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Mutant p53 proteins alter signaling pathways involved in autophagy and redox regulation in cancer cells

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TABLE OF CONTENTS

1. ABSTRACT

Mutations in the *TP53* gene occur in over 50% of the human cancers and most of them are missense mutations that result in the expression of mutant forms of p53. In addition, p53 mutated proteins acquire new biological properties referred as gain-of-function (GOF) that contribute to the induction and maintenance of cancer. Autophagy is an intracellular degradative process by which damaged macromolecules and organelles are targeted to lysosomes via autophagic vesicles and it is crucial to maintain primary biological activities during cellular stresses, such as nutrient starvation. Reactive oxygen species (ROS) are highly reactive byproducts of mitochondrial oxidative phosphorylation and are implicated in a plethora of biological events addressed to sustain each aspect of human cancer being able to act as second messengers in cellular signaling. The aim of this thesis was to dissect the molecular mechanisms by which oncogenic mutant p53 proteins promote cancer cell proliferation and chemoresistance in cancer cells by altering crucial signaling pathways involved in autophagy and redox homeostasis. We unveiled that GOF mutant p53 proteins, contrarily to its wild-type p53 counterpart, inhibit the autophagic pathway and enhance mitochondrial ROS in cancer cells, leading i) antiapoptotic effects, ii) proliferation and iii) chemoresistance of pancreas and breast cancer cells. We found that mutant p53 significantly counteracts the formation of autophagic vesicles and their fusion with lysosomes throughout the repression of some key autophagy-related proteins and enzymes with the concomitant stimulation of mTOR signaling. Consequently to the deregulation of AMPK signaling, the expression of its effector PGC-1α was also affected, driving a reduction of the antioxidant UCP2 protein expression and an increase of mitochondrial superoxide that acts as a critical mediator of oncogenic proprieties of mutant p53. As a paradigm of this mechanism, we showed that *atg12* gene repression was mediated by the recruitment of the p50 NF-kB/mutant p53 protein complex onto two regions of the *atg12* promoter suggesting the involvement of the p50-p50 homodimer as a transcriptional repressor of mutant p53 target genes. We have further correlated the low expression levels of the autophagic genes (*atg12, becn1, sesn1,* and *dram1*) with reduced relapse free survival (RFS) and distant metastasis free survival (DMFS)

of breast cancer patients carrying *TP53* gene mutations conferring a prognostic value to this mutant p53- and autophagy-related signature. Intriguingly, we demonstrated that mutant p53-driven mTOR stimulation, beyond its role on autophagy repression, sensitize cancer cells carrying mutant *TP53* gene to the treatment with the mTOR inhibitor everolimus. The data reported in this thesis reveal novel mechanisms by which mutant p53 sustains tumor progression and lightened on the importance that play the redox cellular status and autophagy regulation in the human tumors carrying oncogenic mutant p53 proteins.

2. SOMMARIO

Mutazioni nel gene *TP53* occorrono in oltre il 50% dei tumori umani e la maggior parte di loro sono mutazioni missenso che causano l'espressione di isoforme mutanti di p53. Inoltre, proteine p53 mutanti acquisiscono nuove proprietà biologiche chiamate gain-of-function (GOF) le quali contribuiscono alla sviluppo della patologia neoplastica. L'autofagia è un processo degradativo intracellulare attraverso il quale macromolecole e organelli citosolici danneggiati sono trasferiti ai lisosomi attraverso vescicole autofagiche. L'autofagia è un fenomeno biologico cruciale nel mantenere una corretta attività biologica durante vari stress cellulari, in particolare la deprivazione di nutrienti. Le specie reattive dell'ossigeno (ROS) sono sottoprodotti del metabolismo cellulare altamente reattivi ed agendo come secondi messaggeri nel "signaling" intracellulare sono implicati in una vastità di eventi che promuovono la tumorigenesi e la progressione tumorale. Lo scopo di questa tesi è stato quello di identificare i meccanismi molecolari attraverso i quali proteine p53 mutanti promuovono la proliferazione e la chemioresistenza delle cellule tumorali in seguito ad un'alterazione di vie di segnalazione intracellulari coinvolte nella regolazione nell'autofagia e dello status redox delle cellule. In seguito ai nostri studi abbiamo scoperto che la GOF di proteine p53 mutanti, a differenza alla loro controparte "wild-type", inibisce il processo autofagico ed incrementa le ROS mitocondriali nelle cellule tumorali, risultando in un effetto antiapoptotico, iperproliferativo e di chemioresistenza in cellule tumorali di pancreas e mammella. Abbiamo dimostrato che la proteina p53 mutante contrasta in maniera significativa la formazione di vescicole autofagiche e la loro fusione con i lisosomi attraverso la repressione di alcune proteine ed enzimi chiave del processo autofagico e promuove concomitantemente la stimolazione del "signaling" di mTOR. Come conseguenza della disregolazione del "signaling" dell'AMPK, anche l'espressione del suo effettore PGC-1α risulta inibita, provocando una riduzione dell'espressione della proteina disaccoppiante mitocondriale UCP2, la quale ha una nota funzione antiossidante. Questo causa un incremento di superossido mitocondriale, il quale funge da mediatore chiave delle proprietà oncogeniche della proteina p53 mutante. Come paradigma di questo

meccanismo, abbiamo mostrato che la repressione del gene *atg12* dipende dal reclutamento del complesso proteico p50 NF-kB/p53 mutante su due regioni del promotore di *atg12* stesso suggerendo il coinvolgimento dell'omodimero p50-p50 come repressore della trascrizione di geni target di p53 mutante. In seguito, abbiamo correlato i bassi livelli di espressione di alcuni geni autofagici (*atg12, becn1, sesn1,* and *dram1*) con la ridotta sopravvivenza libera da malattia (RFS) e libera da metastasi distanti (DMFS) di pazienti affetti da tumore al seno che esprimono la mutazione nel gene *TP53*, la quale conferisce un forte valore prognostico ed una firma molecolare autofagica associata all'espressione di p53 mutante. Al fine di identificare un potenziale approccio terapeutico per i pazienti con neoplasie che esprimono p53 mutante, abbiamo dimostrato che la stimolazione della via di "signaling" mTOR indotta da p53 mutante, oltre a reprimere il macchinario autofagico, sensibilizza anche le cellule tumorali al trattamento con everolimus, un inibitore farmacologico di mTOR. Nel complesso, i dati riportati in questa tesi rivelano nuovi meccanismi con cui p53 mutante sostiene la progressione tumorale e sottolineano l'importanza che riveste lo status ossidativo e la regolazione dell'autofagia nei tumori umani che esprimono proteine oncogeniche p53 mutanti.

3. INTRODUCTION

Cancer is a complex set of diseases characterized by drastic alterations in the cell metabolism and by dynamic changes in the genome that allow the cells to bypass the regulatory pathways that orchestrate normal cell proliferation and tissue homeostasis. However, despite many individual differences, all human tumors share common features, such as i) self-sustained proliferative abilities, ii) insensitivity to growth inhibitors and apoptosis, iii) unlimited replicative potential, iv) sustained angiogenesis, and v) the capability to invade surrounding tissues and metastasize (Hanahan and Weinberg, 2000). Importantly, in addition to these features, human tumors may take advantage from an inflammatory microenvironment (Mantovani, 2009), and by reprogramming cellular metabolism to sustain deregulated cell proliferation (Hanahan and Weinberg, 2011). From a molecular point of view, tumourigenesis is a multistep process characterized by a gradual modification of normal cells into tumours cells and requires specific alterations in various cellular processes. In general, these alterations occur in proto-oncogenes or in tumour suppressor genes. In the first group there are genes like Ras, Myc, Src and erbB2, which once mutated display an enhanced and uncontrolled expression and/or activity, thus promoting growth and survival of cancer cells. Conversely, tumours need to inactivate tumour suppressor genes like p53, Rb or PTEN that would be physiologically activated in stress conditions to limit cellular proliferation and survival. (Hanahan and Weinberg, 2000).

3.1 TUMOR SUPPRESSOR p53: THE GUARDIAN OF THE GENOME

One of the most important signaling pathways that counteract tumour formation and progression is the p53 tumour suppressor pathway. P53 is a transcription factor, with a modular structure formed by distinct functional domains: i) N-terminal transactivation domain (amino acid aa 1-73), interacting with the transcriptional machinery (Unger, 1993); ii) proline reach domain (aa 63-97), required for p53 stabilization; iii) DNA binding domain (aa 93-312), that recognizes and binds the responsive element on DNA and proteins that positively or negatively affect p53 activity, such as MDM2 or 53BP1 respectively (el-Deiry,

1992); iv) oligomerization domain (aa 325-355), essential for tetramer formation that represents the active form of p53 (Shieh, 1999) and v) C-terminal regulatory domain, containing residues post-translationally modified involved in modulation of its stability (Kruse and Gu, 2009) (figure 1).
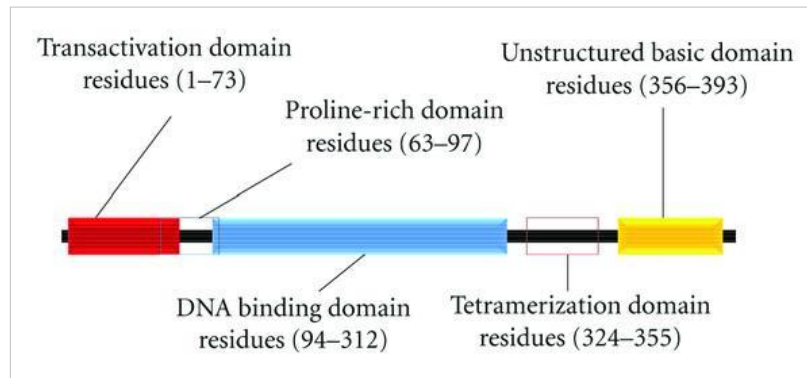
Figure 1. **Multifunctional domains of p53.** The p53 monomer consists of various multifunctional domains (Robbins and Zhao 2011).

After more than thirty years of researches, extensive studies envision p53 at the centre of a highly interconnected network that conveys and transduces biochemical signals deriving by stress conditions. These signals may strongly compromise genomic stability and promote neoplastic transformation and can originate from external factors, such as γ-rays, UV light, DNA damaging agents or chemotherapeutic drugs, as well internal ones, like oncogene activation, high levels of reactive oxygen species (ROS), or nutrient deprivation (Levine and Oren, 2009). In response to these stresses p53 becomes stabilized and activated, events that are governed by a refined combination of post-translational modifications and interacting protein partners (Kruse and Gu, 2009). (figure 2).
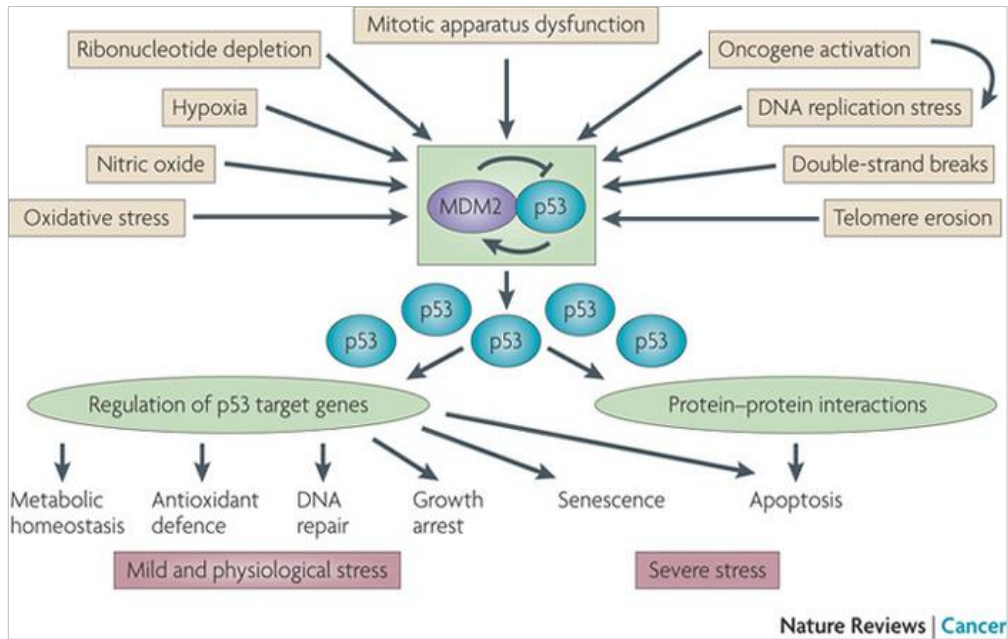
Figure 2. **The p53 pathway.** The p53–MDM2 feedback loop is the "hub" of the p53 pathway. Under normal conditions, it maintains constantly low steady-state p53 levels and activity. Various stress signals, related in many ways to carcinogenesis, impinge on this central loop to release p53 from MDM2-mediated inhibition. This increases p53 protein levels and activity, inducing drastic phenotypic changes (Levine and Oren, 2009)

One of the most crucial events that occur to p53 following several injuries is its escape from the ubiquitination by its major E3 ubiquitin ligase MDM2 and the subsequent degradation by the 26S proteasome (Haupt, 1997; Kubbutat, 1997). Once activated, p53 acts essentially as a transcription factor and is became able to promote the coordinated expression of a plethora of target genes that are the executors of p53-induced cellular responses, such as cell cycle arrest, senescence or apoptosis (Levine and Oren, 2009). Despite all these activities are believed to rely mainly on the ability of p53 to function as a transcription factor, transcription-independent activities can also contribute to its apoptotic functions (Marchenko, 2007; Moll, 2005). 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*" (Lane, 1992). Moreover, p53 was recently found implicated also in the regulation of other cellular processes ranging from

metabolism, autophagy, and stemness (Cicalese, 2009; Crighton, 2006; Green and Kroemer, 2009; Vousden and Ryan, 2009). However, the main significance of the p53 pathway in tumor suppression is strongly highlighted by the observation that mutations of the *TP53* gene are very frequent in human cancers. Indeed, whereas somatic *TP53* mutations contribute to sporadic cancer, germline *TP53* mutations cause a rare type of cancer predisposition known as Li-Fraumeni Syndrome (LFS) which is not associated with site-specific tumours, but rather with a variety of tumour types occurring at a relatively early age (Li and Fraumeni, 1969). Accordingly, pioneering studies have demonstrated that the absence of p53 predisposes to spontaneous development of neoplastic disease, as observed in p53 knockout mouse models (Donehower et al., 1992). Furthermore, somatic mutations in the *TP53* gene are one of the most common alterations in human cancers, occurring in more than 50% of cases (Soussi and Wiman, 2007). In the other half of tumours, the p53 pathway is functionally restrained because of either the amplification of negative regulators, such as MDM2 (Muthusamy, 2006) or the inactivation of upstream factors, like Chk2, ATM or p14ARF (Carr, 2006; Grochola, 2010; Vahteristo, 2001). Recent studies clearly demonstrated that the restoration of p53 levels is able to interfere with tumour progression *in vivo*: the re-expression of p53 in tumours lacking p53 expression triggered a fast and massive regression of established tumours caused by induction of p53-dependent apoptosis or senescence (Martins, 2006; Ventura, 2007; Xue, 2007).

3.2 ONCOGENIC MUTANT p53 VARIANTS

Mutations in the TP53 gene are among the most common gene-specific alterations in human cancers. The frequency of *TP53* gene mutations can vary considerably between cancer types, ranging from 10% in haematopoietic malignancies (Peller and Rotter, 2003) to 50–70% in ovarian (Schuijer and Berns, 2003), colorectal (Iacopetta, 2003), pancreas (Moore, 2003) and head and neck (Blons and Laurent-Puig, 2003) cancers. Even if inactivation of the *TP53* gene may be obtained through several types of mutations, including missense and nonsense mutations or insertions/deletions of several nucleotides, missense mutations account for more

than 70% of them (IARC TP53 Database, www.p53.iarc.fr). As a consequence, in the vast majority of tumours with mutations in the *TP53* gene, cells express a full-length mutant form of the protein, which differs from the wild-type counterpart in a single amino acid substitution. Both germline mutations and sporadic somatic mutations show the same distribution in the *TP53* gene (Varley, 2003). Indeed, they are not casually dispersed along the whole sequence, but they generally occur in some hot-spots located at the region corresponding to the DBD of the protein (figure 3).
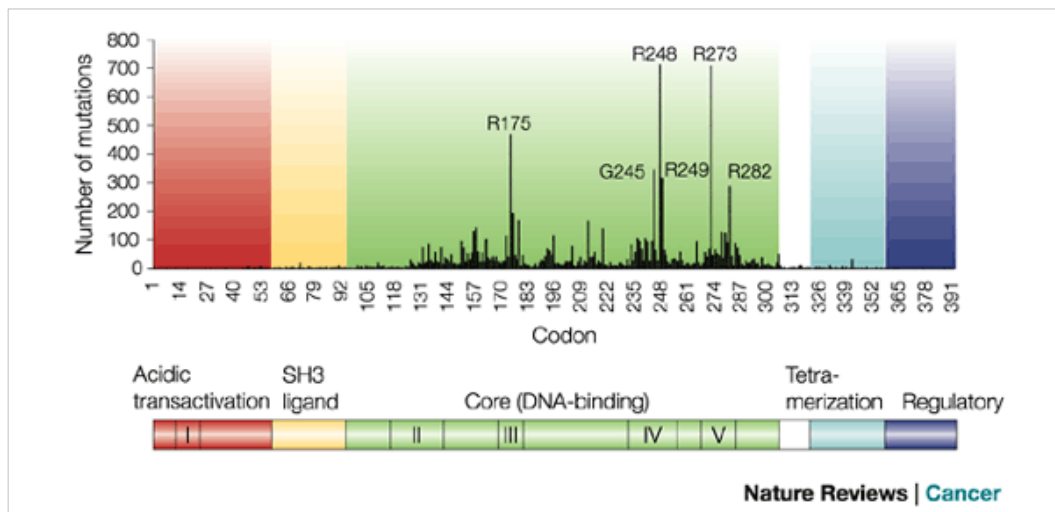
Figure 3. The distribution of reported missense mutations along the p53 sequence. A histogram of p53 missense mutations shows that 95% of mutations occur in the core domain; six labelled residues are hot-spots for mutation. (Bullock and Fersht, 2001).

As shown in figure 3, the majority of all missense mutations are found in six "hotspot" codons (Hollstein, 1991; Petitjean, 2007). The p53 missense mutations can be classified into two main categories according to their effect on the thermodynamic stability of the p53 protein (Bullock and Fersht, 2001). These two mutation categories are commonly referred to as "DNA‑contact" and "conformational" mutations. The first group includes mutations in residues directly involved in DNA binding, such as R248Q and R273H. The second group

comprises mutations that cause local (such as R249S and G245S) or global (such as R175H and R282W) conformational distortions.

Biological impact of the TP53 gene mutations

Unlike other tumor suppressor genes, which are predominantly inactivated by deletion or truncation, the missense mutations on p53 cause a strong functional impact (figure 4).
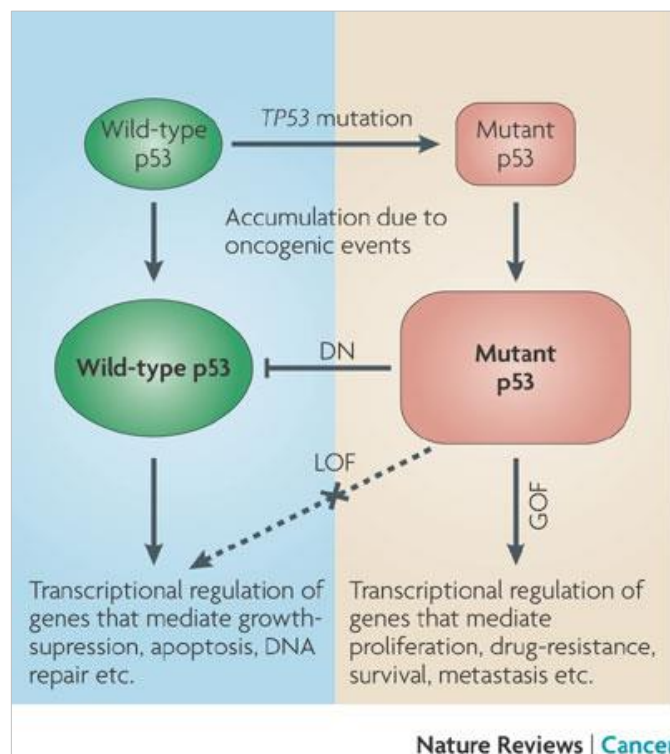
Figure 4. Schematic representation of the functional impacts of the *TP53* mutations. LOF (loss of function); DN (dominant-negative effects); GOF (gain of function). (Brosh and Rotter, 2009).

First, mutations can abrogate the tumor suppressor function of p53 (a phenomenon is called Loss of Function; LOF) resulting in absent activation of downstream effector, and loss of normal checkpoint control (Figure 4) (Brosh and Rotter, 2009). This loss of function is due to reduction of p53 binding to its consensus DNA sequence and, consequently, hampered transcriptional activation of p53 target genes (Kato, 2003). Despite loss of function occurs among missense

mutants, it is particularly relevant in truncating, splicing and nonsense mutations, as well as in gene deletions. If in the same cell also the remaining allele becomes mutated or is lost (loss of heterozygosity), such a cell will be totally deprived of anticancer protection by p53. In addition, most of mutant p53 forms are able to oligomerize with the wildtype protein encoded by the second allele, inhibiting its function, forming a heterotetramer unable to bind DNA (Dominant negative Function) (Figure 4) (Brosh and Rotter, 2009). Hence, even if the wild-type allele is retained, the cell may be rendered practically devoid of wildtype p53 functions through such a mechanism.

Finally, several mutations were shown to confer new oncogenic functions to mutp53 (Gain of Function; GOF), (Figure 5) totally independent on wtp53, and actively contribute to various aspects during tumor progression (Aschauer and Muller, 2016).
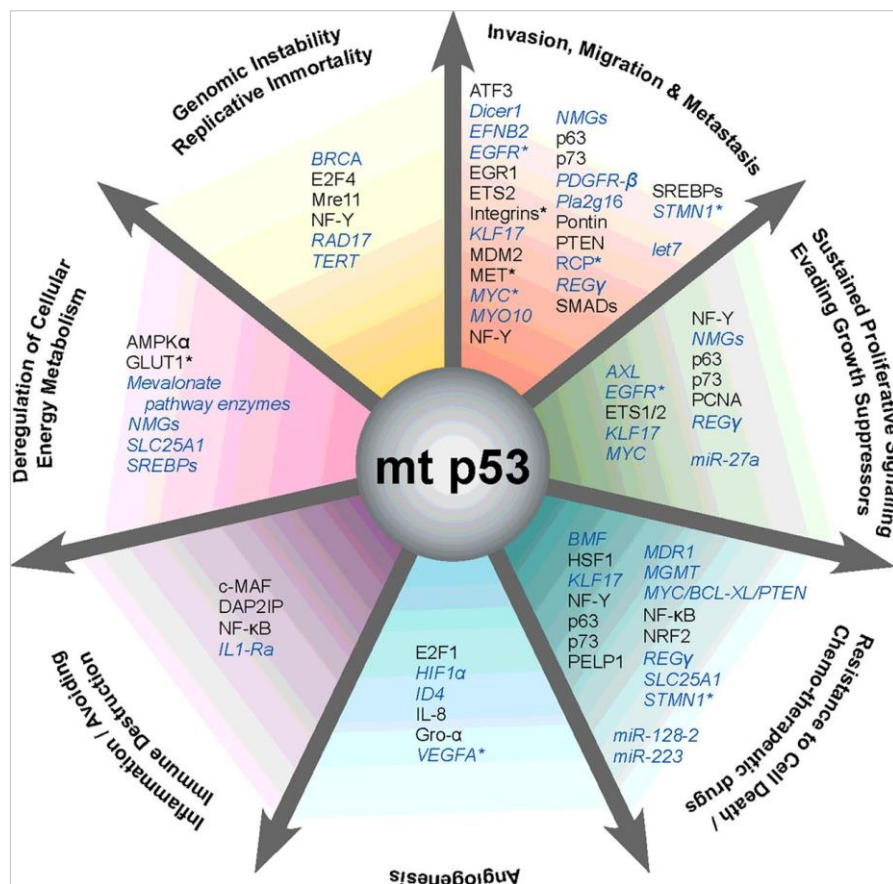
Figure 5. Selected oncogenic properties of mutant p53 proteins and their underlying mechanisms. Key cancer hallmarks and selected molecules that are associated with mutant p53 GOF. Mutant p53 GOF contributes to cancer progression through direct interaction with proteins altering their activity or through the transcriptional activation or repression of target genes and downstream molecules. (Aschauer and Muller, 2016).

Malignant transformation

The first evidence regarding mutant p53 gain of function was obtained in 1993, when it was shown that p53 mutants of both human and mouse origin, but not their wild-type counterparts, can transform p53-null cells and provide them with an increased ability to form colonies in soft agar and tumors in mice (Dittmer, 1993). Accordingly, experiments performed knocking down mutant p53 in several human cancer cell lines, demonstrated that down-regulation of endogenous mutant p53 rendered those cells less tumorigenic (Bossi, 2006; Bossi, 2008). Mutant p53 was shown to cooperate with activated oncogenic Ras in transformation of primary mouse embryo fibroblasts (MEFs) (Lang, 2004). This property is due to the ability of different p53 mutants to bind and inactivate the p53 family members, p63 and p73 (Di Como, 1999; Strano, 2000). The cooperation between mutant p53 and oncogenic Ras was described *in vivo* by using mouse models of skin (Caulin, 2007), lung (Jackson, 2005) and pancreatic cancers (Hingorani, 2005). Such mice exhibited increased tumour formation, accelerated tumour progression and elevated rates of metastasis relative to their p53-null counterparts.

Chemoresistance, escape from apoptosis and genomic instability

One distinctive feature of many mutant p53 proteins is their ability to confer an elevated resistance to cells for a variety of apoptotic signals. This effect of mutant p53 was revealed for the first time by showing that it can suppress c-Myc-induced apoptosis in leukemic cells (Lotem and Sachs, 1995). Interestingly, the overexpression of various tumour-associated p53 mutants can render cancer cells

more resistant to the effect of chemotherapeutic drugs (Blandino, 1999; Fiorini, 2015), whereas knockdown of endogenous mutant p53 sensitizes cancer cells to killing by such molecules (Bossi, 2006). The mutant p53 ability to protect cells from chemotherapeutic drugs is due by various ways, including its capability to inhibit p73 pro-apoptotic functions (Bergamaschi et al., 2003a). Accordingly, short peptides disassembling the mutant p53/p73 complex restore p73 activity and re-sensitize cells harboring mutant p53 to chemotherapy (Di Agostino, 2008). High NF-E2-related factor 2 (Nrf2) expression has been shown to promote resistance to different anticancer drugs in human cancers (Wang XJ, 2008; Niture SK and Jaiswal 2013). However, the underlying mechanism of an increase in Nrf2 expression is not fully understood although some NF-kB and mutant K-ras-mediated mechanisms have been reported (Rushworth, 2012; Tao, 2014). Recently, it has been suggested that mutant p53 may confer cisplatin resistance in lung cancer cells via upregulating Nrf2 transcription (Tung, 2015). Importantly, it has been demonstrated that mutp53 sustains NF-kB activation in colorectal cancer promoting a chronic inflammation status (Cooks, 2013). Since NF-kB can act as a powerful inhibitor of apoptotic stimuli, the stimulation of NF-kB signaling may be a further mechanism by which mutp53 proteins inhibit the apoptotic signaling.

A number of study revealed that mutp53 counteracts normal spindle checkpoint control, resulting in chromosomal aberration and polyploidy (Gualberto, 1998). In addition, mutp53 interferes with DNA repair, by attenuating base excision repair (Offer, 1999), by binding topoisomerase I, fostering DNA rearrangements and aberrant homologous recombination, but above all it inhibits double strand repair system. Mutp53 binds Mre11 preventing its recruitment at level of dsDNA breaks, avoiding Rad50-NBS1 binding and thus ataxia teleangectasia mutated (ATM) dependent DNA damage activity (Song, 2007). Intriguingly, has been recently shown that mutp53 proteins, through the recruitment of mutp53/E2F4 complex onto specific regions of BRCA1 and RAD17 promoters, lead to the inhibition of their expression, whose derived proteins play a pivotal role in DNA damage repair (Valenti, 2015).

Stimulation of the Warburg effect

Cancer is a complex disease characterized by dramatic metabolic alterations, and the Warburg effect, also called aerobic glycolysis, is the best known metabolic shift that occurs in cancer cells (Hanahan, 2011; Warnurg, 1956). During this phenomenon, even under physiological oxygen concentrations, tumour cells adopt glycolysis for their energy requirements and undergo both high rate glucose uptake and lactate production, as compared with normal cells (Feng, 2010; Levine and Puzio-Kuter, 2010). Many studies highlighted that the Warburg effect significantly contributes to tumorigenesis and that its impairment could be considered as a therapeutic anticancer opportunity (Christofk, 2008; Fantin, 2006). Recently, it has been clearly demonstrated that mutp53 proteins stimulate the Warburg effect, in both cultured cells and mutp53 knock-in mice (Zhang, 2013) contrary to wtp53, which is known to repress glycolysis and the Warburg effect through transcriptional regulation of genes involved in energy metabolism, including SCO2, TIGAR, GLS2 and Parkin (Bensaad, 2006; Matoba, 2006; Zhang, 2011). This metabolic-related oncogenic function of mutp53 proteins mostly occurs by promoting glucose transporter 1 (GLUT1) translocation to the plasma membrane through activation of RhoA/ROCK signalling, thus resulting in increased cancer cells glucose uptake and, consequently, in increased glycolytic rate and lactate production (Zhang, 2013).

Alteration of tumor microenvironment and implication in cancer progression

Several studies recently reviewed in Cordani, 2016a demonstrated the involvement of mutp53 proteins in the regulation of proteins/enzymes/molecules secreted by cancer cells and recognized to functionally alter tumour microenvironment. Mutp53 proteins trigger the production and release of cytokines addressed to stimulate an inflammatory cancer-associated microenvironment and concomitantly to repress the immune system. Remarkably, stimulation of expression and release of these cytokines by mutp53 proteins are in stark contrast to the inhibitory functions of the tumour suppressor wtp53. Another

crucial element supporting the motility of tumour cells and their metastatic potential is covered by extracellular pH decrease. This phenomenon is mainly related to the stimulation by mutp53 proteins of secreted lactate and implies a global alteration of metabolism of cancer cells carrying mutant *TP53* gene. The extracellular acidification is also involved in the stimulation of the activity of matrix metalloproteinases (MMPs), which, in turn, promote extracellular matrix (ECM) degradation favoring tumour invasion and cancer motility. Furthermore, mutp53 proteins are also described to stimulate secretion of VEGF, which is conversely inhibited by wtp53. This is likely the main known event to promote the pro-angiogenic feature associated to enhanced metastasis of cancer cells bearing mutant *TP53* gene

Cell migration and invasion

Although p53 knockout mice are highly tumor prone, these lesions do not metastasize frequently nor generally display invasive pathology (Attardi and Jacks, 1999). On the contrary, the presence of mutant p53 leads to a marked increase in the incidence of highly metastatic carcinomas in various mouse models (Doyle, 2010; Heinlein, 2008; Lang, 2004; Liu, 2000). Indeed, another aspect of mutant p53 gain of function that has recently emerged is its ability to drive cell migration and invasion (Adorno, 2009; Dhar, 2008; Muller, 2009). This is achieved by two non-mutually exclusive mechanisms of inhibition of p63 anti-metastatic functions (figure 6).
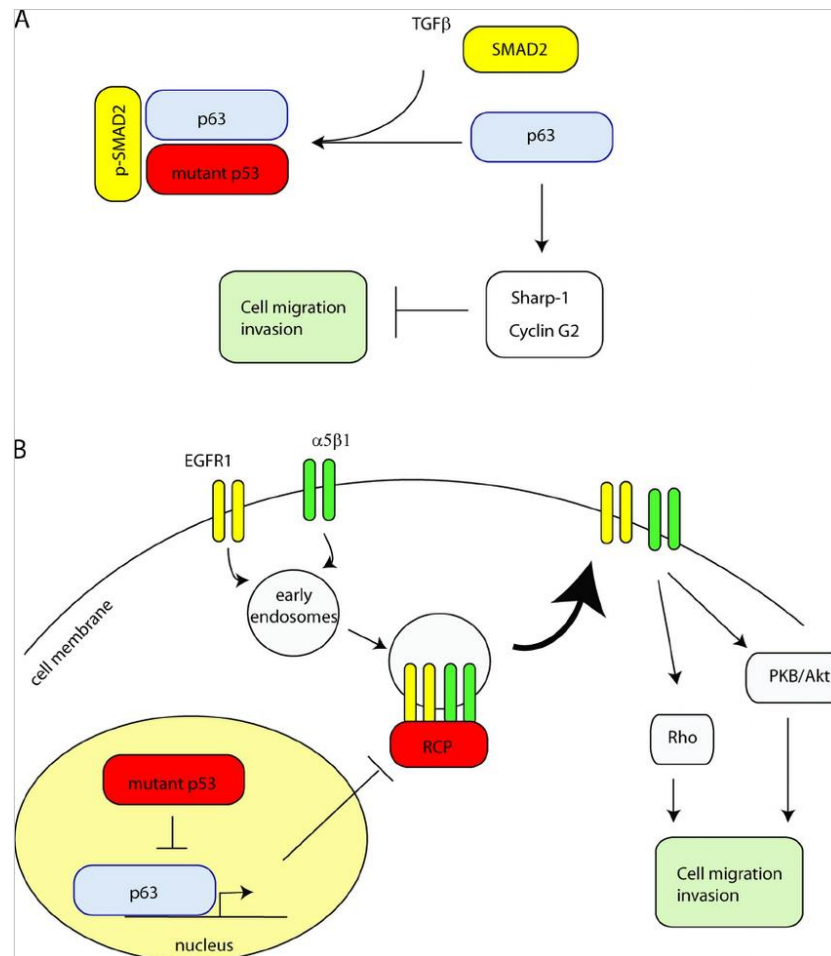
Figure 6. Mutant p53 promote cell migration and invasion by inhibiting p63. (A) Upon TGF-β induction, SMAD2 is phosphorylated and promotes binding of mutant p53 to p63, alleviating p63-mediated suppression of Sharp-1 and Cyclin G2 to allow for cell migration and invasion. (B) p63 inhibits activation of RCP (through transcriptional targets that are currently unknown) to prevent α5-β1 integrin and EGFR recycling to the plasma membrane. Upon expression of mutant p53, p63 activity is suppressed, resulting in enhanced RCP-driven recycling of α5-β1 integrin and EGFR. This activates Rho and PKB/Akt to promote cell migration and invasion (Muller, 2011).

*Transcriptional regulation of mutant-p53 Gain of Function*

Missense mutation that occurs on p53, especially in DBD disrupts its ability to bind p53 consensus. The molecular basis by which mutant p53 regulates its target genes involved in different aspects of tumor progression, such as i) proliferation, ii) inhibition of apoptosis, iii) chemo-resistance and iv) metabolism is not fully

understood. However, it has been shown that changes in interaction with others proteins, with transcription factor, but also with proteins that are not directly involved in gene expression regulation, drastically affect gene expression (Kato, 2003). Moreover, accumulating evidences show that many loci missing for wtp53 responsive elements are directly induced by mutp53, thus suggesting that mutp53 acquires distinct DNA-binding and transactivation activities. Indeed, a number of *in vitro* evidence shows that mutp53 conserves the ability to bind DNA with high affinity. Mutp53, but not wtp53, has been specifically found to bind with high affinity to a variety of nuclear matrix attachment region DNA elements (MARs) (Weissker, 1992; Will, 1998). Due to the importance of MARs in nuclear processes such as gene expression and DNA replication, it is possible to hypothesize that this ability of mutp53 to modulate gene expression and DNA replication might represent the molecular basis for its oncogenic potential in cancer.

A pivotal mechanism by which mutp53 exerts its gain of function, is the direct binding to the transcription factors to enhance or prevent their activities. Among them, the interaction of mutp53 with NF-Y results in the activation of genes involved in cell cycle promotion, such as cyclins and cyclin-dependent kinases, and cancer growth (Di Agostino, 2006). Some researchers identified a lot of mutp53 partners, including Sp1, NF-kB, VDR (Stambolsky, 2010), which upon their interaction with mutp53 can regulate the expression of their target genes; or SREBP, promoting the mevalonate pathway gene expression (Freed-Pastor, 2012); or Est-1 that is directly involved in MDR1 transcription and chemo-resistance (Sampath, 2001). Importantly, mutp53 has been demonstrated to bind with members of its own family, p63 and p73, preventing their binding to DNA responsive elements, thus preventing their anti-metastatic or pro-apoptotic activities. Moreover, as previously mentioned, mutp53 can interact with other protein non-directly involved in transcription, such as MRE11 (Song, 2007) or Topoisomerase1 (Liu, 2011) both resulting in enhanced genomic instability.

3.3 AUTOPHAGY: WHEN CELLS EAT THEMSELVES

Autophagy is an intracellular degradative process by which damaged macromolecules and organelles are targeted by autophagic vesicles to lysosomes and then eliminated. Autophagy is crucial to maintain primary biological activities during cellular stresses, such as nutrient starvation (Mizushima, 2009). Once autophagy is activated, the cellular components are embedded into double-membrane vesicles (autophagosomes), which fuse with lysosomes to form an autophagolysosome structure to degrade its contents by lysosomal hydrolases providing a nutrient source to maintain vital cellular activities (Figure 7) (Mizushima, 2007; Yang, 2009).
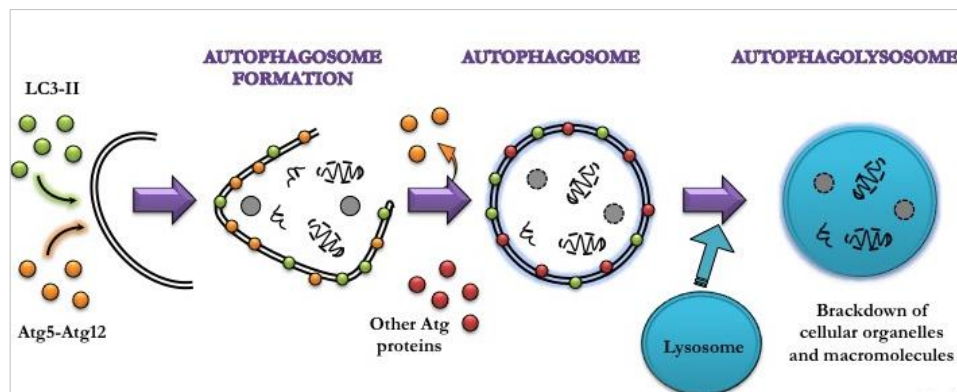
Figure 7 Model of various steps of autophagy. Autophagy is a cellular catabolic process during which a double isolation membrane engulfs cytoplasmic material including proteins and organelles. The membrane forms a vesicle termed autophagosome, which fuses with the lysosome to form the autophagolysosome. After fusion the inner membrane is lysed and autophagic cargo is digested by the lysosomal proteases (Cordani, 2016).

Autophagy requires the activation of a number of autophagy-related genes (ATGs), which play a pivotal role in the formation of double-membrane autophagosome vesicles and in the stimulation of the autophagy machinery (Klionsky, 2007). Under energetic stresses, ATG1 kinase interacts with ATG13 and ATG17 in order to initiate the phagophore formation in yeast, probably by activating the transmembrane protein ATG9 that serves to recruit lipids to the

expanding phagophore (Klionsky, 2007). Vesicular protein sorting 34 (Vps34), belonging to the class III PI-3 kinases, has been described to play a critical role in various cellular membrane-sorting processes. However, when Vps34 interacts with Beclin-1 and other autophagy-related proteins, it mediates the phagophore formation and autophagy initiation (Backer, 2008). In addition to the formation of several protein-protein complexes which are indispensable for the phagophore generation and vesicle nucleation on membranes (Mizushima, 2011) the ubiquitin-like conjugation systems are necessary for certain ATG proteins to execute their functions (Figure 7) (Geng, 2008). In particular, the mammalian homologue of ATG8, also called LC3B, is expressed as a full-length cytosolic protein that, upon induction of autophagy, is proteolytically cleaved by ATG4, a cysteine protease, in order to generate LC3B-I. The carboxy-terminal glycine exposed by ATG4-dependent cleavage is then activated in an ATP-dependent manner by the E1-like ATG7 and transferred to ATG3, a different E2-like carrier protein, to generate the active isoform LC3B-II. The recruitment and integration of LC3B-II into the growing phagophore is dependent on ATG5–ATG12 interaction, favoring the binding of LC3B-II on both internal and external surface of autophagosomes, where it plays a role in both fusion of membranes and in selecting cargo for lysosomal degradation (Figure 7) (Kirkin, 2009).

The role of autophagy in regulating cancer cell death or survival remains highly debated. A number of studies support the idea that constitutive autophagy may have a protective role in cancer cells removing damaged organelles or recycling misfolded macromolecules. In support of this hypothesis, several studies report that autophagy is rapidly upregulated to maintain metabolic homeostasis in cancer cells exposed to stressful conditions, such as nutrient deprivation, oxidative stress, hypoxia, or in response to therapy (Kondo, 2005). However, it is also well established that uncontrolled autophagy can also lead to cell death, called also cell death-type II, likely due to excessive degradation of cellular constituents and organelles required for homeostasis of the cells. Furthermore, cancer cells are able to escape from extensive autophagic cell death resulting in the enhancement of radical oxygen species (ROS) production, genomic instability, and tumor progression. Hence, the role of autophagy in cancer is highly controversial and it

is likely reliant on the tumor type, the stage of neoplasia and the cellular context (Kondo, 2005). Several studies indicate that wild-type p53 protein, in order to react to genotoxic or environmental stimuli, triggers autophagy in cancer cells through various mechanisms, as the stimulation of the nutrient energy sensor AMP-activated protein kinase (AMPK), the inhibition of the mammalian target of rapamycin (mTOR) by up-regulation of PTEN and TSC1, and the induction of the autophagy-related gene DRAM1 (Crighton, 2007).

3.4 REACTIVE OXYGEN SPECIES: THE ACHILLES HEEL OF CANCER CELLS

Reactive oxygen species (ROS) are radicals, ions or molecules, which have a single unpaired electron in their outermost shell of electrons and for this character, they are highly reactive. In cancer cells high levels of ROS can result from increased metabolic activity, mitochondrial dysfunction, peroxisome activity, increased cellular receptor signaling, oncogene activity, increased activity of oxidases, cyclooxygenases, lipoxigenases and thymidine phosphorylase, or through crosstalk with infiltrating immune cells (Storz, 2005; Szatrowski, 1991; Babio, 1999). In mitochondria, ROS are produced as an inevitable byproduct of oxidative phosphorylation (Figure 8). The electron transport chain encompasses complexes I-IV and ATP synthase on the mitochondrial inner membrane. Superoxide is generated at complexes I and III and released in the intermembrane space or in the mitochondrial matrix (Ha, 2011).
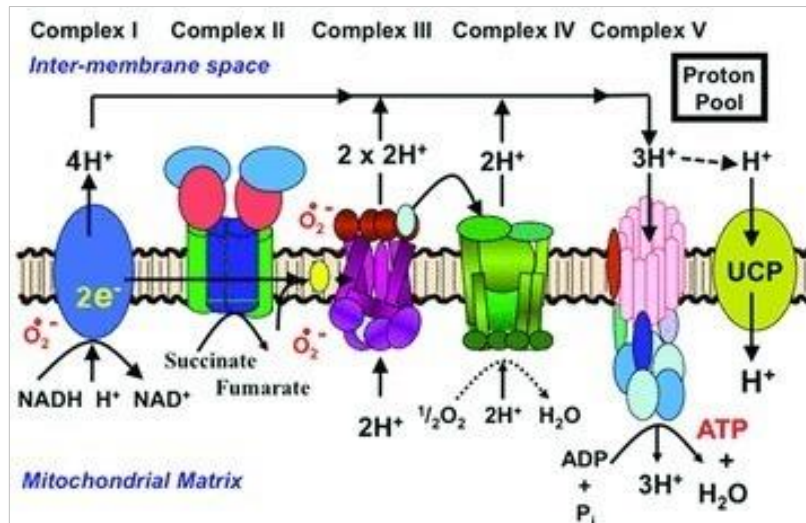
Figure 8. Oxidative phosphorylation in mitochondrial electron transport chain (ETC), and proton leak via uncoupling proteins (UCPs). UCP dissipates mitochondrial membrane potential by facilitating proton leak across the inner membrane, thereby minimizing superoxide ($O_2\bullet{-}$) formation from undesirable interaction between molecular oxygen (O_2) and high-energy electrons ($e-$) (Ramsden, 2012).

However, in addition to the mitochondria, peroxisomes are other major sites of cellular ROS generation (Danse, 2001). In these organelles, superoxide and H_2O_2 are generated through xanthine oxidase in the peroxisomal matrix and peroxisomal membranes (del Rio, 1992). The production of ROS can be induced by hypoxia, metabolic defects, endoplasmic reticulum (ER) stress and oncogenes. However, under normal physiological conditions, the intracellular levels of ROS are steadily maintained to prevent cells from damage. Detoxification from ROS is facilitated by non-enzymatic molecules, including glutathione, flavonoids and vitamins A, C and E or through antioxidant enzymes, such as the transcription factor NRF2, breast cancer susceptibility 1 (BRCA1), p53, phosphatase and tensin homolog (PTEN), ataxia telangiectasia mutated (ATM) and the action of dietary antioxidants which specifically scavenge different kinds of ROS (figure 9).
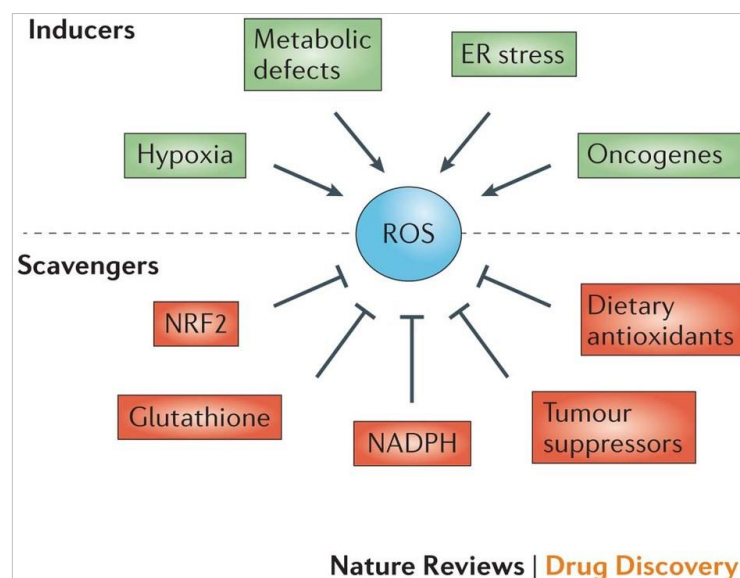
Figure 9. Determination of cellular redox status by a balance between levels of ROS inducers and ROS scavengers (Gorrini, 2013).

ROS-sensitive signaling pathways are persistently elevated in many types of cancers, where they participate in cell growth/proliferation, differentiation, protein synthesis, glucose metabolism, cell survival and inflammation (Storz, 2005). Reactive oxygen species, particularly hydrogen peroxide, can act as second messengers in cellular signaling (Sundaresan, 1995; Colavitti, 2002). H_2O_2 regulates protein activity through reversible oxidation of its targets including protein tyrosine phosphatases, protein tyrosine kinases, receptor tyrosine kinases and transcription factors (Storz, 2005). A great amount of studies has deeply unveiled the involvement of ROS as regulators of several cellular pathways, including the i) mitogen-activated protein (MAP) kinase/ERK cascade, ii) phosphoinositide-3-kinase (PI3K)/ Akt-regulated signaling cascades, as well as the iii) IκB kinase (IKK)/nuclear factor κ-B (NF-κB)-activating pathways (Liu and Storz, 2010). Oxidative stress-mediated signaling events play exceptional relevance in the development and progression of several human cancers (Fiaschi and Chiarugi, 2012). Indeed, ROS are involved in a plethora of biological events addressed to sustain each aspect of cancer progression, including i) cell cycle progression and proliferation, ii) cell survival and apoptosis, iii) genomic

instability and mutations, iv) change in energy metabolism, v) cell motility, invasion and higher metastatic potential, vi) angiogenesis, vii) escaping from immune destruction and viii) maintenance of tumor stemness. A detailed scheme showing the mechanistic contribution of ROS in cancer is reported in figure 10 (Fiaschi and Chiarugi, 2012).
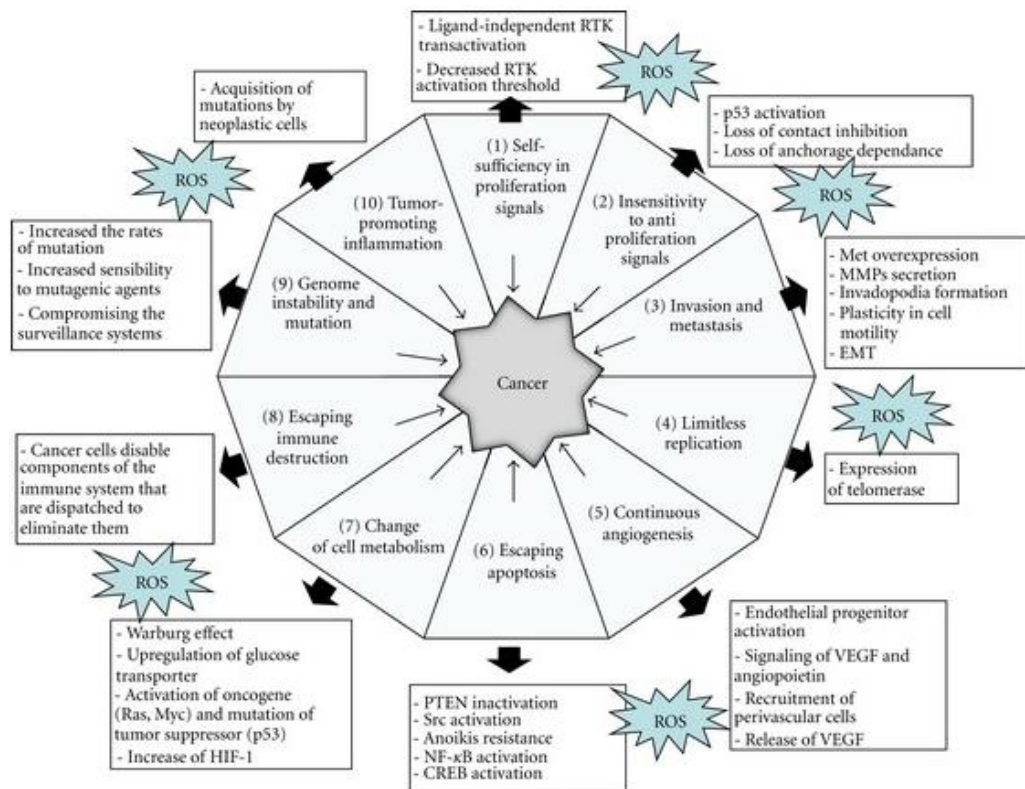
Figure 10. ROS play multiple roles in the hallmarks of cancers. Contribution of oxidants is indicated for each point (Fiaschi and Chiarugi, 2012).

However, the role of ROS in cancer biology is ambiguous, indeed despite many studies attributed to ROS a pivotal role in promoting many events aimed to sustain cancer progression, by acting as signaling molecules (figure 10), many others have highlighted that a severe increase in ROS can induce cell death following a "non-specific" damage of macromolecules such as the irreversible oxidation of lipids, proteins or DNA. (Kamata, 1999). Therefore, ROS represent

an "Achilles heel" of cancer cells and new therapeutic improvement could be reached playing on this sophisticated redox cellular balance. The majority of the damaging effects on DNA are due to hydroxyl ions, which are generated via the Fenton reaction (Imlay, 1988). In this reaction transition metals such as iron and copper donate or accept free electrons during intracellular reactions and use H_2O_2 to catalyze free radical formation. Hydroxyl radicals attack DNA rapidly due to their high diffusibility which results in formation of DNA lesions including oxidized DNA bases, single strand and double strand breaks (Maynard, 2009). DNA adducts are usually removed by either the base excision repair (BER) or the nuclear excision repair (NER) pathways (Mitra, 2001). Cells incapable to completely repair DNA lesions (i.e. due to deficient DNA repair enzymes) undergo apoptosis to ensure these mutations will not be passed on to progeny cells. However, under certain circumstances, the cells harboring DNA mutations successfully escape programmed cell death, which raises a high chance for cancerous growth. The oxidative modification of proteins by ROS is implicated in the etiology or progression of various disorders and diseases. The major damage of ROS to proteins is modification in their amino acid residues, resulting in altered functions. The oxidized amino acid residues of proteins can influence their ability in signal transduction mechanisms (Meng, 2002), as well to impact DNA repair efficiency, the fidelity of DNA polymerase during replication/synthesis and transcriptional activity, which tightly associates with cancer onset. (Storz, 2005; Burhans, 2007). Lipids are others cellular targets of ROS attacks. ROS react with polyunsaturated or polydesaturated fatty acids to initiate lipid peroxidation (Gardner, 1989). Lipid oxidation generates numerous genotoxic molecules such as malondialdehyde, 2-alkenals and 4-hydroxy-2-alkenals (Burcham, 1998; Catala, 1999). Interestingly, the importance that plays ROS-induced lipid peroxidation in human tumor is corroborated by some clinical studies, which have shown its potential use as a tumor marker (Lauschke, 2002).

Oncogenic properties of mutant p53 alter oxidative status in cancer cells

A high ROS level is one of the hallmarks of the human cancers. However, nowadays very little is known about the regulation of oxidative stress by oncogenic mutant p53 proteins. An interesting study elucidates that GOF p53-R273H mutant interferes with the normal response of human cells to oxidative stress (Kalo, 2012). It has been shown that, upon oxidative stress, mutant p53-R273H attenuates the activation and function of NRF2, a transcription factor that induces the antioxidant response, in several human cancer cell lines, highlighting the general nature of this phenomenon. This oncogenic propriety of mutant p53 is manifested by the decreased expression of some detoxifying enzymes, as NQO1 and HO-1, and high ROS levels.

NADPH oxidase enzymes (NOX family) are some powerful intracellular ROS-generating sources and represent critical mediators of redox signaling (Thannickal and Fanburg, 2000). Some studies have indicated that NADPH-oxidase-dependent ROS production can alter cell motility or potentiate metastatic progression (Schroder, 2007; Sadok, 2008; Sadok, 2009). An intriguing study reported that wild type p53 and mutant p53 differentially regulate NOX4 expression and activity, corroborating the statement that GOF mutant p53 plays a role in promoting high ROS levels in cancer cells. Indeed, it has been found that while wtp53 has an inhibitory effect, mutant p53 proteins are able to enhance NOX4 expression, enhancing in turn ROS levels, which sustain an invasive phenotype of cancer cells.

UCP2: a crucial antioxidant player

Mitochondrial ATP production occurs by coupling the electron transport chain (ETC) with the phosphorylation of ADP into ATP, the so-called oxidative phosphorylation. These two processes are not always efficiently coupled, mainly because of the presence of mitochondrial transporters in the inner membrane, such as uncoupling proteins (UCPs). The UCPs belong to the superfamily of anion transport carriers of the mitochondrial inner membrane (Hughes, 2008) and some of them are involved in thermogenesis and regulation of mitochondrial ROS.

While UCP1 tissue expression is localized and abundant in brown adipose tissue (BAT), whereas it is involved in the non-shivering thermogenesis activity (Enerbäck, 1997). UCP2 has been found in several tissues, including liver, brain, pancreas, adipose tissue, immune cells, spleen, kidney, and the central nervous system (Donadelli, 2014) and UCP3 is mainly present in the skeletal muscle (Boss, 1997). Unlike UCP1, UCP2 and 3 are involved in a number of postulated functions in energy regulation, including regulation of insulin secretion (Zhang, 2001) or ROS production and control of the immune response (Arsenijevic, 2000). The other two members of the UCP superfamily, UCP4 and UCP5, are expressed in a tissue-specific manner and are involved in mitochondrial membrane potential reduction (Hoang, 2012). The UCP system represents an efficacy mechanism to decrease ROS production in mitochondria (Mailloux, 2011). The existence of a strong correlation between mitochondrial membrane potential and ROS production is well known (Mailloux, 2012). The uncoupling of oxidative phosphorylation is a short circuit in which the transport of protons from the intermembrane space to the matrix bypasses ATP synthase. Minor increases in the mitochondrial membrane potential induce ROS formation, whereas slight decreases can substantially diminish their production, without greatly lowering the efficiency of oxidative phosphorylation. Hence, the mild uncoupling of mitochondrial oxidative phosphorylation may represent the first line of defense against oxidative stress (Brand, 2005). According to this pattern, UCP2 can dissipate the proton gradient to prevent the proton-motive force from becoming excessive, thus decreasing ROS produced by electron transport (Garlid, 2000). Mitochondrial superoxide ion is considered the initial and leading molecule of ROS signaling and it is generally converted into hydrogen peroxide (H_2O_2) by superoxide dismutase. In addition, superoxide ion can generate hydroxyl radicals (•HO) implicated in lipid damage and protein oxidation (Cadenas, 2000).
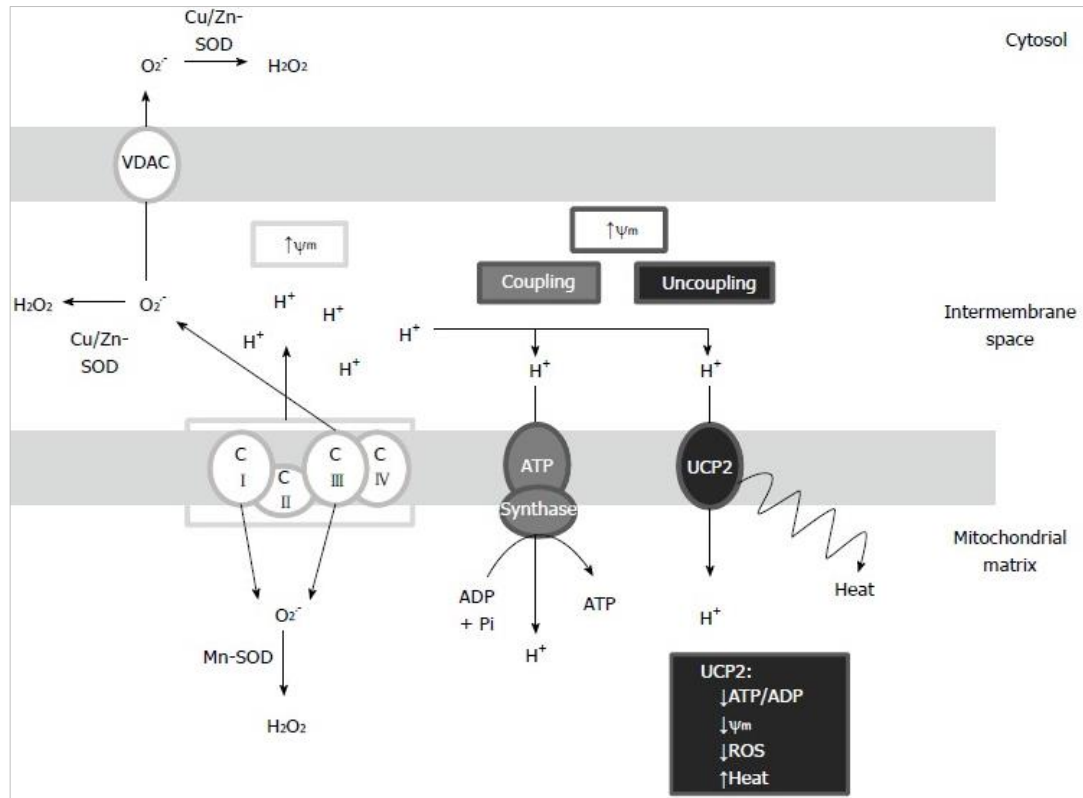
Figure 11. Uncoupling protein 2 uncoupling activity in oxidative phosphorylation. ROS: Reactive oxygen species; UCP2: Uncoupling protein 2; SOD: Superoxide dismutase; Mn-SOD: Manganese-superoxide dismutase (Donadelli, 2015).

Therefore, UCP2 acts as a sensor of mitochondrial oxidative stress and constitutes an important component of local feedback mechanisms generally implicated in cyto-protective activities controlling the production of mitochondrial ROS and regulating redox-sensitive cytosolic signaling pathways. Since UCP2 plays an essential role in the maintenance of cellular ROS homeostasis, it is reasonable to expect that it undergoes to sophisticates regulation at multiple levels, including i) *Ucp2* gene mutation (single nucleotide polymorphisms, SNP), ii) *Ucp2* mRNA and protein expression (transcriptional, translational, and protein turn-over regulation), iii) UCP2 proton conductance (ligands and post-transcriptional modifications), and iv) nutritional and pharmacological UCP2 regulation, as described in Donadelli, 2014. One of the most important mechanisms of the transcriptional regulation of UCP2 is mediated by the peroxisome proliferator-

activated receptor gamma coactivator1-alpha (PGC-1α), which has been shown to stimulate TR-mediated human *Ucp2* gene expression via two TREs located in the proximal *Ucp2* promoter region (figure 12) (Oberkofler, 2006). PGC-1α can also indirectly induce Ucp2 gene expression by co-activating liver X receptor-mediated expression of sterol regulatory element binding protein (SREBP)-1c as well as dexamethasone-stimulated SREBP-2 expression. SREBP isoforms are known to regulate Ucp2 gene expression via either one of the two E-box motifs present on Ucp2 promoter (Fukuchi, 2002).
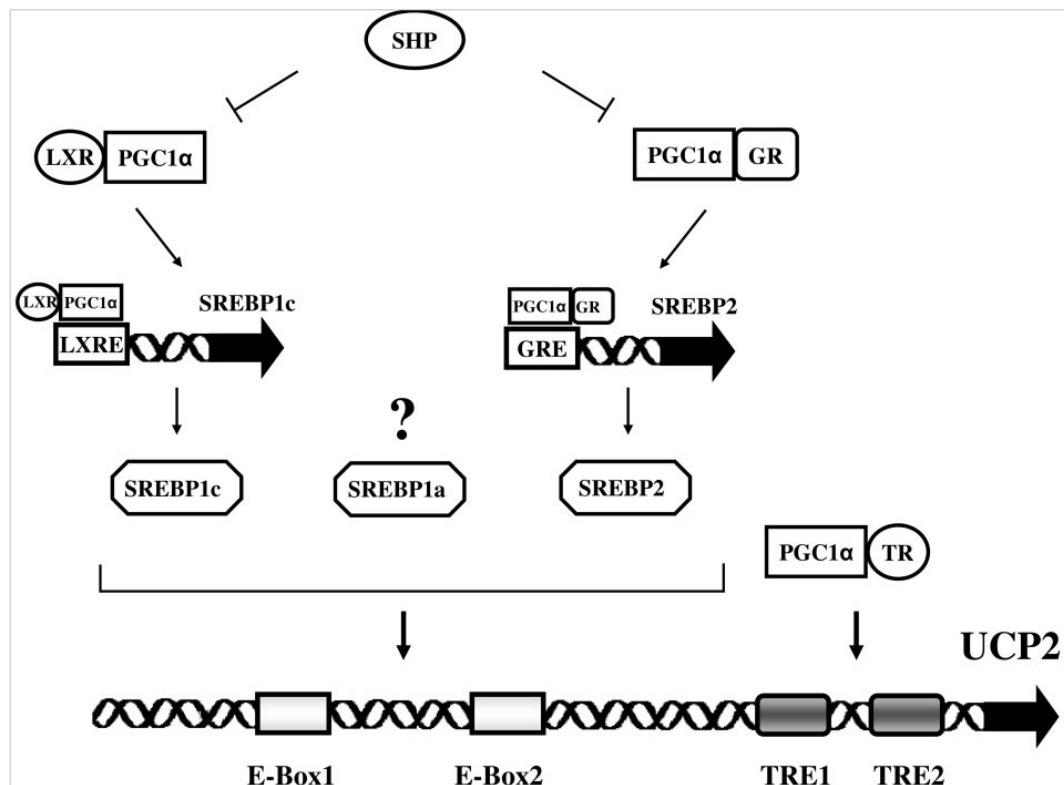
Figure 12. Role of PGC-1α in the regulation of human UCP2 gene expression. Two E-Box motifs mediate the stimulatory effects of SREBP isoforms on human UCP2 gene transcription. PGC-1α up-regulates SREBP-1c via coactivation of the LXR and increases SREBP-2 gene expression via coactivation of the GR, thereby indirectly increasing UCP2 mRNA expression. These interactions of PGC-1 with LXR and GR are antagonized by the transcriptional corepressor SHP. Furthermore, PGC-1α potentiates human UCP2 gene expression as a coactivator of the TR, which binds to two TREs in the

proximal UCP2 promoter region. GRE, Glucocorticoid hormone response element (Oberkofler, 2006).

Sestrins: crucial role in antioxidant defenses

Sestrins are encoded by genes whose expression is upregulated in cells exposed to a variety of environmental stresses including i) DNA damage, ii) hypoxia, iii) hypernutrition and chronic mTORC1 activation, iv) oxidative stress and v) hypoxia. Since all members of the Sestrin's family are induced by oxidative stress, it is reasonable to expect their involvement in the metabolism of ROS and other reactive metabolites. Silencing any of Sesn1-3 by shRNA causes accumulation of ROS in various cell lines (Budanov, 2004), leading to DNA damage and chromosomal instability (Kopnin, 2007; Sablina, 2005) or cell death (Budanov, 2002; Nogueira, 2008 Budanov, 2004; Hagenbuchner, 2012). Sestrins contribute to redox homeostasis through regulation of the AMPK-mTORC1 signaling pathway and prevent mTORC1 hyperactivation that stimulates ROS production through its effects on metabolism and mitochondrial function (Lee, 2010). Sestrin-dependent activation of AMPK and suppression of mTORC1 activity are critical for maintaining basal autophagy (Maiuri, 2009). Thus, Sestrins can be important for autophagic elimination of dysfunctional mitochondria that leak electrons and produce pathogenic amounts of ROS (Ishihara, 2013). Sestrin-dependent inhibition of mTORC1 can be also important for autophagy-mediated degradation of Keap1, an inhibitor of Nrf2-dependent antioxidant gene expression (Bae, 2013). A recent report also demonstrated that, through AMPK activation, Sestrin2 can inhibit NADPH oxidase 4 (NOX4) that generates pathogenic amounts of cytosolic ROS (Eid, 2010; Eid, 2013). The Sestrins were also shown to mediate the antioxidant activities associated with the p53 and FoxO transcription factors (Hagenbuchner, 2012; Nogueira, 2008; Sablina, 2005). While high levels of oxidative stress can lead to cell death through p53- and FoxO-dependent apoptotic gene transcription, low levels of oxidative stress cause moderate activation of p53 and FoxO that can induce Sestrins to reduce oxidative stress and prevent cell death (Lee, 2013). Sestrins are also able to directly bind to

AMPK, as well as to indirectly activate AMPK gene transcription stimulating AMPK signaling and its phosphorylation by upstream kinases, such as LKB1 (Budanov and Karin, 2008; Chen, 2010). Despite a lot of work is still needed to reveal the detailed molecular functions of the Sestrins, genetic studies have clearly shown that Sestrins maintain metabolic homeostasis and protect cells and organisms from oxidative stress, mainly through the regulation of the AMPK-TORC1 axis.

3.5 AMPK: A NUTRIENT AND ENERGY SENSOR

Many studies reported that AMPK signaling coordinates multiple metabolic pathways, acting as a main cellular energy sensor and a master regulator of the cellular metabolic homeostasis (figure 13) (Hardie, 2007).
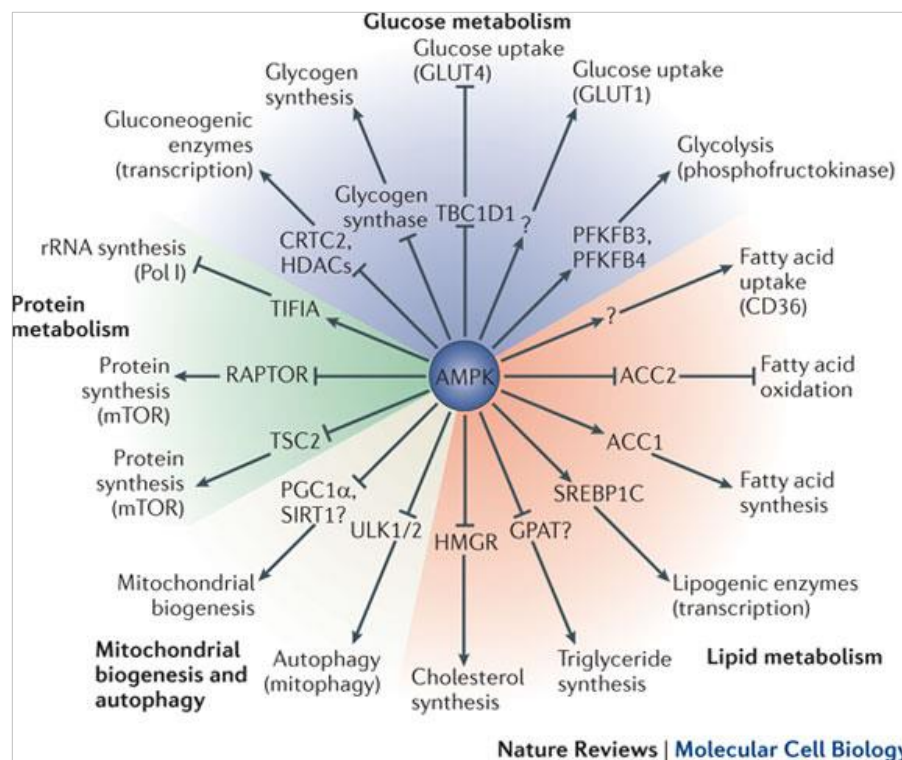
Figure 13. **Effects of activation of AMPK on cellular metabolism**. In the figure the proteins that are supposed to mediate the metabolic effects of AMPK are shown, as well as the final metabolic outcomes. (Hardie et al., 2012).

AMPK is a highly evolutionarily conserved serine/threonine protein kinase complex consisting of three subunits, α, β and γ. The α-subunit carries out the catalytic function, while β and γ subunits have a regulatory role (Hardie, 1998). AMPK is activated by metabolic stresses that inhibit ATP production or stimulate ATP consumption. Once activated, AMPK exerts its function stimulating catabolism and inhibiting anabolism by phosphorylating a number of downstream transcription factors related to metabolism and metabolic enzymes (Hardie, 2007). Many studies showed that activated AMPK increases PGC-1α gene expression in cultured muscle cells, in umbilical vein endothelial cells, in fat, and in skeletal muscle (Jorgensen, 2005; Terada, 2002; Girii, 2006; Terada, 2004; Suwa, 2003; Winder, 2006; Kuhl, 2006; Sriwijitkamol, 2006; Ojuka, 2004; Kukidome, 2006; Taylor, 2005). Interestingly, AMPK binds and activates PGC-1α in muscle cells by direct phosphorylation on two critical residues, threonine-177 and serine-538 (Sibylle, 2007). Therefore, since PGC-1α can function as a regulator of its own gene expression in muscle, in a feedforward loop (Handschin, 2003) it is conceivable that the PGC-1α phosphorylation by AMPK is involved in the induction of the PGC-1α gene. Therefore, posttranslational modifications of PGC-1α by AMPK play an important role in integrating environmental changes into the corresponding metabolic adjustments.

Notably, AMPK is also a master regulator of autophagy. In fact, it inhibits mTORC1 through phosphorylation of TSC2 and Raptor in response to cellular energy cues (Gwinn, 2008; Herrero-Martin, 2009; Inoki, 2006; Matsui, 2007; Meley, 2006). Moreover, Kim *et al.* demonstrated that AMPK-dependent ULK1 phosphorylation is a required step to trigger the autophagy machinery (Kim, 2011). Under energetic stress, ULK1 promotes autophagy by targeting several downstream crucial autophagy effectors involved in the initiation of the process, such as the actin-associated motor protein myosin II and ATG9 [39]. Recent studies reported that phosphorylation of Beclin-1 by ULK1 enhances the activity of ATG14L-containing Vps34 complex, which is crucial for the full autophagic induction in mammalian cells (Russell, 2013). The functional interplay between AMPK and wtp53 is a well-described mechanism involved in tumor suppression. Indeed, the stimulation of AMPK by energy stress promotes the phosphorylation

and activation of wtp53 (Jones, 2005; Okoshi, 2008). Moreover, AMPK is able to increase both the activity and the stability of wtp53 through direct phosphorylation of p53 and inactivation of MDMX-mediated ubiquitination process, thus prolonging half-life of wtp53 itself (He, 2014). Wtp53 may in turn increase AMPK activity through transcriptional activation of the gene encoding the β subunit of the enzymatic complex (Feng, 2007) and Sestrins (Budanov, 2008), providing a positive feedback that sustains AMPK signaling.

Regulation of AMPK signaling by mutant p53 proteins

It has been recently shown by Zhou *et al.* that mutp53 proteins, in contrast to their wild type counterpart, are able to inhibit AMPK signaling in head and neck cancer cells directly binding to the AMPKα subunit, thus gaining their oncogenic function and stimulating anabolic growth of cancer cells (Zhou, 2014). Interestingly, in response to glucose deprivation and metabolic stress, the phosphorylation on Thr172 of AMPKα, an event required for a full AMPK activation (Hawley, 1996), is inhibited in cancer cells expressing several GOF mutp53s, as R175H, P151S, G245C and R282W when compared with control cells lacking mutp53 expression. Moreover, Zhou *et al.* observed that mutp53s fail to regulate the expression level of the AMPK subunits excluding a transcriptional control in mutant p53-mediated AMPK inhibition (Zhou, 2014). After knocking-down of endogenous mutp53 variants the authors observed an induction of both AMPK Thr172 phosphorylation and of its direct downstream target acetyl-CoA carboxylase in response to glucose deprivation or metabolic stress, providing further evidence that GOF mutp53s inhibit AMPK signaling. Furthermore, the authors demonstrated that energy stress strongly induces AMPKα-mutp53 interaction *in vivo* and that mutp53s can directly interact with both AMPKα1 and AMPKα2 mainly through their DNA-binding domain (DBD), while the N-terminus of mutp53s is responsible for blocking the interaction between AMPKα and its upstream kinase LKB1 inhibiting its Thr172 phosphorylation and consequently preventing its activation (Zhou, 2014). Moreover, since AMPK is a master energy sensor and a regulator of metabolic homeostasis, the demonstration

that mutp53s are actively involved in the sustaining of cell growth and regulation of cancer metabolism through direct inhibition of AMPK (figure 15) further extends the understanding of the molecular mechanisms of GOF mutp53s in cancers. Importantly, AMPK was recently identified as a negative regulator of the Warburg effect through inhibition of the hypoxia-induced factor 1 (HIF-1) pathway (Faubert, 2013). Therefore, the inhibition of AMPK by GOF mutp53s, which relieves the suppression of HIF-1 by AMPK, is expected to increase HIF-1 protein expression and thus lead to increased glucose influx and glycolysis. This could represent a further mechanism of GOF mutp53 to promote the Warburg effect and drastic metabolic changes in cancer cells, in addition to that mentioned previously. The inhibition of AMPK signaling by mutp53 provides an excellent example of how a gene mutation in tumor cells can transform an agonistic relationship (i.e., wtp53 activating AMPK signaling) into an antagonistic relationship (i.e., mutp53 inhibiting AMPK). In this case, p53 mutation transforms a signaling network with tumor-suppressing functions into a network with oncogenic potential to promote tumor growth and progression.
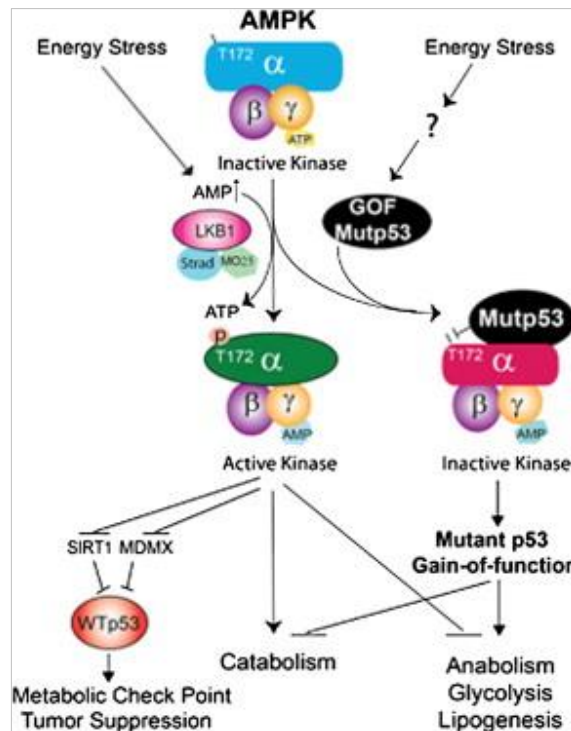
Figura 15. GOF mutp53s with high AMPKα-binding affinity gain oncogenic functions to promote cell growth and cancer cell metabolism through direct inhibition of AMPK activation (Zhou, 2014)

3.6 mTOR: A CELLULAR HUB DRIVING CELL GROWTH

Mammalian target of rapamycin (mTOR), a serine/threonine protein kinase with a large molecular size, belongs to the phosphatidylinositol kinase related kinase (PIKK) family and it is implicated in the regulation of multiple cellular processes including cell growth, cell cycle, cell survival, as well as autophagy. The observation that treatment with rapamycin is sufficient to induce autophagy even in the presence of nutrients represents a valid evidence for the conclusion that mTOR complex is a powerful repressor of autophagy (Noda, 1998). Genetic and biochemical studies demonstrated that the inhibition of ULK1 by mTOR is a crucial mechanism by which it explicates its repressive function on autophagy (Chan, 2009; Mizushima, 2010). mTOR is composed by two multiprotein enzymatic functional complexes, mTORC1 and mTORC2 (Loewith, 2002). Contrarily to mTORC2, mTORC1 is sensitive to the inhibition by rapamycin and it is directly regulated by the cellular nutrient status, including growth factors and amino acids availability, playing essential roles in the regulation of protein translation and autophagy (figure 16).
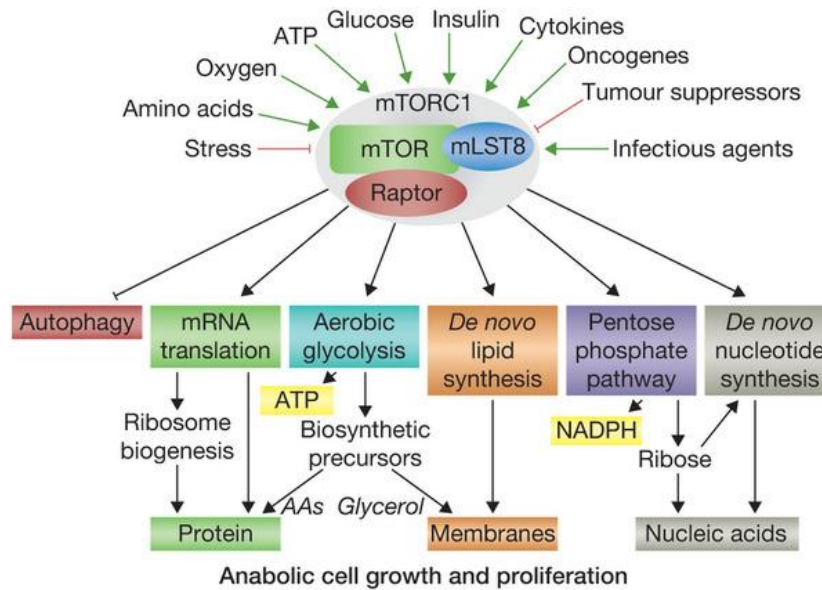
Figure 16. mTORC1 signaling links cellular growth conditions with metabolic processes underlying anabolic cell growth and proliferation. Many physiological and pathological signals affect the activation status of mTORC1, including cellular nutrients and energy, growth factors, oncogenes and tumor suppressors, and a variety of intracellular pathogens. When activated, mTORC1 regulates a number of cellular processes, with those affecting the metabolic state of the cell emphasized in this model.. (Dibble and Manning, 2013).

Raptor, a component of mTORC1, functions as a scaffolding protein in order to recruit different substrates such as p70S6 kinase-1 (S6K1) and eIF4E binding proteins (4EBPs) for phosphorylation by mTORC1 (Nojima, 2003; Schalm, 2003). The ribosomal protein S6K1 is phosphorylated on Thr389 by mTORC1 and this post-translational modification recruits phosphoinositide-dependent kinase-1 (PDK1) and activates many downstream targets such as eIF4B, PDCD4, Skar, and S6, thereby regulating mRNA processing, protein translation, and cell growth (Dorrello, 2006; Shahbazian, 2006). Importantly, recent studies elucidated the mechanism by which mTOR regulates ULK1 in autophagy blockage. Kim *et al.* unveiled that mTOR can phosphorylate ULK1 on Ser757 disrupting its interaction with AMPK, which is a required event to activate ULK1 and to induce the autophagic pathway (Kim, 2011). Therefore, mTOR complex represents a key factor in the cell growth process and its dysregulation, primarily due to

inactivating mutation in TSC1/TSC2 or constitutive activation of the PI3K upstream pathway, drastically contributes to tumorigenesis. Both TSC2 and Sestrins are key inhibitors of mTOR activity and direct targets of wtp53-directed transcription (Feng, 2007). TSC2 inhibits mTOR by facilitating the conversion of the essential mTORC1-activator Rheb-GTP to the inactive Rheb-GDP (Tee, 2003). Sestrins have been shown to inhibit mTOR kinase activity in different ways, including the stimulation of AMPK activity and the suppression of mTOR translocation to the lysosomes (Parmigiani, 2014; Peng, 2014). Moreover, it has been reported that after DNA damage wtp53 exerts a negative control in the regulation of cancer cell growth stimulated by mTOR through the intermediate activation of the AMPK (Feng, 2005)

PKM2 is a key multifaceted enzyme regulated by mTOR frequently enhanced in cancer

Pyruvate kinase is an enzyme that catalyzes the conversion of phosphoenolpyruvate (PEP) and ADP to pyruvate and ATP in glycolysis and plays a role in regulating cell metabolism. The M2 isoform of pyruvate kinase (PKM2) supports anabolic metabolism and is mainly expressed in cancer tissues. Preferential expression of PKM2 in tumor cells is implicated in cancer cell survival and drug resistance, for instance, by increasing the level of the anti-apoptotic protein Bcl-xL via enhancing NF-κB p65 stabilization (Li, 2014). The activity of PKM2 is regulated by both intracellular pathways, including mTOR signaling, and metabolites. Indeed, it has been demonstrated a direct connection between mTOR signaling and PKM2 in cancer. (Sun, 2011). mTOR activation simulates HIF-1α expression, and HIF-1α in turn enhances PKM2 expression through the collaboration with c-Myc–hnRNPs splicing regulators (Sun, 2011). Subsequently, along with other glycolytic enzymes, PKM2 stimulates aerobic glycolysis, a metabolic shift often observed in pseudo-hypoxic tumors. The existence of mTOR/HIF1α/Myc–hnRNPs/PKM2 glycolysis cascade is essential for cell proliferation and tumor growth and the frequent hyperactivation of mTOR signaling during the course of the multistep oncogenesis might contribute to the

development of the Warburg effect in human cancers. However, the involvement of PKM2 in cancer is not constrained to the regulation of glycolysis. Indeed, beyond its dominant role in glycolysis to achieve the nutrient demands of cancer cell proliferation, PKM2 contributes to tumorigenesis through crucial non-metabolic functions. It has been reported that PKM2 can translocate into the nucleus of the cells and directly interact with HIF-1α subunit promoting transactivation of HIF1-target genes by enhancing its binding and recruitment with the transcriptional coactivator p300 (Luo, 2011). PKM2 can further interact with β-catenin phosphorylated by EGFR signaling and this interaction is required for the two proteins to be recruited to the cyclin D1 (CCND1) promoter (Yang, 2011). Thus, PKM2-dependent β-catenin transactivation is described to be required for EGFR-promoted tumor cell proliferation and brain tumor development (Wu, 2014). Intriguingly, PKM2 in addition to play a role as a transcriptional coactivator may function as a protein kinase that directly phosphorylates signal transducer and activator of transcription 3 (STAT3) stabilizing its nuclear localization (Gao, 2012). The activation of STAT3 in malignant cells is possibly one of the most important molecular signatures for promoting the progression of cancer (Yang, 2014). Recently, it has been described that the double N340Q/L344R mutant p53 promotes hepatocarcinogenesis through upregulation of PKM2 (Wu, 2016). Mechanistically, mutant p53 (N340Q/L344R) forms a complex with cancer up-regulated drug resistant (CUDR), a novel noncoding RNA, and the complex binds and stimulates the promoter regions of the PKM2 gene.

Regulation of mTOR signaling by mutant p53 proteins

An increasing number of evidence highlighted that mutp53 proteins acquire the oncogenic ability to stimulate mTOR signaling, representing an essential turning point to sustain cancer cell proliferation and growth. Agarwal *et al.* demonstrated that the ectopic expression of three hot-spot p53 mutant proteins, as R175H, R248W and R273H, causes the hyper-phosphorylation of the mTORC1 targets S6K1 and 4EBP1 in both p53-null HCT116 colon carcinoma cells and H1299

non-small cell lung carcinoma cells, while wtp53 reduces mTORC1 activity as revealed by the decreased phosphorylation of S6K1 and 4EBP1 (Agarwal, 2016). PI3K/Akt signaling pathway plays a crucial role on tumorigenesis regulating relevant cellular functions including survival, proliferation and metabolism. PI3K/Akt pathway is generally activated by a number of growth factor receptors including EGFR, MET and TGFβR, which promote the phosphorylation of Akt that propagates the kinase cascade by phosphorylating in turn many downstream effectors, including its direct target mTORC1. Hyper-activation of PI3K/Akt signaling is often detected in many cancers and hence represents a valid therapeutic target currently adopted in tumor therapy (Li, 2016; Yang, 2010). Importantly, a number of studies demonstrated that mutp53s exert their oncogenic function enhancing Akt activity and this may result in a consequent hyperactivation of mTOR signaling with an inhibitory effect on autophagy. Tan *et al.* reported that depletion of endogenous hot-spot R273H, R280K and R280T mutp53 isoforms can downregulate Akt phosphorylation in MDA-MB-468, MDA-MB-231 and CNE-1 cancer cell lines respectively, suggesting the involvement of mutp53s in the promotion of Akt signaling, which leads cancer cell survival (Tan, 2015). Moreover, the authors showed a direct correlation between high expression of mutp53s and strong phospho-Akt staining in primary human breast cancers providing a further suggestion of the clinical relevance of mutp53s in the PI3K/Akt axis stimulation (Tan, 2015). Many studies demonstrated that mutp53s can drive tumorigenesis by activating a number of growth factor receptors implicated in the activation of PI3K/Akt signaling, including TGF-β receptor (Adorno, 2009), EGFR (Sauer, 2009; Wang, 2013), and MET (Grugan, 2013; Muller, 2013). Furthermore, Xu *et al.* reported that MDA-MB-468, HT29 and A431 cancer cell lines expressing high levels of EGFR are susceptible to Akt de-phosphorylation following endogenous mutp53 depletion, suggesting a direct role of mutant p53 proteins in sustaining Akt pathway through overexpression of growth factor receptors (Xu, 2005). Accordingly, Guo *et al.* recently showed that the hot-spot R273H mutp53 enhances breast cancer cells migration by transcriptional downregulation of miR-30a, which can cover a role as tumor suppressor in non-small cell lung cancer (NSCLC) through the

downregulation of the Akt pathway by targeting Insulin-like Growth Factor 1 Receptor (IGF-1R) (Guo, 2016; Wen, 2015). Functionally, the suppression of miR-30a by R273H mutp53 prevents the inhibitory effect of miR-30a on the IGF-1R expression, thus leading to elevated activation of IGF-1R/Akt signaling cascade in tumor cells (Guo, 2016). Overall, many evidence support the hypothesis that the activation of PI3K/Akt and mTOR pathways by mutp53 proteins might be a direct consequence of an oncogenic stimulation of growth factor receptors driven by mutp53s.

4. AIM OF THE STUDY

Mutations in the *TP53* gene occur in over 50% of human cancers, where most of them are missense mutations resulting in the expression of mutant forms of p53 (Vousden and Lu, 2002; Waddell, 2014). In addition, p53 mutated proteins acquire new biological properties referred to as gain-of function (GOF) that contribute to the induction and maintenance of cancer (Santoro, 2014). In many human tumors, p53 mutations are associated with high genomic instability, poor prognosis, poor response to chemotherapy and accelerated tumor recurrence (Ganci, 2013; Liu, 2012; Walerych, 2012). Recently, we have documented that DNA damage induced by gemcitabine stabilized mutant p53 proteins in cell nuclei triggering chemoresistance and inducing the expression of cell cycle-related genes, as Cdk1 and CCNB1, and increased cell growth (Fiorini, 2015). However, despite different models have been proposed to explain the GOF activities of mutant p53 in cancer, the detailed mechanisms remain largely unknown. In this thesis we would dissect novel molecular mechanisms by which gain of function mutant p53 proteins promote cancer cell proliferation and chemoresistance through the dysregulation of some signaling pathways crucial for metabolic homeostasis of the cells. In particular, we aimed to investigate whether mutant p53 oncogenic functions in cancer cells might counteract the autophagy pathway and alter the redox status of the cells through the aberrant regulation of Sestrins/AMPK axis and mTOR signaling, thus promoting cancer cell proliferation and chemoresistance.

The main aims of this work can be summarized as follows:

-To identify the phenotypic effect on autophagy and redox status of the cells driven by mutant p53 proteins in a panel of cancer cell lines (i.e. autophagosome formation, LC-3II autophagy marker, mitochondrial ROS levels);

-To investigate the detailed molecular mechanisms underlying this regulation (i.e. transcriptional gene regulation and signaling pathways involved);

-To identify the functional role of autophagy and ROS regulation by mutant p53 in cancer cells (i.e. stimulation of cell proliferation, chemoresistance, patient's clinical outcome);

-To propose a personalized therapy for patients carrying mutant *TP53* gene, based on the detailed understanding of signaling pathways altered by mutp53s.

A better understanding of the molecular basis underlying GOF mutant p53 in cancer are required to develop new effective personalized treatments that could improve the clinical outcome of cancer's patients bearing mutant p53 proteins.

5. RESULTS

Gain of function mutant p53 proteins inhibit the autophagic vesicles formation in cancer cells

To study the functional role of GOF mutant p53 proteins in the autophagic mechanism, we first analyzed the endogenous level of the autophagosome vesicle formation by staining various cancer cell lines with the fluorescent probe MDC. Cancer cells with different missense mutations of the *TP53* gene shoewd the endogenous level of the autophagosome vesicles about 5-6 folds lower than cells with wild-type *TP53* alleles (Figure 17A). When PaCa3 and MCF7 cell lines (expressing the wild-type p53 protein) were knocked down for p53 expression, the autophagosome formation was inhibited, accordingly with the literature (Figure 17B and C) (Crighton, 2007). Conversely, the autophagosome formation was significantly increased after knock-down of GOF mutant p53 in six cancer cell lines (Figure 17B and C). Consistent with this, overexpression of mutant p53 (R175H or R273H) proteins in AsPC1 cells (null for p53 expression) produced a drastic reduction of MDC probe incorporation revealing an inhibitory role of mutant p53 on the autophagic process (Figure 17B and C). To further support the idea that mutant p53 may have a role in the inhibition of the autophagic event, we analyzed the expression of the autophagic receptor/adaptor SQSTM1/p62 (sequestosome 1), whose massive accumulation is usually a consequence of autophagy impairment and accumulation of aggregated structures for ubiquitinilation (Katsuragi, 2015). Accordingly, we observed that mutant p53 knockdown decreased p62 expression (Figure 17D). Furthermore, we evaluated the expression levels of the lipidated and truncated isoform II of the light chain 3 protein (LC3-II), which is functionally involved in the formation and maturation of autophagic vesicles. Our findings showed that LC3-II protein expression levels considerably decreased in AsPC1 cells after ectopic expression of mutant R175H or R273H p53 proteins while increased in mutant p53-knocked down cells (Figure 17D). We also observed that chloroquine (CQ) further increased the amount of LC-II in silencing mutant p53 conditions (Figure 17D), accordingly with the

hypothesis of the autophagic inhibitory flux together with the blockage of the lysosomal degradation. According to this, confocal microscopy analysis revealed that mutant p53 counteracts the fusion between autophagic vesicles (identified by LC3-GFP overexpression) and lysosomes (identified by lysotracker red probe) since the merged image of Panc1- shp53 cells showed a significant co-localization of LC3-II with lysosomes represented by yellow puncta and confirmed by the RGB profile (Figure 17E). Furthermore, we have examined the formation of autolysosomes (autophagic vesicles joined with lysosomes) using the fluorescent probe acridine orange (AO), which changes its fluorescence emission from green to red upon accumulation into lysosomal acidic compartments. The typical pH acidification of lysosomes during autophagic stimulation has been examined by AO probe staining in knocked down mutant p53 conditions (Figure 17F and G). Figure 17F showed that mutant p53 cell lines (Panc1 and SKBr3) had a high red/green fluorescence ratio in the sh-p53 condition, conversely wild-type p53 cells (MCF7) showed a decrease of the autolysosome formation (Figure 17F). The microscopy experiments confirmed these results revealing the presence of red areas (acidic lysosomes containing accumulation of AO probe) into the cytoplasm of the cells interfered for mutant p53 expression (Figure 17G). Overall these data strongly support the statement that GOF mutant p53 proteins may contribute to prevent both the formation of the autophagic vesicles and their fusion with lysosomes in cancer cells.
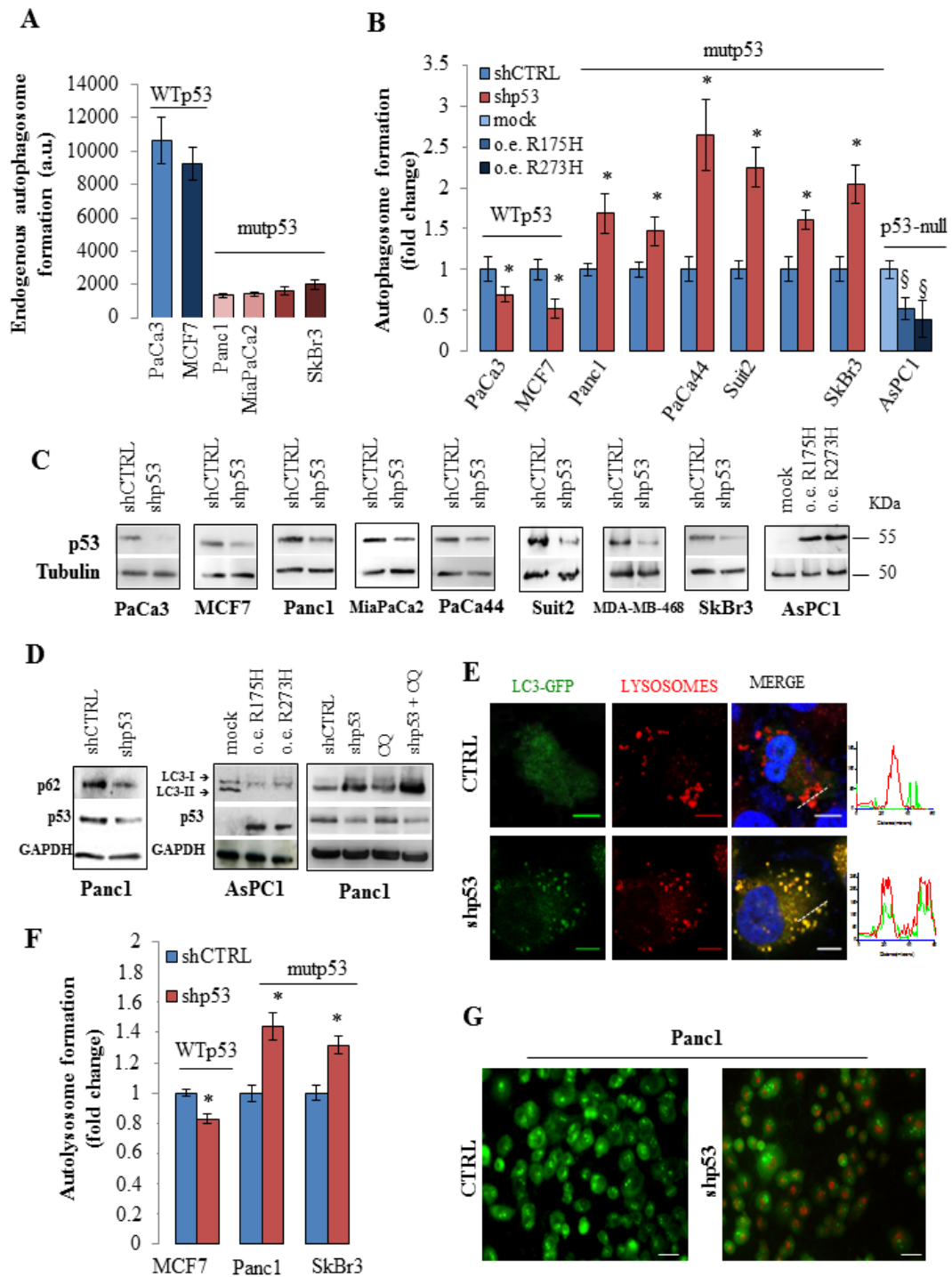
Figure 17. Mutant p53 counteracts autophagy. (A) The endogenous basal level of autophagosomes in cancer cells carrying WT or mutant p53 was analyzed using the incorporation of the fluorescent probe monodansylcadaverine (MDC). (B) Autophagosome formation assay was performed using the incorporation of MDC probe.

(C) Western blot of p53 using the protein extract of the samples used for (B) to test the effective knock down of WT or mutant p53 and the overexpression of mutant p53 in the various cell lines indicated. (D) Whole-cell extracts were used for Western blot analysis of the autophagic proteins p62/SQSTM1 and LC3 (isoforms I and II), p53 and GAPDH (as control loading). (E) 48 h after co-transfection with plasmids coding for LC3-GFP and pRSuper-p53 vector (shp53) or LC3-GFP and the empty vector (negative control; CTRL), Panc1 cells were fixed. Lysosomes and nuclei were stained with Lysotracker (red) and Hoescht (blue), respectively. The RGB profile plotted along the dashed line drawn in the merge image is also shown. Merge and single channel images come from a single z-plane. Scale bar 10 mm. (F) Autolysosome formation analyzed by red/green fluorescence intensity ratio quantification of acridine orange (AO) staining in the indicated cells transfected with pRSuper-p53 vector (shp53) or with its negative control (shCTRL). (G) 48 h after the p53 depletion Panc1 cells were stained with an AO solution and observed at 40X magnification. Scale bar 40 μm. All the experiments presented in this figure are representative of three biological replicates. P-values were calculated with two-tailed t-test. Statistical analysis: $*p < 0.05$ shp53 vs shCTRL; $p < 0.05$ R175H or R273H vs mock.

Autophagy inhibition by mutant p53 increases the proliferation of cancer cells

To unravel the biological role of autophagy inhibition in the aberrant proliferation induced by mutant p53, we knocked-down the essential autophagic protein Atg5 in Panc1, MiaPaCa2, and MDA-MB-468 cells (Mizushima, 1998). The MDC assay established that Atg5 knockdown effectively repressed the autophagosome formation triggered by mutant p53 downregulation (Figure 18A). Importantly, the depletion of Atg5 caused a reversion of cell growth inhibition (Figure 18B) and a decrease of apoptosis (Figure 18C) stimulated by mutant p53 knockdown. These findings indicate that autophagy inhibition strongly contributed to the increased proliferation and to the concomitant down-regulation of the apoptotic stimuli driven by mutant p53.
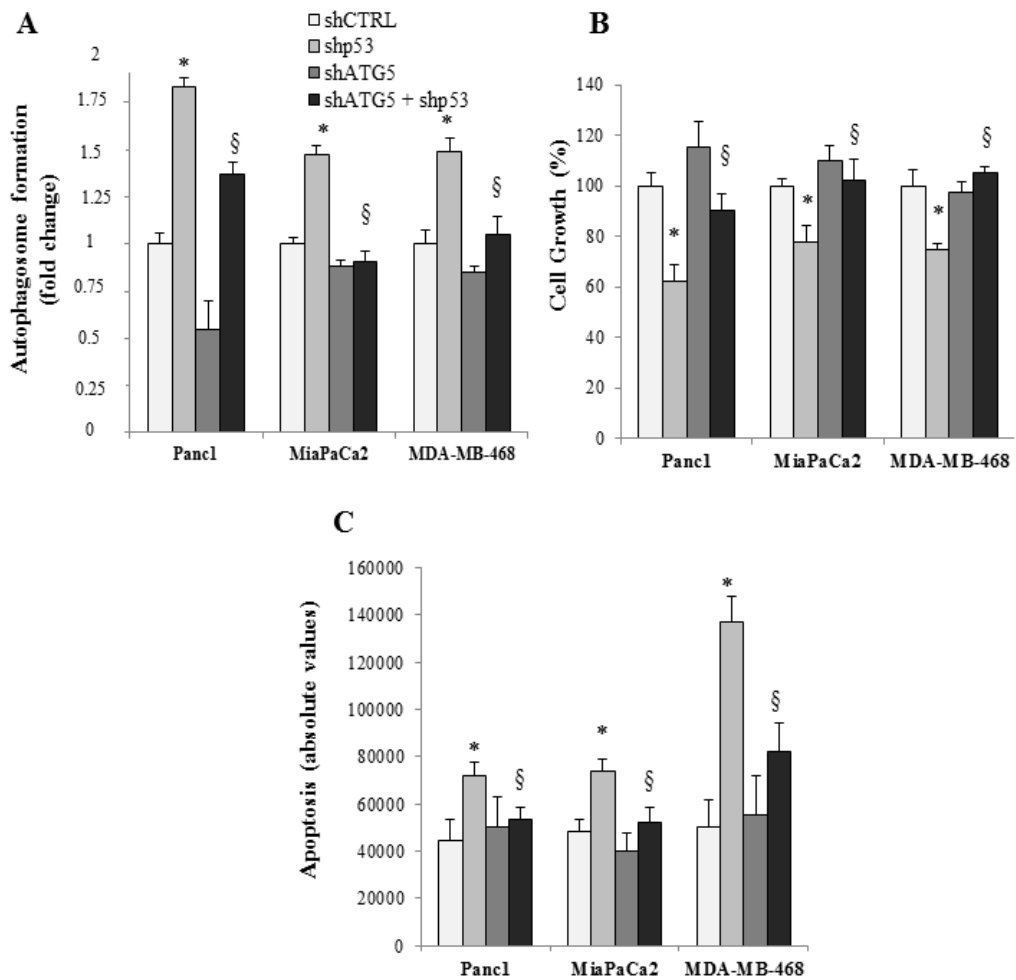
Figure 18. Impact of autophagy inhibition by mutant p53 on cell growth and apoptosis. Panc1, MiaPaCa2 and MDA-MB-468 cell lines were seeded in 96-well plates and transfected with pRSuper-p53 vector (shp53) and/or pMSCV-Puro-miR30-Atg5 (shAtg5), or their relative negative controls (shCTRL) for 48 h. Autophagosome formation (A), cell growth (B), and apoptosis (C) were determined using MDC assay, crystal violet colorimetric assay and annexinV/FITC binding assay, respectively. All the experiments presented in this Figure are representative of three biological replicates. P-values were calculated with two-tailed t-test. Statistical analysis: * $p < 0.05$ shp53 vs shCTRL; § $p < 0.05$ shp53 vs shATG5 vs shp53.

Mutant p53 protein inhibits the expression of autophagic genes in tumoral cell lines

The autophagic pathway is a complex sequence of biological events leading to formation, maturation and fusion of autophagosomes with lysosomes to allow the degradation and recycling of cellular components (Viry, 2014). These events are driven by a number of autophagy-related genes (ATGs), which are mostly transcriptionally induced by autophagic stimuli such as nutritional deprivation, infections or metabolic and oncogenic stress (Galluzzi, 2015). In the present thesis, we have shown that tumor cells carrying mutant p53 proteins escaped autophagic activity (Figures 17). In order to investigate the role of mutant p53 in autophagy inhibition, we studied whether mutant p53 proteins might control the expression of some crucial ATGs, as atg12, becn1, sesn1, sesn2 and dram1 genes. The depletion of mutant p53 by p53 siRNA smart pool oligos transfection (sip53), determined an increase of the mRNA expression in most of these ATGs in Panc1, MDA-MB-468 and SKBr3 cell lines (Figure 19A). In these cell lines, we have checked the CCNB1 mRNA expression as a control of our cellular systems (Figure 19B). Indeed, Cyclin B protein is important for the control of the cell cycle progression and it is a well-established mutant p53 target gene (Di Agostino, 2006). Conversely, the depletion of WTp53 expression in MCF7 breast cancer cell line led to a general decrease of the expression of ATGs (Figure 19A). Accordingly, ectopic expression of mutp53R175H or mutp53R273H in p53-null H1299 cells led to a significant reduction of ATGs transcripts (Figure 19A). Moreover, in Figure 19C, we reported that atg12 and becn1 transcripts increased substantially in three separate biological clones of MDA-MB-468 breast cancer cells (carrying mutp53R273H) where the mutant p53 knock-down was induced by doxycycline (DOX) [our elaboration of the data set from Freed- Pastor and colleagues published in (Freed-Pastor, 2012)].
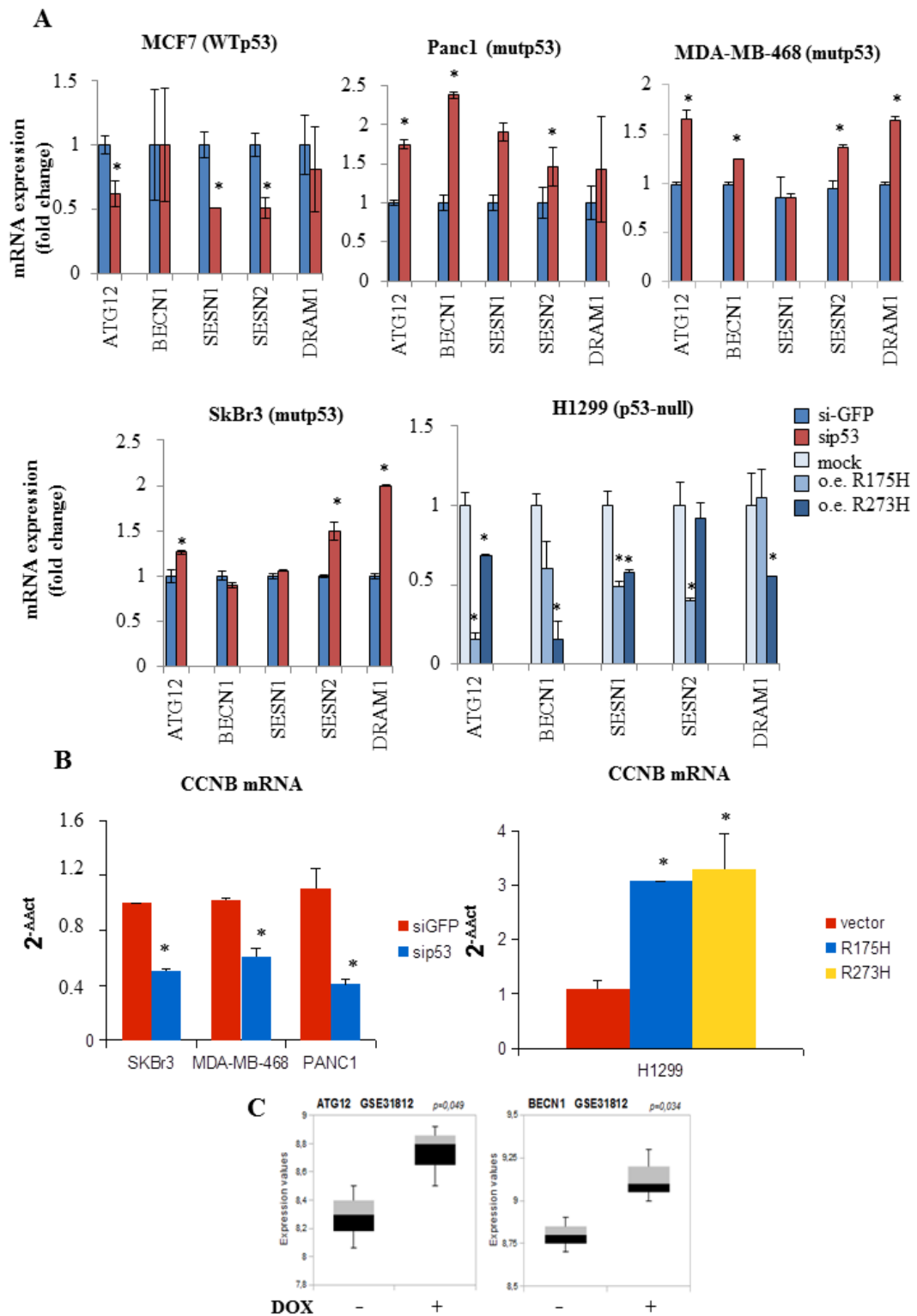
Figure 19. Autophagy-related gene regulation by mutant p53. A) The indicated cell lines were transfected with si-p53 and si-GFP (as control) oligonucleotides. H1299 cells were transfected with mutp53R175H, mutp53R273H or pcDNA3 (mock) vectors for 48 h. Gene expression analysis of the autophagy-related genes ATG12, BECN1, SESN1,

SESN2, and DRAM1 was performed by RT-qPCR and was normalized to GAPDH mRNA. *p < 0.05 si-p53 vs si-GFP and R175H or R273H vs mock. All the experiments are representative of three biological replicates. P values were calculated with two-tailed t-test. B) Indicated cell lines were transfected with pRSuper-p53 vector (shp53), with pCDNA-p53R175H, pCDNA-p53R273H plasmids or their negative controls (empty pRSuper and pCDNA3 mock vector, respectively). Gene expression analysis of CCNB1 was performed by RT-qPCR and was normalized to GAPDH mRNA. * p<0.05 sip53 vs siGFP; R175H or R273H vs vector. (C) Box plot of ATG12 and BECN1 mRNAs expression obtained in MDA-MB-468 breast cancer cells by elaboration of data set from Freed-Pastor and colleagues (Freed-Pastor, 2012; Rhodes, 2004).

Mutant p53 protein and autophagic genes expression in cancer patients

In order to further investigate the inverse relationship between the expression of mutant p53 and two representative ATGs (atg12 and becn1 transcripts), we queried public gene expression data repositories (http://www.oncomine.org/) (Rhodes, 2004). Our analysis of the data set from Gluck *et al.* (Gluck, 2012) showed that atg12 and becn1 transcripts were significantly down-regulated in breast cancer patients carrying mutant p53, compared to those with WTp53 (Figure 20A). These data strongly confirm the inhibitory role of mutant p53 *in vitro* and *in vivo* on the expression level of ATGs. Moreover, to test the potential prognostic value of ATGs in cancer patients, we considered a gene expression signature (atg12, becn1, sesn1, and dram1) by selecting autophagy-related genes that were upregulated by mutant p53 depletion in Figure 19A. To assess the potential correlation between the experimental autophagic gene signature and the patient's clinical outcome, we focused our attention on a subgroup of breast cancer patients expressing mutant p53 proteins independently by intrinsic subtype (luminal, basal-like, Her+) (Figure 20B). We analyzed gene expression datasets and survival information downloaded from GEO and sorted for the presence of *TP53* gene mutations (www.ncbi.nlm.nih.gov/geo/) (www.kmplot.com) (Gyorffy, 2010). To analyze the prognostic value of the signature, we split patients into two groups (high and low expression levels of autophagic gene signature) by the median of the expression values. The two groups were then compared in terms of

relapse free survival (RFS) and distant metastasis free survival (DMFS). KM analysis in the Figure 20B showed that low atg12, dram1, sesn1 and becn1 signature expression was significantly associated with a poor prognosis in the mutant p53 breast cancer subgroup (RFS Statistically significant results with p-value=0.02, N=188; DMFS Statistically significant results with p-value=0.0028, N=83).
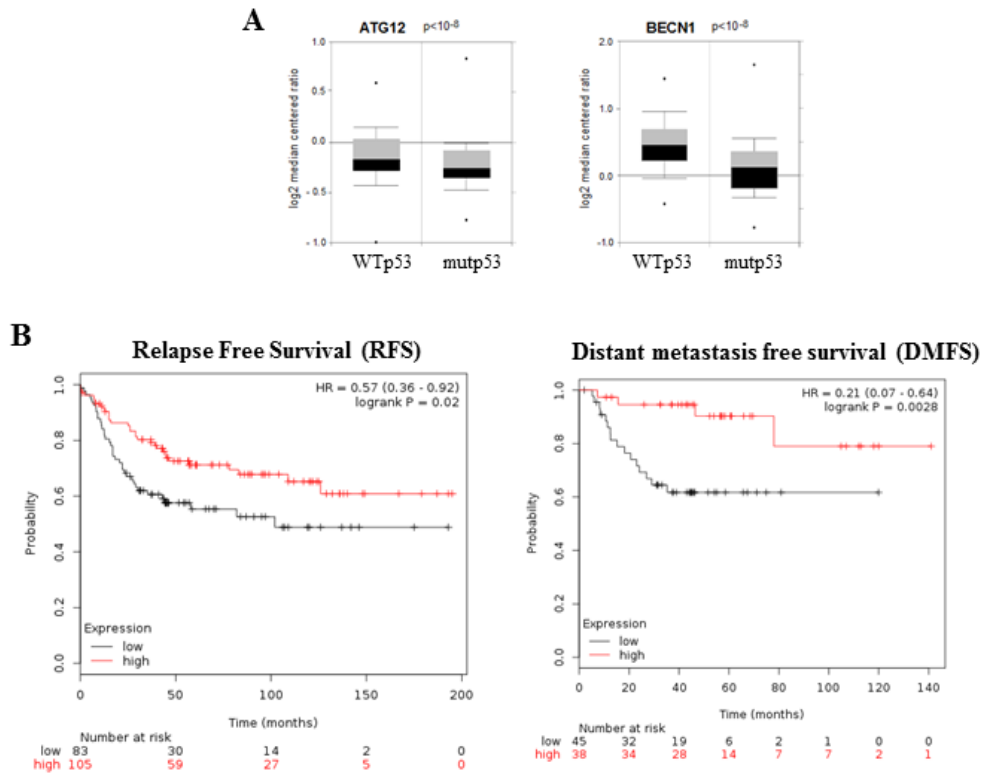
Figure 20. (A) Box plots of ATG12 and BECN1 mRNAs expression in breast cancer patients (n=72 for wt-p53 and n=72 for mut-p53). Data from Gluck and colleagues were obtained from www.oncomine.org website (Gluck et al., 2012). (B) Kaplan-Meier survival curves of relapse free survival (RFS) and distant metastasis free survival (DMFS) of breast cancer patients bearing mutant *TP53* gene classified according to the expression of atg12, becn1, sesn1, and dram1 signature. RFS statistically significant results with p-value=0.02, N=188; DMFS statistically significant results with p-value=0.0028, N=83. The two compared groups are the patients with the highest expression (red) levels of the signature versus the patients with the lowest expression (black).

Mutant p53 inhibits atg12 expression by interaction with the NF-κB p50 subunit

Mutant p53 proteins bind to the promoters of their target genes through cooperating with other transcription factors, such as E2F1, NF-Y, Sp1 and others (Di Agostino, 2006; Stambolsky, 2010; Strano, 2007; Valenti, 2015; Weisz, 2007). Since ATG12 is an essential mediator of the initial phases of autophagosome vesicle formation, we studied this gene looking for consensus regions in the atg12 gene promoter binding potential transcriptional repressors. Our *in silico* analysis by using MatInspector software (www.genomatix.de) revealed the presence of two NF-κB p50 specific consensus sequences in the atg12 promoter: atg12 promoter Region 1 (-1055 bp) and atg12 promoter Region 2 (-513 bp) (Figure 21A). MatInspector software uses TRANSFAC matrix that was able to discriminate between the specificity of binding between NF-κB p50 and NF-κB p65 factors conferring different and specific position frequency to the nucleotides belonging to the promotorial consensus sequence (data not shown). As the NF-κB homodimer p50-p50 usually acts as a transcriptional repressor (Elsharkawy, 2010; Tong, 2004), we studied its recruitment onto the atg12 promoter. Chromatin immunoprecipitation experiments (ChIP) in SKBr3 cells revealed that p50 was actually bound to both the two binding regions of atg12 promoter and that this protein-DNA interaction was removed by transfection of siRNA p50 oligos (Figure 21B). Notably, ChIP assay showed that also mutant p53 protein was recruited onto p50 consensus sites and its binding decreased after p50 depletion (Figure 21B). As expected by the *in silico* analysis, NF-κB p65 did not bind the two atg12 promoter regions that recruited NF-κB p50 (Figure 21B). In order to support new evidence that mutant p53 and p50 bind onto the atg12 gene promoter, we aimed to investigate the existence of a novel floating mutp53/p50 protein complex. Coimmunoprecipitation experiments from whole protein extracts of Panc1 and SKBr3 cancer cell lines endogenously expressing mutant p53 proteins revealed the formation of a mutp53/p50 protein complex (Figure 21C). Again, NF-κB p65 isoform was not detected in this protein complex in any coimmunoprecipitation conditions, confirming its absence on the regions of the atg12 promoter as previously discussed (Figure 21C). We supported this result by also showing the formation of the complex between the overexpressed

mutp53R175H and endogenous p50 proteins in H1299 cells (Figure 21C). Strikingly, the depletion of p50 led to the resumption of atg12 mRNA transcription in mutant p53 Panc1 and MDA-MB-468 cells (Figure 21D). According to the above results, the p50 knock down had a functional effect on the increase of autophagosome formation only in SKBr3 and Panc1 cells (bearing mutant p53), thus supporting the inhibitory role of mutant p53/p50 complex in the autophagic process (Figure 21E).
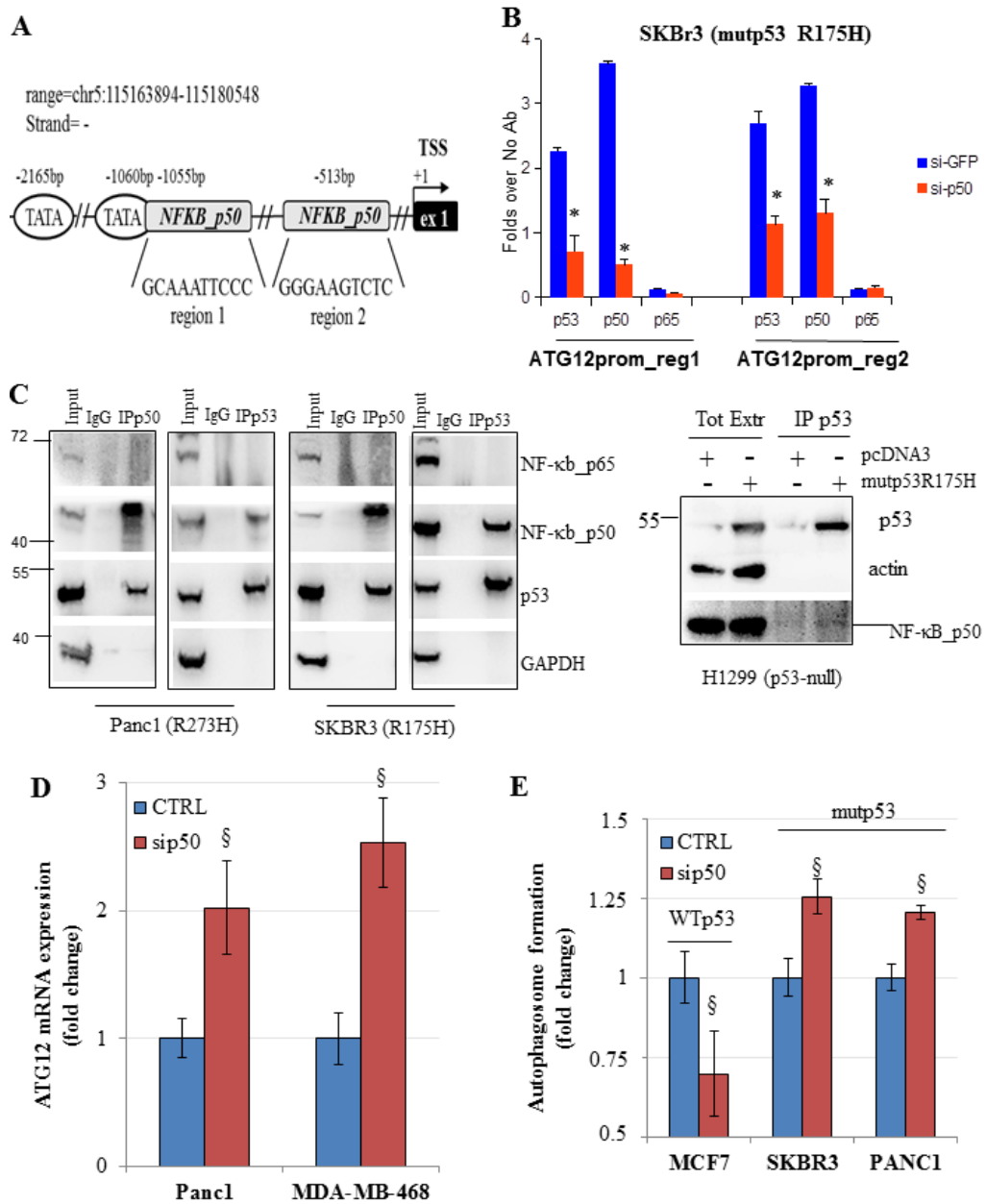
Figure 21. Mutant p53 inhibits atg12 expression by interaction with NF-κB p50 subunit. (A) Schematic representation of the atg12 promoter regions containing NF-κB p50 consensus box sequences analyzed in ChIP assays. Atg12 promoter: two regions at 1055 bp (region 1) and 513 bp (region 2) upstream the first exon of ATG12 gene. The TSS is indicated and it was predicted by Eponine software (Down and Hubbard, 2002). (B) Cross-linked chromatin derived from sip50-treated SKBr3 cells and from siGFP-transfected control, was immunoprecipitated with the indicated antibodies or in the absence of antibody, and analyzed by RT-qPCR with specific primers (see Material and Methods) for the indicated regions in the Figure 4A. The P-values were calculated with two-tailed t-test. * $p < 0.05$. (C) (Left panel). Immunoprecipitations of NF-κB p50 or NF-κB p65 proteins and western blot analysis for p53 binding are performed from lysates of the indicated cancer cell lines expressing mutant p53 proteins (described in Material and Methods). NF-κB p50 or NF-κB p65 proteins were immunoprecipitated with their relative rabbit polyclonal antibody and the same amount of rabbit IgG was used as negative control of IP. (Right panel) Cell lysate from H1299 cells over-expressing mutp53R175H protein was immunoprecipitated with p53 sheep polyclonal antibody. Cells transfected with the empty vector (pcDNA3) was used as negative control. (D) Cells were transfected with the siRNAp50 or its negative control for 48 h. Gene expression analysis of ATG12 mRNA was performed by RT-qPCR and normalized to GAPDH mRNA. The P-values were calculated with two-tailed t-test. Statistically significant results were with p-value < 0.05. (E) Cells were transfected with siRNAp50 or its relative negative control. Autophagosome formation assay was performed using the incorporation of MDC probe. Statistical analysis: * p<0.05 p53 or p50 vs No Ab; § sip50 vs CTRL.

Mutant p53 proteins inhibit AMPK and stimulate mTOR signaling

In order to examine whether mutant p53 impinged in the crucial regulatory kinase complexes of the autophagic process we analyzed the protein expression and the phosphorylation state in different cancer cell lines expressing wild type or mutant p53 proteins. We found that the knock down of mutant p53 in either Panc1 and SKBr3 cell lines led to an increase of Sestrin1 and Sestrin2 protein levels, while silencing of wild type p53 in PaCa3 and MCF7 cancer cells resulted in a general down-regulation of Sestrin protein levels (Figure 22A). Sestrins are a class of proteins that can directly interact with AMPK subunits favouring their

phosphorylation by upstream kinases and thereby resulting in AMPK signaling stimulation (Budanov and Karin, 2008; Morrison, 2015; Sanli, 2012). To determine whether mutant p53 could affect the phosphorylation of AMPK, we analyzed the level of phospho-AMPKα (Thr172) after modulation of mutant or wild type p53 expression. We observed that depletion of mutant p53 was able to increase the phosphorylation level of AMPK [P(Thr172)-AMPK/AMPK] and to decrease that of p70S6K [P(Thr)389- p70S6K/p70S6K], a direct target of mTOR signaling (Figure 22A). Accordingly, the ectopic expression of mutp53R175H or mutp53R273H clearly determined the repression of AMPK phosphorylation and the increase of p70S6K phosphorylation (Figure 22A). To better represent our results, we quantified P-AMPK/P-p70S6K ratio (Figure 22B), highlighting that wild type and mutant p53 proteins produced inverse effects on these signaling pathways.
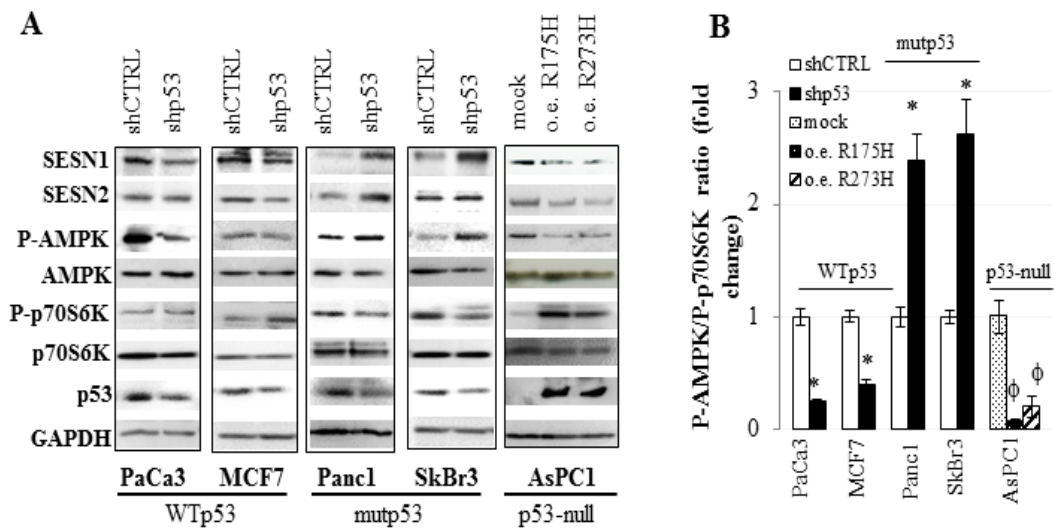
Figure 22. Mutant p53 inhibits AMPK and stimulates mTOR. (A) Western blotting analysis was performed with 50 μg of whole cell extracts and probed with the indicated antibodies. This analysis was representative of three biological replicates. (B) Quantitative analysis of P-AMPK/P-p70S6K ratio.

To address the role of mutant p53-dependent AMPK de-phosphorylation, we inhibited AMPK activity treating cancer cells with compound C (CC) or

overexpressing a dominant negative (DN) AMPK isoform in mutant p53 knock-down conditions (shp53) (Figures 23A and 23B). Strikingly, AMPK signaling inhibition by both CC and DN-AMPK strongly rescued the autophagosome formation induced by mutant p53 knockdown (Figure 23A). Consistently, in the same conditions of AMPK signaling inhibition the oncogenic proliferation impaired by mutant p53 knock down was restored in all cell lines tested (Figure 23B). Overall, these results provide robust evidence that mutant p53 exerts its inhibitory activity on the autophagy throughout the constitutive blockage of the AMPK signaling.
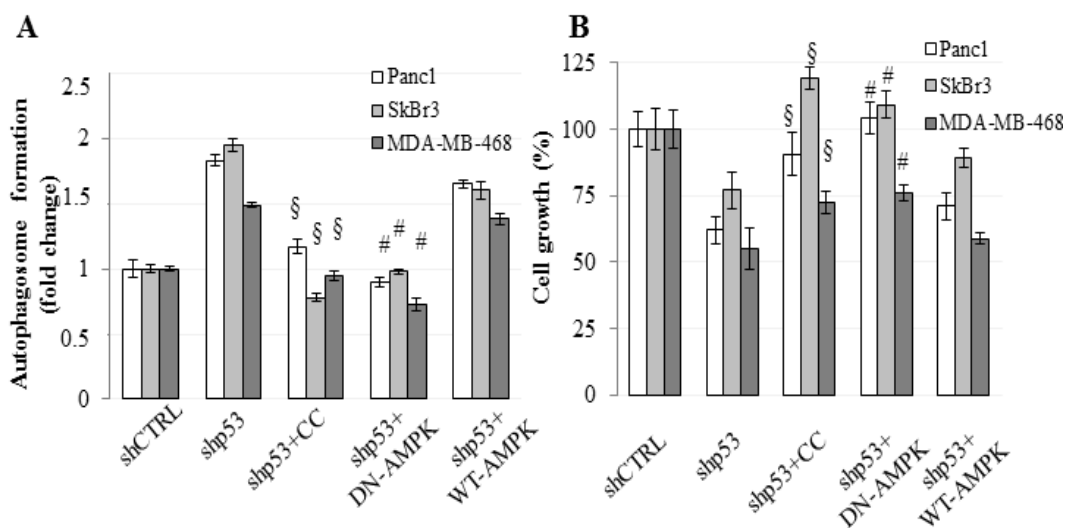
Figure 23. The indicated cell lines were transfected for 48 h with the pRSuper-p53 vector (shp53) or its negative control (shCTRL), in the absence or presence of 20 μM Compound C (CC) or together the expression vector coding for WT or dominant negative (DN; mutant R531G) AMPK γ2 subunit. Autophagosome formation (A) and cell growth (B) were analyzed by using MDC and crystal violet staining, respectively. Statistical analysis: * $p<0.05$ shp53 *vs* CTRL; □ R175H or R273H *vs* mock; § $p<0.05$ shp53+CC *vs* shp53; # $p<0.05$ shp53+DN-AMPK *vs* shp53+WT-AMPK

Mutant p53 inhibits Beclin1 phosphorylation by mTOR and its expression level

Since mTOR pathway was triggered by mutant p53 (Figure 22), we hypothesized that mTOR complex induced by mutp53 might de-phosphorylate Beclin1 via ULK1 inhibition impairing the formation of Beclin1-mediated autophagic protein

complexes and autophagy maturation (Russell, 2013). To gain insight into the molecular mechanisms controlling the Beclin1 expression, we observed that the overexpression of mutp53R175H or mutp53R273H proteins in AsPC1 cells decreased both Beclin1 phosphorylation in Serine 15 and its total protein expression level (Figure 24A). Accordingly, the depletion of mutant p53 from Panc1 cells exerted the inverse results (Figure 24A). These data correlated with the Beclin1 mRNA expression shown in Figure 19. In line with the findings from Russel and colleagues, we also investigated whether the stimulation of mTOR signaling by mutant p53 may be responsible for the decrease of Beclin1 phosphorylation. We observed that the addition of everolimus (RAD001), an inhibitor of mTOR currently used in the therapy against several human cancers, was able to completely restore Beclin1 phosphorylation repressed by the overexpression of both R175H or R273H mutant p53 proteins, indicating that mTOR stimulation by mutant p53 is highly involved in Beclin1 de-phosphorylation (Figure 24A). Since Beclin1 phosphorylation has been described to be crucial for ATGs interaction during autophagosome vesicles formation, we investigated the role of Beclin1 repression on overall autophagy inhibition by mutant p53. We observed that the knockdown of Beclin1 (siBeclin1) significantly reduced the autophagosome formation induced by the depletion of mutant p53 protein (Figure 24B), thus suggesting a role for Beclin1 repression on the autophagy pathway inhibition driven by mutant p53. According to this, the concomitant depletion of mutant p53 and Beclin1 led to a partial rescue of proliferation ability of cancer cells as compared to mutant p53 knockdown conditions (Figure 24C). Overall, our data strongly support the negative effect of mutant p53 on the Beclin1 functionality throughout the inhibition of both its expression and phosphorylation via mTOR stimulation.
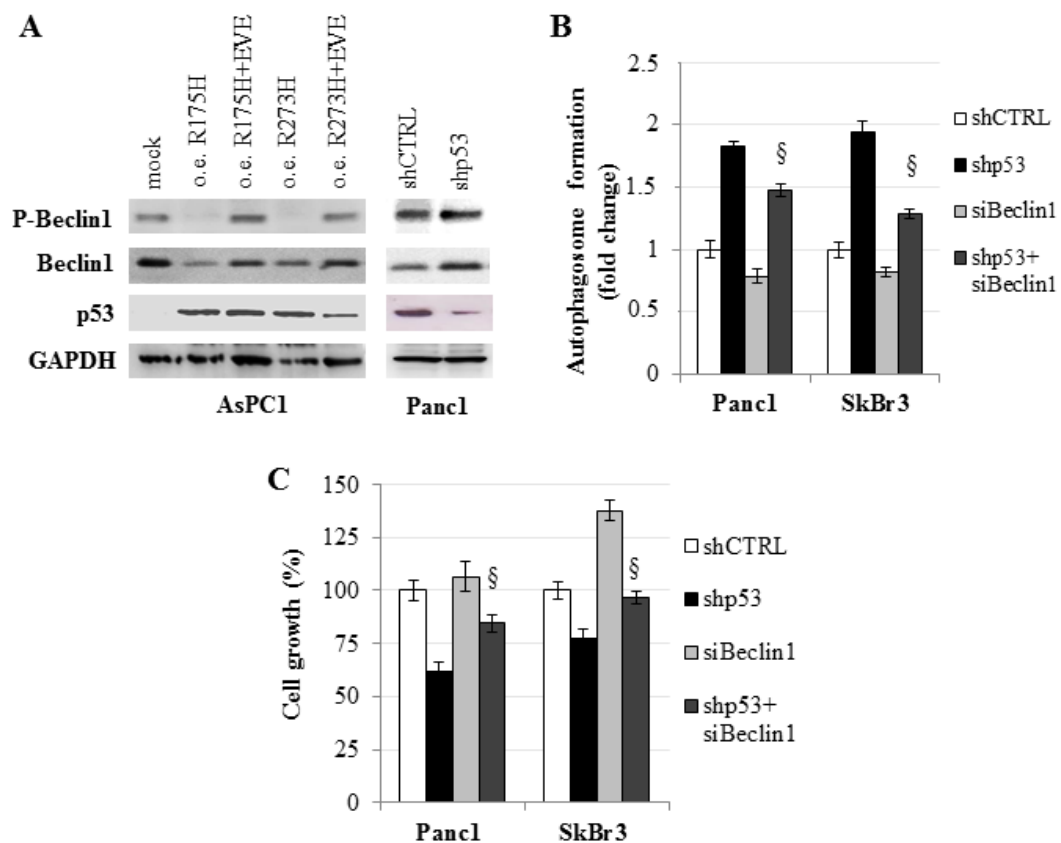
Figure 24. Mutant p53 inhibits Beclin1. (A) AsPC1 cells were transfected for 48 h with the plasmids coding for mutant p53 proteins (R175H or R273H), or with pCDNA3 as negative control (mock), in the absence or presence of 5 μM everolimus (EVE). Panc1 cells were knocked-down for mutant p53 expression by 48 h of pRSuper p53 vector transfection (shp53). Whole-cell extracts were used for Western blot analysis using the indicated antibodies. (B and C) Panc1 and SkBr3 cells transfected with the pRSuper-p53 vector (shp53) and/or si-Beclin1 oligos (siBeclin1). Autophagosome formation (B) and cell growth (C) were analyzed after 48 h after transfection by using MDC and crystal violet staining, respectively. The P-values were calculated with two-tailed t-test. Statistical analysis: § $p<0.05$ shp53+siBeclin1 *vs* shp53.

Mutant p53 regulates mTOR-dependent PKM2 and STAT3 expression

Mutant p53 has been previously described to stimulate the aerobic glycolysis pathway in cancer cells (i.e. Warburg effect) (Zangh, 2013) and mTOR kinase

complex regulation is known to be strictly linked with metabolism regulation. Data presented so far indicate that mutant p53 plays an important role in the modulation of mTOR pathway to repress the autophagy biological process. Consistent with this concept, our next question was to know whether mutant p53/mTOR axis was able to interconnect with other metabolic pathways functionally linked to autophagy. We investigated whether the expression levels of pyruvate kinase type M2 (PKM2), an isoform of pyruvate kinase commonly over-expressed in tumors (Yang, 2013) can be influenced by mutant p53. Pyruvate kinase (PK) is a rate-limiting enzyme in the final step of the glycolytic pathway that catalyzes the conversion from phosphoenolpyruvate and ADP to pyruvate and ATP. Notably, we observed that mutant p53 knockdown inhibited both PKM2 and P(Tyr105)-PKM2 expression levels while they resulted unchanged after WTp53 knockdown (figure 25A). To investigate the involvement of mTOR kinase complex in PKM2 regulation, we have over-expressed R175H or R273H p53 mutants in p53-null AsPC1 cells in the absence or presence of the mTOR inhibitor everolimus. Figure 25B shows that mutant p53 proteins significantly increased both P-PKM2 and PKM2 expression. Furthermore, PKM2 phosphorylation was rescued after the incubation with everolimus, thus demonstrating the crucial role of mTOR on PKM2 regulation by mutant p53. Intriguingly, beyond its metabolic roles, PKM2 can trigger the expression of genes related to cancer cell migration, such as the signal transducer and activator of transcription 3 (STAT3) (Yang, 2014). On turn, STAT3 has been also described to sustain aerobic glycolysis and the Warburg effect in cancer cells (Li, 2015), as well as to inhibit autophagy by down-regulating LC3 gene expression (Gong, 2014). In this context, we found that mutant p53 proteins up-regulated both STAT3 and P(Tyr705)-STAT3 expression levels in a mTOR-dependent manner (figure 25B).
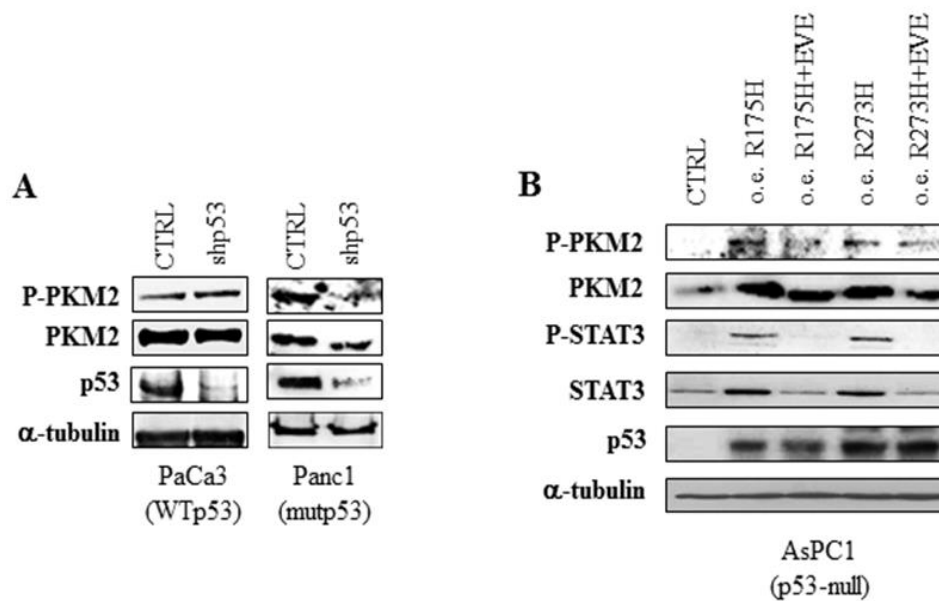
Figure 25. Mutant p53 regulates PKM2 and STAT3 by mTOR pathway. (A) Cells were transfected for 48 h with pRSuper-p53 vector or with the empty vector as negative control (CTRL). Whole-cell extracts were used for Western blot analysis using the indicated antibodies. (B) Cells were transfected for 48 h with the plasmids coding for mutant p53 proteins (R175H or R273H), or with empty pCDNA3 as negative control (CTRL), in the absence or presence of 5 μM everolimus. Whole-cell extracts were used for Western blot analysis using the indicated antibodies.

Mutant p53 sensitizes cancer cells to mTOR inhibition

Everolimus is an inhibitor of mammalian target of rapamycin (mTOR) routinely used in the targeted therapy of many cancers. We aimed to investigate whether cancer cells bearing mutant p53 might be more responsive to everolimus than wild type p53 cancer cells, accordingly with the inhibitory role of mutant p53 on autophagy via mTOR pathway induction (Figures 24 and 25). To demonstrate this idea, we incubated Panc1, SkBr3 and MCF7 cell lines with everolimus and observed that the cells expressing mutant p53 (Panc1 and SKBr3) were more sensitive than cells with wild type p53 (MCF7) to the treatment as reported by the IC_{25} and IC_{50} values (Figure 26A). To functionally demonstrate the involvement of mutant p53 on cancer cell sensitivity to everolimus, we evaluated the response

of the cells after knockdown or overexpression of p53 mutant proteins. Our data reported in Figure 26B, showed that mutant p53 knockdown significantly decreased Panc1 and SkBr3 cell response to everolimus treatment if compared to controls. To corroborate this result, we assessed that the overexpression of R175H or R273H mutant p53 conferred to p53-null AsPC1 cells a strong sensitization to everolimus incubation, as compared to its negative control (mock vector). Moreover, the dominant negative effect of mutant p53 proteins ectopically expressed in WTp53 cells (PaCa3), allowed a stronger impact resulting in a higher response to everolimus incubation in comparison to PaCa3 cells under control conditions (Figure 26B). Overall, these results demonstrated that targeting the mTOR pathway represents an effective therapeutic strategy for cancer patients bearing mutations in the *TP53* gene.

Cell lines	IC$_{25}$ (µM)	IC$_{50}$ (µM)
PaCa3 (WTp53)	4.5±0.35	>10
Panc1 (mutp53)	0.26±0.02	5.76±0.25

Cell lines	IC$_{25}$ (µM)	IC$_{50}$ (µM)
MCF7 (WTp53)	2.03±0.23	>10
SkBr3 (mutp53)	0.09±0.01	7.8±0.31
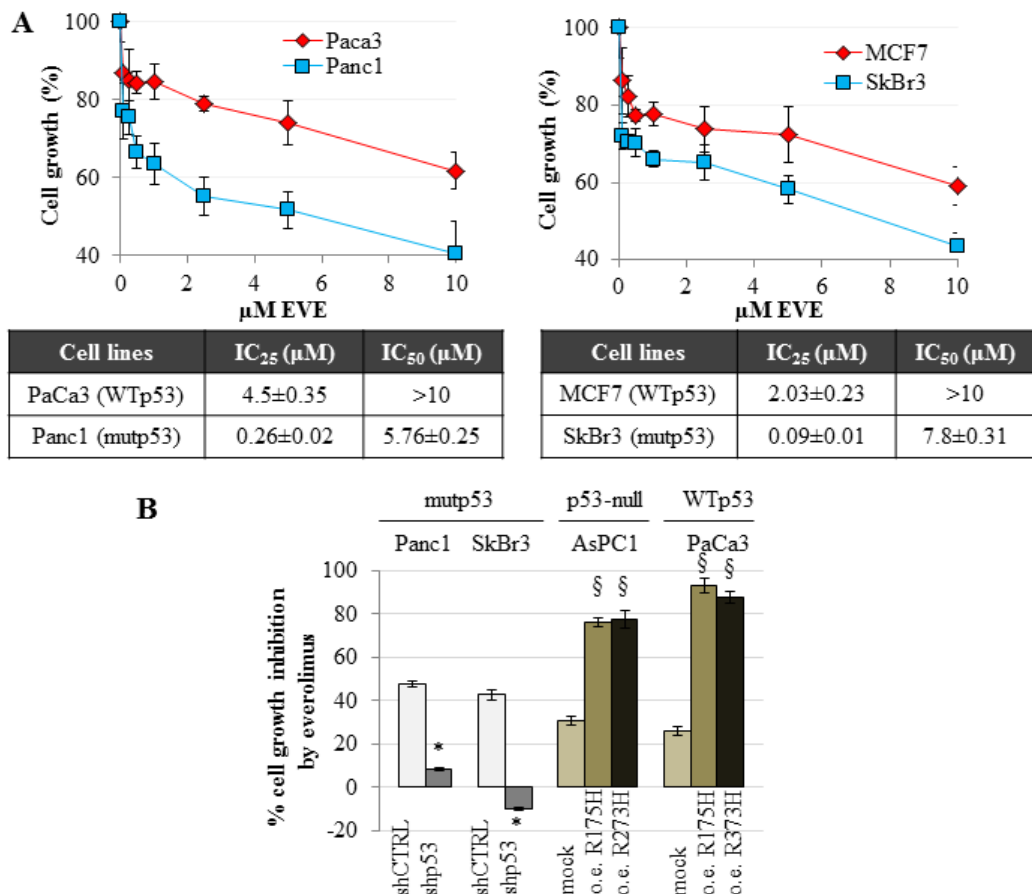
Figure 26. Mutant p53 sensitizes cancer cells to everolimus. (A) Cells were treated for 48 h with increasing concentrations of everolimus. Cell growth was measured

using the crystal violet colorimetric assay and IC_{25} and IC_{50} values for each cell line tested have been reported. (B) Cells were transfected with pRSuper-p53 vector (shp53) and with the plasmids coding for mutant p53 proteins (R175H or R273H), or with empty pRSuper (shCTRL) or pCDNA3 (mock), respectively, as negative controls. For each experimental condition, cells were untreated or treated with 5 μM everolimus for 48 h. Cell growth was determined using the crystal violet colorimetric assay. The rate of cell growth inhibition corresponds to the effect of everolimus, as compared to vehicle (untreated), in each indicated transfection condition. The P-values were calculated with two-tailed t-test. Statistical analysis: * $p<0.05$ shp53 *vs* shCTRL; § $p<0.05$ R175H or R273H *vs* mock.

Mutant p53 proteins enhance ROS production in cancer cells

In order to investigate the effect of mutant p53 on the redox balance of cancer cell we have transiently transfected pancreatic and breast cancer cell lines with pRSuper-p53 vector and subsequently measured the cytosolic ROS level by using the dichlorodihydrofluorescein (DCF) probe. We found that the knock-down of mutant p53 in Panc1 pancreas cancer cells and MDA-MB-468 breast cancer cells (both carrying mutp53R273H), or in SKBr3 breast cancer cells (bearing mutp53R175H), led to a decrease of cytosolic ROS levels (figure 27A and C). In contrast, silencing of wild-type p53 in PaCa3 pancreas cancer cells and MCF7 breast cancer cells resulted in an increased oxidative stress, according with the antioxidant effect of p53 (figure 27A and C). GSH serves numerous important functions, including antioxidant defense, storage of cysteine, maintenance of intracellular redox state, and modulation of cell growth (DeLeve, 1991). Therefore, in order to further investigate the effect of mutant p53 on the oxidative status of cancer cells we have determined the intracellular reduced glutathione (GHS) levels (in nanomole-per-milligram protein). We observed that the transient silencing of R273H-mutp53 leads a significant decrease in the intracellular GSH. In contrast, silencing of its wild-type p53 counterpart leads an increase of GSH (figure 27B). These observations were in accordance with the ability of mutant p53 to induce ROS.
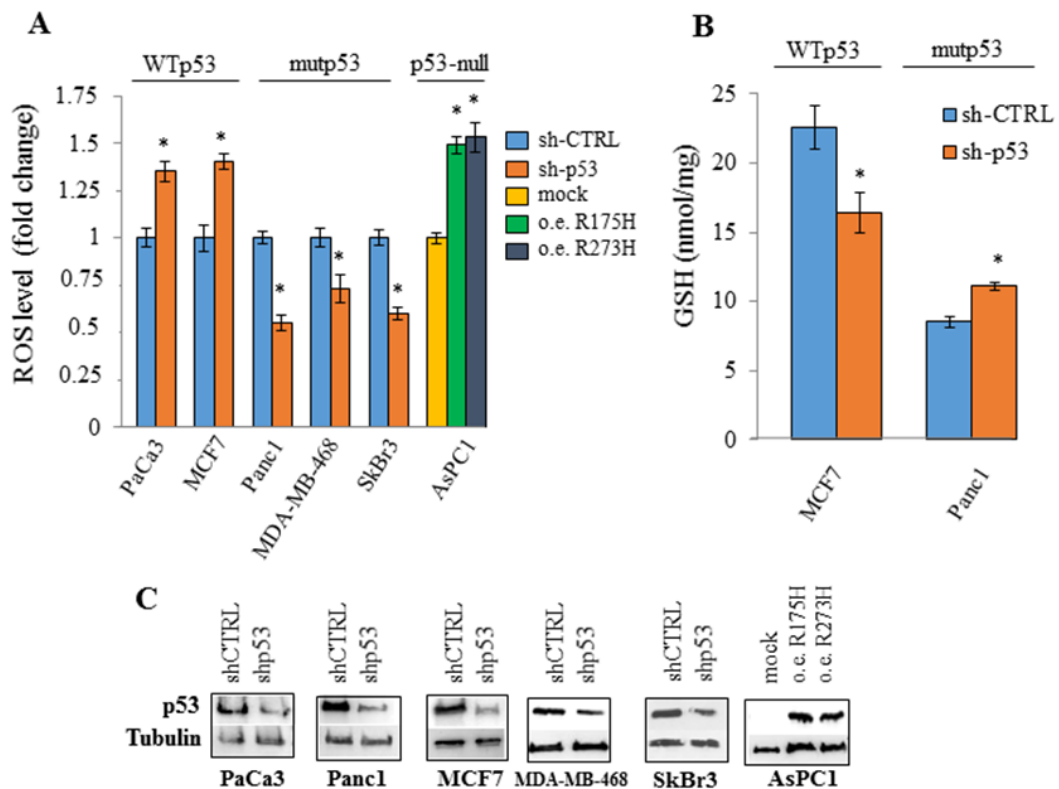
Figure 27. Mutant p53 proteins enhance ROS production in cancer cells. (A) DCF fluorescence intensity, corresponding to ROS production level, measured by a multimode plate reader. The cells line indicate were seeded in 96-well plates, incubated overnight, and transfected with the pRSuper-p53 vector and with plasmids for the ectopic expression of mutant p53 (o.e. R175H; o.e. R273H), or their relative negative control (CTRL) (A). (B) The intracellular oxidized and reduced glutathione were determined in MCF7 and Panc1 cell lines after transient transfection with the pRsuper-p53 vector. Data are presented as means ± SD of results from four independent experiments. Significant difference compared to control group (*p≤0.05). (C) Western blot of p53 using the protein extract of the samples used for (A and B) to test the effective knock down of WT or mutant p53 and the overexpression of mutant p53 in the various cell lines indicated.

In order to collaborate our previous results and investigate the effect of mutant p53 on mitochondrial ROS we performed a confocal microscopy analysis on AsPC1 pancreas cancer cell line which revealed that ectopic expression of mutant p53 (R175H and 273H) is able to strongly induce mitochondrial superoxide ion production (identified by MitoSox red probe), in contrast to cells transfected with

empty vector (figure 28A). We also demonstrated a significant co-localization between mitochondrial superoxide ions and mitochondria (identified by Mitotracker green probe) as shown in the merged images of AsPC1 cells that ectopically over-express both R175H and R273H mutant p53 by yellow puncta and confirmed by the RGB profile (figure 28A). The quantification of superoxide production using MitoSox Red probe was determined with a multimode plate reader and is reported in figure 28B. Overall, our results strongly support that mutant p53 isoforms were able to strongly induce mitochondrial superoxide production, thus affecting redox balance in cancer cells.
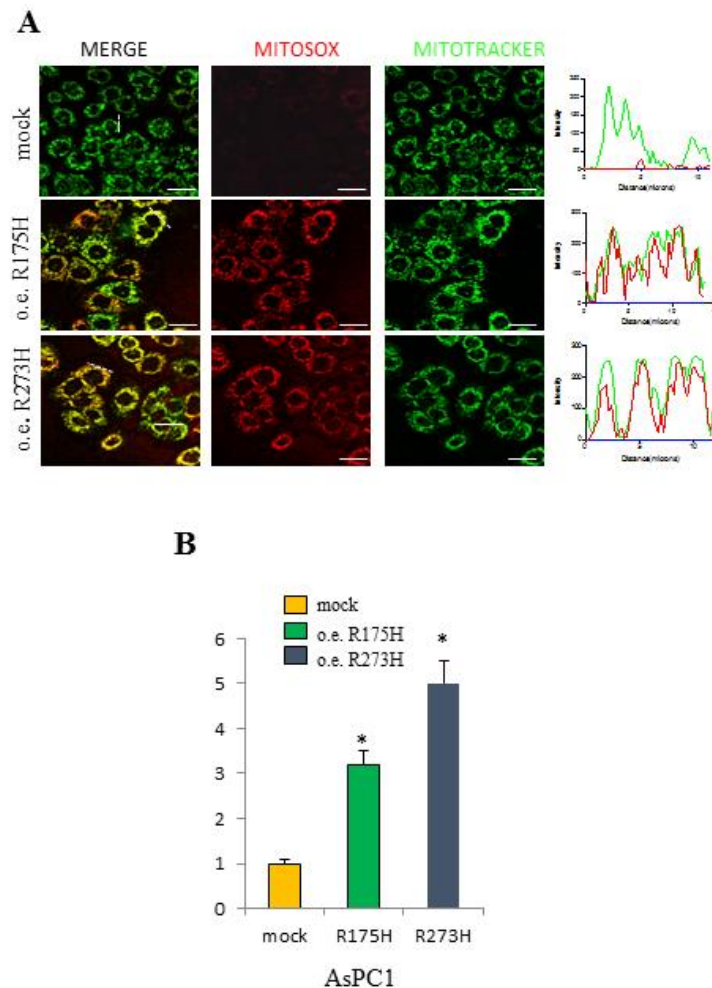
Figure 28. Mutant p53 proteins enhance mitochondrial ROS production. A) 48 h after transfection with either plasmids coding for R175H and R273H mutant p53 vector

and the empty vector (negative control; CTRL), cells were incubated with MitoSox probe (red) for 1h and subsequently fixed. Mitochondria and nuclei were stained with Mitotracker (green) and Hoescht (blue), respectively. The RGB profile plotted along the dashed line drawn in the merge image is also shown. Merge and single channel images come from a single z-plane. Scale bar 10 μm. (B) Mitochondrial superoxide production determined with the MitoSox Red probe measured by a multimode plate reader. Cells were seeded in 96-well plates, incubated overnight, and treated with 100 μg/ml ONC for 72 h.

ROS induced by mutant p53 proteins are critical to mediate their oncogenic proprieties

We have previously established that mutant p53 has a crucial effect in the dysregulation of the redox balance in cancer cells by enhancing ROS production. It has been described that ROS can act as second messengers in cellular signaling (Finkel, 2011) and a great amount of studies has deeply unveiled the existence of a ROS-mediated regulation in many cellular pathways (reviewed in Liu and Storz, 2010). Moreover, as we have previously described, oxidative stress-mediated signaling events play exceptional relevance in the development and progression of many human cancer, since have been reported to affect all characters of cancer cell behavior (Fiaschi and Chiarugi, 2012). Since the existence of these evidences, we moved to investigate the functional role of the ROS induced by mutant p53. Herein, we demonstrated that the increase of cancer cell proliferation induced by ectopic expression of both R175H and R273H p53 isoforms in AsPC1 p53-null cells was abolished when they were concomitantly treated with the antioxidant compound N-Acetyl-Cysteine (NAC) (figure 29A). Moreover, we observed that the reduction of apoptosis due to ectopic expression of both R175H and R273H in AsPC1 cells was rescued when the cells were treated with NAC (figure 29B), corroborating our hypothesis by which ROS might play as signaling molecules modulating specific pathways resulting in an inhibitory effect on apoptosis. We have previously described that mutant p53 drive chemoresistance in pancreatic cancer cells following gemcitabine treatment (GEM) (Fiorini et al., 2015). Intriguingly, we showed herein that ROS also are keys mediators of the

chemoresistance induced by mutant p53 proteins play a role in the chemoresistance induced by mutant p53. Confirming our previous data, AsPC1 p53-null pancreas cancer cells treated with GEM gained a significant chemoresistance when transfected with R175H and R273H p53 proteins, and this chemoresistance were drastically abolished when the cells were concomitantly treated with NAC (figure 29C) suggesting a key role of ROS in the induction of chemoresistance induced by mutant p53. Overall, these intriguing results confirm that ROS are critical mediators of the GOF mutant p53 in cancer cells.
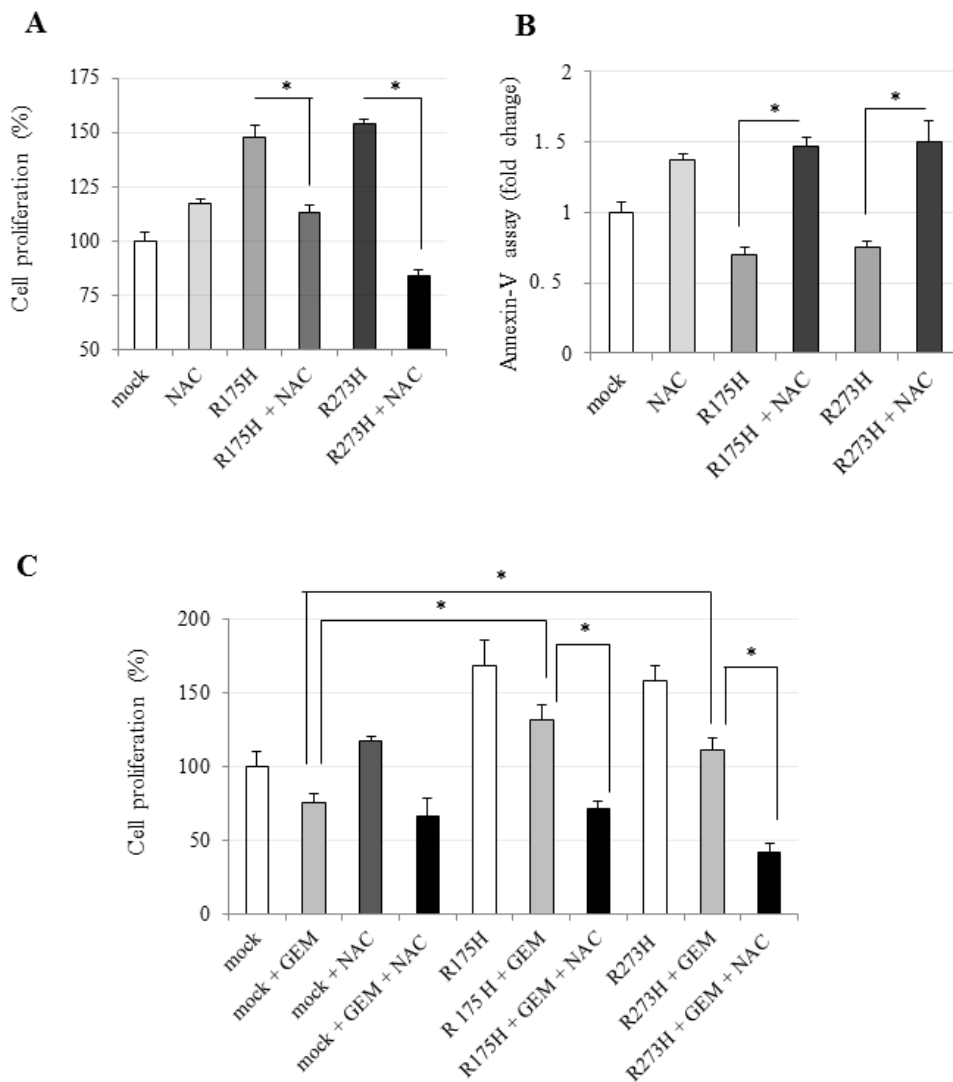
Figure 29. ROS induced by mutant p53 proteins are critical to mediate their oncogenic proprieties. (A) AsPC1 p53 null cells were transfected with R175H and R273H vector, or negative control, and concomitantly treated with NAC at 7 mM for 24h. Cell proliferation was measured by Cristal Violet assay, as described in material and methods. (B) AsPC1 p53 null cells were transfected with R175H and R273H vector, or negative control, and concomitantly treated with NAC at 7 mM for 24h. Apoptosis was determined by annexinV/FITC binding assay. Statistical analysis: *CTRL vs R175H and R273H. *$p<0.05$. # R175H and R273H vs R175H + NAC and R273H + NAC. #$p<0.05$ P-values were calculated with two-tailed t-test. (C) AsPC1 p53 null cells were transfected with R175H and R273H vector, or negative control, and concomitantly treated with GEM at 1uM and NAC at 7 mM for 24h. Cell proliferation was measured by Cristal Violet assay. Statistical analysis: *$p<0.05$

Mutant p53 downregulates UCP2 and PGC1-α

PGC-1α is a multiple-function transcription coactivator that regulates the activity of multiple nuclear receptors and transcription factors involved in the mitochondrial biogenesis and in the maintaining of ROS homeostasis (reviewed in: Puigserver, 2003). We have reported above that its activity is linked to AMPK since it can phosphorylate PGC1-α sustaining a feedforward loop that increases its gene transcription (Lin, 2005). UCP2 plays a crucial role in the limiting mitochondrial ROS that are generated by electron leakage form oxidative phosphorylation (Garlid, 2000) and its expression is intimately linked to the activity of PGC-1α (Oberkofler, 2004). In pancreatic beta-cells, PGC-1α has been shown to stimulate human *UCP2* gene expression via two TREs located in the proximal *UCP2* promoter region (Oberkofler, 2006). Therefore, PGC-1α/UCP2 axis is a relevant player in the maintenance of a correct redox balance in the cells. Since we have previously reported that mutant p53 was able to downregulate SESN1 and SESN2 (figure 19) and this resulted in an inhibition of AMPK phosphorylation (figure 22), we moved to investigate whether this could have any implication in the regulation of UCP2 and PGC-1α expression, which are two downstream effectors of SESN/AMPK signaling pathway. Herein, we show that the depletion of R273H mutant p53 determined an induction of both PGC-1α and

UCP2 mRNA levels in Panc-1 pancreas cancer cells (figure 30A), consistently with its inhibitory activity on PGC-1α/UCP2 axis. In contrast, silencing of wild-type p53 doesn't have a significant effect on their expression (figure 30B). Accordingly, ectopic expression of mutp53R273H in p53-null AsPC1 cells led to a significant reduction of PGC-1α and UCP2 transcripts (figure 30C). To further investigate the effect of mutant p53 on UCP2 protein expression we performed western blot experiments in p53-null AsPC1 after over-expression of mutp53R175H and mutp53R273H that confirmed a significant reduction of UCP2 protein (figure 30 D). Therefore, our data strongly suggest that mutant p53 is able to inhibit PGC-1α and UCP2 expression.
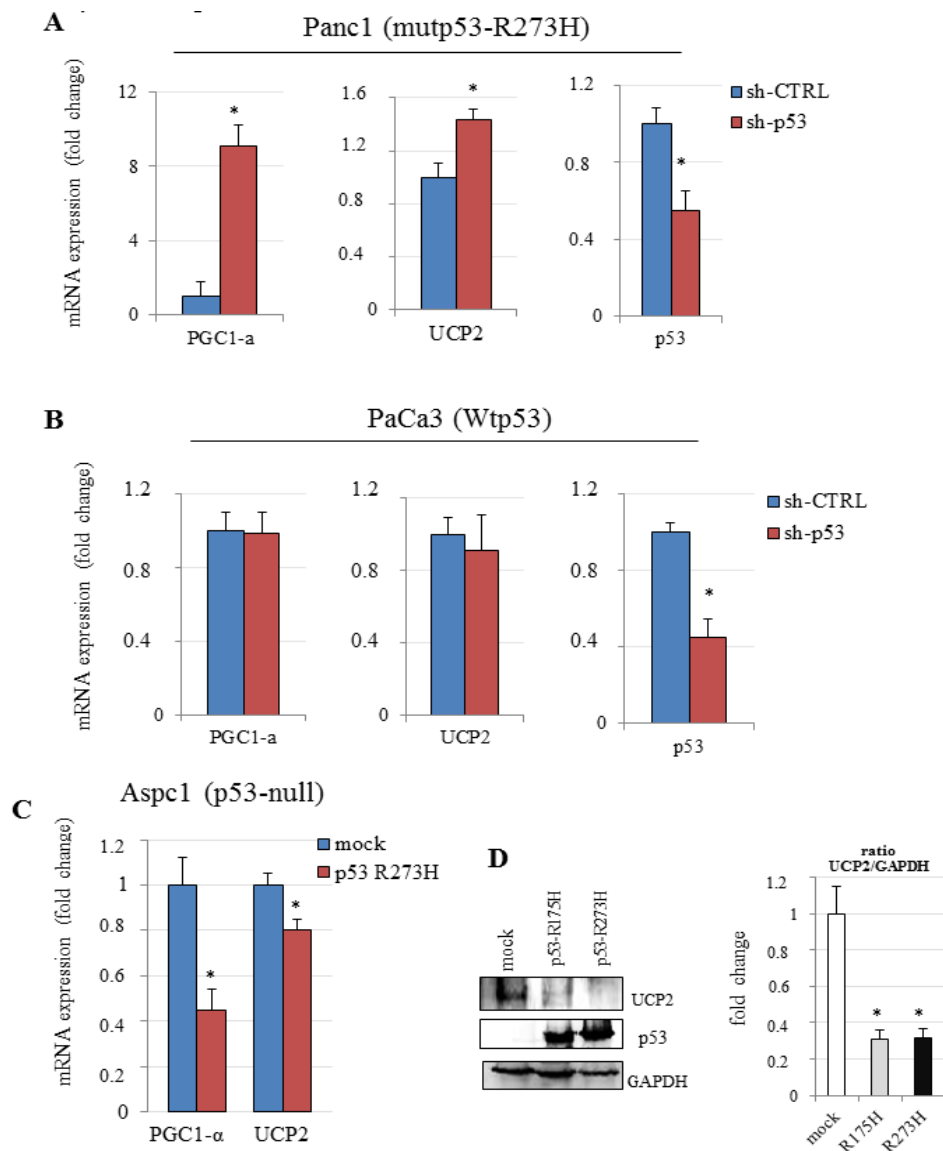
Figure 30. (A-B) The indicated cell lines were transfected for 48 h with the pRSuper-p53 vector or its relative negative control (CTRL). Gene expression analysis of the p53, UCP2 and PGC-1α was performed by RT-qPCR and was normalized to GAPDH mRNA. * $p<0.05$. C) Gene expression analysis of the UCP2 and PGC-1α was performed by RT-qPCR and was normalized to GAPDH mRNA. * $p<0.05$. (D) Western blotting analysis was performed with 50 μg of whole cell extracts, probed with the indicated antibodies and quantified with ImageJ. All the experiments are representative of three biological replicates.

Inhibition of PGC-1α/UCP2 axis by mutant p53 leads an enhancement of ROS production in cancer cells

Since we have reported that UCP2 inhibition by mutant p53 plays a critical role in regulation of oxidative status in cancer cells (figure 31), we moved to investigate the mechanisms underlying the regulation of PGC-1α/UCP2 axis mediated by mutant p53. Herein, we reported that the inhibition of UCP2 and PGC-1α transcripts obtained after ectopical expression of R273H mutant p53 in AsPC1-p53 null cells was completely abolished when the cells were concomitantly treated with 5-aminoimidazole-4-carboxamide ribonucleotide (AICA-R), a stimulator of AMPK activity (figure 31A). As positive control, we observed that the AICA-R treatment induced UCP2 and PGC-1α more than double, in according with the previous observation by Jager *et al.*, 2007. Moreover, we demonstrated that the inhibition of AMPK activity, after transient expression of its dominant negative (DN) isoform, was able to rescue the induction of both PGC1α and UCP2 consequent to knock-down of mutant p53 in Panc1 cancer cells (Figure 31B). In order to corroborate the existence of a PGC-1α/UCP2 axis regulated by mutant p53 we showed that the silencing of PGC-1α was able to strongly rescue the induction of UCP2 consequent to knock-down of mutant p53 in Panc1 cancer cells (Figure 31C). These evidence reinforce our model, which indicates that mutant p53 proteins act as a negative upstream regulator of AMPK-phosphorylation inhibiting PGC-1α/UCP2 expression with relevant impact on cellular ROS homeostasis.
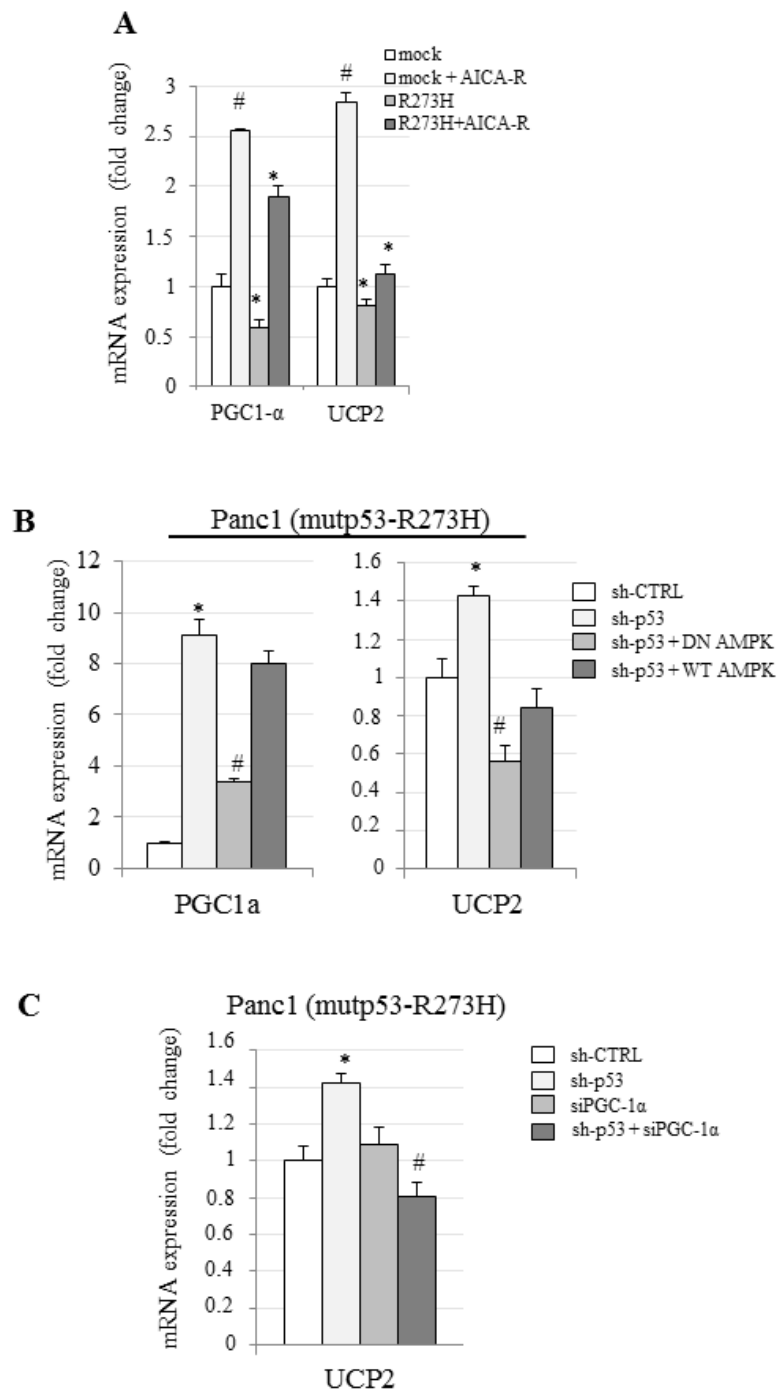
Figure 31. (A) AsPC1-p53 null cells were transfected with the vectors for p53-R273H expression and its mock control and treated with 1 mM AICA-R for 48h. Gene expression analysis of the UCP2 and PGC-1α was performed by RT-qPCR and normalized to GAPDH mRNA. # mock vs mock + AICA-R # p<0.05. * mock vs R273H and R273H vs R27H + AICA-R. * p<0.05. (B-C) Panc1 cells were transfected for 48h with the indicated vectors and their relative negative controls. Gene expression analysis

of UCP2 and PGC-1α was performed by RT-qPCR and was normalized to GAPDH mRNA. * and # p<0.05. P-values were calculated with two-tailed t-test.

Furthermore, we have shown that the regulation of ROS levels by mutant p53 is AMPK-mediated and that the inactivation obtained with the expression of AMPK-dominant negative (DN) isoform largely abolishes the decrease of ROS that we observed after knock-down of mutant p53 (figure 32A). Intriguingly, we have also observed that the reduction of intracellular ROS levels after silencing of mutant p53 was completely rescued when the cells were co-transfected with specific siRNA against UCP2 and concomitantly with pRSuperp53 in order to have a dual knock-down of both UCP2 and mutant p53 (figure 32B). This result reinforces the statement that mutant p53 induces ROS production through the inhibition of UCP2. A similar effect was obtained when UCP2 was inactivated by genipin treatment, a specific inhibitor of UCP2 activity (figure 32B). Moreover, AsPC1 p53-null cells were transfected with the vector for ectopic mutant p53 R175H or R273H expression and concomitantly with a vector for UCP2 over-expression. In these conditions, we observed that the increase of ROS levels due to ectopic expression of p53 mutant was restrained when the cells were co-transfected with the UCP2 vector (figure 32B). Finally, we reported that the enhancement of mitochondrial superoxide ion production due to ectopic expression of R175H or R273H mutant p53 in AsPC1 p53-null cells was significantly rescued by the concomitant transfection with the UCP2 (figure 32C). The results strongly suggest that the inhibitory role that mutant p53 exerts on UCP2 expression represents a key mechanism to alter the oxidative status in cancer cells by enhancing mitochondrial ROS.
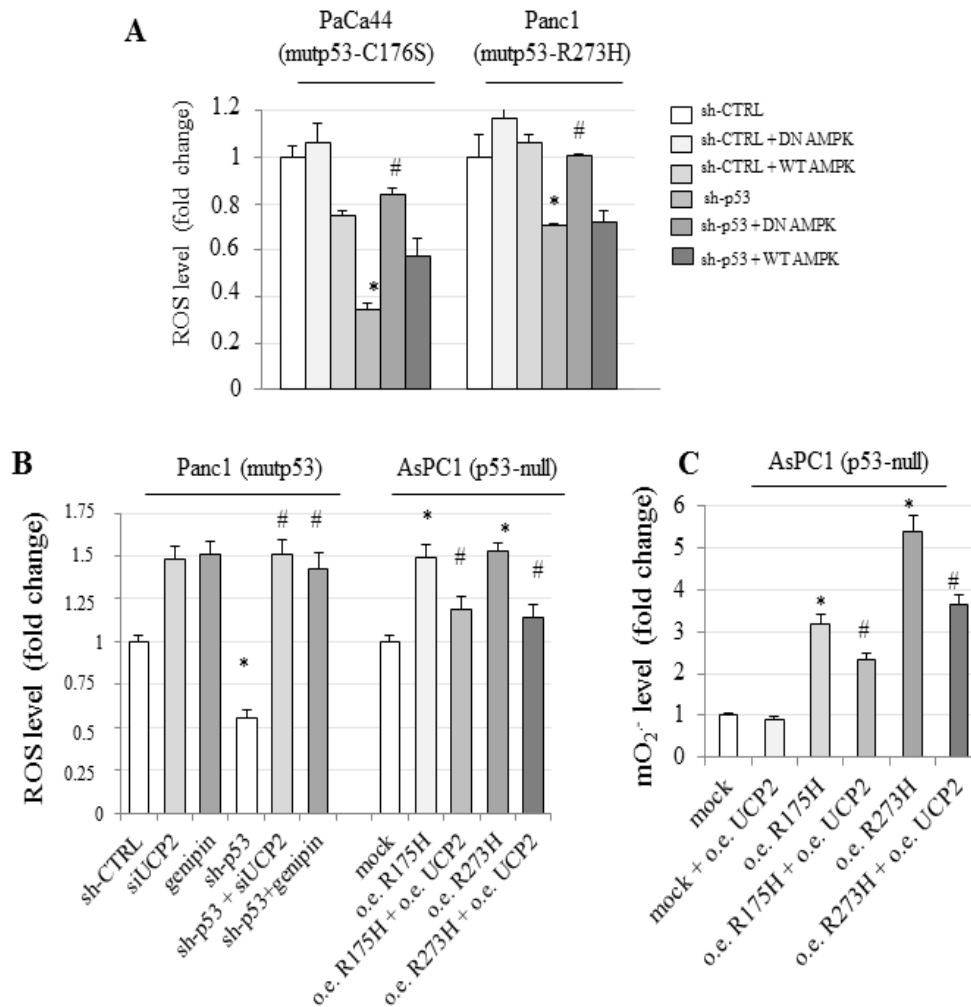
Figure 32. (A) The indicated cells were transfected with the pRSuper-p53 vector or its negative control. DCF fluorescence intensity, corresponding to overall ROS levels was measured by a multimode plate reader. *CTRL vs sh-p53; *p<0.05. # sh-p53 vs sh-p53 + DN-AMPK; #p<0.05. P-values were calculated with two-tailed t-test. (B) Panc1 mutR273H p53 and AsPC1 p53 null cells were transfected with pRSuperp53 and vector for mutant p53 ectopic expression respectively and their controls. In addition, these cell lines were co-transfected with siRNA against UCP2, and treated with genipin for 24h. DCF fluorescence intensity, corresponding to ROS production level, was measured by a multimode plate reader. *CTRL vs sh-p53 or CTRL vs R175H or R273H; *p<0.05. #sh-p53 vs sh-p53 + si-UCP2 or genipin. #R175H and R273H vs R175H + UCP2 and R273H + UCP2; #p<0.05. P-values were calculated with two-tailed t-test. (C) AsPC1-p53 null cells were transfected with the vectors for mutant p53 expression and its mock control. Mitochondrial superoxide production was determined with the MitoSox Red probe

74

measured by a multimode plate reader. *CTRL vs R175H or R273H; *p<0.05. #R175H and R273H vs R175H + UCP2 and R273H + UCP2; #p<0.05. P-values were calculated with two-tailed t-test.

6. MATERIALS AND METHODS

Chemicals

Chloroquine diphosphate (CQ), 3-methyladenine (3MA), N-acetyl-L-cysteine (NAC) and everolimus (EVE; RAD-001) were obtained from Sigma (Milan, Italy). Compound C (CC) solution was obtained from Calbiochem (Merck; Darmstadt, Germany).

Cell culture

PaCa3 (WTp53), Panc1 (mutant p53-R273H), PaCa44 (mutant p53-C176S), MiaPaCa2 (mutant p53-R248W), Suit-2 (mutant p53-R273H) and AsPC1 (p53-null) human pancreatic adenocarcinoma cell lines were grown in RPMI medium (Life Technologies, Milan, Italy), while lung cancer H1299 (p53-null), breast cancer MCF7 (WTp53), SKBr3 (mutant p53-R175H), MDA-MB-468 (mutant p53-R273H) and MDA-MB-231 (mutant p53-R280K) cell lines were cultured in DMEM medium (Life Technologies, Milan, Italy). Both culture media were supplemented with 10% FBS, and 50 μg/ml gentamicin sulfate (BioWhittaker, Lonza, Bergamo, Italy). Cell lines were incubated at 37°C with 5% CO_2. The list of the cell lines used in this study and their p53 status are summarized in Table 1.

Cancer Cell lines	Tissue origin	p53 status	mutation
PaCa3	Pancreas	wild-type	none
MCF7	Breast	wild-type	none
MiaPaca2	Pancreas	mutated	G248W
PaCa44	Pancreas	mutated	C176S
Panc1	Pancreas	mutated	R273H
Suit-2	Pancreas	mutated	R273H
MDA-MB-468	Breast	mutated	R273H
SkBr3	Breast	mutated	R175H
MDA-MB-231	Breast	mutated	R280K
AsPC1	Pancreas	null	gene deleted
H1299	Lung	null	gene deleted

Tab. 1 Tissue of origin and p53 status of cancer cell lines

Cell proliferation assay

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

Transient transfection assays

Exponentially growing cells were seeded at 5×10^3 cells/well in 96-well plates or at 2.5×10^5 cells in 60 mm cell culture plates. The ectopic expression of mutant p53 in p53-null cancer cells was carried out transfecting pcDNA3-mutp53R273H or pcDNA3-mutp53R175H expression vectors, or their relative negative control (pcDNA3). The ectopic expression of wild type or dominant negative (DN)-AMPK subunit γ2 was obtained by transfection of the pcDNA5/FRT expression vector containing the human wild type AMPK γ2 subunit or the mutated R531G protein which were kindly provided by Dr. Hawley (University of Dundee, Scotland, UK). The ectopic expression of UCP2 was obtained with a pCMV expression vector containing the human cDNA of UCP2 (OriGene Technologies, Rockville, MD) using Lipofectamine 2000 (Life Technologies), according to the manufacturer's instructions. Cells transfected with the empty vector were used as a negative control. Wild-type and mutant p53 protein expression was transiently knocked-down by transfection with pRSUPER-p53 or pLVTHM-p53 vectors or their negative controls (pRSUPER or pLVTHM), kindly provided by Dr. Agami (The Netherlands Cancer Institute, Amsterdam) (Brummelkamp et al., 2002) and by Dr. Sergio Ruiz (CNIO, Madrid, Spain), respectively. Commercial siRNA smart pool of three oligonucleotides (sip53) transiently targeting p53 (Santa Cruz Biotech. sc-29435) was used in the experiments of RT-qPCR to exclude back-side effects and to confirm the robustness of the data. A si-GFP as non-silencing control 5'-GGCTACGTCCAGGAGCGCACC-3' was used as a negative control.

The silencing transfections were carried out for 48 hrs using Lipofectamine 2000 (Life Technologies), according to the manufacturer's instructions. Knockdown of Atg5 expression was performed by shRNA-Atg5 cloned in pMSCV-Puro-miR30 vector (or its negative empty vector) kindly provided by Dr. Hans-Uwe Simon (University of Bern, Switzerland) (Maskey, 2013). Knock-down of Beclin1 expression was obtained by transfecting cells with specific Beclin1 small interfering (si) RNA having the following sequences: 5′-ACAGUGAAUUUAAACGACAGCAGCU-3′ and 5′-AGCUGCUGUCGUUUAAAUUCACUGU-3′ and with a siRNA-CTRL (negative control): 5′- CAGUCGCGUUUGCGACUGG-3′ purchased from Life Technologies. Knock-down of p50 NF-κB subunit expression was obtained by transfecting cells with a specific 21-nt-long interfering RNA duplex with two 3'-end overhang dT nucleotides. The sequence of the antisense strand of the anti p50 siRNA was 5'-AGUCCAGGAUUAUAGCCCCdTdT-3' (MWG Biotec, Ebersberg, Germany; or Dharmacon, Lafayette, CO) as reported (Laderach, 2003). Knock-down of UCP2 expression was obtained by transfecting cells with a UCP2 small interfering (si) RNA having the following sequences: 5′-ACAGUGAAUUUAAACGACAGCAGCU-3′ and 5′-AGCUGCUGUCGUUUAAAUUCACUGU-3′ and with a siRNA-CTRL (negative control): 5′- CAGUCGCGUUUGCGACUGG-3′ purchased from Life Technologies. Knock-down of PGC1-α expression was obtained by transfecting cells with a PGC1-α small interfering (si) RNA having the following sequences: 5′-ACAGUGAAUUUAAACGACAGCAGCU-3′ and 5′-AGCUGCUGUCGUUUAAAUUCACUGU-3′ and with a siRNA-CTRL (negative control): 5′- CAGUCGCGUUUGCGACUGG-3′ purchased from Life Technologies. Cells were transfected by siRNAs at a final concentration of 50 nM using Lipofectamine 2000 (Life Technologies), according to the manufacturer's instructions.

RNA isolation and quantitative real-time PCR analysis

Total RNA was extracted from cells using TRIZOL Reagent (Life Technologies) in accordance with the manufacturer's instructions. Five micrograms of total RNA were reverse-transcribed at 37°C for 60 min in the presence of random hexamers and Moloney murine leukemia virus reverse transcriptase (Life Technologies). Transcripts were measured by real-time PCR using the SYBR Green assay (Applied Biosystems, Carlsbad, CA, USA) with a StepOne instrument (Applied Biosystems). PCR analysis was carried out using specific oligonucleotides for the genes listed in Table 2. The primers were designed with Primer3 version 0.4.0 (http://frodo.wi.mit.edu/primer3/). All primer sets worked under identical quantitative PCR cycling conditions with similar efficiencies to obtain simultaneous amplification in the same run. The 2-ΔΔCT method for relative quantification of gene expression was used to determine mRNA expression levels. GAPDH gene expression was used as endogenous control to standardize mRNA expression. All reactions were performed in duplicate from three independent experiments.

Genes	Primer sequences
atg12	for: 5' gtcttccgctgcagtttcc 3'
	rev: 5' tcgctctactgcccacttct 3'
becn1	for: 5' gtagaccggacttgggtgac 3'
	rev: 5' ctgcatggtgctgttgttg 3'
sesn1	for: 5' ggacgaggaacttggcatta 3'
	rev: 5' atgcatctgtgcgtcttcac 3'
sesn2	for: 5' gcctgctacccagagaagac 3'
	rev: 5' cctccaggagcagcaagtt 3'
dram1	for: 5' ggacagtggcctttggttt 3'
	rev: 5' gcctgcgacattcactgag 3'
p53	for: 5' ggcccacttcaccgtactaa 3'
	rev: 5' gtggtttcaaggccagatgt 3'
pgc1-α	for: 5' tgactggcgtcattcaggag 3'
	rev: 5' ccagagcagcacactcgat 3'
ucp2	for: 5' ctcctgaaagccaacctcat-3'
	rev: 5' cccaaaggcagaagtgaagt- 3'

Table 2. Sequences of primers used for qPCR analysis of autophagy- and ROS-related genes.

ChIP experiments

1% formaldehyde cross-linking and chromatin immunoprecipitations were performed as described in (Di Agostino, 2006; Valenti, 2011). The chromatin solution was immunoprecipitated with sheep anti-p53 Ab7 (Millipore, Billerica, MA, USA), rabbit anti-p50 (Santa Cruz Biotech. SC-7178), rabbit anti-p65 (Santa Cruz Biotech SC-372), or no antibody as negative control. The immunoprecipitations were performed using Pierce ChIP-grade Protein A/G magnetic beads (Thermo Fisher Scientific, Rockford, IL, USA). Primers used for the amplification of the different regulatory regions are listed in Table 3. NF-κB p50 Region 2 consensus sequence on ATG12 promoter was selected from the

literature and NF-κB p50 Region 1, the consensus sequence was identified by MatInspector software (www.genomatix.de). The promoter occupancy was analyzed by RT-qPCR using the Fast SYBR Green assay (Applied Biosystems, Carlsbad, CA, USA) and the StepOne™ Real-Time PCR System (Applied Biosystems). Normalization was performed to the amount of input chromatin.

atg12 promoter regions	Primer sequences
region1	for: 5' ccttaatgcctcctgcactt 3'
	rev: 5' ttgactgattggagctgtgg 3'
region2	for: 5' gtgggctcttgggggactg 3'
	rev: 5' ggtgggctgcatgctttc 3'

Table 3. Sequences of primers used for ChIP analysis in the region1 and region2 of the atg12 promoter

Immunoblot analysis

Cells were harvested, washed in PBS, and re-suspended in lysis buffer in the presence of phosphatase and protease inhibitors (50 mM Tris–HCl pH 8, 150 mM NaCl, 1% Igepal CA-630, 0.5% Na-Doc, 0.1% SDS, 1 mM Na_3VO_4, 1 mM NaF, 2.5 mM EDTA, 1 mM PMSF, and 1× protease inhibitor cocktail). After incubation on ice for 30 min, the lysates were centrifuged at 14,000 × g for 10 min at 4°C and the supernatant fractions were used for Western blot analysis. Protein concentration was measured by Bradford reagent (Pierce, Milan, Italy) using bovine serum albumin as a standard. Protein extracts (50 μg/lane) were resolved on a 12% SDS-polyacrylamide gel and electro-blotted onto PVDF membranes (Millipore, Milan, Italy). Membranes were blocked in 5% low-fat milk in TBST (50 mM Tris pH 7.5, 0.9% NaCl, 0.1% Tween 20) for 1 h at room temperature and probed overnight at 4°C with a mouse polyclonal anti-p53 (1:500) (Santa Cruz, #sc-263), rabbit monoclonal anti-LC3 (1:1,000) (Cell Signaling, #2775), rabbit polyclonal antiphospho(Ser15)Beclin1 (1:1,000) (Cell Signaling #13825) rabbit polyclonal anti-Beclin1 (1:1,000) (GeneTex, #GTX113039), rabbit monoclonal glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1,000) (Cell

Signaling, #5174S), rabbit monoclonal anti-phospho (Thr172)AMPK (1:1,000) (Cell Signaling #2535), rabbit monoclonal anti-AMPK (1:1,000) (Cell Signaling #2603), mouse monoclonal anti-phospho (Thr389)p70S6K (1:1,000) (Cell Signaling #9206), rabbit monoclonal anti-p70S6K (1:1,000) (Cell Signaling #2708), rabbit polyclonal anti-SESN1 (1:1,000) (GeneTex #GTX116926), rabbit polyclonal anti-SESN2 (1:1,000) (GeneTex#118141), rabbit polyclonal anti-NF-κB p50 (1:1,000) (Santa Cruz #sc-7178), rabbit polyclonal anti-NF-κB p65 (1:1,000) (Santa Cruz #sc-372), mouse monoclonal anti-alpha-tubulin (1:2,500) (Oncogene #CP06), or rabbit monoclonal anti-phospho (Tyr705)STAT3 (1:1,000) (Cell Signaling #9131), goat polyclonal antiUCP2 (1:2,000) (Abnova #PAB7242) antibodies. Horseradish peroxidase conjugated anti-mouse or anti-rabbit IgGs (1:8,000 in blocking solution) (Upstate Biotechnology, Milan, Italy) were used as secondary antibodies. Immunodetection was carried out using chemiluminescent substrates (Amersham Pharmacia Biotech, Milan, Italy) and recorded using a HyperfilmECL (Amersham Pharmacia Biotech). ECL results were scanned and the amount of each protein band quantified using NIH Image J software (http://rsb.info.nih.gov/nihimage/).

Immunoprecipitation assay

Cell extracts were solubilized in lysis buffer with 150 mM Hepes pH 7.5, 300 mM NaCl, 1% Triton-X100, phosphatase and protease inhibitors. Cells were harvested and lysed by sonication in lysis buffer and cleared by centrifugation. Protein concentrations were determined by colorimetric assay (Bio-Rad, Hercules, CA, USA). For each immunoprecipitation, 1 microg of antibody and 1 microg of rabbit or sheep IgG (Santa Cruz Biotech) were used as controls. To immunoprecipitate, we used rabbit polyclonal anti-p50 (sc-7178) from Santa Cruz Biotech and sheep polyclonal anti-p53 Ab7 (PC35-1EA) from Millipore. 1 mg of pre-cleared extracts were diluted in lysis buffer containing 0.05% BSA and incubated with protein A/G-Agarose beads (Thermo Fisher Scientific, Rockford, IL, USA) and antibodies, under constant shaking at 4°C for 3 hours. Bead-bound immunocomplexes were rinsed with lysis buffer and eluted in 50 μl of SDS

sample buffer for Western blotting. Western blotting was performed using the following primary antibodies: mouse monoclonal p53 (DO1), GAPDH (Santa Cruz Biotech), rabbit polyclonal anti-NF-□B p65 (1:1,000) (Santa Cruz #sc-372), rabbit polyclonal anti-p50 (sc-7178) and actin (Sigma) antibodies. Western blot analysis was performed with the aid of the enhanced chemiluminescence system (Thermo Fisher Scientific, Rockford, IL, USA) and the acquisition of the images by Uvitec technology (Eppendorf).

Apoptosis assay

Cells were seeded in 96-well plates (5×10^3 cells/well). Twenty-four hours later, cells were treated as indicated in the figure legends. At the end of the treatment, cells were fixed with 2% paraformaldehyde in PBS for 30 min at room temperature, washed twice with PBS and stained with annexin V/FITC (Bender MedSystem, Milan, Italy) in binding buffer (10 mM HEPES/NaOH pH 7.4, 140mM NaCl, and 2.5mM $CaCl_2$) for 10 min at room temperature in the dark. Cells were then washed with binding buffer and fluorescence was measured using a multimode plate reader (Ex485nm and Em535nm) (GENios Pro, Tecan, Milan, Italy). The values were normalized on cell proliferation by Crystal Violet assay.

Monodansylcadaverine staining and autophagosome formation assay

To quantify the induction of autophagy, cells were incubated with the fluorescent probe monodansylcadaverine (MDC; Sigma, Milan, Italy), accordingly with the guidelines for studying autophagy (Klionsky, 2016). MDC is a selective marker for acidic vesicular organelles, such as autophagic vacuoles. Briefly, cells were seeded in 96-well plates (5×10^3 cells/well) and treated with various compounds as indicated in the figure legends. At the end of the treatments, cells were incubated in culture medium containing 50 μM MDC at 37°C for 15 min. Cells were then washed with Hanks buffer (20 mM Hepes pH 7.2, 10 mM glucose, 118 mM NaCl, 4.6 mM KCl, and 1 mM $CaCl_2$) and fluorescence was measured using

a multimode plate reader (Ex340nm and Em535nm) (GENios Pro, Tecan, Milan, Italy). The values were normalized for cell proliferation by Crystal Violet assay.

Acridine orange staining and autolysosome detection

Cells were seeded in a 24-well plate on glass cover-slips at a density of 6 x 104/well. After 24 hours, cells were transfected with pRSUPER-p53 or with the empty pRSUPER vector by using Lipofectamine 2000 according to the manufacturer's instructions. 48 hours after transfection cells were rinsed in phosphate buffer saline (PBS) and stained with 6 μg/ml acridine orange (AO). Cell images were captured using a fluorescence microscope Leica DM6000 (Leica Microsystem, Manheim, Germany) at 40× magnification and processed using Adobe Photoshop and NIH ImageJ software. To quantify autolysosome formation, cells transfected with pRSUPER-p53 vector or its negative control (pRSUPER) were stained with AO following the manufacturer's instructions. Quantification of autolysosomes was performed measuring the red/green fluorescence intensity ratio of AO staining (AO green fluorescence Ex485nm and Em535nm; AO red fluorescence Ex430nm and Em590nm) with a multimode plate reader (GENios Pro, Tecan, Milan, Italy). Values were normalized on cell proliferation by crystal violet assay.

LC3-GFP and lysosome co-localization analysis

Cells were seeded in a 24-well plate on glass cover-slips at a density of 6 x 10^4/well. After 24 hours, cells were co-transfected with pEGFP-LC3B and pRSUPER-p53 vector or with pEGFP-LC3B and the pRSUPER empty vector by using Lipofectamine 2000, according to the manufacturer's instructions. 48 hours after transfection, lysosomes were stained with 50 nM Lysotracker-RED DN99 (Life Technologies) and nuclei were stained with 0.1 μg/ml Hoechst (Life Technologies). Cells were than rinsed in PBS and fixed in 4% (w/v) paraformaldehyde. Cover-slips were mounted over slides in AF1 medium (Dako).

Cell images were captured using a confocal laser-scanning fluorescence microscope Leica SP5 (Leica Microsystem, Manheim, Germany) at 63× magnification and processed using Adobe Photoshop and NIH Image J software.

GEO expression data

We used the publicly available data sets: GSE22358 (Gluck, 2012) and GSE31812 (Freed- Pastor, 2012). The data sets were analyzed by the Oncomine platform www.oncomine.org (Rhodes, 2004).

Kaplan-Meier analysis

Gene expression dataset raw data and survival information of 108 BCL patients with mutant *TP53* gene were downloaded from GEO (http://www.ncbi.nlm.nih.gov/geo/) and the Kaplan-Meier analysis was performed by using the on-line tool www.kmplot.com (Gyorffy, 2010) where eight public datasets were considered. Gene expression profiles were performed by using Affymetrix HGU133A (GPL96) and HG-U133 Plus 2.0 (GPL570) microarrays were only considered. The latter are the most frequently used and have 22,277 probe sets in common. To analyse the prognostic value of genes, the cohorts were divided into two groups according to the median (or upper/lower quartile) expression of gene signature considered. The two groups were compared in terms of relapse free survival (RFS) and distant metastasis free survival (DMFS). A survival curve was displayed, and the hazard ratio with 95% confidence intervals and logrank P value are calculated. P values < 0.05 were considered statistically significant.

Analysis of ROS

The non-fluorescent diacetylated 2,7-dichlorofluorescein (DCF-DA) probe (Sigma), which becomes highlyfluorescent upon oxidation, was used to evaluate

intracellular ROS production. Cells were plated in 96-well plates (5×10^3 cells/well) and treated with various compounds 24 h later, as indicated in the legends to the figures. Then, cells were incubated with 10μM DCF-DA for 15 min at 37 °C, and DCF fluorescence was measured (λexc 485 nm and λem 535 nm) in a multimode plate reader (GENios Pro, Tecan, Milan, Italy). Values were normalized on cell proliferation by the crystal violet assay.

To evaluate mitochondrial superoxide ($O_2^{\bullet-}$) production, cells were incubated at 37 °C for 15 min in a culture medium supplemented with 1 μM non-fluorescent MitoSox Red probe (Molecular Probes, Invitrogen). Cells were w ashed with Hanks buffer (20 mM Hepes pH 7.2, 10 mM glucose, 118 mM NaCl, 4.6 mM KCl, and 1 mM $CaCl_2$) and fluorescence was measured in a multimode plate reader (λ_{exc} 430 nm and λ_{em} 590 nm). The probe is live-cell permeant and is rapidly and selectively targeted to mitochondria where it becomes fluorescent after oxidation by $O_2^{\bullet-}$. The usage of 430 ± 35 nm excitation wavelengths allowed us to selectively detect mitochondrial $O_2^{\bullet-}$ and to strongly reduce the recognition of other oxidants (e.g., –OH, $ONOO^-$). Values were normalized on cell proliferation by the crystal violet assay.

Mitotracker and MitoSox colocalization analysis

Cells were seeded in a 24-well plate on glass coverslips at the density of 6×10^4/well. After 24 hours cells were co-transfected with plasmid expressing p53 R175H and p53 R273H and its control (empty vector) by using Lipofectamine 2000, according to the manufacturer's instructions. 48 hours after transfection, mitochondria were stained with 50 nM Mitotracker (Life Technologies), MitoSox (Life Technologies) and nuclei were stained with 0.1 μg/ml Hoechst (Life Technologies). Cells were than rinsed in PBS and fixed in 4% (w/v) paraformaldehyde. Coverslips were mounted over slides in AF1 medium (Dako). Cell images were captured using a confocal laser-scanning fluorescence microscope Leica SP5 (Leica Microsystem, Manheim, Germany) at 63× magnification and processed using Adobe Photoshop and NIH ImageJ software.

Statistical analysis

ANOVA (post hoc Bonferroni) analysis was performed by GraphPad Prism 5 software. Student's test (two-tailed) was also conducted. P value < 0.05 was indicated as statistically significant. Values are the means of three independent experiments (±SD).

7. DISCUSSION AND CONCLUSION

Mutations in the *TP53* gene occur in over 50% of the human cancers (Vousden, 2002; Waddell, 2014) and most of them are missense mutations that result in the expression of mutant forms of p53, which acquire new biological properties referred as gain-of-function (GOF) that contribute to the induction and maintenance of cancer (Santoro, 2014). In many human tumors, p53 mutations are associated with high genomic instability, poor prognosis, poor response to chemotherapy and accelerated tumor recurrence (Ganci, 2013; Liu, 2012; Walerych, 2012). Different models have been proposed to explain the GOF activities of mutant p53, including binding and inactivation of the p53 family members p63 and p73; modulation of the activity of the transcription factors NF-Y, E2F1, E2F4, p65 NF-κB, and vitamin D receptor (VDR); or the inactivation of the DNA damage sensor ataxia telangiectasia mutated (ATM) (Di Agostino, 2006; Strano, 2007; Valenti, 2015; Stambolsky, 2010). Recently, we have documented that the DNA damaging drug gemcitabine stabilized mutant p53 protein in cell nuclei inducing chemoresistance and the mutant-p53 dependent expression of cell cycle genes, as Cdk1 and CCNB1, resulting in a hyperproliferation effect (Fiorini, 2015). This broad spectrum of molecular properties indicates that mutant p53 proteins are involved in a plethora of different cellular pathways focused on cancer progression and aggressiveness. The high frequency of GOF mutations in the *TP53* gene in cancer cells and the relevance of mutp53s in the clinical outcome of cancer patients have solicited worldwide scientists working in molecular oncology to deeply investigate the events associated to cancer progression driven by mutp53 proteins. Autophagy is a tightly regulated catabolic process of cellular self-digestion by which cellular components are targeted to lysosomes for their degradation. However, the role of autophagy in modulating cell death is highly dependent on the metabolic context and on the microenvironmental conditions in which the cells lie (Lorin, 2013). Key functions of autophagy are aimed to provide energy and metabolic precursors under conditions of starvation and to alleviate cellular stress removing damaged proteins and organelles, which are deleterious for cell survival. Therefore, autophagy appears to serve as a pro-survival stress response in most settings (Fiorini, 2013).

However, increasing evidence show the pivotal role of autophagy in cell death after treatment with anti-cancer drugs or under particular stressful conditions (Fiorini, 2015; Fulda and Kogel, 2015). Thus, despite the net effect of autophagy in cancer cells is highly contextual, substantial results describe that enforced over-activation of autophagy can lead to excessive cellular self-digestion via the autophagosomal-lysosomal pathway determining autophagic cell death (also named cell death type II) (Galluzzi, 2012). The importance of autophagic cell death becomes evident by the consideration that it is able to bypass drug resistance, especially in apoptosis refractory tumors, offering new therapeutic options in overcoming cancer resistance mechanisms (Fulda and Kogel, 2015; Schonthal, 2009).

The high mutation rate and the severe genomic instability are the most important features of the cancers expressing mutant p53 proteins (Soussi and Wiman, 2007). Reactive oxygen species are radicals, ions or molecules that have a single unpaired electron in their outermost shell of electrons and for this character, they are highly reactive. Mitochondria and peroxisomes are the major sites of cellular ROS generation (Danse, 2001; Ha, 2011) and while under normal physiological conditions, the intracellular levels of ROS are steadily maintained to prevent cells from damage, in cancer cells the high metabolic rate, inflammatory status or oncogene activity results in a high ROS levels compared to normal cells (Storz, 1999). A great amount of studies has deeply unveiled the existence of a ROS-mediated regulation in several cellular pathways (Liu and Storz, 2010) that sustain a plethora of biological events involved in tumor progression (Fiaschi and Chiarugi, 2012).

In this thesis we report new mechanisms by which GOF mutp53 proteins sustain aggressiveness of human cancer through a sophisticate dysregulation of some signaling pathways involved in the response of energy stresses and in the maintaining of ROS homeostasis in cancer cells. We clearly demonstrated that GOF mutant p53 proteins are able to orchestrate a plethora of events addressed to counteract autophagy in the various phases of the process, thus contributing to inhibit apoptosis and to sustain the oncogenic activity of mutant p53. Notably, we showed that mutant p53 inhibits AMPK-signaling by down-regulating Sestrin1/2

expression and stimulates mTOR signaling, resulting in a strong inhibition of autophagy in cancer cells. As paradigma of the transcriptional regulation of mutant p53 in autophagic process we unveiled that it is able to interact with the p50 NF-κB subunit onto the atg12 promoter inhibiting its expression and contributing in the repression of the autophagic machinery. Concerning the metabolic regulation of mutant p53 in tumors, we further demonstrated that PKM2 expression and its Tyr105 phosphorylated form were strongly induced by mTOR stimulation. Our data are consistent with the study of Zhang *et al*., which documented that mutant p53 was responsible for the raise of the glycolytic rate in cancer cells (Zhang, 2013), and with the study of Hitosugi *et al*. which revealed that mutation at Tyr105 of PKM2 determined both the decrease of the cellular proliferation and the increase of oxidative phosphorylation and lactate production (Hitosugi, 2009). Altogether these findings strongly suggest the involvement of PKM2 regulation on lactate secretion and on the glycolytic cancer metabolism supported by mutant p53 activity. In addition, we showed that mutant p53 was able to induce mTOR-dependent STAT3 pathway which has been already described to stimulate the Warburg effect (Li, 2015) and cancer progression (Yu, 2014), and to inhibit autophagy by down-regulating LC3 gene (Gong, 2014). Interestingly, we point out that cancer cells bearing endogenous mutant p53 are more sensitive to the mTOR inhibitor everolimus than cancer cells expressing wild-type p53 protein, providing a conceivable personalized therapy for cancer patients carrying GOF mutations of the *TP53* gene. Our further unpublished data revealed that through the down-regulation of AMPK signaling, mutant p53 proteins effectively contrast PGC-1α/UCP2 axis that is a key regulator of mitochondrial ROS homeostasis in the cells. As a consequence of the inhibition of these antioxidant players, we observed that mutant p53 enhances mitochondrial and cytosolic ROS production and hence oxidative stress, resulting in the stimulation of chemoresistance and proliferation of cancer cells. It is tempting to speculate about the existence of novel mechanisms by which GOF mutant p53 proteins are able to orchestrate cancer cell proliferation, that we have found to be i) the inhibition of autophagy-apoptosis axis and ii) by enhancing oxidative stress,

which might provide new therapeutic opportunity to treat patients bearing mutant *TP53* gene.

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9. ANNEXS

During the period of my PhD I have been collaborating in the following publications: (* equally contribution, co-first name)

1) Claudia Fiorini; **Marco Cordani**; Chiara Padroni; Giovanni Blandino; Silvia Di Agostino; Massimo Donadelli. Mutant p53 stimulates chemoresistance of pancreatic adenocarcinoma cells to gemcitabine. BIOCHIMICA ET BIOPHYSICA ACTA MOLECULAR CELL RESEARCH 2015. 1853(1):89-100. doi:10.1016/j.bbamcr. 2014.10.003. **IF: 5.128**

2) Claudia Fiorini; **Marco Cordani**; Giovanni Gotte; Delia Picone; Massimo Donadelli. Onconase induces autophagy sensitizing pancreatic cancer cells to gemcitabine and activates Akt/mTOR pathway in a ROS-dependent manner. BIOCHIMICA ET BIOPHYSICA ACTA MOLECULAR CELL RESEARCH 2015. 1853(3):549-60. doi:10.1016/j.bbamcr.2014.12.016. **IF: 5.128**

3) Ilaria Dando*****; **Marco Cordani***; Elisa Dalla Pozza; Giulia Biondani; Massimo Donadelli; Marta Palmieri. Antioxidant mechanisms and ROS-related in Cancer Stem Cells. OXIDATIVE MEDICINE AND CELLULAR LONGEVITY. 2015. 2015:425708. doi:10.1155/2015/425708. **IF: 4.492**

4) Ilaria Dando; Elisa Dalla Pozza; Giulia Biondani; **Marco Cordani**; Marta Palmieri; Massimo Donadelli. The metabolic landscape of cancer stem cells. INTERNATIONAL UNION OF BIOCHEMISTRY AND MOLECULAR BIOLOGY. 2015. 67(9):687-93. doi: 10.1002/iub.1426. **IF: 2.653**

5) José J. Bravo-Cordero*****, **Marco Cordani***, Silvia F. Soriano*****, Begoña Diez, Carmen Muñoz-Agudo, María Casanova-Acebes, César Boullosa, Marta C. Guadamillas, Iakes Ezkurdia, David Gonzalez-Pisano, Miguel Angel Del Pozo, and Maria C. Montoya. A novel High Content Analysis tool reveals Rab8-driven actin and FA reorganization through

RhoGTPases and calpain/MT1. JOURNAL OF CELL SCIENCE. 2016. 129(8):1734-49. doi: 10.1242/jcs.174920. **IF: 4.706**

6) **<u>Marco Cordani</u>**, Raffaella Pacchiana, Giovanna Butera, Gabriella D'Orazi, Aldo Scarpa, Massimo Donadelli, Mutant p53 proteins alter cancer cell secretome and tumour microenvironment: involvement in cancer invasion and metastasis, CANCER LETTERS 2016. 376(2):303-9 doi: 10.1016/j.canlet.2016.03.046. **IF: 5.992**

7) **<u>Marco Cordani</u>**, Elisa Oppici, Ilaria Dando, Elena Butturini, Elisa Dalla Pozza, Mercedes Nadal-Serrano, Jordi Oliver, Pilar Roca, Sofia Mariotto, Barbara Cellini, Giovanni Blandino, Mara Palmieri, Silvia Di Agostino, Massimo Donadelli. Mutant p53 proteins counteract autophagic mechanism sensitizing cancer cells to mTOR inhibition. MOLECULAR ONCOLOGY 2016. 10(7):1008-29. doi: 10.1016/j.molonc.2016.04.001. **IF: 5.367**

8) Ilaria Dando, **<u>Marco Cordani</u>**, Massimo Donadelli. Mutant p53 and mTOR/PKM2 regulation in cancer cells. INTERNATIONAL UNION OF BIOCHEMISTRY AND MOLECULAR BIOLOGY. 2016. 68(9):722-6. doi: 10.1002/iub.1534. **IF: 2.653**

9) Jessica Brandi*****, Daniela Cecconi*****, **<u>Marco Cordani</u>***, Margalida Torrens, Raffaella Pacchiana, Elisa Dalla Pozza, Giovanna Butera, Marcello Manfredi, Emiliano Marengo, Oliver Jordi, Pilar Roca, Ilaria Dando, Massimo Donadelli. Uncoupling protein 2 stimulates GLUT1 and pyruvate kinase isoform M2 expression sensitizing pancreas cancer cells to glycolysis inhibition. FREE RADICAL BIOLOGY & MEDICINE.2016.101:305-316.doi: 10.1016/j.freeradbiomed.2016.10.499. **IF: 5.784**

10) **<u>Marco Cordani</u>**, Raffaella Pacchiana, Giovanna Butera, Massimo Donadelli. Molecular interplay between mutant p53 proteins and autophagy in cancer cells. BBA REVIEWS ON CANCER. pii: S0304-419X(16)30102-0. 003doi: 10.1016/j.bbcan.2016.11.003. **IF: 7.841**

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