CtIP-mediated resection is essential for viability and can operate independently of BRCA1

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Homologous recombination (HR) is initiated by DNA end resection, a process in which stretches of single-strand DNA (ssDNA) are generated and used for homology search. Factors implicated in resection include nucleases MRE11, EXO1, and DNA2, which process DNA ends into 3′ ssDNA overhangs; helicases such as BLM, which unwind DNA; and other proteins such as BRCA1 and CtIP whose functions remain unclear. CDK-mediated phosphorylation of CtIP on T847 is required to promote resection, whereas CDK-dependent phosphorylation of CtIP-S327 is required for interaction with BRCA1. Here, we provide evidence that CtIP functions independently of BRCA1 in promoting DSB end resection. First, using mouse models expressing S327A or T847A mutant CtIP as a sole species, and B cells deficient in CtIP, we show that loss of the CtIP-BRCA1 interaction does not detectably affect resection, maintenance of genomic stability or viability, whereas T847 is essential for these functions. Second, although loss of 53BP1 rescues the embryonic lethality and HR defects in BRCA1-deficient mice, it does not restore viability or genome integrity in CtIP−/− mice. Third, the increased resection afforded by loss of 53BP1 and the rescue of BRCA1-deficiency depend on CtIP but not EXO1. Finally, the sensitivity of BRCA1-deficient cells to poly ADP ribose polymerase (PARP) inhibition is partially rescued by the phospho-mimicking mutant CtIP (CtIP-T847E). Thus, in contrast to BRCA1, CtIP has indispensable roles in promoting resection and embryonic development.

Individuals with mutations in BRCA1 have a high risk of developing breast and ovarian cancer. Although BRCA1 is implicated in many cellular processes, its role in HR (Moyahan et al., 1999; Stark et al., 2004) is thought to be critical for tumor suppression and maintenance of genomic stability (Silver and Livingston, 2012). Tumor suppressor functions of BRCA1 are mediated by the BRCA1 carboxyl-terminal (BRCT) domain (Shakya et al., 2011), a motif that binds phosphorylated serine motifs in three different DNA repair proteins: BACH1 (BRIP1), ABRAXAS (CCDC98), and CtIP (Rbbp8; Li and Greenberg, 2012). Among these complexes, BRCA1 association with CtIP has been implicated in nucleolytic resection of DNA double strand breaks (DSBs; Sartori et al., 2007; Chen et al., 2008).
Although the BRCA1–CtIP complex is not known to possess nuclease activity itself, it enhances the nuclease activity of the MRE11–RAD50–NBS1 DSB sensor complex, which is required for effective resection (Sartori et al., 2007). According to the current “two-step” model, the BRCA1–CtIP–MRE11 complex initiates end processing, then EXO1 and BLM generate longer stretches of ssDNA, which are stabilized by RPA (Symington and Gautier, 2011). Finally, formation of the RAD51–ssDNA nucleoprotein filament promotes strand invasion and HR. In the absence of BRCA1 or CtIP, RAD51 binding to DSB sites and HR are reduced, resulting in mutagenic DNA repair, genome instability, and tumorigenesis (Bunting and Nussenzweig, 2013).

CtIP is directly phosphorylated by cyclin-dependent kinases (CDKs; Ferretti et al., 2013). CDK phosphorylation of CtIP at T847 promotes ssDNA generation, RPA recruitment, and chromosome integrity (Huertas and Jackson, 2009). Whereas changing T847 to alanine impairs resection, mutating T847 to glutamic acid mimics constitutive phosphorylation, which promotes limited resection (Huertas and Jackson, 2009). The BRCA1–CtIP interaction (Wong et al., 1998; Yu et al., 1998) is mediated by CDK phosphorylation of CtIP at S327 (equivalent to S326 in mouse) during G2 phase of the cell cycle (Yu and Chen, 2004). In chicken DT40 cells expressing human CtIP, mutation of S327 into a nonphosphorylatable residue inhibits HR repair (Yun and Hiom, 2009). Moreover, in mammalian cells, CtIP–BRCA1 complex formation facilitates removal of 53BP1 binding protein RIF1 from DSB regions, which otherwise blocks resection (Escri­bano-Díaz et al., 2013). However, the physiological role of S327 phosphorylation has been questioned by the finding that the chicken CtIP–S332A protein can efficiently promote DSB repair by HR, apparently independently of BRCA1 interaction (Nakamura et al., 2010) and by the fact that that knock-in mice homozygous for CtIP–S326A allele are neither tumor prone or HR deficient (Reczek et al., 2013). Thus, the biological importance of the BRCA1–CtIP interaction remains unclear.

Further progress in understanding how BRCA1 promotes HR was made by demonstrating that loss of 53BP1 rescues the lethality, tumorigenesis, and genome instability of BRCA1-deficient mice (Cao et al., 2009; Bouwman et al., 2010; Bunting et al., 2010). It was shown that 53BP1 inhibits resection, but that BRCA1 antagonizes 53BP1, allowing the nucleolytic processing of DNA ends in S phase (Bunting et al., 2010; Chapman et al., 2012). Furthermore, PTIP and RIF1, both of which act downstream of 53BP1, inhibit BRCA1-associated DNA metabolism (Callen et al., 2013; Chapman et al., 2013; Escri­bano-Díaz et al., 2013; Feng et al., 2013; Zimmermann et al., 2013). Nevertheless, the mediators of resection in BRCA1–53BP1, BRCA1–PTIP, and BRCA1–RIF1 double–deficient cells have not been identified.

Here, we establish that both the increased resection associated with the loss of 53BP1 and the rescue of HR defects in doubly deficient 53BP1/BRCA1 cells are dependent on CtIP but independent of EXO1. Loss of CtIP or its ability to be phosphorylated on T847 leads to embryonic lethality, constitutive DNA damage signaling, genome instability, and defective resection. In contrast, constitutive activation of CtIP by expressing a CtIP phospho-mimicking mutant CtIP (CtIP-T847E) reverts the embryonic lethality, and partially rescues BRCA1-deficient chromosomal instability even in the presence of 53BP1. Thus, whereas BRCA1 is dispensable for HR under some conditions, CtIP-mediated resection is an essential activity that can proceed independently of BRCA1.

RESULTS AND DISCUSSION
Loss of CtIP in B cells is compatible with limited cellular proliferation
The analysis of CtIP functions in vivo has been hampered by the lethality of CtIP knockout mice, with mutant embryos dying at embryonic day E3.5 (Chen et al., 2005). To circumvent this, we generated a B cell–specific deletion of CtIP by intercrossing CD19<sup>+co/+</sup> mice (Ricktert et al., 1997) with mice carrying conditional and null alleles (CtIP<sup>-/-+</sup>; Chen et al., 2008; Bothmer et al., 2013). The resulting CtIP-null (CtIP<sup>−/-</sup>) B cells expressed little or no CtIP protein as determined by Western blot analysis (Fig. 1A). Upon ex vivo stimulation with LPS and IL-4 | cells, splenic B cells enter the cell cycle and undergo class switch recombination (CSR). Despite the fact that CtIP-null mice invariably experience early embryonic death (Chen et al., 2005), CtIP<sup>−/-</sup> B cells were able to proliferate, exhibited a normal cell cycle distribution (Fig. 1B), and underwent a similar number of cellular divisions as WT cells, as determined by CFSE dye dilution (Fig. 1C). Moreover, CtIP-deficient B cells have similar level of class switching compared with WT (Bothmer et al., 2013).

Although CtIP deletion in B cells did not lead to a marked defect of cellular proliferation in live cells, we detected heightened phosphorylation of KAP-1 and p53 (Fig. 1A), indicative of constitutive DNA damage signaling. To determine whether there was a selection against CtIP-deficient cells in culture, we compared the efficiency of CD19-CRE–mediated excision in CtIP<sup>-/-</sup> versus CtIP<sup>−/-</sup> B cells by crossing these mice with the reporter line ROSA26-STOP-EYFP, in which YFP expression is induced upon CRE mediated excision. We found that there was a gradual depletion of ROSA26<sup>EYFP/− CD19<sup>−/−</sup> CtIP<sup>-/-</sup> but not ROSA26<sup>EYFP/− CD19<sup>−/−</sup> CtIP<sup>−/-</sup> B cells after 2 d in culture, indicating that the CtIP deletion confers a growth disadvantage (Fig. 1D). We therefore restricted our analyses to day 2 stimulated B cells in subsequent experiments to maximize the number of CtIP-deficient and actively cycling cells.

Spontaneous and PARPi-induced genome instability in CtIP-deficient cells
The observed constitutive phosphorylation of KAP-1 and p53 (Fig. 1A) suggested that loss of CtIP might lead to spontaneous chromosomal damage. To test this, we analyzed metaphase spreads generated from stimulated WT or CtIP<sup>−/-</sup> B cells. We observed that CtIP-deficient B cells showed spontaneous accumulation of chromosome aberrations (Fig. 1E and F). Indeed, ~40% of CtIP<sup>−/-</sup> B cells exhibited chromosomal aberrations (Fig. 1F), and on average, there were 1.5 aberrant chromosomes...
B cells do not express AID and do not undergo CSR. We found similar levels of spontaneous genomic instability in CtIP−/− B cells independent of stimulation conditions (unpublished data). Because CtIP has been implicated in HR, we monitored IR induced Rad51 focus formation in CtIP−deficient B cells. Upon RP105 activation, per mutant metaphase, whereas no instability was observed in controls (Fig. 1 E). To exclude the possibility that the instability we observed is dependent on AID expression induced by activating B cells with IL-4 and LPS, we stimulated CtIP−deficient and WT B cells with RP105. Upon RP105 activation, B cells do not express AID and do not undergo CSR. We found similar levels of spontaneous genomic instability in CtIP−/− B cells independent of stimulation conditions (unpublished data). Because CtIP has been implicated in HR, we monitored IR induced Rad51 focus formation in CtIP−deficient B cells.
We found that the percentage of RAD51 focus-positive cells was decreased on average to 50% of the WT level (Fig. 1 G), which is consistent with decreased resection activity in CtIP-deficient cells (Sartori et al., 2007). Thus, CtIP loss leads to considerable DNA damage accumulation associated with defective HR.

Cells with impaired HR are hypersensitive to compounds that induce replication-associated breaks. For example, BRCA1-deficient cells are hypersensitive to PARPi (Bryant et al., 2005; Farmer et al., 2005; Jackson and Bartek, 2009). Consistent with this, treating cells with the PARPi (olaparib) resulted in a nearly twofold increase in the total amount of chromosomal aberrations in CtIP-null B cells (Fig. 1 H), whereas such PARPi treatment led to little or no induction of aberrations in control cells. Although the total amount of DNA damage induced by PARPi was equivalent in both CtIP-deficient and BRCA1-deficient cells, radial structures accumulated at a greater frequency in the absence of BRCA1 (Fig. 1, H and I). We conclude that BRCA1- and CtIP-deficient B cells are both hypersensitive to PARPi, but CtIP-deficient cells accumulate considerably more spontaneous damage.

**53BP1 or Ku80 deficiency does not rescue embryonic lethality of CtIP knockout mice**

Because loss of 53BP1 rescues the viability of BRCA1-deficient mice (Cao et al., 2009), we tested whether deletion of 53BP1 could similarly rescue the embryonic lethality of CtIP−/− mice. However 53BP1−/−/CtIP−/− intercrosses did not generate any of the expected 15 CtIP/53BP1 double knockout mice out of 60 live births (Table 1). In Schizosaccharomyces pombe, the hypersensitivity of CtIP (Cpt1)-deficient cells to camptothecin (which yields DSBs during S-phase that rely on resection and HR for their repair) is rescued by loss of Ku (Langerak et al., 2011). To see whether a similar relationship exists in mammals, we intercrossed CtIP−/−/+ and 53BP1−/−/+ mice to generate triple CtIP−/−/53BP1−/− mutant B cells. Consistent with the decrease of ssDNA in CtIP−/−/+ MEFs (Fig. 2 D), we found that CtIP deficiency in B cells led to a decrease in IR induced RAD51 foci formation relative to WT (Fig. 2 E). Moreover, PARPi treatment of CtIP−/−/53BP1−/−/+ MEFs (Fig. 2 F), we found that CtIP deficiency in B cells led to a decrease in IR induced RAD51 foci formation relative to WT (Fig. 2 E). Moreover, PARPi treatment of CtIP−/−/53BP1−/−/+ MEFs led to a 13-fold increase in the level of DNA damage relative to BRCA1Δ11Φ 53BP1−/−/+ (Fig. 2 F). Together with the finding that the IR-induced increase in resection in BRCA1−/−/53BP1−/−/+ double-knockout cells was CtIP dependent (Fig. 2 D), these results suggest that CtIP-mediated resection is critical for the rescue of HR in BRCA1−/−/53BP1−/−/+ mutant cells.

### Table 1. Loss of either 53BP1 or Ku80 does not rescue CtIP knockout mouse lethality

| Genotypes | CtIP+/−/53BP1−/− x CtIP+/− | CtIP+/−/53BP1−/− x CtIP+/−/Ku80−/− |
|-----------|--------------------------|-------------------------------|
| Live-born pups |                           |                               |
| Observed     | 25                        | 35                            |
| Expected     | 15                        | 15                            |
| Total mice screened | 60                       | 129                           |

CtIP+/−/53BP1−/− mice and CtIP+/−/Ku80−/− were intercrossed. Genotypes and number of animals generated by the breeding are indicated.
CDK mediated phosphorylation of CtIP at T847 but not S327 is essential for viability

The aforementioned findings suggested that CtIP could act independently of BRCA1 to promote resection. Consistent with this, we found that BRCA1 was not required for recruitment of CtIP to DNA damage sites (Fig. 3A). To explore the physiological role of the CtIP–BRCA1 interaction mediated by CDK-dependent phosphorylation of CtIP at S327 (Yu and Chen, 2004), we generated BAC transgenic mouse models. By introducing the human gene coding for CtIP (hereafter, referred to as CtIP$^{WT}$) as a transgenic copy carried by a bacterial artificial chromosome (BAC RP11-104H10), we were able to rescue the lethality caused by homozygous deletion of mouse CtIP (Table 2). CtIP$^{WT}$ was expressed at a similar level compared with endogenous CtIP (Fig. 3B). Moreover, B cells in CtIP$^{WT}$/CtIP$^{-/-}$ mice were equivalent to CtIP$^{+/+}$ with respect to RAD51 foci formation and genome stability (Fig. 3, C and D).

In the two-step model for resection, CtIP carries out initial end processing, which is followed by EXO1- or DNA2/BLM-mediated extension of the resected tracks (Symington and Gautier 2011). Because EXO1 recruitment and exonuclease activity are reported to be CtIP dependent (Eid et al., 2010), we tested whether the rescue of HR in BRCA–53BP1-deficient cells was EXO1 dependent. To do this, EXO1$^{+/−}$ mice were intercrossed with CD19$^{+/−}$/BreaΔ11$^{+/−}$/53BP1$^{−/−}$ mice, and the resulting CD19$^{−/−}$/BreaΔ11$^{−/−}$/53BP1$^{−/−}$/EXO1$^{+/−}$ mice were intercrossed to generate triple BRCA1–53BP1–EXO1 mutant B cells. We found that BRCA1Δ11$^{−/−}$/53BP1$^{−/−}$/EXO1$^{−/−}$ cells were as insensitive to PARPi as BRCA1Δ11$^{−/−}$/53BP1$^{−/−}$/EXO1$^{−/−}$/CtIP$^{−/−}$ cells and much less sensitive than BRCA1Δ11$^{−/−}$/53BP1$^{−/−}$/EXO1$^{−/−}$/CtIP$^{−/−}$/BRCA1$^{−/−}$ cells (Fig. 2, F and G). These data thus establish that CtIP but not EXO1 mediates the rescue of genomic stability of BRCA1–53BP1 deficient cells.

**Figure 2. Increased resection of 53BP1$^{−/−}$ cells is CtIP dependent.** (A) Analysis of spontaneous genomic instability in metaphases from B cells isolated from mice of the indicated genotypes. Genomic instability measured in five independent experiments is normalized to levels seen in CtIP-deficient B cells ($n > 200$ metaphases/genotype; $P = 0.4179$; two-tailed paired Student’s t test). (B) Constitutive levels of p53 phosphorylation in the CtIP$^{+/+}$ and CtIP$^{−/−}$/53BP1$^{−/−}$ double mutant B cells. (C) Western blot analysis showing levels of CtIP expression in MEFs infected with CtIP shRNA (shCtIP). (D) Fold increase of mean BrdU fluorescence of irradiated (30 Gy) versus unirradiated MEFs of the indicated genotypes that were infected or not with shCtIP. The mean and SD of three independent experiments are shown (significance tests were analyzed with one-tailed Student’s t test; *, $P < 0.05$). (E) Percentage of positive RAD51 foci after 10 Gy irradiation relative to WT (16.6% of WT cells were positive for RAD51 foci). For each experiment, >600 cells were counted. (F) Analysis of genomic instability in metaphases from B cells treated with PARPi (1 µM; 16 h; $n > 200$ metaphases/genotype were counted; the mean of 4 independent experiments is shown relative to BRCA1-deficient cells; significance tests were analyzed with two-tailed Student’s t test; *, $P < 0.05$; n.s., not significant). (G) Analysis of genomic instability in B cells after PARPi treatment (1 µM for 16 h). The graph shows the average number of aberrant chromosome structures per cell measured in three independent experiments relative to genome instability in BRCA1-deficient cells ($n = 150$ metaphases/genotype; significance tests were analyzed with two-tailed Student’s t test; *, $P < 0.05$; n.s., not significant). For each independent experiment, one mouse per genotype was used.
Figure 3. CDK-dependent phosphorylation of CtIP<sup>T847</sup> is essential for CtIP function. (A) WT and BRCA1<sup>Δ11Δ11</sup> MEFs were irradiated with a 364-nm laser line derived from a LSM510 microscope. After 10-min recovery, cells were processed for immunofluorescence analysis of CtIP (red) and γH2AX (green). Bar, 15 µm. (B) Western blot showing expression of transgenic CtIP<sup>WT</sup> and CtIP<sup>S327A</sup> protein and phosphorylated KAP1 and p53 in B cells. (C) Percentage of cells with >5 RAD51 foci after 10 Gy irradiation. Three independent experiments are reported. For each experiment, >600 cells were counted (significance tests were analyzed with two-tailed paired Student’s t test; *, P < 0.05; n.s., not significant). (D) Analysis of total genomic instability in metaphases from B cells isolated from mice of indicated genotypes after treatment for 16 h with 1 µM PARPi. The mean number of chromosome aberrations per cell measured in 3 independent experiments is shown (n = 250 metaphases; significance tests were analyzed with two-tailed paired Student’s t test; *, P < 0.05; n.s., not significant). (E) Levels of transgenic CtIP<sup>WT</sup>, CtIP<sup>T847E</sup>, and CtIP<sup>T847A</sup> protein expression and phosphorylated KAP1 and p53 in mutant B cells detected by Western blotting. (F) Analysis of total genomic instability in metaphases from B cells with or without PARPi treatment. Mean of three independent experiments is shown (significance tests were analyzed with two-tailed paired Student’s t test; *, P < 0.05; n.s., not significant). (G) Percentage of B cells with >5 RAD51 foci after 10 Gy IR. Three independent experiments are reported. >600 cells were counted for each genotype (P = 0.0251; two-tailed paired t test). (H) Western blot showing the level of CtIP expression in infected cells using anti-FLAG antibody (top). WT MEFs expressing FLAG-CtIP<sup>WT</sup>, FLAG-CtIP<sup>S327A</sup>, or FLAG-CtIP<sup>T847E</sup> were treated with Hoechst 33342 and irradiated with the 364-nm laser line and after 20-min recovery FLAG (red) and 53BP1 (green) were detected by immunofluorescence (bottom). Bar, 15 µm. (I) Western blot showing the levels of CtIP protein in B cells from mice of the indicated genotype (top). Genomic instability in B cells isolated from mice of the indicated genotypes, relative to total instability measured in BRCA1-deficient B cells. Cells were treated with 1 µM PARPi for 16 h (bottom). Three independent experiments are reported (significance tests were analyzed with two-tailed paired Student’s t test; *, P < 0.05; n.s., not significant). For each independent experiment, one mouse per genotype was used.
We next generated BAC transgenic mice expressing a CtIP mutant in which Ser-327 was substituted for Ala (CtIPS327A) to abrogate phosphorylation by CDK at this site. Like CtIPWT, expression of mutant CtIPS327A rescued the lethality of CtIP knockout mice (Table 2). Indeed, CtIPS327A/CtIP−/− mice developed normally, without notable defects in growth, weight, lymphocyte development, or fertility (unpublished data). Moreover, in stark contrast to CtIP deficiency, CtIPS327A/CtIP−/− B cells were not markedly hypersensitive to PARPi, did not harbor constitutive DNA damage signaling, and formed normal levels of IR–induced RAD51 foci (Fig. 3, B–D). These results are consistent with a recent characterization of CtIP-S326A knockout mice, which concluded that CDK–dependent phosphorylation of CtIP–BRCA1 interaction domain is dispensable for HR (Reczek et al., 2013).

In addition to S327, the other CDK–dependent phosphorylation site of CtIP implicated in DSB resection was Thr-847 (Huertas and Jackson, 2009). To assess whether this residue affects CtIP function, we used BAC recombineering to mutate the Thr-847 residue to Ala (CtIPT847A) or a potentially phospho-mimic version of the protein (CtIPT847E). Phosphorylation of CtIP–BRCA1 interaction domain is dispensable for HR in mice (Reczek et al., 2013).

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To do so, we crossed CD19<sup>pro</sup> BRCA1<sup>A11F<sup>2</sup>/</sup> mice with either CtIP<sup>WT</sup> or CtIP<sup>T847E</sup> transgenic mice to generate BRCA1-deficient B cells expressing either CtIP<sup>WT</sup> or CtIP<sup>T847E</sup> protein (Fig. 3I). We found that the genomic instability induced by PARPi treatment was significantly reduced in BRCA1<sup>A11F<sup>2</sup>/</sup> mutant B cells expressing CtIP<sup>T847E</sup>, relative to that found in Brca1<sup>A11F<sup>2</sup>/</sup>-<sup>ko</sup> cells (Fig. 3I). CtIP<sup>WT</sup>, which expressed slightly lower levels of CtIP than CtIP<sup>T847E</sup> (Fig. 3I), also reduced the level of chromosomal aberrations in BRCA1-deficient mice, but this was not significant (Fig. 3I). These data therefore indicate that overexpression of CtIP can partially overcome 53BP1's block to resection in BRCA1-deficient cells.

**Model for functions of BRCA1 and CtIP in DSB resection**

Previous studies have suggested that BRCA1 plays a role in resection and that this is mediated by the CtIP-BRCA1 interaction. These conclusions were based on the findings that BRCA1-deficient cells exhibit a mild decrease in IR-induced RPA focus formation (Chen et al., 2008; Escribano-Díaz et al., 2013), that BRCA1 and CtIP inhibit RIF1 end-blocking activity in S/G2 (Escribano-Díaz et al., 2013; Zimmermann et al., 2013), and that cells expressing CtIP-327A are defective in HR (Yun and Hiom, 2009). However, subsequent studies in chicken (Nakamura et al., 2010), frog (Peterson et al., 2011), and mouse cells (Reczek et al., 2013; analogous to our CtIP-null cells reconstituted with CtIP-S327A), and direct measurements of gene conversion (Chandramouly et al., 2013), argue that the CtIP-BRCA1 interaction is dispensable for HR. Moreover, our finding that restoration of HR in BRCA1–53BP1-deficient cells and increased resection that occurs in the absence of 53BP1 are CtIP dependent, demonstrates that CtIP-mediated resection is BRCA1 independent. These results likely explain why loss of 53BP1 cannot reverse the embryonic lethality or genomic instability of CtIP-deficient mice. Localization of CtIP to damage sites must take place by mechanisms other than association with BRCA1, such as direct association of CtIP with DNA (You et al., 2009) or through phosphorylation by the ATR kinase (Peterson et al., 2013).

If CDK-mediated phosphorylation of CtIP is essential for its function (Table 1 and Fig. 3, E–G), whereas BRCA1 is dispensable for resection, how does BRCA1 promote HR? We have previously suggested that BRCA1's principal role in DSB repair is to antagonize 53BP1-dependent end protection (Bunting et al., 2010). In this model, in the absence of BRCA1, CtIP activity is limited by 53BP1 and downstream cofactors RIF1 and PTIP, which results in a modest defect in RPA focus formation (Escribano-Díaz et al., 2013). Nevertheless, CtIP-mediated resection can partly overcome 53BP1 end blocking activity, evidently by our finding that mimicking constitutive CtIP phosphorylation (CtIP<sup>T847E</sup>) partially alleviates genomic instability of BRCA1-deficient/53BP1<sup>−/−</sup> cells. Furthermore, although 53BP1 antagonizes CtIP function, complete absence of 53BP1 allows full access and activity of CtIP, which significantly increases ssDNA formation. Thus, we propose that BRCA1 facilitates CtIP-mediated resection in the presence of 53BP1 but is no longer needed in its absence. In addition, BRCA1 plays functions in meiosis and cross-link repair that are 53BP1 independent (Bunting et al., 2012).

The pro–NHEJ (non-homologous end joining) functions that promote class switch recombination, and the anti–recombination functions that lead to loss of genome stability in BRCA1-deficient cells are mediated by distinct phospho–dependent interactions with 53BP1 (Callen et al., 2013). PTIP associates with phosphorylation sites at the extreme N terminus of 53BP1 to suppress HR in S phase, whereas RIF1 binds to independent residues to promote NHEJ in G1 (Callen et al., 2013). We have proposed that PTIP and RIF1 could interfere with a distinct set of nucleases (Callen et al., 2013). Consistent with this idea, we have found that the rescue of genome stability in BRCA1–53BP1-deficient cells is dependent on ATM (Bunting et al., 2010) and CtIP (Fig. 2F), but occurs independently of EXO1 (Fig. 2G). In contrast, extensive resection during class switching in 53BP1<sup>−/−</sup> cells is ATM-independent (Yamane et al., 2013), and is mediated by both CtIP and EXO1 (Bothmer et al., 2013). Similarly, unrepaired G1-phase breaks during V(D)J recombination are processed by CtIP (Helmink et al., 2011). These results suggest that, whereas CtIP promotes nucleolytic processing of DSBs in all cell cycle phases, other factors that stimulate or antagonize the extension of resected tracks may be distinct and regulated in a cell cycle–dependent manner.

**MATERIALS AND METHODS**

**Generation of mice.** CDIP<sup>pro</sup> (Bothmer et al., 2013), CDIP<sup>pro/−</sup> (Chen et al., 2005), 53BP1<sup>−/−</sup> (Ward et al., 2004), BRCA1<sup>B10d10/A11F</sup> (here reported BRCA1<sup>A11F</sup>; NCI mouse repository), KoKO<sup>pro/−</sup> (Nussenzweig et al., 1996), CDIP pro/− (Rickert et al., 1997), EXO1<sup>−/−</sup> (Wei et al., 2003), and ROSA26-STOP-EYFP (Srinivas et al., 2001) mice have been described. BAC RP11-104H10 containing human CtIP genomic sequence was used to generate transgenic mice. S327A, T847A, and T847E mutations were targeted using BAC recombineering as previously described (Ditllimpanontho et al., 2005). Transgenic CtIP was detected by PCR screening of tail DNA using the following primer pairs: hEx1F 5'-AGCA-CACACACTGAATGC-3' and hEx1R 5'-CACACACGGTTATTCCTCA-CACGG-3', which amplify a product of 305 bp in the intronic sequence specific for the human gene. All experiments with mice were conducted in accordance with protocols approved by the National Institutes of Health Institutional Animal Care and Use Committee.

**B cell culture, flow cytometry, metaphase analysis.** B cells were isolated from WT or mutant spleen with anti-CD43 Microbeads (anti-Ly48; Miltenyi Biotec) and were cultured with LPS (25 µg/ml; Sigma-Aldrich), IL-4 (5 ng/ml; Sigma-Aldrich), and RP105 (0.5 µg/ml; BD). Cell proliferation was analyzed by CFSE (5 µM; Molecular Probes) labeling at 37°C for 10 min. For cell cycle analysis, B cells were fixed in methanol and stained with propidium iodide (PI). BrdU incorporation and detection was performed as described (Bunting et al., 2012). Samples were acquired on a FACSCalibur (BD). For genomic instability analysis, B cells were harvested after 2 d in culture; metaphase spreads were generated and processed for FISH analysis as previously described (Callen et al., 2013). PARP inhibitor (KU55948; Astra Zeneca) was added to cells stimulated ex vivo for one day.

**Generation of CtIP retroviral clones.** Coding sequences of the mouse CtIP were cloned into PMX-IRES-GFP plasmid (CDIP<sup>pro</sup>). A FLAG-tag was cloned by PCR at the N terminus of CtIP coding sequence (FLAG–CDIP<sup>pro</sup>). Mutant FLAG–CtIP<sup>T847E</sup> and FLAG–CtIP<sup>S327A</sup> were generated by targeting Flag–CtIP<sup>pro</sup> using QuikChange II XL Site-Directed Mutagenesis kit (Agilent Technologies), and mutations were confirmed by sequencing. Infected MEFs
were grown and selected in medium containing 2 µg/ml puromycin for 1 wk, and then maintained in 1 µg/ml puromycin.

**Immunoblotting, immunofluorescence, and laser microirradiation.**
Western blotting was performed with the following primary antibodies: mouse anti-tubulin (Sigma-Aldrich), mouse anti–FLAG-M2 (Sigma-Aldrich), mouse anti-CTIP (mAb 14–1; gift from R. Baer, Columbia University, New York, NY), rabbit anti–CTIP (developed in collaboration with Epitomics), rabbit anti–KAP-1 pS824 (Bethyl Laboratories), rabbit anti–p53 pS15 (Cell Signaling Technology), and rabbit anti–53BP1 (Novus). For RAD51 immunofluorescence, cells were irradiated at 10 Gy allowed to recover for 4 h, and then fixed and processed as previously described (Celeste et al., 2003). For microirradiation, cells were presented in DMEM media containing 0.1 µg/ml of Hoechst 33342 for 60 min before replacing with phenol red free media containing 5 mM Hepes, and then irradiated with the 364-nm laser line on a LSM510 confocal microscope (Carl Zeiss, Inc.) equipped with a heated stage. Cells were allowed to recover for the indicated time before processing for immunofluorescence.

Immunoblotting, immunofluorescence, and laser microirradiation.

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