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CtIP is required for DNA damage-dependent induction of *P21*

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Abbreviations: DSB, DNA double-strand break; HR, homologous recombination; MMEJ, micro-homology-mediated end joining; CtBP, adenovirus E1A C-terminal binding protein; ADR, Adriamycin; CDK, cyclin-dependent kinase; CPT, camptothecin; DDR, DNA damage response

DNA endonuclease CtIP is involved in both DNA double-strand break (DSB) repair and transcriptional repression/activation. The cyclin-dependent kinase inhibitor P21, which is induced at transcription level in response to a variety of stresses, controls G_1/S transition. In this report, we found that CtIP bound to the *P21* promoter, and this binding was enhanced in response to DNA damage. Concomitantly, ectopic expression of CtIP increased *P21* promoter activity, and this increment was enhanced upon camptothecin treatment. Conversely, DNA damage failed to induce *P21* gene expression in *CtIP*-deficient cells. Taken together, our data demonstrate that CtIP is required for DNA damage-induced *P21* induction.

Introduction

DNA endonuclease CtIP (CtBP-interacting protein) is a multifunctional protein involved in cell cycle control, transcriptional regulation, DNA damage response, and tumorigenesis.[1] Upon DNA damage, CtIP is phosphorylated and recruited to the DNA damage sites, cooperating with the MRE11-RAD50-NBS1 complex to promote DNA double-strand break (DSB) end resection and repair by homologous recombination (HR) and micro-homology-mediated end joining (MMEJ).[2,3]

CtIP was originally identified as a CtBP (adenovirus E1A C-terminal binding protein)-interacting protein, while CtBP is a transcriptional repressor.[4] The heterotrimeric complex of CtIP, the breast tumor suppressor BRCA1, and the zinc finger and BRCA1-interacting protein ZBRK1 inhibits expression of a group of genes, which are all proliferation markers, including *ANG1*, *bFGF*, *HMGA2*, *LIMK1*, and *RFC1*. The serine-327-phosphorylated CtIP interacts with the tandem BRCT domains within BRCA1, and this phosphorylation-dependent interaction is required for the transcriptional repression by this heterotrimeric complex. Impairment of this complex leads to an acceleration in tumor growth.[5]

In addition to its roles in transcriptional repression, CtIP exhibits activities of a transcriptional activator. During G_1/S transition, CtIP binds to promoters of the Rb-E2F downstream target genes, causing the dissociation of Rb and resulting in the expression of genes required for G_1/S transition.[6] CtIP, BRCA1, and E2F form a complex that binds to the *ATM* promoter and activates its transcription. Adriamycin (ADR) treatment releases CtIP and BRCA1 from the *ATM* promoter, while E2F was kept recruited, and the *ATM* transcription was repressed.[7]

The cyclin-dependent kinase (CDK) inhibitor P21 plays an important role in cell cycle progression, DNA replication, and apoptosis.[8] It exerts its functions primarily through inhibition of CDK2 and CDK1.[9,10] P21 binds to CDKs through its N-terminal Cy motif, and thereby inhibits phosphorylation and activation of CDKs and their substrates.[10] In response to a variety of stimuli, such as DNA damage and oxidative stress, *P21* expression is induced by P53-dependent and -independent mechanisms.[11] Increased P21 levels control the G_1/S transition mainly through CDK2 inhibition and inhibit DNA replication through interacting with the proliferating cell nuclear antigen (PCNA).[12] P21 also is a major inhibitor of P53-dependent as well as P53-independent apoptosis.[13]

As such a crucial regulator, *P21* expression is tightly regulated at transcriptional and post-translational levels through mechanisms involving RNA stabilization, phosphorylation, and ubiquitination. P53 is a major transcriptional activator for *P21*. Upon stimuli, P53 transactivates *P21* expression through binding to 2 highly conserved P53 response elements within promoter region.[14] As a matter of fact, many transcriptional factors contribute to *P21* expression through regulating P53 stability or activity. For example, upon DNA damage, on one hand, P53 is phosphorylated and thus activated by ATM/Chk2 or ATR/Chk1; on the other hand, P53 is stabilized through inhibition

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of its E3 ligase MDM2 as well as activation of its deubiquitinase USP7.[15,16] BRCA1 interacts with P53 and transactivates *P21* through P53-dependent and -independent pathways.[17-19]

In addition, conserved binding sites of transcription factors identified in the *P21* promoter also give us clues about how *P21* is regulated in a P53-independent pathway. Sp1/Sp3 activates *P21* expression through 6 Sp1/Sp3 binding sites in the proximal region of *P21* promoter.[20] Several E2F-binding sites have been identified on the promoter, and ectopic expression of E2F1 transactivated *P21*. P21 expression can also be repressed by the Myc-dependent pathway.[21] In Myc overexpressed cancers, Myc represses P21 expression by competing Sp1 binding sites and inducing expression of transcriptional repressor AP4, which suppressed P53-dependent induction of P21 upon DNA damage and the TGF-β-induced *P21* transactivation.[22,23]

Here, we report that CtIP binds to the *P21* promoter and facilitates activation of the *P21* promoter and is required for P21 induction upon DNA damage.

Results

The heterotrimeric complex of CtIP, BRCA1, and ZBRK1 represses transcription of a group of genes involved in cell proliferation, whereas BRCA1 transactivates *P21* expression both in P53-dependent and in P53-independent manners.[5,18,19] We sought to determine if CtIP modulates *P21* promoter activity. We found that ectopic expression of HA-CtIP increased the *P21* promoter activity in HCT116 cells, and this increment was enhanced upon treatment with camptothecin (**Fig. 1A**). Induction of *P21* promoter activity by CtIP was also observed

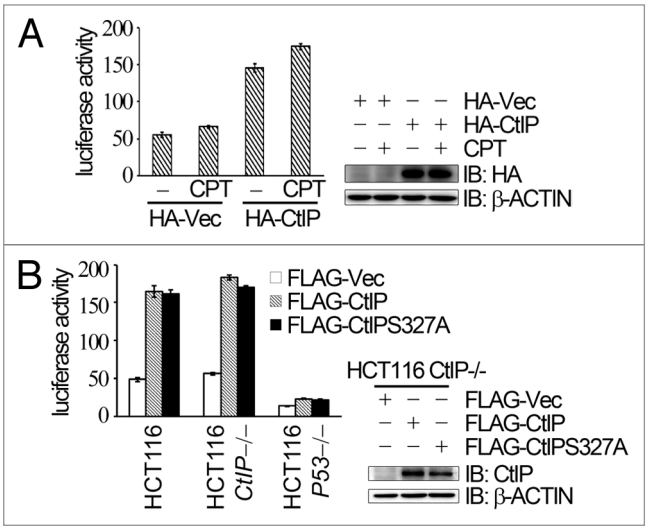

Figure 1. CtIP promotes *P21* promoter activity. (**A**) Ectopic expression of CtIP transactivates *P21* promoter activity. HCT116 cells were transfected with indicated plasmids and *P21* promoter luciferase reporter construct. Total cell lysates were harvested 48 h after transfection. The relative luciferase activity was determined, and protein expression was detected by immunoblotting with antibodies as indicated. (**B**) Both wild-type CtIP and phosphorylation-deficient mutant CtIP(S327A) transactivate *P21* promoter activity. Experiments were performed in HCT116, HCT116*CtIP*–/–, and HCT116*P53*–/– cells as described in (**A**).

in the breast cancer cell line MCF7 (**Fig. S1**). To reveal if CtIP is required for P21 promoter activity, we generated HCT116*CtIP*–/– cells, in which both alleles of *CtIP* were deleted (data not shown). Immunoblotting analysis confirmed that HCT116*CtIP*–/– cells did not express CtIP protein (**Fig. S2**). *P21* promoter activity in HCT116 cells was similar to that in HCT116*CtIP*–/– cells, and ectopic expression of FLAG-CtIP in HCT116 or HCT116*CtIP*–/– cells increased *P21* promoter activity about 3-fold (**Fig. 1B**). These results suggest that CtIP is dispensable for the basal activity of P21 transcription, while its expression promotes P21 transcription.

Given that P21 transcription is largely P53-dependent, we sought to determine if CtIP-mediated induction of *P21* transcription requires P53. Immunoblotting analysis confirmed that HCT116*P53*–/– cells, in which both alleles of *P53* were deleted, did not express P53 (**Fig. S2**). The *P21* promoter basal activity in HCT116*P53*–/– was about one-third to one fourth of that in HCT116 (**Fig. 1B**), confirming that *P21* promoter activity is mainly P53-dependent. Ectopic expression of CtIP in HCT116*P53*–/– led to an increase of about 1.6-fold of *P21* promoter activity (**Fig. 1B**). These results suggest that CtIP-mediated induction of *P21* transcription is possibly occurring through both P53-dependent and -independent mechanisms.

Next, we determined which region of CtIP is required to activate the *P21* promoter. We generated 3 truncation mutants, namely CtIP(1–304), CtIP(300–604), and CtIP(600–897). Ectopic expression of CtIP(1–304), like wild-type CtIP, but not CtIP(300–604) or CtIP(600–897), efficiently activated *P21* promoter activity (**Fig. 2A**). There are several domains/motifs within the N terminus of CtIP. It was reported that the first coiled-coil domain (residues 20–45) mediates CtIP dimerization that is required for HR-mediated DSB repair.[24] Through sequence alignment, we identified another coiled-coil domain (residues 99–144) (data not shown) with unknown function. Phosphorylation on serine-327 was reported to promote binding to BRCA1 and HR-mediated DSB repair.[25] The Rb-binding motif (residues 153–157, LECEE) mediated binding of CtIP to Rb.[26] Thus, in the context of full-length CtIP, we generated deletion mutants CtIPΔCC1, in which the first coiled-coil domain was deleted; CtIPΔRb, in which the Rb-binding motif was deleted; and the point mutant CtIP(S327A), in which the phosphorylation site was mutated to alanine. As shown in **Figure 1**, phosphorylation on serine-327 in CtIP was not required for *P21* induction. Ectopic expression of CtIP(S327A), CtIPΔCC1, or CtIPΔRb resulted in about 3-fold increase of the *P21* promoter activity (**Fig. 2A and B**). Analysis of further deletion mutants identified that the N terminus (residues 1–152) was sufficient to induce the *P21* promoter activity (**Fig. 2C**). In the context of full-length CtIP, deletion of the second coiled-coil domain (residues 99–144) within the N-terminus abolished induction of *P21* transcription by CtIP (**Fig. 2C**). These results demonstrate that the coiled-coil domain (residues 99–144) is required for induction of *P21* transcription by CtIP.

P21 promoter activity is tightly regulated by a number of transcription factors. To identify the minimal region for CtIP-mediated activation of *P21* transcription, we constructed a series

of nested deletion mutants of the *P21* promoter and tested their luciferase activity driven by expression of HA-CtIP or HA-BRCA1 in HCT116*CtIP*–/– cells. We found that *P21* promoter activity decreased, while the induction of *P21* promoter activity by CtIP or BRCA1 did not change when the *P21* promoter region was shortened from position –2920 to position –143 (**Fig. 3A**). We further narrowed down the BRCA1-responsive region to the region between –117 and –93 on the *P21* promoter (**Fig. 3A**). This is consistent with the previous report that the BRCA1-responsive element locates between –143 and –93 of the *P21* promoter (**Fig. 3A**).[18] Meanwhile, the induction of the *P21* promoter activity by CtIP did not diminish until the promoter was truncated beyond position –117 (**Fig. 3A**). In the context of the full-length *P21* promoter (–2920 to +37), deletion of the putative CtIP/BRCA1-responsive region (–143 to –93) partially impaired CtIP-mediated and BRCA1-mediated activation of *P21* (**Fig. 3A**). These results suggest that the CtIP responsive region is located between –143 and –117 and is independent of the BRCA1-responsive region on the *P21* promoter.

We further examined if CtIP resides on the *P21* promoter. The ChIP assays showed that both endogenous CtIP and ectopically expressed CtIP were present on the region between –200 to +28 of the *P21* promoter and were enriched upon treatment with camptothecin for 2 h (**Figs. 3B and C**). Ectopically expressed HA-CtIPΔCC2, in which the second coiled-coil domain (residues 99–144) was deleted, failed to enrich on the *P21* promoter (**Fig. 3D**). Since CtIP is both a DDR factor and a transcription regulator, we wanted to further test if CtIP is involved in DNA damage-induced *P21* transcription induction. As shown in **Figure 4**, treatment of HCT116 cells, but not of HCT116*CtIP*–/– cells, with camptothecin for 2 h resulted in a small but significant increase of the *P21* promoter activity (**Fig. 4A**), mRNA levels determined by real-time RT-PCR (**Fig. 4B**) and protein levels (**Fig. 4C**) as well. Taken together, these results suggest that CtIP is required for *P21* induction upon DNA damage.

Discussion

In this study, we have uncovered that CtIP is a positive transcriptional inducer of *P21* in response to DNA damage. It is not required for the basal levels of *P21* transcription, because the *P21* promoter activity, mRNA levels, and protein levels in the CtIP-deficient cells are similar to those in the *CtIP*-proficient cells (**Figs. 1 and 4**).

Physical interaction with BRCA1 is not necessary for CtIP-mediated induction of *P21*, since the phosphorylation mutant CtIP(S327A), which cannot bind BRCA1, activated the *P21* promoter. The CtIP-responsive region (–143 to –117) within the *P21* promoter completely overlaps with the previously identified BRCA1-responsive region (–143 to –93). However, our data pinpointed the BRCA1-responsive region to the position –117 to –93. Nonetheless, co-expression of CtIP and BRCA1 did not have any additive or synergistic effect on the *P21* promoter activity in *CtIP*-deficient cells (data not shown), suggesting that CtIP-mediated *P21* induction is not independent of BRCA1,

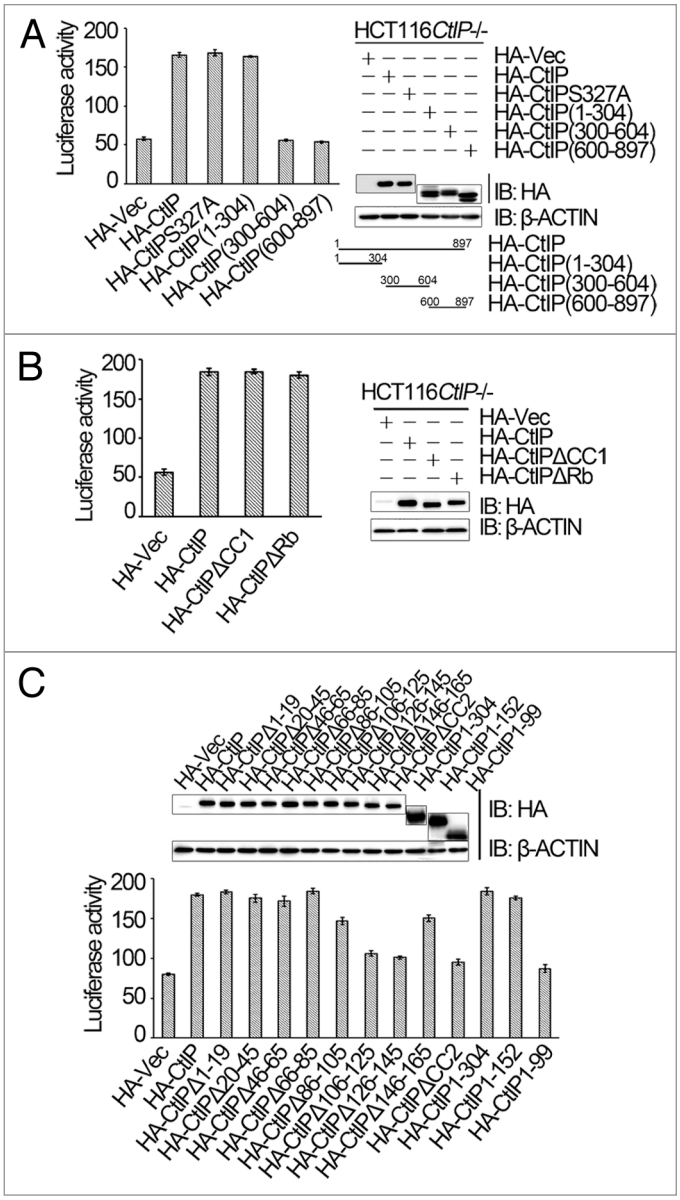

Figure 2. The second putative coiled-coil domain (residues 99–144) is required for P21 induction by CtIP. (**A**) The N terminus of CtIP was sufficient to transactivate *P21* promoter activity. Wild-type CtIP, phosphorylation-defective mutant CtIP(S327A), or truncation mutants (CtIP1–304, 300–604, and 600–897) were cotransfected with the *P21* promoter luciferase reporter construct into HCT116*CtIP*–/– cells. Relative luciferase activity and protein expression levels were determined 48 h after transfection. (**B**) Both Rb binding motif and the first coiled-coil domain are dispensable for CtIP-mediated *P21* transactivation. HA-CtIP, HA-CtIPΔCC1 in which the first putative coiled-coil domain was deleted, or HA-CtIPΔRb in which the Rb-binding motif was deleted was cotransfected with the *P21* promoter luciferase reporter construct into HCT116*CtIP*–/– cells. Relative luciferase activity and protein expression levels were determined 48 h after transfection. (**C**) Deletion of the second putative coiled-coil domain (residues 99–144) diminished P21 induction by CtIP. Experiments were performed as described in (**A**) except that different truncation/deletion CtIP mutants were used. Experiments were performed at least 3 times independently, and each combination was tested in triplicate or quadruplicate wells. One representative experiment is shown here.

though their responsive regions on *P21* promoter are adjacent yet distinct. It would be very interesting to test the hypothesis that binding of BRCA1 to the *P21* promoter may be a prerequisite for CtIP-mediated P21 induction.

Low or loss of expression of CtIP is associated with poor prognosis (disease free survival) and metastasis status in human breast cancer, and patients with progressive disease express significantly lower CtIP protein in their primary breast carcinomas than those who respond to tamoxifen treatment.[27] Furthermore, re-expression of CtIP in tamoxifen-resistant breast cancer cells sensitizes to the inhibitory effects of tamoxifen.[27] On the other hand, loss of expression of P21 contributes to tamoxifen-stimulated growth in tamoxifen-resistant breast cancer cells.[28] These would allow us to speculate that the CtIP-P21 transcriptional axis may be an important target of the tamoxifen therapy. Given that the P53–P21 transcriptional axis is an essential responder to radiation therapy and chemotherapeutic agents targeting DNA, while our data suggest that CtIP-mediated P21 induction is both P53-dependent and -independent, it is logical to believe that the CtIP-P21 transcriptional axis is a potential and promising target and outcome predictor as well for cancer therapy.

Materials and Methods

Cell lines, antibody, and plasmids

HeLa and MCF7 were purchased from ATCC. HCT116 wild-type and HCT116*P53*−/− cell lines were kindly provided by Dr Weiguo Zhu (Peking University). HCT116*CtIP*−/− cell line, in which both CtIP alleles were somatically inactivated, will be described elsewhere. All the cell lines were cultured in Dulbecco modified Eagle medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS, Hyclone) and standard antibiotics (Hyclone), at 37 °C in a 5% CO_2 incubator.

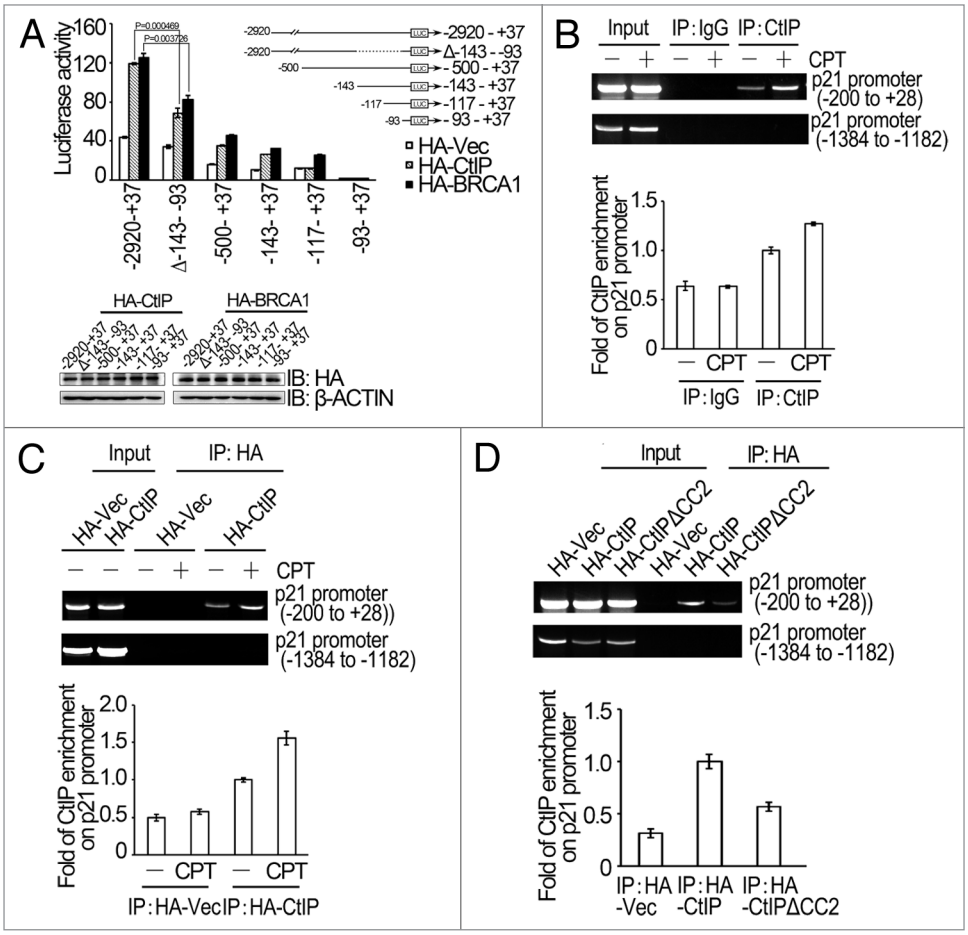

Figure 3. CtIP resides on the region between –143 and –117 of *P21* promoter. (**A**) Mapping the CtIP-responsive region on *P21* promoter. HA-vector, HA-CtIP, or HA-BRCA1 was cotransfected with nested deletion mutants of *P21* promoter into HCT116*CtIP*–/– cells. Relative luciferase activity and protein expression levels were determined 48 h after transfection. Experiments were performed at least 3 times independently, and each combination was tested in triplicate or quadruplicate wells. One representative experiment is shown here. (**B**) CtIP binds to *P21* promoter. The ChIP assay was performed using U2OS cells without or with camptothecin (CPT) treatment for 2 h. The chromatin-associated DNA was incubated with control antibody (rabbit normal IgG) or rabbit polyclonal anti-CtIP. An aliquot of the total chromatin DNA was used for input. Immunoprecipitates were subjected to PCR with a primer pair specific to *P21* promoter containing the putative CtIP-responsive region (–200 to +28) and another primer pair upstream of this region (–1384 to –1182). (**C and D**) Experiments were performed as described in (**B**), except that cells were co-transfected with wild type HA-CtIP (**C**) or HA-CtIPΔCC2 mutant (**D**) in which the region responsive for binding to the promoter was deleted with the *P21* promoter construct and the chromatin-associated DNA was incubated with rabbit polyclonal anti-HA.

Antibodies against CtIP (A300–488A) and HA (A190–208A) were purchased from Bethyl Laboratories Inc. Antibodies against Flag and β-actin were purchased from Sigma. Antibodies against BRCA1 and P21 were purchased from Cell Signaling Technologies.

P21 promoter luciferase reporter construct pGL3-P21 (–2920–+37) was kindly provided by Dr Wei-Guo Zhu (Peking University), *P21* promoter fragments were amplified by PCR and subcloned into KpnI and XhoI sites of pGL3-Basic. Human CtIP cDNA was reverse transcribed and PCR amplified from HeLa RNA and cloned into pCDNA 3.0. CtIP(S327A), and deletion mutants were generated using quick-change mutagenesis kit (Strategene) according to the manufacturer's instruction.

Transfection and luciferase reporter assay

Cells were transfected with plasmids using FuGene (Promega) or PEI (Polyscience). siRNA oligos were purchased from Dharmacon. For the luciferase reporter assay, a luciferase reporter construct, an expression construct of HA-CtIP or its mutants, or BRCA1, and a Renilla luciferase reporter construct under the control of the *TK* promoter for normalization of transfection efficiency were cotransfected, in triplicate or quadruplicate into cells. Luciferase activity was determined 48 h posttransfection with the Dual-Luciferase® Reporter Assay System (Promega). Relative light units were determined using a luminometer Centro LB960 (Berthold). Experiments were performed at least 3 times independently, and each combination was tested in triplicate or quadruplicate wells.

Chromatin immunoprecipitation (ChIP)

ChIP assay was performed as described before.[29] Primers used to amplify *P21* promoter were P21.S: CTCCTGCAGC ACGCGAGGTT C (from –200 to –180) and P21.A: GACTTCGGCA GCTGCTCACA CC (from +7 to +28). Primers used to amplify the non-responsive region were TCTACCTCAC ACCCCTGACC (from –1182 to –1161) and CTGGGCAGAG ATTTCCAGAC (from –1384 to –1363).

Real-time RT-PCR

Total RNA was extracted from HCT116 wide-type and HCT1116*CtIP*–/– cells using TriZol reagent (Invitrogen). Reverse transcription PCR was performed using iScript™ Reverse Transcription Supermix (Bio-Rad) according to the manufacturer's instruction. Real-time PCR was performed using iQ Supermix and iQ5. PCR program was 95 °C for 15 min for initial denaturation; 95 °C 20 s/60 °C 20 s/72 °C 20s repeated for 45 cycles. Primers used were GAPDH.S: TGGTATCGTG GAAGGACTCA, GAPDH.A: CCAGATGAGG CAGGGATGAT; P21.S: ACCATGTGGA CCTGTCACTG T; P21.A: TTAGGGCTTC CTCTTGGAGA A.

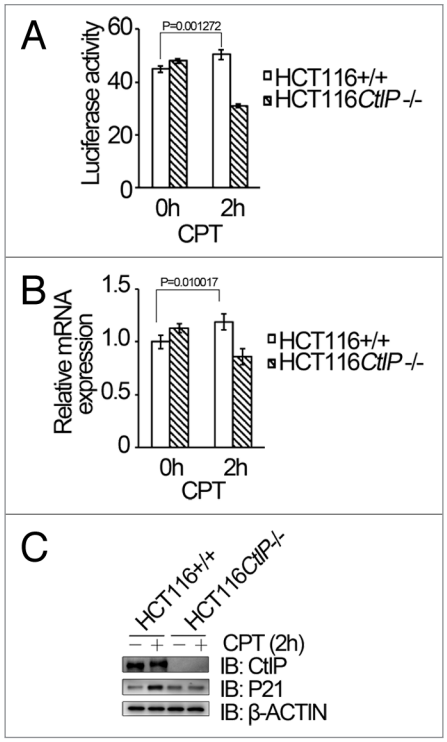

Figure 4. CtIP is required for DNA damage-induced P21 induction. (**A**) CtIP deficiency failed to induce *P21* promoter activity. HCT116 cells and HCT116*CtIP*–/– cells were transfected with the *P21* promoter reporter construct. Relative luciferase activity was determined 48 h after transfection, without or with CPT treatment for 2 h. Experiments were performed at least 3 times independently, and each combination was tested in triplicate or quadruplicate wells. One representative experiment is shown here. (**B**) *CtIP* deficiency failed to induce *P21* mRNA levels. HCT116 cells and HCT116*CtIP*–/– cells were treated with CPT for 2 h. Total RNA was extracted and used for real-time RT-PCR analysis. (**C**) *CtIP* deficiency failed to induce P21 protein levels. HCT116 cells and HCT116*CtIP*–/– cells were treated with CPT for 2 h. Total cell lysate was extracted and used for immunoblotting with antibodies indicated.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Supplemental Materials

Supplemental materials may be found here:
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