

# Chromodomain Helicase DNA-binding Protein 4 (CHD4) Regulates Homologous Recombination DNA Repair, and Its Deficiency Sensitizes Cells to Poly(ADP-ribose) Polymerase (PARP) Inhibitor Treatment<sup>\*S</sup>

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**Background:** Identification of DNA repair regulators is important for gaining new insights into cancer development and treatment.

**Results:** CHD4 interacts with BRIT1 and regulates its loading onto chromatin, which requires CHD4 chromatin remodeling activity.

**Conclusion:** CHD4 functions as a proximal HR regulator, and its deficiency sensitizes cells to PARP inhibitor treatment.

**Significance:** Our discoveries provide a novel approach, by inducing synthetic lethality, to target on CHD4-deficient tumors with PARP inhibitors.

To ensure genome stability, cells have evolved a robust defense mechanism to detect, signal, and repair damaged DNA that is generated by exogenous stressors such as ionizing radiation, endogenous stressors such as free radicals, or normal physiological processes such as DNA replication. Homologous recombination (HR) repair is a critical pathway of repairing DNA double strand breaks, and it plays an essential role in maintaining genomic integrity. Previous studies have shown that BRIT1, also known as MCPH1, is a key regulator of HR repair. Here, we report that chromodomain helicase DNA-binding protein 4 (CHD4) is a novel BRIT1 binding partner that regulates the HR repair process. The BRCA1 C-terminal domains of BRIT1 are required for its interaction with CHD4. Depletion of CHD4 and overexpression of the ATPase-dead form of CHD4 impairs the recruitment of BRIT1 to the DNA damage lesions. As a functional consequence, CHD4 deficiency sensitizes cells to double strand break-inducing agents, reduces the recruitment of HR repair factor BRCA1, and impairs HR repair efficiency. We further demonstrate that CHD4-depleted cells are more sensitive to poly(ADP-ribose) polymerase inhibitor treatment. In response to DNA damage induced by poly(ADP-ribose) polymerase inhibitors, CHD4 deficiency impairs the recruitment of DNA repair proteins BRIT1, BRCA1, and replication protein A at early steps of HR repair. Taken together, our find-

ings identify an important role of CHD4 in controlling HR repair to maintain genome stability and establish the potential therapeutic implications of targeting CHD4 deficiency in tumors.

Failure to appropriately repair damaged DNA contributes to tumorigenesis. To ensure maintenance of genome integrity, cells have developed a complex DNA repair system to sense DNA damage, amplify signaling, and initiate DNA repair (1–3). Homologous recombination (HR)<sup>3</sup> and nonhomologous end joining are two major repair pathways for double strand breaks (DSBs), and the choice of specific repair depends on cell cycle stage and the type of DNA damage. Nonhomologous end joining occurs in the G<sub>0</sub>/G<sub>1</sub> phase and is within the context of a single DNA molecule, whereas HR occurs exclusively in the S and G<sub>2</sub> phases to facilitate an intact sister chromatid as a template for DNA repair. In contrast to error-prone repair mediated by nonhomologous end joining, by using the genetic information in homologous sequences, HR is considered as an error-free repair pathway and represents an essential mechanism to maintain high fidelity transmission of genetic information. Dysfunctional HR repair proteins such as mutated BRCA1 and BRCA2 have been well established in promoting tumor initiation and progression (4–8). Most recently, several lines of clinical studies (9–11) have shown a new class of drugs, poly(ADP-ribose) polymerase (PARP) inhibitors, to serve as a powerful targeting therapy for tumors with HR deficiency, particularly in breast and ovarian cancers with BRCA1/BRCA2 mutations. PARP is a key enzyme for the base excision repair

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<sup>3</sup> The abbreviations used are: HR, homologous recombination; DSB, double strand break; PARP, poly(ADP-ribose) polymerase; CHD4, chromodomain helicase DNA-binding protein 4, NuRD, nucleosome remodeling and deacetylase; PHD, plant homeodomain; RPA, replication protein A.

that utilizes DNA single strand breaks as a template. Inhibition of PARP enzymatic activity causes DNA damage lesions that are repaired by HR in normal cells (12, 13). However, HR-deficient cancer cells cannot cope with DNA damage induced by PARP inhibitors, which leads to a synthetic lethality effect to specifically kill HR-deficient cancer cells. Therefore, identifying HR regulators is important for us not only to understand the complex mechanisms of the HR repair process but also to gain new insights into cancer development and treatment.

Among various regulators in controlling HR, BRIT1 has recently been shown to play a key role in HR repair. Our previous studies indicated that BRIT1 interacts with the evolutionarily conserved ATP-dependent chromatin remodeling factors SWI/SNF and functions in controlling chromatin relaxation to facilitate the recruitment of DNA repair proteins to DNA damage sites (14). In the absence of BRIT1, BRCA1 protein levels and the recruitment of HR repair proteins Rad51/BRCA2 were impaired. Consistent with *in vitro* data, *BRIT1* knock-out mice also exhibit HR repair defects (15–17). In line with the crucial role of HR in maintaining genomic stability and preventing tumorigenesis, aberrations of BRIT1 have been found in a variety of human cancers, suggesting a tumor suppressor role of BRIT1 (18). However, the mechanism mediating BRIT1 recruitment to DNA lesions remains largely unknown. To fully elucidate the mechanisms by which BRIT1 is regulated in response to DNA damage and to identify novel proteins potentially involved in HR repair, we conducted a proteomic analysis to systematically identify proteins that interact with BRIT1.

To our surprise, we identified chromodomain helicase DNA-binding protein 4 (CHD4, also known as Mi2 $\beta$ ) as a previously unknown binding partner of BRIT1. CHD4 is a major subunit of repressive nucleosome remodeling and deacetylase (NuRD) complex that contains a helicase/ATPase domain that facilitates the deacetylation of histone in controlling chromatin reorganization and transcriptional regulation (19, 20). Recently, several groups reported a role of CHD4 in signaling DNA damage response and regulating cell cycle checkpoint activation (21–24). Here, our study shows a previously unknown function of CHD4 in regulating HR repair protein BRIT1. CHD4 interacts with BRIT1 and is required for the recruitment of repair proteins BRIT1, RPA, and BRCA1 at early stages of HR repair. Consistent with its regulatory role in HR repair, CHD4-deficient cells have increased sensitivity to PARP inhibitor treatment.

## EXPERIMENTAL PROCEDURES

**Cells and Antibodies**—MCF10A cells were grown in DMEM/F-12 medium supplemented with 5% horse serum, 10  $\mu$ g/ml insulin, 20 ng/ml EGF, 0.5  $\mu$ g/ml hydrocortisone, and 100 ng/ml cholera toxin. U2OS cells were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. 293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Anti- $\gamma$ -H2AX and anti-histone H3 antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY); anti-FLAG antibody and anti-FLAG agarose beads were purchased from Sigma; anti-p-Chk2, anti-Chk2, and anti-HA antibodies were purchased

from Cell Signaling Technology (Beverly, MA); and anti-CHD4 antibody was purchased from Bethyl Laboratories (Montgomery, TX). Anti-RPA2, anti-p-RPA2pS4/S8, anti-BRIT1, and anti-BRCA1 antibodies were described previously (14, 25).

**Plasmids, siRNAs and Transfection**—GFP-CHD4 was provided by Dr. Claudia Lukas (Institute of Cancer Biology and Centre for Genotoxic Research, Denmark). The full-length construct and deletion constructs of FLAG-BRIT1 were described previously (14). The N-terminal BRIT1 plasmid was kindly provided by Dr. Junjie Chen (26). The C-terminal BRIT1 was generated by subcloning with PCR products (1924–2469 bp) containing HindIII and EcoRI sites. An ATPase-dead form of CHD4 was generated by a QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the oligonucleotides (forward) 5'-GATGGGCTTGGGGCAACTGTACAGACAGC-3' and (reverse) 5'-GCTGTCTGTACAGTTGCCCAAGGCCATC-3'. Plasmids were verified by DNA sequencing. The siRNA duplexes were 19 base pairs long with a 2-base deoxynucleotide overhang. ON-TARGET SMARTpool siRNAs against CHD4, BRIT1, Rad51, and BRCA1 were purchased from Dharmacon Research, Inc. (Lafayette, CO). The sequences of CHD4 siRNA2 and siRNA4 oligonucleotides were GAGCGGCAGUUC UUUGUGA and GGUGUUAUGUC-UUUGAUUC, respectively. Control siRNAs were also purchased from Dharmacon. U2OS cells were transfected with siRNA duplexes by using Oligofectamine (Invitrogen), following the manufacturer's instructions. Plasmid transfections were performed by using FuGENE 6 (Roche Applied Science). MCF10A cells were transfected with siRNA duplexes by using Lipofectamine 2000 (Invitrogen).

**Immunoblotting, Immunoprecipitation, and Immunofluorescence Analyses**—For immunoblotting, cells were sonicated in urea buffer (8 M urea, 150 mM  $\beta$ -mercaptoethanol, and 50 mM Tris/HCl (pH 7.5)), and cellular debris was removed by centrifugation. Protein concentration was determined by using the Bio-Rad protein determination reagent. Proteins were loaded on an SDS-polyacrylamide gel and transferred to nitrocellulose, and immunoblotting was performed by using the appropriate antibodies. For phosphatase and DNase assay, 293T cells were lysed by modified RIPA buffer (50 mM Tris/HCl (pH 7.4), 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, 1 mM PMSF, protease inhibitors). Then cell lysis was treated with  $\lambda$ -phosphatase (Upstate Biotechnology and Sigma) with 20 mM MnCl<sub>2</sub> at 37 °C for 5 min based on commercial instruction or DNase (Ambion) at 37 °C for 15 min as recommended by the manufacturers. Cell lysis was then subjected to 3 $\times$ FLAG beads, and the immunocomplex was eluted with 3 $\times$ FLAG peptide (Sigma). Immunofluorescence staining was performed as described previously (14).

**RT-PCR**—Total RNA was extracted using RNeasy mini kit (Qiagen, Valencia, CA). RT-PCR was performed using the following primer pairs: BRIT1 (forward) 5'-GTCATGACAAGC-ATGCCATC-3' and (reverse) 5'-AAGACCCATTTCTCAGACAG; and CHD4 (forward) 5'-AAATCTAGATCACTGCTGCTGGGCTACCTG-3' and (reverse) 5'-CCTTTGGCAGAA-TGAAGAGC-3'. The PCRs were performed in several different cycle numbers to ensure linear amplification in all cases.

## CHD4 Regulates HR Repair

**Chromatin Isolation**—Cells were harvested and extracted as described previously (25). In brief, cytoplasmic proteins were removed by low speed centrifugation (4 min at  $1300 \times g$  at  $4^\circ\text{C}$ ) after cells were resuspended in solution A (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM  $\text{MgCl}_2$ , 0.34 M sucrose, 10% glycerol, 1 mM dithiothreitol, 0.1% Triton X-100, 10 mM NaF, 1 mM  $\text{Na}_2\text{VO}_3$ , and protease inhibitor mixture). Chromatin was extracted from nuclei by centrifugation (4 min at  $1700 \times g$  at  $4^\circ\text{C}$ ) in solution B (3 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol, and protease inhibitor mixture). Finally, chromatin was resuspended in Laemmli sample buffer and analyzed by using immunoblotting.

To detect the mobility of BRIT1 in CHD4-depleted cells, cell lysis was isolated in different fractions. Cells were lysed in Norel lysis buffer (20 mM HEPES (pH 7.0), 10 mM KCl, 2 mM  $\text{MgCl}_2$ , 0.5% Nonidet P-40, 1 mM  $\text{Na}_3\text{VO}_4$ , and protease inhibitor mixture) and were homogenized by using Dounce homogenizer. Dounced cells were then centrifuged, and supernatant was retained as a non-nuclear fraction. We then extracted nuclear proteins in NETN buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris (pH 8.0), 0.5% Nonidet P-40, 2 mM  $\text{Na}_3\text{VO}_4$ , and protease inhibitor mixture) by freeze-and-thaw method. After centrifugation, supernatant contained the nuclear fraction, and the pellet was chromatin-enriched fraction. Chromatin-enriched fraction was extracted from the pellet in SDS sample buffer by sonication.

**HR Repair Analysis**—A clone was isolated from U2OS cells that stably express a single copy of the HR repair reporter substrate DR-GFP as described previously (14). In this assay system, the DR-GFP reporter substrate includes the SceGFP region that contains an I-SceI endonuclease site within the coding region and the iGFP region that contains homologous sequences for the SceGFP. Expression of I-SceI induces a single DSB in the genome. When this DSB is repaired by HR, the expression of GFP can be restored to indicate the efficiency of HR repair. U2OS cells transfected with siRNA were re-seeded, and the next day they were transfected with mock or pCBASce plasmid. Cells were analyzed 48 h later to detect GFP-positive cells using a Flow cytometer with CellQuest software (BD Biosciences) at the M. D. Anderson Cancer Center Flow Cytometry Facility.

**Colony Formation Assay**—MCF 10A cells transfected with siRNA were seeded at 500 cells per 6-cm dish and irradiated with various doses of AZD 2281. Cells were incubated for 14 days at  $37^\circ\text{C}$  to allow colonies to form. Colonies were stained with 2% crystal violet and counted. Colonies were defined as groups of 50 or more cells.

**Statistical Analysis**—All statistical analyses were done using one-tailed Student's *t*-tests.

## RESULTS

**CHD4 Affects the Recruitment of BRIT1 to DNA Damage Sites**—BRIT1 is an early DNA damage response protein that quickly forms ionizing radiation-induced foci after DNA is damaged (25, 27). Our previous study has shown that BRIT1 mediates the recruitment of chromatin remodeling complex SWI-SNF to DNA damage sites in coordinating DNA repair (14). However, it is still unclear exactly how BRIT1 is regulated at the sites of

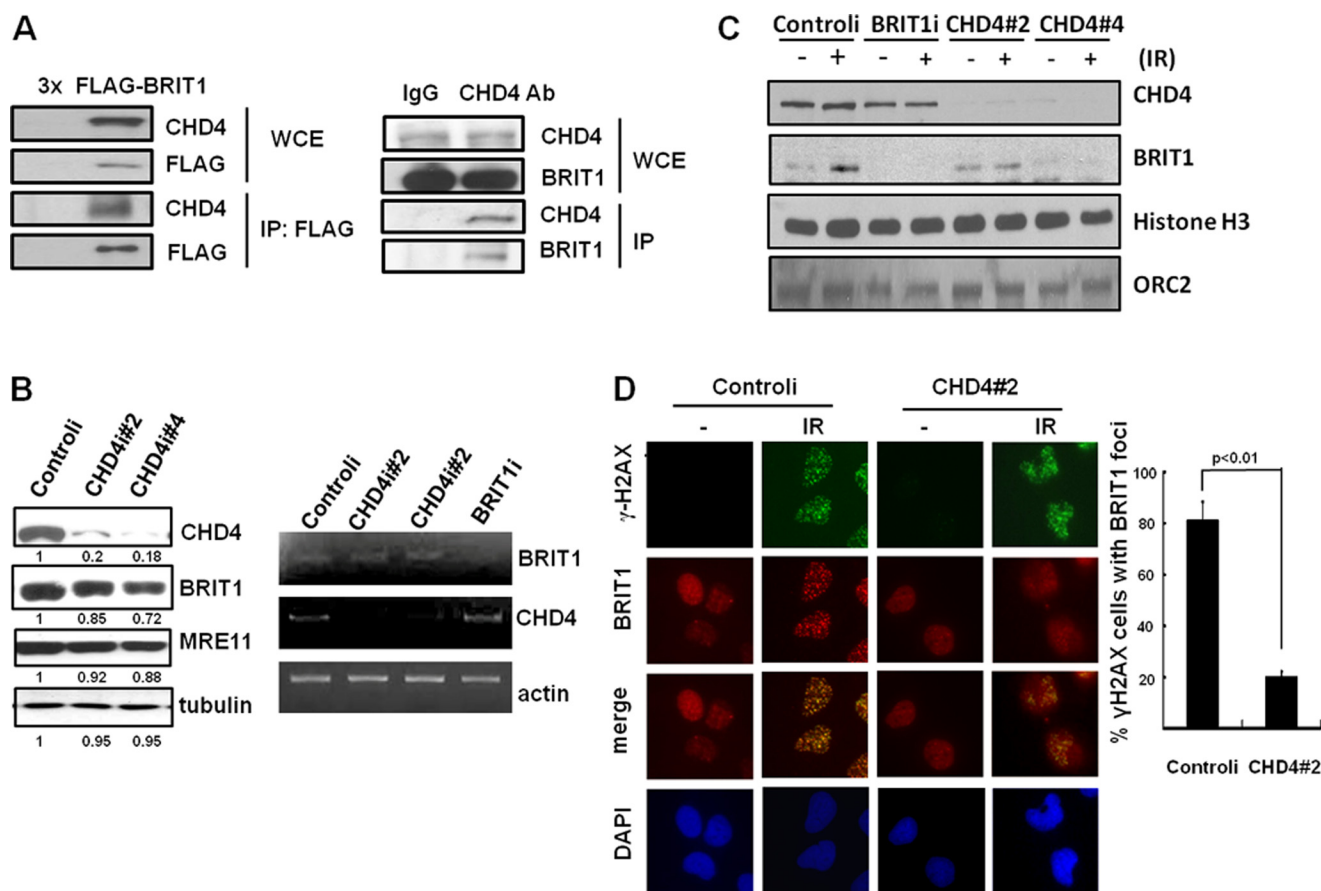
DNA damage. To address the question, we sought to identify the binding partners of BRIT1. In our previous proteomic analysis, we had found CHD4 to be one of the novel BRIT1-associated proteins.<sup>4</sup> To confirm this interaction, we performed immunoprecipitation assays. As shown in Fig. 1A (*left panel*), 293T cells were transfected with FLAG-BRIT1, and CHD4 was detected in the FLAG-BRIT1 protein complex but not in cells without the expression of FLAG-BRIT1. In parallel, we confirmed the endogenous interaction between BRIT1 and CHD4. As shown in Fig. 1A (*right panel*), BRIT1 was detected in CHD4 immunoprecipitates but not in control IgG immunoprecipitates. By using DNase and phosphatase treatment, we showed that CHD4-BRIT1 interaction is independent of the presence of the DNA component or phosphorylation event (*supplemental Fig. S1*). Consistent with our previous proteomic analysis, these data indicated that CHD4 specifically interacts with BRIT1. Our previous study indicated a link between chromatin remodeling complex SWI-SNF and BRIT1 to DNA damage response. These results raise an interesting question of the functional significance of the interaction between BRIT1 and CHD4, another chromatin remodeling protein.

To answer this question, we examined the effects of CHD4 depletion on the function of BRIT1. CHD4 is a major subunit of the repressive NuRD complex. It contains a helicase/ATPase domain that facilitates the deacetylation of histone in the process of chromatin reorganization and transcriptional regulation (19, 20). Therefore, we first examined whether CHD4 regulates the expression level of BRIT1. We found that both the protein level and the RNA level remained unchanged in the CHD4-depleted cells (Fig. 1B). Next, we purified the chromatin-enriched fraction to test whether CHD4 is required for the loading of BRIT1 onto chromatin. As shown in Fig. 1C, depletion of CHD4 by CHD4 siRNA increased the solubility of BRIT1 as detected by cellular fractionation assay, particularly when cells were treated with radiation. Quantitative analysis of the insoluble chromatin-enriched fraction of BRIT1 in CHD4-depleted cells is shown in *supplemental Fig. S2*. In addition, we observed an increased mobility of BRIT1 to non-nuclear fraction in CHD4-deficient cells (*supplemental Fig. S3*). By using ionizing radiation-induced foci staining, we further tested whether CHD4 affects the recruitment of BRIT1 to sites of DNA damage after exposure to radiation. We found that depletion of CHD4 significantly inhibited the formation of BRIT1 foci at the DNA damage sites.  $\gamma$ -H2AX was used as a DSB marker (Fig. 1D) (28). In summary, these data indicated that CHD4 plays a critical role in regulating the recruitment of BRIT1 to DNA damage sites.

**CHD4 Regulates BRIT1 Function in an ATPase-dependent Manner**—According to the results of protein structural analysis, BRIT1 contains an N-terminal BRCT domain (the C-terminal domain of BRCA1) and two C-terminal BRCT domains. The BRCT domain has been well established as a phospho-protein-binding domain, which mediates protein-protein interactions in the phosphorylation signaling cascade initiated by DNA damage response kinases ataxia telangiectasia mutated (ATM), and ATM and Rad3-related protein, and DNA-PK (29,

<sup>4</sup> S.-Y. Lin, unpublished data.





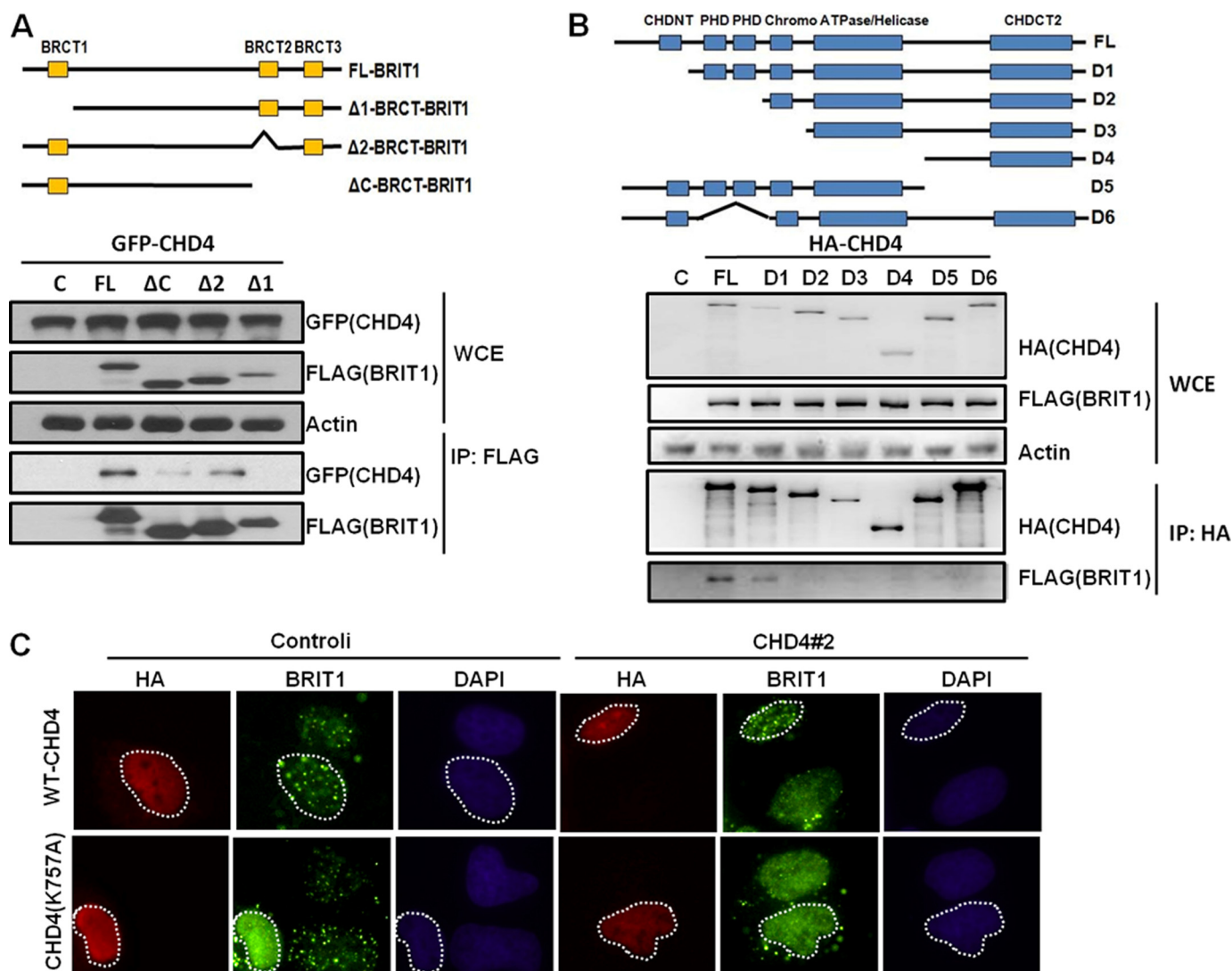
**FIGURE 1. CHD4 is required for BRIT1 recruitment to the DNA damage site by DSB-inducing agents.** *A*, CHD4 as a novel BRIT1-associated binding partner. *Left panel*, co-immunoprecipitation (IP) of BRIT1 and CHD4 from 293T cells transfected with FLAG-BRIT1 was analyzed by Western blotting using the indicated antibodies. *Right panel*, endogenous immunoprecipitation was performed in U2OS cells with anti-CHD4 antibody. *WCE*, whole cell extract. *B*, depletion of CHD4 does not affect expression levels of BRIT1. Cells were transfected with control or CHD4 siRNA for 72 h. Levels of protein (*left panel*) and RNA (*right panel*) were separately analyzed by immunoblotting and RT-PCR assay. Data from densitometry analysis was shown at the *bottom* of the images. *C*, CHD4 is required for the loading of BRIT1 onto chromatin. U2OS cells were transfected with control or CHD4 siRNA. Seventy two hours later, cells were exposed to ionizing radiation (IR) (4 gray) and harvested 2 h later, and then chromatin-enriched fractions were subjected to immunoblotting assay with the indicated antibodies. *D*, U2OS cells were transfected with control siRNA or CHD4 siRNA. Seventy two hours later, cells were exposed to ionizing radiation (4 gray). 2 h later, cells were analyzed by immunofluorescence assay with the indicated antibodies. The quantitative results from multiple experiments were shown next to the images (Student's *t* test  $p < 0.01$ ).

30). The C-terminal BRCT domain is required for the recruitment of BRIT1 to DNA damage sites (27). As shown in Fig. 1C, the recruitment of BRIT1 to DNA lesions is impaired in cells with CHD4 knockdown. To further illustrate the interaction between CHD4 and BRIT1, we examined whether the BRCT domain of BRIT1 is a critical region that mediates interaction of BRIT1 with CHD4. Consistent with our previous hypothesis, co-immunoprecipitation assays indicated that the C-terminal BRCT domains were required for these interactions (Fig. 2A). In addition, we found that the N-terminal BRCT domain was also involved in the interaction. To confirm this finding, we further performed co-immunoprecipitation analysis with BRIT1 deletions containing N- or C-terminal BRCT domains. As shown in supplemental Fig. S4, the N- and C-terminal BRCT domains of BRIT1 are sufficient for binding with CHD4. These data suggest that CHD4 regulates the function of BRIT1 in DNA damage response through its interaction with BRCT domains of BRIT1. Moreover, to further characterize the interaction between BRIT1 and CHD4, we sought to identify the critical regions of CHD4 to mediate the interaction. CHD4 contains two plant homeodomains (PHDs), two chromodomains,

and an ATPase/helicase domain. We then generated a series of deletion mutants on CHD4 and found that PHDs of CHD4 were required for its interaction with BRIT1 (Fig. 2B). The ATPase/helicase domain is necessary for the function of CHD4 in altering the composition of histone during nucleosome remodeling via ATP hydrolysis (31). We next examined whether the ATPase activity of CHD4 is required for ionizing radiation-induced foci formation of BRIT1. As shown in Fig. 2C, expression of an ATPase-dead form of CHD4 (GFP-CHD4 K757R) reduced BRIT1 foci formation. This result suggests that ATP-dependent chromatin remodeling driven by the CHD4 ATPase is required for the recruitment of BRIT1 to DNA damage sites. These data collectively showed that BRCT domains of BRIT1 specifically mediate its interaction with CHD4, and BRIT1 foci formation in the DNA damage response is dependent on the chromatin remodeling activity of CHD4, mediated by its function as an ATPase.

**Loss of CHD4 Impairs HR Repair**—The above findings revealed that CHD4 acts as an upstream regulator of BRIT1. It is well known that BRIT1 plays a critical role in repairing DSBs via HR pathways (14). We thus considered that it is very likely

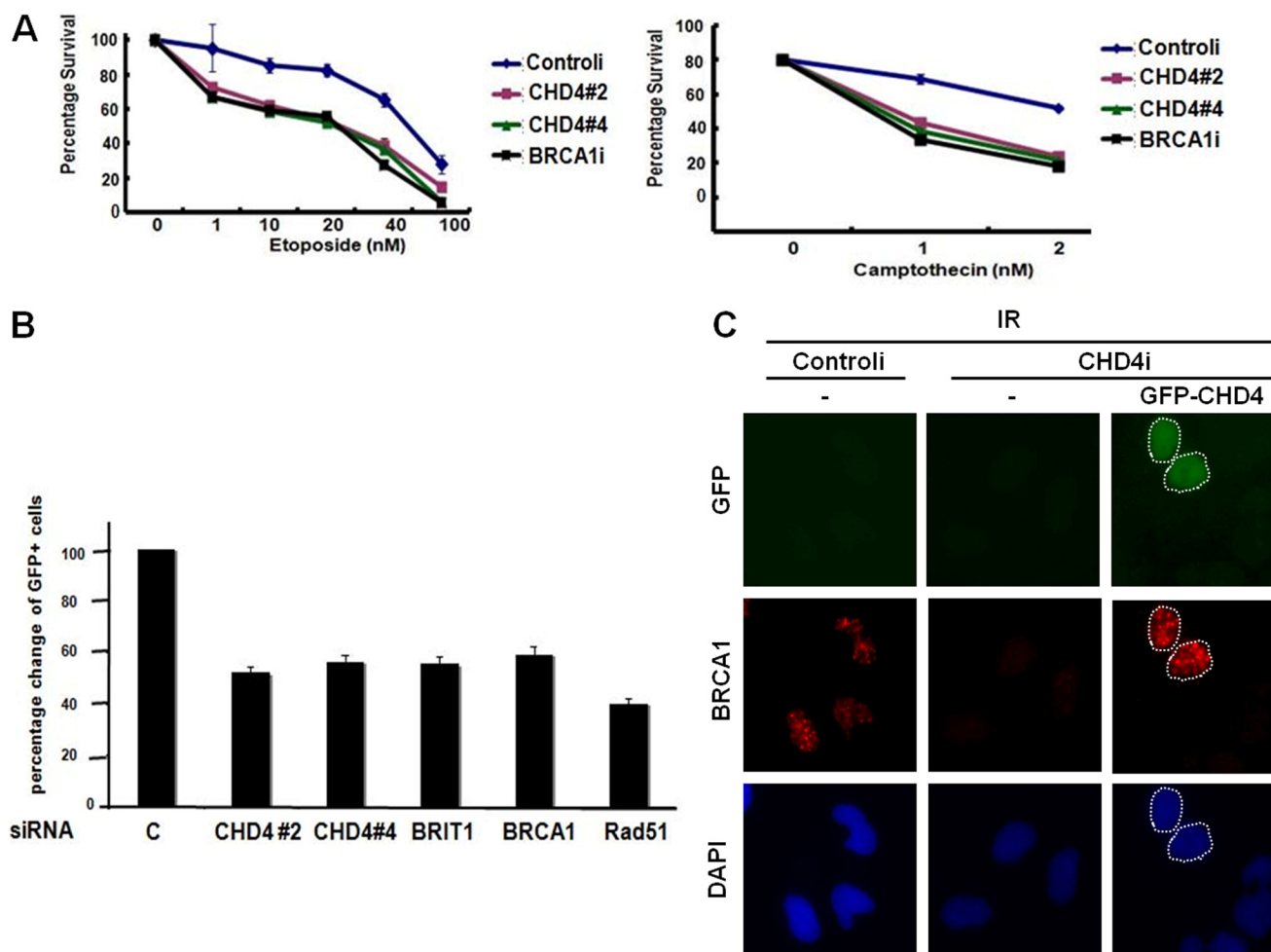
## CHD4 Regulates HR Repair



**FIGURE 2. BRCT domain of BRIT1 specifically mediates its interaction with CHD4.** *A*, BRCT domain is required for interaction between BRIT1 and CHD4. *Upper panel*, diagram of different FLAG-BRIT1 deletion mutants. *Lower panel*, co-immunoprecipitation (IP) of BRIT1 and CHD4 from 293T cells transfected with indicated plasmids was analyzed by Western blotting using the indicated antibodies. *WCE*, whole cell extract. *B*, PHDs of CHD4 specifically mediate its interaction with BRIT1. *Upper panel*, diagram of different HA-CHD4 deletion mutants. *Lower panel*, co-immunoprecipitation of BRIT1 and CHD4 from 293T cells transfected with indicated plasmids was analyzed by Western blotting using the indicated antibodies. *C*, CHD4 ATPase activity is required for BRIT1 ionizing radiation (IR)-induced foci formation. Cells were transfected with siRNA-resistant wild-type CHD4 or the dominant negative form of CHD4 (K757A) in the depletion of CHD4 from cells, and then the cells were exposed to 4 gray of ionizing radiation. After 2 h, BRIT1 foci were analyzed by immunofluorescence assay with the indicated antibodies.

that CHD4 also functions as a regulator of HR repair. To test this possibility, we first examined whether its depletion affects the clonogenic survival of MCF 10A cells with DSB-inducing agents. The topoisomerase inhibitors camptothecin and etoposide induce DSBs associated with replication in the S phase, which are mainly repaired by the HR repair pathway. Therefore, we tested whether CHD4 depletion affects cellular sensitivity to camptothecin and etoposide. As shown in Fig. 3A, cells with CHD4 knockdown showed modestly increased sensitivity to camptothecin and etoposide treatment (Fig. 3A), suggesting that CHD4 impaired cell survival when DSBs were generated in the S phase and needed to be repaired by the HR pathway. To further confirm the function of CHD4 in HR repair, we utilized a classic I-SceI-mediated HR reporter system to assess the efficiency of HR repair in CHD4-depleted cells. In this assay system, the DR-GFP reporter substrate includes an SceGFP region

that contains an I-SceI endonuclease site within the coding region and an iGFP region that contains homologous sequences for the SceGFP. Expression of I-SceI induces a single DSB in the genome. When the DSB is repaired by HR, expression of GFP can be restored to indicate the efficiency of HR repair (14, 32). We found that the depletion of CHD4 resulted in a significant decrease in GFP-positive cells, which were induced by successful HR repair events in cells. As shown in Fig. 3B, depletion of CHD4 and of key HR repair-associated enzymes BRIT1, BRCA1, and Rad51 decreased HR frequencies to a similar extent. To further confirm this result, we tested the effect of CHD4 knockdown on the recruitment of BRCA1 to DNA damage sites by overexpression of siRNA-resistant CHD4 in CHD4-deficient cells. As shown in Fig. 3C, BRCA1 foci formation could be efficiently restored in the rescue experiments. These data strongly suggest that CHD4 may play an important role in



**FIGURE 3. CHD4 depletion impairs HR repair.** *A*, CHD4 depletion increases sensitivity of cells to topoisomerase inhibitor. MCF 10A cells were transfected with control or CHD4 siRNA or BRCA1 siRNA and exposed to etoposide and camptothecin at the indicated doses. Fourteen days later, cells were stained with crystal violet. Colonies containing more than 50 cells were counted. Each value represents the mean  $\pm$  S.D. of three independent experiments. *B*, CHD4 is required for efficient HR repair. U2OS-DR-GFP cells were transfected with the indicated siRNAs and then transfected with pCBASce plasmids. After 48 h, cells were analyzed for GFP expression by FACS. *Column C*, control siRNA. *C*, CHD4 regulates the function of BRCA1 in response to ionizing radiation (IR). U2OS cells expressing GFP or GFP-CHD4 resistant to siRNA were transfected with control or CHD4 siRNA as indicated. The ionizing radiation-induced foci were analyzed using antibodies against BRCA1 and GFP antibodies after irradiation (4 gray, 2 h).

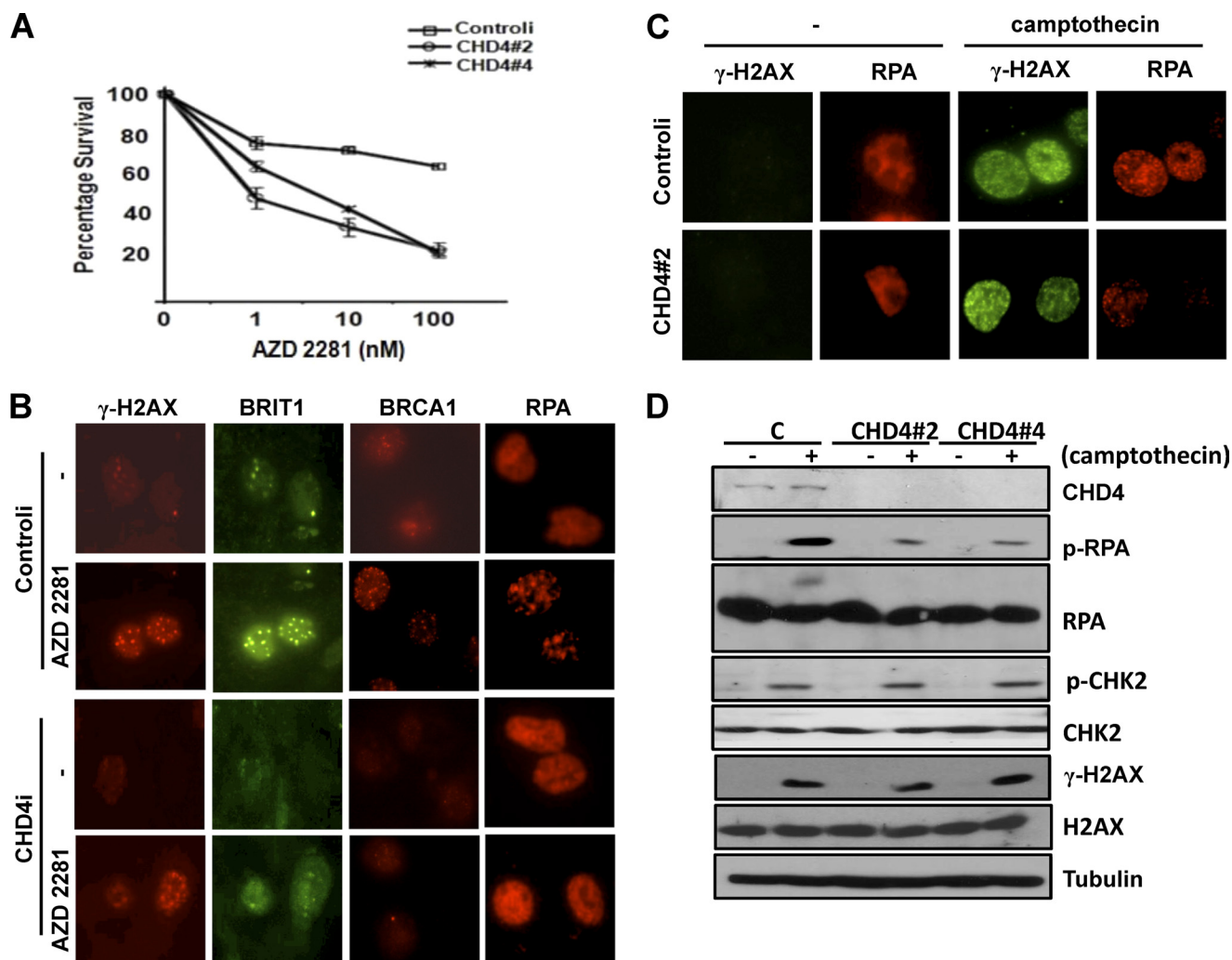
HR repair via regulation of the function of BRCA1 in response to PARP inhibitors.

**CHD4 Depletion Causes Hypersensitivity to PARP Inhibitor Treatment**—Given the role of CHD4 in HR repair, we next tested whether HR deficiency caused by depletion of CHD4 leads to a synthetic lethality interaction with PARP inhibitors. We first examined PARP inhibitor sensitivity in CHD4-depleted cells. Clonogenic cell survival assays showed that CHD4-deficient cells were significantly more sensitive to PARP inhibitors than controls (Fig. 4A).

To gain more insight into the mechanisms by which the dysfunction of CHD4 regulates HR repair of PARP inhibitor-induced DSBs, we first assessed whether depletion of CHD4 impaired the recruitment of BRIT1 to DNA damage sites. As shown in Fig. 4B, treatment with PARP inhibitors induced BRIT1 foci formation in control cells. However, CHD4 knockdown cells showed significantly reduced BRIT1 foci formation. Moreover, CHD4 depletion decreased BRCA1 foci formation induced by PARP inhibitors (Fig. 4B). Recent studies have shown that BRCA1, a well known HR repair protein, plays a

critical role in the detection and resection of DSBs to initiate HR repair (8). The resection of DSBs allows the binding of RPA to the single strand DNA that is generated at the broken ends. Then RPA facilitates the recruitment of BRCA2/Rad51 to initiate recombination and complete the HR repair process. As shown in Fig. 4B, in control cells, PARP inhibitors led to the formation of RPA foci. Compared with the cells treated with control siRNA, CHD4-deficient cells had significantly impaired RPA foci formation (Fig. 4B). Consistent with this observation, CHD4 depletion also markedly reduced RPA foci formation induced by camptothecin (Fig. 4C). RPA is primarily phosphorylated at DNA damage lesions by DNA damage response kinases ataxia telangiectasia mutated (ATM), and ATM and Rad3-related protein, and DNA-PK (34, 35). To further assess the recruitment of RPA detected by foci formation analysis, we also performed Western blot analysis to examine the phosphorylation status of RPA (at Ser-4 and Ser-8). Notably, RPA phosphorylation was significantly impaired in CHD4 knockdown cells (Fig. 4D), although  $\gamma$ -H2AX formation was not affected. These data therefore indicate that CHD4 depletion does not





**FIGURE 4. CHD4 depletion renders cells hypersensitive to PARP inhibitors and impairs RPA phosphorylation and RPA recruitment to camptothecin-induced DSBs.** A, CHD4 depletion increases sensitivity of cells to PARP inhibitors, AZD 2281. MCF 10A cells were transfected with control or CHD4 siRNA and exposed to different PARP inhibitors at the indicated doses. Fourteen days later, cells were stained with crystal violet. Colonies containing more than 50 cells were counted. Each value represents the mean  $\pm$  S.D. of three independent experiments. B, CHD4 is required for PARP inhibitor-induced BRIT1, RPA, and BRCA1 foci. MCF 10A cells were transfected with control siRNA or CHD4 siRNA. After 72 h, cells were exposed to 1  $\mu$ M AZD 2281 for 48 h and analyzed by immunofluorescence assay with the indicated antibodies. C, RPA immunofluorescence staining in MCF 10A cells after CHD4 depletion. MCF 10A cells were transfected with control siRNA or CHD4 siRNA. After 72 h, cells were exposed to 1  $\mu$ M camptothecin for 2 h and analyzed by immunofluorescence assay with anti-RPA antibody. D, CHD4 depletion impairs RPA phosphorylation but not CHK2 or H2AX phosphorylation. MCF 10A cells were transfected with the indicated siRNAs. Analysis of RPApS4/S8, p-CHK2, and  $\gamma$ -H2AX was performed by immunoblotting after exposure to 1  $\mu$ M camptothecin for 2 h. Lane C, control siRNA.

affect the initial recognition of DSBs, as indicated by  $\gamma$ -H2AX formation, but it does play a key role in HR repair by regulating the recruitment of BRIT1, BRCA1, and RPA at the early steps of this process.

### DISCUSSION

CHD4 has been well characterized as a critical transcriptional regulator. Recent studies have highlighted its novel role in DNA damage response and genome maintenance (21–24). It has been shown that CHD4 regulates the G<sub>1</sub>/S cell cycle transition via mediating deacetylation of p53 (23). CHD4 is also shown to be required for activation of the G<sub>2</sub>/M checkpoint because of its regulatory role in controlling RNF8- and RNF168-mediated histone ubiquitylation pathways (21, 22). There is also evidence that depletion of CHD4 results in hypersensitivity to DNA damage resulting from ionizing radiation exposure (21–23), suggesting its general role in DNA repair. This study showed the role of CHD4 specifically in HR repair

pathways via regulating the function of repair proteins BRIT1, BRCA1, and RPA. In addition, we found that, as a consequence of impaired HR repair, depletion of CHD4 renders cells significantly hypersensitive to DSB-inducing agents and PARP inhibitors.

BRIT1 is an important regulator of HR that controls the recruitment of multiple DNA repair proteins to DNA damage sites to maintain chromosomal integrity (14–17, 36–39). Our previous study indicated that BRIT1 interacts with chromatin remodeling complex SWI/SNF, facilitates the loading of SWI/SNF to DNA damage sites, and therefore regulates DNA repair at the chromatin level (14). In this study, we identified CHD4 as a new binding partner of BRIT1. The chromatin remodeling activity mediated by CHD4 is required for the recruitment of BRIT1 to DNA damage sites, and the BRCT domains of BRIT1 are necessary for its interaction with CHD4. Interestingly, the N-terminal BRCT domain of BRIT1 is also involved in the

interaction of BRIT1 with SWI/SNF (14). These data suggested that the N-terminal BRCT domain of BRIT1 may play a general role in mediating the interaction of BRIT1 with ATP-dependent chromatin remodeling complexes. These findings further reveal the complexity of the chromatin remodeling process involved in the DNA repair process. Multiple chromatin remodeling complexes are required to facilitate efficient DNA repair at different stages of the process. Unlike the SWI/SNF complex, CHD4-associated chromatin remodeling and histone deacetylation activity functions upstream of HR repair by recruiting BRIT1 to the damaged sites. In addition, it is notable that the loss of PHDs on CHD4 impairs its interaction with BRIT1. Previous studies have shown that the PHDs of CHD4 are required for the interaction between CHD4 and acetylation or methylation of Lys-9 (H3K9ac and H3K9me) on histone H3 (40, 41). We therefore speculate that PHDs might mediate the recruitment of BRIT1 to DNA damage sites as a consequence of its binding to histone H3. Potential future research interest would be to determine whether and how BRIT1 is regulated by histone modifications.

CHD4 is an integral component of the NuRD complex, which includes two enzyme activities, ATP-dependent chromatin remodeling activity and histone deacetylase activity. Aberrant function of the NuRD complex subunits is closely associated with cancer development (42). As our study identified CHD4 as a key regulator of the HR repair process, we would anticipate that the loss of CHD4 may be identified in tumors with characterized features of chromosomal instability. Indeed, reduced expression of CHD4 has been found in gastric and colorectal cancer samples with microsatellite instability (33). Our studies indeed support a role of CHD4 in preventing tumorigenesis via maintaining genomic integrity. Recent studies indicate that the genetic defects of the HR repair process in tumors provide a great therapeutic opportunity for PARP inhibitors. PARP inhibitors have been demonstrated to be one of the breakthroughs in targeted treatment of breast and ovarian cancers that are deficient in BRCA1 or BRCA2. Based on their synthetic lethality interaction with HR deficiency, PARP inhibitors could be more broadly applied to treat many tumors with compromised HR repair, not just those with BRCA1/BRCA2 mutations. Our study further showed that CHD4 deficiency leads to cellular sensitivity to PARP inhibitors; thus, the expression of CHD4 may provide an effective biomarker for using PARP inhibitors. In summary, our study of CHD4 in HR repair is important for understanding tumorigenesis and for designing effective therapeutics to treat CHD4-deficient tumors.

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