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DNA Damage-inducible Phosphorylation of p53 at Ser20 is Required for p53 Stabilization

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The p53 tumor suppressor gene is among the most frequently mutated and studied genes in human cancer, but the mechanisms by which it suppresses tumor formation remain unclear. DNA damage regulates both the protein levels of p53 and its affinity for specific DNA sequences. Stabilization of p53 in response to DNA damage is caused by its dissociation from Mdm2, a downstream target gene of p53 and a protein that targets p53 for degradation in the proteosome. Recent studies have suggested that phosphorylation of human p53 at Ser20 is important for stabilizing p53 in response to DNA damage through disruption of the interaction between Mdm2 and p53. We generated mice with an allele encoding changes at Ser20, known to be essential for p53 accumulation following DNA damage, to enable analyses of p53 stabilization *in vivo*. Our data showed that the mutant p53 was clearly defective for full stabilization of p53 in response to DNA damage. We concluded that Ser20 phosphorylayion is critical for modulating the negative regulation of p53 by Mdm2, probably through phosphorylation-dependent inhibition of p53-Mdm2 interaction in the physiological context.

The p53 tumor suppressor gene is one of the most commonly altered genes in human malignancy (Hollstein et al., 1996). The tumor suppresive function of p53 can be attributed in part to its participation in the cellular response to DNA damage. Functional analysis of the p53 protein has shown that it is a transcription factor with sequence-specific DNA binding activity (Farmer et al., 1992; Kern et al., 1992; Zambetti et al., 1992). When DNA damage occurs, p53 proteins are accumulated through a post-transcriptional mechanism and then they activate the transcription of several downstream target genes, including *p21*, *Bax*, *Mdm2*, and *PERP*, etc. (Levine, 1997; Attardi et al., 2000; Ljungman, 2000).

p53 activity is tightly controlled through a complex series of events including [1] interaction with regulatory proteins such as Mdm2 and CBP/p300, [2] a series of post-translational modifications such as multi-site phosphorylation and acetylation, and [3] translational regulation (Giaccia and Kastan, 1998; Meek, 1999; Prives and Hall, 1999). The increased p53 protein level, following DNA damage, is regulated post-transcriptionally, due primarily to increased protein stability (Ko and Prives, 1996). Diverse signals that activate p53 converge at a single critical interaction, between p53 and its negative regulator Mdm2. Through complex formation with the N-terminus of p53 (amino acids 19-26), Mdm2 represses p53 transcriptional activity (Oliner et al., 1993) and mediates the degradation of p53 through the ubiquitin pathway (Haupt et al., 1997; Kubbutat et al., 1997). Interfering with the interaction between p53 and Mdm2 leads to the accumulation of p53 protein and the subsequent initiation of p53-dependent processes such as transactivation, cell cycle arrest, or apoptosis (Bottger et al., 1997).

Recent studies have proposed that a number of protein kinases phosphorylate p53 *in vitro* or *in vivo*, in which DNA-PK (Ser15 and Ser37; Lees-Miller et al., 1992), ATM (Ser15; Banin et al., 1998; Canman et al., 1998), ATR (Ser15 and Ser37; Tibbetts et al., 1999), Chk2 (Ser20; Hirao et al., 2000), CDK7-cyclin H-p36MAT1 complex (Ser33; Ko et al., 1997), CKI (Ser6 and Ser9; Milne et al., 1992), and JNK (Ser33; Milne et al., 1995) are included. DNA damage-induced phosphorylation of p53 at Ser15 attenuates the p53-Mdm2 interaction (Shieh et al., 1997; Unger et al., 1999). However, there is controvercy over the function and consequences of Ser15 phosphorylation. Other laboratories have published evidence suggesting that Ser15 phosphorylation of p53 is not essential for DNA damage-induced p53 stabilization (Dumaz and Meek, 1999; Chehab et al., 1999). A further possibility is that Ser15 phosphorylation of p53 contribute to p53 activation without necessarily inducing an increase at the level of the protein (Dumaz and Meek, 1999).

Recently, it has been suggested that phosphorylation of human p53 at Ser20 is important for stabilizing p53

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after DNA damage (Chehab et al., 1999; Shieh et al., 1999; Unger et al., 1999). Ser20 lies directly within the region of the p53 transactivation domain that interacts with Mdm2 (Kussie et al., 1996; Uesugi and Verdine, 1999), and this interaction is required for Mdm2-mediated degradation of p53. Since Mdm2-mediated ubiquitination represents a major pathway for rapid p53 degradation, the disruption of p53-Mdm2 interaction through phosphorylation of Ser20 could be important for stabilizing p53. The Chk1 and Chk2 kinases, which are activated by ATM after exposure to ionizing radiation, phosphorylate human p53 at Ser20 *in vitro* (Chehab et al., 2000; Shieh et al., 2000). Therefore, phosphorylation of p53 at Ser20 by Chk1/2 kinases might represent another ATM-dependent pathway that stabilizes p53. Consistent with this notion, Chk2-null mouse cells are defective in p53 stabilization and activation after ionizing radiation (Hirao et al., 2000). However, the role of the Ser20 phosphorylation *in vivo* is still unclear. Similar *ex vivo* experiments have shown that multiple phosphorylation sites, including Ser15 and Ser20, can be mutated to Ala without dramatic effect on the ability of p53 to be stabilized (Ashcroft et al., 1999; Blattner et al., 1999). Interpretation of the data from experiments using ectopically expressed p53 might be confounded by possible non-physiological regulation of p53 in this context.

To investigate the physiological role(s) of p53 phosphorylation at Ser20 in p53 responses to DNA damage, we point-mutated Ser20 to Ala, targeted it into the endogenous p53 locus, generated mouse embryonic fibroblasts (MEFs), and then investigated p53 response to DNA damage. Interestingly, the p53 Ser20 to Ala20 mutation had a clear effect on the stability of p53 following DNA damage in mutant MEFs. Therefore, we concluded that the Ser20 phosphorylation on p53 is important for p53 stabilization after DNA damage.

Materials and Methods

Materials and general methods

Restriction endonucleases, calf intestinal alkaline phosphatase, the Klenow fragment of DNA polymerase I, and T4 DNA ligase were purchased from New England Biolabs. Preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis, DNA ligation, and bacterial transformations were carried out using standard methods (Sambrook et al., 1989).

Targeting construction

The p53 S20A targeting vector was constructed by cloning fragments of the murine p53 gene isolated from BALB/c strain and 129sv into pBSKII vector (Stratagene). Fragments of mouse genomic p53 sequence extending from intron 1 through exon 6 were cloned into the pBSKII vector. A single base pair mutation leading to an Ala substitution from Ser20 was introduced by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). A puromycin resistance gene driven by the PGK promoter and flanked by *loxP* sites was introduced into an *Xho*I site of intron 1. The presence of the correct mutation was verified by DNA sequencing. The complete insert of the targeting vector was sequenced to exclude the presence of additional unexpected mutations in the p53 encoding sequences and exon/intron boundaries.

Generation of mutant ES cells

The targeting vector was linearized by digestion with *Not*I and electroporated into J1 ES cells derived from strain 129/sv by using standard procedures (Jacks et al., 1994). Puromycin-resistant ES clones were analyzed for homologous recombination of the targeting vector by Southern blot analysis. The probe was used with *Bsm*I digest of a fragment of genomic DNA from intron 1. GFP-Cre was transiently transfected into ES cells to excise the puro cassette, leaving a single *loxP* site in intron 1. The excision of puro cassette, was confirmed by Southern blot analysis. Clones with homologous integration of the targeting vector were checked for the presence of the mutation by a PCR/DNA sequencing-based method.

Generation of mutant mice

C57BL/6 blastocyst-stage embryos were injected with 10-15 p53 S20A ES cells and subsequently transferred to pseudopregnant CD1 females essentially as described (Jacks et al., 1994). Chimeric mice were mated to C57BL/6 animals and F1 agouti offsprings were genotyped. Germline transmission of the mutant allele was detected by either Southern blot analysis or PCR analysis of tail DNA obtained at weaning. PCR genotyping was based on the presence of a single *loxP* site remaining in intron 1 of the correctly targeted locus. The primers 1 (5′ AGCCTGCCTAGCTTCCTCAGG 3′) and 2 (5′ CTTGGAGACATAGCCACACTG 3′) were used for PCR amplification of p53 gene. These primers amplify a 540 bp mutant band in the presence of a single *loxP* site, and a 420 bp as a wild-type band.

Mouse embryonic fibroblast culture

Primary MEFs were isolated from E13.5 embryos and maintained in DMEM supplemented with 10% heat -inactivated fetal calf serum (Gibco-BRL), penicillin, and streptomycin in a humidified atmosphere of 5% CO_2 at 37℃ as described (Attardi et al., 2000).

Western blot analysis

Mouse embryonic fibroblast lysates were prepared by extraction in lysis buffer (50 mM Tris.HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM PMSF, and 1 μg/mL leupeptin). Samples corresponding to 20 μg of

protein were separated on a SDS-polyacrylamide gel and transferred to Immobilon-P membranes (Millipore). p53 protein was detected using the rabbit polyclonal anti-p53 antibody CM5 (Novocastra Immunohistochemistry).

Results and Discussion

Multiple phosphorylation sites in the N-terminal domain of p53

DNA damage induces multiple p53 post-translational modifications, including phosphorylation of serines 6, 9, 15, 20, 33, 37, and 392, phosphorylation of threonine 18, dephosphorylation of seine 376, and acetylation of lysines 320, 373, and 382 (Knippschild et al., 1997; Ko et al., 1997; Shieh et al., 1997; Siliciano et al., 1997; Blaydes and Hupp, 1998; Kapoor and Lozano, 1998; Lu et al., 1998; Sakaguchi et al., 1998; Waterman et al., 1998; Liu et al., 1999; Shieh et al., 1999). The representative phosphorylation sites on the N-terminal domain of p53 are schematically presented in Fig. 1.

Although the Mdm2-dependent p53 degradation event after DNA damage is inhibited by dissociation of p53 from Mdm2 (Shieh et al., 1997), the critical modification sites has not been revealed. It has been shown that ATM and ATR directly phosphorylate p53 on Ser15 in response to DNA damage (Banin et al., 1998; Canman et al., 1998; Tibbetts et al., 1999). The replacement of Ser15 with Ala compromises the apoptotic activity of p53 (Unger et al., 1999), without its stabilization after DNA damage (Ashcroft et al., 1999; Blattner et al., 1999). Thus, this result indicates that other phosphorylation event(s) must be involved in stabilizing p53. According to a recent report, Chk2 directly phosphorylates p53 on Ser20 in response to DNA damage (Hirao et al., 2000). Upon transient transfection of p53 phosphorylation site mutants into various human cell lines, mutation of Ser20 to Ala completely prevented induction of p53 in response to γ- or UV-irradiation (Chehab et al., 1999; Unger et al., 1999).

Although p53 phosphorylation has been proposed to be critical for p53 stabilization following DNA damage, there has been conflicting information as to which post-translational modifications weaken the interaction of p53 with Mdm2. Similar experiments have performed that multiple phosphorylation sites, including Ser15 and Ser20, can be mutated to Ala without dramatic effect on the ability of p53 to be stabilized (Ashcroft et al., 1999; Blattner et al., 1999). We guess that this discrepancy comes from the interpretation of the data from experiments using ectopically expressed p53. The p53 signal transduction cascade is highly sensitive to the level of interacting proteins, yet most analyses of p53 function have been carried out under conditions in which its abundance exceeds that present in normal cells. Additionally, many studies have used transformed cell lines either known or likely to contain genomic

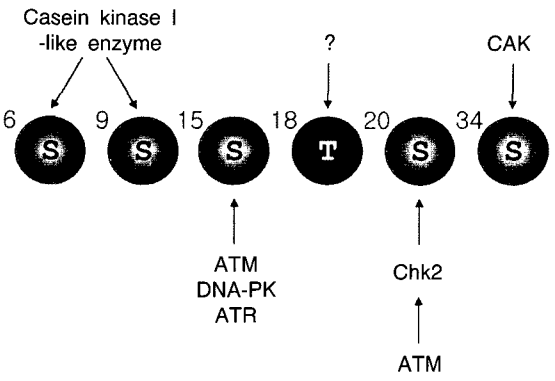

Fig. 1. Location of potential phosphorylation sites in p53 N-terminal domain. The N-terminal phopshosphorylation sites of mouse p53 protein is presented schematically, showing the location of phosphorylation sites and kinases that phosphorylate these sites.

alterations that may affect p53 activation or signaling. Thus, we decided to generate knock-in mouse with p53 point mutation at Ser20. A central goal of these studies is to develop a biologically relevant system to discern which of the phosphorylation site(s) proposed for p53 is/are essential for p53 stabilization following DNA damage.

Generation of ES cells and knock-in mouse with p53 S20A mutation

Genomic clone of mouse *p53* gene has been described before (Bienz et al, 1984). Briefly, *p53* gene is composed of 11 distinct exons which span a DNA region of ~14 kb, as compared with a mature p53 mRNA size of 2.0 kb (Oren et al., 1983). The first exon is noncoding and the translation start site is within exon 2. Interestingly, two independent promoters for the full-length *p53* have been identified. The first promoter is located upstream of exon 1 and a second promoter is identified in the first intron region. As illustrated in Fig. 2, the targeted construct used to create the p53 S20A mice contained the Ser20 to Ala20 missense mutation in exon 2 and *puro* gene flanked by *loxP* sites.

Homologous recombination introduced the altered allele into the p53 genomic locus of ES cells. Southern blot analysis was performed to confirm the homologous recombination in the targeted ES cells (Fig. 3). Genomic DNA from each ES cell was digested with *Eco*RI and separated on 0.8% agarose gel. As shown in Fig. 3 (lanes 1, 3, and 4), the [^{32}P]-labeled DNA probe hybridized to a 14.5 kb *Eco*RI-digested wild-type genomic DNA. Correctly targeted heterozygous ES cells surviving puromycin selection also show the 10.4 kb *Eco*RI fragment (lanes 2 and 5, Fig. 3).

Cre is the 38 kDa product of *cre* (cyclization recombination) gene of bacteriophage P1 (Sternberg, 1979; Sternberg et al., 1986) and is a site-specific DNA recombinase of the *Int* family (Argos et al., 1986).

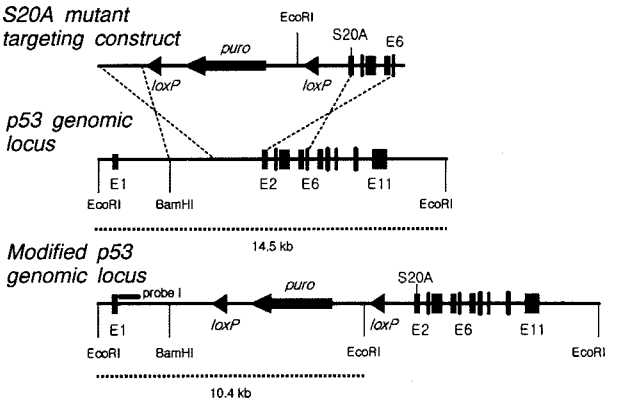

Fig. 2. Targeting of the p53 S20A mutation to the p53 genomic locus. The targeting construct contains base changes from ACATTTTCAGG-CTTA (encoding aa 18-22) to ACATTTGCAGGCTTA, which introduces the mutation S20A. Only exons 1, 2, 6, and 11 are labeled. The Ser20 to Ala20 mutation in exon 2 is indicated as S20A. The targeting construct also contains a loxP-flanked Puro cassette in intron 1. The black boxes represent the p53 exons and the filled bar represents the probe site for Southern blot analysis. Theoretical crossovers for homologous recombination are presented. The germ-line 14.5 kb and mutant 10.4 kb EcoRI fragments are indicated.

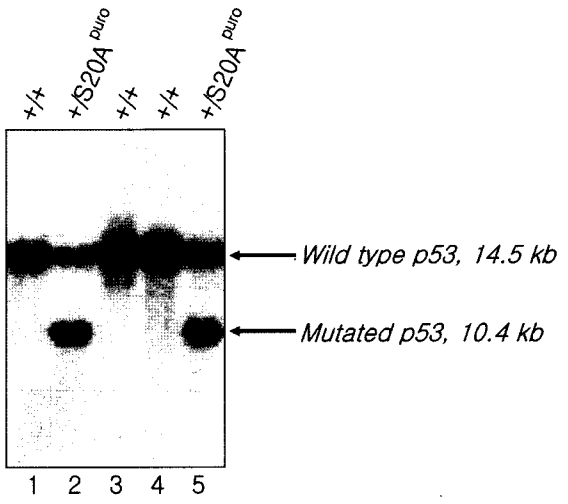

Fig. 3. Southern blot hybridization of genomic DNA derived from the wild type (lanes 1, 3, and 4) and targeted S20A ES cells (lanes 2 and 5), in which homologous recombination had occurred between the germ line allele and the targeted vector. Genomic DNA was digested with EcoRI and hybridized to probe I shown in Fig. 2. The position of germ line and mutant alleles are indicated with arrows. The wild type allele and targeted allele yielded the 14.5 and 10.4 kb EcoRI fragments, respectively.

Cre recognizes a 34 bp site on the P1 genome called *loxP* (locus of X-over of P1) and efficiently catalyzes reciprocal conservative DNA recombination between pairs of *loxP* sites (Hoess and Abremski, 1990). The *loxP* site consists of two 13 bp inverted repeats flaking an 8 bp nonpalindromic core region. Treatment of targeted ES cells by transient transfection with a Cre-expression plasmid led to excision of the puromycin cassette, which was also confirmed by Southern blotting (data not shown). PCR reaction for p53 was performed on genomic DNA derived from the targeted ES cell and sequencing of the PCR product demonstrated the presence of the targeted mutation (data not shown). These ES cells were injected into blastocysts to generate chimeras. The chimeras transmitted the mutant allele to the germline and their progeny were used in the experiments.

Accumulation of p53 after DNA damage is defective in homologous p53$^{S20A/S20A}$ mutant MEF

We generated wild-type (+), +/S20A, and S20A/S20A MEFs to determine whether Ser20 phosphorlation was required for p53 induction following DNA damage. Activation of p53 in response to DNA damage involves an increase in p53 protein levels, which is caused by stabilization of the p53 protein (Maltzman and Czyzyk, 1984; Kastan et al., 1991; Fritsche et al., 1993). In turn, stabilization of p53 is caused by dissociation of p53 from Mdm2 (Shieh et al., 1997), a protein that targets p53 for degradation (Haupt et al., 1997; Kubbutat et al., 1997; Midgley and Lane, 1997). The involvement of Mdm2 in the regulation of p53 by DNA damage is well characterized, because modified p53 proteins that cannot associate with Mdm2 are not subject to Mdm2-dependent degradation and are not stabilized after DNA damage (Haupt et al., 1997; Kubbutat et al., 1997; Midgley and Lane, 1997; Ashcroft et al., 1999; Blattner et al., 1999). The mechanism by which DNA damage leads to dissociation of p53 from Mdm2 has been difficult to resolve, because regulation of p53 by DNA damage is very complex. Biochemical and genetic experiments have attempted to resolve which of these modifications are critical for p53 stabilization, but have yielded somewhat conflicting results.

To determine whether DNA damage induces p53 through Ser20 phosphorylation, we examined the level of p53 in cell extracts from +/+, +/S20A, or S20A/S20A MEFs. After doxorubicin treatment, p53 levels were assessed by Western blot analysis using anti-p53 polyclonal antibody (CM5). As expected, endogeneous levels of p53 from three different MEFs were undetectably low (lanes 1, 3, 5, and 7, Fig. 4), but treatment with DNA damaging agent produced a large increase in the wild-type p53 protein (lane 2 compared to lane 1, Fig. 4). However, p53 induction level in S20A/S20A MEFs following the treatment with DNA damaging agent clearly decreased compared to WT or +/S20A MEFs (lanes 2, 4, 6, and 8, Fig. 4). The blot was stripped and reprobed using an antibody to actin protein to confirm equal loading (Fig. 4, bottom panel).

These data strongly indicate that phosphorylation of p53 at Ser20 is important for stabilizing p53 in response to DNA damage. However, we can not rule out the existence of Ser20 phosphorylation-independent pathway leading to stabilizing p53 (Fig. 5). It includes [1] enhanced translation efficiency of p53 mRNA in response to DNA damage (Kastan et al., 1991; Mosner et

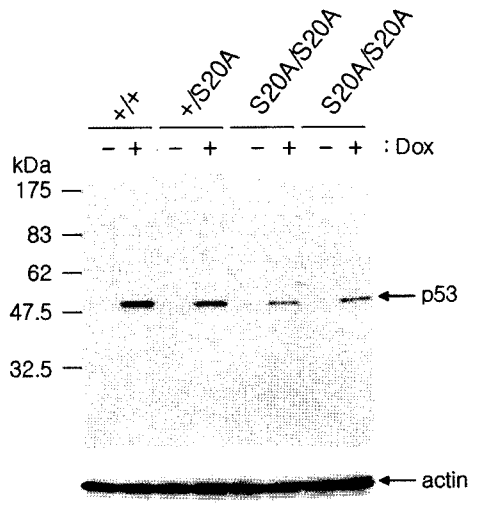

Fig. 4. Defective DNA damage-induced p53 stabilization in S20A/S20A mutant MEFs. Quantitative analysis of p53 induction was performed at 12 h after doxorubicin treatment in the MEFs. Cell extracts corresponding to 20 μg total protein were prepared from untreated cells (-), or from cells treated with doxorubicin. p53 levels were determined by Western blot analysis using polyclonal antibody CM5. Equal loading of the gel was confirmed by stripping the blot and reprobing with anti-β-actin antiserum.

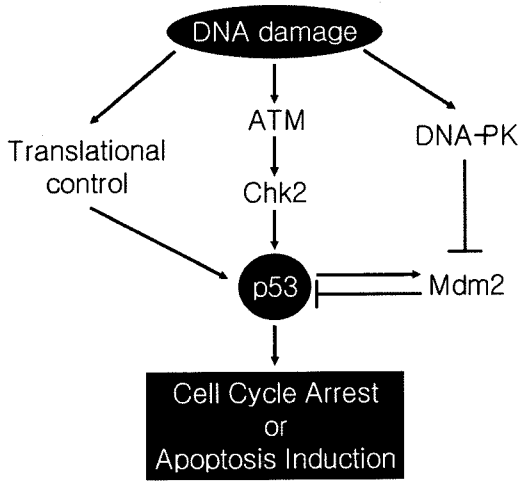

Fig. 5. Model predicting how DNA damage induces p53 stabilization. DNA damage signals to p53, causing it to become more stable and active as a transcription factor. This allows p53 to carry out its function as a tumor suppressor through a number of growth controlling endpoints, including cell cycle arrest, apoptosis, senescence, differentiation, and anti-angiogenesis.

al., 1995; Fu and Benchimol, 1997), [2] inhibition of p53-Mdm2 interaction by Mdm2 phosphorylation following DNA damage (Mayo et al., 1997), and [3] activation of deubiquitination pathway of p53 (Li et al., 2002). Consistent with these reports, the induction level of p53 in the S20A mutant MEF was dramatically reduced, but not completely abolished, following DNA damage (lanes 6 and 8, Fig. 4).

DNA damage-induced signaling pathways to p53

Upon DNA damage, p53 protein accumulates rapidly through (a) posttranscriptional mechanism(s) and is also activated as a transcription factor, which then leads to growth arrest or apoptosis. Stabilization of p53 after exposure to IR requires ATM, a kinase implicated in DNA damage signaling (Kastan et al., 1992; Khanna and Lavin, 1993; Canman et al., 1994; Savitsky et al., 1995). In response to UV light, stabilization of p53 is ATM-independent and may require ATR, an ATM-related kinase (Tibbetts et al., 1999).

ATM and ATR phosphorylate p53 on Ser15 *in vitro* and possibly *in vivo* (Banin et al., 1998; Canman et al., 1998; Khanna et al., 1998; Tibbetts et al., 1999); nevertheless, p53 stabilization cannot be mediated by direct phosphorylation of p53 on Ser15 by ATM or ATR, because replacement of Ser15 with Ala or Asp does not compromise p53 stabilization (Ashcroft et al., 1999; Blattner et al., 1999). Rather, p53 stabilization requires phosphorylation of Ser20, and neither ATM nor ATR can phosphorylate p53 on Ser20 (Banin et al., 1998; Canman et al., 1998; Khanna et al., 1998; Tibbetts et al., 1999). Because stabilization of p53 is dependent on ATM and ATR in response to IR and UV light, respectively, it has been proposed that ATM and ATR activate other kinases that in turn directly phosphorylate p53 on Ser20.

Such suggestion is consistent with the recent report on Chk2 (Hirao et al., 2000). Chk2 is a protein that is involved in cell cycle checkpoint control (Blasina et al., 1999; Furnari et al., 1999; Tominaga et al., 1999; Falck et al., 2001). Interestingly, the Ser20 of p53 is phosphorylated by Chk2, and thereby interrupts the binding of p53 to Mdm2 and p53 ubiquitination, resulting in greater stability of p53 (Hirao et al., 2000). As shown in Fig. 5, Chk2 is activated upon DNA damage by the phosphorylation signaling from ATM kinase (Matsuoka et al., 1988; Chatuvedi et al., 1999; Ahn et al., 2000; Hirao et al., 2000; Matsuoka et al., 2000; Melchionna et al., 2000). Interestingly, germ-line mutations in Chk2 are decreased in some Li-Fraumeni families lacking p53 mutation (Bell et al., 1999). This observation suggests that a loss of Chk2 might have an effect functionally equivalent to mutation of p53. Such a model is compatible with Chk2-dependent phosphorylation of seine 20 of p53 in response to DNA damage. Our data are also similar to findings on Chk2-null MEFs in which p53 stabilization was completely defective in response to DNA damage (Hirao et al., 2000).

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