptional activity in nucleus and also interrupts the interaction of p53 with BCL2 family of proteins in cytoplasm, thus counteracting the nuclear and cytosolic roles of wild-type p53 as a tumor suppressor. It has been shown that mutp53 represses apoptosis by directly getting involved with caspase dependent apoptotic pathway. By various stress stimuli, the initiator caspases activate MOMP or directly activating downstream caspases, as 3/6/7 leading to caspase-dependent apoptosis [60]. Other than that, mutp53 directly binds to these proteins, hampering the activation of caspase 8/9 [61] or impeding the cleavage of caspase 3 [62] (figure 11). Another mechanism by which mutp53 inhibits apoptosis is through NF-kB pathway. Mutp53 sustains NF-kB activation in colorectal cancer leading to chronic

inflammation [63]. Since NF-kB acts as a strong inhibitor of apoptotic stimuli, the stimulation of NF-kB signalling may be a further mechanism by which mutp53 proteins inhibit the apoptotic signalling.

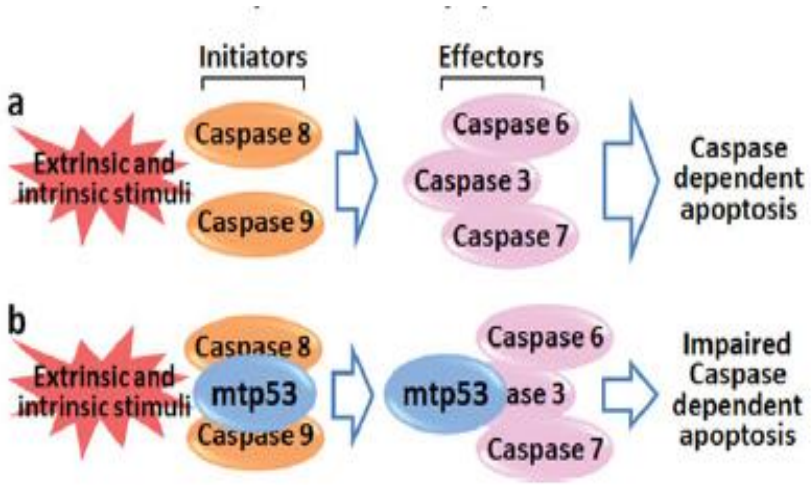

Figure 11: Cell death related mechanisms: apoptosis and autophagy [62]

3.3. THERAPEUTIC STRATAGIES FOR MUTp53 RELATED ONCOGENESIS

Mutp53 is considered as a major obstacle in cancer therapy due to LOH, DN and GOF properties. But some of the treatments or strategies have been implemented to improve the conventional treatment methodologies [37]. Many different mutations and phenotypes allow a variety of strategies are being explored to target tumors expressing mutp53. Some of the important therapeutic strategies can be: *i)* restoration of WTp53 to mutp53; *ii)* promoting mutp53 degradation; *iii*) targeting mutp53 regulated pathways; *iv)* lethality caused by mutp53 [29] (figure 12).

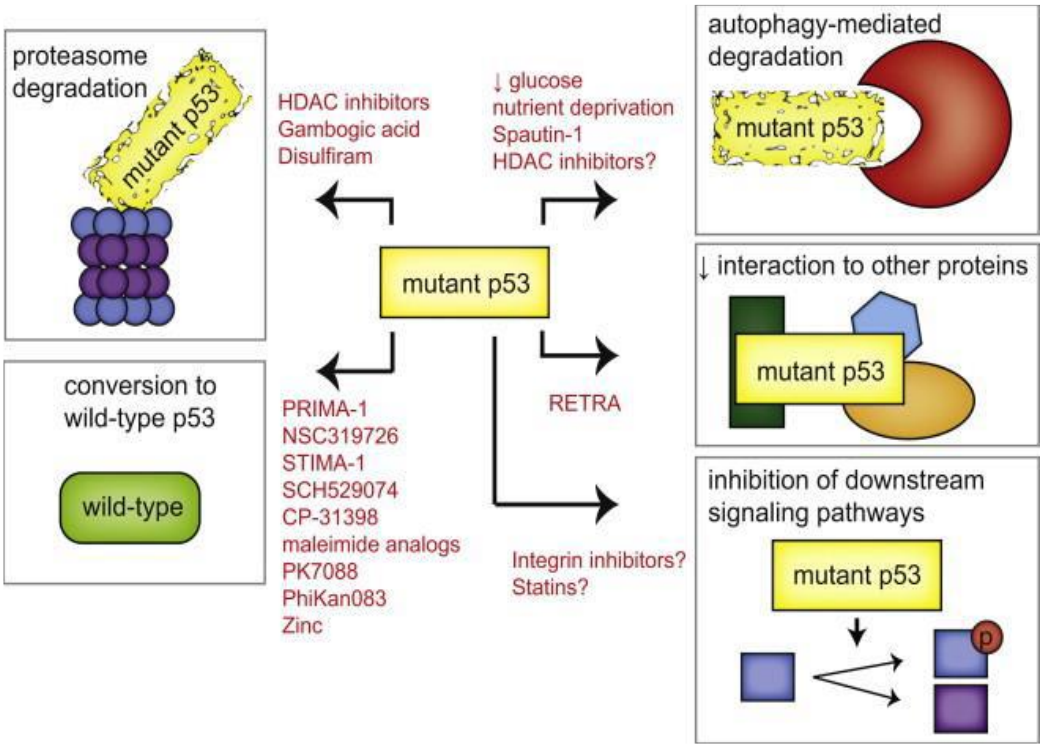

Figure 12: Therapeutic strategies for mutp53 related oncogenesis. These strategies include promotion of mutp53 degradation through the proteasome and autophagy pathways, restoration of wild-type p53 activity, interference with the interaction between mutp53 and other proteins, and interference in signalling pathways downstream of mutp53 [29].

3.3.1 Restoration of wild-type to mutp53

An attractive strategy by targeting mutp53 for cancer therapy is by converting to a protein conformation that can mimic WTp53. WTp53 is a strong inducer of apoptosis and senescence when expressed in cancer cells [37]. Refolding 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 [64]. A variety of compounds that might restore wild-type p53 conformation and function have been characterized [64]. Compounds as CP-31398, P53R3 [65], NSC319726 [66], PK7088 [67], PEITC [68], RITA [69] have been identified to stabilize mutp53 in its wild-type state. Recently, some compounds as COTI-2, which is a thiosemicarbazone-related compound that shows significant efficiency in decreasing cancer growth by retrieving wild-type from a panel of cancer cell lines that contain mutp53-R175H, Y220C, R248Q, I255N, and R273H

[70] and APR-246/PRIMA-1MET, are under clinical trials [71]. p53 Reactivation and Induction of Massive Apoptosis (PRIMA-1) is able to restore sequence specific DNA binding capability to the mutp53 proteins and present *in vivo* tumor suppressive activity with no evident toxicity [72]. Treatment with this drug activates WTp53 target genes as BAX, PUMA, and NOXA [66] activating apoptotic pathway. APR-246 or PRIMA-1 MET is a methylated form of PRIMA-1, but showing high efficiency in eliciting apoptosis [73]. The mechanism of APR-246 is quite similar to molecule PK11007, reactivating p53 mutants Y220C and V143A, but also sensitizes mutp53 containing cancer cells to oxidative stress[74] (figure 12). Molecules including APR-246, PRIMA-1 are converted to a intermediate compound methylene quinuclidinone (MQ), which binds to core domain of p53 [60] (figure 13). Due to aberrant folding, mutp53 proteins expose cysteine residues, which are hidden in wild type p53 conformation [75]. Due to this expose, inter- and intramolecular disulphide bonds are formed, locking mutp53 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 [60].

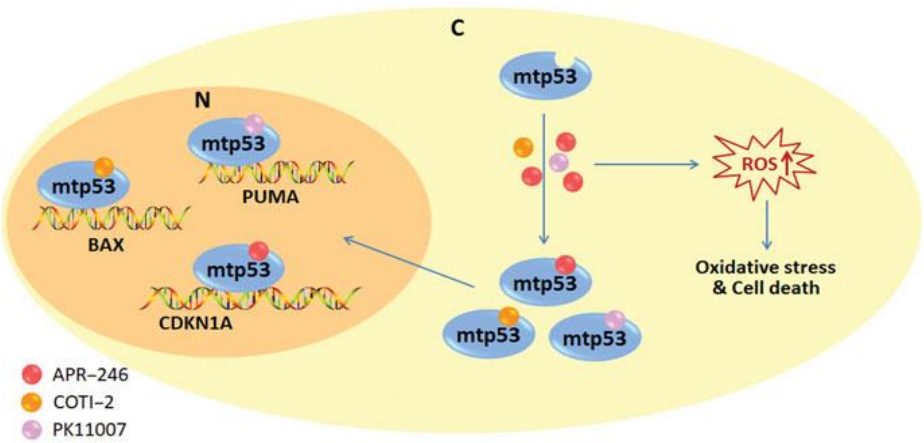

Figure 13: Restoration of wild-type to mutp53 as a therapeutic strategy for mutp53 related oncogenesis[37].

3.3.2 Deprivation and degradation of mutp53

Inhibition of mutp53 protein by promoting its degradation is found to be efficient strategy that sustains mutp53. The idea of depleting mutp53 is that mutp53 proteins are highly unstable in normal cells [76] and stabilised by HSP90 which is overexpressed in cancer cells [77]. HSP90 triggers mutp53 degradation with the help of E3 ubiquitin ligases as CHIP or MDM2 [78]. Also, its shown that mutp53 proteins including p53-R175H/C/D, S241F, G245C, R248Q/W/L, E258K, R273H/L, R280K, and R282W are also degraded through the chaperone mediated pathway (CMA) triggered by spautin-1, a molecule designed for inhibition of macroautophagy [79]. Very recently, a small molecule named MCB-613 has been found to preferentially target p53-R175H for lysosomal degradation by subverting the deubiquitinase USP15 mediated mutp53 stabilization [80] (figure 14). Hence, targeting mutp53 for degradation by small molecule compounds could be potentially beneficial to the improvement of therapeutic sensitivity of human cancers that harbour mutp53.

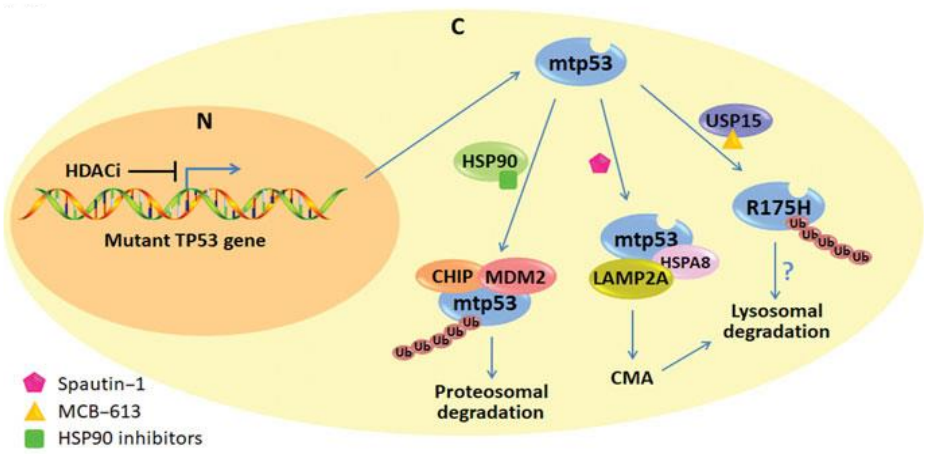

Figure 14: Deprivation of mutp53 / Degradation of mutp53 as a therapeutic strategy for mutp53 related oncogenesis [37].

3.3.3 Synthetic lethality imposed by mutp3

The idea of synthetic lethality is emerged from the studies of Drosophila in which the mutations of two or more separate and non-essential genes result in cell death [37]. In cancer cells, this defect is explained by the fact that oncogenic mutation or tumor suppressor defect offers secondary survival dependency. Cancer

cells are very much vulnerable to attack by targeting the secondary survival signal [81]. In moderate genotoxic stress, WTp53 shows G1 arrest which helps in protection of genome and repair of damaged DNA. But in the case of mutp53, DNA damage response pathway is interrupted and blocked at G1 checkpoint and rely on G2 checkpoints. Supporting this idea, G2 or S checkpoint associated ATR/CHK1, ATM/CHK2, p38, MAPK/MK2 is inactivated to attain synthetic lethality to p53 deficient cancer cells [71]. It seems efficient to target secondary survival-dependant signals to eliminate cancer cells. One of the most successful synthetic lethal agent is WEE1, which is a kinase inhibitor to arrest cell cycle at G2 phase in response to DNA damage stress by phosphorylating CDK1/Cyclin B1 complex in cancer cells with deficient G1 checkpoint [82] (figure 15). AZD1775, which is a WEE1 inhibitor, has been shown to improve tumor treatment and increase carboplatin efficacy in ovarian patients, who are refractory to first line therapy and harbour mutp53 [83][84].

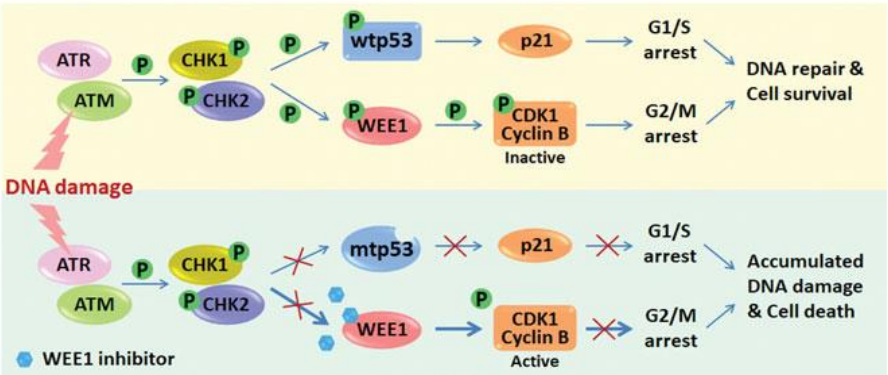

Figure 15: Synthetic lethality imposed by mutp53 as a therapeutic strategy for mutp53 related oncogenesis [37].

3.4 CANCER METABOLISM: THE WARBURG EFFECT

The metabolism of glucose, which is a crucial nutrient for all the cells, helps to acquire energy in the form of ATP through the oxidation of its carbon atoms via the glycolytic pathway [85]. In mammals, the end product of glycolysis is pyruvate, which can be transformed in lactate or CO_2 via respiration in mitochondria, on the basis of oxygen availability.

Cancer cells rewire their metabolism as a direct and indirect consequence of oncogenic mutations to promote growth, survival, proliferation, and long term maintenance [85]. 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 signalling molecules, such as reactive oxygen species (ROS) [86]. The common feature of this altered metabolism is increased glucose uptake into cancer cells and fermentation of glucose to lactate, even in normoxia [85]. This phenomenon called "the Warburg Effect" or aerobic glycolysis, is the best known metabolic shift that occurs in cancer cells to support the biosynthetic requirements of uncontrolled proliferation [85]. In normal cells, ATP is obtained through both oxidative and glycolytic mechanism, where oxidative metabolism yields ~32/34 ATP in the presence of oxygen while glycolysis yields only 2 ATP per molecule of glucose. Usually, this less efficient glycolytic pathway is utilised during the conditions of hypoxia [87]. During Warburg Effect, even in the presence of oxygen and totally functioning mitochondria, tumor cells adopt glycolysis for their energy necessities and undergo both high rate glucose uptake and lactate production and secretion, as compared with normal cell [88]. 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 [89] (figure 16). In this situation, the increased glucose consumption is used as a carbon source for anabolic processes needed to support cell proliferation [90]. 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 pentose phosphate pathway (PPP) [90]. 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 [91]. Finally, the Warburg Effect is also able to induce acidification of the microenvironment and other metabolic crosstalk, favouring cancer cell growth and invasion [91].

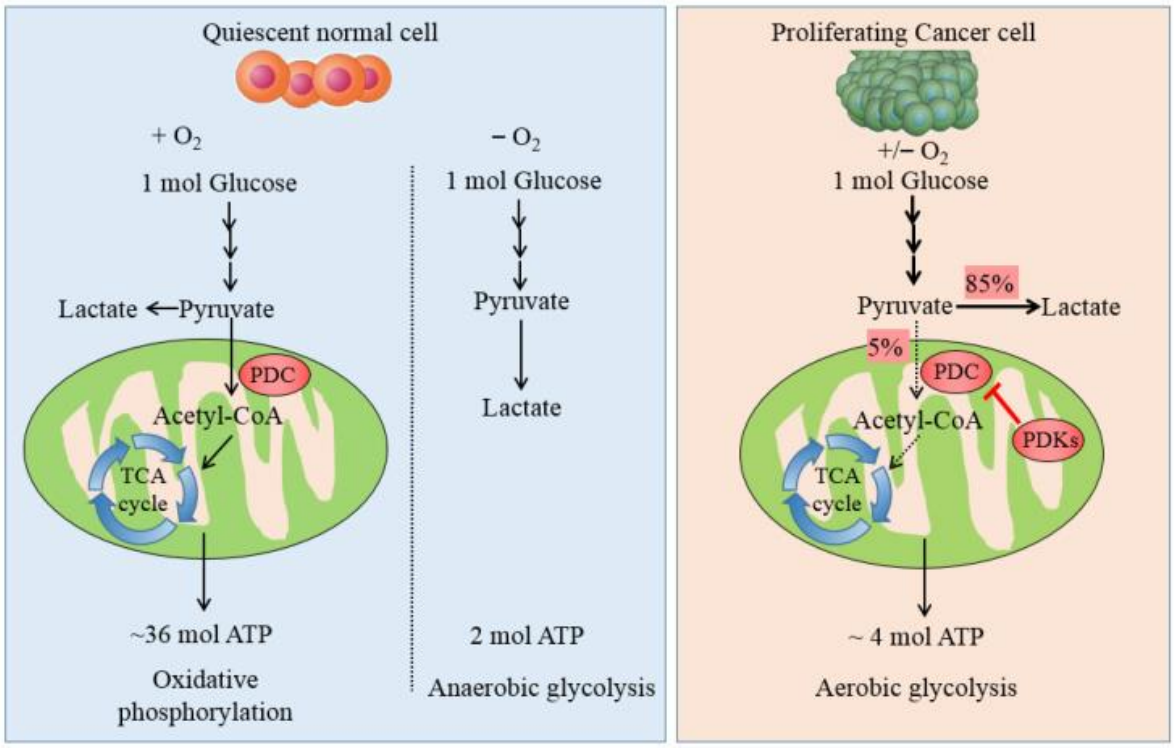

Figure 16: The Warburg Effect. Schematic representation of the differences between oxidative phosphorylation, anaerobic glycolysis, and aerobic glycolysis [92]

3.4.1 The opposite regulation of the Warburg Effect by wild-type and mutp53

Tumor-associated mutant p53 was reported to promote tumor metabolic changes as a novel gain-of-function in promoting tumor development. Mutp53 supports the Warburg Effect and thereby cell proliferation has been demonstrated in both cultured cells and mutp53 knock-in mice [93]. This has been noticed in evidence with the translocation of GLUT1 (glucose transporter 1) to the plasma membrane, mediated by activated RhoA/ROCK signalling [94][93]. In addition, mutp53 was reported to induce the expression of the glycolytic enzyme hexokinase II, which could promote glycolysis [95].

Recent studies have shown that regulating energy metabolism is a critical role of WTp53 in tumor suppression [96]. Indeed, WTp53 is described to regulate glycolysis, mitochondrial oxidative phosphorylation, pentose phosphate pathway (PPP) and lipid metabolism in cells. Functionally, WTp53 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 [97][98]. 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 [98].

3.5 GLYCOLYSIS, GLYCOLYTIC ENZYMES AND NON-METABOLIC FUNCTIONS

Glycolysis is a highly conserved crucial metabolic pathway in most of organisms. This pathway supplies various anabolic pathways with metabolic precursors and cellular energy in form of ATP [99]. It can be aerobic in the presence of oxygen and anaerobic in the absence of oxygen, with the production of pyruvate, acetyl-CoA and finally CO_2 or lactate as end-product in aerobic or anaerobic conditions, respectively. Glycolysis consists of ten sequential enzymatic steps for converting glucose to pyruvate, which is accomplished with the involvement of specific glycolytic enzymes (figure 17). In aerobic conditions, glucose is converted to pyruvate yielding 2 ATP and most of the pyruvate is transported to mitochondria via transporters, undergoing Krebs cycle and mitochondrial oxidative phosphorylation, finally producing 32/34 ATP per one molecule of glucose oxidized [100]. However, cancer cells generally convert pyruvate into lactate, even in the presence of oxygen as previously described as the Warburg Effect.

Glycolytic enzymes can be responsible for conversion of each molecule to another with or without help of co-factors. There are 10 essential steps in glycolytic pathway yielding pyruvate with the involvement of the following enzymes: hexokinase (HK), phosphoglucose isomerase (PGI), phosphofructokinase (PFK), aldolase, triosephosphate isomerase (TPI), glyceraldehydes 3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), phosphoglycerate mutase (PGM), enolase and pyruvate kinase (PK) [101]. The steps in glycolysis can be divided into energy investment or priming phase, splitting phase, and energy generation phase. In the energy investment phase, glucose is converted to glucose-6-phosphate and then to fructose-6-phosphate and to fructose-1,6-bisphosphate

with the help of HK,PGI, PFK respectively, utilising two ATP and then producing ADP [102]. In the energy generation phase, fructose-1,6-bisphosphate is converted to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate by aldolase enzyme. In the ATP generation step, glyceraldehyde-3-phosphate is converted to pyruvate passing through various intermediate complexes with the help of enzymes, as GAPDH, PGK, PGM, enolase and PK [102].

Intriguingly, all these glycolytic enzymes have also roles other than in energy metabolism. These enzymes take part in various other roles, as the regulation of transcription, apoptosis, cell motility and invasion. Some of the roles have been mentioned in the figure 16. For the transcriptional regulation, HK, LDH, GAPDH, enolase 1 have found to be involved [103]. In the studies performed in yeast, it has been reported that glucose represses genes for glucose metabolism via nuclear Hk2 (Hexokinase2) [104]. Similarly, nuclear LDH and GAPDH are component of OCA-S(Oct-1 co-activator in S phase), a transactivator of H2B (Histone 2B) [105]. ENO1 helps in the alternative splicing form of ENO1, which is MBP-1, transcriptionally represses MYC [106]. In regards with apoptosis, HK and GAPDH are involved. In mammals, mitochondrial bound HKs inhibits apoptosis by modulating Bax, Bak and Bad [106] and nuclear GAPDH might be a regulator or indicator of apoptosis [107]. For the cellular motility and invasion, secreted extracellular GPI binds its receptor gp78 and promotes cell motility and invasion [108].

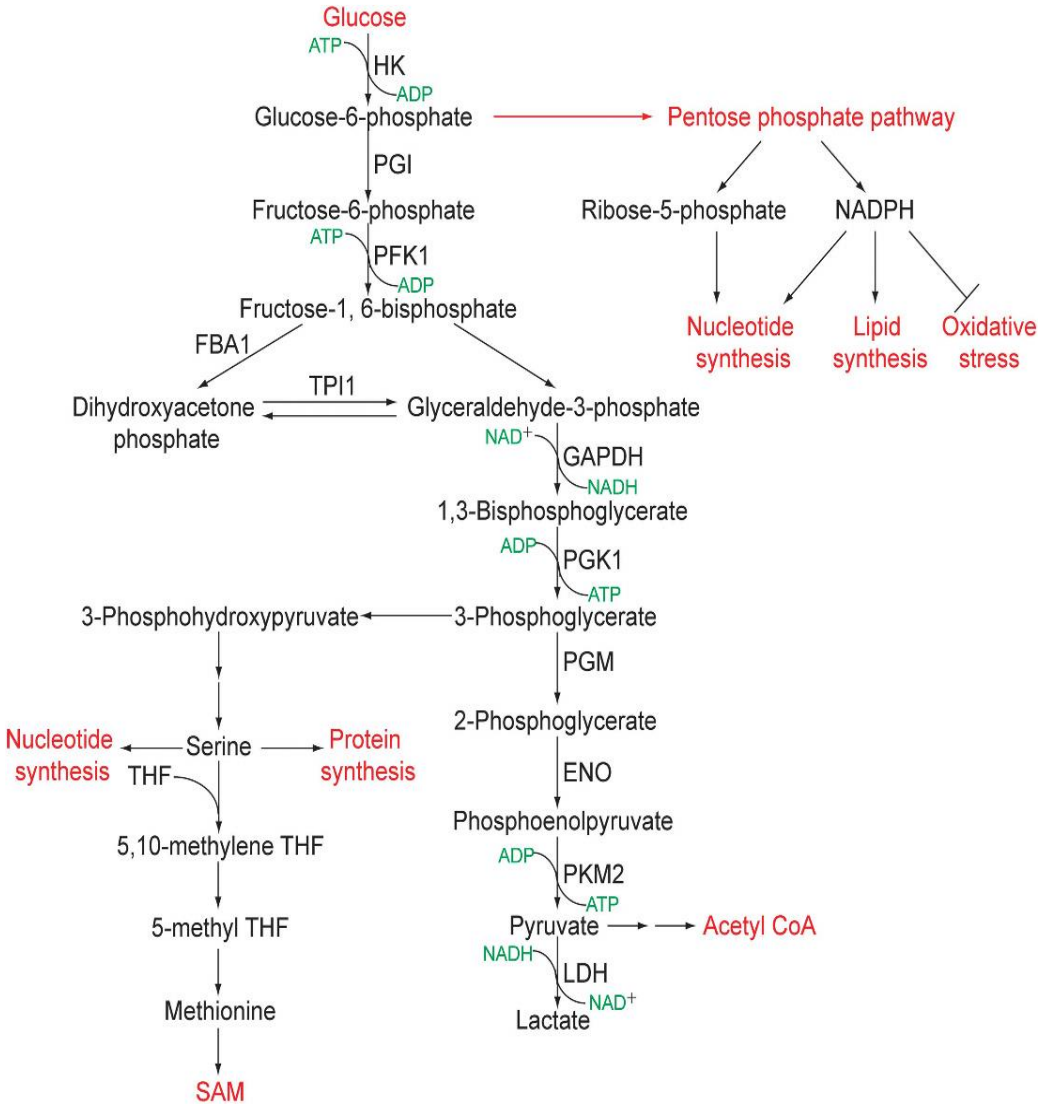

Figure 17: Schematic representation of glycolysis pathway. It consists of various sequential steps for converting glucose to pyruvate, which is accomplished with the help of various glycolytic enzymes [100]

Interestingly, other than mentioned roles (figure 18), some of the enzymes has emerging role in tumorigenesis which opened as a better target for therapeutic interventions [100]. 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 [109]. 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 [103]. The trafficking of metabolic enzymes to the nucleus could be caused by covalent modifications or by forming new protein-protein interactions or protein complexes [110]. However, the precise mechanisms at the basis of the regulation of these non-metabolic functions of the glycolytic enzymes are still largely unknown.

Protein	Glycolytic function	New function	Biological significance of new function
HK	Phosphorylation of glucose	Transcriptional regulation	In yeast, glucose represses genes for glucose metabolism via nuclear Hxk2
		Apoptotic regulation	In mammals, mitochondrial bound HKs inhibit apoptosis by modulating Bax, Bak and Bad
		Glucose homeostasis	In mammals, mitochondrial bound HKs coordinate glycolysis and oxidative phosphorylation
LDH	Oxidation of NADH to NAD^+ by reducing pyruvate to lactate	Transcriptional regulation	Nuclear LDH is a component of OCA-S, a transactivator of H2B
GAPD	NAD^+-dependent oxidation of G3P to 1,3BPG and NADH	Transcriptional regulation	Nuclear GAPD, as a key component of OCA-S, directly binds and transactivates the gene encoding H2B
		Apoptosis	Nuclear GAPD might be a regulator or indicator of apoptosis
		Other functions[b]	
ENO1	Conversion of 2PG to PEP	Transcriptional regulation	Alternative splicing form of ENO1, MBP-1 transcriptionally represses MYC
GPI	Isomerization of G6P to F6P	Cell motility and invasion	Secreted extracellular GPI binds its receptor gp78 and promotes cell motility and invasion

Figure 18: Glycolytic functions, novel functions and biological significance of glycolytic enzymes [103]

3.5.1 GAPDH: a moonlight protein

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a homo tetramer glycolytic enzyme containing four identical 37 kDa subunits. It catalyzes the reversible conversion of Glyceraldehyde-3-phosphate (G-3-P) to 1,3-diphosphoglycerate in the cytosol of the cells [111]. GAPDH is considered a glycolytic and housekeeping enzyme, but it has been discovered to play roles which are independent of energy metabolism, especially in DNA repair, tRNA export, membrane fusion and transport, cytoskeletal dynamics, and cell death [112]. The sub cellular and glycolytic-independent redistribution of the enzyme can mainly occur in the nucleus, mitochondria, and intra- and extra-cellular vesicles. Oligomerisation, post transcriptional modifications and protein-protein interactions

are the main reasons for compartmentalization of GAPDH and its differential regulation [113] (figure 19).

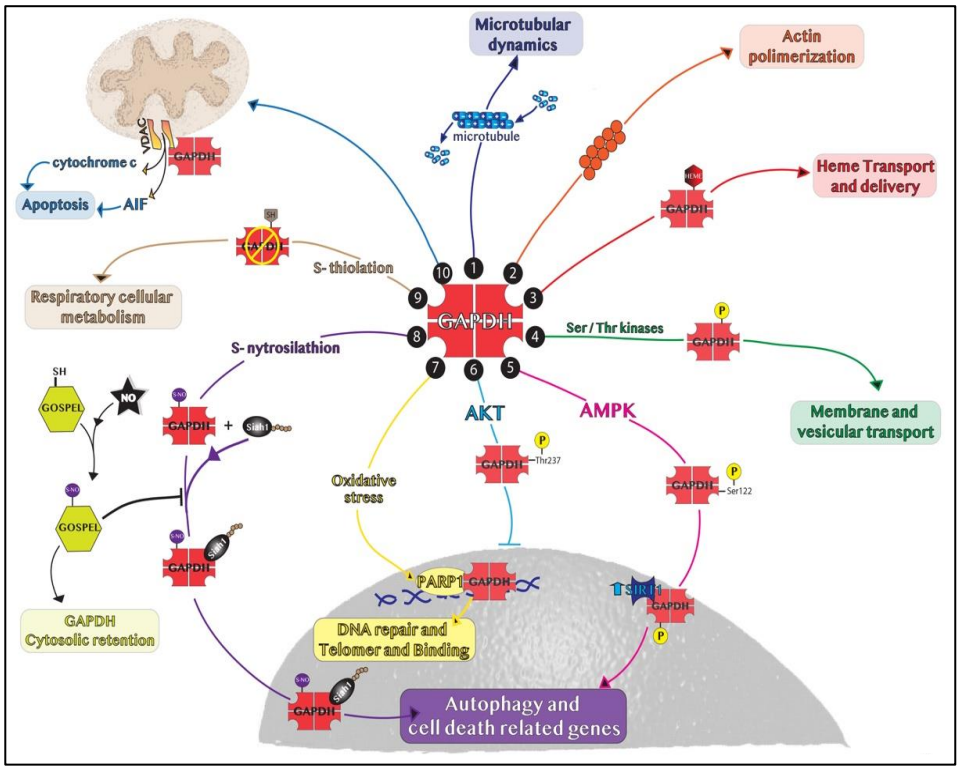

Figure 19: GAPDH, a moonlight protein and its functions [113]

It has been also discovered that GAPDH acts as a key component co-activator complex of OCT-1 in the transcriptional induction of histone H2B gene during the S phase of the cell cycle [105]. Related to autophagy, GAPDH acts as a glucose sensor in the cells stimulating autophagic degradation. Also, during the glucose starvation, AMPK dependent phosphorylation is essential for SIRT1 activation, which helps in autophagic stimulation [114]. One of the nuclear function of GAPDH is associated to the NAD^+ binding domain of GAPDH determining telomere binding and is regulated by the sphingolipid ceramide in a cell cycle dependent manner [115]. In pancreatic cancer, strong increase in GAPDH nuclear translocation due to oxidation of its redox sensitive Cys residues by increased ROS after chemotherapy treatment have been clearly described [116]. In mouse embryonic fibroblasts, following the disruption of glucose intake, GAPDH was found to be translocated from cytosol to nucleus followed by formation of LC3 positive puncta, a marker of autophagy [114]. Also, GAPDH has been exclusively

noted in the neuron disorders like Huntington's Disease (HD) and Parkinsons Disorder (PD) and other neuro degenerative disorders [117]. In ovarian cancer cells, cytosolic GAPDH have been phosphorylated by Akt2 at Thr237 near to the binding site of Siah1, leading to formation of GAPDH/Akt complex, enhancing cell growth and avoiding apoptosis [118]. Furthermore, many studies have demonstrated a functional link between cytosolic GAPDH and microtubules dynamics, vesicular trafficking and membrane recruitment and fusion [119].

Due to its well characterised role in role in glycolysis, GAPDH is rate limiting for the Warburg effect in cancer cells. Also, GAPDH is a rate limiting enzyme for cells which not only rely on glycolysis like protozoans. Hence, GAPDH represents a polyhedral target and its inhibition, especially if achieved with a covalent mechanism, can be a valuable approach for the treatment of several pathological conditions and several diseases like cancer. Covalent inhibitors are powerful tool since their ability to strongly bind to the target, primarily on cysteine residues, can lead to an irreversible inhibition that lasts until the re-synthesis of the protein. The inhibitors belonging to class 3-Bromo-Δ^2-isoxazoline, low cytotoxicity against human endothelial cells (HMEC-1) was observed with very good selectivity indices for *P. falciparum* [120].

3.5.2 GAPDH as a target of cancer

GAPDH has been tested as therapeutic target in various underlying carcinomas from decades. GAPDH targeting has paved the way for better therapeutic strategies in the perspective of cell growth and proliferation. GAPDS (GAPDH segregator) is a triazine-based molecule targeting GAPDH which has been discovered for diabetic drug discovery. This molecule is capable of reducing GAPDH activity, ATP production and thus inducing cancer cell death, blocking migration and inducing cytoskeletal modifications [121]. 3-BrOP(2-Bromopyruvate propyl ester), which is a potential target of GAPDH enzymatic activity, has been shown to decrease the ATP pool and caused cell death in colon cancer cells, better than inhibiting hexokinase (HK) activity [122]. It has been shown that nitroxyl (HNO) acted as an inhibitor of GAPDH and suppressed

proliferation of both estrogen receptor positive ER^+ and ER^- human breast cancer cells [123]. Moreover, treatment with nitroxyl led to a decrease in the expression of the α subunit of HIF1. Inhibition of GAPDH can be attained by targeting the thiol groups, which are non-bound cysteine residues, as Cys152 [124][125] by compounds like chloramines and HOCl that can oxidize cellular receptors and modulate cell proliferation, phosphorylation cascades, transcription factors and cytokine expression [126]. 4-phenylbutyrate(4-PB) inactivates GAPDH at the level of gene transcription. It has been shown to inhibit glioma cell proliferation, to induce apoptosis and to decrease mRNA expression of GAPDH in a concentration-dependent manner [127].

3.6 MITOCHONDRIA AND MITOCHONDRIAL DYNAMICS

Mitochondria are considered the powerhouse of the cell producing the energy required for cell metabolism through oxidative phosphorylation. From the structural point of view, each mitochondrion consists of outer mitochondrial membrane (OMM), inter membrane space, inner mitochondrial membrane (IMM) and the matrix, which has mtDNA [128]. IMM is highly folded into cristae, which contain the electron transport chain and ATP synthase controlling the basic rates of cellular oxidative metabolism. Other than the physiological energy-related functions of mitochondria, these organelles are involved in metabolism, signalling pathways, cell cycle regulation, development, neuronal functions and other crucial cellular roles [129].

From the past 30-40 years, mitochondrial studies evolved drastically and showed that they are not static or isolated intracellular structures. Instead, mitochondrion tries to modulate its structure and morphology making it more tubular or fragmented, depending on various cellular conditions, and then coordinated by fission and fusion events. The balance between these two opposite processes regulates mitochondrial number, size, functionality and positioning within the cytoplasm and is referred as ‘mitochondrial dynamics’ [130]. Mitochondrial fission corresponds to division of one mitochondrion into two daughter mitochondria while, on the contrary, mitochondrial fusion is the union of

two mitochondria to form one longer mitochondrion. The alteration of these events can result in fragmented network by forming several small round shaped mitochondria or hyper-fused and connected network of elongated mitochondria. Mitochondrial dynamics seems to be in a balanced dynamic state, to ensure correct mitochondrial function but also to respond to cellular needs by adapting the network to nutrient availability and to the metabolic state of the cell [131]. Moreover, different morphological states correspond to multiple physiological or patho-physiological conditions [132]. High level of mitochondrial fragmentation is often linked with mitochondrial dysfunction, as this morphological state predominates during cellular death and elevated stress levels [133]. But it has been also noted in the G2/M phase of the cell cycle and is needed for mitochondrial motility, quality control and mtDNA inheritance [134]. Fused mitochondrial network, which is also called as tubular, allows matrix component distribution and stimulation of OXPHOS activity [135] and confers protection against phagophore engulfment during autophagy triggered by nutrient starvation, thus it is mainly associated with cell survival mechanisms [136] (figure 20).

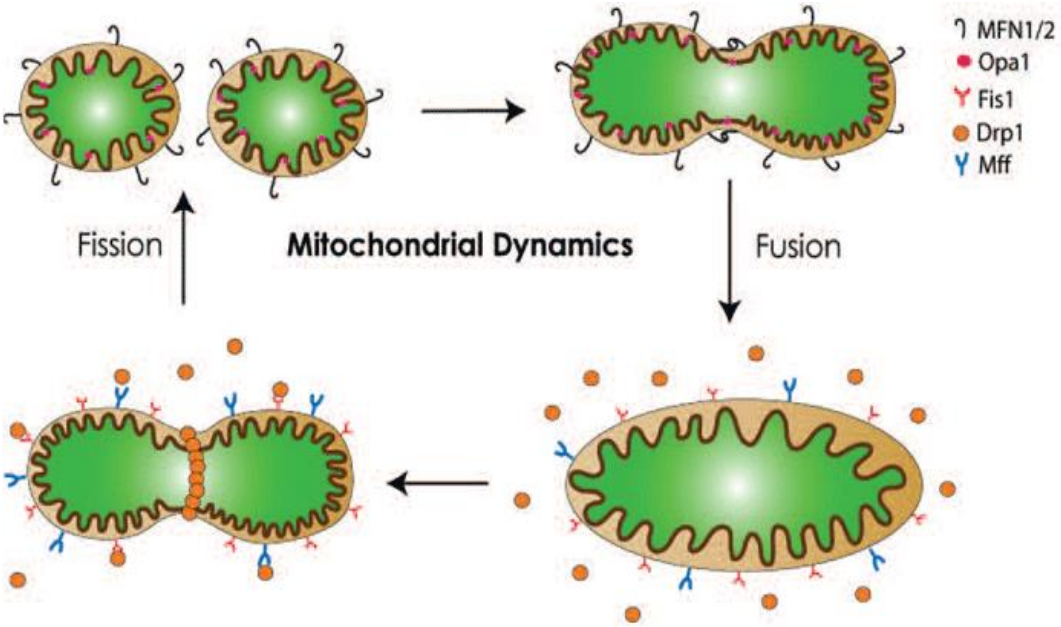

Figure 20: A schematic representation of mitochondrial dynamics and its regulatory proteins [128]

3.6.1 Proteins involved in mitochondrial dynamics

The main proteins which are involved in mitochondrial dynamics core machinery are large GTPase proteins belonging to the dynamin family. These mechano-enzymes oligomerize and change their conformational structure favouring the membrane remodelling, constriction, scission and fusion [137]. Mitochondrial constriction and scission take place with the help of Dynamin related like protein 1 (Drp1), Dynamin 2 (Dnm2) [138]. Mitochondrial fission-1 (FIS1) is also involved in mitochondrial fission. Mitochondrial fusion involves proteins like mitofusins 1 / 2 (Mfn1 /Mfn2) and Optic atrophy1 (OPA1) which helps in the OMM and IMM fusion respectively [139]. Along with these core machinery proteins, Mitochondrial fission factor (MFF), Mid49, MSTO1, Ganglioside-induced differentiation associated protein 1 (GDAP1), SLC25A46 have shown to have involved in these events [140]. Except for Dnm2 knockout, knockout of any of these core machinery GTPase genes determined embryonic lethality in mice and embryonic fibroblasts derived from these mice showed drastic mitochondrial morphological defects [141].

DRP1 is a cytosolic protein driven to mitochondrial and peroxisomal membranes that oligomerises and is involved in constriction or fission of mitochondria in a GTP-dependant manner [142]. DRP1 has four distinct domains, an N-terminal GTPase domain followed by the middle domain, variable domain (or B-insert) and the GTPase effector domain (GED) in C-terminal. Drp1 also contains bundle signalling elements (BSE) and stalk regions, but does not harbour the pleckstrin homology (PH) domain or the proline and arginine rich domain (PRD) at the C-terminal [143] (figure 19). Middle and GED domains are responsible for the mitochondrial targeting determined by phosphorylation and as well as for assembly of Drp1 multimeric collar structure helping in constriction [144]. Increased expression of Drp1 and successive fragmentation of mitochondria has been observed in various neurodegenerative disorders [145], as Huntington [146], Parkinson [147], Alzheimer [148]. Other than this, DRP1 plays a role in apoptosis, mitophagy and cancer development regulation [149].

Mitofusins 1/2 (Mfn1/2) belong to core protein of mitochondrial dynamics and are involved in fusion of mitochondria forming tubular network. Structure of

Mfns consist of N-terminal GTPase, Helix bundle (HB) region, two transmembrane (TM) segments and a C-terminal tail (CT) [150] (figure 19). Despite that both mitofusins are quite similar, the depletion of each results in different morphology of mitochondria. Indeed, depletion of MFN1 forms highly fragmented mitochondria and depletion of MFN2 forms much bigger mitochondrial fragments forming into clusters [151]. Mfns have roles in autophagy [152] and mitophagy [153] as well. Along with that, deregulation of Mfns has been reported in various diseases, as cardiac dysfunction [154], Charcot-Marie-Tooth neuropathy type 2A (CMT2A neuropathy) [155] and Type 2 [156].

Mitochondrial fission1 protein, or Fis1, mediates the assembly of fission complexes which include Drp1, Dnm1 and adaptor proteins as Mdv1 or Caf4 [157]. The cytosolic part of Fis1 forms six helix bundle, in which central four helix has two tandem tetratri co-peptide repeat (TPR)-like motifs [158] (figure 19). It has been reported that Mdv1 and Caf4 use helix-loop-helix motif to attach to surfaces of Fis1. Fis1 has been extensively involved in autophagy and related mechanisms. It's also reported that mitochondria fragmented by Fis1 can be easily targeted by autophagosomes. Fis1 is also involved in Alzheimer's disease [159] and amyotrophic lateral sclerosis [160] by interacting with Drp1.

OPA1 is one of the key protein involved in mitochondrial fusion machinery, which is a dynamic related protein associated with IMM and maintenance of cristae structure [161]. OPA1 composes an N-terminal mitochondrial targeting sequence (MTS), a following transmembrane domain (TM), a coiled coil domain, a highly conserved GTPase domain, a middle domain and a C-terminal GTPase effector domain (GED), and it has eight different spliced variations at the region between the TM and coiled coil domain [162][163] (figure 21).

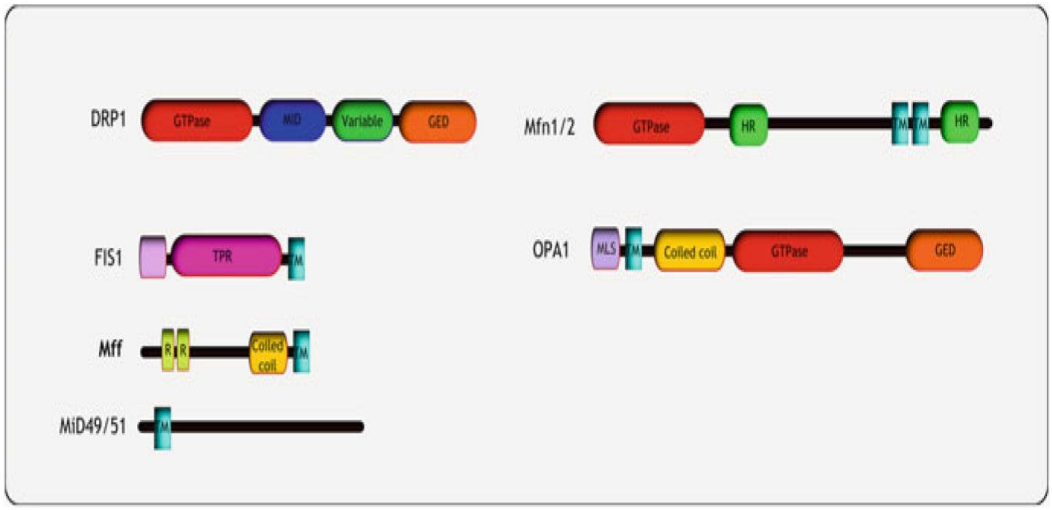

Figure 21: Mitochondrial regulatory proteins and their domains [164]

Mutations in the *OPA1* gene in humans are a common cause of autosomal dominant optic atrophy, a genetic disorder that affects the optic nerve [165] causing extraocular systemic manifestations such as deafness, myopathy, ataxia, peripheral neuropathy [161].

3.6.2 Molecular mechanism of mitochondrial fusion

The proteins Mfn1/2 and OPA1 are involved in mitochondrial fusion. Mfn1 has been noted to have higher GTP-dependent membrane tethering activity than Mfn2 [166]. Mfn2, instead, acts as a key regulator of mitochondria-endoplasmic reticulum (ER) contact sites tethering [167].

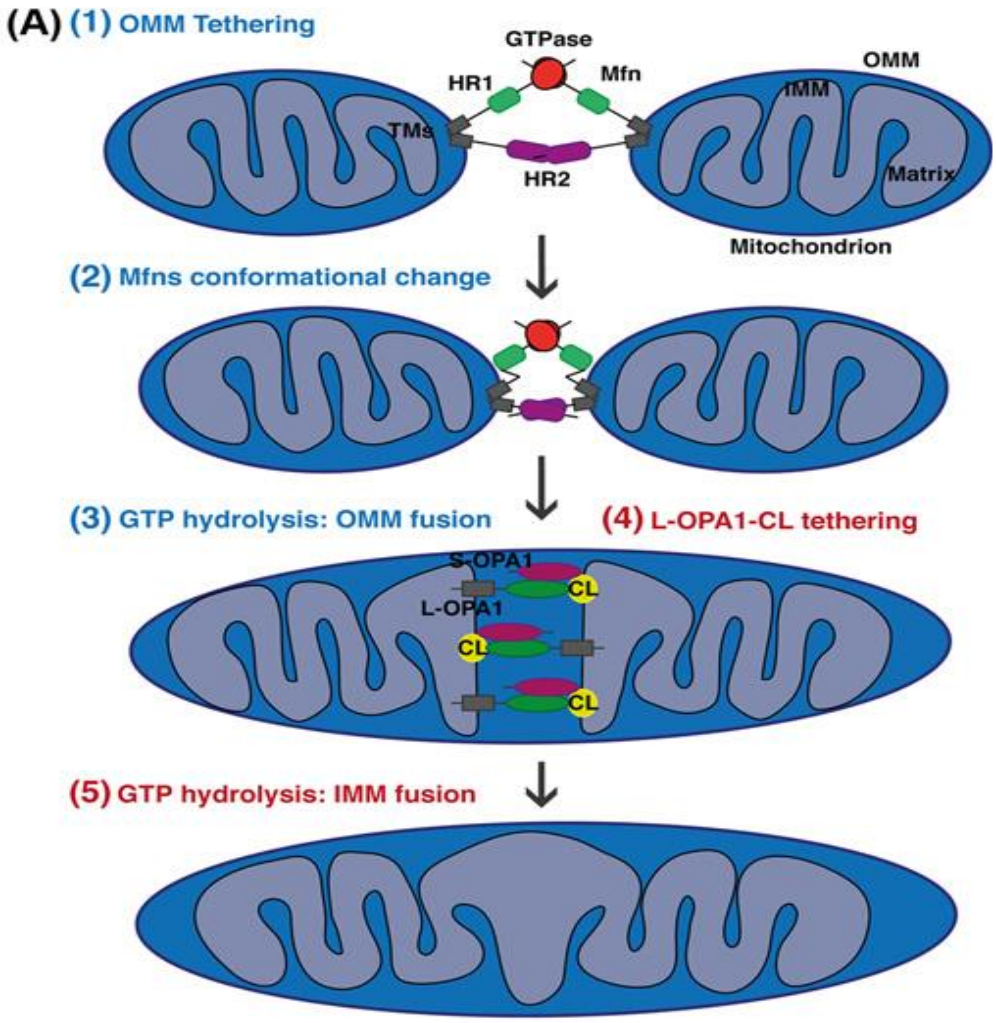

Figure 22: Mechanism of mitochondrial fusion [140]

The Mfns were inserted in the OMM via transmembrane domains (TM) separated by a short loop exposing N-terminal region having GTPase and a coil-coil repeat heptad 1 (HR1) and C-terminal region having HR2 domain in the cytosol [168][169]. Previous studies revealed that Mfns dimeric anti parallel trans interaction was established through HR2 domains [170]. Next step is followed by conformational changes of Mfns and then fusion of adjacent OMM was ensured by GTPase-dependent power stroke or GTP-dependent oligomerisation [171], which also includes GTP hydrolysis. This step is followed by the action of large GTPase OPA1 and other IMM lipid components helping in IMM fusion which is a downstream event of OMM fusion [172]. S and L isoforms of OPA1 along with

cardiolipin present on the opposite sides of OPA1, further helps in the tethering of IMMs together, bringing it closer and undergoing GTP hydrolysis [173] (figure 22).

3.6.3 Molecular mechanism of mitochondrial fission

Mitochondrial fission is a multi-step procedure by which GTPase Drp1 plays an important role [174]. Before the constriction or division of mitochondria begins, replication of mtDNA, which marks the site for endoplasmic reticulum (ER) recruitment, takes place in the mitochondrial matrix [175]. At the same time, Drp1 oligomers are in constant balance between cytosol and mitochondria [176]. Also, before Drp1 oligomerization and maturation, IMM constriction occurs at mitochondria–ER contacts in a Ca^{2+}-dependent process [177]. This step is followed by the accumulation of Drp1 oligomers at the ER-sites where pre-constriction has already been initiated. The actin nucleation and polymerization at mitochondria–ER contact sites is induced by ER bound inverted formin-2 (INF2) [178] and mitochondrial spire 1C [179]. The mechanical force to drive mitochondrial pre–constriction has been ensured by actin cable contraction especially by myosin II a [180]. Then in the pre-constriction site, MFF and adaptors as MiDs recruit DRP1 undergoing oligomerization in a ring like structure [181]. GTP hydrolysis occurs at a faster pace, enhancing conformational changes and mitochondrial constriction. Drp1 assembly and activity is also enhanced by the phospholipids present in the OMM [182]. Later, Dnm2 is recruited to Drp1 mediated constriction site where it assembles and terminates membrane scission, leading to two daughter mitochondria[183] (figure 23). The mechanism of disassembly of fission machinery is not yet fully and clearly understood.

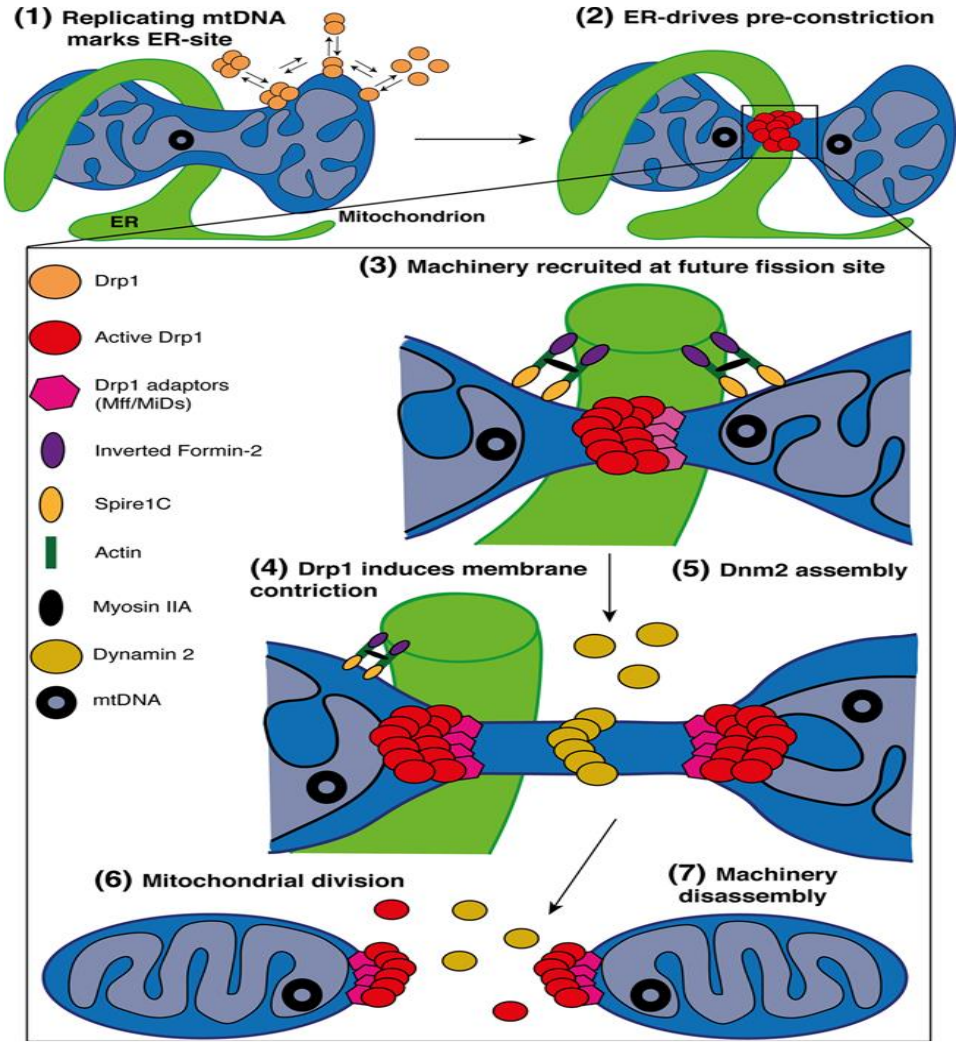

Figure 23: Molecular mechanism of mitochondrial fission[140]

3.6.4 Other functional roles of OPA1

There are other roles of OPA1 independent from mitochondrial dynamics. One of them to be noted is apoptotic cristae remodelling. In the studies performed by Frezza et al, it is confirmed that OPA1, without the help of Mitofusins protect cells from apoptosis and undergo cristae remodelling by blocking intramitochondrial cytochrome C redistribution. Oligomerization of OPA1, which is dependent on the cleavage by rhomboid protease PARL, appears to be a mechanism that regulates apoptosis by maintaining the tightness of cristae junctions [184]. A recent study suggests that OPA1 has been found also on surface of lipid droplets (LD) of adipocytes. OPA1 was noted as a A-Kinase anchoring protein which is involved in lipolysis of the neutral lipids that are stored in the LDs of the

adipocytes [185]. OPA1 is also involved in mitochondrial calcium homeostasis at mitochondrial associated membranes (MAMs) [186] and helps in maintaining Calcium retention capacity [187]. The role of cristae shape in respiratory chain super complexes (RCS) assembly and stability has also been demonstrated by conditional ablation and over expression of OPA1 suggesting that shape of biological membranes can influence membrane protein complexes [188][189].

3.7 MITOCHONDRIAL DYNAMICS AND CANCER

Mitochondrial dynamics which is a powerful mechanism has been shown to be involved in various cancers. Contribution of mitochondrial dynamics in tumor initiation and progression has been studied from long time, although the correct explanation of cancer progression through dynamics has not been understood yet. It's been observed that increased fission and reduced fusion is a hallmark of many cancer [190].

Mitochondrial dysfunction and dysregulation of dynamics has been involved in survival of gynaecologic cancer causing chemoresistance. In chemoresistant gynaecologic cancer cells, Drp1 is neither activated or targeted to the mitochondria and OPA1 is not processed resulting in suppressed mitochondrial fission [191]. Post translational Drp1 activation caused by an increased ratio of Ser616 to Ser637 phosphorylation has been noted in lung cancer patients who have not received any medications yet [190]. In lung cancer patients with increased expression of Drp1 shows a possibility of recurrence by a factor of 3.5 [192] and by decreasing the Drp1 level, decreases proliferation, increases apoptosis in cancer cells and leads to regression of human lung tumors [190][193]. In human pancreatic cancer, expression of oncogenic Ras or activation of MAPK pathway leads to Erk2 mediated phosphorylation of Drp1 on Ser616 that leads to mitochondrial fragmentation and its note that inhibition of this phosphorylation in Xenografts results in decrease of tumor growth [194][195]. In brain tumor initiating cells, cyclin dependent kinase-5 (CDK-5) mediated Drp1 activation shows massive fragmentation and affects AMPK pathway resulting in poor prognosis[196]. There has been enough data which shows the relationship between Drp1 and migration / invasion in tumor cells. Breast cancer cells require Drp1 dependent mitochondrial

fission for relocalising the organelles to lamellipodial region where they need to satisfy the growing energy demand, marking this as a critical early developmental process in metastatic breast cancer [197] (figure 24). In pancreatic cancer cells, induction of mitochondrial fusion by blocking Drp1 or overexpressing Mfn2 reduces oxidative phosphorylation and further suppresses the cell proliferation and growth in both *in vitro* and *in vivo* studies[198] .

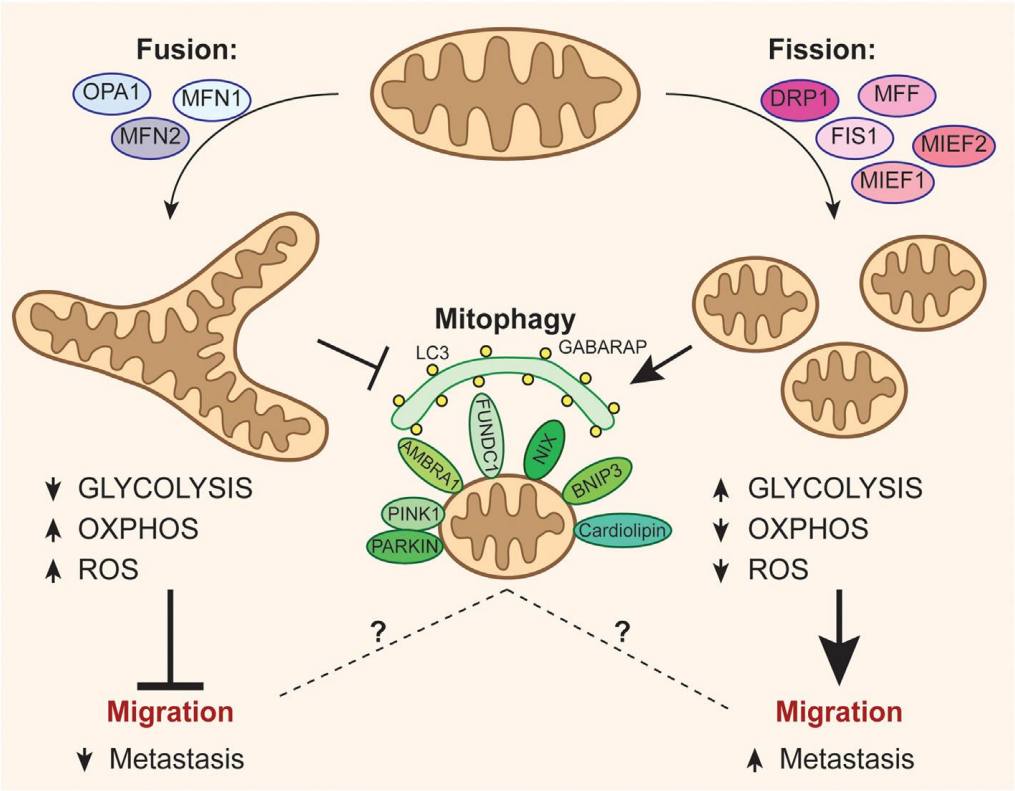

Figure 24: Role of mitochondrial dynamics in metastasis and migration [199]

Ubiquitin ligases acts as an emerging regulators of mitochondrial dynamics in cancer has been extensively studied [200]. Expression of Siah2 which is activated by stress signals like hypoxia, stress response to ER, p38 activity are often activated in cancer and Siah2 controls mitochondrial fission through the regulation of AKAP121 under stress conditions [201].

Excessive mitochondrial fragmentation upon ablation of Mfn1 has been associated with invasive cancer types [197], induces a curvature of the OMM, which is not compatible with Bax association, thus causing resistance to apoptosis [202]. With the Fis1, both positive and negative regulation has been reported in

cancer. Overexpression of Fis1 promotes apoptosis[203] and depends on ER to mitochondria Ca^{2+} transfer [204]. Specifically, Fis1 interacts with Bap31 localized at the ER, facilitating its cleavage into the pro-apoptotic p20Bap31 and building a platform for procaspase-8 recruitment and activation [205]. Overexpression of Fis1 has been observed in oncocytic thyroid tumors [206] and has been associated with poor prognosis in patients with acute myeloid leukemia [207]. OPA1 has been reported to induce cancer in OPA1 transgenic mice likely due to anti-apoptotic effect of OPA1 overexpression [208][209].

With all these data showing same mitochondrial dynamics core machinery protein having both positive and negative impact for the cancer cell survival, can be assumed as new target for cancer therapy.

3.8 MITOCHONDRIAL DYNAMICS AND REGULATION BY MUTp53

The tumor suppressor *TP53* gene is often mutated in cancers and is associated with chemo-responsiveness. The reconstitution of p53 in mutant or null chemoresistant ovarian cancer cells (OVCA) induced L-OPA1 processing, mitochondrial fragmentation and further apoptosis [210]. There are not many reports suggesting the influence of mutp53 in mitochondrial dynamics. But instead, p53, which has tumor suppressor functions, has been reported to influence the mitochondrial dynamics in many ways. Mitofusin2 (Mfn2), which is an integral membrane bound component of fusion is a direct downstream target of p53 implicated in stopping cellular proliferation and sensitizing cell death [211]. In the muscle physiology studies, p53 has been reported to regulate alterations in fission and fusion by knocking out p53 in mice, displaying alterations in mitochondrial morphology and respiratory capacity [212]. In breast cancer cells, p53 is reactivated by chemotherapy or radiotherapy to induce mitochondria mediated cell death [213]. In the absence of stress, p14ARF reactivation of p53 pathway induced cell death in a mitochondrial independent manner and promoted major metabolic reprogramming in hormone-dependent breast cancer cells in favour of increased cellular function and viability [214][215]. Mitochondrial biomass, membrane potentiality, and mitochondria activity were enhanced, as well as metabolic and cellular morphological changes [214][215]. Therefore, p53 may be regarded as a

pendulum between mitochondrial survival on one hand and mitochondrial-induced cell death on the other, mainly depending on the cellular environment.

4. AIMS OF THE STUDY

The main aim of this thesis was to identify altered metabolic pathways in PDAC cells and to investigate related therapeutic options that can be used for efficient treatment of PDAC cancer patients. In particular:

- in order to study the metabolic pathways, we tested a panel of novel GAPDH inhibitors in PDAC cells *in vitro* and *in vivo* and we investigated whether these molecules may efficiently counteract the Warburg effect and proliferation of PDAC cells by blocking GAPDH activity.
- in order to investigate the relationship between mitochondrial dynamics and mutp53 isoforms in PDAC cells, we analyzed whether the expression of mutp53 isoforms may alter mitochondrial dynamics and functionality, further enhancing the knowledge of mutp53-mediated oncogenic properties.

5. MATERIALS AND METHODS

5.1 Cell cultures

Human adenopancreatic cancer cell lines PANC-1, MIA PaCa-2, PaCa-3 and non-transformed fibroblasts were grown in DMEM-Glutamax (ThermoFisher Scientific, Milan, Italy) and AsPC-1 cells was grown in RPMI along with 10% FBS and 50 μg/ml gentamicin sulfate (BioWhittaker, Lonza, Bergamo, Italy).
Cell lines were incubated at 37°C with 5% CO_2.

5.2 Production of cancer stem cells (CSCs) and its maintenance

The PDAC cell lines PANC-1, MIA PaCa-2 were grown in RPMI-1640 supplemented with 10% FBS and 50 μg/mL gentamicin sulfate (all from Gibco/Life Technologies, USA), here reported as differentiated-cell medium (DM), and were maintained at 37°C with 5% CO_2. CSCs were obtained as follows. Briefly, adherent cells were washed twice in 1X PBS (Gibco/Life Technologies, USA) and then cultured in stem-specific medium (SsM), i.e., DMEM/F-12 without glucose (US Biological Life Sciences, USA) supplemented with 1 g/l glucose, B27, 1 μg/mL fungizone, 1% penicillin/streptomycin (all from Gibco/Life Technologies, USA), 5 μg/mL heparin (Sigma/Merck), 20 ng/mL epidermal growth factor (EGF), and 20 ng/mL fibroblast growth factor (FGF) (both from PeproTech, United Kingdom). PANC-1, MIA PaCa-2 CSCs were cultured in flasks with a hydrophobic surface specifically designated for the growth of suspension cells and were maintained at 37°C with 5% CO_2 in the SsM until 8–12 weeks, refreshing twice a week with new medium. Before each experiment, the cells were passed through a cell strainer (40 μm) to separate and maintain only the cell aggregates/spheres, which were trypsinized to obtain single cell suspension.

5.3. Chemicals

Gemcitabine (2′,2′-difluoro-2′-deoxycytidine; Jemta; GEM) was provided by Sandoz Italia and was solubilized in sterile water. Chloroquine diphosphate (CQ) was obtained from Sigma. The inhibitors (s) AXP-1007, AXP-3009, AXP-3018, AXP-3019 were synthesized in University of Milan. The inhibitors were solubilized

in absolute methanol at a final concentration of 100 mM. Monodansyl-cadaverine (MDC) probe was provided by Sigma-Aldrich.

5.4. Cell proliferation assay

Cells were seeded in 96 well plate ($5x10^3$ cell/well), treated with inhibitors after 24 hours at 1, 10, and 100 µm concentration, and incubated further for time indicated. After the treatment time, cells were stained with Crystal violet solution (Sigma, Milan, Italy), then the dye was solubilised in PBS+SDS (1%) and measured photometrically (595 nm) to determine cell growth using GENios Pro Microplate reader (Tecan, Männedorf, Svizzera).

Cell viability for CSCs was evaluated using resazurin cell viability assay kit (Immunological Science), which is based on the reduction of oxidized non-fluorescent blue resazurin to a red fluorescent dye (resorufin) by the mitochondrial respiratory chain in live cells. Resazurin solution was added in each well and, after 1 hour, fluorescence was measured by GENios Pro Microplate reader (Ex_{535}nm, Em_{590}nm). The amount of resorufin produced is directly proportional to the number of living cells. Three independent experiments were performed for each assay condition. CSCs were treated with inhibitors at 1 and 10 µm concentrations.

5.5 Autophagosome formation assay

In order to quantify autophagy induction, cells were incubated with the fluorescent probe monodansyl-cadaverine (MDC; Sigma, Milan, Italy). MDC powder is dissolved in water at 50 mM concentration. Cells were seeded in 96 well plate ($5x10^3$ cell/well), treated with various inhibitors (10 µM) after 24 h, and incubated further for 48 h. After the treatment, cells were incubated with MDC (50 µM) at 37°C for 15 minutes. Cells were washed with HANKS solution and fluorescence was measured (Ex_{340}nm, Em_{535}nm) (GENios Pro, Tecan). The values were normalised with cell proliferation crystal violet assay.

5.6 Apoptotic assay

Cells were seeded in 96 well plate ($5x10^3$ cell/well), treated with inhibitors after 24 hours, and further incubated for time indicated. After the time period, cells were fixed with paraformaldehyde (4%) and washed with PBS for two times.

Further, the cells were incubated with ANNEXIN V/FITC (eBioscience, Thermo Fisher Scientific) probe in binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl and 2.5 mM CaCl2) for about 10 minutes. The cells were washed, and fluorescence was measured (Ex_{485}nm Em_{535}nm) and the values were normalized on cell proliferation by crystal violet assay.

5.7 Lactate assay

Cells were seeded in 96 well plate ($5x10^3$ cell/well), treated with 10 µM inhibitors after 24 hours, and further incubated for time indicated. Supernatant was collected and used for experiment. The assay was performed using the L-Lactic acid kit from Megazyme. The amount of NADH was measured by the increase in absorbance (A_{340}nm). The amount of NADH formed in the reactions is stoichiometric with the amount of L-lactic acid. L-lactic acid concentration (g/L) has been calculated according to the manufacturer's instructions: L-lactic acid secreted by the cells in each sample was calculated by subtracting the amount of L-lactic acid in the culture 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 sample by cell proliferation assay.

5.8 Synergism analysis and drug combination studies

Cells were seeded in 96 well plate ($5x10^3$ cell/well), to check the synergism between the GAPDH inhibitors (AXPs) and gemcitabine (GEM). After 24 hours the cells were treated with 10 µM AXP-3009, AXP-3019 and GEM alone and a combination of them was performed using the molar ratio [AXP(s)]:[GEM] = 1:1.

The combination index (CI) was calculated using the Chou-Talalay equation, which considers both the potency (IC_{50}) and the shape of the dose-effect curve, taking advantage of the CalcuSyn software (Biosoft, Cambridge, UK). The general equation for the classic isobologram is given by CI = (D)1/(Dx)1 + (D)2/(Dx)2 + [(D)1 × (D)2]/[(Dx)1 × (Dx)2], where (Dx)1 and (Dx)2 in the denominators are the doses (or concentrations) of drug 1 and drug 2 alone that give x% growth inhibition, whereas (D)1 and (D)2 in the numerators are the doses of drugs 1 and 2 in combination that also inhibited x% cell growth inhibition (ie, isoeffective).

CI<0.3, 0.3<CI<0.7, 0.7<CI<1.0 values indicate a moderate synergism, synergism or a strong synergism, respectively; whereas CI = 1 and CI>1 indicate additive and antagonism, respectively. CI/effect curves represent the CI versus the fraction (0→1) of cells killed by drug combination. The isobologram graphs using IC_{25}, IC_{50}, and IC_{75} values indicate the equipotent combinations of the two drugs and can be used to further analyse synergism, additivity or antagonism. Dose reduction index (DRI) represents the measure of how much the dose of each drug in a synergistic combination may be reduced at a given effect level compared with the doses of each drug alone. Crystal violet was performed to check the cell proliferation.

5.9 Xenograft mice studies

All procedures involving mice were performed in compliance with our institutional animal care guidelines and following national and international directives (D.L. 4 March 2014, no. 26: directive 2010/63/EU of the European parliament and of the council). MIA PaCa-2 cells (1×10^6 cells/mouse) were subcutaneously injected into the dorsal flank of female nude mice (Charles River Laboratories, Inc.). Twelve days after cell inoculation, 5 randomized animals chosen for each group: control group received 200 µl of vehicle solution (PBS), experimental group received 20 mg/kg of drugs (AXP-3009 and AXP-3019) diluted in DMSO, by intraperitoneal injection biweekly for 7 weeks. Body mass was recorded weekly for each animal. Tumor size was monitored weekly in two perpendicular dimensions parallel to the surface of the mouse using a calliper. Tumor volume was calculated using the formula of V = π/6 x [(w x L)^(3/2)]. Animals were sacrificed at the end of the 7-week study period. After euthanizing the mice, the tumors were resected and weighed.

5.10 Liposome-mediated transient cell transfection

Exponentially growing cells were seeded in 96-well plates or in 60mm cell culture plates. The ectopic expression of mutp53 isoforms in AsPC1 p53-null cells was carried out transfecting pcDNA3- mutp53 R273H or pcDNA3-mutp53 R175H expression vectors, pCMV-wild-type-p53 or their relative mock vector (pcDNA3).

Wild-type and mutp53 protein expression was transiently knocked-down by transfection with sip53 (Santa Cruz Biotechnology, #sc-29436). The expression of OPA1 was transiently knocked-down by transfection with siRNA-OPA1 (Santa Cruz Biotechnology, #106808). The silencing transfections were carried out for 48h using Lipofectamine 3000 (Thermo Fisher), according to the manufacturer's instructions. Cells were transfected by siRNA at a final concentration of 50 nM using Lipofectamine 3000.

5.11 RNA isolation and quantitative real-time PCR analysis

Total RNA was extracted from cells using TRIZOL reagent (Sigma) and reverse-transcribed at 37°C for 50 min in the presence of random hexamers and Moloney murine leukemia (MMLV) virus reverse transcriptase (Life Technologies). Transcripts were measured by real-time qPCR using the SYBR Green assay (Applied Biosystems, Carlsbad, CA, USA) with a 7900 HT Fast Real-Time PCR System (Thermo Fisher). PCR analysis was carried out using specific oligonucleotides for the genes listed in Table 1. 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. GAPDH gene expression was used as endogenous control to standardize mRNA expression. All reactions were performed in triplicate from three independent experiments.

Primers	**Sequences**
DRP1-F	AAGAACCAACCACAGGCAAC
DRP1-R	GTTCACGGCATGACCTTTTT
FIS1-F	CTTGCTGTGTCCAAGTCCAA
FIS1-R	GCTGAAGGACGAATCTCAGG
MFN1-F	TTGGAGCGGAGACTTAGCAT
MFN1-R	TTCGATCAAGTTCCGGATTC
MFN2-F	AGAGGCATCAGTGAGGTGCT
MFN2-R	GCAGAACTTTGTCCCAGAGC
OPA1-F	GGCCAGCAAGATTAGCTACG
OPA1-R	ACAATGTCAGGCACAATCCA
GAPDH-F	ATCAGCAATGCCTCCTGCAC
GAPDH-R	GGTCATGAGTCCTTCCACG

Table 1: Primer used for the qPCR reactions.

5.12 Immunoblot analysis

Cells were lysed in the presence of phosphatase and protease inhibitors [50mM TrisHCl pH 8, 150mM NaCl, 1% Igepal CA-630, 0.5% Na-Doc, 0.1% sodium dodecyl sulphate (SDS), 1mM Na_3VO_4,1mM NaF, 25mM ethylenediaminetetraacetic acid (EDTA), 1mM phenylmethylsulfonylfluorid, and 1× protease inhibitor cocktail]. Protein extracts (50 μg/lane) were resolved on SDS-polyacrylamide gel and electro-blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Milan, Italy). Primary antibodies against p53 (Santa Cruz Biotechnology, Dallas, Texas, USA, #sc-263), GAPDH (Cell Signaling Technology, #5174 s), Mitochondrial dynamics kit (Cell Signaling Technology, #74792) and secondary anti-mouse or anti-rabbit IgGs (Upstate Biotechnology, Milan, Italy) horseradish peroxidase-conjugated antibodies were used. All antibodies were diluted in 5% (w/v) non-fat milk or bovine serum albumin (BSA) in TBS–Tween. The immunocomplexes were visualized by chemiluminescent substrates (Amersham Pharmacia Biotech, Milan, Italy) using Chemidoc XRS Imaging System (Bio-Rad Laboratories, Milan, Italy) and the intensity of the chemiluminescence response was measured by processing the images with NIH ImageJ software (http://rsb.info.nih.gov/nihimage/).

5.13 Confocal microscopy imaging

After the transfection of 48 h, cells were incubated with 1:5000 dilution Mitotracker green FM (Thermo Fisher) and with 1:1000 Hoescht 33258 solution (Sigma, #94403) for 30 min. The cells were observed in confocal microscopy (Leica SP5 inverted confocal microscope). The minimum of about hundred mitochondria were counted and statistically analysed.

5.14 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 INHIBITORS OF GAPDH: NEW INSIGHTS TO TREAT PANCREATIC CANCER

6.1.1. Novel GAPDH inhibitors and its activity

We tested on PDAC cell lines a panel of novel GAPDH inhibitors, namely AXP-1007, AXP-3009, AXP-3018, AXP-3019, which were synthesized in the lab of Prof. Paola Conti (University of Milan, Italy) [216]. The experiments were performed in human recombinant GAPDH enzyme *in vitro* to check the effective capability to inhibit GAPDH activity. Moreover, experiments were also performed in PANC-1 and MIA PaCa-2 PDAC cell lines and in normal fibroblasts, as non-tumoral control cells. The chemical structures of the inhibitors are shown in Fig. 25A. Fractional GAPDH activity of all inhibitors tested was analyzed in human recombinant GAPDH enzyme *in vitro* and represented in Fig. 25B. Fractional activity of GAPDH in the presence of AXP-1007 was almost similar to control, while other inhibitors strongly inhibited recombinant GAPDH enzyme. Also, GAPDH activity was measured for all the inhibitors in fibroblasts, PANC-1 and MIA PaCa-2 cells at 10 µM concentration (Fig. 25 C, D, E).

The selective covalent binding of a drug to the desired target can increase efficiency and lower the inhibitor concentration required to achieve a therapeutic effect. GAPDH is inactivated by a number of compounds that alkylate thiols. 3-Bromo-isoxazolines exhibited this reactivity toward cytidine triphosphate synthetase (CTPS), a glutamine amido transferase endowed with a catalytic Cys within the active site. The irreversible inhibition or inactivation of GAPDH were tested and formulated by our collaborators Paola Conti & Stefano Bruno [216].

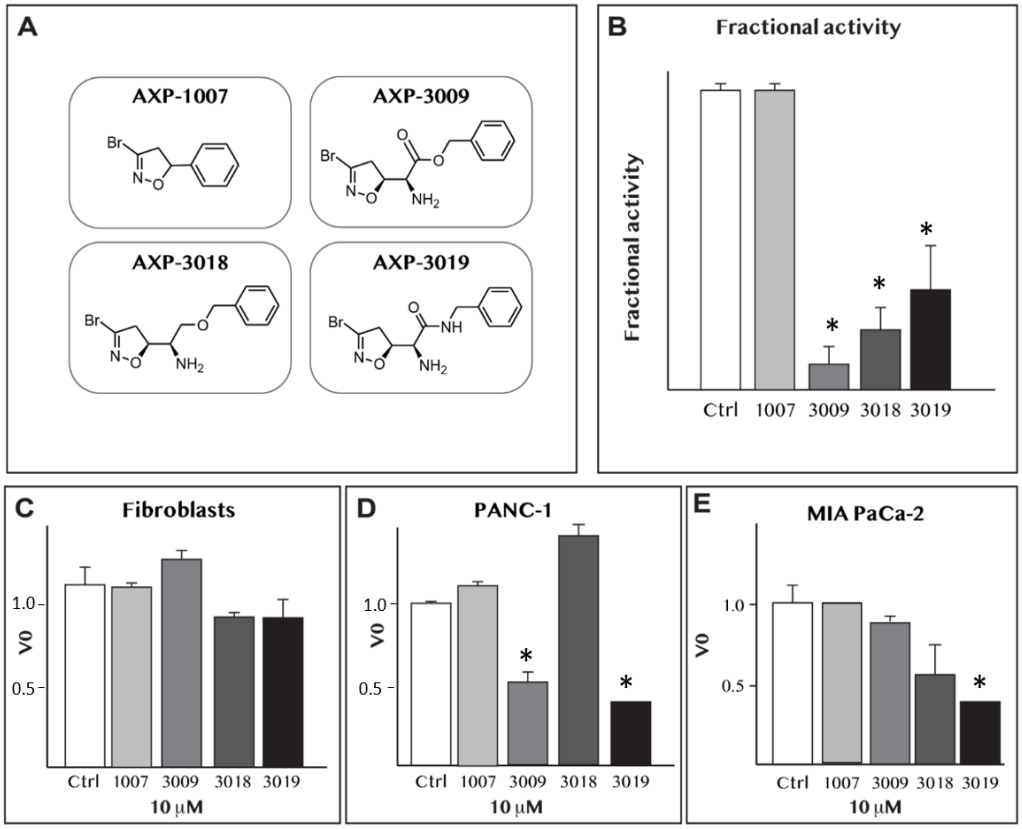

Figure 25: Inhibitors and Fractional activity; GAPDH inhibitors can link the enzyme interfering with its activity: A) Representation of chemical structure of GAPDH inhibitors namely AXP-1007, -3009, -3018, -3019. **B)** Human recombinant GAPDH activity was performed in the absence or in the presence of the various inhibitors (Arbitrary units). **C)** GAPDH activity inside cells were analyzed in fibroblasts. **D)** in PANC-1, **E)** and in MIA PaCa-2 at 10 µM concentration. t test- *p<0.01

Notably, a non-significant reduction was observed in fibroblasts, while in PANC-1 and MIA PaCa-2, about 75-80% GAPDH activity decrease was observed when cells were treated with AXP-3019 (Fig. 25 D, E).

6.1.2. Inhibitors AXP-3009 and AXP-3019 strongly inhibited PDAC cell proliferation

In order to investigate the GAPDH inhibitors effect on cell proliferation, fibroblasts, PANC-1 and MIA PaCa-2 cell lines were treated with 4 inhibitors (AXP-1007; AXP-3009; AXP-3018 and AXP-3019) at three different concentrations (1 µM; 10 µM; 100 µM) (Fig. 26). The percentage of viable cells

was calculated by Crystal Violet assay. The absorbance values of treated cell lines, with each inhibitor, were normalized on the absorbance values obtained on untreated cells.

Regarding fibroblasts (Fig. 26A), no anti-proliferative effect was observed, even at highest concentrations. On the contrary, the most interesting results were obtained from the PDAC cells: in PANC-1 cells (Fig. 26B), the AXP-1007 and AXP-3018 treatments have not caused any cell growth inhibition at any concentrations used, while the anti-proliferative effect was clearly observed with the two inhibitors, AXP-3009 and AXP-3019. In particular, the latter has caused 30% of mortality starting from the lowest concentration, 1 µM, up to reaching 55% of dead cells at the highest concentration (100 µM). The cytotoxic effect caused by two inhibitors (AXP-3009 and AXP-3019) has been mostly observed in MIA PaCa-2 cell line (Fig. 26C), in which the cell proliferation percentage has significantly decreased with 10 µM (around 50%) and 100 µM (around 30%).

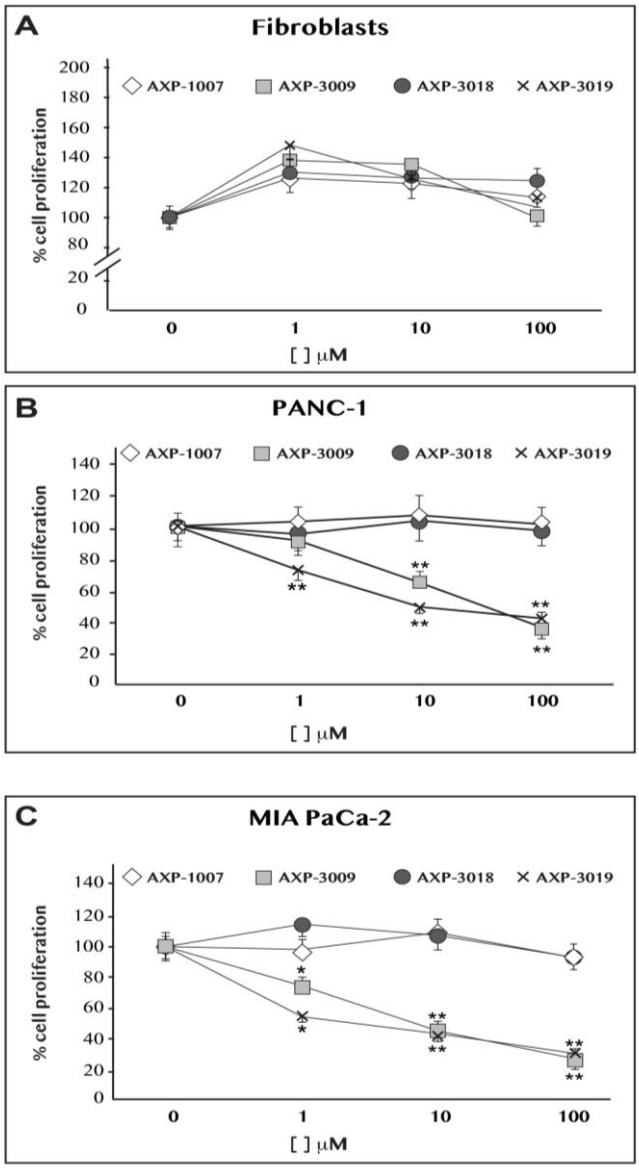

Figure 26: Inhibitors AXP-3009 and AXP-3019 strongly inhibited PDAC cell proliferation: Cell proliferation was determined by Crystal Violet assay with increasing concentrations of inhibitors for 48 h in **A)** fibroblasts **B)** PANC-1 **C)** MIA PaCa-2. t test- *p<0.01, **p<0.001

In both cancer cell lines; the greatest anti-proliferative effect was obtained with AXP-3019 inhibitor.

We also investigated whether pancreatic cancer stem cells (CSCs), previously obtained in our lab [217], had a similar behaviour to parental cells from which CSCs were derived (see Materials & Method). Representative images of

parental and CSCs of PANC-1 and MIA PaCa-2 are shown in fig 27A. The CSCs of PANC-1 and MIA PaCa-2 are completely different in their physical appearance from their parental cells. In CSCs, we can notice the sphere formation or clustering of cells similar to stem cell state. In Fig. 27B, we show PANC-1 parental and CSCs after 48 h of treatments with GAPDH inhibitors AXP-3018 and AXP-3019 at two different concentrations (1 µM and 10 µM). The results for CSCs clearly indicate a decrease of cell proliferation due to treatments with both inhibitors, whereas in the parental cell line, only AXP-3019 caused cytotoxic effect. This result suggests that CSCs derived from PANC-1 cells acquired sensitivity to the AXP-3018 compound, probably due to a different membrane composition and molecule uptake between parental and staminal cell type. In Fig. 27C, MIA PaCa-2 CSCs are shown. Also, in this cell line we observe a different response to GAPDH inhibitors between parental and CSCs, likely due to the marked difference in the membrane composition. We can assume that these results may be due to a different lipid composition of CSCs plasma membrane [218]. In fact, our *in vitro* studies on recombinant enzyme have demonstrated that the AXP-3018 molecule was able to inhibit recombinant GAPDH activity, as AXP-3009 and AXP-3019 molecules (Fig. 25B), supporting the concept that the membrane permeability is a crucial element in allowing the entry of analyzed drugs.

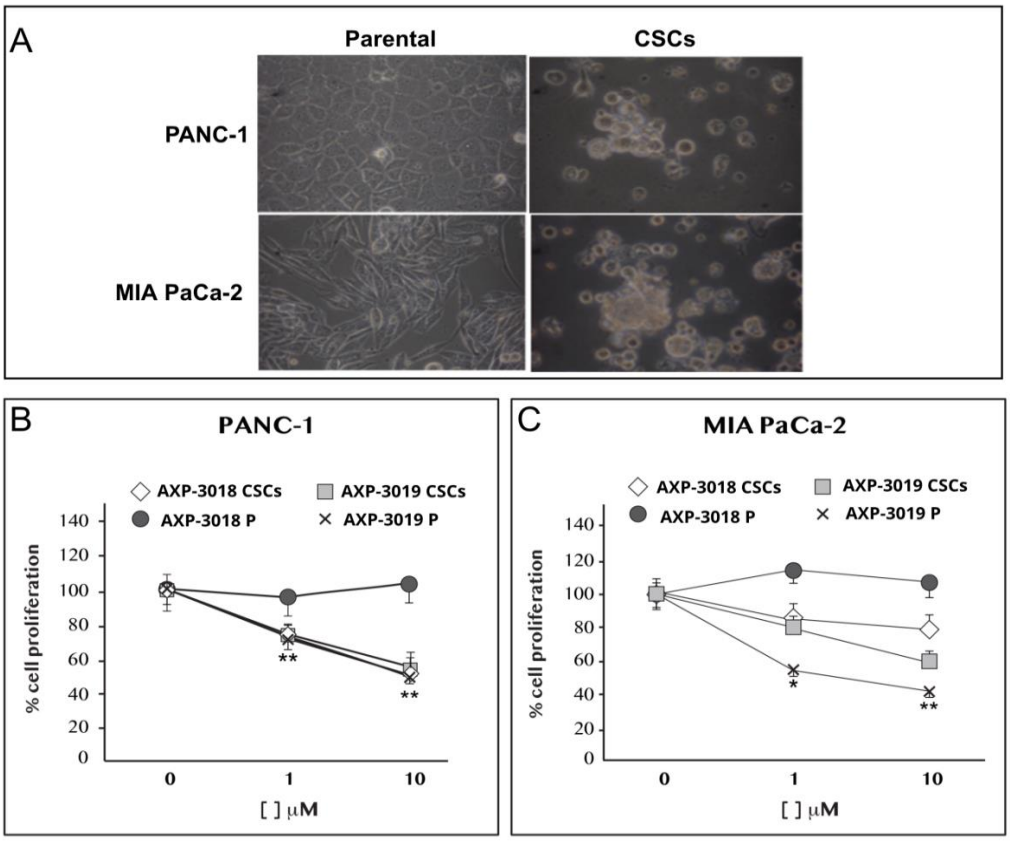

Figure 27: Different response of AXP-3018 and AXP-3019 in PDAC parental and CSCs. AXP-3018 and -3019 were used in parental (P) cancer cells and cancer stem cells (CSCs) at different concentration. Cell proliferation (%) was determined by Crystal Violet assay. In **A)** Representative images of PANC-1 and MIA PaCa-2, Parental and CSCs (as described in 5.2 paragraph). PANC-1 and MIA PaCa-2 cell proliferation was represented in **B)** and **C)** respectively. t-test-*p<0.01, **p<0.001

6.1.3. AXP-3019 blocks L-lactic acid secretion in PDAC cell lines

L-Lactic acid, the metabolic compound generated by the glycolytic pathway, and its secretion was determined in the media of PANC-1 and MIA PaCa-2 cell lines as described in Materials and Methods section. Since cancer cells display increased glucose uptake and therfore an increase in the glycolytic metabolic intermediates or end products, as L-Lactic acid, we decided to analyse the secretion of L-lactic acid in the media after 48 h of treatment with GAPDH inhibitors. Cells were treated with the three GAPDH inhibitors at 10 µM concentration for 48 h. As expected, AXP-3018 inhibitor did not produce a significant decrease compared to

untreated cells, while AXP-3009 and -3019 showed a marked reduction in L-lactic acid secretion, demonstrating these inhibitors, as already highlighted *in vitro*, can bind and inhibit the GAPDH enzyme, thus reducing the glycolytic flux and its metabolic products (Fig. 28).

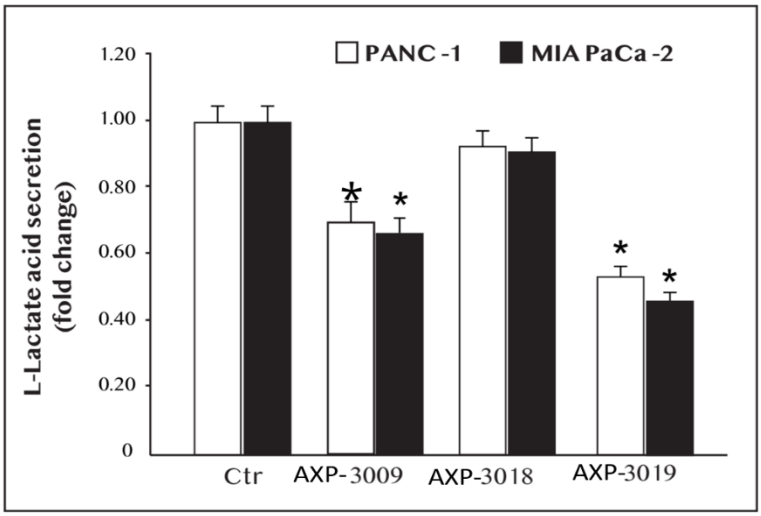

Figure 28: AXP-3019 blocks L-lactate acid secretion in PDAC cell lines: A) L-lactic acid secretion was detected in the media after indicated GAPDH inhibitors in PANC-1 and MIA PaCa-2. t test-*p<0.01

AXP-3019, proved as a better candidate by comparing both cell proliferation and L-lactic acid secretion, which is in line with each other, followed by GAPDH inhibition. In conclusion, since L-lactic acid is the end product of anaerobic glycolysis in cancer cells, these data suggest that reduction of L-lactate secretion by AXP-3019 inhibitor in the direct consequence to GAPDH inhibition and glycolityc flux interrumption.

6.1.4 PDAC cell growth inhibition is caused by apoptotic and autophagic cell death

In the previous experiments, only two inhibitors proved to be particularly effective when tested the GAPDH activity inside cells and we decided to focus our attention only on AXP-3009 and AXP-3019 inhibitors. We used AXP-3018 as a control since it is chemically similar to these inhibitors but inactive *in vivo*, likely for its low cell membrane penetrance.

Since we demonstrated that cell proliferation inhibition was very high, we further investigated whether apoptosis was involved in PDAC cell growth inhibition after treatments. As shown in Fig. 29A, annexin V/FITC assay revealed that PDAC cells, after 48 hours AXP-3009 and AXP-3019-treatments at10 µM, showed a higher amount of phosphatidylserine in the external surface of cell membranes, as compared to AXP-3018 treatment or untreated control. Particularly, apoptotic cell death was significantly marked after AXP-3019 inhibitor treatment in both PDAC cell lines, about three and six times in PANC-1 and MIA PaCa-2, respectively, whereas AXP-3009 inhibitor caused significant apoptotic cell death only in MIA PaCa-2 with a three-fold change.

Similarly, autophagosome formation was determined using MDC assay with AXP-3018 and AXP-3019 inhibitors (10 µM), keeping AXP-3018 as negative control. After treating with AXP-3019, (Fig. 29B), we observed a strong increase in autophagosome formation confirming the data that PDAC cell growth inhibition is related to both apoptotic and autophagic events. Since autophagy can be considered a double edge sword having survival or cell death related features, we decided to test the effect of chloroquine (CQ), which acts as autophagy inhibitor in PANC-1 and MIA PaCa-2 (Fig. 29C, D). The cells were pre-treated with CQ and then treated with GAPDH inhibitors for 48 h. Since AXP-3018 remains as negative control, we did not observe much significant rescue in cell proliferation. On the contrary, using AXP-3019, we observed significant increase in cell proliferation with CQ pre-treatment, suggesting that CQ was able to block autophagy and rescue the cell proliferation, indicating that in our system autophagy induced by GAPDH inhibitors has cell death related properties.

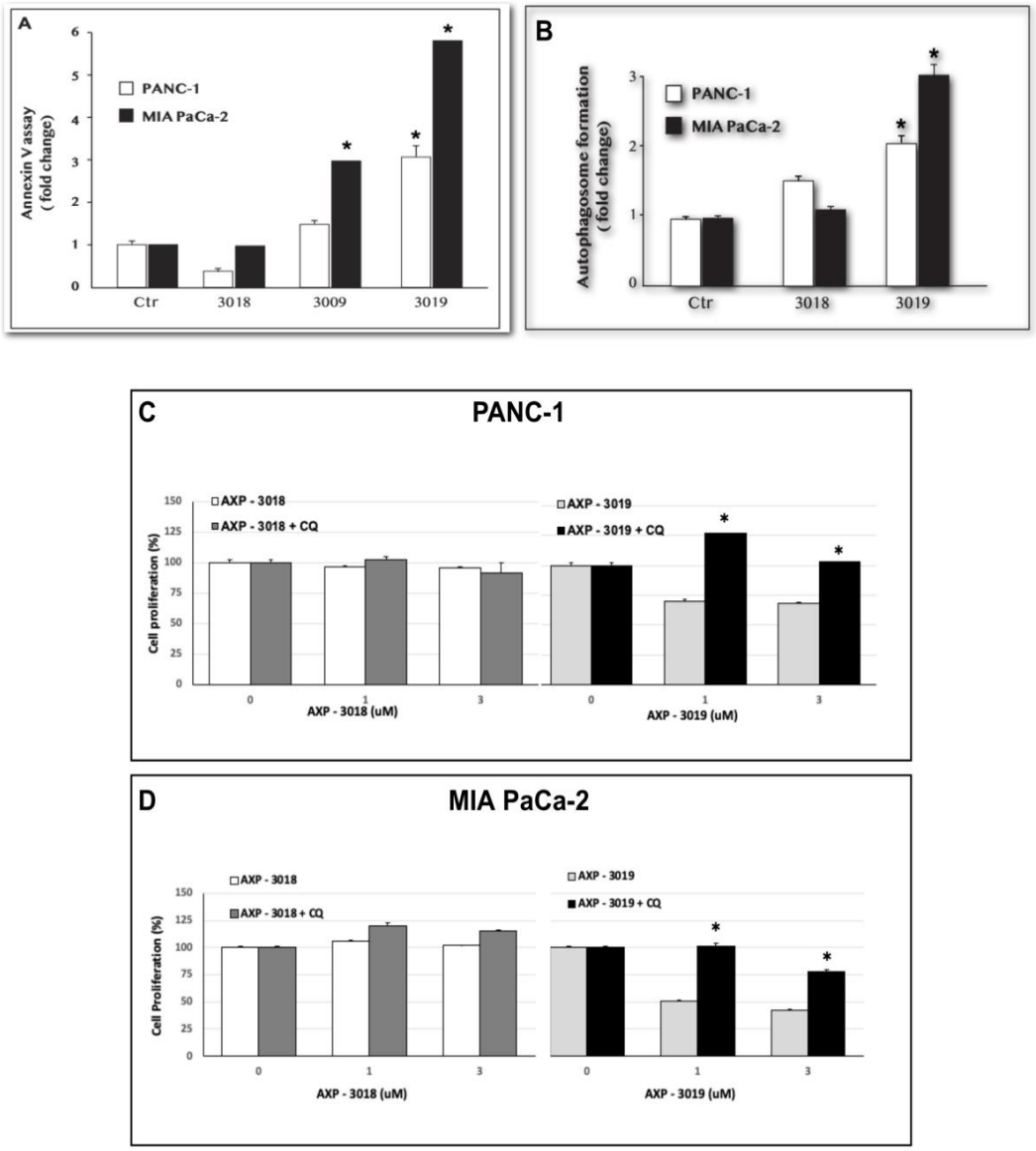

Figure 29: PDAC cell growth inhibition is caused by apoptotic and autophagic cell death. A) Apoptosis was determined using Annexin V assay **B)** Autophagy/autophagosome formation was determined using MDC assay. **C, D)** Chloroquine, which is an autophagy inhibitor, was used in PANC-1 and MIA PaCa-2 cell lines to inhibit autophagy recovering cell proliferation. t test-*p<0.01

6.1.5. AXP-3019 and GEM synergistically inhibited PDAC cell proliferation.

Furthermore, we investigated whether the most effective GAPDH inhibitor test, AXP-3019, may synergistically inhibit PDAC cell proliferation when used in combination with the standard drug GEM. The combination of GEM+AXP-3019

in equal molar ratio decreased cell proliferation stronger than with single drug treatment with GEM or AXP-3019 (Fig. 30A). About 70% decrease in cell proliferation was noted in PANC-1 and MIA PaCa-2 after treating with GEM + AXP-3019 at high concentrations (25 µM). By using CalcuSyn software, the synergistic effect of combination drugs of GEM and AXP-3019 were determined. The combination index (CI) values and the isobologram graphs obtained were plotted using the software and represented in Fig. 30B and C and Fig. 30D and E, respectively.

The fractional effect is the percentage of growth inhibition (0-1) obtained with each combination of the two drugs. CI values lower than 1.0, 0.7 or 0.3 indicate moderate synergism, synergism or strong synergism, respectively. Most of the values obtained were in the strong synergistic region, especially in the low drug concentration range, indicating that the combination of these two drugs can make a strong effect during the treatments, even reducing their dosage. The Table1 represents the values of CI_{25}, CI_{50},CI_{75}, DRI_{50}, which is Dose Reduction Index at 50% cell growth inhibition, and the linear correlation coefficient(r) for the median-effect plot. These data indicate that for the better treatment efficacy, the drug concentration can be reduced of 11.86-fold for GEM and 8.70-fold forAXP-3019 and of 15.61-fold for GEM and 20.94-fold for AXP-3019, in PANC-1 and MIA PaCa-2, respectively.

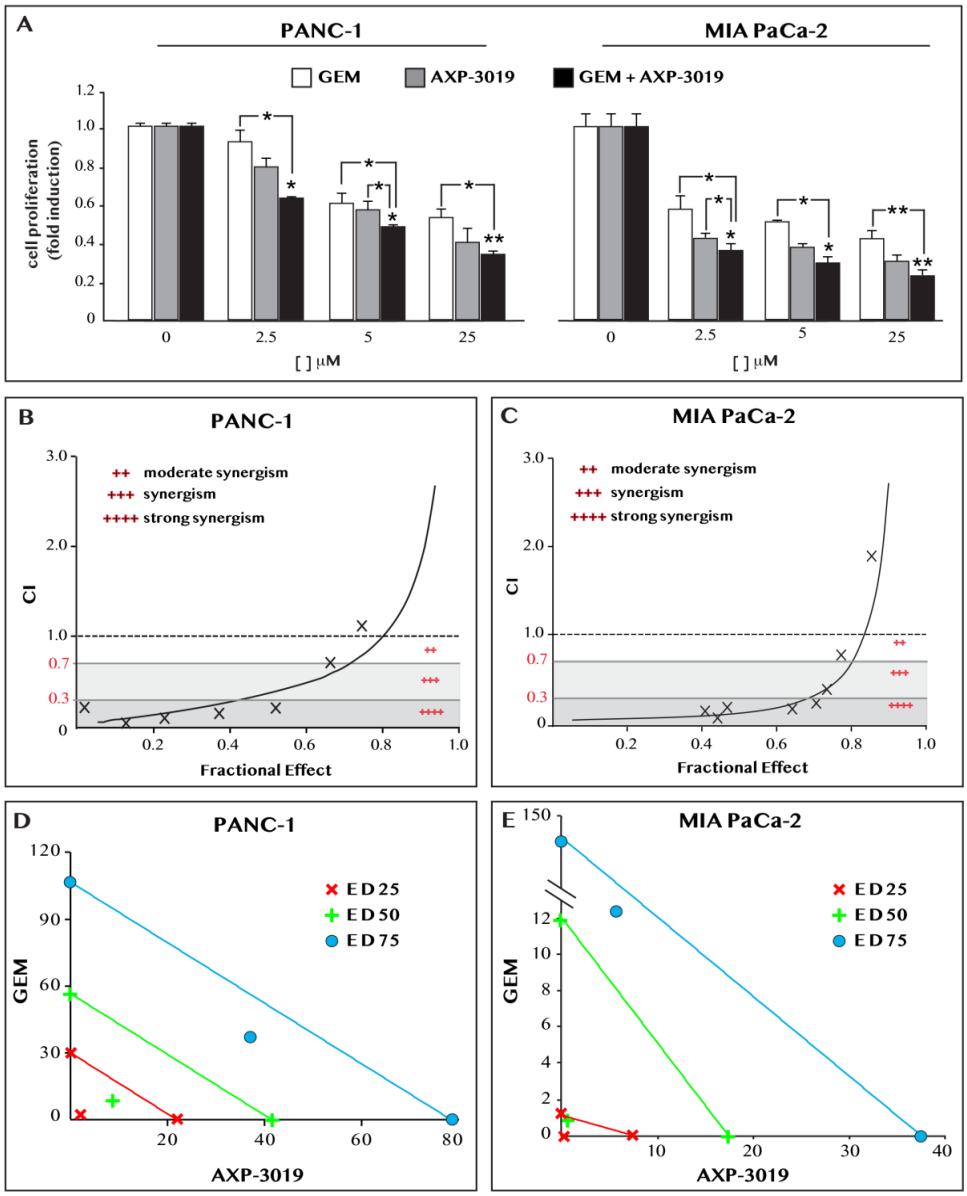

Figure 30: AXP-3019 and GEM synergistically inhibited PDAC cell proliferation: **A)** cell proliferation was determined by crystal violet assay after treating cells with GEM, AXP-3019, or AXP-3019+GEM in both PANC-1 and MIA PaCa-2 cell lines with increasing drug concentrations. The graph of the Combination index (CI) and the fractional effect were plotted by using the software CalcuSyn to show the level of synergism between GEM and AXP-3019 in **B)** PANC-1, and **C)** MIA PaCa-2 cells. Strong synergism was observed when PANC-1 and MIA PaCa-2 cells were treated with low concentrations of both drugs. Isobolograms were plotted from median-effect plot obtained by CalcuSyn and represented in **D)** PANC-1, and **E)** MIA PaCa-2 cells. t test-*p<0.01, **p<0.001

As reported in Table 2, using 1:1 (GEM:AXP-3019) molar ratio, we obtained three different CI values for PANC-1 and MIA PaCa-2: **(1)** CI_{25} (the CIs calculated for 25% cell growth inhibition by isobologram analysis) were respectively 0.164 and 0.052 for PANC-1 and MIA PaCa-2 cells; **(2)** CI_{50} values (the CIs calculated for 50% cell growth inhibition by isobologram analysis) were respectively, 0.366 and 0.111; **(3)** CI_{75} values (the CIs calculated for 75% cell growth inhibition by isobologram analysis) were respectively 0.817 and 0.433, further supporting the concept that the strongest synergism was observed at low drug concentrations.

Cells	Drugs	CI25	CI50	CI75	DRI_{50}	*r*
PANC-1	GEM + AXP-3019 (1:1)	0.164	0.366	0.817	GEM: 11.86 AXP-3019: 8.70	0.90
MIA PaCa-2		0.052	0.111	0.433	GEM: 15.61 AXP-3019: 20.94	0.98

Table 2: Representation table of data from CalcuSyn. The table represents the data obtained using CalcuSyn which shows CI_{25}, CI_{50},CI_{75}, DRI_{50} and *r* value, which confirm the efficiency of the combination of drugs.

6.1.6. AXP-3019 acts as anti-cancer inhibitor.

After these data on PDAC cell lines *in vitro*, we further investigated the effectiveness of GAPDH inhibitors *in vivo*. In the light of our results, we decided to use MIA PaCa-2 as cell line and AXP-3009 and AXP-3019 inhibitors for the treatment of nude mice.

During the 50 days of treatment, the body weight of the animals remained rather constant after various treatments, except some not significantly variations at the end of the treatment (Fig. 31A), suggesting that drugs did not induce apparent toxicity. On the contrary, the tumor volume graph highlights AXP-3019-treated mice have a smaller tumor volume, compared to untreated and AXP-3009-treated mice (Fig. 31B). The inefficiency of the AXP-3009 molecule to reduce tumor volume in mice is in contrast with cell culture results, demonstrating that the compound inhibits cell proliferation, but once injected into the animal, it may go toward other phenomena

or be inactivated or metabolized, counteracting its antitumoral effect and allowing the tumor progression.

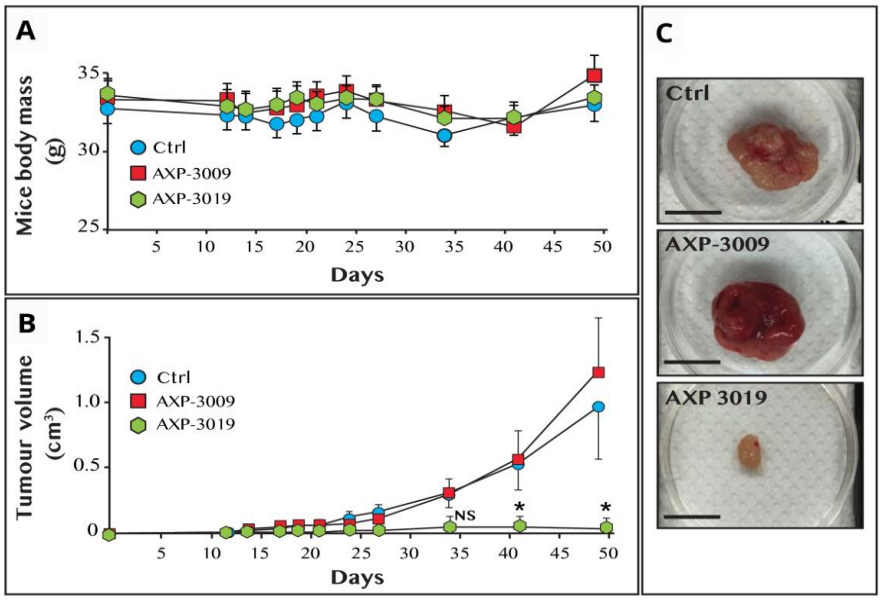

Figure 31: AXP-3019 acts as an anti-cancer inhibitor. *In vivo* studies were performed in mice on a continous treatament with inhibitors AXP-3009 and AXP-3019 for 50 days. Mice body masses **(A)** and the volumes of the tumors growing subcutaneosly **(B)** were measured during the experimental period (50 days). **C)** representative images of tumor mass obtained from mice after 50 days of treatment. AXP-3019 showed drastic decrease in tumor growth after treatment for 50 days. t test-*$p<0.01$

On the contrary, the results from the AXP-3019-treated mice are in line with cell proliferation data obtained with cell cultures, supporting the selection of AXP-3019 for further *in vivo* investigations. Fig. 31C shows some representative images from CTR- (top image), AXP-3009-treated- (middle image) and AXP-3019-treated mice (bottom image). The images reproduce the results obtained from the tumor volume graph. It is clear the AXP-3019-treated mice tumor mass is quite totally disappeared, supporting our hypothesis about the efficacy of this inhibitor as potential new chemotherapy drug based on metabolic features of cancer cells.

6.2 MITOCHONDRIAL DYNAMICS: A NOVEL INSIGHT TO TREAT PANCREATIC CANCER

6.2.1 Mutp53 induces fragmentation of mitochondria

In order to study the role of mutp53 protein in the regulation of mitochondrial dynamics, we modulated the p53 expression in PaCa3 and PANC-1 PDAC cell lines having WTp53 or mutp53 (R273H) , respectively, by using liposome mediated transient transfection assay. We stained mitochondria with Mitotracker probe, analysed the cells under confocal microscopy and classified mitochondria based on their morphology as fragmented, intermediate or elongated mitochondria (Fig. 32A).

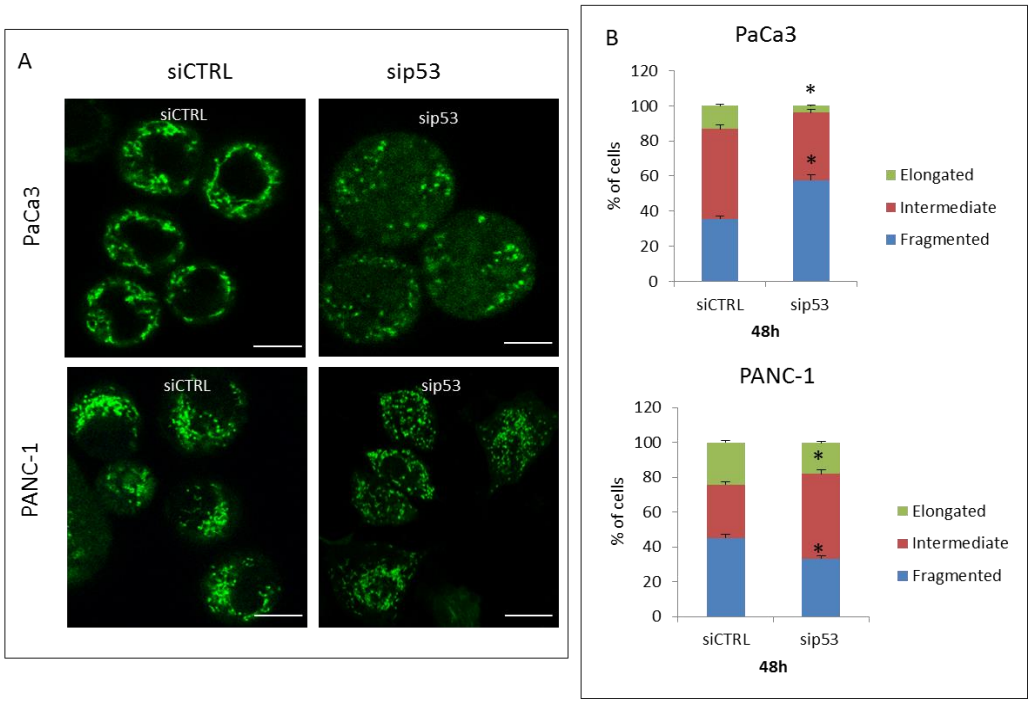

Figure 32: Analysis of mitochondrial morphology. **A)** Representative images of siRNA-CTRL and siRNA-p53 in PaCa3, and PANC-1 cell lines. **B)** Classification of mitochondrial morphology in fragmented, intermediate or elongated. PaCa3 and PANC-1 cells were transfected with siCTRL and sip53 for 48h. After transfection, cells were stained with Mitotracker probe and observed under confocal microscopy in order to classify fragmented, intermediate, or elongated mitochondria. The quantification of the percentage of cells in the three categories was performed in both siCTRL and sip53 conditions. Statistical analysis *$p < 0.05$. *Scale bar 20µm.*

When *TP53* gene is knocked-down, mitochondria in PaCa3 and PANC-1 cells shows an opposite behaviour. Fragmented mitochondria increased in PaCa3 cells silenced for WTp53cells, while, decreased in PANC-1 cells silenced for mutp53, indicating that WTp53 inhibited mitochondria fragmentation (fission) while mutp53 acquired the opposite function, such as the stimulation of mitochondria fragmentation. Consistent with this data, we observed a decrease of intermediate and significant decrease of elongated mitochondria in PaCa3 and a slight increase of intermediate mitochondria in PANC-1 cells (Fig. 32B). Representative images of fragmented, intermediate and elongated mitochondria are shown in Fig. 33 (A, B and C).

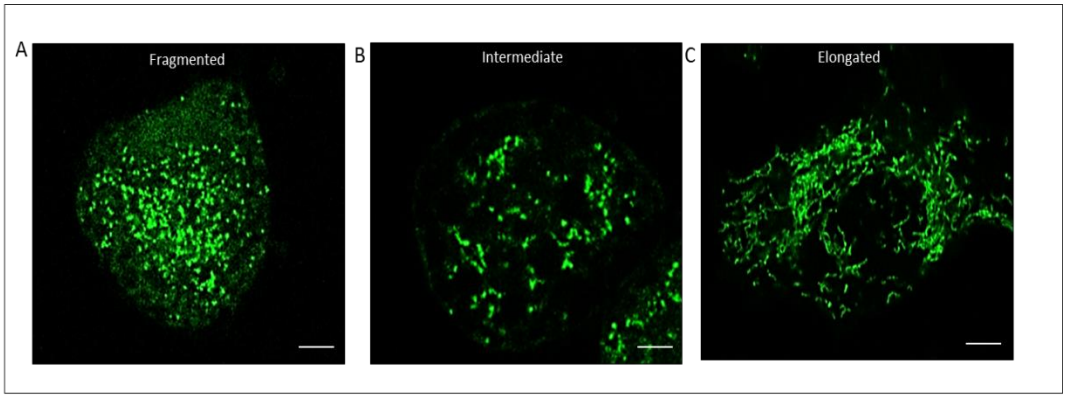

Figure 33: Representative images of mitochondrial morphology A) Fragmented **B)** Intermediate **C)** Elongated. PANC-1 cells were seeded and were acquired from confocal microscopy after 48h. *Scale bar 20µm.*

6.2.2 Mutp53 inhibits OPA1 expression

To study the functional role of mutp53 in the expression of mitochondrial dynamics genes, which includes DRP1, FIS1, MFN1/2, OPA1, we first analyzed mRNA expression level by modulating p53 levels in PaCa3, PANC-1 and AsPC-1 cells, which are WT, mut- or p53-null respectively, by liposome mediated transient transfection. p53 was knocked-down in PaCa3 and PANC-1 cells, while R273H and R175H-p53 mutant isoforms and WTp53 were over expressed in p53-null AsPC-1 cells.

When we knocked-down p53 gene in WTp53-PaCa3 cells, no significant changes were observed in DRP1, MFN1 and MFN2. A slight increase or decrease

in mRNA expression was noted in FIS1 and OPA1 respectively (Fig. 34A). After p53 knocked-down in mutp53-PANC-1, significant increase was noted in DRP1 and OPA1 and reduction in FIS1 (Fig. 34B). To strengthen these data, we over-expressed R273H and R175H p53 mutants and WTp53 in AsPC1 cells. In accordance with the results observed in PaCa3 and PANC-1, a significant decrease and increase in OPA1 expression was evident after over-expressing two different mutants and WTp53 respectively (Fig. 34C).

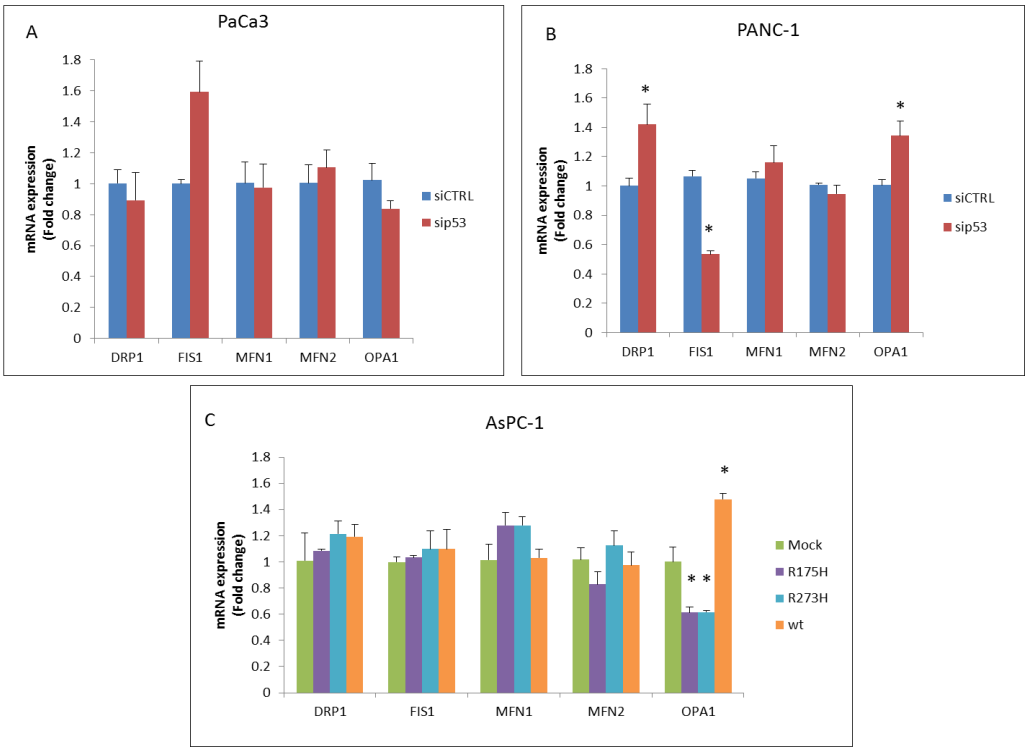

Figure 34: Mutp53 inhibits OPA1 expression. mRNA expression levels of mitochondrial dynamics genes were determined by Real Time-qPCR. PaCa3 **(A)** and PANC-1 **(B)** cells were transfected with siCTRL and sip53 while AsPC-1 cells **(C)** were transfected with mock, R175Hp53, R273Hp53 and WTp53 in order to overexpress respective p53 isoforms. After 48 h, RNA was extracted and qPCR was performed. Paca3 and PANC-1 gene expressions were compared with respective siCTRL. In AsPC1 cell line, the other conditions were compared with mock. Statistical analysis $*p < 0.05$.

As a control, the mRNA expression levels of p53 gene after its knocked–down in PaCa3 and PANC-1, over expression of mutants and WTp53 in AsPC1 were represented in Fig. 35B and C. To support the mRNA expression data, protein expression was confirmed by western immunoblotting. The proteins OPA1, DRP1,

P-DRP1 (Ser-616), MFN1/2 and p53 were blotted and normalized by GAPDH (Fig. 36A, B and C). After the knockdown of p53, OPA1 expression was decreased and increased in PaCa3 and PANC-1, respectively. This was confirmed by overexpressing the p53 isoforms (R175H, R273H and WT). After overexpression of mutants in ASPC-1 cells, the expression level was decreased and WTp53 showed an increase in expression of OPA1, which confirms the data observed in PaCa3 and PANC-1. As stated, OPA1 protein expression levels were in accordance with the mRNA expression levels and confirms that mutp53 inhibits OPA1 expression.

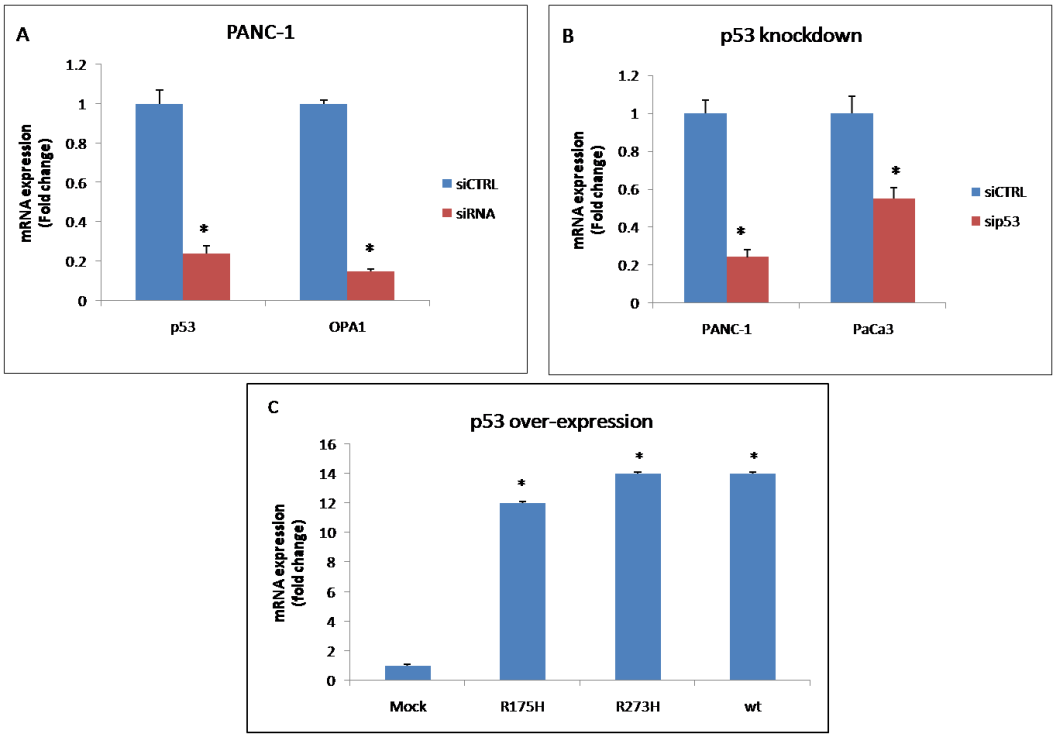

Figure 35: mRNA expression of A) p53 and OPA1 knock-down. PANC-1 cells were transfected with respective siCTRL, sip53, and siOPA1 for 48h. **B)** p53 knockdown. PANC-1 and PaCa3 cell lines were transfected with respective siCTRL and sip53 for 48 h. **C)** Over-expression of p53 in AsPC-1 cell line. Cells were transfected with plasmids mock, R175H, R273H and WTp53 for 48 h. RNA was extracted and qPCR was performed. In PaCa3 and PANC-1 gene expression was compared with respective siCTRL. In AsPC-1, over expression of p53 was compared with mock. Statistical analysis $*p < 0.05$

Concerning the MFN1/2, there were no significant differences in the expression levels. Total DRP1 protein expression was in accordance with its mRNA expression levels. A significant increase of expression level can be seen in PANC-

1 after knocked-down of mutp53, which was further confirmed by over expression of R175H and R273H mutants in AsPC-1 cells.

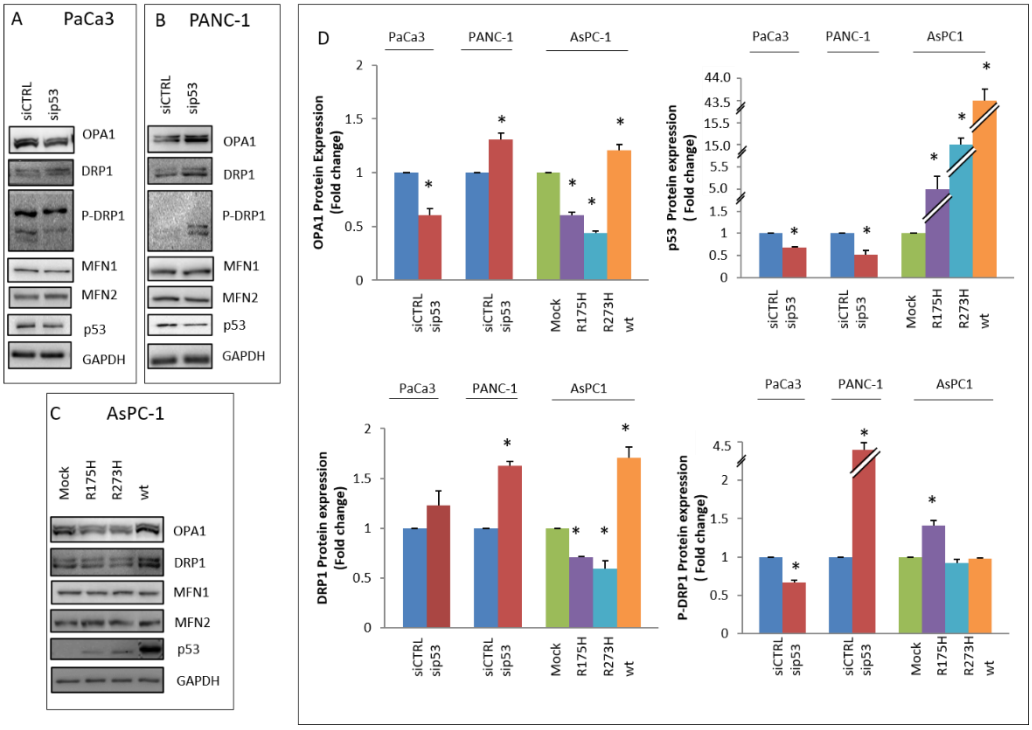

Figure 36: Western Immunoblotting. Whole cell protein extracts from **A)** PaCa3 and **B)** PANC-1and **C)** AsPC-1 were quantified and probed with indicated antibodies. PaCa3 and PANC-1 cells were transfected with siCTRL and sip53, AsPC-1 cells transfected with plasmids for over expression of R175H, R273H mutp53, WTp53 or its negative control (mock). GAPDH was used as a control of equal protein loading. **D)** Analysis of OPA1, p53, DRP1, P-DRP1(Ser-616). Each band was quantified using NIH Image J software and normalized to the amount of GAPDH. Protein expression of PaCa3 and PANC-1 cell lines was compared with respective siCTRL. In AsPC-1, over expression of plasmids was compared with the mock. Statistical analysis $*p < 0.05$.

A no significant increase was observed in DRP1 protein expression after knocked-down in PaCa3 cells. Phosphorylation of Ser616 was observed and notable decrease and increase in phosphorylation was observed in PaCa3 and PANC-1 respectively after knocked-down.

Quantification of western immunoblotting which includes OPA1, p53, DRP1 and P-DRP1(Ser616) are represented in Fig. 36D. In addition, the

representative knocked-down expression of p53 in PaCa3 and PANC-1 cells, over expression of mutants and WTp53 in AsPC-1 cells Fig. 37A.

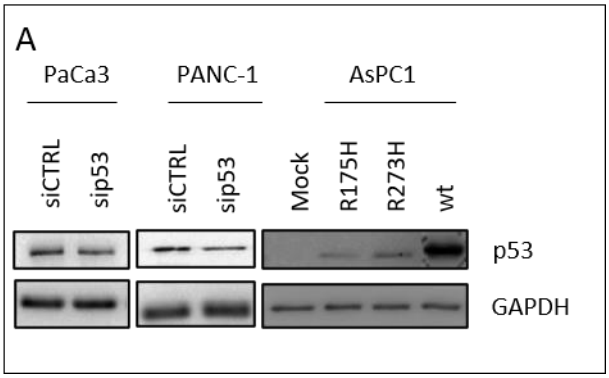

Figure 37: Protein expression of p53. A) Western immunoblotting analysis for p53 expression after knockdown in PaCa3 and PANC-1 cells, and after over expression of mutants and WT in AsPC-1 cells. In PaCa3 and PANC-1 cells protein expression was compared with respective siCTRL. In AsPC-1, over expression of p53 was compared with mock.

6.2.3 Double knocked-down of p53 and OPA1 recovers the numbers of fragmented mitochondria

To investigate the role of OPA1 inhibition by mutp53, we observed that OPA1 and mutp53 double knocked-down in PANC-1 cells was able to recover the percentage of cells with fragmented mitochondria. The representative images of siCTRL, sip53, siOPA1, and sip53+siOPA1 knocked-down are shown in Fig. 38A. Fragmented, intermediate and elongated mitochondria can be seen in siCTRL condition and intermediate can be observed in sip53. Significant decrease in number of cells with more fragmented mitochondria is observed after knocked-down of p53. After the knocked-down of OPA1, about 80% of cells have fragmented mitochondria confirming that OPA1 is involved in fusion.

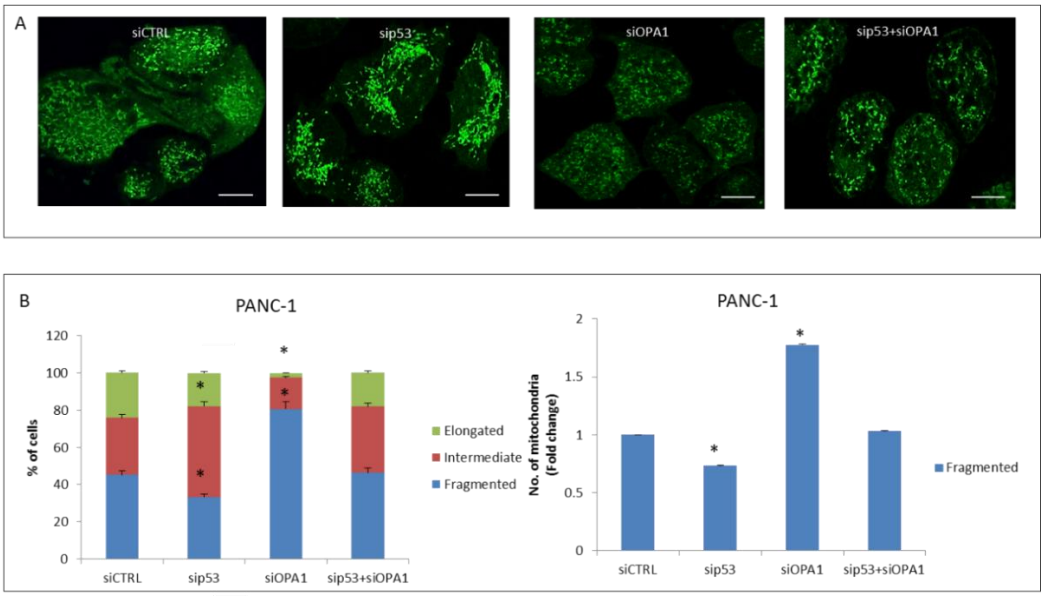

Figure 38: Representative images and quantification of confocal microscopy in PANC-1. PANC-1 cells were transfected with siCTRL, sip53, siOPA1, and co-transfected with sip53+siOPA1 for 48h in order to visualize the effect of the silencing of these genes in mitochondrial dynamics. **A)** Representative confocal images from siCTRL, sip53, siOPA1, sip53+siOPA1 samples. **B)** Left panel, quantification of cell number shows elongated, intermediate and fragmented mitochondria for each analyzed sample; right panel, quantification of cell number shows only fragmented mitochondria, where number of fragmented mitochondria was recovered after co-transfection with sip53+siOPA1 in comparison with siOPA1. Fragmented, intermediate and elongated mitochondria of sip53, siOPA1 and sip53+siOPA1 were compared with the respective of siCTRL sample. Statistical analysis *$p < 0.05$. *Scale bar 20µm.*

The number of fragmented mitochondria was recovered after sip53+siOPA1 knocked-down. Statistical analysis of morphology of mitochondria is represented in Fig. 38B. The figure shows the number of cells having fragmented, intermediate and elongated mitochondria, as compared to control. Also, the figure represents that the number of cells with fragmented mitochondria after mutp53 silencing was recovered double knocked down of p53 and OPA1. This indicates that the inhibition of OPA1 mRNA and protein expression by mutp53 has a role in the stimulation of mitochondrial fragmentation by mutp53.

6.2.4 Inhibition of OPA1 by mutp53 induces cell growth and inhibits apoptosis

In order to identify the functional role of OPA1 in cell growth and apoptosis cell death, we demonstrated that double knocked-down of OPA1 and p53 was able to recover the effect on apoptosis and cell growth compared with sip53. We demonstrated that cell growth inhibition by mutp53 was recovered after double knocked down of p53 and OPA1 (Fig. 39A). An increase in annexin V/FITC fluorescence signal has been observed after p53 knocked-down demonstrating that mutp53 is able to inhibit apoptosis as clearly demonstrated in our previous studies[219][220]. From our results, OPA1 induces apoptosis and it has been demonstrated by the reduction of annexin V/FITC signal after OPA1 silencing (Fig. 39B).

We studied the effect of p53 and OPA1 knocked-down, thereby significantly recovering apoptosis when compared to sip53 (Fig 39B). mRNA expression of p53 + OPA1 knocked-down is represented in Fig 35A.

These results clearly demonstrate that inhibition of OPA1 by mutp53 is a novel mechanism by which the hot-spot mutant isoforms of p53 expressed in many cancers can promote their oncogenic properties triggering mitochondrial fragmentation and counteracting apoptotic stimuli. Further metabolic features of these regulations need to be further investigated *in vitro* and *in vivo*.

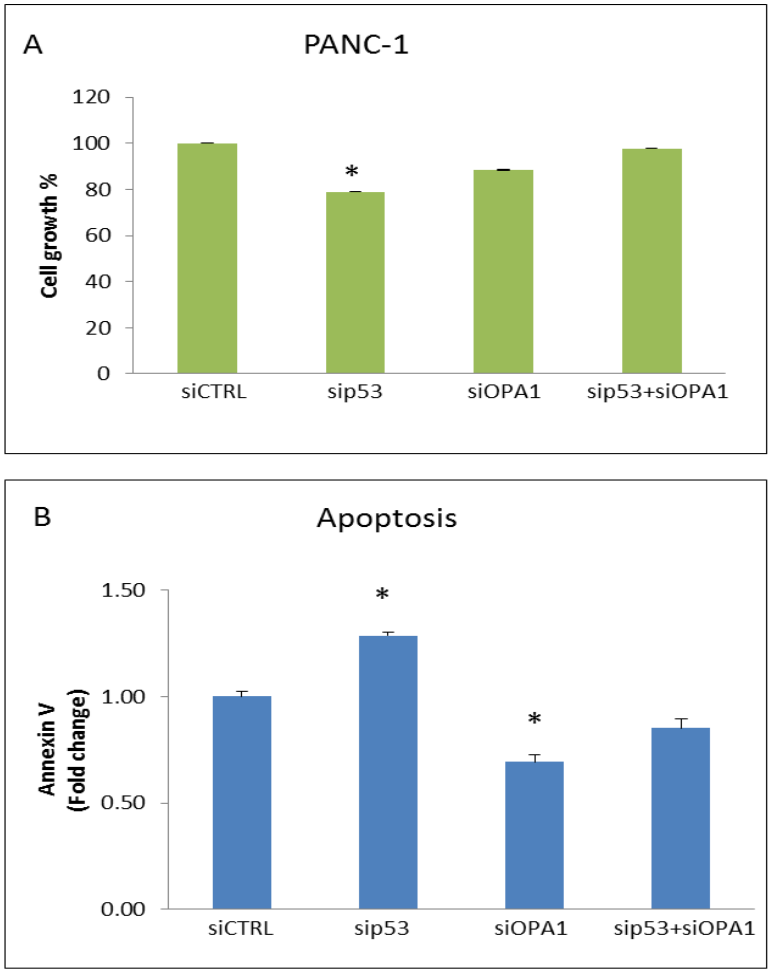

Figure 39: **Inhibition of OPA1 by mutp53 induces cell growth and work towards anti apoptosis effect. A)** Cell growth in PANC-1 cells was measured by Crystal Violet assay after 48 h of indicated treatment. **B)** Apoptosis in PANC-1 cells was determined by the Annexin V/FITC binding assay. Cells were transfected with respective siRNAs and were compared with siCTRL. Statistical analysis *$p < 0.05$.

7. DISCUSSION AND CONCLUSIONS

7.1 GAPDH regulation: new insight to treat PDAC

Pancreatic cancer being one of the most frequent causes of tumor-associated deaths, and its incidence has recently increased in the whole world. 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%. 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%. Therefore, the identification of effective targets and novel therapeutic strategies to improve GEM effects in PDAC has been the topic of extensive investigation in the last few years. PDAC presents genetic heterogeneity with a high number of mutations. Among the various mutations, the *TP53* gene is found altered in about 25-70% of PDAC patients [221].

The main role of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) enzyme is in the ubiquitous process of glycolysis, by which it catalyzes the phosphorylation and oxidation of glyceraldehyde-3-phosphate to 1,3-biphosphoglycerate (G-3-P), using NAD^+ as the electron acceptor. The glycolytic function mainly relies on key amino acids which include Cys152 and His179. GAPDH, being a moonlight protein has various subcellular localizations associated with different functions, which includes the regulation of cell growth and proliferation. GAPDH was used as target for various disorders and diseases until now [222][223]. A growing interest in the study of aerobic glycolysis in cancer (the Warburg Effect) as a key mechanism for cancer cell energetic metabolism, favouring tumor progression and invasion, has contributed to consider GAPDH an effective drug target to specifically hit cancer cells. In this study, we have investigated the anti-proliferative effect of a panel of new synthesized compounds, specifically designed to bind and inhibit GAPDH enzyme, in pancreatic ductal cancer cells (PDAC) and normal fibroblasts.

In this context, therefore, the interest of GAPDH is of particular importance, in order to isolate a molecule capable of reducing or blocking the activity of this enzyme as therapy against pancreatic cancer keeping the activity of non-cancer cells unchanged. To this end, Prof. Paola Conti (University of Milan) in collaboration with Prof. Stefano Bruno (University of Parma) have recently identified a new scaffold of covalent 80 inhibitors based on the 3-Br-isoxazoline group that are active towards GAPDH of *Plasmodium falciparum* (*Pf*GAPDH). In the present study, we tested some of these inhibitors in PADC cells in order to identify the best inhibitors able to bind and inhibit human GAPDH, thus blocking the glycolytic pathway leading cancer cells to death.

We have observed that all tested compounds inhibited the activity of the human recombinant GAPDH enzyme *in vitro*. However, into the cells, the drugs have shown different behaviour: GAPDH activity and cell viability assays have confirmed that only the compounds able to inhibit GAPDH activity inside the cells can also exert an anti-proliferative effect, suggesting that drug entrance into the cells could be a limiting event. Interestingly, compounds affecting cancer cells are not effective on both GAPDH activity and cell proliferation in normal fibroblasts. The overall results support the hypothesis that the GAPDH inhibition alters the glycolytic metabolism, favouring cancer cell death without affecting normal cells, suggesting new strategies to identify the contexts in which glycolysis targeted agents might be most effective. Among the novel GAPDH inhibitors tested, AXP-3019 was the most active compound able to inhibit GAPDH enzyme *in vitro* and *in vivo*. In Table 3 shown below, it emerges that AXP-3019 can reduce the *h*GAPDH *in vitro* activity, intracellular GAPDH activity in cancer cells, L-lactic acid secretion and cancer cell proliferation in both PANC-1 (GEM-resistant) and MIA PaCa-2 (GEM-sensitive) cell lines. In addition, this compound can strongly reduce tumor mass in nude mice without apparent toxicity and to synergistically inhibit PDAC cell proliferation in combination with the standard chemotherapeutic agent GEM.

Inhibitor	In vitro Activity	Intracellular GAPDH activity			L-Lactic acid (%)		Cell proliferation(10 µM-48h)					Mice
		Fibroblast	PANC-1	MIA PaCa-2	PANC-1	MIA PaCa-2	Fibroblast	PANC-1		MIA PaCa-2		
								P	CSC	P	CSC	
AXP-1007	=	=	=	=	nd	nd	=	=	nd	=	nd	nd
AXP-3009	↓	=	↓	↓	↓	↓	=	↓	nd	↓	nd	=
AXP-3018	↓	=	=	=	=	=	=	=	↓	=	↓	nd
AXP-3019	↓	=	↓	↓	↓	↓	=	↓	↓	↓	↓	↓

Table 3: Table showing the various effects of selected GAPDH inhibitors in PANC-1 and MIA PaCa-2 cells.

7.2 Mitochondrial Dynamics Regulation: New insight to treat PDAC having TP53 mutant gene

Regarding mitochondrial dynamics, it is important to state that these phenomena play a major role in regulating mitochondrial functionality and cell viability, by bringing the fission and fusion to a dynamic equilibrium. In addition, alteration of the mitochondrial dynamics has been found to be involved in different oncogenic properties of the cells. The contribution of mitochondrial dynamics in tumor initiation and progression has been studied from long time, although the correct explanation of cancer progression through dynamics has not been understood yet [190]. In human pancreatic cancer, expression of oncogenic Ras or activation of MAPK pathway leads to Erk2 mediated phosphorylation of Drp1 on Ser-616 that leads to mitochondrial fragmentation and it's worth noting that inhibition of this phosphorylation results in decrease of tumor growth in xenograft cancer models [194][195]. Furthermore, it is emerging that mutp53 proteins, contrarily to their wild-type p53 counterpart, has major roles in activating or inhibiting some crucial mitochondrial regulatory genes [224][225]. Our data show that mutant p53 favours fragmentation of mitochondria as observed after knocking down of mutp53 in PANC-1, contrary to WTp53, as represented in fig 30. Indeed, mutp53 inhibits OPA1 expression at both mRNA and protein level (Figg. 34, 36). Also, the number of fragmented mitochondria, which was recovered after the

double knock-down of p53 and OPA1, confirms the role of OPA1 in mutp53 patients (Fig. 36). Further, this inhibition of OPA1 by mutp53 has roles in improving cell growth and in anti-apoptotic stimuli, as shown in fig. 37. This clearly suggests that overexpression of OPA1 in mutp53 bearing patients could retrieve the oncogenic properties and sensitize cancer cells to apoptotic stimuli and inhibition of cell growth. In the scientific literature, it has been reported that besides the already mentioned role of OPA1 on mitochondrial dynamics, this protein can also perturb the mitochondrial inner membrane structure and integrity, leading to cytochrome *c* release and apoptosis [226]. Further studies should be performed to further confirm the role of OPA1 inhibition by mutp53 in regulating apoptosis and alteration of mitochondrial metabolism in PDAC cells.

In conclusion, it maybe hypothesized that the selective inhibition of the glycolytic pathway can potentially be an effective therapy for pancreatic tumor by targeting the Warburg Effect, which is supported by the presence of mutp53 proteins with oncogenic functions. In addition, the counteraction of the mutp53-dependent inhibition of OPA1 and of the regulation of mitochondrial dynamics induced by mutp53 may become a potential novel strategy for treating pancreatic cancer bearing mutp53 in patients, as schematically reported in fig. 39.

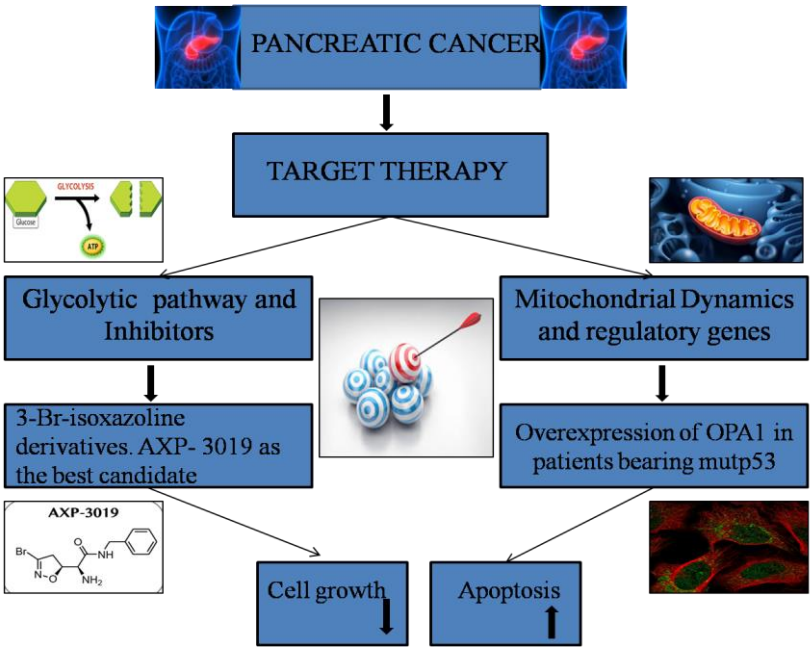

Figure 39: Representative summary of the data described in the thesis.

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9. ANNEXES

During the period of my PhD, I studied the mitochondrial dynamics involved in the pancreatic cancer bearing mutant p53 gene. I focused my attention on studying the molecular mechanism induced by mutp53. Specifically, I unveiled the relationship between mutp53 and mitochondrial dynamics. A part of the main project was carried out for three months in the Lab of translational oncology under the guidance of Prof. Jordi Oliver and Prof. Roca Pilar and I had won grant named mobilita Internazionale -2019, from University of Verona for the same. A subsequent phase of my research also has an ongoing project which focuses on the attention on GAPDH inhibition, an effective strategy against tumor proliferation. During PhD, I presented my projects in various international and national conferences and I collaborated for the following publications:

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, and Massimo Donadelli. BBA- Molecular Cell Research, *Biochim. Biophys. Acta - Mol. Cell Res., 2018*, 1865: 1914–1923 https://doi.org/10.1016/j.bbamcr.2018.10.005.

2) 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 and Massimo Donadelli, *Biol. Rev.*, 2019, 000-000, doi: 10.1111/brv.12513

3) Regulation of autophagy by nuclear GAPDH and its aggregates in cancer and neurodegenerative disorders, Giovanna Butera, **Nidula Mullappilly**, Francesca Masetto, Marta Palmieri, Maria Teresa Scupoli, Raffaella Pacchiana, Massimo Donadelli, International Journal of Molecular Sciences , *Int. J. Mol. Sci.* ,2019 20: 2062, 1–17, doi:10.3390/ijms20092062

4) Mutant p53 induces SIRT3/MnSOD axis to moderate ROS production in melanoma cells, Margalida Torrens-Mas, Marco Cordani, **Nidula Mullappilly**,

Raffaella Pacchiana, Chiara Riganti, Marta Palmieri, Daniel G. Pons, Pilar Roca, Jordi Oliver, Massimo Donadelli, *Arch. Biochem. Biophys.* ,2020, 679:108219, https://doi.org/10.1016/j.abb.2019.108219

5) Mutant p53-associated molecular mechanisms of ROS regulation in cancer cells, Cordani Marco, Butera Giovanna, Pacchiana Raffaella, Masetto Francesca, **Mullappilly Nidula**, Chiara Riganti, Donadelli Massimo, *Biomolecules 2020*, 10;361, 1–22, doi:10.3390/biom10030361

6) MRP5 nitration by NO-releasing gemcitabine encapsulated in liposomes confers sensitivity in chemoresistant pancreatic adenocarcinoma cells, Francesca Masetto, Konstantin Chegaev, Elena Gazzano, **Nidula Mullappilly**, Barbara Rolando, Silvia Arpicco, Roberta Fruttero, Chiara Riganti, Massimo Donadelli, *Biochim. Biophys. Acta - Mol. Cell Res., 2020,* 1867: 118824 https://doi.org/10.1016/j.bbamcr.2020.118824.

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