Published in final edited form as:
Mol Cell. 2008 July 11; 31(1): 21–32. doi:10.1016/j.molcel.2008.04.028.

Chk2 suppresses the oncogenic potential of DNA replication-associated DNA damage

Travis H. Stracker[1], **Suzana S. Couto**[2], **Carlos Cordon-Cardo**[3], **Tulio Matos**[3], and **John H. J. Petrini**[14]

1*Molecular Biology Program, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center and Cornell University Graduate School of Medical Sciences, 1275 York Avenue, New York, NY10021, USA*

2*Pathology and Laboratory Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA*

3*Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA*

Summary

The Mre11 complex (Mre11, Rad50, and Nbs1) and Chk2 have been implicated in the DNA damage response, an inducible process required for the suppression of malignancy. The Mre11 complex is predominantly required for repair and checkpoint activation in S phase, while Chk2 governs apoptosis. We examined the relationship between the Mre11 complex and Chk2 in the DNA damage response via the establishment of $Nbs1^{\Delta B/\Delta B}$ $Chk2^{-/-}$ and $Mre11^{ATLD1/ATLD1}$ $Chk2^{-/-}$ mice. Chk2 deficiency did not modify the checkpoint defects or chromosomal instability of Mre11 complex mutants; however, the double mutant mice exhibited synergistic defects in DNA damage-induced p53 regulation and apoptosis. $Nbs1^{\Delta B/\Delta B}$ $Chk2^{-/-}$ and $Mre11^{ATLD1/ATLD1}$ $Chk2^{-/-}$ mice were also predisposed to tumors. In contrast, DNA-PKcs deficient mice, in which G1-specific chromosome breaks are present, did not exhibit synergy with $Chk2^{-/-}$ mutants. These data suggest that Chk2 suppresses the oncogenic potential of DNA damage arising during S and G2 phases of the cell cycle.

Keywords

Mre11; Nbs1; Rad50; Chk2; Atm; p53; apoptosis; tumorigenesis

Introduction

The Mre11 complex, consisting of Mre11, Rad50 and Nbs1 (Xrs2 in S. cerevisiae), plays a central role in the cellular response to DNA double-strand breaks (DSBs). In addition to promoting recombinational DNA repair (Bressan et al., 1999), the complex acts as a DSB sensor and modulates the activity of the primary transducing kinases Ataxia-telangiectasia mutated (ATM) and ATM and RAD3 related (ATR) (Lee and Paull, 2007; Petrini and Stracker, 2003). The Mre11 complex is required for the activation of ATM and also functions downstream of ATM to facilitate the phosphorylation of numerous substrates including Smc1 and Chk2 (Lee and Paull, 2007). ATM is required for checkpoint activation throughout the cell cycle (Shiloh, 2003) and controls the intra-S phase checkpoint via the effector kinases Chk1

4Please direct all correspondence to petrinij@mskcc.org.

and Chk2 as well as Nbs1 and the cohesin subunit SMC1, with the latter two proteins acting in a parallel pathway to Chk2 (Falck et al., 2002; Kitagawa et al., 2004).

ATM is required for p53 regulation (Kastan et al., 1992) and promotes apoptosis in response to diverse stimuli including DSBs (Xu and Baltimore, 1996), oncogene expression (ie. *Myc* and *E2F1*)(Powers et al., 2004; Pusapati et al., 2006; Rogoff et al., 2004), and genotoxic stress induced by the $Rad50^{S}$ allele (Morales et al., 2005). As a result, $Atm^{-/-}$ mice exhibit apoptotic defects in numerous tissues, including lymphoid organs and the central nervous system (CNS) (Herzog et al., 1998; Westphal et al., 1997; Xu and Baltimore, 1996).

Several lines of evidence support the view that the Mre11 complex exerts a significant influence on p53-dependent apoptosis via its functional interaction with ATM. First, the precipitous apoptotic attrition of hematopoietic and spermatogenic cells in $Rad50^{S/S}$ mice is acutely dependent on ATM; loss of a single *ATM* allele (in $Rad50^{S/S}$ $Atm^{+/-}$ mice) mitigates $Rad50^{S/S}$-induced cellular attrition (Morales et al., 2005). We have recently shown that apoptosis is blocked in multiple tissues of $Rad50^{S/S}$ mice that express a C-terminally truncated Nbs1 variant lacking an ATM interaction domain ($Nbs1^{\Delta C}$) (Falck et al., 2005; Stracker et al., 2007; You et al., 2005), and that this C-terminal Nbs1 domain is required for ATM-dependent apoptosis in response to ionizing radiation (IR) in lymphoid cells (Stracker et al., 2007).

Chk2 also contributes to the regulation of p53-dependent apoptosis. $Chk2^{-/-}$ mice exhibit defects in p53 phosphorylation, stabilization, and p53-dependent transcriptional events in response to damaging agents (Hirao et al., 2002; Hirao et al., 2000; Takai et al., 2002). Accordingly, apoptosis is attenuated in the brain, thymus and other tissues of $Chk2^{-/-}$ mice (Hirao et al., 2002; Takai et al., 2002). Chk2 phosphorylates p53 on S20 (Chehab et al., 2000) (S23 in the mouse) and mice harboring the $p53^{S23A}$ allele showed an apoptotic phenotype similar to that of $Chk2^{-/-}$ mice (MacPherson et al., 2004). Although $Chk2^{-/-}$ mice are not predisposed to spontaneous tumors (Hirao et al., 2002), a non-null Chk2 allele predisposes to tumorigenesis in transgenic mice (Kwak et al., 2006) and several Chk2 mutations have been identified as low penetrance alleles in human malignancies including breast and prostate cancer (Nevanlinna and Bartek, 2006).

Single gene disorders arising from mutations in the *ATM*, *MRE11*, or *NBS1* genes present with extensive phenotypic overlap (Ataxia-telangiectasia, A-T, *ATM* deficiency; Ataxia-telangiectasia like disorder, A-TLD, *Mre11* hypomorphism; and Nijmegen breakage syndrome, NBS, *NBS1* hypomorphism) (Shiloh, 2003; Stracker et al., 2004). Cell lines from these human patients, or from murine models of the human diseases (Figure 1A), exhibit defective responses to DNA double-strand break inducing agents, including cell cycle checkpoint defects, chromosomal instability, and increased sensitivity (Barlow et al., 1996; Theunissen et al., 2003; Williams et al., 2002; Xu and Baltimore, 1996). Given the functional interdependence of the Mre11 complex and ATM, and the fact that ATM regulates both Chk2 and p53, it has been suggested that defects in the activation of Chk2 is among the pathologic consequences of these disorders (Bartek et al., 2001).

To test this idea, and to further define the relative disposition of Chk2 and the Mre11 complex in the DSB response, we generated $Nbs1^{\Delta B/\Delta B}$ $Chk2^{-/-}$ and $Mre11^{ATLD1/ATLD1}$ $Chk2^{-/-}$ mice. Loss of *Chk2* did not strongly affect the checkpoint phenotypes, damage sensitivity, or chromosomal instability resulting from Mre11 complex hypomorphism. However, both $Nbs1^{\Delta B/\Delta B}$ $Chk2^{-/-}$ and $Mre11^{ATLD1/ATLD1}Chk2^{-/-}$ double mutant animals exhibited apoptotic defects and were predisposed to a wide spectrum of tumors. Tumors were not evident in $Prkdc^{scid/scid}$ $Chk2^{-/-}$ double mutant mice in which DSBs generated in G0/G1 phase lymphoid cells are stabilized (Roth et al., 1992). These results suggest that Chk2 is required to suppress the oncogenic potential of DNA replication-associated DNA damage.

Results

Chk2 deficiency does not exacerbate the cell cycle checkpoint and DNA repair defects of Mre11 complex hypomorphs

Chk2 activation appears to require phosphorylation by ATM (Ahn et al., 2000; Falck et al., 2001; Melchionna et al., 2000). ATM activity is attenuated in $Nbs1^{\Delta B/\Delta B}$ and $Mre11^{ATLD1/ATLD1}$ cells; this is correlated with decreased Chk2 phosphorylation upon IR treatment in those mutants (Theunissen et al., 2003). Were Mre11 complex hypomorphism to abolish Chk2 activation, Chk2 deficiency would not be expected to exacerbate the phenotypes of $Mre11^{ATLD1/ATLD1}$ and $Nbs1^{\Delta B/\Delta B}$ cells. Conversely, if those mutant settings retain some degree of Chk2 function, then phenotypic additivity would be observed in Chk2 deficient Mre11 complex mutants. To test these predictions, mice were intercrossed to generate $Nbs1^{\Delta B/\Delta B}$ $Chk2^{-/-}$ and $Mre11^{ATLD1/ATLD1}$ $Chk2^{-/-}$ mice and the ability of double mutant cells mount a DNA damage response was assessed.

The intra-S phase checkpoint suppresses the rate of DNA synthesis in response to DNA damage. Whereas DNA synthesis was suppressed following IR in $Chk2^{-/-}$ murine embryo fibroblasts (MEFs) to the same extent as *WT* cells, it was suppressed to a lesser extent in $Nbs1^{\Delta B/\Delta B}$ and $Mre11^{ATLD1/ATLD1}$ cells as previously reported (Figure 1B) (Takai et al., 2002; Theunissen et al., 2003; Williams et al., 2002). Loss of *Chk2* increased the severity of the intra-S phase defect in $Nbs1^{\Delta B/\Delta B}$ $Chk2^{-/-}$ (DNA synthesis suppression of 26%) compared to $Nbs1^{\Delta B/\Delta B}$ (DNA synthesis suppression of 38%) after 10 Gy but did not exacerbate the phenotype of $Mre11^{ATLD1/ATLD1}$ $Chk2^{-/-}$ cells (Figure 1B).

Inhibition of the G2/M transition following DNA damage (the G2/M checkpoint), which is defective in $Nbs1^{\Delta B/\Delta B}$ and $Mre11^{ATLD1/ATLD1}$ cultures, occurred normally in $Chk2^{-/-}$ MEFs 1 hour after IR treatment (Figure 1C) (Takai et al., 2002; Theunissen et al., 2003; Williams et al., 2002). Chk2 deficiency did not enhance the G2/M checkpoint defects associated in Mre11 complex hypomorphism in $Nbs1^{\Delta B/\Delta B}$ $Chk2^{-/-}$ and $Mre11^{ATLD1/ATLD1}$ $Chk2^{-/-}$ cultures (Figure 1C).

DNA damage-induced cell cycle arrest at the G1/S transition (the G1/S checkpoint) is primarily governed by p53 (Kuerbitz et al., 1992), and is partially impaired by ATM deficiency (Xu et al., 1998). Although, like ATM, Chk2 regulates p53, differing results regarding its role in the G1/S checkpoint have been reported (Cao et al., 2006; Hirao et al., 2002; Jack et al., 2002; Takai et al., 2002). To examine the G1/S checkpoint in Chk2 deficient Mre11 complex mutants, cells were exposed to IR and the percentage of cells in S phase 14hrs post treatment was measured by BrdU incorporation. Whereas greater than 90% of $p53^{-/-}$ cells had entered S phase, only 38% of *WT* had resumed cell cycle (Figure 1D). $Chk2^{-/-}$ and $Mre11^{ATLD1/ATLD1}$ $Chk2^{-/-}$ were both mildly defective in the G1/S arrest with 50–60% of cells in S phase 14 hrs post IR (Figure 1D). These data indicate that neither $Mre11^{ATLD1/ATLD1}$ nor Chk2 deficiency exerts a pronounced effect on the G1/S checkpoint.

Consistent with the lack of additivity in DNA damage dependent cell cycle checkpoint responses, Chk2 deficiency did not modify the IR sensitivity of either $Nbs1^{\Delta B/\Delta B}$ (data not shown) or $Mre11^{ATLD1/ATLD1}$ (Figure 1E). Similarly, $Chk2^{-/-}$ splenocytes did not show increased spontaneous or IR induced chromosomal lesions and both $Mre11^{ATLD1/ATLD1}$ and $Mre11^{ATLD1/ATLD1}$ $Chk2^{-/-}$ cells showed similar levels of chromosomal instability, primarily chromatid type breaks (Figure 1F and Supplemental Table S1). Together these data demonstrate that loss of *Chk2* does not exacerbate the checkpoint deficiency, DSB sensitivity or chromosomal instability conferred by the hypomorphic $Mre11^{ATLD1/ATLD1}$ or $Nbs1^{\Delta B/\Delta B}$ alleles.

The Mre11 complex and Chk2 effect p53-dependent apoptosis through parallel pathways

We previously showed that deficiencies in ATM and Chk2 confer additive apoptotic defects in IR-treated thymocytes, suggesting that they act in parallel to effect p53-dependent apoptosis (Stracker et al., 2007). That ATM activity is impaired in $Nbs1^{\Delta B/\Delta B}$ and $Mre11^{ATLD1/ATLD1}$ predicts that Mre11 complex hypomorphism would also be additive with Chk2 deficiency for apoptotic defects. Whereas $Mre11^{ATLD1/ATLD1}$ exhibited apoptotic defects at IR doses ranging from 0.5 to 8 Gy, $Nbs1^{\Delta B/\Delta B}$ was indistinguishable from *WT* over the same range (Figure 2A). The apoptotic defect in $Mre11^{ATLD1/ATLD1}$ was markedly enhanced by Chk2 deficiency, as thymocytes from $Mre11^{ATLD1/ATLD1}$ $Chk2^{-/-}$ were as defective as $p53^{-/-}$ thymocytes for IR-induced apoptosis (Figure 2B). In contrast, $Nbs1^{\Delta B/\Delta B}$ $Chk2^{-/-}$ was not significantly different than that of $Chk2^{-/-}$ (Figure 2B). No differences in dexamethasone induced apoptosis, which is p53 independent, were evident in any of the genotypes (Supplemental Figure S1).

Despite their differing effects on apoptosis, IR-induced Chk2 phosphorylation was markedly impaired in both $Mre11^{ATLD1/ATLD1}$ and $Nbs1^{\Delta B/\Delta B}$ thymocytes (Figure 3A). The Chk2 phosphorylation defect was not correlated with defects in ATM activation (inferred from S1987 phosphorylation (Bakkenist and Kastan, 2003)), as ATM autophosphorylation was impaired in $Mre11^{ATLD1/ATLD1}$ thymocytes but unaffected in $Nbs1^{\Delta B/\Delta B}$ (Figure 3A) (Morales et al., 2005). Hence, both hypomorphic Mre11 complex alleles impair the modification of ATM substrates such as Chk2 (Figure 3A) and the pro-apoptotic effector BID (Supplemental Figure S2)—a reflection of ATM activity—whereas the $Mre11^{ATLD1/ATLD1}$ allele also affects ATM activation.

The effects of $Nbs1^{\Delta B/\Delta B}$ and $Mre11^{ATLD1/ATLD1}$ on p53 phosphorylation support this interpretation. Phosphorylation and stabilization of p53 is influenced by both ATM and Chk2 (Barlow et al., 1997; Hirao et al., 2002; Kastan et al., 1992; Takai et al., 2002). We observed reduced levels of S18-phosphorylated (ATM consensus site) and total p53 in $Mre11^{ATLD1/ATLD1}$ thymocytes after IR treatment (Figure 3B), whereas p53 phosphorylation and stabilization was normal in $Nbs1^{\Delta B/\Delta B}$ cells (Supplemental Figure S3) (Morales et al., 2005). These data suggest that impaired ATM activation in $Mre11^{ATLD1/ATLD1}$ accounts for reduced p53 phosphorylation and may further account for the additive defects of $Mre11^{ATLD1/ATLD1}$ and $Chk2^{-/-}$ in apoptotic induction.

p53 responses in $Mre11^{ATLD1/ATLD1}$ $Chk2^{-/-}$ mice

The apoptotic defect in $Mre11^{ATLD1/ATLD1}$ $Chk2^{-/-}$ thymocytes is correlated with the virtually complete loss of DNA damage induced-p53 stabilization. Stabilization of p53 was slightly diminished in each of the single mutants at 2 and 4 hrs post IR, but p53 was barely detectable at those time points in $Mre11^{ATLD1/ATLD1}$ $Chk2^{-/-}$ double mutants (Figure 3C). Loss of DNA damage-dependent p53 regulation, as opposed to basal p53 expression, underlies the observed defect as $Mre11^{ATLD1/ATLD1}$ $Chk2^{-/-}$ thymocytes treated with the proteasome inhibitor MG132 exhibited similar levels of p53 as *WT* cells (Supplemental Figure S4).

Central to p53's influence on the DNA damage response and tumor suppression is its role in transcriptional regulation. Using quantitative PCR (Q-PCR), the induction of the proapoptotic genes *Bbc3/Puma* and *Bax* in irradiated thymocytes was analyzed. In *WT* cells these p53 target genes are induced approximately 12 and 8 fold respectively, and unchanged by IR in $p53^{-/-}$ cells as expected (Figure 4A). Whereas their induction was 30–50% of *WT* in $Chk2^{-/-}$, $Mre11^{ATLD1/ATLD1}$, and $Atm^{-/-}$ single mutants, *Bbc3/Puma* and *Bax* induction was similar to $p53^{-/-}$ in $Mre11^{ATLD1/ATLD1}$ $Chk2^{-/-}$ double mutants, consistent with the observed p53 stabilization defect (Figure 4A).

The transcriptional effects on *Bbc3/Puma* and *Bax* seen by Q-PCR in $Chk2^{-/-}$ and $Atm^{-/-}$ were a reflection of global effects on DNA damage-induced transcriptional responses in these mutants. Microarray analysis of transcriptome changes in *WT*, $Atm^{-/-}$ and $Chk2^{-/-}$ thymocytes following IR treatment was carried out. When compared to *WT* cells, few statistically significant changes that were specific to $Chk2^{-/-}$ or $Atm^{-/-}$ were noted in 2338 probe sets that were induced or repressed after IR treatment (Figure 4B, 4C, Supplemental Table S2) Together these data indicate that Chk2 and the ATM/Mre11 complex pathway have independent, global effects on p53 transcriptional programs and apoptosis and argue against p53 transcriptional targets that are specifically regulated by either arm of the apoptotic response (Figure 4D).

Chk2 is required to suppress tumorigenesis in diverse tissues of animals expressing hypomorphic Mre11 complex alleles

Whereas none of the single mutants were predisposed to malignancy (Supplemental Figure S5, S6 and Takai et al., 2002; Theunissen et al., 2003; Williams et al., 2002) survival of both $Nbs1^{\Delta B/\Delta B}$ $Chk2^{-/-}$ and $Mre11^{ATLD1/ATLD1}$ $Chk2^{-/-}$ was reduced, with roughly 40% of each cohort succumbing to malignancy within 2 years (Figure 5A, 5B, Supplemental Figure S6 and Supplemental Tables S3, S4). In contrast, no reduction in the tumor latency or spectrum seen in $Atm^{-/-}$ was evident in $Atm^{-/-}$ $Chk2^{-/-}$ (Figure 5C).

Contrary to expectations based on the defects in p53 regulation observed in $Nbs1^{\Delta B/\Delta B}$ $Chk2^{-/-}$ nor $Mre11^{ATLD1/ATLD1}$ $Chk2^{-/-}$ mice, neither displayed the penetrant, early onset lymphoma characteristic of $p53^{-/-}$ or $Atm^{-/-}$ animals (Barlow et al., 1996; Donehower et al., 1992; Xu et al., 1996). Instead, both double mutants developed a wide variety of tumor types. $Atm^{-/-}$ (or $Atm^{-/-}$ $Chk2^{-/-}$) developed only T-cell lymphomas, whereas $Mre11^{ATLD1/ATLD1}$ $Chk2^{-/-}$ and $Nbs1^{\Delta B/\Delta B}$ $Chk2^{-/-}$ mice developed lymphomas that expressed T, B, or macrophage markers (histiocytic sarcoma) as well as biphenotypic lymphoblastic lymphomas that were positive for both T and B cell markers (CD3 and B220- Supplemental Figure S7). $Mre11^{ATLD1/ATLD1}$ $Chk2^{-/-}$ and $Nbs1^{\Delta B/\Delta B}$ $Chk2^{-/-}$ mice also developed a wide range of highly proliferative solid tumors including carcinomas and sarcomas in multiple tissues in which apoptosis was absent or detected to a very minimal extent (Figure 5D, Supplemental Figure S7, Supplemental Table S4, and data not shown).

To determine whether tumorigenesis in $Nbs1^{\Delta B/\Delta B}$ $Chk2^{-/-}$ and $Mre11^{ATLD1/ATLD1}$ $Chk2^{-/-}$ mice was associated with p53 inactivation, DNA or RNA from 13 representative tumors was used for sequence analysis of *p53*. For genomic DNA samples, exons 5–10 of p53, encompassing the p53 DNA binding domain that contains the nucleotides most frequently mutated in tumors (UMD p53 database 2007_R1; http://p53.free.fr/; (Soussi and Wiman, 2007)), was sequenced and from cDNA preparations we analyzed the entirety of the p53 coding sequence (Exons 2–11). These analyses revealed no *p53* mutations in any of the tumor samples analyzed (Table 1). As mutations in $p19^{ARF}$ could also reduce the selective pressure for *p53* mutations, we amplified and sequenced $p19^{ARF}$ from cDNA prepared from 7 tumors. In all cases, *p53* was wild type and in 5 of 7 tumors $p19^{ARF}$ was wild type (Table 1) and was not amplifiable from the remaining 2 (presumably due to mutation or deletion). These results are consistent with a diminished selective pressure against p53 function during tumorigenesis in the context of Chk2 deficiency and impaired Mre11 complex activity.

The tumor suppressive effects of Chk2 are specific for replication associated chromosome instability

As chromosomal instability arises predominantly during S-phase in Mre11 complex hypomorphs, the tumor predispostion of $Nbs1^{\Delta B/\Delta B}$ $Chk2^{-/-}$ and $Mre11^{ATLD1/ATLD1}$ $Chk2^{-/-}$ mice indicates that Chk2 suppresses the oncogenic potential of replication-associated damage. To determine if Chk2 was also responsive to lesions arising in G1, we generated mice

doubly deficient for *Chk2* and the catalytic subunit of DNA-PK (DNA-PKcs), encoded by the *Prkdc* gene. DSBs induced in G1/G0 by the RAG proteins during antigen receptor gene assembly are stabilized in $Prkdc^{scid/scid}$ animals (Roth et al., 1992). This triggers p53-dependent apoptosis in developing B and T lymphocytes, resulting in severe combined immune deficiency (Guidos et al., 1996). p53 deficiency partially mitigates the attrition of lymphocytes in $Prkdc^{scid/scid}$ mice, and $Prkdc^{scid/scid}$ $p53^{-/-}$ mice exhibit early onset B and T lymphoma (Guidos et al., 1996; Nacht et al., 1996).

In contrast, Chk2 does not appear to respond to RAG-induced DSBs, as no rescue of lymphoid organ cellularity was evident (thymus, spleen, and lymph nodes), and although survival was reduced due to severe immunodeficiency, no tumors developed in $Prkdc^{scidscid}$ $Chk2^{-/-}$ mice (Figure 6A, 6B, and data not shown). These results suggest that Chk2 activity is not required for the DSB response in G1 cells, and support the interpretation that its function is critical for reducing the oncogenic potential of replication associated DNA damage (Figure 6C).

Discussion

Genetic Interactions Between the *Mre11* Complex and *Chk2*

We previously established evidence that Nbs1 and Chk2 acted in parallel pathways to regulate the intra S-phase checkpoint (Falck et al., 2002). This conclusion is supported by enhancement of the $Nbs1^{\Delta B/\Delta B}$ intra-S phase checkpoint defect by *Chk2* deficiency reported here (Figure 1B). Other data suggest that the Mre11 complex and Chk2 act in the same pathway; Chk2 activation appears to require ATM-dependent phosphorylation of T68 (Ahn et al., 2000; Melchionna et al., 2000) that is dependent upon the Mre11 complex (Carson et al., 2003) and Chk2 phosphorylation is reduced in cells established from NBS and A-TLD patients (Uziel et al., 2003) and murine mutants that model Mre11 complex hypomorphism (Theunissen et al., 2003).

To examine this issue genetically, we generated $Nbs1^{\Delta B/\Delta B}$ $Chk2^{-/-}$ and $Mre11^{ATLD1/ATLD1}$ $Chk2^{-/-}$ double mutant mice and examined several phenotypic outcomes in cell cultures derived from them. The data demonstrate that Chk2 has substantial effects on apoptosis that are independent of the Mre11 complex and ATM, as double mutants exhibited apoptotic defects that far exceeded that of the single mutants (Figure 2B and (Stracker et al., 2007)). Further, $Nbs1^{\Delta B/\Delta B}$ $Chk2^{-/-}$ and $Mre11^{ATLD1/ATLD1}$ $Chk2^{-/-}$ mice developed a diverse spectrum of tumors, indicating that the penetrance of potentially oncogenic lesions arising in Mre11 complex mutants is suppressed by Chk2. Because Chk2 deficiency had a limited effect on cell cycle checkpoint, damage sensitivity, and chromosomal instability phenotypes in $Nbs1\Delta B/\Delta B$ and $Mre11^{ATLD1/ATLD1}$ mice (Figure 1), a parsimonious interpretation of this outcome is that the apoptotic function of Chk2 is the primary determinant of its tumor suppressive effect.

Our genetic data clearly demonstrates that Chk2 is functional for apoptosis in settings where its hyperphosphorylation is compromised ($Atm^{-/-}$, $Nbs1^{\Delta B/\Delta B}$ and $Mre11^{ATLD1/ATLD1}$). Reconstitution of $Chk2^{-/-}$ animals with mutant Chk2 (7 SQ/TQ sites mutated to alanine) is insufficient to complement the apoptotic defect, implicating phosphorylation as a prerequisite for Chk2 apoptotic activity (Hirao et al., 2002). Chk2 may be activated by other ATM-related kinases, such as ATR or DNA-PKcs that have been demonstrated to target Chk2 *in vitro* (Li and Stern, 2005; Matsuoka et al., 2000; Wang et al., 2006). As ATM and ATR show differences in their preference for Chk2 target residues (Matsuoka et al., 2000), this alternative mode of activation may involve the utilization of phosphorylation sites that do not produce the slow-migrating species present in wild type cells. Alternatively, Chk2 may have kinase-independent roles in apoptotic signaling. Previous biochemical analysis demonstrated that Chk2 kinase activity is severely impaired in the absence of ATM (Falck et al., 2001; Falck et al., 2002) and

our genetic results indicate that it is largely functional for apoptosis in that context (Stracker et al., 2007).

Chk2 and the DNA damage response

In *Nbs1*$^{\Delta B/\Delta B}$ and *Mre11*$^{ATLD1/ATLD1}$, DNA replication-associated chromosome instability is evident at the cytologic level, and is indicated genetically by the effect of these mutations on *p53*$^{+/-}$ mice in which tumor latency and spectrum are shifted to resemble *p53*$^{-/-}$ (Theunissen et al., 2003). Despite the chromosome instability and the intra-S and G2/M checkpoint defects caused by Mre11 complex hypomorphism, *Nbs1*$^{\Delta B/\Delta B}$ and *Mre11*$^{ATLD1/ATLD1}$ are not predisposed to malignancy (Theunissen et al., 2003; Williams et al., 2002). On this basis, we proposed that a high rate of chromosome breakage-induced cell death may contribute to suppressing the penetrance of potentially oncogenic chromosome rearrangements (Petrini and Theunissen, 2004).

The occurrence of multiple and diverse tumors in *Nbs1*$^{\Delta B/\Delta B}$ *Chk2*$^{-/-}$ and *Mre11*$^{ATLD1/ATLD1}$ *Chk2*$^{-/-}$ mice clearly demonstrates that Chk2 also contributes substantially to suppressing the oncogenic potential of *Mre11*$^{ATLD1/ATLD1}$ and *Nbs1*$\Delta^{B/\Delta B}$. The phenotypic presentation of cells established from *Chk2*$^{-/-}$ mice is not consistent with the view that Chk2 plays a significant role in DSB-induced cell cycle checkpoint regulation (Hirao et al., 2002; Jack et al., 2002; Takai et al., 2002). This is somewhat paradoxical given the primary role of the yeast Chk2 orthologs Rad53 (*S. cerevisiae*) and Cds1 (*S. pombe*) in affecting checkpoint functions in their respective contexts (Allen et al., 1994; Boddy et al., 1998; Lindsay et al., 1998; Paulovich and Hartwell, 1995; Zheng et al., 1993). However, in both yeast and mice, these orthologous proteins influence DNA damage-dependent transcriptional changes (Allen et al., 1994). The apparent cell cycle specificity of Chk2 tumor suppressive functions also may provide some reconciliation of this issue. Like Rad53 and Cds1, Chk2 may indeed affect the S-phase DNA damage response, but in mammals, its role in the response to DNA replication-associated chromosome breakage would be confined to tumor suppression and apoptosis.

This interpretation is supported by the phenotypes of both *Brca1*$^{\Delta 11/\Delta 11}$ *Chk2*$^{-/-}$ and *Prkdc*$^{scid/scid}$ *Chk2*$^{-/-}$ mice. Like the Mre11 complex, Brca1 functions in promoting homologous recombination mediated repair and contributes to S and G2/M phase checkpoint responses (Boulton, 2006). *Brca1*$^{\Delta 11/\Delta 11}$ *Chk2*$^{-/-}$ mice developed tumors with a similar latency as *Nbs1*$^{\Delta B/\Delta B}$ *Chk2*$^{-/-}$ and *Mre11*$^{ATLD1/ATLD1}$ *Chk2*$^{-/-}$ double mutants, but exhibited a more circumscribed tumor spectrum (Cao et al., 2006). Similarly, conditional deletion of Brca1 in the thymus or mammary gland of *Chk2*$^{-/-}$ mice predisposed to tumorigenesis (McPherson et al., 2004).

The PRKDC gene product, DNA-PKcs, is required for the rejoining of DSBs created during antigen receptor gene assembly (V(D)J) recombination (Bassing and Alt, 2004). The induction of these breaks is restricted to the G1 phase of the cell cycle (Lin and Desiderio, 1995), and failure to rejoin them in a timely manner results in p53 activation and apoptotic death of the developing B and T cells. In *Prkdc*$^{scid/scid}$ *p53*$^{-/-}$ double mutant mice, early onset (7–12 weeks; earlier than *p53*$^{-/-}$) pro-B and immature-T lymphoma is observed. This, as well as studies utilizing both Artemis and Ligase 4 deficient mice, indicates that p53 suppresses the oncogenic potential of V(D)J-associated DSBs (Gao et al., 1998; Guidos et al., 1996; Nacht et al., 1996; Rooney et al., 2004). In contrast, Chk2 deficiency had no effect on the attrition of *Prkdc*$^{scid/scid}$ B and T cell progenitors and *Prkdc*$^{scid/scid}$ *Chk2*$^{-/-}$ double mutant mice are not tumor prone (Figure 6 and data not shown). These data demonstrate that DSBs induced in G1 lymphocytes do not trigger Chk2-dependent apoptosis, and that the oncogenic potential of DSBs in G1 cells is not suppressed by Chk2.

Apoptosis and Checkpoints in Tumor Suppression

Chk2 deficiency strongly attenuated DNA damage-induced p53-dependent transcriptional changes (Figure 4) (Hirao et al., 2002;Takai et al., 2002). Given this result, the lack of overt cancer predisposition in *Chk2* null mice was surprising. This is likely to reflect a broader role in the DNA damage response for p53 than Chk2 with respect to the types of genotoxic stress it responds to or particular DNA lesions such as those generated during V(D)J recombination (Guidos et al., 1996).

An alternative explanation is that the G1/S checkpoint, which remains intact in $Chk2^{-/-}$, may play a significant role in tumor suppression. The significance of the G1/S checkpoint in this regard has been previously suggested from the analysis of mice expressing the $p53^{515C/515C}$ (R172P) allele (Liu et al., 2004). In these mice, the G1/S checkpoint is relatively normal but apoptotic induction is severely compromised. $p53^{515C/515C}$ mice do not present with early onset lymphomas, and instead develop sarcomas and other malignancies at 7 to 13 months of age. Tumors that arise in $p53^{515C/515C}$ do not exhibit the aneuploidy characteristic of $p53^{-/-}$. Attenuation of the G1/S checkpoint in these mice by deletion of the *p21* gene decreases tumor latency and enhances aneuploidy in the resulting tumors (Barboza et al., 2006). These data suggest that the G1/S checkpoint is critical for maintenance of euploidy and suppression of certain types of malignancy, and demonstrate that apoptotic induction and tumor suppression can be uncoupled.

Thus, it is conceivable that the absence of tumors in $Prkdc^{scid/scid}$ $Chk2^{-/-}$ mice may be partially attributable to the tumor suppressive effect of the G1/S checkpoint. The relatively long latency of tumorigenesis in $Nbs1^{\Delta B/\Delta B}$ $Chk2^{-/-}$ and $Mre11^{ATLD1/ATLD1}$ $Chk2^{-/-}$ mice may similarly be attributed to the fact that the G1/S checkpoint is largely intact in those double mutants.

Multiple roles of the Mre11 complex in apoptosis

The influence of the Mre11 complex on apoptosis can be parsed into two general categories: i. promoting events at the site of DNA damage that facilitate ATM activity; ii. promoting DSB-induced ATM activation. Our data indicated that both of these molecular events are required for the promotion of Mre11 complex-dependent apoptosis. We observed a deficit in IR-induced ATM activation in $Mre11^{ATLD1/ATLD1}$ mice (Figure 3A), which is correlated with reduced phosphorylation of p53 and Chk2 in response to DNA damage. As is true for $Mre11^{ATLD1/ATLD1}$ $Chk2^{-/-}$, mice expressing *p53* alleles in which the ATM and CHK2 consensus sites at S18 and S23 are altered ($p53^{S18A/S23A}$) have a substantial apoptotic defect, fail to fully stabilize p53 in thymocytes following IR, and develop a broad spectrum of malignancies with a long latency period (Chao et al., 2006). These data support the interpretation that reduced ATM activation associated with $Mre11^{ATLD1/ATLD1}$ contributes to the apoptotic defects observed. Conversely, $Nbs1^{\Delta C/\Delta C}$ mice exemplify the effect of attenuating ATMs access to its pro-apoptotic substrates. In these mice, ATM activation is unaffected, but ATM activity and apoptosis is defective, and this is associated with a reduction in the phosphorylation of certain ATM substrates (Stracker et al., 2007).

Chk2 hypomorphism and deficiency

The data presented herein clearly support of a global role for Chk2 as a tumor suppressor; however, Chk2 deficiency alone does not appreciably increase spontaneous tumor risk. Mice expressing either a kinase dead allele of *Chk2* (D347A) or the equivalent of the human $CHEK2^{*}$1100delC allele, identified in several types of human cancers, have been established (Bahassi el et al., 2007; Kwak et al., 2006; Nevanlinna and Bartek, 2006). Unlike Chk2 deficient mice, mice expressing the *Chk2- D347A* allele from the MMTV promoter in the spleen and mammary gland developed spontaneous tumors (Kwak et al., 2006). Analogously, checkpoint

defects not apparent in *Chk2*$^{-/-}$ mice or cell lines with acute *Chk2* deletion have been described in human or mouse cells expressing hypomorphic *Chk2* alleles or in which siRNA-mediated depletion has been induced (Bahassi el et al., 2007; Falck et al., 2001; Falck et al., 2002; Jallepalli et al., 2003; Matsuoka et al., 1998; Takai et al., 2002). These data are consistent with the idea that the protein products of certain *Chk2* alleles may exert an inhibitory effect on DNA damage responses. These observations are also consistent with the possibility that compensating mutations arise during development of *Chk2*$^{-/-}$ mice and may suppress some aspects of Chk2 deficiency.

This study demonstrates that the Mre11 complex and ATM function together to promote apoptosis in a pathway that parallels Chk2. The Mre11 complex is required for both ATM activation and to facilitate access of the active kinase to its substrates (Lee and Paull, 2007). In addition, the study elucidates a mechanism by which the oncogenic potential of DNA replication-associated chromosome breakage is suppressed. We show that Chk2 plays a heretofore, unrecognized role in responding to DNA replication-associated DNA damage. Chk2's influence in that context comprises apoptosis as well as tumor suppression, supporting the view that Chk2 is a tumor suppressor.

This interpretation resonates with the observation that the DNA damage response is activated in pre-malignant lesions in humans; this is indicated by several parameters, including the phosphorylation of Chk2 (Bartkova et al., 2005; Gorgoulis et al., 2005). Tumor progression correlates with the attenuation of DNA damage response (DDR) markers, indicating that the damage response acts as an inducible barrier to tumorigenesis. Our data is consistent with this view and demonstrate that the loss of multiple facets of the DDR results in the predisposition to spontaneous tumorigenesis in diverse tissues. In this regard, the data presented define a mechanistic basis for such a barrier to be effected in response to DNA replication stress and genome instability.

Experimental Procedures

Mice

Chk2$^{-/-}$, *p53*$^{-/-}$, and *Atm*$^{-/-}$ mice were obtained from T. Mak, T. Jacks, and T. Wynshaw Boris, and *Prkdc*$^{+/scid}$ were purchased (Jackson Laboratories). *Nbs1*$^{\Delta B}$ and *Mre11*^{ATLD1} mice were described (Theunissen et al., 2003; Williams et al., 2002). Mice were raised in a pathogen free facility and genotyped by PCR (details upon request).

Immunoreagents and Western Blotting

Antibodies and Western blotting techniques are described in the Supplemental Data.

Cellular Assays

The generation of MEFs, checkpoint assays and analysis of chromosome spreads were performed as described (Theunissen and Petrini, 2006). Sensitivity of SV40 transformed MEFs to IR was assessed using a colony formation assay as described (Williams et al., 2002).

Apoptosis Assays

Thymi were dissected from 6–9 wk old animals and 1×10^6 cells were irradiated using a Cs-137 source (dose rate 220 cGy/min- Shepherd Mark-1) or treated with 1mM dexamethasone (Sigma). Viable (double negative, DN) cells were identified by flow cytometry after propidium iodide and anti-AnnexinV-FITC antibody (BD Biosciences) staining. A viability ratio (%DN treated or mock/%DN mock) is plotted.

Pathology, Histology, and Immunohistochemistry

Moribund mice were sacrificed, tissues fixed in 10% neutral buffered formalin, and processed for immunohistochemistry (IHC) (described in Supplemental Data).

Sequencing of *p53* and p^{19ARF}

For sequencing of *p53* or *Arf*, genomic DNA or cDNA was amplified by PCR (Primers described in Supplemental Data). Genomic DNA was prepared from tumors by digestion with proteinase-K, phenol/chloroform extraction and DNA precipitation. Some samples were isolated using a PixCell laser capture microscopy (LCM) system (Arcturus) or from paraffin by macro-dissection of tumor-enriched lesions. DNA was isolated from LCM samples using the Pico Pure DNA extraction kit (Arcturus). RNA was extracted from tumor tissue using Trizol reagent (Invitrogen) and cDNA was prepared using the Superscript RT-PCR kit (Invitrogen).

Microarray analysis

Thymocytes were cultured *in vitro* and treated with RNAlater (Ambion) 12 hours post-IR. Whole RNA was extracted using RNAeasy columns (Qiagen). Generation of cDNA, labeling and hybridization to MOE430A_2 gene arrays (Affymetrix) was performed in the Sloan-Kettering Genomics Core Facility. Analysis of microarray data is described in the Supplemental Data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank S. Shelly, H. Hussein and A. Baker for diligent maintenance of our animal colony. Thanks to N. Draghi and P. Savage for advice on B-cell proliferation, J. Theunissen for technical assistance and discussions, G. Lozano, S. Post, and R. Maser for *p53* or p^{19ARF} primer and sequencing information, A. Viale and N. Socci for microarray analysis, and A. Koff for critical reading of the manuscript. J.H.J.P was 22 funded by grants from the NIH and the Joel and Jean Smilow Initiative. T.H.S. was funded by a NIH National Research Service Award and is a Leukemia and Lymphoma Society Special Fellow.

References

Ahn JY, Schwarz JK, Piwnica-Worms H, Canman CE. Threonine 68 phosphorylation by ataxia telangiectasia mutated is required for efficient activation of Chk2 in response to ionizing radiation. Cancer Res 2000;60:5934–5936. [PubMed: 11085506]

Allen JB, Zhou Z, Siede W, Friedberg EC, Elledge SJ. The SAD1/RAD53 protein kinase controls multiple checkpoints and DNA damage-induced transcription in yeast. Genes & Dev 1994;8:2401–2415. [PubMed: 7958905]

Bahassi el M, Penner CG, Robbins SB, Tichy E, Feliciano E, Yin M, Liang L, Deng L, Tischfield JA, Stambrook PJ. The breast cancer susceptibility allele CHEK2* 1100delC promotes genomic instability in a knock-in mouse model. Mutat Res 2007;616:201–209. [PubMed: 17174984]

Bakkenist CJ, Kastan MB. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature 2003;421:499–506. [PubMed: 12556884]

Barboza JA, Liu G, Ju Z, El-Naggar AK, Lozano G. p21 delays tumor onset by preservation of chromosomal stability. Proc Natl Acad Sci U S A 2006;103:19842–19847. [PubMed: 17170138]

Barlow C, Brown KD, Deng CX, Tagle DA, Wynshaw-Boris A. Atm selectively regulates distinct p53-dependent cell-cycle checkpoint and apoptotic pathways. Nat Genet 1997;17:453–456. [PubMed: 9398849]

Barlow C, Hirotsune S, Paylor R, Liyanage M, Eckhaus M, Collins F, Shiloh Y, Crawley JN, Ried T, Tagle D, Wynshaw-Boris A. Atm-deficient mice: a paradigm of ataxia telangiectasia. Cell 1996;86:159–171. [PubMed: 8689683]

Bartek J, Falck J, Lukas J. CHK2 kinase--a busy messenger. Nat Rev Mol Cell Biol 2001;2:877–886. [PubMed: 11733767]

Bartkova J, Horejsi Z, Koed K, Kramer A, Tort F, Zieger K, Guldberg P, Sehested M, Nesland JM, Lukas C, et al. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature 2005;434:864–870. [PubMed: 15829956]

Bassing CH, Alt FW. The cellular response to general and programmed DNA double strand breaks. DNA Repair (Amst) 2004;3:781–796. [PubMed: 15279764]

Boddy MN, Furnari B, Mondesert O, Russell P. Replication checkpoint enforced by kinases Cds1 and Chk1. Science 1998;280:909–912. [PubMed: 9572736]

Boulton SJ. Cellular functions of the BRCA tumour-suppressor proteins. Biochem Soc Trans 2006;34:633–645. [PubMed: 17052168]

Bressan DA, Baxter BK, Petrini JH. The Mre11-Rad50-Xrs2 protein complex facilitates homologous recombination-based double-strand break repair in Saccharomyces cerevisiae. Mol Cell Biol 1999;19:7681–7687. [PubMed: 10523656]

Cao L, Kim S, Xiao C, Wang RH, Coumoul X, Wang X, Li WM, Xu XL, De Soto JA, Takai H, et al. ATM-Chk2-p53 activation prevents tumorigenesis at an expense of organ homeostasis upon Brca1 deficiency. Embo J 2006;25:2167–2177. [PubMed: 16675955]

Carson CT, Schwartz RA, Stracker TH, Lilley CE, Lee DV, Weitzman MD. The Mre11 complex is required for ATM activation and the G(2)/M checkpoint. Embo J 2003;22:6610–6620. [PubMed: 14657032]

Chao C, Herr D, Chun J, Xu Y. Ser18 and 23 phosphorylation is required for p53-dependent apoptosis and tumor suppression. Embo J 2006;25:2615–2622. [PubMed: 16757976]

Chehab NH, Malikzay A, Appel M, Halazonetis TD. Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53. Genes Dev 2000;14:278–288. [PubMed: 10673500]

Donehower LA, Harvey M, Slagle BL, Mc Arthur MJ, Montgomery CAJ, Butel JS, Bradley A. Mice deficient for p53 are developmentally normal but susceptible to spontaenous tumours. Nature (London) 1992;356:215–221. [PubMed: 1552940]

Falck J, Coates J, Jackson SP. Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage. Nature 2005;434:605–611. [PubMed: 15758953]

Falck J, Mailand N, Syljuasen RG, Bartek J, Lukas J. The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. Nature 2001;410:842–847. [PubMed: 11298456]

Falck J, Petrini JH, Williams BR, Lukas J, Bartek J. The DNA damage-dependent intra--S phase checkpoint is regulated by parallel pathways. Nat Genet 2002;30:290–294. [PubMed: 11850621]

Gao Y, Sun Y, Frank KM, Dikkes P, Fujiwara Y, Seidl KJ, Sekiguchi JM, Rathbun GA, Swat W, Wang J, et al. A critical role for DNA end-joining proteins in both lymphogenesis and neurogenesis. Cell 1998;95:891–902. [PubMed: 9875844]

Gorgoulis VG, Vassiliou LV, Karakaidos P, Zacharatos P, Kotsinas A, Liloglou T, Venere M, Ditullio RA Jr. Kastrinakis NG, Levy B, et al. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. Nature 2005;434:907–913. [PubMed: 15829965]

Guidos CJ, Williams CJ, Grandal I, Knowles G, Huang MTF, Danska JS. V(D)J recombination activates a p53-dependent DNA damage checkpoint in *scid* lymphocytes. Genes & Dev 1996;10:2038–2054. [PubMed: 8769647]

Herzog KH, Chong MJ, Kapsetaki M, Morgan JI, McKinnon PJ. Requirement for Atm in ionizing radiation-induced cell death in the developing central nervous system. Science 1998;280:1089–1091. [PubMed: 9582124]

Hirao A, Cheung A, Duncan G, Girard PM, Elia AJ, Wakeham A, Okada H, Sarkissian T, Wong JA, Sakai T, et al. Chk2 is a tumor suppressor that regulates apoptosis in both an ataxia telangiectasia mutated (ATM)-dependent and an ATM-independent manner. Mol Cell Biol 2002;22:6521–6532. [PubMed: 12192050]

Hirao A, Kong YY, Matsuoka S, Wakeham A, Ruland J, Yoshida H, Liu D, Elledge SJ, Mak TW. DNA damage-induced activation of p53 by the checkpoint kinase Chk2. Science 2000;287:1824–1827. [PubMed: 10710310]

Jack MT, Woo RA, Hirao A, Cheung A, Mak TW, Lee PW. Chk2 is dispensable for p53-mediated G1 arrest but is required for a latent p53-mediated apoptotic response. Proc Natl Acad Sci U S A 2002;99:9825–9829. [PubMed: 12097646]

Jallepalli PV, Lengauer C, Vogelstein B, Bunz F. The Chk2 tumor suppressor is not required for p53 responses in human cancer cells. J Biol Chem 2003;278:20475–20479. [PubMed: 12654917]

Kastan MB, Zhan Q, El-Diery WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B, Fornace AJ. A mammalian cell cycle checkpoint pathway utilizing p53 and *GADD45* is defective in ataxia-telangiectasia. Cell 1992;71:587–597. [PubMed: 1423616]

Kitagawa R, Bakkenist CJ, McKinnon PJ, Kastan MB. Phosphorylation of SMC1 is a critical downstream event in the ATM-NBS1-BRCA1 pathway. Genes Dev 2004;18:1423–1438. [PubMed: 15175241]

Kuerbitz SJ, Plunkett BS, Walsh WV, Kastan MB. Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc Natl Acad Sci USA 1992;89:7491–7495. [PubMed: 1323840]

Kwak EL, Kim S, Zhang J, Cardiff RD, Schmidt EV, Haber DA. Mammary tumorigenesis following transgenic expression of a dominant negative CHK2 mutant. Cancer Res 2006;66:1923–1928. [PubMed: 16488990]

Lee JH, Paull TT. Activation and regulation of ATM kinase activity in response to DNA double-strand breaks. Oncogene 2007;26:7741–7748. [PubMed: 18066086]

Li J, Stern DF. Regulation of CHK2 by DNA-dependent protein kinase. J Biol Chem 2005;280:12041–12050. [PubMed: 15668230]

Lin WC, Desiderio S. V(D)J recombination and the cell cycle. Immunol Today 1995;16:279–289. [PubMed: 7662097]

Lindsay HD, Griffiths DJ, Edwards RJ, Christensen PU, Murray JM, Osman F, Walworth N, Carr AM. S-phase-specific activation of Cds1 kinase defines a subpathway of the checkpoint response in Schizosaccharomyces pombe. Genes Dev 1998;12:382–395. [PubMed: 9450932]

Liu G, Parant JM, Lang G, Chau P, Chavez-Reyes A, El-Naggar AK, Multani A, Chang S, Lozano G. Chromosome stability, in the absence of apoptosis, is critical for suppression of tumorigenesis in Trp53 mutant mice. Nat Genet 2004;36:63–68. [PubMed: 14702042]

MacPherson D, Kim J, Kim T, Rhee BK, Van Oostrom CT, DiTullio RA, Venere M, Halazonetis TD, Bronson R, De Vries A, et al. Defective apoptosis and B-cell lymphomas in mice with p53 point mutation at Ser 23. Embo J 2004;23:3689–3699. [PubMed: 15343266]

Matsuoka S, Huang M, Elledge SJ. Linkage of ATM to Cell Cycle Regulation by the Chk2 Protein Kinase. Science 1998;282:1893–1897. [PubMed: 9836640]

Matsuoka S, Rotman G, Ogawa A, Shiloh Y, Tamai K, Elledge SJ. Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro. Proc Natl Acad Sci U S A 2000;97:10389–10394. [PubMed: 10973490]

McPherson JP, Lemmers B, Hirao A, Hakem A, Abraham J, Migon E, Matysiak-Zablocki E, Tamblyn L, Sanchez-Sweatman O, Khokha R, et al. Collaboration of Brca1 and Chk2 in tumorigenesis. Genes Dev 2004;18:1144–1153. [PubMed: 15131084]

Melchionna R, Chen XB, Blasina A, McGowan CH. Threonine 68 is required for radiation-induced phosphorylation and activation of Cds1. Nat Cell Biol 2000;2:762–765. [PubMed: 11025670]

Morales M, Theunissen JW, Kim CF, Kitagawa R, Kastan MB, Petrini JH. The Rad50S allele promotes ATM-dependent DNA damage responses and suppresses ATM deficiency: implications for the Mre11 complex as a DNA damage sensor. Genes Dev 2005;19:3043–3054. [PubMed: 16357220]

Nacht M, Strasser A, Chan YR, Harris AW, Schlissel S, Bronson RT, Jacks T. Mutations in the *p53* and *SCID* genes cooperate in tumorigenesis. Genes & Dev 1996;10:2055–2066. [PubMed: 8769648]

Nevanlinna H, Bartek J. The CHEK2 gene and inherited breast cancer susceptibility. Oncogene 2006;25:5912–5919. [PubMed: 16998506]

Paulovich AG, Hartwell LH. A checkpoint regulates the rate of progression through S phase in S. cerevisiae in response to DNA damage. Cell 1995;82:841–847. [PubMed: 7671311]

Petrini JH, Stracker TH. The cellular response to DNA double-strand breaks: defining the sensors and mediators. Trends Cell Biol 2003;13:458–462. [PubMed: 12946624]

Petrini JH, Theunissen JW. Double Strand Break Metabolism and Cancer Susceptibility: Lessons from the Mre11 Complex. Cell Cycle 2004;3

Powers JT, Hong S, Mayhew CN, Rogers PM, Knudsen ES, Johnson DG. E2F1 uses the ATM signaling pathway to induce p53 and Chk2 phosphorylation and apoptosis. Mol Cancer Res 2004;2:203–214. [PubMed: 15140942]

Pusapati RV, Rounbehler RJ, Hong S, Powers JT, Yan M, Kiguchi K, McArthur MJ, Wong PK, Johnson DG. ATM promotes apoptosis and suppresses tumorigenesis in response to Myc. Proc Natl Acad Sci U S A 2006;103:1446–1451. [PubMed: 16432227]

Rogoff HA, Pickering MT, Frame FM, Debatis ME, Sanchez Y, Jones S, Kowalik TF. Apoptosis associated with deregulated E2F activity is dependent on E2F1 and Atm/Nbs1/Chk2. Mol Cell Biol 2004;24:2968–2977. [PubMed: 15024084]

Rooney S, Sekiguchi J, Whitlow S, Eckersdorff M, Manis JP, Lee C, Ferguson DO, Alt FW. Artemis and p53 cooperate to suppress oncogenic N-myc amplification in progenitor B cells. Proc Natl Acad Sci U S A 2004;101:2410–2415. [PubMed: 14983023]

Roth DB, Menetski JP, Nakajima PB, Bosma MJ, Gellert M. V(D)J recombination: broken DNA molecules with covalently sealed (hairpin) coding ends in scid mouse thymocytes. Cell 1992;70:983–991. [PubMed: 1356077]

Shiloh Y. ATM and related protein kinases: safeguarding genome integrity. Nat Rev Cancer 2003;3:155–168. [PubMed: 12612651]

Soussi T, Wiman KG. Shaping genetic alterations in human cancer: the p53 mutation paradigm. Cancer Cell 2007;12:303–312. [PubMed: 17936556]

Stracker TH, Morales M, Couto SS, Hussein H, Petrini JH. The carboxy terminus of NBS1 is required for induction of apoptosis by the MRE11 complex. Nature. 2007

Stracker TH, Theunissen JW, Morales M, Petrini JH. The Mre11 complex and the metabolism of chromosome breaks: the importance of communicating and holding things together. DNA Repair (Amst) 2004;3:845–854. [PubMed: 15279769]

Takai H, Naka K, Okada Y, Watanabe M, Harada N, Saito S, Anderson CW, Appella E, Nakanishi M, Suzuki H, et al. Chk2-deficient mice exhibit radioresistance and defective p53-mediated transcription. Embo J 2002;21:5195–5205. [PubMed: 12356735]

Theunissen JW, Kaplan MI, Hunt PA, Williams BR, Ferguson DO, Alt FW, Petrini JH. Checkpoint failure and chromosomal instability without lymphomagenesis in Mre11(ATLD1/ATLD1) mice. Mol Cell 2003;12:1511–1523. [PubMed: 14690604]

Theunissen JW, Petrini JH. Methods for studying the cellular response to DNA damage: influence of the Mre11 complex on chromosome metabolism. Methods Enzymol 2006;409:251–284. [PubMed: 16793406]

Uziel T, Lerenthal Y, Moyal L, Andegeko Y, Mittelman L, Shiloh Y. Requirement of the MRN complex for ATM activation by DNA damage. Embo J 2003;22:5612–5621. [PubMed: 14532133]

Wang XQ, Redpath JL, Fan ST, Stanbridge EJ. ATR dependent activation of Chk2. J Cell Physiol 2006;208:613–619. [PubMed: 16741947]

Westphal CH, Rowan S, Schmaltz C, Elson A, Fisher DE, Leder P. atm and p53 cooperate in apoptosis and suppression of tumorigenesis, but not in resistance to acute radiation toxicity. Nat Genet 1997;16:397–401. [PubMed: 9241281]

Williams BR, Mirzoeva OK, Morgan WF, Lin J, Dunnick W, Petrini JH. A murine model of nijmegen breakage syndrome. Curr Biol 2002;12:648–653. [PubMed: 11967151]

Xu Y, Ashley T, Brainerd EE, Bronson RT, Meyn MS, Baltimore D. Targeted disruption of ATM leads to growth retardation, chromosomal fragmentation during meiosis, immune defects, and thymic lymphoma. Genes & Dev 1996;10:2411–2422. [PubMed: 8843194]

Xu Y, Baltimore D. Dual roles of ATM in the cellular response to radiation and in cell growth control. Genes Dev 1996;10:2401–2410. [PubMed: 8843193]

Xu Y, Yang EM, Brugarolas J, Jacks T, Baltimore D. Involvement of p53 and p21 in cellular defects and tumorigenesis in Atm−/− mice. Mol Cell Biol 1998;18:4385–4390. [PubMed: 9632822]

You Z, Chahwan C, Bailis J, Hunter T, Russell P. ATM activation and its recruitment to damaged DNA require binding to the C terminus of Nbs1. Mol Cell Biol 2005;25:5363–5379. [PubMed: 15964794]

Zheng P, Fay DS, Burton J, Xiao H, Pinkham JL, Stern DF. SPK1 is an essential S-phase-specific gene of Saccharomyces cerevisiae that encodes a nuclear serine/threonine/tyrosine kinase. Mol Cell Biol 1993;13:5829–5842. [PubMed: 8355715]

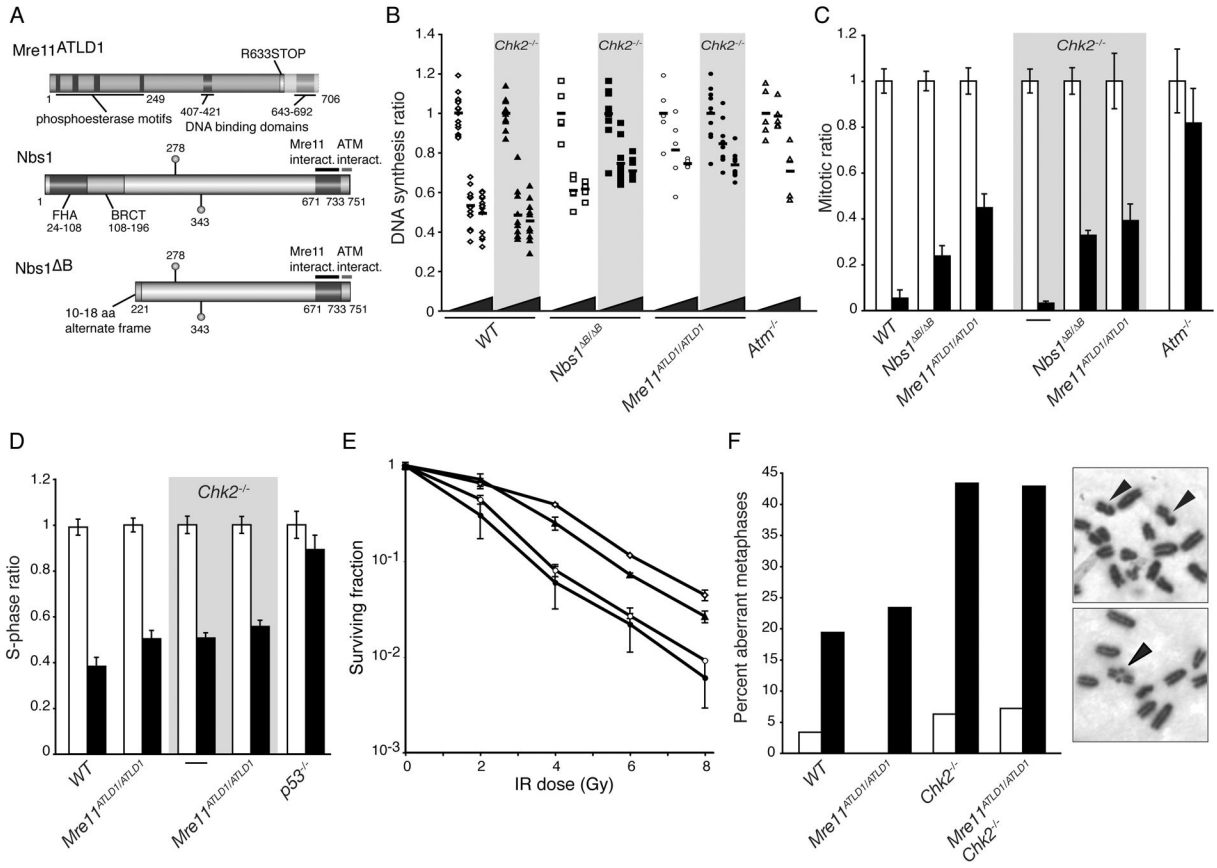

Figure 1. Loss of *Chk2* does not exacerbate the checkpoint defects, damage sensitivity, or chromosome instability conferred by Mre11 complex hypomorphism

(A) Diagram of the hypomorphic $Mre11^{ATLD1}$ and $Nbs1^{\Delta B}$ alleles (reviewed in Stracker et al., 2004).

(B) Intra-S phase checkpoint response of *WT* (◇), $Chk2^{-/-}$ (◆), $Nbs1^{\Delta B/\Delta B}$ (□), $Nbs1^{\Delta B/\Delta B}Chk2^{-/-}$ (■), $Mre11^{ATLD1/ATLD1}$ (○), $Mre11^{ATLD1/ATLD1}Chk2^{-/-}$ (●) and $Atm^{-/-}$ (△) fibroblasts. The DNA synthesis ratios (untreated and IR treated cultures, normalized to untreated samples) are plotted. Wedge denotes increasing IR dose (0, 10, or 20 Gy) and horizontal bars indicate the average value of replicate experiments. $Chk2^{-/-}$ cultures are not significantly different than *WT* (P=0.33 at 10 and 0.82 at 20 Gy, *WT* vs. $Chk2^{-/-}$) and loss of *Chk2* does not exacerbate the defect of $Mre11^{ATLD1/ATLD1}$ (P=0.88 at 10 and 0.55 at 20 Gy, $Mre11^{ATLD1/ATLD1}$ vs. $Mre11^{ATLD1/ATLD1}Chk2^{-/-}$). However, $Nbs1^{\Delta B/\Delta B}Chk2^{-/-}$ cultures showed increased RDS compared to $Nbs1^{\Delta B/\Delta B}$ (P=1.5×10^{-3} at 10 and 2.5×10^{-3} at 20 Gy, $Nbs1^{\Delta B/\Delta B}$ vs. $Nbs1^{\Delta B/\Delta B}Chk2^{-/-}$). Wilcoxon rank sum test, 2-sided.

(C) G2/M checkpoint responses of exponentially growing MEFs. The mitotic ratios (mock or IR treated normalized to mock treated) of mock (white) or 10 Gy IR (black) treated cells at 1 hr post treatment are presented. Results are the average of 3–6 experiments performed in triplicate for each genotype. Error bars denote standard deviation.

(D) G1/S checkpoint in early passage MEF cultures. The S-phase ratios (%BrdU positive of irradiated or unirradiated/ average %BrdU positive unirradiated) are plotted. Results are the average of 2–4 experiments performed in triplicate for each genotype. Error bars denote standard deviation.

(E) IR sensitivity determined by clonogenic survival assay. *WT* (◇), *Chk2−/−* (▲), $Mre11^{ATLD1/ATLD1}$ (○), and $Mre11^{ATLD1/ATLD1}Chk2^{-/-}$ (●) SV40 transformed MEF cultures

were exposed to IR at the indicated doses and plated for colony formation. Results from 2 representative experiments performed in triplicate are shown. Error bars denote standard deviation.

(F) Genomic instability in mock and irradiated primary proliferating splenocytes. Examples of metaphases from IR treated *Mre11*$^{ATLD1/ATLD1}$ *Chk2*$^{-/-}$ spreads are shown: 2 chromatid breaks (top) and a rearrangement (bottom) are indicated. The number of spreads analyzed and aberrations identified are described in Supplementary Table S1.

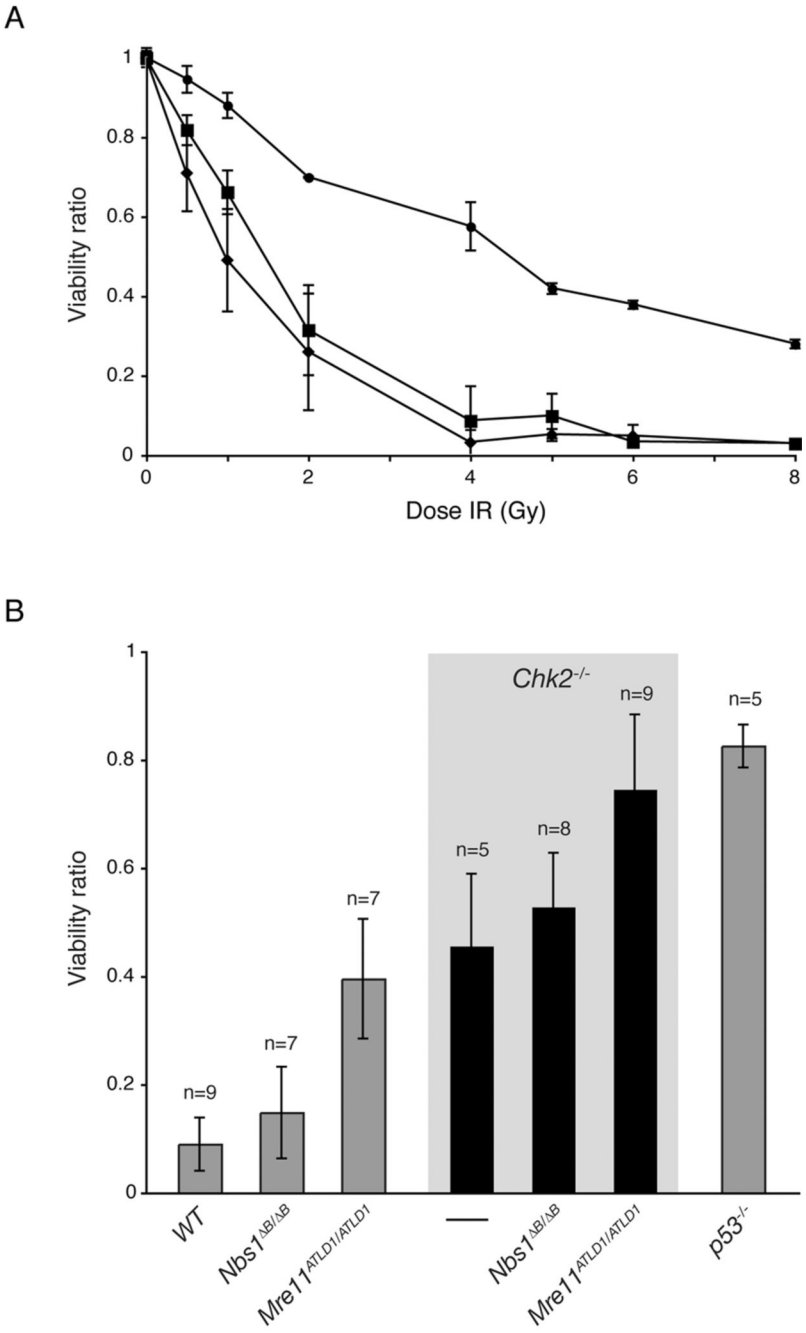

Figure 2. The Mre11 complex and Chk2 regulate apoptosis in parallel pathways

(A) Apoptosis in irradiated *WT* (◆), *Nbs1*$^{\Delta B/\Delta B}$ (■), and *Mre11*$^{ATLD1/ATLD1}$ (●) thymocytes 20 hours post exposure to the indicated IR dose. Viability ratios are plotted for each dose (% viable cells mock or IR treated/ % viable cells mock treated). Triplicate results of 2 representative experiments are plotted. Error bars denote standard deviation.

(B) Thymocytes from the indicated genotypes were mock treated or exposed to 5 Gy of IR in culture and analyzed 20 hours post treatment. Viability ratios are plotted for each dose and experiments were performed in triplicate with n indicating the number of animals represented and error bars denoting standard deviation. The viability ratio differences between

Nbs1$^{\Delta B/\Delta B}$ and *WT*, or *Chk2*$^{-/-}$ and *Nbs1*$^{\Delta B/\Delta B}$ *Chk2*$^{-/-}$ are not statistically significant (Wilcoxon rank sum test, 2-sided).

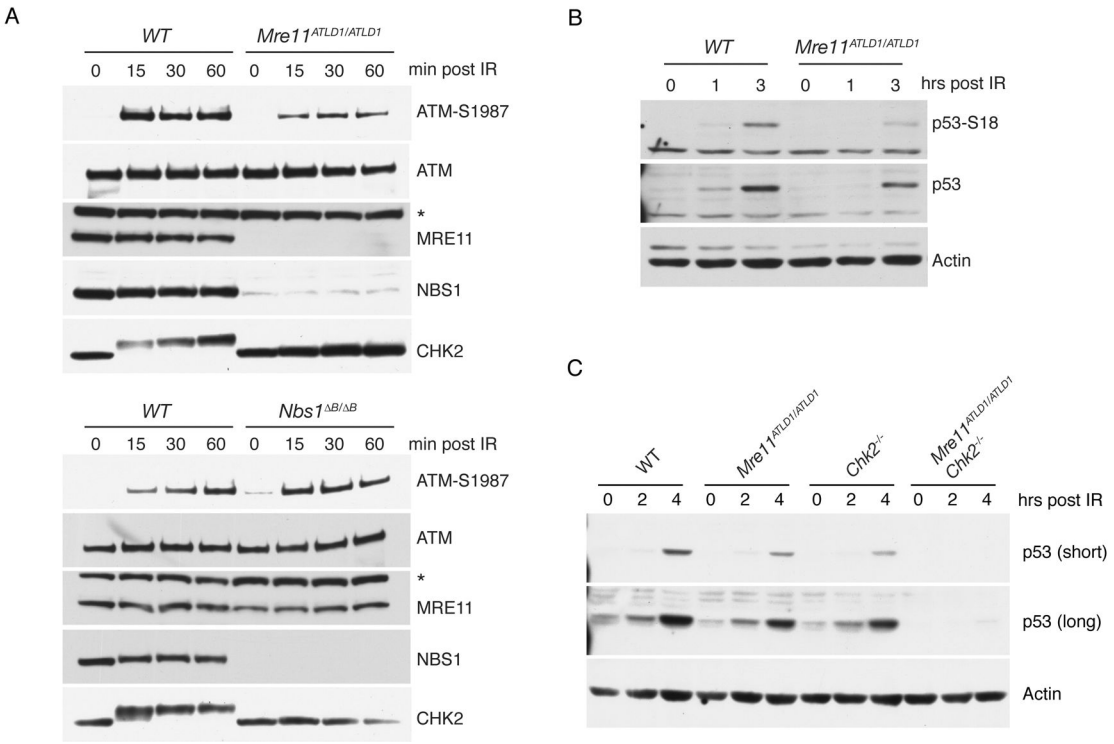

Figure 3. The Mre11 complex and Chk2 regulate p53 in parallel pathways

(A) Western blot of thymocyte lysates from the indicated genotypes. Membranes were probed with antibodies for ATM-S1981, ATM, Nbs1, Mre11, and Chk2 at various times following 5 Gy of IR treatement. A non-specific band is indicated by *.

(B) Western blot of thymocyte lysates probed with antibodies for p53-S18, p53 and Actin (loading control) at the indicated times following 5 Gy of IR.

(C) Western blot of thymocyte lysates probed for p53 or Actin (loading control) at the indicated times post 5 Gy of IR.

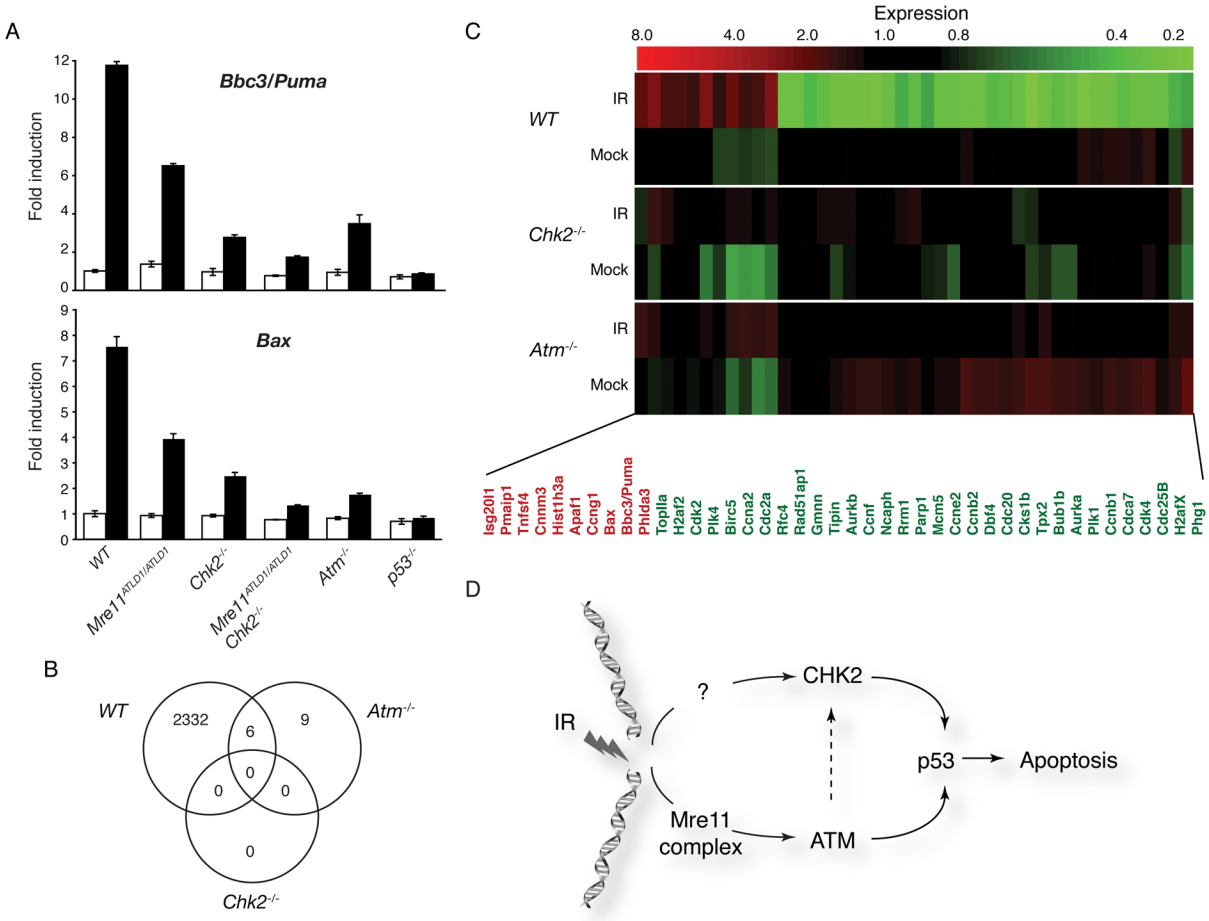

Figure 4. The Mre11 complex-ATM and Chk2 regulate global p53 transcriptional responses

(A) Quantitative PCR analysis of p53 target genes *Bbc3/Puma* and *Bax*. Thymocytes were harvested 8 hours after exposure to 5 Gy of IR. Representative experiments performed in triplicate are shown: mock (white) or IR treated (black).

(B) Analysis of microarray data for the indicated genotypes. 2338 probe sets showed statistically significant expression changes following IR treatment in *WT* thymocytes. No changes were significant in $Chk2^{-/-}$ and only 15 in $Atm^{-/-}$ (listed in Supplementary Table S2). The number of common genes altered in both *WT* and $Atm^{-/-}$ are indicated by the Venn diagram (GEO accession number pending).

(C) Transcriptional regulation of genes involved in the cell cycle and apoptosis are impaired in both $Atm^{-/-}$ and $Chk2^{-/-}$ thymocytes. The heatmap indicates the induction (red) or repression (green) of selected genes with known roles in cell cycle regulation or apoptosis following IR treatment.

(D) Model of p53-dependent apoptotic signaling. The Mre11 complex is required for activation of ATM and this function is attenuated in $Mre11^{ATLD1/ATLD1}$ thymocytes. While both ATM and the Mre11 complex influence Chk2 phosphorylation, the apoptotic defects of $Mre11^{ATLD1/ATLD1}$ or $Atm^{-/-}$ are additive with loss of Chk2 indicating that they function through a largely parallel signaling pathway.

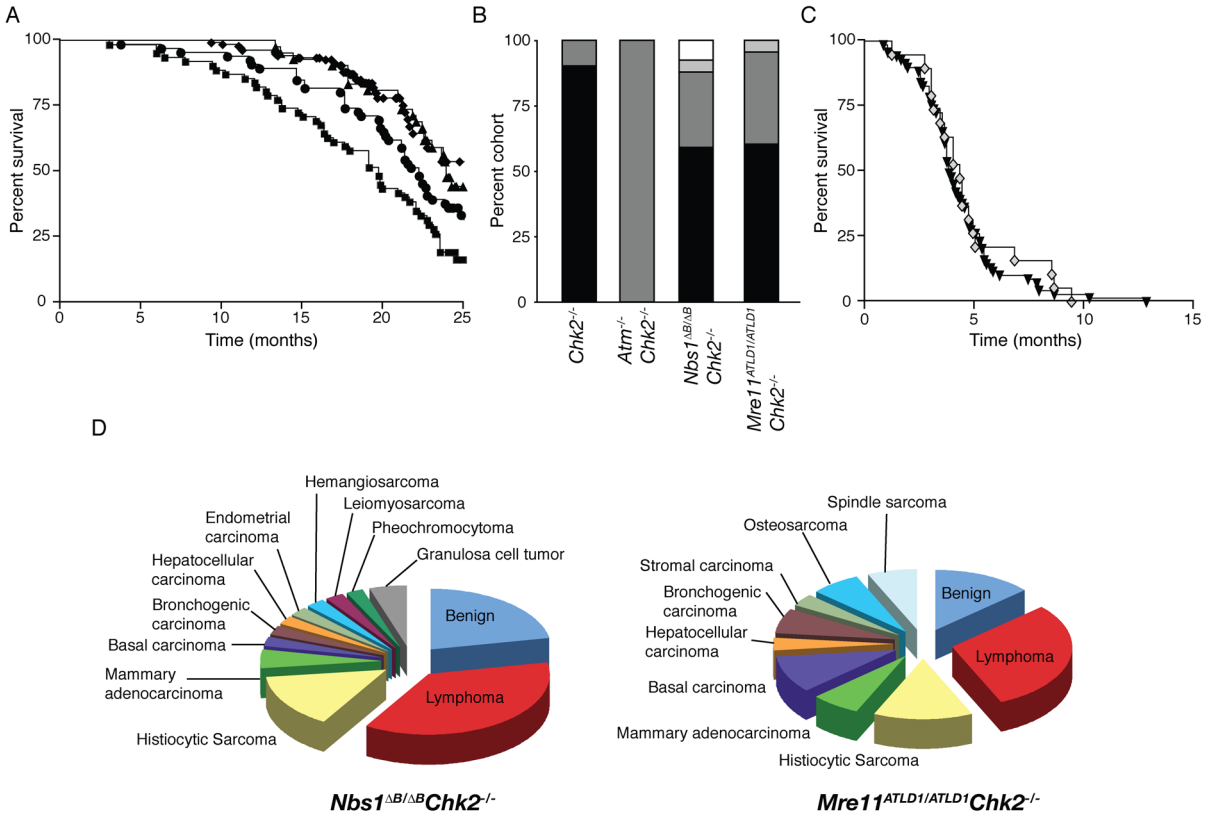

Figure 5. ***Nbs1*$^{\Delta B/\Delta B}$ *Chk2*$^{-/-}$ and *Mre11*$^{ATLD1/ATLD1}$ *Chk2*$^{-/-}$ double mutants are predisposed to tumorigenesis**

(A) Cohort survival of *WT*, *Mre11*^{ATLD1ATLD1} and *Nbs1*$^{\Delta B/\Delta B}$ (*combined n=172, ◆), *Chk2*$^{-/-}$ (n=41, ▲), *Nbs1*$^{\Delta B/\Delta B}$ *Chk2*$^{-/-}$ (n=66, ■), and *Mre11*^{ATLD1ATLD1} *Chk2*$^{-/-}$ (n=67, ●) is shown on a Kaplan Meier curve. *Individual survival curves, tumor free survival curves, and statistical analysis are included in Supplemental Figure S5, S6, and Table S3.

(B) Animals succumbing to tumors are shown as a percentage of their cohort: tumor free (black), 1 tumor (dark gray), 2 tumors (light gray), more than 2 tumors (white).

(C) Survival of *Atm*$^{-/-}$ (n=71, ▼) and *Atm*$^{-/-}$ *Chk2*$^{-/-}$ (n=19, ◇) cohorts plotted on a Kaplan Meier curve.

(D) The percentage of tumor bearing animals with a specific tumor type is plotted for the indicated genotypes.

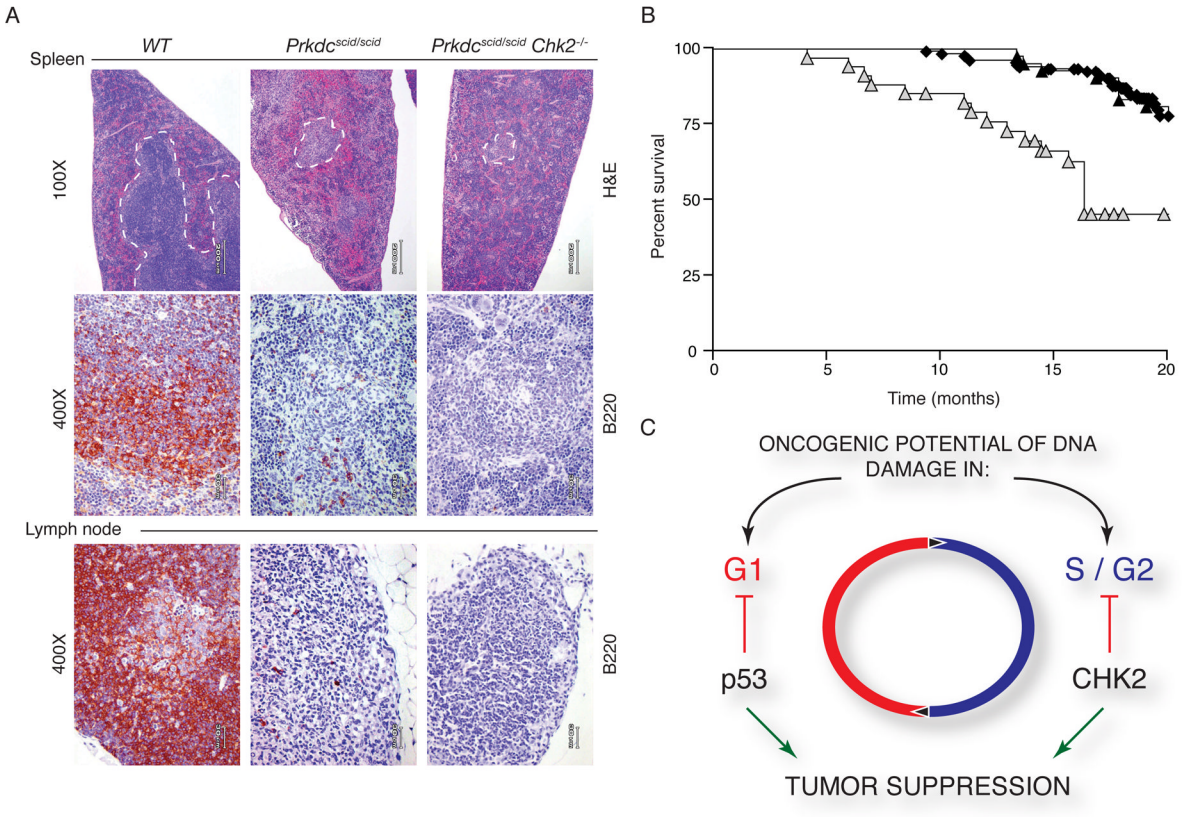

Figure 6. *Prkdc*$^{scid/scid}$ *Chk2*$^{-/-}$ animals are not tumor prone

(A) Immunohistochemical staining of the spleen and lymph nodes of *WT*, *Prkdc*$^{scid/scid}$ and *Prkdc*$^{scid/scid}$ *Chk2*$^{-/-}$ mice. White pulp (WP) of the spleen is outlined in broken lines (top panels). Regions of WP are smaller and contain few lymphocytes in *Prkdc*$^{scid/scid}$ and *Prkdc*$^{scid/scid}$ *Chk2*$^{-/-}$. B220 staining of B-cells in the spleen (middle panels) and lymph nodes (bottom panels). In *Prkdc*$^{scid/scid}$ and *Prkdc*$^{scid/scid}$ *Chk2*$^{-/-}$ mice, both tissues have few B220 positive cells compared to *WT*.

(B) Survival of *WT* (◆, n=62), *Chk2*$^{-/-}$ (▲, n=41), and *Prkdc*$^{scid/scid}$ *Chk2*$^{-/-}$ (▲, n=34) is plotted on a Kaplan Meier curve. Survival of *Prkdc*$^{scid/scid}$ *Chk2*$^{-/-}$ animals was decreased due to infections and related pathology but no tumors were evident.

(C) Model for the role of Chk2 in tumor suppression. Chk2 is required for suppressing the oncogenic potential of DNA-replication associated breaks, such as those arising in *Nbs1*$^{\Delta B/\Delta B}$ or *Mre11*^{ATLD1ATLD1} mutants, but not for breaks in G0/G1 phase cells resulting from defects associated with the *Prkdc*$^{scid/scid}$ mutation.

Table 1

Analysis of *p53* and p^{19ARF} sequence in tumor samples

Exons 5–10 of *p53* genomic DNA and exons 2–11 of *p53* cDNA were PCR amplified and sequenced. The coding sequence of $p19^{ARF}$ was amplified and sequenced (exons 1–3) from cDNA samples. All sequencing was performed multiple times and PCR products were sequenced in both directions.

***p53* exon amplification from genomic DNA**								
ID	**genotype**	**tumor type**	**Ex. 5**	**Ex. 6**	**Ex.7**	**Ex. 8**	**Ex. 9**	**Ex. 10**
22790	M-C2	basal carcinoma	WT	WT	WT	WT	WT	WT
23186	N-C2	adeno/squamous carcinoma	WT	WT	WT	WT	WT	WT
23182	M-C2	osteosarcoma	WT	WT	WT	WT	WT	WT
23828	N-C2	granulosa cell tumor	WT	WT	WT	WT	WT	WT
21872	N-C2	histiocytic sarcoma	WT	WT	WT	WT	WT	WT
23646	N-C2	lymphoma	WT	WT	WT	WT	WT	WT
23649	N-C2	mammary carcinoma	WT	WT	WT	WT	WT	ND
24098	N-C2	basal carcinoma	WT	WT	WT	WT	WT	WT

***p53* and $p19^{ARF}$ amplification from cDNA**				
ID		**tumor type**	***p53* Cdna exons 2–11**	**$p19^{ARF}$ cDNA exons 1–3**
23902	M-C2	basal carcinoma	WT	NA
24071	N-C2	lymphoma	WT	NA
23828	N-C2	granulosa cell tumor	WT	WT
23129	M-C2	histiocytic sarcoma	WT	WT
23186	N-C2	adeno/squamous carcinoma	WT	WT
25094	N-C2	uterine leiomyosarcoma	WT	WT
23646	N-C2	lymphoma	WT	WT

(M-C2=$Mre11^{ATLD1/ATLD1}$ $Chk2^{-/-}$, N-C2=$Nbs1^{\Delta B/\Delta B}$ $Chk2^{-/-}$, WT=wild type, ND=not determined, NA=not amplifiable)