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Activation of DSB Processing Requires Phosphorylation of CtIP by ATR

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SUMMARY

DNA double-strand breaks (DSBs) activate a DNA damage response (DDR) that coordinates checkpoint pathways with DNA repair. ATM and ATR kinases are activated sequentially. Homology-directed repair (HDR) is initiated by resection of DSBs to generate 3′ single-stranded DNA overhangs. How resection and HDR are activated during DDR is not known, nor are the roles of ATM and ATR in HDR. Here, we show that CtIP undergoes ATR-dependent hyperphosphorylation in response to DSBs. ATR phosphorylates an invariant threonine, T818 of *Xenopus* CtIP (T859 in human). Nonphosphorylatable CtIP (T818A) does not bind to chromatin or initiate resection. Our data support a model in which ATM activity is required for an early step in resection, leading to ATR activation, CtIP-T818 phosphorylation, and accumulation of CtIP on chromatin. Chromatin binding by modified CtIP precedes extensive resection and full checkpoint activation.

INTRODUCTION

DNA double-strand breaks (DSBs) can arise during normal cell metabolism as a consequence of interaction with reactive oxygen species, the collapse of stalled replication forks, telomere dysfunction, or chromosome breakage during anaphase or following programmed genomic rearrangements during immune and germ cell maturation. Additionally, DSBs are formed after exposure to exogenous insults such as ionizing radiation (IR) or chemotherapeutic agents. Cells have evolved pathways, collectively termed the DNA damage response (DDR), to sense, signal, and repair these lesions. Failure to repair DSBs properly is associated with cancer development, radiation sensitivity, immune deficiencies, and developmental disabilities (Hoeijmakers, 2009).

DSBs are sensed by the Mre11-Rad50-Nbs1 complex (MRN), which binds to DNA ends and activates the ATM protein kinase (Lee and Paull, 2007). ATM, ATR, and DNA-PK are all members of the PIKK family of kinases that controls the DDR. ATM activation triggers multiple signaling pathways, causing changes in cell-cycle progression (damage checkpoints), gene expression, chromatin structure, and recruitment of repair proteins to sites of DNA damage (Derheimer and Kastan, 2010). DSBs can be repaired by nonhomologous end-joining (NHEJ), which requires very minimal or no end-processing. Alternatively, DNA ends are resected to form 3′ single-stranded DNA (ssDNA) overhangs that allow annealing of the ends or strand invasion and homology search during homology-directed repair (HDR) (Symington and Gautier, 2011). Repair pathway choice depends on cell-cycle phase, the structure of the damaged DNA ends, and the availability of DNA homologous to the damaged sequence (Shrivastav et al., 2008). HDR and NHEJ compete for DNA ends: binding of the NHEJ factor Ku impairs resection, whereas resection prevents Ku binding (Langerak et al., 2011; Sun et al., 2012). By generating RPA-coated ssDNA filaments, resection also activates a second protein kinase, ATR, which is recruited to ssDNA-RPA through the ATRIP adaptor protein (Zou and Elledge, 2003). Activation of Chk1 downstream of ATR requires a signaling complex that includes TopBP1, Rad9-Rad1-Hus1, and claspin. Activated Chk1 then spreads the checkpoint signal throughout the nucleus (Nam and Cortez, 2011). Thus, resection promotes a switch from ATM to ATR activation that reflects the conversion of double-stranded DNA (dsDNA) to ssDNA (Shiotani and Zou, 2009).

There are at least three distinct resection pathways. MRN-CtIP initiates resection, whereas Exo1 exonuclease both initiates and extends resection tracts. In addition, DNA2 nuclease, in association with a RecQ helicase homolog (Sgs1 in yeast, WRN or BLM in vertebrates) and Top3-Rmi1/2, can extend resection tracks. Studies of DSB repair often utilize restriction endonucleases to create DSBs with a free 5′ phosphate and 3′ hydroxyl group. Repair of these DSBs can occur in the absence of CtIP or MRN (Sartori et al., 2007) and is due to the activity of Exo1 exonuclease and the RecQ helicase, in cooperation with

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DNA2-Top3α-Rmi1/2 (Budd and Campbell, 2009; Liao et al., 2008a; Tomimatsu et al., 2012; Zhu et al., 2008). In contrast, resection of DSBs induced by IR, chemotherapeutic agents, or meiotic recombination, as well as those containing modified bases, altered chemistry, or covalent protein adducts (Barker et al., 2005; Henner et al., 1983; Keeney and Neale, 2006; Lawley and Phillips, 1996), must be initiated by the endonucleolytic activity provided by MRN in complex with CtIP (Paull, 2010). Thus, cells defective in Mre11 endonuclease activity or CtIP are highly sensitive to topoisomerase poisons and IR and are unable to repair Spo11-capped meiotic DSBs (Akamatsu et al., 2008; Hartsuiker et al., 2009b; Langerak et al., 2011; Limbo et al., 2007; Milman et al., 2009; Rothenberg et al., 2009; Sartori et al., 2007; Williams et al., 2008).

CtIP activation requires Cdk2 or Cdk1 phosphorylation of a conserved residue (T847 in humans, T806 in *Xenopus*). This modification restricts CtIP activity to the S, G2, and M phases of the cell cycle (Huertas and Jackson, 2009; Peterson et al., 2011), ensuring that HDR is not initiated before DNA replication provides a homologous template for repair. Many substrates of ATM and ATR have been identified, including proteins that regulate DSB repair (such as Mre11, Nbs1, or CtIP), but the functional impact of these modifications on HDR is not known. *S. cerevisiae* Sae2, the budding yeast ortholog of CtIP, is phosphorylated by ATM (Tel1) as well as ATR (Mec1)—principally by the latter—and these modifications are required for Sae2 activity (Baroni et al., 2004). The functional consequences of CtIP phosphorylation by PIKKs are not known.

The DDR can be recapitulated in cell-free extracts derived from *Xenopus laevis* eggs (Garner and Costanzo, 2009; Srinivasan and Gautier, 2011). For example, DSB resection has been studied in this setting through the use of small DNA templates (Liao et al., 2012b, 2008a, 2011; Taylor et al., 2010), as well as in the context of chromosomal DSBs (Peterson et al., 2011; You et al., 2009). Low-speed supernatant (LSS), a cytosolic egg extract that contains all soluble proteins and lipids, supports nuclear envelope assembly around added sperm chromatin, which then undergoes semiconservative DNA replication. High-speed supernatant (HSS), obtained by ultracentrifugation of LSS, is membrane-free and does not replicate DNA. However, HSS still supports many DNA transactions, such as pre-replication complex formation, ATM and ATR activation, resection, and checkpoint signaling in response to DSBs.

Here, we use HSS and LSS extracts to evaluate the contributions of the three different resection pathways and the roles of ATM and ATR. We show that EXO1 can initiate resection of DSBs generated by endonucleases in the absence of ATM. In contrast, ATM is essential for CtIP-MRN-dependent resection in EXO1-defective extract. We identify CtIP-T818 (corresponding to human T859) by mass spectrometry as a conserved target of phosphorylation by PIKKs. Characterization of this site using phosphospecific antibodies and mutational analysis reveals that it is phosphorylated by ATR and is required for the binding of CtIP to chromatin and subsequent processive resection. ATM activation precedes ATR phosphorylation of CtIP, which allows DNA2-dependent long-range resection. Therefore, our work provides a functional link between the activation of checkpoint kinases and the repair of DSBs.

RESULTS

Initiation of DSB Resection in Cell-free *Xenopus* Extracts

Studies in yeast and our work in *Xenopus* extracts suggest that at least two pathways can initiate DNA DSB resection in the context of chromatin. In LSS inactivation of the CtIP-MRN pathway delays but does not abrogate resection (Peterson et al., 2011), suggesting that other factors could initiate resection. In contrast, membrane-free cytosol, referred to as HSS, exclusively initiates resection via the CtIP-MRN pathway (Peterson et al., 2011; You et al., 2009). Our studies also established that the EXO1, DNA2, and CtIP-MRN pathways all participate in processing DSBs on small DNA templates (Liao et al., 2012a, 2008b, 2011). We wished to elucidate the molecular basis for the difference between extracts and to delineate the contribution of the three known resection pathways (CtIP-MRN, EXO1, and DNA2) toward initiation and long-range chromosomal DNA resection. We quantitatively depleted EXO1 from LSS using specific antibodies (Liao et al., 2011). EXO1 depletion inhibited, but did not abolish, ssDNA formation from PflMI-induced DSBs, as detected by chromatin binding of RPA (Figure 1A). As we reported previously (Peterson et al., 2011), CtIP depletion likewise reduced, but did not abrogate, resection (Figure 1A). Importantly, no resection was detected in the absence of both EXO1 and CtIP. These results indicate that CtIP-MRN and EXO1 independently initiate resection of DSBs (Figure 1A). We next evaluated the contribution of the DNA2 pathway, which processively extends resection tracts but, unlike EXO1, cannot initiate resection. Depletion of DNA2 nearly abrogated resection (Figure 1B), as did immunodepletion of both EXO1 and DNA2 (Figure 1B). The resection defect in EXO1-depleted extracts was rescued by addition of purified recombinant EXO1 protein. However, the addition of purified DNA2 protein did not rescue DNA2 depletion, presumably because DNA2 functions in a complex with BLM/RMI1/TOP3α or WRN/RMI1/TOP3α. We conclude that EXO1 and DNA2 pathways are not redundant for resection, that DNA2 resection depends upon initial DSB processing, and that resection in LSS can be accounted for by the CtIP-MRN, DNA2, and EXO1 pathways.

In contrast to LSS, CtIP depletion abrogates resection in HSS (Peterson et al., 2011; You et al., 2009). This suggests that the EXO1 pathway is inactive in HSS and that processive recession is promoted by DNA2. As predicted, the depletion of DNA2 from HSS eliminated resection (Figure 1C). This establishes that DNA2 requires CtIP in order to perform long-range resection in the absence of EXO1.

Hyperphosphorylation of CtIP in Response to DNA DSBs

Phosphorylation of CtIP following DNA damage has been reported in yeast and mammalian cells, both in functional studies as well as in phosphoproteomic screens (Baroni et al., 2004; Kim et al., 1999; Li et al., 2000; Matsuoka et al., 2007). We therefore asked whether CtIP was also phosphorylated in a DSB-dependent manner in *Xenopus* extracts. LSS was incubated with or without linear DNA fragments to trigger DSB signaling. The phosphatase inhibitor okadaic acid (OA) was added to trap labile phosphorylation intermediates (Figure 2A). In the
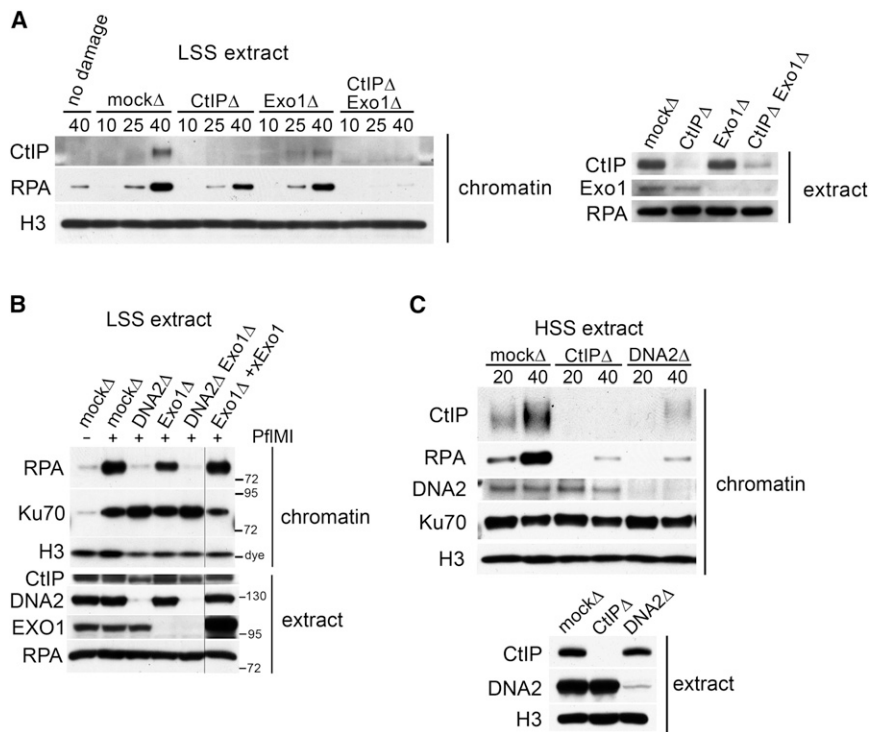
Figure 1. Contribution of Individual Resection Pathways to Resection of Chromosomal DNA DSBs

(A) Chromatin was incubated in extract that was depleted of CtIP, Exo1 or both, or mock-depleted and isolated at the indicated time (minutes) after addition of 0.05 U/μL PfIMI restriction endonuclease, and then immunoblotted with the indicated antibodies ("chromatin," left panels). Aliquots of the depleted extract were immunoblotted with the indicated antibodies to assess immunodepletion ("extract," right panels).

(B) Chromatin was incubated in mock-depleted LSS or LSS immunodepleted of DNA2, Exo1, or DNA2 and Exo1, and chromatin isolation was performed as in (A). Recombinant Exo1 was added to Exo1-depleted extract as indicated.

(C) HSS extract was mock-depleted or depleted of either CtIP or DNA2, and chromatin isolation was performed as in (A).

absence of DSBs, OA treatment revealed a single species of phosphorylated CtIP, whereas DSBs and OA induced a striking electrophoretic mobility shift of soluble CtIP. This mobility shift was reduced with caffeine, an inhibitor of ATM and ATR. Remarkably, all isoforms of CtIP collapsed to a single, fast-migrating band following λ phosphatase treatment, confirming that the electrophoretic mobility change was due exclusively to phosphorylation (Figure 2A).

CtIP Is Phosphorylated at T818 by ATR in Response to DSBs

We then identified the critical damage-dependent phosphorylation sites in CtIP. Recombinant FLAG-tagged CtIP was incubated in LSS with OA in the presence or absence of DSBs, then affinity-purified and subjected to tandem mass spectrometry analysis. The complete list of phosphorylation sites induced by DSBs is shown in Figure S1A (available online). Notably, two DSB-induced phosphorylation sites, T200 and T818, conformed to the canonical TQ PIKK consensus sequence (Figure 2B). Although T200 is not conserved phylogenetically, T818 lies within the evolutionarily conserved C terminus of CtIP. Moreover, this site exists within a TQ sequence among CtIP orthologs ranging from budding yeast Sae2 (T279) to human CtIP (T859) (Figure 2C). Therefore, we produced and affinity-purified a phosphospecific antibody against phospho-T818 (pT818) and tested its specificity for phosphorylated T818 in *Xenopus* and the equivalent T859 in human. Recombinant wild-type (WT) CtIP or CtIP-T818A (*Xenopus*) and WT CtIP or CtIP-T859A (human) proteins were incubated in extracts with OA in the presence of DSBs and then probed for total CtIP and pT818-CtIP (αpT818, Figure 2D). The antibody recognized phosphorylated *Xenopus*

and human CtIP, but not the nonphosphorylatable T818A and T859A variants, demonstrating the specificity of the antibody. Importantly, treatment with λ phosphatase eliminated the pT818 signal (Figure S1B). To identify the protein kinase(s) responsible for CtIP-T818 phosphorylation in response to DSBs, we used small molecule inhibitors specific for ATM (KU55933; Hickson et al., 2004), ATR (ETP-46464; Toledo et al., 2011), or DNA-PK (NU7441; Leahy et al., 2004) and monitored CtIP electrophoretic mobility shifts in LSS supplemented with DSB-containing DNA (Figure 2E). Inhibition of DNA-PK or ATM only slightly increased the mobility of phosphorylated CtIP. In contrast, inhibition of ATR strongly reduced CtIP phosphorylation. Notably, ATR inhibition also abrogated detection of pCtIP with the anti-pT818 antibody, indicating that ATR phosphorylates CtIP on T818 (Figure 2E). T818 phosphorylation of soluble CtIP is transient and is undetectable in the absence of phosphatase inhibitor. Since ATR activates Chk1, we also treated extracts with a Chk1 inhibitor (SB-218078), which did not affect DSB-dependent phosphorylation of T818. Our data are therefore consistent with ATR phosphorylating T818 of CtIP (Figure S1).

ATR is activated by RPA-coated ssDNA (Costanzo et al., 2003; Zou and Elledge, 2003). This intermediate is generated by resection of DSBs, as well as by replication fork stalling and subsequent uncoupling of the replicative helicase. To test whether RPA-coated ssDNA in the absence of DSBs was sufficient to cause chromatin recruitment of CtIP, we treated replicating chromatin with aphidicolin to trigger fork stalling and ATR activation (Byun et al., 2005). As a control, we used a HaeIII restriction enzyme to create DSBs, which are resected to form RPA-ssDNA (Figure S1D). As anticipated, CtIP was recruited to chromatin-containing DSBs but not to RPA-ssDNA filaments that form in the absence of DSBs.

We then tested whether DNA damage induced phosphorylation of CtIP in human cells. Since the CtIP domain containing
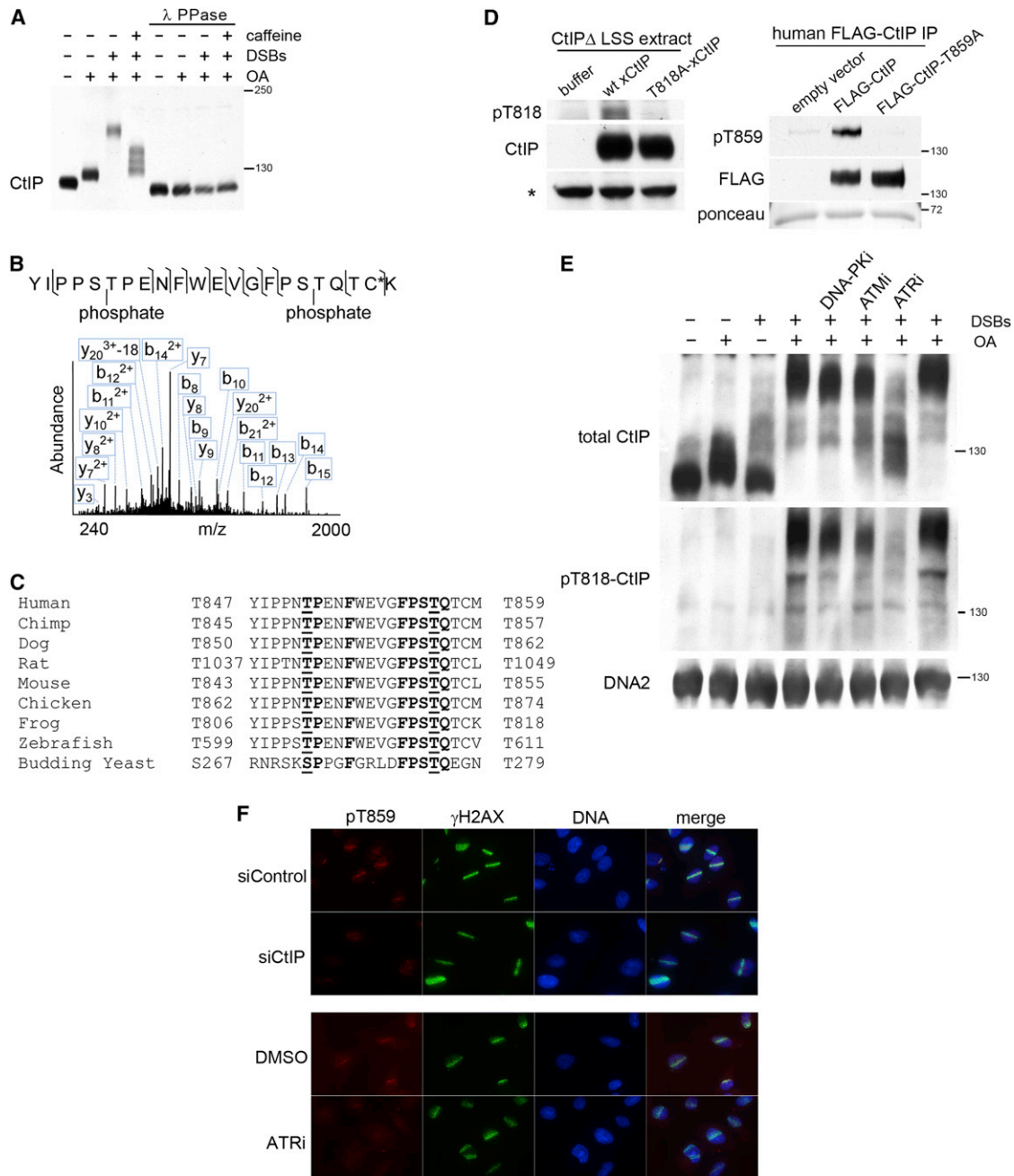
Figure 2. CtIP Is Hyperphosphorylated by ATR in Response to DSBs, Including Conserved Residue T818

(A) LSS was incubated with okadaic acid (OA), BstEII-digest of λ DNA (DSBs), and 5 mM caffeine, as indicated. Samples were split and half were treated with λ phosphatase. Protein size markers are indicated at right (kD).

(B) FLAG-xCtIP was incubated in LSS treated with OA (with or without DSBs), recovered by affinity purification, and subjected to tandem MS. Shown is a spectrum of a triply protonated diphosphopeptide-spanning residues 801–822. The fragmentation pattern localizes a phosphate group to S805/T806, and a second to S817/T818 (*, alkylated cysteine).

(C) The site of the TP and TQ motif for each model organism is listed to the left and the right of the alignment, respectively.

(D) In the left panels, WT or T818A-xCtIP was incubated in CtIP-depleted LSS in the presence of OA and DSBs, as in (A) (*, nonspecific, cross-reactive band used as a loading control). In the right panels, human WT or T859A-CtIP was expressed in 293 cells, isolated with anti-FLAG beads, and incubated in LSS in the presence of OA and DSBs. Western blots were probed as indicated.

(E) Experiment performed as in (A), but with 50 μM DNA-PKi, 100 μM ATMi, or 50 μM ATRi, as indicated.

(F) U2OS cells treated with control or CtIP siRNA 2 days prior to irradiation and treated with DMSO or 10 μM ATRi 1 hr prior to localized "stripe" irradiation with a high-power UV laser. Cells were fixed and stained with anti-pT818-CtIP and anti-γH2AX antibodies.
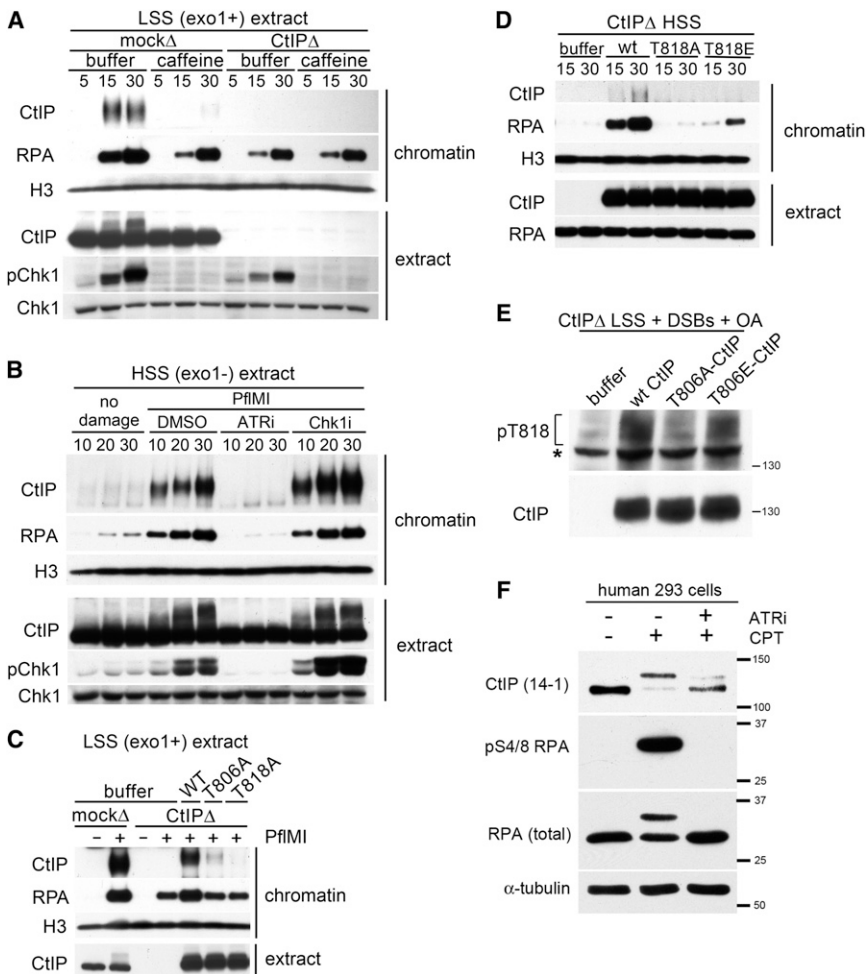
See also Figure S1.

Figure 3. ATR Phosphorylation of T818 Is Required for CtIP Chromatin Binding

(A) Chromatin was isolated (at the indicated time after addition of PflMI) from mock- or CtIP-depleted LSS that was incubated with either 5 mM caffeine or buffer.

(B) Chromatin was incubated in HSS in the presence of 50 μM ATRi, 50 μM Chk1i (SB-218078), or DMSO and isolated at the indicated time (min) after addition of PflMI or buffer (no damage). Samples were processed as in (A).

(C) Mock- or CtIP-depleted LSS was incubated with 80 nM recombinant WTCtIP, T806A-CTIP, T818A-CtIP, or buffer, as indicated. Chromatin was isolated 30 min after the addition of PflMI (+) or buffer (−).

(D) CtIP-depleted HSS was incubated with 40 nM recombinant WT CtIP, T818A-CtIP, T818E-CtIP, or buffer, and chromatin was isolated as in (A).

(E) Experiment performed as in Figure 2D with WT CtIP, T806A-CtIP, or T806E-xCtIP incubated in CtIP-depleted extract (*, nonspecific cross-reacting band).

(F) A total of 293 cells were incubated with 10 μM ATRi prior to treatment with DMSO or CPT. Cell lysates were probed with the indicated antibodies. See also Figure S2.

To determine whether ATR was the PIKK responsible for activating CtIP-dependent resection in extracts, we used small molecule inhibitors of ATR (or its effector kinase, Chk1) and monitored RPA recruitment to DSBs (Figure 3B). To circumvent resection by EXO1, we performed these experiments in HSS. Notably, we found that ATR inactivation abolished both recruitment of CtIP to damaged chromatin and resection. Interestingly, inhibition of Chk1, which is downstream of ATR, had no effect on CtIP phosphorylation, chromatin recruitment, or DSB resection (Figure 3B). To confirm the specificity of the small-molecule ATR inhibitor, we immunodepleted ATR from HSS (Costanzo et al., 2003). As with chemical inhibition, immunodepletion of ATR abrogated CtIP-dependent resection (Figure S2A).

To test the functional significance of phosphorylation on T818, we compared recombinant xCtIP proteins with or without substitutions at T818, including nonphosphorylatable T818A and phosphomimic T818E. The recombinant proteins were purified from baculovirus-infected insect cells and added to CtIP-depleted LSS. As expected, the purified recombinant proteins (Figure S2B) did not react with the pT818 antibody (Figure S2C). Unlike WT CtIP, the CtIP-T818A mutant neither bound to chromatin nor stimulated resection (Figure 3C). Similarly, CtIP-T818A did not support resection in CtIP-depleted HSS. In contrast, CtIP-T818E partially restored resection in depleted HSS (Figure 3D), indicating that T818 phosphorylation by ATR is required for CtIP chromatin binding and resection of damaged chromatin. CtIP-T818E did not restore resection in
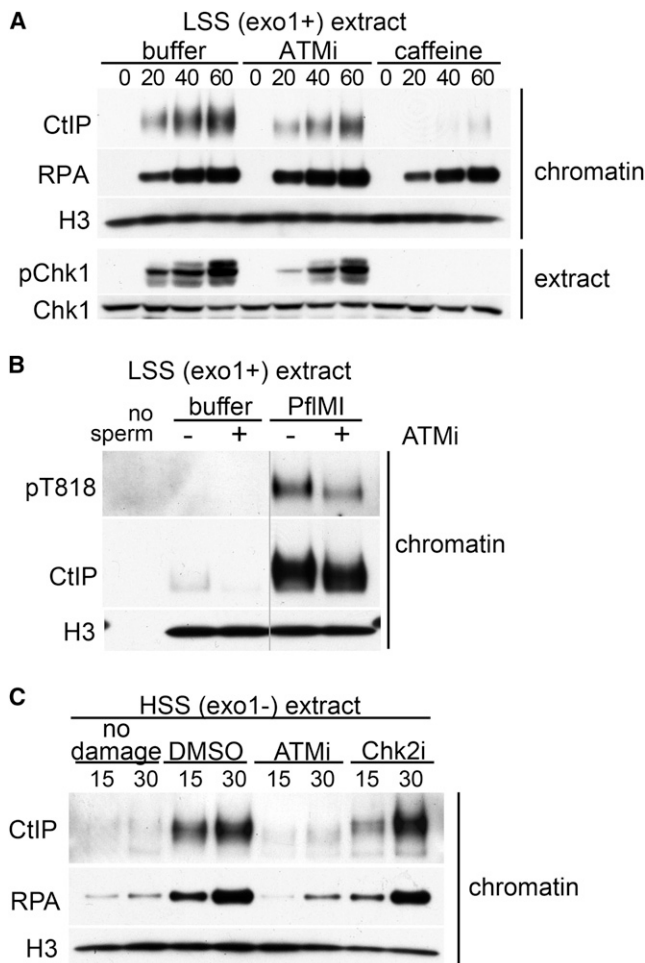
T818 is highly conserved (Figure 2C) and the pT818 antibody reacts with phosphorylated T859 in human CtIP (Figure 2D), we induced DSBs with the use of a high-power UV laser in human cells in the absence of OA and probed them with anti-pT818 antibody (Peterson et al., 2011). The pT859-CtIP signal colocalized with the DSB marker γH2AX. pT859-CtIP signal was absent in cells depleted of CtIP via small interfering RNA (siRNA), establishing the specificity of the phosphospecific antibody (Figure 2F). Furthermore, treatment with ATRi prior to irradiation significantly decreased the pT859-CtIP signal compared to treatment with DMSO, despite the presence of DSBs (Figure 2F). This establishes that regulation of CtIP by ATR phosphorylation of T818/T859 is conserved among vertebrates.

Phosphorylation of CtIP by ATR Is Required for Resection

To determine the functional consequences of DSB-dependent CtIP phosphorylation, we evaluated the effect of caffeine on CtIP-dependent resection in LSS. Caffeine almost entirely abolished CtIP chromatin binding and partially inhibited resection, similar to the depletion of CtIP (Figure 3A). In contrast, resection in CtIP-depleted LSS was not inhibited by caffeine, indicating that EXO1 does not require ATM or ATR activity (Figure 3A).

Figure 4. ATM Activity Is Required for CtIP-Dependent Resection Initiation

(A) Chromatin was incubated in mock- or CtIP-depleted LSS with buffer, 100 μM ATMi or 5 mM caffeine, then isolated and probed with the indicated antibodies.

(B) 100 μM ATMi (+) or DMSO (−) was added to LSS extract treated with PflMI or buffer and chromatin was isolated after 40 min.

(C) Chromatin was incubated in HSS in the presence of 100 μM ATMi, 100 μM Chk2i (PV1019), or DMSO and then isolated and probed with the indicated antibodies.

See also Figure S3.

CtIP-depleted extract in the presence of ATRi, suggesting either that additional ATR-dependent phosphorylation events also regulate CtIP activity or that CtIP-T818E is a weak phosphomimic of pT818 (data not shown).

Phosphorylation of CtIP by CDK2 at the onset of S phase is essential for CtIP-dependent resection (Huertas and Jackson, 2009). In *Xenopus*, this phosphorylation takes place at T806 and is required for CtIP association to chromatin (Peterson et al., 2011). Since T818 and T806 are in close proximity within the highly conserved domain of CtIP (Figure 2C), we asked whether CDK phosphorylation is a prerequisite for ATR phosphorylation. We showed previously that CtIP-T806A is defective in chromatin binding, whereas CtIP-T806E is functional (Peter-

son et al., 2011). Therefore, we generated and purified CtIP-T806A and CtIP-T806E and compared phosphorylation of T818 in these mutants to WT CtIP upon incubation in extracts containing DSBs (Figure 3E). Notably, WT CtIP and CtIP-T806E became phosphorylated at T818, whereas phosphorylation of CtIP-T806A was greatly reduced, indicating that phosphorylation of T806 by CDK facilitates T818 phosphorylation by ATR.

Lastly, we tested the impact of ATR inhibition on resection in human cells. U2OS cells were treated with camptothecin (CPT), which generates DSBs during S phase, in the presence or absence of ATRi (Figure 3F). CPT treatment triggered phosphorylation of CtIP, as evidenced by its reduced electrophoretic mobility, and the initiation of DNA resection, as indicated by Ser4/Ser8 phosphorylation of RPA2 (Figure 3F) (Bunting et al., 2010; Sartori et al., 2007). Moreover, CPT-induced DNA resection was abolished by siRNA-mediated CtIP depletion, indicating that resection is dependent on CtIP (data not shown). Notably, ATRi treatment abrogated both CtIP phosphorylation and CPT-induced DNA resection (Figure 3F). Therefore, we conclude that phosphorylation of CtIP by ATR is required for resection in human cells.

A Distinct Role for ATM in Resection Initiation

Previous studies in HSS extracts implicated ATM in chromosomal DSB resection (You et al., 2009). We therefore probed the role of ATM in CtIP-dependent resection in HSS as well as in LSS. First, we tested the impact of ATM inhibition on CtIP binding to chromatin in LSS. Inhibition of ATM decreased, but did not abolish, CtIP binding. In contrast, inhibition of both ATM and ATR with caffeine entirely abrogated CtIP binding (Figure 4A). Inhibition of ATR alone using ATRi in LSS gave the same result (Figure S3), which is consistent with a requirement of ATR for stable CtIP association to chromatin in HSS (Figure 3B). Furthermore, ATM inhibition reduced, but did not abolish, phosphorylation of T818, supporting the notion that T818 is modified by ATR (Figures 2E and 4B).

The decrease in pT818 upon exposure to ATMi was commensurate with reduction in chromatin-bound CtIP (Figure 4A). We suggest that this reflects the delayed kinetics of ATR activation when ATM is inhibited (compare the rates of CtIP chromatin binding to Chk1 activation in Figure 4A).

In contrast to LSS, inhibition of ATM in HSS abrogated CtIP chromatin binding and resection. This is a direct effect of ATM, since inhibition of the ATM effector Chk2 had no impact on CtIP binding or resection (Figure 4C). We conclude that ATM is required for initiating resection in HSS but not in LSS. As mentioned above, a critical difference between these extracts is their EXO1 status. EXO1 is active in LSS and can initiate resection in the absence of active ATM (Figure 3A, CtIPΔ; compare presence versus absence of caffeine), generating ssDNA-RPA and activating ATR. We hypothesize that in EXO1-deficient HSS, ATM activity is required for initial processing of DSBs.

DISCUSSION

We have investigated the roles of ATM and ATR in DSB resection of chromosomal DNA using two types of cell-free *Xenopus*
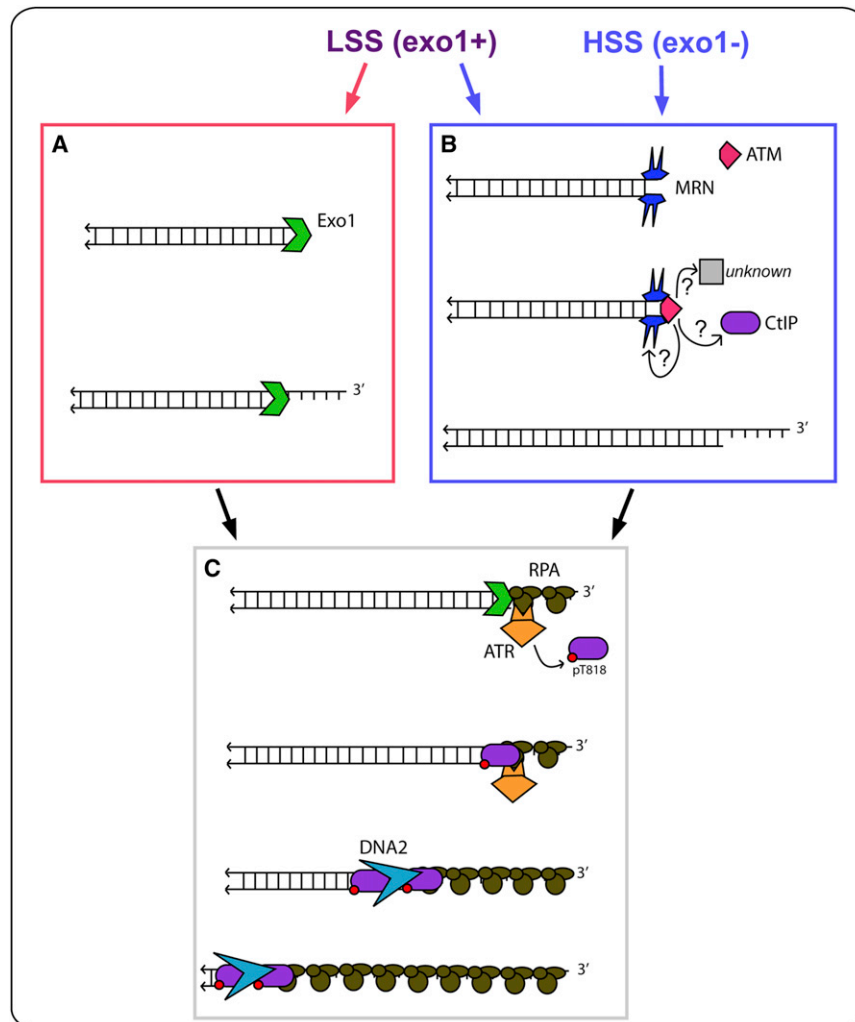
Figure 5. Model for Resection of Neat DSBs in LSS and HSS

(A and B) In LSS, resection can be initiated by Exo1 in an ATM-independent manner (A) or by CtIP-MRN in an ATM-dependent manner (B). In HSS, Exo1 is not active and resection is initiated by CtIP-MRN in an ATM-dependent manner (B).

(C) Initiation by either pathway allows clipping of DSBs to provide sufficient RPA-ssDNA to locally activate ATR. ATR then phosphorylates CtIP on T818, which is essential for stable CtIP chromatin binding and downstream extension of resection tracts by a DNA2-dependent mechanism.

mutants in yeast (Lewis et al., 2002; Moreau et al., 2001). Additionally, EXO1 and CtIP may cooperate to promote efficient resection, since these proteins can interact (Eid et al., 2010). In contrast, membrane-free cytosol (HSS), derived from high-speed centrifugation of LSS, supports resection only when CtIP is present and ATM and ATR are active (Figures 1and 4C), supporting the notion that HSS is EXO1-defective (Figure 5B).

The requirement for ATM in the absence of EXO1 (Figure 4C) (You et al., 2009) suggests that initiation of resection requires ATM phosphorylation of a component of MRN, CtIP, or of another factor (Figure 5B). In support of this notion, phosphoproteomic screens in humans reveal phosphorylation of CtIP, Mre11, Rad50, and Nbs1 in response to DNA damage (Kim et al., 1999; Matsuoka et al., 2007; Mu et al., 2007). However, the functional consequences of these phosphorylation events have not been determined. Consistent with a role for ATM in initiating resection, ATM-deficient mammalian cells have a defect in RPA foci formation and Chk1 activation in response to IR (Cuadrado et al., 2006; Jazayeri et al., 2006; Myers and Cortez, 2006; Shiotani and Zou, 2009).

There is ample evidence in yeast and vertebrates that CtIP-MRN promotes limited processing of DSBs. Budding yeast cells lacking EXO1 and Sgs1 undergo slow, Sae2-MRX-dependent degradation of DSBs, indicating that Sae2-MRX can clip oligonucleotides from the 5′ strand (Mimitou and Symington, 2008; Shim et al., 2010; Zhu et al., 2008). Moreover, Sae2 is required for processing Spo11-capped DSBs during meiosis (McKee and Kleckner, 1997; Prinz et al., 1997), which yields a short oligonucleotide covalently linked to Spo11 at the 5′ terminus (Hartsuiker et al., 2009a; Neale et al., 2005). This suggests that Sae2-MRX initiates resection by endonuclease attack on the 5′ strand. A similar mechanism has been proposed for *S. pombe* Ctp1 and vertebrate CtIP in removal of protein adducts from 5′ DNA ends (Hartsuiker et al., 2009b; Milman et al., 2009; Nakamura et al., 2010; Rothenberg et al., 2009). Furthermore, Mre11-bound oligonucleotides have been detected in *Xenopus*

extracts, one lacking active EXO1 (HSS) and one containing active EXO1 (LSS). We show that CtIP is phosphorylated at T818 (T859 in humans) by ATR and that ATR phosphorylation at T818 and other sites promotes stable association of CtIP to damaged chromosomes, allowing CtIP-dependent resection. This finding is significant, as it establishes a direct functional link between a PIKK and DSB repair by HDR in vertebrates. Moreover, our observation has several implications for the current model of DSB repair, as will be discussed below. Our findings are summarized in Figure 5.

ATM and DSB Resection

Crude extract (LSS) supports resection in the absence of CtIP or active ATM and ATR (Figures 1A, 3A and 4A), whereas codepletion of EXO1 and CtIP from LSS abolishes all resection (Figure 1A). Furthermore, depletion of DNA2 nearly abrogates resection. Together, these results suggest that EXO1 can initiate restriction-enzyme-generated DSBs independently of CtIP, ATM, or ATR but cannot support long-range resection in the absence of DNA2 (Figure 5A). This is consistent with the fact that EXO1 overexpression can rescue the IR defect of MRX

extracts following incubation with DSB-containing DNA. These complexes are sufficient to activate ATM (Jazayeri et al., 2008). Finally, in *S. pombe*, the endonuclease activity of Mre11 (but not its exonuclease activity) is important for resistance to IR, which requires processing of modified DNA ends (Williams et al., 2008).

We cannot detect chromatin-bound CtIP in the absence of ATR activity (Figures 3B and S3), suggesting that the initial ATM-dependent step does not require stable chromatin association of CtIP. However, EXO1-defective HSS depleted of DNA2 supports modest recruitment of CtIP to chromatin (Figure 1C), suggesting that slow processing of DSBs by CtIP-MRN generates sufficient ssDNA to allow binding of RPA and ATR and subsequent stabilization of CtIP. However, we cannot rule out the possibility that this effect is due to incomplete depletion of DNA2.

ATR and DSB Resection

We propose that CtIP-MRN-dependent initial processing generates sufficient ssDNA to allow binding of RPA and ATRIP-ATR, which requires at least 60 nt of ssDNA (Zou and Elledge, 2003). Limited activation of ATR is then responsible for CtIP phosphorylation at T818, which in turn promotes stable chromatin association of CtIP and robust resection. We note that T818 is conserved within a TQ sequence from yeast Sae2 to human CtIP. Damage-induced T859 phosphorylation is observed in *Xenopus* extracts and human cells (Figures 2D and 2F), suggesting that phosphorylation of CtIP by ATR is an evolutionarily conserved mechanism linking DDR to activation of DSB repair by HDR. Indeed, inhibition of CtIP phosphorylation with ATRi abrogates resection in human cells (Figure 3F). Mutation of all nine putative ATM/ATR phosphorylation sites, including the invariant threonine (*S. cerevisiae* T279, *Xenopus* T818), inactivates *S. cerevisiae* Sae2, whereas mutation of any single site, including the T818 equivalent (T279), has no functional consequence (Baroni et al., 2004). Phosphorylation of human CtIP at two ATM/ATR sites, S664 and S745, has been reported (Li et al., 2000; Matsuoka et al., 2007), but whether modification of these sites affects CtIP activity is not known (Li et al., 2000; Wu-Baer and Baer, 2001). Phosphorylation of human CtIP-T859 (corresponding to *Xenopus* T818) has not been previously described.

Given the importance of CtIP in DDR, redundant mechanisms for its recruitment to damaged chromatin may exist. You et al. (2009) described a "DR motif" within the central region of the protein that promotes CtIP recruitment to sites of DNA damage but does not contain T818 or T859. Thus, both elements of CtIP (T818/T859 phosphorylation and the DR motif) may contribute to the recruitment of CtIP to damage sites. We note that the DR motif is not fully conserved in *Xenopus* and is entirely absent in yeast. In contrast, the T818/T859 phosphorylation site is conserved as a TQ sequence from yeast to human (Figure 2C). The greater conservation of the T818/T859 phosphorylation site may reflect its key role as a reversible modification that allows CtIP recruitment in response to ATR signaling. Moreover, structural data show that CtIP interaction with Nbs1—through an N-terminal motif of CtIP—is critical for CtIP recruitment (Lloyd et al., 2009; Williams et al., 2009).

ATM inhibition does not prevent CtIP chromatin binding when resection is initiated by EXO1; i.e., when generation of the ssDNA-RPA substrate to activate ATR occurs independently of ATM activity (Figures 4A and 4B). Thus, if ATM phosphorylates CtIP, this phosphorylation is not required for ATR-dependent association of CtIP with chromatin.

Implications for ATR Activation

Our model suggests that ATR can promote its own activation by phosphorylating and activating CtIP, which in turn generates additional ssDNA-RPA (Figures 3B and 5C) (Cimprich and Cortez, 2008). Starting this feedback loop would require some initial ATR activation. We favor the idea that this initial step is triggered by limited end-processing of DSBs dependent on Exo1 (Figure 5A) and/or ATM, MRN, and CtIP (Figure 5B).

A positive feedback loop for ATR activation is consistent with the fact that ATR kinase activity is important for the localization of RPA and ATR to IR-induced foci (Barr et al., 2003). Furthermore, ssDNA-RPA and ATRIP are sufficient for ATR autophosphorylation, which leads to full ATR activation by providing a docking site for TopBP1 recruitment prior to checkpoint activation (Liu et al., 2011). We propose that Chk1 activation occurs only after CtIP stably binds to chromatin and initiates processive resection.

CtIP or EXO1 can initiate resection but do not support processive resection. Conversely, DNA2-dependent resection requires initiation by either Exo1 or CtIP-MRN (Figures 1A and 5C). CtIP depletion abolishes resection in EXO1-defective HSS, despite the presence of active DNA2 (Figure 1C). In the absence of DNA2, resection is nearly abolished in EXO1-proficient LSS. By initiating resection, CtIP-MRN could generate the preferred substrate for DNA2 recruitment. Indeed, yeast DNA2 requires Sgs1 and RPA to function in vitro (Cejka et al., 2010), implying that DNA2 starts resection on an RPA-ssDNA intermediate (Figure 5C). However, it is also possible that DNA2 recruitment requires chromatin-bound CtIP. In support of this notion, incubation of linear DNA containing 3′ overhangs with purified *Xenopus* DNA2 and RPA does not promote resection (Yan et al., 2011), suggesting that RPA-bound ssDNA alone cannot promote DNA2-dependent resection.

Mutations Resulting in Truncation of Either ATR or CtIP Cause Seckel Syndrome in Humans

ATR is an essential gene, but inherited hypomorphic mutations in ATR have been identified. These mutations cause Seckel syndrome in humans, which is characterized by dwarfism, microcephaly, and mental retardation (O'Driscoll et al., 2003). ATR mutant Seckel cells display defects in the G2/M damage checkpoint, reduced phosphorylation of ATR targets, and replication-stress-induced genome instability, characterized by nuclear fragmentation (Alderton et al., 2004). Seckel syndrome is genetically heterogeneous, with five loci identified to date. Recently, SCKL2 was identified as a recessive mutation in *RBBP8* (the human gene encoding for CtIP) (Qvist et al., 2011). The mutation alters splice-site selection and produces low-level expression of a truncated form of CtIP in which the last 134 amino acids are replaced by 20 amino acids of novel sequence (truncation after H762). Notably, the deleted region includes

both the ATR and CDK phosphorylation sites. Therefore, the same human disease is caused by either hypomorphic mutation of ATR or expression of a truncated CtIP lacking the two phosphorylation sites required for ATR activation. This suggests that regulation of CtIP activity at this site by ATR and/or CDK has profound cellular as well as physiological consequences.

EXPERIMENTAL PROCEDURES

Extract and Sperm Chromatin Preparation, Assay to Monitor the Response to Chromosomal DSBs

Xenopus leavis were handled in accordance to guidelines provided by the institutional Animal Care and Use Committee at Columbia University. Preparation of extracts (LSS and HSS) and resection assays were performed as described in Peterson et al., 2011. See also Supplemental Information.

Generation and Purification of Phosphospecific pT818-CtIP Antibody

Rabbits were handled in accordance to guidelines provided by the institutional Animal Care and Use Committee at Columbia University. Rabbits were immunized with pT818 -CtIP peptide (CWEVGFPSpTQTCKDRG), and serum was purified by two-step affinity chromatography with use of a SulfoLink Immobilization Kit (Thermo cat. no. 44999). One column was conjugated to the pT818 peptide and another was conjugated to the non-pT818 peptide (CWEVGF PSTQTCKDRG), according to the manufacturer's instructions. Next, 0.5 ml of crude serum was diluted to 4 ml with 0.1M sodium phosphate buffer, 150 mM NaCl (pH 7.2). Diluted serum was incubated with pT818 column and flowthrough was discarded. Antibody was eluted sequentially by acid (0.1 M glycine [pH 2.5]) then base (0.1 M triethylamine [pH 11.5]) into 1 M Tris (pH 7.5). This eluate was incubated with the non-pT818 column and the flowthrough was saved as the purified anti-pT818-CtIP antibody. The purified antibody is a 1:20 dilution relative to the serum, and it is used at a final dilution of 1:200 for western blotting and immunofluorescence.

Immunodepletions and Antibodies

Immunodepletions were performed as described in Peterson et al., 2011. See also Supplemental Information.

Site-Directed Mutagenesis of xCtIP

xCtIP mutants were generated by two-step PCR, as described previously in Peterson et al., 2011. See Supplemental Information for details about the oligonucleotides.

Recombinant Protein Purification

See Peterson et al., 2011. See also Supplemental Information.

Immunofluorescence of UV Laser-Irradiated Human U2OS Cells

Immunofluorescence and laser irradiation were performed as described in Peterson et al., 2011. See also Supplemental Information.

Isolation of Recombinant xCtIP from Control and DSB-Containing Extract and Tandem MS/MS Analysis of xCtIP

Purification of recombinant xCtIP from extract and mass spectrometry (MS) analysis were performed as described in Peterson et al., 2011.

ATRi Inhibition of Damage-Induced CtIP Phosphorylation in Human Cells

Human 293 cells were seeded at 0.3×10^6 cells per 100 mm plate. Two days later, the cells were treated with or without 10 uM ATRi for 1 hr and then incubated for an additional hour with or without 1uM of camptothecin (CPT) prior to harvest. The cells lysed in NETN150 buffer (20 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, 0.5% NP40) were supplemented with complete Mini EDTA-free protease inhibitor cocktail (Roche), 0.5 mM PMSF, 1 mM DTT, and 50 mM NaF. We then subjected 50 μg of each protein lysate to SDS-PAGE and western analysis.

Phosphorylation of Recombinant Proteins

CtIP-depleted LSS extract was incubated with 10 nM recombinant xCtIP and treated with 4 μM OA and 33.3 μg/mlBstEII digest of λ DNA, or buffer, for 30 min at 21°C. For phosphorylation of human proteins, anti-FLAG beads were added to 50 μl of lysate from U20S cells that were treated with siRNA against CtIP and transfected with vector alone, WT CtIP, or T859A-CtIP (as described above). The washed FLAG beads were incubated in *Xenopus* LSS extract treated with OA and BstEII digest of lambda DNA, as described above. The beads were washed three times, boiled in Laemmli buffer, and processed for western blotting with anti-pT818 and anti-FLAG antibodies.

SUPPLEMENTAL INFORMATION

Supplemental Information contains Supplemental Experimental Procedures and three figures and can be found online with this article at http://dx.doi.org/10.1016/j.molcel.2012.11.020.

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