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Identification of mutant p53 inhibitors by high-content screening

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XXVII CICLO DEL DOTTORATO DI RICERCA IN BIOMEDICINA MOLECOLARE

Identification of mutant p53 inhibitors by high-content screening

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This thesis is dedicated to my parents for teaching me the importance of passion and sacrifice and who always supported me unconditionally
Thank you.

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ABSTRACT

The TP53 gene is the most commonly altered gene in human cancer. The majority of p53 mutations are missense and result in the accumulation of dysfunctional p53 protein in cancer cells. These mutant proteins frequently acquire neomorphic functions (defined as Gain of Function, GOF) including the induction of malignant properties of cancer cells, such as uncontrolled cell proliferation, metastasis and drug resistance. A number of evidence reveals that stabilization of mutant p53 proteins in tumours is required for their GOF properties, while reduction of mutant p53 levels mitigates the malignant potential of cancer cells. Thus, targeting mutant p53 represents a tumour attractive strategy for cancer therapy. Several small-molecule compounds that specifically target mutant p53 have been identified and are now in preclinical or clinical development. Some of them induce instability of mutant p53 proteins, leading to inhibition of multiple downstream pathways of GOF mutant p53. In this thesis, I tested a collection of FDA-approved drugs to identify molecules able to reduce the levels of mutant p53 in a triple negative breast cancer cell line. This screening allowed the identification of statins as class of drugs strongly inhibiting mutant p53 accumulation. Further investigation demonstrated that mutant p53 protein stability depends on the activation of the metabolic mevalonate pathway and that statins inhibit mutant p53 GOF in cancer cells. Mechanistically, the mevalonate pathway intermediate geranylgeranyl pyrophosphate inhibits the Mdm2-dependent mutant p53 protein degradation. In particular, my data show that mutant p53 stability is controlled by geranylgeranylated proteins and that geranylgeranyl transferase inhibitors behave as statins. These results on a novel crosstalk between the metabolic mevalonate pathway and mutant p53 support the notion that these two signals are strongly intertwined and together concur to the malignant phenotype of different tumours. The data of this thesis provide the experimental-based rational for the use of mevalonate pathway inhibitors as adjuvant treatment in the therapy of tumours bearing sporadic or inherited mutations of p53.

INTRODUCTION

The tumour suppressor p53

One of the most important signalling pathways against tumour formation and progression is the p53 tumour suppressor pathway. p53 was discovered in 1979 by three independent groups as an interacting partner of the viral SV40 T-antigen (Lane & Crawford, 1979) (Linzer & Levine, 1979) (Kress, May, Cassingena, & May, 1979). For almost a decade, p53 was considered to be a tumour antigen with transforming capabilities. Only during the late 1980s it was revealed that p53 is indeed a tumour suppressor and that the evidence for its supposed oncogenic functions had been erroneously collected from tumour-derived mutant clones (Weisz et al. 2007). Throughout about 30 years of intensive studies, extensive knowledge has been achieved on the p53 pathway and a great extent of complexity has been unveiled. Embedded within a complex signaling pathway, p53 in response to a variety of stress signals that originate both from external factors (such as γ-radiation, UV-light, DNA damaging agents etc) or internal once (oncogene activation, high levels of reactive oxygen species, ribonucleotide depletion etc.) acts essentially as a transcription factor able to promote the coordinated expression of an array of target genes that are the executors of p53-induced cellular responses, such as cell cycle arrest, senescence or apoptosis (Levine and Oren, 2009) that may compromise genomic stability and promote neoplastic transformation (Figure1).

Given its crucial role as key integrator in translating diverse stress signals into different cellular outcomes, p53 has been called “guardian of the genome” (Levine & Oren 2009; Müller et al. 2001).

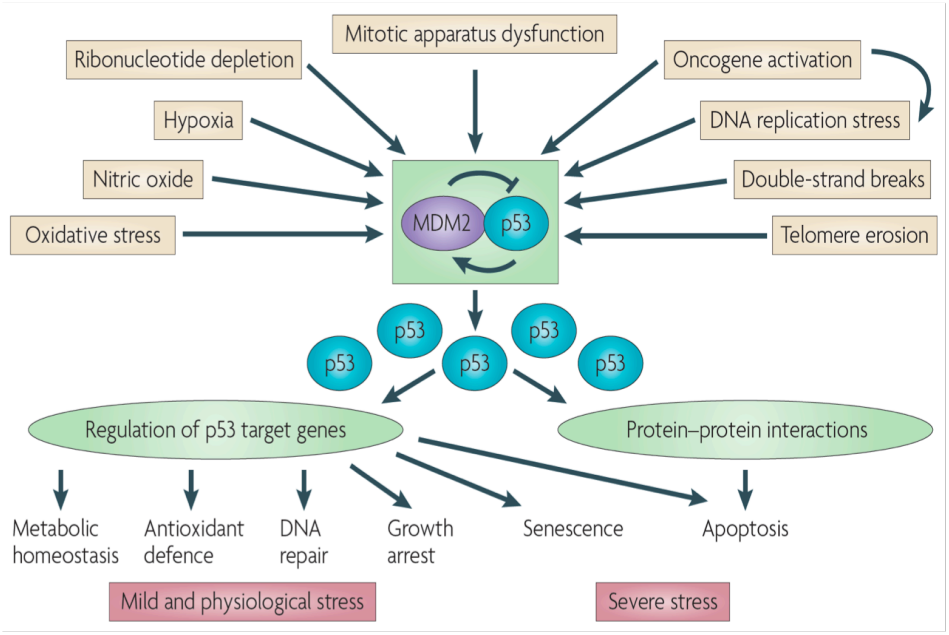

Figure 1. Simplified scheme of the p53 pathway. The p53–Mdm2 feedback loop is the "heart" of the p53 pathway. Under normal conditions, it maintains constantly low steady-state p53 levels and activity. Various stress signals, related in many ways to carcinogenesis, impinge on this central loop to release p53 from Mdm2-mediated inhibition. This increases p53 protein levels and activity, inducing various phenotypic changes. The nature of the phenotypic response to p53 activation is, at least partially, proportionate to the amplitude, duration and nature of the activating signal. Recent evidence indicates that p53 has an important role also in enabling the cell to adjust its metabolism in response to mild normal physiological fluctuations, including those in glucose and other nutrient levels, oxygen availability and reactive oxygen species levels. (Levine and Oren, 2009)

The tumour suppressor protein p53 is expressed at very low levels in normal cells but accumulates in response to stress. The E3 ubiquitin ligase, Mdm2, is the most critical regulator of p53 and is also a p53 target gene (Harris & Levine 2005; Brooks & Gu 2006). Mdm2 is able to maintain p53 protein at low levels promoting its ubiquitylation and consequent proteasomal degradation (Haupt et al. 1997; Kubbutat et al. 1997). However under stress condition, the increase of p53 levels are accompanied by transcriptional upregulation of Mdm2 expression, which can in turn inhibit p53 dependent transcriptional activation, creating a negative feedback loop resulting in down-regulation of p53 activity (Figure 1).

A complex role in the p53-Mdm2 loop is played by the Mdm2-related protein Mdmx (known as Mdm4 in mouse), which, although lacking ubiquitin ligase activity on its own, has been reported to complex with Mdm2 and stimulate its E3 activity towards

p53, thus maintaining p53 at low levels under normal conditions (Linares et al. 2003).

p53 undergoes a great variety of post-translational modifications, such as phosphorylation, acetylation and ubiquitination, that influence its stability and its transcriptional activity. Many kinases, (for example ATM, ATR, Chk1, Chk2, CK1, CK2, JNK, Erk, p38, Aurora Kinase A, GSK3β, HIPK2 and DYRK2) have been shown to phosphorylate p53 after DNA damage (Kruse & Gu 2009; Vousden & Prives 2009). The best-characterized p53 phosphorylations (Ser15, Thr18, Ser20 and Ser46) occur in the N-terminus of the protein and are know to upregulate p53's transcriptional activity in response to stress condition; while Thr55, Ser376 and Ser378 residues seem to be constitutively phosphorylated in unstressed cells (Gatti et al. 2000; Waterman et al. 1998). Moreover, p53 can be acetylated at several lysines by different histone acetyl-transferases (HATs) such as p300/CBP (on Lys 370, 372, 373, 381 and 382) and PCAF (p300/CBP-associated factor) (on Lys320 and Lys305), which have been shown to acetylate p53 in response to DNA damaging agents, such as UV- and γ-irradiation (Carter & Vousden 2009). Thus, in response to a variety of stress stimuli, p53 is rapidly stabilized and activated through a complex repertoire of post-translational modifications and protein–protein interactions to allow both direct and indirect transactivation of many coding and non-coding genes (Kruse & Gu 2009).

The p53 family

For a long time, p53 was believed to be a unique protein without any paralogue. However, later it became clear that p53 belongs to a multigene family that includes other two members and also transcription factors, namely p63 and p73 (Kaghad et al. 1997; Yang et al. 1998) that are structurally similar and functionally related to p53. Although all three proteins are able to bind to DNA in a sequence specific manner (recognizing similar *consensus* sequences) and have a similar modular organization, their primary structures do not share an elevated homology: it is about 30% for the whole sequence, but it reaches 65% when only the DNA binding domain is considered (*IARC TP53 Database*, www.p53.iarc.fr).

As a consequence of the partial structural homology, p53 family members have some overlapping functions mediated by the transactivation of common target genes (Stiewe 2007). Indeed, p63 and p73 are able to trigger apoptosis upon DNA damage and in

response to chemotherapeutic drugs (Yang & McKeon 2000; Lang et al. 2004; Lin et al. 2009) and to induce senescence both *in vitro* and *in vivo* (Fang et al. 1999; Guo et al. 2009). Recently, a critical role for p63 in metastasis suppression has emerged (Adorno, Cordenonsi, Montagner, Dupont, Wong, Hann, Solari, Bobisse, Rondina, Guzzardo, Anna R Parenti, et al. 2009; Muller et al. 2009). The silencing of p73 and p63 increases the transforming potential of p53-null mouse embryonic fibroblast (Lang et al. 2004). Consistently, analysis of the tumour predisposition of p63 and p73 heterozygous mice revealed a consistent connection with cancer: p63+/- and p73+/- mice develop spontaneous tumours and survive similarly to p53+/- mice (Flores et al. 2005). Even if there are not evidence of mutations that compromise their functions in cancer, p63 and p73 are aberrantly expressed in tumour.

Thus the entire p53 family may be considered as a unique signaling network in which all p53 family proteins are involved in the response to oncogenic stress and physiological inputs, sharing also many oncosuppressive functions.

Functional domains of p53 protein

The human p53 gene span 20 Kbases on chromosome 17 (17p13.1) and consists of 11 exons, which encode a protein of 393 residues (el-Deiry et al. 1992). As many other transcription factors, p53 has a modular structure composed by evolutionarily conserved functional domains: an N-terminal transactivation domain (aa 1-61), a proline-rich domain (aa 64-93), a central DNA-binding domain (aa 93-292), an oligomerization domain (aa 325-355) and a C-terminal regulatory domain (figure 2).

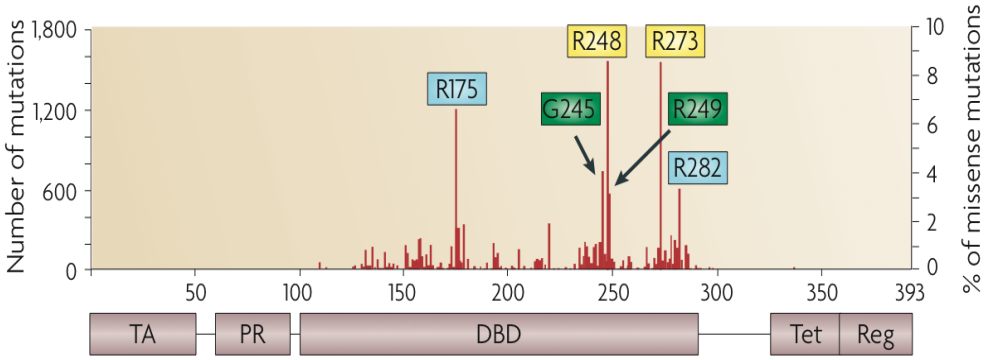

Figure 2. The distribution of reported missense mutations along the p53 sequence. The six most common hotspot mutations are highlighted in yellow for DNA-contact mutations, green for locally distorted mutants and blue for globally denatured mutants. The domain architecture of p53 is aligned below. TA, transactivation domain; PR, proline-rich domain; DBD, DNA binding domain; Tet, tetramerization domain; Reg, carboxy-terminal regulatory domain. Data derived from the IARC TP53 Database.

The N-terminal transactivation domain of p53 contains two acidic domains (TAD1), aa 1-40 (Unger et al. 1993) and TAD2, aa 40-61 (Candau et al. 1997) interacting with the transcriptional machinery (Unger et al. 1993) such as TBP (TATA box binding protein), TAFs (TBP-associated factors) (Chang et al. 1995) and the transcriptional coactivators CBP (CREB binding protein) and p300 (Avantaggiati et al. 1997) (Scolnick et al. 1997). This region contains also the binding site for the major negative regulator of p53, the ubiquitin ligase Mdm2, and the related transcriptional repressor Mdmx (Toledo et al. 2007).

The transactivation domain is followed by a proline-rich domain (PRD, aa 64-93), which contains five repeats of the amino acid motif PXXP (P= proline; X= any amino acid) and it is required for p53 stabilization. Indeed, the prolyl isomerase Pin1 binds to Thr81-Pro82 site, upon Thr81 phosphorylation, and induces the isomerization of the peptide bond, leading to Mdm2 displacement (Berger et al. 2005; Zacchi et al. 2002; Zheng et al. 2002). The central core of p53 contains its DNA binding domain (aa 93-292), that recognizes and binds the p53-responsive element on DNA, and also proteins that positively affect p53 acitvity (e.g. 53BP1, Hzf, ASPP1 and ASPP2). The oligomerization domain (OD, aa 325-355) is required for the formation of a high-affinity DNA binding and transcriptional competent p53 tetramer.

The C-terminal domain of p53 contains several residues targeted by post-translational modifications that modulate p53 stability and function (Kruse & Gu 2009). In the C

terminus of p53 is present a cluster of three nuclear localization signals (NLS) that mediate the shuttling of the protein into the cell nucleus (Shaulsky et al., 1990). p53 contains also two putative nuclear export signals (NES), one in the N-terminus (nNES, aa 11-27) and the other in the OD (cNES, aa 340-351) (Stommel et al. 1999; Zhang & Xiong 2001).

p53 isoforms

Few years ago, it has been revealed the existence of a complex pattern of different p53 isoforms due to use of several promoters and to alternative splicing (figure 3).

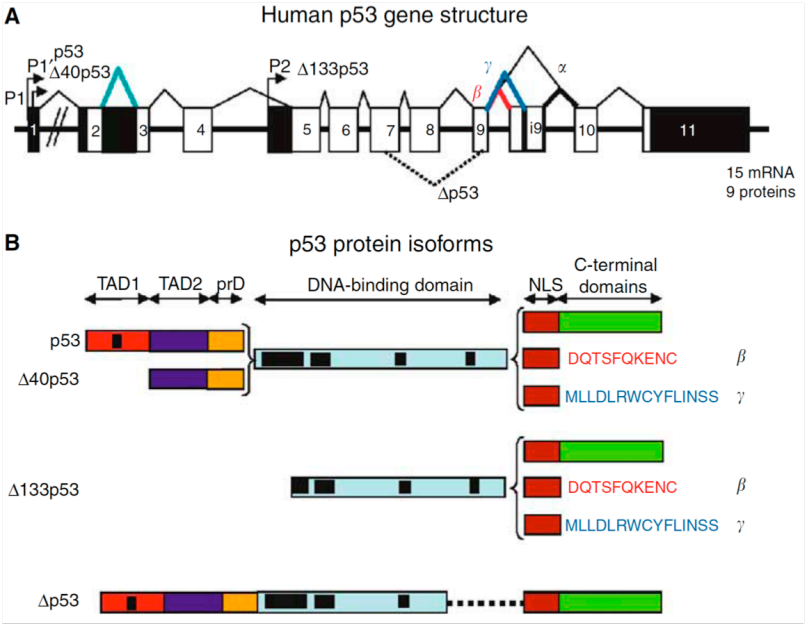

Figure 3. (A) Scheme of the human p53 gene structure. Altrenative splicing (α, β,γ) and alternative promoters (P1, P1', P2) are indicated. **(B)** Stuctural and functional domains of p53 protein isoforms.

The p53 protein can be codified from transcripts that can be initiated from two distinct sites upstream of exon 1 (P1 and P1'), but also from an internal promoter located in intron 4 (P2). The use of alternative promoters can lead to the expression of two different N-terminally truncated p53 proteins: Δ40p53 and Δ133p53. Δ40p53, which misses part of the transactivation domain (TAD1), can be obtained also by alternative initiation of translation or alternative splicing of the intron 2. The usage of P2 mediates the expression of Δ133p53, isoform that initiates at codon 133 and lacks the entire

transactivation domain (TAD1 and TAD2), the proline rich domain and part of the DNA-binding domain. Moreover, the alternative splicing of intron 9 can produce three isoforms different in their C-terminus: p53 (or p53α), p53β and p53γ, with p53β and p53γ isoforms lacking the oligomerization domain (Bourdon et al. 2005; Ghosh et al. 2004). Interestingly, an additional p53 isoform (Δp53) has been described, which is characterized by a deletion of 66 aminoacids within the core domain (corresponding to aa 257-322) and showed a transcriptional activity different from that of full-length p53 (Rohaly et al. 2005). Indeed, it induces only p53 target genes involved in cell- cycle arrest, thereby participating in a specific intra-S phase checkpoint.

Until now, the spatial and temporal expression levels of the different p53 isoforms are largely unknown and their functional roles still await further characterization.

Tumour suppressor activities of p53

Once activated, the transcription factor p53 exerts its activity by directly binding to specific target sequences on DNA to regulate the expression of several genes involved in a wide variety of biological process such as DNA repair, development, cell death, metabolism, senescence to prevent tumourgenesis and to maintain genomic integrity (Levine & Oren 2009).

Among the tumour suppressive activities fostered by p53, apoptosis is undoubtedly the most studied mechanism *in vitro* and *in vivo*. Under genotoxic stress, activated p53 rapidly regulates the expression of pro-apoptotic members of this pathway such as, Bax, Bid, Puma, Noxa, p53AIP1 (Nakano & Vousden 2001) and repress anti-apoptotic genes Bcl2 and Bcl-xL (Green & Kroemer 2009; Chipuk et al. 2004) with consequent permeabilization of the mitochondrial outer membrane and release of cytocrome c (Vousden, 2005). Furthermore, recent studies have shown that p53 is also able to induce apoptosis in a transcription-independent way (Vaseva & Moll 2009); indeed, a fraction of p53 is able to translocate to mitochondria, where it directly binds and inhibits anti-apoptotic Bcl-xL and Bcl-2 factors, inducing release of cytochrome c (Mihara et al. 2003).

The tumour suppressor p53 was found also to regulate apoptosis in a Ca2+-dependent manner at the endoplasmic reticulum. Mechanistically, p53, upon activation induced by

genotoxic stress, was found directly to bind to the sarco/ER Ca2+-ATPase (SERCA) pump at the ER, changing its oxidative state and thus leading to an increased Ca2+ load, followed by an enhanced transfer to mitochondria. The consequent mitochondrial Ca2+ overload causes in turn alterations in the morphology of this organelle and induction of apoptosis (Giorgi et al. 2015).

Interestingly, in response to oxidative stress, the tumour suppressor p53 can induce necrosis instead of apoptosis. Excess cytosolic Ca2+ and ROS induce p53 activation and its mitochondrial translocation, leading to VDAC oligomerization and permeability transition pore (PTP) formation, causing H2O intake, swelling of mitochondria and necrosis (Vaseva et al. 2012). According to cell type and the kind of stress, the activation of the p53 pathway can also induce cell cycle arrest, at different cell cycle checkpoints (G1 or G2), mostly promoting the induction of three critical target genes: p21, 14-3-3σ and GADD45 to prevent the propagation and accumulation of DNA damage and mutations (Kastan et al. 1992; Hermeking et al. 1997; El-Deiry et al. 1993).

Induction of senescence is another important function of the tumour suppressor p53. Indeed, in response to oncogenic stress and chemotherapy, p53 activation can also lead to cell cycle arrest and senescence instead of apoptosis and necrosis. Interestingly, it was recently reported that reactivation of p53 in p53-deficient tumours completely represses tumour growth through senescence in a mouse liver tumour model (Xue et al. 2007). In the first moment after the oncogenic stimulus, p53 activation leads to senescence by inducing p21, while the permanent growth arrest is then maintained by p16 expression (Chen et al. 2005; Ventura et al. 2007).

In the recent years it has also been found that p53 can promote the process of autophagy through different mechanism, which may contribute to the role of p53 in tumour prevention. When exposed to stress, nuclear p53 can induce autophagy by inhibiting the master negative regulator of autophagy mTOR, acting at multiple levels of the AMPK-mTOR axis (Feng et al. 2005; Feng et al. 2007). Furthermore, p53 is also able to induce autophagy regulating the expression of genes such as DRAM, PUMA, Ei24 (Crighton et al. 2006; Zhao et al. 2012). Regulation of cell cycle arrest, senescence and apoptosis are the most well studied functions of p53, which have been accepted as the main mechanisms for p53 to function as a tumour suppressor. Interestingly, recent studies have revealed that p53 regulates cellular energy metabolism (Bensaad et al. 2006;

Vousden & Ryan 2009), and antioxidant defense (Budanov et al. 2004) which contribute greatly to the role of p53 in tumour suppression.

The activity of p53 in metabolism involves several aspects, spanning from oxidative stress regulation to the orchestration of glucose metabolism (Maddocks & Vousden 2011) resulting from a coordination of transcriptional and cytoplasmic activities of p53. The tumour suppressor is able to promotes both the mitochondrial oxidative phosphorylation (OXPHOS), activating synthesis of cytochrome c oxidase 2 (SCO2) expression, cytochrome c oxidase (COX) I subunit, and AIF (Vahsen et al. 2004) and down-regulates glycolysis in cells to maintain the homeostasis of energy metabolism. The reactive oxygen species (ROS), produced by the enhancement of OXPHOS, could be detrimental for cell survival, thus p53 transcriptionally induces a group of antioxidant genes, including sestrins 1/2, TIGAR, MnSOD, GPX1, ALDH4, GLS2, and Parkin (Hu et al. 2010; Suzuki et al. 2010; Budanov et al. 2010; Pani & Galeotti 2011), to reduce the intracellular levels of ROS and prevent DNA damage induced by ROS (Liang et al. 2013; Bensaad & Vousden 2007). Interestingly, it has been recently demonstrated that p53 binds to and reduces the activity of glucose-6-phosphate dehydrogenase, a rate-limiting enzyme in the pentose phosphate pathway, to down-regulate glucose metabolism and the Warburg effect (Jiang et al. 2011).

Thus, the tumour suppressor p53 is a transcription factor that in response to a plethora of stress stimuli activates a complex and context-dependent cellular response, ultimately protecting genome integrity and preventing tumourgenesis.

Mutant p53

The TP53 gene is frequently altered gene in human cancers (Cyriac Kandoth, Michael D. McLellan, Fabio Vandin, Kai Ye 2013). Mutations in the TP53 gene occur in over 50% of all tumours with frequencies that vary considerably between cancer types, raging from 10% in hematopoietic malignancies (Peller et al. 2003), to 50-70% in ovarian (Schuijer & Berns 2003), colorectal (Iacopetta 2003), and head neck cancers (Blons & Laurent-Puig 2003). Clinical studies revealed that certain mutations in the TP53 gene have been associated with poor clinical outcome in a variety of malignancies, including breast cancer, and are also associated with an even worse

prognosis (Olivier et al. 2006; Petitjean et al. 2007). TP53 gene is mutated in ~35% of total breast cancer, but this percentage is increased to 54% in triple negative breast cancers (TNBC) (Polyak & Metzger Filho 2012).

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).

Unlike most tumour-suppressor genes that usually undergo gene-expression inactivation in carcinogenesis by deletions or truncating mutations, the TP53 gene is frequently inactivated (74%) by missense mutations (IARC TP53 Database). As a consequence, in the vast majority of tumours, cells express a stable full-length mutant form of p53, which differs from the wild-type counterpart in a single amino acid substitution. Most of these missense mutations occur within the DNA binding domain (DBD) and particularly six "hot spot" mutated residues have been identified (Hollstein et al., 1991) and classified into two main categories according to their effect on the thermodynamic stability of the p53 protein (Bullock and Fersht, 2001). These two categories are commonly referred to as "DNA-contact" mutant, where mutations occur on amino acids directly binding to the DNA (e.g. $p53^{R273H}$ and $p53^{R280K}$) and "conformational" mutant in which the structure of p53 protein is altered by mutations, thus abolishing its DNA-binding ability (e.g. $p53^{R175H}$ and $p53^{R249S}$) (Joerger & Fersht 2008).

The functional effects of TP53 mutations can be classified into three non-mutually exclusive groups (Brosh and Rotter, 2009) (Figure 4):

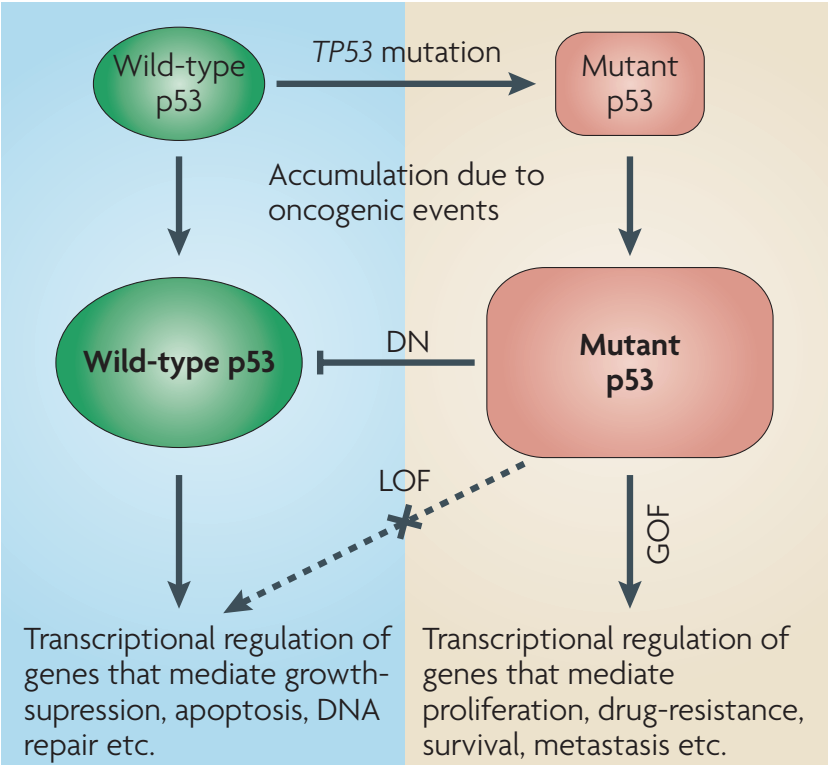

Figure 4. Schematic representation of the functional impacts of TP53 mutations. LOF (loss-of-function); DN (dominant-negative effects); GOF (gain-of-function). From Brosh and Rotter, 2009.

- Loss of functions (LOF): most missense mutations have abrogated the tumour suppressor functions of the affected allele. 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 et al., 2003) (Figure 4).
- Dominant-negative effect: mutant p53 is capable of inhibiting, to varying degrees, the function of the wild-type protein encoded by the second allele. This "dominant-negative" effect is achieved by oligomerization of mutant and wild-type proteins, forming a heterotetramer defective in sequence specific DNA binding (Dittmer et al., 1993; Milner and Medcalf, 1991). Furthermore, p53mutations are usually followed by loss of heterozygosity in human cancer, leading to deletion or mutation of the rest wild-type p53 allele (Figure 4).
- Gain of functions (GOF): the aberrant expression of several missense mutant p53 proteins leads the cells to acquire a complex repertoire of oncogenic traits such as increased proliferation, increased migration and resistance to

chemotherapy treatment.

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Gain-of-Function of mutant p53 in cancers

As the field of p53 research evolves, it is increasingly evident that many mutant p53 forms not only lose their tumour suppressive functions and acquire dominant-negative activities, but also gain oncogenic properties that can actively contribute to various aspects of tumour progression such as migration and invasion, angiogesnsis and chemioresistance, rendering the tumour cells harboring mutant p53 more aggressive. (figure 5).

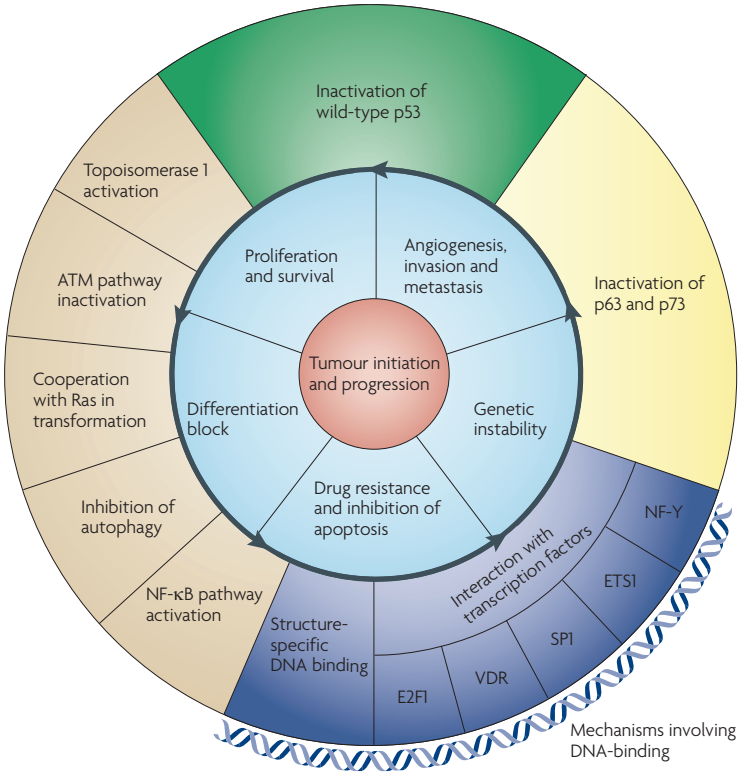

Figure 5. Selected oncogenic properties of mutant p53 and their underlying mechanisms. The inner circle (shaded blue) represents oncogenic phenotypes associated with the activities of mutant p53 proteins. The outer circle depicts key mechanistic properties of p53 mutants that underlie the phenotypes listed in the inner circle. Each of the phenotypic effects can be attributed to almost each of the mechanistic properties; hence the inner blue circle can be freely rotated. (Brosh and Rotter, 2009)

In these years numerous evidences clearly established the pro-oncogenic role of p53 missense mutants p53 (Oren & Rotter 2010). The first evidence that mutant p53 may

show neomorphic gain of functions, was proved from the findings that the introduction of mutant p53 protein in p53-null tumour cells greatly increased the oncogenic potential of those cells in nude mice (Wolf et al. 1984; Dittmer et al. 1993). Mutant p53 was also shown to cooperate in oncogenesis with activated oncogenic Ras, both in primary mouse embryo fibroblasts (MEFs) (Lang et al. 2004) and *in vivo* experiments, demonstrating that this cooperation increased tumour formation and progression with elevated rates of metastasis (Caulin et al. 2007; Jackson et al. 2005).

Although p53 knockout mice are highly tumour prone, these lesions do not metastasize frequently nor generally display invasive pathology (Attardi & Jacks 1999). On the contrary, knock-in mouse models harboring two tumour-derived mutants of p53 (equivalent to $p53^{R175H}$ and $p53^{R273H}$ in humans), display an altered tumours spectrum and more metastatic tumours when compared to p53 null mice (Olive et al. 2004; Lang et al. 2004). In addition it was unveiled, that mouse embryonic fibroblasts (MEFs) from mice with one or two copies of the $p53^{515A}$ allele *(*corresponding to the $p53^{R175H}$ hot spot mutation in human cancers) showed increased growth rates than $p53^{+/-}$ and $p53^{-/-}$ cells. This suggests that the $p53^{515A}$ mutation gives to cells a growth advantage, allowing cells to bypass contact inhibition.

More recently, mutant p53 was found to enhance the metastatic potential of human tumour cell lines by facilitating cell migration and invasion (Adorno, Cordenonsi, Montagner, Dupont, Wong, Hann, Solari, Bobisse, Rondina, Guzzardo, Anna R Parenti, et al. 2009; Dhar et al. 2008; Müller et al. 2001).

One distinctive feature of many p53 mutants is the ability to confer an elevated resistance to cells to a variety of pro-apoptotic signals such as cMyc-induced apoptosis in leukemic cells (Lotem & Sachs 1995). Overexpression of various tumour-associated mutant p53, compromise the efficacy of cancer chemotherapy rendering some cell types more resistant to therapeutic drugs such as doxorubicin, etoposide, and cisplatin (Bossi et al. 2006; Blandino et al. 1999; Li et al. 1998). The antiapoptotic activities of mutant p53 may thus not only accelerate tumour progression but also hinder the response of cancer patients to anticancer therapy.

Several p53 mutations were reported to disrupt normal spindle checkpoint control, leading to genomic instability, manifested by inter-chromosomal translocations (Gualberto et al. 1998). The existence of this connection was established also *in vivo*

(Caulin et al. 2007; Hingorani et al. 2005).

Alterations in cellular metabolism (metabolic reprogramming) are known to be a hallmark of cancer cells and a key contributor to tumour development (Hanahan & Weinberg 2011; Haupt et al. 1997; Feng et al. 2007; Levine & Puzio-Kuter 2010). Recently, it was demonstrated that stimulation of the Warburg effect (or aerobic glycolysis), the best-characterized metabolic change observed in cancer, is a crucial GOF of tumour-associated mutant p53. The Warburg effect is a phenomenon in which most tumour cells primarily utilize glycolysis for their energy, even under normal oxygen concentrations and is characterized by a much higher rate of glucose uptake and higher lactate production in tumour cells compared with normal cells (Feng et al. 2007; Haupt et al. 1997; WARBURG 1956). Mechanistically, mutant p53, both in cultured cells and knock-in mice, stimulates the Warburg effect mainly through promoting the translocation of GLUT1 (glucose transporter 1) to the plasma membrane (PM). This effect has been proved to be mediated by RhoA and its downstream effector ROCK (Rho-associated protein kinase) (Zhang et al. 2013).

Recent studies showed that mutant p53 is also involved in the disruption of mammary tissue architecture via the mevalonate pathway, a metabolic pathway responsible for *de novo* cholesterol biosynthesis. It was observed that non malignant breast epithelial cells, in three dimensional culture model (3D), form spheroids reminiscent of acinar structures found *in vivo*, whereas breast cancer cells display highly disorganized morphology. Interestingly, it was unveiled that expression of mutant p53 in non-malignant mammary epithelial cells is sufficient to induce an alteration of the three-dimensional architecture of breast acini, disrupting their morphology in 3D cultures (Freed-Pastor et al., 2012). In particular, through a genome-wide expression analysis the mevalonate pathway was identified as significantly upregulated by mutant p53 and experiment performed with supplement of statins and sterol biosynthesis intermediates, reveal that this pathway is both necessary and sufficient for the phenotypic effects of mutant p53 on breast tissue architecture (Freed-Pastor et al. 2012).

Mechanisms of mutant p53 Gain-of-function

Several mechanisms of mutant p53 GOF have been described and can be subdivided in three main categories (Figure 5):

Mutant p53 binds to DNA to alter gene expression

Despite mutant p53 proteins are unable to recognize wt-p53 consensus on DNA (Kato et al., 2003), modulation of gene transcription by mutant p53 is well documented and the list of its target genes is constantly growing (Brosh and Rotter, 2010). Mut-p53 proteins typically retain an intact transactivation domain (TAD), which may still operate exactly as it does within the wt-p53 protein (Lin et al. 1995), but can now be targeted to different sites on the chromatin. More evidences unveiled that several p53 mutants, although defective in sequence-specific DNA binding, retain the ability to bind specific non-B DNA structures with high affinity, even if different mutants bind various DNA structures through distinct mechanisms and with different affinities (Gohler et al., 2005). Therefore, the specificity of mutant p53 to certain regulatory sequences is perhaps mediated by preferential binding to structural DNA motifs and not consensus sequences.

Mutant p53 binds to transcription factors to regulate their functions

Aberrant transcriptional regulation is a major event in human cancers, and this may occur through unscheduled activity of specific transcription factors, or aberrant recruitment of transcription co-activators, thus regulating their uncontrolled gene activation or repression. It has been reported that mutant p53 can interact with several transcription factors to enhance or prevent their activities operating as a co-factor able to sustain the expression of several pro-oncogenic genes.

A central point in the mutant p53 gain-of-function mechanisms is the ability of p53 mutants to bind and inactivate the p53 family members p63 and p73 (Di Como et al. 1999; Gaiddon et al. 2001). Evidence supporting this notion, besides numerously experiment in cells, has come from the recently developed knock-in mouse model expressing mutant p53 isoform ($p53^{R172H}$) which was shown to bind p63 and p73, consequently inhibiting their abilities to induce cell-cycle arrest and to suppress tumour formation (Lang et al. 2004). p63 and p73 proteins bind to and activate many wild-type p53 target genes, and mediate cell cycle arrest, apoptosis, and senescence in response to stress. On the contrary mut-p53 proteins can engage in direct protein–protein interactions with p73 and p63, rendering them transcriptionally inactive (Marin et al.

2000; Strano et al. 2002). Consequently, genes that are normally controlled by p63 or p73 will become deregulated. Moreover, the p73-binding capacity, is correlated with the ability of p53 mutants to protect cells from chemotherapeutic agents and, accordingly, with less favorable response to chemo-radiotherapy in patients with head and neck cancer (Li & Prives 2007). However, the interaction between mut-p53 and p63/p73 can be regulated by cell-intrinsic and extrinsic signals and by additional partner proteins. In particular, in breast cancer cells, mutant p53 acts as a molecular switch for TGF-β-induced metastasis by curbing the p63 transcriptional activity through the formation of a ternary complex with Smad proteins (mutant p53/p63/Smad) (Adorno et al., 2009).

In particular, mutant p53 has been found to enhance migration, through inhibition of the p63-mediated transcriptional induction of metastasis suppressor genes (Sharp1 and CCNG2). Consistently these genes were found associate with metastasis risk in a large cohort of breast cancer patients (Adorno, Cordenonsi, Montagner, Dupont, Wong, Hann, Solari, Bobisse, Rondina, Guzzardo, Anna R Parenti, et al. 2009).

Nevertheless, more evidences unveiled that mut-p53 binds to several transcription factors to enhance their activity. The transcription factor NF-Y, was previously shown to associate with mutant p53, as well as wild-type p53, and regulate the transcriptional activation of cell cycle-regulated genes (cyclin A, cyclin B, CDC25C, and CDK1) (Di Agostino et al. 2006). Conversely to wild-type p53, which recruits HDAC1 on the promoters of NF-Y target genes, mutant p53/NF-Y complex is associated to p300 upon adriamycin treatment (Di Agostino et al. 2006) (Figure 6b). This complex was proposed to support the growth promoting properties of mutant p53 as well as the chemoresistance of some mutant p53 bearing tumours (Aas et al. 1996; Bergh et al. 1995; Lu & El-Deiry 2009). Moreover, very recently studies unveiled that the effect of the cross-talk between NF-YB and mutant p53 is maximized by the transcriptional co-activator YAP (Yes associated protein), with profound impact on cell proliferation. In particular, YAP physically interacts with mut-p53 proteins and promotes its binding to the heterotrimeric transcription factor NF-Y thus increasing cell proliferation (S. Di Agostino et al. 2015).

Interestingly, it has been reported that mutant p53 expression leads to high expression of sterol biosynthesis genes in human breast tumours. Indeed, mutant p53 is associated

with sterol responsive elements (SRE) within promoters of sterol-regulated genes, acting as transcriptional coactivator for the sterol regulatory element-binding protein transcription factors, SREBP (Freed-Pastor et al. 2012) to enhance the expression of many mevalonate pathway genes and genes involved in protein prenylation (e.g. HMGCR, HMGCS1, GGT-1)(Figure 6a).

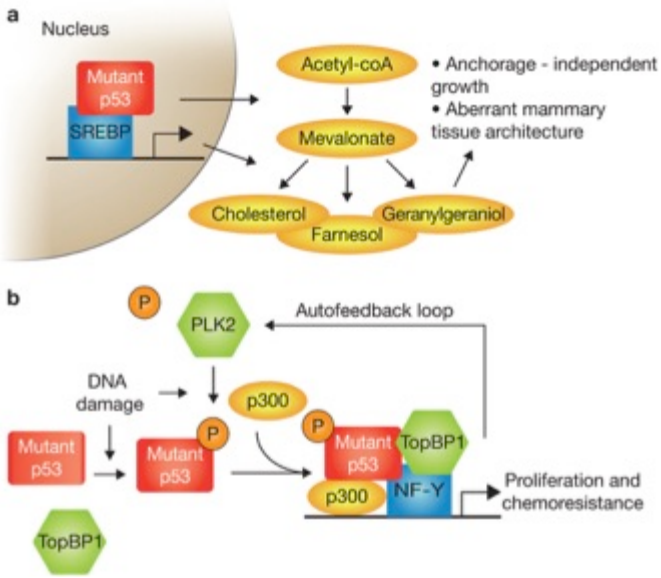

Figure 6. Mutant p53 binds to numerous proteins to enhance or inhibit their function. a)Mutant p53 enhance SREBP function to increase sterol biosyntesis, leading to enhanced anchorage-independent growth and disruption of mammary tissue architeture. b) In response to DNA damage,TopBP1 and PLK2 facilitate the recruitment of mutant p53 toNF-Y, leading to increased expression of genes involved in proliferation (Muller and Vousden 2013).

Downstream of the mevalonate pathway act the pro-oncogenic transcription co-activators YAP/TAZ, effectors of the Hippo pathway (Sorrentino et al). In this context, missense mutant-p53 and SREBP trigger unscheduled activation of YAP/TAZ, in both cancer cells and human primary tumours, by sustaining the mevalonate pathway. For this reason, YAP/TAZ have been suggested as critical effector of the pro-oncogenic function of mutant p53 (Sorrentino et al. 2014).

Another example of transcription factor able to interact with mutant p53 is the vitamin D receptor (VDR). By ChIP-on-chip analysis, the VDR response element was found to be over-represented in promoters bound by mutant p53 R175H. Mutant p53 is indeed

recruited on VDR-regulated genes and modulates their expression, thus converting vitamin D receptor into an anti-apoptotic factor (Stambolsky et al. 2010).

Mutant p53 interacts with other proteins

A crucial role in mut-p53 GOF is certainly played by protein-protein interaction with partners other than DNA binding transcription factors. Indeed, mut-p53 can bind MRe11, a DNA nuclease required for homologous recombination DNA repair, and consequently inhibits the cellular response to DNA double-stranded breaks, promoting genomic instability and tumour progression (Song et al. 2007). Another example is the interaction between p53 and topoisomerase I (Topo I) which leads to an increase in aberrant homologous DNA recombination events and mutagenic DNA rearrangements, spawning an additional type of genomic instability (Restle et al. 2008). Moreover, it has been identified that promyelocytic leukemia (PML) protein, a well known tumour suppressor, is also a mutant p53 interacting protein (Haupt et al. 2009).

Interestingly, the prolyl isomerase Pin1, which regulates conformational changes of proteins to affect their stability and activity, was reported to be an additional mutant p53 binding protein. In particular, it has been described that Pin1 binds to phosphorylated mutant-p53 (on Ser46 and Ser315) in breast cancer cell lines, and together regulate a transcriptional program of 10 genes (DEPDC1, BUB1, CENPA, CCNE2, FAM64A, C21orf45, CPSF6, EPB41L4B, NCAPH, WDR67) that promotes migration and metastasis formation *in vivo* and correlates with poor prognosis in triple negative breast cancer patients. Moreover, Pin1 was demonstrated to enhance the oncogenic activity of mutant p53 through mutant p53-dependent inhibition of p63 (Javier E. Girardini et al. 2011). This suggest that Pin1 contributes to mutant p53 oncogenic properties both by potentiating its ability to block p63 transcriptional activity and by influencing other mutant p53 transcriptional functions (Javier E. Girardini et al. 2011).

Regulation of mutant p53 protein stability

Although a drastic difference between the stability of mutant and wt p53 has been reported in cancer cells, many aspects of their regulation are shared. The majority of the positive and negative regulators of wt p53 have a similar regulatory effect on mutant

p53 in normal cells (Terzian et al. 2008; Meek & Anderson 2009; Alsheich-Bartok et al. 2008). However, the tightly controlled myriad of positive and negative auto-regulatory loops, which govern wt p53 levels, is uncoupled to mutant p53 proteins in the context of cancer cells.

Regulation of mutant p53 protein degradation

High levels of mutant p53 are generally found in tumours (Bártek et al. 1991). However, mutation by itself is not sufficient to explain the greater stability of mutant p53 compared to its wild-type counterpart in cancer cells. Indeed, several murine and zebrafish models, that are homozygous for mutant p53, display no stabilization of the protein in normal tissues but have high levels in tumours arising in these backgrounds (Lang et al. 2004; Lee et al. 2008; Olive et al. 2004). Similarly, mutant p53 does not accumulate in normal tissues from patients with Li-Fraumeni syndrome, but only in derived tumours (Soussi & Béroud 2001).

Interestingly, Mdm2–/– mice harboring knock-in TP53 mutants accumulate mutant p53 also in some normal tissues (Terzian et al. 2008), thus indicating that Mdm2, besides acting on wild-type p53, is a pivotal regulator of mutant p53 stability *in vivo*.

Despite mutant p53 is susceptible to Mdm2-mediated degradation (Haupt et al. 1997; Lukashchuk & Vousden 2007), contrary to wild-type isoform the mutant protein does not form a feedback loop with Mdm2, and thus is incapable of inducing the transcription of the E3 ubiquitin ligase (Midgley & Lane 1997). Therefore, following stress-induced stabilization of wt and mutant p53, only the wtp53 recovers to basal levels under the influence of Mdm2. Furthermore, the E3 ubiquitin ligase Mdm2 is able to interact with multiple domains of p53, which allows it to bind to both conformational p53 mutants as DNA contact (Shimizu et al. 2002; Wallace et al. 2006).

In vitro biochemical experiments have demonstrated that mutant p53 is a substrate of another ubiquitin ligase that targets also wild-type p53: the C-terminus of HSP70-interacting protein (CHIP) (Esser et al. 2005; Lukashchuk & Vousden 2007). CHIP- and Mdm2-mediated ubiquitylation of mutant p53 is counteracted by the chaperone HSP90, which binds to mutant p53 increasing its stability (Esser et al. 2005; Nagata et al. 1999). HSP90 is frequently over-activated in tumours (Kamal et al. 2003), thus the dependency

of mutant p53 on HSP90 may partly account for its specific accumulation in tumours and underlies the therapeutic potential of HSP90 inhibitors (Solit & Rosen 2006).

Autophagy may also play a role in mutant p53 degradation following proteasomal inhibition (Choudhury et al. 2013; Rodriguez et al. 2012). In fact, it has been reported that glucose restriction-induced macro-autophagy leads to mutant p53 depletion as effect of its de-acetylation and ubiquitination (Rodriguez et al. 2012). Nevertheless, this degradation is Mdm2-dependent, but interestingly does not involve the proteasome (Morselli et al. 2008; Tasdemir et al. 2008). Additionally, glucose starvation combined with confluent growth conditions could promote mutant p53 degradation by a specialized form of autophagy known as chaperone-mediated autophagy (CMA) (Vakifahmetoglu-Norberg et al. 2013), a mechanism that induce degradation of protein through a lysosomal-dependent machinery. In particular, the inhibition of autophagy leads to increased cytosolic levels of mutant p53 proteins, which interact with heat shock protein Hsc70 which in turn promotes its degradation in a lysosome-dependent manner.

The degradation of mutant p53 can be also induced through the 20S proteasome after inhibition of NADH quinone oxidoreductase 1 (NQO1), for example by dicoumarol, in an Mdm2-independent manner. Specifically, mutant p53 interacts with NQO1, rendering cells resistant to NQO1 inhibitors (Tsvetkov et al. 2010). Consistently, NADH quinone oxidoreductase 1 is elevated in many cancers, which may contribute to the stabilization of mutant p53 (Belinsky & Jaiswal 1993).

Post-translational modifications of mutant p53

In response to a variety of stress stimuli, p53 is rapidly stabilized and activated through a complex repertoire of post-translational modifications that inhibit its interaction with Mdm2 (Kruse & Gu 2009; Vousden & Prives 2009)

Although the mechanism of hyperstabilizzation of mutant p53 is not completely known several evidences showed that, like for wt p53, PTMs in p53 mutant proteins are also required for its stabilization and activation .

Indeed most of the PTMs of wild-type p53, such as phosphorylation and acetylation, are non-discriminatory between wt and mutant p53 proteins (Terzian et al. 2008; Meek & Anderson 2009; Alsheich-Bartok et al. 2008) and contribute to mutant p53 stability

protecting it from degradation by Mdm2.

Interestingly, the phosphorylation on Ser15, which has major role in the control of DNA damage-induced p53 stabilization, is also induced on mutant p53 in response to genotoxic stress (i.e. camptothecin) (Dun Li et al. 2011; Alsheich-Bartok et al. 2008).

In particular, it was found that mutant p53, expressed in UV induced primary mouse skin tumours and in cell lines established from primary tumours, is constitutively phosphorylated on Ser15, inducing mutant p53 protein stabilization and its accumulation in cell nuclei. Constitutive phosphorylation of mutant p53 at Ser15 is mediated by ERK1/2 MAP kinase (mitogen-activated protein kinase), which physically interacts with mutant p53 in the nucleus (Melnikova et al. 2003).

Another important PTM to stabilize p53 protein is acetylation. Although mutant p53 is known to be acetylated (Perez et al. 2010), the roles of these acetylations on mutant p53 still need further investigation. It has been unveiled that the activation of surtuins (SIRTs), an evolutionally conserved enzyme family acting as protein deacetylases/ADP ribosyltransferases, induces wt and mutant p53 deacetylation on Lys382 residue, leading to reduction of their stability in TNBC cell lines. This evidence revealed an important role of acetylation in controlling the stability of mutant p53 proteins (Yi et al. 2013; Z. Y. Zhang et al. 2015).

In addition to mutant p53 itself, modifications of Mdm2/ MdmX contribute to the protection of mutant p53 from these key inhibitors. In response to DNA damage, ATM and c-Abl phosphorylate Mdm2 and MDMX compromising their ability to degrade p53 (Maya et al. 2001; Goldberg et al. 2002; Cheng et al. 2009).

Mutant p53 addiction

Several evidence have observed that mutant p53 proteins exhibit GOF activities (Dittmer et al., 1993), and accumulating studies unveiled that knockdown of mutant p53 in cancer cells attenuates their malignant properties, suggesting that their oncogenic potential is dependent on the presence of high mutant p53 protein levels.

Experiments in several cancer cell lines expressing mutant p53 and implanted in nude mice showed that the depletion of p53 mutant protein using either stable or conditional shRNA-mediated knockdown, rendered those cells significantly less tumourigenic by

compromising mutant p53 GOF activities (Bossi et al. 2006; Bossi et al. 2008). In agreement with the impact of mutant p53 on the response to genotoxic anticancer drugs *in vitro*, the knockdown of mutant p53 sensitized several cancer cells to chemotherapeutic agents such as doxorubicin, etoposide, and cisplatin, inducing a higher apoptotic response. These results confirmed the existence of GOF activity of some human tumour-derived p53 mutants (Bossi et al.,2006). Remarkably, constitutive inhibition of mutant p53 reduced tumour growth in nude mice and showed reduced stromal invasion and angiogenesis, suggesting a positive role of mutant p53 in the regulation of this process (Bossi et al. 2008). Furthermore, it has been reported that knockdown of mutant p53 in human triple negative breast cancer cell lines did not alter primary tumour growth, but strongly reduced metastasis to both lymph nodes and lung (Adorno, Cordenonsi, Montagner, Dupont, Wong, Hann, Solari, Bobisse, Rondina, Guzzardo, Anna R. Parenti, et al. 2009).

Nonmalignant breast epithelial cells in 3D cultures form spheroids reminiscent of acinar structures found *in vivo*, whereas breast cancer cells display highly disorganized morphology. Prives and colleagues showed that mutant p53 depletion in breast cancer cell lines (MDA-MB-231cells and in MDA-MB-468) grown in 3D cultures is sufficient to phenotypically revert breast cancer cells to a more acinar-like morphology (Freed-Pastor et al. 2012). Inhibition of mutant p53 by siRNA significantly reduced the transcriptional activity of the SREBPs transcription factors leading to a strong reduction of the expression of multiple enzymes involved in the mevalonate pathway and consequent inhibition of YAP/TAZ transcriptional activity (Sorrentino et al. 2014) (Freed-Pastor et al. 2012).

More recently, Moll and colleagues using a novel mutant p53 mouse model expressing an inactivatable $p53^{R248Q}$ hotspot mutation, showed that tumours depend on sustained mutant p53 expression. In particular tamoxifen-induced mutant p53 ablation reduced tumour growth and extended the survival of host mice. Importantly, clinically advanced tumours responded to mutant p53 ablation with regression or stagnation due to marked tumour apoptosis. (Alexandrova et al. 2015).

Pharmacological strategy targeting mutant p53

A number of evidence reveals that stabilization of mutant p53 proteins in tumours is

required for its GOF properties, while its reduction mitigates the malignant potential of cancer cells, hence targeting mutant p53 represents an attractive strategy for cancer therapy. In these years several small-molecule compounds, that specifically target mutant p53 have been identified and are now in preclinical or clinical development. According to the mechanism of how these compounds target mutant p53 proteins they can be divided in several groups (Figure 7).

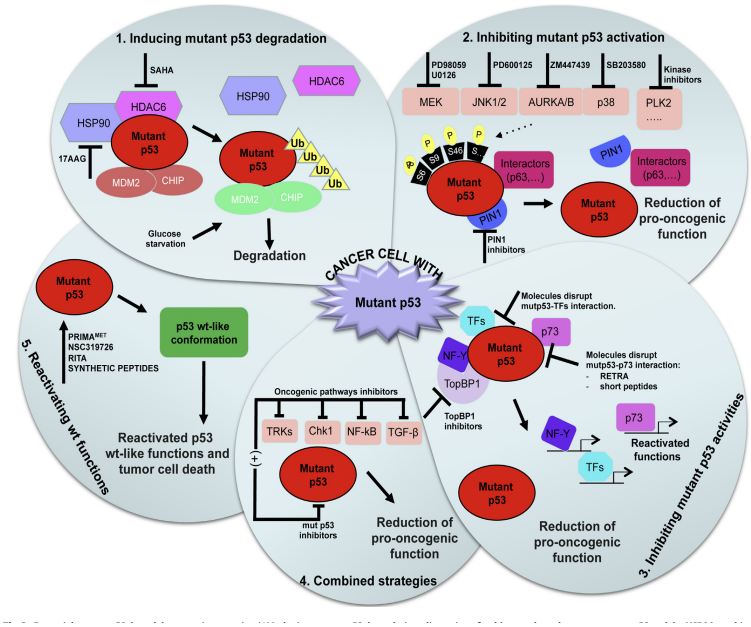

Figure 7. potential mutant p53-based therapeutics strategies. (1) Inducing mutant p53 degradation: disruption of stable complex between mutant p53and Hsp90 machinery by 17AAG (HS90 inhibitor) or SAHA (HDAC6 inhibitor) may release MDM2 and CHIP E3 ubiquitin ligases from inhibition. Mutant p53 degradation may also be enhanced by glucose restriction; **(2)** Inhibiting mutant p53 activation: pharmacological inhibition of kinases or mutant p53 activators as Pin1 may restrain its pro-oncogenic activities by avoiding proper activation. **(3)** Inhibiting mutant p53 activities: mutant p53 functions may be restrained by avoiding the interaction with partners or interfering with the functional significance of mutant p53 complexes; **(4)** Combined strategies: using simultaneously drugs that target mutant p53 and inhibitors of pathway that cooperate with its malignant functions may synergize to hinder tumour progression; **(5)** Reactivating wt (wild type) functions: pharmacological reactivation of wt-like functions in p53point mutants may recover tumour suppressor capabilities specifically in tumour cells. TFs(transcription factors); TRKs (tyrosine kinase receptors) (Girardini et al. 2014).

-Compound that restore wild-type p53 activity

Recent studies clearly demonstrated that restoration of wt p53 activity is able to interfere with tumour progression *in vivo*: the re-introduction 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 et al., 2006; Ventura et al., 2007; Xue et al., 2007). These observations suggest that p53 restoration would be effective in tumour therapy and boost the search for new strategies to activate the p53 pathway in tumours. In the last years, several approaches have been proposed to increase the wt p53 stability and activity with peptides, natural compounds or small molecules, and to restore the wild-type properties of p53 mutants in cancer cells (Brown et al., 2009; Grinkevich et al., 2009; Issaeva et al., 2004; Lain et al., 2008; Vassilev et al., 2004) (Figure 5).

The evidence that suggest that functional reactivation of p53 can be restored from mutant p53 are: (1) many p53 mutants are temperature sensitive and restore the p53 activity at the permissive temperature (Zhang et al. 1994; Selivanova et al. 1998); (2) synthetic peptides, derived from C-terminal domain of p53, restore the sequence-specific DNA binding and transcriptional activity of p53 (Friedler et al. 2002; Selivanova et al. 1997) and (3) insertion of second-site mutations or a N-terminal deletion in several p53 mutants restore the p53 transcriptional activities (Nikolova et al. 2000; Bykov, Issaeva, Shilov, et al. 2002; Liu et al. 2001).

Recently, attempts have been made to identify small molecules that reactivate mutant p53 (Table1).

CP-31398 and STIMA-1 *(SH Group-Targeting Compound That Induces Massive Apoptosis)*

The first p53-reactivating compound, CP-31398 (styrylquinazoline), was identified through a structure-based screening as a compound that stabilize the wild type p53 and enhances its transcriptional activities in cells (Wang et al. 2003). CP-31398 promotes mitochondrial translocation of p53, leading to changes in mitochondrial membrane permeability pore transition (MPT) and consequent cytochrome c release in human skin carcinoma cells expressing mutant p53 (Tang et al. 2007). However, a recent study reported that CP-31398 causes toxicity in liver and other tissues in animal models, suggesting the necessity to modify the structure of CP-31398 to reduce its toxicity (Johnson et al. 2011).

STIMA-1 [2-vinylquinazolin-4-(3H)-one] is a low molecular weight compound that was

identified as one of the CP-31398 derivatives, which induced mutant p53 ($p53^{R175H}$ and $p53^{R273H}$)-dependent growth suppression. STIMA-1 was showed to stimulate mutant p53 binding to DNA, *in vitro*, and to induce expression of p53 target proteins (*p21*, *PUMA,* and *BAX*), triggering apoptosis in mutant p53-expressing human tumour cells. Mechanistically, both CP-31398 and STIMA-1 bind to the cysteine residues in the core domain of mutant p53, leading to stabilization of wild-type p53 conformation and subsequent restoration of transcriptional activity (Zache, Lambert, Rökaeus, et al. 2008).

PRIMA-1 and PRIMA-1MET

PRIMA-1 [2,2-bis (hydroxymethyl)-3-quinuclidinone] and its methylated analog PRIMA-1^{MET} are molecules that can restore sequence-specific DNA binding and convert mutant p53 conformation to wild-type, thereby leading to the transactivation of p53 target genes (Bykov, Issaeva, Selivanova, et al. 2002; Bykov, Issaeva, Shilov, et al. 2002; V. J. Bykov et al. 2005).

PRIMA-1 was first identified through a screening as a compound that suppressed proliferation of osteosarcoma cell line expressing $p53^{R273H}$ with little effect on the parental cells. In particular PRIMA-1 and PRIMA-1^{MET} were unveiled as compounds capable of inducing apoptosis in human tumour cells through restoration of the transcriptional transactivation function to mutant p53 cells (Bykov, Issaeva, Shilov, et al. 2002; V. J. Bykov et al. 2005). Interestingly, it has been also reported that mutant p53 reactivating compound PRIMA-1^{MET} acts synergistically with several chemotherapeutic drugs, such as cisplatin, to induce tumour cell apoptosis and to inhibit human tumour xenograft growth *in vivo* (V. J. N. Bykov et al. 2005). The molecular mechanism by which PRIMA-1 and PRIMA-1^{MET} lead to refolding of mutant p53 (both DNA contact and structural mutants isoforms) involves the conversion of these compounds to products which form adducts with thiol groups in the mutant p53 core domain, leading to restoration of wild-type conformation and induction of apoptosis in tumour cells (Lambert et al. 2009; Lambert et al. 2010). Several studies have successfully validated their tumour suppressive effects in mouse models of multiple types of cancer (Rao et al. 2013; Zache, Lambert, Wiman, et al. 2008) and importantly,

the PRIMA-1^{MET} is currently in phase I of clinical trials in liver or prostate cancer patients (Farnebo et al. 2010).

MIRA-1 and its structure analogs

Using the same screening strategy as PRIMA-1, MIRA-1 (NSC19630) was identified as a compound that suppressed proliferation of osteosarcoma cell lines expressing p53^{R273H} (V. J. Bykov et al. 2005). MIRA-1 and its structural analogs (MIRA-2 and MIRA-3) were showed to inhibit proliferation and also to induce cell death in cancer cells expressing mutant p53 (V. J. Bykov et al. 2005)and in mouse models.

In particular, MIRA-1 restores native wild-type p53 conformation, leading to enhanced DNA-binding activity of mutant p53 (p53R^{175H} and p53^{R248Q}) and increased expression of p53 downstream target genes (*Mdm2* and *p21*) in several mutant p53-carrying cancer cell lines.

RITA (NSC6522887)

RITA (reactivation of p53 and induction of tumour cell apoptosis) was identified through cell proliferation assay-based screening as a compound that suppresses the growth of cancer cell lines expressing wild-type p53 with minimum effect on p53-null cells.

Initially, RITA was found to induce expression of p53 target genes and massive apoptosis in several tumour cells lines expressing wild-type p53 and to suppress the growth of human fibroblasts and lymphoblasts only upon oncogene expression and showed substantial p53-dependent antitumour effect *in vivo* (Issaeva et al. 2004; Nieves-Neira et al. 1999). Mechanistically, RITA disrupts the p53-Mdm2 complex by binding to p53 (Issaeva et al. 2004) and induces HIPK2 (proapoptotic homeodomain-interacting protein kinase-2) stabilization with consequent phosphorylation of p53 on Ser46 (Rinaldo et al. 2009). Subsequent prolyl-isomerization by Pin1 of the Ser46-Pro47, leads to decreased polyubiquitination of p53 in favor of its monoubiquitination, with consequent relocalization of cytosolic p53 to mitochondria to induce p53 transcription-independent apoptosis (Sorrentino et al. 2013).

Later, RITA was found to suppress also the growth of cancer cell lines carrying various p53 mutants (p53^{R175H}, p53$^{R213Q/Y234H}$, p53^{R248W}, p53^{R248Q}, p53^{I254D}, p53^{R273H}, and

$p53^{R280K}$) by restoration of p53 transcriptional activity (p21, NOXA, PUMA, and GADD45) and induction of apoptosis through upregulation of pro-apoptotic proteins and downregulation of several oncogenes or anti-apoptotic proteins (Zhao et al. 2010; Burmakin et al. 2013; Grinkevich et al. 2009). However, the exact mechanism by which RITA activates both wild type and mutant p53 to induce apoptosis remains unclear.

NUTLIN-3a

Nutlin is a potent and selective pharmacological Mdm2 inhibitor that prevents Mdm2-p53 interaction inducing wild-type p53 activation, and it is currently being tested in phase 1 clinical trail (Vassilev et al. 2004)

It was unveiled that nutlin competitively binds to Mdm2 in the p53-binding pocket within the N-terminus of Mdm2, thereby leading the activation and stabilization of p53 in cancer cells, without causing major conformational changes in the Mdm2 molecule and preserving its E3-ligase activity (Vassilev et al. 2004). As a consequence of the reactivation of wild-type p53 induced by Nutlin-3a, the tumour suppressor p53 was showed to induce cell cycle arrest, apoptosis and growth inhibition in human cancer cells harboring wild-type p53, but not mutant p53, and in tumour xenograft in nude mice (Vassilev et al. 2004; Tovar et al. 2006; Xia et al. 2008).

Although the apoptotic effects of Nutlin-3a have been initially linked exclusively to the transcriptional activities of p53 in the nucleus (Vassilev et al. 2004), it has recently emerged how the transcription-independent activity of p53 could be a major contributor to the apoptosis induced by this drug in human cancer cell lines. Indeed, Vaseva and others found that p53 rapidly translocates to the mitochondria after treatment of cells with Nutlin-3a, and activates a pro-apoptotic signal that is strongly independent from its transcriptional activities (Vaseva et al. 2009; Steele et al. 2008).

Mechanistically, the Mdm2-p53 complexes are only partially disrupted by Nutlin and the remaining low levels of complex formation between Mdm2 and p53 are sufficient for mediating p53 moubiquitination which is known to promote p53 trafficking to mitochondria (Vaseva et al. 2009).

NSC319726/ZMC1 (Zinc Metallochaperone-1)

Zinc is required for proper folding of p53 protein, while lack of zinc in the central core domain of p53 leads to its unfolding (Bykov, Issaeva, Shilov, et al. 2002; Margalit et al. 2012; Pintus et al. 2013). Several evidence reported that administration of zinc, in combination with Adriamycin, restore activity of misfolded p53 and enables induction of its proapoptotic and tumour suppressor functions *in vitro* and *in vivo*. Indeed, NSC319726 [zinc metallochaperone-1 (ZMC1)], a thiosemicarbazone derivative, was identified in a screen of the NCI60 panel of human tumour cell lines, as a compound able to facilitate the binding of mutant p53 to zinc, exhibiting selective toxicity to cells carrying $p53^{R175H}$ with minimum effects on cells expressing wild-type p53 and other p53 mutants (p53R248Q and p53R273H) (Yu et al. 2012).

Chetomin

Chemotin (CTM) is a small-molecule that reactivates mutant p53 and restores the WT-like function, through the p53-heat-shock protein 40 (Hsp40) axis (Hiraki et al. 2015).
The chemotin was identified trough a high-throughput chemical library screening using a luciferase reporter with the p53 response element promoter in p53-null cells carrying mutant p53, as a compound that increased luciferase activity. In particular, CTM was found to induce p53 target genes (*p21*, *PUMA*, and *Mdm2)* and showed anticancer effects in $p53^{R175H}$ specific manner *in vitro* and *in vivo* (Hiraki et al. 2015)
Mechanistically, Chemotin does not bind directly to mutant $p53^{R175H}$ protein, but was found that CTM binds to Hsp40 and increases the binding capacity of Hsp40 to the $p53^{R175H}$ mutant protein, causing a potential conformational change to a wild-type-like p53 (Hiraki et al. 2015).

Other Compound that restore wild-type p53 activity

Several other small molecules have been reported as mutant p53 reactivators.
-*Stictic acid* (Wassman et al. 2013) was identified through an ensemble-based virtual screening approach, and its mechanism of p53 reactivation is through docking of the small molecule in the open L1/S3 p53 binding pocket around Cys124, Cys135, and Cys141 (Wassman et al. 2013).

-*The p53 reactivator (P53R3)* restores sequence-specific DNA binding of several p53 mutants ($p53^{R175H}$, $p53^{M237I}$, and $p53^{R273H}$) and induces p53-dependent anti-proliferative effects in mutant p53 cancer cells with increase in mRNA expression of many p53 target genes (p21,GADD45, BAX,PUMA) but also in wild type p53 cancer cells (Weinmann et al. 2008).

-*SCH529074* was identified by a DNA-binding assay-based screening as a compound that enabled $p53^{R273H}$ to bind to a consensus p53 DNA-binding site . The small molecule SCH529074, acting as a chaperone, binds specifically to the p53 DBD restoring DNA binding activity to mutant p53 and inhibits HDM2-mediated ubiquitination (Demma et al. 2010).

-*WR1065* is an aminothiol used to protect tissues against the damaging effects of radiation and chemotherapeutic drugs. WR1065 has been shown to induce wild-type p53 accumulation and activation in cultured cells, suggesting a role of p53 in cytoprotection. WR-1065 was showed to increas wild-type p53 activity through a JNK-dependent signaling pathway, but not through genotoxic mechanisms (Shen et al. 2001; Pluquet, North, Bhoumik, et al. 2003; Pluquet, North, Richard, et al. 2003).

TABLE 1 | Compounds that induce reactivation of mutant p53.

Compound	Type of mutant	Mechanism
CP-31398	V173A, S241F, R249S, R273H	Stabilize the DNA-binding core domain and induce conformational change
STIMA-1, structural similarity to CP-31398	R175H, R273H	Bind to the cysteine residues in the core domain and stabilize wild-type p53 conformation
PRIMA-1 and the methylated analog (APR-246/PRIMA-1^{MET})	R175H, R273H	Bind to thiol groups in the core domain and restore wild-type conformation
MIRA-1 (NSC19630), and its analogs MIRA-2 and -3	R175H, R248Q, R273H	Prevent unfolding of wild-type and mutant p53 and restore native wild-type p53 conformation
RITA (NSC652287)	R175H, R248W, R273H, R280K	Restore p53 transcriptional activity and induce apoptosis
NSC319726/ ZMC 1 (zinc metallochaperone-1)	R175H, R172H (mouse)	Restore wild-type p53 conformation and activity with MDM2-dependent degradation
Chetomin (CTM)	R175H	Increase Hsp40 (DNAJB1) levels and Hsp40-$p53^{R175H}$ binding, restoring wild-type p53 conformation, activity, and MDM2-dependent degradation
PK7088	Y220C	Bind to a $p53^{Y220C}$-specific surface cavity and stabilize $p53^{Y220C}$ with restored wild-type p53 conformation
Stictic acid (NSC87511)	R175H, G245S	Target cysteine 124 at the p53 core domain and restore wild-type p53 activity
p53R3	R175H, M237I, R273H	Restore sequence-specific DNA binding and p53 transcriptional activities
SCH529074	R175H, L194F, R248W, R249S, R273H	Restore sequence-specific DNA binding and p53 transcriptional activities
WR-1065	V272M	Restore DNA binding and transcriptional activities of $p53^{V272M}$

(Parrales & Iwakuma 2015)

Peptides and Aptamers

The equilibrium between the properly folded and misfolded states of p53 may be affected by molecules that interact with p53, stabilizing its native folding and restoring wild type p53 activity to cancer cells such as peptides and aptamers (Guida et al. 2008) (Tal et al. 2016).

Peptides libraries have proved a useful approach to identify drugs that specifically target mutant p53 but not wild-type form to restore p53 activity.

-*Peptide aptamers (PA)*

Peptide aptamers are combinatorial protein molecules with specific bind affinity to given target proteins under intracellular conditions. The typical structure of peptide aptamers is a short peptide region inserted within a scaffold protein. The short peptide region is responsible for binding with its target protein and the scaffold protein helps to enhance the binding affinity and specificity through restriction on the conformation of the binding-peptide (J. Li et al. 2011).

Using the yeast two-hybrid method and molecular modeling analysis, the peptides aptamers have been identified able to interact with both p53 conformational mutants ($p53^{R175H}$, $p53^{D281G}$) and contact mutants (R273H, R248W) but not wild-type p53 (Guida et al. 2008).

Moreover, PAs were showed to break also the complexes between the mutant p53 and p63/, p73 and the respective isoform proteins, increasing in this way the free p73 and p63 and restoring the oncosuppressoiver activity of these proteins (Guida et al. 2008).

In tumour cells expressing mutant p53, but not in cells harboring wt p53 or in p53-null cells, PAs were observed to trigger apoptosis while the ablation of endogenous mutant p53 reduced PA-induced cell death, further suggesting that the induction of apoptosis depends on the presence of mutant p53. Interestingly, the apoptotic response induced by PAs was comparable with that exerted by PRIMA-1 (Guida et al. 2008).

- *p53 conformation activating peptides (pCAPs)*

Very recently, Tal P., et al., (Tal et al. 2016) have described an innovative approach for the identification of mutation p53 reactivating peptides through functional screening of phage display libraries. Through a phage display technology, based on the interactions between mutant p53 and random peptide libraries presented on phages and enriched for phage that favor the correctly folded p53 conformation, they obtained a large database of potential reactivating peptides. A total of close to 350 peptides, deduced from the phage sequences and known as pCAPs (p53 conformation activating peptides), were synthesized and subjected to several alternative complementary methods of semi high-throughput functional screening. Analyzing the ability of these peptides to induce p53 transcriptional activity, several candidate lead peptides (pCAP-24R, pCAP-54, pCAP-

60R, pCAP-97R) were found to increase the expression of p53 target genes. Importantly, lead peptides elicited dramatic regression of aggressive tumours in mouse xenograft models (Tal et al. 2016).

-Compounds that deplete mutant p53

Although many p53-reactivating compounds seem to target more than one p53 mutant, it remains unclear if these can reactivate all p53 mutants or specific mutant proteins. Another important strategy to target oncogenic p53 is to use compounds that induce mutant p53 degradation without altering wild-type p53. These classes of molecules can be used as effective therapeutic strategies for both cancers carrying only mutant p53 and those retain a heterozygous status. Moreover, these compounds may be valuable, for elucidating the mechanisms of stabilization or of mutant p53 in cancer cells (Figure 5) (Table 2).

Hsp90 inhibitors: Geldanamycin, 17-AAG, Ganetespib

The heat shock protein 90 (Hsp90) signaling pathway is ubiquitously upregulated in cancer cells where plays an important role in stabilizing mutant p53 by protecting it from both CHIP and Mdm2-mediate ubiquitination and degradation (Wang & Chen 2003; Dun Li et al. 2011). 17-AAG (17-allylamino-17-demethoxygeldanamycin) is a potent and highly specific Hsp90 inhibitor currently in phase I to III clinical trials for refractory multiple myeloma and several solid cancers including breast cancer (Trepel et al. 2010; Dun Li et al. 2011). It has been reported that 17AAG specifically induces the release of mutant p53 from hsp90, promoting degradation of varieties of p53 mutants ($p53^{R175H}$, $p53^{L194F}$, $p53^{R273H}$, and $p53^{R280K}$) and reducing cell viability (Dun Li et al. 2011).

Ganestespib, another Hsp90 inhibitor, is 50-fold more potent than 17-AAG in destabilizing mutant p53 with little effect on wild-type p53 levels which induces mutant p53 depletion with increased apoptosis in tumours *in vivo* (Dun Li et al. 2011).

Histone Deacetylase inhibitors: vorinostat/ SAHA, Romidepsin/Depsipeptide

Histone deacetylase (HDAC) inhibitors (HDACi) are a new class of promising anti-cancer drugs to compromise mutant p53 protein stabilization.

SAHA (suberoylanilide hydroxamic acid) also know as Vorinostat is the only FDA-approved HDACi that acts by inhibiting HDAC6, a positive regulator of Hsp90, disturbing the physically interaction of HDAC6/Hsp90/mutant p53 complex, leading to mutant p53 ubiquitination by Mdm2 and CHIP and degradation (D Li et al. 2011; Marks 2007). In particular, it was unveiled that SAHA exhibits preferential cytotoxicity in mutant p53 tumour cells, whereas wtp53 and p53 null tumour cells are much less sensitive (D Li et al. 2011). The cytotoxicity of SAHA in mutant p53 cancer cells, despite being pleiotropic drug, is largely due to the destabilization of mutant p53 protein via Hsp90/HDAC6 inhibition. The mutant p53 destabilization induced by SAHA is at least as efficiently as 17AAG, but a synergistic effect of both drugs was showed to be correlate with further mutant p53 degradation in some mutant p53 breast-cancer cells lines (D Li et al. 2011; Alexandrova et al. 2015). This synergistic cytotoxicity could be explain that the inhibition of HDAC6 by SAHA, that in turn causes hyperacetylation of HSP90, further lowers the threshold of inhibiting HSP90 by 17AAG, resulting in enhanced ubiquitination of HSP90 client proteins including mutant p53.

Moreover pharmacological degradation of mutant p53 via HSP90 targeting by SAHA it was showed also mediate chemosensitization in response to conventional genotoxic drugs such as topoisomerase inhibitor camptothecin (Alexandrova et al. 2015).

Arsenic Compounds

Arsenic trioxide (ATO), a drug for patients with acute promyelocytic leukemia, is found to target and degrade a class of proteins with high levels of cysteine residues and vicinal thiol groups, such as promyelocytic leukemia protein (PML) and PML-retinoic acid receptor α fusion protein (Beauchamp & Üren 2012).

Some evidence revealed that wild type p53 is induced by arsenic trioxide in tumour cells, presumably due to arsenic-induced oxidative stresses(Jiang et al. 2010). In addition, Yan et al., unveiled that arsenic compounds target mutant p53 for degradation and inhibit the proliferation of tumour cells harboring a mutant p53. In particular, ATO induces proteasomal-dependent degradation of several mutants p53 proteins ($p53^{R175H}$, $p53^{H179Y/R282W}$, $p53^{R248W}$, and $p53^{R273H}$) and also increases E3 ubiquitin ligase Pirh2 expression, leading to ubiquitination and degradation of several mutant p53 (Yan et al. 2014). However, it should be noted that arsenic compounds have carcinogenic effects

and are known to induce several types of cancer (Hughes et al. 2011).

Disulfiram

Disulfiram (DSF) is a FDA approved drug for the treatment of alcoholism and available for clinical use since over 5 decades for some types of cancer including glioblastoma multiforme and metastatic non-small cell lung cancer (Kona et al. 2011; Nechushtan et al. 2015). Paranjpe et al. (Paranjpe 2013) reported that DSF induced degradation of both wild-type p53 and p53R273H through the 26S proteasome pathway (Paranjpe 2013).

Spautin

The small molecule Spautin is a derivative of MBCQ (4-((3,4-methylenedioxyben-zyl)amino)-6-chloroquinazoline) and an inhibitor of autophagy.

Spautin-1 promotes the degradation of Vps34 PI3 kinase complexes by inhibiting two ubiquitin-specific peptidases, USP10 and USP13. Since USP10 also deubiquitinates wild-type p53, Spautin-1 promotes the degradation of wild-type p53 (Liu et al. 2011; Yuan et al. 2010). Moreover, suppression of macroautophagy by Spautin-1 under glucose-free and confluent conditions was found to induce degradation of several p53 mutants ($p53^{R158InF}$, $p53^{R175H}$, $p53^{R248Q}$, $p53^{S241F}$, $p53^{G266E}$, $p53^{R280L}$, and $p53^{R273H}$) through the chaperone-mediated autophagy (CMA) pathway (Vakifahmetoglu-Norberg et al. 2013). Mutant p53 degradation induced by Spautin-1 is dependent on nuclear export of mutant p53 and independent of Mdm2 and the ubiquitin proteasome pathway (Vakifahmetoglu-Norberg et al. 2013; Vakifahmetoglu-Norberg & Yuan 2013)

Other compounds that deplete mutant p53

Several other small molecules have been reported to induce mutant p53 destabilization and degradation.

-*Gambogic Acid* (GA) was showed to reduce viability of mutant p53-expressing cancer cells and to increase cytotoxic effects of several chemotherapy drugs in human breast cancer cell lines (Wang et al. 2011) GA prevents the mutant p53-Hsp90 interaction and induces mutant p53 nuclear exports and subsequent degradation by CHIP ubiquitin ligase (Wang et al. 2011).

-*YK-3*-237 was found to inhibit the proliferation of breast cancer cell lines carrying both

mutant and wild-type p53. YK-3-237 also decreased the levels of mutant p53 proteins ($p53^{V157F}$, $p53^{M237I}$, $p53^{R249S}$, $p53^{R273H}$, and $p53^{R280K}$) through reduction in acetylation at lysine 382 (K382) of mutant p53, a target site of a NAD+-dependent protein deacetylase SIRT1 (also known as sirtuin 1) (Yi et al. 2013). Indeed *YK-3-237* activates SIRT1 enzyme activity (Yi et al. 2013).

-NSC59984 induces degradation of several p53 mutants ($p53^{R175L}$, $p53^{R175H}$, $p53^{S241F}$, and $p53^{R273H/P309S}$) through Mdm2-mediated ubiquitination (S. Zhang et al. 2015).

TABLE 2 | Compounds that deplete mutant p53.

Compound	Type of mutant	Mechanism
Hsp90 inhibitors: 17-AAG, geldanamycin, ganetespib	R175H, L194F, R248Q, R273H, R280K, R172H (mouse)	Reverse the Hsp90's function to inactivate MDM2 and CHIP
HDAC inhibitors: vorinostat/ SAHA, romidepsin/ depsipeptide	R175H, R280K, V247F/P223L	Inhibit HDAC6 and disrupt the HDAC6/ Hsp90/mutant p53 complex
Arsenic compounds	R175H, R248W, H179Y/R282W, R273H	Increase transcripts of Pirh2 and induce degradation of mutant p53
Gambogic acid	R175H, G266E, R273H, R280K	Inhibit the mutant p53-Hsp90 complex and induce CHIP-dependent degradation
Spautin-1	R158lnF, R175H, S241F, R248Q, G266Q, R280L, R273H	Induce mutant p53 degradation via the CMA pathway activated by the suppression of macroautophagy under glucose-free and confluent conditions
YK-3-237	V157F, M237I, R249S, R273H, R280K	Decrease mutant p53 levels through deacetylation at lysine 382 by activating SIRT1
NSC59984	R175L, R175H, S241F, R273H/ P309F	Induce MDM2-mediated mutant p53 degradation and activate p73
Disulfiram (DSF)	R273H	Induce degradation of both wild-type p53 and $p53^{R273H}$ via the 26S proteasome pathway

(Parrales & Iwakuma 2015)

-Compounds that affect downstream targets of mutant p53

Another approach to target oncogenic activity of mutant p53 is to reactivate tumour suppressive pathways that are inhibited by mutant p53 or to inhibit tumour-promoting pathways that are activated by mutant p53.

RETRA

Compounds that impair the interaction of mutantp53 with other target proteins could be a more general strategy to prevent the oncogenic effect of mutant p53s that share binding partners.

The small-molecule RETRA (Reactivate transcriptional activity) as been reported to destabilize the p73-mutant p53 interaction (Kravchenko et al. 2008) thereby restoring the p73-mediated apoptosis.

RETRA has been shown also to induce the transactivation of p53 target genes in mutant p53-bearing tumours and to prevent the growth of xenograft tumours in mice (Kravchenko et al. 2008).

Statins

Statins are a class of drugs largely used to lower the cellular cholesterol levels in patients with hypercholesterolemia by inhibiting the enzyme HMG-CoA-reductase (HMGCR) (Clendening & Penn 2012; Demierre et al. 2005).

This enzyme catalyzes the production of mevalonic acid (MVA), which represents the rate-limiting step of cholesterol biosynthesis (the mevalonate pathway) and also regulates prenylation/ lipidation (farnesylation and geranyl-geranylation) of proteins.

Prenylation is a post-translational modification of proteins by which a farnesyl- or geranylgeranyl moieties are added to a cysteine residue of target proteins involved in cellular adhesion, migration, and proliferation signaling (e.g., Rho, Rac, Cdc42, Ras) facilitating their attachment to cell membranes (Shimoyama 2011).

In breast cancer cells, mutant p53 acts as a positive transcriptional cofactor for SREBPs, (Freed-Pastor et al., 2012), to regulate expression of several enzymes involved in the mevalonate pathway (Brown and Goldstein, 1997), leading to elevated expression of cholesterol biosynthesis enzymes and enhanced prenylation of proteins associated with

cancer progression; hence, inhibition of protein prenylation by statins leads to reduced malignancy of human breast cancer cells (Freed-Pastor et al. 2012). Interestingly, it was unveiled that activation and subcellular localization of YAP/TAZ, the main downstream effectors of the Hippo pathway, is mediated by prenylation and activation of Rho-GTPases and inhibition of the rate-limiting enzyme of this pathway (HMG-CoA reductase) by statins opposes YAP/TAZ nuclear localization and transcriptional responses (Sorrentino et al. 2014).Thus, YAP/TAZ represent important executors of mutant p53 gain-of-function, acting downstream of the mutant p53-induced metabolic reprogramming is cancer cells.

-Compounds that induce Synthetic Lethality

Synthetic lethality is generally used for the condition where a mutation in a gene is not lethal by itself, but its combination with a drug or other gene mutations leads to cell death (Kaelin 2005). Induction of synthetic lethality for mutant p53 is an attractive approach as a therapeutic strategy since over 50% of human cancers have mutations in the p53 gene. In this regard, compounds that induce synthetic lethality should selectively kill cancer cells expressing mutant p53 without affecting normal cells carrying wild-type p53.

BI-2536 PLK1 (polo-like kinase 1) inhibitors

Another compound that is synthetic lethal to mutant p53 is BI-2536, an inhibiter of polo-like kinase 1 (PLK1), an enzyme that controls G2/M checkpoint.

Transcriptome analyses revealed a consistent up-regulation of polo-like kinase 1 (PLK1), as well as other genes controlling the G_2/M transition, in breast (King et al. 2012) cancers with mutant p53 expression; the presence of both coincided with a worse prognosis than cancers with either PLK1 upregulation or mutant p53 expression alone (King et al., 2012). Inhibition of PLK1 by BI-2536 significantly enhances cytotoxic effect of ionizing radiation in mutant p53 ($p53^{S241F}$) and ($p53^{R248W}$) overexpressing cancer cell line but it does not do so in parental (wild-type p53) cells (Sur et al. 2009).

UCN01

UCN0 is a protein kinase C inhibitor and a potent blocker of G2/M checkpoint of the

cell cycle. Interestingly, UCN-01 treatment enhances the cytotoxicity of gamma irradiation and induced G2/M cell cycle arrest in human lymphoma cells CA46 ($p53^{R248Q}$) and human colon carcinoma HT29 ($p53^{R273H}$). Moreover human breast carcinoma MCF-7 cells defective for p53 function were more sensitive to cisplatin, upon treatment with UCN-01, with little effect on MCF7 cells having normal p53 function (Wang et al. 1996).

AIM OF THE THESIS

Mutation of the *TP53* gene is the most frequent genetic lesion in human cancers and contributes to malignant transformation. Ninety percent of p53 alterations are missense mutations in the DNA-binding domain, which do not only abrogate its tumour suppressive functions, but also lead to the acquisition of new oncogenic functions (overall called gain of function - GOF), such as the ability to foster tumour progression, metastatic potential and drug resistance. Common features of mutant p53 proteins are their constitutive hyper-stabilization in cancer cells and their ability to establish aberrant protein complexes with several partners such as p63/p73, NF-Y, SREBPs, SP1 and Ets, affecting their biological activities. However, the pathways leading to mutant p53 stabilization in cancer cells have been only partially investigated and are still not completely understood.

Inhibition of the pathways that cancer cells exploit for their survival and metastatic dissemination is a rational approach to selectively hit transformed cells with minimal effects on normal tissues. Thus, targeting mutant p53, which specifically accumulates in the majority of tumour cells, represents an attractive strategy for cancer therapy, in particular *via* the identification of molecules that reduce its protein amount. This notion is supported by the evidence that knocking-down mutant p53 from cancer cells that are addicted to its expression either by small molecules or by RNA interference is able to reduce their malignant progression. In this context, the discovery that HDACs and HSP90 proteins protect mutant p53 from proteasome-mediate degradation not only unveiled two important mechanisms of mutant p53 stabilization in cancer, but also led to the identification of 17-AAG and SAHA (respectively Hsp90 and HDAC inhibitors) as anticancer small molecules acting, at least in part, by blocking mutant p53 GOF. Unfortunately these drugs, due to the pleiotropic activities of their targets, cause large toxic effects in cancer patients and have failed several clinical trials. The identification of efficient and well-tolerated drugs that specifically target mutant p53 remains challenging; however, elucidation of mechanisms of mutant p53 stabilization in cancer cells would open new chances for the design of anticancer therapeutic strategies.

The work presented in this thesis aimed to identify small molecules, from a library of FDA-approved compounds, able to reactivate the Mdm2-dependent degradation of

mutant p53 in cancer cells Since the mechanisms of action of all the used FDA-approved compounds are well known, we reasoned that the identification of such molecules could also reveal new mechanisms of regulation of mutant p53 protein stability in cancer cells. The identified lead-compound could also clinically tested clinically tested it in breast cancer with high levels of mutant p53.

RESULTS

Identification of FDA-approved drugs that reduce mutant p53 levels

To identify drugs able to reactivate the mutant p53 negative regulator Mdm2 in cancer cells, we performed a high-content, fluorescence-microscopy-based, high-throughput screening using a library of FDA (Food and Drug Administration)-approved drugs composed of a collection of 640 clinically-used compounds with known and well-characterized bioactivity, safety and bioavailability (Sorrentino et al. 2014). Nuclear accumulation of mutant p53 proteins is a hallmark of a variety of human cancers, moreover tumour-specific mutant p53 hyperstabilization is crucial for manifestation of its GOF (Soussi & Béroud 2001; Rotter et al. 1983; Shaulsky et al. 1990). We thus monitored the effect on nuclear mutant p53 protein levels of each compound of the library added at two different concentrations (1 and 10 μM) to the culture medium of the breast cancer cell line MDA-MB-231 (Supplementary Figure A). SAHA, which belongs to the class of histone deacetylase inhibitors and has been found to strongly reduce the stability of mutant p53, was used as positive control (D Li et al. 2011). After 24h, mutant p53 positive cells were detected by immunofluorescence and quantified through automated image analysis. As expected, MDA-MB-231 cells exhibited a strong mutant p53 nuclear staining, unaffected by most compounds. However, we were able to identify several drugs that significantly reduced the number of mutant p53 positive cells. In particular, filtering the results based on reproducibility, on dose-dependence and on manual image analysis, we identified as the best hits (Figure 8A-C):

- Ouabain, a cardiac glycoside used for the treatment of congestive heart failure and atrial fibrillation. Ouabain is an inhibitor of Na^+/K^+-ATPases and increases the intracellular calcium concentration (Newman et al. 2008). In addition, the drug was also found to be beneficial to breast cancer patients (Stenkvist et al. 1979) and was associated with a lower risk for leukemia, lymphoma, as well as kidney and urinary tract cancer (Haux et al. 2001);
- Spiperone, a butyrophenone antipsychotic agent with dopamine and serotonin (5-HT) receptor antagonist properties (Gundlach et al. 1984)(Leysen et al. 1978). Spiperone is a calcium regulator that specifically blocks canonical Wnt signaling by elevating intracellular calcium levels (Lu & Carson 2009);
- Ivermectin, a derivative of avermectin B1 used for the treatment of the parasitic infections. It was unveiled that ivermectin leads cell death in leukemia cells

through chloride-dependent membrane hyperpolarization and generation of reactive oxygen species (ROS) (Sharmeen et al. 2010);

- Thioridazine, an antipsychotic drug and D_2 dopamine receptor antagonist.

Moreover, we found that two classes of drugs, namely adrenergic agonists (e.g. Salmeterol) and mevalonate pathway inhibitors (statins), were strongly associated with reduction of mutant p53 levels (Figure 8 A-C). Indeed, many of the adrenergic agonists and all the statins present in the library scored positive as drugs able to reduce the number of mutant p53 positive cells. Of note, adrenergic agonists (e.g. Isoprenaline) and Ouabain have been already identified as p53 destabilizing agents, thus confirming the reliability of our screening (Wang et al. 2009).

Statins reduce mutant p53 proteins stability in an Mdm2-dependent manner

To gain insight into the mechanism of mutant p53 inhibition by the identified drugs, we monitored p53 mRNA levels in MDA-MB-231 cells upon drugs treatment. Interestingly, none of these compounds was able to reduce p53 mRNA levels, suggesting that the identified drugs reduce mutant p53 protein amount acting at the post-transcriptional level (Figure 9A).

The aberrant stability of mutant p53 observed in cancer is not due to its inability to transactivate the Mdm2 gene. In fact, experiments performed in knock-in (KI) mice expressing mutant p53 R172H, demonstrated that mutant p53 protein is inherently unstable in normal tissues while only tumour cells display constitutive stabilization of mutant p53 (Haupt et al. 1997; Terzian et al. 2008; Lukashchuk & Vousden 2007). Therefore, during oncogenic transformation, one or more still not completely understood events occur that stabilize mutant p53. In this context, study by different groups demonstrated that the aberrant activation of Hsp90 chaperone machinery induces Mdm2 functional inactivation thus sustaining mutant p53 hyper stability (Figure 10G). Based on these premises, we hypothesized that the identified drugs could reduce mutant p53 levels by reactivating the inhibitory function of Mdm2 on mutant p53. To test this hypothesis, we used Nutlin, a cis-imidazoline drug which specifically inhibits the Mdm2-p53 interaction. Of note, Nutlin treatment rescued mutant p53 levels only in cells treated with the adrenoceptor agonist Salmeterol and the mevalonate pathway

inhibitor Cerivastatin, thus suggesting that β2-adrenergic receptor pathway and the mevalonate pathway control mutant p53 protein levels in a Mdm2-dependent manner (Figure 9B). Interestingly, activation of β2-adrenergic pathway has been already shown to trigger AKT-mediated activation of Mdm2 and also to promote Mdm2-dependent degradation of wild type p53 (Pääjärvi et al. 2005). Therefore, we focused our attention on the mevalonate pathway.

Statins are a class of drugs clinically used to lower the plasma cholesterol levels in patients with cardiovascular diseases by inhibiting the enzyme HMG-CoA reductase (HMGCR). This enzyme catalyses the biosynthesis of mevalonic acid (MVA), which represents the rate-limiting step of cholesterol biosynthesis.

Interestingly, mutant p53 has been identified as a crucial upstream activator of the mevalonate pathway in cancer cells by binding to and inducing the transcriptional activity of SREBPs (Sterol Regulatory Element Binding Proteins) transcription factors (Freed-Pastor et al. 2012). Activation of the mevalonate pathway, in turn, leads to increased protein prenylation, aberrant cell growth in 3D culture and disruption of mammary tissue architecture. However, our results suggest that mutant p53 could be not only an upstream regulator but also a downstream target of the mevalonate pathway. To assess whether the mevalonate pathway is a general regulator of mutant p53 proteins in different cellular contexts, we treated a panel of human tumour cell lines, derived from different tumour types, with Cerivastatin and monitored mutant p53 protein levels. As shown in Figure 10A, inhibition of the mevalonate pathway caused a strong reduction of mutant p53 protein levels in all the cell lines tested. Similar results were obtained treating cells with Simvastatin (Supplementary Figure B). The reduction of mutant p53 levels was time-dependent and was maximal after 48 hours of treatment (Figure 10B). Moreover, statin treatment reduced also the level of exogenously overexpressed mutant p53 R280K in MDA-MB-231 cells, thus confirming that the mevalonate pathway sustains mutant p53 stability through post-transcriptional mechanisms (Figure 10C).

All the mutant p53 proteins have been found to show different degrees of altered conformation. To understand whether statins act specifically on mutant p53 proteins with strong structural alteration, we stably expressed siRNA-resistant mutant p53 R175H (which has a strong altered conformation) or R280K (which has an almost intact conformation) proteins in isogenic normal breast epithelial cells MCF10A depleted of

endogenous wild type p53. Of note, both mutant p53 proteins were affected by statin treatment, suggesting that the mevalonate pathway sustains the stability of mutant p53 proteins, irrespectively of their structural features (Figure 10D).

To further explore the mechanisms of mevalonate-dependent mutant p53 stabilization we performed cycloheximide (chx) experiments in MDA-MB-231 cells. Strikingly, Cerivastatin treatment strongly shortened mutant p53 half-life in a proteasome-dependent manner (Figure 10E). Again, Mdm2 inhibition by means of Nutlin-3 treatment rescued mutant p53 degradation (Figure 10E), thus suggesting that Mdm2 is responsible for mutant p53 degradation upon mevalonate pathway inhibition.

Ubiquitination is a major mechanism by which mutant p53 is controlled by Mdm2 (Esser et al. 2005; Nagata et al. 1999). In line with this, we found that statin treatment strongly increased ubiquitination of mutant p53 in MDA-MB-231 cells (Figure 10F).

In cancer cells, mutant p53 proteins are engaged in stable complexes with Hsp90 chaperone machinery, which is often upregulated in cancer (Wang & Chen 2003; Dun Li et al. 2011) (Kamal et al. 2003). This interaction results in a marked reduction of mutant p53 ubiquitination and degradation by the E3 ligases Mdm2 and CHIP (Esser et al. 2005; Nagata et al. 1999) (Figure 10G). Based on this premises, we hypothesized that statins might act by destroying the mutant p53-Hsp90 interaction thus reactivating the inhibitory activity of Mdm2 on mutant p53. Strikingly, we found that in MDA-MB-231 cells, Cerivastatin treatment led to strong dissociation of mutant p53 from Hsp90, suggesting that the mevalonate pathway supports mutant p53 stabilization by promoting its interaction with the Hsp90 chaperone machinery (Figure 10H).

Taken together, these data suggest that the mevalonate pathway inhibitors treatment of cancer cells restore the inherent mutant p53 instability by functionally reactivating the Mdm2 inhibitory effect on mutant p53.

The Mevalonate Pathway is required for mutant p53 proteins stability

To investigate whether the effects of statin were specifically mediated by the inhibition of HMGCR enzymatic activity and consequent reduction of intracellular mevalonate levels (Figure 11A), we added MVA to the culture medium of MDA-MB-231 cells treated with statin, thus bypassing the requirement of HMGCR enzymatic activity. Importantly, mutant p53 protein levels were completely rescued by MVA addition into

the medium, thus confirming that mutant p53 stability depends on intracellular mevalonate levels (Figure 11B).

The identification of the mevalonate pathway as a key controller of mutant p53 stability, led us to investigate the upstream regulators of this metabolic signalling. All the enzymes of the mevalonate pathway, among which HMGCR, are under direct transcriptional control of SREBPs (sterol regulatory element-binding proteins) transcription factors (Figure 11A) (Brown & Goldstein 1997). SREBP1 and SREBP2 localize into the endoplasmic reticulum as inactive membrane-bound precursors. However, when cells require sterols—for example, after reduction of lipoprotein concentration in plasma—SREBPs translocate to the Golgi where they undergo maturation by proteolytic cleavage, and enter the nucleus to transcribe the enzymes of the mevalonate pathway thus restoring cellular lipids homeostasis (Edwards et al. 2000; Horton et al. 2002). Based on our results, it is conceivable that SREBPs may regulate mutant p53 levels by modulating the mevalonate pathway. To investigate this, we inhibited SREBP1 and SREBP2 expression by siRNA transfection in MDA-MB-231 cells maintained in medium with lipoprotein-depleted serum and we observed, along with reduction of the SREBP1/2 target gene SCD, a significative reduction of mutant p53 levels (Figure 11C).

The Mevalonate kinase (MVK) gene encodes for a crucial kinase within the mevalonate pathway that phosphorylates MVA into mevalonate-5-phosphate. Mevalonate kinase deficiency is a pathologic condition caused by several mutations on the MVK gene, which causes accumulation of MVA and impairment of the metabolites produced from MVA. In particular, the mutation N301T in the MVK gene has been identified as a dominant-negative mutation able to inhibit the wild type MVK enzyme thus impairing the mevalonate pathway flow (Gibson et al. 1989). In order to inhibit the mevalonate pathway using a naturally occurring mutation on MVK, we transfected the MVK N301T construct in MDA-MB-231 cells. Of note, immunofluorescence analysis demonstrated that cells expressing the mutated form of MVK were also negative for mutant p53 expression (Figure 11D), confirming that inhibition of mevalonate pathway reduces mutant p53 levels.

Downstream of mevalonate-5-phosphate, the enzyme farnesyl diphosphate synthase (FDPS) is required for the biosynthesis of geranyl pyrophosphate, a key precursor of

several mevalonate pathway products. Bisphosphonates (BPs) are FDA-approved compounds able to inhibit the mevalonate pathway by blocking the enzymatic activity of FDPS (Shipman et al. 1998) (Räikkönen et al. 2010) (Cl??zardin 2011). BPs are clinically used to treat osteoporosis, however several preclinical and clinical data suggest that BPs exert anticancer effects in different tumours, among which breast cancer. To test whether inhibition of mevalonate pathway by means of BPs affects mutant p53 levels in cancer cells, we treated MDA-MB-231 cells with Zoledronic Acid (ZA), a clinically-used BPs. As expected, similarly to statin treatment, ZA induced a strong reduction of mutant p53 protein levels (Figure 11E).

To investigate whether the mevalonate pathway controls mutant p53 protein levels *in vivo*, we orthotopically injected MDA-MB-231 cells in the flank of immunocompromised mice and, after tumour establishment, we treated mice with zoledronic acid. As shown in Figure 11F, tumours from mice receiving ZA showed a robust reduction of mutant p53 levels.

These results demonstrate that mutant p53 hyper stability is under metabolic control by the mevalonate pathway and that the genetic or pharmacological inhibition of this metabolic signal, at different levels, prevents mutant p53 accumulation in cancer cells.

Geranylgeranyl pyrophosphate mediates mevalonate-dependent mutant p53 stabilization

The mevalonate pathway is essential for the *de novo* biosynthesis of cholesterol but is also in charge for the production of other key metabolites among which isoprenoids (e.g. farnesyl pyrophosphate and geranylgeranyl pyrophosphate) (Figure 12A) (Repko & Maltese 1989). To gain insights into the molecular mechanisms controlling mutant p53 stabilization downstream of MVA, we inhibited specific enzymes of the mevalonate pathway to identify the metabolic intermediate responsible for mutant p53 regulation (Figure 12A). In particular, we inhibited cholesterol biosynthesis by means of ym-53601 (inhibitor of squalene synthase); protein farnesylation by means of fti-277 (inhibitor of the farnesyl transferase); and protein geranylgeranylation by means of ggti-298 (inhibitor of geranylgeranyl transferase I). Interestingly, only the geranylgeranyl transferase I (GGT-I) inhibitor GGTI-298 was able to reproduce the effect of statins on mutant p53, whereas the inhibition of squalene synthase or of farnesyl transferase had

no effect (Figure 12B). Moreover, GGTI-298 treatment reduced the interaction between Hsp90 and mutant p53 (Figure 12C).

These results suggest that the mevalonate pathway intermediate responsible for mutant p53 stability is geranygeranyl pyrophosphate. Consistent with this, GGPP addiction to culture medium almost completely rescued the mutant p53 levels in statin-treated cells (Figure 12D). These data indicate that protein geranylgeranylation is required for aberrant mutant p53 stability.

Geranylgeranylation is a crucial post-translational modification (PTM) of proteins that requires the attachment of GGPP, a 20-carbon lipophilic isoprene unitss derived from the mevalonate pathway, to one cysteine residue at the carboxy-terminal of several proteins (Shimoyama 2011). The main biological function of geranylgeranylation is to anchor proteins to cellular membranes. Rho-GTPases represent one of the main families of proteins which are geranylgeranylated and this PTM is required for their activation. Indeed, statin-induced GGPP reduction has been found to inhibit the enzymatic activity of RhoA (Figure 12E) (Sorrentino et al. 2014). Interestingly, RhoA has been recently identified as a key mediator of the anti-tumour activity of statins (Sorrentino et al. 2014) Therefore, we tested whether statins were able to reduce mutant p53 levels by affecting the activity of RhoA. To this aim we decided to experimentally bypass the requirement of geranylgeranylation for RhoA membrane attachment, and verify whether mutant p53 protein levels were rescued upon geranylgeranyl transferase inhibition. For this, we used a mutant GFP–RhoA bearing a C-terminal consensus for farnesylation (RhoA-F) instead of its natural geranylgeranylation motif (Supplementary Figure C). Indeed, farnesylation is an alternative PTM for membrane localization of several small GTPases (for example, Ras). As shown in Figure 12G, although RhoA-F protein was completely insensitive to geranylgeranyl transferase I inhibition (Figure 12F), mutant p53 levels were still reduced by GTI-298 treatment. These results demonstrate that RhoA is not involved in the regulation of mutant p53 stability downstream of mevalonate pathway and suggest that another geranylgeranylated protein controls mutant p53 levels in cancer cells.

Statins exert antitumour effects preferentially in mutant p53-harbouring tumour cells

Genetic and pharmacological ablation of mutant p53 proteins in tumours have been shown to have positive therapeutic effects *in vivo*, since those tumours show addiction to mutant p53 gain-of-function (Bossi et al. 2006) (Bossi et al. 2008) (Alexandrova et al. 2015) (Freed-Pastor et al. 2012). So far, the pharmacological inhibition of Hsp90 chaperone is the only reliable clinical strategy to achieve this goal. Indeed, Hsp90 inhibition in mice significantly extended the survival of mutant p53 knock-in mice by inducing mutant p53 degradation, growth arrest and apoptosis in tumour cells (D Li et al. 2011) (Alexandrova et al. 2015). Our results suggest that mevalonate pathway inhibitors could significantly curb the GOF activities of mutant p53 proteins, and thus might exert anticancer activity preferentially in p53-harbouring tumours. To test this hypothesis, we employed different assays on multiple human cancer cell lines with different p53 status. Interestingly, colony formation assay demonstrated that Cerivastatin reduces cell proliferation by reducing intracellular GGPP levels (Figure 13A and 13B). Moreover, colony formation and BrdU assay showed that Cerivastatin is significantly more active in cancer cell lines harbouring mutant p53 (Figure 13A-C). Interestingly, Cerivastatin induced massive apoptosis in mutant p53-harbouring cells as demonstrated by Caspase 3 cleavage (13D), while wild type p53 and p53-null cancer cells were almost unaffected by Cerivastatin treatment (Figure 13A-D).

To understand whether statins induce cell growth arrest and apoptosis by reactivating the wild type functions of mutant p53 proteins, we monitored the expression of p21, the most relevant p53 target gene, after statin treatment. As shown in Figure 14E, Cerivastatin was able to induce only a slight increase of p21 expression, which was not due to wild type p53 reactivation, as demonstrated by the fact that p21 levels increase also after knocking-down p53 in statin-treated cells.

Taken together, these results indicate that statins exert strong anti-tumour activity in mutant p53-expressing human cancer cell lines, with reduced effects in tumours harbouring wild type p53.

Statins counteract mutant p53 gain-of-function in cancer cells

One of the most critical aspects of mutant p53-dependent metastatic phenotype is the induction of migration and invasion. Mechanistically, mutant p53 has been found to enhance migration by sequestering and blocking the p63-mediated transcriptional induction of the anti-metastatic genes Sharp1 and CCNG2 (Adorno, Cordenonsi, Montagner, Dupont, Wong, Hann, Solari, Bobisse, Rondina, Guzzardo, Anna R Parenti, et al. 2009) (Figure 14A). In line with this, we found that Cerivastatin treatment in highly metastatic MDA-MB-231 cells, strongly rescued the expression of Sharp1 and CCNG2, likely caused by reduction of mutant p53 levels and consequent reactivation of p63 (Figure 14B). A crucial determinant of mutant p53-p63 interaction is the Prolyl-isomerase Pin1. Indeed, Pin1 promotes mutant p53-dependent aggressiveness in breast cancer by reinforcing mutant p53-p63 interaction and thus fostering the repression of p63-target genes (Javier E Girardini et al. 2011) (Figure 15A). Moreover, Pin1 and mutant p53 induce a specific set of target genes to fully establish tumour aggressive behaviour (Figure 14A). In particular, ten mutant p53/Pin1 target genes have been identified as key effectors of mutant p53 GOF and strongly correlate with poor clinical outcome. Among them, the DEPDC1 gene was identified as a the main mediator of mutant p53-induced cell migration (Javier E Girardini et al. 2011). Interestingly, we found that Cerivastatin treatment reduced the expression of mutant p53/Pin1 target genes, in particular of DEPDC1 (Figure 14C and 14D). In line with this, Cerivastatin treatment strongly suppressed the migratory capability of MDA-MB-231 cells (Figure 14E), confirming that mevalonate pathway inhibitors efficiently blunt mutant p53 GOF in cancer cells.

DISCUSSION AND FUTURE PERSPECTIVES

Missense p53 mutants accumulate in almost 50% of human tumours and their aberrant expression, due to extended half life, is necessary to confer oncogenic features to cancer cells (gain-of-function) (Cyriac Kandoth, Michael D. McLellan, Fabio Vandin, Kai Ye 2013). Therefore molecules able to target the mechanism governing the protein stability of mutant p53 could have great clinical value. Although the molecular events responsible for mutant p53 stabilization in cancer cells are still poorly understood, several studies pointed out a crucial role for the Hsp90 chaperone machinery in controlling the mutant p53 protein stability. The multi-complex Hsp90 chaperone machinery is often altered in several human cancers and this evidence, at least in part, accounts for mutant p53 hyper stabilization specifically in tumour tissues.

Genetic and pharmacological reduction of mutant p53 protein levels in human tumour cells showed strong therapeutic potential, in preclinical settings (Bossi et al. 2008; Bossi et al. 2006; D Li et al. 2011), in terms of reduction of cell proliferation and migration and increased chemosensitivity. Based on this notion, the systematic identification of well tolerated small molecules capable of reducing mutant p53 levels in human tumour cells could provide a great opportunity to blunt mutant p53 gain of function activities in cancer. Furthermore, the dissection of the mechanism of mutant p53 stabilization in cancer cells could expand our understanding of this process unveiling new pathways potentially targetable. Along this line, the discovery that mutant p53 is stabilized by a chaperone machinery composed of several proteins, among which HDAC6 and HSP90, led to the identification of 17-AAG and SAHA (respectively Hsp90 and HDAC inhibitors) as anticancer small molecules acting, at least in part, by blocking mutant p53 (Alexandrova et al. 2015; D Li et al. 2011).

In this thesis we describe the identification of novel regulators of mutant p53 stabilization in cancer cells, identified trough a screening of a library of FDA-approved compounds for their ability to reduce mutant p53 levels. This approach allowed us to identify several known and unknown mutant p53-destabilizing drugs. We focused on statins and show, for the first time, that the metabolic mevalonate pathway has a strong impact on stabilization of several p53 mutants both *in vitro* (in a variety of human cancer cells lines) and *in vivo* (in xenograft experiments).

The mevalonate pathway is essentially required for the biosynthesis of crucial metabolites such as cholesterol, dolichol, ubiquinone and isoprenoids. Despite this

pathway has been extensively studied in the context of cardiovascular diseases, recent reports have pointed out a crucial role of a dysregulated mevalonate pathway in human cancers. Interestingly, mutant p53 has been found to associate with mevalonate pathway gene promoters, via SREBP transcription factors, and to aberrantly activate the biosynthesis of isoprenoids (Freed-Pastor et al. 2012).

Our results clearly demonstrate that mutant p53 and the mevalonate pathway are engaged in a positive circuit with mutant p53. Indeed mutant p53 acts as transcriptional cofactor of SREBP proteins, thus increasing the levels of mevalonate pathway intermediates which in turn sustain mutant p53 protein stability. Therefore, mutant p53 acts not only as an upstream activator but also as a downstream mediator of aberrant activation of this metabolic pathway in cancer cells.

Recently, we and others demonstrated that the transcription cofactor YAP, the main effector of the Hippo pathway, is positively controlled by the mevalonate pathway through prenylation of Rho-GTPases (Sorrentino et al. 2014). We also characterised the physical and functional association of YAP with mutant p53 proteins which is functional for activation of NF-Y transcriptional activity on the promoters of cell cycle-related genes (Silvia Di Agostino et al. 2015; Di Agostino et al. 2006). Thus, the mevalonate pathway could sustain the oncogenic actions of mutant p53 in several ways, by increasing its stability and by favouring its recruitment into pro-oncogenic transcriptional complexes.

Mechanistically, our results suggest that activation of the mevalonate pathway protects mutant p53 from Mdm2-mediated degradation by the proteasome. In particular, we found that inhibition of the mevalonate pathway (by using statins, bisphosphonates and geranylgeranyl transferase inhibitors) reduces the engament of mutant p53 in the Hsp90 chaperone machinery thus restoring the ability of Mdm2 to poly-ubiquitinate mutant p53.

Although in our experiments we did not investigated the effects of mevalonate levels on Mdm2 enzymatic activation, work done in cells from liver cancer harbouring wild type p53, showed that statins trigger AKT-mediated Mdm2 phosphorylation, which is known to be required for its enzymatic activity. Thus, it is possible that the mevalonate pathway acts at different levels to control mutant p53 stability by both controlling mutant p53-Hsp90 interaction and the enzymatic activity of Mdm2.

Beyond Mdm2, the E3 ubiquitin ligase CHIP has been identified as a negative regulator of mutant p53 proteins. Similarly to Mdm2, binding of mutant p53 to Hsp90 protects mutant p53 from CHIP-mediated ubiquitination and degradation. Our results that statins inhibit the Hsp90-mutant p53 interaction suggest that reduction of mevalonate levels could also restore the negative activity of CHIP over mutant p53 and further investigation to test this hypothesis will be performed.

Experiments aimed at identifying the specific intermediate of the mevalonate pathway responsible for the mutant p53-Hsp90 interaction and consequent mutant p53 hyper stabilization, showed that in cancer cells mutant p53 turnover depends on the intracellular amount of geranylgeranyl pyrophosphate (GGPP), which is required for protein prenylation. The mechanisms by which GGPP controls the interaction of mutant p53 with Hsp90 are still unknown, and might involve the alteration of post-translational modification patterns on mutant p53 or Hsp90, such as phosphorylation and acetylation, or alteration of protein structure and folding. However, our results with geranylgeranyl transferase inhibitors implicate that protein geranylgeranylation is involved in the control of mutant p53 stability. In this context, we recently found that RhoGTPases, in particular RhoA, mediate the effects of GGPP on YAP/TAZ. However, our data exclude a role for RhoA in controlling mutant p53 stability and implicate that at least another geranylgeranylated protein controls mutant p53 protein levels. Our ongoing experiments are focused on the identification of this protein.

Cancer cells are addicted to mutant p53 and induction of mutant p53 degradation represents an effective approach to blunt its GOF. Indeed, reduction of mutant p53 protein levels results in the release and activation of the oncosuppressors proteins such as p73 and p63, triggering their antitumoural transcription program. In line with this we demonstrate that statins exert anti-proliferative activity preferentially in those cells in which p63 oncosuppressive activities are restrained by the aberrant expression of mutant p53 proteins.

Several studies attempted to demonstrate a significative correlation between statin use and reduced cancer-related(Chan et al. 2003).

However, the evidences are still debatable and context-dependent. Our results suggest that in order to evaluate the possible antitumour activity of statins in human patients, the mutational status of p53 should be taken into account. Moreover, our results suggest the

possibility to assess the antitumour activities of statins, in combination with standard chemotherapy, specifically in patients with mutant p53-bearing tumours.

One elegant and powerful approach to inhibit mutant p53 in cancer cells is provided by several compounds able to bind mutant p53 proteins and reactivate its wild type function (Bykov, Issaeva, Shilov, et al. 2002; Parrales & Iwakuma 2015; Guida et al. 2008; Bykov, Issaeva, Selivanova, et al. 2002; V. J. Bykov et al. 2005). Conversely, statins were unable to reactivate the oncosuppressive transcriptional program of p53 as demonstrated by the fact that the induction of the p53-target gene p21 by statins was largely p53-independent. Nevertheless, statins triggered activation of anti-metastatic p63 target genes and inhibition of the pro-oncogenic mutant-p53/Pin1 target genes expression.

All in all, the discovery that mutant p53 stability is controlled by the mevalonate pathway via protein geranylgeranylation reveals an unexpected metabolic layer of regulation of mutant p53 and provides the rational for the clinical use of mevalonate pathway inhibitors in cancers harbouring missense mutant p53.

EXPERIMENTAL PROCEDURES

Cell lines, culture conditions, and treatments

MDA-MB-231 (p53R280K), MDA-MB-468 (p53R273H), SUM149 (mutp53M237I) and BT-549 (mutp53R249S) are triple-negative breast cancer cells (TNBC). SK-BR-3 (p53R175H) are HER2-overexpressing breast cancer cells. T47D (mutp53L194F) human ductal breast carcinoma breast cancer cell line. Mahlavu (mutp53R249S) are hepatocellular carcinoma cell line. U2OS, osteosarcoma cell line, and MCF-7, human adenocarcinoma cell line, express wild-type p53, while H1299, a non-small cell lung cancer cell line, are p53 null. MCF10A ,mammary ephitelial cells expressing wild-type p53.

MDA-MB-231, MDA-MB-468, BT-549, SKBR-3, U20S and T47D cells were cultured in DMEM (LONZA) supplemented with 10% FBS (Fetal Bovine Serum) and with 1% antibiotics (penicillin 100U/mL and streptomycin 10μg/mL). SUM 149 cells were cultured in DMEM/F12 (LONZA) (1:1) supplemented with 5% HS (Horse Serum) and with 1% antibiotics. Mahlavu cells were cultured in EMEM (Sigma) supplemented with 10% FBS (Fetal Bovine Serum), with 1% antibiotics (penicillin 100U/mL and streptomycin 10μg/mL), 1% MEM NEAA (Minimum essential medium non-essential amino acids) and 1% Glutamax. H1299 cells were cultured in RPMI medium RPMI 1640 with 10% FBS and 1% antibiotics. MCF7 cells were cultured in EMEM (Sigma) supplemented with 10% FBS (Fetal Bovine Serum), with 1% antibiotics (penicillin 100U/mL and streptomycin 10μg/mL) and 1% MEM NEAA (Minimum essential medium non-essential amino acids. MCF10A (sh stable and mutant p53 expressing cell lines) cells were mainetened in DMEM:F12 Ham's medium 1:1, supplemented with 5% horse serum, insulin (10 μg/ml), hydrocortinose (0,5 μg/ml) and epidermal growth factor (EGF 20ng/ml) and with addition of selection antibiotics.

Treatment with inhibitors: Nutil-3 (10μM), Zoledronic Acid (ZA) (50μM), FTI-277 (1μM), YM-53601 (1μM), GTI-298 (1μM), Mevalonic acid (MVA) (0.5 mM), GGPP (20μM) alone or with Cerivastatin, Cycloheximide (CHX) (50 μM), MG132 (50mM).

Reagents and plasmids:

The library of FDA-approved drugs (Screen-Well FDA-Approved Drug Library, 640 chemical compounds dissolved at 10mM in DMSO) was obtained from Enzo Life Sciences (Enzo Life Sciences Inc.,Plymouth Meeting, PA, USA).

The following compounds were purchased from Sigma Aldrich: Cerivastatin (SML0005), Simvastatin (S6196), FTI-277 (F9803), GGTI-298 (G5169), DL-Mevalonic Acid 5-Phosphate (79849), Geranylgeranyl Pyrophosphate (#G6025), Zoledronic Acid (SML0223), YM-53601 (18113) was purchased from Cayman.

pSR-shRNAp53 puroR used to stably silence TP53 expression was a kind of R.Agami.

N-terminally HA-tagged p53 constructs: pMSCV-HA-P53R280H was generated by first introducing 4 silent point mutations in the region targeted by p53 siRNA I/shRNA (the same target sequence) by site directed mutagenesis in pcDNA-HA-p53, subsequent introduction of missense point mutation and subcloning of sequenced p53 cds constructs to pMSCV-HA-BlastR retroviral vectorto obtain pMSCV with N-terminally HA-tagged p53 cds.

High Content Screening

For the screening experiments, MDA-MB-231 cells (3.0×10^3 per well) were seeded on black clear-bottom 384-well plates (PerkinElmer). Twenty-four hours later, the FDA-approved drugs were transferred robotically from library stock plates (0.1mM and 1mM in DMSO) to the plates containing the cells; controls were added to columns 1, 2, 23 and 24 of each plate. Cells were fixed at 48 h after plating, i.e. 24h after addition of drugs, and processed immediately for immunofluorescence. Briefly, cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.5% Triton X-100 in phosphate buffered saline (PBS) solution for 10 min, followed by 30 min blocking in 3% FBS. Cells were then incubated with a mouse antibody against mutant p53 (Santa Cruz Biotechnology) diluted in blocking solution for 1 h. Cells were further washed with PBS and incubated for 1h with a secondary antibody conjugated to Alexa Fluor-594 (Life Technologies), and stained with Hoechst 33342 (Life Technologies).

Image acquisition was performed using an ImageXpress Micro automated high-content screening fluorescence microscope (Molecular Devices) at a 10x magnification; a total of 16 images were acquired per wavelength, well and replicate, corresponding to ca.

4,500 cells analyzed per experimental condition and replicate. Image analysis to identify cells presenting mutant p53 signal was performed using the 'Multi-Wavelength Translocation' application module implemented in MetaXpress software (Molecular Devices).
Screening was performed in duplicate, at two drug concentrations (1μM and 10μM); final concentration of DMSO in the culture medium was 1% (v/v) for all experimental conditions. The screening was performed at the ICGEB High-Throughput Screening Facility (http://www.icgeb.org/high-throughput-screening.html).

Transfections

siRNA transfections were performed with Lipofectamine RNAi-MAX (Life technologies) in antibiotics-free medium according to manufacturer instructions.
siRNAs were previously described and sequences are:

Target	siRNA sense sequence
Control	Allstars neg.control siRNA Quiagen #1027281
SREBP-1	5'-AUCUCUGAAGGAUCUGGUG-3'
SREBP-2	5'GCCCUCUAUUGGAUGAUGC-3'

Negative control siRNA was: AllStars negative control siRNA Qiagen 1027281.
In detail RNAi-MAX Lipofectamine was diluited in Optimem medium (Invitrogen) and, separately, siRNA-p53, siSREBP1,SREBP2 and siRNA-control (siRNA Qiagen 1027281) were also diluited in Optimem medium; after 5 minutes of incubation, RNAi-MAX Lipofectamine solution was added to siRNA solutions and they were incubated for 20 minutes; mixed solutions were finally added to the medium of cells, plated 24 hours before. After 48 hours from transfections, cells were analysed.
DNA transfections were performed in MDA-MB 231 cell with Lipofectamine 2000 (Invitrogen) in antibiotic-free medium according to the manufactured instructions.
Transfection was performed with Lipofectamine 2000 (Invitrogen) in antibiotic-free medium according to the manufactured instructions. In details, Lipofectamine was diluited in Optimem medium (Invitrogen) and separately another solution with pLPC-

GFP or pLPC-GFP-RhoA and Optimem was prepared; after 5 minutes of incubation, Lipofectamine solution was added to pLPC-GFP or pLPC-GFP-RhoA or Myc-MVK-N301T solution and they were incubated for 20 minutes; mixed solutions were finally added to the medium of cells, plated 24 hours before. After 24 hours from transfection, cells transfected with pLPC-GFP or pLPC-GFP-RhoA were treated with GTI-298 or cerivastatin for 24 hours, and then examined by fluorescence microscopy.

Quantitative Real-Time PCR

Cells were harvested in Qiazol lysis reagent (Qiagen) for total RNA extraction, and contaminant DNA was removed by DNase treatment. qRT-PCR analyses were carried out on retrotranscribed cDNAs with Quantitect reverse transcription kit (Qiagen) and analyzed with Biorad CFX Manager software. Experiments were performed at least three times, with duplicate replicates. Expression levels are always given relative to histone H3. Primers sequences have been previously described (Girardini J., et al., 2011)

Proteins extraction

Plated cells were lysed with Lysis Buffer (NP40 1%, Tris-HCL pH=7.5% 50mM, NaCl 300mM, EDTA 1mM) solution, supplemented with protease inhibitors (CLAP 0.1mM and PMSF 1mM) and with phosphatase inhibitors (NaF 5mM and Na3VO4 1mM), and were harvested. Cells were then centrifuged at 10,000 rpm for 10 minutes at 4°C. Concentration of proteins in the lysate was then quantified with the spectroscopic analytical procedure Bradford Protein Assay (Bio> Rad). Samples obtained were denatured in Laemmli Sample Buffer 2X or 6X and boiled for Electrophoresis.

Western blot

Western blotting allows the antibody detection of specific proteins from extracts made from cells. In order to make the proteins accessible to antibody detection they were moved from within the gel onto a membrane made of nitrocellulose with the blotter Trans-Blot Transfer Cell. The membrane was incubated in Blotto-Tween 20 solution (milk powder 5% w/v in PBS solution, added with Tween20 0.2% w/v) for 30 minutes and then incubated with primary antibody over-night. The next day, membrane was

incubated with secondary antibody for at least 30 minutes and finally developed in photographic plates with the solution kits ECL or ECL-Plus (Amersharm).
The antibodies used for western blot were: mouse monoclonal p53 (DO1) (1:1000) (Santa Cruz Biothecnology); Anti-actin (1:2000) is C11 (Sigma); Anti-vinculin (1:5000) is V4505 (Sigma);

Co-immuno precipitation.

Co-IP experiments with endogenous proteins were performed using Co-IP buffer (NaCl 120mM, Tris-HCl pH8 20mM, EDTA 1mM, NP40 0,5%) with protease inhibitors. Samples were cleared by centrifugation for 30 min at 13,000g at 4 °C and incubated for 2 h at 4 °C with anti-p53 antibody (DO-1; Santa Cruz). After 1 h incubation with protein G-Sepharose (GE Healthcare), immunoprecipitates were washed three times in Co-IP buffer, resuspended in sample buffer, and analyzed by immunoblotting. To avoid cross-reaction with Ig heavy chains, immunoprecipitated p53 was detected using HRP-conjugated DO-1 monoclonal antibody (Santa Cruz).
For ubiquitination assays, cells were lysed in 2% SDS, 150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM PMSF, 5 mM NaF, 1mM Na3VO4, 0,5% (v/v) sodium deoxycholate with protease inhibitor cocktail (Sigma-Aldrich) and Ubiquitin Aldeyde 50 ng/ml. Cell lysates were diluted in IP buffer: 10 mM Tris-HCl, pH8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton. The anti-p53 antibody (DO-1; Santa Cruz) was covalently bound to protein G Sepharose (Amersham Biosciences, GE Healthcare, Munich, Germany) using 5 mg/ml dimethylpimelimidate (Pierce Biosciences, Thermo Fisher Scientific, Bonn, Germany).

Colony formation assay

Cells ($5x10^3$) were plated on 6cm plates. The day after the medium was supplemented with cerivastatin (0,1 μM) alone or in combination with geranygeranyl pyrophosphate (GGPP) 20μM. After 6 days, cells were fixed with 4% paraformaldehyde (PFA) and stained for 30 min with Giemsa (FLuka) diluted solution 1:5 in water. Plates washed with water and dried were scanned.

Migration and invasion assays. For migration analysis, transfected cells ($1x10^5$) were plated on 24 well PET inserts (8.0 μm pore size, Falcon), according to the manufacturer's instructions. For invasion assays cells ($1x10^5$) were plated on matrigel-coated filters (8.0 μm pore size, Falcon) and the lower part of the chamber was filled with DCCM medium. After 16 h cells on the upper part of the membrane were removed with a cotton swab and cells that passed through the filter were fixed in 4% PFA, stained with 0.05% crystal violet and counted.

Viability assay

Cells (10^4 per well) were plated in 96-well plates and treated with cerivastatin (0,1 μM) for 96h. Cell viability was assayed with ATPlite (Perkin Elmer) according to manufacturer instructions using *EnSpire* Multilabel Reader (Perkin Elmer).

Brdu incorporation assay

Cells ($3x10^4$) were plated in 24-well plates and treated with cerivastatin (0,1 μM).

After 24 h from the treatment, the DNA precursor bromodeoxyuridine (BrdU) (1:1000) was added to the medium for 12-2h before fixation.

Briefly, cells were fixed in 4% paraformaldehyde for 10 min, whashed in PBS, permeabilized with Triton 0.1% for 10 min and washed 3 times with NaOH 50mM solution and washed in PBS. Primary anti BrdU antibody solution (1:2 dilution), to detect bromodeoxyuridine (BrdU) incorporated, was used for 2h at 37°C and Goat anti-mouse Alexa Fluor 568 (Life Technologies) as secondary antibody for 1h a 37°C. Nuclei were counterstained with Hoechst 33342 (Life Technologies).

Mice and animal care

For *in vivo* studies, one million of MDA-MB-231 cells were resuspended in 100 μl of DMEM, injected into the mammary fat of previously anesthetized 7 weeks old SCID female mice (1-3% isoflurane, Merial Italia S.p.A, Italy) as previously described. At day 12 after cell injection, mice were subjected to intravenous injection of zoledronic acid ([1-hydroxy-2- (1H-imidazoledronic acid-1-yl) ethylidene] (200μg/Kg body weight), every 4 days until the end of the experiment (day 40). The mice were used and housed in a specific pathogen-free (SPF) animal facility. Procedures involving animals and

their care were performed in conformity with institutional guidelines (D.L. 116/92 and subsequent complementing circulars) and all experimental protocols were approved by the ethical Committee of the University of Padua (CEASA). At day 40 the animals were sacrificed and the primary tumours were extracted and directly frozen in liquid nitrogen to perform molecular analyses.

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FIGURES

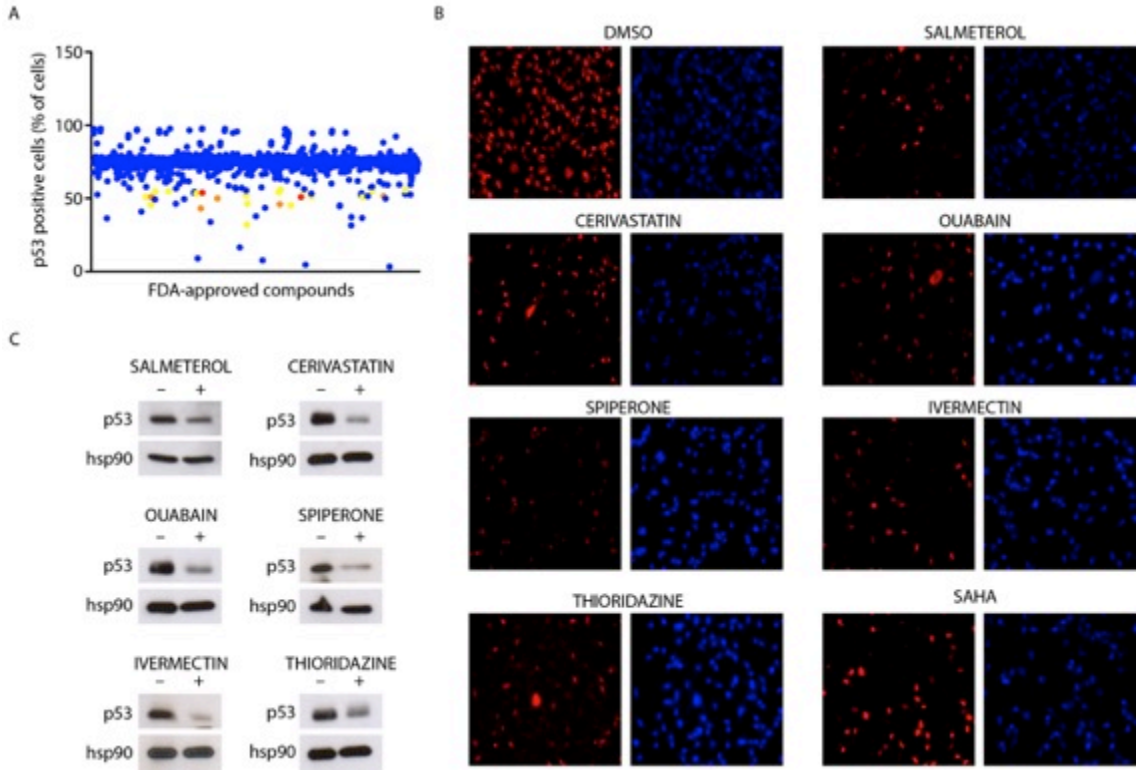


Figure 8. Identification of FDA-approved drugs that reduce mutant p53 levels.
(A) Results of high-content screening. Mutant p53 positive cells were detected by immunofluorescence and quantified through automated image analysis. Yellow circles are adrenergic agonists; orange circles are statins; red circles is SAHA. (**B)** Representative images of immunofluorescence from the screening. MDA-MB-231 cells stained for Hoechst (blue) and mutant p53 (red) treated with dimethylsulphoxide (DMSO) or with 10μM of the indicated drugs for 48h. (**C**) p53 protein levels in MDA-MB-231 cells treated with dimethylsulphoxide (DMSO)(-) or with the indicated drugs from the screening (+).

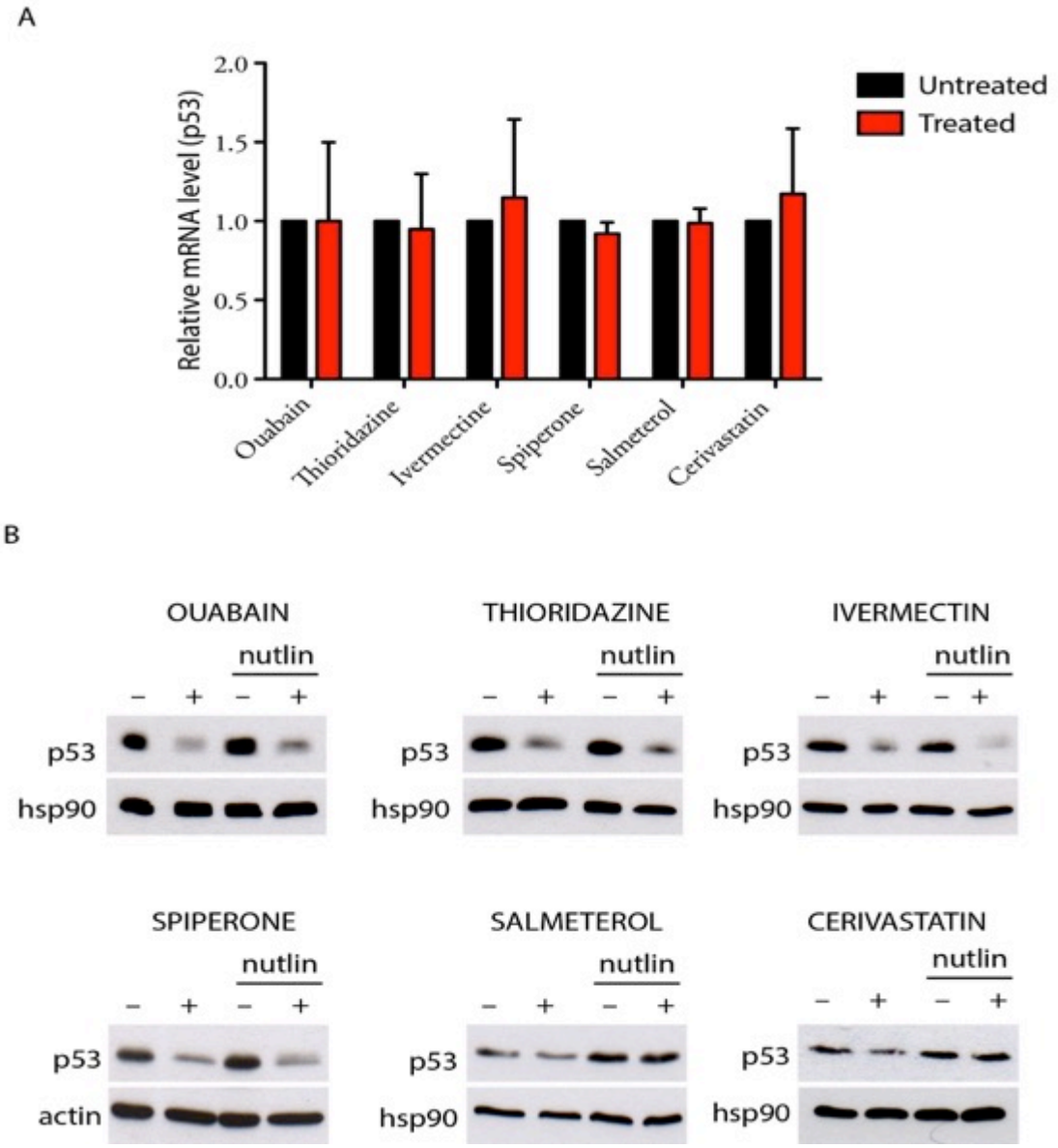


Figure 9. Statins reduce mutant p53 proteins stability in an Mdm2-dependent manner.

(A) p53 mRNA levels were analyzed by qRT-PCR in cDNA obtained from MDA-MB 231-cells untreated (black bars) or treated (red bars) with the identified drugs. Cells were treated as in Figure 1B. (**B)** Western blot of p53 protein levels from lysates of MDM-MB-231 cells treated with Nutlin-3 alone or in combination with the indicated drugs. Cells were treated with Nutlin-3 10μM for 12h before the treatment with the single hit drugs for 48h.

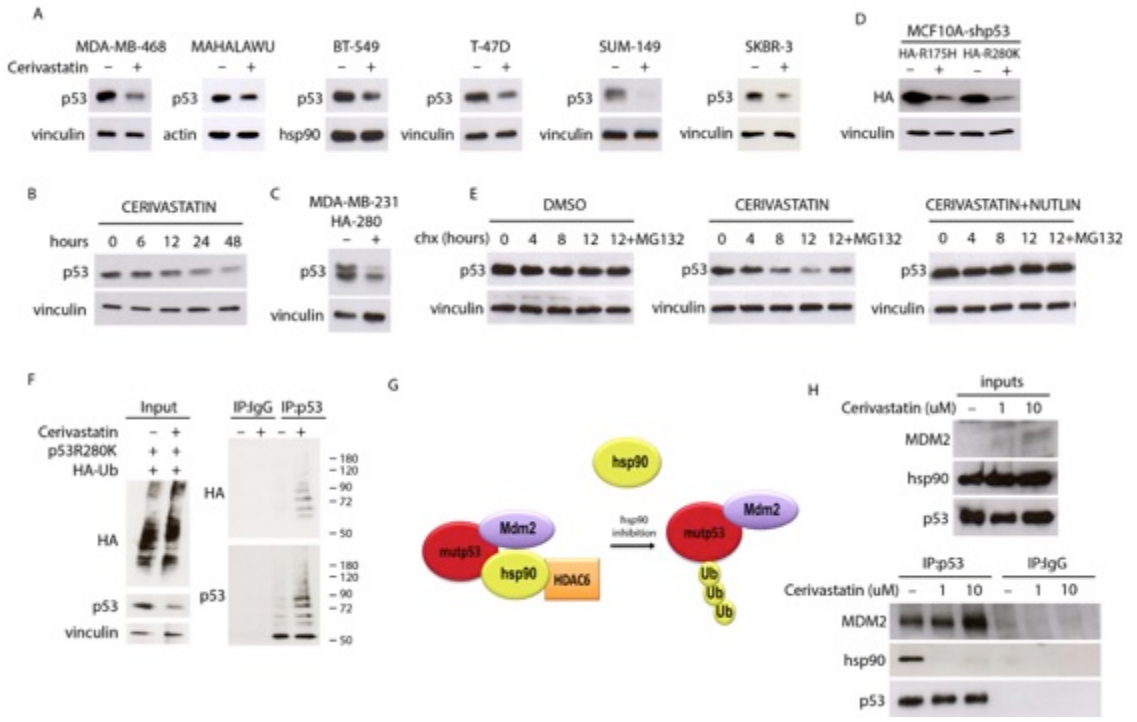


Figure 10. Inhibition of the Mevalonate pathway destabilizes mutant p53 proteins via proteasome-mediated degradation in different cellular contexts.
(A) p53 protein levels were analyzed by Western blot in a panel of human tumour cell lines. Cells were treated with Cerivastatin 1 µM for 48 h. **(B)** Western blot analysis of p53 protein levels upon treatment of MDA-MB-231 cells with Cerivastatin 1 µM for the indicated times. **(C)** Western blot analysis of p53 protein levels in MDA-MB-231 cells expressing pcDNA3-HA-p53R280K vector untreated (-) or treated (+) with Cerivastatin 1 µM for 48 h. **(D)** p53 protein levels were analyzed by Western blot in normal breast epithelial cells MCF10A depleted of endogenous p53 and stably expressing either siRNA-resistant mutant p53 R175H or R280K. Cells were treated for 48h with Cerivastatin 1 µM (+). **(E)** Cycloheximide (CHX) experiments in MDA-MB-231 cells. Cells were pre-treated with Cerivastatin (1 µM), alone or with nutlin (10 µM) and after 24h cells were treated with CHX (50 µM) for the indicated times. The proteasome inhibitor MG132 (50mM) was added to the last time point. **(F)** MDA-MB-231 cells were transfected with constructs expressing HA-ubiquitin and pcDNA3-p53R280K, then treated with Cerivastatin 1 µM for 48h. Ubiquitylated mutant p53 was detected by IP followed by anti-HA western blot. (G) Schematic representation of the mechanism of mutant p53 stabilization in cancer cells. **(H)** Mutant p53 was immunoprecipitated from lysates of MDA-MB-231 cells untreated (-) or treated (+) with Cerivastatin 1 or 10 µM for 24h. Co-immuniprecipitated Hsp90 and Mdm2 were detected by Western blot.

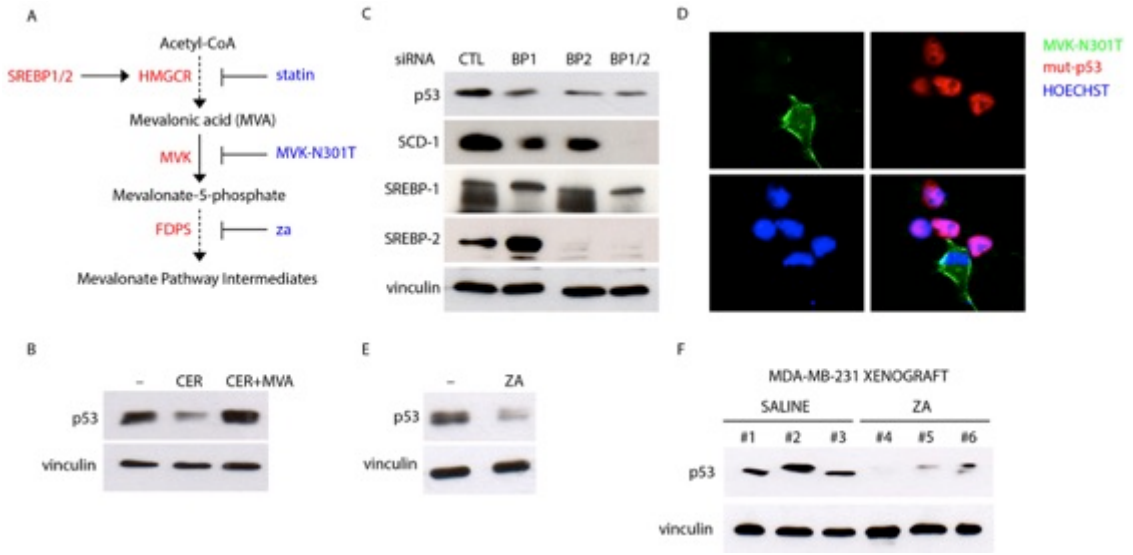


Figure 11. The Mevalonate Pathway is required for mutant p53 proteins stability. **(A)** Schematic overview of the enzymes of the mevalonate pathway. Enzymes are shown in red and inhibitors in blue. **(B)** Western blot analysis of p53 in MDA-MB-231 cells treated with Cervistatin 1µM (CER) alone or with mevalonic acid (MVA) 0,5 mM for 48h. **C)** Western blot analysis of p53 in MDA-MB-231 cells transfected with siRNAs specific for SREBP1 (BP1) or SREBP2 (BP2) and SREBP1/2 together (BP1/2) for 48h. **D)** Myc-MVK-N301T was transiently expressed in MDA-MB-231. p53 expression was analysed by immunofluorescence. **E**) p53 protein levels are show by western blot analysis in cells untreated or treated with Zoledronic Acid (ZA) (50 µM) for 48h. **F)** Lysates of tumours from control (saline) or zoledronic acid-treated mice were immunoblotted to detect p53 expression.

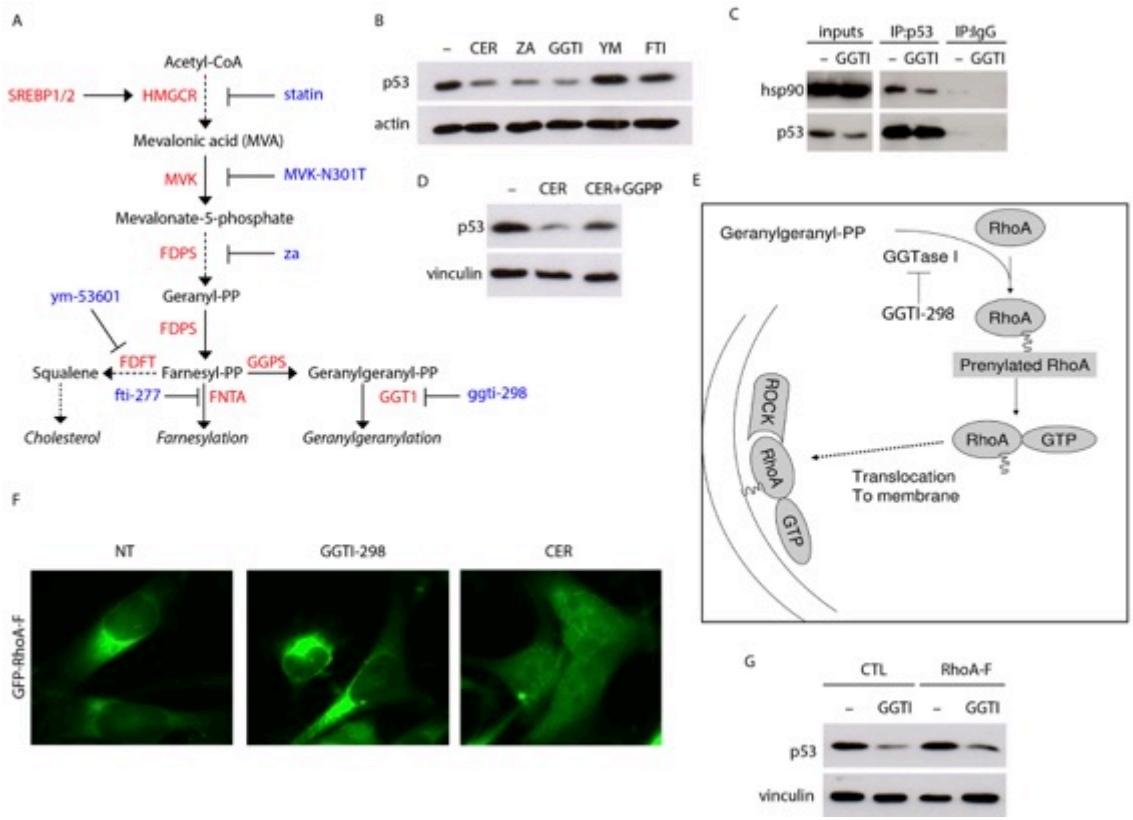


Figure 12. Geranylgeranyl pyrophosphate mediates mevalonate-dependent mutant p53 stabilization.

(A) Schematic overview of the mevalonate pathway. Enzymes are shown in red and inhibitors in blue. **(B)** Western blot of p53 levels in MDA-MB-231cells untreated (-) or treated with different inhibitors: cerivastatin (CER) 1μM, Zoledronic Acid (ZA) 50μM, geranylgeranyl transferase I inhibitor (GGTI-298) 1μM, squalene synthase inhibitor (YM-53601) 20μM, farnesyl transferase (FTI-277) inhibitor 20μM, for 48 h. **(C)** Mutant p53 was immunoprecipitated from lysates of MDA-MB-231 cells untreated (-) or treated (+) with GGTI-298 1μM for 24h. Co-immunoprecipitated Hsp90 was detected by western blot. **(D**) Western blot analysis of p53 levels in MDA-MB-231 cells treated with Cervistatin 1μM (CER) alone or with geranygeranyl pyrophosphate (GGPP) 20μM for 48h. **(E)** Schematic representation of geranyl-geranylation of Rho-GTPases. **(F)** MDA-MB-231 cells stably expressing the construct (pLPC-GFP-RhoA-F) coding for a mutant RhoA bearing a farnesylation consensus sequence (Cys-Val-Leu-Ser). Cells were left untreated (-) or treated with GTI-298 1 μM or with Cerivastatin 1 μM for 48h **(G)** Western blot of p53 levels from MDA-MB-231 cells stably expressing RhoA-F and treated with GTI-298 1μM for 48h.

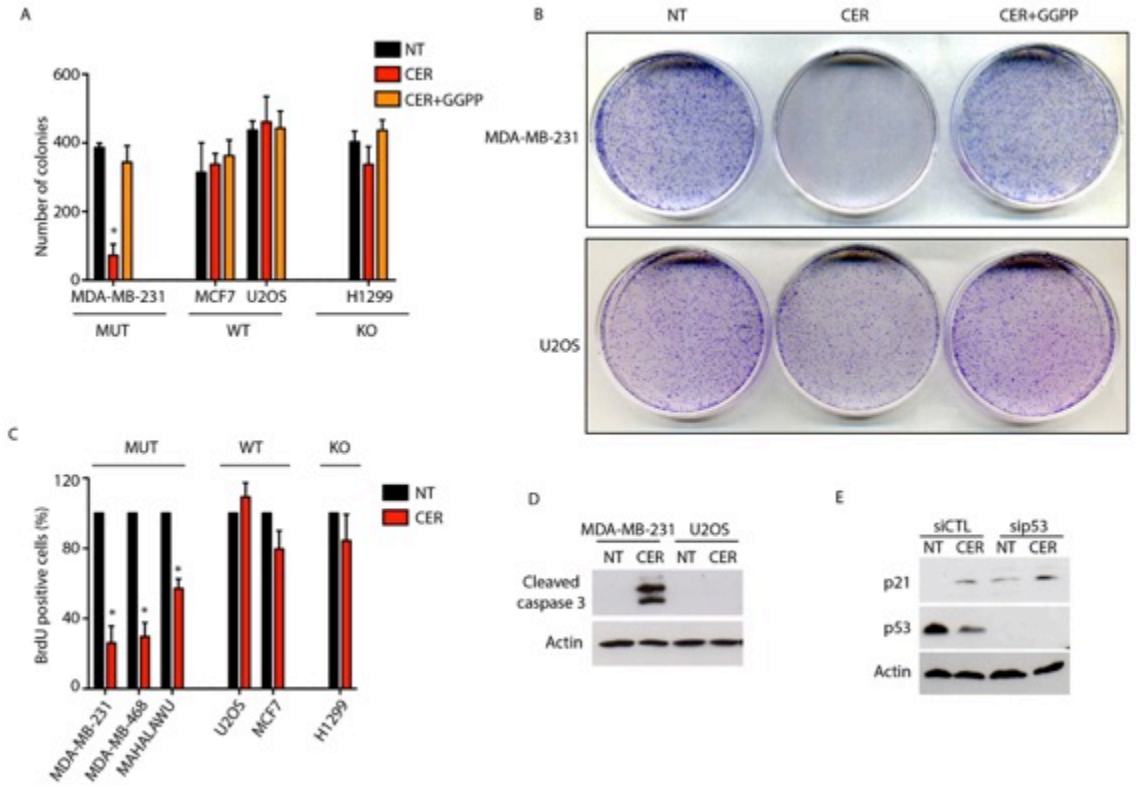


Figure 13. Statins exert antitumour effects preferentially in mutant p53-harbouring tumour cells.

(A-B) Colony formation assay. The indicated cell lines were treated with Cerivastatin (CER) 0,1μM alone or in combination with geranygeranyl pyrophosphate (GGPP) 20μM for 6 days. Quantification (A) and representative images (B) are shown. The status of p53 in the different cell lines is indicated. Error bars represent mean ± s.d. from n=3 biological replicates. **(C)** Quantification of BrdU-positive cells. The indicated cell lines were treated with Cerivastatin (CER) 0,1 μM for 24h. Error bars represent mean ± s.d. from n=3 biological replicates. **(D)** Cleaved caspase 3 protein levels are show by western blot analysis. MDA-MB-231 cells were left untreated (-) or treated with Cerivastatin (CER) 0,1 μM for 96 h. **(E)** MDA-MB-231 cells were transfected with control siRNA (siCTL) of with p53 siRNA for 24h. After 24h cells were left untreated (NT) or treated with Cerivastatin 1μM for 48h.

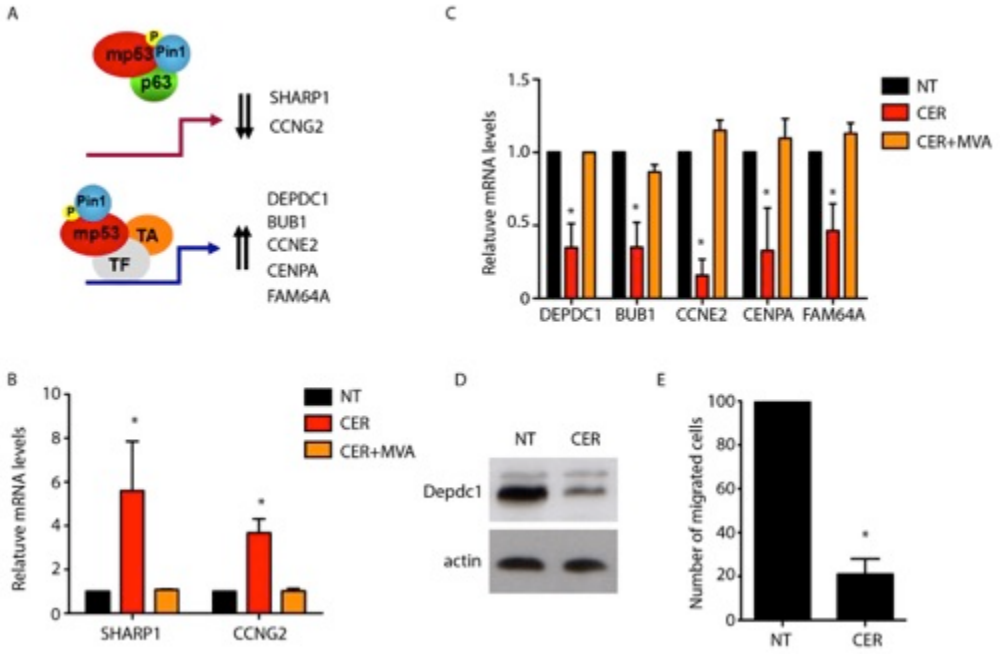
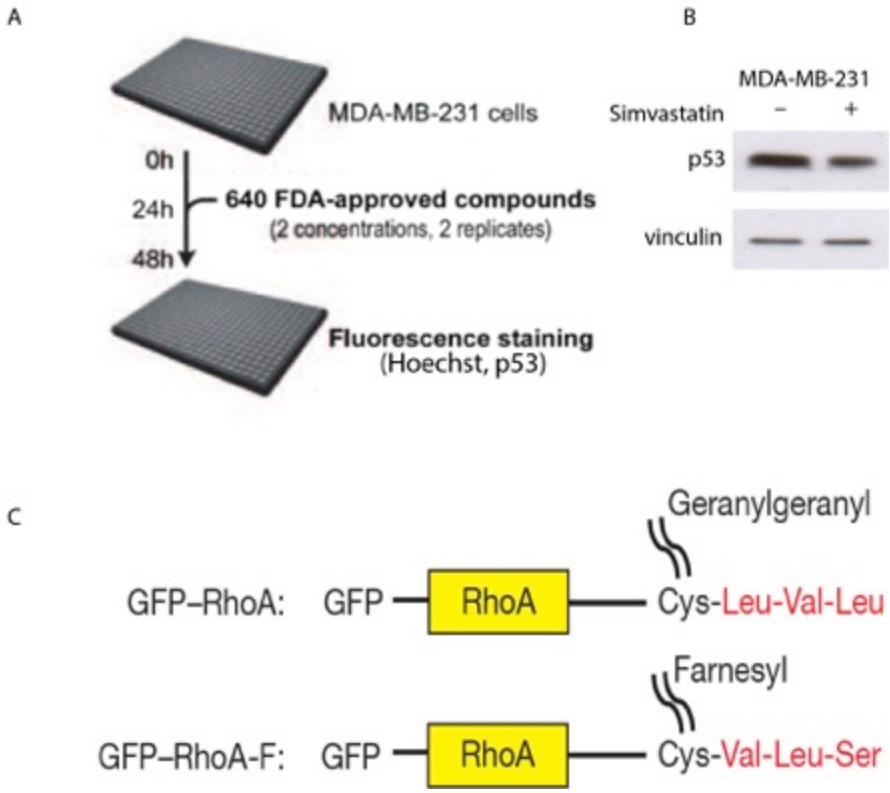


Figure 14. Statins counteract mutant p53 gain-of-function in cancer cells.
(A) Schematic representation of the pro-oncogenic role of Pin1 on mutant p53 activity. **(B)** Relative mRNA levels of *SHARP1* and *CCNG2* genes in MDA-MB-231 cells non-treated (NT) or treated with Cerivastatin (CER) alone or in combination with mevalonic acid (MVA) for 48h. **(C)** Relative mRNA levels of mutant p53/Pin1 target genes (*DEPDC1, BUB1,CCNE2, CENPA, FAM64A*) in MDA-MB-231 cells non-treated (NT) or treated with Cerivastatin 1 μ M (CER) alone, or in combination with mevalonic acid (MVA) for 48h. **(D)** DEPDC1 protein levels were analyzed by western blot analysis. MDA-MB-231 cells untreated (NT) or treated with Cerivastatin (CER) 1 μ M for 48h. **(E)** Transwell migration assays of MDA-MB-231. Cells were treated with Cerivastatin 1 μM for 24h, then plated for transwell assay and allowed to migrate for 12h.



Supplementary Figure.
(A) Schematic representation of the high-content screening. MDA-MB-231 cells were seeded in 384-well plates and 24h later the FDA-approved compounds were added to cells at 1 or 10 uM. 24h after the treatment, cells were fixed and processed for immunofluorescence for p53 and stained with Hoechst. **(B)** p53 protein levels were analyzed by western blot after treatment with Simvastatin 1 μM for 48 h. **(C)** Schematic representation of GFP–RhoA with a geranylgeranylation consensus sequence (Cys-Leu-Val-Leu) and the mutant GFP–RhoA-F with a farnesylation consensus sequence (Cys-Val-Leu-Ser)

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APPENDIX

During my PhD I have been involved in the following publications:

- Sorrentino G., Ruggeri N., Zannini A., **Ingallina E.,** Bertolio R., Marotta C., Neri C., Forcato M., Rosato A., Mano M., Bicciato S. and Del Sal G. Glucocorticoid receptor signalling activates YAP in breast cancer (*Submitted to Nature Communications)*

- Walerych D., Lisek k., Sommaggio R., Piazza S., Ciani Y., Dalla E., Rajkowska K., Gaweda-Walerych K., **Ingallina E**, TonelliC.,, Morelli M. J., Amato A, Eterno V., Zambelli A., Rosato A., Amati B., Wisniewski J.R., Del Sal G. Proteasome machinery is instrumental in a common gain-of-function program of the p53 missense mutants in cancer. (*Accepted to Nature Cell Biology)*

- Di Agostino S.*, Sorrentino G.*, **Ingallina E**., Valenti F., Ferraiuolo M., Bicciato S., Piazza S., Strano S., Del Sal G.§ and Blandino G.§ (2015). YAP enhances the pro-proliferative transcriptional activity of mutant p53 proteins. *EMBO reports 17, 188-201*

- Sorrentino, G., Ruggeri, N., Specchia, V., Cordenonsi, M., Mano, M., Dupont, S., **Ingallina E**., Piazza, S., Rosato A.; Piccolo S. and Del Sal G. (2014). Metabolic control of YAP and TAZ by the mevalonate pathway. *Nature Cell Biology 16, 357-366*.