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Trypanosoma brucei AP endonuclease 1 has a major role in the repair of abasic sites and protection against DNA-damaging agents

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Abbreviations: AP, apurinic/apyrimidinic; TBAPE1, *Trypanosoma brucei* AP endonuclease 1; LMAP, *Leishmania major* AP endonuclease; exoIII, exonuclease III; endoIV, endonuclease IV; OGG1, 8-oxoguanine DNA glycosylase 1; NTH1, endonuclease III; BSD, blasticidin S transferase; HYG, hygromycin phosphotransferase; THF, tetrahydrofuranyl residue; 3′-PG, 3′-phosphoglycolate; 3′-P, 3′-phosphate; MXA, methoxyamine; MTX, methotrexate; PLM, phleomycin; CPT, camptothecin; MMS, methyl methanesulphonate; NO, nitric oxide; Tdp1, tyrosyl-DNA-phosphodiesterase; PNKP, polynucleotide kinase phosphatase.

ABSTRACT

DNA repair mechanisms guarantee the maintenance of genome integrity, which is critical for cell viability and proliferation in all organisms. As part of the cellular defenses to DNA damage, apurinic/apyrimidinic (AP) endonucleases repair the abasic sites produced by spontaneous hydrolysis, oxidative or alkylation base damage and during base excision repair (BER). *Trypanosoma brucei*, the protozoan pathogen responsible of human sleeping sickness, has a class II AP endonuclease (TBAPE1) with a high degree of homology to human APE1 and bacterial exonuclease III. The purified recombinant enzyme cleaves AP sites and removes 3′-phosphoglycolate groups from 3′-ends. To study its cellular function, we have established TBAPE1-deficient cell lines derived from bloodstream stage trypanosomes, thus confirming that the AP endonuclease is not essential for viability in this cell type under *in vitro* culture conditions. The role of TBAPE1 in the removal of AP sites is supported by the inverse correlation between the level of AP endonuclease in the cell and the number of endogenously generated abasic sites in its genomic DNA. Furthermore, depletion of TBAPE1 renders cells hypersensitive to AP site and strand break-inducing agents such as methotrexate and phleomycin respectively but not to alkylating agents. Finally, the increased susceptibility that TBAPE1-depleted cells show to nitric oxide suggests an essential role for this DNA repair enzyme in protection against the immune defenses of the mammalian host.

1. Introduction

Abasic sites are one of the most common DNA lesions that arise in DNA and if left unrepaired, block DNA replication and have both mutagenic and cytotoxic effects [1]. The loss of purine or pyrimidine bases in cellular DNA occurs by spontaneous hydrolysis due to the inherent lability of the N-glycosyl bond. AP sites are also generated by the action of endogenous factors such as reactive oxygen species produced by the normal metabolism of the cell or by exposure to chemical and physical exogenous agents. Moreover, in the BER pathway, damaged bases are excised by a DNA glycosylase, giving rise to abasic sites [2, 3]. DNA strand breaks are also a major threat to genetic stability. Strand breaks containing modified 3′-ends such as 3′-phosphoglycolate (3′-PG) and 3′-phosphate (3′-P) may arise by the attack of DNA by free radicals [4, 5] or caused by the action of some antitumoral agents such as neocarzinostatin and bleomycin [6]. In addition, certain glycosylases can incise AP sites generating single-strand breaks with blocked 3′-ends. Human 8-oxoguanine-DNA glycosylase (OGG1) and the endonuclease III (NTH1) have intrinsic associated β-lyase activity that cleaves the phosphodiester backbone 3′ to the abasic site through a β-elimination reaction to produce a 3′-α, β-unsaturated aldehyde [7, 8] while endonuclease VIII-like glycosylases incise the abasic site by β,∂-elimination generating a 3′-P terminus [9]. Abasic sites and blocked-ends damages must be removed prior to DNA repair synthesis. The BER pathway is then completed by the coordinated actions of DNA polymerase and DNA ligase activities [10-12].

AP site repair is initiated by class II AP endonucleases, which catalyze the hydrolytic cleavage of the phosphodiester bond 5′ to the AP site [2]. There are two structurally unrelated families of class II AP endonucleases. The first family is comprised by AP endonuclease 1 (Apn1) from *Saccharomyces cerevisiae* and the homologous

endonuclease IV from *Escherichia coli* (endoIV) [13]. In contrast, human APE1 is homologous to *E. coli* exonuclease III (exoIII) [14]. APE1 has a broad specificity for AP sites but can also act as a 3′-phosphodiesterase and 3′-phosphatase [15, 16]. APE1 is also an activator of oxidized transcription factors [17]. This function relies on the cysteine 65 located at the N-terminal domain [18]. The AP endonuclease is essential in mammalian cells as mice lacking both *Ape1* alleles die during early embryonic development [19]. Recent studies involving downregulation of APE1 by RNAi in several tumour cell lines or by generation of conditional *Ape1* knockout mouse embryonic fibroblasts have revealed that cell death caused by the APE1 defect can be prevented by the independent expression of its DNA repair or redox regulatory functions [20, 21].

Human cells possess a second exoIII AP endonuclease named APE2, which is endowed with strong 3′ to 5′ exonuclease and 3′-phosphodiesterase but very low AP endonuclease activity [22]. Similarly to human cells, kinetoplastid parasites have two genes that code for putative AP endonucleases of the exoIII family. We have previously characterized the *Leishmania major* AP endonuclease LMAP whose C-terminal domain shares a 38% sequence identity with APE1 [23]. Unlike the human enzyme, LMAP lacks the cysteine responsible for the redox activity. Instead, the protozoan protein has AP endonuclease and 3′-phosphodiesterase activities equally efficient and its expression in AP endonuclease-deficient *E. coli* mutants confers resistance to killing by oxidative and alkylating agents. In comparison to human APE1, the parasite AP endonuclease has subtle aminoacid changes in its active site that confer a higher capacity to remove 3′-blocking ends [24].

Trypanosoma brucei, the African trypanosome, is the protozoan parasite transmitted by the tsetse fly vector and responsible for human African trypanosomiasis (sleeping

sickness) and a related disease in livestock. During its life cycle, *T. brucei* alternates between an insect and a mammalian host (procyclic and bloodstream forms respectively) and the adaptation to the environmental conditions requires significant structural and physiological changes. Our recent work has focused on characterizing the DNA repair mechanisms employed by the parasite to counteract the deleterious effects of host-generated oxidative stress. In *T. brucei*, we have identified the corresponding APE1 ortholog, named TBAPE1 henceforth. Our study shows that TBAPE1 is a nuclear enzyme with both AP endonuclease and 3′-phosphodiesterase activity *in vitro*. To further understand how the parasite cell deals with AP sites, we have generated TBAPE1-deficient cells derived from the bloodstream form. Depletion of the TBAPE1 protein is not lethal but results in a significant increase in abasic DNA damage. The deficiency in AP endonuclease renders cells hypersensitive to methotrexate, methoxyamine, phleomycin and nitric oxide. Our findings provide the first direct evidence that TBAPE1 is the major AP endonuclease activity present in *T. brucei* cells and exerts protection against abasic and DNA strand break-inducing agents. Therefore, kinetoplastid AP endonuclease proteins warrant further investigation regarding their involvement in drug resistance mechanisms and survival in the host.

2. Materials and Methods

2.1. Cloning, expression and purification of Trypanosoma brucei TBAPE1

The *TBAPE1* gene was amplified by PCR from *Trypanosoma brucei* genomic DNA with the following primers: 5′-AGG GCA ATT AAT ATG CCA CCG AAA AAA CTA TCC-3′ and 5′-GTC ATC GGA TCC TTA CTT ACG GAG CCA CAT CTG -3′, digested with AseI and BamHI and cloned into NdeI and BamHI sites of pET28a vector (Novagen) to generate pET28-TBAPE1. The N287A mutant gene was generated by PCR amplification with primers 5′-C ATA TGG GCT GGA GAC CTT GCT GTA GCG GAG CGC GAT TAT G′ and 5′-GTC ATC GGA TCC TTA CTT ACG GAG CCA CAT CTG -3′ on the plasmid template pET28-TBAPE1. The DNA fragment containing the desired mutation (underlined) was digested with NdeI and BamHI and cloned into pET28-TBAPE1 replacing the corresponding NdeI-BamHI fragment. Plasmids pET28-TBAPE1 and pET28-N287A were transformed into BL21(DE3) for subsequent purification. Histidine-tagged TBAPE1 proteins were purified on Ni^{2+}-charged HiTrap Chelating HP columns (GE Healthcare) as described [23]. The histidine-tagged protein containing eluates were concentrated and applied to a Superdex-75 gel filtration column (GE Healthcare). The fusion proteins were eluted in phosphate buffer (50 mM sodium phosphate pH 7.0, 150 mM NaCl and 10 mM 2-mercaptoethanol), aliquoted and stored in 20% (v/v) glycerol at −80 °C.

2.2. Enzymatic assays

Oligonucleotide substrates were generated as previously described [24]. A 21-mer oligonucleotide containing a containing a tetrahydrofuranyl residue (THF) (Eurogentec) or a single uracil base (U) (Trevigen) were employed as substrate for AP endonuclease and uracil DNA-glycosylase activity assays respectively. The oligonucleotides

harboring a 3′-PG residue and the oligonucleotide with a 5′-phosphate were synthesized by Eurogentec and used to determine 3′-phosphodiesterase activity. The lesion-containing oligonucleotides were labeled at the 5′-end using [γ-^{32}P]ATP (3000 Ci/mmol; Perkin Elmer) and T4 polynucleotide kinase (New England Biolabs). The ^{32}P-labeled oligonucleotides were purified using MicroSpin G25 columns (GE Healthcare) and annealed to 1.5-fold molar excess of the complementary strand by incubating at 95ºC for 2 min, followed by slow cooling to room temperature. THF and U were annealed to a complementary oligonucleotide containing adenine (A) or guanine opposite the lesion respectively; 3′-PG was annealed to A and 5′-P to produce a single-strand break.

In a standard reaction (10 µl final volume), 50 fmol of the corresponding ^{32}P-labeled duplex DNA was incubated in reaction buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 1% (w/v) bovine serum albumin, BSA) with increasing amounts of TBAPE1, N287A or protein from total cell extracts as indicated in the Figures. Reactions were carried out at 37 °C for 5 min. and stopped by adding 10 µl of formamide dye, followed by heating for 5 min. at 95 °C before loading onto the gels. To measure uracil glycosylase activity, the total amount of AP sites generated was determined by incubating the reaction mixture with 5 µl of a solution containing 0.1 N NaOH and 30 mM EDTA. The samples were then heated for 5 min at 95 ºC, neutralized with 5 µl of a solution containing 0.1 N HCl, mixed with 20 µl of formamide dye and heated for 5 min. at 95 ºC before loading. The products of the reactions were resolved by denaturing 20% (w/v) polyacrylamide gel electrophoresis (PAGE) (19:1 acrylamide/bisacrylamide). Gels were scanned and band intensities quantified using a Storm PhosphorImager (Molecular Dynamics).

2.3. Trypanosome cell lines, culture conditions and drug sensitivity assays

All the cell lines used in this study are derivatives of *Trypanosoma brucei brucei* bloodstream form S16 [25] and the procyclic cell line 449 [26]. Bloodstream trypanosomes were cultured at 37 ºC and 5% CO_2 in HMI-9 with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and procyclics were cultured at 28 ºC in SDM-79 supplemented with 10% FBS and 7.5 µg/mL Haemin.

Log-phase parasites at $7.5x10^4$ cells/mL in HMI-9 with 10% FBS were exposed to increasing concentrations of the genotoxic agent for 24 h. at 37 ºC. Cells were counted using a Z1 Coulter counter and experiments for each compound were performed at least three times, in duplicate. Methotrexate, camptothecin, methoxyamine, phleomycin and temozolide were purchased from Sigma-Aldrich; hydrogen peroxide and methyl methanesulfonate were obtained from Merck.

2.4. TBAPE1 replacement and overexpression constructs

To generate the *TBAPE1* gene replacement cassettes, a 5′-UTR fragment of 624 bp and a 3′-UTR fragment of 600 bp of the regions flanking the open reading frame of *T. brucei TBAPE1* gene (Tb927.8.5510, GeneDB database) were amplified by PCR from *T. brucei* 449 genomic DNA. The primers used to amplify the 5′-UTR region were 5′-ATC GCG GCC GCC GTT GTC TTG TTG TCA CTC-3′ (NotI restriction site underlined) and 5′-ATC CTC GAG ACC TCG CCA CCG GAT GAA AC-3′ (XhoI site underlined). For the 3′-UTR region, primers 5′-TAC GCA TGC GTG AGG TGA TGG GG -3′ (SphI site underlined) and 5′-ATC GCT AGC GGC CGC ACC ACG AGC ACA TAC CAT -3′ (NheI and NotI sites underlined) were used. To remove one of the two *TBAPE1* alleles, both 5′-UTR and 3′-UTR regions of *TBAPE1* gene were cloned in the

pHD887 plasmid (provided by Christine Clayton, ZMBH, Heidelberg, Germany), flanking the blasticidin S transferase (BSD) resistance gene as a selectable marker yielding the construct PAV35. To substitute the second *TBAPE1* allele, a XbaI-SphI DNA fragment containing the hygromycin phosphotransferase (HYG) selectable marker was obtained from plasmid pGR19 [27] and subcloned into pAV35 digested with XbaI and SphI, thus replacing the BSD resistance gene and yielding plasmid pAV62.
To generate cell lines that overexpress the TBAPE1 protein fused to a *c-myc* epitope at the N-terminus, the *TBAPE1* coding sequence was amplified by PCR from *T. brucei* 449 genomic DNA using specific primers containing NdeI and BamHI restriction sites. The amplified fragment was cloned in pGBKT7 (Clontech) that provides the *c-myc* sequence at the 5′-end of the cloning site. Subsequently, *myc-TBAPE1* was amplified from pGBKT7-TBAPE1 with primers 5′- AGG CCA ATT AAT ATG GCG GAG CAG AAG CTG ATC-3′ (including an AseI site) and 5′- GTC ATC GGA TCC TTA CTT ACG GAG CCA CAT CTG- 3′ (including a BamHI site), digested with AseI and BamHI and cloned into pGRV23b (unpublished) previously digested with NdeI-BamHI to generate pAV61. This plasmid is an expression plasmid which affords regulated expression of the corresponding gene from a procyclin promoter responsive to tetracycline and a puromycin N-acetyl-transferase resistance gene as selectable marker.

2.5. Transfection

Ten micrograms of linear targeting fragments obtained by NotI digestion and containing the Blasticidin S deaminase, hygromycin phosphotransferase or puromycin N-acetyl-transferase genes were transfected into bloodstream parasites by electroporation as previously described [25, 28]. Recombinants were selected in the presence of 5µg/mL of blasticidin (Invitrogen), 5µg/mL of hygromycin (Sigma-Aldrich) or 0.1 µg/mL of

puromycin (Sigma-Aldrich). Clone selection was completed after 7–15 days.

2.6. PCR primers used to confirm TBAPE1 replacement

Integration of the resistance markers was checked by PCR amplification with one of the primers directed to marker-specific sequences and the other directed to sequences upstream and downstream of the *TBAPE1* locus. Upstream sequence-specific primer (5′): 5′-GGC GG TGGA AGA ACT GGC-3′; BSD-specific primer (3′): 5′- TTG AGA CAA AGG CTT GGC C-3′; HYG-specific primer (3′): 5′- GTG ATA CAC ATG GGG ATC AGC AAT CGC GC-3′; BSD-specific primer (5′): 5′-TGG TTA TGT GTG GGA GGG C-3′; HYG-specific primer (5′): 5′-GCG CGA TTG CTG ATC CCC ATG TGT ATC AC-3′; Downstream sequence-specific primer (3′): 5′-GGG CGT AGT AAT ATG CGG C-3′.

2.7. Measurement of AP sites

Genomic DNA was isolated from $5x10^7$ cells by using DNAzol Reagent (Invitrogen). DNA samples (0.5 µg) were analyzed by using OxySelect™ Oxidative Damage Quantitation Kit (AP sites) (Cell Biolabs) following the manufacturer's instructions.

2.8. Antibody generation

Polyclonal antibody specific for *T. brucei* TBAPE1 was raised by immunizing rabbits with purified recombinant TBAPE1 protein. Before injecting the antigen into the rabbit, 300 µg of protein was mixed with Freund′s adjuvant at 1:1 ratio. Four inoculations were performed before obtaining the anti-TBAPE1 serum. The antibody was affinity-purified using homogeneous recombinant protein coupled to Affi-Gel® 15 Gel (Bio-Rad) resin, following the manufacturer's instructions.

2.9. Immunofluorescence studies

Cells from log-phase cultures were centrifuged and resuspended at $1x10^7$ cells/mL in 4% *p*-formaldehyde diluted in washing solution (1X PBS, 0.2% Tween® 20). The cells were fixed on poly-l-lysine coated slides for 20 min. at room temperature, washed twice and incubated for 75 min. in blocking/permeabilizing solution (1% Roche Blocking Reagent, 1% IGEPAL). The coverslips were incubated with primary antibody against TBAPE1 (1:100 diluted in blocking solution) for 1 h. and washed six times for 5 min. Subsequently, cells were incubated with FITC-conjugated anti-rabbit secondary antibody (Sigma, 1:40 diluted in blocking solution) for 1 h. and washed six times for 5 min. Next, the slide was dehydrated in methanol for 1 min., stained and mounted with Vectashield-DAPI (Vector Laboratories, Inc.). Vertical stacks of 15–25 slices (0.2 μm steps) were captured using an Olympus microscope and Cell R IX81 software. Deconvolution and pseudo-colouring of images were carried out using Huygens Essential software (version 3.3; Scientific Volume Imaging) and ImageJ software (version 1.37; National Institutes of Health), respectively.

3. Results

3.1. Enzymatic properties and cellular localization of TBAPE1

According to the genome sequence database of *Trypanosoma brucei*, the gene Tb927.8.5510 codes for a putative apurinic/apyrimidinic endonuclease. To verify the enzymatic properties of this uncharacterized protein, the gene was amplified by PCR and cloned into an *E. coli* vector that allow expression of the protein fused to a hexahistidine peptide. The histidine-tagged *T. brucei* AP endonuclease protein (TBAPE1) was overexpressed in *E. coli* BL21 (DE3) and purified to over 99% purity by metal-affinity and gel filtration chromatography. To confirm that the activity is intrinsic to the purified protein and does not come from a bacterial source, we also generated by site-directed mutagenesis and purified the active site mutant form N287A. Residue N287 aligns with N212 of the human enzyme APE1, which is essential for AP site recognition and whose substitution for alanine abolishes the AP endonuclease activity [29]. Both wild-type and mutant proteins had similar chromatographic behaviour and were purified to the same level (Figure 1A).

The purified recombinant proteins were assayed for AP endonuclease activity using a 5′-radiolabeled 21-nucleotide DNA substrate, in which one strand contained a THF residue. THF is a more stable substrate than regular AP sites that cannot be cleaved by class I endonucleases [30]. Reaction conditions were the same previously used for the AP endonuclease LMAP from *Leishmania major*. In the presence of increasing concentrations of TBAPE1, an AP site incision product was generated corresponding to a nine-nucleotide oligomer (Figure 1B). As control, the reaction product formed by incubation with an excess amount of endonuclease IV is shown. The bacterial AP endonuclease cleaves DNA at 5′ of the abasic site leaving a 3′-hydroxyl end (3′-OH).

The N287A mutant has a greatly reduced cleavage activity and only minor incision product (less than 5%) was detected at the highest enzyme concentration tested.

A second catalytic function associated to AP endonucleases is the 3′-phosphodiesterase activity that excises 3′-modified ends at DNA strand breaks. These 3′-end lesions are refractory to gap-filling by DNA polymerase or rejoining by DNA ligases so they need to be cleaned up to complete the repair process. The 3′-phosphodiesterase activity of TBAPE1 was examined by monitoring the conversion of a 3′-PG end to the corresponding 3′-OH form at a DNA single-strand break (Figure 1C). Upon incubation at 37 °C with low concentrations of TBAPE1 (0.1-1 nM), most of the 3′-PG ends were converted into a moiety with lower electrophoretic mobility which corresponds to the 3′-OH product. However, while TBAPE1 is able to excise the 3′-PG from single-strand breaks, the N287A mutant lacks such activity.

The cellular localization of TBAPE1 was determined by immunofluorescence microscopy using an affinity-purified antibody directed against pure recombinant TBAPE1 protein. As shown in Figure 1D, endogenous TBAPE1 protein was exclusively located in the nucleus of procyclic and bloodstream *T. brucei* cell lines (449 and S16 respectively). The same subcellular localization has been previously shown for the AP endonuclease of *Leishmania major*, suggesting that their repair function is restricted to nuclear DNA [24]. However, we cannot rule out the possibility that a minor undetectable fraction of TBAPE1 is constitutively associated to the kinetoplast or present in the cytosol.

3.2. Double replacement of TBAPE1 in the bloodstream form of T. brucei.

In order to perform the double replacement of the *TBAPE1* gene, two DNA constructs were made consisting on the blasticidin S deaminase (BSD) and hygromycin

phosphotransferase (HYG) resistance markers flanked by approximately 600 base pairs of the *TBAPE1* 5′-UTR and 3′-UTR sequences. Linear targeting fragments were transfected sequentially into bloodstream S16 cells (WT) and after each transfection, recombinant clones were selected with the appropriate drug. Integration of the BSD and HYG markers was monitored by PCR using primers that hybridize either upstream or downstream of the inactivation cassette of *TBAPE1* in combination with marker-specific primers. The first round of transfections yielded blasticidin-resistant clones all of which had correctly integrated the BSD marker (*TBAPE1/*Δ*tbape1*::BSD, abbreviated SKO). One of these clones was subjected to a second round of inactivation with the HYG cassette obtaining this way transfectants resistant to blasticidin and hygromycin (Δ*tbape1*::HYG/Δ*tbape1*::BSD, abbreviated KO). Figure 2 shows the analysis by PCR of the clones chosen for further analysis. Genomic DNA from single and double-replacement clones where BSD has integrated at the right locus produces 1260 and 1396 bp PCR fragments with the two different sets of primers above indicated (Figure 2A). On the other hand, PCR analysis of clones obtained after a second-round inactivation where the HYG cassette has correctly recombined yields 1613 and 2145 bp amplification products (Figure 2B). Only the double knockout clone does not retain any copy of *TBAPE1* as shown by the absence of PCR product (1191 bp) after the PCR assay with internal *TBAPE1*-specific primers (Figure 2C). The level of protein expression of the selected clones was determined by western blot using the purified anti-TBAPE1 antibody. Protein quantity standardization was carried out using an antibody directed against *T. brucei* dUTPase [31] (Figure 2D). In cell line SKO, TBAPE1 expression was partially decreased consistently with having a single allelic replacement. As expected, no TBAPE1 protein was detected in KO cells extracts. The fact that both chromosomal alleles of *TBAPE1* can be successfully replaced provides

direct evidence that the AP endonuclease coded by the Tb927.8.5510 gene is not essential for trypanosome viability.

3.3. TBAPE1 is the major class II abasic site endonuclease activity in Trypanosoma brucei cells.

We have examined the contribution of TBAPE1 to the AP site processing by measuring AP site incision activity of whole cell extracts from TBAPE1-deficient cells lines. A 21-mer DNA duplex containing a THF residue was used as substrate. In addition to TBAPE1, the genomic sequence database indicates that *Trypanosoma brucei* possesses a second putative class II AP endonuclease with homology to yeast Apn2 and human APE2. Having assayed the AP endonuclease activity over a wide range of protein concentrations, no cleavage product was produced by the TBAPE1-deficient (KO) cell extract (Figure 3A). Consistently, the reduced TBAPE1 expression in SKO was correlated with a 2-fold decrease in the AP site incision activity respect to wild-type (WT) cells (according to the product formed at 10 ng of protein extract). In addition to western blot analysis, these biochemical data further validate our KO constructs. No other AP site cleavage activity was detected under the conditions tested indicating that TBAPE1 is the major and probably the only class II AP endonuclease activity of trypanosome cells.

Cell extracts were also assayed for cleavage activity using a DNA substrate harboring a single-strand break with a 3′-PG end. With this substrate, a clear correlation between the expression level of TBAPE1 protein and excision activity was observed (Figure 3B). At 10 ng of protein extract, the 3′-phosphodiesterase activity present in SKO cells was 1.7-fold lower respect to the endogenous activity of parental S16 cells. However, while endonuclease activity was absent, the excision of phosphoglycolate was greatly

decreased but not completely abolished in the absence of TBAPE1. Our data indicate that TBAPE1 is indeed the main enzyme that excises 3′-phosphoglycolate at single-strand breaks but not the only one and other excision activities might also participate in the removal of these 3′-blocking ends.

The excision of uracil from DNA is an important source of endogenous abasic sites in the genome of many organisms [32]. Consequently, we speculated that the enzymatic processes that control uracil removal might be downregulated in the AP endonuclease-deficient background as part of an adaptation mechanism to alleviate the burden of AP sites. In *T. brucei* there is a single uracil DNA-glycosylase activity coded by the *UNG* gene (unpublished results). We checked the levels of uracil excision by TBAPE1-depleted or wild-type cell extracts *in vitro*. In comparison to the other activities tested, the uracil excision activity was not significantly modified by the presence or absence of TBAPE1 indicating that the proposed mechanism to reduce the load of AP sites does not operate in the selected trypanosome knockout cells (Figure 3C).

3.4. Effect of TBAPE1 depletion on cell proliferation.

Although cells lacking TBAPE1 were viable, in the course of clone selection we noticed that the *TBAPE1*-KO cell lines exhibited a lower proliferation rate than WT cells. To confirm that the growth defect was indeed due to the loss of TBAPE1, we proceeded to generate WT and KO cell lines where the AP endonuclease was ectopically expressed. To this purpose, we used a DNA construct containing the *TBAPE1* gene with a c-myc epitope sequence at the 5′-end. The fusion gene was flanked by sequences that allow gene integration at rDNA regions, selection by puromycin and regulation of TBAPE1 expression by doxycycline. The WT and KO cell lines were transfected and several clones resistant to puromycin and harboring at least one *TBAPE1* copy were obtained

(WT/TBAPE1 and KO/TBAPE1 respectively). All of the clones selected expressed myc-TBAPE1 constitutively even in the absence of doxycycline. Figures 4A and 4B show the PCR and western blot analysis of one of the clones chosen for further characterization. The myc-tagged version of the AP endonuclease is enzymatically active since higher levels of AP endonuclease activity can be detected in cell extracts from selected clones after induction with doxycycline (Figure 4C) and localizes correctly in the nucleus as the wild-type enzyme (Figure 4D). Figure 4E shows cell proliferation of the various cell lines generated over a 4-day period. Growth of KO cells was clearly inhibited respect to parental cells (Figure 4E). We have estimated a 30% increase in the doubling time for KO with regard to WT cells during logarithmic phase (9.4 vs 6.4 hours). Expression of myc-TBAPE1 in the TBAPE1-deficient background did not reverted the growth delay even when protein expression was induced by doxycycline, indicating that poor growth was not due to the absence of the TBAPE1 enzymatic function. A toxic effect due to the overexpression of myc-tagged TBAPE1 is not probable since WT/TBAPE1 cells proliferate similar to parental wild-type cells (Figure 4E). Moreover, the myc-TBAPE1 enzyme is active as shown in the oligonucleotide-based cleavage assay *in vitro*. Our data suggest that the proliferation rate is probably affected by genetic changes acquired during the knockout selection process. Indeed, the absence of the DNA repair function of TBAPE1 might increase the mutagenesis rate in these cells thus favoring the generation of adaptive mutations to compensate the loss of AP endonuclease.

3.5. TBAPE1-depleted cells accumulate AP sites and are sensitive to methoxyamine.

In human cells, depletion of APE1 by RNAi generates an increase in AP site damage [20]. Our hypothesis predicts that a similar accumulation of DNA damage should take

place in the absence of the AP endonuclease TBAPE1. By using an aldehyde reactive probe, we determined abasic sites in genomic DNA from various trypanosome cell lines (Figure 5A). As expected, the number of AP sites detected in SKO and KO backgrounds was 1.6 and 2-fold higher respectively than in the parental S16 cell line. Upon expression of the myc-TBAPE1 protein, the number of abasic sites diminished and no significant differences were appreciated in comparison to the wild-type basal level of DNA damage. The data presented above strongly suggest that TBAPE1 participates in the removal of abasic sites from DNA.

Methoxyamine (MXA) specifically binds to AP sites [33] and prevents their processing by both AP endonucleases and AP lyases [34]. Treatment of WT cell line with 0.1 mM MXA resulted in 10% growth inhibition, indicating that MXA itself is mildly toxic to trypanosome cells (Figure 5B). In contrast, the antiproliferative effect was significantly higher when SKO and KO cells were exposed to the same dose of MXA, producing a 40% and 60% inhibition respectively. The growth defect was fully reverted by the ectopic expression of myc-TBAPE1. It is worth noting that the level of toxicity induced by MXA strongly correlates with the basal number of unrepaired AP sites in the genome of these cells. The results further suggest that chemical protection by MXA prevents abasic site cleavage by AP lyases when TBAPE1 is not present, leading to an accumulation of these DNA lesions that results toxic for the cell.

3.6. TBAPE1 protects cells against methotrexate but not against alkylation-induced DNA damage.

In order to gain insight into the cellular function of the trypanosomal AP endonuclease, KO cells were exposed to several DNA damage inducing agents and their effect on the proliferation rate was determined. Antifolates such as methotrexate (MTX) have been

shown to produce an increase in the intracellular levels of dUTP due to impaired dTMP synthesis and dUMP accumulation. As a result of the altered dTTP/dUTP ratio, dUTP is preferentially incorporated into DNA during replication, which activates the BER pathway [35]. KO cells exposed to MTX showed a significant decrease in proliferation in comparison to parental WT or SKO cells (Figure 6A). The expression of myc-TBAPE1 in the AP endonuclease-deficient background not only restored the resistance to the antifolate but also conferred an increased protection to MTX in comparison to WT cells. These results may indicate that AP site cleavage is rate-limiting under antifolate treatment. To confirm that AP sites are indeed the DNA repair intermediate upon which TBAPE1 is acting, we measured the steady-state level of abasic damage after MTX treatment in repair-proficient and -deficient cells (Figure 6B). At the dose used for the study (5 μM MTX), the proliferation rates decreased 30% and 50% in WT and KO cells respectively. The exposure to the antifolate induced an overall increase in the number of AP sites over the endogenous level of DNA damage shown in Figure 5A. The total number of AP sites induced by MTX was higher in the absence of TBAPE1 and dropped to wild-type level in KO/TBAPE1 cells. These data would further support that TBAPE1 is involved in AP site removal in trypanosome cells.

To further assess the role of TBAPE1 on the repair of cellular AP sites, we next examined the resistance of the different cell lines to the alkylating agent methyl methanesulfonate (MMS). This genotoxic agent alkylates mainly purine bases in DNA and produces abasic sites [36]. Strikingly, we did not find significant differences between growth rates of the various cell lines at any of the doses assayed (Figure 6C). Similar results were obtained with the antitumour methylating drug temozolomide (Figure 6D). Our data indicate that alkylation DNA damage is repaired by an AP endonuclease-independent pathway, suggesting that alkylation base damage such as N^3-

methyladenine is processed by mechanisms other than BER in *T. brucei*. Treatment with MMS and TMZ causes indeed a proliferation defect, which is probably due to the block of DNA replication by alkylated DNA adducts or DNA repair intermediates other than AP sites.

3.7. Role of TBAPE1 in the repair of DNA strand break damage.

Kinetoplastid AP endonucleases are endowed with efficient 3′-phosphodiesterase and 3′-phosphatase activities that have been postulated to participate in 3′-end processing during DNA strand break repair [23]. We therefore investigated whether KO cells were more sensitive to this type of damage by exposing the cells to phleomycin (PLM) and camptothecin (CPT). These DNA-damaging agents induce distinct types of DNA strand breaks by different mechanisms of action. PLM is a structurally related form of bleomycin that binds to DNA and, through a free radical-based mechanism, produces DNA base loss and single and double-strand breaks with 3′-phosphoglycolate [37]. After PLM treatment, repair-deficient cells experienced important proliferation defects with growth rates below 50% at 0.25 µg/mL of PLM (Figure 7A), while at the same dose WT and KO/TBAPE1 cells exhibited 90% of the growth rate of non-treated cells. CPT blocks the rejoining step of the cleavage/religation reaction catalyzed by DNA topoisomerase I, resulting in accumulation of a reaction intermediate where the enzyme is covalently linked to the 3′ end of the broken DNA [38]. The stalled topoisomerase I-DNA adducts are initially processed by tyrosyl-DNA phosphodiesterase 1 (Tdp1) that hydrolyzes the phosphodiester bond between the tyrosine of topoisomerase and the 3′-phosphate of DNA [39]. Subsequent removal of the 3′-phosphate is probably performed by polynucleotide kinase phosphatase (PNKP) [40]. Trypanosomatid genomes code for Tdp1 and PNKP repair enzymes although they have not yet been biochemically

characterized with the exception of the Tdp1 enzyme in the parasite *Leishmania donovani* [41]. Treatment of trypanosome cells with increasing concentrations of CPT did not reveal significant differences in sensitivity between cell lines, which indicates that the parasite AP endonuclease does not intervene in the repair of CPT-induced DNA damage (Figure 7B).

3.8. AP endonuclease deficiency sensitizes cells to nitric oxide

Nitric oxide (NO) is an important effector molecule of the immune system that possesses antiparasitic activity. *T. brucei* is an extracellular parasite highly susceptible to nitric oxide (NO)-dependent killing by gamma interferon-activated macrophages [42]. In addition, several NO donors have been shown to induce cell death of *Trypanosoma brucei in vitro* [43]. We have investigated the role of BER and TBAPE1 in particular, in nitric oxide resistance by treating trypanosomes with Spermine NONOate, as this compound spontaneously dissociates liberating two molecules of NO per mole of parent compound. Upon exposure to the NO donor, trypanosome cells lacking one or two *TBAPE1* alleles were more sensitive than their parental cell line. However, ectopic expression of TBAPE1 in the KO background reverted the proliferation defect caused by the NO donor. Therefore, the parasite AP endonuclease counteracts part of the toxic effect of Spermine NONOate suggesting that some of its trypanocidal activity is due to NO-induced DNA damage.

4. Discussion

Human african trypanosomiasis or sleeping sickness is a disabling and fatal disease caused by infection with the protozoan parasite *Trypanosoma brucei*. As one of the most neglected tropical diseases, it requires the development of novel effective

strategies for chemotherapy. The mechanisms of protection against oxidative stress have been considered a valid drug target as suggested by the high sensitivity of this protozoan parasite to activated macrophages [44]. One of these mechanisms is the base excision repair pathway required to eliminate cytotoxic oxidative DNA damage such as base lesions, strand breaks and base-free sites. Here, we show that *Trypanosoma brucei TBAPE1* gene codes for an active class II abasic site endonuclease, an essential component of the BER pathway. The protein is expressed in both procyclic and bloodstream forms of *T. brucei* and localizes in the parasite nucleus. The exclusive localization of TBAPE1 contrasts with the ubiquitous presence of mammalian APE1 in nucleus, cytosol and mitochondria. We cannot exclude that TBAPE1 is constitutively present at organelles other than the nucleus, particularly at the highly active mitochondrion of insect-stage procyclics, but at concentrations too low to be detected by immunological techniques. Moreover, several studies have shown that the amount of APE1 protein increases and undergoes a translocation to the nucleus and mitochondria from the cytosol in response to exogenous oxidative stress [45, 46]. Hence, the parasite enzyme might experience a similar dynamic behavior under oxidative stress conditions. This possibility is currently under study.

To address the cellular role of TBAPE1, we have measured the accumulation of DNA damage in chromosomal DNA. The inverse correlation between the amount of TBAPE1 protein and the number of endogenous abasic sites in genomic DNA supports its role in the removal of AP sites *in vivo*. In addition, AP endonuclease-deficient trypanosomes are more sensitive to antifolates in a similar manner to *S. cerevisiae* Apn1 mutants [47]. Indeed, we have confirmed that the MTX treatment produces an overall increase in the steady-state level of abasic sites in the absence of AP endonuclease. In apparent contradiction, KO cells were equally sensitive to MMS than repair-proficient cells even

though methylated purines are known to be the substrate of specific DNA glycosylases and consequently a source of AP sites. Treatment of trypanosome S16 cells with sublethal doses of MMS did not induce an adaptive response to alkylating agents as in bacteria (data not shown). It is well established that prokaryotic and eukaryotic cells deficient in AP endonuclease activity have an increased sensitivity to the toxic effects of MMS due to their inability to repair alkylation damage via BER [48-50]. All these organisms possess specific DNA-glycosylases (the so-called methyladenine glycosylases) that excise methylated purines generating AP sites. Our data suggest that trypanosome cells lack of functional methyladenine glycosylase activity. This exceptional observation is supported by the fact that no methyladenine glycosylase orthologs have been identified in the genome sequence database of *T. brucei* and other related kinetoplastid parasites such as *Trypanosoma cruzi* and *Leishmania major*.
DNA single-strand breaks are also frequent lesions in DNA and their repair of vital importance for the cell. *In vitro*, TBAPE1 removes 3′-phosphoglycolate groups in an efficient manner suggesting its role in the processing of this type of blocked 3′-ends. We have investigated this function by challenging repair-deficient cells with phleomycin, a genotoxic agent that specifically induces strand-breaks with 3′-PG termini. In agreement with previous reports on the human APE1 [50, 51], our results show that depletion of AP endonuclease renders trypanosome cells hypersensitive to PLM. This sensitivity to PLM clearly suggests a link between the specific reduction of TBAPE1 levels and a deficient repair of 3′-PG ends *in vivo*. However, other DNA repair enzymes could be involved in the repair of 3′-PG lesions and in fact, we have detected a significant 3′-PG excision activity in TBAPE1-deficient cell extracts. This activity might belong to *T. brucei*'s APE2, aprataxin or Tdp1 orthologs whose human counterparts have been biochemically characterized as potential DNA repair factors capable to initiate the

processing of DNA strand breaks [22, 52, 53].

Unlike the APE1 essential role in human cells, our work provides evidence that the main cellular AP endonuclease activity is dispensable in *T. brucei* bloodstream life stage forms. The accumulation of endogenous AP sites does not appear sufficient to provoke cell death. This conclusion is in agreement with a recent study of high-throughput phenotyping screens using RNAi target sequencing (RIT-seq) which reported that TBAPE1 depletion does not impair significantly the growth of both bloodstream and procyclic trypanosomes [54]. A similar phenotype is displayed by *S. cerevisiae* AP endonuclease mutant where the combined activity of Apn1 and Apn2 is not essential for survival. In this organism, viability in the absence of AP endonuclease strictly depends on nucleotide excision repair since mutations affecting genes involved in this pathway cause cell death [55]. According to the available genetic data, the repair of abasic sites in AP endonuclease-deficient yeast cells would involve AP site cleavage by an AP lyase activity associated to DNA glycosylases [55]. The cleavage reaction results in a 3′-unsaturated aldehyde product that would require further excision by the NER protein RAD1/RAD10. *T. brucei*'s genome contains two DNA-glycosylase/AP-lyases with a high degree of homology to NTH1 and OGG1 and also the RAD1/RAD10 orthologs. The finding that knockout cells for TBAPE1 are sensitive to methoxyamine suggests that the modification of abasic sites by this chemical agent is blocking the AP lyase activities required as part of an alternative pathway to initiate AP site repair when the AP endonuclease is not present.

Depletion of TBAPE1 confers the trypanosome cells a higher sensitivity to abasic and strand break damage, which suggests that the enzyme could confer resistance to pharmacologic therapies where the mode of action involves genotoxic damage. Also, the increased susceptibility that TBAPE1-depleted cells show to NO could indicate an

essential role for this DNA repair enzyme in protection towards the oxidative stress generated by the immune defenses of the mammalian host. Additional studies are in progress in order to confirm this protective function and establish the role of TBAPE1 in parasite proliferation *in vivo* and its use as a potential drug target.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure Legends

1. DNA repair activities and intracellular localization of the *Trypanosoma brucei* AP endonuclease TBAPE1. A) Coomassie blue stained SDS-PAGE gel showing purified TBAPE1 (WT) and mutant N287A proteins (1 μg); M, molecular mass standard. B) The AP endonuclease and C) 3′-phosphodiesterase activity of TBAPE1 and N287A was tested by incubating increasing concentrations of the proteins (0-1 nM) with 5 nM of the THF and 3′-PG substrates respectively at 37 ºC for 5 minutes. The products of the reactions were separated by denaturing 20% PAGE. Positions in the gel of the substrates and the 3′-OH product of the reaction are indicated. EndoIV, Endonuclease IV. D) Immunofluorescence analysis was performed to establish the intracellular localization of TBAPE1 from *T. brucei* in both procyclic (PF) and bloodstream forms (BF). Nuclear and kinetoplast DNA were stained with DAPI and TBAPE1 was detected with anti-TBAPE1 and FITC conjugated anti-rabbit secondary antibody. Images were collected with an Olympus microscope and Cell R IX81 software.

2. *TBAPE1* inactivation by double replacement in the bloodstream stage form. A) Schematic drawing and PCR tests confirming the replacement of the *TBAPE1* locus by blasticidin S deaminase (BSD). Integration of the BSD marker was monitored by PCR using primers complementary to sequences of *loci* located upstream or downstream of the inactivation cassette in combination with BSD-specific primers. B) Inactivation of the second *TBAPE1* allele with the hygromycin phosphotransferase (HYG) cassette. C) Detection of the *TBAPE1* open reading frame by PCR amplification with specific primers. A, B and C) The figure shows the amplification assays carried out with genomic DNA from clones selected for further study. The position of the amplification

primers is shown by arrows. The expected sizes of the PCR products are also indicated. M, Molecular mass standard. D) TBAPE1 content was determined by western blot analysis on protein extract from $5x10^6$ parasites. As loading control, the level of dUTPase protein was measured in the three cell lines. WT, wild-type; SKO, *TBAPE1/*Δ*tbape1*::BSD; KO, Δ*tbape1*::HYG/Δ*tbape1*::BSD.

Figure 3. Comparison of DNA repair activities in whole cell extracts from TBAPE1-proficient or deficient cells. TBAPE1-proficient (WT) or TBAPE1-deficient (SKO and KO) cell lines were used to prepare extracts. Reactions were carried out as described in Materials and Methods with increasing amounts of total extract protein as indicated. A) AP endonuclease activity was measured using an oligonucleotide harboring a single tetrahydrofuranyl residue as substrate (THF). The 9-nucleotide oligomer reaction product is shown (9 nt). B) 3′-phosphodiesterase was tested on 21-mer DNA duplex containing a nick with a 3′-phosphoglycolate residue (3′-PG). The reaction products are shown (3′-OH and -1 nt). C) Uracil glycosylase activity was tested on a 21-mer DNA duplex containing a uracil residue (U). For each activity and cell line, the plot of the percentage of DNA substrate cleaved versus the total amount of protein used in the reaction is represented. WCE, whole cell extract. Cell lines are represented with the following symbols: WT (filled circle), SKO (grey circle), KO (open circle).

Figure 4. Proliferation rate in the presence or absence of AP endonuclease. A) Integration of the myc-TBAPE1 expressing construct in parental (WT) and KO cell lines was checked by PCR amplification of *TBAPE1* with primers directed against sequences within the *TBAPE1* open reading frame. M, Molecular mass standard. B) TBAPE1 expression was determined by western blot analysis on 10 µg of protein extract obtained from doxycycline-induced (+) or uninduced cells (-). The level of

dUTPase protein was measured as loading control. C) The AP endonuclease activity of protein extracts from cell lines WT/TBAPE1 and KO/TBAPE1 grown in the presence (+) or absence of doxycycline (-) was measured using an oligonucleotide harboring a single THF residue. 10 ng of total protein were incubated for 30 min. at 37 ºC with the oligonucleotide substrate under standard reaction conditions. The products of the reactions were separated by denaturing 20% PAGE. The position of the substrate (THF) and the reaction product (9 nt) are shown. D) Nuclear localization was confirmed by immunofluorescence analysis of the *T. brucei* KO/TBAPE1 cell line. Nuclear and kinetoplast DNA were stained with DAPI and TBAPE1 was detected with anti-TBAPE1 and FITC conjugated anti-rabbit secondary antibody. E) Cell proliferation was quantified over a period of 4 days. Cultures were initiated with $5x10^3$ cells/mL in HMI-9 with 10% (v/v) heat-inactivated fetal bovine serum and doxycycline in the case of WT/TBAPE1 and KO/TBAPE1. Cells were counted using a Z1 Coulter counter. Plotted data are the average of three independent experiments. Cell lines are represented with the following symbols: WT (filled circle), SKO (grey circle), KO (open circle), WT/TBAPE1 (filled triangle), KO/TBAPE1 (open triangle).

Figure 5. Abasic site damage in genomic DNA from TBAPE1-deficient cells. A) Genomic DNA from wild-type (WT), single TBAPE1 knockout (SKO), double TBAPE1 knockout (KO) and double knockout cell overexpressing myc-TBAPE1 (KO/TBAPE1) was purified and AP sites quantified by using an aldehyde reactive probe. The error bars correspond to standard deviations (n=3). The asterisk indicates significant differences with WT data ($p<0.05$). B) Log-phase parasites at $7.5x10^4$ cells/mL were exposed to increasing concentrations of methoxyamine (MXA) for 24 h at 37 ºC. Proliferation was calculated relative to cell growth in the absence of the

genotoxic agent for each cell line. Experiments for each compound were performed at least three times, in duplicate. Cell lines are represented with the following symbols: WT (filled circle), SKO (grey circle), KO (open circle), KO/TBAPE1 (open triangle).

Figure 6. Sensitivity of trypanosome repair-deficient cells to methotrexate but not to alkylating agents. Log-phase parasites at $7.5x10^4$ cells/mL were exposed to increasing concentrations of A) methotrexate (MTX), C) methyl methanesulfonate (MMS) or D) temozolomide (TMZ) for 24 h. at 37 ºC. Proliferation was calculated relative to cell growth in the absence of the genotoxic agent for each cell line. Experiments for each compound were performed at least three times, in duplicate. Cell lines are represented with the following symbols: WT (filled circle), SKO (grey circle), KO (open circle), KO/TBAPE1 (open triangle). B) AP site quantification after exposure to MTX. Log-phase cells at $7.5x10^4$ cells/mL were incubated with 5 μM MTX for 24 h. After treatment, cells were collected and their genomic DNA isolated. AP sites were quantified as indicated in Material and Methods.

Figure 7. Protective role of TBAPE1 towards DNA damage induced by phleomycin. Log-phase *T. brucei* cells at $7.5x10^4$ cells/mL were exposed to increasing concentrations of A) phleomycin (PLM) and B) camptothecin (CPT) for 24 h. at 37 ºC. Proliferation was calculated relative to cell growth in the absence of the genotoxic agent for each cell line. Experiments for each compound were performed at least three times, in duplicate. Cell lines are represented with the following symbols: WT (filled circle), SKO (grey circle), KO (open circle), KO/TBAPE1 (open triangle).

Figure 8. Trypanocidal activity of nitric oxide in the absence of AP endonuclease.

Log-phase *T. brucei* cells at $7.5x10^4$ cells/mL were exposed to increasing concentrations of Spermine NONOate (SperNO) for 24 h. at 37 ºC. Proliferation was calculated relative to cell growth in the absence of the NO donor for each cell line. Experiments for each compound were performed at least three times, in duplicate. Cell lines are represented with the following symbols: WT (filled circle), SKO (grey circle), KO (open circle), KO/TBAPE1 (open triangle).

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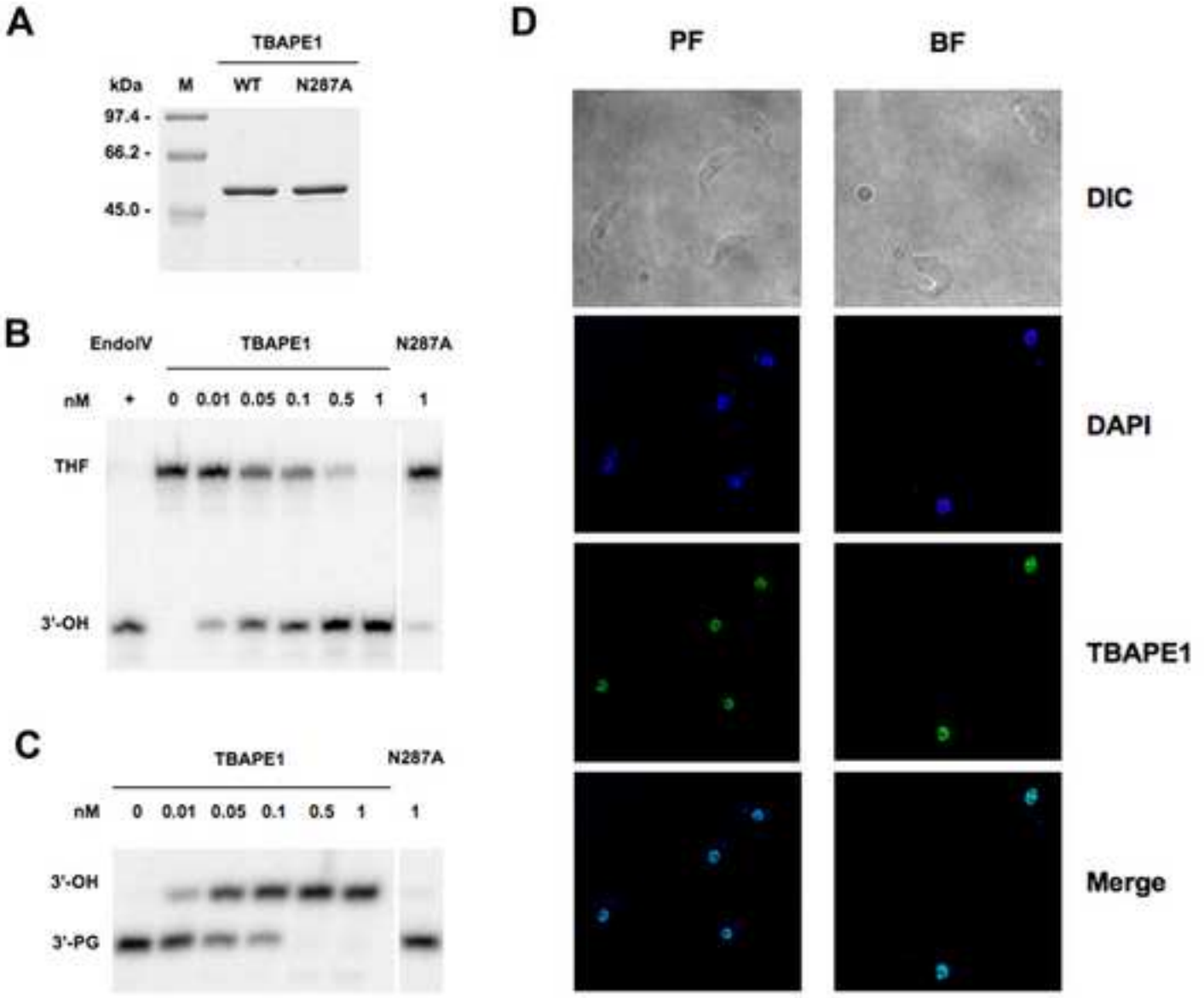

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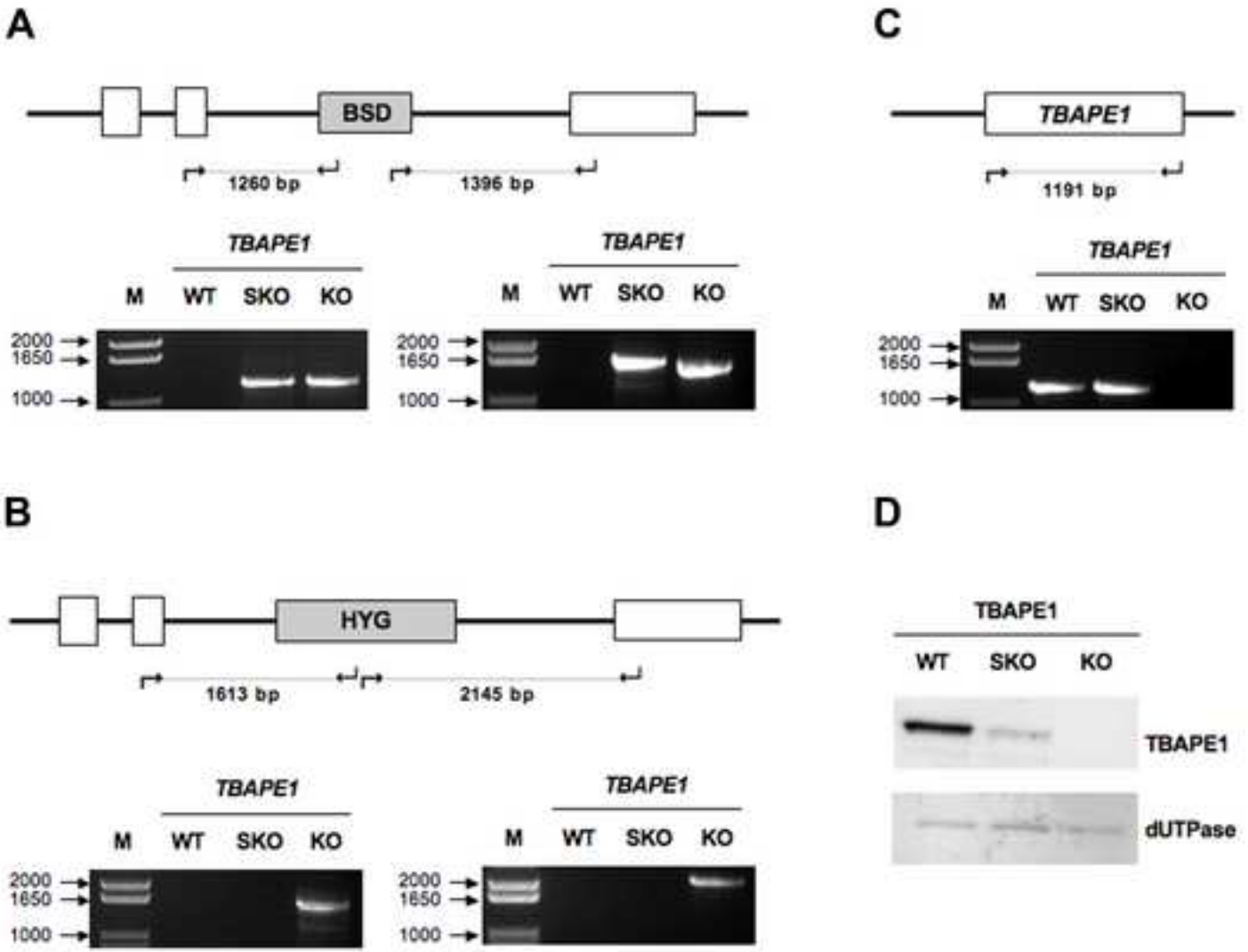

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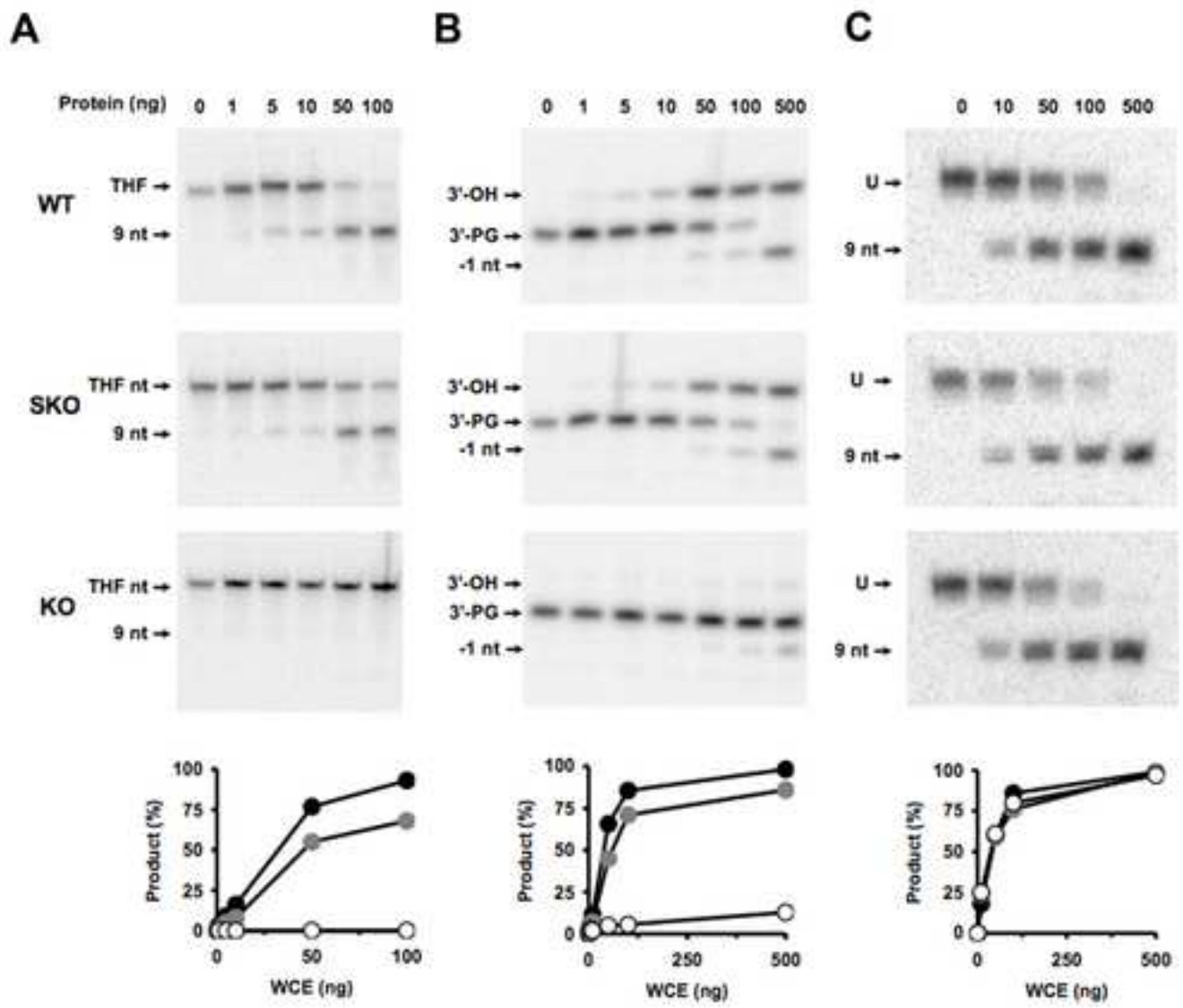

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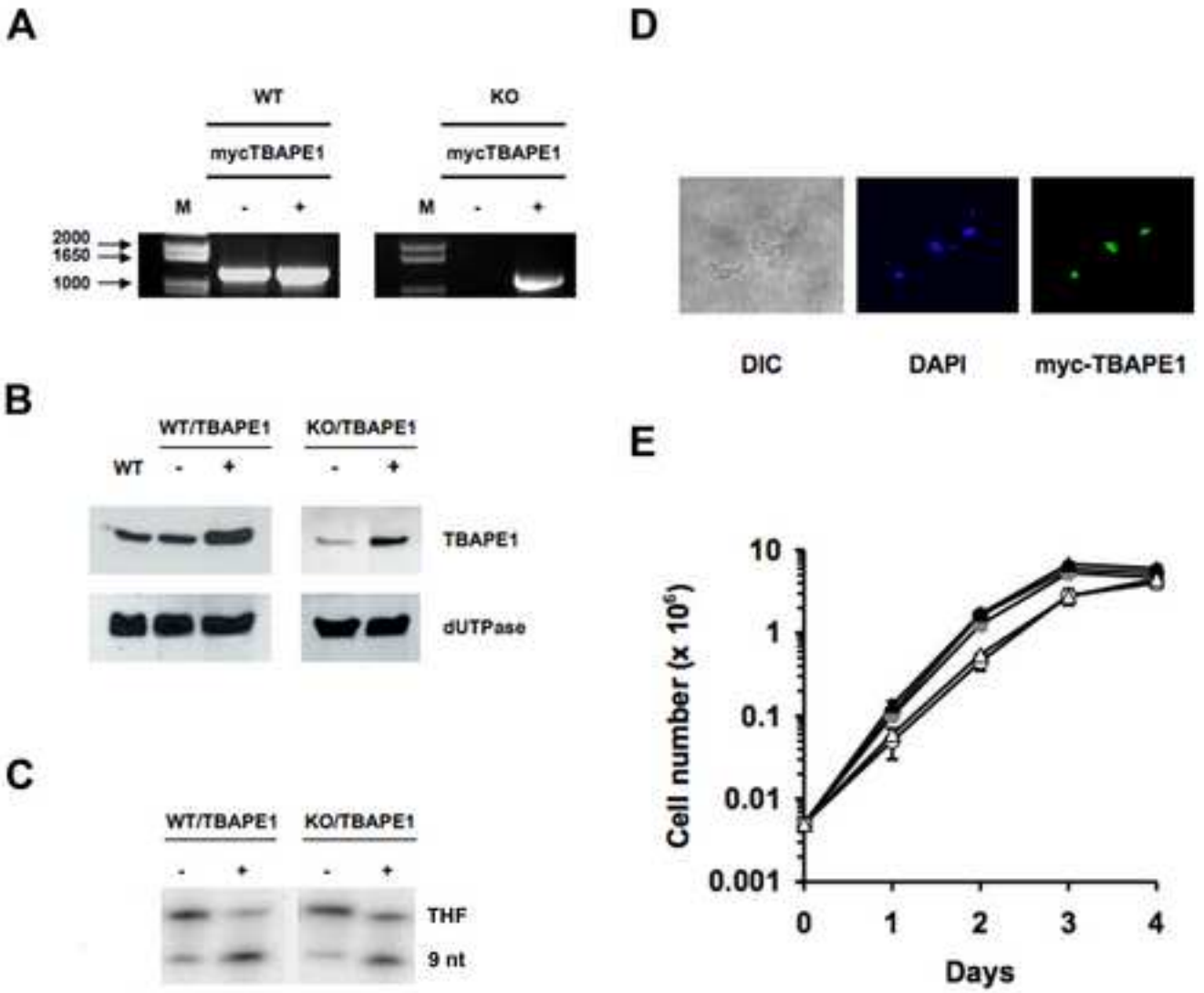

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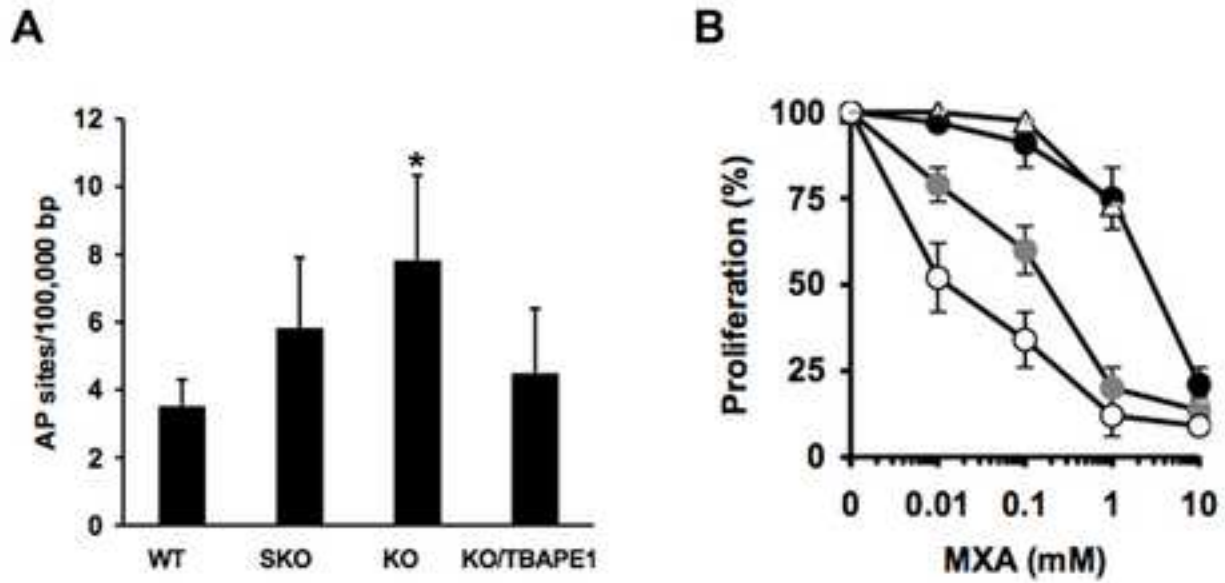

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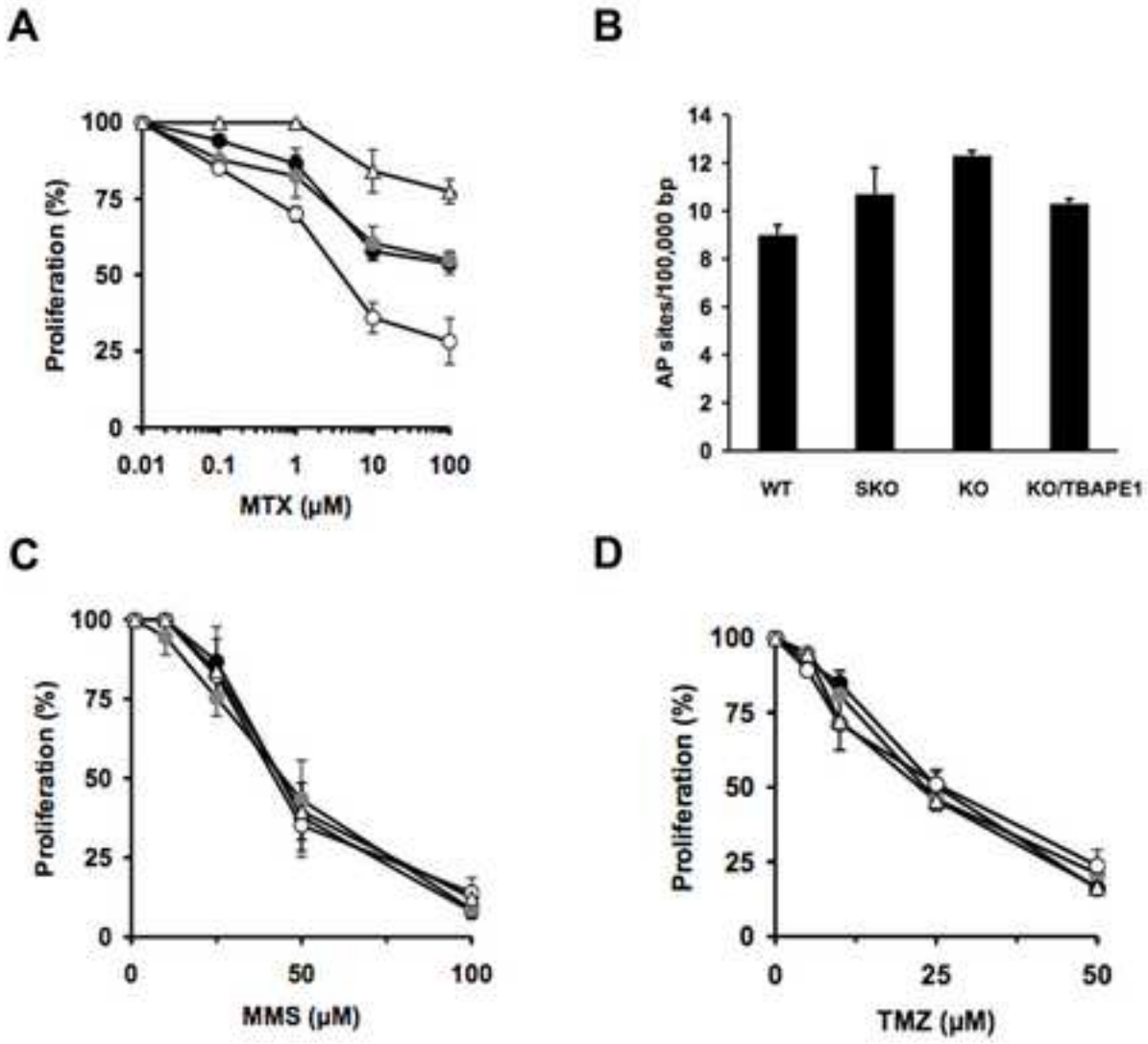

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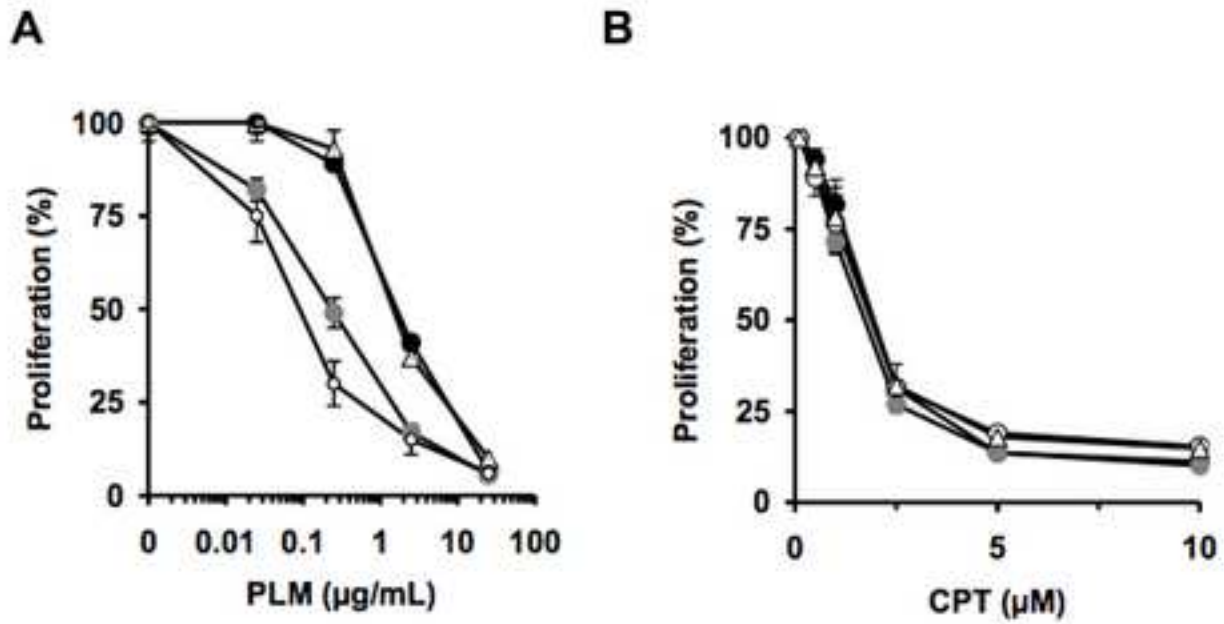

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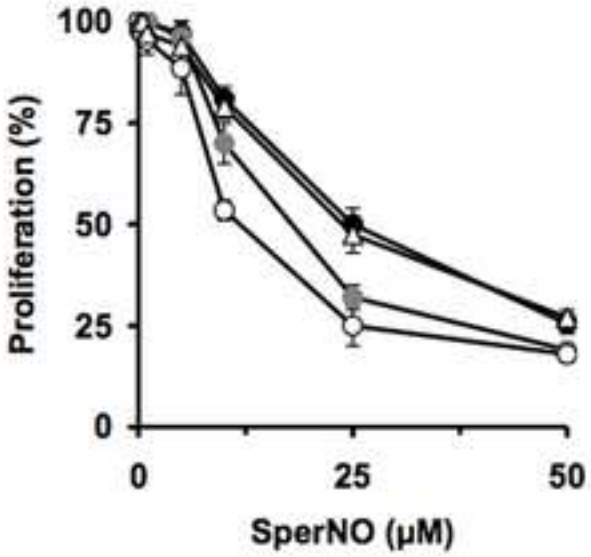

Figure 8