DNA-damage-induced degradation of EXO1 exonuclease limits DNA end resection to ensure accurate DNA repair

Received for publication, December 14, 2016, and in revised form, May 11, 2017 Published, Papers in Press, May 17, 2017, DOI 10.1074/jbc.M116.772475

Nozomi Tomimatsu, Bipasha Mukherjee, Janelle Louise Harris, Francesca Ludovica Boffo, Molly Catherine Hardebeck, Patrick Ryan Potts, Kum Kum Khanna, and Sandeep Burma

From the Department of Radiation Oncology, University of Texas Southwestern Medical Center, Dallas, Texas 75390, Signal Transduction Laboratory, QIMR Berghofer Medical Research Institute, Brisbane, Queensland 4006, Australia, Department of Molecular Medicine and Medical Biotechnology, Università Federico II, Napoli 80131, Italy, and Department of Cell and Molecular Biology, St. Jude Children’s Research Hospital, Memphis, Tennessee 38105

End resection of DNA double-strand breaks (DSBs) to generate 3’-single-stranded DNA facilitates DSB repair via error-free homologous recombination (HR) while stymieing repair by the error-prone non-homologous end joining (NHEJ) pathway. Activation of DNA end resection involves phosphorylation of the 5’ to 3’ exonuclease EXO1 by the phosphoinositide 3-kinase-like kinases ATM (ataxia telangiectasia-mutated) and ATR (ATM and Rad3-related) and by the cyclin-dependent kinases 1 and 2. After activation, EXO1 must also be restrained to prevent over-resection that is known to hamper optimal HR and trigger global genomic instability. However, mechanisms by which EXO1 is restrained are still unclear. Here, we report that EXO1 is rapidly degraded by the ubiquitin-proteasome system soon after DSB induction in human cells. ATR inhibition attenuated DNA-damage-induced EXO1 degradation, indicating that ATR-mediated phosphorylation of EXO1 targets it for degradation. In accord with these results, EXO1 became resistant to degradation when its SQ motifs required for ATR-mediated phosphorylation were mutated. We show that upon the induction of DNA damage, EXO1 is ubiquitinated by a member of the Skp1-Cullin1-F-box (SCF) family of ubiquitin ligases in a phosphorylation-dependent manner. Importantly, expression of degradation-resistant EXO1 resulted in hyper-resection, which attenuated both NHEJ and HR and severely compromised DSB repair resulting in chromosomal instability. These findings indicate that the coupling of EXO1 activation with its eventual degradation is a timing mechanism that limits the extent of DNA end resection for accurate DNA repair.

DNA double-strand breaks (DSBs) are arguably the most dangerous of all types of DNA lesions that can arise in the cell. These breaks arise as a result of both exogenous (for e.g. ionizing radiation and chemotherapeutic drugs) and endogenous (for e.g. reactive oxygen species and stalled replication forks) insults. DSBs can be repaired by one of two major pathways in eukaryotes: 1) non-homologous end joining (NHEJ), an error-prone process wherein the DNA ends are directly rejoined after limited end processing (1), and 2) homologous recombination (HR), an error-free pathway that uses the undamaged sister chromatid as a template for repair (2). Correct “repair pathway choice” is critical for the maintenance of genomic integrity (for review, see Refs. 3–5). Recent evidence suggests that cyclin-dependent kinases (CDKs) that are active in S and G2 phases regulate repair pathway choice by promoting DNA end resection that stymies NHEJ and facilitates HR (for review, see Ref. 6). End resection results in the generation of 3’-ended single-stranded DNA (ssDNA) that is rapidly coated by replication protein A (RPA), which is then replaced with Rad51 to generate a nucleoprotein filament that copies information from the sister chromatid. DNA end resection occurs in a two-step manner (for review, see Refs. 7 and 8). First, resection is initiated by the removal of ~50–100 bases of DNA from the 5’ end by the MRX/MRN complex (Mre11-Rad50-Xrs2 in yeast and MRE11-RAD50-NBS1 in mammals) in concert with Sae2/CtIP (9–13). Next, long range resection is carried out by two alternate pathways involving either EXO1 alone or the helicase Sgs1/BLM working in conjunction with EXO1 or the nuclease DNA2 (14–16). Research from a number of laboratories has established that CDKs 1 and 2 promote the initiation of resection by phosphorylating Sae2/CtIP (12, 17–21) and NBS1 (22), thereby coupling HR to S and G2 phases of the cell cycle.

Recent results from our laboratory established that CDK1 and CDK2 also promote long-range resection via phosphorylation of EXO1 (23; for review, see Refs. 8 and 24). EXO1 is a 5’ to 3’ exonuclease with key roles in DNA mismatch repair, mitotic and meiotic recombination, replication, and telomere homeo-

---

This work was supported, in whole or in part, by National Institutes of Health Grants R01CA197796, R01CA149461, and R21CA202403 (to S.B.). This work was also supported by National Aeronautics and Space Administration Grant NNX16AD78G. The authors declare that they have no conflict of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views or policies of the National Institutes of Health.

This article contains supplemental Tables 1–3 and Figs. 1–5.

1 Both authors contributed equally to this work.

2 To whom correspondence should be addressed: Division of Molecular Radiation Biology, Dept. of Radiation Oncology, University of Texas Southwestern Medical Center, 2201 Inwood Rd., NC7.208, Dallas, TX 75390. Tel.: 214-648-7440; Fax: 214-648-5995; E-mail: sandeep.burma@utsouthwestern.edu.

3 The abbreviations used are: DSB, double-strand break; NHEJ, non-homologous end joining; ssDNA, single-stranded DNA; RPA, replication protein A; ATM, ataxia telangiectasia-mutated; ATR, ATM and Rad3-related; CPT, camptothecin; CHX, cycloheximide; SCF, Skp1-Cullin1-F-box; DN, dominant negative; AUC, area under the curve; RFP, red fluorescent protein; Ub, ubiquitin; CDK, cyclin-dependent kinase.
EXO1 degradation prevents hyper-resection of DNA breaks

Figure 1. EXO1 was rapidly degraded after DNA damage. a, HEK-293 cells were treated with CPT for the indicated times, and EXO1 protein levels were assessed by Western blotting. Phosphorylation of KAP-1 and p53 were assayed by Western blotting with phospho-specific antibodies to confirm DNA-damage induction. Actin served as a loading control. b, HEK-293 cells were pretreated with the proteasome inhibitor MG-132 or with DMSO as control for 4 h, then treated with CPT for the indicated times. EXO1 levels were assessed by Western blotting. c, HEK-293 cells were treated with DMSO, ionizing radiation (IR), CPT, okadaic acid, or calyculin A as indicated immediately after the addition of the protein synthesis inhibitor CHX. EXO1 levels were assessed by Western blotting at the indicated times after CHX treatment. The plot shows relative EXO1 protein levels after CHX treatment (y axis) versus time (x axis) as determined by scanning and quantifying EXO1 bands and normalizing to actin bands using ImageJ software. All experiments were replicated three times. Error bars depict S.E.

Results

EXO1 is rapidly degraded after DNA damage

In the course of our studies on EXO1 we were intrigued to find that EXO1 levels rapidly declined in HEK-293 cells treated with the topoisomerase I poison camptothecin (CPT), which induces replication fork-associated DSBs (43) (Fig. 1a). A reduction in EXO1 levels was also seen in additional human cell lines treated with CPT (supplemental Fig. 1a) or other genotoxic agents (supplemental Fig. 1b). By following EXO1 levels over a longer time period after CPT treatment, we found that the decrease in EXO1 was transient, showing recovery by ~16 h post-CPT treatment (supplemental Fig. 1c). By synchronizing cells in different phases of the cell cycle, we determined that EXO1 levels were cell cycle-dependent, with the highest levels observed in phases where HR is operative, i.e. in S and G2 (supplemental Fig. 1d). However, the rapid reduction in EXO1 levels after DNA damage could not be attributed to cell-cycle...

stasis (for review, see Refs. 25–27). Research from our laboratory has established that EXO1 plays a major role in DNA end resection in human cells and not only promotes a switch from NHEJ to HR but also facilitates a transition from ATM- to ATR-mediated checkpoint signaling (15, 16, 23, 28, 29). The nuclease domain of EXO1 is highly conserved (30), whereas its C-terminal region is divergent and unstructured and mediates interactions with multiple DNA repair proteins (25, 31–34). The C terminus of EXO1 is phosphorylated at four (S/T)P sites by CDKs 1 and 2 in the S/G2 phases of the cell cycle (23). Phosphorylation of EXO1 by CDKs stimulates DNA end resection by promoting the recruitment of EXO1 to DNA breaks via interactions with BRCA1 (23). The C terminus is also phosphorylated at serine 714 by ATM (35) and ATR (36), which are the central kinases triggering the DNA-damage response to DSBs and DNA replication stress, respectively (37, 38). The functional consequences of serine 714 phosphorylation are not well understood.

Given that EXO1 is a key HR exonuclease in eukaryotic cells, it is important to understand how such an enzyme is kept on a tight rein after it is activated to prevent excessive DNA end resection. Excessive ssDNA would pose a threat to genomic integrity as they would be prone to breakage and could even trigger global genomic instability by exhausting the existing pool of RPA (39, 40). Furthermore, extensive DNA end resection would also cause a switch in the DSB repair mode from error-free HR to the highly deleterious single-strand-annealing pathway (41, 42). Here, we describe a mechanism by which resection is restrained in human cells that involves the degradation of EXO1 after DNA damage in a phosphorylation- and ubiquitination-dependent manner.
EXO1 degradation prevents hyper-resection of DNA breaks

Figure 2. Phosphorylation of EXO1 by ATR triggered its degradation. a, HEK-293 cells were treated with the ATM/ATR inhibitor caffeine or with DMSO as the control for 1 h before treatment with CPT for the indicated times. EXO1 protein levels were assessed by Western blotting. Phosphorylation of KAP-1 and p53 were assayed by Western blotting with phospho-specific antibodies to confirm DNA-damage induction. Phosphorylation of CHK1 was assessed to confirm ATR inactivation. b, HEK-293 cells were treated with the ATR inhibitor VE822 or with DMSO as the control for 1 h before treatment with CPT. EXO1 protein levels were assessed by Western blotting. c, EXO1 levels were assessed after CPT treatment of HEK-293 cells with siRNA-mediated knockdown of ATR. d, HEK-293 cells were depleted of endogenous EXO1 using siRNA and complemented with V5-tagged, siRNA-resistant wild-type EXO1 (WT) or EXO1 mutated at serine 714 (S714A). Cells were treated with CPT, and EXO1 levels were assessed by Western blotting with anti-V5 antibody. e, HEK-293 cells were depleted of endogenous EXO1 using siRNA and complemented with siRNA-resistant EXO1 mutated at all six SQ sites (Ser-432, Ser-652, Ser-674, Ser-676, Ser-694, and Ser-714) in its C-terminal domain (6A). EXO1 levels after CPT treatment were assessed by Western blotting. All experiments were replicated three times.

Changes, as no major differences in cell-cycle distribution were observed for at least 8 h post-CPT treatment (supplemental Fig. 1f). No changes in EXO1 transcript levels were seen after CPT treatment (supplemental Fig. 1f), indicating that post-transcriptional mechanisms were involved in the decrease in EXO1 levels. Indeed, the decrease in EXO1 appeared to be due to proteasomal degradation as this could be inhibited by treatment of cells with the proteasomal inhibitor MG-132 (Fig. 1b). By treating cells with the protein synthesis inhibitor cycloheximide (CHX), we found that the half-life of EXO1 was considerably shortened upon the induction of DNA damage by ionizing radiation (IR) or CPT (Fig. 1c). Interestingly, treatment of cells with the phosphatase inhibitors okadaic acid or calyculin A in the absence of DNA damage also resulted in destabilization of EXO1, indicating that phosphorylation of EXO1 might trigger its degradation (Fig. 1c).

Phosphorylation of EXO1 by ATR triggers its degradation

EXO1 is known to be phosphorylated at serine 714 by ATM and ATR in response to ionizing radiation and DNA replication inhibitors, respectively (35, 36). CPT-induced EXO1 degradation was attenuated by pretreatment of cells with caffeine, a broad-specific inhibitor of both ATM and ATR kinases (44) (Fig. 2a), KU55933, a specific inhibitor ATM (45), or NU7026, a specific inhibitor of DNA-PKcs (46), had no effect on EXO1 degradation (supplemental Fig. 2a). On the other hand, pretreatment of cells with VE822, a specific inhibitor of ATR (47), or siRNA-mediated knockdown of ATR resulted in stabilization of EXO1 (Fig. 2, b and c). Taken together, these observations indicated that EXO1 degradation might be triggered by ATR-mediated phosphorylation events. Knockdown of second-tier kinases that function downstream of ATR and ATM, CHK1 and CHK2, respectively (48), had no effect on EXO1 levels (supplemental Fig. 2b), implying that direct phosphorylation of EXO1 by ATR might trigger its degradation. To test this possibility, we mutated serine 714 of EXO1 to alanine, and expressed siRNA-resistant V5-tagged S714A-EXO1 in HEK-293 cells with siRNA-mediated knockdown of endogenous EXO1 (23). However, upon challenging these cells with CPT, we found that mutation of the serine 714 phosphorylation site did not affect EXO1 degradation appreciably (Fig. 2d). We reasoned that EXO1 might be phosphorylated at additional SQ sites upon DNA damage. This line of reasoning was based upon the observation that EXO1 with mutated serine 714 (S714A-EXO1) was still recognized by anti-phospho-(Ser/Thr) ATM/ATR substrate antibody in immunoprecipitation/Western blotting, whereas EXO1 with mutation of all six potential SQ phosphorylation sites in its C-terminal tail (S432A/S652A/S674A/S676A/S694A/S714A; henceforth referred to as 6A-EXO1) was no longer recognized by this antibody (supplemental Fig. 2e). Moreover, CPT-induced phosphorylation of S714A-EXO1 was attenuated by pretreatment of cells with the ATR inhibitor VE822 (47), thereby implicating ATR in EXO1 phosphorylation (supplemental Fig. 2f). Significantly, unlike wild-type EXO1, 6A-EXO1 was very stable after the induction
EXO1 degradation prevents hyper-resection of DNA breaks

Figure 3. EXO1 was ubiquitinated in a phosphorylation-dependent manner. a, HeLa cells expressing V5-tagged EXO1 (V5-EXO1) and His<sub>6</sub>-tagged wild-type (WT) or conjugation-defective (ΔGG) ubiquitin were treated with CPT for the indicated periods of time. His<sub>6</sub>-ubiquitin-conjugated proteins were captured under denaturing conditions using Ni<sup>2+</sup>-agarose beads, and ubiquitinated forms of EXO1 were detected by Western blotting with anti-V5 antibody. b, HeLa cells expressing V5-EXO1 and His-Ub were treated with caffeine or DMSO as control before the addition of CPT for 4 h. EXO1 ubiquitination was assessed by Ni<sup>2+</sup>-capture followed by Western blotting. c, HeLa cells expressing wild-type (WT) or phosphorylation site-mutant (Δα) V5-EXO1 and His-Ub were treated with CPT for 4 h. EXO1 ubiquitination was assessed by Ni<sup>2+</sup>-capture followed by Western blotting. d, HeLa cells expressing V5-EXO1 were treated with the cullin-RING ubiquitin ligase inhibitor MLN4924 or DMSO for 4 h before the addition of CPT for 4 h. EXO1 ubiquitination was assessed by Ni<sup>2+</sup>-capture followed by Western blotting. e, HEK-293 cells were treated with DMSO or MLN4924 for 4 h before treatment with CPT for the indicated times. EXO1 protein levels were assessed by Western blotting. f, HeLa cells expressing V5-EXO1 and His-Ub in the presence or absence of dominant negative Cullin1 (DN-Cullin1) were treated with CPT for 4 h. EXO1 ubiquitination was assessed by Ni<sup>2+</sup>-capture followed by Western blotting. Cells were transfected with a V5-β-gal vector as control (Mock). g, HEK-293 cells expressing DN-Cullin1 were treated with CPT for the indicated times. EXO1 levels were assessed by Western blotting. All experiments were replicated three times.

of DNA damage (Fig. 2e). We, therefore, concluded that DNA-damage-induced phosphorylation of the C terminus of EXO1 triggers its degradation.

EXO1 is ubiquitinated in a phosphorylation-dependent manner

As EXO1 appears to be degraded by the ubiquitin-proteasome system (Fig. 1b), we next sought to determine if EXO1 phosphorylation upon DNA damage serves as trigger for its ubiquitination and subsequent degradation. To determine whether EXO1 was directly ubiquitinated in vivo, we co-expressed V5-EXO1 and His<sub>6</sub>-ubiquitin in HeLa cells (49). The cells were exposed to CPT, His<sub>6</sub>-ubiquitin-conjugated proteins were captured under denaturing conditions using Ni<sup>2+</sup>-agarose beads, and ubiquitinated forms of EXO1 were detected by Western blotting with anti-V5 antibody. We found that EXO1 was ubiquitinated at low levels in unstressed cells and that EXO1 ubiquitination increased dramatically in response to DNA damage (Fig. 3a). The modified forms of EXO1 were not seen in cells expressing conjugation-defective His<sub>6</sub>-ubiquitin (ΔGG) (50), confirming that they represented ubiquitinated EXO1. Importantly, the DNA-damage-induced increase in EXO1 ubiquitination was not seen in cells treated with the ATR inhibitor caffeine (Fig. 3b) or in cells expressing 6A-EXO1 (Fig. 3c), indicating that phosphorylation of EXO1 by ATR serves to target it for ubiquitination.

In light of the apparent link between EXO1 phosphorylation and ubiquitination, we were interested in the Skp1-Cullin1-F-box (SCF) family of ubiquitin ligases, which typically recognize and ubiquitinate phosphorylated substrates (51, 52). SCF ubiquitin ligases regulate multiple DNA-damage-response pathways (51, 53). Notably, a Cullin1-containing SCF has been reported to target CHK1 for degradation after it is phosphorylated by ATR in a manner similar to that observed for EXO1 (54, 55). To examine if a cullin-based E3 ligase was involved in EXO1 ubiquitination, we first treated cells with MLN4924, which is a general inhibitor of the cullin-RING subtype of ubiquitin ligases (56). Treatment with MLN4924 ablated EXO1 ubiquitination (Fig. 3d) and resulted in failure of CPT-induced destabilization of EXO1 (Fig. 3e). To specifically examine the role of Cullin1 in EXO1 ubiquitination, we transfected HeLa cells with V5-EXO1 and His<sub>6</sub>-ubiquitin along with dominant negative Cullin1 (DN-Cull1) (57) and assayed for EXO1 ubiquitination by Ni<sup>2+</sup>-pulldown under denaturing conditions. Expression of DN-Cull1 attenuated EXO1 ubiquitination (Fig. 3f) and suppressed degradation of EXO1 protein triggered by CPT exposure (Fig. 3g). As a control, we co-expressed dominant negative Cullin4A and Cullin4B (DN-Cull4) (57) in HeLa cells but saw no effect on EXO1 stability (supplemental Fig. 3). These results implicate a Cullin1-containing SCF complex in phosphorylation-dependent EXO1 degradation.

EXO1 degradation prevents hyper-resection and genomic instability

We next asked whether EXO1 degradation might serve to prevent unbridled DNA resection, as the generation of exces-
EXO1 degradation prevents hyper-resection of DNA breaks

It is now well understood that several mechanisms exist to promote DNA end resection, a critical step that is essential for HR repair of DSBs, especially replication-associated DSBs like those induced by CPT (66). However, how resection is restrained or terminated is not well understood. Our results uncover a novel mechanism by which resection is negatively regulated via the degradation of EXO1, an exonuclease with critical functions in long-range resection (25, 26). Our results indicate that EXO1, although vital for resection and HR, is rapidly degraded after DNA damage in a phosphorylation- and ubiquitination-dependent manner. The rapid degradation of EXO1 serves to prevent hyper-resection. Hyper-resection could promote genomic instability because 1) extensive resection would shift DSB repair from RAD51-dependent HR to RAD52-dependent single-strand annealing, a highly mutagenic DNA repair pathway (41, 42), and 2) excessive ssDNA would exhaust the existing pool of RPA thereby triggering global genomic instability (39, 40). The net effect of these two phenomena would be the accumulation of CPT-induced DNA breaks and chromosome shattering that we observe in cells expressing degradation-resistant EXO1.

In light of these data and our previously published results (23), we envisage that phosphorylation of EXO1 by CDKs in S/G2 primes EXO1 to respond to DSBs, especially replication-associated breaks that can be correctly repaired only by HR. However, upon the induction of such breaks, EXO1 is phosphorylated by ATR, which marks it for SCF-mediated ubiquitination and subsequent degradation (Fig. 5). A delay between EXO1 phosphorylation and its degradation possibly creates a temporal window that is sufficient for EXO1 to optimally resect DNA ends before it is destroyed to prevent excessive resection or collateral damage to other regions of the genome.

The degradation of EXO1 that we observe appears to be mediated by a Cullin1-containing SCF ubiquitin ligase complex (51, 52). Ubiquitination along with SUMOylation are emerging as important regulators of DNA-damage response (for review, see Refs. 67 and 68). Most published studies have focused on the role of ubiquitination and SUMOylation in the recruitment of proteins to DSBs, notably CtIP, 53BP1, RIF1, RAD18, and BRCA1 (68–70). Interestingly, recent reports also implicate these modifications in the degradation of cell-cycle checkpoint and DNA-repair proteins, notably CHK1, CtIP, 53BP1, MDC1, RPA, BRCA1, and FANCA (54, 71–77), and in the release of the NHEJ factor Ku from DSBs (78–80). Among these proteins, a very close analogy can be drawn between the degradation of EXO1 and that of the cell-cycle checkpoint kinase CHK1 (54, 55). Phosphorylation of CHK1 by ATR serves to initiate cell-cycle checkpoint arrest but also limits the duration of checkpoint signaling via SCF-mediated degradation of the kinase. In a similar manner, phosphorylation of EXO1 by ATR limits the extent of DNA end resection by SCF-mediated degradation of the potentially genotoxic nuclease. Of relevance to our findings, SCFs have been implicated in the phosphorylation-triggered degradation of a number of additional DNA repair and cell-cycle checkpoint proteins including BRCA1 (76), Ku (78, 79), and Cdc25A (81).
EXO1 degradation prevents hyper-resection of DNA breaks

(a) Western blot of V5-tagged EXO1 in WT and 6A cell lines. Actin staining as a loading control.

(b) Immunostaining for RPA (green) and DAPI (blue) in WT and 6A cells treated with DMSO or CPT. Bar graph showing the number of RPA foci per cell for each condition.

(c) Immunostaining for BrdU (green) and DAPI (blue) in WT and 6A cells following CPT treatment. Time course of fluorescence intensity over 120 minutes.

(d) Bar graph showing relative NHEJ-directed repair in WT and 6A cells with or without I-SceI.

(e) Bar graph showing relative HR-directed repair in WT and 6A cells with or without I-SceI.

(f) Immunostaining for 53BP1 (green) in WT and 6A cells treated with CPT. Bar graph showing the number of 53BP1 foci per cell for each time point.

(g) Comparison of normal and shattered chromosomes. % metaphases with shattered chromosomes.

(h) Graph showing survival curves for WT and 6A cells treated with various concentrations of CPT.
Lending credence to our observation of DNA-damage-induced EXO1 ubiquitination, EXO1 was recently identified as a SCF target in a quantitative proteomic screen for UV-induced ubiquitination events in human cells (82). This screen and others (57, 83, 84) identified the following ubiquitination sites on EXO1 (Lys-214, Lys-292, Lys-391, Lys-548, and Lys-796). However, mutation of these five sites, singly or in combination, was not sufficient to affect EXO1 ubiquitination. It is possible that there are additional, yet unidentified, ubiquitination sites on EXO1 or that mutation of the above sites simply resulted in promiscuous ubiquitination on nearby lysines (85). Future studies will hopefully shed more light on the exact ubiquitination site(s) on EXO1 that trigger its degradation.

Our results showing that ATR triggers EXO1 degradation in response to replication-associated DSBs are of interest in light of a previous report showing that ATR triggers EXO1 degradation upon treatment of cells with the DNA replication inhibitor hydroxyurea (36). Given that treatment with hydroxyurea induces DSBs due to replication fork collapse (86, 87), it is plausible that the actual trigger for EXO1 degradation in the previous study might have been replication fork-associated DSBs. Also, in the previous study, as in ours, mutation of serine 714 was not sufficient to stabilize EXO1 (36), which bolsters our assessment that multiple phosphorylation sites on the C terminus of EXO1 are needed for its ubiquitination and subsequent degradation. In a recent study from the same group, it was reported that EXO1 is also SUMOylated in response to DNA replication stress, although mutation of the identified SUMO sites did not affect EXO1 stability (88). It is, therefore, clear that EXO1 stability is probably regulated by an interplay of post-translational modifications (PTM)—phosphorylation, SUMOylation, and ubiquitination—but the exact choreography of these PTMs and the exact roles of specific modifications in EXO1 regulation still need to be elucidated.

Regulatory mechanisms impinging on EXO1 are of significance from a cancer standpoint, as multiple SNPs in EXO1 have been identified in patients with colorectal cancers (89–91), and a specific polymorphism, K589E (rs1047840), has been linked with an increased risk of breast (92), gastric (93), oral (94), lung (95), and brain (96) cancer development. Another polymorphism, N279S (rs4149909), was recently linked to an increased risk of breast cancer in a genome-wide association analysis of >120,000 individuals (97). It would be of interest to determine in the future if some of these SNPs affect the degradation of EXO1 elucidated in our study and whether blocking EXO1 degradation might be a viable cancer therapeutic strategy.

### Experimental procedures

#### Cell culture

Cell lines were obtained from the American Type Culture Collection. U2OS cells were maintained in α-MEM (α-minimum Eagle’s medium) and HEK-293 and HeLa cells in DMEM medium supplemented with 10% fetal bovine serum and penicillin/streptomycin in a humidified atmosphere with 5% CO₂. All cells were mycoplasma free.

#### Drug treatments

Cells were treated with 1 μM camptothecin (Sigma), 10 μM etoposide (Selleckchem), or 50 μM hydroxyurea (Sigma) for the indicated periods of time to induce DNA damage. Cells were treated with 10 μM MG-132 (Selleckchem) for 4 h before treat-
EXO1 degradation prevents hyper-resection of DNA breaks

EXO1 degradation was carried out using Pfx50 polymerase (Invitrogen) and Taq ligase (New England BioLabs); see supplemental Table 2 for primer sequences. EXO1 was then transferred to pLenti6.3/V5-DEST (Invitrogen) using the Gateway LR Clonase II enzyme mix (Invitrogen) for C-terminal V5-tagged fusion protein expression, as described before (23). Serine 432, 652, 674, 676, and 714 of EXO1 were mutated to alanine to generate 6A-EXO1.

Transfection of cells

Depletion of EXO1, ATR, CHK1, or CHK2 was carried out by transfection with the appropriate siRNAs (supplemental Table 3; Invitrogen) using electroporation (Lonza) for U2OS cells and Lipofectamine2000 (Invitrogen) for HEK-293 cells; cells were harvested 48 h later to verify knockdown by Western blotting. Cells were transfected with scrambled siRNA as control; see supplemental Table 3 for siRNA sequences. For ectopic expression of proteins, cells were transfected with the appropriate expression plasmid and assayed after 24–48 h. pHAGE DN Cullin1, pHAGE DN Cullin4A, and pHAGE DN Cullin4B were gifts from Stephen Elledge (Addgene plasmid #41911, 41914, 41915). For ectopic expression of siRNA-resistant EXO1 in cells with knockdown of endogenous EXO1, cells were transfected with the expression plasmid 24 h after siRNA transfection.

Ubiquitination assay

HeLa cells were transfected with His-Ub (WT or ΔGG) (49) and V5-EXO1 (WT or 6A) expression plasmids using Effectene (Qiagen). After 48 h, cells were treated with 10 μM MG-132 for 4 h before the addition of 1 μM camptothecin. Cells were harvested at the indicated times post-camptothecin treatment. Harvested cells were resuspended in denaturing lysis buffer (6 M guanidine-HCl, 100 mM Na2HPO4/NaH2PO4, pH 8, 10 mM Tris-HCl, pH 8, 5 mM imidazole, and 10 mM β-mercaptoethanol). Extracts were clarified and incubated with 100 μl of nickel-nitrilotriacetic acid beads for 4 h with rotation. Beads were serially washed with lysis buffer without imidazole, wash buffer A (8 M urea, 100 mM Na2HPO4/NaH2PO4, pH 8, 10 mM Tris-HCl, pH 8, and 10 mM β-mercaptoethanol), wash buffer B (8 M urea, 100 mM Na2HPO4/NaH2PO4, pH 8, 10 mM Tris-HCl, pH 8, and 10 mM β-mercaptoethanol), wash buffer C (8 M urea, 100 mM Na2HPO4/NaH2PO4, pH 8, 10 mM Tris-HCl, pH 6.8, 0.2% Triton-X, and 10 mM β-mercaptoethanol) and wash buffer D (8 M urea, 100 mM Na2HPO4/NaH2PO4, pH 8, 10 mM Tris-HCl, pH 6.8, 0.1% Triton-X, and 10 mM β-mercaptoethanol). Bound proteins were eluted with elution buffer (200 mM imidazole, 150 mM Tris-HCl, pH 6.8, 30% glycerol, 5% SDS, and 720 mM β-mercaptoethanol) and analyzed by Western blotting.

Immunoprecipitation

Whole cell extracts of HEK293 cells expressing V5-tagged EXO1 were made in lysis buffer (20 mM Tris-HCl, pH 7.5, 80 mM NaCl, 2 mM EDTA, 10% glycerol, and 0.2% Nonidet P-40) supplemented with protease and phosphatase inhibitors. Anti-V5 antibodies were bound to Dynabeads (Invitrogen), and beads were incubated for 4 h with cell extracts following the manufacturer’s protocol. Beads were washed extensively in lysis buffer and then boiled in 1× SDS loading buffer before Western

Cloning and mutagenesis

EXO1b was subcloned from Flag2-EXO1 (15) into pDONR221 (Invitrogen) using the Gateway BP Clonase II enzyme mix (Invitrogen). Site-directed mutagenesis of EXO1 was carried out using Pfx50 polymerase (Invitrogen) and Taq ligase (New England BioLabs); see supplemental Table 2 for primer sequences. EXO1 was then transferred to pLenti6.3/V5-DEST (Invitrogen) using the Gateway LR Clonase II enzyme mix (Invitrogen) for C-terminal V5-tagged fusion protein expression, as described before (23). Serines 432, 652, 674, 676, and 714 of EXO1 were mutated to alanine to generate 6A-EXO1.
EXO1 degradation prevents hyper-resection of DNA breaks

blotting. Normal mouse IgG (Santa Cruz) was used for control immunoprecipitations.

Exonuclease assay

V5-tagged EXO1 proteins were immunoprecipitated from HEK293 cells using Dynabeads as described previously (23). Nuclease activity of wild-type or mutant EXO1 was assessed by analyzing the degradation of a linearized 3'-radioactively labeled 7.8-kb plasmid (pLVX-Tight Puro). Each nuclease reaction contained EXO1 protein equivalent to the immunoprecipitate from 2 mg of cell lysate and 50 ng of substrate in a final volume of 35 μl of nuclease assay buffer (20 mM HEPES, pH 7.5, 40 mM KCl, 5 mM MgCl2, 0.05% Triton X-100, 5% glycerol, 100 μg/μl BSA, 0.5 mM DTT, and 1 mM ATP). Reactions were incubated at 37 °C, and samples were removed at the indicated intervals. Samples were resolved on 0.8% agarose gels and transferred onto a Hybond-XL nylon membrane (Amersham Biosciences). Images were acquired using phosphorimaging screens and a Fujiﬁlm Staron scanner. Quantitation was carried out using Multi Gauge software (Fujiﬁlm). Gel lanes were marked and used to generate line traces, and peaks were detected automatically using default settings. Area under the curve (AUC) for the 7.8-kb reagent peak was calculated for each lane. The fraction of substrate remaining was calculated by dividing AUC of each sample by AUC of the starting material. Three independent time courses were quantified for each protein.

Immunofluorescence staining

To stain for RPA foci, cells were seeded onto glass chamber slides (Lab-Tek) and treated with 0.1 mM camptothecin for 4 h. The cells were ﬁxed with 4% paraformaldehyde/PBS and permeabilized with 0.5% Triton-X/PBS before incubation with antibodies (60). To obtain clear RPA foci, cells were subject to in situ fractionation (98). The average number of RPA foci per nucleus was determined after scoring at least 50 nuclei and subtracting background (average numbers of foci in mock-treated cells). For quantifying DSB repair kinetics, cells were treated with 0.1 mM camptothecin for 2 h and immunostained with anti-53BP1 or H2AX antibody at different time points post-camptothecin treatment as described previously (61). Images were captured using a Leica DH5500B ﬂuorescence microscope (X40 objective lens) coupled to a Leica DFC340 FX camera using Leica Application Suite v4 acquisition software.

BrdU/ssDNA assay

Cells grown in the presence of 10 μM BrdU (Sigma) for 16 h were treated with 0.1 mM camptothecin for 4 h and immunofluorescence stained with anti-BrdU antibody under non-denaturing conditions (to detect BrdU incorporated into ssDNA) (60). For clarifying BrdU/ssDNA foci, cells were subject to in situ fractionation (98). The average number of BrdU/ssDNA foci per nucleus was determined after scoring at least 50 nuclei and subtracting background (average numbers of foci in mock-treated cells).

Laser live cell imaging

Cells were transfected with GFP-RPA construct, laser micro-irradiated and time-lapse-imaged, and the ﬂuorescence intensities of micro-irradiated areas were plotted after background subtraction (ﬂuorescence intensities of un-irradiated areas) as described before (16). Cells were irradiated, and live cell images were taken with a pulsed nitrogen laser (Spectra-Physics; 365 nm, 10 Hz) coupled to a Carl Zeiss Axiovert 200M microscope (×63 oil immersion objective). Mean ﬂuorescence intensities for each time point were determined using Axiovision software v4 from at least 30 independent measurements, and total increase in ﬂuorescence signal was plotted versus time.

NHEJ and HR assays

To measure HR, GFP expression in HEK-293 cells with an integrated DR-GFP reporter was quantitated by ﬂow cytometry (62). To measure NHEJ, red ﬂuorescent protein (RFP) expression in HEK-293 cells with an integrated GFP-to-RFP reporter was quantitated by ﬂow cytometry (61). For both assays, cells were depleted of endogenous EXO1 using siRNA and transfected with the V5-EXO1 (WT or 6A) expression plasmid; after 24 h, cells were transfected with an I-Sce1 expression vector, and GFP or RFP expression was quantitated by ﬂow cytometry after an additional 72 h. GFP-positive or RFP-positive frequencies were corrected for transfection efﬁciencies (quantitated by parallel transfection with a wild-type GFP expression vector).

Metaphase chromosome preparations

Cells were treated with 0.1 μM camptothecin for 2 h after which the media was replaced with camptothecin-free media. Colcemid (Sigma), along with 1 mM caffeine (Sigma) to bypass G2/M arrest, was added to cells 24 h after camptothecin treatment. Metaphase chromosome spreads were prepared 16 h later as described before (99).

Colony formation assay

Cells were treated with the indicated doses of camptothecin for 4 h and plated in triplicate onto 60-mm dishes (1000 cells/dish). Surviving colonies were stained with crystal violet ~10–14 days later as described before (100).

Statistical analyses

Statistical analyses were performed using GraphPad Prism7 software. Two-tailed Student’s t test was used to determine statistical signiﬁcance (ns, not signiﬁcant; *, p < 0.05; **, p < 0.005; ***, p < 0.001; ****, p < 0.0001).

Author contributions—N. T., B. M., M. C. H., F. L. B., J. L. H., and S. B. conducted the experiments. N. T., P. R. P., J. H., K. K. K., and S. B. designed the experiments and interpreted the results. B. M. and S. B. wrote the paper.

References

1. Burma, S., Chen, B. P., and Chen, D. J. (2006) Role of non-homologous end joining (NHEJ) in maintaining genomic integrity. DNA Repair 5, 1042-1048
EXO1 degradation prevents hyper-resection of DNA breaks


EXO1 degradation prevents hyper-resection of DNA breaks


DNA-damage-induced degradation of EXO1 exonuclease limits DNA end resection to ensure accurate DNA repair

Nozomi Tomimatsu, Bipasha Mukherjee, Janelle Louise Harris, Francesca Ludovica Boffo, Molly Catherine Hardebeck, Patrick Ryan Potts, Kum Kum Khanna and Sandeep Burma

*J. Biol. Chem.* 2017, 292:10779-10790. doi: 10.1074/jbc.M116.772475 originally published online May 17, 2017

Access the most updated version of this article at doi: 10.1074/jbc.M116.772475

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2017/05/17/M116.772475.DC1

This article cites 100 references, 29 of which can be accessed free at http://www.jbc.org/content/292/26/10779.full.html#ref-list-1