UNIVERSITY OF CALIFORNIA, SAN DIEGO

Functional analysis of p53 N-terminal phosphorylation and C-terminal multiple posttranslational modifications in regulating p53 responses to DNA damage

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by

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The dissertation of Lijin Feng is approved, and it is acceptable in quality and form for publication on microfilm:

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Chair

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DEDICATION

To

My parents and Grandpa

for their love and support

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ABSTRACT OF THE DISSERTATION

Functional analysis of p53 N-terminal phosphorylation and C-terminal multiple posttranslational modifications in regulating p53 responses to DNA damage

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Lijin Feng

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Professor Cornelis Murre, Chair

p53 is the most commonly mutated tumor suppressor gene in human cancers. Activation of p53 maintains the genomic integrity and protects the organism against the propagation of cells that carry damaged DNA with potentially oncogenic mutations. While p53 protein is normally short-lived and kept at low levels in a relatively inactive form, upon genotoxic and cellular stresses p53 is transiently stabilized and activated as a transcription factor. The increase in protein level and transcriptional activity is attributed largely to the various posttranslational modifications of p53, including the N-terminal phosphorylation, and the multiple C-terminal modifications such as ubiquitination, acetylation, sumoylation, neddylation

and methylation. Specifically, the six Lysine residues at p53 C-terminus can be posttranslationally modified by diverse mechanisms, among which the ubiquitination of those residues has been thought to be required for Mdm2-mediated ubiquitin-directed proteasomal degradation, and the acetylation is suggested to activate p53 transcriptional activity and contribute to its stabilization. To investigate the physiological functional outcome of the C-terminal modifications in regulating p53 stability and activity, we introduced Lysine to Arginine missense mutations at the six Lysine residues (K6R) into the endogenous p53 locus in mouse embryonic stem cells (ESCs). Unexpectedly, analysis of mouse ESCs, mouse embryonic fibroblast (MEFs) and thymocytes has concluded that ubiquitination of C-terminal Lysine residues is not required for efficient p53 degradation either before or after DNA damage. The outcome of loss of all potential posttranslational modifications is a modestly impaired p53 activity after DNA damage in a promoter-specific manner. Phosphorylation of Ser46 of human p53 is suggested to play an important role in activating p53-dependent apoptosis. To address the physiological role of Ser46 phosphorylation, we introduced Ser46 to Alanine mutation (S46A) into the human p53 knock-in (HUPKI) allele in mice. Consistent with the previous cell line studies, transactivation of p53 targeting apoptotic genes is preferentially affected by the mutation and p53-dependent apoptosis after DNA damage is partially impaired in mutant thymocytes and in E1A/Ras-overexpressed MEFs. In addition, Ser46 phosphorylation may contribute to preventing spontaneous immortalization of cultured MEFs and to oncogene Ras-induced premature senescence of MEFs.

Chapter 1

Introduction

The p53 gene is the most commonly mutated tumor suppressor gene in human cancers (1). Around 50% of all human cancers show mutations in p53, and a further fraction show a functional inactivation of the protein (2). During the past twenty-seven years, the roles of p53 in cellular responses to various stresses and in tumor suppression have been well established.

p53: the early years

p53 was firstly discovered in transformed cell lines as a 54kD protein co-immunoprecipitated with SV40 large T antigen, which is needed for the ongenic property of SV40 virus (3, 4). Another report found the same 54kD protein associated with adenovirus protein E1B-55Kd (5). In addition, elevated p53 protein level was detected in several human tumor cell lines compared to its undetectable level in primary cells, leading to the hypothesis that p53 might be a proto-oncogene (6). The identification of p53 as a tumor suppressor occurred after several groups showing that expression of wild-type mouse p53 gene can inhibit tumor progression, by controlling the cell growth or by mediating apoptosis (7-13).

Sequence analysis of p53 in various human tumors indicated that mutations in the p53 gene were common events in diverse human malignancies, which included point mutations resulting in single amino acid substitutions, base-pair deletions, and chromosome 17p allelic deletions leaving only mutant forms of p53 (14-17). Most of the point mutations are missense mutations, the majority of which

are clustered within highly conserved region of p53, and there are at least three mutation 'hot spots' affecting residues 175, 248 and 273 (18). Several subsequent protein analysis suggested that the mutant p53 protein expressed in tumor cell lines had a much longer half-life and greater level compared to the wild-type p53, which was kept at a low steady-state level, resulting in large amounts of the mutant protein in transformed cells or tumors, and that mutant p53 usually lost or reduced its transcriptional activity (19, 20). Some mutants were also shown to have an altered protein conformation, which could drive the wild-type p53 into the mutant form through oligomerization (21). The notion that p53 may play an important role in suppressing many common human tumors was further demonstrated by the finding that germ line p53 mutations were detected in the families of Li-Fraumeni syndrome (LFS), a rare autosomal dominant syndrome characterized by diverse neoplasms occurring at many different sites in the body of affected individuals (22, 23). And its importance in tumor suppression is conclusively demonstrated by the creation of the $p53^{-/-}$ mice that are highly prone to spontaneous tumor formation and develop a large spectrum of tumors (24, 25). Since these early findings were published, numerous groups have focused on studying the biological function of this molecule and the mechanisms of its tumor suppression.

Overview of p53 structure and function

The full-length human p53 gene encodes a 393-amino acid protein, which

is composed of four functionally interdependent domains: N-terminal transactivation and proline-rich domain (amino acids 1-63 and 64-92, respectively), the central sequence-specific DNA-binding domain (amino acids 110-286), the tetramerization domain (amino acids 326-355) and the C-terminal regulatory domain (amino acids 373-393) (2, 26). Besides the tumor suppressor p53, the p53 family also contains p63 and p73, both showing strong homology with p53 in terms of overall domain structure and conformation (27, 28). However, distinct from p53, both p63 and p73 have multiple isoforms resulting from variant splicing or alternative internal promoters (27, 28). Recently, it was discovered that p53 also has an N-terminally truncated isoform, ΔNp53 or p44, which uses an alternative translation start site located in exon 4 at codon 40 in human mRNA (mouse codon 41) (29, 30). The resultant p44 protein, which lacks the N-terminal transactivation domain of p53, is suggested to regulate both p53 transcriptional activity and its ubiquitination by Mdm2 and that the overexpression of p44 can accelerate the aging process and reduce the mice life span (31-33). Besides the alternative initiation of translation, Bourdon *et al* also reported that the human p53 gene contains an alternative promoter in intron 3 and transcribes various splice variants (34).

The N-terminal transactivation domain mediates p53 interaction with the basal transcriptional machinery and with the proteins that positively or negatively regulate its function as a transcription factor. It consists of two subdomains with complementary functions, the first domain (1-41) mainly carrying out

transactivation function and the second one (42-63) specifying the target genes (35, 36). Mdm2 and Mdmx, which are two critical negative regulators of p53, interact with p53 through its N-terminal transactivation domain, specifically including amino acids 19-26, inhibiting p53 transactivity or regulating its stability (37-40). The N-terminal domain also binds to the TATA-binding protein (TBP), TATA-associated factors $TAF_{II}70$ and $TAF_{II}31$, both of which are subunits of TFIID and activate transcription of target genes (41). Coactivators CBP/p300 bind to p53 through its N-terminal domain and acetylate p53 during DNA damage response, which is required for its full activation (42, 43). The role of the proline-rich region (PXXP) is not clear, although evidence suggest that it may regulate Mdm2-mediated p53 degradation and p53-dependent apoptosis (44, 45).

The central domain of p53 can bind to DNA in a sequence-specific manner. The consensus p53 DNA binding site is composed of two copies of 10bp sequence RRRC(A/T)(T/A)GYYY separated by 0-13 bp, where R is a purine and Y is a pyrimidine. It is estimated that human genome may contain two to three hundred of such p53 responsive elements. The importance of the central DNA-binding domain is further underscored by the fact that the majority of tumor-derived p53 mutations occurs in this region and disrupt the sequence-specific DNA-binding activity of p53 (1). Crystallography study of p53 structure suggested that those mutations located within DNA-binding domain could either change the conformation of the central domain or directly change the amino acids that mediate p53 interaction with DNA.

Most of p53 mutations in Li-Fraumeni syndrome are missense mutations located in the central DNA binding domain. Two recent papers, by analyzing transgenic mice, have shown that the mutant p53 allele harboring hot-spot mutations within DNA-binding domain gained additional properties to promote tumorigenesis *in vivo* and developed different spectrum of tumors than $p53^{-/-}$ mice (46, 47). One mechanism of this gain-of-function phenotype has been proposed to be inactivation of p53 family members, p63 and p73, by the dominant negative mutant p53 allele (46, 48).

p53 exists in a latent form in unstressed cells, and transforms to its active tetrameric form upon various cellular and genotoxic insults. The oligomerization of p53 is specifically mediated by its tetramerization domain (residues 324-355). The structure of this domain contains a dimer of a dimer with two β sheets and two α helices, which are held together to form a four-helix bundle (49). It was suggested that the active p53 tetramer could bind to DNA with much higher affinity and interact with other proteins more efficiently, which is required to fully activate p53 function (50). On the other hand, the oncogenic forms of p53 can inhibit the tumor suppressor activity of coexpressed wild-type p53 through forming heterotetramers (51).

Different from the other typical transcription factors, such as stimulatory factor 1 (Sp1) and homeodomain proteins, p53 contains two DNA-binding domains with distinct properties. The central DNA-binding domain of p53 binds to DNA in a

sequence-specific manner, while the C-terminus (363-393aa) possesses sequence non-specific DNA-binding ability (52, 53). To date the role of the extreme C-terminus of p53 in regulating p53 sequence-specific DNA-binding activity remains controversial. Earlier studies established the negative regulator role of the p53 C-terminus, with the evidence that deletion of the last 30 amino acids, mild proteolysis of full-length p53, casein kinase II (phosphorylation of C-terminal residues), or binding to an antibody (pAb421) whose epitope resides within the C-terminus significantly stimulated the sequence-specific DNA binding, as measured by an electrophoretic mobility shift assay (EMSA) using short DNA oligonucleotides as p53 binding substrates (54). Further support came from the studies demonstrating that by microinjecting a basic peptide spanning the epitope of pAb421 in the C-terminus region into the cells, p53-dependent transactivation and pro-apoptotic activity can be markedly enhanced *in vivo*, or even be restored in at least some p53 mutant living cells (55-58). Further, the p53 C-terminus can undergo extensively posttranslational modifications in response to genotoxic stresses, including phosphorylation at Ser315 by cyclin-dependent kinase (CDK) and Ser378 by protein kinase C, and the multiple modifications of the six C-terminal lysine residues, such as acetylation, ubiquitination, neddylation and methylation (59-66). Specifically, Gu *et al.* suggested that CBP/p300 mediated acetylation of p53 can increase p53 sequence-specific DNA-binding activity *in vitro* by electrophoretic mobility-shift assay (EMSA) using short oligonucleotides (62). Several subsequent

studies also supported that the acetylation of p53 can dramatically stimulates its sequence-specific DNA-binding activity both *in vitro* and *in vivo*, possibly caused by an acetylation-induced conformational change (63, 67, 68). Therefore, it was hypothesized that the p53 C-terminus acts as a negative regulator of p53 DNA-binding activity and the C-terminal modification can cause allosteric changes in the conformation of the protein, thereby converting it from a latent form to an activated state for DNA binding (62, 69).

However, this hypothesis was challenged by several following studies. By EMSA assay with long carrier DNA molecules, Anderson et al. draw the conclusion that only the interaction of long, nonspecific DNA with p53 C-terminus strongly negatively regulates p53 DNA-binding by steric hindrance (70). Once the long nonspecific DNA molecules are removed, addition of pAb421 can no longer stimulate p53 sequence-specific DNA binding activity, which contradicts the model that the C-terminus regulates the conformation of p53. Furthermore, Ayed ***et al.*** use NMR experiments to show that the conformation of dimeric p53 remains the same both containing and lacking the C-terminus and that there is no interaction between the C-terminal domain and any other domains of p53 (71). Another recent study indicates that p53 binds to its cognate promoters constitutively *in vivo* and genotoxic stresses fails to increase the DNA binding level of p53 by chromatin immunoprecipitation (ChIP) (72). In addition, C-terminal acetylation does not increase the p53 DNA-binding activity when assayed by binding to an artificially

reconstituted chromatin (73). And the deleted form of p53 (p53ΔC30), which lacks the last 30 amino acids, exhibits a weaker binding to p53 target promoters, implying the C-terminus is required for in vitro transcription from p53 target promoters. This and other studies instead show that p53 acetylation is important for the recruitment of co-activators (73, 74). Further, it is suggested that C-terminus is required for high-affinity binding of p53 to minicircular DNA (75) and stem-loop structures (76). Recent studies further indicate that p53 C-terminus is required for p53 linear diffusion on chromatin and its efficient DNA binding as well as transactivation of target promoters *in vivo* (77). However, acetylation and other modifications of C-terminus do not increase this p53 activity, which support the positive role of p53 C-terminus in regulating p53 DNA-binding both *in vitro* and *in vivo*.

p53 stabilization and activation after cellular stresses

1. Signaling to p53

The mechanism of how upstream signals transmitted from damaged DNA or insulted cellular microenvironment to activate the p53 pathway is not completely understood, yet it's now clearer that p53 can be activated through multiple ways in response to a variety of cellular and genotoxic stresses, including those disrupting the integrity of the DNA template – Gamma and UV irradiation, alkylation of bases, depurination of DNA, and reaction with oxidative free radicals, and those altering the physiological environment - hypoxia, ribonucleotide depletion, change of

temperature and pH, mitotic spindle damage, and oncogene activation (78). In unstressed cells, p53 is present in latent state and in extremely small quantities in most cells and displays a rapid turnover rate, while both p53 stability and transcriptional activity are greatly induced following stresses in a cell-type and stress-type dependent manner (79).

Among all examined forms of genotoxic damages that activate p53, DNA damage such as ionizing irradiation (IR) and ultraviolet (UV) has been extensively studied. IR, causing DNA single strand or double strand breaks, activates p53 mainly through ATM and CHK2 kinase, while UV which induces DNA base damage is more engaged in ATR, CHK1 and casein kinase-2 pathway (79). Several studies have pointed out the important differences in the cellular responses to IR and UV. Although p53 protein level increases in cells exposed to either IR or UV, UV radiation can induce much greater p53 transcriptional activity than IR (80). In mouse and human fibroblasts and epithelial cells, UV activates p53-dependent apoptosis more effectively than IR (80, 81). Oxidative stress, mainly the reactive oxygen species (ROS) produced by hydrogen peroxide (H_2O_2) or other peroxide, can also cause DNA damage (82). For example, H_2O_2 can oxidize DNA and induce DNA single strand breaks, which activates p53 through ATM-dependent signaling pathway (83). Doxorubicin (DOX), a widely used chemotherapeutic agent, exhibits cardiotoxicity as an adverse side effect in cancer patients, which is linked to its ability to induce apoptosis in endothelial cells and cardiomyocytes by activation of

reactive oxygen species and p53 activity (84). DOX can induce apoptosis in human primary endothelial cells through a p53-dependent mitochondrial-mediated mechanism (85). Adriamycin can also induce p53-dependent apoptosis in oncogene E1A transformed mouse embryonic fibroblasts (86). In addition, p53 accumulation and activation can occur in response to several physiological processes that are not associated with DNA damage, including hypoxia and nucleotide deprivation (79). Hypoxia was suggested to decrease the expression of Mdm2, resulting in an inhibition of p53 export from nucleus to cytoplasm and therefore the accumulation of p53 activity in nucleus (87).

2. ubiquitin-mediated regulation of p53 protein levels

The growth inhibitory activities of p53 that play an important role in preventing the outgrowth of cells with malignant tendencies are also extremely detrimental to normal growth and development. p53 activity is therefore usually kept under very tight control, in large part by the rapid turnover of the p53 protein, which is normally maintained in tissues at extremely low level. The ubiquitin-mediated proteasomal degradation of p53 plays a critical role in regulating p53 protein stability in both unstressed and DNA damaged cells. Ubiquitin is a highly conserved 76 amino acid eukaryotic protein that can be covalently attached to Lysine residue on target protein through the sequential actions of enzymes E1, E2, and E3, called ubiquitination. Ubiquitination is a series

of highly specific enzymatic reactions, involving several E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and a great number of E3 (ubiquitin protein ligase) enzymes (88). Once modified with chains of four or more ubiquitin molecules through Lys 48 of ubiquitin, known as polyubiquitination, proteins can be efficiently targeted to the 26S proteasome where they are degraded (88). During ubiquitination, E3 ubiquitin ligases can recognize substrates and therefore are the primary specificity determining factors.

The first *in vivo* evidence of a role for ubiquitination in regulating p53 activity came from the study that discovered E6-associated protein (E6-AP) as a p53 ubiquitin ligase which can regulate p53 level in cells infected by human papillomavirus (HPV) (89). Soon after this finding, another cellular E3 ligase, mouse double-minute 2 protein (Mdm2), was discovered to be able to ubiquitinate and degrade p53 in the absence of exogenous factors (38, 90, 91). Mdm2 was firstly discovered as one of the three genes located on extrachromosomal amplifications in a spontaneously transformed murine cell line (3T3-DM) and thus named Mdm2 (92). *Mdm*2, as a proto-oncogene, is amplified in 7% of all human cancers observed in 19 tumor types (92, 93). The ubiquitin ligase activity of Mdm2 is centered within the RING finger domain at the C-terminus, which is capable of both self-ubiquitination and targeted-ubiquitination of other proteins (94). Mdm2 protein itself is dynamically regulated in cells and was thought for some time to be the sole E3 ligase responsible for p53 degradation both under normal physiologic and

stressed conditions, while the basal expression of Mdm2 in murine tissues might not depend on p53 activity (95). Notably, p53 can transcriptionally activate Mdm2 expression which in turn inhibit p53 stability and activity, thus forming a negative autoregulatory feedback loop (96). The critical role of Mdm2 in negatively regulating p53 is highlighted by the studies carried out in mice where Mdm2-deficiency leads to embryonic lethality that can be completely rescued by loss of p53 (37, 97). Consistent with this notion, recent data have shown that a single nucleotide polymorphism (SN309) found in the *Hdm2* promoter results in higher *Hdm2* mRNA level and thus protein level, leading to attenuation of the p53 pathway and accelerated tumor formation in human (98).

The ability of Mdm2 to inactivate p53 relies on a direct interaction between the two proteins, where Mdm2 can either promote ubiquitin-mediated degradation of p53, or repress p53 transcriptional activity by concealing p53 from basic transcriptional machinery and its co-activators or by recruiting transcriptional co-repressors (64, 99). For example, mice with a hypomorphic allele of Mdm2 reveal increase in both p53 transcriptional activity and apoptotic function, however without an obvious increase in p53 protein level (100). CBP/p300 mediated acetylation of p53 can be inhibited by Mdm2 *in vitro* and *in vivo* through forming p300-Mdm2-p53 ternary complex (101).

Several mechanisms exist to modulate the p53-mdm2 pathway during cellular stresses, which include posttranslational modifications of p53 and Mdm2

and interactions with other cellular factors such as ARF (102). ARF (known as $p14^{ARF}$ in human and $p19^{ARF}$ in mouse) is encoded by the INK4a locus that also encodes the cyclin dependent kinase (CDK) inhibitor $p16^{INK4a}$ (103). ARF can suppress aberrant cell growth in response to oncogene activation, partly by inducing the p53 pathway through inhibiting the activities of either Mdm2 (104) or a recently identified ARF binding protein ARF-BP1 (105). The ways ARF negatively regulates Mdm2 remain controversial, including the direct association and inhibition of Mdm2 E3 ligase activity by ARF (106), or restricting Mdm2 localization to nucleolus and inhibiting its interaction with p53 (107), or promoting Mdm2-mediated ubiquitination of MdmX since MdmX is shown to be important for Mdm2 stability (108). In addition to ARF, several ribosome proteins such as L11 and L23 can also inhibit the Mdm2 activity upon nucleolar stress and lead to efficient stabilization of p53 (109, 110).

Multiple forms of posttranslational modifications of p53 and Mdm2 have been identified for their important roles in regulating p53 ubiquitination and degradation by Mdm2 under cell stress, including ubiquitination, phosphorylation, acetylation, sumoylation, neddylation and methylation (64-66, 111). Posttranslational modifications of p53 play an essential role in both stabilization and activation of the protein (112). Phosphorylation at several p53 N-terminal residues was shown to disrupt the p53-Mdm2 interaction, while acetylation at p53 C-terminus might contribute to p53 stabilization by competing with

Mdm2-mediated ubiquitination (64). In addition to the modification of p53, Mdm2 can be modified posttranslationally to prevent its interaction with p53. Mdm2 phosphorylation by Ataxia telangiectasia (ATM) was shown to disrupt p53-Mdm2 interaction (112). On the other hand, treatment of cells with IR resulted in a dramatic decrease in Mdm2 phosphorylation and this hypophosphorylation preceded p53 accumulation (113).

MdmX (also known as Mdm4) is a p53-binding protein that shares extensive homology with Mdm2, including a RING finger domain at its C-terminus and p53-binding domain at N-terminus. Unlike Mdm2, MdmX is not a p53 transcriptional target, nor is increased following genotoxic stimulation, nor does it promote p53 ubiquitination (114). The ubiquitination and degradation of MdmX is shown to be mediated by Mdm2 (108). Nevertheless, as is the case for Mdm2, MdmX is a critical negative regulator for p53 during embryonic development, since *MdmX*-null embryos exhibit lethal phenotype and loss of p53 can completely rescue the embryonic lethality (37, 97). MdmX amplification and overexpression are found in certain human malignant gliomas (115). The physiological function of MdmX remains ambiguous. It is reported that overexpression of MdmX can lead to p53 stabilization as polyubiquitinated forms of p53 readily accumulate within the nucleus but without obvious effect on p53 ubiquitination by Mdm2 (114). In addition, MdmX overexpression can also enhance p53 transactivation (116, 117). On the other hand, MdmX and Mdm2 negatively regulate p53 activity in a

non-redundant and cooperative manner, where Mdm2 is critical for regulating p53 level, MdmX is critical for the fine-tuning of p53 transcriptional activity (118). When the ratio of MdmX:Mdm2 is low, these proteins cooperatively decrease p53 levels (119). MdmX can also act as a transcriptional repressor, and inhibit the acetylation of p53 by p300/CBP (120).It remains controversial whether Mdm2 by itself is sufficient to mediate formation of poly-ubiquitin chains on p53. Both mono- and poly-ubiquitination can occur on p53. Whereas polyubiquitination can target protein for degradation by providing a recognition signal for the 26S proteasome, monoubiquitination has been implicated in a number of degradation-independent processes (121). Mdm2 was recently found to differentially catalyze mono- and poly-ubiquitination of p53 in a dosage-dependent manner (122). Therefore, low levels of Mdm2 activity induce monoubiquitination and nuclear export of p53, whereas high levels promote polyubiquitination and nuclear degradation of p53. It was proposed that distinct mechanisms are employed under different physiological settings, where Mdm2-mediated polyubiquitination and nuclear degradation may play a dominant role in suppressing p53 function when Mdm2 is malignantly overexpressed (102, 121, 123). Another possibility is that Mdm2 needs another factor to add multiubiquitin chain onto p53 in nucleus and such a factor that facilitates p53 polyubiquitination is referred as E4 ubiquitin ligase (124). For example, it was reported that p300/CBP has ubiquitin ligase activity for p53 and promotes polyubiquitination of p53 together with Mdm2, suggesting that p300

could be an E4 for p53 (125).

Whereas Mdm2 is a critical E3 ubiquitin ligase for p53, other proteins are emerging implicated in the regulation of p53 stability. For example, COP1 has been described as a direct ubiquitin ligase for p53 and promote p53 ubiquitination and degradation, and at the same time is also a p53 inducible gene (126). Similarly, Pirh2, another RING finger domain-containing protein, has been shown to interact with p53 and mediate p53 ubiquitination and degradation as an E3 ligase (127). Since Pirh2 binds to p53 at a region (residues 82-292) distinct from that to which Mdm2 binds (residues 1-51), it is conceivable that Mdm2 and Pirh2 can bind to a single p53 molecule and function in a cooperative manner.

Besides the ubiquitin-directed 26S proteasome degradation, p53 stability can also be regulated in ubiquitin-independent manner. Studies have demonstrated that native p53 without ubiquitination can be degraded by 20S proteasome and quinone oxidoreductase 1 (NQO1) can stabilize p53 by physically binding to it and preventing proteasomal degradation, which is an Mdm2-independent process (128, 129).

3. Stress-induced posttranslational modification of p53

In normal unstressed cells, p53 is maintained as a relatively inactive form at low protein levels through rapid degradation by its negative regulators, such as Mdm2. Upon various cellular and genotoxic stresses, p53 protein stability and

transcriptional activity are both significantly induced, leading to downstream effects including cell cycle arrest, DNA repair, apoptosis, senescence and differentiation. Accumulating evidence indicates that various types of posttranslational modifications play important role in regulating p53 stability and activity by modulating p53 interaction with its positive or negative regulators (130).

(1). The N-terminus: DNA-damage induced phosphorylation of p53

Human p53 can be phosphorylated at multiple Serine and Threonine sites at both the N-terminus and C-terminus by a number of kinases following DNA damage *in vitro* and *in vivo*, demonstrated by polyclonal and monoclonal antibodies recognizing specific modified sites developed by several groups (79). Studies have reported that human p53 can be phosphorylated on at least 18 sites, including seven serines, Ser 6, 9, 15, 20, 33, 37, 46 and two threonines, Thr 18 and 81 within the N-terminus (residues 1-89), and Ser 315 and Ser 392 in the C-terminal domain of human p53 (112).

In this context, phosphorylation of Ser15 can be induced both *in vitro* and *in vivo* rapidly after DNA damage by several ATM family kinases, including ATM, ATR and DNA-PK (131, 132). A number of groups have sought to elucidate the role of phosphorylation of Ser15 in regulating p53 stability and activity. One study reported that phosphorylation at Ser15 disrupted p53 interaction with Mdm2 and thereby led to p53 stabilization (133). Several other studies argued that Ser15

phosphorylation is required for recruitment of coactivators CBP/p300 and for enhancing the acetylation at C-terminal Lys residues, but dispensable for p53-Mdm2 interaction (134-136). Study from Xu lab has shown that Ser18 (mouse equivalent of human Ser15) phosphorylation is required for full p53 activation in both differentiated ES cells and MEFs in order to induce G1/S arrest, and for efficient C-terminal acetylation in MEFs but not in ES cells, and that Ser18 phosphorylation is not required for p53-Mdm2 disruption upon DNA damage (137, 138). In addition, Ser15 phosphorylation can be dramatically induced in human fibroblasts undergoing replicative senescence or oncogene Ras-induced premature senescence, implicating that this modification may be critical in regulating p53 responses to DNA damage (139, 140).

Ser20 is another N-terminal residue discovered to be phosphorylated after IR or UV, and its phosphorylation was suggested to be important for stabilizing p53 after DNA damage (141, 142). Two serine/threonine checkpoint kinases, CHK1 and CHK2, which are activated by ATM after IR, can phosphorylate human p53 at Ser20 *in vitro* (143). Two subsequent mouse model studies argued that Ser23 phosphorylation is not required for p53 stability and activity in ES cells and MEFs after DNA damage, but may be required for protein stabilization and p53-dependent apoptosis in thymocytes (144, 145). Since phosphorylation of Ser15 and Ser20 occur simultaneously after various types of DNA damage, and might be mediated through ATM signaling pathways induced by DNA double-strand breaks, the two

phosphorylation events are postulated to have synergistic functions in modulating p53 stability and activity. Chao *et al* reported that the combination of Ser18 and Ser23 phosphorylation is crucial for p53-dependent apoptosis in IR treated thymocytes and important for suppression of spontaneous tumorigenesis, but dispensable for suppression of DNA damage caused tumorigenesis (146).

Ser33 was shown to be phosphorylated by cyclin-dependent kinase (CDK)-activating kinase (CAK) *in vitro* and by p38 MAPK *in vivo* (147). Of particular interest, human p53 can be phosphorylated at Ser46 after UV by p38 MAPK or Homeodomain Interacting Protein Kinase-2 (HIPK2) *in vivo* (147, 148). In response to IR, Ser46 phosphorylation may be mediated by ATM signaling pathway (149). In addition, p53DINP1, which is encoded by a p53-inducible gene, can recruit PKCδ, mediate Ser46 phosphorylation and regulate p53-dependent apoptosis upon exposure to genotoxic stress (148, 150). Cell line transfection studies have suggested that Ser46 phosphorylation might be specifically important for p53-dependent apoptosis after DNA damage by preferentially activating pro-apoptotic target genes. For example, Oda *et al* have identified a novel p53-dependent apoptotic gene *p53AIP1* in human, induction of which may be dependent on phosphorylation of Ser46 (151). Phosphorylation of Ser46 may also be involved in UV-induced p53-dependent apoptosis, functioning synergistically with Ser33 phosphorylation (147). It is recently reported that Ser46 phosphorylation is correlated with activation of p53-dependent apoptotic genes, such as *PTEN*, to

promote apoptosis instead of cell cycle arrest (152, 153). Another *in vivo* study showed that codon47 polymorphism interrupts Ser46 phosphorylation and decreases p53-dependent apoptotic activity (154), however, the codon47 polymorphism could have other impacts on p53 activity in addition to the loss of Ser46 phosphorylation. Thr81 can be phosphorylated by Jun-N-terminal kinase (JNK) in response to DNA damage-inducing agents and the phosphorylation contributes to p53 transcriptional activity as well as stability (155).

At the C-terminus, the cyclin B-dependent kinase, p34 (Cdc2), phosphorylates p53 at Ser315 *in vivo* after DNA damage, which coincide with increased p53-dependent transcription activity (156). Interestingly, a recent study indicates that the rapid downregulation of Nanog mRNA during ES cells differentiation correlates with induction of p53 transcriptional activity and Ser315 phosphorylation, and that the absence of this phosphorylation event largely impairs p53 activity in ES differentiation possibly due to failure to recruit co-repressor mSin3a to the Nanog promoter (157). Phosphorylation of p53 at Ser392 can be induced by overexpression of cyclin E in high-density cells or induced by UV light but not IR, which may stimulate DNA-binding activity of p53 *in vitro* and mediate G1 arrest *in vivo* (158, 159).

Two novel studies uncover a potential mechanism of how p53 posttranslational modifications lead to its conformational change and regulate its transcriptional activity. Pin1 is a peptidyl-prolyl isomerase catalyzing the switch of

peptide bond between *trans* and *cis* configuration, for example, the X-Pro peptide bond. When the amino acid X in X-Pro motif is serine or threonine, it is subjected to modification by phosphorylation (160). The addition of a phosphate group to X will change the energetic constraints of the Ser-Pro/Thr-Pro bond, resulting in the selective catalyzation of a conformational change through Pin1 binding to the phosphorylated Ser/Thr-Pro motif (161). There are three phosphorylation sites involved in Ser/Thr-Pro motif, Ser33, Thr81 and Ser315, and might be the targets of regulation by Pin1. Both groups show that Pin1 can bind to phosphorylated p53 and stimulate the p53 transcriptional activity. Also, the phosphorylation of all these three sites is required for the efficient binding of Pin1 to p53 (162, 163).

(2). Regulation at p53 C-terminus

The multiple Lysine residues at the extreme carboxyl-terminal domain of p53 (the last 30aa) can be posttranslationally modified by multiple mechanisms, including acetylation, ubiquitination, sumoylation, neddylation and methylation, in response to DNA damage and other cellular stresses (62-66) (164).

Human p53 undergoes acetylation at multiple lysine residues within its C-terminal domain after a variety of stresses (165). CBP/p300, both containing histone acetyltransferase (HAT) activity, was previously discovered to be able to acetylate nonhistone proteins, such as transcription factors (62). Specifically, human p53 can potentially be acetylated by CBP/p300 at five Lysine residues, Lys370, Lys372,

Lys373, Lys381 and Lys382, both *in vitro* and *in vivo* (62, 68). Using acetylation-specific antibody, it has been shown that the acetylation of p53 at Lys373 and Lys382 is significantly induced in response to DNA damage *in vivo* (63, 67, 165). In addition, human p53 can also be acetylated by P/CAF (p300/CBP associated factor) at Lys320 *in vitro* and this acetylation event is induced by DNA damage *in vivo* (63, 67).

The role of p53 acetylation has been studied extensively. Gu *et al* firstly demonstrated that CBP/p300 mediated acetylation of p53 can increase p53 sequence-specific DNA-binding activity *in vitro* by electrophoretic mobility-shift assay (EMSA) using short oligonucleotides (62). Several subsequent studies also supported that the acetylation of p53 can dramatically stimulates its sequence-specific DNA-binding activity both *in vitro* and *in vivo*, possibly caused by an acetylation-induced conformational change (63, 67, 68). This result is consistent with the general idea that the C-terminal tail of p53 acts as a negative autoregulator and the multiple posttranslational modifications at this region, including phosphorylation and acetylation, can increase p53 DNA-binding ability and induce its transcriptional activity (54). However, the hypothesis is challenged by a recent study showing that p53 binds to its cognate promoters constitutively (72). In addition, acetylation does not increase the p53 DNA-binding activity when assayed by chromatin immunoprecipitation assay (ChIP) using an artificially reconstituted chromatin (73). Instead, the p53 acetylation was suggested to promote

the recruitment of its coactivators such as p300/CBP and to increase p53-dependent transcriptional activity (74). Recent study also showed that p53 C-terminus is actually required for p53 linear diffusion on chromatin and required for p53 efficient DNA binding and transactivation of target promoters *in vivo* (75, 77). However, acetylation and other modifications of C-terminus do not increase this p53 activity.

Several studies also suggested that ubiquitination and acetylation at the C-terminus of p53 can regulate p53 stability. Early study indicated that p53 C-terminus was required for Mdm2-mediated degradation of p53 but not Mdm2-p53 interaction (166). Since the lysine residues at the C-terminus might be ubiquitinated by Mdm2, two studies tested the importance of the C-terminal lysine residues in p53 stabilization. In one study, all six lysine residues were changed to Arginines (6KR mutant) to prevent ubiquitination but preserve the structure of p53 (167). While those 6KR mutants interact with Mdm2 normally, they were not able to undergo Mdm2-mediated ubiquitination and degradation in tumor cell lines, leading to p53 stabilization and activation (167). In addition, mutation of four lysine residues (Lys372, 373, 381 and 382) into Alanine (A4 mutant) also abrogated the p53 ubiquitination and degradation, while the individual single lysine mutants could be degraded efficiently, indicating that none of these lysine residues is essential for p53 ubiquitination (168). Since acetylation of the same lysine residues might prevent ubiquitination at these sites, it has been suggested that p53 acetylation can stabilize

p53. In support of this notion, increased levels of p53 acetylation by deacetylase inhibitors could inhibit p53 degradation *in vivo* and the p53 degradation requires deacetylation (165, 169).

In addition to acetylation and ubiquitination, the C-terminal lysine residues can also be modified by other mechanisms. Lys386 of p53 can be modified by conjugation to a small ubiquitin-like protein SUMO-1 *in vitro* and *in vivo* (111, 164), although the role of this modification remains controversial. Recent studies also showed that ubiquitin-like protein Nedd8 can be covalently linked to p53 at Lys370, 372 and 373, and Mdm2-dependent neddylation of p53 negatively regulates its transcriptional activity (65). Additionally, methylation of p53 at Lys372 by Set9 methyltransferase has been identified and suggested to restrict p53 in the nucleus and stabilize p53 (66).

p53 downstream effects

Upon various cellular and genotoxic stresses, p53 is activated to be a tetrameric transcription factor, binds to its cognate responsive elements within the genome, activates the numerous downstream target genes, and contributes to multiple biological effects, such as cell cycle arrest, DNA repair, apoptosis, senescence and differentiation. Although how p53 can make its choice to exert specific downstream effects is not clear, more evidence indicates that the cell context, including the intracellular and extracellular microenvironment may play a

role in determining p53 responses, among which the presence of extracellular survival factors may predispose cells to undertake p53-dependent cell cycle arrest instead of apoptosis (170). Additionally, the genetic alterations of cells that affect the activity of either p53 or other proteins associated with apoptosis, cell cycle progression or DNA repair can also modulate the p53 responses (170). Besides all these, the p53 choice between life and death can also be determined by the ability of p53 to preferentially activate its particular subsets of targets. While conditions favor the activation of cell cycle related genes will lead to p53-dependent cell growth arrest, more severe situations more likely to cause cell death will specifically activate p53 targeting pro-apoptotic genes. The mechanism how p53 can preferentially activate specific subsets of genes under different levels or types of stresses is not completely understood, however, the differential posttranslational modifications of p53 and its ability to recruit different coactivators or corepressors may explain to some extent. Multiple posttranslational modifications of p53 may modulate p53 conformational change and thereby alter p53 affinity for some DNA binding sites, resulting in different DNA-binding specificity (60, 62, 162, 163). But the hypothesis was challenged by the study showing that the DNA-binding capacity of p53 is not significantly changed by posttranslational modifications and the binding affinity has remained to be similar with or without stresses using ChIP assay (72). Ser46 phosphorylation seems to specifically activate p53-dependent apoptotic activity by targeting the apoptotic genes including p53AIP1 and PTEN,

therefore bias the transcriptional repertoire of p53 to favor cell death (151, 152, 154). Particular p53 modifications may also regulate the interaction of p53 with other proteins. For example, phosphorylation of Ser15 is suggested to be required for efficient recruitment of coactivators p300/CBP, which may lead to elevated transactivation of target genes (135, 138). ASPP family protein and p300 cofactor JMY have both been demonstrated to interact with p53 and specifically enhance p53 apoptotic activity by upregulating specific p53-dependent apoptotic genes in response to cellular stresses (171, 172). Most interestingly, studies show that the p53 family members, p63 and p73 are required for p53-dependent apoptosis after DNA-damage (173). Therefore, the differential protein interaction with p53 may also contribute to the specific activation of some p53 targeting genes.

1. Cell cycle checkpoint

Cell cycle checkpoint is a control mechanism by which cell arrests progression through the cell cycle to make sure that the initiation of the late events is dependent on the completion of the early events (174). In response to DNA damage, p53 can mediate the delay or arrest at checkpoints preceding DNA replication (the G1/S checkpoint), and is involved in delaying damaged cells prior premitotic chromosome condensation (the G2/M check-point) and actual chromosome partition (the spindle check-point). Checkpoint failure leads to accumulation of mutations and chromosomal aberrations, which in turn increase the

probability of developmental malformations or genetic syndromes and diseases including cancer (175).

Upon DNA damage, activation of the PI(3)k-like kinase (PIKKs), ATM (ataxia telangiectasia mutated) and ATR (ATM- and Rad3- related) is conceived to be the first step in the activation of signaling pathway that inhibits cell cycle progression. While ATR seems critical in mediating cellular response to arrest of DNA replication forks, ATM is primarily activated following DNA damage (174). ATM kinase is present as homodimer and held at minimal activity in unstressed cells, yet undergoes rapid activation through auto-phosphorylation and dimmer dissociation after DNA damage (176). Activated ATM cooperates with checkpoint mediator proteins such as 53BP1 and MDC1, and with checkpoint kinase CHK2/CHK1 to trigger cell cycle delays at various transitions of the cell division cycle, including G1 and G1/S checkpoint, S phase checkpoint and the G2 checkpoint (177-180).

The ATM/CHK2-p53/MDM2-p21 pathway plays a dominant role in mediating G1 checkpoint response to DNA damage in mammalian cells, as p53-null MEFs fail to undergo G1 arrest upon DNA damage treatment (174). p53 is transiently stabilized and activated as a transcriptional factor by ATM phosphorylation within its N-terminal transactivation domain, stimulating the transcription of cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$, whose accumulation is essential for silencing the cyclinE/Cdk2 and thereby inhibiting cell cycle entry

into S phase (181, 182). Entry into mitosis is blocked by the G2 checkpoint when DNA is completed replication but contains damage. G2/M checkpoint is exerted in both p53-dependent and p53-independent manner (183). The mechanism by which p53 regulates the G2/M transition may involve the regulation of the cyclin dependent kinase Cdc2 which is essential for entry into mitosis, partly through upregulating the CDK inhibitors p21, GADD45a and 14-3-3 sigma proteins (183).

2. DNA repair

DNA can be damaged in a variety of ways, either by normal metabolic processes or exposure to an external source of ionizing radiation or chemicals, and result in many different types of DNA lesions. DSBs are most severe type of DNA damage, in which the sugar backbones of both DNA strands are broken close enough to disrupt the base pairing, resulting in two DNA ends. While lesions on a single DNA strand are repaired by base excision repair (BER) and nucleotide excision repair (NER), the DNA double strand breaks (DSBs) are repaired via either the nonhomologous end joining (NHEJ) or homologous recombination (HR) pathways (184).

Besides DNA damage induced double-strand breaks, DSBs occur normally during DNA replication, meiosis and immune system development, which is termed as programmed DSBs (185). For example, DSB formation is a conserved step in the initiation of meiotic recombination in eukaryocytes, which is repaired by HR and

ultimately leads to exchange of genetic materials between two homologous chromosomes (186). During lymphocytes development, both V(D)J recombination and IgH class switch recombination employ DSB and the subsequent repair through NHEJ to generate genomic rearrangement (187).

The proteins involved in the two major pathways of DSB repair, NHEJ and HR, are highly conserved across all eukaryotes and ubiquitously expressed in multi-cellular organisms (185). The four necessary components of NHEJ pathways, Ku70, Ku80, XRCC4 and DNA ligase IV, are conserved from yeast to human, where Ku70 and Ku80 are subunits of DNA-dependent protein kinase (DNA-PK) and may function to remodel broken ends, the DNA ligase IV and XRCC4 function as a complex to ligate DNA ends (187). There are a large number of proteins known to be involved in the recognition and response to DSBs in HR pathway, including several Rad proteins, Mre11 and Nbs1, and proteins encoded by breast cancer susceptibility genes 1 and 2 (Brca1 and Brca2) (188). ATM functions as a master regulator of the response to DSBs induced by exogenous and endogenous factors, and MRN complex (Mre11, Rad50 and Nbs1) seems to be a break sensor and required for both ATM activation itself and the ability of ATM to activate a number of downstream proteins involved in DNA repair and cell cycle checkpoint (189).

While it is essential to modulate higher order chromatin structure in order for NHEJ and HR repair proteins to access the broken DNA ends, phosphorylation of histone H2AX (termed as γ-H2AX) has been extensively studied regarding to its

critical function in cellular response to DSBs (190). Phosphorylation of H2AX is mediated by the PI3K-like protein kinase family members ATM, ATR, and DNA-PK, and γ-H2AX largely forms in the vicinity of DNA breaks that colocalize with many known DSB repair proteins MRN complex, Brca1, 53BP1, MDC1 and Rad51 (191). H2AX phosphorylation has been shown to be critical for protecting the genome from spontaneous, irradiation and V(D)J recombination induced DSBs, although it probably does not directly mediate enzymatic DNA repair (192). In addition, γ-H2AX plays an important role in suppressing oncogenic translocation, which is supported by the fact that H2AX-haploinsufficiency can cause genomic instability and in a p53-deficient background can dramatically increase the tumorigenesis in mice (190, 192).

Several studies have suggested the in vivo role of XRCC4 by generating *XRCC4*-deficient mice, that XRCC4 deficiency leads to embryonic lethality with massive apoptosis of newly generated, postmitotic neurons during the nervous system development, and also that progenitor lymphocyte development is arrested due to impaired V(D)J recombination, possibly caused by impaired DNA end joining (193). Further investigation has shown that p53 deficiency or severely impaired p53-dependent apoptotic activity can rescue the embryonic lethality observed in XRCC4$^{-/-}$ mice, implying that the increased neuronal apoptotic death and cell proliferation defects of XRCC4$^{-/-}$ mice mainly result from a p53-dependent response to DNA damage that has occurred but not repaired during embryo

development (146, 194).

3. Apoptosis

The p53 tumor suppressor gene is considered as “guardian of genome” in terms of its nodal role in linking many different pathways that safeguard the genomic integrity of cells in response to various genotoxic and cellular insults (195). It is widely accepted that p53 exerts its tumor suppression activity mainly through activating apoptosis (196). Early evidence has come from the study of thymocytes from p53 knockout mice indicating that p53 was required for radiation-induced apoptosis, but not several other types of stimuli; and from the study of p53-null MEFs indicating that p53 was required for MEFs apoptosis induced by oncogene overexpression or chemotherapeutic agents (86, 197, 198). The *in vivo* study shows that aggressive tumor development in the absence of p53 function corresponds to loss of p53-dependent apoptosis (199). Another elegant *in vivo* study supportively shows that mice develop p53-null-like myc-driven lymphomas by disruption of apoptosis downstream of p53 either by Bcl-2 or dominant negative caspase 9, while these apoptosis defective lymphomas that retain wild-type p53 do not display checkpoint defects or aneuploidy (200). Similarly, mice harboring R172P missense mutation, which abrogates p53 apoptotic activity and maintains cell cycle arrest, developed many tumors but did not exhibit chromosomal instability (201). These studies altogether imply that impaired p53-dependent apoptosis is a critical step in

tumor development. Consistently, because of their loss of apoptotic potential, p53 deficient cells are highly enriched under hypoxia condition, which is common in solid tumors (202).

Apoptosis proceeds through two major pathways, extrinsic pathway via the death receptors and intrinsic pathway mediated by the mitochondria (203). The extrinsic pathway is initiated by ligand binding to the death receptor on cell surface, followed by recruitment of the adaptor molecules, and finally resulting in activation of caspase cascade (204). The extrinsic pathway consists of cell surface death receptors, their inhibitory counterparts, and the associated cytoplasmic proteins, in which the regulation is either by altering the number of different types of receptors or by modulating the expression levels of activating ligands and cytoplasmic adaptors (205). The intrinsic pathway is engaged when cells are exposed to stresses, such as DNA damage or growth factor withdrawal, and is mediated by the mitochondria. The Bcl-2 family proteins are key regulators of this pathway. Upon genotoxic or cellular stresses, cells regulate the interaction of the Bcl-2 family members, initiate mitochondrial outer membrane permeabilization, and thereby control the release of apoptogenic proteins, such as cytochrome *c*, SMAC/Diablo, AIF and Omi/HtrA2, eventually leading to cell death through caspase-dependent and –independent mechanisms (196).

CD95 (*Fas*/*APO-1*) and *Killer*/*DR5* are among the best characterized members of death receptors. CD95 receptor is a member of tumor necrosis factor

(TNF) superfamily, normally being expressed on cell surface as a preassociated homotrimer, containing a cytoplasmic protein-protein interaction domain termed the death domain (DD) which mediates the formation of death-inducing signaling complex (DISC) and initiates the death signaling (206). Killer/DR5 is also a member of TNFR family with the typical cytoplasmic death domain, and its expression can be induced by cytotoxic chemotherapy (207). Both *CD95* and *Killer/DR5* expression were shown to be upregulated by p53 and trigger or enhance apoptosis upon DNA damage treatment or upon overexpression of p53 in a tissue-specific manner, although CD95 can also be activated by DNA damage independent of p53 (207, 208). *PIDD*, encoding another death domain containing protein, was discovered in transformed mouse erythroleukaemia cells to be a p53-regulated gene and contribute to p53-dependent apoptosis (209). PERP, a PMP-22/gas family protein localized to both plasma membrane and Golgi membrane, was a transcriptional target of p53 and highly induced in E1A-transformed MEFs following DNA damage, partly contributing to p53-dependent apoptosis (210). PERP was also shown to play a role in p53-mediated apoptosis in thymocytes and neurons upon ionizing irradiation (211).

The intrinsic pathway centers on the mitochondria and directly impacts on Bcl-2 family proteins. The Bcl-2 family members can be transcriptionally activated, transcriptionally repressed or subjected to posttranslational modifications to enhance or repress function. Cells that have engaged p53-dependent apoptosis

typically follow the intrinsic cell death pathway (212).

Bcl-2 family has at least 20 members, all of which share at least one conserved Bcl2 homology (BH) domain. Bcl-2 family contains anti-apoptotic proteins that promote cell survival such as Bcl-2 and Bcl-x_L, and pro-apoptotic proteins including the Bax family and the BH3-only family members, such as Bax, Bak, Bad, Bid, Noxa and Puma (213). The pro-apoptotic proteins are thought to act by binding to their anti-apoptotic relatives and inhibiting the anti-apoptotic activity (196). The Bax family members, Bax and Bak, mainly function at the mitochondria. Bax was the first identified p53-regulated proapoptotic Bcl-2 family member (214). Bax is a cytosolic monomer in unstressed cells, but changes conformation during apoptosis, integrates into the outer mitochondria membrane and oligomerizes (213). Knockout studies indicate that Bax is mutated in some human gastric and colorectal cancers and that Bax is largely responsible for p53-dependent apoptosis induced by 5-fluorouracil in colorectal cancer cells (215). It was also shown that loss of Bax accounts for almost half of the accelerated tumor growth which results from p53 deficiency in a mouse brain tumor model (216). However, *Bax* expression is not significantly induced in thymocytes after ionizing irradiation (our unpublished data) and *Bax* is proposed to be dispensable in γ-irradiation induced apoptosis in thymocytes and intestinal epithelial cells (217).

Both *Noxa* and *Puma* are BH3-only members of the Bcl-2 family. Among the numerous p53-dependent pro-apoptotic genes, *Puma* and *Noxa* are indicated to

be critical mediators of the apoptotic responses induced by p53. In *Noxa* knockout mice, the apoptosis in response to DNA-damaging agent treatment was significantly compromised in both the primary MEFs and E1A-overexpressing MEFs (218). Additionally, the absence of *Noxa* resulted in resistance to X-ray-induced apoptosis in epithelial cells of small intestinal crypts (219). In *Puma* knockout mice, the large resistance to p53-dependent apoptosis was not only detected in MEFs and E1A-transfected MEFs, but also in primary thymocytes and developing postnatal CNS upon DNA damage, and hematopoietic cells triggered by hyperproliferative signals, such as c-Myc (218, 220). Therefore, Puma was considered as a principal mediator of cell death in response to diverse apoptotic signals, since *Puma*-knockout mice recapitulated several key apoptotic deficiencies observed in the p53-knockout mice, which has not been observed in the knockout mice of most other p53 targets. *Puma* (p53 up-regulated modulator of apoptosis) was initially identified by differential gene expression studies as a p53 target gene and a potent inducer of apoptosis (221). PUMA acts by binding to Bcl-2 and BcL-x_L, and activating Bax to facilitate cytochrome *c* release from the mitochondria, thereby triggering the apoptotic cascade (220, 222). However, *Puma*-deficient mice do not appear to be inherently predisposed to developing spontaneous tumors, further supporting the notion that inactivation of multiple p53 effectors is critical for tumorigenesis (220). Consistent with the notion, the requirement for specific p53 targeted apoptotic gene in mediating cellular apoptosis response to DNA damage is

highly selective and clearly cell type- and tissue-dependent. For example, *In situ* hybridization assay in mouse tissues shows that although *Killer/DR5*, *Bid*, *Puma* and *Noxa* are all induced in small intestine, the key effector of apoptosis in the red pulp of spleen is Bid, while in thymus and the while pulp of spleen is Puma, and that the target gene activation is even different between transverse and descending colon (223).

p53AIP1 is another p53 target that mediates p53-dependent apoptosis (151). p53AIP1 is localized at mitochondria and ectopic expression of *p53AIP1* can induce dissipation of mitochondrial potential, leading to cytochrome *c* release, whose activity can be blocked by overexpression of Bcl2 (224). Interestingly, phosphorylation of p53 at Ser46 seems to be specifically required for p53AIP1 induction after severe DNA damage (151).

Although a large number of genes are transcriptionally regulated by p53 during induction of apoptosis, none of the target genes has been identified whose altered expression or genetic deficiency can sufficiently phenocopy p53 deficiency *in vivo* (225). As an additional mechanism of p53-dependent pro-apoptotic activity, recent studies have suggested the non-transcriptional apoptotic activity of p53. The initial finding has shown that in response to a broad spectrum of apoptotic stimuli, a fraction of p53 can translocate to mitochondria in primary cells and *in vivo* in mice, and that forced targeting of p53 to mitochondria is sufficient to induce mitochondria outer membrane permeabilization (MOMP) by forming complex with the Bcl2 and

Bcl-x_L proteins, liberating pro-apoptotic Bcl2 family members such as Bax and Bak, and promoting cytochrome *c* release (226). Subsequent study on the direct role of p53 at mitochondria indicated that p53 can physically interact with Bak and directly promote the pro-apoptotic activity of Bak by causing Bak oligomerization and disrupting the interaction between Bak and Mcl1 (227). The other elegant *in vitro* study provided compelling evidence that cytosolic accumulation of p53 directly activated Bax in the absence of other proteins or p53 transcriptional activity and led to cytochrome *c* release (228). Consistently, the first *in vivo* evidence by analysis in mouse fibroblasts indicated that high amount of activated p53 in cytosol is required to promote UV-induced fibroblasts apoptosis in a transcriptional-independent way (229). In accordance with this model, the difference of apoptotic potentials of two human p53 polymorphic variants (codon 72, Arg/Pro) was suggested to be caused by greater ability of Arg72 variant to localize to the mitochondria, and the cytoplasmic accumulation of p53 was correlated with Mdm2-mediated ubiquitination (230). Thus, the physical appearance of p53 in cytoplasm but not its transcriptional activity is responsible for the apoptosis.

4. Senescence

Since Hayflick discovered for the first time that human diploid fibroblasts only have limited division potential in culture, the phenomenon of cellular senescence or replicative senescence has been extended to many other cell types,

which is defined as the irreversible cell growth arrest at the G1 stage of the cell cycle (231, 232). The senescent cells exhibit distinct phenotypic characteristics with dramatic changes in chromatin structure and gene expression, with enlarged and flattened cell morphology and increased β-galactosidase (SA-β-gal) activity (233). The process is now also known as replicative senescence, which is mainly caused by telomere shortening. Telomere is a heterochromatin structure present at the end of linear chromosome structure, differentiating chromosomal end from broken DNA end, preventing chromosome fusion and instability (234). Owing to the nature of DNA replication, 50-200 bp of 3' telomeric DNA is left unreplicated during S phase; and since most human cell types do not express telomerase, telomeres shorten with each cell division. Replicative senescence phenotype occurs in human cells when the length of one or more telomeres shortens to an average size of 4-6 kb, before short enough to cause chromosome instability (235).

Besides telomere shortening, certain types of DNA damage, such as DNA strand breaks and oxidative lesions due to environmental or endogenous insults can also cause cells to senesce, with little or no impact on telomeres, which is termed as premature senescence (235-237). This might also explain why mouse embryonic fibroblasts, which have longer telomeres and frequently express telomerase, are instead easier to undergo senescence in culture than human fibroblasts, because they are more sensitive to supraphysiological oxygen stress (238) (239). In addition, the excess mitogenic signals resulted from overexpression of certain oncogenes, such as

Ras and other components of RAS-RAF-MEK pathway, or the epigenetic changes to chromatin structures can lead to premature cellular senescence (240-242). The replicative senescence and stress-induced premature senescence may represent two disparate signals eliciting one common cellular response (243). Consistent with the role of cellular senescence in tumor suppression, p53 and pRB, both of which are critical tumor suppressors, play a major role in mediating cell senescence response. In mouse embryonic fibroblasts (MEFs), p53 or ARF deficiency is sufficient for cells to bypass both the replicative senescence and the premature senescence induced by DNA damage, oxidative stress, oncogenes or intense mitogenic stress (244).

Primary murine cells in culture undergo senescence in response to overexpression of the *Ras* oncogene or of downstream effectors such as Raf, activated MAP kinase and PML (245-247). Although the mechanism of how premature senescence stimulates p53 activation is not clear, many studies indicated that two major pathways can be crucial for p53 activation by cell senescence (248). One is the ATM/ATR and Chk1/Chk2 mediated p53 phosphorylation and activation in response to DNA damage; the other is the activation of p53 by hyperproliferative signals from oncogenes such as *Ras*, *Raf* and *c-Myc*, mainly mediated through p53/ARF pathway (248). Previous studies have identified a number of posttranslational modifications of p53 during replicative senescence (139). In this context, increased phosphorylation of human p53 is detected at Ser15, Thr18 and

Ser376. Our study also showed that in Hupki MEFs Ser46 is phosphorylated upon overexpression of Ras, and that mutant p53hki^{S46A} MEFs escape Ras-induced cellular senescence (data not published). p14^{ARF} (p19^{ARF} in mouse) is encoded by an alternative transcript of the *INK4a* locus, which shares two exons with the p16^{INK4a} transcript but has a separate promoter and is translated in a different reading frame (103). In MEFs, Ras induced cellular senescence is mediated primarily through p53/ARF pathway (244, 245). p19^{ARF} can be activated by hyperproliferating signals, such as overexpressed Ras, Myc, E2F1 and E1A, and activation of p19^{ARF} can prevent negative regulation of p53 by Mdm2, either by sequestering Mdm2 in nucleolus of cells or by inhibiting Mdm2 E3 ubiquitin ligase activity, therefore stimulating p53 activity (103). In addition, the p38/MAPK pathway has been shown to play a crucial role in mediating *Ras*-induced senescence and in activating p53 (249). Interestingly, it has been suggested that *Ras* overexpression induces senescence through the generation of reactive oxygen species (ROS), which could lead to p38MAPK activation (243). Therefore, overexpressed Ras may also activate p53 response by producing high levels of DNA-damaging ROS.

p53 then promote cellular senescence by controlling the activity of its effectors, including p21 and PML (250). p21 is a well characterized downstream effector of p53-mediated growth arrest following DNA damage (251, 252). The expression level of p21 has also been shown to increase dramatically when human

diploid fibroblasts undergo replicative senescence, which is correlated with increased p53 transcriptional activity (253). Moreover, p21 expression is significantly elevated in oxidative stress and oncogene induced senescent cells, and may play an important role for p53-dependent initiation of cellular senescence (254, 255). Inactivation of p21 failed to arrest cell cycle in response to DNA damage and was sufficient to bypass senescence in human fibroblasts (256). Overexpression of PML (promyelocytic leukemia) can induce cell growth arrest or apoptosis depending on cell types and/or PML expression level (257). Recently, a link between premature senescence, PML and p53 has been established. Oncogene *Ras* upregulates PML expression and overexpression of PML can recruit p53 and CBP colocalization to nuclear bodies and activate p53 through acetylation on Lys382 and phosphorylation on Ser15, inducing p53-dependent premature senescence (140, 247). In addition, it has been shown that overexpression of PML isoform IV can cause cellular senescence by activation of p53 through acetylation at Lys382 as well as phosphorylation at Ser46, while it is independent of nuclear bodies (258). Another recent study indicated that PML not only acts upstream of p53, but itself is a p53 target gene mediating p53 antiproliferative effects, and that PML deficiency compromises p53 activated premature senescence and apoptosis (259).

It has been shown that overexpression of oncogene can lead to senescence in *in vitro* cultured cells, which may serve as protection against cell transformation and thereby a tumor suppressive mechanism (238). However, whether cellular

senescence represents a physiological process in living tissues *in vivo* remains poorly understood. Four recent papers indicated that endogenous oncogene *Ras* or Ras downstream effectors can trigger premature senescence *in vivo* which may be p53-dependent, and that by analyzing tumors induced by oncogenes, cellular senescence did exist in premaligmant but not malignant tumor cells which is detected by SA-β-gal enzymatic activity (260-263). Therefore, oncogene-induced premature senescence occurrs *in vivo* and they can provide a barrier to oncogene induced tumorigenesis.

Recent studies suggest a tight link between p53 and PTEN, another major tumor suppressor. The *PTEN* (phosphatase and tensin homolog deleted on chromosome 10) tumor suppressor gene was identified to be frequently mutated or deleted in various human cancers, and germline mutations in the *PTEN* gene have been associated with sporadic and heritable syndromes, including Cowden syndrome and Bannayan-Riley-Ruvalcaba syndrome (264, 265). The major function of the tumor suppressor PTEN relies on its phosphatase activity and subsequent antagonism of the PI3K/AKT pathway (266-268). Loss of PTEN function can result in activation of PI3K/AKT kinase activity and subsequent activation of its downstream effectors, which is an important cell survival signaling pathway. Early cell line transfection data showed that the PI3K/AKT pathway can phosphorylate Mdm2 *in vivo* and thereby promote nuclear translocation of Mdm2, resulting in decreased p53 protein level and transcriptional activity (269). Another

group by using primary mouse embryonic fibroblasts indicated that Pten can not only regulate p53 protein level by retaining Mdm2 in the cytoplasm through its phosphatase activity on PI3K/Akt, but can also physically interact with p53 and control the transcriptional activity of p53 by modulating its DNA binding activity, which is independent of its phosphatase activity (270). A recent study further supported the notion that Pten can increase p53 stability and its transactivation of *Pten* itself, both dependent and independent of Pten phosphatase activity (271). Interestingly, Chen et al debated that acute loss of *Pten* in mouse embryonic fibroblasts (MEFs) can increase both p53 stability and activity, especially activating p53-dependent senescence response and therefore restricting tumorigenesis in Pten-deficient prostate (261).

5. Aging

Recent studies have suggested that p53, despite a tumor suppressor, can contribute to organism aging or age-related diseases, such as late-life cancer (272). More studies have suggested a tight link between aging and p53-induced cellular senescence. By using both p16 and SA-β-gal markers, Campisi group show that cells characteristic of senescence accumulate with age in multiple human and mouse tissues, or present at sites of certain age-related pathologies (273). They also indicate that the accumulation of non-dividing senescent cells can compromise tissue renewal and repair by depleting stem cells pool or affecting stem cell niches,

and can alter tissue microenvironment, tissue structures and functions at least partly by fibroblasts secretory factors resembling phenotypes of wounding responses or fibroblast associated carcinomas (272).

However, the relationship between p53 activity and aging has remained to be controversial. Tyner *et al* generated a truncated p53 allele lacking the first six exons and suggested that this mutant p53 allele can augment the wild-type p53 activity, which is shown by enhanced tumor resistance in $p53^{+/m}$ mice, enhanced p53 stability and transcriptional activity in response to both DNA damage and oncogenes (274). Unexpectedly, accompanied with the elevated p53 activity is the early aging phenotypes. But it is worthy to note that in the $p53^{+/m}$ mouse model Tyner *et al* analyzed there were additional 23 genes deleted, therefore suggesting that the age-related phenotype might not be solely caused by p53 mutation (275). Another group generated the transgenic mice overexpressing a naturally occurred N-terminal truncated isoform of p53 (p44), and showed that the mice had reduced size because of slower growing rate both pre- and postnatally, and had reduced lifespan with premature aging phenotypes possibly due to upregulated IGF signaling (31). However, Mendrysa group argue against that elevated p53 activity caused by reduced Mdm2 expression resulted in significantly reduced tumor formation without causing reduction in lifespan or any age-related phenotypes (276). In addition, the “super p53”, in which an extra copy of p53 is expressed in the context of wild-type p53 alleles, has been shown to enhance p53-mediated response

to DNA damage and increase resistance to tumor development, yet have normal lifespan without premature aging (277). The possible reason why decreased Mdm2 level or additional p53 allele does not cause shortened lifespan or early aging phenotype while overexpression of short isform or mutant form of p53 does could be that it is the altered ratio between different p53 isoforms and thereby the imbalanced expression of p53 target genes, but not the overall increased p53 activity, that lead to aging. (278).

6. Differentiation

Besides playing a role in regulating cell proliferation and apoptosis, p53 has also been implicated in controlling stem cell self-renew and differentiation (157, 279). The hallmark features of stem cells are the pluripotency and capacity of infinite self-renewal. The extensive self-renewal of stem cells is in many ways resembling the expansion of cancer cells, and in this context several important tumor suppressors, such as p53, PTEN and Bmi-1 are involved in controlling embryonic stem cell or normal tissue stem cell self-renew (157, 280, 281). Studies have shown that p53 protein stability decreases while p53 activity dramatically increases during ES cell differentiation *in vitro* induced by retinoic acid (RA) (157, 282). Lin *et al* further indicate that upregulated p53 activity in ES cells in response to DNA damage or RA-induced differentiation is accompanied by Ser315 phosphorylation and results in direct suppression of Nanog expression, mainly by

p53 recruiting co-repressor mSin3A to Nanog promoter (157). Therefore, while DNA damage induced cell cycle arrest or apoptosis are not efficient in ES cells, p53-dependent suppression of Nanog expression exhibits an alternative mechanism to maintain genetic stability by promoting the ES cell differentiation (283). It is also reported that p53 can negatively regulate neural stem cell proliferation, survival and thereby self-renewal possibly functioning through its downstream target p21, therefore suggesting that p53-mediated suppression of stem cell self-renewal may be general although the mechanisms can be different depending on the stem cell types (279).

p53 knock-in mouse model

Since the early conclusive demonstration that p53 plays a central role in tumor suppression by analyzing p53 knock-out mice which are highly prone to tumorigenesis, the study of p53 in mice through transgenic, knock-out and knock-in approaches, as well as through various crosses to other mouse strains, has greatly enhanced our understanding of the genetics and the contexts in which it plays a role in tumor suppression (24, 25, 284). Among them, the knock-in strategy is a powerful approach to investigate p53 function *in vivo*, especially for those with subtle changes in the p53 locus. The advantage of this strategy is that a particular mutant gene is expressed from the endogenous promoter at physiological levels, with the correct spatial and temporal profile. This approach is very useful for

functional analysis of p53 activities *in vivo*, and in some cases, it can mimic a *p53* mutation found in human tumors to better understand how such a mutation contributes to tumorigenesis (46, 47). Specifically, to assess the role of p53 posttranslational modification *in vivo*, several groups of investigators, including our laboratory, have generated knock-in mice with single phosphorylation site or combination of modification sites altered, in order to study the physiological importance of single as well as combinatorial posttranslational modifications in suppressing tumor (138, 144, 146, 285).

One knock-in mouse model was created to study the human p53 activity in the experimental *in vivo* system, known as HUPKI (human p53 knock-in) model (286). In this model, exons 4 to 9 and the intervening introns of mouse p53 are replaced by the corresponding human p53 sequence. This HUPKI p53 allele has been demonstrated to maintain normal biological functions equivalent as mouse p53 allele and HUPKI mice do not develop spontaneous tumor at early age (286). More importantly, our unpublished data have suggested the signaling pathway leading to phosphorylation of human p53 at Ser46, the residue only appearing in human, is conserved in various types of mouse cells. In spite of the high homology between mouse and human *p53* genes, they have different nucleotide sequence and thus differential amino acid sequence, therefore this knock-in model can provide a tractable *in vivo* system for mutagenesis studies to define the p53 mutation profiles in their contribution to tumor initiation and development. In addition, this mouse

model could be useful in providing a system for testing potential cancer therapeutics for their ability to modulate human p53 DNA binding in vivo, a step that is important for validating the efficacy of such therapy agents in tumor treatment (284).

References

1. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. p53 mutations in human cancers. Science 1991;253:49-53.

2. Levine, A. J. p53, the cellular gatekeeper for growth and division. Cell 1997;88:323-331.

3. Lane, D. P. and Crawford, L. V. T antigen is bound to a host protein in SV40-transformed cells. Nature 1979;278:261-263.

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

5. Sarnow, P., Ho, Y. S., Williams, J., and Levine, A. J. Adenovirus E1b-58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54 kd cellular protein in transformed cells. Cell. 1982;28:387-394.

6. Crawford, L. V., Pim, D. C., Gurney, E. G., Goodfellow, P., and Taylor-Papadimitriou, J. Detection of a common feature in several human tumor cell lines--a 53,000-dalton protein. Proc Natl Acad Sci U S A. 1981;78:41-45.

7. Braithwaite, A. W., Sturzbecher, H. W., Addison, C., Palmer, C., Rudge, K., and Jenkins, J. R. Mouse p53 inhibits SV40 origin-dependent DNA replication. Nature. 1987;329:458-460.

8. Finlay, C. A., Hinds, P. W., and Levine, A. J. The p53 proto-oncogene can act as a suppressor of transformation. Cell 1989;57:1083-1093.

9. Eliyahu, D., Michalovitz, D., Eliyahu, S., Pinhasi-Kimhi, O., and Oren, M. Wild-type p53 can inhibit oncogene-mediated focus formation. Proc Natl Acad Sci U S A. 1989;86:8763-8767.

10. Baker, S. J., Markowitz, S., Fearon, E. R., Willson, J. K., and Vogelstein, B. Suppression of human colorectal carcinoma cell growth by wild-type p53. Science. 1990;249:912-915.

11. Diller, L., Kassel, J., Nelson, C. E., Gryka, M. A., Litwak, G., Gebhardt, M., Bressac, B., Ozturk, M., Baker, S. J., Vogelstein, B., and et al. p53 functions as a cell cycle control protein in osteosarcomas. Mol Cell Biol. 1990;10:5772-5781.

12.Ginsberg, D., Michael-Michalovitz, D., and Oren, M. Induction of growth arrest by a temperature-sensitive p53 mutant is correlated with increased nuclear localization and decreased stability of the protein. Mol Cell Biol. 1991;11:582-585.

13.Yonish-Rouach, E., Resnitzky, D., Lotem, J., Sachs, L., Kimchi, A., and Oren, M. Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. Nature. 1991;352:345-347.

14.Moll, U. M., Riou, G., and Levine, A. J. Two distinct mechanisms alter p53 in breast cancer: mutation and nuclear exclusion. Proc Natl Acad Sci U S A. 1992;89:7262-7266.

15.Sidransky, D., Von Eschenbach, A., Tsai, Y. C., Jones, P., Summerhayes, I., Marshall, F., Paul, M., Green, P., Hamilton, S. R., Frost, P., and et al. Identification of p53 gene mutations in bladder cancers and urine samples. Science. 1991;252:706-709.

16.Baker, S. J., Fearon, E. R., Nigro, J. M., Hamilton, S. R., Preisinger, A. C., Jessup, J. M., vanTuinen, P., Ledbetter, D. H., Barker, D. F., Nakamura, Y., White, R., and Vogelstein, B. Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science. 1989;244:217-221.

17.Nigro, J. M., Baker, S. J., Preisinger, A. C., Jessup, J. M., Hostetter, R., Cleary, K., Bigner, S. H., Davidson, N., Baylin, S., Devilee, P., and et al. Mutations in the p53 gene occur in diverse human tumour types. Nature. 1989;342:705-708.

18.Levine, A. J., Momand, J., and Finlay, C. A. The p53 tumour suppressor gene. Nature. 1991;351:453-456.

19.Zambetti, G. P. and Levine, A. J. A comparison of the biological activities of wild-type and mutant p53. Faseb J. 1993;7:855-865.

20.Olson, D. C. and Levine, A. J. The properties of p53 proteins selected for the loss of suppression of transformation. Cell Growth Differ. 1994;5:61-71.

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

22.Srivastava, S., Zou, Z. Q., Pirollo, K., Blattner, W., and Chang, E. H. Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. Nature. 1990;348:747-749.

23.Malkin, D., Li, F. P., Strong, L. C., Fraumeni, J. F., Jr., Nelson, C. E., Kim, D. H., Kassel, J., Gryka, M. A., Bischoff, F. Z., Tainsky, M. A., and et al. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science. 1990;250:1233-1238.

24.Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Jr., Butel, J. S., and Bradley, A. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature 1992;356:215-221.

25.Jacks, T., Remington, L., Williams, B. O., Schmitt, E. M., Halachmi, S., Bronson, R. T., and Weinberg, R. A. Tumor spectrum analysis in p53-mutant mice. Curr Biol 1994;4:1-7.

26.Ko, L. J. and Prives, C. p53: puzzle and paradigm. Genes Dev 1996;10:1054-1072.

27.Kaghad, M., Bonnet, H., Yang, A., Creancier, L., Biscan, J. C., Valent, A., Minty, A., Chalon, P., Lelias, J. M., Dumont, X., Ferrara, P., McKeon, F., and Caput, D. Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. Cell 1997;90:809-819.

28.Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M. D., Dotsch, V., Andrews, N. C., Caput, D., and McKeon, F. p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Mol Cell 1998;2:305-316.

29.Courtois, S., Verhaegh, G., North, S., Luciani, M.-G., Lassus, P., Hibner, U., Oren, M., and Hainaut, P. DeltaN-p53, a natural isoform of p53 lacking the first transactivation domain, counteracts growth suppression by wild-type p53. Oncogene 2002;21:6722-6728.

30.Yin, Y., Stephen, C. W., Luciani, M. G., and Fahraeus, R. p53 Stability and activity is regulated by Mdm2-mediated induction of alternative p53 translation products. Nature Cell Biology 2002;4:462-467.

31.Maier, B., Gluba, W., Bernier, B., Turner, T., Mohammad, K., Guise, T., Sutherland, A., Thorner, M., and Scrable, H. Modulation of mammalian life

span by the short isoform of p53. Genes Dev. 2004;18:306-319.

32.Ghosh, A., Stewart, D., and Matlashewski, G. Regulation of Human p53 Activity and Cell Localization by Alternative Splicing. Mol. Cell. Biol. 2004;24:7987-7997.

33.Rohaly, G., Chemnitz, J., Dehde, S., Nunez, A. M., Heukeshoven, J., Deppert, W., and Dornreiter, I. A Novel Human p53 Isoform Is an Essential Element of the ATR-Intra-S Phase Checkpoint. Cell 2005;122:21-32.

34.Bourdon, J.-C., Fernandes, K., Murray-Zmijewski, F., Liu, G., Diot, A., Xirodimas, D. P., Saville, M. K., and Lane, D. P. p53 isoforms can regulate p53 transcriptional activity. Genes Dev. 2005;19:2122-2137.

35.Candau, R., Scolnick, D. M., Darpino, P., Ying, C. Y., Halazonetis, T. D., and Berger, S. L. Two tandem and independent sub-activation domains in the amino terminus of p53 require the adaptor complex for activity. Oncogene. 1997;15:807-816.

36.Venot, C., Maratrat, M., Sierra, V., Conseiller, E., and Debussche, L. Definition of a p53 transactivation function-deficient mutant and characterization of two independent p53 transactivation subdomains. Oncogene. 1999;18:2405-2410.

37.Jones, S. N., Roe, A. E., Donehower, L. A., and Bradley, A. Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. Nature. 1995;378:206-208.

38.Haupt, Y., Maya, R., Kazaz, A., and Oren, M. Mdm2 promotes the rapid degradation of p53. Nature 1997;387:296-299.

39.Fuchs, S. Y., Adler, V., Buschmann, T., Wu, X., and Ronai, Z. Mdm2 association with p53 targets its ubiquitination. Oncogene 1998;17:2543-2547.

40.Parant, J., Chavez-Reyes, A., Little, N. A., Yan, W., Reinke, V., Jochemsen, A. G., and Lozano, G. Rescue of embryonic lethality in Mdm4-null mice by loss of Trp53 suggests a nonoverlapping pathway with MDM2 to regulate p53. Nat Genet 2001;29:92-95.

41.Lu, H. and Levine, A. J. Human TAFII31 protein is a transcriptional coactivator of the p53 protein. Proceedings Of The National Academy Of Sciences Of The United States Of America 1995;92:5154-5158.

42.Lill, N. L., Grossman, S. R., Ginsberg, D., DeCaprio, J., and Livingston, D. M. Binding and modulation of p53 by p300/CBP coactivators. Nature 1997;387:823-827.

43.Grossman, S. R. p300/CBP/p53 interaction and regulation of the p53 response. Eur J Biochem 2001;268:2773-2778.

44.Venot, C., Maratrat, M., Dureuil, C., Conseiller, E., Bracco, L., and Debussche, L. The requirement for the p53 proline-rich functional domain for mediation of apoptosis is correlated with specific PIG3 gene transactivation and with transcriptional repression. EMBO J. 1998;17:4668-4679.

45.Toledo, F., Krummel, K. A., Lee, C. J., Liu, C. W., Rodewald, L. W., Tang, M., and Wahl, G. M. A mouse p53 mutant lacking the proline-rich domain rescues Mdm4 deficiency and provides insight into the Mdm2-Mdm4-p53 regulatory network. Cancer Cell. 2006;9:273-285.

46.Lang, G. A., Iwakuma, T., Suh, Y. A., Liu, G., Rao, V. A., Parant, J. M., Valentin-Vega, Y. A., Terzian, T., Caldwell, L. C., Strong, L. C., El-Naggar, A. K., and Lozano, G. Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome. Cell. 2004;119:861-872.

47.Olive, K. P., Tuveson, D. A., Ruhe, Z. C., Yin, B., Willis, N. A., Bronson, R. T., Crowley, D., and Jacks, T. Mutant p53 Gain of Function in Two Mouse Models of Li-Fraumeni Syndrome. Cell 2004;119:847-860.

48.Gaiddon, C., Lokshin, M., Ahn, J., Zhang, T., and Prives, C. A subset of tumor-derived mutant forms of p53 down-regulate p63 and p73 through a direct interaction with the p53 core domain. Molecular And Cellular Biology 2001;21:1874-1887.

49.Jeffrey, P. D., Gorina, S., and Pavletich, N. P. Crystal structure of the tetramerization domain of the p53 tumor suppressor at 1.7 angstroms. Science 1995;267:1498-1502.

50.Halazonetis, T. D. and Kandil, A. N. Conformational shifts propagate from the oligomerization domain of p53 to its tetrameric DNA binding domain and restore DNA binding to select p53 mutants. Embo J. 1993;12:5057-5064.

51.Kern, S. E., Pietenpol, J. A., Thiagalingam, S., Seymour, A., Kinzler, K. W., and

Vogelstein, B. Oncogenic forms of p53 inhibit p53-regulated gene expression. Science. 1992;256:827-830.

52.Bakalkin, G., Selivanova, G., Yakovleva, T., Kiseleva, E., Kashuba, E., Magnusson, K. P., Szekely, L., Klein, G., Terenius, L., and Wiman, K. G. p53 binds single-stranded DNA ends through the C-terminal domain and internal DNA segments via the middle domain. Nucleic Acids Res. 1995;23:362-369.

53.Ahn, J. and Prives, C. The C-terminus of p53: the more you learn the less you know. Nat Struct Biol 2001;8:730-732.

54.Hupp, T. R., Meek, D. W., Midgley, C. A., and Lane, D. P. Regulation of the specific DNA binding function of p53. Cell 1992;71:875-886.

55.Hupp, T. R., Sparks, A., and Lane, D. P. Small peptides activate the latent sequence-specific DNA binding function of p53. Cell 1995;83:237-245.

56.Abarzua, P., LoSardo, J. E., Gubler, M. L., and Neri, A. Microinjection of monoclonal antibody PAb421 into human SW480 colorectal carcinoma cells restores the transcription activation function to mutant p53. Cancer Res. 1995;55:3490-3494.

57.Selivanova, G., Iotsova, V., Okan, I., Fritsche, M., Strom, M., Groner, B., Grafstrom, R. C., and Wiman, K. G. Restoration of the growth suppression function of mutant p53 by a synthetic peptide derived from the p53 C-terminal domain. Nat Med. 1997;3:632-638.

58.Kim, A. L., Raffo, A. J., Brandt-Rauf, P. W., Pincus, M. R., Monaco, R., Abarzua, P., and Fine, R. L. Conformational and molecular basis for induction of apoptosis by a p53 C-terminal peptide in human cancer cells. J Biol Chem. 1999;274:34924-34931.

59.Takenaka, I., Morin, F., Seizinger, B. R., and Kley, N. Regulation of the sequence-specific DNA binding function of p53 by protein kinase C and protein phosphatases. The Journal Of Biological Chemistry 1995;270:5405-5411.

60.Wang, Y. and Prives, C. Increased and altered DNA binding of human p53 by S and G2/M but not G1 cyclin-dependent kinases. Nature 1995;376:88-91.

61.Waterman, M. J., Stavridi, E. S., Waterman, J. L., and Halazonetis, T. D. ATM-dependent activation of p53 involves dephosphorylation and association

with 14-3-3 proteins. Nat Genet 1998;19:175-178.

62.Gu, W. and Roeder, R. G. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell 1997;90:595-606.

63.Sakaguchi, K., Herrera, J. E., Saito, S. i., Miki, T., Bustin, M., Vassilev, A., Anderson, C. W., and Appella, E. DNA damage activates p53 through a phosphorylation-acetylation cascade. Genes Dev. 1998;12:2831-2841.

64.Brooks, C. L. and Gu, W. Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation. Curr Opin Cell Biol 2003;15:164-171.

65.Xirodimas, D. P., Saville, M. K., Bourdon, J. C., Hay, R. T., and Lane, D. P. Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity. Cell 2004;118:83-97.

66.Chuikov, S., Kurash, J. K., Wilson, J. R., Xiao, B., Justin, N., Ivanov, G. S., McKinney, K., Tempst, P., Prives, C., Gamblin, S. J., Barlev, N. A., and Reinberg, D. Regulation of p53 activity through lysine methylation. Nature 2004;3:3.

67.Liu, L., Scolnick, D. M., Trievel, R. C., Zhang, H. B., Marmorstein, R., Halazonetis, T. D., and Berger, S. L. p53 sites acetylated in vitro by PCAF and p300 are acetylated in vivo in response to DNA damage. Mol Cell Biol 1999;19:1202-1209.

68.Luo, J., Li, M., Tang, Y., Laszkowska, M., Roeder, R. G., and Gu, W. Acetylation of p53 augments its site-specific DNA binding both in vitro and in vivo. Proc Natl Acad Sci U S A 2004;101:2259-2264.

69.Hupp, T. R. and Lane, D. P. Allosteric activation of latent p53 tetramers. Current Biology 1994;4:865-875.

70.Anderson, M. E., Woelker, B., Reed, M., Wang, P., and Tegtmeyer, P. Reciprocal interference between the sequence-specific core and nonspecific C-terminal DNA binding domains of p53: implications for regulation. Mol Cell Biol. 1997;17:6255-6264.

71.Ayed, A., Mulder, F. A., Yi, G. S., Lu, Y., Kay, L. E., and Arrowsmith, C. H. Latent and active p53 are identical in conformation. Nat Struct Biol. 2001;8:756-760.

72.Kaeser, M. D. and Iggo, R. D. Chromatin immunoprecipitation analysis fails to support the latency model for regulation of p53 DNA binding activity in vivo. Proc Natl Acad Sci U S A 2002;99:95-100.

73.Espinosa, J. M. and Emerson, B. M. Transcriptional regulation by p53 through intrinsic DNA/chromatin binding and site-directed cofactor recruitment. Mol Cell 2001;8:57-69.

74.Barlev, N. A., Liu, L., Chehab, N. H., Mansfield, K., Harris, K. G., Halazonetis, T. D., and Berger, S. L. Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases. Molecular Cell 2001;8:1243-1254.

75.McKinney, K. and Prives, C. Efficient specific DNA binding by p53 requires both its central and C-terminal domains as revealed by studies with high-mobility group 1 protein. Mol Cell Biol 2002;22:6797-6808.

76.Gohler, T., Reimann, M., Cherny, D., Walter, K., Warnecke, G., Kim, E., and Deppert, W. Specific interaction of p53 with target binding sites is determined by DNA conformation and is regulated by the C-terminal domain. The Journal Of Biological Chemistry 2002;277:41192-41203.

77.McKinney, K., Mattia, M., Gottifredi, V., and Prives, C. p53 Linear Diffusion along DNA Requires Its C Terminus. Mol Cell 2004;16:413-424.

78.Giaccia, A. J. and Kastan, M. B. The complexity of p53 modulation: emerging patterns from divergent signals. Genes Dev. 1998;12:2973-2983.

79.Appella, E. and Anderson, C. W. Signaling to p53: breaking the posttranslational modification code. Pathol Biol (Paris) 2000;48:227-245.

80.Saito, S., Yamaguchi, H., Higashimoto, Y., Chao, C., Xu, Y., Fornace, A. J., Jr., Appella, E., and Anderson, C. W. Phosphorylation site interdependence of human p53 posttranslational modifications in response to stress. J Biol Chem 2003;14:14.

81.Meyer, K. M., Hess, S. M., Tlsty, T. D., and Leadon, S. A. Human mammary epithelial cells exhibit a differential p53-mediated response following exposure to ionizing radiation or UV light. Oncogene. 1999;18:5795-5805.

82.Das, K. C. and Dashnamoorthy, R. Hyperoxia activates the ATR-Chk1 pathway and phosphorylates p53 at multiple sites. Am J Physiol Lung Cell Mol Physiol 2004;286:L87-97.

83.Shackelford, R. E., Innes, C. L., Sieber, S. O., Heinloth, A. N., Leadon, S. A., and Paules, R. S. The Ataxia telangiectasia Gene Product Is Required for Oxidative Stress-induced G1 and G2 Checkpoint Function in Human Fibroblasts. J. Biol. Chem. 2001;276:21951-21959.

84.Wang, S., Konorev, E. A., Kotamraju, S., Joseph, J., Kalivendi, S., and Kalyanaraman, B. Doxorubicin Induces Apoptosis in Normal and Tumor Cells via Distinctly Different Mechanisms: INTERMEDIACY OF H2O2- AND p53-DEPENDENT PATHWAYS. J. Biol. Chem. 2004;279:25535-25543.

85.Lorenzo, E., Ruiz-Ruiz, C., Quesada, A. J., Hernandez, G., Rodriguez, A., Lopez-Rivas, A., and Redondo, J. M. Doxorubicin induces apoptosis and CD95 gene expression in human primary endothelial cells through a p53-dependent mechanism. J Biol Chem 2002;277:10883-10892. Epub 12002 Jan 10884.

86.Lowe, S. W., Ruley, H. E., Jacks, T., and Housman, D. E. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 1993;74:957-967.

87.Alarcon, R., Koumenis, C., Geyer, R. K., Maki, C. G., and Giaccia, A. J. Hypoxia induces p53 accumulation through MDM2 down-regulation and inhibition of E6-mediated degradation. Cancer Res. 1999;59:6046-6051.

88.Weissman, A. M. Themes and variations on ubiquitylation. Nat Rev Mol Cell Biol. 2001;2:169-178.

89.Scheffner, M., Huibregtse, J. M., Vierstra, R. D., and Howley, P. M. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell. 1993;75:495-505.

90.Honda, R., Tanaka, H., and Yasuda, H. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS Lett 1997;420:25-27.

91.Kubbutat, M. H., Jones, S. N., and Vousden, K. H. Regulation of p53 stability by Mdm2. Nature 1997;387:299-303.

92.Cahilly-Snyder, L., Yang-Feng, T., Francke, U., and George, D. L. Molecular

analysis and chromosomal mapping of amplified genes isolated from a transformed mouse 3T3 cell line. Somatic Cell And Molecular Genetics 1987;13:235-244.

93.Momand, J., Jung, D., Wilczynski, S., and Niland, J. The MDM2 gene amplification database. Nucl. Acids Res. 1998;26:3453-3459.

94.Fang, S., Jensen, J. P., Ludwig, R. L., Vousden, K. H., and Weissman, A. M. Mdm2 Is a RING Finger-dependent Ubiquitin Protein Ligase for Itself and p53. J. Biol. Chem. 2000;275:8945-8951.

95.Mendrysa, S. M. and Perry, M. E. The p53 tumor suppressor protein does not regulate expression of its own inhibitor, MDM2, except under conditions of stress. Mol Cell Biol. 2000;20:2023-2030.

96.Wu, X., Bayle, J., Olson, D., and Levine, A. The p53-mdm-2 autoregulatory feedback loop. Genes Dev. 1993;7:1126-1132.

97.Montes de Oca Luna, R., Wagner, D. S., and Lozano, G. Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. Nature. 1995;378:203-206.

98.Bond, G. L., Hu, W., Bond, E. E., Robins, H., Lutzker, S. G., Arva, N. C., Bargonetti, J., Bartel, F., Taubert, H., Wuerl, P., Onel, K., Yip, L., Hwang, S. J., Strong, L. C., Lozano, G., and Levine, A. J. A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans. Cell 2004;119:591-602.

99.Moll, U. M. and Petrenko, O. The MDM2-p53 Interaction. Mol Cancer Res 2003;1:1001-1008.

100.Mendrysa, S. M., McElwee, M. K., Michalowski, J., O'Leary, K. A., Young, K. M., and Perry, M. E. mdm2 Is critical for inhibition of p53 during lymphopoiesis and the response to ionizing irradiation. Mol Cell Biol. 2003;23:462-472.

101.Kobet, E., Zeng, X., Zhu, Y., Keller, D., and Lu, H. MDM2 inhibits p300-mediated p53 acetylation and activation by forming a ternary complex with the two proteins. PNAS 2000;97:12547-12552.

102.Brooks, C. L. and Gu, W. p53 Ubiquitination: Mdm2 and Beyond. Molecular

Cell 2006;21:307-315.

103.Lowe, S. W. and Sherr, C. J. Tumor suppression by Ink4a-Arf: progress and puzzles. Curr Opin Genet Dev. 2003;13:77-83.

104.Pomerantz, J., Schreiber-Agus, N., Liegeois, N. J., Silverman, A., Alland, L., Chin, L., Potes, J., Chen, K., Orlow, I., and Lee, H.-W. The Ink4a Tumor Suppressor Gene Product, p19Arf, Interacts with MDM2 and Neutralizes MDM2's Inhibition of p53. Cell 1998;92:713-723.

105.Chen, D., Kon, N., Li, M., Zhang, W., Qin, J., and Gu, W. ARF-BP1/Mule Is a Critical Mediator of the ARF Tumor Suppressor. Cell 2005;121:1071-1083.

106.Honda, R. and Yasuda, H. Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. Embo J. 1999;18:22-27.

107.Llanos, S., Clark, P. A., Rowe, J., and Peters, G. Stabilization of p53 by p14ARF without relocation of MDM2 to the nucleolus. 2001;3:445-452.

108.Pan, Y. and Chen, J. MDM2 promotes ubiquitination and degradation of MDMX. Mol Cell Biol 2003;23:5113-5121.

109.Lohrum, M. A. E., Ludwig, R. L., Kubbutat, M. H. G., Hanlon, M., and Vousden, K. H. Regulation of HDM2 activity by the ribosomal protein L11. Cancer Cell 2003;3:577-587.

110.Dai, M.-S., Zeng, S. X., Jin, Y., Sun, X.-X., David, L., and Lu, H. Ribosomal Protein L23 Activates p53 by Inhibiting MDM2 Function in Response to Ribosomal Perturbation but Not to Translation Inhibition. Mol. Cell. Biol. 2004;24:7654-7668.

111.Rodriguez, M. S., Desterro, J. M. P., Lain, S., Midgley, C. A., Lane, D. P., and Hay, R. T. SUMO-1 modification activates the transcriptional response of p53. EMBO J. 1999;18:6455-6461.

112.Appella, E. and Anderson, C. W. Post-translational modifications and activation of p53 by genotoxic stresses. Eur J Biochem 2001;268:2764-2772.

113.Blattner, C., Hay, T., Meek, D. W., and Lane, D. P. Hypophosphorylation of Mdm2 augments p53 stability. Mol Cell Biol 2002;22:6170-6182.

114.Stad, R., Little, N. A., Xirodimas, D. P., Frenk, R., van der Eb, A. J., Lane, D. P., Saville, M. K., and Jochemsen, A. G. Mdmx stabilizes p53 and Mdm2 via two distinct mechanisms. EMBO Rep 2001;2:1029-1034.

115.Riemenschneider, M. J., Buschges, R., Wolter, M., Reifenberger, J., Bostrom, J., Kraus, J. A., Schlegel, U., and Reifenberger, G. Amplification and overexpression of the MDM4 (MDMX) gene from 1q32 in a subset of malignant gliomas without TP53 mutation or MDM2 amplification. Cancer Res. 1999;59:6091-6096.

116.Jackson, M. W. and Berberich, S. J. MdmX protects p53 from Mdm2-mediated degradation. Mol Cell Biol 2000;20:1001-1007.

117.Mancini, F., Gentiletti, F., D'Angelo, M., Giglio, S., Nanni, S., D'Angelo, C., Farsetti, A., Citro, G., Sacchi, A., Pontecorvi, A., and Moretti, F. MDM4 (MDMX) overexpression enhances stabilization of stress-induced p53 and promotes apoptosis. J Biol Chem 2003;1:1.

118.Francoz, S., Froment, P., Bogaerts, S., De Clercq, S., Maetens, M., Doumont, G., Bellefroid, E., and Marine, J. C. Mdm4 and Mdm2 cooperate to inhibit p53 activity in proliferating and quiescent cells in vivo. Proc Natl Acad Sci U S A. 2006;103:3232-3237. Epub 2006 Feb 3221.

119.Gu, J., Kawai, H., Nie, L., Kitao, H., Wiederschain, D., Jochemsen, A. G., Parant, J., Lozano, G., and Yuan, Z.-M. Mutual Dependence of MDM2 and MDMX in Their Functional Inactivation of p53. J. Biol. Chem. 2002;277:19251-19254.

120.Sabbatini, P. and McCormick, F. MDMX inhibits the p300/CBP-mediated acetylation of p53. DNA Cell Biol 2002;21:519-525.

121.Brooks, C. L., Li, M., and Gu, W. Monoubiquitination: The Signal for p53 Nuclear Export? Cell Cycle 2004;3:436-438.

122.Li, M., Brooks, C. L., Wu-Baer, F., Chen, D., Baer, R., and Gu, W. Mono- versus polyubiquitination: differential control of p53 fate by Mdm2. Science 2003;302:1972-1975.

123.Shirangi, T. R., Zaika, A., and Moll, U. M. Nuclear degradation of p53 occurs during down-regulation of the p53 response after DNA damage. The FASEB

Journal: Official Publication Of The Federation Of American Societies For Experimental Biology 2002;16:420-422.

124.Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H. D., Mayer, T. U., and Jentsch, S. A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. Cell. 1999;96:635-644.

125.Grossman, S. R., Deato, M. E., Brignone, C., Chan, H. M., Kung, A. L., Tagami, H., Nakatani, Y., and Livingston, D. M. Polyubiquitination of p53 by a ubiquitin ligase activity of p300. Science 2003;300:342-344.

126.Dornan, D., Wertz, I., Shimizu, H., Arnott, D., Frantz, G. D., Dowd, P., K, O. R., Koeppen, H., and Dixit, V. M. The ubiquitin ligase COP1 is a critical negative regulator of p53. Nature 2004;429:86-92.

127.Leng, R. P., Lin, Y., Ma, W., Wu, H., Lemmers, B., Chung, S., Parant, J. M., Lozano, G., Hakem, R., and Benchimol, S. Pirh2, a p53-induced ubiquitin-protein ligase, promotes p53 degradation. Cell 2003;112:779-791.

128.Asher, G., Lotem, J., Sachs, L., Kahana, C., and Shaul, Y. Mdm-2 and ubiquitin-independent p53 proteasomal degradation regulated by NQO1. Proc Natl Acad Sci U S A. 2002;99:13125-13130.

129.Asher, G., Tsvetkov, P., Kahana, C., and Shaul, Y. A mechanism of ubiquitin-independent proteasomal degradation of the tumor suppressors p53 and p73. Genes Dev. 2005;19:316-321.

130.Xu, Y. Regulation of p53 responses by post-translational modifications. Cell Death Differ 2003;10:400-403.

131.Canman, C. E., Lim, D.-S., Cimprich, K. A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M. B., and Siliciano, J. D. Activation of the ATM Kinase by Ionizing Radiation and Phosphorylation of p53. Science 1998;281:1677-1679.

132.Banin, S., Moyal, L., Shieh, S.-Y., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Prives, C., Reiss, Y., Shiloh, Y., and Ziv, Y. Enhanced Phosphorylation of p53 by ATM in Response to DNA Damage. Science 1998;281:1674-1677.

133.Shieh, S. Y., Ikeda, M., Taya, Y., and Prives, C. DNA damage-induced

phosphorylation of p53 alleviates inhibition by MDM2. Cell 1997;91:325-334.

134.Avantaggiati, M. L., Ogryzko, V., Gardner, K., Giordano, A., Levine, A. S., and Kelly, K. Recruitment of p300/CBP in p53-dependent signal pathways. Cell 1997;89:1175-1184.

135.Lambert, P. F., Kashanchi, F., Radonovich, M. F., Shiekhattar, R., and Brady, J. N. Phosphorylation of p53 Serine 15 increases Interaction with CBP. J. Biol. Chem. 1998;273:33048-33053.

136.Dumaz, N. and Meek, D. W. Serine15 phosphorylation stimulates p53 transactivation but does not directly influence interaction with HDM2. Embo J 1999;18:7002-7010.

137.Chao, C., Saito, S., Anderson, C. W., Appella, E., and Xu, Y. Phosphorylation of murine p53 at ser-18 regulates the p53 responses to DNA damage. Proc Natl Acad Sci U S A 2000;97:11936-11941.

138.Chao, C., Hergenhahn, M., Kaeser, M. D., Wu, Z., Saito, S., Iggo, R., Hollstein, M., Appella, E., and Xu, Y. Cell type- and promoter-specific roles of Ser18 phosphorylation in regulating p53 responses. J Biol Chem 2003;8:8.

139.Webley, K., Bond, J. A., Jones, C. J., Blaydes, J. P., Craig, A., Hupp, T., and Wynford-Thomas, D. Posttranslational modifications of p53 in replicative senescence overlapping but distinct from those induced by DNA damage. Mol Cell Biol 2000;20:2803-2808.

140.Ferbeyre, G., de Stanchina, E., Querido, E., Baptiste, N., Prives, C., and Lowe, S. W. PML is induced by oncogenic ras and promotes premature senescence. Genes Dev. 2000;14:2015-2027.

141.Shieh, S.-Y., Taya, Y., and Prives, C. DNA damage-inducible phosphorylation of p53 at N-terminal sites including a novel site, Ser20, requires tetramerization. EMBO J. 1999;18:1815-1823.

142.Chehab, N. H., Malikzay, A., Stavridi, E. S., and Halazonetis, T. D. Phosphorylation of Ser-20 mediates stabilization of human p53 in response to DNA damage. PNAS 1999;96:13777-13782.

143.Shieh, S.-Y., Ahn, J., Tamai, K., Taya, Y., and Prives, C. The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple

DNA damage-inducible sites. Genes Dev. 2000;14:289-300.

144.Wu, Z., Earle, J., Saito, S. i., Anderson, C. W., Appella, E., and Xu, Y. Mutation of Mouse p53 Ser23 and the Response to DNA Damage. Mol. Cell. Biol. 2002;22:2441-2449.

145.MacPherson, D., Kim, J., Kim, T., Rhee, B. K., Van Oostrom, C. T., DiTullio, R. A., Venere, M., Halazonetis, T. D., Bronson, R., De Vries, A., Fleming, M., and Jacks, T. Defective apoptosis and B-cell lymphomas in mice with p53 point mutation at Ser 23. Embo J. 2004;23:3689-3699.

146.Chao, C., Herr, D., Chun, J., and Xu, Y. Ser18 and 23 phosphorylation is required for p53-dependent apoptosis and tumor suppression. Embo J. 2006;25:2615-2622.

147.Bulavin, D. V., Saito, S. i., Hollander, M. C., Sakaguchi, K., Anderson, C. W., Appella, E., and Fornace Jr, A. J. Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. EMBO J. 1999;18:6845-6854.

148.D'Orazi, G., Cecchinelli, B., Bruno, T., Manni, I., Higashimoto, Y., Saito, S., Gostissa, M., Coen, S., Marchetti, A., Del Sal, G., Piaggio, G., Fanciulli, M., Appella, E., and Soddu, S. Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser 46 and mediates apoptosis. Nat Cell Biol 2002;4:11-19.

149.Saito, S., Goodarzi, A. A., Higashimoto, Y., Noda, Y., Lees-Miller, S. P., Appella, E., and Anderson, C. W. ATM mediates phosphorylation at multiple p53 sites, including Ser(46), in response to ionizing radiation. J Biol Chem 2002;277:12491-12494.

150.Yoshida, K., Liu, H., and Miki, Y. Protein kinase C delta regulates Ser46 phosphorylation of p53 tumor suppressor in the apoptotic response to DNA damage. J Biol Chem. 2006;281:5734-5740.

151.Oda, K., Arakawa, H., Tanaka, T., Matsuda, K., Tanikawa, C., Mori, T., Nishimori, H., Tamai, K., Tokino, T., Nakamura, Y., and Taya, Y. p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. Cell 2000;102:849-862.

152.Mayo, L. D., Rok Seo, Y., Jackson, M. W., Smith, M. L., Rivera Guzman, J. R.,

Korgaonkar, C. K., and Donner, D. B. Phosphorylation of human p53 at serine 46 determines promoter selection and whether apoptosis is attenuated or amplified. J Biol Chem 2005;20:20.

153.Stambolic, V., MacPherson, D., Sas, D., Lin, Y., Snow, B., Jang, Y., Benchimol, S., and Mak, T. W. Regulation of PTEN Transcription by p53. Molecular Cell 2001;8:317-325.

154.Li, X., Dumont, P., Della Pietra, A., Shetler, C., and Murphy, M. E. The codon 47 polymorphism in p53 is functionally significant. J Biol Chem 2005;25:25.

155.Buschmann, T., Potapova, O., Bar-Shira, A., Ivanov, V. N., Fuchs, S. Y., Henderson, S., Fried, V. A., Minamoto, T., Alarcon-Vargas, D., Pincus, M. R., Gaarde, W. A., Holbrook, N. J., Shiloh, Y., and Ronai, Z. Jun NH2-terminal kinase phosphorylation of p53 on Thr-81 is important for p53 stabilization and transcriptional activities in response to stress. Mol Cell Biol 2001;21:2743-2754.

156.Blaydes, J. P., Luciani, M. G., Pospisilova, S., Ball, H. M.-L., Vojtesek, B., and Hupp, T. R. Stoichiometric Phosphorylation of Human p53 at Ser315 Stimulates p53-dependent Transcription. J. Biol. Chem. 2001;276:4699-4708.

157.Lin, T., Chao, C., Saito, S., Mazur, S. J., Murphy, M. E., Appella, E., and Xu, Y. p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. Nat Cell Biol 2004;26:26.

158.Kapoor, M., Hamm, R., Yan, W., Taya, Y., and Lozano, G. Cooperative phosphorylation at multiple sites is required to activate p53 in response to UV radiation. Oncogene 2000;19:358-364.

159.Hao, M., Lowy, A. M., Kapoor, M., Deffie, A., Liu, G., and Lozano, G. Mutation of Phosphoserine 389Affects p53 Function in Vivo. J. Biol. Chem. 1996;271:29380-29385.

160.Lu, K. P., Liou, Y. C., and Zhou, X. Z. Pinning down proline-directed phosphorylation signaling. Trends Cell Biol 2002;12:164-172.

161.Ryo, A., Liou, Y. C., Lu, K. P., and Wulf, G. Prolyl isomerase Pin1: a catalyst for oncogenesis and a potential therapeutic target in cancer. J Cell Sci 2003;116:773-783.

162.Zheng, H., You, H., Zhou, X. Z., Murray, S. A., Uchida, T., Wulf, G., Gu, L., Tang, X., Lu, K. P., and Xiao, Z. X. The prolyl isomerase Pin1 is a regulator of p53 in genotoxic response. Nature 2002;419:849-853.

163.Zacchi, P., Gostissa, M., Uchida, T., Salvagno, C., Avolio, F., Volinia, S., Ronai, Z., Blandino, G., Schneider, C., and Del Sal, G. The prolyl isomerase Pin1 reveals a mechanism to control p53 functions after genotoxic insults. Nature 2002;419:853-857.

164.Gostissa, M., Hengstermann, A., Fogal, V., Sandy, P., Schwarz, S. E., Scheffner, M., and Del Sal, G. Activation of p53 by conjugation to the ubiquitin-like protein SUMO-1. Embo J 1999;18:6462-6471.

165.Ito, A., Lai, C.-H., Zhao, X., Saito, S. i., Hamilton, M. H., Appella, E., and Yao, T.-P. p300/CBP-mediated p53 acetylation is commonly induced by p53-activating agents and inhibited by MDM2. EMBO J. 2001;20:1331-1340.

166.Kubbutat, M. H. G., Ludwig, R. L., Ashcroft, M., and Vousden, K. H. Regulation of Mdm2-Directed Degradation by the C Terminus of p53. Mol. Cell. Biol. 1998;18:5690-5698.

167.Rodriguez, M. S., Desterro, J. M., Lain, S., Lane, D. P., and Hay, R. T. Multiple C-terminal lysine residues target p53 for ubiquitin-proteasome-mediated degradation. Molecular And Cellular Biology 2000;20:8458-8467.

168.Nakamura, S., Roth, J. A., and Mukhopadhyay, T. Multiple lysine mutations in the C-terminal domain of p53 interfere with MDM2-dependent protein degradation and ubiquitination. Mol Cell Biol 2000;20:9391-9398.

169.Li, M., Luo, J., Brooks, C. L., and Gu, W. Acetylation of p53 inhibits its ubiquitination by Mdm2. J Biol Chem 2002;277:50607-50611.

170.Oren, M. Decision making by p53: life, death and cancer. Cell Death Differ. 2003;10:431-442.

171.Samuels-Lev, Y., O'Connor, D. J., Bergamaschi, D., Trigiante, G., Hsieh, J. K., Zhong, S., Campargue, I., Naumovski, L., Crook, T., and Lu, X. ASPP proteins specifically stimulate the apoptotic function of p53. Mol Cell. 2001;8:781-794.

172.Shikama, N., Lee, C. W., France, S., Delavaine, L., Lyon, J., Krstic-Demonacos, M., and La Thangue, N. B. A novel cofactor for p300 that regulates the p53

response. Mol Cell 1999;4:365-376.

173.Flores, E. R., Tsai, K. Y., Crowley, D., Sengupta, S., Yang, A., McKeon, F., and Jacks, T. p63 and p73 are required for p53-dependent apoptosis in response to DNA damage. Nature 2002;416:560-564.

174.Kastan, M. B. and Bartek, J. Cell-cycle checkpoints and cancer. 2004;432:316-323.

175.Khanna, K. K. and Jackson, S. P. DNA double-strand breaks: signaling, repair and the cancer connection. 2001;27:247-254.

176.Bakkenist, C. J. and Kastan, M. B. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature 2003;421:499-506.

177.DiTullio, R. A., Mochan, T. A., Venere, M., Bartkova, J., Sehested, M., Bartek, J., and Halazonetis, T. D. 53BP1 functions in an ATM-dependent checkpoint pathway that is constitutively activated in human cancer. 2002;4:998-1002.

178.Wang, B., Matsuoka, S., Carpenter, P. B., and Elledge, S. J. 53BP1, a Mediator of the DNA Damage Checkpoint. Science 2002;298:1435-1438.

179.Lou, Z., Minter-Dykhouse, K., Wu, X., and Chen, J. MDC1 is coupled to activated CHK2 in mammalian DNA damage response pathways. 2003;421:957-961.

180.Bartek, J. and Lukas, J. Chk1 and Chk2 kinases in checkpoint control and cancer. Cancer Cell 2003;3:421-429.

181.Sherr, C. J. and Roberts, J. M. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev. 1999;13:1501-1512.

182.Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. p21 is a universal inhibitor of cyclin kinases. Nature 1993;366:701-704.

183.Taylor, W. R. and Stark, G. R. Regulation of the G2/M transition by p53. Oncogene. 2001;20:1803-1815.

184.Bartek, J. and Lukas, J. DNA repair: Damage alert. Nature 2003;421:486-488.

185.Bassing, C. H. and Alt, F. W. The cellular response to general and programmed DNA double strand breaks. DNA Repair. 2004;3:781-796.

186.Richardson, C., Horikoshi, N., and Pandita, T. K. The role of the DNA double-strand break response network in meiosis. DNA Repair. 2004;3:1149-1164.

187.Rooney, S., Chaudhuri, J., and Alt, F. W. The role of the non-homologous end-joining pathway in lymphocyte development. Immunol Rev. 2004;200:115-131.

188.Wyman, C., Ristic, D., and Kanaar, R. Homologous recombination-mediated double-strand break repair. DNA Repair. 2004;3:827-833.

189.Stracker, T. H., Theunissen, J.-W. F., Morales, M., and Petrini, J. H. J. The Mre11 complex and the metabolism of chromosome breaks: the importance of communicating and holding things together. DNA Repair. 2004;3:845-854.

190.Celeste, A., Petersen, S., Romanienko, P. J., Fernandez-Capetillo, O., Chen, H. T., Sedelnikova, O. A., Reina-San-Martin, B., Coppola, V., Meffre, E., Difilippantonio, M. J., Redon, C., Pilch, D. R., Olaru, A., Eckhaus, M., Camerini-Otero, R. D., Tessarollo, L., Livak, F., Manova, K., Bonner, W. M., Nussenzweig, M. C., and Nussenzweig, A. Genomic Instability in Mice Lacking Histone H2AX. Science 2002;296:922-927.

191.Celeste, A., Fernandez-Capetillo, O., Kruhlak, M. J., Pilch, D. R., Staudt, D. W., Lee, A., Bonner, R. F., Bonner, W. M., and Nussenzweig, A. Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks. Nat Cell Biol 2003;5:675-679.

192.Celeste, A., Difilippantonio, S., Difilippantonio, M. J., Fernandez-Capetillo, O., Pilch, D. R., Sedelnikova, O. A., Eckhaus, M., Ried, T., Bonner, W. M., and Nussenzweig, A. H2AX haploinsufficiency modifies genomic stability and tumor susceptibility. Cell 2003;114:371-383.

193.Gao, Y., Sun, Y., Frank, K. M., Dikkes, P., Fujiwara, Y., Seidl, K. J., Sekiguchi, J. M., Rathbun, G. A., Swat, W., and Wang, J. A Critical Role for DNA End-Joining Proteins in Both Lymphogenesis and Neurogenesis. Cell 1998;95:891-902.

194.Gao, Y., Ferguson, D. O., Xie, W., Manis, J. P., Sekiguchi, J., Frank, K. M.,

Chaudhuri, J., Horner, J., DePinho, R. A., and Alt, F. W. Interplay of p53 and DNA-repair protein XRCC4 in tumorigenesis, genomic stability and development. Nature 2000;404:897-900.

195.Lane, D. P. p53, guardian of the genome. 1992;358:15-16.

196.Cory, S. and Adams, J. M. THE BCL2 FAMILY: REGULATORS OF THE CELLULAR LIFE-OR-DEATH SWITCH. Nat Rev Cancer 2002;2:647-656.

197.Clarke, A. R., Purdie, C. A., Harrison, D. J., Morris, R. G., Bird, C. C., Hooper, M. L., and Wyllie, A. H. Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature 1993;362:849-852.

198.Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A., and Jacks, T. p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature 1993;362:847-849.

199.Symonds, H., Krall, L., Remington, L., Saenz-Robles, M., Lowe, S., Jacks, T., and Van Dyke, T. p53-Dependent apoptosis suppresses tumor growth and progression in vivo. Cell 1994;78:703-711.

200.Schmitt, C. A., Fridman, J. S., Yang, M., Baranov, E., Hoffman, R. M., and Lowe, S. W. Dissecting p53 tumor suppressor functions in vivo. Cancer Cell 2002;1:289-298.

201.Liu, G., Parant, J. M., Lang, G., Chau, P., Chavez-Reyes, A., El-Naggar, A. K., Multani, A., Chang, S., and Lozano, G. Chromosome stability, in the absence of apoptosis, is critical for suppression of tumorigenesis in Trp53 mutant mice. Nat Genet 2004;36:63-68.

202.Graeber, T. G., Osmanian, C., Jacks, T., Housman, D. E., Koch, C. J., Lowe, S. W., and Giaccia, A. J. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. 1996;379:88-91.

203.Fridman, J. S. and Lowe, S. W. Control of apoptosis by p53. Oncogene. 2003;22:9030-9040.

204.Adams, J. M. and Cory, S. Apoptosomes: engines for caspase activation. Current Opinion in Cell Biology 2002;14:715-720.

205.Peter, M. E. and Krammer, P. H. The CD95(APO-1/Fas) DISC and beyond.

Cell Death Differ. 2003;10:26-35.

206.Schulze-Osthoff, K., Ferrari, D., Los, M., Wesselborg, S., and Peter, M. E. Apoptosis signaling by death receptors. Eur J Biochem. 1998;254:439-459.

207.Wu, G. S., Burns, T. F., McDonald, E. R., 3rd, Jiang, W., Meng, R., Krantz, I. D., Kao, G., Gan, D. D., Zhou, J. Y., Muschel, R., Hamilton, S. R., Spinner, N. B., Markowitz, S., Wu, G., and el-Deiry, W. S. KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. Nat Genet 1997;17:141-143.

208.Muller, M., Wilder, S., Bannasch, D., Israeli, D., Lehlbach, K., Li-Weber, M., Friedman, S. L., Galle, P. R., Stremmel, W., Oren, M., and Krammer, P. H. p53 Activates the CD95 (APO-1/Fas) Gene in Response to DNA Damage by Anticancer Drugs. J. Exp. Med. 1998;188:2033-2045.

209.Lin, Y., Ma, W., and Benchimol, S. Pidd, a new death-domain-containing protein, is induced by p53 and promotes apoptosis. Nat Genet 2000;26:122-127.

210.Attardi, L. D., Reczek, E. E., Cosmas, C., Demicco, E. G., McCurrach, M. E., Lowe, S. W., and Jacks, T. PERP, an apoptosis-associated target of p53, is a novel member of the PMP-22/gas3 family. Genes Dev. 2000;14:704-718.

211.Ihrie, R. A., Reczek, E., Horner, J. S., Khachatrian, L., Sage, J., Jacks, T., and Attardi, L. D. Perp Is a Mediator of p53-Dependent Apoptosis in Diverse Cell Types. Current Biology 2003;13:1985-1990.

212.Chipuk, J. E. and Green, D. R. Dissecting p53-dependent apoptosis. 2006;13:994-1002.

213.Reed, J. C. Bcl-2 family proteins. Oncogene 1998;17:3225-3236.

214.Miyashita, T. and Reed, J. C. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 1995;80:293-299.

215.Zhang, L., Yu, J., Park, B. H., Kinzler, K. W., and Vogelstein, B. Role of BAX in the Apoptotic Response to Anticancer Agents. Science 2000;290:989-992.

216.Yin, C., Knudson, C. M., Korsmeyer, S. J., and Dyke, T. V. Bax suppresses tumorigenesis and stimulates apoptosis in vivo. 1997;385:637-640.

217.Yu, J. and Zhang, L. The transcriptional targets of p53 in apoptosis control. Biochemical and Biophysical Research Communications p53 in Apoptosis Control 2005;331:851-858.

218.Villunger, A., Michalak, E. M., Coultas, L., Mullauer, F., Bock, G., Ausserlechner, M. J., Adams, J. M., and Strasser, A. p53- and Drug-Induced Apoptotic Responses Mediated by BH3-Only Proteins Puma and Noxa. Science 2003;18:18.

219.Shibue, T., Takeda, K., Oda, E., Tanaka, H., Murasawa, H., Takaoka, A., Morishita, Y., Akira, S., Taniguchi, T., and Tanaka, N. Integral role of Noxa in p53-mediated apoptotic response. Genes Dev. 2003;17:2233-2238.

220.Jeffers, J. R., Parganas, E., Lee, Y., Yang, C., Wang, J., Brennan, J., MacLean, K. H., Han, J., Chittenden, T., and Ihle, J. N. Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. Cancer Cell 2003;4:321-328.

221.Nakano, K. and Vousden, K. H. PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell 2001;7:683-694.

222.Yu, J., Zhang, L., Hwang, P. M., Kinzler, K. W., and Vogelstein, B. PUMA induces the rapid apoptosis of colorectal cancer cells. Mol Cell 2001;7:673-682.

223.Fei, P., Bernhard, E. J., and El-Deiry, W. S. Tissue-specific Induction of p53 Targets in Vivo. Cancer Res 2002;62:7316-7327.

224.Matsuda, K., Yoshida, K., Taya, Y., Nakamura, K., Nakamura, Y., and Arakawa, H. p53AIP1 Regulates the Mitochondrial Apoptotic Pathway. Cancer Res 2002;62:2883-2889.

225.Moll, U. M., Wolff, S., Speidel, D., and Deppert, W. Transcription-independent pro-apoptotic functions of p53. Current Opinion in Cell Biology 2005;17:631-636.

226.Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P., and Moll, U. M. p53 Has a Direct Apoptogenic Role at the Mitochondria. Molecular Cell 2003;11:577-590.

227.Leu, J. I.-J., Dumont, P., Hafey, M., Murphy, M. E., and George, D. L. Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex.

Nature Cell Biology 2004;6:443-450.

228.Chipuk, J. E., Kuwana, T., Bouchier-Hayes, L., Droin, N. M., Newmeyer, D. D., Schuler, M., and Green, D. R. Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. Science 2004;303:1010-1014.

229.Speidel, D., Helmbold, H., and Deppert, W. Dissection of transcriptional and non-transcriptional p53 activities in the response to genotoxic stress. 2005;25:940-953.

230.Dumont, P., Leu, J. I.-J., Della Pietra, A. C., George, D. L., and Murphy, M. The codon 72 polymorphic variants of p53 have markedly different apoptotic potential. 2003;33:357-365.

231.Hayflick, L. and Moorhead, P. S. The serial cultivation of human diploid cell strains. Exp Cell Res. 1961;25:585-621.

232.Smith, J. R. and Pereira-Smith, O. M. Replicative senescence: implications for in vivo aging and tumor suppression. Science. 1996;273:63-67.

233.Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linskens, M., Rubelj, I., and Pereira-Smith et, a. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proceedings Of The National Academy Of Sciences Of The United States Of America 1995;92:9363-9367.

234.van Steensel, B., Smogorzewska, A., and de Lange, T. TRF2 protects human telomeres from end-to-end fusions. Cell. 1998;92:401-413.

235.Campisi, J. Cancer, aging and cellular senescence. In Vivo. 2000;14:183-188.

236.Hasty, P., Campisi, J., Hoeijmakers, J., van Steeg, H., and Vijg, J. Aging and Genome Maintenance: Lessons from the Mouse? Science 2003;299:1355-1359.

237.Samper, E., Nicholls, D. G., and Melov, S. Mitochondrial oxidative stress causes chromosomal instability of mouse embryonic fibroblasts. Aging Cell. 2003;2:277-285.

238.Campisi, J. Cellular senescence as a tumor-suppressor mechanism. Trends Cell Biol 2001;11:S27-31.

239.Parrinello, S., Samper, E., Krtolica, A., Goldstein, J., Melov, S., and Campisi, J. Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. 2003;5:741-747.

240.Bringold, F. and Serrano, M. Tumor suppressors and oncogenes in cellular senescence. Exp Gerontol. 2000;35:317-329.

241.Bandyopadhyay, D. and Medrano, E. E. The emerging role of epigenetics in cellular and organismal aging. Exp Gerontol. 2003;38:1299-1307.

242.Narita, M. and Lowe, S. W. Executing cell senescence. Cell Cycle. 2004;3:244-246.

243.Ben-Porath, I. and Weinberg, R. A. The signals and pathways activating cellular senescence. Int J Biochem Cell Biol 2005;37:961-976.

244.Itahana, K., Dimri, G., and Campisi, J. Regulation of cellular senescence by p53. Eur J Biochem 2001;268:2784-2791.

245.Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 1997;88:593-602.

246.Lin, A. W., Barradas, M., Stone, J. C., van Aelst, L., Serrano, M., and Lowe, S. W. Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. Genes & Development 1998;12:3008-3019.

247.Pearson, M., Carbone, R., Sebastiani, C., Cioce, M., Fagioli, M., Saito, S., Higashimoto, Y., Appella, E., Minucci, S., Pandolfi, P. P., and Pelicci, P. G. PML regulates p53 acetylation and premature senescence induced by oncogenic Ras. Nature 2000;406:207-210.

248.Wahl, G. M. and Carr, A. M. The evolution of diverse biological responses to DNA damage: insights from yeast and p53. Nat Cell Biol. 2001;3:E277-286.

249.Wang, W., Chen, J. X., Liao, R., Deng, Q., Zhou, J. J., Huang, S., and Sun, P. Sequential activation of the MEK-extracellular signal-regulated kinase and MKK3/6-p38 mitogen-activated protein kinase pathways mediates oncogenic ras-induced premature senescence. Molecular And Cellular Biology

2002;22:3389-3403.

250.Lowe, S. W., Cepero, E., and Evan, G. Intrinsic tumour suppression. Nature 2004;432:307-315.

251.el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. WAF1, a potential mediator of p53 tumor suppression. Cell 1993;75:817-825.

252.el-Deiry, W. S., Harper, J. W., O'Connor, P. M., Velculescu, V. E., Canman, C. E., Jackman, J., Pietenpol, J. A., Burrell, M., Hill, D. E., Wang, Y., and et al. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res 1994;54:1169-1174.

253.Atadja, P., Wong, H., Garkavtsev, I., Veillette, C., and Riabowol, K. Increased activity of p53 in senescing fibroblasts. Proc Natl Acad Sci U S A 1995;92:8348-8352.

254.Noda, A., Ning, Y., Venable, S. F., Pereira-Smith, O. M., and Smith, J. R. Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. Exp Cell Res. 1994;211:90-98.

255.Stein, G. H., Drullinger, L. F., Soulard, A., and Dulic, V. Differential roles for cyclin-dependent kinase inhibitors p21 and p16 in the mechanisms of senescence and differentiation in human fibroblasts. Mol Cell Biol. 1999;19:2109-2117.

256.Brown, J. P., Wei, W., and Sedivy, J. M. Bypass of senescence after disruption of p21CIP1/WAF1 gene in normal diploid human fibroblasts. Science. 1997;277:831-834.

257.Pearson, M. and Pelicci, P. G. PML interaction with p53 and its role in apoptosis and replicative senescence. Oncogene. 2001;20:7250-7256.

258.Bischof, O., Kirsh, O., Pearson, M., Itahana, K., Pelicci, P. G., and Dejean, A. Deconstructing PML-induced premature senescence. Embo J 2002;21:3358-3369.

259.de Stanchina, E., Querido, E., Narita, M., Davuluri, R. V., Pandolfi, P. P., Ferbeyre, G., and Lowe, S. W. PML is a direct p53 target that modulates p53 effector functions. Mol Cell 2004;13:523-535.

260.Braig, M., Lee, S., Loddenkemper, C., Rudolph, C., Peters, A. H. F. M., Schlegelberger, B., Stein, H., Dorken, B., Jenuwein, T., and Schmitt, C. A. Oncogene-induced senescence as an initial barrier in lymphoma development. 2005;436:660-665.

261.Chen, Z., Trotman, L. C., Shaffer, D., Lin, H. K., Dotan, Z. A., Niki, M., Koutcher, J. A., Scher, H. I., Ludwig, T., Gerald, W., Cordon-Cardo, C., and Pandolfi, P. P. Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. Nature. 2005;436:725-730.

262.Collado, M., Gil, J., Efeyan, A., Guerra, C., Schuhmacher, A. J., Barradas, M., Benguria, A., Zaballos, A., Flores, J. M., Barbacid, M., Beach, D., and Serrano, M. Tumour biology: Senescence in premalignant tumours. 2005;436:642.

263.Michaloglou, C., Vredeveld, L. C. W., Soengas, M. S., Denoyelle, C., Kuilman, T., van der Horst, C. M. A. M., Majoor, D. M., Shay, J. W., Mooi, W. J., and Peeper, D. S. BRAFE600-associated senescence-like cell cycle arrest of human naevi. 2005;436:720-724.

264.Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S. H., Giovanella, B. C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M. H., and Parsons, R. PTEN, a Putative Protein Tyrosine Phosphatase Gene Mutated in Human Brain, Breast, and Prostate Cancer. Science 1997;275:1943-1947.

265.Liaw, D., Marsh, D. J., Li, J., Dahia, P. L. M., Wang, S. I., Zheng, Z., Bose, S., Call, K. M., Tsou, H. C., Peacoke, M., Eng, C., and Parsons, R. Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. 1997;16:64-67.

266.Simpson, L. and Parsons, R. PTEN: Life as a Tumor Suppressor. Experimental Cell Research 2001;264:29-41.

267.Sun, H., Lesche, R., Li, D.-M., Liliental, J., Zhang, H., Gao, J., Gavrilova, N., Mueller, B., Liu, X., and Wu, H. PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway. PNAS 1999;96:6199-6204.

268.Wu, X., Senechal, K., Neshat, M. S., Whang, Y. E., and Sawyers, C. L. The PTEN/MMAC1 tumor suppressor phosphatase functions as a negative regulator

of the phosphoinositide 3-kinase/Akt pathway. PNAS 1998;95:15587-15591.

269.Mayo, L. D. and Donner, D. B. A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. PNAS 2001;98:11598-11603.

270.Freeman, D. J., Li, A. G., Wei, G., Li, H.-H., Kertesz, N., Lesche, R., Whale, A. D., Martinez-Diaz, H., Rozengurt, N., and Cardiff, R. D. PTEN tumor suppressor regulates p53 protein levels and activity through phosphatase-dependent and -independent mechanisms. Cancer Cell 2003;3:117-130.

271.Tang, Y. and Eng, C. PTEN Autoregulates Its Expression by Stabilization of p53 in a Phosphatase-Independent Manner. Cancer Res 2006;66:736-742.

272.Campisi, J. Senescent Cells, Tumor Suppression, and Organismal Aging: Good Citizens, Bad Neighbors. Cell 2005;120:513-522.

273.Krtolica, A. and Campisi, J. Cancer and aging: a model for the cancer promoting effects of the aging stroma. Int J Biochem Cell Biol. 2002;34:1401-1414.

274.Tyner, S. D., Venkatachalam, S., Choi, J., Jones, S., Ghebranious, N., Igelmann, H., Lu, X., Soron, G., Cooper, B., Brayton, C., Hee Park, S., Thompson, T., Karsenty, G., Bradley, A., and Donehower, L. A. p53 mutant mice that display early ageing-associated phenotypes. 2002;415:45-53.

275.Gentry, A. and Venkatachalam, S. Complicating the role of p53 in aging. Aging Cell. 2005;4:157-160.

276.Mendrysa, S. M., O'Leary, K. A., McElwee, M. K., Michalowski, J., Eisenman, R. N., Powell, D. A., and Perry, M. E. Tumor suppression and normal aging in mice with constitutively high p53 activity. Genes Dev. 2006;20:16-21.

277.Garcia-Cao, I., Garcia-Cao, M., Martin-Caballero, J., Criado, L. M., Klatt, P., Flores, J. M., Weill, J. C., Blasco, M. A., and Serrano, M. "Super p53" mice exhibit enhanced DNA damage response, are tumor resistant and age normally. Embo J. 2002;21:6225-6235.

278.Poyurovsky, M. V. and Prives, C. Unleashing the power of p53: lessons from mice and men

10.1101/gad.1397506. Genes Dev. 2006;20:125-131

279.Meletis, K., Wirta, V., Hede, S. M., Nister, M., Lundeberg, J., and Frisen, J. p53 suppresses the self-renewal of adult neural stem cells. Development. 2006;133:363-369.

280.Groszer, M., Erickson, R., Scripture-Adams, D. D., Lesche, R., Trumpp, A., Zack, J. A., Kornblum, H. I., Liu, X., and Wu, H. Negative Regulation of Neural Stem/Progenitor Cell Proliferation by the Pten Tumor Suppressor Gene in Vivo. Science 2001;294:2186-2189.

281.Molofsky, A. V., Pardal, R., Iwashita, T., Park, I. K., Clarke, M. F., and Morrison, S. J. Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. Nature. 2003;425:962-967.

282.Sabapathy, K., Klemm, M., Jaenisch, R., and Wagner, E. F. Regulation of ES cell differentiation by functional and conformational modulation of p53. Embo J 1997;16:6217-6229.

283.Xu, Y. A new role for p53 in maintaining genetic stability in embryonic stem cells. Cell Cycle. 2005;4:363-364.

284.Attardi, L. D. and Donehower, L. A. Probing p53 biological functions through the use of genetically engineered mouse models. Mutation Research 2005;576:4-21.

285.Feng, L., Lin, T., Uranishi, H., Gu, W., and Xu, Y. Functional analysis of the roles of posttranslational modifications at the p53 C terminus in regulating p53 stability and activity. Mol Cell Biol 2005;25:5389-5395.

286.Luo, J. L., Yang, Q., Tong, W. M., Hergenhahn, M., Wang, Z. Q., and Hollstein, M. Knock-in mice with a chimeric human/murine p53 gene develop normally and show wild-type p53 responses to DNA damaging agents: a new biomedical research tool. Oncogene 2001;20:320-328.

Chapter 2

Functional analysis of the roles of posttranslational modifications at the p53 C terminus in regulating p53 stability and activity

Abstract

Posttranslational modification of tumor suppressor p53 plays important roles in regulating its stability and activity. Six lysine residues at p53 C-terminus can be posttranslationally modified by various mechanisms, including acetylation, ubiquitination, neddylation, methylation and sumoylation. Previous cell line transfection studies show that ubiquitination of these lysine residues is required for ubiquitin-dependent degradation of p53. In addition, biochemical and cell line studies suggested that p53 acetylation at the C-terminus might stabilize p53 and activate its transcriptional activities. To investigate the physiological functional outcome of these C-terminal modifications in regulating p53 stability and activity, we introduced missense mutations (Lysine to Arginine) at the six Lysine residues (K6R) into the endogenous p53 gene in mouse embryonic stem (ES) cells. K6R mutation prevents all posttranslational modifications at these sites but conserves the structure of p53. In contrast to the conclusion of previous studies, analysis of p53 stability in K6R ES cells, mouse embryonic fibroblasts (MEFs) and thymocytes showed normal p53 stabilization in K6R cells both before and after DNA damage, indicating that ubiquitination of these lysine residues is not required for efficient p53 degradation. However, p53-dependent gene expression was impaired in K6R ES cells and thymocytes in a promoter-specific manner after DNA damage, indicating that the net outcome of the posttranslational modifications at the C-terminus is to activate p53 transcriptional activities after DNA damage.

Introduction

p53 is one of the most frequently mutated tumor suppressor genes in human cancers (1). It is a transcriptional factor composed of four functional domains: the N-terminal transactivation domain, through which p53 interacts with coactivators or corepressors, the central sequence-specific DNA binding domain, the tetramerization domain and the extreme C-terminal regulatory domain (2, 3). In unstressed cells, p53 is present in a latent form and is maintained at low levels through rapid protein degradation. Recent studies showed that Mdm2, Pirh2 and COP1 can all facilitate p53 degradation via the ubiquitin-proteasome pathway by functioning as ubiquitin ligase (4-8). In response to genotoxic and cellular stresses, the stability and activity of p53 are greatly induced, leading to cell cycle arrest, DNA repair, and/or apoptosis, depending on the cell types (2, 9-13).

While the mechanism how p53 responses are activated after various genotoxic and cellular stresses remains to be established, accumulating evidence indicates that posttranslational modifications of p53 play important roles in regulating its stability and transcriptional activity (10). Specifically, the multiple lysine residues at the extreme carboxyl-terminal domain of p53 (the last 30aa) can be posttranslationally modified by multiple mechanisms, including phosphorylation, acetylation, ubiquitination, neddylation and methylation, in response to DNA damage and other cellular stresses (14-20). In this context, human p53 can potentially be acetylated by CBP/p300 at five lysine residues, Lys370, Lys372,

Lys373, Lys381 and Lys382 (16, 21). Using acetylation-specific antibody, it has been shown that the acetylation of p53 at Lys373 and Lys382 is significantly induced in response to DNA damage *in vivo* (17, 22, 23). In addition, human p53 can also be acetylated by P/CAF (p300/CBP associated factor) at Lys320 *in vitro* and this acetylation event is induced by DNA damage *in vivo* (17, 22).

The roles of p53 acetylation have been studied extensively. Gu et al. suggested that CBP/p300 mediated acetylation of p53 can increase p53 sequence-specific DNA-binding activity *in vitro* by electrophoretic mobility-shift assay (EMSA) using short oligonucleotides (16). Several subsequent studies also supported that the acetylation of p53 can dramatically stimulates its sequence-specific DNA-binding activity both *in vitro* and *in vivo*, possibly caused by an acetylation-induced conformational change (17, 21, 22). However, recent studies showed that p53 binds to its cognate promoters constitutively (24). In addition, acetylation does not increase the p53 DNA-binding activity when assayed for binding to an artificially reconstituted chromatin (25). Instead, this and other studies showed that p53 acetylation is important for the recruitment of co-activators (25, 26). Recent studies also showed that p53 C-terminus is required for p53 linear diffusion on chromatin and its efficient DNA binding as well as transactivation of target promoters *in vivo* (27). However, acetylation and other modifications of C-terminal do not increase this p53 activity.

Several studies also suggested that ubiquitination and acetylation at the C-terminus

of p53 can regulate p53 stability. In this context, one study showed that p53 C-terminus was required for Mdm2-mediated degradation of p53 but not Mdm2-p53 interaction (28). Since the lysine residues at the C-terminus might be ubiquitinated by Mdm2, two studies tested the importance of the C-terminal lysine residues in p53 stabilization. In one study, all six lysine residues were changed to Arginine (6KR mutant) to prevent ubiquitination but preserve the structure of p53 (29). While 6KR mutant interacts with Mdm2 normally, it could not undergo Mdm2-mediated ubiquitination and degradation in transfected tumor cell lines, leading to p53 stabilization and activation (29). In addition, mutation of four lysine residues (Lys372, 373, 381 and 382) into Alanine (A4 mutant) also abrogated the p53 ubiquitination and degradation (30). Since acetylation of the same lysine residues might prevent ubiquitination at these sites, it has been suggested that p53 acetylation can stabilize p53. In support of this notion, increased levels of p53 acetylation by deacetylase inhibitors could inhibit p53 degradation *in vivo* and the p53 degradation requires deacetylation (23, 31).

In addition to acetylation and ubiquitination, the C-terminal lysine residues can also be modified by other mechanisms. Lys386 of p53 can be modified by conjugation to a small ubiquitin-like protein SUMO-1 *in vitro* and *in vivo* (32, 33), although the role of this modification remains controversial. Recent studies also showed that ubiquitin-like protein Nedd8 can be covalently linked to p53 at Lys370, 372 and 373, and Mdm2-dependent neddylation of p53 negatively regulates its

transcriptional activity (19). Additionally, methylation of p53 at Lys372 by Set9 methyltransferase has been identified and suggested to restrict p53 in the nucleus and stabilize p53 (20).

To further study the physiological roles of the posttranslational modifications at the C-terminal lysine residues in regulating p53 stability and activity, we employed homologous recombination and LoxP/Cre-mediated deletion to introduce six Lysine to Arginine mutations (Lys367, 369, 370, 378, 379 and 383 to Arg; K6R) into the endogenous mouse p53 gene in ES cells. Analysis of p53 stability and activity before and after DNA damage indicated that ubiquitination of the six C-terminal lysine residues is not required for p53 degradation. However, the net effects of these modifications increase p53 transcriptional activities in a cell type-dependent manner.

Materials and Methods

Construction of targeting vector and generation of p53K6R mutant ES cells

The six lysine residues, Lys367, Lys369, Lys370, Lys378, Lys379 and Lys383 are encoded by exon11 of murine p53 gene. A fragment of mouse p53 genomic sequence extending from exon 2 through exon 11 was cloned into pBS vector. Site-directed mutagenesis was performed to introduce Lys to Arg mutations into cloned exon 11. To facilitate screening of ES cell clones, an *Eco*RI restriction site was introduced to intron 1 and a neomycin resistance gene (PGK-neo^r) flanked by loxP sites was inserted into intron 7. This targeting vector was then linearized with *Not*I and electroporated into AY ES cells. To excise the PGK-neo^r gene flanked by loxP sites, 20μg of a circular plasmid expressing Cre enzyme was transiently transfected into the mutant ES cells; and the loxP/Cre-mediated deletion was screened by PCR using primers shown in Fig. 1C and D. The PGK-neo^r deleted ES cell clones identified by PCR were then subcloned and the Cre deletion was further confirmed by Southern blot using the same probe.

Derivation, culture and treatment of p53^{K6R} MEFs

We employed the Hprt-deficient blastocyst complementation approach to generate MEFs from the mutant ES cells as previously described (34). MEFs recovered from the embryos at E12.5 were cultured and selected in Dulbecco's

modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, glutamine, antibiotics, 50 μM β-mecaptoethanol and HAT (0.016mg/ml hypoxanthine, 0.01mM aminopterin and 0.0048mg/ml thymidine). After we confirmed that all the live MEFs were derived from mutant ES cells, the HAT selection was removed and MEFs were cultured in normal medium. MEFs were treated with 60J/M^2 UV-C light or 0.25 μM Doxorubicin (Sigma) and harvested at different time points for the analysis of protein levels or gene expression.

Culture and treatment of ES cells

ES cells were cultured on feeder layer in DMEM supplemented with 15% fetal bovine serum, glutamine, nonessential amino acids, sodium pyruvate, antibiotics, 100 μM 2-mecaptoethanol, and recombinant leukemia inhibitory factor (LIF). Before experiments, ES cells were split and plated on gelatin-coated plates in the presence of LIF but without feeder layer cells. ES cells were exposed to 60J/M^2 UV-C light and harvested at different time points for protein levels or at 8hr for gene expression analysis.

Ubiquitination Assay

AY, K6R and $p53^{-/-}$ ES cells were cultured in 10 cm plates. The cells were treated for 6h with proteasome inhibitors, 25μM LLNL and 25μM MG132, before harvested, and were then lysed with RIPA buffer (1% Nonidet P-40, 0.1% SDS,

Tris-HCl, pH 7.8, 150mM NaCl, 1mM DTT, 0.5mM EDTA, 25μM LLNL, 25μM MG132, 5mM NEN, and fresh proteinase inhibitors) with mild sonication. Protein extract of 2×10^6 cells samples was incubated with 1μg p53 antibody against the full-length protein (Santa Cruz Biotechnology) for 1h at 4°C. 30μl of protein A/G agarose beads were added, and the reactions were further incubated overnight at 4°C. The immunoprecipitates were subsequently resolved by 8% SDS-PAGE and analyzed by Western blot with anti-Ubiquitin antibody (P4D1, Santa Cruz Biotechnology) or anti-p53 antibody (Pab240, Santa Cruz Biotechnology).

IR-induced apoptosis in thymocytes

Thymocytes were recovered from 4-6 weeks-old mice and cultured in DMEM supplemented with 5% FCS and 25mM HEPES (pH 7.4) before treatment. Thymocytes were treated with 5Gy of ionizing radiation (IR) and harvested at different time points for analysis of p53 protein levels. For apoptosis assay, thymocytes were exposed to 2.5, 5, 10 or 20 Gy of IR and apoptotic cells were identified 10hr after treatment by staining with Annexin V. For Real-time PCR, thymocytes were irradiated with 5Gy of IR and RNA was harvested 8hr after treatment.

Western blot analysis

Protein extract from 2×10^4 ES cells and MEFs or 2×10^5 thymocytes were

separated on 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% dry milk and probed with CM5, a polyclonal antibody against p53 (Novo laboratories, Inc.) or the polyclonal antibodies specifically against mouse p53 phosphorylated at Ser18 or Ser389 (Cell Signaling Technology). The membranes were subsequently incubated with horseradish peroxide-conjugated secondary antibody, developed with ECL Plus (Amersham Biosciences) and exposed to X-ray film. To determine whether the total amount of proteins loaded in each lane was comparable, the membranes were probed with a goat polyclonal antibody against β-actin (Santa Cruz Biotechnology).

RNA preparation and quantitative real-time PCR analysis

Total RNA was prepared from the frozen cell pellets by following RNeasy RNA Cleanup protocol (Qiagen). Up to 1μg RNA from individual samples was reversely transcribed to cDNA using Superscript II First-strand Synthesis kit (Invitrogen). Real-time PCR was performed on ABI PRISM® 7000 sequence detection system with SYBR Green PCR MasterMix (ABI). The average threshold cycle (C_t) for each gene was determined from triplicate reactions, and the levels of gene expression were determined relatively to the average C_t value of *glyceraldehyde-3- phosphate-dehydrogenase* (GAPDH) as previously described (35). The primer sequences used in real-time PCR are described previously (36).

Statistic analysis

To determine statistical significance, quantitative RT-PCR data and apoptosis data were subjected to a two-tailed Student's *t*-test analysis. Significance was noted for p values less than 0.05.

Results

Generation of K6R mutant murine ES cells.

A DNA fragment harboring murine p53 exon 11 was cloned into pBluescript and the nucleotides encoding the six lysine residues (Lys367, 369, 370, 378, 379 and 383) were mutated to those encoding arginine by site-directed mutagenesis. The knock-in vector was constructed by replacing the germline exon 11 of the cloned mouse p53 genomic DNA with the K6R exon, followed by inserting the PGK-neo^r gene flanked by loxP sites into intron 7 (Fig. 1B). To facilitate the mutagenesis process, the knock-in vector was electroporated into AY ES cells, which contain one wild type p53 allele and the AY allele with exons 2 to 4 replaced with a loxP site (Fig. 1A) (34). The AY allele does not produce any truncated p53 protein, therefore, the genotype of AY ES cell line is p53$^{+/-}$. Homologous recombination between the wild type allele of AY ES cells and the targeting vector was screened by Southern blotting analysis of genomic DNA with *Eco*RI digestion and hybridization to probe 1 (Fig. 1C and E). The PGK-neo^r gene in the mutant ES cells was deleted through transient expression of the Cre enzyme as previously described (37). The LoxP/Cre-mediated deletion was confirmed by Southern Blot analysis with *Eco*RI digestion and hybridization to the probe 1 (Fig. 1F). The final knock-in ES cells were denoted p53K6R. Both the genomic DNA and cDNA from p53K6R ES cells were sequenced to confirm that the p53 gene expressed in p53K6R ES cells harbored the six lysine to arginine mutations but no other mutations.

p53 responses to DNA damage in p53K6R ES cells.

Since previous cell line transfection studies indicated that the C-terminal lysine residues of p53 are important for ubiquitin-mediated degradation of p53 (29, 30), we determined the p53 stability in the p53K6R ES cells before and after DNA damage induced by UV radiation. p53 protein levels were similarly low before DNA damage but greatly induced in both AY and p53K6R ES cells after UV radiation (Fig. 2A). While the protein levels of p53 were slightly reduced at 4 hrs after UV radiation, the peak levels of p53 induction were similar between AY and p53K6R ES cells at 8 hrs after UV radiation (Fig. 2A). Therefore, K6R mutation had no significant impact on p53 stabilization in ES cells with or without DNA damage. In addition, K6R mutation had no significant impact on p53 phosphorylation at N-terminal (Ser18) or C-terminal (Ser389) (Fig. 2A). The normal stability of p53 in p53K6R ES cells in the absence of DNA damage indicates that other lysine residues of p53 can also be ubiquitinated and contribute to the destabilization of p53. In support of this notion, p53 was ubiquitinated in p53K6R ES cells (Fig. 2B). However, the levels of p53 ubiquitination appeared to be slightly reduced in p53K6R ES cells, indicating that the lysine residues at the C-terminus are ubiquitination targets.

Activation of p53 transcriptional activities by DNA damage is important to maintain genetic stability in ES cells (38, 39). To determine whether K6R affects

p53 transcriptional activities, we analyzed the p53-dependent gene expression in p53K6R and control AY ES cells after UV radiation. While there was no significant difference in the p53-dependent induction of *perp* mRNA in p53K6R and AY ES cells after UV radiation, the expression of several other p53 target genes, including *p21*, *Mdm2*, *Noxa*, *Pidd* and *PUMA*, was significantly reduced in p53K6R ES cells 8 hrs after UV radiation when compared with that in AY ES cells (Fig. 2C). As internal controls, no significant difference in the mRNA levels of *p53* or *actin* was detected in p53K6R and AY ES cells after UV radiation (Fig. 2C). Since the protein levels of p53 were similar in K6R and AY ES cells 8 hrs after UV radiation, these findings indicated that K6R mutation impaired p53-dependent transcriptional activity in a promoter-specific manner in ES cells after DNA damage.

p53 responses to DNA damage in p53K6R MEFs.

MEFs undergo p53-dependent cell cycle arrest after DNA damage. Therefore, we examined the effects of K6R mutation on p53 stability and activity in p53K6R MEFs after DNA damage. We employed the Hprt-deficient blastocyst complementation to derive p53K6R MEFs from ES cells as previously described (34). Since the genotype of AY ES cells is $p53^{+/-}$, p53 protein levels in p53K6R and the control $p53^{+/-}$ MEFs before and after DNA damage were analyzed. Consistent with the findings in ES cells, the protein levels of p53 were very low before UV treatment and similarly induced in both p53K6R and $p53^{+/-}$ MEFs after UV

radiation (Fig. 3A). p53 stabilization also appeared to be normal in p53K6R MEFs after DNA damage induced by Doxorubicin (Fig. 3B). Consistent with the findings in ES cells, K6R mutation did not affect the phosphorylation of p53 at Ser18 or Ser389 after UV radiation and Doxorubicin treatment (data not shown). To test whether K6R mutation affects p53-dependent transcriptional activities, we analyzed the expression levels of several p53 target genes, including *p21*, *Mdm2*, *Bax* and *Pidd*, after UV radiation and Doxorubicin treatment (Fig. 3C and D). There is no significant difference in the p53-dependent gene expression between p53K6R and control MEFs after these types of DNA damage. In addition, there was no significant difference in the mRNA levels of *p53* and *actin* between p53K6R and $p53^{+/-}$ MEFs after DNA damage (Fig. 3C, D). Therefore, K6R mutation has no apparent impact on the p53 stability and activity in MEFs after DNA damage.

p53 responses to IR in p53K6R thymocytes.

Mouse thymocytes undergo p53-dependent apoptosis in response to IR. To determine the effects of K6R mutation on p53-dependent apoptotic activities in thymocytes, we derived the p53K6R and control AY thymocytes by Rag2-deficient blastocyst complementation assay as described (34, 40). In addition, $p53^{+/-}$ thymocytes were also used as controls because the genotype of AY cells is $p53^{+/-}$ and previous studies indicated that p53 responses to IR is the same between AY thymocytes and $p53^{+/-}$ thymocytes. Consistent with the notion that K6R mutation

does not affect p53 stabilization, p53 protein levels were similar in p53K6R and control thymocytes before and after IR (Fig. 4A). p53K6R thymocytes were slightly more resistant to the p53-dependent apoptosis after IR than control AY thymocytes (Fig. 4B). However, while the p53-dependent induction of *Mdm2*, *Bax* and *Pidd* was similar between p53K6R and control thymocytes, induction of p53 targeted apoptotic genes, including *Killer/DR5* and *PUMA,* was significantly impaired in p53K6R thymocytes after IR (Fig. 4C). Therefore, we concluded that K6R mutation impaired p53 transcription activities in a promoter-specific manner in thymocytes after IR.

Discussion

Multiple lysine residues at p53 extreme C-terminal domain can be posttranslationally modified by several mechanisms. To investigate the physiological importance of these posttranslational modifications, we introduced the six Lysine to Arginine missense mutations into the endogenous p53 gene in ES cells, which can be differentiated *in vivo* into MEFs and thymocytes. Previous cell line transfection studies reached the conclusion that the C-terminal lysine residues are ubiquitination sites by Mdm2 and are required for ubiquitin-dependent p53 degradation (29, 30). In contrast to these previous findings, our studies indicated that the K6R mutation has no significant impact on p53 stability either before or after DNA damage in mouse ES cells, MEFs and thymocytes. While our findings do not argue against the notion that these C-terminal lysine residues could be ubiquitinated and involved in regulating p53 stability, the finding that p53 was ubiquitinated in p53K6R ES cells clearly indicate that ubiquitination of lysine residues residing in other regions of p53 could play redundant roles in ubiquitin-dependent p53 degradation. One likely explanation for apparent discrepancy between previous findings and our findings is that the $p53^{-/-}$ tumor cell lines or mouse cells used in previous studies might harbor additional mutations that disrupt the redundant pathways involved in ubiquitination of p53 at other lysine residues. In addition, previous studies only focused on the involvement of Mdm2 in the ubiquitination of p53 by overexpressing Mdm2 with p53K6R mutant, and drew

the conclusion that the six C-terminal lysine residues are the primary targets for Mdm2-mediated ubiquitination (29, 30). However, recently identified additional E3 ligases for p53, including p53 targets Pirh2 and COP1, might ubiquitinate p53 at alternative lysine residues and thus destabilize p53K6R (7, 8). In this context, overexpression of Pirh2/COP1/Mdm2 with p53K6R might lead to the ubiquitination and degradation of p53K6R. Consistent with this notion, while p53K6R is as unstable as the wild type p53, $p53^{Gln25Ser26}$, which cannot interact with Mdm2 and is essentially abolished in its p53-dependent expression of genes such as Pirh2 and COP1, is constitutively stable (38).

p53 acetylation is correlated with p53 stabilization after DNA damage (41). Previous studies have suggested that acetylation of lysine residues, particularly the ones at the C-terminus, might prevent ubiquitination of the same residues, thus leading to p53 stabilization (23, 42). Since our findings indicate that ubiquitination at the C-terminal lysine residues is not required for efficient p53 degradation, the importance of the competition of the acetylation and ubiquitination at the p53 C-terminal lysine residues remains questionable. However, our findings do not argue against the notion that p53 acetylation at multiple lysine residues, including those within the extreme C-terminus, can prevent ubiquitination, leading to p53 stabilization.

In addition to their impact on p53 stability, the functions of the posttranslational modifications at C-terminal lysine residues in regulating p53

activities have been extensively studied in biochemical and cell line transfection studies. In this context, various posttranslational modifications of p53 at the C-terminus have been suggested for different roles in regulating p53 activities. Acetylation of p53 at C-terminal has been thought to recruit co-activators to the p53-dependent promoters and thus to activate p53-dependent transcription (25, 26). However, neddylation of p53 at the C-terminal lysine residues might inhibit p53 transcriptional activities (19). Our analysis of p53-dependent transcriptional activities in K6R mutant ES cells, MEFs and thymocytes indicated that K6R mutation impaired p53 activities after DNA damage in ES cells and thymocytes. The lack of defects in MEFs might be due to the activation of functionally redundant posttranslational modification pathways that are induced by stresses of *in vitro* culturing, as indicated by the findings that p53 activities are normally increased during the continuous passage of MEFs eventually leading to cellular senescence (43, 44). In conclusion, our findings are consistent with the notion that p53 acetylation at C-terminus activates p53 transcriptional activation in a promoter-specific manner. In addition, the partial defects observed in K6R mutant mouse cells might underestimate the whole impact of the lack of C-terminal acetylation, since the simultaneous disruption of neddylation at C-terminal by K6R mutation might compensate by increasing p53 activities.

Figure 1. Generation of p53K6R ES cells. (A) Genomic configuration of the endogenous p53 genes in AY ES cells. AY cell line has one wild-type p53 allele and one mutant p53 allele (AY allele) with exons 2 to 4 replaced with a LoxP site. The filled boxes represent p53 exons, and the filled bar represents the probe for Southern blot analysis. The 14-kb germline *EcoRI* fragment and 6-kb *Eco*RI fragment of AY allele are indicated. (B) The targeting construct. The PGK-neo^r gene flanked by LoxP sites was inserted into intron 7. K6R mutation within exon 11 is indicated by an asterisk. (C) Targeted configuration after homologous recombination between the wild-type p53 allele and the targeting vector. The 9.8-kb mutant *Eco*RI fragment is shown. The positions of the primer sets used to screen for LoxP/Cre-mediated deletion are shown by arrowheads. (D) p53K6R knock-in allele. The size of the mutant *Eco*RI fragment is indicated. (E) Southern blotting analysis of the genomic DNA derived from targeted AY ES cells before PGK-neo^r deletion. Genomic DNA was digested with *Eco*RI and hybridized to the probe. The positions of *Eco*RI fragments derived from the germline, AY allele and targeted allele are indicated by arrows. (F) Southern blot analysis of genomic DNA from wild-type ES cells (lane 1), AY ES cells (lane 2), targeted AY ES cells before LoxP/Cre deletion (lane 3) and p53K6R knock-in ES cells after the deletion of PGK-Neo^r gene (lane 4). Genomic DNA was digested with *Eco*RI and hybridized to the probe. The positions of the 14-kb germline, 12.5-kb PGK-neo^r-deleted, 9.8-kb PGK-neo^r-inserted and 6-kb AY *EcoRI* fragments are indicated.

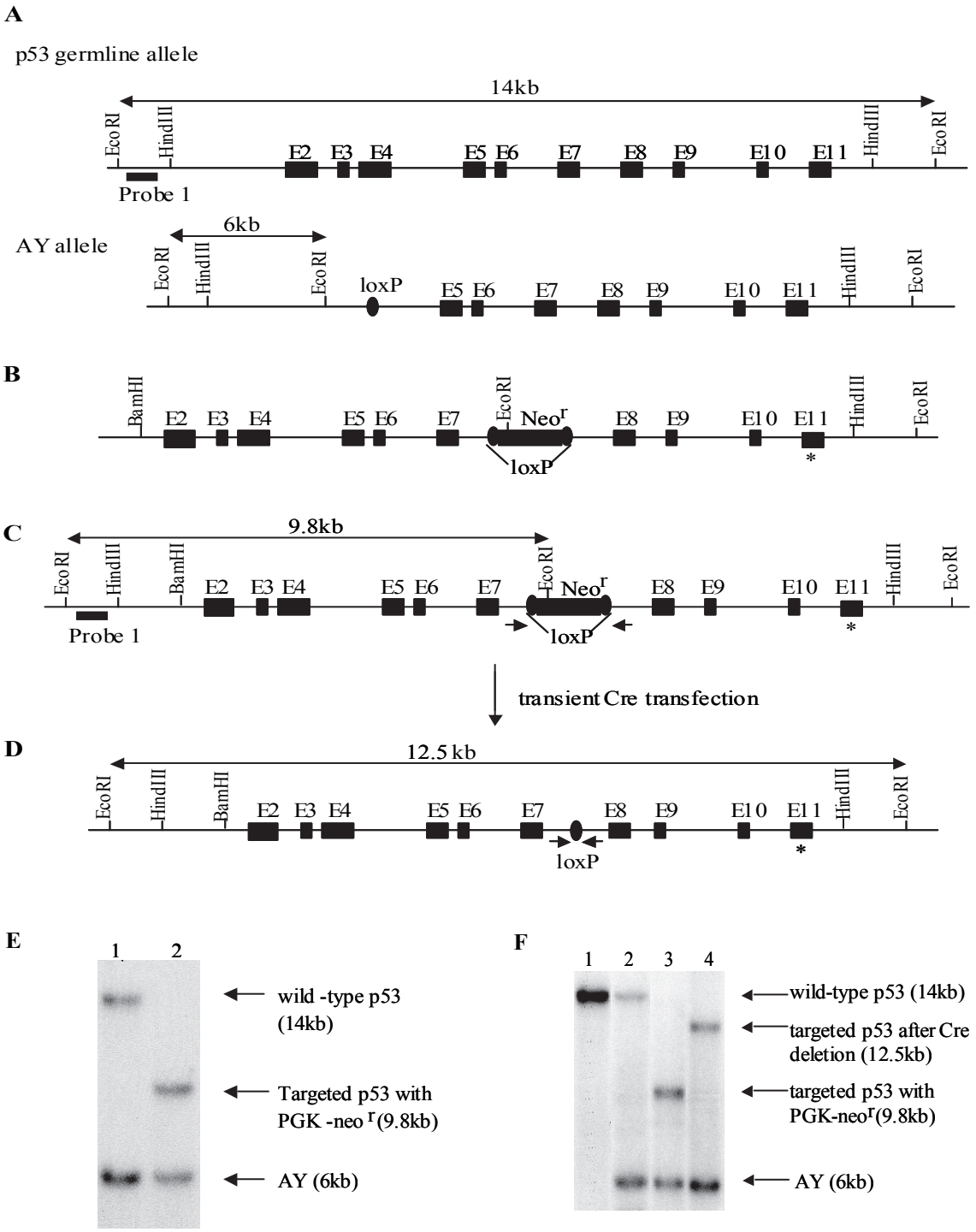

A
p53 germline allele
14kb
EcoRI
HindIII
E2 E3 E4 E5 E6 E7 E8 E9 E10 E11
HindIII
EcoRI
Probe 1
AY allele
6kb
EcoRI
HindIII
EcoRI
loxP
E5 E6 E7 E8 E9 E10 E11
HindIII
EcoRI
B
BamHI
E2 E3 E4 E5 E6 E7
EcoRI
Neo^r
E8 E9 E10 E11
HindIII
EcoRI
loxP
*
C
9.8kb
EcoRI
HindIII
BamHI
E2 E3 E4 E5 E6 E7
EcoRI
Neo^r
E8 E9 E10 E11
HindIII
EcoRI
Probe 1
loxP
*
transient Cre transfection
D
12.5 kb
EcoRI
HindIII
BamHI
E2 E3 E4 E5 E6 E7 E8 E9 E10 E11
HindIII
EcoRI
loxP
*
E
1 2
wild -type p53 (14kb)
Targeted p53 with PGK -neo r(9.8kb)
AY (6kb)
F
1 2 3 4
wild-type p53 (14kb)
targeted p53 after Cre deletion (12.5kb)
targeted p53 with PGK-neo^r(9.8kb)
AY (6kb)

Figure 2. p53 responses to DNA damage in p53K6R ES cells. (A) Induction of p53 in AY and p53K6R ES cells. ES cells were cultured on gelatinized plates in the presence of LIF but without feeder layer cells before UV radiation. Cell extracts were prepared from AY and p53K6R ES cells at the indicated time points after $60J/M^2$ UV-C irradiation and analyzed for p53 protein levels or p53 phosphorylation levels at Ser18 and Ser389. Genotypes of ES cells are shown on top and the p53/actin indicated on the right. (B) Ubiquitination levels of p53 in p53K6R ES cells. Western blot analysis of the ubiquitination levels of p53 immunoprecipitated from AY and p53K6R ES cells either not treated (lanes 3 and 5) or treated with proteasome inhibitors (25μM MG132 + 25μM LLNL) for 6h (lanes 4 and 6) as indicated. Unsaturated amount of anti-full length p53 antibody (1μg) was used to ensure similar total amount of immunoprecipitated p53. Ubiquitinated p53 and total p53 are indicated. (C) p53-dependent transcriptional activity in p53K6R ES cells after UV radiation. The mRNA levels of several p53 target genes in AY and p53K6R ES cells 8 hrs after $60J/M^2$ UV radiation were analyzed by quantitative real-time PCR. The mRNA levels of each gene were standardized by the mRNA level of GAPDH. *p53* and *actin* mRNA levels were also analyzed as non p53-dependent controls. The ratio of mRNA levels in p53K6R cells after UV radiation *versus* those in AY cells is presented. Mean values from three independent experiments are presented with standard deviation. The *p* value representing the statistical significance of the difference between each mean value and 1 is shown on top of each ratio bar. The approximate folds of induction for each p53 target gene in AY ES cells after UV treatment are: *p21*, 10 folds; *Mdm2*, 3.5 folds; *Noxa*, 7 folds; *Perp*, 7.5 folds; *Pidd*, 3 folds; *PUMA*, 5 folds.

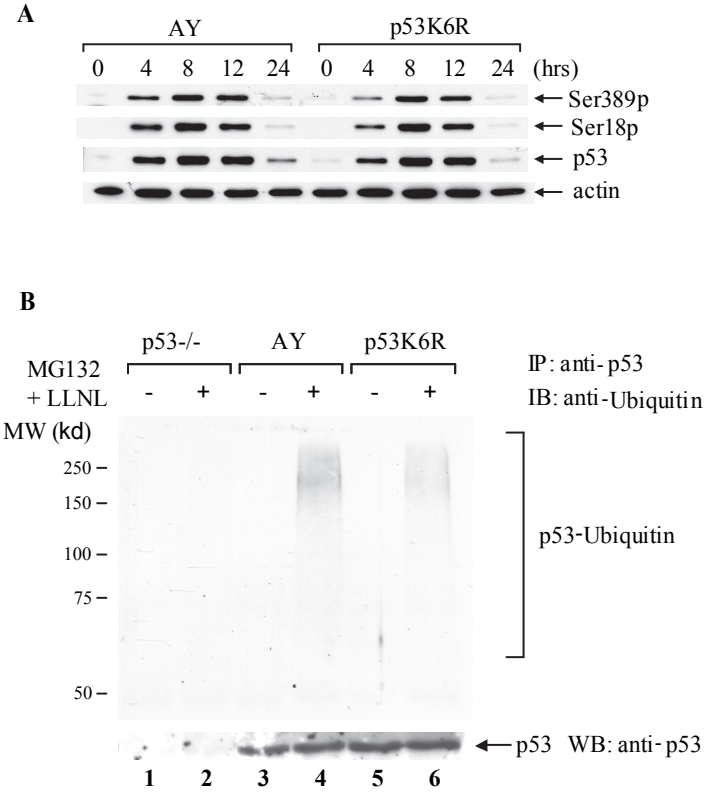

A
AY
p53K6R
0 4 8 12 24 0 4 8 12 24 (hrs)
Ser389p
Ser18p
p53
actin
B
p53-/-
AY
p53K6R
MG132
+ LLNL
- + - + - +
IP: anti-p53
IB: anti-Ubiquitin
MW (kd)
250
150
100
75
50
p53-Ubiquitin
p53 WB: anti-p53
1 2 3 4 5 6
C
p53K6R ES cell gene expression after UV
AY ES cell gene expression after UV
1.2
1
0.8
0.6
0.4
0.2
0
0.028
0.005
0.005
0.137
0.0003
0.049
0.080
0.965
p21
Mdm2
Noxa
perp
Pidd
PUMA
p53
Actin

Figure 3. p53 responses to DNA damage in p53K6R MEFs. Induction of p53 protein levels in $p53^{+/-}$ and p53K6R MEFs after $60J/M^2$ UV treatment (A) or 0.25 μM Doxorubicin treatment (B). The genotype and time points after DNA damage are indicated on the top. p53/actin are indicated on the right. p53-dependent transcriptional activity in p53K6R and control MEFs 18 hrs after treatment with $60J/M^2$ UV radiation (C) or 24 hrs after treatment with 0.25 μM Doxorubicin (D). The mRNA levels of p53 target genes were analyzed by quantitative real-time PCR. In addition, p53 and actin mRNA levels were analyzed as internal controls. Ratio of mRNA levels in treated p53K6R MEFs *versus* those in treated $p53^{+/-}$ MEFs is indicated. Mean values from at least three independent experiments are presented with standard deviation. The *p* values are shown on top of each bar. The folds of induction for each p53 target gene in $p53^{+/-}$ MEFs after UV radiation are: *p21*, 5 folds; *Mdm2*, 5 folds; *Bax*, 1.5 folds; *Pidd*, 7 folds. The folds of induction after Doxorubicin treatment are: *p21*, 11 folds; *Mdm2*, 7 folds; *Bax*, 2.5 folds; *Pidd*, 8.5 folds.

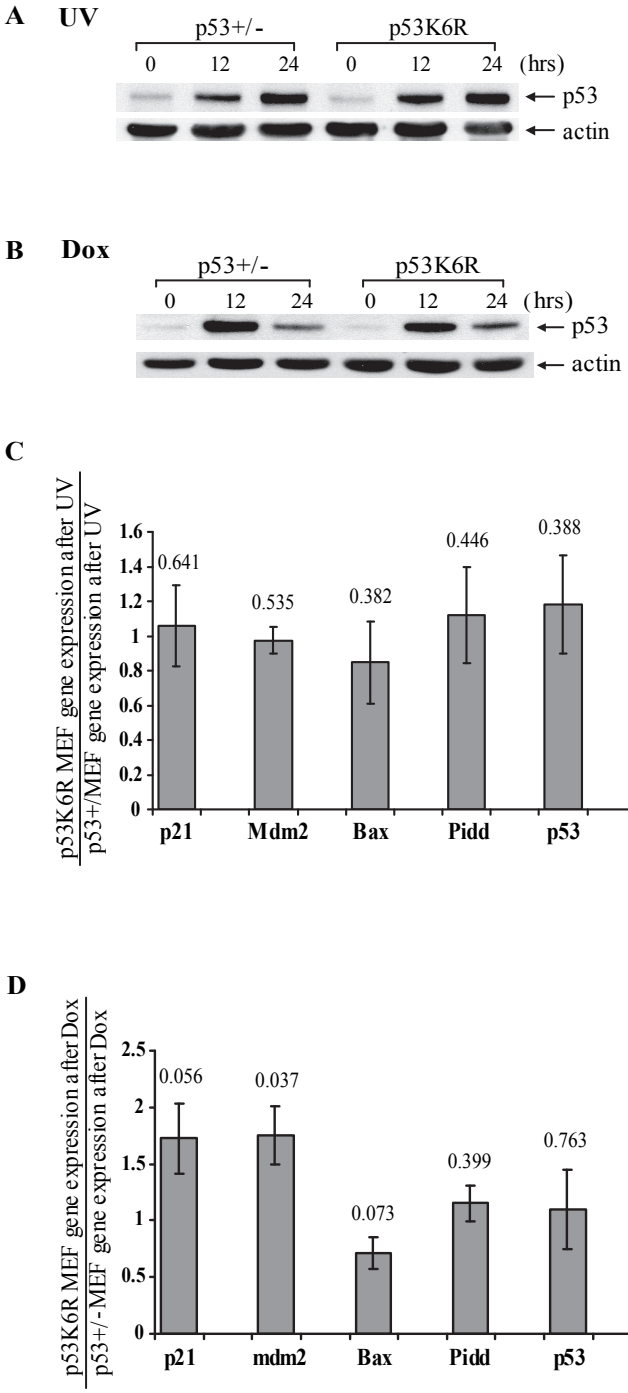
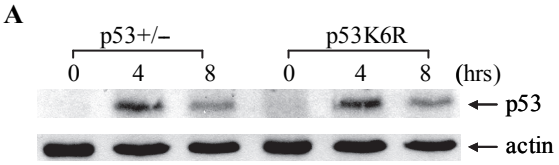
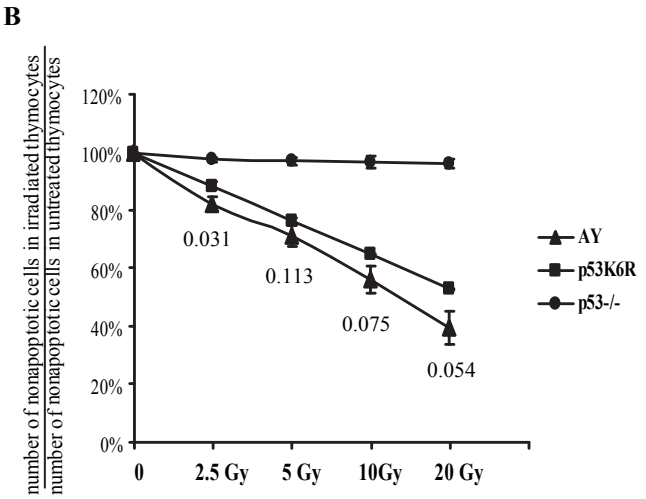
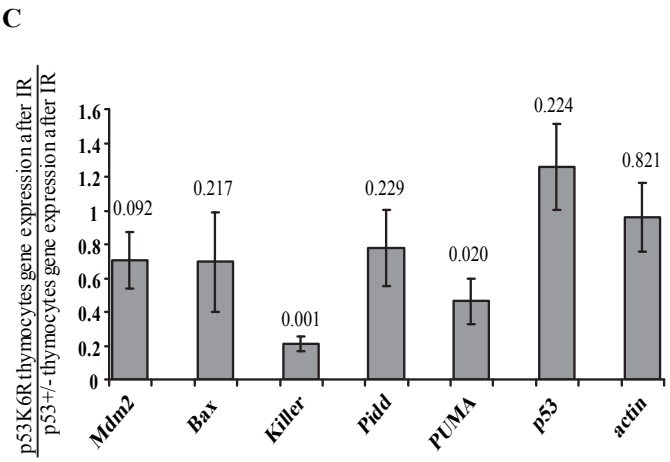

A
UV
p53+/-
p53K6R
0
12
24
0
12
24
(hrs)
p53
actin
B
Dox
p53+/-
p53K6R
0
12
24
0
12
24
(hrs)
p53
actin
C
p53K6R MEF gene expression after UV
p53+/MEF gene expression after UV
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.641
0.535
0.382
0.446
0.388
0.187
p21
Mdm2
Bax
Pidd
p53
actin
D
p53K6R MEF gene expression after Dox
p53+/- MEF gene expression after Dox
2.5
2
1.5
1
0.5
0
0.056
0.037
0.073
0.399
0.763
0.523
p21
mdm2
Bax
Pidd
p53
actin

Fig. 4. p53 responses to DNA damage in p53K6R thymocytes. (A) Induction of p53 protein levels in $p53^{+/-}$ and p53K6R thymocytes after IR. The genotype and time points after IR are indicated on the top. p53/actin are indicated on the right. (B) p53-dependent apoptosis in p53K6R and control AY thymocytes 10 hours after increasing dosages of IR. The *p* values representing the statistical significance of the difference in apoptosis between p53K6R and AY thymocytes are also indicated. (C) p53-dependent transcriptional activity in p53K6R and control $p53^{+/-}$ thymocytes 8hrs after 5Gy of IR. The ratio of mRNA levels in irradiated p53K6R thymocytes *versus* those in irradiated $p53^{+/-}$ thymocytes is shown. Mean value from three independent experiments were shown with standard deviation. *p* value is indicated. The folds of induction of each p53 target gene in $p53^{+/-}$ thymocytes after IR are: *p21*, 17 folds; *Mdm2*, 3 folds; *Bax*, 15 folds; *Killer/RD5*: 4 folds; *Pidd*, 5 folds; *PUMA*, 28 folds.


A
p53+/–
p53K6R
0
4
8
0
4
8
(hrs)
p53
actin


B
number of nonapoptotic cells in irradiated thymocytes / number of nonapoptotic cells in untreated thymocytes
120%
100%
80%
60%
40%
20%
0%
0.031
0.113
0.075
0.054
0
2.5 Gy
5 Gy
10Gy
20 Gy
AY
p53K6R
p53-/-


C
p53K6R thymocytes gene expression after IR / p53+/- thymocytes gene expression after IR
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.092
0.217
0.001
0.229
0.020
0.224
0.821
Mdm2
Bax
Killer
Pidd
PUMA
p53
actin

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References

1. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. p53 mutations in human cancers. Science 1991;253:49-53.

2. Ko, L. J. and Prives, C. p53: puzzle and paradigm. Genes Dev 1996;10:1054-1072.

3. Levine, A. J. p53, the cellular gatekeeper for growth and division. Cell 1997;88:323-331.

4. Haupt, Y., Maya, R., Kazaz, A., and Oren, M. Mdm2 promotes the rapid degradation of p53. Nature 1997;387:296-299.

5. Honda, R., Tanaka, H., and Yasuda, H. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS Lett 1997;420:25-27.

6. Kubbutat, M. H., Jones, S. N., and Vousden, K. H. Regulation of p53 stability by Mdm2. Nature 1997;387:299-303.

7. Dornan, D., Wertz, I., Shimizu, H., Arnott, D., Frantz, G. D., Dowd, P., K, O. R., Koeppen, H., and Dixit, V. M. The ubiquitin ligase COP1 is a critical negative regulator of p53. Nature 2004;429:86-92.

8. Leng, R. P., Lin, Y., Ma, W., Wu, H., Lemmers, B., Chung, S., Parant, J. M., Lozano, G., Hakem, R., and Benchimol, S. Pirh2, a p53-induced ubiquitin-protein ligase, promotes p53 degradation. Cell 2003;112:779-791.

9. Appella, E. and Anderson, C. W. Signaling to p53: breaking the posttranslational modification code. Pathol Biol (Paris) 2000;48:227-245.

10. Appella, E. and Anderson, C. W. Post-translational modifications and activation of p53 by genotoxic stresses. Eur J Biochem 2001;268:2764-2772.

11. Vogelstein, B., Lane, D., and Levine, A. J. Surfing the p53 network. Nature 2000;408:307-310.

12. Vousden, K. H. and Lu, X. Live or let die: the cell's response to p53. Nat Rev Cancer 2002;2:594-604.

13. Meek, D. W. The p53 response to DNA damage. DNA Repair (Amst)

2004;3:1049-1056.

14. Steegenga, W. T., van der Eb, A. J., and Jochemsen, A. G. How phosphorylation regulates the activity of p53. J Mol Biol 1996;263:103-113.

15. Waterman, M. J., Stavridi, E. S., Waterman, J. L., and Halazonetis, T. D. ATM-dependent activation of p53 involves dephosphorylation and association with 14-3-3 proteins. Nat Genet 1998;19:175-178.

16. Gu, W. and Roeder, R. G. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell 1997;90:595-606.

17. Sakaguchi, K., Herrera, J. E., Saito, S. i., Miki, T., Bustin, M., Vassilev, A., Anderson, C. W., and Appella, E. DNA damage activates p53 through a phosphorylation-acetylation cascade. Genes Dev. 1998;12:2831-2841.

18. Brooks, C. L. and Gu, W. Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation. Curr Opin Cell Biol 2003;15:164-171.

19. Xirodimas, D. P., Saville, M. K., Bourdon, J. C., Hay, R. T., and Lane, D. P. Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity. Cell 2004;118:83-97.

20. Chuikov, S., Kurash, J. K., Wilson, J. R., Xiao, B., Justin, N., Ivanov, G. S., McKinney, K., Tempst, P., Prives, C., Gamblin, S. J., Barlev, N. A., and Reinberg, D. Regulation of p53 activity through lysine methylation. Nature 2004;3:3.

21. Luo, J., Li, M., Tang, Y., Laszkowska, M., Roeder, R. G., and Gu, W. Acetylation of p53 augments its site-specific DNA binding both in vitro and in vivo. Proc Natl Acad Sci U S A 2004;101:2259-2264.

22. Liu, L., Scolnick, D. M., Trievel, R. C., Zhang, H. B., Marmorstein, R., Halazonetis, T. D., and Berger, S. L. p53 sites acetylated in vitro by PCAF and p300 are acetylated in vivo in response to DNA damage. Mol Cell Biol 1999;19:1202-1209.

23. Ito, A., Lai, C.-H., Zhao, X., Saito, S. i., Hamilton, M. H., Appella, E., and Yao, T.-P. p300/CBP-mediated p53 acetylation is commonly induced by p53-activating agents and inhibited by MDM2. EMBO J. 2001;20:1331-1340.

24. Kaeser, M. D. and Iggo, R. D. Chromatin immunoprecipitation analysis fails to support the latency model for regulation of p53 DNA binding activity in vivo. Proc Natl Acad Sci U S A 2002;99:95-100.

25. Espinosa, J. M. and Emerson, B. M. Transcriptional regulation by p53 through intrinsic DNA/chromatin binding and site-directed cofactor recruitment. Mol Cell 2001;8:57-69.

26. Barlev, N. A., Liu, L., Chehab, N. H., Mansfield, K., Harris, K. G., Halazonetis, T. D., and Berger, S. L. Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases. Molecular Cell 2001;8:1243-1254.

27. McKinney, K., Mattia, M., Gottifredi, V., and Prives, C. p53 Linear Diffusion along DNA Requires Its C Terminus. Mol Cell 2004;16:413-424.

28. Kubbutat, M. H. G., Ludwig, R. L., Ashcroft, M., and Vousden, K. H. Regulation of Mdm2-Directed Degradation by the C Terminus of p53. Mol. Cell. Biol. 1998;18:5690-5698.

29. Rodriguez, M. S., Desterro, J. M., Lain, S., Lane, D. P., and Hay, R. T. Multiple C-terminal lysine residues target p53 for ubiquitin-proteasome-mediated degradation. Molecular And Cellular Biology 2000;20:8458-8467.

30. Nakamura, S., Roth, J. A., and Mukhopadhyay, T. Multiple lysine mutations in the C-terminal domain of p53 interfere with MDM2-dependent protein degradation and ubiquitination. Mol Cell Biol 2000;20:9391-9398.

31. Li, M., Chen, D., Shiloh, A., Luo, J., Nikolaev, A. Y., Qin, J., and Gu, W. Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. Nature 2002;416:648-653.

32. Rodriguez, M. S., Desterro, J. M. P., Lain, S., Midgley, C. A., Lane, D. P., and Hay, R. T. SUMO-1 modification activates the transcriptional response of p53. EMBO J. 1999;18:6455-6461.

33. Gostissa, M., Hengstermann, A., Fogal, V., Sandy, P., Schwarz, S. E., Scheffner, M., and Del Sal, G. Activation of p53 by conjugation to the ubiquitin-like protein SUMO-1. Embo J 1999;18:6462-6471.

34. Wu, Z., Earle, J., Saito, S. i., Anderson, C. W., Appella, E., and Xu, Y. Mutation

of Mouse p53 Ser23 and the Response to DNA Damage. Mol. Cell. Biol. 2002;22:2441-2449.

35. Boley, S. E., Wong, V. A., French, J. E., and Recio, L. p53 heterozygosity alters the mRNA expression of p53 target genes in the bone marrow in response to inhaled benzene. Toxicol Sci 2002;66:209-215.

36. Chao, C., Hergenhahn, M., Kaeser, M. D., Wu, Z., Saito, S., Iggo, R., Hollstein, M., Appella, E., and Xu, Y. Cell type- and promoter-specific roles of Ser18 phosphorylation in regulating p53 responses. J Biol Chem 2003;8:8.

37. Chao, C., Saito, S., Anderson, C. W., Appella, E., and Xu, Y. Phosphorylation of murine p53 at ser-18 regulates the p53 responses to DNA damage. Proc Natl Acad Sci U S A 2000;97:11936-11941.

38. Chao, C., Saito, S., Kang, J., Anderson, C. W., Appella, E., and Xu, Y. p53 transcriptional activity is essential for p53-dependent apoptosis following DNA damage. Embo J 2000;19:4967-4975.

39. Lin, T., Chao, C., Saito, S., Mazur, S. J., Murphy, M. E., Appella, E., and Xu, Y. p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. Nat Cell Biol 2004;26:26.

40. Inlay, M., Alt, F. W., Baltimore, D., and Xu, Y. Essential roles of the kappa light chain intronic enhancer and 3' enhancer in kappa rearrangement and demethylation. Nat Immunol 2002;3:463-468.

41. Xu, Y. Regulation of p53 responses by post-translational modifications. Cell Death Differ 2003;10:400-403.

42. Li, M., Luo, J., Brooks, C. L., and Gu, W. Acetylation of p53 inhibits its ubiquitination by Mdm2. J Biol Chem 2002;277:50607-50611.

43. Serrano, M. and Blasco, M. A. Putting the stress on senescence. Current Opinion in Cell Biology 2001;13:748-753.

44. Campisi, J. Senescent Cells, Tumor Suppression, and Organismal Aging: Good Citizens, Bad Neighbors. Cell 2005;120:513-522.

Chapter 3

Ser46 phosphorylation regulates p53-dependent apoptosis and cellular senescence

Abstract

Posttranslational modification such as phosphorylation of p53 plays important roles in activating p53 responses to various cellular and genotoxic stresses. Cell line studies have shown that phosphorylation of Ser46 is correlated with the activation of p53 apoptotic activities. To address the physiological roles of Ser46 phosphorylation, we employed homologous recombination and LoxP/Cre-mediated deletion to introduce Ser46 to Ala missense mutation into the human p53 knock-in (HUPKI) allele in mice (p53hki^{S46A}). p53 stabilization in response to various types of DNA damage is modestly reduced in p53hki^{S46A} embryonic stem (ES) cells, mouse embryonic fibroblasts (MEFs) and thymocytes. In addition, p53-dependent apoptosis is partially impaired in p53hki^{S46A} thymocytes and E1A/Ras-expressing mouse embryonic fibroblasts (MEFs) after DNA damage. Consistent with this finding, transcription of p53 target apoptotic genes is preferentially affected by S46A mutation after DNA damage. p53hki^{S46A} MEFs proliferate and reach senescence normally but can be spontaneously immortalized more easily than wild type MEFs. In addition, p53hki^{S46A} MEFs more readily escapes from RAS-induced senescence. Therefore, Ser46 phosphorylation activates p53-dependent apoptosis and cellular senescence induced by oncogenic stress.

Introduction

p53 is one of the most frequently mutated tumor suppressor genes in human cancers (1). Structural and functional analyses show that full-length human *p53* gene encodes a 393-amino acid transcription factor composed of N-terminal transactivation domains that interact with coactivators or corepressors, a sequence-specific DNA-binding domain, a tetramerization domain and the extreme carboxy-terminal regulatory domain (2). In normal unstressed cells, p53 is inactive and maintained at low levels through rapid protein degradation. In this context, p53 transcriptional targets, including Mdm2, Pirh2 and COP1, can all function as ubiquitin ligases to facilitate p53 degradation via the ubiquitin-proteasome pathway (3-7). Upon the introduction of the cellular or genotoxic stresses, p53 protein levels are upregulated and its transcriptional activity induced, leading to cell cycle arrest, senescence, or apoptosis, partially depending on the cell types and the strength of the stress stimuli (8). In addition, activation of p53 can induce cellular differentiaton (9-11).

Accumulating evidence indicates that various types of posttranslational modifications play important roles in regulating p53 stability and activities by modulating the interactions between p53 and its positive and negative regulators (12). For example, studies of the N-terminal phosphorylation of p53 have indicated that phosphorylation of human p53 at Ser15 (Ser18 of mouse p53) is important to recruit co-activators to p53 target promoters and activate p53 responses to DNA

damage in a promoter- and cell type- specific manner (13-17). While Ser23 phosphorylation is important to stabilize p53 in a cell type-specific manner (18, 19), recent studies indicate that phosphorylation of mouse p53 at Ser18 and Ser23 are critical to activate p53-dependent apoptosis after DNA damage and suppress tumorigenesis in aging animals (20).

Human p53 can also be phosphorylated at Ser46 after DNA damage (21). Cell line transfection studies have suggested that Ser46 phosphorylation might be important for p53-dependent apoptosis after DNA damage by activating apoptotic target genes. For example, Oda *et al.* have identified a novel p53-dependent pro-apoptotic gene *p53AIP1* in humans, induction of which might be dependent on Ser46 phosphorylation (22). In addition, phosphorylation of p53 at Ser46 and Ser33 might be involved in UV-induced p53-dependent apoptosis (23). Phosphorylation at Ser46 is also shown to transcriptionally activate *PTEN* to promote p53-dependent apoptosis instead of cell cycle arrest (24, 25). A recent study also showed that codon 47 polymorphism interrupts Ser46 phosphorylation and decreases p53-dependent apoptotic activity (26), however, the codon 47 polymorphism could have many impacts on p53 stability and activity in addition to the loss of Ser46 phosphorylation.

To address the physiological role of Ser46 phosphorylation in regulating p53 stability and activity, we employed homologous recombination and LoxP/Cre-mediated deletion to introduce Ser46 to Ala mutation into the

endogenous human p53 knock-in (Hupki) allele in mice. Hupki (or p53hki as named in this paper) allele includes the core domain of human p53 gene spanning exons 4 through 9 and the intervening introns (codons 33-332 representing over 75% of human p53 sequence) and the highly conserved extreme N- and C-terminal domain of mouse p53 (27). p53hki allele was used because there is no apparent mouse counterpart of human Ser46 but the signaling pathways leading to Ser46 phosphorylation is conserved in mice (21). In addition, p53hki is functionally equivalent to the endogenous mouse p53 since the p53hki mice exhibit normal p53-dependent activities and tumor suppression in mice (27). Analysis of p53-dependent functions in p53hki^{S46A} mice indicates the roles of Ser46 phosphorylation in regulating p53 activities in apoptosis and cellular senescence.

Materials and Methods

Generation of p53hki^{S46A} mutant mice

Ser46 is encoded by exon 4 of the human p53 gene. A DNA fragment of human p53 genomic DNA covering exon 4 through exon 6 was cloned into pBS vector. Site-directed mutagenesis was performed to introduce Ser46 (TCC) to Ala46 (GCC) missense mutation. The targeting vector was made by replacing the corresponding germline genomic sequence of the Hupki construct with the genomic DNA fragment harboring S46A mutation (27). This targeting vector was linearized with *Not*I and electroporated into ES cells by electroporation. Homologous recombination between the targeting vector and the endogenous mouse p53 allele in ES cells was screened by Southern blotting with *Bgl*II/*Bam*HI digestion of genomic DNA and hybridization to the probe 1. To excise the PGK-neo^r gene from the targeted allele, 20μg of a circular plasmid expressing Cre enzyme was transiently transfected into the mutant ES cells; and the loxP/Cre-mediated deletion was screened by PCR using primers (Fig. 6C). The PGK-neo^r deleted ES cell clones were subcloned and the deletion event was further confirmed by Southern blotting. The heterozygous p53hki^{S46A}/+ mutant ES cells were used to generate chimeric mice that transmit the mutant allele into mouse germline. The confirmed germline-transmitted mice were p53hki^{S46A}/+. Heterozygous mutant mice were bred to obtain homozygous p53hki^{S46A} offsprings. To ensure that the control p53hki cells have the same genetic background as p53hki^{S46A} cells, p53hki$^{S46A/+}$ mice were

intercrossed to produce littermate embryos or offsprings of p53hki and p53hki^{S46A} genotypes.

Culture and treatment of ES cells and MEFs

ES cells were cultured on feeder layer in DMEM supplemented with 15% fetal bovine serum (ES cell qualified), glutamine, nonessential amino acids, sodium pyruvate, antibiotics, 100μM β-mecaptoethanol, and leukemia inhibitory factor. ES cells were cultured in the absence of feeder layer, exposed to 60J/m^2 UV-C and harvested at different time points after treatment.

MEFs derived from E13 embryos were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, glutamine, antibiotics and 50μM β-mecaptoethanol MEFs were treated with 60J/m^2 UV-C light, 200μM H_2O_2, 400μM paraquat (Sigma) or 0.25μM Doxorubicin (Sigma) and harvested at the indicated time points for the analysis of protein or mRNA levels.

To obtain retroviral E1A/Ras or Ras-expressing MEFs, early passage (P3 or P4) p53hki, p53hki^{S46A} and p53$^{-/-}$ MEFs were infected with retrovirus-containing supernatant in the presence of 4μg/ml polybrene (Orbigen Phoenix^{TM} Retroviral System) for 24hr. Media were changed on the following day and puromycin (2μg/ml, Sigma) was added to select for stably infected cells.

For MEF proliferation assay, we followed the standard 3T3 growth protocol (28). Briefly, passage 2 MEFs derived from littermate p53hki and p53hki^{S46A}

embryos were serially passaged on duplicate 60mm dishes once every three days at a density of 3×10^5 cells/plate. The total cell number was counted at each passage and averaged. The accumulative cell number at each passage = (the increase fold of the cells) × (the total cell number of the last passage).

For colony formation assay of Ras-expressing MEFs, Ras-overexpressed MEFs were plated on 10cm dish at a low density of 4×10^3 cells/plate after two days of puromycin selection post-infection. The colony was visualized by staining with Crystal Violet (Sigma) 14 days after plating.

Western blotting analysis

Protein extract from 2×10^4 MEFs or 2×10^5 thymocytes were separated on 10% (for p53) or 12% (for p21) SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in 5% dry milk in TBST and probed with a polyclonal antibody CM1 against p53 (Novo laboratories) or a polyclonal antibody against p21 (Santa Cruz Biotechnology). The membrane was subsequently incubated with horseradish peroxide-conjugated secondary antibody, developed with ECL Plus (Amersham biosciences) and exposed to X-ray film (Kodak). To determine whether the total amount of proteins loaded in each lane was comparable, the membranes were probed with a goat polyclonal antibody against β-actin (Santa Cruz Biotechnology, Inc.). Quantification of Western blots was performed using an AlphaImager 2200 (Alpha Innotech, San Leandro, CA).

Flow cytometry

For thymocytes apoptosis assay, thymocytes were recovered from p53hki^{S46A} and control mice (age 4-6 weeks), and plated at a density of 1×10^6 cells/ml in freshly made medium (DMEM supplemented with HEPES, 25mM, pH7.4, 5% FCS and glutamine). Cells were then treated with different dosages of IR and stained with FITC-conjugated AnnexinV (Pharmingen) 24 hour later for apoptotic cells by flow cytometry as previously described (29).

For E1A/Ras transformed MEFs apoptosis assay, E1A/Ras-MEFs were plated at a density of 2×10^5 cells/well and treated with 0.5 or 0.75μg/ml Doxorubicin (sigma) for 24 hours and analyzed for apoptotic percentage by flow cytometry following AnnexinV-FITC staining.

RNA preparation and Real-time PCR analysis

Total RNA was prepared from the frozen cell pellets by RNeasy RNA Cleanup (Qiagen). Up to 1μg RNA from each sample was reverse transcribed to cDNA using Superscript II First-strand Synthesis kit (Invitrogen). Real-time PCR was performed on ABI PRISM® 7000 sequence detection system with SYBR Green PCR MasterMix (ABI). The average threshold cycle (C_t) for each gene was determined from triplicate reactions, and the levels of gene expression were determined relatively to the average C_t value of *glyceraldehyde-3-*

phosphate-dehydrogenase (GAPDH) as previously described ((30)). The primer sequences used in real-time PCR are described previously (17).

Statistics

To determine statistical significance, quantitative RT-PCR data and apoptosis data were subjected to a two-tailed Student's *t*-test analysis. Significance was noted for *p* values less than 0.05.

Results

Generation of p53hki^{S46A} mutant mice.

Ser46 is located within the proline-rich domain of human p53 and is not conserved in mouse p53 gene. While certain phosphorylation site of mouse p53 such as Ser37 might be functionally equivalent to Ser46 of human p53, it is difficult to predict the counterpart of Ser46 in mouse p53. Since the pathways leading to Ser46 phosphorylation after DNA damage is conserved in mice (Fig. 5), we took advantage of the Hupki (human p53 knock-in) mouse model to analyze the physiological importance of Ser46 phosphorylation. The strategy to introduce the Ser46Ala mutation into the endogenous HUPKI allele is described in Figure 6. Homologous recombination event was screened by Southern blot analysis with *Bgl*II/*Bam*HI digestion of genomic DNA and hybridization to probe 1, giving rise to a 4.2kb germline band and a 6.2kb mutant band (Fig. 6A, C and E). The PGK-neo^r gene in the targeted allele was deleted through transient expression of the Cre enzyme as previously described (29). The LoxP/Cre-mediated deletion was screened by PCR using primers shown as arrows (Fig. 6D and E). Both the genomic DNA and cDNA from p53hki^{S46A}/+ ES cells were sequenced to confirm that only Ser46Ala mutation was present in the p53 gene expressed from the p53hki^{S46A} allele. In addition, Ser46 phosphorylation could be easily detected in p53hki/+ ES cells after UV radiation but was undetectable in p53hki^{S46A}/+ ES cells, further supporting the success of the knock-in mutation (Fig. 6F). When analyzed with antibody

specific for human p53, the protein levels of p53hki in p53hki/+ ES cells after UV radiation were higher than those in p53hki^{S46A}/+ ES cells, indicating that Ser46 phosphorylation may be important for p53 stabilization in ES cells after DNA damage.

The offspring from heterozygous p53$^{hkiS46A/+}$ mice intercross yielded 29 p53$^{+/+}$, 41 p53$^{hkiS46A/+}$, 22 p53$^{hkiS46A/S46A}$ mice, close to the expected 1:2:1 Mendelian ratio, indicating that p53hki^{S46A} embryos develop normally.

p53 responses to DNA damage in p53hki^{S46A} MEFs.

To determine the roles of Ser46 phosphorylation in regulating the p53 responses to DNA damage, we examined p53 stabilization in p53hki^{S46A} and control p53hki MEFs after various types of DNA damage. The protein levels of p53 in p53hki^{S46A} MEFs at some time points after UV radiation were less than those in p53hki MEFs (Fig. 7A, B). Since oxidative stress can induce p53 stability and activity in MEFs, leading to p53-dependent apoptosis or senescence (31, 32), p53 protein levels in p53hki and p53hki^{S46A} MEFs treated with H_2O_2 or paraquat were also examined. The protein levels of p53 in p53hki^{S46A} MEFs were consistently lower than those in p53hki MEFs at various time points after oxidative stress (Fig. 7C, D). Therefore, Ser46 phosphorylation contributes to p53 stabilization after DNA damage.

Previous studies suggest that phosphorylation of human p53 at Ser46 may

be important for activating apoptosis by upregulating p53 target apoptotic genes (22, 23, 26, 33). To examine the impact of Ser46Ala mutation on p53-dependent transcription activities, the expression levels of a number of p53 targets, including *p21*, *Mdm2*, and pro-apoptotic genes *Noxa*, *Killer/DR5*, *Perp*, *Pidd*, and *Puma* in p53hki and p53hki^{S46A} MEFs after UV treatment were analyzed by quantitative real-time PCR. While the expression of several p53 target genes was not affected in response to UV, p53-dependent transcription of the pro-apoptotic gene *Noxa* and *Perp* was significantly reduced in p53hki^{S46A} MEFs (Fig. 7E). Therefore, Ser46 phosphorylation was required for the expression of p53 target genes in a promoter-dependent manner.

To further examine the DNA-damage induced apoptosis in MEFs, p53hki as well as p53hki^{S46A} MEFs were infected with retrovirus expressing E1A/Ras, which sensitized MEFs to p53-dependent apoptosis after DNA damage (34-36). While E1A-p53$^{-/-}$ MEFs were largely resistant to Dox-induced apoptosis as previously reported, E1A-p53hki^{S46A} MEFs exhibited significant less apoptotic levels than E1A-p53hki MEFs after different dosages of Doxorubicin treatment (Fig. 8B). To determine whether the reduced p53-dependent apoptosis in E1A-p53hki^{S46A} MEFs is caused by the defective p53 transcriptional activity, both the p53 protein stabilization and the p53-dependent gene expression level were examined after Dox treatment. The p53 protein level was only slightly reduced at some time points in E1A-p53hki^{S46A} MEFs when compared with that in E1A-p53hki MEFs after Dox

treatment (Fig. 8A). The mRNA levels of two p53-dependent pro-apoptotic genes, *Noxa* and *Puma*, were significantly impaired in E1A-p53hki^{S46A} MEFs after DNA damage (Fig. 8C). Therefore, phosphorylation of Ser46 is important for p53-dependent transcription in a promoter-specific manner.

p53 responses to DNA damage in p53hki^{S46A} thymocytes.

To further test the importance of Ser46 phosphorylation in activating p53 apoptotic activities, we determine the effects of S46A mutation on p53-dependent apoptotic activities in thymocytes after IR. p53 protein levels in p53hki^{S46A} thymocytes at various time points after IR were consistently reduced compared to those in p53hki thymocytes (Fig. 9A). In addition, p53-dependent apoptosis was modestly but statistically significantly lower in p53hki^{S46A} thymocytes after IR than that in p53hki thymocytes, indicating that Ser46 phosphorylation is important for optimally activating p53 apoptotic activity (Fig. 9B). Consistent with this notion, p53-dependent transcription of target genes, including *Noxa*, *Bax* and *Puma*, was all modestly reduced in p53hki^{S46A} thymocytes after IR (Fig. 9C).

S46A mutation cannot rescue the embryonic lethality of XRCC4$^{-/-}$ mice.

XRCC4 is required for non-homologous end joining and XRCC4-deficient mice die during embryonic development due to massive p53-dependent apoptosis in the nervous system (37). Significant loss of p53-dependent apoptosis, such as

p53-deficiency, can rescue the neuronal apoptosis and embryonic lethality of XRCC4$^{-/-}$ mice (37). In addition, recent studies show that p53$^{S21/23A}$ mutation greatly impairs p53 apoptotic activities and completely rescues the embryonic lethality of XRCC4$^{-/-}$ mice (20). To test whether S46A mutation can rescue the embryonic lethality of XRCC4$^{-/-}$ mice, the p53hki^{S46A} mice were bred with XRCC4$^{+/-}$ mice. So far, no p53hki^{S46A}XRCC4$^{-/-}$ double mutant mice were observed in 114 offsprings from p53hki$^{S46A/+}$XRCC4$^{+/-}$ intercross (7 expected based on Mandelian ratio) nor 27 offspring from p53hki^{S46A}XRCC4$^{+/-}$ intercross (6 expected). Therefore, the absence of Ser46 phosphorylation did not abrogate p53-dependent apoptosis enough to rescue the embryonic lethality of XRCC4$^{-/-}$ mice.

Cellular senescence in p53hki and p53hki^{S46A} MEFs.

MEFs undergo cellular senescence after a few number of doublings in culture possibly due to culture stress such as oxidative stress (38). p53 is required for senescence in MEFs induced by diverse stimuli, including DNA damage, intense mitogenic signals and oxidative stress (32, 39). To test the potential role of Ser46 phosphorylation in cellular senescence, we employed the standard 3T3 assay to compare the proliferation of p53hki and p53hki^{S46A} MEFs over the course of 20 passages. At early passages, both p53hki and p53hki^{S46A} MEFs proliferated at similar rate and reached senescence around passage 8 (Fig. 10A). However, p53hki^{S46A} MEFs was more readily immortalized than p53hki MEFs (Fig. 10A).

Therefore, phosphorylation of p53 at Ser46 might play a role in activating p53 responses to suppress spontaneous immortalization.

Oncoprotein Ras induces p53-dependent cellular senescence and the phosphorylation of p53 at Ser46 (40-45). To examine the roles of Ser46 phosphorylation in p53 responses to oncogenic stresses, Ras was overexpressed in p53hki and p53hki^{S46A} MEFs via retroviral infection and its impact on senescence also analyzed by the 3T3-type proliferation assay. As expected, Ras-expressing p53$^{-/-}$ MEFs did not undergo senescence and proliferated robustly (Fig. 10B). While Ras-expressing p53hki MEFs underwent cellular senescence after only a few doublings, Ras-expressing p53hki^{S46A} MEFs escaped from cellular senescence but proliferated with a much slower rate than Ras-expressing p53$^{-/-}$ MEFs (Fig. 10B). Consistent with this notion, Ras-expressing p53hki^{S46A} MEFs formed three times as many colonies as Ras-expressing p53hki MEFs by the quantitative colony formation assay (10C, and D). Therefore, phosphorylation at Ser46 contributed to activation of p53 responses to oncogenic stresses induced by Ras.

Discussion

Various stress stimuli activate distinct as well as overlapping signaling pathways, leading to distinct phosphorylation patterns of p53 that eventually activate p53 responses (12). These distinct combinations of phosphorylation events after various stresses could modulate the p53 responses in a manner that dictate the cellular outcome. Therefore, it is important to understand the roles of individual as well as combinatorial phosphorylation events in regulating p53 responses to stresses. The phosphorylation event of human p53 at Ser46 appears to be a common one after many types of genotoxic and cellular stresses (21). While Ser46 is not completely conserved in p53 of other species, the stress-induced signaling pathways leading to the phosphorylation of human p53 at Ser46 are conserved between human and mice, suggesting the importance of these pathways in regulating p53 responses after stresses (Fig. 5). Previous cell line studies have shown that phosphorylation of human p53 at Ser46 is correlated with the p53-dependent apoptosis (22-24, 26).

To address the physiological roles of Ser46 phosphorylation, we employed the human p53 knock-in allele to construct the Ser46Ala knock-in mutant mice. Our results conclusively demonstrate that Ser46 phosphorylation is important for the optimal p53-dependent apoptosis and efficient induction of some p53-dependent pro-apoptotic genes, such as *Noxa* and *perp*, after DNA damage. However, the defects in p53-dependent transcription are different in MEFs and thymocytes. Of

the seven p53 target genes analyzed in p53hki^{S46A} MEFs after UV radiation, only the induction levels of *Noxa* and *Perp* were significantly reduced. However, the reduced apoptosis in p53hki^{S46A} thymocytes is correlated with the reduced induction of all examined p53 targets, possibly as a result of the impaired p53 stabilization. Therefore, Ser46 appears to play overlapping as well as distinct roles in modulating p53 activities in different cell types. Similar scenario has been seen in the studies of Ser18 phosphorylation that plays cell type-specific roles in activating p53 responses to DNA damage (17).

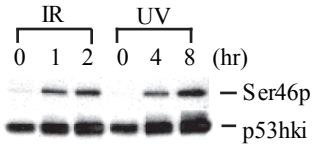
Ser46 phosphorylation appears to be important for p53 stabilization after some types of DNA damage. The reduced p53 protein levels appear to be the result of the decreased p53 stability. p53 stability is negatively regulated by several E3 ligases, including Mdm2, Pirh2 and COP1, which are p53 transcriptional targets and can ubiquitinate p53 (6, 7). We did not observe any difference in the interaction between the endogenous p53 and Mdm2 in p53hki^{S46A} MEFs before and after DNA damage, indicating that Ser46 phosphorylation is not required to disrupt the interaction between Mdm2 and p53 after DNA damage (data not shown). Since Pirh2 might interact with the proline-rich domain of p53 (7), it is possible that Ser46 phosphorylation disrupt the interaction between p53 and Pirh2. However, this hypothesis remains to be tested when better Pirh2 antibodies that can detect endogenous Pirh2 become available. In contrast to the p53 response to stresses induced by UV radiation and oxidative stresses, a full p53 response to DNA damage

induced by Doxorubicin does not require Ser46 phosphorylation (data not shown). Therefore, Doxorubicin treatment might induce other phosphorylation events of p53 that play redundant roles of Ser46 phosphorylation in stabilizing p53 in MEFs.

Our study has also identified another important role of Ser46 phosphorylation in suppressing spontaneous transformation and inducing p53-dependent cellular senescence in response to oncogenic stresses. In this context, the absence of Ser46 phosphorylation confers the MEFs the ability to be immortalized more easily. More importantly, Ser46 phosphorylation is crucial for Ras-induced p53-dependent senescence in MEFs. Previous studies indicated that oncogenic Ras-induced cellular senescence is associated with p53 acetylation at Lysine 382 (43, 44). In addition, another study indicated that overexpression of PML isoform IV can also cause premature senescence by activation of p53 through posttranslational modifications such as acetylation at Lysine 382 and phophorylation at Ser46 (45). Since acetylation of lysine 382 is not important in activating p53 responses during cellular senescence (29, 46), our findings indicate important roles of Ser46 phosphorylation in cellular senescence induced by in vitro culture stress as well as oncogene activation. Therefore, p53hki^{S46A} mice will be employed to determine the roles of Ser46 phosphorylation in suppressing oncogene-induced tumorigenesis *in vivo*.

Figure 5. DNA damage induced phosphorylation of p53hki at Ser46 in mouse cells. (A) Ser46 phosphorylation in p53hki/+ ES cells. ES cells were irradiated with UV-C ($60J/m^2$) or IR (5Gy) and harvested at the indicated time points. p53hki protein was immunoprecipitated with pAb1801 from the harvested cell lysates and Ser46 phosphorylation was detected by Western Blot using the antibody specifically recognizing human p53 phosphorylated at Ser46. The primary antibodies pAb1801 (anti-p53) (Santa Cruz Biotechnology) and pAbSer(P)46 (Cell Signaling) are indicated on the right. Both irradiated and unstressed cells were treated with proteasome inhibitor ALLN (20μM) for 4hr before harvest so that comparable amount of p53hki was present in cells harvested at different time points. (B) Ser46 phosphorylation in p53hki MEFs in response to Doxorubicin treatment. MEFs were harvested from E12.5 p53hki embryos, treated with 0.25μM Doxorubicin, and cell lysates were directly analyzed for p53 phosphorylation and p53 protein levels by Western Blotting at different time points as shown. Ser46 phosphorylation was also detected in p53hki MEFs upon UV-C radiation and in thymocytes upon IR treatment (data not shown). (C) Ser46 phosphorylation in p53hki MEFs after paraquat treatment. p53hki MEFs were exposed to 200μM paraquat (Sigma) and analyzed for Ser46 phosphorylated and p53 total protein levels at different time points.

A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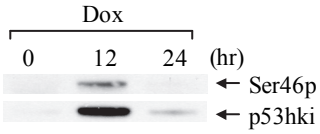
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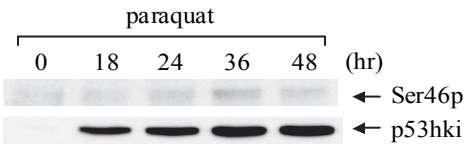
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Ser46p
p53hki

Figure 6. Generation of p53hki^{S46A} mice. (A) Schematic diagram showing the structure of endogenous mouse p53 gene in ES cells. The filled boxes represent mouse p53 exons, and the filled bar within exon 11 represents the probe for Southern blot analysis. The 4.2-kb *Bgl*II/*Bam*HI digestion fragment is indicated. (B) The targeting construct. The sequence indicated within arrow A range indicates the human p53 gene fragment encompassing exons4 to 9 (aa 33-332). The filled boxes represent mouse p53 exons, and the open boxes represent human p53 exons. The PGK-neo^r gene flanked by LoxP sites was inserted within intron 3. *, the S46A mutation in exon 4. (C) Targeted p53 locus after homologous recombination between the wild-type p53 allele and the targeting vector. The knock-in allele 6.2-kb *Bgl*II/*Bam*HI fragment is shown. The position of the primer sets that are used to screen for LoxP/Cre-mediated deletion is shown in arrowheads. (D) Knock-in allele after PGK-neo^r deletion. The size of the *Bgl*II/*Bam*HI fragment is indicated. (E) Southern blot analysis of genomic DNA from targeted ES cells after PGK-neo^r deletion. Genomic DNA was digested with *Bgl*II/*Bam*HI and hybridized to the probe 1. Homologous recombination occurred between the germline mouse p53 allele and the targeting vector, which is indicated as a 6.2-kb and a 4.2-kb *Bgl*II/*Bam*HI fragment (lane2) compared to one 4.2-kb fragment in untargeted ES cells (lane 1). Lane3, *Bgl*II/*Bam*HI fragments from homozygous p53hki^{S46A} genomic DNA. The positions of germline allele and targeted allele *Bgl*II/*Bam*HI fragments are indicated by arrows. (F) Induction of p53 protein level in p53hki/+ and p53hki^{S46A}/+ ES cells. Cell extracts were prepared at the indicated time points after 60J/m^2 of UV-C irradiation and analyzed for p53 protein level by Western Blot. Quantification of p53 protein level from Western blots was performed using AlphaImager 2200. The signal intensity of protein p53hki^{S46A} was normalized to that of p53hki at each corresponding time points, and the ratios were shown under the blot as 1.4 at 0hr, 0.55 at 4hr and 0.53 at 8hr after treatment. The absence of induced Ser46 phosphorylation in p53hki^{S46A}/+ ES cells further confirmed the success of the knock-in strategy. Genotypes of ES cells are shown on top of the panel and the primary antibodies used are indicated on the right.

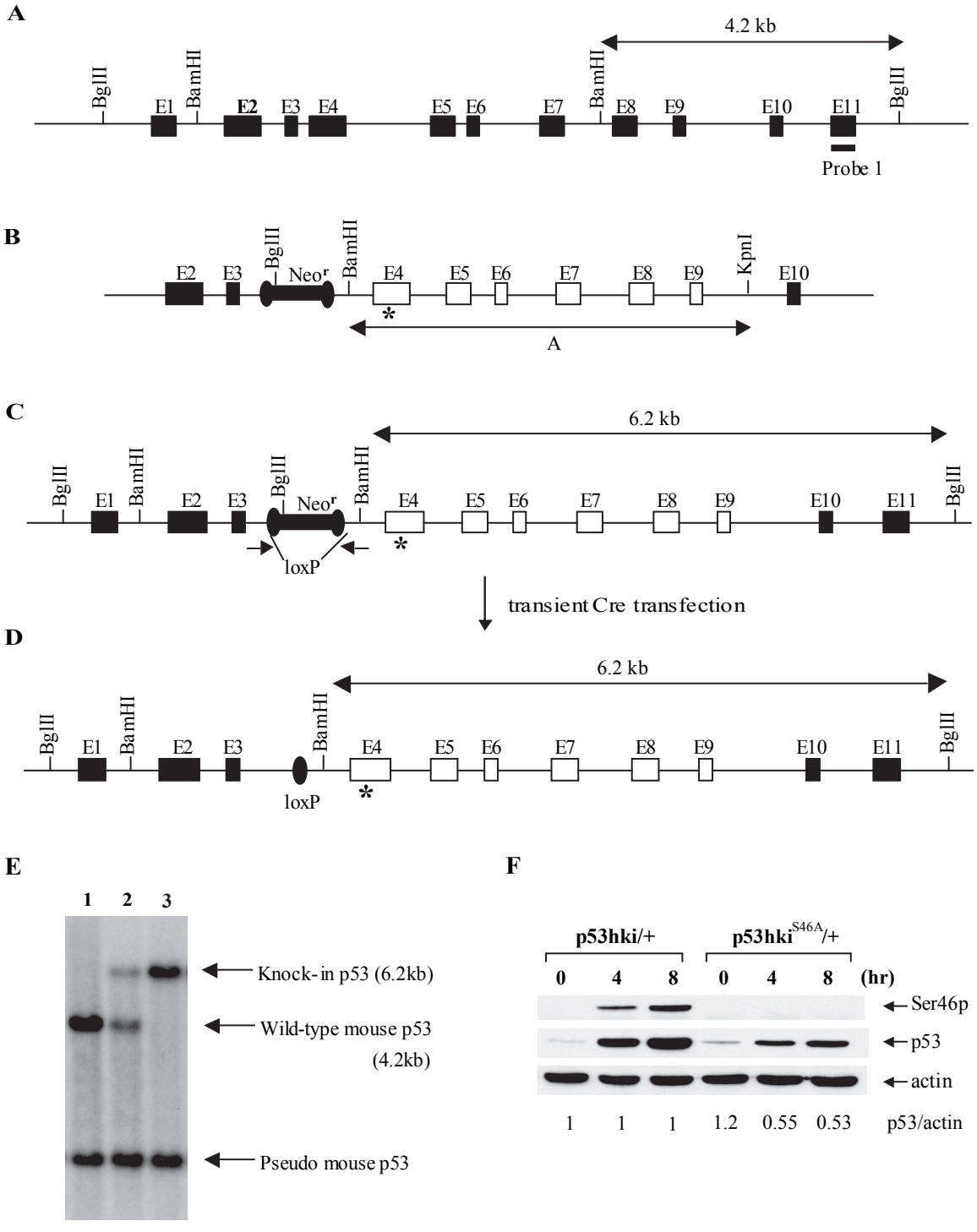
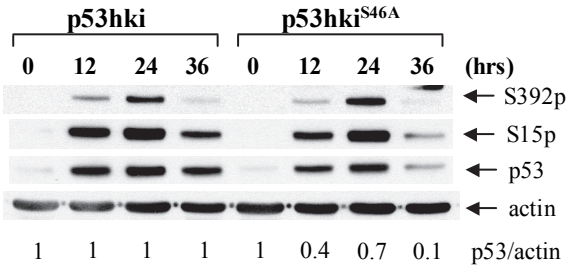
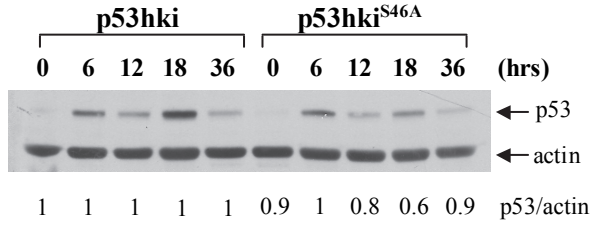

A
4.2 kb
BglII
BamHI
E1
E2
E3
E4
E5
E6
E7
BamHI
E8
E9
E10
E11
BglII
Probe 1
B
E2
E3
BglII
Neor
BamHI
E4
E5
E6
E7
E8
E9
KpnI
E10
*
A
C
6.2 kb
BglII
E1
BamHI
E2
E3
BglII
Neor
BamHI
E4
E5
E6
E7
E8
E9
E10
E11
BglII
*
loxP
transient Cre transfection
D
6.2 kb
BglII
E1
BamHI
E2
E3
BamHI
E4
E5
E6
E7
E8
E9
E10
E11
BglII
loxP
*
E
1
2
3
Knock-in p53 (6.2kb)
Wild-type mouse p53
(4.2kb)
Pseudo mouse p53
F
p53hki/+
p53hkiS46A/+
0
4
8
0
4
8
(hr)
Ser46p
p53
actin
1
1
1
1.2
0.55
0.53
p53/actin

Figure 7. p53 responses to DNA damage in p53hki^{S46A} MEFs. p53 protein level in p53hki and p53hki^{S46A} MEFs before and after UV treatment. MEFs are irradiated with UV-C light at the dosage of 60J/m2 (A) or 30J/m2 (B), harvested at the indicated time points and analyzed for protein levels by Western blot. Genotypes are shown on top of the panels, and the primary antibodies used CM1 (anti-p53), pAbSer(P)18, pAbSer(P)389 and actin as indicated on the right. p53hki^{S46A} signals were normalized to the intensity of p53hki protein bands at each time point using AlphaImager 2200, and the calculated numbers were shown under the blot as 1 at 0hr, 0.4 at 12hr, 0.7 at 24hr and 0.1 at 36hr after 60J/m2 of UV-C, and 0.9 at 0hr, 1 at 6hr, 0.8 at 12hr, 0.6 at 18hr and 0.9 at 36hr after 30J/m2 of UV-C respectively. (C) p53 protein level in p53hki and p53hki^{S46A} MEFs after H_2O_2 treatment. MEFs are treated with 400μM H_2O_2 and analyzed for protein levels at different time points as shown. The p53hki^{S46A} signal volume ratios are 0.9 at 0h, 0.4 at 1hr, 0.8 at 2hr, and 0.7 at 4hr after H_2O_2 treatment. (D) p53 induction level in p53hki and p53hki^{S46A} MEFs after 200μM Paraquat treatment. The p53hki^{S46A} verses p53hki signal ratios are 1, 0.6, 0.7 and 0.9 at each time point following treatment. (E) p53-dependent transcriptional activity in p53hki^{S46A} MEFs. Total RNA from p53hki and p53hki^{S46A} MEFs was harvested at 18hr after treatment with 30J/m2 of UV. The mRNA levels of several p53 targets were analyzed by quantitative real-time PCR. The values are represented as the ratios of mRNA level in UV treated p53hki^{S46A} MEFs *versus* that in treated p53hki MEFs. Each value is the average from at least three independent experiments, with error bars indicating the standard deviation. *p* values resulted from Student's *t*-test are shown on top of each bar, and statistical significance is noted for *p* values less than 0.05. The approximate folds of induction of each p53 target gene in p53hki cells after UV treatment are: *p21*, 6 folds; *Mdm2*, 7 folds; *Noxa*, 12 folds; *Killer/DR5*, 2 folds; *Perp*, 33 folds; *Pidd*, 8 folds; *PUMA*, 2.5 folds.

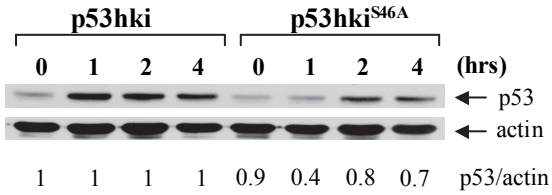
A


p53hki
p53hki^S46A
0 12 24 36 0 12 24 36 (hrs)
S392p
S15p
p53
actin
1 1 1 1 1 0.4 0.7 0.1 p53/actin

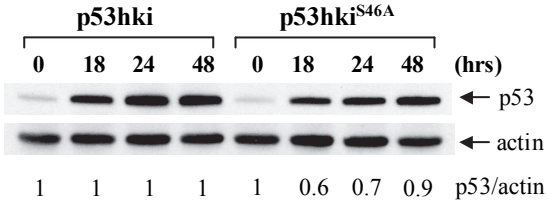
B


p53hki
p53hki^S46A
0 6 12 18 36 0 6 12 18 36 (hrs)
p53
actin
1 1 1 1 1 0.9 1 0.8 0.6 0.9 p53/actin

C


p53hki
p53hki^S46A
0 1 2 4 0 1 2 4 (hrs)
p53
actin
1 1 1 1 0.9 0.4 0.8 0.7 p53/actin

D


p53hki
p53hki^S46A
0 18 24 48 0 18 24 48 (hrs)
p53
actin
1 1 1 1 1 0.6 0.7 0.9 p53/actin

E

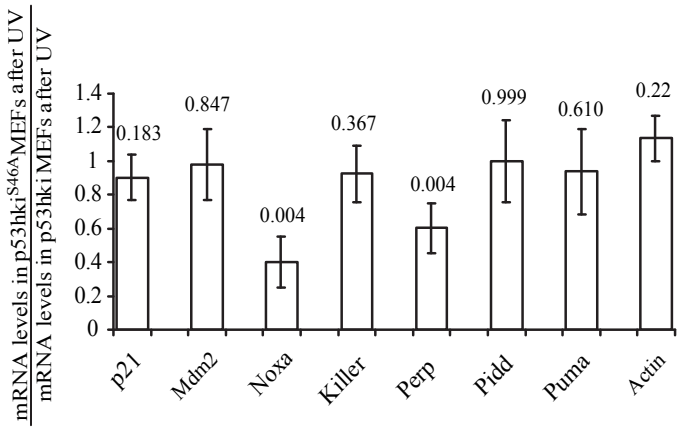
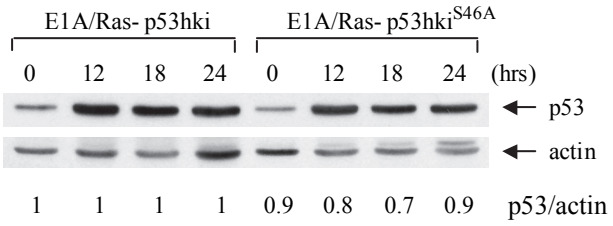
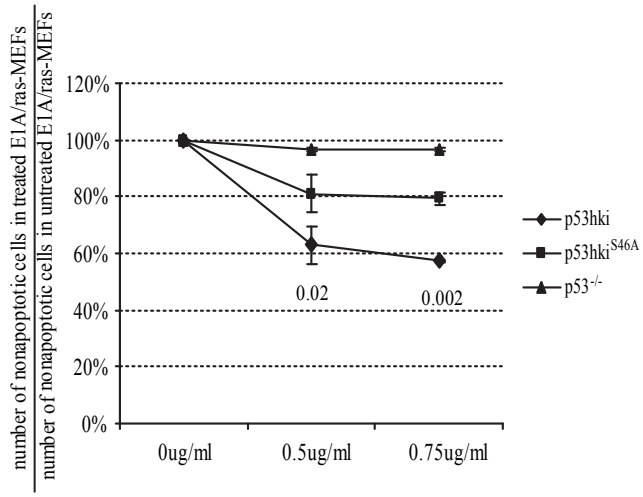

mRNA levels in p53hki^S46A MEFs after UV
mRNA levels in p53hki MEFs after UV
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.183
0.847
0.004
0.367
0.004
0.999
0.610
0.22
p21
Mdm2
Noxa
Killer
Perp
Pidd
Puma
Actin

Figure 8. p53-dependent apoptosis in E1A/Ras-MEFs. (A) p53 protein levels in E1A/Ras transfected p53hki and p53hki^{S46A} MEFs before and after Doxorubicin treatment. Cell extracts were harvested at the indicated time points after Dox treatment and analyzed by Western blotting. The quantification of p53 signals indicates 0.9, 0.8, 0.7 and 0.9 at each time point in E1A/Ras-p53hki^{S46A} MEFs. (B) p53-dependent apoptosis in E1A/Ras-p53hki^{S46A} MEFs after Dox treatment. E1A/Ras-expressing p53hki and p53hki^{S46A} MEFs were treated with 0.5μg/ml or 0.75μg/ml Dox for 24hr and followed by Annexin-FITC staining and flow cytometry. The mean percentage of viable cells and the SD from at least three independent experiments for each genotype is shown. *p* values indicates the significance of the apoptosis difference between p53hki and p53hki^{S46A} samples. (C) p53-dependent expression of target genes in E1A/Ras-expressing MEFs. The p53hki and p53hki^{S46A} E1A/Ras-MEFs were treated with 0.75μg/ml Dox, and total RNA harvested 12hrs after treatment. The mRNA levels of several p53 targets were analyzed by quantitative real-time PCR. Values are presented as the ratio of mRNA level in Dox treated E1A/Ras-expressing p53hki^{S46A} cells *versus* that in Dox treated E1A/Ras-p53hki MEFs. Each value is the average from three independent experiments, with error bars indicating the standard deviation.

A


E1A/Ras- p53hki
E1A/Ras- p53hki^{S46A}
0 12 18 24 0 12 18 24 (hrs)
p53
actin
1 1 1 1 0.9 0.8 0.7 0.9 p53/actin

B


number of nonapoptotic cells in treated E1A/ras-MEFs / number of nonapoptotic cells in untreated E1A/ras-MEFs
120%
100%
80%
60%
40%
20%
0%
0.02
0.002
0ug/ml
0.5ug/ml
0.75ug/ml
p53hki
p53hki^{S46A}
p53$^{-/-}$

C

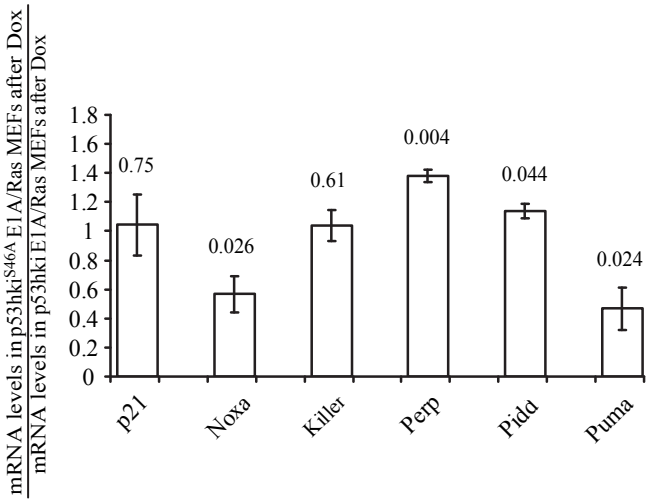
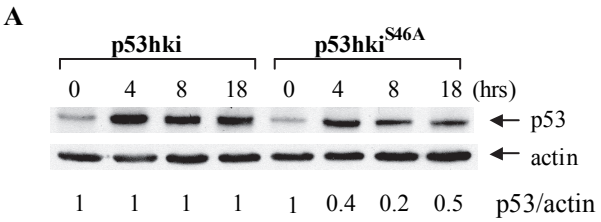
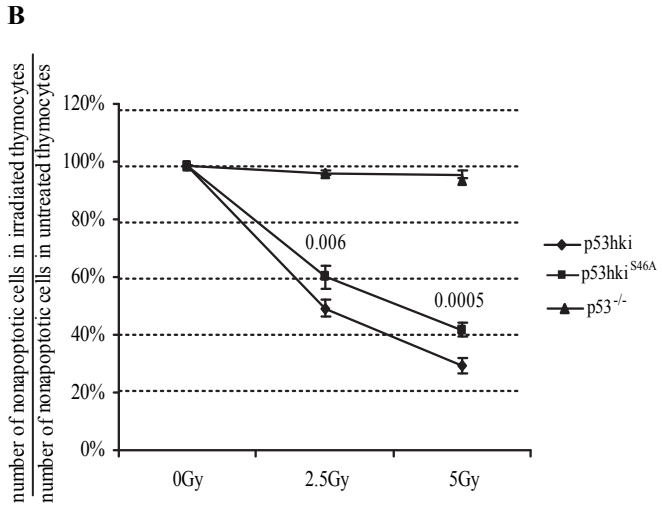

mRNA levels in p53hki^{S46A} E1A/Ras MEFs after Dox / mRNA levels in p53hki E1A/Ras MEFs after Dox
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.75
0.026
0.61
0.004
0.044
0.024
p21
Noxa
Killer
Perp
Pidd
Puma

Figure 9. p53 responses to DNA damage in p53hki^{S46A} thymocytes. (A) p53 protein levels in p53hki and p53hki^{S46A} thymocytes before and after IR treatment. Thymocytes from one-month old p53hki or p53hki^{S46A} littermate mice were isolated and exposed to 5Gy of ionizing radiation (IR). Cell extracts were harvested at the indicated time points after IR treatment and analyzed by Western blotting. Quantitative p53hki^{S46A} protein level was 1, 0.4, 0.2 and 0.5 compared to p53hki respectively. (B) p53-dependent apoptosis in thymocytes after IR. Apoptosis in p53hki and p53hki^{S46A} thymocytes irradiated with different dosages of IR (2.5 or 5Gy) as shown was analyzed 24hr after IR by annexin V-FITC staining. The values represented are the average from four independent experiments, with error bars indicating the standard deviation and *p* values indicating the significance of the apoptosis difference between p53hki and p53hki^{S46A} samples. (C) p53-dependent gene expression in thymocytes. The p53hki and p53hki^{S46A} littermate thymocytes were isolated and irradiated with 5Gy of IR, and total RNA harvested 18hrs after treatment. The gene expression levels of several p53 targets were analyzed by quantitative real-time PCR. Values are represented as the ratio of mRNA level in IR treated p53hki^{S46A} cells *versus* that in IR treated p53hki thymocytes. Each value is the average from at least three independent experiments, with error bars indicating the standard deviation. The approximate folds of induction of each p53 target gene in p53hki thymocytes after IR are: *p21*, 15 folds; *Mdm2*, 3.8 folds; *Noxa*, 5 folds; *Killer/DR5*, 2.8 folds; *Bax*, 10 folds; *Pidd*, 9 folds; *PUMA*, 14 folds.


A
p53hki
p53hki S46A
0 4 8 18 0 4 8 18 (hrs)
p53
actin
1 1 1 1 1 0.4 0.2 0.5 p53/actin


B
number of nonapoptotic cells in irradiated thymocytes / number of nonapoptotic cells in untreated thymocytes
120%
100%
80%
60%
40%
20%
0%
0Gy
2.5Gy
5Gy
0.006
0.0005
p53hki
p53hki S46A
p53 -/-

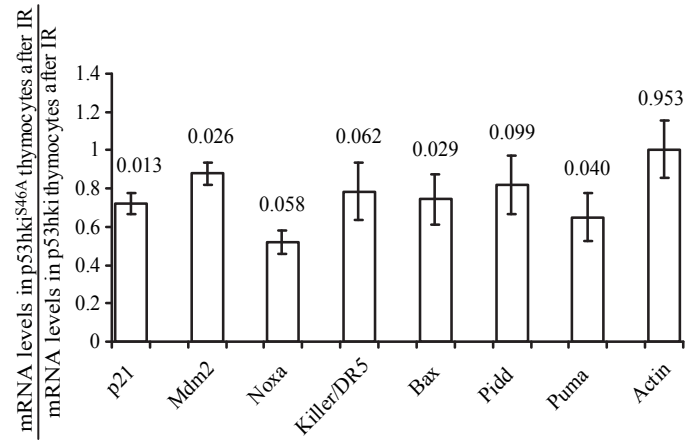
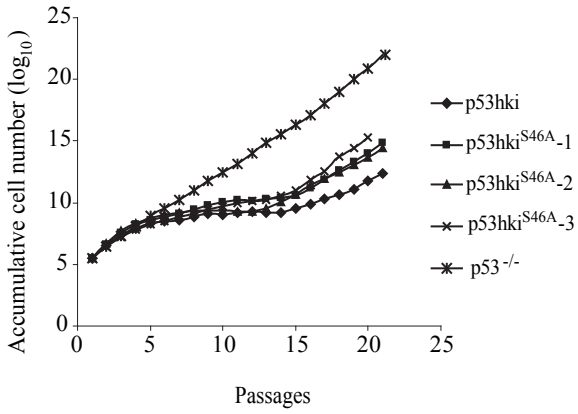
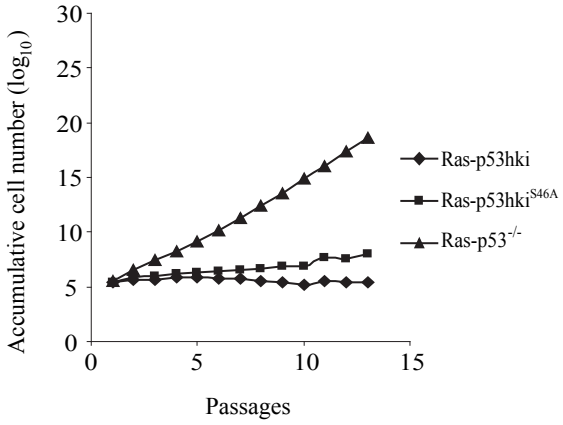

C
mRNA levels in p53hki S46A thymocytes after IR / mRNA levels in p53hki thymocytes after IR
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.013
0.026
0.058
0.062
0.029
0.099
0.040
0.953
p21
Mdm2
Noxa
Killer/DR5
Bax
Pidd
Puma
Actin

Figure 10. Cellular proliferation of p53hki and p53hki^{S46A} MEFs. (A) Proliferation of MEFs analyzed by standard 3T3 assay. The accumulative cell number at each passage (log_{10}) is plotted against passage numbers. Data of p53hki^{S46A} MEFs from 3 different embryos are shown. (B) Proliferation of Ras-transfected MEFs analyzed by 3T3-type assay. (C) Colony formation assay. Picture of colonies in Ras-expressing p53hki (left), p53hki^{S46A} (right) and p53$^{-/-}$ (top) MEFs that were plated at a low density of 4×10^3 cells per 10 cm plate. (D) Quantitative summary of colony formation. Each bar represents the average colony number of four samples. The average colony number is 10 (p53hki), 28 (p53hki^{S46A}) and 146 (p53$^{-/-}$), respectively, and is presented with standard derivation. The *p* value for the colony numbers of Ras-p53hki vs. p53hki^{S46A} MEFs is 0.001.

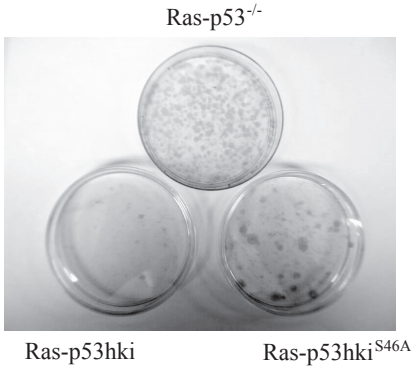
A


Accumulative cell number (log_{10})
25
20
15
10
5
0
0
5
10
15
20
25
Passages
p53hki
p53hki^{S46A}-1
p53hki^{S46A}-2
p53hki^{S46A}-3
p53$^{-/-}$

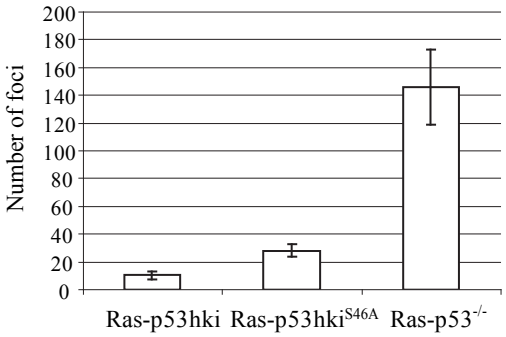
B


Accumulative cell number (log_{10})
30
25
20
15
10
5
0
0
5
10
15
Passages
Ras-p53hki
Ras-p53hki^{S46A}
Ras-p53$^{-/-}$

C


Ras-p53$^{-/-}$
Ras-p53hki
Ras-p53hki^{S46A}

D


Number of foci
200
180
160
140
120
100
80
60
40
20
0
Ras-p53hki
Ras-p53hki^{S46A}
Ras-p53$^{-/-}$

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References

1. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. p53 mutations in human cancers. Science 1991;253:49-53.

2. Courtois, S., de Fromentel, C. C., and Hainaut, P. p53 protein variants: structural and functional similarities with p63 and p73 isoforms. Oncogene 2004;23:631-638.

3. Haupt, Y., Maya, R., Kazaz, A., and Oren, M. Mdm2 promotes the rapid degradation of p53. Nature 1997;387:296-299.

4. Honda, R., Tanaka, H., and Yasuda, H. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS Lett 1997;420:25-27.

5. Kubbutat, M. H., Jones, S. N., and Vousden, K. H. Regulation of p53 stability by Mdm2. Nature 1997;387:299-303.

6. Dornan, D., Wertz, I., Shimizu, H., Arnott, D., Frantz, G. D., Dowd, P., K, O. R., Koeppen, H., and Dixit, V. M. The ubiquitin ligase COP1 is a critical negative regulator of p53. Nature 2004;429:86-92.

7. Leng, R. P., Lin, Y., Ma, W., Wu, H., Lemmers, B., Chung, S., Parant, J. M., Lozano, G., Hakem, R., and Benchimol, S. Pirh2, a p53-induced ubiquitin-protein ligase, promotes p53 degradation. Cell 2003;112:779-791.

8. Ko, L. J. and Prives, C. p53: puzzle and paradigm. Genes Dev 1996;10:1054-1072.

9. Lin, T., Chao, C., Saito, S., Mazur, S. J., Murphy, M. E., Appella, E., and Xu, Y. p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. Nat Cell Biol 2004;26:26.

10. Xu, Y. A new role for p53 in maintaining genetic stability in embryonic stem cells. Cell Cycle. 2005;4:363-364.

11. Meletis, K., Wirta, V., Hede, S. M., Nister, M., Lundeberg, J., and Frisen, J. p53 suppresses the self-renewal of adult neural stem cells. Development. 2006;133:363-369.

12. Xu, Y. Regulation of p53 responses by post-translational modifications. Cell

Death Differ 2003;10:400-403.

13. Lambert, P. F., Kashanchi, F., Radonovich, M. F., Shiekhattar, R., and Brady, J. N. Phosphorylation of p53 Serine 15 increases Interaction with CBP. J. Biol. Chem. 1998;273:33048-33053.

14. Dumaz, N. and Meek, D. W. Serine15 phosphorylation stimulates p53 transactivation but does not directly influence interaction with HDM2. Embo J 1999;18:7002-7010.

15. Borges, H. L., Chao, C., Xu, Y., Linden, R., and Wang, J. Y. Radiation-induced apoptosis in developing mouse retina exhibits dose-dependent requirement for ATM phosphorylation of p53. Cell Death Differ 2004;11:494-502.

16. Chao, C., Saito, S., Anderson, C. W., Appella, E., and Xu, Y. Phosphorylation of murine p53 at ser-18 regulates the p53 responses to DNA damage. Proc Natl Acad Sci U S A 2000;97:11936-11941.

17. Chao, C., Hergenhahn, M., Kaeser, M. D., Wu, Z., Saito, S., Iggo, R., Hollstein, M., Appella, E., and Xu, Y. Cell type- and promoter-specific roles of Ser18 phosphorylation in regulating p53 responses. J Biol Chem 2003;8:8.

18. Wu, Z., Earle, J., Saito, S. i., Anderson, C. W., Appella, E., and Xu, Y. Mutation of Mouse p53 Ser23 and the Response to DNA Damage. Mol. Cell. Biol. 2002;22:2441-2449.

19. MacPherson, D., Kim, J., Kim, T., Rhee, B. K., Van Oostrom, C. T., DiTullio, R. A., Venere, M., Halazonetis, T. D., Bronson, R., De Vries, A., Fleming, M., and Jacks, T. Defective apoptosis and B-cell lymphomas in mice with p53 point mutation at Ser 23. Embo J. 2004;23:3689-3699.

20. Chao, C., Herr, D., Chun, J., and Xu, Y. Ser18 and 23 phosphorylation is required for p53-dependent apoptosis and tumor suppression. Embo J. 2006;25:2615-2622.

21. Saito, S., Yamaguchi, H., Higashimoto, Y., Chao, C., Xu, Y., Fornace, A. J., Jr., Appella, E., and Anderson, C. W. Phosphorylation site interdependence of human p53 posttranslational modifications in response to stress. J Biol Chem 2003;14:14.

22. Oda, K., Arakawa, H., Tanaka, T., Matsuda, K., Tanikawa, C., Mori, T.,

Nishimori, H., Tamai, K., Tokino, T., Nakamura, Y., and Taya, Y. p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. Cell 2000;102:849-862.

23. Bulavin, D. V., Saito, S. i., Hollander, M. C., Sakaguchi, K., Anderson, C. W., Appella, E., and Fornace Jr, A. J. Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. EMBO J. 1999;18:6845-6854.

24. Mayo, L. D., Rok Seo, Y., Jackson, M. W., Smith, M. L., Rivera Guzman, J. R., Korgaonkar, C. K., and Donner, D. B. Phosphorylation of human p53 at serine 46 determines promoter selection and whether apoptosis is attenuated or amplified. J Biol Chem 2005;20:20.

25. Stambolic, V., MacPherson, D., Sas, D., Lin, Y., Snow, B., Jang, Y., Benchimol, S., and Mak, T. W. Regulation of PTEN Transcription by p53. Molecular Cell 2001;8:317-325.

26. Li, X., Dumont, P., Della Pietra, A., Shetler, C., and Murphy, M. E. The codon 47 polymorphism in p53 is functionally significant. J Biol Chem 2005;25:25.

27. Luo, J. L., Yang, Q., Tong, W. M., Hergenhahn, M., Wang, Z. Q., and Hollstein, M. Knock-in mice with a chimeric human/murine p53 gene develop normally and show wild-type p53 responses to DNA damaging agents: a new biomedical research tool. Oncogene 2001;20:320-328.

28. Chen, S., Kazim, D., Kraveka, J., and Pollack, R. E. Skin Fibroblasts from Individuals Hemizygous for the Familial Adenopolyposis Susceptibility Gene Show Delayed Crisis in vitro. PNAS 1989;86:2008-2012.

29. Feng, L., Lin, T., Uranishi, H., Gu, W., and Xu, Y. Functional analysis of the roles of posttranslational modifications at the p53 C terminus in regulating p53 stability and activity. Mol Cell Biol 2005;25:5389-5395.

30. Boley, S. E., Wong, V. A., French, J. E., and Recio, L. p53 heterozygosity alters the mRNA expression of p53 target genes in the bone marrow in response to inhaled benzene. Toxicol Sci 2002;66:209-215.

31. Sharpless, N. E. and DePinho, R. A. p53: Good Cop/Bad Cop. Cell 2002;110:9-12.

32. Lowe, S. W., Cepero, E., and Evan, G. Intrinsic tumour suppression. Nature 2004;432:307-315.

33. Saito, S., Goodarzi, A. A., Higashimoto, Y., Noda, Y., Lees-Miller, S. P., Appella, E., and Anderson, C. W. ATM mediates phosphorylation at multiple p53 sites, including Ser(46), in response to ionizing radiation. J Biol Chem 2002;277:12491-12494.

34. Lowe, S. W., Ruley, H. E., Jacks, T., and Housman, D. E. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 1993;74:957-967.

35. Debbas, M. and White, E. Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. Genes Dev. 1993;7:546-554.

36. Lowe, S. W. Activation of p53 by oncogenes. Endocr Relat Cancer 1999;6:45-48.

37. Gao, Y., Ferguson, D. O., Xie, W., Manis, J. P., Sekiguchi, J., Frank, K. M., Chaudhuri, J., Horner, J., DePinho, R. A., and Alt, F. W. Interplay of p53 and DNA-repair protein XRCC4 in tumorigenesis, genomic stability and development. Nature 2000;404:897-900.

38. Parrinello, S., Samper, E., Krtolica, A., Goldstein, J., Melov, S., and Campisi, J. Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. 2003;5:741-747.

39. Campisi, J. Cellular senescence as a tumor-suppressor mechanism. Trends Cell Biol 2001;11:S27-31.

40. Eliyahu, D., Michalovitz, D., Eliyahu, S., Pinhasi-Kimhi, O., and Oren, M. Wild-type p53 can inhibit oncogene-mediated focus formation. Proc Natl Acad Sci U S A. 1989;86:8763-8767.

41. Finlay, C. A., Hinds, P. W., and Levine, A. J. The p53 proto-oncogene can act as a suppressor of transformation. Cell 1989;57:1083-1093.

42. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 1997;88:593-602.

43. Ferbeyre, G., de Stanchina, E., Querido, E., Baptiste, N., Prives, C., and Lowe, S. W. PML is induced by oncogenic ras and promotes premature senescence. Genes Dev. 2000;14:2015-2027.

44. Pearson, M., Carbone, R., Sebastiani, C., Cioce, M., Fagioli, M., Saito, S., Higashimoto, Y., Appella, E., Minucci, S., Pandolfi, P. P., and Pelicci, P. G. PML regulates p53 acetylation and premature senescence induced by oncogenic Ras. Nature 2000;406:207-210.

45. Bischof, O., Kirsh, O., Pearson, M., Itahana, K., Pelicci, P. G., and Dejean, A. Deconstructing PML-induced premature senescence. Embo J 2002;21:3358-3369.

46. Krummel, K. A., Lee, C. J., Toledo, F., and Wahl, G. M. The C-terminal lysines fine-tune P53 stress responses in a mouse model but are not required for stability control or transactivation. Proc Natl Acad Sci U S A 2005;8:8.

Chapter 4

Conclusion

The tumor suppressor gene p53 has been extensively studied since it becomes clear that more than 50% of human cancers harbor mutations or functional inactivation in this gene (1, 2). p53 sits in the hub of a complex network composed of genes and their products that are involved in regulating cellular homeostasis in response to a variety of intrinsic and extrinsic stress signals. Upon various stresses, p53 is activated, where the increased protein half-life leads to p53 stabilization in cells and enhanced ability of sequence-specific DNA-binding and co-activators recruitment leads to increased p53 transcriptional activity for target genes. Accumulating evidence indicates that multiple posttranslational modifications of p53 play a critical role in regulating p53 responses to stress such as DNA damage, impacting on both p53 stability and activity (3, 4). In this context, p53 knock-in mouse model provides an invaluable source for assessing the physiological importance of p53 posttranslational modifications *in vivo* in modulating p53 tumor suppression (5).

The six Lysine residues at the extreme C-terminal domain of human p53 (Lys 370, 372, 373, 381, 382 and 386) can be posttranslationally modified by multiple mechanisms, including ubiquitination, acetylation, sumoylation, neddylation and methylation, in response to DNA damage (6-12). To investigate the physiological role of these modifications, we introduced six Lysine to Arginine missense mutations into the endogenous p53 gene in mouse ES cells which can be further differentiated *in vivo* into MEFs and thymocytes, thus knocking out all the

potential modifications on those Lysine residues while reserving the C-terminus conformation (13). In contrast to the previous finding that the C-terminal Lysine residues are major ubiquitination sites of Mdm2 and required for ubiquitin-mediated p53 degradation (6, 7), our studies indicate that the K6R mutation has no significant impact on p53 stability either before or after DNA damage in ES cells, MEFs and thymocytes, which is consistent with the finding from another group that the mutant mice are viable and phenotypically normal and mutant p53 protein has normal half-life and functions in both MEFs and thymocytes where they mutate all the seven Lysine at C-terminus of mouse p53 to Arginine (13, 14). The apparent discrepancy between our study and previous cell line studies may partly be explained as that the specific tumor cell lines used by early studies could contain some unknown mutations which impaired redundant pathways involved in p53 ubiquitination and degradation. Also, since the previous studies mainly focused on analyzing Mdm2-mediated p53 degradation by overexpression of Mdm2 and p53, it is possible that other E3 ligases for p53 such as Pirh2 and COP1 may contribute to ubiquitination of p53 at alternative Lysine residues and thereby destabilizes p53K6R (15, 16). The ubiquitination assay from our study (Figure 2, Chapter 2) clearly shows that p53K6R in ES cells can be efficiently ubiquitinated although the ubiquitination level is slightly reduced than that in wild-type control, implying that other Lysine residues at the other regions of p53 can play an essential role for ubiquitin-mediated p53 degradation, at least in the absence of the

C-terminal ubiquitination. A recent study suggested that several conserved lysine residues in the DNA-binding domain of p53 could be critical for p53 ubiquitination (17).

Our analysis of p53-dependent transcriptional activities in p53K6R ES cells, MEFs and thymocytes indicate that loss of all the potential C-terminal Lysine modifications results in modestly impaired p53 activity after DNA damage in ES cell and thymocytes but not in MEFs (Figure 2, 3, and 4, Chapter 2). The lack of defects in MEFs might be due to different regulatory mechanisms in MEFs since p53 transcriptionally activate its target genes in a cell type-specific manner (18, 19), or due to the activation of redundant pathways induced by *in vitro* culturing stresses, as suggested by the finding that p53 activity is normally increased during the continuous passage of MEFs eventually leading to cellular senescence (20, 21). Since our studies did not distinguish the physiological role of individual posttranslational modifications in regulating p53 transcriptional activity, we do not argue against the notion that p53 acetylation at C-terminus can significantly stimulate p53 transactivation in a promoter-specific way (10, 22). In addition, the partial defects observed in K6R mutant cells may underestimate the whole impact of the lack of C-terminal acetylation, since the simultaneous disruption of neddylation might compensate by increasing p53 activities (11). Unexpectedly, the studies from Wahl group point out that while cell cycle arrest and apoptosis are normal in p53(7KR) MEFs, p53(7KR) is activated more easily by DNA damage in

thymus than wild-type p53 and that p53(7KR) MEFs was not escaped to spontaneous immortalization in a 3T3 growth assay (14). The discrepancy in p53 activity between p53K6R and p53(7KR) could be caused by different mouse genetic backgrounds and/or by the additional mutation (mouse Lys 384) in p53(7KR) which might by itself contribute to p53 activation after cellular stress.

The N-terminus of p53 can be phosphorylated at multiple Serine and Threonine sites after DNA damage, modulating the p53 interactions with its positive and negative regulators (19). For example, phosphorylation of human p53 at Ser15 is indicated to be important for recruitment of co-activators to p53 target promoters and activate p53 responses in a promoter- and cell type- specific manner (18, 23). Of particular interest, Ser46 of human p53 can be induced to phosphorylation after a variety of genotoxic and cellular stresses (24). And cell line studies have shown that phosphorylation of Ser46 is specifically correlated with p53-dependent apoptosis through activating p53 targeting pro-apoptotic genes (25-28). Although Ser46 is not conserved in mouse p53 or p53 of other species, we demonstrate that the stress-induced signaling pathways leading to Ser46 phosphorylation are conserved between human and mouse cells, suggesting the important role of these pathways in mediating p53 responses upon stresses (Figure 1, Chapter 3). To further address the physiological importance of Ser46 phosphorylation, we employed the human p53 knock-in (HUPKI) allele to introduce Ser46 to Alanine mutation into the endogenous p53 locus in mice (Figure

2, Chapter 3). Our studies of p53-dependent transcriptional activity are generally consistent with the previous reports that Ser46 phophorylation is important for optimal induction of p53-dependent apoptosis and for efficient activation of p53-dependent apoptotic genes after DNA damage, in both E1A-MEFs and thymocytes (Figure 3, 4 and 5, Chapter 3). However, we were not able to analyze the expression level of *p53AIP1*, a critical p53 downstream pro-apoptotic gene whose induction seems to specifically require phosphorylation at Ser46, since there is no homolog of *p53AIP1* in mouse genome (26). The defects in p53 transcriptional activation of specific target genes may be caused by different mechanisms in MEFs and thymocytes. Only the induction levels of *Noxa* and *Perp* in MEFs, or *Noxa* and *Puma* in E1A-MEFs were obviously reduced, while the induction of almost all the p53 targets examined in $p53^{S46A}$ thymocytes was reduced (Figure 3, 4 and 5, Chapter 3). Since the p53 protein levels in MEFs and E1A-MEFs after DNA damage is only slightly reduced, and p53 protein concentration in MEFs does not clearly affect its transcriptional activation of several downstream target genes including *p21*, *Mdm2* and *Noxa* compared between $p53^{+/+}$ and $p53^{+/-}$ MEFs (data not shown), we postulate that in MEFs loss of Ser46 phosphorylation may affect p53 transactivity through modest defect in recruiting co-activators to specific promoters or reduced affinity to sequence-specific DNA-binding. However in thymocytes the global reduction of p53-dependent activation of target genes may be caused by the impaired p53 stabilization, since the

gene expression induction fold seems to be dramatically affected by p53 protein level in thymocytes (data not shown). Therefore, Ser46 phosphorylation appears to play overlapping as well as distinct roles in modulating p53 activity in different cell types.

Ser46 phosphorylation also seems important for p53 stabilization after some types of DNA damage treatment, and the reduced p53 protein level may be the result of the decreased p53 stability, as suggested by protein half-life assay (data not shown). Mdm2 is a critical negative regulator of p53 stability and activity, however, we did not observed any difference in the interaction between endogenous p53 and Mdm2 proteins in MEFs either before or after DNA damage, indicating that Ser46 phosphorylation is not required for efficient disruption of p53-Mdm2 interaction upon DNA damage (data not shown). Besides Mdm2, Pirh2 and COP1 are both E3 ubiquitin ligases, physically interact with p53 and can ubiquitinate p53 *in vivo* independent of Mdm2 (15, 16). Since Pirh2 might interact with the proline-rich domain of p53 (15), it is possible that Ser46 phosphorylation disrupts the interaction between p53 and Pirh2. It will be further investigated when better Pirh2 antibody is available to analyze the interaction between endogenous Pirh2 and p53.

Our study also identified another important aspect of Ser46 phosphorylation in suppressing spontaneous immortalization of cultured MEFs and inducing p53-dependent cellular senescence in response to oncogene activation. Previous reports indicated that overexpression of PML isoform IV can cause premature

senescence in part by activation of p53 through posttranslational modifications, including acetylation at Lysine 382 and phosphorylation at Ser46 (29, 30). The absence of Ser46 phosphorylation causes MEFs easier to escape replicative senescence due to *in vitro* culture stress and be spontaneously immortalized; more importantly, loss of Ser46 phosphorylation confers the MEFs the ability to escape Ras-induced premature senescence much faster and more easily (Figure 6, Chapter 3). Since acetylation of Lysine 382 may not be important in activating p53 responses during cellular senescence (13, 14), our finding indicates potential role of Ser46 phosphorylation in replicative senescence as well as oncogene *Ras* induced premature senescence. In this context, it will be more informative when p53hki^{S46A} mice are employed to cross with other transgenic lines such as *Ras*-transgenic mice to determine the role of this phosphorylation event in suppressing oncogene-induced tumorigenesis *in vivo*.

References

1. Levine, A. J., Momand, J., and Finlay, C. A. The p53 tumour suppressor gene. Nature. 1991;351:453-456.

2. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. p53 mutations in human cancers. Science 1991;253:49-53.

3. Appella, E. and Anderson, C. W. Signaling to p53: breaking the posttranslational modification code. Pathol Biol (Paris) 2000;48:227-245.

4. Appella, E. and Anderson, C. W. Post-translational modifications and activation of p53 by genotoxic stresses. Eur J Biochem 2001;268:2764-2772.

5. Attardi, L. D. and Donehower, L. A. Probing p53 biological functions through the use of genetically engineered mouse models. Mutation Research 2005;576:4-21.

6. Nakamura, S., Roth, J. A., and Mukhopadhyay, T. Multiple lysine mutations in the C-terminal domain of p53 interfere with MDM2-dependent protein degradation and ubiquitination. Mol Cell Biol 2000;20:9391-9398.

7. Rodriguez, M. S., Desterro, J. M., Lain, S., Lane, D. P., and Hay, R. T. Multiple C-terminal lysine residues target p53 for ubiquitin-proteasome-mediated degradation. Molecular And Cellular Biology 2000;20:8458-8467.

8. Gostissa, M., Hengstermann, A., Fogal, V., Sandy, P., Schwarz, S. E., Scheffner, M., and Del Sal, G. Activation of p53 by conjugation to the ubiquitin-like protein SUMO-1. Embo J 1999;18:6462-6471.

9. Rodriguez, M. S., Desterro, J. M. P., Lain, S., Midgley, C. A., Lane, D. P., and Hay, R. T. SUMO-1 modification activates the transcriptional response of p53. EMBO J. 1999;18:6455-6461.

10. Gu, W. and Roeder, R. G. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell 1997;90:595-606.

11. Xirodimas, D. P., Saville, M. K., Bourdon, J. C., Hay, R. T., and Lane, D. P. Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity. Cell 2004;118:83-97.

12. Chuikov, S., Kurash, J. K., Wilson, J. R., Xiao, B., Justin, N., Ivanov, G. S., McKinney, K., Tempst, P., Prives, C., Gamblin, S. J., Barlev, N. A., and Reinberg, D. Regulation of p53 activity through lysine methylation. Nature 2004;3:3.

13. Feng, L., Lin, T., Uranishi, H., Gu, W., and Xu, Y. Functional analysis of the roles of posttranslational modifications at the p53 C terminus in regulating p53 stability and activity. Mol Cell Biol 2005;25:5389-5395.

14. Krummel, K. A., Lee, C. J., Toledo, F., and Wahl, G. M. The C-terminal lysines fine-tune P53 stress responses in a mouse model but are not required for stability control or transactivation. Proc Natl Acad Sci U S A 2005;8:8.

15. Leng, R. P., Lin, Y., Ma, W., Wu, H., Lemmers, B., Chung, S., Parant, J. M., Lozano, G., Hakem, R., and Benchimol, S. Pirh2, a p53-induced ubiquitin-protein ligase, promotes p53 degradation. Cell 2003;112:779-791.

16. Dornan, D., Wertz, I., Shimizu, H., Arnott, D., Frantz, G. D., Dowd, P., K, O. R., Koeppen, H., and Dixit, V. M. The ubiquitin ligase COP1 is a critical negative regulator of p53. Nature 2004;429:86-92.

17. Chan, W. M., Mak, M. C., Fung, T. K., Lau, A., Siu, W. Y., and Poon, R. Y. C. Ubiquitination of p53 at Multiple Sites in the DNA-Binding Domain. Mol Cancer Res 2006;4:15-25.

18. Chao, C., Hergenhahn, M., Kaeser, M. D., Wu, Z., Saito, S., Iggo, R., Hollstein, M., Appella, E., and Xu, Y. Cell type- and promoter-specific roles of Ser18 phosphorylation in regulating p53 responses. J Biol Chem 2003;8:8.

19. Xu, Y. Regulation of p53 responses by post-translational modifications. Cell Death Differ 2003;10:400-403.

20. Serrano, M. and Blasco, M. A. Putting the stress on senescence. Current Opinion in Cell Biology 2001;13:748-753.

21. Campisi, J. Senescent Cells, Tumor Suppression, and Organismal Aging: Good Citizens, Bad Neighbors. Cell 2005;120:513-522.

22. Barlev, N. A., Liu, L., Chehab, N. H., Mansfield, K., Harris, K. G., Halazonetis, T. D., and Berger, S. L. Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases. Molecular Cell

2001;8:1243-1254.

23. Chao, C., Herr, D., Chun, J., and Xu, Y. Ser18 and 23 phosphorylation is required for p53-dependent apoptosis and tumor suppression. Embo J. 2006;25:2615-2622.

24. Saito, S., Yamaguchi, H., Higashimoto, Y., Chao, C., Xu, Y., Fornace, A. J., Jr., Appella, E., and Anderson, C. W. Phosphorylation site interdependence of human p53 posttranslational modifications in response to stress. J Biol Chem 2003;14:14.

25. Bulavin, D. V., Saito, S. i., Hollander, M. C., Sakaguchi, K., Anderson, C. W., Appella, E., and Fornace Jr, A. J. Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. EMBO J. 1999;18:6845-6854.

26. Oda, K., Arakawa, H., Tanaka, T., Matsuda, K., Tanikawa, C., Mori, T., Nishimori, H., Tamai, K., Tokino, T., Nakamura, Y., and Taya, Y. p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. Cell 2000;102:849-862.

27. Li, X., Dumont, P., Della Pietra, A., Shetler, C., and Murphy, M. E. The codon 47 polymorphism in p53 is functionally significant. J Biol Chem 2005;25:25.

28. Mayo, L. D., Rok Seo, Y., Jackson, M. W., Smith, M. L., Rivera Guzman, J. R., Korgaonkar, C. K., and Donner, D. B. Phosphorylation of human p53 at serine 46 determines promoter selection and whether apoptosis is attenuated or amplified. J Biol Chem 2005;20:20.

29. Pearson, M., Carbone, R., Sebastiani, C., Cioce, M., Fagioli, M., Saito, S., Higashimoto, Y., Appella, E., Minucci, S., Pandolfi, P. P., and Pelicci, P. G. PML regulates p53 acetylation and premature senescence induced by oncogenic Ras. Nature 2000;406:207-210.

30. Bischof, O., Kirsh, O., Pearson, M., Itahana, K., Pelicci, P. G., and Dejean, A. Deconstructing PML-induced premature senescence. Embo J 2002;21:3358-3369.