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Molecular and functional characterization of a putative PA28 γ proteasome activator orthologue in *Schistosoma mansoni*

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Abstract

PA28 γ is a proteasome activator involved in the regulation of the cellular proliferation, differentiation and growth. In the present study, we identified and characterized a cDNA from *Schistosoma mansoni* exhibiting significant homology to PA28 γ of diverse taxa ranging from mammals (including humans) to simple invertebrates. Designated SmPA28 γ , this transcript has a 753 bp predicted ORF encoding a protein of 250 amino acid residues. Alignment of SmPA28 γ with multiple PA28 γ orthologues revealed an average similarity of ~40% among the investigated organisms, and 90% similarity with PA28 γ from *Schistosoma japonicum*. In addition, phylogenetic analysis demonstrated a close linkage between SmPA28 γ to its sister group that contains well-characterized PA28 γ sequences from *Drosophila* spp., as well as sharing the same branch with PA28 γ from *S. japonicum*. Gene expression profiling of SmPA28 γ using real-time quantitative PCR revealed elevated steady-state transcript levels in the eggs, miracidia and paired adult worms compared to other stages. In parallel with gene expression profiles, an affinity-purified anti-SmPA28 γ antibody produced against recombinant protein exhibited strongest reactivity in Western blot analyses to endogenous SmPA28 γ from miracidia, sporocysts and paired adult worms. Given its known regulatory function in other organisms, we hypothesized that the high level of SmPA28 γ transcript and protein in these stages may be correlated with an important role of the PA28 γ in the cellular growth and/or development of this parasite. To address this hypothesis, miracidia were transformed *in vitro* to sporocysts in the presence of SmPA28 γ double-stranded RNAs (dsRNAs) and cultivated for 4 days, after which time steady-state transcript and protein levels, and phenotypic changes were evaluated. SmPA28 γ dsRNA treatment resulted in gene and protein knockdown of ~60% and ~80%, respectively, which were correlated with a significant decrease in larval length compared to its controls. These findings are consistent with a putative role of SmPA28 γ in larval growth/development of the *S. mansoni*.

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Author Contributions

Conceived and designed the experiments: CSS, VR, TPY. Performed the experiments: CSS, ERM, FRT. Analyzed the data: CSS, LGM, CBM. Contributed reagents/materials/analysis tools: VR, TPY. Wrote the paper: CSS. Reviewed and revised the manuscript: EBCM, TPY.

Keywords

Schistosoma mansoni; proteasome activator; PA28 γ subunit; protease; stage-specific expression; molecular phylogeny; RNAi

1. Introduction

Helminth parasites of the genus *Schistosoma* are the causative agents of schistosomiasis, one of the most prevalent of parasitic diseases in humans and animals [1, 2]. It is estimated that approximately 200 million people are infected in over 70 developing countries [3], with an additional 770 million worldwide at risk of becoming infected [4]. It is responsible for more than 280,000 deaths per year [5]. Praziquantel is the only approved drug currently available for the efficacious treatment of all schistosomiasis-causing species [6, 7]. However, the possible emergence of resistant strains [8, 9], as evidenced by reductions in cure rates and the treatment failures following praziquantel administration [10], reinforce the need to develop new safe and effective methods for controlling or preventing schistosome infections. Using high-throughput screening approaches, several new drugs have been found to show chemotherapeutic promise [11–16], although further testing is still needed before any new drugs can be considered as true replacements for praziquantel. Likewise, identification and testing of various antigens as potential vaccine targets is being actively pursued [17, 18], but progress in this area has been slow and prospects are not good for developing an effective, highly protective vaccine in the foreseeable future [19, 20]. With a limited repertoire of tools for effectively combating schistosomiasis, the completion and continued annotation of the *Schistosoma mansoni* genome [21], now serves as a critically important resource for identifying genes with potential as drug or vaccine targets as well for exploring new functional-genomic technologies such as gene-transfer/transgenetics [22–24], RNA interference (RNAi) [25–27] and high-throughput RNAseq methodologies for detailed gene expression/interactome analyses [28, 29].

Schistosomes have a complex life cycle in which extensive somatic remodeling occurs during its metabolic adaptation to differing environments presented by the molluscan intermediate host, an aquatic transmission phase, and the mammalian host [30]. It is presumed that different signaling and protein metabolic pathways must be involved in the differentiation, growth, migration and reproduction in each stage of its life cycle, although a comprehensive understanding of such pathways, especially the genes involved in their regulation, is still lacking. Controlled protein degradation plays an essential role in parasite development, through regulation of such processes as tissue remodeling, apoptosis and signal transduction. Central to these processes is the proteasome system [31, 32]. The presence of a subunit of the 20S proteasome in *S. mansoni* was first identified by Harrop *et al.* [33], but a functional 20S proteasome has only been shown to be required for development of schistosomulae in the vertebrate host by Guerra-Sá *et al.* [34]. Nabhan *et al.* showed that some 20S Proteasome subunits are differentially expressed among cercaria, schistosomula and adult worm stages, besides to develop a transfection-based RNA interference method to knockdown the expression of the proteasome subunits in *S. mansoni* schistosomula [35]. In other eukaryotic organisms the 20S proteasome is a large multi-catalytic protease involved in a variety of intracellular functions centered on the degradation of aberrant or damaged proteins, or inactivation of regulatory proteins that are no longer needed for specific cellular processes [32, 36, 37]. The 20S proteasome is composed of four rings, each of which contains seven subunits that form a barrel-like structure with a α 1–7, β 1–7, β 1–7, α 1–7 stoichiometry [38]. The 20S proteasome is associated with different subcomplexes that contribute to the overall functioning of the proteasome complex. These include: the 19S regulator complex forming the ATP-dependent 26S proteasome [39, 40],

PA200, a nuclear proteasome activator involved in DNA repair [41], and PA28 proteasome activator [42], which is the focus of the present study.

The PA28 is a complex of proteins consisting of ring-shaped heptamers formed by small 28-kDa proteins designated PA28 α , β or γ [31, 43]. PA28 was first identified by Ma *et al.* [44] and Dubiel *et al.* [45] as an ATP-independent proteasome activator that stimulates the degradation of small peptides but not intact or polyubiquitinated proteins. PA28 $\alpha\beta$ is comprised of a heptameric ring with three α and four β subunits, while the PA28 γ complex is proposed to be a homopolymer of seven identical γ subunits [43, 46]. PA28 $\alpha\beta$ subunits are particularly abundant in immune tissues, are regulated by IFN γ and are associated with adaptive immune response, with a proposed contribution to MHC class I peptide presentation [47–49]. The PA28 γ appears to function in the regulation of the cell proliferation and growth [50–52] and has been well characterized in such model invertebrates as *Drosophila melanogaster* [51], *Dictyostelium discoideum* [53] and *Caenorhabditis elegans* [48].

In the present study, we have cloned, expressed and characterized a PA28 γ gene in *S. mansoni* designated herein as SmPA28 γ . In addition to performing transcript expression profiling and phylogenetic analyses, an affinity-purified anti-SmPA28 γ antibody was produced to analyze the abundance and distribution of endogenous SmPA28 γ protein at different developmental stages of the *S. mansoni* life cycle, and to test the effects of SmPA28 γ knockdown by RNA interference on early primary sporocyst protein expression and development under *in vitro* culture conditions.

2. Materials and Methods

2.1. *In silico* analysis and identification of a PA28 orthologue in the *S. mansoni* databases

In order to identify homologous sequences of PA28 in *S. mansoni* the annotation term “proteasome activator AND Schistosoma” was searched in the National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov>), revealing a sequence of PA28 from *S. japonicum* (accession number AY274583). This sequence was used to query sequence in a BLASTn search of the GeneDB *S. mansoni* database (<http://www.genedb.org/genedb/smansoni/>) and SchistoDB database (<http://www.schistodb.net/schistodb20/>), both of which are publically available compilations of clustered ESTs and gene predictions from the parasite’s transcriptome [54] and genome [21]. The putative recovered *S. mansoni* PA28 sequences were subjected to a further Basic Local Alignment Search Tool (BLASTp) search in the NCBI database to confirm annotation features and identity with other organisms. The PA28 sequence in *S. mansoni* characterized in this present work was named SmPA28.

2.2. Parasites

In the study, we used two different strain of *S. mansoni*: LE strain from School of Medicine, Ribeirão Preto, University of São Paulo, Brazil and NMRI strain from Biomedical Research Institute, Rockville, MD, USA.

The *S. mansoni* parasites were maintained by serial passage through *Biomphalaria glabrata* snails and BALB/c mice. Cercariae were obtained from infected snails by exposure to artificial illumination at 26°C for 1 h to induce larval shedding. To obtain schistosomulae, cercariae were mechanically transformed by vortexing [55] followed by cultivation in sterile 24-well TC plates containing RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 25 mM HEPES pH 7.5, antibiotics (100 IU/mL penicillin and 100 μ g/mL streptomycin) and 10% bovine fetal serum (Gibco, Carlsbad, CA) for 24 h at 37°C in 5% CO₂. Adult worms were recovered from mice by aseptic perfusion of the mesenteric veins 56 days after

infection with 100 cercariae [56]. Eggs were obtained by homogenization of 7–8 weeks infected mouse livers in extraction buffer (0.89 g Na₂HPO₄ 0.04 g KH₂PO₄ and H₂O to 100 mL), followed by trypsinization for 3 h at 37°C, and recovery by double sieving (0.30 mm upper sieve and 0.180 mm lower sieve) [57]. Miracidia were obtained from eggs isolated from homogenates of infected mouse livers. For experiments requiring *in vitro* cultivation, following hatching in sterile artificial “pond water” [58], miracidia were immobilized on ice for 15 min, washed twice in cold pond water by centrifugation (1 min, 700 × *g*), gently resuspended in Chernin’s balanced saline solution plus (CBSS⁺) [59]. Miracidia were then aliquoted into sterile 24-well TC plates and cultured for 48 h at 26°C in CBSS⁺ to permit complete transformation to primary sporocysts [60] or 4 days for RNA interference experiments (see below).

2.3. Ethics statement

In accordance with accepted national and international principles for laboratory animal welfare all experiments performed in Brazil were authorized by the Ethical Committee for Animal Care of University of São Paulo, while the research protocols involving mice at the University of Wisconsin-Madison were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) under assurance no. A3368-01.

2.4. PCR, cloning, sequencing and expression of SmPA28 cDNA

Total RNA from miracidia (LE strain) was extracted and isolated using Trizol reagent (Invitrogen). The isolated RNA was subjected to DNase treatment using the DNA-Free kit (Ambion, Austin, TX) to eliminate any contaminating genomic DNA. RNA concentration was quantified and its purity assessed on a Nanodrop Spectrometer ND-1000 (NanoDrop Technologies Inc., Wilmington, DE). One µg of DNase-treated RNA was used as template to synthesize cDNA using the Superscript III cDNA Synthesis kit (Invitrogen) following the manufacturer's protocol. The full length cDNA sequence of SmPA28 was amplified by PCR using primers flanking the entire open reading frame (ORF) of its predicted gene (Acc. No. Smp_017660; SchistoDB database). The primer sequences used were as follows: F 5' ***CACC*** ATGAACACAGAAGTGGGT 3' and R 5' GTACATATTGATTGCATT 3'. A 5' extension (in bold and italic) was included in the forward primer for application in the directional TOPO cloning method according to protocols of the Invitrogen directional TOPO Vectors (Invitrogen). PCR amplifications were performed using 3 ng of miracidial cDNA as template and the following protocol: 94°C for 2 min, followed by 35 cycles at 94°C/15 sec, 50°C/30 sec, and 72°C/2 min and a final extension at 72°C/7 min. Reactions were performed using an Eppendorf Mastercycler EP/Gradient Thermal Cycler and the Promega PCR Master Mix (Promega, Madison, WI). The PCR product was purified using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and cloned into the vector pET K101-01 directional TOPO (Invitrogen) and the resulting plasmid used to transform One Shot TOP10 competent *E. coli* (Invitrogen). All protocols were run according to manufacturer's recommendations. After an overnight culture, five colonies were picked and used as samples to select the positive clones by two different PCR reactions: 1) using the same SmPA28 primer set described above and 2) using a T7 promoter 5' TAATACGACTCACTATAGGG 3' and the same SmPA28 reverse. Positive clones were again transformed in One Shot TOP10 cells and their plasmids purified using the QIAprep Spin Miniprep Kit (Qiagen). The purified recombinant plasmid, pET-(K101-01)-SmPA28, was confirmed by DNA sequencing and transformed in BL21 Star™ (DE3) competent *E. coli* (Invitrogen) to protein expression.

2.5. Bioinformatic analysis

Initially the cloned nucleotide sequence of SmPA28 was analyzed using the ORF Finder Tool in the NCBI website (<http://www.ncbi.nlm.nih.gov/projects/gorf/>) in order to predict its

ORF. This was followed by BLASTp analysis to recover protein sequence homology and identity using the SmPA28 and putative Smp_017660 (SchistoDB) sequences. BLASTp results recovered several PA28 sequences from different organisms in the NCBI database. The SmPA28 sequence and its retrieved orthologues were then subjected to searches of conserved protein domains or motifs using Pfam (v24.0) (<http://pfam.sanger.ac.uk/>). These sequences were aligned using the multiple sequence alignment program ClustalW 2.0 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>) and phylogenetic analyses were performed using the MEGA 4 software [61]. The neighbor-joining method [62], followed by bootstrap analyses using 1,000 replicates were used to construct a consensus tree of the PA28 sequences from various organisms to infer the evolutionary history of the taxa analyzed. Molecular weight and isoelectric point of SmPA28 were predicted using ExPASy Proteomics Server (<http://www.expasy.org/>).

2.6. Production of affinity-purified anti-SmPA28 γ antibody

The recombinant plasmid, pET-(K101-01)-SmPA28 γ , was transformed in BL21 StarTM (DE3) competent *E. coli* (Invitrogen) and the SmPA28 γ -6xHis recombinant protein expression was induced by IPTG treatment for 4 h at 37°C. The ~32 kDa SmPA28 γ -6xHis protein was purified by nickel column and its correct expression was confirmed by western blotting using the anti-His-tag antibody (1:2500) and by mass spectrometry (nanoLC-MS/MS). The polyacrylamide gel band containing ~100 μ g of the purified 32 kDa SmPA28 γ - 6xHis protein was excised, finely ground and emulsified in complete Freund's adjuvant. This emulsion was injected subcutaneously/intramuscular into a New Zealand rabbit. After 3 weeks, a second SC/ID booster injection (~100 μ g) of purified recombinant protein in incomplete Freund's adjuvant was administered. This was followed 3 weeks later by a third booster, administered SC/IM, of purified protein in saline alone. Seven days following the third injection, the rabbit serum was obtained and processed using methods of Harlow & Lane (1988) [63]. Anti-SmPA28 γ antibody was affinity-purified from rabbit serum by a Sepharose-matrix (GE Healthcare, Pittsburgh, PA) onto which the purified SmPA28 γ -6xHis protein had been covalently linked [63]. Bound antibody was eluted with 100 mM glycine buffer, pH 2.8, immediately neutralized, and stored at 4°C for use in Western blot and immunocytochemical studies.

2.7. Protein analysis of different *S. mansoni* life cycle stages

The affinity-purified rabbit anti-SmPA28 γ antibody was used to analyze the endogenous SmPA28 γ protein levels in (NMRI strain) miracidia, sporocysts and paired adult worms. The parasites were lysed in 1x RIPA buffer (0.1M Tris pH 7.2, 0.1% Sodium deoxycholate, 0.2% SDS, 2% NP40, 0.3 M NaCl) containing a cocktail of protease and phosphatase inhibitors, and sonicated on ice for 40 sec. The lysates were obtained as the supernatant fractions after centrifugation at $20,800 \times g$ for 10 min. One hundred micrograms of protein from each lysate was separated by SDS-PAGE gel electrophoresis, transferred onto nitrocellulose membrane (BioRad Laboratories, Hercules, CA). After transfer, the membrane was incubated in a mixture of affinity-purified rabbit anti-SmPA28 γ (1:1000) and rabbit anti- β -tubulin (loading control, 1:1000 dilution; Santa Cruz Technology, Dallas, TX) primary antibodies. A goat anti-rabbit IgG (Fc) alkaline phosphatase conjugated (AP) secondary antibody (1:7500, Promega) was used to detect the primary antibodies. Colorimetric immunoreactivity was detected with the chromogen substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT), diluted in AP buffer (0.1 M Tris, 0.1 M NaCl, 0.05 M MgCl₂, pH 9.5). The β -tubulin-relative levels of anti-SmPA28 γ antibody in miracidial, sporocyst and paired adult worm extracts were scanned and quantitative comparisons were analyzed by Image J software [64]. The experiment was performed on 3 independent biological replicates and the results were compared by one-way

analysis of variance (ANOVA) followed by Tukey's pairwise comparisons. Differences between groups were considered statistically significant at $p = 0.05$.

2.8. Real-time quantitative PCR (q-PCR) analysis in the different stages of the *S. mansoni* life cycle

Total RNA isolation and cDNA synthesis from *S. mansoni* (LE strain) eggs, miracidia, sporocysts, cercariae, schistosomulae and paired or unpaired adult worms were performed as described in Section 2.4. Steady-state SmPA28 γ transcript levels from different stages of *S. mansoni* were quantified relative to α -tubulin (normalization control) using the AB 7500 real-time PCR system (Applied Biosystems). SmPA28 γ primer set: F 5' CGTTACATTCTGGTGGTG 3' and R 5' CGATACCCGATGCTTCAC 3'; α -Tubulin primer set: F 5' GCTAATAACTACGCTCGCGGTCATT 3' and R 5' TGCAATTTGTGGTGGTGGATAA 3'. Total reaction volume was 25 μ L with 10 μ L of 600 nM primer mix, 12.5 μ L of SYBR green PCR Master Mix (Applied Biosystems, Carlsbad, CA), and 2.5 μ L of cDNA as template (or water as a negative control). The experiment was performed on 3 independent biological replicates. The AB 7500 software was used to calculate the SmPA28 γ transcript levels normalized with the endogenous α -tubulin control according to the $2^{-\Delta C_t}$ method [65]. The results were compared by one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparisons to test for significance between selected groups. The differences observed were considered to be statistically significant at $p = 0.05$.

2.9. SmPA28 γ double-stranded (ds) RNA synthesis

A SmPA28 γ PCR primer set containing a T7 promoter extension (in bold and italic) (F 5' ***TAATACGACTCACTATAGGG*** ATGGAAGACATATTAGCGTCGGAC 3', and R 5' ***TAATACGACTCACTATAGGG*** GTGGATATTTGCCACTTTACCCA 3') was designed to amplify a ~500 base pair (bp) segment. The vector pAcGFP (Clontech, Mountain View, CA) and a set of PCR primers also containing a T7 promoter extension (F 5' ***TAATACGACTCACTATAGGG*** GTGCCCATCCTGGTCGAG 3', and R 5' ***TAATACGACTCACTATAGGG*** CCGTTCTTCTGCTTGTCGG 3') were used to synthesize a 500-bp green fluorescent protein (GFP) nonspecific dsRNA treatment control [66]. The PCR products were separated on a 1% agarose gel, purified using QIAquick gel extraction kit (Qiagen) according to manufacturer's recommendations and submitted for DNA sequencing. All sequences were subsequently confirmed using the NCBI BLASTn application software. SmPA28 γ dsRNA was synthesized from isolated miracidial cDNA (NMRI strain) using the T7 RiboMAX Express RNAi Kit (Promega) as outlined by the manufacturer.

2.10. Effect of SmPA28 γ dsRNA treatment on larval gene expression

In order to determine if larval treatment with specific SmPA28 γ dsRNA can modulate (knockdown) SmPA28 γ transcript expression, q-PCR analysis was used to compare steady-state transcript levels between SmPA28 γ dsRNA-treated sporocysts and those treated with control GFP dsRNA [66]. In these experiments, miracidia (NMRI strain) were cultured in the presence of SmPA28 γ or GFP control dsRNA for 4 days at 26°C prior RNA extraction. Parallel cultures containing no dsRNA also were run as a non-treatment control for use in later phenotypic comparisons. Following treatment, sporocysts were washed 3 times with 1 mL CBSS in order to eliminate unabsorbed dsRNAs and shed ciliary epidermal plates, followed by total RNA extraction and cDNA synthesis (see 2.4). In the 96-Well Optical Reaction Plates (Applied Biosystems), the reaction mixtures consisting of 2.5 μ L of cDNA, 12.5 μ L of SYBR Green PCR Master Mix and 10 μ L of 600 nM primers were added for amplification and quantification in a AB7500 Real Time PCR System (Applied

Biosystems). In order to avoid false amplification of the dsRNA previously added to sporocyst cultures, pairs of SmPA28 γ primers were designed outside of this region (F 5' CCGTAATCACTATGCTACCTTAC 3' and R 5' GATTGCATTATTGGAACGAGGCA 3') and used to quantify the SmPA28 γ transcript levels. Each q-PCR run was performed with 2 internal controls assessing both potential genomic DNA contaminations (no reverse transcriptase added) and purity of the reagents used (no cDNA added). SmPA28 γ transcript levels between the dsRNA treatment and control groups were normalized using the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase – GAPDH (F 5' TCGTTGAGTCTACTGGAGTCTTTACG 3' and R 5' AATATGAGCCTGAGCTTTATCAATGG 3') as an endogenous control and for analysis by the comparative $2^{-\Delta\Delta C_t}$ method [67]. Statistical analyses were carried out using the unpaired T-test, with significance set at $p = 0.05$. Each experiment was repeated 3 times ($N = 3$) on independent biological samples.

2.11. Effect of SmPA28 γ dsRNA treatment on larval protein expression

Using an affinity-purified rabbit anti-SmPA28 γ antibody, western blotting and immunolocalization experiments were performed to analyze SmPA28 γ protein levels in SmPA28 γ dsRNA-treated 4-day (NMRI strain) sporocysts. For western blot analyses, SmPA28 γ dsRNA- or GFP dsRNA-treated 4-day sporocysts were lysed in 1x RIPA buffer. The lysates were subjected to SDS-PAGE gel electrophoresis, transferred to a nitrocellulose membrane and probed with rabbit anti-SmPA28 γ and rabbit anti- β -tubulin primary antibodies (see Section 2.7). The goat anti-rabbit IgG AP-conjugate secondary antibody was detected with BCIP/NBT. Anti-SmPA28 γ -reactive bands in SmPA28 γ dsRNA- and control GFP dsRNA-treated 4-day sporocyst extracts were scanned and, using concurrently measured β -tubulin-levels to normalize sample loads, quantitative comparisons of treated and control sporocysts were analyzed by Image J software [64]. The experiment was repeated 3 times ($N = 3$) on independent biological replicates. Statistical analyses were carried out using the unpaired T-test, with significance set at $p = 0.05$. In immunolocalization experiments, SmPA28 γ dsRNA- or GFP dsRNA-treated 4-day cultured (NMRI strain) sporocysts were fixed in PT buffer (2% paraformaldehyde and 1% Triton-X100 in sPBS), washed in sPBS, blocked in 5% BSA/ 0.02% azide/ sPBS, and incubated with rabbit anti-SmPA28 γ (1:100 dilution in blocking buffer) primary antibody, followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:300 in blocking buffer) secondary antibody, and then mounted on coverslips according to Mourão *et al.* (2009) [66]. The samples were imaged and analyzed using a Nikon Eclipse TE 300 inverted epifluorescent microscope (Nikon Instrument Inc., Melville, NY).

2.12. Effects of SmPA28 γ dsRNA treatment on *in vitro* *S. mansoni* sporocyst development

In order to gain a better understanding of the function of the SmPA28 γ in *S. mansoni* we also tested whether dsRNA treatment of *in vitro* cultured sporocysts (NMRI strain) had any effect on larval phenotype. Using 12-well TC plates (Costar, Corning Incorporated, Tewksbury, MA) ~ 6,000 miracidia were added to each well containing 200 nM of SmPA28 γ dsRNA or control green fluorescent protein (GFP) dsRNA in a total volume of 1250 μ L CBSS⁺ or medium lacking any dsRNA (no dsRNA control). These parasites were incubated at 26°C for 4 days, and over that period they were monitored for the following phenotypes: failure or delay in transformation, loss of motility, tegumental lysis and granulation (lethality) and changes in larval growth. A Nikon Eclipse TE 300 inverted epifluorescent microscope (Nikon Instrument Inc.) was used to monitor daily morphological changes presented by cultured parasites. For detailed observations of larval morphology and to quantify sporocyst growth (length) in treated vs. control groups at day 4, digital images were captured using a CoolPix EZ digital camera (Nikon Instruments Inc.) and larval length measurements from these images were analyzed by Metamorph software version 7.0 (Meta

Imaging series, Molecular Devices). Larval growth datasets for each experimental replicate were compared by one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparisons with significance set at $p = 0.05$. Three independent replicates of miracidia isolated from different batches of infected mouse livers were performed.

3. Results and discussion

The primary goal of our present work was to validate the existence of a PA28 in *S. mansoni* establish its subunit identity and characterize this protein both structurally and functionally. Using the available schistosome genome and transcriptome databases, we initially recovered two sequences: 1) a predicted gene designated as “*Schistosoma mansoni* proteasome subunit, putative” (Acc. No. Smp_017660, SchistoDB database) and 2) a cluster of ESTs identified as a “putative proteasome activator PA28 subunit” (Acc. No. Sm04870, GeneDB *S. mansoni* database) that only encompassed the C-terminal half of the Smp_017660 sequence (data not shown). Using a set of primers designed to flank the predicted ORF of Smp_017660, we were able to determine by RT-PCR the entire open reading frame of our sequence, which was given the name SmPA28. The nucleotide sequence from the amplified transcript of SmPA28 contained 753 bp and was confirmed to be the predicted spliced DNA sequence deposited as Smp_017660. The complete coding sequence for SmPA28 obtained in this study has been deposited in the NCBI GenBank (<http://www.ncbi.nlm.nih.gov>) under accession number **HQ235026**.

The predicted SmPA28 ORF revealed a protein of 250 amino acid residues (Fig. 1, Fig. S1) with an isoelectric point and molecular mass of 7.08 and 27.75 kDa, respectively. In humans, the 3 different subunits of PA28, namely -alpha (α), -beta (β), and -gamma (γ), exhibit ~45% similarity between each other [68] and belong to the PA28-beta superfamily, identified by the presence of a conserved PA28-beta-like domain. The SmPA28 protein sequence, when aligned with the 3 different subunits of PA28 (α , β , and γ) from *Homo sapiens*, displays greatest similarity with PA28 γ (41%) when compared with PA28 α (29%) or PA28 β (24%) (Fig. 1). In addition, SmPA28 lacks the KEKE motif that characterizes the PA28 α protein (Fig. 1) [68]. As confirmed by Pfam analysis, SmPA28 contains the PA28-beta-like domain demonstrating its membership in the PA28-beta superfamily (Fig. S1). SmPA28 when compared to various PA28 γ orthologues exhibited an average similarity of approximately 40% among the PA28 γ sequences from *Drosophila melanogaster*, *Caenorhabditis elegans*, *Dictyostelium discoideum*, *Mus Musculus* and *Homo sapiens* and 90% of similarity to PA28 γ from *S. japonicum* (Fig. S1). Overall sequence analyses and homologous structural associations strongly support the inclusion of SmPA28 as a gamma (γ) subunit. Importantly, the functional role of the PA28 γ in regulating cell proliferation and/or development in these model organisms [48, 50–53] supports the SmPA28 γ as a likely orthologue with predicted similar role(s) in *S. mansoni*.

SmPA28 γ orthology is supported by our phylogenetic analysis (Fig. 2) showing that SmPA28 γ is closely linked to its sister *Drosophila melanogaster* PA28 γ sequence, a protein involved in regulating cellular growth [51], and not surprising, it also shares the same branch with PA28 γ from *S. japonicum* and is presumed to possess similar, if not identical functions in both species. However, only weak linkage is demonstrated to the *Dictyostelium discoideum* sequence, a well-characterized PA28 γ localized to the nucleus and present in growing as well as starving cells [53], as evidenced by the low bootstrap values at the major branch junctions. The apparent absence of PA28 α and β subunits in *S. mansoni* may be predicted as these proteasome activator proteins are best known for their role in adaptive immune responses, especially in the processing of protein antigens for MHC class I peptide presentation [47–49]. Because invertebrate metazoans, including helminths [69], only have an innate immune system [70], one might expect immunoproteasomes (and their activators)

to be absent. As mentioned above, as PA28 γ subunits have been functionally linked to growth in well-studied invertebrates such as *D. melanogaster* [51] and the *D. discoideum* [53], it is likely that this gene may play a similar functional role in *S. mansoni*.

In order to gain further insight into SmPA28 γ activity during schistosomosome development, we assessed by real-time qPCR the expression profiles of SmPA28 γ transcripts at different stages of the parasite's life cycle (eggs, miracidia, sporocysts, cercariae, schistosomulae and paired or unpaired adult worms). Steady-state SmPA28 γ transcript levels were greatest in the egg, miracidial and paired adult worm stages, while they were significantly reduced in stages associated with intramolluscan development (sporocysts and cercariae) and unpaired adult worms, and nearly absent in schistosomulae (Fig. 3). This transcript distribution among stages strongly suggests a potentially important role associated with sexual maturation, egg production and/or *in ovo* embryonic development. Given the high levels of cellular proliferation and metabolic demands during this phase of development, the turnover of proteins may be assumed to be high as well. Thus, an increase in proteasome activity, including PA28 γ , would be expected to play an essential role in cellular development during this reproductively active period. Furthermore corroborating with our data, Nabhan *et al.* [35], using real-time qPCR analysis, showed that of *S. mansoni* proteasome subunit expression levels were also differentially expressed among the mammalian life cycle stages. Similar to SmPA28 γ in our study, specific subunits of the 19S lid (e.g., SmRPN8, SmRPN11), as well as 19S base subunits, were downregulated in schistosomula and subsequently upregulated in adult worms, suggesting that different proteasome subunits may be assuming different functional roles that are parasite stage specific.

To provide a more complete molecular and functional characterization of SmPA28 γ , we expressed a recombinant SmPA28 γ protein and used it to produce an affinity-purified rabbit anti-SmPA28 γ antibody, which was then used in follow up SmPA28 γ 's experiments. Firstly, we induced the SmPA28 γ -6xHis recombinant protein expression with IPTG for 4h and affinity purified it on a nickel column (Fig. S2A). Expression of the expected ~32 kDa recombinant protein (28 kDa SmPA28 γ + 4 kDa tag sequence) was confirmed by western blot analysis using the anti-His-tag antibody (Fig. S2B) and by mass spectrometry (LC-MS/MS, Orbitrap, [71]) (Fig. S2C) that detected 6 different peptides covering 48% of the complete SmPA28 γ protein sequence. Serum antibodies from a rabbit immunized with this recombinant protein and affinity-purified using a SmPA28 γ -Sephadex-matrix recognized the endogenous ~28 kDa SmPA28 γ protein from sporocysts (Fig. S3), and therefore, was subsequently used to compare the SmPA28 γ protein level in the different stages of *S. mansoni* by western blotting. Rabbit pre-immune serum obtained prior to immunization displayed little to no immunoreactivity and was used as a negative control serum (Fig. S3).

In contrast to the robust SmPA28 γ gene expression exhibited by miracidia and paired adult worms compared to the intramolluscan sporocyst stage, the endogenous SmPA28 γ protein level in sporocysts was similar to those of miracidia and paired adult worms (Fig. 4). This quantitative difference in gene and protein expression between stages may not be too surprising since sporocysts exhibit low to moderate SmPA28 γ transcript levels (Fig. 3), and depending on the endogenous half-life of this protein, sporocysts may have retained residual SmPA28 γ produced by miracidia prior to *in vitro* sporocyst transformation. Given that sporocysts also are characterized as a rapidly developing, asexual reproductive stage of *S. mansoni* we sought to investigate the putative function of the PA28 γ in this stage of *S. mansoni* by using a RNA interference (RNAi) approach.

Phenotypic changes have been widely studied in different organisms by functional gene knockdowns using RNAi as a reverse-genetic tool [72–74], and when combined with complete and well-annotated genome databases, RNAi has been effective in assigning

putative functions to unknown genes, or providing direct functional confirmation of genes identified by sequence homology (orthologues) [75]. RNAi libraries are available and have been used for genome-wide functional analyses in some model invertebrates like *D. melanogaster* [76] and *C. elegans* [77]. Although still in early stages of technical development, RNAi approaches also have been successfully applied to schistosomes [25, 27, 78]. Nabhan *et al.* were the first to knockdown a proteasome gene using a transfection-based RNA interference method in *S. mansoni* schistosomula [35], and showed a significant reduction (~80%) in gene expression of SmRPN11, a subunit of the 19S lid assembly, that led to decrease (~78%) of the parasite viability.

In our initial experiments, we tested whether the *S. mansoni* sporocyst treatment with specific SmPA28 γ dsRNA would result in a significant knockdown in SmPA28 γ transcript expression. As shown in Figure 5, in comparison with a corresponding control treatment (GFP dsRNA-treated group), the normalized-level of steady-state SmPA28 γ mRNA was significantly reduced (~60%) in the SmPA28 γ dsRNA-treated larval group. Previously, Mourão *et al.* [66] showed that some genes presented a greater decrease in the transcript levels using shorter dsRNA exposure times (e.g. 2 or 4 days, rather than 7 days), and we found the same to be true in our study. That is, in order to achieve a significant knockdown of steady-state transcripts, a shorter time of dsRNA exposure (4 days), rather than 7 days as reported previously [79], was required for SmPA28 γ knockdown. Possible reasons for this observation may be that RNA degradation in culture is avoided by a shortened incubation period [80], or perhaps may be due to the transient nature of knockdown exhibited by specific dsRNAs [66].

In addition to assessing the effect of SmPA28 γ dsRNA-treatment on SmPA28 γ transcript levels, we also used western blot analysis and immunofluorescence imaging to determine the effect of RNAi on SmPA28 γ protein expression. By western blotting, we showed that SmPA28 γ dsRNA-treated 4-day sporocyst extracts were significantly reduced in SmPA28 γ (28 kDa band) compared to the GFP dsRNA-treated control group (Fig. 6). The 55 kDa β -tubulin band was present in both the gene specific and GFP control dsRNA-treated samples confirming that the SmPA28 γ dsRNA treatment produced a significant and specific protein knockdown (Fig. 6). This result was quantitatively confirmed by densitometric analysis showing that the normalized-level of SmPA28 γ protein was highly reduced (~80%) in the SmPA28 γ dsRNA-treated sample compared to the GFP dsRNA control (Fig. 6). Immunofluorescence microscopic observations corroborated these western blot results by showing that SmPA28 γ dsRNA-treated sporocysts displayed little immunoreactivity, while strong anti-SmPA28 γ -reactivity was evident in larvae treated with control GFP dsRNA (Fig. 7). The distribution of immunoreactive SmPA28 γ appeared similar to untreated and GFP-dsRNA treated sporocysts, with SmPA28 γ immunoreactivity being localized in various tissues including some resembling germinal cell clusters and subtegumental parenchyma within sporocysts (Fig. 7). It has been noted that in *Drosophila* and *Dictyostelium* cells, the PA28 γ protein may be present in the nucleus [81, 53], but we were unable to confirm this localization pattern in sporocysts.

In order to gain a better understanding of the function of the SmPA28 γ and to correlate the down-regulation of the SmPA28 γ transcript and protein expression to possible phenotypic changes, the dsRNA-treated larvae were monitored for morphological/behavioral changes during the 4 days of the dsRNA treatment. No differences between SmPA28 γ dsRNA-treated larvae and controls (GFP dsRNA-treated and no dsRNA-treated sporocysts) were observed relative to mortality, larval motility or miracidial transformation rates during the *in vitro* cultivation period. The only phenotype observed was a significantly greater number of shortened sporocysts, suggesting a possible functional linkage to growth or development (Fig. 8). Because sporocysts represent a range of sizes, live sporocysts were imaged and

their lengths were measured, digitally analyzed using Metamorph software. Analysis of sporocyst length measurements in each of 3 independent biological replicates demonstrated that the median length of sporocysts in SmPA28 γ dsRNA-treatment group was significantly less ($p = 0.05$) than that of both the GFP dsRNA and the blank (no dsRNA) controls (Fig. 9). This sporocyst growth-retarding phenotype is consistent with previous findings in both vertebrates and invertebrates. Murata *et al.* [50] showed PA28 $\gamma^{-/-}$ mice were born without appreciable abnormalities in all tissues examined, but their growth after birth was retarded compared with that of PA28 $\gamma^{+/-}$ or PA28 $\gamma^{+/+}$ mice, while Masson *et al.* [51] showed a partial arrest in the G1->S cell cycle transition in *Drosophila* cells depleted of PA28 γ by RNAi. Moreover, consistent with this role, over-expression of PA28 γ in thyroid neoplasm was found to be directly associated with an accelerated cellular growth rate [52]. As stated previously, the application of RNAi can be used in the direct support of putative functions of genes identified by sequence homology alone. Evidence presented herein is consistent with involvement of SmPA28 γ in the cellular development in *S. mansoni* has been shown for other PA28 γ protein orthologues. However, regulation and maintenance of cellular growth and differentiation within metazoan organisms is extremely complex, involving a myriad of genes. Thus it is not surprising that the growth “defect” in sporocysts has been observed with knockdown of other genes as well [66, 82].

In summary, this study is the first to describe the isolation, structural characterization and functional role of a PA28 γ proteasome activator gene, designated SmPA28 γ in *S. mansoni*. In addition, the phylogenetic analysis showed that the SmPA28 γ homologue closely clusters with PA28 γ protein sequences from invertebrates that have been well-characterized functionally as regulators of cellular growth and development, thereby predicting similar role(s) for this gene in *S. mansoni*. Steady-state transcript levels were highest in eggs, miracidia and paired adult worms, significantly decreased in the sporocyst, cercariae and unpaired adult worms, and nearly absent in schistosomulae. However, at the protein level SmPA28 γ was similarly expressed at elevated levels in sporocysts, miracidia and paired adult worms, suggesting retention of residual protein between stages, in spite of lowered transcript abundance. Using cultured sporocysts as an *in vitro* model for intramolluscan development, functional studies incorporating RNAi demonstrated a significant correlation between SmPA28 γ transcripts and protein knockdown and a growth defect in sporocysts, suggesting a putative role in larval growth/development.

Supplementary Material

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Acknowledgments

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Highlights

- First molecular characterization of the proteosome activator PA28 γ from *S. mansoni*
- SmPA28 γ transcripts are differentially expressed in *S. mansoni* life cycle stages
- SmPA28 γ expression is functionally linked to sporocyst growth/development *in vitro*

SmPA28	--- --MNTGVRLNAYKEKT KVDGEELVLNVFPKRVFEMED I L ASDMFFLEGKYVH	51
Hsgamma	MAS - - L LKVDQEVKLKVD SFRERI TSEAEDLVANFFPKLLELDSFLKEP I LNIHD - - - L	55
Hsalpha	MAM - - LRVQPEAQAKVDVFREDL CTKTENLLGSYFPKKISELDAFLKEP ALNEAN - - - L	54
Hsbeta	MAK P CGVRL SGEARKQVEVFRQNL FQEAEFFLYRFLPKII IYLNQLLQED SLNVAD - - - L	57
SmPA28	SDV NV P Y P V N P H N S L H S G G D M D S D L S N A A D K L T N D K Q Q F P S K G - S F V Y A L P N G S V S Y N K H	110
Hsgamma	TQ I H S D M N L P V P D P I L L T N S H D G L D G P T Y K K R R L D E C E A F Q G - T K V F V M P N G M L K S N Q Q	114
Hsalpha	S N L K A P L D I P V P D P V K E K E K E E - - - - R K K Q E K E D K D E K K G E D E D K G P P C G P V N C N E K	109
Hsbeta	T S L R A P L D I P I P D P P K D D E M E - - - - T D K Q E K K E - - - - - V P K C G F L P G N E K	99
SmPA28	I K A M I E K T K P C L T O L M I E A Q L V R L W V Q F N I P R I E D G N N F G V G I Q E E I L G E A S G I E R D A C T	170
Hsgamma	L V D I I E K V K P E I R L L I E K C N T V K M V V Q L L I P R I E D G N N F G V S I Q E E T V A E L R T V E S E A A S	174
Hsalpha	I V V L L Q R L K P E I K D V I E Q L N L V T T W L Q L Q I P R I E D G N N F G V A V Q E K V F E L M T S L H T K L E G	169
Hsbeta	V L S L L A L V K P E V W T L K E K C I L V I T W I Q H L I P K I E D G N D F G V A I Q E K V L E R V N A V K T K V E A	159
SmPA28	F L D Q V T R Y Y A S R G K L V G K V A K Y P H I E D Y R E C I R D M D E K Q A I T M R Y I I M E I R N H Y A T L H D I	230
Hsgamma	Y L D Q I S R Y Y I T R A K L V S K I A K Y P H V E D Y R R T V T E I D E K E Y I S L R L I I S E L R N Q Y V T L H D M	234
Hsalpha	F H T Q I S K Y F S E R G D A V T K A A K Q P H V G D Y R Q L V H E L D E A E Y R D I R L M V M E I R N A Y A V L Y D I	229
Hsbeta	F Q T T I S K Y F S E R G D A V A K A S K E T H V M D Y R A L V H E R D E A A Y G E L R A M V L D L R A F Y A E L Y H I	219
SmPA28	I I K N L D R I K M P R S N N A I N M Y	250
Hsgamma	I L K N I E K I K R P R S S N A E T I L Y	254
Hsalpha	I L K N F E K L K K P R G E T K G M I Y	249
Hsbeta	I S S N L E K I V N P K G E E K P S M Y	239

Figure 1. SmPA28 protein sequence aligned with PA28-alpha (α), -beta (β) and -gamma (γ) from *Homo sapiens*

PA28 (α , β and γ) from *Homo sapiens* recovered from the NCBI database were aligned with SmPA28 using ClustalW. By Pfam analysis, all PA28 (α , β and γ) sequences from humans and SmPA28 exhibited the PA28-beta-like domain (see Fig. S1), confirming that all sequences belong to PA28-beta superfamily. The name, GenBank accession number and % similarity with SmPA28 for each sequence are as follows: SmPA28 (*S. mansoni* HQ235026); Hsgamma (PA28 γ *Homo sapiens* AAH08020, 41%); Hsalpha (PA28 α *Homo sapiens* AAV38144, 29%); Hsbeta (PA28 γ *Homo sapiens* AAH19885, 24%). Note that SmPA28 lacks the KEKE motif that characterizes all PA28 α protein sequences (boxed sequence). The black box represents identical amino acids, and grey box shows the presence of conserved residues.

Figure 2. Phylogenetic comparison of SmPA28 γ with well-characterized PA28- γ orthologues of other taxonomic groups

SmPA28 γ was aligned with PA28 γ protein sequences representing a diversity of organisms using ClustalW 2.0, and a bootstrap consensus tree was constructed using the neighbor-joining method. The most of the sequences retrieved from NCBI database by BLASTp are PA28 γ sequences, with all sequences presenting the PA28-beta-like domain, confirming their inclusion in the PA28-beta superfamily. Note that SmPA28 γ shares the same branch with PA28 γ from *S. japonicum* (90% of similarity) and is closely linked to its sister well-characterized PA28 γ sequence from *D. melanogaster* (39% of similarity).

Figure 3. Steady-state transcript levels of SmPA28γ associated with different stages of the *Schistosoma mansoni* life cycle

Quantification of SmPA28γ transcript levels relative to an endogenous Sma-tubulin control (comparative $2^{-\Delta C_t}$ method) was performed on eggs, miracidia, sporocysts, cercariae, schistosomulae, and paired or unpaired adult worms using real-time quantitative PCR. Transcript levels for each stage, determined in 3 independent biological replicates, were statistically compared by 2-way analysis of variance (ANOVA, $F_{6,14} = 99.4$; $p < 0.05$) followed by Tukey's pairwise comparisons. Parasite stages displaying the same letters are not significantly different from each other at the $p < 0.05$ level.

Figure 4. Endogenous SmPA28 γ protein levels remained constant in sporocysts, miracidia and paired adult worms

The protein extracts from sporocysts, miracidia and paired adult worms were subjected to western blot analysis using an affinity-purified rabbit anti-SmPA28 γ to detect endogenous SmPA28 γ and a rabbit anti- β -tubulin antibody as loading control (A). Quantification of endogenous SmPA28 γ protein expression levels between the stages was accomplished by optical densitometry comparing protein band intensities following anti- β -tubulin normalization. No statistical difference was observed in the endogenous SmPA28 γ protein level from sporocysts, miracidia and paired adult worms (B). $F_{1,3} = 4.962$; $p = 0.112$; $N = 3$.

Figure 5. SmPA28γ expression profile in *Schistosoma mansoni* primary sporocysts after 4 days of SmPA28γ dsRNA-treatment

Relative transcript abundance (mean steady-state transcript level) of SmPA28γ in sporocysts after 4 days of SmPA28γ dsRNA-treatment compared to the GFP dsRNA control. SmPA28γ and GFP transcript levels were calibrated using the constitutively expressed SmGAPDH as an endogenous control according to the comparative 2^{-ΔΔCt} method followed by statistical analysis using the unpaired t-test. (**) indicates a statistical different in SmPA28γ expression between SmPA28γ and GFP dsRNA-treated groups (t=6.066; df=4; p=0.0037). Data were generated from 3 independent experiments.

Figure 6. SmPA28γ protein level significantly decreases in sporocysts after 4 days of SmPA28γ dsRNA-treatment

Western blot analysis of SmPA28γ from *S. mansoni* sporocysts cultured for 4 days in medium containing SmPA28γ - or GFP-dsRNA. An affinity-purified rabbit anti-SmPA28γ was used to detect endogenous 28 kDa *S. mansoni* SmPA28γ, while an anti-β-tubulin antibody served as loading control marker. The presence of SmPA28γ protein in GFP dsRNA-treated sporocysts is noted, but reduced in SmPA28γ dsRNA-silenced parasites (A). SmPA28γ protein knockdown (~80%) in the test group was confirmed by optical densitometry comparing band intensities of SmPA28γ dsRNA- and GFP dsRNA-treatment

groups following anti- β -tubulin normalization (B). Statistical analyses were performed using unpaired t-test. $t=14.85$; $df=4$; $*p=0.0001$; $N = 3$.

Figure 7. Corroboration of SmPA28 γ protein knockdown in *Schistosom. mansoni* primary sporocysts following SmPA28 γ RNAi

Immunofluorescence photomicrographs of untreated sporocysts (no-dsRNA control), GFP dsRNA-treated (GFP control) and SmPA28 γ dsRNA-treated sporocysts showing localization of SmPA28 γ protein in each larval treatment group. Strong immunoreactivity (green fluorescence) is distributed among various tissues (e.g., putative germinal cells, subtegumentary parenchyma) of both control sporocyst groups, compared to only weak reactivity in SmPA28 γ dsRNA-treated sporocysts. Paired brightfield and epifluorescent microscope images at 200 $\times$ final magnification. Images were normalized for fluorescence intensities by equal scaling in Metamorph v.7.0.

Figure 8. *In vitro* cultured *Schistosoma mansoni* primary sporocyst 4 days post-dsRNA treatments

Shown are brightfield images of *in vitro* cultured *S. mansoni* sporocysts after 4 days of treatments with no dsRNA (A), GFP dsRNA (B) and SmPA28 γ dsRNA (C), illustrating the effects of SmPA28 γ dsRNA-exposure on sporocyst lengths. White arrows indicate examples of shortened sporocysts, while black arrows show normal elongate sporocysts in the different treatments. Asterisks indicate rounded ciliated epidermal plates that were released from the miracidial surface after transformation. (100 $\times$ final magnification).

Figure 9. *Schistosoma mansoni* sporocyst length measurements after 4 days of SmPA28 γ dsRNA-exposure

Graphs represent sporocyst length measurements (μm) after 4 days of treatment with SmPA28 γ dsRNA, GFP dsRNA (specificity control) or no dsRNA (blank control). Each dot represents the length of an individual sporocyst and the median of all lengths with each group is indicated by a horizontal black bar. An overall significant decrease in larval length was detected as a result of SmPA28 γ dsRNA-treatment (open circles) when compared to the matched control groups; GFP dsRNA (gray triangles) and no treatment “Blank” (black squares). This was consistent in all 3 independent experiments. Significant one-way analyses of variance (ANOVA) were followed up by Tukey’s pairwise comparisons with significance set at $p < 0.05$ (**). ANOVA values for each experiment were as follows: *Experiment 1*: $F_{2,246}=9.462$, $p=0.027$; *Experiment 2*: $F_{2,280}=16.81$, $p=0.0001$; *Experiment 3*: $F_{2,258}=25.69$, $p=0.0069$.