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NEDD8 conjugation in *Schistosoma mansoni*: Genome analysis and expression profiles

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ABSTRACT

NEDD8 is an ubiquitin-like molecule that covalently binds to target proteins through an enzymatic cascade analogous to ubiquitylation. This modifier is known to bind to p53 and p73, as well as all Cullin family proteins, which are essential components of Skp1/Cul-1/F-box protein (SCF)-like Ub ligase complexes. Here, we focused on a genomic analysis of the genes involved in the NEDD8 conjugation pathway in *Schistosoma mansoni*. The results revealed seven genes related to NEDD8 conjugation that are conserved in *Schistosoma japonicum, Caenorhabditis elegans, Drosophila melanogaster* and *Homo sapiens*. We performed quantitative RT-PCR (qRT-PCR), which showed differential profiles for *Smnedd8*, *Smapp1*, *Smuba3*, *Smube2f*, *Smdcn1*, *Smrbx* and *Smsenp8* throughout the life cycle of *S. mansoni*. Upregulation was observed in 3-day-old schistosomula and adult worms for all analysed genes. We also analysed the transcription levels of Cullin family members *Smp63* and *Smp73*, and observed upregulation in early schistosomula, while cercariae and adult worms showed expression levels similar to one another. Taken together, these results suggest that the NEDDylation/DeNEDDylation pathway controls important cellular regulators during worm development from cercariae to schistosomula and, finally, to adult.

1. Introduction

Posttranslational modifications (PTMs), such as ubiquitylation and ubiquitin-like protein (Ubl) modifications, play critical roles in a variety of cellular processes. The ubiquitin–proteasome pathway is one of the best-characterised systems involved in protein regulation in eukaryotes [1,2]. A number of small Ubls such as SUMO (Small ubiquitin modifier), NEDD8 (neural precursor cell-expressed developmentally downregulated-8) and ISG15 (interferon-induced ubiquitin-like modifier) have been found covalently attached to their targets in a manner similar to the ubiquitylation process. Among the Ubls, the NEDD8 protein has been found to be evolutionarily conserved in eukaryotes and has the highest identity with the ubiquitin protein (59%) [3,4]. Studies have revealed that NEDD8 is essential for cell viability in fission yeast and is required for development processes in both mouse and *Drosophila* [5–7].

The NEDDylation pathway, analogous to the ubiquitin pathway, involves a set of enzymes working together to conjugate the NEDD8 protein to specific target proteins [8–10]. In the first step, the NEDD8-specific E1 activating enzyme (APP-BP1/Uba3 heterodimer) activates the NEDD8 protein by adenylating the carboxyl terminal glycine residue. This is followed by the formation of a high-energy thioester bond with the catalytic cysteine residue of UBA3. In the next step, NEDD8 is transferred from the E1 enzyme to a catalytic cysteine residue of the NEDD8-specific E2 conjugation enzymes UBC12 (also known as UBE2M) or UBE2F, again using a thioester linkage. Finally, the activated NEDD8 on the E2 enzyme is subsequently transferred and conjugated to the E3 ligase substrates. The RING-finger protein ROC1, a subunit of the Cullin Ring Ligases (CRLs), interacts with UBC12, acting as the NEDD8 E3 ligase for Cullins. The most significant and best-studied substrates of NEDD8 are the Cullin proteins, which serve as scaffolds in E3 CRLs [11,12].

NEDD8 contains seven conserved lysine residues, similar to the ubiquitin protein. The NEDD8 residues K11, K22, K48 and K60 can allow NEDD8 to form chains *in vivo*, whereas the NEDD8 residues K22 and K48 can be NEDDylated *in vitro*, suggesting that chain formation of ubiquitin and ubiquitin-like proteins might be a general phenomenon for these modifications [13]. In addition, the NEDD8 modification is a dynamic and reversible process that is controlled by NEDD8-specific proteases that remove NEDD8 protein from distinct targets. A specific activity of the NEDD8-specific protease has been reported to be associated with a metalloprotease motif in JAB1/CSN5, a subunit of the COP9 signalosome. The whole COP9 signalosome complex is required for CSN5 activity, as evidenced by the fact that the isolated protein did not display NEDD8 protease

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activity [14,15]. NEDD8, similar to the UBL proteins, is synthesised as an inactive precursor and has to be processed by a NEDD8-specific protease (SENP8). This processing exposes the diglycine motif at the carboxyl-terminus, which is required for the conjugation process. SENP8 is a highly conserved and specific protease for NEDD8. Neither ubiquitin nor SUMO are utilised as SENP8 substrates, although both share COOH-terminal extensions [16].

Although the NEDDylation process shares similarities to the ubiquitylation process, the biological impact of this modification appears to be different. Ubiquitylation has been shown to be involved in trafficking, signalling, endocytosis and proteasome-dependent proteolysis, whereas NEDDylation has been implicated in modulation of the biochemical activity of target proteins to affect their stabilities [17]. Recently, a set of NEDD8 target proteins have been identified, including pVHL, p53, BCA3, the EGF receptor and ribosomal proteins [18].

Previous investigations revealed the importance of ubiquitylation during *S. mansoni* development [19,20] and evidenced the complexity of the proteasome-dependent intracellular protein degradation in adult worms [21]. Subsequently, our group reported the existence of two SUMO paralogs and UBC9 in *S. mansoni*, confirming the importance of these posttranslational protein modifications in this parasite [22,23]. In this work we aimed to investigate the importance of post-translational modifications in this parasite, focusing on the NEDDylation system. We analysed the conservation of the proteins predicted to be related to this pathway using *S. mansoni* databases. We then evaluated the gene expression of the components of the NEDD8 machinery, as well as the putative NEDD8 targets (Cullins, p63 and p73), using qRT-PCR at the cercariae, adult worm and mechanically transformed schistosomula (MTS) stages of development. The current study provides for the first time information about the NEDDylation machinery in the *S. mansoni* parasite. Investigations involving posttranslational regulation may help to better understand the biology of the parasite, as well as its relationship with the host.

2. Materials and methods

2.1. Ethics statement

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

2.2. Parasites

The LE strain was maintained by 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 by sedimentation on ice. Adult worm parasites were obtained by liver perfusion of mice after 50 days of infection. Mechanically transformed schistosomula (MTS) were prepared as described by Harrop and Wilson [24]. Briefly, cercariae were recovered and washed in RPMI 1640 medium (Invitrogen) before vortexing at maximum speed for 90 s and then were immediately cultured for 3.5h at 37 °C, 5% CO_2. The recovered schistosomula were then washed with RPMI 1640 until no tails were detected. For subsequent incubations, the parasites were maintained in M169 medium supplemented with 10% FBS, 100 μg/mL penicillin, 100 μg/mL streptomycin and 5% Schneider's medium [25] at 37 °C on 5% CO_2 for 3.5 h, 1, 2, 3, 5 and 7 days.

2.3. Identification and computational analysis of the NEDDylation pathway and possible targets

The sequences for *Sm*NEDD8, *Sm*APP1-UBA3, *Sm*UBE2F, *Sm*DCN1, *Sm*RBX, *Sm*SENP8 and possible targets (*Sm*Cullins, *Sm*p63 and *Sm*p73) were retrieved through BLAST searches from the *S. mansoni* genome database version 5.0, available at http://www.genedb.org/genedb/smansoni/. Amino acid sequences from the orthologs in *Drosophila melanogaster*, *Caenorhabditis elegans* and *Homo sapiens* were used as queries. The BLASTp algorithm, underpinned by Pfam (v26.0), allowed detection of conserved protein domains or motifs from *S. mansoni* sequences. Multiple alignments of *Sm*UBE2F and *Sm*Cullins were performed by ClustalX 2.0 [26]. Phylogenetic analysis was conducted by MEGA 5 [27]. A phylogenetic tree of these sequences was inferred using the Neighbor-Joining method [28]. The bootstrap consensus tree inferred from 1000 replicates was used to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% of the bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset.

2.4. Expression analysis of NEDDylation pathway components and possible targets

Total RNA from adult worms, cercariae and schistosomula were obtained using a combination of the Trizol reagent (GIBCO-BRL) and chloroform for extraction and then were on-column purified using the "SV total RNA Isolation System" (Promega, Belo Horizonte, Brazil). The preparation was treated with 3 different rounds of RNase-free DNase I (RQ1 DNase; Promega). RNA was quantified using a spectrophotometer and an aliquot containing 1 μg of total RNA reverse transcribed using an oligodT primer from the Thermoscript RT-PCR System (Invitrogen São Paulo, Brazil), as described by the manufacturer. The efficiency of DNAse I treatment was evaluated by PCR amplification of the cDNA reaction mix without the addition of the Thermoscript enzyme. *S. mansoni*-specific primers were designed using the program GeneRunner®. The sequence accession numbers and their pair of primers are in the supplementary materials (Table S1). Reverse-transcribed cDNA samples were used as templates for PCR amplification using SYBR Green Master Mix UDG-ROX® (Invitrogen) and the 7300 Real Time PCR System (Applied Biosystems, Rio de Janeiro, Brazil). Specific primers for *S. mansoni* EIF4E were used as an endogenous control (GeneDB ID: Smp_001500) (forward 5′ TGTTCCAACCACGGTCTCG3′, reverse 5′TCGCCTTCCAATGCTTAGG3′) [29]. The efficiency of each pair of primers was evaluated according to the protocol developed by the Applied Biosystems application (cDNA dilutions were 1:10, 1:100 and 1:1000). For all investigated transcripts three biological replicates were performed. The gene expression was normalised to the *Sm*EIF4E transcript using the Applied Biosystems 7300 software according to the $2^{-\Delta Ct}$ method [30].

2.5. Statistical analysis

Statistical analysis was performed using the GraphPad Prism version 5.0 software package (Irvine, CA, USA). Normality of the data was established using one way analysis of variance (ANOVA). Tukey post tests were used to investigate significant differential expression of transcripts throughout the investigated stages. In all cases, the differences were considered significant when p values were <0.05.

3. Results

3.1. NEDDylation pathway conservation in S. mansoni

Using both a homology-based method and de novo prediction on *S. mansoni* sequences deposited in the genome database, we were able to characterise the components of the NEDDylation machinery *in silico*. This analysis identified seven genes involved in the NEDDylation

mechanism in this parasite. These genes were the following: *Sm*NEDD8 (GeneDB ID: Smp_130170), components of the E1 activation heterodimer *Sm*APP1 (GeneDB ID: Smp_087920) and *Sm*UBA3 (GeneDB ID: Smp_151760), E2 conjugating enzyme *Sm*UBE2F (GeneDB ID: Smp_027370), E3 ligases *Sm*DCN1 (GeneDB ID: Smp_105330) and *Sm*RBX (GenBank ID: DQ466078.1) and protease *Sm*SENP8 (GeneDB ID: Smp_121890). The number of genes found in *S. mansoni* that were related to the NEDDylation pathway was compared with the number of genes from *S. japonicum*, *C. elegans*, *D. melanogaster* and *H. sapiens*. Our results demonstrate that the *S. mansoni* genes are very similar to those found in the Protostome organisms *C. elegans* and *D. melanogaster*, and identical to *S. japonicum*, reinforcing that the NEDDylation pathway is conserved in these organisms.

The conserved domains of *Sm*NEDD8, *Sm*UBE2F and *Sm*SENP8 were assigned in these enzymes and compared with their orthologs in *D. melanogaster*, *C. elegans* and *H. sapiens* (Fig. 1). The predicted *Sm*NEDD8 protein has 77 amino acid residues and contains a main domain, Rad60 (PF 11976). The predicted *Sm*UBE2F protein has 186 amino acid residues and a UQ domain (PF 00179), characteristic of conjugation enzymes. Furthermore, *Sm*UBE2F contains a critical

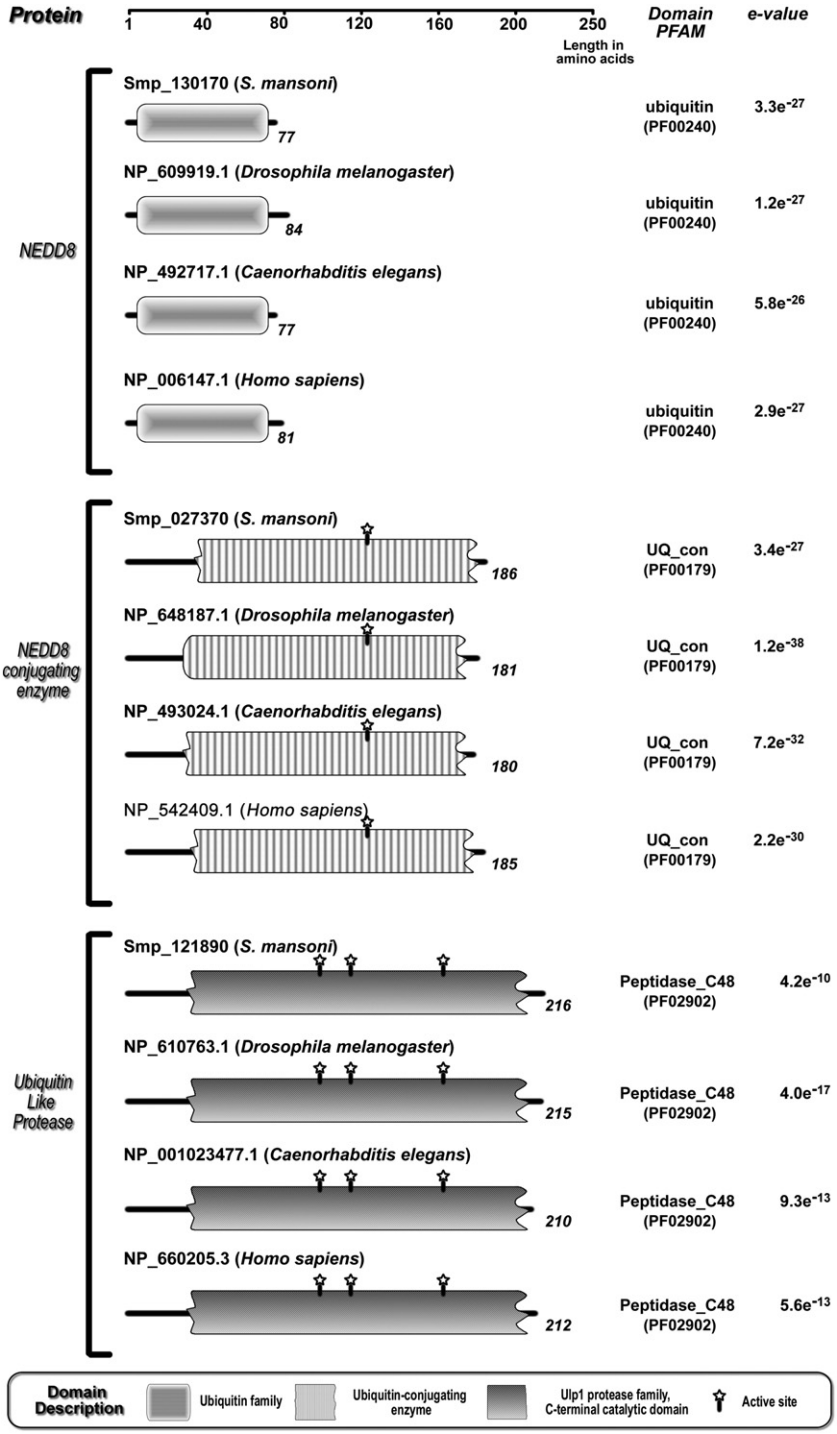

Fig. 1. Schematic diagram of conserved protein domains in *S. mansoni*. A comparative analysis of the domains in *Sm*NEDD8 (ubiquitin), *Sm*UBE2F (UQ-con) and *Sm*SENP8 (peptidase C-48) with their orthologs in *D. melanogaster*, *C. elegans* and *H. sapiens*.

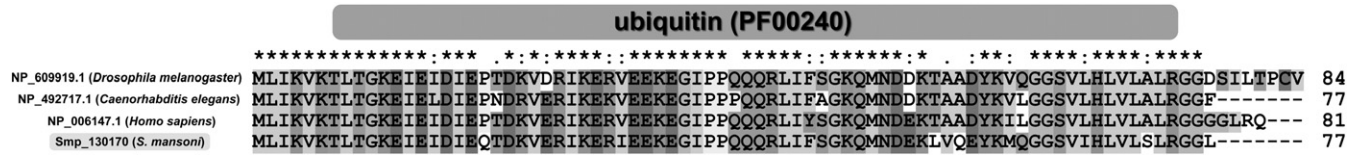

Fig. 2. Global alignment of *Sm*NEDD8 and its orthologs, using the ClustalX 2.0 program and boxshade. Identical residues are shaded in black and similar residues in grey.

Cysteine residue at position117, similar to orthologous E2 conjugation enzymes. The predicted protein *Sm*SENP8 has 216 amino acid residues and a conserved C-48 domain (PF 02902), which includes the enzyme active site residues histidine, aspartic acid and cysteine at the positions 102, 119 and 162, respectively (Fig. 1). Comparing *Sm*NEDD8 with its orthologs, we noticed that this protein was highly conserved with at least 74% identity when compared to the proteins from *D. melanogaster*, *C. elegans* and *H. sapiens* (Fig. 2). The alignment of the proteins from *S. mansoni* and *S. japonicum* displayed 100% identity, affirming the evolutionary conservation of this species (data not shown). The gene encoding the NEDD8 E2 conjugation enzyme has been found duplicated in several organisms (UBE2F and UBE2M or UBC12 isoforms). The phylogenetic analysis displayed that the NEDD8-E2 enzyme in *S. mansoni* is unique and has a higher homology with UBE2F orthologs than UBE2M orthologs (Fig. 3). The global alignment between E2-NEDD8 orthologs reinforced this conservation and the differences between the two E2 conjugation enzymes. The cysteine residue characteristic of the UQ_con domain is shown highlighted (Fig. 4).

3.2. NEDDylation machinery is differentially regulated in S. mansoni

The gene expression of the NEDDylation pathway components was analysed using the qRT-PCR technique with three biological replicates. The results were expressed using RNA levels in relation to EIF4E, a constitutively expressed gene. Regarding the expression of *Smnedd8*, we observed a 1.5-fold increase in MTS-3 d compared to the other developmental stages and similar levels when comparing cercariae to adult worm (Fig. 5A). The expression of *Smapp1* and *Smuba3* subunits that form the NEDD8-activating E1 enzyme demonstrated no relevant changes among the subunits. Both components of the E1 heterodimer were upregulated at least 2-fold in MTS-3.5h, 1d, 3 days and adult worm (Fig. 5B). The conjugation enzyme UBE2F is the only enzyme able to conjugate NEDD8 in *S. mansoni*. While verifying the expression of this transcript in the parasite stages, we found an increase of 2-fold in MTS-3.5h in relation to other schistosomula and an increase of 1.5-fold in adult worm compared to cercariae (Fig. 5C). The expression of the E3 ligases (*Smdcn1* and *Smrbx*) was very similar in all stages, except in adult worms, where RBX was upregulated 2-fold compared to other developmental stages (Fig. 5D). The NEDD8-specific protease SENP8 has been found to be capable of both processing the inactive precursor of NEDD8 and freeing NEDD8 monomers from the substrates they are bound to [18]. There was a stage-specific expression pattern of this enzyme's transcript during the cercariae-schistosomula transition and a 3-fold increase in MTS-1d and adult worm compared to other evolutionary stages (Fig. 5E). Together, the results showed a higher expression of all NEDDylation machinery components in adult worm, suggesting that this modification pathway is important during this stage of the parasite cycle.

3.3. Cullins and tumour suppressor proteins (p63 and p73) are highly expressed in MTS-3.5h

We identified five Cullins and their conserved domains in *S. mansoni*: *Sm*Cullin1 (GeneDB ID: Smp_150270), *Sm*Cullin2 (GeneDB ID:

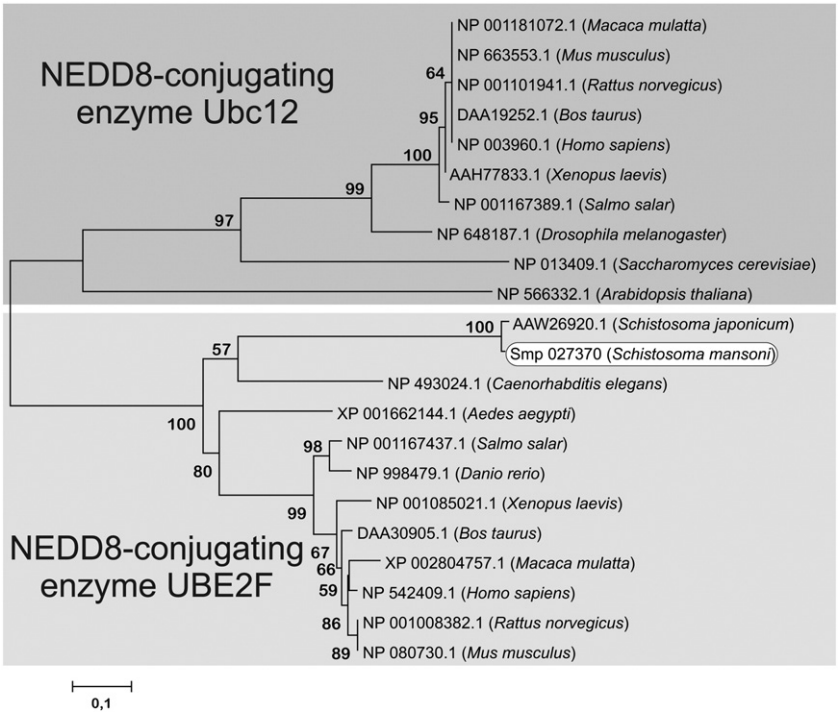

Fig. 3. Consensus phylogenetic tree based on the amino acid sequence of *Sm*UBE2F. The tree construction and bootstrap analysis were performed using ClustalX 2.0 and MEGA 5.0. To validate the consensus tree and reliability of the branches formed, a phylogenetic bootstrap test was used with 1000 replicates for each sequence, and reproduction of the branch in 50% of the runs considered the minimum for a reliable branch.

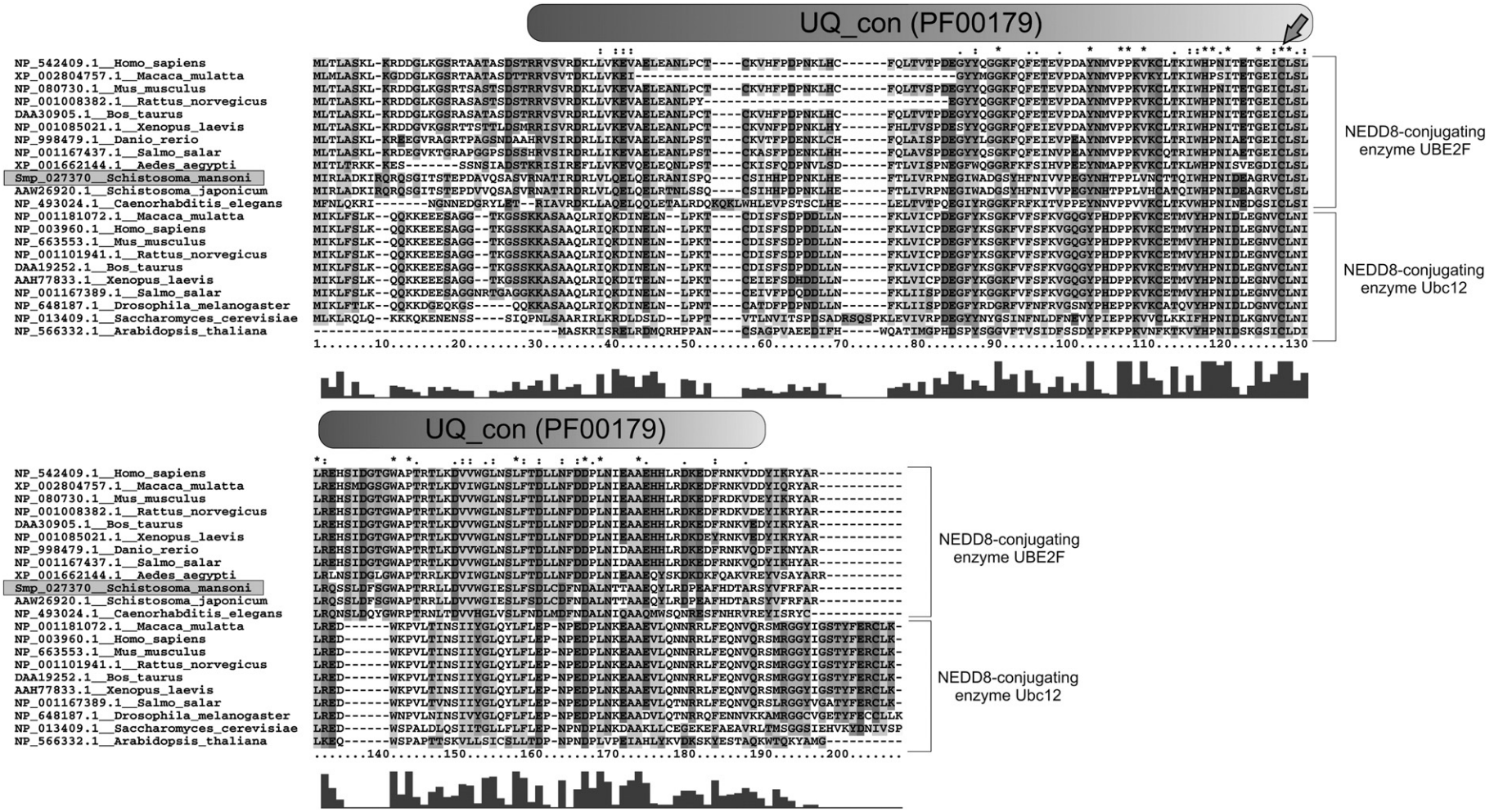

Fig. 4. CLUSTALX 2.0 multiple alignment of predicted amino acid sequences of *Sm*UBE2F, showing the putative conserved domain UQ_con (PF00179), obtained by using the Pfam algorithm.

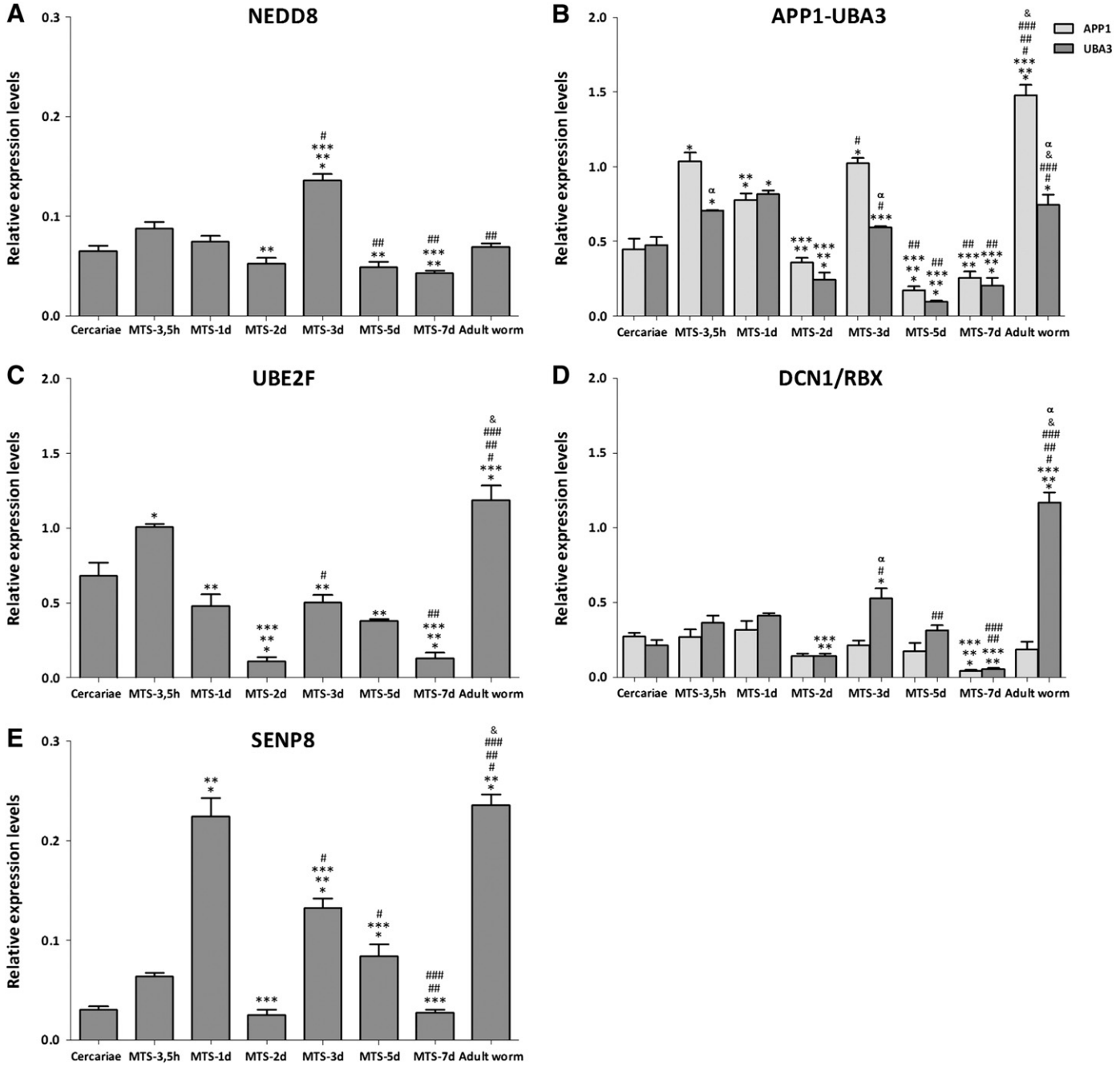

Fig. 5. NEDDylation pathway components are differentially expressed throughout the *S. mansoni* life cycle. The *Smnedd8*, *Smapp1*, *Smuba3*, *Smube2f*, *Smdcn1*, *Smrbx* and *Smsenp8* mRNA expression levels were measured using quantitative RT-PCR based on three replicates for each of the following stages: adult worms, cercariae, MTS-3.5h, 1, 2, 3, 5, and 7 days. Expression levels were calibrated according to the comparative $2^{-\Delta Ct}$ method, using the constitutively expressed *Sm*EIF4E as an endogenous control (one-way variance analysis followed by Tukey pairwise comparison $p<0.05$). * Different from cercariae, ** different from MTS-3.5h, *** different from MTS-1d, # different from MTS-2d, ## different from MTS-3d, ### different from MTS-5d and $^{\delta}$ different from MTS-7d. *t* Tests were performed to compare UBA3 to APP1 and RBX to DCN1 ($^{\alpha}$ different from APP1 or DCN1).

Smp_079740), *Sm*Cullin3 (GeneDB ID: Smp_117580), *Sm*Cullin4 (GeneDB ID: Smp_082850) and *Sm*Cullin5 (GeneDB ID: Smp_143030). The phylogenetic analysis indicated that the Cullins' sequences are conserved among their orthologs (Fig. 6). We analysed the expression levels of these Cullins in cercariae, adult worms, MTS-3.5h, 1, 2, 3, 5 and 7 days. In MTS-3.5h, the levels of these Cullins were upregulated approximately 3-fold when compared with other stages. *Smcullin 2, 3, 4* and *5* were expressed at the same levels in cercariae and adult worm stages (Fig. 7A–E). Regarding *Smcullin1*, we verified upregulation of approximately 2-fold in cercariae in relation to adult worms.

Furthermore, we analysed the two tumour suppressor proteins *Sm*p63 (GeneDB ID: Smp_139530) and *Sm*p73 (GeneDB ID: Smp_136160.2), which have been found to be putative targets of the NEDD8 conjugation pathway (Fig. 7F). The p63 protein was characterised in *S. mansoni* and its expression was observed in adult worm [31]. In this article, this protein was denominated as p53. However, a recent study revealed that invertebrates do not have p53, only the orthologs p63 and p73 [32]. Therefore, in this article we used p63 instead of p53. The p63 and p73 proteins had similar expression levels to one another at all analysed stages. Both proteins were upregulated in MTS-3.5h and MTS-5 d (2-3-fold) in relation to the other analysed stages. *Sm*p63 transcript was 3-fold higher in adult worm in relation to cercariae, whereas *Sm*p73 mRNA was found upregulated 2-fold in cercariae compared to adult worm.

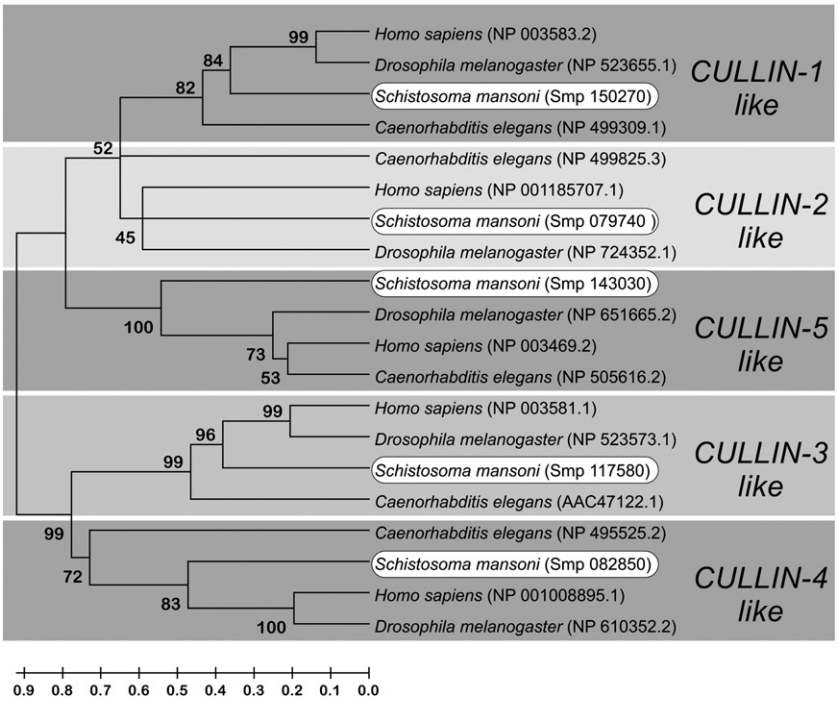

Fig. 6. Consensus phylogenetic tree based on amino acid sequences of *Sm*Cullins. Global alignment of *Sm*Cullin1, *Sm*Cullin2, *Sm*Cullin3, *Sm*Cullin4 and *Sm*Cullin5. The tree construction and bootstrap analysis were performed using ClustalX 2.0 and MEGA 5.0. To validate the consensus tree and reliability of the branches formed, a phylogenetic bootstrap test was used with 1000 replicates for each sequence, and reproduction of the branch in 50% of the runs considered the minimum for a reliable branch.

4. Discussion

Despite the advances in genome sequencing, transcriptome and proteome exploration, PTM in parasites is still poorly understood. This is a subject of fundamental importance because these pathways are involved in many cellular processes [18]. With regards to biochemical mechanisms, the NEDD8 pathway is similar to the PTM processes ubiquitylation and SUMOylation [33,34].

Our *in silico* analysis revealed that the number of predicted NEDDylation pathway components in the *S. mansoni* genome is in agreement with other invertebrate genomes, suggesting an important functional conservation among these organisms. Regarding the *S. japonicum* genome, the number of enzymes involved in the NEDD8 conjugation process is the same as is found in *S. mansoni*, reinforcing the evolutive proximity between these species [35]. During this analysis the RBX protein was not found in the parasite EST database (http://www.genedb.org/genedb/smansoni/), despite the fact that this E3 ligase has been characterised in this helminth previously [36]. Furthermore, we have observed errors in the assembled genome sequence for a subunit of the E1 heterodimer (APP1), reinforcing the importance of re-annotating the putative proteins deposited in the genome database to increase data accuracy.

The NEDDylation pathway is conserved and essential for the viability of most model organisms, including *Schizosaccharomyces pombe*, *C. elegans*, *Drosophila*, *Arabidopsis* and mouse, supporting the hypothesis that NEDDylation is involved with important cellular processes in eukaryotic cells [37,8,6,38,7,39]. Based on these data, we decided to evaluate the conserved domains in predicted NEDD8 pathway components: NEDD8 (PF 11976), UBE2F (PF 00179) and SENP8 (PF 02902) (Fig. 1). Moreover, we analysed the conservation of E2 conjugation proteins by phylogenetic analysis and multiple sequence alignment. Two enzymes, UBC12 and UBE2F, have been previously identified as E2 conjugation proteins. By observing the phylogenetic tree, we have noted that the NEDD8-E2 from *S. mansoni* is unique and has higher identity with UBE2F than with UBE2M. In addition, they might share a common ancestral protein, suggesting that these proteins have been the result of gene duplication events. Biochemical and structural analyses have indicated how plasticity of hydrophobic E1–E2 interactions and E1 conformational flexibility allow one E1 to charge multiple E2s [40]. The E2 proteins have displayed distinct functions, with UBE2M/RBX1 and UBE2F/RBX2 each targeting different Cullins. UBE2M and UBE2F pairs specifically regulate NEDDylation of Cullins 1–4 and Cullin 5, respectively, in an E2-RING-dependent manner [40]. This specific mechanism in *S. mansoni* is still unclear because the parasite has a unique E2 and E3 ligase RBX. Furthermore, we found enhanced expression of *Smube2f*, a conjugation enzyme and partner of E1, at all examined developmental stages, and all components of the NEDDylation machinery were upregulated in the adult worm.

Recently it has been demonstrated that NEDDylation of Cullin targets is correlated with cell cycle progression. All the Cullin proteins are NEDDylated and involved in formation of various SCF complexes. The modification by NEDD8 induces a conformational change that inhibits the interaction with the inhibitory protein CAND1 and activates the ubiquitin E3 ligase [18]. Accordingly, we analysed the members of the Cullin family (Cullins 1–5), the possible NEDDylation machinery targets. The conserved domains were characterised (Cullin: Pfam PF 00888 and Cullin-NEDD8: Pfam PF10557), confirming the presence of five Cullins in the *S. mansoni* genome, as well as their conservation. In addition, the expression profiles of these proteins during the transition from cercariae to adult worms were evaluated. Cullins showed similar expression profiles among analysed stages. Comparative analyses showed that *Smcullin1* was the highest-expressed among the Cullins. Cullin1 is the most important among Cullins and has been shown to be involved in most E3ubiquitin ligase complexes [41], suggesting that most of the Cullin-RING ligases in *S. mansoni* have Cullin1 as their scaffold.

In addition to the Cullins, other NEDDylation machinery targets were analysed, such as the tumour suppressor proteins p63 and p73. Some reports showed NEDDylation inhibits transcriptional activities of these proteins, which are negative regulators of the cell cycle [18,42]. Recent studies showed that NEDDylation's role in p53 activity remains unclear. The NEDD8/ubiquitin interplay controls p53 subcellular

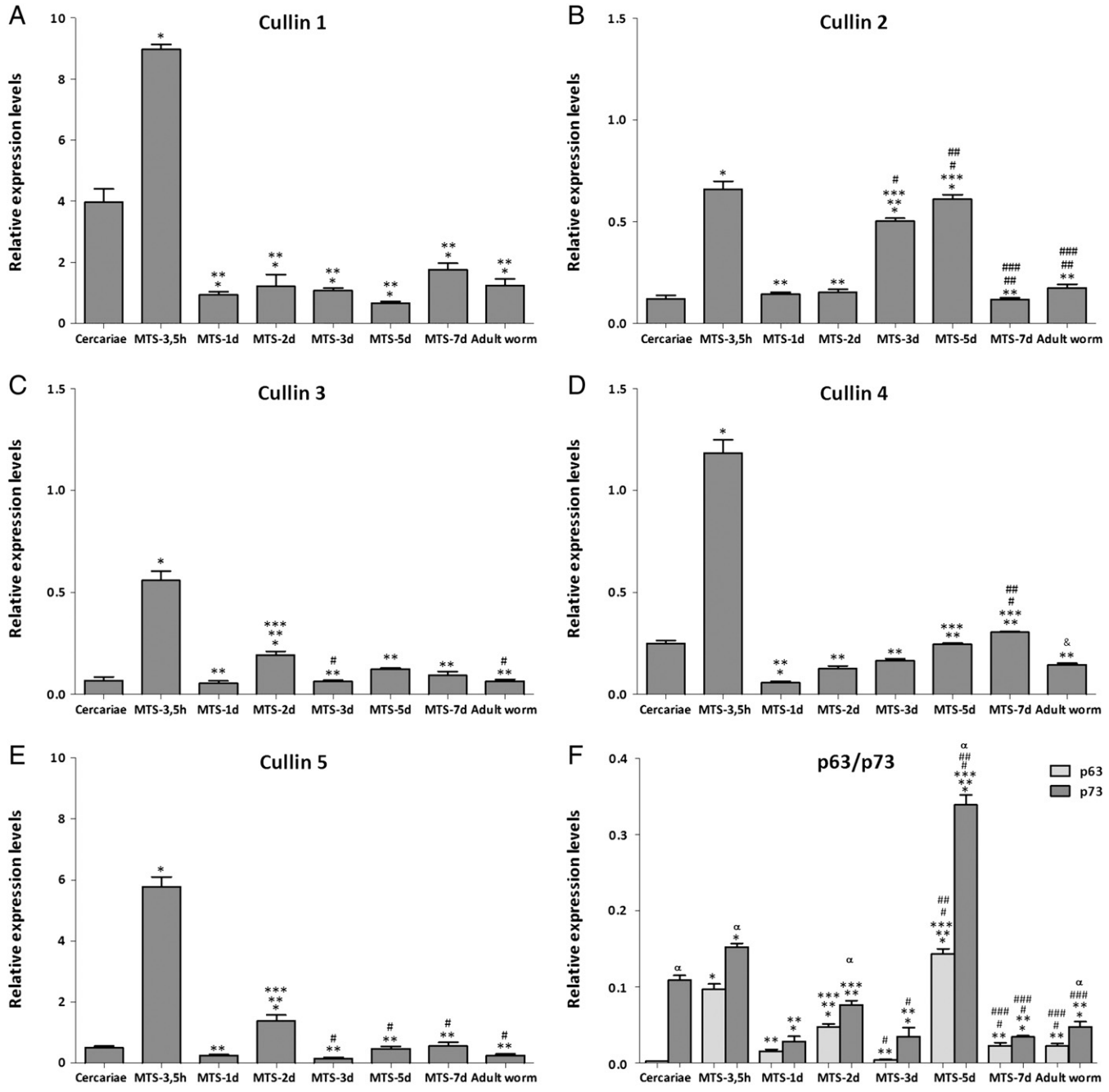

Fig. 7. Possible NEDDylation targets are differentially expressed throughout the *S. mansoni* life cycle. The *Smcullin1*, *Smcullin2*, *Smcullin3*, *Smcullin4*, *Smcullin5*, *Smp63* and *Smp73* mRNA expression levels were measured using quantitative RT-PCR based on three replicates, for each of the following stages: adult worms, cercariae, MTS-3.5h, 1, 2, 3, 5, and 7 days. Expression levels were calibrated according to the comparative $2^{-\Delta Ct}$ method, using the constitutively expressed *Sm*EIF4E as an endogenous control (one-way variance analysis followed by Tukey pairwise comparison $p<0.05$). * Different from cercariae, ** different from MTS-3.5h, *** different from MTS-1d, # different from MTS-2d, ## different from MTS-3d, ### different from MTS-5d and ◊ different from MTS-7d. *t* Tests were performed to compare p73 to p63 (α different from p63).

localisation, suggesting that NEDD8 can control protein function [43,44]. By analysing the expression profiles of these transcripts, we observed that *Smp73* is highly expressed compared to *Smp63* in all parasite stages. Moreover, there was a 2-fold increase in p73 gene expression in cercariae levels compared to adult worms.

SENP8 has both hydrolase and isopeptidase activities, so this protease might process the NEDD8 precursor and promote deconjugation of NEDDylated substrates. However, under physiological conditions SENP8 is not able to DeNEDDylate Cullins, the major substrates of NEDDylation pathway. This process is catalysed by the CSN-5 subunit of the Cop9 signalosome complex (CSN) [18]. CSN is able to DeNEDDylate monoNEDDylated Cul1 under physiological conditions, but is not as efficient in deconjugating hyper- or polyNEDDylated Cul1. In contrast SENP8 is able to process hyper- but not mono-NEDDylated Cul1 and is able to activate the NEDD8 pro-protein [16]. HyperNEDDylated Cul1 is induced in the cell under non-physiological conditions, and thus may not represent a physiological target of either SENP8 or CSN-5. However, the ability of SENP8 to deconjugate hyperNEDDylated Cul1 likely derives from the ability of SENP8 to accommodate large leaving groups at the C terminus of NEDD8. Although, nothing is known about the nature of the interactions between the Cullin component of these substrates and SENP8 or CSN-5 [45,46]. In the near future, this subunit of CSN will be investigated in this parasite.

In conclusion, we described that NEDDylation machinery and presumable substrates were differentially expressed in *S. mansoni*. Considering that the transformation from cercariae to schistosomula requires the adaptation of the parasite to different environments and subsequent large scale cellular differentiation and growth, our data reinforce that the NEDD8 pathway is involved in parasite development. Additional studies for the mechanisms of NEDD8 conjugation in *S. mansoni* will provide further insights into the biological activities of this pathway and the role of NEDD8 conjugation as a rapid response to cercariae/schistosomula/adult worm development.

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