Metnase Mediates Loading of Exonuclease 1 onto Single Strand Overhang DNA for End Resection at Stalled Replication Forks*

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Hyun-Suk Kim1, Elizabeth A. Williamson1, Jac A. Nickoloff2, Robert A. Hromas3, and Suk-Hee Lee4

From the 1Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana 46202, the 2Department of Medicine, University of Florida and Shands Health Care System, Gainesville, Florida 32610, and the 3Department of Environmental and Radiological Health Sciences, Colorado State University, Fort Collins, Colorado 80523

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Stalling at DNA replication forks generates stretches of single-stranded (ss) DNA on both strands that are exposed to nucleolytic degradation, potentially compromising genome stability. One enzyme crucial for DNA replication fork repair and restart of stalled forks in human is Metnase (also known as SETMAR), a chimeric fusion protein consisting of a su(var)3–9, enhancer-of-zeste and trithorax (SET) histone methylase and transposase nuclease domain. We previously showed that Metnase possesses a unique fork cleavage activity necessary for its function in replication restart and that its SET domain is essential for recovery from hydroxyurea-induced DNA damage. However, its exact role in replication restart is unclear. In this study, we show that Metnase associates with exonuclease 1 (Exo1), a 5’-exonuclease crucial for 5’-end resection to mediate DNA processing at stalled forks. Metnase DNA cleavage activity was not required for Exo1 5’-exonuclease activity on the lagging strand daughter DNA, but its DNA binding activity mediated loading of Exo1 onto ssDNA overhangs. Metnase-induced enhancement of Exo1-mediated DNA strand resection required the presence of these overhangs but did not require Metnase’s DNA cleavage activity. These results suggest that Metnase enhances Exo1-mediated exonuclease activity on the lagging strand DNA by facilitating Exo1 loading onto a single strand gap at the stalled replication fork.

DNA-damaging agents as well as inhibitors of deoxyribonucleotide synthesis and DNA polymerase action block the progression of replication forks (1, 2). Replication fork stalling induces uncoupling between DNA polymerases and the replicative DNA helicases and generates stretches of ssDNA on both strands that are exposed to nuclease attack (1–7). Replication fork repair and restart in eukaryotes are complex and poorly understood (1, 5, 7, 8). Upon replication fork arrest from deoxyribonucleotide depletion after hydroxyurea (HU) treatment, uncoupling of DNA polymerases and helicase generates ssDNA overhangs on both leading and lagging strands. Restart of stalled replication forks can occur via the realignment of ssDNA, or forks can undergo regression and pairing of the newly synthesized strands to form a Holliday junction structure (“chicken foot”). When free 5’-ends are present, end resection creates 3’-ssDNA on which Rad51 can load, and Rad51-mediated DNA displacement loop formation eventually allows reloading of the replication machinery for fork restart. Rad51-mediated homologous recombination (HR) also mediates fork reversal for restart of replication (9, 10). Holliday junctions can also be processed into a one-ended double strand break (DSB), and fork restart is then achieved through Rad51-mediated homologous recombination repair (1, 5, 7, 8). Although homologous recombination repair is a preferred pathway in restart of stalled replication forks (1, 5, 8, 11, 12), the detailed mechanism(s) and the factors involved are not well understood.

Stalled replication forks often require the generation of an intrinsic DSB to begin the 5’-resection that initiates recombination-mediated fork repair (1, 7, 8, 13, 14). The stressed fork can do this in at least two ways: the fork can reverse into a chicken foot structure with a one-sided DSB, or a nuclease can generate a DSB at the stalled fork as part of the restart process. If a stalled fork is not repaired, it can collapse into a variety of structures that make restart difficult (8, 15–17) and can result in genomic instability, leading to cell death or neoplastic transformation (2, 8, 11). Repair pathway choice at stalled forks is important for genomic stability because unopposed classical non-homologous end joining (cNHEJ), such as seen in malignancies with inherited BRCA1 or BRCA2 deficiencies, results in fusion of the one-sided DNA ends at damaged replication forks (12, 18–22). These chromosomal fusions at stalled forks can cause severe genome instability, resulting in catastrophic mitoses with gross nuclear abnormalities, such as nuclear bridges and micronuclei (8, 12, 18, 21, 23). Preventing cNHEJ by

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1To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Indiana University Simon Cancer Center, Indiana University School of Medicine, Indianapolis, IN 46202. Tel.: 317-278-3464; Fax: 317-274-8046; E-mail: slee@iu.edu.

2The abbreviations used are: ss, single-stranded; Exo1, exonuclease 1; HU, hydroxyurea; HR, homologous recombination; DSB, double strand break; cNHEJ, classical non-homologous end joining; alt-EJ, alternative end join; C-TBP (C-terminal binding protein) interacting protein.
repressing 53BP1 rescues these nuclear defects (19). Thus, HR is the preferred repair pathway for stalled replication forks to prevent genomic instability (1, 5, 8, 11, 12). There is accumulating evidence that DSB pathway choice between cNHEJ and HR is mediated by 5′-end resection (13, 14, 24). End resection for HR is likely a two-step process where CtIP and Mre11 nucleolytically resect short 5′-tracks with Dna2 and especially Exo1 resecting longer tracks (13, 14, 17, 25, 26), generating long 3′-ss-tracts. The choice between different DSB repair pathways is tightly regulated, and DNA end resection represents a primary regulatory step (27). The ssDNA tails created at DSBs also play a critical role in DNA damage (checkpoint) response (27, 28). DNA resection decreases cNHEJ efficiency (27) but is essential for the homology-mediated DSB repair pathways, alternative end joining (alt-EJ), and HR (17, 29–31). The balance between cNHEJ, alt-EJ, and HR is controlled by key DNA end resection factors (27). Alt-EJ and HR share the initial end resection step of a limited resection at DSB sites by Mre11/CtIP (17, 22, 25, 31–35), which is necessary for subsequent recruitment of Exo1 along with Bloom syndrome protein (BLM) and its paralog Werner syndrome ATP-dependent helicase (WRN) to mediate extensive end resection (22, 25, 34, 36–38). However, alt-EJ in contrast to HR does not seem to require γ-H2AX (17), suggesting that the initial end resection event in the alt-EJ pathway is distinct from the HR repair pathway.

Metnase is a SET-transposase domain chimeric protein with endonuclease activity that plays a crucial role in restart of stalled replication forks (39–41). In this study, we found that Metnase interacts with Exo1 and is involved in DNA processing at the stalled replication fork. Metnase not only mediates loading of Exo1 onto single strand overhangs but also enhances Exo1-mediated resection, suggesting that Metnase enhances Exo1’s 5′-exonuclease activity on the lagging daughter DNA by mediating Exo1 loading onto gapped DNA.

Results

Metnase Interacts with Exo1 and Promotes DNA Processing at Stalled Forks—Metnase’s SET domain and its DNA cleavage activity derived from the transposase domain are essential for Metnase’s function in restart of stalled replication forks (40, 41). This suggests that Metnase has multiple roles in replication restart. In an effort to clarify these roles, we examined association of Metnase with DNA end-processing nucleases that function at stalled replication fork (25, 26, 34, 37, 42–44). Metnase expression was not affected by HU, but the Exo1 level was markedly reduced after HU treatment (Fig. 1A, lane 1 versus lane 2). This observation is in keeping with the previous finding by others that human Exo1 is phosphorylated and rapidly degraded through ubiquitin-proteasome pathways in response to HU treatment (45). We found that Metnase associates with Exo1 in the presence of DNase I treatment regardless of replication stress (Fig. 1A). A physical interaction was also observed between two purified proteins (Fig. 1B), indicating that there is a direct interaction between Metnase and Exo1. To further analyze the Metnase-Exo1 interaction, we examined deletion mutants of Metnase and Exo1 for physical interaction. A mutant Metnase lacking the C terminus (ΔTransposase) failed to interact with Exo1 (Fig. 1C), whereas a mutant Exo1 lacking the N terminus (Δ1–404) did not pull down Metnase in the co-immunoprecipitation experiment (Fig. 1D). These findings suggest that Metnase directly interacts with Exo1, and these two proteins may function together at replication forks.

We next examined whether Metnase affects DNA processing at stalled replication forks. For this, we measured HU-induced generation of ssDNA by immunostaining of newly incorporated BrdU when the cells were not treated with agents that denature DNA as described previously (34, 46). In this analysis, only BrdU present in ssDNA was detected. As shown in Fig. 2A, there were fewer BrdU foci in Metnase-repressed cells compared with control cells after HU treatment, suggesting that DNA end resection is attenuated when Metnase is repressed. We also saw a marked reduction in BrdU foci with Exo1 knockdown; a further reduction in BrdU foci was observed in Metnase- and Exo1-repressed cells (Fig. 2, A and B). To further assess Metnase involvement in the processing of stalled replication forks, cells transfected with scrambled siRNA or si-Metnase were labeled with IdU for 45 min prior to prolonged exposure to HU, and DNA resection on nascent DNA was analyzed by measuring lengths of IdU-labeled fibers (10) (Fig. 2C). Nascent IdU tracks were substantially longer in Metnase-depleted cells compared with control when replication forks were stalled, and IdU tracks were also shorter in Exo1- and doubly depleted cells (Fig. 2, D and E). After 10 h of HU treatment, the mean track lengths of control and Metnase-depleted cells were 5.9 and 9.3 μm, respectively (p < 0.001; Fig. 2E), suggesting that Metnase plays a crucial role in the processing of stalled replication forks. Metnase also promoted Exo1-mediated resection at DSB sites following ionizing radiation (IR) (Fig. 2F), indicating that Metnase’s action would be more general and not limited to forks.

To see whether Metnase’s involvement in DNA resection is linked to recombination repair at stalled forks, we examined fork restart and formation of Rad51 foci after HU treatment. Formation of replication protein A foci is also a common readout for ssDNA at stalled forks (41) but was not included here because they can be formed at replication forks without DNA resection (47, 48). Repression of Exo1 had very little impact on fork restart after a short (1-h) HU exposure (Fig. 2, G and H), whereas si-Metnase showed a marked delay after 1-h HU treatment (Fig. 2, G and H). In contrast, a marked reduction in formation of Rad51 foci was observed with Metnase- and Exo1-repressed cells compared with control cells after a long HU exposure (Fig. 2, I and J). Together, these results suggest that Exo1 is not a major player at stalled fork following a short HU exposure but may play a crucial role at collapsed forks that require recombination repair for fork restart. Metnase, in contrast, could be involved in preventing fork reversal in response to a short exposure to HU (9).

Metnase Enhances Exo1-mediated 5′-End Resection on Model Replication Fork Structures—Metnase possesses DNA endonuclease activity that cleaves replication fork structures at the ssDNA gap of the lagging strand (40). End resection nucleases, such as Exo1, Dna2, and CtIP, cleave these structures poorly because they require a free 5′-OH end to begin exonucleolysis (49–52). Metnase-mediated fork endonucleolytic cleavage not only generates a free 5′-OH end on the lagging
parental DNA but also produces the 3’-ss-overhang for 5’-end resection of the daughter strand DNA (Fig. 3B) (40, 41), suggesting that Metnase may play a unique role in initiating 5’-end resection at stalled replication forks. To explore this further, we analyzed nucleolytic processing of model replication fork structures in vitro by WT Metnase and/or WT Exo1. Metnase displayed minor end cleavage activity that was unaffected by Exo1 (Fig. 3, C, lanes 2 and 3 versus lanes 6 and 7, and D). In contrast, Exo1’s nuclease activity on the lagging nascent DNA was markedly enhanced in the presence of Metnase (Fig. 3, C, lane 4 versus lanes 6 and 7, and D). No DNA resection was observed with a nuclease-dead mutant of Exo1 activity (D173A) (37) even in the presence of WT Metnase (Fig. 3, C and D). Because Metnase’s catalytic motif (DDN) was necessary to promote restart of stalled forks (40), we examined whether Metnase’s DNA cleavage activity plays a role in Exo1-mediated resection of the lagging daughter DNA. Exo1-mediated resection of the lagging daughter DNA was enhanced by both WT Metnase and the catalytically dead D483A mutant, (Fig. 3E), suggesting that Metnase enhances Exo1-mediated resection by a mechanism that is independent of lagging daughter strand cleavage. Although Metnase’s DNA cleavage activity is not involved in Exo1-mediated resection of the lagging daughter DNA (Fig. 3, E and F), cells overexpressing a Metnase mutant lacking DNA cleavage activity (D483A) showed a marked reduction in generation of ssDNA as measured by formation of BrdU foci after...
HU treatment (Fig. 3, G and H), suggesting that Metnase’s DNA cleavage activity does play an important role in resection of stressed forks in vivo.

Metnase Mediates Loading of Exo1 onto Single Strand Overhang DNA—Because enhancement of Exo1-mediated resection by Metnase does not require its DNA cleavage activity (Fig. 3E), we reasoned that Metnase might be involved in loading Exo1 onto DNA. To test this, we performed a pulldown experiment in which WT Metnase was incubated with 3′-biotinylated DNA to assay protein-DNA binding by using streptavidin-agarose beads, and the protein-DNA interaction was analyzed by Western blotting. WT Metnase was effectively pulled down with single strand overhangs but not with blunt-ended dsDNA (Fig. 4A), suggesting that Metnase requires ss-overhang for binding to dsDNA. Exo1 alone showed little or no interaction with single strand overhangs (Fig. 4B, top panel, lanes 2 and 3), but the Exo1-DNA interaction was markedly enhanced in the presence of WT Metnase (Fig. 4B, top panel, lanes 4 and 5). A substitu-
tion mutation at conserved Arg residues within the HTH motif of DNA binding domain (R339A and R371A; Fig. 4C) not only destroyed Metnase’s DNA binding activity (Fig. 4E) but also led to a failure in Exo1 loading onto single strand overhangs (Fig. 4F). A nuclease-dead mutant of Metnase (D483A), however, was effective in loading Exo1 onto DNA (Fig. 4F). A mutation at a key DNA binding or DNA cleavage site of Metnase has no effect on the Metnase-Exo1 interaction (Fig. 4G). Taken together, these results suggest that Metnase HTH and NUMOD1 DNA binding domains mediate Metnase binding to single strand overhangs and Exo1 loading onto DNA.

Enhancement of Exo1-mediated Resection by Metnase Does Not Require Metnase’s DNA Cleavage Activity but Depends on the Presence of ss-Overhang DNA—Metnase’s role in the loading of Exo1 onto single strand overhangs might be directly linked to enhancement of Exo1-mediated 5’ resection at fork...
DNA. To test this, we first examined WT Exo1 for cleavage of blunt-ended dsDNA and dsDNA with ss-overhang in the presence and absence of WT Metnase. Exo1 exhibited no DNA cleavage activity with a blunt-ended dsDNA even in the presence of WT Metnase (Fig. 5A). A duplex DNA with single strand overhangs not only supported Exo1’s 5’-resection activity (Fig. 5B, lanes 2 and 3) but also was necessary for enhancement of Exo1’s resection activity by Metnase (Fig. 5, B, lanes 2 and 3 versus lanes 5 and 6, and C). We next examined Exo1’s cleavage activity with dsDNA containing various sizes of 3’-ss-overhangs. A duplex DNA with a ≥9-nucleotide 3’-ss-overhang was able to support Exo1’s 5’-resection activity that was further stimulated 5–10-fold in the presence of WT Metnase (Fig. 5, D and E), suggesting that 3’-ss-overhang is not only essential for Exo1’s 5’-end resection activity but also crucial for Metnase’s role in enhancing Exo1 activity. Similar to Exo1 loading onto single strand overhangs by Metnase (Fig. 4F), Metnase mutants defective in DNA binding (R339A and R371A) failed to enhance Exo1’s end resection activity (Fig. 5, F and G), whereas
a nuclease-dead mutant (D483A) showed the same activity as WT Metnase (Fig. 5, H and I), suggesting that Metnase enhances Exo1’s end resection activity by mediating Exo1 loading onto single strand overhangs.

**Discussion**

The mechanism of DNA replication fork restart upon stress is complex. Many components, including DNA helicases, translocases, and nucleases, are thought to play a role in this process (1, 2, 5, 6, 11, 53). How these distinct activities cooperate to orchestrate and/or facilitate fork restart is far from clear. We previously demonstrated that a SET-transposase chimeric protein called Metnase (or SETMAR) participates in fork restart after HU treatment (39–41, 54). Metnase possesses a unique DNA cleavage activity toward fork DNA (40, 55), and its catalytic motif (DDN) is necessary for Metnase’s function in repli-
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Our previous study showed that Metnase promotes resolution of γ-H2AX (41) but is not required for formation of Rad51 foci in response to HU treatment of cells for 4 h (39). In this study, however, we found that Metnase is involved in formation of Rad51 foci after a long exposure (8–24 h) to HU (Fig. 2, I and J), suggesting that Metnase’s role in the loading of Exo1 not only enhances Exo1’s resection activity but is also strategically beneficial for formation of Rad51 foci in recombination repair at stalled forks.

Metnase associated with Rad9 following HU treatment (39), indicating that it works with a damage response factor(s) at the stalled forks. Metnase also interacts with topoisomerase IIα and stimulates relaxation of positive supercoils (39), which may contribute to Metnase’s function in promoting fork restart. In this work, we found that Metnase interacts with Exo1, a key DNA resection factor with 5′-exonuclease activity (Fig. 1). Although the Metnase–Exo1 interaction occurs independently of HU treatment, Exo1’s 5′ resection activity was significantly enhanced in the presence of Metnase (Fig. 3, C and D), suggesting that the Metnase–Exo1 interaction likely occurs on DNA. Metnase requires an ss-overhang for its binding to DNA (Fig. 4A) as well as for fork cleavage activity (Fig. 5, A and B). Given that Metnase’s DNA binding, not DNA cleavage, activity is required for enhancement of Exo1-mediated 5′-end resection on a gapped DNA (Fig. 5, F and H), Metnase, once bound to a gapped DNA, recruits Exo1 and stabilizes the Exo1-gapped DNA interaction (Fig. 6). This Metnase–Exo1 interaction on single strand overhangs would eventually enhance Exo1’s 5′-end resection activity at stalled forks. Exonuclease/endonuclease/phosphatase domain-1 (EEPDD1), similar to Metnase, possesses DNA cleavage activity on a replication fork structure, interacts with Exo1, and plays a role in replication restart (59). Given that Metnase and its DNA cleavage activity play a crucial role in restart of replication (40), however, the replication fork nucleases may have a defined role for different situations.

DNA damage or replication stress causes uncoupling of DNA synthesis at replication forks and generates ssDNA gaps (62). The ssDNA gap could collapse into DSBs that are repaired by a recombination pathway (Fig. 6). Metnase knockdown sensitizes cells to HU treatment and delays restart of the replication fork (39–41, 61); its endonuclease activity (40) likely plays an initiating role in 5′-end resection by cleaving the ssDNA gap on the lagging strand template of a fork structure where both daughter branches are double-stranded flap forks prior to the stimulatory effect of Exo1 (Figs. 3B and 6). In the following step, Metnase interacts with Exo1 and promotes Exo1-mediated 5′-end
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resection (Figs. 1 and 2), generating 3’-ssDNA for Rad51-mediated strand invasion (Fig. 6). Dna2, BLM, and the 9-1-1 clamp interact with Exo1 (25, 26, 43, 60, 63), which may also play a role in enhancing Exo1 activity. Metnase has several key characteristics that make it uniquely qualified for an initiating role in 5’-end resection on lagging strand. First, Metnase possesses endonuclease activity that preferentially cleaves a single strand gap on the lagging parent strand of fork DNA (40), whereas other end resection nucleases, such as CtIP, Exo1, and Dna2, cleave the ssDNA gap of a replication fork structure poorly because they require a free 5’-end to begin exonucleaseysis (49–52). Given that Metnase interacts with Exo1 (Fig. 1) and mediates loading of Exo1 onto the ss-overnhang of duplex DNA (Fig. 4), cleavage of an ssDNA gap on the lagging parent strand by Metnase would be strategically beneficial for Rad51-mediated strand transfer of the lagging template DNA at stalled replication forks (Fig. 6). Without Metnase present, HU induces far fewer single strand breaks and formation of Rad51 foci (Fig. 2G), consistent with that in our model of Metnase function (Fig. 6). Stalled forks that have collapsed such that they cannot be repaired often cause cell death by terminating in a DSB (2, 11, 6). Stalled forks that have collapsed such that they cannot be repaired often cause cell death by terminating in a DSB (2, 11, 6). Stalled forks that have collapsed such that they cannot be repaired often cause cell death by terminating in a DSB (2, 11, 6). Stalled forks that have collapsed such that they cannot be repaired often cause cell death by terminating in a DSB (2, 11, 6). Stalled forks that have collapsed such that they cannot be repaired often cause cell death by terminating in a DSB (2, 11, 6).

Experimental Procedures

Chemicals, Antibodies, and DNA Substrates—[α-32P]dCTP (3000 Ci/mmol) was obtained from Perkin Elmer Life Sciences and protein markers and Bradford reagents were purchased from Bio-Rad. An anti-FLAG M2 antibody was obtained from Sigma, and Clidu and Idu nucleotide analogs were obtained from Sigma. Oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA).

Purification of Metnase and Exo1—WT Metnase and the mutants were purified from HEK293 cells stably expressing FLAG-tagged Metnase as described (66, 67). FLAG-Metnase was detected in cell extracts by Western blotting using a monoclonal antibody (Sigma) as described previously (66, 67). Cells overexpressing WT or mutant Metnase were suspended in 20 ml of buffer E (50 mM Tris-HCl, pH 7.5, 5 mM DTT, 1% Nonidet P-40, 10% glycerol, 1 mM EDTA), and mammalian protease inhibitor mixtures containing 0.2 mM NaCl and centrifuged (100,000 × g) for 30 min. The supernatant was filtered through a Whatman paper and incubated at 4°C for 60 min with anti-FLAG affinity gel pre-equilibrated with buffer E. The beads were washed three times with buffer E containing 2.0 M NaCl prior to elution of the protein with buffer E containing FLAG peptide (500 µg/ml). The eluant was diluted with 10 volumes of buffer E and loaded onto a heparin-Sepharose 6 Fast Flow column (Amersham Biosciences) pre-equilibrated with buffer E. After washing the column, Metnase was fractionated using a linear gradient (0–2.0 M NaCl) in buffer E. The eluted protein was dialyzed against buffer E containing 50 mM NaCl and stored at −80°C.

For preparation of V5-Exo1, cells were harvested; lysed with cold lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5% Nonidet P-40, 5 µg/ml leupeptin, 5 µg/ml antipain, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM DTT, and 1 mM PMSF) for 30 min at 4°C; and clarified by centrifugation. The cell lysates were precloned with protein G-agarose beads (Millipore) for 1 h at 4°C with rotation, anti-V5 antibody (Invitrogen) was added and incubated overnight at 4°C, and then protein G-agarose beads (Millipore) were added and incubated for 2 h at 4°C. Agarose beads were collected and washed eight times with washing buffer (50 mM Tris-HCl, pH 7.5, 1.5 mM NaCl, 10% glycerol, 1% Nonidet P-40, and 1 mM EDTA). Proteins were eluted with 0.2 M glycine, pH 2.5, into 1.5 M Tris-HCl, pH 8.8. The eluent was dialyzed overnight into dialysis buffer (25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 20% glycerol, 1 mM EDTA, 0.05% Nonidet P-40, and 1 mM DTT) and concentrated using Amicon Ultra centrifugal filter units (Millipore).

Preparation of 32P-Labeled DNA Substrates—For 3'-32P labeling of DNA, 40 pmol of the indicated ssDNA was incubated with 30 units of terminal transferase (Perkin Elmer Life Sciences) in the presence of [α-32P]dCTP according to the manufacturer’s protocol. The 32P-labeled ssDNA was then annealed to non-labeled DNAs to prepare the indicated DNA substrates for the DNA cleavage assay.

DNA Cleavage Assay in Vitro—For preparation of DNA substrates used in DNA cleavage assays, oligonucleotides were mixed and annealed together for fork DNA (5'-32P-labeled 5'-CTAGACTCGAGATGTCAAGCAGTCCTAACTTTGAGG-CAGAGTCCGTGACGCTCAGTATG-3'), 5'-CGTACTGAGGTGGTTGGGTCTACAGGCTTGTATAGTATTAGGTTGGTGACCCCC-GTAAAGAAATTTTT-3' and 5'-AAAACTTTTCTTACTACGGGTCACCAACAATACATATACGATAGCCACTGC-3'), and 3'-overhang partial duplex DNA (5'-TATAGTGTTGTGACCAGCAAAGTGAAGAGTTTTTT-3' and 5'-AAACATTCTTACCTACGCGGCTCAAGACGGTAGTCAACGTGTTACAGCTTGATG-3').

The DNA cleavage assay was carried out using the previously described procedure with modification (40). Briefly, reaction mixtures (20 μl) containing 50 mM Tris-HCl, pH 7.5, 5 mM DTT, 5% glycerol, 2 μg of BSA, 2 mM MgCl2, 0.05% Triton X-100, and 25 mM KCl were incubated with Metnase (2–4 pmol) and/or Exo1 (1–2 fmol) in the presence of 240 fmol of radiolabeled DNA. After incubation at 37°C for the indicated amount of time, reaction mixtures were analyzed by 12% polyacrylamide gel electrophoresis containing 8 μM urea for DNA.
cleavage. The cleavage product was quantified using a PhosphorImager and ImageQuant software (GE Healthcare).

*Protein-DNA Interaction Using DNA Pulldown Assay—* 3′-Biotinylated 5′-flap DNA (25 pmol) was incubated with streptavidin-agarose pretreated with BSA and rotated for 30 min at room temperature in the presence of 1.0 ml of buffer (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 1.0 mM DTT, and 0.1 μg/ml BSA). After adding 1.0 μg of WT Metnase to the DNA-streptavidin-agarose, the mixtures were rotated for 30 min at room temperature and centrifuged for 5 min at 5,000 rpm. The precipitates were collected and washed once with 1.0 ml of the washing buffer (50 mM Tris-HCl, pH 7.5, and 25 mM KCl). After rotation for 5 min, the pellets were centrifuged for 5 min at 5,000 rpm at room temperature and analyzed by Western blotting.

DNA Resection in Vivo Using DNA Fiber Analysis after Single Labeling of Chromosomal Replication with IdU—DNA fiber analysis was carried out as described (10) with some modifications. Briefly, cells were grown in 6-well dishes (6 × 10^5/well), and 20 μM IdU was added to the growth medium and incubated for 20 min at 37 °C. After washing with fresh medium, cells were treated with 5 mM HU for 60 min or mock-treated and further incubated for the indicated times at 37 °C. HU stalls replication forks without generating additional DNA structural damage. Cells were harvested and resuspended in PBS, and ~1000 cells were transferred to a positively charged microscope slide (Superfrost/Plus, Daigler) and processed for DNA fiber analysis as described previously (40). Slides were mounted in PermaFluor aqueous, self-sealing mounting medium (Thermo Scientific), and DNA fibers were visualized using a confocal microscope (Olympus FV1000D, 63× oil immersion objective). Images were analyzed using Olympus Fluoview software.

*DNA Resection Analysis Using Anti-BrdU Immunofluorescence—* DNA resection analysis was carried out as described (61) with slight modifications. HEK293 cells were transfected with si-control, si-Exo1, si-Metnase, or si-Exo1 + si-Metnase and seeded onto poly-d-lysine coverslips (18-mm diameter; neuVitro, catalog number GG-18-PDL) with fresh medium containing 40 μM BrdU for 36 h. After treatment with 10 mM HU for various times, cells were pre-extracted with 0.5% Triton X-100 and PBS for 5 min on ice and fixed with 4% paraformaldehyde for 20 min. The coverslips were blocked for 1 h with 1% BSA and PBS and incubated with mouse anti-BrdU antibody (1:250; BD Biosciences) overnight in a wet chamber at 4 °C. After washing four times, coverslips were incubated with secondary antibody donkey anti-mouse Alexa Fluor 488 (1:500; Invitrogen) for 1 h. After washing four times with 0.2% Tween 20 and PBS, the samples were mounted in Vectashield HardSet mounting medium with DAPI on a microscope slide (Fisherfinest Premium Superfrost microscope slides) and analyzed using a confocal microscope (Olympus2 confocal microscope with 63× water immersion objective). In the second approach, we carried out DNA fiber analysis as described previously (40). Cells were grown in 6-well dishes (6 × 10^5/well), and 20 μM IdU was added to growth medium and incubated for 20 min at 37 °C. After washing with fresh medium, cells were treated with 5 mM HU for 60 min or mock-treated. Medium was replaced with fresh medium containing 100 μM CldU, and cells were further incubated for the indicated times at 37 °C. Cells were harvested and resuspended in PBS, and ~1000 cells were transferred to a positively charged microscope slide (Superfrost/Plus) and processed for DNA fiber analysis as described previously (40). Slides were mounted in PermaFluor aqueous, self-sealing mounting medium, and DNA fibers were visualized using a confocal microscope (Olympus FV1000D, 63× oil immersion objective). Images were analyzed using the Olympus Fluoview software.

*Statistical Analysis—* All experiments were performed at least three times. Data are the means ± S.E. Statistical significance between groups was determined using analysis of variance with Tukey’s post hoc test or Student’s t test as indicated.

### Author Contributions—S.-H. L. and H.-S. K. conceived the project, designed the study, and performed and analyzed all the experiments. H.-S. K., E. A. W., R. A. H., and S.-H. L. contributed reagents and materials. S.-H. L., J. A. N., R. A. H., E. A. W., and H.-S. K. analyzed the data and wrote the paper.

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