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


PLOS Genetics

DOI: 10.1371/journal.pgen.1000828

Published: 22/01/2010

Publisher's PDF, also known as Version of record

Cyswllt i'r cyhoeddiad / Link to publication

Dyfyniad o’r fersiwn a gyhoeddwyd / Citation for published version (APA):

Hawliau Cyffredinol / General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.
Collaborative Action of Brca1 and CtIP in Elimination of Covalent Modifications from Double-Strand Breaks to Facilitate Subsequent Break Repair

Kyoko Nakamura1, Toshiaki Kogame1, Hiroyuki Oshiumi2, Akira Shinohara2, Yoshiki Sumitomo1, Keli Agama3, Yves Pommier3, Kimiko M. Tsutsui4, Ken Tsutsui4, Edgar Hartsuiker5, Tomoo Ogi6, Shunichi Takeda1, Yoshihito Taniguchi1*

1 Department of Radiation Genetics, Graduate School of Medicine, Kyoto University, Kyoto, Japan, 2 Institute for Protein Research, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan, 3 Laboratory of Molecular Pharmacology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, United States of America, 4 Department of Neurogenomics, Department of Genome Dynamics, Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama University, Okayama, Japan, 5 Cancer Biology, North West Cancer Research Fund Institute, Bangor University, Bangor, United Kingdom, 6 Department of Molecular Medicine, Atomic Bomb Disease Institute, Nagasaki University, Nagasaki, Japan

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 CtIPΔ332/Δ332 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 wild-type cells. Finally, CtIPΔ332/Δ332 and 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.

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

Author Summary

Induction of double-strand breaks (DSBs) in chromosomal DNA effectively activates a program of cellular suicide and is widely used for chemotherapy on malignant cancer cells. Cells resist such therapies by quickly repairing the DSBs. Repair is carried out by two major DSB repair pathways, homologous recombination (HR) and nonhomologous end-joining. However, these pathways cannot join DSBs if their ends are chemically modified, as seen in the DSB ends that would arise after the prolonged treatment of the cells with topoisomerase inhibitors such as camptothecin and etoposide. These anti-cancer drugs can produce the polypeptides covalently attached to the 3’ or 5’ end of DSBs. It remains elusive which enzymes eliminate these chemical modifications prior to repair. We here show evidence that the BRCA1-CtIP complex plays a role in eliminating this chemical modification, thereby facilitating subsequent DSB repair. Thus, BRCA1 and CtIP have dual functions: their previously documented roles in HR and this newly identified function. This study contributes to our ability to predict the effectiveness of chemotherapeutic agents prior to their selection by evaluating the activity of individual repair factors. Accurate prediction is crucial, because chemotherapeutic agents that cause DNA damage have such strong side effects.

Results

CtIP is required for the assembly of Rad51 at DNA damage sites

In order to determine the function of CtIP, we conditionally disrupted the CtIP gene in chicken DT40 cells, using a chicken CtIP transgene under the control of a tetracycline-repressible promoter (tetCtIP transgene, Figure S1A). We designed CtIP gene-disruption constructs, so that the amino acid sequences from 96 to 335 would be replaced by selection-marker genes. Since the gene is encoded on chromosome 2, which is in trisomy in DT40, we disrupted three CtIP alleles (Figure S1B and S1C). The resulting CtIP<sup>+/−</sup>/− tetCtIP cells tended to grow more slowly than did wild-type cells, presumably due to overexpression of the tetCtIP transgene (Figure 1A and 1B). To deplete the CtIP in the CtIP<sup>+/−</sup>/− tetCtIP cells, we added doxycycline (modified tetracycline) to the culture medium. One day after the addition of doxycycline, the amount of CtIP was reduced to around 20% of wild-type cells (Figure 1B), and the cells started dying as evidenced by an increase in the sub-G1 fraction (Figure 1C). This lethality can be attributed to abolished HR, because the cells showed a significant increase in the number of spontaneous chromosomal breaks (Table 1), as do Mre11- and Rad51-depleted cells [23,25]. By day 3, the vast majority of the CtIP<sup>−/−</sup>/− tetCtIP cells had stopped growing and died (Figure 1A and 1C). We therefore conclude that CtIP is essential for maintenance of chromosomal DNA and cellular proliferation.

To assess the HR capability of CtIP<sup>+/−</sup>/− tetCtIP cells, we monitored the recruitment of Rad51 and RPA to DNA damage sites one day after addition of doxycycline. Clear Rad51 foci appeared in wild-type cells one hour after ionizing radiation (IR), whereas Rad51 foci were hardly detectable in the CtIP-depleted cells (Figure 2A). Likewise, the depletion of CtIP abolished the accumulation of RPA on DNA lesions induced by microcosmic treatment (Figure 2B). This is consistent with a phenotype shown in the previous report [3]. Thus, CtIP plays an essential role in the resection of DSBs during HR in DT40 cells as well as in mammalian cells.

We next investigated whether or not CtIP facilitates the activation of BRCA1 at DSBs. To this end, we measured the formation of conjugated-ubiquitin foci at DSBs, since Brca1 promotes extensive ubiquitylation at IR-induced DSBs [26]. Previous studies showed that BRCA1<sup>−/−</sup>/− DT40 cells exhibit a prominent defect in 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<sup>S332A/−/−</sup> DT40 clones

To functionally analyze the interaction of CtIP with BRCA1, we generated CtIP<sup>S332A/−/−</sup> cells, in which the critical amino acid in the binding interface has been mutated (Figure S2A, S2B, S2C). The CtIP<sup>S332A/−/−</sup> DT40 clones were capable of proliferating at a rate similar to the CtIP<sup>+/−</sup>/− 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<sup>S332A/−/−</sup> cells, we integrated an artificial substrate, S<sub>Caco</sub>, into the Ovalbumin locus [28], and measured the efficiency of I-SceI-induced gene conversion. The CtIP<sup>S332A/−/−</sup> clones showed no significant decrease in the...
appearance of neomycin-resistant colonies compared to CtIP+/−/− cells (Figure 3B). The proficient HR in CtIPS332A+/−/− DT40 clones is in marked contrast to the severe phenotype of the 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 SCneo substrate [29]. Next, we measured the frequency of gene targeting at the CENP-H and Ovalbumin loci. In contrast to I-SceI-induced gene conversion, the gene-targeting frequency of the CtIPS332A+/−/− clones decreased moderately in comparison with 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 γ-irradiation was indistinguishable between CtIPS332A+/−/− cells and the CtIP+/−/− control cells, while BRCA1+/− cells showed the significant reduction in the Rad51 focus formation at 1–6 h after irradiation (Figure 4A). Furthermore, the CtIP+/−/− mutants displayed laser-induced RPA accumulation as did the 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 Ubc13 deficient cells [27]. This suggests that CtIP332A−/− cells are proficient in resection at DSB sites. Taken together, we conclude that the S332A mutation of CtIP does not significantly compromise HR.

**Figure 1. CtIP is essential for cell survival.** (A) Defective proliferation of CtIP−/−/− tetCtIP cells following addition of doxycycline (2 μg/ml) to deplete CtIP, from time zero. Data shown are the average results from three separate clones. Points indicate mean (n = 3); bars indicate S.D. (B) Western blot analysis of chicken CtIP expression. Whole cell lysates were prepared from CtIP−/−/− tetCtIP cells 24 h after addition of doxycycline. β-actin was used as a loading control. (C) Cell cycle distribution of CtIP−/−/− tetCtIP cells at the indicated time after addition of doxycycline. 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). Each square on the left-hand side represents apoptic cells (sub-G1 fraction). The small rectangle at the bottom of the tubular arch, the tubular arch, and the small square to the right represent cells in the G1, S, and G2/M phases, respectively. doi:10.1371/journal.pgen.1000828.g001

<table>
<thead>
<tr>
<th>Genotype Chromatid-type Chromosome-type</th>
<th>IR 0 Gy Wild-type</th>
<th>CtIP−/−/− tetCtIP Dox(−)</th>
<th>CtIP−/−/− tetCtIP Dox(+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>6</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>CtIP−/−/− tetCtIP Dox(−)</td>
<td>4</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>CtIP−/−/− tetCtIP Dox(+)</td>
<td>7</td>
<td>28</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genotype Chromatid-type Chromosome-type</th>
<th>IR 2 Gy Wild-type</th>
<th>CtIP−/−/− tetCtIP Dox(−)</th>
<th>CtIP−/−/− tetCtIP Dox(+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>10</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>CtIP−/−/− tetCtIP Dox(−)</td>
<td>6</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>CtIP−/−/− tetCtIP Dox(+)</td>
<td>20</td>
<td>71</td>
<td></td>
</tr>
</tbody>
</table>

γ-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. doi:10.1371/journal.pgen.1000828.001
CtIP$^{332A/-/-}$ 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$^{+/+}$ 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$^{+/+}$ 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$^{+/+}$ cells exhibited about 80% reductions in CtIP mRNA level compared to the wild-type level (Figure S2G). In contrast to CtIP$^{+/+}$ cells, CtIP$^{332A/-/-}$ 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$^{332A/-/-}$ mutants, in comparison with the CtIP$^{+/+}$ 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$^{332A/-/-}$ 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...
CtIP and VP16, independently of HR or NHEJ, most likely by concluding that CtIP can therefore contribute to cellular tolerance to clones exhibited no decrease in the efficiency of HR or NHEJ. We significantly higher sensitivity to CPT and VP16, although these comparison with BRCA1−/− clones (doubling time ± SD: 8.3±0.2 h for wild-type, 9.3±0.3 h for BRCA1−/−, 11±0.3 h for CHP4/−/−/BRCA1−/−, 12.6±0.9 h for CHP832A/−/−/BRCA1−/−). The viability of CHP832A/−/−/BRCA1−/− cells is in marked contrast with the lethality of CIP-mall cells, supporting the idea that the CIP-BRCA1 interaction works independently from the function of CIP in resorption.

We next examined the sensitivity of double mutant cells to CPT and VP16. To this end, we measured the number of viable 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 CHP4/−/−/BRCA1−/− and CHP832A/−/−/BRCA1−/− clones grew very badly from a single cell in semi-solid methylcellulose medium. The number of viable cells cultured in the presence of CPT was significantly decreased for CHP832A/−/− and BRCA1−/− clones compared to the wild-type cells, whereas CHP4/−/− cells grew to the similar extent to the wild-type cells in the presence of CPT (Figure 6A). The sensitivity of CHP4/−/−/BRCA1−/− cells to CPT was greater than that of BRCA1−/− clones. This observation is in agreement with the idea that BRCA1 and CtIP can independently contribute to HR, where CIP promotes the resection of DSBs, while BRCA1 subsequently loads Rad51 at resected ssDNA overhang. Importantly, although the CHP832A mutation significantly increased cellular sensitivity to CPT in the presence of BRCA1, the CHP832A/−/−/BRCA1−/− and CHP4/−/−/BRCA1−/− clones exhibited a very similar sensitivity to CPT (Figure 6A). Likewise, the CHP832A/−/−/BRCA1−/− and CHP4/−/−/BRCA1−/− 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 S32A 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 CHP4/−/− cells, the CHP832A/−/− clones 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 polyamines from DSBs. Hence, CHP832A/−/− clones are the novel separation-of-function mutants where CtIP-dependent resection is proficient, whereas the second function required for the tolerance to topoisomerase inhibitors is deficient.

In this study, we demonstrated that the inactivation of CTP 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 CTP promotes G1/S progression by releasing relationship between Ser332 phosphorylation of CtIP and BRCA1, we disrupted the BRCA1 gene in the CHP832A/−/− and CHP4/−/− clones (Figure S4A and S4B), as was done previously [31]. Both the CHP832A/−/−/BRCA1−/− and the CHP4/−/−/BRCA1−/− clones proliferated with similar rates at significantly reduced growth rates, in comparison with BRCA1−/− cells (doubling time ± SD: 0.3±0.2 h for wild-type, 9.3±0.3 h for BRCA1−/−, 11±0.3 h for CHP4/−/−/BRCA1−/−, 12.6±0.9 h for CHP832A/−/−/BRCA1−/−). The viability of CHP832A/−/−/BRCA1−/− cells is in marked contrast with the lethality of CIP-mall cells, supporting the idea that the CTP-BRCA1 interaction works independently from the function of CTP in resorption.

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 AS:

Figure 3. Proficient homologous recombination in CTP832A/−/− clones. (A) Growth kinetics of the indicated CtIP mutant clones. (B) I-Sce1-induced DSBs stimulate homologous recombination (HR) in an artificial substrate, SCneo. 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 CTP832A/−/− clones. In summary, in comparison with CTP4/−/− cells, CTP832A/−/− 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 polyamines from the DSBs.

Table 2. Targeted integration frequencies of CTP832A/−/− clones.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Targeted loci</th>
<th>CENP-H</th>
<th>Ovalbumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>38/61 (62.2%)</td>
<td>24/26  (92.3%)</td>
<td></td>
</tr>
<tr>
<td>CTP4/−/−</td>
<td>42/64 (65.6%)</td>
<td>25/30  (83.3%)</td>
<td></td>
</tr>
<tr>
<td>CTP832A/−/−</td>
<td>25/71 (35.2%)</td>
<td>30/47  (63.8%)</td>
<td></td>
</tr>
<tr>
<td>BRCA1−/−</td>
<td>N.D.</td>
<td>5/37 (13.5%)</td>
<td></td>
</tr>
</tbody>
</table>

Gene targeting efficiencies were determined by flow cytometry for CENP-H locus and by Southern blot analysis for Ovalbumin locus. N.D. indicates not determined.

doI:10.1371/journal.pgen.1000828.t002
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 CtIP(S332A) 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 CtIP(S332A)−/− 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
the representative results are shown. The error bars indicate S.D.

vertebrate and yeast systems. First, yeast Ctp1 or Sae2 seem to be
to vertebrate cells although there are significant differences between
of DSBs [20]. Our study indicates that this conclusion is also relevant
dependent removal of covalently-bound polypeptides from the 5
of DSB ends as demonstrated for DNA damage induced by TOP-53
increased during treatment with TOP-53, one of the VP16

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 phosphodiester-
ase1 (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 oligonucleo-
tides 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 CtIP mutant (ctp1A) showed that the level
of Top2 protein covalently bound to DNA in the ctp1A 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 Sae2 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 Spo11 [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 CtIP. In summary, we here show compelling
evidence that the collaborative action of BRCA1 and CtIP 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 10−5 M β-mercaptoethanol, penicillin, streptomycin, 10% fetal
calf serum (FCS), and 1% chicken serum (Sigma, St Louis,
MO, USA) at 39.5°C.

Generation of CtIP conditional mutant DT40 cells

To generate CtIP gene disruption constructs, genomic DNA
sequences of DT40 cells were amplified using primers 5’-
GGATGCGGAGAGGCTTGAAGAGTTTTACAC-3’ and 5’-
GGAGCTTCTAGCAATACGCGGAACAACTCA-
9 arm, and 5’-GGAGCTTCTAGCAATACGCGGAACAACTCA-
9 arm, and 5’-GGAGCTTCTAGCAATACGCGGAACAACTCA-
9 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
CtIP+/−/− cells, linearized CtIP gene-disruption constructs were
transfected sequentially by electroporation (BioRad). The genomic
DNA of the transfectants was digested with SaeI and the targeted
clones were confirmed by Southern blot analysis. The 0.5-kb
fragment was amplified using primers 5’-GATTGTATGCTTGAGAGGCTCCTGC-3’
and 5’-GAAATTCCCAACCTCTTAGCT-
puromycin-resistant clones were selected to isolate the
In the first pathway, tyrosyl-DNA phosphodiesterase1 (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 δ (Polδ), and aprataxin (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 phosphodiesterase1 (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 δ (Polδ), and aprataxin (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.

cccccttggac-3' and used as a probe. To construct the CtIP expression plasmid, chicken the CtIP open reading frame was amplified by PCR, using the primers 5'-ggggacaggttttg-tacaaaaagacagcttggcaaccataagatgggtcttg-gggactttgtg-3' and 5'-ggggacacctttgtgtaacaa-gaaagctgggtctttgctctttgcccttttg-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 a tetracycline-repressible promoter, pDONR207 (Invitrogen, CA, USA), by BP reaction. The CtIP gene was 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'-ctcggacttttcgctttttggga-3' and 5'-ttttgctttttggccttttg-3'. After the deletion of the puromycin-resistance gene by transiently expressing Cre recombinase by nucleofection (Amaxa, 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+/−/− and CtIPS332A+/−/− clones

The puromycin-resistant cassette in the targeting vector for the Brca1 gene [31] was replaced with the neomycin-resistant cassette. CtIP+/−/− and CtIPS332A+/−/− cells 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 instruction. CtIP cDNA was amplified using primers 5'-gggaatcctgagagacaagcag-3' and 5'-ggaacctcactttgccttttg-3'. The expression level of...
CiIP was normalized against β-actin using the comparative CT method.

Western blotting analysis
For Western blot analysis, the antibodies specific for CiIP (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 × 10^5) were seeded onto 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 Luminescence 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 luminescence signal. Luminescence was measured by Fluorskan 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 SColo cassette [28] and CENP-H-EGFP was performed as described previously [47]. After the I-Sce-I 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 (~3x10^7) 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 (GeneTex, 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+/−/−tetCtIP 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 SacI-digested genomic DNA using the probe shown in (A).

Found at: doi:10.1371/journal.pgen.1000828.s001 (11.61 MB TIF)

Figure S2 Generation of CtIPS332A+/−/− mutants. (A) The strategy for the generation of CtIPS332A+/−/− mutants. The knock-in vectors shown in Figure S3B were introduced into the CtIP+/+/−/− cells. The insertion of the S332A mutation was verified by Southern blot analysis of HindIII-digested genomic DNA. Cre recombinase were transiently expressed in the resulting CtIPS332A+/−/− clones to delete the drug-resistant marker. The remaining intact CtIP allele was targeted by the CtIP disruption construct to obtain CtIPS332A+/−/−−/− clones. (B) The knock-in constructs for the generation of CtIPS332A+/−/−−/− mutants. Black and white boxes represent exons and the drug-marker cassettes, respectively. (C) Nucleotide sequence analysis of CtIP cDNAs derived from the CtIP+/+/−/− and CtIPS332A+/−/−−/− mutants. 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 CtIPS332A+/−/−−/− 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 CtIPS332A+/−/−−/− DT40 clones. β-actin was used as a loading control. (F) FLAG-BRCA1 association with CtIP is dependent on Ser332. 293T cells were transfected with plasmids encoding FLAG-tagged chicken BRCA1 together with either wild-type CtIP or S332A CtIP. Cell lysates were subjected to immunoprecipitation with anti-FLAG antibody, and the precipitated proteins were detected with anti-FLAG or anti-CtIP antibody. (G) Quantitative real time PCR of CtIP mRNA in wild-type, CtIP+/+/−/− and CtIPS332A+/−/−−/− DT40 clones. PCR amplification was performed in triplicate. The expression level of CtIP was normalized against β-actin using the comparative CT method.

Found at: doi:10.1371/journal.pgen.1000828.s002 (19.89 MB TIF)

Figure S3 Sensitivity of CtIP+/+/−/−−/− 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.

Found at: doi:10.1371/journal.pgen.1000828.s003 (10.15 MB TIF)

Figure S4 Generation of CtIP+/+/−/−−/− and CtIPS332A+/−/−−/− 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 was used for PCR amplification of Brca1 (upper panel) or β-actin (lower panel) as a control. Found at: doi:10.1371/journal.pgen.1000828.s004 (7.59 MB TIFF).

Acknowledgments

We thank A. Lehmann for his critical reading of the manuscript. The gene disruption construct for chicken Brca1 was kindly provided by D. Bishop from the University of Chicago. We thank G. Anderson from the University of Birmingham (UK) for his critical reading of the manuscript.

Author Contributions

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

References

24. Brca1 and CtIP Collaborate in DNA Repair
