CtIP is essential for early B cell proliferation and development in mice

Xiangyu Liu1,2,*, Xiaobin S. Wang1,3,*, Brian J. Lee3, Foon K. Wu-Baer1, Xiaohui Lin1,*, Zhengping Shao1, Verna M. Estes1, Jean Gautier4, Richard Baer1,4, and Shan Zha1,5,6,*

B cell development requires efficient proliferation and successful assembly and modifications of the immunoglobulin gene products. CtIP is an essential gene implicated in end resection and DNA repair. Here, we show that CtIP is essential for early B cell development but dispensable in naive B cells. CtIP loss is well tolerated in G1-arrested B cells and during V(D)J recombination, but in proliferating B cells, CtIP loss leads to a progressive cell death characterized by ATM hyperactivation, G2/M arrest, genomic instability, and 53BP1 nuclear body formation, indicating that the essential role of CtIP during proliferation underscores its stage-specific requirement in B cells. B cell proliferation requires phosphorylation of CtIP at T847 presumably by CDK, but not its interaction with CtBP or Rb or its nuclease activity. CtIP phosphorylation by ATM/ATR at T859 (T855 in mice) promotes end resection in G1-arrested cells but is dispensable for B cell development and class switch recombination, suggesting distinct roles for T859 and T847 phosphorylation in B cell development.

Introduction

The diversity and specificity of the adaptive immune system depend on the somatic assembly and subsequent modifications of the antigen receptor gene products. In particular, B lymphocyte development is achieved by multiple rounds of clonal expansion and two programmed DNA double-strand break (DSB) repair events at the Ig gene loci. V(D)J recombination assembles the exons that encode the variable region of the Ig genes in immature B cells, occurs exclusively in the G1 phase of the cell cycle, and is mediated exclusively by the nonhomologous end joining (NHEJ) pathway of DSB repair. Class switch recombination (CSR) modifies the constant region of the Ig heavy chain and results in different isotypes and thus effector function for the antibody, requires cell proliferation, and can be achieved by either NHEJ or the alternative end-joining (Alt-EJ) pathway that preferentially uses sequence microhomology (MH) to align the DSB junctions for repair. DNA recombination, which converts DSB ends into 3′ single-stranded DNA (ssDNA) overhangs, promotes Alt-EJ by exposing flanking MH (McVey and Lee, 2008; Zhang and Jasien, 2011), and suppresses NHEJ by limiting KU binding (Mimitou and Symington, 2008; Symington and Gautier, 2011). Therefore, end resection is a critical determinant of the repair pathway choice in developing lymphocytes. In addition, end resection is also necessary for homologous recombination (HR), which is often necessary to support rapid cell proliferation.

C-terminal binding protein (CtBP)–interacting protein (CtIP) is best known as the mammalian orthologue of yeast Sae2, which initiates DNA end resection together with the MRE11–RAD50–NBS1 complex (Sartori et al., 2007; Mimitou and Symington, 2008; Cannavo and Cejka, 2014; Deshpande et al., 2016). In addition to DNA end resection, CtIP/Sae2 has also been implicated in nucleolytic processing of DNA hairpins (Lengsfeld et al., 2007; Makharashvili et al., 2014; Wang et al., 2014; Chen et al., 2015), removal of protein–DNA adducts (Nakamura et al., 2010; Aparicio et al., 2016; Deshpande et al., 2016), and termination of checkpoint signaling (Lengsfeld et al., 2007; Makharashvili et al., 2014; Wang et al., 2014; Chen et al., 2015).

CtIP protein contains several functional domains. Despite their primary sequence divergence, the N-terminal region of CtIP and Sae2 both mediate oligomerization necessary for end resection (Dubin et al., 2004; Wang et al., 2012; Andres et al., 2015). CtIP (897 amino acids in human) is much larger than Sae2 (345 amino acids). The middle of CtIP contains several motifs

---

1Institute for Cancer Genetics, Vagelos College of Physicians and Surgeons, Columbia University, New York, NY; 2Guangdong Key Laboratory of Genome Instability and Human Disease Prevention, Shenzhen University Carson Cancer Center, Department of Biochemistry and Molecular Biology, School of Medicine, Shenzhen University, Shenzhen, China; 3Pathobiology and Human Disease Graduate Program, Vagelos College of Physicians and Surgeons, Columbia University, New York, NY; 4Department of Pathology and Cell Biology, Vagelos College of Physicians and Surgeons, Columbia University, New York, NY; 5Division of Pediatric Oncology, Hematology and Stem Cell Transplantation, Department of Pediatrics, Vagelos College of Physicians & Surgeons, Columbia University, New York, NY.

*X. Liu and X.S. Wang contributed equally to this paper; Correspondence to Shan Zha: sz2296@cumc.columbia.edu; X. Liu’s present address is Department of Biochemistry and Molecular Biology, Shenzhen University Medical Center, Shenzhen, China.

© 2019 Liu et al. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike–No Mirror Sites license for the first six months after the publication date (see http://www.nupress.org/terms/). After six months it is available under a Creative Commons License (Attribution–Noncommercial–Share Alike 4.0 International license, as described at http://creativecommons.org/licenses/by-nc-sa/4.0/).
unique for CtIP, including those essential for its interaction with CtBP transcriptional repressor (through PLDLS motif; Schaeper et al., 1998), BRCA1 (S327) (Wong et al., 1998; Yu et al., 1998), and retinoblastoma-associated protein (Rb; EI57; Liu and Lee, 2006) tumor suppressors, as well as its proposed intrinsic nuclelease activities (Makharashvili et al., 2014; Wang et al., 2014). The C-terminus of CtIP shares the most homology with Sae2 (Sartori et al., 2007), including two conserved phosphorylation sites implicated in end resection. Specifically, CtIP is phosphorylated by cyclin-dependent kinase (CDK) and possibly the Pole-like kinases at T847 (S267 in Sae2) in S and G2 phases of the cell cycle (Chen et al., 2008; Huertas et al., 2008; Barton et al., 2014), and by ataxia telangiectasia and Rad3-related protein (ATR)/ataxia telangiectasia mutated (ATM) at T859 (S279 in Sae2) upon DNA damage (Peterson et al., 2013; Wang et al., 2013). Whether CtIP is essential for B cell development and how the specific domains/interaction partners of CtIP contribute to B lymphocyte development and Ig gene assembly and modification are not yet fully understood, in part due to the early embryonic lethality associated with the complete loss of CtIP (Chen et al., 2005). During V(D)J recombination, the hairpin coding ends (CEs) must be opened nucleolytically before end ligation, providing a unique opportunity to investigate whether mammalian CtIP can open hairpins outside the S/G2 phase.

Several attempts have been made to address the function of CtIP in B cells, especially during CSR. Knockdown of CtIP using shRNA in purified splenic B cells compromises CSR, which has been attributed to its direct contribution to Alt-EJ or its indirect effects on CDK2 activation or cell viability (Lee-Theilen et al., 2011; Buis et al., 2012; Polato et al., 2014). Knockin mouse models expressing S327A CtIP that cannot interact with BRCA1 are able to support both embryonic development (Reczek et al., 2013) and B cell CSR (Polato et al., 2014). In transgenic mouse models that express exogenous human CtIP proteins, embryogenesis and CSR can be rescued by expression of the phospho-mimetic CtIP-T847E mutant, but not the phospho-deficient CtIP-T847A mutant (Polato et al., 2014). In proliferating human cells, CtIP phosphorylation at T847 was proposed as a prerequisite for ATR/ATM-mediated hyperphosphorylation of CtIP upon DNA damage (Wang et al., 2013). However, in G1-arrested murine B cells, CtIP can still resect unrepaired DSBs in an ATM-dependent manner, but only in the absence of H2AX or 53BP1 (Helmink et al., 2011; Zha et al., 2011a; Liu et al., 2012; Oksenych et al., 2012), suggesting that CtIP can function outside S/G2, when CDK activity is minimal. However, it is unclear which CtIP residues, such as T859 (Peterson et al., 2013; Wang et al., 2013) or other potential ATM sites (Makharashvili et al., 2014), are relevant in G1. In Xenopus extract, phosphorylation of CtIP at T818 (corresponding to T859 in human CtIP) is essential for the recruitment of CtIP to the damaged chromatin (Peterson et al., 2013). In human cells, the expression of T859A mutant CtIP compromises end resection and HR (Wang et al., 2013), but the role of CtIP in early B cell development remains unknown, and how phosphorylation of CtIP at T859 contributes to lymphocyte development through the myriad functions of CtIP is also not known.

Using development stage-specific Cre recombinase and a conditional CtIP allele, we found that CtIP is essential for early B cell development, independent of V(D)J recombination, in part due to its ability to support continued proliferation at the G2/M transition. A genetic complementation screen reveals that the interaction of CtIP with CtBP, Rb, and Brca1 and its own nuclelease activity are dispensable for B cell proliferation. Instead, the T847, but not the T859, phosphorylation of CtIP is essential for B cell development and switch recombination. Together these data indicate that in murine B cells, phosphorylation of CtIP at T859 has a cell cycle-independent role in end resection but is dispensable for B cell development in vivo.

**Results**

**CtIP is essential for early B cell development but dispensable for the survival of peripheral naive B cells** To circumvent the embryonic lethality of Ctip-null mice, a conditional Ctip allele (Ctip<sup>−/−</sup>) was inactivated specifically in precursor B cells using an Mb1/CD79a-driven Cre transgene (Mb1<sup>Cre/−/Ctip<sup>−/−</sup> mice) or in naive B cells using a CD21-driven Cre transgene (CD21Cre<sup>−/−/Ctip<sup>−/−</sup> mice; Kraus et al., 2004; Hobeika et al., 2006; Polato et al., 2014). Given B cell–specific deletion of CtIP, the relative frequency of B cells among all the hematologic lineages provides a measurement for B cell development in these mouse models. In contrast to CD19Cre (Rickert et al., 1997) used in prior studies, CD21Cre turns on only in IgM-positive naive B cells and achieves robust depletion of targeted protein in splenic B cells (Kraus et al., 2004). Consistent with prior studies (Lee-Theilen et al., 2011; Buis et al., 2012; Polato et al., 2014), CtIP deletion is well tolerated in naive mature B cells, as evidenced by the normal frequency and number of B220<sup>+</sup>IgM<sup>+</sup> B cells in the bone marrow and spleen of CD21Cre<sup>−/−/Ctip<sup>−/−</sup> mice (Fig. 1 A and Table S1 A). However, upon activation, these Ctip-null B cells display severe proliferation defects and accumulate in the sub-G1 and G2 phases of the cell cycle (Fig. 1, B and C). Unlike V(D)J recombination, which is completed within the G1 phase, CSR requires cell proliferation (Franco et al., 2006). Thus, the severe proliferation defects of activated CtIP-deficient B cells likely contribute to the initial delay and moderate reduction of CSR (Fig. 1 B). Accordingly, CSR junctions recovered from activated CD21Cre<sup>−/−/Ctip<sup>−/−</sup> B cells are not significantly different from those of control CD21Cre<sup>−/−</sup> cells (Fig. 3 B). In contrast to naive B cells, the Ctip-null pre-B cells of Mb1Cre<sup>−/−/Ctip<sup>−/−</sup> mice fail to thrive (Fig. 1 D), leading to severe reductions in the pro-, naive, and mature B cell frequency in the bone marrow and number and frequency in the spleen (Fig. 1, D and E; Fig. S1 A; and Table S1 A). There is also a notable reduction of pre-B/pro-B ratio in the bone marrow of Mb1Cre<sup>−/−/Ctip<sup>−/−</sup> mice (Table S1 A). Although this observation is consistent with potential V(D)J recombination defects, the initiation of Cre expression in Mb1Cre<sup>−/−/Ctip</sup> mice also occurs at the pro-B to pre-B transition. A specific role of CtIP in V(D)J recombination was then tested in a cell-based system (see below). Meanwhile, T cell development and thymus cellularity
Figure 1. CtIP is essential for immature B cells yet dispensable for naive B cells. (A) Left: Representative flow cytometry analyses of lymphocytes in bone marrow and spleen from CD21Cre<sup>+</sup> Ctip<sup>-/-</sup> and CD21Cre<sup>+</sup> Ctip<sup>+/+</sup> mice. The boxes marked the IgM<sup>-</sup>B220<sup>+</sup> mature B cells. Middle: Representative flow cytometry analyses of purified CD43<sup>-</sup> splenic B cells stimulated with LPS plus IL-4 for 4 and 5 d. The numbers indicate the percentage of IgG1<sup>+</sup> B cells among all B cells (B220<sup>+</sup>). The percentage of IgG1<sup>+</sup> cells among all B220<sup>+</sup> cells was marked on each blot. Right: Total splenocytes and B220<sup>-</sup>IgM<sup>-</sup> B cells in the spleens of CD21Cre<sup>+</sup> Ctip<sup>-/-</sup> and CD21Cre<sup>+</sup> Ctip<sup>+/+</sup> mice (n ≥ 4 for each group). (B) Combo graph for CSR efficiency (percentage of IgG1<sup>+</sup> cells, left y axis) and cell density (×10<sup>6</sup>/ml, right y axis) from CD21Cre<sup>+</sup> Ctip<sup>-/-</sup> and CD21Cre<sup>+</sup> Ctip<sup>+/+</sup> mice (n ≥ 4 for each group). (C) Representative cell cycle analyses of activated CD21Cre<sup>+</sup> Ctip<sup>-/-</sup> and CD21Cre<sup>+</sup> Ctip<sup>+/+</sup> B cells pulse labeled with BrdU for 30 min before fixation and staining. Representative results from n = 3 biological repeats are shown. The boxes mark the sub-G1, G1, S (BrdU<sup>-</sup>), and G2 populations. P.I., propidium iodide. (D) Representative flow cytometry analyses of lymphocytes from the bone marrow and spleens of Mb1<sup>+</sup>/Cre control and Mb1<sup>+</sup>/Cre Ctip<sup>-/-</sup> mice. Different B cell subtypes that were used to calculate the frequency and counts (Table S1 A) are marked in Fig. 1 D; they are pro-B cells (B220<sup>-</sup>CD43<sup>+</sup>), recirculating B cells (IgM<sup>-</sup>B220<sup>high</sup>), naive B cells (IgM<sup>+</sup>B220<sup>+</sup>) and pre/pro-B cells (IgM<sup>-</sup>B220<sup>-</sup>). (E) The frequency of B cell subtype in bone marrow. Artemis<sup>-/-</sup> (Atr<sup>-/-</sup>) mice were used here as NHEJ-deficient control. Pro-B cells were defined as B220<sup>-</sup>CD43<sup>+</sup>. Pre-B cell frequency was calculated by subtracting the frequency of pro-B cells from all IgM<sup>-</sup> B cells in the bone marrow. Naive B cells are IgM<sup>+</sup>B220<sup>+</sup> cells and the recirculated B cells are IgM<sup>-</sup>B220<sup>high</sup>. Two-tailed Student’s t test was used to calculate P values (n ≥ 3 for each group). The calculated absolute cell counts for each population were included in Table S1 A. Statistical significance was assessed using a two-tailed Student’s t test (not significant [n.s.], P > 0.05; *, P < 0.05; **, P < 0.01). The error bars represent SD from three independent experiments.
Ctip is dispensable for V(D)J recombination and NHEJ in G1 cells Next, we examined whether the severe B cell developmental defects of MbC/Tip/th/co, but not CD21/Cre Tip/th/co, mice reflect a role for Ctip in V(D)J recombination, including the opening of hairpin CEs (Fig. 1, B and C; Moshous et al., 2001; Rooney et al., 2002). Abelson murine leukemia virus–transformed pre-B cells (hereafter referred to as v-abl cells) containing an integrated V(D)J recombination reporter (pMX-INV) were derived from Ctip/th/co mice, as described previously (Bredemeyer et al., 2006; Zha et al., 2011b). To induce Ctip inactivation, the Ctip/th/co v-abl cells were infected with retrovirus-encoded WT CreER or recombinase-dead CreERMut(R173K) (Jonkers et al., 2001). Induction of nuclear translocation of Cre with 4-hydroxytamoxifen (4OHT) effectively ablated the expression of WT Ctip in CreER-expressing cells, but not in CreERMut(R173K)-containing cells (Fig. 2 B). Induction of G1 arrest and the expression of recombination-activating gene (RAG) recombinase via the v-abl kinase inhibitor STI571 (imatinib) in Ctip-deleted cells leads to successful chromosomal V(D)J recombination at the integrated pMX-INV reporter (Fig. 2 A; as measured by the formation of both coding joins [CJs] and signal joins [SJs]), as well as expression of GFP, regardless of Ctip depletion (Fig. 2, C and D). No measurable accumulation of unjoined CEs was detected in Ctip/th cells (Fig. 2 D). Similar results were obtained in Rosa26/CreER-T2 Ctip/th/co v-abl cells, which harbor a knocked-in CreER-T2 allele in the Rosa26 locus (Fig. S1 D). Sequence analyses of the SJs and CJs formed in the presence or absence of Ctip were also undistinguishable, with similar fidelity measured by ApaL1 digestion (Fig. 2 E) and a similar number of base-pair deletions and insertions (including both palindromic and non-template nucleotide addition; Fig. 2 F and Table S1 B). Moreover, ATM kinase inhibition similarly delays the joining phase of V(D)J recombination in both WT and Ctip-null cells without abolishing end joining or hairpin opening (Fig. S1 D). Artemis endonuclease is responsible for opening CE hairpins during V(D)J recombination (Moshous et al., 2001; Ma et al., 2002). In Artemis−/− cells, ectopic expression of the T847E human Ctip, but not the corresponding phospho-deficient mutant (T847A), leads to significant reduction of hairpin accumulation (Fig. 2 G), suggesting that phosphorylation of Ctip at T847 promotes Ctip-dependent hairpin opening. The low level of Ctip protein and the limited CDK activities in G1-arrested cells undergoing V(D)J recombination may explain why Ctip does not contribute to hairpin opening during physiological V(D)J recombination. Moreover, chromosomal breaks generated by the I-Ppol endonuclease is also efficiently repaired in Ctip−/− cells, in contrast to the lack of repair in Xcc4−/− cells (Fig. S1 E). Taken together, these findings indicate that Ctip is dispensable for hairpin opening, general NHEJ, and RAG-mediated V(D)J recombination in murine B cells and that phosphorylation of Ctip at T847 promotes Ctip-mediated hairpin opening in lymphocytes.

Phosphorylation of Ctip at T859 is important for end resection in G1-arrested cells To determine whether T859 phosphorylation of Ctip can promote end-resection in G1 cells independent of CDK dependent phosphorylation, we generated Rosa26/CreER-T2 Xcc4−/− 53BPI−/− Ctip/th/co cells in which the un repaired (due to XRCC4 deficiency)
RAG-generated DSBs in G1-arrested (STI571-treated) cells are rapidly degraded in the absence of 53BP1 or H2AX (Fig. 3 E, lane 2; Helmink et al., 2011; Zha et al., 2011a; Liu et al., 2012; Oksenych et al., 2012). In these cells, inactivation of CtIP (via +4OHT) completely prevented end degradation and restored normal levels of unrepaired CEs (lane 4), providing a system to measure CtIP-dependent end resection in G1, presumably independent of CDK phosphorylation. Moreover, ATM inhibition partially inhibits CE degradation in Ctip+/+ cells (lane 3) without affecting CE levels in Ctip−/− cells (lane 5), supporting an epistatic relationship between

Figure 2. CtIP is not required for NHEJ and chromosomal V(D)J recombination. (A) Schematic of pMX-INV V(D)J recombination substrates. The un-rearranged substrate, hybrid join, coding/signal end intermediates, and CJs/SJs are diagrammed. The recombination signal sequence (triangle) and hCD4 probe (dashed lines) are indicated. (B) Western blot for CtIP and actin on Ctip+/+ cells carrying ER-Cre (WT or mutant) treated with 4OHT (0.2 µM). (C and D) FACS (C) and Southern blot analyses (D) of pMX-INV rearrangement products (expressed GFP) in ER-Cre+ Ctip+/+ cells treated with 4OHT. The percentage of GFP+ cells are marked on the histograms. UR, unrearranged. (E) Semiquantitative PCR analyses (10-fold dilution) of the SJs formed in RosaERCreT2/+Ctip+/+ cells treated with STI571 (STI) with or without pretreat with 4OHT (for 24 h) to inactivate CtIP. Precise SJs formed in the pMX-INV substrate can be digested by ApalL. (F) Comparison of the number of nucleotide deletion (Del) and insertion (Ins; including both nontemplate and palindromic elements) in de novo CJs formed in Ctip+/+ (WT) and Ctip−/− cells. Each dot represents a unique CJ (n = 14 for WT and n = 12 for Ctip−/− cells). The junction sequences are presented in Table S1 B (P = 0.8655 for insertion and P = 0.9576 for deletion). (G) Diagram (left) and results (right) of the TdT-mediated ligation PCR assay. If the hairpin is opened, then TdT adds a poly(A) tail to the ends, which can be subsequently amplified via PCR using universal primer complementary to the poly(A) tail. The PCR product (∼500 bp) was detected with p32-labeled oligo probes against hCD4. PCR corresponding to the Rosa26 locus was used as loading control. Two biological repeats support the same conclusion. One representative result is shown. Statistical significance was assessed using a two-tailed Student’s t tests (n.s., not significant). F, forward; N, nontemplated nucleotide addition; P, palindromic nucleotide addition; R, reverse. The error bars represent SD from three independent experiments.

Liu et al. Journal of Experimental Medicine 1652
CtIP is essential for early B cell development
https://doi.org/10.1084/jem.20181139
Figure 3. CtIP is essential for continued proliferation of B cells. (A) Representative FACS analyses of Rosa26+/CreER-T2 Ctipco/co cells treated with 4OHT and with/without concurrent treatment with STI571 (STI; 3 µM). FSC, forward scatter; SSC, side scatter. (B) The percentage of live cells after 4OHT treatment was determined by forward and side scatter. The data represent the average and SD of three or more independent experiments collected at day 6 after 4OHT treatment. (C) Diagram for the CtIP rescue experiment. Retrovirus encoding Flag-tagged human CtIP-IRES-hCD2 was used to infect Rosa26+/CreER-T2 Ctipco/co cells. The fold of enrichment for hCD2+ cells after 4OHT treatment (deletion of endogenous CtIP; for 6–9 d) compared with before 4OHT treatment was plotted. Cells with the expression of WT-CtIP or mutants that support cell viability accumulate after the deletion of endogenous CtIP, leading to ∼10-fold enrichment for the percentage of hCD2+ cells in the culture (e.g., from 2.5% to nearly 25% among the live cells). (D) Statistical analyses of the rescue experiments for indicated CtIP mutants. The rescue experiments were repeated four to six times for each CtIP mutant. EV, empty vector. (E) Southern blot analyses of CEs of pMX-INV substrate in Rosa26+/CreER-T2 XRCC4−/−53BP1−/−Ctipco/co cells mock infected (lanes 1–5) or infected with retrovirus encoding either WT human CtIP (lanes 6–9) or the indicated CtIP mutants (lanes 10–17). Individual cultures were then treated with or without ATM inhibitor (ATMi), 4OHT, and/or STI571, as indicated. (F) pMX-INV substrate analyses in Xrcc4−/−53BP1−/−CtipT855A/T855A cells and controls. Statistical significance was assessed using a two-tailed Student’s t test (n.s., P > 0.05; **, P < 0.01). RV, EcoRV; UR, unrearranged.
ATM and CtIP with respect to end resection in these cells. Complete
degradation of CEs in the Ctipt−/− cells (+4OHT) was restored
by the expression of WT human CtIP (lane 8), but not the CtIP-
T847A (lane 12) or CtIP-T859A mutants (lane 16). ATM inhibition
further promoted CEs accumulation in CtIP-T847A- or CtIP-
T859A-expressing cells (lanes 13 and 17), consistent with the exis-
tence of other ATM phosphorylation sites on CtIP. B cell lines
derived from mouse carrying the knockin mutation correspond-
ting to T859A (T855A in mouse) in the germline (CtipT855A/T855A)
also show reduced end resection (see below; Fig. 3 F). Together,
these observations suggest that phosphorylation of CtIP at T859
(T855 in mouse) promotes end resection in G1 phase cells together
with other ATR/ATM phosphorylation sites.

Normal lymphocyte development in CtipT855A/T855A mice

Consistent with the ability of exogenous human CtIP-T859A to
partially support lymphocyte proliferation in culture (Fig. 3 D),
mice with an alanine substitution at the corresponding T855
residue (CtipT855A/T855A) are of normal size (Fig. S3 A) and normal
thymus and spleen cellularity (Fig. S3 D) and have normal de-
velopment of B cells and T cells (Fig. 4 A; Fig. S3, B and C; and Table S1 A). A minor yet consistent reduction in the fre-
cquency of CD3hiCD4+CD8− immature thymocytes was noted in
CtipT855A/T855A mice (Fig. 4 A). In previous studies, similar reduc-
tions of surface CD3 levels in thymocytes from ATM- or
53BP1-deficient mice have been attributed to defects in matura-
tion from double-positive to single-positive T cells (Borghesani
et al., 2000; DiFilippantonio et al., 2008). Sequence analyses of
the V(D)J recombination junctions in both B cells (Igh) and T cells
(TCβ) did not find any significant difference in SJ fidelity or CJ
palindromic and nonpalindromic nucleotide addition or deletion
(Fig. S3 E; and Table S1, C and D). Moreover, despite a moderate
and transient delay at day 3, CtipT855A/T855A B cells undergo robust
CSR upon activation in vitro (Figs. 4 B and S3 F). Consistent with
the partial rescue in the reconstitution experiments (Fig. 3 D),
activated CtipT855A/T855A B cells show a moderate reduction in
overall proliferation (Fig. 4 C). However, the frequency of IgG1+ B %
cells does not significantly differ in CtipT855A/T855A B cells versus
the control (P = 0.74) at each cell division, suggesting T855A-Ctip
does not directly compromise IgG1 switching beyond cell prolif-
eration. Moreover, high-throughput sequencing analyses of
>10,000 CSR junctions between Sµ and downstream switch
regions show that the usage of peripheral switch regions (an
indicator of end resection) and the number of MHs at the junc-
tion (an indicator of Alt-EJ) are also not affected by the T855A
mutation of CtIP (Fig. 4, D and E). Taken together, these find-
ings indicate that ATR/ATM-mediated phosphorylation of CtIP at
T859 promotes end resection but is largely dispensable for CSR,
V(D)J recombination, and lymphocyte viability in vivo.

Ctip-deficient cells accumulate at the G2/M checkpoint

We next investigated how Ctip inactivation compromises the viabil-
ity of proliferating immature B cells. The loxP recombination
signals of the conditional Ctipt−/− allele flank exon 2, which encodes
the initiator methionine codon and most of the CtIP tetrameri-
zation domain (Bothmer et al., 2013). In v-abl-transformed
Rosa26+/CreER-T2Chipt−/− cells, 4OHT leads to complete absence of
full-length CtIP protein by 24 h and expression of a truncated CtIP
protein lacking the N-terminal tetramerization domain (Fig. S4
A). Consistent with the essential role of oligomerization in CtIP
function (Davies et al., 2015; Forment et al., 2015), this truncated
Ctip polypeptide does not support embryonic development
(Bothmer et al., 2013), immature B cell development (Fig. 1 C), or
the viability of proliferating immature B cell in culture (Fig. 3 A).

Within 24 h of Cre induction, Rosa26+/CreER-T2Chipt−/− cells
displayed a marked increase in phosphorylation of the ATM
substrates KAP1 and CHK2, as well as a decrease in mitosis-
specific phosphorylation events (e.g., pH3Ser10 and pCdc2;
Fig. 5 A and Fig. S4, A and B). Cell cycle analyses revealed pro-
gressive loss of BrdU+ S phase cells (from 62.5% to 2.3%),
prominent G2/M accumulation (BrdU−, 4N cells; from 5.8% to
39.6%; Fig. 5 B), and decreased mitotic cell (pH3Ser10+) frequency
in the G2/M pool (Fig. 5 C), all of which are consistent with G2/M
cell cycle arrest. Phosphorylation of CHK1 at S345, a substrate of
ATR kinase, increases transiently 2 d after Ctip inactivation (Fig. 5
A), while total CHK1 protein levels are reduced by day 3, con-
sistent with phosphorylation-dependent ubiquitination and deg-
radation of CHK1 during persistent G2/M arrest (Zhang et al.,
2005, 2009). Meanwhile, Ctip-deleted cells arrested in G1 (by
either CDK4/6 inhibitor or SI751) can enter S phase and initiate
dNA synthesis upon release, despite a moderate delay (Fig. S4, F
g and F), suggesting that Ctip is not essential for the initiation of S
phase of DNA replication. Ctip-deleted cells also harbor high
levels of 53BP1 nuclear bodies, which typically arise from aber-
rant processing of underreplicated DNA during the previous
mitosis (Lukas et al., 2011; Fig. 5 D). As observed for Ctip-deficient
splenic B cells (Polato et al., 2014), cytogenetic analysis of im-
mature B cells harvested 24 h after Ctip inactivation showed in-
creased levels of chromosome and chromatid breaks (Figs. 5 E and
S4 E), as well as frequent accumulation of micronuclei (Fig. 5 F).

In mammalian cells, the G2/M cell cycle checkpoint is in-
duced in response to DNA damage by ATM kinase (Beamish
et al., 1994; Xu et al., 2002). Interestingly, the Sae2 protein in
yeast acts to attenuate checkpoint signaling by limiting Tell
activation (the yeast ATM ortholog; Chen et al., 2015). As a
consequence, Sae2-deficient cells exhibit reduced survival due
to persistent checkpoint signaling and cell cycle arrest. To as-
certain if a similar mechanistic contribution to the reduced via-
bility of Ctip-deleted lymphocytes, we tested whether inhibition
of ATM signaling would delay cell death upon Ctip loss. In
contrast to yeast, in B lymphocytes, ATM inhibition and ATM
deletion failed to restore the viability of Ctip-deleted cells (Figs.
5 G and S4 D). Instead, ATM inhibition greatly increased the
frequency of nuclear fragmentation in Ctip-deleted cells
(Fig. 5 F), suggesting that the ATM-mediated G2/M checkpoint
protects Ctip-deficient cells from mitotic DNA fragmentation.
Together, these findings suggest that Ctip inactivation leads to
an accumulation of aberrant recombination/replication inter-
mediates, which activate ATM and induce G2/M cell cycle arrest.

Ku70 deletion enhances G2/M arrest and delays cell death
associated with Ctip deficiency

Next, we tested whether partial restoration of DNA end resec-
tion via KU deletion or EXO1 activation can restore the viability

Liu et al.
Figure 4. T859 phosphorylation of CtIP is dispensable for normal B cell development and CSR. (A) Representative flow cytometry analyses of lymphocyte development of 7–8-wk-old Ctip+/+ and CtipT855A/T855A mice (n ≥ 3 mice of each genotype). The boxes on the B220-IgM plots represent IgM−B220− pre- or pro-B cells, IgM−B220high recirculating B cells, and IgM+B220− naive B cells, respectively. The boxes on the B220−CD43 plots represent B220−CD43− double negative T cells. (B) Statistical analyses of IL-4 plus LPS-stimulated B cells (CD43− splenocytes) from Ctip+/+ and CtipT855A/T855A mice (n ≥ mice of each genotype). The bar graphs represent the average and SE (P = 0.064 at day 2, 0.023 at day 3, and 0.11 at day 4). (C) The CTV labeling for cell proliferation in activated splenic B cells from Ctip+/+ and CtipT855A/T855A mice. The switch percentage in each respective cell division is plotted on the right (P = 0.74 via Student’s t test). (D) The spatial distribution of prey-break sites in Sy1 presented as the frequency (%) of Sy1 prey-break sites that falls into each 100-bp bin. The pool of all data from each genotype was plotted. All (+, red, from telomere to centromere orientation) and (−, blue, from centromere to telomere orientation) strand prey breaks add up to 100%. For each genotype, the number of total (+) strand (blue) and (−) strand (red) junctions are marked on the right. The dashed lines indicate the percentage of (−) strand Sy1 prey that fall outside the core Sy1 region. (E) The distribution of IgH junctions by junctional sequence features (blunt). This panel represents the pool of IgH junctions from each genotype. The numbers of junctions from each genotype are listed in the small table in the figure (n > 2,300 for each genotype). Statistical significance was assessed using a two-tailed Student’s t test (n.s., P > 0.05; *, P < 0.05).
Figure 5. Hyperactivation of ATM and G2/M cell cycle arrest upon Ctip inactivation in B cells. (A) Western blotting analyses of Abelson cells upon Ctip inactivation via 4OHT (days 1–3). HU-treated Ctip\textsuperscript{co/co} cells are shown as positive control. (B) Representative cell cycle analyses and quantification of cell cycle changes upon Cre activation in Rosa26\textsuperscript{+/CreER-T2}Ctip\textsuperscript{co/co} B cells. The boxes represent the G1, S, and G2 populations. The data represent the average and SD of $n \geq 3$ biological repeats. PI, propidium iodide. (C) Representative flow cytometry analyses and quantification of pHistone3-Ser10–positive cells among BrdU–G2/M cells. Experiments were repeated three times on two independently derived lines. The boxes mark the G2 (upper) and mitotic (lower, pH3Ser10+) cells. (D) Representative images show the 53BP1 nuclear body in Ctip\textsuperscript{−/−} cells. Representative images from two independent experiments and $n \geq 6$ cells acquired were shown. Scale bars, 5 µm. (E) Quantification of metaphases with abnormalities (including chromosomal and chromatid breaks) in Rosa26\textsuperscript{+/CreER-T2} Ctip\textsuperscript{co/co} B cells with (Ctip\textsuperscript{−/−}) or without (Ctip\textsuperscript{co/co}) Cre activation. The data represent the average and SD of $n \geq 3$ biological repeats. (F) The frequency of the cells with micronuclei or fragmented nucleus in WT and Ctip-deficient Abelson cells. The data represent the average and SD of $n \geq 3$ biological repeats. (G) Representative cell viability analyses for Rosa26\textsuperscript{+/CreER-T2} Ctip\textsuperscript{co/co} cells treated with ATM inhibitor (ATMi) or 4OHT to active Cre recombination. The data represent the average and SD of $n \geq 3$ biological repeats. Statistical significance was assessed using a two-tailed Student’s $t$ test (*, $P < 0.05$).
of CtIP-deficient cells (Mimitou and Symington, 2010; Symington and Gautier, 2011). Although KU deletion does not rescue the embryonic development of CtIP-deficient mice (Polato et al., 2014), we observed a significant delay in cell death upon deletion of Ku in Rosa26<sup>CreER-T2</sup>/Ku70<sup>−/−</sup>Ctip<sup>Co/co</sup> v-abl cells (Fig. 6 A). This delay is not due to the lack of DNA-dependent protein kinase (DNA-PK) activity, since DNA-PK kinase inhibitor does not delay cell death upon CtIP inactivation (Fig. S3 G). Ku deletion in CtIP-deficient cells elicits hyperphosphorylation of the ATM substrates KAP1 and CHK2 (Fig. 6 B), enhances G2/M arrest, and delays the loss of S phase and BrdU<sup>+</sup> populations. ATR kinase inhibition also delays cell death in CtIP-deleted cells and appears to act epistatically to CtIP deletion (Fig. 6 D), suggesting that defects in end resection potentially suppress basal ATR activity (as measured by CHK1 phosphorylation) during normal cell proliferation to affect the viability of CtIP-deleted cells. Using ATM- or ATR-specific inhibitors, we confirmed that in CtIP-deficient cells, ATM is responsible for hyperphosphorylation of KAP1 and CHK2, while ATR is responsible for CHK1 phosphorylation (S345; Fig. 6 E). Notably the level of γH2AX and pRPA(T21) also increased in Ku70-deficient cells, consistent with the requirement for Ku70 in NHEJ and in preventing end resection, which likely enhance ATM and ATR activation and G2/M arrest. Together, these findings suggest that CtIP-mediated end resection might be necessary to process certain types of replication intermediates to generate ssDNA and basal ATR signaling during replication.

**Ctip contributes to the processing of Top1cc-like lesions**

To identify the features of lesions that trigger ATM activation and G2/M arrest in CtIP-deleted cells, we challenged Ctip<sup>−/−</sup> B cells with several genotoxins with defined mechanisms of action. WT and CtIP-deleted cells were treated with ionizing radiation (IR; Fig. S3 H), hydroxyurea (HU; Fig. 7 A), or the topoisomerase I inhibitor camptothecin (CPT; Fig. 7 B) at 24 h after Cre activation, a time point at which CtIP is deleted, the cell cycle distribution of Ctip<sup>−/−</sup> cells is not significantly altered (Figs. 5 B and 6 C), and there is no significant DNA DSBs detected via neutral comet assay (Fig. S4 C). Ctip inactivation did not affect HU-induced CHK1 phosphorylation (Fig. 7 A) or IR-induced phosphorylation of KAP1 and CHK2 (Fig. S3 H), suggesting that CtIP does not directly affect ssDNA-induced ATR activation or DSB-induced ATM activation. In contrast, CtIP deficiency markedly attenuates CPT-induced RPA (replication protein A) and CHK1 phosphorylation while enhancing KAP1 and CHK2 phosphorylation in a dose-dependent manner (Fig. 7 B), similar to what was found in Ctip<sup>−/−</sup> proliferating B cells at later time points (Fig. 3 E). Ku70 deletion partially restores RPA and CHK1 phosphorylation and intensifies KAP1 and CHK2 phosphorylation in CPT-treated CtIP-deficient cells, consistent with the notion that the end-resection defect of these cells is ameliorated by the loss of Ku70 (Fig. 7 D). Ctip<sup>T855A/T855A</sup> B cells do not show spontaneous hyperphosphorylation of KAP1/CHK2 or reduced CHK1/RPA phosphorylation under these conditions. Although CPT-induced pRPA and pCHK1 occurs reproducibly in Ctip<sup>T855A/T855A</sup> cells and much higher than those in Ctip<sup>−/−</sup> cells, there are moderate reductions in comparison to Ctip<sup>+/−</sup> B cells (Fig. 7 E). Meanwhile, IR- and CPT-induced chromatin accumulation of CtIP is also moderately reduced in Ctip<sup>T855A/T855A</sup> B cells (Fig. 7 F), consistent with the previous finding in Xenopus extracts (Peterson et al., 2012). Taken together, our findings suggest that CtIP normally processes replication/recombination intermediates (e.g., Top1cc-like lesions) during S/G2 phase in a T847 phosphorylation-dependent but largely T859 phosphorylation-independent manner. In the absence of CtIP, the failure to process these replication intermediates contributes to loss of cellular viability, in part through hyperactivation of ATM and prolonged G2/M arrest and premature entry into mitosis with unrepaired and/or underreplicated DNA.

**Discussion**

Although the role of CtIP in DNA end resection has been extensively studied at the biochemical level, the in vivo functions of CtIP in lymphocyte development are less clear owing to its requirement for embryonic development. Using a conditional allele, we found that CtIP is dispensable in peripheral naive B cells but essential for early B cell development. We further showed that this requirement for CtIP in early B cells is independent of its role in V(D)J recombination, reflecting its critical role in continuous proliferation. Using G1-arrested B cells, we found that ectopic expression of T847E CtIP, but not endogenous CtIP or T847A CtIP, is able to open CEs hairpins. These data suggest that hairpin opening might be regulated in a cell cycle-dependent manner, mediated exclusively by Artemis during G1 and by Artemis and/or CtIP in S and G2 cells. CtIP binds both CtBP and Rb. While tissue-specific interactions in other cell lineages cannot be excluded, the essential role of CtIP in B cell proliferation does not require its readily detected interaction with CtBP or Rb. While phosphorylation of CtIP at T847 (likely by CDK) is required both for CtIP-mediated DNA resection of unrepaired DSBs and for the viability of proliferating lymphocytes, phosphorylation of CtIP at T859 (presumably by ATR/ATM) is important for CtIP-mediated resection in G1-arrested cells (Fig. 3 E) and cycling cells (Fig. 7 E; Peterson et al., 2013) but is not essential for proliferating lymphocytes. It is possible that CtIP is essential for the nucleolytic processing of complex DNA substrates beyond simple DSBs. In this context, we found that T847 phosphorylation is uniquely required for hairpin opening. Activation of ATM in Artemis<sup>−/−</sup> cells is not sufficient to activate the hairpin-opening activity of CtIP (Fig. 2 F). Other studies have highlighted an important role of CtIP in complex genomic structures (Makharashvili et al., 2014; Wang et al., 2014), including the CPT lesion we verified in murine lymphocytes here (Fig. 7 E). In this case, CtIP does not seem to play a role in the resection of IR-generated breaks (at least not at the early time points measured here). In addition, the amount of resection in G1 might be much lower than what was needed in S/G2 and during proliferation. This hypothesis is supported by the dramatic increase of CtIP protein levels in S/G2 phase cells (Yu et al., 1998), in addition to the CDK-mediated phosphorylation at T847. Thus, T859 phosphorylation, while dispensable for lymphocyte development and CSR, provides a means to fine-tune CtIP activity upon DNA damage in S/G2 phase.
Several mechanisms have been proposed to explain the impact of CtIP on CSR, including its ability to modulate activation-induced cytidine deaminase function, DNA end joining, CDK2 regulation or, less directly, cell proliferation (Lee-Theilen et al., 2011; Buis et al., 2012; Polato et al., 2014). CtIP-knockdown or CtIP-depleted cells have major proliferation defects, which could indirectly affect CSR. Indeed, sensitive CellTrace Violet (CTV) labeling (Fig. 4 C) suggest that even \( Ctip^{T855A/T855A} \) cells have a slight proliferation disadvantage. Yet, the CSR efficiency of \( Ctip^{T855A/T855A} \) B is not significantly different from \( Ctip^{+/+} \) B cells with the same doubling (Fig. 4 C). Together with the normal distribution of the CSR junctions and MH usage in the \( Ctip^{TR55A/TR55A} \) B cells (Fig. 4, D and E), we conclude that the CSR defects in CtIP-deficient cells in large part reflect the inability of these cells to proliferate normally. In the case of \( Ctip^{TR55A/TR55A} \) B cells, the switching frequency in cells with six or more divisions is slightly higher than that of \( Ctip^{+/+} \) B cells (Fig. 4 C), suggesting the transient CSR delay in early time points might be compensated by extensive proliferation of successfully switched \( Ctip^{TR55A/TR55A} \) B cells.

Finally, using the conditional allele and acute inactivation of CtIP in proliferating B cells, we show that while not essential for the initiation of DNA replication, CtIP processes spontaneous replication/recombination intermediates during S or G2 phase. While the exact nature of these intermediates remains elusive, previous studies suggest that CtIP can process protein–DNA adducts (Nakamura et al., 2010; Aparicio et al., 2016), DNA secondary structures (e.g., common fragile sites or palindromic sequences; Makharashvili et al., 2014; Wang et al., 2014), and DNA hairpins (Lengsfeld et al., 2007; Makharashvili et al., 2014; Wang et al., 2014; Chen et al., 2015). Deletion of the BRCA2 tumor suppressor, specifically loss of its HR function, elicits a similar mode of cell lethality as \( Ctip^{−/−} \) cells, marked by ATM hyperactivation, G2/M arrest, and formation of 53BP1 nuclear bodies (Feng and Jasin, 2017). As HR is also dependent on the DNA resection activity of CtIP (Sartori et al., 2007), similar replication/recombination intermediates may be responsible for the loss of cell viability in both Brca2- and Ctip-deleted cells. The normal response of

---

**Figure 6.** Ku deletion and ATR inhibition delay cell death upon Ctip inactivation. (A) Representative cell viability analyses for \( Rosa26^{+/CreER-T2} Ctip^{+/co} \) and \( Rosa26^{+/CreER-T2} Ctip^{−/−} \) cells treated with 4OHT. The data represent the average and SD of \( n \geq 3 \) biological repeats. Ku70 deficiency significantly \((P < 0.01)\) rescued the viability of Ctip-deficient cells at all time points measured. (B) Western blotting analyses of \( Rosa26^{+/CreER-T2} Ctip^{+/co} \) and \( Rosa26^{+/CreER-T2} Ctip^{+/-} \) Abelson cells upon Ctip inactivation (days 1 and 3). (C) Representative cell cycle analyses of \( Rosa26^{+/CreER-T2} Ctip^{+/co} \) and \( Rosa26^{+/CreER-T2} Ctip^{−/−} \) Abelson cells after Cre activation. The boxes mark the sub-G1, G1, S (BrdU+), and G2 populations. The data represent the average and SD of \( n \geq 3 \) biological repeats. (D) Representative cell viability analyses for \( Rosa26^{+/CreER-T2} Ctip^{+/co} \) cells treated with ATR kinase inhibitor (ATRi) and/or 4OHT to active Cre recombination. The data represent the average and SD of \( n \geq 3 \) biological repeats. (E) Western blotting analyses of \( Rosa26^{+/CreER-T2} Ctip^{+/co} \) treated with ATM (ATMi) or ATR (ATRI) kinase inhibitors.
CtIP-deficient B cells to HU- or IR-induced breaks, but not CPT-induced breaks, further suggests that CtIP processes complex replication intermediates, including a potential hairpin structure, to support early B cell development and CSR in a CDK-dependent (i.e., CtIP-T847 phosphorylation) and ATR/ATM-independent (e.g., CtIP-T859 phosphorylation) manner.

Figure 7. DNA damage response of CtIP-deficient B cells. (A and B) Western blotting analyses of (A) HU (0.1, 0.2, 0.5, and 1 mM, 1 h)– or (B) CPT (10, 25, 50, 100, and 500 nM)–induced DNA damage responses in Rosa26<sup>CreER-T2</sup>Ctip<sup>−/−</sup> cells (with or without 4OHT for 24 h). (C) Cell cycle analyses of Rosa26<sup>CreER-T2</sup>Ctip<sup>−/−</sup>Ku70<sup>−/−</sup> cells with or without 4OHT for 24 h. The boxes mark the sub-G1, G1, S (BrdU+), and G2 populations. (D) Western blotting analyses of CPT (10, 25, 50, 100, and 500 nM)–induced DNA damage responses in Rosa26<sup>CreER-T2</sup>Ctip<sup>−/−</sup>Ku70<sup>−/−</sup> cells (with or without 4OHT for 24 h). (E) Western blotting analyses of CPT (10, 25, 50, 100, 500 nM)–induced DNA damage responses in CtIP<sup>T855A/T855A</sup> and control 4OHT-treated Rosa26<sup>CreER-T2</sup>Ctip<sup>−/−</sup> cells (24 h). (F) Western blotting analyses of the chromatin and soluble fractionation assay of CtIP<sup>+/−</sup> (WT) and CtIP<sup>T855A/T855A</sup> v-abl cells after CPT (1 µM, 2 h) or IR (10 Gy, ~15 min) treatment. The experiments were repeated three times, and one representative result is shown.

**Materials and methods**

**Mice**

Ctip<sup>+/−</sup> (Bothmer et al., 2013), CD2iCre (Kraus et al., 2001), MblCre (Hobeika et al., 2006), and Rosa26<sup>CreER-T2</sup> (Guo et al., 2007) mice were previously characterized. Artemis-deficient mice (Rooney et al., 2002) were used as controls for B cell and thymocyte development. All animal work was conducted in a...
DNA was digested with EcoRV or EcoRV+NcoI and probed with Southern blotting for rearrangement. Specifically, genomic DNA was isolated from FACS analyses of GFP expression were treated with STI571 (3 µM; Novartis Pharmaceuticals) and v-abl (Ku55933; Selleckchem) was added to a final concentration of 5 µM. To perform the assay, 15% (vol/vol) FBS for 6–8 wk before the cells were infected with retrovirus encoding the V(abl) kinase (Bredemeyer et al., 2006). Cells were maintained in DMEM (GIBCO) supplemented with 15% (vol/vol) FBS and 25 ng/ml LPS plus 25 ng ml⁻¹ of LPS plus 25 ng ml⁻¹ of IL-4 (R&D). Cultured cells were maintained daily at a density of 0.5–1.0 × 10⁶ cells ml⁻¹. Cells were collected 2–4 d after activation for flow cytometry with FITC-conjugated anti-IgG1 (BD Pharmingen) and Cy3-conjugated anti-murine B220 (eBioscience). Flow cytometry was performed on a FACSCalibur flow cytometer (BD Bioscience) and the data processed using FlowJo software package.

Construction of plasmids
pBMN-Flag-IRES-hCD2 plasmid was constructed as described before (Liu et al., 2012). An expression vector encoding Flated full-length WT human CtIP (F13-siCtIP/pCIN4) was subjected to site-directed mutagenesis to generate vectors expressing CtIP derivatives bearing the T847A, T847E, T859A, T859E, or E157K missense mutations, or the ΔPLDLS deletion mutation. A human CtIP expression plasmid containing the combined S231A/S646A/S745A missense mutation was a gift from Dr. Tanya Paull (The University of Texas at Austin, Austin, TX; Lengsfeld et al., 2007; Makharashvili et al., 2014; Wang et al., 2014; Chen et al., 2015). The pBMN-Flag-CtIP-IRES-hCD2 plasmid was used to transfect Phoenix cells and generate retrovirus. Infections were performed with ~3 × 10⁶ cells in the presence of 12 µg/ml polybrene for 3 h with spin (~500 g) at room temperature.

Chromatin fractionation, western blot, and antibodies
Cells were lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, and fresh protease inhibitor cocktail) following standard Western blotting protocols and blotted with antibodies specific for Flag (M2, 1:5,000, F3165; Sigma-Aldrich), CtIP (1:1,000; Yu and Baer, 2000), anti-phoKAP1 (1:1,000, A300-767A; Bethyl Laboratories), anti-KAP1 (1:1,000, 4124S; Cell Signaling), anti-CHK1 (1:1,000, 2348S; Cell

Terminal deoxynucleotidyl transferase (TdT)-assisted PCR assay
2 µg genomic DNA was treated with TdT (New England Biolabs) according to the manufacturer’s protocol in the presence of 5 µM dATP, and the reaction was terminated by heating to 70°C for 15 min. 2% of the reaction was used for primary amplification (15 cycles) with primers IRES-REV5 (5′-REV5; S′-CTCGACTAAACACATGTAAAGCCTGACTATAACATGTAAGC-3′) and T17-UNIV (5′-GTAAAACGACGGCCAGTCGACTTTTTTTTTTTTTTTTTT-3′). 2% of the products of the primary amplification and serial 1:5 dilutions were used as templates for a second amplification step (17 cycles) using primers IRES-REV4 (5′-AGTGAATAATTCCCTTTGTGTTAATGCTGCTTG-3′) and UNIV (5′-AGACCAGGATCCGTAAAAGCGGCGCAG-3′). The secondary reaction products (~500 bp) were analyzed on 1% agarose gel, transferred onto a Zeta-Probe membrane (Bio-Rad), and hybridized with the 32P-labeled hCD4 probe as described above. A PCR product (~700 bp) from the Rosa26a locus was amplified as control as described previously (jiang et al., 2015).

Lymphocyte development and CSR
Lymphocytes of the thymus, bone marrow, spleen, and LN from 4–6 wk-old mice of the described genotypes were made into ~1 × 10⁶ cells ml⁻¹ single-cell suspensions, which were stained using fluorescence-conjugated antibodies before analyzed by flow cytometry. For the CSR assay, CD43+ splenic B cells were purified using anti-murine CD43 magnetic beads (MACS; Miltenyi Biotech) and cultured at 0.5 × 10⁶ cells ml⁻¹ in RPMI medium supplemented with 15% FBS and 25 ng ml⁻¹ of LPS plus 25 ng ml⁻¹ of IL-4 (R&D). Lymphocytes of the thymus, bone marrow, spleen, and LN from 4–6 wk-old mice of the described genotypes were made into ~1 × 10⁶ cells ml⁻¹ single-cell suspensions, which were stained using fluorescence-conjugated antibodies before analyzed by flow cytometry. For the CSR assay, CD43+ splenic B cells were purified using anti-murine CD43 magnetic beads (MACS; Miltenyi Biotech) and cultured at 0.5 × 10⁶ cells ml⁻¹ in RPMI medium supplemented with 15% FBS and 25 ng ml⁻¹ of LPS plus 25 ng ml⁻¹ of IL-4 (R&D). Cultured cells were maintained daily at a density of 0.5–1.0 × 10⁶ cells ml⁻¹. Cells were collected 2–4 d after activation for flow cytometry with FITC-conjugated anti-IgG1 (BD Pharmingen) and Cy3-conjugated anti-murine B220 (eBioscience). Flow cytometry was performed on a FACSCalibur flow cytometer (BD Bioscience) and the data processed using FlowJo software package.

Construction of plasmids
pBMN-Flag-IRES-hCD2 plasmid was constructed as described before (Liu et al., 2012). An expression vector encoding Flaged full-length WT human CtIP (F13-siCtIP/pCIN4) was subjected to site-directed mutagenesis to generate vectors expressing CtIP derivatives bearing the T847A, T847E, T859A, T859E, or E157K missense mutations, or the ΔPLDLS deletion mutation. A human CtIP expression plasmid containing the combined S231A/S646A/S745A missense mutation was a gift from Dr. Tanya Paull (The University of Texas at Austin, Austin, TX; Lengsfeld et al., 2007; Makharashvili et al., 2014; Wang et al., 2014; Chen et al., 2015). The pBMN-Flag-CtIP-IRES-hCD2 plasmid was used to transfect Phoenix cells and generate retrovirus. Infections were performed with ~3 × 10⁶ cells in the presence of 12 µg/ml polybrene for 3 h with spin (~500 g) at room temperature.
High-throughput genome-wide translocation sequencing (HTGTS)

HTGTS was performed as previously described (Dong et al., 2015; Hu et al., 2016; Panchakshari et al., 2018). Genomic DNA was collected from activated B cells after 4 d, sonicated (Digestion of Chromatin, Novegan) and incubated on ice for 30 min. After slow centrifugation (500 g, 5 min), the supernatant was collected into a new tube for a 15-min top-speed centrifugation. The supernatant from the high-speed spin was saved as “soluble fraction.” The pellet from the first slow-centrifugation step was washed once with 0.1% NP-40 lysis buffer, without fully disturbing the pellet. After another slow centrifugation, the supernatant was discarded and the pellet was resuspended with 0.5% NP-40 lysis buffer with 125 U/ml Benzonase (Novegan) and incubated on ice for 1 h. The reaction was stopped by adding EDTA to a 5 µM final concentration, followed by a 15-min top-speed centrifugation, and the supernatant was collected as chromatin fraction. For each sample, the volume of the 0.5% NP-40 lysis buffer used for chromatin fraction was one tenth of the 0.05% NP-40 lysis buffer for the soluble fraction, and the two fractions were loaded the same volume for Western blot analysis.

References


Accept: 24 April 2019