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# CtIP and MRN promote non-homologous end-joining of etoposide-induced DNA double-strand breaks in G1

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## ABSTRACT

**Topoisomerases class II (topoII) cleave and re-ligate the DNA double helix to allow the passage of an intact DNA strand through it. Chemotherapeutic drugs such as etoposide target topoII, interfere with the normal enzymatic cleavage/re-ligation reaction and create a DNA double-strand break (DSB) with the enzyme covalently bound to the 5′-end of the DNA. Such DSBs are repaired by one of the two major DSB repair pathways, non-homologous end-joining (NHEJ) or homologous recombination. However, prior to repair, the covalently bound topoII needs to be removed from the DNA end, a process requiring the MRX complex and ctp1 in fission yeast. CtIP, the mammalian ortholog of ctp1, is known to promote homologous recombination by resecting DSB ends. Here, we show that human cells arrested in G0/G1 repair etoposide-induced DSBs by NHEJ and, surprisingly, require the MRN complex (the ortholog of MRX) and CtIP. CtIP's function for repairing etoposide-induced DSBs by NHEJ in G0/G1 requires the Thr-847 but not the Ser-327 phosphorylation site, both of which are needed for resection during HR. This finding establishes that CtIP promotes NHEJ of etoposide-induced DSBs during G0/G1 phase with an end-processing function that is distinct to its resection function.**

## INTRODUCTION

DNA double-strand breaks (DSBs) are highly cytotoxic lesions, posing a major threat to genomic integrity. Following DSB induction, cells elicit an orchestrated DNA damage response which encompasses pathways of DSB repair, the initiation of cell cycle checkpoints and, in some cells, the induction of apoptosis (1,2). DSBs can be repaired by two major pathways, homologous recombination (HR) and non-homologous end-joining (NHEJ) (3–5). NHEJ is the predominant repair pathway throughout the cell cycle and is particularly important in the G1 phase of the cell cycle (6–8). HR, in contrast, is important for repairing stalled or collapsed replication forks (9,10), and can also repair two-ended DSBs in S and G2 phase when the presence of a sister chromatid provides a template for repair (11).

Mre11 is part of the Mre11-Rad50-Nbs1 (MRN) complex which is important for HR-mediated DSB repair and damage signaling (12). The MRN complex, besides being a target of ATM, is a direct inducer of ATM kinase activity which is particularly important for efficient damage signaling (13). Mre11 from human and yeast possesses nuclease activity and contributes to DSB end resection to generate single stranded DNA (ssDNA), the intermediate for HR repair processes (14). The role of the MRN complex in NHEJ is perhaps less clear (15) but Mre11 and Nbs1 are required for an end-joining pathway that repairs a sub-set of ionizing radiation induced DSBs in G1 (16). This subset represents DSBs localizing to heterochromatic DNA regions and also requires ATM (17). Further, cells synchronized at G0/G1 phase contain phospho-Nbs1 foci following etoposide treatment, suggesting the involvement of MRN in NHEJ of etoposide-induced DSBs (18).

CtIP is a critical player in multiple molecular pathways. It was originally identified as a binding partner of the transcriptional suppressor CTBP (C-terminal binding protein) (19) and interacts with the Brca1 BRCT domains in a manner that is dependent on the phosphorylation of CtIP at serine 327 (20,21). CtIP promotes HR by initiating DSB end resection and the formation of ssDNA (22). Mutating the CtIP site threonine 847 to alanine (T847A) prevents its phosphorylation and results

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in impaired resection (23) but serine 327 phosphorylation also seems to be required for resection and HR (24). Both Ser-327 and Thr-847 are CDK1 phosphorylation sites. Although CtIP promotes HR in S and G2 phase, there is evidence that it can also function in G1 in a specialized end-joining pathway called microhomology-mediated end-joining (MMEJ) (24). Since MMEJ involves short regions of sequence homology at the break site, CtIP may promote MMEJ by initiating (limited) resection similar to its role in HR.

DNA topoisomerases are responsible for the conversion of DNA topology via their cleavage/re-ligation equilibrium (25,26). Topoisomerase II (topoII) is a homo-dimeric enzyme. Each subunit cleaves one strand of the DNA double helix creating a transient DSB to allow the passage of an intact DNA strand through it (27). Chemotherapeutic drugs such as etoposide target topoII and interfere with the normal enzyme reaction. Disruption of the cleavage/re-ligation reaction stabilizes cleavage complexes, intermediates in the catalytic cycle of the enzyme which can be converted to DSBs with the enzyme covalently bound to the 5′-end of the DNA (28,29). Importantly, the covalently bound enzyme needs to be removed from the DNA end before repair can ensue, a process requiring the MRX complex and ctp1 in fission yeast (orthologs of mammalian MRN and CtIP) (30). Consistent with this requirement, chicken DT40 cells defective in CtIP are hyper-sensitive to etoposide treatment (31). However, the repair pathway utilized following enzyme removal is unclear. Paradoxically, NHEJ seems to play a major role in the resistance to topoII-mediated DNA damage (32–34) raising the possibility that CtIP and MRN promote the repair of etoposide-induced DSBs by NHEJ.

Here, we measure the repair of DSBs after etoposide treatment specifically in G1 phase and show that NHEJ-deficient cells are unable to repair etoposide-induced DSBs. Importantly, cells deficient in Mre11 or Nbs1 but not ATM also exhibit a major repair defect. Furthermore, CtIP depletion leads to a repair defect in G1 which is epistatic to the Mre11 repair defect and involves NHEJ. Finally, we show that CtIP's function in promoting repair of etoposide-induced DSBs by NHEJ in G1 requires the Thr-847 but not the Ser-327 phosphorylation site. Since both CtIP phosphorylation sites are required for resection during HR, this separates CtIP's end-processing from its resection function. Our findings provide new mechanistic insight into the repair pathways conferring resistance to the anti-cancer drug etoposide.

## MATERIALS AND METHODS

### Cells and cell culture

Primary human fibroblasts utilized were HSF1 [wild-type (wt)], C2886 (wt), AT1BR (ATM deficient), HSC62 (Brca2 deficient) (IVS19-1 G to A) (35), ATLD2 (Mre11 deficient), 180BR (LigIV deficient) (36), CZD82CH and GM07166A (Nbs1 deficient); immortalized and transformed cell lines utilized were 82-6 hTert (wt) and 2BN hTert (XLF defective) and HeLa. ATLD2 cells were grown in Dulbeccos minimal essential medium (DMEM) supplemented with 20% FCS, 1% non-essential amino acids (NEAA) and 1% antibiotics (penicillin–streptomycin). AT1BR cells were cultured in HAM'S F10 buffer, supplemented with 15% FCS and 1% antibiotics and human HeLa cells in DMEM, supplemented with 10% FCS and 1% NEAA. All other cells were cultured in MEM supplemented with 20% FCS (10% for HSF1), 1% NEAA and with 1% antibiotics (82-6 hTert cells without antibiotics). All cells were maintained at 37°C in a 5% $CO_2$ incubator.

### RNA interference

siRNA transfection of HeLa cells was carried out using HiPerFect Transfection Reagent (Qiagen) following the manufacturer's instructions. Mre11, CtIP and Rad51 siRNAs were used at a final concentration of 20, 50 and 10 nM, respectively. Experiments were performed 48 h after transfection (120 h for Mre11). The knock-down efficiencies were determined by immunofluorescence analysis or immunoblotting. siRNA sequences were as follows: Mre11 (ACA GGA GAA GAG ATC AAC T); CtIP1 (TCC ACA ACA TAA TCC TAA T); CtIP2 (AAG CTAAAACAGGAACGAATC); Rad51 (AAG GGA ATT AGT GAA GCC A); control (AAT TCT CCG AAC GTG TCA CGT).

### Random plasmid integration

After 24 h incubation with CtIP2 siRNA, HeLa cells were transfected with Effectene (Qiagen) following the manufacturer's protocol to integrate various GFP-tagged siRNA-resistant CtIP plasmids. On the following day, cells were treated with etoposide (Sigma), fixed and stained for γH2AX foci and GFP. Only GFP-positive G1 cells were analyzed. 82-6 hTert cells were transfected by electroporation with siRNA and plasmid in the same reaction according to the manufacturer's protocol 48 h prior to etoposide treatment (Amaxa).

### Chemical treatment and irradiation

Cells were treated with 20 or 100 μM of etoposide (Sigma) and incubated for 1 h (for primary and hTert immortalized cells) or half an hour (for HeLa cells). After incubation, cells were washed with PBS and fresh medium was added (in case of non-confluent cells with aphidicolin (Calbiochem) at a concentration of 3 μg/ml). ATM inhibitor (Tocris) and DNA-PK inhibitor (Sigma) were added at 10 mM 1 h prior to etoposide treatment, during etoposide incubation and repair time. Aclarubicin at 5 μM was added immediately before etoposide treatment. X-irradiation at 90 kV and 19 mA was performed at a dose rate of 2 Gy/min. Dosimetry considered the increase in dose for cells grown on glass coverslips relative to plastic surfaces (37).

### Immunofluorescence

All cells were grown on glass coverslips for immunofluorescence microscopy. HeLa cells and 82-6 hTert cells were fixed with 2% formaldehyde in PBS for 15 min, washed three times for 10 min in PBS, permeabilized in 0.2% Triton

X-100 in PBS for 10 min at 4°C and washed three times for 10 min with PBS/1% FCS. All other cells were fixed for 30 min with methanol at −20°C, dipped for 1 min in ice cold acetone for permeabilization and washed three times for 10 min with PBS/1% FCS. Non-specific antigens were blocked for 30 min in 5% BSA (AppliChem) in PBS/1% FCS. Samples were incubated with primary antibodies in PBS/1% FCS over night at 4°C, washed three times in PBS/1% FCS and incubated for 1 h at room temperature with Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies (1 : 500, Invitrogen). After three times of washing in PBS, cells were DAPI (Sigma) stained and mounted using Vectashield mounting medium (Vector Laboratories). All cells were examined using a Zeiss microscope and Metasystems software (Altlussheim, Germany).

### Immunoblotting

Cells were harvested and sonicated three times for 1 min in RIPA lysis buffer (50 mM Tris/HCl, pH 8, 150 mM NaCl, 0.5% Natriumdesoxycholat, 1% Triton X-100, 0.1% SDS and fresh added protease inhibitor cocktail 1 : 25) and incubated for 30 min at 4°C. After centrifugation of the cell extracts for 30 min at 4°C with 15.7 *g*, the protein concentration was determined and the cell lysates were boiled with SDS Laemmli loading buffer [4% (w/v) SDS, 200 mM DTT, 120 mM Tris/HCl, pH 6.8, 10 mM β-Mercaptoethanol, 20% (v/v) Glycerin, 0.02% Bromphenol blue] for 5 min at 95°C (target proteins >200 kD at 80°C). Proteins were separated via SDS–PAGE and transferred to PVDF membrane. The membrane was blocked for 1 h in 5% low fat milk in TBS/0.1% Tween-20 and immunoblotting was carried out with primary antibody in TBS/0.1% Tween-20/1% low fat milk over night at 4°C or for 1 h at room temperature, followed by HRP-conjugated secondary antibody incubation in PBS/0.1 % Tween-20/1% low fat milk for 1 h. The immunoblots were developed using ECL (Roche). Signal detection was carried out with a chemi smart system (Vilber Lourmat).

### Antibodies

Antibodies for immunofluorescence were: mouse monoclonal α-γH2AX, 1 : 1000 (Upstate); rabbit polyclonal α-γH2AX, 1 : 2000 (Abcam); mouse monoclonal α-GFP, 1 : 200 (Roche); rabbit polyclonal α-CENP-F, 1 : 2000 (Santa Cruz); rabbit polyclonal α-RAD51 (PC130), 1 : 15 000 (Calbiochem). Antibodies for immunoblotting were: polyclonal rabbit α-GAPDH, 1 : 1000 (Santa Cruz); mouse monoclonal α-Mre11, 1 : 1000 (Abcam); rabbit polyclonal α-CtIP, 1 : 1500 (Bethyl Laboratories); rabbit polyclonal α-RAD51, 1 : 2000 (Abcam); mouse monoclonal α-Tubulin, 1 : 3000 (Santa Cruz).

## RESULTS

### Repair of etoposide-induced DSBs in G1/G0 involves NHEJ and Mre11/Nbs1 function

We used confluent primary human fibroblasts to investigate G1/G0 phase cells and scored γH2AX foci as a marker for DSBs. Etoposide is an established inducer of DSBs (38). Consistent with this, etoposide-induced foci formation is abolished in cells treated with specific ATM and DNA-PK inhibitors, indicating that ATM and DNA-PK but not ATR phosphorylate H2AX (Figure 1A). Furthermore, pre-treatment with aclarubicin, an intercalative antibiotic that efficiently inhibits the catalytic activity of topoII (39,40), completely abolishes etoposide-induced foci formation (Figure 1B). This establishes that γH2AX foci after etoposide treatment represent DSBs arising from topoII activity.

Wt cells repair ~90% of the γH2AX foci induced by 20 or 100 μM etoposide within 4 h post treatment. In contrast, 180 BR cells deficient in the NHEJ factor DNA ligase IV (LigIV) exhibit a substantial repair defect (Figure 1C; Supplementary Figure S1), consistent with the hypersensitivity of NHEJ mutant cells to etoposide (32,33). Further, HSC62 cells deficient in the HR factor Brca2 (35), repair etoposide-induced DSBs similar to wt cells (Figure 1C). These results establish that etoposide-induced DSBs in G1/G0 are repaired by NHEJ.

We next investigated the contribution of the MRN complex to DSB repair after etoposide treatment. ATLD2 cells defective in Mre11 and two Nbs1 deficient cell lines show a significant repair defect with unrepaired DSBs up to 8 h post treatment. In contrast, AT1BR cells defective in ATM show normal repair kinetics demonstrating that the role of the MRN complex after etoposide treatment is independent of ATM (Figure 2; Supplementary Figure S2).

### Repair of etoposide-induced DSBs in G1/G0 involves CtIP

To study the role of CtIP in etoposide-induced DSB repair we treated HeLa cells with CtIP siRNA. Since HeLa cells do not readily arrest in G0/G1, we utilized cell cycle markers to distinguish the different cell cycle phases (11). In short, G2-phase cells show a strong pan-nuclear CENP-F staining pattern while S-phase cells show weak and G1-phase cells no CENP-F staining. Aphidicolin is a specific inhibitor of the replicative DNA polymerases α and δ and was used to prevent S-phase cells from progressing into G2 and G1 during analysis. It causes pronounced pan-nuclear γH2AX phosphorylation in S-phase cells due to replication stalling but no damage in G1 and G2 cells (11,41). G2-phase cells show a very strong punctuate γH2AX signal after etoposide treatment probably due to high numbers of etoposide-induced DSBs (Figure 3A). Thus, G2- and S-phase cells could be clearly identified and were excluded from analysis.

G1-phase cells depleted for CtIP show a DSB repair defect after etoposide treatment similar to Mre11-depleted cells. Importantly, down-regulation of both factors does not confer a defect greater than inhibition of each factor alone, suggesting an epistatic relationship between CtIP and Mre11 for the repair of etoposide-induced DSBs (Figure 3B). In contrast, down-regulation of Rad51, a key HR protein (42), does not affect repair kinetics after etoposide treatment in G1 and depletion of CtIP does not affect repair of radiation-induced DSBs in G1 (Supplementary Figure S3A and SB). These data

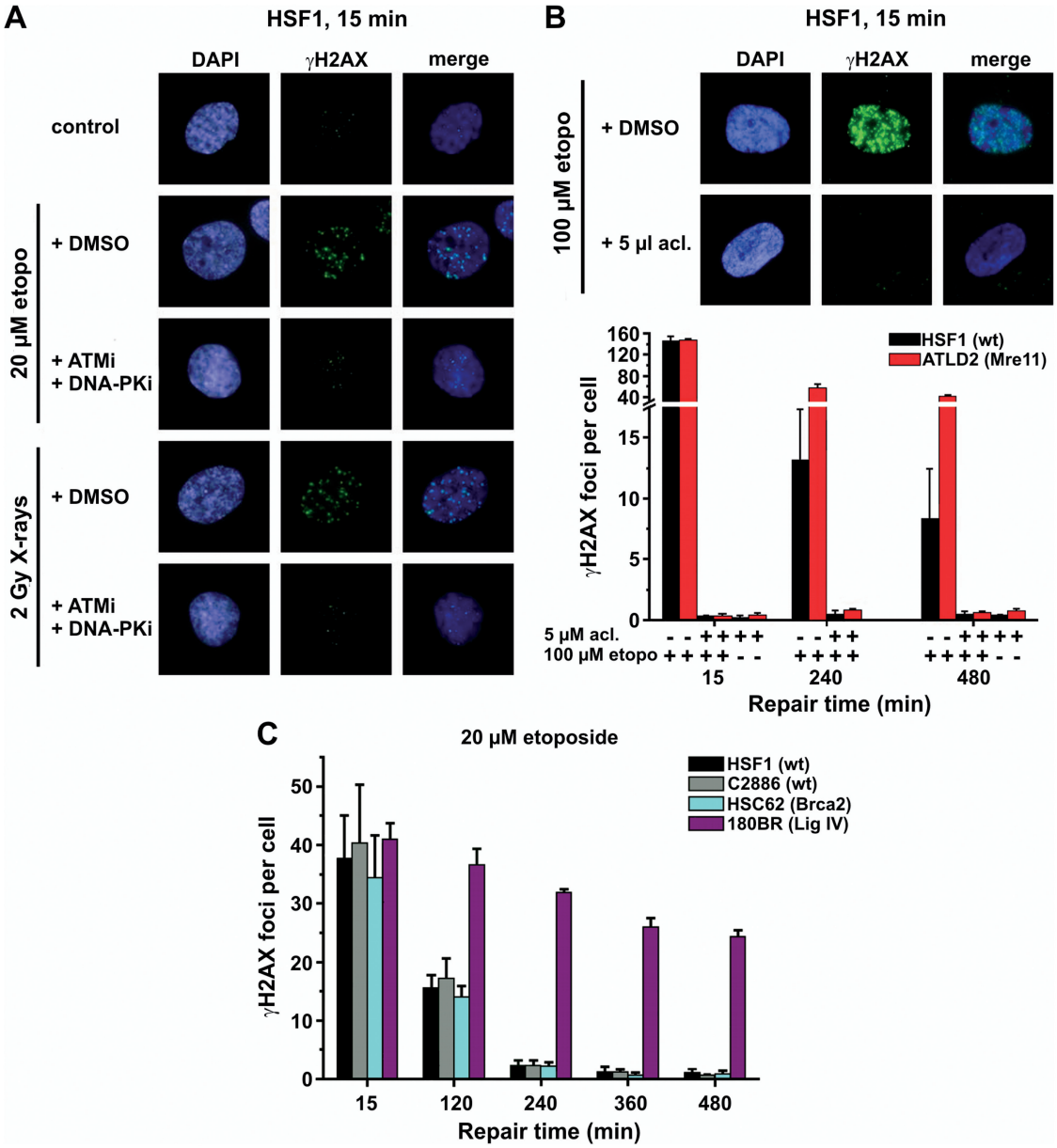


**Figure 1.** Etoposide-induced DSBs are repaired by NHEJ. (**A**) HSF1 cells were incubated with a specific ATM and DNA-PK inhibitor 1 h prior to etoposide treatment or irradiation. Foci formation is abolished by combined inhibitor treatment, showing that the kinases ATM and DNA-PK but not ATR phosphorylate H2AX. (**B**) γH2AX foci due to etoposide (etopo) treatment require topoII activity. Pre-treatment with aclarubicin (acl.), a topoII inhibitor, abolishes the formation of etoposide-induced γH2AX foci. Aclarubicin alone does not form γH2AX foci. (**C**) γH2AX foci kinetics in primary human fibroblasts. Wt (HSF1 and C2886) and Brca2-deficient cells (HSC62) show similar repair kinetics whereas LigIV-deficient cells (180 BR) exhibit elevated γH2AX foci levels after 20 μM etoposide treatment in G0/G1. Background foci numbers were subtracted. Error bars represent the standard deviation (SD) from at least three different experiments.

suggest that CtIP is involved in etoposide-induced DSB repair in G1.

To substantiate the notion that CtIP is involved in etoposide-induced NHEJ, we depleted CtIP in hTert immortalized human fibroblasts deficient for the NHEJ factor XLF (2BN hTert cells) (43,44). Repair proficient hTert cells show a repair defect after siRNA mediated CtIP depletion similar to CtIP-depleted HeLa cells (Figure 3C). 2BN hTert cells exhibit a substantial repair defect similar to that of LigIV-deficient 180BR cells. Down-regulation of CtIP in 2BN hTert cells does not further elevate the γH2AX foci level, demonstrating an epistatic relationship between CtIP and XLF (Figure 3C). These data establish that CtIP is involved in etoposide-induced DSB repair in G1 by NHEJ.

### CtIP function during etoposide-induced DSB repair in G1 requires Thr-847 phosphorylation

To gain further mechanistic insight into the role of CtIP in NHEJ of etoposide-induced DSBs, we analyzed different CtIP derivatives. We transfected CtIP-depleted HeLa cells

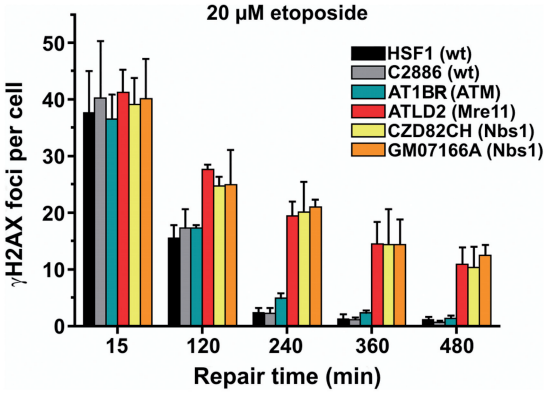


**Figure 2.** Etoposide-induced DSB repair by NHEJ involves the MRN complex. γH2AX foci kinetics were assessed in primary human fibroblasts. Mre11-defective (ATLD2) and Nbs1-defective (CZD82CH and GM07166A) but not ATM-defective primary human fibroblasts (AT1BR) exhibit elevated foci levels after 20 μM etoposide treatment in G0/G1 phase. Background foci numbers were subtracted. Error bars represent the SD from at least three different experiments.

transiently with different GFP-tagged siRNA resistant plasmids, each of them carry a certain mutation of CtIP. The consensus site Thr-847 was mutated to alanine (T847A) which prevents its phosphorylation and hence activation. To investigate the effect of CtIP/Brca1 complex formation on the repair of etoposide-induced DSBs, we expressed a mutated form of CtIP in which Ser-327 was substituted by alanine (S327A) which also results in prevention of phosphorylation and the disability to interact with Brca1. A wt CtIP plasmid and an empty vector carrying GFP were transfected as positive and negative controls, respectively. We only evaluated GFP-positive G1-phase cells (Figure 4A) and distinguished cell cycle phases on the basis of their DNA content as described previously (11).

CtIP siRNA treated cells transfected with wt CtIP repair etoposide-induced DSBs similar to control siRNA treated cells. CtIP siRNA treated cells transfected with T847A CtIP show the same repair defect as CtIP-depleted cells transfected with an empty vector, which demonstrates the necessity of Thr-847 phoshorylation for CtIP function in G1. Interestingly, the S327A mutant form of CtIP shows no repair defect (Figure 4B) suggesting that CtIP/Brca1 complex formation is dispensable for NHEJ of etoposide-induced DSBs in G1. Higher etoposide concentrations and data obtained with hTert immortalized human cells substantiate these observations (Figure 4C and Supplementary Figure S4A). In contrast to their differential requirement for etoposide-induced DSB repair in G1, both T847A and S327A mutants are deficient in Rad51 foci formation after irradiation in G2 (Figure 4D). Thus, CtIP is differentially regulated and possibly has different roles during the repair of etoposide-induced DSBs by NHEJ in G1 and the repair of radiation-induced DSBs by HR in G2.

Thr-847, which is important for repair in G1, represents a CDK1 phosphorylation site but CDK1 activity in G1 is low (45,46). Therefore, we examined if CDK1 activity is required for repair of etoposide-induced DSBs in G1 and analyzed HeLa cells treated with roscovitine, a selective CDK inhibitor (47). CDK inhibition 3 h prior to treatment significantly reduces Rad51 foci formation after irradiation in G2 but does not affect the repair of etoposide-induced DSBs in G1, suggesting that Thr-847 phosphorylation and hence CtIP activation is dependent on other kinases in G1 (Figure 4E). To exclude the possibility that CtIP phosphorylation occurs in G2 and is maintained until cells reach G1, we treated cells with roscovitine 6 and 9 h prior to etoposide treatment or irradiation and obtained the same result (Supplementary Figure S4B).

## DISCUSSION

The major finding of our work is that CtIP and the MRN complex promote NHEJ of etoposide-induced DSBs in G1. Both CtIP and the MRN complex have important roles in resecting DSB ends during HR (48,49) and in the removal of covalently bound topoII from DSB sites (30,50,51). Cell survival studies suggested that NHEJ is a major repair pathway for etoposide-induced DSBs; however, HR also contributes to resistance leaving unclear how CtIP and the MRN complex interplay with NHEJ to provide repair of etoposide-induced DSBs (32–34,52). We have addressed this question by specifically analyzing G1/G0-phase cells which, we show, repair etoposide-induced DSBs exclusively by NHEJ with no contribution of HR. Hence, the uncovered functions of CtIP and the MRN complex in G1/G0 phase are distinct to their function in HR. In support of this prediction, CtIP's roles during removal of topoII from the break site in G1 and resection of DNA ends during G2 have distinct phosphorylation requirements. We have used γH2AX foci analysis to measure DSB repair kinetics which served in this and several other previous publications as a highly sensitive, accurate and reliable method for assessing DSB levels in non-replicating G1/G0-phase cells (53–56). Although we have previously provided extensive evidence for a 1 : 1 relationship between foci numbers and DSBs [summarized in (57)] we here confirm that the foci analyzed arise from the enzymatic property of the topoII enzymes.

Nucleolytic processing by Mre11 is an essential function of fundamental importance for DNA repair, distinct from MRN-mediated control of ATM signaling (58). The nuclease activity is important for DSB end resection during HR as well as for the removal of topo II from the 5′-end of etoposide-induced DSBs (30,50,51). Similarly, the *Saccharomyces cerevisiae* Spo11 protein which initiates meiotic recombination must be removed before repair can occur, a process performed by the endonucleolytic activity of the Mre11 subunit of the MRX complex (49,59). Although the MRN complex is not a core component of NHEJ (58,60,61), we show here that it has a clear requirement for the repair of etoposide-induced breaks by NHEJ which is independent of ATM. However, some breaks are repaired in

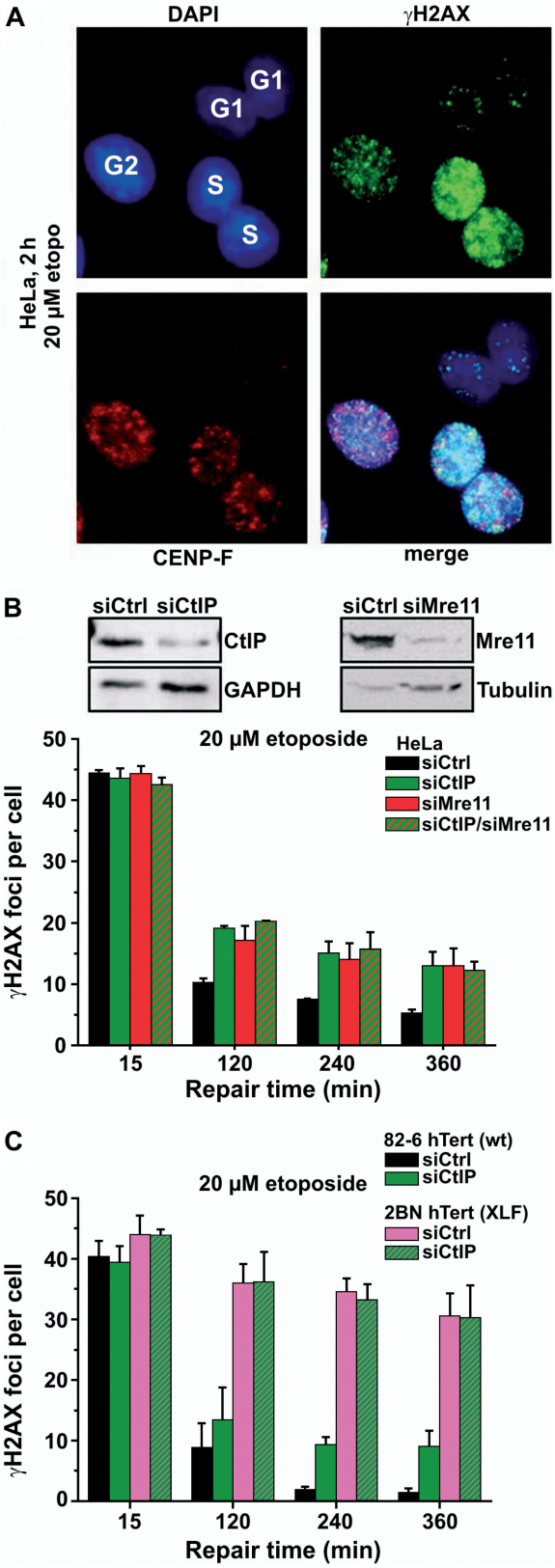


**Figure 3.** NHEJ of etoposide-induced DSBs in G1/G0 involves CtIP. (**A**) Identification of cell cycle phases in HeLa cells (see text for explanation). (**B**) γH2AX foci kinetics in siRNA treated HeLa cells analyzed 48 h after transfection. Down-regulation of Mre11 alone, CtIP alone or Mre11 and CtIP in combination results in similarly elevated γH2AX foci levels after etoposide treatment in G1-phase cells. Background foci numbers were subtracted. Error bars represent the SD from at least three different experiments. (**C**) γH2AX foci kinetics in hTert immortalized human fibroblasts. CtIP down-regulation in wt cells (82-6 hTert) results in a modest but significant repair defect. XLF-deficient cells (2BN hTert) exhibit a substantially higher repair defect. CtIP depletion in XLF-defective cells has no additional effect. Efficient CtIP down-regulation was confirmed by the abolishment of Rad51 foci formation after irradiation (data not shown). Background foci numbers were subtracted. Error bars represent the SD from at least three different experiments.

Mre11-deficient cells perhaps reflecting the elimination of topoII from the break sites via Tdp2 (62).

CtIP controls the initiation of DNA end resection and was described as an endonuclease that is stimulated by the MRN complex (48,49). Hartsuiker *et al.* (30,63) provided evidence that Ctp1 in *S. pombe* is responsible for the removal of 5′-linked proteins such as Rec12 (Spo11) and topoII, confirming functional conservation between Ctp1 and the more distantly related Sae2 protein from *S. cerevisiae* (CtIP in humans). Sae2 seems to be particularly important for the initiation of resection at DSBs with covalently bound proteins since sae2Δ mutants are defective in removing Spo11–DNA adducts (48,59). Loss of CtIP results in a dramatic defect in processing mitotic DSBs and down-regulation of CtIP decreases HR frequencies (22,64). Our observed involvement of CtIP in G1-phase cells is perhaps surprising since CtIP levels in human cells are highest during S/G2 and low during G1 (65). However, CtIP in chicken cells does function in G1 during MMEJ, a specialized end-joining pathway (24).

Huertas and Jackson (23) showed that the function of CtIP during HR is activated by CDK-dependent phosphorylation on Thr-847. Here, we show that Thr-847 phosphorylation is also needed for the repair of etoposide-induced DSBs in G1 by NHEJ. However, in contrast to its role in G2, Thr-847 phosphorylation in G1 can occur in the presence of the CDK inhibitor roscovitine suggesting that phosphorylation on this site is performed by other kinases in the absence of CDKs. Ser-327 is another CtIP site which is needed for CtIP function during HR in G2 but not for MMEJ in G1 (24). However, a more recent paper reported that resection measured by Rad51 foci formation is independent of Ser-332 phosphorylation in DT40 cells (Ser-327 in humans) and that S332A mutants exhibit sensitivity to etoposide treatment (31). We observed normal repair of etoposide-induced DSBs in G1 in the non-phosphorylatable S327A mutant suggesting that repair of etoposide-induced DSBs in G1 does not involve the resection function of CtIP in G2. This might also explain why CDK activity, which is essential for recombinational repair (45), is not required for etoposide-induced DSB repair.

Taken together, our results show that etoposide-induced DSBs in G1 are repaired by NHEJ with a requirement for the MRN complex and CtIP. We further show that the function of CtIP in this process has a phosphorylation requirement which is distinct to its role in resecting DSBs during HR. We suggest that the MRN complex and

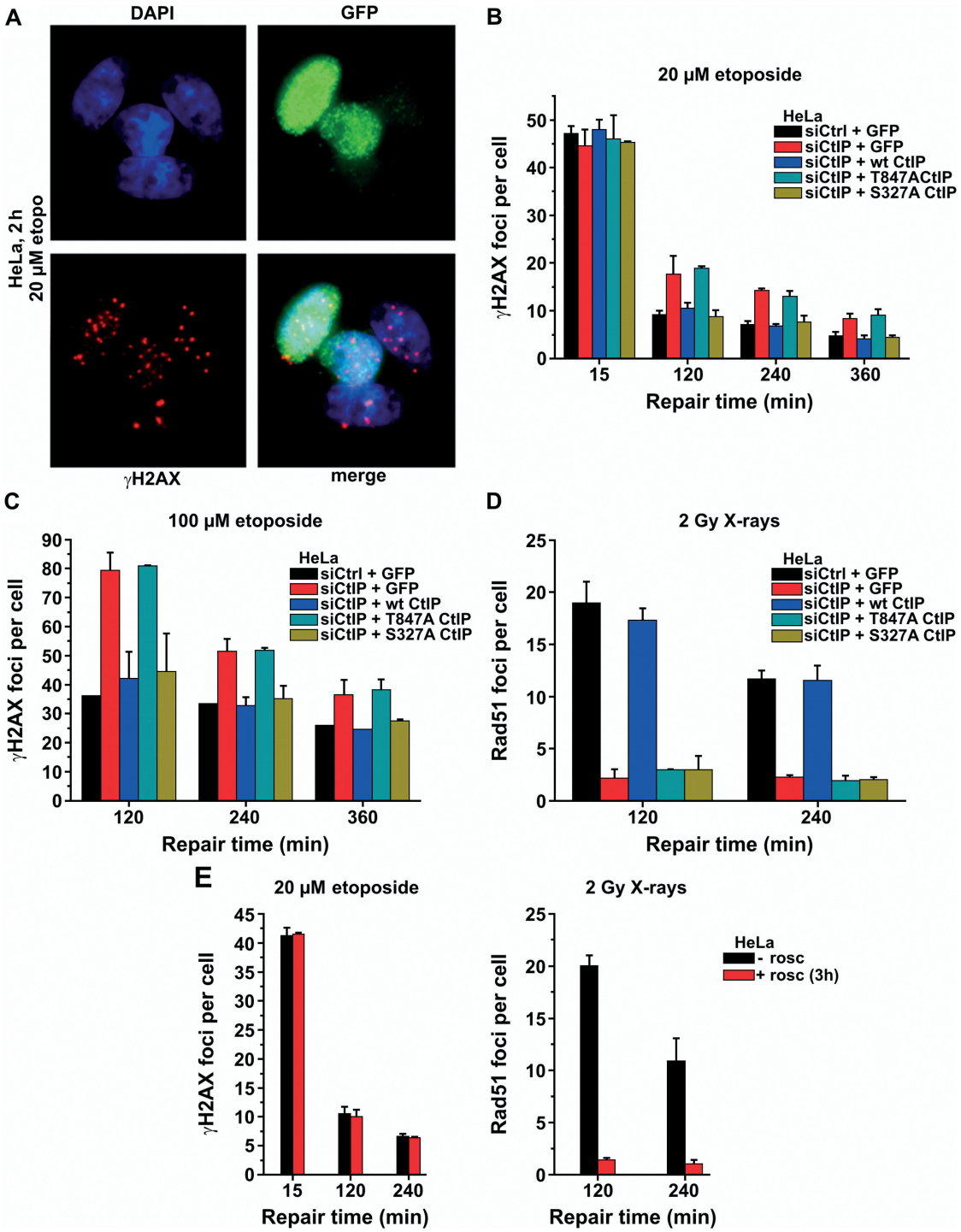


**Figure 4.** CtIP function during repair of etoposide-induced DSBs in G1 requires Thr-847 phosphorylation. (**A**) HeLa cells were depleted for endogenous CtIP by siRNA and transfected with various GFP-tagged CtIP plasmids. Only GFP-positive cells in G1 were analyzed. (**B**) γH2AX foci kinetics in HeLa cells after 20 μM etoposide. Cells transfected with the CtIP mutation T847A but not the mutation S327A exhibit a repair defect. Background foci numbers were subtracted. Error bars represent the SD from at least three different experiments. (**C**) γH2AX foci kinetics in HeLa cells after 100 μM etoposide. Background foci numbers were subtracted. Error bars represent the SD from at least two different experiments. (**D**) Rad51 foci in CENP-F positive G2-phase HeLa cells after 2 Gy X-rays. Cells transfected with the CtIP mutation T847A or the mutation S327A exhibit a defect in the formation of Rad51 foci. Background foci numbers were subtracted. Error bars represent the SD from at least two different experiments. (**E**) γH2AX and Rad51 foci analysis in HeLa cells treated with the CDK inhibitor roscovitine (rosc) for 3 h prior to etoposide treatment or irradiation. CDK inhibition does not affect γH2AX foci levels after etoposide treatment in G1-phase cells but inhibits Rad51 foci formation after 2 Gy X-irradiation in G2-phase cells. Background foci numbers were subtracted. Error bars represent the SD from at least two different experiments.

CtIP remove topoII from the DSB site prior to repair by NHEJ.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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