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Base excision repair apurinic/apyrimidinic endonucleases in apicomplexan parasite *Toxoplasma gondii*

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Abstract

DNA repair is essential for cell viability and proliferation. In addition to reactive oxygen produced as a byproduct of their own metabolism, intracellular parasites also have to manage oxidative stress generated as a defense mechanism by the host. The spontaneous loss of DNA bases due to hydrolysis and oxidative DNA damage in intracellular parasites is great, but little is known about the type of DNA repair machineries that exist in these early-branching eukaryotes. However, it is clear processes similar to DNA base excision repair (BER) must exist to rectify spontaneous and host-mediated damage in *Toxoplasma gondii*. Here we report that *Toxoplasma gondii*, an opportunistic protozoan pathogen, possesses two apurinic/apyrimidinic (AP) endonucleases that function in DNA BER. We characterize the enzymatic activities of *Toxoplasma* exonuclease III (ExoIII, or Ape1) and endonuclease IV (EndoIV, or Apn1), designated TgAPE and TgAPN, respectively. Over-expression of TgAPN in *Toxoplasma* conferred protection from DNA damage, and viable knockouts of TgAPN were not obtainable. We generated an inducible TgAPN knockdown mutant using a ligand-controlled destabilization domain to establish that TgAPN is critical for *Toxoplasma* to recover from DNA damage. The importance of TgAPN and the fact that humans lack any observable APN family activity highlights TgAPN as a promising candidate for drug development to treat toxoplasmosis.

Keywords

BER; DNA damage; APE1; APN1; protozoa; malaria

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1. Introduction

The Base Excision Repair (BER) pathway is responsible for the repair of oxidative and alkylation DNA damage, resulting in protection against the deleterious effects of endogenous and exogenous agents encountered on a daily basis. It is the major DNA repair pathway that remedies oxidative DNA damage with little backup or alternative pathways [1]. Removal of the incorrect or damaged base by a DNA glycosylase comprises the first step of the BER pathway. The second step is mediated by an apurinic/apyrimidinic (AP) endonuclease, which hydrolyzes the phosphodiester backbone immediately 5′ to an AP site. This incision generates a normal 3′-hydroxyl group and an abasic deoxyribose-5-phosphate, which is processed by DNA polymerase β and ligase.

AP sites can arise spontaneously or be generated following exogenous treatment of cells by oxidative and alkylating agents as well as ionizing radiation [2–3]. If not repaired by AP endonucleases in dividing cells, AP sites have dramatic consequences as they can lead to single and subsequently double DNA strand breaks, which are lethal to the cell [4]. Bacteria, yeast, or human cells lacking AP endonuclease repair activity are hypersensitive to agents (e.g. alkylating or oxidizing) that induce the formation of AP sites [2]. Similar observations have been documented for the kinetoplastid protozoan, *Leishmania major*; in this case, over-expression of its AP endonuclease conferred protection against various agents known to induce DNA damage [5].

Class II endonucleases are specific for AP sites and lack DNA glycosylase activity. There are two families of class II AP endonucleases based on homology to the *E. coli* enzymes that are structurally unrelated and differ in their requirement for Mg^{2+}. Exonuclease III (ExoIII) is Mg^{2+}-dependent and exhibits a four-layered α/β-sandwich fold [6–7] while endonuclease IV (EndoIV) is Mg^{2+}-independent and has a α8/β8 TIM-barrel fold [8–9] including three tightly bound Zn^{2+} ions. Human Ape1 shares sequence homology with *E. coli* ExoIII and, like ExoIII in *E. coli*, serves as the predominant AP endonuclease in humans [10–11]. There is no evidence of an EndoIV AP endonuclease in mammals. In the yeast *Saccharomyces cerevisiae*, the primary AP endonuclease is an EndoIV homologue called APN1 [12]. The importance of EndoIV AP endonucleases on cell viability has been explored in some species. *S. cerevisiae* lacking APN1 are viable but hypersensitive to both oxidative and alkylating agents that damage DNA [13]. Knockdown of APN1 in *Caenorhabditis elegans* diminishes genomic stability, increases sensitivity to DNA damaging agents, and causes defective development [14]. In general, however, APN1 AP endonucleases have not been thoroughly characterized.

In addition to generating reactive oxygen species (ROS) during metabolism, intracellular protozoan parasites are also exposed to oxidative stress produced by immune effector cells [15]. It has been established that such parasites are highly sensitive to ROS [16]. We hypothesized that AP endonucleases in the obligate intracellular parasite *Toxoplasma gondii* (phylum Apicomplexa) would be critical for viability by protecting against DNA damage and particularly in the context of host immune insults on the parasite. *Toxoplasma* causes congenital birth defects and serious opportunistic disease in immunocompromised individuals, and is a relative of the malaria parasite *Plasmodium*. Current treatments to control acute toxoplasmosis caused by the proliferative tachyzoite stage are inadequate as these drugs have serious toxic side effects. Thus, an urgent need exists to identify new drug targets that may be exploited in the development of safer therapies. In this study we establish that apicomplexan parasites have both APE1 and APN1 homologues (TgAPE and TgAPN, respectively). We present multiple lines of evidence that identify TgAPN as an abundant and critical AP endonuclease in *Toxoplasma* tachyzoites. Through regulated

knockdown of the TgAPN protein, we also establish that this DNA repair enzyme is required for recovery from DNA damage.

2. Materials and methods

2.1. Parasite culture and growth assays

Toxoplasma gondii tachyzoites were cultured in human foreskin fibroblasts (HFF) in Dulbecco Modified Eagle's Media (Invitrogen) supplemented with 1% heat inactivated fetal bovine serum at 37°C and 5% CO_2. For plaque assays, 1000 parasites were inoculated into confluent HFF monolayers in 24-well plates and allowed to grow undisturbed for 7 days at 37°C. Infected monolayers were fixed in cold 100% methanol for 10 min prior to staining and counting. All plaque assays were performed in triplicate. For B1 assays, genomic DNA was collected at various time points from infected HFFs using the DNeasy kit (Qiagen) and analyzed as described [17]. Parasite doubling assays were performed in T-25 cm^2 flasks containing confluent HFF monolayers. 10^7 parasites inoculated into the flask and 50 random vacuoles were counted at each of the indicated time points.

2.2. Chemicals

Methyl methanesulfonate (MMS) was obtained from Sigma (M4016) in DMSO at a concentration of 11.8 M (1.3 g/ml) and stored at 4 °C. Shield-1 (CheminPharma, New Haven, catalog # CIP-S1) was dissolved in 100% ethanol at a concentration of 0.5 mM and stored at −20 °C. 200 nM Shield-1 was added to designated parasite cultures to stabilize the indicated fusion protein.

2.3. Cloning of TgAPE and TgAPN

The TgAPE and TgAPN open reading frames were amplified from an RH strain tachyzoite cDNA library using primers designed to the predicted gene at the *Toxoplasma* genomics resource (ToxoDB, www.toxodb.org). 5′- and 3′-rapid amplification of cDNA ends (RACE) was performed with the GeneRacer kit (Invitrogen) using tachyzoite mRNA as a template for reverse transcription (RT) with the provided random primers or oligo dT primer. RT-PCRs were performed using the SuperScript™ One-Step RT-PCR kit from Invitrogen following manufacturer's instructions.

2.4. Generation of parasites over-expressing TgAPE and TgAPN

FLAG-tagged TgAPE and TgAPN were ligated into the *p*tub_{FLAG}::HX expression vector using the NdeI and AvrII restriction sites [18]. $_{FLAG}$APN was amplified using primers (sense) 5′-
TCGATCGCATATGAAAATG*GACTACAAGGACGACGACGACAAG*ACGGCTGCAGCG
TCCCTAAGAAAAACCAAGG and (antisense) 5′-
TCGATCGCCTAGGTCACTCTTCGATGATGAATTTGTACATCATCTCC, where the underscored sequences represent the NdeI and AvrII restriction sites, respectively, and FLAG sequence is italicized. $_{FLAG}$APE was amplified using primers (sense) 5′-
ATCATATGAAAATG*GACTACAAGGACGACGACGACAAG*TCTGTTCACAGAGCAGT
GAACTCA and (antisense) 5′-
ATCGAACCTAGGTCAACTGGAGGCCTGGACGTTTCGCG. The N-terminal FLAG-tagged TgAPE and TgAPN constructs were linearized with NotI and electroporated into RH strain tachyzoites lacking hypoxanthine-xanthine-guanine phosphoribosyltransferase (RHΔHX) [19]. Transgenic parasites were selected in mycophenolic acid (25μg/ml) and xanthine (50μg/ml) and cloned by limited dilution.

2.5. Generation of parasites with regulatable TgAPN protein

We tagged the 3′ end of the endogenous TgAPN genomic locus with two hemagglutin (HA) epitopes and destabilization domain (DD). 1504 bp of the 3′ end of the TgAPN genomic locus was amplified minus the stop codon using Phusion® High-Fidelity DNA Polymerase (New England Biolabs) and the following primers: (sense) 5′-TACTTCCAATCCAATTTAATGCCCTCCACAGTGTACAGACAGTTCCTCTAGGC and (antisense) 5′-TCCTCCACTTCCAATTTTAGCCTCTTCGATGAATTTGTACATCATCTCCG. The amplified product was cloned into the LIC-HA2X-DD-DHFR-TS vector as described [20]. The completed plasmid was linearized with BlpI and transfected into ΔKu80 RH strain parasites; pyrimethamine resistant clones were examined for the loss of TgAPN-DD protein in the absence of Shield-1.

2.6. Purification of recombinant TgApe and TgApn

An expression vector for TgAPE was constructed in pET28a including an N-terminal cleavable hexa-histidine tag using NdeI and NotI restriction sites and verified by DNA sequencing. Following transformation into the Rosetta *E. coli* strain, a 1 liter culture was induced at 37 °C for 4 hrs, after which cells were pelleted and stored at −80 °C prior to purification. The cell pellet was resuspended in 25 ml of Lysis buffer, Buffer A (50 mM Na phosphate pH 7.8, 0.3M NaCl) with 10 mM imidazole, sonicated, and centrifuged at 35K for 35 min in an ultracentrifuge. The crude extract was incubated with 1 ml of a Ni-NTA agarose slurry for 45 minutes at 4 °C, placed in a column, washed with 10 ml of wash Buffer A with 20 mM imidazole and eluted with 2 × 1.5 ml of elution buffer, Buffer A with 250 mM imidazole. Fractions were diluted to 0.05M NaCl with Buffer B (50 mM HEPES pH 7.5, 1 mM DTT) and applied to a Q-Sepharose column using an AKTA FPLC system. The protein was then eluted from the Q-Sepharose column using a NaCl gradient (0.05M NaCl to 1M NaCl). The fractions containing TgAPE were combined and digested overnight with thrombin in order to remove the N-terminal hexa-His affinity tag. The sample was then diluted to a final salt concentration of 0.05M NaCl with Buffer B and subjected to a NaCl gradient separation on the Q-Sepharose column as before. Fractions including TgAPE were combined, concentrated, and stored at −80 °C. The purified sample is greater than 95% pure as judged by SDS-PAGE analysis

An expression vector for TgAPN was constructed in pET15b using NdeI and BamHI restriction sites and verified by DNA sequencing. Expression and purification were similar to that described for TgAPE with the same buffers used for Ni-NTA affinity purification followed by S-Sepharose ion-exchange chromatography. The fractions containing TgAPN from the Ni-NTA purification were pooled, diluted in 50 mM HEPES pH 7.0, 1 mM DTT, and eluted using a NaCl gradient 0.05–1 M in the same buffer. The fractions containing TgAPN were combined and digested overnight with thrombin in order to remove the N-terminal hexa-His affinity tag. The sample was then diluted to a final salt concentration of 0.05M NaCl with Buffer B and subjected to a NaCl gradient separation on the S-Sepharose column as before. Fractions including TgAPN were combined, concentrated, and stored at −80 °C. The purified sample is approximately 95% pure.

2.7. Generation of antisera recognizing TgApe and TgApn

Purified recombinant TgApe and TgApn (with N-terminal poly-His tag removed) were injected into rabbits for production of polyclonal antisera at Quality Controlled Biochemicals (Hopkinton, MA). Raw antiserum was affinity-purified over antigen-based columns. Recombinant TgApe was immobilized on Affi-Gel-15 and TgApn was immobilized on Affi-Gel-10 (Bio-Rad), and the respective antisera were concentrated using Amicon centrifugal columns.

2.8. Immunofluorescence assays

HFFs grown in 12-well plates containing glass coverslips were infected with parasites. 24 hrs post-infection the cultures were fixed with 3% paraformaldehyde for 15 min at room temperature. The infected cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min and blocked in 3% BSA (fraction V, OmniPur) in PBS for 30 min. Primary antibodies used included affinity-purified polyclonal TgAPN 1:10,000 and TgAPE 1:1,000, monoclonal anti-FLAG antibody 1:5,000 (Sigma F1804). DNA intercalator 4′,6-diamidino-2-phenylindole (DAPI, Molecular Probes) was applied at a concentration of 0.3 μM for 5 min as a co-stain to visualize nuclei. Coverslips were prepared for viewing using mounting media with 50% glycerol containing 48μg/ml Mowiol (Calbiochem) and 10μg/ml DABCO (Sigma) to reduce photobleaching. The slides were analyzed using a Leica DMLB fluorescent microscope at 100X with a HCX Plan Apo oil immersion objective. Images were captured with a monochrome SPOT-RTSE camera and Spot Advance software (version 4.6).

2.9. Immunoblotting

Affinity-purified antisera recognizing TgApe and TgApn were diluted in Tris-buffered saline tween (TBST) to a final concentration of 1:10,000. The nitrocellulose membranes were blocked in 3% BSA for 2 hrs prior to being probed with the respective antibodies. Donkey anti-rabbit conjugated to horseradish peroxidase diluted 1:5,000 was used as a secondary antibody (GE Health Care). The signal was detected using an ECL chemiluminescent system (Thermo Scientific).

2.10. DNA repair assays

AP endonuclease activity for recombinant proteins was measured in a fluorescent-based kinetic assay using a duplex DNA substrate containing a tetrahydrofuran abasic site mimic as the substrate. The assay employs fluorescein and dabcyl as a fluor-quench pair in the following oligonucleotides (5′-**6-FAM-** GCC GAC FGA GGA CGT ACG CG -3′ and 5′-CGC GTA CGT CCT CTG TCG GC **-Q-**3′) from Eurogentec Ltd (San Diego, CA) [21], where FAM indicates fluorescein and Q, Dabcyl. The oligonucleotides were diluted in water to a final concentration of 100 μM and annealed in a 4:5 ratio of fluorescein vs. Dabcyl labeled oligonucleotides. Release of the fluorescein labeled oligonucleotide following cleavage at the abasic site by an AP endonuclease is achieved by thermal denaturation at 37°C and followed in the kinetic mode on a Tecan Ultra 384 instrument using an excitation wavelength of 485 nm and emission of 535 nm. TgAPN was assayed in 50 mM MOPS 7.5, 50 mM NaCl, and 0.8% DMSO. An optimal concentration of 4 nM TgAPN was determined by varying the enzyme concentration with fixed substrate concentration of 25 nM. The substrate was then varied from 0 to 75 nM to determine V_{max} and K_m values for this enzyme. Similarly, 0.05 nM TgAPE was used with substrate concentrations of 0 to 75 nM in 50 mM MOPS 7.5, 50 mM NaCl, 1 mM $MgCl_2$, and 0.8% DMSO. DMSO was included in the assay in preparation for screening of small molecule inhibitors dissolved in 0.8% DMSO and found not to affect the activity of the enzyme. Kinetic parameters were calculated from the average of quadruplicate measurements for each substrate concentration using the Enzyme Kinetics module in SigmaPlot (SigmaPlot 11.2).

Similar assays were used to measure AP endonuclease activities from wild-type parasite lysate. Wild-type RH strain *Toxoplasma* tachyzoites were filter-purified and washed extensively in PBS to exclude host cell material. No detectable levels of human APE1 were observed by immunoblot of the purified parasite lysate, even after a 15 min overexposure (data not shown). To measure total AP endonuclease activity, a master mix was made with buffer (50 mM HEPES, 1 mM $MgCl_2$, 50 mM KCl and 2 mM DTT), 25 nM final HEX-labeled oligo and aliquoted in 15 μL increments. To measure TgAPN activity, a buffer without $MgCl_2$ containing 10 mM final EDTA was used. 100, 250, 500, 1000, 1500 or 3000

ng parasite lysate in a volume of 5.0 μL was added to each aliquot of master mix and incubated in a 37 °C water bath. Ten nM recombinant human APE1 or recombinant TgAPN, along with a sample run with the master mix alone, were run as positive and negative controls for each assay, respectively. After 30 min incubation, 10 μL of formamide was added to stop the reaction. The samples were then electrophoresed in a 20% denaturing (7 M urea) polyacrylamide gel in 1X Tris-borate EDTA at 300 V for 40 min. The resulting bands were detected and quantified using the FMBioII fluorescence imaging system and software (Hitachi Genetic Systems, South San Francisco, CA). Each assay was repeated two times.

3. Results

3.1. Apicomplexan parasites have ExoIII and EndoIV AP endonucleases

A search of the *Toxoplasma* genome web site (ToxoDB.org) revealed that accession number TGGT1_098640/35.m00892 (chromosome XI) is a homologue of human APE1 (hAPE1, ExoIII class) and TGGT1_034710/80.m00015 (chromosome IX) a homologue of yeast APN1 (EndoIV class). We subsequently cloned the full-length cDNA and confirmed the sequences for each (GenBank accession numbers are HM593513 for TgAPE and HM593514 for TgAPN). 5′- and 3′-RACE (rapid amplification of cDNA ends) were performed, revealing that TgAPE has a 5′UTR (untranslated region) of 582 nt and 3′UTR of 320 nt; TgAPN has a 5′UTR of 254 nt and 3′UTR of 424 nt.

BLASTp searches with the deduced amino acid sequence for TgAPE reveal most significant matches to other apicomplexan APE1 homologues, followed by plant species (rice, sorghum, and *Arabidopsis*), which are ~35% identical and ~50% similar. In contrast, TgAPE is less similar to hAPE1. TgApe is comprised of 513 amino acids while hApe1 only has 318 residues; TgAPE contains 10 insertions and one deletion as compared to the human sequence, which may have important structural consequences. A PSI-BLAST alignment of TgAPE to hAPE1 sequences is shown in Supplemental Figure 1. Most but not all of the residues thought to play an important role in the repair active site are conserved in TgAPE, including E96, Y171, N174, D210, N212, D282, and H309 (numbering refers to the human sequence). Notably, three residues that are not conserved are W280 and F266, which form the walls of the abasic deoxyribose binding site, and R177, which fills the position of the missing base in the duplex. These residues in TgAPE are M379, S361, and A253, respectively. The fact that some of the residues in the active site are not conserved suggests there may be important structural differences in the repair active site.

BLASTp searches with the deduced 472 amino acid sequence of TgAPN reveal that the most significant matches are to other apicomplexan APN1 homologues, followed by fungal species (e.g. *Chaetomium globosum*, *Fusarium graminearum*, *Neurospora crassa*, *Cryptococcus neoformans*) which are ~40–50% identical and ~60–70% similar. Residues 187 to 463 of TgAPN are 43% identical to *E. coli* EndoIV, which includes 285 residues (Supplemental Figure 2). Residues involved in coordinating the three metal ions in the active site of *E. coli* EndoIV are conserved in TgAPN. These residues include H255, H295, and E331 (numbering for TgAPN), which coordinate Zn_1, E448, H392, and D365, Zn_2, and D415, H417, and H368, Zn_3. In addition, recognition loop residues, R222 and Y258 [22] are also conserved suggesting that mechanistically TgAPN is very similar to *E. coli* EndoIV. In contrast to the APE family enzymes in which the larger TgAPE has a number of sequence inserts throughout the enzyme that contribute to its significantly larger mass, the difference in size between TgAPN and *E. coli* endonuclease IV results primarily from an N-terminal insertion in TgAPN.

Previous studies and comparative genomics reveal additional insight into the AP endonucleases in Apicomplexa. Biochemical evidence suggested that *Plasmodium*

falciparum (malaria) contains both Mg^{2+}-dependent (APE1) and Mg^{2+}-independent (APN1) AP endonuclease activities [23]; we have verified that the *Plasmodium* genome sequence harbors genes for both APE1 (chr3.glimmerm_170) and APN1 (chr13.genefinder_182r) homologues. Survey of the *Cryptosporidium* genome also reveals both APE1 (CpIOWA_EAK87392) and APN1 (CpIOWA_EAK88592) homologues. Phylogenetic analysis shows that apicomplexan APN1 homologues form a clade with yeast APN1, while apicomplexan APE1 homologues are more closely related to those found in plants rather than mammals (Supplemental Figure 3).

3.2. TgAPE and TgAPN AP DNA repair activities

We expressed recombinant TgAPE and TgAPN in *E. coli* and purified the proteins for enzymatic analysis. The DNA repair assay utilizes a 5′-hexachloro-fluorescein phosphoramidite (HEX)-labeled terahydrofuranyl (THF) oligo (the 26 bp oligonucleotide substrate contains a single THF residue in the middle, yielding a HEX-labeled 13mer fragment upon incision). THF is a stable synthetic abasic site [24]. The reaction products are visualized after resolving on 20% polyacrylamide gels. As predicted from their structural similarities to hAPE1 and yeast APN1, the TgAPE enzyme should require Mg^{2+} while TgAPN should be Mg^{2+}-independent. As shown in Figure 1A, TgAPE is capable of cleaving the THF oligo as evidenced by the accumulation of the lower 13 bp product with increasing concentrations of enzyme and is only active in the Mg^{2+}-containing buffer. TgAPN has similar AP endonuclease activity and is active in the presence or absence of Mg^{2+} (Figure 1B). Further steady state enzymatic characterizations of TgAPN and TgAPE were performed using a fluorescent-based solution assay as described in the Materials and Methods. The THF-containing oligonucleotide substrate concentration was varied from 0 to 75 nM, and Michaelis-Menton K_m values of 26.2 and 21.1 nM and V_{max} values of 1458 and 110,070 RFU/μg/s, respectively for TgAPN vs. TgAPE, were calculated (Figure 1C, D), where RFU is a relative fluorescent unit. Thus, while the K_m values of each enzyme for the substrate are similar, the maximum velocity is approximately 75 times faster for TgAPE.

3.3. Size and localization of *Toxoplasma* AP endonucleases

Polyclonal antisera were generated in rabbits using the recombinant TgAPE and TgAPN purified from bacteria as antigen. Polyhistidine tags were removed by proteolytic cleavage prior to immunization. Antibodies were affinity-purified and used to probe Western blots containing lysates from *Toxoplasma* tachyzoites or the recombinant protein as control (Fig. 2A). Recombinant TgAPE migrates at the expected MW of 57 kD, but also appears at 52 kD, possibly a breakdown or proteolytic product. Native TgAPE expressed in *Toxoplasma* also migrates as two proteins as seen when produced in *E. coli*, making it likely that this is protein processing rather than an alternatively spliced product. Native TgAPN migrates slightly higher than the expected 51 kD in *Toxoplasma* lysate, possibly due to a post-translational modification(s).

The antibodies were used in immunofluorescence assays (IFA) to localize the native protein in *Toxoplasma* tachyzoites growing inside their host cells. Native TgAPE is present in the parasite nucleus and cytoplasm while native TgAPN is nuclear (Fig. 2B). Arrowheads in Fig. 2B point to potential TgAPE present in the apicoplast, a specialized organelle housing a 35 kb extrachromosomal element. We also expressed recombinant FLAG-tagged versions of TgAPE or TgAPN (tagged at N-terminus) in RH strain tachyzoites ($_{FLAG}$APE or $_{FLAG}$APN). Expression of each ectopic copy was driven by the same strong tubulin promoter as previously described [18]. Following selection and cloning, we performed IFA to determine the subcellular localization of each tagged *Toxoplasma* AP endonuclease. When anti-APE or anti-APN antibodies are used in IFAs of the parasites over-expressing FLAG-tagged TgAPE or TgAPN, the staining pattern is identical to that seen for wild-type

parasites (compare Fig. 3, green to Fig. 2B). Moreover, anti-FLAG IFAs of parasites expressing $_{FLAG}$APN confirm nuclear localization of this protein (Fig. 3, red). In contrast to IFAs with anti-APE, anti-FLAG IFAs of parasites expressing $_{FLAG}$APE show almost exclusive localization to the nucleus (Fig. 3, red). This suggests that the cytosolic staining for native TgAPE is due to the fact that anti-APE recognizes both full-length and truncated forms (see Fig. 2A, Western blot). In other words, full-length TgAPE is nuclear, but the shorter TgAPE (which anti-FLAG would not recognize) is cytoplasmic.

3.4. TgAPN is more abundant than TgAPE in tachyzoites

In other species possessing more than one AP endonuclease, one enzyme is predominant for BER [25–26]. We therefore sought to establish which AP endonuclease was predominant in *Toxoplasma* tachyzoites. An analysis of the expressed sequence tags (ESTs) data at the ToxoDB Release 6.0 (www.toxodb.org) shows that only 2 ESTs represent TgAPE whereas >30 ESTs were identified for TgAPN, indicating that TgAPE message levels are very low compared to those encoding TgAPN. Analysis at the protein level also suggests that TgAPN is the more prevalent AP endonuclease (Fig. 2A). Proteomics data at the ToxoDB mirrors our data and what we observed for ESTs in that a large number of peptides in various independent datasets suggest TgAPN is present in abundance at the protein level; in contrast, only 2 peptides have been reported matching to TgAPE. Considered together, these multiple lines of evidence indicate that TgAPN is more predominant than TgAPE at both the RNA and protein levels in tachyzoites.

We measured total AP endonuclease activity levels in wild-type *Toxoplasma* lysate using the AP endonuclease gel assay (Fig. 2C). The percent activity was expressed as a percent of the value of the cleaved oligo to the total value of the cleaved and uncleaved oligo [24]. TgAPN (Mg^{2+}-independent) activity levels were measured by using Mg^{2+}-free buffer containing EDTA (Fig. 2D). In both cases, a dose-dependent increase of activity was seen with increasing amounts of parasite lysate present in the reaction. The results demonstrate that TgAPN contributes substantially (~50%) to the total amount of AP endonuclease activity detected in parasite lysate.

3.5. Parasites over-expressing TgAPN are protected from DNA damage

We examined if our transgenic parasites over-expressing recombinant forms of each AP endonuclease under control of the same heterologous promoter (tubulin) exhibited cytoprotection against the alkylating agent methyl methanesulfonate (MMS), a well-established DNA-damaging agent used to study of BER. MMS was chosen to induce DNA damage rather than H_2O_2 since *Toxoplasma* possesses peroxidases and a highly active catalase [27]. Using three independent growth assays, we determined that *Toxoplasma* tachyzoites exposed to MMS have increased viability only when TgAPN is stably over-expressed; no protection is conferred when TgAPE is over-expressed (Fig. 4). Figure 4A is a PCR-based assay for the parasite B1 gene that monitors growth over 5 days following exposure to an insult of 400 μM MMS for 2 hrs. A plaque assay performed at day 7 following 400 or 800 μM MMS produced similar results (Fig. 4B). Finally, a *Toxoplasma* doubling assay performed on parasites over a time course following exposure to 800 μM MMS verified that those over-expressing TgAPN were remarkably refractory to this insult (Fig. 4C). It should be noted that the growth rates of each over-expressing line are virtually identical to each other as well as wild-type under vehicle-treated conditions (Fig. 4). We also examined the relative levels of ectopic $_{FLAG}$APN and $_{FLAG}$APE; while driven by the same promoter, it is possible that a higher copy number could explain the protection seen in TgAPN over-expressers. However, immunoblots demonstrated that there is more $_{FLAG}$APE compared to $_{FLAG}$APN in the over-expressing lines (Supplemental Figure 4). Also note that only the full-length TgAPE appeared on the immunoblot probed with anti-FLAG

(Supplemental Figure 4), confirming that the shorter form of TgAPE is missing a portion of its N-terminal region.

3.6. Impaired response to DNA damage in TgAPN knockdown parasites

To determine if the predominant AP endonuclease is essential for *Toxoplasma* viability, we first attempted knockouts in type I RH strain parasites using conventional recombination. We did not obtain viable clones, possibly because the TgAPN gene is essential. To test this idea, we created a conditional knockout for TgAPN in the TATi parasite background, which expresses a transactivator protein that can be regulated by anhydrotetracycline (ATc) [28]. Ectopic $_{\text{HA-MYC}}\text{APN}_{\text{FLAG}}$ driven by the ATc-responsive SAG1 promoter was stably expressed in TATi parasites. Next, the entire endogenous locus encoding TgAPN was eliminated through homologous recombination. Over 130 clones were screened and a single knockout was obtained and verified (data not shown). Unfortunately, addition of 1.0 μg/ml ATc for 48 hrs did not significantly reduce the amount of ectopic TgAPN in the conditional knockout clone (higher levels of ATc proved toxic). A modest slow growth phenotype accompanied the conditional knockout treated with 1.0 μg/ml ATc (data not shown), suggesting again that TgAPN may be important for *Toxoplasma* viability.

As an alternative approach, we engineered a “knock in” vector designed to place a fusion tag onto the C-terminal end of TgAPN consisting of two HA epitopes and a destabilization domain (2xHA-DD), which allows for regulated expression of the fusion protein through addition of the stabilizing ligand Shield-1 [29]. RH strain parasites lacking Ku80 (Ku80-KO), which display a significantly higher degree of homologous recombination [20,30], were used to create *Toxoplasma* parasites expressing endogenous TgAPN fused to the 2xHA-DD tag (called TgAPN-2xHA-DD). We were able to obtain several clones tagged in this fashion that behaved similarly. The ability to regulate levels of TgAPN-2xHA-DD protein with Shield-1 was verified using IFA and immunoblotting (Fig. 5A and B). TgAPN-2xHA-DD parasites cultured under normal conditions without Shield (TgAPN knockdown) are viable, as reported for other species [12,31]. We then analyzed the ability of purified, extracellular TgAPN knockdown parasites to recover from a direct 2 hr exposure to 25 μM MMS. Relative to the Ku80-KO parental line, the TgAPN knockdown parasites were significantly impaired in recovering from the MMS insult (Fig. 6A). We also examined the ability of intracellular TgAPN knockdown parasites to proliferate in the presence of MMS in tissue culture. In these conditions, 25 μM MMS has no significant effect on parental or TgAPN knockdown parasites (data not shown), possibly because the parasites are sheltered by the host cells. But as shown in Fig. 6B, the parental Ku80-KO parasites show ~40% reduction in growth in the presence of 100 μM MMS. In contrast, TgAPN knockdown parasites show ~80% reduction in growth in the presence of 100 μM MMS (Fig. 6B). Together these data further support that TgAPN plays a major role in the DNA damage response in *Toxoplasma*.

Discussion

A number of protozoal species in phylum Apicomplexa have a tremendous impact on human health and vitality of livestock. In addition to *Toxoplasma*, other important pathogens in this phylum include *Plasmodium*, *Cryptosporidium*, and *Eimeria*. *Plasmodium* is the causative agent of malaria and kills up to 2.7 million persons per year, over 75% of them children [32]. *Cryptosporidium* causes acute diarrheal illness that can be extremely serious in the young and immunocompromised [33]. *Eimeria* is a coccidian parasite of poultry, causing losses in excess of $3 billion annually to US agribusiness [34]. A combination of too little research and rapid spread of resistance to frontline drugs is grossly hindering our control of these infectious diseases.

The frontline treatment for *Toxoplasma*, pyrimethamine and sulfadiazine, is limiting for a number of reasons. The treatment must be given in combination, the DHFR inhibitor pyrimethamine causes bone marrow suppression, and numerous patients have or develop allergies to sulfa drugs. The adverse effects are more pronounced in the immunocompromised, the patient population that most commonly suffers from recurring episodes of acute toxoplasmosis [35]. There is also a paucity of treatments that can be safely administered during pregnancy to treat toxoplasmosis acquired *in utero*. In short, there is an urgent need to develop novel therapies against *Toxoplasma* that are more selectively toxic. Additionally, it is hoped that such novel therapies would also have utility against other apicomplexan parasites, particularly if the therapies are targeted against crucial molecular targets that are similar in protozoa.

Like other apicomplexans, *Toxoplasma* is an intracellular parasite exposed to an oxidative burst generated by the host immune response in addition to ROS generated through its own metabolism [15,36]. Consequently, intracellular organisms may incur extensive damage to DNA that must be repaired for genomic stability and survival [16]. Especially relevant to *Toxoplasma* are the generation of abasic sites in DNA, which result from removal of oxidized or alkylated bases. These sites are repaired by AP endonucleases during BER. Limited work has been performed to date to characterize AP endonucleases in protozoa. AP endonucleases have been studied in kinetoplastid parasites *Trypanosoma cruzi* and *Leishmania major* [37]. Over-expression of the *L. major* AP endonuclease (LMAP), which is an APE1 (ExoIII) homologue, protects the protist from DNA damage [5]. The crystal structure for LMAP has recently been solved, and it largely resembles other APE1 orthologues [38]. It has been reported that *Plasmodium falciparum* predominantly uses long-patch BER as opposed to short-patch BER, which is the predominant pathway in most other species including human, and biochemical activities for class II, Mg^{2+}-dependent and independent AP endonucleases have been described [23,39]. Our study confirms the presence of two AP endonucleases in fellow apicomplexan *Toxoplasma*, the Mg^{2+}-dependent TgAPE and Mg^{2+}-independent TgAPN.

TgAPE does not appear to be abundant in tachyzoites, but it may be upregulated during another life cycle stage. It should be noted that recombinant TgAPE displays fast substrate turnover rate (Fig. 1C), which may offset its low abundance in tachyzoites. The fast turnover rate observed for TgAPE may result from fast product release. In human APE1, R177 has been shown to slow product release potentially facilitating recruitment of β polymerase to the site [7,9]. Thus, the R177A human APE1 enzyme actually has a faster turnover rate than the wild-type enzyme. In TgAPE, the residue equivalent to R177 in TgAPE1 is A253, which by analogy may also result in faster product release. It is also interesting that over-expression of TgAPE failed to provide protection against MMS (Fig. 4). There are at least two possible explanations for this result. It is possible that TgAPE requires additional factors *in vivo* that are not present in stoichiometric amounts to over-expressed TgAPE. A second possibility is that TgAPE has evolved to serve a different DNA repair related function in *Toxoplasma*. Although TgAPE efficiently cleaves an oligonucleotide substrate including an abasic site mimic, it lacks the residues found in other APE1 enzymes that confer specificity for this substrate, namely W280 and F266. Substitution of residues equivalent to F266 and W280 in the APE-like enzyme from the hyperthermophilic archaeon *Archaeoglobus fulgidus* resulted in an increase in non-specific endonucleolytic activity and exonucleolytic activity [40]. In contrast, all of the residues that confer specificity for abasic site specificity are conserved in the TgAPN enzyme.

Another interesting finding from our studies is that TgAPE exists as two forms, the shorter one being in the cytoplasm and possibly the apicoplast. The presence of truncated APE1 in the cytoplasm is not without precedent; the distribution of human APE1 in the nucleus and

cytosol is in a dynamic equilibrium and the nuclear transport of human APE1 requires its 20 N-terminal residues [41]. Localization of TgAPE to the apicoplast could be a significant finding as this plastid-like organelle houses its own 35 kb genome that is essential for *Toxoplasma* viability [42]. In addition to its endonuclease activities, human APE1 can regulate transcription by virtue of its redox activity; the TgAPE homologue, however, has a valine residue in the position equivalent to cysteine residue 65, which is essential for redox activity in the human enzyme [43–45]. Further investigation into the precise role of TgAPE in the parasite is an important future study.

Alteration of TgAPN levels alters susceptibility of the parasite to DNA damage, marking the first demonstration that such enzymes may be attractive drug targets against intracellular protozoan pathogens. A knockdown of TgAPN was viable, but significantly impaired in recovering from DNA damage (Fig. 6). As in humans, the total knockout of the main AP endonuclease is not viable and only cells with low levels of APE can escape death. This appears to be similar to our findings that we have been unable to knockout or knockdown TgAPN completely. Therefore, it would be of interest to determine if complete inhibition of TgAPN by a small molecular inhibitor would be detrimental to parasite proliferation. Humans do not possess APN1 homologues, and the TgAPN enzyme is structurally unrelated to human APE1, making it likely that compounds will be identified that specifically inhibit *Toxoplasma* BER without inhibiting its human counterpart allowing for a large therapeutic index with predicted limited toxicity. These predictions must wait for the identification of small molecule inhibitors, which is currently underway in our laboratories.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Friedberg, EC.; Walker, GC.; Siede, W. DNA Repair and Mutagenesis. ASM Press; Washington, D. C.: 1995.
2. Demple B, Harrison L. Repair of oxidative damage to DNA: Enzymology and Biology. Ann Rev Biochem. 1994; 63:915–948. [PubMed: 7979257]
3. Loeb LA, Preston BD. Mutagenesis by apurinic/apyrimidinic sites. Ann Rev Genet. 1986; 20:201–230. [PubMed: 3545059]
4. Evans AR, Limp-Foster M, Kelley MR. Going APE over ref-1. Mutat Res. 2000; 461:83–108. [PubMed: 11018583]
5. Gallego C, Estevez AM, Farez E, Ruiz-Perez LM, Gonzalez-Pacanowska D. Overexpression of AP endonuclease protects Leishmania major cells against methotrexate induced DNA fragmentation and hydrogen peroxide. Mol Biochem Parasitol. 2005; 141:191–197. [PubMed: 15850702]
6. Gorman MA, Morera S, Rothwell DG, de La Fortelle E, Mol CD, Tainer JA, Hickson ID, Freemont PS. The crystal structure of the human DNA repair endonuclease HAP1 suggests the recognition of extra-helical deoxyribose at DNA abasic sites. Embo J. 1997; 16:6548–6558. [PubMed: 9351835]

7. Mol CD, Izumi T, Mitra S, Tainer JA. DNA-bound structures and mutants reveal abasic DNA binding by APE1 and DNA repair coordination [corrected]. Nature. 2000; 403:451–456. [PubMed: 10667800]
8. Hosfield DJ, Guan Y, Haas BJ, Cunningham RP, Tainer JA. Structure of the DNA repair enzyme endonuclease IV and its DNA complex: double-nucleotide flipping at abasic sites and three-metal-ion catalysis. Cell. 1999; 98:397–408. [PubMed: 10458614]
9. Mol CD, Hosfield DJ, Tainer JA. Abasic site recognition by two apurinic/apyrimidinic endonuclease families in DNA base excision repair: the 3′ ends justify the means. Mutat Res. 2000; 460:211–229. [PubMed: 10946230]
10. Chen DS, Herman T, Demple B. Two distinct human DNA diesterases that hydrolyze 3′-blocking deoxyribose fragments from oxidized DNA. Nucleic Acids Res. 1991; 19:5907–5914. [PubMed: 1719484]
11. Weiss B. Terminal cross-linking of DNA strands by a mixture of exonuclease III of Escherichia coli and polynucleotide ligase of bacteriophage T4. J Mol Biol. 1976; 103:669–673. [PubMed: 781281]
12. Popoff SC, Spira AI, Johnson AW, Demple B. Yeast structural gene (APN1) for the major apurinic endonuclease: homology to Escherichia coli endonuclease IV. Proc Natl Acad Sci U S A. 1990; 87:4193–4197. [PubMed: 1693433]
13. Ramotar D, Popoff SC, Gralla EB, Demple B. Cellular role of yeast Apn1 apurinic endonuclease/3′-diesterase: repair of oxidative and alkylation DNA damage and control of spontaneous mutation. Mol Cell Biol. 1991; 11:4537–4544. [PubMed: 1715020]
14. Zakaria C, Kassahun H, Yang X, Labbe JC, Nilsen H, Ramotar D. Caenorhabditis elegans APN-1 plays a vital role in maintaining genome stability. DNA Repair (Amst). 2009
15. Nathan C, Shiloh MU. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. Proc Natl Acad Sci U S A. 2000; 97:8841–8848. [PubMed: 10922044]
16. Vonlaufen N, Kanzok SM, Wek RC, Sullivan WJ Jr. Stress response pathways in protozoan parasites. Cell Microbiol. 2008; 10:2387–2399. [PubMed: 18647172]
17. Costa JM, Pautas C, Ernault P, Foulet F, Cordonnier C, Bretagne S. Real-time PCR for diagnosis and follow-up of Toxoplasma reactivation after allogeneic stem cell transplantation using fluorescence resonance energy transfer hybridization probes. J Clin Microbiol. 2000; 38:2929–2932. [PubMed: 10921953]
18. Bhatti MM, Sullivan WJ Jr. Histone acetylase GCN5 enters the nucleus via importin-alpha in protozoan parasite Toxoplasma gondii. J Biol Chem. 2005; 280:5902–5908. [PubMed: 15591057]
19. Donald RG, Carter D, Ullman B, Roos DS. Insertional tagging, cloning, and expression of the Toxoplasma gondii hypoxanthine-xanthine-guanine phosphoribosyltransferase gene. Use as a selectable marker for stable transformation. J Biol Chem. 1996; 271:14010–14019. [PubMed: 8662859]
20. Huynh MH, Carruthers VB. Tagging of endogenous genes in a Toxoplasma gondii strain lacking Ku80. Eukaryot Cell. 2009; 8:530–539. [PubMed: 19218426]
21. Madhusudan S, Smart F, Shrimpton P, Parsons JL, Gardiner L, Houlbrook S, Talbot DC, Hammonds T, Freemont PA, Sternberg MJ, Dianov GL, Hickson ID. Isolation of a small molecule inhibitor of DNA base excision repair. Nucleic Acids Res. 2005; 33:4711–4724. [PubMed: 16113242]
22. Garcin ED, Hosfield DJ, Desai SA, Haas BJ, Bjoras M, Cunningham RP, Tainer JA. DNA apurinic-apyrimidinic site binding and excision by endonuclease IV. Nat Struct Mol Biol. 2008; 15:515–522. [PubMed: 18408731]
23. Haltiwanger BM, Karpinich NO, Taraschi TF. Characterization of class II apurinic/apyrimidinic endonuclease activities in the human malaria parasite, Plasmodium falciparum. Biochem J. 2000; 345(Pt 1):85–89. [PubMed: 10600642]
24. Kreklau EL, Limp-Foster M, Liu N, Xu Y, Kelley MR, Erickson LC. A novel fluorometric oligonucleotide assay to measure O(6)-methylguanine DNA methyltransferase, methylpurine DNA glycosylase, 8-oxoguanine DNA glycosylase and abasic endonuclease activities: DNA repair

status in human breast carcinoma cells overexpressing methylpurine DNA glycosylase. Nucleic Acids Res. 2001; 29:2558–2566. [PubMed: 11410664]

25. Shatilla A, Ishchenko AA, Saparbaev M, Ramotar D. Characterization of Caenorhabditis elegans exonuclease-3 and evidence that a Mg2+-dependent variant exhibits a distinct mode of action on damaged DNA. Biochemistry. 2005; 44:12835–12848. [PubMed: 16171399]
26. Ramotar D. The apurinic-apyrimidinic endonuclease IV family of DNA repair enzymes. Biochem Cell Biol. 1997; 75:327–336. [PubMed: 9493955]
27. Ding M, Kwok LY, Schluter D, Clayton C, Soldati D. The antioxidant systems in Toxoplasma gondii and the role of cytosolic catalase in defence against oxidative injury. Mol Microbiol. 2004; 51:47–61. [PubMed: 14651610]
28. Meissner M, Schluter D, Soldati D. Role of Toxoplasma gondii myosin A in powering parasite gliding and host cell invasion. Science. 2002; 298:837–840. [PubMed: 12399593]
29. Herm-Gotz A, Agop-Nersesian C, Munter S, Grimley JS, Wandless TJ, Frischknecht F, Meissner M. Rapid control of protein level in the apicomplexan Toxoplasma gondii. Nat Methods. 2007; 4:1003–1005. [PubMed: 17994029]
30. Fox BA, Ristuccia JG, Gigley JP, Bzik DJ. Efficient gene replacements in Toxoplasma gondii strains deficient for nonhomologous end joining. Eukaryot Cell. 2009; 8:520–529. [PubMed: 19218423]
31. Zakaria C, Kassahun H, Yang X, Labbe JC, Nilsen H, Ramotar D. Caenorhabditis elegans APN-1 plays a vital role in maintaining genome stability. DNA Repair (Amst). 2010; 9:169–176. [PubMed: 20036200]
32. Breman JG. The ears of the hippopotamus: manifestations, determinants, and estimates of the malaria burden. Am J Trop Med Hyg. 2001; 64:1–11. [PubMed: 11425172]
33. Davies AP, Chalmers RM. Cryptosporidiosis. BMJ. 2009; 339:b4168. [PubMed: 19841008]
34. Dalloul RA, Lillehoj HS. Poultry coccidiosis: recent advancements in control measures and vaccine development. Expert Rev Vaccines. 2006; 5:143–163. [PubMed: 16451116]
35. Wong SY, Remington JS. Biology of Toxoplasma gondii. AIDS. 1993; 7:299–316. [PubMed: 8471191]
36. Halliwell B. Oxidants and human disease: some new concepts. FASEB J. 1987; 1:358–364. [PubMed: 2824268]
37. Perez J, Gallego C, Bernier-Villamor V, Camacho A, Gonzalez-Pacanowska D, Ruiz-Perez LM. Apurinic/apyrimidinic endonuclease genes from the trypanosomatidae leishmania major and Trypanosoma cruzi confer resistance to oxidizing agents in DNA repair-deficient Escherichia coli. Nucleic Acids Res. 1999; 27:771–777. [PubMed: 9889272]
38. Vidal AE, Harkiolaki M, Gallego C, Castillo-Acosta VM, Ruiz-Perez LM, Wilson K, Gonzalez-Pacanowska D. Crystal structure and DNA repair activities of the AP endonuclease from Leishmania major. J Mol Biol. 2007; 373:827–838. [PubMed: 17870086]
39. Haltiwanger BM, Matsumoto Y, Nicolas E, Dianov GL, Bohr VA, Taraschi TF. DNA base excision repair in human malaria parasites is predominantly by a long-patch pathway. Biochemistry. 2000; 39:763–772. [PubMed: 10651642]
40. Schmiedel R, Kuettner EB, Keim A, Strater N, Greiner-Stoffele T. Structure and function of the abasic site specificity pocket of an AP endonuclease from Archaeoglobus fulgidus. DNA Repair (Amst). 2009; 8:219–231. [PubMed: 19015049]
41. Jackson EB, Theriot CA, Chattopadhyay R, Mitra S, Izumi T. Analysis of nuclear transport signals in the human apurinic/apyrimidinic endonuclease (APE1/Ref1). Nucleic Acids Res. 2005; 33:3303–3312. [PubMed: 15942031]
42. Fichera ME, Roos DS. A plastid organelle as a drug target in apicomplexan parasites. Nature. 1997; 390:407–409. [PubMed: 9389481]
43. Bapat A, Fishel ML, Kelley MR. Going ape as an approach to cancer therapeutics. Antioxid Redox Signal. 2009; 11:651–668. [PubMed: 18715143]
44. Georgiadis MM, Luo M, Gaur RK, Delaplane S, Li X, Kelley MR. Evolution of the redox function in mammalian apurinic/apyrimidinic endonuclease. Mutat Res. 2008; 643:54–63. [PubMed: 18579163]

45. Kelley MR, Fishel ML. DNA repair proteins as molecular targets for cancer therapeutics. Anticancer Agents Med Chem. 2008; 8:417–425. [PubMed: 18473726]

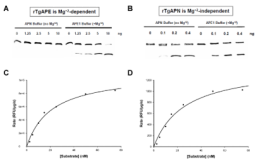

Figure 1. TgAPE and TgAPN AP endonuclease assays

A and B. Reactions contained 1 mM DTT and 0.05 pmol HEX-THF oligonucleotide substrate in a total volume of 10 μl and were incubated at 37 °C for 15 min. The reactions were analyzed on a 20% denaturing polyacrylamide gel. The lower band on the gel is cleaved AP oligo substrate, and its generation represents active AP endonuclease activity. The upper band is uncleaved substrate. A. The AP activity of purified recombinant TgAPE is dependent on Mg^{2+}. Increasing amounts of TgAPE protein (ng) were incubated with AP activity assay buffer for hAPE1 protein (50 mM Hepes, 50 mM KCl, 10 mM $MgCl_2$, 1 μg/ml BSA, 0.05% TritonX-100, pH 7.5), or APN buffer, which is identical but without $MgCl_2$, and includes 20 mM EDTA. B. Purified recombinant TgAPN is active in both buffers since it does not require Mg^{2+}. C and D. A fluorescent-based solution assay was used to characterize the steady state kinetic properties of TgAPE (C) and TgAPN (D). Using optimized concentrations of TgAPE (0.05 nM) and TgAPN (4 nM) and substrate concentrations from 0 to 75 nM, K_m values of 21.1 and 26.2 nM, respectively, were determined. V_{max} values for the two enzymes differ by a factor of 75, with TgAPE exhibiting the more rapid rate of endonuclease activity.

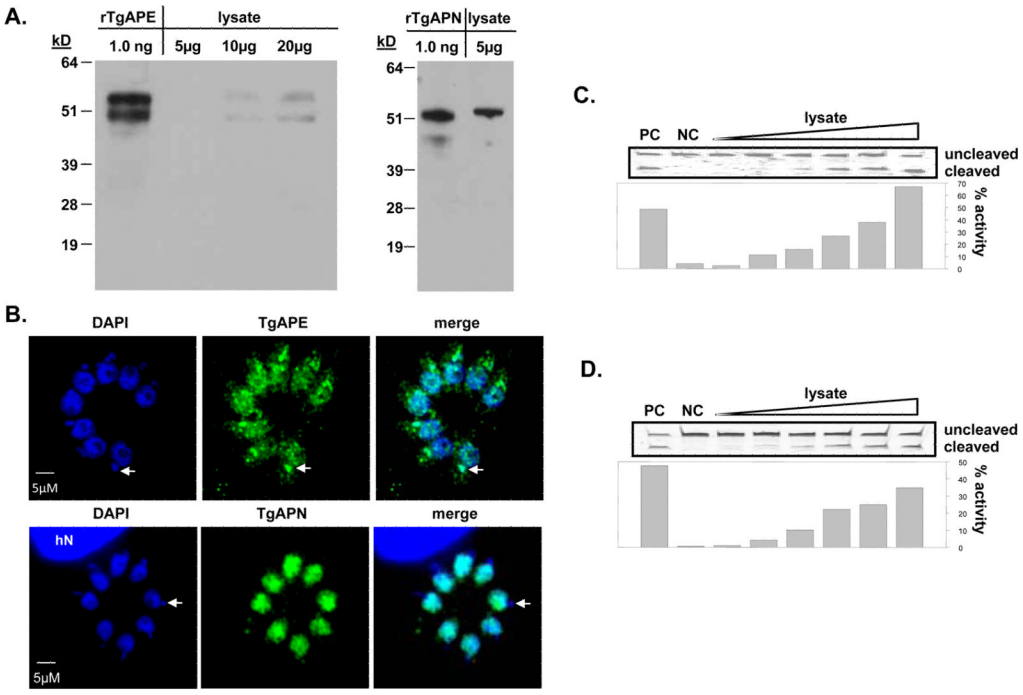

Figure 2. Characterization of *Toxoplasma* AP endonucleases

A. Western analysis with affinity-purified anti-TgAPE or anti-TgAPN, each used at a 1:10,000 dilution. The designated amount of *Toxoplasma* protein lysate is shown. Recombinant (r) protein expressed and purified from *E. coli* was run as control, and suggests anti-TgAPE binds its protein with greater affinity than anti-TgAPN, yet TgAPN is much more readily detected in lysates derived from tachyzoites. B. IFA of intracellular parasites probed with 1:1,000 dilution of anti-TgAPE (top panels, green) or 1:10,000 dilution anti-TgAPN (lower panels, green). 4′,6-diamidino-2-phenylindole (DAPI) was used as a co-stain to highlight the nuclear compartment (blue). hN= host cell nucleus. Arrowheads point to parasite apicoplast organelle. C. Total AP endonuclease activity in wild-type *Toxoplasma* lysate, performed in presence of 1.0 mM $MgCl_2$. Lane 1, 10 nM recombinant human APE1 (PC, positive control); lane 2, DNA alone (NC, negative control); lanes 3–8, increasing amounts of *Toxoplasma* lysate (100, 250, 500, 1000, 1500 and 3000 ng, respectively). The columns on the graphs below each gel correspond to the percent activity of the sample directly above and are representative of the average percent activity of two independent assays. D. APN activity levels in parasite lysate, as determined by degree of cleaved oligonucleotide. AP endonuclease assays were performed in the presence of 10 mM EDTA to chelate Mg^{2+}. Lane 1, 10 nM recombinant TgAPN (PC, positive control); lane 2, DNA alone (NC, negative control); lanes 3–8, increasing amounts of *Toxoplasma* lysate (100, 250, 500, 1000, 1500 and 3000 ng, respectively).

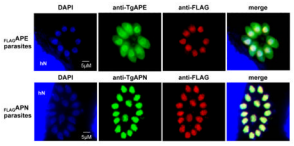

Figure 3. Localization of FLAG-tagged AP endonucleases

IFA was performed on intracellular parasites expressing $_{FLAG}$APE (top panels) or $_{FLAG}$APN (bottom panels). Parasites were probed with anti-FLAG (1:5,000) and either anti-TgAPE (1:1,000) or anti-TgAPN (1:10,000). DAPI was used as a co-stain to highlight the parasite nucleus (blue).

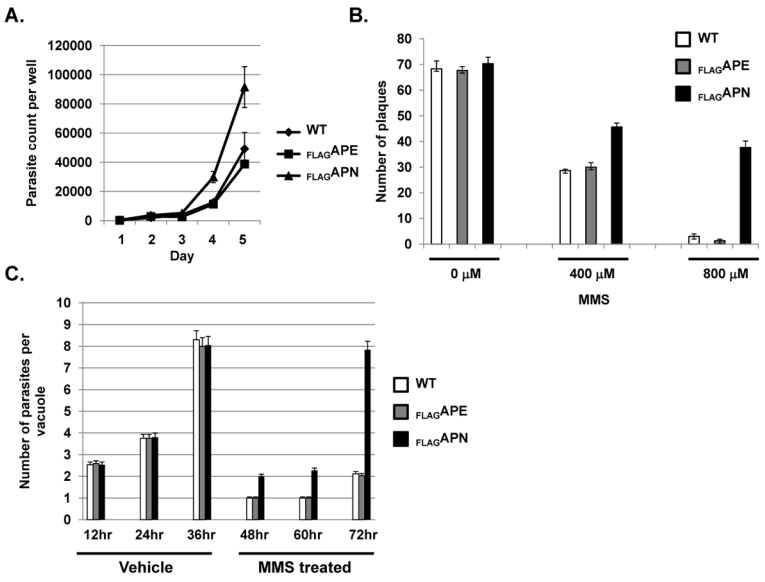

Figure 4. DNA damage recovery assays

Extracellular parasites (wild-type (WT), $_{FLAG}$APE and $_{FLAG}$APN) were subjected to DNA damage induced by MMS or vehicle control (DMSO) for 2 hr and then allowed to infect fresh HFF monolayers under normal culture conditions. A. Parasite growth was monitored by PCR-based B1 assay following exposure to 400 μM MMS. B. Parasite growth was measured using a plaque assay in either 400 or 800 μM MMS. C. Parasite doubling assays were performed following exposure to 800 μM MMS. Error bars indicate the SD of the mean. The times designated for the MMS treated samples are a reflection of the lag in parasite proliferation due to recovery from the MMS treatment.

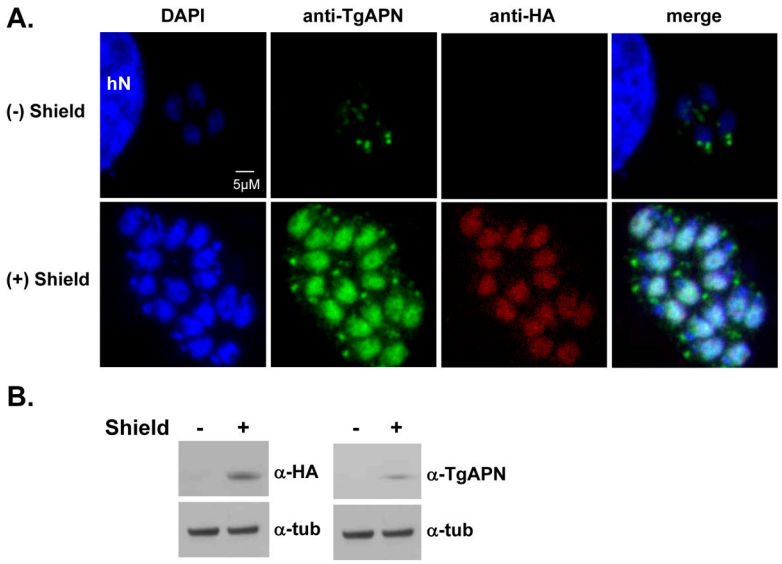

Figure 5. Knockdown of TgAPN protein using ligand-controlled destabilization domain

A parasite clone was generated to express endogenous TgAPN fused to a tag containing two HA epitopes and a destabilization domain (2xHA-DD). A. TgAPN-2xHA-DD is rapidly degraded unless stabilized through the inclusion of 100 nM Shield-1 in the media. IFA of TgAPN-2xHA-DD parasites grown with (+) or without (−) Shield, probed with anti-TgAPN (1:10,000, green) or anti-HA (1:2,000, red). DAPI was used as a nuclear co-stain (blue). hN = host cell nucleus. B. Western blot analysis of TgAPN-2xHA-DD parasites grown with (+) or without (−) Shield, probed with anti-HA (1:3,000, left) or anti-TgAPN (1:10,000, right). Ten μg of parasite lysate was used for the immunoblots. Tubulin was monitored as a loading control.

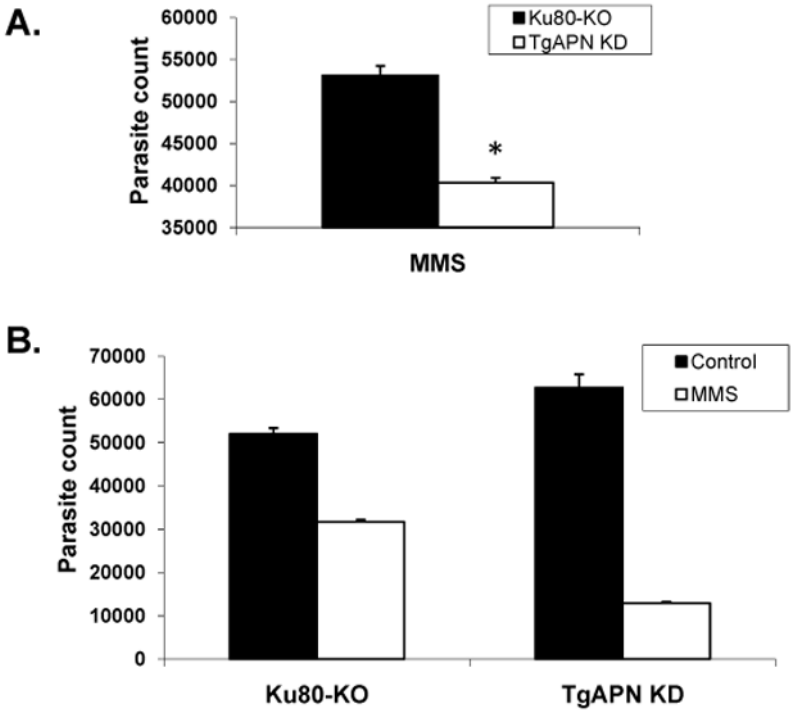

Figure 6. TgAPN knockdown parasites exhibit impaired response to DNA damage

A. Parental (Ku80-KO) and TgAPN-2xHA-DD parasites cultured without Shield (TgAPN knockdown, KD) were purified and subjected to 25 μM MMS for 2 hrs prior to being placed back in culture to infect fresh host cells. Growth was monitored at day 5 using the PCR-based B1 assay (* denotes p=0.000463, student t-test). B. Parental (Ku80-KO) and TgAPN-2xHA-DD parasites cultured without Shield were allowed to infect HFF monolayers incubated in the presence of 100 μM MMS (white bars) or vehicle control (black bars). Growth was monitored at day 7 using the B1 assay. Error bars indicate the SD of the mean.