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

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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, HNPPC), 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 (MutSo) and MLH1/PMS2 (MutLo) 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-
istence of redundant nucleases, as well as by the existence of an EXO1-independent, but MLH1/PM2- and polymerase-6-dependent strand displacement mechanism of mismatch correction (24), led to the expectation that mutations in EXOI in LS families are likely to be rare and linked to late cancer onset and low penetrance. Germline mutations in EXOI 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 EXOI, displayed phenotypic traits [e.g. wild-type-like mutation (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 EXOI 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<sub>x</sub>1 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 EXOI 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<sup>TM</sup> Kit, NEB) encoding the wild-type EXOI cDNA and the D173A mutant, both with a C-terminal intein tag, were kindly provided by Stefano Ferrari. The E109K mutation was introduced using a following primers: 5'-CTTAAGGGAAGAACCTTCTCGTAGGAAAAGTCTCGG-3' and 5'-CCGAGACTTTCTCCCCTTAAGAAGATGTGCTTTCCCTTAAG-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<sub>600</sub> of 0.3 in LB medium supplemented with chloramphenicol (25 µg/ml) and ampicillin (100 µg/ml). 0.2 mM isopropylβ-D-galactoside (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, 15 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<sub>2</sub>, 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<sub>2</sub> and 1 mM DTT for 30 min at 37°C. Forty-eight femtomoles of the substrate were incubated in 40 mM Tris-HCl, 10 mM NaCl, 6 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub> 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'-CAAGGCCUAUUCUGAUUUTT-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 Sall 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 Exol wt, E109K or D173A in 20 mM Tris-HCl pH 7.6, 110 mM KCl, 5 mM MgCl<sub>2</sub>, 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 MgCl2, 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′-32P labelled 5′ recessed oligonucleotide 5′-GAGATATTCCTGGTACGC GTGACCCGAGCTGAAAG-3′ annealed with a 2-fold excess of the unlabelled oligonucleotide 5′-CTTTACGCTCGTACGCTGACCAGAATATCTC-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′-32P labelled oligonucleotide 5′-TCAAAGTCAAGCCTA CACTGCGAAGCTCAGTACCCTC-3′ with a 2-fold excess of the unlabelled oligonucleotide 5′-GAGGTCACTCCAGTGAATTCGAGCTCGCAG CGGTCACGCTGACCAGGAATATCTCTA-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.

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Far-western analyses

This experiment was carried out as described (30) without modification, using recombinant MutSα (31) or CtfIP (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-
CtIP (bottom panel). M, pre-stained molecular size marker. Revealed upon hybridization of the membrane with anti-FLAG to detect and incubated with recombinant FLAG-tagged CtIP. The interaction was SDS-PAGE (Coomassie staining top panel), transferred onto a membrane between EXO1 and CtIP. EXO1 wild-type and mutants were separated by molecular size marker. (Figure 2A, all three variants interacted efficiently with MutS\(^{\text{A}}\) heterodimer with the MSH2 subunit of MutS\(^{\text{A}}\) 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 MutS\(^{\text{A}}\) as reported previously (6,9,10,22). We separated the MSH6 and MSH2 subunits of purified recombinant MutS\(^{\text{A}}\) 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 Sall, 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). Sall/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 Sall-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 MutS\(^{\text{A}}\)/MutL\(^{\text{A}}\) 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-\(\beta\) (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
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′ 32P-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 spliced-arm substrate (see ‘Materials and Methods’ section) labelled with 32P 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 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).
Phenotypic comparison of the wild-type- and E109K-gels.EXO1-catalyzed cleavage liberates the 19-mer 32P-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 32P-labelled 5′ flap. The experiment was repeated four times and the figure shows an autoradiograph of a representative gel.

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 Exo1null/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- and 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 His6 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.

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