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Collaborative Action of Brca1 and CtIP in Elimination of Covalent Modifications from Double-Strand Breaks to Facilitate Subsequent Break Repair

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Abstract
Topoisomerase inhibitors such as camptothecin and etoposide are used as anti-cancer drugs and induce double-strand breaks (DSBs) in genomic DNA in cycling cells. These DSBs are often covalently bound with polypeptides at the 3’ and 5’ ends. Such modifications must be eliminated before DSB repair can take place, but it remains elusive which nucleases are involved in this process. Previous studies show that CtIP plays a critical role in the generation of 3’ single-strand overhang at “clean” DSBs, thus initiating homologous recombination (HR)–dependent DSB repair. To analyze the function of CtIP in detail, we conditionally disrupted the Ctip gene in the chicken DT40 cell line. We found that CtIP is essential for cellular proliferation as well as for the formation of 3’ single-strand overhang, similar to what is observed in DT40 cells deficient in the Mre11/Rad50/Nbs1 complex. We also generated DT40 cell line harboring Ctip with an alanine substitution at residue Ser332, which is required for interaction with BRCA1. Although the resulting CtIPS332A/−/− cells exhibited accumulation of RPA and Rad51 upon DNA damage, and were proficient in HR, they showed a marked hypersensitivity to camptothecin and etoposide in comparison with Ctip+/−/− cells. Finally, CtIPS332A/−/−/BRCA1−/− and CtIPS332A/−/−/BRCA1+/− showed similar sensitivities to these reagents. Taken together, our data indicate that, in addition to its function in HR, CtIP plays a role in cellular tolerance to topoisomerase inhibitors. We propose that the BRCA1-CtIP complex plays a role in the nuclease-mediated elimination of oligonucleotides covalently bound to polypeptides from DSBs, thereby facilitating subsequent DSB repair.

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Introduction
CtIP was isolated as a binding partner of CtBP (C-terminal binding protein), and has subsequently been shown to interact with a number of molecules, including BRCA1 (Breast Cancer Susceptibility Gene 1) [1]. CtIP is a functional homolog of yeast Sae2 (Sporulation in the Absence of Spo Eleven), and acts at the initial step of homologous recombination (HR)–dependent double-strand break (DSB) repair [2,3]. HR is initiated by forming 3’ single-strand (ss) overhangs at DSBs. In this resection step, Sae2/CtIP works together with a complex composed of Mre11/Rad50/Xrs2 in budding yeast, or with Mre11/Rad50/Nbs1 in mammals [4–7]. The Rad51 recombinase protein polymerizes on the ss DNA overhang, and the resulting ssDNA-Rad51 complex undergoes homology search. Resection activity is upregulated by phosphorylation of a conserved residue in Sae2 by the cyclin-dependent kinase (CDK) [8]. This phosphorylation site is conserved in human CtIP (Thr847), and is also phosphorylated by CDK [7].

BRCA1 was originally identified as a tumor suppressor gene associated with familial breast and ovarian cancer [9]. BRCA1 contains an N-terminal RING-finger domain, and is associated with structurally related BARD1 to form an E3-ubiquitin ligase. BRCA1/BARD1 forms three distinct complexes with Abraxas, Bach1 and CtIP, and plays a role in DNA repair [10]. BRCA1 binds to CtIP in a manner that is dependent on the phosphorylation of CtIP at Ser327 [11,12]. Following DNA damage, the ubiquitylation of CtIP by BRCA1 causes the migration of CtIP towards a chromatin fraction [12]. However, the biological significance of the complex formed between BRCA1 and CtIP has not yet been clarified.

Topoisomerases 1 and 2 (Topo1 and Topo2) have been drawing increasing attention as important targets for cancer therapy, since the inhibition of these enzymes causes DSBs during DNA repair.
Replication [13]. Topo1 and Topo2 induce single-strand breaks (SSBs) and DSBs, respectively. Covalent bonds are transiently formed between Topo1 and the 3’ end of the SSB and between Topo2 and the 5’ end of the DSB [14]. The anti-cancer agent camptothecin (CPT) inhibits Topo1 by stabilizing the Topo1-cleavage complex, which interferes with replication, and thereby induces DSBs in one of the sister chromatids [15]. Topo2 inhibitors such as etoposide (VP16) and ICRF-193 also kill cycling cells and are used in cancer therapy. VP16 stabilizes the Topo2-cleavage complex, while ICRF-193 stabilizes the closed clamp which forms after the strand passage [16,17]. Topo1-mediated DNA damage caused by CPT is repaired primarily by homologous recombination (HR), while Topo2-mediated DNA damage caused by VP16 or ICRF-193 is mainly repaired by nonhomologous end joining (NHEJ) [18,19]. It should be noted that the repair of CPT- and VP16-induced DSBs requires an additional step: the elimination of covalently bound polypeptides from the DNA ends. Hartsuiker et al. demonstrated that Topo2 is removed from DNA by the collaborative action of the MRX complex and ctp1 (the ortholog of CtIP) in fission yeast [20]. It remains to be seen whether vertebrate CtIP shares the same function as in yeast, which does not have BRCA1 ortholog.

To understand the role of the BRCA1-CtIP interaction, we substituted the Ser332 residue (equivalent to human Ser327) of CtIP with alanine in the chicken DT40 B lymphocyte line [21,22]. In addition, to analyze the function of CtIP, we conditionally depleted CtIP in DT40 cells. We here show that the depletion of CtIP is lethal to cells as it is the inactivation of Mre11, Rad50, and Nbs1 [23,24], indicating the critical role played by CtIP in HR. Remarkably, although the CtIP S332A mutation had no effect on the survival of wild-type cells, it did not reduce the viability of DNA damage sites [S2E]. As expected, given the results of a previous study [12], the depletion of CtIP abolished the formation of conjugated-ubiquitin foci [27]. In contrast, CtIP depletion did not reduce the ubiquitylation of DNA damage sites (Figure 2C), suggesting that CtIP is not required for the activation of Brca1.

Proficient HR in CtIP$^{S332A/-/-}$ DT40 clones

To functionally analyze the interaction of CtIP with BRCA1, we generated CtIP$^{S332A/-/-}$ cells, in which the critical amino acid in the binding interface has been mutated (Figure S2A, S2B, S2C). The CtIP$^{S332A/-/-}$ DT40 clones were capable of proliferating at a rate similar to the CtIP$^{+/+}$ cells without a prominent change in the cell-cycle profile (Figure 3A and Figure S2D). Western blot analysis showed that the S332A CtIP proteins were expressed at the similar level to the wild-type protein, indicating that amino acid substitutions do not affect the stability of the CtIP protein (Figure S2E). As expected, given the results of a previous study [12], the S332A mutation of CtIP indeed inhibited its interaction with BRCA1 (Figure S2F).

To evaluate the capability of HR in CtIP$^{S332A/-/-}$ cells, we integrated an artificial substrate, S_Cas, in the Ovalbumin locus [28], and measured the efficiency of I-SceI-induced gene conversion. The CtIP$^{S332A/-/-}$ clones showed no significant decrease in the
appearance of neomycin-resistant colonies compared to \( \text{CtIP}^{+/+} \) cells (Figure 3B). The proficient HR in \( \text{CtIP}^{S332A/2} \) DT40 clones is in marked contrast to the severe phenotype of the \( \text{Nbs1p70} \) hypomorphic mutant, which exhibited a 10-fold reduction of the gene-targeting frequency and a 103-fold decrease in the efficiency of HR in the \( \text{SCneo} \) substrate [29]. Next, we measured the frequency of gene targeting at the \( CENP-H \) and \( \text{Ovalbumin} \) loci. In contrast to \( \text{I-SceI} \)-induced gene conversion, the gene-targeting frequency of the \( \text{CtIP}^{S332A/2} \) clones decreased moderately in comparison with \( \text{CtIP}^{+/+} \) cells (Table 2). We speculate that this is because unknown recombination intermediates that require processing by CtIP/BRCA1 may arise during gene targeting event (see Discussion).

Fluorescent immunostaining revealed that the kinetics of Rad51 focus formation after \( \gamma \)-irradiation was indistinguishable between \( \text{CtIP}^{S332A/2} \) and the \( \text{CtIP}^{+/+} \) control cells, while \( \text{BRCA1}^{2/2} \) cells showed the significant reduction in the Rad51 focus formation at 1–6 h after irradiation (Figure 4A). Furthermore, the \( \text{CtIP}^{S332A/2} \) mutants displayed laser-induced RPA accumulation as did the \( \text{CtIP}^{+/+} \) cells (Figure 4B). Laser-generated RPA accumulation following BrdU incorporation largely arises from the resection rather than other routes of single-strand formation such as the damage caused by laser itself or replication-associated single-strand formation, because RPA accumulation is abolished specifically in \( \text{Ubc13}^{-/-} \) cells [27]. This suggests that \( \text{CtIP}^{S332A/2} \) cells are proficient in resection at DSB sites. Taken together, we conclude that the S332A mutation of CtIP does not significantly compromise HR.

**Table 1. Chromosomal aberrations in \( \text{CtIP}^{S332A/2} \) mutants.**

| Genotype | Chromatid-type | Chromosome-type |
|----------|----------------|----------------|
| IR 0 Gy  | Wild-type      | 6              |
|          | \( \text{CtIP}^{S332A/2}/\text{tetCtIP} \) Dox(−) | 4              |
|          | \( \text{CtIP}^{S332A/2}/\text{tetCtIP} \) Dox(+) | 7              |
| IR 2 Gy  | Wild-type      | 10             |
|          | \( \text{CtIP}^{S332A/2}/\text{tetCtIP} \) Dox(−) | 6              |
|          | \( \text{CtIP}^{S332A/2}/\text{tetCtIP} \) Dox(+) | 20             |

\( \gamma \)-ray irradiated (2 Gy) and non-irradiated cells were treated with colcemid for 3 h to enrich mitotic cells prior to fixation of cells for chromosome analysis. Data are presented as the number of aberrations per 100 cells.

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CtIP<sup>S332A/−/−</sup> cells display a marked hypersensitivity to both CPT and VP16, which stabilize the Topo-cleavage complexes.

To determine the role of CtIP in the cellular response to DNA damage, we measured the sensitivity of the CtIP mutant cells to various genotoxic agents using a colony survival assay. CtIP<sup>S332A/−/−</sup> cells exhibited the slightly elevated sensitivity toward CPT and VP16 (Figure 5A and 5B), though they expressed the similar level of CtIP protein to the wild-type cells (Figure S2E). It is possible that the difference in the amount of CtIP protein between CtIP<sup>S332A/−/−</sup> and wild-type cells is too subtle to detect, and that even the suboptimal level of CtIP protein renders the cells sensitive to genotoxic stimuli.

A compensatory post-translational regulation may be present because CtIP<sup>S332A/−/−</sup> cells exhibited about 80% reductions in CtIP mRNA level compared to the wild-type level (Figure S2G). In contrast to CtIP<sup>S332A/−/−</sup> cells, CtIP<sup>S332A/−/−</sup> mutants showed a significantly increased sensitivity to VP16 and MMS (Figure 5B and 5D), but not to γ-rays (data not shown). Furthermore, the sensitivity to CPT was dramatically elevated in the CtIP<sup>S332A/−/−</sup> mutants, in comparison with the CtIP<sup>S332A/−/−</sup> cells (Figure 5A).

The contribution of CtIP to the cellular tolerance to VP16 indicated that CtIP might play a role in NHEJ [18]. To test this hypothesis, we evaluated NHEJ by measuring the sensitivity of CtIP mutant cells to ICRF-193, because ICRF193-induced DNA lesions are repaired exclusively by NHEJ, whereas a fraction of the VP16-induced DSBs are repaired by HR [18]. The CtIP<sup>S332A/−/−</sup> clones exhibited no increased ICRF193 sensitivity (Figure 5C). NHEJ can also be evaluated by measuring the IR sensitivity of the cell population at the G1 phase, where NHEJ plays a dominant role in DSB repair [30]. The CtIP hypomorphic mutants synchronized at the G1 phase did not show significant IR hypersensitivity (Figure S3). These observations indicate that...
Clones exhibited no decrease in the efficiency of HR or NHEJ. We significantly higher sensitivity to CPT and VP16, although these comparison with tolerance to CPT and VP16 Epistatic relationship of CtIP to BRCA1 in cellular phosphatase of Ser332 [12]. In order to assess the functional relationship between Ser332 phosphorylation of CtIP and BRCA1, we disrupted the BRCA1 gene in the CtIP<sup>S332A</sup>−/− and CtIP<sup>+/−</sup> clones (Figure S4A and S4B), as was done previously [31]. Both the CtIP<sup>S332A</sup>−/−BRCA1<sup>+/−</sup> and the CtIP<sup>+/−</sup>BRCA1<sup>/−</sup> clones proliferated with similar rates at significantly reduced growth rates, in comparison with BRCA1<sup>−/−</sup> cells (doubling time ± SD; 8.3±0.2 h for wild-type, 9.3±0.3 h for BRCA1<sup>/−</sup>, 11±0.3 h for CtIP<sup>/−</sup>BRCA1<sup>/−</sup>, 12.6±0.9 h for CtIP<sup>S332A</sup>−/−BRCA1<sup>/−</sup>). The viability of CtIP<sup>S332A</sup>−/−BRCA1<sup>/−</sup> cells is in marked contrast with the lethality of CIP-mall cells, supporting the idea that the CtIP-BRCA1 interaction works independently from the function of CtIP in resorption.

We next examined the sensitivity of double mutant cells to CPT and VP16. To this end, we measured the number of live cells after 48-hour continuous exposure to the DNA-damaging agents [32], during which the double mutant cells are able to divide four to five times. We did not use a conventional colony formation assay for this purpose, because CtIP<sup>/−</sup>BRCA1<sup>−/−</sup> and CtIP<sup>S332A</sup>−/−BRCA1<sup>/−</sup> clones grew very badly from a single cell in semi-solid methycellulose medium. The number of viable cells cultured in the presence of CPT was significantly decreased for CtIP<sup>S332A</sup>−/−BRCA1<sup>/−</sup> and BRCA1<sup>/−</sup> cells compared to the wild-type cells, whereas CtIP<sup>/−</sup>cells grew to the similar extent to the wild-type cells in the presence of CPT (Figure 6A). The sensitivity of CtIP<sup>/−</sup>BRCA1<sup>/−</sup> cells to CPT was greater than that of BRCA1<sup>/−</sup> clones. This observation is in agreement with the idea that BRCA1 and CtIP can independently contribute to HR, whereas CtIP promotes the resection of DSBs, while BRCA1 subsequently loads Rad51 at resected ssDNA overhang. Importantly, although the CtIP<sup>S332A</sup> mutation significantly increased cellular sensitivity to CPT in the presence of BRCA1, the CtIP<sup>S332A</sup>−/−BRCA1<sup>/−</sup> and CtIP<sup>/−</sup>BRCA1<sup>−/−</sup> clones exhibited a very similar sensitivity to CPT (Figure 6A). Likewise, the CtIP<sup>S332A</sup>−/−BRCA1<sup>/−</sup> and CtIP<sup>/−</sup>BRCA1<sup>−/−</sup> clones exhibited indistinguishable cellular sensitivities to VP16 (Figure 6B). These observations suggest that CtIP and BRCA1 can act in collaboration to repair DSBs that are chemically modified by topoisomerases.

**Discussion**

We here show that conditional depletion of CtIP protein led to cellular lethality with increased frequency of chromosomal aberrations in DT40 cells. CtIP depletion abolished the accumulation of RPA and Rad51 at DNA damaged sites, suggesting that it is required for the resection of DSBs during HR, and that this function is essential for the proliferation of cells. These results are in agreement with previous reports [3]. In contrast, the DT40 cells harboring S332A mutation in CtIP showed the accumulation of RPA and Rad51 upon DNA damage, and were able to proliferate with normal kinetics. Remarkably, compared to the CtIP<sup>/−</sup> cells, the CtIP<sup>S332A</sup>−/− cells exhibited significantly increased sensitivity to CPT and VP16, both of which stabilize the Topo-DNA cleavage complex. These observations support the proposition that, in addition to the resection of DSBs, CtIP has the second function, most likely the removal of covalently-bound polypeptides from DSBs. Hence, CtIP<sup>S332A</sup>−/− clones are the novel separation-of-function mutants where CtIP-dependent resection is deficient, whereas the second function required for the tolerance to topoisomerase inhibitors is deficient.

In this study, we demonstrated that the inactivation of CtIP in DT40 cells results in cellular death. We speculate that the defective DSB repair during S phase is the primary cause of cellular death rather than the misregulation of RB/E2F pathway [33,34]. It has been reported that CtIP promotes G1/S progression by releasing

![Figure 3. Proficient homologous recombination in CtIP<sup>S332A</sup>−/− clones.](image)

(A) Growth kinetics of the indicated CtIP mutant clones. (B) I-Sce1-induced DSBs stimulate homologous recombination (HR) in an artificial substrate, Sceo. The recombination frequency was calculated by dividing the number of neomycin-resistant colonies by the number of the total colonies. Error bars indicate S.D. doi:10.1371/journal.pgen.1000828.g003

NHEJ is not impaired in CtIP<sup>S332A</sup>−/− clones. In summary, in comparison with CtIP<sup>/−</sup> cells, CtIP<sup>S332A</sup>−/− clones exhibited a significantly higher sensitivity to CPT and VP16, although these clones exhibited no decrease in the efficiency of HR or NHEJ. We conclude that CtIP can therefore contribute to cellular tolerance to CPT and VP16, independently of HR or NHEJ, most likely by eliminating covalently bound polypeptides from the DSBs.

**Epistatic relationship of CtIP to BRCA1 in cellular tolerance to CPT and VP16**

CtIP physically interacts with BRCA1 in a manner dependent on phosphorylation of Ser332 [12]. In order to assess the functional relationship between Ser332 phosphorylation of CtIP and BRCA1, we disrupted the BRCA1 gene in the CtIP<sup>S332A</sup>−/− and CtIP<sup>+/−</sup> clones (Figure S4A and S4B), as was done previously [31]. Both the CtIP<sup>S332A</sup>−/−BRCA1<sup>+/−</sup> and the CtIP<sup>+/−</sup>BRCA1<sup>/−</sup> clones proliferated with similar rates at significantly reduced growth rates, in comparison with BRCA1<sup>−/−</sup> cells (doubling time ± SD; 8.3±0.2 h for wild-type, 9.3±0.3 h for BRCA1<sup>/−</sup>, 11±0.3 h for CtIP<sup>/−</sup>BRCA1<sup>/−</sup>, 12.6±0.9 h for CtIP<sup>S332A</sup>−/−BRCA1<sup>/−</sup>). The viability of CtIP<sup>S332A</sup>−/−BRCA1<sup>/−</sup> cells is in marked contrast with the lethality of CIP-mall cells, supporting the idea that the CtIP-BRCA1 interaction works independently from the function of CtIP in resorption.

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**Discussion**

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In this study, we demonstrated that the inactivation of CtIP in DT40 cells results in cellular death. We speculate that the defective DSB repair during S phase is the primary cause of cellular death rather than the misregulation of RB/E2F pathway [33,34]. It has been reported that CtIP promotes G1/S progression by releasing
RB-imposed repression and by upregulating the genes required for S phase entry such as cyclin D1. MEF from CtIP-deficient mice and NIH3T3 cells transfected with CtIP siRNA arrest at G1 phase of cell cycle. In contrast, DT40 cells that are depleted of CtIP showed a marked reduction in S phase and an increase in sub-G1 population with the spontaneous chromosomal aberrations. We speculate that DT40 cells have a lower threshold to enter the S phase in the presence of DNA damage compared to the other types of cells owing to their character that they lack p53 expression [35] and overexpress c-myc [36].

The phenotype of our CtIP-depleted DT40 cells was remarkably different from that of the CtIP-deficient DT40 cells generated by Hiom’s group [37]. Surprisingly, their CtIP-depleted DT40 cells were capable of proliferating. However, we believe that CtIP is essential for cellular proliferation because it has been shown that CtIP works together with Mre11/Rad50/Nbs1 complex in budding and fission yeasts as well as in mammalian cells [3,6,38], and the increased spontaneous chromosomal aberrations and cellular death observed in our CtIP-depleted cells are consistent with our previous reports that deficiency of either one of Mre11, Rad50, or Nbs1 was all lethal to DT40 clones [23,24]. The viability of the CtIP-deficient DT40 cells generated by Hiom’s group might be due to the occurrence of suppressor mutations during the disruption of the three allelic CtIP genes. Another possibility is that the disruption of exons 1 and 2 in Hiom’s group might still allow the residual expression of an N-terminal-truncated
CtIP protein, as is observed for the expression of an N-terminal-truncated Nbs1 protein in patients with Nijmegen syndrome [39].

Another critically different point between our study and Hiom’s group is that they conclude that the phosphorylation of CtIP-S332 promotes the resection of DSBs, whereas our data do not support this conclusion. The discrepancy between the two studies may be attributable to the different ways of introducing the S332A mutation into the DT40 cells. They randomly integrated wild-type and CtIPS332A transgenes at different loci in their “CtIP-null” cells, while we inserted the S332A mutant into one of the CtIP allelic genes. This knock-in approach is essential for the accurate quantitative evaluation of HR and NHEJ, because the endogenous promoter expresses CtIP transcripts differently in each phase of the cell cycle, and this differential expression accounts for the reduced usage of HR in the G1 phase in fission yeast [38]. Alternatively, the difference between our results could be because Hiom’s group re-introduced human CtIP cDNA (wild type or mutants) instead of that derived from chicken into DT40 cells to create individual clones. The human protein may act differently or incompletely in chicken DT40 cells.

The exact function of BRCA1 in HR is controversial. The discovery of the BRCA1-CtIP interaction has led to a proposal that BRCA1 might facilitate the resection step of HR [11,37,40]. However, RPA foci are not completely abolished in BRCA1 mutant cells in these reports, suggesting that ssDNA does form in the absence of functional BRCA1. We found that RPA accumulated at the sites of laser microirradiation in BRCA1−/− and CtIPS332A−/− cells, while Rad51 focus formation is impaired in BRCA1−/− cells. These results indicate that the BRCA1-CtIP interaction is not involved in the promotion of HR including the resection step, and are in agreement with the idea that BRCA1 facilitates the loading of Rad51 on resected ssDNA as does BRCA2 [1,29,41]. Recently, it was found that BRCA1 forms a complex with BRCA2 [42], further supporting the collaborative and overlapping function of BRCA1 and BRCA2. Although we cannot formally exclude the possibility that the RPA accumulation is delayed in BRCA1−/− cells (the extent of RPA accumulation induced by laser irradiation cannot be quantified, and we failed to induce RPA foci by other genotoxic stimuli in DT40 cells), our data, together with the fact that BRCA1 deficiency does not lead

Figure 5. Colony survival assay of CtIP mutant cells to various genotoxic agents. Colony survival assay of asynchronous populations of cells exposed to (A) camptothecin (CPT), (B) etoposide (VP16), (C) ICRF-193, and (D) methyl methanesulfonate (MMS). The dose is displayed on the X axis on a linear scale, while the percent fraction of surviving colonies is displayed on the Y axis on a logarithmic scale. To count the surviving colonies, cells were grown for 8–10 days in methylcellulose medium containing various concentrations of DNA-damaging agents. At least three independent experiments were performed with triplicate samples, and the representative results are shown. The error bars indicate S.D.

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to cellular lethality in DT40 cells, indicate that BRCA1 has only a minor role, if any, in the resection step. The discrepancies among researchers may arise from different experimental settings including how BRCA1 is inactivated (by gene targeting, siRNA knockdown, or C-terminal truncation), the cell-cycle distribution of each cell type, and the extent of DSB end modifications induced by laser or γ-ray irradiation. Further studies will clarify the differences among each group.

Accumulating evidence indicates that there are two parallel pathways to eliminate chemical modifications from single-strand breaks and DSBs (Figure 7). Firstly, tyrosyl-DNA phosphodiesterase 1 (Tdp1) removes polypeptides covalently bound at the 3′ end of DSBs [43]. Polynucleotide kinase 3′-phosphatase (PNKP) and AP endonuclease I (APE1) are also involved in this process. Likewise, PNKP, DNA polymerase β, and aprataxin remove aberrant chemical modifications from the 5′ ends of DSBs [44]. These enzymes may be capable of accurately repairing damaged bases at DSBs. On the other hand, the second pathway involves endonucleases and removes damaged bases along with proximal intact oligonucleotides from the 3′ or 5′ ends of DSBs. Our study showed that this pathway could contribute to cellular tolerance to alkylating agents such as MMS as well as to topoisomerase inhibitors.

A well-known precedent involving the second pathway is the Mre11/Rad50/Nbs1-complex-dependent elimination of oligonucleotides as well as the covalently associated topoisomerase-like protein (Spo11) from DSBs during meiotic HR in S. cerevisiae [2]. A more recent study of the S. pombe Ctp1 mutant (ctp1Δ) showed that the level of Top2 protein covalently bound to DNA in the ctp1Δ mutant increased during treatment with TOP-53, one of the VP16 derivatives, suggesting that Ctp1 plays a role in the endonuclease-dependent removal of covalently-bound polypeptides from the 5′ end of DSBs [20]. Our study indicates that this conclusion is also relevant to vertebrate cells although there are significant differences between vertebrate and yeast systems. First, yeast Ctp1 or Sacl2 seem to be important only for the removal of the peptide covalently bound to 5′ of DSB ends as demonstrated for DNA damage induced by TOP-53 or Spol1 [2,20]. Second, yeast does not have BRCA1 counterpart.

BRCA1 is involved in degradation of trapped Topo1 cleavage complexes along with proteasome [45]. We hypothesize that BRCA1 may facilitate the removal of Topo1 by degrading them to small polypeptides, which in turn are removed with oligonucleotides by the nuclease activity of Ctp1. In summary, we here show compelling evidence that the collaborative action of BRCA1 and Ctp1 plays a critical role in the endonuclease-dependent removal of damaged nucleotides from DSBs, and acts on the processed DSBs for subsequent HR and NHEJ.

Materials and Methods

Cell culture

DT40 cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), and 1% chicken serum (Sigma, St Louis, MO, USA) at 39.5°C.

Generation of Ctp conditional mutant DT40 cells

To generate Ctp conditional disruption, genomic DNA sequences of DT40 cells were amplified using primers 5′- GGATGCGGAGAGGCTTGAAGAGTTTTACAC-3′ and 5′-TTACAGCACAAACGAAGCATCGATATAATCCGCCGTCGTC-3′ for the 5′ arm, and 5′-GGAGCTTCCTACGAATACGCCGGAACACTCA-3′ and 5′-GCTTCCCCCTCCAATTCTTGACTGAGAATCA-3′ for the 3′ arm. The amplified PCR products were cloned into the pCR2.1-TOPO vector (Invitrogen, CA, USA). The BamHI site in the plasmid that contains the 5′ arm was disrupted by blunt self-ligation. The 1.6-kb HindIII fragment was ligated into the partially-digested HindIII site of the 3.0-kb 3′ arm containing the plasmid. A drug-resistance gene (hisD or bar) was inserted into the BamHI site of the pCR2.1 vector containing both the 5′ and 3′ arms. To generate CtpΔΔ cells, linearized Ctp Δ gene-disruption constructs were transfected sequentially by electroporation (BioRad). The genomic DNA of the transfectants was digested with SalI and the targeted clones were confirmed by Southern blot analysis. The 0.5-kb fragment was amplified using primers 5′-GATTGTATGCTTCACG-3′ and 5′-GAAATTCACCAACTTGGCTTACG-3′.
puromycin-resistant clones were selected to isolate the various chemical modifications from the 3’ and 5’ ends of DSBs, and thereby inhibit subsequent DSB repair. There are two distinct pathways to eliminate such chemical modifications from DSBs. In the first pathway, tyrosyl-DNA phosphodiesterase 1 (Tdp1), polynucleotide kinase 3’-phosphatase (PNKP), and AP endonuclease 1 (APE1) remove various chemical modifications from the 3’ ends of DSBs, while PNKP, DNA polymerase β (Polβ), and apratexin (APTX) remove those from the 5’ ends of DSBs (the arrows on the left). In the second pathway, MRN-CtIP-BRCA1 may act as an endonuclease and eliminate oligonucleotides covalently bound to polypeptide (the arrows on the right). The resulting processed DSBs are subject to homologous recombination (HR) and nonhomologous-end-joining (NHEJ)-dependent DSB repair.

Figure 7. Model for processing the modified DNA ends. Topoisomerase inhibitors induce chemical modification (indicated by a circle) at 3’ and 5’ ends of DSBs, and thereby inhibit subsequent DSB repair. There are two distinct pathways to eliminate such chemical modifications from DSBs. In the first pathway, tyrosyl-DNA phosphodiesterase 1 (Tdp1), polynucleotide kinase 3’-phosphatase (PNKP), and AP endonuclease 1 (APE1) remove various chemical modifications from the 3’ ends of DSBs, while PNKP, DNA polymerase β (Polβ), and apratexin (APTX) remove those from the 5’ ends of DSBs (the arrows on the left). In the second pathway, MRN-CtIP-BRCA1 may act as an endonuclease and eliminate oligonucleotides covalently bound to polypeptide (the arrows on the right). The resulting processed DSBs are subject to homologous recombination (HR) and nonhomologous-end-joining (NHEJ)-dependent DSB repair.

cccccttgac-3’ and used as a probe. To construct the CtIP expression plasmid, the CtIP open reading frame was amplified by PCR, using the primers 5’-GGGAGCAGCTTGG-TACAAAAAGCAGCTTGGACCATGATGCCTGAGGGAACTTGTG-GAGCACTTTGTACAA-GAAAGCTGGGTCTTATGTCTTCTGCTCTTTGCCTTTTGG-3’ and cloned into a Gateway donor vector, pDONR207 (Invitrogen, CA, USA), by BP reaction. The CtIP gene in the donor vector was transferred to an expression vector (pA-puro) containing the Gateway conversion cassette under the β-actin promoter by LR reaction. To construct a CtIP expression vector under the control of a tetracycline-repressible promoter, CtIP cDNA was amplified by PCR, using the primers 5’-CTCGAGAATATGCGTCTGG-GGAACTTTGTACAA-GAAAGCTGGGTCTTATGTCTTCTGCTCTTTGCCTTTTGG-3’ and cloned into a Gateway donor vector, pDONR207 (Invitrogen, CA, USA), by BP reaction. The CtIP gene in the donor vector was transferred to an expression vector (pA-puro) containing the Gateway conversion cassette under the β-actin promoter by LR reaction. To construct a CtIP expression vector under the control of a tetracycline-repressible promoter, CtIP cDNA was amplified by PCR, using the primers 5’-CTCGAGAATATGCGTCTGG-GGAACTTTGTACAA-GAAAGCTGGGTCTTATGTCTTCTGCTCTTTGCCTTTTGG-3’ and cloned into a Gateway donor vector, pDONR207 (Invitrogen, CA, USA), by BP reaction. The CtIP gene in the donor vector was transferred to an expression vector (pA-puro) containing the Gateway conversion cassette under the β-actin promoter by LR reaction. To construct a CtIP expression vector under the control of a tetracycline-repressible promoter, CtIP cDNA was amplified by PCR, using the primers 5’-CTCGAGAATATGCGTCTGG-GGAACTTTGTACAA-GAAAGCTGGGTCTTATGTCTTCTGCTCTTTGCCTTTTGG-3’ and cloned into a Gateway donor vector, pDONR207 (Invitrogen, CA, USA), by BP reaction. The CtIP gene in the donor vector was transferred to an expression vector (pA-puro) containing the Gateway conversion cassette under the β-actin promoter by LR reaction. To construct a CtIP expression vector under the control of a tetracycline-repressible promoter, CtIP cDNA was amplified by PCR, using the primers 5’-CTCGAGAATATGCGTCTGG-GGAACTTTGTACAA-GAAAGCTGGGTCTTATGTCTTCTGCTCTTTGCCTTTTGG-3’ and cloned into a Gateway donor vector, pDONR207 (Invitrogen, CA, USA), by BP reaction. The CtIP gene in the donor vector was transferred to an expression vector (pA-puro) containing the Gateway conversion cassette under the β-actin promoter by LR reaction. To construct a CtIP expression vector under the control of a tetracycline-repressible promoter, CtIP cDNA was amplified by PCR, using the primers 5’-CTCGAGAATATGCGTCTGG-GGAACTTTGTACAA-GAAAGCTGGGTCTTATGTCTTCTGCTCTTTGCCTTTTGG-3’ and cloned into a Gateway donor vector, pDONR207 (Invitrogen, CA, USA), by BP reaction. The CtIP gene in the donor vector was transferred to an expression vector (pA-puro) containing the Gateway conversion cassette under the β-actin promoter by LR reaction. To construct a CtIP expression vector under the control of a tetracycline-repressible promoter, CtIP cDNA was amplified by PCR, using the primers 5’-CTCGAGAATATGCGTCTGG-GGAACTTTGTACAA-GAAAGCTGGGTCTTATGTCTTCTGCTCTTTGCCTTTTGG-3’ and cloned into a Gateway donor vector, pDONR207 (Invitrogen, CA, USA), by BP reaction. The CtIP gene in the donor vector was transferred to an expression vector (pA-puro) containing the Gateway conversion cassette under the β-actin promoter by LR reaction.

CtIP<sup>+/−</sup> cells were introduced into the tetracycline-controlled trans-activator (tTA) gene through retrovirus infection. Infected cells were sub-cloned, and tTA expression was confirmed by western blot analysis. The resulting tTA-expressing CtIP<sup>+/−</sup> cells were transfected with the pTRE2 puro<sup>+/−</sup>CtIP<sup>−/−</sup> cells, and puromycin-resistant clones were selected to isolate the CtIP<sup>+/−</sup> cells. The puromycin-resistance gene was then deleted by transiently expressing the Cre recombinase (Amazax solution T, program B-23). Puromycin-sensitive CtIP<sup>+/−</sup> cells were transfected with the CtIP gene-disruption construct carrying the puromycin-resistant cassette to generate CtIP<sup>−/−</sup> cells.

Generation of CtIP<sup>S332A−/−</sup> mutant cells

The targeting vectors for the CtIP S332A mutants were generated by site-directed mutagenesis. To generate the S332A knock-in vector, genomic DNA was amplified by PCR, using primers 5’-ATTATGCCCTGAAGAAGCGAAAC-3’ and 5’-TTTCTGGGTTTGTCTTGTATTTT-3’, and cloned into the pCR2.1-TOPO vector (Invitrogen, CA, USA). Site-directed mutagenesis was performed using primers 5’-GATTTCGAG-TAGTTTGCTCTTGGTTTGCAGGA-3’ and 5’-TCCGAAAA-CAGGAGCAACTTCAGGAGTCGTTG-3’. The puromycin-resistance gene was inserted into the HpaI site of the resulting plasmid. After transfection of the S332A knock-in vector into the CtIP<sup>+/−</sup>-cell lines, the targeted clones were selected against puromycin and then identified by Southern blot analysis of genomic DNA digested with HindIII. To make probe DNAs, the 0.6-kb fragments were amplified using primers 5’-GACTAACAGATCACCCCTTG-3’ and 5’-GTCGACTATAGTTTTTGCTTG-3’. After the deletion of the puromycin-resistance gene by transiently expressing Cre recombinase by nucleofection (Amazax, Germany), the third allele of the CtIP gene was disrupted by transfecting the CtIP gene-disruption construct carrying the puromycin-resistance gene. The insertion of the S332A mutation into the endogenous CtIP gene was confirmed by RT-PCR followed by sequencing amplified DNA.

Generation of CtIP<sup>+/−</sup>- Brca1<sup>−/−</sup> and CtIP<sup>S332A−/−</sup>- Brca1<sup>−/−</sup> clones

The puromycin-resistant cassette in the targeting vector for the Brca1 gene [31] was replaced with the neomycin-resistant cassette. CtIP<sup>+/−</sup>- and CtIP<sup>S332A−/−</sup>- cell lines were sequentially transfected with targeting vectors containing the puromycin- and neomycin-resistant gene, and selected against G418 and puromycin, respectively. The clones with the disrupted Brca1 gene were identified by Southern blot analysis as described previously [31].

Real-time PCR quantification of gene expression

Quantitative real-time PCR was performed in an ABI Prism 7000 sequence detector (Applied Biosystems) using SYBR Green PCR Master Mix reagent (Applied Biosystems) according to the manufacturer’s instructions. CtIP cDNA was amplified using primers 5’-GGAATTTGAGAGCAGCAAGAC-3’ and 5’-GGAATCTCACTGTTGCTTTTG-3’. The expression level of
CtIP was normalized against β-actin using the comparative CT method.

Western blotting analysis
For Western blot analysis, the antibodies specific for CtIP (BL1914, Bethyl, TX, USA), β-actin (Sigma, MO, USA), Rad51 (Ab-1, Calbiochem, CA, USA) were used for detection of each protein. Secondary antibodies were horseradish peroxidase (HRP)-conjugated antibodies to mouse Ig (GE Healthcare, MA, USA) and HRP-conjugated antibody to rabbit Ig (Santa Cruz, CA, USA).

Chromosome aberration analysis
Karyotype analysis was performed as described previously [25]. To measure the number of γ-ray-induced chromosome breaks in mitotic cells, we exposed cells to 2 Gy γ-rays and immediately added colcemid. At 3 hours after irradiation, mitotic cells were harvested and subjected to chromosome analysis.

Measurement of cellular sensitivity to DNA-damaging agents
Methylcellulose colony formation assays were performed as described previously [30,46]. Since in this assay the plating efficiency of BRCA1-deficient cells was less than 50%, we used a different assay to measure cellular sensitivity to DNA-damaging agents. Cells (1×10⁶) were seeded into 24-well plates containing 1 ml culture medium per well and the DNA-damaging agents, and then incubated at 39.5°C for 48 hours. To assess the number of live cells, we measured the amount of ATP in the cellular lysates. We confirmed that the number of live cells was closely correlated with the amount of ATP. This ATP assay was carried out with 96-well plates using a CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega Corporation, WI, USA). Briefly, we transferred 100 μl of cell suspension to the individual wells of the plates, placed the plates at room temperature for approximately 30 minutes, added 100 μl of CellTiter-Glo Reagent, and mixed the contents for 2 minutes on an orbital shaker to induce cell lysis. The plate was then incubated at room temperature for 10 minutes to stabilize the luminescent signal. Luminescence was measured by Fluoroskan Ascent FL (Thermo Fisher Scientific Inc., MA, USA).

I-SceI–induced gene conversion and targeted integration frequencies
The measurement of homologous recombination frequencies using a SCneo cassette [28] and CENP-H-EGFP was performed as described previously [47]. After the I-SceI vector was transfected into the cells, the frequency of neomycin-resistant colony formation was measured.

Synchronization of cells
To enrich DT40 cells in the G1 phase, cells were synchronized by centrifugal counterflow elutriation (Hitachi Industrial, Japan). The cell suspension (~5×10⁷) was loaded at a flow rate of 11 ml/min into an elutriation chamber running at 2,000 rpm. The first 50 ml was discarded, and the following 100 ml was used as a G1-phase cell fraction.

Microscopy imaging and generation of DNA damage
Fluorescence microscopy was carried out and images were obtained and processed using the IX81 (Olympus, Japan). Cells were cultured in medium containing BrdU (10 μM) for 24–48 h to sensitize them to DSB generation by means of a 405 nm laser from a confocal microscope (FV-1000, Olympus, Japan). During laser treatment, cells were incubated in phenol-red-free Opti medium (GIBCO, NY, USA) to prevent the absorption of the laser’s wavelength. γ-irradiation was performed using 137C (Gammacell 40, Nordion, Kanata, Ontario, Canada). Antibodies against Rad51 (Ab-1, Calbiochem, CA, USA), FK2 (Nippon Biotest Laboratories, Japan), RPA p32 (GeneFex, TX, USA), rabbit Ig (Alexa 488-conjugated antibody, Molecular Probe, OR, USA), and mouse Ig (Alexa 594-conjugated antibody, Molecular probe, OR, USA) were used for visualization.

Supporting Information
Figure S1 Generation of CtIP+/−/−tetCIP mutants. (A) CtIP gene disruption strategy. The map shows the organization of the CtIP gene (top), targeting construct (middle), and targeted allele (bottom). Black and white boxes represent exons and the drug-marker cassettes, respectively. (B) Genomic PCR analysis at the disrupted site using primers p1 and p2, as shown in (A). (C) Southern blot analysis of Sce-digested genomic DNA using the probe shown in (A).

Figure S2 Generation of CtIPS322A+/−/− mutants. (A) The strategy for the generation of CtIPS322A+/−/− mutants. The knock-in vectors shown in Figure S3B were introduced into the CtIP+/−/− cells. The insertion of the S322A mutation was verified by Southern blot analysis of HindIII-digested genomic DNA. Cre recombinase were transiently expressed in the resulting CtIPS322A+/−/− clones to delete the drug-resistant marker. The remaining intact CtIP allele was targeted by the CtIP disruption construct to obtain CtIPS322A+/−/− clones. (B) The knock-in constructs for the generation of CtIPS322A+/−/− mutant clones. Black and white boxes represent exons and the drug-marker cassettes, respectively. (C) Nucleotide sequence analysis of CtIP cDNAs derived from the CtIP+/−/− and CtIPS322A+/−/− mutant. The total RNA was subjected to reverse transcription. The regions spanning the mutations were amplified by PCR and the sequence was determined. (D) Cell cycle profiles of CtIPS322A+/−/− and CtIP+/−/− mutant cells. Cells were pulse-labeled with BrdU for 10 min and subsequently stained with FITC-conjugated anti-BrdU antibody (Y axis, log scale) and propidium iodide (PI) (X axis, linear scale). (E) Western blot analysis of wild-type, CtIP+/−/− and CtIPS322A+/−/− DT40 clones. β-actin was used as a loading control. (F) FLAG-BRCA1 association with CtIP is dependent on Ser322. 293T cells were transfected with plasmids encoding FLAG-tagged chicken BRCA1 together with either wild-type CtIP or S322A CtIP. Cell lysates were subjected to immuno-precipitation with anti-FLAG antibody, and the precipitated proteins were detected with anti-FLAG or anti-CtIP antibody.

Figure S3 Sensitivity of CtIPS322A+/−/− mutant to IR at G1 phase. Cells at G1 phase were separated by centrifugal elutriation and were γ-irradiated for colony survival assay. The dose of γ-ray irradiation is displayed on the X axis on a linear scale, while the percent fraction of surviving colonies is displayed on the Y axis on a logarithmic scale.

Figure S4 Generation of CtIP+/−/−BRCA1−/− and CtIPS322A+/−/−BRCA1−/− mutants. (A) Southern blot analysis of double mutant...
clones. The genomic DNA of indicated genotype was digested with HindIII and hybridized with the probe which detects the 3’ of the targeted BRCA1 site. The top band at 10 kb and the bottom band at 4.5 kb correspond to the BRCA1 allele disrupted with neomycin (NEO)- and puromycin (Puro)-resistance cassette, respectively. The middle band at 5.7 kb is the non-targeted internal allele of BRCA1 gene. (B) RT–PCR of double mutant clones. cDNA was synthesized from each genotype and used for PCR amplification of BRCA1 (upper panel) or β-actin (lower panel) as a control. 

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Author Contributions

Conceived and designed the experiments: KN ST YT. Performed the experiments: KN TK HOYS YT. Analyzed the data: KN TK HOYS. Contributed reagents/materials/analysis tools: AS KA YP KMT KT EH TO. Wrote the paper: KN ST YT.

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