DNA end resection requires constitutive sumoylation of CtIP by CBX4

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DNA breaks are complex DNA lesions that can be repaired by two alternative mechanisms: non-homologous end-joining and homologous recombination. The decision between them depends on the activation of the DNA resection machinery, which blocks non-homologous end-joining and stimulates recombination. On the other hand, post-translational modifications play a critical role in DNA repair. We have found that the SUMO E3 ligase CBX4 controls resection through the key factor CtIP. Indeed, CBX4 depletion impairs CtIP constitutive sumoylation and DNA end processing. Importantly, mutating lysine 896 in CtIP recapitulates the CBX4-depletion phenotype, blocks homologous recombination and increases genomic instability. Artificial fusion of CtIP and SUMO suppresses the effects of both the non-sumoylatable CtIP mutant and CBX4 depletion. Mechanistically, CtIP sumoylation is essential for its recruitment to damaged DNA. In summary, sumoylation of CtIP at lysine 896 defines a subpopulation of the protein that is involved in DNA resection and recombination.
Maintenance of genomic integrity is an essential priority for all living organisms. In humans, genomic instability predisposes the appearance of several rare diseases and cancer, and could have grave consequences for progeny. A major cause of such instability is the erroneous repair of chromosome breaks. To avoid this, the choice between different DNA double-strand break (DSB) repair pathways is tightly regulated, mainly through the DNA end processing known as DNA resection. Indeed, DNA DSBs can be simply re-rejoined with little or no processing by the non-homologous end-joining (NHEJ) machinery. However, when specific criteria are met, DNA resection is activated. Such DNA processing consists on the degradation of one strand of the DNA, with a 5′–3′ polarity, that leaves a tail of protruding ssDNA, which is immediately coated by the protecting complex RPA. This resected DNA can then pair with a homologous sequence, prompting homologous recombination and blocking NHEJ. Thus, resection acts as a molecular switch that regulates how a DNA break will be repaired. Hence, DNA end resection is a tightly regulated mechanism. The actual network of signals controlling resection is still poorly understood. A critical factor is the protein known as CtIP, a multifunctional protein that receives and somehow deciphers cellular and environmental cues to activate resection. For DNA end resection, especially relevant is the carboxy-terminal part of the protein, that is loosely related with its functional counterparts in budding and fission yeast, Sae2, and Cpt1, respectively. Moreover, such region mediates the interaction with pro and anti-resection factors such as the MRN complex and CCA2. Finally, it is critical to interpret the cellular status and activate resection via post-translational modifications (PTMs) such as CDK phosphorylations.

The dynamic modulation of PTMs has been extensively reported to coordinate DNA repair and the global DNA damage response (DDR) (for reviews, see refs. 10–15). Among them, in recent years sumoylation has emerged as a key element of the DDR. Sumoylation is a reversible process involving several enzymatic steps in which specific protein targets are modified by the covalent conjugation of a small peptide known as SUMO. In mammals, the E1 SUMO-activating complex (SAE1 and SAE2) initiates the process, and a unique E2 conjugating enzyme (UBC9) binds the activated SUMO molecule to transfer it to the substrate together with one of the many E3 ligases present in the cell, which provide substrate specificity. While numerous proteins have been proposed to act as a SUMO E3 ligase, the best characterized ones are the PIAS family, RanBP2, TOPORS, hMMS21, and CBX4 (also known as Pc2). PIAS1 and PIAS4 are important in the DDR and DSB repair pathways. Despite the importance for the DDR, the specific roles of sumoylation in DNA end resection are still object of investigation. In budding yeast, proteomic studies have identified key resection factors as potential damage-dependent sumoylation targets. Moreover, such global upregulation of sumoylation depends on the resection machinery itself. In mammals, the effect of sumoylation in resection is less well understood. Recruitment of both pro- and anti-resection proteins requires sumoylation.

Here we show that the sumoylation of a single residue in the carboxy-terminal tail of the critical resection factor CtIP (Lys-896) by the SUMO E3 ligase CBX4 is essential for DNA resection, homologous recombination, and the maintenance of genomic stability. CBX4 depletion impairs CtIP constitutive sumoylation and DNA end processing. Importantly, mutating the single lysine at position 896 in CtIP recapitulates the CBX4-depletion phenotype, renders cells mildly sensitive to PARP inhibitor, reduces homologous recombination and increases genomic instability. Artificial fusion of CtIP with SUMO suppresses the effects of both the non-sumoylatable CtIP mutant and CBX4 depletion. Notably, CtIP sumoylation and its CDK-dependent phosphorylation at Threonine 847 are functionally connected. Mechanistically, sumoylation of CtIP is required for its recruitment to damaged DNA. Thus, CBX4-mediated constitutive sumoylation establishes the pool of CtIP that can be recruited to damaged chromatin, hence available for DNA resection and homologous recombination, effectively controlling DNA DSB repair.

**Results**

The **SUMO ligase CBX4 is required for DNA end resection.** Given the relevance of sumoylation in the DDR, we wondered if such PTM might be directly involved in DNA repair and, more specifically, in DNA resection. To find SUMO E3 ligase(s) involved in DNA processing, we selected those that had either been previously linked to DDR or that are located in the nucleus. We knocked down each selected E3 ligase or, as a control, the key resection factor CtIP in the human U2OS cell line, and assessed the proficiency of resection upon induction of DNA damage with ionizing radiation using RPA foci formation as a readout. Several of the single E3 ligase knockdowns reduced resection proficiency.
measured as percentage of RPA positive cells (Fig. 1a, Supplementary Fig. 1a–c). However, extensive DNA resection is known to be limited to the S and G2 phases of the cell cycle1, 22, and there were significant alterations of the cell cycle progression upon depletion of some E3s (Supplementary Fig. 1d). Thus, to minimize any effects of cell cycle arrest on RPA foci, we normalized the percentage of resection-proficient cells to the population of S/G2 cells in each case (Fig. 1b). Again, several single E3 knockdowns were able to slightly reduce resection proficiency, but only that of CBX4 caused a clear decrease in RPA foci formation regardless of cell cycle distribution, suggesting that CBX4 may play a specific, relevant role in DNA end resection. It is noteworthy that such normalization did not prompt the appearance of a resection phenotype upon downregulation of any of the E3s, but actually reduced the effect in most of them (including CBX4). Even though, to be absolutely sure of the effect of CBX4 depletion on DNA end resection, we reanalysed RPA accumulation by a different parameter. We decided to perform a computer based quantification of the average number of RPA foci per cell upon CBX4 downregulation. Indeed, the average number of foci per cell was also reduced in CBX4 depleted cells, validating our earlier observation (Fig. 1c).

CBX4 constitutively sumoylates CtIP. CBX4 is known to be recruited rapidly to DNA damage, and its depletion sensitizes cells to ionizing radiation23. CBX4 activity as a SUMO E3 ligase was firstly identified with CtBP1 and CtBP2 as targets17, 24. Strikingly, CtIP was also identified as an interactor of CtBP25. Hence, we decided to test if CBX4 and CtIP interact. Indeed, we observed a DNA damage-independent interaction of both proteins by both co-immunoprecipitation and the proximity ligation assay (PLA), using both endogenous and tagged proteins (Fig. 2a–c). Such interaction occurred in the absence of an exogenous source of DNA damage.

This CBX4–CtIP interaction suggested that CtIP might be targeted for CBX4-mediated sumoylation in a constitutive, DNA damage-independent manner. We transiently co-expressed 6×His-tagged-SUMO1 or -SUMO2 and GFP-CtIP in HEK293T cells and performed pulldowns under stringent conditions (with 8 M urea buffer). Indeed, several bands

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corresponding to a modified form of CtIP appeared among the purified sumoylated proteins, both with SUMO1 and SUMO2 isoforms (Supplementary Fig. 2). Interestingly, this CtIP modification was constitutive and occurred at similar levels both in the presence and the absence of DNA damage (Fig. 2d). Sumoylation of endogenous CtIP was confirmed in U2OS cell lines stably expressing 10×His-SUMO1 (Fig. 2e). Moreover, CBX4 depletion reduced the amount of sumoylated endogenous CtIP (Fig. 2e), indicating that this E3 contributes to CtIP sumoylation.

**Sumoylation at Lys896 is essential for CtIP activity.** Analyzing in silico the amino acid sequence of CtIP using the GPS-SUMOsp 2.0 software, we found seven putative sumoylation sites (Fig. 3a, Supplementary Fig. 3a). We mutated all seven putative sumoylatable lysines to a non-modifiable arginine in the GFP-CtIP plasmid to produce the 7KR mutant. Importantly, this mutant was very close to the Thr-847 essential phosphorylatable site 22 and its yeast homologs Sae2 and Ctp1. Moreover, such residue could reassociate with DNA in the presence and the absence of DNA damage (Fig. 2d).

Sumoylation of K896 in CtIP is essential for DNA resection. a) Diagram of human CtIP protein. The predicted sumoylation sites and the CDK-mediated phosphorylation site used in this study are depicted. b) U2OS cells stably expressing the indicated variants of GFP-CtIP, or GFP as a control, and depleted of endogenous CtIP were irradiated with 10 Gy. RPA foci were detected 1 h after ionizing radiation. c) Resection length measured with SMART assay using DNA fibers extracted from U2OS downregulated for endogenous CtIP and expressing GFP-CtIP, GFP-CtIP-K896R or GFP as control. The length of at least 200 fibers and the median length (in red) of a representative experiment in each case are plotted in the left graph. The average and SEM of the median length in three independent experiments are plotted in the right graph. d) Student’s t-test was performed in each condition compared to control. e) Same as b but with cells expressing wild-type CtIP or the K896R mutant transcriptionally fused (WT-S and K896R-S) or not (WT, K896R) to SUMO1. f) RPA foci formation was analyzed as in b and d, but in cells expressing the mutant E894A and its SUMO1 fusion (E894A-S). g) For c and d, one-way ANOVA was used to analyze significance. h) Cells depleted for the indicated genes and expressing either GFP-CtIP or GFP-CtIP-SUMO1 were irradiated and RPA foci-positive cells was scored as in b. Values of RPA foci-positive cells were normalized to the percentage of S/G2 cells (Supplementary Fig. 6b), as in Fig. 1b. Statistically significant differences were analyzed using two-way ANOVA. For b-e, the average and SEM of at least three independent experiments were plotted. For all statistical analyses; *P < 0.1; **P < 0.05; ***P < 0.001.
mutant was completely proficient for RPA foci formation (Fig. 3d, Supplementary Fig. 4). To unequivocally show that the effect of K896R mutation and SUMO1 fusion were indeed caused by affecting sumoylation at this sumoylation site, we mutated the key acidic amino acid E894 to alanine. Acidic residues are essential for recognition of the SUMO binding machinery of canonical and inverted sumoylation sites[28], so we reasoned that E894A mutant would phenocopy the K896R mutation, including the suppression of endogenous CtIP but bearing a GFP-CtIP version mutated at Glu 894 were as deficient in DNA resection as the K896 mutant, but that defect disappeared when a SUMO1 sequence was constitutive sumoylation (Fig. 4a, b, Supplementary Fig. 7a, b). Strikingly, constitutive sumoylation of CtIP not only rescued the formation of RAD51 foci (Fig. 4a, b, Supplementary Fig. 7a, b). Strikingly, constitutive sumoylation of CtIP at K896, and that this modification is absolutely required for DNA end resection. As only a small fraction of CtIP is normally sumoylated (Fig. 2d, e, Supplementary Fig. 2), we propose that this represents the only pool of CtIP protein available for DNA end resection.

K896 sumoylation is required for genomic stability. Defective sumoylation of CtIP by K896 or E894 mutations or CBX4 depletion seemed almost as defective in DNA end resection as CtIP depletion itself. We decided first to investigate the consequences for homologous recombination of impairing such PTM. We used the recruitment of the recombinase RAD51 by immunofluorescence as a proxy of homologous recombination. In agreement with a reduced DNA end resection, both K896R mutation and CBX4 depletion impaired recombination and reduced the formation of RAD51 foci (Fig. 4a, b, Supplementary Fig. 7a, b). Strikingly, such defects were rescued by CtIP constitutive sumoylation (Fig. 4a, b, Supplementary Fig. 7a, b). Defective DNA repair usually leads to an increase in genomic instability. Indeed, a 4-fold increase in the irradiation-dependent accumulation of micronuclei could be observed in cells downregulated of endogenous CtIP and expressing GFP-CtIP-K896R mutant, a similar level of those cells expressing only GFP (Fig. 4c;
see Supplementary Fig. 7c for an example of a micronucleus). As other phenotypes, this was completely suppressed when a SUMO1 sequence was fused to the construct (Fig. 4c). Interestingly, no increase in spontaneous micronuclei formation was observed in any cells. To complement this approach, we quantified gross chromosomal aberrations (GCAs) in cells bearing different CtIP variants using metaphase spreads. As expected, K896R mutation increases the number of GCA to a similar extent as the GFP-bearing control (Fig. 4d, Supplementary Fig. 7d). Such effect was almost completely reversed by constitutive CtIP sumoylation (Fig. 4d, Supplementary Fig. 7d).

Thus, GFP-CtIP-K896R mutation showed phenotypes similar to the cells expressing only GFP for DNA end resection, RAD51 accumulation and genomic instability. However, those defects caused only a mild cellular hyper-sensitivity to the PARP inhibitor (PARPi) Olaparib, a source of replication-dependent DNA lesions that are independent of DNA repair. As expected, the constitutive phosphorylation at T847 (T847E mutant) suppressed the phenotype of the K896R sumoylation mutant. However, constitutive sumoylation was not sufficient to overcome the defect in resection caused by the T847A phosphomutant (Fig. 5a, compare T847A with T847-S). These data suggest that the modifications occur sequentially, with CDK-mediated phosphorylation downstream of K896 sumoylation. Accordingly, the presence of the phosphomimetic version of CtIP completely abolished the CBX4-depletion phenotype of the resection defect but not the other phenotypes, such as the G1 arrest (Fig. 5b, Supplementary Fig. 8d, e).

K896 sumoylation controls CtIP recruitment to damaged DNA. We wondered how CtIP constitutive sumoylation might impact in its role on DNA repair. One tantalizing idea was that it controls its ability to be recruited to DSBs, explaining why only the sumoylated subpopulation of the protein can engage in DNA processing. Thus, we hypothesized that CtIP interaction with damaged DNA was modulated by sumoylation. To test this idea, we analyzed its recruitment to a doxycycline-inducible DSB created by the nuclease I-SceI. This I-SceI cleavage site is flanked by 256 repeats of the lac operator DNA sequences (lacO), allowing the locus to be visualized at the lacO sequences by FISH (Fig. 6a). We compared the co-localization of either the DSB marker γH2AX or Flag-Cherry-CtIP with the lacO array in either a wild-type, a K896R, constitutive sumoylation or the combination of both mutations background (Fig. 6b–d, Supplementary Fig. 9). Upon induction of the break by doxycycline addition, we observed a twofold increase of wild-type CtIP (Fig. 6b, c). Strikingly, no increase in recruitment above background levels was observed in the K896R mutant (Fig. 6b, c). As before, CtIP recruitment was restored in a K896R mutant when a SUMO1 sequence was fused at the end of the protein (Fig. 6b, c). In fact, constitutive sumoylation of CtIP slightly increased its recruitment to the I-SceI induced break, even in combination with the K896R mutation, reinforcing the idea that sumoylation at the carboxy-terminal part of CtIP stimulates its recruitment to damaged chromatin (Fig. 6c). None of those differences were caused by a difference in DSB induction, as similar amounts of
γH2AX recruitment were observed in cells expressing any of the CtIP variants, as measured by immuno-FISH (Fig. 6b, c).

**Discussion**

Protein sumoylation is considered a critical way to fine-tune the cellular response to DNA damage, especially in the sensing and signaling of DSBs. This prompted us to try to resolve its implication in the modulation of the actual repair of the breaks, particularly at the level of DNA processing. Strong evidence in *Saccharomyces cerevisiae* suggested that sumoylation and resection are related. Indeed, many HR factors, including the essential resection factor MRX complex (Mre11–Rad50–Xrs2), are sumoylated in response to DNA damage and, strikingly, such sumoylation induction depends both on the MRX complex and DNA resection itself. In higher eukaryotes, however, the
relevance of SUMO conjugation in resection was less well established. Sumo ligases such as PIA1 and PIA4 are known to promote DSB repair and radiosensitivity, and, indeed, a two-fold reduction on RPA recruitment to laser lines in cells depleted of any of those E3s has been reported. Interestingly, only PIA4 depletion showed a similar effect in our hands, arguing that different types of lesions might have a distinct set of SUMO ligases requirements for proficient resection.

We decided to analyze a small subset of SUMO E3 specifically for their impact on resection. In order to compile the list of candidates, we reasoned that any that was described as nuclear or already related with the DDR might be involved. An important consideration when studying DNA resection is cell cycle. Extensive resection is only activated in S and G2 cells, most likely to avoid recombination when the sister chromatid is not present 3, 30. In G1 cells, some breaks undergo limited DNA processing to allow alternative NHEJ to proceed. However, this confined resection is slower and does not render a global RPA accumulation easily visible as RPA positive cells (see Supplementary Fig. 1 for examples of RPA positive and negative cells). Thus, cell cycle modifications rendered by the E3s depletion had to be accounted somehow. We normalized the RPA foci data with cell cycle profile. It is noteworthy that such approach did not result in any amplification of the difference with control cell, but the opposite. By such approach, we uncovered CBX4 as a critical factor for resection activation. CBX4 is a fine example of why considering cell cycle is so important in these studies. The effect of CBX4 was overestimated by the strong G1 accumulation in cells downregulated of this E3. However, even with the cell cycle correction, the effect was clear enough to make us suggest that CBX4 is required for DNA resection. We reason that the fact that constitutive sumoylated CtIP can rescue CBX4 depletion resection defect without suppressing the cell cycle arrest is proof enough that the lack of RPA positive cells is indeed due to hampered resection and not to an altered cell cycle progression. Thus, the carboxy-terminal tail of CtIP seems to act as a hub in which several cellular signals converge in order to regulate DNA resection. Indeed, loss of such portion of the protein renders the protein inactive and acts as a dominant negative, causing the appearance of Seckel and Jawad syndromes 31. We have shown that, in addition to the aforementioned interactions and PTMs, sumoylation at the very end of the protein is essential for its resection function. Strikingly, those PTMs, or at least T847 CDK phosphorylation and K986 sumoylation, are functionally connected and they seem to act sequentially rather that in parallel. Interestingly, budding yeast Sae2 is also sumoylated and phosphorylated by CDK, but in that case, they seem to act independently as double mutants Sae2-S267A K97A showed an additive effect in resection when compared with the single mutations 32. Cascades of consecutive PTMs in proteins have been proposed before. For example, FEN1 nuclease sumoylation requires prior phosphorylation and in turn causes ubiquitylation 33. Although we cannot observe such sequential appearance of sumoylation and phosphorylation, the fact that a mutation that mimics constitutive phosphorylation such as T847E can suppress both CtIP K986R mutant and CBX4 depletion, strongly agree with this idea that SUMO conjugation predecates CDK phosphorylation.

Only a small subpopulation of the protein is indeed conjugated to SUMO, as it is typical for sumoylation 33. This low abundance of the modified form of the protein is usually attributed to local or temporal constrains. Most usually, sumoylation of proteins involved in dealing with DNA lesions are restricted to the proteins that are already recruited to damaged chromatin and/or induced by the presence of DNA damage. In stark contrast, CtIP sumoylation on K986 seems to be a constitutive modification that predecates the appearance of DNA damage and CtIP recruitment to DNA breaks. In that regard, it is very different to the DNA damage induced phosphorylation and more similar to the CDK-dependent modification of the protein. CtIP is known to have many different roles within the cell, including transcription regulation, replication, checkpoint activation or the G1/S transition. We propose that sumoylation of CtIP defines a limited pool of the protein that can engage in resection. But, why limit the amount of CtIP that can be readily used to process DNA breaks? We reasoned that for most DNA breaks, NHEJ would suffice for repair, especially for clean breaks or the ones that appear in G1. Thus, limiting resection might be important to facilitate NHEJ on those circumstances. More importantly, resection might activate unscheduled single strand annealing or alt-NHEJ, both mutagenic repair pathways. So, by reducing the pool of CtIP that can be engaged in end processing, cells would modulate the decision between NHEJ and HR, but also the balance between different HR subpathways.

This resection control absolutely reflects in the appearance of recombination centers and in the formation of GCAs. But surprisingly, this does not correlate perfectly with cell survival to camptothecin or PARP inhibitor. This uncoupling between resection phenotype and cell survival to damaging agents have been observed before by us and others. For example, mutation of serine 327, that modulates CtIP interaction with BRCA1, showed mild resection defects but a hyper-sensitivity to DNA damaging agents similar to CtIP depletion. Together with those described earlier, these data seem to suggest that CtIP might play additional roles downstream in the DSB repair pathways that are independent on its resection activity and that K986R and S327A modification might act as separation of function mutants. However, we cannot exclude the possibility...
that camptothecin and PARP inhibitor-induced DNA damage does not require CtIP sumoylation due to the nature of the break. Along those lines, the nuclease activity is dispensable for clean breaks but essential for ragged ends.

Mechanistically, CtIP sumoylation controls resection at the level of CtIP recruitment to DNA damage. K896R mutant fails to be recruited to a single, I-SceI induced DSB, whereas the constitutively sumoylated form concentrates more avidly there. CtIP is known to bind DNA directly. Interestingly, binding of several DDR factors to DNA is either increased or decreased by sumoylation. Indeed, sumoylation of Tdp1, Top2, Yku70, and SLX4 stimulates their association with DNA. However, we cannot conclude if the effect we observe is mediated by the direct binding to DNA or to damaged chromatin or even mediated by interaction with other factors. Sumoylation is a potent regulator of protein-protein interaction. In some cases, it might act as a molecular glue that stabilizes them. Indeed, this has been documented for several DDR factors and, more relevant, for the MRX complex. Interestingly, Mre11, the catalytic subunit of the MRX complex, possesses several SUMO interacting motifs that are likely involved in MRX assembly. Alternatively, sumoylation might disrupt the binding of CtIP to anti-resection factors, allowing CtIP recruitment or retention to DSBs. This would parallel what is observed in budding yeast, in which Sae2 is maintained in inactive aggregates and sumoylation of the protein solubilizes it.

In summary, CBX4 has emerged as a critical component of the DNA end resection machinery. Strikingly, and in contrast to other SUMO ligases previously described to act during the response to DNA damage, such as PIAS1 or PIAS4, CBX4 functions in a constitutive manner by sumoylating CtIP. We can circumscribe CBX4’s role in resection to its sumoylation of a single factor at a single residue—namely, CtIP at K896. Our data suggest that further sumoylation occurs in CtIP, either by CBX4 or other E3 ligases, but that this has little or no effect on DNA end resection. We hypothesize that only the pool of CtIP that has been previously sumoylated by CBX4 at K896 can be recruited to DSBs, hence making it readily available for resection. We propose that the rest of the CtIP protein pool might be involved in its other functions, such as transcription and DNA replication. By reducing the levels of CtIP that can be engaged in DNA end processing, the cells limit the availability of the resection machinery, thus regulating the balance between homologous recombination and NHEJ, a critical element in the maintenance of genomic integrity.

**Methods**

**Cell lines and growth conditions.** U2OS (S. P. Jackson, University of Cambridge, UK), HEK293T (S. P. Jackson, University of Cambridge, UK), and U2OS 10 × His-SUMO1 cell lines (A. C. Verteaga, Leiden University Medical Center) were grown in DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 100 units ml⁻¹ penicillin, and 100 μg ml⁻¹ streptomycin (Sigma-Aldrich). U2OS stably expressing GFP-CtIP plasmids were grown in standard U2OS medium supplemented with 0.5 μg ml⁻¹ G418. U2OS19pht13 cells 42 were grown in the absence of phenol red tetracyclines, and supplemented with 0.8 mg ml⁻¹ puromycin.

**siRNAs, plasmids and transfections.** siRNA duplexes were obtained from Sigma-Aldrich or Dharmacon (Supplementary Table 1) and were transfected using RNAiMax Lipofectamine Reagent Mix (Life Technologies), according to the manufacturer’s instructions.

Plasmid transfection of U2OS cells was carried out using FuGENE 6 Transfection Reagent (Promega) according to the manufacturer’s protocol.

HEK293T cells were transfected with a standard calcium phosphate transfection protocol.

pRSV 6 × His-SUMO1 and SUMO2 and GFP-CtIP plasmids were previously published 43, 44. Point mutants of GFP-CtIP were produced using the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies), according to the manufacturer’s instructions. To obtain GFP-CtIP-SUMO1 constructs, the stop codon of GFP-CtIP was substituted by a NolI restriction site, to have this sequence next to the BamHI site in which CtIP was cloned originally. SUMO1 cDNA was cloned from pRSV 6 × His-SUMO1 by PCR, adding NolI and BamHI sites to 5’ and 3’ ends of the sequence, respectively. The modified plasmid and the cloned sequence were then cut with NolI and BamHI and ligated. The SUMO1 sequence was then produced at the C-terminus of CtIP, thus leaving free the C-terminus of CtIP. To make this SUMO unavailable for conjugation, the two terminal glycines were removed by mutagenesis. Flag-Cherry-CtIP plasmids (WT and K896R mutant) were assembled by golden gate cloning.

**Lentiviral infection.** A plasmid containing shRNA targeting CtIP (TRCN0000318738, Sigma) was transfected into HEK293T cells to produce lentiviruses as previously described 45. Briefly, lentiviral particles were generated using 10 μg p8.91, 5 μg pVSV-G and 15 μg of the shRNA bearing plasmids by calcium phosphate transfection in A293T cells. After 48 h, lentiviruses were collected from the media by 100,000 × g centrifugation for 2 h at 4 °C. These lentiviruses were used to infect U2OS cells in the presence of 8 μg ml⁻¹ polybrene (Sigma), and CtIP knocked-down cells were then selected for 72 h with 1 μg ml⁻¹ puromycin.

**RT-qPCR.** RNA was extracted from U2OS cells using RNeasy Mini Kit (Qiagen), and cDNA was produced from RNA samples with QuantiTect Reverse Transcription Kit (Qiagen), according to the manufacturer’s instructions. qPCR was performed using iTag Universal SYBR Green Supermix (Bio-Rad) and the primers indicated in Supplementary Table 2.

**SDS-PAGE and western blot analysis.** Protein extracts were prepared in 2 × Laemmli buffer (4% SDS, 20% glycerol, 125 mM Tris-HCL, pH 6.8) and passed 10 times through a 0.25 mm needle-mounted syringe to reduce viscosity. Proteins were resolved by SDS-PAGE and transferred to low fluorescence PVDF membranes (Immobilon-FL, Millipore). Membranes were blocked with Odyssey Blocking Buffer (LI-COR) and blotted with the appropriate primary antibody and incubated with secondary antibody (LI-COR) (Supplementary Tables 1). Antigens were detected in blocking buffer supplemented with 0.1% Tween-20. Membranes were air-dried and scanned in an Odyssey Infrared Imaging System (LI-COR), and images were analyzed with ImageStudio software (LI-COR).

**His-tag pulldowns.** To perform His-tag pulldowns, HEK293T cells transiently transfected with pRSV 6 × His-SUMO1 or -SUMO2, or U2OS cells stably expressing GFP-CtIP-SUMO1 or -SUMO2, were gently scraped off in PBS and lysed in binding buffer (20 mM Tris-HCL, pH 7.9, 150 mM NaCl, 8 M urea). Protein extracts (1 mg) were incubated with 50 μl of cobalt-based magnetic Dynabeads (Novex, Life Technologies) on a roller for 2 h at room temperature. Beads were then washed 3 × with binding buffer supplemented with 5 mM imidazol, and the final product was eluted by boiling the beads at 100 °C for 1 min with SDS sample buffer 2 × containing 200 mM imidazol.

**Immunoprecipitation.** For GFP immunoprecipitation, U2OS cells expressing GFP or GFP-CtIP were harvested in lysis buffer (50 mM Tris-HCL, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.2% Triton X-100, 1 x protease inhibitors [Roche] and 1 x phosphatase inhibitor cocktail 1 [Sigma]). Protein extract (1 mg) was mixed with 50 μl of washed magnetic anti-GFP beads (GFP-Trap_M, Chromotek) and incubated overnight at 4 °C with gentle rocking. Beads were then washed 3 × with lysis buffer, and the precipitate was eluted in SDS sample buffer by boiling the beads and loaded onto a gel.

To immunoprecipitate endogenous proteins, U2OS were scirpped in lysis buffer (50 mM Tris-HCL, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 x protease inhibitors [Roche] and 1 x phosphatase inhibitor cocktail 1 [Sigma]). To degrade DNA, 100 U ml⁻¹ Benzonase (Mercer Millipore) was added to protein extracts and incubated for 30 min on ice. Protein extracts (1 mg) were then precleared with magnetic protein A Dynabeads (Novex) under gentle agitation at 4 °C for 30 min. Precleared samples were then incubated with anti-CBX4 antibody or a rabbit IgG (Sigma) as a control for 1.5 h at 4 °C and Dynabeads were added afterwards and were incubated 1 h at 4 °C under gentle agitation. Beads were then washed three times with lysis buffer, and the precipitate was eluted in Laemmli buffer.

**Immunofluorescence, PLA, immuno-FISH and microscopy.** For RPA and RAD51 foci visualization, U2OS cells knocked-down for different proteins were seeded on coverslips. At 1 h (for RPA) or 3 h (for RAD51) after irradiation (10 Gy), coverslips were washed once with PBS followed by treatment with pre-extraction buffer (25 mM Tris-HCL, pH 7.5, 50 mM NaCl, 1 mM EDTA, 3 mM MgCl₂, 8: 113 | DOI: 10.1038/s41467-017-00183-6 | www.nature.com/naturecommunications
300 mM sucrose, and 0.2% Triton X-100 for 5 min on ice. Cells were fixed with 4% paraformaldehyde (w/v) in PBS for 20 min. Following three washes with PBS, cells were blocked in 1% BSA with 0.2% Triton X-100 and incubated in the appropriate primary antibodies (Supplementary Table 3) in blocking solution overnight at 4 °C or for 2 h at room temperature, washed again with PBS and then co-immunostained with the appropriate secondary antibodies (Supplementary Table 3) in blocking buffer. After washing with PBS, coverslips were mounted in glass slide mounting medium with DAPI (Vector Laboratories). For tubulin staining to count binucleated cells, fixation was performed with methanol for 10 min and acetone for 30 s on ice. Cells were then incubated with 5% FBS in PBS for 1 h, immunostained with anti-tubulin antibody for 2 h at room temperature, washed with PBS and incubated with the secondary antibody for 1 h at room temperature. Coverslips were mounted as described above.

For PLAs, U2OS cells were irradiated (10 Gy) or mock treated and fixed 1 h after with methanol for 10 min followed by acetone incubation for 30 s. After that, cells were blocked with blocking solution from the Duolink PLA Kit (Olink Bioscience) for 30 min at 37 °C and incubated with primary antibodies against CTIP and CBX4 (Supplementary Table 3) overnight at 4 °C. Samples were then incubated with MINUS and PLUS secondary PLA probes for 1 h at 37 °C, and the detection of these probes was carried out with the Duolink Detection Kit Red (Olink Bioscience). The number of PLA signals per nucleus was scored in at least 100 cells per sample.

For immuno-FISH, U2OS18ptight13 cells were fixed in 4% paraformaldehyde for 15 min, permeabilized in 0.5% Triton for 15 min, blocked in 3% BSA in PBS 0.1% Tween and incubated with primary and secondary antibodies (Supplementary Table 3) for 1 h each. Cells were then post-fixed in 4% formaldehyde for 20 min, and the FISH protocol was carried out as previously described 29. Briefly, samples were washed for 5 min in 2 × SSC (saline-sodium citrate) buffer and 45 min in 2 × SSC with a increasing temperature from room temperature to 72 °C, washed once in 70% ethanol and twice in absolute ethanol. Samples were then dehydrated in 90% ethanol for 5 min at room temperature, air-dried, and incubated with 0.1 N NaOH for 5 min in 2 × SSC for 5 min. Coverslips were washed again in 70% ethanol and twice with absolute ethanol. After drying, cells were hybridized with a DNA probe for 30 s at 85 °C and incubated overnight at 37 °C. The probe was prepared by nick-translation from the lacO-I-SceI plasmid. DNA probe (0.3 mg) was mixed with 9 mg of ssDNA and 3 mg of Cot1 human DNA (Roche) and precipitated with 2.53 vol of ethanol and 1/10 vol of 2.5 M sodium acetate for 30 min at −80 °C. After 20 min of centrifugation, the supernatant was discarded, and the pellet was washed with 70% ethanol and centrifuged again for 5 min. The supernatant was discarded, and the pellet was dissolved in 20 ml of hybridization solution (50% formamide, 43 SSC, 10% dextran sulfate) per coverslip by vortexing for 1 h. The probe was denatured for 5 min at 90 °C and preannealed for at least 15 min at 37 °C before hybridization with cells.

The day after hybridization, immuno-FISH was revealed. Coverslips were washed twice for 20 min at 42 °C in 2 × SSC and then incubated with secondary antibody and fluorescein anti-biotin (Vector Laboratories, SP-3040) at 1:100 dilution for 45 min. Vectashield Antifade mounting medium (Vector Laboratories, H-1200) containing DAPI. Immuno-FISH samples were analyzed using a confocal microscopy.

**Cell cycle analysis.** Cells were fixed with cold 70% ethanol overnight, incubated with 250 μg/ml RNase A (Sigma) and 10 μg/ml 1-propidium iodide (Fluka) at 37 °C for 30 min and analyzed with a FACScalibur (BD). Cell cycle distribution data were further analyzed using ModFit LT 3.0 software (Verity Software House Inc.).

**Proteinase K assay.** U2OS cells expressing GFP-tagged wild-type or K896R CtIP were subjected to GFP immuno precipitation as described above. The immuno-precipitation product was divided into four parts, which were digested with increasing amounts of proteinase K (0, 5, 15 or 50 mg/ml) for exactly 3 min at room temperature. Samples were then boiled with SDS sample buffer for 5 min at 100 °C and loaded into a gradient SDS polyacrylamide gel.

**Cell fractionation.** U2OS cells expressing GFP-tagged wild-type or K896R CtIP were knocked down for endogenous CtIP by siRNA transfection and then, 48 h later, irradiated or not with 10 Gy ionizing radiation. At 1 h post irradiation, cells were harvested in resuspension buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.34 M sucrose, 10% glycerol, 1 mM DTT and protease inhibitor cocktail). To disrupt cellular membranes, Triton X-100 was added to cell suspensions to a final 5% FBS centration of 0.1%, and samples were incubated on ice for 8 min. Cells were then centrifuged at 4 °C at 1300 × g for 5 min to separate the cytoplasmic fraction from the nuclei pellet. Nuclei were then washed once with resuspension buffer and incubated with resuspension buffer supplemented with 90 U/ml benzazene and 0.1 mg/ml BSA for 1 h on ice. Cytoplasmic and nuclear fractions were boiled in SDS sample buffer and loaded into SDS polyacrylamide gel.

**Chlonogenic survival assay.** U2OS expressing GFP and GFP-CtIP variants were transfected with siCtIP, seeded in low confluency and treated with Olaparib (AstraZeneca) at the indicated doses or mock treated with DMSO for 1 h. After treatment, cells were washed two times with PBS and cultured under standard conditions to allow colony formation for the next 8–10 days. Colonies were then counted after staining with 0.5% crystal violet/20% ethanol. The results were normalized to DMSO treatment.

**Micronuclear assay.** Cells were seeded onto coverslips and treated with 10 Gy ionizing radiation. Following treatment, cytoclastasin B (Sigma) was added at 4 μg ml−1. 24 h post treatment, cells were fixed and subjected to DAPI staining. Only binucleated cells were scored, which was confirmed by visualization of the cytoplasm with Tubulin immunofluorescence (performed as described in the immunofluorescence section).

**Chromosomal aberrations.** Aberrant chromosomes were counted on DAPI-stained mitotic spreads. Following CtIP depletion, GFP or GFP-CtIP expressing U2OS cells were exposed to 2 Gy of IR and then allowed to recover for 8 h in fresh medium before chromosome preparation. Within these 8 h, cells were treated with caffeine (2 mM) for the last 5 h to allow cells with gross chromosomal aberrations (GCAUs) to enter mitosis, and for the last 3 h, they were treated with Colcemid (Sigma) to induce chromosome condensation. Cells were then harvested and treated with 0.075 M KCl for 10 min at 37 °C, fixed in methanol/acetic acid (3/1), washed twice with methanol/acetic acid (3/1), and then spread on a glass microscope slide, air-dried, and DAPI-stained.

**Statistical analysis.** Statistical significance was determined with a Student’s t-test, one-way ANOVA or two-way ANOVA as indicated in each case, using PRISM software (Graphpad Software Inc.). Statistically significant differences were labeled with one, two or three asterisks if P < 0.05, P < 0.01 or P < 0.001, respectively.

**Data availability.** The data that support the findings of this study are available from the corresponding author.

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Author contributions

I.S.-B. performed all the experiments. C.C.-G. helped with the resection analysis of CtIP SUMO mutants and fusions and recruitment studies of CtIP. C.C.-R. did and analyzed the mitotic spreads. CtIP recruitment was performed by I.S.-B. in the E.S. laboratory with her help. B.R.-S.-M. and V.H. created the different CtIP constructs. P.H. supervised the work and wrote the manuscript. All authors read and commented on the manuscript.

Additional information

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