CTCF prevents genomic instability by promoting homologous recombination-directed DNA double-strand break repair

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Edited by Douglas Koshland, University of California, Berkeley, CA, and approved September 1, 2017 (received for review March 11, 2017)

CTCF is an essential epigenetic regulator mediating chromatin insulation, long-range regulatory interactions, and the organization of large topological domains in the nucleus. Phenotypes of CTCF haploinsufficient mutations in humans, knockout in mice, and depletion in cells are often consistent with impaired genome stability, but a role of CTCF in genome maintenance has not been fully investigated. Here, we report that CTCF maintains genome stability, is recruited to sites of DNA damage, and promotes homologous recombination repair of DNA double-strand breaks (DSBs). CTCF depletion increased chromosomal instability, marked by chromosome breakage and end fusions, elevated genotoxic stress-induced genomic DNA fragmentation, and activated the ataxia telangiectasia mutated (ATM) kinase. We show that CTCF could be recruited to drug-induced 53BP1 foci and known fragile sites, as well as to I-SceI endonuclease-induced DSBs. Laser irradiation analysis revealed that this recruitment depends on ATM, Nijmegen breakage syndrome (NBS), and the zinc finger DNA-binding domain of CTCF. We demonstrate that CTCF knockdown impaired homologous recombination (HR) repair of DSBs. Consistent with this, CTCF knockdown reduced the formation of γ-radiation–induced Rad51 foci, as well as the recruitment of Rad51 to laser-irradiated sites of DNA lesions and to I-SceI–induced DSBs. We further show that CTCF is associated with DNA HR repair factors MDC1 and AGO2, and directly interacts with Rad51 via its C terminus. These analyses establish a direct, functional role of CTCF in DNA repair and provide a potential link between genome organization and genome stability.

CTCF | chromatin | genome stability | DNA repair | homologous recombination

The insulator protein CTCF organizes the genome into local chromatin loops and large topological domains and carries out numerous diverse nuclear functions by binding to thousands of sites in the genome (1–3). CTCF mutations in humans leads to microcephaly and intellectual disability (4). Its conditional knockout in mice results in the loss of neuronal cells (5, 6), blocked differentiation and proliferation in lymphocytes, and massive apoptosis in the developing limb (7, 8), while mice with a CTCF hemizygous mutation are predisposed to cancer (9). In *Drosophila*, CTCF mutations lead to instability of the ribosomal RNA gene loci (10). Experiments in cultured cells have implicated CTCF in the protection against UV-induced apoptosis (11). While these complex phenotypes could result from the diverse reported nuclear functions of CTCF, they are also consistent with impaired genome stability. However, whether and, if so, how CTCF plays a role in genome maintenance have not been systematically investigated. Here we report that CTCF has a direct role in the maintenance of genome stability, and that its depletion results in chromosomal instability and activation of the DNA damage response (DDR). CTCF is recruited to sites of DNA damage in a process that depends on ataxia telangiectasia mutated (ATM) and Nijmegen breakage syndrome (NBS), and CTCF facilitates the homologous recombination directed DNA double-strand break (DSB) repair by interacting with Rad51 and promoting the formation of Rad51 repair foci.

Results

CTCF Prevents Genomic Instability. To directly test whether CTCF depletion increases genomic instability, we performed cytogenetic analysis of chromosomes in CTCF-depleted B-cell leukemia BALL-1 cells. We found a higher incidence of chromosomal abnormalities, including twofold increases in chromosomal breakage (Fig. 1 *A* and *C*) and end fusions (12, 13) (Fig. 1 *B* and *D*). As chromosomal instability usually manifests as nuclear buds (NBUDs), nucleoplasmic bridges (NPBs), micronuclei (MNi), and increased apoptosis, we evaluated the effect of CTCF depletion on these markers (14) and found significant increases in NBUD, NPB, and MNi formation, along with an elevated percentage of apoptotic cells (Fig. 1 *E*–*K*). These results suggest an increased level of genomic instability and DNA damage in CTCF-depleted cells.

We next measured the effect of CTCF depletion on UV-induced genomic DNA fragmentation using the comet assay, and observed that the UV-treated cells produced significant amounts

Significance

CTCF interacts with the genome through thousands of sites and organizes the genome into topological domains, but whether CTCF has direct functions in the maintenance of genome stability is not known. Here, we report that CTCF depletion increases chromosomal instability and activates the DNA damage response. We show that CTCF is recruited to sites of DNA lesions in a process that depends on DNA damage signaling and the DNA-binding domain of CTCF, and that CTCF participates in homologous recombination repair of DNA double-strand breaks by interacting with Rad51 and promoting Rad51 repair foci formation. Thus, CTCF maintains genome stability by participating in DNA repair, highlighting a potential link between genome organization and genome stability.

Author contributions: F.L. and J.Z. designed research; F.L., X.L., W.Z., Z.L., D.L., G.C., D.G., L.Y., and J.F. performed research; and F.L., Z.L., P.S., and J.Z. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1704076114/-/DCSupplemental.

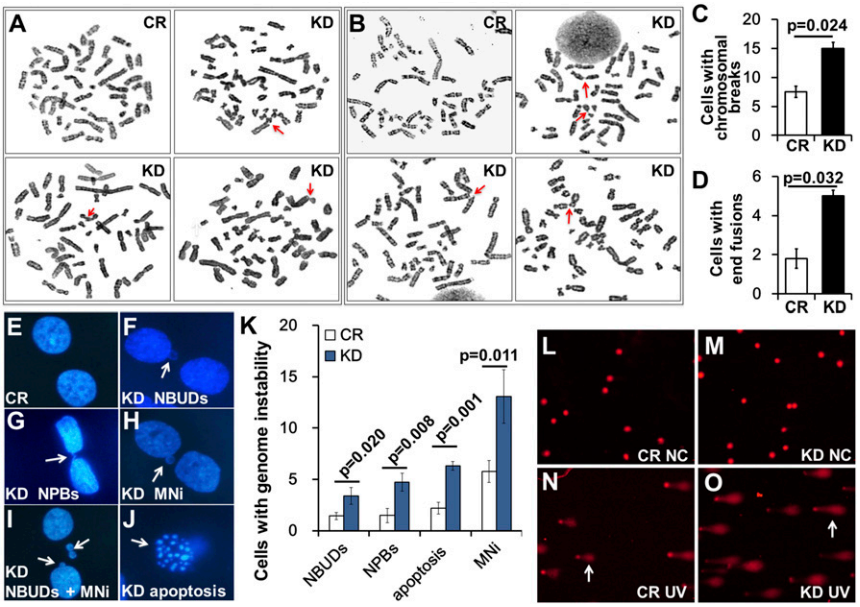

Fig. 1. CTCF knockdown led to chromosomal instability and aggravated drug-induced genome fragmentation. (*A* and *C*) Cytogenetic analysis of chromosomal breaks in control (CR) or CTCF knockdown (KD) BALL-1 cells (arrows). (*B* and *D*) Cytogenetic analysis for telomere fusions before and after CTCF depletion in BALL-1 cells. Arrows indicate fused chromosomal ends. (*E*–*K*) Representative images (arrows) of NBUDs, NPBs, and MNi, as well as apoptotic cells, in the CTCF-depleted BALL-1 cells. Statistical results from *E*–*J* were obtained from analysis of >100 mitotic cells each. (*L*–*O*) U2OS cells were transfected with siCR (CR) or siCTCF (KD) for 72 h, then plated in six-well plates for another 24 h before being treated with 60 J/cm^2 of UV, followed by collection for comet analysis. Arrows point to genomic DNA migrated away from the original location in the nucleus.

of fragmented genomic DNA that migrated away from the original locations, while untreated cells showed little or no migration after electrophoresis (Fig. 1 *L* and *N*). CTCF depletion increased the amount and migration distance of this fragmented genomic DNA (Fig. 1 *M* and *O*). Statistical analysis confirmed that the percentage of tail DNA and the extent of tail movement increased after CTCF knockdown, indicative of more DNA breaks (Fig. S1*A*). A similar effect of CTCF knockdown was seen in these cells treated with etoposide (ETO), a topoisomerase II inhibitor known to induce a large amount of DSBs (Fig. S1 *B* and *C*). Because genotoxic stress treatments will result in apoptosis in treated cells, we measured the effects of CTCF depletion on UV- and ETO-induced apoptosis, and found that the knockdown indeed increased apoptosis in these treated cells (Fig. S1 *D* and *E*). Taken together, these results demonstrate that CTCF prevents genomic instability.

CTCF Knockdown Activates the DDR. Defects in genome maintenance cause the accumulation of unrepaired genomic lesions, the increased expression of common fragile sites (CFS), and the activation of the DDR (15, 16). To determine whether CTCF depletion increases DDR activation, we examined the immunofluorescence signal of phosphorylated histone variant H2AX (γH2AX) in CTCF-depleted cells, as γH2AX is an indicative of ongoing DNA repair and unresolved DNA lesions (17). We found a strong increase in the number of γH2AX foci in CTCF knockdown cells compared with control cells (Fig. 2*A* and Fig. S2*A*). Consistent with this, CTCF depletion increased ETO and camptothecin (CPT; a topoisomerase I inhibitor inducing single-stranded DNA breaks) increased the number of γH2AX foci (Fig. S2 *C* and *D*). We also examined 53BP1 immunofluorescence signals in CTCF-depleted cells, as 53BP1 is a key factor in repairing DSBs generated by fragile site expression and other types of DNA damage (18, 19). We saw similar increases in 53BP1 foci number by CTCF knockdown in untreated cells (Fig. 2*B* and Fig. S2*B*) and in ETO-treated cells (Fig. S2*E*). To further test whether CTCF prevents genomic instability, we examined the effect of CTCF depletion on γH2AX occupancy at several known fragile sites by chromatin immunoprecipitation (ChIP) followed by quantitative PCR (20). Indeed, knockdown of CTCF in human primary fibroblast BJ cells led to increased γH2AX binding at all sites examined except site chr21-2 (Fig. 2*C*). These results suggest that CTCF maintains genome stability, and that its depletion activates DDR signaling.

To directly measure the activation of DDR, we analyzed key DDR proteins by Western blot, and found that the phosphorylation level of ATM was strongly increased by CTCF knockdown in CPT- and ETO-treated cells (Fig. 2*D* and Fig. S2*F*), while total ATM expression remained unchanged. In addition, Rad51, a single-stranded DNA-binding protein responsible for HR repair (21, 22), showed modest increases in CTCF depleted cells. In contrast, the ATR kinase was only minimally affected by the knockdown (Fig. 2*D* and Fig. S2*F*), as was the level of PARP1, a protein that mediates single-stranded DNA repair. Thus, CTCF knockdown led to a stronger activation of the ATM kinase in drug-treated cells and thus an elevated level of DDR.

CTCF Is Recruited to Sites of DNA Damage. To determine whether CTCF is present at sites of DNA damage, we combined immunofluorescence with fluorescence in situ hybridization (immune-FISH) and observed increased colocalization of CTCF, 53BP1, and chr7 following fragile site expression-inducing aphidicolin

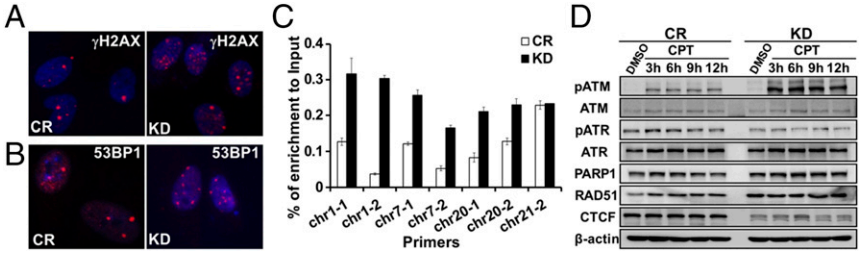

Fig. 2. CTCF knockdown activates the DNA damage response. (*A* and *B*) γH2AX (*A*) and 53BP1 (*B*) immunofluorescence in CR and CTCF knockdown (KD) BJ cells. (*C*) γH2AX ChIP experiment in CR and CTCF KD cells. The genomic regions amplified were as described previously (20) ($P < 0.05$, except for chr21-2). Primers sequences are listed in Table S1). (*D*) Western blot analysis in CR or CTCF KD U2OS cells treated with DMSO or CPT. β-actin was used as a loading control.

(APH) treatment (Fig. 3 *A*–*C*). Statistical analysis showed that APH treatment produced a 45% increase in the colocalization frequency of CTCF, 53BP1, and chr7 (from 6.0% to 8.7%; Fig. 3*D*), suggesting that CTCF is recruited to expressed fragile sites. The localization of CTCF to sites of DNA damage is also supported by immunofluorescence analysis of either APH-treated (to induce DNA breaks at fragile sites in the genome) or ETO-treated (to induce DSBs) cells, where CTCF colocalized with most of the 53BP1 foci in both untreated and drug-treated cells, suggesting that CTCF was recruited to the induced DNA damage sites (Fig. 4 *A* and *B*).

To test whether CTCF directly interacts with sites of DNA lesions, we measured CTCF occupancy at CFS 1q23.2, FRA7D, 20q13.33, and 21q22.3 by ChIP in U2OS cells, as these sites have been shown to be enriched with γH2AX in U2OS (20). We found significant of CTCF enrichment at these tested fragile sites compared with the control site, chr21-2, a site with little or no CTCF binding (Fig. 3*E*). Importantly, CTCF occupancy at these sites increased following APH treatment, suggesting that CTCF binding to these fragile sites was induced as a result of increased DDR signaling and fragile site expression. In particular, the induced CTCF binding to FRA 7D (tested by primer chr7-2; Fig. 3*E*) on chromatin 7 after APH treatment is consistent with the immuno-FISH experiments shown in Fig. 3 *A*–*C*. These results suggest that CTCF interacts with the DNA sequences near the sites of DNA lesions, and that the binding could be induced by the DDR.

To obtain more direct evidence supporting the interaction of CTCF at sites of DNA damage, we investigated whether CTCF interacts with the DNA near DSB ends by ChIP in a U2OS cell line carrying the DR-GFP reporter (23, 24) (Fig. S3*C*). Binding of CTCF to the DNA sequences around the I-Sce I endonuclease

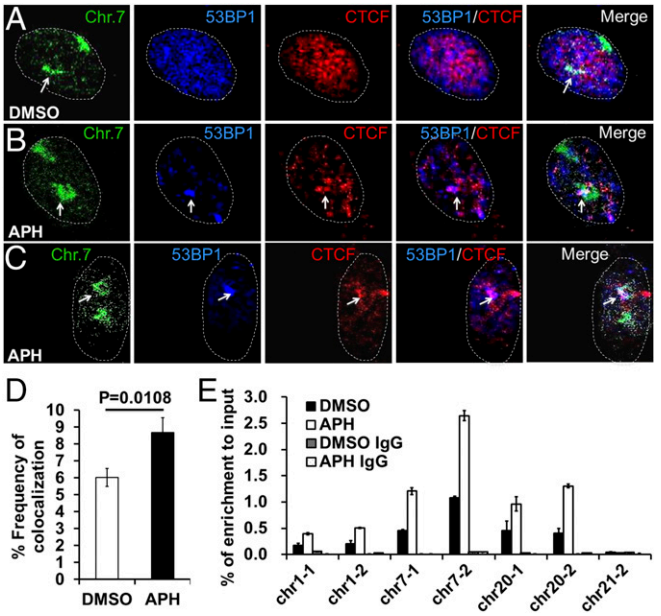

Fig. 3. Interaction of CTCF with fragile sites following APH treatment. (*A*–*C*) BJ cells were treated with DMSO (*A*) or 0.4 μM APH (*B* and *C*) for 24 h, followed by serum starvation for another 24 h. DNA immuno-FISH was performed with whole chromosome painting probes. The procedure was combined with immunofluorescence using antibodies against 53BP1 and CTCF. Representative images of colocalization of CTCF, 53BP1, and chr7 are shown. (*D*) Colocalization frequency of 53BP1 and CTCF immunofluorescence signals, and the FISH signals of chr7 as shown in *A*–*C*. (*E*) CTCF ChIP conducted in U2OS cells that had been incubated in DMSO or 0.4 μM APH for 24 h before being fixed with 1% formaldehyde. The genomic regions amplified were chosen according to previously published ChIP sequencing data (20) ($P < 0.05$, except for the control site chr21-2).

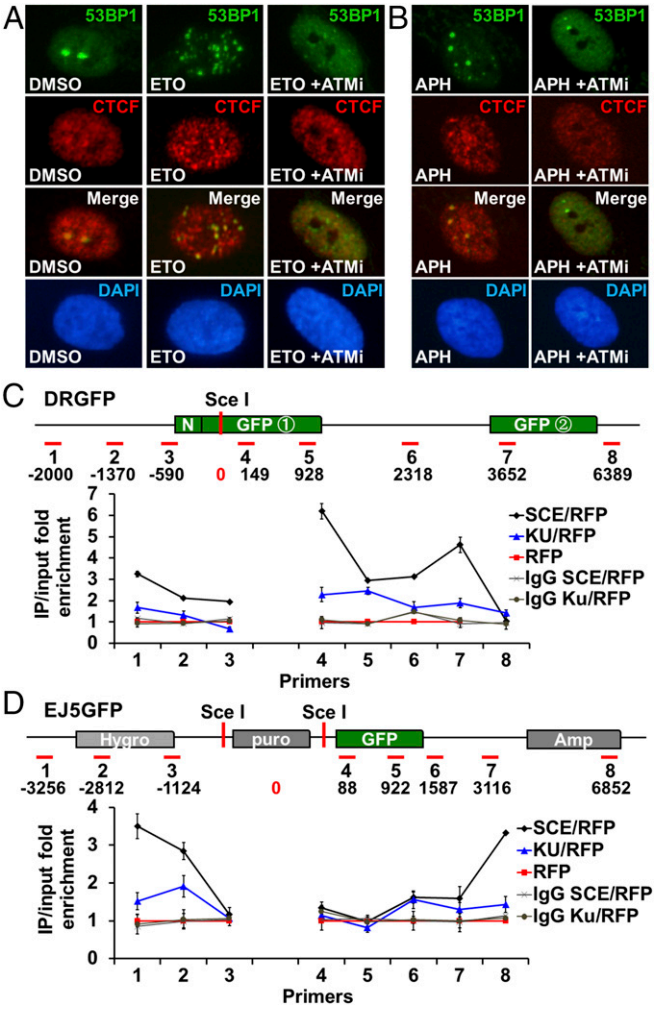

Fig. 4. CTCF is recruited to sites of DNA damage. (*A*) Immunofluorescence using anti-53BP1 and anti-CTCF antibodies in BJ cells treated with DMSO and 25 μM ETO in the presence or absence of ATMi or KU55933 for 1 h. (*B*) CTCF and 53BP1 immunofluorescence in BJ cells treated with 0.4 μM APH for 24 h in either the presence or absence of ATMi for 3 h. (*C* and *D*) DR-GFP U2OS (*C*) and EJ5-GFP-U2OS (*D*) cells were transfected with either a blank plasmid plus an RFP plasmid or an I-SceI expression plasmid plus the RFP plasmid. For ATM inhibition, these cells were pretreated with ATMi (25 mmol/L) for 1 h and then incubated with the inhibitors for an additional 8 h during I-SceI transfection. ChIP was performed 24 h later with CTCF antibody. The ChIP DNA was then analyzed by quantitative PCR using primers designed for different regions on the DR-GFP and EJ5-GFP plasmids. The red bars and the coordinates represent distances from the I-SceI cutting sites. ChIP signals are presented here as fold of enrichment over control.

cutting site was minimal in the absence of the I-Sce I enzyme, but was strongly induced by introducing I-Sce I, as shown in Fig. 4*C*. A similar result was also using a second inducible DSB system in a U2OS cell line carrying the EJ5-GFP reporter (23), where CTCF is also recruited to most sites flanking the I-Sce I induced break point (Fig. 4*D*). These results confirm that CTCF was recruited to DSB ends, consistent with the increased recruitment of CTCF seen in drug- induced sites of DNA damage (Fig. 3). Of interest is the apparent absence of a CTCF-binding motif in DR-GFP and EJ5-GFP reporters, suggesting that the interaction between CTCF and sites of DNA damage is independent of a known DNA-binding consensus in the experiments described in Fig. 4 *C* and *D*, unlike most other reported CTCF-binding sites in the genome, where a consensus is usually present (1).

CTCF Recruitment Depends on ATM, NBS, and the DNA-Binding Domain of CTCF. To determine whether DDR signaling is necessary for CTCF recruitment to sites of DNA damage, we used an inhibitor of the ATM kinase (ATMi), KU55933, to block the DDR in ETO- and APH-treated cells. We observed a strong inhibition of CTCF recruitment to the 53BP1 foci, along with simultaneous reductions of both 53BP1 foci number and size (Fig. 4 *A* and *B* and Fig. S3 *A* and *B*), suggesting that CTCF recruitment to the drug-induced DNA damage sites is ATM-dependent. To confirm this, we examined CTCF binding to DNA sequences near DSB ends by ChIP in the presence of ATMi, and indeed observed significant reductions in CTCF occupancy near DSBs in both DR-GFP and EJ5-GFP reporters (Fig. 4 *C* and *D*). Thus, CTCF recruitment to DSBs requires DDR signaling initiated by ATM.

To further characterize CTCF recruitment to sites of DNA damage, we used a laser microbeam to induce DNA lesions in U2OS cells, followed by double-immunofluorescence staining with γH2AX and CTCF antibodies. Laser microirradiation resulted in colocalization of CTCF with the DSBs tracks in the irradiated nucleus (indicated by γH2AX signals; Fig. 5*A*), but this colocalization was absent in ATMi-treated and ATM-deficient cells (Fig. 5 *B* and *C*), confirming the observations in Fig. 4.

Using this method, we then examined CTCF recruitment in cell lines deficient for NBS1, 53BP1, or RNF8 (Fig. 5 *D*–*H*), and found that cells deficient in NBS1 also failed to recruit CTCF to the laser-irradiated regions (Fig. 5*D*), while 53BP1 or RNF8 deficiency did not affect this recruitment (Fig. 5 *E*–*H*). This result suggests that the Mre11-Rad50-Nbs1 (MRN) complex (25) is necessary for the recruitment of CTCF to sites of DNA damage.

To dissect the domains of CTCF responsible for its recruitment, we created fusion proteins between sections of CTCF and the RFP protein. The full-length CTCF molecule was divided into several sections, and RFP tags were added (26, 27) (Fig. S3*D*). The full- length N- and C-terminal truncations colocalized with laser-induced DNA damage regions, while the N and C termini alone showed no or minimal recruitment into the laser-induced regions of DNA damage (Fig. 5*I*). The zinc finger region showed weak but detectable recruitment, and when one to seven zinc finger domains were removed from CTCF (28), recruitment was essentially abolished (Fig. 5*I*). Thus, the zinc finger region is necessary for targeting CTCF to laser-induced sites of DNA damage. Taken together, the results from Figs. 4 and 5 demonstrate that CTCF recruitment to regions of DNA damage is ATM, NBS1, and CTCF DNA-binding domain dependent.

CTCF Promotes HR-Directed DNA DSB Repair. To test whether CTCF functions in DSB repair, we used two established assay systems, DR-GFP and EJ5-GFP (Fig. S3*C*), to measure the effect of CTCF depletion on HR and nonhomologous end-joining repair (NHEJ), respectively. We found that when CTCF level was reduced by siRNA, there was a 47% reduction in relative HR efficiency, from 1.0 to 0.53 (Fig. 6*A*), suggesting a function of CTCF in HR. In contrast, knockdown elevated the efficiency of NHEJ from 1.0 to 1.34, a 34% increase (Fig. 6*A*), indicating that CTCF is not necessary for NHEJ. Since NHEJ is a compensatory repair choice when HR is blocked, this result is also consistent with a role of CTCF in HR, a phenotype similar to the depletion of several repair factors in the HR pathway (29–31). In these experiments, an RFP expression plasmid was included to control for transfection efficiency, while cell cycle distributions were assessed before and after CTCF knockdown in the modified U2OS cell lines to control for cell cycle effects. We found no significant differences in transfection efficiency between control cells and knockdown cells (Fig. 6*B*), or any significant changes in the cell population at different stages of the cell cycle after CTCF knockdown (Fig. 6*C*). Thus, these experiments suggest that CTCF promotes an HR type of DNA repair.

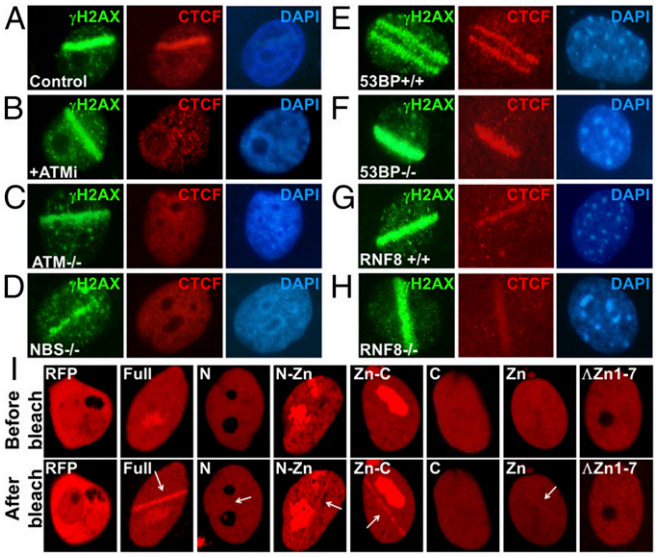

Fig. 5. CTCF recruitment depends on ATM, NBS, and the DNA-binding domain of CTCF. (*A*–*H*) Laser irradiation analysis was done with an Olympus FluoView FV1000 confocal microscope in U2OS cells (*A*), U2OS cells in the presence of ATMi (*B*), human ATM-deficient cells (*C*), NBS1- deficient cells (*D*), wild-type MEF cells from a 53BP1 control mouse (*E*), 53BP1-deficient mouse MEF cells (*F*), wild-type MEF cells from an RNF8 control mouse (*G*), and RNF8-deficient MEF cells (*H*). These cells were scanned with a 405-nm laser and then fixed for immunofluorescence. γH2AX antibody was used to detect laser-induced sites of DNA lesions, while CTCF antibody was used to detect potential enrichment of CTCF at sites of damage. (*I*) RFP-tagged full-length CTCF and domains of CTCF were transfected into U2OS cells, followed by laser microirradiation and observation under the confocal microscope.

CTCF Interacts with Rad51 and Is Necessary for Formation of Rad51-Labeled DNA Repair Foci. To determine how CTCF functions in the HR, we analyzed X-ray irradiation-induced Rad51 foci formation in the CTCF knockdown cells, since Rad51 is a key repair factor in HR (32–34), and aggregates into DNA repair foci after ionizing irradiation or DNA damage-inducing drug treatment (35, 36). We found that the knockdown decreased the percentage of cells with five or more X-ray–induced Rad51 foci decreased from 42.13% to <10.17% (Fig. 6 *D* and *E*). A similar result was obtained in cells treated with methyl methanesulphonate (MMS), a drug that can trigger single-stranded DNA breaks. Here CTCF depletion also led to a decrease in the percentage of Rad51 foci-positive cells (Fig. S4 *A* and *B*). We further tested the effect on Rad51 recruitment by CTCF depletion in the laser-induced DNA lesion experiments. Cells depleted of CTCF consistently showed deficient Rad51 recruitment to laser-induced DNA damage regions (Fig. 6 *F* and *G*). In contrast, XLF, a protein in the NHEJ repair pathway, was not affected by the knockdown (Fig. S4 *C* and *D*). To confirm the effect of CTCF depletion on the recruitment of Rad51, we performed ChIP using an Rad51 antibody, and observed decreased Rad51 binding in two tested sites (primers 4 and 6 in the DR-GFP reporter; Fig. 4*C*) after CTCF knockdown (Fig. 6*H*). We also examined the effect of CTCF knockdown on RPA S4/S8 foci formation, and found that these foci were unaffected (Fig. S5). Taken together, these results suggest that CTCF promotes HR by facilitating the recruitment of Rad51.

To determine whether CTCF physically interacts with any repair components, we conducted in vivo coimmunoprecipitation (co-IP) experiments and found that endogenous CTCF interacts with MDC1, Rad51, and AGO2 but not with other tested DDR components, including BRCA1, 53BP1, RPA32, ATR, ATRIP, and TOPBP1 (Fig. S6*A*). Analysis of anti-CTCF immunoprecipitates from U2OS cells incubated with antibody to Rad51,

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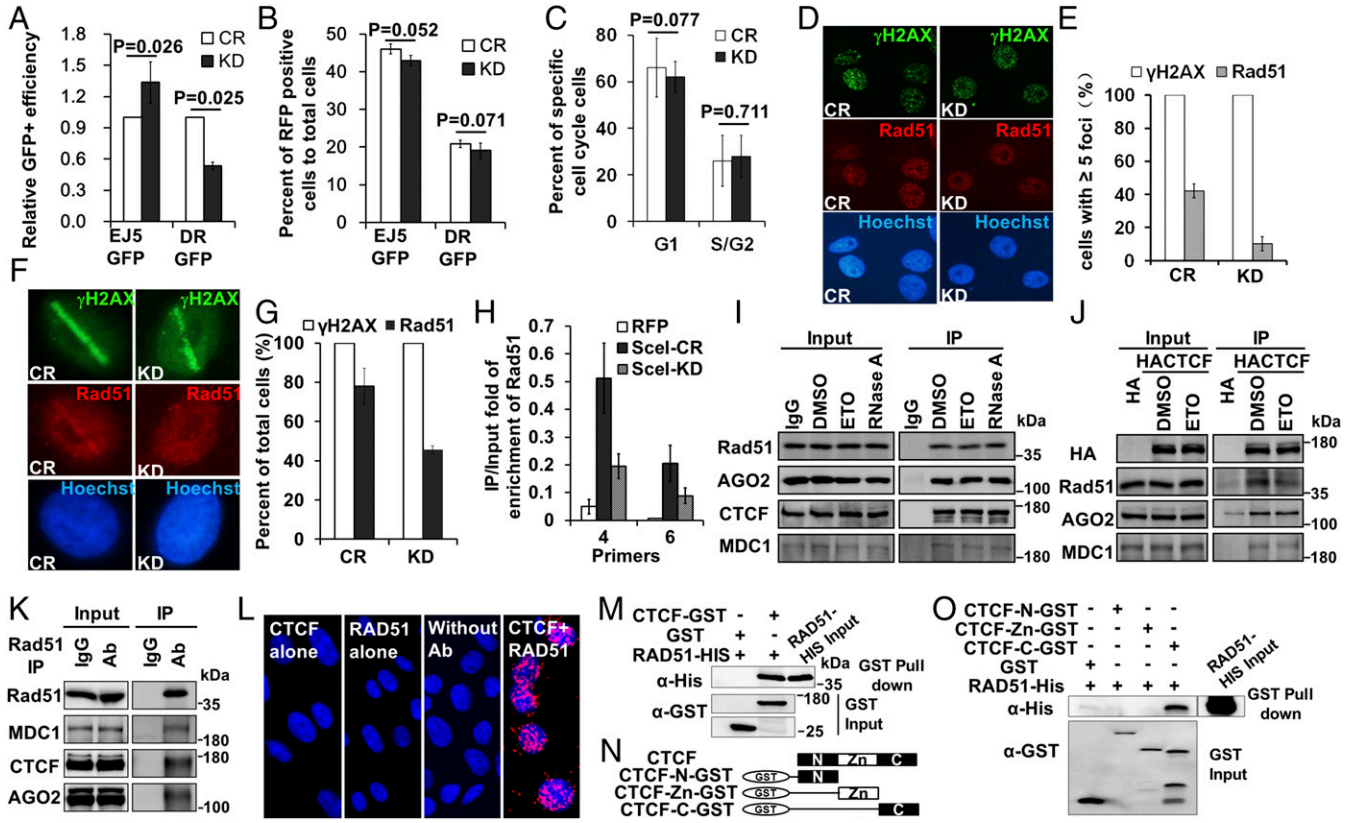

Fig. 6. CTCF promotes HR-directed DSB repair. (*A*) After CTCF depletion for 48–72 h, DR-GFP U2OS or EJ5-GFP-U2OS cells were transfected with I-SceI plasmid. After another 48 h, GFP expression was detected with FACS. (*B*) Plasmids expressing RFP were also transfected into the aforementioned cells to monitor for transfection efficiency. (*C*) Propidium iodide (PI) was used to stain the DNA, and DNA content was quantified by FACS to monitor the cell cycle distribution of these cells. (*D* and *E*) CTCF was knocked down for 48–72 h in U2OS cells, followed by X-ray irradiation (with 5 Gy). After a 1-h recovery, the cells were fixed for immunostaining to detect Rad51 recruitment ($P < 0.001$). (*F* and *G*) U2OS cells were transfected with siCR (NC) and siCTCF (KD) for 72 h before be subjected to laser scanning and immunostaining to detect Rad51 recruitment ($P < 0.05$). (*H*) ChIP analysis of Rad51 recruitment at two locations of high CTCF occupancy (Fig. 4*C*) near I-SceI–induced breaks in the DR-GFP U2OS cell line before and after CTCF knockdown ($P < 0.05$). (*I*) U2OS cells were treated with DMSO or 25 μM ETO for 1 h, and the lysates were untreated or treated with 100 μg/mL RNase A before being subjected to immunoprecipitation using CTCF antibody- or IgG-preincubated Dynabeads. Immunoprecipitates (IP) and whole-cell extracts (Input) were immunoblotted with the indicated antibodies. (*J*) HA-CTCF expression plasmid or HA vector transfected in U2OS cells were treated with DMSO or 25 μM ETO for 1 h. The cell lysates were then subjected to immunoprecipitation using HA antibody-preincubated Dynabeads, and Western blot analysis was performed with the indicated antibodies. (*K*) U2OS cells lysates were subjected to immunoprecipitation using Rad51 antibody- or IgG-preincubated Dynabeads, followed by Western blot analysis with the indicated antibodies. (*L*) CTCF and Rad51 interactions in U2OS cells detected by PLA, shown as red spots. (*M*) GST or GST-CTCF was preincubated with GST-tagged protein purification resin for 2 h at 4 °C, followed by the addition of His-Rad51 and overnight incubation at 4 °C. Complexes were washed six times with binding buffer. Proteins were visualized by Western blot analysis using the indicated antibodies. (*N*) Diagram of GST-tagged regions of CTCF. (*O*) GST or GST-tagged truncations of CTCF were preincubated with GST-tagged protein purification resin, followed by the addition of His-Rad51 and incubation for pull-down analysis. Proteins were visualized by Western blot analysis using the indicated antibodies.

AGO2, and MDC1 revealed that these interactions were not affected by ETO or RNase A treatment, suggesting that the interactions are constitutive and independent of RNA (Fig. 6*I*). In agreement with interaction between endogenous CTCF and these DNA repair factors, the HA-tagged exogenous CTCF also exhibited interactions with Rad51, AGO2, and MDC1 (Fig. 6*J*). In the reciprocal experiments, anti-Rad51, -AGO2, and -MDC1 immunoprecipitates immunoblotted with antibody to CTCF also showed associations between CTCF and Rad51, AGO2, and MDC1 (Fig. 6*K* and Fig. S6 *B* and *C*). The CTCF–Rad51 interaction suggests that CTCF promotes HR repair foci formation by facilitating Rad51 recruitment.

The in vivo CTCF–Rad51 interaction was further confirmed by co-IP using cotransfected HA-tagged CTCF and His-tagged Rad51 (Fig. S7*A*), and confirmed by a proximity ligation assay (PLA) (37), which allows direct visualization of in vivo protein–protein interactions (Fig. 6*L*). To determine whether the CTCF–Rad51 interaction is direct, we performed an in vitro GST pull-down analysis, and found that GST-CTCF can pull down His-Rad51 (Fig. 6*M*) and that GST-Rad51 can pull down His-CTCF (Fig. S7*B*). To determine the domain of CTCF responsible for the interaction, we examined the N and C termini and the zinc finger domain of CTCF, and found that the C terminus of CTCF directly interacts with Rad51 in both GST pull-down and co-IP analyses (Fig. 6 *N* and *O* and Fig. S7 *C* and *D*). These experiments suggest that CTCF may promote Rad51 repair foci formation by facilitating Rad51 recruitment.

Discussion

Here we have provided several lines of evidence supporting a previously undescribed function of CTCF in the maintenance of genome stability and DNA repair. CTCF knockdown increased genomic instability, marked by elevated levels of chromosomal breakage and fragile site expression. In addition, CTCF was physically recruited to sites of DNA lesions, with recruitment dependent on DNA damage signaling, the MRN complex, and the zinc finger DNA-binding domain of CTCF. Furthermore, CTCF participated in the HR type of DSB repair by promoting the recruitment and thus the formation of Rad51-dependent DNA repair foci. These activities suggest a potential link between genome organization and genome maintenance.

Considering its multiple reported nuclear activities, CTCF also could contribute to genome maintenance indirectly, for example, by regulating gene transcription. A recent study reported that CTCF maintains p53 expression and promotes p53 function in the DNA damage response through epigenetic regulation and binding to the antisense transcript originated from the p53 locus (27), suggesting that the RNA-binding properties of CTCF may play a role in DNA damage repair. Here we found that recruitment of CTCF to sites of DNA damage did not depend on the RNA-binding domain of CTCF, and that RNase A did not disrupt the association of CTCF and other DDR proteins (Fig. 6*I*), suggesting that the DNA repair activity of CTCF that we observed is separable from the effect of CTCF on p53.

The interaction of CTCF and sites of DNA damage exhibits different characteristics from most reported CTCF binding in the genome, in that it is induced by DNA damage and requires DNA damage signaling, but does not appear to require a known DNA-binding motif. This finding leads to the interesting possibility that CTCF binding to the genome could be far more dynamic and regulated than has been reported previously (2, 3, 38). Precisely how CTCF acts in DNA repair will require extensive further investigation. Since CTCF directly interacts with Rad51, and CTCF knockdown disrupted the formation of Rad51 repair foci but did not affect single-stranded DNA formation (RPA S4/S8 foci formation was unaffected; Fig. S5), we believe that CTCF acts downstream of RPA but upstream of Rad51 repair foci formation, possibly by facilitating Rad51 recruitment via the C terminus of CTCF.

We hypothesize that CTCF promotes DNA repair by recruiting Rad51, and that together with Rad51, CTCF stabilizes the DSB ends and/or homologous DNA sequences with the local chromosome architectural network, which could prevent the ends of DSBs from drifting apart and could facilitate the pairing of homologous sequences necessary for repair. Such an activity is supported by a "homing" phenomenon of *P* elements in *Drosophila* whereby transposable elements carrying genomic sequences containing CTCF-binding sites tend to integrate back to the very genomic regions from which these sequences derive (39).

Materials and Methods

BJ, BALL-1, and U2OS cells were obtained from American Type Culture Collection. DR-GFP-U2OS and EJ5-GFP-U2OS cell lines were obtained from Jeremy Stark, Beckman Research Institute of the City of Hope, Duarte, CA. ATM$^{-/-}$, NBS1$^{-/-}$, 53BP1$^{-/-}$, and RNF8$^{-/-}$ cell lines were obtained from Matthew Weitzman, Children's Hospital of Philadelphia, Philadelphia. Human ATM$^{-/-}$ cells were originally from Yosef Shiloh, Sackler School of Medicine, Tel Aviv University, Tel Aviv, human NBS$^{-/-}$ cells were originally from Patrick Concannon, Genetics Institute, University of Florida, Gainesville, FL, and 53BP1 and RNF8 MEFs were originally from Junjie Chen, The University of Texas MD Anderson Cancer Center, Houston. The sources of antibodies, plasmids, and reagents and detailed methodology for immunofluorescence, immuno-FISH, cytokinesis-block micronucleus cytome assay, laser irradiation analysis, CTCF deletion mutant cloning, siRNA- and shRNA-mediated knockdown of CTCF, cell cycle analysis, apoptosis assays, immunoprecipitation and Western blot analyses, ChIP, recombinant protein expression and purification, GST pull-down assays, and PLA are described in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Matthew Weitzman, Ping Zheng, and Yun Zhang for reagents and Jeremy Stark for reagents, insightful discussions, and a critical reading of the manuscript. This work was supported by Strategic Priority Research Program of the Chinese Academy of Sciences Grants XDB13020400 and KSZD-EW-Z-009; Chinese Academy of Sciences Grant KSCXZ-EW-BR-6; Yunnan Provincial Government Grants 2011HA005, 2013FA051, and 2016FB039; and National Natural Science Foundation of China Grants 81471966, 81672040, U1602226, 2016YFC1200404 (to J.Z.), and 31601157 (to F.L.).

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