

Defective DNA Ligation during Short-Patch Single-Strand Break Repair in Ataxia Oculomotor Apraxia 1[∇]

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Ataxia oculomotor apraxia 1 (AOA1) results from mutations in aprataxin, a component of DNA strand break repair that removes AMP from 5' termini. Despite this, global rates of chromosomal strand break repair are normal in a variety of AOA1 and other aprataxin-defective cells. Here we show that short-patch single-strand break repair (SSBR) in AOA1 cell extracts bypasses the point of aprataxin action at oxidative breaks and stalls at the final step of DNA ligation, resulting in the accumulation of adenylated DNA nicks. Strikingly, this defect results from insufficient levels of nonadenylated DNA ligase, and short-patch SSBR can be restored in AOA1 extracts, independently of aprataxin, by the addition of recombinant DNA ligase. Since adenylated nicks are substrates for long-patch SSBR, we reasoned that this pathway might in part explain the apparent absence of a chromosomal SSBR defect in aprataxin-defective cells. Indeed, whereas chemical inhibition of long-patch repair did not affect SSBR rates in wild-type mouse neural astrocytes, it uncovered a significant defect in *Aptx*^{-/-} neural astrocytes. These data demonstrate that aprataxin participates in chromosomal SSBR in vivo and suggest that short-patch SSBR arrests in AOA1 because of insufficient nonadenylated DNA ligase.

Oxidative stress is an etiological factor in many neurological diseases, including Alzheimer's disease, Parkinson's disease, and Huntington's disease. One type of macromolecule damaged by reactive oxygen species is DNA, and oxidative damage to DNA has been suggested to be a significant factor in these and other neurological conditions (2). In particular, a number of rare hereditary neurodegenerative disorders have provided direct support for the notion that unrepaired DNA damage causes neural dysfunction. Not least of these are the recessive spinocerebellar ataxias, a number of which are associated with mutations in DNA damage response proteins (17). The archetypal DNA damage-associated spinocerebellar ataxia is ataxia-telangiectasia (A-T), in which mutations in ATM protein result in defects in the detection and signaling of DNA double-strand breaks (DSBs) (3). A-T-like disorder is a related disease that exhibits neurological features similar to those of A-T, resulting from mutation of Mre11, a component of the MRN complex that operates in conjunction with ATM during DSB detection and signaling (28).

Two additional spinocerebellar ataxias are spinocerebellar ataxia with axonal neuropathy 1 (SCAN1) and ataxia oculomotor apraxia 1 (AOA1), in which the TDP1 and aprataxin proteins are mutated, respectively (9, 19, 27). Both TDP1 and aprataxin are components of the DNA strand break repair machinery (recently reviewed in references 6 and 24). Whereas SCAN1 is currently limited to nine individuals from a single family, AOA1 is one of the commonest recessive spinocere-

bellar ataxias. Aprataxin is a member of the histidine triad superfamily of nucleotide hydrolases/transferases and has been reported to remove phosphate and phosphoglycolate moieties from the 3' termini of DNA strand breaks (26). Aprataxin can also remove AMP from a variety of ligands in vitro, including adenosine polyphosphates, AMP-lysine, AMP-NH₂ (adenine monophosphoramidate), and adenylated DNA in which AMP is covalently attached to the 5' terminus of a DNA single-strand break (SSB) or DSB (1, 16, 23, 25). To date, aprataxin activity is greatest on AMP-DNA, suggesting that this may be the physiological substrate of this enzyme.

In vitro, DNA strand breaks with 5'-AMP termini can arise from premature DNA ligase activity. DNA ligases adenylate 5' termini at DNA breaks to enable nucleophilic attack of the resulting pyrophosphate bonds by 3'-hydroxyl termini, thereby resealing the breaks. However, DNA adenylation by DNA ligases can occur prematurely, before a 3'-hydroxyl terminus is available. Aprataxin reverses these premature DNA adenylation events, in vitro at least, effectively "resetting" the DNA ligation reaction to the beginning (1). Whether or not 5'-AMP arises in DNA in vivo or is a physiological substrate of aprataxin, however, is unknown. Moreover, attempts to measure DNA strand break repair rates in vivo are conflicting and have failed to identify a consistent defect in DNA SSB repair (SSBR) or DSB repair (DSBR) in AOA1 cells (14, 15, 20). It is thus not clear whether or not defects in DNA strand break repair can account for this neurodegenerative disease.

Here we have resolved the discrepancy between the requirements for aprataxin in vitro and in vivo by identifying the stage at which SSBR reactions fail in vitro and by carefully analyzing chromosomal SSBR rates in vivo. We show that short-patch SSBR reactions are defective in AOA1 cell extracts at the final step of DNA ligation, resulting in the accumulation of adeny-

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lated DNA nicks, and that this defect can be rescued in AOA1 extracts independently of aprataxin by addition of recombinant DNA ligase. We also find that treatment with aphidicolin, an inhibitor of DNA polymerase δ (Pol δ) and Pol ϵ , unveils a measurable defect in chromosomal SSB in *Aptx*^{-/-} primary neural astrocytes, suggesting that the adenylated nicks that arise from the short-patch repair defect can be channeled into long-patch repair in vivo. These data demonstrate that aprataxin participates in chromosomal SSB and suggest that this process arrests in AOA1, at oxidative SSBs, due to insufficient levels of nonadenylated DNA ligase.

MATERIALS AND METHODS

Preparation of adenylated DNA substrates. Oligonucleotide duplexes containing an SSB with a 5'-AMP terminus and either a 1-bp gap with a 3'-phosphate terminus or a nick with a 3'-hydroxyl terminus were prepared as follows. A 25-mer (5'-GACATACTAACTTGAGCGAAACGGT) was labeled by phosphorylation with T4 PNK and [γ -³²P]ATP and was annealed with a 43-mer (5'-CCG TTTCGCTCAAGTTAGTATGTCAAAGCAGGCTTCAACGGAT) and a 17-mer with a 3'-phosphate terminus (17-mer-P) (5'-TCCGTTGAAGCCTGTCT-P). The labeled 25-mer in this duplex was then either adenylated at the 5' terminus by incubation with 1 U T4 DNA ligase at 30°C for 1 h in ligation buffer (50 mM Tris-HCl [pH 7.5], 10 mM MgCl₂, 5 mM dithiothreitol [DTT], 25 μ g/ μ l bovine serum albumin, 1 mM ATP) or mock adenylated in the absence of T4 DNA ligase. The labeled adenylated 25-mer was then separated from the labeled nonadenylated 25-mer (and from the 17-mer and 43-mer) on a 15% denaturing polyacrylamide gel electrophoresis (PAGE) gel, excised, and eluted from the crushed gel in elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA [pH 8.0]) overnight at room temperature. The eluted DNA was then ethanol precipitated. The labeled mock-adenylated 25-mer was purified in parallel. The labeled adenylated 25-mer was then reannealed with the 43-mer and either the 17-mer-P (containing a 3' phosphate) or an 18-mer (5'-TCCGT TGAAGCCTGCTT) to produce oligonucleotide duplexes harboring an adenylated SSB with either a 1-bp gap and a 3'-phosphate terminus or a nick with a 3'-hydroxyl terminus, respectively. To measure the repair of 3' termini by PNK or gap filling by Pol β , the 17-mer-P or a 17-mer oligonucleotide lacking a 3' phosphate, respectively, was labeled with T4 PNK (phosphatase dead) and [γ -³²P]ATP and was annealed to the 25-mer and 43-mer. The substrate was either mock adenylated or adenylated as described above and was purified on a 15% native PAGE gel. The final substrate contained a 1-bp gap with a 5' AMP and either a 3' P or a 3' OH.

Purification of recombinant human SSB proteins. Pol β was expressed in *Escherichia coli* and purified by anion and cation exchange as previously described (29), using a 1.6-ml Poros column on a Biocad Sprint chromatography system (Applied Biosystems, United Kingdom). His-tagged PNK and His-tagged DNA ligase III α (Lig3 α) were expressed in the *E. coli* strain BL21(DE3) for 90 min at 20°C following addition of 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG). PNK was purified by cation-exchange chromatography followed by immobilized metal chelate affinity chromatography (IMAC) as previously described (30). Lig3 α was purified by IMAC as described previously (7), followed by cation exchange as described previously for PNK. His-tagged aprataxin was expressed from pB352 (8) in BL21(DE3) by addition of 1 mM IPTG and incubation at 30°C for 3 h. Aprataxin was purified by IMAC followed by cation exchange as described above for PNK and Lig3 α .

Preparation of human lymphoblastoid cell extracts. Total-cell extracts were prepared from 5×10^6 ConR2 (wild type [WT]) or Ap5 (AOA1) lymphoblastoid cells or from *Aptx*^{+/+} and *Aptx*^{-/-} primary quiescent mouse astrocytes by lysis in 0.2 ml 20 mM Tris-HCl (pH 7.5), 10 mM EDTA, 1 mM EGTA, 100 mM NaCl, 1% Triton X-100, and 1/100 dilution of protease inhibitor cocktail (Sigma). The extracts were clarified by centrifugation, quantified with a Bio-Rad DC protein assay kit using bovine serum albumin as a standard, and then aliquoted and snap-frozen.

In vitro repair of adenylated SSB substrates by recombinant proteins. For reactions employing recombinant human proteins, a 25 nM concentration of the indicated ³²P-labeled substrate was incubated with the indicated concentrations of recombinant protein for 1 h at 30°C in 25 mM HEPES (pH 8.0), 130 mM KCl, 1 mM DTT, 10 mM MgCl₂, 100 μ M deoxynucleoside triphosphates (dNTPs), and 1 mM ATP unless otherwise indicated. Reactions were stopped by addition of 1/3 volume of formamide gel loading buffer, and reaction products were fractionated on 15% denaturing PAGE gels and analyzed by a phosphorimager.

For reactions employing cell extracts, 5 μ g of extract from WT lymphoblastoid cells (ConR2) or AOA1 lymphoblastoid cells (Ap5) or from *Aptx*^{+/+} and *Aptx*^{-/-} primary quiescent mouse astrocytes was incubated with a 100 nM concentration of the indicated ³²P-labeled substrate for 1 h at 30°C in 25 mM HEPES (pH 8.0), 130 mM KCl, 1 mM DTT, and, unless otherwise indicated, 10 mM MgCl₂, 100 μ M dNTPs, and 1 mM ATP. A 1,000-fold excess of a competitor oligonucleotide with an unrelated sequence and a 5' phosphate (5'-P-TCTGCTAGCATCGATCCATG-3') was added to reduce degradation of the labeled substrate by non-specific nucleases. Reactions were stopped and products analyzed as described above.

Cell culture. (i) **Human cells.** 1BR3 cells are primary human fibroblasts, and ConR and ConR2 cells are lymphoblastoid cells, all from unrelated normal individuals. fAp1 and fAp4 cells are AOA1 primary fibroblasts, and Ap3 and Ap5 cells are AOA1 lymphoblasts. ConR, Con R2, fAp1, fAp4, Ap3, and Ap5 cells were kindly provided by Malcolm Taylor and, except for Ap5 and ConR2, have been described previously (8). Ap1, Ap3, and Ap4 cells harbor the homozygous *APT*X mutation W279X, which results in little or no aprataxin protein or activity (1, 8), and Ap5 cells harbor a genomic deletion of *APT*X (M. Taylor, unpublished data). Cells were cultured as described previously (8).

(ii) **Primary mouse astrocytes.** *Aptx*^{-/-} mice have been described previously (1). The generation and characterization of nestin-Cre conditional *Xcc1*^{-/-} mice will be described elsewhere. All animals were housed in individually ventilated cabinets and were maintained in accordance with the guidelines of the institutional animal care and ethical committee at the University of Sussex. Mouse astrocytes were prepared from postnatal day 3 (P3) to P4 brains. Cortices were dissociated by passage through a 5-ml pipette, and cells were resuspended in Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture (1:1; Gibco-BRL) supplemented with 10% fetal bovine serum, 1 \times Glutamax, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 20 ng/ml epidermal growth factor (Sigma). Primary astrocytes were established in Primeria T-25 tissue culture flasks (Falcon) at 37°C in a humidified oxygen-regulated (9%) incubator. The culture medium was changed after 3 days, and astrocyte monolayers reached confluence 3 days later. The purity of the astrocyte cultures was confirmed by immunofluorescence using an anti-GFAP antibody (Sigma).

Alkaline comet assays. Cells ($\sim 3 \times 10^5$ /sample) were incubated with 75 μ M (mouse astrocytes), 50 μ M (human lymphoblastoid cells), or 75 μ M (primary human fibroblasts) H₂O₂ for 10 min on ice or with the indicated doses of methyl methanesulfonate (MMS) for 10 min at 37°C. Where indicated, cells were then incubated in complete medium for the indicated repair periods at 37°C. To inhibit long-patch DNA polymerases, cells were incubated with 50 μ M aphidicolin (Sigma) in complete medium for 30 min at 37°C. Cells were then either mock treated or treated with H₂O₂ or MMS as described above in the continued presence or absence of aphidicolin, as appropriate. Chromosomal DNA strand breaks were then measured using alkaline comet assays as previously described (5). Statistical analyses were performed using SPSS software.

RESULTS

Aprataxin interacts with the SSB scaffold protein XRCC1, raising the possibility that a defect in chromosomal SSB underlies the neuropathology of this disease. However, evidence for a defect in SSB in aprataxin-defective AOA1 cells is conflicting (14, 15, 20). To resolve this issue, we employed alkaline comet assays to compare chromosomal SSB rates in a variety of different aprataxin-defective cell types. It should be noted that while alkaline comet assays measure both SSBs and DSBs, these assays primarily measure SSBs following short periods of treatment with H₂O₂ or MMS, because these agents induce several orders of magnitude more SSBs than DSBs (4, 18). In contrast to the findings of a previous report (15), we failed to observe a significant difference in chromosomal SSB rates between either lymphoblastoid cells (Fig. 1A) or primary fibroblasts (Fig. 1B) from AOA1 patients and those from normal individuals following H₂O₂-induced oxidative stress. Because individual genetic variability might mask differences in SSB rates, we also compared chromosomal SSB in quiescent primary astrocytes from *Aptx*^{-/-} mice and isogenic *Aptx*^{+/+} littermate controls. Astrocytes were chosen because

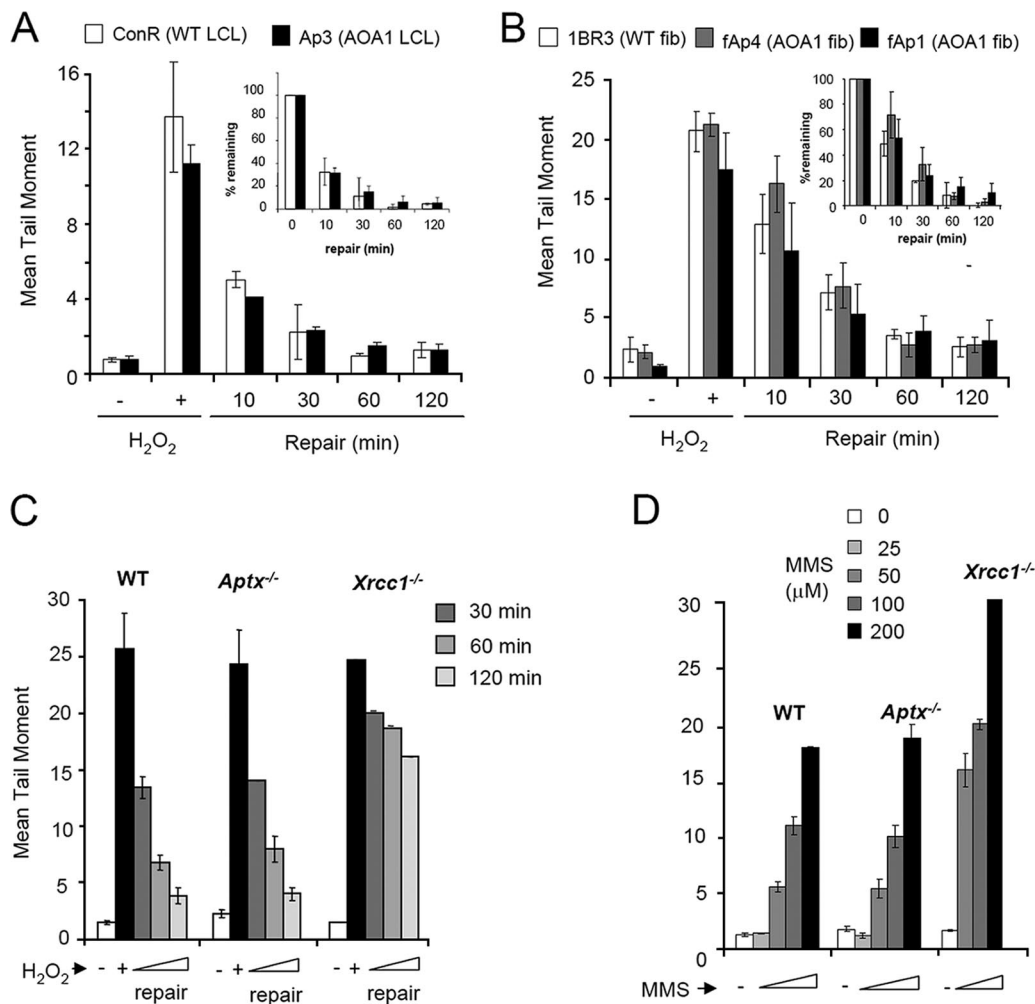


FIG. 1. Normal rates of chromosomal SSB in cells lacking aprataxin. (A) AOA1 lymphoblastoid cells were incubated in the absence (–) or presence (+) of 50 μ M H₂O₂ (15 min on ice), followed by incubation in a drug-free medium for the indicated repair period at 37°C. Chromosomal DNA strand breaks were then measured by alkaline comet assays. (B) AOA1 primary fibroblasts were treated in the absence (–) or presence (+) of 75 μ M H₂O₂ (15 min on ice) and processed as described for panel A. Insets in panels A and B contain the same data as in the main panels but plotted as percent damage remaining. (C) WT, *Aptx*^{-/-}, and *Xrcc1*^{-/-} primary quiescent astrocytes were incubated in the absence (–) or presence (+) of 75 μ M H₂O₂ (10 min on ice), followed by incubation in a drug-free medium for the indicated repair period at 37°C. Chromosomal DNA strand breaks were then measured by alkaline comet assays. (D) WT, *Aptx*^{-/-}, and *Xrcc1*^{-/-} primary quiescent astrocytes were incubated in the absence (–) or presence of the indicated concentration of MMS (10 min at 37°C). Chromosomal DNA strand breaks were then measured by alkaline comet assays.

they are neural in origin and thus better reflect the type of cells affected in AOA1. However, SSB rates were similar in *Aptx*^{+/+} and *Aptx*^{-/-} astrocytes following treatment with H₂O₂ (Fig. 1C). *Aptx*^{+/+} and *Aptx*^{-/-} astrocytes also accumulated SSBs at similar rates during incubation with the alkylating agent MMS, suggesting that the rate of SSB rejoining during DNA base excision repair is also normal in *Aptx*^{-/-} cells (Fig. 1D). In contrast, astrocytes lacking the core SSB factor *Xrcc1* exhibited greatly reduced SSB rates following treatment with either H₂O₂ or MMS (Fig. 1C and D). It should be noted that we also observed normal SSB kinetics in *Aptx*^{-/-} postmitotic cerebellar granule neurons (data not shown). We conclude from these experiments that AOA1 and other aprataxin-defective cells lack a measurable defect in the global rate of chromosomal SSB.

In contrast to the findings of cellular SSB assays, protein

extracts from *Aptx*^{-/-} astrocytes and *Aptx*⁻ chicken DT40 cells are unable to support short-patch repair of adenylated oxidative SSBs in vitro (1). To resolve this discrepancy, we examined whether this was also true in AOA1 lymphoblastoid cell extracts. To measure short-patch SSB, we employed an oligonucleotide duplex harboring a 1-bp gap with 3'-phosphate termini, a substrate that mimics one of the commonest types of oxidative SSB arising in cells (Fig. 2A, top). The SSB also possesses a 5' AMP and is therefore a substrate for aprataxin. In agreement with previous experiments with neural extracts (1), WT extracts efficiently removed AMP from 5' termini and repaired the SSBs, as indicated by the respective appearance of the ³²P-labeled 25-mer and the ³²P-labeled 43-mer (Fig. 2A, lanes 6 to 8). In contrast, AOA1 extracts did not (Fig. 2A, lanes 3 to 5). Importantly, SSB was proficient in AOA1 extracts if the initial SSB lacked 5' AMP (Fig. 2B). A number of trun-

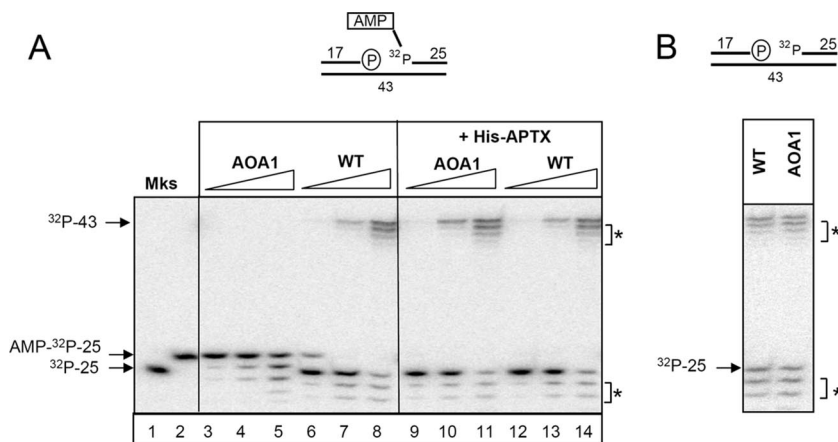


FIG. 2. Aprataxin-dependent short-patch SSB repair of 5'-AMP SSBs in AOA1 lymphoblastoid extracts. (A) Defective short-patch SSB repair in AOA1 extracts and its complementation by recombinant aprataxin. (Top) Cartoon of the oligonucleotide duplex employed for these experiments, containing a 1-bp gap, a 3'-phosphate terminus, and a 5'-AMP terminus. The 5' phosphate to which AMP is covalently linked is labeled with ^{32}P . The position of this label restricts the assay to measurements of short-patch repair events only. Nucleotide lengths are shown. (Bottom) Portions (0.1 μg , 1.0 μg , or 5.0 μg) of either WT (ConR2) or AOA1 (Ap5) cell extracts were incubated for 60 min at 30°C with a ^{32}P -labeled 5'-AMP SSB substrate (25 nM) in the absence (left) or presence (right) of recombinant human aprataxin (APT) (100 nM). Reaction products were fractionated and detected as described in Materials and Methods. The ^{32}P -labeled 25-mer containing (AMP- ^{32}P -25) or lacking (^{32}P -25) 5' AMP was fractionated in parallel for size markers (Mks). The position of the repaired ^{32}P -labeled 43-mer is also indicated. Asterisks indicate nonspecific nucleolytic products. (B) Normal short-patch repair of SSBs lacking 5' AMP in AOA1 cell extracts. (Top) Cartoon of the oligonucleotide duplex employed for these experiments, containing a 1-bp gap, a 3'-phosphate terminus, and a normal ^{32}P -labeled 5'-phosphate terminus. WT (ConR2) or AOA1 (Ap5) cell extracts (5 μg) were incubated for 60 min at 30°C with a ^{32}P -labeled SSB substrate lacking 5' AMP (100 nM). Reaction products were fractionated and detected as described in Materials and Methods.

cated oligonucleotide fragments were generated by nonspecific nucleolytic activity in these experiments, which represented degradation of the 3' terminus of the ^{32}P -labeled 25-mer. However, this activity did not account for the short-patch repair defect in AOA1 cell extracts, which was fully complemented by the addition of recombinant aprataxin (Fig. 2A, lanes 9 to 11). The appearance of ligated product in these experiments was dependent on the presence of dNTPs, confirming that these experiments measured gap repair rather than ligation across the 1-bp gap (data not shown).

The predicted pathway for short-patch repair of SSBs harboring 5' AMP in WT cells is depicted in Fig. 3A. It was considered likely that this pathway arrests in AOA1 extracts at the very beginning, prior to 3'-DNA end processing by PNK and/or DNA gap filling by Pol β , because the presence of AMP at the 5' terminus might occlude access to the 3' terminus. Surprisingly, however, the 3'-phosphatase activity of PNK was similar irrespective of whether the SSB possessed 5' AMP or not, under conditions in which PNK was limiting (Fig. 3B). Similar results were observed for DNA gap filling by Pol β , with similar amounts of the ^{32}P -labeled 17-mer converted into a ^{32}P -labeled 18-mer irrespective of the presence or absence of 5' AMP (Fig. 3C). These experiments suggested that neither PNK nor Pol β activity is affected by the presence of 5' AMP at these time points, and thus short-patch SSB repair might fail in AOA1 extracts at the final step of DNA ligation, resulting in the accumulation of adenylated DNA nicks (Fig. 3D).

To confirm this hypothesis, we compared WT and AOA1 extracts for their abilities to ligate adenylated nicks. Indeed, whereas adenylated nicks were efficiently ligated by extracts from WT cells, they remained largely unligated in reaction mixtures containing AOA1 cell extracts (Fig. 4A). The finding that short-patch repair in AOA1 arrests during DNA ligation is

surprising, because adenylated nicks are normal intermediates of DNA ligation and are normally rapidly resealed by nonadenylated DNA ligase (11). Normally, the nonadenylated DNA ligase required for this purpose is created during, and tightly coupled with, adenylation of the 5' terminus of the break by an adenylated DNA ligase (Fig. 4B). However, in the absence of aprataxin, DNA nicks may accumulate in a preadenylated state and therefore require preexisting pools of nonadenylated DNA ligase. While both nonadenylated and adenylated DNA ligases exist in cells, the former may be very limited because the readenylation of DNA ligases by cellular ATP is very rapid. Consequently, we reasoned that the DNA ligation defect in AOA1 may reflect insufficient cellular levels of nonadenylated DNA ligase. To test this hypothesis, we examined the impact of supplementing short-patch SSB repair reaction mixtures in AOA1 cell extracts and *Aptx*^{-/-} mouse neural astrocyte extracts with recombinant DNA ligase. Strikingly, addition of T4 ligase restored short-patch SSB repair efficiency in both AOA1 cell extracts and *Aptx*^{-/-} neural cell extracts to a level similar to that observed in WT extracts (Fig. 4C and D, compare lanes 6 and 10). Importantly, complementation was more efficient in the absence of ATP than in its presence, supporting the notion that it was the nonadenylated subfraction of T4 ligase that was responsible for complementation (Fig. 4C and D, compare lanes 8 and 10). Similar results were observed for AOA1 cell extracts if T4 ligase was replaced with human DNA ligase 3 α , albeit to a lesser extent (Fig. 5A).

The data described above suggest that short-patch repair of some adenylated SSBs can occur independently of aprataxin if sufficient nonadenylated DNA ligase is available. To confirm this idea, we attempted to reconstitute this aprataxin-independent SSB repair pathway using recombinant proteins. As expected, the repair of adenylated gaps by PNK, Pol β , and Lig3 α was

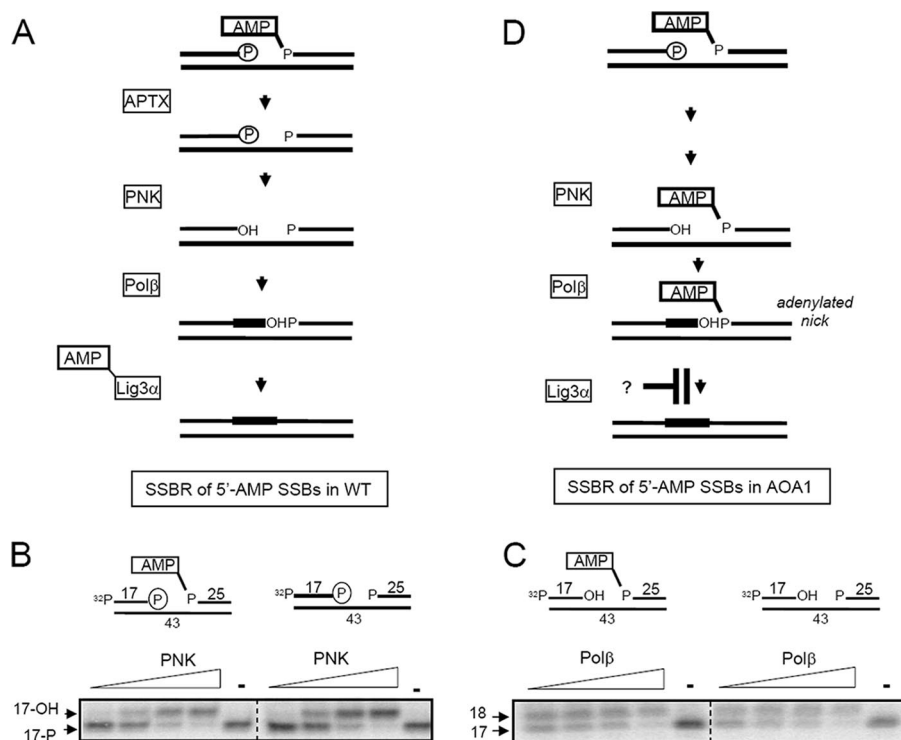


FIG. 3. Removal of 5' AMP by aprataxin is not required for end processing by PNK or gap filling by Pol β . (A) Cartoon of aprataxin-dependent short-patch repair of adenylated SSBs in WT cells. (B) 5' AMP does not affect processing of 3'-phosphate termini by PNK. An SSB substrate (25 nM) lacking or containing 5' AMP as indicated (top) was incubated either with 25 nM, 50 nM, 125 nM, or 250 nM recombinant human PNK or without PNK (–) for 1 h at 30°C. Reaction products were fractionated by denaturing PAGE and detected by phosphorimaging. Note that the 5' terminus of the 17-mer is labeled with ^{32}P in these experiments to allow detection of 3' end processing. The position of the 17-mer harboring 3' phosphate (17-P) or 3' hydroxyl (3'-OH) is indicated. (C) 5' AMP does not affect gap filling by Pol β . An SSB substrate (25 nM) lacking or containing 5' AMP as indicated (top) was incubated either with 5 nM, 15 nM, 30 nM, or 100 nM purified recombinant human Pol β or without Pol β (–) for 1 h at 30°C. Reaction products were fractionated by denaturing PAGE and detected by phosphorimaging. The positions of the 17-mer and 18-mer are indicated as markers. (D) Cartoon depicting APTX-independent repair of adenylated SSBs in AOA1. Note the hypothetical blockage of this process at the final step of DNA ligation.

dependent on aprataxin in the presence of ATP, conditions under which Lig3 α is largely adenylated (Fig. 5B). However, in the absence of ATP, short-patch repair of 5'-AMP SSBs occurred independently of aprataxin. Taken together, these experiments indicate that short-patch repair arrests in AOA1 cell extracts at the final step of DNA ligation, due to insufficient levels of nonadenylated DNA ligase.

Importantly, the finding that short-patch repair failed during DNA ligation provides a possible explanation for the absence of a measurable defect in chromosomal SSB in AOA1, following oxidative stress. This is because adenylated DNA nicks might be channeled into long-patch SSBR reactions, during which the adenylated 5' terminus is displaced by extended DNA gap filling and then clipped off nucleolytically, avoiding the requirement for either aprataxin or preexisting pools of nonadenylated DNA ligase. While our *in vitro* assay was not capable of measuring long-patch repair reactions, this process might operate efficiently at adenylated nicks within a chromosomal context. To examine this possibility, we compared chromosomal SSBR rates in WT and *Aptx*^{–/–} quiescent mouse neural astrocytes in the presence and absence of aphidicolin, an inhibitor of the DNA polymerases Pol δ and Pol ϵ , which are implicated in long-patch repair reactions (10, 13, 22). Strikingly, whereas aphidicolin did not measurably reduce SSBR

rates in WT astrocytes following H₂O₂ treatment, it significantly slowed SSBR in *Aptx*^{–/–} astrocytes (Fig. 6A). Aphidicolin also selectively slowed short-patch base excision repair in AOA1 cells, as indicated by the accumulation of higher steady-state levels of chromosomal DNA strand breaks in *Aptx*^{–/–} astrocytes than in WT astrocytes during short incubations with the DNA-alkylating agent MMS (Fig. 6B). The fact that SSBR in *Aptx*^{–/–} cells was slowed rather than prevented by aphidicolin most likely reflects the fact that only a subset of H₂O₂- and MMS-induced SSBs possess adenylated 5' termini. Nevertheless, the presence of a measurable defect in chromosomal SSBR in *Aptx*^{–/–} cells indicates that a significant fraction of endogenous SSBs arising from oxidative stress and DNA base damage are substrates for aprataxin.

In summary, we demonstrate here that aprataxin is required for SSBR *in vivo* and suggest that short-patch SSBR is defective in AOA1 cells, at oxidative SSBs, due to insufficient levels of nonadenylated DNA ligase.

DISCUSSION

AOA1 is one of the commonest recessive hereditary spinocerebellar ataxias identified to date. This disease results from mutations in aprataxin, a component of the DNA strand break

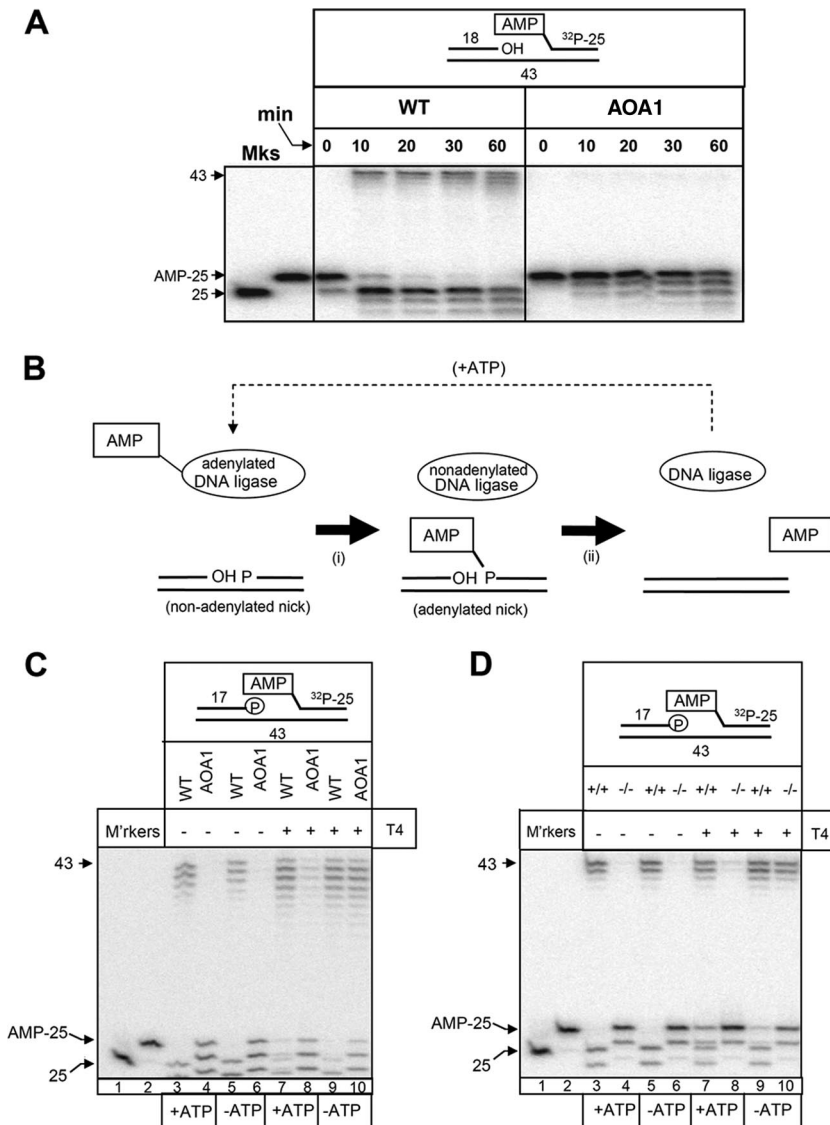


FIG. 4. Short-patch SSB repair in AOA1 cells. (A) AOA1 cell extracts cannot efficiently ligate adenylated DNA nicks. Total WT (ConR2) or AOA1 (Ap5) lymphoblastoid cell extracts (6.25 μ g) were incubated with a 32 P-labeled adenylated nicked substrate (25 nM) (top) for the indicated times at 30°C. Reaction products were fractionated by denaturing PAGE and detected by phosphorimaging. A 32 P-labeled 25-mer containing 5' AMP (AMP-25) and one lacking 5' AMP were fractionated for size markers (Mks). The position of the 43-mer ligated product is indicated. (B) Cartoon depicting ligation of a nonadenylated DNA nick by DNA ligase. (i) An adenylated DNA ligase molecule transfers AMP to the 5'-phosphate terminus of a DNA nick, resulting in an adenylated nick and a nonadenylated ligase molecule. (ii) The nonadenylated DNA ligase molecule rapidly catalyzes a nucleophilic attack of the pyrophosphate bond linking AMP to DNA, resulting in nick ligation and the release of AMP. The nonadenylated DNA ligase molecule is readenylated (dashed arrow) in readiness for the next ligation event. (C) Complementation of the SSB defect in AOA1 lymphoblastoid extracts by recombinant T4 DNA ligase. Total WT (ConR2) or AOA1 (Ap5) lymphoblastoid cell extracts (5 μ g) were incubated with a 32 P-labeled 5'-AMP SSB substrate (25 nM) for 1 h at 30°C in the presence or absence of 1 mM ATP and/or 2 U of T4 DNA ligase, as indicated. Reaction products were fractionated by denaturing PAGE and detected by phosphorimaging. The positions of the 32 P-labeled 43-mer reaction product and the 32 P-labeled 25-mer containing or lacking 5' AMP are indicated. M'rkrs, markers. (D) Complementation of the SSB defect in *Apx*^{-/-} primary astrocyte extracts by recombinant T4 DNA ligase. *Apx*^{+/+} (+/+) or *Apx*^{-/-} (-/-) mouse astrocyte extracts (5 μ g) were incubated with a 32 P-labeled 5'-AMP SSB substrate (25 nM) for 1 h at 30°C in the presence or absence of 1 mM ATP and/or 2 U of T4 DNA ligase, as indicated. Reaction products were fractionated by denaturing PAGE and detected by phosphorimaging. The positions of the 32 P-labeled 43-mer reaction product and the 32 P-labeled 25-mer containing or lacking 5' AMP are indicated.

repair machinery that removes AMP from 5' termini during short-patch SSB reactions in vitro (1, 9, 19). However, whether or not aprataxin is required for chromosomal DNA strand break repair in vivo is unclear. This is because the occurrence of DNA strand breaks with 5' AMP has not yet

been demonstrated. Moreover, attempts to detect a chromosomal DNA repair defect in AOA1 cells have proved conflicting and unclear (14, 15, 20). To resolve the latter issue, we carefully measured chromosomal SSB rates in a variety of aprataxin-defective cell lines, including genetically matched

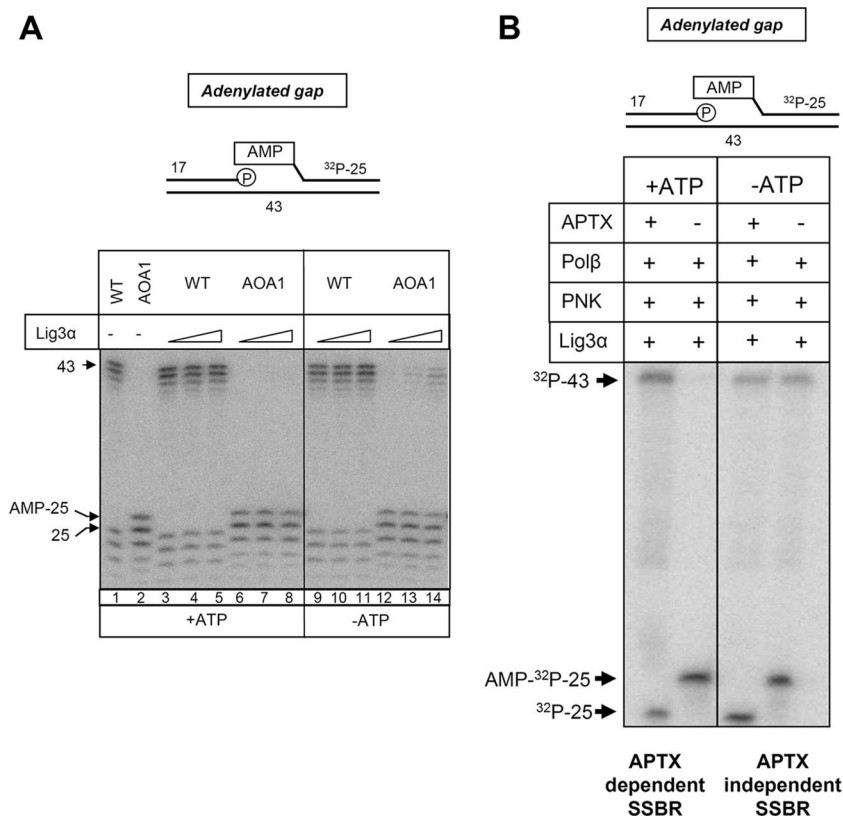


FIG. 5. Complementation of the SSBR defect in AOA1 lymphoblastoid cell extracts by recombinant human Lig3 α and reconstitution of aprataxin-independent SSBR with recombinant human proteins. (A) Complementation of the SSBR defect in AOA1 lymphoblastoid cell extracts by recombinant DNA ligase III α (Lig3 α). Total WT or AOA1 lymphoblastoid cell extracts (5 μ g) were incubated with a 32 P-labeled 5'-AMP SSB (100 nM) substrate for 1 h at 30°C in the presence or absence of 1 mM ATP and/or 8 nM, 24 nM, or 72 nM Lig3 α , as indicated. (B) Reconstitution of aprataxin-independent SSBR with recombinant human proteins. A 5'-AMP SSB substrate (60 nM) was incubated with 250 nM recombinant PNK, 100 nM Pol β , and 80 nM Lig3 α in the presence or absence of 100 nM aprataxin (APTX) and 1 mM ATP, as indicated, for 1 h at 30°C. Reaction products were fractionated by denaturing PAGE and detected by phosphorimager.

WT and *Aptx*^{-/-} quiescent neural cells. These experiments revealed that the overall rate of SSBR is normal in aprataxin-defective cells. Notably, we also failed to detect reduced rates of DSBR in AOA1 (unpublished observations).

To resolve the discrepancy between the requirement for aprataxin for SSBR in vitro versus that in vivo, we identified the stage at which short-patch SSBR fails in AOA1 in vitro. Notably, 3'-DNA end processing and DNA gap filling occurred normally during SSBR in the absence of aprataxin, despite the persistence of AMP on the 5' terminus. However, SSBR reactions subsequently stalled at the final step of DNA ligation, resulting in the accumulation of adenylated DNA nicks. This was surprising, because adenylated DNA nicks are normal, indeed prerequisite, intermediates of DNA ligation reactions, requiring only nonadenylated DNA ligase to reseal the breaks. We therefore reasoned that DNA ligation might fail in AOA1 cell extracts because of insufficient levels of nonadenylated DNA ligase. This would be consistent with the notion that while DNA ligases exist in both adenylated and nonadenylated states, the former predominates in cells because of the cellular ATP concentration. This would not pose a problem in WT cells, in which aprataxin activity ensures that DNA nicks arising during SSBR are not preadenylated and thus are substrates for adenylated DNA ligase. However, in AOA1 cells, DNA

nicks can arise in a preadenylated state during SSBR which thus require nonadenylated DNA ligase. In short, we propose that while all cells possess low levels of nonadenylated ligase, due to the rapid adenylation of free ligase molecules by cellular ATP, only in aprataxin-defective cells do preadenylated nicks arise at a level that exceeds the availability of nonadenylated ligase.

Confirmation that short-patch SSBR fails in AOA1 due to insufficient levels of nonadenylated DNA ligase emerged from our finding that addition of recombinant T4 DNA ligase or human Lig3 α restored short-patch SSBR in AOA1 extracts and *Aptx*^{-/-} quiescent astrocyte extracts in the absence of aprataxin. Importantly, complementation was more efficient if ATP was omitted from the reaction mixtures, supporting the notion that the complementing factor was nonadenylated DNA ligase. This may also explain the apparent difference between the abilities of T4 DNA ligase and Lig3 α to complement the defect (compare Fig. 4C and 5A), since it is likely that the relative amounts of nonadenylated ligase present in the two DNA ligase preparations were different.

The finding that adenylated nicks accumulate during short-patch SSBR in AOA1 provides a possible explanation for the normal rate of chromosomal SSBR observed in this disease. This is because adenylated nicks can be channeled into long-

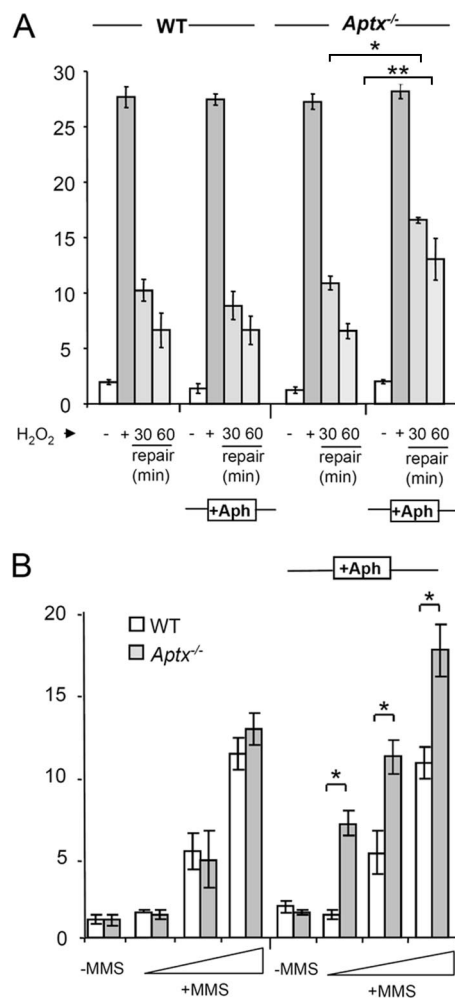


FIG. 6. Defective short-patch SSB repair in quiescent primary *Apx*^{-/-} mouse neural cells. (A) Quiescent WT and *Apx*^{-/-} mouse astrocytes were either left untreated (-) or treated with 75 μ M H₂O₂ for 10 min on ice (+) and, where indicated, then incubated in drug-free medium for 30 or 60 min. Where indicated (+Aph), cells were preincubated with 50 μ M aphidicolin for 30 min at 37°C, and repair was conducted in the continued presence of aphidicolin. Chromosomal DNA strand breaks were then quantified by alkaline comet assays. Each bar represents the average mean tail moment from three independent experiments (error bars, ± 1 standard deviation). Asterisks indicate statistically significant differences (*, $P \leq 0.05$; **, $P \leq 0.01$). (B) Quiescent WT and *Apx*^{-/-} mouse astrocytes were either left untreated (-) or treated with 25, 50, or 100 μ M MMS for 10 min at 37°C with (+Aph) or without preincubation with 50 μ M aphidicolin. Chromosomal DNA strand breaks were then quantified by alkaline comet assays. Each bar represents the average mean tail moment from three independent experiments (error bars, ± 1 standard deviation). Asterisks indicate statistically significant differences (*, $P \leq 0.05$; **, $P \leq 0.01$).

patch SSB. In this pathway, damaged 5' termini are displaced as a single-stranded flap during gap filling from the 3' terminus and cleaved off by Flap endonuclease 1 (FEN1) (reviewed in reference 12). This process would not be detected by the short-patch repair assays employed in the current work, because oligonucleotide duplexes of the type employed here are not good substrates for long-patch repair reactions and because cleavage of the single-strand flap would remove the ³²P label in

our substrates. However, to determine whether long-patch repair might compensate for the loss of short-patch repair in aprataxin-defective cells, we exploited the observation that long-patch SSB repair employs the aphidicolin-sensitive DNA polymerases Pol δ and/or Pol ϵ (10, 13, 22). Because inhibition of Pol δ and/or Pol ϵ also prevents DNA replication during S phase and can thus impact DNA metabolism independently of long-patch SSB, we employed quiescent primary astrocytes for these experiments. Strikingly, whereas preincubation with aphidicolin did not affect SSB rates in WT cells, it revealed a significant chromosomal SSB defect in *Apx*^{-/-} primary quiescent astrocytes following treatment with either H₂O₂ or MMS. SSB was slowed rather than prevented by aphidicolin, perhaps reflecting the fact that only a subset of DNA strand breaks induced by these agents possess 5'-AMP termini. Alternatively, it is possible that *Apx*^{-/-} quiescent astrocytes possess additional, partially overlapping SSB pathways. Nevertheless, these data demonstrate, for the first time, the involvement of aprataxin in chromosomal SSB *in vivo*.

It remains to be determined whether long-patch repair can also compensate for defective short-patch repair in AOA1 cells, as appears to be the case in *Apx*^{-/-} mouse neural cells. However, if this is the case, why do aprataxin mutations result in disease? One possibility is that a subset of SSBs arise at which long-patch repair cannot operate. For example, it is possible that aprataxin is also required to repair specific types of damaged 3' termini (26). Long-patch repair would be unable to operate at such breaks, because no 3'-hydroxyl primer terminus is available for DNA gap filling. Alternatively, since aprataxin is associated with the DSB repair machinery (8), it is possible that unrepaired DSBs might account for AOA1. It should be noted, however, that we have so far failed to detect a DSB defect in AOA1 or *Apx*^{-/-} cells (unpublished observations). Finally, it is possible that long-patch repair is not operative or is attenuated in the specific cell types that are affected in AOA1. For example, a number of replication-associated proteins, including several of those implicated in long-patch repair, are downregulated in certain differentiated cell types (21). Thus, it will now be of interest to expand the experiments described in this study to other cell types, including those neural cell types most likely affected in AOA1.

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