EXD2 promotes homologous recombination by facilitating DNA end resection

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Repair of DNA double-strand breaks (DSBs) by homologous recombination (HR) is critical for survival and genome stability of individual cells and organisms, but also contributes to the genetic diversity of species. A vital step in HR is MRN–CtIP-dependent end resection, which generates the 3’ single-stranded DNA overhangs required for the subsequent strand exchange reaction. Here, we identify EXD2 (also known as EXDL2) as an exonuclease essential for DSB resection and efficient HR. EXD2 functionally interacts with the MRN complex to accelerate resection through its 3’-5’ exonuclease activity, which efficiently processes double-stranded DNA substrates containing nicks. Finally, we establish that EXD2 stimulates both short- and long-range DSB resection, and thus, together with MRE11, is required for efficient HR. This establishes a key role for EXD2 in controlling the initial steps of chromosomal break repair.

DNA double-strand breaks (DSBs) are extremely cytotoxic lesions that can arise during normal cellular processes or are induced by exogenous factors such as ionizing radiation (IR) as well as many commonly used anticancer drugs. The faithful repair of DSBs is essential for cell survival and organismal development, as defective repair can contribute to a plethora of inherited human syndromes with life-threatening symptoms including cancer, neurodegeneration or premature ageing.¹² The two major pathways involved in the repair of DSBs in eukaryotic cells are non-homologous end joining and homologous recombination (HR; refs 3–5). A key initial step in HR is resection of the DNA ends on either side of the break, which is carried out initially by the MRE11–RAD50–NBS1 complex (MRN) and CtIP (also known as RBBP8, retinoblastoma binding protein 8) to generate short stretches of single-stranded DNA (ssDNA; refs 6–8). Subsequently, the EXO1 (exonuclease 1) or DNA2 (DNA replication helicase/nuclease 2) nucleases, in conjunction with the Bloom syndrome helicase (BLM), extend these to generate longer 3’ ssDNA tails.⁸–¹⁵ These ssDNA strands are then bound by replication protein A (RPA; refs 10–12,16–18), which is subsequently replaced by RAD51 in a BRCA2 (breast cancer 2)-dependent manner, leading to the formation of ssDNA–RAD51 nucleoprotein filaments essential for the strand exchange process.⁸–¹⁹ In vitro, MRE11 displays a weak endo- and exonuclease activity, which may be due to the lack of accessory factors.⁶–²⁰ Accordingly, work from multiple laboratories has shown that CtIP, or its yeast homologue Sae2 (sporulation in the absence of spo eleven), can stimulate MRE11’s endonuclease activity.⁸–¹⁶,¹⁸ Interestingly, MRE11 has also been shown to nick the DNA strand to be resected in multiple positions, as far as 300 base pairs (bp) from the break itself, suggesting that resection could proceed from several entry points that are distal to the DSB (refs 21,22). However, it is unclear whether this would enhance MRE11-dependent nucleolytic processing of DNA ends, thus generating a better substrate for subsequent processing of the break by BLM–DNA2 and/or BLM–EXO1 complexes, or allow access for additional factors accelerating the initial strand processing. Indeed, the inhibition of MRE11’s endonuclease activity confers a stronger resection defect than inhibition of its exonuclease activity, suggesting perhaps that initial break processing might be also carried out by other exonucleases.²³ Here we identify EXD2 (exonuclease 3’–5’ domain containing 2) as a cofactor of the MRN complex required for efficient DNA end resection, recruitment of RPA, HR and suppression of genome instability.

EXD2 is required for repair of damage to DNA

In an effort to identify factors required to promote HR, we carried out an unbiased proteomic approach to define the CtIP interactome.
Here, we have identified EXD2, a largely uncharacterized protein with a putative exonuclease domain, as a candidate CtIP binding partner (Fig. 1a). We validated this interaction by co-immunoprecipitations from human cell extracts and found that we could readily detect endogenous EXD2 by western blotting of green fluorescent protein (GFP)–CtIP immunoprecipitates (Fig. 1b). Endogenous CtIP, as well as its known interactors MRE11 and BRCA1, were detected in reciprocal Flag–EXD2 immunoprecipitates (Fig. 1c; lysates were treated with Benzonase to prevent DNA bridging). Therefore, we conclude that the two proteins probably exist in the same complex in cells.

EXD2 is highly conserved across vertebrates (Supplementary Fig. 1) and was recently identified in the screen for suppression of sensitivity to mitomycin C (ref. 24). However, the biological and biochemical features of this protein are unknown. As we identified EXD2 as its known interactors MRE11 and BRCA1, were detected in reciprocal Flag–EXD2 immunoprecipitates (Fig. 1c; lysates were treated with Benzonase to prevent DNA bridging). Therefore, we conclude that the two proteins probably exist in the same complex in cells.

EXD2 promotes DNA end resection and the generation of ssDNA

CtIP is essential for efficient DNA end processing during DSB repair, with cells depleted for this factor showing a defect in the generation of ssDNA and the subsequent formation of RPA foci16,25,26. Thus we hypothesized that EXD2 may promote DNA end resection. To test this, we analysed RPA focus formation in response to both CPT and IR in wild-type (WT) and EXD2-depleted cells. Strikingly, cells depleted for EXD2 showed severely impaired kinetics of RPA focus formation in response to both treatments (Fig. 2a,b and Supplementary Fig. 2c,d). RPA2 phosphorylation at Ser4 and Ser8 has been widely used as a marker for the generation of ssDNA by DNA end resection27. Consistent with the data above, EXD2-depleted cells showed impaired RPA Ser4, Ser8 phosphorylation in response to DNA damage (Fig. 2c and Supplementary Fig. 2e). Treatment with both agents resulted in a robust phosphorylation of histone H2AX (also known as H2AFX) and CHK2 (also known as CHEK2) (Fig. 2c and Supplementary Fig. 2e), confirming induction of DNA damage in cells. Moreover, since these responses were intact in EXD2-depleted cells, EXD2 is most likely not required for initial sensing of the DNA damage. Failure to generate RPA foci could be associated either with a defect in exonucleolytic...
were not due to changes in the cell cycle, as EXD2 depletion had little effect on the overall cell-cycle distribution profile (Supplementary Fig. 2f). Despite multiple attempts, we were unable to visualize EXD2 recruitment to DNA damage foci. In this regard, we note that certain DDR proteins in U2OS cells 72 h post transfection with control siRNA or an siRNA oligonucleotide targeting EXD2 (EXD2 siRNA) treated with 1 μM CPT for 1 h as indicated and stained for BrdU and DAPI. BrdU staining was carried out under non-denaturing conditions, with foci indicating the presence of ssDNA. Scale bars, 5 μm.

Quantification of the percentage of U2OS cells treated as in d exhibiting more than 15 BrdU foci per nucleus. n = 311 cells (control siRNA, untreated), 300 cells (EXD2 siRNA, untreated), 300 cells (control siRNA, 1 μM CPT) and 300 cells (EXD2 siRNA, 1 μM CPT), grouped from three independent experiments. Error bars represent ± s.e.m. The Chi-square test was used to determine statistical significance.  

Chromatin fractionation of HeLa cells untreated or treated with 500 μM phleomycin for 1 h as indicated. γH2AX and RPA2 pSer4, Ser8 are used as markers of DNA damage and histone H3 acts as a loading control. This experiment was carried out twice independently. Unprocessed original scans of blots are shown in Supplementary Fig. 7.
EXD2 promotes HR and suppresses genome instability

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In *vivo* RPA is required for RAD51 focus formation, and *in vitro* has been shown to promote RAD51-mediated strand exchange. Consistent with this, treatment of U2OS cells with IR generated large numbers of RAD51 foci (Fig. 3a,b). In contrast, EXD2 depletion significantly impaired RAD51 focus formation (Fig. 3a,b). RAD51–ssDNA nucleoprotein filament formation is a crucial step in DSB repair by HR (refs 30–32). To examine if EXD2 is also required for efficient HR, we used a U2OS cell line carrying an integrated HR reporter transgene and an I-SceI recognition sequence. Transient expression of I-SceI enzyme was analysed by fluorescence-activated cell sorting (FACS) and EXD2 siRNA samples were compared with the control siRNA (normalized to 100%). Data represent the mean ± s.e.m. Statistical significance was determined using Student’s t-test. EXD2 treatment was carried out using either control siRNA or siRNA targeting EXD2 (EXD2 siRNA) in cells treated with the indicated doses of olaparib. Survival data represent mean ± s.e.m. (n = 3 independent experiments). Statistical significance was determined using Student’s t-test.

The MRN complex processes DSBs to generate ssDNA, which requires MRE11’s 3’–5’ exonuclease activity. Interestingly, EXD2 has a predicted exonuclease fold, which has sequence homology to the 3’–5’ exonuclease domain of the Werner syndrome protein (WRN). Analysis of the alignment between EXD2 and WRN identified two key amino acids (Asp 108 and Glu 110) within the putative exonuclease domain of EXD2, which are also highly conserved in other DnaQ type exonucleases, including WRN, that coordinate the binding of metal ions within the active site (Supplementary Fig. 3a). Mutation of the equivalent residues in WRN (Asp 82 and Glu 84) renders
Figure 4 EXD2 displays 3′–5′ exonuclease activity in vitro. (a) 5′-radiolabelled ssDNA 50-mer substrate (10 nM molecules) was incubated for the indicated lengths of time with EXD2 WT or EXD2 D108A–E110A mutant protein (70 nM). Samples were resolved on a 20% Tris-boric acid–EDTA (TBE)–urea polyacrylamide gel and visualized by phosphorimaging. This experiment was carried out twice independently. (b) 3′-radiolabelled ssDNA 50-mer substrate (0.25 μM molecules) was incubated for the indicated amounts of time with EXD2 WT or EXD2 D108A–E110A (70 nM) mutant protein. Samples were resolved by thin layer chromatography in 1 M sodium formate at pH 3.4 and visualized by phosphorimaging. This experiment was carried out twice independently. (c) 5′ dsDNA 50-mer substrates (10 nM molecules) were incubated for the indicated lengths of time with EXD2 WT protein (70 nM). Samples were resolved on a 20% TBE–urea polyacrylamide gel and visualized by phosphorimaging. This experiment was carried out twice independently. (d) 5′-radiolabelled ssDNA or dsDNA with 5′ overhang substrate (3 nM molecules) was incubated for indicated lengths of time with EXD2 WT (Lys76–Val564) protein (25 nM). Samples were resolved on a 20% TBE–urea polyacrylamide gel and visualized by phosphorimaging. This experiment was carried out twice independently. (e) 5′-radiolabelled ssDNA or dsDNA (3 nM molecules) with the 3′ end blocked by biotin–streptavidin was incubated for the indicated time with EXD2 WT (Lys76–Val564) protein (25 nM) in buffer supplemented with 1 mM ATP. Samples were resolved on a 20% TBE–urea polyacrylamide gel and visualized by phosphorimaging. This experiment was carried out twice independently. (f) Upper panel: EXD2 WT (Lys76–Val564) gel-filtration (GF) fractions were tested for nuclease activity against 5′-radiolabelled ssDNA (10 nM molecules). Reactions were incubated for 30 min, resolved on a 15% TBE–urea polyacrylamide gel and visualized by phosphorimaging. Lower panel: Coomassie blue-stained gel depicting the EXD2 protein in gel-filtration fractions analysed in the upper panel. This experiment was carried out twice independently.

the protein devoid of nuclease activity. Therefore, we hypothesized that the equivalent residues in EXD2 may be also required for its putative nuclease activity. To test this, we expressed the full-length glutathione S-transferase (GST)-tagged EXD2 and the D108A and E110A mutant protein in bacteria, and purified them to apparent homogeneity (Supplementary Fig. 3b). Next, we tested the activity of these purified proteins on ssDNA radiolabelled on the 3′ or 5′ end (Fig. 4a,b). We found that purified EXD2, but not the D108A and E110A mutant, exhibited a robust nuclease activity on short 5′ labelled ssDNA (Fig. 4a). Furthermore, a time course of the 3′ labelled substrate digest indicates that EXD2 degrades the labelled DNA strand from the 3′ end, as evidenced by the release of the single labelled nucleotide (Fig. 4b). This data shows that EXD2 displays a 3′–5′ exonuclease activity in vitro. Moreover, under these conditions the WT protein exhibited only weak activity towards blunt end double-stranded DNA (dsDNA; Fig. 4c). To verify this data, we also identified a highly soluble truncated form of EXD2 (spanning residues Lys76 through to Val564, containing the predicted exonuclease domain) that can be produced at very high yields and purity in a three-step procedure (Supplementary Fig. 3c–e). This version of EXD2, and its D108A E110A variant, behaved indistinguishably from full-length EXD2 (Supplementary Fig. 3f). In addition, the protein showed only a weak activity towards dsDNA with a resected 3′ end (Fig. 4d), and did not display any endonuclease activity on ssDNA or dsDNA with a biotin/streptavidin-blocked 3′ end (Fig. 4e). Importantly, purified EXD2 displayed a robust exonuclease activity, which co-elutes with the protein (Fig. 4f). Thus our data identify EXD2 as a bone fide exonuclease with a 3′–5′ polarity.

To address the potential biological significance of EXD2’s exonuclease activity, we tested whether this activity was required to promote DNA end resection in vivo. To this end, we examined the phenotypes of two independently derived U2OS clones stably expressing WT or the nuclease-dead (D108A and E110A) EXD2 mutant. The endogenous protein was depleted with siRNA targeting the 3′ untranslated region (UTR) of EXD2 (Supplementary Fig. 4a). Notably, cells expressing the nuclease-dead protein did not correct...
Figure 5 Nuclease activity of EXD2 is required for DSB repair in vivo. (a) U2OS control cells or cells stably expressing Flag–HA–EXD2 WT or D108A–E110A mutant fusion proteins 72 h post transfection with an siRNA targeting EXD2 3′UTR or without siRNA were treated with 1 μM CPT for 1 h. Quantification of the percentage of cells with more than 15 BrdU foci per nucleus is represented. n = 223 cells (U2OS – EXD2 siRNA), 235 cells (U2OS + EXD2 siRNA), 309 cells (clone 1 – EXD2 siRNA), 170 cells (WT clone 1 + EXD2 siRNA), 183 cells (WT clone 2 – EXD2 siRNA), 203 cells (WT clone 2 + EXD2 siRNA), 200 cells (D108A–E110A clone 1 – EXD2 siRNA), 190 cells (D108A–E110A clone 1 + EXD2 siRNA), 282 cells (D108A–E110A clone 2 – EXD2 siRNA) and 223 cells (D108A–E110A clone 2 + EXD2 siRNA), pooled from three independent experiments. (b) U2OS control cells or cells stably expressing Flag–HA–EXD2 WT or D108A–E110A mutant fusion proteins 72 h post transfection with an siRNA targeting EXD2 3′UTR or without siRNA were treated with 1 μM CPT for 1 h as indicated. Cells were fixed and stained for RPA by immunofluorescence with quantification of the percentage of cells with more than 15 RPA foci per nucleus represented. n = 308 cells (U2OS – EXD2 siRNA), 308 cells (U2OS + EXD2 siRNA), 327 cells (WT clone 1 – EXD2 siRNA), 320 cells (WT clone 1 + EXD2 siRNA), 308 cells (WT clone 2 – EXD2 siRNA), 353 cells (WT clone 2 + EXD2 siRNA), 321 cells (D108A–E110A clone 1 – EXD2 siRNA), 368 cells (D108A–E110A clone 1 + EXD2 siRNA), 370 cells (D108A–E110A clone 2 – EXD2 siRNA) and 337 cells (D108A–E110A clone 2 + EXD2 siRNA), pooled from three independent experiments. At least 100 cells were scored for each experiment. (c) U2OS control cells or cells stably expressing Flag–HA–EXD2 WT or D108A–E110A mutant fusion proteins 72 h post transfection with an siRNA targeting EXD2 3′UTR or without siRNA were irradiated with 8 Gy. Cells were fixed and stained for RAD51 6 h post treatment and the percentage of RAD51-positive cells quantified. n = 213 cells (U2OS – EXD2 siRNA), 254 cells (U2OS + EXD2 siRNA), 169 cells (WT clone 1 – EXD2 siRNA), 176 cells (WT clone 1 + EXD2 siRNA), 191 cells (WT clone 2 – EXD2 siRNA), 184 cells (WT clone 2 + EXD2 siRNA), 195 cells (D108A–E110A clone 1 – EXD2 siRNA), 224 cells (D108A–E110A clone 1), 191 cells (D108A–E110A clone 2 – EXD2 siRNA) and 198 cells (D108A–E110A clone 2 + EXD2 siRNA), pooled from three independent experiments. (d) Survival assay in U2OS cells complemented with WT or D108A–E110A mutant EXD2 protein transfected with control siRNA (sample 1) or siRNA targeting 3′UTR of EXD2 (samples 2–6). Cells were treated with 5 μg ml⁻¹ phleomycin, and the resistance of cells transfected with control siRNA was set at 100% (n = 4 independent experiments). For all panels, bars represent mean ± s.e.m. Statistical significance was determined using the Chi-square test (a–c) or Student’s t-test (d).

EXD2 cooperates with MRE11 in the repair of DSBs

The in vivo resection that initiates DSB repair is catalysed by the MRN complex. To test whether or not EXD2 collaborates in this process with MRE11 we analysed the kinetics of RPA focus formation (a marker of resection) in cells depleted for either of these proteins or concomitantly depleted for both EXD2 and MRE11. We found that combined depletion resulted in a comparable inhibition of resection as observed for depletion of MRE11 alone (Fig. 6a). A similar relationship was also observed for RAD51 foci (Supplementary Fig. 4b,c). Interestingly, the defect observed in EXD2-depleted cells was slightly weaker than that observed in MRE11 alone, suggesting that MRE11 functions upstream of EXD2 in DNA resection, perhaps initiating resection through its endonuclease activity. Indeed, it has been suggested recently that MRE11 may create multiple nicks on the strand...
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Figure 6 EXD2 promotes resection through a common mechanism with MRE11. (a) Quantification of the signal intensity of RPA foci in U2OS cells depleted for EXD2, MRE11 or both by siRNA (as indicated) at various time points following treatment with 8 Gy IR. ImageJ was used to quantify signal intensity per nucleus (using RPA as a marker of resection; DAPI staining marks the nucleus). n = 154, 155, 150 and 161 cells for control siRNA 0, 30, 60 and 120 min post treatment, respectively. n = 177, 187, 189 and 173 cells for EXD2 siRNA 0, 30, 60 and 120 min post treatment, respectively. n = 182, 167, 184 and 187 cells for MRE11 siRNA 0, 30, 60 and 120 min post treatment, respectively. n = 214, 182, 150 and 163 cells for EXD2–MRE11 siRNA 0, 30, 60 and 120 min post treatment, respectively. In all cases cells were pooled from three independent experiments. Error bars represent ±s.e.m. Statistical significance was determined using the Mann–Whitney test. (b) EXD2 stimulates MRN complex-dependent nuclease activity. MRN complex (50 nM) was incubated with ΦX174 substrate DNA being resected that could serve as additional exonuclease entry sites to further enhance nucleolytic processing. We tested this notion in several ways. First, we analysed if MRN-dependent DNA resection is accelerated in the presence of EXD2. Purified EXD2 and the MRN complex (MRE11, RAD50, NBS1) were incubated together with circular single-stranded ΦX174 DNA. This substrate requires initial endonuclease-dependent nicking by the MRN complex to undergo resection. As previously reported, the MRN complex exhibited nuclease activity under these conditions. Importantly, combining EXD2 with the MRN complex resulted in increased ssDNA degradation in vitro (Fig. 6b). As expected, addition of exonuclease-dead EXD2 protein to the MRN complex resulted in DNA degradation similar to that observed for MRN alone (Fig. 6c). Second, we predicted that EXD2 should be able to initiate resection from a nicked and a gapped duplex substrate designed to mimic the substrates generated by MRE11 endonuclease activity during the initial stage of DNA end resection. Strikingly, EXD2 exhibited robust exonuclease activity on both the nicked and gapped substrates (Fig. 6d–f and Supplementary Fig. 4d,e). Taken together, these data show that EXD2 functionally collaborates with the MRN complex in promoting DNA degradation.

To gain more functional insight into the role of EXD2’s exonuclease activity in DNA end resection in vivo, we took advantage of the recently developed small molecule inhibitors targeting the exo- or endonuclease activity of MRE11 (ref. 23). Inhibition of MRE11 endonuclease activity resulted in almost total inhibition of resection, whereas cells treated with the MRE11 exonuclease inhibitor showed a milder resection defect (Fig. 7a), as reported previously. Knockdown of EXD2 alone resulted in a resection defect significantly stronger (p < 0.0001) than that observed in cells treated with the MRE11 exonuclease inhibitor alone. Depletion of EXD2 in the presence of the MRE11 exonuclease inhibitor did not decrease efficiency of resection further than was achieved with EXD2 depletion alone (Fig. 7a). Interestingly, some residual resection was still observed in cells concomitantly depleted for EXD2 and incubated with the
MRE11 exonuclease inhibitor, indicating either an involvement of another exonuclease in this step of DNA end processing or that EXD2 knockdown or MRE11 exonuclease inhibition was not complete. Nevertheless, these data suggest that both the exonuclease activity of EXD2 and that of MRE11 function within the same pathway and that of both proteins did not further potentiate the resection defect. Given that both EXD2 and MRE11 regulate DSB resection, we tested the effect of their combined depletion on HR using the DR-GFP construct with concomitant depletion of EXD2 and MRE11 by siRNA following transient expression (4-OHT) for 1 h; genomic DNA was extracted and digested or mock digested by BsrGI overnight. DNA end resection adjacent to the DSB was measured by qPCR. The percentage of ssDNA was calculated and related to the control siRNA treated sample, which was set as 100%. Bars represent mean values ± s.e.m. (n = 5 independent experiments). Student’s t-test was used to determine statistical significance.

Figure 7 EXD2 is required for efficient HR. (a) Quantification of the signal intensity of RPA foci in U2OS cells treated with dimethylsulphoxide or small molecule inhibitors specifically targeting MRE11’s exo- or endonuclease activity (EXO or ENDO inhibitors, as indicated) 72 h post transfection with siRNA targeting EXD2 (EXD2 siRNA) or control siRNA. Cells were pretreated for 30 min with 100 μM PFM39 (EXO inhibitor) or 100 μM PFM01 (ENDO inhibitor) before irradiation with 3 Gy IR. Cells were collected 2 h post irradiation and stained for RPA foci. The intensity of RPA signal per cell nucleus was analysed using ImageJ. n = 231 cells (control siRNA, EXO–ENDO–), 226 cells (control siRNA, EXO+,) 177 cells (control siRNA, ENDO+), 233 cells (EXD2 siRNA EXO–ENDO–), 201 cells (EXD2 siRNA, EXO+), and 158 cells (EXD2 siRNA, ENDO+), pooled from three independent experiments. Error bars represent ± s.e.m. The Mann–Whitney test was used to determine statistical significance. (b) Schematic diagram of the resection assay in human cells using the ER-AsiSI system. Arrows indicate qPCR primers for measurement of resection efficiency following induction of the DSB. (c) ER-AsiSI U2OS cells were treated with 300 nM 4-hydroxytamoxifen for the induction of a DSB in a specific genomic locus in vivo. Then we analysed the efficiency of DNA resection at this DSB by quantitative PCR (qPCR) at two positions: one located close to the break (335 bp downstream of the break—short-range resection) and the other located at 1,618 bp from the break (long-range resection; ref. 40). Interestingly, we found that EXD2 depletion affected both short-range and long-range resection (Fig. 7a and Supplementary Fig. 4f). Knockdown of MRE11 resulted in a similar albeit stronger resection phenotype, thus providing further evidence to support its upstream function in this process. Importantly, concomitant depletion of both proteins did not further potentiate the resection defect.

Given that both EXD2 and MRE11 regulate DSB resection, we tested the effect of their combined depletion on HR using the DR-GFP...
In support of their role in this process, we observed that combined depletion of EXD2 and MRE11 did not decrease HR efficiency further than observed in the single knockdowns (Fig. 6d and Supplementary Fig. 4g).

These findings therefore show that EXD2 promotes DNA end resection and HR by enhancing the generation of ssDNA through a common mechanism with the MRN complex. Furthermore, it seems likely that MRE11 functions upstream of EXD2 in this process, probably initiating resection through its endonuclease activity.

To verify and extend the above conclusions, we used CRISPR/Cas9 nickase based gene editing41 in HeLa cells to generate EXD2−/− clones (Supplementary Fig. 5a). The use of Cas9 nickase has been recently shown to minimize any off-target effects42. Comparable to siRNA-treated U2OS cells, EXD2−/− HeLa cells showed dramatically decreased RPA focus formation in response to CPT (Supplementary Fig. 5b,c), diminished RPA2 phosphorylation on Ser4, Ser8 (Supplementary Fig. 5d) and decreased survival in response to CPT (Supplementary Fig. 5e). We also tested if EXD2 depletion affects the MRE11 or CtIP protein stability and/or their recruitment to DSBs. We found this not to be the case, as cells lacking EXD2 had similar levels of endogenous MRE11 or CtIP to the WT control (Supplementary Fig. 5f). Likewise, MRE11 or GFP–CtIP localization to DSBs induced by microirradiation43 was not affected (Supplementary Fig. 6a–c). These findings therefore establish EXD2 as an important regulator of DSB resection.

**DISCUSSION**

We have shown that EXD2 facilitates DSB resection, thus promoting recruitment of RPA and HR. Accordingly, cells depleted for EXD2 show spontaneous chromosomal instability and are sensitive to DNA damage induced by agents that generate DSBs. Furthermore, we establish that EXD2 functionally interacts with the MRN complex, utilizing its 3′–5′ exonuclease activity to accelerate DSB resection and promote efficient HR. In line with this, complementation experiments showed that exonuclease-dead mutant protein failed to complement these phenotypes. Interestingly, EXD2 seems to be dispensable for the initial sensing of the break, as evidenced by efficient γH2AX and CHK2 phosphorylation, and most likely acts downstream of MRE11. Finally we reveal that both EXD2 and MRE11 function in the same pathway for DSB resection and HR. It is unclear at present why cells would need two exonucleases with the same polarity. However, a paradigm for such a requirement is evident from the fact that cells have two alternative machineries, consisting of BLM–DNA2–RPA–MRN and EXO1–BLM–RPA–MRN, that carry out long-range resection4,10,13,44. Thus, by analogy EXD2 may function together with MRE11 to accelerate resection in the 3′–5′ direction to efficiently produce short 3′ ssDNA overhangs. This could promote faster generation of longer stretches of ssDNA, which in turn may serve as a better substrate for BLM–DNA2 or BLM–EXO1 to initiate long-range resection. Accordingly, depletion of EXD2 adversely impacts on this process. Ultimately, efficient generation of ssDNA with minimal homology length required for productive HR would suppress unscheduled deleterious recombination events. This may be particularly important in vertebrates, as they require significantly longer stretches of ssDNA (200–500 bp) to initiate productive HR (ref. 45), in contrast to yeast, where as little as 60 bp of 3′ ssDNA is sufficient to support HR (ref. 46). Not mutually exclusive is the possibility that EXD2 could also augment resection efficiency under specific circumstances, for instance in the presence of modifications to the damaged DNA and/or polypeptides bound at the 5′ ends. In line with this, we show that in vivo EXD2 depletion impairs short-range resection.

Recently, it has been proposed that MRE11 may create multiple incisions on the DNA strand undergoing resection up to 300 bp distal to the break, which could allow for more efficient resection21,22. Indeed, inhibition of MRE11’s endonuclease activity seems to be dominant in promoting the generation of ssDNA over its exonuclease activity23. Thus, we postulate a model whereby EXD2 functionally collaborates with the resection machinery, most likely utilizing DNA nicks generated by MRE11’s endonuclease activity 3′ of the DSB. This would enhance the generation of ssDNA tails required for efficient homologous recombination (model Fig. 6d).

In summary, our work identifies EXD2 as a critical factor in the maintenance of genome stability through HR-dependent repair of DSBs, including those induced by commonly used anticancer agents, such as IR or CPT. This highlights EXD2 itself and/or its enzymatic activity as a potential candidate for development of anticancer drugs.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Note:** Supplementary Information is available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

R.B. and J.N. carried out the majority of experimental work with contributions from W.N.; H.T.B., P.J.M. and O.G. contributed to the purification and analysis of the biochemical activities of EXD2. R.A.D. and T.T.P. purified the MRN complex. W.N. conceived the project and wrote the manuscript with editing contributions from R.B., J.N., T.T.P. and P.J.M.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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METHODS

Cell lines. HEK293FT cells were a gift from G. Stewart (Institute of Cardiovascular Sciences, University of Birmingham, UK) and HeLa and U2OS cells were a gift from F. Esaul (Sir William Dunn School of Pathology, University of Oxford, UK). The initial Chinese hamster ovary (CHO) cells were obtained from Life Technologies and ATCC, respectively, and were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and standard antibiotics. U2OS cells stably expressing GFP–Citrine and U2OS cells harbouring the HR reporter DR-GFP were a gift from S. P. Jackson (Wellcome Trust/Cancer Research UK Gurdon Institute, University of Cambridge, UK) and were maintained in media supplemented with 500 μg ml⁻¹ G-418. The ER-ASiJ U2OS cell line, a gift from G. Legube (Center for Integrative Biology, Université Paul Sabatier, France), was maintained in DMEM media without phenol red supplemented with 10% dialysed FBS (Life Technologies) and 1 μM 1-pyromycin. Cell lines stably expressing Flag–HA–EXD2 WT or D108A–E110A fusion proteins were generated by transfection of U2OS cells with these plasmid constructs, followed by clonal selection of cells grown in media containing 0.5 μg ml⁻¹ pyromycin (Life Technologies). All cell lines have been verified mycoplasma free by a PCR-based test (Takara).

Plasmids. The open reading frame (ORF) of human EXD2 was purchased as a gateway entry clone in the pDONR221 plasmid backbone from DNAX Plasmid Repository (HsCD00295838). Discrepancies in the amino-acid sequence in comparison to the reference sequence for human EXD2 (NM_001193360.1) were corrected by site-directed mutagenesis. Site-directed mutagenesis was then employed to generate EXD2 D108A–E110A in pDONR221. Flag–HA–EXD2 WT and D108A–E110A as well as GST–EXD2 WT and D108A–E110A plasmid constructs were generated by recombinase of the WT or D108A–E110A EXD2 ORF in pDONR221 by LR Clonase recombination into either the pHAGE-N-Flag–HA destination vector (a gift from R. Chapman, The Wellcome Trust Centre for Human Genetics, University of Oxford, UK), or the pDEST-pGEX6P-1 destination vector (a gift from C. Green, The Wellcome Trust Centre for Human Genetics, University of Oxford, UK), respectively. LR Clonase reactions were carried out using the Gateway LR Clonase II enzyme mix according to the instructions of the manufacturer (Life Technologies). The pCMV-I-SceI plasmid was a gift from V. Macaulay (Department of Oncology, University of Oxford, UK). pmCherry-C1 was obtained from Clontech. pX335-GFP plasmid (pX335 vector containing PGK-EGFP-P2A-Neo-pA) was a gift from J. Riesmeaue and M. de Brujin (MRC Molecular Haematology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, UK).

His–EXD2 (Lys76–Ser589) construct was generated by cloning of truncated human EXD2 (Lys76–Ser589) in the expression vector pNICE28-Bsa4 (ref. 49), containing an amino-terminal His tag followed by a tobacco etch virus protease cleavage site. The construct was subsequently subjected to site-directed mutagenesis to introduce the D108A–E110A mutations. Plasmids were transfected into human cells using Lipofectamine 2000 (Life Technologies), according to the manufacturer's instructions.

Immunoblotting. Cell extracts were prepared by lysing cells in urea buffer (9 M urea, 50 mM Tris-HCl, pH 7.3, 150 mM NaCl, 5% BSA in IP lysis buffer for 1 h at 4°C) before centrifugation at 16,000 g for 5 min. The resultant supernatant fraction was collected and pre-treated for 24 h with ultraviolet C light using a Stratagene UV Stratalinker 2400. Membrane filters were blocked in 10% dialysed FBS in PBS for 30 min on top of the coverslips and cells were exposed to 30 μM ultraviolet C light using a Stratagen UV Stratalinker 2400. Membrane filters were removed and media placed back on the cells, which were allowed to recover for the indicated times before fixation. Cells were fixed, permeabilized and blocked as previously indicated. Fixed cells were permeabilized and blocked as described above. Cells expressing GFP–Citrine were stained for GFP (using the GFP-Booster reagent, ChromoTek, 1:200) and visualized on a Zeiss LSM 510 laser scanning confocal microscope with Zen 2009 software using a 63 objective. Image analysis was carried out using Fiji (ImageJ) software.

Microirradiation experiments. Induction of localized DSBs in human cells was carried out as described previously44. Briefly, cells were grown on coverslips and pre-treated for 24 h with 10 μM BrdU before microirradiation. To induce localized DSBs, the medium was removed and cells were washed once in PBS, with subsequent removal of excess PBS. Isopore membrane filters (Millipore TMTFP2500, 0.5 μM pore size) were placed on top of the coverslips and cells were exposed to 30 μM ultraviolet C light using a Stratagen UV Stratalinker 2400. Membrane filters were removed and media placed back on the cells, which were allowed to recover for the indicated times before fixation. Cells were fixed, permeabilized and blocked as described above. Cells expressing GFP–Citrine were stained for GFP (using the GFP-Booster reagent, ChromoTek, 1:200) and H2AX using the indicated secondary antibody. Images of microirradiated cells were acquired using a DeltaVision DV Elite microscope using a ×40 objective. Image analysis was carried out with Fiji (ImageJ) and Huygens Professional (Scientific Volume Imaging) software. U2OS cells were stained for MRE11 and H2AX and visualized on a Zeiss LSM 510 confocal microscope using a ×40 objective. Image analysis was carried out using Fiji (ImageJ).

Immunoprecipitation experiments. Lysates for co-immunoprecipitation experiments were prepared as follows: cells were washed twice in PBS and then lysed in IP buffer (100 mM NaCl, 0.2% IGEPSAL CA-630, 1 mM MgCl₂, 10% glycerol, 5 mM NaF, 50 mM Tris–HCl, pH 7.5), supplemented with complete EDTA-free protease inhibitor cocktail (Roche) and 25 U ml⁻¹ Benzonase (Novagen). After Benzonase digestion, the NaCl and EDTA concentrations were adjusted to 200 mM and 2 mM, respectively, and lysates cleared by centrifugation (16,000 g for 5 min). Lysates were then incubated with 20 μl of GFP-Trap agrose beads (ChromoTek) blocked with 5% BSA in IP lysis buffer for 1 h at 4°C in the case of GFP-Trap IPs or with 20 μl of anti-Flag M2 affinity gel in the case of Flag IPs for 2 h with end-to-end mixing at 4°C. Complexes were washed extensively in IP buffer (including 200 mM NaCl and 2 mM EDTA) before elution. In the case of GFP-Trap IPs, beads were resuspended in ×2 SDS sample buffer and boiled for 5 min before centrifugation at 5,000 g for 5 min. The resultant supernatant fraction was retained as the eluate. In the case of Flag IPs, beads were incubated for 30 min with gentle agitation at 4°C in IP buffer supplemented with 400 μM ×3 Flag peptide (Sigma), followed by centrifugation at 5,000 g for 5 min. The resultant supernatant fraction was collected as the eluate.

For mass spectrometry analyses, eluates from IP experiments were analysed by the Mass Spectrometry Laboratory (BBS PAS) using the Thermo Orbitrap Velos system and protein hits were identified by Mascot.

Chromosomal aberrations. Cells were prepared for analyses of chromosomal aberrations as described previously46. Briefly, colcemid (0.1 μg ml⁻¹) was added 4 h before cell harvesting. Cells were trypsinized and incubated in 0.075 M KCl for 20 min. After fixing in methanol:acetic acid (3:1) for 30 min, cells were dropped onto slides and stained with Leishman’s solution for 2 min. Slides were then coded and scored blind to the experimenter.

Homologous recombination DR-GFP assay. 48 h after siRNA transfection, U2OS DR-GFP cells were co-transfected using Ammax nucleofection with an I-SceI expression vector (pCMV-I-SceI) and a vector expressing mCherry fluorescent protein (pmCherry-C1). 24 h after I-SceI transfection cells were collected and analysed by flow cytometry (CyAn ADP Analyzer, Beckman Coulter). The percentage of GFP-positive cells among transected cells (mCherry-positive cells) was determined using Summit 4.3 software. The control siRNA treated sample was set as 100%. Statistical significance was determined with Student’s t-test.

Recombinant protein purification. GST-tagged proteins were purified as described47 with some modifications. Briefly, GST protein expression was induced with 0.1 mM IPTG (isopropyl-β-D-thiogalactoside) (Sigma-Aldrich) at 16°C for 18 h. Bacteria were collected by centrifugation and resuspended in lysis buffer containing 50 mM Tris–HCl at pH 8.0, 150 mM NaCl, 2 mM EDTA, before mounting with VECTASHIELD mounting medium (Vector Laboratories) with DAPI.

To visualize ssDNA foci, the same protocol as for RPA focus staining was used, preceded by treatment of cells growing on coverslips with 10 μM BrdU for 24 h before fixation. To visualize RAD51 foci, cells grown on coverslips were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature followed by permeabilization with 0.5% Triton-X100 in PBS for 10 min at room temperature. Cells were then blocked, incubated with primary and secondary antibodies and mounted for analysis as described above. Confocal microscopy was carried out using a Zeiss LSM 510 laser scanning confocal microscope with Zen 2009 software using a ×63 objective. Image analysis was carried out with Fiji (ImageJ) software.

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1 mM dithiothreitol (DTT), 1% Triton-X100, and protease inhibitors. Lysates were sonicated and cleared by centrifugation. Supernatants were incubated with Glutathione HiCap Matrix (Qiagen) for 2 h with rotation at 4 °C. Beads were washed with lysis buffer containing increasing NaCl concentration, elution buffer (50 mM Tris-HCl at pH 7.0, 130 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.2% Triton-X100), resuspended in elution buffer supplemented with PreScission Protease (50 units ml⁻¹) (GE Healthcare) and incubated for 18 h with rotation at 4 °C. Eluates were dialysed to buffer containing 20 mM HEPES-KOH at pH 7.2, 100 mM NaCl, 1 mM DTT and 10% glycerol, aliquoted and stored at −80 °C.

His–EXD2 (Lys76–Ser589) protein and the corresponding D108A–E110A mutant protein were expressed in Escherichia coli BL21 (DE3)–R3–pRARE2 cells grown in TB medium and induced with 0.5 mM IPTG at 18 °C overnight. Cells were collected by centrifugation and resuspended in a lysis buffer containing 50 mM HEPES, pH 7.5, 500 mM NaCl, 10 mM imidazole, 5% glycerol, 1 mM tris(2-carboxyethyl)phosphine (TCEP), 0.5% Triton, supplemented with a protease inhibitor mixture (Roche Applied Science). The cells were sonicated, polyethyleneimine was added to 0.15% (w/v) from a 5% pH 7.5 stock solution, and lysates were cleared by centrifugation. The supernatant was applied to a Ni-Sepharose resin, washed with 50 mM HEPES, pH 7.5, 300 mM NaCl, 45 mM imidazole, 5% glycerol, 1 mM TCEP, 0.5% Triton, 1 mM phenylmethylsulphonyl fluoride (PMSF) and 2 mM benzamidine, and eluted in 50 mM HEPES, pH 7.5, 300 mM NaCl, 300 mM imidazole, 5% glycerol, 1 mM TCEP, 1 mM PMSF and 2 mM benzamidine. The eluate was further purified on two sequential Superdex 200 gel-filtration columns in GF buffer (50 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 1 mM TCEP, 0.1% Triton, 1 mM PMSF and 2 mM benzamidine). At each stage the presence of protein was confirmed on an InstantBlue-stained SDS–PAGE gel, and the identity of the final preparation was confirmed using electrospray ionization–time of flight mass spectrometry. Mass spectrometry indicated that both the WT and D108A–E110A mutant proteins had a lower mass than predicted.

Analysis using PAWS software (Genomic Solutions) suggests that the proteins were lacking amino acids 565–589 at the carboxy-terminus, probably due to proteolysis resulting in an EXD2 protein (WT or mutant) that consisted of amino acids Lys76–Val564. Recombinant human MRN was purified as previously described.

In vitro nuclease assay. Sequences of DNA oligonucleotides used are listed in Supplementary Table 3. To generate 3’ end labelled substrates, the indicated sDNA oligonucleotide was labelled using [α-32P]dATP and TdT enzyme (New England Biolabs). To generate 5’ end labelled substrates, the indicated sDNA oligonucleotide was labelled using [γ-32P]dATP and T4 polynucleotide kinase (New England Biolabs). To obtain dsDNA substrates, complementary sDNA oligonucleotides (as indicated in Supplementary Table 3) were mixed in an equimolar ratio and annealed by heating at 100 °C for 5 min followed by gradual cooling to room temperature. Where indicated DNA substrates with biotin label at the 5’ end were used and pre-incubated for 5 min at room temperature with a tenfold molar excess of streptavidin (Sigma).

Exonuclease assays were performed as described 69 with some modifications. Briefly, reactions were carried out in a buffer containing 20 mM HEPES-KOH, pH 7.5, 50 mM KCl, 0.5 mM DTT, 10 mM MgCl₂, 0.05% Triton-X, 0.1 mg ml⁻¹ BSA, 5% glycerol and 50 ng of EXD2 protein, initiated by adding the indicated amount of substrate and incubated at 37 °C for the indicated lengths of time. Reactions were stopped by addition of EDTA to a final concentration of 20 mM and 1/5 volume of formamide. The samples were resolved on denaturing 20% polyacrylamide TBE–urea gels. Gels were fixed, dried and visualized using a Typhoon FLA 9500 instrument (GE Healthcare).

Thin layer chromatography was performed as described 70. Briefly, exonuclease reactions were terminated by addition of stop solution (2% SDS, 120 mM EDTA) and 1 μl of reaction mixtures was spotted on polyethylenimine cellulose thin layer plates (Merck). Plates were developed in 1.0 M sodium formate at pH 3.4 and subsequently visualized using a Typhoon FLA 9500 instrument (GE Healthcare).

The ΦX174 circular single-stranded substrate (30 μM nucleotides) from New England Biolabs was incubated with MRN complex (50 nM) in the presence or absence of His–EXD2 (Lys76–Ser589) (350 nM) or corresponding mutant protein in buffer 2 (20 mM HEPES-KOH, pH 7.5, 50 mM NaCl, 0.5 mM DTT, 3 mM MnCl₂, 0.05% Triton-X, 0.1 mg ml⁻¹ BSA, 5% glycerol and 1 mM ATP. After 2 h the reaction was stopped by adding 1/5 volume of stop solution (2% SDS, 50 mM EDTA). Reactions were resolved on agarose gels, stained with SYBR Gold and visualized using a Typhoon FLA 9500 instrument (GE Healthcare).

ER-AsiSI resection assay. The level of resection adjacent to a specific DSB (Chr 1: 89231183) was measured as described 69 with some modifications. Briefly ER-AsiSI U2OS cells were treated with 300 nM of 4-OHT (Sigma) for 1 h to allow the AsiSI enzyme to enter the nucleus and induce DSBs. Cells were then collected and genomic DNA was extracted as previously described. 69 Genomic DNA was then digested with the BsrGI enzyme or mock digested and was used as a template for qPCR performed using Taq Universal SYBR Green Mix (Bio-Rad) and a Rotor-Gene (Corbett Research) qPCR system. Primers used are listed in Supplementary Table 4 (ref. 40). The percentage of ssDNA was calculated as previously described. All data were then related to the control siRNA treated sample, which was set to 100%. Statistical significance was determined with Student’s t-test.

Generation of EXD2−/− cells by CRISPR/Cas9. The following guide RNA sequences targeting the first exon of EXD2 were selected using the Optimized CRISPR Design tool (http://crispr.mit.edu; ref. 55): gRNA1: AAGGCCATGCAGGCGCGGA, gRNA2: CCACTACAGCCACACCGAGA. DNA oligonucleotides were purchased from IDT and cloned into px335-GFP vector 68 to generate targeting constructs that were subsequently co-transfected in an equimolar ratio into HeLa cells using Lipofectamine. 24 h after transfection, cells were sorted using a MoFlo cell sorter (Beckman Coulter) for cells expressing Cas9 nickase (GFP-positive cells) and left to recover for 6 days before sorting for single cells and allowing colonies to form. EXD2 expression was analysed by western blotting. Two clones showing loss of all detectable EXD2 were selected for subsequent analysis.

Chromatin fractionation. HeLa cells were treated with 500 μM phenol red for 1 h, washed with ice cold PBS and scraped into PBS, and the chromatin fractionation was performed as described 47. Briefly, cells were resuspended in buffer A (10 mM HEPES-KOH at pH 7.9, 10 mM KCl, 1.5 MgCl₂, 340 mM sucrose, 10% glycerol, 1 mM DTT, protease inhibitors) and Triton-X100 was added to final concentration 0.1%. After 5 min incubation on ice, nuclei were spun down at 1,300g for 4 min. Pelleted nuclei were washed with buffer A, resuspended in buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, protease inhibitors) and lysed for 20 min on ice before centrifugation at 1,700g for 5 min. The supernatant (nuclear soluble fraction) was saved, and the pellet (chromatin fraction) was washed with buffer B, resuspended in urea buffer (9 M urea, 50 mM Tris-HCl, pH 7.3) and sonicated.

Statistics and reproducibility. Microsoft Excel or Prism 6 software was used to perform statistical analyses. Detailed information (statistical tests used, number of independent experiments, P-values) are listed in individual figure legends. All experiments were repeated at least twice unless stated otherwise.

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Supplementary Figure 1 Cartoon illustrating the phylogenetic tree for the EXD2 gene.
**Supplementary Figure 2** Additional characterisation of resection defect in EXD2-depleted cells. a and b) Western blotting confirming depletion of endogenous EXD2 72h post-transfection with either control siRNA (siControl) or siRNAs targeting EXD2 (siEXD2-1 and 2). α-Tubulin acts as a loading control. These experiments were carried out three times independently. RPA foci in U2OS cells 72h post-transfection with control siRNA (siControl) or an siRNA oligo targeting EXD2 (siEXD2). Cells were either untreated or were exposed to 8 Gy IR and left to recover for 1h prior to fixation and then stained for RPA and DAPI as indicated. Scale bar = 20μm. Quantification of the percentage of cells treated as in (c), exhibiting greater than 15 RPA foci per nucleus. n=300 cells (siControl untreated), 390 cells (siEXD2 untreated), 355 cells (siControl 8Gy IR) and 403 cells (siEXD2 8gy IR) respectively, pooled from three independent experiments. Bars represent mean values +/- SEM. The Chi-square test was used to determine statistical significance. Western blotting of various DDR proteins in U2OS cells 72h post-transfection with control siRNA (siControl) or an siRNA oligo targeting EXD2 (siEXD2) with cells treated with 8 Gy IR. Samples were acquired at the indicated time points post-IR treatment. Chk2-p T68 acts as a control for ATM activation, RPA2 pS4/S8 acts to indicate resection efficiency, γH2AX serves to indicate DSB induction with RPA and histone H3 serving as loading controls. This experiment was carried out two times independently. Quantification of the percentage of G1, S or G2/M U2OS cells as analysed by propidium iodide staining and FACS analysis. Cells were analysed 72h post-transfection with control siRNA (siControl) or siRNA targeting EXD2 (siEXD2) (mean values +/- SEM, n= 5 independent experiments).
Supplementary Figure 3 Additional characterisation of purified EXD2.

Alignment of the partial amino acid sequences of EXD2 proteins from various vertebrate organisms (human, mouse, chicken and xenopus are depicted) with a partial amino acid sequence of the exonuclease domain of human Werner helicase protein (WRN). Highly conserved residues shown to mediate the exonuclease activity of WRN protein which are also conserved in human EXD2 and its vertebrate homologues are indicated with red arrows. Coomassie stained SDS-PAGE gel of EXD2 WT or D108A E110A mutant protein ectopically expressed in E. coli and purified to homogeneity for use in in vitro biochemistry experiments. InstantBlue stained SDS-PAGE gel of the truncated EXD2 proteins (WT and D108A E110A mutant) used in this study. Mass spectrum of truncated EXD2 (K76 - V564) WT protein confirming sample purity. Mass spectrum of truncated EXD2 (K76 - V564) D108A E110A protein confirming sample purity. f) 5' radiolabeled ssDNA or dsDNA substrate (10 nM molecules) was incubated for indicated amounts of time with EXD2 WT (K76 - V564) or EXD2 (K76 - V564) D108A E110A (EXD2 mut) protein (100 nM). Samples were resolved on a 20% TBE-Urea polyacrylamide gel and visualised by phosphorimaging. This experiment was carried out two times independently.
Supplementary Figure 4 Western blots confirming EXD2 knockdown efficiency and additional characterisation of EXD2 in vivo and in vitro. Western blotting determining the relative levels of expression of FLAG-HA-EXD2 WT or D108A E110A mutant proteins in U2OS cells stably expressing these fusion proteins. Two independent clones for each construct are shown. Cells were transfected with control siRNA (siControl) or siRNA targeting endogenous EXD2 (siEXD2 3’UTR) as indicated and harvested for western blotting. MCM2 serves as a loading control. This experiment was carried out three times independently. Quantification of the frequency of RAD51 focus-positive U2OS cells 72h post-transfection with the indicated siRNA. Cells were either untreated or exposed to 8 Gy IR and left to recover for 6h prior to fixation. Cells were stained with DAPI and RAD51 as indicated. The percentage of cells exhibiting RAD51 foci was quantified. n=366 cells (siControl 0 min), 386 cells (siEXD2 0 min), 337 cells (siMRE11 0 min), 315 cells (siEXD2/siMRE11, 0 min), n=308 cells (siControl 360 min), 321 cells (siEXD2 360 min), 337 cells (siMRE11 360 min), 374 cells (siEXD2/siMRE11, 346 min), respectively, pooled from three independent experiments. Bars represent mean values +/- SEM. The Chi-square test was used to determine statistical significance. Western blotting confirming the depletion of EXD2 and MRE11 in U2OS cells 72h post-transfection with control siRNA (siControl) or siRNA targeting EXD2 or MRE11 as indicated. MCM2 serves as a loading control. This experiment was carried out three times independently. d and e) 5’ radiolabeled ssDNA or dsDNA 50-mer substrates (1 nM molecules) (d) or 5’ radiolabeled dsDNA substrates (1 nM molecules) containing a nick or 1 nucleotide gap (e) were incubated for the indicated amounts of time with EXD2 WT protein (70 nM). Samples were resolved on a 20% TBE-Urea polyacrylamide gel and visualised by phosphorimaging. These experiments were carried out once. f and g) Western blotting confirming the depletion of EXD2 and MRE11 in ER-AsiSI U2OS cells (f) and DR-GFP U2OS cells (g) 72h post-transfection with control siRNA (siControl) or siRNA targeting EXD2 or MRE11 as indicated. MCM2 serves as a loading control. These experiments were carried out three times independently.
Supplementary Figure 5 Generation and characterisation of EXD2 knockout cell lines. EXD2 knockout generation strategy using the CRISPR-Cas9 nickase. Schematic representation of the human EXD2 genomic locus with guide RNAs sequences highlighted in green and predicted cut sites marked by red arrows. Representative images of RPA foci in HeLa control cells and in two independent clones of HeLa EXD2−/− cells treated with 1 µM CPT for 1h. Scale bar = 5 µm. Quantification of the percentage of HeLa or HeLa EXD2−/− cells treated as in b. exhibiting greater than 15 RPA foci per nucleus. Data from two independent HeLa EXD2−/− clones are represented. n = 617 cells (HeLa), 433 cells (HeLa EXD2−/− cl.1) and 429 (HeLa EXD2−/− cl.2) respectively, pooled from three independent experiments. Bars represent mean values +/- SEM. The Chi-square test was used to determine statistical significance. Western blot of HeLa EXD2−/− cl.1 and parental cells treated with 1 µM CPT for 1h. RPA2 pS4/S8 acts as an indicator of resection, MCM2 acts as a loading control. This experiment was carried out two times independently. Survival of HeLa control cells and HeLa EXD2−/− cells treated with the indicated doses of CPT. Survival data from two independent EXD2−/− clones is depicted. Survival data represent mean +/- SEM, (n= 3 independent experiments). Western blot of HeLa EXD2−/− cl.1 and parental cells probed with antibodies against MRE11 and CtIP. α-Tubulin acts as a loading control. This experiment was carried out three times independently.
Supplementary Figure 6  EXD2 is not required for CtIP or MRE11 recruitment to site of DNA damage. Immunofluorescence microscopy of U2OS cells stably expressing GFP-CtIP treated with control siRNA (siControl) or siRNA targeting EXD2 (siEXD2) following the induction of localized DSBs by microirradiation. DAPI serves as a marker for the cell nucleus and γH2AX serves as a marker for DSB induction. Scale bar = 20μm. This experiment was carried out three times independently. Western blotting confirming EXD2 knock down efficacy in samples from (a). α-Tubulin serves as a loading control. This experiment was carried out three times independently. Immunofluorescence microscopy of WT HeLa and HeLa EXD2−/− clones stained for MRE11 using an antibody recognising the endogenous protein following the induction of localized DSBs by microirradiation. γH2AX serves as a marker for DSB induction. Scale bar = 20μm. This experiment was carried out three times independently.
Supplementary Figure 7 Original uncropped images of western blots.
| Antibodies for Western blotting | Company | Accession Number/Clone ID | Dilution | Gifted by |
|--------------------------------|---------|---------------------------|----------|-----------|
| α-Tubulin                      | Sigma    | B-5-1-2; T5168            | 1 in 100000 | Dr. G. Stewart |
| γH2AX                          | Millipore| JBW301; 05-636             | 1 in 2000 | Dr. F. Esashi |
| BRCA1                          | n/a      | n/a                       | 1 in 1000 |           |
| CHK2 p-T68                     | Cell Signalling| n/a             | 1 in 500 |           |
| CtIP                           | n/a      | n/a                       | 1 in 2000 |           |
| EXD2                           | Sigma    | HPA005848                 | 1 in 1000 |           |
| GFP                            | Roche    | 118144460001              | 1 in 1000 |           |
| Histone H3                     | Abcam    | ab4461                    | 1 in 100000 |           |
| MCM2                           | Abcam    | ab214                     | 1 in 1000 |           |
| NBS1                           | BD Biosciences| 34/NBS1; 611871       | 1 in 1000 |           |
| RPA2                           | Calbiochem| Ab-2; NA18               | 1 in 1000 |           |
| RPA2 pS4/S8                    | Bethyl   | A-300 245A                | 1 in 1000 |           |

| Antibodies for Immunofluorescence | Company | Accession Number/Clone ID | Dilution | Gifted by |
|----------------------------------|---------|---------------------------|----------|-----------|
| γH2AX                            | Millipore| 07-627                    | 1 in 200 |           |
| γH2AX                            | Millipore| JBW301; 05-636             | 1 in 500 |           |
| BrdU                             | GE Healthcare Life Sciences| BU-1; RPN202          | 1 in 200 |           |
| MRE11                            | Abcam    | ab214                     | 1 in 200 |           |
| RAD51                            | Santa Cruz| H-92                    | 1 in 100 |           |
| RPA2                             | Calbiochem| Ab-2; NA18               | 1 in 200 |           |

Supplementary Table 1. Antibodies.
| Name             | Sequence or catalog number                                                                 |
|------------------|-------------------------------------------------------------------------------------------|
| siEXD2-1         | L-020899-02-0005, Dhharmacon (ON-TARGETplus EXD2 siRNA)                                    |
| siEXD2-2         | CAGAGGACCAGGUAUUUA                                                                        |
| siEXD2 (3’ UTR)  | GAACAGAGUCUUUUUUUU                                                                        |
| siMRE11-1        | L-009271-00-0005, Dhharmacon (ON-TARGETplus MRE11A siRNA)                                 |
| siMRE11-2        | GGAGGUCUCGCUUUCAGAACUUGGUCACUAAAG                                                        |
| siControl        | D-00180-10-20, Dhharmacon (ON-TARGETplus Non-targeting Pool)                               |
| siLuciferase     | CGTACGCAGTACTTCGAG                                                                       |

Supplementary Table 2. siRNA sequences.
| Name    | Sequence                                                                 | Substrate (indicates labeled strand)                                                                 |
|---------|--------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------|
| BS40    | ATAAATTTTTTAATTTAAAAATAGATCACCTTTCTTTCTCTCCCTTT                      | ssDNA*; dsDNA*; 5'overhang*                                                                            |
| UA1     | AAGGGGAAGAGAGAGAGAGAGAGAGAGATCTATTATTAATAAAAAATATTAT                          | dsDNA; dsDNA nick; dsDNA 1 nt gap                                                                  |
| BS401   | ATAAATTTTTTAAAAATAGATCACC                                                | dsDNA nick *; dsDNA 1 nt gap*                                                                         |
| BS402   | TTCTTTCTTTCTTTCTCCCTT                                                   | dsDNA nick                                                                                           |
| BS403   | TTCTTTCTTTCTTTCTCCCTT                                                   | dsDNA 1nt gap                                                                                         |
| UA1_10  | AAGGGAGAAGAGAGAGAGAGAGAGAGAGATCTATTATTAATAAAAAAATTTTTATAGCTTGGA            | 5'overhang                                                                                           |

Supplementary Table 3. DNA oligonucleotides used in *in vitro* nuclease assays.
| Name          | Sequence                                      |
|---------------|-----------------------------------------------|
| DSB-335 FW    | GAATCGGATGTATGCGACTGATC                       |
| DSB-335 REV   | TTCCAAAGTTATTCCAACCCGAT                      |
| DSB-1618 FW   | TGAGGAGGTGACATTAGAACTCAGA                    |
| DSB-1618 REV  | AGGACTCACTTACACGGCCTTT                      |
| NoDSB FW      | ATTGGGTATCTCGTCTAGTGAGG                      |
| NoDSB Rev     | GACTCAATTACATCCCTGCAGCT                    |

Supplementary Table 4. qPCR primer sequences used in ER-AsiSI resection assay.