Aprataxin resolves adenylated RNA–DNA junctions to maintain genome integrity

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Faithful maintenance and propagation of eukaryotic genomes is ensured by three-step DNA ligation reactions used by ATP-dependent DNA ligases1,2. Paradoxically, when DNA ligases encounter nicked DNA structures with abnormal DNA termini, DNA ligase catalytic activity can generate and/or exacerbate DNA damage through abortive ligation that produces chemically adducted, toxic 5′-adenylated (5′-AMP) DNA lesions3–6. Aprataxin (APTX) reverses DNA adenylation but the context for deadenylation repair is unclear. Here we examine the importance of APTX to RNase-H2-dependent excision repair (RER) of a lesion that is very frequently introduced into DNA, a ribonucleotide. We show that ligases generate adenylated 5′ ends containing a ribose characteristic of RNase H2 incision. APTX efficiently repairs adenylated RNA–DNA, and acting in an RNA–DNA repair (RER) of a lesion that is very frequently introduced into DNA, acts in an RNA–DNA ligase1,2. Paradoxically, when DNA ligases encounter nicked DNA structures with abnormal DNA termini, DNA ligase catalytic events producing sealed DNA ends (39-nucleotide product, blue bars) are impaired at incised RNA–DNA junctions1,14. We compared the ability of human DNA ligase I to seal a nick containing canonical 3′-OH and 5′-P termini to a nick containing a 3′-OH and a 5′-P attached to a rG that mimics a nick generated when RNase H2 initiates RER (Fig. 1b). Greater than 95% of the nicked DNA substrate containing the 3′-OH and 5′-P termini was ligated within 10 min. In contrast, the presence of a single ribonucleotide (rG) on the 5′ side of the nick (5′ RNA substrate, Fig. 1b) significantly impaired generation of the 39-nucleotide ligation product (<1% ligation at 10 min, Extended Data Fig. 1a). Ligase I processing of the 5′ RNA substrate also produced an additional species migrating at a size of ~20 nucleotides, that corresponds to a bona fide 5′-adenylated product (5′-AMPDNA-P) (Fig. 1b and Extended Data Fig. 1b). The adenylated product comprises greater than 50% of all DNA ligase I catalytic events on the 5′ RNA substrate at all time points measured (Fig. 1c and Extended Data Fig. 1a). Also, human DNA ligase III and bacteriophage T4 DNA ligase, but not Escherichia coli NAD-dependent LigA, generated similar amounts of ribonucleotide-triggered abortive ligation products (Fig. 1c). Thus, incised RNA–DNA junctions

Figure 1 | Abortive ligation at RNA–DNA junctions is resolved by APTX.

a. ATP-dependent DNA ligation: (1), ATP-dependent DNA ligase adenylation; (2), AMP is transferred to the DNA 5′ phosphate to form 5′-AMP; (3), alignment of a DNA 3′-hydroxyl with 5′-AMP within the ligase active site facilitates the nick-sealing reaction. Ligase encounter with distorting termini triggers abortive ligation. 

b. DNA ligation is aborted at RNA–DNA junctions.

c. Substrates containing DNA or RNA are incubated with human APTX and ligation reactions are resolved on polyacrylamide gels. Ligase I or II (lanes 1 and 2) was added to unmodified DNA substrates (lanes 1–4). AMP accumulation is reduced in the presence of APTX (lane 5).

d. Human APTX DNA-adenylate hydrolysis. Reactions contained 2 nM human APTX and 10 nM of the indicated substrate.
are poor substrates for eukaryotic DNA ligase nick-sealing reactions, and also trigger abortive ligation at high frequency in vitro.

Apratxin deadenylase (APTX in mammals and Schizosaccharomyces pombe, and Hnt3 in Saccharomyces cerevisiae) reverses DNA adenylation3–5. Inactivation of APTX in ataxia oculomotor apraxia 1 (AOA1)16–17 suggests that persistent adenylylated DNA strand breaks drive cerebellar degeneration in neurological disease6. However, the molecular context for APTX deadenylation remains uncertain. To examine a role for APTX during RER, we compared steady-state kinetic parameters for deadenylation by human APTX on gel-purified abortive ligation substrates arising from metabolism of RNA–DNA junctions (5′-AMP–DNA) to those representative of deadenylation on DNA single-strand breaks created by reactive oxygen species (5′-AMPSSB) (Fig. 1d and Extended Data Fig. 1c). Both substrates were efficiently processed with comparable rates (kcat = 0.31 versus 0.37 s−1) with catalytic efficiencies that are ~30,000-fold higher than those reported on nucleotide substrates18. A ~6-fold higher kcat/Km for 5′-AMP–DNA versus 5′-AMPSSB indicates that human APTX displays an in vitro preference for the RNA–DNA-derived substrates.

Both S. pombe Apx and S. cerevisiae Hnt3Apts also harbour 5′-AMP–DNA deadenylase activity (Extended Data Fig. 1d, e). To determine whether Apx deadenylates abortive ligation products generated at RNA–DNA junctions in vivo, we examined whether the phenotypes of budding yeast strains with varying capacity to incorporate and repair ribonucleotides were altered by Hnt3Apts deficiency (Fig. 2). A M644G variant of the leading strand replicase, DNA polymerase ε (Pol ε, encoded by the POL2 gene, see Extended Data Table 1), has increased capacity to incorporate ribonucleotides into DNA in vitro and in vivo13,19. We generated heterozygous diploids in which one copy of HNT3 was replaced with the hnt3Δ mutant (reduced genomic ribonucleotides) is consistent with the model wherein Hnt3Aptx deadenylates genotoxic abortive ligation intermediates arising during RER of ribonucleotides incorporated by Pol ε during DNA replication (Fig. 2e). A genetic interaction between HNT3 and RNH201 is not apparent in a POL2 strain, possibly because ade- nylated RNA–DNA junctions may be removed by alternative nucleolytic processing, for example, mediated by Rad2720 and Mre11/Rad50/Xrs221 nuclease.

Having implicated apratixins in processing 5′-AMP–DNA in vitro and in vivo, we aimed to define the molecular basis for 5′-AMP–DNA processing by human APTX. Structural analysis of the S. pombe APTX DNA complex revealed the architecture of the yeast Apx Hit-2nfd domain, and a basis for engagement of DNA ends3. However, the molecular basis for the APTX RNA–DNA interactions, and the mechanism of the APTX DNA damage direct reversal catalytic reaction, remain

Figure 2 | Yeast Hnt3Apts is critical for resolving abortive ligation intermediates that arise after incision at genomic ribonucleotides by Rnase H2. a, Tetrad analysis of hnt3Δ/ntagMX diploids. 1–8 are tetrad dissections and A–D are haploid spore colonies. Right: day 3 microscopic spore colonies in the pol2-M644G hnt3Δ strains. b, Tetrad analysis of HNT3/ hnt3ΔntagMX diploids in the pol2-M644G nsh201Δ background. Plates imaged at 3 days. c, Deletion of HNT3 in the pol2-M644G mutator confers a slow growth phenotype that is eliminated by deleting RNH201. Doubling times (D) were calculated from cultures in the logarithmic phase of growth in rich medium at 30 °C. Average doubling time ± s.d. are calculated from four biological replicates (eight for the pol2-M644G hnt3Δ genotype. *P < 0.0007; **P < 0.0011 (two-tailed t-test). d, Immunoblotting of whole-cell extracts was performed using an antibody to Rnr3. e, Rnase H2 cleavage at ribonucleotides incorporated during Pol ε leading-strand DNA synthesis leads to abortive ligation intermediates requiring APTX processing. Deletion of HNT3 (hnt3Δ) or APTX deficiency in ataxia oculomotor apraxia 1 (AOA1) creates persistent adenylylated strand breaks.

Notably, deleting RNH201 (nsh201Δ) largely mitigated the growth defect of the pol2-M644G hnt3Δ mutant (Fig. 2b, c). This observation indicates that incision of ribonucleotides in DNA by Rnase H2 generates an RER intermediate leading to production of 5′-AMP–DNA that requires deadenylation by Hnt3Apts (see model, Fig. 2e).

Increased Rn3 protein level is a sensitive indicator of S-phase checkpoint activation12,20. An increased level of the Rn3 subunit of ribonucleotide reductase was detected in pol2-M644G hnt3Δ cells (Fig. 2d, lane 6), but was reduced in the triple mutant pol2-M644G hnt3Δ nsh201Δ strain (lane 8) to a level equivalent to that of a pol2-M644G nsh201Δ mutant (lane 7). This suggests that failure of Hnt3Apts to deadenylate 5′-AMP–DNA lesions activates the S-phase checkpoint. We also tested hnt3Δ mutant strains for sensitivity to genotoxic stress caused by hydroxyurea (HU). HU treatment increases rNMP incorporation16 and induces replication fork stalling. Growth of the pol2-M644G hnt3Δ mutant on rich medium was slowed, and survival in the presence of HU was reduced (Extended Data Fig. 2b, c). Notably, deleting RNH201 reduced HU sensitivity to a level comparable to pol2-M644G nsh201Δ cells (Extended Data Fig. 2c).

Next we examined the consequences of loss of Hnt3 function in yeast strains containing a Pol ε variant with reduced capacity to incorporate ribonucleotides, pol2-M644L (ref. 13). With fewer ribonucleotides in the genome, the pol2-M644L hnt3Δ mutant displayed normal growth (Fig. 2c) and was unaffected by deleting RNH201. The stark contrast between the consequences of loss of Hnt3 function in the pol2-M644G variant (high genomic ribonucleotides) versus the pol2-M644L mutant (reduced genomic ribonucleotides) is consistent with the model wherein Hnt3Aptx deadenylates genotoxic abortive ligation intermediates arising during RER of ribonucleotides incorporated by Pol ε during DNA replication (Fig. 2e). A genetic interaction between HNT3 and RNH201 is not apparent in a POL2 strain, possibly because adenylylated RNA–DNA junctions may be removed by alternative nucleolytic processing, for example, mediated by Rad2720 and Mre11/Rad50/Xrs221 nuclease.

Having implicated apratixins in processing 5′-AMP–DNA in vitro and in vivo, we aimed to define the molecular basis for 5′-AMP–DNA processing by human APTX. Structural analysis of the S. pombe APTX DNA complex revealed the architecture of the yeast Apx Hit-2nfd domain, and a basis for engagement of DNA ends3. However, the molecular basis for the APTX RNA–DNA interactions, and the mechanism of the APTX DNA damage direct reversal catalytic reaction, remain
The APTX-bound RNA–DNA junction is significantly distorted from the 5'–3' representation helices (cylinders) and β-strands. DNA is displayed as magenta duplex, with a green 5'-ribonucleotide and yellow AMP lesions. The four conserved elements dictate interactions with the 5'-ribonucleotide (green) and 5'-AMP (yellow with orange/red phosphate group). The β2-β3 loop (orange), HIT ξ1 (gold), Znf ζ3 (blue) and ΗΗΗΦΗ loop (dark green) completely orient and envelop the 5'-adenylated ribonucleotide lesion for catalytic processing.

The first step of the APTX reaction is proposed to generate a covalent enzyme–AMP intermediate, via an enzyme–nucleic acid transition state that poses a significant challenge to protein structural interrogation. To trap this transition state, we developed reaction conditions under which APTX activity is inhibited when co-incubated with adenosine, orthovanadate and a 5'-phosphorylated RNA–DNA junction duplex (Extended Data Fig. 3g, h). Reaction of human APTX with these reagents in crystallo produced a mimic of the enzyme–RNA–DNA–AMP transition state intermediate for step 1 of a two-step deadenylation reaction (Fig. 4a and Extended Data Fig. 3i, j).

The ΗΗΗΦΗ loop completely encircles the adenylated 5'-ribonucleotide lesion (Fig. 4), with His 260 covalently bonded to a pentavalent metal ion. Four conserved elements dictate interactions with the 5'-ribonucleotide (green) and 5'-AMP (yellow with orange/red phosphate group). The β2-β3 loop (orange), HIT ξ1 (gold), Znf ζ3 (blue) and ΗΗΗΦΗ loop (dark green) completely orient and envelop the 5'-adenylated ribonucleotide lesion for catalytic processing.

The HIT domain is displayed as cartoon representation helices (cylinders) and β-strands. DNA is displayed as magenta duplex, with a green 5'-ribonucleotide and yellow AMP lesions. The four conserved elements dictate interactions with the 5'-ribonucleotide (green) and 5'-AMP (yellow with orange/red phosphate group). The β2-β3 loop (orange), HIT ξ1 (gold), Znf ζ3 (blue) and ΗΗΗΦΗ loop (dark green) completely orient and envelop the 5'-adenylated ribonucleotide lesion for catalytic processing.
Human APTX is found in two markedly different conformations in the product-bound structure. The first conformation (the ‘assembled active site’) Fig. 4b and Extended Data Fig. 7a,b) has an intact active site characterized by close interactions between HIT α1 (Leu 171 and Trp 167) and the ΗΦΗΦΗ loop, and correct positioning of His 260 for catalysis. This state has the His 260 imidazole ring hydrogen bonded to the His 268 main-chain carbonyl oxygen. In the second state (the ‘disassembled active site’), α1 is displaced by ~4 Å relative to a rearranged ΗΦΗΦΗ loop, and His 260 is flipped out of alignment for nucleophilic attack (compare Fig. 4b and c). Structural overlays (Fig. 4e) and interpolations between these two states (Supplementary Videos 1 and 2) indicate that concerted conformational rearrangements sculpt the ΗΦΗΦΗ loop, and may be linked to RNA–DNA substrate binding by α1 and ΗΦΗΦΗ (Extended data Fig. 7b–c). We propose that interactions between RNA–DNA and protein proximal to the active site regulate active-site conformations involving HIT α1. RNA/DNA-regulated assembly of the APTX active site may ‘license’ catalytic activity and also prevent inappropriate, nonspecific hydrolysis of nucleotides (for example, ATP or ADP hydrolysis). Discrimination against ATP cleavage may be critical for mitochondrial APTX isoforms that have previously been implicated in DNA damage repair in mitochondria, because off-target catalysis could imbalance nucleotide pool levels.

Both missense and truncating APTX substitutions are linked to neurodegenerative disease. On the basis of the human APTX structures determined here, we predict that most AOA1 mutations (D185E, A198V, P206L, G231E, R247X, V263G, D267G, W279X, W279R and R306X) will decrease protein stability by truncating the polypeptide or by altering the protein-folding core (Extended Data Fig. 8a). Conformational differences between our RNA–DNA bound structures extend into the protein core (Extended Data Fig. 7a). APTX conformational changes may thus be subject to mutagenic modulation in disease. We posit that differential impacts on protein folding, active-site chemistry and substrate induced-fit active site assembly may all contribute to the variable clinical outcomes observed in patients with APTX defects.

One AOA1 mutation is found in the RNA–DNA substrate interaction cleft (K197Q) and two participate directly in active-site chemistry (H201R and H201Q) (Fig. 4a–c and Extended Data Fig. 8a). The late-onset AOA1 variant APTX(K197Q) displays significantly impaired deamination activity on both the 5’-AMP<sub>KSR</sub> and 5’-AMP<sub>RNA-DNA</sub> substrates (Extended Data Fig. 6b). To understand the molecular basis for the K197Q defect, we determined a 1.90 Å X-ray structure of APTX(K197Q) bound to RNA–DNA and AMP that reveals the mutant protein harbours a distorted active-site pocket (Fig. 4f and Extended Data Fig. 8b, c). In the wild-type protein, Lys 197 participates in salt-bridging interactions with the 5’-terminal sugar-phosphate backbone and the AMP lesion 2’-hydroxyl. In the mutant, Gln 197 is rotated away from the substrate-binding pocket and substitutes direct protein–substrate interaction with a protein–water–substrate nucleic acid binding interaction, thus revealing that distortions in the APTX(K197Q) substrate-binding pocket underlie AOA1.

Our data indicate that during repair of non-canonical ribonucleotides introduced into DNA during replication of the nuclear genome, DNA ligases generate 5’-adenylated RNA–DNA junctions that can elicit a DNA damage checkpoint response unless this is prevented by APTX deamidase. In addition to frequent ribonucleotide incorporation by DNA replicases, rNTPs are used by RNA primase to initially
synthesize ~5% of the nascent lagging strand, and rNTPs are also incorporated during mitochondrial DNA replication25-26, during trans- lesion synthesis27, and during DNA repair28. Ribonucleotide incorporation during DNA repair may be more prevalent in non-proliferating cells because dNTP concentrations are lower29,30 thereby increasing rNTP:dNTP ratios30. Thus, the late onset of AA01 might partly reflect failure to deadenylate RNA–DNA junctions resulting from ribonucleotides incorporated in DNA transactions occurring over many years in quiescent neurons. It will be important in future work to establish quantitative measurements of RNA–DNA adenylation to explore this hypothesis.

In this context, APTX acts in a nucleic acid transaction that is not exclusively DNA or RNA. Instead, using a reaction mechanism that is finely tuned to operate on RNA–DNA junctions, APTX acts in an RNA–DNA damage response (RDDR) to protect the genome from a compound insult, a ribosylated, adenylated 5’ terminus. In a broader sense, it seems probable that other enzymes may also modulate the RDDR via the detection, processing and signalling of RNA–DNA-derived structures posing threats to genomic integrity.

METHODS SUMMARY

Proteins were expressed in Escherichia coli and purified with standard procedures. All crystals were grown using sitting-drop vapour diffusion. X-ray diffraction data were all collected at 100 K at the Advanced Photon Source, beamlines 22-ID and 22-BM. Initial DNA-bound human APTX structures were solved by molecular replacement with the S. pombe APTX–DNA complex (PDB code 3SZQ). RNA–DNA-bound wild-type and mutant human APTX structures were solved by molecular replacement using the refined human APTX–DNA-bound model. S. cerevisiae strain construction and growth assays were performed as described2-4.

Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions P.T. performed biochemical studies and crystallization. M.J.S. and R.S.W. solved and refined X-ray structures. J.S.W. performed S. cerevisiae experiments. All authors contributed to experimental design, data analysis and preparation of the manuscript.

Author Information Molecular coordinates and structure factors for X-ray structures reported here have been deposited in the RCSB Protein Data Bank under accession codes 4Q72 (human APTX–RNA–DNA–AMP–Zn complex), 4NDG (human APTX–RNA–DNA–adenosine–vanadate–Zn complex), 4NDH (human APTX–DNA–AMP–Zn complex) and 4NDI (human APTX(K197Q)–RNA–DNA–AMP–Zn complex). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to R.S.W. (williamsrs@niehs.nih.gov).
**METHODS**

**APTX protein expression and purification.** An *Escherichia coli* codon-optimized coding sequence (GenScript) facilitated robust recombinant overexpression of human APTX. Human APTX full-length protein and truncated variants were expressed from pET15b as N-terminal His-tagged proteins in *E. coli* BL21(DE3) codon-plus cells (Novagen). APTX modifications were introduced using the Quickchange system (Stratagene). Cell cultures were grown at 37 °C in LB medium containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) until *A. oak* reached 0.8 to 1, at which time cells were cooled to 16 °C and grown for an additional 8–12 h, without IPTG induction. Cells were lysed by sonication in lysis buffer (50 mM Tris, pH 8.5, 500 mM NaCl, 10 mM imidazole, 0.01 g/l lysozyme, with Roche mini EDTA-free protease inhibitor). The soluble lysate was applied to Ni-NTA column (5 ml, Qiagen) and 6× His-tagged human APTX proteins were eluted in lysis buffer with 300 mM imidazole. The 6×His tag was removed with overnight thrombin digestion (50 U) (Sigma) at 4 °C. Subsequent purification was achieved by size-exclusion chromatography (Superdex 75, GE healthcare in 50 mM Tris, pH 7.5, 500 mM NaCl, 5% glycerol, 0.1% β-mercaptoethanol) and calcium exchange chromatography on a 5-ml HiTrap SP HP (GE Healthcare). S. pombe APTx was expressed and purified as previously described.

**DNA ligation reactions.** For DNA ligation experiments, human DNA ligase I (500 nM), oligonucleotides 1, 2, and 4, Extended Data Fig. 3d) or 5′-DNA (50 nM, oligonucleotides 1, 2 and 5, Extended Data Fig. 3d) in 10 mM Tris, pH 7.5, 2 mM MgCl₂, 1 mM DTT, and DNA ligase III b (20 U, New England Biolabs), 50 mM Tris-HCl, pH 7.5, 1 mM β-mercaptoethanol with an equal volume (300 nl) of the precipitant solution (100 mM NaCl, 20 mM Tris-HCl, pH 7.5 and 0.1% β-mercaptoethanol) with an equal volume (300 nl) of precipitant solution (150 mM magnesium acetate, 8% w/v polyethylene glycol 3350). Single crystals appeared overnight after incubation at 4 °C. Crystals of the human APTX–DNA–AMP product complex (oligonucleotide 9, Extended Data Fig. 3d) were grown by mixing complex solution 2 (10 mg/ml human APTX(165–342), 1 mM AMP, 1:1 DNA:protein ratio, and 150 mM NaCl, 20 mM Tris-HCl, pH 7.5 and 0.1% β-mercaptoethanol) with an equal volume (300 nl) of precipitant solution 2 (150 mM magnesium acetate, 8% w/v polyethylene glycol 3350). Single crystals appeared overnight after incubation at 4 °C. Crystals of the human APTX–DNA–AMP product complex (oligonucleotide 6 and 7, Extended Data Fig. 3d) were grown by mixing complex solution 3 (10 mg/ml human APTX(165–342), 1 mM AMP, 1:1 DNA:protein ratio, and 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, and 0.1% β-mercaptoethanol) with an equal volume (300 nl) of precipitant solution 3 (200 mM potassium sodium tartrate, 20% w/v polyethylene glycol 3350). Single crystals appeared overnight after incubation at 4 °C. Crystals of the human APTX–RNA–DNA–vanadate–adenosine–Zn transition state complex (oligonucleotides 6 and 7, Extended Data Fig. 3d) were grown by mixing complex solution 4 (10 mg/ml human APTX(165–342), 1 mM adenosine, 1:1 DNA:protein ratio, and 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, and 0.1% β-mercaptoethanol) with an equal volume (300 nl) of precipitant solution 4 (100 mM sodium formate, 20% w/v polyethylene glycol 3350). The adenosine–RNA–DNA bound crystals were then soaked and reacted for 1 h at 4 °C in cryoprotectant solution supplemented with 0.5 mM orthovanadate. Crystals of the human APTX(197Q)–RNA–DNA–AMP product complex (oligonucleotides 6 and 7, Extended Data Fig. 3d) were grown by mixing complex solution 5 (10 mg/ml human APTX(165–342)/K197Q), 1 mM AMP, 1:1 RNA–DNA:protein ratio, and 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, and 0.1% β-mercaptoethanol) with an equal volume (300 nl) of precipitant solution 5 (200 mM potassium acetate, 20% w/v polyethylene glycol 3350). All crystals were washed in cryo-protectant (precipitant solutions supplemented with 12% glycerol) and flash frozen in liquid nitrogen for data collection. Diffraction data were processed with HKL2000 (ref. 32).

**Yeast strains.** *Saccharomyces cerevisiae* strains used are isogenic derivatives of strain Δ(-1)-2/*B-YUN300 (MATa CAN1 his7-2 leu2-3 a::kanMX ura3-1 trp1-289 ade2-1 lys2-1 D28G2299-2900 alpha1::URA3-OR1 or OR2)*. Relevant strain genotypes are listed in Extended Data Table 1. HNT3 deletion strains were generated by two experimental strategies. For the POL2 (Pol ε-wild type) and pol2-M446S strains, diploids homozygous for the polymerase mutation (and rnh201A, where applicable) were made heterozygous for *HNT3* (*Hnt31A*) by PCR-based targeted gene disruption. Deletion of one copy of the *HNT3* gene was verified by PCR, haploids were obtained from tetrads and were verified by PCR, appropriate drug-resistance and sequencing of the POL2 locus. Dissection plates were photographed after 3 days growth on rich medium (YPD: 1% yeast extract, 2% bacto-peptone, 250 mg l⁻¹ adenine, 2% dextrose, 2% agar for plates). For the pol2-M644L strains (±RHN201), deletion-replacement of *HNT3* was performed via transformation with a PCR product containing the nourseothricin-resistance cassette (matnMX) amplified from pAG25 and flanked by 60 nucleotides of sequence homologous to the intergenic regions upstream and downstream of the *HNT3* open reading frame. Transformants that arose from homologous recombination were confirmed by proper drug resistance and PCR analysis.

**Human APTX structure solution and refinement.** Molecular replacement phases for the human APTX–DNA–AMP–Zn complex (complex 1) to 3.0 Å were obtained by using a systematic molecular replacement search varying ~190 model and data parameters in PHASER, and testing multiple molecular constructs based on an S. pombe APTx–DNA complex (3SSQ) (~29% identity with human APTX). The successful solution (PHASER Tetz = 0.6, LLC = 195, Refmac Rfree = 48.9%) was obtained with a Phasor quality score of 699, and molecular replacement phases were improved and extended to 2.5 Å using prime-and-switch density modification with NCS averaging in RESOLVE<sup>18</sup>.<sup>19</sup> The initial model was fit manually in O (v10.0) and partially refined in Refmac<sup>20</sup>. This model was then used for molecular replacement searches to phase the human APTX–RNA–DNA–AMP–Zn (at 1.95 Å, 2 molecules per ASU), human APTX–DNA–AMP (at 1.85 Å, 2 molecules per ASU), and human APTX(1K97Q)–RNA–DNA–AMP–Zn product complexes (at 1.90 Å, 2 molecules per ASU). After autobuilding using PHENIX Autobuild<sup>36</sup>, models were fit manually in Coot<sup>38</sup> and autobuild-and-refine-and-translusion at 1.90 Å, 2 molecules per ASU. After autobuilding using PHENIX Autobuild, models were fit manually in Coot<sup>38</sup> and autobuild-and-refine-and-translusion at 1.90 Å, 2 molecules per ASU. After autobuilding using PHENIX Autobuild, models were fit manually in Coot<sup>38</sup> and autobuild-and-refine-and-translusion at 1.90 Å, 2 molecules per ASU.
Strain doubling times ($D_t$) were calculated from measurements of the OD$_{600}$ nm of cultures in the logarithmic phase of growth in rich medium at 30 °C over a 17-h time course. $D_t$ measurements were made using data from four independent biological replicates (eight for the pol2-M644G hnt3Δ genotype) and the average $D_t$ ± standard deviation is displayed. Statistical analysis (Fig. 2d) was performed in GraphPad Prism. D’Agostino and Pearson omnibus normality test (alpha = 0.05) is passed using doubling time determinations for the pol2-M644G hnt3Δ strain ($n = 8$). The sample size is too small to run a normality test on the doubling time measurements for the hnt3Δ or pol2-M644G hnt3Δ rnh201Δ strains ($n = 4$ for each). The coefficients of variations in the data are as follows: 11.4% for the hnt3Δ strain, 19.1% for the pol2-M644G hnt3Δ strain and 15.2% for the pol2-M644G hnt3Δ rnh201Δ strain. A spot dilution assay was performed by spotting tenfold serial dilutions of mid-log phase cultures onto YPDA agar. Plates were incubated at 30 °C and photographed after 3 days of growth. A quantitative HU-survival assay was performed as described previously. Exponentially-growing cells were arrested in G1 for 3 h with α-factor (5 μg ml$^{-1}$). Approximately 500 cells from each strain were plated onto YPDA agar (untreated) or YPDA agar containing 100 mM HU, incubated for 3–6 days at 30 °C and counted. Percentage survival was calculated as the percentage of surviving cells compared to the untreated control. The experiment was performed in triplicate; data are displayed as the average ± standard deviation.

**Immunoblotting.** Whole-cell extracts were prepared from exponentially growing cells as described. The positive control is an extract prepared from wild-type cells treated with 200 mM HU for 3 h. Actin was probed as a loading control. Proteins were resolved on a 10% Bis-Tris gel (Life Technologies) and western blotting was performed using a 1:1,000 dilution of an affinity-purified rabbit polyclonal antibody against Rnr3 (Agrisera; AS09574). The anti-actin antibody (Millipore, MAB1501) was used at 1:1,000.

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Extended Data Figure 1  APTX homologues efficiently process products of abortive DNA ligation at RNA–DNA junctions. a, Abortive DNA ligation reaction time course. DNA ligation reactions for human DNA ligase I were monitored at the indicated time points. The per cent ligation was measured by quantifying the total amount of 39-nucleotide ligation product evolved as a percentage of total unprocessed substrate (Fig. 1b, 5’-P species). The per cent adenylation was measured by quantifying the total amount of 5’-AMP product evolved as a percentage of total unprocessed substrate. Substrates used are those described in Fig. 1b. Mean ± s.d. (n = 2 technical replicates) is displayed.

b, Recombinant human APTX specifically processes the adenylated product of DNA ligase I abortive ligation at an incised RNA–DNA junction. DNA ligation reactions (see Fig. 1b) on 5’-RNA substrates were stopped with addition of 5 mM EDTA at 240 min. Recombinant human APTX(165–342) catalytic domain was added after abortive ligation (lane 4). Human APTX specifically processed the 5’-AMPRNA–DNA species generated by abortive ligation at the RNA–DNA junction, but does not process the 19-nucleotide 5’-P substrate, or the 39-nucleotide ligation product, confirming the 5’-AMPRNA–DNA species is a bona fide adenylation product.

c, Kinetic parameters of human APTX processing of oxidative DNA damage and RNA–junction-derived adenylated substrates. Mean ± s.e.m. (n = 2 technical replicates) is displayed.

d, 5’-AMPRNA–DNA deadenylation activity is an evolutionarily conserved function of fission and budding yeast Aptx/Hnt3. Reaction conditions used were as for Fig. 1d.

e, Kinetic parameters of S. cerevisiae Hnt3/Aptx (Hnt3) deadenylation processing. Kinetic experiments were performed as described in the Methods. Mean ± s.e.m. (n = 2 technical replicates) is displayed.
Extended Data Figure 2 | Deletion of HNT3 in the pol2-M644G strain impairs growth and causes genotoxin sensitivity.  

a, Tetrad analysis of the HNT3/hnt3::natMX4 diploid in the pol2-M644G strain background reveals the severe growth defect of the haploid pol2-M644G hnt3Δ mutant. The plate was scanned after 6 days growth on rich medium. 

b, Deleting HNT3 in the pol2-M644G strain impairs growth and causes genotoxin sensitivity that is reduced on deletion of RNH201. b, Serial (tenfold) dilutions of cells were plated on rich medium with or without 100 mM HU and photographed after 3 days of growth at 30 °C. c, A quantitative HU-survival assay was performed as in ref. 12. Data are displayed as the mean ± s.d. (n = 3 independent experiments) with per cent survival calculated as the percentage of surviving cells (grown on YPDA agar + 100 mM HU) compared to the untreated control. *P < 0.0002 (two-tailed t-test).
Extended Data Figure 3 | Human APTX domain mapping and structure determination. a, APTX protein domain schematic. b, Protease stable domains in human APTX were identified with limited tryptic and chymotryptic digests. Left: chymotryptic proteolysis of full-length human APTX produces clustered cut sites (C1 through C3) flanking the N terminus of the human APTX histidine triad (HIT) domain helix. Right: tryptic proteolysis of full-length human APTX produces a major cut site (T1) near the N terminus of the HIT domain. This analysis reveals a meta-stable HIT-Znf domain encompassing residues 152–342, and a chymotrypsin resilient core bounding residues 172–342. c, A comparison of the DNA 5’-deadenylation activities of the chymotryptic C2 fragment (amino acids 168–342), a smaller tryptic fragment (T1, 180–342), and two designed recombinant fragments (HIT-Znf1, 155–342, and HIT-Znf2, 165–342) shows that the HIT-Znf2 fragment, but not the smaller C2 and T1 fragments, retain robust deadenylation activity. Furthermore, the N-terminal boundary of the minimal catalytic domain in the absence of damaged DNA substrate is accessible to proteolytic digest. The HIT-Znf2 (165–342) fragment was used for crystallization studies. d, Table of crystallization and assay oligonucleotides. e, The product complex crystallographic asymmetric unit contains two copies of the APTX–RNA–DNA–AMP–Zn complex. An omit (the RNA–DNA duplex and AMP were excluded from the model for electron density map calculation) σ-A weighted 1.95 Å $F_o - F_c$ map is displayed contoured at 3.0σ overlaid upon the AMP and RNA–DNA duplex for complex 1. DNA unwinding of the terminal base pair also facilitates crystallization and formation of a one-nucleotide base pair between the two complexes. f, An omit (the RNA–DNA and AMP were excluded from the model for electron density map calculation) σ-A weighted 1.95 Å $F_o - F_c$ map is displayed contoured at 3.5σ overlaid upon the terminal 5’-rG, showing clearly the location of the 2’-OH and the C3’-endo sugar pucker of the 5’-terminal nucleotide. g, Assembly of the APTX–RNA–DNA–adenosine–vanadate transition state mimic complex. Soaking of an adenosine RNA–DNA-bound crystal form with orthovanadate facilitates an in crystallo reaction, and formation of the APTX–RNA–DNA–adenosine–vanadate covalent complex. h, Adenosine and orthovanadate addition in the presence of a 5’-phosphorylated RNA–DNA duplex inhibits APTX activity on 5’-AMPRNA–DNA, suggesting formation of a specific APTX–RNA–DNA–adenosine–vanadate covalent complex in solution. i, Model phased anomalous difference fourier for the transition state mimic complex is calculated from a 2.85 Å data set for the transition state mimic complex crystal collected on the NIEHS rotating anode home source ($\lambda = 1.5418$ Å). A 3σ peak marks the position of the vanadium. j, Unbiased electron density for the enzyme transition state mimic covalent complex. The σ-A weighted 2$F_o - F_c$ electron density 2.5 Å map (contoured at 1.0σ) is calculated using a model in which the displayed atoms were not included, and before refinement.
Extended Data Figure 4 | APTX structure-based sequence alignment. The positions of AOA1 mutations are marked by red text corresponding to the missense single amino acid substitutions or nonsense (marked by red X) coding truncating mutations.
Extended Data Figure 5 | Human APTX RNA–DNA interactions.

a, Structural distortions of the human APTX-bound RNA–DNA. A cartoon representation of the duplex from the human APTX–RNA–DNA–AMP–Zn reaction product complex (magenta and green) is shown superimposed on an ideal B-form DNA duplex (grey), showing distortion of the 5’-terminal nucleotides from B-form geometry.

b, Electrostatic potential representation of the human APTX HIT–Znf DNA interaction interface is displayed with electropositive (blue), electronegative (red) and hydrophobic (white) surfaces. An extended positively charged surface of the Znf mediates sequence nonspecific RNA–DNA contacts. Hydrophobic base stacking stabilizes the exposed ribonucleotide base.

c, Trp 167 and Tyr 195 anchor the terminal ribonucleotide (green), and envelop the adenylate lesion (yellow). An omit σ-A weighted 1.95 Å F_o − F_c map is displayed contoured at 3.0σ overlaid upon the AMP and RNA–DNA duplex product complex. The RNA–DNA duplex and AMP were excluded from the model for electron density map calculation.

d, Human APTX protein RNA–DNA and AMP lesion binding contacts are displayed schematically.

e, The HIT (tan) and Znf (blue) surfaces mould a contiguous RNA–DNA damage interacting surface.

f, Molecular details of the Znf structure-specific DNA damage binding interface. DNA–protein contacts are mediated by four basic side chains (Lys 276, Lys 277, His 278, Lys 314) of Znf helices α3 and α5. Additional sugar-phosphate backbone contacts from Pro 330 and Ile 329, as well as the electropositive helix dipole of helix α6, also engage the undamaged strand.

g, Zn-binding by the human APTX C2H2 zinc finger subdomain. The Zn is coordinated with tetrahedral geometry by four zinc-binding residues (Cys 319, Cys 322, His 335 and His 339).
Extended Data Figure 6 | Recognition of RNA–DNA and DNA by human APTX. a, Structural overlays of DNA-bound (blue–grey) and RNA–DNA-bound (brown) human APTX complex structures. Inset: interactions at the 5’ terminus reveal similar modes of engagement of RNA–DNA and DNA-bound substrates. The β2-β3 loop residues Tyr 195 (Y195) and Lys 197 (K197) orient the 5’ terminus for catalysis. b, Effects of β2-β3 loop mutants on APTX deadenylation activity. Tenfold dilutions of APTX mutant proteins were tested for DNA adenylation activity on 5’-AMP<sub>RNA-DNA</sub> or 5’-AMP<sub>SSB</sub> substrates. Mean ± s.d. (n = 2 technical replicates) is displayed. Fold increase of protein to reach 50% activity is relative to the wild-type human APTX for each substrate.
**Extended Data Figure 7 | DNA end binding by α1 bridges active site conformations to DNA end sensing status.**

a, Structural overlays of three states reported in this study. b, Conformational rearrangements localize to the HIT N-terminal helix and the HIT HPHPH loop. HIT α1 is found in variable conformations, and movement of α1 is linked to rearrangement of the active site HPHPH loop. The conformation of Met 256, His 260 and His 258 is modulated by van der Waals interactions with Leu 171 and Trp 167 from α1 that together flank the HPHPH loop. In the disassembled active site conformer of the product complex, α1 migrates $-4\text{Å}$ away from HPHPH, with concomitant morphing of His 260 conformation into an inactive state.

c, Transition state complex $\sigma$-A weighted $2.55\text{Å}^2 F_o - F_c$ electron density map is displayed contoured at $1.0\sigma$ for the active site. A yellow sphere, ‘V’, marks the position of the vanadium covalently bonded to His 260, the 5'-phosphate of 5'-rG and adenosine.

d, Product complex (assembled active site) $\sigma$-A weighted $1.95\text{Å}^2 F_o - F_c$ electron density map is displayed contoured at $1.5\sigma$ for the active site.

e, Product complex (disassembled active site) $\sigma$-A weighted $1.95\text{Å}^2 F_o - F_c$ electron density map is displayed contoured at $1.5\sigma$.
Extended Data Figure 8 | Ataxia oculomotor apraxia (AOA1) mutations.
a, The positions of APTX mutations found in AOA1 are mapped onto the structure of human APTX. b, Omit 2Fo – Fc electron density for the K197Q variant. A solvent molecule occupies the position of the Lys 97 epsilon amino group of the wild-type protein, and Gln 197 rotates away from the active site pocket, into the protein core. c, Surface representation of the K197Q mutant (pink) overlaid upon wild-type APTX (grey). In K197Q, the active site pocket is distorted, and rearrangements in the protein core proximal to Gln 197 are also observed, and involve Leu 273.
### Extended Data Table 1 | *Saccharomyces cerevisiae* strains

| Name | Strain | Relevant Genotype | Source |
|------|--------|-------------------|--------|
| wt   | SNM8   | POL2              | ref. 13|
| mh201Δ | SNM106 | POL2 mh201::hphMX4 | ref. 13|
| pol2-M644G | SNM70 | pol2-M644G | ref. 13|
| pol2-M644L | SNM82 | pol2-M644L | ref. 13|
| pol2-M644G mh201Δ | SNM120 | pol2-M644G mh201::hphMX4 | ref. 13|
| pol2-M644L mh201Δ | SNM132 | pol2-M644L mh201::hphMX4 | ref. 13|
| pol2-M644G mh201Δ hnt3Δ | YJW122 | pol2-M644G mh201::hphMX4 hnt3::natMX4 | This study|
| hnt3Δ | YJW131 | POL2 hnt3::natMX4 | This study|
| hnt3Δ mh201Δ | YJW132 | POL2 hnt3::natMX4 mh201::hphMX4 | This study|
| pol2-M644G hnt3Δ | YJW177 | pol2-M644G hnt3::natMX4 isolate 1 | This study|
| pol2-M644G hnt3Δ | YJW208 | pol2-M644G hnt3::natMX4 isolate 2 | This study|
| pol2-M644G hnt3Δ | YJW210 | pol2-M644G hnt3::natMX4 isolate 3 | This study|
| pol2-M644L hnt3Δ | YJW183 | pol2-M644L hnt3::natMX4 | This study|
| pol2-M644L mh201Δ hnt3Δ | YJW154 | pol2-M644L mh201::hphMX4 hnt3::natMX4 | This study|
Extended Data Table 2 | X-ray data collection and refinement statistics

| Data set | ApbtxDNA/AMP/Zn | Apbtx/RNA--DNA/AMP/Zn | Apbtx-K197Q/RNA--DNA/AMP/Zn | Apbtx/RNA--DNA/adenosine/transition state/Zn | Apbtx/RNA--DNA/adenosine/vanadate/Zn/Cu Kα | Apbtx/DNA/AMP/Zn Molecular Replacement |
|----------|----------------|----------------------|----------------------------|---------------------------------|----------------------------------------|--------------------------------------|
| Reaction state | Product Complex | Product Complex | Product Complex | Transition state | Transition state | Product Complex |
| Data collection | | | | | | |
| Space group | P2₁2₁2₁ | P2₁2₁2₁ | P2₁2₁2₁ | P2₁2₁2₁ | P2₁2₁2₁ | P2₁ |
| Cell dimensions | | | | | | |
| a, b, c (Å) | 40.40, 117.08, 90.90 | 40.43, 116.21, 90.90 | 40.47, 116.17, 90.90 | 40.28, 114.58, 90.90 | 40.23, 114.46, 90.90 | 40.31, 119.25, 90.90 |
| α, β, γ (°) | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 | 90, 91.93, 90 |
| Resolution (Å) | 50.1-1.85 | 50.1-1.95 | 50.1-1.90 | 50.2-5.55 | 50.2-8.5 | 50.2-5.0 |
| Wavelength (Å) | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 1.5418 | 1.0000 |
| Rmerge | 0.063 (0.436) | 0.078 (0.588) | 0.102 (0.551) | 0.079 (0.562) | 0.074 (0.548) | 0.079 (0.338) |
| I / σI | 18.9 (2.5) | 16.8 (2.2) | 13.0 (2.4) | 15.6 (2.4) | 16.6 (2.1) | 16.1 (2.5) |
| Completeness (%) | 99.4 (98.5) | 99.9 (99.9) | 99.8 (100) | 99.3 (100) | 99.4 (99.3) | 95.7 (76.0) |
| Redundancy | 3.8 (3.3) | 4.0 (3.9) | 3.9 (3.9) | 4.0 (4.1) | 4.4 (4.0) | 3.4 (2.6) |

Each data set was collected from a single crystal. Values in parentheses are for highest-resolution shell (1.95 to 1.85 Å for the APTX-DNA-AMP product data set, 1.97 to 1.90 Å for the APTX(K197Q)-DNA-AMP product data set, 2.02 to 1.95 Å for the APTX-RNA-DNA-AMP product data set, 2.64 to 2.55 Å for the APTX-RNA-DNA-adenosine-vanadate transition state data set, 2.95 to 2.85 Å for the APTX-RNA-DNA-adenosine-vanadium-Cu Kα data set, and 2.59 to 2.50 Å for the APTX-DNA-AMP product molecular replacement data set). R_{free} calculated from 5% subset of randomly selected reflections.