

In silico analysis and developmental expression of ubiquitin-conjugating enzymes in *Schistosoma mansoni*

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Abstract Ubiquitin-conjugating enzymes (Ub-E2) perform the second step of ubiquitination and, consequently, are essential for regulating proteolysis and for modulating protein function, interactions and trafficking. Previously, our group demonstrated the crucial role of ubiquitination and the Ub-proteasome pathway during the *Schistosoma mansoni* life cycle. In the present investigation, we used a homology-based genome-wide bioinformatics approach to identify and molecularly characterise the Ub-E2 enzymes in *S. mansoni*. The putative functions were further investigated through molecular phylogenetic and expression profile analyses using cercariae, adult worms, eggs and mechanically transformed schistosomula (MTS) cultured in vitro for 3.5 h or 1 or 3 days. We identified, via in silico analysis, 17 Ub-E2 enzymes with conserved structural characteristics: the beta-sheet and the helix-2 form a central core bordered by helix-1 at one side and helix-3 and helix-4 at the other. The observed quantitative differences in the steady-state transcript levels between the cercariae and adult worms may contribute to the differential protein ubiquitination observed during the parasite's life cycle. This study is the first to identify and characterise the E2 ubiquitin

conjugation family in *S. mansoni* and provides fundamental information regarding their molecular phylogenetics and developmental expression during intra-mammalian stages.

Keywords *Schistosoma mansoni* · Ubiquitination · Ubiquitin-conjugating enzyme · E2 · Differential expression

Introduction

Ubiquitin (Ub) is a highly conserved 76-amino acid protein expressed in all eukaryotic cells. The multi-step process in which Ub is covalently attached to the lysine residue in a target protein is known as ubiquitination. Mono-ubiquitination, which is the conjugation of a single or multiple Ub moieties to distinct lysine residues on the substrate, is important for protein trafficking and modulating protein function. Poly-ubiquitination, which is the covalent assembly of a chain of ubiquitin molecules on one or multiple lysine residues of the substrate, regulates proteolysis of the substrate, its functions and other post-translational modifications (Kaiser and Huang 2005; Komander and Rape 2012).

The conjugation of a Ub monomer to its target is first catalysed by a ubiquitin-activating enzyme (Ub-E1). This is an ATP-dependent reaction that forms a high-energy thioester bond between a conserved cysteine residue on the Ub-E1 enzyme and a glycine in the C-terminus of Ub. Once the ubiquitin is activated, it is transferred to a ubiquitin-conjugating enzyme (Ub-E2), forming a thioester bond between a cysteine residue in the catalytic core of the enzyme and the glycine residue in the Ub C-terminus. The final step of ubiquitination involves a ubiquitin ligase (Ub-E3). These

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enzymes specifically recognise targets and facilitate the attachment of Ub (Ye and Rape 2009).

Seven lysine residues are found in Ub (K⁶, K¹¹, K²⁷, K²⁹, K³³, K⁴⁸, K⁶³), and in theory, poly-ubiquitination can occur via the creation of a bond between the C-terminal glycine of one Ub and the ϵ -amine of any Ub lysine residue. The Ub-E2 enzymes play an important role in selecting the target lysine that Ub is linked to (David et al. 2010). Ub-E2 enzymes are present in all eukaryotes and are characterised by the presence of a highly conserved ubiquitin-conjugating domain (UBC). A catalytic cysteine residue that allows the conjugation of Ub is embedded within the domain, accepting the activated molecule via a thioester bond. Polypeptide extensions to the C- or N-termini of the UBC domain are used as criteria to structurally classify Ub-E2s into four classes (I–IV). As ubiquitination normally occurs in the cytoplasm or inside a cellular compartment, some Ub-E2 enzymes are found both in the cytoplasm and in the nucleus, while others localise to a specific compartment, such as the lumen of the endoplasmic reticulum (van Wijk and Timmers 2010).

Previous investigations from our group revealed the importance of ubiquitination and the role of the Ub-proteasome pathway during *Schistosoma mansoni* development (Castro-Borges et al. 2007; Guerra-Sa et al. 2005). These studies suggested a stage-specific regulation of these processes, but little is known about the expression pattern of the Ub-E2 family in the parasite, particularly during the transition from larvae to the adult stage. In the present investigation, we attempted to reveal the structural characteristics and the phylogenetic conservation of Ub-E2 to evaluate the gene expression profile and correlate it with the ubiquitination profile in crude protein extracts from larvae and adult *S. mansoni* (Pereira et al. 2014). Therefore, we mined the *S. mansoni* databases for Ub-E2 enzymes by assigning the UBC domain. The phylogenetic relationships were determined and the expression levels were evaluated via qRT-PCR using cercariae, adult worms and mechanically transformed schistosomula (MTS) cultured in vitro for 3.5 h or 1 or 3 days.

Materials and methods

Ethics statement

All of the experiments were authorised by the Ethical Committee for Animal Care of the University of Ouro Preto, as well as being in accordance with the accepted national and international regulations for laboratory animal use and care.

Parasites

The *S. mansoni* parasite (LE strain) was maintained via routine passage through *Biomphalaria glabrata* snails and

BALB/c mice. The infected snails were induced to shed cercariae under light exposure for 2 h, and the cercariae were recovered via sedimentation on ice. The adult worm parasites and eggs were obtained by liver perfusion of the mice after being infected for 50 days. The mechanically transformed schistosomula (MTS) were prepared as described by Harrop and Wilson (Harrop and Wilson 1993). Briefly, the cercariae were recovered and washed in RPMI 1640 medium (Invitrogen, Sao Paulo, Brazil) before vortexing at maximum speed for 90 s. The samples were then immediately cultured for 3.5 h at 37 °C in a 5 % CO₂ incubator. Then, the recovered schistosomula were washed with RPMI 1640 until no tails were detected. For the subsequent incubations, the parasites were maintained in M169 medium supplemented with 10 % foetal bovine serum (FBS), penicillin (100 µg/mL), streptomycin (100 µg/mL) and 5 % Schneider's medium (Basch and DiConza 1977) at 37 °C in a 5 % CO₂ incubator for 3.5, 24 or 72 h.

Identification and computational analysis of the ubiquitin-conjugating enzymes (Ub-E2)

The sequences of the ubiquitin-conjugating enzyme (Ub-E2) genes were searched using the *S. mansoni* genome database version 5.0 from GeneDB (<http://www.genedb.org/genedb/smansoni/>) using the ubiquitin-conjugating enzyme catalytic domain (UBCc) of the orthologue LET-70 (NP_502065) from *C. elegans* (accession: NP_502065) as a query. The BLASTp algorithm, underpinned by the Pfam (v26.0) database, was used for searches of the conserved protein domains or motifs from the *S. mansoni* sequences. The selected genes showed probability values less than e^{-11} . The position of the UBC (Uq_con in Pfam) domain in each sequence was determined by alignment using the Pfam database, and the putative Ub-E2 enzymes were classified based on the existence of additional extensions to the catalytic core.

The prediction of the signal peptides was performed using SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>) with the default D-cutoff (0.45) (Petersen et al. 2011).

The 3D structural models of the *S. mansoni* Ub-E2 enzymes and their human homologues were created by homology using Swiss-Model (<http://swissmodel.expasy.org/>) (Biasini et al. 2014).

Alignment and phylogenetic trees

Multiple alignments of the *S. mansoni* putative E2 were performed using ClustalX 2.1 (available at <http://www.clustal.org/clustal2/>). The phylogenetic analyses were conducted in the Phylogeny Inference Package (PHYLIP) (available at <http://evolution.genetics.washington.edu/phylip.html>) (Tamura et al. 2011). The phylogenetic trees of the sequences were inferred using the neighbour-joining method (Saitou and

Nei 1987). The bootstrap consensus tree inferred from 2000 replicates was used to represent the evolutionary history of the analysed taxa. The trees were drawn to scale with Figtree v1.4.0 (<http://tree.bio.ed.ac.uk/software/>), with the branch length units corresponding to the evolutionary distances used to infer the phylogenetic tree. All of the positions containing gaps and missing data were eliminated from the dataset.

Expression analysis of the 17 identified Ub-E2 enzymes

Total RNA samples from the cercariae, schistosomula, adult worms and eggs were obtained using a combination of TRIzol reagent (Sigma, Belo Horizonte, MG, Brazil) and chloroform for extraction. The samples were purified on a column using the SV Total RNA Isolation System (Promega, Belo Horizonte, MG, Brazil). The preparation was treated with RNase-free DNase I in two rounds. Quantification of the RNA was performed using a spectrophotometer, and 1 µg of total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Sao Paulo, Brazil), according to the manufacturer's instructions. Primers were designed for the less conserved regions of the sequences using the programme GeneRunner®. The sequence accession numbers and their primer pairs can be found in the online resource (Online Resource Table 1). The reverse-transcribed cDNA samples were used as templates for the PCR amplification using SYBR Green Master Mix UDG-ROX® (Invitrogen) and a 7300 Real-Time PCR System (Applied Biosystems). *S. mansoni* EIF4E was used as an

endogenous control (GeneDB ID: Smp_001500) (forward 5'-TGTTCCAACACGGTCTCG-3', reverse 5'-TCGCCTTCCAATGCTTAGG-3'). The efficiency of each pair of primers was evaluated according to the protocol developed by the Applied Biosystems application (the cDNA dilutions used were 1:4, 1:16, 1:64, 1:256 and 1:1024). For all of the investigated transcripts, three biological and technical replicates were performed, and their gene expression levels were normalised to the EIF4E transcript according to the $2^{-\Delta C_t}$ method (Livak and Schmittgen 2001).

Statistical analysis

The statistical analyses were performed using the GraphPad Prism version 5.0 software package (Irvine, CA, USA). The data were normalised using one-way analysis of variance (ANOVA). Tukey's post-test was used to investigate the significant differentially expressed transcripts throughout the investigated stages. The differences were considered to be significant when the *p* values were <0.05.

Results

The *S. mansoni* Ub-E2 family is composed of 17 members organised into genomic clusters

All of the putative members of the *S. mansoni* Ub-E2 family were retrieved by mining the parasite databases. We identified

Table 1 The molecular characteristics of the *S. mansoni* Ub-E2s

GeneDB ID	Annotation	Protein length (aa)	Gene length (bp)	UBC domain position	Active site cysteine	Class
Smp_004350	UBE2L	156	4930	5–146	85	I
Smp_028100	UBE2G	168	1114	10–160	90	I
Smp_041390	UBE2G	168	12,062	8–159	89	I
Smp_051010	UBE2C	151	4614	9–138	89	I
Smp_055920	UBE2E1	299	4869	55–192	135	IV
Smp_055960	UBE2R	289	1971	14–170	94	III
Smp_059750	UBE2J	223	5568	16–124	94	III
Smp_067980	ND	149	1035	5–142	85	I
Smp_067990	ND	149	1350	5–142	85	I
Smp_068000	ND	149	1094	5–142	85	I
Smp_082050	UBE2K	225	10,246	34–175	118	III
Smp_082820	UEV	374	1400	200–338	n/a	IV
Smp_083030	UBE2Z	282	6886	41–157	126	IV
Smp_083400	UBE2N	151	4329	7–144	87	I
Smp_118220	UBE2A	156	471	8–145	88	I
Smp_169440	UBE2A	245	3780	108–236	179	II
Smp_174670	UBE2J	573	13,332	14–122	91	III
Smp_180170	UBE2S	206	1869	11–149	92	III

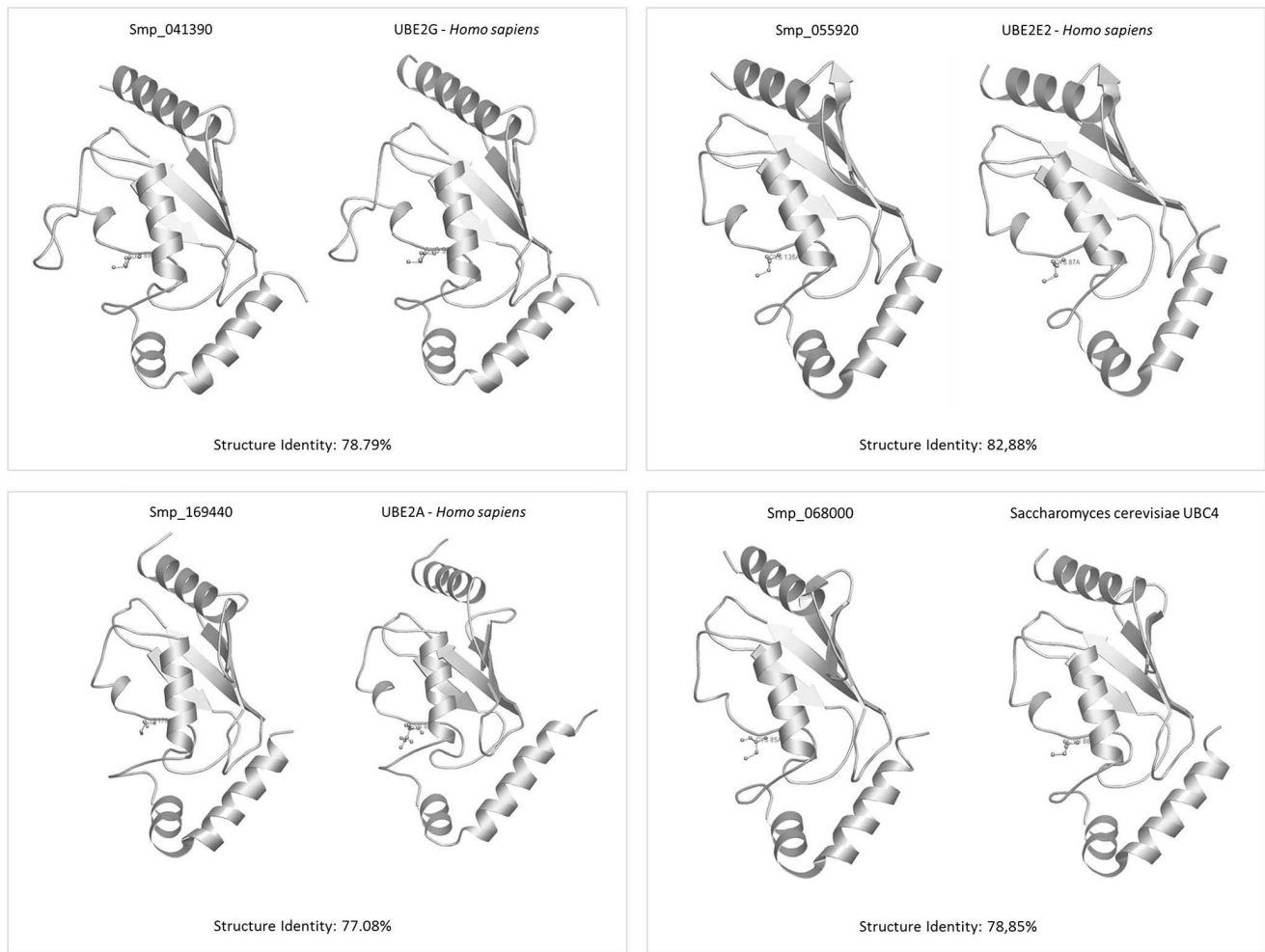


Fig 2 The homology-based tertiary structures of the *S. mansoni* Ub-E2 enzymes and their homologues. The active UBC cysteine is shown in the structure

MTS-24 h, MTS-48 h, MTS-72 h, adult worms and eggs, performing three technical and biological replicates. A total of nine of the 17 genes showed at least a twofold change in expression and were considered to be biologically relevant: Smp_041390, Smp_051010, Smp_055960, Smp_082050, Smp_082820, Smp_083030, Smp_083400, Smp_174670, Smp_180170 (Fig. 4). We could not design primers to perform qRT-PCR on Smp_004350 or to individually analyse the expression of three genes, Smp_067980, Smp_067990 and Smp_068000, because of the high similarity between the sequences. The gene sequences alignment can be found in the online resource (Online Resource Fig. SV).

None of the Ub-E2s are upregulated in the cercariae. Smp_118220 and Smp_169440 showed similar expression levels in all of the analysed stages ($p < 0.05$). Six genes were upregulated in MTS-24 h: Smp_028100, Smp_041390 and Smp_174670, which were at least 2.5-folds higher compared to the cercariae, MTS-3.5 h and the eggs; Smp_051010 and Smp_059750, which were at least 2.1-folds higher compared to the other larval stages; and Smp_180170, which was at least

2.7-fold higher compared to MTS-3.5 h ($p < 0.05$). The combined expression of Smp_067980, Smp_067990 and Smp_068000 showed levels that were at least threefolds higher in MTS-24 h compared to the other larval stages ($p < 0.05$). Five genes showed higher expression levels in the adult worms: Smp_083030, Smp_083400, which were at least 2.3-folds higher compared to the cercariae, MTS-3.5 h and MTS-24 h; Smp_082050 and Smp_174670, which were at least 3.3-folds higher compared to the cercariae and MTS-3.5 h; and Smp_055960, which was 7-folds greater than MTS-72 h ($p < 0.05$). Smp_055920 was upregulated at least 3-folds in the eggs and Smp_055960 from MTS-3.5 h was at least twofolds greater than the cercariae and MTS-72 h.

Upon examining the expression levels of all of the Ub-E2 genes, we observed that the enzymes were more abundant in the schistosomula and adult worms when compared to the cercariae and eggs. Overall, the expression of Ub-E2 in the parasite evaluated in this study increased following the transformation from cercariae to schistosomula at the 24-h stage and decreased progressively until the egg stage.

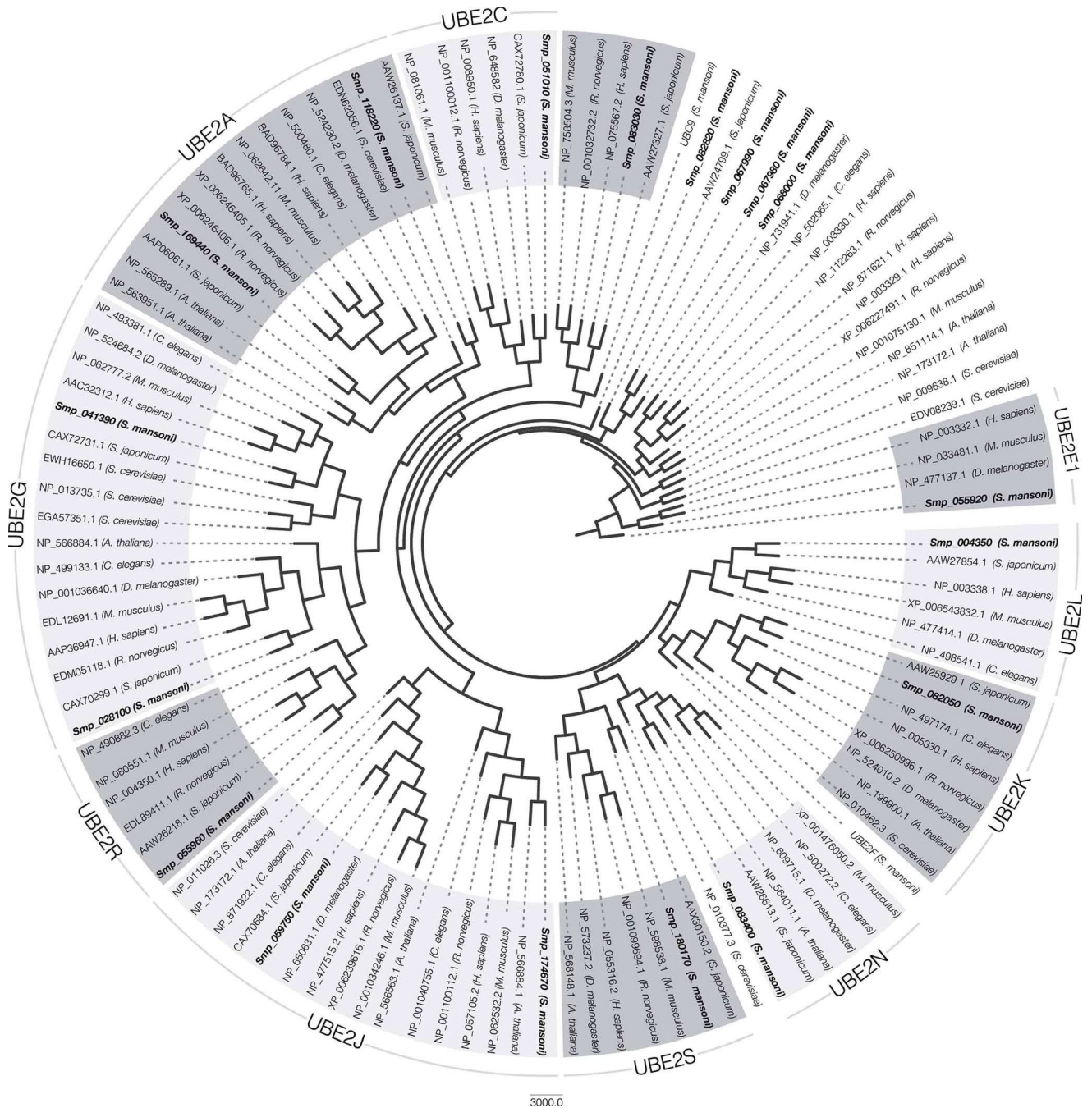


Fig 3 The phylogenetic tree of the *S. mansoni* E2s. Multiple alignments were performed using Mega 5.0 with a bootstrap analysis. The tree was drawn to scale, with the branch lengths representing the evolutionary distances used to infer the phylogenetic tree

Discussion

The Ub-E2 enzymes play a central role in the ubiquitination pathway, coupling the activation of Ub to its conjugation. We identified 17 genes that encode for Ub-E2 in the *S. mansoni* genome, and all of the sequences contain the active cysteine residue, and the region surrounding the UBC active site is demarcated by two invariant proline (P) residues and a tryptophan (W) residue. Phylogenetic analysis confirms the

conservation of the parasite Ub-E2s when compared to their orthologues, suggesting duplication events. Six Ub-E2 genes are clustered into chromosome 1, although no Ub-E2 gene organisation pattern has been observed in other eukaryotes (van Wijk and Timmers 2010). The number of Ub-E2 genes found in *S. mansoni* is in agreement with other eukaryote genomes: lower eukaryotes have fewer E2 enzymes, mostly resulting from gene duplication (van Wijk and Timmers 2010). We identified, using the presence of the UBC domain

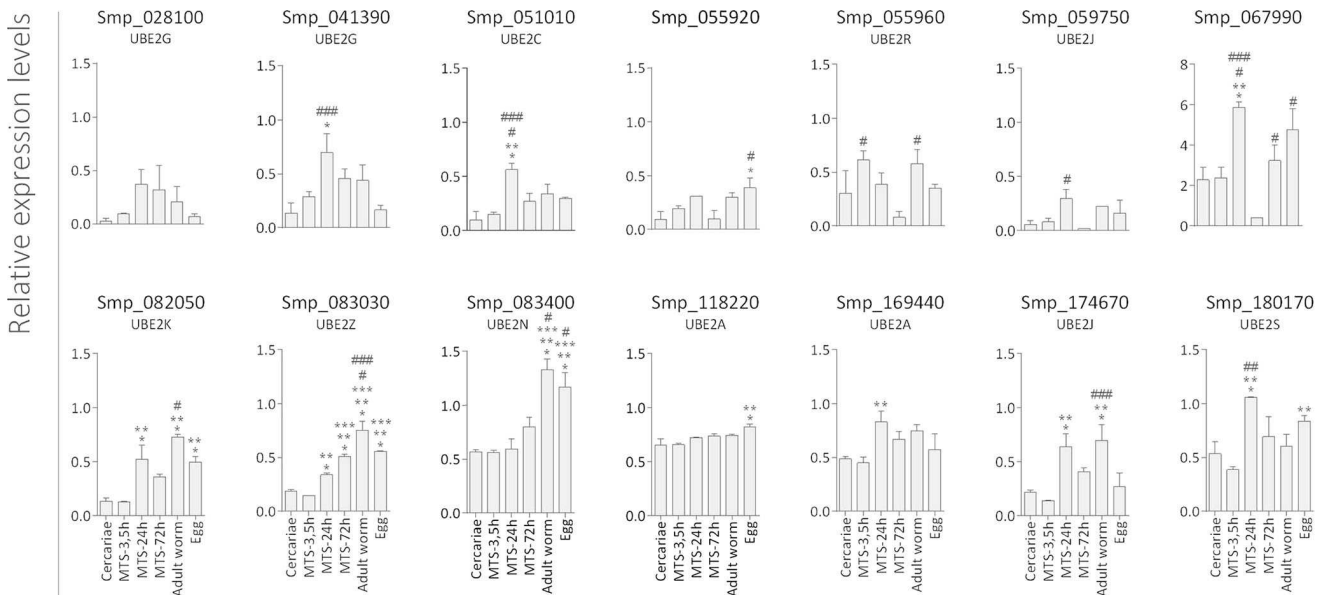


Fig 4 The differential expression patterns of the E2 genes during the various developmental stages of *S. mansoni*. The mRNA expression levels were measured, using three replicates, using qRT-PCR in the following stages: cercariae, MTS-3.5 h, MTS-24 h, MTS-72 h, adult worms and eggs. The expression levels of Smp_068000, Smp_067980

and Smp_067990 have been combined, labelled as Smp_067990. *Different from the cercariae, **different from MTS-3.5 h, ***different from MTS-24 h, #different from MTS-72 h, ##different from the adult worm, ###different from the egg

and the active cysteine residue within the domain as criteria, two Ubl-E2 enzymes, related to small ubiquitin-like modifier (SUMO) and neural-precursor-cell-expressed developmentally down-regulated 8 (NEDD8), confirming the previous data from our group (Pereira et al. 2011, 2013). In addition, one gene, Smp_055920, is an orthologue of human and murine UBE2E1, an ISG15-conjugating enzyme. The gene for ISG (a Ubl) is not found in the *S. mansoni* genome, but UBE2E1 conjugates both ISG15 and ubiquitin (van Wijk and Timmers 2010), which can explain, at least in part, its presence and transcription in the parasite.

14 putative Ub-E2 proteins contain the histidine–proline–asparagine (HPN) motif approximately 8 or 9 residues away from the active cysteine, a general signature of the E2 superfamily (Cottee et al. 2006). The histidine residue within the HPN motif is known to interact with tyrosine residues, which is important for proper folding of the active-site region (Haas and Siepmann 1997). Smp_083030 has a variation in the HPN tripeptide, becoming NPN, and Smp_055920 contains the variation HCN. These non-canonical motifs are shared by the Ub-E2 proteins of *H. sapiens* and *C. elegans*. Smp_059750 and Smp_174670 contain the sequence TPNGR, another variation that also occurs in *Drosophila*, *C. elegans* and humans. The effect of this variation in the structure is unknown (Jones et al. 2002). Smp_028100, Smp_041390 and Smp_055960 have a 13 amino acid insertion between the catalytic cysteine and the conserved tryptophan. The Ub-E2 homologues of *H. sapiens*, *C. elegans*, *M. musculus*, and *A. thaliana* for these proteins also present a similar modification in the protein sequence.

These insertions can be seen in the predicted Ub-E2 structures as a larger polypeptide chain within loop-7, between alpha-helix h-3₁₀ and H2, which is accommodated on the surface of the protein. Smp_083030 has a 5 amino acid insertion in the N-terminal side of the NPN motif, also shared by its orthologues in the above mentioned organisms. Thirteen of the 17 Ub-E2s showed a greater than 60 % structural identity with their orthologues in PDB. The catalytic cysteine residue is found in the loop connecting the beta-sheet and helix-2, and 14 structures showed a conserved structure where the beta-sheet and helix-2 form a central core bordered by helix-1 on one side and helix-3 and 4 on the other (Burroughs et al. 2008).

The level of ubiquitinated (Ub-) conjugates within the cell is controlled by a balance in the ubiquitination, deubiquitylating and proteolysis systems. Our group has shown the accumulation of Ub-conjugates in cercariae, compared to intra-mammalian stages (Pereira et al. 2014) and the proteasome inhibition in the same stage (Guerra-Sa et al. 2005). None of the Ub-E2 genes are upregulated in cercariae, suggesting that the higher levels of Ub-conjugates are not related to the upregulation of the ubiquitination pathway enzymes and reinforcing the idea that this accumulation is related to a decrease in 26S proteasome activity. The expression levels of two Ub-E2 genes, Smp_118220 and Smp_169440, showed no significant variation among the analysed stages, suggesting that these enzymes are important throughout the parasite's life cycle, including within the mammalian host. According to the phylogenetic analysis, both genes code for

a UBE2A enzyme that has a central role in the maintenance of the healthy population in the cell's mitochondria by reducing the oxidative stress response (Haddad et al. 2013). Smp_174670 and Smp_059750 are both UBE2J enzymes, which are upregulated in MTS-24 h. This family of enzymes is anchored to the endoplasmic reticulum (ER) by a short hydrophobic C-terminal transmembrane segment, with the active site facing the cytosol (Liu and Ye 2011). Uwe et al. suggested that these Ub-E2s play an important role in the quality control of ER-associated degradation (ERAD) by the Ub-proteasome system, and that this is a conserved characteristic from yeast to mammals (Lenk et al. 2002). Future experiments will be conducted to analyse the conservation of ERAD components in *S. mansoni* as well as the role of Smp_174670 and Smp_059750 in the ubiquitin proteasome system. UBE2C and UBE2S, which are both upregulated in MTS-24 h, are essential for the regulation of the APC-mediated cell cycle. UBE2C was shown to be the cognate E2 for the anaphase-promoting complex (APC) function, enhancing the regulation of the APC and the substrate selection through a conserved N-terminal domain (Summers et al. 2008). UBE2S is important for making K-11-linked Ub on the APC substrates (Garnett et al. 2009; Wu et al. 2010). Overexpression of UBE2C is associated with chromosome instability and missegregation (van Ree et al. 2010). We did not find high levels of expression of the UBE2C gene, Smp_051010, in the analysed stages. Many of the Ub-E2 families found in the parasite are being functionally characterised by different research groups in various cell types, but the structural and functional diversity of these enzymes make further *S. mansoni* in vitro investigations necessary.

In conclusion, the repertoire of ubiquitin-conjugating enzymes in *S. mansoni* contains 17 members. The high conservation at the structural level of the predicted proteins with human and mouse orthologues suggests that the mechanism of action, including the interactions with the E1 and E3 enzymes, are likely preserved in *S. mansoni*. The differential gene expression patterns that were observed when we compared the larval and adult stages of the parasite reinforce the complexity of ubiquitination and reflect stage-specific profiles for the protein conjugates. Therefore, this hypothesis will be investigated next.

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Conflict of interest The authors declare that they have no competing interests.

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