Biochemical characterization of a cancer-associated E109K missense variant of human exonuclease 1

Stephanie Bregenhorn and Josef Jiricny[*]

Institute of Molecular Cancer Research of the University of Zurich and the ETH Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

ABSTRACT

Mutations in the mismatch repair (MMR) genes *MSH2*, *MSH6*, *MLH1* and *PMS2* are associated with Lynch Syndrome (LS), a familial predisposition to early-onset cancer of the colon and other organs. Because not all LS families carry mutations in these four genes, the search for cancer-associated mutations was extended to genes encoding other members of the mismatch repairosome. This effort identified mutations in *EXO1*, which encodes the sole exonuclease implicated in MMR. One of these mutations, E109K, was reported to abrogate the catalytic activity of the enzyme, yet, in the crystal structure of the EXO1/DNA complex, this glutamate is far away from both DNA and the catalytic site of the enzyme. In an attempt to elucidate the reason underlying the putative loss of function of this variant, we expressed it in *Escherichia coli*, and tested its activity in a series of biochemical assays. We now report that, contrary to earlier reports, and unlike the catalytic site mutant D173A, the EXO1 E109K variant resembled the wild-type (wt) enzyme on all tested substrates. In the light of our findings, we attempt here to reinterpret the results of the phenotypic characterization of a knock-in mouse carrying the E109K mutation and cells derived from it.

INTRODUCTION

Postreplicative mismatch repair (MMR) improves replication fidelity by two to three orders of magnitude and is thus one of the key guardians of genomic stability in replicating cells (1,2). The clinical importance of MMR is clearly apparent from its association with Lynch Syndrome (LS, also known as hereditary non-polyposis colon cancer, HNPCC), in which inheritance of mutated alleles of *MMR* genes predisposes to cancer of the colon, endometrium and other organs (3). The majority of *MMR* gene mutations affect the *MLH1* and *MSH2* loci, but families carrying mutations in *MSH6* and *PMS2* have also been identified. However, as these fail to account for all known LS kindreds, attention was focused on other genes that encode MMR proteins, and this effort culminated in the identification of mutations in *EXO1* (4). This gene encodes a member of the evolutionarily highly conserved RAD2 family of structure-specific nucleases, first discovered in *Schizosaccharomyces pombe*, where it was shown to play a role in meiotic recombination (5) and later also in MMR (6). Since then, EXO1 has been the subject of numerous genetic and biochemical studies, predominantly in *Saccharomyces cerevisiae*, but also in mouse and human cells. These findings implicated the enzyme in several additional processes of DNA metabolism, including replication, mitotic recombination, double strand break repair, antibody diversification and telomere maintenance [for reviews, see (7,8)].

Yeast two-hybrid assays and biochemical pull-down experiments showed that EXO1 can interact with MLH1 and MSH2 (9–13), as well as with Proliferating Cell Nuclear Antigen (PCNA) (14,15), all major players in MMR. Implication of the *S. cerevisiae* enzyme in MMR *in vivo* proved to be more challenging, given that the mutator phenotype of EXO1-deficient cells was substantially weaker than that of *MSH2*- or *MLH1*-disrupted strains. The nuclease activity of the enzyme was shown to be required for suppression of the conditional viability of a MSH2-deficient *S. cerevisiae* strain expressing a proofreading-deficient polymerase-δ (16), but it was only through the use of hypomorphic *S. cerevisiae MMR* alleles that EXO1 could be shown to be epistatic with other *MMR* genes (13,17,18). Subsequent studies indicated that full functionality of EXO1 in MMR requires both the nuclease and the MLH1-interacting domains (12,19), and data obtained with the human system reconstituted from purified recombinant proteins, which demonstrated that the activity of EXO1 during MMR is controlled by interaction with the MSH2/MSH6 (MutSα) and MLH1/PMS2 (MutLα) heterodimers (20,21), provided additional evidence suggesting that the protein plays a catalytic as well as a structural role in the processing of biosynthetic errors.

The modest mutability associated with EXO1 deficiency in both yeast (12,22) and mice (23), explained by the ex-

[*]To whom correspondence should be addressed. Tel: +41 44 635 3450; Fax: +41 44 635 3484; Email: jiricny@imcr.uzh.ch

istence of redundant nucleases, as well as by the existence of an EXO1-independent, but MLH1/PMS2- and polymerase-δ-dependent strand displacement mechanism of mismatch correction (24), led to the expectation that mutations in *EXO1* in LS families are likely to be rare and linked to late cancer onset and low penetrance. Germline mutations in *EXO1* were indeed identified, but their link to cancer was less than convincing. Thus, in the first study, the tumours were reported to have lost preferentially the mutant- rather than the wild-type allele, and some displayed microsatellite instability (MSI) at both mono- and dinucleotide markers, a phenotype characteristic of full MMR defect, which differs from findings in EXO1-deficient yeast (12,22) and mice (23), where only a subset of mononucleotide runs was unstable. A follow-up study reported that the identified mutations often affected non-conserved residues and were observed with similar frequencies also in controls (25). Most importantly, two families affected with multiple cutaneous and uterine leiomyomatosis, carrying germline deletions on chromosome 1 that included *EXO1*, displayed no predisposition to colon cancer and their tumours displayed no MSI (26). Surprisingly, although the above findings suggested that *EXO1* mutations are not cancer predisposing, biochemical defects were described for eight of the reported EXO1 variants (27).

We became interested in the E109K mutant, which was found to be enzymatically inactive, even though the amino acid change did not affect the catalytic site. Moreover, recently described knock-in mice harbouring the EXO1 E109K mutation and cells derived from these animals (28) displayed phenotypic traits [e.g. wild-type-like mutation rates and frequency of somatic hypermutation and class switch recombination, yet elevated resistance to the S_N1 methylating agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG)] that were incompatible with our current understanding of the role of this enzyme in DNA metabolism. We, therefore, expressed the EXO1 E109K variant in *Escherichia coli* and studied its biochemical properties in an attempt to learn how this mutation affects its enzymology.

MATERIALS AND METHODS

EXO1 expression and purification

The pTXB1 vector (IMPACT™ Kit, NEB) encoding the wild-type *EXO1* cDNA and the D173A mutant, both with a C-terminal intein tag, were kindly provided by Stefano Ferrari. The E109K mutation was introduced using the following primers: 5′-CTTAAGGGAAAG CAACTTCTTCGTAAGGGGAAAGTCTCGG-3′ and 5′-CCGAGACTTTCCCCTTACGAAGAAGTTGCT TTCCCTTAAG-3′. The template was incubated in the presence of these primers (30 μM) in 1× Phusion buffer, 0.8 mM deoxynucleoside triphosphates (dNTPs) and 2 U Phusion polymerase (NEB), subjected to polymerase chain reaction (PCR) amplification (95°C, 2 min, then 35 cycles of 95°C, 30 s; 55°C, 1 min, 72°C, 8 min; followed by a final elongation step at 72°C, 10 min) and the product was digested with 40 U DpnI (NEB) for 1 h, 37°C.

The plasmids were transformed into the *E. coli* strain BL21 and grown to an OD_{600} of 0.3 in LB medium supplemented with chloramphenicol (25 μg/ml) and ampicillin (100 μg/ml). 0.2 mM isopropylthiogalactoside (IPTG) was added to induce protein expression overnight at 18°C with shaking at 250 rpm. Subsequently, the bacteria were harvested by centrifugation (4000 rpm, 10 min, 4°C, Sorvall SLA-3000 rotor), washed with 1× phosphate buffered saline (PBS) and snap-frozen. Bacterial pellets were suspended in CH buffer (20 mM Tris pH 8.0, 500 mM NaCl, 0.1% Triton X100, 1 mM EDTA (ethylenediaminetetraacetic acid), 10% glycerol) and sonicated prior to loading on chitin beads. The beads were then washed with CH buffer and the proteolytic cleavage of the intein tag was induced by incubation with CH buffer supplemented with 30 mM dithiothreitol (DTT) overnight at 4°C. The slurry was spun down (3220 × g, 10 min, 4°C) and the supernatant was clarified (20 000 × g, Eppendorf 5810R centrifuge, 45 min, 4°C) and aliquotted in liquid nitrogen.

Non-specific endonuclease assay

Forty-eight femtomoles of a supercoiled plasmid DNA substrate were incubated with 10 nM EXO1. The reaction was carried out in a total volume of 20 μl in 20 mM Tris·HCl pH 7.6, 110 mM KCl, 5 mM MgCl$_2$, 1 mM glutathione and 75 μg/ml BSA (bovine serum albumin), for 30 min at 37°C, followed by heat inactivation (5 min, 80°C). The nicking reaction was carried out with 48 fmol of the substrate and 5 U Nt·BstNBI (NEB) in 100 mM NaCl, 50 mM Tris·HCl, 10 mM MgCl$_2$ and 1 mM DTT for 30 min at 37°C. Fortyeight femtomoles of the substrate were incubated in 40 mM Tris·HCl, 10 mM NaCl, 6 mM MgCl$_2$, 1 mM CaCl$_2$ with 3 U DNaseI (Roche) for 30 min at 37°C. The reaction products were separated on a 1% agarose gel eluted with Tris acetate EDTA (TAE) buffer and stained with GelRed. The image was quantified with ImageQuantTL, and expressed as a ratio of nicked versus total DNA.

Knock-down of EXO1

HEK293 cells were transfected with siRNA-EXO1 (5′-CAAGCCUAUUCUCGUAUUUTT-3′; Microsynth) (7) at 60% confluency, using a standard calcium phosphate precipitation protocol. The cells were harvested after 72 h, and nuclear extracts were prepared as described in (29).

Mismatch repair assays

The *in vitro* MMR assays were carried out as described previously (29). Briefly, the heteroduplex phagemid DNA substrate (48 fmol) containing a T/G mismatch in its unique SalI site and a single nick generated by Nt·BstNBI 361 nucleotides 5′ from the mispaired T was incubated with 100 μg of nuclear extracts of HEK293 cells pretreated or not with EXO1 siRNA and supplemented with 40 nM Exo1 wt, E109K or D173A in 20 mM Tris·HCl pH 7.6, 110 mM KCl, 5 mM MgCl$_2$, 1 mM glutathione, 1.5 mM ATP, 50 μg/ml BSA and 100 μM dNTPs for 30 min in a total volume of 25 μl. The reactions were terminated by a 30-min incubation with a stop solution (final concentrations: 0.5 mM EDTA, 1.5% SDS (sodium dodecyl sulfate), 2.5 mg/ml proteinase K), cleaned up on a MinElute column (Qiagen), and the recovered phagemid was subjected to restriction digest with

6 U SalI and 20 U DraI (NEB). RNase A (40 ng, Sigma-Aldrich) was then added and, following an overnight incubation at 37°C, the reaction products were separated on a 1% agarose gel eluted with TAE buffer and stained with GelRed.

Exonuclease assays

To measure enzyme processivity, supercoiled homoduplex C/G DNA substrate (48 fmol) generated by primer extension on single-stranded phagemid template and purified on a CsCl gradient (29) was nicked with Nt·BstNBI (NEB) and incubated with the indicated amounts of EXO1 or its variants in a total volume of 20 µl in 20 mM Tris·HCl pH 7.6, 110 mM KCl, 5 mM $MgCl_2$, 1 mM glutathione and 75 µg/ml BSA, for the indicated times at 30°C, followed by heat inactivation (5 min, 80°C). The reaction products were separated on 1% agarose gels eluted with TAE buffer and visualized with GelRed. The 5′ exonuclease activity was measured using 100 fmol of a 5′-^{32}P labelled 5′ recessed oligonucleotide 5′-GAGATATTCCTGGTCAGC GTGACCGGAGCTGAAAG-3′ annealed with a 2-fold excess of the unlabelled oligonucleotide 5′-CTTTCAGCTC CGGTCACGCTGACCAGGAATATCTCTCTA-3′ (Microsynth) in a 10 µl total volume under the above conditions. The products were separated on 20% denaturing polyacrylamide gels, which were fixed, dried and exposed to PhosphoImager screens. The images were quantified with ImageJ (Fig. 4AB) or ImageQuantTL (Fig. 4C), whereby the degraded DNA was plotted against the total amount of DNA.

Endonuclease assays

To test the endonucleolytic activity of EXO1, we used a splayed-arm substrate generated by annealing the 5′ ^{32}P-labelled oligonucleotide 5′-TCAAAGTCACGACCTAGA CACTGCGAGCTCGAATTCACTGGAGTGACCTC-3′ with a 2-fold excess of the unlabelled oligonucleotide 5′-GAGGTCACTCCAGTGAATTCGAGCTCGCAG CAATGAGCACATACCTAGT-3′ (Microsynth) in 1× NEB buffer 2. The substrate (500 fmol) was incubated with 5 nM EXO1 variants in a total volume of 50 µl in 20 mM Tris·HCl pH 7.6, 110 mM KCl, 5 mM $MgCl_2$, 1 mM glutathione and 75 µg/ml BSA at 30°C and samples were taken at the indicated time points. The reaction products were isolated by ethanol precipitation, separated on 20% denaturing polyacrylamide gels, analyzed by exposing the dried gels to PhosphoImager screens and quantified with ImageQuantTL.

Far-western analyses

This experiment was carried out as described (30) without modification, using recombinant MutSα (31) or CtIP (32). The anti-Exo1 antibody was purchased from Neomarkers (Ab-4), the anti-FLAG antibody was from Sigma (Anti-FLAG M2 monoclonal antibody).

RESULTS

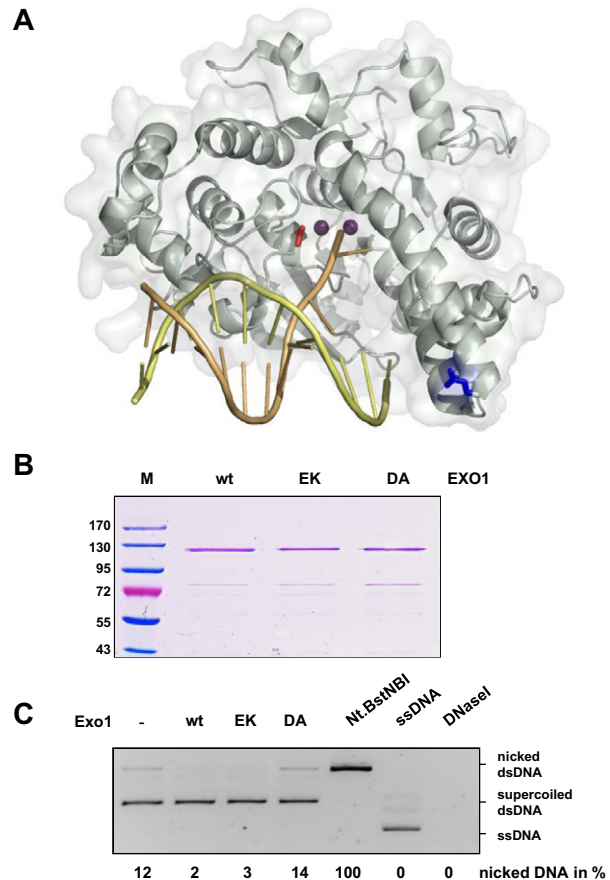Exonuclease 1 belongs to the RAD2 family of structure-specific endonucleases (33), the N-terminal and internal nu-

Figure 1. Structure, expression and purification of wild-type EXO1 and its E109K and D173A variants. (**A**) Structure of human EXO1 bound to DNA [3QEB.pdb; (36)]. Catalytic metal ions are depicted as purple spheres, the catalytic aspartate 173 is indicated in red and glutamate 109 in blue. (**B**) Recombinant EXO1 wild-type (wt), E109K (EK) and D173A (DA) variant proteins used in this study. The figure is a scan of a 4–15% SDS-polyacrylamide gel stained with Coomassie blue. M, pre-stained molecular size marker. (**C**) EXO1 wild-type and its E109K and D173A variants are devoid of non-specific endonucleolytic activity, as revealed by their inability to convert supercoiled phagemid DNA into a nicked, open circular form upon 30 min incubation with 10 nM variants. As a control, the plasmid was digested with NtBstNBI, a nickase with a single recognition site in this vector, or with DNaseI. Single-stranded phagemid DNA was included as a control.

clease domains of which are highly conserved from bacteriophage to man (33,34). EXO1 possesses 5′→3′ exonuclease and flap endonuclease activities (35) that share the same active site, composed of two Mg^{2+} atoms coordinated by five aspartate residues. In human EXO1, these are D30, D152, D171, D173 and D225 (36). Glutamate 109 is not part of the active site; it resides in a flexible loop between α-helices α4 and α5 (Figure 1A) that form, together with the β-sheet β3, a mobile microdomain, which was postulated to mediate protein/protein interactions (36). As the E109K mutation replaces a negatively charged residue with a positively charged one, we wanted to test whether it perturbs intermolecular protein/protein interactions between EXO1 and its partners during MMR.
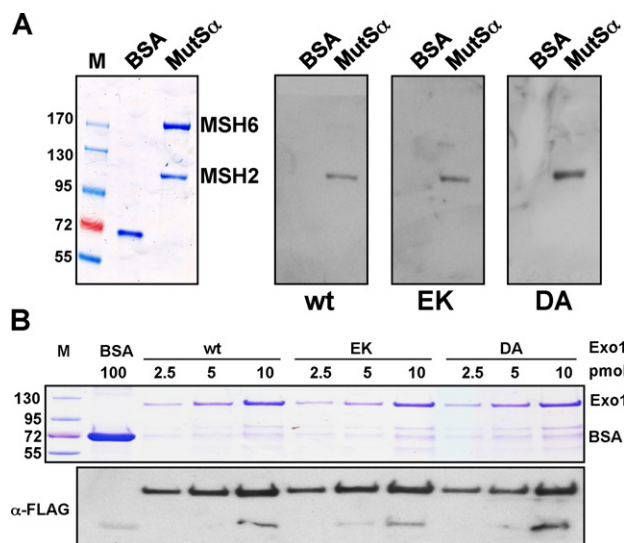
Figure 2. Wild-type EXO1 and its E109K and D173A variants interact directly with MSH2 and CtIP. (**A**) Far-western blot showing a direct interaction between EXO1 and MSH2. The MSH2/MSH6 (MutSα) heterodimer was separated by SDS-PAGE (left panel) and the MSH6 and MSH2 subunits were transferred onto a membrane, which was subsequently incubated with the recombinant EXO1 variants (right panels). Hybridization with an anti-EXO1 antibody revealed specific interactions of the variants with the MSH2 subunit of MutSα. BSA was run on the SDS-PAGE gel and used as a negative control for interaction with EXO1. M, pre-stained molecular size marker. (**B**) Far-western blot showing a direct interaction between EXO1 and CtIP. EXO1 wild-type and mutants were separated by SDS-PAGE (Coomassie staining top panel), transferred onto a membrane and incubated with recombinant FLAG-tagged CtIP. The interaction was revealed upon hybridization of the membrane with anti-FLAG to detect CtIP (bottom panel). M, pre-stained molecular size marker.

Using site-directed mutagenesis (see 'Materials and Methods' section), we modified the bacterial expression plasmid pTXB1 encoding the C-terminally intein-tagged EXO1 to generate vectors encoding the variants E109K (Supplementary Figure S1A) and D173A (Supplementary Figure S1B), the latter of which was reported to be nuclease-dead (18). We then expressed the tagged wild-type protein and the two mutants in *E. coli* BL21 and isolated the recombinant polypeptides by chitin bead affinity chromatography, during which the intein tag was cleaved off with DTT. As shown in Figure 1B, the proteins were >85% homogeneous. The preparations were free of contaminating unspecific endonucleases, as shown by the lack of nicking of supercoiled phagemid DNA (Figure 1C).

We then asked whether the EXO1 variants interacted with the MSH2 subunit of the mismatch recognition factor MutSα as reported previously (6,9,10,22). We separated the MSH6 and MSH2 subunits of purified recombinant MutSα on a denaturing polyacrylamide gel, electrotransferred the polypeptides onto a nitrocellulose membrane, denatured them in 6 M guanidinium hydrochloride, allowed them to renature and then incubated them with the recombinant EXO1 variants. Following extensive washing, we visualized the bound EXO1 variants with an anti-EXO1 antibody. BSA was used as the negative control. As shown in Figure 2A, all three variants interacted efficiently with MSH2 on these far-western blots.

EXO1 was also described to interact with CtIP, a polypeptide involved in the resection of DNA double-strand breaks (DSBs) (37). As mouse embryonic fibroblasts expressing the E109K mutant were reported to be hypersensitive to camptothecin (28), which induces DSBs during replication, we wanted to test whether the interaction of this variant with CtIP might be perturbed. However, as shown by far-western blotting (Figure 2B), all three EXO1 variants behaved similarly in this assay. Both interactions were highly selective, given that EXO1 failed to bind to BSA even when used in large excess. Moreover, the fact that all variants interacted to similar extents with their target proteins even after denaturation and renaturation indicated that the mutations did not alter the stability of the polypeptides.

Rather than examine further individual interactions between EXO1 and purified MMR proteins MLH1 and PCNA, which EXO1 has also been reported to bind (11,13,15,19), we decided to test the functionality of the EXO1 variants in an *in vitro* MMR assay, in which a phagemid heteroduplex substrate carrying a single T/G mismatch and a strand discrimination signal (a single nick generated by Nt.BstNBI) 361 nucleotides 5′ from the mispaired T was incubated with extracts of human cells (29). In this assay, the mismatch makes the phagemid refractory to cleavage with SalI, but correction of the mismatch to C/G through EXO1-mediated degradation of the nicked T-strand and repair synthesis restores the restriction site (29). SalI/DraI digestion of the repaired phagemid recovered from the extract thus gives rise to four fragments of 1324, 1160, 694 and 19 bp, whereas the uncorrected phagemid is cleaved only by DraI into fragments of 2484, 694 and 19 bp (Figure 3A).

When the nicked T/G phagemid was incubated with HEK293 nuclear extracts (Figure 3B, lane 1), ∼60% of the substrate were corrected to C/G as indicated by the relative intensity of the 1324 and 1160 bp bands. In contrast, incubation of the phagemid heteroduplex with extracts of HEK293 cells treated with EXO1 siRNA (see 'Materials and Methods' section) yielded only background levels of SalI-cleavable substrate (lane 2), similarly to the extract supplemented with the recombinant EXO1 D173A mutant (lane 5). Supplementation of the extract with recombinant wild-type enzyme resulted in full restoration of MMR activity (lane 3), as did complementation with the EXO1 E109K variant (lane 4). This result was anticipated from the data obtained with the *Exo1* E109K knock-in mouse, which was deemed to have no MMR defect based on a phenotypic comparison with the wild-type- and the knock-out animals (28).

In the latter work, the E109K variant was assumed to have no nuclease activity and it was therefore proposed that EXO1 played a structural rather than a catalytic role in MMR. This hypothesis was, however, incompatible with the currently accepted mechanism of eukaryotic MMR (1,2), which posits that mismatch-stimulated MutSα/MutLα recruits and activates EXO1 to degrade the nicked strand up to and past the mispaired nucleotide, generating thus a long tract of single-stranded DNA that is subsequently filled-in by DNA polymerase-δ (38). We, therefore, wished to verify that the EXO1 E109K variant indeed lacked nuclease activity as reported by others (27,28). To this end, we incubated
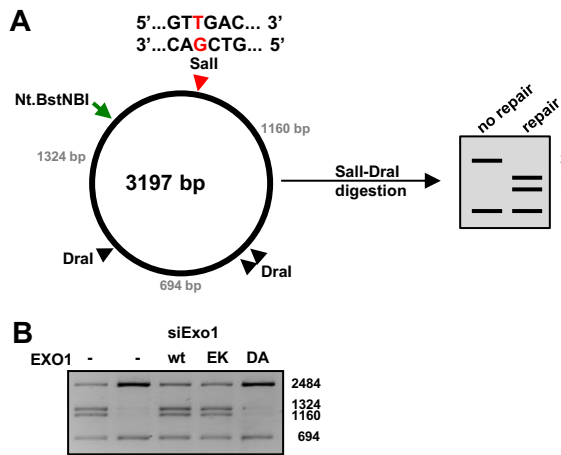
Figure 3. Wild-type EXO1 and its E109K variant complement the MMR defect of EXO1-depleted extracts of HEK293 cells. (**A**) Schematic representation of the T/G MMR substrate. The SalI restriction site contains a T/G mismatch, which renders the site refractory to cleavage. Repair of the T/G mismatch to C/G restores a *bona fide* SalI site. The SalI- and the three DraI restriction sites are indicated. The restriction patterns seen upon agarose gel electrophoresis before and after repair are shown on the right. The Nt.BstNBI nicking site is located 316 nucleotides 5′ from the mispaired T. (**B**) Mismatch repair assay using HEK293 siEXO1 extracts supplemented with recombinant EXO1, either wild-type, or the E109K or D173A variants. The reactions were stopped after 30 min and the recovered substrates were digested with SalI/DraI. In the absence of repair, the substrate gives rise to fragments of 2484, 694 and 19 bp, while the repaired substrate generates fragments of 1324, 1160, 694 and 19 bp. The figure shows a scan of a 1% agarose gel stained with GelRed. The image is representative of three independent experiments.

the nicked phagemid substrate with the three recombinant enzymes. No degradation was detectable upon incubation of the substrate with the D173A variant, but addition of increasing amounts of wild-type EXO1 or its E109K variant resulted in substantial degradation of the nicked strands, both enzymes yielding the single-stranded phagemid, which is indicative of substantial processivity (Figure 4A). Similar results were obtained in a time-course study at a constant enzyme concentration (Figure 4B), as well as under varying pH and salt concentrations (Supplementary Figure S2). In all these experiments, the two enzymes behaved comparably, even though the E109K mutant appeared to be reproducibly slightly less efficient than the wild-type protein at low enzyme concentrations. This result indicated that the EXO1 E109K variant is not catalytically dead as previously reported; rather, it possesses similar exonucleolytic activity to the wild-type protein. This observation could be confirmed using a second substrate, an oligonucleotide duplex with a 5′ recessed end (see 'Materials and Methods' section), where removal of the 5′ ^{32}P-labelled nucleotide took place with similar kinetics upon incubation with the wild-type- or the E109K-variant (Figure 4C).

We next decided to test whether the E109K mutation might have affected the flap endonuclease activity of the enzyme (35), using a 50-mer oligonucleotide splayed-arm substrate (see 'Materials and Methods' section) labelled with ^{32}P on the unannealed 5′-terminus. As shown in Figure 5A, incubation of this substrate with the wild-type enzyme released the labelled 19-mer flap, while the D173A variant was
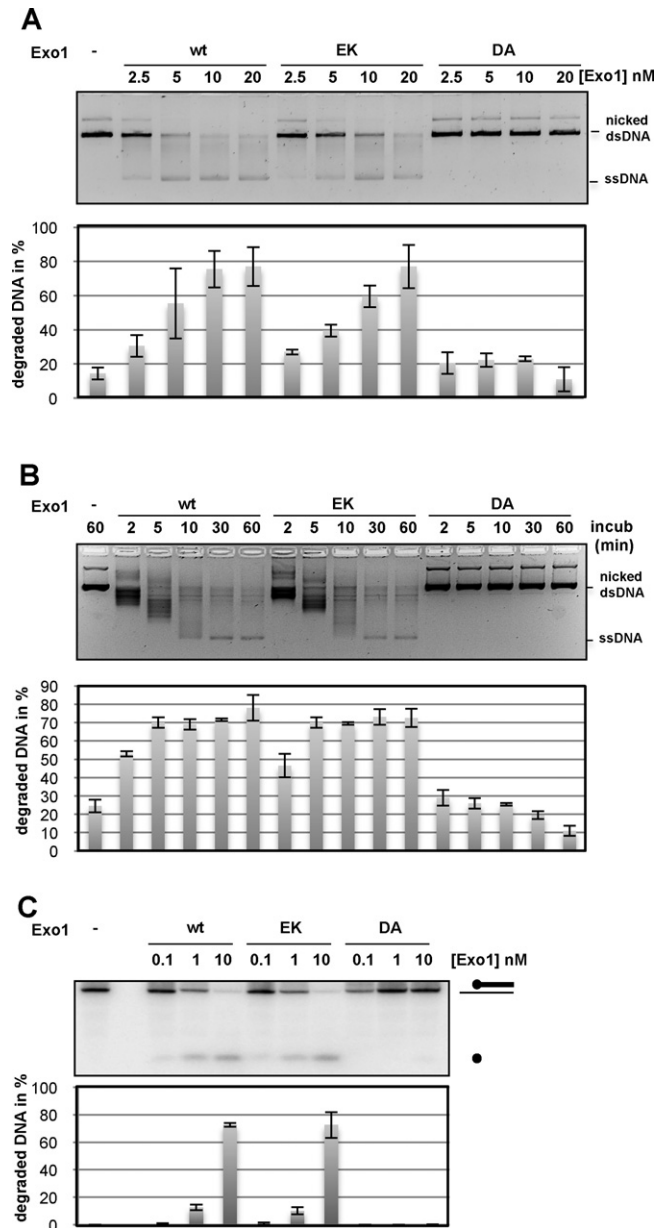

Figure 4. Wild-type EXO1 and its E109K variant have comparable exonucleolytic activities. (**A**) Exonucleolytic degradation of a nicked C/G homoduplex substrate in the presence of the indicated concentrations of the recombinant EXO1 variants for 10 min at 30°C. Degradation of the nicked strand gives rise to the faster-migrating single-stranded phagemid DNA. (**B**) Time course of an exonucleolytic degradation of a nicked C/G homoduplex substrate in the presence of 10 nM recombinant EXO1 variants. (**C**) Exonucleolytic degradation of a 5′- [32]P labelled oligonucleotide duplex with a recessed 5′ terminus in the presence of the indicated amounts of EXO1 or its variants (0.1–10 nM) for 10 min at 30°C. The substrate is schematically shown on the right of the figure. The labelled strand is indicated by a thicker line, with the ^{32}P-labelled 5′ phosphate indicated by a dot. Degradation liberates the rapidly migrating 5′ terminal nucleotide (indicated by the dot on the right).

inactive in this assay. Interestingly, the EXO1 E109 variant was slightly, but reproducibly, more active in this assay than the wild-type enzyme. This could be explained by the proposed role of the α4 helix in accommodating the 5′ flap (36),
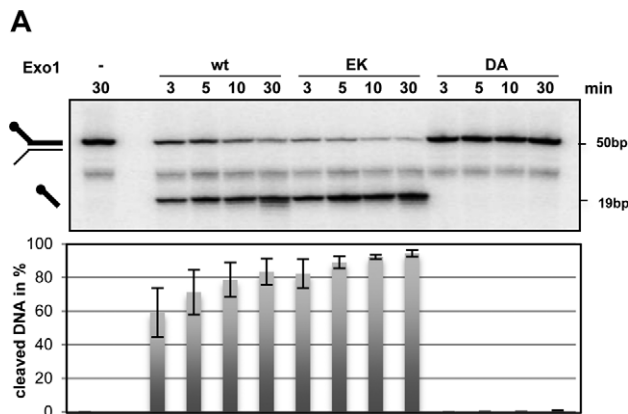
Figure 5. Endonucleolytic activities of wild-type EXO1 and its E109K variant on a 50-mer splayed arm substrate. The substrate is schematically shown on the left of the figure. The labelled strand is indicated by a thicker line, with the ^{32}P-labelled 5′ phosphate indicated by a dot. The substrate (100 fmol) was incubated with 5 nM EXO1 variants for 3–30 min at 30°C. The reaction products were separated on 20% denaturing polyacrylamide gels. EXO1-catalyzed cleavage liberates the 19-mer ^{32}P-labelled 5′ flap. The experiment was repeated four times and the figure shows an autoradiograph of a representative gel.

whereby the replacement of the acidic glutamate 109 for lysine might stabilize this interaction.

DISCUSSION

Search for *EXO1* mutations in LS/HNPCC identified 14 patients: one belonging to a typical HNPCC family who carried a splice-site mutation in the gene and the others belonging to atypical LS families who carried missense mutations that were not found in 200 control individuals and were therefore judged to be disease-associated (4). However, the causative nature of these mutations was uncertain; in autosomal-dominant syndromes such as LS, tumourigenesis requires that the allele containing the germline mutation be retained and the wild-type allele be silenced, mutated or lost through loss of heterozygosity. Unexpectedly, the wild-type allele was retained in all 13 analyzed tumours, and a loss of the mutated allele was detected in 12 of these. In an attempt to define the role of EXO1 in tumourigenesis, Edelmann *et al.* generated an *Exo1* knock-out mouse, which lacked exon 6 of the gene (23). As anticipated, extracts of embryonal fibroblasts from these mice lacked MMR activity, displayed elevated microsatellite instability at a mononucleotide repeat marker and increased mutation rates at the *Hprt* locus. The animals were susceptible to lymphoma development, which implied that *Exo1* is a tumour suppressor gene. That both genders were sterile confirmed the role of *Exo1* in meiosis, as shown earlier in yeast (5,39,40). However, the very subtle predisposition of these mice to tumourigenesis raised the possibility that deletion of exon 6 may not have resulted in full inactivation of the gene. The authors therefore generated an *Exo1*[null/null] mouse, as well as a knock-in animal carrying the E109K mutation (28), which was identified in an atypical LS family (4). Phenotypic comparison of the wild-type-, E109K- and null homozygous animals showed that the E109K mutant resembled the wild-type in terms of fertility, mutation

frequency in the liver, spleen and small intestine, microsatellite instability, somatic hypermutation, class switch recombination and chromosomal stability or longevity in a p53-deficient background. Based on the report that the E109K mutant lacked nuclease activity (27), a finding also substantiated by the authors (28), these similarities were interpreted to mean that Exo1 plays an essential structural, rather than an enzymatic, role in these processes (28). Our finding that the EXO1 E109K mutant is fully enzymatically active as an exo- and endonuclease contradicts this notion and argues that the above processes do indeed require the enzymatic function of EXO1, hence the phenotypic similarities between the E109K knock-in and wild-type mice, and the differences to the knock-out animals, which displayed defects in the above functions. The reason underlying the lack of enzymatic activity of the EXO1 E109K variant seen in previous studies (27,28) might be linked to the presence of the His$_6$ tag at its N-terminus, or to the purification protocol, which involved denaturation of the polypeptide in urea and refolding. Our protein was expressed with an intein tag, but this was cleaved off during elution from the chitin beads.

Small differences in tumour spectra of the three mouse models were reported, but the major differences were found among the phenotypes of embryonal fibroblast cell lines established from these mice. Thus, cells from the null- and E109K mice were reported to be slightly more resistant to MNNG, and displayed lower DNA damage response to this chemical, which is normally indicative of a MMR defect. This finding was unexpected, given that the knock-in animals did not display an elevated mutation frequency and that no MMR defect was seen in our *in vitro* experiments (Figure 3).

The embryonal fibroblasts generated from the knock-in mice displayed also somewhat greater chromosomal instability and sensitivity to camptothecin, a topoisomerase I inhibitor that stabilizes single strand breaks made by this enzyme. When such lesions reach replication forks, they are converted to DSBs, which can cause cell cycle arrest and trigger apoptosis. The repair of these breaks requires CtIP and EXO1 for resection. We, therefore, wondered whether the E109K mutant failed to interact with CtIP as reported (37). As shown in Figure 2B, no difference between the variants was detected by far-western blotting. The above evidence, coupled with the finding that the biochemical parameters of the wild-type and the E109K variant proteins were comparable, suggests that the phenotype of the embryonal fibroblasts was not linked to the lack of a nuclease activity of the E109K variant. One possible explanation concerns the stability of the proteins *in vivo*. EXO1 is extensively post-translationally modified and is highly-regulated by proteolysis during the cell cycle and in response to DNA damage (41). Given that the mutation at E109 generates a new lysine residue, and thus a possible novel site for modifications ranging from ubiquitylation and SUMOylation to methylation and acetylation, the possibility that the mutation alters the properties of the variant EXO1 enzyme and thus its biological function *in vivo* should not be disregarded. Unfortunately, levels of the variant EXO1 proteins were not described in these cell lines (28).

The data obtained in the course of this work show that the biochemical properties of the EXO1 E109K variant are

similar to that of the wild-type enzyme. Thus, in contrast to Edelmann *et al.* (28), we conclude that the biological functions of the enzyme require its nuclease activity. However, it is possible that the E109K mutation alters the behaviour of the protein *in vivo*, which clearly deserves further study.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

FUNDING

REFERENCES

1. Jiricny,J. (2006) The multifaceted mismatch-repair system. *Nat. Rev. Mol. Cell Biol.*, **7**, 335–346.
2. Modrich,P. (2006) Mechanisms in eukaryotic mismatch repair. *J. Biol. Chem.*, **281**, 30305–30309.
3. Abdel-Rahman,W.M. and Peltomaki,P. (2008) Lynch syndrome and related familial colorectal cancers. *Crit. Rev. Oncog.*, **14**, 1–22; discussion 23–31.
4. Wu,Y., Berends,M.J., Post,J.G., Mensink,R.G., Verlind,E., Van Der Sluis,T., Kempinga,C., Sijmons,R.H., van der Zee,A.G., Hollema,H. *et al.* (2001) Germline mutations of EXO1 gene in patients with hereditary nonpolyposis colorectal cancer (HNPCC) and atypical HNPCC forms. *Gastroenterology*, **120**, 1580–1587.
5. Szankasi,P. and Smith,G.R. (1992) A DNA exonuclease induced during meiosis of Schizosaccharomyces pombe. *J. Biol. Chem.*, **267**, 3014–3023.
6. Szankasi,P. and Smith,G.R. (1995) A role for exonuclease I from S. pombe in mutation avoidance and mismatch correction. *Science*, **267**, 1166–1169.
7. Liberti,S.E. and Rasmussen,L.J. (2004) Is hEXO1 a cancer predisposing gene? *Mol. Cancer Res.*, **2**, 427–432.
8. Tran,P.T., Erdeniz,N., Symington,L.S. and Liskay,R.M. (2004) EXO1-A multi-tasking eukaryotic nuclease. *DNA Repair*, **3**, 1549–1559.
9. Rasmussen,L.J., Rasmussen,M., Lee,B., Rasmussen,A.K., Wilson,D.M. 3rd, Nielsen,F.C. and Bisgaard,H.C. (2000) Identification of factors interacting with hMSH2 in the fetal liver utilizing the yeast two-hybrid system. In vivo interaction through the C-terminal domains of hEXO1 and hMSH2 and comparative expression analysis. *Mutat. Res.*, **460**, 41–52.
10. Schmutte,C., Marinescu,R.C., Sadoff,M.M., Guerrette,S., Overhauser,J. and Fishel,R. (1998) Human exonuclease I interacts with the mismatch repair protein hMSH2. *Cancer Res.*, **58**, 4537–4542.
11. Schmutte,C., Sadoff,M.M., Shim,K.S., Acharya,S. and Fishel,R. (2001) The interaction of DNA mismatch repair proteins with human exonuclease I. *J. Biol. Chem.*, **276**, 33011–33018.
12. Tran,P.T., Fey,J.P., Erdeniz,N., Gellon,L., Boiteux,S. and Liskay,R.M. (2007) A mutation in EXO1 defines separable roles in DNA mismatch repair and post-replication repair. *DNA Repair*, **6**, 1572–1583.
13. Tran,P.T., Simon,J.A. and Liskay,R.M. (2001) Interactions of Exo1p with components of MutLalpha in Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. U.S.A.*, **98**, 9760–9765.
14. Chen,X., Paudyal,S.C., Chin,R.I. and You,Z. (2013) PCNA promotes processive DNA end resection by Exo1. *Nucleic Acids Res.*, **41**, 9325–9338.
15. Liberti,S.E., Andersen,S.D., Wang,J., May,A., Miron,S., Perderiset,M., Keijzers,G., Nielsen,F.C., Charbonnier,J.B., Bohr,V.A. *et al.* (2011) Bi-directional routing of DNA mismatch repair protein human exonuclease 1 to replication foci and DNA double strand breaks. *DNA Repair*, **10**, 73–86.
16. Sokolsky,T. and Alani,E. (2000) EXO1 and MSH6 are high-copy suppressors of conditional mutations in the MSH2 mismatch repair gene of Saccharomyces cerevisiae. *Genetics*, **155**, 589–599.
17. Amin,N.S., Nguyen,M.N., Oh,S. and Kolodner,R.D. (2001) exo1-Dependent mutator mutations: model system for studying functional interactions in mismatch repair. *Mol. Cell. Biol.*, **21**, 5142–5155.
18. Tran,P.T., Erdeniz,N., Dudley,S. and Liskay,R.M. (2002) Characterization of nuclease-dependent functions of Exo1p in Saccharomyces cerevisiae. *DNA Repair*, **1**, 895–912.
19. Dherin,C., Gueneau,E., Francin,M., Nunez,M., Miron,S., Liberti,S.E., Rasmussen,L.J., Zinn-Justin,S., Gilquin,B., Charbonnier,J.B. *et al.* (2009) Characterization of a highly conserved binding site of Mlh1 required for exonuclease I-dependent mismatch repair. *Mol. Cell. Biol.*, **29**, 907–918.
20. Genschel,J. and Modrich,P. (2003) Mechanism of 5′-directed excision in human mismatch repair. *Mol. Cell*, **12**, 1077–1086.
21. Genschel,J. and Modrich,P. (2009) Functions of MutLalpha, replication protein A (RPA), and HMGB1 in 5′-directed mismatch repair. *J. Biol. Chem.*, **284**, 21536–21544.
22. Tishkoff,D.X., Boerger,A.L., Bertrand,P., Filosi,N., Gaida,G.M., Kane,M.F. and Kolodner,R.D. (1997) Identification and characterization of Saccharomyces cerevisiae EXO1, a gene encoding an exonuclease that interacts with MSH2. *Proc. Natl. Acad. Sci. U.S.A.*, **94**, 7487–7492.
23. Wei,K., Clark,A.B., Wong,E., Kane,M.F., Mazur,D.J., Parris,T., Kolas,N.K., Russell,R., Hou,H. Jr, Kneitz,B. *et al.* (2003) Inactivation of Exonuclease 1 in mice results in DNA mismatch repair defects, increased cancer susceptibility, and male and female sterility. *Genes Dev.*, **17**, 603–614.
24. Kadyrov,F.A., Genschel,J., Fang,Y., Penland,E., Edelmann,W. and Modrich,P. (2009) A possible mechanism for exonuclease 1-independent eukaryotic mismatch repair. *Proc. Natl. Acad. Sci. U.S.A.*, **106**, 8495–8500.
25. Jagmohan-Changur,S., Poikonen,T., Vilkki,S., Launonen,V., Wikman,F., Orntoft,T.F., Moller,P., Vasen,H., Tops,C., Kolodner,R.D. *et al.* (2003) EXO1 variants occur commonly in normal population: evidence against a role in hereditary nonpolyposis colorectal cancer. *Cancer Res.*, **63**, 154–158.
26. Alam,N.A., Gorman,P., Jaeger,E.E., Kelsell,D., Leigh,I.M., Ratnavel,R., Murdoch,M.E., Houlston,R.S., Aaltonen,L.A., Roylance,R.R. *et al.* (2003) Germline deletions of EXO1 do not cause colorectal tumors and lesions which are null for EXO1 do not have microsatellite instability. *Cancer Genet. Cytogenet.*, **147**, 121–127.
27. Sun,X., Zheng,L. and Shen,B. (2002) Functional alterations of human exonuclease 1 mutants identified in atypical hereditary nonpolyposis colorectal cancer syndrome. *Cancer Res.*, **62**, 6026–6030.
28. Schaetzlein,S., Chahwan,R., Avdievich,E., Roa,S., Wei,K., Eoff,R.L., Sellers,R.S., Clark,A.B., Kunkel,T.A., Scharff,M.D. *et al.* (2013) Mammalian Exo1 encodes both structural and catalytic functions that play distinct roles in essential biological processes. *Proc. Natl. Acad. Sci. U.S.A.*, **110**, E2470–2479.
29. Baerenfaller,K., Fischer,F. and Jiricny,J. (2006) Characterization of the "mismatch repairosome" and its role in the processing of modified nucleosides in vitro. *Methods Enzymol.*, **408**, 285–303.
30. Wu,Y., Li,Q. and Chen,X.Z. (2007) Detecting protein-protein interactions by Far western blotting. *Nat. Protoc.*, **2**, 3278–3284.
31. Dufner,P., Marra,G., Raschle,M. and Jiricny,J. (2000) Mismatch recognition and DNA-dependent stimulation of the ATPase activity of hMutSalpha is abolished by a single mutation in the hMSH6 subunit. *J. Biol. Chem.*, **275**, 36550–36555.
32. Sartori,A.A., Lukas,C., Coates,J., Mistrik,M., Fu,S., Bartek,J., Baer,R., Lukas,J. and Jackson,S.P. (2007) Human CtIP promotes DNA end resection. *Nature*, **450**, 509–514.

33. Lieber,M.R. (1997) The FEN-1 family of structure-specific nucleases in eukaryotic DNA replication, recombination and repair. *BioEssays*, **19**, 233–240.

34. Ceska,T.A. and Sayers,J.R. (1998) Structure-specific DNA cleavage by 5′ nucleases. *Trends Biochem. Sci.*, **23**, 331–336.

35. Lee,B.I. and Wilson,D.M. 3rd. (1999) The RAD2 domain of human exonuclease 1 exhibits 5′ to 3′ exonuclease and flap structure-specific endonuclease activities. *J. Biol. Chem.*, **274**, 37763–37769.

36. Orans,J., McSweeney,E.A., Iyer,R.R., Hast,M.A., Hellinga,H.W., Modrich,P. and Beese,L.S. (2011) Structures of human exonuclease 1 DNA complexes suggest a unified mechanism for nuclease family. *Cell*, **145**, 212–223.

37. Eid,W., Steger,M., El-Shemerly,M., Ferretti,L.P., Pena-Diaz,J., Konig,C., Valtorta,E., Sartori,A.A. and Ferrari,S. (2010) DNA end resection by CtIP and exonuclease 1 prevents genomic instability. *EMBO Rep.*, **11**, 962–968.

38. Constantin,N., Dzantiev,L., Kadyrov,F.A. and Modrich,P. (2005) Human mismatch repair: reconstitution of a nick-directed bidirectional reaction. *J. Biol. Chem.*, **280**, 39752–39761.

39. Zakharyevich,K., Ma,Y., Tang,S., Hwang,P.Y., Boiteux,S. and Hunter,N. (2010) Temporally and biochemically distinct activities of Exo1 during meiosis: double-strand break resection and resolution of double Holliday junctions. *Mol. Cell*, **40**, 1001–1015.

40. Tsubouchi,H. and Ogawa,H. (2000) Exo1 roles for repair of DNA double-strand breaks and meiotic crossing over in Saccharomyces cerevisiae. *Mol. Biol. Cell*, **11**, 2221–2233.

41. El-Shemerly,M., Janscak,P., Hess,D., Jiricny,J. and Ferrari,S. (2005) Degradation of human exonuclease 1b upon DNA synthesis inhibition. *Cancer Res.*, **65**, 3604–3609.