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PRINCIPAL INVESTIGATOR: Stephen J. Elledge, Ph.D.

CONTRACTING ORGANIZATION: Brigham and Women's Hospital, Inc.
Boston, Massachusetts 02115

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Introduction

DNA damage activates cellular responses that promote DNA repair, arrest the cell cycle, and in some cases, induce apoptosis (56). Cell cycle arrest allows time for the repair of damaged DNA while apoptosis eliminates cells harboring abnormal DNA. It is widely believed that these DNA damage responses are required for the maintenance of genomic stability and prevention of tumor development (20). The ataxia telangiectasia (A-T) mutated (ATM) gene, which is homologous to the yeast checkpoint gene Tel1, plays a critical role in sensing DNA double strand breaks (DSBs) in mammalian DNA. ATM is a kinase involved in activating the appropriate damage response pathway, leading to either cell cycle arrest or apoptosis, and is therefore a key checkpoint molecule in regulating cell cycle responses to DNA damage (37, 45). Indeed, the majority of phosphorylation events induced by ionizing radiation (IR) are carried out by ATM. Both A-T patients and ATM-deficient mice show defective cell cycle arrest, hypersensitivity to DNA DSBs, and tumor predisposition (4, 21, 52, 53). When cells are damaged by IR, ATM phosphorylates and activates the protein kinase Chk2 (1, 35, 36, 55). Chk2 is a homologue of the Rad53 gene in budding yeast and of the Cds1 gene in fission yeast. Once phosphorylated, activated Chk2 phosphorylates multiple Cdc25 molecules which are thought to inhibit the activation of cyclin-dependent kinases (7, 10, 34). However, in response to damage induced by UV-irradiation or hydroxyurea, Chk2 is phosphorylated in an ATM-independent manner, possibly by A-T and *rad3*_related (ATR) (35, 46). Notably, ATM, ATR, and Chk2 are each able to phosphorylate the tumor suppressor gene p53 (2, 9, 11, 26, 42, 49).

p53 is the most frequently mutated cancer-associated gene identified to date (29). In response to DNA damage, p53 undergoes phosphorylation and conformational changes which result in increased levels and activity of the protein (23). Increased p53 activity enhances the rate of transcription of numerous target genes (such as p21, Mdm2, GADD45, and Bax) that mediate the plethora of p53-dependent functions (19, 54). These functions include the promotion of apoptosis and the induction of G1 cell cycle arrest. The p53 protein can be modified by many different protein kinases and acetylases, resulting in modulation of p53 function (39). In particular, the phosphorylation or dephosphorylation of various serine residues can have a significant impact on p53 stability. Recent studies with phospho-specific antibodies have established that serines (Ser) 6, 9, 15, 20, 33, 37, and 46 of p53 are sites of de novo phosphorylation in cells following DNA damage and that phosphorylation of different sites has different effects (2, 9, 12, 25, 38, 43, 44, 47). For example, it has been proposed that the phosphorylation of the N-terminal Ser 15, 33, and 37 residues permits subsequent modification of the distant C-terminal lysine residues of p53 through enhanced recruitment of the coactivator protein p300/CBP/PCAF (28, 41). In contrast, phosphorylation of Ser20 is required for stability of p53 in response to DNA damage (11). Ser20 comprises part of the site used by Mdm2 to bind p53 and target it for ubiquitination, and phosphorylation of Ser20 interferes with Mdm2 binding.

Previous studies have demonstrated that, in response to IR, Ser15 on p53 is phosphorylated by ATM (2, 9), whereas Ser20 is phosphorylated by Chk2 (11, 26, 42). There is abundant evidence that ATM controls p53 stabilization either directly or indirectly via Chk2, and it is also now clear that p53-mediated G1 arrest is suppressed in

ATM^{-/-} thymocytes. However, it is more controversial whether ATM is involved in p53-mediated apoptosis of damaged cells. While some laboratories have shown that ATM^{-/-} thymocytes are resistant to IR-induced apoptosis (51, 53), others have found that these cells exhibit normal p53-mediated cell death (3, 21, 24). It appears that the pathways governing p53-dependent cell cycle arrest and apoptosis may be distinct and that ATM plays a major role in regulating only the former.

Previous work on Chk2-deficient thymocytes reconstituted from Chk2^{-/-} embryonic stem (ES) cells in Rag1^{-/-} mice showed that Chk2 contributes to p53 stabilization following exposure to IR (26). We have now created mutant mice in which the Chk2 gene was disrupted through homologous recombination. We report on the phenotype of Chk2^{-/-} mice and compare it to that of ATM^{-/-} mice. Using various Chk2^{-/-} cell types, we show that Chk2 is involved in both ATM-dependent and ATM-independent regulation of IR-induced p53-mediated apoptosis.

More recently we have concentrated on Aim 2 which includes finding Chk2 associated proteins and potential Chk2 substrates. To identify Chk2 associated proteins, we took the FHA domain (Forked-Head Associated) of Chk2 which is known to be involved in protein-Protein interaction and produced it in *E. coli* as a GST fusion. We then went on to use this GST-FHA domain protein as an affinity column to purify associated proteins. In this way we identified a known DNA damage regulated protein 53BP1. We provide the data for this experiment below in the Body section. 53BP1 was originally identified through its ability to bind to the tumor suppressor protein p53 through 53BP1's C-terminal BRCT (Brca1 carboxyl terminus) repeats (57, 58) which are found in many DNA damage response proteins (59-64). 53BP1 responds to DNA double strand breaks (9-12), quickly relocating to discrete nuclear foci upon exposure to IR. These foci colocalize with those of the Mre11/Nbs1/Rad50 complex and phosphorylated γ -H2AX which are thought to facilitate recruitment of repair factors to damaged DNA (65-68). In response to IR, 53BP1 is phosphorylated in an ATM (ataxia telangiectasia mutated) dependent manner (66-68), but its role in the DNA damage response is unclear..

Body

Generation of Chk2^{-/-} mice. The Chk2 gene was disrupted by replacing a region of the genomic sequence containing exons 8 to 11 with a neomycin resistance cassette (Fig. 1A). Following transfection, selection of targeted clones, and blastocyst microinjection, chimeric mice were produced that successfully transmitted the Chk2 mutation to the germ line. F1 heterozygote mice were intercrossed to generate animals homozygous for the mutant Chk2 allele. Chk2^{-/-} mice were viable and born at the expected Mendelian ratio (-/- : -/+ : +/+ :: 0.20:0.53:0.26). The expression of Chk2 protein was assessed in Chk2^{-/-}

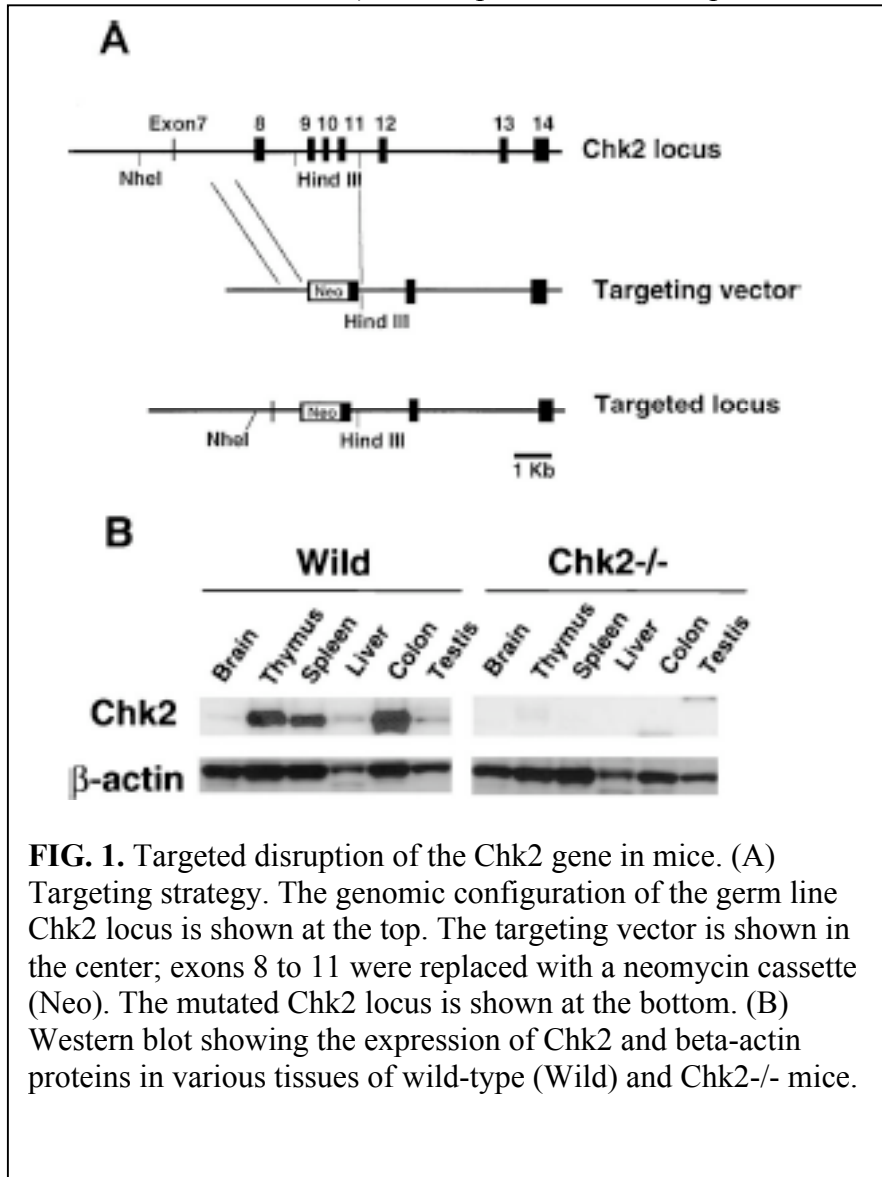


FIG. 1. Targeted disruption of the Chk2 gene in mice. (A) Targeting strategy. The genomic configuration of the germ line Chk2 locus is shown at the top. The targeting vector is shown in the center; exons 8 to 11 were replaced with a neomycin cassette (Neo). The mutated Chk2 locus is shown at the bottom. (B) Western blot showing the expression of Chk2 and beta-actin proteins in various tissues of wild-type (Wild) and Chk2^{-/-} mice.

and Chk2^{-/-} mice. The Chk2 protein is ubiquitously expressed in wild-type mouse tissues with its highest levels in the thymus, spleen, and colon (Fig. 1 B). The protein was not detected in Chk2^{-/-} tissues, confirming the null mutation.

Chk2 is not essential for somatic growth, fertility, or immunological development.

Since Chk2 activation in response to DNA DSBs is dependent on ATM (34), we anticipated that Chk2^{-/-} mice might display

overlapping phenotypes with those of ATM^{-/-} mice. We therefore investigated several of the most obvious phenotypes of ATM^{-/-} mice in Chk2^{-/-} animals. ATM^{-/-} mice are smaller in size and weigh less than their wild-type or heterozygous littermates (4, 21, 52). However, Chk2^{-/-} mice were not smaller than control mice at birth, at weaning, or

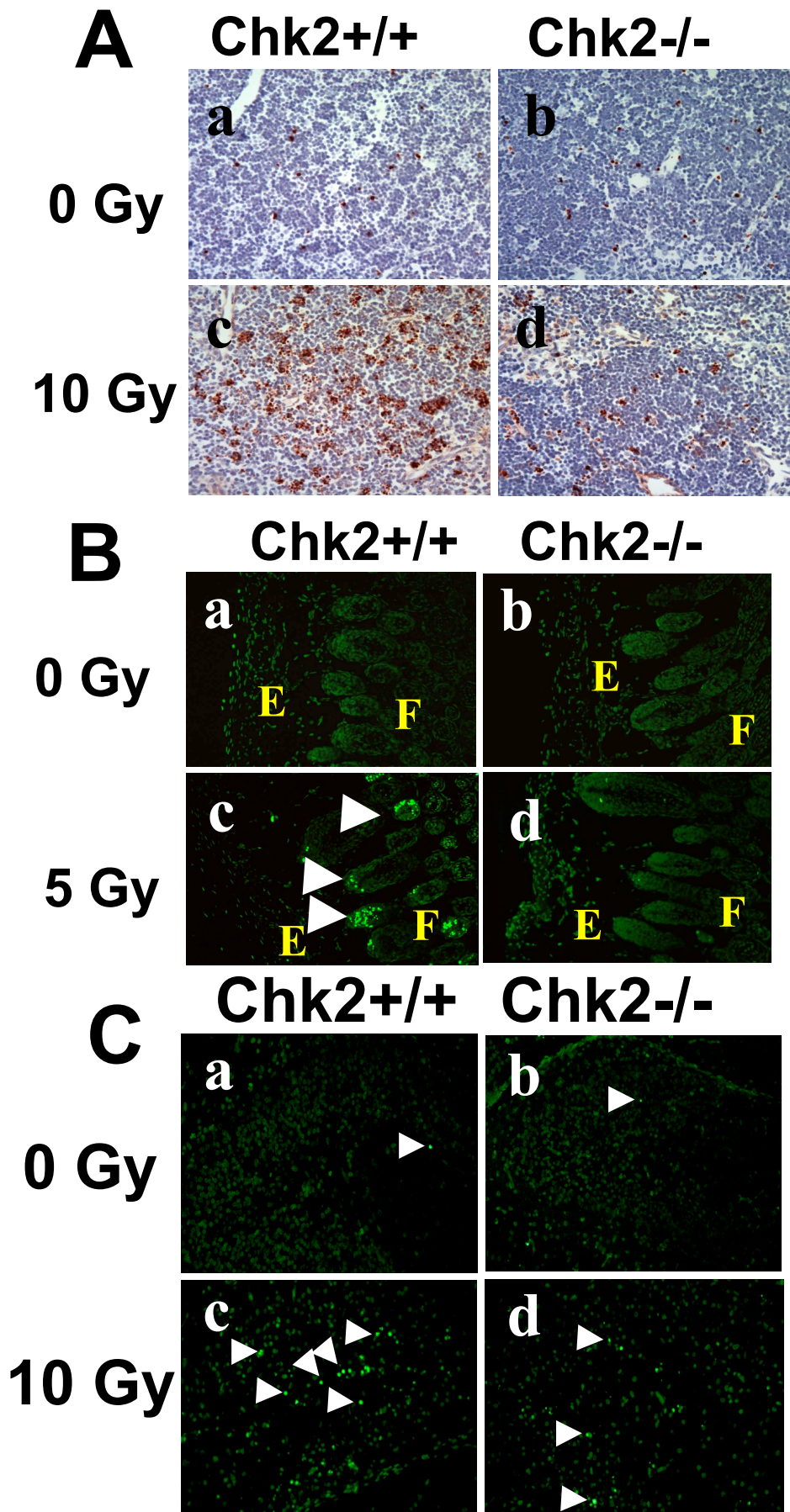


FIG. 2. Induction of apoptosis in response to gamma-irradiation.

(A) Mice (8 weeks old) were mock irradiated (a and b) or irradiated with 10 Gy of irradiation (c and d), and apoptosis in thymi was evaluated at 10 h post-IR.

(B) Mice (P7) were mock irradiated (a and b) or irradiated with 5 Gy of irradiation (c and d), and apoptosis in skin was evaluated at 6 h post-IR by in situ TUNEL staining. E and F indicate epidermis and hair follicle, respectively.

(C) Mice (P5) were mock irradiated (a and b) or irradiated with 10 Gy of irradiation (c and d), and apoptosis in the hippocampal dentate gyrus was evaluated at 24 h post-IR by in situ TUNEL staining. Arrows indicate apoptotic TUNEL-positive cells.

through adulthood (male, +/+versus +/- versus -/-: 19.5 ± 3.4 g versus 23.1 ± 2.1 g versus 22.0 ± 2.6 g, respectively; female, +/+versus +/- versus -/-: 18.2 ± 3.2 g versus 17.8 ± 1.3 g versus 17.9 ± 3.1 g, respectively) (42 days of age). In addition, while cultured MEFs from ATM-/- mice showed extremely poor growth consistent with the animals' growth retardation, cultured Chk2-/- fibroblasts did not show an obvious growth deficit (data not shown). ATM-/- mice are infertile due to a defect in germ cell development. However, Chk2-/- male and female mice are fertile, and their gonads are histologically normal (data not shown).

Several immunological abnormalities have been reported in ATM-/- mice, including a defect in T-lymphocyte maturation. However, lymphoid tissues from Chk2-/- mice were similar in gross size to those of their Chk2-/- and wild-type littermates, and no histological abnormalities were observed in the thymus, spleen, or lymph nodes. The numbers of thymocytes and spleen cells isolated from Chk2-/- mice were comparable to those of the controls at 8 to 10 weeks of age. Analysis of cell surface markers of isolated Chk2-/- lymphoid cells showed that populations of cells bearing CD3, CD4, CD8, Thy1, or T-cell receptor were not affected by the loss of Chk2 in either the thymus or the spleen. The development of immature T cells, as evaluated by CD25 and CD44 expression, was also normal. Analysis of the expression of B220, immunoglobulin D (IgD), IgM, and CD43 in spleen and bone marrow cells revealed no abnormalities in B-cell development. The proliferation of peripheral lymph node T cells stimulated with anti-CD3 Ab, anti-CD28 Ab, and interleukin-2 was normal, as was that of splenic B cells stimulated with anti-CD40 Ab, anti-IgM Ab, interleukin-4, or lipopolysaccharide. Immunoglobulin levels in Chk2-/- mice were normal.

Apoptosis and cell cycle arrest induced by IR are defective in Chk2-/- mice.

In our previous work, we showed that CD4⁺ CD8⁺ thymocytes isolated from Chk2-/- Rag1-/- chimeric animals were resistant to IR-induced apoptosis because of a lack of p53 stabilization. In this study, we evaluated the role of Chk2 in regulating IR-induced cell death in vivo. Wild-type and Chk2-/- mice were subjected to whole-body gamma-irradiation, and their thymic tissues were evaluated for apoptosis by using the TUNEL assay. Thymi of unirradiated wild-type and Chk2-/- mice showed very few apoptotic cells (Fig. 2A). As expected, substantial apoptosis was observed in the thymi of irradiated wild-type mice. However, the thymi of irradiated Chk2-/- mice showed significantly less apoptosis.

We next examined the effect of the Chk2 mutation on hair follicular matrical cells since these cells have been shown to be susceptible to IR-induced apoptosis (4, 48). Four hours after 5 Gy of irradiation, a large number of cells in the wild-type matrix were undergoing apoptosis, as evaluated by an in situ TUNEL assay (Fig. 2B). In contrast, there was an almost complete absence of IR-induced apoptosis in the follicular matrices of Chk2-/- mice. Finally, we examined IR-induced apoptosis in the developing CNS. In wild-type perinatal mice (P5), significant apoptosis was observed in many regions of the

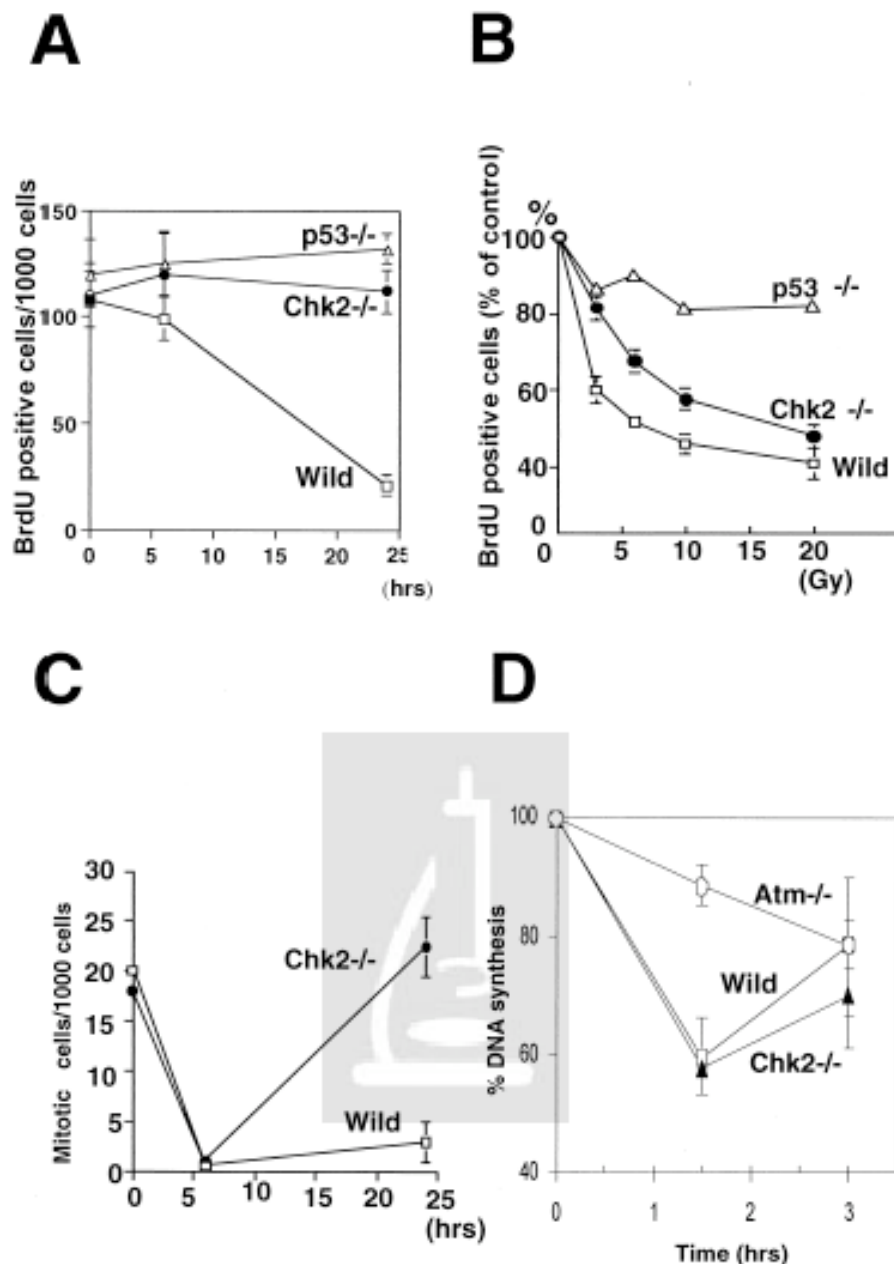


FIG. 3. Cell cycle checkpoints induced by gamma-irradiation of wild-type (wild) and Chk2^{-/-}-mice. (A) G1/S checkpoint in the epidermis. Mice (P7) were irradiated with 5 Gy of irradiation, and BrdU was injected 1 h prior to sacrifice. BrdU-positive cells were taken as those in S phase. Each value represents the mean number $\pm$ standard deviation (SD) of BrdU-positive cells from 3 mice per group. (B) G1/S checkpoint in MEFs. MEFs were synchronized in G0 by serum starvation and irradiated with 0 to 20 Gy of irradiation as indicated. The irradiated cells were incubated with BrdU, and S-phase cells were detected by flow cytometric analysis of anti-BrdU Ab binding. Each value represents the mean percentage $\pm$ SD of BrdU-positive cells present after IR in 5 cultures per group relative to the unirradiated controls. (C) G2/M checkpoint in epidermis. Mice (P7) were irradiated with 5 Gy of irradiation, and mitotic cells in the epidermis were detected with anti-phosphohistone H3 Ab. Each value represents the mean number $\pm$ SD of mitotic cells from 3 mice per group. (D) S-phase checkpoint in primary MEFs. Exponentially growing primary MEFs were irradiated with 10 Gy of irradiation and sampled at the post-IR times indicated. [³H]thymidine was added for 30 min prior to sampling, and [³H]thymidine incorporation was determined in the cell lysates. The results are expressed as percentages of DNA synthesis relative to label incorporated in unirradiated cells. Each point represents the mean $\pm$ SD of 3 to 5 samples per group.

CNS 24 h after 5 Gy of gamma-irradiation, consistent with previous reports. Strikingly, very little apoptosis was observed in the CNS, including the cerebellum and dentate gyrus, of irradiated Chk2^{-/-} mice (Fig. 2C and data not shown). Since IR induces cell cycle arrest at several distinct cell cycle transitions, we studied the effect of the absence of Chk2 on the G1/S, S, and G2/M checkpoints. First, we examined the G1/S checkpoint in epidermal cells in vivo. P7 mice were irradiated with 5 Gy of irradiation followed by BrdU injection 1 h prior to sacrifice. The frequency of BrdU-positive cells in Chk2^{-/-} epidermis was comparable to that in wild-type tissue (wild type versus Chk2^{-/-}: 108 ± 13 per 1,000 cells versus 110 ± 15 per 1,000 cells). Although epidermal cells are not susceptible to IR-induced apoptosis, there was a clear reduction in the BrdU-positive population in the wild type at 24 h post-irradiation (21 ± 5 per 1,000 epidermal cells), likely due to the operation of the G1/S checkpoint (Fig. 3A). In contrast, there was no reduction in the BrdU-positive population at 24 h in either Chk2^{-/-} epidermis (112 ± 10 per 1,000 cells) or in p53^{-/-} epidermis (132 ± 7 per 1,000 cells). G1 arrest was also evaluated in cultured wild-type, p53^{-/-}, and Chk2^{-/-} MEFs. Serum-starved cells were irradiated and stimulated to enter the cell cycle by the addition of serum. BrdU was added with the serum to allow the detection of S-phase entrance. In response to increasing doses of IR, p53^{-/-} MEFs failed to arrest in G1, as expected (Fig. 3B) (18). Wild-type MEFs arrested normally in G1 as evidenced by a dose-dependent reduction in the number of BrdU-positive cells. Interestingly, Chk2^{-/-} MEFs were significantly defective in their ability to arrest in G1 at low IR doses but behaved like wild-type cells at higher doses. To examine the G2/M checkpoint, we used an anti-phosphohistone H3 Ab which specifically recognizes mitotic cells to evaluate the frequency of mitotic cells in the epidermis following IR. In the absence of IR, the frequency of mitotic cells was comparable in wild-type and Chk2^{-/-} mice (wild type versus Chk2^{-/-}: 18 ± 4 per 1,000 cells versus 20 ± 4 per 1,000 cells) (Fig. 3C). Almost no mitotic cells were observed in wild-type or Chk2^{-/-} mice 6 h after exposure to 5 Gy of irradiation, indicating that the induction of G2 arrest is intact in Chk2^{-/-} mice. However, mitosis had resumed in Chk2^{-/-} cells by 24 h post-IR (21 ± 4 per 1,000 cells), whereas wild-type cells still showed suppression of mitosis (3 ± 2 per 1,000 cells) at this time. These data indicate that the loss of Chk2 curtails the duration of the G2/M checkpoint. An intra-S-phase checkpoint occurs in MEFs in response to IR, and this checkpoint requires ATM function (4). We evaluated the effect of the Chk2 mutation on this checkpoint by examining the inhibition of DNA synthesis in MEFs. Both wild-type and Chk2^{-/-} primary MEFs showed equivalent levels of DNA synthesis inhibition following IR, whereas ATM^{-/-} cells showed a characteristic profile of radiation-resistant DNA synthesis (Fig. 3D). These findings show that the intra-S-phase checkpoint is ATM dependent but Chk2 independent.

Incidence of DMBA-induced skin tumors is enhanced in Chk2-deficient mice. Since the loss of Chk2 leads to suppressed apoptosis and cell cycle arrest in response to IR, one would expect to find a higher incidence of tumor formation in Chk2^{-/-} mice, perhaps comparable to that in p53^{-/-} mice. However, by age 1 year, Chk2^{-/-} mice had not developed tumors of any kind. We speculate that tumors caused by the loss of Chk2 are either too rare to be detected or require a prolonged period to develop. We therefore challenged Chk2^{-/-} mice with the chemical carcinogen DMBA, an agent that damages

DNA and efficiently induces skin tumors (15, 40). More Chk2^{-/-} mice than wild-type animals developed skin tumors in response to DMBA treatment (wild type versus Chk2^{-/-} : 6 of 14 versus 12 of 14 at 25 weeks) (Fig. 4A). The onset of tumors in Chk2^{-/-} mice occurred earlier than in wild-type animals, and the total number of tumors was increased in Chk2^{-/-} mice compared to wild-type mice (Fig. 4B). However, the size of individual tumors and the frequency of malignant tumors (carcinomas) were comparable between wild-type and Chk2^{-/-} mice. We conclude that Chk2 has a suppressive effect on tumor development induced by at least some types of DNA damage.

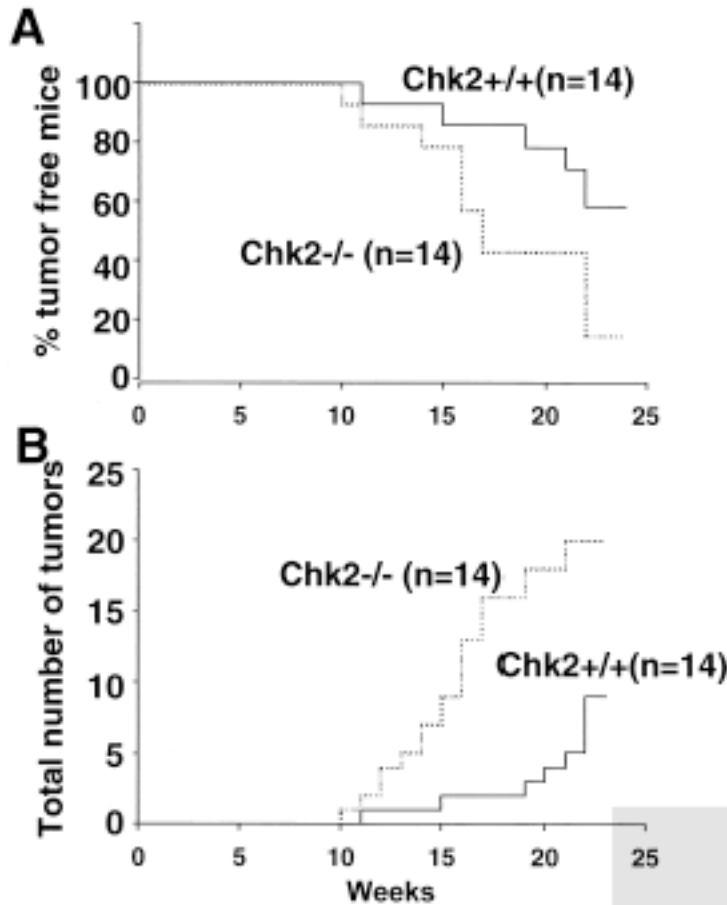


FIG. 4. Skin tumor formation in mice treated with DMBA. The back skin of 6- to 8-week-old mice was shaved and painted with 10 ug of DMBA once a week for 25 weeks. Tumors in skin were scored once a week.

(A) Kaplan-Meier plot of tumor incidence in wild-type ($n = 14$) and Chk2^{-/-} ($n = 14$) mice.

(B) Total numbers of tumors in mice for which data are shown in panel A.

Chk2 selectively regulates apoptosis in an ATM-independent manner. Although Chk2 acts downstream of ATM in yeast and mammals, the loss of Chk2 does not result in many of the phenotypes observed in ATM^{-/-} mice. In fact, the only shared phenotype is defective p53 function in response to IR. This finding prompted us to explore the possibility that Chk2 and ATM might have different regulatory effects on p53 function. Whereas Chk2^{-/-} cells have a clear defect in IR-induced apoptosis, this phenotype is variable in ATM^{-/-} cells. We therefore carefully compared the effect of the loss of Chk2 or ATM on the regulation of p53 activation in thymocytes. p53- mediated cell cycle

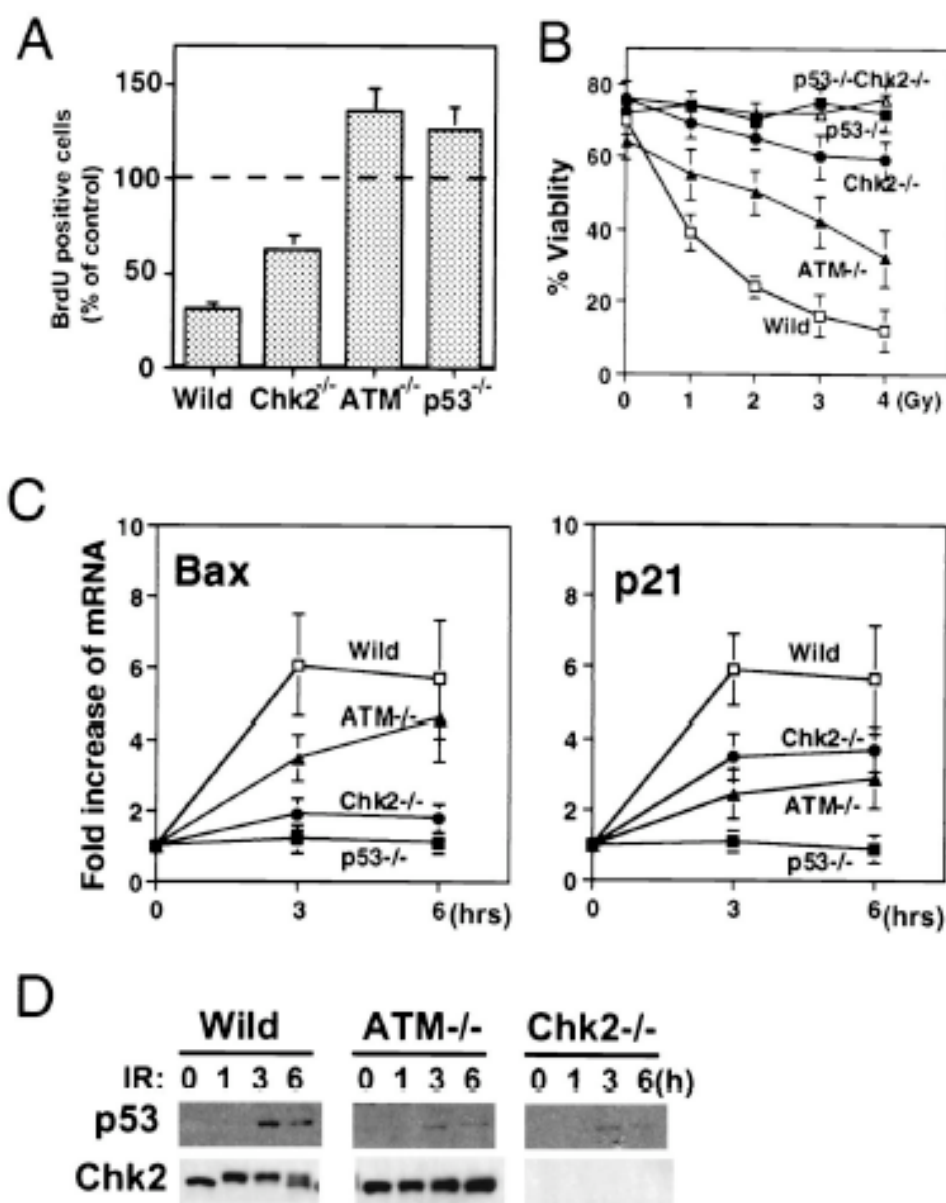


FIG. 5. IR-induced p53 activation and p53 stabilization in Chk2^{-/-} and ATM^{-/-} thymocytes. (A) G1/S checkpoint induced by gamma-irradiation. The indicated strains of mice were irradiated with 10 Gy of irradiation and injected 1 h later with BrdU. At 2 h post-IR, thymocytes were isolated, stained with anti-BrdU Ab, and subjected to flow cytometry. Each value represents the mean percentage $\pm$ standard deviation (SD) of BrdU-positive (S phase) cells present after IR in 4 samples per group relative to the unirradiated controls. (B) Apoptosis induced by IR. Isolated thymocytes were treated with the indicated doses of IR, and apoptotic cells were evaluated by flow cytometry after Annexin V and PI staining. Each value represents the mean percentage $\pm$ SD of viable cells (Annexin V negative and PI negative) for 4 samples per group. (C) Induction of p53 downstream molecules. Total RNA was isolated from thymocytes before and after treatment with 5 Gy of irradiation. The expression of mRNA for p21, Bax, and actin was evaluated by Northern blotting. The amount of p21 or Bax mRNA was quantified by using a PhosphorImager analyzer and normalized to actin expression. Each value represents the mean increase (n -fold) $\pm$ SD of the expression of Bax or p21 mRNA after IR in 3 to 5 samples per group relative to the unirradiated controls. (D) p53 protein stabilization and Chk2 phosphorylation in thymocytes after irradiation. Isolated thymocytes were irradiated with 5 Gy of irradiation and lysed at the indicated times. p53 and Chk2 proteins were detected by Western blotting with anti-p53 or anti-Chk2 Ab. Wild, wild type.

arrest and apoptosis have been well characterized in thymocytes, and it is possible to precisely quantify these phenotypes in this cell type. When wild-type mice were subjected to 10 Gy of γ -irradiation, the number of thymic BrdU-positive S-phase cells was reduced to 35% $\pm$ 4% of that in the nonirradiated controls (Fig. 5A). The G1/S-phase checkpoint was defective in ATM $^{-/-}$ and p53 $^{-/-}$ mice (ATM $^{-/-}$, 132% $\pm$ 10%; p53 $^{-/-}$, 128% $\pm$ 11%), consistent with previous reports (3). In contrast to irradiated ATM $^{-/-}$ mice, irradiated Chk2 $^{-/-}$ mice showed only a partial defect that resulted in milder G1 arrest (65% $\pm$ 5%). However, when thymocytes were isolated from Chk2 $^{-/-}$ mice and subjected to IR in vitro, apoptosis was dramatically impaired (Fig. 5B). ATM $^{-/-}$ thymocytes were more resistant than wild-type thymocytes to IR-induced apoptosis but considerably more sensitive than either Chk2 $^{-/-}$ or p53 $^{-/-}$ thymocytes. It should be noted that the IR-induced apoptosis and inhibition of the G1/S transition observed in this study are p53 dependent because they are completely inhibited by the loss of p53 function. Dying p53 $^{-/-}$ thymocytes can be detected within 48 h of IR treatment, and irradiated Chk2 $^{-/-}$ p53 $^{-/-}$ thymocytes behave in the same manner as irradiated p53 $^{-/-}$ cells (data not shown). These results indicate that Chk2 acts in the pathway leading to p53-dependent apoptosis rather than in the general apoptosis program.

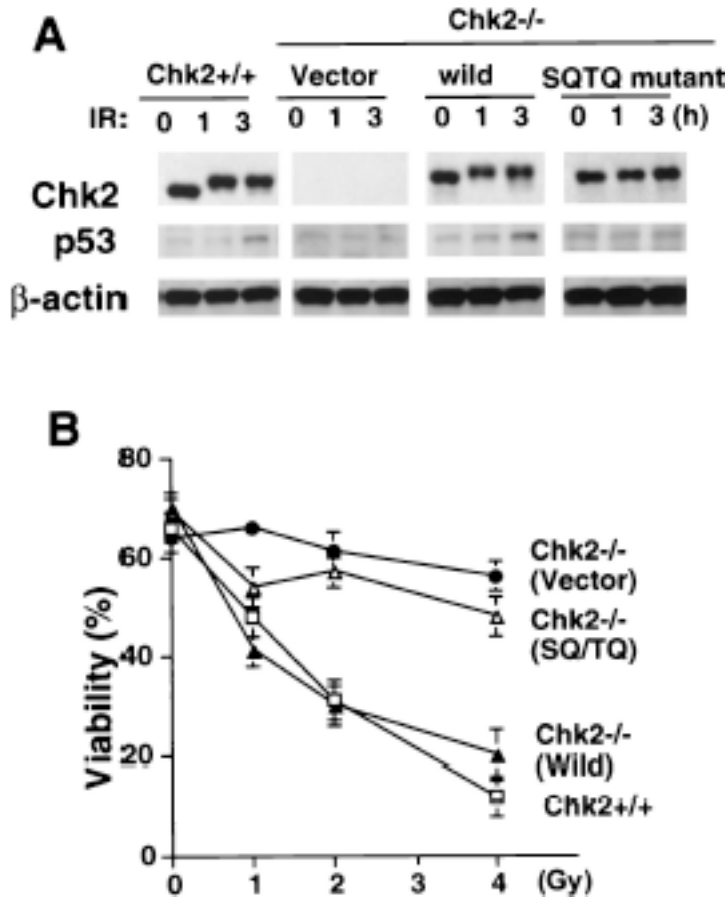


FIG. 6. Effect of reintroduced mutant Chk2 on IR-induced apoptosis. Chk2 $^{-/-}$ ES cells transfected with either empty vector (vector), vector containing wild-type Chk2 (wild), or vector containing the Chk2 SQ/TQ mutant gene (SQ/TQ mutant) were injected into blastocysts from Rag1 $^{-/-}$ mice to generate chimeric animals expressing the corresponding proteins in thymocytes. (A) Expression of reintroduced Chk2 protein and stabilization of p53 following 5 Gy of IR for the indicated times. (B) Thymocytes isolated from the chimeric mice for which data are shown in panel A were irradiated at the indicated doses. Apoptosis was analyzed by Annexin V-PI staining at 24 h post-IR. Each value represents the mean percentage standard deviation of viable cells in 3 cultures per group.

Our findings led us to hypothesize that Chk2 selectively regulates p53 activity leading to apoptosis. To address this question, we used Northern blotting to evaluate the transactivation of mRNA expression for known p53 target genes. Although the expression of many molecules is induced by p53 activation, p21 and Bax are the most prominent p53-responsive genes in mouse primary thymocytes (8). Loss of p21 in thymocytes leads to a clear defect of the G1 checkpoint induced by IR (18). Although Bax^{-/-} thymocytes are not resistant to IR-induced apoptosis, Bax^{-/-} Bak^{-/-} thymocytes fail to die in response to IR (32). Since Bak^{-/-} thymocytes also show normal responses to IR, the induction of Bax must be critical for IR-induced apoptosis. In wild-type thymocytes subjected to 5 Gy of IR, Bax mRNA was increased by (6.05 ± 1.44) -fold over the baseline at 3 h and by (5.68 ± 1.64) -fold at 6 h (Fig. 5C, left panel). Strikingly, irradiated Chk2^{-/-} thymocytes showed defective induction of Bax ($[1.9 \pm 0.45]$ -fold at 3 h and $[1.80 \pm 0.40]$ -fold at 6 h). These results represent statistically significant decreases compared to the wild type at 3 h ($P < 0.01$) and 6 h ($P < 0.01$). In contrast, irradiated ATM^{-/-} thymocytes were slower than the wild-type to induce Bax mRNA synthesis at 3 h ($P < 0.05$) but had caught up by 6 h. p21 mRNA induction was significantly suppressed compared to that of the wild type ($P < 0.05$) in both ATM^{-/-} and Chk2^{-/-} irradiated thymocytes at 3 h (Fig. 5C, right panel), but there were no significant differences in the level of suppression between these two genotypes. Consistent with a previous report (3), neither p21 nor Bax was induced in irradiated p53^{-/-} thymocytes. These results suggest that thymic apoptosis induced by IR depends on p53 function and is controlled mainly by Chk2 rather than ATM. Stabilization of p53 induced by IR was suppressed in both ATM^{-/-} and Chk2^{-/-} thymocytes (Fig. 5D). Taken together, the data in Fig. 5 demonstrate that Chk2, rather than ATM, controls p53-mediated apoptosis, and that p53-mediated apoptosis does not correlate with stabilization of the p53 protein.

We next determined whether Chk2 phosphorylation is required for the activation of p53 leading to apoptosis. Mouse Chk2 has seven N-terminal SQ/TQ sites in the N-terminal region of the protein. In response to IR in vivo, ATM phosphorylates several of these SQ/TQ sites in Chk2, including Thr68 (1, 35, 36). Phosphorylation of Chk2 following IR is abolished by mutation of these SQ/TQ sites, and most of the endogenous Chk2 is not phosphorylated in ATM^{-/-} cells (Fig. 5D); nevertheless, p53-mediated apoptosis can occur under these circumstances. To determine whether the phosphorylation of Chk2 SQ/TQ sites is required for Chk2-mediated regulation of p53-mediated apoptosis, we reintroduced into Chk2^{-/-} thymocytes a mutant form of Chk2 in which all Nterminal SQ/TQ sites were replaced with AQ and analyzed apoptosis. In a previous study (35), the mutated SQ/TQ kinase had the same level of kinase activity as the wild-type enzyme when transfected into unirradiated Chk2^{-/-} MEFs. To reintroduce the mutated Chk2 gene into Chk2^{-/-} thymocytes, we generated somatic chimeras by Rag1^{-/-} blastocyst complementation. An expression vector carrying wild-type or mutant Chk2 was transfected into Chk2^{-/-} ES cells, and clones with high expression of Chk2 protein were selected and used for blastocyst complementation. The size and cellularity of thymi in Chk2^{-/-} mice bearing an empty vector, Chk2^{-/-} mice bearing wild-type Chk2, and Chk2^{-/-} mice bearing the mutated Chk2 were all comparable to those of control wild-type mice (data not shown). Moreover, the expression level of the exogenous Chk2 protein was comparable to that of the endogenous Chk2 protein. Exogenous wild-type

Chk2 was phosphorylated in response to IR, whereas the mutated Chk2 protein failed to show a mobility shift (Fig. 6A). Thus, the defective p53 stabilization in Chk2^{-/-} thymocytes was restored by the introduction of exogenous wild-type Chk2 but not by mutant Chk2. Reintroduction of wild-type Chk2 also clearly restored the defective apoptosis observed in Chk2^{-/-} thymocytes (Fig. 6B), whereas thymocytes receiving mutant Chk2 behaved similarly to control Chk2^{-/-} thymocytes. These data suggest that phosphorylation of the SQ/TQ sites in Chk2 is required for this protein to induce ATM-independent p53 activation leading to apoptosis.

Identification of 53BP1 as a Chk2 FHA-domain interacting protein. To search for Chk2 associated proteins we attempted to immunoprecipitate large quantities of Chk2 from human extracts. While we could isolate large amounts of Chk2, we could not identify large quantities of associated proteins in sufficient levels to identify them by mass spectroscopy. In an alternative approach we used a GST-Chk2 fusion construct that contain the FHA domain of Chk2. We made an affinity column of this Gst-FHA fusion protein and poured extracts from hela cells over the column. Proteins specifically associated with this matrix were eluted and analyzed by mass spectroscopy. In this analysis we isolate 53BP1 (data not shown). We then set out to understand the function of 53BP1 in this pathway.

Analysis of the genetic role of 53BP1.

To determine 53BP1's role, small interfering RNAs (siRNA) in the form of two independent, non-overlapping 21-base pair RNA duplexes targeting 53BP1, were used to inhibit its expression (69, 70). U2OS cells were transfected with these siRNA oligos and, within three days post transfection, a portion of cells had undergone cell death. A similar phenotype was also observed in two other cell lines, Hct116 and Saos2 (data not shown).

To determine whether 53BP1 plays a role in DNA damage cell cycle checkpoints, we examined the response of 53BP1-inhibited cells to IR. IR induces the intra-S-phase checkpoint which reduces DNA synthesis. Unlike the control cells, 53BP1-inhibited cells showed radio-resistant DNA synthesis (Fig. 7A). This was also seen in Saos2 and Hela cells with both siRNAs (71, data not shown) and indicates a role of 53BP1 in the intra-S phase checkpoint.

To assess the G2/M checkpoint, 53BP1-inhibited and control cells were irradiated with 3 or 10 Gy of ionizing radiation. Approximately three-fold more 53BP1-inhibited cells entered into mitosis than the control cells treated with 3 Gy (Fig. 7B). However, inhibition of 53BP1 had no effect following 10 Gy IR. Therefore, 53BP1-inhibited cells also displayed an IR-induced G2/M checkpoint defect. The fact that 53BP1-inhibited cells were only defective in response to lower doses of irradiation indicates the existence of an alternative signaling pathway that operates at higher doses of IR.

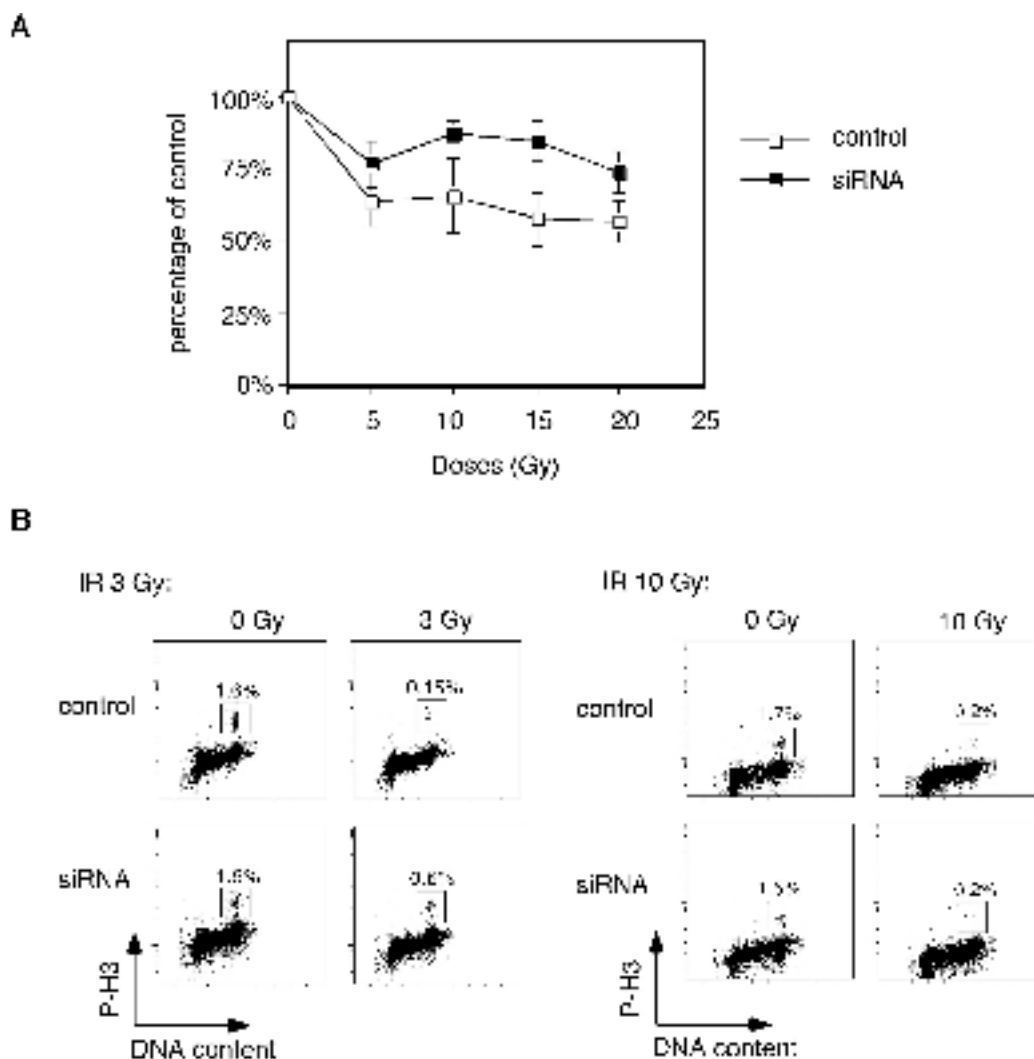


Fig. 7. 53BP1 inhibition results in defective IR-induced intra-S-phase and G2/M checkpoints. **(A)** IR-induced intra-S-phase checkpoint. Replicative DNA synthesis was assessed 30 min after various doses of ionizing irradiation in U2OS cells transfected with oligos. The DNA synthesis in unirradiated cultures was set to 100% for cells transfected with control oligos or siRNA oligos against 53BP1 (14). Error bars represent the standard deviation of triplicate samples. **(B)** Analysis of the G2/M DNA damage checkpoint. Cells were either untreated or irradiated with either 3 Gy or 10 Gy as indicated, then incubated for 1 hour at 37 °C prior to fixation. Cells in mitosis were determined by staining with propidium iodide and antibody to phospho-histone H3 (Cell Signaling), followed by FITC-conjugated secondary antibody (Jackson ImmunoResearch Laboratories), and percentage of the M-phase cells was determined by flow cytometry.

response to IR. P53 induction in response to IR was significantly decreased in 53BP1-inhibited cells (Fig. 8A). We then examined Chk2, a checkpoint protein

implicated in p53 regulation that is phosphorylated on Thr68 and forms foci in response to IR (72,73). Quantification of the ratio of Chk2 phosphorylated on Thr 68 to the total amount of Chk2 revealed that Chk2 phosphorylation at Thr 68 was reduced 2-fold at 2h in response to IR in the 53BP1-inhibited cells (Fig. 8B). The reduction of Chk2 phosphorylation at Thr 68 was reproducibly observed at 1 h or 2h after IR in different experiments (71). A much stronger effect was observed in the formation of IR-induced foci recognized by antibodies raised against P-T68 of Chk2 (17), which were nearly completely abolished in 53BP1 siRNA-treated cells, but were unaffected in control cells (Fig. 8C).

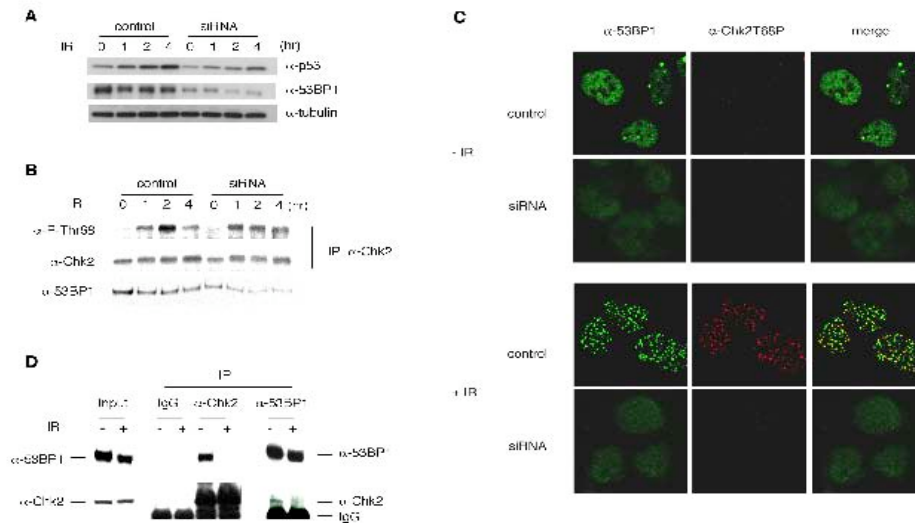


Fig. 8. 53BP1 regulates p53 and Chk2 in response to IR. **(A)** IR-induced p53 stabilization. U2OS cells were transfected with siRNA oligos against 53BP1 or control oligos for two days, then exposed to 10Gy ionizing irradiation. Cell lysates were made from samples at indicated times recovered from irradiation and separated on SDS-PAGE gel. Western blots were performed using anti-53BP1, anti-tubulin and anti-p53 (Oncogene) antibodies. **(B)** Chk2 phosphorylation at Thr 68 is reduced in 53BP1-inhibited cells. Chk2 immunoprecipitates were prepared from U2OS cells at indicated hours after exposure to 10 Gy irradiation. Western blots were performed using anti-Chk2 (14) and anti-T68P-Chk2 antibodies (14). **(C)** IR-induced phospho-foci recognized by antibodies against P-T68 of Chk2 depend on 53BP1. SiRNA transfected U2OS cells were irradiated with 10 Gy irradiation, and 2 hour later were fixed with paraformaldehyde, permeabilized with Triton X-100, and then immunostained with antibodies against Chk2T68P (provided by J. Chen) and 53BP1 (provided by T. D. Halazonetis) and the appropriate FITC- (Molecular Probes) and Cy3-conjugated secondary antibodies (Amersham). **(D)** 293T cells were untreated (-) or treated (+) with 20 Gy IR and harvested after 1h. Cell extracts were incubated with control IgG, anti-Chk2 or anti-53BP1 antibodies and protein A Sepharose. Immunoprecipitates were separated by SDS-PAGE, and then immunoblotted with anti-53BP1 and anti-Chk2 antibodies as indicated.

53BP1 resembles the Rad9 BRCT-repeat protein of budding yeast, which binds to and is required for the DNA-damage induced activation of Rad53, a homologue of Chk2 (72). Like Rad9 and Rad53, we found that antibodies to Chk2, but not control antibodies, could efficiently immuno-precipitate 53BP1 and that Chk2 dissociates from 53BP1 in response to IR (Fig. 8D). This association was also detected in the reciprocal IP using 53BP1 antibodies. These data suggest that 53BP1 may act as an adaptor that facilitates Chk2 phosphorylation. It is likely that 53BP1 facilitates Chk2 activation in a transient complex, and upon activation, Chk2 dissociates from the 53BP1 complex.

The discrepancy between the partial dependency of 53BP1 for Chk2 phosphorylation and its major role in the formation of phospho-foci could be explained if

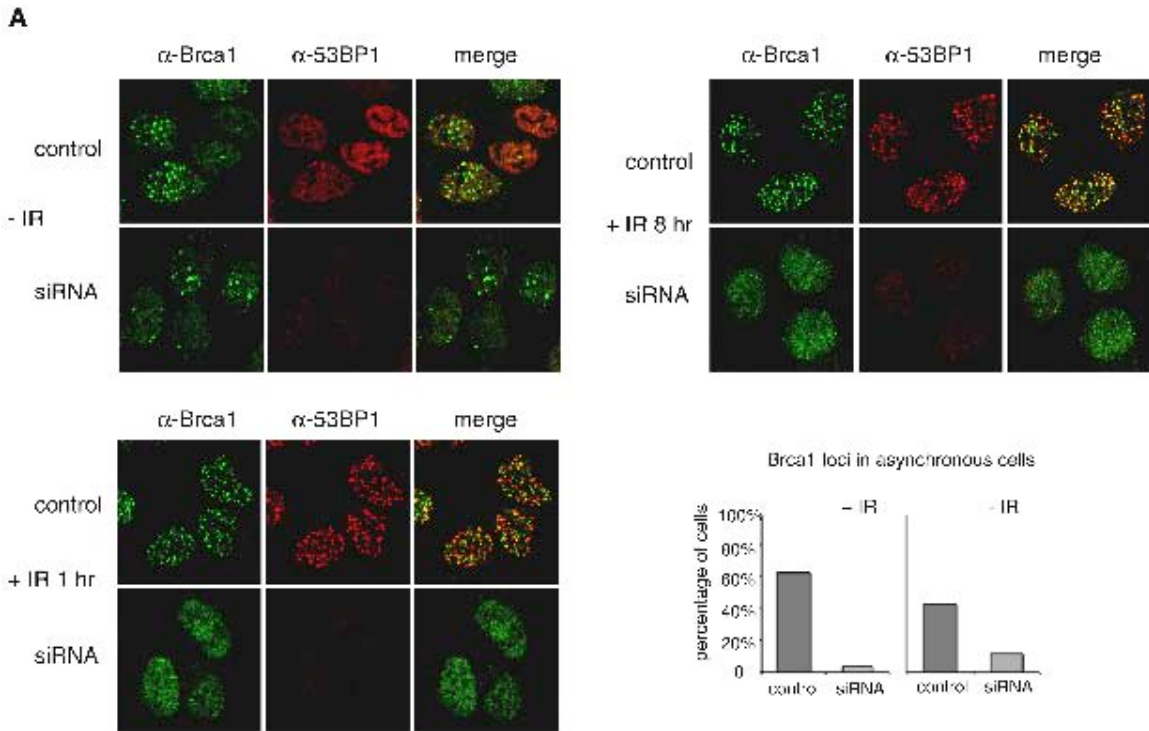
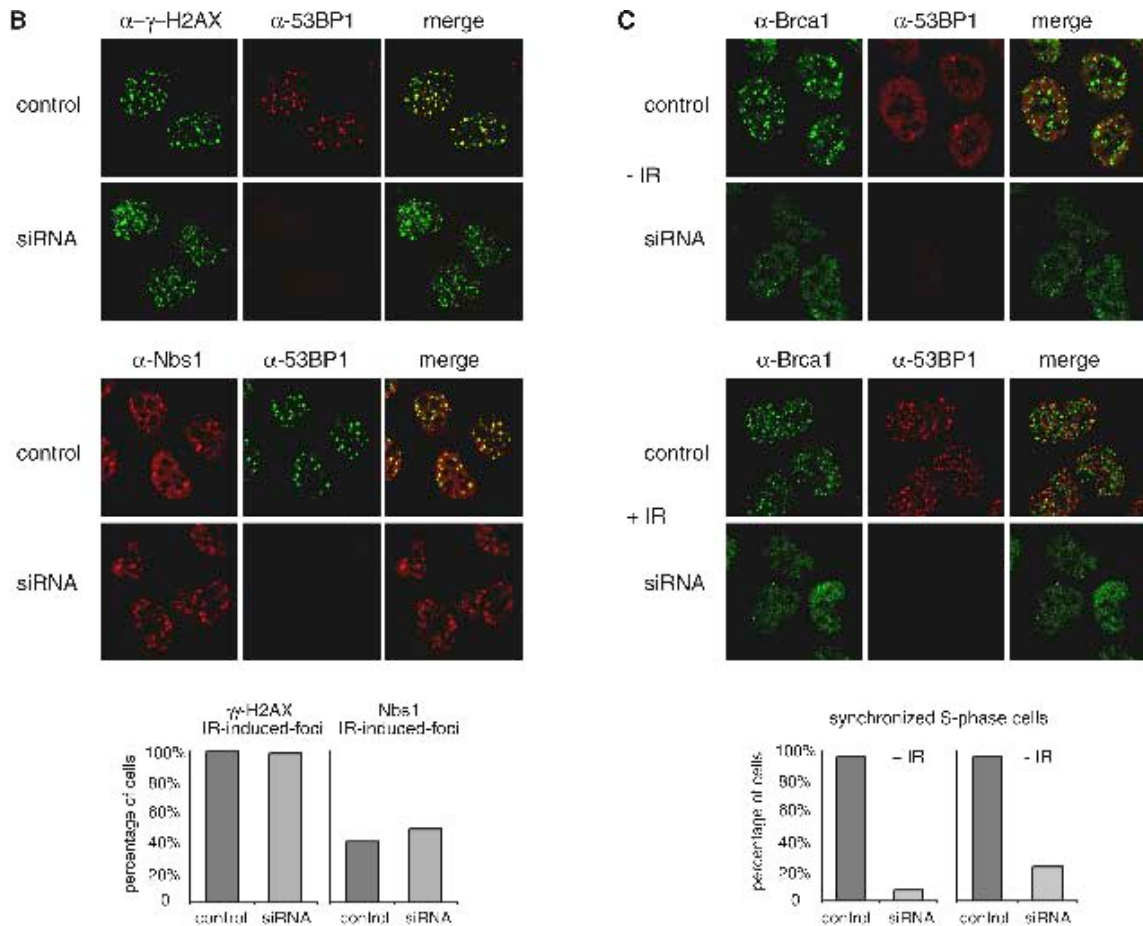


Fig. 9. Brca1 localization in S-phase and relocalization in response to IR is dependent on 53BP1. (A) Brca1 localization in the presence and absence of 10 Gy IR. U2OS cells were transfected with siRNA against 53BP1 or control oligos and 2 days later exposed to 10 Gy IR. At the indicated times after IR, cells were permeabilized with paraformaldehyde and fixed with Triton X-100. Immunostaining were performed using antibodies against 53BP1 and Brca1. Images were taken using a Zeiss confocal microscope. Quantitation of the BRCA1 foci are shown. These data were obtained using siRNA oligo pair #1 against 53BP1.

Chk2 or other proteins phosphorylated via the 53BP1 pathway were solely responsible for the foci recognized by these antibodies. Alternatively, 53BP1 might function as a general regulator of foci formation. To test this, we examined the ability of other proteins to form foci, in the absence of 53BP1. Brca1, Nbs1, and γ -H2AX all form foci in response to IR (72). IR-induced Brca1 foci formation was largely abolished in

53BP1-inhibited cells. Brca1 showed diffuse staining and rarely formed distinctive foci in response to IR at different time points (Fig. 9A). In an asynchronous cell population, at



2 hr post-IR, only 4% of the cells formed Brca1 nuclear foci when cells were treated with 53BP1siRNA, compared to 60% of the control cells (Fig. 9A). Similar results were obtained in Hct116 and Hela cells with both oligo pairs (71). In contrast, formation of γ -H2AX foci or Nbs1 foci after IR remained unchanged in cells treated with control oligos or siRNA oligos (Fig. 9B). Rad51 foci were also unchanged (71).

When asynchronous control cells were analyzed for Brca1 foci formation in the absence of IR, approximately 40% contained more than 20 Brca1 foci, reflecting the S phase and G2 population. In 53BP1-inhibited cells, both the number of foci and the percentage of cells containing foci were reduced. Only 12% of 53BP1-inhibited cells contained more than 20 Brca1 foci (Fig. 9A). To control for cell cycle differences, we synchronized cells using a double-thymidine block (70), and S-phase cells (4 hours after release from the block) were used for immunostaining. BRCA1 foci were also dependent on 53BP1 in S-phase cells in the presence or absence of ionizing irradiation (Fig. 9C).

Although the IR-induced foci formation of Brca1 is dependent on the presence of 53BP1, Brca1 foci did not show complete colocalization with 53BP1 foci at early times (Fig. 9A). The strong effect on BRCA1 foci formation, coupled with the fact that the 53BP1 and BRCA1 foci do not initially fully overlap suggests that 53BP1 may regulate BRCA1 through a mechanism other than direct recruitment to foci. One means by which this might be achieved is through regulation of BRCA1 phosphorylation. In IR-treated

cells, Brca1 phosphorylation was reduced in the samples prepared from cells treated with siRNA oligos against 53BP1 relative to controls (Fig. 10A). As with the G2/M checkpoint, the strongest dependency of Brca1 phosphorylation appeared to be at lower

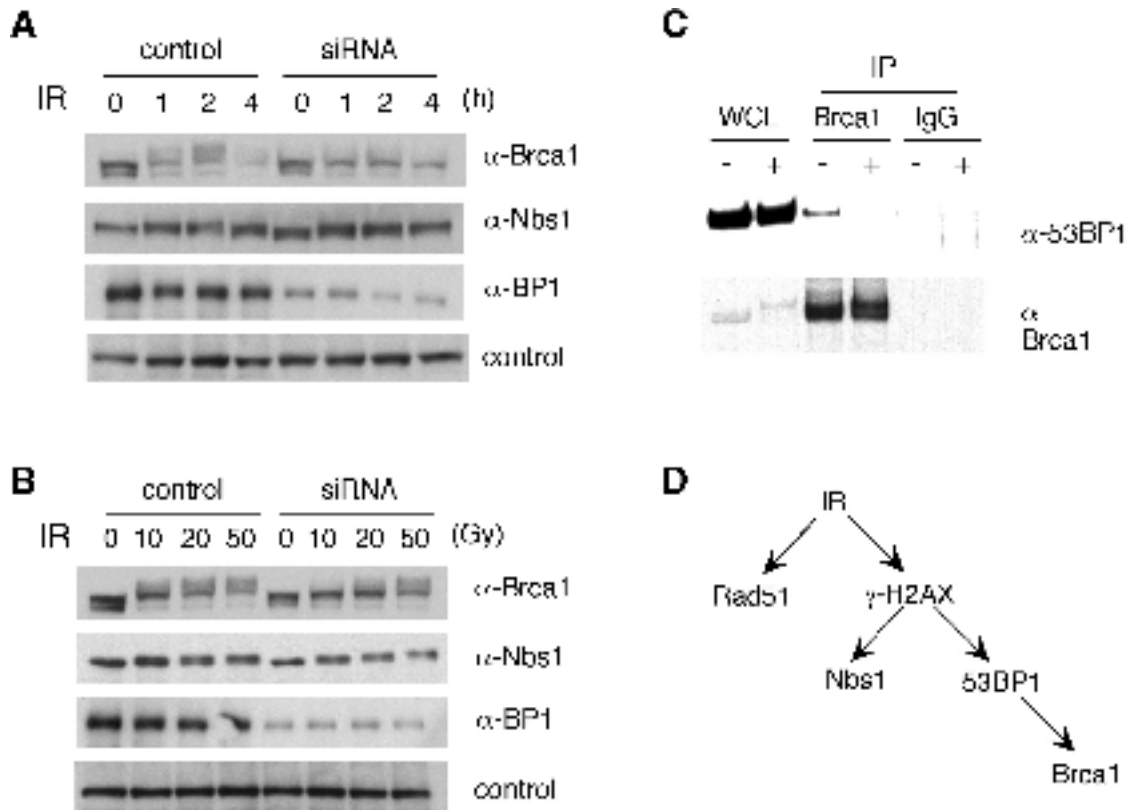


Fig. 10. 53BP1 regulation of Brca1. **(A)** Brca1 phosphorylation is reduced in the absence of 53BP1. U2OS cells were treated with siRNA oligos against 53BP1 or control oligos for two days. Cells were exposed to 10 Gy irradiation and cell lysates were prepared at indicated times after irradiation. Immunoblots were performed with antibodies against Brca1 (Oncogene), Nbs1 (Norvus) and 53BP1. The control band is a non-specific band from the same blot that was incubated with antibodies against Brca1. **(B)** Brca1 phosphorylation in response to different doses of irradiation. U2OS cells were transfected with siRNA oligos against 53BP1 or control oligos for two days, then treated with different doses of irradiation. Cell lysates were prepared at 2h after irradiation. **(C)** 53BP1 associates with Brca1. Cell lysates from untreated U2OS cells or 2 hour after exposure to 10 Gy IR were incubated with antibodies against Brca1 or rabbit IgG as a control. Western blots were performed using anti-53BP1 and anti-Brca1 antibodies (Oncogene). Ten percent of the cell lysate used for immunoprecipitation were loaded in the control lanes (WCL). **(D)** A schematic showing the genetic dependence for formation of nuclear foci for different proteins in response to IR.

doses of IR (Fig. 10B).

High levels of IR have been shown to obscure BRCA1 regulation by other proteins such as ATM (74). Loss of 53BP1 did not have a general effect on the DNA damage-inducible phosphorylation of other proteins. Nbs1 phosphorylation was not affected (Fig. 10A and 10B). Furthermore, while BRCA1 phosphorylation showed less dependency on 53BP1 at 50Gy IR, these cells still failed to form foci (71).

Next we examined whether 53BP1 associated with BRCA1. Brca1 interacts with 53BP1 in vivo, and like Chk2, this interaction was abolished in response to IR (Fig. 10C).

Thus, this dynamic association is likely to be important for regulation of 53BP1's ability to regulate both Chk2 and BRCA1 function in response to DNA damage.

We have continued efforts to identify additional Chk2 associated proteins but were unable to identify anything novel. In addition, we began to look for Chk2 substrates by a new phospho-proteomic methods we are currently collaborating on these new methods with other investigators here at Harvard. We are trying to identify all DNA Damage induced phospho-peptides in cells and hope to be able to sort out which belong to Chk1, Chk2, Atm and Atr in the future. This is a large undertaking and we do not yet know the boundaries of the effort. However, in summary we learned quite a bit about how Chk2 works and gets activated in response to Dna damage during the course of this award.

Key research Accomplishments

- A) Making a Chk2 knockout mouse in collaboration with T. MAK who did all the mouse work.
- B) Discovery that Chk2 controls apoptosis in ATM-dependnet and independent ways.
- C) Discovery that Chk2 interacts with 53BP1.
- D) Discovery that 53BP1 controls Chk2.

Reportable Outcomes

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Conclusions

We have shown in this study that there are differences between Chk2^{-/-} and ATM^{-/-} cells in the IR-induced activation of checkpoint and apoptotic responses, despite the fact that ATM regulates IR-induced Chk2 activity. ATM and Chk2 both modify p53 and activate it; however, our results show that at least some p53 phosphorylation is Chk2 dependent and ATM independent. Moreover, Chk2-dependent and ATM-dependent p53 phosphorylation events may differentially affect downstream p53-dependent transactivation targets. Specifi- cally, we have demonstrated that Chk2 predominantly regulates IR-induced apoptosis rather than the G1/S checkpoint in thymocytes, whereas ATM is predominantly involved in the regulation of the G1/S checkpoint. Contradictory results have been reported regarding the effect of ATM on p53-mediated apoptosis (3, 21, 24, 53). The discrepancies among these reports are not caused by strain differences among experimental mice but could be due to differences in methods used to detect apoptosis or to irradiation conditions (in vivo versus ex vivo). The in situ TUNEL assay may not be sensitive enough to detect differences in apoptosis in a thymus taken from an ATM^{-/-} mouse treated with wholebody irradiation, whereas in vitro irradiation of isolated ATM^{-/-} thymocytes reveals an apoptotic defect. In contrast, Chk2^{-/-} thymocytes examined using either method show obvious resistance to IR-induced apoptosis. In our

study, ATM^{-/-} thymocytes were more resistant than the wild type to IR but significantly less resistant than Chk2^{-/-} thymocytes. Consistent with the results of the apoptosis assay, the induction of Bax mRNA was more profoundly impaired in Chk2^{-/-} thymocytes than in ATM^{-/-} cells. This tight correlation between the induction of apoptosis and the transactivation of p53 downstream molecules suggests that the inhibition of apoptosis induced by the loss of Chk2

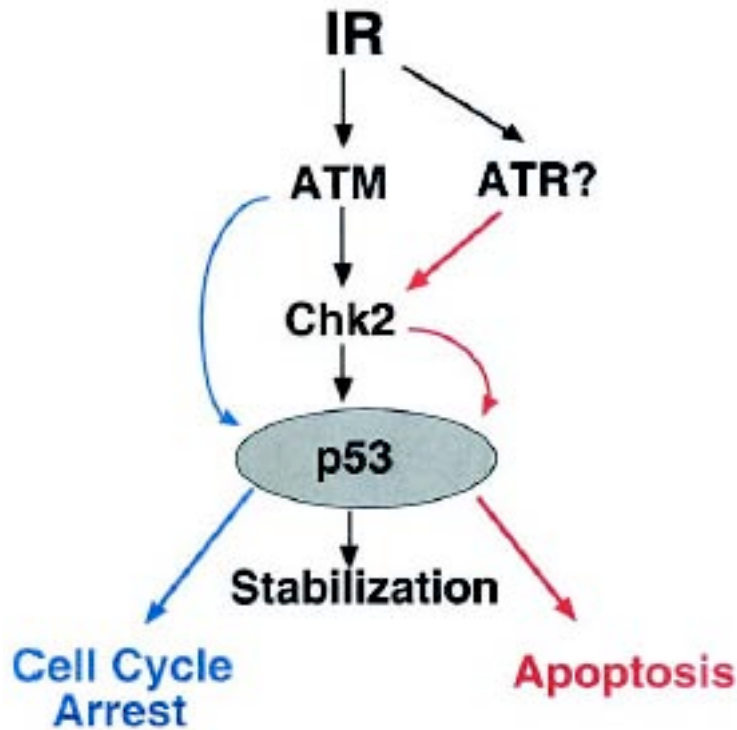


FIG. 11. Model of the regulation of p53 activation by Chk2 in response to IR. Chk2-mediated stabilization of p53 induced by IR and leading to apoptosis is controlled independently of ATM, possibly by ATR. ATM appears to stabilize p53, leading to cell cycle arrest without involving Chk2.

is caused by the suppression of p53 activation itself and not by effects on molecules further downstream in the apoptosis pathway.

We and others have proposed a model of IR-induced p53 activation leading to apoptosis

via Chk2. Mutation of the SQ/TQ sites on Chk2 abolished p53 activation leading to apoptosis, demonstrating that the phosphorylation of the SQ/TQ sites in the N-terminal region of Chk2 is essential for this process. Most of the phosphorylation of Chk2 induced by IR is abolished if ATM is absent. However, our data suggest that there is some ATM-independent phosphorylation at SQ/TQ sites of Chk2 in response to IR. It is possible that the very low level of ATM-independent phosphorylation of Chk2 that occurs is insufficient to alter the mobility of the protein. The most likely agent of ATM-independent Chk2 phosphorylation is ATR, although other members of the phosphatidylinositol 3-kinase family are also possibilities. ATR is a phosphatidylinositol 3-kinase-related kinase which contains a protein kinase domain similar in sequence to a region of *Schizosaccharomyces pombe rad3* (6, 13). Matsuoka et al. have reported that ATR phosphorylates Thr26, Ser50, and Thr68 in the SQ/TQ cluster domain of human Chk2 in vitro (35). Although ATR is believed to act primarily in response to a DNA replication block or UV-irradiation, it is also involved in responses to IR. Cells lacking ATR die within several days of exposure to IR; however, prior to their deaths, a profound defect in the IR-induced G2/M checkpoint can be demonstrated (16). Other studies have shown that overexpression of a kinase-dead mutation of ATR causes increased sensitivity to IR and a defect in the G2/M arrest and S-phase checkpoints (14). Furthermore, ATR mutation also abolishes DNA damage-induced phosphorylation of Ser15 on p53 (49).

These data suggest a potential functional overlap between ATM and ATR with respect to IR responses, consistent with our hypothesis that ATM and ATR cooperate in regulating p53 activity (Fig. 11). We theorize that ATR selectively regulates p53 activation leading to apoptosis via Chk2, while ATM governs cell cycle arrest in a Chk2-independent manner. In addition to phosphorylating p53, Chk2 is known to phosphorylate Cdc25. It has been reported that ATM and Chk2 are required for the S-phase checkpoint induced by IR and that this induction depends on Chk2-dependent phosphorylation of Cdc25A (22). In our study, we confirm that ATM is required for the S-phase checkpoint in primary MEFs but we also demonstrate that Chk2 is dispensable for this checkpoint in this cell type. In experiments with immortalized MEFs, we have observed a slightly slower onset and shorter duration of the Sphase checkpoint in the absence of Chk2 (unpublished data). This small difference may reflect differences in cell cycle parameters between primary and transformed cells. On balance, however, we believe that Chk2 is not essential for S-phase arrest in normal cells. It is possible that Chk1 can substitute for Chk2 in the phosphorylation of Cdc25 and that Chk2 thus has a redundant function in the intra-S-phase checkpoint. Previous reports concluding that Chk2 was required for the intra-Sphase checkpoint utilized overexpression constructs containing mutated Chk2 genes identified in sporadic colon cancer and as a germ line mutation in Li-Fraumeni syndrome (LFS). The data showed that these mutations had a dominant-negative impact in wild-type cells. Constructs containing such mutations could therefore have an inhibitory effect on a downstream component of the S-phase checkpoint (such as Cdc25), thereby preventing the operation of any compensatory Chk1-dependent process. Alternatively, differences in Chk2 dependency in different tissues or between mice and humans may underlie the discrepancy between the previous reports and our data. Failures in the transcriptional response to damage, cell cycle arrest and apoptosis induced by IR should lead to a higher incidence of tumor development. Unexpectedly, however, Chk2^{-/-} mice do not have obvious tumors, unlike ATM^{-/-} mice, which die within 4 months of birth with thymic lymphomas. Interestingly, the thymic lymphomas in ATM^{-/-} mice are critically dependent on V(D)J recombination, whereas thymic lymphomas in p53^{-/-} mice arise independent of V(D)J recombination (30). These observations indicate that at least two different mechanisms of lymphoma development are at work in these mutant animals, such that ATM-mediated p53 activation may not be required for the development of lymphomas. ATM phosphorylates many target substrates in addition to Chk2 and p53, including Nbs1 and Brcal (17, 31). These molecules, which act downstream of ATM, may contribute in an unknown way to the prevention of spontaneous lymphoma development. Mutations of Chk2 are found more frequently in patients with variant LFS, which has a moderate phenotype, than in patients with classical LFS (5). In contrast, mutations of p53 have been reported in 70% of classical LFS cases and 20% of variant LFS patients (50). Although the tumor-suppressive effect of Chk2 appears to be milder than that of p53, loss of Chk2 clearly increased the incidence of DMBA-induced skin tumors in mice. DBMA treatment resembles UV-irradiation in that both cause DNA adducts that are repaired by the nucleotide excision repair system. This similarity in DNA lesions suggests that the DBMA-induced skin tumors appearing in Chk2^{-/-} mice could be the result of a failure in the tumor-suppressive effect by ATR.

In conclusion, the results of this part of the study have shown that p53 activation leading to cell cycle arrest is regulated differently from that leading to apoptosis. Chk2 is required for p53-mediated apoptosis of thymocytes and must undergo phosphorylation in order to function, but this phosphorylation is not carried out by ATM. We propose a new model for the IR pathway that emphasizes the independent effects of Chk2 and ATM.

The major finding of the second half of these studies is that 53BP1 is a critical transducer of the DNA damage signal and is required for both the intra-S phase and G2/M checkpoints and similar results have been obtained by others (R. DiTullio and T. Halazonetis, personal communication). It is clearly part of a partially redundant branch of the signaling apparatus and its loss results in a partial decrease in phosphorylation of key checkpoint target proteins. As it binds to p53, Chk2 and Brca1 and controls the phosphorylation of at least two of these proteins, it has the property of a mammalian adaptor or mediator that might recruit a subset of substrates to the ATM/ATR-ATRIP checkpoint kinases.

A second key finding of this part of the study is that the pathway leading to the assembly of repair/signaling foci in response to damage is branched and shows a regulatory hierarchy in which H2AX is required for Nbs1 and 53BP1 foci (75) and 53BP1 controls the ability of at least BRCA1 but not Nbs1 to form foci as depicted in the pathway model. The nature of this disruption in foci formation is unknown but may be related to the role of 53BP1 in control of phosphorylation of these or other proteins. Regardless of the mechanism, it is clear that 53BP1 is a central transducer of the DNA damage signal to p53 and other tumor suppressor proteins and is likely to play an important role in the maintenance of genomic stability and prevention of cancer (76, 77). We have also recently helped make 53BP1 mutant cells which have many of the properties we observed for the siRNA treated cells. These cells were derived from a 53BP1 mutant mouse generated by Phil Carpenter. We did not contribute to making the mouse, we worked with only the mouse embryonic fibroblasts from these mice. That work is described in Morales et al. (2003) *J. Biol. Chem.* 278:14971-7. We played only a small role in that project.

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