Schistosoma haematobium extracellular vesicle proteins confer protection in a heterologous model of schistosomiasis

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Abstract

Helminth parasites release extracellular vesicles which interact with the surrounding host tissues, mediating host-parasite communication and other fundamental processes of parasitism. As such, vesicle proteins present attractive targets for the development of novel intervention strategies to control these parasites and the diseases they cause. Herein, we describe the first proteomic analysis by LC-MS/MS of two types of extracellular vesicles (exosome-like, 120k pellet vesicles and microvesicle-like, 15k pellet vesicles) from adult *Schistosoma haematobium* worms. A total of 57 and 330 proteins were identified in the 120k pellet vesicles and larger 15k pellet vesicles, respectively, and some of the most abundant molecules included homologues of known helminth vaccine and diagnostic candidates such as *Sm*-TSP2, *Sm*23, glutathione S-tranferase, saposins and aminopeptidases. Tetraspanins were highly represented in the analysis and found in both vesicle types. Vaccination of mice with recombinant versions of three of these tetraspanins induced protection in a heterologous challenge (*S. mansoni*) model of infection, resulting in significant reductions (averaged across two independent trials) in liver (47%, 38% and 41%) and intestinal (47%, 45% and 41%) egg burdens. These findings offer insight into the mechanisms by which anti-tetraspanin antibodies confer protection and highlight the potential that extracellular vesicle surface proteins offer as anti-helminth vaccines.

Keywords: *Schistosoma haematobium*, schistosomiasis, extracellular vesicles, exosome-like vesicles, microvesicles, tetraspanin, vaccine

1. Introduction

Schistosomiasis is the second most important parasitic disease, only after malaria, in terms of social, economic and public health impact [1]. *Schistosoma haematobium*, the causative agent of urogenital schistosomiasis, is highly prevalent in 53 Middle East and African countries [1] and it is also sporadically seen in India [2] and France [3]. Urogenital schistosomiasis affects more than 90 million people, mostly in sub-Saharan Africa where 180 million inhabitants are at risk, and is responsible for 150,000 deaths per year [4]. Furthermore, the most common complications associated with this disease include schistosomal haematuria, bladder wall pathology, hydronephrosis, and dysuria [4].

The current control programs against schistosomiasis are aimed at reducing the morbidity caused by the parasite by regularly treating infected populations with praziquantel [5]. Despite the efforts made to control this devastating disease, schistosomiasis is still spreading to new geographical areas [3,6]. Furthermore, praziquantel has shown reduced efficacy in field studies [7] and is not effective against the immature stages of the parasite [8,9]. Hence, a vaccine that reduces disease severity and/or reduces transmission is needed to control and eliminate schistosomiasis [10,11]. Despite efforts over decades, there is no licensed vaccine [1,12]. Even though various vaccine candidates have advanced into clinical trials targeting *Schistosoma mansoni*, the causative agent of intestinal schistosomiasis, the only vaccine candidate against *S. haematobium* to have progressed into clinical trial is a glutathione S-transferase recombinant protein, Sh28GST. However, results from a phase 3 trial conducted from 2009 to 2012 in *S. haematobium* infected children did not report any significant efficacy due to the vaccine [13]. Therefore, it is important to continue identifying new target antigens in the effort to develop a vaccine against *S. haematobium* and the other species of schistosomes [10,12].

S. haematobium adult worms live in the perivesicular veins where they can survive for years using evasion strategies to remain undetected by the host immune system. One of the main strategies the parasite's employ to hijack the immune effector response is to release a suite of immunomodulatory excretory/secretory (ES) products. ES products comprise different proteins, glycans, lipids, and nucleic acids [14], and have been the focus of different studies aiming at understanding the molecular basis of host-parasite interactions and the subsequent use of this information to develop novel therapeutics and diagnostics [15-18]. Recently, it has been documented that the ES products from different helminths (including schistosomes) contain extracellular vesicles (EV)s [19-22]. EVs are membrane-bound organelles released by cells that can act as mediators of intercellular communication by transferring molecular signals mediated by proteins, lipids, metabolites, mRNAs, microRNAs and other non-coding RNA species [23,24]. In addition to the transmission of information between cells within the same organism, recent studies have shown that EVs secreted by parasitic helminths are taken up by host cells within the parasite's niche tissue and provide a means of inter-species communication [19,25-38]. For instance, EVs from trematodes and nematodes can be internalised by host cells whereupon they suppress effector immune responses [37,39,40], or in contrast, some helminth EVs contribute to pathogenesis by promoting cell proliferation and inflammatory cytokine production [29].

EVs from helminths also contain vaccine candidate antigens. For example, EVs from *S. mansoni* contain molecules that have known vaccine efficacy in animal models of schistosomiasis [27], and vaccination of mice with helminth EVs stimulates the production of protective immune responses that significantly reduce faecal egg counts, worm burdens and symptom severity and mortality induced by infection after parasite challenge [25,41-44]. Moreover, antibodies produced against recombinant forms of

Opisthorchis viverrini EV surface proteins hinders the uptake of EVs by cholangiocytes and suppresses the immune response that fuels pathogenesis [29,42].

In addition to other molecules, helminth EVs contain tetraspanins (TSPs), which have been shown to be effective vaccine candidates against *Schistosoma* spp. [21,27]. The TSPs *Sm*23, *Sm*-TSP-1 and *Sm*-TSP-2, all found in the membrane of *S. mansoni* EVs [20,27], have displayed partial efficacy in when used as adjuvanted subunit vaccines [45-47], and *Sm*-TSP-2 has successfully completed phase I clinical trials [48]. TSPs from other helminth species, including *S. japonicum* and *O. viverrini*, have also been shown to be efficacious vaccine candidates in animal challenge models of infection [42,49].

Although there are reports describing EVs and the vaccine efficacy of EV-derived molecules from other schistosomes, no studies have been conducted on *S. haematobium*. Herein, we have characterized for the first time the proteomic composition of small (120k pellet) and large (15k pellet) subclasses of EVs from this parasite, and selected three of the EV surface TSPs for assessment as subunit vaccines in a heterologous challenge mouse challenge model of schistosomiasis.

2. Materials and methods

2.1 Parasite material and experimental animals

Bulinus truncatus snails infected with *S. haematobium* (Egyptian strain) were provided by the Biomedical Research Institute, MD, USA, and maintained in aquaria in a 27°C incubator. Male BALB/c mice were purchased from the Animal Resource Centre, Canningvale, Western Australia, and maintained at the Australian Institute of Tropical Health and Medicine (AITHM) animal facility in cages under controlled temperature and light with free access to pelleted food and water. All experimental procedures performed

126 on animals in this study were approved by the James Cook University (JCU) animal ethics
127 committee (A2391). All experiments were performed in accordance with the 2007
128 Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and
129 the 2001 Queensland Animal Care and Protection Act.

130 To obtain cercariae, snails were removed from the tank with a pair of forceps and
131 washed several times with water to remove debris and rotifers, transferred to a Petri dish
132 and incubated without water at 27°C in the dark for 2 h. Water was then added and the
133 snails were placed under light for 1.5 h at 28°C. Cercariae were concentrated using a 20
134 µm pore size sieve and, finally, each BALB/c mouse (6 week-old) was infected with
135 1,000 cercariae by tail penetration [50].

136

137 *2.2 Adult worm culture, ES collection and EV purification*

138 *S. haematobium* adult worms were obtained by perfusion of mice at 16 weeks
139 post-infection and parasites were washed several times with serum-free modified Basch
140 media supplemented with 4× antibiotic/antimycotic (SFB) [20] and then incubated in SFB
141 (50 pairs/5 ml) at 37°C in with 5% CO_2 for 2 weeks. ES products were harvested daily,
142 differentially centrifuged at 4°C (500 *g*, 2,000 *g* and 4,000 *g* for 30 min each) to remove
143 large parasite material such as eggs and tegumental debris and stored at -80°C until use.
144 EVs were isolated using established methods previously described for *S. mansoni*
145 [20]. Stored supernatants were thawed on ice, concentrated at 4°C using a 10 kDa spin
146 concentrator (Merck Millipore, USA) and centrifuged for 1 h at 15,000 *g* at 4°C. The
147 resultant pellet (containing 15k vesicles) was washed with 1 ml of PBS, centrifuged at
148 15,000 *g* for 1 h at 4°C, resuspended in 200 µl PBS and stored at -80°C. The supernatant
149 was ultracentrifuged at 120,000 *g* for 3 h at 4°C using an MLS-50 rotor (Beckman

Coulter, USA) to collect 120k pellet vesicles. The resultant pellet was resuspended in 70 µl of PBS and subjected to Optiprep® density gradient (ODG) separation. The ODG was prepared by diluting a 60% Iodixanol solution (Optiprep®, Sigma-Aldrich, USA) with 0.25 M sucrose in 10 mM Tris-HCl pH 7.2 to make 40%, 20%, 10% and 5% iodixanol solutions, and 1.0 ml of these solutions was layered in decreasing density in an ultracentrifuge tube. The resuspended 120k pellet vesicles were added to the top layer and ultracentrifuged at 120,000 *g* for 18 h at 4°C. A control tube was similarly prepared using PBS instead of the 120k pellet vesicle sample to measure the density of the different fractions recovered from the gradient. Fractions obtained from the ODG were diluted with 8 ml of PBS containing 1 × EDTA-free protease inhibitor cocktail (Santa Cruz, USA), and concentrated using a 10 kDa spin concentrator to remove the excess Optiprep® solution.

The density of different fractions obtained from the ODG (12 each for the sample and control) was calculated by measuring the absorbance of each fraction at 340 nm using a POLARstar Omega (BMG Labtech, Australia) spectrophotometer and interpolating the absorbance in a standard curve as previously shown [27]. The protein concentration of all fractions was quantified using the Quick Start™ Bradford Protein Assay Kit (Bio-Rad Laboratories, Inc. Life Science Research, USA) following the manufacturer's instructions.

2.3 Determination of the size and concentration of EVs

The size distribution and particle concentration of the different fractions recovered after ODG, as well as the 15k vesicle fraction, was measured using tunable resistive pulse sensing (TRPS) using a qNano instrument (Izon, New Zealand) following an established

protocol [34]. A Nanopore NP150 and a NP400 (Izon, New Zealand) were used to measure each fraction containing 120k pellet vesicles and the 15k pellet vesicle fraction, respectively. Thirty-five µl of measurement electrolyte (Izon, New Zealand) was added to the upper fluid well and maximum pressure was applied; the shielding lid was clicked 5 to 10 times to wet the Nanopore. Then, 75 µl of measurement electrolyte was added to the lower fluid well, maximum pressure and an appropriate voltage (0.1 V) was applied and Nanopore current was checked for stability. Thirty-five (35) and 75 µl of filtered coating solution (Izon, New Zealand) was loaded in the upper and lower fluid well, respectively, and maximum pressure was applied for 10 min followed by maximum vacuum for another 10 min. The coating solution was flushed out of the upper and lower fluid wells two to three times with measurement electrolyte, maximum pressure was applied for 10 min and the voltage was increased until the current reached between 120 and 140 nA and the baseline current was stable. Then, 35 µl of calibration particles (CP200 carboxylated polystyrene calibration particles; Izon, New Zealand) was loaded to the upper fluid well at a 1:200 dilution when calibrating for 120k pellet vesicle fractions and 1:1,500 when calibrating for the 15k pellet vesicle fraction, incubated for 2 min at maximum pressure and the stretch was reduced and the calibration particles were measured at 2 different pressures (P10 and P5). Then, the 120k and 15k pellet vesicle fractions were diluted 1:5, applied to the Nanopore and measured similarly to the calibration particles. The size and concentration of particles were determined using the software provided by Izon (version 3.2).

198 *2.4 In- gel trypsin digestion of EVs*

199 Fractions containing 120k pellet vesicles of sufficient size and concentration, and
200 the 15k pellet vesicle fraction, were resuspended in 1 × loading buffer (10% glycerol, 80
201 mM Tris-HCl, 2% SDS, 0.01% bromophenol blue and 1.25% beta-mercaptoethanol, pH
202 6.8), boiled at 95°C for 5 min and electrophoresed in a 15% SDS-PAGE gel at 100 V.
203 The gel was stained with 0.03% Coomassie Brilliant Blue (40% methanol, 10% acetic
204 acid and 50% water) for 30 min at room temperature (RT) with gentle shaking and
205 destained using destaining buffer 1 (60% water, 10% acetic acid and 30% methanol) for
206 1 h at RT with gentle shaking. Each lane was sliced into 3 pieces with a surgical blade
207 and placed into a fresh Eppendorf tube. Then, slices were further destained 3 times using
208 destaining buffer 2 (50% acetonitrile (ACN), 20% ammonium bicarbonate and 30%
209 milliQ water) by adding 200 µl of buffer to the gel slice, and incubating at 37°C for 45
210 min. Supernatants were discarded and, finally, gel slices were dried in a vaccum
211 concentrator (LabGear, Australia) on low/high medium heat (< 45°C). One hundred (100)
212 µl of reduction buffer (20 mM dithiothreitol, 25 mM ammonium bicarbonate) was added
213 to each dried slice, incubated at 65°C for 1 h and supernatants were discarded. Alkylation
214 was achieved by adding 100 µl of alkylation buffer (50 mM iodoacetamide, 25 mM
215 ammonium bicarbonate) to each gel slice, which were further incubated in darkness for
216 40 min at RT. Gel slices were washed with 200 µl of wash buffer (25 mM ammonium
217 bicarbonate) and incubated at 37°C for 15 min twice after the gel slices were dried in a
218 speedivac. For trypsin digestion, a total of 2 µg of trypsin (Sigma-Aldrich, USA) was
219 added to each gel slice and incubated for 5 min at RT. Finally, 50 µl of trypsin reaction
220 buffer (40 mM ammonium bicarbonate, 9% ACN) was added to gel slices and incubated
221 overnight at 37°C. Peptides were extracted in 50% acetonitrile with 0.1% trifluoroacetic
222 acid. The last step was performed three times to maximize peptide recovery. All peptides

were finally dried in a vacuum concentrator. Samples were then resuspended in 10 µl of 0.1% trifluoroacetic acid and tryptic peptides were desalted using a Zip-Tip® column (Merck Millipore) pipette tip according to the manufacturer's protocol and dried in a vacuum concentrator before analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

2.5 LC-MS/MS analysis, database search and bioinformatic analysis

Each ELV and MV preparation were reconstituted in 10 µl of 5% formic acid and injected onto a 50 mm 300 µm C18 trap column (Agilent Technologies, USA). The samples were then desalted for 5 min at 30 µl/min using 0.1% formic acid and the peptides were then eluted onto an analytical nano-HPLC column (150 mm × 75 µm 300SBC18, 3.5 µm, Agilent Technologies, USA) at a flow rate of 300 nL/min. Peptides were separated using a 95 min gradient of 1–40% buffer B (90/10 ACN/0.1% formic acid) followed by a steeper gradient from 40 to 80% buffer B in 5 min. A 5600 ABSciex mass spectrometer operated in information-dependent acquisition mode, in which a 1 s TOF-MS scan from 350–1400 m/z was used, and for product ion ms/ms 80–1400 *m/z* ions observed in the TOF-MS scan exceeding a threshold of 100 counts and a charge state of +2 to +5 were set to trigger the acquisition of product ion. Analyst 1.6.1 (ABSCIEX, Framingham, MA, USA) software was used for data acquisition.

For database search and protein identification, a database was built using a concatenated target/decoy version of the *S. haematobium* predicted proteome [51,52] sequences; Bioproject PRJNA78265 downloaded from Parasite WormBase (www.parasite.wormbase.org) and concatenated to the common repository of adventitious proteins (cRAP, https://www.thegpm.org/crap/), as well as to the *Sh*-TSP-2

protein (Genbank QCO69687.1). Database search was performed using a combination of four search engines - X! Tandem version X! Tandem Vengeance (2015.12.15.2) [53], MS-GF+ version Release (v2018.04.09) [54], OMSSA and Tide [55]) using SearchGUI version v3.3.3[56]. The identification settings were as follows: Trypsin, Specific, with a maximum of 2 missed cleavages 10.0 ppm as MS1 and 0.2 Da as MS2 tolerances; fixed modifications: Carbamidomethylation of C (+57.021464 Da), variable modifications: Deamidation of N (+0.984016 Da), Deamidation of Q (+0.984016 Da), Oxidation of M (+15.994915 Da). Peptides and proteins were inferred from the spectrum identification results using PeptideShaker version 1.16.38[56]. Peptide Spectrum Matches (PSMs), peptides and proteins were validated at a 1.0% False Discovery Rate (FDR) estimated using the decoy hit distribution. Only proteins having at least two unique peptides were considered as identified. The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD019462 and 10.6019/PXD019462 (Username: reviewer46995@ebi.ac.uk Password: 03jNpGFk),

Protein family (Pfam) domains were classified using HMMER v3.1b1 [57] and protein gene ontology (GO) categories were classified using Blast2GO v5.2 [58]. ReviGO was used to visualise GO terms using semantic similarity-based scatterplots [59]. TMHMM [60] and SignalP 4.1 [61] software were used to predict transmembrane domains and putative signal peptides, respectively.

2.6 Cloning and expression of Sh-TSP2 and MS3_09198 in Pichia pastoris

DNA sequences encoding the large extracellular loop (LEL) regions of the TSPs *Sh*-TSP2 and MS3_01370 (predicted using TMPRED) were codon optimized based on

yeast codon usage preference and synthesized by GenScript (NJ, US). The synthesized coding DNAs with a 6-His-tag expressed at the C-terminus were cloned into the pPinkα-HC expression vector (Thermofisher, US) using *Xho*I/*Kpn*I restriction sites. The recombinant plasmids with correct insert confirmed by double-strand DNA sequencing were linearized with *Afl*II, and then transformed by electroporation into PichiaPink™ Strain 4 with endogenous proteinases A and B knocked out to prevent degradation of expressed proteins. The transformants were selected on PAD (Pichia Adenine Dropout) agar plates and the expression yield of picked colonies was evaluated in 10 mL BMMY medium with 0.5% methanol for 72h. The clones with highest expression yield were used to express recombinant *Sh*-TSP2 and MS3_01370 in 2L BMMY under induction of 0.5% methanol at 30°C with 250 rpm shaking for 72 h. The culture medium containing the secreted proteins was harvested by centrifugation (5000 *g* for 20 min at RT) and filtered through a 0.22 μm membrane filter (Millipore). Recombinant proteins were purified by immobilized metal affinity chromatography (IMAC) using a prepacked 5 ml His-Trap HP column (GE Healthcare). The purified recombinant proteins were buffer-exchanged in 1 × PBS, pH7.4. The purity of the proteins was analyzed by SDS-PAGE and the concentration was measured by OD_{280}. The low endotoxin contamination was confirmed by Endosafe® cartridge (Charles River). The purified recombinant proteins were aliquoted and stored at -80°C.

2.7 Cloning and expression of MS3_01370 in Escherichia coli

Unlike *Sh*-TSP2 and MS3_09198, we were unsuccessful at expressing the LEL region of MS3_01370 in soluble form in *P. pastoris*, so the protein was expressed as a thioredoxin (TrX) fusion in *E. coli*. Primers incorporating *Nco*I (forward primer) and *Xho*I restriction enzyme sites (reverse primer) were used to amplify the LEL of MS3_09198

296 from *S. haematobium* cDNA and the amplicon was cloned into the pET32a expression
297 vector (Novagen), in-frame with the N-terminal TrX tag. Protein expression was induced
298 for 24 h in *E. coli* BL21(DE3) by addition of 1 mM Isopropyl beta-D-1-
299 thiogalactopyranoside (IPTG) using standard methods. The culture was harvested by
300 centrifugation (8,000 *g* for 20 min at 4°C), re-suspended in 50 ml lysis buffer (50 mM
301 sodium phosphate, pH 8.0, 300 mM NaCl, 40 mM imidazole) and stored at -80°C. The
302 cell pellet was lysed by three freeze-thaw cycles at -80°C and 42°C followed by
303 sonication on ice (10 × 5 sec pulses [70% amplitude] with 30 s rest periods between each
304 pulse) with a Qsonica Sonicator. Insoluble material was pelleted by centrifugation at
305 20,000 *g* for 20 min at 4°C. The supernatant was diluted 1:4 in lysis buffer and filtered
306 through a 0.22 µm membrane (Millipore). MS3_01370 was purified by IMAC by loading
307 onto a prepacked 1 ml His-Trap HP column (GE Healthcare) equilibrated with lysis buffer
308 at a flow rate of 1 ml/min using an AKTA-pure-25 FPLC (GE Healthcare). After washing
309 with 20 ml lysis buffer, bound His-tagged protein was eluted using the same buffer with
310 a stepwise gradient of 50-250 mM imidazole (50 mM steps). Fractions containing
311 MS3_1370 (as determined by SDS-PAGE) were pooled and concentrated using Amicon
312 Ultra-15 centrifugal devices with a 3 kDa MWCO and quantified using the Pierce BCA
313 Protein Assay kit. The final concentration of MS3_01370 was adjusted to 1 mg/ml and
314 the protein was aliquoted and stored at -80°C.
315

316 *2.8 Vaccine formulation and immunization schedule*

317 Numbers of *S. haematobium* cercariae were insufficient to perform appropriately
318 powered vaccination trials using an *S. haematobium* model of infection so vaccine
319 experiments were instead performed using an *S. mansoni* challenge model [62]. Four
320 groups of 10 BALB/c mice (6-8 weeks) were immunized intraperitoneally on day 1 with

either recombinant *Sh*-TSP-2, MS3_09198, MS3_01370 or TrX control protein (50 µg/mouse), each formulated with an equal volume of Imject alum adjuvant (Thermofisher) and 5 µg of CpG ODN1826 (InvivoGen). Immunizations were repeated on days 15 and 29 and each mouse was challenged (tail penetration) with 120 *S. mansoni* cercariae on day 43. Blood was sampled at day 42 to determine pre-challenge antibody titers. Two independent trials were performed.

2.9 Necropsy and estimation of parasite burden

Mice were necropsied at day 91 (7 weeks post-infection) [62]. Blood was collected and worms harvested by vascular perfusion and counted. Livers were removed, weighed and digested for 5 h with 5% KOH at 37°C with shaking. Schistosome eggs from digested livers were concentrated by centrifugation at 1,000 *g* for 10 min and re-suspended in 1 ml of 10% formalin. The number of eggs in a 5 µl aliquot was counted in triplicate and the number of eggs per gram (EPG) of liver tissue was calculated. Small intestines were removed and cleaned of debris before being weighed and digested as per the livers. Eggs were also similarly concentrated and counted to calculate intestinal EPG.

2.10 Statistics

All statistics were performed using GraphPad Prism 7.0. The reductions in worm and egg numbers were analysed using a Student's *t* test and results were expressed as the mean ± standard error of the mean. For antibody titers, the reactivity cut-off values were determined as the mean + 3SD of the naive serum.

3. Results

3.1 Density, protein concentration, particle concentration and purity of 120k and 15k pellet vesicles from Schistosoma haematobium

S. haematobium adult worm 120k pellet vesicles were purified using an iodixanol gradient. The density of the 12 fractions obtained after gradient separation ranged from 1.039 to 1.4 g/ml (Supplementary Table S1). The protein and particle concentration of the fractions ranged from 1.6 to 25.35 µg/ml and 3.72×10^{6} to 2.06×10^{8} particles/ml, respectively, while the protein and particle concentration of the 15k pellet vesicle fraction was 18.00 µg/ml and 1.48×10^{7} particles/ml, respectively. The size of the particles in the gradient-separated fractions ranged from 135 nm ± 19.3 to 342 nm ± 113.9 and size of particles in the 15k pellet vesicle fraction was 274 nm ± 40.7. Gradient-separated fractions having an appropriate purity and density corresponding to 120k pellet vesicles (1.09-1.22 g/ml) [20,27] (fractions 5–9) were selected for further analysis (Fig. 1).

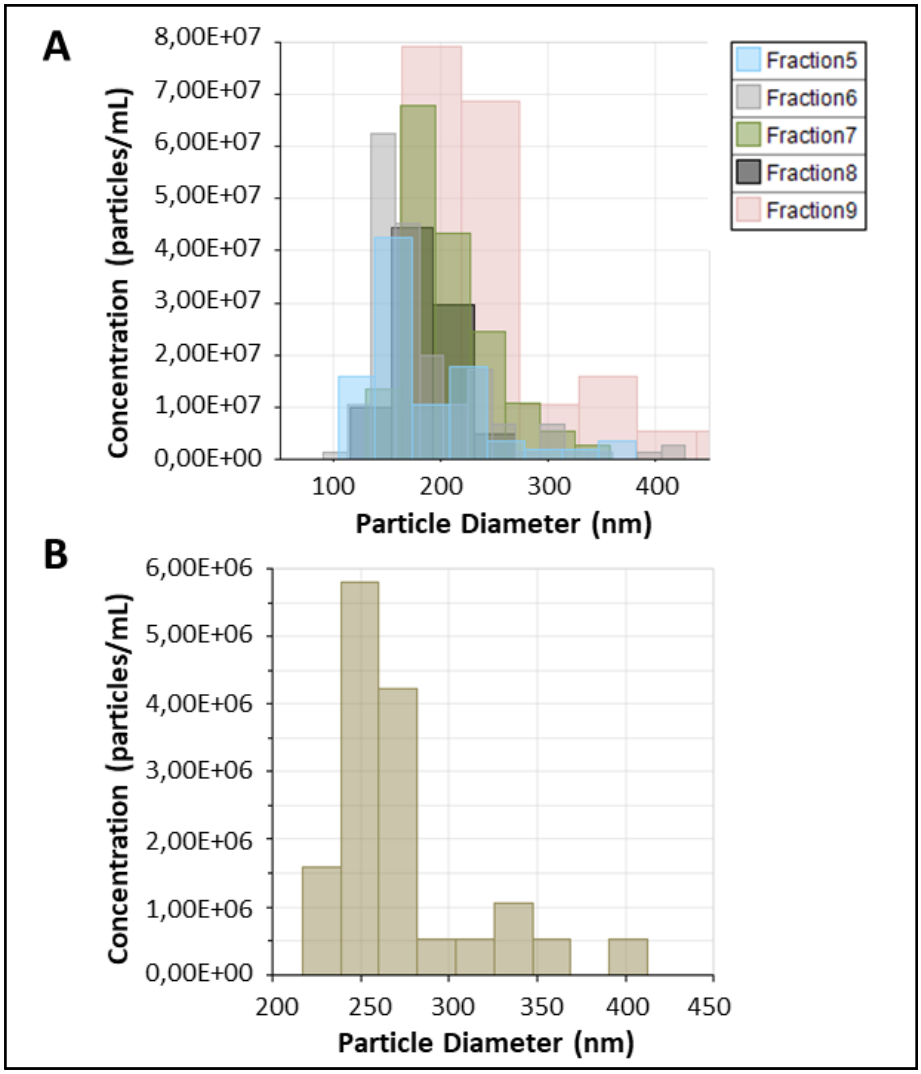

Figure 1. Tunable resistive pulse sensing analysis of 120k pellet vesicles and 15k pellet vesicles from *Schistosoma haematobium*. Size and number of EVs secreted by *S. haematobium* was analysed by qNano (iZon). (A) Size and concentration of particles in fractions containing 120k pellet vesicles (5-9). (B) Size and particle concentration of *S. haematobium* 15k pellet vesicles.

3.2 Proteomic analysis of Schistosoma haematobium 120k and 15k pellet vesicles

The proteome composition of *S. haematobium* adult worm EVs was characterised by LC-MS/MS. After combining the protein identified in fractions 5-9 (fractions containing the highest purity of 120k pellet vesicles), a total of 133 proteins matching *S. haematobium* proteins and common contaminants from the cRAP database were identified. From these, 80 proteins were identified with at least two validated unique

peptides and 57 of them matched *S. haematobium* proteins. From the 57 identified proteins, 8 (14%) contained a transmembrane domain and 7 (12%) had a signal peptide. In a similar fashion, 506 proteins were identified from analysis of the 15k pellet vesicles. From these, 344 proteins were identified with at least two validated unique peptides and 330 matched *S. haematobium* proteins. From these identified proteins, 54 (16.3%) contained a transmembrane domain and 30 (9%) had a signal peptide. Forty proteins were identified in both types of vesicles. The identity of the most abundant proteins in each type of vesicle, as well as proteins having homologues typically found in other helminth EVs, are shown in Table 1. A full list of proteins identified in both 15k and 120k pellet vesicles is shown in Supplementary Tables S2 and S3.

397 **Table 1.** Proteins found in 120k and 15k pellet vesicles isolated from the ES products of adult *S. haematobium**.

Protein category	Protein accession numbers
120k vesicles	
Proteasome subunit	MS3_10249.1, MS3_05734.1, MS3_01483.1, MS3_06009.1, MS3_04526.1, MS3_08808.1, MS3_07240.1, MS3_02807.1, MS3_09236.1, MS3_03070.1
GAPDH	MS3_10141.1
Papain family cysteine protease	MS3_08498.1
C-terminal domain of 1-Cys peroxiredoxin	MS3_08460.1
Ferritin-like domain	MS3_08059.1
S-adenosyl-L-homocysteine hydrolase	MS3_04449.1
Cytosol amino peptidase	MS3_01749.1
Trefoil (P-type) domain-containing protein	MS3_00004.1
15k vesicles	
EF hand	MS3_05735.1, MS3_00180.1, MS3_09846.1, MS3_05877.1, MS3_05317.1, MS3_04536.1, MS3_10043.1, MS3_05959.1, MS3_05150.1, MS3_04275.1, MS3_05958.1 MS3_05952.1, MS3_00361.1, MS3_02003.1
Ras family	MS3_10193.1, MS3_05953.1, MS3_05910.1, MS3_05976.1, MS3_07854.1, MS3_11139.1, MS3_02375.1, MS3_01653.1, MS3_04355.1, MS3_09110.1, MS3_09593.1, MS3_03443.1
TCP-1/cpn60 chaperonin family	MS3_03054.1, MS3_06928.1, MS3_01627.1, MS3_10572.1, MS3_06669.1, MS3_07556.1, MS3_08399.1, MS3_00785.1, MS3_08926.1
Tetraspanins	MS3_01905.1, MS3_01370
Heat-like repeat	MS3_08696.1, MS3_01642.1, MS3_09658.1, MS3_10590.1, MS3_05814.1, MS3_02928.1, MS3_06293.1
Calponin homology (CH) domain	MS3_07481.1, MS3_05505.1, MS3_01744.1, MS3_00852.1, MS3_00361.1, MS3_03766.1, MS3_10701.1
Dynein light chain type 1	MS3_05351.1, MS3_08569.1, MS3_05345.1, MS3_01173.1, MS3_05342.1, MS3_04412.1, MS3_05960.1
Actin	MS3_07374.1, MS3_04014.1, MS3_00351.1, MS3_02465.1, MS3_04907.1, MS3_01922.1
HSP-70 protein	MS3_10713.1, MS3_11293.1, MS3_11411.1, MS3_10049.1, MS3_02688.1, MS3_02787.1

Immunoglobulin domain	MS3_03027.1, MS3_01271.1, MS3_03208.1, MS3_07594.1, MS3_01223.1
Annexin	MS3_08725.1, MS3_08723.1, MS3_04598.1, MS3_01964.1, MS3_01952.1
AAA domain	MS3_03802.1, MS3_02581.1, MS3_01139.1, MS3_01650.1, MS3_07031.1
14-3-3 protein	MS3_03977.1, MS3_05219.1, MS3_00047.1, MS3_01871.1, MS3_03976.1
120k and 15k vesicles	
Tetraspanins	MS3_09198, *Sh*-TSP-2, MS3_05226, MS3_05289, MS3_01153
Ferritin-like domain	MS3_07972.1, MS3_07178.1
14-3-3 protein	MS3_03977.1, MS3_00047.1
Elongation factor Tu C-terminal domain	MS3_08479.1
EF hand	MS3_08446.1
Actin	MS3_07374.1
GST, N-terminal domain	MS3_06482.1
Cytosol aminopeptidase family, catalytic domain	MS3_08450.1
Lipocalin/cytosolic fatty-acid binding protein family	MS3_04307.1
Immunoglobulin domain	MS3_03208.1
Saposin-like type B, region 2	MS3_02805.1
Enolase, N-terminal domain	MS3_02425.1

398 *proteins listed are members of protein families typically found in helminth EVs.

3.3 Protein families present in Schistosoma haematobium 120k and 15k pellet vesicles

Identified proteins were subjected to a Pfam analysis using default parameters in HMMER v3.1b1 and proteins containing an identified Pfam domain with an E-value < 1E-05 were selected. A total of 70 and 387 domains were identified from 120k and 15k pellet vesicles, respectively. In 120k pellet vesicles, the three most abundant domains were proteasome subunit domains (PF00227) (14%), TSP family domains (PF00335) (7%) and ferritin-like domains (PF12902) (4%) (Fig. 2A). The most abundant protein domains from 15k pellet vesicles were EF-hand domains (PF00036) (3%), Ras family domains (PF00071) (3%), TCP-1/cpn60 chaperonin family domains (PF00118) (2%) and TSP family domains (PF00335) (2%) (Fig. 2B). From these, TSP family domains, ferritin-like domains and 14-3-3 protein domains were common to both types of vesicles.

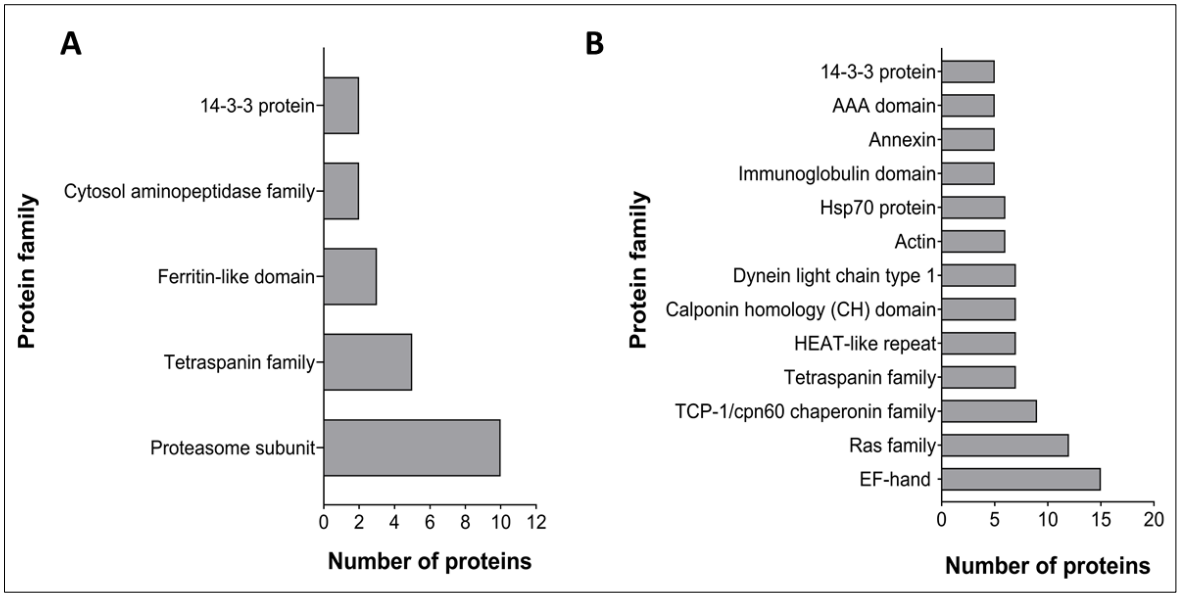

Figure 2. Pfam analysis of the most abundant *Schistosoma haematobium* vesicle proteins. The X-axis represents the number of proteins containing at least one of those domains. (A) 120k pellet vesicles (B) 15k pellet vesicles.

3.4 Gene ontology of proteins identified from Schistosoma haematobium 120k and 15k pellet vesicles

The proteins of adult *S. haematobium* 120k and 15k pellet vesicles were annotated using Blast2GO [241]. To avoid redundancy in the analysis and better comprehend the represented GO terms in the vesicles, the parental GO terms were removed and children GO terms were visualised using ReviGO based on semantic similarity-based scatterplots [242]. The GO terms were ranked by the nodescore provided by Blast2GO and plotted using their nodescore and frequency. Semantically similar GO terms plot close together and increasing heatmap score signifies increasing nodescore from Blast2GO. The circle size denotes the frequency of the GO term from the underlying database. In 120k pellet vesicles, several biological processes were highly represented, such as the ubiquitin-dependent protein catabolic process, oxidation-reduction process and gluconeogenesis and glycolytic process (Fig. 3A). Similarly, in 15k pellet vesicles, several biological processes were highly represented, such as the carbohydrate metabolic process, transport process, organonitrogen compound metabolic process and microtubule-based process (Fig. 3B). The oxidation-reduction process was common to both types of vesicles; 6 proteins from 120k pellet vesicles and 22 proteins from 15k pellet vesicles were predicted to be involved in this process.

In *S. haematobium* 120k vesicles, several molecular functions were highly represented, such as threonine-type endopeptidase activity, protein binding activity, endopeptidase activity and transition metal ion binding activity. In 15k vesicles, molecular functions such as protein binding activity, ATP binding activity, nucleoside-triphosphatase activity and calcium ion binding activity were highly represented. From these highly represented molecular function terms, protein binding was common to both 120k and 15k vesicles.

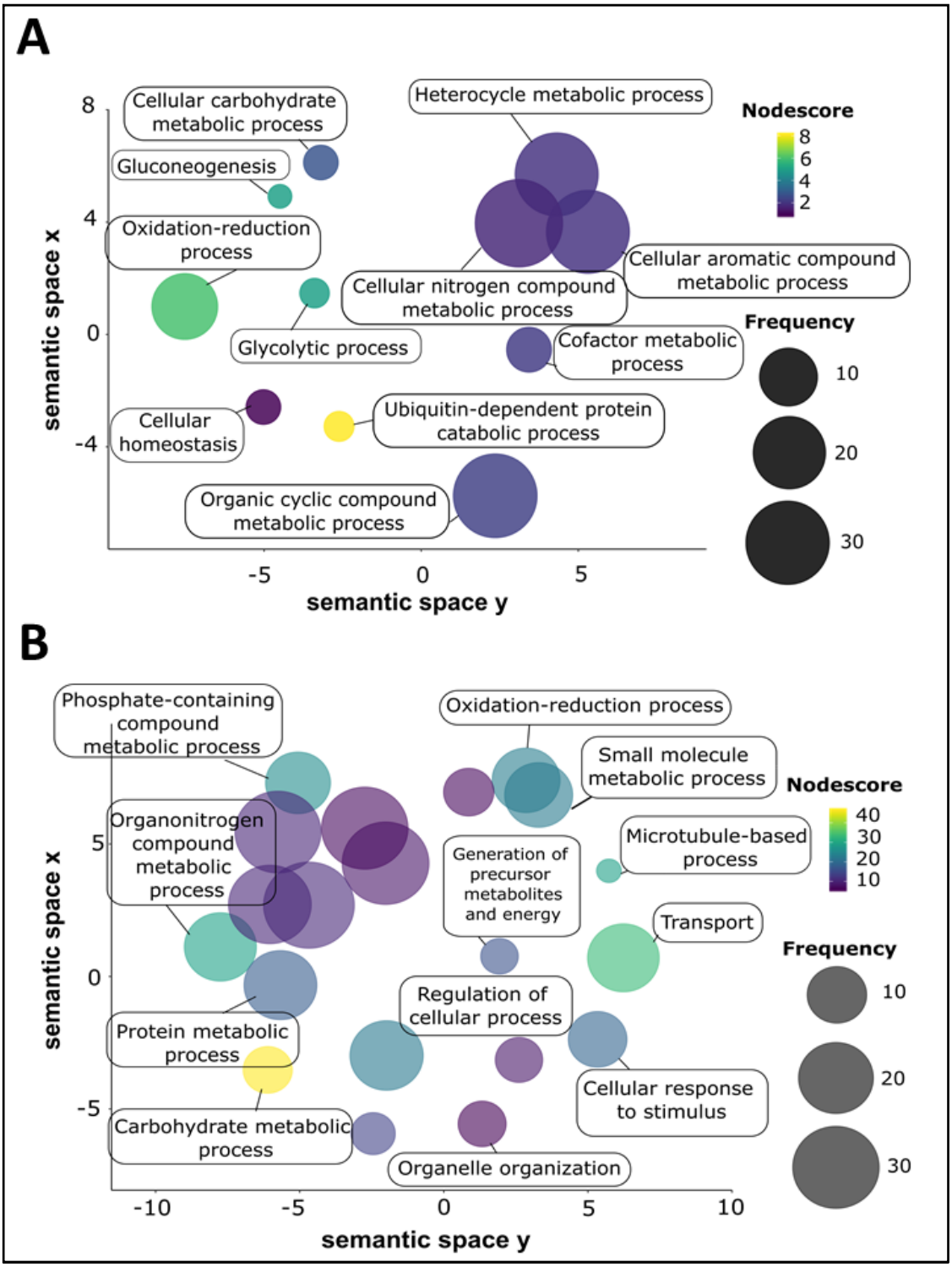

Figure 3. Biological process GO term categories of adult *Schistosoma haematobium* vesicle proteins. Biological processes were ranked by nodescore (Blast2GO) and plotted using REViGO. Semantically similar GO terms plot close together, increasing heatmap score signifies increasing nodescore from Blast2GO, while circle size denotes the frequency of the GO term from the underlying database. (A) 120k pellet vesicles (B) 15k pellet vesicles.

3.5 Parasite burdens in vaccinated and control mice

Vaccination of mice with MS3_01370, MS3_09198 showed a trend towards reduced adult *S. mansoni* burden in trial 1 by 22% and 12%, respectively, and in trial 2 by 14% and 5%, respectively, compared to controls. *Sh*-TSP2-vaccinated mice showed a reduction in trial 1 only of 2%, compared to controls. None of these differences were statistically significant except for MS3_01370 in trial 1 ($P<0.05$) (Fig. 4A and B). However, in trial 1, vaccination of mice with MS3_01370, MS3_09198 and *Sh*-TSP-2 significantly reduced liver egg burdens by 39% ($P<0.05$), 49% ($P<0.001$) and 32% ($P<0.01$), respectively (Fig. 4C), and in trial 2, MS3_01370, MS3_09198 and *Sh*-TSP-2 vaccination significantly reduced liver egg burdens by 54% ($P<0.001$), 27% ($P<0.05$) and 49% ($P<0.001$), respectively (Fig. 4D). Similarly, immunisation of mice with MS3_01370, MS3_09198 and *Sh*-TSP-2 reduced the intestinal egg burden by 57% ($P<0.01$), 51% ($P<0.01$) and 54% ($P<0.001$) in trial 1, respectively (Fig. 4E), and in trial 2, MS3_01370, MS3_09198 and *Sh*-TSP-2 vaccination reduced the intestinal egg burden by 36% ($P<0.01$), 39% ($P<0.05$) and 27% ($P<0.05$), respectively (Fig. 4.2F).

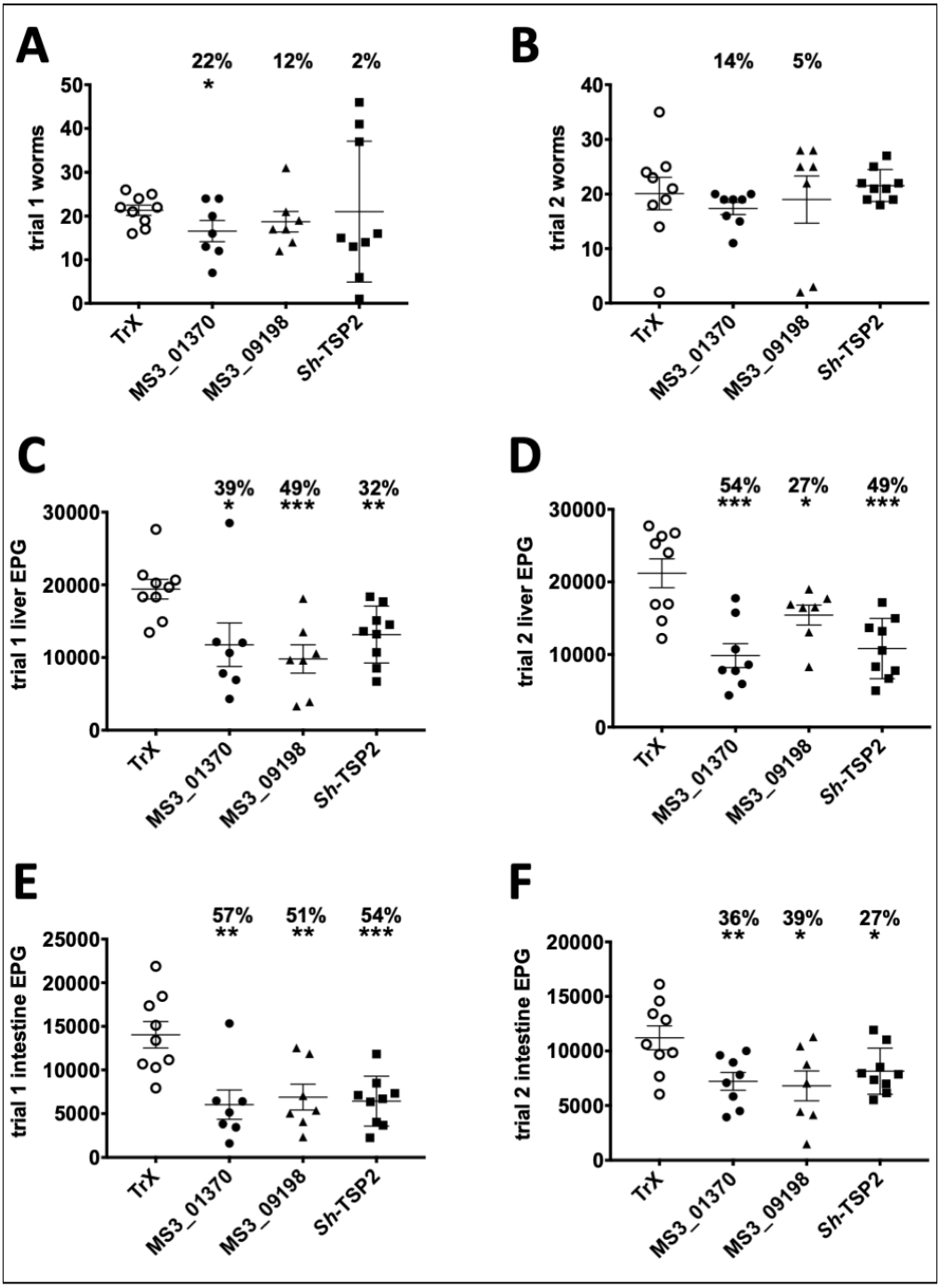

Figure 4. *Schistosoma mansoni* worm and egg burden reduction of vaccinated and control mice vaccinated with *S. haematobium* recombinant tetraspanins. (A) Adult worm reduction trial 1, (B) Adult worm reduction trial 2, (C) liver egg reduction trial 1, (D) liver egg reduction trial 2, (E) intestinal egg reduction trial 1, (F) intestinal egg reduction trial 2. Percent reductions in parasite burden are above each dataset. Differences between each vaccinated group and the control group were analysed with a student's t-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

4. Discussion

More than 100 million people are infected with *S. haematobium* and 150,000 people die every year [4]. Efforts have been made to reduce the prevalence of schistosomiasis but the parasite is spreading to new areas [3] and is now the second most prevalent of the neglected tropical diseases [63]. Praziquantel is the only drug available for schistosomiasis treatment, although it doesn't protect against re-infection and the risk of resistance emerging in the field is high [64,65]. Furthermore, there is no licensed and effective vaccine for this devastating disease, and there is an urgent need to develop one to eliminate urogenital schistosomiasis as well as the other species of schistosomes.

We have shown that *S. haematobium* secretes at least two populations of vesicles with distinct differences in size and proteomic composition, similar to other schistosome species like *S. mansoni* [20]. The most represented domains contained within 120k pellet vesicle proteins were the proteasome subunit domains, TSP domains and ferritin-like domains, among others, while the most represented domains in the 15k pellet vesicles were EF-hand, Ras family, TCP-1/cpn60 chaperonin family and TSP family domains. The proteasome is involved in the biogenesis of EVs [66] and also controls protein homeostasis and degradation of damaged proteins [67]. Furthermore, in schistosomes, the proteasome plays an important role in the cellular stress response and survival of the parasite [68]; it has been shown that treating mice with a proteasome inhibitor prior to infection with *S. mansoni* cercariae significantly impaired parasite development [69] and *in vitro* treatment of schistosomula with siRNAs targeting a deubiquitinase subunit of the 19S regulatory particle significantly reduced parasite viability [70].

Ferritins are iron-storage proteins, involved in maintaining intracellular iron balance [71], which minimize free-radical reactions and prevent cellular damage caused by iron accumulation in the cell [72]. Iron also plays an important role in the eggshell

499 formation of schistosomes, and fer-1, one of the two ferritin isoforms of the parasite, is
500 highly expressed in female worms in comparison to males [73]. Since, female worms
501 produce many eggs per day and eggs are the primary cause of pathology, vaccination
502 using ferritins could disrupt the formation of eggs and reduce egg-induced disease.
503 Indeed, ferritins have been tested as vaccine candidates against schistosomes;
504 immunisation of mice with the recombinant Fer-1 of *S. japonicum* caused 35.5% and
505 52.1% reduction in adult worm and liver egg burden, respectively [74]. Ferritins have been
506 identified in the proteomic analysis of other blood feeding helminth EVs [20,27],
507 suggesting a role for EVs in iron storage and acquisition.

508 Proteins containing EF-hand domains are involved in a number of protein-protein
509 interactions for the uptake and release of calcium [75]. The influx of calcium in the cell
510 induces the redistribution of phospholipids in the cell membrane, resulting in increased
511 release of microvesicles [76]. EF-hand domains are also among the most predominant
512 protein domains found within other helminth EV proteins [77]. This is consistent with the
513 GO analysis herein, in which proteins involved in protein binding and calcium ion binding
514 are the highest represented molecular function terms.

515 Ras proteins serve as signalling nodes activated in response to diverse
516 extracellular stimuli [78] and are involved in biogenesis and release of microvesicles [79].
517 In *S. mansoni*, Ras proteins are involved in the male-directed maturation of the female
518 worms [80], which could suggest a potential role of EVs in parasite-parasite
519 communication.

520 TCP-1/cpn60 chaperonin family proteins play an important role in the folding of
521 proteins, including actin and tubulin [81], which bind and hydrolyse ATP using
522 magnesium ions [82]. This is consistent with the molecular function GO terms, in which
523 ATP binding and nucleoside-triphosphatase was the most represented.

The second and fourth most abundant protein domains in 120k and 15k pellet vesicles, respectively, were the TSPs. TSPs are involved in EV biogenesis [79], are present on the surface membrane of EVs from many different organisms and are considered a molecular marker of EVs [83]. TSPs are also found from the proteomic analysis of other helminth EVs (reviewed in [77]). In trematodes, TSPs are involved in tegument development [84-86]. TSP LELs have been tested as vaccine candidates in trematode models of infection [42,47,87] and antibodies produced against TSP vaccine candidates present in *S. mansoni* and *O. viverrini* EVs blocked the internalisation of EVs by host cells in both parasites, and decreased pathogenesis in the case of *O. viverrine* [29,42,88], suggesting a possible mechanism of vaccine efficacy.

Since we identified TSPs in both types of vesicles described in this study and there is evidence that these molecules orchestrate interactions between parasite EVs and host tissues, we aimed to assess the vaccine efficacy of some of the most abundant EV TSPs in a challenge model of schistosome infection. Difficulties in generating the large amount of cercariae needed for mouse infection (the use of hamsters is prohibited in Australia) precluded the use of *S. haematobium* as the challenge parasite and so we used an *S. mansoni* challenge model instead. Although this meant that a heterologous infection model would be employed to assess the efficacy of *S. haematobium* proteins, we reasoned that the homology of the candidates between the two species could be sufficiently high (*Sh*-TSP2 – 69.6% identity, MS3_09198 – 98.6% identity and MS3_01370 – 79.5% identity) (Figure S1) as to afford a level of cross-species protection and provide informative data with regards to the vaccine efficacy of these candidates.

Vaccination with any of the TSPs did not elicit significant reductions in worm burden compared to controls (except for MS3_01370 in trial 1), however, significant reductions in tissue egg burdens (both liver and intestinal) were observed in all groups of

549 vaccinated mice in both trials. Decreases in tissue egg loads are arguably the most
550 important hallmarks of an effective vaccine against schistosomiasis given that (1)
551 pathology due to the disease is egg-induced [89] and (2) disease transmission is dependent
552 on the excretion of eggs from the host into the environment [10], so a vaccine which
553 reduces egg burden in the host would ameliorate both disease pathology and transmission.

554 Other schistosome antigens have been reported to elicit a primarily anti-fecundity
555 protective effect upon vaccination. Glutathione-S-transferases (GSTs) from *S. japonicum*,
556 *S. haematobium* and the bovine schistosome *S. bovis* have all been reported to induce
557 decreases in tissue egg loads, despite no observable reduction in worm burden [90-92].
558 Interestingly, GSTs have been prominently identified from the vesicles in this study and
559 other proteomic analyses of schistosome EVs [20,27,28]. This manifestation of protective
560 immunity is seen in cattle which have had repeated field and laboratory exposure to *S.*
561 *bovis* [93,94] and the closely-related *S. matheei* [95], in Rhesus monkeys experimentally
562 infected with *S. mansoni* [96], and in baboons following laboratory exposure to *S.*
563 *haematobium* [97]. In the cattle/*S. bovis* and baboon/*S. haematobium* models, this
564 observation was confirmed by surgically transplanting "suppressed" worms into hosts
565 with no prior exposure and showing that the parasites resumed high levels of egg
566 production [91,97]. It could be that vaccination with the EV molecules described herein is
567 eliciting similar immune mechanisms to engender the observed anti-fecundity effects,
568 which may be manifesting in a reduction of the fitness and fecundity of adult worms
569 and/or a shortening of the lifespan of eggs embolised in the tissues [91]. Further, the ability
570 of antibodies against helminth EV proteins to block vesicle uptake, which has subsequent
571 impacts on host cell immune effector mechanisms [20,98], provides a plausible mechanism
572 for protective efficacy.

573 In this study we provide the first characterisation of EVs secreted by *S.*
574 *haematobium*, their expression as vaccine candidates present on the surface of these EVs
575 and the evaluation of these candidates in a heterologous challenge model of
576 schistosomiasis. The significant reduction in tissue egg burden described here indicates
577 that these EV-derived vaccine candidates could be effective in reducing the pathology
578 and transmission of *S. mansoni* and *S. haematobium* (due to the cross-protective efficacy
579 observed in the heterologous vaccine/challenge model herein) and they could potentially
580 be incorporated into a pan-schistosome vaccine, due to the geographical overlap between
581 the two species.

582

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593 **References**

594 1. McManus, D.P.; Dunne, D.W.; Sacko, M.; Utzinger, J.; Vennervald, B.J.; Zhou,
595 X.N. Schistosomiasis. *Nature Review Disease Primers* **2018**, *4*, 13,
596 doi:10.1038/s41572-018-0013-8.

2. Chitsulo, L.; Engels, D.; Montresor, A.; Savioli, L. The global status of schistosomiasis and its control. *Acta Tropica* **2000**, *77*, 41-51, doi:10.1016/s0001-706x(00)00122-4.

3. Boissier, J.; Grech-Angelini, S.; Webster, B.L.; Allienne, J.-F.; Huyse, T.; Mas-Coma, S.; Toulza, E.; Barré-Cardi, H.; Rollinson, D.; Kincaid-Smith, J., et al. Outbreak of urogenital schistosomiasis in Corsica (France): an epidemiological case study. *The Lancet Infectious Diseases* **2016**, *16*, 971-979, doi:10.1016/S1473-3099(16)00175-4.

4. Berger, S. *Schistosoma haematobium 2017 edition*; GIDEON Informatics Inc: Los Angeles, CA, **2017**.

5. World Health Organization. Schistosomiasis: number of people treated worldwide in 2014. *Weekly Epidemiological Record* **2016**, *91*, 53-60.

6. Berry, A.; Moné, H.; Iriart, X.; Mouahid, G.; Aboo, O.; Boissier, J.; Fillaux, J.; Cassaing, S.; Debuisson, C.; Valentin, A., et al. *Schistosomiasis haematobium*, Corsica, France. *Emerging Infectious Diseases* **2014**, *20*, 1595-1597, doi:10.3209/eid2009.140756.

7. Wang, W.; Wang, L.; Liang, Y.S. Susceptibility or resistance of praziquantel in human schistosomiasis: a review. *Parasitology Research* **2012**, *111*, 1871-1877, doi:10.1007/s00436-012-3151-z.

8. Vale, N.; Gouveia, M.J.; Rinaldi, G.; Brindley, P.J.; Gartner, F.; Correia da Costa, J.M. Praziquantel for Schistosomiasis: Single-Drug Metabolism Revisited, Mode of Action, and Resistance. *Antimicrobial Agents and Chemotherapy* **2017**, *61*, doi:10.1128/AAC.02582-16.

620 9. Gönnert, R.; Andrews, P. Praziquantel, a new board-spectrum antischistosomal
621 agent. *Zeitschrift fur Parasitenkunde* **1977**, *52*, 129-150,
622 doi:10.1007/bf00389899.
623 10. McManus, D.P.; Bergquist, R.; Cai, P.; Ranasinghe, S.; Tebeje, B.M.; You, H.
624 Schistosomiasis-from immunopathology to vaccines. *Seminars in*
625 *Immunopathology* **2020**, 10.1007/s00281-020-00789-x, doi:10.1007/s00281-020-
626 00789-x.
627 11. Wilson, R.A.; Coulson, P.S. Why don't we have a schistosomiasis vaccine?
628 *Parasitology Today* **1998**, *14*, 97-99, doi:10.1016/S0169-4758(97)01198-8.
629 12. Tebeje, B.M.; Harvie, M.; You, H.; Loukas, A.; McManus, D.P. Schistosomiasis
630 vaccines: where do we stand? *Parasites and Vectors* **2016**, *9*, 1-15,
631 doi:10.1186/s13071-016-1799-4.
632 13. Riveau, G.; Schacht, A.M.; Dompnier, J.P.; Deplanque, D.; Seck, M.; Waucquier,
633 N.; Senghor, S.; Delcroix-Genete, D.; Hermann, E.; Idris-Khodja, N., et al. Safety
634 and efficacy of the rSh28GST urinary schistosomiasis vaccine: A phase 3
635 randomized, controlled trial in Senegalese children. *PLoS Neglected Tropical*
636 *Diseases* **2018**, *12*, e0006968, doi:10.1371/journal.pntd.0006968.
637 14. Crowe, J.; Lumb, F.E.; Harnett, M.M.; Harnett, W. Parasite excretory-secretory
638 products and their effects on metabolic syndrome. *Parasite Immunology* **2017**, *39*,
639 e12410, doi:10.1111/pim.12410.
640 15. Delcroix, M.; Medzihradsky, K.; Caffrey, C.R.; Fetter, R.D.; McKerrow, J.H.
641 Proteomic analysis of adult *S. mansoni* gut contents. *Molecular and Biochemical*
642 *Parasitology* **2007**, *154*, 95-97, doi:10.1016/j.molbiopara.2007.03.008.
643 16. Hall, S.L.; Braschi, S.; Truscott, M.; Mathieson, W.; Cesari, I.M.; Wilson, R.A.
644 Insights into blood feeding by schistosomes from a proteomic analysis of worm

vomitus. *Molecular and Biochemical Parasitology* **2011**, *179*, 18-29, doi:10.1016/j.molbiopara.2011.05.002.

17. Liu, F.; Cui, S.-J.; Hu, W.; Feng, Z.; Wang, Z.-Q.; Han, Z.-G. Excretory/secretory proteome of the adult developmental stage of human blood fluke, *Schistosoma japonicum*. *Molecular and Cellular Proteomics* **2009**, *8*, 1236-1251, doi:10.1074/mcp.M800538-MCP200.

18. Sotillo, J.; Pearson, M.S.; Becker, L.; Mekonnen, G.G.; Amoah, A.S.; van Dam, G.; Corstjens, P.L.A.M.; Murray, J.; Mduluza, T.; Mutapi, F., et al. In-depth proteomic characterization of *Schistosoma haematobium*: Towards the development of new tools for elimination. *PloS Neglected Tropical Diseases* **2019**, *13*, e0007362, doi:10.1371/journal.pntd.0007362.

19. Eichenberger, R.M.; Sotillo, J.; Loukas, A. Immunobiology of parasitic worm extracellular vesicles. *Immunology and Cell Biology* **2018**, *96*, 704-713, doi:10.1111/imcb.12171.

20. Kifle, D.W.; Pearson, M.S.; Becker, L.; Pickering, D.; Loukas, A.; Sotillo, J. Proteomic analysis of two populations of *Schistosoma mansoni*-derived extracellular vesicles: 15k pellet and 120k pellet vesicles. *Molecular and Biochemical Parasitology* **2020**, *236*, 111264, doi:10.1016/j.molbiopara.2020.111264.

21. Mekonnen, G.G.; Pearson, M.; Loukas, A.; Sotillo, J. Extracellular vesicles from parasitic helminths and their potential utility as vaccines. *Expert Review of Vaccines* **2018**, *17*, 1-9, doi:10.1080/14760584.2018.1431125.

22. Kifle, D.W.; Sotillo, J.; Pearson, M.S.; Loukas, A. Extracellular vesicles as a target for the development of anti-helminth vaccines. *Emerging Topics in Life Sciences* **2017**, *1*, 659-665, doi:10.1042/ETLS20170095.

670 23. Kim, K.M.; Abdelmohsen, K.; Mustapic, M.; Kapogiannis, D.; Gorospe, M. RNA
671 in extracellular vesicles. *Wiley Interdisciplinary reviews: RNA* **2017**, *8*, 1757-
672 7012 doi:10.1002/wrna.1413.
673 24. Pathan, M.; Fonseka, P.; Chitti, S.V.; Kang, T.; Sanwlani, R.; Van Deun, J.;
674 Hendrix, A.; Mathivanan, S. Vesiclepedia 2019: a compendium of RNA, proteins,
675 lipids and metabolites in extracellular vesicles. *Nucleic Acids Research* **2019**, *47*,
676 D516-D519, doi:10.1093/nar/gky1029.
677 25. Coakley, G.; Maizels, R.M.; Buck, A.H. Exosomes and Other Extracellular
678 Vesicles: The New Communicators in Parasite Infections. *Trends in Parasitology*
679 **2015**, *31*, 477-489, doi:10.1016/j.pt.2015.06.009.
680 26. Tritten, L.; Geary, T.G. Helminth extracellular vesicles in host-parasite
681 interactions. *Current Opinion in Microbiology* **2018**, *46*, 73-79,
682 doi:10.1016/j.mib.2018.08.002.
683 27. Sotillo, J.; Pearson, M.; Potriquet, J.; Becker, L.; Pickering, D.; Mulvenna, J.;
684 Loukas, A. Extracellular vesicles secreted by *Schistosoma mansoni* contain
685 protein vaccine candidates. *International Journal for Parasitology* **2016**, *46*, 1-5,
686 doi:10.1016/j.ijpara.2015.09.002.
687 28. Nowacki, F.C.; Swain, M.T.; Klychnikov, O.I.; Niazi, U.; Ivens, A.; Quintana,
688 J.F.; Hensbergen, P.J.; Hokke, C.H.; Buck, A.H.; Hoffmann, K.F. Protein and
689 small non-coding RNA-enriched extracellular vesicles are released by the
690 pathogenic blood fluke *Schistosoma mansoni*. *Journal of Extracellular Vesicles*
691 **2015**, *4*, 1-16, doi:10.3402/jev.v4.28665.
692 29. Chaiyadet, S.; Sotillo, J.; Smout, M.; Cantacessi, C.; Jones, M.K.; Johnson, M.S.;
693 Turnbull, L.; Whitchurch, C.B.; Potriquet, J.; Laohaviroj, M., et al. Carcinogenic
694 liver fluke secretes extracellular vesicles that promote cholangiocytes to adopt a

tumorigenic phenotype. *Journal of Infectious Disease* **2015**, *212*, 1636-1645, doi:10.1093/infdis/jiv291.

30. Cwiklinski, K.; de la Torre-Escudero, E.; Trelis, M.; Bernal, D.; Dufresne, P.J.; Brennan, G.P.; O'Neill, S.; Tort, J.; Paterson, S.; Marcilla, A., et al. The extracellular vesicles of the helminth pathogen, *Fasciola hepatica*: Biogenesis pathways and cargo molecules involved in parasite pathogenesis. *Molecular and Cellular proteomics* **2015**, *14*, 3258-3273, doi:10.1074/mcp.M115.053934.

31. Zhu, L.; Liu, J.; Dao, J.; Lu, K.; Li, H.; Gu, H.; Liu, J.; Feng, X.; Cheng, G. Molecular characterization of *S. japonicum* exosome-like vesicles reveals their regulatory roles in parasite-host interactions. *Scientific Reports* **2016**, *6*, 25885, doi:10.1038/srep25885.

32. Zheng, Y.; Guo, A.; Guo, X.; Su, M.; Ding, J.; Yang, J.; Xiang, H.; Cao, X.; Zhang, S.; Ayaz, M., et al. Regulatory effects of *Echinococcus multilocularis* extracellular vesicles on RAW264.7 macrophages. *Veterinary Parasitology* **2017**, *235*, 29-36, doi:10.1016/j.vetpar.2017.01.012.

33. Nicolao, M.C.; Rodriguez Rodrigues, C.; Cumino, A.C. Extracellular vesicles from *Echinococcus granulosus* larval stage: Isolation, characterization and uptake by dendritic cells. *PloS Neglected Tropical Diseases* **2019**, *13*, e0007032, doi:10.1371/journal.pntd.0007032.

34. Eichenberger, R.M.; Ryan, S.; Jones, L.; Buitrago, G.; Polster, R.; de Oca, M.M.; Zuvelek, J.; Giacomin, P.R.; Dent, L.A.; Engwerda, C.R., et al. Hookworm secreted extracellular vesicles interact with host cells and prevent inducible colitis in mice. *Frontiers in Immunology* **2018**, *9*, 850, doi:10.3389/fimmu.2018.00850.

35. Eichenberger, R.M.; Talukder, M.H.; Field, M.A.; Wangchuk, P.; Giacomin, P.; Loukas, A.; Sotillo, J. Characterization of *Trichuris muris* secreted proteins and

extracellular vesicles provides new insights into host-parasite communication. *Journal of Extracellular Vesicles* **2018**, *7*, 1428004-1428016, doi:10.1080/20013078.2018.1428004.

36. Bernal, D.; Trelis, M.; Montaner, S.; Cantalapiedra, F.; Galiano, A.; Hackenberg, M.; Marcilla, A. Surface analysis of *Dicrocoelium dendriticum*. The molecular characterization of exosomes reveals the presence of miRNAs. *Journal of Proteomics* **2014**, *105*, 232, doi:10.1016/j.jprot.2014.02.012.

37. Buck, A.H.; Coakley, G.; Simbari, F.; McSorley, H.J.; Quintana, J.F.; Le Bihan, T.; Kumar, S.; Abreu-Goodger, C.; Lear, M.; Harcus, Y., et al. Exosomes secreted by nematode parasites transfer small RNAs to mammalian cells and modulate innate immunity. *Nature Communications* **2014**, *5*, 5488, doi:10.1038/ncomms6488.

38. Hansen, E.P.; Fromm, B.; Andersen, S.D.; Marcilla, A.; Andersen, K.L.; Borup, A.; Williams, A.R.; Jex, A.R.; Gasser, R.B.; Young, N.D., et al. Exploration of extracellular vesicles from *Ascaris suum* provides evidence of parasite-host cross talk. *Journal of Extracellular Vesicles* **2019**, *8*, 1578116, doi:10.1080/20013078.2019.1578116.

39. Wang, L.; Li, Z.; Shen, J.; Liu, Z.; Liang, J.; Wu, X.; Sun, X.; Wu, Z. Exosome-like vesicles derived by *Schistosoma japonicum* adult worms mediates M1 type immune- activity of macrophage. *Parasitology Research* **2015**, *114*, 1865-1873, doi:10.1007/s00436-015-4373-7.

40. Zamanian, M.; Fraser, L.M.; Agbedanu, P.N.; Harischandra, H.; Moorhead, A.R.; Day, T.A.; Bartholomay, L.C.; Kimber, M.J. Release of small RNA-containing exosome-like vesicles from the human filarial parasite *Brugia malayi*. *PloS*

Neglected Tropical Diseases **2015**, *9*, e0004069, doi:10.1371/journal.pntd.0004069.

41. Shears, R.K.; Bancroft, A.J.; Hughes, G.W.; Grencis, R.K.; Thornton, D.J. Extracellular vesicles induce protective immunity against *Trichuris muris*. *Parasite Immunology* **2018**, *40*, e12536, doi:10.1111/pim.12536.

42. Sujittra, C.; Sotillo, J.; Krueajampa, W.; Thongsen, S.; Brindley, P.J.; Sripa, B.; Loukas, A.; Laha, T. Vaccination of hamsters with *Opisthorchis viverrini* extracellular vesicles and vesicle-derived recombinant tetraspanins induces antibodies that block vesicle uptake by cholangiocytes and reduce parasite burden after challenge infection. *PloS Neglected Tropical Diseases* **2019**, *13*, e0007450, doi:10.1371/journal.pntd.0007450.

43. Trelis, M.; Galiano, A.; Bolado, A.; Toledo, R.; Marcilla, A.; Bernal, D. Subcutaneous injection of exosomes reduces symptom severity and mortality induced by *Echinostoma caproni* infection in BALB/c mice. *International Journal for Parasitology* **2016**, *46*, 799-808, doi:10.1016/j.ijpara.2016.07.003.

44. Coakley, G.; Maizels, R.M.; Buck, A.H. Exosomes and other extracellular vesicles: The new communicators in parasite infections. *Trends in Parasitology* **2015**, *31*, 477-489, doi:10.1016/j.pt.2015.06.009.

45. Da'dara, A.A.; Skelly, P.J.; Wang, M.; Harn, D.A. Immunization with plasmid DNA encoding the integral membrane protein, Sm23, elicits a protective immune response against schistosome infection in mice. *Vaccine* **2001**, *20*, 359-369, doi:10.1016/S0264-410X(01)00374-7.

46. Pearson, M.S.; Pickering, D.A.; McSorley, H.J.; Bethony, J.M.; Tribolet, L.; Dougall, A.M.; Hotez, P.J.; Loukas, A. Enhanced protective efficacy of a chimeric

form of the schistosomiasis vaccine antigen *Sm*-TSP-2. *PloS Neglected Tropical Diseases* **2012**, *6*, e1564, doi:10.1371/journal.pntd.0001564.

47. Tran, M.H.; Pearson, M.S.; Bethony, J.M.; Smyth, D.J.; Jones, M.K.; Duke, M.; Don, T.A.; McManus, D.P.; Correa-Oliveira, R.; Loukas, A. Tetraspanins on the surface of *Schistosoma mansoni* are protective antigens against schistosomiasis. *Nature Medicine* **2006**, *12*, 835-840, doi:10.1038/nm1430.

48. Keitel, W.A.; Potter, G.E.; Diemert, D.; Bethony, J.; El Sahly, H.M.; Kennedy, J.K.; Patel, S.M.; Plieskatt, J.L.; Jones, W.; Deye, G., et al. A phase 1 study of the safety, reactogenicity, and immunogenicity of a *Schistosoma mansoni* vaccine with or without glucopyranosyl lipid A aqueous formulation (GLA-AF) in healthy adults from a non-endemic area. *Vaccine* **2019**, *37*, 6500-6509, doi:10.1016/j.vaccine.2019.08.075.

49. Yuan, C.; Fu, Y.-j.; Li, J.; Yue, Y.-f.; Cai, L.-l.; Xiao, W.-j.; Chen, J.-p.; Yang, L. *Schistosoma japonicum*: Efficient and rapid purification of the tetraspanin extracellular loop 2, a potential protective antigen against schistosomiasis in mammalian. *Experimental Parasitology* **2010**, *126*, 456-461, doi:10.1016/j.exppara.2010.05.018.

50. Tucker, M.S.; Karunaratne, L.B.; Lewis, F.A.; Frietas, T.C.; Liang, Y.-S. Schistosomiasis. In *Current Protocols in Immunology*, Coico, R., Ed. John Wiley and Sons, Inc.: 2013; 10.1002/0471142735.im1901s103pp. 19.11.11-19.11.57.

51. Stroehlein, A.J.; Korhonen, P.K.; Chong, T.M.; Lim, Y.L.; Chan, K.G.; Webster, B.; Rollinson, D.; Brindley, P.J.; Gasser, R.B.; Young, N.D. High-quality *Schistosoma haematobium* genome achieved by single-molecule and long-range sequencing. *Gigascience* **2019**, *8*, doi:10.1093/gigascience/giz108.

792 52. Young, N.D.; Jex, A.R.; Li, B.; Liu, S.; Yang, L.; Xiong, Z.; Li, Y.; Cantacessi,
793 C.; Hall, R.S.; Xu, X., et al. Whole-genome sequence of *Schistosoma*
794 *haematobium*. *Nature Genetics* **2012**, *44*, 221-225, doi:10.1038/ng.1065.
795 53. Craig, R.; Cortens, J.P.; Beavis, R.C. Open source system for analyzing,
796 validating, and storing protein identification data. *Journal of Proteome Research*
797 **2004**, *3*, 1234-1242, doi:10.1021/pr049882h.
798 54. Kim, S.; Pevzner, P.A. MS-GF+ makes progress towards a universal database
799 search tool for proteomics. *Nature Communications* **2014**, *5*, 5277,
800 doi:10.1038/ncomms6277.
801 55. Diament, B.J.; Noble, W.S. Faster SEQUEST searching for peptide identification
802 from tandem mass spectra. *Journal of Proteome Research* **2011**, *10*, 3871-3879,
803 doi:10.1021/pr101196n.
804 56. Vaudel, M.; Barsnes, H.; Berven, F.S.; Sickmann, A.; Martens, L. SearchGUI: An
805 open-source graphical user interface for simultaneous OMSSA and X!Tandem
806 searches. *Proteomics* **2011**, *11*, 996-999, doi:10.1002/pmic.201000595.
807 57. Finn, R.D.; Clements, J.; Eddy, S.R. HMMER web server: interactive sequence
808 similarity searching. *Nucleic Acids Research* **2011**, *39*, W29-37,
809 doi:10.1093/nar/gkr367.
810 58. Conesa, A.; Gotz, S.; Garcia-Gomez, J.M.; Terol, J.; Talon, M.; Robles, M.
811 Blast2GO: a universal tool for annotation, visualization and analysis in functional
812 genomics research. *Bioinformatics* **2005**, *21*, 3674-3676,
813 doi:10.1093/bioinformatics/bti610.
814 59. Supek, F.; Bosnjak, M.; Skunca, N.; Smuc, T. REVIGO summarizes and
815 visualizes long lists of gene ontology terms. *PLoS One* **2011**, *6*, e21800,
816 doi:10.1371/journal.pone.0021800.

817 60. Sonnhammer, E.L.; von Heijne, G.; Krogh, A. A hidden Markov model for
818 predicting transmembrane helices in protein sequences. *Proceedings of the*
819 *International Conference on Intelligent Systems in Molecular Biology* **1998**, *6*,
820 175-182.
821 61. Petersen, T.N.; Brunak, S.; von Heijne, G.; Nielsen, H. SignalP 4.0:
822 discriminating signal peptides from transmembrane regions. *Nature Methods*
823 **2011**, *8*, 785-786, doi:10.1038/nmeth.1701.
824 62. Tedla, B.A.; Pickering, D.; Becker, L.; Loukas, A.; Pearson, M.S. Vaccination
825 with *Schistosoma mansoni* Cholinesterases Reduces the Parasite Burden and Egg
826 Viability in a Mouse Model of Schistosomiasis. *Vaccines (Basel)* **2020**, *8*,
827 doi:10.3390/vaccines8020162.
828 63. WHO. *Fact sheet on neglected tropical diseases. Available at*
829 *https://www.who.int/en/news-room/fact-sheets/detail/schistosomiasis. Accessed*
830 *on 20 Jan 2019*.
831 64. Bergquist, R.; Utzinger, J.; Keiser, J. Controlling schistosomiasis with
832 praziquantel: How much longer without a viable alternative? *Infect Disease and*
833 *Poverty* **2017**, *6*, 74, doi:10.1186/s40249-017-0286-2.
834 65. Cioli, D. Praziquantel: is there real resistance and are there alternatives? *Current*
835 *Opinion in Infectious Diseases* **2000**, *13*, 659-663, doi:10.1097/00001432-
836 200012000-00014.
837 66. Tucher, C.; Bode, K.; Schiller, P.; Classen, L.; Birr, C.; Souto-Carneiro, M.M.;
838 Blank, N.; Lorenz, H.M.; Schiller, M. Extracellular Vesicle Subtypes Released
839 From Activated or Apoptotic T-Lymphocytes Carry a Specific and Stimulus-
840 Dependent Protein Cargo. *Frontiers in Immunology* **2018**, *9*, 534,
841 doi:10.3389/fimmu.2018.00534.

842 67. Bhattacharyya, S.; Yu, H.; Mim, C.; Matouschek, A. Regulated protein turnover:
843 snapshots of the proteasome in action. *Nature Reviews Molecular Cell Biology*
844 **2014**, *15*, 122-133, doi:10.1038/nrm3741.
845 68. de Paula, R.G.; de Magalhaes Ornelas, A.M.; Morais, E.R.; de Souza Gomes, M.;
846 de Paula Aguiar, D.; Magalhaes, L.G.; Rodrigues, V. Proteasome stress responses
847 in *Schistosoma mansoni*. *Parasitology Research* **2015**, *114*, 1747-1760,
848 doi:10.1007/s00436-015-4360-z.
849 69. Guerra-Sa, R.; Castro-Borges, W.; Evangelista, E.A.; Kettelhut, I.C.; Rodrigues,
850 V. *Schistosoma mansoni*: functional proteasomes are required for development in
851 the vertebrate host. *Experimental Parasitology* **2005**, *109*, 228-236,
852 doi:10.1016/j.exppara.2005.01.002.
853 70. Nabhan, J.F.; El-Shehabi, F.; Patocka, N.; Ribeiro, P. The 26S proteasome in
854 *Schistosoma mansoni*: bioinformatics analysis, developmental expression, and
855 RNA interference (RNAi) studies. *Experimental Parasitology* **2007**, *117*, 337-
856 347, doi:10.1016/j.exppara.2007.08.002.
857 71. Arosio, P.; Elia, L.; Poli, M. Ferritin, cellular iron storage and regulation. *IUBMB*
858 *Life* **2017**, *69*, 414-422, doi:10.1002/iub.1621.
859 72. Orino, K.; Lehman, L.; Tsuji, Y.; Ayaki, H.; Torti, S.V.; Torti, F.M. Ferritin and
860 the response to oxidative stress. *Biochemical Journal* **2001**, *357*, 241-247,
861 doi:10.1042/0264-6021:3570241.
862 73. Jones, M.K.; McManus, D.P.; Sivadorai, P.; Glanfield, A.; Moertel, L.; Belli, S.I.;
863 Gobert, G.N. Tracking the fate of iron in early development of human blood
864 flukes. *International Journal of Biochemistry and Cell Biology* **2007**, *39*, 1646-
865 1658, doi:10.1016/j.biocel.2007.04.017.

74. Chen, L.Y.; Yi, X.Y.; Zeng, X.F.; Zhang, S.K.; McReynolds, L. [Mucosal immunization of recombinant Schistosoma japonicum ferritin]. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi* **2004**, *22*, 129-132.

75. Nagamune, K.; Moreno, S.N.; Chini, E.N.; Sibley, L.D. Calcium regulation and signaling in apicomplexan parasites. *Subcellular Biochemistry* **2008**, *47*, 70-81, doi:10.1007/978-0-387-78267-6_5.

76. Bucki, R.; Bachelot-Loza, C.; Zachowski, A.; Giraud, F.; Sulpice, J.C. Calcium induces phospholipid redistribution and microvesicle release in human erythrocyte membranes by independent pathways. *Biochemistry* **1998**, *37*, 15383-15391, doi:10.1021/bi9805238.

77. Sotillo, J.; Robnson, M.W.; Kimber, M.J.; Cucher, M.; Ancarola, M.E.; Nesjum, P.; Marcilla, A.; Eichenberger, R.M.; Tritten, L. The protein and microRNA cargo of extracellular vesicles from parasitic helminths - current status and research priorities. *International Journal for Parasitology* **2020**, in press.

78. Repasky, G.A.; Chenette, E.J.; Der, C.J. Renewing the conspiracy theory debate: does Raf function alone to mediate Ras oncogenesis? *Trends in Cell Biology* **2004**, *14*, 639-647, doi:10.1016/j.tcb.2004.09.014.

79. Abels, E.R.; Breakefield, X.O. Introduction to Extracellular Vesicles: Biogenesis, RNA Cargo Selection, Content, Release, and Uptake. *Cellular and Molecular Neurobiology* **2016**, *36*, 301-312, doi:10.1007/s10571-016-0366-z.

80. Schussler, P.; Grevelding, C.G.; Kunz, W. Identification of Ras, MAP kinases, and a GAP protein in *Schistosoma mansoni* by immunoblotting and their putative involvement in male-female interaction. *Parasitology* **1997**, *115 (Pt 6)*, 629-634, doi:10.1017/s003118209700173x.

890 81. Gomez-Puertas, P.; Martin-Benito, J.; Carrascosa, J.L.; Willison, K.R.; Valpuesta,
891 J.M. The substrate recognition mechanisms in chaperonins. *Journal of Molecular*
892 *Recognition* **2004**, *17*, 85-94, doi:10.1002/jmr.654.
893 82. Frydman, J.; Nimmesgern, E.; Erdjument-Bromage, H.; Wall, J.S.; Tempst, P.;
894 Hartl, F.U. Function in protein folding of TRiC, a cytosolic ring complex
895 containing TCP-1 and structurally related subunits. *EMBO Journal* **1992**, *11*,
896 4767-4778.
897 83. Andreu, Z.; Yanez-Mo, M. Tetraspanins in extracellular vesicle formation and
898 function. *Frontiers in Immunology* **2014**, *5*, 442, doi:10.3389/fimmu.2014.00442.
899 84. Chaiyadet, S.; Krueajampa, W.; Hipkaeo, W.; Plosan, Y.; Piratae, S.; Sotillo, J.;
900 Smout, M.; Sripa, B.; Brindley, P.J.; Loukas, A., et al. Suppression of mRNAs
901 encoding CD63 family tetraspanins from the carcinogenic liver fluke Opisthorchis
902 viverrini results in distinct tegument phenotypes. *Scientific Reports* **2017**, *7*,
903 14342, doi:10.1038/s41598-017-13527-5.
904 85. Piratae, S.; Tesana, S.; Jones, M.K.; Brindley, P.J.; Loukas, A.; Lovas, E.;
905 Eursitthichai, V.; Sripa, B.; Thanasuwan, S.; Laha, T. Molecular characterization
906 of a tetraspanin from the human liver fluke, *Opisthorchis viverrini*. *PLoS*
907 *Neglected Tropical Diseases* **2012**, *6*, e1939, doi:10.1371/journal.pntd.0001939.
908 86. Tran, M.H.; Freitas, T.C.; Cooper, L.; Gaze, S.; Gatton, M.L.; Jones, M.K.; Lovas,
909 E.; Pearce, E.J.; Loukas, A. Suppression of mRNAs encoding tegument
910 tetraspanins from *Schistosoma mansoni* results in impaired tegument turnover.
911 *PLoS Pathogens* **2010**, *6*, e1000840, doi:10.1371/journal.ppat.1000840.
912 87. Zhang, W.; Li, J.; Duke, M.; Jones, M.K.; Kuang, L.; Zhang, J.; Blair, D.; Li, Y.;
913 McManus, D.P. Inconsistent protective efficacy and marked polymorphism limits

914 the value of *Schistosoma japonicum* tetraspanin-2 as a vaccine target. *PloS*
915 *Neglected Tropical Diseases* **2011**, *5*, e1166, doi:10.1371/journal.pntd.0001166.
916 88. Kifle, D.W.; Chaiyadet, S.; Waardenberg, A.; Wise, I.; Cooper, M.; Becker, L.;
917 Doolan, D.; Laha, T.; Javier, S.; Pearson, M.S., et al. Uptake of *Schistosoma*
918 *mansoni* extracellular vesicles by human endothelial and monocytic cell lines and
919 impact on vascular endothelial cell gene expression. *International Journal for*
920 *Parasitology* **2020**, in press.
921 89. Burke, M.L.; Jones, M.K.; Gobert, G.N.; Li, Y.S.; Ellis, M.K.; McManus, D.P.
922 Immunopathogenesis of human schistosomiasis. *Parasite Immunology* **2009**, *31*,
923 163-176, doi:10.1111/j.1365-3024.2009.01098.x.
924 90. Beaumier, C.M.; Gillespie, P.M.; Hotez, P.J.; Bottazzi, M.E. New vaccines for
925 neglected parasitic diseases and dengue. *Translational Research* **2013**, *162*, 144-
926 155, doi:10.1016/j.trsl.2013.03.006.
927 91. Bushara, H.O.; Bashir, M.E.N.; Malik, K.H.E.; Mukhtar, M.M.; Trottein, F.;
928 Capron, A.; Taylor, M.G. Suppression of *Schistosoma bovis* egg production in
929 cattle by vaccination with either glutathione S-transferase or keyhole limpet
930 haemocyanin. *Parasite Immunology* **1993**, *15*, 383-390, doi:10.1111/j.1365-
931 3024.1993.tb00623.x.
932 92. Mbanefo, E.C.; Kumagai, T.; Kodama, Y.; Kurosaki, T.; Furushima-
933 Shimogawara, R.; Cherif, M.S.; Mizukami, S.; Kikuchi, M.; Huy, N.T.; Ohta, N.,
934 et al. Immunogenicity and anti-fecundity effect of nanoparticle coated glutathione
935 S-transferase (SjGST) DNA vaccine against murine *Schistosoma japonicum*
936 infection. *Parasitology International* **2015**, *64*, 24-31,
937 doi:10.1016/j.parint.2015.01.005.

938 93. Bushara, H.O.; Majid, A.A.; Saad, A.M.; Hussein, M.F.; Taylor, M.G.; Dargie,
939 J.D.; Marshall, T.F.; Nelson, G.S. Observations on cattle schistosomiasis in the
940 Sudan, a study in comparative medicine. II. Experimental demonstration of
941 naturally acquired resistance to *Schistosoma bovis*. *American Journal of Tropical*
942 *Medicine and Hygiene* **1980**, *29*, 442-451, doi:10.4269/ajtmh.1980.29.442.
943 94. Saad, A.M.; Hussein, M.F.; Dargie, J.D.; Taylor, M.G.; Nelson, G.S. *Schistosoma*
944 *bovis* in calves: the development and clinical pathology of primary infections.
945 *Research in Veterinary Science* **1980**, *28*, 105-111.
946 95. Massoud, J. Parasitological and pathological observations on *Schistosoma bovis*
947 Sonsino, 1876, in calves, sheep and goats in Iran. *Journal of Helminthology* **1973**,
948 *47*, 155-164, doi:10.1017/s0022149x00023853.
949 96. Cheever, A.W.; Powers, K.G. *Schistosoma mansoni* infection in rhesus monkeys:
950 changes in egg production and egg distribution in prolonged infections in intact
951 and splenectomized monkeys. *Annals of Tropical Medicine and Parasitology*
952 **1969**, *63*, 83-93, doi:10.1080/00034983.1969.11686603.
953 97. Webbe, G.; James, C.; Nelson, G.S.; Smithers, S.R.; Terry, R.J. Acquired
954 resistance to *Schistosoma haematobium* in the baboon (*Papio anubis*) after
955 cercarial exposure and adult worm transplantation. *Annals of Tropical Medicine*
956 *and Parasitology* **1976**, *70*, 411-424, doi:10.1080/00034983.1976.11687140.
957 98. Meningher, T.; Barsheshet, Y.; Ofir-Birin, Y.; Gold, D.; Brant, B.; Dekel, E.; Sidi,
958 Y.; Schwartz, E.; Regev-Rudzki, N.; Avni, O., et al. Schistosomal extracellular
959 vesicle-enclosed miRNAs modulate host T helper cell differentiation. *EMBO*
960 *Reports* **2020**, *21*, e47882, doi:10.15252/embr.201947882.

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963 **SUPPLEMENTARY DATA**

964 **Supplementary Table 1.** Density, protein concentration, particle concentration, purity
965 and size of the 15k pellet vesicle fraction and gradient-separated 120k vesicle pellet
966 fractions.

967

968 **Supplementary Table 2.** Proteins identified from *Schistosoma haematobium*-derived
969 15k pellet vesicles.

970

971 **Supplementary Table 3.** Proteins identified from *Schistosoma haematobium*-derived
972 120k pellet vesicles.

973

974 **Supplementary Figure 1**. Protein alignment of the large extracellular loop from each of
975 the *Schistosoma haematobium* tetraspanins used in the vaccine trials with their
976 homologue from *Schistosoma mansoni*. (A) *Sh*-TSP2; (B) MS3_09198 and (C)
977 MS3_01370.

978

979 Figure S1

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