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Structural Maintenance of Chromosomes Flexible Hinge Domain Containing 1 (SMCHD1) Promotes Non-homologous End Joining and Inhibits Homologous Recombination Repair upon DNA Damage*

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Background: The role of SMCHD1 in DNA damage response is largely unknown.
Results: SMCHD1 recruitment to DNA damage foci is regulated by 53BP1. Knocking out SMCHD1 compromised cell survival, and decreased the efficiency of non-homologous end joining (NHEJ) while elevating the efficiency of homologous recombination (HR).
Conclusion: SMCHD1 regulates both NHEJ and HR.
Significance: Our findings should further understanding of how cells adopt different repair pathways.

Structural maintenance of chromosomes flexible hinge domain containing 1 (SMCHD1) has been shown to be involved in gene silencing and DNA damage. However, the exact mechanisms of how SMCHD1 participates in DNA damage remains largely unknown. Here we present evidence that SMCHD1 recruitment to DNA damage foci is regulated by 53BP1. Knocking out SMCHD1 led to aberrant γH2AX foci accumulation and compromised cell survival upon DNA damage, demonstrating the critical role of SMCHD1 in DNA damage repair. Following DNA damage induction, SMCHD1 depletion resulted in reduced 53BP1 foci and increased BRCA1 foci, as well as less efficient non-homologous end joining (NHEJ) and elevated levels of homologous recombination (HR). Taken together, these results suggest an important function of SMCHD1 in promoting NHEJ and repressing HR repair in response to DNA damage.

Members of the structural maintenance of chromosomes (SMC)[3] family of ATPases are conserved through evolution and play critical roles in chromosomal duplication and segregation (1–3). Different heterodimers of SMC proteins carry out distinct function. For example, the core of the cohesin complex consists of SMC1 and SMC3, which takes part in sister chromatid cohesion (4); SMC2 and SMC4 are essential condensin complex components and mediate chromosome assembly and segregation (4); and SMC5 and SMC6 have been shown to participate in DNA damage response and homologous recombination (5).

Unlike canonical members of the SMC family, the structural maintenance of chromosomes hinge domain-containing protein 1 (SMCHD1) contains a GHKL (gyrases, Hsp90, histidine kinase, and MutL) domain (6). Originally identified as an epigenetic modifier, it was later shown to localize to the inactive X chromosome and play a critical role in controlling CpG island methylation associated with X chromosome inactivation (7–9). Interestingly, SMCHD1 has also been reported to be enriched at long telomeres, suggesting possible function in global chromatin structure maintenance in addition to X chromosome inactivation (10).

Recent studies of human SMCHD1 as well as the *Arabidopsis thaliana* SMCHD1 homologue GMI1 found recruitment of SMCHD1 to laser micro-irradiated damage sites along with DNA repair factors such as Ku70 and RAD51, suggesting an important role for SMCHD1 in double strand break (DSB) repair (11, 12). These findings point to evolutionary conservation of SMCHD1 function, but the precise mechanism of SMCHD1-mediated DNA damage repair remains to be elucidated.

Cells are constantly exposed to endogenous and environmental agents that cause DNA damage. Of the different types of DNA damage, DSBs are considered the most detrimental, because unrepaired DSBs will lead to genome changes such as chromosomal deletion, inversion, and translocation, and ultimately growth arrest and cell death (13–15). In mammalian cells, DSBs induce a complex and multiple-step cascade of events, mediated by a network of DNA damage response (DDR) proteins. Some proteins are recruited early to DSB lesions, such as ATM/ATR that phosphorylate the histone variant H2A.X

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[3] The abbreviations used are: SMC, structural maintenance of chromosomes; SMCHD1, structural maintenance of chromosomes flexible hinge domain containing 1; NHEJ, non-homologous end joining; HR, homologous recombination; DSB, double strand break; gRNA, guide RNA; HU, hydroxyurea; KO, knockout.

(γH2AX) and signal further assembly of DDR complexes, while others act as scaffolds and facilitate DSB repair (*e.g.* 53BP1 and BRCA1) (13, 16–19).

In this report, using Hela cells individually knocked out (KO) for SMCHD1, 53BP1, and BRCA1 that were generated with the CRISPR/Cas9 technology (20, 21), we found that the localization of human SMCHD1 to DNA DSB lesions was regulated by 53BP1 but not BRCA1. Upon DSB induction, formation of 53BP1 foci, not BRCA1 foci, was defective in SMCHD1 KO cells, indicating dysregulated DNA damage response and repair in these cells. Furthermore, RNAi depletion of SMCHD1 decreased non-homologous end joining (NHEJ) but enhanced homologous recombination (HR)-mediated DSB repair. Our data place SMCHD1 downstream of γH2AX foci formation, where it contributes to the adoption of DSB repair mechanisms (NHEJ *versus* HR), adding further evidence to the complex nature of DNA damage response and repair pathways.

EXPERIMENTAL PROCEDURES

Cell Culture and KO Cell Lines—Hela and U2OS cells were maintained in DMEM medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin. Zeocin was added at 100 μg/ml (Invitrogen), and hydroxyurea was added at 2 mM (Sigma). KO cell lines were established by co-transfecting vectors encoding guide RNAs against SMCHD1, 53BP1, or BRCA1 together with Cas9 into Hela cells. Cells were then individually sorted by FACS. The gRNA and Cas9 vectors (Addgene) were the same as described by the Church Laboratory (21). Individual KO clones were isolated, and their genomic DNA extracted for sequencing. Successful targeting was also confirmed by both immunofluorescence and Western blotting. The gRNA sequences are: SMCHD1: GAAATTACC-TGTGATAATTT; 53BP1: GAAAGTTCGGCTTACCTTGC; BRCA1: GTGATATTAACTGTCTGTAC.

Immunofluorescence (IF), Western Blotting, and Antibodies—Immunofluorescence and Western blotting were carried out as previously described (22). For IF, cells grown on glass coverslips were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and then blocked with 5% BSA before incubation with primary and secondary antibodies. The following antibodies were used in this study: anti-SMCHD1 (Ab31865, Abcam), anti-BRCA1 (a gift from Dr. Junjie Chen), anti-trimethyl-Histone H3 (Lys-9) (05-1242, Millipore), anti-HP1α (39977, Active Motif), anti-53BP1 (NB100-304, Novus), anti-γH2AX (05-636, Millipore), anti-actin (M20010M, Abmart), anti-GAPDH (M20006M, Abmart), and anti- β-tubulin (sc-9104, Santa Cruz Biotechnology).

Cell Survival Assay—Cells (*e.g.* Hela and U2OS) were exposed to different concentrations of Zeocin for 2 h before washing with 1× PBS and maintained in fresh medium. At the indicated time points, cells were fixed and stained with 0.1% Coomassie Brilliant Blue R250 in 25% isopropanol. Experiments were done in triplicate. Colonies were counted and normalized to plating efficiencies.

I-SceI-based NHEJ and HR Assays—The U2OS cell line containing a single copy of the DR-GFP reporter (U2OS-DR-GFP) was a kind gift from Dr. Junjie Chen. The I-SceI-based U2OS/DR-GFP reporter HR assay was carried out as previously described (23). The NHEJ reporter cassette used as previously described (24) was modified with another selection marker hygromycin. It consists of the *GFP* gene with an engineered intron from the rat *Pem1* gene, interrupted by an adenoviral exon (Ad). The adenoviral exon is flanked by two I-SceI recognition sites in inverted orientation for induction of DSBs. HEK293 cells carrying stably integrated NHEJ reporter cassette were generated by hygromycin selection and used as NHEJ reporter cells. The I-SceI-based NHEJ assay was carried out similarly to the HR assay. Briefly, cells were transfected first with appropriate siRNA oligos, and then with the I-SceI-expressing plasmid (a kind gift from Dr. Junjie Chen) at 24 h after siRNA transfection. After 48 h of incubation, cells were collected for flow cytometry analysis to determine GFP signals.

The siRNA used here were: siSMCHD1–1: CCGUUAUCA-UCCAUUCUUA dTdT; siSMCHD1–2: GUCCAUUCCAGU-GAUUAAU dTdT; si53BP1: GAAGGACGGAGUACUAAUA dTdT; siBRCA1: AGAUAGUUCUACCAGUAAA dTdT (25); siRAP80: GUAUUGACUCGGAGACAAA dTdT (25).

Cell Cycle Analysis—Cells were synchronized by double thymidine block. At 12 h after the 2nd thymidine treatment. Zeocin was added to cells for 2 h and then washed. At different time points following Zeocin treatment, cells were collected, fixed in 70% ethanol overnight, and then incubated in propidium iodide (PI) (50 μg/ml) with RNase A (100 μg/ml) for 30 min. Cell cycle profiles were determined by flow cytometry on FACSCalibur (BD Biosciences).

Real-time Quantitative RT-PCR (qRT-PCR)—Real-time qRT-PCR was carried out as described previously (26). Briefly, total RNA was isolated with Trizol (Invitrogen) and used for reverse transcription with the iScriptcDNA Synthesis kit (Bio-Rad). Real-time qPCR amplification reactions were carried out using the SYBR Green mix (Invitrogen) and the ABI StepOnePlus real-time PCR system (Applied Biosystems). The primers used were: SMCHD1 FP: TAACAACTGGGCCGTGTATAGG; SMCHD1RP: TGGTACTGGACGAACATATCCTG; BRCA1 FP: ACCTTGGAACTGTGAGAACTCT; BRCA1 RP: TCTT-GATCTCCCACACTGCAATA; RAP80 FP: TGCCAGTTGG-AGGTTTATCAAAA; RAP80 RP: GAGAAGGAGGTCTAG-GTAACACT; Actin FP:TGTACGCCAACACAGTGCTG; Actin RP: GCTGGAAGGTGGACAGCGA.

RESULTS

Generation of SMCHD1 Knock-out Hela Cells using the CRISPR/Cas9 Technology—To better define the role of SMCHD1 in DNA damage responses, we first generated SMCHD1 KO Hela cells using the CRISPR/Cas9 technology (20, 21). Our guide RNA (gRNA) sequences targeted exon 2 of human SMCHD1, which encodes sequences N-terminal to the ATPase domain (Fig. 1*A*). We selected two mutant clones (KO1 and KO2) for PCR amplification of the targeted regions and sequencing (Fig. 1*B*). The KO1 clone contained one allele with an 11 base pair deletion and the other allele with a single nucleotide insertion. The KO2 clone was homozygous for the deletion of 8 base pairs. Both lines lost expression of endogenous SMCHD1 proteins as determined by Western blot analysis (Fig. 1*C*). SMCHD1 has been reported to co-localize with trimethylated H3K9 (H3K9me3) on heterochromatin (7). We therefore examined

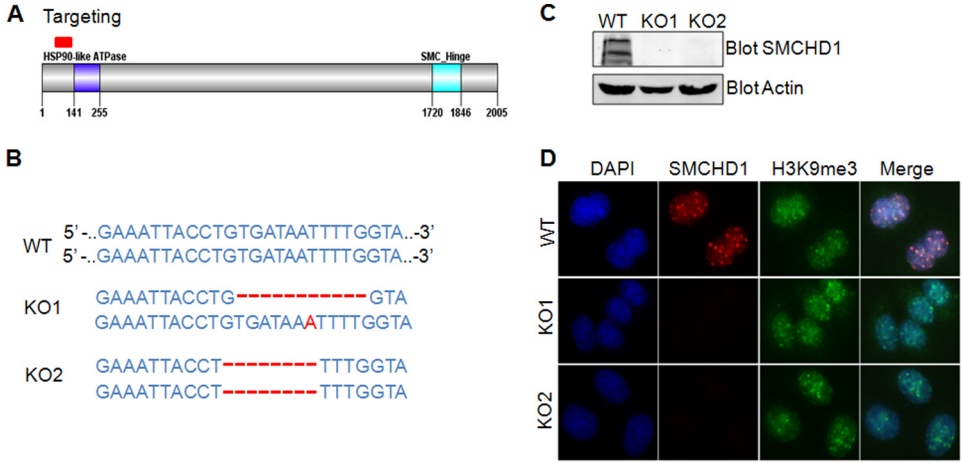

FIGURE 1. **Generation of SMCHD1 homozygous knock-out (KO) Hela cells using the CRISPR/Cas9 technology.** *A*, schematic representation of the human SMCHD1 protein. The ATPase and SMC hinge domains are highlighted. The *red bar* indicates the position of the targeting sequences for the gRNAs (exon 2). *B*, two SMCHD1 homozygous KO clones (KO1 and KO2) were expanded and their genomic DNA extracted for sequencing. The resulting indels for each allele are shown, with nucleotide insertions indicated by *red letters* and deleted bases by *red dashed lines*. *C*, SMCHD1 homozygous KO cells from *B* were analyzed by Western blotting with the indicated antibodies. Actin served as loading control. *D*, KO cells from *B* were examined by immunostaining analysis using the indicated antibodies. DAPI was used to mark the nuclei.

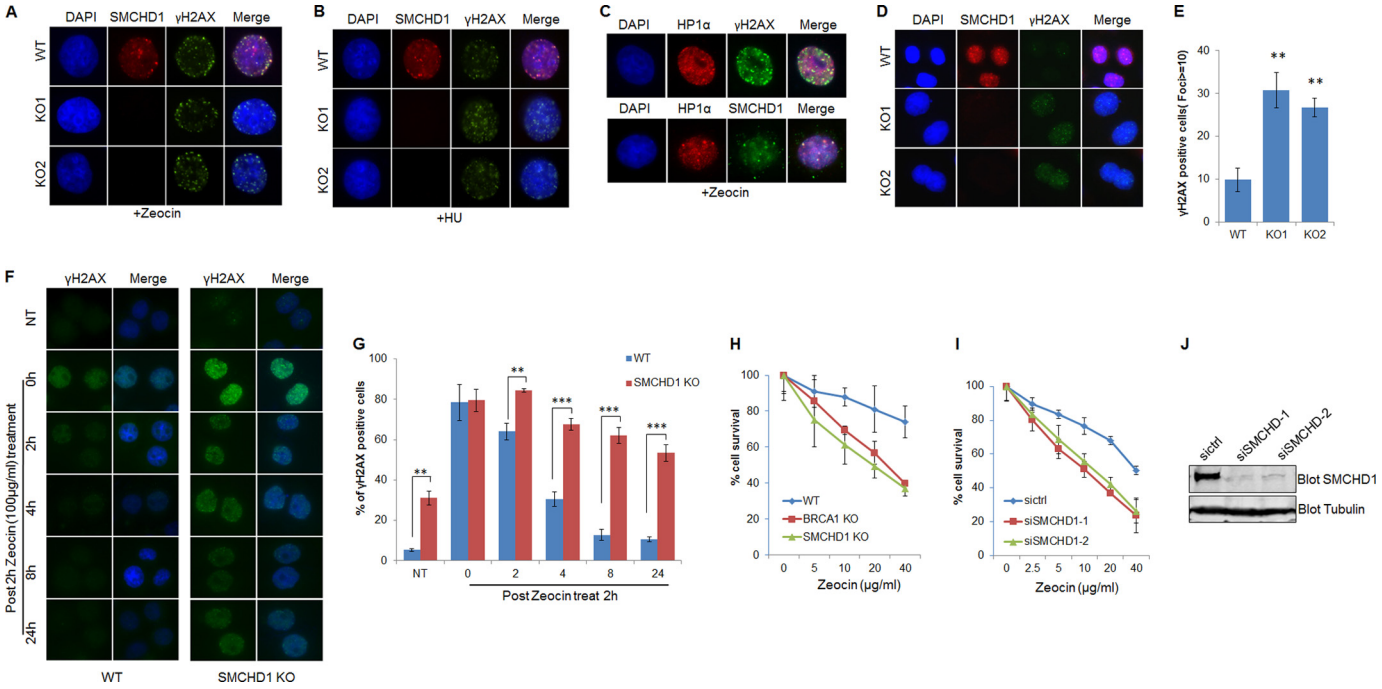

FIGURE 2. **SMCHD1 localizes to DNA damage foci and is required for proper response and cell survival following DNA DSB induction.** *A* and *B*, wild-type (*WT*) and SMCHD1 KO cells were treated for 2 h with Zeocin (100 μg/ml) (*A*) or HU (2 mM) (*B*) before immunostaining analysis with the indicated antibodies. DAPI was used to mark the nuclei. *C*, WT cells were treated with Zeocin (100 μg/ml) for 2 h before immunostaining analysis with the indicated antibodies. HP1α was used to mark the heterochromatin. DAPI was used to stain the nuclei. *D*, WT and SMCHD1 KO cell lines were examined by immunostaining analysis using the indicated antibodies. *E*, quantification of data from *D*. Only cells with $\geq$10 foci were counted as positive. Error bars represent standard error ($n = 3$). *p* values were determined by Student's *t* test. **, $p < 0.01$. *F*, WT and SMCHD1 KO cells were similarly treated as in *A* before immunostaining analysis at the indicated times points (hours) post Zeocin treatment. *NT*, no treatment. *G*, quantification of data from *F*. Error bars represent standard error ($n = 3$). *p* values were determined by Student's *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *H*, WT, SMCHD1 KO, and BRCA1 KO Hela cells were treated with Zeocin (100 μg/ml) for 2 h. Colony formation assays were then carried out in triplicate to determine cell survival following DSB induction. Error bars represent standard error ($n = 3$). *I*, U2OS cells were transfected with control (sictrl) or two different SMCHD1 siRNA oligos. At 24 h after transfection, cells were treated with varying doses of Zeocin for 2 h. Colony formation assays were then carried out as in *H*. Error bars represent standard error ($n = 3$). *J*, cells from *I* were examined by Western blotting using ani-SMCHD1 antibodies to determine SMCHD1 knockdown efficiency. Tubulin was used as loading control.

the localization of SMCHD1 in these KO cells by immunostaining. As expected, wild-type cells exhibited extensive co-staining of SMCHD1 and H3K9me3 signals (Fig. 1*D*). In comparison, we could not detect SMCHD1 foci in the two SMCHD1 KO cell lines, indicating homozygous inactivation of SMCHD1 in these cells and further confirming the specificity of the anti-SMCHD1 antibody.

In addition, to better determine the mechanism of SMCHD1 action in DNA damage responses, we also generated BRCA1 and 53BP1 KO Hela cells using the CRISPR/Cas9 technology

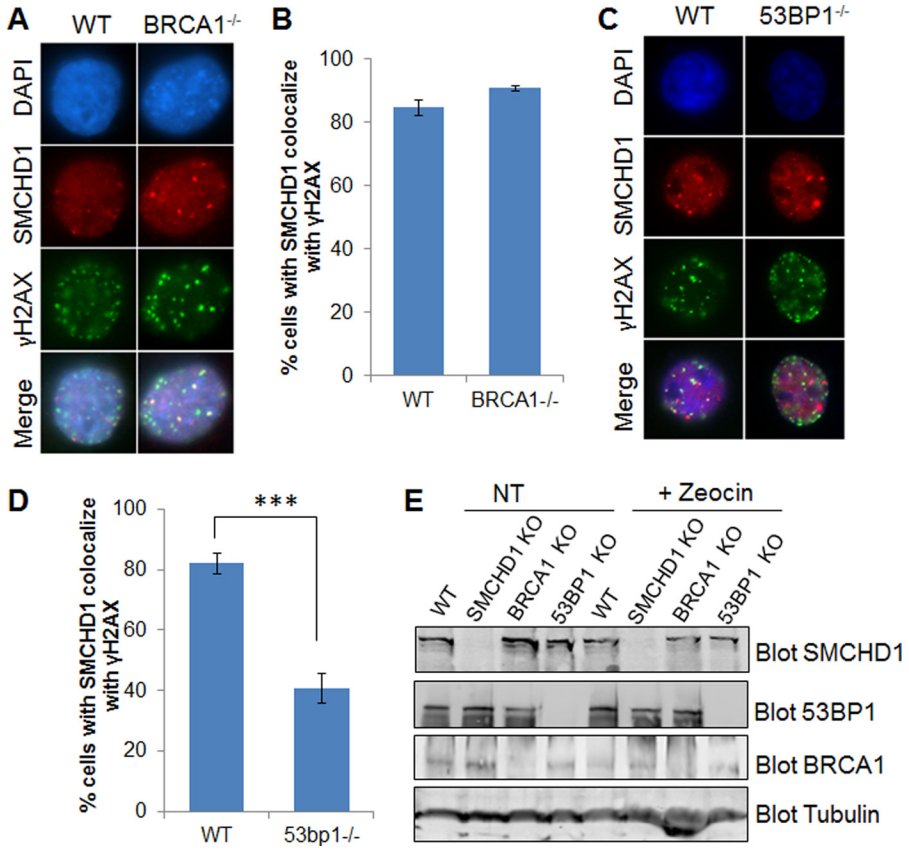

FIGURE 3. **SMCHD1 localization to DNA damage foci is regulated by 53BP1 not BRCA1.** *A*, WT and BRCA1 KO cells were treated with Zeocin (100 μg/ml) for 2 h before immunostaining analysis with the indicated antibodies. *B*, quantification of data from *A*. Two independent experiments were carried out. Error bars represent standard deviation ($n = 2$). *p* values were determined by Student's *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *C*, WT and 53BP1 KO Hela cells were treated with Zeocin (100 μg/ml) for 2 h before immunostaining analysis with the indicated antibodies. *D*, quantification of data from *C*. Two independent experiments were carried out. Error bars represent standard deviation ($n = 2$). *p* values were determined by Student's *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *E*, WT and cells knocked out for SMCHD1, 53BP1, and BRCA1 were treated with Zeocin (100 μg/ml) for 2 h before analysis by Western blotting with the indicated antibodies. *NT*, no treatment. Tubulin served used as loading control.

(Figs. 3*E* and 4). These two proteins have distinct function during DNA damage response and repair, the KO cell lines would therefore serve as important tools to probe SMCHD1 function.

SMCHD1 Is Targeted to DNA Damage Foci and Regulates DNA Damage Repair and Cell Survival—To determine the role of SMCHD1 in DSB damage response, we first investigated the localization of SMCHD1 when cells were treated with two different DSB-inducing drugs, Zeocin and hydroxyl urea (HU). Zeocin is an antibiotic that can cause DNA damage by cleaving both DNA strands (28), while HU is a potent inhibitor of ribonucleotide reductase and inhibits DNA replication (29). As shown in Fig. 2, *A* and *B*, treatment of Zeocin (for 2 h) and HU (for 2 h) led to accumulation of γH2AX foci, a well-known marker for unrepaired DSBs (30, 31). In ~80% cells, SMCHD1 foci could be superimposed with γH2AX signals, indicating localization of SMCHD1 to γH2AX foci in these cells. This observation supports findings from the *A. thaliana* homolog GMI1 (11) and the involvement of human SMCHD1 in DSB damage response. The SMCHD1 and γH2AX co-localization foci were likely sites of heterochromatin, because nearly all of the SMCHD1 foci could co-stain with H3K9Me3 or HP1α (Figs. 1*D* and 2*C*), suggesting that SMCHD1-mediated DNA damage response may happen on the heterochromatin.

If SMCHD1 is a critical component of the DDR network, SMCHD1 deletion should disrupt normal DNA damage repair pathways and increase genotoxic stress even in the absence of exogenous DNA damage agents, which should cause accumulation of γH2AX foci in SMCHD1 KO cells. Indeed, when we compared γH2AX staining in wild-type *versus* SMCHD1 KO cells (in the absence of exogenous DSB induction), noticeable elevation of γH2AX staining could be observed in SMCHD1 KO cells compared with wild-type cells (Fig. 2, *D* and *E*). Approximately 30% of SMCHD1 KO cells exhibited bright γH2AX staining compared with ~10% in WT cells. These observations suggest sustained endogenous DNA damage and defects in repair in SMCHD1 KO cells.

Next, we monitored γH2AX staining in these cells at different time points following Zeocin treatment. While untreated wild-type cells had barely detectable γH2AX foci, the majority (~80%) of wild-type cells stained positive for γH2AX foci after 2 h of treatment (Fig. 2, *F* and *G*). Accumulation of DNA damage foci began to taper off after that, with ~40% cells being γH2AX foci positive at 4 h post-treatment. By 8 h, γH2AX foci staining had returned to basal levels, indicating active and efficient DSB repair in these cells. Similar to wild-type cells, accumulation of γH2AX foci in SMCHD1 KO cells also peaked 2 h after Zeocin treatment (Fig. 2, *F* and *G*). However, SMCHD1 KO cells exhibited prolonged and sustained γH2AX staining, even at 24 h post-treatment. These observations further sup-

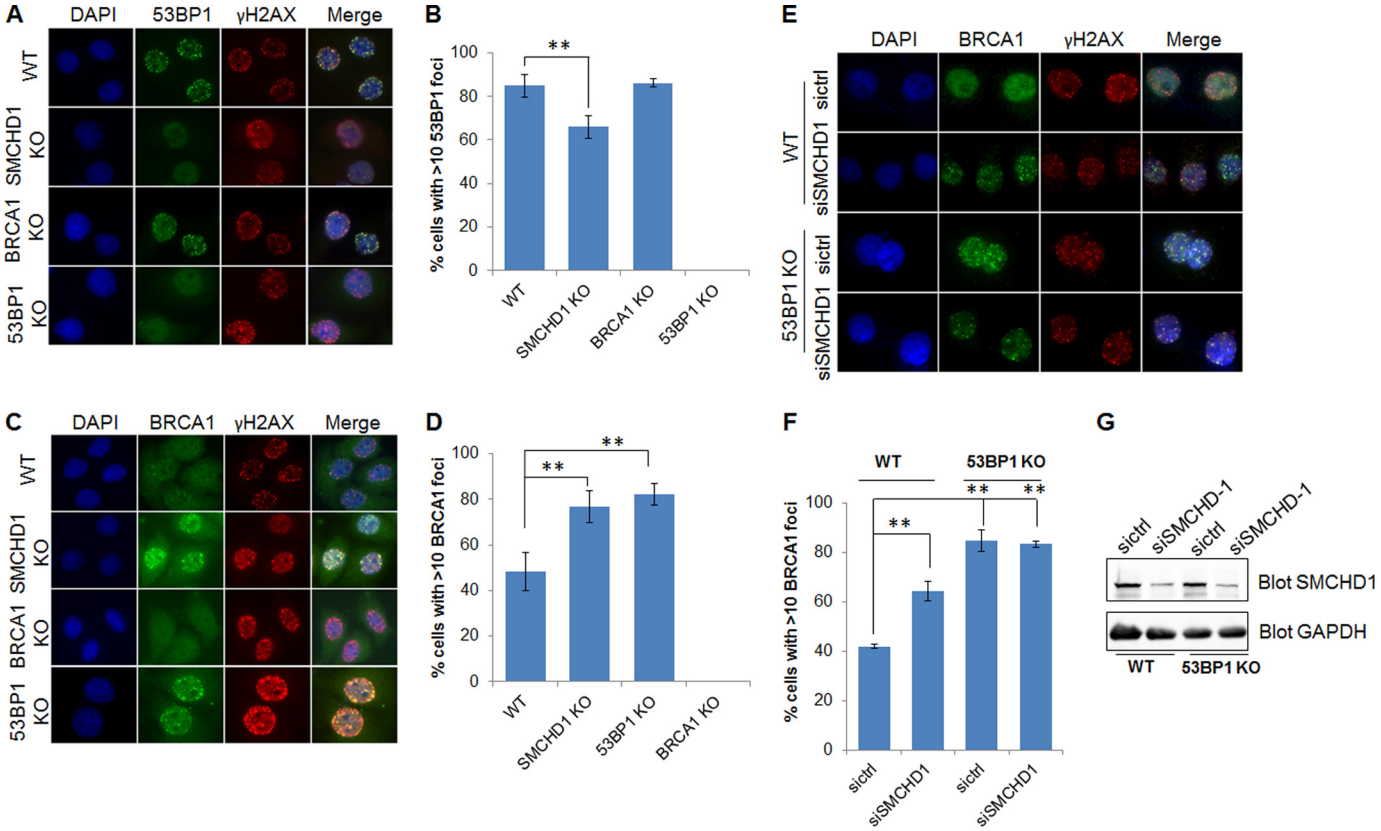

FIGURE 4. **SMCHD1 is important for 53BP1 and BRCA1 foci formation during DNA damage responses.** *A*, WT and cells knocked out for SMCHD1, 53BP1, and BRCA1 were treated with Zeocin (100 μg/ml) for 2 h and then immunostained using antibodies against 53BP1 and γH2AX. DAPI was used to stain the nuclei. *B*, quantification of data from *A*. Error bars represent standard error ($n = 3$). *p* values were determined by Student *t* test.*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *C*, Zeocin-treated cells from *A* were also immunostained with antibodies against BRCA1 and γH2AX. DAPI was used to stain the nuclei. *D*, quantification of data from *C*. Error bars represent standard error ($n = 3$). *p* values were determined by Student's *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *E*, WT and 53BP1 KO cells were transfected with control (*sictrl*) or SMCHD1 (*siSMCHD-1*) siRNA oligos. At 48 h after transfection, the cells were treated with Zeocin (100 μg/ml) for 2 h and then immunostained using antibodies against BRCA1 and γH2AX. DAPI was used to stain the nuclei. *F*, quantification of data from *E*. Error bars represent standard error ($n = 3$). *p* values were determined by Student *t* test.*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *G*, cells from *E* were examined by Western blotting using anti-SMCHD1 antibodies. GAPDH was used as loading control.

port a critical role for SMCHD1 in suppressing endogenous DNA damage and promoting efficient repair.

We speculated that extended DSB accumulation as a result of SMCHD1 ablation following DNA damage induction would negatively affect cell viability. To test this idea, we carried out colony formation assays using wild-type and SMCHD1 KO cells treated with Zeocin. The BRCA1 KO cells were also included as a positive control, as it has been found that BRCA1-deficient cells are sensitive to ionizing radiation and drugs that produce DSBs (32–35). Consistent with previous studies, exposure to Zeocin led to dose-dependent cell death in BRCA1 KO cells. SMCHD1-deficient cells were similarly sensitive to Zeocin-induced DSBs and exhibited more death with drug treatment (Fig. 2, *H*, *I*, and *J*), adding additional evidence to the importance of SMCHD1 in DNA damage response and repair.

SMCHD1 Recruitment to DNA Damage Foci Is Regulated by 53BP1—Cells primarily utilize classical NHEJ and HR for DNA DSB repair (36–38). HR is template-driven, of high fidelity, and more prevalent during DNA replication. NHEJ is more error-prone, directly ligating DNA termini without relying on significant homology, and occurs throughout the cell cycle (39, 40). Numerous studies have highlighted the pivotal roles of 53BP1 and BRCA1 in NHEJ and HR, respectively, and how their interplay impacts the equilibrium of HR *versus* NHEJ in cells. For instance, oligomerization of 53BP1 at DSBs can inhibit DSB resection and promote NHEJ (41–43), while recruitment of BRCA1 to DSB lesions on the chromatin (*e.g.* during S phase) can suppress 53BP1-mediated NHEJ and enhance HR-dependent repair (44).

Given the distinct roles of 53BP1 and BRCA1 in DSB repair, we set out to determine whether SMCHD1 targeting to damage foci depended on BRCA1 or 53BP1 by examining SMCHD1 localization in Zeocin-treated wild-type *versus* BRCA1/53BP1 KO cells. Again, the majority of wild-type cells exhibited co-staining of SMCHD1 and γH2AX with Zeocin treatment (Fig. 3, *A–D*). Interestingly, knocking out 53BP1, but not BRCA1, led to a ~50% reduction in the percentage of cells that showed SMCHD1 recruitment to DNA damage foci, implicating SMCHD1 in the NHEJ repair pathway. This decrease was likely not due to reduced expression of SMCHD1 (Fig. 3*E*), as SMCHD1 levels appeared similar between 53BP1 and BRCA1 KO cells.

SMCHD1 Participates in 53BP1 and BRCA1 Foci Formation upon DNA Damage—Early during DSB damage response, the MRE11-RAD50-NBS1 (MRN) sensor complex recruits ATM family kinases to breaks, which in turn phosphorylate H2A.X. Subsequent recruitment of factors such as MDC1, RNF8, and RNF168 to γH2AX foci helps to initiate the complex, feedback-

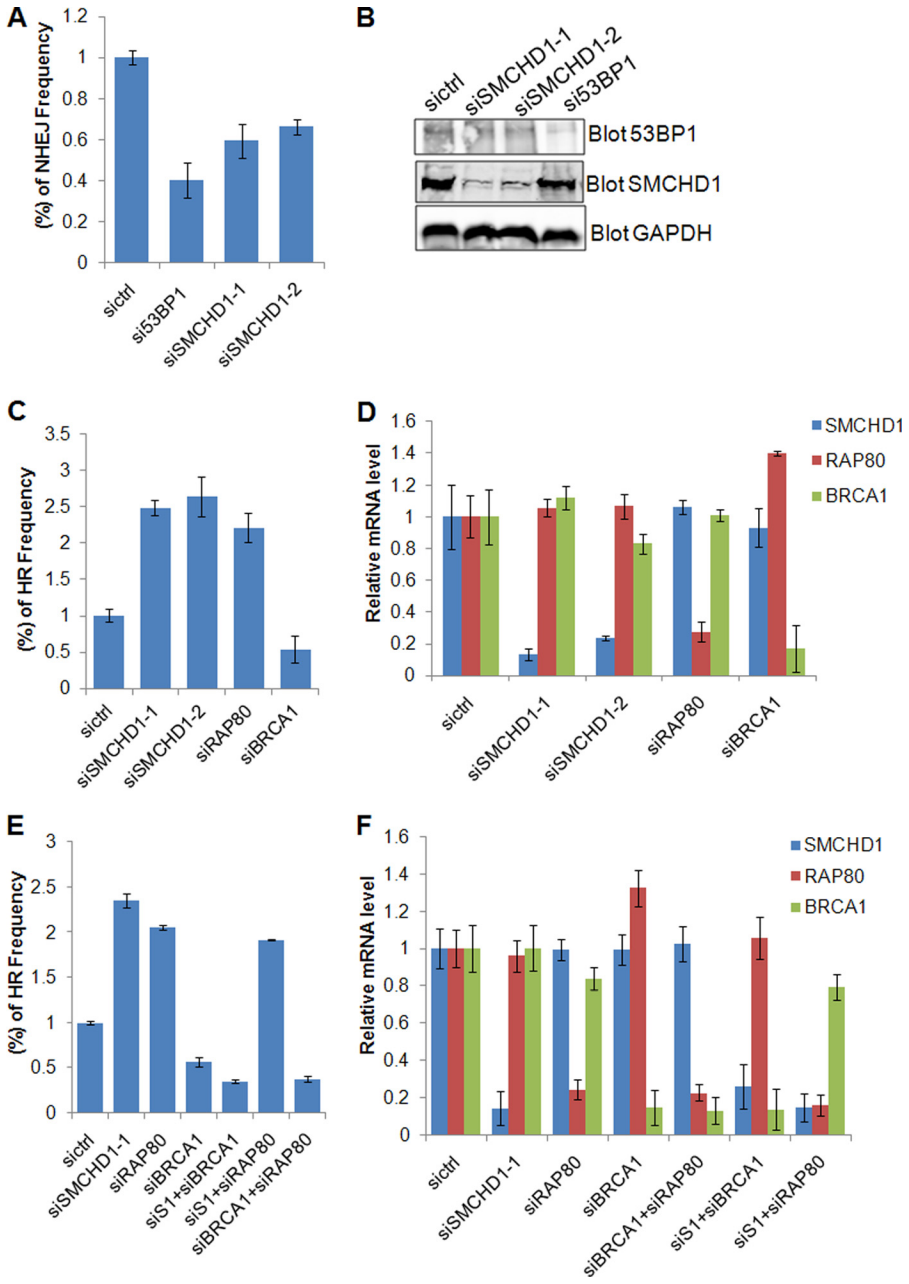

FIGURE 5. **SMCHD1 depletion affects the efficiency of DNA repair through both NHEJ and HR.** *A*, HEK293 NHEJ reporter cells were transfected first with siRNAs targeting the indicated genes, and then the plasmid encoding I-SceI (24 h after siRNA transfection). Cells were then analyzed after incubation for another 48 h by flow cytometry. Experiments were done in triplicate. *B*, cells from *A* were examined by Western blotting using anti-SMCHD1 and anti-53BP1 antibodies to determine SMCHD1 and 53BP1 knockdown efficiency. GAPDH was used as loading control. *C* and *D*, U2OS DR-GFP cells were transfected first with siRNAs targeting the indicated genes, and then the plasmid encoding I-SceI (24 h after siRNA transfection). Cells were then analyzed after incubation for another 48 h by flow cytometry (*C*) or real-time quantitative RT-PCR (qRT-PCR) (*D*). Experiments were done in triplicate. *E* and *F*, U2OS DR-GFP cells were similarly analyzed in HR assays as in *C* following transfection with siRNA oligos against the indicated genes. Data from flow cytometry (*E*) and qRT-PCR (*F*) analysis are similarly plotted.

regulated DNA damage and repair signaling cascades (17, 45–47). RNF168 in turn ubiquitylates H2A, which triggers the recruitment of 53BP1 as well as BRCA1 (17). Because γH2AX foci could still form in SMCHD1 deficient cells (Fig. 2, *D* and *E*), we postulated that SMCHD1 most likely acted at later steps in the damage response cascade, for example, stabilizing 53BP1 and/or BRCA1.

To more precisely pinpoint the steps at which SMCHD1 functions in DNA damage response, we compared foci numbers of 53BP1 and BRCA1 to DSB lesions in wild-type *versus* SMCHD1 KO cells following Zeocin treatment. Unlike in wild-type cells, where 53BP1 foci could be readily detected in >80% of the cells, only ~60% of SMCHD1 KO cells exhibited 53BP1 foci (Fig. 4, *A* and *B*), suggesting SMCHD1 may be important for 53BP1 foci formation in response to DNA damage, perhaps by stabilizing 53BP1 foci on DNA breaks. This may further strengthen the possibility that SMCHD1 is involved in 53BP1 mediated NHEJ repair. In comparison, the percentage of BRCA1 foci-positive cells significantly increased for both SMCHD1 KO and 53BP1 KO cell lines (~80%) compared with wild-type con-

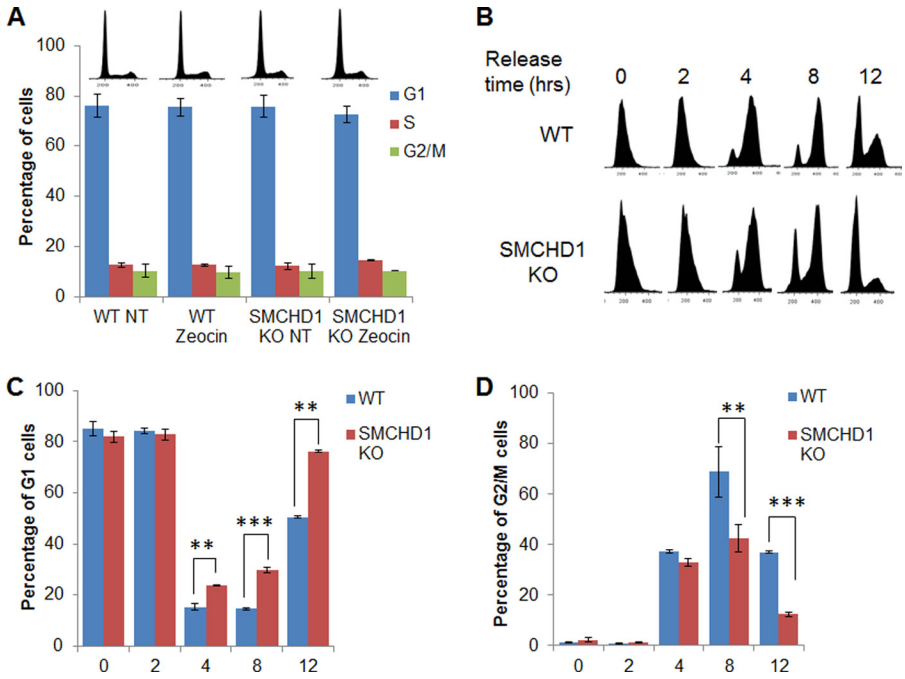

FIGURE 6. **SMCHD1 depletion impacts cell cycle progression in response to DNA damage induction.** *A*, asynchronous WT and SMCHD1 KO cells with or without Zeocin treatment (100 μg/ml) were stained with PI and analyzed by flow cytometry (*top*). Quantification of cells at different stages of the cell cycle is shown (*bottom*). *B*, WT and SMCHD1 KO cells were synchronized by double thymidine block and then treated with Zeocin (100 μg/ml) for 2 h before being released into the cell cycle. At the indicated time points following release, the cells were collected for PI staining and analysis by flow cytometry. *C* and *D*, quantification of cells from *B* for different cell cycle stages (G1(*C*) and G2/M (*D*)). Error bars represent standard error ($n = 3$). *p* values were determined by Student's *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

trol cells (~50%) (Fig. 4, *C* and *D*). The changes observed here were unlikely the results of altered 53BP1 and BRCA1 protein levels in SMCHD1 KO cells (Fig. 3*E*). Additionally, further depletion of SMCHD1 in 53BP1 KO cells had little effects on BRCA1 foci formation (Fig. 4, *E–G*), compared with individual KO of SMCHD1 (Fig. 4, *C* and *D*) or 53BP1, suggesting that SMCHD1 may act downstream of 53BP1. These findings indicate that SMCHD1 deficiency disrupted 53BP1 and BRCA1 recruitment to DNA damage foci, and support dual roles for SMCHD1 following DSB induction, promoting 53BP1 foci formation and suppressing BRCA1 foci formation.

SMCHD1 Inhibition Enhances DNA Damage Repair through Homologous Recombination—Our data thus far suggest that loss of SMCHD1 may disrupt the equilibrium between the two major repair pathways: HR *versus* NHEJ, leading to defective DSB repair and accumulation of DNA damage. To test this hypothesis, we utilized the I-SceI-based HEK293 NHEJ and U2OS/DR-GFP reporter systems to determine the effect of SMCHD1 knockdown on NHEJ and HR (23, 24). The reporter cells were transfected with siRNA oligos against SMCHD1 together with an I-SceI-expressing plasmid. siRNAs against 53BP1 served as controls for inhibition of NHEJ pathway, because 53BP1 is essential for NHEJ (38). siRNAs against BRCA1 and receptor-associated protein 80 (RAP80) were used as controls for examining HR, because BRCA1 knockdown should inhibit HR, whereas RAP80 knockdown should have the opposite effect (25). The ubiquitin-binding protein RAP80 is part of the complex that also contains BRCC36 and ABRAXAS, which has been shown to inhibit DSB resection and promote NHEJ (48–50). In these assays, successful NHEJ or HR-mediated repair would result in GFP fluorescence that could be detected by a flow cytometer. As shown in Fig. 5, *A* and *B*, similar to depletion of 53BP1, knocking down SMCHD1 led to ~40% reduction in GFP-positive cells compared with control cells, supporting the notion that SMCHD1 is required for NHEJ repair. In U2OS-DR/GFP cells, the siRNAs were able to reduce endogenous gene expression by >70% based on RT-PCR results (Fig. 5*D*). As expected, BRCA1 knockdown abrogated the ability of cells to repair I-SceI induced DSBs by HR, and RAP80 inhibition enhanced HR efficiency (Fig. 5*C*). In comparison, knocking down SMCHD1 by siRNAs significantly increased the frequency of HR (>2-fold) (Fig. 5*C*), to levels comparable to cells depleted for RAP80. This increase in HR efficiency was unlikely caused by increased BRCA1 protein levels, because knocking down SMCHD1 did not affect BRCA1 or RAP80 expression (Fig. 5*D*), and suggests that SMCHD1 can suppress HR-mediated DSB repair pathway.

Taken together with our findings that knocking out SMCHD1 could facilitate BRCA1 foci formation (Fig. 4, *C* and *D*), we speculated that increased HR efficiency in SMCHD1-depleted cells might be BRCA1 dependent. To test this notion, we examined how simultaneous knockdown of SMCHD1 and BRCA1 would affect the rate of HR. U2OS/DR-GFP reporter cells were similarly transfected with an I-SceI-expressing plasmid together with siRNA oligos against SMCHD1 as well as BRCA1. siRNA oligos against RAP80 served as controls. Similar to single knockdown cells, the siRNA oligos were able to achieve significant inhibition of their target genes in double knockdown cells (>70%) (Fig. 5*F*). Importantly, further depletion of BRCA1 abolished the ability of SMCHD1 knockdown cells to enhance HR, whereas additional inhibition of RAP80 had little effect on SMCHD1 depleted cells (Fig. 5*E*). These

findings indicate that SMCHD1 may inhibit BRCA1-driven HR activity, and point to SMCHD1 as an important factor in helping to determine which repair pathway to undertake after DSB induction.

SMCHD1 Depletion Impacts Cell-Cycle Progression in Response to DNA Damage—Depletion of SMCHD1 should affect both NHEJ and HR repair pathways that are intimately linked to cell cycle, with NHEJ more prevalent during G1 while HR primarily occurring during S/G2. We therefore investigated how SMCHD1 inhibition might impact cell cycle progression in response to DNA damage. We found depletion of SMCHD1 to have no detectable effect on cell cycle stages of asynchronous cells, regardless of Zeocin treatment (Fig. 6*A*). However, when we synchronized cells by double thymidine block and monitored cell cycle progression following Zeocin treatment, SMCHD1-deficient cells displayed a significant enrichment of G1 phase cells and a concomitant decrease of cells in G2/M (Fig. 6, *B–D*). These observations indicate that depleting SMCHD1 can delay G1 exit while enhancing G2/M progression, and provide further support for a role of SMCHD1 in promoting NHEJ and inhibiting HR.

DISCUSSION

A complex network of proteins from diverse pathways participate in DNA damage response and repair. In our study, we found SMCHD1 could localize to DNA damage foci following induction of DSBs by drug treatment, consistent with a recent study using female fibroblast cells that were subjected to laser micro-irradiation (51). Unlike complexes of canonical SMC family members SMC1/SMC3 and SMC5/SMC6, which have been reported to promote HR by keeping damaged and intact sister chromatids in close proximity (1–5), SMCHD1 appears to facilitate the cellular process that commits cells to utilizing NHEJ rather than HR for damage repair by increasing 53BP1 recruitment while inhibiting BRCA1 targeting to DSB sites.

Studies have shown that depleting 53BP1 could enhance HR (42), whereas cells knocked down for BRCA1 were HR deficient (40). Increasing data suggest that one early and key event in the NHEJ *versus* HR decision is resection regulation. It has been proposed that BRCA1 promotes HR by overcoming 53BP1 imposed resection restriction on DSBs (44). In addition to BRCA1, the RAP80/BRCC36/ABRAXAS complex also participates in regulating HR *versus* NHEJ decisions (25, 50). Inhibition of RAP80 has been shown to greatly up-regulate resection and HR (25). Perhaps SMCHD1 can cooperate with 53BP1 and other factors at DSBs to inhibit DNA strand resection.

Heterochromatin maintenance is required for diverse cellular processes, including chromosome segregation, dosage compensation in females, and meiotic progression in males (51, 52). The tightly packed heterochromatin is also a barrier to the detection and repair of DNA damage (52). As a member of the SMC family, SMCHD1 participates in epigenetic regulation and chromatin organization (7, 27, 53). The co-localization of SMCHD1 with H3K9me3 and HP1α points to the possibility that SMCHD1 may regulate DNA damage responses at the heterochromatin. Future studies will be necessary to unravel the mechanisms of SMCHD1-dependent DNA damage responses on heterochromatin, and to probe how SMCHD1 regulates the balance between NHEJ and HR pathways.

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