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Functional role of p53 N-terminal phosphorylation in regulating the p53 response to DNA damage

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by

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DEDICATION

This work is dedicated to my parents, Davy and Josephine Chao, for lovingly making the sacrifices to provide my sister and me with a first-rate education, and for instilling in us the notion that nothing is impossible.

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ABSTRACT OF THE DISSERTATION

Functional role of p53 N-terminal phosphorylation in regulating the p53 response to DNA damage

by

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Since the p53 tumor suppressor is mutated in over 50% of human cancers, understanding the mechanisms that regulate its function holds great importance for cancer therapy. In response to diverse stresses, p53 is activated as a potent transcription factor, and induces signals that prevent the proliferation of mutation-harboring cells, which may otherwise lead to cancer. Because of its protective role in maintaining genetic stability, p53 is aptly named "guardian of the genome." Exposure to DNA damage and other cellular stresses results in p53 phosphorylation at multiple

serine and threonine residues at its N-terminus. These events are believed to play critical roles in regulating p53 stability and activity. Of particular interest is phosphorylation of mouse p53 at Ser18, which is mediated by members of the ATM family kinases that are also the master regulators of the cellular response to DNA damage. To address the physiological relevance of this event, we introduced a Ser18 to Alanine missense mutation into the endogenous p53 gene of mouse embryonic stem cells and generated germline $p53^{S18A}$ mice. Our analysis of cells from the $p53^{S18A}$ mice indicate that Ser18 phosphorylation is dispensable for p53 accumulation, but important for regulating p53 transcriptional activity in a cell type and promoter-specific manner. The defect in p53-dependent transactivation could be attributed to impaired recruitment of transcriptional coactivators, since the DNA binding activity of p53 after DNA damage to endogenous promoters was similar in both $p53^{+/+}$ and $p53^{S18A}$ cells. To address whether p53 may be synergistically regulated by Ser18 and Ser23 phosphorylation, we simultaneously introduced Ser18/Ser23 to Ala mutations into the mouse germline. Our findings demonstrate that Ser18/Ser23 phosphorylation is important for activating p53-dependent apoptotic activity. Furthermore, while Ser18/Ser23 phosphorylation is important for spontaneous tumor suppression in aging animals, it is dispensable for p53-dependent tumor suppression in the presence of persistent genetic instability, such as that acquired through DNA repair deficiency. Thus p53 tumor suppressor activity is regulated through different mechanisms depending on the physiological context.

Chapter 1

Introduction

p53 is the most frequently mutated tumor suppressor gene found in human cancers (Hollstein et al., 1991). Accordingly, germline p53 mutations and/or loss of p53 pathway function correspond to the increased cancer risk and early cancer onset of the genetic Li-Fraumeni syndrome (LFS)(Royds and Iacopetta, 2006). Based on structural and biochemical studies, the full-length p53 protein consists of an N-terminal transactivation domain, a central sequence specific DNA binding domain, and a multifunctional C- terminal regulatory domain. Importantly, the majority of p53 mutations observed in human tumors localize to the core DNA binding domain (Magali Olivier, 2002), indicating that activation of the p53 DNA binding activity is crucial for p53 mediated tumor suppression.

While normally in a latent state, p53 becomes activated in response to DNA damage and other cellular insults that include oxidative stress, hypoxia, oncogene activation and nucleotide depletion (Ko and Prives, 1996). The biological role of p53 as a tetrameric transcription factor enables it to activate or repress a vast number of genes that regulate cell growth, cell death and DNA repair processes (Vogelstein et al., 2000) (Lin et al., 2005). In particular, p53-mediated G1 cell cycle arrest and apoptosis are two characteristic outcomes of p53 activation, and help prevent the propagation of harmful mutations to daughter cells that may potentially develop into cancer.

Cellular exposure to DNA damage induces a wide array of p53 post translational modifications, including phosphorylation, acetylation, ubiquitination, sumoylation, neddylation and methylation (Appella and Anderson, 2001; Chuikov et al., 2004; Gostissa et al., 1999; Kahyo et al., 2001; Kwek et al., 2001; Rodriguez et al.,

1999; Xirodimas et al., 2004; Xu, 2003). Numerous studies have implicated important roles for these events in modulating p53 stability and activity, however it was not until recently, with the ability to generate "knock-in" mice expressing site-specific mutant p53 alleles, that it has become possible to study the impact of these events in a physiologically relevant context. Elucidating the precise mechanism by which p53 becomes activated in the aftermath of cellular and genotoxic stresses could lead to important advances in understanding how carcinogenesis can be suppressed and lead to novel therapies for cancer treatment.

Negative regulation of p53 by the Mdm2 oncoprotein

Increased stabilization of p53 protein following exposure to DNA damage and other stresses is critical for its activation as a tumor suppressor. However, because of the growth suppressive activities of p53 that may threaten normal cellular viability, p53-mediated responses must be tightly regulated. One important regulator of p53 stability and activity is the p53 transcriptional target Mdm2. Since Mdm2 expression is dependent on p53 transactivation after exposure to DNA damage, the p53-Mdm2 autoregulatory feedback loop represents a critical mechanism for the efficient downregulation of p53-dependent responses. A severe consequence of deregulated p53 activity is illustrated by the early embryonic lethality of $Mdm2^{-/-}$ mice due to unabated p53 dependent apoptosis (de Oca Luna et al., 1995; Jones et al., 1995) (Chavez-Reyes et al., 2003; de Rozieres et al., 2000). Concomitant deletion of p53, however, completely rescues the lethality of $Mdm2^{-/-}$ embryos. In this context, Mdm2

is not only a critical regulator of p53 activity in vivo, its expression is also essential to ensure proper embryonic development (de Oca Luna et al., 1995; Jones et al., 1995).

In homeostatic tissues, Mdm2 basal expression is independent of p53 transcriptional regulation; however Mdm2 expression is still important for inhibiting aberrant p53 activation. (Mendrysa et al., 2003; Mendrysa and Perry, 2000). Mice expressing reduced levels of Mdm2, due to the presence of a hypomorphic Mdm2 allele, were significantly runted due to developmental and proliferative defects associated with increased p53 transcriptional activity (Mendrysa et al., 2003). These mice were anemic, due to the reduced lymphocyte populations compared to wild type mice, radiosensitive, and the epithelial cells of the small intestine were highly sensitized to undergo apoptosis. Further genetic manipulation that allowed for a more subtle reduction in endogenous Mdm2 expression, led to the generation of relatively healthy mice expressing constitutively higher levels of p53, but without the gross side effects (Mendrysa et al., 2006). Strikingly, the increased p53 expression afforded these mice protection against tumorigenesis. Conversely, it would be expected that increased Mdm2 expression in mice could attenuate the p53 responses, and thereby enhance tumorigenesis (Jones et al., 1998). In support of this, approximately 30% of human soft tissue sarcomas that harbor wild type p53 alleles are attributed to mutations that prompt Mdm2 over expression (Oliner et al., 1992). Furthermore, a single nucleotide polymorphism in the human Mdm2 promoter that results in increased Mdm2 expression is associated with early cancer risk in Li-Fraumeni patients, and also increases the susceptibility to spontaneous cancers (Bond et al.,

2004). Overall these findings reinforce the importance of the p53–Mdm2 interplay and demonstrate that disruptions in the p53 signaling pathway may also facilitate cancer progression. These studies are also important in that they offer new strategies for cancer treatment by honing in on the pathways that can lead to p53 activation.

Role of Mdm2-mediated p53 ubiquitination

Mdm2 negatively regulates p53 through various mechanisms. It inhibits p53 transcriptional activity by binding to the p53 transactivation domain and preventing its association with basal transcription components including TBP and TAFs (Buschmann et al., 2001; Thut et al., 1997). The p53-Mdm2 interaction inhibits p53 ability to recruit transcriptional co-activators like p300/CBP and PCAF, that also acetylate p53, and enhance p53-dependent transcription (Kobet et al., 2000; Wadgaonkar and Collins, 1999). Lastly, Mdm2 functions as an E3 ligase for p53 (Honda et al., 1997), and promotes its degradation in a ubiquitin dependent manner (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997). Mdm2 mediated ubiquitination of several evolutionarily conserved lysine residues in the p53 C-terminus can facilitate p53 nuclear export, a process thought to be important for the efficient degradation of p53 on cytoplasmic 26S proteasomes (Gu et al., 2001) (Lohrum et al., 2001; Nakamura et al., 2000; Rodriguez et al., 2000). Even so, accumulating evidence indicates that p53 is likely to be degraded on nuclear proteasomes as well (Li et al., 2003). Increased Mdm2 activity has been shown to facilitate polyubiquitination of p53. As mentioned earlier, p53 and Mdm2 generate a

negative feedback loop in response to DNA damage. Since prolonged activation of p53 could be detrimental to the cell, the nuclear degradation of p53 could be an important mechanism to ensure that p53 activity is rapidly suppressed, particularly during the late stages of the p53 response when DNA repair has already taken place (Shirangi et al., 2002).

While it is evident Mdm2 plays a critical role in regulating p53 stability, recent studies have challenged the notion that the C-terminal lysine residues ubiquitinated by Mdm2 are crucial for regulating p53 stabilization and subsequent activation (Feng et al., 2005; Krummel et al., 2005). In these studies, subtle point mutations, in which the C terminal lysine residues were simultaneously mutated to arginine, were introduced into the endogenous p53 locus of mouse embryonic stem cells, making it possible to study the impact of these ubiquitination events in a physiologically relevant environment. Despite the loss of these lysine residues, p53 stabilization was completely normal both before and after DNA damage. Moreover, the mutant p53 could still be ubiquitinated, suggesting that other ubiquitination sites are involved in regulating p53 stability. In this context, a recent study has identified two conserved lysine residues located in the core DNA binding domain that can also be targeted for ubiquitination (Chan et al., 2006). Characterization of these sites in a functionally relevant system, and their ability to regulate p53 protein stability in the presence or absence of the C-terminal ubiquitination sites awaits further studies.

Other cellular factors that contribute to p53 stabilization and activation

Apart from Mdm2, Pirh2 and COP1 are additional E3 ligases that can promote p53 degradation in a proteasome dependent manner. Pirh2 was demonstrated to interact directly with the p53 core DNA binding domain (Leng et al., 2003). Thus Pirh2 may suppress p53 dependent transcription by impairing its DNA binding activity (Leng et al., 2003). Over expression of the COP1 E3 ligase also led to a dramatic reduction of p53 transactivational activity. Conversely, COP1 depletion by siRNA enhanced p53 stabilization, and induced a G1 cell cycle arrest, furthering supporting its role as a negative regulator of p53 stability and activity (Dornan et al., 2004). Both Pirh2 and COPI appear to function independently of Mdm2 as inhibitors of p53 activity. Furthermore Pirh2 and COP1 may also be transcriptionally regulated by p53 in response to DNA damage. Thus p53 activity can targets its own suppression through two additional pathways. Nevertheless, the functional relevance of the p53-Pirh2 and p53-COP1 negative feedback loop remains to be elucidated, particularly since neither can compensate for Mdm2 loss during mouse embryogenesis. In this context, Pirh2 and COPI may be important for regulating p53 stability and activity during later stages of development, in specific cellular contexts, or after specific types of stress signals.

Many cellular components may interfere with the balance of the p53-Mdm2, Pirh2, or COPI autoregulatory network, and therefore contribute to the regulation of p53 stability and activity. One such protein is the YY1 transcription factor. YY1 stabilizes the p53-Mdm2 complex, thereby enhancing Mdm2-mediated ubiquitination

and degradation of p53. (Gronroos et al., 2004; Sui et al., 2004). In this context, overexpression of YY1 significantly inhibits p53 accumulation. Furthermore, YY1 inhibits p53 transcriptional activity by preventing the association of p53 with the p300/CBP transcriptional co-activator (Gronroos et al., 2004). Other factors, such as the deubiquitinating enzymes ATF3 and HAUSP enhance p53 stability and activity by removing the ubiquitin sidechains (Li et al., 2002a; Yan et al., 2005). The inactivation of ATF impairs p53 upregulation and activity in response to stress. The partial reduction of HAUSP levels through siRNA decreased p53 stabilization. Unexpectedly though, complete inactivation of HAUSP stabilized p53 significantly, contradicting its role as a p53 deubiquitinating enzyme (Cummins et al., 2004; Li et al., 2004). Further studies, though, revealed that the deubiquitination activity of HAUSP can preferentially target Mdm2 over p53. In this manner, HAUSP stabilizes Mdm2 and enhances Mdm2-dependent negative regulation of p53, thus providing an interesting level of regulation in the p53-Mdm2 interplay.

The competition between p53 ubiquitination and acetylation , at the same C-terminal lysine residues, also have a direct impact on p53 stabilization and activation (Ito et al., 2001; Li et al., 2002b). In this context, tumor suppressors such as ARF and PML can stabilize p53 by simultaneously promoting p53 acetylation and blocking p53 ubiquitination. ARF directly inhibits the E3 ligase activity of Mdm2 (Honda and Yasuda, 1999). Both ARF and PML have been shown to facilitate the nucleolar sequestration of Mdm2 (Bernardi et al., 2004; Weber et al., 1999), thereby preventing its interaction with p53.

Regulation of p53 by MdmX

Another important regulator of p53 is the Mdm2 related protein MdmX. Inactivation of MdmX in mice results in embryonic lethality that can also be completely rescued through simultaneous deletion of p53 (Finch et al., 2002; Migliorini et al., 2002; Parant et al., 2001a). The embryonic lethality of MdmX-/- mice occurs at a later stage of development than $Mdm2^{-/-}$ mice, and since neither Mdm2 nor MdmX expression can compensate for the loss of the other, the regulation of p53 by these two proteins occurs through independent pathways. Even so, it appears that both MdmX and Mdm2 rely on each other to inhibit p53 activity. In this context, MdmX and Mdm2 form stable heterodimers, which increases the half-life of Mdm2 and enhances its inhibitory effects on p53 (Ghosh et al., 2003; Gu et al., 2002). Conversely Mdm2 enables MdmX-negative regulation of p53 by facilitating MdmX nuclear localization (Gu et al., 2002)Both proteins associate directly with the p53 transactivation region; however MdmX does not ubiquitinate p53, facilitate p53 nuclear export nor promote its degradation. Furthermore, MdmX is not transcriptionally regulated by p53, nor is it induced in response to DNA damage (Parant et al., 2001b; Shvarts et al., 1996). In this context, the mechanism by which MdmX regulates p53 has not been fully elucidated. However, a short isoform of MdmX (MdmX-S), that is expressed in transformed cells, was found to efficiently localize to the nucleus and bind much more strongly to p53 than full-length MdmX (Rallapalli et al., 1999). Binding of MdmX-S to p53 interferes with p53 acetylation,

and strongly inhibits p53 dependent gene expression . To further add to the complexity of MdmX mediated regulation of p53, studies have shown that overexpression of MdmX may also increase p53 stability and activity (Jackson and Berberich, 2000; Mancini et al., 2004). Given that MdmX and Mdm2 compete with each other to bind p53, excessive levels of MdmX may prevent the Mdm2-p53 interaction and therefore block Mdm2-mediated degradation of p53 (Gu et al., 2002).

Upstream activation of p53 by multiple kinases

Phosphorylation events at the p53 N- terminus are coupled with increases in p53 stability and activity. Both human and mouse p53 sequences are highly conserved. Recently a "humanized" p53 mouse model ($p53^{Hupki}$) was generated, in which exons 4-9 of mouse p53 were replaced with the homologous human sequences (Luo et al., 2001b). Many of the phosphorylation sites, and the kinases that mediated these events, are also evolutionarily conserved between humans and mice. Since the p53 response in $p53^{Hupki}$ mice was normal, these findings indicate that human and mouse p53 are regulated through similar mechanisms, and validate the use of knock-in mouse models to examine how p53 modifications may regulate p53 responses to DNA damage.

The ataxia-telangiectasia-mutated (ATM) family kinases including ATM, ATR (ATM/Rad3 related) and DNA-PK (DNA- dependent protein kinase) phosphorylate Ser15 of human p53 (corresponding to Ser18 of mouse p53)(Appella and Anderson, 2001; Shieh et al., 1997; Siliciano et al., 1997); ATR and DNA-PK also phosphorylate

Ser37 (Shieh et al., 1997; Tibbetts et al., 1999), which is not conserved in mice. As primary sensors of DNA damage, ATM family members integrate a large network of signal transduction pathways important for genomic maintenance (reviewed by Shiloh, 1996). Inactivation of ATM gives rise to the human autosomal recessive disorder ataxia-telangiectasia (A-T) that is characterized by neurodegeneration, immunodeficiency, radiosensitivity and cancer predisposition. Cells deficient in ATM demonstrate impaired accumulation of p53 concomitant with reduced levels of Ser15 phosphorylation after IR (Siliciano et al., 1997). In this context, defective G1 cell cycle arrest in A-T cells and the resistance to p53 mediated apoptosis in the developing central nervous system and retinas of $ATM^{-/-}$ mice may be attributed to the impaired stabilization and activation of p53 following IR (Borges et al., 2004; Chong et al., 2000) Thus, ATM represents an important upstream regulator of p53.

The Chk1 and Chk2 checkpoint kinases are activated by ATM family members in response to DNA damage and have been demonstrated to phosphorylate p53 at Ser20 (Ser23 of mouse p53) in response to IR and UV. More recently Chk1 and Chk2 have been shown to target additional sites at the p53 C terminus, including Ser366, Ser378 and Thr387 that may also impact p53 acetylation and transcriptional activity (Ou et al., 2005). A role for Chk2 in tumor suppression is highlighted by the fact that Chk2 mutations are frequently found in Li-Fraumeni patients who harbor wild type alleles of p53 (Meijers-Heijboer, 2002 #311). p53 responses are moderately impaired in $Chk2^{-/-}$ mice including reduced p53 accumulation and impaired p53- dependent apoptosis (Hirao et al., 2002; Takai et al., 2002) . However, recent studies

demonstrate that Chk2 may not be essential for mediating Ser20 phosphorylation since this site can still be modified in Chk2 deficient cells (Ahn et al., 2003; Takai et al., 2002); thus it is possible that Chk1, or other yet unidentified kinases, can mediate this event when Chk2 is inactivated.

Other kinases that contribute to p53 signaling include the casein kinase 1 (CK1) which phosphorylates Ser6, Ser9, and Thr18 (which corresponds to Thr23 of mouse p53). (Appella and Anderson, 2001; Bode and Dong, 2004)The Thr18 phosphorylation event also requires prior phosphorylation of Ser15(Dumaz et al., 1999) (Saito et al., 2003). Since Thr18 lies within the Mdm2 binding region of p53, phosphorylation at this site may interfere with the Mdm2-mediated negative regulation (Craig et al., 1999). Members of the mitogen activated protein kinase (MAPK) family phosphorylate p53 at multiple residues following diverse stresses. p38 MAPK phosphorylates Ser15, as well as Ser33 and Ser46, which are not conserved in mice, but have been shown to regulate p53 dependent apoptosis (Saito et al., 2003; Sakaguchi et al., 1998). Ser46, however, is also phosphorylated by HIPK2 (homeodomain interacting protein kinase 2), and has been demonstrated to enhance p53 dependent apoptosis through transcriptional activation of AIP (Oda et al., 2000b; Saito et al., 2002; Thompson et al., 2004). Several major phosphorylation sites, particularly Ser34 phosphorylated by JNK after UV, and Thr73 and Thr83 phosphorylated by ERK in response to growth factors, are found in mice but not in humans (Hu et al., 1997; Wu, 2004). Despite these discrepancies between the two

species, these events can still be considered functionally relevant considering that human and mouse p53 are both targeted by MAPK signaling pathways (Wu, 2004).

Phosphorylation of N terminal sites has been shown impact p53 stability by destabilizing the interaction between p53 and its negative regulators. In particular phosphorylation of Ser15, Thr18, Ser20 and/or Ser37 were shown to increase p53 accumulation and activation by disrupting the p53- Mdm2 interaction (Craig et al., 1999; Scolnick et al., 1997; Shieh et al., 1997; Unger et al., 1999). However the requirement for these events was also challenged since mutating the N- terminal residues individually or in combination did not impair p53 stabilization in response to DNA damage (Ashcroft et al., 1999; Blattner et al., 1999). The physiological roles of Ser15 and Ser20 phosphorylation have recently been examined through the introduction of either a Ser18Ala ($p53^{S18A}$) or Ser23Ala ($p53^{S23A}$) missense mutation into the endogenous p53 gene of mouse ES cells (Chao et al., 2000; Wu et al., 2002). $p53^{S18A}$ ES cells demonstrated delayed and reduced accumulation of p53 (Chao et al., 2000), whereas $p53^{S23A}$ ES cells were completely normal in p53 stabilization (Wu et al., 2002). In this context, phosphorylation of Ser18 may be important for efficient upregulation of p53 in response to DNA damage. However neither phosphorylation of Ser18 or Ser20 alone is sufficient to induce p53 stability in ES cells (Chao et al., 2000; Wu et al., 2002). Detailed analyses of germline $p53^{S18A}$ and $p53^{S18/23A}$ mice and the p53 dependent responses in cell types derived from these animals will be discussed in detail in Chapters II and III.

In addition to enhancing p53 stability, DNA damage induces a phosphorylation-acetylation cascade leading to modifications of the p53 C- terminus that can modulate p53 transcriptional activity (Sakaguchi et al., 1998). In addition phosphorylation events can affect the subcellular localization of p53. Phosphorylation at Ser392 of human p53 has been shown to stabilize p53 tetramer formation (Sakaguchi et al., 1997). The tetramerization domain of p53 is located at the p53 C-terminus and includes a nuclear export sequence (NES). In this context, stress induced tetramerization of p53 could lead to enhanced nuclear localization by masking the NES signal, thereby preventing nuclear export of p53 (Stommel et al., 1999). A second NES signal located in the transactivation domain can also be impaired by p53 phosphorylation events at the N terminus (Zhang and Xiong, 2001) (Martinez et al., 1997; Sakaguchi et al., 1997) (Stommel et al., 1999). Since N-terminal phosphorylation disrupts p53-Mdm2 binding this may further enhance p53 activity by inhibiting the ubiquitination and nuclear export of p53.

Role of p53 acetylation

p53 transcriptional activity is dramatically increased in the presence of the transcriptional co-activators p300/CBP and PCAF that possess intrinsic histone acetyltransferase activity (Avantaggiati et al., 1997; Scolnick et al., 1997). In this context, p53 recruitment of these co-activators can induce histone acetylation, which is thought to enable nucleosome remodeling and facilitate access of transcription factors to the chromatin template. Additionally, p300/CBP and PCAF directly acetylate the

p53 C terminus, which has been shown to augment p53 transcriptional activity (Gu and Roeder, 1997). p53 is acetylated at Lys382, and to a lesser extent at Lys373 at the extreme C terminal regulatory domain by p300/CBP (Liu et al., 1999). PCAF acetylates Lys320 (mouse Lys317) in the core DNA binding region (Liu et al., 1999).

DNA damage induced phosphorylation at the N terminus enables the interaction between p300/CBP and the p53 transactivation domain, and leads to increased levels of p53 acetylation (Lambert et al., 1998; Sakaguchi et al., 1998). Early studies indicated that C terminal post translational modifications increased DNA binding activity. Specifically p53 acetylation stimulated binding of p53 to short oligomeric sequences in vitro, prompting those speculations that its main role was to activate p53 DNA binding activity (Gu and Roeder, 1997). It was postulated that acetylation neutralized the charges within the p53 C terminus, which could relieve the inhibitory interaction of this domain on the core DNA binding domain. Alternatively, p53 acetylation could induce conformational changes in p53 by converting it to a form more favorable for DNA binding.

Recent studies have challenged these notions that acetylation enhances DNA binding, by demonstrating that both acetylated and unacetylated forms of p53 bind with similar efficiency to a chromatinized p21 promoter element (Espinosa and Emerson, 2001). Furthermore, mutation of C terminal acetylation sites did not impair p53 binding to the endogenous p21 promoter, but reduced levels of transcription were observed. In addition p53 was shown to bind non-specifically to random pieces of DNA due to the presence of a non-sequence specific DNA binding element within the

p53 C terminus(McKinney et al., 2004; Yakovleva et al., 2001). Recent studies reported that the p53 C terminus is important for enabling p53 to slide across linear strands of DNA (McKinney et al., 2004). In this manner, regardless of acetylation status, p53 can efficiently locate its target sequences leading to enhanced transactivation of downstream genes. Taken together, these findings suggest that DNA damage induced p53 acetylation, is dispensable for DNA binding activity, but still nonetheless required for efficient transcriptional activity (Barlev et al., 2001; Espinosa and Emerson, 2001). In further support of this, increased levels of p53 occupancy on target promoters after DNA damage correlated with increases in p53 protein levels, indicating that the relative binding affinity of p53 is not augmented by genotoxic stress (Kaeser and Iggo, 2002).

The cellular exposure to IR resulted in dramatic increases in the overall levels of p53, transcriptional co-activators and histone acetylation found at the endogenous p21 promoter (Barlev et al., 2001). This suggests that acetylation might be important for co-activator recruitment leading to histone acetylation (Barlev et al., 2001), and is underscored by findings that p53 DNA binding exposes the acetylation motif of p53 (Dornan et al., 2003). In this regard p300 efficiently acetylated p53 that was bound to the chromatized p21 promoter (Espinosa and Emerson, 2001). Since p53 binds with high affinity to the p21, Mdm2 and Puma promoters, but with significantly lower affinity to others, such as the Bax promoter (Kaeser and Iggo, 2002), it is possible that p53 acetylation facilitates the recruitment of specific co-activators to enhance transactivation of the different promoters (Liu et al., 2003)

While the mechanism of how acetylation regulates p53 activity still needs to be fully elucidated, the effects of factors that stabilize levels of p53 acetylation leading to enhanced p53 responses have been reported. For example, the pro-myoletic leukemia (PML) tumor suppressor stabilizes p53 by sequestering Mdm2 in the nucleolus and facilitating p53 acetylation at Lys382 after DNA damage (Bernardi et al., 2004). Furthermore, colocalization of p53/CBP/PML in so called PML bodies leads to premature senescence in response to oncogenic Ras expression.(Ferbeyre et al., 2000; Pearson et al., 2000) The ING2 tumor suppressor can also induce replicative senescence in a p53 dependent manner by enhancing p53 acetylations (Pedeux et al., 2005).

Conversely, deacetylation of p53 represents another cellular mechanism by which p53 responses may be regulated. Histone deacetylases (HDACs) are functionally linked with transcriptional silencing and have been implicated in facilitating p53 dependent gene repression (Murphy et al., 1999). The SIRT1 and Sir2 HDACs can specifically deacetylate p53, and repress p53 transactivation (Cheng et al., 2003; Vaziri et al., 2001) . SIRT1 was shown to rescue cells from premature senescence following oncogenic Ras or PML overexpression. Furthermore, p53 in SIRT1 deficient cells were hyperacetylated, and the SIRT1-/- mice demonstrated overall growth defects attributed to increased p53 activity (Luo et al., 2001a; Luo et al., 2000; Vaziri et al., 2001). In this context, treatment of cells with deacetylase inhibitors can increase the levels of p53 acetylation and potentiate p53 mediated growth arrest and apoptosis (Luo et al., 2000).

The cellular outcome of p53 activation

p53 is essential for inhibiting the inappropriate growth of cells carrying harmful mutations that can lead to cancer. The type of response elicited by p53 activation, however, is dependent on multiple factors, including the cellular context, the nature and magnitude of the stress signal, and the level of p53 that may be induced. The exposure of MEFs to DNA damage, such as IR or UV, induces a G1 cell cycle arrest, and requires p53 dependent transactivation of the p21 cyclin/cyclin kinase dependent inhibitor. Activation of p53 can also induce a G2/M checkpoint through transactivation of p21, GADD45, 14-3-3 and cyclin G (Stark and Taylor, 2004). Overall, the transient p53-mediated cell cycle arrest allows cells to repair the damaged DNA before entry into mitosis and completion of cell division. p53 may also induce a permanent growth arrest or cellular senescence in MEFs, in the presence of mitogenic signals, such as oncogenic Ras expression.

Another cellular outcome of p53 activation is the induction of apoptosis, or programmed cell death, which has been detailed in thymocytes, epithelial cells of the small intestine, and the developing central nervous system of mice (Clarke et al., 1994; Clarke et al., 1993; Lowe et al., 1993). The transcriptional activation of numerous p53 target genes including Bax, Puma, Noxa, are all members of the pro-apoptotic group of Bcl2 family members which facilitates cytocrome c release from the mitochondria. However their roles in facilitating p53 death signals occur in a stress and/or tissue-specific manner (Fei et al., 2002). For example, Bax, is required

for mediating p53 dependent apoptosis in the developing central nervous system of mice, but not for the IR-induced cell death of thymocytes (Chong et al., 2000; Clarke et al., 1993; Lowe et al., 1993). Noxa$^{-/-}$ thymocytes are likewise sensitive to IR-induced apoptosis, whereas oncogene- expressing MEFs and intestinal epithelial cells from the Noxa$^{-/-}$ mice were protected from cell death following DNA damage (Oda et al., 2000a; Schuler et al., 2003; Shibue et al., 2003; Villunger et al., 2003). Puma appears to play a more universal role in mediating p53–dependent apoptosis, since numerous cell types, including thymocytes, neurons and MEFs, derived from Puma$^{-/-}$ mice are virtually resistant to p53-dependent apoptosis (Jeffers et al., 2003; Villunger et al., 2003; Yu and Zhang, 2003). Interestingly, however the defective apoptosis in Puma-/- mice do significantly impact tumor susceptibility, unlike the majority of p53$^{-/-}$ mice which succumb to thymic lymphomas within 6 months of age. Likewise, Noxa$^{-/-}$ mice are also resistant to spontaneous tumorigenesis, indicating that p53 dependent tumor suppression is not completely dependent on its ability to induce apoptosis.

Mouse cells expressing a transactivation-deficient mutant of p53 (p53^{QS}) fail to undergo p53-dependent apoptosis in response to DNA damage, which strongly suggests that p53 transcriptional activity is required for its apoptotic functions. However, a new study shows that p53^{QS} retains the ability to transactivate Bax, and may explain why introducing p53^{QS} into the mouse germline results in embryonic lethality (Johnson et al., 2005). In this context, hypoxic stress stimuli, such as that presented *in utero* may activate transcriptional-independent functions of p53 to induce apoptosis. A number of studies have demonstrated the ability of p53 to rapidly

translocate to the mitochondria, following exposure to DNA damage. (Chipuk et al., 2004; Erster et al., 2004). As these events are demonstrated to precede p53 nuclear accumulation and activation of its transcriptional activity, this represents a first wave of apoptosis (Erster et al., 2004) (Zhao et al., 2005). By engaging the pro-survival factors Bcl-2 or Bcl-X, it is thought that p53 can directly activate Bax and thereby promote cytochrome c release (Marchenko et al., 2000; Sansome et al., 2001) (Mihara et al., 2003). Whether or not p53 post-translational modifications regulate this process remains unclear.

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Chapter 2

Cell type- and promoter-specific roles of Ser18phosphorylation in regulating p53 responses

Abstract

Phosphorylation of mouse p53 at Ser18 (corresponding to Ser15 of human p53) occurs after various types of DNA damage and was suggested to activate p53 responses to DNA damage (Chao et al., 2000a). To determine the physiological roles of this phosphorylation event in p53-dependent DNA damage responses, we introduced a Ser18 to Ala missense mutation into the endogenous p53 gene in mice. Analysis of p53 responses to ionizing radiation (IR) in $p53^{S18A}$ mouse thymocytes indicated that this phosphorylation event is not required for p53 stabilization but important for p53-dependent apoptosis. Microarray analysis of p53-dependent gene expression in thymocytes after IR indicated that the function of this phosphorylation event is promoter-specific. p53 was more stabilzed in p53 ^{S18A} mouse embryonic fibroblasts (MEFs) than in wild-type MEFs after UV radiation, but p53-dependent functions were impaired and p53 acetylation at C-terminal greatly reduced in p53 ^{S18A} MEFs after UV radiation. However, chromatin immunoprecipitation analysis showed normal *in vivo* binding of p53 ^{S18A} to p53-dependent promoters, strongly supporting the notion that neither Ser18 phosphorylation nor acetylation at C-terminal are required for in vivo binding of p53 to p53-dependent promoters. These findings have important implications for future functional studies of p53 posttranslational modifications.

Introduction

p53 is the most commonly mutated tumor suppressors in human cancers and plays multiple roles in responses to stresses: cell cycle arrest or apoptosis, partly depending on the cell types (Hollstein et al., 1991; Ko and Prives, 1996). Both functions of p53 could protect the genome from accumulating mutations and passaging these mutations to the daughter cells. Structural and functional analysis of p53 have shown that p53 is a transcription factor with a sequence-specific DNA-binding domain in the central region and a transcriptional activation domain at the N-terminal. Three additional domains, including a nuclear localization signal, a tetramerization domain and a extreme C-terminal regulatory domain, are present in the C-terminal of p53 (Ko and Prives, 1996). The transcriptional activities of p53 are essential for both p53-dependent cell cycle arrest and apoptosis (Chao et al., 2000b; Jimenez et al., 2000)

In response to DNA damage and other cellular stresses, the protein levels of p53 are greatly upregulated and its transcriptional activities significantly induced (Ko and Prives, 1996). However, it remains unclear how these stresses signal p53 responses. The p53 protein level is regulated post-transcriptionally and upregulation of p53 following DNA damages is mainly due to increased protein stability (Appella and Anderson, 2001). The interaction between p53 and MDM2, a transcriptional target of p53, represents a feedback negative regulatory mechanism for the rapid turnover of p53 in normal cells(Haupt et al., 1997; Kubbutat et al., 1997). In this context, p53 is

degraded through a ubiquitin-dependent pathway and MDM2 functions as the p53-specific E3 ubiquitin ligase (Maki et al., 1996) (Honda et al., 1997).

Accumulating evidence suggests that phosphorylation of p53 might play important roles in regulating p53 stability and activity (Appella and Anderson, 2001). Both human and mouse p53 are phosphorylated at multiple sites at the N- and C-terminal both in vivo and in vitro by a number of kinases. In this context, phosphorylation of p53 at Ser15 occurs rapidly following DNA damage and requires ATM family protein kinase (Banin et al., 1998) (Canman et al., 1998). A number of studies have tried to understand the functions of Ser15 phosphorylation in regulating p53 stability and activity. One study reported that phosphorylation of p53 at Ser15 disrupted its interactions with MDM2, leading to p53 stabilization (Shieh et al., 1997), others have argued that Ser15 phosphorylation is required for the acetylation of p53 at C-terminal Lys residues but dispensable for p53-Mdm2 interaction (Avantaggiati et al., 1997; Dumaz and Meek, 1999; Lambert et al., 1998).

To address the importance of Ser15 phosphorylation in p53 responses to DNA damage, we introduced a missense Ser18 (corresponding to Ser15 of human p53) to Ala mutation into the endogenous p53 gene of mouse embryonic stem (ES) cells. Analysis of homozygous mutant $p53^{S18A}$ ES cells indicated that phosphorylation of mouse p53 at Ser18 is important for the maximum induction of p53 protein levels after DNA damage (Chao et al., 2000a). In addition, p53-dependent cell cycle G1 arrest and induction of p21was impaired in $p53^{S18A}$ differentiated ES cells (Chao et al., 2000a). However, p53 acetylation at extreme C-terminal is normal in $p53^{Ser18Ala}$ ES cells.

Therefore, these findings support a role for Ser18 phosphorylation in regulating p53 responses to DNA damage. However, considering that ES cell is an atypical cell type in the context of regulation of p53 stability and activity (Aladjem et al., 1998), the $p53^{Ser18Ala}$ mutation was introduced into mouse germline. Analysis of the p53 responses to various types of DNA damage in distinct primary cell types derived from $p53^{S18A}$ mice indicated cell-type and promoter-specific roles of this phosphorylation event in regulating p53 responses to DNA damage.

Results

Stabilization and activation of p53 in p53^{S18A} thymocytes after IR

Mouse thymocytes undergo p53-dependent apoptosis after IR (Clarke et al., 1993; Lowe et al., 1993). Therefore, to test the functions of p53 phosphorylation at Ser18 in regulating p53 apoptotic activities, we analyzed p53 stabilization and p53-dependent apoptosis in p53 ^{S18A} thymocytes after IR essentially as described (Wu et al., 2002). The protein levels of p53 were induced to similar maximum levels in p53 ^{S18A} and wild-type thymocytes after IR, indicating that Ser18 phosphorylation is not requred for the maximum p53 induction in thymocytes after IR (Fig. 1a). However, the p53-dependent apoptosis was significantly impaired in p53 ^{S18A} thymocytes after IR, indicating that Ser18 phosphorylation is required to activate p53 apoptotic activities (Fig. 1b).

Expression profiles of p53-dependent genes in wild-type and p53^{Ser18Ala} thymocytes after IR

To understand the basis for the impaired p53-dependent apoptosis in p53 ^{S18A} thymocytes after IR, we profiled the global gene expression in wild-type and p53 ^{S18A} thymocytes before and after IR by microarray analysis. Scatter graphs of microarray analysis from duplicate experiments (two sets of treated wild-type and p53 ^{S18A} thymocytes and two sets of untreated controls) show excellent reproducibility (data not shown). Upon challenge by IR, clear differences between wild-type and p53 ^{S18A} thymocytes were revealed with respect to overall transcriptional response to IR (Fig.

2a). Specifically, the induction or reduction of most p53-dependent genes was impaired in p53 ^{S18A} thymocytes after IR (Fig. 2b, c). In this context, mRNA levels of the known p53-inducible downstream genes p21, Perp, SNK, 14-3-3-sigma and TNFα were increased to considerably higher levels in wild-type thymocytes than in p53 ^{S18A} thymocytes after IR (Table 1). However, the impacts of p53^{S18A} mutation on p53-dependent gene expression appear to be promoter-specific, since a number of known p53 downstream traget genes, including Mdm2, Noxa, Bax, Apaf1, and Wig1, were equally or even more vigorously induced in p53^{S18A} thymocytes than in wild-type thymocytes after IR (Table 1). The differential induction of a number of p53-dependent genes, including p21, Mdm2, Noxa, Perp, Cyclin G and 14-3-3sigma in wild-type and p53^{S18A} thymocytes after IR was further confirmed by real-time PCR analysis (Fig. 3a).

The promoter-specific effects of Ser18Ala mutation on the expression of p53 target genes could be due to the differentiatial requirement of Ser18 phosphoylation in the recruitment of co-activators to the distinct p53-dependent promoters. Therefore, we employed chromatin immunoprecipitation assay to analyze the histone H3 acetylation at two promoters of p53-dependent genes, Mdm2 and p21, in wild-type and p53^{S18A} thymocytes with or without IR treatment. Histone acetylation of Mdm2 promoter was increased to similar extent in both wild-type and p53^{S18A} thymocytes after IR (Fig. 3b). However, histone acetylation of p21 promoter was reduced in p53^{S18A} thymocytes after IR when compared with that of wild-type thymocyte (Fig. 3b). The correlation between the expression levels of Mdm2 and p21 and histone

acetylation of their promoters suggested that Ser18 phosphorylation is important to recruit co-activators to some but not all p53-dependent promoters.

Hyper-stabilization of p53 in p53 ^{S18A} MEFs after DNA damage

To study the roles of Ser18 phosphorylation in p53 responses to DNA damage in MEFs, p53 protein levels in p53^{S18A} MEFs at different time points after IR and UV radiation were analyzed by Western blotting. Unexpectedly, these studies indicated that p53 was induced to higher peak levels in p53^{S18A} MEFs than in wild-type MEFs (Fig. 4a, b). Since Mdm2-p53 interaction plays a major role in regulating p53 stability, we analyzed p53-Mdm2 interaction by co-immunoprecipitation assay as described in wild-type and p53 ^{S18A} MEFs at different time points after UV radiation (Wu et al., 2002). This analysis indicated that p53-Mdm2 interaction was weaker in p53^{S18A} MEFs than that in wild-type MEFs at various time points after UV radiation, thus providing the basis for the higher protein levels of p53 in p53 ^{S18A} MEFs after UV radiation (Fig. 4c). To further understand the basis for the reduced p53-Mdm2 interaction in p53^{S18A} MEFs after UV radiation, we analyzed the induction of Mdm2 mRNA in p53^{Ser18Ala} and wild-type MEFs after UV radiation by quantitative real-time PCR. In contrast to the findings that higher Mdm2 mRNA levels were detected in p53^{S18A} thymocytes after IR, Mdm2 mRNA levels were significantly lower in p53^{S18A} MEFs than in wild-type MEFs after UV radiation (Fig. 3a). In addition, Mdm2 mRNA levels were also reduced in p53^{S18A} MEFs after IR, indicating that the differential impacts of Ser18Ala mutations on the Mdm2 expression in thymocytes and MEFs

were not due to the distinct types of DNA damage (Fig. 3a). Together, these findings could account for the higher protein levels of p53 in p53 ^{S18A} MEFs after UV radiation. In addition, these findings also indicated that Ser18 phosphorylation plays cell type-specific roles at least in the context of transcriptional regulation of Mdm2.

Impaired p53-dependent functions in p53 ^{S18A} MEFs after UV radiation

To test whether the high levels of p53 protein are functional in p53^{S18A} MEFs after UV radiation, we assayed the G_1 cell cycle arrest in p53^{S18A} MEFs after UV radiation as described (Xu et al., 1998). The G_1/S cell cycle arrest was significantly impaired in p53^{S18A} MEFs after UV radiation, indicating that Ser18 phosphorylation is required for the activation of p53 activities after DNA damage in MEFs (Fig. 5a). Consistent with this notion, p53-dependent induction of p21 was reduced in p53^{S18A} MEFs after UV radiation (Fig. 3b).

Phosphorylation of p53 at N-terminal was suggested to be involved in the nuclear retention of p53 (Zhang and Xiong, 2001). Therefore, one possibility for the impaired p53 activities in p53^{S18A} MEFs after UV radiation was that p53^{S18A} is mostly cytoplasmic. To test this notion, we analyzed the subcellular localization of p53 in MEFs after UV by immunochemical staining with anti-p53 antibody as described (Chao et al., 2000b) Our analysis indicated that p53 accumulated in the nucleus of p53^{S18A} MEFs after UV radiation (Fig. 5b).

Acetylation of p53 at C-terminal has been suggested to activate p53-dependent transcriptional activities by recruiting co-activators (Barlev et al., 2001; Espinosa and

Emerson, 2001). Therefore, we analyzed the p53 acetylation at C-terminal in $p53^{Ser18Ala}$ MEFs after UV radiation as described (Chao et al., 2000a). In contrast to what was observed in $p53^{S18A}$ ES cells (Chao et al., 2000a), acetylation of p53 at C-terminal was greatly reduced in $p53^{S18A}$ MEFs after UV radiation, providing potential mechanism for the impaired p53 activities in $p53^{S18A}$ MEFs after UV radiation (Fig. 5C).

Chromatin immunoprecipitation analysis of bindings of p53 to p53-dependent promoters in wild-type and $p53^{S18A}$ MEFs

While one published study suggested that p53 acetylation at C-terminal can activate the sequence-specific DNA binding activities of p53 (Gu and Roeder, 1997). However, latter published studies argued against this proposed role of p53 acetylation (Barlev et al., 2001; Espinosa and Emerson, 2001). Since p53 acetylation was significantly reduced in $p53^{S18A}$ MEFs after UV radiation, we assayed the in vivo binding of p53 to various p53-depenent promoters in wild-type and $p53^{S18A}$ MEFs using chromatin immunoprecipitation assay as described (Kaeser and Iggo, 2002). This analysis indicated that the binding of p53 to p53-dependent promoters was normal in $p53^{S18A}$ MEFs after UV radiation (Fig. 6). Therefore, our findings support the notion that p53 acetylation at C-terminal is not required for its binding to p53-dependent promoters. In addition, the impaired p53 activities in $p53^{S18A}$ MEFs after UV radiation are not due to impaired binding of p53 to p53-dependent promoters.

Tumorigenesis in $p53^{S18A}$ mice

Modulation of p53 activities appears to be required during early mouse embryogenesis (de Oca Luna et al., 1995). Gnotyping of the offsprings from the intercrossing of heterozygous mutant mice showed a ratio of $p53^{+/+}$:$p53^{+/S18Aa}$:$p53^{S18A}$ of, close to the expected 1:2:1 Mendelian ratio. Therefore, Ser18 phosphorylation is not required for the modulation of p53 activities during embryonic development. $p53^{S18A}$ mice were viable and matured into adulthood without any apparent developmental defects. Since p53-dependent responses to DNA damage were impaired in $p53^{S18A}$ mice, $p53^{S18A}$ mice might be cancer proned. Therefore, we monitored the onset of tumorigenesis in more than 55 pairs of $p53^{S18A}$ and littermate or age-matched wild-type controls, which are presently between 1 to 2-years old. However, no apparent increase in the spontaneous tumorigenesis was observed in $p53^{S18A}$ mice, suggesting that Ser18Ala mutation did not significantly affect p53 tumor suppression activities.

Discussion

To investigate the functions of Ser18 phosphorylation in regulating p53 stability and activity, we analyzed the p53 responses to various types of DNA damage in a number of primary cell types, including MEFs, thymocyte and ES cells. These studies indicated that this phosphorylation event might play multiple roles in regulating p53 stability in response to genotoxic stresses in different cell types. In this context, the delayed and reduced p53 induction in p53 ^{S18A} ES cells after DNA damage suggested that phosphorylation of mouse p53 at Ser18 is important for p53 stabilization in ES cells after DNA damage (Chao et al., 2000a). However, the maximum levels of p53 induction induced by IR are similar between wild-type and p53 ^{S18A} thymocytes, therefore, Ser18 phosphorylation is dispensible for p53 stabilization in thymocytes. In addition, higher than normal levels of p53 proteins accumulate in p53 ^{S18A} MEFs after UV radiation, likely due to weaker interaction between p53-Mdm2 in these cells upon UV radiation. The difference in the regulation of p53 stability between thymocyte and MEFs after DNA damage could be due to differential regulation of Mdm2 expression. While higher than normal levels of Mdm2 were expressed in p53 ^{S18A} thymocytes after IR, significantly lower than normal levels of Mdm2 were detected in p53 ^{S18A} MEFs after both IR and UV radiation. Therefore, the roles of Ser18 phosphorylation in regulating certain target genes such as Mdm2 could be cell type specific.

The normal or higher than normal levels of p53 induction in p53^{S18A} MEFs and thymocytes after DNA damage make it feasible to draw conclusions on the importance

of Ser18 phosphorylation in activating p53 functions. The findings that p53-dependent cell cycle G_1 arrest and apoptosis are impaired in $p53^{S18A}$ cells after DNA damage indicate that Ser18 phosphorylation is important for the activation of p53 activities after these genotoxic stresses. The impaired p53 activities in $p53^{S18A}$ cells after DNA damage are not due to abnormal subcellular localization of p53 after DNA damage, as p53 accumulated in the nucleus of wild-type and $p53^{S18A}$ cells after DNA damage. The impaired p53 activities are also not due to defective sequence-specific DNA-binding of $p53^{S18A}$ to p53-dependent promoters since p53 binds to p53-dependent promoters normally in $p53^{S18A}$ cells after DNA damage. Greatly reduced acetylation of p53 at C-terminal in $p53^{S18A}$ MEFs after UV radiation might account for the impaired p53 activities, since these acetylation events were suggested to be required to recruit transcriptional co-activators to the p53-dependent promoters (Barlev et al., 2001; Espinosa and Emerson, 2001). These findings also indicate that p53 acetylation at C-terminal is not required to activate *in vivo* sequence-specifc DNA-binding activities of p53, a conclusion consistent with some of published studies (Barlev et al., 2001; Espinosa and Emerson, 2001). Contrary to what was observed in MEFs, our analysis of $p53^{S18A}$ ES cells indicated that p53 acetylation is normal in $p53^{S18A}$ ES cells (Chao et al., 2000a). This difference could be an additional indication of abnormal regulation of p53 in ES cells as suggested by Aladjem et al. (Aladjem et al., 1998) or represents a cell type-specific role of Ser18 phosphorylation.

Another novel finding from our studies is that the roles of Ser18 phosphorylation in regulating the transcription of p53 target genes appear to be

promoter-specific. In this context, while the induction of many p53-dependent genes was impaired in $p53^{S18A}$ thymocytes after IR, induction of some p53-dependent genes was normal or even more robust in $p53^{S18A}$ thymocytes after IR when compared with those in wild-type thymocytes after IR. It is likely that the unaffected promoters contain DNA-binding motifs bound by other transcriptional factors, which function redundantly with p53 in the recruitment of transcriptional machinery. In addition, this promoter-specific role sometimes depend on cell types, for example, the induction of Mdm2 is enhanced in $p53^{S18A}$ thymocytes but reduced in $p53^{S18A}$ MEFs after DNA damage.

The findings of the promoter-specific roles of Ser18 phosphorylation have several implications. As revealed by the analysis of p53-dependent responses to DNA damage, p53-dependent functions appear to be intermediately defectve in $p53^{S18A}$ mice. However, no apparent increase in spontaneous tumorigenesis was observed in $p53^{S18A}$ mice. Therefore, it is possible that the unaffected p53-dependent gene products in $p53^{S18A}$ cells could still suppress tumorigenesis. A number of p53-dependent gene products are involved in apoptosis (Vousden and Lu, 2002). The promoter-specific roles of Ser18 could also help us to identify p53-dependent genes which are involved in IR-induced thymocyte apoptosis. Finally, most published studies on the impacts of p53 phosphorylation used only a few well-known p53 target genes, such as p21 and Mdm2, as readouts for p53 functions. Our findings of the promoter-specific roles of Ser18 phosphorylation indicate the requirements to analyze

a more comprehensive list of p53 target genes in future functional studies of p53 posttranslational modifications.

Methods

Construction of p53 ^{S18A} mice

p53^{S18A} ES cells were described previously (Chao et al., 2000a). The heterozygous mutant ES cells were injected into C57/BL6 blastocysts to generate chimeric mice which transmitted the mutation into mouse gemline. The germline transmitted mice were bred with CMV-Cre mice to excise the PGK-neo^{r} gene from the targeted alleles. Heterozygous mutant mice were intercrossed to generate p53 ^{S18A} mice.

Western blotting analysis

Protein extracts from 4x 10^5 MEFs or 5x 10^6 mouse thymocytes were separated by sodium dodecyl sulfate-polyacrimide gel electrophoresis (SDS-PAGE) on 8% (for Mdm2), 12% (p53) or 15% (p21) polyacrimide gels and transferred to nitrocellulose membrane. The membrane was blocked with 5% dry milk and probed with a monoclonal antibody against p53 (pAb240; Santa Cruz biotechnology, Inc.) or a polyclonal antibody against p53 (CM-5;Vector Laboratories Inc.) or a polyclonal antibody against p21 (Santa Cruz Biotechnology, Inc.) or a monoclonal antibody against Mdm2 (2A10; Oncogene Science). The filter was then incubated with a horse-radish peroxidase conjugated secondary antibody, developed with ECL PLUS from Amersham. To determine if the amount of proteins in each lane were compatible, the filter was stripped and probed with a rabbit polyclonal antibody against β-actin (Santa Cruz Biotechnology, Inc.).

To detected p53 acetylation at C-terminal, MEFs were exposed to UV light (60 J/M^2) and treated with 5 mM Trichostatin A for 4 hours before harvesting. Cell extract from the irradiated and untreated controls were analyzed by Western blot with antibodies specific for p53 or p53 acetylated at Lys-379. Acetylated p53 and total p53 are indicated with arrowheads. Rabbit polyclonal antibody against murine p53 acetylated at Lys379 was described previously (Chao et al., 2000b).

Cell Cycle Analysis

Cell cycle G_1/S arrest was performed as previously described (Xu et al., 1998). Briefly, subconfluent MEFs were synchronized at G_0 by serum starvation in DMEM supplemented with 0.1% FCS for 96 hours. Irradiated or untreated G_0 synchronized cells were trysinized and plated in 10-cm-diameter plates at a density of $1X10^6$ cells/plate in normal growth medium supplemented with 10 mM bromodeoxyuridine (BrdU). After 24 hours of BrdU labeling, cells were harvested, fixed in 70% ethanol and stored at -20 ºC. DNA content and DNA synthesis were analyzed by flow cytometry as previously described (Xu et al., 1998).

p53-dependent apoptosis in mouse thymocytes

Single cell suspension of thymocytes were plated in DMEM supplemented with 5% FCS and 10 mM Hepes (pH 7.0) at a density of 10^6 cells/ ml and treated with increasing dosages of IR. Ten hours after treatment, the percentage of apoptotic

thymocytes were determined by staining with FITC-conjugated Annexin V (Pharmingen) and analyzed by flow cutometry as described (Chao et al., 2000b).

Microarray analysis

Total RNA was extracted from thymocytes with a Qiagen Rneasy Extraction Kit. RNA amount and quality were monitored with Agilent Nano6000 RNA chips (Agilent Technologies). Ten micrograms of total RNA from each thymocyte sample were reverse-transcribed using Superscript II reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD), converted into double-stranded cDNA, and then biotin-labelled during in vitro transcription using the ENZO RNA Transcript labelling kit (Affymetrix, Sunnydale, CA) according to Affymetrix recommended protocols. Each sample was pre-tested for RNA integrity by hybridization to Affymetrix Test3 chips, and then hybridized to Affymetrix Murine Genome U74Av2 expression arrays, which interrogate approximately 12,600 murine sequences (listed at www.affymetrix.com). Data from microarray experiments were evaluated by Affymetrix MAS 5.0 and DMT software. Following scanning of each oligonucleotide array, the average signal intensity (sum of all intensities/number of sequence) was set to 100.

Real Time PCR Analysis

Total RNA was isolated from MEFs or thymocytes with the combination of Trizol (Invitrogen) and RNeasy RNA Cleanup (Qiagen). RNA was treated with RNase-free DNase for 20 min (Roche Applied Science) at room temperature before being reverse

transcribed using Superscript II RT (Invitrogen). Real time PCR was performed on an MX-400 machine (Stratagene) with SyBr Green PCR Master Mix (ABI). PCR conditions consisted of a 10-min hot start at 95 °C, followed by 40 cycles of 30 s at 95 °C and 1 min at 61 °C. The average threshold cycle (C_t) for each gene was determined from triplicate reactions, and levels of gene expression relative to glyceraldehyde-3-phosphate dehydrogenase were determined as previously described (Boley et al., 2002). The primer sequences for p21, Bax, and cyclin G were described (Boley et al., 2002). Other primers are listed below: Mdm2 primers, 5'ATTGCCTGGATCAGGATTCAGTT-3' and ACCTCATCATCCTCATCTGAGA-3'; Noxa primers, 5'-CCACCTGAGTTCGCAGCTCAA-3' and 5'-GTTGAGCACACTCGTCCTTCAA-3'; Perp primers, 5'-TCATCCTGTGCATCTGCTTC-3' and 5'-GGGTTATCGTGAAGCCTGAA-3'; 14-3-3σ primers, 5'-GCCGAACGGTATGAAGACAT-3' and 5'-CTCCTCGTTGCTCTTCTGCT-3'; and glyceraldehyde-3-phosphate dehydrogenase primers, 5'-CCAGTATGACTCCACTCACG-3' and 5'-GACTCCACGACATACTCAGC-3'.

Chromatin Immunoprecipitation Assay

ChIP analysis of in vivo binding of p53 to p53-dependent promoters was performed essentially as described (Kaeser, 2002 #149). For ChIP, 1.6×10^6 MEFs in a 10-cm dish were fixed with 1% formaldehyde for 10 min at room temperature. Formaldehyde was neutralized by the addition of 125 mM glycine for 5 min. The cells were washed

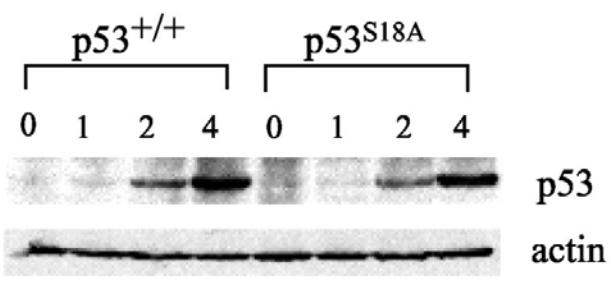
twice in ice-cold phosphate-buffered saline and collected by scraping in 1 ml of 1% SDS 50 mM Tris, pH 8.0, 100 mg/liter sonicated salmon sperm DNA and protease inhibitors (15 mg/liter aprotinin, 2 mg/liter leupeptin, and 0.2 g/liter phenylmethylsulfonyl fluoride). The lysates were vortexed, and insoluble material was collected by centrifugation at 4 °C for 5 min. The pellets were resuspended in 1 ml of 0.25% SDS, 200 mM NaCl, 50 mM Tris, pH 8.0, 100 mg/l sonicated salmon sperm DNA, and protease inhibitors and sonicated to an average fragment size of 0.6 kb using a Microtip on a Branson sonicator. The remaining insoluble material was removed by centrifugation at 4 °C for 5 min. The supernatant was diluted with 2 volumes of 1% Nonidet P-40, 350 mM NaCl and split in three samples. Each ChIP was incubated for 12 h at 4 °C with 10 µl of antibody-coated paramagnetic protein G beads (Dynal). One µg PAb248 (Yewdell, 1986 #304) was used per ChIP. Immune complexes were collected with a magnet, washed four times in 1% Nonidet P-40, 350 mM NaCl, 50 mM Tris, pH 8.0, resuspended in 125 µl of 1% SDS, and eluted by heating to 85 °C for 10 min. Cross-linking was reversed by incubation of the eluate for 6 h at 65 °C. The samples were diluted with 125 µl of water containing 160 mg/liter proteinase K and incubated for 1 h at 50 °C. DNA was purified by extraction with phenolchloroform and precipitated with isopropanol and glycogen. Quantitative PCR was performed on a PE5700 PCR machine using a SYBR Master Mix (Perkin Elmer). The PCR cycles were 50 °C for 2 min to digest dUTP-containing DNA and 95 °C for 10 min to activate Taq Gold polymerase, and then there were cycles of 95 °C for 15 s and 60 °C for 1 min repeated 40 times, except for the Mdm2 primers where the

annealing temperature was 62 °C. The primer sequences are: Mdm2, forward primer 5'-GGTCAAGTTGGGACACGTCC-3' and reverse primer 5'-AGCGTTTAAATAACCCCAGCTG-3'; PERP distal promoter, forward primer 5'-CACCTATATAAGGCTATGTGTAGAGG-3' and reverse primer 5' TACAATATCATATGGAGGAATCTAGTCTGTG 3'; and PERP proximal promoter, forward primer 5'-TCACAGGCTATTGGGATGTCC-3' and reverse primer 5'-AACACAGTGTAAGGAGCATGGCT-3'.

For ChIP analysis of histone acetylation at p53-dependent promoters, anti-acetyl histone H3 rabbit polyclonal antibody (Upstate Biotechnology, Inc.) was used to immunoprecipitate acetylated chromatin. The primers to detect the promoters of Mdm2, p21, and β-actin are: p21, forward primer 5'-CCTTTCTATCAGCCCCAGAGGATA-3' and reverse primer 5'-GGGACATCCTTAATTATCTGGGGT-3'; Mdm2, forward primer 5'-GGTGCCTGGTCCCGGACTCGC-3' and reverse primer 5'-AGAGGGTCCCCCAGGGGTGTC-3'; and β-actin, forward primer 5'-TTACGCCTAGCGTGTAGACTCT-3' and reverse primer 5'-AATACTGTGTACTCTCAAGATGGA-3'.

Figure 1. p53 stability and activities in wild type and $p53^{S18A}$ thymocytes after IR. (A), the protein levels of p53 in wild type and $p53^{S18A}$ thymocytes at various time points after exposure to 5 grays of IR. The time points and genotypes are labeled at the tops of the lanes. p53 and actin are indicated on the right. (B) The percentile ratio of nonapoptotic thymocytes in wild type and $p53^{S18A}$ thymocytes treated with 5, 10, and 20 grays of IR to the nonapoptotic thymocytes from untreated controls. The mean values from three independent experiments are presented with error bars.

A


p53+/+
p53S18A
0 1 2 4 0 1 2 4
p53
actin

B

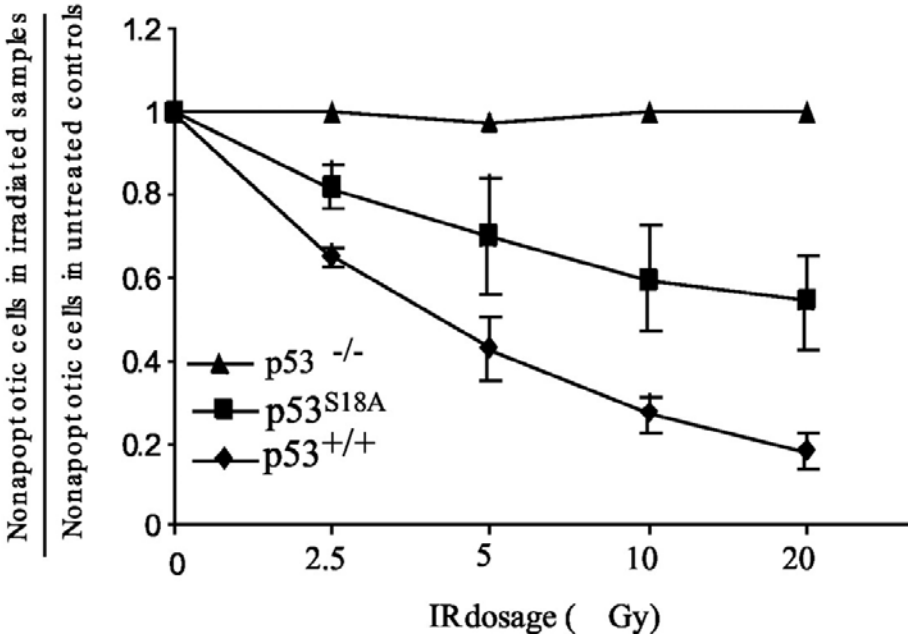
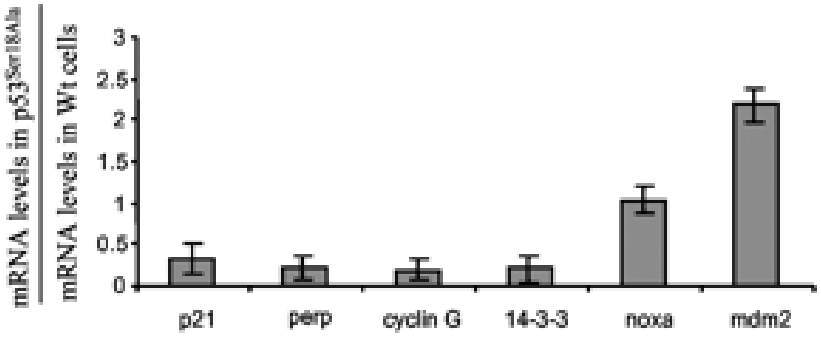

Nonapoptotic cells in irradiated samples
Nonapoptotic cells in untreated controls
1.2
1
0.8
0.6
0.4
0.2
0
0
2.5
5
10
20
p53 -/-
p53S18A
p53+/+
IR dosage (Gy)

TABLE I. Fold induction of the mRNA levels of well known p53-dependent genes in wild type and $p53^{S18A}$ thymocytes after IR as determined by Affymetrix gene chip system. Data from two independent experiments are given. Expt., experiment.

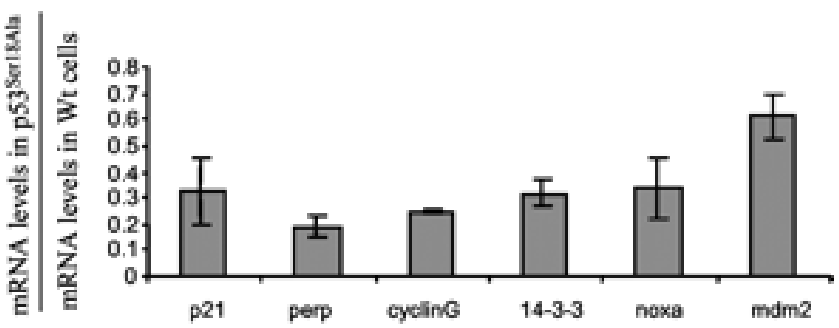
Genes	Folds of induction in thymocytes after IR					
	Wild type			$p53^{S18A}$		
	Expt. 1	Expt. 2	Average	Expt. 1	Expt. 2	Average
p21	12	15	13.5	2.8	6	4.4
Mdm2	1.7	1.5	1.6	1.5	2.5	2
14-3-3σ	6.2	6	6.1	1.9	2.1	2
TNF-α	4.7	8	6.4	0.7	1.2	0.95
C/EBP	14	11.5	12.75	2.2	1.3	1.75
SNK	16	7.5	11.75	3.4	3.3	3.3
Calcyclin	18	14	16	0.5	0.8	0.7
Perp	8.5	4.5	6.5	3	1	2
Apaf1	4	2	3	3	3.5	3.25
Bax	4	4.5	4.25	5	6	5.5
BLK	1	2	1.5	2	4	3
Wig-1	6	4	5	6.5	8	7.25
Pig8	3.5	2.5	3	2.7	3.2	2.95

Figure 2. p53-dependent transcription after DNA damage. The ratio of the mRNA levels of several p53 target genes in $p53^{S18A}$ thymocytes 8 h after IR treatment (A) or in MEFs 18 h after UV radiation (B) versus those in wild type cells as determined by quantitative real time PCR. mRNA levels for each gene were standardized by glyceraldehyde-3-phosphate dehydrogenase as described under "Experimental Procedures." (C) Chromatin immunoprecipitation analysis of histone H3 acetylation at the promoters of Mdm2, Noxa, and p21 in thymocytes before and after IR. Histone acetylation at the β-actin promoter was also analyzed as an internal control. The genotypes and time points are labeled on the top. Mdm2, p21, and β-actin are indicated on the right. Wt, wild type.

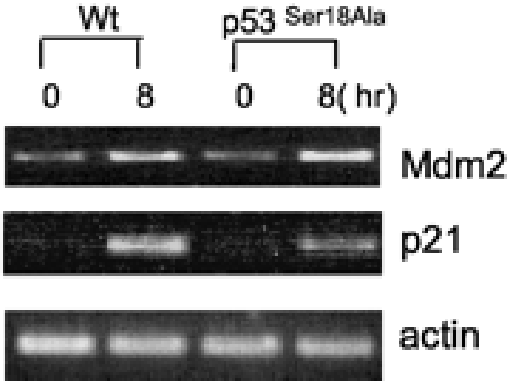
A


mRNA levels in p53Ser18Ala
mRNA levels in Wt cells
3
2.5
2
1.5
1
0.5
0
p21
perp
cyclin G
14-3-3
noxa
mdm2

B


mRNA levels in p53Ser18Ala
mRNA levels in Wt cells
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
p21
perp
cyclinG
14-3-3
noxa
mdm2

C


Wt
p53 Ser18Ala
0
8
0
8(hr)
Mdm2
p21
actin

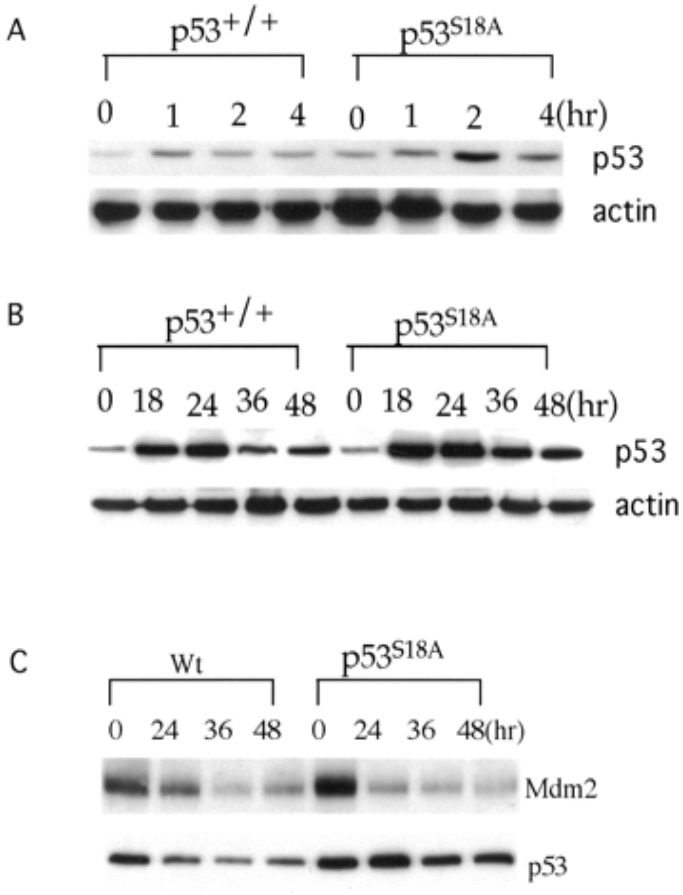


Figure 3. p53 stabilization in wild type and $p53^{S18A}$ MEFS after DNA damage. Protein levels of p53 in wild type and $p53^{S18A}$ MEFs at various time points after 5 grays of IR (A) and 60 J/M^2 UV radiation (B). The time points and genotypes are labeled at the tops of the lanes. p53 and actin are indicated on the right. (C) Co-immunoprecipitation analysis of p53-Mdm2 in wild type and $p53^{S18A}$ MEFs before and after UV radiation. p53 was immunoprecipitated from the cellular extract, and the amounts of p53 and Mdm2 in the immunoprecipitates were analyzed by Western blotting. The genotypes and time points are labeled at the tops of the lanes. p53 and Mdm2 are indicated on the right. Untreated wild type (Wt) and $p53^{S18A}$ MEFs were incubated with the proteasome inhibitor N-acetyl-Leu-Leu-Nle-CHO for 4 h before harvest to ensure detectable levels of p53.

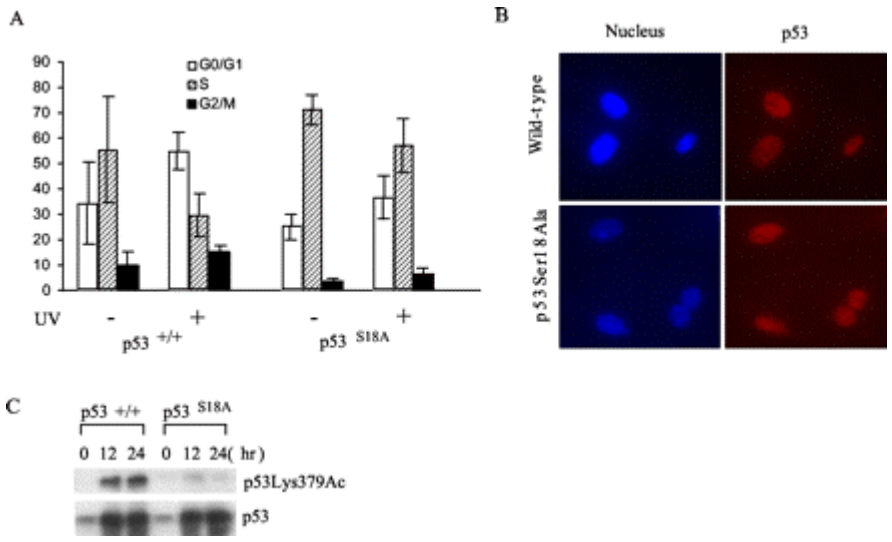


Figure 4. p53-dependent cell cycle G_1/S arrest in wild type and $p53^{S18A}$ MEFs after UV radiation. (A) Cell cycle profile of wild type and $p53^{S18A}$ MEFs before and 24 h after UV radiation. The mean values of the percentage of cells in each phase of the cell cycle from three independent experiments are shown with error bars. (B) Subcellular localization of p53 in wild type and $p53^{S18A}$ MEFs after UV radiation. The genotypes are indicated on the left. Nuclear and p53 staining indicated on the top. (C) Acetylation of p53 at Lys^{379} in wild type and $p53^{S18A}$ MEFs after UV radiation (60 J/M^2). The time points are indicated at the top. Total p53 and acetylated p53 are indicated on the right.

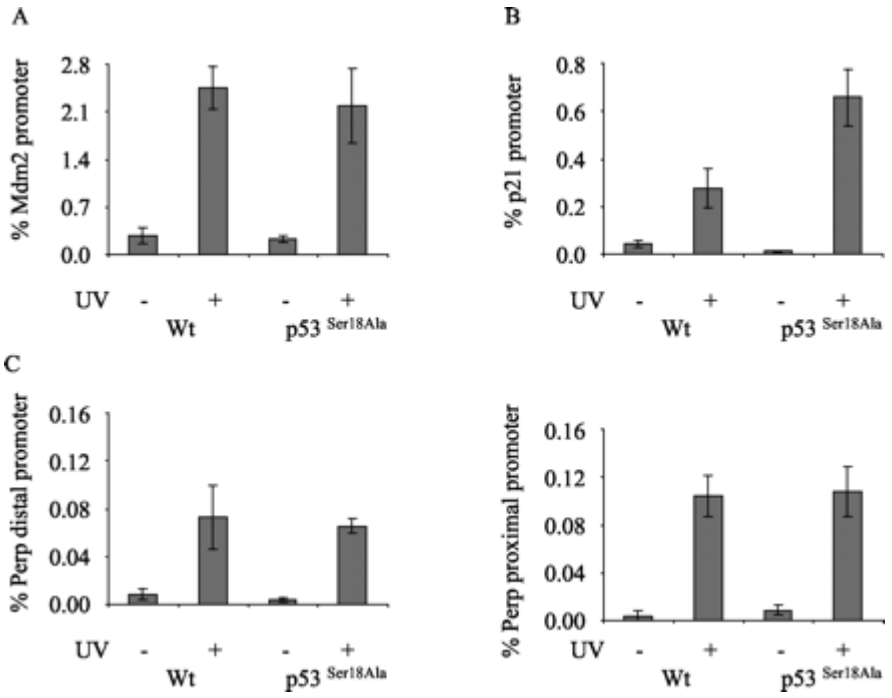


Figure 5. ChIP analysis of DNA binding of p53 to p53-dependent promoters in wild type and $p53^{S18A}$ MEFs 18 h after UV radiation (60 J/M^2). Shown are the results of quantitative PCR analysis of the percentage of promoters of p53-dependent genes Mdm2 (A), p21 (B), and Perp (C) bound by p53 in wild type (Wt) and $p53^{S18A}$ MEFs 18 h after UV radiation. The mean values from three independent experiments are presented with error bars.

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Chapter 3

Ser18 and 23 phosphorylation is required for p53-dependent apoptosis and tumor suppression

Abstract

Mouse p53 is phosphorylated at Ser18 and Ser23 after DNA damage. To determine whether these two phosphorylation events have synergistic functions in activating p53 responses, we simultaneously introduced Ser18/23 to Ala mutations into the endogenous p53 locus in mice. While partial defects in apoptosis are observed in $p53^{S18A}$ and $p53^{S23A}$ thymocytes exposed to IR, p53-dependent apoptosis is essentially abolished in $p53^{S18/23A}$ thymocytes, indicating that these two events have critical and synergistic roles in activating p53-dependent apoptosis. In addition, $p53^{S18/23A}$, but not $p53^{S18A}$, could completely rescue embryonic lethality of $Xrcc4^{-/-}$ mice that is caused by massive p53-dependent neuronal apoptosis. However, certain p53-dependent functions, including G_1/S checkpoint and cellular senescence, are partially retained in $p53^{S18/23A}$ cells. While $p53^{S18A}$ mice are not cancer prone, $p53^{S18/23A}$ mice developed a spectrum of malignancies distinct from $p53^{S23A}$ and $p53^{-/-}$ mice. Interestingly, $Xrcc4^{-/-}$ $p53^{S18/23A}$ mice fail to develop tumors like the pro-B cell lymphomas uniformly developed in $Xrcc4^{-/-}p53^{-/-}$ animals, but exhibit developmental defects typical of accelerated ageing. Therefore, Ser18 and Ser23 phosphorylation is important for p53-dependent suppression of tumorigenesis in certain physiological context.

Introduction

The importance of p53 in tumor suppression is highlited by studies demonstrating that p53 is the most commonly mutated tumor suppressor gene in human cancers (Hollstein et al., 1991). This essential role of p53 is evolutionarily conserved as mice lacking p53 uniformly develop and die of cancers (Donehower et al., 1992) (Jacks et al., 1994). While p53 is inert and relatively unstable in cells, the stability and activity of p53 are significantly induced after various cellular and genotoxic stresses, leading to cell cycle arrest, apoptosis and DNA repair (Ko and Prives, 1996). In this context, the interaction between p53 and Mdm2, Pirh2, or COPI, all E3 ubiquitin ligases and p53 transcriptional targets, leads to p53 ubiquitination and degradation (Dornan et al., 2004; Haupt et al., 1997; Kubbutat et al., 1997; Leng et al., 2003). In response to various stresses, p53 undergoes post-translational modifications, including phosphorylation and acetylation, leading to the disruption of its interaction with negative regulators and enhanced stability and activity (Dornan et al., 2004; Leng et al., 2003; Martinez et al., 1997).

Multiple phosphorylation sites at the N-terminus of p53, including Ser3, 6, 9, 15, 20, 33, 37 and Thr18 could be phosphorylated in response to DNA damage (Saito et al., 2002). Ser15 and Ser20 are highly conserved evolutionarily and correspond to Ser18 and Ser23 of mouse p53. Biochemical and cell line transfection studies suggest that these phosphorylation sites are involved in p53 stabilization and activation. The physiological roles of these phosphorylation sites have also been addressed in the knock-in mice with either Ser18 or Ser23 to Ala mutations (p53S18A and p53S23A,

respectively). These studies indicate an important role for Ser18 phosphorylation in activating p53 dependent apoptosis and cell cycle arrest after DNA damage (Borges et al., 2004; Chao et al., 2003; Sluss and Jones, 2003). However p53S18A mice are not predisposed to spontaneous tumorigenesis, indicating that p53 Ser18 phosphorylation is not required for p53 dependent tumor suppression in ageing animals (Chao et al., 2003; Sluss and Jones, 2003). Studies of p53 responses in p53S23A embryonic stem cells and mouse embryonic fibroblasts (MEFs) indicate that Ser23 phosphorylation is dispensable for p53 stabilization and activation in response to DNA damage (Wu et al., 2002). Consistent with this finding p53 responses to DNA damage in MEFs derived from germline p53S23A mice are also normal (MacPherson et al., 2004). However in this study p53 stabilization is modestly impaired in thymocytes after IR but significantly impaired in neurons of p53S23A mice after DNA damage. Therefore Ser23 phosphorylation appears to stabilize p53 in a cell type dependent manner.

Since phosphorylation at Ser15 and Ser20 occur simultaneously after various types of DNA damage, and might be mediated by ATM dependent signaling pathways, after DNA double strand breaks, the two phosphorylation events might have synergistic roles in activating p53 dependent tumor suppression. To address this issue in a physiological context, we introduced S18/23A mutations into the endogenous p53 gene in mice. Our findings indicate that the two phosphorylation events are critical to activate p53 dependent apoptosis. In addition they are important for p53 dependent suppression of spontaneous tumorigenesis, but dispensable for p53 dependent tumor suppression induced by excess DNA damage as a result of DNA repair deficiency.

Results

Generation of $p53^{S18/23A}$ germline mice.

The knock-in strategy to introduce S18/23A mutations into the p53 gene is described in Figure 1. Heterozygous mutant ES cells were used to generate chimeric mice that transmitted the $p53^{S18/23A}$ allele into the germline. The F1 mutant heterozygous mice were bred with CMV-Cre transgenic mice to excise the LoxP-flanked PGK-Neo^R gene from the targeted allele. The PGK-Neo^R gene deleted heterozygous mice were intercrossed to generate homozygous mutant mice. The entire p53 cDNA derived from homozygous mutant mouse embryonic fibroblasts (MEFs) was sequenced to verify that only the S18/23A mutations but not other mutations were present in the p53 gene of $p53^{S18/23A}$ mice. $P53^{S18/23A}$ mice were born at an expected Mendelian ratio (Figure 1F), and displayed no overt developmental abnormalities.

p53 responses to DNA damage in $p53^{S18/23A}$ MEFs

Neither p53 Ser18 nor Ser23 phosphorylation affects p53 stability in MEFs after DNA damage (Chao et al., 2003; MacPherson et al., 2004; Sluss et al., 2004; Wu et al., 2002). To test whether these two phosphorylation sites have synergistic roles in p53 stabilization in MEFs after DNA damage, protein levels of p53 in $p53^{S18/23A}$ MEFs at various time points after DNA damage were examined. Similarly to $p53^{S18A}$ MEFs (Chao et al., 2003) p53 protein levels were slightly higher in the $p53^{S18/23A}$ MEFs than in wild type (WT) control MEFs in response to DNA damage induced by

doxorubicin or UVC light (Figure 2A and 2B). To test whether Ser18 and Ser23 phosphorylation have synergistic roles in activating p53 transcription activities in MEFs, the mRNA levels of a number of p53 target genes in $p53^{S18/23A}$ MEFs after DNA damage were determined by quantitative real time PCR (Figure 2C). When compared with those in WT MEFs, the mRNA levels of p21, Noxa, Bax and K/DR5 were similarly reduced in $p53^{S18/23A}$ MEFs and $p53^{S18A}$ MEFs after doxorubicin treatment (Figure 2C) as well as after IR (data not shown). In addition, similarly to $p53^{S18A}$ MEFs (Chao et al., 2003), p53-dependent cell cycle G_1/S checkpoint is partially impaired in $p53^{S18/23A}$ MEFs after IR (Figure 2D, Chao et al., 2003). Therefore, Ser18 and Ser23 phosphorylation have no apparent functional synergy in p53 stabilization and activation in MEFs after DNA damage.

Cellular proliferation and genetic stability of $p53^{S18/23A}$ cells

To test whether $p53^{S18/23A}$ mutation affects lymphocyte proliferation, the proliferation of thymocytes derived from WT, $p53^{S18/23A}$ and $p53^{-/-}$ mice was analyzed at various time points after stimulation with PMA and Ionomycin as described (Chao et al., 2000c; Kang et al., 2005). $p53^{S18/23A}$ thymocytes proliferated similarly to WT thymocytes but more slowly than $p53^{-/-}$ thymocytes at the later time point (Figure 3C). p53 is required for the replicative senescence of MEFs. Therefore, to test whether $p53^{S18/23A}$ mutation impacts on p53-dependent cellular senescence, we examined the proliferation and senescence of WT, $p53^{S18/23A}$ and $p53^{-/-}$ MEFs following a standard 3T3 proliferation protocol. As expected, $p53^{-/-}$ MEFS completely escaped senescence

in culture (Figure 3A). $p53^{S18/23A}$ MEFs proliferated modestly faster than WT MEFs but significantly slower than $p53^{-/-}$ MEFs (Figure 3B). In addition, $p53^{S18/23A}$ MEFs reached senescence similarly to WT MEFs, but underwent spontaneous immortalization more easily than WT MEFs (Figure 3B).

Polyploidy is the type of genetic instability consistently observed in $p53^{-/-}$ cells (Bischoff et al., 1990; Bouffler et al., 1995; Fukasawa et al., 1996)Therefore, we examined the polyploidy in the WT, $p53^{S18/23A}$ and $p53^{-/-}$ MEFs before and after DNA damage. In contrast to $p53^{-/-}$ MEFs that had high levels of polyploidy especially after IR, $p53^{S18/23A}$ MEFs exhibited normal diploid DNA content both before and after IR (Figure 3C). Therefore, p53-dependent suppression of polyploidy is retained in $p53^{S18/23A}$ cells.

p53-dependent apoptosis in $p53^{S18/23A}$ thymocytes

To test whether Ser18 and Ser23 play synergistic roles in p53 apoptotic activities, p53-dependent apoptosis in WT, $p53^{S18A}$ and $p53^{S18/23A}$ thymocytes was compared as previously described (Chao et al., 2003). Consistent with previous findings, p53-dependent apoptosis was partially impaired in $p53^{S18A}$ thymocytes after IR (Chao et al., 2003)(Figure4A). In contrast, similarly to $p53^{-/-}$ thymocytes, $p53^{S18/23A}$ thymocytes were essentially resistant to p53-dependent apoptosis after IR (Figure 4A). Since p53-dependent apoptosis is only modestly reduced in $p53^{S23A}$ thymocytes after IR (MacPherson et al., 2004), these findings indicate that Ser18 and Ser23 phosphorylation play critical and synergistic roles in activating p53-dependent

apoptosis after DNA damage. Consistent with this finding, when compared with that in WT thymocytes, p53-dependent induction of target genes was much more dramatically impaired in $p53^{S18/23A}$ thymocytes than in $p53^{S18A}$ thymocytes after IR (Figure 4B). In addition, with the exception of Puma, the extent of reduction of other analyzed p53 target genes was similar between $p53^{S18/23A}$ and $p53^{-/-}$ thymocytes after IR, further underscoring the importance of Ser18 and Ser23 phosphorylation in activating p53 activities in thymocytes after IR.

To understand this functional synergy, p53 protein levels in $p53^{S18/23A}$ thymocytes after IR were compared to those in WT and $p53^{S18A}$ thymocytes. As expected, Ser18Ala mutation has no apparent impact on p53 stabilization in thymocytes after IR (Chao et al., 2003). When compared to those in WT and $p53^{S18A}$ thymocytes, p53 protein levels were modestly reduced in $p53^{S18/23A}$ thymocytes at earlier time points after IR but much more dramatically reduced at later time points after IR (Figure 4C, D). Therefore, the combination of the reduced p53 protein levels and its activities contribute to the loss of p53-dependent apoptosis in $p53^{S18/23A}$ thymocytes after IR.

$p53^{S18/23A}$ completely rescued embryonic lethality of $Xrcc4^{-/-}$ mice

Xrcc4 is a critical member of the mammalian non-homologous end joining pathway (Li et al., 1995). Xrcc4-deficiency results in extensive DNA damage, accompanied by massive neuronal apoptosis in the embryo, leading to embryonic lethality (Gao et al., 2000). Both the neuronal apoptosis and embryonic lethality

phenotypes can be completely rescued by p53 deficiency, indicating that neuronal apoptosis in Xrcc4$^{-/-}$ mice is p53-dependent (Gao et al., 2000). To test whether p53$^{S18/23A}$ mutation impairs p53 apoptotic activities in developing neurons in the presence of unrepaired DNA damage, p53$^{S18/23A}$ mutation was introduced into Xrcc4$^{-/-}$ mice. Similarly to Xrcc4$^{-/-}$p53$^{-/-}$ mice, Xrcc4$^{-/-}$p53$^{S18/23A}$ mice were born at the expected Mendelian ratio, indicating that p53$^{S18/23A}$ mutation can suppress the p53-dependent neuronal apoptosis in Xrcc4$^{-/-}$ embryos (Figure 5A). In support of this notion, the levels of apoptosis in the brain of Xrcc4$^{-/-}$p53$^{S18/23A}$ embryo were similar to those in WT embryo but much lower than those in Xrcc4$^{-/-}$p53$^{+/+}$ embryonic brain (Figure 5C). Therefore, p53$^{S18/23A}$ mutation essentially abolishes the p53-dependent apoptosis in the developing neurons in the presence of DNA damage. Consistent with the findings that Ser18A mutation only partially impairs p53 apoptotic activities (Chao et al., 2003), S18A mutation could rescue the embryonic lethality of Xrcc4$^{-/-}$ mice only at a very low frequency (Figure 5B).

p53$^{S18/23A}$ mice are cancer prone

Since p53 apoptotic activities are greatly compromised in p53$^{S18/23A}$ mice, we monitored the spontaneous tumorigenesis in p53$^{S18/23A}$ and control wild type mice. p53$^{-/-}$ animals primarily develop thymic lymphomas within 6 months of age (Donehower et al., 1992; Jacks et al., 1994). p53$^{S18/23A}$ mice were also cancer prone but developed tumors in a wide spectrum of tissues with a significantly delayed onset (Figure 6A). In this context, lymphomas of the spleen and lymph node, but not thymus

are common in $p53^{S18/23A}$ mice. In addition, tumors rarely detected in $p53^{-/-}$ mice, including leukemias, fibrosarcomas, adenomas and granuloma, had also been observed in $p53^{S18/23A}$ mice (Figure 6B, supplementary Figure 1). Therefore, Ser18 and Ser23 phosphorylation are important for p53-dependent suppression of spontaneous tumorigenesis in aging animals.

$p53^{S18/23A}$ was able to suppress tumorigenesis in $Xrcc4^{-/-}$ mice

$Xrcc4^{-/-}p53^{-/-}$ animals uniformly develop and die of B cell lymphomas by 8 weeks of age (Gao et al., 2000). The accelerated tumorigenesis is caused by widespread genetic instability since p53-deficiency allows the survival of cells with extensive DNA damage caused by DNA repair deficiency. While p53-dependent apoptosis is greatly reduced in $p53^{S18/23A}$ mice, leading to the complete rescue of embryonic lethality of Xrcc4-deficient mice, $Xrcc4^{-/-}p53^{S18/23A}$ mice were not highly prone to tumors, since only 2 mice out of a cohort of 34 animals developed thymic tumors at 93 and 128 days of age. Despite the increased longevity of $Xrcc4^{-/-}p53^{S18/23A}$ mice compared to $Xrcc4^{-/-}p53^{-/-}$ animals, the median life span of $Xrcc4^{-/-}p53^{S18/23A}$ mice was still significantly reduced compared to the $Xrcc4^{+/-}p53^{S18/23A}$ controls (Figure 5D). In this context, $Xrcc4^{-/-}p53^{S18/23A}$ mice were runted and displayed several aging-related phenotypes, including an acute spinal curvature, reduced thickness of the skin and testicular atrophy (Figure 7). In addition, $Xrcc4^{-/-}$ mice are immunodeficient due to the lack of mature lymphocytes (Gao et al., 2000).

These findings suggest that Xrcc4$^{-/-}$p53$^{S18/23A}$ mice die prematurely due to ageing associated phenotypes.

Discussion

Phosphorylation of human p53 at Ser15 and Ser20 occurs simultaneously after DNA damage (Saito et al., 2002). To address the physiological roles of these phosphorylation events, we introduced Ser18/23 (corresponding to Ser15 and Ser20 of human p53) to Ala mutations into the endogenous p53 gene in mice. p53-dependent apoptotic activities are greatly reduced in $p53^{S18/23A}$ thymocytes after IR. In addition, $p53^{S18/23A}$ mutation completely rescues the embryonic lethality of Xrcc4-deficient mice, which die of massive p53-dependent apoptosis in the embryonic neurons (Gao et al., 2000). Therefore these two phosphorylation events are critical to activate p53-dependent apoptotic activities after DNA damage. The extent of the defective p53-dependent apoptosis after DNA damage appears to be much more severe in $p53^{S18/23A}$ mice than in $p53^{S18A}$ or $p53^{S23A}$ mice, indicating that Ser18 and Ser23 play synergistic roles in activating p53-mediated apoptotic activities after DNA damage (Chao et al., 2003; MacPherson et al., 2004). In this context, Ser18 phosphorylation has been shown to be important for activating p53-dependent transcription by recruiting co-activators to p53 target promoters (Chao et al., 2003; Dumaz and Meek, 1999; Lambert et al., 1998), and Ser23 phosphorylation is important for p53 stabilization in a cell type dependent manner (MacPherson et al., 2004). However, p53 stability and activity are similar between $p53^{S18/23A}$ and $p53^{S18A}$ MEFs after DNA damage, indicating that the functional synergy between these two phosphorylation events is cell-type specific.

Despite significant impairment of p53 responses to DNA damage in $p53^{S18A}$ mice, these mice are resistant to spontaneous tumorigenesis (Chao et al., 2003). In contrast, aging $p53^{S18/23A}$ mice spontaneously develop tumors at a high frequency. While the loss of p53-dependent apoptosis in $p53^{S18/23A}$ mice is similar to that in $p53^{-/-}$ mice, the onset of tumorigenesis in $p53^{S18/23A}$ mice is much slower than that in $p53^{-/-}$ mice, likely due to the partial retention of other p53-dependent functions. In this context, p53-dependent cell cycle G_1/S checkpoint and cellular senescence are partially retained in $p53^{S18/23A}$ cells. In addition, $p53^{S18/23A}$ is sufficient to suppress polyploidy typically associated with p53-deficiency. The tumor spectrum in $p53^{S18/23A}$ mice is also different from that in $p53^{-/-}$ mice which predominantly develop thymic lymphomas (Donehower et al., 1992; Jacks et al., 1994). The spectrum of tumors detected in $p53^{S18/23A}$ is much more close to the published tumor spectrum in $p53^{+/-}$ mice (Jacks et al., 1994). While $p53^{S23A}$ mice are also cancer prone and predominantly develop B cell lymphomas (MacPherson, et al., 2004), $p53^{S18/23A}$ mice develop lymphomas as well as tumors in a number of other cell types. Therefore, the impact of Ser23Ala mutation on p53 tumor suppression activities might be more cell type-restricted than Ser18/23Ala mutations. Alternatively, the difference in tumor spectrum between the $p53^{S18/23A}$ and $p53^{S23A}$ mice could be partially due to the different genetic background of the two mutant strains of mice.

Non-homologous end joining machinery is critical to maintain genetic stability in lymphocytes (Xu, 2006). In the absence of NHEJ and a functional p53, widespread genetic instability, particularly chromosomal translocations, leads to facilitated

tumorigenesis in B lineage cells (Frank et al., 2000; Gao et al., 2000). In this context, p53 deficiency completely rescues the embryonic lethality of Xrcc4$^{-/-}$ mice, and Xrcc4$^{-/-}$p53$^{-/-}$ mice uniformly die of B cell tumors by three months of age (Gao et al., 2000). p53$^{S18/23A}$ can also completely rescue the embryonic lethality of Xrcc4$^{-/-}$ mice. Interestingly the Xrcc4$^{-/-}$p53$^{S18/23A}$ do not develop tumors, but succumb to ageing associated phenotypes. These findings indicate that despite the increased levels of DNA damage as a result of DNA repair deficiency, p53-dependent tumor suppression activity is retained in p53$^{S18/23A}$ mice. Since p53-dependent apoptosis upon damage is greatly reduced in p53$^{S18/23A}$ mice, these findings support the recent conclusion that p53-dependent apoptosis is not essential for p53-dependent tumor suppression (Liu et al., 2004). Considering that p53$^{S18/23A}$ animals are susceptible to spontaneous tumorigenesis, these findings also suggest that the type and/or level of stimuli to activate p53 in aging animals are different from those in DNA repair deficient mice. In conclusion, our studies demonstrate that Ser18 and Ser23 phosphorylation function synergistically in activating p53 apoptotic activities and are required for p53-dependent tumor suppression in certain contexts.

Materials and Methods

Generation of p53S18/23A knock-in ES cells

Ser18 and Ser23 are both encoded by exon 2 of mouse p53. A mouse genomic fragment including p53 exon 2 was isolated, and nucleotides encoding Ser18 and Ser23 were simultaneously mutated to those encoding Ala residues by site-directed mutagenesis. The PGK-neomycing resistance gene (PGK-NeoR) flanked by two LoxP sites was inserted into the intron 4. The targeting construct was linearized with XbaI and electroporated in mouse J-1 ES cells, which were derived from 129 strain of inbred mice. Homologous recombination was confirmed by Southern blotting.

Introduction of p53 mutant alleles into Xrcc4-deficient mice

Xrcc4+/- mice were bred with either $p53^{S18A}$ or $p53^{S18/23A}$ homozygous mutant mice to generate $Xrcc4^{+/-}p53^{S18/+}$ or $Xrcc4^{+/-}p53^{S18/23A/+}$ mice, which were subsequently intercrossed to generate the double mutant mice.

Western blot analysis

Whole cell lysates from 4×10^5 MEFs or 5×10^6 mouse thymocytes were separated by a 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane. The membranes were blocked in 5% nonfat milk and probed with a polyclonal antibody against p53 (CM-5; Vector Laboratories Inc) overnight at 4 C. The filter was then incubated with a horseradish peroxidase-conjugated secondary antibody, and

developed with ECL Plus (Amersham Biosciences). To ensure that the protein amounts in all lanes were similar, the membrane was stripped and probed with a goat polyclonal antibody directed against B-actin (I-19; Santa Cruz Biotechnology).

Proliferation and cell cycle assays

The cell cycle G1/S checkpoint was analyzed as previously described (Xu et al., 1998). Briefly, 1.5 x 10^6 MEFs were synchronized at G_0 by culturing in DMEM supplemented with 0.5% FCS for 4 days. Cells were either untreated or irradiated with 20 Gy IR and simultaneously released in normal growth media (10% FCS in DMEM) in the presence of 10 μM BrdU. After 24 hours, MEFs were harvested and fixed in 70% ethanol, stained for BrdU and DNA content and analyzed by flow cytometry.

Proliferation of MEFs was analyzed following a standard 3T3 proliferation protocol. Briefly 3 x 10^5 MEFs were serially passaged in 6 cm dishes and counted every three days up to 20 passages. Two plates of each genotype were counted at each passage.

Thymocyte proliferation was performed as previously described (Chao et al., 2000c). Briefly, single cell suspension of thymocytes was cultured in growth media alone (RPMI 1640 media supplemented with 10% FCS, and 100ug/ml penicillin/streptomycin) or in the presence of 5 ng/ml PMA and 500ng/ml Ionomycin at a density of 10^6 cells/ml. Cell numbers were analyzed after 2, 4, or 6 days in culture using the CellTiter 96 Cell Proliferation kit (Promega).

p53-dependent apoptosis in mouse thymocytes

p53-dependent apopotosis was analyzed as previously described (Chao et al., 2003). Single cell suspension of thymocytes was cultured in Dulbecco's modified Eagle's medium supplemented with 5% FCS an 10mM helpes (pH 7.0) at a density of 10^6 cells/ml. The cells were subsequently exposed to 0, 2.5 and 5 Gy of IR. The percentage of apoptotic cells was analyzed 24 hours following IR by staining with flourescein isothiocyanate-labeled Annexin V (Pharmingen) and flow cytometry.

Quantitative real time PCR analysis

Total RNA from MEFs or thymocytes were isolated using Trizol Reagent (invitrogen) followed by Rneasy RNA cleanup (Qiagen) and on-column Dnase digestion (Qiagen). One microgram of RNA was reverse transcribed using First Strand Superscript Synthesis System (Invitrogen). Real time PCR was performed on an ABI Prism 7000 Sequence Detection System with SYBR Green PCR Master Mix (Applied Biosystems). The following PCR conditions were used : 10 min hot start at 95 C, followed by 40 cycle of 30 sec at 95 C and 1 min at 60 C. Samples were run in triplicate and the mean threshold cycle (Ct) for each target gene analyzed was normalized to the Ct for the housekeeping gene Gapdh. Primer sequences for p21, Noxa, K/DR5, Bas, Puma and Gapdh were described previously.

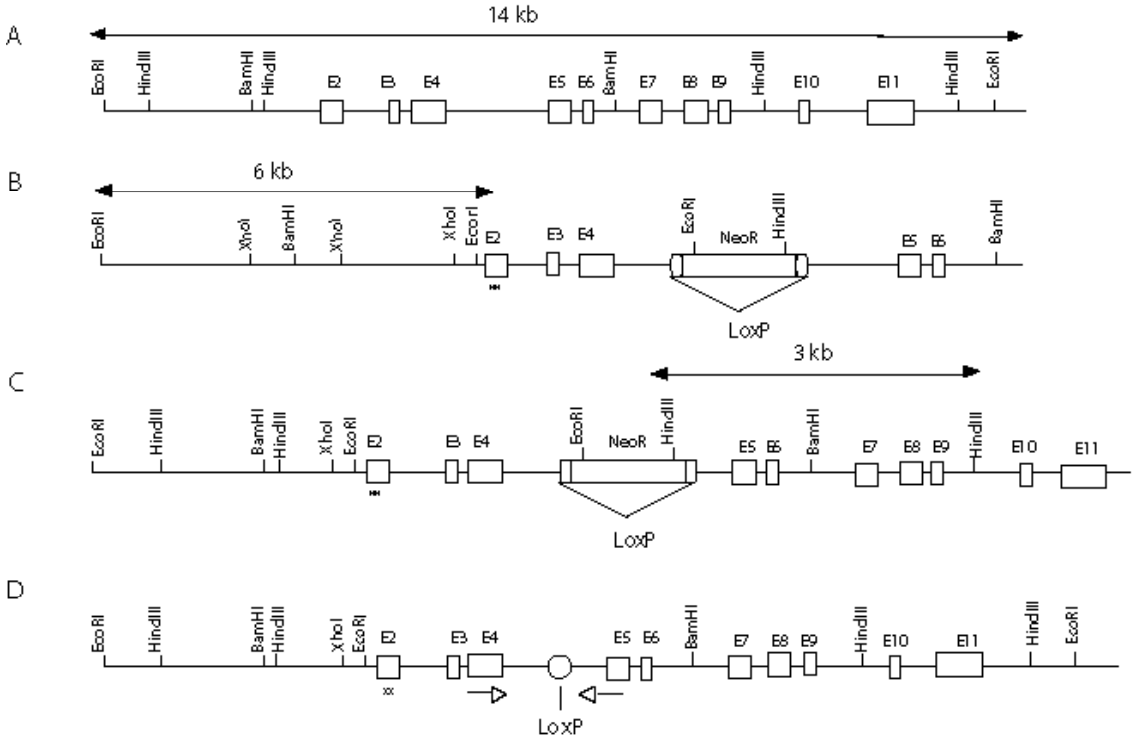
Histology Analysis

Tissues or tumor samples were harvested from animals upon death and fixed in 10% buffered formalin, embedded in paraffin and sliced in 6uM sections. All sections were stained with hematoxylin and eosin for histological assessment. X-ray images of euthanized animals were taken using a Faxitron X-ray system.

Detection of Neuronal apoptosis

In situ end-labeling plus (ISEL+) was performed essentially as described previously (Anne J. Blaschke, 1998; Blaschke et al., 1996) Briefly, 20 μm-thick sections were obtained from freshly-frozen embryos and collected on Superfrost plus slides (Fisher), fixed in 4% paraformaldehyde, acetylated, dehydrated through an ethanol series, and either used fresh or stored at -80^{o}C. DNA was end-labeled with digoxygenin-11-dUTP (Baker et al.) by incubation with terminal deoxynucleotidyl transferase (Invitrogen) for 1 hour at 37^{o}C. dUTP incorporation was detected by binding with an alkaline phosphatase-conjugated sheep anti-digoxygenin antibody (1:2,000)(Baker et al.) and visualized by reacting with 5-bromo-4-chloro-3-indoxyl phosphate/tetranitroblue tetrazolium (Chemicon). Images were captured under direct illumination using a Zeiss Axio Imager.

Figure 1. Generation of $p53^{S18/23A}$ knock-in mice. (A) The endogenous mouse p53 locus. (B) The targeting construct. The PGK-Neo^R cassette flanked by two loxP sites was inserted into intron 4. S18/23A mutations are indicated by asterisks. (C) The targeted p53 locus following homologous recombination between the endogenous p53 allele and targeting vector. (D) Knock-in p53 allele following LoxP/Cre-mediated deletion of the PGK-Neo^R gene. (E) PCR analysis of the genomic DNA isolated from the tails of wild type (lane 1), $p53^{S18/23A/+}$ (lane 2) and $p53^{S18/23A}$ mice (lane 3). The PCR products depicting the wild type and knock-in mutant allele are indicated to the right. The primers are indicated by arrowheads. (F) The number of offsprings in various genotypes derived from the breeding of $p53^{S18/23A/+}$ mice. The expected number based on the Mendelian ratio is also shown.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E3
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HindIII
E10
E11
HindIII
EcoRI
B
6 kb
EcoRI
XhoI
BamHI
XhoI
XhoI
EcoRI
E2
E3
E4
EcoRI
NeoR
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E10
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EcoRI
E2
E3
E4
E5
E6
BamHI
E7
E8
E9
HindIII
E10
E11
HindIII
EcoRI
LoxP
E
1
2
3
mutant allele
wild type allele
F
Genotype
Observe (Expect)
p53+/+
39 (40)
p53S18/23A/+
81 (80)
p53S18/23A
40 (40)

Figure 1

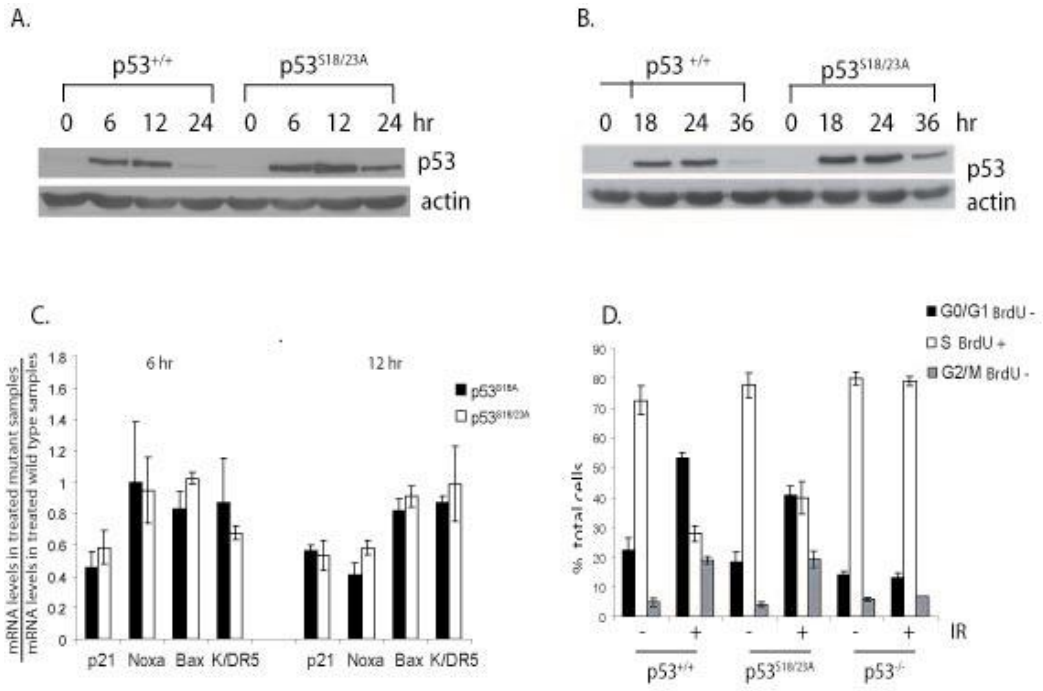
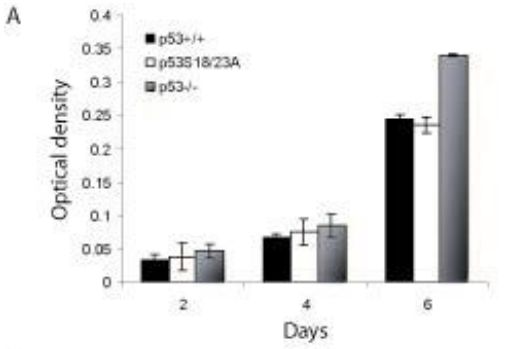
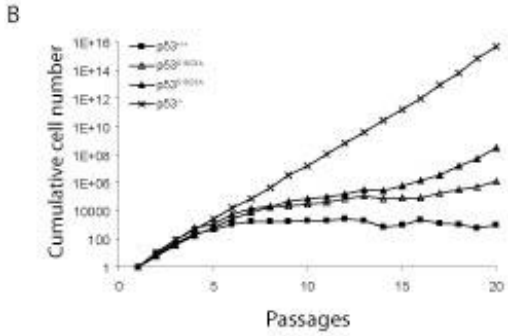
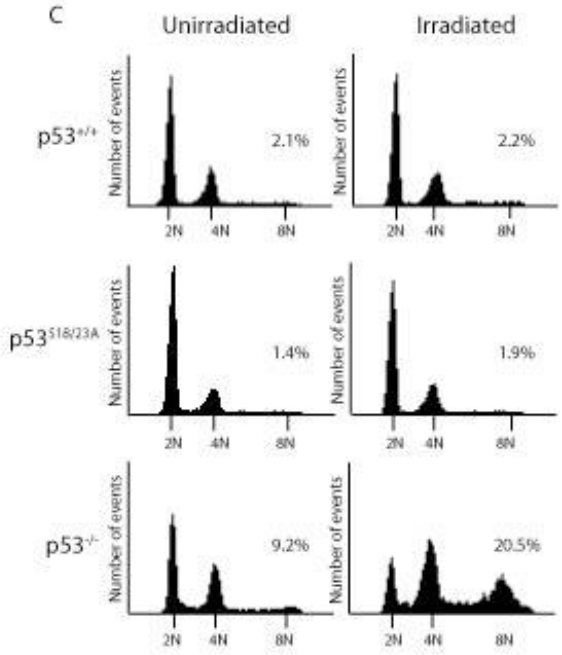


Figure 2. p53 stabilization and activity in $p53^{S18/23A}$ MEFs after DNA damage. Protein levels of p53 in the wild type and $p53^{S18/23A}$ MEFs at various time points after exposure to 0.25μM doxorubicin (A) or 60 J/M^2 UVC (B). Time points and genotypes are indicated on the top. p53 and actin are indicated on the right. (C) Real time PCR analysis of the p53-dependent transcription of p21, Noxa, Bax and K/DR5 at 6 and 12 hrs after doxorubicin treatment. The ratio of the mRNA levels in the untreated $p53^{S18A}$ or $p53^{S18/23A}$ MEFs versus those in treated wild type MEFs is shown. Mean values from three independent experiments are shown with standard deviation.
(D) Cell cycle G_1/S arrest in wild type, $p53^{S18/23A}$ and $p53^{-/-}$ MEFs after 20 Gy of IR.

Figure 3. Cellular proliferation and polyploidy of $p53^{S18/23A}$ cells. (A) Proliferation of thymocytes derived from wild type, $p53^{S18/23A}$, and $p53^{-/-}$ mice. Thymocytes were activated with 5ng/ml PMA, 500ng/ml ionomycin, and proliferation was measured after 2, 4,or 6 days of stimulation. Mean values from triplicate wells are shown with error bars. (B) 3T3 proliferation assay of wild type, $p53^{S18/23A}$ and $p53^{-/-}$ MEFs. MEFs were serially passaged with a plating density of 3×10^5 cells per 6cm plate and counted once every three days up to 20 passages. Cumulative cell numbers, based on the average of duplicate plates for each passage, are shown. Data from two independent $p53^{S18/23A}$ MEFs are presented. (C) Polyploidy in wild type, $p53S^{18/23A}$ and $p53^{-/-}$ MEFs before (left panels) and 24 hours after IR (right panels). Genotypes are indicated to the left. Histograms show DNA content on X-axis versus cell number on Y-axis. The peaks representing 2N, 4N, and 8N cells are indicated. The percentage of total cells with over 8N DNA content is shown.


A
Optical density
p53+/+
p53S18/23A
p53-/-
Days


B
Cumulative cell number
Passages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Unirradiated
Irradiated
p53+/+
p53S18/23A
p53-/-
Number of events
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20.5%
2N
4N
8N

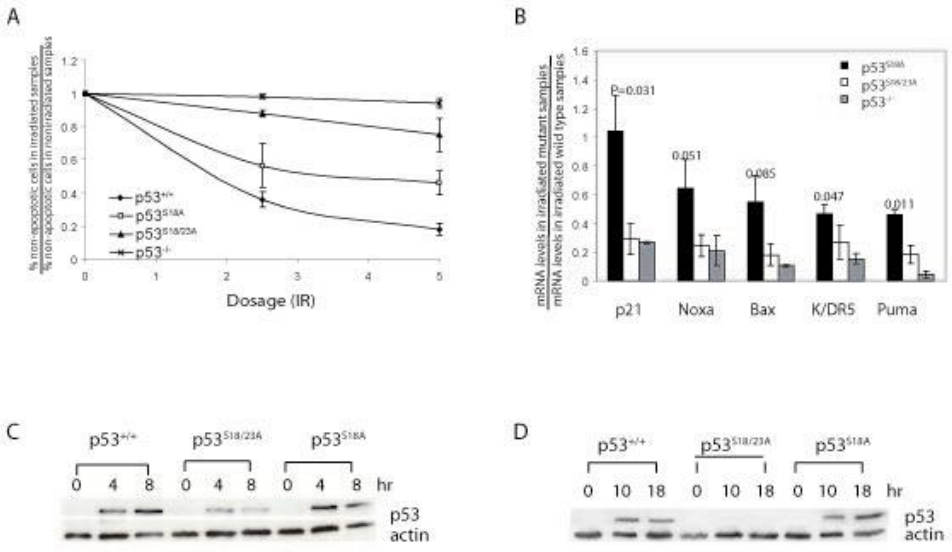


Figure 4. p53 stabilization and activity in $p53^{S18/23A}$ thymocytes after IR. (A) p53-dependent apotosis of wild type, $p53^{-/-}$, $p53^{S18A}$ and $p53^{S18/23A}$ thymocytes 24 hrs after exposure to 2.5 Gy and 5 Gy IR. Mean values from three independent experiments are shown with standard deviation. (B) p53-dependent transcription of its target genes in $p53^{S18A}$, $p53^{S18/23A}$, and $p53^{-/-}$ thymocytes as compared to that in wild type thymocytes 18 hrs after 5 Gy IR. Mean values from two independent experiments are shown with error bars. P values between the levels of reduction in $p53^{S18/23A}$ thymocytes and $p53^{S18A}$ thymocytes are given. Protein levels of p53 in wild type, $p53^{S18/23A}$ and $p53^{S18A}$ thymocytes at earlier (C) or later time points (D) after 5 Gy IR. Genotypes and time points are indicated on the top. p53 and actin are indicated on the right.

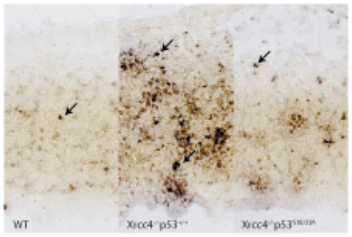
A

Genotype:	Xrcc4 $^{+/-}$ p53$^{+/+}$	Xrcc4 $^{+/-}$ p53$^{S18/23A/+}$	Xrcc4 $^{+/-}$ p53$^{S18/23A}$	Xrcc4 $^{-/-}$ p53$^{+/+}$	Xrcc4 $^{-/-}$ p53$^{S18/23A/+}$	Xrcc4 $^{-/-}$ p53$^{S18/23A}$
Observed	38	119	57	0	8	29
Expected	18	115	61	18	48	30

B

Genotype:	Xrcc4 $^{+/-}$ p53$^{+/+}$	Xrcc4 $^{+/-}$ p53$^{S18A/+}$	Xrcc4 $^{+/-}$ p53^{S18A}	Xrcc4 $^{-/-}$ p53$^{+/+}$	Xrcc4 $^{-/-}$ p53$^{S18A/+}$	Xrcc4 $^{-/-}$ p53^{S18A}
Observed	8	34	21	0	0	2
Expected	4	34	22	4	15	11

C



D

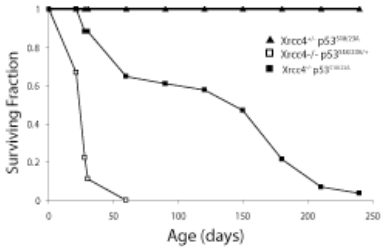
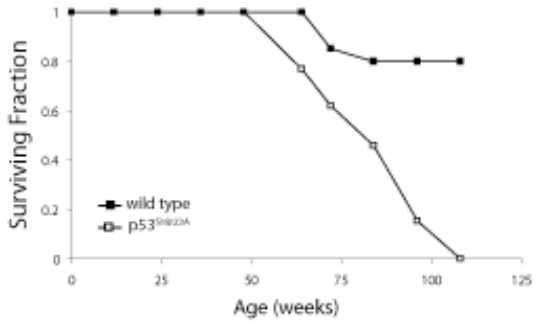


Figure 5. p53$^{S18/23A}$ mutation completely rescues the embryonic lethality of Xrcc4$^{-/-}$ mice (A) The number of offspring in various genotypes derived from the following breeding: Xrcc4$^{+/-}$p53$^{S18/23A/+}$ x Xrcc4$^{+/-}$p53$^{S18/23A/+}$ or Xrcc4$^{+/-}$p53$^{S18/23A}$ x Xrcc4$^{+/-}$p53$^{S18/23A}$. The expected number based on the Mendelian ratio is also shown. (B) The number of offsprings in various genotypes derived from either Xrcc4$^{+/-}$p53$^{S18A/+}$ x Xrcc4$^{+/-}$p53$^{S18A/+}$ or Xrcc4$^{+/-}$p53^{S18A} x Xrcc4$^{+/-}$p53^{S18A} breeding. (C) p53-dependent apoptosis in the embryonic cerebral cortex as shown by in situ end-labeling. Saggital sections from E12.5 embryos were shown. The apoptotic cells are indicated by arrowheads. (D) The surviving percentage of Xrcc4$^{+/-}$p53$^{S18/23A}$ (n=16), Xrcc4$^{-/-}$p53$^{S18/23A/+}$ (n=10) and Xrcc4$^{-/-}$p53$^{S18/23A}$ (n=34) mice at various times after birth. N represents the number of mice monitored. P value is 0.0004 for the comparison between Xrcc4$^{-/-}$p53$^{S18/23A/+}$ and Xrcc4$^{+/-}$p53$^{S18/23A}$ mice and 0.001 for the comparison between Xrcc4$^{-/-}$p53$^{S18/23A}$ and Xrcc4$^{+/-}$p53$^{S18/23A}$ mice.

A



B

Tumor Type	Number of cases
Lymphoma:	
spleen	5
thymus	1
lymph node	3
Leukemia	2
Fibrosarcoma	2
Adenoma	3
Granuloma	1
Other malignancies:	
kidney	1
small intestines	1
ovaries	1

Figure 6. Aging $p53^{S18/23A}$ mice are prone to spontaneous cancer. (A) Survival curve of 20 pairs of wild type and $p53^{S18/23A}$ mice. P value is 0.04 for the comparison of the survival rate of $p53^{S18/23A}$ mice and wild type controls. Animals were monitored for spontaneous tumorigenesis over the course of two years. (B) The spectrum of tumors developed in $p53^{S18/23A}$ mice.

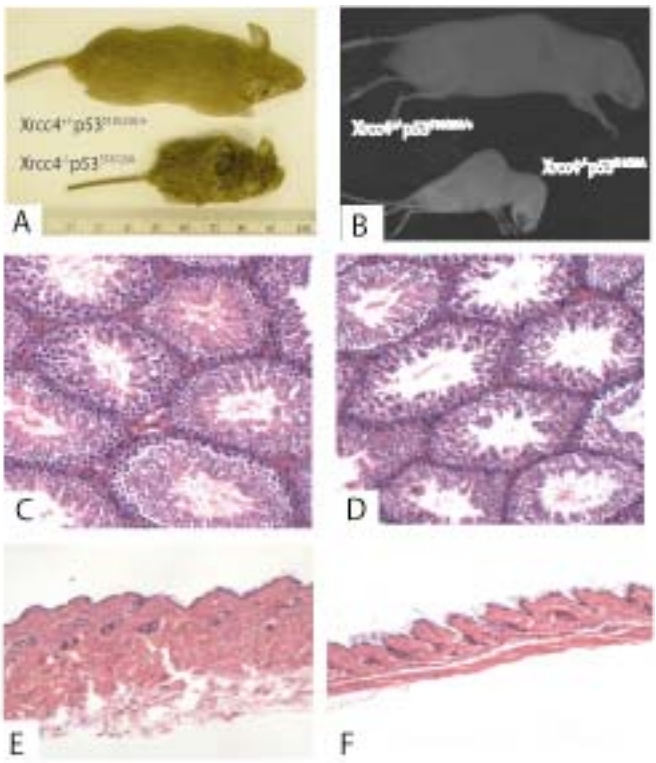
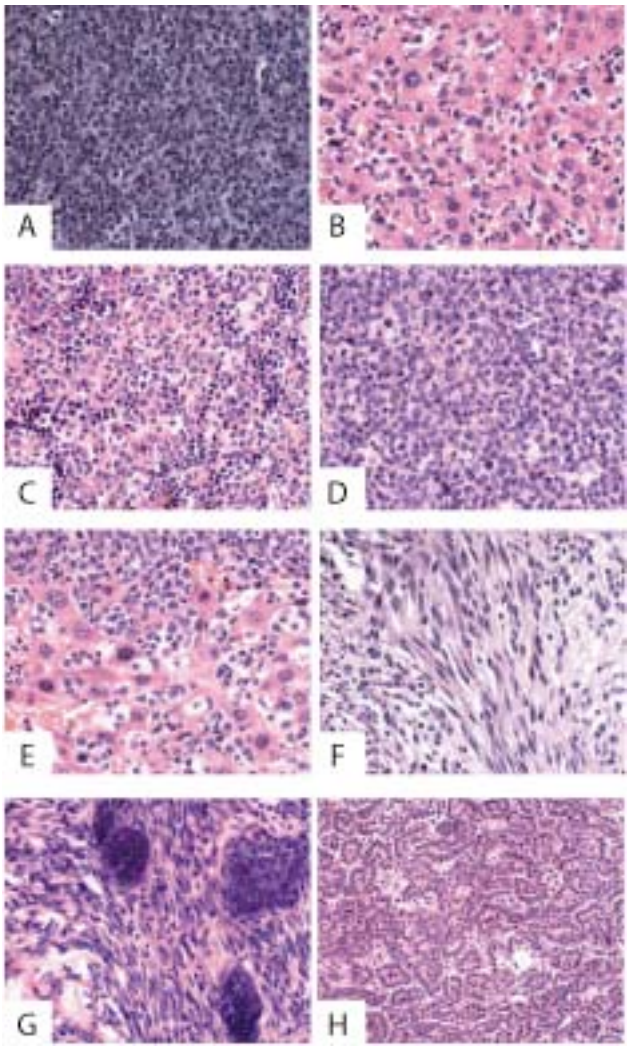


Figure 7. Xrcc4$^{-/-}$p53$^{S18/23A}$ mice show accelerated aging phenotype. (A) Picture of eight-week-old male Xrcc4$^{+/-}$p53$^{S18/23A/+}$ and Xrcc4$^{-/-}$p53$^{S18/23A}$ littermates. Histological analysis of testes (C and D) and skin (E and F) of the eight-week-old Xrcc4$^{+/-}$p53$^{S18/23A/+}$ (C, E) and Xrcc4$^{-/-}$p53$^{S18/23A}$ (D, F) littermates. (B) X-ray image showing the spine curvature of seventeen-week-old Xrcc4$^{+/-}$p53$^{S18/23A/+}$ and Xrcc4$^{-/-}$ p53$^{S18/23A}$littermate.



Supplementary Figure 1. Histological analysis of tumors observed in p53S18/23A animals, including Lymph node lymphoma (A), Leukemia infiltrated into the liver (B), Leukemia infiltrated into spleen (C), Splenic lymphoma and the infiltration into the liver (D and E), fibrosarcomas (F andG), and Bronchial adenoma (H).
All magnifications are at 400X except (H), which is at 200X.

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Chapter 4

Conclusions

p53 knock-in mice are an invaluable resource for elucidating the pathways that regulate p53 in vivo. In light of findings that the p53 "hot spot" mutations most frequently observed in human cancers target the sequence-specific DNA binding activity of p53, it is postulated that the tumor suppressive role of p53 is due to its potent transcriptional properties. Therefore, an understanding of how p53 becomes stabilized and activated in response to cellular and genotoxic stresses would be helpful in regarding therapeutic approaches for fighting cancer.

As mentioned previously, the N terminus of p53 is heavily phosphorylated in response to DNA damage. Our studies were focused on elucidating the physiological role of Ser18 phosphorylation, as well as the impact of Ser18/Ser23 phosphorylation, which occurs simultaneously after DNA damage. To do so we generated germline $p53^{S18A}$ mice and germline $p53^{S18/23A}$ mice, to help us better understand how the N-terminal phosphorylation events contribute to the regulation of p53 DNA damage responses. Importantly, the knock-in mice allowed us to elucidate whether or not Ser18 and Ser23 are required for p53 dependent tumor suppression.

The normal accumulation of p53 in $p53^{S18A}$ MEFs and thymocytes definitively demonstrate that Ser18 phosphorylation is not required to disrupt the p53-Mdm2 interaction that inhibits p53 stabilization following exposure to DNA damage. However, p53 stabilization was significantly reduced in $p53^{S18/23A}$ thymocytes in comparison to wild type , indicating that different pathways regulate p53 stability in the two cell types in response to DNA damage. Moreover, $p53^{S23A}$ thymocytes, and neurons, were also reported to be impaired in p53 stability, suggesting that Ser23

phosphorylation plays a role in disrupting the p53-Mdm2 interaction in certain cell types. In this regard, it is unclear whether the simultaneous loss of Ser18 and Ser23 had a synergistic effect on p53 stabilization. Thus quantitative analysis of the p53 protein levels in $p53^{S18A}$ $p53^{S23A}$, and $p53^{S18/23A}$ thymocytes after IR would be informative to determine specifically which sites are required for regulating p53 stability.

Ser18 phosphorylation is important for activating p53 transcriptional activity. However a caveat to these findings is that Ser18 phosphorylation is required for regulating p53 dependent transactivation from a subset of target promoters in certain cell types. In this context p53 dependent gene expression of p21, Mdm2, and Noxa, for example, were partially reduced in $p53^{S18A}$ MEFs compared to $p53^{+/+}$ MEFs following IR. However, in IR treated $p53^{S18A}$ thymocytes, p21 induction was significantly impaired, Mdm2 expression was elevated, and Noxa expression was similar to the levels of expression in irradiated $p53^{+/+}$ thymocytes. Since $p53^{+/+}$ and $p53^{S18A}$ bound to endogenous target promoters with similar efficiency, the defective transactivation in $p53^{S18A}$ cells may be attributed to impaired recruitment of transcriptional coactivators. Although this was not formally tested through co-immunoprecipitation studies, the reduced level of p53 acetylation in $p53^{S18A}$ MEFs after UV is consistent with this notion. In addition, the levels of histone acetylation at the p21 and Mdm2 promoters in $p53^{S18A}$ thymocytes reflected the differences in their expression levels (Figure 2, Chapter II). However it would be informative to see which transcriptional coactivators are recruited to various promoters in response to

Ser18 phosphorylation through chromatin immunoprecipitation experiments. Additionally the impact of Ser18 in regulating p53-dependent gene repression was not thoroughly investigated, given the possibility that Ser18 phosphorylation may also be important for the recruitment of corepressors like mSin3A to its target promoters.

Despite the defective p53 responses in $p53^{S18A}$ and $p53^{S18/23A}$ cells, only the $p53^{S18/23A}$ mice were prone to spontaneous tumorigenesis. Compared to $p53^{-/-}$ animals, $p53^{S18/23A}$ mice developed tumors later in life, and the frequency of thymic lymphomas was low. $p53^{S23A}$ mice were reported to develop mostly B cell lymphomas, however $p53^{S18/23A}$ mice developed a broader range of malignancies. The differences in tumor spectra may be influenced by the differences in the mouse genetic backgrounds. However the loss of Ser18/Ser23 phosphorylation could have a broader impact on p53 activity in various cell types, and thus facilitate tumorigenesis. In addition recent studies demonstrate that mice expressing a hot-spot mutant of p53 develop a wider range of tumors with a high rate of metastasis, in comparison to the p53-deficient mice, which mostly develop lymphomas (Lang et al., 2004; Olive et al., 2004). It is not uncertain whether $p53^{S18/23A}$ possesses any intrinsic gain of function activity, given that transcriptional activation is essentially lost, as shown in the $p53^{S18/23A}$ thymcotes (Figure 4, Chapter III). However our studies were limited to a small subset of p53-dependent genes. Detailed analysis of p53 activity, including micro-array analysis, from other tissues derived from the $p53^{S18/23A}$ and $p53^{S23A}$ and $p53^{S18A}$ animals may provide clues into how the differences in the level of p53 transcriptional activity may affect spontaneous tumorigenesis as well as tumor spectrum.

Despite the requirement of Ser18/Ser23 phosphorylation to suppress spontaneous tumorigenesis in aging mice, they are dispensable for tumor suppression after the onset of persistent genetic instability due to Xrcc4 inactivation. This was surprising given that $p53^{S18/23A}$ completely rescued $Xrcc4^{-/-}$ embryonic lethality. Whereas the majority of $Xrcc4^{-/-}p53^{-/-}$ mice eventually succumb to B-cell lymphomas within 3 months, $Xrcc4^{-/-}p53^{S18/23A}$ mice were highly resistant to tumors. However, $Xrcc4^{-/-}p53^{S18/23A}$ mice manifested signs of premature aging and died within 8 months of age. In light of recent findings that tumor latency is significantly delayed in mice expressing the hot spot mutant p53R172P, that is completely impaired in apoptotic function, it is possible that tumors may have arisen in $Xrcc4^{-/-}p53^{S18/23A}$ mice if they too survived longer.

It has been postulated that increased genetic instability generated by DNA repair deficiency, as well those brought about by telomere erosion and mitotic checkpoint defects, may lead to accelerated ageing because of persistent activation of p53, which can induce cellular senescence (Baker et al., 2004; Dumble et al., 2004; Lim et al., 2000; Tyner et al., 2002). Our findings are consistent with this notion, given that p53-dependent tumor suppression is intact in the $Xrcc4^{-/-}p53^{S18/23A}$ mice. Introducing additional stresses into the $Xrcc4^{-/-}p53^{S18/23A}$ background, such as hypoxia or activated oncogene may reveal the mechanism for p53- dependent tumor suppression in these mice, as well as provide insight into how p53 activation factors in the organismal ageing processes.

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