Supplementary Information for "Human CtIP promotes DNA end resection"

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This pdf file includes:

Supplementary Methods
Supplementary Figs. S1 to S6 with Legends.
Supplementary References

Supplementary Methods

siRNA transfection

All siRNA duplexes were purchased from MWG-Biotech, with the exception of CtIP-1 and MRE11 siGENOME Smart Pool (main ref. 33) which were obtained from Dharmacon. Sequences were as follows: Luciferase (CGUACGCGGAAUACUUCGA); CtIP-1 (GCUAAAACAGGAACGAAUC, main ref. 16); CtIP-2 (UCCACAACAUAAUCCUAAU, this study); XRCC4

(AUAUGUUGGUGAACUGAGA)[1]; BRCA1 (GGAACCUGUCUCCACAAAG)[2] and RAD51 (GAGCUUGACAAACUACUUC)[3]. Transfections of siRNA were done with 50 nM final concentration of oligos by using Oligofectamine (Invitrogen). In the laser micro-irradation experiments, the control siRNA was against HSP70B[4] and siRNA transfections were done with lipofectamine RNA MAX (Invitrogen). In the Homologous recombination assay, RISC-Free siRNA (Dharmacon) was used as a negative control. Aphidicolin, camptothecin and etoposide were purchased from Sigma. BLEOCIN™ was purchased from Calbiochem.

Antibodies

R. Baer provided a mouse monoclonal antibody to CtIP[5]. Other antibodies used in this study were purchased from Cell Signaling Technology (H2AX-pS139, Chk1-pS345, Chk1-pS317), Sigma (Tubulin), Abcam (XRCC4, Chk2, RAD50, MRE11, NBS1), Upstate (H2A acidic patch), Calbiochem (RPA2 (Ab-3), Chk2-pT68), Oncogene Research Products (BRCA1), Bethyl Laboratories (RPA2-pS4/S8, Chk1, SMC1, SMC1-pS966, SMC3-pS1083[6]) and Santa Cruz Biotechnology (RAD51, Cyclin A, GFP). Polyclonal antisera used in immunoprecipitation experiments were as follows: CtIP-Ct (residues 690-897) and CtIP-Nt (residues 58-369)[5], MRE11 and RAD50[7] and Histone H1 (Abcam).

Lambda-Phosphatase treatment

U2OS cells were scraped in 250 µl lysis buffer containing Benzonase and indicated samples were incubated with lambda-Phosphatase (400 U, New England Biolabs) for 30 min at 30 °C.

FACS and S-phase index analysis

To determine cell-cycle distribution, cells were fixed with 70% ethanol, incubated for 30 min with RNase A (250 µg/ml) and propidium iodide (10 µg/ml) at 37 °C and analysed by FACS. S-phase index (measuring cell growth and viability) in U2OS was determined by immunofluorescent detection of incorporated BrdU. In brief, cells grown on coverslips were pulsed with BrdU (RPN201, 1/500, GE Healthcare) for 30 min, fixed in 3.5%

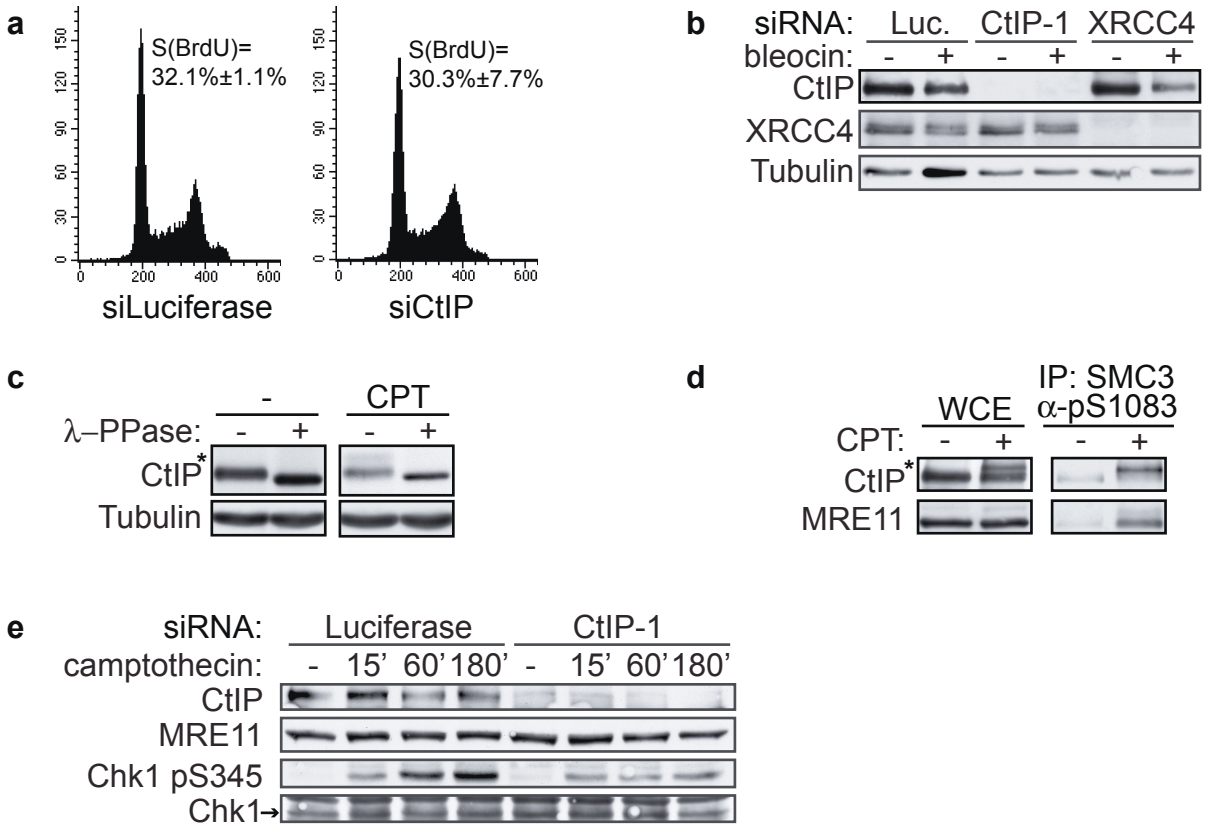
paraformaldehyde for 1 h, washed and permeabilized in 1% Triton X-100 for 5 min. After extensive washing in PBS, cells were blocked in 10% FCS in PBS and incubated for 1 h with a solution containing a mouse monoclonal anti-BrdU antibody (RPN202, 1/100, GE Healthcare) and a nuclease. A mixture of Alexa Fluor-594 (red) conjugated secondary antibody and TOTO-3 stain (Molecular Probes, UK) to detect incorporated BrdU and total DNA in cells was then incubated for 60 min at room temperature in the dark. Coverslips were washed, mounted on glass-slides and imaged immediately for analysis. Alternatively, cells grown on coverslips were pulsed with 100 μM BrdU for 30 min, washed and fixed by adding 70% ethanol. Double-stranded DNA was denatured by incubating the cells in 2N HCl for 30 min. Cells were neutralized by repeated washings in PBS, blocked in 5% FBS in PBS for 1 hour and then incubated with a mixture of anti-BrdU-FITC conjugated antibody (BD Biosciences) and TOTO-3 (Molecular Probes) for 30 min. The average percentage of BrdU positive cells, measured by using these two methods and analyzing at least 600 cells, is shown in Supplementary Figure 1a (inset).

Supplementary References

1. Ahnesorg, P., Smith, P. and Jackson, S. P. XLF interacts with the XRCC4-DNA ligase IV complex to promote DNA nonhomologous end-joining *Cell* **124,** 301-313 (2006).
2. Bruun, D.*, et al.* siRNA depletion of BRCA1, but not BRCA2, causes increased genome instability in Fanconi anemia cells *DNA Repair (Amst)* **2,** 1007-1013 (2003).
3. Ito, M.*, et al.* Rad51 siRNA delivered by HVJ envelope vector enhances the anti-cancer effect of cisplatin *J. Gene Med.* **7,** 1044-1052 (2005).
4. Bekker-Jensen, S.*, et al.* Dynamic assembly and sustained retention of 53BP1 at the sites of DNA damage are controlled by Mdc1/NFBD1 *J. Cell Biol.* **170,** 201-211 (2005).

5. Yu, X. and Baer, R. Nuclear localization and cell cycle-specific expression of CtIP, a protein that associates with the BRCA1 tumor suppressor *J. Biol. Chem.* **275,** 18541-18549 (2000).
6. Matsuoka, S.*, et al.* ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage *Science* **316,** 1160-1166 (2007).
7. Goldberg, M.*, et al.* MDC1 is required for the intra-S-phase DNA damage checkpoint *Nature* **421,** 952-956 (2003).

Sartori *et al*., Supplementary Figure S1



Supplementary Figure S1.

a, Flow cytometric analysis and S-phase index of CtIP downregulated cells.
U2OS cells were transfected with siRNA oligonucleotides and 3 days later later subjected to propidium iodide staining for cell cycle analysis. The S-phase index was determined by immunofluorescent detection of incorporated BrdU. Histograms of DNA content are displayed together with the S-phase index in untreated cells (inset).

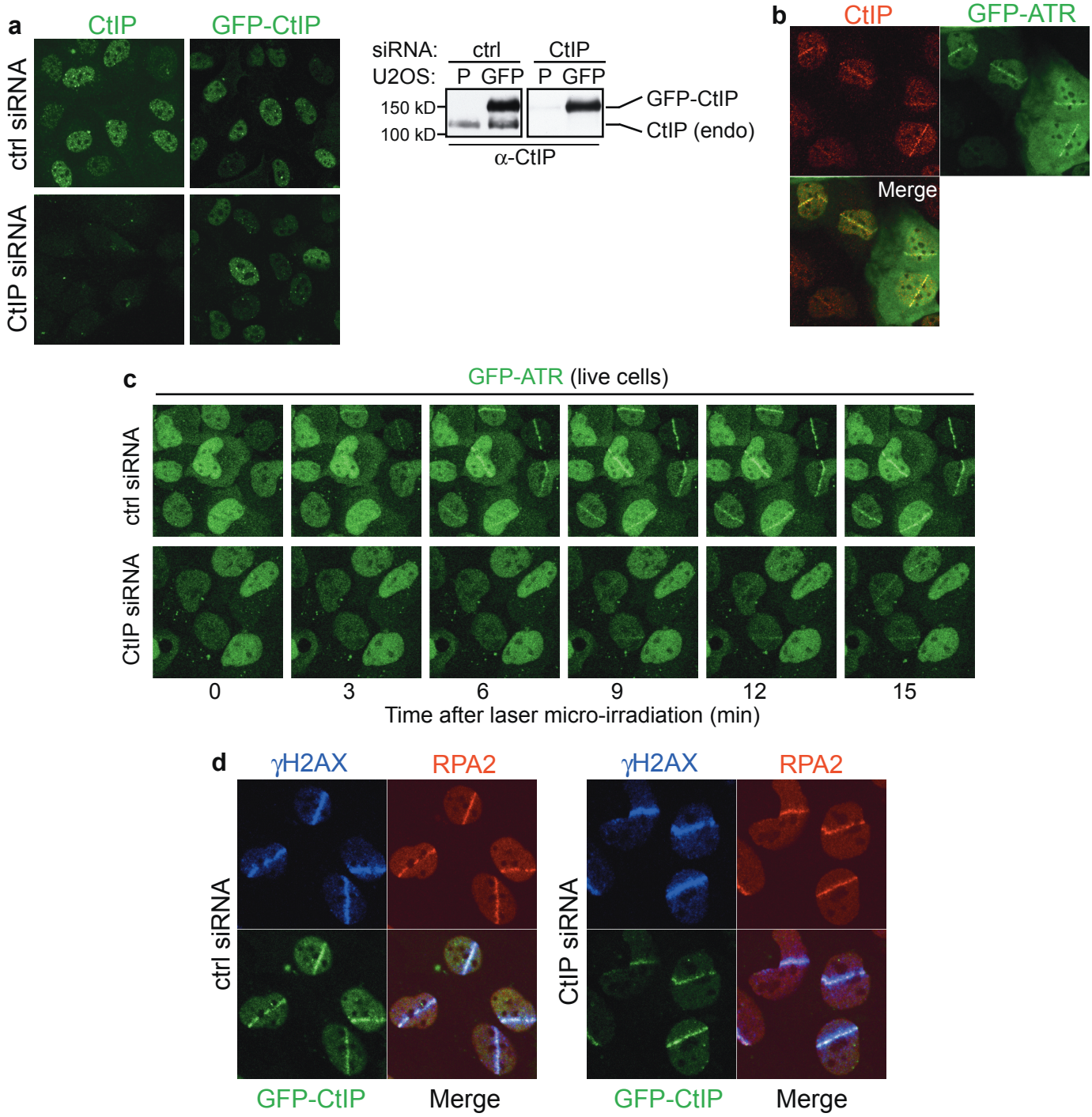
b, Efficient downregulation of CtIP and XRCC4 in U2OS cells.
Extracts were prepared from the same cells as in Figure 1c treated for 1 h with 10 μg/ml bleocin and analyzed by immunoblotting with the indicated antibodies.

c, Changes in CtIP mobility are due to DNA damage-induced phosphorylation.
Whole cell lysates from untreated (-) or camptothecin (CPT)-treated U2OS cells were untreated or treated with lambda-phosphatase and immunoblotted with anti-CtIP and anti-tubulin antibodies.

d, Phosphorylated species of CtIP and MRE11 are co-immunprecipitated with a phospho-specific antibody.
U2OS cells were either left untreated or treated with 1 μM CPT for 1 h. RIPA lysates were pre-cleared with Protein A-Sepharose beads and then incubated with anti-phospho-SMC3 S1083[6] antibody for 2 h. Immunoprecipitated proteins were subjected to immunoblotting with antibodies against CtIP and MRE11. Asterisks in c and d: hyperphosphorylated CtIP.

e, CtIP depletion effects Chk1 phosphorylation at later time-points after CPT treatment.
Extracts from siRNA transfected cells were prepared at different time points upon continous CPT exposure (1 μM) and immunoblotted as indicated. Arrowhead indicates Chk1.

Sartori *et al.*, Supplementary Figure S2



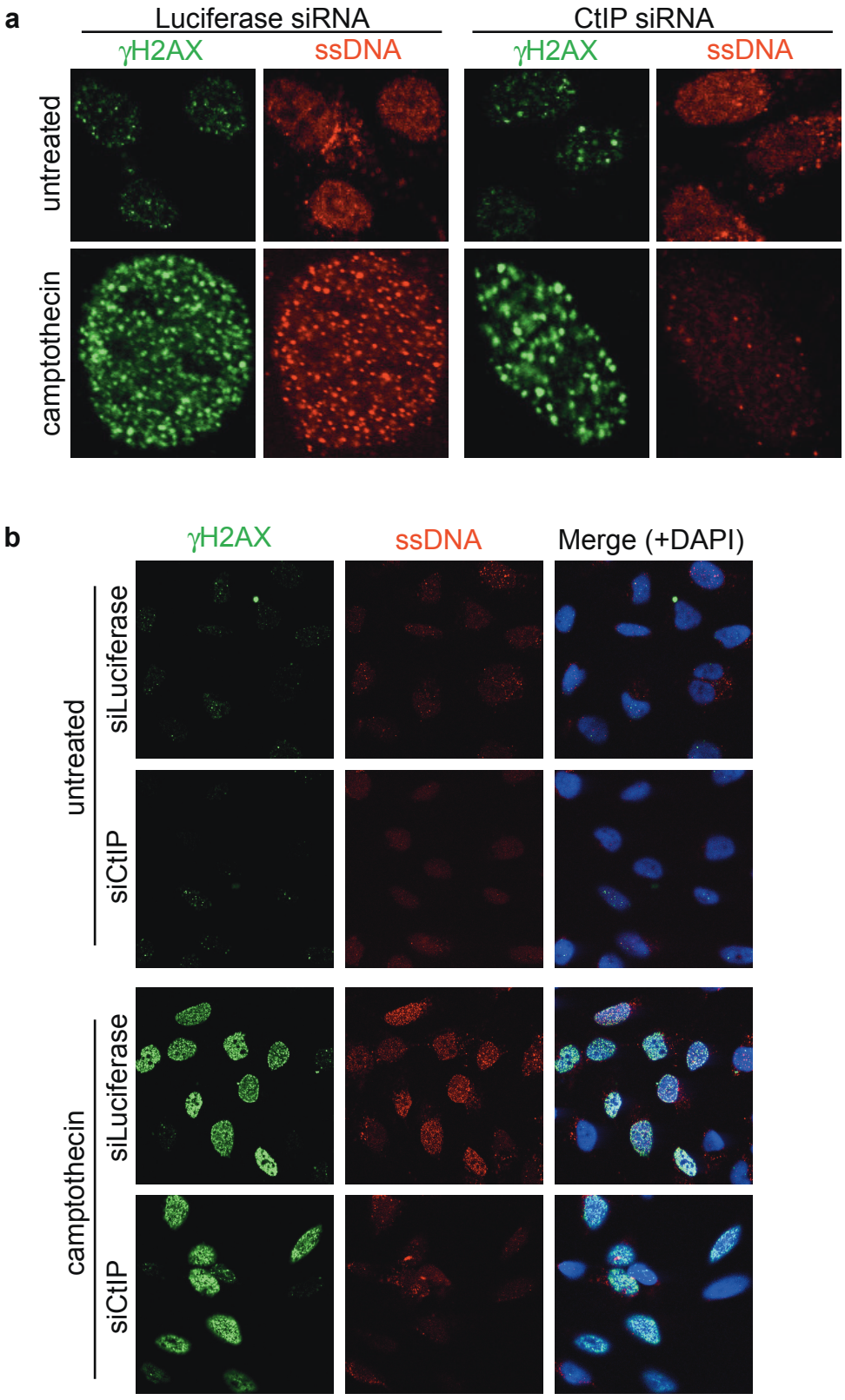
Supplementary Figure S2.

a, Generation of a U2OS cell line stably expressing siRNA-resistant GFP-CtIP.
U2OS cells stably expressing GFP-CtIP and the corresponding parental U2OS cell line ('P') were transfected with either control or CtIP siRNA and two days later analyzed by immunofluorescence (left) and immunoblotting (right) using CtIP monoclonal antbodies. Full-length GFP-tagged CtIP is expressed and resistant to transfection with CtIP siRNA oligonucleotides (lane 4).

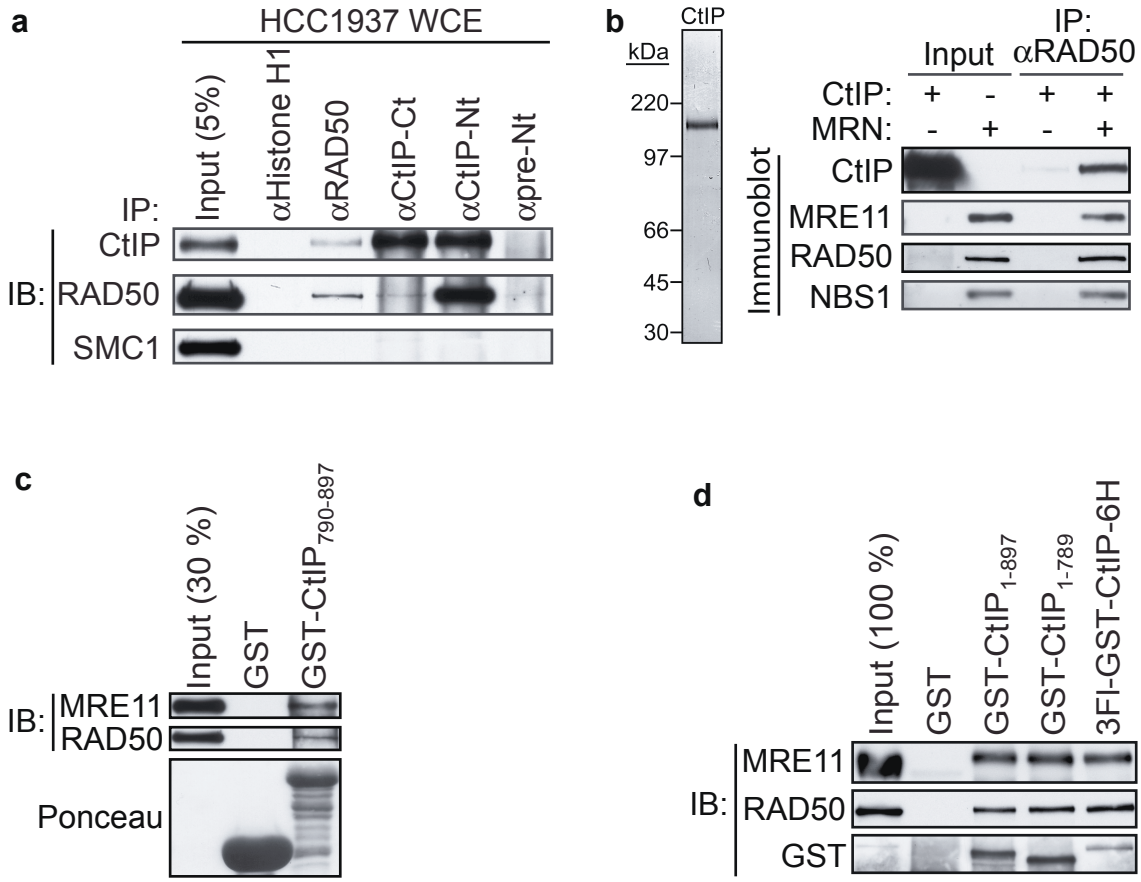
b, CtIP co-localizes with GFP-ATR in DSB tracks. U2OS cells stably expressing GFP-ATR were micro-irradiated and immunostained with CtIP antibodies

c, CtIP downregulation impairs ATR recruitment to DNA damage.
GFP–ATR expressing cells were treated, micro-irradiated and monitored as indicated.

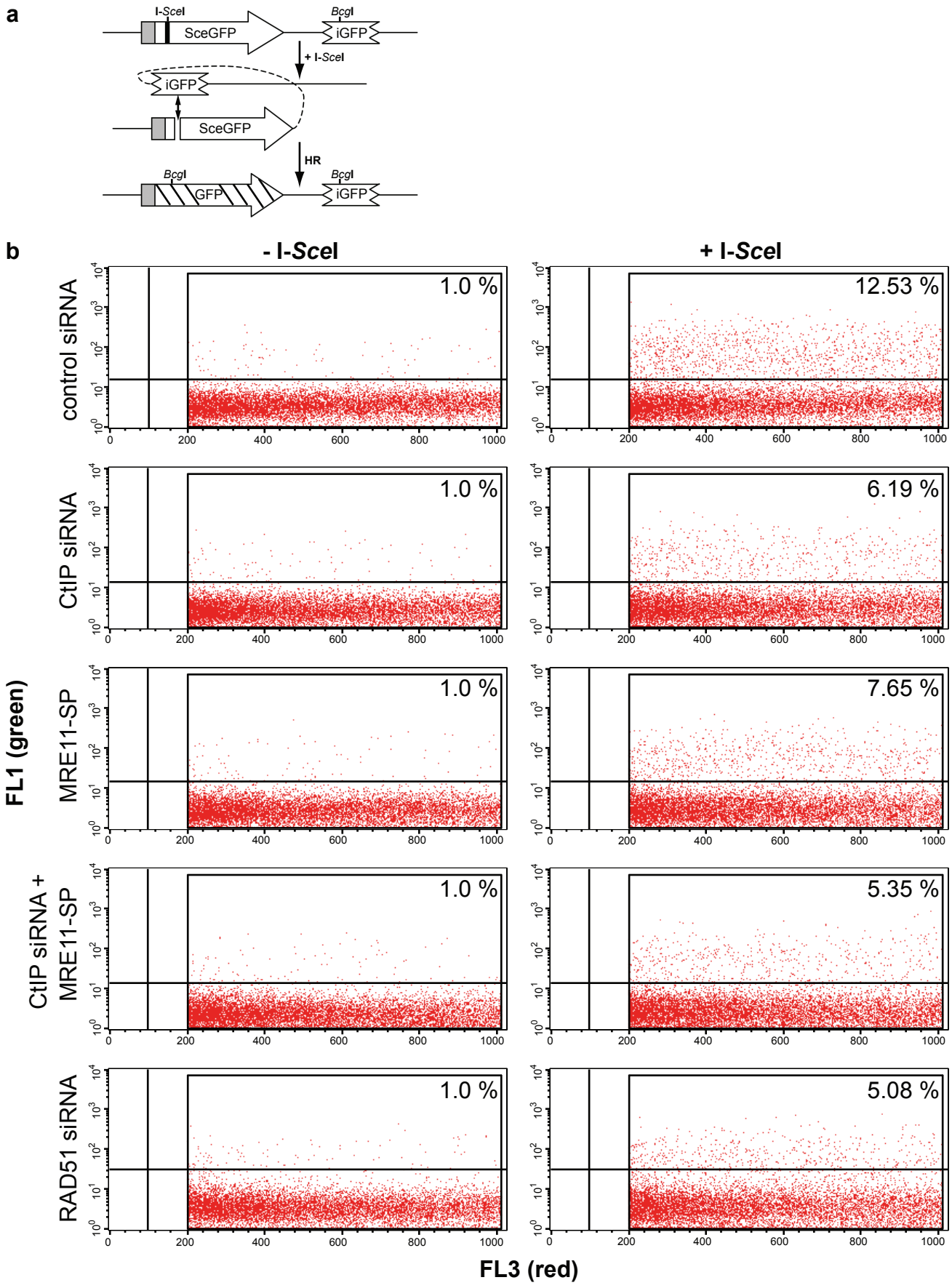
d, siRNA resistant GFP-CtIP expression rescues RPA recruitment to microlaser-induced DSB tracks in the absence of endogenous CtIP. U2OS cells stably expressing siRNA resistant GFP-CtIP were microirradiated and co-immunostained for RPA2 and γH2AX.



Supplementary Figure S3.
a, **b**, CtIP is required for ssDNA formation after camptothecin-incuded DSBs.
U2OS cells were treated as in Figure 3c and co-immunostained using γ-H2AX and BrdU antibodies.

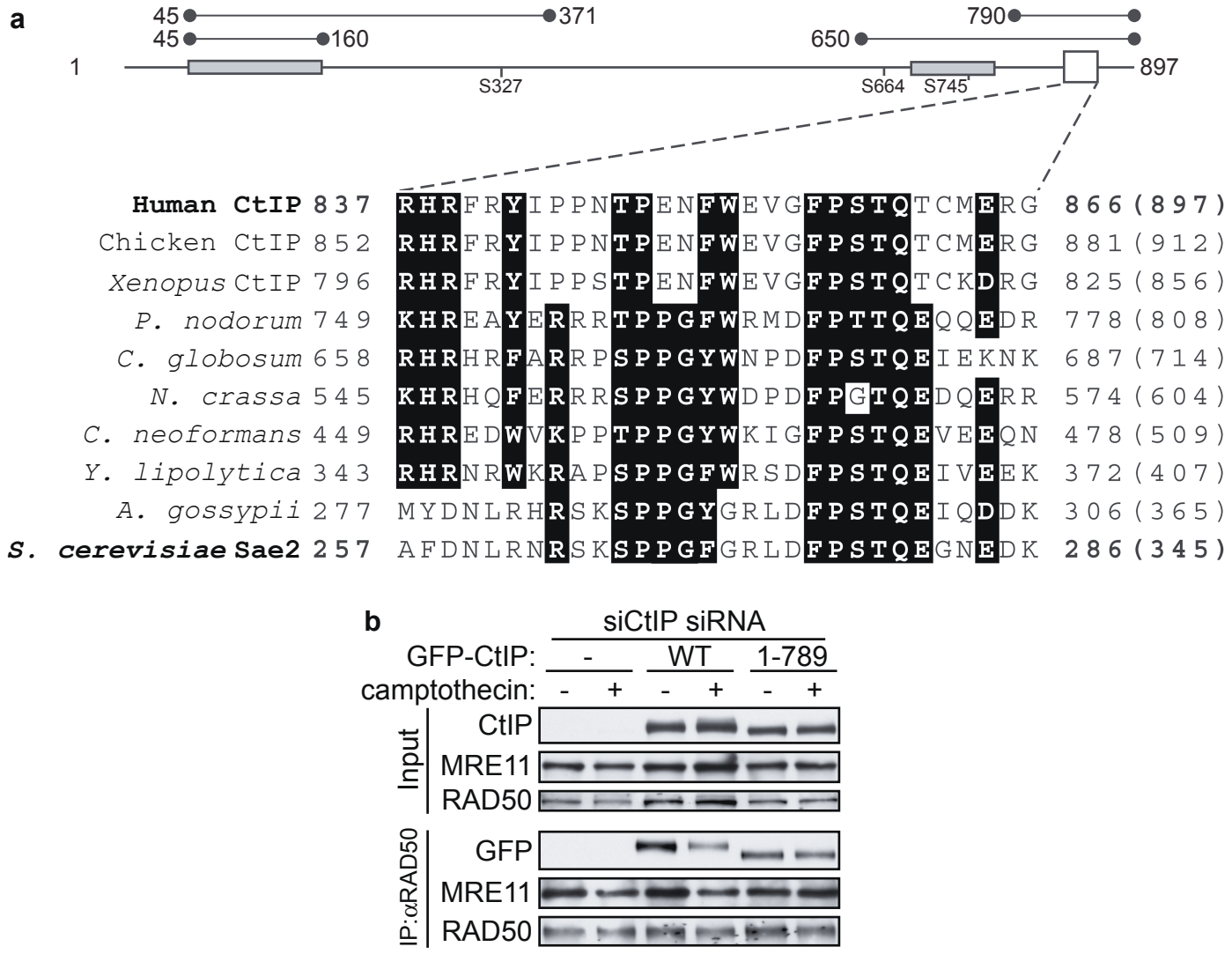


Supplementary Figure S4.
a, RAD50 and CtIP co-immunoprecipitate from HCC1937 extracts.
b, Interaction between purified MRN and CtIP.
Left, Silver-stained gel of recombinant CtIP. *Right*, Purified baculovirus-expressed human MRN and CtIP, or CtIP alone, was immunoprecipitated and immunoblotted.
c, **d**, GST-CtIP fragments bind MR.
Purified human MR was incubated with GST fusions or purified recombinant CtIP bound to sepharose beads, then detected by immunoblotting.



Supplementary Figure S5.
a, Schematic of HR assay.
b, Representative images from the flow cytometric analysis scoring GFP and RFP positive cells in the upper right quadrant as a direct measurement for DSB repair by HR in U2OS-DR-GFP cells (see Methods). 'Risc-free control si-RNA' (Dharmacon) was used as a negative control.

Sartori *et al*., Supplementary Figure S6



Supplementary Figure S6.

a, Evolutionary conservation of the CtIP C-terminus.

Top, Schematic of human CtIP with conserved domains and known phosphorylation sites (main refs. 16 and 43) . Bars are CtIP regions used in pulldown experiments and light grey indicates putative coiled-coil domains. *Bottom*, alignment of the CtIP C-terminus with CtIP/Sae2 homologues from different species. Genbank accessions: Human CtIP (AAC14371), Chicken CtIP (XP_419158), *Xenopus laevis* CtIP (AAH73395), *Phaeosphaeria nodorum* (EAT90897), *Chaetomium globosum* (XP_001224668), *Neurospora crassa* (XP_957865), *Cryptococcus neoformans* (EAL19137), *Yarrowia lipolytica* (XP_502193), *Ashbya gossypii* (NP_984048) and *Saccharomyces cerevisiae* Sae2 (NP_011340).

b, C-terminal deletion of CtIP does not abolish interaction with the MRE11 complex .

Three days after siRNA transfection, U2OS cells stably expressing siRNA resistant GFP-tagged wild-type (WT) or truncated CtIP (1-789) were mock-treated or treated with 1 μM camptothecin for 1 h and then lysed in NP40-buffer containing benzonase. Whole cell extracts were immunoblotted either directly (5 % Input) or after immunoprecipitation with a polyclonal anti-RAD50 antibody with the indicated antibodies.