# UNIVERSITÀ DEGLI STUDI DI TRIESTE

## XXVIII CICLO DEL DOTTORATO DI RICERCA IN

## BIOMEDICINA MOLECOLARE

# Serine 249 phosphorylation and Pin1-induced isomerization activate mutant p53 R249S gain-of-function and promote cross-talk with JAK/STAT1 signalling in hepatocellular carcinoma

Settore scientifico-disciplinare: **BIO/13**


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*To all People who believe in me*

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# ABSTRACT

Evidences from basic and clinical studies clearly established that mutations of the tumour suppressor TP53 contribute to carcinogenesis. Unlike the majority of tumour suppressors, the *TP53* gene is often found to undergo missense mutations, with a strong dominance in the DNA-binding domain. Mutant p53 proteins contribute to carcinogenesis by losing tumour suppressor activities, by exerting dominant negative effects over the wild-type allele and by acquiring gain-of-function properties. These oncogenic activities enforce tumour growth, EMT, metastasis and chemoresistance. It has been clearly demonstrated that mutations on p53 are not sufficient to drive tumorigenesis *per se* but mutant p53 needs to be activated by specific oncogenic signalling produced by a pro-tumorigenic cellular background to be active as an oncogene. Moreover, understanding what are the environmental factors that account for the selection of specific p53 mutant forms in different tumours is critical to identify novel determinants of carcinogenesis and to find new putative targets for cancer therapy.

Among the frequently mutated residues in p53, Arginine 249 is replaced in about 65% of cases with a Serine (mutp53 R249S). Since Arginine 249 regulates the secondary structure of p53 loops 2 and 3, its substitution is responsible for a local distortion of the molecule, which reaches a dynamic equilibrium between a native and a native-like conformation. Although mutp53 R249S displays an overall low frequency in tumours bearing missense mutations in p53, it is detected in up to 90% of hepatocellular carcinomas (HCC) occurring in populations exposed to HBV infections and aflatoxin $B_1$ food-contamination, which represent two major risk factors for the HCC in the east of Asia and sub-Saharan Africa. In these areas, since R249S mutation occurs early in the pathway leading to HCC, it is considered an early biomarker of hepatocarcinogenesis. Interestingly, R249S mutation is associated to a bad prognosis and to the expression of a stem cell-associated gene signature. Although *in vitro* studies show that mutant p53 R249S is able to promote the proliferation of HCC cell lines and that the co-expression with the HBV-oncogene HBx provides further oncogenic properties, a specific mechanism of gain of function is still missing.

In this thesis, it is shown that mutation of the Arginine 249 introduces a residue phosphorylated in liver and breast cancer cells, and that the Dual-specificity Tyrosine regulated kinase 2, DYRK2, is the main kinase for Serine 249 phosphorylation. Moreover, it is provided evidence that Serine 249 phosphorylation creates a new binding site for Pin1,

which is responsible for the distorted conformation of mutant p53 R249S. Cancer cells depleted of mutp53 R249S show an impairment in their proliferation, which is unleashed by Pin1 activity over mutant p53. By analyzing the transcription profile of Mahlavu cells upon knocking down of mutp53 R249S, it has been identified the JAK/STAT pathway as molecular axis which sustains the mutant p53 R249S activity.

This study unveils a novel post-translational modification signalling converging in mutant p53 R249S required for the gain-of-function of the protein and suggests a possible opportunity for therapeutic intervention.

# INTRODUCTION

The first cause of death worldwide is cancer, with 14,1 million new cancer cases and 8,2 million cancer deaths in 2012 (IARC p53 Database, http://p53.iarc.fr/). Cancer develops through a multistep mutagenic process that allows normal cells to progressively evolve towards a neoplastic state. Along this process cancer cells acquire common features, known as the "Hallmarks of Cancer" including: sustained proliferative signalling, evasion of growth suppressive checkpoints, resistance to cell death, replicative immortality, induction of angiogenesis, activation of invasion and metastasis, deregulation of cellular metabolism and evasion of the immune system (Hanahan and Weinberg, 2011). Accumulation of mutations in cancer genomes mainly occur in proto-oncogenes and tumour suppressor genes. The first group features well-known drivers of tumorigenesis like Ras, Myc or PI3K, in the second class we can find gatekeeper genes like Rb or PTEN, as well as checkpoint genes, crucial to preserve genomic stability and coordinate tumour suppressive responses to oncogenic stress conditions. A prototypical tumour suppressor belonging to this last group is p53, widely known as the guardian of the genome. The *TP53* gene is the most commonly mutated gene in human cancers, and its pathway results inactivated in most human tumors (Murray-Zmijewski et al., 2008).

# The tumour suppressor p53

The p53 protein, encoded by the *TP53* gene, is able to integrate different physiologic and pathologic *stimuli* and to orchestrate adequate cellular responses in order to maintain genomic stability and restrain oncogenic transformation and cancer outgrowth. In response to a variety of *stimuli* that a cell might encounter during malignant progression such as genotoxic stress, oncogene activation, loss of normal cell contacts and hypoxia, p53 is able to elicit antiproliferative cellular responses (Mantovani et al., 2015). These include, either transient or permanent cell cycle arrest and cell death. The importance of these p53-induced responses for tumour suppression has been demonstrated in different mouse models (Martins, Brown-Swigart, & Evan, 2006). Restoration of p53 expression in p53 KO lymphomas led to their widespread apoptosis, whereas restoration of p53 in sarcomas and hepatocarcinomas led to a senescence-type response. p53 growth inhibitory functions are normally held dormant, and the p53 protein, present at low levels in the cells, gets stabilized and activated in response to a variety of tumour-related stress conditions (Mantovani et al., 2015). In unstressed cells, p53 is maintained at low levels through degradation, mainly mediated by the MDM2 E3-ubiquitin ligase in complex with the related protein MDM4 (known as MDMX in humans) (Finch et al., 2002; Wadgaonkar & Collins, 1999). Stress-induced post-translational modifications of both p53 and MDM2/MDM4 abolish their interaction leading to p53 accumulation and induction of its transcriptional activity (Toledo and Wahl, 2006); once active, p53 can mediate different biological processes depending on the entity and type of damage. The best characterized p53 responses are temporary cell cycle arrest to favour DNA repair, programmed cell death (apoptosis) and permanent cell cycle arrest (senescence). In addition to these classical responses, p53 can regulate several cellular processes such as metabolic reprogramming, accumulation of reactive oxygen species (ROS), autophagy, stem cell self-renewal, invasion and metastasis (Figure 1). Regulation of these processes by p53 may directly promote tumour suppression or may impinge on the canonical functions, such as apoptosis or senescence (Bieging et al., 2014).

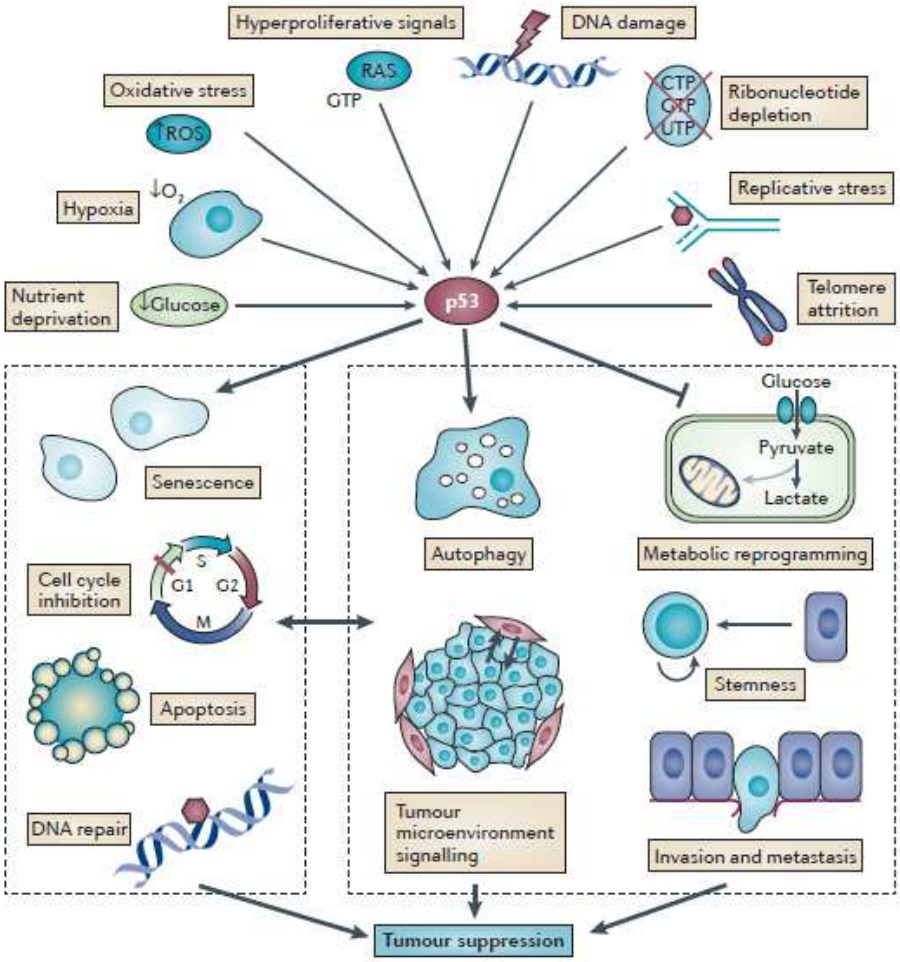



**Figure 1. p53-activating signals and responses that are important for tumour suppression** (Bieging et al., 2014).

Independently of the specific function, p53 mainly acts as a transcription factor regulating both positively and negatively the expression of an elevated number of genes, through its ability to direct bind as a tetramer to specific DNA target sequences (p53-responsive elements, p53RE) (el-Deiry, Kern, Pietenpol, Kinzler, & Vogelstein, 1992; Laptenko & Prives, 2006) (Millau et al., 2009). However, several transcription-independent activities of p53 have been described, mainly involved in potentiating the pro-apoptotic response acting in the cytoplasm and at mitochondria (Yee & Vousden, 2005).

## p53 functional domains

The human *TP53* gene, localized on the short arm of chromosome 17 (17p13.1), encodes twelve different p53 isoforms. p53 mRNA variants are expressed in a tissue-dependent manner, indicating that the internal promoter and the alternative splicing forms of p53 can be regulated (Jean-Christophe Bourdon et al., 2005). In addition, p53 belongs to a family of

related proteins, which includes p63 and p73; these proteins exert important roles in organism development and both possess tumour suppressive activities (Murray-Zmijewski et al. 2008). The p53 protein is characterized by five functional domains (Figure 2): the trans-activation domain (TAD), the proline-rich domain (PRD), the DNA-binding domain (DBD), the oligomerization domain (OD) and the C-terminal domain (CTD).

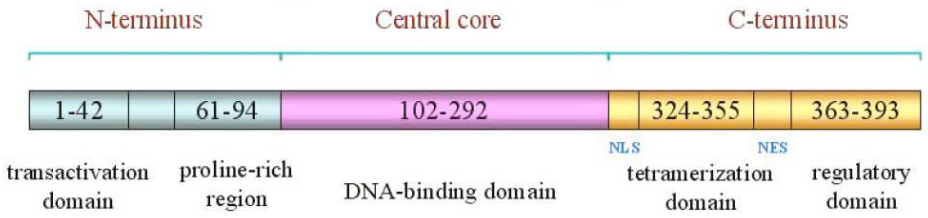



**Figure 2. Schematic representation of p53 domains**. p53 contains 393 amino acids, consisting of three main functional domains: N-terminal activation domain, DNA binding domain and C-terminal tetramerization domain. The N-terminal domain includes trans-activation sub-domain and a PXXP region that is a proline-rich fragment. The central DNA binding domain is required for sequence-specific DNA binding and amino acid residues within this domain are frequently mutated in human cancer cells and tumour tissues. The C-terminal region is considered to perform a regulatory function. Residues on this basic C-terminal domain undergo post-translational modifications including phosphorylation and acetylation. Numbers indicate residue number. NLS, nuclear localization signal sequence; NES, nuclear export signal sequence. (Bai et al., 2006)

- The TAD is in the N-terminal region of the protein and it contains two different acidic domains (TAD1 and TAD2), both required for induction of transcription. This domain also contains the binding site for the major negative regulator of p53, the ubiquitin ligase Mdm2 (Toledo & Wahl, 2006).
- The PRD contains five repeats of the amino acid motif PXXP (where P is proline and X any amino acid) (Walker & Levine, 1996). This domain is involved in the stabilization of the protein, through the activity of the prolyl isomerase Pin1, that binds to Thr81-Pro82 site upon phosphorylation, and induces a conformational change, thus reducing the Mdm2 binding, increasing p53 stability (Zacchi et al., 2002).
- The DBD is required for p53 function as a transcriptional activator (el-Deiry et al., 1992). The importance of sequence specific DNA binding for p53 to function as a tumour suppressor is highlighted by the fact that 97% of tumour-associated mutations are clustered in this domain (Sigal & Rotter, 2000).
- The OD is necessary for tetramerization that is required for high-affinity DNA binding and transcriptional activation.
- The CTD is a regulatory domain, due to the great number of post-translational modifications it undergoes upon stress signalling, able to finely modulate p53 activity.

Moreover the CTD contains a cluster of three nuclear localization signals (NLS) that mediate the migration of the protein into the cell nucleus.

## Regulation of p53 through post-translational modifications

Post-translational modifications (PTMs) can have strong effects on the proteins functions, through the conformational changes. p53 undergoes a great variety of PTMs that influence its stability and its transcriptional activity. These include phosphorylation of Serines and Threonines, acetylation, mono- and poly-ubiquitination, sumoylation, neddylation and methylation of Lysines. The pattern of phosphorylation in the N-terminal domain shows significant redundancy: a single site can be phosphorylated by multiple kinases and a single kinase can phosphorylate multiple sites (Dai & Gu, 2010). In general many kinases that phosphorylate p53 after DNA damage have been found, such as ATM, ATR, JNK, HIPK2 and DYRK2. The majority of the phosphorylation sites detected are phosphorylated following cellular stress, although a few are constitutively phosphorylated in unstressed cells and de-phosphorylated following stress, as Thr55 and Ser376 (Bode & Dong, 2004). The most extensively studied pattern of PTMs is the N-terminal domain one. Among the phoshorylable sites there is Ser15, phosphorylated by ATM, that is important for the modulation of the interaction between p53 and Mdm2 (Gu & Zhu, 2012), so for the p53 stability. Another site is Ser46, whose phosphorylation is involved in the transcription of pro-apoptotic genes. It is phosphorylated mainly by HIPK2 and DYRK2, but also by ATM, p38 MAPK and others, upon stress conditions. The redundancy indicates that this phosphorylation site is really important for the regulation and the functions of p53 (Smeenk et al., 2011). Another kind of PTMs is the acetylation on the lysine residues. This PTM occurs in response to DNA damage agents, and it is exerted by different HAT (Histone Acetyl Transferase). The role of acetylations is to facilitate the recruitment of co-factors for p53 transcriptional activation, indeed this modification is involved in the choice of the targets gene activated by p53 (Dai & Gu, 2010). The complicated pattern of PTMs occurring on p53 protein is difficult to analyse and study because its redundancy and its variability, indeed it is still an open and interesting field of research.

# Mutant p53 proteins

*TP53* mutations are widespread in cancer, with frequencies that vary between 10% to nearly 96% (Rivlin, Brosh, Oren, & Rotter, 2011) in different human tumours. Alterations have been found in every region of the protein, but only a handful of the most frequently occurring mutations have been studied in depth for their contribution to cancer progression (Leroy et al., 2013). The point mutations are the most frequently found, and are divided in transitions and transversions. In some cases, frameshift or nonsense mutations result in the loss of p53 protein expression, as seen with other tumour suppressors. More frequently, the alterations associated with tumours are missense mutations, leading to the substitution of a single amino acid in the p53 protein, that can be stably expressed in the tumour cells (P. a J. Muller & Vousden, 2014). The spectrum of *TP53* missense mutations is extremely broad with more than 1800 different amino-acid changes reported, despite most of them are very rare in tumours (Soussi, 2011). Among the over 25,000 *TP53* mutations currently reported, almost a third arises in six "hot-spot" residues. These include "DNA contact" mutations in residues directly involved in DNA binding, such as R248Q and R273H, and "conformational" mutations, which cause local or global conformational distortions (R175H, R282W, R249S and G245S) (Figure 3).

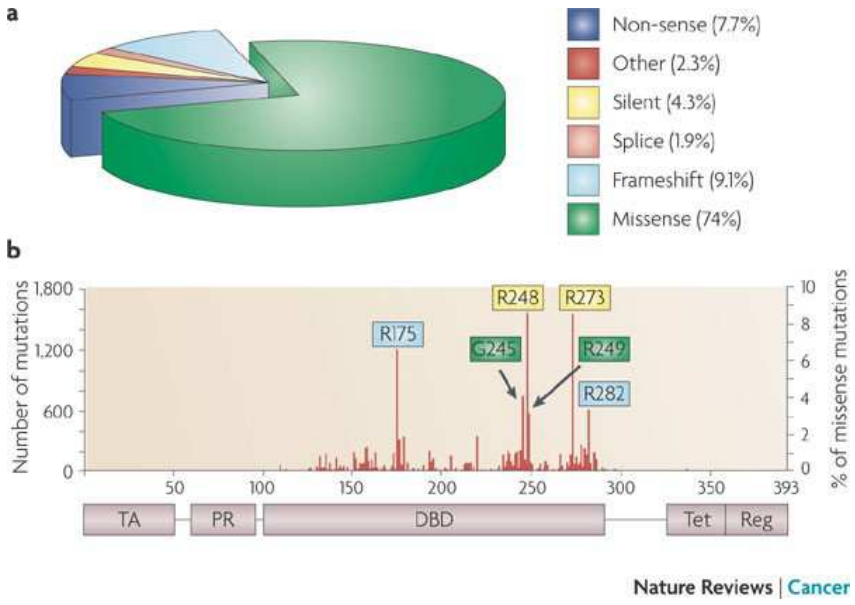



**Figure 3. Distribution of *TP53* somatic mutations according to the IARC TP53 Mutation Database.** A) Pie chart representing the different tumour-derived mutation types reported in the IARC TP53 Mutation Database. B) The distribution of reported missense mutations along the 393 amino-acid sequence of p53. The six most common hotspot mutations are highlighted in yellow for DNA-contact mutations, green for locally distorted mutants and blue for globally denatured mutants. The domain architecture of p53 is aligned below. PR, proline-rich domain; Reg, carboxy-terminal regulatory domain; TA, transactivation domain; Tet, tetramerization domain. (Brosh and Rotter, 2009).

Whereas somatic p53 mutations are associated with sporadic cancer, germline mutations cause a rare type of cancer predisposition known as Li-Fraumeni Syndrome (LFS), which is not associated with site-specific tumours, but rather with a variety of tumour types occurring at a relatively early age (Li & Fraumeni, 1969).

In contrast to wild-type p53, that is maintained at very low levels and is a short-lived protein, mutant p53 shows a prolonged half-life and often accumulates in tumour cells (Strano et al., 2007). The overlapping ways in which mutant p53 (mutp53) can contribute to cancer development are: loss of the wild-type functions, dominant negative effects over the remaining wild-type p53 and gain-of-function (Figure 4).

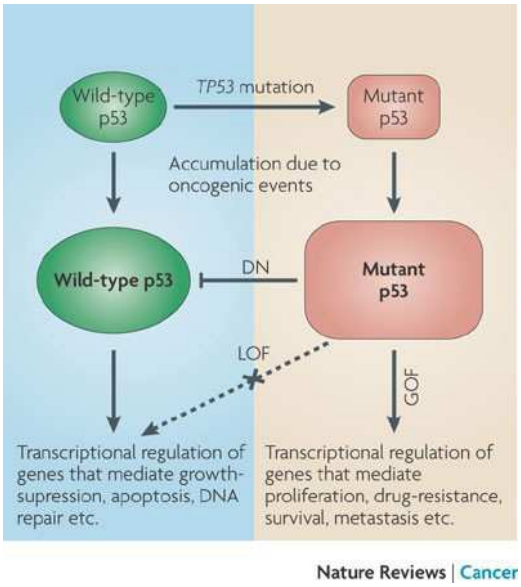



**Figure 4. Functional impact of *TP53* mutations.** First, most mutations observed in human tumours abrogate or attenuate the binding of p53 to its consensus DNA sequence and, consequently, impede the transcriptional activation of p53 target genes. Second, most missense mutations, but usually not the 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. Moreover, several mutations were shown to confer mutant p53 with new functions that are independent of wild-type p53 (Brosh and Rotter, 2009).

Mutp53 is unable to bind p53-consensus sites in target gene promoters, so the wild-type p53 trans-activation functions are lost (P. a J. Muller & Vousden, 2014). Coherenlty p53-null mice are extremely tumour-prone, indicating that loss of wild-type p53 activity is a significant component of tumour predisposition (Freed-pastor & Prives, 2012). Mutant p53 can further exert a dominant negative effect on the protein produced by the remaining wild-type allele, abrogating the ability of wild-type p53 to inhibit cellular transformation, particularly when the mutant protein is expressed in stoichiometric excess of its wild-type counterpart (Oren & Rotter, 2010). This effect on wild-type p53 can be caused by the formation of mutant/wild-type p53 co-tetramers or by the incorporation of wild-type p53 into mutant p53 supra-tetrameric aggregates (Xu et al., 2011). Loss of wild-type p53 functions is clearly

tumorigenic. Beyond this, however, tumor cells acquire a selective advantage by retaining only the mutant form of the protein, since this is able to actively promote transformation by virtue of neomorphic features known as Gain of Function (GOF). The existence of GOF activities is supported by *in vivo* evidence showing that mice expressing mutant p53 display a tumour profile that is more aggressive and metastatic than p53-null or wild-type p53 mice (Olive et al., 2004). Numerous cell-based assays also demonstrate implication of mutant p53 in actively supporting different aspects of tumorigenesis, including proliferation, invasion, migration, metastasis formation, chemo-resistance, somatic cell reprogramming, disruption of tissue architecture, angiogenesis and others (Freed-Pastor & Prives, 2012).

## Mutant p53 gain-of-function

Mutant p53 drives the acquisition of many cancer hallmarks both through transcriptional and non-transcriptional processes (Brosh & Rotter, 2009) (Girardini et al., 2013), mainly by forming protein complexes with several interacting partners (Figure 5).

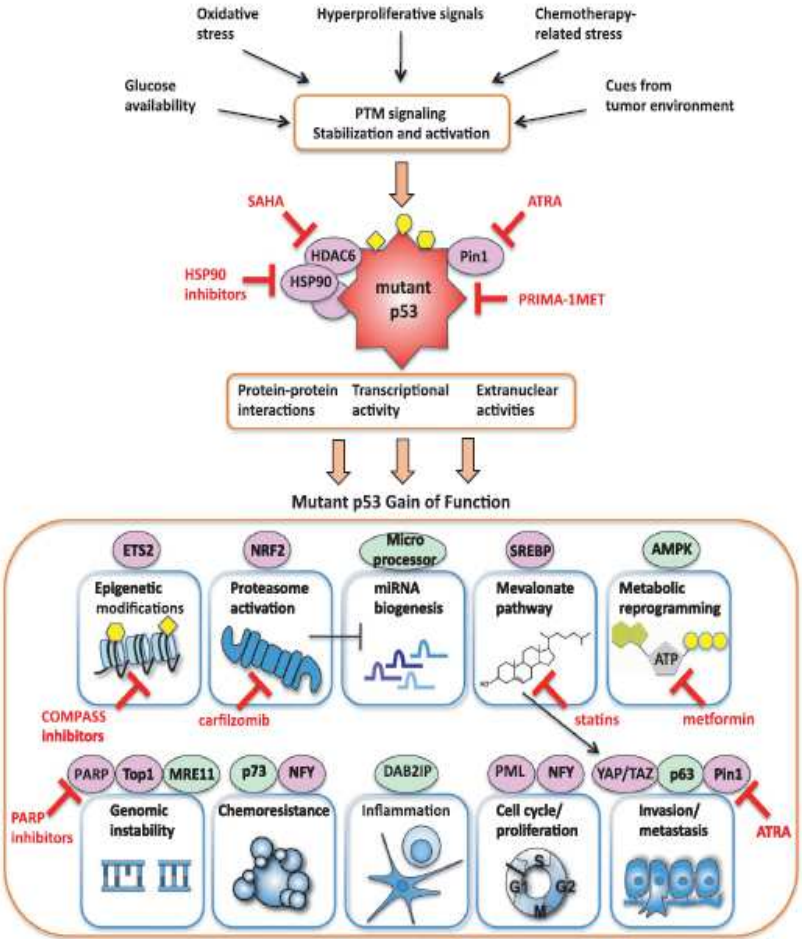



**Figure 5. The mutant p53 oncogenic network**. Several cancer-related conditions concur to stabilization and activation of mutant p53 in tumor cells, including hyper-proliferative signals (also promoting chronic DNA

damage and oxidative stress), glucose availability, and chemotherapy-related stress. Signal transduction cascades lead to post-translational modifications (PTM) of mutant p53 and enable its gain-of-function activities (GOF). Oncogenic functions of p53 missense mutants are highly pleiotropic (square boxes) and rely on partnerships with multiple cellular factors (pink circles indicate active partners of mutant p53, while green circles indicate proteins inhibited by mutant p53). Regulation of cellular processes by p53 missense mutants may directly promote tumorigenesis (e.g., transcription-mediated increase of proliferation, metastatic spread, and chemoresistance) or may indirectly impinge on oncogenic pathways through rewiring energy metabolism, mevalonate pathway, affecting proteasome activity and miRNA biogenesis, and impacting the tumor stroma. Integration of multiple GOF activities determines mutant p53-dependent tumorigenicity in a given tumor context. Importantly, mutant p53 GOF mechanisms disclose actionable targets and molecules for precision therapies (examples are indicated in red) (Mantovani et al., 2016).

The available mutant p53 ChIP-sequencing data and other DNA-interaction informations have failed to define a mutant p53 target site analogous to that of wild-type p53, and currently the main hypothesis is that mutant p53 dependent trans-activation takes place through interaction with several partner transcription factors and cofactors (Walerych et al., 2015). One of these is NF-Y. The interaction between mutant p53 and NF-Y results in up-regulation of NF-Y target genes, driving deregulation of cell cycle check-points following induction of DNA damage (Di Agostino et al., 2006). Moreover, mutant p53 has been identified as a tumour specific, YAP-transcriptional partner. Associated to YAP, mutant p53 forms a complex with NF-Y, and aberrantly promotes the expression of cell cycle-related genes (Di Agostino et al., 2015). Another transcription cofactor is Sp1; the interaction of mutant p53 with Sp1 was shown to elicit cooperative effects and amplify the activating effects of Sp1 on transcription (Strano et al., 2007). In normal conditions both NF-Y and Sp1 interact with wild-type p53. Interestingly, the interactions of these proteins with wild-type p53 often result in opposite transcriptional outcomes than those observed with mutant p53. Recently, a combination of "omics" methods allowed to identify mutant p53 as partner of the transcription factor NRF2 in the induction of proteasome genes thus increasing proteasome activity in several cancer models and in triple negative breast cancer (TNBC) patients (Walerych et al., 2016). p53 mutants can also establish neomorphic interactions with transcriptional regulators that are not bound by wild-type p53. For instance, the altered DNA-binding domain of mutant p53 mediates interaction with the p53 homologs p63 and p73 (Strano et al., 2000 and 2002), resulting in inhibition of p73-dependent apoptosis and chemosensitivity (Bergamaschi et al., 2003), as well as suppression of TA-p63 anti-metastatic target genes (Adorno et al., 2009; Girardini et al., 2011).

Interestingly, it has been reported that mutant p53 leads to high expression of sterol biosynthesis genes in human breast tumours. Indeed, mutant p53 associates with sterol responsive elements (SRE) within promoters of sterol-regulated genes, acting as transcriptional co-activator for the sterol regulatory element-binding protein transcription

factors, SREBP (Freed-Pastor et al., 2012) to enhance the expression of genes involved in the mevalonate pathway and in protein prenylation. Downstream of the mevalonate pathway act the pro-oncogenic transcription co-activators YAP/TAZ, effectors of the Hippo pathway. In this context, mutant p53 and SREBP, sustaining the mevalonate pathway, trigger unscheduled activation of YAP/TAZ in both cancer cells and human primary tumours. For this reason, YAP/TAZ have been suggested as critical effector of the pro-oncogenic function of mutant p53 (Sorrentino et al. 2014).

Recently it has been discovered that mutant p53 exerts its gain-of-function also through the direct interaction with ETS2 allowing the up-regulation of some epigenetic regulators, as MLL1, MLL2 and MOZ. The effect is a genome-wide increase of histone methylation and acetylation, that promotes cancer cell proliferation (Zhu et al., 2015).

In addition to the effects on the transcriptional machinery, mutant p53 can interact with other proteins to change their function directly. For example, interacting with MRE11, mutant p53 prevents the MRE11-RAD50-NSB1 complex from activating ATM, leading to impaired homologous recombination (Song et al., 2007).

Although less investigated, cytoplasmic activities of mutant p53 are relevant for its oncogenic potential. Alteration of cancer cell metabolism by mutant p53 entails direct inhibition of AMPK in the cytoplasm (Zhou et al., 2014). Cytoplasmic localization of mutant p53 was identified as an important feature for its ability to suppress autophagy (Morselli et al., 2008), thus supporting tumor cell survival. In addition, mutant p53 has also been reported to inhibit apoptosis through cytoplasmic activities (Frank et al., 2011; Chee et al., 2013). Moreover, the extranuclear functions of mutant p53 include regulation of the DAB2IP protein, which affects TNFα-dependent signalling (Di Minin et al. 2014).

## Regulation of mutant p53 by the tumour context

Clearly established through the study of mutant p53 knock-in mouse models (Olive et al. 2004; Lang et al. 2004), a remarkable property of p53 mutants is the dependency on a transformed cell context for full activation of their malignant potential. A paradigmatic aspect is the selective accumulation of p53 mutant proteins in tumors, as opposed to their inherent instability in normal tissues (Terzian et al., 2008). Constitutive inhibition of mutant p53 degradation occurs exclusively in transformed cells, and is critical for reaching the high protein amounts required for GOF manifestation. The molecular mechanisms acting in tumor cells to shelter mutant p53 from ubiquitin-mediated degradation are only partially understood.

Different groups have indicated the Hsp90 chaperone machinery as a major player (Blagosklonny et al., 1996). This system includes Hsp90, Hsp70 and other co-chaperones, and is aberrantly activated with high frequency during oncogenic transformation. Hsp90 causes the functional inactivation of the ubiquitin ligases MDM2 and CHIP bound to mutant p53, thus sustaining its stability (Li et al., 2011).

A field of intense research concerns the pathways that transduce the signaling *milieu* generated within the tumor context into cues that unleash mutant p53 oncogenic potential. Notably, many of the signal transduction pathways that induce post-translational modifications (PTMs) on wild-type p53 are altered in cancers. Importantly, analysis of the TP53 tumour mutation databases reveals that the vast majority of residues subjected to post-translational modification within wild-type p53 are infrequently mutated in human tumours, suggesting that modification of the same residues that are required for wild-type p53 tumour suppressor activities may be exploited to foster mutant p53 gain-of-function. There is indeed evidence that in tumour cells mutant p53 proteins receive PTMs on the same residues as in the wild-type counterpart, and that some of these events contribute to fine-tune the functions of mutant p53 by modifying its stability, cellular localization, target gene selection and protein interactome, contributing to oncogenic GOF properties (Girardini et al., 2014). Phosphorylation on Ser15, which has a major role in the control of DNA damage-induced wild-type p53 stabilization, is also induced on mutant p53 by ERK1/2 MAP kinase (mitogen-activated protein kinase) (Melnikova et al. 2003) in response to genotoxic stress (Dun Li et al. 2011; Alsheich-Bartok et al. 2008). In conditions of chemotherapy-related stress, mutant p53 was shown to induce Polo-like kinase-2 (PLK2) gene expression. In turn, PLK2-dependent phosphorylation promotes mutant p53 acetylation and stimulates its interaction with p300 and NFY, thereby enhancing transactivation of a gene set that sustains cell proliferation and chemoresistance. Adorno et al. (2009) showed that activated RAS signalling promotes the phosphorylation of mutant p53 R280K at Ser6 and Ser9 and results in the formation of a mutant p53/SMAD complex, which in turn inhibits p63 antimetastatic activities.

Our research group demonstrated that oncogenic RAS signalling drives phosphorylation of Ser/Thr-Pro sites within mutant p53, thus promoting its interaction with the prolyl-isomerase Pin1. In triple negative breast cancer cells PIN1 enhanced mutant p53 GOF activities, in particular cooperating towards activation of a mutp53-dependent transcriptional program that increases cell migration and invasiveness (Girardini et al., 2011).

# The prolyl isomerase Pin1

Phosphorylation-dependent isomerization of cellular proteins at Ser/Thr-Pro (S/T-P) motifs is a crucial event in many signal transduction pathways, particularly those regulating cell cycle, proliferation and cellular responses to cytotoxic and genotoxic stress conditions. The structural changes resulting from proline isomerization affect protein stability, PTMs, interaction profile, subcellular localization, thus finely modulating the biological functions of key cellular proteins. Ser or Thr residues that precede Pro (Ser/Thr-Pro) are a major regulatory phosphorylation motif in cells. Enzymes that are responsible for their phosphorylation belong to a large superfamily of Pro-directed protein kinases, which include cyclin-dependent protein kinases (CDKs), extracellular signal-regulated kinases (ERKs), stress-activated protein kinases/c-Jun‑N-terminal kinases (SAPKs/ JNKs), p38 kinases, glycogen synthase kinase‑3 (GSK3) and Polo-like kinases (PLKs). These kinases have a crucial role in diverse cellular processes such as cell growth regulation, stress responses and neuronal survival, as well as in human diseases such as cancer and Alzheimer's disease (Lu et Zhou, 2007). Phosphorylated Ser/Thr-Pro motifs are recognized and isomerized by a unique member of the parvulin family of prolyl-isomerases, the conserved Pin1 enzyme. Pin1 is a protein of 163 amino acids with a mass of 18kDa that is composed of two domains: an amino terminal WW domain (amino acids 1-39) and a carboxy-terminal PPIase domain (amino acids 45-163), which are separated by a short flexible linker region. The N-terminal WW region is characterized by two conserved tryptophan residues and mediates the interaction with the substrates on pSer/Thr-Pro sites, which cannot be bound by any other isomerase (Ranganathan, Lu, Hunter, & Noel, 1997). As a consequence of the interaction, the PPIase domain can isomerize specific pSer/Thr-Pro motifs and induce conformational changes of its target proteins, resulting in alteration of their enzymatic activities, phosphorylation status, protein-protein interaction pattern, sub-cellular localization and protein stability (Cheng, Leong, & Tse, 2016). By modification of a plethora of cellular substrates Pin1 governs a variety of cellular processes including cell cycle, transcription and splicing, RNA editing, DNA damage and oxidative stress responses, germ cell development, stem cells self renewal/expansion and neuronal survival (Rustighi et al., 2014; Yeh and Means, 2007) (Figure 6).

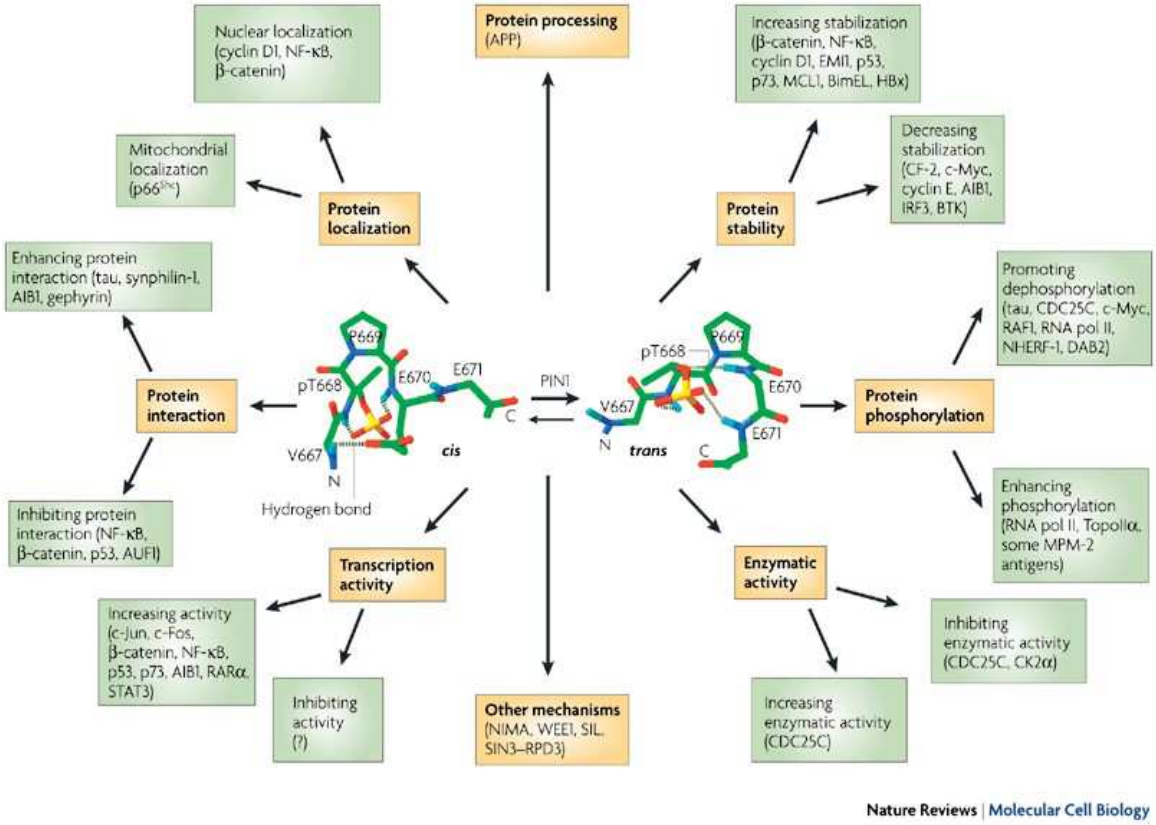



**Figure 6. PIN1-catalysed prolyl isomerization regulates a spectrum of target activities.** Phosphorylation dramatically reduces *cis/trans* isomerization of certain regulatory Ser/Thr-Pro motifs between the two completely distinct *cis* and *trans* conformations, but converts the peptide bonds as substrates specifically for Pin1. Pin1 greatly accelerates *cis* to *trans* or *trans* to *cis* isomerization depending on specific target sites and local structural constraints, thereby regulating protein conformation after phosphorylation between two distinct structures. Such conformational changes have profound effects on phosphorylation signalling by regulating a spectrum of target activities (Lu and Zhou, 2007).

In contrast to other PPIases, Pin1 is tightly regulated at multiple levels. With a few exceptions such as neurons, in normal human tissues PIN1 expression correlates with the cell proliferative potential and indeed the protein levels decrease with ageing. Conversely, the analysis of 60 different human tumours showed that PIN1 is aberrantly up-regulated or over-activated in cancer (Bao et al., 2004).

The transcription factor E2F, enhanced by oncogenic Neu or Ras, is a critical regulator of PIN1 expression (Ryo et al., 2002). Interestingly, Pin1 has also been found to interact with Rb and promote its hyper-phosphorylation, therefore inducing E2F activation (Tong et al., 2015). Moreover, Pin1 interacts with phosphorylated NOTCH1 enhancing its transcriptional activity; since in turn NOTCH1 increases PIN1 transcription, the two proteins create a positive feedback promoting tumorigenesis. In addition, PIN1 is regulated post-transcriptionally through the activity of miRNA 200b/c and miRNA-296-5P, which suppress its expression in breast and prostate cancer, respectively. Finally, PIN1 protein levels and activity are regulated

by post-translational modifications such as phosphorylation and sumoylation (Cheng et al., 2016).

## Pin1 in cancer

Pin1 has been found overexpressed in breast, prostate, lung, colon, oesophageal, ovarian and cervical cancers, human oral squamous cell cancer, glioblastoma and melanoma (Atkinson et al., 2009). Conversely, PIN1 single nucleotide polymorphisms (SNPs) decrease PIN1 expression and are associated to a reduced cancer risk (Li et al., 2013). Moreover, Pin1-null mice are resistant to tumorigenesis even upon oncogenes over-expression (HER2 or HRAS) or after mutation of tumour suppressors such as TP53 (Zhou & Lu, 2016). All these evidences underscore a prominent role of Pin1 in up-regulating key cellular pathways that are central during oncogenesis (Lu & Zhou, 2007): indeed, Pin1 has been reported to activate more than 40 oncogenes and and inactivate 20 tumour suppressors.

Pin1 is able to raise cyclin D1 levels by fostering its transcription; moreover up-regulation of cyclin D1 expression induced by oncogenic *stimuli* such as Ras, Her2/Neu and Wnt signalling pathways and by NF-κB requires the presence of Pin1 which in turn is indispensable for full activity of these pathways (Lu & Zhou, 2007; Ryo, Liou, Lu, & Wulf, 2003). Pin1 can bind to phosphorylated c-Jun, β-catenin and NF-κB boosting their protein levels and transcriptional activity, with the consequence of increasing cyclin D1 gene expression. Moreover, Pin1 is able to shield β-catenin and NF-κB from their negative regulators, APC (adenomatous polyposis coli gene product) and IkB, respectively.

One of the key signalling pathways induced upon activation of growth factor receptors is the MAPKs cascade. In response to growth *stimuli* Ras activates the Raf kinase, which in turn controls the MAPKs. As for other signalling pathways, this kinases cascade must be turned off by a negative feedback mechanism in which MAPKs phosphorylate and inactivate Raf. Notably, Pin1 prevents this negative feedback, by promoting Raf dephosphorylation and consequently maintaining the MAPKs cascade activated (Dougherty et al., 2005).

In the context of triple negative breast cancer Pin1 has been demonstrated to cooperate with Notch1 (Rustighi et al., 2009). Pin1 interacts with the Notch1 receptor and increases its *stimulus*-induced cleavage mediated by γ-secretase, allowing full activation of the pathway and boosting its activity both *in vitro* and *in vivo*. Also in this case a positive loop is generated since activated Notch is directly recruited on the Pin1 gene promoter thus inducing Pin1 expression. As a consequence, in human breast cancer samples there is a strong correlation

between high levels of activated Notch and Pin1 overexpression (Rustighi et al., 2009). Moreover it was recently demonstrated that Pin1 can modulate Notch levels also post-transcriptionally by regulating the detachment from its negative regulator Fbxw7 (Rustighi et al., 2014).

## Cooperation of Pin1 with mutant p53

As abovementioned *in vivo* studies underscored the requirement of an oncogenic context to activate mutant p53 function (Lang et al., 2004; Olive et al., 2004). Notably, the association between mutant p53 GOF and the oncogenic phosphorylation signaling converging on it is provided by Pin1-dependent prolyl-isomerization. Work by our group established that in the mono-allelic R172H p53 mouse model of LFS, Pin1 expression is required for tumor formation (Girardini et al., 2011) In particular markedly reduced tumor frequency, complete absence of carcinomas and a reduced number of lymphomas have been observed in the absence of Pin1. Prolyl isomerization of mutant p53 increases the ability of the protein to engage in complexes and inhibit the metastasis suppressor p63. In addition, a new mutant p53-dependent transcriptional program promotes metastatic progression and predicts poor prognosis of breast cancer patients. Given that the prognostic value provided by the presence of mutations in TP53 is strongly improved when combined with Pin1 levels, the components of the Pin1/mutant p53 axis might be exploited as diagnostic and therapeutic tools. These data indicate that Pin1 acts as an essential co-factor of mutant p53. It is worth noting that, depending on the TP53 status, Pin1 activity leads to opposite outcomes. This is due to the enzymatic nature of Pin1 that recognizes and modifies S/T-P sites in p53, irrespective of the presence of mutations at other sites. Thus, Pin1 provides the necessary framework for both wild-type p53 pro-apoptotic activities and mutant p53 aberrant functions (Mantovani et al., 2015).

# Targeting mutant p53 for cancer therapy

TP53 is one of the most frequently mutated genes in cancer: targeting mutant p53, which specifically accumulates in tumour cells, represents an attractive strategy for cancer therapy. This notion is supported by the evidence that knocking-down mutant p53 from cancer cells that are addicted to its expression either by small molecules or by RNA interference is able to reduce their malignant progression (Parrales 2015; Alexandrova 2015). Among the first drugs developed to target mutp53 proteins were the inhibitors of Hsp90, a molecular chaperone that stabilizes mutant p53 variants. Another possible approach is to act towards upstream activators of mutp53 oncogenic functions such as the Pin1 enzyme (Girardini et al. 2011). Pin1 appears particularly attractive for design of inhibitors: it is highly specific, over-expressed in cancers, and essential for tumor growth and progression, while being largely dispensable for normal tissue homeostasis. Unfortunately, none of the available Pin1 inhibitors has reached clinical trials so far. An exception is the discovery that All-*trans* Retinoic Acid (ATRA), used for treatment of acute promyelocytic leukemia (APL), directly interacts with the substrate-binding pockets in the Pin1 active site. It has been shown that ATRA exerts inhibition and degradation of Pin1 in tumor cells, blunting Pin1-dependent oncogenic mechanisms and breast cancer growth *in vivo*. However, possibly due to its low potency as Pin1 inhibitor, ATRA showed moderate efficacy against advanced breast cancer in clinical trials, leaving open the search for more effective molecules (Mantovani et al., 2016). Another strategy is the use of molecules that restore a wild-type conformation in mutp53, thus reactivating its oncosuppressive potential. Among compounds restoring the wild type p53 activities the best described and studied drug is PRIMA-1 (Bykov et al. 2002) and its more potent and less toxic derivative PRIMA-1MET/APR-246 (Bykov & Wiman 2014). This molecule is able to bind directly and modify mutant p53, transforming it into a wild-type-like protein conformation (Lambert et al. 2009), thus allowing it to activate wild-type p53 transcriptional targets, inducing *in vitro* and *in vivo* cell cycle arrest or apoptosis in human cancer cells (Zache et al. 2008; Lambert et al. 2010). The problem with application of the therapies based on mentioned drugs is that despite the results of their work are promising, the limited knowledge of their performance in various mutant p53 context as well as different genetic backgrounds is making them difficult to apply. To date the only drug that reached the clinical stage is PRIMA-1MET/APR-246.

# Mutant p53 R249

Different tumour types show peculiar *spectra* of *TP53* mutations, reflecting the mutagenic events that can contribute to that type of cancer. The most striking example of this association is the mutation of p53 on residue 249 within hepatocellular carcinoma (HCC). Specifically, R249S substitution is detected in up to 90% of hepatocellular carcinomas (HCC) occurring in populations exposed to Aflatoxin B1 ($AFB_1$), a mycotoxin growing on improperly stored grains that is metabolized by the liver to create intermediates forming promutagenic DNA adducts (Xia et al., 2013). Importantly, HCCs with this aetiology account for approximately a half of the annual deaths caused by HCC worldwide. Even if site-specific mutagenesis by Aflatoxin B1 on codon 249 represents a unique association between a mutagen and a specific *TP53* mutation, mutant p53 R249S (mutp53 R249S) is also found at relatively high frequency in other cancer types, including lung cancer caused by cigarette smoke (figure 7). In particular Aflatoxin B1 and benzo-α-pyrene induce a G->T transversion, that changes the codon encoding for Arginine 249 (AGG) into a codon encoding for Serine (AGT) in more than 60% of the cases (Hainaut, 2002) (Figure 7B).

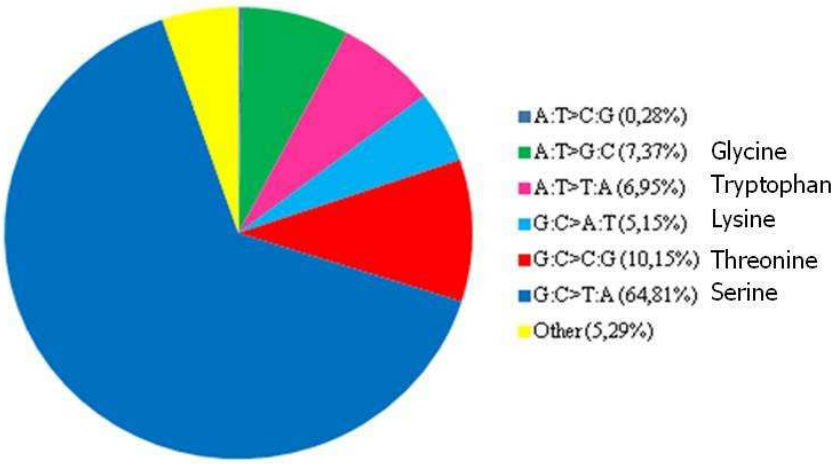



Figure 7. **Mutational profile of Arginine 249 in cancer.** Different mutation types found at residue 249 within p53 (IARC p53 Database, http://p53.iarc.fr/).

Arginine 249 is located in Loop 3 of the p53 DNA binding domain, which is folded in a common immunoglobulin motif with a DNA-binding surface formed by two β-turn loops, L2 and L3, and a loop-sheet-helix motif (Cho et al., 1994). Loops 2 and 3 lack regular secondary structure and are stabilized by a zinc coordination and side-chain interactions. The guanidinium group of Arg-249 is salt-bridged to the carboxylate group of Glu-171 in L2 and backbone oxygens of Gly-245 and Met-246. Replacement of Arginine with Serine at 249

position removes these interactions which are important in stabilizing the conformation of both loops (Wong et al., 1999).

## Epidemiology and aetiology of mutant p53 R249S

As said, Arginine 249 substitution in Serine is very frequent in hepatocellular carcinoma from eastern Asia and sub-Saharan Africa, where it associates with Aflatoxin B1 exposure and HBV infection (Figure 8).

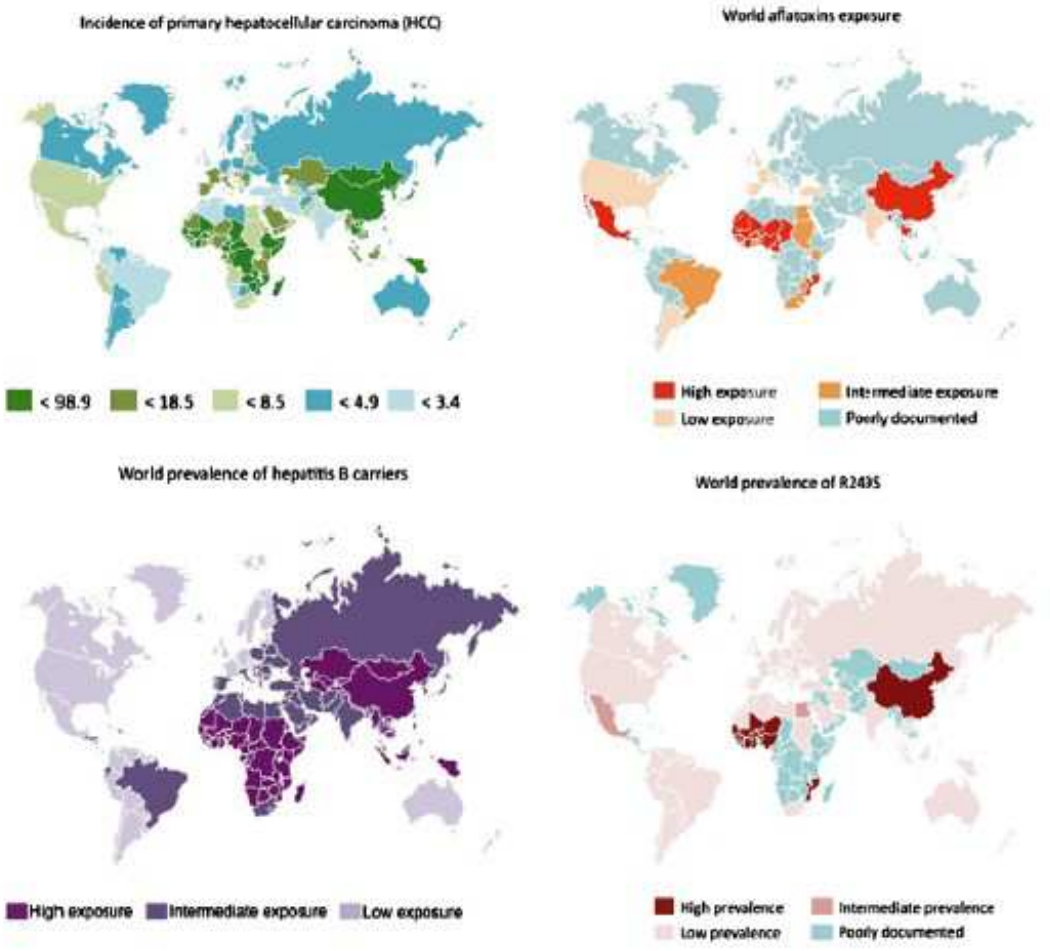



**Figure 8. Geographical distribution of incidence of HCC, exposure to aflatoxin, Hepatitis B Virus chronic carriage and prevalence of R249S mutation.** HCC incidence data are from GLOBOCAN 2002, IARC (http://www-dep.iarc.fr) and R249S prevalence data are from IARC TP53 Mutation Database, R12 [10] (http://www-p53.iarc.fr). Data on chronic HBV carriage are from World Health Organization and data on aflatoxin exposure are referenced in the text. Maps drawn thanks to Servier Medical Art (http://www.servier.fr/smart/homesmart.asp).

Aflatoxins are compounds produced by the *fungi Aspergillus flavus* and *Aspergillus parasiticus,* and the human exposition happens mainly through the consumption of contaminated foods, consequence of an inappropriate storage of the aliments. Aflatoxin B1

(AFB1) is one of the most potent hepatocarcinogens known and it can cause DNA damage that can result in mutations (Gouas, Shi, & Hainaut, 2009). AFB1, in human bodies, is oxidized by CYP450 (principally CYP450 1A2 and CYP450 3A4), and this event generates four different metabolites, among which AFB1-exo-8,9-epoxide, that can easily interact with DNA and forms unstable adducts, leading to DNA damage (Gouas et al., 2009; Wong et al., 1999). Although the abovementioned mechanism was demonstrated in HepG2 cells (Aguilar et a., 1993), *in vivo* studies showed that AFB1 does not induce mutation in codon 249 of Hupki mice (Human p53 knock-in), suggesting that other factors may be involved (Tong, Lee, Galendo, Wang, & Sabapathy, 2006). There are several evidences that mutant p53 R249S somatic change is a primary genetic event in hepatocarcinogenesis occurring in the contest of high Aflatoxin B1 exposure (Kirk et al., 2005). Indeed it was demonstrated that p53 R249S mutation is detectable in the plasma of HCC patients 1 to 5 years prior to HCC diagnosis (Jakson et al. 2003). This opens the intriguing possibility that circulating free DNA containing mutant p53 R249S may serve as a biomarker of exposure to AFB1 or a predictor of liver cancer, depending upon levels and temporal variation, such that low and transient plasma concentrations may reflect seasonal exposure to $AFB_1$, whereas high and sustained plasma concentrations may indicate the presence of a developing cancer lesion. Interestingly, it was observed that R249S mutation can occur in patient who develop HCC without clinical evidences of pre-existing or simultaneous liver cirrhosis (Villar et al., 2011).

Importantly Aflatoxin B1 was shown to induce mutation also in other hot-spot residues within p53, but the only alteration found in Aflatoxin B1-induced tumours is the one at residue 249. The reason of this may be linked to the functional properties of mutant p53 R249S, hypothesizing that it could provide special advantages to liver cancer cells (Hussain et al., 2007).

## Functional properties of mutant p53 R249S

Due to the fact that mutant p53 R249S is the predominant form of mutant p53 in liver cancer, several studies have tried to assess whether it has a specific and context dependent gain-of-function. It is known that mutant p53 R249S has lost DNA-binding and trans-activation capacities towards most, if not all, promoters that contain p53 consensus binding sequence (Forrester et al., 1995). In yeast assays, its residual activity on p53-dependent promoters is of less than 20% of that of wild-type p53, similar to most other hot-spot p53 mutants. When transfected in p53-null cancer cell lines, mutant p53 R249S shows a wide range of loss-of-

function, including loss of cell-cycle arrest and loss of pro-apoptotic capacity.

Dominant-negative effects over wild-type p53 have been observed in several studies, but they seem to be highly dependent upon the particular cellular context. For example, mutant p53 R249S has been reported to block wild-type p53 activity in Saos2 osteosarcoma cells but not in 21PT breast cancer cells. Conversely, it was shown that p53 R246S, the mouse equivalent of human p53 R249S mutant, is capable of dominant negative effects over wild-type p53 proteins both in undifferentiated and differentiated embryonic stem cells *in vitro* and *in vivo* (M. K. Lee & Sabapathy, 2008).

There is limited evidence for a gain-of-function activity in the absence of wild-type p53. Mutant p53 R249S appears to be capable of modest interactions with p73 in Saos2 and H1299 lung cancer cells, and of binding with low affinity to p63 in H1299. None of these properties have been demonstrated so far in the context of normal or transformed hepatocytes. There are also controversial results about the new capabilities acquired by the mutant p53 R249S. Transfection of p53-null hepatoma cells (Hep3B) with mutant p53 R249S led to the acquisition of a new phenotype with an increased *in vitro* survival and mitotic activity, but not of tumorigenicity in nude mice (Ponchel et al., 1994). Another study demonstrated that mutant p53 can bind directly the P4 promoter of the insulin-like growth factor II (IGF-II), despite the lack of p53 consensus binding site, increasing its transcription. In this context mutant p53 R249S stimulates phosphorylation of Sp1 and enhances the formation of Sp1/TBP complex, that causes an increase transcription of the IGF-II gene, through the binding on the P4 promoter (Y. I. Lee et al., 2000). The increased expression of this protein is linked with tumour progression in several tumours, such as in the liver cancer, where IGF-II is involved in mechanisms of growth control during malignant transformation (Daughaday & Rotwein, 1989). Recently, it has been demonstrated that mutp53 R249S shares with other missense mutations a common GOF program based on the promotion of proteasome activity and consequent degradation of tumour suppressors (Walerych et al., 2016).

As mutant p53 R249S has been demonstrated to be associated mainly to HBV-driven HCC, it should be considered that there could be an interplay between the viral infection and mutant p53. Some studies have demonstrated that silencing of mutant p53 R249S leads to a down-regulation of the proliferation and survival only in cells lines with the HBV genome integrated. Moreover, it was shown that mutant p53 R249S and HBx proteins form a detectable complex. All these findings taken together suggest that mutant p53 R249S may exerts its gain-of-function through the interaction with HBV (Gouas et al., 2010). Studies performed using knock-in mice strains showed that mutant p53 R249S, unlike the others “hot-

spot" mutations, is not able to induce an increase of the tumour formation through the typical mechanisms of GOF, such as the inhibition of p73 activity (M. K. Lee et al., 2012).

Despite all this experimental controversial evidence, however, data from cohorts of liver cancer cases clearly demonstrate that patients with mutant p53 have a shorter overall survival than patients with the wild-type p53 protein (Woo et al., 2011) (Figure 9). In mutant p53 R249S-bearing tumours was observed an increase of the expression of the proliferation related genes and stem cell genes.

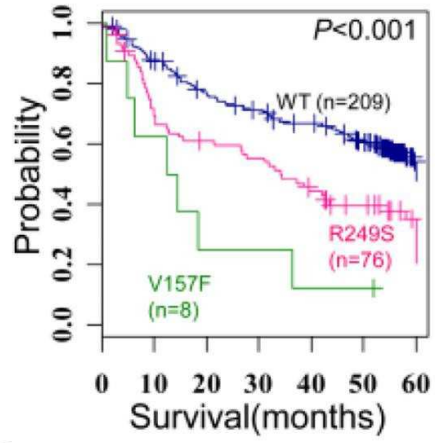



**Figure 9. Prognostic values of *TP53* mutation sites**. Kaplan-Meir plot for survival between the tumors with R249S and V157F mutations *versus* WT p53 in a Chinese cohort of HCC patients (n=334) (Woo et al., 2011).

# Role of mutant p53 R249S in Hepatocellular carcinoma

## Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) constitutes 85-90% of all the primary liver tumours and represents, with 500.000-600.000 deaths per year, the second cause of cancer related death worldwide (Levrero & Zucman-rossi, 2016). Its development is due to the interaction between genetic predisposition and environmental factors. Risk factors for HCC are well defined: cirrhosis, alcohol abuse, metabolic syndrome, intake of Aflotoxin B1 and hepatitis B/C infection. Notably, the combined exposure to Aflotoxin B1 and HBV leads to the high frequency of hepatocellular carcinoma in Asia and in the sub-Saharan Africa.

Other causes of HCC are amenable to metabolic syndrome due to diabetes and obesity, the non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH). These latter together with HBV infection represent the exception to the rule that HCC is always associated to fibrosis or cirrhosis (Levrero & Zucman-rossi, 2016; Llovet et al., 2016).

The neoplastic evolution of HCC proceeds through a multi-step histological process that is less defined than other cancer types. HCC-inducing aetiologies provoke continuous rounds of hepatocyte damage and regeneration, culminating in chronic liver disease. These lesions can progress to pre-malignant dysplastic nodules, which have abnormal cytological features including clear cell changes and nuclear crowding. These lesions are associated with the increased thickening of the *trabeculae*, which indicates abnormal liver architecture. These dysplastic nodules can evolve to frank HCC which is endowed with the capacity to invade the surrounding fibrous stroma and vessels, and occasionally have metastatic potential (Farazi & Depinho, 2006) (Figure 10).

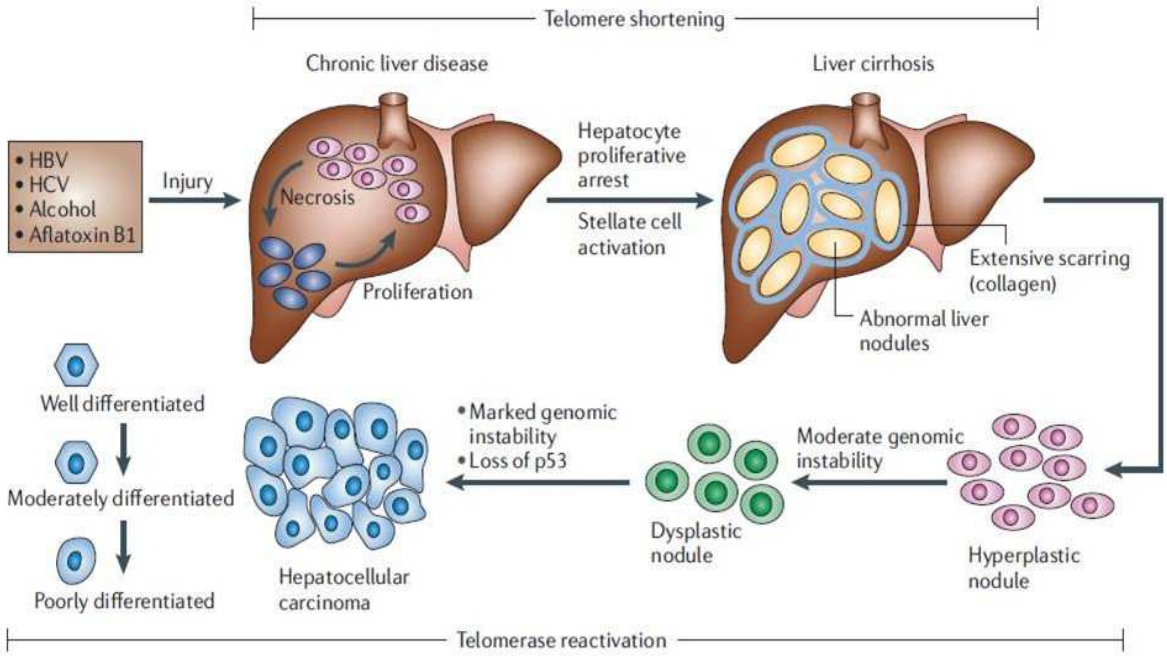



**Figure 10. Histopathological progression and molecular features of HCC.** Hepatic injury, due to any one of several factors is followed by a state of chronic liver disease in which continuous cycles of necrosis and proliferation occur. This destructive–regenerative process culminates in liver cirrhosis. Cirrhosis is characterized by abnormal liver nodule formation surrounded by collagen deposition and scarring of the liver. Subsequently, hyperplastic nodules are observed, followed by dysplastic nodules and ultimately hepatocellular carcinoma (HCC), which can be further classified into well differentiated, moderately differentiated and poorly differentiated tumours; the last of which represents the most malignant form of primary HCC. Telomere shortening is a feature of chronic liver disease and cirrhosis. Telomerase reactivation has been associated with hepatocarcinogenesis. Loss and/or mutation of p53 and genomic instability also characterize hepatocarcinogenesis. p53 loss and/or mutation is shown to occur during progression to HCC, however, there is some evidence that loss and mutation of p53 might also occur in the initial stages of hepatocarcinogenesis. (Faranzi et al., 2006)

Over the past decade genome wide technologies and next generation sequencing allowed the identification of molecular signatures to classify HCC subgroups, stratify patients according to prognosis and discover the role of pathways previously under-explored. This genomic approach revealed the main drivers responsible for tumour initiation and progression. All etiological factors seem to act through similar mechanisms, such as point mutations, chromosomal aberrations and epigenetic changes that converge to common pathways. The integrative transcriptomic analysis performed by Hoshida et al., revealed that hepatocellular carcinoma can be divided in 2 major molecular subclasses. One, defined "Proliferation class", is characterized by an enrichment of signals related to cell proliferation and progression in the cell cycle; the second one, "non-proliferation class", shows molecular properties which resemble normal hepatic physiology.

The first is genomically and phenotypically heterogeneous with high rates of chromosomal instability, aberrant epigenetic changes and activations of multiple pathways such as AKT/mTOR, IGF, TGF-β, NOTCH. Proliferation class tumours are aggressive, moderately/poor differentiated, and show high alpha fetoprotein (AFP) expression, frequent vascular invasion, higher risk of recurrence after resection and lower survival rates. Non-proliferation class tumours are less aggressive, better histologically differentiated, with low AFP levels and activation of WNT-β catenin signalling in up to 25% of cases (Hoshida et al., 2009). Interestingly, HCV and alcohol-related HCC are prevalent in the non-proliferation class, whereas HBV-related HCCs predominantly belong to the proliferation subclass (Zucman-rossi, Villanueva, Nault, & Llovet, 2015).

## Role of wild-type and mutant p53 in hepatocellular carcinoma

The most frequent mutations found in HCC affect: telomere maintenance, WNT pathway activation, chromatin remodelling, RAS, mTOR and oxidative stress signalling (Figure 11).

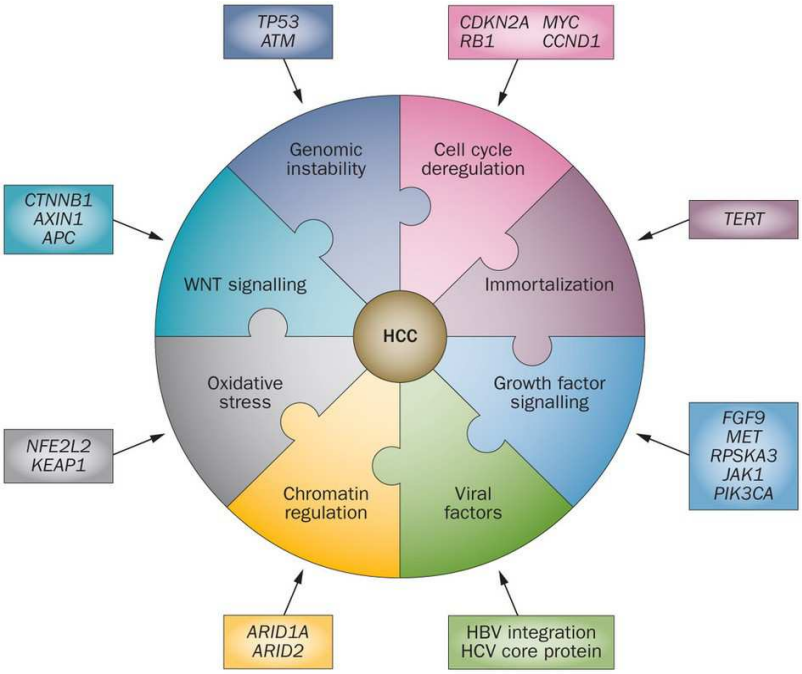



**Figure 11. Core oncogenic pathways in hepatocellular carcinoma.** Representative genes involved in each pathway are indicated. (Shibata and Aburatani, 2014)

Among the recurrent mutated genes, *TP53* represents the first one (Amaddeo et al., 2014; Llovet et al., 2016), indeed TP53 mutations have been found in 12-48% of HCC cases. Alterations of other genes upstream or downstream in the p53 pathway, such as recurrent mutations of ATM and CDKN1A have also been reported. Notably, mutations of IRF2 gene,

which is a positive regulator of p53 expression, are mutually exclusive to the TP53 mutations in HCC patients (Shibata and Aburatani 2014). Importantly, *TP53* mutation and cell cycle activating mutations are associated with aggressiveness and poor prognosis (Levrero & Zucman-rossi, 2016). In humans, analyses of HBV- and HCV-related HCCs have shown a greater frequency of p53 mutations in advanced malignancies (43%) than in regenerative nodules (~7%) (Minouchi et al., 2002). Regions of high Aflatoxin B1 exposure show frequent p53 R249S mutations in early-stage HCC lesions (Aguillar et al., 1994), whereas regions of low Aflatoxin B1 exposure show p53 mutations in much later stages. It seems plausible that p53 mutation might operate in either HCC initiation or progression, depending on the context. In the setting of Aflatoxin B1, this mutation might serve to drive "initiation" with other cooperating events. In the context of other aetiologies (such as those that provoke regeneration, oxidative stress and telomere erosion) the loss of p53 might have a more prominent role in HCC progression by facilitating continued proliferative potential. Unlike HCV- and alcohol-induced hepatocarcinogenesis, there is no clear connection between Aflatoxin B1 exposure and the development of cirrhosis, indicating that the mutational actions of this toxin might be a primary driver of HCC development. It is worth noting that Aflatoxin B1 exposure often coexists with HBV infection, and such individuals possess a 5–10-fold increased risk of developing HCC compared with exposure to only one of these factors. The mechanistic basis for this synergy is not known, although it seems plausible that cooperation would derive from continuous hepatocyte turnover during chronic HBV infection and Aflatoxin-B1-induced mutagenesis (Faranzi 2006). Study of the role of p53 in hepatocarcinogenesis showed that HBV large envelope protein (HBsAg) transgenic mice have an HCC-prone condition that is accelerated when combined with Aflatoxin B1 exposure and the p53 Ser246 allele. Similarly, increased progression to high grade HCC is observed in Aflatoxin-B1-treated HBsAg mice heterozygous for a p53 null allele (Ghebranious and Sell, 1998). These data support the idea that mutant p53 R249S contributes to the Aflotoxin B1-induced hepatocarcinogenesis in a yet unknown manner.

# Interferon and JAK-STATs signalling

Interferons (IFNs) are a group of cytokines exerting antiviral and growth-inhibitory effects; they represent the first line of defence against viral infections and have an important role in immuno-surveillance against malignant cells. IFNs are classified in type I (IFN α1-12, β, ε, κ and ω), type II (IFNγ), and type III (IFNλ1-4); they act through differentiated receptor complexes and give rise to important and distinct outcomes which vary for Interferon Stimulated Genes (ISGs) profiles, kinetics of induction, antiviral and anti-proliferative activity, immuno-modulatory potential (Schneider, Chevillotte, & Rice, n.d.).

A common property of IFNs is the activation of the Janus activated kinase - Signal Transducer and Activator of Transcription (JAK-STAT) pathway, that in mammals is central for the transmission of signals from cytokines and growth factor receptors to the cell nucleus, affecting cell proliferation, differentiation, migration and apoptosis.

Both type I and II IFN receptors have multi-chain structures; they are composed of at least two subunits which interact with a member of the Janus activated Kinase family. In type I IFN pathway, IFNAR1 and IFNAR2 receptors associate respectively with Tyk2 and Jak1, whereas in type II IFN receptor, the IFNGR1 associates with JAK1 and IFNGR2 binds JAK2. Upon IFN binding, receptors are brought into close proximity; the two kinases juxtapose and undergo trans-phosphorylation and activation. Once activated, JAKs phosphorylate IFN receptor chains on highly conserved tyrosine residues, creating phosphoTyrosine-based docking sites for the Src-homology 2 (SH2) domain of STAT proteins. After the recruitment to the IFN receptors, STATs are phosphorylated by JAKs on conserved Tyrosine and consequently engaged in omo- or heterodimers through SH2 domain interactions. As result, STATs release the receptors and move to the nucleus (Schneider et al., n.d.)(Böhmer & Friedrich, 2014) (fig.12). The STAT family comprises seven members, STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6, each sharing the same five-domain structure: an N-terminal, a coil-coiled, a DNA-binding, an SH2, and a C-terminal domain. The STATs activated by type I IFNs are STAT1, STAT2, STAT3 and STAT5. The Interferon Stimulated Gene Factor 3 (ISGF3) is a complex that represents the canonical transcription factor activated by type I IFNs; it is composed of phosphorylated STAT1 and STAT2, and Interferon regulated factor 9 (IRF9). ISGF3 binds palindromic cognate sequence known as Interferon-Stimulated Response Elements (ISRE) in the gene promoters.

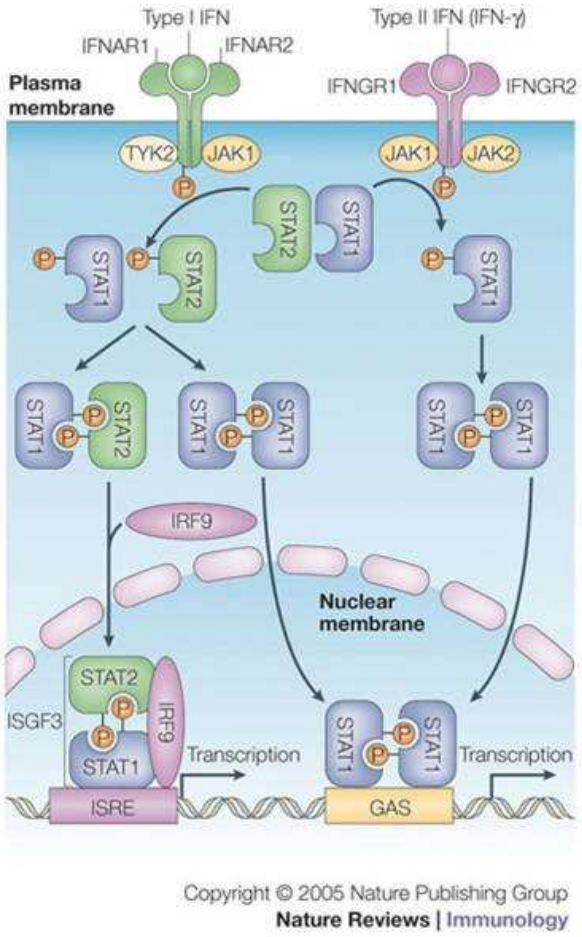



**Figure 12. Signal transduction by the interferon (IFN) receptors**. Binding of type I and type II IFNs to their respective receptors (IFNAR and IFNGR) activates the Janus kinases (JAKs) that are permanently bound to these receptors (JAK1 and tyrosine kinase 2 (TYK2) for IFNAR, and JAK1 and JAK2 for IFNGR). The activated JAKs phosphorylate tyrosine residues in the receptor. Receptor phosphorylation creates docking sites for signal transducer and activator of transcription 1 (STAT1) (for IFNGR) or a STAT1–STAT2 complex (for IFNAR). Receptor-associated STATs are phosphorylated on tyrosine residues by the receptor-associated JAKs. Tyrosine phosphorylated STATs form STAT1 homodimers downstream of both IFNAR and IFNGR stimulation and STAT1–STAT2 heterodimers downstream of IFNAR stimulation. These STAT dimers move to the nucleus. STAT1 homodimers bind to IFN--activated-site (GAS) promoter sequences and STAT1–STAT2 heterodimers associate with a third protein, IFN regulatory factor 9 (IRF9), to form IFN-stimulated gene factor 3 (ISGF3) and to bind to the IFN-stimulated response element (ISRE) promoter sequence to stimulate IFN-stimulated gene transcription (Decker et al., 2005).

JAK-STAT signalling is attenuated at different stages. Key regulators are SOCS (suppressors of cytokine signalling), which counteract JAKs phosphorylation, PTP (protein tyrosine phosphatases), acting on JAKs and STATs, and PIASs (protein inhibitor of activated STAT), that specifically bind STATs and halt their activity. Interestingly, different conditions regulate the expression of the negative regulators; for instance SOCS1 can be repressed epigenetically through promoter hypermethylation (Calvisi et al., 2006), as well as transcriptionally up-regulated in a mutant p53-dependent manner. Notably, the latter mechanism is part of a regulatory loop between mutant p53 and IFN β: when present in cancer cells, mutant p53

promotes inhibition of STAT1 phosphorylation, but in turn IFN β reduces mutant p53 RNA levels by attenuating the expression of its RNA stabilizer WIG1 (Madar et al., 2013).

## JAK-STATs pathway in cancer

In cancers where JAK/STAT signalling is abnormally induced, the mechanism underlying inappropriate activation of the pathway is not well defined. Analysing the instances in which a mechanism has been identified, it was observed that cancer cells employ diverse strategies to activate the JAK-STAT pathway. Gain-of-function mutations in JAKs have been observed to cause signalling activation in haematological malignancies. More recently, large-scale sequencing efforts identified genetic changes affecting JAKs in certain solid tumours. Missense mutations in JAK1 have been detected in 9% of patients with Hepatitis B-associated hepatocellular carcinoma, and validation in cell culture showed that these mutations increase phosphorylation of JAK1 and STAT3 (Kan et al, 2013). Moreover, JAK-STAT activation represents the principal feature of IHCAs (inflammatory hepatocellular adenomas) where mutations in IL6ST/gp130 and *STAT3* act by a similar mechanism called "oncogene-induced inflammation" that leads to constitutive STAT3 activation (Nault et al., 2013). In addition to STAT3 and STAT5, well-known pro-tumour proteins, increasing evidences show that STAT1, STAT2 and IRF9, together in ISGF3 complex or not, can exert oncogenic roles.

STAT1 modulates different cellular processes: proliferation, differentiation, cell death and immune response driven by viral infection. Although STAT1 was extensively studied for its antiviral and tumour suppressive actions, several studies pointed out STAT1 cancer promoting properties, principally exerted through evasion and suppression of tumour immune surveillance, increase in invasiveness and metastasis, resistance against irradiation and chemotherapy (Meissl, Macho-maschler, Müller, & Strobl, 2015). In squamous cell carcinoma, serial transplantation of cancer cells in combination with irradiation led to activation of STAT1 signalling and acquisition of radio-resistance. Gene profiling showed that these cells express high levels of a specific subset of ISGs, defined as IFN-related DNA damage resistance signature (IRDS). IRDS induction after irradiation was also observed in xenografts from different tumour types, including breast, head and neck, and colon cancer (Kodarev et al., 2007). Coherently, IRDS correlates with poor prognosis in glioblastomas, breast and lung cancer, head and neck tumours (Weichselbaum et al., 2008). Interestingly, Stark and colleagues found that un-phosphorylated STAT1 can activate the transcription of a group of genes which overlaps with IRDS (Cheon and Stark, 2009). Based on this

observation, it could be possible that pro-survival ISGs are preferentially transcribed by un-phosphorylated protein, and in this case the equilibrium between phospho- and un-phosphorylated STAT1 might be critical for its oncogenic roles.

Additional mechanisms may promote STAT1 expression and activity in cancer cells. Up-regulation of STAT1 and ISGs can occur via concomitant loss of p53 and p14 in breast cancer, as demonstrated by studies performed in triple negative breast cancer patients (Forys et al., 2014). Interestingly, down-regulation of JAK-STAT inhibitors, resulting in persistent pathway activation, has been found to support tumorigenesis in hepatocellular carcinoma. Phosphorylation of Janus kinases was not detected in normal livers, but it increased from surrounding non neoplastic livers to HCC, as well as the expression levels of JAK/STAT target genes such as Bcl-xl and Mcl-1. (Calvisi et al., 2006).

STAT2 is the most structurally and functionally divergent member of the STAT family. Type I IFNs activate STAT2 through phosphorylation on the Tyrosine 690, promoting the creation of ISGF3 complex, in which STAT1 and IRF9 provide DNA binding of the complex, whereas STAT2 contains a trans-activation domain (TAD) essential for its transcriptional activity. STAT2-TAD has been shown to bind and recruit transcriptional co-activators such as p300/CBP, GCN5, DRIP150 and pp32 (Qureshi et a., 1996). The direct role of STAT2 in tumour development and progression is not clear. Some evidences suggest that STAT2 might be involved in the malignant transformation by regulating the production of IL-6, which in turn activates STAT3 oncogenic pathway (Gamero et al., 2010). Moreover, increased STAT2 staining was more frequently detected in cervical cancer specimens than in the non-cancerous and pre-cancerous lesions (Zeng et al., 2012). Recent studies also point out at the involvement of STAT2 in drug resistance through the regulation of specific ISGs (Cheon et al., 2013).

IRF9 is well known for its integral role in type I IFN-mediated cellular responses, to which it contributes by recognizing ISRE sequences and forming the docking site for the ISGF3 complex. Interestingly, it has been reported that IRF9 is directly activated by c-Myc, and cells lacking IRF9 expression are more susceptible to cytotoxic chemotherapeutic drugs. Recently, Kolosenko showed that IRF9 is up-regulated in Multicellular spheroids (MCS) as well as in confluent 2D cultures of cancer cells. Although the mechanism of IRF9 induction by cell crowding remains to be investigated, its over-expression in HCT116 cells was sufficient to up-regulate genes belonging to the IRDS and induce resistance to cisplatin, docetaxel, oxaliplatin, 5FU and etoposide (Kolosenko et a., 2014).

# AIM OF THE THESIS

Mutations in the *TP53* tumour suppressor gene represent the most common genetic alterations found in human malignancies. In most cases *TP53* undergoes missense mutations causing aminoacid substitutions that prevalently fall in the DNA-binding domain, thus impairing transcriptional activity. In addition to loss of tumour suppressor functions, mutant p53 proteins contribute to carcinogenesis by exerting dominant negative effects over wild-type p53, where present, and by neomorphic oncogenic properties (gain-of-function). Importantly, mutant p53 oncoproteins become activated specifically in a tumour cell context. In line with this evidence, understanding the environmental factors that account for the selection and activation of specific p53 mutants in different tumours is critical to identify determinants of carcinogenesis and to find new putative targets for cancer therapy.

Among the frequently mutated residues in p53, in human tumours Arginine 249 is replaced in about 65% of cases with a Serine. Although this event occurs with an overall low frequency, mutant p53 R249S is detected in up to 90% of hepatocellular carcinomas (HCC) occurring in populations exposed to HBV infections and aflatoxin $B_1$, two major risk factors for the HCC in the east of Asia and sub-Saharan Africa. From a molecular point of view, Aflatoxin B1 causes the specific mutation R249S, whose presence associates with stem cell traits and poor prognosis in cohorts of HCC patients. However, these epidemiologic data are not supported by clear indications of gain-of-function activities of mutant p53 R249S from *in vitro* and *in vivo* studies. On a critical reflection, this apparent discrepancy could rely to a poor understanding of the particular interaction of mutant p53 R249S with the specific tumour context where it is selected, its biochemical regulation and the biological interactions in which the mutant protein is involved in HCC.

Starting from the consideration that in HCC associated to Aflatoxin B1 and HBV exposure Arginine 249 is most frequently mutated to a phosphorylable amino acid (either Serine or Threonine), and that its selection in tumours may be due to a specific oncogenic signalling involving this residue, this thesis aims at: 1) identifying the possible post-translational modification network affecting mutant p53 R249S and impacting its oncogenic activities; 2) understanding the molecular events driving mutant p53 R249S oncogenic functions in HCC; 3) verifying whether targeting mutant p53 R249S modification could block its gain-of-function activities.

# RESULTS

## p53 R249S mutation generates a novel phosphorylation site that mediates a Pin1-dependent conformational switch

In human cancers, the p53 residue Arginine 249 is mutated to a phosphorylable amino acid (either Serine or Threonine) in more than 70% of the cases (IARC, http://gco.iarc.fr/today/home). This is of particular interest given that several reports have stated that mutant p53 activation entails post-translational modifications, particularly phosphorylation (Muller et al., 2009 (Girardini et al., 2011) (Walerych, Napoli, Collavin, & Del Sal, 2012). However, whether substitution of Arg249 with Ser or Thr may be indeed associated with phosphorylation of the mutated residue in cancer cells, has not been investigated yet. In order to address this issue we performed a mass spectrometry analysis of mutant p53 from Mahlavu liver cancer cells and BT 549 breast cancer cells, both bearing endogenous mutant p53 R249S (Figure 13A). We observed phosphorylation of Serine 249 in both cell lines, demonstrating for the first time that a mutation in p53 can introduce a new functional post-translational modification (Figure 13B).

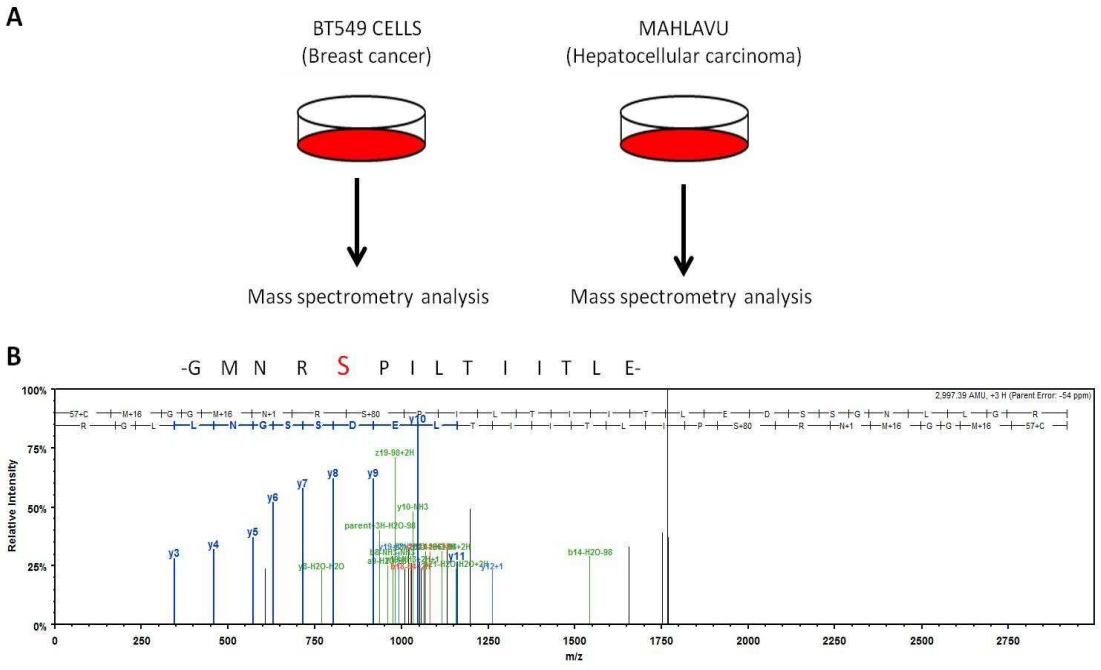



**Figure 13. Mass spectrometry analysis of mutant p53 R249S.** A) Scheme of the mass spectrometry experiment. Endogenous p53 was immunoprecipitated from 2mg of total cell lysate and subjected to trypsin digestion followed by mass spectrometry. B) Annotated spectra for p53 phospho-Serine 249. Scaffold was used to annotate the spectra matching to p53 phospho-Ser249 (C-M-G-G-M-N-R-S-P-I-I-L-T-I-I-I-T-I-E-D-S-S-G-N-L-L-G-R). The b- and y- ion series are indicated at the top and the matching peaks from the y-ion series are shown in blue. Neutral loss of water in the b-ion series is shown in green and neutral loss of methane sulphenate is shown in red.

To validate this result, we performed western blot analysis of Mahlavu cells protein lysates employing a specific anti-phospho Serine 249 antibody. In line with the previous observations, we detected phosphorylation on Serine 249 of mutant p53 isolated from Mahlavu cells (Figure 14).

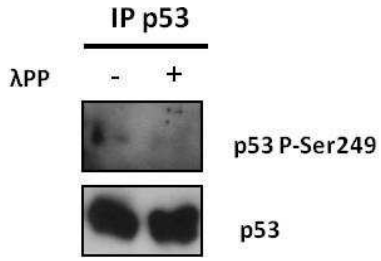



**Figure 14. Serine 249 phosphorylation in Mahlavu cells**. Total p53 was immunoprecipitated from equal amounts of Mahlavu cell lysates treated (+) or not (-) with lambda phosphatase (λPP) and analyzed by Western Blot with anti-phospho Ser249 antibody.

Serine 249 is followed by a Proline (Figure 15A). Thus, Ser 249 phosphorylation generates a canonical consensus binding site for the prolyl-isomerase Pin1 (Zhou and Lu, 2016). Importantly, our research group demonstrated that Pin1 is required to foster the oncogenic activities of other mutant p53 forms commonly found in tumors (Girardini et al., 2011). Based on these data, we reasoned that Pin1 might regulate mutant p53 R249S by specifically binding and modifying the new target site Serine 249-Proline 250 that is introduced upon mutation. This event may be particularly relevant for hepatocellular carcinoma (HCC), since Pin1 is overexpressed in more than 50% of cases in this tumour type (Pang et al 2007).

We first assessed that mutant p53 R249S and Pin1 were indeed able to interact in our cellular models, as proved by co-immunoprecipitation experiments in both Mahlavu (Figure 15B) and BT 549 (Figure SI 1) cells.

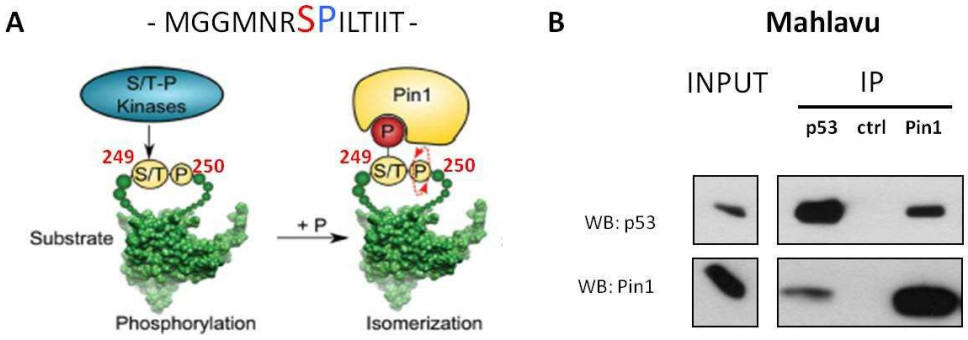



**Figure 15. p53 R249S mutation creates a new binding site for the prolyl-isomerase Pin1.** A) Hypothetical model in which Serine 249 phosphorylation by Proline-directed kinases generates a novel binding site for Pin1 (Adapted from Polonio-Vallon et al., 2014); in the amino acid sequence of the peptide containing R249S mutation Ser249 and Pro250 are depicted in red and blue, respectively B) Co-immunoprecipitation between mutant p53 R249S and Pin1. Total lysate from Mahlavu cells was subjected to immunoprecipitation with anti-p53 and anti-Pin1 antibodies.

To assess whether Serine 249 phosphorylation creates a novel Pin1 binding site we took advantage of an available p53 expression construct with point mutations at all six Pin1 consensus sites (Ser/Thr to Ala point mutant, p53 6M) (Mantovani et al., 2007), where we introduced the mutation R249S, generating a p53 6M R249S expression construct (Figure 16A). In this mutant protein, the only Pin1 consensus site is Ser249-Pro250. Importantly, when expressed in p53-null H1299 cells p53 6M R249S was phosphorylated on Serine 249, as demonstrated by Western Blot with anti-phospho Serine 249 antibody (Figure 16B). We then tested the ability of Pin1 WW domain to associate with p53 6M R249S. We performed a GST-pull-down assay upon overexpression of p53 6M or p53 6M R249S in H1299 cells. The Pin1 WW domain bound p53 6M R249S with high affinity compared to the 6M protein. This interaction relied entirely on Serine 249 phosphorylation, as demonstrated by the fact that p53 de-phosphorylation by phosphatase treatment abolished the binding (Figure 16C).

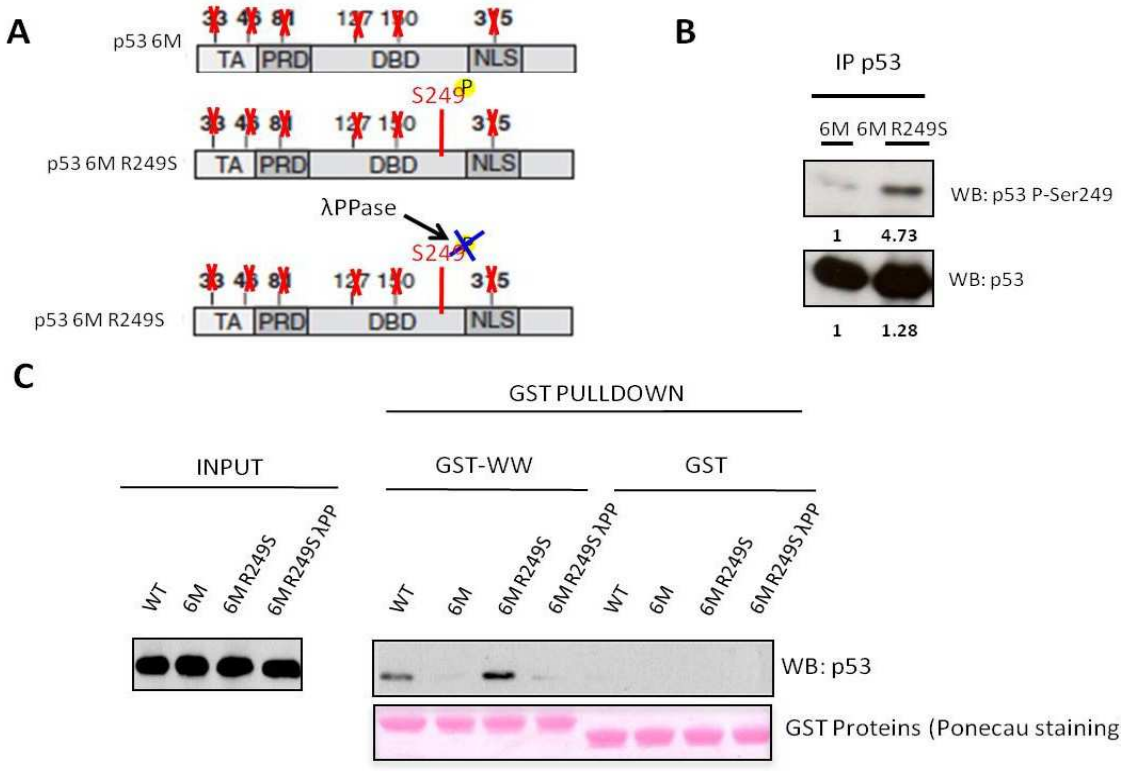



**Figure 16. Mutant p53 R249S interacts with Pin1 in a phosphorylation-dependent manner**. A) The mutagenized residues within p53 6M and p53 6M R249S are indicated in red. B) p53-null H1299 cells were transfected with p53 6M (all six canonical S/T-P sites within p53 were mutated to Alanine) or p53 6M R249S constructs. Immunoprecipitated p53 was analysed by Western Blot with anti-phospho Ser249 antibody. Signal intensity was quantified by ImageJ. C) H1299 were transfected with p53 WT, p53 6M and p53 6M R249S; half of the lysate from p53 6M R249S was treated with lambda phosphatase (λPP) for 90' at 30°C and the binding with Pin1 was assessed by incubation with GST-WW or GST as a control.

Pin1-mediated isomerization of Serine/Threonine-Proline sites is often reported to regulate the overall conformation of its client proteins. This is of particular interest for mutant p53 R249S, which displays a mildly destabilized conformation as compared to other mutant forms

of p53, and is in a dynamic equilibrium between native and native-like status (Friedler et al., 2004). Based on this, we reasoned that phosphorylation and Pin1-dependent isomerization could contribute in shifting the balance towards a more distorted form, similar to conformational p53 mutants (Joerger and Fersht, 2007). To test this hypothesis, we treated Mahlavu cells with the Pin1 inhibitor ATRA (All *trans* Retinoic Acid [Wei et al., 2015]) and immunoprecipitated p53 with the conformation-specific anti-p53 antibody Pab240, which specifically recognizes highly distorted mutant p53 structure in native conditions (Gannon et al., 1990). Interestingly, inhibition of Pin1 catalytic activity caused a reduction of Pab240 binding, demonstrating that Pin1 is required to maintain mutant p53 R249S in a highly distorted conformation (Figure 17).

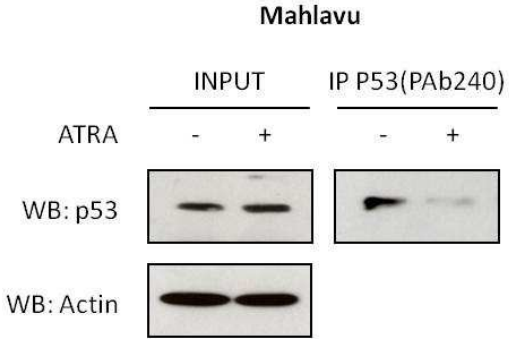



**Figure 17**. **Pin1 catalytic activity is required to stabilize mutant p53 R249S in a highly distorted conformation**. Immunoprecipitation (IP) for the misfolded form of mutant p53 R249S (PAb240) in Mahlavu cells. Following 24h of 25 μM ATRA treatment, p53 was immunoprecipitated and the amount of misfolded p53 was evaluated by Western blot analysis with anti-p53 total antibody DO-1.

We then sought to understand whether Pin1-dependent isomerization of pSer249-Pro250 simply assists folding of mutant p53 R249 in a highly destabilized conformation, or it also required to stabilize this structure. To this aim we performed molecular simulations of the thermodynamic stability of the various modified forms of mutant p53 R249S. To represent all the possible states of the Ser249-Pro250 bond, we modelled the mutated R249S in its native *trans* conformation (Bullock et al., 2000; Joerger et al., 2005), the mutated and phosphorylated form (R249pS), the mutated, phosphorylated and isomerised mutant (*cis*R249pS), and, as Pin1 substrates frequently undergo de-phosphorylation by cellular phosphatases, the isomerised and de-phosphorylated form (*cis*R249S). Notably, the isomerised, de-phosphorylated *cis*R249S shows the highest stability among all the forms, as demonstrated by the average number of hydrogen bonds within the protein (Figure 18). Taken together, these data show that Serine 249 mutation creates a new binding site for Pin1 on the p53 protein, and suggest that Pin1 activity may be crucial to stabilize a highly unfolded structure of mutant p53 R249S.

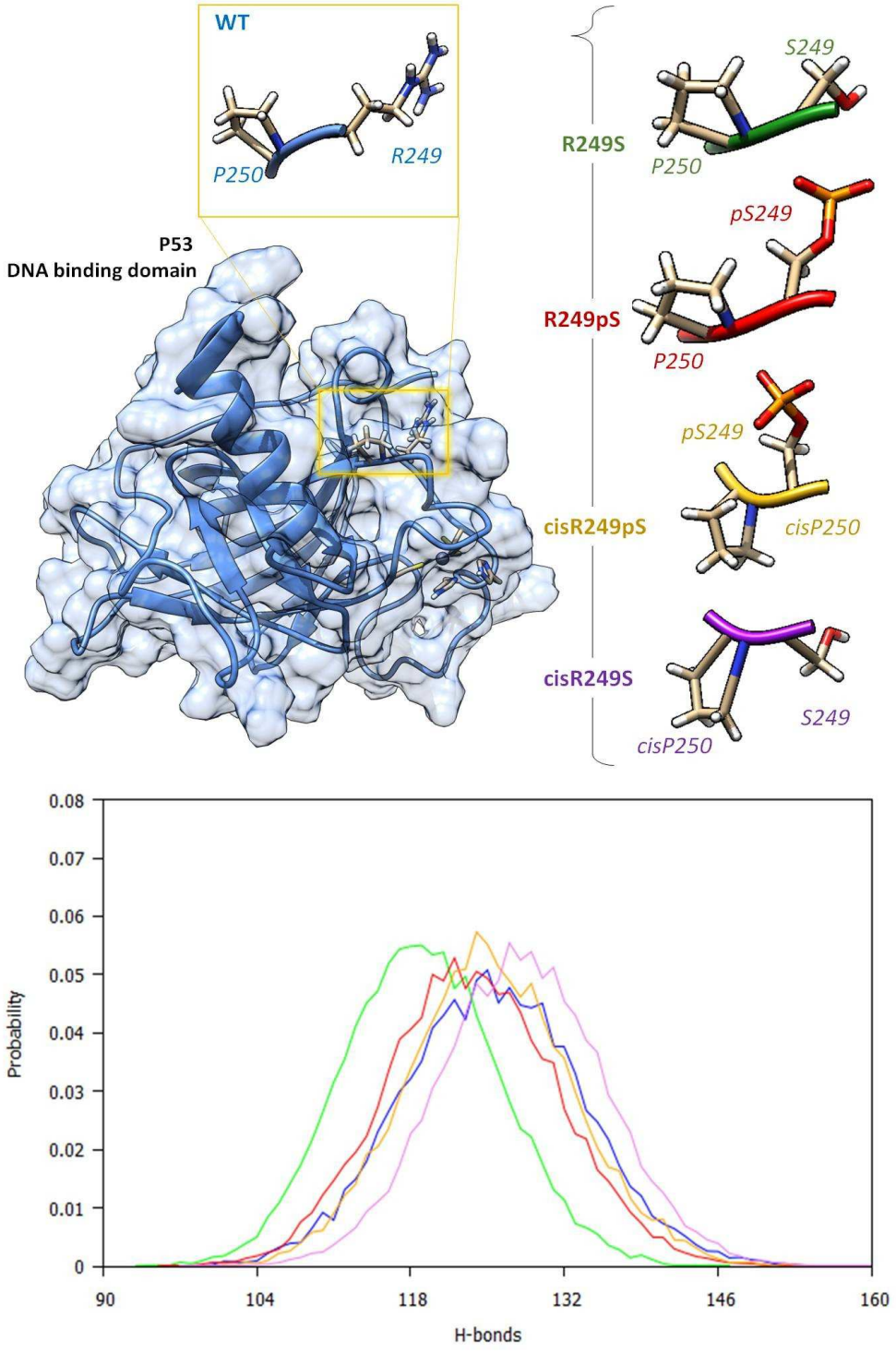
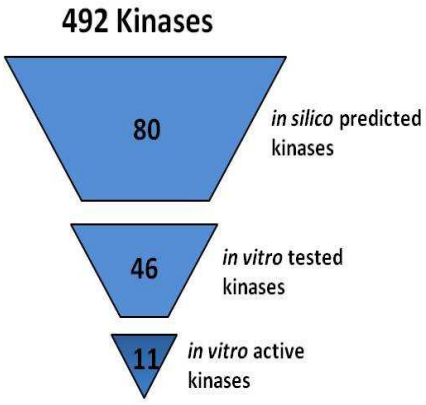



**Figure 18. Molecular simulations of mutant p53 R249S stability upon *trans* to *cis* isomerisation of the pSer249-Pro50 peptide bond.** Top: the p53 DNA binding domain adopts the fold of a beta sandwich, formed by two antiparallel beta-sheets that pack against each other. Its DNA binding interface features a $Zn^{2+}$ ion. The latter is coordinated by C176, H179, C238 and C242 (represented in sticks). The residue mutated here into serine (R249) is highlighted in a yellow box. A detail of the residues in position 249 and 250 (shown in sticks) is offered. From top to bottom: wild type R249 (WT), the mutated S249 (R249S), the mutated and phosphorylated pS249 (R249pS), the mutated and phosphorylated pS249 plus the following proline isomerized in cis (cisR249pS), the mutated S249 and isomerized proline, cisR249S. Bottom: hydrogen bonds distributions for the various forms of mutant p53 R249S, compared with wild-type p53.

# DYRK2 is the main kinase responsible for phosphorylating Serine 249 and modulates the interaction of mutant p53 R249S with Pin1

In order to identify the kinase(s) responsible for Serine 249 phosphorylation, we employed a two-step approach combining *in silico* prediction of kinases putatively targeting the residue and a subsequent *in vitro* screening of a panel of predicted kinases on Ser249 phosphorylation. This procedure was performed by the Kinexus Bioinformatics Corporation (www.kinexus.ca). The prediction was performed employing the company patented algorithm (Safaei et al., 2011) obtaining a list of 80 kinases putatively targeting Ser249 (Figure SI 2). In the second step, the activity of 46 kinases selected based on the *in silico* prediction step was tested on two peptide substrates, one bearing wild-type p53 sequence and the other containing mutated Ser249. 11 recombinant kinases, including members of DYRK, CDK, GSK3, CLK and MAPK kinase families, displayed a significant activity on the mutant peptide (Figure 19). The first hit was the kinase DYRK2.

A




B

| No. | Target ID | p53 [R249S] MGGMNRSPIL-pT-IRR | | | p53 Wild Type MGGMNRRPIL-pT-IRR | | | Difference / PhosphoNET Blank Value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| # | Kinase | CPM 1 | CPM 2 | Average | CPM 1 | CPM 2 | Average | Difference | Score | Blank |
| 1 | DYRK2 | 170,470 | 169,674 | 170,072 | 22,229 | 19,138 | 20,684 | 149,389 | 112.9 | 933 |
| 2 | CDK1/Cyclin A1 | 85,547 | 89,521 | 87,534 | 7,536 | 6,871 | 7,204 | 80,331 | 159.9 | 926 |
| 3 | CDK5/p25 | 74,080 | 77,146 | 75,613 | 6,649 | 4,740 | 5,695 | 69,919 | 145.6 | 837 |
| 4 | GSK3b | 69,834 | 73,977 | 71,906 | 4,937 | 3,823 | 4,380 | 67,526 | 122.2 | 947 |
| 5 | DYRK3 | 61,444 | 63,396 | 62,420 | 6,085 | 3,731 | 4,908 | 57,512 | 119.3 | 1063 |
| 6 | CDK2/Cyclin A1 | 39,323 | 43,732 | 41,528 | 5,033 | 4,194 | 4,614 | 36,914 | 146.4 | 1063 |
| 7 | GSK3a | 38,192 | 42,876 | 40,534 | 5,979 | 5,155 | 5,567 | 34,967 | 122.3 | 1055 |
| 8 | CLK2 | 30,867 | 32,620 | 31,744 | 8,142 | 7,635 | 7,889 | 23,855 | 88.1 | 936 |
| 9 | DYRK1a | 26,372 | 28,366 | 27,369 | 4,404 | 4,025 | 4,215 | 23,155 | 129.2 | 902 |
| 10 | CLK1 | 28,304 | 27,046 | 27,675 | 6,779 | 5,766 | 6,273 | 21,403 | 96.3 | 839 |
| 11 | p38d | 18,143 | 19,138 | 18,641 | 3,121 | 2,810 | 2,966 | 15,675 | 139.5 | 926 |
| 12 | p38g | 10,841 | 10,277 | 10,559 | 1,897 | 1,625 | 1,761 | 8,798 | 154.4 | 1004 |
| 13 | CDK3/Cyclin E1 | 10,530 | 11,448 | 10,989 | 2,377 | 2,623 | 2,500 | 8,489 | 158.1 | 944 |
| 14 | ERK2 | 9,339 | 9,716 | 9,528 | 2,012 | 2,326 | 2,169 | 7,359 | 160.8 | 905 |
| 15 | HIPK1 | 10,703 | 11,597 | 11,150 | 4,880 | 4,924 | 4,902 | 6,248 | 132.3 | 931 |
| 16 | HIPK2 | 8,150 | 8,146 | 8,148 | 4,324 | 4,254 | 4,289 | 3,859 | 132.9 | 899 |

**Figure 19**. **Screening for kinases responsible for the phosphorylation of Serine 249 identifies DYRK2 as the main Serine 249 kinase.** A) Scheme of the performed screenings. B) Result of the *in vitro* screening performed with the recombinant kinases incubated with WT and mutant peptides as indicated. The kinases are listed from highest to lowest score, based on the difference (in cpm) from the average counts of the p53 R249S mutant peptide minus the counts of the p53 wild-type peptide. Counts that have differences greater than 30,000 cpm are highlighted in red, and counts with differences greater than 15,000 and less than 30,000 cpm are highlighted in blue.

The dual-specificity tyrosine phosphorylation-regulated kinases (DYRKs) are evolutionary conserved enzymes weakly related to mitogen-activated protein kinases and cyclin-dependent protein kinases (Becker and Joost, 1999; Himpel et al., 2001). DYRKs consist of a conserved kinase domain and adjacent DYRK homology box with variable N- and C-terminal regions. All DYRKs auto-phosphorylate a critical Tyrosine residue in the activation loop in order to

attain the full catalytic activity, but mature and active kinases only phosphorylate substrates on Serine or Threonine residues followed by Proline. DYRK family members regulate key developmental and cellular processes such as neurogenesis, cell proliferation, cytokinesis and cellular differentiation (Soppa & Becker, 2015). The best hit obtained by the screening, DYRK2, was reported to act as an oncosuppressive kinase (Becker, 2012); it can induce degradation of GLI2, the main transcription factor of the Hedgehog pathway, and prime c-Jun and c-Myc for ubiquitin-dependent degradation (Taira et al., 2012). Importantly, it has been demonstrated that, in cells exposed to genotoxic stress, DYRK2 phosphorylates wild-type p53 at Serine 46, promoting apoptosis (Taira et al., 2007). Despite these reports, the DYRK2 locus has been found amplified in esophageal and lung adenocarcinoma (Miller et al., 2003). In line with this, recent data have highlighted that DYRK2 exerts a pro-proliferative role in triple negative breast cancer cells by fostering the 26S proteasome-dependent degradation of cell-cycle inhibitors (Guo et al. 2015).

To test whether DYRK2 was able to directly phosphorylate Serine 249 residue within full-length mutant p53 protein, we performed an *in vitro* phosphorylation assay on GST-p53 proteins. As DYRK2 is a Proline-directed kinase and has already been reported to phosphorylate p53 on Serine 46, to assess if DYRK2 was specifically acting on Serine 249 we employed the 6M and 6M R249S p53 constructs (Figure 20).

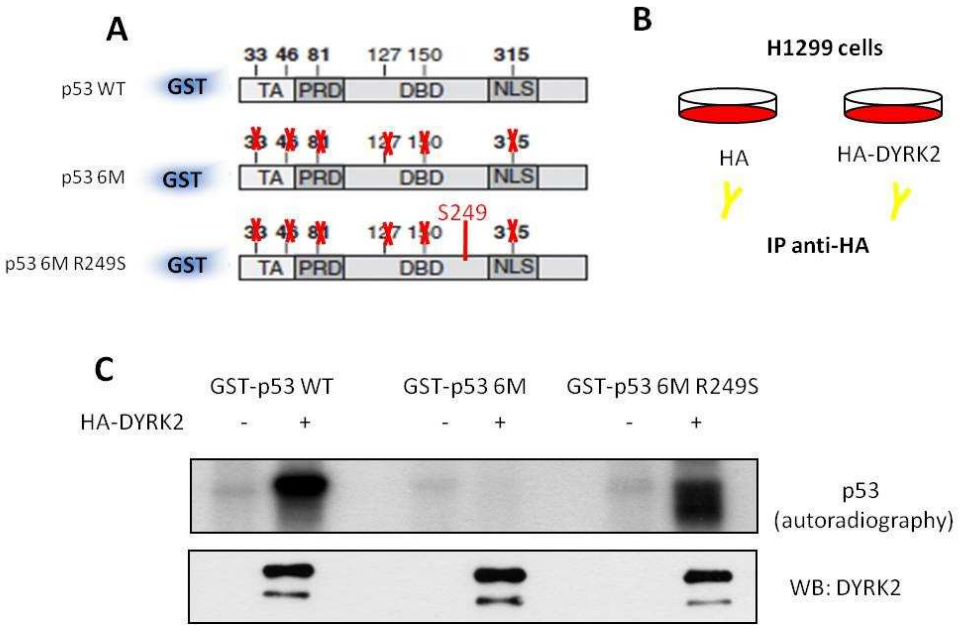



**Figure 20**. **DYRK2 phosphorylates Serine 249 in *in vitro* phosphorylation experiments.** A) Scheme of the GST-p53 protein used as substrates for the *in vitro* phosphorylation experiment. B) H1299 p53-null cells were transfected with either HA-tagged DYRK2 or empty vector as a control; the kinase was immunoprecipitated with anti-HA antibody. C) GST-proteins were incubated with HA-DYRK2 or HA in presence of $\gamma$-ATP$^{32}$ for 30' at 37°C. The reaction was run on SDS-PAGE and the presence of the phosphorylation was detected by autoradiography.

These experiments confirmed that DYRK2 acts as a direct kinase of Serine 249. Then we tested whether DYRK2 was able to bind mutant p53 R249S by co-immunoprecipitation experiments upon co-overexpressing the two proteins in p53-null H1299 cells. This analysis confirmed the interaction between DYRK2 and mutant p53 R249S (Figure 21).

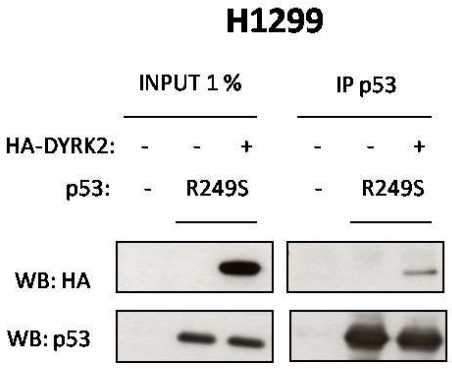



**Figure 21. DYRK2 interacts with mutant p53 R249S.** Co-immunoprecipitation of overexpressed DYRK2 and mutant p53 R249S from p53-null H1299 cells. Lysates were immunoprecipitated with anti-p53 antibody and proteins detected with the indicated antibodies.

Since Serine 249 phosphorylation creates a Pin1 binding site, we postulated that modulation of DYRK2 levels or activity should have an impact on the binding between p53 6M R249S and Pin1. To test this hypothesis we overexpressed p53 6M R249S in H1299 cells and either overexpressed or silenced DYRK2. As shown in Figure 22, DYRK2 overexpression was able to stimulate the binding of p53 6M R249S to the WW domain of Pin1, while DYRK2 silencing strongly reduced the binding. DYRK2-dependent modulation of Serine 249 phosphorylation was verified employing a phospho-S/T-Pro antibody. Moreover, GST pulldown between mutant p53 R249S and Pin1 WW domain in presence of two inhibitors of the DYRK family of kinases, INDY and Acridine, further demonstrated that DYRK2 catalytic activity is required for the interaction between p53 6M R249S and the Pin1-WW domain (Figure SI 4).

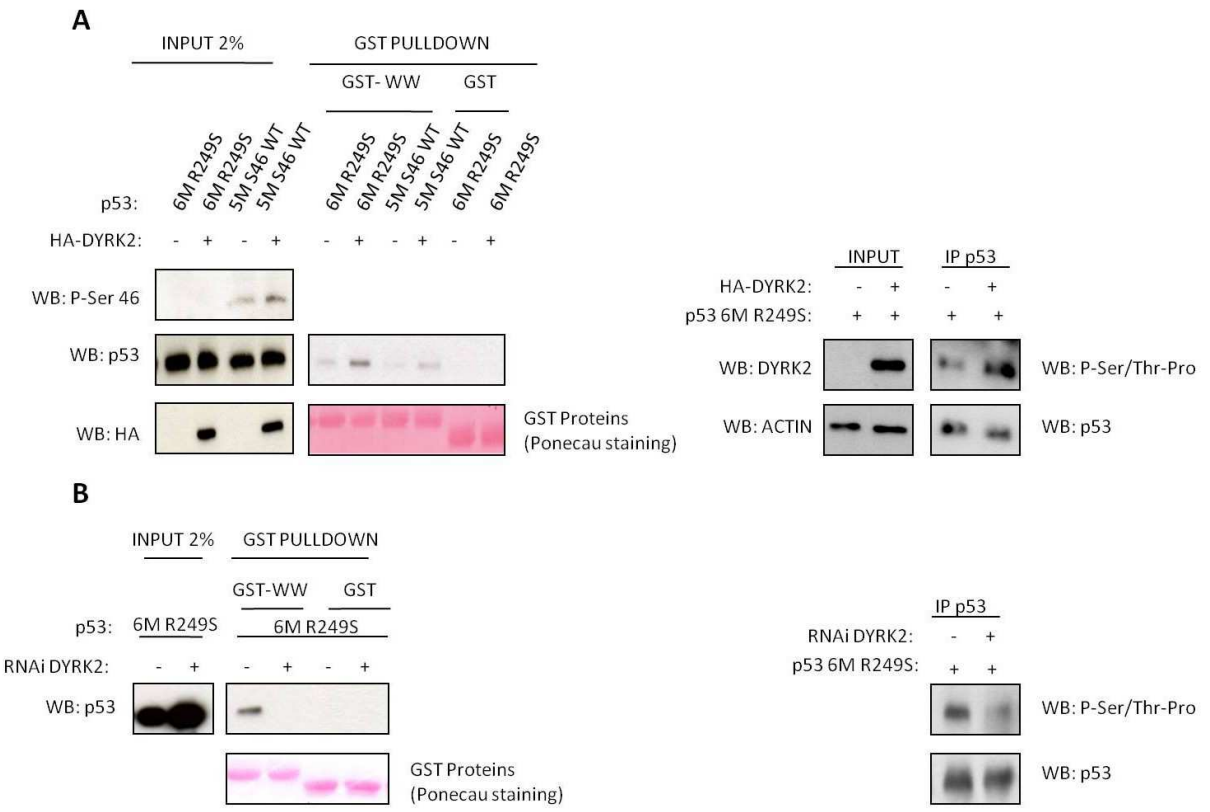



**Figure 22. Modulation of DYRK2 perturbs the binding of p53 6M R249S to the WW domain of Pin1.** A) H1299 cells were transfected with p53 6M, p53 6M R249S and p53 5M Serine 46 wild-type (used as a control) along with HA-DYRK2 or empty vector. The interaction of p53 with recombinant WW domain of Pin1 was analysed by GST pulldown of cell lysates normalized for p53 levels. The phosphorylated Serine 46 within p53 5M Serine 46 wild-type after DYRK2 overexpression is shown as a readout of DYRK2 activity. B) H1299 cells were silenced for DYRK2 and then transfected with p53 6M R249S. The interaction of p53 with recombinant Pin 1 WW domain was analysed by GST pulldown. The silencing of DYRK2 was checked by qRT-PCR (Figure SI 3). The effect of DYRK2 overexpression or depletion on the phosphorylation status of p53 6M R249S was verified with a phospho-S/T-Pro antibody.

Taken together these data demonstrate that DYRK2 phosphorylation of Serine 249 is required for the interaction of mutant Serine249-Pro250 and Pin1.

# Mutant p53 R249S regulates the expression of a subset of Interferon Stimulated Genes

Although epidemiologic data support a strong association between mutant p53 R249S and hepatocellular carcinoma, the knowledge on the mechanisms of mutant p53 R249S gain-of-function is very poor, thus leaving unanswered the question about the reason for selection of this p53 mutant in HCC. To understand which molecular events mediate mutant p53 R249S activity in hepatocellular carcinoma, we performed gene expression analysis by cDNA arrays in Mahlavu cells upon depletion of mutant p53 R249S. Functional analysis was performed on this data by Ingenuity Pathway Analysis, highlighting that among the genes downregulated

upon depletion of mutant p53 R249S, the most represented category was the one comprising interferon responsive genes (Figure 23).

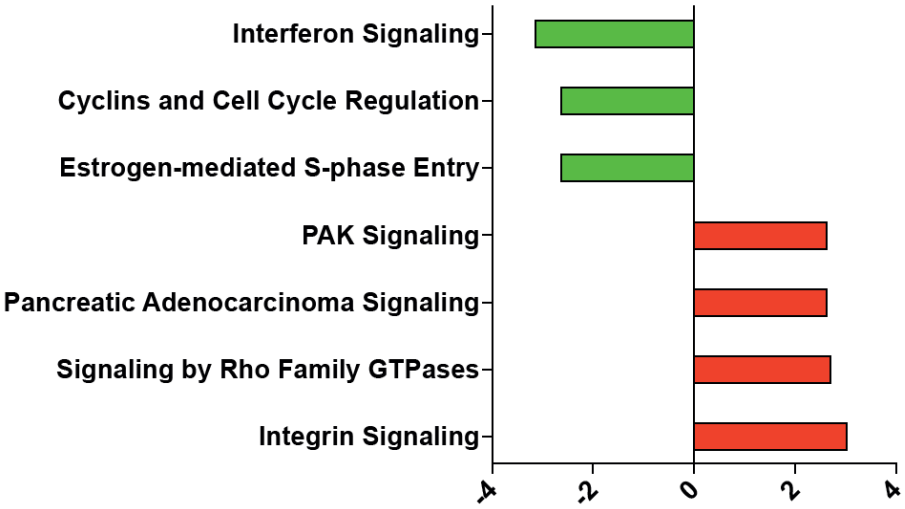



**Figure 23. Major pathways altered by mutant p53 R249S silencing in Mahlavu cells.** RNA from Mahlavu cells depleted of mutant p53 R249S expression was subjected to Illumina expression microarray in comparison with control silenced cells. Analysis of perturbed cellular pathways was performed by Ingenuity Pathway Analysis (iPA) and is shown as activation z-score (<2,5 downregulated pathways in green, >2,5 activated pathways in red). Interferon signalling resulted as the most repressed pathway after mutant p53 R249S silencing.

We built a signature of interferon stimulated genes affected by mutant p53 R249S expression, which we validated in Mahlavu (Figure 24) but also partially in other cellular contexts such as BT 549 cells (Figure SI 5).

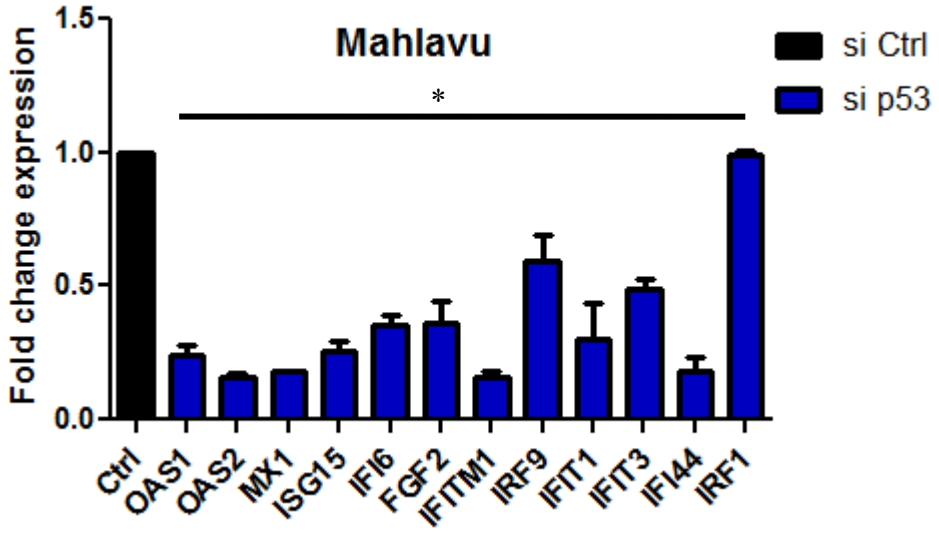



**Figure 24. mRNA level of selected targets upon silencing of mutant p53 R249S..** Changes in gene expression in Mahlavu cells are shown as fold change upon mutant p53 depletion comparing to cells transfected with control siRNA. IRF1, an IFN/STAT1 regulated gene not affected by mutant p53 R249S, was amplified as control. Error bars indicate s.d. (n=3). * *P* value <0,05.

Analysis of upstream activators of the interferon gene signature performed by Ingenuity Pathway Analysis unveiled that all the genes selected are regulated by IFNα and STAT1. To

validate these results we analysed the expression of the IFN responsive gene set after IFNα stimulation in Mahlavu cells. We observed that, although with variable activation, the selected interferon responsive genes were all upregulated by IFNα, representing the most important type I interferon (Figure SI 6). IFNα exerts its function through the activation of JAK/STAT signalling and specifically by the recruitment of the Interferon Stimulated Genes Factor 3 (ISGF3) at the Interferon-Stimulated Response Elements (ISRE) of the Interferon Stimulated Genes (ISGs). The ISGF3 complex is composed by STAT1, STAT2 and IRF9. qRT-PCR analysis of mRNA extracted from Mahlavu cells showed that the IFN signature modulated by mutant p53 R249S is affected by STAT1 silencing (Figure 25), as well as STAT2 and IRF9 silencing (Figure SI 7).

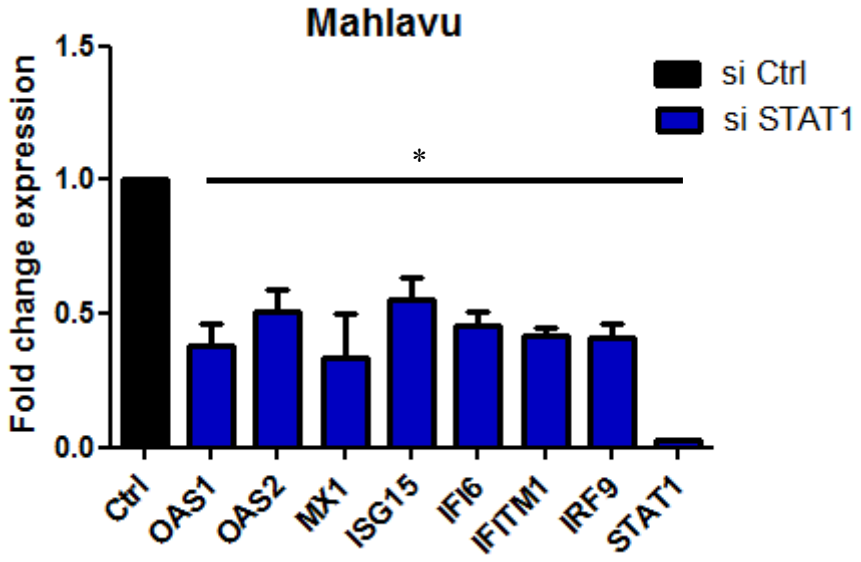



**Figure 25**. **mRNA level of selected targets upon silencing of STAT1.** Changes in gene expression are shown as fold change upon STAT1 silencing compared to control silencing. Error bars indicate s.d. (n=3). * *P* value <0,05.

ISGs are commonly defined as mediators of immune responses against viruses (Schoggins and Rice, 2011) and anti-tumour factors. However, increasing evidences show that interferon stimulated genes can exert more functions, also opposite each other. Interestingly, high expression levels of ISGs, including mutant p53 R249S modulated genes, have been reported to be involved in tumour growth, promoting cell proliferation and therapy resistance (Khodarev et al., 2004, 2007, 2012; Cheon et al., 2014, Li et al., 2014: Jin et al., 2017; Ogony et al., 2016).

# Mutant p53 R249S binds the promoters of interferon responsive genes mediating STAT1 recruitment to its target sites in a Pin1-dependent manner

The above analysis raised the question whether the transcriptional effect of mutant p53 R249S on interferon stimulated genes was mediated by interaction with STAT1. Indeed, co-immunoprecipitation experiments showed that mutant p53 R249S was able to interact with STAT1 in Mahlavu cells (Figure 26) and with STAT2 upon co-overexpression in H1299 cells (Figure SI 8).

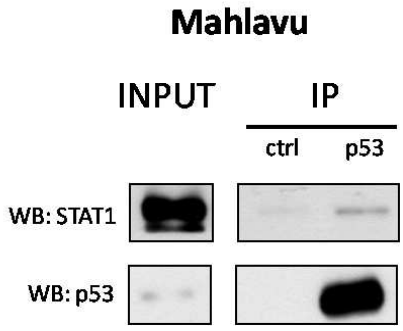



**Figure 26. Mutant p53 R249S interacts with STAT1.** Co-immunoprecipitation assay between endogenous STAT1 and mutant p53 from Mahlavu cells.

Prompted by these observations we tested whether the conformational switch mediated by Pin1 on mutant p53 R249S may affect its interaction with STAT1. Inhibition of Pin1 in H1299 cells over-expressing mutant p53 R249S was able to reduce the binding between the two proteins (Figure 27), suggesting that a highly destabilized conformation of p53 R249S, supported by Pin1 activity, is required for interaction with STAT1.

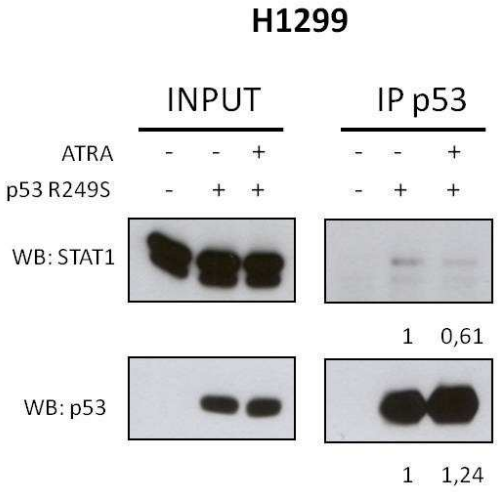



**Figure 27. Pin1 regulates the interaction between mutant p53 R249S and STAT1.** Co-immunoprecipitation assay between endogenous STAT1 and mutant p53 overexpressed in H1299 cells. Pin1 inhibitor ATRA was used 25 μM for 24h.

Having observed that mutant p53 R249S was able to interact with STAT1 and regulate its target genes, we sought to investigate if the activity of mutant p53 R249S on interferon gene signature was dependent on direct transcriptional regulation. We therefore performed ChIP experiments to determine whether mutp53 R249S was capable to bind STAT1 ISRE sites in these promoters, taking the IRF9 promoter as a paradigm since its regulation could affect the expression of all the other genes. These experiments indeed demonstrated that in Mahlavu cells mutant p53 R249S binds the STAT1-target sequence within the IRF9 promoter (Figure 28A). To assess whether Pin1 affected the binding, we performed the experiment also upon Pin1 inhibition. Treatment of the cells with ATRA was able to reduce the binding of mutant p53 R249S to IRF9 promoter, thus suggesting that the activation of interferon responsive genes by mutant p53 R249S may require the phosphorylation and isomerisation of Serine 249-Proline 250 bond. Coherently, IRF9 mRNA levels were reduced by ATRA treatment (Figure 28 B), as well as by DYRK2 depletion (Figure SI9) in Mahlavu cells.

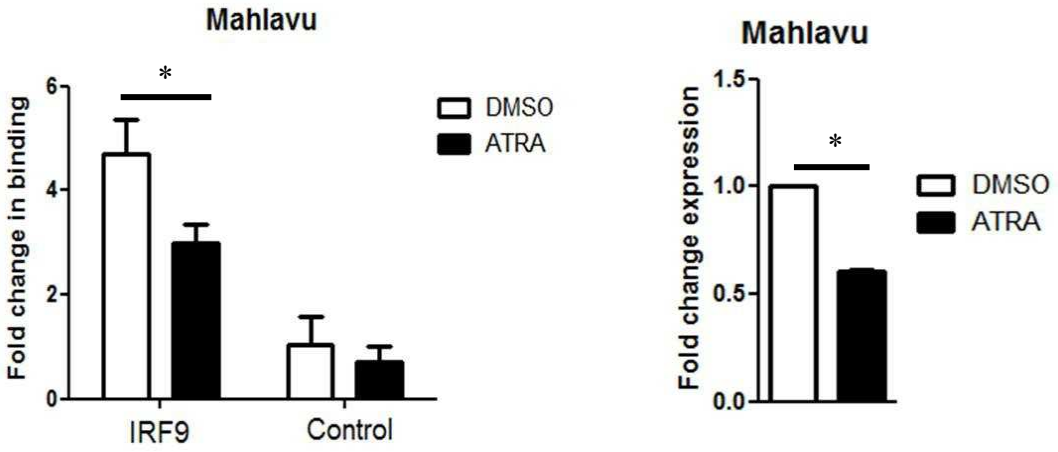



**Figure 28. Mutant p53 R249S binds to IRF9 promoter in a Pin1-dependent manner. A)** Following 48h of treatment with ATRA 50 µM, Mahlavu cells were subjected to ChIP with either anti-p53 antibody or normal Mouse IgG. Binding of p53 to the IRF9 promoter was quantified by calculating the fold increase of p53-immunoprecipitated chromatin over the control IgG-immunoprecipitated chromatin after specific qRT-PCR amplification of the ISRE-containing region. The amplification of a genomic region not bound by mutant p53 (control) was performed as control of the specificity of the binding. B) mRNA levels of IRF9 upon 48h of ATRA treatment. Error bars indicate s.d. (n=3). **P* value <0,05.

In agreement with the results of ChIP experiments, in p53-null H1299 cells the overexpression of mutant p53 R249S was able to induce IRF9 (Figure 29).

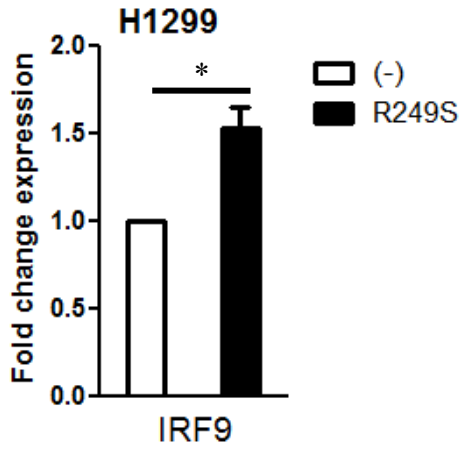



**Figure 29. mRNA levels of IRF9 in H1299 expressing mutant p53 R249S.** Error bars indicate s.d. (n=3). * *P* value <0,05.

The Interferon Regulatory Factor 9 is well known for its integral role in the ISGF3, in which it constitutes the molecular platform binding ISRE elements, therefore affecting the transcriptional activity of the whole complex (Yanai et al., 2012). By increasing IRF9 expression levels, mutant p53 R249S could foster association of IRF9 with STAT1 and STAT2, thus promoting the assembly and recruitment of ISGF3 at the ISGs promoter. To verify this hypothesis, we tested whether mutant p53 R249S was able to directly affect the amount of STAT1 bound to IRF9 promoter. To this aim we performed ChIP experiments on STAT1 in Mahlavu cells silenced for p53 expression. Importantly, STAT1 binding to its target sites was diminished in cells in which mutant p53 expression was knocked down, indicating that mutant p53 R249S was required for proper STAT1 recruitment at chromatin target sites (Figure 30).

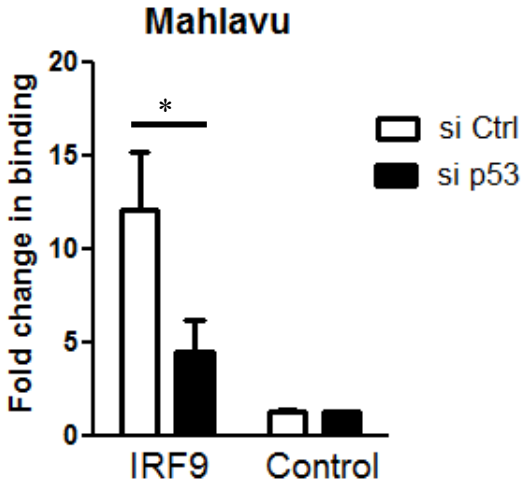



**Figure 30. STAT1 binding to its target sequences on chromatin is regulated by mutant p53 R249S.** After p53 silencing, Mahlavu cell lysates were subjected to ChIP with either anti-STAT1 antibody or normal Rabbit IgG. Binding of STAT1 to IRF9 promoter was quantified by calculating the fold increase of chromatin

immunoprecipitated by STAT1 over chromatin immunoprecipitated by control IgG after specific qRT-PCR amplification of the ISRE-containing region. The amplification of a genomic region not bound by STAT1(Control) was performed as a control of the specificity of the binding. Error bars indicate s.d. (n=3).* *P* value <0,05.

# Phosphorylation of Serine 249 and Pin1-mediated isomerization are required for mutant p53 R249S gain-of-function

Evidences of gain-of-function activities of mutant R249S include a reported pro-proliferative role in lung cancer (Vaughan et al., 2012) as well as HCC (Gouas et al., 2010). Interestingly, integrative transcriptomic analysis revealed that HBV-related hepatocellular carcinoma, which is strongly associated to p53 R249S mutation, belongs to a "Proliferation class", characterized by enrichment of pathways related to proliferation and cell cycle progression (Hoshida et al., 2009). Given these premises, we investigated the ability of mutant p53 R249S to stimulate proliferative activity in our cellular models. We observed that depletion of mutant p53 from Mahlavu cells strongly reduced cell proliferation measured by BrdU incorporation assay; in line with this effect, reconstitution of mutant p53 R249S expression with a siRNA resistant construct was able to rescue cell proliferation (Figure 31).

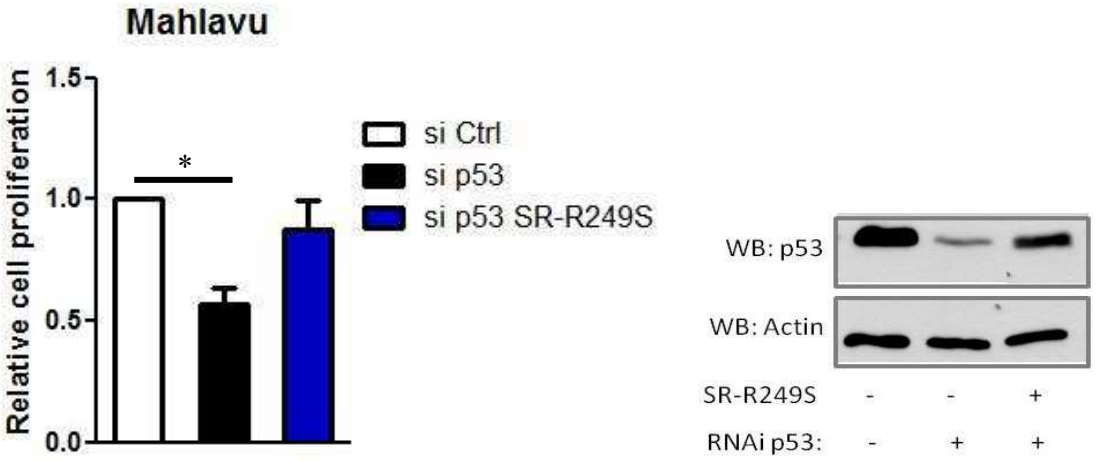



**Figure 31. Mutant p53 R249S promotes proliferation of Mahlavu cells.** BrdU cell proliferation assay in Mahlavu cells was performed after transient depletion of mutant p53 R249S; concomitant transfection of a siRNA-resistant p53 R249S overexpressing construct (SR-R249S) resulted in the rescue of the phenotype, thus confirming the direct effect of mutant p53 in promoting cell proliferation. Error bars indicate s.d. (n=3). * *P* value <0,05.

Having demonstrated that induction of cell proliferation is an aspect of mutant p53 R249S gain-of-function, we asked whether this phenotype is affected by the phosphorylation status of Serine 249. Therefore we compared the ability of mutant p53 R249S and mutant p53 R249A (mimicking unphosphorylated Serine 249) to induce proliferation in p53-null H1299 cells.

Interestingly, cells bearing the mutant p53 R249S displayed a gain in proliferation compared to the corresponding control cells, whereas overexpression of mutant p53 R249A was ineffective to induce the same phenotype (Figure 32).

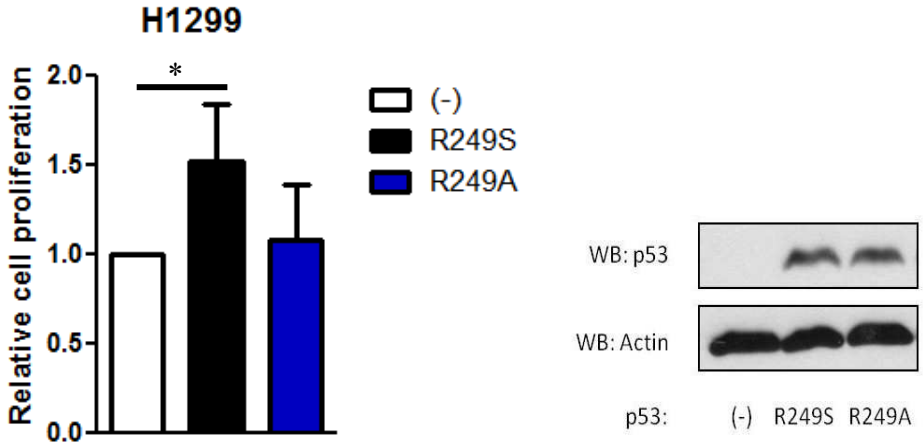
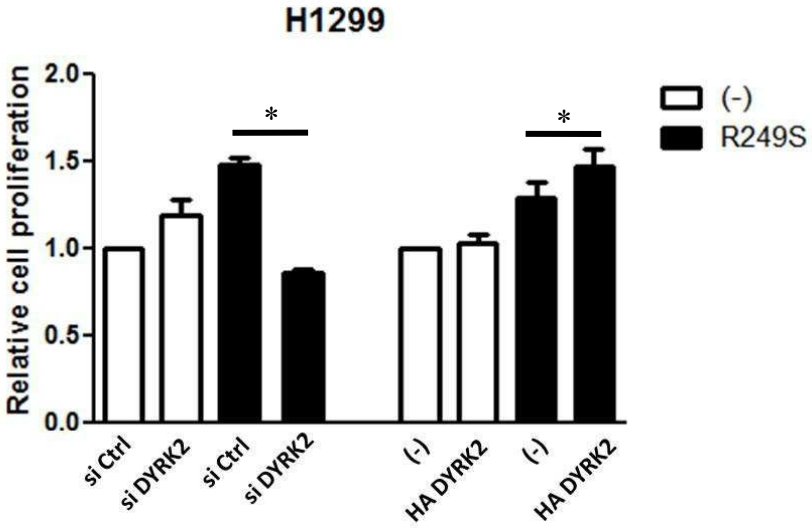



**Figure 32. Phosphorylation of Serine 249 within mutant p53 R249S promotes cell proliferation.** BrdU proliferation assay in H1299 cells was performed after overexpression of p53 mutants with Serine or Alanine residue in 249 position. The increase of cell proliferation caused by mutant p53 R249S overexpression is absent in the case of unphosphorylable Serine 249 (R249A). Error bars indicate s.d. (n=3). * *P* value <0,05.

To further investigate the role of Serine 249 phosphorylation for mutant p53 R249S gain-of-function, we verified whether modulation of DYRK2 levels was able to regulate mutant p53-dependent induction of proliferation. Consistent with DYRK2 acting as an upstream regulator of mutant p53 R249S pro-proliferative activity, both DYRK2 overexpression and silencing impacted on the proliferation of cells expressing mutant p53 R249S, while not showing significant effects in a p53-null background (Figure 33).



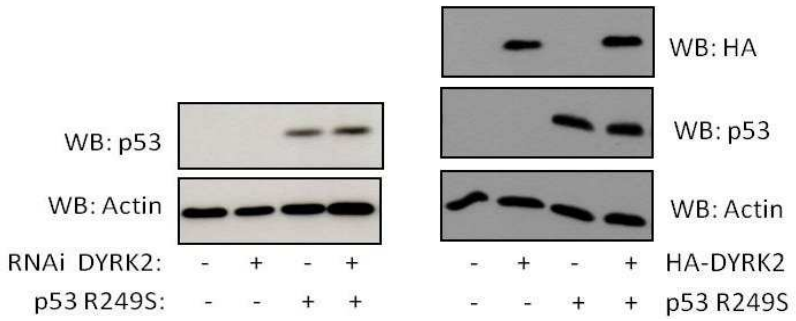



**Figure 33. Mutant p53 R249S requires DYRK2 for its pro-proliferative activity.** BrdU proliferation assay in H1299 cells was performed after DYRK2 depletion or over-expression in a p53-null (-) or mutant p53 R249S (p53 R249S) background. The effect of DYRK2 modulation on cell proliferation is effective only in a mutant R249S context. DYRK2 depletion was checked by qRT-PCR and shown in figure SI 3. Error bars indicate s.d. (n=3). * *P* value <0,05

In a similar manner, overexpression of catalytically active Pin1 was able to foster the proliferation induced by mutant p53 R249S, instead it had no effect on R249A mutant, suggesting that Pin1-dependent isomerization is required to switch Serine 249 phosphorylation into a conformational change responsible for mutant p53 R249S gain-of-function (Figure 34). Conversely, overexpression of catalytically inactive Pin1 S67E mutant was ineffective in this respect, independently of p53 status of H1299 cells.

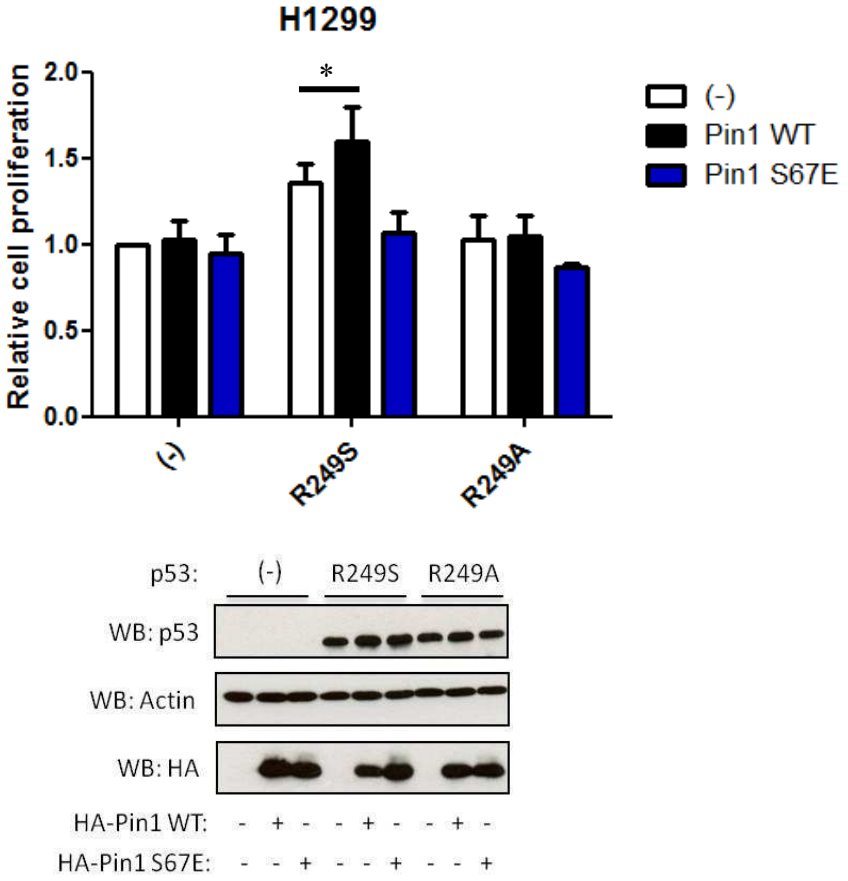



**Figure 34. Pin1 fosters mutant p53 R249S pro-proliferative capacity through its catalytic activity.** BrdU cell proliferation assay in H1299 expressing empty vector, mutant p53 R249S or mutant p53 R249A was

performed after co-overexpression of empty vector, Pin1 WT or Pin1 catalytic inactive S67E. Pin1 WT promotes proliferation of p53 R249S expressing cells, without affecting the proliferation R249A expressing H1299. Pin1 S67E was ineffective in fostering the proliferation independently of p53 context. Error bars indicate s.d. (n=3).* *P* value <0,05.

Since DYRK2, through Serine 249 phosphorylation, modulates the interaction of mutant p53 with Pin1, we reasoned that this kinase could be required for Pin1 to promote mutant p53 R249S gain-of-function. Thus we investigated the functional interplay between DYRK2 and Pin1. In the context of mutant p53 R249S-overexpressing H1299 cells we overexpressed Pin1 and concomitantly silenced DYRK2; importantly, Pin1 overexpression had no effect on the pro-proliferative activity of mutant p53 R249S in the absence of DYRK2, thus demonstrating that DYRK2-Pin1 coordinated activity is required for mutant p53 R249S gain-of-function (Figure 35).


**Figure 35. Pin1 requires DYRK2 to foster mutant p53 R249S pro-proliferative activity.** H1299 cells co-overexpressing p53 R249S and Pin1 were transiently silenced for DYRK2 expression. The gain in cell proliferation induced by overexpression of Pin1 in p53 R249S background is abolished upon silencing of DYRK2. DYRK2 depletion was checked by qRT-PCR shown in figure SI 3. Error bars indicate s.d. (n=3). * *P* value <0,05.

We demonstrated that DYRK2 and Pin1 are required for mutant p53 R249S pro-proliferative activity and for its ability to modulate an interferon gene signature associated with tumour growth. To test whether mutant p53 R249S pro-proliferative activity relies on regulation of STAT1-dependent transcription, we performed STAT1 silencing and monitored mutant p53 R249S-induced proliferation. This experiment highlighted that STAT1 depletion could abolish the gain in proliferation induced by mutant p53 R249S overexpression in H1299 cells (Figure 36). This result strongly suggests that mutant p53 R249S gain-of-function relies on the regulation of the transcription of pro-proliferative STAT1 target genes.

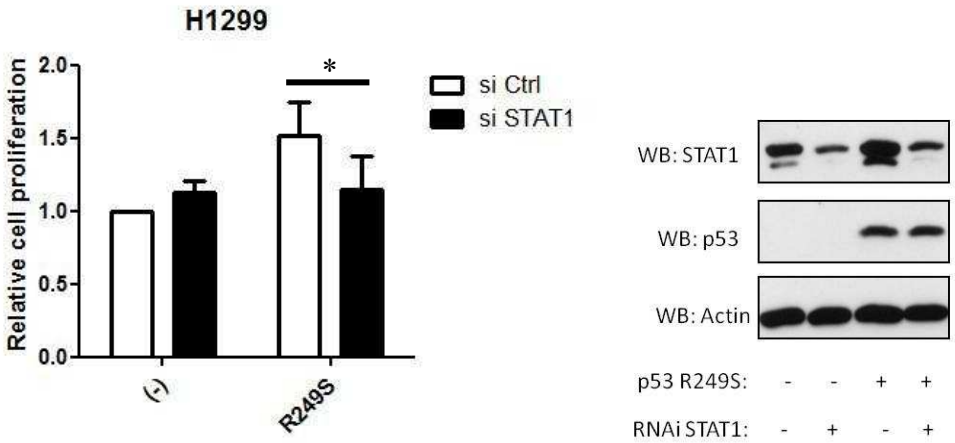



**Figure 36. STAT1 is required for the pro-proliferative activity of mutant p53 R249S.** H1299 cells overexpressing mutant p53 R249S or empty vector were transiently silenced for STAT1. Depletion of STAT1 was effective in reducing the proliferation of mutant p53 expressing cell, but not of p53-null cells. Error bars indicate s.d. (n=3). * *P* value <0,05

# DISCUSSION

In this Thesis we demonstrated for the first time that a mutation in p53, specifically the replacement of Arginine 249 with a Serine, introduces a novel post-translational modification of an oncoprotein. Moreover, p53 mutation and subsequent phosphorylation of the mutated residue create a phosphoSer249-Pro250 motif that is recognized by the prolyl-isomerase Pin1, an enzyme able to promote structural changes responsible for fine-tuning target proteins' biochemical and biological properties such as localization, stability and activity. Indeed, our results indicate that by isomerising the phosphoSer249-Pro250 bond, Pin1 promotes the stabilization of mutant p53 unfolded structure, and fosters its interaction with the transcription factors STAT1 and STAT2. In turn, cooperation between mutant p53 R249S and the JAK-STAT pathway results in the induction of Interferon-stimulated genes, that support the proliferation of cancer cells bearing p53 R249S mutation (Figure 37).

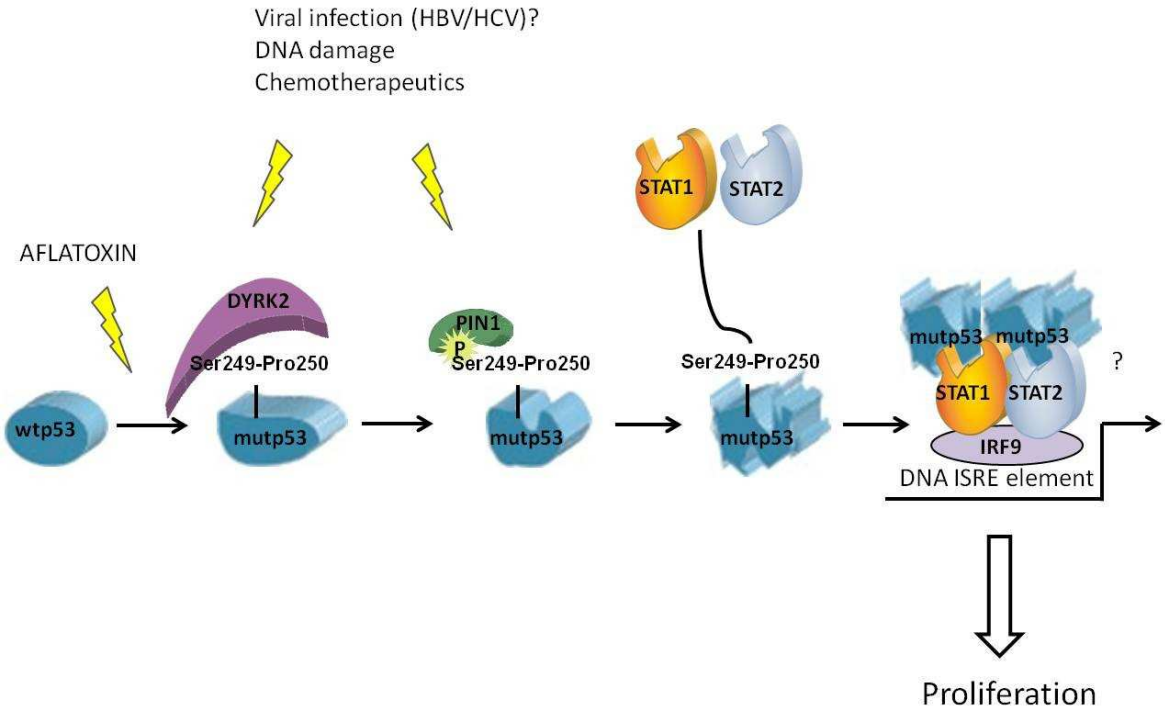



**Figure 37. Working model describing the pro-proliferative activation of mutant p53 R249S.** Mutation of p53 Arginine 249 to Serine induced by the food contaminant Aflatoxin B1 is targeted by the kinase DYRK2, whose mechanisms of stabilization and activation are still under investigation but putatively promoted by various *stimuli* such as HBV/HCV infection or DNA damage. Serine 249 phosphorylation generates a binding site for the prolyl-isomerase Pin1. The conformational change fostered by this enzyme distorts p53 in a way that it becomes capable of binding STAT1 and STAT2. This association potentiates the interferon-mediated transcription of STAT1-target genes to unleash a transcriptional program that mediates proliferation of cancer cells.

The selection for p53 R249S mutation during hepatocarcinogenesis cannot be justified exclusively by sequence-specific DNA damage induced by Aflatoxin B1. It is conceivable that the specific context in which this mutation occurs could offer further explanation for its strong recurrence in HCC. The R249S mutation has been detected in non-cancer liver tissues of aflatoxin-exposed subjects (Aguilla et al., 1994) and in circulating DNA of individuals without clinically detectable liver disease from Gambia (Kirk et al., 2005). Interestingly in the Gambian study the prevalence of R249S mutation increased with the severity of liver disease, supporting the hypothesis that after its induction by Aflatoxin, the R249S mutation is selected during hepatocarcinogenesis to facilitate HCC progression. Interestingly, in HBsAg transgenic mice treated with AFB, mutant p53 R249S appeared to cooperate with toxins in inducing HCC (Ghebranious and Sell, 1998). This evidence supports the hypothesis that p53 R249S activity is also related to HBV infection. Coherently, the proportion of HCCs with R249S that are HBV-negative is very small in high incidence areas (Gouas et al., 2012). *In vitro* studies showed that the viral HBx protein is able to bind mutant p53 R249S; moreover, cooperation between tumour-derived HBx mutant proteins and mutp53 R249S regulates cell proliferation and anchorage-independent growth in human hepatocyte-derived cell line (Jiang et al., Int J Cancer, 2010). All together these evidences support the possibility of a liason between HBV and mutant p53, specifically mediated by HBx.
In this section we will analyze all the open aspects of the activation pathway that we identified with particular attention on the interaction of molecular players with HBV.

By means of *in silico* and *in vitro* screenings we identified several putative kinases for Serine 249 phosphorylation, among which DYRK2 showed the strongest effect. DYRK2 has been long considered an oncosuppresive kinase, due to its ability to promote degradation of oncogenes such as c-Myc and c-Jun and to positively regulate wild-type p53 functions (Taira et al., 2012). In particular, DYRK2-dependent phosphorylation of wild-type p53 Serine 46 upon genotoxic stress is intriguing since it was demonstrated that Ser46-Pro47 phosphorylation and consequent isomerization by Pin1 are crucial for gain-of-function of several p53 mutants frequently found in tumours (Girardini et al., 2011). According to this model and based on our findings, it is conceivable that the role of DYRK2 in fostering mutant p53 R249S gain-of-function is mediated by an interplay between modification of both Serine 46 and Serine 249 in concert with Pin1. We aim to address this issue by assessing if Serine 46 phosphorylation is required for mutant p53 R249S gain-of-function.

Given the role of DYRK2 in modulating both wild-type and mutant p53 functions it would be extremely important to understand the mechanisms of DYRK2 activation in pre-cancerous and tumor tissues. It has been demonstrated that genotoxic stress stabilizes DYRK2 via ATM-mediated phosphorylation in p53 wild-type cells (Taira et al., 2010). Further investigations will be required to understand the signalling inducing DYRK2 in mutant p53-bearing tumours and in particular in the p53 R249S HCC context, where viral infection and persistence of DNA damage signalling are expected.

It has been recently reported that DYRK2 exerts a pro-proliferative role in triple negative breast cancer cells by promoting the activity of the 26S proteasome through direct phosphorylation of one of its subunits (Guo et al., 2015 and 2016). Interestingly, activation of proteasome via transcriptional partnership with NRF2 has been recently recognized as a common gain-of-function activity of mutant p53 proteins, including p53 R249S (Walerych et al., 2016). These data support a model in which on one hand DYRK2 directly fosters proteasome activity and on the other it may support mutant p53 ability to stimulate proteasome gene transcription. We will verify this hypothesis by modeling the interaction of modified mutant p53 R249S with NRF2 as well as testing the ability of DYRK2 to modulate proteasome gene transcription. We may similarly investigate the contribution of mutant p53 Serine 249 modification to modulate the interactions with other transcription factors important for mutant p53 gain-of-function (e.g. NF-Y).

In addition to DYRK2, other kinases were able to phosphorylate Serine 249, including CDK1 and CDK2 and mTOR. Interestingly, inhibition of several CDKs has been proven effective to halt HCC progression (Bisteau, 2014), while PI3K/AKT/mTOR pathway has been found to be activated in 5-10% of HCC, via activating mutations of PIK3CA or inactivating mutations of TSC1 or TSC2 (Zucmann-Rossi et al., 2015). These evidences may suggest that different signalling pathways, which in turn activate several kinases, might concomitantly operate to unleash the gain-of-function of mutp53 R249S.

We demonstrated that Serine 249 phosphorylation within mutant p53 creates a new binding site for Pin1, which fosters the stabilization of the distorted structure that characterizes the mutant conformation of p53 R249S. This observation is particularly important given the relatively mild structural distortion of this mutant compared to others, that could account for the limited and conflicting evidence of its gain-of-function (Lee et al., 2011). Our results suggest that Pin1-dependent isomerisation and subsequent de-phosphorylation by yet unknown phosphatases stabilizes p53 R249S mutant conformation. We will perform a

dedicated *in vitro* screening to identify the phosphatases that act on Serine 249 stabilizing its mutant conformation and thus promoting its gain-of-function.

The finding that Pin1 is fundamental in promoting mutant p53 R249S gain-of-function is of particular importance given the link between Pin1 and HBV. In particular Pin1 has been reported to increase HBx protein stability and to enhance HBx-dependent transcriptional activity, synergyzing to induce HepG2 cell proliferation and growth of tumour xenografts in mice (Pang et al., 2007). The relevance of the HBx-Pin1 interplay is supported by the fact that Pin1 overexpression occurs in more than 50% of HBx positive HCC Chinese patients (Bao et al., 2004). These evidences and our results support the idea that in HBV-related hepatocellular carcinoma Pin1 could promote tumorigenesis fostering the activity of HBx and mutant p53 R249S, which separately or synergistically can foster tumor growth.

We demonstrated that the structural distortion imposed by Pin1 on mutant p53 R249S is required for its binding to STAT1 and STAT2 to induce the expression of a subset of interferon stimulated genes. While our results demonstrate direct binding of mutant p53 R249S to DNA ISRE sequences, it may also act at different levels on the JAK/STAT pathway. It was shown that mutant forms of p53 were able to interact with STAT1, promoting its phosphorylation (Youlyouz-Marfak, I). Although from our transcriptomic data and from preliminary results we can exclude a direct effect of mutant p53 R249S on the expression of the Janus kinases, it could be possible that mutant p53 R249S impacts on the phosphorylation status of STAT proteins acting in a subsequent step, and in particular regulating the phosphorylation of the fraction of the protein bound to DNA; this could be particularly important to explain the selectivity of mutant p53 R249S in inducing a particular subset of ISGs, as unphosphorylated STAT1 was observed to regulate the expression of genes involved in tumour growth and to be associated to poor survival (Cheon et al., 2011).

It is important to underline that all our data, that we demonstrated significant in a mutant p53 R249S context, could be relevant also in tumours bearing other mutant forms of p53. Indeed, it was observed that induction of STAT1 signaling in esophageal tumor microenvironment is related to the cooperation between mutant p53 R175H and Periostin (POSTN). Invasive esophageal cells expressing POSTN and p53 R175H mutation display activation of STAT1 target genes, that in turn foster a permissive microenvironment that facilitates invasion of esophageal epithelial cells into the extracellular matrix (Wong et al., 2013). Interestingly, our preliminary data demonstrate that in MDA-MB 231 cells the depletion of mutant p53 (R280K) affects the expression of some of the genes validated in Mahlavu cells.

All the abovementioned steps of mutant p53 R249S activation towards its pro-proliferative gain-of-function could provide possible targets for therapeutic intervention for HCC that can poorly benefit from chemotherapy.

Drugs commonly employed to target altered conformation of mutant p53 in tumours have been proven to be only slightly effective in mutant p53 R249S-bearing cells. In particular, out of the several synthetic polypeptides and small molecules that have been identified as having the capacity to restore wild-type functions to mutant p53, only two of them, PRIMA-1 and CDB3, have been specifically tested for their capacity to restore the activity of mutant p53 R249S. A study on HCC cells overexpressing mutant p53 R249S showed that PRIMA-1 had a cytotoxic effect in liver cancer cells, enhanced by mutant p53; however, inhibition of p53 by RNA interference in PLC/PRF/5 did not prevent the activity of PRIMA-1, indicating that the effect is p53 R249S independent. The evidence that the presence of mutant p53 R249S appears to protect cells against PRIMA-1 toxicity suggests that mutant p53 possesses a GOF that promotes cell survival, and that PRIMA-1 may act blocking this function (Shi et al., 2008). CDB3 is a synthetic peptide that binds *in vitro* to native or partially denatured forms of p53 core domain and promotes its folding into a native wild-type form. Studies with mutant p53 R249S have shown that CDB3 could bind to mutant p53 and decrease the local distortion in the DNA binding region. However, the proof of restoration of wild-type p53 biological effects is still lacking (Friedler et al., 2002). Given these premises and based on our results, it could be suggested a strategy to target mutant p53 R249S structure by acting on DYRK2 and Pin1.

Unluckily, no specific DYRK2 inhibitor is available to date; however, as we identified several other kinases putatively acting on Serine 249, inhibitors of those enzymes could be tested for their efficacy in inhibiting Serine 249 phosphorylation.

Several Pin1 inhibitors have been patented in the recent years, but only few of them have undergone clinical trials (Zhou and Lu, 2016). Recently it has been reported that Pin1 can be effectively inhibited by a drug already employed in clinic for the treatment of acute promyelocytic leukemia, All *Trans* Retinoic Acid (ATRA). ATRA exerts inhibition and degradation of Pin1 in tumour cells, blunting Pin1-dependent oncogenic mechanisms and breast cancer growth *in vivo* (Wei et al., 2015). However ATRA showed only moderate efficacy against advanced breast cancer in clinical trials (Mantovani et al., 2016), suggesting that combinatorial therapy with kinase inhibitors could represent a better option to efficiently counteract mutant p53 R249S activity.

Apart from the possible inhibition of mutant p53 R249S gain-of-function via interference with its upstream regulatory pathways, our data also raise important observations about the clinical use of interferons in the treatment of mutant p53-bearing tumours. The ability of type I IFNs to induce pro-apoptotic and anti-proliferative responses in a variety of cell types has led to exploration of their potential as antitumor therapeutic agents. IFNα is approved for the treatment of haematological malignancies, solid tumours and viral infections (Platanias et al., 2005). However, recent studies showed that constitutive exposure of cells to a low level of type I IFN leads to steady-state expression of interferon responsive genes linked to chemoresistance (the so-called IRDS signature) that, via still unknown mechanisms, favour EMT and metastasis, suppression of T cell toxicity and resistance to chemotherapies (Wallance et al., 2011). Coherently, increased expression of interferon stimulated genes has been identified in cancer cells compared to corresponding normal primary cells or normal tissues, and associated with tumour invasion (Andersen et al., 2006; Hatano et al., 2008; Suomela et al., 2004), and metastatic properties (Khodarev et al., 2009). These observations are in line with our data which demonstrate that the mutant p53 R249S oncogene can intercept JAK/STAT1 pathway to promote pro-proliferative interferon gene expression in cancer cells. Based on our data, it is also conceivable that mutant p53 R249S (but also other forms of mutant p53) might potentiate the effect of exogenous IFNα administration in promoting pro-proliferative gene expression, thereby converting anti-proliferative IFNα activities into dangerous tumour promoting actions. In this respect, understanding how mutant p53 R249S contributes to selectively induce a specific subset of interferon responsive genes might be of fundamental importance to redirect the response to exogenous interferon administration towards the expression of ISGs encoding pro-apoptotic and anti-proliferative proteins.

# MATERIALS AND METHODS

## Reagents and plasmids

The following compounds were purchased from Sigma Aldrich: DYRKs inhibitors Indy (SML1011) and Acridine (A23609), Pin1 inhibitor ATRA (R2625). Interferon α 2A was purchased from Abcam (ab187225).

$pcDNA_3$ p53 wild-type, $pcDNA_3$ p53 6M, $pcDNA_3$, pGEX 4T1 plasmid containing Pin1 WW domain (GST-Pin1 WW) have been previously described (Zacchi et al., 2002). $pcDNA_3$ p53 R249S siRNA resistant was obtained by site-directed mutagenesis (Agilent technologies, quick change lightning site-directed mutagenesis kit #210518-5) on $pcDNA_3$ p53 wild-type siRNA resistant plasmid (Walerych et al 2016). $pcDNA_3$ p53 6M R249S was generated by site-directed mutagenesis of $pcDNA_3$ p53 6M plasmid. pGEX 4T1 p53 WT, p53 6M and p53 6M R249S were constructed by subcloning the corresponding cDNAs from $pcDNA_3$. cDNA encoding for DYRK2 variant 2 was purchased from Addgene in the form of pRV DYRK2 vector and subcloned into $pCDNA_3$ HA. $pCDNA_3$HA Pin1 and $pCDNA_3$HA Pin1 S67E have been previously described (Rustighi et al., 2009).

## Cell Lines

Mahlavu liver cancer cells were obtained from IARC and cultured in Eagle's minimal essential medium (EMEM) supplemented with 10% FBS, 1% Glutamax, 1% non essential aminoacids, 1% antibiotics. BT549 cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% FBS, 1% antibiotics. H1299 were cultured in Roswell Park Memorial Institute medium supplemented with 10% FBS, 1% antibiotics.

## Transfections and Infection

Transfection of DNA was performed with Lipofectamine 2000 (Invitrogen) for H1299 and Lipofectamine LTX (Invitrogen) for Mahlavu. For siRNA transfection, double-stranded RNA oligos (40 nM) were transfected using Lipofectamine RNAiMax (Invitrogen). siRNA oligonuceotides are listed below.

| **siRNA** | **Sequence** |
|---|---|
| DYRK2 | GAACAAGCAATGAAGCAAT |
| IRF9 | GCAGAGACUUGGUCAGGUA |

| | |
|---|---|
| p53 | GACUCCAGUGGUAAUCUAC |
| Pin1 | CGGGAGAGGAGGACUUUGA |
| STAT1 | GCGUAAUCUUCAGGAUAAU |
| STAT2 | GGACUGAGUUGCCUGGUUA |

## Liquid chromatography tandem mass spectrometry (LC_MS/MS)

The frozen beads were thawed on ice and then 20 µl of trypsin (Promega), diluted to 50 µg/ml with 20 mM triethylammonium bicarbonate, was added to the beads and then incubated for 12h at room temperature. The supernatant was removed and the beads washed with an additional 50 µl trypsin. The supernatant and wash were pooled and incubated at 56°C for 1h. The digest was reduced, alkylated and purified using standard procedures. The purified peptides were subjected to LC-MS/MS using an Easy Nano LC II (Bruker Daltonics) and an Amazon ETD ion trap mass spectrometer (Bruker Daltonics). The acquisition cycle of the mass spectrometer was set to perform ETD and pseudo-$MS^3$ when triggered by the neutral loss of phosphate. The resulting data was searched using the MASCOT (Matrix Science, version 2.4) and the GPM (version 2.2.1) search engines. Scaffold (Proteome Software, version 3_00_08) was used to filter the results at a false discovery rate of <5% for both peptides and proteins.

## *In Vitro* Binding

GST pull-down assays were performed using recombinant GST-Pin1 WW domain or GST purified from bacteria. H1299 cells were transfected with different p53 expression constructs and after 24 h cells were lysed in 150 mM NaCl, 50 mM Tris/HCl pH7.5, 10% glycerol, 0.1% NP-40, supplemented with protease inhibitor cocktail (Sigma), 1 mM PMSF, 5 mM NaF, 1 mM $Na_3VO_4$. Protein extracts were incubated with 2 µg of GST proteins to detect p53 binding. For mutant p53 6M R249S binding to GST-WW domain upon DYRK2 silencing, cells were transfected with p53 construct after 24 h from the silencing. For mutant p53 6M R249S binding to GST-WW domain upon DYRK2 overexpression, cells were transfected with p53 expression construct along with $pcDNA_3$ HA-DYRK2 or empty vector. For mutant p53 6M R249S binding to GST-WW domain upon DYRK2 inhibition, cells were transfected with p53 expression construct, serum starved for 24 h and treated with Acridine or INDY 10 µM for 16 h. For lambda phosphatase (λPP) treatment, cell lysates were incubated with 1000 U of λPP (NEB P0753S) at 30°C for 1 h and 30 minutes.

For co-IP of ectopically expressed mutant p53 R249S and ectopically expressed Dyrk2, mSTAT2 and endogenous STAT1, H1299 cells were transfected with the appropriate constructs and after 36 h harvested and lysed in 50 mM Tris-HCl pH8, 150 mM NaCl, 1% NP-40, 1mM EDTA with protease inhibitor cocktail [Sigma], 1 mM PMSF, 5 mM NaF, 1 mM $Na_3VO_4$. Monoclonal anti-p53 DO-1 (Santa Cruz, sc-126) was used for immunoprecipitation.

For co-IP of endogenous mutant p53 and Pin1 in Mahlavu and BT 549, cells were harvested and lysed in 20 mM Tris-HCl pH8, 120 mM NaCl, 1mM EDTA, 0,5% NP-40 with protease inhibitor cocktail [Sigma], 1 mM PMSF, 5 mM NaF, 1 mM $Na_3VO_4$. Monoclonal anti-p53 DO-1 and monoclonal anti-Pin antibodies were used for immunoprecipitation, normal Mouse IgG (Santa Cruz, sc-2025) and normal Rabbit IgG (Santa Cruz, sc-2027) were used as negative controls.

For co-IP of endogenous mutant p53 and STAT1, Mahlavu cells were harvested and lysed in 50 mM Tris-HCl pH7.5, 150 mM NaCl, 0,5% NP-40, 1mM EDTA, 10% glycerol with protease inhibitor cocktail [Sigma], 1 mM PMSF, 5 mM NaF, 1 mM $Na_3VO_4$. Monoclonal anti-p53 DO-1, was used for immunoprecipitation and normal Mouse IgG as negative control.

For conformational immunoprecipitation, Mahlavu cells were lysed in 50 mM Tris-HCl pH8, 150 mM NaCl, 1% NP-40, 1mM EDTA with protease inhibitor cocktail [Sigma], 1 mM PMSF, 5 mM NaF, 1 mM $Na_3VO_4$. 2 ug of anti-p53 Pab240 (Santa Cruz, sc-99) were used for imunoprecipitation.

For detection of phospho-Serine 249, cells were lysed in 50 mM Tris-HCl pH8, 150 mM NaCl, 1% NP-40, 1mM EDTA with protease inhibitor cocktail [Sigma], 1 mM PMSF, 5 mM NaF, 1 mM $Na_3VO_4$. 1 ug of anti-p53 DO-1 was used for immunoprecipitation. After incubation with the primary antibody, the non-phosphorylated peptide GMNRSPILTI-cys was employed for 2 h at the final concentration of 10 µg/ml to block non specific signals before secondary antibody was used.

All the antibodies used for co-ip experiments were covalently bound to protein G–Sepharose (Amersham) using 5 mg/ml dimethylpimelimidate (Pierce).

## Antibodies

Antibodies used for Western Blot: monoclonal anti-HA (Roche, 11867423001), polyclonal anti-p53 (raised against human p53 wild-type expressed in bacteria as MBP fusion protein and affinity-purified by standard procedures), polyclonal anti-Pin1 (Zacchi et al., 2002), monoclonal anti-Flag (Sigma F3165), polyclonal anti-STAT1 (Santa Cruz, E23, sc-346), anti-

p53pS15 (Cell Signaling, 9284S), anti-p53pS46 (BD, 558545), polyclonal anti-pS-P/pT-P (Abcam, 9344). Anti-phospho p53 Serine 249 was kindly provided by Hua Lu.

## BrdU Proliferation assay

Cells, transfected with the indicated plasmids or RNAi dsRNA, were pulsed for 3 h with 30 μM bromodeoxyuridine (BrdU; Sigma). Cells were then fixed, permeabilized and treated with NaOH to denature DNA. BrdU incorporation was measured by immunofluorescence with an anti-BrdU antibody (GE Healthcare), and the nuclei were stained with Hoechst. At least 300 cells were scored for BrdU incorporation.

## Quantitative Real-Time PCR

Cells were harvested in Qiazol lysis reagent (Qiagen) for total RNA extraction. cDNA was obtained using iScript Advanced cDNA Kit (BIOARD) in accordance with the manifacturer's protocol. qRT-PCR was performed using iTaq Universal SYBR Green Supermix (BIORAD). Actin and H3 mRNA were used as internal controls. Primer sequences are listed below.

| **GENE** | **Sequence FORWARD** | **Sequence REVERSE** |
|---|---|---|
| β-Actin | CGCCGCCAGCTCACCATG | CACGATGGAGGGGAAGACGG |
| DYRK2 | CCTACCCGACCGATTGGC | TTGGTTGTGTCGTGAGCACT |
| FGF2 | GCTGTACTGCAAAAACGGGG | TAGCTTGATGTGAGGGTCGC |
| H3 | GTGAAGAAACCTCATCGTTAC AGGCCTGGT | CTGCAAAGCACCAATAGCTGCAC TCTGGAA |
| IFI6 | CTTGTGCTACCTGCTGCTCT | TTTCTTACCTGCCTCCACCC |
| IFI44 | CCACCGAGATGTCAGAAAGAG | TGGTACATGTGGCTTTGCTC |
| IFIT1 | CCTCAGTCTTGCAGCCTCTC | TCACCATTTGTACACATCTCCACT |
| IFIT3 | GAACATGCTGACCAAGCAGA | CAGTTGTGTCCACCCTTCCT |
| IFITM1 | CCGTGAAGTCTAGGGACAGG | GGTAGACTGTCACAGAGCCG |
| IRF1 | CAAATCCCGGGGCTCATCTGG | CTGGCTCCTTTTCCCCTGCTTTGT |
| IRF9 | TTCTGTCCCTGGTGTAGAGCCT | TTTCAGGACACGATTATCACGG |
| ISG15 | GGTGGACAAATGCGACGAAC | TCGAAGGTCAGCCAGAACAG |
| MX1 | ACCATTCCAAGGAGGTGCAG | TGCGATGTCCACTTCGGAAA |
| OAS1 | GATTCTGCTGGCTGAAAGCAA | CTGGGATCGTCGGTCTCATC |
| OAS2 | AGCCAGCTGAGAGCAATGG | GAGCCACCTATGGCCACTCC |
| p53 | CTCCTCTCCCCAGCCAAAGA | GGAACATCTCGAAGCGCTCA |

| | | |
|---|---|---|
| PIN1 | CTGGAGCTGATCAACGGCTAC ATCC | GCAGCGCAAACGAGGCGTCT |
| STAT1 | CTTTGGTTGAATCCCCAGGC | GTGCTCCCAGTCTTGCTTTTC |
| STAT2 | CCGGGACATTCAGCCCTTTT | CTCATGTTGCTGGCTCTCCA |

## *In vitro* phosphorylation assay

GST-p53 WT, GST-p53 6M and GST-p53 6M R249S were incubated with HA DYRK2 or HA overexpressed and immunoprecipitated from H1299 cells for 30 min at 37°C. The reaction buffer was composed as follows: 20mM Hepes pH7, 10mM $MgCl_2$, 2mM DTT, 1 mM $Na_3VO_4$, 5mM β-glycerophosphate and 200μM ATP. To each reaction $^{32}$P-γ-ATP (5 μCi per sample) was added. The reaction was stopped by addition of 6x SDS sample buffer and SDS-PAGE was performed, followed by fixation of the gel, drying and autoradiography.

## Microarray hybridization and IPA anlysis

For gene expression profiling in Mahlavu cell lines, we used the Illumina HumanHT-12-v4-BeadChip (Illumina). Total RNA isolated from the cell lines transfected with control or p53 siRNA was reverse transcribed and amplified according to standard protocols and *in vitro* transcription was then carried out to generate cRNA. cRNA was hybridized onto each array and then labeled with Cy3-streptavidin (Amersham Biosciences). The array was then scanned using a BeadStation 500 system (Illumina). The probe intensities were calculated and normalized using GenomeStudio Data Analysis Software's Gene Expression Module (GSGX) Version 1.9 (Illumina). Further data processing was performed in the R computing environment version 3.2 (http://www.r-project.org/), with BioConductor packages (http://www.bioconductor.org/). Full transcriptomic expression datasets have been imported to Ingenuity Pathway Analysis (IPA) software (Qiagen, www.ingenuity.com) and Pathway analysis module of IPA was further used to associate analyzed gene list with molecular pathways.

## Chromatin Immunoprecipitation assay

After specific treatments, Mahlavu cells were cross-linked for 15 min with 1% formaldehyde, neutralized with 125 mM glycine pH2.5 and washed in PBS. Cells were scraped and centrifuged at 4000 rpm for 10 min at 4°C.

For STAT1 chromatin immunoprecipitation, cellular pellets were resuspended in hypotonic lysis buffer (20 mM Hepes, pH7.9, 10 mM KCl, 1 mM EDTA pH8, 10% glycerol, 1 mM

DTT, protease inhibitor cocktail [Sigma], 1 mM PMSF, 5 mM NaF 0.5 mM) and incubated for 10 min at 4°C. Cell lysates were homogenized with 30 strokes in Dounce homogenizer. Nuclear pellets were collected by centrifugation (3000 rpm for 5 min at 4°C) and resuspended in lysis buffer (10 mM Tris-Cl pH8, 140 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxycholic acid, 1 mM DTT, protease inhibitor cocktail [Sigma], 1 mM PMSF, 5 mM NaF 0.5 mM).

For mutant p53 chromatin immunoprecipitation, cellular pellets were resuspended in lysis buffer (50 mM Hepes pH7.9, 140 mM NaCl, 1 mM EDTA, 10% Glycerol, 0.5% NP-40, 0.25% Triton X-100, protease inhibitor cocktail [Sigma], 1 mM PMSF, 5 mM NaF) and incubated for 10 min at 4°C. The crude nuclei were collected by centrifugation (3000 rpm for 10 min at 4°C) and washed (10 mM Tris-HCl at pH8, 1 mM EDTA, 0.5 mM EGTA, 200 mM NaCl, protease inhibitor cocktail [Sigma], 1 mM PMSF, 5 mM NaF) for 5 min at 4°C. Washed nuclei were centrifuged as described earlier and resuspended in RIPA 100 mM buffer (20 mM Tris-HCl pH7.5, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.5% Na-Deoxycholate, 0.1% SDS, protease inhibitor cocktail [Sigma], 1 mM PMSF, 5 mM NaF).

Chromatin was sonicated (power setting 5) with a Misonix Microson in 10" bursts followed by 50" of cooling on ice for a total sonication time of 2 min per sample. Chromatin was pre-cleared for 1 h at 4°C with protein A/G PLUS-Agarose (Santa Cruz Biotechnologies). After overnight immunoprecipitation at 4°C, DNA protein complexes were recovered with protein A/G PLUS-Agarose and washed sequentially with RIPA 100 mM buffer, RIPA 250 mM buffer (20 mM Tris-HCl pH7.5, 250 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.5% NaDeoxycholate and 0.1% SDS), LiCl solution (10 mM Tris-HCl pH8, 1 mM EDTA, 250 mM LiCl, 0.5% NP-40 and 0.5% Na-Deoxycholate) and TE. RNase treatment was performed in TE for 30 min at 37°C and to reverse cross-linking samples were treated overnight at 68°C adding an equal volume of proteinase K solution (200 mM NaCl, 1% SDS and 0.3 mg/ml proteinase K [Invitrogen]). In parallel, inputs were treated in the same way. After phenol/chloroform extraction and ethanol precipitation samples were resuspended in $H_2O$.

Immunoprecipitations were performed using 2 μg of anti-STAT1 (Santa Crutz E23, sc-346), anti-p53 DO-1 (Santa Cruz, sc-126), normal Rabbit IgG (Santa Cruz, sc-2027) or normal Mouse IgG (sc-2027 Santa Cruz). Real-time PCR was performed by using iTaq Universal SYBR Green Supermix (BIORAD). Promoter occupancy was calculated as the fold increase of normalized immunoprecipitated chromatin over the control IgG with the $2^{-\Delta\Delta Ct}$ method. Primer sequences are listed below.

| GENE | Sequence FORWARD | Sequence REVERSE |
|---|---|---|
| Ctrl region | CAAAGGCAGTCTGGGAGAGG | CTGTTCTTTGGCCTTTGGGC |
| IRF9 | CCTCCCTGGAGGAGAACTGA | TCTCTCCGCCCCTTTCTACA |

## Molecular dynamics simulations in aqueous solution

The models were built based on the NMR structure 2FEJ. The DeepView/Swiss-Pdb program was used to replace R249 with S and to introduce the phosphate group. The proline 250 was isomerized from trans to cis by Adiabatic Bias MD simulations (ABMD) implemented in PLUMED. ABMD provides a simple and reasonably inexpensive route to generate MD trajectories joining points in conformational space separated by activation barriers. The two points are here represented by the P torsional angles in *cis* and *trans* respectively. It evolves the system towards the target point (P in *cis* conformation) using a harmonic potential moving with the thermal fluctuations of the main degrees of freedom.

The models were inserted in an octahedral box of ~7.6 x ~7.2 x ~6.2 nm edges containing ~10,000 water molecules. Na+ Cl- ions were added so as to insure the electroneutrality in the systems as well as a ionic strength of 1.5 mM. The GROMACS 4.5.5 MD algorithm were used for the MD simulations. The all-atom AMBER ff99SB-ILDN force field was used for the protein, phosphorylated serine residues (249S), $Cl^-$, $Na^+$ and $NAD^+$. The bonded plus electrostatics model and corresponding zinc AMBER force field (ZAFF) parameters in Ref was used for the zinc and the four coordinated residues (C176, C238, C242, H179). The van der Waals parameters for zinc ion were taken from AMBER ff99SB-ILDN force field. The TIP3P force field was used for the water molecules. Periodic boundary conditions were applied. Electrostatic interactions were calculated using Particle Mesh-Ewald (PME) method, and van der Waals and Coulomb interactions were truncated at 1.2 nm. Chemical bonds involving hydrogen atoms were constrained by LINCS algorithm. The systems underwent 1000 steps of steepest-descent energy minimization with 1000 $kJ \cdot mol^{-1} \cdot Å^{-2}$ harmonic position restraints on the protein, followed by 2500 steps of steepest-descent and 2500 steps of conjugate-gradient minimization without restraints. The systems were then gradually heated from 0 K up to 298 K in 20 steps of 2 ns. After that, 50 ns long equilibration was performed in the NPT ensemble (298 K, 1 bar and 2 fs time-step) by coupling the systems with a Nose-Hoover thermostat and an Andersen-Parrinello-Rahman barostat. Finally, 400 ns-long MD simulations were carried out for each system in the same NPT ensemble.

Analyses were performed by considering the last 130 ns of the simulation. The following properties were calculated: (i) PAD (Protein Angular Dispersion), (ii) The root mean square

deviation (RMSD), (iii) Secondary structure content according to the DSSP algorithm. For (i), an in house program was used. For (ii)-(iv) we used the GROMACS 4.6.5 package.

# SUPPLEMENTARY INFORMATION

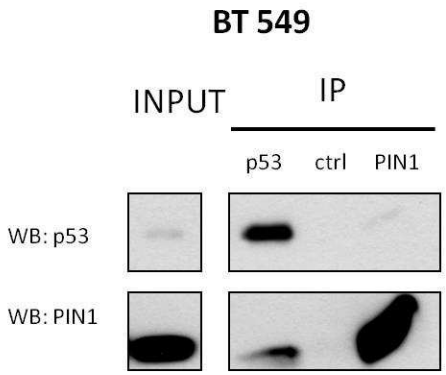



**Figure S1. Mutant p53 R249S associates with Pin1 in BT549 cells.** Co-immunoprecipitation between mutant p53 R249S and Pin1. Total lysate from BT549 cells was subjected to immunoprecipitation with anti-p53 and anti-Pin1 antibodies.

| No. | Kinase Uniprot | Kinase Name | Specificity Score |
|---|---|---|---|
| 1 | P42345 | mTOR/FRAP | 191,3 |
| 2 | O94921 | PFTAIRE1 (PFTK1) | 169,4 |
| 3 | P45983 | JNK1 (MAPK8) | 168,6 |
| 4 | P53779 | JNK3 (MAPK10) | 168,6 |
| 5 | P45984 | JNK2 (MAPK9) | 163,7 |
| 6 | Q15759 | p38b MAPK (MAPK11) | 162,0 |
| 7 | Q9UBE8 | NLK | 161,8 |
| 8 | Q16539 | p38a MAPK (MAPK14) | 161,0 |
| 9 | P28482 | ERK2 (MAPK1) | 160,8 |
| 10 | P27361 | ERK1 | 160,7 |
| 11 | P06493 | CDK1 (CDC2) | 159,9 |
| 12 | Q96Q40 | PFTAIRE2 (ALS2CR7) | 158,1 |
| 13 | Q00526 | CDK3 | 158,1 |
| 14 | Q07002 | PCTAIRE3 | 156,9 |
| 15 | Q00537 | PCTAIRE2 (PCTK2) | 156,8 |
| 16 | Q13164 | ERK5 (MAPK7) | 155,9 |
| 17 | P53778 | p38g MAPK (MAPK12) | 154,4 |
| 18 | Q8TD08 | ERK7 (BI916334) | 149,0 |
| 19 | P24941 | CDK2 | 146,4 |
| 20 | Q00535 | CDK5 | 145,6 |
| 21 | Q00536 | PCTAIRE1 (PCTK1) | 144,5 |
| 22 | O15264 | p38d MAPK (MAPK13) | 139,5 |
| 23 | Q9UPZ9 | ICK | 138,6 |
| 24 | P20794 | MAK | 138,2 |
| 25 | Q8IVW4 | CDKL3 | 138,2 |
| 26 | Q9UQ07 | MOK (RAGE) | 135,7 |
| 27 | Q9H2X6 | HIPK2 | 132,9 |
| 28 | Q9H422 | HIPK3 | 132,6 |
| 29 | Q86Z02 | HIPK1 (BE180036) | 132,3 |
| 30 | Q9Y463 | DYRK1B | 130,0 |
| 31 | Q8IZL9 | CCRK | 129,4 |
| 32 | Q13627 | DYRK1A | 129,2 |
| 33 | Q00532 | CDKL1 | 127,6 |
| 34 | Q5MAI5 | CDKL4 (AA626859) | 122,5 |
| 35 | P49840 | GSK3A | 122,3 |
| 36 | P49841 | GSK3B | 122,2 |
| 37 | Q8TAS1 | KIS (BC014917) | 120,0 |
| 38 | O43781 | DYRK3 | 119,3 |
| 39 | Q00534 | CDK6 | 118,2 |
| 40 | Q9NR20 | DYRK4 | 118,0 |

| No. | Kinase Uniprot | Kinase Name | Specificity Score |
|---|---|---|---|
| 41 | Q8N5S9 | CaMKK1 (DKFZP761M0423) | 116,4 |
| 42 | P11802 | CDK4 | 116,3 |
| 43 | Q8NE63 | HIPK4 (BG105231) | 115,2 |
| 44 | Q92630 | DYRK2 | 112,9 |
| 45 | Q9UQ88 | PITSLRE (CDC2L1) | 109,7 |
| 46 | P50613 | CDK7 | 109,0 |
| 47 | P50750 | CDK9 | 105,9 |
| 48 | Q13523 | PRP4 | 105,7 |
| 49 | P78362 | SRPK2 | 105,1 |
| 50 | Q92772 | CDKL2 | 104,9 |
| 51 | Q96SB4 | SRPK1 | 104,116 |
| 52 | Q9UPE1 | MSSK1 (STK23) | 102,9345 |
| 53 | Q9UHY1 | NRBP1γ | 101,2477 |
| 54 | Q96RR4 | CAMKK2 | 99,2041 |
| 55 | Q14004 | CHED (CDC2L5) | 99,1428 |
| 56 | O76039 | CDKL5 (STK9) | 98,0193 |
| 57 | O00311 | CDC7 (CDC7L1) | 96,6478 |
| 58 | P49759 | CLK1 | 96,3013 |
| 59 | Q9BWU1 | CDK11 (DPK) | 95,981 |
| 60 | P49336 | CDK8 | 95,9322 |
| 61 | Q15131 | CDK10 | 95,4618 |
| 62 | Q9NYV4 | CRK7 (CRKRS) | 95,2645 |
| 63 | Q16659 | ERK3 (MAPK6) | 95,2085 |
| 64 | Q15831 | LKB1 (STK11) | 93,6131 |
| 65 | Q13873 | BMPR2 | 90,7825 |
| 66 | Q16671 | MISR2 | 88,9466 |
| 67 | P49760 | CLK2 | 88,0667 |
| 68 | O43353 | RIPK2 | 87,8627 |
| 69 | P46734 | MEK3 (MAP2K3) | 87,4158 |
| 70 | P52564 | MEK6 (MAP2K6) | 87,0683 |
| 71 | Q8NCX8 | NRBP2 (DKFZP434P086) | 86,9472 |
| 72 | P00540 | MOS | 84,6282 |
| 73 | P49761 | CLK3 | 84,4177 |
| 74 | O15530 | PDK1 (PDPK1) | 84,3816 |
| 75 | P31152 | ERK4 (MAPK4) | 83,893 |
| 76 | Q9HAZ1 | CLK4 | 83,2541 |
| 77 | P33981 | TTK | 82,4985 |
| 78 | P37173 | TGFBR2 | 81,4137 |
| 79 | Q504Y2 | SgK493 | 80,3143 |
| 80 | Q6SA08 | TSSK4 (BF510751) | 80,2832 |

**Figure S2 Results from the prediction of protein kinases phosphorylating residue 249.** The 15 amino acid sequence CMGGMNRSPILTIIT corresponding to mutant p53 R249S was analyzed by the Kinexus Kinase Substrate Predictor Version 1.0 algorithm. The Table shows the ranking of the top protein kinases predicted to phosphorylate Serine 249. The evidenced kinases were chosen for the validation according to their availability.

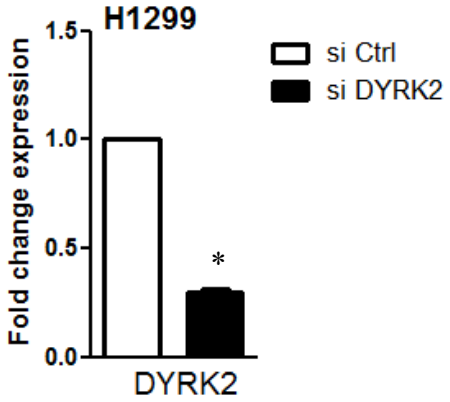



**Figure S3. Evaluation of DYRK2 silencing by qRT-PCR.** Change in DYRK2 expression is shown as fold change upon silencing compared to cells transfected with control siRNA. Error bars indicate s.d. (n=3). * *P* value <0,05.

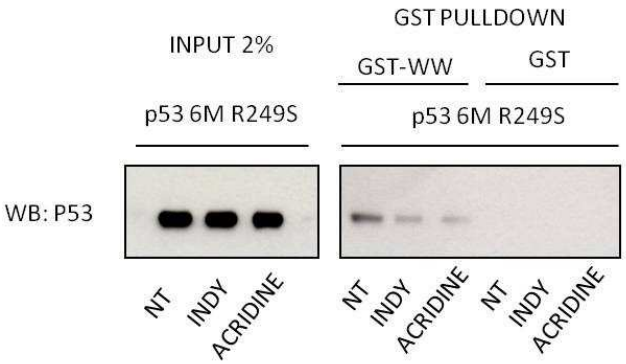



**Figure S4**. **Inhibition of DYRK2 perturbs the binding of p53 6M R249S to the WW domain of Pin1.** H1299 cells were transfected with p53 6M R249S and treated with INDY or ACRIDINE inhibitors 10µM for 16h. The interaction of p53 with recombinant WW domain of Pin1 was analysed by GST pulldown of cell lysates normalized for p53 levels.

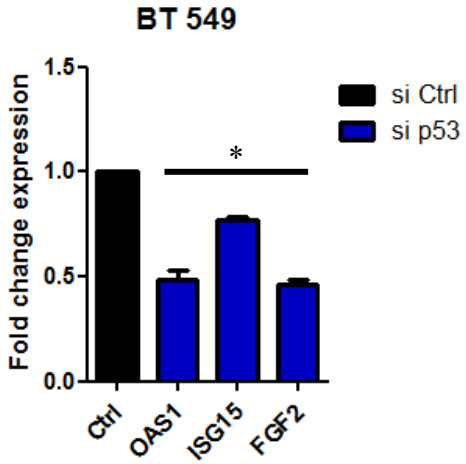



**Figure S5. mRNA level of selected targets upon silencing of mutant p53 R249S.** Changes in gene expression in BT549 cells are shown as fold change upon mutant p53 depletion comparing to cells transfected with control siRNA. Error bars indicate s.d. (n=3). * *P* value <0,05.

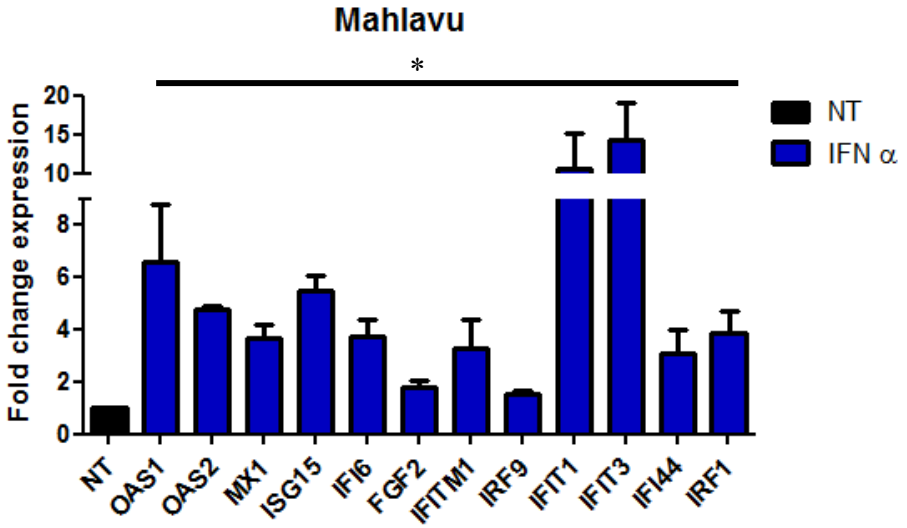



**Figure S6. Induction of mutant p53 R249S-target genes upon Interferon stimulation.** Changes in gene expression in Mahlavu cells are shown as fold change upon Interferon alpha 2a stimulation (1h, 1000UI/ml) compared to untreated cells. Error bars indicate s.d. (n=4). * *P* value <0,05.

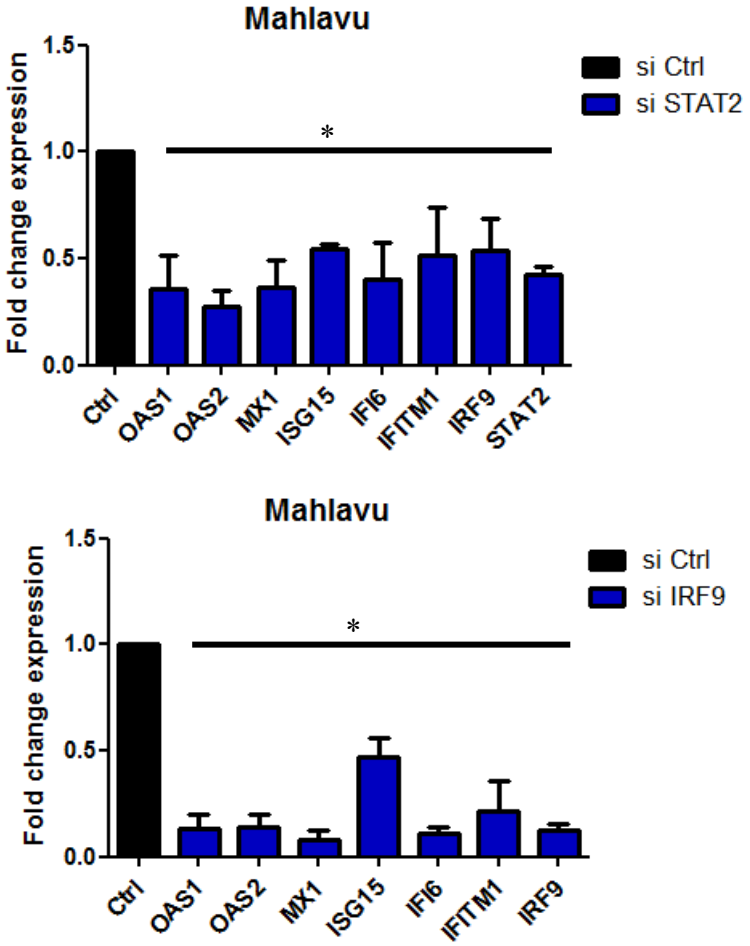



**Figure S7**. **mRNA levels of selected mutant p53 R249S targets upon silencing of STAT2 or IRF9.** Changes in gene expression are shown as fold change upon STAT2 (top) and IRF9 (bottom) depletion comparing to cells transfected with control siRNA. Error bars indicate s.d. (n=3). * *P* value <0,05.

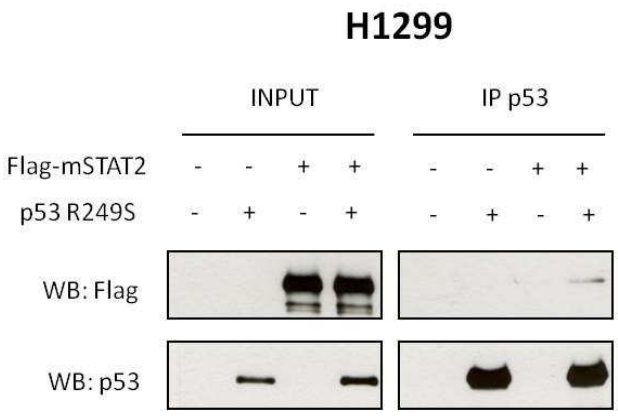



**Figure S8. Mutant p53 R249S interacts with STAT2.** Co-immunoprecipitation assay between murineSTAT2 and mutant p53 R249S co-overexpressed in H1299 cells. Lysates were immunoprecipitated with anti-p53 DO1 antibody.

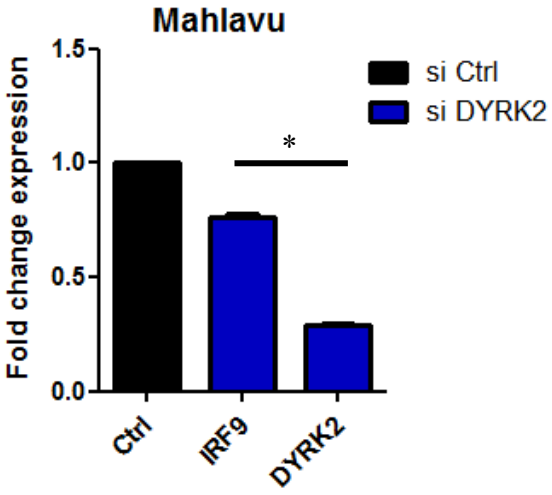



**Figure S9. mRNA levels of IRF9 upon DYRK2 depletion.** Changes in gene expression are shown as fold change upon DYRK2 silencing compared to cells transfected with control siRNA. Error bars indicate s.d. (n=3). * *P* value <0,05.

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# APPENDIX

During the period of my PhD I have been involved in the following publication:

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