MODULATION OF p53 ACTIVITIES BY THE PROLYL-ISOMERASE PIN1 AND THE BROMODOMAIN PROTEIN BRD7

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DOTTORANDA

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Cancer is a complex and frequently lethal disease. Due to the great variety and complexity of tumors, for a long time cancer treatment has been approached only with blunt therapies aimed at simply blocking the proliferation or inducing apoptosis of rapidly dividing cells, thus frequently causing as much damage to the patient as to the tumour. Yet, rapid advances in cancer research have been achieved and the mechanisms of the tumorigenic process have been increasingly unveiled. This great knowledge has been exploited to design targeted therapies that block the growth of cancer cells by interfering with specific mechanisms and molecules needed for carcinogenesis and tumor growth and leave normal cells unaffected.

According to classical cancer genetics, the cancer-causing mutations occur mostly against two major categories of genes, oncogenes and tumor suppressors. Oncogenes like Ras, myc, erb-2, Bcl-2, hTERT, derive from normal cellular genes (proto-oncogenes) that exert different functions in promoting cell growth and survival. Thus, mutations enhancing their expression or activity can cause a cell to divide in an unregulated manner. Conversely, tumor suppressor genes like p53, Rb or PTEN that limit cellular growth, proliferation and survival processes, are often inactivated in cancer so leading to a loss of inhibitory growth control. The actual picture is furthermore complicated by the fact that malignant tumors often display multiple mutations, harbour epigenetic abnormalities, and contain chromosomal aberrations that include aneuploidy and loss of heterozygosity at numerous loci.

Molecular dissection of the signalling networks that drive and maintain tumours, although confirming their complexity and subtlety, has shown that tumour cells harbour the means of their own potential destruction. It has been reported that many tumors, even when full established, rely for their maintenance on the persistent activation of certain cancer-promoting genes. This dependence, described by Weinstein as “oncogene addiction” (Weinstein and Joe, 2006) has been demonstrated in various mouse models by generating transgenic mice that over express an oncogene in a specific target tissue that can be conditionally switched on or off. By this means it was observed that activation of an
oncogene lead to the development of tumors, however, when this gene was subsequently switched off the tumor cells stopped dividing and displayed differentiation and apoptosis (Felsher and Bishop, 1999); (Huettner et al., 2000); (Fujita et al., 1999); (Jackson et al., 2001). This dependency that renders tumors vulnerable to the inhibition of a cancer-promoting agent, although seeming counterintuitive in the beginning, has been largely exploited in drug design leading to clinical application of molecular targeted therapeutic agents.

Indeed, several such inhibitors have already proven to be effective in cancer treatment. Examples include imatinib mesylate (Gleevec®), which targets the oncogenic BCR/ABL protein in chronic myeloid leukaemia (Druker et al., 1996) and the EGFR-targeted drugs gefitinib (Iressa) and erlotinib (Tarceva) in non-small-cell lung carcinoma (NSCLC), pancreatic cancer, and glioblastoma (Grunwald and Hidalgo, 2002).

Although the concept of oncogene addiction has proven to be true in several tumors, it is apparent from clinical experience with molecular targeted agents that cancers can ‘escape’ from this state of dependence probably due to their genomic instability.

Combination therapy directed against multiple targets within the tumour cells, has proven to be more effective than the use of a single molecular targeted agent and has rendered possible to achieve long lasting remissions in many human cancers. Synergistic or additive effects on tumor growth inhibition might be obtained by combining therapies that exploit oncogene addiction to others aimed at restoring the loss of a tumor suppressor gene.

Among the tumor suppressor genes candidate for targeted therapy, p53 is one of the most studied. Indeed, p53 constitutes a central node in a complex signalling pathway evolved to sense a great variety of cytotoxic and genotoxic stresses which may compromise genomic stability and promote neoplastic transformation. Once activated by a stress and depending on the cellular context, p53 may mediate a series of cellular outcomes that vary from cell-cycle arrest to DNA-repair, senescence and apoptosis.

The key role played by p53 in tumor suppression is furthermore highlighted by the observation that direct inactivation of this gene is the most common mutation in human cancer, occurring in more than 50% of malignancies. Moreover, individuals affected by Li Fraumeni syndrome, in which one mutant allele of p53 is germ-line inherited, show 25-fold increase in the chance of developing early-onset cancers, compared with the general population (Evans and Lozano, 1997). In addition, other components of the p53 pathway
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are frequently altered in tumors bearing wild type p53. It is thus possible to maintain that the p53 pathway is compromised to some degree in all human cancers.

Three new studies published in the last year clearly demonstrated that restoration of p53 is an effective means to induce tumor regression in vivo. Like in “oncogene addiction”, it was in fact demonstrated that tumors remain addicted to the loss of p53 function thus assessing the key role of p53 in inhibiting not only tumor development but also tumor maintenance.

With three distinct genetic approaches the three groups obtained engineered mice in which lack of p53 function gene could be conditionally reverted by treating the animals with a particular chemical.

Despite the different technical approaches and tumour types examined, the reinstatement of p53 expression led universally to a prompt and impressive regression of established, in situ tumours. How p53 carries out its anticancer function seems to differ according to the tumor type and its context. For example, restoring p53 function in p53-deficient lymphomas (blood cancers) rapidly induces apoptosis (Martins et al., 2006; Ventura et al., 2007). By contrast, p53 reactivation in solid soft tissue sarcoma and hepatocellular carcinoma induces a potent growth arrest characterized by the hallmarks of cellular senescence (Ventura et al., 2007; Xue et al., 2007). It is not clear which features of a cancer determine whether its response to p53 activation is apoptosis or senescence but both outcomes are associated with tumour regression. Most importantly p53 activation occurred only in cancer cells and not in normal ones that probably lack the specific environment to activate the p53 pathway (Ventura et al., 2007). This furthermore suggests that p53 restoration would be effective in tumor therapy to develop interventions that selectively kill tumor cells relative to normal cells.

Indeed, the last years, several approaches to reactivate the p53 pathway in human cancers have been proposed. p53 signalling could be reactivated in tumours by Nutlins, small molecules that highly increase p53 stability (Thompson et al., 2004). DNA-methyl transferases inhibitors such as azacytidin have been exploited to reverse epigenetic silencing of genes positively affecting the p53 pathway (Fang et al., 2004). Moreover, restoration of wild type properties in mutant p53 has been achieved by means of small molecules like PRIMA-1 and CP-31398 (Bykov et al., 2002; Demma et al., 2004; Rippin et al., 2002; Wang et al., 2006).
1. The tumor suppressor p53

p53 was first identified in 1979 in association with simian virus 40 (SV40) large T-antigen. Throughout nearly 30 years of intensive studies a great knowledge has been achieved on the p53 pathway and a great extent of complexity has been unveiled. Due to its potentially lethal effect, in normal conditions p53 is maintained at low levels by continuous ubiquitylation and subsequent degradation by the 26S proteasome (Haupt et al., 1997; Kubbutat et al., 1997). Yet, upon stimulation by stress signals such as DNA damage, hypoxia, unscheduled oncogene expression, viral infection and ribonucleotide depletion, p53 is rapidly stabilized and exerts its role of "guardian of the genome" and acts as a transcription factor coordinating a program that eventually leads to cell cycle arrest, senescence or apoptosis (Vousden and Lu, 2002). Obviously this is a very simplified view of p53 activity in fact it has been recently demonstrated to exert also transcription-independent functions within the apoptotic response and to be involved in other cellular processes as metabolism, autophagy, DNA repair or antioxidant response to increased ROS levels (Crighton et al., 2006; Sablina et al., 2005; Zhou et al., 2001).

p53 activity is tightly regulated and, depending on the stimulus, the protein undergoes a series of post-translational modifications at specific residues that affect its stability, subcellular localization and its ability to interact with different co-factors and to bind to its target promoters (Gostissa et al., 2003; Vousden and Lu, 2002). Despite the huge knowledge achieved on p53 and its pathway in the last years, many issues about its functions and regulation remain partly unsolved. What are the stimuli leading to the activation of p53 tumor suppressive activity, which modifications and interactions are essential for p53 functions, what are the mechanisms by which p53 specifically selects among different subsets of target genes leading to distinct cellular outcomes, are some out of many interesting topics on which research needs to focus in order to achieve greater insights on p53 biology.
2. The p53 family

p53, together with its homologues p63 and p73 belongs to a family of related transcription factors (Kaghad et al., 1997; Yang et al., 1998). p53 family members have high structural similarity (Fig. 1). All the three contain a central DNA binding domain (DBD), an N-terminal transactivation domain (TA) and an oligomerization domain (OD). Since the recent identification of p53 isoforms (described in detail below) it was believed that only p63 and p73 could be expressed in different variants. p63 and p73 are in fact transcribed from two major promoters thus can be expressed with (TA forms) or without (ΔN forms) the amino terminal TA domain (Stiewe et al., 2002). Moreover, p63 and p73 splicing variants, designated by greek letters, differ in the regions at the carboxy terminal end. Here also resides the most obvious structural difference between p53 and its siblings consisting in the fact that the α-forms of p63 and p73 contain a sterile α motif (SAM) that is absent in p53 (Kim and Bowie, 2003). The homology shown by p53, p63 and p73 has at first suggested that the products of this gene family share similar or even redundant functions. However, difference in regulation and activity among the p53 members emerged quite rapidly. Many reports highlighted that the three members of p53 family might be critical regulator of different processes. In particular, p53 is mainly involved in DNA-damage response while p73 and p63 play key roles in development. This is strikingly highlighted by the phenotypes of mice with targeted deletions for each of the three family members. While p53-null mice are tumor-prone, highlighting the key function of p53 in tumor suppression, they lack a clear developmental phenotype that is instead evident upon disruption of p63 and p73.

The phenotype of p63-null mice demonstrates that p63 expression is absolutely essential for limb formation and epidermal morphogenesis. p63-null animals show in fact absence of limbs or severe limb truncations, absence of skin and craniofacial malformations. They also fail to develop skin and most epithelial tissues (e.g., prostate and mammary glands). Clearly, the animals do not survive beyond a few days postnatally (Yang et al., 1999). Notably, heterozygous germ line point mutations of p63 in humans cause six rare autosomal dominant developmental disorders with alterations reminescent of the knock-out phenotype in mice (Brunner et al., 2002). Importantly, basal cells of normal human epithelium including the epidermis strongly express p63 proteins but lose them as soon as these cells withdraw from the stem cell compartment. Consistent with this notion, keratinocyte differentiation is associated with the disappearance of ΔNp63α, a variant that
Figure 1. (A) Homology between the functional domains in the p53 family proteins: % denotes percent identity. AD, activation domain; PRD, proline-rich domain; DBD, DNA-binding domain; NLS, nuclear localization signal; TD, tetramerization domain; BD, basic domain; SAM, sterile-a-motif domain. (B) p63 protein isoforms: TAp63 proteins encoded from promoter P1 contain the conserved N-terminal transactivation domain (TA). ΔNp63 proteins encoded from promoter P2 are amino-truncated proteins containing an N-terminal domain different from TAp63 proteins. Alternative splicing originates the α, β, and γ isoforms, numbers indicate the exons encoding p63 protein isoforms. (B) p73 protein isoforms: TAp73 proteins encoded from promoter P1 contain the conserved N-terminal transactivation domain (TA). Ex2p73 proteins are due to alternative splicing of exon 2. They have lost the conserved N-terminal TA domain, but still contain part of the transactivation domain (Exon-3). Ex2/3p73 proteins are due to alternative splicing of exons 2 and 3. They have entirely lost the TA domain and are initiated from exon 4. The protein encoded by ΔN′p73 mRNA has not been described. ΔN′p73 variant is often overexpressed at the mRNA level in tumours. ΔN′p73 is due to alternative splicing of exon 30 contained in intron 3. Theoretically, ΔN′p73 mRNA would encode either for a short p73 protein or p73 protein isoforms identical to ΔNp73. DN′p73 mRNA contains the normal initiation site of translation in exon 2 (ATG in perfect kozak sequence) and a stop codon in exon-30. Therefore, it could encode for a short p73 protein composed only of the transactivation domain. It is possible that translation of ΔN′p73 mRNA is initiated from the third ATG available present in exon 30 and leading to p73 protein identical to ΔNp73 protein isoforms. ΔNp73 proteins encoded from promoter P2 are amino-truncated proteins containing an N-terminal domain different from TAp73 proteins. Alternative splicing generates 7 different isoforms denoted by greek letters, numbers indicate the exons encoding p73 protein isoforms.
shows a negative role in p21 regulation (Pellegrini et al., 2001). Together, these data clearly establish a fundamental role of p63 in epithelial stem cell biology and in skin development. p73 also has distinct developmental roles. Its expression is required for neurogenesis of specific neural structures, for pheromonal signalling, and for normal fluid dynamics of cerebrospinal fluid. Indeed, p73-null mice exhibit hippocampal dysgenesis and failure in cortex organization, have severe malformations of the limbic telencephalon and suffer from hydrocephalus. Moreover, they show hyper-inflammatory response of the respiratory mucosa likely due to mucus hyper secretion. Alterations in the neuroepithelium of the vomero-nasal organ causes defects in pheromone detection and is responsible of altered social and reproductive behaviour in p73-null mice (Yang et al., 2000). Contrary to p63, no human genetic disorders have been associated yet with germ line mutation of the p73 gene.

Interestingly, phylogenetic analysis demonstrated that the primordial ancestor of all three genes is actually much more related to p63 and p73, while p53 appears as the most recently evolved member of its family (Yang et al., 2002). It might be hypothesized that p53 lost some of its ancestral functions that are instead carried out by its homologues, yet it has gained the ability to protect cells from tumorigenesis. This seems also to be confirmed by the evidence that p53 is mutated in almost 50% of all human malignancies, while mutations in the p63 and p73 genes are rare (Irwin and Kaelin, 2001; Nomoto et al., 1998). However, there are reports showing that also p63 and p73 have tumor suppressive activities in human tumors. On the one hand they contribute to p53 tumor suppression, as it has been shown that mice heterozygous for mutations in both p53 and p63 or p53 and p73, lead to a more aggressive tumor phenotype (Flores et al., 2005) and that p53 requires at least one of its homologues to function properly as an inducer of pro-apoptotic genes upon DNA damage (Flores et al., 2002). One the other hand they might have a specific role in tumor suppression as their loss or down-expression leads to tumorigenesis at specific tissues (Ahomadegbe et al., 2000; Park et al., 2000; Park et al., 2004; Puig et al., 2003; Urist et al., 2002) with a tumor spectrum that differs from that of p53 and that reflects their pattern of expression (mostly epithelial tissues).
3. p53 isoforms

While the p53 gene was cloned almost thirty years ago, the existence of splice variants has been ignored for long time. Only recently it has been established that the human TPp53 gene has a gene structure similar to p73 and p63 genes (Fig.2) (Bourdon et al., 2005). p53 gene transcription can be initiated from two distinct sites upstream of exon1 and from an internal promoter located in intron 4. The alternative promoter leads to the expression of an N-terminally truncated p53 protein initiated at codon 133 (Δ133p53) and lacking the entire transactivation domain (TAD1 and TAD2) and proline rich domain (PRD) and part of the DNA-binding domain (DBD). The intron 9 can be alternatively spliced to produce three isoforms: p53, p53β and p53γ, where the p53β and p53γ isoforms lack the oligomerization domain (OD). Therefore, the human p53 gene can encode at least nine different p53 protein isoforms which are named accordingly to p63/p73 nomenclature p53, p53β, p53γ, Δ133p53, Δ133p53β and Δ133p53γ due to alternative splicing of the intron 9 and usage of the alternative promoter in intron 4, and also Δ40p53, Δ40p53β, Δ40p53γ deleted of part of the transactivation domain (TAD1) due to alternative splicing of the intron 9 and alternative initiation of translation or alternative splicing of the intron 2 (Ghosh et al., 2004). Interestingly, an additional p53 isoform (Δp53) has been described, which bears a deletion of 66 amino acids within the core domain (corresponding to aa 257-322).

p53 mRNA variants are expressed in several normal human tissues in a tissue-dependent manner, indicating that the internal promoter and the alternative splicing of p53 can be regulated (Bourdon et al., 2005). Moreover, p53 isoforms can have distinct biological activities. p53β was shown to hetero-dimerize with wt p53 and to preferentially bind to the promoters of the Bax and p21 genes but not to Mdm2. p53β appeared to act in concert with wt p53 on the Bax-gene promoter, particularly under conditions of stress. However, this interaction seemed not to correlate with apoptosis induction, as co expression of wt p53 together with p53β did not lead to increased apoptosis, when compared with cells over expressing wt p53 alone. Conversely, a different isoform of p53, Δ133p53 clearly acted as a potent dominant negative for wt p53 in apoptosis induction (Bourdon et al., 2005) Finally, the recently described Δp53 appears to induce only p53-target genes involved in cell-cycle arrest, thereby participating in a specific intra-S phase checkpoint (Rohaly et al., 2005).

p53, p53β, p53γ, and Δ133p53 were found to be differentially expressed in normal versus tumor breast as well as in different breast tumors (Bourdon et al., 2005). Possibly,
deficiency in appropriate regulation of expression of p53 isoforms may have a role in tumor formation. In particular, expression or loss of expression of certain p53 isoforms could impair p53 function in cells that do not harbour inactivating mutations of the parental p53 gene.

At the moment, it is unclear what the biological function of the individual p53 isoforms might be, however it seems likely that the interplay between p53 isoforms on specific targets may play a role in controlling p53 activity in normal and transformed conditions.

Fig 2. Schematic representation of p53 structure (A) Genomic structure of p53: Alternative splicing (α, β, γ) and alternative promoters (P1, P10 and P2) are indicated. (B) p53 protein isoforms: p53, p53 β and p53 γ proteins encoded from P1 or P10 promoters contain the conserved N-terminal domain of transactivation (TA). Δ133p53 isoforms encoded from promoter P2 are amino-truncated proteins deleted of the entire TA domain and deleted of part of the DNA-binding domain. Translation is initiated at ATG-133. Δ40p53 protein isoforms encoded from P1 or P10 promoters are amino-truncated proteins due to alternative splicing of exon 2 and/or alternative initiation of translation at ATG-40. Δ40p53 protein isoform have lost the conserved N-terminal domain of transactivation, but still contain part of the transactivation domain. Δp53 protein isoform is due to noncanonical alternative splicing between the exon 7 and 9. Δp53 has lost 66 residues including the highly conserved domain V of the DNA-binding domain. The isoforms Δp53 β, Δp53 γ, Δ40Δp53, Δ40 Δp53 β, Δ40 Δp53 γ, Δ133 Δp53, Δ133 Δp53β and Δ133 Δp53 γ should theoretically be generated. (adapted from Bourdon, 2007)
4. Structure of the p53 protein

The p53 protein, like many other transcription factors, has a modular structure characterized by the presence of evolutionarily conserved functional domains (Fig.3). This organization is common to the other p53 family members p63 and p73 (Kaghad et al., 1997; Yang et al., 1998).

Fig. 3. Schematic representation of p53 functional domains. TAD1 and 2, transactivation domain 1 and 2; PRD, proline-rich domain; DBD, DNA-binding domain; OD, tetramerization domain; CT, C-terminal domain; NES, Nuclear export signal; NLS, nuclear localization signal

4.1. Transactivation Domain.

Early experiments already demonstrated that the N-terminal acidic domain of p53 is responsible for transcriptional activity (Fields and Jang, 1990). This portion contains two transactivation domains (TA1 aa 1-42 and TA2 aa 43-62) that are independently sufficient to activate transcription when fused to a heterologous DNA-binding domain (DBD) (Unger et al., 1992) and that interact with components of the basal transcriptional machinery as the TATA-binding protein (TBP) and TBP associated proteins (TAFs) as well as with p300/CBP (Liu et al., 1993). These two domains are both necessary for p53 full transcriptional activity but with different functions, the TA1 is in fact required for inducing p21 transcription and G1 arrest while the TA2, together with the proline rich domain (PRD), is essential for apoptotic response (Sabbatini et al., 1995; Zhu et al., 2000).

The transcriptional activation domain is finely regulated both by post-translational modifications and by interaction with protein partners such as p300/CBP, Mdm2 and Pin1 (see below). Mdm2, the most critical negative regulator of p53, interacts with residues 17–27 of TA1 and mediates the ubiquitination of p53 C-terminal lysine residues. Moreover
Mdm2, together with MdmX, prevents the binding of transcriptional co-activators such as p300 and interferes with the transactivation of TA1-dependent target genes.

### 4.2. Proline Rich Domain

Adjacent to the transactivation domain there is a proline-rich domain (PRD, aa 61-94) containing five repeats of the amino acid motif PXXP (where P designates proline and X any amino acid) in humans while only two in mice (Walker and Levine, 1996). This region was thought to be significant for p53 regulation since PXXP motifs create binding sites for Src homology 3 (SH3) domain-containing proteins and can modulate signal transduction (Kay et al., 2000). Indeed, the p53 PXXP motifs may contribute to interactions with the transcription co-activator p300 (Dornan et al., 2003a) thus influencing p53 acetylation. Furthermore, the prolyl isomerase Pin1 binds to Thr81-Pro82 site upon Thr81 Phosphorylation and induces a conformational change on Pro82. This may reduce Mdm2 binding thus influencing p53 stability (Wulf et al., 2002; Zacchi et al., 2002), consistently with other evidence indicating that the PRD modulates Mdm2 binding (Berger et al., 2001; Dumaz et al., 2001). Furthermore, the functional importance of the PRD is suggested by studies showing that this domain is dispensable for cell cycle arrest but is essential for apoptosis (Sakamuro et al., 1997; Venot et al., 1998) being required both for transcriptional activation of pro-apoptotic genes (Bergamaschi et al., 2006; Venot et al., 1999; Zhu et al., 2003) and for direct apoptotic function of p53 at mitochondria (Chipuk et al., 2004). Yet, a recent study (Toledo et al., 2007) conducted in mice indicated that, although the PRD may play some role in the regulation of p53, as mouse p53<sup>ΔPR</sup> (that bear p53 lacking the PRD) displays increased Mdm2-mediated degradation and decreased transactivation capacity (Toledo et al., 2007), the PXXP motifs are not essential for p53 tumor suppressor functions, as their depletion does not significantly affect p53 accumulation and transactivation, and exhibits only little effect on cell cycle control or apoptosis.

A common polymorphism is located within p53’s PRD at codon 72, encoding either proline or arginine. This polymorphism is unique to humans while in the majority of vertebrates proline is encoded in the corresponding residue. In early studies, the differential electrophoretic gel mobility of the two isoforms raised the possibility that these variant could differ structurally and therefore also functionally (Matlashewski et al., 1987). Indeed, many reports have shown that the Arg72 variant is more efficient than Pro72 at inducing apoptosis (Bonafe et al., 2004; Dumont et al., 2003; Sullivan et al., 2004; Thomas et al., 2004).
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The mechanistic basis appears to involve, on one hand, the enhanced localization of p53 Arg72 to the mitochondria as compared to p54 Pro72 (Dumont et al., 2003) and on the other hand its lower affinity for the iASPP protein, which binds preferentially to the PRD and selectively blocks access of p53 to the promoters of apoptosis-related genes such as Bax and PIG3 (Bergamaschi et al., 2006).

Extensive studies have been carried out to investigate the link between the expression of p53 polymorphic variants at codon 72 (p53Pro72 and p53Arg72) and cancer susceptibility. The results are controversial, reflecting a lack of understanding of how p53Pro72 and p53Arg72 function *in vivo* (de Oliveira et al., 2004; Granja et al., 2004; Matakidou et al., 2003). Homozygosity for the highly apoptotic wt p53-Arg72 variant is associated with greater sensitivity of tumor cells to anticancer drugs, and is predictive of a more favourable clinical response to chemotherapy in squamous carcinomas of the head and neck (Sullivan et al., 2004). Yet Homozygosity for wtPro72 variant has shown to increase longevity suggesting that enhanced lifespan associated with the lesser active Pro72 allele may outweigh the deleterious effects of cancer susceptibility.

4.3. DNA-binding Domain.

The central third of p53 (aa 100-300) contains the sequence-specific DNA binding domain (DBD) required for p53 to function as a transcriptional activator (el-Deiry et al., 1992). The canonical p53-responsive element contains two decamers or half sites, PuPuPuC(A/T)(A/T)GPyPyPy, which are separated by a spacer of 0–13 bp. Several studies indicate that a p53 monomer binds the pentameric sequence and that a tetramer binds the full consensus site (Cho et al., 1994; Ma et al., 2005). Notably, variation within the individual p53 responsive elements (REs) resulted in up to 1000-fold difference in transactivation when tested in a yeast model system (Inga et al., 2002; Resnick and Inga, 2003). Besides, p53 may also recognize DNA sequences that differ from its canonical consensus. For instance, a gene that is weakly induced by p53 is AQP3, in which, the RE is made up of three pairs of pentamers (Zheng and Chen, 2001). Another example is the repressive p53 RE located within the promoter of the MDR1 gene that shows orientation of pentamers “head-to-tail” instead of classical “head-to-head”(Johnson et al., 2001). Moreover, the microsatellite region within the PIG3 gene that was shown to function even more effectively as a p53 RE than a more canonical binding site within the same promoter (Contente et al., 2002). Also DNA topology is an important determinant of sequence-
specific DNA binding by p53 (Gohler et al., 2002). Indeed, many p53REs deviating from the consensus display an internal twofold symmetry that allows for intra-strand pairing and formation of stem-loop structures. When presented in linear conformation, these sites are poorly recognized by p53, while in stem-loop conformation, they are bound with high affinity. It has been shown that p53 specifically binds to CTG_CAG trinucleotide repeats that undergo topological alterations (Walter et al., 2005) or to cruciform-forming sequences (Jett et al., 2000) that have no resemblance to the p53RE consensus.

The crystal structure of the p53-DBD bound to DNA has revealed that the conserved regions are crucial for the p53–DNA interaction (Cho et al., 1994). The larger part of the DBD forms an antiparallel β-sandwich. This β-sandwich serves as a scaffold that supports the structures important for the interaction with DNA, specifically two large loops and a loop-sheet-helix motif.

The two large loops are held together by a zinc atom which is coordinated by three cysteines and one histidine (Cys176, His179, Cys238, and Cys242). The loop-sheet-helix motif contains residues that contact the DNA phosphate backbone (Arg273, Ala276, Arg283) as well as the major groove (Cys277 and Arg280). One additional residue, Lys120, is important for the interaction with the major groove and the DNA phosphate backbone. Importantly, all of the residues that are essential for the interaction with DNA are conserved in p63 and p73 except R283, which is a Lysine in p63 and p73. However, the proteins also possess unique functions and regulate distinct downstream target genes. Subtle differences in the DBD are likely to contribute to the distinct functions of the transcription factors (Harms and Chen, 2006).

Although the DBD is less involved than the N-term and C-terminal domains in protein interactions, some proteins have been found to interact with it, among them 53BP1, Hzf, ASPP1 and ASPP2 positively affect p53 activity. 53BP1 was demonstrated to enhance p53 transcriptional activation (Iwabuchi et al., 1998). Moreover it localizes at sites of DNA damage (Anderson et al., 2001) where it participates in DNA damage signalling (Rappold et al., 2001; Schultz et al., 2000) and repair (Iwabuchi et al., 2003). The roles of ASPP1/2 and Hzf will be treated in detail below. The p53-DBD also interacts with proteins that negatively regulate p53. The ubiquitin ligase Mdm2 has been shown to interact with residues in the DBD(Shimizu et al., 2002) besides its well known interaction with the N-terminus of p53 (Oliner et al., 1993). In line with this observation, it was shown that the binding of Mdm2 to full-length p53 is 10-fold stronger than binding to the N-terminal
domain alone (Dawson et al., 2003). The SV40 oncprotein large T antigen impairs p53 transcriptional functions by binding to its DBD (Jiang et al., 1993; Tan et al., 1986). The importance of sequence-specific DNA binding for p53 to function as a tumor suppressor is highlighted by the fact that 97% of tumor-associated mutations cluster in this domain (Sigal and Rotter, 2000). The mutations situated in the DBD can disrupt specific DNA binding by several possible ways. Mutant at some positions (e.g. Arg273His) lose direct contacts with DNA and are therefore denominated “contact mutants” (Bullock et al., 1997; Cho et al., 1994). Some other mutants, defined “conformational mutants” (e.g. Arg175His, Gly245Ser, Arg249Ser, Arg282Trp) show reduced binding due to destabilization of the tertiary structure of p53 DBD (Bullock et al., 2000; Wong et al., 1999). Mutation at position 248 (Arg248Trp), in addition to breaking DNA-protein contacts, also introduces extensive structural changes into the DBD (Wong et al., 1999).

Notably the p53 DBD requires a significant level of flexibility within the tetramer in order to grant recognition and contact with the different bases of a consensus RE. The same flexibility, though, seems to be responsible for the ability of some p53 mutants to bind and inactivate the other two p53 relatives, p63 and p73, as well as to force wild-type p53 molecules (translated from the remaining wild-type p53 allele) into a mutant conformation (Bensaad et al., 2003; Di Como et al., 1999; Gaiddon et al., 2001).

It has been recently found that at least three DNA-binding defective molecules of p53 are needed in order to effectively inactivate the tetramer (Chan et al., 2004; Shieh et al., 1999). The existence of partially active tetramers in the cell may lead to a new phenotype via appearance of an unusual binding surface on a p53 molecule, which may change the pattern of coactivators/corepressors interacting with p53. Alternatively, mutations in the DBD may change the relative affinity for target genes either resulting in loss of binding or in recognition of novel DNA sequences with consequent alterations in the normal global transcriptional response to p53.

4.4. Oligomerization Domain

The tetramerization required for high-affinity DNA binding and transcriptional activation is mediated through the oligomerization domain (OD, aa 326-356). Tetramerization appears to be essential for p53 tumor suppressor activities in fact it has been reported that DNA damage-induced signalling to p53 mediated by phosphorylation of Ser15, Ser20, and Ser33 requires the OD but not other domains (Shieh et al., 1999). Moreover p53 is unable
to bind DNA *in vitro* if the hydrophobic core that stabilizes the dimer-dimer interaction is disrupted. The OD’s are highly conserved among of the p53 family proteins, however they show distinct properties that prevent the heterotetramerization of the p53 family proteins. This is significant for the function of these proteins since p53, p63, and p73 are likely to be expressed simultaneously, at least in some tissues (Chene, 2001). Interestingly, mutant forms of p53 are able to heterotetramerize with p73 thus inhibiting its apoptotic functions (Strano et al., 2000). Moreover, the heterotetramerization of wild-type and mutant p53 is likely causative of the dominant-negative activities of mutant p53. It will be interesting to learn how heterotetramerization of full-length p53 and the other recently identified isoforms could affect p53 activity and if the transcriptional activity of the heterotetramer is different from that of the homotetramer.

4.5. C-terminal Domain
The last 30 amino acids of p53 constitute a basic C-terminal domain (CT, aa 364-393) that has been regarded as a regulatory domain due to its ability to influence p53 activity and to the great number of post-translational modifications it undergoes upon stress-signalling. Nearly every residue within this domain is in fact subjected to at least one post-translational modification. This domain does not form a regular secondary structure and is able to interact directly with DNA and RNA (Ayed et al., 2001; Lee et al., 1995). In particular CT is able to bind ssDNA ends, insertion/deletion mismatches, recombination intermediates and γ-irradiated DNA *in vitro* thus implying an ability to recognize damaged DNA and DNA repair intermediates *in vivo* (Bakalkin et al., 1995; Lee et al., 1995; Zotchev et al., 2000). Moreover some recent works demonstrated that the CT is important for binding to non-linear DNAs (Fojta et al., 2004; McKinney and Prives, 2002; Palecek et al., 2004) and is involved in the ability of p53 to diffuse linearly on DNA (McKinney et al., 2004). These studies also revealed that the CT is required for efficient promoter activation by p53. The role of the CT and its post-translational modifications in directing p53 activities will be treated more in detail below.

4.6. Nuclear localization and nuclear export signals
p53 is known to shuttle between the nucleus and the cytoplasm. Since tetrameric p53 is too large to passively diffuse across the nuclear pore, its nucleo-cytoplasmic shuttling is facilitated by nuclear import and export signals (Middeler et al., 1997). The C terminus of
INTRODUCTION

the p53 molecule contains a cluster of three nuclear localization signals (NLS) that mediate the migration of the protein into the cell nucleus. NLSI (aa 316–322), the most active domain, is highly conserved in genetically diverged species and shares perfect homology with consensus NLS sequences found in other nuclear proteins (Shaulsky et al., 1990). This NLS has a bipartite structure (Liang and Clarke, 1999a) consisting in the canonical NLS sequence (aa 315-322) and a basic sequence (aa 305-306) separated by a spacer (Liang and Clarke, 1999b). The basic residues at the N- and C-termini of the NLS (Lys305-Arg306/Lys319-Lys320-Lys321) are necessary and sufficient for the complete nuclear localization of a cytoplasmic reporter protein (Liang and Clarke, 1999a) The other two NLSs, II and III (aa 370-384) appear to be less effective and less conserved (Shaulsky et al., 1990). Moreover, p53 has two putative nuclear export signals NES, one in the N-terminus and another in the oligomerization domain (aa 11–27 and 340–351, respectively). As the NLS is adjacent to the OD and the NES is contained within the OD, it has been proposed that the oligomerization of p53 may regulate its nucleo-cytoplasmic transport by affecting the accessibility of the NLS and/or NES to their respective receptors (Liang and Clarke, 2001; Stommel et al., 1999).

5. Upstream events engaging the p53 pathway

p53 is the central node of a complex web of incoming stress signals and outgoing effector pathways. The great variety of stress signals that lead to p53 activation illustrate the multiplicity of p53 functions in responding to potentially oncogenic insults to prevent tumor development (Fig. 4)
5.1. DNA damage

DNA damage was the first type of stress found to activate p53 and, based on this, p53 has been widely regarded as “the guardian of the genome” (Lane, 1992). DNA damage signalling is triggered by a great variety of exogenous and endogenous events that might compromise genome integrity both by altering the primary structure of DNA thus generating mutations, and by causing double strand breaks (DSB) with consequent genomic rearrangements or loss of genetic information. Exogenous damage might be caused by external agents such as UV radiation, ionizing radiations or chemical mutagenic compounds. Endogenous DNA damage derives instead from normal cellular processes linked to metabolism and replication. For instance, reactive oxygen species (ROS) produced from normal metabolic by-products can lead to nucleotide oxidation. Replication stress due to premature termination of replication fork progression can result in fork collapse and DNA breakage (Branzei and Foiani, 2005). Moreover, telomere erosion, consequent to continuous replication, is perceived by mammalian cells as a form of DSB (d’Adda di Fagagna et al., 2003; Takai et al., 2003).

Remarkably, in human cancers the DNA damage signalling cascade is permanently activated, suggesting that the cancerous state is intrinsically associated to the generation of DNA damage (Bartkova et al., 2005; Gorgoulis et al., 2005). The constitutive DNA damage present in cancer cells is shown to emanate primarily from the DNA replication stress due to aberrant firing of DNA replication origins that occurs upon activation of oncogenes or loss of tumor suppressors (Bartkova et al., 2006; Di Micco et al., 2006; DiTullio et al., 2002; Mallette and Ferbeyre, 2007). The strong generation of reactive oxygen species detected in cells transformed by various oncogene (Mallette and Ferbeyre, 2007) may also contribute to activation and maintenance of the DNA damage response.

DNA damage signals of different nature are transduced to p53 through a cascade of Ser/Thr kinases that play key roles in p53 activation by mediating the phosphorylations necessary to promote its stabilization and its transcriptional activity (Lambert et al., 1998). ATR and ATM, the two DNA damage sensor kinases and their respective downstream kinases Chk1 and Chk2, phosphorylate p53 at different sites. Specifically, ATM and Chk2 act in response to ionizing radiation and DSBs leading to phosphorylation of p53 at Ser15 Thr 18, and Ser20. ATR and Chk1 appear to be required in UV damage response (Banin et al., 1998; Chehab et al., 1999; Hirao et al., 2000) but are also involved in response to hypoxia (Hammond et al., 2002), to fork stalling and single strand DNA formation (Zou
Upon activation ATR phosphorylates p53 at Ser15 and Ser37 while Chk1 at Ser6, Ser9 and Ser20.

Ionizing radiations induce also the activation of DNA-dependent protein kinase (DNA-PK), another member of a protein kinase family that includes ATR and ATM. DNA-PK has been shown phosphorylate p53 at Ser15 and Ser37 and to interact with it at sites of DNA-damage (Okorokov et al., 2002). Interestingly, when the severity of DNA-damage is elevated, DNA-PK activation is required in combination with Chk2 to induce an apoptotic response (Woo et al., 2002). p38, is activated in response to stress stimuli and cytokines (Pearson et al., 2001) and has been shown to phosphorylate p53 at Ser15, Ser33, Ser37, Ser46 and Ser389 upon different stimuli and with different outcomes. Activation of p38 upon UV irradiation or nitric oxide treatment leads to apoptosis that is abrogated upon treatment with p38 inhibitors (She et al., 2002). Upon osmotic shock p38 phosphorylates p53 at Ser33 causing a G1 arrest (Kishi et al., 2001), while its inhibition during UV irradiation leads to a decrease in phosphorylation at Ser15, Ser33, Ser37 and Ser46 and to a reduced p53-dependent apoptotic response (Bulavin et al., 1999). Moreover, during UV radiation p38 has been also reported to phosphorylate p53 at Ser389 (Huang et al., 1999). Notably, the phosphatase Wip1, a p53 transcriptional target, is known to inhibit p38 activity upon UV radiation (Fiscella et al., 1997) by participating in a negative feedback loop that controls the MAPK signalling to p53 (Takekawa et al., 2000).

The stress-activated protein kinase JNK phosphorylates p53 at Ser15 upon UV radiation (Buschmann et al., 2000a) and at Ser20 under oxidative stress (Buschmann et al., 2000c) leading to an increase in p53 transcriptional activity. In addition, JNK-mediated phosphorylation at Thr 81 (Buschmann et al., 2001) is important for p53 stabilization through a mechanism involving the prolyl-isomerase Pin1 (See also below).

The Ser/Thr kinase HIPK2 is an important inducer of p53 apoptotic response (D'Orazi et al., 2002). Indeed, high doses of DNA-damaging agents lead to HIPK2-mediated phosphorylation of human p53 at Ser46, a modification that is required for p53 to engage an effective apoptotic response (Di Stefano et al., 2004b; Mayo et al., 2005; Oda et al., 2000). HIPK2 stimulates the apoptotic response by stabilizing p53 (D'Orazi et al., 2002). Moreover, it leads to p53 accumulation by antagonizing Mdm2-mediated nuclear export and ubiquitination of p53 (Di Stefano et al., 2004a) thus granting the presence of high levels of p53 necessary to activate apoptotic promoters (Chen et al., 1996b). It has recently
been reported that upon mild DNA-damage, p53 may act in negative feedback loop inducing HIPK2 degradation by Mdm2 (Rinaldo et al., 2007). Thus, under conditions that do not require the triggering of the apoptotic response, p53 may indirectly repress its phosphorylation at Ser46 and consequently its pro-apoptotic activation.

5.2. Oncogenic signalling

Oncogenic signalling activates p53 not only through the DDR but also through the transcriptional activation of p14ARF (ARF) (de Stanchina et al., 1998; Palmero et al., 1998). The expression of ARF is up-regulated by E2F-1 (Zhu et al., 1999) and beta-catenin (Damalas et al., 2001). Moreover, the levels of ARF protein are found to be increased upon Ras (Lin and Lowe, 2001) and Myc (Zindy et al., 1998) activation. One of the key roles of the ARF protein is to bind to Mdm2 and inhibit its ubiquitin ligase activity thus favouring p53 stabilization (Stott et al., 1998). In human tumors ARF is inactivated with an extraordinarily high frequency. However loss of ARF occurs almost invariably in combination with loss of p16INK4a thus generating ambiguity on which is the key targeted tumor suppressor. It was demonstrated that p16INK4A is the major tumor suppressor of the human INK4A locus. In detail, p16INK4A synergizes with p53 to protect primary cells from unrestricted growth and from oncogenic transformation. Instead, ARF regulates growth of primary human cells in normal culture conditions through p53 but loss of ARF has little tumorigenic effect in human cells transformed with oncogenes (Voorhoeve and Agami, 2003). This finding is consistent with the observation that mutations that inactivate p16INK4a only, sparing ARF, outnumbers of a factor of 20 those which inactivate ARF alone (Kim and Sharpless, 2006). In mice, instead, lack of ARF leads to a remarkable tumor-prone phenotype, although not to the extent of p53-deficient mice (Jacks et al., 1994; Kamijo et al., 1999).

The great relevance of oncogenic signalling in inducing the tumor suppressor functions of p53 was recently highlighted by the observation that in mice, the cancer-protective activity of p53 was abolished in the absence of ARF even in the presence of an additional p53 allele (“super”p53 mice) leading to an increased response to DNA damage (Efeyan et al., 2006). Moreover, in a study exploiting p53-null mice ingennerized so that p53 expression could be conditionally restored, it was observed that reinstatement of p53 during the phase of acute genotoxic damage did not lead to increased tumor protection respect to its complete absence. Conversely, p53 restoration at later times, after the acute DNA damage
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response had subsided, lead to decreased tumorigenesis that was abolished in the absence of ARF (Christophorou et al., 2006). Although these evidences suggest that oncogenic signalling play a fundamental role in inducing p53 tumor suppressive functions, yet this does not call into question the relevance of DDR. Indeed, other studies demonstrated that mice deficient in DDR are tumor-prone that DNA-damage signalling in premalignacies activates p53 preventing the formation of tumors (Bartkova et al., 2005; Gorgoulis et al., 2005). Taken together this data indicate that acute DNA damage does not lead to a persistent signalling and might result predominantly in toxicity that eliminate most of the damaged cells. The surviving cells may acquire oncogenic mutations and generate an incipient tumor. On the one hand, sustained oncogenic signalling leads to ARF activation while on the other hand results in replication stress and DNA damage that activate the DDR through the ATR–Chk1 and ATM–Chk2 kinase signalling pathways respectively. These two responses converge in p53 activation and are both necessary for sustained p53 tumor suppressive activity in order to prevent tumor progression to malignancy.

5.3. Other kinds of stimuli

Other kinds of stimuli have been shown to participate in p53 activation. Interestingly, nucleolar disruption has shown to be a key element in the signalling to p53. A model has been proposed according to which the nucleolus is a central stress sensor for a variety of agents and nucleolar disruption is required besides DNA-damage for p53 stabilization (Rubbi and Milner, 2003a). A growing body of literature has demonstrated that nucleolar morphology is diagnostic for the general metabolism of the cell and that nucleolar structure depends on rDNA transcription (Schwarzacher and Wachtler, 1991). After inhibition of rDNA transcription by drugs blocking PolII, such as actinomycin D, or by physiological stimuli that include serum starvation and cell-cell contact growth inhibition, the nucleolar components are more or less rapidly rearranged and the nucleolar structure disintegrates. This causes the release of ribosomal proteins, such as L5, L11, or L23, all of which bind to Mdm2 and stabilize p53 through inhibiting the E3 ligase activity of Mdm2 (Bhat et al., 2004; Dai et al., 2004; Gilkes et al., 2006; Lohrum et al., 2003; Zhang et al., 2003).

To further support the role of the nucleolus in p53 activation there is the observation that the nucleolar protein nucleophosmin binds to p53 after leaving the nucleolus upon DNA-damage and mediates p53 stabilization (Colombo et al., 2002).
Hypoxia, a condition faced by the cells at the centre of expanding tumors, has shown to activate p53 to its apoptotic response through a yet not clear mechanism that involves ATR, HIF1α (Hypoxia inducible factor) and VHL (Von Hippel-Lindau) (Liu et al., 2007; Roe et al., 2006; Roe and Youn, 2006). Interestingly, after hypoxia induction p53 shows a different pattern of target gene activation than seen following DNA-damage suggesting that under hypoxic signalling p53 might mediate a quite different response compared to other stress signals (Hammond and Giaccia, 2005; Krieg et al., 2006).

In addition to acute insults, p53 responds to a variety of milder, constitutive stresses such as the generation of ROS by normal metabolism. p53 was known to be a potent pro-oxidant protein, inducing a set of ROS-generating genes that contribute to p53-mediated apoptosis (Achanta and Huang, 2004; Macip et al., 2003). Yet an unexpected anti-oxidant function mediated by p53 has recently emerged. Through the activation of antioxidant target genes p53 functions to allow survival and repair of the damage rather than to eliminate damaged cells. In this way, p53 exerts its tumor suppression function by decreasing the incidence of genetic alterations even contributing to the longevity of the organism. (Sablina et al., 2005).

In addition, many other stresses that will result in a loss of fidelity in the cellular duplication process have recently been shown to communicate with the p53 pathway. Heat and cold shock (Ohnishi et al., 1998a; Ohnishi et al., 1998b), presence of denaturated proteins, depletion of ribonucleotidiphosphate pool in the cell (Khan et al., 2000), spindle damage leading to faulty chromosomal segregation (Lanni and Jacks, 1998; Peng et al., 2007), nitric oxide production associated with infections and inflammation (Hofseth et al., 2003) all activate p53 protein and its response.

### 6. Outcomes of p53 activation

Once the p53 protein is activated, the ultimate outcome can be quite different, ranging from the induction of reversible cell cycle arrest, apoptosis and senescence to protective antioxidant activities and DNA repair. How the choice can be driven toward a specific response depends on different factors. First, the induction of p53 activation has different outcomes depending on cell type. In primary fibroblasts it is in fact usually associated with cell cycle arrest (Di Leonardo et al., 1994; Kuerbitz et al., 1992) whereas the activation of
p53 in hematopoietic cells (e.g., thymocytes) generally results in apoptosis (Lowe et al., 1993). Moreover, even within a particular cell type, the p53 response can be influenced by many factors such as the nature of the stimuli that activate p53 and a plethora of protein partners that affect its stability and activity.

Probably the best understood way by which p53 mediates its response is to act as a transcription factor with sequence-specific DNA binding ability and the potential to induce the expression of a large number of genes (Table I). Jointed bio-informatic and ChIP based studies have suggested that the number of genes containing p53 binding sites may vary between 500 and 1600 (Cawley et al., 2004; Wei et al., 2006). Certainly, genes involved in the well-established responses of cell-cycle arrest and apoptosis are largely represented, yet the identification of genes involved in other processes such as metabolism or cell adhesion, indicate that p53 might play further roles in governing cellular homeostasis (Wei et al., 2006). Moreover p53 has shown to play also roles that are not related to transcriptional activation, such as the induction of apoptosis through direct interaction with mitochondrial proteins.

### 6.1. Cell cycle arrest

The ability of p53 to induce cell-cycle arrest mostly depends on three critical target genes: p21, 14-3-3σ and GADD45 (el-Deiry et al., 1993; Hermeking et al., 1997; Kastan et al., 1992). The cyclin-dependent kinase inhibitor p21\textsuperscript{Waf1/Cip1} was the first transcriptional target identified and its transactivation results in cell cycle arrest in G1 phase due to inhibition of cyclinE/CDK2, CyclinA/CDK2 and cyclinD/CDK4 (Harper et al., 1993; Prives and Hall, 1999). The role of p21 in G1-arrest is further underlined by the observation that cells lacking p21 fail to arrest in response to DNA-damage, yet they can still undergo cell death (Brugarolas et al., 1995). Although the most prominent function of p21 is the mediation of G1 arrest, evidence has been presented that it also participates in the G2/M arrest after DNA damage, presumably by blocking PCNA function at replication forks (Ando et al., 2001; Bunz et al., 1998). However, the p53-induced G2 arrest is mostly mediated by the activation of genes such as GADD45 and 14-3-3σ. 14-3-3σ has been shown to prevent nuclear import of cyclin B1 and CDC2, through their sequestration in the cytoplasm (Chan et al., 1999), whereas GADD45 destabilizes CDC2/cyclinB complexes and these two processes cooperate to prevent initiation of mitosis (Jin et al., 2002a; Zhan et al., 1999).
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Table I. Classes of genes induced by p53. Adapted from *The Biology of Cancer* (© Garland Science 2007)

<table>
<thead>
<tr>
<th>CLASS OF GENES</th>
<th>NAME OF GENES</th>
<th>FUNCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 antagonist</td>
<td>Mdm2</td>
<td>Induces p53 ubiquitination</td>
</tr>
<tr>
<td>Growth arrest genes</td>
<td>p21Cip1</td>
<td>Inhibitor of CDKs</td>
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<tr>
<td></td>
<td>Slah-1</td>
<td>Aids β-catenin degradation</td>
</tr>
<tr>
<td></td>
<td>14-3-3-Σ</td>
<td>Sequesters cyclin B/CDC2 in cytoplasm</td>
</tr>
<tr>
<td></td>
<td>Reprimin</td>
<td>G2 arrest</td>
</tr>
<tr>
<td>DNA repair genes</td>
<td>p53R2</td>
<td>Ribonucleotide reductase</td>
</tr>
<tr>
<td></td>
<td>XBE/DBB2</td>
<td>Global NER</td>
</tr>
<tr>
<td></td>
<td>XPG</td>
<td>Global NER</td>
</tr>
<tr>
<td></td>
<td>XPGG</td>
<td>Global NER, TCR</td>
</tr>
<tr>
<td></td>
<td>GADD45</td>
<td>Global NER</td>
</tr>
<tr>
<td></td>
<td>DNA Pol k</td>
<td>Epsilon-prime DNA polymerase</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>Bax</td>
<td>Mitochondrial pore protein</td>
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<tr>
<td></td>
<td>Noxa</td>
<td>Bcl-2 only mitochondrial pore protein</td>
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<tr>
<td></td>
<td>Puma</td>
<td>Bcl-2 only mitochondrial pore protein</td>
</tr>
<tr>
<td></td>
<td>p53AIP1</td>
<td>Dissipates mitochondrial membrane potential</td>
</tr>
<tr>
<td></td>
<td>Killer/DR5</td>
<td>Cell surface death receptor</td>
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<tr>
<td></td>
<td>PIDD</td>
<td>Death domain protein</td>
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<tr>
<td></td>
<td>PERP</td>
<td>Pro-apoptotic transmembrane protein</td>
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<tr>
<td></td>
<td>APAF-1</td>
<td>Activator of caspase-9</td>
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<tr>
<td></td>
<td>NF-κB</td>
<td>Transcription factor, mediator of TNF signalling</td>
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<tr>
<td></td>
<td>FAS/APO1</td>
<td>Death receptor</td>
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<tr>
<td></td>
<td>PTGS</td>
<td>Mitochondrial redox control</td>
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<tr>
<td></td>
<td>PTEN</td>
<td>Reduces levels of the anti-apoptotic PIPJ</td>
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<tr>
<td></td>
<td>Bcl2</td>
<td>Anti-apoptotic (repressed)</td>
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<tr>
<td></td>
<td>IGF-1R</td>
<td>Anti-apoptotic (repressed)</td>
</tr>
<tr>
<td></td>
<td>Stat3</td>
<td>ER membrane protein, transduces ER stress</td>
</tr>
<tr>
<td></td>
<td>IGFBP-3</td>
<td>IGF-1 sequestering protein</td>
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<tr>
<td>Autophagy</td>
<td>DRAM</td>
<td>lysosomal protein</td>
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<tr>
<td>Pro-survival</td>
<td>SLUG</td>
<td>Inhibits PUMA expression</td>
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<tr>
<td></td>
<td>Myosin VI</td>
<td>Maintains Golgi integrity</td>
</tr>
<tr>
<td>Anti-oxidant</td>
<td>ALDH1</td>
<td>Mitochondrial matrix NADH-dependent protein</td>
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<tr>
<td></td>
<td>sestrins</td>
<td>Restore peroxidases</td>
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<tr>
<td></td>
<td>TIGAR</td>
<td>Inhibitor of glycolysis</td>
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</table>

6.2. DNA-damage repair

Part of tumor suppressor functions of p53 is exerted by preventing propagation of deleterious mutations arising from DNA damage. Indeed, p53 plays an indirect role also in DNA repair through the induction of ribonucleotide reductase subunits (Hwang et al., 1999; Xue et al., 2003). Another p53-regulated gene, GADD45, that was originally proposed to participate in global genomic repair GGR downstream of p53 (Smith et al., 1994) has been later reported to have more likely functions in remodelling chromatin to give access to the sites of DNA (Smith et al., 2000). Furthermore, two mismatch repair genes MLH1 and PMS2 have recently been shown to contain p53-response elements (p53
RE) within their first intron and to be responsive to p53 activation after DNA-damage. These two genes may provide a sensor in DNA repair mechanisms and constitute a critical determinant for the decision between cell-cycle arrest and apoptosis (Chen and Sadowski, 2005).

A direct participation of p53 in DNA repair was suggested by a number of biochemical observations. For instance, the C-terminal 30 amino acids of p53 were shown to recognise several DNA damage-related structures, such as DNA ends, gaps, and insertion/deletion mismatches (Bakalkin et al., 1995; Jayaraman and Prives, 1995; Lee et al., 1995). p53 was also demonstrated to catalyze reannealing of short stretches of single- and double-stranded DNA and to promote strand exchange between them (Oberosler et al., 1993; Brain and Jenkins, 1994). In addition to p53’s biochemical activities, numerous reports on physical and functional protein interactions further strengthened the proposal of a direct role of p53 in nucleotide excision repair (NER), base excision repair (BER), and double-strand break (DSB) repair (Albrechtsen et al., 1999; Bertrand et al., 2004).

Although it is well documented that efficient nucleotide excision repair (NER) requires p53 its exact role has been difficult to define (Hanawalt, 2001). It has been suggested that p53 may function in NER by facilitating access to the chromatin to the repair machinery thus favoring DNA repair. It was further demonstrated that p53 is required for global chromatin relaxation induced by UV-irradiation (Rubbi and Milner, 2003b). In concordance with this, the histone deacetylase inhibitor trichostatin A overcomes the requirement for p53 suggesting that p53 may induce global chromatin relaxation through changes in histone acetylation (Rubbi and Milner, 2003b). Moreover the histone acetyltransferase p300 co localizes with p53 to sites of NER and inhibition of p300 by antibody microinjection inhibits NER suggesting that p53-dependent recruitment of p300 histone acetyl transferase (HAT) activity may be mechanistically involved in the ability of p53 to induce global chromatin relaxation to foster DNA repair (Rapic-Otrin et al., 2002).

p53 is also directly involved in inhibiting homologous recombination (HR). Two studies demonstrated that p53 inhibits HR in response to replication fork stalling (Janz and Wiesmuller, 2002; Saintigny and Lopez, 2002). Consistently, it was further noticed that p53 prevents the accumulation of DSBs at stalled-replication forks induced by UV or hydroxyurea treatment Kumari et al., 2004; Squires et al., 2004). When DNA replication is blocked, p53 becomes phosphorylated on serine 15 and associates with key enzymes of HR (Linke et al., 2003; Sengupta et al., 2003; Zink et al., 2002). Notably, during replication
arrest p53 remains inactive in transcriptional transactivation (Gottifredi et al., 2001; Restle et al., 2005) supporting the idea that p53 is involved in HR regulatory functions unrelated to transcriptional transactivation activities.

6.3. Senescence

Several lines of evidence support the idea that p53 tumor suppressor activity is partly mediated through the induction of senescence, a program leading to irreversible arrest of cell growth accompanied by a characteristic set of phenotypic changes in the cell. Senescence can be triggered by the shortening of telomeres due to proliferation (replicative senescence) or by other exogenous or endogenous acute and chronic stress signals (telomere-independent or premature senescence) such as cytokine signalling (TGFβ) oxidative damage (Chen et al., 2000; Di Leonardo et al., 1994), mitogenic oncoprotein over-expression (Ras, Raf) (Ferbeyre et al., 2002; Michaloglou et al., 2005; Serrano et al., 1997; Zhu et al., 1998), loss of anti-oncogenic tumor suppressors (PTEN) (Chen et al., 2005b) or supra-physiological mitogenic signals (over expressed MAPK or E2F1) (Dimri et al., 2000; Lin et al., 1998). In both replicative and premature senescence a key role is mediated by tumor suppressor pathways involving p53 and p16-pRB as demonstrated by a general refractoriness of human cells to multiple senescence-inducing stimuli upon loss of p53 and pRB function (Serrano et al., 1997; Dimri et al., 2000). These two pathways interact but can also independently halt cell-cycle progression and to some extent they respond to different stimuli in a cell-type or species-specific fashion. Recent work has revealed the importance of DNA-damage response (DDR) in initiating both replicative and premature senescence. A common signal is the occurrence of double strand breaks caused by telomere erosion or by oncoprotein activation through DNA hyper replication (Bartkova et al., 2006; d'Adda di Fagagna et al., 2003; Di Micco et al., 2006; Hemann and Narita, 2007; Herbig et al., 2004; Mallette and Ferbeyre, 2007; Takahashi et al., 2006) Consistently, in models of cellular senescence induced by DNA damaging agents causing double strand breaks, ATR/ATM mediates the activation of cell-cycle checkpoints via CHK1/CHK2 and p53, with the participation of p21, p16 and Rb (Itahana et al., 2004).

While p53 involvement in senescence and the signals that trigger it are well established much less is known on the actual mechanisms that contribute to this outcome. p21 is certainly a crucial transcription target in mediating p53-induced senescence (Brown et al., 1997) Interestingly, at early phases p21 levels are transiently elevated and gradually fall
while p16 levels rise (Alcorta et al., 1996; Stein et al., 1999). This suggests that p53 is crucial for the onset of senescence at least by inducing p21, then p16 may maintain the growth arrest. What needs to be defined in much detail is how p53 can mediate both reversible cell-cycle arrest and senescence through the same effector, p21. One possibility is that rapid DNA-repair quickly terminates p53-mediated p21 induction, while slow, incomplete or faulty repair results in sustained signalling and senescence. This might be sustained by the fact that proteins involved in DNA-repair mechanisms, such as PARP, play a role in post-translational activation of p53 during senescence (Vaziri et al., 1997). An indication that signalling to p53 may be different depending on the response is given by the observation that p53 phosphorylation pattern in senescence is distinct from that of DNA damage. In fact, in both cases there is an increase in Ser-15 phosphorylation but senescent cells have additional phosphorylation in Thr18 and Ser-376 and decrease in Ser-392 phosphorylation (Webley et al., 2000). This signature might be fundamental in recruiting co-factors that specifically direct p53 response.

Some key p53 regulators involved in senescence have been identified. Association among p53 senescence and PML has been reported by several groups. PML is up regulated upon oncogenic Ras expression and induces senescence in a p53-dependent manner by promoting p53 acetylation at Lys-382 by p300 in the nuclear bodies (NBs) (Pearson et al., 2000; Webley et al., 2000). In addition, the deacetylase SIRT1 also co-localizes in PML NBs and inhibits PML- and p53- induced senescence by deacetylating p53 (Langley et al., 2002).

Finally, the recent identification of two chromatin remodelling proteins, namely p400 and BS69, whose knock-down causes premature senescence starts to shed light on the regulation of p53 transcriptional activity within the senescence programme (Chan et al., 2005; Zhang et al., 2007).

6.4. Apoptosis

Clearly the main role of p53 is to prevent the outgrowth of damaged or stressed cells that may develop into malignancies if left unchallenged. This can be achieved by eliminating any aberrant cells through apoptosis (Fig.5), indeed the more ancient and evolutionarily conserved function also found in p53 orthologues from simpler organisms. Indeed, the best known transcriptional targets of p53 include a large number of pro-apoptotic genes that can be divided into several categories depending on their specific functions. These target genes
are generally classified on the basis of their involvement either in the extrinsic or the intrinsic apoptotic pathway. The extrinsic apoptotic pathway is triggered upon the engagement of particular death-receptors belonging to TNF-receptor family by their specific ligands and leads to the induction of a cascade of caspase activation which in turn induces apoptosis (Attardi et al., 2000; Nagata and Golstein, 1995; Wu et al., 1997). The intrinsic pathway instead is activated in response to different signals such as DNA damage, oncogenic signalling, hypoxia or endoplasmic reticulum stress, and is associated with mitochondrial depolarization and release of cytochrome C (CytC) from the mitochondrial intermembrane space into the cytoplasm. This event leads to the formation of the apoptosome, a complex of CytC, APAF-1 and pro-caspase-9, that activates the caspase cascade, thus converging in the effector phase with the extrinsic pathway (Cory and Adams, 2002).

p53 can promote apoptosis via the extrinsic pathway by activating the transcription of the death receptors located at the plasma membrane, including Fas, DR4 and KILLER/DR5, PERP and PIDD. Both DR5 and DR4 can trigger apoptosis or enhance apoptosis induced by their ligand TRAIL and by chemotherapeutic agents (Liu et al., 2004b). Fas can be activated by p53, yet its induction upon DNA damage is tissue specific and often does not require p53 (Bouvard et al., 2000). Moreover, Fas is dispensable for p53-dependent apoptosis in most tissues (O'Connor et al., 2000). PERP, a PMP-22/gas family protein, is activated in transformed MEFs following DNA damage (Attardi et al., 2000) and contributes to the p53-dependent apoptosis induced by γ-irradiation in thymocytes and neurons, but not to that induced by oncogene activation (Ihrie et al., 2003; Reczek et al., 2003). PIDD was identified as a p53-regulated gene in mouse erythroleukemia cells and shown to promote apoptosis. Its induction by ionizing radiation is p53-dependent also in MEFs (Lin et al., 2000). It has been described that PIDD can form an activating complex with caspase 2 (Tinel and Tschopp, 2004). Yet, it remains unclear whether PIDD is required for p53-dependent apoptosis as caspase 2 deficiency does not abrogate p53 responses in vivo.

Several other p53-regulated genes such as Bax, Noxa and PUMA enhance the release of cytochrome c into cytoplasm from mitochondria to initiate the intrinsic apoptotic pathway. Bax was the first identified p53-regulated pro-apoptotic Bcl-2 family member (Miyashita and Reed, 1995). Loss of Bax accounts for nearly half of the accelerated tumor growth which resulted from the loss of p53 in a brain tumor model (Schmitt et al., 2002). Bax is
also responsible for nearly half of p53-dependent apoptosis induced by 5-FU in colorectal cancer cells (Zhang et al., 2000). Nevertheless, Bax is dispensable for the apoptosis induced by γ-irradiation in thymocytes and intestinal epithelial cells and its induction is not strictly dependent on p53 in many tissues (Bouvard et al., 2000).

p53 regulates the expression of several BH3 domain-only proteins that function upstream of Bax to induce apoptosis. Some of these proteins are shown to be critical mediators of p53-dependent apoptosis. PUMA and Noxa are activated in a p53-dependent manner following DNA damage (Han et al., 2001; Nakano and Vousden, 2001; Yu et al., 2001). PUMA mediates apoptosis induced by p53 in response to hypoxia, DNA damaging agents, and endoplasmic reticulum (ER) stress in human colorectal cancer cells (Reimertz et al., 2003; Yu et al., 2003). Remarkably, PUMA-knockout mice recapitulated several key apoptotic deficiencies observed in the p53-knockout mice, including deficiencies in the apoptosis induced by γ-irradiation in thymocytes, by oncogenes in MEFs, and by DNA damage in developing neurons (Jeffers et al., 2003, Villunger et al., 2003). This suggests that the ability of PUMA to mediate apoptosis and tumor suppression is context-dependent. Indeed, other studies demonstrated that the ability of PUMA to act as a tumor suppressor can be dependent on other oncogenic events such as myc or E1A activation. Notably, PUMA depletion cooperated with myc and E1A induced tumorigenesis but not with Ras as this last oncogene induces a senescence response rather than apoptosis. Similarly, Noxa-deficient mice develop normally but their MEFs are strongly resistant to apoptosis induced by oncogenes and UV radiation while only slightly resistant to etoposide induced cell death (Shibue et al., 2003; Villunger et al., 2003). In vivo, the absence of Noxa resulted in resistance to X-ray-induced apoptosis in the small intestinal crypts (Shibue et al., 2003; Villunger et al., 2003). Moreover, p53 contributes to the formation of the apoptosome also through the transcriptional activation of APAF-1 (Kannan et al., 2001; Moroni et al., 2001) and is involved in the more downstream phases of apoptosis by activating the transcription of caspase-6 (MacLachlan and El-Deiry, 2002).

p53 can also induce the expression of a mitochondrial protein encoded by p53AIP1 gene following severe DNA damage (Oda et al., 2000). Interestingly, this induction is regulated by the phosphorylation of p53 at Serine 46 which might be mediated by several kinases such as p38, HIPK2, PKC and another p53 target p53DINP1 (Taira et al., 2007; Yoshida et al., 2006). p53 may sense the alterations in ROS levels and can activate numerous REDOX genes like PIG3, POX2/PIG6 and ferredoxin reductase (Donald et al., 2001;
Hwang et al., 2001). This results in an increased generation of ROS and originates a positive loop feeding p53 activation that further contributes to apoptosis (Johnson et al., 1996; Li et al., 1999; Martindale and Holbrook, 2002; Polyak et al., 1997). Moreover, p53 can contribute to the apoptotic pathway also through repressing the transactivation of anti-apoptotic genes such as Bcl-2 (Haldar et al., 1994; Miyashita et al., 1994a; Miyashita et al., 1994b), Bcl-X_L (Cherbonnel-Lasserre and Dosanjh, 1997) and survivin (Hoffman et al., 2002).

Interestingly p53 appears also to provide a connection between the extrinsic death receptor pathway and the triggering of mitochondrial disruption processes through the activity of its transcriptional target Bid. In fact Bid is activated by caspase-8 upon triggering of the extrinsic pathway and translocates to the mitochondria where it activates Bax and consequently the initiation of the intrinsic pathway (Sax et al., 2002). Among the pro-apoptotic p53 target proteins Scotin (Bourdon et al., 2002), that is located in the ER and the nuclear membrane, has been shown to be required for ER-stress mediated apoptosis. As a protein-folding compartment, the ER is extremely sensitive to alterations in homeostasis that disrupt its functions (Ferri and Kroemer, 2001; Kaufman, 1999). Prolonged ER stress can result in the activation of caspase-12 that in turn activates caspase-9 thus executing apoptosis. It has been proposed that, upon ER-stress, the release of calcium from the ER triggers mitochondrial depolarization thus allowing the release of CytC to the cytosol and the activation of the caspase cascade (Ichas et al., 1997; Ichas and Mazat, 1998; Jouaville et al., 1998). Notably, upon ER-stress p53 is up regulated and induces transcription of two other pro-apoptotic genes, Noxa and Puma (Li et al., 2006). These evidences clearly suggest that p53 mediates the apoptotic response at multiple levels and that the apoptotic pathways are not independent but rather involved in a complex interplay.

p53 participates in apoptosis also in a transcription-independent fashion by acting directly at mitochondria where it can perturb or modulate the functions of proteins implicated in the apoptotic machinery. This was thought to account for the reported ability of a transactivation incompetent p53 mutant to trigger apoptosis via the mitochondrial death pathway (Regula and Kirshenbaum, 2001). A fraction of p53 protein rapidly translocates to mitochondria in response to genotoxic, hypoxic, and oxidative stresses (Marchenko et al., 2000) and it has recently been demonstrated that this translocation is dependent on its monoubiquitination by Mdm2 (Marchenko et al., 2007). At the mitochondria, p53 has been found to interact with the Bcl-X_L and Bcl-2 protective proteins and to prevent them from...
inhibiting on the oligomerization among Bax and Bak (Mihara et al., 2003). There is a crosstalk among p53 functions at the mitochondria and its transcriptional activity, in fact upon stress-induced-stabilization and activation within the nucleus, p53 induces the transcription of Puma and this one is able to release cytoplasmic p53 from the inhibitory interaction with Bcl-XL, thus allowing it to directly activate Bax (Chipuk et al., 2005). p53 interacts also with Bad and the mitochondrial p53/Bad complex promotes apoptosis via activation and oligomerization of Bak (Jiang et al., 2006). Moreover, p53 acts directly on the pro-apoptotic Bak promoting its dissociation from the anti-apoptotic protein MCL1 (Leu et al., 2004). Once the inhibitory interactions upon Bax and Bak are relieved, they oligomerize to form a transmembrane pore for the release of cytochrome C from mitochondria.

Figure 5  Schematic representation of the different apoptotic pathways controlled by p53. In response to cellular stress, p53 transactivates proapoptotic genes activating (1) the death receptor pathway (pidd, DR4, DR5, Bid), (2) the mitochondrial pathway (Bax, Noxa, PUMA, p53AIP1, Apaf-1) and (3) the ER pathway (Scotin). All the different pathways converge to a common downstream pathway (4), where caspase-6 is directly transactivated by p53, thus modulating the sensitivity of the cell to die. p53 can also repress the transcription of relevant prosurvival genes such as Bcl2 (5). Moreover, p53 can mediate apoptosis by acting directly at mitochondria. (6). Finally, it has been shown that p53 participates in maintaining mtDNA stability by interacting with Poly and mtDNA.
Notably, upon translocation at mitochondria, p53 has been shown to play a role in maintaining mitochondrial genetic stability. Indeed, in response to mitochondrial damage p53 interacts with Polγ and enhances its DNA replication functions (Achanta et al., 2005). Moreover it has been proposed that p53 might interact with mtDNA (Heyne et al., 2004) and that it might participate in mitochondrial base excision repair (mtBER) (de Souza-Pinto et al., 2004).

The precise contribution of the transcriptional-independent apoptotic activities of p53 to the overall apoptotic response needs to be figured out much more in detail. However they seem to be important, as demonstrated by the fact that the stronger capacity of the isoform p53Arg72 to activate apoptosis as compared to the Pro72 form, is associated at least in part with the greater ability of Arg72 isoform to localize to mitochondria (Dumont et al., 2003). Recent reports showed that p53 can also modulate autophagy, a cellular process triggered by nutrient starvation and genotoxic agents that may function in different contexts to either promote or inhibit cell survival (Crighton et al., 2006). DRAM (damage-regulated autophagy modulator), a p53-induced protein, has been identified to be a likely linker between p53-dependent apoptosis and autophagy. In fact p53 induces autophagy in a DRAM-dependent manner and DRAM, which has been found to be down regulated in human cancer, has proven to be also critical for p53-induced cell death.

6.5. Pro-survival and antioxidant functions
Growing evidence of pro-survival roles for p53 suggests that it has key functions not only under condition of exceptionally severe stress, but also in response to the milder but more constitutive stress encountered during everyday life of the organism. Indeed, pro-survival genes induced by p53 have been identified. Among these, SLUG acts as inhibitor of PUMA expression (Wu et al., 2005) while Myosin VI, an unconventional motor protein, is induced by p53 to aid survival by maintaining Golgi-complex integrity (Jung et al., 2006). Many recent studies have also proposed a role for p53 in determining cell-survival through the regulation of intracellular reactive oxygen species (ROS) (Macip et al., 2003). It has been suggested that p53 may exert this function by inducing the expression of proteins, such as sestrins or ALDH4, that function to lower ROS levels. This antioxidant function of p53 is important in preventing DNA damage and tumor development under low-stress conditions (Budanov et al., 2004; Sablina et al., 2005). Furthermore, the recently discovered p53-inducible protein TIGAR (TP53-induced glycolysis and apoptosis...
regulator), has shown to regulate glycolysis and protect against oxidative stress (Bensaad et al., 2006). TIGAR can lower ROS levels and decrease sensitivity to p53 and other ROS-associated apoptotic signals and is likely to be an important component in mediating the pro-survival and tumor-suppressive effects of p53.

An attractive model would be that the low levels of p53 that are induced under conditions of normal proliferation and daily exposure to stress promote the expression of genes that induce cell cycle arrest, lower ROS levels and promote DNA damage repair. This will temporarily halt the proliferation of the stressed cell and allow damage repair to occur thus granting cell survival and organismal longevity. More severe and irreparable damage, or stress that is associated with oncogene activation or loss of survival signals, would conversely result either in activation of the apoptotic target genes, and elimination of the errant cell or in activation of the senescence program thus preventing its proliferation (Vousden, 2006).

7. Regulation of p53

When cells do respond adequately to p53-activating signals, the consequent biological outcome may vary considerably. As it appears from a large number of studies, much of the choice does not depend on p53 itself. Rather it is the cellular context, as defined by the balance of intracellular and extra cellular signalling events which dictates the direction undertaken by p53 response. Multiple proteins contribute to the activation of p53 in response to diverse stress stimuli. These include proteins that modify p53 for both stabilization and transcriptional activation, that reverse these modifications, that enhance the translation of p53 mRNA or that alter its sub cellular localization.

7.1. Regulation of translation

Regulation of p53 abundance has long been thought to rely exclusively on its stabilization as a consequence of reduced ubiquitination and proteasome degradation. However, regulation of p53 mRNA translation has also been shown to play a role in determining p53 expression levels. A negative auto-regulation of p53 mRNA translation is mediated by both a secondary structure of the 5’UTR in p53 mRNA and an element within its 3’UTR (Fu and Benchimol, 1997; Fu et al., 1996). Besides, other proteins such as tymidilate-syntase
inhibit translation of p53 mRNA through direct binding while Hu antigen R stimulates it after UV radiation (Mazan-Mamczarz et al., 2003). A recent study showed that the translational regulation of p53 is modified in response to DNA damage by the ribosomal protein L26 (RPL26) and nucleolin. These two proteins are part of the nucleolin-containing ribonucleocomplex and compete for binding to the 5'UTR of p53 mRNA co-regulating its abundance with opposing effects (Takagi et al., 2005). RPL26 preferentially binds to the 5'UTR after DNA damage, and its over-expression enhances association of p53 mRNA with heavier polysomes, increases the rate of p53 translation, induces G1 cell-cycle arrest, and augments irradiation-induced apoptosis. In contrast, nucleolin over-expression suppresses p53 translation and induction after DNA damage.

7.2. Regulation by the Mdm2/MdmX proteins.

In the absence of stress, p53 abundance is maintained at very low levels. Multiple lines of evidence indicate that Mdm2 and MdmX play a major role in regulating p53 stability.

Mdm2 was first isolated from a mouse double minute chromosome (hence the name) that was present at a high copy number in a spontaneously transformed derivative of mouse 3T3 cells (Cahilly-Snyder et al., 1987). Its role as an oncogene was assessed after the observation that its over-expression leads to immortalization of rodent primary fibroblasts and induced a fully transformed phenotype in cultured cells. Early studies ascribed the transforming capabilities of Mdm2 to its ability to form a complex with p53 (Momand et al., 1992) and to the observation that its over-expression leads to abrogation of both p53-mediated G1 phase cell cycle arrest and induction of apoptosis in cultured cells (Chen et al., 1996a; Haupt et al., 1996). The MdmX oncogene was identified because of its ability to interact with p53 (Shvarts et al., 1996) and later as a Mdm2 partner (Tanimura et al., 1999). Like Mdm2, MdmX acts as an oncogene and both are found amplified in many cancers including soft tissue, breast, lung and small intestine cancer (Danovi et al., 2004; Momand et al., 1998). The two proteins share a great structural similarity and are able to homo- and heterodimerize through their RING-finger domain (Tanimura et al., 1999). Importantly, the highest similarity among the two proteins resides in the region encompassing the p53 binding domain where the amino acid residues necessary for the interaction p53 are strictly conserved (Freedman et al., 1997). Conversely, the same amino acids on p53 are required for both the interaction with Mdm2 and with MdmX (Bottger et al., 1997). Yet, an important difference between Mdm2 and MdmX is that the RING domain of Mdm2 is
essential for its action as an E3-ubiquitin ligase, whereas MdmX apparently has no intrinsic ubiquitin-ligase activity.

Genetic studies revealed that germ line inactivation of either Mdm2 or MdmX leads to early embryonic lethal phenotypes that are completely overcome by concomitant inactivation of the p53 gene (Finch et al., 2002; Jones et al., 1995; Migliorini et al., 2002; Montes de Oca Luna et al., 1995; Parant et al., 2001) These studies also indicated that Mdm2 and MdmX are non-redundant p53 inhibitors, as normal levels of either regulator cannot compensate for the loss of the other. These evidences clearly indicate that p53 is a key downstream target of Mdm2/MdmX and that Mdm2/MdmX are essential negative regulators of p53.

Mdm2 and MdmX mediate the inactivation of p53 through different molecular mechanisms. Both Mdm2 and MdmX interfere with the transcriptional activity of p53. This was initially thought to occur by virtue of their binding to the N-terminal domain of p53 that masks the transactivation domain of p53 (Oliner et al., 1993), disabling its interactions with the basal transcriptional machinery and essential co-factors (Chen et al., 1995). Indeed, Mdm2/MdmX bind to the N-terminus of p53 within a domain that serves to recruit the histone acetyltransferases p300 and CBP (Dumaz and Meek, 1999; Lambert et al., 1998) thus interfering with p53 acetylation by p300 (Kobet et al., 2000; Sabbatini and McCormick, 2002). Mdm2 also associates with the histone deacetylase HDAC1, targeting it toward p53 deacetylation (Ito et al., 2002); Mdm2 has also been shown to localize with p53 at its responsive elements on the p21 and Mdm2 genes in normal growth conditions and to dissociate upon DNA damage (White et al., 2006) thus suggesting that the recruitment of a Mdm2-HDAC1 complex to chromatin might potentially promote histone deacetylation and transcriptional silencing. In addition, it has been reported that Mdm2 is capable of binding directly to histones and promoting their monoubiquitylation leading to a transcriptional repression (Minsky and Oren, 2004). Moreover, Mdm2 further inhibits p53’s transcriptional functions by catalyzing the conjugation of NEDD8, a small ubiquitin-like protein, to p53 Lys-370, 372 and 373 (Xirodimas et al., 2004).

The most important role played by Mdm2 is however the regulation of p53 turn-over. Mdm2 is in fact an E3-ubiquitin ligase that upon binding to p53 can catalyze either mono- and poly-ubiquitination of p53, leading to nuclear export or proteasomal degradation, respectively (Li et al., 2003). In contrast to Mdm2, MdmX does not act as an E3 ubiquitin ligase and by itself cannot stimulate p53 ubiquitination and proteasome-dependent
degradation (Jackson and Berberich, 2000; Stad et al., 2001). However MdmX can stabilize Mdm2 preventing its auto-ubiquitination (Sharp et al., 1999; Stad et al., 2001), while Mdm2 mediates MdmX poly-ubiquitination (Linares et al., 2003; Pan and Chen, 2003).

It has been shown that Mdm2 mediates both monoubiquitination or polyubiquitination of p53 depending on its relative amounts respect to p53 (Li et al., 2003): when Mdm2 is abundant, it promotes effective p53 polyubiquitination, leading to subsequent p53 degradation by the 26S proteasome. Several proteins have been demonstrated to assist the ubiquitination of p53 by Mdm2. Gankyrin increases the ubiquitin-ligase activity of Mdm2 and p53 degradation by favouring the association of the Mdm2-p53 complex to the 26S proteasome (Higashitsuji et al., 2005). Notably, gankyrin is frequently over expressed in hepatocellular carcinomas (HCC), therefore it may constitute an important mechanism for inactivating the p53 pathway in these tumors usually retaining wild-type p53 (Fu et al., 2002). Moreover, transcription factor Yin Yang 1 (YY1), a protein that plays a key role in development, increases p53 degradation by stabilizing the complex p53-Mdm2 (Sui et al., 2004). Intriguingly, it has been shown that p300 cooperates with Mdm2 to promote p53 poly-ubiquitination (Grossman, 2001) by acting as a E4 ubiquitin ligase (Grossman et al., 2003). This reveals a complex interplay between the two proteins that needs further elucidation.

Conversely, when Mdm2 is scarce, p53 becomes monoubiquitinated and rather than being degraded, p53 is exported into the cytoplasm. Notably, Mdm2 and p53 do not leave the nucleus together, it seems rather that mono-ubiquitination of p53 by Mdm2 leads to an unmasking of the NES within the C-terminus of p53 (Gu et al., 2001; Li et al., 2003). Movement of p53 into the cytoplasm has been thought for long to have an inhibitory effect on p53 activity. Clearly this translocation prevents p53 from transcriptional activation of its target genes, however it might be important for transcription-independent functions of p53 such as interactions with mitochondrial proteins in the apoptosis response (Chipuk and Green, 2004; Mihara et al., 2003). Indeed it has recently been reported that Mdm2-mediated monoubiquitylation of p53 greatly promotes its mitochondrial translocation and thereby its direct mitochondrial apoptosis (Marchenko et al., 2007). Upon arrival at the mitochondria, p53 undergoes rapid deubiquitination by mitochondrial HAUSP via a stress-induced mitochondrial p53–HAUSP complex, thus generating the apoptotically active non-ubiquitinated p53. A novel model for mitochondrial p53 targeting has been proposed,
whereby a distinct cytoplasmic pool of stabilized monoubiquitinated p53, generated in resting cells by basal levels of Mdm2, is subject to a binary switch from a fate of inactivation via subsequent polyubiquitination and degradation in unstressed cells, to a fate of activation via mitochondrial trafficking upon stress stimuli (Marchenko et al., 2007).

Mdm2-mediated monoubiquitination and subsequent cytoplasmic translocation of p53 may represent an important aspect in p53 regulation in unstressed cells, where Mdm2 is maintained at low levels (Boyd et al., 2000; Geyer et al., 2000; Jimenez et al., 1999; Stommel et al., 1999). On the other hand, Mdm2-mediated polyubiquitination and nuclear degradation may play a critical role in suppressing p53 function during the later stages of DNA damage response or when Mdm2 is over expressed in tumors (Shirangi et al., 2002; Xirodimas et al., 2001). Indeed, in many tumors bearing wild type p53, the p53 pathway is attenuated due to Mdm2 over-expression that may derive either from gene amplification or from the presence of a single nucleotide polymorphism in the Mdm2 promoter (SNP309 T/G). The G-allele of this polymorphism originates a stronger binding site for the transcriptional activator SP1 leading to a 2-3 fold increase in Mdm2 expression and correlates with accelerated tumor formation. Notably, this occurs in a gender-specific and hormonal dependent manner (Bond et al., 2006a; Bond et al., 2006b) probably due to the cooperation of ER with SP1 on Mdm2 promoter.

Under stress condition p53 activity increases and an early step in this process is the abrogation of Mdm2-mediated inhibition. Stress signalling directly communicates with the p53/Mdm2/MdmX axis and the activation of ATM-Chk2/ATR-Chk1 kinases leads to phosphorylation of p53, Mdm2 and MdmX (Kawai et al., 2003; Meek, 2002; Pereg et al., 2005). On the one hand, ATM-mediated phosphorylation of p53 at Ser15 and indirectly (through Chk2) at Ser20 inhibits the interaction between p53 and its ubiquitin-ligase allowing the stabilization of p53 (Sakaguchi et al., 1998; Shieh et al., 1997; Unger et al., 1999). Moreover, in response to DNA-damage the Jun NH₂-term Kinase (JNK) phosphorylates p53 within the PRD at Thr81 (Buschmann et al., 2001). Upon phosphorylation of this residue (Phospho-Thr81/Pro82) p53 can be recognized and isomerized by the prolyl isomerase Pin1 to promote Chk2-dependent phosphorylation of p53 on Ser20, thereby stimulating its dissociation from Mdm2 (Berger et al., 2005).

On the other hand phosphorylation of Mdm2 and MdmX enhances their degradation by auto-ubiquitination and by reduction of their association with the de-ubiquitinating enzyme HAUSP, further releasing p53 from its negative regulation (Brooks et al., 2007; Li et al.,
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2004; Li et al., 2002). Notably HAUSP can de-ubiquitinate p53 directly, yet its main function in the p53 pathway seems to be in the de-ubiquitination and stabilization of Mdm2 and MdmX (Cummins and Vogelstein, 2004; Li et al., 2004; Li et al., 2002) resulting in enhanced degradation of p53.

The p53-Mdm2 interplay is tightly regulated in a negative feedback loop wherein p53 stimulates Mdm2 synthesis through transactivation of its promoter (which contains two adjacent p53 binding sites) while Mdm2 inhibits p53 activity. Interestingly MdmX is not a p53 transcriptional target and its abundance is mainly regulated through the interaction with HAUSP.

The interplay between p53 and Mdm2/MdmX determining p53 stability and activation has been elegantly summarized by G. Wahl into a model (Fig. 6) that divides p53 regulation in four phases (Wahl, 2006). In unstressed cells p53 is maintained at low levels and in an inactive state by interaction with Mdm2 and MdmX (homeostasis). In this phase Mdm2 levels are sustained through the interaction with HAUSP, through mitogen-dependent post-translational modifications (Ashcroft et al., 2002; Gottlieb et al., 2002) and, through transcriptional activation (Lahav et al., 2004); (Ries et al., 2000). Mdm2 can ubiquitinate MdmX that is however de-ubiquitinated and stabilized via interaction with HAUSP.

Moreover, Mdm2-mediated ubiquitination of histones may provide an additional means of controlling p53 transcription functions. DNA damage leads to the activation of ATM and other kinases within minutes (early activation), as well as phosphorylation of p53’s N-terminal serines that may modulate association with Mdm2/X and co activators. Of equal importance, these kinases also rapidly phosphorylate Mdm2 and MdmX thus accelerating their degradation by preventing their interaction with HAUSP. In this phase MdmX levels do not decline immediately thus p53 transcriptional activity is still inhibited to some extent and p53 target genes are activated weakly.

Full p53 activation occurs only after a lag of about 2h and requires Mdm2 and MdmX phosphorylation to decrease HAUSP interaction with both proteins, the net effect of which is to increase Mdm2-mediated self-ubiquitylation and ubiquitylation of MdmX. This decreases the levels of Mdm2 at early time points and as a consequence activates p53. As a consequence of p53 transcriptional activation, the increased levels of Mdm2 can then degrade MdmX, resulting in full activation of p53. Notably p53 activation requires a positive feedback loop in which increasing Mdm2 abundance titrates the amount of MdmX degradation to assure the fine-tuning of the timing and magnitude of the p53 transcriptional
response (see Figure 6). The components of this system also provide the mechanism for attenuating p53 signalling after stress dissipation or damage repair. ATM and p53 phosphorylation at Ser15 return to background levels within 4–6 h of induction of DNA damage (Stommel and Wahl, 2004). In normal human fibroblasts, Mdm2 levels are high at 4 h, and start to decline thereafter, along with a parallel decrease in p53 transactivation. Reasonably, the damage-phosphorylated Mdm2 pool is replaced with a non-phosphorylated pool as a consequence of new synthesis and diminished abundance of activated ATM. The elevated levels of Mdm2 should be able to interact with and inactivate p53. Furthermore, in the absence of DNA damage-activated kinases, MdmX will be allowed to interact with HAUSP, leading to deubiquitination and stabilization thus providing a second barrier to continued p53 activation.

p53 stabilization and activation is furthermore regulated by other pathways that crosstalk with the p53-Mdm2 axis. A feedback loop governing p53 activity involves the AKT kinase and the PIP-3 phosphatase PTEN. In detail, AKT phosphorylates Mdm2 and induces its translocation into the nucleus, where it down-regulates p53 (Mayo et al., 2002; Zhou et al., 2003a). By inhibiting AKT and by blocking the nuclear entry of Mdm2, PTEN sustains p53 activity (Mayo and Donner, 2002). On the other hand, in damaged cells, p53 activates the transcription of PTEN (Stambolic et al., 2001) thus facilitating formation of a cycle in which p53 induces PTEN and PTEN stabilizes p53.

Upon increased oncogenic signalling, p53 activation and stability is regulated also by the tumor suppressor ARF. ARF increases p53 stability as it down-modulates Mdm2 ubiquitin ligase activity both by sequestering it to the nucleolus (Damalas et al., 2001; Honda and Yasuda, 1999; Lowe, 1999) and by interfering with its interaction with YY1 (Chen et al., 2005). Notably p53 itself has been shown to down-regulate ARF expression (Robertson and Jones, 1998). In this way increased levels of p53 act in a negative feedback, resulting in a down-regulation of ARF transcription and concomitant decreases in ARF levels. This will then allow MDM2 to bind to p53 and reduce its stability by targeting it for degradation.

Fig. 6. Model of the attenuation and destabilization of p53 before and after stress stimuli, and for its activation and stabilization in response to stress. p53 regulation is presented in four phases as described in detail in the text. (From Wahl, 2006)
7.3. Regulation by other ubiquitin ligases

The ubiquitination of p53 was first discovered in papilloma-virus-infected cells, where the HPV protein E6 was found to associate to an E3 ubiquitin ligase, E6-AP thus mediating p53 degradation. E6-AP-mediated disruption of p53 is important in the aetiology of HPV associated cervical cancer and anogenital malignancies as it prevents p53 from activating the apoptotic program, that would be induced upon oncogenic signalling by E7 and that constitute a major obstacle to viral replication. Other oncogenic viruses exploit this mechanism to prevent p53 response, for instance the adenoviral proteins E1B55K and E4orf6 have shown to cooperate in promoting p53 ubiquitination and degradation through a SCF E3 ubiquitin-ligase (Querido et al., 2001).

c-JUN NH2-terminal kinase (JNK) mediates another Mdm2-independent p53 degradation pathway that seems to have a dual role. Under unstressed conditions, JNK binds to p53 preferentially in the G0/G1 cell-cycle phase and the interaction results in the ubiquitylation and degradation of p53. Conversely, under stress conditions, JNK phosphorylates p53 at Thr81, resulting in its stabilization and activation (Buschmann et al., 2001; Fuchs et al., 1998a; Fuchs et al., 1998b). Other proteins that act as E3 ubiquitin ligase have been shown to target p53 and promote its proteasome-mediated degradation. Pirh2, a RING-H2 domain-containing protein, interacts with p53 and promotes Mdm2-independent p53 ubiquitination and degradation (Leng et al., 2003). Similar to Mdm2, Pirh2 is a p53 responsive gene and participates in a comparable auto regulatory negative feedback loop.

Another E3 ligase, COP1, has also been described recently as a direct ubiquitin ligase for p53 (Dornan et al., 2004). COP1 is also a p53-inducible gene and can ubiquitinate and degrade p53. Further, COP1 depletion by siRNA enhances p53-mediated G1 arrest and can sensitize cells to ionizing radiation. COP1 is phosphorylated by ATM upon DNA damage thus leading to its auto-degradation (Dornan et al., 2006).

ARF-BP1/Mule/HectH9 was recently identified as a HECT domain E3 ligase that can ubiquitinate and degrade p53 (Chen et al., 2005a). ARF interacts with ARF-BP1/Mule/HectH9 and inhibits its activity thus leading to p53 stabilization.

Another route to degradation for p53 is mediated by the ubiquitin ligase CHIP (chaperone-associated ubiquitin ligase), that has been shown to poly-ubiquitinate p53 upon transient binding to HSP90 and HSP70. This mechanism might contribute to maintaining low levels of p53 under physiological conditions as CHIP depletion significatively augment p53 basal
levels (Esser et al., 2005). Notably CHIP has been recently shown to play a role in mutant p53 ubiquitin-mediated degradation (Muller et al., 2008).

Recently, p53 was shown to be substrate of another E3 ubiquitin ligase, TOPORS (Rajendra et al., 2004; Zhong et al., 2005). Interestingly, TOPORS has shown to localize into PML nuclear bodies and to enhance the conjugation of the small ubiquitin-like modifier 1 (SUMO-1) to p53 fashion (Rasheed et al., 2002). The role of TOPORS in regulating p53 activity is controversial. As there are reports indicating that TOPORS has an inhibitory effect on p53 transcriptional activity (Shinbo et al., 2005) while others suggesting that it is a p53 co-activator (Lin et al., 2005).

Together, Mdm2, COP1, Pirh2, ARF-BP1 and Topors represent an array of E3 ligases that act to regulate and maintain p53 levels (Fig.7). This redundancy suggests that multiple mechanisms are used cooperatively by the cell for tight p53 regulation. It might be suggested that the Mdm2/MdmX-independent pathways of degradation might serve for

Fig. 7. Schematic representation of the regulation of p53 degradation.
Fine tuning of p53 response. For example, they might be activated in different cellular contexts or upon specific stimuli. Alternatively, they might backup Mdm-2/MdmX during the phases of p53 full activation, when Mdm-2/MdmX levels are low, in order to avoid unbalancing of the system and to favour the attenuation phase when the stress stimuli are dissolved. However, further investigation on the specific regulation of these proteins is needed in order to fully understand their actual role.

Two other ubiquitin-ligases, namely Cullin 7 (CUL7) and WWP1 can promote the cytoplasmic localization of p53 yet without targeting p53 for proteasomal degradation (Andrews et al., 2006; Laine and Ronai, 2007). Similarly, regulation of p53 ubiquitination by the E2 ubiquitin-conjugating enzyme Ubc13 has been shown to drive nuclear export of p53 (Laine et al., 2006). Curiously, ubiquitination of p53 by E4F1 results in neither degradation nor nuclear export, but enhances transcriptional activation resulting selectively in cell cycle arrest (Le Cam et al., 2006).

In addition to ubiquitin-conjugation it has been shown that p53 can be degraded as well through an ubiquitin-independent mechanism. This pathway involves the 20S proteasome and is regulated by the NAD(P)H quinone oxido-reductase 1 (NQO1) (Asher et al., 2001). NQO1 can bind directly to p53 thus preventing it from being degraded by 20S proteasome. Binding of NQO1 to p53 is augmented in the presence of NADH and disrupted by dicoumarol, an inhibitor of NQO1 (Asher et al., 2005). Genotoxic insults of various nature lead to increased association between P53 and NQO1 and to consequent p53 stabilization (Asher and Shaul, 2005). Although NQO1 regulates p53 stability via a distinct pathway that is both Mdm2 and ubiquitin independent, this pathway is still responsive to some of the regulators of Mdm2-ubiquitin dependent degradation such as ARF, which in addition to inhibiting the ability of Mdm2 to target p53 for degradation also inhibits dicoumarol-induced p53 degradation (Asher et al., 2002).

7.4. Localization at the Nuclear Bodies (NBs)

The promyelocytic leukaemia (PML) tumour-suppressor protein is localized either in the nucleoplasm or in subnuclear structures referred to as nuclear bodies (NBs). Although their function is not totally clear, NBs are dynamic multiprotein-complexes comprising numerous transient or permanently localized proteins among which figure p53 and many of its effectors (Borden, 2002; Guo et al., 2000; Zhong et al., 2000a; Zhong et al., 2000b) The co localization of p53, CBP, and HIPK2 in PML NBs contributes to the regulated
phosphorylation (by HIPK2) and acetylation (by CBP) of p53 in response to DNA damage (D'Orazi et al., 2002; Guo et al., 2000; Hofmann et al., 2002). Protein inhibitor of activated STAT (PIAS) and HAUSP also accumulate in NBs, further indicating that NBs could provide a scaffold for the regulation of p53 post-translational modification.

It has been reported that NBs are required for Ras-induced senescence through a mechanism involving p53 acetylation at Lys382 by CBP (Pearson et al., 2000). In addition, SIRT1, a p53 deacetylase, also co-localises with acetylated p53 in NBs and seems to be involved in mediating cellular senescence (Langley et al., 2002). PML protects p53 from Mdm2-mediated ubiquitylation and degradation by inducing the auto-phosphorylation and activation of CHK2 that consequently phosphorylates p53 on Ser20 (Yang et al., 2006a).

### 7.5. Post-translational modifications

p53 undergoes a great variety of post-translational modifications 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 actual pattern of post-translational modifications is complicated since the same residue might be modified in different ways by different enzymes. Notably nearly all of these modifications occur in the N-terminal or in the C-terminal domains. In general N-terminal phosphorylations seem to be important for stabilizing p53 and crucial for acetylation to occur in the C-terminal sites which in combination lead to full p53 activation. An intriguing model for the function of the C-terminal region of the p53 protein, corroborated by the observation that nearly every residue of it is subject to a post-translational modification, is that it acts like a histone tail, progressively accumulating modifications that permit the sequential recruitment of chromatin modifying enzymes, for example through binding of acetylated p53 residues to bromodomain proteins (Agalioti et al., 2002).

#### 7.5.1. Phosphorylation

Many kinases, including ATM, ATR, Chk1, Chk2, casein kinase1 and 2 (CK1, CK2), c-JUN NH2-terminal kinase (JNK), Erk, p38, Aurora Kinase A (AurKA), glycogen synthase kinase-3β (GSK3β), HIPK2 and DYRK2, have been shown to phosphorylate p53 after DNA damage. So far, 17 phosphorylation/dephosphorylation sites have been detected in
human cells following DNA damage induced by ionizing radiation or ultraviolet (UV)-light irradiation (Fig 8). In humans, these include serines 6, 9, 15, 20, 33, 37 and 46 and threonines 18 and 81 in the amino-terminal region; Ser315 and Ser392 in the C-terminal domain; and Thr150, Thr155 and Ser215 in the central core. In addition, Thr55, Ser376 and Ser378 seem to be constitutively phosphorylated in unstressed cells (Gatti et al., 2000; Waterman et al., 1998). At this time, only a few p53 sites are reported to be phosphorylated by one specific protein kinase. Ser6, Ser9 and Thr18 are phosphorylated only by casein kinase 1 (CK1). Furthermore, the phosphorylation of Thr18 requires previous phosphorylation of Ser15 (Dumaz et al., 1999; Sakaguchi et al., 2000). Moreover, Thr81 is reported to be phosphorylated only by JNK (Buschmann et al., 2001). Clearly, significant redundancies are observed, yet this multiplicity may provide a failsafe mechanism and a distinctive combination of phosphorylated residues could be required for further modifications, leading to maximal activation.

The phosphorylation of the amino-acid residues Ser15, Thr18 and Ser20 in the N-terminal domain of human p53 is the most extensively studied. These residues are located in, or close to, the region of p53 that also binds to Mdm2/MdmX (Appella and Anderson, 2001; Bode and Dong, 2004; Ito et al., 2001; Sabbatini and McCormick, 2002) Ser15 is phosphorylated in an ATM-dependent manner early in response to γ-irradiation but is not phosphorylated upon exposure to UV-light (Banin et al., 1998; Waterman et al., 1998).
Conversely p38 phosphorylates p53 at this residue in response to UV radiation (Bulavin et al., 1999). Data from *in vitro* or over-expression studies indicate that phosphorylation at Ser15 stimulates p53-dependent transactivation, growth arrest and apoptosis in response to DNA damage (Fiscella et al., 1993; Shieh et al., 1997). However, there exist conflicting data on whether Ser15 phosphorylation affects Mdm2 binding or not (Dumaz and Meek, 1999; Shieh et al., 1997) but it is known that it increases the interaction with the acetyltransferase CBP (Lambert et al., 1998). Phosphorylation of other residues, including Thr18 and Ser20, which occurs later in the response to DNA damage, was shown to depend on initial phosphorylation at Ser15 and seems to interfere with the interaction of p53 with Mdm2, thus promoting p53 stabilization (Chehab et al., 1999; Dumaz et al., 1999; Unger et al., 1999). Notably, mice with a serine-to-alanine substitution at serine 23 (corresponding to Ser20 in humans) develop a wide spectrum of B-cell tumours thus indicating that B-cells are most sensitive to activating p53 through phosphorylation of serine 23 (MacPherson et al., 2004).

Phosphorylation of Ser46 by the kinases HIPK2, p38MAPK and DYRK2 was reported to mediate selectivity in promoter binding by p53 and to specifically promote the induction of apoptosis inducing genes, such as p53-regulated apoptosis-inducing protein1 (p53AIP1) (D’Orazio et al., 2002; Oda et al., 2000; Taira et al., 2007). Moreover, HIPK2-mediated phosphorylation of p53 at Ser 46 is required for the acetylation of p53 at Lys 382 by CBP (Hofmann et al., 2002). Notably, the stronger apoptotic potential of the polymorphic variant p53-Arg72 as compared to p53-Pro72 has been associated with phosphorylation of Ser46 as mutation of this residue abolishes the differences between these two isoforms (Sullivan et al., 2004). Another coding region polymorphism exists in p53 just at codon 47 encoding either a proline or, less frequently, a serine residue (Felley-Bosco et al., 1993). The Ser47 polymorphism shows impaired phosphorylation on Ser46 as proline-directed kinases like p38-MAPK and HIPK2 require an adjacent proline to direct phosphorylation. Consistently, the p53-Ser47 variant has impaired ability to transactivate the p53 target genes p53AIP1 and PUMA, further confirming that reduced phosphorylation on Ser46 leads to impaired ability to induce apoptosis (Li et al., 2005).

Phosphorylation at Ser46 has been recently shown to be important in p53 promoter selection. It is in fact sufficient for p53 to induce the PTEN tumor suppressor protein in preference to Mdm2 (Mayo et al., 2005). The shift of p53 gene targeting from Mdm2 to
PTEN results in sustained p53 activation and diminished survival as p53 and PTEN coordinate cell death by facilitating one another’s expression and functions (see above). Conversely, in normal conditions p53 represses its own phosphorylation at Ser46 due to Mdm2-mediated HIPK2 degradation (Rinaldo et al., 2007). This creates another positive feedback loop in which Mdm2 degrades HIPK2 thus decreasing p53 phosphorylation at Ser46. Under this condition p53 will activate Mdm2 transcription rather than that of PTEN. These findings support the notion that the cell-cycle-arresting functions of p53 include active inhibition of the apoptotic ones, and furthermore indicates that phosphorylation at Ser46 is an important determinant of p53 choice as it acts like a switch between two opposite feedback loops.

Phosphorylation at Thr81 by JNK generates a binding site for the prolyl isomerase Pin1 (Thr81/Pro82) that influences p53 stabilization and activation (Buschmann et al., 2001). The relevance of this modification will be treated in detail below.

Within the DNA binding domain Ser 215 phosphorylation by means of Aurora Kinase A, abolishes the DNA binding ability of p53 (Liu et al., 2004a). Besides, Thr 150 and Thr 155 are phosphorylated by two COP9 signalosome-associated kinases, CK2 and protein kinase D, leading to ubiquitin-dependent degradation of p53 (Uhle et al., 2003). Interestingly, a recent report demonstrated that Ser 149 of p53 is O-GlcNAcylated and that this modification is associated with decreased phosphorylation of p53 at Thr 155, resulting in decreased p53 ubiquitination (Yang et al., 2006b). This offers another demonstration of the dynamic interplay between different post-translational modifications in p53.

Phosphorylation of p53 at serine 315 and serine 376, mediated by GSK-3β upon ER stress has been shown to increase its cytoplasmic localization and reduce its stability (Pluquet et al., 2005; Qu et al., 2004; Qu and Koromilas, 2004). In this way the cell may adapt to ER-stress and engage mechanism that limit accumulation of unfolded proteins in the ER. However, when ER stress is prolonged the apoptotic pathway is triggered, both by direct activation of caspase-12 and by mechanisms that involve the p53 target gene Scotin (see above).

Two serine residues are phosphorylated in the CTD of p53. Phosphorylation of Ser315 by the cyclinB-dependent kinase, p34(Cdc2), was reported to increase the transactivation potential of human p53 in response to irradiation damage, possibly by promoting nuclear retention, mediated via interaction of p53 with E2F1 (Blaydes et al., 2001; Fogal et al., 2005a). Phosphorylation at the same site by aurora kinase A, however, was suggested to
promote Mdm2-dependent ubiquitination and proteolysis of p53, arguing for an inhibitory role of this modification (Katayama et al., 2004). Ser392 (Ser389 in mouse p53) is phosphorylated by p38 MAPK or casein kinase 2 in response to UV-irradiation, but only very inefficiently after \(\gamma\)-irradiation. An UV-activated protein kinase complex that phosphorylates Ser-392 of p53 in vitro has been identified. This kinase complex contains casein kinase 2 (CK2) and the chromatin transcriptional elongation factor FACT (a heterodimer of hSpt16 and SSRP1). FACT seems to alter the specificity of CK2 in the complex such that it selectively phosphorylates p53 over other substrates including casein. Phosphorylation by the kinase complex enhances p53 activity (Keller et al., 2001). It has been demonstrated that damage-stimulated phosphorylation of p53 at Ser392 by CK2 has an important impact on p53 structure as it can increase intrinsic thermostability of the core DNA-binding domain thus enhancing p53 affinity for its consensus DNA binding site. Mice with a serine-to-alanine mutation of p53 amino acid 389 reveal a slight decrease in DNA binding, reduced and delayed transcriptional activation of several p53-responsive genes, and decreased apoptosis in response to UV (Bruins et al., 2004).

In addition to stress-induced phosphorylation there are also a few sites in p53 which are dephosphorylated in response to stress signals, such as Ser-376 and Thr-55. The loss of phosphorylation from Ser-376 creates a consensus binding site for 14-3-3\(\sigma\) and in turn increases the affinity of p53 for sequence-specific binding sites on DNA (Waterman et al., 1998). Dephosphorylation by PP2A at Thr55 instead has been shown to stabilize p53 (Li et al., 2007b). It is likely that there exists an ordered pattern and interdependence of stress-induced modifications to p53. However, the relevance and the precise role of phosphorylations on p53 in its stabilization and activation remain controversial. While it seems likely that the complex network of stress-induced modifications to p53 is important in generating a functional molecule, there is also evidence that p53 can be activated without these changes. It was demonstrated that p53 stabilization occurs as well upon mutation of a series of known stress-induced phosphorylation sites. (Blattner et al., 1999; Fuchs et al., 1995). Further evidence pointing to a lack of requirement for post-translational modification in p53 stabilization comes from the use of small molecule inhibitors of the p53–Mdm2 interaction. In fact, p53 accumulates in cells treated with Nutlin-1 (a cis-imidazoline analogue that displaces p53 from its complex with Mdm2) and this is followed by an increase in the levels of both p21 and Mdm2 consistent with activation of the p53 pathway. Yet, unlike DNA damage, nutlins did not induce phosphorylation of p53 but this
stabilized, unphosphorylated form of p53 was equally efficient at sequence-specific DNA binding and the induction of apoptosis (Thompson et al., 2004). These controversial results does not mean however that phosphorylations on p53 are irrelevant or do not occur in vivo but nature appears to be able to compensate quite easily for the loss of one or even two or more of these modifications in p53.

7.5.2. Prolyl-isomerization

Tightly connected to phosphorylation is the prolyl-isomerization mediated by Pin1. Pin1 is a phosphorylation-dependent prolyl isomerase (PPIase) that specifically recognizes and catalyzes cis/trans isomerization at Ser-Pro or Thr-Pro motifs in which the first amino acid is phosphorylated (pSer/pThr-Pro). These motifs are present in many proteins involved in controlling cell proliferation and transformation, such as Cyclin D1, β-catenin, NF-κB, p53, p73 and p66Shc (Mantovani et al., 2004a; Ryo et al., 2002; Zacchi et al., 2002; Zheng et al., 2002). It has been demonstrated that Pin1 has a central role in transducing phosphorylation of p53 into conformational changes that affect its stability and function. Indeed, p53 stabilization and activation upon genotoxic stresses are impaired in the absence of Pin1 (Mantovani et al., 2004b; Zacchi et al., 2002; Zheng et al., 2002). Within the p53 sequence six Ser/Thr-Pro sites are present (namely Ser33-Pro34, Ser46-Pro47, Thr81-Pro82, Ser127-Pro128, Thr150-Pro151 and Ser315-Pro316). Ser-33, Thr-81 and Ser-315 residues are phosphorylated upon UV radiation while Ser-46 upon ionizing radiation (See above) thus generating consensus sites for Pin1. The structural change mediated by Pin1 can trigger various functional outcomes. Data from Berger et al. suggest that the residues Thr81-Pro82 within the PRD are crucial for Pin1 to promote Chk2-dependent phosphorylation on Ser-20 (Berger et al., 2005) and this might explain the ability of Pin1 to induce dissociation of the p53-Mdm2 complex and favor p53 stabilization. Moreover, mutations of Thr81-Pro82 have been reported in cancer patients with Li-Fraumeni syndrome and sporadic breast tumors (Berger et al., 2005; Sun et al., 1996). In addition to a role in the dissociation of Mdm2 from p53, in a recent report from our group it has been demonstrated that Pin1 is involved also in regulation of p53 acetylation by p300 (Mantovani et al., 2007). It has been shown that mutants of p53 unable to bind to Pin1 (upon substitution of Ser/Thr residues at Pin1 consensus binding sites with alanines) interact with p300 markedly less than wild type p53 and are consequently less acetylated. Notably, in an in vitro assay it was demonstrated that a Pin1 catalytic mutant (C113A,
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described in Zhou et al., 2000) that binds p53 but cannot isomerize it, is not able to increase p53 acetylation by p300 as its wild type counterpart does, thus indicating that the conformational change induced by Pin1 on p53 upon prolyl-isomerization is required for effective acetylation by p300. It is likely that Pin1 might regulate p53 acetylation by p300 through phosphorylation induced isomerization of Thr81-Pro82 within p53 PRD as this domain has been proven to be essential for p300-mediated acetylation of p53 upon DNA binding (Dornan et al., 2003a). This is also supported by the evidence that tumor-associated mutations at Thr81 or Pro82 result in impaired acetylation at Lys 373 and Lys382.

Notably, it has been also demonstrated that the prolyl-isomerase Pin1 is required for efficient binding of p53 to its REs on p21 and Bax promoters upon stress (Mantovani et al., 2007). Moreover, it was shown that Pin1 itself is recruited by p53 on its cognate promoters on chromatin where it acts enhancing p53 acetylation by p300. Indeed, it was observed that acetylation of chromatin-bound p53 on Lys373 and Lys382 increased upon stress only in the presence of Pin1. This effect of Pin1 may be mediated through isomerization of the Thr81-Ser82 site within p53 PRD as, as described above, the integrity of these residues proved to be fundamental to allow p53 acetylation (Bushmann et al., 2001; Berger et al., 2005).

Another site which may be important for Pin1 effect on p53-mediated apoptosis is Ser46-Pro47, as also confirmed by the reduced apoptotic potential displayed by the Ser47 allele of the Pro/Ser47 polymorphism (Li et al., 2005).

Pin1 is also an essential co-factor of the p53-family member p73 pro-apoptotic function. Indeed, it has been demonstrated by our group that the binding between Pin1 and p73 is stimulated upon stress conditions by c-Abl and p38 kinases and that this association increases p73 acetylation by p300, thereby stimulating its transcriptional activity towards apoptotic target genes, such as p53AIP1 (Mantovani et al., 2004b).

7.5.3. Acetylation/Deacetylation

p53 can be acetylated at several lysines by different HAT. Acetylation of p53 by p300/CBP and PCAF occurs in response to DNA damaging agents, such as UV- and γ-irradiation. CBP and p300 acetylate p53 at lysines within the C-terminal domain (Lys370, 372, 373, 381, 382) (Avantaggiati et al., 1997; Gu et al., 1997; Lill et al., 1997). Moreover, Lys320 and Lys305 in the nuclear localization domain are acetylated by PCAF (p300/CBP-
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associated factor) and p300, respectively (Liu et al., 1999; Sakaguchi et al., 1998). Finally, the MYST family acetyl transferases, hMOF and TIP60, were recently shown to acetylate p53 at Lys120 in the DBD (Berns et al., 2004; Doyon et al., 2004; Legube et al., 2002) (Fig. 9).

The role of acetylation in mediating p53 binding to DNA has been largely debated and is still controversial (Gu and Roeder, 1997) yet, what is commonly assessed is that it potentiates the recruitment of co-factors thus favoring p53 transcriptional activation. Consistently, reports showing that acetylated p53 is enriched at target promoters in a stress dependent manner (Liu et al., 2003; Mantovani et al., 2007) suggest a role for p53 acetylation in affecting its transcriptional activation. While some studies could show that acetylation favours the recruitment of co-factors and histone acetyltransferases (TTRAP and CBP) thus increasing p53 transcriptional activity (Barlev et al., 2001) other suggested that the enhancing effect of p300/CBP on p53 transactivation function was independent of p53 acetylation (Espinosa and Emerson, 2001)

Nevertheless, a number of recent studies have indicated that acetylation of specific lysine residues on p53 has differential effects on the choice of the target genes activated by p53. Using acetylation-mimicking lysine to glutamine mutations, functional differences between acetylation of Lys320 versus Lys373 were reported. Acetylation of Lys320 in p53 was shown to favour interaction with high-affinity p53-binding sites in target genes, promoting cell survival and cell cycle arrest. In contrast, acetylation of Lys373 led to a stronger interaction of p53 with low-affinity binding sites, which are found in pro-apoptotic target genes and therefore promoted cell death (Knights et al., 2006).

Acetylation on Lys120 by the MYST family acetyl transferase Tip60 has also been shown to modulate p53 transcriptional activity and to be necessary for apoptotic response. Notably, Lys120 is specifically acetylated by Tip60 (Legube et al., 2004). Lys120-acetylated p53 was reported to accumulate upon DNA damage preferentially on the promoters of pro-apoptotic target genes, including Bax and Puma (Tang et al., 2006). Moreover, mutants of p53 that can no longer be modified at this residue showed impaired pro-apoptotic activity, whereas cell-cycle arrest and induction of Mdm2 were not affected. Interestingly, Lys120 is conserved in all species that contain functional p53 genes and, more importantly, it is mutated in human tumors (IARC TP53 Mutation Database).

Acetylation might also enhance p53 transcriptional activity through promoting the assembly of an active transcriptional apparatus. It is known that TFIID binding to the core
promoter element is required for assembly of a functional transcriptional initiation complex (Thomas and Chiang, 2006). TAF1, the larger subunit of TFIID has been shown to interact with the C-terminal domain of p53 on p21 promoter (Espinosa et al., 2003; Li and Wu, 2004). In a recent study it has been demonstrated that the acetylation of two lysine residues in p53, Lys373 and Lys382, leads to a direct interaction with the Double Bromodomain (DBrD, a structural protein module that recognizes acetylated lysines) of TAF1. Transcription of the human p21 gene is regulated through two p53 binding elements at -2.3 kb (5’ site) and -1.4 kb (3’ site) relative to the transcription start site (+1). Upon DNA damage, TAF1 is initially recruited to the 3’ site and then through DNA looping is brought to the TATA-box-containing core promoter. Site-directed mutants in p53 or in the TAF1 bromodomains disrupt acetyl-p53/bromodomain interaction and fail to recruit TAF1 to both the distal p53-binding site and the core promoter. These results suggest that the diacetyllysine/DBrD interaction is required for the recruitment of TAF1 to the p21 core promoter and consequent assembly of a complete TFIID complex. Notably, also the recruitment of CBP is mediated through the binding of its single bromodomain to p53 acetylated on Lys382 (Mujtaba et al., 2004). Recruitment of HATs has been shown both to enhance p53 acetylation and to favour the acetylation of histones on p53 responsive promoters thus favouring transcriptional activation.

Interestingly, it has been proposed that DNA-binding is required to allow p53 acetylation by p300. In the absence of DNA the C-terminal domain of p53 is not accessible for acetylation by p300 and this provides an intrinsic negative regulatory mechanism to prevent acetylation until the tetramer is promoter bound. After DNA binding, allosteric effects mediate an exposure of the acetylation motif to allow DNA-dependent acetylation of the tetramer. Notably, the conformational change allows p300 to contact p53 on its PRD and this interaction is essential for DNA-dependent acetylation of p53 (Dornan et al., 2003a). Indeed, upon PRD deletion, p300 can still bind to p53 on its N-terminal LXXLL motif encompassing Thr18-Ser20, but cannot acetylate DNA-bound p53. It has been suggested that the prolyl-isomerase Pin1 may assist structural rearrangements that occur upon DNA binding thus promoting the DNA-dependent acetylation of p53 by p300. This may occur at least in part through phosphorylation induced isomerization of Thr81-Pro82 within the PRD. Indeed, it has been demonstrated that mutations affecting this site, result in impaired acetylation of p53 at Lys 373 and Lys382 (Mantovani et al., 2007). Moreover, Pin1-mediated Pro82 isomerization within the PRD has been shown to be required for
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Ser20 phosphorylation by Chk2 (Berger et al., 2005), which promotes p53 binding to p300 (Dornan et al., 2001). DNA-dependent acetylation of p53 stabilizes p300 binding (Dornan et al., 2003b) and directs the organization of transcription complexes on the p53 bound promoters thus promoting the transcriptional functions of p53.

The acetylation status of p53 can be regulated via deacetylation by an HDAC1 (histone deacetylase 1)-containing complex (Murphy et al., 1999) or by the NAD-dependent histone deacetylase SIRT1 (silent information regulator 2a, Sir2a) (Vaziri et al., 2001). Deacetylation was shown to repress p53-dependent transcriptional activation, apoptosis and growth arrest (Luo et al., 2001; Luo et al., 2000). Moreover, as the lysine residues within the CTD of p53 are also targets for ubiquitination, it was proposed that acetylation of these residues may promote the stabilization of p53 by interfering with proteasomal degradation (Brooks and Gu, 2003). In addition to mediating p53 ubiquitination, Mdm2 also seems to interfere with the acetylation of p53. Indeed, it was shown to inhibit p53 acetylation by p300 and PCAF (Ito et al., 2001; Jin et al., 2002b) and to promote the HDAC1-mediated deacetylation of p53 (Ito et al., 2002). It has been observed that many tumor-associated proteins can mediate p53 deacetylation. The oncogenic form of PML, PML-RAR (Insigna et al., 2004), and the metastasis-associated MTA2/PID (Luo et al.,

Fig. 9. Schematic representation of post-translational modifications occurring on p53 at Lysine residues. Ac, acetylation; M, methylation; Ub, Ubiquitination; N, NEDDylation; SU, Sumoylation. The enzymes mediating the modification are indicated. (Olsson et al., 2007)
2000), have been shown to recruit HDACs to exert their negative control on p53 function. Moreover, the tumor antigen MAGEA2 interacts with p53 and represses its transactivation functions by recruiting HDAC3 to the MAGEA2-p53 complex (Monte et al., 2006). Notably, mouse mutants that express p53 in which the C-terminal 6 or 7 lysines (Feng et al., 2005; Krummel et al., 2005) are mutated to arginine residues, result almost similar to mice with wild-type p53, thus suggesting that lysines in the CTD are not essential for p53 function, but instead fine-tune stress responses (Krummel et al., 2005).

7.5.4. Other modifications affecting Lysine residues (Fig. 9) Lysine residues are also targeted by methyl transferases. Methylation of p53 can occur at least at two different sites, reported to lead to opposing effects on p53 function. Methylation at Lys372 by the methyltransferase Set9, increases the stability of p53, restricts it to the nucleus and enhances p53 dependent transcription, whereas methylation of Lys370, mediated by another methyl transferase, Smyd2, leads to repression of transcriptional activity (Chuikov et al., 2004; Huang et al., 2006). In human cells, the histone lysine-specific demethylase LSD1 interacts with p53 to repress p53-mediated transcriptional activation and to inhibit the role of p53 in promoting apoptosis (Huang et al., 2007). While in vitro LSD1 removes both monomethylation (K370me1) and dimethylation (K370me2) at K370, in vivo, LSD1 shows a strong preference to reverse K370me2, which is performed by an unknown methyltransferase, distinct from Smyd2. Interestingly, methylation at K370me2 has a different role in regulating p53 from that of K370me1: K370me1 represses p53 function, whereas K370me2 promotes association with the co activator 53BP1 (p53-binding protein 1) through tandem Tudor domains in 53BP1. Thus, LSD1 represses p53 function through the inhibition of interaction of p53 with 53BP1. Taken together, these observations show that p53 is dynamically regulated by lysine methylation and demethylation and that the methylation status at a single lysine residue confers distinct regulatory output.

Lysine residues are also subjected to other post-translational modifications such as ubiquitination, sumoylation and NEDDylation. p53 is ubiquitinated by several E3 ubiquitin-ligase among which Mdm2 is the best characterized (see above). Mdm2 can induce both monoubiquitination, leading to nuclear export (Gu et al., 2001) and mitochondrial localization (Marchenko et al., 2007) of p53 and polyubiquitination that leads to degradation by the 26S proteasome (Li et al., 2003).
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Three lysines targeted for ubiquitination (Lys 370, Lys372 and Lys373) are also subjected to NEDDylation. The C-terminal glycine residue of the ubiquitin-like protein NEDD8 is covalently linked to p53 by Mdm2 thus inducing transcriptional repression (Xirodimas et al., 2004). Sumoylation is also similar to ubiquitylation in that an isopeptide bond is formed between the C-terminal carboxy group of the small ubiquitin-like protein SUMO1 and the ε-amino group of Lys386 (Gostissa et al., 1999; Rodriguez et al., 1991). Sumoylation was reported to positively modulate p53 transcriptional activity (Gostissa et al., 1999; Rodriguez et al., 1991). Other data indicate that sumoylation of p53 has a repressive effect (Buschmann et al., 2000b; Chen and Chen, 2003), leaving the issue of whether sumoylation of p53 results in activation or repression of p53 activities controversial. Although the effect on p53 is not completely elucidated several other key regulators of p53 activation are subjected to sumoylation, including PML, Mdm2, HIPK2 and p300 (Girdwood et al., 2003; Kim et al., 1999; Xirodimas et al., 2002). In particular sumoylation of PML is important in the formation of the nuclear bodies (Ishov et al., 1999).

7.6. Regulation by co-factors

An increasing array of co-factors is known to influence p53 activity in different ways. Co-activator and co-repressors may affect p53 transcriptional activity by inducing modifications in chromatin surrounding the p53RE, by mediating or preventing the assembly of the transcriptional machinery or by directing p53 activation towards a particular subset of target genes thus leading to a specific response. It has been suggested that co-factors may also influence p53 loading on its target promoters.

The need for additional partners that assist p53 loading on its target promoters might be of particular importance in the case of genes harbouring low-affinity p53BS, which may fail to be engaged effectively by the limited concentrations of p53 attained under physiological conditions (Szak et al., 2001). It is noteworthy that many, albeit not all, pro-apoptotic p53 target genes harbour p53RE of rather low binding affinity that need elevated and sustained p53 activation. It might be hypothesized that the requirement of specific rate-limiting co-factors may serve well the need to call on the apoptotic option sparingly, only under conditions where death is the best solution, rather than any time that a cell is exposed to a p53-activating stress.
An important role in regulating the apoptotic functions of p53 at the transcriptional level is played by the highly conserved ASPP family of proteins, composed of three members ASPP1, ASPP2 and the inhibitory iASPP that all bind to p53 both on its DNA-binding and its Proline Rich domain (Bergamaschi et al., 2006). Notably ASPP1/2 bind preferentially to the DBD while iASPP to the PRD of p53. The importance of the ASPP family in human malignancies is underscored by the observation that down-regulation of ASPP1 and ASPP2 is a frequent event in human tumors expressing wild-type p53, and to a lesser extent, in tumor cells expressing mutant p53 (Samuels-Lev et al., 2001). Notably, studies conducted on ASPP2+/− and ASPP2+/− demonstrated that ASPP2 is a haploinsufficient tumor suppressor gene that cooperates with p53 in tumor suppression (Vives et al., 2006). ASPP2 is activated upon DNA damage while in the absence of damage, p53 suppresses transcription of the ASPP2 gene. Significantly, after UV treatment the ability of ASPP2 to interact with p53 increases (Samuels-Lev et al., 2001). Notably, ASPP1/2 transcription is up regulated upon oncogenic signalling by the E2F family of transcription factors (Fogal et al., 2005b; Hershko et al., 2005). When ASPP1 or ASPP2 bind to p53, they can specifically enhance the ability of p53 to induce apoptosis but not cell cycle arrest (Samuels-Lev et al., 2001). Chromatin immunoprecipitation assays have shown that the binding of ASPP1 and ASPP2 to p53 can selectively stimulate the loading of p53 on promoters of the pro-apoptotic genes, such as Bax and PIG3, but not to the promoters of the cell cycle arrest gene p21 or the Mdm2 gene (Samuels-Lev et al., 2001). Similarly, the binding of ASPP1 and ASPP2 to the p53 family members, p63 and p73, also enhance their apoptotic function by selectively stimulating their transcriptional activities on pro-apoptotic promoters (Bergamaschi et al., 2004). Moreover, ectopic ASPP2 is predominantly cytoplasmic where it partially co-localises and interacts with Bcl-2. Some evidences suggest that Bcl-2 and p53 are not capable of simultaneously binding to ASPP2, rather Bcl-2 may compete with ASPP2 for p53 binding (Naumovski et al., 1996).

Another member of the ASPP protein family, iASPP (inhibitory member of the ASPP family), an oncoprotein that cooperates with Ras, E1A and E7 to transform cells in vitro (Bergamaschi et al., 2003). iASPP shows opposite effects to ASPP1 and ASPP2, in fact depletion of iASPP expression increases p53-dependent apoptosis while its over-expression inhibits it. Moreover, iASPP is a nuclear protein that competes with ASPP1 and ASPP2 for binding to p53 (Bergamaschi et al., 2003b). Interestingly, iASPP binds preferentially and inhibits the isoform wt p53 Pro72, that indeed shows lower apoptotic potential.
compared with wt p53 Arg72 in cells with elevated iASPP levels (Bergamaschi et al., 2003a; Bergamaschi et al., 2006). It is thus likely that the greater apoptosis-inducing activity of p53Arg72 results from its ability to escape iASPP inhibition in addition to a greater ability to locate to the mitochondria (Dumont et al., 2003). Notably, iASPP is frequently over expressed in tumors retaining wt p53 Pro72 but only rarely in those expressing wt p53 Arg 72. This indicates that there would be no advantage for tumor cells to select for iASPP over-expression in presence of wt p53 Arg72. Conversely, in tumors over expressing iASPP the isoform p53 Pro 72 is rarely mutated, while the p53 Arg72 allele is preferentially targeted for mutations that inactivate its functions (Bergamaschi et al., 2003a; Bergamaschi et al., 2006).

In a report by Das et al. hematopoietic zinc finger (Hzf), a p53 target gene, was identified as a modulator of p53 activity (Das et al., 2007). Hzf is induced by p53 and interacts with its DNA-binding domain, resulting in preferential transactivation of pro-arrest p53 target genes over its pro-apoptotic target genes. It has been demonstrated that Hzf facilitates p53 binding to the p53REs in p21waf1 and 14-3-3σ promoters, while it inhibits p53 loading on Bax, Perp and Noxa genes. Consistently, Hzf knockdown has shown to compromises p53’s ability to promote expression of cell-cycle arrest target genes whilst enhancing the transactivation of pro-apoptotic genes. Hzf expression levels may play a critical role in this decision, in fact following prolonged DNA damage both the total protein levels and the amount of Hzf bound to p53 rapidly declined and this correlated with the up-regulation of pro-apoptotic genes and induction of apoptosis. Notably, Hzf does not influence its own expression levels by acting in a loop with p53 on its promoter. It would be therefore interesting to determine how Hzf protein abundance is regulated and which are the mechanisms through which it determines p53’s choice. As Hzf effects are dependent on its abundance it might be supposed that it competes with other factors for the interaction with p53 and the binding of either leads to different outcomes. Putative competitors of Hzf are ASPP1/2 protein that also interact with p53 DBD and that lead to selective activation of pro-apoptotic genes.

In apparent contrast with the above observations many studies highlighted that there is no shift in p53 loading on promoters that control growth arrest versus those implied in apoptosis upon specific stimuli. Instead, high affinity sites (p21, Mdm2, Puma) are efficiently bound by p53 in conditions leading to either cell cycle arrest or apoptosis, whereas low affinity sites (Bax, Pig3, p53Aip1) are poorly bound regardless of the ultimate
cellular outcome adopted (Kaeser and Iggo, 2002; Szak et al., 2001). This suggested that selective binding of p53 to a promoter is not a determinant for choice of cell fate. It is therefore likely that recruitment of a specific array of transcriptional co-activators dictates selective activation of p53 target genes in response to specific stimuli (Fig. 10).

A number of transcriptional co-activator and co-repressor complexes that bind p53 in cells possess histone modifying activities suggesting that targeted chromatin remodelling may be important for p53 to function as a transcription factor. In fact, although euchromatin (in which most promoters are located) is transcriptionally competent, access of the basal transcriptional machinery to the DNA is severely restricted and transcription of genes located within euchromatin still requires local chromatin remodelling in order to proceed. p53-dependent changes in histone acetylation have been observed at the promoters of a number of p53 target genes (Kaeser and Iggo, 2004; Shan et al., 2003).

The role of CBP/p300 in regulating p53-dependent transcription is well established. CBP/p300 belong to a group of ubiquitous transcriptional co-activators with HAT activity that interact with a plethora of sequence-specific transcription factors (Avantaggiati et al., 1997; Gu and Roeder, 1997; Lill et al., 1997; Scolnick et al., 1997). As described above, the C-terminal region of p53 contains two sites for acetylation by CBP/p300 and one for P/CAF (Liu et al., 1999). Chromatin immunoprecipitation (ChIP) experiments suggest that p53 recruits p300 to the p21 promoter thereby enabling targeted acetylation of chromatin-assembled core histones H2A, H2B, H3 and H4 (Espinosa and Emerson, 2001). Consistently, recruitment of CBP and TRRAP (a component of multiple co-activator complexes with HAT activity) by acetylated p53 correlated with elevated levels of acetylated H3 and H4 at the endogenous p21 promoter (Barlev et al., 2001). In another study, ChIP analysis revealed that variations in p53 ability to activate transcription at the p21 promoter correlate directly with the extent of histone acetylation and levels of p300/CBP (Liu et al., 1999).

Many reports indicate that p300 takes part into multi-component co-activator complexes. The p300 cofactor JMY regulates the physiological response to p53 activation by augmenting p53-dependent transcription and apoptosis (Shikama et al., 1999). JMY and p300 associate in physiological conditions and the p300/JMY complex is recruited to
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Fig 10 Model for p53 transcriptional activation in different chromatin contexts accounting for the differential induction kinetics among subsets of target genes. p53 binding to most response elements does not result in altered gene expression, as only a minor fraction of p53-bound loci display changes in their transcriptional status. Before p53 activation, p53 target loci display many differences, as manifested by different concurring cis-regulatory elements, distinct combinations of histone modifications and DNA methylation (blue, red and turquoise lollipops), even different amounts of preloaded paused RNAP II. These differences impose gene-specific requirements for transcriptional coregulators, such as chromatin modifying and remodeling activities, variants of the Mediator and SAGA complexes, specific subunits of general transcription factors (GTFs), even defined sets of elongation and RNA processing factors. For example, the presence of a repressive histone methylation mark at a given locus would impose the requirement for a specific histone demethylase, whereas the presence of fixed nucleosomes at the promoter would impose a requirement for a chromatin remodeling complex. Alternatively, promoters not carrying preloaded RNAP II will require the recruitment of GTFs and assembly of a pre-initiation complex (PIC) before they could be readily activated. In turn, the requirement for specific subunits of the PIC may be dictated by the core promoter elements (CPEs) present at a given locus (for example, TATAbox, Initiator, downstream promoter element) (blue, red and turquoise barrels). Finally, effective gene activation will require conversion of paused RNAP II into an elongating form, as well as correct processing of the nascent mRNA, all of which may be also regulated in a gene-specific manner. Importantly, the quality of these combinatorial ‘filters’ could vary with the signaling scenario (that is, cell type or type of p53 activating stimuli), as the levels and activity of coregulators in each category could be modulated, thus allowing for great flexibility within the p53 transcriptional program. (Espinosa, 2008)
activated p53 during the stress response. Furthermore, protein isoforms of JMY that arise through alternative splicing display selective activation of distinct p53 target genes (Shikama et al., 1999). In fact an isofrom lacking an N-terminal stretch rich in Prolines (ΔP) showed impaired ability to induce p53-dependent apoptosis but increased G1-arrest. Besides, the tandem tetra-tricopeptide (TPR) co-factor Strap is involved in regulation of the p300/JMY complex formation (Demonacos et al., 2001). As Strap undergoes stress-responsive protein stabilization upon exposure to diverse genotoxic insults it has been suggested that it might be a mediator of the DNA-damage signalling pathway directly communicating with p53 to promote its transcriptional activation by p300 (Demonacos et al., 2004). In further support of the importance of chromatin remodelling-proteins in mediating regulation of p21 transcription, is the observation that p53 and the histone deacetylase HDAC1 are antagonistic regulators of p21 gene expression and that p53 can displace HDAC1 from the p21 promoter (Lagger et al., 2003). Besides p21, also transcriptional activation of Mdm2 promoter is dependent on recruitment of TRRAP (Ard et al., 2002), and correlates with increased histone acetylation suggesting that chromatin remodelling is quite a general feature in regulation of p53-dependent transcription.

p53 also interacts with histone deacetylase complexes in vivo and this markedly influences its transcriptional repression activity. p53 recruits the transcriptional co-repressor mSin3a, bound to HDAC1, to Map4 promoter. This correlates with histone deacetylation and reduced transcription (Murphy et al., 1999). Moreover, the histone deacetylase inhibitor trichostatin A (TSA) can abrogate the ability of p53 to repress transcription of the p53 target genes Map4 and stathmin.

The arginine methyltransferases CARM1 and PRMT1 have also been implicated in p53 transcriptional activity, in that they co-operate with p300 in activating the GADD45 gene and assemble with it on the GADD45 promoter during the p53 response (An et al., 2004). Finally, several subunits of the ATP-dependent remodelling complex SWI/SNF have been shown to bind p53. p53 is able to recruit hSNF and hBRG1 to p21 promoter. While over-expression of hSNF5 and BRG1 stimulated p53-dependent transcription of a reporter construct, dominant-negative forms of hSNF5 and BRG1 repressed transcription, inhibiting p53-induced growth arrest and apoptosis (Lee et al., 2002).

It is apparent from these and other studies that regulation of local chromatin structure, mediated through the varying actions of histone acetyltransferases and deacetylases, histone methyltransferases and chromatin remodelling complexes, is important for p53
function as a transcription factor. This might be a general mechanism occurring at p53-responsive promoters, yet the factors determining the selection between different cellular outcomes, are far less understood.

A recent study (Wei et al., 2005) revealed a mechanism by which growth arrest and survival responses may be selected in respect of cell death. It was shown that upon DNA damage the oncoprotein MUC1 is loaded with p53 on the p53REs in the p21 promoter. Thus, MUC1 co-activates p21 transcription at least in part through recruitment of CBP (and displacing of HDAC1) to the p21 promoter with consequent increase in acetylation of histone H4. Interestingly, MUC1 also binds to Bax proximal promoter in response to stress and attenuates Bax transcription in a p53-independent way. In tumors MUC1 may provide a survival advantage against genotoxic stresses in that activation of MUC1 might contribute to a transient p53-mediated growth arrest, p53-independent suppression of Bax activation and attenuation of the intrinsic apoptotic pathway as it is confirmed by the high incidence of MUC1 over-expression in human tumors.

The molecular mechanism for selective pro-apoptotic promoter activation was concomitantly addressed by another group (Tanaka et al., 2007). A complex experimental approach combining ChIP and sucrose gradient ultracentrifugation followed by mass spectrometry allowed isolating functionally distinct promoter-bound p53 assemblies \textit{in vivo}. This assessed that different promoter-bound p53 multiprotein complexes are present in the cell and that each fraction, identified by differential sedimentation, may contain unique proteins. This study also led to the identification of hCAS/CSE1L as a component of some chromatin-bound p53 transcriptional complexes associated to PIG3, p53AIP1 and p53R2 but not p21, PUMA and 14-3-3σ promoters. hCAS/CSE1L is required in these sites to reduce repressive Lys27 methylation on histone H3 possibly by inhibiting an unknown histone methyltransferase (HMT). A putative model suggests that hCAS/CSE1L co-regulates a subset of genes with p53 and the two proteins function together to facilitate the formation of active chromatin at selected target promoter in human cells.

In another study aimed at investigating the mechanisms of stimulus-specific p53 transcriptional responses leading to cell-cycle arrest or apoptosis it was observed that sustained transcriptional activation of p21 occurs in Nutlin-treated cells that undergo cell-cycle arrest, but not in cells that undergo apoptosis in a stress-specific fashion (Donner et al., 2007). This turned out to depend on differential assembly of the Mediator complex, a multiprotein complex that functions as a coactivator and binds to the C-terminal domain of
RNA polymerase II holoenzyme, acting as a bridge between this enzyme and transcription factors (Bjorklund and Gustafsson, 2005). In particular, CDK8, a subunit of the CDK module of Mediator, proved to function as a stimulus-specific transcriptional co-activator within the p53 transcriptional program. Histone acetylation and association with core Mediator subunits MED1 and MED17 is observed with in the p21 locus under both conditions. In contrast, CDK8, cyclin C and MED12 are recruited to the p21 locus exclusively after Nutlin 3 treatment, when p53-dependent p21 transcription is strongly activated. CDK8 recruitment is also observed during p53-dependent activation of the Mdm2 locus, and the levels of CDK8 occupancy at p53 target genes correlate positively with gene activity. These data indicate that p53 binding to chromatin and p53-mediated histone acetylation is not sufficient for transcriptional activation of p21, suggesting that downstream events define the ultimate transcriptional status of this gene. A recent report (Mattia et al., 2007) reached similar conclusions. Upon hydroxyurea (HU) treatment, p53 showed to be stabilized, to bind to the p21 locus, and to mediate histone acetylation. However, p21 transcriptional activation was drastically impaired in HU-treated cells as compared to cells in which p53 was activated by the DNA-damaging agent daunorubicin. RNA Pol II was effectively recruited to the p21 promoter in response to HU treatment but it failed to elongate into the intragenic region, revealing the importance of post-initiation mechanisms in regulation of p21 transcription.

An elegant study by Espinosa et al. demonstrated that p53 is present before activation on p21 promoter together with different amounts of poised PolII (Espinosa et al., 2003). Although PolII is already loaded on promoters before activation, it is transcriptionally paused and recruitment of elongation factors is needed in order to allow efficient transcription. It has been proposed that in a stepwise process, p53 recognizes high-affinity chromatin binding sites and mediates pre-initiation complex (PIC) assembly at some promoters, but this action is not sufficient to achieve maximum transcriptional activation due to RNA Pol II arrest. After DNA damage, increased p53 activity leads to completion of the transactivation process by stimulating elongation of arrested RNA Pol II. Notably, among p53 target promoters there are differences in RNA Pol II occupancy which might establish different thresholds for activation of each particular gene. Indeed, growth arrest genes like p21 and GADD45 show rapid induction kinetics and require little increase in p53 levels to achieve activation, whereas most pro-apoptotic are expressed with intermediate or late kinetics (Zhao et al., 2000).
Taken together all these evidences suggest that many regulatory forces are involved in p53 transcriptional activation. The signal generated by p53 binding is filtered by the availability of chromatin modifying/remodelling complexes and other coregulators of RNA Pol II activity at a given promoter. Consequently, only a small subset of p53 target genes is expressed in a given context. It might be hypothesized that p53 post-translational modifications provide the means for selective coregulator recruitment. This is also supported by the evidence of a correlation between particular post-translational modifications and specific cellular outcomes. Indeed, besides the well established association of Ser46 phosphorylation with apoptotic response, two recent reports indicated that upon p53 binding to DNA, acetylation of p53 at Lys120 by MOF and TIP60 favors selective activation of apoptotic genes (Sykes et al., 2006; Tang et al., 2006).

### 7.7. Cooperation with other transcription factors

The cooperation between p53 and other transcription factors that interact with discrete DNA-binding sites within different promoters has shown to hugely influence the pattern of gene expression in response to p53. An example of such cooperation is seen between p53 and NF-κB, another transcriptional activator that plays an important role in the regulation of apoptosis. The cooperation between p53 and NF-κB reflects their ability to function together to induce expression of apoptotic target genes regulated by promoters containing both p53- and NF-κB-binding sites, such as that of the death receptor DR5 (Shetty et al., 2005). Similarly, both p53 and Miz are required for the activation of expression of p21WAF1/CIP1 (Seoane et al., 2002; Vousden, 2002).

An important contribution to the regulation of p53 activity is made by E2F1, a transcription factor often implicated in apoptosis. It was previously known that E2F1 associates directly to p53 thus modifying its biochemical properties and that the two cooperate in the induction of apoptosis (Hsieh et al., 2002; Wu and Levine, 1994). In addition, E2F1 can synergize with p53 to activate particular pro-apoptotic genes containing adjacent binding sites for each of these two transcription factors, as best illustrated for the Apaf1 gene (Moroni et al., 2001). This is probably an example of a broader generic mechanism, where the availability of a particular transcription factor is expected to affect selectively the ability of p53 to trigger transcription from genes containing responsive elements for both p53 and that factor. This is further confirmed by the observation that, on the other way
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round, p53 is required for TGF-β response. p53 and SMADs in fact interact on separate cis binding elements on a target promoter thus synergistically activating TGF-beta induced transcription (Cordenonsi et al., 2003). Moreover p53 and Smad proteins have been found to simultaneously occupy overlapping p53- and Smad-regulatory elements (SRE/p53RE) to repress transcription of the onco-developmental tumor marker alpha-fetoprotein (AFP) (Wilkinson et al., 2005; Wilkinson et al., 2008).
8. Bromodomain proteins in transcriptional regulation

Bromodomains comprise an extensive family of evolutionarily conserved protein modules of 110aa that have the ability to bind to acetylated lysines with high specificity (Haynes et al., 1992; Zeng and Zhou, 2002). Bromodomain are found in many chromatin-associated proteins and derive their name from the *Drosophila* protein Brahma, where it was identified first (Elfring et al., 1998; Haynes et al., 1992). The finding that this domain have specific acetyl-lysine binding properties (Dhalluin et al., 1999; Hudson et al., 2000; Jacobson et al., 2000; Owen et al., 2000) highlighted how some protein-protein interaction can be modulated by lysine-acetylation, with broad implications in a wide variety of cellular processes including chromatin remodelling and transcriptional activation (Dyson et al., 2001; Winston and Allis, 1999). Histone acetylation emerges as a central switch that allows interconversion between repressive and permissive chromatin domains in terms of transcriptional competence. Acetylation can loosen chromatin structure by neutralizing the positive charges of histones and attenuating their interaction with (negatively charged) DNA. This possibly increases the exposure of DNA to transcription factors and thus correlates with gene activation (Horn and Peterson, 2002). Consistent with this notion, transcriptional co-activators and co-repressors have been found to posses intrinsic HAT and HDAC activities, respectively (Carrozza et al., 2003; Grozinger and Schreiber, 2002; Sterner and Berger, 2000). Moreover, HATs and HDACs have shown to exert fundamental roles in developmental processes and their deregulation has been linked to the progression of diverse human disorders, including cancer.

Bromodomain containing proteins are important for sequential chromatin modifications by HATs, HDACs and other chromatin remodelling proteins such as Swi2/Snf2, the ATPase subunit of the SWI/SNF complex (Carrozza et al., 2003; Fry and Peterson, 2001; Pazin and Kadonaga, 1997). Some bromodomain proteins in fact present additional domains with specific enzymatic activity, through which they may directly mediate chromatin modifications. Among these p300/CBP (Kraus et al., 1999; Manning et al., 2001), PCAF (Dhalluin et al., 1999), TAFII250 (Jacobson et al., 2000) have HAT activity, the transcriptional silencer Ash1L (Gregory et al., 2007) and the tumor suppressor RIZ1 (Steele-Perkins et al., 2001) have histone methyl transferase (HMT) activity and the SNF2α and β subunits of the SWI/SNF complex have ATP-dependent remodelling functions (Smith and Peterson, 2005). Conversely, other bromodomain proteins such as MTA1 (Nicolson et al., 2003) and ACF1 (Eberharter et al., 2001), that have no catalytic activity,
may mediate communication between chromatin and multiprotein remodelling complexes. These scaffolding functions contribute to epigenetic regulation of transcription through a mechanism called the histone code (Rea et al., 2000; Strahl and Allis, 2000; Turner, 2000), wherein combination of specific modifications of histones, such as acetylation, mediate the recruitment of different chromatin-remodelling machinery.

By recognizing acetylated lysines at the flexible N-terminal tails of histones bromodomains can also contribute at a larger scale, to the physical organization of chromosomes and chromatin domains. An example of this last function is given by Bdf1, a yeast member of the Brd/BET family of double bromodomain proteins, that binds to acetylated histone H4 and imposes a physical barrier between euchromatin and heterochromatin (Matangkasombut and Buratowski, 2003). Brd2 and Brd4, two other members of the Brd/BET family, are instead involved in cell cycle regulation. Brd2 activates cyclinA transcription synergistically with oncogenic Ras leading to increased proliferation (Sinha et al., 2005) while Brd4 arrests cell-cycle progression to S-phase through inhibitory interaction with Replication factor C (Maruyama et al., 2002).

Lysine acetylation is also known to occur on many sequence-specific transcription factors such as p53, c-Myb, and MyoD affecting their DNA-binding activity, association with co-regulator, nuclear localization and stability (Avantaggiati et al., 1997; Barlev et al., 2001; Polesskaya and Harel-Bellan, 2001; Sano and Ishii, 2001). Bromodomain-containing proteins might mediate these processes by interacting with acetylated lysines on transcription complexes. Interestingly it has been recently demonstrated that the acetylation of Lys373 and Lys382 on p53 promotes the recruitment of the TFIID subunit TAF1 to the p21 promoter through its double bromodomain. In this way TAF1 may function as a specific transcriptional co-activator to bridge enhancer-binding proteins to the core transcription machinery (Li et al., 2007a).

An important characteristic of bromodomains-acetyl lysine interaction is its specificity in fact the presence of acetylated lysines is necessary but not sufficient for efficient binding by bromodomains. Indeed, the bromodomain of CBP, but not that of p300 or PCAF, specifically binds to Lys382-acetylated p53 being the flanking residues His380, Lys381 and Leu383 crucial for efficient and selective binding (Mujtaba et al., 2004).

Similar to other protein-interaction modules, such as SH2 domains for phospho-tyrosines, the specific association of bromodomains with acetylated lysines might be a further mean to grant dynamicity and regulability to the interaction networks existing within the cell.
9. The Bromodomain-containing protein Brd7

Brd7 (Fig. 11) is a 75kDa protein, highly conserved from *C. elegans* to humans, that displays a characteristic bromodomain moiety. A Brd7 homolog in *C. elegans* is called C01H6.7 and two stretches of high homology with C01H6.7 are present in the C-terminal portion of Brd7.

Brd7 bromodomain shares the highest similarity to the bromodomains of a human zinc finger protein, Peregrin or BR140, abundant in testes and spermatogonia (Cuppen et al., 1999; Thompson et al., 1994). The gene is located on chromosome 16q12, a region frequently deleted in Nasal-Pharyngeal Carcinoma, an epithelial neoplasm whose pathogenesis is related to Epstein-Barr virus infection and genetic abnormalities. Interestingly these tumors bear wild-type p53 and gain metastatic properties upon p53 mutation (Chan et al., 2002).

Brd7 mRNA is ubiquitously expressed (Staal et al., 2000) and its expression has been found reduced in NPC in respect to normal tissue (Yu et al., 2000). Brd7 localization is predominantly nuclear due to the two bipartite NLS present at the N-terminal portion of the protein within a basic domain rich in lysine residues (Zhou et al., 2006a). Brd7 contains a single Bromodomain located between aa 129 and 239 and that suggests a putative function in chromatin remodelling and transcriptional regulation.

It has been demonstrated that Brd7 co-localizes with hyper acetylated histones H3 and H4 (Peng et al., 2006; Staal et al., 2000) and that binds strongly to histone H3 acetylated on Lys14 while only weakly to non-acetylated or to Lys9 acetylated form. The bromodomain is fundamental for the selective interaction as a mutant depleted of the bromodomain is impaired in binding to acetylated histones (Peng et al., 2006). Brd7 has also been shown to co-localize with active Pol II but not with BRG1, a component of a SWI/SNF chromatin remodelling complex (Staal et al., 2000). Brd7 has no HAT activity and its over-expression does not lead to an increase in histone acetylation thus its involvement in chromatin remodelling at large scale is not likely (Peng et al., 2007). Moreover, Brd7 interacts with
proteins involved in transcriptional regulation such as IRF-2, a transcription factor that binds to ISRE and modulates cell-cycle dependent histone H4 gene expression (Staal et al., 2000) and E1B-AP5, a member of the hnRNP family that negatively regulates transcription (Kzhyshkowska et al., 2003). Thus the interaction with “open” chromatin rather than chromatin modification might be the means by which Brd7 exerts its biological function possibly by mediating the assembly of a functional transcriptional apparatus. Yet, there are no strong evidences confirming this hypothesis.

An involvement of Brd7 in cell cycle regulation is suggested by a report where inducible expression of Brd7 in a Nasal-Pharyngeal carcinoma cell line (HNE1) resulted in inhibition of cell growth and cell cycle progression from G1 to S phase, therefore suggesting that down-regulation of Brd7 found in this tumor type may provide a proliferative advantage. Brd7 induction affected the expression of 13 genes belonging to ras/MEK/ERK and Rb/E2F pathways. Among them P19-INK4D was up regulated while MEK, ERK1/2, cyclinD1 and E2F3 were down regulated (Peng et al., 2007; Zhou et al., 2004). The presence of the bromodomain was demonstrated to be essential for transcriptional inhibition of E2F3 promoter in reporter assays, yet Brd7 could not be detected on the endogenous E2F3 promoter by ChIP assay, possibly suggesting an indirect role for Brd7 in regulation of E2F3 (Peng et al., 2006). Interestingly, in a study aimed at investigating the mechanisms regulating Brd7 gene expression it emerged that binding sites for E2F and the repressive E2F6 are present (together with SP1 binding site) within Brd7 promoter. This suggests that Brd7 might be transcriptionally regulated during cell-cycle and that an auto-regulatory loop may occur among Brd7 and E2F.

Brd7 interacts with two other bromodomain containing proteins, Brd2 and Brd3 (Zhou et al., 2003b; Zhou et al., 2006b). These two proteins belong to the BET family and contain a double bromodomain. While little is known about Brd3 several reports have characterized Brd2 as a serine/threonine kinase that has elevated activity in human leukaemias. Brd2 transforms NIH/3T3 cells and is activated by mitogenic signals. Moreover its over-expression leads to transactivation of the promoters of cyclin D1, cyclin A, cyclin E, and dihydrofolate reductase (dhfr) genes in a manner dependent on Ras signalling, probably through the formation of a complex with E2F1 and E2F2. All these evidence suggest that, together with Brd7, it may play an opposite role in cell cycle-responsive transcription.

In agreement with the anti-proliferative role indicated for Brd7, a recent report showed that Brd7 could block accumulation of β-catenin in the nucleus through up-regulation of α-
catenin (Peng et al., 2007) Indeed, abnormal nuclear β-catenin accumulation is a common event in NPC and this might be a consequence of Brd7 down-expression (Morrison et al., 2004; Zheng et al., 1999)

In contrast, in a different cell context, it has been shown that Brd7 can enhance Wnt signalling. Upon binding to the DIX domain of Dvl-1, Brd7 induces dephosphorylation of GSK-3β at Tyr216. This negatively regulates kinase activity thus favouring β-catenin activation (Kim et al., 2003). The actual mechanism is not known but it has been hypothesized that Brd7 could target a Tyr-Phosphatase to the Dvl-1/Axin/APC/GSK-3β/β-catenin complex to mediate dephosphorylation of GSK-3β. A candidate phosphatase is PTP-BL that has been demonstrated to interact both with Brd7 and with APC (Cuppen et al., 1999; Erdmann et al., 2000). Notably, these data suggest that Brd7 might exert functions also in the cytoplasm, independent of its activities as transcriptional regulator. Therefore, it appears that Brd7 can exert different roles in control of proliferation depending on cell context and further analysis is needed to better define Brd7 biological functions.
AIM OF THE THESIS

The p53 tumor suppressor plays key roles in preventing cancer development, as underscored by the fact that its pathway is compromised to some extent in almost all human tumors. Significant advances have been made in understanding p53 functions, yet a greater insight is needed on the specificity of p53 response.

Aim of this thesis is to understand the molecular mechanisms by which p53 response is finely orchestrated and regulated. The first part of this study will focus on the role of the prolyl isomerase Pin1 in regulating p53 apoptotic activity. In the second part, the functional interaction between p53 and the novel interactor Brd7 will be characterized, as the presence of the bromodomain and evidences from literature make it a promising candidate for modulating the p53 pathway at the transcriptional level.

These studies are conducted in the light to achieving greater knowledge on the regulation of the p53 pathway. This might be of relevance for understanding the tumorigenesis process and could be exploited to design new targeted therapies for cancer treatment.
The prolyl-isomerase Pin1 has been shown to interact with p53 upon phosphorylation induced by DNA damage (Zacchi et al., 2002; Zheng et al., 2002). Six Ser/Thr-Pro are present on p53 sequence, namely Ser33/Pro34, Ser46/Pro47, Thr81/Pro82, Ser127/Pro128, Thr150/Pro151 and Ser315/Pro316. Among these, Ser33, Ser46, Thr 81 and Ser315 are phosphorylated by DNA damage-activated kinases (Bulavin et al., 1999; Bushmann et al., 2001; Hofmann et al., 2001; D’Orazi et al., 2001, Radhakrishnan and Gartel, 2006) therefore generating putative binding sites for Pin1. Pin1 has been shown to generate conformational changes in p53 and to enhance its stability and transcriptional activity (Wulf et al., 2002; Zacchi et al., 2002; Zheng et al., 2002). Both mouse and human cells lacking Pin1 show impaired accumulation of p53 upon DNA damage (Zacchi et al., 2002; Zheng et al., 2002; Mantovani et al., 2004) owing to its inability to efficiently dissociate from Mdm2. Pin1 isomerase activity is in fact required for p53 stabilization (Zacchi et al, 2002). Notably, the residues Thr81-Pro82 within the PRD have been shown to be crucial for Pin1 to promote Chk2-dependent phosphorylation on Ser-20 (Berger et al., 2005), that favors dissociation of the p53-Mdm2 complex (Sakaguchi et al., 1998; Shieh et al., 1997; Unger et al., 1999). In a recent report from our group it has been demonstrated that the conformational change induced by Pin1 on p53 upon prolyl-isomerization promotes p53 interaction with p300. Indeed, p53 mutants unable to bind Pin1 were impaired in binding and acetylation by p300 as compared to wild type p53. Notably, the Thr81-Pro82 site within p53 PRD appeared to be important for p53 mediated acetylation. Although these events are important for general p53 activation, Pin1 appears to be particularly required for p53-dependent apoptosis. Indeed, MEF Pin1<sup>−/−</sup> are strikingly impaired in apoptotic response and in the induction of the pro-apoptotic p53 target genes Bax and Killer/DR5 upon genotoxic-stress and oncogene expression (Zacchi et al., 2002). Thus it was of interest to investigate which critical steps in the activation of p53-dependent apoptotic response are controlled by Pin1.
Pin1 is required for p53-dependent apoptosis in response to DNA-damage in human cells

To verify the requirement of Pin1 for an efficient p53-mediated apoptosis also in human cells, Pin1 expression was depleted in HCT116 human colon carcinoma cells by means of RNA interference. Cells were transfected with two different double-stranded small interfering RNA oligonucleotides targeting Pin1 (RNAi PinII and PinIII) or with a scrambled double-stranded RNA as control (C) and then treated with either 50µM Etoposide or 40 Jm⁻² UV in order to induce the apoptotic response. The percentage of apoptotic cells was estimated after 18 hrs by AnnexinV/Propidium Iodide (PI) staining and FACS analysis. In order to discriminate p53-dependent effects, both HCT116 wt and an isogenic cell line lacking the p53 gene (HCT116 p53⁻⁻) were analyzed in parallel assays.

As shown in Figure 12, Pin1 appeared to be required for p53-mediated apoptosis, as upon its depletion in HCT116 wild type cells a significant impairment in stress-induced apoptotic response was observed. Conversely, no significant difference could be appreciated in HCT116 p53⁻⁻ cells upon Pin1 silencing with respect to control-treated cells, thus indicating that Pin1 depletion affects apoptosis in a p53-dependent manner.

Fig. 12 Analysis of the effect of Pin1 depletion on p53-dependent apoptosis.
HCT116 cells were transfected with Pin1-specific siRNA (I) or (II), or control siRNA (C) before treatment with UV (40 Jm⁻²) or Etoposide (Et) (50µM). The percentage of apoptotic cells was estimated after 18hrs by annexin V/propidium iodide staining and FACS analysis. Graphs show means and standard deviations from three independent experiments. *P*-values are relative to RNAi C data.
Pin1 is required for dissociation of the iASPP-p53 complex upon DNA damage

It was of interest to investigate the mechanisms by which Pin1 could specifically assist p53 in mediating apoptosis. It is known from literature that the inhibitory factor iASPP leads to downregulation of p53-mediated apoptosis by interacting with p53 both on its DBD and, with higher affinity, on its PRD (Bergamaschi et al., 2006). Yet, the mechanism by which this inhibition is relieved upon stress stimuli that induce p53 apoptotic response is not clear. Interestingly, it has been demonstrated that iASPP interacts preferentially with the polymorphic variant p53-Pro72 as compared with p53-Arg72. Besides the effect of the aminoacid substitution on direct contact between the iASPP SH3 domain and p53 PRD predicted by docking analysis (Bergamaschi et al., 2006), this might also depend on different conformation of the two isoforms, that has been suggested by their different electrophoretic mobility (Matlashewski et al., 1987). Since a Pin1 binding site within the PRD has been shown to be important for p53 activation (see above), it was hypothesized that a conformational change induced by Pin1 on p53 upon cytotoxic stress, might affect the p53-iASPP interaction. To test this hypothesis a co-immunoprecipitation assay of endogenous p53 and iASPP proteins was performed by F. Mantovani in the lab upon blocking Pin1 expression by RNA interference in U2OS cells either treated with UV radiation or not. In order to compare similar input levels of p53 in different conditions, p53 was stabilized in unstressed cells with the CBZ proteasome inhibitor. Immunoprecipitation was performed with a polyclonal anti-iASPP antibody and the immunoprecipitated complexes were analyzed for p53 presence by western blot with anti-p53 monoclonal antibody (DO-I). Interestingly, in cells transfected with control siRNA (C) the interaction between p53 and iASPP was decreased upon DNA damage (Figure 13). This suggests that pro-apoptotic stress stimuli induce dissociation of p53 from iASPP in order to achieve an effective apoptotic response. Most notably, in the absence of Pin1 stress-induced dissociation of p53 from iASPP was impaired, indicating that Pin1 is required for this process. Similar results were also observed upon Etoposide treatment (data not shown).
Fig 13. Analysis of the effect of Pin1 depletion on the dissociation of p53 from iASPP upon cytotoxic stress
The interaction of endogenous p53 and iASPP proteins was analyzed by co-immunoprecipitation (IP). U2OS cells were transfected with Pin1-specific siRNA (Pin1) or control (C) siRNA and treated with either cytotoxic UV dosage (40 J/m²) or with the proteasome inhibitor CBZ as a control. Immunoprecipitation of endogenous iASPP was performed with a polyclonal anti-iASPP antibody. Co-immunoprecipitated p53 was analyzed by western blot with monoclonal anti p53 antibody (DO-1). An aliquot of each protein lysate used for IP (5% of input) was also loaded as a control.

Pin1 directly mediates dissociation of p53 from iASPP

To understand whether the effect of Pin1 on p53 dissociation from iASPP requires direct interaction with p53, p53 mutants in Pin1 binding sites were employed.

To verify that individual consensus binding sites for Pin1 are effectively recognized by Pin1 when phosphorylated, mutant p53 proteins with only one intact Pin1-consensus site (p53-5M: Ser33-wt, Ser46-wt, Thr81-wt, or Ser315-wt) were generated. This was obtained by disrupting the consensus sites for Pin1 binding (Ser/Thr-Pro) outside the DBD, that are known to be phosphorylated by stress-activated kinases, through substitution of the Ser/Thr residue to Ala by means of site-directed mutagenesis. A schematic representation of the constructs is given in Figure 14A. The p53-5M proteins were then expressed in H1299 cells
and tested for binding to Pin1 in a GST pull-down assay. The mutant p53-6M, where all Pin1 consensus sites are mutated, was used as negative control (the construct for p53 6M was generated by A.Bisso in the lab). Cell lysates were incubated with either GST-Pin1 (or GST alone as negative control) bound to Glutathione-sepharose beads. Parallel experiments were also performed by treating protein lysates with λ-phosphatase prior to GST-pulldown. Proteins associated with the beads were loaded on a SDS-PAGE gel and analyzed for the presence of p53 by western blot with a monoclonal antibody (DO-1). The result indicated that all the sites examined are bound by Pin1, however this binding is lost when these sites are dephosphorylated (Figure 14B).

Therefore, a mutant at all four Pin1 binding sites outside the DBD (p53 4M) was constructed by site-directed mutagenesis as previously described (performed by F.Agostini in the lab). And is shown in Figure 14A. p53 4M and p53 6M were then compared for binding to p53. As expected, it was observed that substitution of Ser33, Ser46, Thr81 and Ser315 to alanine (p53-4M) greatly reduced the binding of p53 to glutathione S-transferase (GST)-tagged Pin1 upon stress. The interaction was completely abolished when Ser127 and Thr150 were additionally mutated to alanine (p53-6M) (Figure 14C) Notably, the residual binding of p53 4M to GST-Pin1 indicates that at least one of the two Pin1 consensus sites located within the DBD (Ser127 and/or Thr150) is also bound by Pin1.

Then, the ability of p53-4M and p53-6M to bind to iASPP was compared to that of p53 wt upon stress treatment. To do this, H1299 cells were transiently transfected with either p53wt, p53-4M or p53-6M expression constructs and treated with etoposide in order to stimulate the dissociation of p53 from iASPP. Upon immunoprecipitation of endogenous iASPP from cell lysates, immunoprecipitated complexes were analyzed by western blot with anti-p53 antibody. As shown in Figure 15A it was observed that p53 mutants impaired in binding to Pin1 displayed a stronger interaction with iASPP as compared to wt p53 upon stress stimuli. This suggests that Pin1 might be directly involved in dissociation of p53 from iASPP.
Subsequently, the ability of p53-4M and p53-6M mutants to induce apoptosis was compared with that of wild-type p53. SaOS-2 p53-null cells were transiently transfected with either wild type p53 (p53-wt), p53-4M or p53-6M expression constructs. pCDNA3 empty vector was also transfected as control. The percentage of apoptotic cells was evaluated by Annexin V/PI staining and FACS analysis. The result presented in Figure 15 B showed that the ability of p53-4M and p53-6M to promote apoptosis was greatly impaired with respect to that of p53-wt, consistent with the different capacity to dissociate from iASPP. Interestingly, the ability to promote apoptosis of p53 mutants was decreased proportionally with their ability to bind to Pin1.
Ser46 is required for Pin1-mediated regulation of p53-iASPP interaction

To identify which Pin1 binding site(s) is directly involved in mediating p53 dissociation from iASPP, we decided to focus on Ser46 and Thr 81. Ser46 has been largely demonstrated to play a key role in p53-mediated apoptotic response as phosphorylation at this site is specifically associated with the induction of pro-apoptotic genes. Thr81 is located within the PRD where the main iASPP binding site lies and is important for Pin1 induced activation of
p53. We speculated that a conformational change at this site could be the means by which Pin1 mediated p53 dissociation from iASPP. To indagate the involvement of Ser46 and Thr81 in the dissociation of p53 from iASPP, H1299 cells were transfected with either wild-type p53 or with two point mutants at Ser46 or Thr81 where Ser or Thr were substituted with Ala (p53-S46A and p53-T81A). Cells were then treated with etoposide to stimulate iASPP dissociation and co-immunoprecipitation with iASPP was performed as described above. As can be observed in Figure 16 A, p53-S46A appeared to bind to iASPP more strongly as compared to wt-p53. In contrast p53-T81A bound iASPP to the same extent as wt-p53. This suggests that phosphorylation at Ser46 might reduce the interaction with iASPP.

In order to confirm these results and to prove that Pin1 is able to induce p53 dissociation from iASPP upon stress-dependent phosphorylation of Ser46, co-immunoprecipitation of wt-p53, p53-S46A or p53-T81A with iASPP upon overexpressing Pin1 was performed. (This experiment was performed by F.Mantovani). As reported in Figure 16B the overexpression of Pin1 resulted in an increased dissociation of wt p53 and p53-T81A from iASPP while it had only a slight effect on p53-S46A. These results together indicate that Ser46 is required for Pin1-mediated regulation of p53-iASPP interaction.

**Fig 16. Analysis of the requirement of specific Pin1 binding sites to mediate p53 dissociation from iASPP**

A. H1299 cells were transfected with p53wt, p53 S46A or p53 T81A and treated with Etoposide 10µM for 12 hrs. The lysates were immunoprecipitated with anti-iASPP polyclonal antibody. Bound p53 was detected by western blot with DO-1 monoclonal antibody

B. H1299 cells were transfected with either wt-p53 or mutant S46A or T81A p53, and with vector encoding HA-Pin1 (+) or empty vector (−) and treated with UV (40 Jm−2). After 12 hrs, the interaction of p53 with endogenous iASPP was analyzed by co-immunoprecipitation with anti iASPP polyclonal antibody and western blot with anti-p53 (DO-1) and anti-iASPP monoclonal antibodies.
Results previously obtained in the lab (Mantovani et al., 2007) have demonstrated that Pin1 directly enhances the acetylation of p53 at Lys382 and Lys373 by p300. This evidence, together with the data from literature indicating that phosphorylation at Ser 46 is required for the acetylation of p53 at Lys 382 by CBP (Hofmann et al., 2002), lead to hypothesize that Pin1-mediated dissociation of p53 from iASPP might depend on the increased acetylation of p53. To test this possibility, a co-immunoprecipitation was performed by comparing the binding of iASPP to p53-wt and a p53 mutant where the C-terminal lysines had been mutated to arginines (p53-9KR, described in Ard et al. 2002). p53-wt and p53-9KR expression constructs were transiently transfected in H1299 cells and p300 was also transfected in order to induce p53 acetylation. The acetylation status of p53 was evaluated by western blot with a polyclonal anti-AcK382 antibody. Co-immunoprecipitation was performed with anti iASPP monoclonal antibody and with an unrelated antibody as a control. As shown in Figure 17 it was observed that iASPP could bind to the same extent to both wild type p53 and non-acetylable p53-9KR (compare lanes 2 and 4) and that acetylation of p53 by p300 did not alter its binding to iASPP (compare lanes 2 and 3). These results indicate that the interaction between p53 and iASPP occurs irrespectively of p53 acetylation on C-terminal lysines. Therefore it can be concluded that Pin1-dependent acetylation of p53 and its dissociation from iASPP are independent events.

**Fig. 17 Analysis of the effect of p53 C-terminus acetylation on iASPP binding.**
H1299 cells were transfected with either wild-type (WT) or 9KR p53 and with GFP-p300 as indicated. The ability of wt and p53-9KR to interact with endogenous iASPP was then compared by co-IP. C: control IP with non-immune serum. Expression of GFP-p300 and acetylation of p53 on Lys382 were analyzed by western blot.
The overall picture of p53 regulation is highly complex yet the discovery of novel co-factors that modulate its functions allows to gain insights on the multiple regulatory levels within the p53 pathway. In order to increase the knowledge on p53 family members’ regulation by analyzing their interaction profiles, a yeast two-hybrid screening of a human foetal brain cDNA library has been previously performed in our laboratory. Wild-type p53, mutant p53R175H, p63 and p73, deleted of their transactivation domains were used as baits. Among the protein partners identified, the Bromodomain containing protein Brd7 demonstrated the capability to interact with all the three members of the p53 family and with mutant p53 R175H. The presence of the bromodomain and the evidences from literature that support a role in transcriptional activation rendered Brd7 an appealing candidate for modulating the p53 pathway.

Analysis of Brd7 expression levels and half-life

Brd7 has been reported to be ubiquitously expressed in mouse tissues (Cuppen et al., 1999). Brd7 expression levels were thus analyzed by western blot in human cell lines of different origin, either transformed or not, that were commonly used in our lab. As shown in Figure 18 it was observed that Brd7 protein could be detected in all cell lines analyzed, with lower expression levels in all primary (WI38) and immortalized (IMR90, HaCat) fibroblasts tested as compared to tumor-derived cell lines (MCF-7, SkBr3, MDAMB468, HeLa, SHSY5Y, 293T, RKO, U2OS, HCT116, H1299, SaOS2 and HL60). Then, HCT1116 human colon carcinoma cell line, expressing high levels of Brd7 protein, was chosen to analyze Brd7 protein stability. Brd7 half life was estimated by means of blocking protein synthesis in HCT116 cells by treatment with ciclohexymide 50µM (CHX) and analyzing the amount of Brd7 protein remaining at different time points by western blotting. p53 half life, known to be of about 3-4 hours in the tumor cell lines examined was also evaluated as a control. As shown in Figure 19, Brd7 protein is very stable, as its protein levels declined only upon 18 hours of CHX treatment and were reduced to a half after 24 hours. Similar results have been obtained also in U2OS osteosarcoma cells (data not shown).
Fig. 19 Analysis Brd7 protein expression and stability
A. Analysis of Brd7 protein levels in different cell lines. Total lysates from various primary cells and immortalized or tumor-derived cell lines were analyzed for Brd7 expression by western blotting.
B. Analysis of Brd7 stability. A time course upon CHX treatment was performed in HCT116 cell lines. Cells were treated with CHX 50 μM and harvested at the time points indicated. Brd7 and p53 protein levels were analyzed by western blotting. Actin was used as a control.

Since the Brd7 promoter contains several E2F binding sites it was sought to test whether Brd7 expression may be influenced by E2F transcription factors. To this aim, analysis of Brd7 mRNA levels upon E2F1 induction was performed. An U2OS/ER-E2F1 inducible cell line, expressing inducible E2F1 in fusion with estrogen receptor ligand binding domain (ER) (kindly provided by K.Helin, Muller et al. 2001) was employed. Cells were treated with 4-hydroxy-tamoxyfen (4OHT) for 24 hours to induce E2F1 activity or left uninduced as control. Upon extraction of total mRNA and retro-transcription, RealTime PCR amplification was performed with primers specific for Brd7 coding sequence and GAPDH as a control. The result reported in Figure 20 indicated that Brd7 mRNA levels increase of about two-fold 24 hrs after E2F1 induction.
RESULTS - Part2

Figure 20. Analysis of the induction of Brd7 expression by E2F1.
U2OS/ER-E2F1 cells were treated with 4-hydroxy-tamoxifen (4OHT) for 24 hours to induce E2F1 activity or left uninduced as control. Brd7 mRNA levels relative to GAPDH were analyzed by Real Time RT PCR.

Brd7 acetylation and oligomerization

Analysis of Brd7 protein sequence by means of the PROSITE protein motif database predicted the presence of a Lysine-rich domain at its N-terminus (aa 3-91) which might be subjected to acetylation. With the aim of determining whether Brd7 is acetylated, pCDNA3-Flag Brd7 expression vector was overexpressed in H1299 cells and purified by immunoprecipitation. Subsequently, western blot analysis was performed with a monoclonal antibody that specifically recognizes acetylated lysines (AcK). To discriminate background signal the p53 non-acetylatable mutant p53 9KR (see above) was used as a negative control. The result presented in Figure 20 shows that Brd7 is indeed subject to acetylation.

Since lysine acetylation can provide a means for recognition and binding by bromodomain motifs, it was hypothesized that upon acetylation Brd7 might oligomerize through its bromodomain. To verify this possibility, in vitro pull-down assays were performed by incubating full-length Brd7 transcribed and translated in vitro in the presence of 35S-methionine (IVTT) with bacterially expressed MBP-fused N-terminal portion of Brd7 containing amino acids 1-236 and comprising the bromodomain (MBP Brd7 1-BD), schematically represented in Figure 21B (upper panel). The result presented in figure 21B demonstrates that Brd7 is able to oligomerize through its N-terminal domain.
Fig 20. Analysis of the acetylation and oligomerization of Brd7
A. H1299 cells were transiently transfected with either pCDNA3 Flag Brd7 or p53 9KR expression constructs. Upon immunoprecipitation with specific polyclonal antibodies, the acetylation status of Brd7 and p53-9KR was evaluated by western blot with a monoclonal antibody recognizing acetylated lysines (AcK). (The non-acetylable p53-9KR was employed as negative control)

B. (Upper panel) Schematic representation of the MBP-fusion protein used in the MBP pull down assay. (Lower panel) Full length Brd7 expression construct was transcribed-translated in vitro (IVTT) in the presence of 35S-Met, and its interaction with MBPBrd7 1-BD was estimated by MBP pull down. 10 μl of the IVTT reaction were assayed for interaction with a recombinant protein encoding the N-term portion of Brd7 up to the bromodomain in fusion with MBP (MB-Brd7 1-BD) or MBP as control. Bound Brd7 was detected by autoradiography. The amounts of MBP proteins employed in the experiment were evaluated by Coomassie staining (right panel)
Kzhyskowska et al. showed that Brd7 is able to bind to acetylated histones *in vitro* by means of its bromodomain. Based on these data and on its prevalent nuclear localization, observed by immunofluorescence microscopy upon overexpression of pCDNA3-Flag Brd7 in U2OS cells (Figure 22A), it is likely that Brd7 might be associated to chromatin in cells. To test this hypothesis, chromatin-bound and free protein fractions were prepared by biochemical fractionation from HCT116 wt cells. Cells were either treated with Etoposide or left untreated in order to evaluate whether Brd7 localization might be influenced by stress stimuli. To control fractionation efficiency, HMGA1b and tubulin were evaluated as markers for chromatin bound and free fractions respectively. The result presented in Figure 22B shows that Brd7 localizes almost completely in the insoluble fraction thus confirming its association with chromatin. Notably this localization is unchanged in normal growing conditions and upon stress.

**Fig 22. Analysis of Brd7 nuclear localization and association with chromatin.**
A. U2OS cells were transiently transfected with pCDNA3-Flag Brd7. After 24 hrs Brd7 localization was evaluated by immunofluorescence microscopy using anti-Flag monoclonal antibody and FITC-conjugated anti-mouse antibody. B. Chromatin-bound and free fractions were obtained by biochemical fractionation of total lysates from HCT116 cells, either treated with 50 µM Etoposide (Et) for 12 hrs or left untreated. Brd7 localization within the two fractions was determined by western blot. Western blot of HMGA1b and Tubulin were used as controls for fractionation efficiency.
Characterization of the interaction between Brd7 and the p53 family proteins.

Validation of the binding between Brd7 and the p53 family proteins in vitro

Brd7 was identified as a common interactor of all the three members of the p53 family and of mutant p53 (R175H) in a yeast two-hybrid screening conducted in our laboratory. The interaction was observed in three independent screenings using as a bait respectively p53, mutp53 (R175H) or p73α lacking the N-terminal domain and was subsequently confirmed also for p63 in secondary screening.

To confirm the interaction of Brd7 with full-length p53 family proteins an MBP-pull down was performed between IVTT human Brd7 and bacterially produced MBP-fused full length p53, p63 and p73. The result shown in Fig. 23 demonstrated that Brd7 is able to interact in vitro with all the p53 family members.

Fig.23 Analysis of the interaction between Brd7 and the p53 family members

A. Full length Brd7 was in vitro transcribed-translated (IVTT) in presence of 35S-Met, and their interaction with the p53 family members was estimated by MBP pull down. 10 µl of each IVTT reaction were pulled down with MBP-fused p53, p63 or p73 recombinant proteins. Bound Brd7 was detected by autoradiography (upper panel). The amounts of MBP proteins used in the experiment were evaluated by Coomassie staining (lower panel)
Mapping of the binding between Brd7 and the p53 family members

To identify the regions of Brd7 that are involved in the interaction with the p53 family proteins, three Brd7 mutant constructs were produced. ΔBD-Brd7, lacks the bromodomain (aa 129-239), Brd7-1-BD bears an N-terminal portion of Brd7 comprising the amino acids from 1 to 239 and containing the bromodomain, and Brd7-381-562 bears the portion of Brd7 used in the yeast two hybrid screening (aa 381-562) (represented in Figure 24A). FL-Brd7, ΔBD-Brd7 Brd7-1-BD and Brd7-381-651 were transcribed and translated in and tested in pull-down assay with MBP-fused full length p53, p63 and p73 and MBP alone as a control. Taken together, the results shown in Figure 24B disclose the presence of two binding sites for the p53 family members on Brd7, one residing within the 129 N-terminal residues and the other mapping between residues 381 and 562. Notably, the bromodomain does not appear to be required for the interaction as its deletion did not alter the extent of the binding.

Fig. 24 Mapping of the binding of Brd7 to the p53 family members
A. Schematic representation of the IVTT proteins employed in the experiments  B. Full-length Brd7, ΔBD-Brd7, Brd7-1-BD and Brd7-381-562 were transcribed-translated in vitro (IVTT) and tested in MBP pull-down for binding to MBP-p53, MBP-p63, MBP-p73 or MBP as a control. Bound IVTT proteins were detected by autoradiography. The amounts of MBP proteins used were evaluated by coomassie staining (right panel).
To confirm that binding to p53 involves two sites on Brd7 two MBP-fusion deletion constructs were generated: MBP-Brd7-NT, comprising aminoacids 1-361 and containing the bromodomain and MBP-Brd7-CT, spanning aminoacids 361-651 and containing the interacting portion identified in the yeast two hybrid assay (schematized in Figure 25A). These were tested for binding to IVTT p53. As can be seen in figure 25B, both MBP Brd7 NT and MBP Brd7 CT were able to bind to p53, thereby confirming that each portion contains a binding site for p53. Interestingly, the N-terminal half of Brd7 appeared to interact with p53 with higher affinity as compared to the C-terminal one.

Due to difficult purification, MBP-fused full-length Brd7 protein could not be used at this stage.

Fig. 25. Mapping of the binding of Brd7 to the p53 family members
A. Schematic representation of the MPB-fusion proteins used in the MBP pull down B. A construct expressing full-length p53 was in vitro transcribed-translated (IVTT) in the presence of 35S-Met, and the interaction with MBP-Brd7-NT and MBP-Brd7-CT was estimated by MBP pull down and detected by autoradiography. The amounts of MBP proteins used in the experiments were evaluated by coomassie staining (lower panel).
To start defining more in detail the regions of p53 involved in the binding to Brd7, MBP pull-down assays were performed with different N-terminal and C-terminal deletions of p53 (schematically represented in Figure 26C).

MBP Brd7 NT and MBP Brd7 CT were tested for binding to \[^{35}S\]-labeled IVTT p53 full-length (p53 FL), p53 1-298 (deleted of the oligomerization domain and the C-terminal domain), p53 1-175 (encoding the N-terminal 175 residues) and p53 175-393 (deleted of the first 175 residues). MBP Brd7 NT was also tested for binding to p53 251-393 (deleted of the first 251 residues).

The results are shown in Figure 26. From these data, it was possible to conclude that p53 C-terminal aa 298-393 are involved in the interaction with Brd7 N-terminal part, containing the bromodomain, while p53 N-terminal aa 1-175 are involved in the interaction with Brd7 C-terminal portion.

As the region of Brd7 containing the bromodomain interacts with p53 C-terminus, where most of the lysine residues known to be acetylated reside, it was hypothesized that this interaction might be mediated by the bromodomain through binding to the acetylated lysines of p53. To assess the role of p53 acetylation in this interaction, Brd7 binding to p53-wt and the p53-9KR non acetylatable was compared by pull-down. Quite surprisingly, p53-9KR appeared to be still able to bind both Brd7-NT and Brd7-CT, thus suggesting that acetylation of p53 is not required for binding to Brd7 (data not shown).
Figure 26: Mapping of the domains involved in the interaction between Brd7 and p53

Constructs expressing full-length p53 or p53 deleted of increasing portions at its C-term or N-term (depicted in C) were in vitro transcribed-translated (IVTT) in the presence of $^{35}$S-Met, and their interaction with (A) the recombinant protein encoding either the N-term portion (MBP Brd7 NT) or (B) the C-terminal portion (MBP Brd7 CT) of Brd7 in fusion with MBP was estimated by MBP pull-down and detected by autoradiography. The amounts of MBP proteins used in the experiments were evaluated by Coomassie staining (lower panels)

C Schematic representation of the p53 deletion mutants and summary of their interaction with MBP Brd7 NT and MBP Brd7 CT
Analysis of the interaction between Brd7 and the p53 family members in human cells.

Co-immunoprecipitation assays were performed on cell lysates from p53-null H1299 cells upon over expression of wt p53, mutant p53R175H, p63α or p73α together with Flag-tagged Brd7 or pCDNA3 as negative control. The lysates were immunoprecipitated with an anti-Flag antibody hence analyzed by western blot with antibodies specific for p53, p63 and p73. Results shown in Figure 27 confirmed that Brd7 interacts with p53 (A), mut p53 R175H (B), p63 (C) and p73 (D) also in cells.

**Fig. 27** Analysis of the interaction between Brd7 and the p53 family members in human cells

H1299 cells were transiently transfected with either p53wt (A), mutant p53 R175H (B), p63 (C) or p73 (D) together with Flag-tagged Brd7. Brd7 was immunoprecipitated by means of the monoclonal antibody α Flag. Lysates of immunoprecipitated proteins were analyzed by SDS-PAGE followed by western blotting. Immunoprecipitated Brd7 was detected with anti-Flag antibody. Co-precipitated p53 (wt and mutant R175H), p63 and p73 were analyzed with the corresponding polyclonal antibodies. An aliquot of total lysate (5% input) was also loaded and analyzed by western blot.
The ability of Brd7 to interact with wild-type p53 and mutant p53 R175H was directly compared by co-immunoprecipitation upon transient overexpression in H1299 cells of either p53 wt or mutant p53 R175H together with Flag-tagged Brd7. As shown in Figure 28, Brd7 appeared to bind more strongly to mutant p53 R175H with respect to wild type p53. Thus it might be hypothesized that the altered conformation of p53 R175H leads to the disclosure of a stronger binding site for Brd7.

Finally, to assess that the binding of Brd7 to p53 occurs in cells among endogenous proteins a co-immunoprecipitation of Brd7 and p53 from HCT116 cell lysates was performed. To this purpose an anti-Brd7 polyclonal antibody was produced (described in Materials and Methods section) and used for co-immunoprecipitation. The result presented in Figure 29 confirms that endogenous Brd7 and p53 interact in cells.
**Analysis of Brd7 biological functions**

Having assessed that Brd7 binds to p53 it was then sought to analyze its possible role in regulating cell cycle progression upon stress stimuli that induce p53 activation. Data from literature had in fact suggested an involvement of Brd7 in cell cycle regulation as inducible overexpression of Brd7 in a Nasal-Pharyngeal carcinoma cell line endogenously expressing very low levels of Brd7, resulted in inhibition of cell cycle progression from G1 to S phase (Zhou et al., 2004).

To this purpose, Brd7 expression was depleted in U2OS cells and the effect on cell proliferation was analyzed by evaluating the percentage of BrdU incorporation both in unstressed conditions and upon challenging with the DNA-damaging drug Etoposide. As expected, Etoposide treatment led to a reduction in the percentage of proliferating cells. This decrease was much reduced upon blocking Brd7 expression by means of RNA interference. In contrast, Brd7 depletion did not affect proliferation in unstressed cells (Figure 30). This indicates that Brd7 is required for stress-induced cell-cycle arrest. Western blot analysis of cell lysates from this experiment indicated that the effect of Brd7 silencing on cell proliferation was accompanied by a reduction in p21 and p53 protein levels (Figure 31B).

**Fig. 30 Effect of Brd7 depletion in U2OS cells**

U2OS cells were transfected with either Brd7 siRNA1 (B) or control RISC FREE siRNA (C). 50 μM Etoposide (Et) or DMSO (-) was administrated for 6 hrs and then cells were incubated with BrdU for 2 hrs. The effects of Brd7 ablation on S-phase entry were then evaluated by counting BrdU incorporating cells (A) The values and the bars represent the mean and s.d. of three independent experiments respectively. The expression levels of Brd7, p53 and p21 were evaluated by western blot (B)
To verify that the reduction observed on p21 protein levels upon using Brd7 small interfering RNA (siRNA B1) was a specific effect of Brd7 depletion, a different siRNA was tested (siRNA B2). As can be seen in Figure 31, a reduction in p21 levels could be observed with both siRNAs thus indicating that there was no off-target effect. siRNA B1 was then used to perform the experiment described below.

To investigate whether Brd7 affects the induction of p21 at the transcriptional level, Brd7 expression was blocked by RNA interference in U2OS cells either treated with etoposide or left untreated. Real Time RT-PCR of different p53 target genes was then performed, using GAPDH housekeeping gene mRNA levels as control. The result shown in Figure 32 highlighted that induction of p21 transcription upon DNA damage was reduced when Brd7 expression was blocked by means of RNA interference. In contrast, there was only little variation in Mdm2 and Puma mRNA levels. Under normal conditions, slight inhibition of p21, Mdm2 and Puma transcription was observed.
Fig 32 Brd7 is required for induction of p21 transcription upon DNA-damage in U2OS cells

U2OS cells were transfected with either Brd7 siRNA (B) or RISCFree control siRNA (C) and Etoposide (50 μM) or DMSO were administrated to cells 18 hrs before harvesting. (A) The amount of p21, Mdm2 and Puma mRNA was evaluated by Real Time RT-PCR and normalized with respect to GAPDH mRNA (housekeeping gene control) by ΔΔCT method. The values and the bars represent the mean and s.d. of three independent experiments respectively. (B) Brd7 silencing efficiency was evaluated by western blot analysis.

Then it was investigated whether the effect of Brd7 on p21 transcriptional activation is p53-dependent. To this aim p53 was either overexpressed or not in p53-null H1299 cells and Brd7 expression was inhibited by RNA interference. Then Real Time RT-PCR of p21 was performed. As can be seen in Figure 33, p21 mRNA levels are upregulated upon overexpression of p53. This upregulation is reduced when Brd7 expression is depleted by RNA interference. Notably Brd7 silencing has no effect on p21 expression in the absence of p53, thus indicating that the downregulation of p21 upon Brd7 silencing is p53-dependent.
It is noteworthy that the above experiments (see western blots in Figures 30B and 32B) highlighted a reduction in p53 levels upon Brd7 depletion. Preliminary data indicated that this reduction does not occur at the transcriptional level, as p53 mRNA levels do not vary upon block of Brd7 expression by means of RNA interference as compared to mock treated control (not shown). This suggests that Brd7 depletion might influence p53 stability and that the effect on p21 expression might, at least in part, be exerted through reduction of p53 levels.

In order to confirm the effect of Brd7 on p21 transcription and to determine whether this effect involves regulation of induction of the p21 promoter by p53 a luciferase reporter assay was conducted. p53-null H1299 cells were co-transfected with constructs containing p21 or Bax promoter upstream of the luciferase gene (p21-prom-luc and Bax-prom-luc) along with p53 expression vectors and with either Brd7 siRNA or control siRNA. However in these conditions no significant variation neither in p21 nor in Bax promoter induction could be observed upon Brd7 depletion (not shown).

A luciferase reporter assay was also performed upon overexpression of increasing amounts of Brd7 together with p53 or pCDNA3 (Figure 34). From the result showed in Figure 34 it appeared that Brd7 over-expression does not significantly alter p53 transcriptional activity towards p21 promoter. Interestingly, it instead caused a reduction of Bax promoter trans-activation by p53. This suggests that Brd7, when present at high levels, might participate in transcriptional repression of Bax promoter.
The absence of an effect observed on induction of p21 promoter by luciferase assays upon either Brd7 silencing or overexpression, might be due to the fact that in this experimental system the p21 promoter is not present in its normal chromatin context, where Brd7 is supposed to exert its functions. From the evidence indicating that Brd7 is bound to chromatin in cells (Figure 22B) it was hypothesized that it could exert a role in p21 transcriptional regulation at the chromatin level. To verify this hypothesis, it was investigated whether Brd7 is localized on the endogenous p21 promoter at the upstream (-2283) high affinity p53-binding site. To this aim, chromatin-immunoprecipitation (ChIP) of both Brd7 and p53 were performed in U2OS cells. Immunoprecipitation of a chromatin region encompassing the p53 upstream binding site of p21 promoter (-2283) was analyzed by RealTime PCR. Preliminary results indicated that, while p53 appeared to bind efficiently to p21 promoter (~1% of input, under stress conditions), the amount of Brd7 at the same site was much lower (estimated to 20-fold less) as compared with p53 (data not shown). This was however not unexpected, since Brd7 is thought to contact chromatin by means of interaction with p53 and acetylated histones, and not to directly bind to DNA. Moreover, the result indicated that while p53 bound selectively its cognate binding site, Brd7 appeared

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**Fig 34. Effect of Brd7 overexpression on p53 transcriptional activity**

H1299 cells were co-transfected in a 24-well plate with 500 ng of p21-prom-luc (left panel, blue) or Bax-prom-luc reporter constructs (right panel, pink) together with, respectively, 2 μg of pCDNA3-HA, 50 ng of pCDNA3 p53 either alone or along with increasing amounts (0,5 1 or 2 μg) of pCDNA3-Flag-Brd7, or with 2 μg of pCDNA3-Flag-Brd7 alone, as reported in the diagrams below. 12.5 ng CMV Renilla-Luc were also introduced for normalization of transfection efficiency. pCDNA3-HA vector was used to normalize the amount of DNA transfected in each sample. Luciferase activity was measured at 36 hrs after transfection. The expression levels of transfected constructs were evaluated by western blot (lower panels). Loading was normalized according to Renilla-luciferase activity. The histograms represent mean results of three independent experiments with standard deviation.
RESULTS - Part 2

Instead to be loaded both on p21 promoter and on GAPDH genomic sequence, thus indicating that it might be widely distributed on chromatin. Nonetheless, a preference for p21 over GAPDH was apparent. Further investigation is needed in order to assess if Brd7 recruitment on p21 promoter is p53 or stress-dependent.

Moreover to investigate whether Brd7 depletion could influence p53 loading on p21 promoter at its upstream (-2283) binding site, ChIP was performed with anti-p53 antibody from U2OS cells transfected with Brd7 specific siRNA or control siRNA and either treated with etoposide or left untreated. Preliminary results indicated that Brd7 is not required for recruitment of p53 to its upstream (-2383) high affinity binding site on p21 promoter upon stress conditions (not shown).

To investigate whether Brd7 could affect p53 transcriptional activity by altering its acetylation status on chromatin, a biochemical fractionation was performed on U2OS cells transfected with either Brd7 specific siRNA or with control siRNA. Cells were either treated with etoposide to induce p53 activation or left untreated. Chromatin-bound fraction was analyzed by SDS-PAGE. Western blot was then performed with a polyclonal antibody that specifically recognizes p53 acetylated at Lys382 as this modification is associated with p53 transcriptional activation. The result shown in Figure 35 indicates that Brd7 is not required for effective acetylation of chromatin-bound p53 at this residue.

<table>
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<tr>
<th>siRNA</th>
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<th>B</th>
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<th>WB: Brd7</th>
<th>WB: HMGA1b</th>
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<td>Et</td>
<td>Et</td>
<td>Et</td>
<td>AcK382</td>
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**Fig. 35 Brd7 does not influence p53 acetylation at Lys-382 on chromatin.**

Biochemical fractionation was performed on lysates from U2OS cells either transfected with Brd7 siRNA (B) or control siRNA (C) and treated with etoposide 50 µM for 12 hrs. Chromatin bound fraction was analyzed by western blot with anti p53 acetyl-lys382. Protein loading was normalized in order to obtain equal levels of total p53 in the samples transfected with Brd7 siRNA or control siRNA that were subjected to the same treatment. Western blot of HMGA1b was used as control for fractionation efficiency.
DISCUSSION

The p53 signal transduction pathway is a complex network finely coordinated to sense a plethora of intrinsic and extrinsic stress signals and to mediate a response in order to preserve genomic stability and cellular homeostasis. When activated in response to different kind of stimuli, p53 may induce the expression of a large number of genes that may be divided into categories depending on their functions. Notably, p53 activities are not only circumscribed within the nucleus where it acts as a transcription factor. Indeed, it has been demonstrated that p53 translocates to mitochondria where it directly mediates the activation of the apoptotic program (Marchenko et al., 2000). Depending on the cellular context and on the nature of the stimuli that activate p53, the ultimate outcome can be quite different, ranging from the induction of reversible cell cycle arrest, apoptosis and senescence to protective antioxidant activities and DNA repair. p53 activities are finely regulated through a large number of post-translational modifications and interactions of other proteins that direct the execution of the appropriate cellular response. Throughout many years of research, considerable knowledge on p53 activities has been achieved. However, less is understood on the mechanisms that dictates the specificity of its response.

New insights on the regulation of p53 apoptotic response have been gained with the discovery of new interactors such as the ASPP protein family, composed by ASPP1, ASPP2 and iASPP. ASPP1/2 interact with p53 and specifically enhance p53-induced apoptosis, while iASPP competes with ASPP1/2 for binding to p53 and has an inhibitory effect on p53-mediated apoptotic response, yet the mechanisms at the basis of these effects are still unclear.

The identification of different co-factors and the discovery of novel mechanisms that regulate p53 activity at different levels by modulating its functions will thus be of great help to understand how p53 activation can lead to different cellular outcomes in response to various stimuli.

Among the various p53 modulators, the prolyl isomerase Pin1 plays key roles in transducing stress signalling-induced phosphorylations into conformational changes that
determine p53 activation (Zacchi et al., 2002; Zheng et al., 2002; Wulf et al., 2002). Upon DNA damage and phosphorylation at Ser/Thr-Pro sites, Pin1 has been shown to interact with p53 and to influence its stability and activity at different levels (Zacchi et al., 2002; Zheng et al., 2002; Wulf et al., 2002). Pin1 isomerase activity is in fact required for p53 accumulation upon stress stimuli, by favoring dissociation of the p53-Mdm2 complex (Zacchi et al., 2002). Recently, data from our group highlighted also a role for Pin1 in assisting p53 efficient loading on its cognate promoters (Mantovani et al., 2007). Moreover, it was demonstrated that Pin1 promotes binding of p300 and subsequent acetylation of p53, and in this way it might enhance p53 transcriptional activity (Mantovani et al., 2007). Notably, data from literature highlighted that the presence of Pin1 is particularly required for p53-dependent apoptosis (Zacchi et al., 2002; Zheng et al., 2002).

In this work a new mechanism was identified by which Pin1 specifically influences p53 apoptotic activity. It has been demonstrated that Pin1 directly binds to its consensus sites at Ser33/Pro34, Ser46/Pro47, Thr81/Pro82, and Ser315/Pro316, and likely also to Ser127/Pro128 and/or Thr150/Pro151 when these sites are phosphorylated. Most importantly it has been shown that upon stress conditions Pin1 directly modulates the dissociation of p53 from the inhibitory factor iASPP. This sheds light on the mechanism through which inhibition of p53-mediated apoptotic response by iASPP is relieved in response to stress stimuli and indicates that Pin1 plays a direct role in this process. We speculated that a conformational change mediated by Pin1 at its Thr81-Pro82 within the PRD, where the main binding site for iASPP lies, could be the means by which Pin1 mediates p53 dissociation from iASPP. Yet this proved not to be the case as mutating Thr81 to Ala did not alter the interaction between p53 and iASPP as compared with wild type p53. Interestingly, it emerged instead that Ser46 is required for Pin1-mediated dissociation of the p53-iASPP complex and this might, at least in part, account for the relevance of this site for p53 mediated apoptosis (Feng et al., 2006). Indeed, phosphorylation of p53 at Ser46 is specifically induced by severe or persistent stress conditions and represents a major event in the specific commitment of the p53-mediated response to apoptotic cell death (D'Orazi et al., 2002; Oda et al., 2000; Taira et al., 2007).

The data presented in this thesis together disclose a mechanism by which, upon genotoxic stress-induced phosphorylation of Ser46, Pin1 is recruited at p53 Ser46-Thr47 site and it mediates dissociation of the p53-iASPP complex thus relieving the inhibition to p53-mediated apoptosis exerted by iASPP. Interestingly, iASPP does not contact p53 in the
DISCUSSION

region encompassing Ser46-Pro47 thus it might be hypothesized that prolyl isomerization at Pro47 induces a conformational change that has long range effect also on the PRD, for example by introducing a structural constraint within the flexible N-terminal domain of p53. The involvement of other Pin1 consensus sites in determining p53 dissociation from iASPP, even if possible, is not likely. The data here reported indicate that Thr81 is not required for this process and this has been assessed also for the two residues located within the DBD as their mutation did not further alter the interaction between p53 and iASPP. Moreover, no association has been reported between the phosphorylation at Ser33 and Ser315 and apoptosis, thus it seems unlikely that they are involved in p53-iASPP interaction.

Taking into consideration the results reported herein and previous evidences it can be envisioned a model according to which Pin1 regulates fundamental events in determining p53 activation upon stress (Figure 36). Pin1-mediated prolyl isomerization of phospho-Ser/Thr-Pro sites on p53, among which particularly relevant is Thr81-Pro82, favors Ser20 phosphorylation by Chk2 (Berger et al., 2005). This in turn promotes p53 dissociation from Mdm2 and binding of p300 (Dornan et al., 2001) with consequent stabilization and acetylation of p53. It might be speculated that Pin1 induces these effects also by acting on p300 itself. Pin1 is in fact able to bind to p300 (our unpublished observation) in a DNA-damage dependent way. Thus, through inducing a conformational change in p300, it might

![Fig. 36 Model for regulation of p53 by Pin1 upon stress.](image)

(A) Upon DNA damage, Pin1-mediated prolyl isomerization of phospho-Ser/Thr-Pro sites (in particular Thr81-Pro82) in p53 favors Ser20 phosphorylation (P) by Chk2 and dissociation of p53 from Mdm2, promoting p53 stabilization. (B) In addition, Pin1 favors the binding of p53 to target promoters and the acetylation of DNA-bound p53 by p300. Upon phosphorylation of Ser46, Pin1 promotes dissociation of p53 from the apoptosis inhibitor iASPP.
favour its association with p53 or even determine a switch from E4-ubiquitin-ligase to acetyl-transferase activity. In this way, upon DNA damage Pin1 would favour p53 stability both by enhancing its dissociation from Mdm2 and by preventing p53 poly-ubiquitination by p300.

Moreover, Pin1 favors the binding of p53 to its target promoters and, being recruited on chromatin with p53 it might assist structural rearrangements of p53 that occur upon DNA binding, exposing its C terminus to p300 (Ceskova et al., 2006) and thus promoting the DNA-dependent acetylation and transcriptional function of p53 as well as the organization of transcription complexes on the promoter (Barlev et al., 2001). Thr81-Pro82 site within p53 PRD seems to play an important role also in these steps as its mutation leads to a decrease in acetylation of p53 bound to its target promoters (Mantovani et al., 2007). In addition, Ser33 and Ser315 have been recently reported to be phosphorylated by CDK9 (Radhakrishnan and Gartel, 2006), a component of the transcription elongation factor pTEFb, thus it might e suggested that if Pin1 directly regulates p53 transcriptional activity upon recruitment on chromatin (Mantovani et al., 2007) also through coordinating the recruitment of specific elongation factors on p53 responsive promoters

In addition to these effects that mediate general activation of p53 response, Pin1 plays a role in specifically regulating p53 dependent apoptosis. Upon Ser46 phosphorylation in response to severe stress stimuli, Pin1 binds to its consensus site at Ser46-Pro47 and may provide the switch that allows p53 dissociation from iASPP thus activating p53 apoptotic response. Interestingly, this effect appears to be independent from Pin1-induced acetylation of p53, further confirming that Pin1 may modulate p53 activity at different levels. Moreover it might not be excluded that Pin1 exerts its effect by acting directly also on iASPP. It might be speculated that Pin1 favors p53 apoptotic activity also by assisting p53 functions at mitochondria were both can localize (Marchenko et al, 2000; our unpublished observations). Interestingly, the pro-apoptotic member of the ASPP family ASPP2 has been found to localize at mitochondria were it interacts with Bax stimulating Cytochrome C release into cytoplasm. It is thus likely that Pin1 might favor the interaction between p53 and ASPP2 to promote apoptosis. Otherwise it is possible that also iASPP localizes at mitochondria thereby preventing p53 from triggering apoptosis. Thus, through the mechanism described in this work, Pin1 would relieve iASPP-mediated inhibition and favor p53 apoptotic activity.
Ser46 phosphorylation (Sullivan et al., 2004) together with iASPP interaction (Bergamaschi et al., 2006) have been indicated as important determinants of the differential apoptotic response of p53 Pro72 and p53 Arg72 isoforms at the transcriptional level. Work from our group demonstrated that more efficient Ser46 phosphorylation occurs on p53 Arg72 than on p53 Pro72 upon stress and indeed Pin1 binds more to p53 Arg72 (Mantovani et al., 2007). This indicates that Pin1 plays a role also in determining the differential apoptotic potential of the two isoforms. In fact, higher efficiency in phosphorylation at Ser 46 would determine an increased Pin1-mediated dissociation of the p53-iASPP complex in the case of p53Arg72 with respect to p53 Pro72. This would add to the already weaker interaction with iASPP of the p53 Arg72 isoform due to the effect of the amino acid substitution on direct contact between p53 PRD and iASPP SH3 domain. Interestingly, in breast cancers Pin1 over-expression correlates inversely with iASPP over-expression. These evidences favours a model where overexpression of Pin1 would be selected against in tumours bearing wt p53 Pro72, as it would counteract iASPP-dependent inhibition of p53-mediated apoptosis. Conversely, in cancers with wt-p53 72 Arg/Arg, there would be no requirement to select against Pin1 over-expression because iASPP does not efficiently target this p53 isoform, thus cancer cells would take advantage of the pro-tumorigenic effects of Pin1 (as the amplification of pro-oncogenic pathways through cyclin D or β-catenin) without being affected by its pro-apoptotic function.

As Pin1 governs the functions of several substrates, and it is itself under control of signalling pathways that could become aberrantly activated in cancers (Wulf et al., 2005) is not surprising that its abundance has been associated both positively and negatively with cancer, highlighting the relevance of the cellular context in determining whether Pin1 inhibits or amplifies cell-growth pathways. Due to its pleiotropic effects, Pin1 might play contrasting roles in different phases of tumorigenesis. In normal cells or at early stages it might be involved in the regulation of cell-cycle check-points (Atchison et al., 2003; You et al., 2002) and its overexpression would be favourable to contrast tumor initiation by enhancing p53-dependent apoptotic response. Yet, at more advanced tumor stages, concomitant with deregulation of signalling pathways, it may instead contribute to the amplification of proliferative signals (Wulf et al., 2005) thus promoting tumor progression and maintenance, in particular in p53wt Arg/Arg contexts. In this case, development of specific Pin1 inhibitors would be potentially effective in cancer treatment to counteract the amplification of proliferative signals. Yet, based on our observations, at early stages,
inhibition of Pin1 would be predicted to inhibit cytotoxicity and hence the clinical activity of agents inducing p53-dependent apoptosis. Thus the disclosure of novel mechanisms in the interplay between p53 and its co-regulators, such as the one reported in this thesis, would be of relevance in designing targeted therapies for cancer treatment.

Analyzing the protein interaction profiles is a powerful system to gain insight on the mechanisms governing p53 regulation. From a yeast two-hybrid screening previously performed in our laboratory, the bromodomain containing protein Brd7 emerged as a common interactor of the p53 family members (as well as of mutant p53 R175H). The presence of the Bromodomain and the evidences from literature supporting a role in transcriptional activation made Brd7 a promising candidate for modulating the p53 pathway at the transcriptional level. This protein and its functional interaction with p53 have been therefore characterized in the second part of this thesis.

The interaction emerged among Brd7 and the p53 family members has been validated both in vitro and in cells, disclosing the presence of two binding sites for p53 on Brd7. One, located in the C-terminal region of Brd7, interacts with p53 N-terminal portion spanning from aa 74 to aa 175. Interestingly, in these assays the p53 deletion mutant 1-175 appear to bind even strongly than p53 full length, it might be hypothesized that a region present in p53 C-terminal portion could exert an inhibitory effect on this interaction, or, most likely, that this deletion, occurring within the p53DBD might cause an improper folding leading to the disclosure of a fictitiously stronger binding site.

A second binding site for p53 maps at the N-terminus of Brd7, in a region that contains the bromodomain. Interestingly, this region has shown to interact with p53 CTD where most of the acetylable lysine residues are located. It was thus supposed that Brd7 bromodomain might bind to acetylated lysines at p53 CTD. Yet, preliminary investigations suggested that the interaction between Brd7 N-terminus and p53 occurs irrespective of p53 acetylation status. Conversely, in vitro assays indicated that the bromodomain might mediate Brd7 oligomerization. This might involve acetylated lysines within Brd7 lysine-rich domain. However, as this interaction has been tested only in vitro, further investigation is needed to asses whether it actually occurs in cells. The functional significance of Brd7 oligomerization has not been investigated, yet an auto-inhibitory effect might be speculated in preventing the interaction with acetylated histones by keeping the bromodomain
inaccessible. Conversely it might stabilize multi-protein complexes on chromatin. Moreover it would be interesting to determine whether Brd7 is constitutively acetylated or this modification is subjected to regulation.

Data from literature suggested an involvement of Brd7 in cell cycle regulation (Peng et al., 2007; Zhou et al., 2004). Overexpression of BRD7 could inhibit NPC cell growth and block cell cycle progression from G1 to S phase. This was ascribed to modulation of the expression of factors involved in Ras/MEK/ERK and Rb/E2F pathways, such as E2F3, cyclin D1 and MEK1 (Zhou et al., 2004). Notably, Brd7 could significantly inhibit E2F3 promoter activity in luciferase assays, yet ChIP analysis did not show the presence of Brd7 on the endogenous promoter. The role for Brd7 as a transcriptional regulator had also been suggested by other reports indicating that Brd7 could interact with other transcription factors such as E1B-AP5 (early adenovirus E1B-55kD association protein) (Kzhyshkowska et al., 2003) and IRF2 (Interferon Regulatory Factor-2) (Staal et al., 2000).

In this thesis it was demonstrated that Brd7 is required for efficient cell-cycle arrest in U2OS cells upon challenging with genotoxic stimuli. This effect was, at least in part, due to a reduction in p21 expression that occurred upon Brd7 depletion and under stress condition. The down-regulation of p21 as a consequence of Brd7 silencing occurred at the transcriptional level and proved to be p53-depedent. Notably, this effect seemed to be specific for p21 as the stress-dependent induction of two other p53 targets, Mdm2 and Puma, appeared to be almost unaffected. Interestingly, Brd7 depletion lead to a decrease in p53 protein levels, thus it might be hypothesized that it exerts its effect also regulating p53 abundance.

On the basis of these data and of the evidence demonstrating that Brd7 is localized within the nucleus in the chromatin bound fraction, it was hypothesized that Brd7 might be involved in regulation of p21 promoter induction by p53. Reporter assays showed no significant variation in p21 promoter induction by p53 upon Brd7 silencing. However the role of Brd7 might require an endogenous chromatin context, and this might explain the lack of effect on a p21 promoter fragment cloned into an expression vector.

We therefore sought to verify the ability of Brd7 to bind endogenous p21 promoter in conditions where its expression is induced by p53 by performing chromatin immunoprecipitation assays. The results highlighted lower levels of Brd7 binding to p21 promoter with respect to p53. This however was not surprising as Brd7 is supposed to contact DNA indirectly, by means of interaction with acetylated histones or with p53. The
precipitation of lower amounts of chromatin might thus be due to a limitation of this technique. Indeed, ChIP experiments conducted on p53 transcriptional co-factors such as p300 lead to similar results (our unpublished observations).

Notably, it also appeared that Brd7 is not required for p53 recruitment on its high affinity binding site on p21 promoter neither it influenced the acetylation status of chromatin-associated p53. However, Brd7 might exert its effect on p53 transcriptional activity by assisting the assembly of a functional transcriptional apparatus or by recruiting other chromatin remodelling factors on chromatin surrounding p53 responsive element thus it would be worth analyzing the chromatin modifications occurring at this site upon Brd7 depletion.

In contrast to p53, Brd7 might be widely distributed on chromatin, consistent with binding to acetylated histones. However, binding to p21 promoter appeared to be preferential with respect to other sites. Thus, it would be interesting to investigate whether Brd7 is recruited on p21 promoter by p53 and if this occurs in response to specific stimuli. Brd7 might be also bound at other sites within p21 promoter or at enhancers governing p21 expression, and this would also explain the lack of effect observed in the reporter assays. It has been demonstrated (Cordenonsi et al., 2003) that p53 and Smad2 interact on separate cis binding elements present on target promoters and synergistically activate various genes, among which p21. Thus it might be speculated that Brd7 may play a role in the cooperation between p53 and the TGFβ signalling pathway in the induction of p21 transcription. Therefore, it would be interesting to investigate whether Brd7 influences also TGFβ-induced transcription of p21 and if it localizes with p53 and Smad2 on p21 promoter.

Consistently, data from literature indicating a role for Brd7 in downregulation of the Wnt pathway (Peng et al., 2007; Kim et al., 2003) suggest that Brd7 may have pleiotropic effects in regulating cell growth and it might be highly interconnected with different signalling pathways.

However, data regarding Brd7 involvement in the Wnt pathways are contrasting. It seems in fact that Brd7 might up-regulate α-catenin expression thus favoring β-catenin sequestering while, it was reported to bind to dishevelled-1 (Dvl-1) and positively regulate Wnt signalling (Kim et al, 2003). As this last function would be exerted within the cytoplasm it might be supposed that Brd7 plays different roles depending on its localization within the cells and that there might be mechanisms that induce its cytoplasmic localization.
DISCUSSION

and that prevent it from mediating its growth suppressive functions. It would thus be of interest to determine if its localization might be altered in some tumor context.

Taken together the data reported in this thesis suggest a role for Brd7 as a positive regulator of p53 transcriptional activity during cell-cycle arrest response. Interestingly it was observed that Brd7 expression is higher in tumor-derived cell lines with respect to normal or immortalized cells. In the light of its role in mediating cell-cycle arrest this seems surprising, as it would be expected that it is generally downregulated in tumors (as in the case of NPC). Yet, it might be supposed that its upregulation is an upstream event in response to altered growth signals and that the disruption of downstream pathways bypasses the induction of cell-cycle arrest mediated by Brd7. Notably, Brd7 seems to be regulated by E2F1, thus it might be speculated that it plays also a role in response to oncogenic signalling (Berkovich and Ginsberg, 2001) leading to the onset of the senescence program. Notably, the p53-p21 axis plays key roles in mediating senescence. Thus, upon activation of oncogenes such as Ras, Brd7 might be upregulated through E2F1 and assist p53 in the induction of p21 to favor the onset of senescence thus offering a barrier to unscheduled cell proliferation. Mutations of this pathway are frequently present in cancer and this might explain why Brd7 overexpression is not selected against. It would thus be of interest to investigate whether Brd7 is required for Ras-induced senescence.

It has also been observed that, when present at high levels (overexpression) Brd7 might cause a reduction in p53-mediated transactivation of Bax promoters. Thus it might be supposed that Brd7 plays a role in p53 choice towards different subset of promoters. Thus its overexpression, when cell-cycle arrest response is impaired, might favor tumor progression by mediating a by-pass of apoptosis.

Interestingly, Brd7 has proven to interact also with mutant p53 R175H and the binding appeared to be stronger as compared to that of wild type p53. This suggests that the altered conformation of p53R175H might disclose a stronger binding site as hypothesized for what observed in the in vitro binding of p53 1-175 deletion mutant with Brd7 C-terminal portion. It might thus be speculated that, as mutant p53R175H binds much strongly to Brd7 with respect to wt p53, it might compete for interaction with Brd7 and lead to a phenotype similar to Brd7 depletion in cells expressing mutant p53 R175H thus Brd7 overexpression would be selected in tumors bearing mutant p53. However, it would be of interest to investigate whether Brd7 might directly influence also mutant p53 functions.
In conclusion, the role of Brd7 in regulating p53 activity needs further investigation in order to be elucidated, yet from the data here reported it seems a promising candidate to better understand how p53 response might be directed towards specific outcomes as a result of the complex cross-talk among different signalling pathways.

The work described in Results - Part1 is contained in the following article (see APPENDIX):

The prolyl isomerase Pin1 orchestrates p53 acetylation and dissociation from the apoptosis inhibitor iASPP. Nat Struct Mol Biol. 14(10); 912-20.
The prolyl isomerase Pin1 orchestrates p53 acetylation and dissociation from the apoptosis inhibitor iASPP.

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The tumor-suppressor function of p53 relies on its transcriptional activity, which is modulated by post-translational modifications and interactions with regulatory proteins. The prolyl isomerase Pin1 has a central role in transducing phosphorylation of p53 into conformational changes that affect p53 stability and function. We found that Pin1 is required for efficient loading of p53 on target promoters upon stress. In addition, Pin1 is recruited to chromatin by p53 and stimulates binding of the p300 acetyltransferase and consequent p53 acetylation. Accordingly, tumor-associated mutations at Pin1-binding residues within the p53 proline-rich domain hamper acetylation of p53 by p300. After phosphorylation of p53 at Ser46 triggered by cytotoxic stimuli, Pin1 also mediates p53’s dissociation from the apoptosis inhibitor iASPP, promoting cell death. In tumors bearing wild-type p53, expression of Pin1 and iASPP are inversely correlated, supporting the clinical relevance of these interactions.
The prolyl isomerase Pin1 orchestrates p53 acetylation and dissociation from the apoptosis inhibitor iASPP

Fiamma Mantovani¹, Francesca Tocco¹, Javier Girardini¹, Paul Smith², Milena Gasco³, Xin Lu⁴, Tim Crook² & Giannino Del Sal¹

The tumor-suppressor function of p53 relies on its transcriptional activity, which is modulated by post-translational modifications and interactions with regulatory proteins. The prolyl isomerase Pin1 has a central role in transducing phosphorylation of p53 into conformational changes that affect p53 stability and function. We found that Pin1 is required for efficient loading of p53 on target promoters upon stress. In addition, Pin1 is recruited to chromatin by p53 and stimulates binding of the p300 acetyltransferase and consequent p53 acetylation. Accordingly, tumor-associated mutations at Pin1-binding residues within the p53 proline-rich domain hamper acetylation of p53 by p300. After phosphorylation of p53 at Ser46 triggered by cytotoxic stimuli, Pin1 directly favors p53 binding on target promoters of p53-Arg72 isoforms, thereby stimulating dissociation of p53 from Mdm2 (ref. 17). This site lies within p53's proline-rich domain (PRD), which modulates the stability, acetylation, transcriptional activity and direct apoptotic function of p53 at mitochondria.

In particular, Thr81-Pro82 is a crucial site through which Pin1 promotes Chk2-dependent phosphorylation of p53 at Ser20 (ref. 20), thereby stimulating dissociation of p53 from Mdm2 (ref. 17). This site lies within p53's proline-rich domain (PRD), which modulates the stability, acetylation, transcriptional activity and direct apoptotic function of p53 at mitochondria.

The tumor-suppressor activity of p53 is also controlled by the apoptosis inhibitor iASPP, which interacts with the PRD and blocks p53 binding to cell death-related promoters. We recently reported that a common single-nucleotide polymorphism within the PRD, encoding either a proline or arginine at residue 72 (refs. 30–32), determines differential binding of iASPP and that this correlates with the different apoptotic potentials of the p53-Pro72 and p53-Arg72 isoforms.

In this report, we dissect the multiple levels at which Pin1 might fine-tune the transcriptional activity of p53 in human cells subjected to genotoxic stress. We investigate the requirement of Pin1 for efficient recruitment of p53 to its target promoters and for acetylation mediated by p300. In addition, we analyze how Pin1 might affect iASPP-dependent regulation of p53 apoptotic activity.

RESULTS
Pin1 directly favors p53 binding on target promoters
To assess the impact of Pin1-induced prolyl isomerization on the transcriptional activity of p53, we performed chromatin immunoprecipitation (ChIP) assays of endogenous p53 from HCT116 cells. Under stress conditions promoting the interaction of p53 with Pin1, prevention of cancer development depends on the ability of p53 to either arrest proliferation or induce apoptosis of damaged cells, and relies substantially on its transcriptional function. The high frequency of mutations in the p53 DNA-binding domain in tumors highlights the importance of loss of p53's transcriptional activity for the ability of cancer cells to evade stress-induced growth suppression. However, the mechanisms by which p53 function is subverted in cancers lacking these mutations remain poorly defined.

The orchestration of appropriate cellular responses to genotoxic and cytotoxic stimuli depends on the ability of stress pathways to finely modulate p53’s functions through regulation of its post-translational modifications, conformation and interactions with other proteins. In particular, whereas stress-induced phosphorylation of N-terminal residues promotes p53 stabilization, full activation of its transcriptional function also involves acetylation of the C-terminal and core domains as well as conformational changes. Phosphorylation-dependent prolyl isomerization induced by Pin1 is a well-conserved and extremely efficient mechanism for transducing post-translational modifications into conformational changes in key cellular proteins. Upon recognizing phosphorylated serine or threonine preceding proline in its substrates, Pin1 catalyzes the switch of the intervening peptide from the cis to the trans conformation or vice versa. The resulting structural rearrangements control the stabilities and functions of several proteins governing cell proliferation and transformation, among which is p53. Upon stress, different kinases phosphorylate p53 at Ser33, Ser46, Thr81 and Ser315, generating sites for recognition by Pin1, which greatly affects p53 stability and activity.
we observed that inhibition of Pin1 expression by RNA interference (RNAi) reduced, by approximately five-fold, the binding of p53 to its upstream responsive site (BS1, Fig. 1a) within the promoter of the endogenous gene encoding p21\(^{WAF1/CIP1}\) (the CDKN1A gene, here called p21); in contrast, only a minor effect was seen in unstressed cells (Fig. 1b). Pin1 was also required for binding of p53 to its downstream site, BS2 (data not shown), allowing efficient induction of p21 upon stress (Fig. 1b). As lack of Pin1 results in increased destabilization of p53 by Mdm2 (refs. 17,20), we then performed ChiP after inhibiting Pin1 expression by RNAi and stabilizing p53 with nutlin-3a, which inhibits Mdm2 binding to p53 (ref. 33). The results demonstrate that Pin1 is required for optimal binding of p53 to both p21 and BAX promoters upon stress, in a manner independent of its ability to promote dissociation of p53 from Mdm2 (Fig. 1c). Accordingly, expression of p21 and BAX remained low in cells transfected with Pin1-specific short interfering RNAs (siRNAs), despite exposure to DNA-damaging stimuli and treatment with nutlin-3a (Fig. 1c). Inefficient BAX induction might contribute to the observed impairment of stress-induced apoptosis of HCT116 cells upon silencing of Pin1 (Fig. 1d). Inhibition of Pin1 expression in cells lacking the TP53 gene (HCT116 p53\(^{-/-}\)) caused, in contrast, a moderate reduction of stress-induced apoptosis (Fig. 1d), consistent with the role of Pin1 in promoting the apoptotic activity of the p53 family member p73 (ref. 34).

Given its role in favoring p53 binding to DNA, we hypothesized that Pin1 might be associated with p53 on chromatin. ChiP assays in HCT116 p53\(^{+/+}\) cells indicated that, although barely detectable under normal conditions, Pin1 is recruited to p53-binding sites within the p21 and BAX promoters as a result of UV and etoposide treatments (Fig. 2), as well as cisplatin and doxorubicin treatments (data not shown), all known to promote its interaction with p53 (refs. 17–19). Notably, DNA damage-induced recruitment of Pin1 to the p21 promoter involves p53-responsive elements, but not upstream promoter regions (Supplementary Fig. 1a online); moreover, it is only slightly evident in HCT116 p53\(^{-/-}\) cells (Supplementary Fig. 1b). This suggests that the presence of Pin1 on p53-target promoters depends on either p53 or p53 family members.

**Pin1-binding sites modulate p53’s transcriptional activity**

To understand whether Pin1 is recruited to chromatin by means of its interaction with p53, we generated p53 mutants unable to bind Pin1 by disrupting Pin1 consensus sites (Ser/Thr-Pro). We and others have previously shown that phosphorylation of Ser33, Ser46, Thr81 and Ser315 mediates p53 binding to Pin1 in response to stress stimuli\(^{17–19}\) (see also Supplementary Fig. 2 online), and Thr150 can also cooperate in this interaction (F.M. and G.D.S., unpublished data). As expected, substitution of Ser33, Ser46, Thr81 and Ser315 with alanine (p53-6M) greatly reduced the binding of p53 to glutathione S-transferase (GST)-tagged Pin1 upon stress, and the interaction was completely abolished when we additionally mutated Ser127 and Thr150 to alanine (p53-6M) (Fig. 3a). ChiP assays for Pin1 were performed with p53-null H1299 cells overexpressing wild-type (WT) p53, p53-4M or p53-6M. Whereas WT p53 recruited about ten-fold more Pin1 to the p21 promoter as compared to recruitment in cells transfected with empty vector, p53-4M had only a slight effect, and p53-6M was completely inactive in this assay (Fig. 3b). Notably, the amino acid substitutions did not seem to cause major changes in p53’s conformation, as judged by the ability of both p53-4M and p53-6M to bind cognate DNA in vitro (Supplementary Fig. 3a online).

We then tested the transcriptional activity of the p53-4M and p53-6M mutants. To avoid transfection-dependent stress, we generated inducible polyclonal cell lines expressing p53-WT, p53-4M or p53-6M in an SaOS-2 p53-null background. Both p53-4M and p53-6M were highly impaired in promoting transcription of endogenous p21 and BAX genes after exposure to etoposide (Fig. 3c), and ChiP assays demonstrated that upon stress, p53-4M bound the endogenous p21 promoter about five-fold less than did WT p53 expressed at comparable levels (Fig. 3d). This is consistent with the reduced ability of endogenous p53 to bind chromatin upon inhibition of Pin1 expression (Fig. 1c), suggesting a direct role of Pin1 in enhancing the interaction of p53 with its target promoters upon DNA damage.

![Figure 1](https://example.com/figure1.png)

**Figure 1** Pin1 is required for binding of p53 to its target promoters. (a) Scheme indicating the positions of PCR primers in the p21 promoter. (b) ChiP of p53 was performed with HCT116 cells transfected with Pin1-specific (RNAi Pin1 (I)) or control (RNAi C) siRNAs, and then left untreated (--) or treated with UV or etoposide (Et). Cell lysates were analyzed by western blotting (WB). (c) Pin1’s effect on p53 binding to DNA is not dependent on p53-Mdm2 interaction. ChiP was performed as in b, using two different Pin1 siRNAs, (I) and (II). Where indicated, pin1 was stabilized with nutlin-3a, p53 binding to p21 and BAX promoters was estimated by real-time PCR and represented as fold difference from the value measured in control-transfected, untreated cells. (d) We estimated the effect of Pin1 silencing on p53-dependent apoptosis by transfecting HCT116 cells with Pin1-specific siRNA (I) or (II), or control siRNA (C) before treatment with UV or Et. Percentage of apoptotic cells was estimated by annexin V/propidium iodide staining and FACS analysis. Graphs in b–d show means and s.d. from three independent experiments. P-values are relative to RNAi C data.
agreement with the requirement of Pin1 for p53-dependent apoptosis (Fig. 1c), the ability of p53-4M and p53-6M to promote apoptosis in response to stress was greatly impaired as compared to WT p53 (Supplementary Fig. 3b,c).

Pin1 enhances p300-mediated acetylation of p53
We hypothesized that prolyl isomerization might affect subsequent post-translational modifications of p53, thereby increasing its binding to chromatin and its transcriptional activity. In particular, as acetylation of p53 C-terminal lysines at positions 373 and 382 by p300 (or its homolog CBP) stimulates these activities, we tested whether p53 mutants unable to bind Pin1 were modified by p300 differently from WT p53. Indeed, p53-4M and p53-6M bound p300 markedly less than WT p53 (Fig. 4a) and were consequently acetylated much less efficiently by p300 (Fig. 4b), particularly on Lys373 and Lys382 (Supplementary Fig. 4a online). Notably, overexpression of p300 did not increase the transcriptional activity of p53-6M (Supplementary Fig. 4b). Moreover, RNAi-mediated inhibition of Pin1 expression in HCT116 cells reduced the interaction of endogenous p53 and p300 (Fig. 4c) as well as the stress-induced acetylation of p53 at Lys373 and Lys382 (Fig. 4d), suggesting a direct role of Pin1 in these events.

Finally, we sought to prove that the contribution of Pin1 to p300-mediated acetylation of p53 depends on its direct activity toward p53, rather than on indirect effects on p300 or other components of stress pathways. Accordingly, we immunoprecipitated in vitro-translated p53 and incubated it with purified catalytically competent Pin1 protein. A control reaction was set up using a catalytically inactive Pin1 mutant (C113A) that binds p53 but cannot isomerize it. After removal of bound Pin1, p53 was subjected to acetylation by purified p300 protein. Treatment of p53 with WT Pin1, but not mutant Pin1, increased the efficiency of subsequent acetylation by p300 (Fig. 4e), suggesting that this depends on isomerization of p53.

Pin1 favors the acetylation of p53 by p300 on chromatin
Acetylation of p53 C-terminal lysines by p300 or CBP has been reported to increase binding of p53 to its target genes, but other studies have indicated that the prevalent role of this acetylation is in promoting the recruitment of transcription cofactors on p53-target promoters. We sought to understand the contribution of Pin1-dependent acetylation of p53 to each of these processes.

To assess whether Pin1 promotes binding of p53 to target chromatin sites by favoring its acetylation, we assayed the effect of silencing Pin1 on promoter binding by WT p53 and p53-9KR, a mutant where nine C-terminal lysines, including those acetylated by p300, are substituted by arginines. This mutant protein bound Pin1 to a similar extent as did WT p53 (Supplementary Fig. 5a online). Notably, in ChIP assays, p53-9KR showed reduced binding to p21 promoter as compared to WT p53 expressed at similar levels (Fig. 5a), suggesting that acetylation of C-terminal lysines modulates DNA binding in vivo. RNAi-mediated inhibition of Pin1 expression reduced the binding of p53-9KR to the p21 promoter, although to a lesser extent than seen with WT p53 (Fig. 5b). This suggests that Pin1 affects binding of p53 to chromatin independently of its impact on p53 acetylation.

To estimate the extent to which Pin1’s ability to promote p53 acetylation affects p53 transcriptional activity, we inhibited either Pin1 expression or isomerase activity and used ChIP assays to analyze the acetylation of p53 bound to p21 promoter under these conditions. Similar amounts of DNA-bound p53 were compared in these experiments, as detailed in Supplementary Figure 5b. The results showed that acetylation of promoter-bound p53 on Lys373 and Lys382 increased upon stress only in the presence of normal levels of Pin1, and could be blocked by inhibition of Pin1’s catalytic activity with the small molecule PiB (Fig. 5c). Acetylation of Lys373 and Lys382 is known to induce the recruitment of p300 to p53-target promoters, and indeed, the binding of p300 to the upstream p53-responsive element of the p21 promoter was reduced upon silencing of Pin1 (Fig. 5d).

Finally, we performed biochemical fractionation of HCT116 cell lysates after inhibiting Pin1 expression by RNAi. This showed that acetylation of mostly the chromatin-bound pool of p53 was reduced; there was only a smaller effect on free p53 protein (Fig. 5e).

The above results suggest that Pin1 favors the recruitment of p300 and the acetylation of p53 on chromatin, which activate transcription. In addition, Pin1 also affects the acetylation of soluble p53, and this might further stimulate its binding to chromatin.

Interplay of Pin1 and PRD in p53 acetylation
It has been shown that the p300-dependent acetylation of p53 that occurs upon binding to cognate DNA involves p53’s PRD domain, which contains a Pin1 target site at residues Thr81-Pro82. We sought to verify the requirement of these residues for p53 acetylation by transfecting cells with plasmids encoding WT or mutant p53 and p300 together with the pG13 plasmid, which contains 13 tandem p53-binding sites. In agreement with previous reports, this consensus DNA sequence stimulated WT p53 acetylation, whereas a plasmid containing mutated p53-binding sites (pM13) did not (Fig. 6a, left blots). Notably, p53 acetylation was reduced by the
Figure 3 Pin1-binding sites are essential for p53 functions. (a) Top, p53 scheme indicating the positions of Pin1 consensus sites (phospho-Ser/Thr-Pro), TA, transactivation domain; DBD, DNA binding domain; NLS, nuclear localization signal. p53 variants Ser/Thr-to-Ala substitutions in Pin1 consensus sites, either at residues 33, 46, 81 and 315 (in bold, p53-4M) or at all six positions (p53-6M). Bottom, binding of p53 variants to Pin1 upon overexpression in Saos-2 cells, etoposide treatment and GST-Pin1 pull-down. G, GST alone; P, GST-Pin1. Binding reactions were compared with 10% of protein inputs by western blotting (WB). (b) Pin1 recruitment to chromatin requires binding to p53, p53-null H1299 cells transfected with plasmids encoding WT p53, p53-4M or p53-6M, or vector alone, were used for ChIP with Pin1-specific or control (HA) antibodies, and the percentage of input p21 promoter bound by Pin1 was estimated by real-time PCR. Graph shows means and s.d. from three independent experiments. p53 levels were determined by western blotting. (c) To assess the role of Pin1-binding sites in p53’s transcriptional activity, polycystic Saos-2 cell lines were generated for PonA-inducible expression of WT p53, p53-4M and p53-6M. Upon induction with PonA, cells were treated with etoposide (Et) or left untreated. Induction of p53 target genes p21 and BAX was evaluated by semiquantitative RT-PCR, with GAPDH serving as a control for efficiency of RNA extraction and transcription. (d) Binding of WT p53 and p53-4M to p21 promoter in inducible Saos-2 cell lines treated as in c was analyzed by ChIP with antibodies (Ab) to p53 or HA (control, C). Bound p21 promoter was estimated by semiquantitative PCR, with relative band intensities reported below.

mutations T81A and P82I (Fig. 6a, right blots), which also weaken p53 binding to Pin1 (refs. 17,20). We then performed ChIP assays with Saos-2 cell lines expressing either WT p53 or p53-T81A. As expected, lower amounts of acetylated p53-T81A than of WT p53 were found on the p21 promoter (Fig. 6b). The above results reinforce the previous observation that the Thr81-Pro82 site is important for p53 transcriptional and apoptotic activity12,20, and in particular for its activation by Pin1 (ref. 20).

To confirm the role of the PRD in p53 acetylation, we then assayed the relevance of the codon 72 proline/arginine polymorphism, which also affects the functions of this domain11,32. Upon stress, p53-Arg72 was more rapidly and efficiently acetylated on Lys373 and Lys382 than was p53-Pro72, and this was observed when comparing cell lines endogenously expressing either p53-Pro72 or p53-Arg72 (Fig. 6c), as well as otherwise isogenic p53-inducible H1299 cell lines (Supplementary Fig. 6a online). The stronger apoptotic potential of p53-Arg72 has been associated with phosphorylation of Ser46 (ref. 32), which enhances p53 acetylation at Lys382 by CBP (homolog of p300)13. Notably, we observed more Ser46 phosphorylation on p53-Arg72 than on p53-Pro72 (Fig. 6d and Supplementary Fig. 6b). Consistent with this, p53-Arg72 bound GST-Pin1 more strongly than did p53-Pro72 upon stress (Supplementary Fig. 6c).

Pin1 stimulates p53 dissociation from IASPP

Our results indicate that Pin1 controls p53’s transcriptional and apoptotic activities. However, although acetylation of Lys373 or Lys382 can promote binding of p53 to cell death–related promoters9, it is not sufficient to activate its apoptotic function, as dissociation from inhibitory factors is also required. Among these inhibitory factors, IASPP28 binds the PRD, interacting preferentially with the p53-Pro72 variant29. We therefore asked whether, upon cytotoxic stress, Pin1 might regulate the p53-IASPP interaction.

Using co-immunoprecipitation experiments, we found that the interaction of IASPP with ectopically expressed p53 was reduced upon irradiation of H1299 cells with cytotoxic UV dosage (Fig. 7a), even though p53 levels were equalized by treatment of unstressed cells with a proteasome inhibitor (CBZ). IASPP, ASPP1 and ASPP2 protein levels remained unchanged (Fig. 7a and data not shown). This result suggests that stress-induced post-translational modifications might regulate the interaction of p53 with IASPP. Indeed, the phosphorylation mutants p53-4M and p53-6M bound IASPP more strongly than did WT p53 upon DNA damage (Supplementary Fig. 7a online). Notably, disruption of single Pin1 target sites on p53 demonstrated that stress-induced dissociation of p53 from IASPP required Ser46 and, to a lesser extent, Thr81 (Fig. 7a).

We therefore reasoned that, by modifying the conformation of p53 in response to cytotoxic stress, Pin1 could specifically modulate p53’s dissociation from IASPP in addition to affecting its acetylation. In support of this hypothesis, we found that in the absence of Pin1, the dissociation of p53 from IASPP upon cytotoxic stress was impaired, despite the fact that Ser46 was phosphorylated (Fig. 7b). Conversely, Pin1 overexpression enhanced p53 dissociation from IASPP in a Ser46-dependent fashion (Fig. 7c). This effect required Pin1 isomerase activity, as it was not observed either upon expression of catalytically inactive Pin1 mutants (Fig. 7d) or upon inhibition of prolyl isomerase activity (Supplementary Fig. 7b). Together, these observations suggest that, upon stress-induced phosphorylation of p53 on Ser46, Pin1 favors its dissociation from IASPP. However, this does not depend on Pin1-induced acetylation of p53, as IASPP bound p53 irrespective of its acetylation status (Supplementary Fig. 8a online). It also does not
Pin1 promotes p300 recruitment and p53 acetylation on chromatin. (a) Results of ChIP of WT p53 or p53-9KR overexpressed in H1299 cells. Percentage of input p21 promoter bound was estimated by real-time PCR. Graphs represent means and s.d. from three independent experiments. (b) Pin1 affects p53 binding to chromatin independently of acetylation. ChIP was done as in (a), after expression of WT p53 or p53-9KR together with either Pin1-specific (P) or control (C) siRNAs. p53 expression and acetylation (ac) on Lys373 and Lys382 was analyzed by western blotting. (c) Pin1 affects p53 acetylation on target DNA. ChIP was performed with HCT116 cells treated with UV or etoposide (Et) and with antibodies specific for p53 acetylated on Lys373 and Lys382. Using real-time PCR, we analyzed the increase in acetylation of p53 bound to p21 promoter (upstream binding site) upon DNA damage, after either blocking Pin1’s expression by RNAi (RNAi Pin1) compared with control RNAi C; black bars) or inhibiting its prolyl isomerase activity (PiB treatment with GST-Pin1). Pin1 affects p300 recruitment to p21 promoter. ChIP of p300 was performed with HCT116 cells transfected with either Pin1-specific (P) or control (C) siRNAs and treated with Et. Binding of p300 to p21 promoter (upstream p53-binding site) was evaluated by real-time PCR as in c. (e) Pin1 controls primarily the acetylation of the chromatin-bound p53 pool. Chromatin-bound and free protein fractions were prepared from HCT116 cells transfected with Pin1-specific siRNA (I) or (II) or with control siRNA, and then treated with Et or left untreated. Acetylation (ac) of p53 on Lys373 was evaluated by western blotting. We loaded excess protein from Pin1-silenced cells relative to control-transfected cells to allow comparison of similar amounts of p53. Western blots of HMGA1b and tubulin were used as controls for fractionation efficiency. Graphs in a–d represent means and s.d. from three independent experiments.
Figure 6 Pin1-target site Thr81-Pro82 and the codon 72 polymorphism affect p53 acetylation. (a) DNA-dependent acetylation of WT p53 and p53 mutants T81A and P82I overexpressed in SaOS-2 cells was induced by cotransfection of a plasmid encoding p300 and either the p53 or pM13 plasmid, which contain 13 WT or mutated p53-binding sites, respectively. Acetylation (ac) of p53 on Lys382 was estimated by western blotting (WB). (b) ChIP was performed, using an antibody specific for p53 acetylated on Lys382, with PolA-inducible polyclonal SaOS-2 cell lines expressing WT p53 or p53-T81A and either treated with UV or etoposide (Et) or left untreated. The increase in acetylation of p53 bound to p21 promoter (upstream binding site) after DNA damage was estimated by semiquantitative PCR (right gels). Relative band intensities are reported below gel. (c) Acetylation of p53 on Lys382 was estimated by western blotting of WM278 and WM115 melanoma cells, homozygous for alleles encoding p53-Pro72 and p53-Arg72, respectively, upon treatment with either UV, Et, doxorubicin (Dox) or deacetylase inhibitors (DI). (d) Phosphorylation of p53 on Ser46 (pSer46) was estimated by western blotting of doxycycline-inducible H1299 cell lines expressing p53-Pro72 and p53-Arg72, upon treatment with doxorubicin for different times.

Figure 7 Pin1 is required for p53 dissociation from iASPP upon cytotoxic stress. (a) The interaction of WT p53 or mutant S46A or T81A p53 expressed in H1299 cells with endogenous iASPP was analyzed by co-immunoprecipitation (IP) after treatment with either cytotoxic UV dosage or the proteasome inhibitor CB2 (as a control). WB, western blot; mAb, monoclonal antibody. (b) The interaction of endogenous p53 and iASPP proteins was analyzed, as in a, in HCT116 cells transfected with Pin1-specific siRNA (I) or (II) or control (C) siRNA and treated with either UV or CB2. (c) H1299 cells were transfected with either WT p53 or mutant S46A or T81A p53, and with vector encoding HA-Pin1 (+) or empty vector (–). After UV treatment, the interaction of p53 with endogenous iASPP was analyzed as in a. (e) Histograms show the percentage of tumors overexpressing iASPP (gray bars) and Pin1 (black bars), assessed by comparing mRNA abundance in breast tumor samples homozygous for the allele encoding WT p53-Pro72 (P/P) or WT p53-Arg72 (R/R) to mRNA abundance in their matched normal samples.

DISCUSSION

As knowledge of the mechanisms of p53 activation increases, different levels of regulation are revealed, which imply ordered sequences of post-translational modifications and protein interactions, ultimately leading to adaptation and differential susceptibility to stress. Previous data have indicated that, upon DNA damage and phosphorylation on Ser/Thr-Pro sites (in particular Thr81), prolyl isomerization of p53 by Pin1 precedes its stabilization and full activation. In this report, we have further highlighted different levels at which Pin1 stimulates p53 functional activation (summarized in Fig. 8). Pin1 is required for p53 loading on target promoters, and is itself recruited to chromatin by p53 to promote binding of p300 and acetylation of p53 on target DNA. Moreover, upon cytotoxic stress, Pin1 induces dissociation of p53 from the apoptosis inhibitor iASPP.

On the basis of our results and previous evidences, we hypothesize that Pin1 might assist structural rearrangements of p53 that occur upon DNA binding, exposing its C terminus to p300 and thus promoting the DNA-dependent acetylation and transcriptional function of p53. In fact, our data suggest that Pin1 regulates these events at least in part through phosphorylation-induced isomerization of Thr81-Pro82 within p53 PRD, supporting the notion that this domain is required for DNA-dependent acetylation of p53 by p300 (ref. 23). This, in turn, stabilizes p300 binding and directs the organization of transcription complexes on the promoter. Indeed, Pin1-mediated Pro82 isomerization is required for Ser20 phosphorylation by Chk2 (ref. 20), which promotes p53 binding to p300 (ref. 43). Thr81 regulates the transcriptional and apoptotic activity of p53, and, notably, mutations of Thr81 and Pro82 have been described in several cancers. Mutations at Pin1 target sites are less frequent than those at residues involved in DNA binding. This is to be expected, however, as we show here that alteration of multiple Pin1 consensus sites is required for complete abrogation of p53 acetylation and transcriptional activity, suggesting cooperative actions of Pin1 on different domains of p53.
Given its pleiotropic effects on numerous substrates\textsuperscript{15,16}, once recruited to chromatin, Pin1 might enhance p53-dependent transcription at several levels—for example, by modifying transcription coactivators (including p300), chromatin-remodeling factors or structural components. In addition, acetylation of Lys120 has recently been reported to direct p53 to low-affinity apoptotic promoters\textsuperscript{29}, and it would be interesting to determine whether Pin1-mediated isomerization influences this event as well.

However, acetylation is not sufficient to activate the apoptotic function of p53 unless it dissociates from the iASPP inhibitor, whose main binding site also lies within the PRD\textsuperscript{29}. Our data implicate Pin1 prolyl isomerase activity in stimulating the release of p53 from iASPP upon cytotoxic stress. Although phosphorylation of Thr81 also seems to be involved in this process, phosphorylation of Ser46 is essential in allowing Pin1 to trigger p53 dissociation from iASPP. This finding is biologically relevant, as Ser46 phosphorylation is specifically induced by severe or persistent stress conditions and represents a major event in shifting the p53 response from cell-cycle arrest to apoptosis\textsuperscript{47,48}. Notably, Ser46 phosphorylation\textsuperscript{32} and iASPP interaction\textsuperscript{29} have been shown to determine the differential apoptotic potentials of p53 Pro72 and Arg72 isoforms at the transcriptional level, and our data indicate that these events are indeed correlated, as we observed more efficient Lys373 and Lys382 acetylation and Ser46 phosphorylation of p53-Arg72 than of p53-Pro72 upon stress. This is in agreement with the reported induction of CBP binding to p53 upon HIPK2-mediated Ser46 phosphorylation\textsuperscript{13}.

It is tempting to speculate that Pin1 might also have a role in this process.

As overexpression of iASPP has been found in several tumor types\textsuperscript{38,49}, the molecular basis of the interplay between p53, Pin1 and iASPP is of potential clinical interest. We have previously reported that iASPP overexpression is common in breast carcinomas bearing WT p53 that arise in a 72 Pro/Pro genetic background\textsuperscript{25}. We have further shown that Pin1 is overexpressed in a substantial proportion of primary breast cancers bearing WT p53; however, this correlates inversely with iASPP overexpression and is more common in cancers arising in 72 Arg/Arg individuals. It is conceivable that, in tumors with p53-Pro72, overexpression of Pin1 might be selected against, as it would relieve p53 inhibition by iASPP. In contrast, the moderate interaction of iASPP with p53-Arg72 might not lead to strong selection pressure against Pin1 in cancers bearing this p53 isoform. Notably, homozygosity for the allele encoding WT p53-Arg72 is associated with greater sensitivity of tumor cells to anticancer drugs and is predictive of a more favorable clinical response to chemotherapy in individuals with head and neck cancer\textsuperscript{32}.

Pin1, together with Pro-directed kinases, governs the functions of several substrates, and it is itself under control of signaling pathways that could become aberrantly activated in cancers\textsuperscript{52}. Indeed, Pin1 abundance has been associated both positively and negatively with cancer, underscoring the relevance of the cellular context in dictating whether Pin1 inhibits or amplifies cell-growth pathways\textsuperscript{18}. In normal cells or at early stages of cancer, Pin1 could be part of a checkpoint system that surveys cell proliferation. At more advanced tumor stages, concomitant with deregulation of signaling pathways, it may instead contribute to the amplification of proliferative signals\textsuperscript{42}.

In the first case, high levels of Pin1 could improve the apoptotic performance of WT p53 (and of p73; see ref. 34) upon drug treatment, particularly when levels of iASPP are low. Our data support this hypothesis. However, at later stages of tumor progression, when cancer cells have acquired additional changes (among them p53 mutations), high levels of Pin1 could amplify proliferative signals. Our results predict that inhibition of Pin1 may counteract the cytotoxicity and hence the clinical activity of drugs that induce p53-dependent apoptosis (for example, the effect of etoposide on p53 acetylation and dissociation from iASPP) if administered at early stages. Conversely, Pin1 inhibition could be beneficial at later stages in tumors that may have also acquired p53 mutations. Therefore, in addition to providing a clearer view into the dynamics of the modifications that allow p53 to mount an efficient response to stress, our observations may also be relevant in designing new strategies for cancer treatment.

**METHODS**

**Cell lines and treatments.** Human HCT116 p53\textsuperscript{1/+} and p53\textsuperscript{-/-} colon carcinoma, U2OS osteosarcoma, WM115 and WM278 melanoma, p53-null SaOS-2 osteosarcoma and H1299 non–small cell lung carcinoma cell lines were cultured as described\textsuperscript{34}. Doxycycline-inducible H1299 cell lines expressing p53-Pro72 or p53-Arg72 have been described\textsuperscript{32}. For generation of ponasterone A (PonA)-inducible SaOS-2 cell lines expressing p53 mutants, see Supplementary Methods online. UV dosage was 20 J m\textsuperscript{-2} for ChIP assays and 40 J m\textsuperscript{-2} for apoptosis and iASPP-binding assays; etoposide was added to cells at 50 \mu M for 12 h. Deacetylase inhibitors (1 \mu M for 4 h; peptidylprolyl isomerase–parvulin proteasome inhibitor (Sigma), 50 \mu M for 4 h; peptidylprolyl isomerase–parvulin inhibitor PIB (diethyl-1,3,6,8-tetrahydro-1,3,6,8-tetraoxobenzoin[3,8]phenanthroline-2,7-diacetate; Calbiochem), 1.5 \mu M for 48 h; PonA (Invitrogen), 2.5 \mu M for 12 h. Deacetylase inhibitors (1 \mu M trichostatin A and 5 \mu M nicotinamide) were added to cells for 4 h before analysis of p53 acetylation. Cells were transfected with Lipofectamine 2000 (Invitrogen). For RNAi, cells were transfected with 50 nM of the following double-stranded siRNA

![Diagram](https://example.com/diagram.png)
oligonucleotides: siRNA Pin1 (I), sense 5'-GCCAUUUGAGAGGCGUCG
GdTdT-3' siRNA Pin1 (II), sense 5'-CGGGAGAGGAGGACUUUGGdTdT-3';
or scrambled control double-stranded RNA. Where not otherwise specified,
siRNA Pin1 (I) was used. For iASPP RNAs, Draconaro SMARTpool siRNA
was used.

Plasmids. pcDNA3-Pin1 and pcDNA3-Pin1-C113A, pGEX-Pin1 (ref. 17),
pGEX-Pin1–C113A27 (also described as C109A27), pcDNA3-Pin1-S67E27,
EGFP-Pin1 (ref. 34), pcDNA3-p53-T81A and pcDNA3-p53-S46A27,
pcDNA3-p53-P8220 and pcDNA3-1aSPP-V5 (ref. 28) have been described
previously. pGFP-3p30 was a gift, as were pcDNA3-p53-Pro72, pcDNA3-p53-
Arg72 and pcDNA3-p53-R175H (see Acknowledgments). SerThr-to-Ala p53
mutations were generated by PCR-based site-directed mutagenesis (Supplemen-
tary Methods).

RNA extraction and reverse-transcription PCR. Total RNA was extracted with
TRizol (Invitrogen), and 5 μg was reverse-transcribed using SuperscriptIII
reverse transcriptase and random primers (Invitrogen). Reverse-transcription
(RT)-PCR primer sequences are listed in Supplementary Methods.

In vitro binding, co-immunoprecipitation assays and western blotting. GST
pull-down assays were done as described27. For p53-p30 co-immunoprecipita-
tion, cells were lysed in 25 mM HEPES-KOH (pH 8), 150 mM KC1, 2 mM
EDTA, 1 mM DTT and 0.1% (w/v) Nonidet P-40, and cleared lysates were
incubated with antibodies specific to p300 or GFP. For p53-iASPP co-
immunoprecipitation, cells were lysed in 50 mM Tris-HCl (pH 8), 150 mM
NaCl, 1 mM EDTA, 1% (w/v) Nonidet P-40 and 0.5% (v/v) sodium deox-
cycholate with protease inhibitor cocktail (Sigma), 1 mM PMSE, 5 mM NaF and
1 mM Na3VO4, and incubated with anti-p53 (FL-393, Santa Cruz Biotech),
anti-iASPP (pAbiASPP1) or anti-V5 (Invitrogen). Where required, antibodies
were covalently bound to protein A– or protein G–Sepharose (Amersham)
using 5 mg/ml dimethylpimelimidate (Pierce). Pin1-specific rabbit polyclonal
antiserum27, pAbiASPP1 rabbit polyclonal antibody and the mAbiASPP93.4
monoclonal antibody to iASPP29 have been described previously. GFP-specific
polyclonal antisera (see Acknowledgments) was affinity-purified. Poly-
clonal antibody to HMGA1b was a gift (see Acknowledgments). Other
antibodies were anti-p53 DO-1 and FL-393 (Santa Cruz Biotech); anti-
phospho-Ser15-p53 and anti-acetyl-Lys382-p53 (Cell Signaling Technology);
anti-acetyl-Lys373-p53 (Upstate); anti-p300 NM11 (BD-Pharmingen); anti-
acetylsine (Ab409/11A1 Abcam); rabbit polyclonal anti-actin (Sigma); and
anti-p21 C-19 (Santa Cruz Biotechnology). Band intensities on autoradiograms
were quantified using ImageJ software (http://rsb.info.nih.gov/iij/).

Apoptosis assays. Apoptosis was determined by cytometerimetry (Bio-Rad
Bryte HS) using the Annexin V-FITC Apoptosis detection kit (Sigma).

Cell fractionation. Proteins were separated into chromatin-bound and free
fractions by standard methods26. Briefly, 106 cells were incubated for 20 min on
ice in 10 mM PIPES (pH 7.0), 100 mM NaCl, 3 mM MgCl2, 300 mM sucrose
and 0.1% (w/v) Nonidet P-40. Soluble and insoluble fractions were collected by
centrifugation at 2,500 g for 5 min at 4 °C and added to Laemmli sample buffer.

Chromatin immunoprecipitation. ChIP was performed as described31 (Sup-
plementary Methods). Antibodies were as follows: Pin1-specific antisera (see
above); anti-p53 FL-393 (Santa Cruz Biotechnology) and polyclonal antibody
421 (Ab-1 Calbiochem); anti-acetyl-Lys373-p53 (Upstate) and anti-acetyl-
Lys382-p53 (Cell Signaling Technology); and anti-p300 NM11 (BD-Pharmin-
gen). Control antibody was anti-HA Y11 (Santa Cruz Biotechnology).
DNA–protein complexes were recovered with protein A/G PLUS-Agarose
(Santa Cruz Biotechnology). Semi quantitative PCR products were quantified
with Kodak Digital Science Id 2.0.2 software. Real-time PCR was performed on
an ABI PRISM 7000 cycler, using TaqMan Universal PCR Master Mix (Applied
Biosystems) as described27. Promoter occupancy was calculated as the per-
centage of input chromatin immunoprecipitated using the 2-ΔΔCt method31. Primer
sequences and PCR settings are available upon request.

In vitro acetylation. HA-p53 was produced in vitro using the TNT T7 Coupled
Transcription/Translation System (Promega) and immunoprecipitated using
anti-HA linked to protein G–Sepharose. Beads were washed in 50 mM Tris-HCl
(pH 7.5), 10 mM MgCl2, 5 mM NaF and 1mM Na3VO4 and incubated with
500 ng of recombinant GST-Pin1 or GST–Pin1–C113A for 20 min at room
environment. Beads were washed extensively with 50 mM Tris-HCl (pH 7.5),
1 mM DTT and 10% (v/v) glycerol and then resuspended in 40 μl of the same
buffer. The acetylation reaction contained 100 μM acetyl-CoA, and 200 ng
purified recombinant p300 (see Acknowledgments) was added. After 1 h
incubation at 30 °C, the beads were washed and resuspended in Laemmli
sample buffer. p53 acetylation and total abundance were detected using the
antibodies anti-acetyl-Lys382–p53 (Cell Signaling) and FL-393 (Santa Cruz).
Recombinant WT GST-Pin1 and GST-Pin1–C113A were produced in bacteria
and purified using glutathione-Sepharose as described27.

Nucleic acid isolation, gene analysis and immunohistochemistry. Primary
breast cancers were all ductal carcinomas of no special type. Tumor tissue
was obtained and used with fully informed patient consent and approval of
the S. Croce e Carle hospital ethical committee. For each cancer, the
diagnosis of invasive ductal carcinoma, not otherwise specified (NOS), was
confirmed by independent histopathology review. Normal and tumor
tissue were obtained from tissue sections by microdissection. The presence
of an adequate proportion of tumor tissue was confirmed histologically
before analysis.

Genomic DNA and messenger RNA were isolated using Qiagen kits. Germline
p53 genomic 72 single-nucleotide polymorphism genotypes were
confirmed by amplification of individual exons and sequencing of multiple
domes. Expression of iASPP and Pin1 were analyzed using quantitative PCR.
Primers sequences and reaction conditions are described in Supplementary
Methods. For both Pin1 and iASPP, overexpression was defined as at least a
three-fold greater mRNA abundance in the cancer relative to matched normal
tissue. In additional experiments in cell lines and primary cancers, we verified
that Pin1 mRNA levels measured in the quantitative PCR assay correlated with
protein expression determined by western blotting and immunocytocmetry,
concluding that a cutoff of three-fold overexpression of mRNA accurately
predicts an increase in Pin1 protein.

Statistical analysis. For ChIP and apoptosis assays, P-values were obtained
from two-tailed unpaired t-tests. For tissue analysis, P values were obtained from
z-tests with continuity corrections.

Note: Supplementary information is available on the Nature Structural &
Molecular Biology website.

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AUTHOR CONTRIBUTIONS

E.M., F.T. and J.G. performed biochemical and cell biology experiments, P.S. and
M.G. analyzed tissue samples, X.L. produced constructs and reagents, and T.C.
supervised P.S. and M.G. and produced reagents. G.D.S. was responsible for the
overall project.
**MATERIALS and METHODS**

**Cell lines and treatments.** WI38 are human lung fibroblasts; IMR90 are immortalized human lung fibroblast; HaCat are immortalized human keratinocytes; HCT116 p53+/+ and RKO are human colon-carcinoma cells, HCT116 p53+/-, derived from wt p53-containing HCT116 by gene disruption (Bunz et al., 1998); U2OS are human osteosarcoma cells; U2OS/ER-E2F1 inducible cell line was kindly provided by K. Helin and is described in (Muller et al., 2001); SaOS-2 are human p53-null osteosarcoma cells; H1299 are human p53-null non–small cell lung carcinoma cells; MCF-7, SkBR-3 and MDAMB468 are human breast carcinoma cells; HeLa are human cervical carcinoma cells; 293T are a derivative of the 293 kidney carcinoma cells and constitutively express the simian virus 40 (SV40) large T antigen; SHSY5Y are human neuroblastoma cells; HL 60 are human promyelocytic leukemia cells.

UV dosage was 40 J m⁻² for 18 h in apoptosis assays and for 12 h in iASPP-binding assays; Etoposide was added to cells at the concentration and time indicated. N-CBZ-Leu-Leu-Leu-al proteasome inhibitor (Sigma), 50 µM for 4 h; Ciclohexymide (CHX), 50 µM for the time indicated.

Cells were transfected with Lipofectamine 2000 (Invitrogen). For RNAi, cells were transfected with Lipofectamine RNAiMAX (Invitrogen) with 50 nM of the following double-stranded siRNA oligonucleotides:

- siRNA Pin1 (I), sense 5’-GCCAUUUGAAGACGCCUCGdTdT-3’
- siRNA Pin1 (II), sense 5’-CGGGAGAGGAGGACUUUGAdTdT-3’
- siRNA Brd7-1, sense 5’-GCACGTATGGAGTTCGAAAdTdT-3’
- siRNA Brd7-2, sense 5’-CCAGAGACCATTATTATATAdTdT-3’

as control a siCONT RISC Free (Dharmacon) double stranded RNA oligonucleotide was used.

**Plasmids.** pcDNA3-p53wt and pcDNA3-p53-R175H were provided by L. Banks; pCDNA3-p53-4M and pCDNA3-p53 6M, pCDNA3-p53T81A, pCDNA3-p53S46A and all the pCDNA3 p535M were produced by site directed mutagenesis from pCDNA3 p53wt; pCDNA3-HA-Pin1 was cloned in the lab by P. Zacchi; pcDNA3-HAp73α and pcDNA3-
myc$p63\alpha$ have been kindly provided by G. Melino and F. McKeon respectively; pCDNA3 Flag Brd7 and pCDNA3-Flag $\Delta BD$ Brd7 were kindly provided by T. Dobner; Brd7 expression plasmid for the expression of MBP-Brd7 fusion proteins in bacteria were all obtained by cloning the entire Brd7 CDS, or its portions, in frame with Maltose binding protein (MBP) gene of pMAL-2X expression vector. To obtain Brd7 deletion derivatives, defined portions of the Brd7 CDS were PCR-amplified with specific primers using as a template pCDNA3-Flag Brd7.

**Antibodies.** Endogenous and overexpressed p53 were detected using DO-1 monoclonal antibody or FL-393 polyclonal antibody (Santa Cruz). Other commercial antibodies were rabbit polyclonal anti-aceyl-Lys382-p53 (Cell Signaling Technology); rabbit polyclonal anti-p63$\alpha$ H-129 (Santa Cruz); mouse monoclonal anti-p300 NM11 (BD-Pharmingen); mouse monoclonal anti-acetyllysine (Ab409/11A1 Abcam); mouse monoclonal anti-Flag (M2 Sigma); rabbit polyclonal anti-actin (Sigma); mouse monoclonal anti-BrdU (GE Healthcare)and anti-p21 C-19 (Santa Cruz Biotechnology). Purification and production of p73 rabbit polyclonal serum and Pin1 polyclonal serum is described in Mantovani et al., 2004 and Zacchi et al., 2002, respectively. Anti iASPP pAbiASPPN1 rabbit polyclonal antibody and the mAbiASPP49.3 monoclonal antibody to were kindly provided by X. Lu and are described in Bergamaschi et al., 2006; polyclonal antibody against HMGA1b was kindly provided by G. Manfioletti.

Anti Brd7 rabbit polyclonal serum was raised against the C-terminal portion (aa361-651) of human Brd7. A recombinant protein bearing Brd7 C-terminal portion (aa 361-651) in fusion with MBP was expressed in BL-21 E.coli bacterial strain and affinity-purified with amiloyd resin (NEB E8021S). The purified protein has been injected in a rabbit to induce immunization. The sera deriving from the bleedings have been tested for the presence of Brd7 antibody by western blot (Figure 36 A). The antiserum derived from the 5th bleed has been affinity purified. Briefly, bacterially expressed Brd7 C-terminal portion (aa 361-651) in fusion with GST was cross-linked to glutathione Sepharose4B beads (GST was used instead of MBP to avoid purification of anti-MBP antibodies) and incubated over-night with the serum. Upon washing with 10 ml PBS, 10 ml Tris pH8, 10 ml SDS 0,1% in PBS, 20 ml PBS, 10 ml NaCl 400mM in PBS and 20 ml PBS, the antibody was eluted from the resin with glycine 0,1M pH 2.8 and pH was adjusted to 7.5-8 with Tris pH8. The antibody purified was then tested for selectivity and affinity in western blot (Figure 36B).
MATERIALS and METHODS

**In vitro pull-down assays.** Cells were lysed in lysis buffer (LY) (300 mM NaCl, 50 mM Tris pH 7.5, 0.5% NP40, 10% glycerol, 1 mM PMSF, 5mM NaF and 1mM Na3VO4) for 20 min at 4°C rocking and lysates were cleared by centrifuging for 10 min at 13000 rpm at 4°C. The supernatant was diluted 1:2 with pull down buffer (PD) (50 mM Tris pH=7.5, 0.5% NP40, 10% glycerol, 1 mM PMSF, 5mM NaF and 1mM Na3VO4). GST-Pin1 fusion protein and GST were extracted from bacteria in GST purification buffer (250mM Tris pH 7.5, 1% Triton X-100, 0.5% NP40, 0.1% Tween-20, 0.2%SDS) and purified by incubation with glutathione-Sepharose 4B beads 4hrs at 4°C and washed three times at 4°C with PBS. The beads were equilibrated with binding buffer (150mM Tris pH 7.5, 0.5% NP40, 10% glycerol). Cell lysates were precleared with glutathione Sepharose 4B beads for 1 h at 4°C and then incubated with GST or GST-Pin1 proteins bound to glutathione Sepharose 4B beads. After incubation at 4°C for 3 hrs, the beads were washed five times in binding buffer, and proteins bound to the beads were eluted into sample buffer, followed by SDS/PAGE, and Western blots. λ-phosphatase treatment was performed where indicated by incubating half of the cell lysate (w/o phosphatase inhibitors) for 1h with 100 units of λ-phosphatase at 30°C.

IVTT proteins were obtained by incubating 1µg of the indicated expression vectors with 12.5 µl rabbit reticulocyte lysate, 5 µl IVTT buffer (2mM MgCl2, 50mM KOAc, 1mM NTP, 1mM creatine phosphate), 20 units of T7 RNA Pol, , aminoacid mix (minus Met) and 35S-Met to a final volume of 25 µl, for 1h at 30°C (Promega) following the manufacturer’s instructions. IVTT proteins were diluted in MBP-PD buffer (150mM Tris pH 0, 50 mM Tris pH=7.5, 1% NP40, 10% glycerol) and pull down was performed as described above. MBP-fusion proteins and MBP were purified as described above with amiloyld resin (NEB).
E8021S) in Column buffer (20mM Tris-HCl pH7.5, 200mM NaCl, 1mM EDTA pH8, and 10mM Beta-mercaptoethanol)

**Co-immunoprecipitation assays.** For p53-iASPP coimmunoprecipitation, cells were lysed in 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 0.5% sodium deoxycholate with protease inhibitor cocktail (Sigma), 1 mM PMSF, 5 mM NaF and 1 mM Na3VO4, and incubated with anti-iASPP (pAbiASPPN1) or anti-V5 (Invitrogen) covalently bound to protein A/G–Sepharose (Amersham) using 5 mg/ml dimethylpimelimidate (Pierce).

For p53-Brd7 coimmunoprecipitation, cells were lysed in 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% NP-40, 10% glycerol, with protease inhibitor cocktail (Sigma), 1 mM PMSF, 5 mM NaF, 1 mM Na3VO4, 1µM TSA and 5 µM nicotinamide and incubated with anti-Flag monoclonal antibody (Sigma) or anti-Brd7 polyclonal antibody covalently bound to protein A- or protein G–Sepharose (Amersham) using 5 mg/ml dimethylpimelimidate (Pierce). For the co-immunoprecipitation of the endogenous p53 and Brd7 pre-immune rabbit serum was used as a control.

**Chromatin immunoprecipitation (ChIP).** Cells were crosslinked with 1% formaldehyde in DMEM for 10 min, neutralized with 125 mM glycine pH 2.5 in PBS and washed in PBS. Nuclei were prepared by hypotonic lysis (5mM Pipes pH 6.8, 85 mM KCl, 0.5% NP40) and centrifugation, and resuspended in RIPA-100 buffer (20 mM Tris HCl, pH 7.5, 100 mM NaCl 1 mM EDTA 0,5% NP-40 0,5% deoxycholate 0,1% SDS) with protease inhibitor cocktail (Sigma), 1mM PMSF and phosphatase inhibitors (NaF 5mM and Na3VO4 1mM). Chromatin was sonicated with Bioruptor (Diagenode) to 500-1000 bp average fragment size and cleared by centrifugation. IP was performed overnight at 4°C with either: anti-Brd7 antiserum (see above) or anti-p53 FL-393 (Santa Cruz Biotechnology) a negative control was performed in the presence of Y-11 anti-HA polyclonal antibody (Santa Cruz). DNA–protein complexes were recovered by protein A/G PLUS-Agarose (Santa Cruz Biotech.) and washed sequentially with RIPA-100 buffer, RIPA-250 buffer (20 mM Tris HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0,5% NP-40, 0,5% deoxycholate, 0,1% SDS) and LiCl solution (10 mM Tris-HCl pH 8, 1mM EDTA, 250 mM LiCl, 0.5% Na-Deoxycholate, 0.5% NP40), then resuspended in TE, digested with 2U DNase-free RNase (Calbiochem) for 30 min at 37°C, and incubated o/n at 68°C with 300 mg/ml Proteinase K
(Invitrogen) in 0.5% SDS, 100 mM NaCl to digest proteins and reverse crosslinks. After purification by phenol-chloroform extraction and ethanol precipitation, DNA was resuspended in H$_2$O and 1/10 volume was used for quantification. Real Time PCR was performed on ABI PRISM 7000, using TaqMan Universal PCR Master Mix (Applied Biosystems), with primers and probes (Applied Biosystems) and amplification settings as described in Kaeser and Iggo, 2002.

p21 fw: 5’- GTGGCTCTGATTGGCTTTCTG -3’,
p21 rv: 5’-CTGAAAAACAGGCAGCACAAG-3’,
p21 probe: 5’-TGGCATAGAAGAGGCAGCTGGCTATTTTG-3’

GAPDH fw: 5’- GTATTCCCCCAGGTATACAT-3’,
GAPDH rv: 5’-TTCTGTCTTCCACCTCACTCC-3’
GAPDH Probe: 5’-TGGCATAGAAGAGGCAGCTGGCTATTTTG-3’

Diluted inputs (1/10 and 1/100) were used as standards for each primer set. Promoter occupancy was calculated as a percent of input chromatin immunoprecipitated using the 2$^\Delta$CT method.

**RNA extraction and RT-PCR.** Total RNA was extracted with TRI Reagent (Sigma) according to manufacturer's instructions. RNA treated with RQ1 RNase-free DNase (Promega) for 30 min at 37°C and reaction was stopped by incubating with 1mM EGTA pH8 for 10 min at 65°C. 500 ng of total RNA were reverse-transcribed using ImProm-II Reverse Transcriptase(Promega) according to the manufacturer’s instructions. Real Time PCR was performed on cDNAs on ABI PRISM 7000, using TaqMan Universal PCR Master Mix (Applied Biosystems) or with Fast SYBR Green Master Mix (Applied Biosystems). Primers and probes for p21 mRNA are

p21 fw 5’- CTGGAGACTCTCACGGGTCGAAA-3’,
p21 rv: 5’GATTAGGGCTTCCTCTTGGAGAA-3’,
p21 probe: 5’-GGCAGACCACAGCATGACAGATTTCTACC-3’

GAPDH primers and probe sequences are:
FW: 5’-TTTGGAGGGATCTCGCTCCT-3’,
RV: 5’-CAACTACATGGTGGTACATGTTCC-3’;
Probe:5’-TGGCATAGAAGAGGCTGGCTATTTTG-3’;
For Mdm2 PRIMER&PROBE 20X mix (Hs00242813_m1) from Applied Biosystems; for PUMA(BBC3): PRIMER&PROBE 20X mix (Hs00248075_m1) from Applied Biosystems;
Brd7 amplification was performed with Fast SYBR Green Master Mix (Applied Biosystems) with primers:

FW: 5'-CTGGAGATGCCGAAGCACAC-3'
RV: 5'-TGGGATCCACAGGATGGAGA-3'.

mRNA abundance was calculated relative to GAPDH housekeeping gene using the $2^{\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

**Transient Transactivation Assays.** H1299 cells were co-transfected in a 24-well plate with 500 ng of p21-prom-luc or Bax-prom-luc reporter constructs together with, respectively, 2 µg of pCDNA3-HA, 50 ng of pCDNA3 p53 either alone or along with increasing amounts (0.5 1 or 2 µg) of pCDNA3-Flag-Brd7, or with 2 µg of pCDNA3-Flag-Brd7. For normalization of transfection efficiency, 12.5 ng of pRL-CMV reporter (Promega), constitutively expressing the *Renilla reniformis* luciferase, was included. After 36 hrs, cells were lysed and assayed for luciferase activity using the Dual Luciferase kit (Promega). H1299 cells in a 60 mm plate were transfected by RNAiMAX (Promega) with 2 µl of 20 µM double-stranded siRNA oligonucleotides specific for Brd7 (siBrd7) or with the same amount of RISC Free siRNA control (siC) (Dharmacon). After 12hrs the cells were splitted to a 24 well plate and transfected by Lipofectamine 2000 (Invitrogen) with 500 ng of Luc reporters, 12.5 ng of pRL-CMV (Promega) together with either siBrd7 or siC. After 36 hrs, cells were lysed and assayed for luciferase activity using the Dual Luciferase kit (Promega).

**Apoptosis assays.** Apoptosis was determined by cytofluorimetry (Bio-Rad Bryte HS) using the Annexin V-FITC Apoptosis detection kit (Sigma).

**BrdU Incorporation Assays.** Cells were plated on a slide in 35 mm dish and transfected with either 6 µl of 20 µM double-stranded siRNA oligonucleotides specific for Brd7 or with the same amount of RISC Free siRNA control. After 60 hrs cells were treated with etoposide 50 µM or left untreated for 12 hrs. Cells were pulsed with 3 µl of 20 mM bromodeoxyuridine (BrdU, Sigma) for 3 hrs. After pulsing, cells were fixed, permeabilized and denatured. BrdU incorporation was measured by immunofluorescence using an anti-BrdU antibody (GE Healthcare) and the nuclei were stained with Hoechst. The stained
cells were visualized under a fluorescence microscope and at least 300 cells were scored for BrdU incorporation.

**Cell fractionation.** Proteins were separated into chromatin-bound and free fractions by standard methods (Geng et al., 2003). Briefly, 10⁶ cells were incubated for 20 min on ice in 10 mM PIPES (pH 7.0), 100 mM NaCl, 3 mM MgCl₂, 300 mM sucrose and 0.1% (v/v) Nonidet P-40. Soluble and insoluble fractions were collected by centrifugation at 2,500g for 5 min at 4°C and added to Laemmli sample buffer.
REFERENCES


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REFERENCES


REFERENCES


anchorage-independent growth in vitro and induces tumour formation and cachexia in vivo. Melanoma research 9, 279-291.


REFERENCES


REFERENCES


REFERENCES


REFERENCES


Liang, S.H., and Clarke, M.F. (1999b). The nuclear import of p53 is determined by the presence of a basic domain and its relative position to the nuclear localization signal. Oncogene 18, 2163-2166.


REFERENCES


REFERENCES


REFERENCES


References


REFERENCES


REFERENCES


REFERENCES


139


She, Q.B., Huang, C., Zhang, Y., and Dong, Z. (2002). Involvement of c-jun NH(2)-terminal kinases in resveratrol-induced activation of p53 and apoptosis. Molecular carcinogenesis 33, 244-250.


REFERENCES


REFERENCES


REFERENCES


REFERENCES


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