# UNIVERSITA' DEGLI STUDI DI TORINO

Dipartimento di *Scienze Oncologiche*

Dottorato di Ricerca in

## "Sistemi Complessi applicati alla Biologia post-genomica"

XXIII ciclo

Titolo della Tesi

## "Does p53 mutant R273H show dominant negative or gain-of-function activity in mediating sensitivity or resistance to anti-cancer drugs?"


| Tesi presentata da: | Tutor interno: | Tutor esterno: |
|---|---|---|
| **Dott.ssa** | **Ch.mo Prof.** | **Dr.** |
| ***Mariangela Russo*** | ***Federico Bussolino*** | ***Christopher Torrance*** |

Coordinatore del Dottorato:
***Ch.mo Prof. Michele Caselle***


Anni Accademici **2008/2010**

SETTORE SCIENTIFICO-DISCIPLINARE: **BIO/11**

**TABLE OF CONTENTS**

## ABSTRACT

Mutation of the p53 tumor suppressor gene is the most common genetic alteration in human cancer. The majority of these mutations are missense mutations in the DNA-binding-domain, which still result in expression of a stable and full-length p53 protein. The primary outcome of these mutations is the loss of tumor-suppressing functions of the wild-type (wt) p53; however there is growing body of evidence that suggests mutant p53 can have a dominant negative effect (DNE) over the wt-p53 and/or gain of function (GOF) activity compared to the wt protein.
Moreover the nature of a p53 mutation in a cell is thought to impact upon clinical responses to therapy and poorer prognosis in cancer patients.
Studies showing DNE or GOF rely mostly on over-expression of the mutated cDNA at non-physiological levels and within p53-null cells, therefore reproducing an artificial situation and, importantly, leaving the heterozygous state of this mutation mainly unstudied.
Dominant negative activity and gain-of-functions have been ascribed also to the p53 hot spot mutant R273H, mainly through over-expression models in p53 null cells.

To explore the basis of these observations, experiments were designed to compare the properties of cells with and without p53 R273H mutation, in the same genetic background and within the endogenous gene locus.
To that end, 'patient-relevant' human isogenic cell-lines were created using recombinant adeno-associated viruses (rAAV)-mediated homologous recombination, to introduce the p53 hot spot mutant R237H within the endogenous p53 locus of the SW48 human colon cancer cell line, which is wt for p53.
SW48 lines in which the R273H mutant is over-expressed were also generated, so as to directly compare our patient-relevant genotypes with cell-lines used in previous published studies.

Expression at physiological levels of mutant p53 in heterozygous cells resulted in the typical response to p53 dependent cellular perturbations, such as activation of cyclin-dependent kinase inhibitor p21 and pro-apoptotic protein Bax after DNA damage. These data indicate that dominant-negative activity of endogenous expressed mut-p53 was not marked in these cells. We also found that patient-relevant expression of mutant p53 had no significant effect on cell proliferation and cell cycle progression, in contrast with previous published data on p53-mutant expression in p53 null cells.

The absence of DNE does not necessarily imply absence of mut-p53 gain-of-function, such as chemoresistance. However p53 R237H mutant in SW48 cell lines failed to induce resistance to common chemotherapies, such as irinotecan, paclitaxel and cisplatin, in contrast with previous data. The only evidence that mut-p53 had GOF came from partial resistance to 5FU treatment and anti-EGFR treatments, but that could also be due to haploinsufficiency. Absence of significant GOF is further demonstrated by almost absent influence of the hot spot mutant on the transcriptional profile of the cells.

In summary, the co-expression of mutant R273H p53 with wt-p53 at physiological levels in a colon cancer cellular model, does not appear to be sufficient to induce a strong dominant negative or gain of function activity.

# 1. BACKGROUD

## 1.1 p53 History

During the 1960s and 70s when studies on tumor viruses and oncogenes were the focus of many cancer research laboratories, several groups reported the existence of a cellular protein with an approximate molecular weight of 53 kDa, called p53, that appeared to be important in tumorigenesis.

The p53 protein was identified as a protein that co-immunoprecipitates with the SV40 large T antigen (Lane and Crawford, 1979; Linzer and Levine, 1979) and subsequently also with other oncoproteins produced by different tumor viruses, including the adenovirus E1B (Sarnow et al., 1982), the human papillomavirus HPV E6 protein (Scheffner et al., 1990), the Epstein-Barr virus nuclear antigen (Szekely et al.,1993), the hepatitis B virus X protein (Wang et al.,1994) and human cytomegalovirus IE84 protein (Speir et al.,1994).

Independently, experimentally induced tumors showed high levels of p53 protein (DeLeo et al., 1979) as did naturally occurring tumors compared to non-transformed cells (Lane and Crawford, 1979; Linzer and Levine, 1979). These data all led to the assumption that p53 was an oncogene, wherein elevated p53 expression was a tumor promoting event.

The molecular cloning of the p53 cDNA (Zakut-Houri et al., 1985) allowed for a greater variety of studies to be performed into the function of p53 in cell transformation in vitro and in tumorigenesis in vivo. These data started to show the opposite function to p53, together providing strong evidence that the loss of normal p53 expression and function may occur in the transformation process. This conclusion was based on the finding that rearrangement and functional inactivation of p53 gene was observed at high frequency in mouse spleen tumors induced by the Friend erythroleukaemia virus (Mowat et al., 1985).

Then this paradox was resolved when it was shown that the originally cloned p53 cDNAs used in the early experiments contained missense mutations within a conserved region of p53 important for both the conformation and biological activity of the protein. This fact clearly explains the initial observations that ectopic p53 expression immortalizes cells (Jenkins et al., 1984) and can transform primary rat embryo fibroblasts in cooperation with Ras (Eliyahu et al., 1984).

The idea of p53 as an oncogene was now in transition and the wild-type (wt) p53 was instead considered a key tumor suppressor gene.

In direct contrast with the mutant forms, wt-p53 cannot cooperate with Ras, and indeed can actually suppress transformation by mutant (mut) p53 gene and Ras (Finlay et al., 1989). Moreover, the transfection of wild-type p53 into human osteosarcoma cells lacking endogenous p53 abrogates the neoplasticity of these cells (Chen et al., 1990). In wide tumor genotyping experiments, p53 was shown to be lost, or to contain mutations that inactivate p53, in about half of almost all human cancer types. Indeed p53 is now known to be the most commonly altered gene in human tumors (Caron de Fromentel and Soussi, 1992; Hollstein et al., 1994). Supporting its close association with tumor development, it has noted that patients with the cancer-prone Li-Fraumeni syndrome, an inherited susceptibility disorder, carry a germline mutation in the p53 allele (Srivastava et al., 1990), had an increased risk of developing a variety of cancers, including soft tissue sarcoma, tumor of

breast, bone, brain and bladder (Malkin et al., 1990). Finally, upon the emergence of forward-genetic tools in transgenic mouse models, direct functional linkage to tumor promotion was demonstrated by p53 deficient mice being susceptible to spontaneous tumorigenesis (Donehower et al., 1992).

### 1.2 The TP53 gene and its product's structure

Localized on the short arm of chromosome 17 (17p13.1), the TP53 gene encodes a protein of 393 amino acids (aa) that is conserved during evolution. The gene is composed of 11exons, the first of which is non coding and localized 8-10 kb away from exons 2 through 11 (Benchimol et al.,1985).
It has five structural and functional domains: an N-terminal transactivation domain (aa 1-50); a proline-rich regulatory domain (aa 63-97); a central DNA-binding core domain (aa 102-292); a C-terminal oligomerization domain (aa 323-356) and a regulatory domain (aa 363-393) (Fig.1). Based on this structure, other functional assays and the mutation spectrum in p53, this protein is believed to operate primarily as a transcription factor to suppress tumor formation.

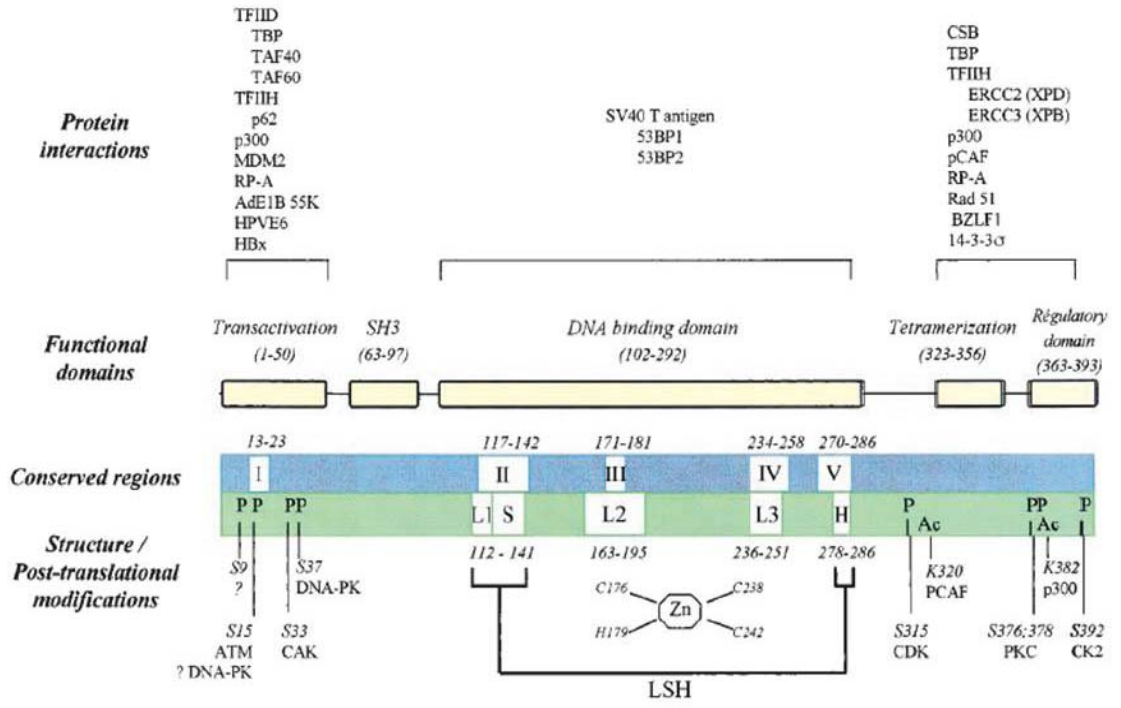



**Fig.1** Schematic representation of the p53 protein (from May and May, 1999). Major functional domains, conserved regions and the positions of post-translational modifications are indicated. In the upper part known interacting viral and cellular proteins are indicated; in the lower part proteins responsible of post-translational modifications are outlined.

The N-terminal region contains the activation domain (aa 1-50) (Fields and Jang, 1990). The acidic N-terminal transcriptional domain allows p53 to recruit the basal transcriptional machinery, including the TATA box binding protein (TBP) and TBP-associated factors (TAF) components of TFIID (Lu and Levine, 1995) (Fig.1). The

human MDM2 protein and hepatitis B virus X protein, also bind to the amino-terminal region of p53 and inhibit its transactivation function (Oliner et al., 1993; Momand et al., 1992; Levine, 1997) (Fig.1).

Further into the N-terminal region is also a proline-rich region (aa 63-97) with similarity to SH3-binding proteins, which is required for p53-mediated apoptosis in some experimental systems (Sakamuro et al., 1997) and for suppressing tumor cell growth (Walker and Levine, 1996) (Fig.1).

The central part of the protein is the area bounded approximately by amino acids 102 to 292 and it contains the DNA Binding Domain (DBD) which recognizes and binds the 10bp consensus sequence 5'-PuPuPuC(A/T)(T/A)GPyPyPY-3' present in two copies separated by 0-13bp in the regulatory region of the target gene promoters. One copy of the motif is not sufficient for binding p53 and subtle alterations of the motif results in loss of affinity for p53 (El-Deiry et al., 1992) (Fig.1). The DNA-binding domain of the p53 protein is a "hot spot" for mutation, as the majority of tumor-associated mutations in p53 occur within this region (Hainaut and Hollstein, 2000). This finding underscores the importance of p53 binding to DNA in a sequence-specific manner.

The C-terminal region (residues 300-393) includes: a flexible linker region (aa 300 -323) connecting the central core domain and the C-terminal region, and a tetramerization domain from amino acids 323-356. It is well established that p53 forms tetramers (Kraiss et al., 1988) via an oligomerization domain (aa 323-356). The structure of this tetramerization domain has been deduced from nuclear magnetic resonance (Clore et al., 1994) and X-ray crystallography (Jeffrey et al., 1995).

Three nuclear localization signals (NLS) have been identified in the C-terminal region. Mutagenesis of the most N-terminal signal (NLS1, aa 316-325) induces the synthesis of a totally cytoplasmic p53 protein, while alteration of the NLS2 (amino acids 369-375) and NLS3 (aa 379-384) leads to both cytoplasmic and nuclear localization (Shaulsky et al., 1990).

### 1.3 p53 stabilization and activation

p53 is stabilized and activated in response to a range of cellular stresses, including DNA damage, hypoxia, nucleotide depletion, hyperoxia and activated oncogenes (Vousden and Lane, 2007). Once induced, p53 regulates the expression of a wide range of genes, leading to a variety of biological outcomes, such as DNA repair, growth arrest or apoptosis (Riley et al., 2008) (Fig.2). Following DNA damage, the p53 protein rapidly accumulates and becomes activated (Kastan et al., 1991), binding strongly to strand breaks by its C-terminal domain (Nelson and Kastan, 1994) and this leads to the stabilization and activation of the protein.

Since active p53 has the ability to determine life (with correction of DNA damage) or death for a cell (if DNA damage is too extensive), by inducing cell cycle arrest or apoptosis respectively, it is clear that the triggering of p53 activation must be tightly regulated. In fact, both p53 stabilization and activation involve a variety of molecular mechanisms, including phosphorylation, acetylation, ubiquitination, glycosylation, binding to regulatory proteins, and alternative splicing (Gu and Roeder 1997; Giaccia and Kastan, 1998) (Fig.1).

The traditional overview describing p53 activation in response to cellular stress comprises three basic steps: stabilization of p53, sequence specific DNA binding and transcriptional activation of target genes.

The first step is the stress-induced stabilization of p53 that can occur through various mechanisms. In response to DNA damage p53 is post-translationally modified, including phosphorylation of the amino-terminus at specific amino acids by various kinases, including the ataxia–telangiectasia mutated (ATM) and ataxia–telangiectasia and Rad3-related (ATR) protein kinases (Meulmeester et al., 2005), members of the phosphatidylinositol-3 kinase like family, which coordinate a complex signaling network in response to various forms of DNA damage (Fig.2).

ATM plays a crucial part in the immediate response to double-strand breaks by coordinating the activation and execution of checkpoint pathways and repair pathways. The response to other forms of DNA damage, such as replication stress and DNA cross linking, is coordinated mainly by ATR (Fig.2). However, there is substantial interplay between the pathways governed by these molecules, and they share downstream targets in the repair and checkpoint pathways, including the kinases Chk1and Chk2.

Specifically ATM and ATR phosphorylate Ser15 of p53 (Banin et al., 1998; Lakin et al., 1999), protein kinase CK1 phosphorylate threonine 18 (Dumaz et al., 1999) and S20 is phosphorylated by Chk2 (which is activated by ATM).

Phosphorylations at T18 and S20 inhibit the association of p53 with Mdm2, its key negative regulator, which in absence of DNA damage mediates an ubiquitin-mediated degradation of p53 (Hirao et al., 2000). The MDM2 binds to the N-terminal (aa residues 17-27) part, a region containing several phosphorylation sites of p53 and inhibits p53-dependent transcription (Oliner et al., 1993).

MDM2 functions as an E3 ligase, the final component of the enzyme cascade that leads to the conjugation of ubiquitin to their substrate proteins and their subsequent transfer to the cytoplasm where protesome-mediated degradation takes place. It also contributes to its own degradation as it can auto-ubiquitinate itself. The promoter of MDM2 gene carries a p53 binding motif and it is transcribed in a p53-dependent manner (Barak et al., 1993). Thus, high levels of MDM2, caused by increased p53 activity generate an autoregulatory loop that lead to rapid turnover of the p53 protein, allowing induced p53 to act during a short time window (6-13min) before it is withdrawn to its normal cellular levels (Haupt et al., 1997) (Fig.2).

P53 is also induced through the ARF tumor suppressor pathway (Sherr, 1998), which is an important inhibitor of MDM2 (Fig.2). ARF is the product of an alternative open reading frame of the tumor suppressor INK4a (also known as p16) locus and it is normally expressed at low levels in the cell. Activation of ARF by activated oncogenes has classically been considered to be the mechanism by which p53 responds to abnormally sustained proliferation: once activated, ARF is able to inhibit Mdm2 by blocking its E3 ubiquitin ligase activity, uncoupling the p53-Mdm2 interaction and sequestering Mdm2 in the nucleolus, thereby separating it from nucleoplasmic p53 (Honda and Yasuda, 1999; de Stanchina et al., 1998; Tao and Levine, 1999).

After its stabilization and sequence-specific DNA binding, p53 activates or represses its target genes. The traditional view suggests that p53 promotes transcriptional activation or repression of target genes by interacting with general transcriptional factors such as TFIID/TAFs.

However many post-translational modifications of p53 can influence the recruitment of p53 binding proteins to specific promoters. The extreme C-terminus of the protein controls its sequence-specific DNA binding and transcriptional activity, and these functions can be influenced by multitude of covalent and non-covalent modifications within the C-terminus. Modifications suggested to be involved in activation of p53 include sumoylation (Muller et al., 2000), phosporylation, dephosphorylation, acetylation and glycosylation (Shaw et al., 1996).

For example p53 can interact with various transcriptional activators, such as histone acetyltransferase CBP/p300 (Iyer et al., 2004) that can mediate acetylation of the C-terminal lysine residues of p53 through interaction with its N-terminal region: DNA damage-induced phosphorylation of p53 in the N-terminal region increase its association with CBP/p300 that augments p53 acetylation leading to a more open chromatin conformation near p53 targets and a more active p53 protein (Lambert et al., 1998).

Other modifications that regulate p53 activity include C-terminal phosphorylation of p53 by CDKs and Protein Kinase C on Ser15 and Ser378 respectively, which also mediate p53 sequence specific binding in vitro (Baudier et al., 1992). Methylation mediated by methyltrasferases, such as Set9 cause hyperstabilization and activation of p53 through lysine methylation on Lys 372 and enhances apoptosis (Chuikov et al., 2004).

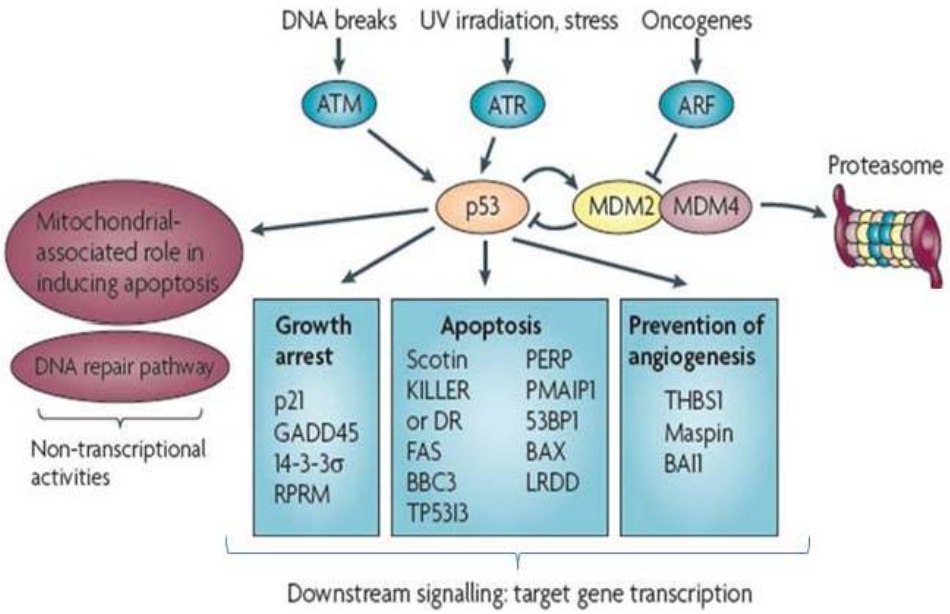



**Fig.2** p53 regulatory pathway (modified from Brown et al., 2009). In normal cells, the transcription factor p53 is inactivated by MDM2, an ubiquitin ligase that targets p53 for degradation in the proteasome. Several types of stress can activate p53, including DNA damage and oncogene activation, hypoxia, depletion of the cell's nucleotide pool or defects in DNA methylation. Each type of stress is communicated to p53 by distinct mechanisms: p53 is the master switch that integrates signals from these pathways and transforms them into a variety of biological outcomes such as growth arrest, DNA repair and apoptosis.

### 1.4 p53 functions

p53'functions are mainly exerted at the transcriptional-level, although some additional transcription independent pathways, based on protein-protein interactions, are also now know (Fig.2). The function of the multiple transcriptional p53 targets are linked to diverse cellular processes including cell cycle arrest, apoptosis, DNA repair, senescence, angiogenesis and metastasis (Fig.2). Cell cycle arrest and apoptosis are the most widely studied effects of p53 activation.

#### 1.4.1 Cell-cycle Arrest

The cell cycle is a complex process and is tightly regulated by multiple factors. Cyclins, their corresponding cyclin dependent kinases (CDKs) and CDK inhibitors being the core components of this process. Successful completion of cell cycle is monitored and controlled by cell cycle checkpoints, which are important control features that ensure the fidelity of cell division by verifying whether the processes at each phase of the cell cycle have been accurately completed before progression into the next phase. In response to various cellular stresses, cells may undergo growth arrest at these checkpoints to prevent the propagation of mutations in the DNA.
It has been observed that mouse embryo fibroblast cells develop tetraploid populations after treatment with spindle inhibitors. Most of the cells arrest without re-entering S phase. In contrast, cells that are p53-null and similarly treated continue to cycle and a much larger proportion of cells become tetraploid or octaploid. These data suggested that p53 is a component of a spindle checkpoint and that the spontaneous development of tetraploid cell populations further indicates that this checkpoint is required during normal cell division (Cross et al., 1995).
Moreover murine embryonic fibroblasts (MEFs) exposed to DNA damage activate the ATM/ATR pathways, leading to the activation of p53 and subsequently undergo G1 arrest. It is well established that wild-type p53 is required for the induction of G1 arrest in response to ionizing radiation (IR) because cell lines engineered to lack p53 activity display an attenuated response (Kuerbitz et al., 1992), but it is also known that p53 transactivates components of the G2/M checkpoints upon physiological stress. Several findings have greatly contributed to the understanding of how p53 governs cell-cycle checkpoints.
Gene targeting strategies have established that p21/Waf1, a cyclin-dependent kinase inhibitor, is a critical mediator of the p53-mediated G1 arrest response. The p21/WAF1 gene product has been identified as a potent inhibitor of several CDKs involved in both G1 and G2 arrest, by inhibiting cyclinE/CDK2 and cyclinB/Cdc2, respectively (Agarwal et al., 1995; Bunz et al., 1998, Waldman et al., 1995). Independent studies have shown that embryonic fibroblast obtained from p21 null mice are only partially defective in their ability to undergo G1 arrest following exposure to IR (Deng et al., 1995; Brugarolas et al., 1995) suggesting that another gene product contribute to a complete response.
One potential downstream target of p21 inhibitory activity in G1 phase is the cell cycle-dependent phosphorylation of the retinoblastoma (RB) protein. RB is in a hypophorylated form during G1. In this state it binds to and sequesters the S phase promoting E2F family of transcription factors.

Advancement through the cell cycle is thought to be mediated by sequential phosphorylation of RB by G1 cyclin-dependent kinases, resulting in the release of active E2F, which then leads to the transcriptional activation of genes required for S phase progression (Giaccia and Kastan, 1998). In this pathway p21 leads to the inhibition of cyclin D-CDK4/6 complexes and subsequent accumulation of the un-phosphorylated form of RB, which arrests cell in G1.
This G1 arrest is supposed to prevent the replication of damaged DNA and to allow DNA repair before entry into S phase.
Also E2F appears to be a critical downstream target within the p53 pathway because overexpression of E2F-1 can overcome IR-induced G1 arrest and p21-mediated inhibition of cdk activity (Degregori et al., 1995). Alteration of any of these downstream components may have an effect similar to that of inactivating p53 itself in preventing the pathway from functioning.

Several studies have suggested that the G2/M block following DNA damage is also p53-dependent (Agarwal et al., 1995). Progression of cells from G2 to mitosis is driven by the maturation-promoting factor (MPF), which comprises a complex of cyclin B1 and cdc2. p53 has been shown to induce G2/M arrest by primarily perturbing the function of the cyclin B1/cdc2 complex through the transcriptional activation of the 14-3-3sigma protein after DNA damage (Hermeking et al. 1997), which prevents proper nuclear localization of cyclin B1/cdc2. Moreover, deletion of 14-3-3sigma in HCT116 cells resulted in cell death in response to DNA damage (Chan et al. 2000).

### 1.4.2 Apoptosis

Although the ability of p53 to trigger cell-cycle arrest was discovered first, its action in controlling apoptosis is the most intensely studied.
The first proof that p53 functions in apoptosis was obtained by using a clone of the mouse myeloid cell line M1 lacking endogenous expression of p53. Cells of this clone were stably transfected with a temperature-sensitive mutant which acquires the conformation of wild-type p53 at permissive temperature (32°C). In these experiments, it was observed that upon downshift to the permissive temperature, the transfectants underwent a rapid loss of viability with all the characteristics of apoptosis (Yonish-Rouach et al., 1991). The observed rapid cell death upon temperature shift to 32°C implicated a role of p53 in cell death. Interestingly, the same mutant induces a growth arrest in fibroblasts at the permissive temperature whereas the M1 cells continue to divide as p53 induces cell death (Yonish-Rouach et al., 1993).
Later, it was shown that oncogenes could activate the p53 tumor suppressor leading to apoptosis, and that p53 is required for apoptosis induced by certain DNA damaging anticancer agents (Lowe and Ruley 1993). Current evidence indicates that the apoptotic activity of p53 is tightly controlled and is influenced by a series of quantitative and qualitative events that determine the outcome of p53 activation. Along these lines, other p53 family members can induce apoptosis, either in concert or in parallel with p53: therefore apoptosis can be integrated into a larger p53 tumor suppressor network controlled by different signals, environmental factors, and cell type (Fridman and Lowe 2003).

Many transcriptional dependent and independent p53 targets have been implicated in p53-mediated apoptosis. Members of both the extrinsic and intrinsic apoptotic pathway have been identified, wherein all of them lead to caspase-mediated apoptosis.

In the extrinsic pathway the initiators are specific transmembrane death receptors and their respective ligands.

Formation of so-called death-receptor-inducing-signalling-complex (DISC) including receptor, ligand, the adapter molecule FADD and caspase-8, triggers a chain of events resulting in activation of effector caspases. The cell surface receptor Fas (CD95) (that is a key inducer of this pathway) and the death-domain-containing receptor DR5/Killer are p53 targets that upon DNA damage can induce apoptosis in a tissue specific manner (Muller et al., 1998; Burns et al., 2001). A third transmembrane protein that is also induced by p53 in response to DNA damage is PERP, which probably in collaboration with E2F1, induces apoptosis (Attardi et al., 2000) (Fig.3).

The intrinsic pathway is regulated by the pro- and anti-apoptotic Bcl-2 proteins family that consists of three subclasses: the pro-survival proteins like Bcl-XL, the pro-apoptotic proteins Bax and Bak and the "BH3-only" pro-apoptotic proteins like PUMA. Several members of all the three groups are p53 targets. Bax is one of the first genes of the Bcl2 family identified, whose expression has been shown to be activated by p53 in response to cellular stress. Upon induction, Bax undergoes a conformational change, forming a homodimer and translocates to the mitochondrial membrane where it promotes cytochrome-c release leading to activation of caspase-9 (Miyashita and Reed, 1995).

p53 can also localize to the mitochondria, where it can induce mitochondrial outer membrane permeabilization (MOMP), thus leading to the release of pro-apoptotic factors from the mitochondrial inter-membrane space. p53 can interact with Bcl2, Bcl-XL, and Bak at the mitochondria, and has been suggested to act like a BH3-only protein, either as a direct activator of Bax and/or Bak, or as a derepressor (Green and Kroemer, 2009) (Fig.3).

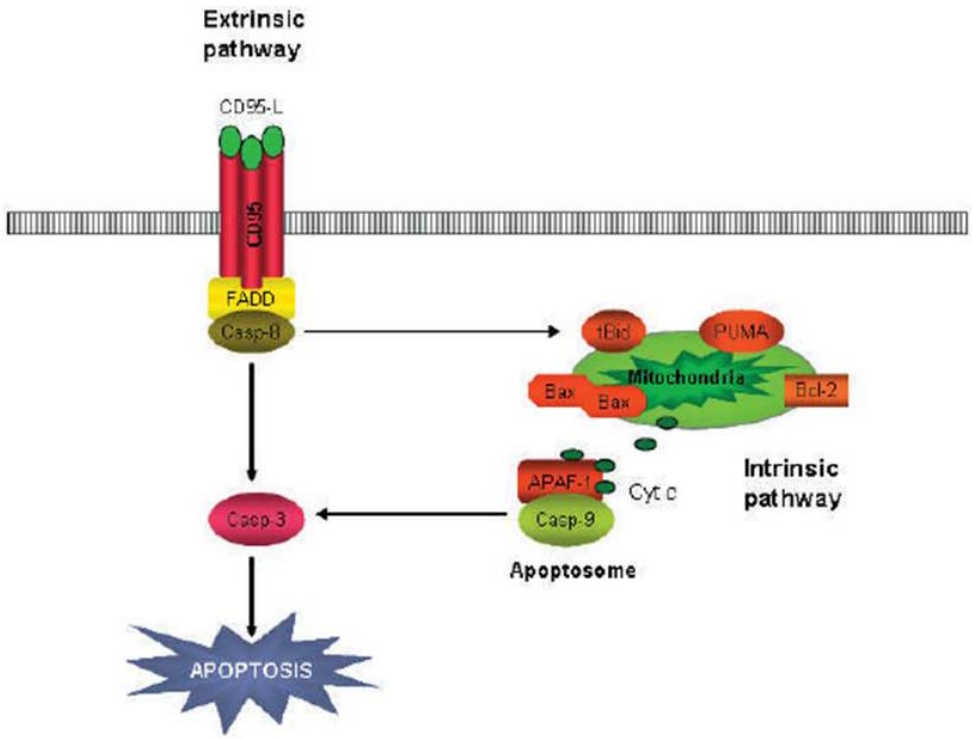



**Fig.3** Model of the extrinsic and intrinsic apoptotic pathway. The p53 targets are shown in orange.

The transcription-dependent and transcription independent mechanisms of p53 have been recently linked through the p53 apoptotic target gene PUMA (Chipuk et al., 2005). Specifically, in response to cellular stress, p53 transactivates PUMA, which then translocates to the mitochondria where it can bind Bcl-XL protein, thus releasing p53 to activate Bax. These data suggest that the transcription-dependent component of the p53 network is essential for the thorough induction of apoptosis, and PUMA plays a critical role in this process. Indeed, PUMA is a unique p53 apoptotic target gene. It is the only p53 target gene whose loss produces a similar apoptotic defect to p53 loss in irradiated T lymphocytes (Jeffers et al., 2003). These data suggest that PUMA is an essential p53 effector during apoptosis at least in this cell type under these conditions. However, PUMA-null mice are not overtly tumor prone, thus suggesting that simultaneous inactivation of multiple p53 effector functions is critical for initiating tumorigenesis.

The importance of p53-mediated apoptosis to provide protection from transformation has been exemplified in tissue culture where p53-/- cells fail to undergo cell death following introduction of a variety of oncogenes with dramatic enhancement of malignant transformation.

It is well established that induction of the p53 tumor suppressor protein can lead to either cell cycle arrest or apoptosis. But how p53 might regulate growth arrest versus apoptosis?

Although the mechanism governing the decision of the cell is not elucidated, deletion of p21Waf1 can cause cells that would otherwise undergo p53-dependent cell cycle arrest to undergo apoptosis. Several factors, including the cell type, the presence or absence of survival factors in the external environment, the extent of DNA damage and the levels of p53 are involved in the choice between cell cycle arrest and

apoptosis (Chen et al., 1996). Also cooperation between the p53 and RB pathways may be of major importance in determining the biological response to DNA damage. In fact, inactivation of RB has been correlated with loss of G1 arrest (Slebos et al., 1994) and induction of apoptosis after DNA damage (Morgenbesser et al., 1994). This might be explained by the release of E2F, which when over-expressed on its own can induce apoptosis (Degregori et al., 1995). Over-expressing RB has also been noted to block p53-dependent apoptosis (Haupt et al., 1995). Thus, modulation of RB and E2F through p53 signalling in response to DNA damage may play a central role in deciding the choice between cell cycle and arrest.

### 1.5 TP53 cancer genetic alterations and consequences

The anti-proliferative role of the p53 protein in response to various stresses and during physiological processes such as senescence, makes it a primary target for inactivation in cancer (Levine, 1997). Unlike most tumor-suppressor genes, which typically undergo biallelic inactivation during carcinogenesis by deletions or truncating mutations, TP53 is frequently (73.6%) inactivated by a single monoallelic missense mutations that cause single amino-acid changes at many different positions (Fig.4). This still results in the formation of a stable full-length protein, which tends to accumulate in the tumor cells (Weisz et al., 2007).
Data on mutation prevalence in human cancer can be conveniently accessed through the IARC TP53 database (http://www-p53.iarc.fr/), a resource that compiles all TP53 gene variations reported in human cancers with annotations on tumor phenotype, patient characteristics, and structural and functional impact of mutations (Petitjean et al., 2007).

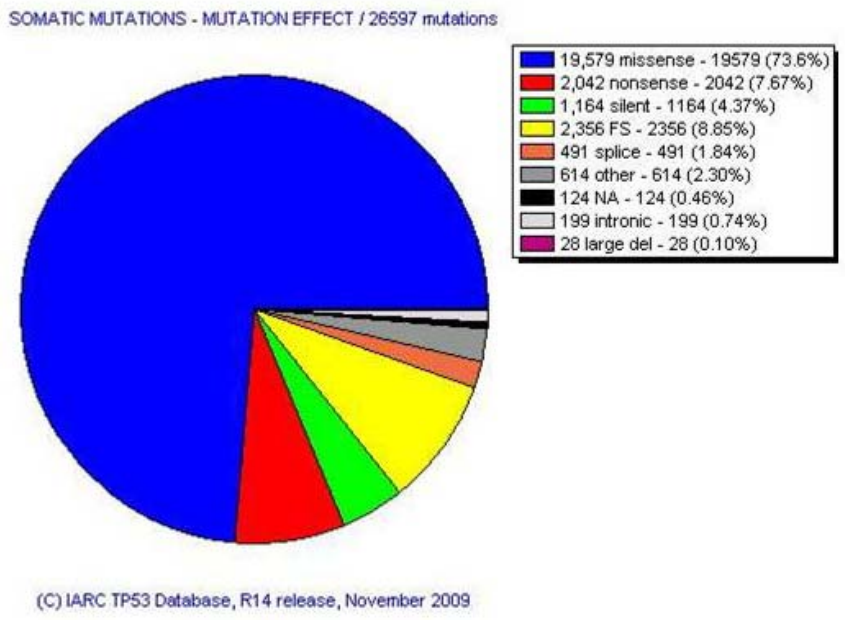



**Fig.4** Somatic TP53 mutations in human cancers. Unlike most tumor-suppressor genes, which typically undergo biallelic inactivation during carcinogenesis, TP53 is frequently (73.6%) inactivated by a single monoallelic missense mutations. Data from the IARC TP53 database (R14, November 2009) (Petitjean et al., 2007).

### 1.5.1 TP53 somatic mutations in human cancers

In cancers of the upper aero-digestive tract (oral, esophageal or bronchial cancers), TP53 is mutant in up to 75% of the cases of invasive cancers, particularly in smokers who are exposed to mutagens, and the mutation is often detectable in early, pre-neoplastic lesions. In cancers of the lower digestive tract, such as colon cancer, TP53 mutations are less common at early stages (polyps or adenomas) but become highly prevalent at the adenoma-carcinoma transition. In breast cancers, mutations are detected in about 25% of the cases, but it has been suggested that other mechanisms than mutations may account for inactivation of p53 in a proportion of the cases.
Cancers in which TP53 mutations are infrequent include cancers of the cervix, testicular cancers, neuroblastoma and malignant melanomas, in which the TP53 mutation prevalence is about 5% (Fig.5) (Data from the IARC TP53 database - Petitjean et al., 2007).

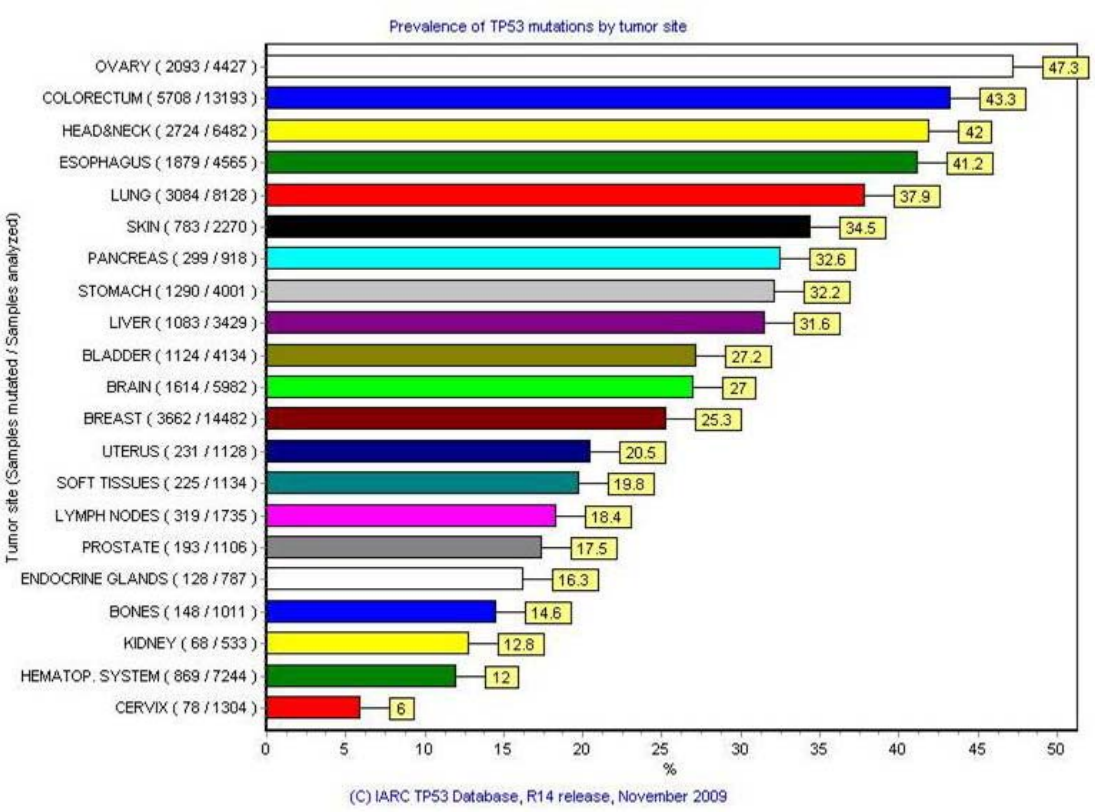



**Fig.5** Somatic TP53 mutations in human cancers. Data from the IARC TP53 database (R14, November 2009) (Petitjean et al., 2007).

Although there are also mutation-independent mechanisms to inactivate p53 function, p53 lies within a pathway and perturbations occurring either upstream or downstream can negatively impact p53-dependent tumor suppression (Cadwell and Zambetti, 1998). One such regulator of this pathway is ARF, which activates p53 by antagonizing its negative regulator Mdm2, by directly binding Mdm2, resulting in the relocalization of Mdm2 to the nucleolus (Tao and Levine, 1999). Generally, there

is an inverse correlation between wild-type p53 and ARF status; tumor cells that maintain wild-type p53 are frequently altered in ARF expression (deletion or methylation of the gene), whereas tumors that are defective in p53 (mutation, deletion or over-expression of Mdm2) typically express high levels of ARF (Quelle et al., 1995). However, as mentioned in the previous intro-section, the p53 pathway may be functionally inactivated also by viral or cellular oncogenes.

Thus, most if not all tumors are functionally defective in p53 tumor suppression either by mutation of the p53 gene or by corruption of the p53 pathway.

The range of p53 mutations is affected not only by selection processes, but also by intrinsic factors that differentially affect specific nucleotides and regions of the gene. In fact among single-base substitutions, about 25% are C:G=>T:A substitutions at CpG sites. CpG dinucleotides mutate at a rate 10 times higher than other nucleotides, generating transitions (Jones et al., 1992). About 3%–5% of cytosines in the human genome are methylated at position 50 by a post replicative mechanism that is restricted to CpG dinucleotides and is catalyzed by DNA methyl transferases. The 5-methylcytosine (5mC) is less stable than cytosine and undergoes spontaneous deamination into thymine at a rate five times higher than the unmethylated base. Among the 22 CpG of the DNA-binding domain (DBD), three hotspot codons (175, 248, and 273) represent 60% of CpG mutations and another five residues (196, 213, 245, 282, and 306) account for 26% of these mutations. The lack of mutations at other CpG sites reflects the fact that substitution at these residues does not generate a dysfunctional protein.

80% of the TP53 missense mutations are located within the sequence encoding the DNA-binding domain (DBD) of the protein (Fig.6). The N-terminus, which contains the transactivation domain, and the C-terminus, which contains regulatory sites, are rarely targeted by mutations (less than 2% of all mutations) with the majority of these mutations being nonsense or frameshift. In the DBD, missense mutations have been reported at almost all residues, but some residues are more frequently mutated than others , with 30% of the mutations falling at five hotspot codons (175, 245, 248, 273, 282) (Hollstein et al., 1991; Petitjean et al., 2007) (Fig.6). Four of these codons correspond to arginine residues (175, 248, 273, 282) involved in protein–DNA interactions, either by direct contact with DNA (residues 248 and 273) or by stabilization of the DNA-binding surface (residues 175, 282) (Cho et al., 1994).

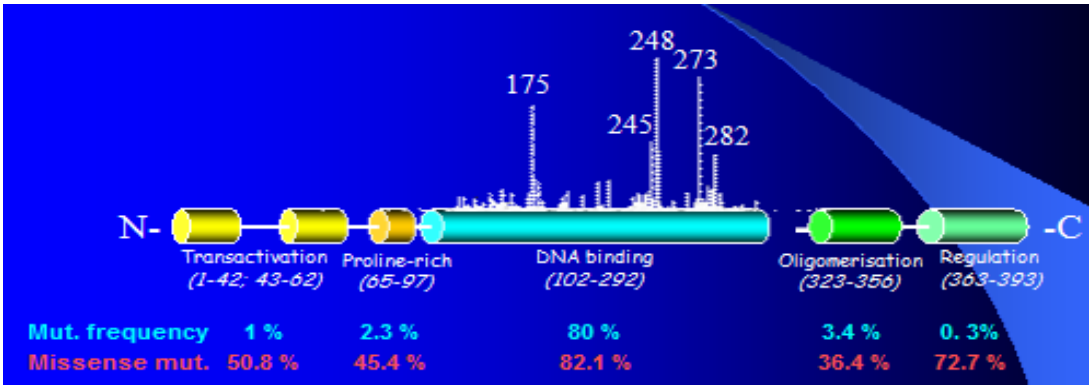



**Fig 6**. Distribution of p53 mutations on the gene. 80% of missense mutations cluster in the DNA-binding domain (DBD). The five most common hotspot mutations are highlighted. The N-terminus

and the C-terminus of the protein are rarely targeted by mutations. Data from the IARC TP53 database (R14, November 2009) (Petitjean et al., 2007).

.

### 1.5.2 TP53 germline mutations: Li-Fraumeni Syndrome

Whereas somatic TP53 mutations contribute to sporadic cancer, germline TP53 mutations cause a rare type of cancer predisposition known as Li–Fraumeni Syndrome (LFS), an inherited susceptibility disorder in which affected individuals are at increased risk of developing a variety of cancers, including soft tissue sarcoma and cancers of the bone, breast, brain and genito-urinary tract (Malkin et al., 1990).
The distribution of germline mutations is similar to somatic mutations, with mostly missense mutations (77%) located at the same hotspots. The proportion of CpG mutations (54% vs. 25% in somatic mutations) may reflect the spontaneous nature of germline mutations. Genotype–phenotype correlations suggest that the most significant defect is loss of function because large deletions including the whole TP53 gene have been found in LFS families with aggressive behavior (Bougeard et al., 2003). Tp53 germline mutations have been initially considered as a rare syndrome (Eeles, 1995), detected in about 500 families or individuals with complete or partial LFS features (Olivier et al., 2003). However, screening for TP53 germline mutation in patients with early onset breast cancer, unselected or by following familial history, has shown TP53 mutations in 2%–3% of the cases (Lalloo et al., 2006). Whereas screening of 525 patients with any kind of cancer family history has identified 91 (17.3%) TP53 mutations (Gonzalez et al., 2009). Based on these results, TP53 mutation may contribute to up to 17% of all familial cancer cases. Breast cancer and soft tissue and bone sarcoma account for over 50% of tumors in TP53 mutation carriers, followed by adreno-cortical carcinomas and brain tumors. Other cancers include hematological malignancies, gastric, colorectal, and ovarian cancers, occurring at earlier ages than in the general population (Olivier et al., 2003).

## 1.6 Structural and functional impact of TP53 mutations

### 1.6.1 Impact on Protein Structure

Hotspot mutations, which cluster mostly in the DBD of p53 gene, occur in residues involved in either making contacts with DNA, or in supporting the structure of the DNA-binding surface. Indeed, they can be distinguished by taking into account their impact on either the protein structure and stabilization or the interaction with DNA:

- Null mutations that completely destroy the functionality of the protein, such as insertion/deletion, nonsense mutations, frameshift mutations.
- Missense mutations that affects residues of the DNA-binding surface and disrupt the protein-DNA contact points (such as those at the mutational hotspots 248R and 273R) (Cho et al., 1994), also known as “contact mutants”.
- Missense mutations that occur in areas important for the conformational stability of p53 protein (such as R175H), leading to conformational changes that expose the mutant-specific epitope of the PAb240 antibody and result in the loss the wild-type-specific epitope detected by PAb1620 (Cho et al., 1994) . These mutants are also known as “structural mutants”.

### 1.6.2 Impact on Transcriptional Activities

Functional assays in yeast and human cells have shown that cancer associated mut-p53 proteins typically retain an intact transactivation domain (TAD), which may still operate exactly as it does within the wt-p53 protein, but can be targeted also to different sites on chromatin. Some mutants, as for example, arginine to proline at codon 175 (R175P) and arginine to cysteine at codon 181 (R181C), retain the capacity to transactivate p21/WAF1 but are defective for BAX activation and fail to induce apoptosis (Ludwig et al., 1996). Recently Kato and coworkers have used a yeast-based functional assay to analyze the transactivation of 2314 different missense mutants on eight TP53-responsive promoters (TP53-REs). The majority of mutants affected in the DNA-binding domain have an impaired transcriptional activity, whereas most of the mutants affected in other regions retain, at least partially or even full, transcriptional activity on various TP53-REs. A small category of mutants even showed increased activity compared with wild type p53 (Kato et al., 2003). Moreover using a different type of yeast based assay, Inga and colleagues further showed that differential transactivation by wild-type and mutant proteins depends on both protein levels and target sequence (Inga et al., 2002).

### 1.6.3 Biological effects of TP53 mutations

The functional effects of TP53 mutations can be classified into three non-mutually exclusive groups (Weisz et al., 2007; Soussi, 2007):

- first, most mutations observed in human tumours abrogate or attenuate the binding of p53 to its consensus DNA sequence and, consequently, impede the transcriptional activation of p53 target genes (Kato et al., 2003). These mutations are known as Loss of function (LOF), they are frequent among missense mutants, but are particularly relevant to truncating, splicing and nonsense mutations, as well as to gene deletions (Fig.7).
- Second, most missense mutations, but usually not the other types of mutations, also produce a full-length mutant p53 capable of inhibiting, to varying degrees, the function of the wild-type protein encoded by the second allele. This dominant-negative effect (DNE) is achieved by oligomerization of the mutant and wild-type proteins, forming a heterotetramer defective in sequence-specific DNA binding (Milner and Medcalf, 1991; Sigal and Rotter, 2000) (Fig.7).
- Finally, several mutations were shown to confer mutant p53 with new functions that are independent of wild-type p53. These gain-of-function (GOF) properties can be experimentally demonstrated in the absence of a functional wild-type p53. Most gain-of-function properties are believed to stem from binding of mutant p53 to cellular proteins such as transcription factors and, consequently, alteration in their activity (Brosh and Rotter, 2009) (Fig.7).

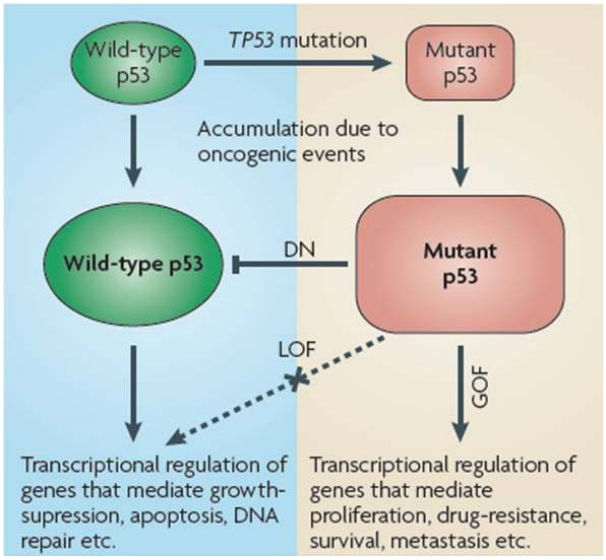



**Fig.7** Representative scheme of p53-mutations biological outcomes (from Brosh and Rotter, 2009). Hotspot mutations can be distinguished taking into account their impact on either the protein structure and stabilization or the interaction with DNA: 1) loss of function mutants that completely destroy the functionality of the protein; 2) dominant negative mutants which are able to heteroligomerize the wild-type counterpart leading to its inactivation; 3) gain-of-function mutants which possess new activity not-related to wt-p53 functions.

### 1.6.4 Dominant negative effect

The simple bi-allelic loss of p53 loci is relatively infrequent (about 2%); notwithstanding, however, the relative lack of bi-allelic p53 loci losses is not prima facie evidence for DNE or GOF roles for p53 mutation.
A high percentage of human cancers show mutations of the p53 gene accompanied by deletion of the other allele, resulting in the hemizygous state in which only mutant p53 is synthesized (Hollstein et a., 1991; Nigro et al., 1989). However, it's also true that relatively little data are available on p53 loss of heterozygosity in human cancers, but it is estimated that 40% of human cancers can still carry the wild type p53 allele (Dearth et al., 2007). These tumors may still ultimately lose the other allele, so functional proof of GOF or DNE is required.
One of the issues for such functional data is that evidence for DNE or GFO come from over-expression models; for example mice that are engineered to express a missense mutant p53 transgene, in addition to the endogenous wt-p53 allele, have an accelerated rate and higher incidence of developing tumors (Lavigueur et al., 1989; Harvey et al., 1995).

The generally accepted mechanism behind mutant p53's trans-dominant suppression is the shutdown of wild-type p53 function because of heteromerization with mutant p53. Wild-type p53 forms a tetramer to perform its tumor suppressor activity, and this oligomerization is mediated by the oligomerization region (aa residues 326-356), that is fully functional in core domain mutants (Chene, 1998). Moreover it appears that the mutant protein has the ability to drive wild-type p53 into a mutant, or perhaps inactive conformation (Milner and Medcalf, 1991).
Thus, when wild-type and mutant p53 were co-translated, wild-type p53 lost the epitope recognized by the PAb1620 antibody and became reactive with the mutant specific PAb240 (Milner and Medcalf, 1991). It is not surprising, therefore, that

contact mutants such as R273H mutant, which seems to retain almost all of the wild-type conformation (98% folding of wild-type p53), may have a weaker dominant negative activity (Chene, 1998). Heteromerization decreases the ability of wild-type p53 to bind to its various specific DNA target sequences (Unger et al., 1993) and to transactivate downstream genes. Interestingly, the half-life of wild-type p53 increases dramatically when it is bound to mutant p53 (Eliyahu et al., 1988), probably the result of a sharp decrease in Mdm2 induction.
Several mutants, including all the principle hot spot mutants, have been shown to interfere with wild-type p53 transactivation, at various degrees (Brachmann et al., 1996), but not without controversial results depending on mutation type, cellular type, genetic background and model systems used; therefore the picture is less than clear.

### 1.6.5 Gain of functions

Initial indications that mutants of p53 may possess a gain-of-function activity came from study in 1984 demonstrating that murine p53-deficient cells transformed with mut-p53 exhibited enhanced plating efficiency and an increased frequency of immortalization and malignant transformation (Wolf et al., 1984). However, the most important discovery came from studies demonstrating that mut-p53 isoforms, of both human and murine origin, but not wt-p53, can transform p53 null cells and endow them with an increased ability to form colonies in soft agar and tumors in mice (Dittmer et al., 1993). In these studies, the data were obtained by over-expressing mut-p53 in cells that already lack endogenous wt-p53, which does not necessarily prove the existence of GOF.

A wide range of oncogenic properties for mutant p53 forms would need to be considered in any analysis. By definition, these properties are not shared by wt-p53 and could be independent of their ability to exert a DNE toward wt-p53.
One feature of many GOF mutations is the ability to confer on cells a resistance to pro-apoptotic signals, such as the suppression of c-Myc induced apoptosis in leukemia cells (Frazier et al., 1998), or through the neo-potentiation of the transactivation of genes such as MDR1 (Chin et al., 1992), EGFR (Ludes-Meyers et al., 1996) and PCNA (Deb et al., 1992), which are not transactivated by the wild-type p53 protein; and do not necessarily contain p53 binding-sites in their regulatory regions (Cadwell and Zambetti, 2001).

Furthermore, just like wt-p53, mut-p53 has been proposed to not only up-regulate specific genes , but also down-regulate others, such as CD95/Fas (Gurova et al., 2003), p21, Gadd45 and PTEN (Vikhanskaya et al., 2007). If true, the subset of genes affected by mutp53 is like to vary greatly among different cell types and cell contexts.

Other gain-of-function activities could be ascribed to protein-protein interactions with mut-p53, such as with various isoforms of p53-related proteins p63 and p73 altering their transcriptional activity, resulting in negation of the p63/p73 functions (Gaiddon et al., 2001).

Again they could promote migration by elevating the steady-state levels of Slug protein, presumably by inhibiting p73-dependent Mdm2 gene expression, thereby blocking Mdm2-mediated Slug degradation (Wang et al., 2009).

Mut-p53 can disrupt ATM-mediated cellular responses to double-stranded DNA brakes through physical interaction of mut-p53 with MRE11 (a component of the MRN complex), preventing its binding to double-stranded DNA brakes and enabling persistence of unrepaired DNA damage (Song et al., 2007; Song and Xu, 2007).

Another important protein-protein interaction is between mut-p53 and topoisomerase-I, that may lead to an increase in aberrant homologous DNA recombination events and mutagenic DNA rearrangements (Restle et al., 2008).

But there are thought to be degrees of "aggressiveness" (Bulloch et al., 1997). Some allelic forms such as R273H are not us unfolded, are not so aggressive in transformation assays, and are sometimes considered to be only DNA-contact mutants. However R273H allele also shows allosteric or conformational defects in some assays (Hupp et al., 1993; Fields and Jang, 1990), complicating a full understanding of what determinants in p53 drive gain-of-function, pro-oncogenic signalling.

The impact of p53 mutations on tumorigenesis probably depends on many factors, including the stage in the process when p53 mutations and mut-p53 accumulation occur. In some types of cancer, p53 mutations are rather late events, correlating with progression to aggressive, advanced desease (Vogelstein and Kinzler, 1993). Moreover, GOF is mainly monitored by over-expressing a particular mutp53 isoform and measuring its impact on the properties of the over-expressing cells, be it in culture, or in mouse tumor model; and this forced over-expression represents an artificial situation that has to be viewed with caution.

### 1.6.6 TP53 mutations and drug resistance: p53 as biomarker in clinic

Finally, p53 mutants may have an important role in response to toxicity, for example in cellular resistance to chemotherapy and ionizing radiation by interfering with the induction of apoptosis. It is reported that over-expression of various tumor-associated mut-p53 isoforms can render the cells resistant to killing by a variety of anticancer agents, such as doxorubicin, cisplatin and etoposide (Li et al., 1998; Blandino et al., 1999; Matas et al, 2001). In contrast, studies using siRNA mediated knock-down of endogenous mut-p53 have shown this to sensitize cancer cells to killing by such anticancer agents and other proapoptotic stimuli (Vikhanskaya et al., 2007; Wong et al., 2007).

Mechanisms could be partially attributed to transcriptional activation of the multi-drug resistance 1 gene (MDR1) and survival factors, such as BAG-1 (Chin et al., 1992; Yang et al., 1999), which may be necessary to counteract the apoptotic response elicited by the induction of c-myc (Frazier et al., 1998).

Due to these experimental properties, the prognostic value of TP53 gene mutations has been investigated in several types of cancer. In breast (Berns et al., 1998; Olivier et al., 2006) and colon cancer (Borresen-Dale et al., 1998; Goh et al., 1995), e.g., there is a strong association between mutations in the DBD and shorter survival or poor response to treatment (Fig.8A). Although, loss of wild-type p53 activity is thought to be a much strong predictor of failure to respond to radiotherapy and chemotherapy in various human cancers.

Noteworthy are the many studies in which TP53 status has been accurately assessed by gene sequencing and immunoistochemical (IHC) staining, which has generated

associations between TP53 mutational status and clinical properties; with the general trend being that TP53 mutations are associated with poor overall and disease free survival rate, as well as with poor drug response. However, many studies still report lack of such associations, and few report opposite trends (Brosh and Rotter, 2009).

Inconsistent data regarding the association of TP53 mutations with survival and drug response have led to a debate over the prognostic and predictive values of TP53 status in cancer. A major reason for the inconsistency is that, until recently, most studies used IHC detection of p53 accumulation in tumor samples as a surrogate marker for TP53 mutations. This occurs because wt-p53 is generally observed to be short lived, whereas mut-p53 is often found to be rather stable. Initially, this was suspected to be a salient feature of mutp53, directly caused by the impact of the mutations on the biochemical properties of the mutant protein.

Subsequent work revealed that mut-p53 is not necessarily intrinsically stable; rather, changes that occur within tumor cells could result in its stabilization. Thus, in primary cells derived from human Li-Fraumeni syndrome patients the levels of mut-p53 are rather low and comparable to those of wt-p53 (Yin et al., 1992). Similarly, mut-p53 protein levels are low in mut-p53 knock in mice, but increase in a fraction of tumors that emerge in such mice (Lang et al., 2004). So this accumulation of mutant p53 protein is not only associated with specific properties of the protein itself but instead depends also on the endogenous genetic background of the tumors.

Additionally, not all tumours with missense TP53 mutations are IHC positive (Langerod et al., 2007; Alsner et al., 2008). Moreover, few tumors accumulate a functional wt-p53 due to persistent stress signals, and some tumors inactivate wt-p53 function by mutation independent mechanisms, such as MDM2 amplification or deregulation of upstream or downstream components of the p53 pathway (Vogelstein et al., 2000; Soussi and Beroud, 2001) (Fig.8B).

The assignment of TP53 status to a tumor sample is therefore inaccurate when IHC is solely used, as many tumours with TP53 mutations do not accumulate mutant p53; this is especially the case for frameshift, nonsense and splicing mutations (Soussi and Beroud, 2001) (Fig.8B). Prognostic and predictive significance of TP53 mutations is therefore extremely variable according to tumor type and/or treatment (Bertheau et al., 2008), and there is no simple, universal clinical message that can be delivered by TP53 mutation analysis. Cellular models that do not rely on viral-promoter driven p53 expression would allow to clarify this important practical aspect of treating p53 mutated tumors.

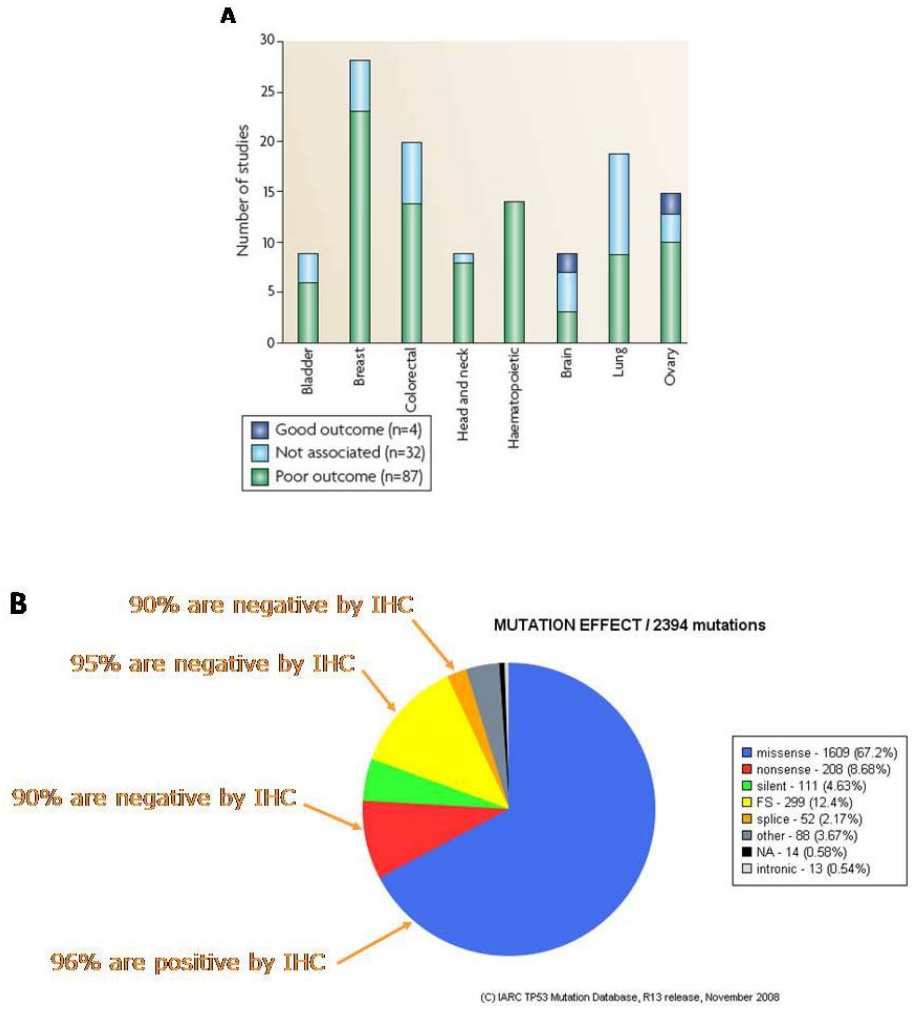



**Fig.8** Clinical relevance of TP53 status. (A) Association of TP53 mutations and clinical outcome (overall survival, disease-free survival or drug response). Only studies that analyzed TP53 mutations by gene sequencing or related methods and with cohorts > 50 patients were considered (From Brosh and Rotter, 2009). (B) Studies using IHC to investigate p53 prognostic value have yielded inconsistant results; more than 23% of TP53 mutations do not accumulate the p53 protein therefore may stain negative to IHC. IHC alone is not suitable for assessing p53 status. Data from the IARC TP53 database (R14, November 2009) (Petitjean et al., 2007).

## 2. AIM THE STUDY

TP53 mutations are the most common genetic alterations in human cancers, with the majority of p53 mutations being clustered within the DBD.
Several lines of investigation suggest that DNA binding domain mutants can act as DNE or GOF proteins. However, previous functional studies rely on over-expression of mutated proteins in a p53 null background, which may not be representative of their true function.
One of the most important questions to answer is whether specific cancer therapies are influenced by p53 mutations. In this regard previous data are fewer, as well as being controversial due to the models systems used.
The aim of this thesis is to assess the role of one of the major hot spot p53 mutations, R273H, in a physiologically relevant model system via endogenous gene knock-in of the mutation; and then ascertan how the isogenic wt vs mutant models systems respond to cancer drugs. Importantly, by modifying endogenous gene, rather than introducing exogenous over-expressed p53 vectors, the mutant allele is studied at the correct expression levels that occur naturally within cancer patients.
We created an isogenic colorectal cellular model in SW48 cell lines, which still retain the wt-p53, to analyze the effect of wild-type/mutant p53 heterozygosity on malignant phenotype, and comparing them with: 1) parental cells expressing wt-p53, and 2) cells lacking one or both p53 alleles.
These cell-lines were investigated for p53 mediated cellular responses (cellular proliferation, cell cycle progression) to further explore putative dominant negative activity; and in particular for their responses to cytotoxic anticancer agents, as well as newer ‘targeted’ agents. Moreover, the effect of the hotspot mutant on the transcriptional profile of the cells was investigated in order to further assess DNE or GOF activity.

# 3. MATERIALS AND METHODS

**Cell cultures**.
SW48 p53 null, SW48 p53 +/- and DLD-derivative cells were kindly provided by laboratory of Prof. B.Vogelstein.
All SW48 and DLD colon rectal cancer cell lines were coultered in DMEM medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 50units/ml penicillin and 50mg/ml streptomycin, in a humified atmosphere of 95% air and 5% $CO_2$ at 37° C.

**pAAV Viral vector.**
The Knock-in (KI) construct has been synthesized from Geneart AG (Regensburg, Germany) as in Fig.9 and subcloned in the pAAV-MCS (Stratagene, La Jolla, CA) using the NotI restriction sites, to obtain the pAAV-p53-R273H viral vector.

**Packaging of the rAVV-p53-R273H vector into viral particles.**
Recombinant adeno-associated viruse (rAAV) has been produced using the AVV Helper- Free System (Stratagene, La Jolla, CA) following manufacturer instructions.
In detail $2x10^6$ of packaging cell line HEK293 were seeded in 10-cm-diameter dishes 24h prior to transfection in DMEM media with 10% fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 mg/ml) in a 5% $CO_2$ incubator; the culture medium was changed 2h prior to transfection.
The pAAV-p53-R273H viral vector was mixed with pAAV-RC and pHelper plasmids from the AAV Helper-Free System (Stratagene, La Jolla, CA) in 2.5 M $CaCl_2$, then adding dropwise Hepes-buffered saline while vortexing and immediately adding the precipitate to the cultures.
The medium was replaced after 14-16 h, and the virus was harvested according to the AAV Helper-Free System instructions. To harvest the virus, the media were aspirated from the flask, together with 293 cells, and subjected to three freeze and thaw cycles. The lysate was clarified by centrifugation at 13000 rpm to remove cell debris, and the supernatant containing rAAV particles was divided into aliquots and frozen at -80°C for subsequent use.

**Infection and clonal selection.**
The SW48 parental cell line were seeded in $10cm^2$ dish 24 h prior to infection in complete medium, so that they were at 70% of confluency at the moment of infection. The day after 200µl of the pAAV-p53-R273H viral lysate were added to the cells in 5ml of fresh medium. After 4h further 5ml of medium were added.
Cells were maintained in 10ml of rAVV containing medium for 12-16h and, after that rAAV containing medium was replaced with fresh medium.
Two days after, the cells were detached with ATV (Trypsin 2.5%, EDTA 1%) and seeded in ten 96well plates in media containing G418 at final concentration of 0.8mg/ml. The G418 resistant colonies were grown in selection for 3-4 weeks, changing the G418 containing medium every 10-12days; when the resistant colonies arrived to 50-60% of confluence, they were detached in 30µl of ATV/well and then the DNA was extracted from each well using the Lyse-N-Go™ PCR Reagent (Pierce). 3µl of detached cells were added to 5µl of Lyse-N-Go™ PCR Reagent and the following thermal cycle program was performed:

| Step | Temperature | Time |
|---|---|---|
| 1 | 65°C | 30 seconds |
| 2 | 8°C | 30 seconds |
| 3 | 65°C | 90 seconds |
| 4 | 97°C | 180 seconds |
| 5 | 8°C | 60 seconds |
| 6 | 65°C | 180 seconds |
| 7 | 97°C | 60 seconds |
| 8 | 65°C | 60 seconds |
| 9 | 80°C | 300 seconds |

After thermal cycle program 30µl/well of water were added.
PCR amplification was set up on lysates in order to assess locus-specific integration using a primer that annealed outside the homology arm and another one that annealed within Neo cassette (Neo screening).
In parallel, in order to perform a quality control on gDNA extract and to further assess locus-specific integration on selected clones, another PCR amplification was set up using a primer that annealed outside the homology region and another one that annealed outside Neo, but on the 3' homology arm of the contruct.

Primers used for both the screenings are listed in the table below:

| | Neo Screening | gDNA screening |
|---|---|---|
| Forward primer | 5'-GAGGGGTTAAGGGTGGTTGT-3' (anneals out of the construct on p53 gDNA) | 5'- caagggtggttgggagtaga-3' ( Anneals on p53 gDNA on right arm of the construct) |
| Reverse primer | 5'-GGCGGAGTTGTTACGACATT-3' ( anneals on Neo cassette) | 5'- AGGCAGATCACAAGGTCAGG-3' (anneals out of the construct on p53 gDNA) |

<u>The following PCR-reagents mix was prepared for each reaction:</u>

10X PCR buffer-MgCl2 (Invitrogen) 1µl

| | |
|---|---|
| MgCl2 50mM | 0,3µl |
| DMSO | 0,6µl |
| dNTPs 2,5mM (each) | 1µl |
| Forward primer | 1µl |
| Reverse primer | 1µl |
| Water | 3µl |
| Platinum Taq DNA Polymerase 5u/µl (Invitrogen) | 0,1µl |
| Sample (lysate) | 2µl |

The following touch-down PCR program was carried out:

| | **Temperature** | **Time** |
|---|---|---|
| 1° step | 94°C | 120 seconds |
| 3 cycles | 94°C | 15 seconds |
| | 64°C | 30 seconds |
| | 70°C | 90 seconds |
| 3 cycles | 94°C | 15 seconds |
| | 61°C | 30 seconds |
| | 70°C | 90 seconds |
| 3 cycles | 94°C | 15 seconds |
| | 58°C | 30 seconds |
| | 70°C | 90 seconds |
| 25 cycles | 94°C | 15 seconds |
| | 57°C | 30 seconds |
| | 70°C | 90 seconds |
| | 70°C | 5 minutes |
| End | 8°C | For ever |

Positive clones were firstly confirmed by two independent rounds of amplifications, then by sequencing the region of locus-specific integration to assess the presence of the allele-specific mutation on genomic DNA.
The positive clones were then plated at limiting dilution in 96-well plates in G418 containing medium, in order to obtain clones derived from a single cell. A PCR screening with the same primers used above was performed on the single colonies. One PCR-screening-positive colony for each original clone diluted was expanded and used for the subsequent experiments.

**RNA extraction and cDNA synthesis.**
To confirm the expression of the mutation at the transcriptional level, total RNA was isolated using Trizol reagent (Invitrogen).

A two-step protocol was employed. Firstly, total RNA was reverse-transcribed using the Promega reverse transcription system (Promega, Southampton, UK) including 1 μg RNA, 0.5 μg Random Primers, 20 units of Recombinant RNasin® Ribonuclease Inhibitor and 15 units of Reverse Transcriptase AMV (Promega) to final 20 μl of reaction.
2 μL of the corresponding cDNA were then directly amplified using Taq DNA Polymerase-mediated PCR reactions (PCR amplification program as above). A forward primer and a reverse primer annealing on the exons around the mutation were used to produce the amplicon containing the mutated expressed sequence:
Forward primer 5'- TGGCTCTGACTGTACCACCA-3'
Reverse Primer 5'- GGAGAGGAGCTGGTGTTGTT-3'
The amplicons were sequenced to verify the expression of the introduced mutation at the RNA level.

**Stable overexpression of p53 R273H mutant.**
pCMV-Neo-Bam-R273H and pCMV-neo-Bam expression vectors, containing the cytomegalovirus constitutive promoter and the neomycin (G418) resistance gene under the control of the Simian Virus 40 promoter, were purchased from Addgene (Cambridge, USA).
SW48 wt cells at 80% of confluency were transfected with pCMV-Neo-R273H or mock (pCMV-vector) vectors using Lipofectamine Plus reagent (Invitrogen, CA) according to the manifacturer's instruction. The selection of stably transfected clones were achieved growing cells in medium containing 0.8mg/ml of G418 for 3 weeks.
Pooled cultures of cells were collected and used for each construct to exclude any variations due to clonal variability.
Transfected cells were then checked for the p53 expression by western blotting.

**Proliferation assay.**
Parental and isogenic cells were seeded in triplicates in 96-well plates at equal density ($3x10^3$) on day 0 and cell number was measured every 24h for 7 days by sulphorhodamine B (SRB) assay.
Briefly, after removing the media, the plates were incubated with 100μl/well of 10% of Tricloric Acedic Acid (TCA) for 30min; after that TCA was removed, the plates were washed twice with water and incubated with 100μl/well of SRB for 15 min.
Then the plates were washed three times with 100μl/well of 1% Acetic Acid to remove the excess of SRB and left to dry overnight in the dark, at room temperature.
The day after the SRB bound the cellular proteins was dissolved with 100μl/well of 10mM Tris base for 10 min and the absorbance was detected with the BMG Labtech plate reader (570nm).

**Western blot analysis**.
Cells were seeded 24 h prior to treatment and were at 70% confluency at the time of treatment with Adriamycin (doxorubicin) at a concentration of 0.2 μg/ml.
Total protein lysates were obtained from cellular lysates using NP-40 buffer (1% NP-40, 10% glycerol, 20mM Tris) supplemented with Cocktail proteinase inhibitors (Complete™ from Roche), phenylmethylsulfonyl fluoride (PMSF, 1mM) and $Na_3VO_4$ (200nM).

The protein extracts were resolved by 10% or 4-12% SDS-PAGE. The primary antibodies used for immunoblotting were: monoclonal anti-p53 DO-1, which recognizes both wild-type and mutant p53 (Santacruz Biothecnology, CA); polyclonal anti-p21 (Santacruz Biothecnology, CA); monoclonal anti-Bax (Santacruz Biothecnology, CA); monoclonal anti-Actin (Sigma); polyclonal anti-Cleaved PARP (Cell Signalling); monoclonal anti-phospho-p44/42 MAPK (Cell Signalling); polyclonal anti-p44/42 MAPK (Cell Signalling); polyclonal anti-phospho EGFR (Y1173) (Cell Signalling); polyclonal anti-ERFR (Cell Signalling); polyclonal anti-phospho-Akt (Ser473) (Cell Signalling) and polyclonal anti-Akt (Cell Signalling).

**FACS analysis.**
Cells were seeded 24h prior to treatment and were at 70% confluency at the time of treatment with Adriamycin (doxorubicin) at a concentration of 0.2 ug/ml.
At indicated times cells were trypsinized and then washed twice in PBS by centrifugations at 800g for 5 min; after that the cells were fixed by resuspending the pellet in 1ml cold 70% ethanol and kept overnight at -20°C.
The day after the cells were centrifuged at 800g for 5min, the supernatant was then removed and the pellet of fixed cells was washed twice with PBS.
After these two washes, $5x10^5$ cells were stained with Propidium Iodide by suspending the pellet in 250µL of the following mix:

| | 1 x | Final |
|---|---|---|
| Propidium Iodide (1mg/ml) | 12.5ul | 50ug/ml |
| 10X Perm buffer (1% Triton$^{X100}$/PBS) | 25ul | 0.1% TritonX |
| RNase A solution (100mg/ml) | 1.25ul | 500ug/ml |
| PBS | 211.25ul | |

After 30 min of incubation in the dark at room temperature, the cell cycle was analysed by Flow Cytometry.

**Compounds**.
Geneticin (G418) was purchased from Gibco (Carlsbad, CA).
The anti-EGFR small molecules Erlotinib and Gefitinib and the Paclitaxel were purchased by LC Laboratories (Woburn, MA).
The AZD7762, the AZD2281 and the PD0325901 were purchased by Axon Medchem (Netherland).
The Cisplatin and SN38 were purchased by Tocris Bioscience (USA).
The Nutlin-3 was purchased from Santacruz Biothecnology (CA).
The 5FU was purchased from Melford Laboratories Ltd (UK).
The Everolimus was purchased from Sigma-Aldrich (St. Louis, MO).
The Cetuximab and the GDC0941 were kindly provided by TGen (Phoenix, AZ) and Genentech (South San Francisco, CA) respectively.
Each compound was diluted in DMSO at appropriate stock dilution and stored at -20°C.

**Drug proliferation assays.**
Cells were seeded in 150µL complete growth medium at 5000 cells/well in 96-well plastic culture plates in triplicates at day 0. The following day, cells were treated with a range of drug concentrations prepared by serial dilution. Plates were incubated at 37° C in 5% CO2 for 72 or 96 h, or 6 days (for anti-EGFR targeted drugs) after which, cell viability was assessed by SRB method, preoviously described in the section "Proliferation assay".

**Microarray analysis.**
Total RNA was extracted from SW48 panel of cells before and after treatment with 0.2µg/ml of doxorubicin over night and then used for microarray analysis on the Agilent platform by Beckman Coulter Genomics (Morrisville, NC).
Briefly labeled cRNA samples were used for hybridization to Agilent SuperPrint Human 8x60K microarrays and scanned as directed by the manufacturer. Data were extracted by using Rosetta Resolver using the Rosetta ratio error model and subsequently analyzed using Microsoft Excel. Genes were filtered based on their expression level: if a gene never reached a signal corresponding to the 60th percentile of any sample, it was removed from further analyses. Then a rank-invariant normalization was run, using SW48 parental-untreated as the normalizing reference. Data quality control indicated that genes with signal below 200 in two samples (p53 KO and WT Doxo) were not correctly measured, therefore these genes were also removed. Statistical selection of genes with differential expression between two subgroups of choice involved a combination of three tests: (i) average fold change > 1.5; (ii) T-test p-value < 0.05; Signal-to-noise ratio (SNR) >2, with SNR = (µgroup0 – µgroup1) / (σgroup0 + σgroup1) where, for each gene, µgroup0 represents the mean value and σgroup0 represents the standard deviation for that gene in all samples of group 0.

**Statics.**
$IC_{50}$ value for each drug was calculated using Prism 5.01 software. Where indicated the results are given as the mean ± standard deviation of three independent experiments.
The Student's t test was used to evaluate the statistical significance of the results. P-values <0.05 (*) and P values<0.01 (**) were considered significant; all reported P-values were two-sided.

# 4. RESULTS

## Synthesis of rAAV-p53-R273H vector and rAAV viral particles production.

p53 mutants have been mainly characterized in the past by over-expressing the different mutated cDNAs at non-physiological levels, under the control of non-endogenous viral promoters in p53-null cells. In contrast, the direct alteration of endogenous gene sequences is the most definitive method to evaluate the role of an allele within a specific cell type or organism.
Endogenous gene-targeting exploits rAAV-mediated homologous recombination (Russell et al., 2002; Kohli et al., 2004) to alter any gene-sequence with high-efficiency in the human cell genome; enabling the better study and characterization of gene function in a physiological, stable and disease-relevant context.
Using rAAV-mediated homologous recombination it is possible to introduce in a single allele a specific nucleotide substitution, or a small deletion, identical to that observed in human tumors, thus generating experimental models recapitulating the molecular alterations present in human tumors. The engineered cell models also generate a matched parental, or 'normal' isogenic cell line, which is genetically identical to the mutant cell-line, except for a normal copy of the gene in question.

In order to better understand the putative functions of mut-p53, we have used rAAV-mediated gene-targeting to create a cancer cell-line (SW48) harbouring a knock-in (KI) mutation of the p53 R237H allele, which is a hot spot mutation with putative dominant-negative or gain-of-function activity.
We focused on the SW48 human colon cancer cell line, as it is wild type for p53, thus mutant p53 alleles, when introduced in the right and equal context into cells, can be studied for its putative effects over wt-p53.

The rAAV-p53-R273H targeting construct comprises the following elements (see also Fig.9): 1) Two homology arms of ~1Kb each, which are perfectly homologous to the p53-gene sequence to be targeted, except for a single nucleotide substitution G818A (R273H) within exon 8 (Fig.9). 2) To enable packaging in AAV-virions, the homology arms are flanked by ITR sequences. 3) A selectable marker gene encoding for G418 (neomycin) resistance, driven by a constitutive promoter, which is placed within the two homology arms so that it is introduced into the intronic sequence of p53 gene, just before the exon to target. The whole Neo cassette was flanked by two LoxP sites, so that subsequent transfection of Cre-Recombinase in the targeted cells will allow the excision of the neomycin cassette from the cells' genome, if required. 4) Finally, two FRT sites are placed in the intronic sequences flanking the targeting exon 8, so that the expression of Flp-Recombinase would allow the excision of the mutated exon, leaving only the wt-p53 in the cells (Fig.9).

The final vector (subcloned into the pAAV-MCS vector harbouring the ITRs sequences) was then cotransfected into the HEK293 packaging cell line, as explained in the Materials and Methods, to get obtain infectious viral particles (rAAV-p53-R273H virus).

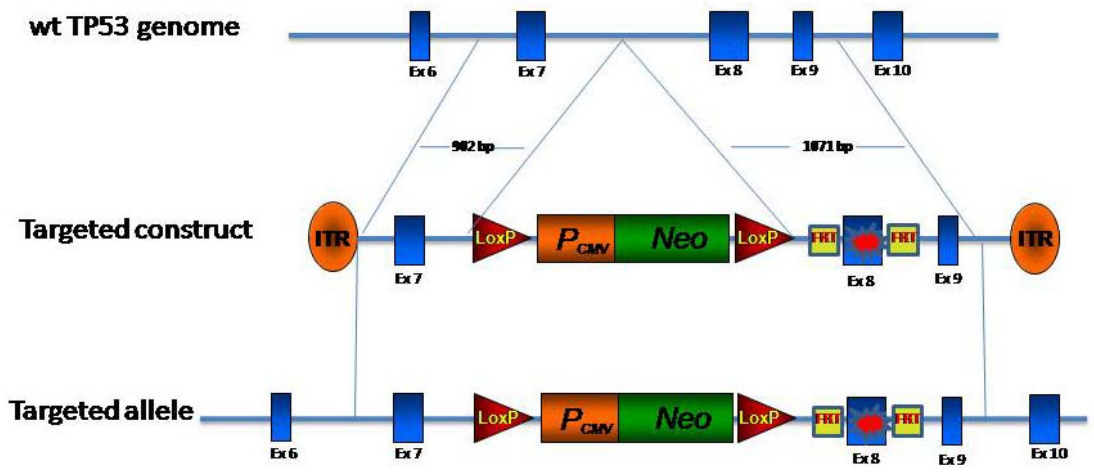



**Fig.9** rAAV-p53-R273H targeted construct. Adeno-associated virus (AAV) is a human, replication-deficient parvovirus of 4.7 Kb. The wild type genome possesses two open reading frames (ORFs), termed rep and cap, flanked by two inverted terminal repeats (ITRs). In the recombinant AAV (rAAV), both of the ORFs were deleted and replaced with exogenous sequences, while the ITRs, necessary for the packaging of the vector into the virion, were the only elements maintained from the wild type virus. The homologous recombination cassette cloned within the ITRs consisted of two 1kb sequences ("homology arms"), one of which containing the mutated exon to be introduced in the target locus. A selectable marker gene coding for G418 (Neomicin) transferase, driven by constitutive active CMV promoter (Pcmv), was introduced between the homology arms into the vector, flanked by two LoxP sites, so that the forced expression of Cre Recombinase in the targeted cells allowed the excision of Neo cassette from the cells' genome. Moreover two Flp-specific FRT sequences were added around the targeting exon 8, to allow the eventual excision of the mutated exon after the expression of the Flp-Recombinase in the cells.

**Generation of SW48 isogenic cellular models carrying the p53 R273H mutation.**

SW48 cells were infected with the rAAV-p53-R273H virus and plated at limiting dilution in multiple 96-well plates. Infected cells were selected for ≈ 3 weeks in G418 sulphate-containing medium. G418-resistant colonies were screened by performing a high-throughput PCR on their genomic DNA in order to assess whether clones contain p53-locus-specific integration events. To this end, a forward primer that annealed within the Neo cassette, and another one that annealed on a 3' sequence outside the homology region were used (Fig.10), a product of 1590bp was expected (Fig.10A). We found that 5% (63 clones) of G418-resistant clones were positive for the correct integration event.
Successful targeting of the mutation was then confirmed by sequencing the region of locus-specific integration using genomic DNA extracted from the clones. Only 1 well was found to be positive for the correct mutated sequence (0.09%).
This rate of targeting is low compared to other gene knock-ins produced in SW48, usually around 5-7%. It has already been reported that repeated elements might adversely affect recombination at the desired locus by favouring promiscuous recombination with repeats in other locations (Topaloglu et al., 2005) and nearly 40% of p53 region is made up of repeated elements. Specifically, the region containing the targeted exon 8 contains a high number of GC elements, which could

impair the accessibility of this region to homologous recombination and also reduce the specificity in PCR screening.

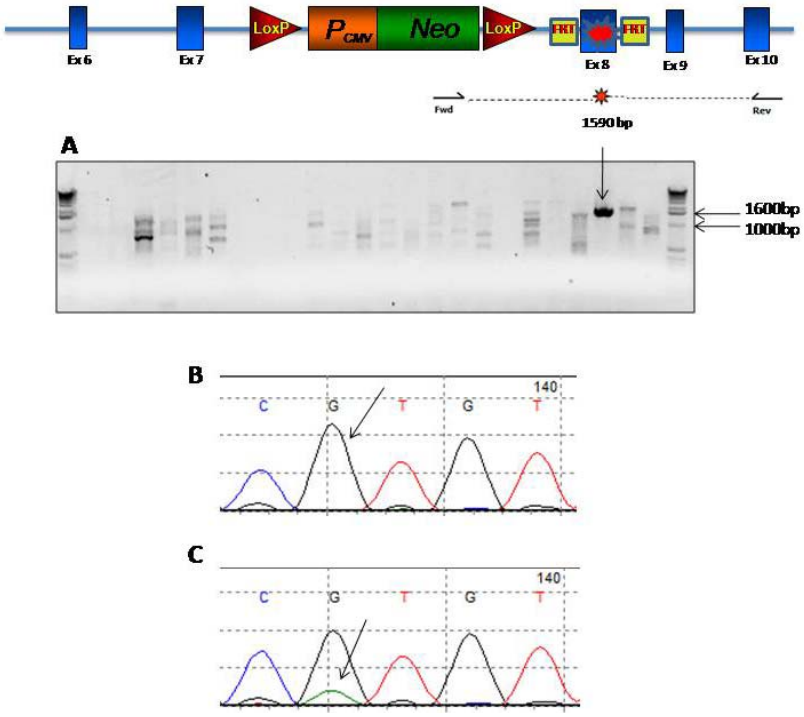



**Fig.10**. Generation of SW48 knock-in p53 R273H cell line. (A) SW48 parental cell line were infected with the rAAV-p53 R273H virus and then selected in G418 containing media for 3 weeks; after that they were screened by performing a high-throughput PCR on their genomic DNA in order to assess the p53-locus-specific integration events. To this end, a forward primer that annealed within Neo cassette, and another one that annealed on a 3' sequence outside the homology region were used; a product of 1590bp was expected. The cells were then checked by sequencing to assess the locus-specific homologous recombination. SW48 parental cells are wt for p53 (B). Only one of the G418-resistant clones screened showed the G818A (R273H) mutation in the exon 8 of TP53 gene, with unbalanced allelic ratio of wt and mutant p53 alleles (C).

The genomic sequence data using primers that allow amplification of both alleles demonstrated that the 'positive clone' was probably not pure, but a mixture of recombinant clones. In order to obtain a pure clonal cell line(s), the positive pool of cells was plated again at limiting dilution in 96-well plates and selected in G418-medium for other 3 weeks. Resistant colonies were screened as previous by PCR; 80% of the G418 resistant clones were positive to PCR locus specific screening. PCR positive clones were then confirmed by sequences for the presence of the G818A (R273H) mutation in exon 8 of TP53 gene and all of these pure clones showed 50% allelic balance mut/wt p53 (Fig.11A).

We then checked by sequence the presence of the FRT sites in the isogenic clones harbouring the R273H mutation. We found that not all the clones harboured both the FRT sites (Fig.11B), highlighting the presence of multiple clones with different homologous recombination events that yield recombinants at different points along the homology arms. We therefore chose two clones different in their FRT status,

which are importantly produced as the consequence of independent homologous recombination events.

To assess whether the mutated allele was expressed, the RNA was extracted from the clonal populations. The corresponding cDNA was reverse transcribed and the region corresponding to the targeted locus was amplified using primers annealing on the cDNA. The amplicon was then sequenced in order to verify the expression of the introduced mutation. All targeted clones expressed the G818A (R273H) substitution at RNA level at 50% allelic balance (Fig. 11C).
Two clones were so chosen, expanded and used as independent clones for the subsequent experiments.

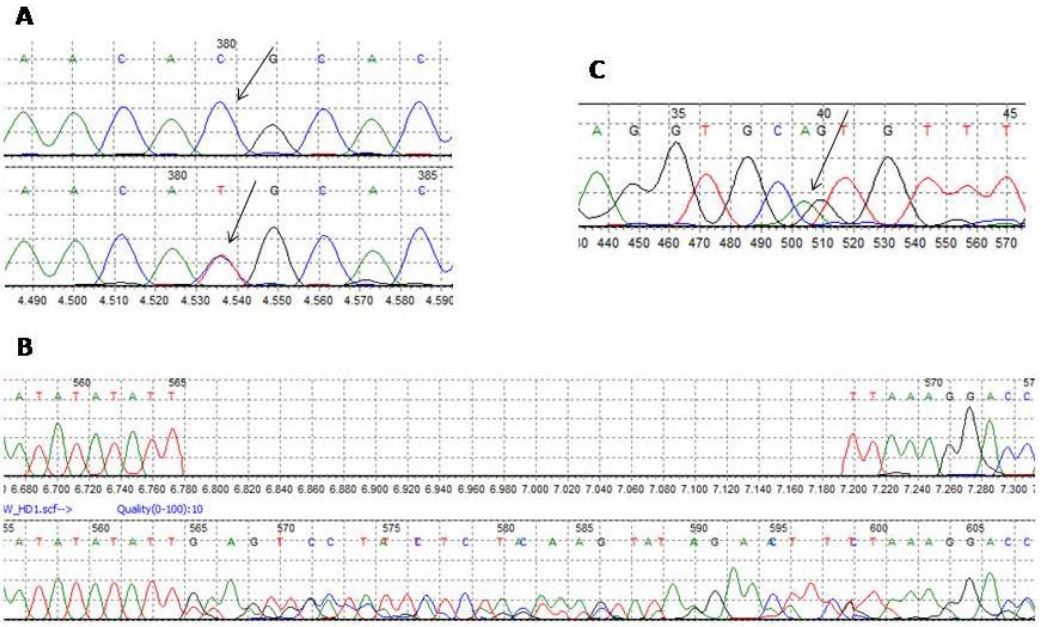



**Fig.11**. Selection of pure clones of SW48 knock-in p53 R273H cell line. (A) In order to obtain pure clones with 50% allelic balance of wt/p53 alleles, the positive cells were seeded at limiting dilution in 96well plates and selected in G418-containing media for 3 weeks. After this period, cells were screened by high-throughput PCR to check the locus-specific integration; all the G418 resistant clones showed 50% allelic balance of wt and mut-p53 alleles (sequenced by reverse primer). (B) The presence of the FRT sequences introduced in the right arm of the homologous construct was then assessed. Not all the clones harboured both the FRT sites, highlighting the occurrence of homologous recombination at different points along the homologous recombination arms. (C) We tested whether the mutation introduced was also expressed at the RNA level, by sequencing the relative cDNA sequence. All the clones showed the expression of the mutation by cDNA sequence with 50% allelic balance of the two p53 alleles (sequenced by forward primer).

## Generation of the control SW48 over-expressing p53 R273H hot spot mutant.

It is known that wt-p53 levels are usually kept very low in the absence of cellular stresses by interaction with its inhibitor MDM2; but following cellular stresses, such as DNA damage, the p53 protein rapidly accumulates and becomes activated (Kastan et al., 1991; Lu and Lane, 1993).

For mutant p53, there is evidence from in-vivo studies and from patients that mut-p53 tends to accumulate in cancer cells. Because of this, all prior studies of mutant p53 have been quite comfortable with the fact of performing over-expression of mutant cDNA, especially in a p53 null background in order to reproduce the LOH event that occurs in a high percentage of human tumours; even if it is estimated that 40% of human cancers still carry the wild type p53 allele (Dearth et al., 2007).
Therefore we also generated SW48 cell lines in which the R273H mutant is over-expressed, so as to directly compare the patient-relevant genotypes and cell-lines with previous published data on p53 characterization (albeit in wt-p53 cells in this case).
SW48 parental cells were transfected either with the vector containing the cDNA of mut-p53 R237H under the control of the constitutive active CMV promoter (pCMV-R273H), or with the pCMV-empty vector (mock). Stable transfected cells were selected for 3 weeks in G418 medium.
Stable clones were checked by western blot for p53 expression. The biochemical analysis showed that SW48 parental cells express a low level of wt-p53 as expected, and control transfection with empty vector did not alter this level (Fig.12). Over-expression of the p53 R273H hot spot mutant (SW48 pCMV R273H), instead, produced higher expression of the mutant protein in the cells, showing higher basal level of p53 comparing to parental and mock cells (Fig.12).
It is important to highlight that, even if basal levels of p53 are higher in over-expressing cells due to the constitutive production of the protein, we may have expected to find a stronger signal. This could be explained by the fact that even if the p53-mutants tend to accumulate to a higher level in the cancer cells, there is a maximal level of total p53 (wt + mutant) allowed in the cell, due to the control of the p53-inhibitor MDM2.

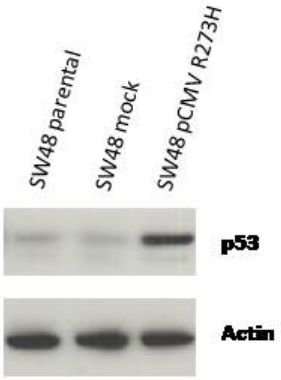



**Fig.12** Western blot analysis of SW48 pCMV R273H overexpressing cells. Total protein lysates from SW48 parental cells, SW48 mock cells and SW48 pCMV R273H over-expressing cells were extracted and resolved by 10% SDS-PAGE and then incubated with Anti-p53 antibody DO-1, which is able to recognize both wt- and mut-p53 proteins. Actin was used as internal control.

### SW48 isogenic p53-R273H mutant shows a small increase in p53 basal level.

Since mutant p53 is retained and accumulated, it has been hypothesized that it plays a role in tumorigenesis. In order to assess the basal level of p53 in the different

SW48 cell lines and how it changes in response to DNA damage, we treated the SW48 cells lines differing in their p53 status with doxorubicin (doxo), a chemotherapeutic drug known to activate p53 by intercalating DNA, causing double strand breaks at the DNA level (Waldman et al., 1995). After doxo-treatment total protein lysates were analysed by western blot.

SW48 parental cells express, as expected, a very low level of p53 under normal conditions; p53 levels are then increased with doxorubicin treatment, indicating that the endogenous wild-type p53 response is functional (Fig.13). SW48 parental cells have 2 wt p53 alleles, when one is disrupted by targeted homologous recombination, creating the heterozygote-null (Sur et al., 2009), there was less accumulation of p53 protein in the cells after doxo treatment. When both alleles were disrupted there was of course no p53 (Fig.13) (Sur et al., 2009).

p53-R273H knock-in clones, instead, showed an increase in the basal level of p53 compared to the parental cells, in absence of doxorubicin (Fig.13). This data is consistent with previous in-vivo data that p53 mutants are usually more stable than wt-p53, causing accumulation of the protein that could be detected in tissue by immunohistochemistry (IHC) (Deng et al., 1994).

Of note, the basal levels of p53 in the ectopic over-expression lines, was only marginally higher that when the mutation was introduced into the endogenous locus (Fig.13). This situation should be born in mind when comparing the phenotype of the lines in this thesis, with previous over-expression studies, which were performed in p53 null cell-lines; wherein the 'fold-increase' in expression would be apparently much larger in comparison to the parental line if the data are viewed in isolation.

After doxo-induction, both isogenic and over-expressing cell lines showed higher and similar levels of total p53 accumulation after 48h treatment compared to parental cells. Once again, the mutant p53 levels had a longer half-life compared to WT p53 after doxo-treatment.

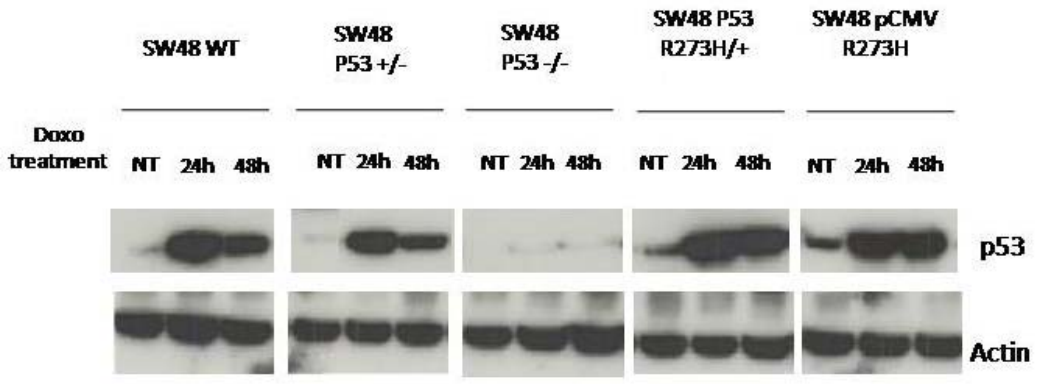



**Fig.13** Western blot analysis of p53 protein levels in SW48-p53 panel of cells. (A)To induce p53 activation SW48 parental, SW48 p53 +/-, SW48 p53 null (-/-), SW48 isogenic p53 R273H/+ and SW48 pCMV R273H over-expressing cells were treated with 0.2µg/ml of doxorubicin (doxo) for 24 and 48h. Total protein lysates were extracted at indicated time pointes from treated and not treated cells, resolved by 10% SDS-PAGE and then incubated with Anti-p53 antibody DO-1, which is able to recognize both wt- and mut-p53 proteins. Actin was used as internal control.

**p53 R273H mutant does not exert DNE with respect to transactivation of wt-p53 targeted genes p21 and Bax upon DNA damage in heterozygous knock-in SW48 isogenic cells.**

Previous cell line studies have shown that some p53 mutants cannot bind to p53 DNA-binding sites and transactivate target gene expression (El-Deiry et al., 1992; Kern et al., 1992). This could result in the inability to activate some p53-dependent promoters. For example, whereas wt-p53 trans-activates both Bax and p21, some p53 mutants can lose the ability to trans-activate Bax, but not p21 promoter, which appears to contain binding sites of higher affinity than the Bax promoter (Flaman et al., 1998). Therefore in order to assess the mut-p53 transcription functionality, we tested the ability of p53 mutant to increase the basal level of p21 and Bax, which are usually upregulated in response to expression of wt-p53, and are responsible for cell cycle arrest and apoptosis, respectively.

To this end, the SW48 parental, SW48 KI p53 R273H and SW48 pCMV R237H cells were analyzed by Western blot for Bax and p21 expression after DNA damage. SW48 parental cells showed expected low levels of p21 and Bax in absence of DNA damage, with strong up-regulation of p21 and moderate induction of Bax following doxo-treatment (Fig.14). The same expression profile was found in both the knock-in and over-expression p53 mutant cells lines. The presence of the p53 mutant R273H does not influence the low basal levels of both these effectors, even if when it is over-expressed, nor their induction upon DNA damage (Fig.14).

Following these results we checked the induction of apoptosis through cleaved PARP using western blot analysis. PARP is a 116 kDa nuclear poly-(ADP-ribose) polymerase that is highly conserved and involved in DNA repair and apoptosis. This protein can be cleaved by many caspases in vitro and is one of the main cleavage targets of caspase-3 and caspase-7 in vivo. The cleavage separates the N-terminal DNA binding domain (24 kDa) from the C-terminal catalytic domain (89 kDa). It has been shown that cleavage of PARP facilitates cellular disassembly and inhibition of PARP cleavage attenuates apoptosis in vitro (Boulares et al., 1999).
We found that presence of mutant R273H does not significantly influence PARP cleavage and therefore initiation of apoptosis process after cellular stress (Fig.14).

These results indicate that there is no clear evidence for dominant negative activity of the mutant p53 R273H over the remaining wt-p53 protein in its principle transactivation functions.

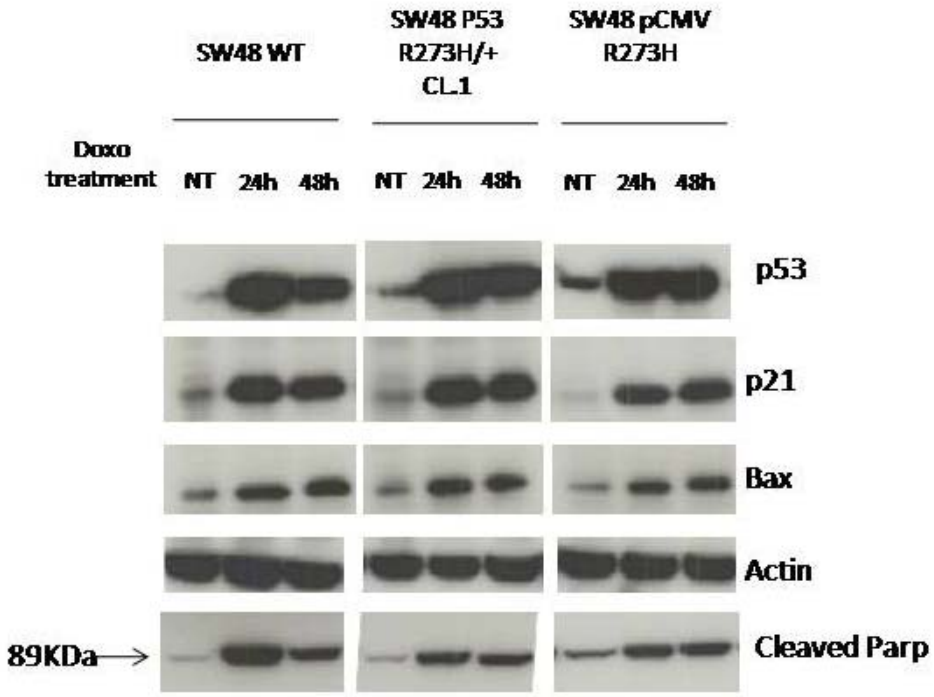



**Fig.14** Transactivation of wt-p53 target genes p21 and Bax. To induce p53 activation, and subsequent activation of its downstream effectors, SW48 parental cells, SW48 isogenic p53 R273H/+ and SW48 R273H pCMV overexpressing cells were treated with 0.2μg/ml of doxorubicin (doxo) for 24 and 48h. Total protein lysates were extracted at indicated time pointes from treated and not treated cells, resolved by 10% SDS-PAGE and then incubated with Anti-p53 antibody DO-1, which is able to recognize both wt- and mut-p53 proteins, Anti-p21, Anti-Bax, anti-Actin and anti-cleaved PARP antibodies.

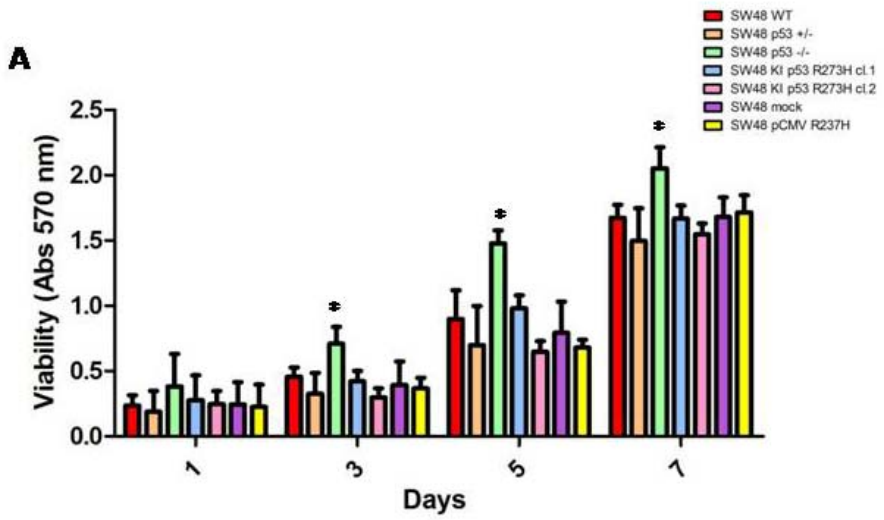
**p53 R273H mutant does not affect proliferation rate and cell cycle progression in heterozygous knock-in SW48 cells.**

The next experiment performed is the analysis of SW48 cell lines with different p53 genetic status, for the acquisition of hallmark malignant phenotypes.
Firstly, proliferation rates of the cells were broadly similar. The cells were seeded in 96 well plate at the same cellular densities at day zero, and the cell viability was assessed for 7 days. As shown in Fig15 panel A, the proliferation rate of the different SW48 p53-derivative cells did not differ significantly. The only exception is the SW48 p53 null cells, which show a significant increase in proliferation rate, especially after 3 and 5 days (P value<0.05). This result demonstrated that R273H mutant co-expressed with the wt-p53, either by stable KI, or by over-expression, is not able to enhance the proliferation of the cells by inhibiting the counterpart wild-type function; therefore differing from the complete p53 loss of function p53 achieved in p53 KO cells.

The next analysis was to compare cell-cycle kinetics across the p53 cell panel. One of the two principal wt-p53 functions is to mediate cell-cycle arrest, primarily in

the G1 phase, in response to DNA damage. However, a significant G2 arrest function has also been reported for p53 (Agarwal et al., 1995; Ryan et al., 1993). P53 null cells, instead, do not arrest in response to spindle inhibitors, but rather undergo multiple rounds of DNA synthesis without the appropriate chromosome segregation resulting in aneuploidy (Bunz et al., 1998).
So, in order to assess if the mutant R273H is able to alter the functionality of these cellular checkpoints, SW48 cell lines were treated with doxo, and then analyzed by FACs sorting for their cell-cycle profiles compared to untreated cells..
As previously known, the presence of 2 wt-p53 alleles in SW48 parental cells causes the arrest of the cell cycle in G1 and G2 phases, 45% and 55.2% respectively, with only 1,8% of cells in S phase. Heterozygous KO cells (SW48 p53 +/-) showed a similar behavior with most of the cells blocked in G1 and G2 phases, 46.8% and 45.6% respectively, and only 6.6% of cells cycling through S phase; synonymous of functional wt-p53 activity. Homozygous KO cells, however, overcome the G1 (2.9%) cell cycle arrest due to the lack of p21 transactivation and tend to accumulate in G2 phase (90.4%) (Waldman et al., 1997) (Fig.15B).
The transfection with the empty vector (SW48 mock) does not alter the p53-wt behavior, showing a similar cellular distribution in the cell cycle phases comparing to parental cells.

The p53 mutant lines (knock-in and over-expression) in contrast, may have a small but registerable movement away from a 'normal' doxo-treatment profile. Whereas wt p53 showed a G1>S>G2% distribution of 45>1.8>55.2, mutant p53 cells, either KI cells either over-expressing cells, showed a small increase in the percentage of cells blocked in G2 phase comparing to parental as well to mock cells: a G1>S>G2% distribution of 27.5>4.1>68.4 was found in KI p53 R273H cells and a G1>S>G2% distribution of 23.8>5.9>70.3 in SW48 pCMV R273H (Fig.15B).
This relative increase in cells blocked in G2 phase, from 57% in S+G2 for wt-p53 cells to an S+G2 of 74.3 ±1.8 % in mutant p53 cells, has already been reported for some p53 mutants, and it could suggest that p53 R273H mutant-related activity increases the S+G2 cell cycle progression (Rieber and Rieber, 2009; Willis et al., 2004).



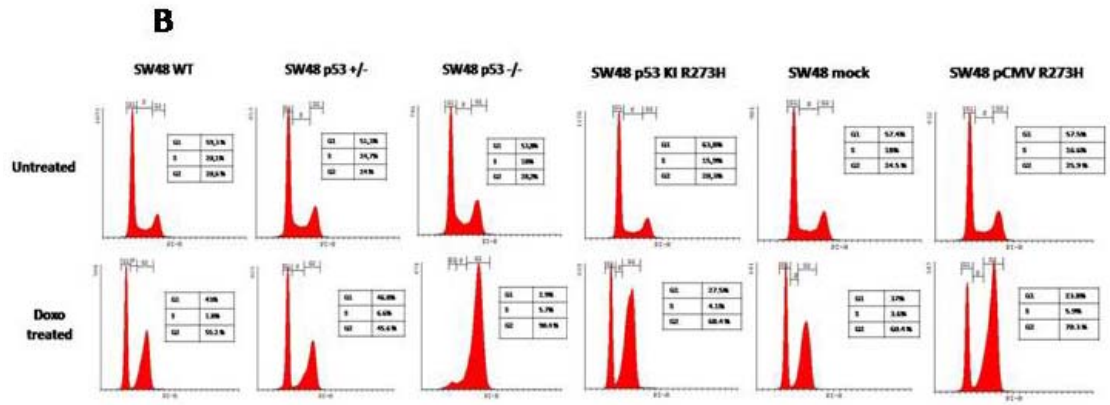



**Fig.15** Effect of R237H p53 mutant on proliferation rate and cell cycle progression. (A) Cells were seeded at day zero at the same cellular density ($3x10^3$ cells/well) in 96 well plate. Cell growth was assessed by SRB staining at days 1, 3, 5 and 7. Only SW48 p53 null (-/-) cells showed a significant increase in proliferation rate (P values <0.05). The bars represent the average and the standard deviation of three independent experiments, each performed in triplicate. (B) SW48 p53 derivative cells were seeded at day zero at the same cellular density and the day after treated with 0.2µg/ml of doxo for 24h to induce DNA damage. After that they have been collected and stained with propidium iodide and analyzed by flow cytometry. Representative data of three independent experiments are presented.

**p53 R273H mutant shows wild type behaviour in response to Nutlin-induced apoptosis.**

As another test for the functionality of p53 mutant, we evaluated the effects of Nutlin-3, a small molecule that binds to MDM2 and disrupts the interaction between MDM2 and p53 proteins (Vassilev, 2004). This drug retards the ability of MDM2 to ubiquitinate p53 and to mark it for degradation.
SW48 panel of cells were treated with increasing doses of Nutlin-3 and its effect on growth rate/survival were assessed. SW48 p53-null cells were less growth inhibited by Nutlin-3 than the other p53-containing cell lines, with an increase of ten-fold in the $IC_{50}$ value (12±0.06µM) compared to wt-p53 cells which show an $IC_{50}$ value of 1.1±0.06µM (P value <0.01) (Fig.16B). No statistical differences in response to Nutlin-3 were observed among all the other cells which harbor at least one wt-p53 allele. Both KI-p53-R237H cells and p53 R273H-overexpressing cells showed Nutlin-sensitivity comparable to parental cells (Fig.16A).
This is in accord with previous study, in which it has been reported that cells harbouring functional wt-p53 were more growth-inhibited by Nutlin-3 than clones without wt-p53 (Sur et al., 2009). We can conclude that cells harbouring the p53 contact mutant R273H in the heterozygous state, or by over-expression, show functional wt-p53 activity, confirming no evidence of DNE.

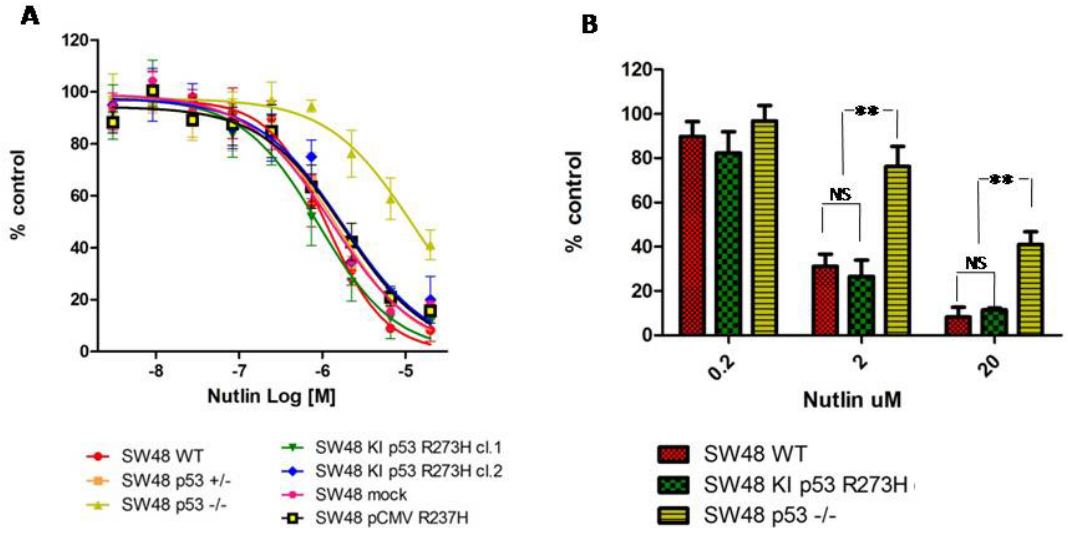



**Fig.16** Nutlin-induced apoptosis. (A) Cells were seeded at same density in 96 well plates at day 0 in complete medium. The day after they were treated with increased doses of Nutlin-3 for 72h. The cellular viability was assessed by SRB method. SW48 p53-null (-/-) cells show a ten-fold increase in $IC_{50}$ value comparing with other cells treated (P value<0.01). Graphs represent average±sd of three independent experiments. (B) Cells were seeded at same density in 96 well plates at day 0 in complete medium. The day after they were treated with 3 different doses of Nutlin-3 for 96h. The cellular viability was assessed by SRB method. SW48 p53 null (-/-) cells showed increased resistance to high doses of Nutlin-3 (P value<0.01). Bars represent avarage±sd of three independent experiments.

### Mutant p53 R273H induces partial protection against 5FU but not Paclitaxel and Cisplatin dependent toxicities.

An important and novel function attributed to mutant p53 is an increase in cellular resistance to chemotherapy and ionizing radiation, by interfering with the induction of apoptosis. It is reported that various tumor-associated mut-p53 isoforms can render the cells resistant to killing by a variety of anticancer agents, such as doxorubicin, cisplatin or 5-FU (Li et al., 1998; Blandino et al., 1999).
Therefore it was studied whether the contact mutant p53 R273H was able to influence the response to apoptosis induced by different chemo drugs (acting with different mechanisms of action) used in clinical treatment of solid tumors such as colorectal cancer.
Firstly the SW48-derived cells were tested for their ability to respond to a wide range of concentrations of the following anticancer drugs: paclitaxel, cisplatin and fluorouracil (5FU).

Paclitaxel acts by stabilizing microtubules and as a result, interferes with the normal breakdown of microtubules during cell division. Sensitivity to paclitaxel-induced toxicity was not affected either by the presence of the mutations, nor by the p53 in the heterozygous state or under complete deletion (Fig.17A). All lines had comparable $IC_{50}$ values (figure 17A).

Cisplatin belongs to a class of chemo-drugs that crosslink DNA, which ultimately triggers apoptosis. p53 genetic alteration caused by R237H mutation did not statistically influenced the response to cisplatin treatment; instead inactivation of p53 cellular functions through complete deletion of both p53 alleles in SW8 p53 null

cells, induced a significant resistance (P value< 0.01) to the cytotoxic effect caused by the drug, with an increase of 20% in the viable cells, especially at the highest drug's doses (Fig.17B,C).

Cells were next treated with 5FU, a pyrimidine analog that works through non-competitive inhibition of thymidylate synthase. Due to its noncompetitive nature and effects on thymidine synthesis, 5-FU is frequently referred to as a "suicide inactivator", it belongs to the family of drugs called antimetabolites.

Complete loss of p53 alleles was able to cause some resistance to drug toxicity with an increase in the $IC_{50}$ from 8±0.06μM of the parental cells to 18±0.18μM of p53-null cells (Fig. 17D, E).

Interestingly, also the SW48 p53 KI R273H cells showed a statistical significant (P value<0.01) increase in the $IC_{50}$ values, which are 12±0.08μM and 18±0.2μM for KI R237H cl.1 and cl.2 respectively, compared to parental cells (Fig. 17D) . P53-mutants-induced resistance to 5FU is marked at high doses of the drug (Fig. 17 E).

Instead when the R237H is over-expressed in SW48 p53-wt background, it did not show the same resistant phenotype, with $IC_{50}$ value comparable both to parental and mock cells (Fig.17 D, E).

Notably deletion of only one p53 allele in SW48 p53 heterozygous KO cells does not interfere with the sensitivity to all of the three drugs tested, meaning that functionality of at least one p53 allele is enough to respond to drug-induced apoptosis.

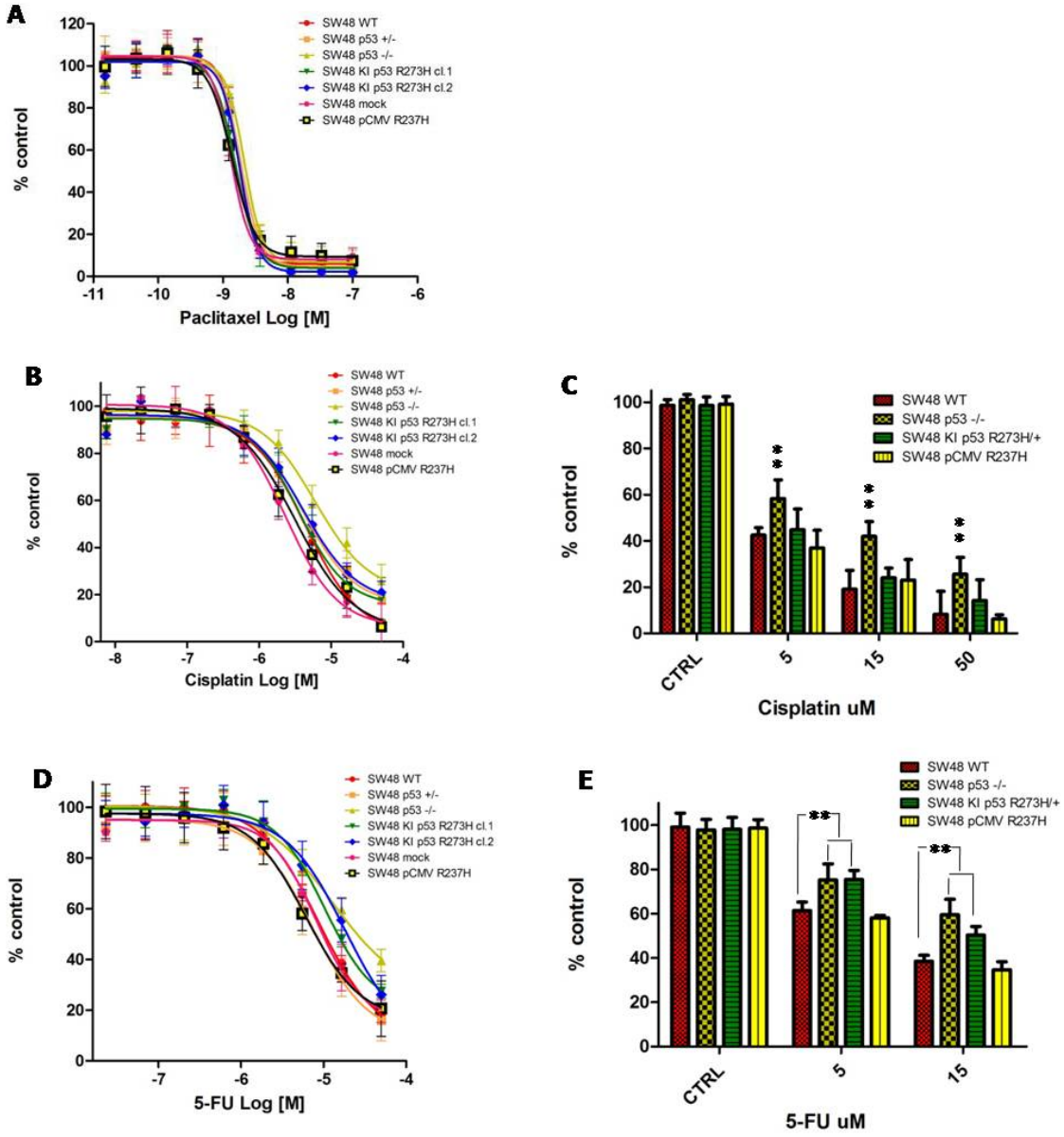



**Fig.17** Chemodrugs-induced apoptosis. Cells were seeded at same density in 96 well plate at day 0 in complete medium. The day after they were treated with increased doses of Paclitaxel (A), Cisplatin (B, C) and 5-FU (D, E) for 72h. The cellular viability was assessed by SRB method. No differences have been detected in Paclitaxel dose responses among the different cell lines (A). SW48 p53-null (-/) cells' increased resistance was instead detected in response to both Cisplatin and 5-FU treatments (P values<0.01) (B, D). p53 null cells showed highest resistance to highest doses of the drugs with more than 20% increase in the viable cells (P value<0.01) (C, E). SW48 KI p53 R273H/+ clones showed partial, but anyway significant (P value<0.01), increase in resistance to 5FU treatment (D), with at least 20% increase in viable cells at highest doses of 5FU (E). The two isogenic clones harbourirng R273H mutants showed similar behavior. Graphs represent average±sd of three independent experiments.

**PARP inhibitor is not able to enhance the toxicity of Cisplatin and 5FU in SW48 derivative cell lines.**

Poly (ADP-ribose) polymerase (PARP) is a nuclear protein involved in a number of cellular processes, but is principally involved in DNA repair and programmed cell death. This protein is important for repairing single-strand breaks and 'nicks' in the DNA. Inhibition of this process has been shown to lead to the accumulation of double-stranded breaks, due to the apparent collapse of stalled replication forks, leading to the death of the cells (Schreiber et al., 2006). PARP inhibitors have been intensively pre-clinically tested as potentiators of chemotherapy or radiotherapy (Plummer, 2006) and several have been recently entered early clinical trials (Ratnam and Low, 2007).

Specifically it has been preoviously reported that PARP inhibition could be clinically relevant in the treatment of the BRAC1/2 tumors; moreover it has been hypothesized that it sensitizes also p53-deficient cells to chemodrugs-induced apoptosis, such as doxorubicin (Muñoz-Gámez et al., 2005).

We therefore checked whether the PARP inhibitor AZD2281 (Olaparib) could enhance the activity of chemodrugs, such as Cisplatin and 5FU. To test this possibility, the SW48 panel of cells were treated with the AZD2281 alone (Fig.18A) or in combination with increasing doses of Cisplatin (Fig.18B) or 5FU (Fig.18C).

We found that all the cell lines treated showed similar pattern of response to treatment with PARP inhibitor alone (Fig.18A).

Moreover PARP inhibitor was not able to enhance the toxicity of both Cisplatin (Fig.18B) and 5FU (Fig. 18C) chemodrugs in the p53-panel of cells.

Of note, in both combination treatments, SW48 p53 null cells showed an increased in resistance to Cisplatin (Fig. 18B) and 5FU (Fig.18C) alone, as already showed previously; moreover SW48 p53 null cells showed higher, but not significant, sensitivity to combination of both chemodrugs with 3μM AZD2281 (Fig.18 B and C).

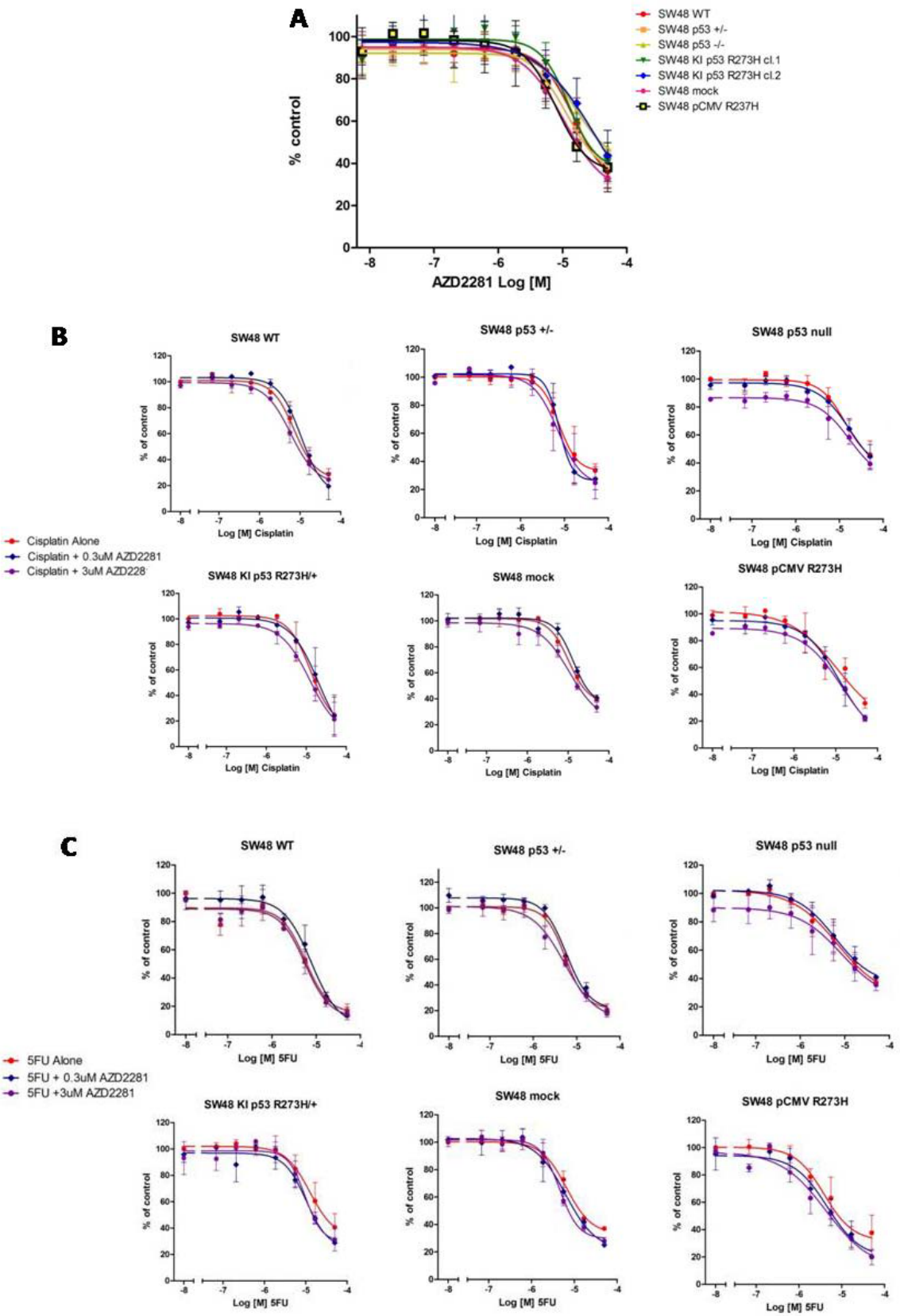

A
SW48 WT
SW48 p53 +/-
SW48 p53 -/-
SW48 KI p53 R273H cl.1
SW48 KI p53 R273H cl.2
SW48 mock
SW48 pCMV R237H
% control
AZD2281 Log [M]
B
SW48 WT
SW48 p53 +/-
SW48 p53 null
SW48 KI p53 R273H/+
SW48 mock
SW48 pCMV R273H
% of control
Log [M] Cisplatin
Cisplatin Alone
Cisplatin + 0.3uM AZD2281
Cisplatin + 3uM AZD228
C
SW48 WT
SW48 p53 +/-
SW48 p53 null
SW48 KI p53 R273H/+
SW48 mock
SW48 pCMV R273H
% of control
Log [M] 5FU
5FU Alone
5FU + 0.3uM AZD2281
5FU +3uM AZD2281

**Fig.18** PARP inhibitor (AZD2281) combination treatments on SW48 cell lines. Cells were seeded at same density in 96 well plates at day 0 in complete medium. The day after they were treated with increased doses of AZD2281 alone (A), or with increased doses of Cisplatin (B) or 5FU (C) in combination with 0.3μM and 3μM of AZD2281 for 96h. The cellular viability was assessed by SRB method. No differences in SW48 panel of cells were detected in response to AZD2281 alone (A). Moreover PARP inhibitor was not able to enhance the toxicity of both Cisplatin (B) and 5FU (C) chemodrugs in SW48 p53-panel of cells.

### Chk-1 inhibitors enahance the activity of SN38 in p53-null and p53-S241F/sil mutant cells, but not in p53-R273H/wt mutant cells.

SN-38 is the active metabolite of the camptothecin analogue, irinotecan (CPT-11), which is the component of the first line chemotherapy against colorectal cancer. SN-38 inhibits topoisomerase-I, a nuclear enzyme involved in transcription, recombination and DNA damage, therefore causing DNA double strand breaks and inhibiting DNA replication, transcription, recombination and repair.
It has been reported that topoisomerase-I interacts directly with wt-p53, this interaction is therefore tightly regulated and takes place only in brief periods of genotoxic stress. In contrast mut-p53 seem to constitutively interact with topoisomerase-I and this could have implications both for cellular stress response and genomic stability (Gobert et al., 1999).
We therefore investigated the effects of SN-38 on SW48 p53 derivative cells to find a potential role of mut-p53 in response to SN38 cytoxicity.
After treatment with increased doses of SN38 alone, no differences were detected in p53-mut cells lines versus parental or p53 deficient cells, with no statistical significant differences in $IC_{50}$ values (fig.19A).

Since SN38 acts mainly blocking the cells in G2 phase, it has been previously showed that ATP-competitive and selective check point kinases (Chk1) inhibitors, such AZD7762, can enhance the toxic activity of chemodrugs such as SN38, preferentially within p53-deficient cells (Zabludoff et al., 2008).
The rationale is that cells with altered p53-functions are G1 check-point deficient, thus they solely rely on Chk1 to maintain S or G2 arrest in response to DNA damage, whereas normal cells have an additional G1 arrest mechanism conferred by functional wtp53. Chk1 inhibition would abrogate S/G2 checkpoint in wt non-functional-p53 cells and drive them to apoptosis.

SW48 p53-derivatve cells were treated with AZD7762 alone, or in combination with increasing doses of SN38 (Fig 19B and 19C respectively).
Treatment with AZD7762 alone did result only in low, but not statistical significant increase in p53-null sensitivity to checkpoint inhibition (fig 19B); instead we found that AZD7762 was able to enhance the SN38 cytotoxic activity in SW48 p53 null cells, with a reduction of the $IC_{50}$ value from 1.3±0.07nM of SN38 alone to 0.8±0.06nM with 25mM AZD7762 and to 0.4±0.08nM with 50nM of this Chk1 inhibitor (P values $< 0.05$).
No statistical differences were detected in the $IC_{50}$ value of the other cell lines (Fig.19C).
Results in p53 null cells do agree with previous studies and confirm that SW48 cells harbouring the p53 contact mutant in heterozygous state still have functional wt-p53 regulation of checkpoints, as already seen in cell cycle analysis.

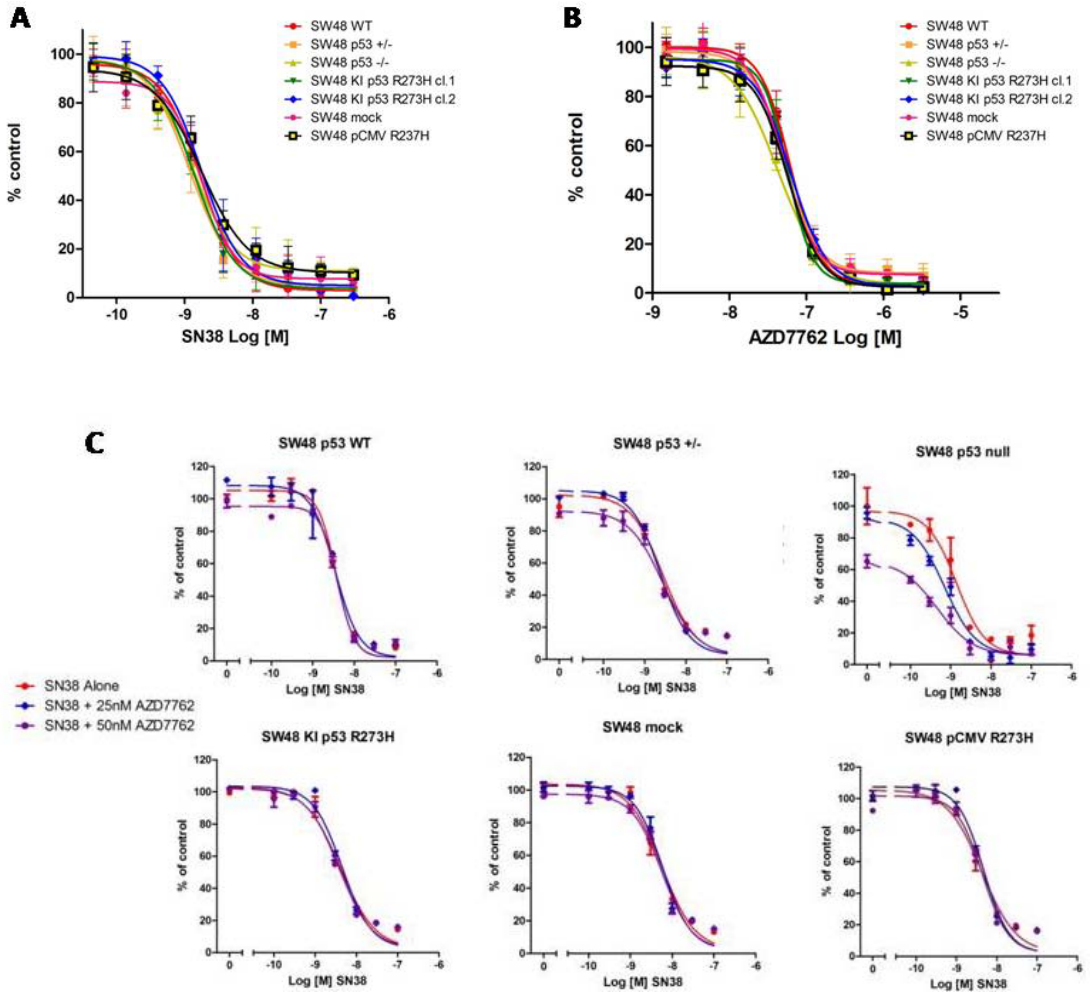



**Fig.19** SN38-Chk1 inhibitor (AZD7762) combination treatment on SW48 cell lines. Cells were seeded at same density in 96 well plates at day 0 in complete medium. The day after they were treated with increased doses of SN38 alone (A), Chk-1 inhibitor AZD7762 alone (B) or with combination of increased doses of SN38 with 25nM or 50nM of AZD772 (C) for 72h. The cellular viability was assessed by SRB method. No differences were detected in single treatments. Chk1 inhibitor is able to enhance SN-38 apoptotic treatment only in SW48 p53 null cells with a reduction of $IC_{50}$ value from 1.3nM (SN38 alone) to 0.8 and 0.4nM for 25mM and 50mM AZD7762 respectively (P values <0.05). Graphs represent average±sd of three independent experiments.

In further confirmation of this result, another panel of isogenic colorectal cancer cell lines in the DLD background was studied. DLD1 colorectal cancer cells harbor a mutated p53 allele (S241F), while the other allele is silenced through methylation of the promoter (genotype S241F/sil) (Sur et al., 2009). Starting from parental cells, Sur and colleagues, using rAAV technology, created two derivative cell lines; one in which the mutated allele has been removed, resulting therefore in the phenotype DLD -/sil, and another one in which the mutated allele has been substituted with the wild type resulting in the DLD wt/sil cell lines.
The DLD derivative cells were treated with the Chk-1 inhibitor AZD7762 in combination with increasing doses of SN38.

We found that AZD7762 is able to enhance the SN38 cytotoxic activity in the DLD1 -/sil genotype (Fig.20B), which are null for p53 functions, with a reduction in the $IC_{50}$ values from 10nM±0.09 in response to SN38 alone to 4±0.07 and 2.9±0.02 in combination with AZD7762 10nM and 100nM, respectively; therefore resembling the result obtained in SW48 p53 null cells. Interestingly, the AZD7762 induces increase in SN38 sensitivity also in the parental cells which harbour the mutated allele co-present with a silenced allele, with $IC_{50}$ values of 3.1±0.05nM and 2.7±0.1nM for combinations treatments with AZD7762 10nM and 100nM respectively, compared to $IC_{50}$ value of 9.5±0.16nM after treatment with SN38 alone (Fig.20C). No increase in SN38 sensitivity is achieved, however, in the DLD wt/sil line (Fig.20A).
These results, coupled with results obtained in SW48 p53-derivative cells, suggest differences in mut-p53 activities when expressed alone, or in presence of the wt counterpart. In DLD parental cells in which the mutant allele is expressed without a wt-functional counterpart, the combination treatment of SN38 and Chk-1 inhibitor showed similar pattern of response to p53 null cells, therefore suggesting that the p53 mutant protein alone is inactive, or even if not completely inactive, it has lost wild type functions such as cell cycle checkpoint regulation.
Instead, the results obtaind in SW48 cells showed functional wt-p53 activity in presence of the p53R273H mutants, suggesting absence of DNE or GOF.

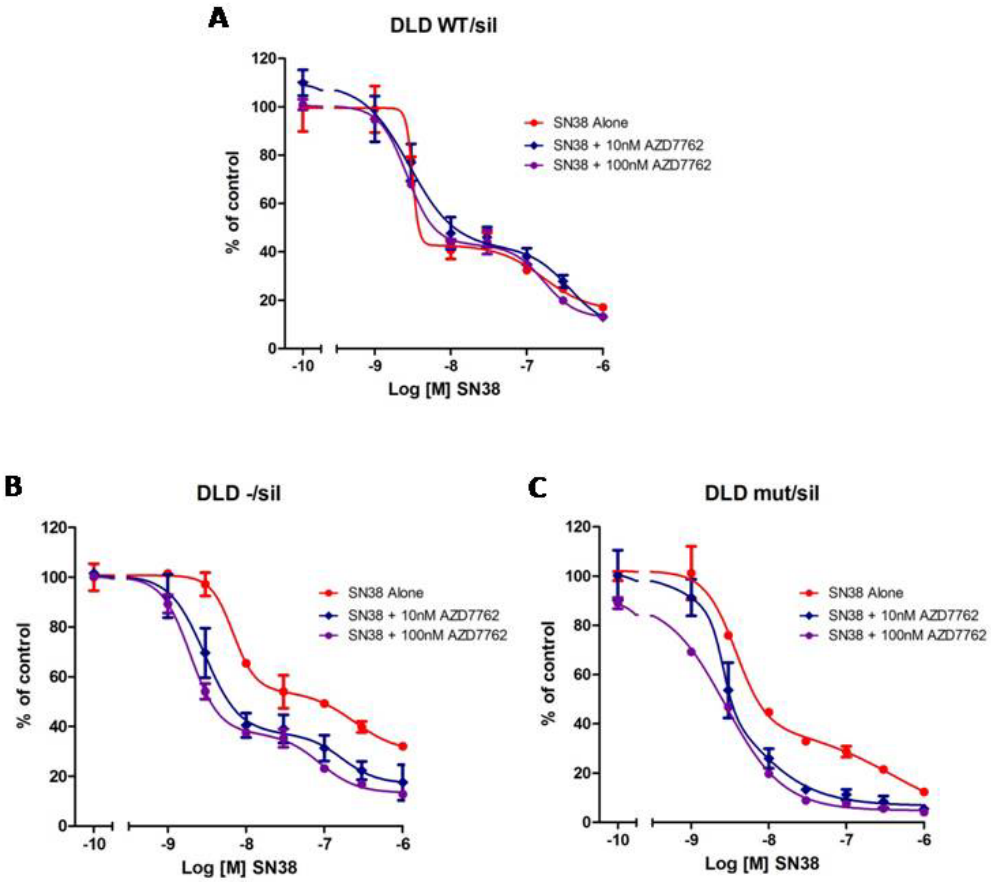



**Fig.20** SN38-Chk1 inhibitor (AZD7762) combination treatment on DLD cell lines. Cells were seeded at same density in 96 well plate at day 0 in complete medium. The day after they were treated with

10nM or 100nM of AZD772 in combination with increasing doses of SN38 for 72h. The cellular viability was assessed by SRB method. Chk1 inhibitor is able to enhance SN-38 apoptotic treatment in DLD -/sil (B) and DLD mut/sil (C) cell lines (P values <0.05). No differences came out from DLD wt/sil cell lines (A). Graphs represent average±sd of three independent experiments.

### p53-genetic alterations induce resistance to cytostatic anti-EGFR treatments.

Following our aim to characterize the influence of p53 mutant R273H in response to cancer treatments in presence of the wt allele, we tested our panel of cells also with novel targeted therapies used in the clinical treatment of cancer patients.
The Epidermal Growth Factor Receptor (EGFR) has emerged as an important therapeutic target in a variety of human cancers, including colorectal cancer, in which between 25% and 77% of tumors over-express EGFR (Mayer et al., 1993).
This brought the active development of anti-EGFR treatment strategies for these patients, including monoclonal antibodies, such as Cetuximab, which target the extracellular domain of the EGFR; and small molecules (tyrosine kinase inhibitors, TKIs), such as Erlotinib (Tarceva) and Gefitinib (Iressa), which target the tyrosine kinase domain of the receptor. These agents have proven to be efficacious as monotherapy and in combination with chemotherapy in patients with metastatic disease.

Treating SW48 derivative cell lines with increasing doses of Erlotinib, we found that p53-genetic alterations caused a relative resistance to cytostatic effect of Erlotinib. In detail, we found that deletion of one of both alleles in the SW48 caused a stepwise resistance to Erlotinib activity, with an increase in the $IC_{50}$ value from 11±0.08nM of the parental cells to 17±0.12nM and 164±0.2nM for heterozygous and homozygous p53 deleted cells respectively (Fig.21 A, B).

The presence of the genetic mutation R273H, both in isogenic or overexpressing models, also caused resistance to Erlotinib activity, but was less marked compared to cells in which alleles were deleted. The p53 mutant effect is more evident at highest doses of Erlotinib curve dosage (Fig.21B), with more than 20% of difference in the cell viability.

To confirm the result obtained with Erlotinib we treated the panel of cells with an analogous EGFR inhibitor, Gefitinib and with the monoclonal antibody Cetuximab. We found that Gefitinib and Cetuximab had in general less marked effect than Erlotinib on SW48 panel of cells, but reproduced the pattern of responses with Erlotinib treatment (Fig. 21C, D).

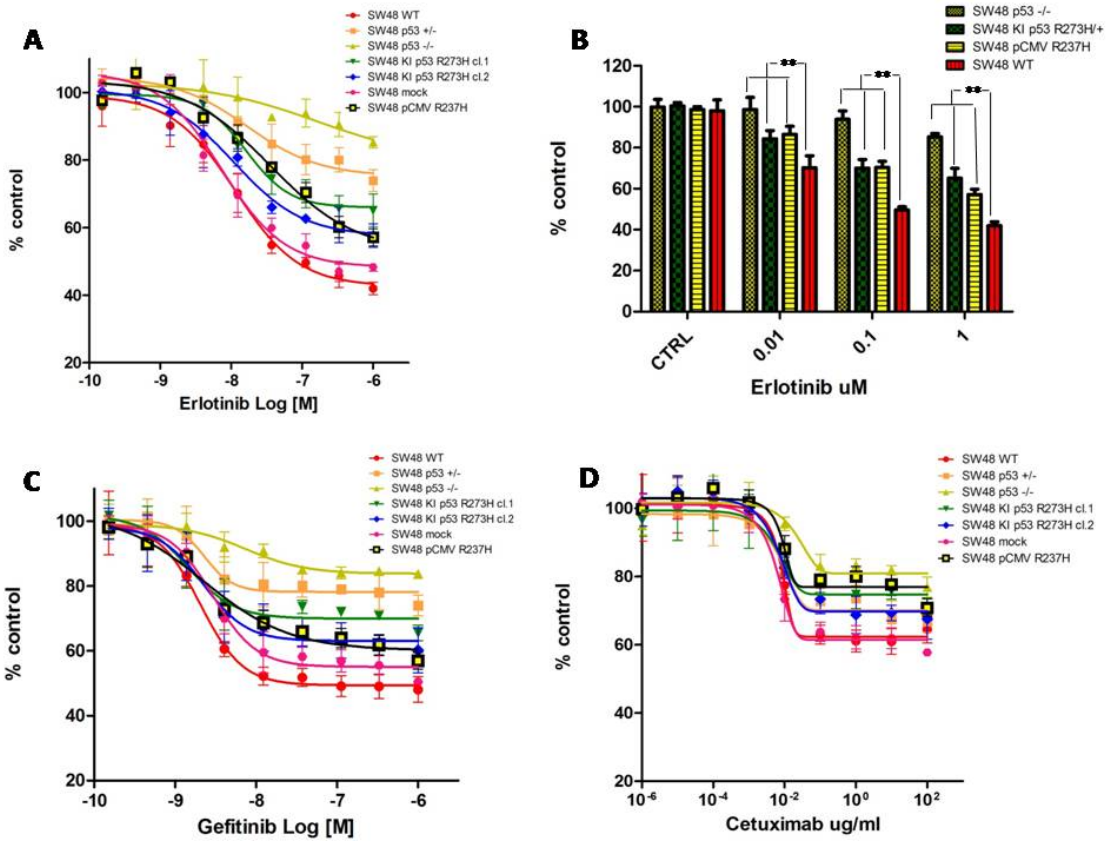



**Fig.21** Response to cytostatic effect of anti-EGFR targeted drugs. Cells were seeded at same density in 96 well plate at day 0 in complete medium. The day after they were treated with increased doses of Erlotinib (A, B), Gefitinib (C) and Cetuximab (D) for 6 days. The cellular viability was assessed by SRB method. p53-genetic alterations caused increase in resistance to the cytostatic effect of all the three drugs treatments (A, C, D). Deletion of both p53 alleles caused strongest resistance (B). p53 R237H mutation showed less marked resistance compared to heterozygous or homozygous p53 deletion, but with a significant (P values<0.01) increase in the viable cells at highest dosages (B). The two isogenic cellular clones showed similar behavior. Graphs represent average±sd of three independent experiments.

**Deletion of one or both p53 alleles induces resistance to PI3K-pathway inhibitors, but not the R273H mutant.**

The epidermal growth factor receptor (EGFR) is a tyrosine kinase protein, which plays an important role both in the signal transduction pathway and cellular function (Carpenter and Cohen, 1990).
Binding of specific ligand, such as the epidermal growth factor (EGF) results in the dimerisation of the receptor with the subsequent initiation of the intracellular signalling pathways cascade. A major downstream signalling route is via the Ras-Raf-MAPK. Activation of Ras initiates a multistep phosphorylation cascade that leads to the activation of MAPKs, ERK1 and ERK2, which ultimately regulate transcription of molecules involved in cell proliferation (Mendelshon and Baselga, 2003). Another important target in EGFR signalling is phosphatidylinositol 3-kinase (P13K) and the downstream protein-serine/threonine kinase Akt. This latter protein

kinase transduces molecular signals triggering crucial steps for cell growth and survival (Carpenter and Cohen, 1990; Mendelsohon and Baselga, 2003).

Following the results obtained with EGFR targeted drugs, we therefore treated the SW48 panel of cells with drugs that specifically target effectors of the PI3K-AKT and RAS-MAPK pathways.

The cells were treated with increasing doses of the PI3K-inhibitor GDC0941 (Fig. 22A), mTOR-inhibitor rapamycin-derivative Everolimus (Fig. 22C) and MEK-inhibitor PD0325901(Fig. 22B).

Notably cells in which one or both p53 alleles have been deleted showed increased resistance to higher doses of the two PI3K-pathway inhibitors GDC0941 and Everolimus (Fig.22 A,C respectively); consistent with the observations with specific EGFR inhibitors (Fig.21 A, C and D).

In detail, knock-out of the p53 gene caused an increase in the $IC_{50}$ value to 23±0.03nM in response to the PI3K inhibitor GDC0941, compared to the $IC_{50}$ value of 11±0.02nM observed from parental treatment. An $IC_{50}$ value of 3.6±0.3nM was observed in p53 null cells compared to 1.7±0.2nM of the parental cells when treated with the mTOR inhibitor Everolimus.

Instead, a similar pattern of response to parental cells was observed in p53 R273H mutant cell lines in response to these two drugs (Fig. 22 A, C).

Moreover no differences were observed among the SW48 cells differing in their p53 status in response to the treatmnet with the MEK inhibitor (Fig.22B).

These results suggest an interaction between p53 protein and the PI3K-AKT pathway that is therefore altered only when the p53 gene is completly deleted, deeply influencing response to EGFR and PI3K targeted drugs.

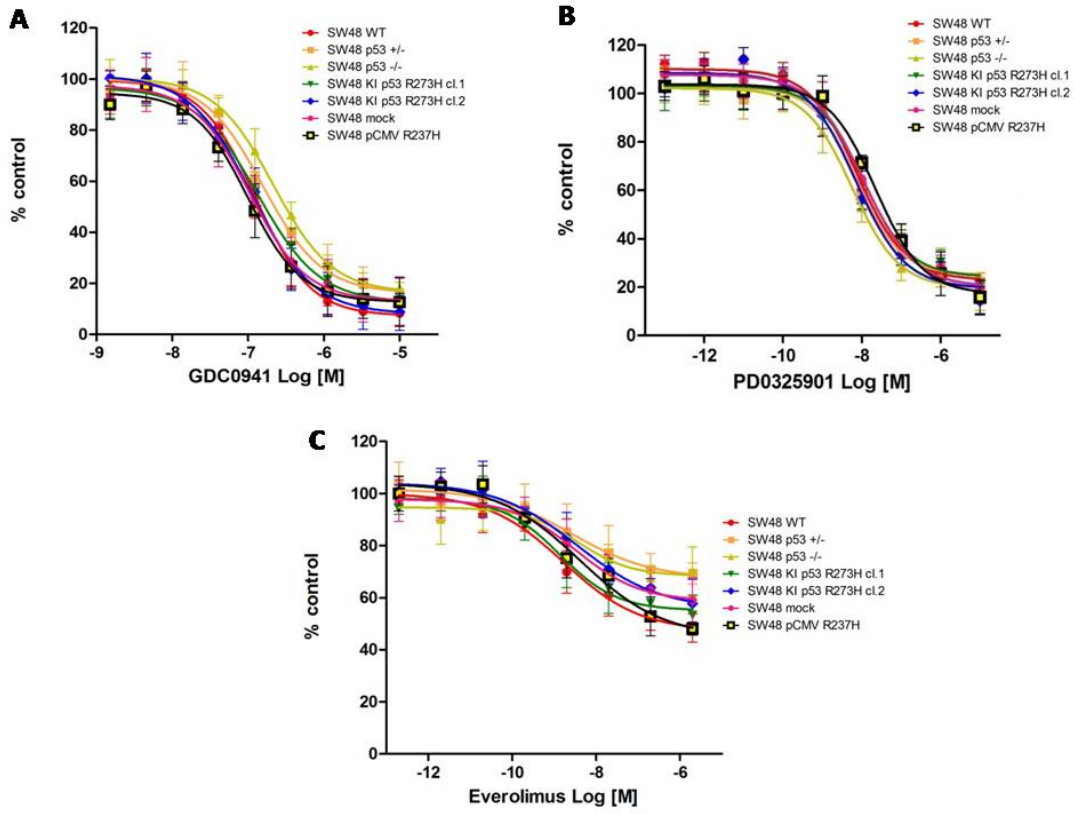



**Fig.22** Response to PI3K and Ras' pathways inhibition. Cells were seeded at same density in 96 well plate at day 0 in complete medium. The day after they were treated with increased doses of PI3K inhibitor GDC0941(A), MEK inhibitor PD0325901(B) and mTOR inhibitor Everolimus (C) for 96h. The cellular viability was assessed by SRB method. Only SW48 p53 +/- and p53 null (-/-) cells showed increased resistance (P values <0.01) to GDC0941 (A) and Everolimus (C). No differences among the different cell lines were detected in response to MEK inhibitor PD0325901 (B). Graphs represent average±sd of three independent experiments.

**Absence of p53 causes activation of Akt therefore inducing resistance to EGFR and PI3K targeted therapies.**

We then checked the effect of the EGFR inhibition at the biochemical level. To this issue the SW48 WT cells, the SW48 p53 KO cells, the SW48 KI p53 R273H cells and the SW48 R273H overexpressing cells were treated with two different concentrations of Erlotinib for 6h, and then levels of downstream effectors of the EGFR-pathway and the p53 status were checked by western blot.

Firstly we confirmed that parental cells show low (almost undetectable) levels of p53 in absence of cellular stress; no protein is obviously present in p53 ko cells. p53 R273H isogenic cells, instead, showed higher p53 basal levels compared to parental cells (as already demonstrated), but not compared to overexpressing cells that showed constitutive production of p53 protein. Notably, treatment with Erlotinib caused activation of the p53 protein, probably as a response to a cellular stress.

Parental cells and p53 R273H mutant cells do not show basal activation of the EGFR pathway effectors as we can see by comparison of p-EGFR, p-MAPK and p-

Akt levels after EGFR stimulation with EGF for 1h (Fig.23). No effect are indeed detectable upon Erlotinb treatments.

Interestingly p53 null cells showed higher basal level of p-Akt compared to the other cell lines, level that is further increased by EGFR stimulation through EGF.

Of interest, Erlotinib was not able to turn-off the Akt activation up to at 1μM concentration, which is ten fold higher then $IC_{50}$ value of p53 null cells, as we can see from high p-Akt level also after Erlotinib treatments (Fig. 23).

The treatments did not affect the total amount of EGFR, MAPK and Akt.

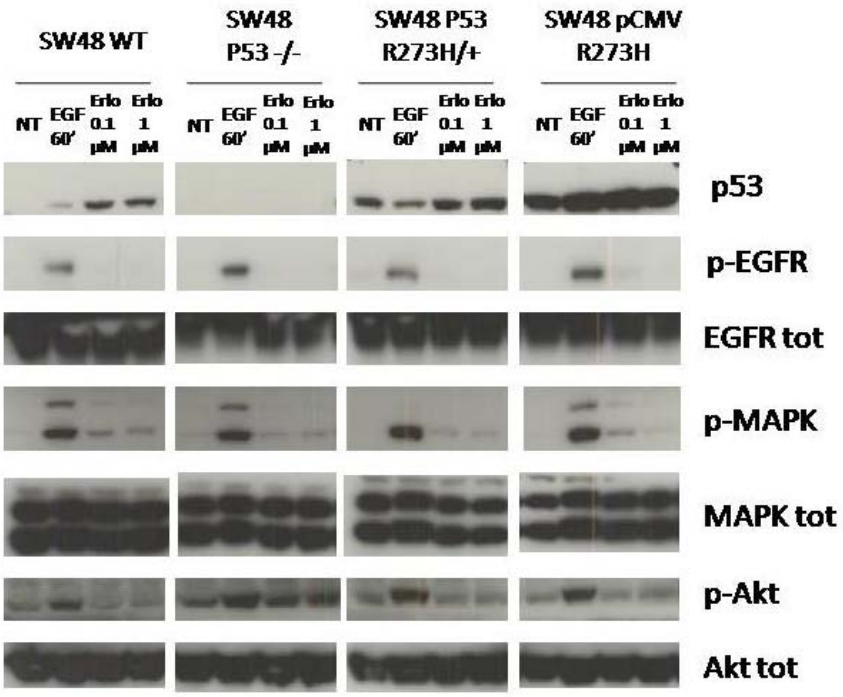



**Fig.23** EGFR pathway' effectors and p53 status**.** SW48 parental cells, Sw48 p53 null (-/-) cells, SW48 p53 R273H/+ isogenic cells and SW48 R273H overexpressing cells (SW48 pCMV R273H) were treated with 50ng/ml of EGF for 60 minutes (lane 2 of each blot); with Erlotinib 0.1μM (lane 3 of each blot) or Erlotinib 1μM (lane 4 of each blot) for 6h. Total protein lysates extracted from treated and not-treated cells were resolved by 4-12% SDS-PAGE and incubated with antibodies anti-p53 DO-1, which recognize both wild type and mutant p53; anti-pEGFR (Y1173); anti-EGFR total; anti-pMAPK; anti-MAPK total; anti-AKT (Ser473) and anti-Akt total.

### Mut-p53 R273H does not markedly affect the transcriptional profile of the cells.

Given that previous data suggest a lack of DNE, in order to verify whether any putative GOF properties of mut-p53 R273H (even subtle ones) can be determined, we compared the broad transcriptional profile of SW48 panel of cells before and after doxo treatment.

Once more, consistent with our previous results, we found that the presence of the p53 R273H mutant has minimal effect on the quantity and magnitude of differentially expressed genes; with only 43 genes going up or down compared to parental cells. Among these, the only relevant and strongest up-regulated transcript

was a "pseudogene similar to hematological and neurological expressed 1". Moreover, this is in stark contrast to the many differentially expressed genes seen in wt-p53 vs p53 null cells (Fig.25).

Of note most of these genes are similarly up- or down-regulated also in the heterozygous cell lines (p53 +/-). This is infact confirmed by the $R^2$value (0.377) that comes out from direct comparison of the expression levels of these 43 genes between SW48 p53-R273H knock-in clones and SW48 p53 +/- cells, which shows a good correlation of expression (Fig.24A). This could be explained, as already seen till now, by partial inactivation of the mutated allele, more than to acquisition of GOF.

The correlation is instead completely reverted in the p53 null cells ($R^2$=0.106), in accord with the complete loss of the gene (Fig.24B).

Interestingly, no correlation was found in the overexpressing cells ($R^2$=0.033) (Fig. 24C); which is in accord with our previous results, in which the overexpression of the mut-p53 R273H in cells harbouring two wt-p53 alleles does not show a differential phenotype, maybe due to the compensation of the of the subtle effect of the mutants by the two wt alleles.

We then checked how the induction of p53 activity through DNA damage could influence the expression of these genes. We found that doxo treatment of the cells reverted the effect of the mut-p53 on the expression of these 43 genes ($R^2$=0.456) (Fig.24D) therefore annulling it ($R^2$=0) (Fig. 24E). This effect could be explained by the strong stabilization and activation of the wt-p53 in response to cellular stress such as DNA damage.

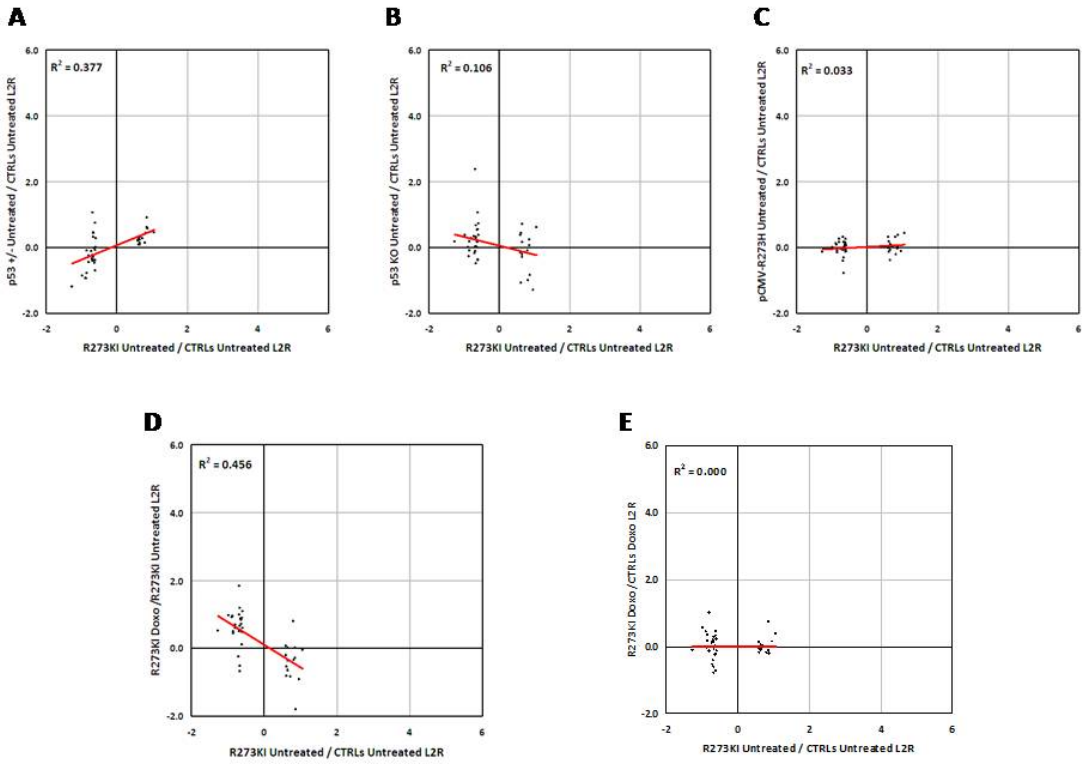



**Fig.24** Knock-in of p53 R273H mutant in SW48 cell lines induces differential expression of only 43 genes. P53 R273H mutant in SW48 cell lines (R273H KI untreated) alters the transcriptional profile of only 43 genes comparing to SW48 parental cells (CTRLs Untreated). The differential expression of these 43 genes is represented as the $Log_2$ratio (L2R) between SW48 KI p53 R273H cells (R273H KI

untreated) and SW48 parental cells (CTRLs untreated) on the X-axis of the five dot-plots represented in the figure. The differential expression of these 43 genes has been compared in: (A) SW48 p53 +/- cells, represented on the Y-asis as the $Log_2$ratio of SW48 p53 +/- on SW48 parental cells (CTRLs untreated); (B) SW48 p53 null cells, represented on the Y-axis as the $Log_2$ratio of SW48 p53 KO on SW48 parental cells (CTRLs untreated); (C) SW48 p53 pCMV R273H overexpressing cells, represented on the Y-asis as the $Log_2$ratio of pCMV R273H on SW48 parental cells (CTRLs untreated); in SW48 KI p53 R273H cells after induction of DNA damage, through treatment with 0.2µg/ml of doxo over night, compared to SW48 KI p53 R273H untreated ($Log_2$ratio R273H KI doxo/R273H untreated) (D) or compared to SW48 parental cells doxo treated ($Log_2$ratio R273H KI doxo/CTRL doxo) (E).

Finally comparing the general heat maps of the different SW48 cells harbouring genetic alteration in TP53 gene, each compared versus parental cells, we found that genetic alterations of one allele, either by deletion or mutation did not alter significantly the transcriptional profile of the cells, without significant differences among the samples, including the parental cells transfected with the empty vector (mock cells) (Fig.25 columns 1-5). Instead only the complete deletion of p53 in p53 null cells deeply affects the expression profile of the cells (Fig.25 column 6).

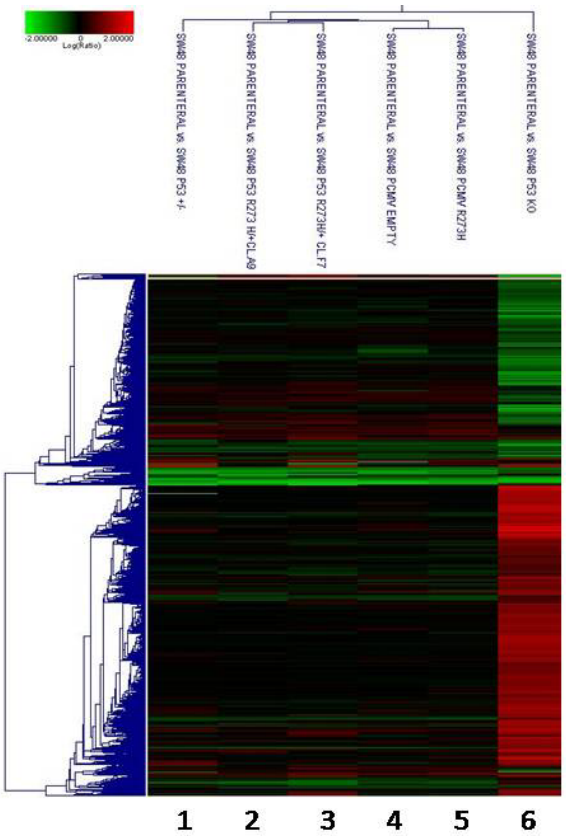



**Fig.25** Transcriptional profile of SW48 p53-derivative cell lines. The SW48 derivative cell lines were analysed by microarray for their transcriptional profile and then all compared vs SW48 parental cells. The data are shown in a heatmap format in which each row represents a gene and each column corresponds to a sample. The colour in each cell reflects the expression level of the corresponding gene in the corresponding sample: red colour indicates a transcriptional level above the expression of that gene in the parental cells; green colour indicates a transcription level below the expression of that gene in parental cells. Column 1: SW48 p53 +/- cells; Column 2: SW48 KI p53 R273H/+ cl.1; Column 3: SW48 KI p53 R273H/+ cl.2; Column 4: SW48 mock cells; Column 5: SW48 pCMV R273H overexpressing cells; Column 6: SW48 p53 null cells.

# 5. DISCUSSION

The p53 tumor suppressor is the most highly mutated gene in human tumors. Dominant negative, or even completely new functions for mutant forms of p53 have been proposed by a large number of studies, including clinical data that suggest a poorer prognosis in cancer patients that possess p53 mutations. However the prognostic and predictive significance of p53 mutations is extremely variable in these studies, which may reflect differences in mutant type, tumor type and/or treatment studied (Bertheau et al., 2008) and consequently, there is no universal clinical message that can be delivered by TP53 mutational analysis.

On one hand, some reports have documented an increased therapy resistance of p53-null tumor cell lines under experimental conditions that relied on transient or stable mutant p53 overproduction. For example, murine myeloblastic cells showed higher resistance to adriamycin and cisplatin (Li et al., 1998) and Saos-2 or H1299 cells to etoposide or cisplatin (Wang et al., 1998; Blandino et al., 1999).

Since these experiments involve artificial components, such as ectopically expressed protein, the concept that p53 mutants are oncogenic should be questioned; specifically they may not reflect the ratio of mutant and wt p53 proteins found in human cancer cells heterozygous for p53 (Sigal and Rotter, 2000; Blagosklonny, 2000). In fact, many p53-mut functions have been studied to date in p53 null background, which does not allow effects over the wt allele to be studied.

On the other hand, some studies have failed to establish correlations between the expression of mutant p53 and anti-apoptotic genes (Reeve et al., 1996). Moreover, several investigators have failed to observe a correlation between p53 mutation and chemo- or radio-resistance (Makris et al., 1995; Cote et al., 1997). For example, work by Cote and colleagues have suggested that patients with transitional cell carcinoma of the bladder, surprisingly, benefit from adjuvant ADR and cisplatin chemotherapy only when their tumors express mutant p53 (Cote et al., 1997).

To perhaps reconcile these data, firstly the p53 mutants categorization, although useful, may be oversimplifying the situation, because mutants may vary in their degree of folding and function; and thus in their oncogenic properties.

Indeed, the conformational mutations have been suggested to be more oncogenic than the DNA contact mutations in several systems. For example, the conformational mutants R175H and R249S, resulted in immortalization of mammary epithelial cells, whereas the DNA contact mutants R248W and R273H did not (Cao et al., 1997).

Moreover when tumor-derived cell lines with missense p53 mutations were examined for wild-type p53 transcriptional activity, lines with the R273H mutant possessed it, whereas lines with mutants R156P, R175H, R248W, R248Q and R280K did not (Park et al., 1994).

In our study we observed that physiological expression of the hot spot contact mutant p53 R273H (to which DNE and GOF activities have been previously attributed when studied in p53 null background) in heterozygous context, resulted in the typical response to p53 dependent cellular perturbations, such as activation of cyclin-dependent kinase inhibitor p21 and pro-apoptotic protein Bax after DNA damage.

It has been shown that wt-p53 could have different affinity for the promoters of its targeted genes; it was demonstrated that wt-p53 has much higher affinity for p21 promoter than for Bax one (Flaman et al., 1998); and some mutants in fact retain the

capacity to transactivate p21, but are defective for BAX activation and fail to induce apoptosis (Ludwing et al., 1996).
In contrast in our study, SW48 p53 R273H cells showed induction of both p21 and Bax and cleavage of the PARP protein after DNA damage, even when the mutant is over-expressed in wt-p53 background, indicating that wt-p53 dependent transactivation activity is still functional and so dominant-negative activity was not so marked in these cells.
This result is more in keeping with the fold change in the protein conformation related to this specific mutant. It has been reported that R273H mutant seems to retain almost all of the wild-type conformation (98% folding of wild-type p53) (Chene, 1998), therefore keeping principle wt-p53 transactivation properties.

When cell cycle progression was analyzed, the SW48-derivative cell lines also showed similar profile of proliferation, without evidence of significant increase in the percentage of proliferating cells induced by R273H mutant, as demonstrate also by proliferation curve. The only detectable difference is an increase in proliferation rate of SW48 p53 null cells, according with complete loss of wt-p53 activity in controlling cell cycle progression. Moreover p53 null cells showed the expected profile at flow cytometry analysis by propidium iodide staining, with all the cells accumulated in G2 phase after DNA damage, due to lack of p21 activation and downstream G1 arrest of the cells. Instead p53 heterozygous (p53 +/-) cells showed similar behavior to parental cells, therefore deletion of only one p53 allele does not influence the cell cycle control by p53.
No significant differences were detected in the profile of p53-mut cells, except that for a small increase in the percentage of the cells blocked in the G2 phase, either in isogenics, or overexpressing models, harbouring the R273H mutation. It has already previously been shown that this, even if small, increase could be due to a pro-proliferation effect of the p53 mutant (Rieber and Rieber, 2009; Willis et al., 2004). If this is true, and this is a characteristic of the p53 mutant, in our cellular models it is clearly attenuated by the presence of the wt-allele, since the cells do not completely accumulate in the G2 phase (as for the p53-null cells), highlighting a lack of a strong DNE.

We can suppose that if peculiar characteristics exist for this p53-mutant, they are more subtle, especially in the presence of the wt allele and endogenous expression levels.

This is also confirmed by Nutlin-treatment of these cells. Nutlin-3 is a small molecule able to inhibit the p53-MDM2 interaction with high degree of specificity, leading to the stabilization of p53 and the activation of p53 pathway (Vassilev et al., 2004). Sur and colleagues have shown that cells harbouring functional wt-p53 are more growth-inhibited by Nutlin-3 than cells without wt-p53. In their hands, cells with completely inactive TP53 gene had Nutlin-3 sensitivities identical to those with p53 missense contact mutant R248W, in cell lines in which the mutant allele is expressed in hemizygous state, due to the deletion or the inactivation of the wt allele (Sur et al., 2009).
We found that p53 null cells induced resistance to Nutlin-3 growth inhibition; instead the p53 contact mutant R273H expressed heterozygously showed a similar pattern of Nutlin sensitivity to parental cells, demonstrating that the wt allele co-expressed with the mutated one is not inhibited in its function.

While all these studies show that physiological levels of mutant p53 have no, or subtle DN effects, these data do not necessary imply lack of GOF. Putative GOF properties are especially concerning given that they can theoretically impart worse prognoses in cancer patients and/or poorer outcomes with therapy. Such a possibility is supported by in vitro studies demonstrating that p53 mutants have a marked protective effect against chemodrugs-induced apoptosis. However, these studies were also based on over-expression data.

We studied one of the most common mutant genotypes, R273H, for GOF activity and observed that the p53 contact mutant R273H failed to induce resistance to paclitaxel and cisplatin, in contrast with p53-null cells, even when over-expressed in p53 wt background.

Some discrepancy may reflect not only methodological differences between the studies, but also the genetic background in which the mutated allele is expressed.

Previous evidences of resistance-induced activity to cancer treatments conferred by mut-p53 were also mainly conducted in p53 null background, whereas leaving unstudied the effect of these mutations in the presence of wt-p53. In fact we failed to observe protective effect against chemo-treatments from p53 mutant R273H even when it is over-expressed in wt-p53 background.

In accord with these data, Blandino and colleagues have documented that H1299 cells are significantly protected from etoposide-induced apoptosis when overproducing the p53 mutant 175H and, to a lesser extent by, 273H, and they failed to observe a protective effect in the low expressor subpopulations of their 175H and 273H transfected cultures.

Moreover, the p53 contact mutant R273H showed a similar pattern of response to parental cells to the topoisomerase-I inhibitor SN38, even when it is used in combination with Chk-1 inhibitor AZD7762; despite previous evidences of constitutive interaction of mut-p53 with topo-I, unlike wt-p53 (Gobert et al., 1999).

SN38 acts mainly blocking the cells in G2 phase, and cells with altered p53-functions are G1 check-point deficient, thus they solely rely on Chk1 to maintain S or G2 arrest in response to DNA damage. In contrast, normal cells have an additional G1 arrest mechanism conferred by functional wt-p53. Chk1 inhibition would therefore abrogate S/G2 checkpoint in wt non-functional-p53 cells and drive them to apoptosis (Zabludoff et al., 2008).

As expected, the Chk-1 inhibitor induced an increase in SN38 activity preferentially in p53 null cells, both in the SW48 p53KO cells than in DLD-/sil colorectal cancer cells. But interestingly we found that the combination treatment showed an equivocal pattern of response in all the other SW48 derivative cells, except for the functionally null DLD mut/sil cells, therefore suggesting a lack of DNE; and that the p53 mutant protein is simply inactive, or even if not completely inactive, it could have lost wild type functions such as cell cycle checkpoint regulation.

There was, instead, one discernable effect of p53 R273H co-expressed at physiological levels along with the wt allele on a cyto-toxic drugs' activity; wherein a small resistance to 5FU treatment was seen compared to that observed in p53 null cells. Specifically, an increase of 20% of viable cells was seen at high doses of the drug compared to parental cells. Surprisingly the over-expression of the mut-p53 in p53 wt cells does not induce the same resistance.

One explanation could be the difference in the allelic ratio due to artificial over-expression of the protein in cells harbouring 2 wt p53 alleles, which maybe could

compensate the over-expression effect; therefore the exact ratio wt/mut p53 cannot be determined. With the isogenic models instead we are able to directly recapitulate the physiological situation find in human cancer patients with a 50% allelic balance between wt and mutated allele, results so obtained are therefore more trustworthy.

This conclusion is also consistent with studying a PARP inhibitor, a drug which is purported to be effective in cells defective in DNA repair systems, but not the p53 KI-cells, therefore highlighting the wt-p53 functionality in p53 R273H mutant cells.

Finally, a clear effect of mutp53 R273H on resistance to EGFR targeted agents was seen (Erlotinib, Gefitinib and Catuximab), but it is less marked when compared to heterozygous and homozygous null cells. It's important to highlight that SW48 parental cells harbour the genetic alteration G719S in EGFR as reported from sanger website (http://www.sanger.ac.uk/genetics/CGP/). This mutation has been reported to be an Erlotinib and Gefitinib-sensitive mutation (Kancha et al., 2009). This can explain the parental sensitivity to EGFR inhibitors.

In order to better elucidate this result we treated SW48 panel of cells with EGFR-downstream PI3K (GDC0941 and Everolimus) and Ras (PD0325901) pathways inhibitors. Cells in which one or both p53 alleles have been deleted showed increased resistance to the two PI3K-pathway inhibitors, but not to the MEK inhibitor PD0325901. The R273H mutants instead did not cause differences in response to all these three treatments. Moreover we found that wt-p53 and mut-p53 R273H does not induce basal activation either of EGFR, MAPK and Akt proteins, in contrast with some previous studies which report EGFR activation by conformational mutant R175H (Dong et al., 2009). SW48 p53 null cells, instead, showed higher basal levels of p-Akt protein when compared with parental and mutant cell lines; and when treated with Erlotinib at concentrations, either similar to the $IC_{50}$ value determined for p53 null cells, or at ten-fold higher concentration, no effect is observed on p-Akt levels. It has been reported previously that cross-talk can exist between p53, Akt and MDM2. It is possible therefore that under conditions leading to an irreversible apoptotic commitment, activation of p53 may contribute to apoptosis by inhibition of Akt. On the other hand, in the presence of survival signals, Akt activation may lead to p53-inhibitor Mdm2 activation through phosphorylation of its Ser166 (Gottlieb et al., 2002; Ogawara et al., 2002). Therefore deletion of p53 could cause an alteration in the regulation of pro- anti-survival signals, leading to a lack in Akt down-regulation in response to pro-survival signals, causing resistance to PI3K and EGFR-inhibitors.

We can therefore interpret that p53-genetic alteration-dependent resistance to EGFR pathway inhibitors is mainly linked to inactivation of the p53 protein (as manifested by the complete p53 KO) or reduction at different levels (through deletion of one allele or through missense mutation in one p53 allele) of p53 wt function.

It's important to highlight that most of the results here reported do not suggest a strong DNE of the p53 contact mutant R273H. Moreover, even if a peculiar characteristic versus parental cells is observed, such as resistance to 5FU and anti EGFR therapies, this is also observed after complete inactivation of one of both alleles of the TP53 gene.

Even if complete deletion of TP53 gene in vivo is rare, these results can nonetheless suggest that the effects generated by the R273H p53 mutant are not necessarily related to DNE or GOF, but also to a reduction of wt-p53 activity of the mutated allele without acquiring new functions.

A final assessment of GOF activity has being conducted using microarray analysis of the transcriptional profile of these cells. We found that R273H mutation in TP53 gene does not strongly influence the transcriptional profile of the cells. Only 43 genes were differentially transcribed in the isogenic mutant cell lines and subsequent comparison of the expression of these genes in the other cells showed a similar effect in p53 +/- cells. This could be explained, as already seen,by partial inactivation of the mutated allele more than the acquisition of GOF properties. Instead, no effect was observed in the over-expressing cells; this is in accord with our previous results in which the overexpression of the mut-p53 R273H in cells harbouring two wt-p53 alleles does not show a differential phenotype, maybe due to the compensations of the of the subtle effect of the mutants by the two wt alleles.
The complete loss of p53 instead showed no correlation with mut-p53 R273H regulation of these genes; therefore highlighting lack of DNE over the wild type functions of p53 by mutant R273H. Moreover the wt-p53 induction through DNA damage completely reverted the mut-p53 subtle effect, due to strong activation of wt-p53 functions. This is not in conflict with the other results showed in this thesis, confirming that p53 mutant R237H does not show relevant DNE and GOF in presence of wt-p53 allele in colorectal cancer cells.

# 6. CONCLUSIONS

There are two inter-related reasons why the study of heterozygotes (mut/wt), which express both the mutant and the wt-p53 alleles is important.
First, as mentioned in the body of this thesis, it is of interest to evaluate the extent to which mutant forms of p53 can down-regulate the wt form of the protein by forming inactive heterotetramers. Second, cancer-prone Li-Fraumeni family members possess a germline mutant form of p53, and generation of such models could genotypically and phenotypically mimic such patients' disease characteristics.

The relationship between specific p53 mutations, structural elements of p53 and clinical outcome should be assessed using as rigorous criteria as possible.

In the case of p53 mutants with no or weak dominant p53 inhibition, presence of the wt allele may indicate a good prognosis cancer, because the p53 pathway is likely to still be somewhat functional; whereas loss of heterozygosity may specify a poor prognosis or cancer highly resistant to cancer therapy

The evidence that some p53 mutants that show no or little interference with wt p53 highlights the importance of determining whether human cancers with p53 mutations still carry the wt p53 allele, or have lost it, and which type of mutant occurs in that specific tumor type.

The currently available data for loss of heterozygosity are too small, emphasizing the need for large clinical studies that not only identify p53 gene mutations but also assess for concordant loss of heterozygosity.
Together these data suggest that certain mutations of p53 could be pathologically significant only in the hemizygous state, including some or all of the p53 mutations inherited in the Li-Fraumeni syndrome.

## 7. ACKNOWLEDGEMENTS

I thank **Prof. Federico Bussolino**, for giving me the opportunity for this stimulating professional experience.

I thank **Dr. Chris Torrance and Prof. Alberto Bardelli,** for their continuous tutorship and support during my doctorate years. Mostly for always being patient despite my constant anxiety. ☺

I thank all my **colleagues at Horizon Discovery** for their help and support.
Especially:
**Bettina**…it was great to sing loud together in the lab! I am looking forward for my first trip with you to your beloved Lisbon…
**Charli**…how can you have everything under control?!?? I'll find out your secret sooner or later ☺!
**Becky**…my "drug screening teacher".

A special thanks to the **Italian Horizon team, the "milestone"**:
to my **Gnappy**…a sister, who shared "my breath" in a period of great change of my life; I'll never thank you enough!
to **Lessi**…a colleague, a friend, THE flatmate of this unusual English experience, we'll always be "gemmate"!
to **Pinotto**…for the energy she gave me and the laughter she shared with me..I will always share the last gel with you! ☺
to **Grattachecca e Fichetto**…only 2 months together…but simply great!

I thank all my **colleagues in the Molecular Genetics lab at IRCC**. I really appreciated the opportunity to work with you all; you made my daily life in Turin joyful from the beginning!
Thanks to **Miriam**…for your madness - dangerously contagious!
Thanks to **Sabrina**…for your energy.
Thanks to **Davide** for…for…to be just Davide…6 letters for a crazy person! ☺
Thanks to **Carlotta**…for your sweetness.
Thanks to **Fonnet**...it was an honour to be your Italian/Neapolitan teacher!
Thanks to **Luca**…my "piece of Naples" in Turin.

A big thanks to my **Feds**…working with you has been amazing! You let me "breath" science, not only as a colleague, but especially as a friend, my first true friend in Turin! Thanks for everything!!

Thanks to **my true Friends**, to have always been with me, even thousands of Km away…you are PRECIOUS!!
Thanks to **gmail and Skype**…our best friends…we could not live anymore without them… isn't it true?
Thanks to **intercontinental phonechats** at 2 am…

Thanks to great surprises from Brazil…
Thanks to "**the event of the 2010**"…it gave me rest and happiness during the most stressful time ever…
Thanks to **B4+1**…even when they are overwhelmed by the Catapecchia's infinite troubles… by Polly Pocket's furniture… tango and new loves…
Thanks to a **hundred daily emails**…they helped me survive…feel "home"…and LAUGH…LAUGH SO MUCH!! :-D

Thanks to **you**… even if now distant from my life…you have always believed in me… contributing so much to this! **You** deserve all the best…

Thanks to my **5 Kg of Nutella** and to **Gioia**, who brought us together!
…they should be provided to every PhD student to survive till the end! ☺

Thanks to **Ruby**…15 years of friendship and you are always with me… in every city or Country...you and all the weird situations which used to follow you…
OMG how can I escape from you????
May be I just have to be resigned….to the oddities of my **"good mood pill"!**

Thanks to the **best movie** ever and his special star **Panzone** to make me dream!

I thank all **my family**, who never left me alone, even when things were too difficult to share… you are my strength… my example…my safe haven!
**Daddy, Mom and Pisolo**…you are my soul…you are ME!!!!

**My grandparents**…here and from heaven… you are…
my roots in the past ...
my teaching in the present ...
my example for the future ...
**My uncles**…my source of family, love and special fish meals in Turin!

Thanks **to my new special person**, able to always say the right words and to do the right things at the right moments; for making me laugh even when I was crying, you are a gift!

I thank **all the people** who came into my life in these years, for their precious encouragement, support and immense affection.

## 8. REFERENCES


Agarwal ML, Agarwal A, Taylor WR, Stark GR. p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. Proc Natl Acad Sci U S A. 1995 Aug 29;92(18):8493-7.

Alsner J, Jensen V, Kyndi M, Offersen BV, Vu P, Børresen-Dale AL, Overgaard J. A comparison between p53 accumulation determined by immunohistochemistry and TP53 mutations as prognostic variables in tumours from breast cancer patients. Acta Oncol. 2008;47:600-7.

Attardi LD, Reczek EE, Cosmas C, Demicco EG, McCurrach ME, Lowe SW, Jacks T. PERP, an apoptosis-associated target of p53, is a novel member of the PMP-22/gas3 family. Genes Dev. 2000 Mar 15;14(6):704-18. Erratum in: Genes Dev 2000; 14(14):1835.

Banin S, Moyal L, Shieh S, Taya Y, Anderson CW, Chessa L, Smorodinsky NI, Prives C, Reiss Y, Shiloh Y, Ziv Y. Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science. 1998 Sep 11;281(5383):1674-7.

Barak Y, Juven T, Haffner R, Oren M. mdm2 expression is induced by wild type p53 activity. EMBO J. 1993;12(2):461-8.

Baudier J, Delphin C, Grunwald D, Khochbin S, Lawrence JJ. Characterization of the tumor suppressor protein p53 as a protein kinase C substrate and a S100b-binding protein. Proc Natl Acad Sci U S A. 1992 Dec 1;89(23):11627-31.

Benchimol S, Lamb P, Crawford LV, Sheer D, Shows TB, Bruns GA, Peacock J.Transformation associated p53 protein is encoded by a gene on human chromosome 17. Somat Cell Mol Genet. 1985;11:505-10.

Berns EM, Klijn JG, van Putten WL, de Witte HH, Look MP, Meijer-van Gelder ME, Willman K, Portengen H, Benraad TJ, Foekens JA. p53 protein accumulation predicts poor response to tamoxifen therapy of patients with recurrent breast cancer. J Clin Oncol. 1998;16:121-7.

Bertheau P, Espié M, Turpin E, Lehmann J, Plassa LF, Varna M, Janin A, de Thé H. TP53 status and response to chemotherapy in breast cancer. Pathobiology. 2008;75:132-9.

Blagosklonny M.V. p53 from complexity to simplicity: mutant p53 stabilization, gain-of-function, and dominant-negative effect. FASEB J. 2000;14:1901-1907.

Blandino G., Levine A. J., Oren M. Mutant p53 gain of function: differential effects of different p53 mutants on resistance of cultured cells to chemotherapy. Oncogene, 1999;18*:* 477-485

Børresen-Dale AL, Lothe RA, Meling GI, Hainaut P, Rognum TO, Skovlund E. TP53 and long-term prognosis in colorectal cancer: mutations in the L3 zinc-binding domain predict poor survival. Clin Cancer Res. 1998;4:203-10.

Bougeard G, Brugières L, Chompret A, Gesta P, Charbonnier F, Valent A, Martin C, Raux G, Feunteun J, Bressac-de Paillerets B, Frébourg T. Screening for TP53 rearrangements in families with the Li-Fraumeni syndrome reveals a complete deletion of the TP53 gene. Oncogene. 2003;22(6):840-6.

Boulares AH, Yakovlev AG, Ivanova V, Stoica BA, Wang G, Iyer S, Smulson M. Role of poly(ADP-ribose) polymerase (PARP) cleavage in apoptosis. Caspase 3-resistant PARP mutant increases rates of apoptosis in transfected cells. J Biol Chem. 1999 Aug 13;274(33):22932-40.

Brachmann RK, Vidal M, Boeke JD. Dominant-negative p53 mutations selected in yeast hit cancer hot spots. Proc Natl Acad Sci U S A. 1996;93(9):4091-5.

Brosh R, Rotter V. When mutants gain new powers: news from the mutant p53 field. Nat Rev Cancer. 2009;9:701-13.

Brown CJ, Lain S, Verma CS, Fersht AR, Lane DP. Awakening guardian angels: drugging the p53 pathway. Nat Rev Cancer. 2009 Dec;9(12):862-73.

Brugarolas J, Chandrasekaran C, Gordon JI, Beach D, Jacks T, Hannon GJ. Radiation-induced cell cycle arrest compromised by p21 deficiency. Nature. 1995;377(6549):552-7.

Bullock AN, Henckel J, DeDecker BS, Johnson CM, Nikolova PV, Proctor MR, Lane DP, Fersht AR. Thermodynamic stability of wild-type and mutant p53 core domain. Proc Natl Acad Sci U S A. 1997;94:14338-42.

Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, Sedivy JM, Kinzler KW, Vogelstein B. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science. 1998 Nov 20;282(5393):1497-501.

Burns TF, Bernhard EJ, El-Deiry WS. Tissue specific expression of p53 target genes suggests a key role for KILLER/DR5 in p53-dependent apoptosis in vivo. Oncogene. 2001 Aug 2;20(34):4601-12.

Cadwell C, Zambetti GP. The effects of wild-type p53 tumor suppressor activity and mutant p53 gain-of-function on cell growth. Gene. 2001;277:15-30.

Cadwell C, Zambetti GP. Regulators and mediators of the p53 tumor suppressor. J Cell Biochem Suppl. 1998;30-31:43-9.

Cao Y., Gao Q., Wazer D. E., Band V. Abrogation of wild-type p53-mediated transactivation is insufficient for mutant p53-induced immortalization of normal human mammary epithelial cells. Cancer Res., 1997; 57*:* 5584-5589

Caron de Fromentel C, Soussi T.TP53 tumor suppressor gene: a model for investigating human mutagenesis. Genes Chromosomes Cancer. 1992;4:1-15.

Carpenter G, Cohen S. Epidermal growth factor. J Biol Chem 1990;265: 7709–7712.

Chan TA, Hwang PM, Hermeking H, Kinzler KW, Vogelstein B. Cooperative effects of genes controlling the G(2)/M checkpoint. Genes Dev. 2000 Jul 1;14(13):1584-8.

Chen PL, Chen YM, Bookstein R, Lee WH. Genetic mechanisms of tumor suppression by the human p53 gene. Science. 1990;250:1576-80.

Chen X, Ko LJ, Jayaraman L, Prives C. p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells. Genes Dev. 1996;10(19):2438-51.

Chène P. In vitro analysis of the dominant negative effect of p53 mutants.J Mol Biol. 1998 Aug 14;281(2):205-9.

Chin KV, Ueda K, Pastan I, Gottesman MM. Modulation of activity of the promoter of the human MDR1 gene by Ras and p53. Science. 1992;255:459-62.

Chipuk JE, Bouchier-Hayes L, Kuwana T, Newmeyer DD, Green DR. PUMA couples the nuclear and cytoplasmic proapoptotic function of p53. Science. 2005;309(5741):1732-5.

Cho Y., Gorina S., Jeffrey P. D., Pavletich N. P. Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. Science (Washington DC), 1994;265: 346-355

Chuikov S, Kurash JK, Wilson JR, Xiao B, Justin N, Ivanov GS, McKinney K, Tempst P, Prives C, Gamblin SJ, Barlev NA, Reinberg D. Regulation of p53 activity through lysine methylation. Nature. 2004;432(7015):353-60.

Clore GM, Omichinski JG, Sakaguchi K, Zambrano N, Sakamoto H, Appella E, Gronenborn AM. High-resolution structure of the oligomerization domain of p53 by multidimensional NMR. Science. 1994;265:386-91.

Cote RJ, Esrig D, Groshen S, Jones PA, Skinner DG. p53 and treatment of bladder cancer. Nature. 1997;385(6612):123-5

Cross SM, Sanchez CA, Morgan CA, Schimke MK, Ramel S, Idzerda RL, Raskind WH, Reid BJ. A p53-dependent mouse spindle checkpoint. Science. 1995;267(5202):1353-6.

de Stanchina E, McCurrach ME, Zindy F, Shieh SY, Ferbeyre G, Samuelson AV, Prives C, Roussel MF, Sherr CJ, Lowe SW. E1A signaling to p53 involves the p19(ARF) tumor suppressor. Genes Dev. 1998 Aug 1;12(15):2434-42.

Dearth LR, Qian H, Wang T, Baroni TE, Zeng J, Chen SW, Yi SY, Brachmann RK. Inactive full-length p53 mutants lacking dominant wild-type p53 inhibition highlight loss of heterozygosity as an important aspect of p53 status in human cancers. Carcinogenesis. 2007;28:289-98.

DeGregori J, Leone G, Ohtani K, Miron A, Nevins JR. E2F-1 accumulation bypasses a G1 arrest resulting from the inhibition of G1 cyclin-dependent kinase activity. Genes Dev. 1995;9(23):2873-87.

DeLeo AB, Jay G, Appella E, Dubois GC, Law LW, Old LJ.Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. Proc Natl Acad Sci U S A. 1979;76:2420-4.

Deng C, Zhang P, Harper JW, Elledge SJ, Leder P. Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. Cell. 1995 Aug 25;82(4):675-84.

Deng G, Chen LC, Schott DR, Thor A, Bhargava V, Ljung BM, Chew K, Smith HS. Loss of heterozygosity and p53 gene mutations in breast cancer. Cancer Res. 1994;54(2):499-505.

Dittmer D, Pati S, Zambetti G, Chu S, Teresky AK, Moore M, Finlay C, Levine AJ. Gain of function mutations in p53. Nat Genet. 1993;4:42-6.

Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA Jr, Butel JS, Bradley A.Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature. 1992;356:215-21.

Dong P, Xu Z, Jia N, Li D, Feng Y. Elevated expression of p53 gain-of-function mutation R175H in endometrial cancer cells can increase the invasive phenotypes by activation of the EGFR/PI3K/AKT pathway. Mol Cancer. 2009 Nov 16;8:103.

Dumaz N, Milne DM, Meek DW. Protein kinase CK1 is a p53-threonine 18 kinase which requires prior phosphorylation of serine 15. FEBS Lett. 1999 Dec 17;463(3):312-6.

Eeles RA. Germline mutations in the TP53 gene. Cancer Surv. 1995;25:101-24.

El-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW, Vogelstein B. Definition of a consensus binding site for p53. Nat Genet. 1992;1(1):45-9.

Eliyahu D, Raz A, Gruss P, Givol D, Oren M. Participation of p53 cellular tumour antigen in transformation of normal embryonic cells. Nature. 1984 Dec 13-19;312(5995):646-9.

Eliyahu D., Goldfinger N., Pinhasi-Kimhi O., Shaulsky G., Skurnik Y., Arai N., Rotter V., Oren M. Meth-A fibrosarcoma cells express two transforming mutant p53 species. Oncogene, 3: 313-321, 1988.

Fields S, Jang SK. Presence of a potent transcription activating sequence in the p53 protein. Science. 1990;249:1046-9.

Finlay CA, Hinds PW, Levine AJ. The p53 proto-oncogene can act as a suppressor of transformation. Cell. 1989;57:1083-93.

Flaman JM, Robert V, Lenglet S, Moreau V, Iggo R, Frebourg T. Identification of human p53 mutations with differential effects on the bax and p21 promoters using functional assays in yeast. Oncogene. 1998 Mar 12;16(10):1369-72.

Forrester K, Lupold SE, Ott VL, Chay CH, Band V, Wang XW, Harris CC. Effects of p53 mutants on wild-type p53-mediated transactivation are cell type dependent. Oncogene. 1995;10:2103-11.

Frazier MW, He X, Wang J, Gu Z, Cleveland JL, Zambetti GP. Activation of c-myc gene expression by tumor-derived p53 mutants requires a discrete C-terminal domain. Mol Cell Biol. 1998;18:3735-43.

Fridman JS, Lowe SW. Control of apoptosis by p53. Oncogene. 2003 Dec 8;22(56):9030-40. Review.

Gaiddon C, Lokshin M, Ahn J, Zhang T, Prives C. A subset of tumor-derived mutant forms of p53 down-regulate p63 and p73 through a direct interaction with the p53 core domain. Mol Cell Biol. 2001;21:1874-87.

Giaccia AJ, Kastan MB. The complexity of p53 modulation: emerging patterns from divergent signals. Genes Dev. 1998 Oct 1;12(19):2973-83.

Gobert C, Skladanowski A, Larsen AK. The interaction between p53 and DNA topoisomerase I is regulated differently in cells with wild-type and mutant p53. Proc Natl Acad Sci U S A. 1999 Aug 31;96(18):10355-60.

Goh HS, Yao J, Smith DR. p53 point mutation and survival in colorectal cancer patients. Cancer Res. 1995;55:5217-21.

Gonzalez KD, Noltner KA, Buzin CH, Gu D, Wen-Fong CY, Nguyen VQ, Han JH, Lowstuter K, Longmate J, Sommer SS, Weitzel JN. Beyond Li Fraumeni Syndrome: clinical characteristics of families with p53 germline mutations. J Clin Oncol. 2009;27(8):1250-6.

Gottlieb TM, Leal JF, Seger R, Taya Y, Oren M. Cross-talk between Akt, p53 and Mdm2: possible implications for the regulation of apoptosis. Oncogene. 2002 Feb 14;21(8):1299-303.

Green DR, Kroemer G. Cytoplasmic functions of the tumour suppressor p53. Nature. 2009 Apr 30;458(7242):1127-30. Review.

Gu W, Roeder RG. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell. 1997 Aug 22;90(4):595-606.

Gurova KV, Rokhlin OW, Budanov AV, Burdelya LG, Chumakov PM, Cohen MB, Gudkov AV. Cooperation of two mutant p53 alleles contributes to Fas resistance of prostate carcinoma cells. Cancer Res. 2003;63:2905-12.

Hainaut P, Hollstein M. p53 and human cancer: the first ten thousand mutations. Adv Cancer Res. 2000;77:81-137.

Harvey M, Vogel H, Morris D, Bradley A, Bernstein A, Donehower LA. A mutant p53 transgene accelerates tumour development in heterozygous but not nullizygous p53-deficient mice. Nat Genet. 1995;9(3):305-11.

Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. Nature. 1997 May 15;387(6630):296-9.

Haupt Y, Rowan S, Oren M. p53-mediated apoptosis in HeLa cells can be overcome by excess pRB. Oncogene. 1995;10(8):1563-71.

Hermeking H, Lengauer C, Polyak K, He TC, Zhang L, Thiagalingam S, Kinzler KW, Vogelstein B. 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. Mol Cell. 1997 Dec;1(1):3-11.

Hirao A, Kong YY, Matsuoka S, Wakeham A, Ruland J, Yoshida H, Liu D, Elledge SJ, Mak TW. DNA damage-induced activation of p53 by the checkpoint kinase Chk2. Science. 2000 Mar 10;287(5459):1824-7.

Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. Science. 1991 Jul 5;253(5015):49-53.

Hollstein M, Rice K, Greenblatt MS, Soussi T, Fuchs R, Sørlie T, Hovig E, Smith-Sørensen B, Montesano R, Harris CC. Database of p53 gene somatic mutations in human tumors and cell lines. Nucleic Acids Res. 1994 Sep;22(17):3551-5.

Honda R, Yasuda H. Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. EMBO J. 1999 Jan 4;18(1):22-7.

Hupp TR, Meek DW, Midgley CA, Lane DP. Activation of the cryptic DNA binding function of mutant forms of p53. Nucleic Acids Res. 1993;21:3167-74.

Inga A, Storici F, Darden TA, Resnick MA. Differential transactivation by the p53 transcription factor is highly dependent on p53 level and promoter target sequence. Mol Cell Biol. 2002 ;22(24):8612-25.

Iyer NG, Chin SF, Ozdag H, Daigo Y, Hu DE, Cariati M, Brindle K, Aparicio S, Caldas C. p300 regulates p53-dependent apoptosis after DNA damage in colorectal cancer cells by modulation of PUMA/p21 levels. Proc Natl Acad Sci U S A. 2004;101(19):7386-91.

Jeffers JR, Parganas E, Lee Y, Yang C, Wang J, Brennan J, MacLean KH, Han J, Chittenden T, Ihle JN, McKinnon PJ, Cleveland JL, Zambetti GP. Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. Cancer Cell. 2003:4(4):321-8.

Jeffrey PD, Bajorath J, Chang CY, Yelton D, Hellström I, Hellström KE, Sheriff S.The x-ray Structure of an anti-tumour antibody in complex with antigen.Nat Struct Biol. 1995 Jun;2(6):466-71.

Jenkins JR, Rudge K, Currie GA. Cellular immortalization by a cDNA clone encoding the transformation-associated phosphoprotein p53. Nature.1984;312:651-4.

Jones PA, Rideout WM 3rd, Shen JC, Spruck CH, Tsai YC. Methylation, mutation and cancer. Bioessays. 1992 Jan;14(1):33-6.

Kancha RK, von Bubnoff N, Peschel C, Duyster J.Functional analysis of epidermal growth factor receptor (EGFR) mutations and potential implications for EGFR targeted therapy. Clin Cancer Res. 2009 Jan 15;15(2):460-7.

Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig RW. Participation of p53 protein in the cellular response to DNA damage. Cancer Res. 1991;51:6304-11.

Kato S, Han SY, Liu W, Otsuka K, Shibata H, Kanamaru R, Ishioka C. Understanding the function-structure and function-mutation relationships of p53 tumor suppressor protein by high-resolution missense mutation analysis. Proc Natl Acad Sci U S A. 2003;100(14):8424-9.

Kern S. E., Pietenpol J. A., Thiagalingam S., Seymor A., Kinzler K. W., Vogelstein B. Oncogenic forms of p53 inhibit p53-regulated gene expression. Science (Washington DC), 1992; 256*:* 827-830.

Kohli M, Rago C. An efficient method for generating human somatic cell gene knockouts. Discov Med. 2004 Feb;4(20):37-40.

Kraiss S, Quaiser A, Oren M, Montenarh M. Oligomerization of oncoprotein p53. J Virol. 1988;62:4737-44.

Kuerbitz SJ, Plunkett BS, Walsh WV, Kastan MB. Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc Natl Acad Sci U S A. 1992 Aug 15;89(16):7491-5.

Lakin ND, Hann BC, Jackson SP. The ataxia-telangiectasia related protein ATR mediates DNA-dependent phosphorylation of p53. Oncogene. 1999 Jul 8;18(27):3989-95.

Lalloo F, Varley J, Moran A, Ellis D, O'dair L, Pharoah P, Antoniou A, Hartley R, Shenton A, Seal S, Bulman B, Howell A, Evans DG. BRCA1, BRCA2 and TP53

mutations in very early-onset breast cancer with associated risks to relatives. Eur J Cancer. 2006;42(8):1143-50.

Lambert PF, Kashanchi F, Radonovich MF, Shiekhattar R, Brady JN. Phosphorylation of p53 serine 15 increases interaction with CBP. J Biol Chem. 1998 Dec 4;273(49):33048-53.

Lane, D.P. & Crawford, L.V. T antigen is bound to a host protein in SV40-transformed cells. Nature 1979;278:261-3.

Lang GA, Iwakuma T, Suh YA, Liu G, Rao VA, Parant JM, Valentin-Vega YA, Terzian T, Caldwell LC, Strong LC, El-Naggar AK, Lozano G. Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome. Cell. 2004;119:861-72.

Langerød A, Zhao H, Borgan Ø, Nesland JM, Bukholm IR, Ikdahl T, Kåresen R, Børresen-Dale AL, Jeffrey SS. TP53 mutation status and gene expression profiles are powerful prognostic markers of breast cancer. Breast Cancer Res. 2007;9:R30.

Lavigueur A, Maltby V, Mock D, Rossant J, Pawson T, Bernstein A. High incidence of lung, bone, and lymphoid tumors in transgenic mice overexpressing mutant alleles of the p53 oncogene. Mol Cell Biol. 1989 Sep;9(9):3982-91.

Levine AJ. p53, the cellular gatekeeper for growth and division. Cell. 1997 Feb 7;88(3):323-31. Review.

Li G, Tang L, Zhou X, Tron V, Ho V. Chemotherapy-induced apoptosis in melanoma cells is p53 dependent. Melanoma Res. 1998;8:17-23.

Linzer D.I.& Levine A.J. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell 1979;17:43-52

Lowe SW, Ruley HE. Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. Genes Dev. 1993 Apr;7(4):535-45.

Lu H, Levine AJ.Human TAFII31 protein is a transcriptional coactivator of the p53 protein. Proc Natl Acad Sci U S A. 1995;92:5154-8.

Lu X, Lane DP. Differential induction of transcriptionally active p53 following UV or ionizing radiation: defects in chromosome instability syndromes? Cell. 1993 Nov 19;75(4):765-78.

Ludes-Meyers JH, Subler MA, Shivakumar CV, Munoz RM, Jiang P, Bigger JE, Brown DR, Deb SP, Deb S. Transcriptional activation of the human epidermal growth factor receptor promoter by human p53. Mol Cell Biol. 1996;16:6009-19.

Ludwig RL, Bates S, Vousden KH. Differential activation of target cellular promoters by p53 mutants with impaired apoptotic function. Mol Cell Biol. 1996 Sep;16(9):4952-60.

Makris A, Powles TJ, Dowsett M, Allred C. p53 protein overexpression and chemosensitivity in breast cancer. Lancet. 1995 May 6;345(8958):1181-2.

Malkin D, Li FP, Strong LC, Fraumeni JF Jr, Nelson CE, Kim DH, Kassel J, Gryka MA, Bischoff FZ, Tainsky MA, et al.Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science. 1990;250:1233-8.

Mendelsohon J, Baselga J. Status of epidermal growth factor receptor antagonists in the biology and treatment of cancer. J Clin Oncol 2003;21: 2787–2799.

Matas D, Sigal A, Stambolsky P, Milyavsky M, Weisz L, Schwartz D, Goldfinger N, Rotter V. Integrity of the N-terminal transcription domain of p53 is required for mutant p53 interference with drug-induced apoptosis. EMBO J. 2001;20:4163-72.

May P, May E. Twenty years of p53 research: structural and functional aspects of the p53 protein. Oncogene. 1999 Dec 13;18(53):7621-36.

Mayer A, Takimoto M, Fritz E, Schellander G, Kofler K, Ludwig H. The prognostic significance of proliferating cell nuclear antigen, epidermal growth factor receptor, and mdr gene expression in colorectal cancer. Cancer. 1993 Apr 15;71(8):2454-60.

Meulmeester E, Pereg Y, Shiloh Y, Jochemsen AG. ATM-mediated phosphorylations inhibit Mdmx/Mdm2 stabilization by HAUSP in favor of p53 activation. Cell Cycle. 2005 Sep;4(9):1166-70.

Milner J., Medcalf E. A. Cotranslation of activated mutant p53 with wild type drives the wild-type p53 protein into the mutant conformation. Cell, 1991;65*:* 765-774

Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell. 1995 Jan 27;80(2):293-9.

Momand J, Zambetti GP, Olson DC, George D, Levine AJ.The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell. 1992;69:1237-45.

Morgenbesser SD, Williams BO, Jacks T, DePinho RA. p53-dependent apoptosis produced by Rb-deficiency in the developing mouse lens. Nature. 1994 Sep 1;371(6492):72-4.

Mowat M, Cheng A, Kimura N, Bernstein A, Benchimol S.Rearrangements of the cellular p53 gene in erythroleukaemic cells transformed by Friend virus. Nature. 1985;314:633-6.

Müller M, Wilder S, Bannasch D, Israeli D, Lehlbach K, Li-Weber M, Friedman SL, Galle PR, Stremmel W, Oren M, Krammer PH. p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs. J Exp Med. 1998 Dec 7;188(11):2033-45.

Muller S, Berger M, Lehembre F, Seeler JS, Haupt Y, Dejean A. c-Jun and p53 activity is modulated by SUMO-1 modification. J Biol Chem. 2000 May 5;275(18):13321-9.

Muñoz-Gámez JA, Martín-Oliva D, Aguilar-Quesada R, Cañuelo A, Nuñez MI, Valenzuela MT, Ruiz de Almodóvar JM, De Murcia G, Oliver FJ.

Nelson WG, Kastan MB.DNA strand breaks: the DNA template alterations that trigger p53-dependent DNA damage response pathways. Mol Cell Biol. 1994 Mar;14(3):1815-23.

Nigro JM, Baker SJ, Preisinger AC, Jessup JM, Hostetter R, Cleary K, Bigner SH, Davidson N, Baylin S, Devilee P, et al. Mutations in the p53 gene occur in diverse human tumour types Nature. 1989;342:705-8.

Ogawara Y, Kishishita S, Obata T, Isazawa Y, Suzuki T, Tanaka K, Masuyama N, Gotoh Y. Akt enhances Mdm2-mediated ubiquitination and degradation of p53. J Biol Chem. 2002 Jun 14;277(24):21843-50. Epub 2002 Mar 28.

Oliner JD, Pietenpol JA, Thiagalingam S, Gyuris J, Kinzler KW, Vogelstein B. Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. Nature. 1993 Apr 29;362(6423):857-60.

Olivier M, Hollstein M, Hainaut P. TP53 mutations in human cancers: origins, consequences, and clinical use. Cold Spring Harb Perspect Biol. 2010;2:a001008.

Olivier M, Langerød A, Carrieri P, Bergh J, Klaar S, Eyfjord J, Theillet C, Rodriguez C, Lidereau R, Bièche I, Varley J, Bignon Y, Uhrhammer N, Winqvist R, Jukkola-Vuorinen A, Niederacher D, Kato S, Ishioka C, Hainaut P, Børresen-Dale AL. The clinical value of somatic TP53 gene mutations in 1,794 patients with breast cancer. Clin Cancer Res. 2006;12:1157-67.

Olivier M.,Goldgar D.E.,Sodha N., Ohgaki H., Kleihues P., Hainaut P.Eeles R.A. Li-Fraumeni and related syndromes: correlation between tumor type, family structure, and TP53 genotype. Cancer Res. 2003;63:6643-6650.

Park D. J., Nakamura H., Chumakov A. M., Said J. W., Miller C. W., Chen D. L., Koeffler H. P. Transactivational and DNA binding abilities of endogenous p53 in p53 mutant cell lines. Oncogene, 1994;9*:* 1899-1906.

Petitjean A, Mathe E, Kato S, Ishioka C, Tavtigian SV, Hainaut P, Olivier M. Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. Hum Mutat. 2007 Jun;28(6):622-9.

Plummer ER. Inhibition of poly(ADP-ribose) polymerase in cancer. Curr Opin Pharmacol 2006; 6: 364–8

Quelle DE, Zindy F, Ashmun RA, Sherr CJ. Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. Cell. 1995 Dec 15;83(6):993-1000.

Ratnam K, Low JA. Current development of clinical inhibitors of poly(ADP-ribose)polymerase in oncology. Clin Cancer Res 2007; 13: 1383–8.

Reeve JG, Xiong J, Morgan J, Bleehen NM. Expression of apoptosis-regulatory genes in lung tumour cell lines: relationship to p53 expression and relevance to acquired drug resistance. Br J Cancer. 1996 May;73(10):1193-200.

Restle A, Färber M, Baumann C, Böhringer M, Scheidtmann KH, Müller-Tidow C, Wiesmüller L. Dissecting the role of p53 phosphorylation in homologous recombination provides new clues for gain-of-function mutants. Nucleic Acids Res. 2008;36:5362-75.

Rieber M, Strasberg Rieber M. DN-R175H p53 mutation is more effective than p53 interference in inducing epithelial disorganization and activation of proliferation signals in human carcinoma cells: role of E-cadherin. Int J Cancer. 2009 Oct 1;125(7):1604-12.

Riley T, Sontag E, Chen P, Levine A. Transcriptional control of human p53-regulated genes. Nat Rev Mol Cell Biol. 2008 May;9(5):402-12.

Russell DW, Hirata RK, Inoue N. Validation of AAV-mediated gene targeting.Nat Biotechnol. 2002 Jul;20(7):658.

Russell DW, Hirata RK. Human gene targeting by viral vectors. Nat Genet. 1998 Apr;18(4):325-30.

Ryan JJ, Danish R, Gottlieb CA, Clarke MF.Cell cycle analysis of p53-induced cell death in murine erythroleukemia cells. Mol Cell Biol. 1993 Jan;13(1):711-9.

Sakamuro D, Sabbatini P, White E, Prendergast GC.The polyproline region of p53 is required to activate apoptosis but not growth arrest. Oncogene. 1997;15:887-98.

Sarnow P, Ho YS, Williams J, Levine AJ. Adenovirus E1b-58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54 kd cellular protein in transformed cells. Cell. 1982;28:387-94.

Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. he E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell. 1990;63:1129-36.

Schreiber V, Dantzer F, Ame J-C, de Murcia G. Poly (ADP-ribose): novel functions for an old molecule. Nat Rev Mol Cell Biol 2006; 7: 517–28.

Shaulsky G, Goldfinger N, Ben-Ze'ev A, Rotter V.Nuclear accumulation of p53 protein is mediated by several nuclear localization signals and plays a role in tumorigenesis.Mol Cell Biol. 1990;10:6565-77.

Shaw P, Freeman J, Bovey R, Iggo R. Regulation of specific DNA binding by p53: evidence for a role for O-glycosylation and charged residues at the carboxy-terminus. Oncogene. 1996 Feb 15;12(4):921-30.

Sherr CJ. Tumor surveillance via the ARF-p53 pathway. Genes Dev. 1998 Oct 1;12(19):2984-91. Review.

Sigal A., Rotter V. Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome. Cancer Res. 2000;60:6788-6793.

Slebos RJ, Lee MH, Plunkett BS, Kessis TD, Williams BO, Jacks T, Hedrick L, Kastan MB, Cho KR. p53-dependent G1 arrest involves pRB-related proteins and is disrupted by the human papillomavirus 16 E7 oncoprotein. Proc Natl Acad Sci U S A. 1994;91(12):5320-4.

Song H, Hollstein M, Xu Y. p53 gain-of-function cancer mutants induce genetic instability by inactivating ATM. Nat Cell Biol. 2007;9:573-80.

Song H, Xu Y. Gain of function of p53 cancer mutants in disrupting critical DNA damage response pathways. Cell Cycle. 2007;6:1570-3.

Soussi T, Béroud C. Assessing TP53 status in human tumours to evaluate clinical outcome. Nat Rev Cancer. 2001;1:233-40.

Soussi T. p53 alterations in human cancer: more questions than answers. Oncogene. 2007; 26(15):2145-56.

Speir E, Modali R, Huang ES, Leon MB, Shawl F, Finkel T, Epstein SE. Potential role of human cytomegalovirus and p53 interaction in coronary restenosis. Science. 1994;265:391-4.

Srivastava S, Zou ZQ, Pirollo K, Blattner W, Chang EH.Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. Nature. 1990;348:747-9.

Sur S, Pagliarini R, Bunz F, Rago C, Diaz LA Jr, Kinzler KW, Vogelstein B, Papadopoulos N.A panel of isogenic human cancer cells suggests a therapeutic approach for cancers with inactivated p53. Proc Natl Acad Sci U S A. 2009 Mar 10;106(10):3964-9. Epub 2009 Feb 18.

Szekely L, Selivanova G, Magnusson KP, Klein G, Wiman KG. EBNA-5, an Epstein-Barr virus-encoded nuclear antigen, binds to the retinoblastoma and p53 proteins. Proc Natl Acad Sci U S A. 1993;90:5455-9.

Tao W, Levine AJ. P19(ARF) stabilizes p53 by blocking nucleo-cytoplasmic shuttling of Mdm2. Proc Natl Acad Sci U S A. 1999 Jun 8;96(12):6937-41.

Topaloglu O, Hurley PJ, Yildirim O, Civin CI, Bunz F. Improved methods for the generation of human gene knockout and knockin cell lines. Nucleic Acids Res. 2005 Oct 7;33(18):e158.

Unger T., Mietz J. A., Scheffner M., Yee C. L., Howley P. M. Functional domains of wild-type and mutant p53 proteins involved in transcriptional regulation, transdominant inhibition and transformation suppression. Mol. Cell. Biol., 13: 5186-5194, 1993.

Vassilev LT, Vu BT, Graves B, Carvajal D, Podlaski F, Filipovic Z, Kong N, Kammlott U, Lukacs C, Klein C, Fotouhi N, Liu EA. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. Science. 2004 Feb 6;303(5659):844-8. Epub 2004 Jan 2.

Vikhanskaya F, Lee MK, Mazzoletti M, Broggini M, Sabapathy K. Cancer-derived p53 mutants suppress p53-target gene expression-potential mechanism for gain of function of mutant p53. Nucleic Acids Res. 2007;35:2093-104.

Vogelstein B, Kinzler KW. The multistep nature of cancer. Trends Genet. 1993;9:138-41.

Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature. 2000;408:307-10.

Vogelstein B, Kinzler KW.p53 function and dysfunction. Cell. 1992;70:523-6.

Vousden KH, Lane DP.p53 in health and disease. Nat Rev Mol Cell Biol. 2007;8:275-83.

Waldman T, Kinzler KW, Vogelstein B. p21 is necessary for the p53-mediated G1 arrest in human cancer cells. Cancer Res. 1995 Nov 15;55(22):5187-90.

Waldman T, Zhang Y, Dillehay L, Yu J, Kinzler K, Vogelstein B, Williams J.Cell-cycle arrest versus cell death in cancer therapy. Nat Med. 1997 Sep;3(9):1034-6.

Walker KK, Levine AJ.Identification of a novel p53 functional domain that is necessary for efficient growth suppression. Proc Natl Acad Sci U S A. 1996;93:15335-40.

Wang LH, Okaichi K, Ihara M, Okumura Y. Sensitivity of anticancer drugs in Saos-2 cells transfected with mutant p53 varied with mutation point. Anticancer Res. 1998 Jan-Feb;18(1A):321-5.

Wang SP, Wang WL, Chang YL, Wu CT, Chao YC, Kao SH, Yuan A, Lin CW, Yang SC, Chan WK, Li KC, Hong TM, Yang PC. p53 controls cancer cell invasion by inducing the MDM2-mediated degradation of Slug. Nat Cell Biol. 2009;11:694-704.

Wang XW, Forrester K, Yeh H, Feitelson MA, Gu JR, Harris CC. Hepatitis B virus X protein inhibits p53 sequence-specific DNA binding, transcriptional activity, and association with transcription factor ERCC3. Proc Natl Acad Sci U S A. 1994 Mar 15;91(6):2230-4.

Weisz L, Oren M, Rotter V. Transcription regulation by mutant p53. Oncogene. 2007 Apr 2;26(15):2202-11. Review.

Willis A, Jung EJ, Wakefield T, Chen X. Mutant p53 exerts a dominant negative effect by preventing wild-type p53 from binding to the promoter of its target genes. Oncogene. 2004 Mar 25;23(13):2330-8.

Wolf D, Harris N, Rotter V. Reconstitution of p53 expression in a nonproducer Ab-MuLV-transformed cell line by transfection of a functional p53 gene. Cell. 1984;38:119-26.

Wong RP, Tsang WP, Chau PY, Co NN, Tsang TY, Kwok TT. p53-R273H gains new function in induction of drug resistance through down-regulation of procaspase-3. Mol Cancer Ther. 2007;6:1054-61.

Yang X, Pater A, Tang SC. Cloning and characterization of the human BAG-1 gene promoter: upregulation by tumor-derived p53 mutants. Oncogene. 1999;18:4546-53.

Yin Y, Tainsky MA, Bischoff FZ, Strong LC, Wahl GM. Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. Cell. 1992;70

Yonish-Rouach E, Grunwald D, Wilder S, Kimchi A, May E, Lawrence JJ, May P, Oren M. p53-mediated cell death: relationship to cell cycle control. Mol Cell Biol. 1993 Mar;13(3):1415-23.

Yonish-Rouach E, Resnitzky D, Lotem J, Sachs L, Kimchi A, Oren M. Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. Nature. 1991 Jul 25;352(6333):345-7.

Zabludoff SD, Deng C, Grondine MR, Sheehy AM, Ashwell S, Caleb BL, Green S, Haye HR, Horn CL, Janetka JW, Liu D, Mouchet E, Ready S, Rosenthal JL, Queva C, Schwartz GK, Taylor KJ,Tse AN, Walker GE, White AM. AZD7762, a novel checkpoint kinase inhibitor, drives checkpoint abrogation and potentiates DNA-targeted therapies. Mol Cancer Ther. 2008 Sep;7(9):2955-66.

Zakut-Houri R, Bienz-Tadmor B, Givol D, Oren M.Human p53 cellular tumor antigen: cDNA sequence and expression in COS cells. EMBO J. 1985;4:1251-5.