



Developmental expression of high molecular weight tropomyosin isoforms in *Mesocostoides corti*

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ABSTRACT

Tropomyosins are a family of actin-binding proteins with diverse roles in actin filament function. One of the best characterized roles is the regulation of muscle contraction. Tropomyosin isoforms can be generated from different genes, and from alternative promoters and alternative splicing from the same gene. In this work, we have isolated sequences for tropomyosin isoforms from the cestode *Mesocostoides corti*, and searched for tropomyosin genes and isoforms in other flatworms. Two genes are conserved in the cestodes *M. corti* and *Echinococcus multilocularis*, and in the trematode *Schistosoma mansoni*. Both genes have the same structure, and each gene gives rise to at least two different isoforms, a high molecular weight (HMW) and a low molecular weight (LMW) one. Because most exons are duplicated and spliced in a mutually exclusive fashion, isoforms from one gene only share one exon and are highly divergent. The gene duplication preceded the divergence of neodermatans and the planarian *Schmidtea mediterranea*. Further duplications occurred in *Schmidtea*, coupled to the selective loss of duplicated exons, resulting in genes that only code for HMW or LMW isoforms. A polyclonal antibody raised against a HMW tropomyosin from *Echinococcus granulosus* was demonstrated to specifically recognize HMW tropomyosin isoforms of *M. corti*, and used to study their expression during segmentation. HMW tropomyosins are expressed in muscle layers, with very low or absent levels in other tissues. No expression of HMW tropomyosins is present in early or late genital primordia, and expression only begins once muscle fibers develop in the genital ducts. Therefore, HMW tropomyosins are markers for the development of muscles during the final differentiation of genital primordia.

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1. Introduction

Tropomyosins (TPMs) are a family of actin-binding proteins, consisting of homo or heterodimers forming a parallel α -helical coiled coil [1–4]. Their structure is unusual in that almost all of the molecule has a coiled coil structure; the primary structure therefore consists of an imperfect heptad repeat, in which positions one and four of the repeat are occupied by hydrophobic residues, and

positions five and seven by charged residues. The chains are held together by the close packing of the hydrophobic residues and by salt bridges between the charged residues. Small regions in which the coiled coil is destabilized by the presence of non-canonical residues have been shown to be important for actin binding [4,5].

TPMs bind cooperatively to actin filaments, forming a polymer in which the C-terminus of a dimer interacts with the N-terminus of the next [4,6,7]. This TPM strand runs continuously over the major groove of the actin filament, typically interacting with seven actin monomers per TPM molecule, although four to six monomers are covered by lower molecular weight isoforms (see below). Binding of TPM results in stabilization and stiffening of actin filaments [1,2]. Furthermore, TPM binding affects the interaction of the actin filaments with other proteins, such as the Arp2/3 complex, ADF/cofilin, gelsolin, calmodulin, tropomodulin and myosin, among others [1,2,7]. This generally results in the stabilization of the actin filament and the inhibition of filament growth or branching, but the precise effects are isoform specific. TPM isoforms are

Abbreviations: EST, expressed sequence tag; HMW, high molecular weight; LMW, low molecular weight; MALDI-TOF, matrix-assisted laser desorption/ionization-time-of-flight; PAGE, polyacrilamide gel electrophoresis; PBS, phosphate buffered saline; SDS, sodium dodecyl-sulfate; TPM, tropomyosin.

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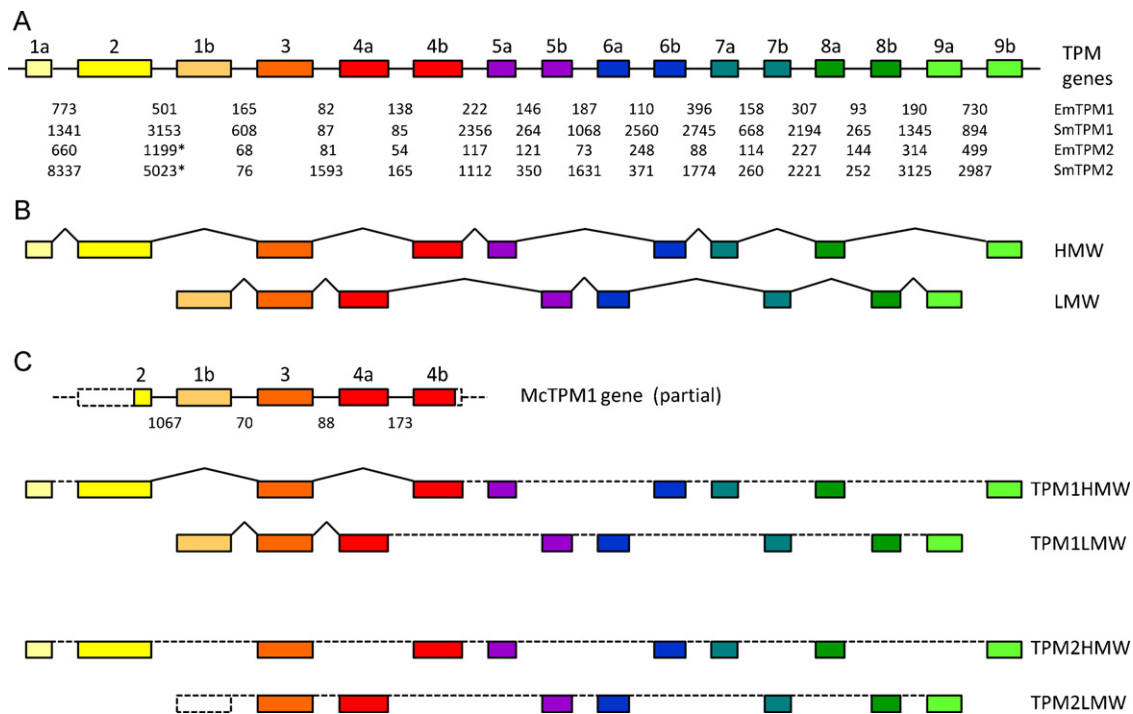


Fig. 1. Structure and isoforms of TPM1 and TPM2 genes from neodematans. (A) General structure of TPM1 and TPM2 genes in *Echinococcus multilocularis* and *Schistosoma mansoni*. Boxes represent exons and are in scale to their lengths. Lines between them represent introns and their sizes are indicated below. Duplicated exons are shown with the same color. The asterisk indicates the position of a possible additional exon between exons 2 and 1b in TPM2 genes. (B) LMW and HMW isoforms derived of TPM1 and TPM2 genes of *E. multilocularis* and *S. mansoni*. (C) Partial structure of *McTPM1* gene and isoforms of *McTPM1* and *McTPM2* in *Mesocostoides corti*. The genomic sequence of *McTPM1* is known between the end of exon 2 and the end of exon 4b. The boxes are in scale with their length and intron sizes are shown below. Part of the genomic sequences of exon 2 and exon 4b are unknown and are shown with dashed boxes. Most of the intron positions are unknown and are shown with dashed straight lines assuming conservation of intron position with other platyhelminthes and bilaterians.

differentially expressed in different cell types and developmental stages, and show differential sub-cellular localization [2,7–9].

The best characterized function of TPM is in vertebrate striated muscle contraction [10]. The striated muscle specific isoform participates in the calcium-dependent regulation of the interaction between thin filaments (actin) and thick filaments (myosin). The troponin complex controls the position of TPM along the actin thin filament; when Ca^{2+} binds to troponin, the TPM strand is displaced, allowing myosin to attach to the thin filaments. This troponin and TPM based calcium-dependent regulation has been found to exist in invertebrate muscles as well (both smooth and striated) [11,12]. A second mechanism of regulation, based in thick filament activation via Ca^{2+} binding to myosin light chain, is also commonly present in invertebrate muscles. The relative importance of each mechanism in each species may vary [11].

The diversity of TPM isoforms is generated from a combination of paralog genes, alternative promoter usage and alternative splicing of mutually exclusive exons [2,7,13,14]. Traditionally, TPM proteins from animals have been divided in high molecular weight (HMW) isoforms, of approximately 284 amino acid residues, and low molecular weight (LMW) isoforms, of approximately 247 amino acid residues. Typically, HMW and LMW TPM isoforms are generated by alternative promoter usage from the same gene [2,7,13]. The HMW TPMs originate from the upstream promoter, and contain exons 1a and 2 linked to exons 3–9, while LMW TPMs originate from a promoter downstream of exon 2 and contain exon 1b linked to exons 3–9 (Fig. 1). The position of introns and their phase is very well conserved across bilaterians [13,14]. TPMs in the thin filaments of the contractile apparatus of muscle cells are always HMW isoforms, while TPMs that are part of actin filaments in the cytoskeleton of all cell types (including muscle cells) can be either HMW or LMW isoforms [7].

In bilaterians, it is common for exon 4, 5, 6, 7, 8 or 9 to be tandemly duplicated [14]. These duplicated exons are spliced in a mutually exclusive fashion (only one is included within each mRNA). Furthermore, only some combinations of mutually exclusive exons are spliced together, and these combinations are correlated with promoter usage (HMW and LMW isoforms). Therefore, from one TPM gene, two proteins can be generated which are encoded by mRNAs that only have few exons in common, resulting in two “internal paralogs” (in comparison with classical paralogs, which result from the duplication of a complete gene) [14]. In some cases, it has been shown that these internal paralogs have been resolved into two different genes, one coding only for a LMW isoform and the other one coding only for a HMW isoform, in a process referred to as “externalization”. In this case, an ancestral gene coding for both HMW and LMW isoforms is duplicated, and the resulting genes selectively lose the promoter region and associated internal exons of either HMW or LMW isoforms [14].

TPMs commonly participate in immunogenic and allergic reactions to invertebrates, including parasites [15]. Because of this, recombinant TPMs have been investigated as possible vaccines against parasitic nematodes, against the trematode *Schistosoma mansoni*, and against the cestode *Echinococcus granulosus* [15–17]. A HMW TPM from *E. granulosus* has been cloned and characterized, and shown to be expressed preferentially in the muscles of the suckers and in sub-tegumental muscles [18,19].

In previous work, we have characterized the formation and development of the genital primordium in the model cestode *Mesocostoides corti* [20]. The genital primordium consists of a mass of cells within each developing proglottid that will differentiate into the reproductive ducts. It is generated as dorso-ventral extensions of a layer of proliferative cells located next to the inner muscle layer. It initially consists of a small and loose accumulation of cells in the

center of each segment. As it grows, it divides into two regions, an inner compact non-proliferative region, and an outer loose proliferative one. Also, it divides into an anterior (presumably male) genital primordium and a posterior (presumably female) genital primordium [20,21].

In this work, we have characterized TPM genes and isoforms in *M. corti*, and studied the developmental expression of HMW TPMs and the distribution of F-actin in order to investigate the later development and differentiation of genital primordia into muscular reproductive ducts and organs (cirrus, cirrus pouch, vagina and uterus). The TPM genes and isoforms found in *M. corti* have also allowed us to predict conserved homolog isoforms in other neodermatans.

2. Materials and methods

2.1. Parasite culture

Mice infected with *M. corti* tetrathyridia were kindly donated by Jenny Saldaña (Laboratorio de Experimentación Animal, Facultad de Química, UdelaR). Parasite removal, culture and induction of strobilization were performed as previously described [20,22].

2.2. Nucleic acids extraction and purification

DNA was extracted with the GenomicPrep Cells and Tissue DNA Isolation Kit (Amersham Biosciences). RNA was purified with TRIreagent® (Sigma–Aldrich) as instructed by the manufacturer.

2.3. RT-PCR, cloning and sequencing of TPM coding sequences

cDNA was synthesized from 1 µg of total RNA from cultured tetrathyridia with the BD Sprint™ Power Script™ Single Shots system (BD Biosciences), using 0.5 µg of poly dT primer, in the presence of 40 units of RNase inhibitor (RNase OUT, Invitrogen) as described by the manufacturer. 3 µL of cDNA was used for RT-PCR with Taq polymerase (Invitrogen) and 0.8 µM of degenerate primers TropoF (5'-AARAARAATGATNGCNATGAA-3', corresponding to the amino acid motif KKKM(I/M)AMK) and TropoR (5'-CKNGCNGCYTCRTCTAYTT-3' corresponding to the amino acid motif KYDEAAR). The cycling conditions were 94 °C for 5 min, 35 cycles of 94 °C for 45 s, 50 °C for 45 s and 72 °C for 45 s, and a final extension of 72 °C for 5 min. A PCR product of the expected size (488 bp) was purified and cloned into pGEM-T-Easy (Promega). Restriction analysis of several clones with *HindIII* showed that two different TPM coding sequences were cloned. One representative clone of each was sequenced (Macrogen, Korea), resulting in partial coding sequences of HMW isoforms from two different genes, named *McTPM1* and *McTPM2*.

2.4. 3' Rapid amplification of cDNA ends (3' RACE)

RNA from tetrathyridia was reverse transcribed with Superscript II reverse transcriptase (Invitrogen) using primer CDS (5'-AAGCAGTGGTATCAACGCAGAGTAC(T)₃₀NN-3'). The PCR reaction was performed using U-Taq polymerase (SBS Genetech) with 3 µL of cDNA as template, primer SmartIII (5'-AAGCAGTGGTATCAACGCAGAGT-3'), which is complementary to the adaptor of the CDS primer, and specific primers for *McTPM1* or *McTPM2* (5'-GCTACTAATGCTGAAGCTGAAGTTGCAG-3' and 5'-TGCTCTTCAGCGTCGCATACGTC-3', respectively). For *McTPM2*, 0.5 µL of the first PCR reaction was used as template in a second nested reaction to improve amplification specificity, using primers SmartIII and 5'-TGCTCTTCAGCGTCGCATACGTC-3'. PCR products were gel-purified and cloned into pGEM-T-Easy (Promega). Several

clones from each 3' RACE experiment were sequenced (Macrogen, Korea). cDNA sequences were deposited in GenBank (accession numbers HQ230017–20).

2.5. Cloning of genomic fragments of *McTPM1*

Fragments of genomic DNA of the *M. corti* gene *McTPM1* were amplified with Long PCR enzyme mix (Fermentas), as described by the manufacturer. One fragment was amplified between putative exons 2 and 3, in order to obtain the intron sequences and the exon 1b sequence found between them, using primers 5'-GCCATCAGTAAGCTTGAAGAGACCG-3' and 5'-AAGACGAATGCGGCGGGTCATG-3'. The other fragment was amplified between putative exons 3 and 4b, in order to confirm the position of exon 4a between them, using primers 5'-CTGAAGCTGAAGTTGCAGCCATGA-3' and 5'-TCGTAATTACGTTACAGCATCTCC-3'. The genomic sequence was deposited in GenBank (accession number HQ229992).

2.6. Search and annotation of TPM genes in *Echinococcus multilocularis*, *S. mansoni* and *Schmidtea mediterranea*

TPM genes were searched by *tblastn* or *blastp* using different TPM HMW and LMW isoforms isolated from *M. corti* (this work) and *Echinococcus* spp. (GenBank accession numbers AJ314792, FM872427.1). Searches were performed against *E. multilocularis* genome contigs (version 07.01.10, produced by the Parasite Genomics group at the Wellcome Trust Sanger Institute, available from <http://www.sanger.ac.uk/resources/downloads/helminths/echinococcus-multilocularis.html>), *S. mansoni* genes [23], and predicted proteins of *S. mediterranea* (SmedGD, <http://smedgd.neuro.utah.edu/> [24]). For *S. mansoni*, genes currently annotated as putative TPMs were analyzed for Pfam hits (<http://pfam.sanger.ac.uk>). Within each gene, alternative exons not detected in the original blast analysis were searched for by looking for similarities with both HMW and LMW TPM isoforms from *M. corti* and *E. granulosus* with pairwise *tblastn*, and by searching for EST coverage. Canonical splice sites (GT/AG) were identified in the borders of exons. Alternative splicing isoforms of genes in *Echinococcus* spp. and *S. mansoni* were determined by manually analyzing ESTs that mapped to each gene. For *Echinococcus* spp., evidence was also included from the related *Taenia* spp., and for *S. mansoni*, from the related *Schistosoma japonicum*. Finally, the LMW isoform of *EgTrp* of *E. granulosus* [18], which was predicted on the basis of sequence homology but which had no EST coverage, was confirmed by RT-PCR using cDNA from protoscoleces isolated from sheep with specific primers 5'-TGGAAAGGGAGATTGCACTC-3' and 5'-GATTCCTTGCGAAGAAGCG-3'.

2.7. Phylogenetic analyses

Phylogenetic trees were constructed by Bayesian Inference method, by means of MrBayes version 3.1 [25]. Markov chain Monte Carlo analyses were run until convergence, sampling trees and parameters every 100 generations. We used the standard deviation of split frequencies as convergence diagnostic of analyses, which ran for more than 5×10^5 generations. We discarded the first 25% of generations as burn in and calculated consensus trees. Fixed rate model of amino acid sequence evolution was estimated using "mixed prior". "Mixed prior" allows the MCMC sampler to explore all of the fixed-rate models included in MrBayes. In all cases WAG [26] was inferred as the most probable model for amino acid sequence evolution. Three different alignments were used as input for analysis, including: (i) the alternatively spliced exons of TPM isoforms (exons 4, 5, 6, 8 and 9 of HMW and LMW isoforms), (ii)

all exons from HMW isoforms and (iii) all exons from LMW isoforms. Equivalent regions were used from *M. corti* isoforms when exon limits were unknown. The strength of the evidence in favor of an inconsistent node was tested using the Bayes Factor Test, comparing the harmonic mean of the likelihood values of the MCMC samples as marginal likelihood of alternative tree topologies. This test was interpreted according to recommendations developed by [27].

2.8. Protein extracts

For 1-D electrophoresis, 100 μ L of tetrathryidia were combined with 100 μ L of extraction buffer 1 (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Triton X-100), homogenized in ice with a pestle homogenizer, and the extract was clarified by centrifugation for 30 min at 15,000 $\times$ g at 4 °C. For 2-D electrophoresis, approximately 2 mL of tetrathryidia were combined with 2 mL of extraction buffer 2 (10 mM phosphate buffer, pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1% Triton X-100, 100 μ M phenylmethylsulfonyl fluoride) and homogenized with a pestle homogenizer in ice. The extract was clarified by two steps of centrifugation (30 min at 15,000 $\times$ g and 30 min at 26,000 $\times$ g, both at 4 °C) and one step of filtration (0.22 μ m pore size). Protein extracts were quantified using the BCA Protein Assay Kit (Thermo Scientific Pierce).

2.9. Electrophoresis and western blot

For 1-D western blot, 3 μ g of *M. corti* protein extract was loaded in a discontinuous 15% SDS-PAGE gel. 2-D electrophoresis was performed by the Unidad Tecnológica de Bioquímica y Proteómica Analíticas of the Institut Pasteur of Montevideo. 150 μ g of *M. corti* protein extract were purified using the 2D-CleanUp kit (GE Healthcare), resuspended in 125 μ L of rehydration solution (7 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.5% IPG Buffer 4-7, 0.002% Bromophenol blue, 17 mM dithiothreitol) and loaded in a 7 cm linear pH 4–7 isoelectric focusing strip (Immobiline DryStrips; GE Healthcare). Details of the first dimension run protocol are available under request. The focused strips were reduced and alkylated by incubation for 15 min at room temperature in equilibration buffer (6 M urea, 75 mM Tris-HCl, pH 8.8, 29.3% glycerol, 2% SDS, 0.002% Bromophenol Blue) supplemented with 1 mg/mL dithiothreitol followed by 15 min at room temperature in equilibration buffer supplemented with 25 mg/mL iodoacetamide. Equilibrated strips were run in the second dimension in a 12.5%, 10 cm $\times$ 10 cm $\times$ 0.1 cm SDS-PAGE. Two 2-D gels were run in parallel, one gel was stained with colloidal Coomassie Blue and the other was electroblotted as described next.

For western blots, gels were electroblotted to a Hybond-C membrane (Amersham Bioscience) using a tank transfer system (BioRad Mini-PROTEAN® TetraCell). The membrane was blocked overnight at 4 °C in blocking buffer (3% BSA, 2% glycine in TBS-T: 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20). The membrane was incubated for 2 h at room temperature with serum raised in rabbit against recombinant *E. granulosus* TPM EgTrpA, a HMW isoform (GenBank accession number ACA14466.1) fragment lacking the N-terminal end (rEgTrp; [19]), diluted 1:2000 or 1:1000 in blocking buffer for 2-D and 1-D gels, respectively. After 12 washes in TBS-T, it was incubated for 1 h with donkey anti-rabbit IgG conjugated to horseradish peroxidase (Amersham) (diluted 1:8000 or 1:5000 in blocking buffer for 2-D and 1-D, respectively). After 12 washes with TBS-T, membranes were visualized using the Chemiluminescent Peroxidase Substrate-3 (Sigma-Aldrich) and exposing them to Biomax MR Film (Kodak).

2.10. Protein identification by mass spectrometry

This was performed by the Unidad Tecnológica de Bioquímica y Proteómica Analíticas of the Institut Pasteur of Montevideo. Peptide mass fingerprinting of protein selected spots was carried out by in-gel trypsin treatment (Sequencing-grade, Promega) overnight at 37 °C. Peptides were extracted from the gels using 60% acetonitrile in 0.2% trifluoroacetic acid, concentrated by vacuum drying and desalted using C18 reverse phase micro-columns (OMIX Pipette tips, Varian). Peptide elution from micro-column was performed directly into the mass spectrometer sample plate with 3 mL of matrix solution (α -cyano-4-hydroxycinnamic acid in 60% aqueous acetonitrile containing 0.2% trifluoroacetic acid).

Mass spectra of digestion mixtures were acquired in a 4800 MALDI-TOF/TOF instrument (Applied Biosystems) in reflector mode and were externally calibrated using a mixture of peptide standards (Applied Biosystems). Collision-induced dissociation MS/MS experiments of selected peptides were performed. Proteins were identified by local database searching with peptide *m/z* values using the MASCOT program and using the following search parameters: monoisotopic mass tolerance, 0.05 Da; fragment mass tolerance, 0.3 Da; methionine oxidation, cysteine carbamidomethylation and protein N-terminal acetylation as possible modifications and one missed tryptic cleavage allowed.

2.11. Immunohistochemistry in paraplasm sections

Specimens were fixed either overnight at 4 °C or for 4 h at room temperature with 4% paraformaldehyde prepared in PBS (PFA-PBS), dehydrated progressively to 100% ethanol and stored at –20 °C until further use. Specimens were embedded in Paraplast (Oxford Labware) as described by the manufacturer and cut into 10 μ m thick sections. These were rehydrated to PBS and processed for immunohistochemistry as follows. Endogenous peroxidase activity was eliminated by incubating in 3% H₂O₂ for 10 min at room temperature, followed by a quick wash with PBS. Sections were blocked with 1% bovine serum albumin (BSA, Sigma-Aldrich) and 5% sheep serum (Sigma-Aldrich) in PBS plus 0.05% Tween-20 for 1 h. They were then incubated overnight at 4 °C with rabbit serum raised against recombinant rEgTrp [19] diluted 1:500 in PBS with 1% BSA and 0.05% Tween-20. After four washes of 15 min each in PBS, sections were incubated for 2 h at room temperature with polyclonal donkey anti-rabbit IgG conjugated to horseradish peroxidase (Amersham, 1:1000 dilution in PBS with 1% BSA). After four washes of 15 min each in PBS, detection was performed with 3-amino-9-ethylcarbazole (AEC, Sigma-Aldrich).

2.12. Immunohistochemistry in cryosections and phalloidin staining

Fixation, embedding and cryosectioning of specimens were performed as previously described [20]; the immunohistochemistry protocol was the same as for paraplasm sections. Staining with Alexa-546 conjugated phalloidin (Invitrogen) in cryosections was previously described [20]. For staining with Alexa-546 conjugated phalloidin in whole mounts, specimens were permeabilized for 20 min at room temperature with 0.1% Triton X-100 in PBS, washed twice in PBS for 5 min each, blocked in 1% BSA in PBS at room temperature for 30 min, and incubated for 60 min in Alexa-546 conjugated phalloidin (Invitrogen, 5 units/mL) in PBS with 1% BSA. Finally, specimens were washed four times (15 min each), in PBS, and mounted in 80% glycerol. Sections and whole mounts were co-stained with 4',6-diamidino-2-phenylindole (DAPI) dilactate (Sigma-Aldrich).

3. Results

3.1. TPM genes and isoforms in *E. multilocularis* and *S. mansoni*

In order to compare and correctly interpret our results with TPM sequences from *M. corti*, we searched for TPM genes in the genomes of *E. multilocularis* and *S. mansoni*, and manually identified exons and alternative splicing isoforms (see materials and methods). For the sake of clarity, these are described prior to the results obtained for *M. corti*.

Two paralog TPM genes were found in *E. multilocularis*, *EmTPM1* and *EmTPM2*, located in contigs 3430 and 6124, respectively. Contig 5882 is 99% identical to contig 6124, and has insertions of undetermined (N) bases within TPM coding regions. We have interpreted this contig as a possible artifact of assembly. Both genes have the same gene structure (Fig. 1A), and code for both HMW and LMW isoforms. The HMW isoforms of both genes are 80% similar and 61% identical to each other at the amino acid level, while the LMW isoforms are only 57% similar and 33% identical to each other. The position and phase of introns is highly conserved with other bilaterians (thus resulting in identical exon length) except for the position of the intron between exons 1a and 2 (Supplementary Information 1). The unusual position of this intron is conserved between both genes in *E. multilocularis*, and with TPM genes in other flatworms (see below).

Strikingly, all exons after exon 3 are duplicated and are used as mutually exclusive exons in both genes. HMW and LMW isoforms only share exon 3, and use different alternative downstream exons (Fig. 1). This results in two “internal paralogs” in which only 16% of their coding sequence is coded by the shared exon. This is similar to cases described by Irimia et al. [14] although in the most extreme cases yet described (such as the TPM gene from *Lotia gigantea*), exon 7 was shared between HMW and LMW isoforms. EST and cDNA support from *Echinococcus* spp. and from the closely related *Taenia* spp. was only found for the existence of one HMW and one LMW isoform from each gene, spliced from equivalent exons in both genes (Fig. 1B; Supplementary Information 2). For the *EmTPM1*-LMW isoform, no support was found in databases. Instead, we confirmed this isoform by RT-PCR for EgTrp-LMW, the LMW isoform of *EgTrp*, the ortholog gene of *E. granulosus* [18] in which the same alternative exons were found. No evidence for “mixed” isoforms with a combination of exons from HMW and LMW isoforms was found.

In *S. mansoni*, four different TPM genes were found. A fifth gene, Smp_032490, is described as a putative TPM in GenBank, but Pfam searches do not recognize the predicted protein as such, and protein length and intron positions also suggest that the gene does not code for a TPM. Two genes, Smp_044010 (*SmTPM1* in this work) and Smp_031770 (*SmTPM2* in this work) code for both HMW and LMW isoforms, with the same exon structure as the genes in *E. multilocularis* (Fig. 1A). The HMW isoforms of both genes are 81% similar and 65% identical to each other at the amino acid level, while the LMW isoforms are 60% similar and 32% identical to each other. *SmTPM1* and *SmTPM2* are most similar to *EmTPM1* and *EmTPM2*, respectively. The other two genes (Smp_022170 and Smp_085290.2) apparently only code for LMW isoforms, which are very divergent but conserve the same intron positions and phases. No duplicated exons were found in these genes.

For *SmTPM1*, EST and cDNA support was found in *Schistosoma* spp. for HMW and LMW isoforms with identical alternative splicing as in the *E. multilocularis* genes (Fig. 1B, Supplementary Information 2; the HMW isoform was previously described by [28]). The alternative exons for the LMW isoform are not included in the gene model in GenBank. No evidence for “mixed” isoforms was found. On the other hand, for *SmTPM2*, evidence was found supporting HMW and LMW isoforms with identical alternative splicing as in

SmTPM1 and in *E. multilocularis* genes (Fig. 1B; the major HMW isoform was described by [29]). However, EST support was also found for minor isoforms with atypical combinations of exons (Supplementary Information 2). Interestingly, two *S. japonicum* ESTs indicate the existence of a *SmTPM2* isoform containing an extra exon situated between exons 2 and 1b (and therefore of higher molecular weight than canonical HMW isoforms); furthermore, this region is conserved in *E. multilocularis*, suggesting a conserved and previously undescribed exon in TPMs from neodermatans (Supplementary Information 2).

3.2. Cloning and characterization of *M. corti* TPM isoforms

We isolated TPM isoforms from *M. corti* by RT-PCR with degenerate primers aimed at motifs present in the beginning of exon 1a and in the limit between exons 4 and 5 in HMW TPMs of cestodes and trematodes. The partial coding sequences of two HMW TPMs (McTPM1-HMW and McTPM2-HMW) were obtained. The downstream region of each isoform was obtained by 3' RACE. The specific primers for 3' RACE were designed within the region usually coded by exon 3 in other metazoans, which is shared by both HMW and LMW isoforms. We found the complete downstream coding region for McTPM1-HMW and McTPM2-HMW, and for their respective LMW isoforms, McTPM1-LMW and McTPM2-LMW (Fig. 1B). HMW and LMW isoforms are only identical in the region typically coded by exon 3, while all downstream sequences clearly originate from different exons, as is the case in *E. multilocularis* and *S. mansoni*. A genomic fragment of *McTPM1* between exons 2 and 4b was sequenced, confirming that McTPM1-HMW and McTPM1-LMW are alternative splicing isoforms of the same gene (with the same exon structure as the *E. multilocularis* genes). This also allowed us to obtain the sequence of exon 1b.

3.3. Phylogenetic analyses

Sequence comparison suggested that *McTPM1*, *EmTPM1* and *SmTPM1* are orthologs (TPM1 genes), and that *McTPM2*, *EmTPM2* and *SmTPM2* are orthologs (TPM2 genes). In order to confirm this, to map the origin of the duplication that generated TPM1 and TPM2 genes, and to map the origin of the tandemly duplicated exons, we performed a series of phylogenetic analyses. TPM genes from the planarian *S. mediterranea* were also searched for and included. Interestingly, seven genes (some incompletely covered in the genome assembly) were found in this genome, three of which code for HMW proteins exclusively, while four code for LMW proteins exclusively (Supplementary Information 3). In most cases, the position and phase of introns is conserved, but in one case (Mk4.008311.00.01, coding for a HMW TPM) exons 2–7 are fused into only one exon. In this case, and in others in which intron size is too small to allow for alternative exons to exist, discarding the existence of alternative splicing isoforms is straightforward. This strongly suggested that TPM genes from *S. mediterranea* originated by gene duplication and “externalization” (selective loss of exons coding for HMW or LMW isoforms, as described in Section 1 [14]). In this case, the expected phylogenetic signal for the mutually exclusive duplicated exons would be that HMW TPM and LMW TPM coding genes from *S. mediterranea* would be more closely related to the HMW and LMW isoforms from dual-encoding genes, respectively [14]. However, for exon 3, which is constitutive, the gene duplicates would be most closely related to each other.

Ideally, phylogenetic analyses should be performed with alignments of each exon individually; however, very little phylogenetic signal is present in these short sequences and trees are unresolved (data not shown; see [14]). Hence, we performed phylogenetic analyses of alternatively spliced exons of TPM isoforms (4, 5, 6, 8 and 9; exon 7 was not included because it is not alternatively

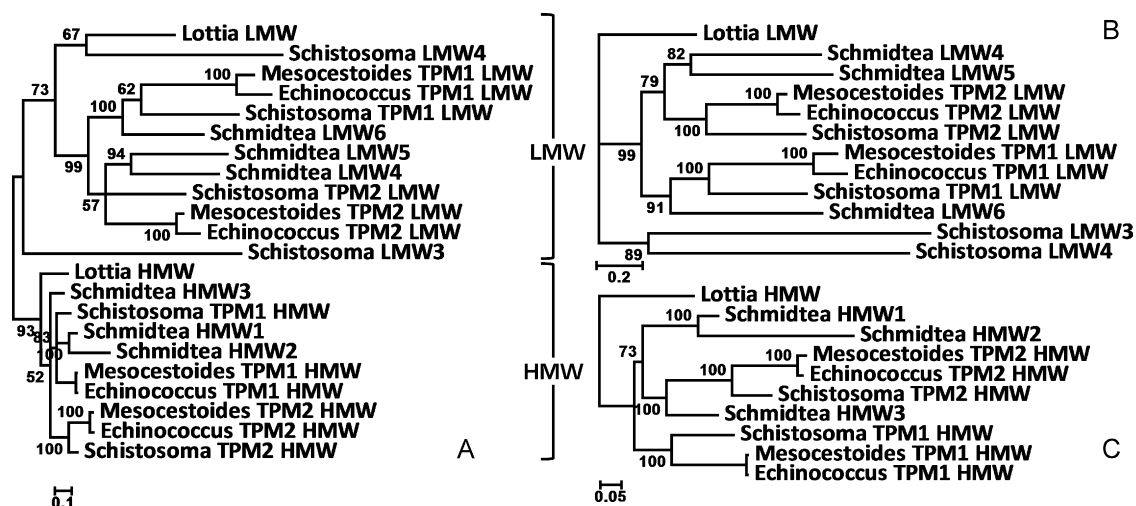


Fig. 2. Bayesian inference phylogenetic analyses of TPM proteins. (A) Analysis of HMW and LMW TPM proteins for a data set including regions encoded by exons 4, 5, 6, 8 and 9. Clusters of HMW and LMW TPMs are indicated. (B) Analysis of complete LMW isoforms. (C) Analysis of complete HMW isoforms. Numbers next to nodes indicate posterior probability values as percentages. Contig and accession numbers for sequences from *Echinococcus multilocularis* and *Schistosoma mansoni* are indicated in [Supplementary Information 2](#), except for *Schistosoma* LMW3 (XP.002573656.1) and *Schistosoma* LMW4 (XP.002579631.1). Accession codes from SmedGD [24] for *Schmidtea* sequences are as follows: HMW1, 000522.00.01; HMW2, 011352.04.01; HMW3, 008311.00.01; LMW4, 001238.00.01; LMW5 (incomplete), 003231.04.01; LMW6, 004209.00.01. The sequences from *Lottia* were previously published by Irimia et al. [14].

spliced in *L. gigantea*, a mollusk included as a representative out-group lophotrochozoan). The results clearly show that all HMW and LMW isoforms form two different clusters (Fig. 2A). HMW and LMW isoforms from TPM1 and TPM2 genes from neodermatans also form highly supported clades, and interestingly, different genes from *S. mediterranea* cluster with either TPM1 or TPM2 genes from neodermatans. The HMW isoform of the mollusk *L. gigantea* is external to all other HMW sequences, while the LMW isoform forms a moderately supported basal clade with one of the divergent LMW coding genes from *S. mansoni*. A simple hypothesis explaining these phylogenetic patterns would be that a dual-encoding TPM gene, with duplicated exons 4, 5, 6, 8 and 9 (and perhaps 7), was present in the last lophotrochozoan ancestor (as previously suggested [14]). In the last common ancestor of triclads and neodermatans, this gene had a shift in the position of the intron between exons 1a and 2, and was then duplicated resulting in TPM1 and TPM2 genes. In the ancestor of the planarian *S. mediterranea* further duplications ensued, coupled with selective loss of exons resulting in genes coding only for HMW or LMW isoforms. The origin of the highly divergent genes that only code for LMW genes in *S. mansoni* is not clear from these results, but could have resulted from another process of externalization.

Further confirmation of this hypothesis was obtained by performing phylogenetic analyses including full length sequences of only LMW or only HMW isoforms (Fig. 2B and C, respectively). In the case of LMW isoforms, the results clearly confirm that LMW TPM encoding genes from *S. mediterranea* are most closely related to LMW isoforms of genes TPM1 or TPM2 from neodermatans. For HMW TPMs, one *S. mediterranea* gene (Smed008311.00.01) is associated with TPM2 genes. The position of the other two HMW TPM genes is less clear, since in this tree they form a moderately supported clade with TPM2 genes (Fig. 2C), but are clustered with TPM1 genes in the tree generated from alternatively spliced exons (Fig. 2A). A likelihood ratio test comparing both alternatives for the complete HMW TPM sequences data demonstrated that they are not significantly different.

Externalization of TPM isoforms was previously described for the annelid *Helobdella robusta* [14]. Phylogenetic analyses including the TPM isoforms of the annelids *Helobdella* and *Capitella* (which has a single gene encoding for both HMW and LMW isoforms) show that the externalized TPMs of *Schmidtea* are more related to

isoforms from TPM1 and TPM2 genes from neodermatans, while the externalized TPMs from *Helobdella* are more related to the isoforms of the gene of *Capitella* ([Supplementary Information 6](#)). The monophyly of HMW isoforms of *Schmidtea* and neodermatans is further supported by the shared change of the position of the intron between exons 1a and 2. Therefore, the process of paralogy externalization occurred convergently in *Helobdella* and *Schmidtea*. Some inconsistencies are observed between the tree including only alternative exons from both HMW and LMW isoforms and the tree of complete HMW isoforms. The tree of alternative exons also indicates convergence of externalization of TPM genes in *Schmidtea* and *Helobdella*, but requires several gene gains and losses that are unlikely, and convergence in the change of the position of the first intron in HMW TPMs of flatworms. Possible reasons for this inconsistency include the lower number of sites and higher number of divergent sequences, and the inclusion in the same analysis of sequences (HMW and LMW isoforms) which are better modeled by different evolutionary models (data not shown). A figure showing the evolutionary events that occurred in the TPM gene family in lophotrochozoans is included as [Supplementary Information 7](#).

3.4. Characterization of TPMs in *M. corti* that are recognized by an anti-EgTrpA polyclonal antibody

We used serum raised against a recombinant C-terminal fragment of EgTrpA [19], the *E. granulosus* ortholog of EmTPM1-HMW, to investigate the expression of TPM isoforms in *M. corti*. The fragment of EgTrpA spanned from part of exon 3 to the end of exon 9b. Therefore, the serum would very likely recognize McTPM1-HMW (98% identical in this region to EgTrpA), and perhaps McTPM1-LMW (which shares exon 3 with the HMW isoform) and McTPM2-HMW (67% identical in this region).

In 1-D western blot of extracts from tetrathyridia, two bands were recognized with apparent molecular weights of 43 and 38 kDa (Fig. 3A). Bands with the same apparent molecular weights were observed in extracts of specimens in different stages of development (data not shown). Because migration of TPMs in SDS-PAGE electrophoresis is anomalous and even TPMs of identical molecular weight show important differences in migration, it is not possible to correlate the apparent molecular weights to specific TPM isoforms [30–32]. In order to identify the proteins recognized by

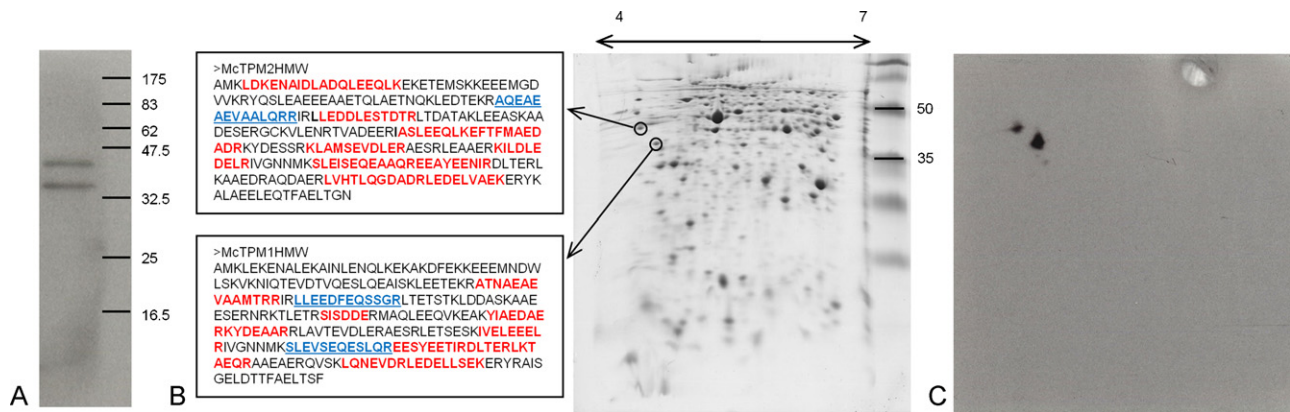


Fig. 3. Western blot and mass spectrometry analysis of TPMs of *Mesocostoides corti*. (A) 1-D western blot of *M. corti* protein extracts with anti-rEgTrp serum. Molecular weights are indicated to the right in kDa. (B) 2-D electrophoresis of *M. corti* protein extracts and mass spectrometry (MS) results of selected spots. Molecular weights are indicated to the right in kDa. Tryptic peptides identified by MS matching with HMW TPM isoforms are shown in bold red and selected peptides identified by collision-induced dissociation MS/MS experiments are shown in bold blue and underlined (C) 2-D western blot of *M. corti* protein extracts with anti-rEgTrp serum. (For interpretation of the references to color in text, the reader is referred to the web version of the article.)

the serum, we performed 2-D western blot of soluble extracts from tetrathryidia, and analyzed the recognized proteins by excising them from a gel run in parallel (Fig. 3B and C). The proteins recognized by the serum were identified by MALDI-TOF/TOF analysis of tryptic fragments (Fig. 3B). The proteins were identified as McTPM2-HMW (apparent molecular weight of 43 kDa; the coverage by identified tryptic fragments was 45%) and McTPM1-HMW (apparent molecular weight of 38 kDa; the coverage by identified tryptic fragments was 37%). For practical purposes, the serum is therefore specific for HMW isoforms in *M. corti*. Some minor spots with similar isoelectric point and molecular weight were detected in western blot when lower antibody dilutions and higher exposure times were used. These could represent partial degradation fragments, post-translational modifications [7,33], or faint detection of LMW-isoforms.

3.5. Distribution of F-actin during the development of *M. corti*

As described in Section 1, during early proglottid development in *M. corti* the genital primordium grows from an initial loose accumulation of proliferative cells into a large primordium in which the cells in the center become compactly distributed and cease to proliferate [20]. It also becomes divided into anterior and posterior regions. To study the subsequent development of the primordium into the genital ducts, we performed phalloidin staining in whole-mount specimens and cryosections, analyzing the development of muscle fibers in series of proglottids in the strobila.

As previously described for tetrathryidia [34], phalloidin staining is very strong in the main muscle layers: the sub-tegumental muscle layer (outer circular and inner longitudinal fibers), the inner muscle layer of longitudinal fibers, and the muscle fibers of the suckers (Figs. 4 and 5). Individual dorso-ventral and transverse muscle fibers are also numerous. Phalloidin staining is also intense in flame cells [34,35] (Fig. 5). Within the early and late genital primordia, and in testes primordia, diffuse phalloidin staining is observed, but no muscle fibers are observed except for transverse and dorso-ventral muscle fibers that show similar abundance in these regions as in other parts of the inner parenchyma (Figs. 4A, B and 5E, F).

During the development of the genital primordium, weak circular muscle fibers are first observed after 12 days of culture in the developing cirrus within the anterior portion of the genital primordium (Fig. 4C and D). In posterior (and therefore more devel-

oped) proglottids, the fibers in the cirrus are more prominent, and inner circular and outer longitudinal fibers are apparent (the position of these layers would be inner longitudinal and outer circular in an everted cirrus; Fig. 4I and J). At the same time, a weakly muscular cirrus pouch develops from the genital primordium surrounding the cirrus, with radial fibers connecting both, and a vagina with poorly developed circular fibers develops from the posterior region of the genital primordium (Fig. 4C and E–H'). The vagina and cirrus converge into the dorsal genital atrium (Fig. 4E). Finally, a uterus with thin circular muscle fibers develops (Fig. 4H and H'). The precise origin of the developing uterus could not be determined.

3.6. Expression of HMW TPMs during the development of *M. corti*

The distribution of HMW TPMs during the development of *M. corti* was determined by immunohistochemistry with the serum raised against rEgTrp. Strong signal was observed in the sub-tegumental and inner muscle layers throughout development (Figs. 5 and 6), and individual fibers in these layers could be clearly discerned (Fig. 6B and D). Weak signal was observed from individual dorso-ventral and transverse muscle fibers and in the parenchyma, while no signal was observed in the tegument or in flame cells. In control experiments in which serum was omitted or in which a pre-immune serum was used, no signal was obtained (data not shown).

In early and late genital primordia, no signal of HMW TPMs is present (although weak staining is seen in the surrounding parenchyma and individual muscle fibers; Figs. 5D and 6A, B). After the cirrus has begun to develop, signal appears in its muscle layers (Fig. 6E). HMW TPMs can also be detected in the weakly muscular cirrus pouch (Fig. 6F). We were unable to detect the presence of HMW TPMs in the developing female ducts. In summary, the staining pattern, although less sensitive, was very similar to the pattern observed for phalloidin, and it appears to be specific for muscle fibers, since very little or no signal is observed in flame cells or genital and testes primordia.

4. Discussion

4.1. TPM genes and isoforms in neodermatans

Searching for TPMs in *M. corti*, an unexpected complexity of TPM isoforms was discovered, which is conserved in

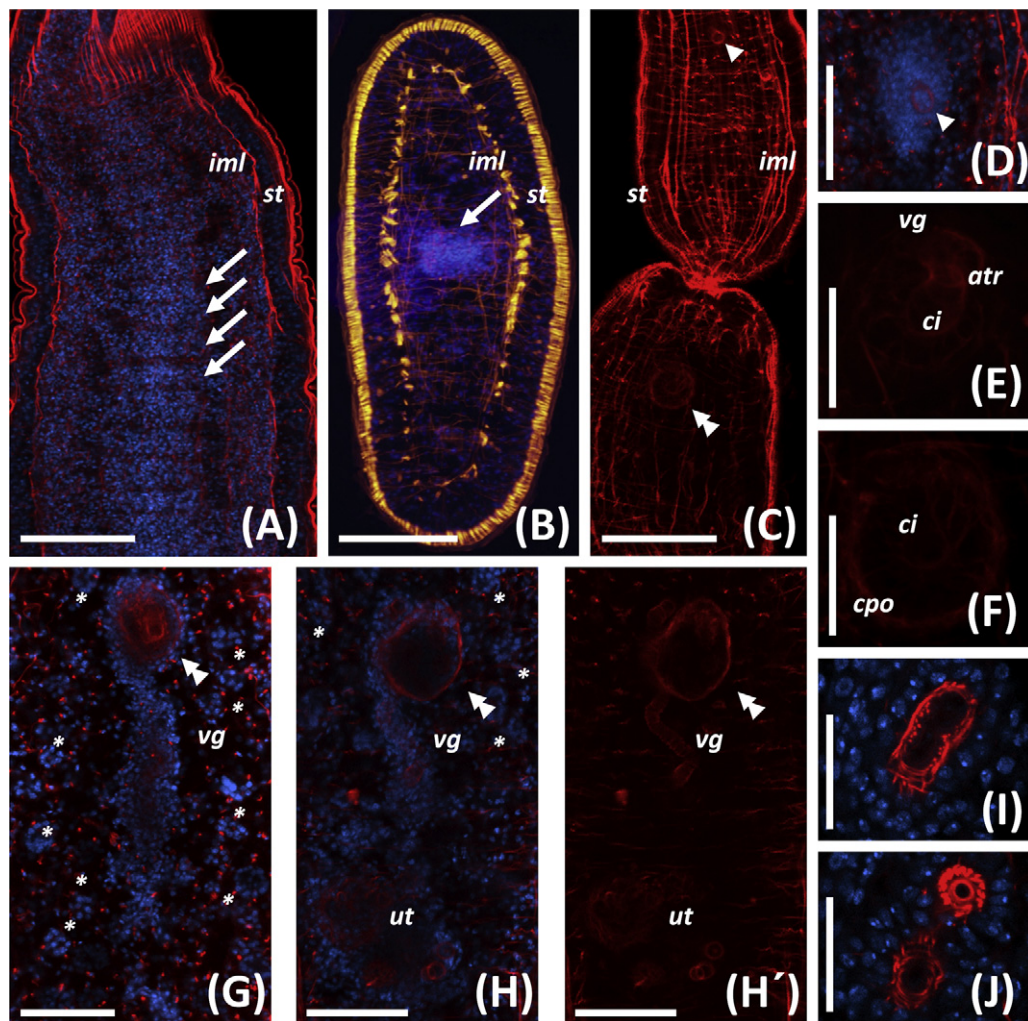


Fig. 4. Phalloidin staining of *M. corti* during different developmental stages of segmentation. Specimens were stained with DAPI (blue) and/or Alexa-546 conjugated phalloidin (orange). (A) Early segments of adult worm, showing early genital primordia (arrows). (B) Transverse section of the strobila through an early genital primordium (arrow). (C) Two consecutive maturing segments, the most anterior one showing the development of muscle fibers in the cirrus (arrowhead) while the most posterior one (and therefore most developed) shows the development of muscular fibers in both the cirrus and the cirrus pouch (double arrowhead). (D) Detail of early developing cirrus in the anterior genital primordium (equivalent to the region indicated by an arrowhead in C). (E) and (F) detail of the region indicated by double arrowhead in (C). (G) Developing genital organs including testes primordia (asterisks), vagina and cirrus and cirrus pouch. (H) and (H') Developing genital organs in a more developed segment showing the development of the uterus; in (H') only phalloidin staining is shown. (I) and (J). Two different planes of a confocal z-stack of a section through a cirrus. Abbreviations: atr, genital atrium; ci, cirrus; cpo, cirrus pouch; iml, inner muscle layer; st, sub-tegumental muscle layer; ut, uterus; vg, vagina. Bars represent 100 μ m (A–C), 50 μ m (D, H–J) or 30 μ m (E, F). (B) is a fluorescence microscopy image of a transverse cryosection. (I) and (J) are confocal microscopy images of a transverse cryosection. All other images are confocal microscopy images of whole mount specimens, either single confocal planes (A, C, D–F) or merges of 4 (G) or 2 (H) confocal planes. (For interpretation of the references to color in text, the reader is referred to the web version of the article.)

E. multilocularis and *S. mansoni*. Our results indicate that TPM1 and TPM2 genes, both coding for HMW and LMW isoforms with extensive alternative splicing, originated before the divergence of cestodes and trematodes. Therefore, it is likely that the same TPM isoforms will be found in other neodermatan parasites. Because TPMs are usually immunogenic and have been investigated as possible vaccines against helminths, these new isoforms could be novel vaccine candidates [15]. Furthermore, because cross-reactivity is likely to occur between HMW isoforms from paralog genes (as is observed with the serum raised against rEgTrp in this work), and perhaps between HMW and LMW isoforms from the same gene (because they share exon 3), caution must be taken in future work when attempting to determine if one particular TPM is the cause of the generation of antibodies.

Four different TPM isoforms were found in *M. corti* and *E. multilocularis*. No evidence was found for other isoforms containing a mixture of exons from HMW and LMW isoforms.

However, the presence of a conserved putative coding region between exons 2 and 1b in *EmTPM2* and *SmTPM2* (coupled with EST support in the case of *SmTPM2*) suggests that at least another isoform, with higher molecular weight, could originate from TPM2 genes. Minor alternative splicing isoforms of *EgTrp* (the *E. granulosus* ortholog of *EmTPM1*) with exon 4 skipping or intron retention between exons 6 and 7 were previously described by Alvite and Esteves [18] (note that we have renumbered the exons for consistency with the usual nomenclature of TPMs of bilaterians). For *SmTPM2*, EST support for isoforms with non-canonical combinations of exons was also found. It is unknown if these are actually translated and of physiological relevance, or if they consist of abnormal splicing events. It is noteworthy that support from several independent ESTs from *S. mansoni* and/or *S. japonicum* was found for some isoforms. Because TPMs are exclusively found as dimers, the actual number of TPM proteins will depend on whether

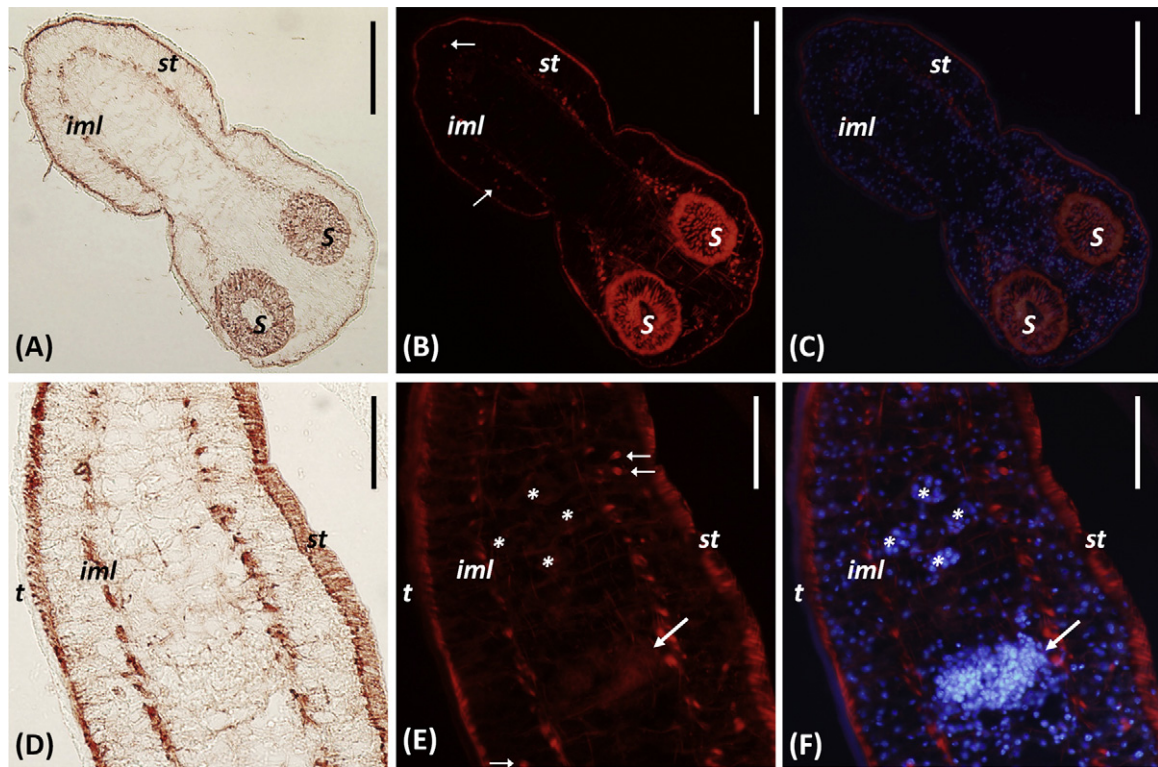


Fig. 5. Double staining with Alexa-546 conjugated phalloidin and immunohistochemistry with anti-rEgTrp serum in cryosections. (A–C) Longitudinal section of a tetrathyridium. (D–F) Longitudinal section of a developing proglottid. (A, D) Anti-rEgTrp staining (bright field microscopy). (B, E) Phalloidin staining (fluorescence microscopy). (C, F) DAPI staining merged with phalloidin staining (fluorescence microscopy). Large arrow points to the genital primordium. Small arrows indicate flame cells. Asterisks indicate testes primordia. Abbreviations: *iml*, inner muscle layer; *s*, suckers; *st*, sub-tegumental muscle layer; *t*, tegument. Bars represent 100 μm (A–C) or 50 μm (D–F).

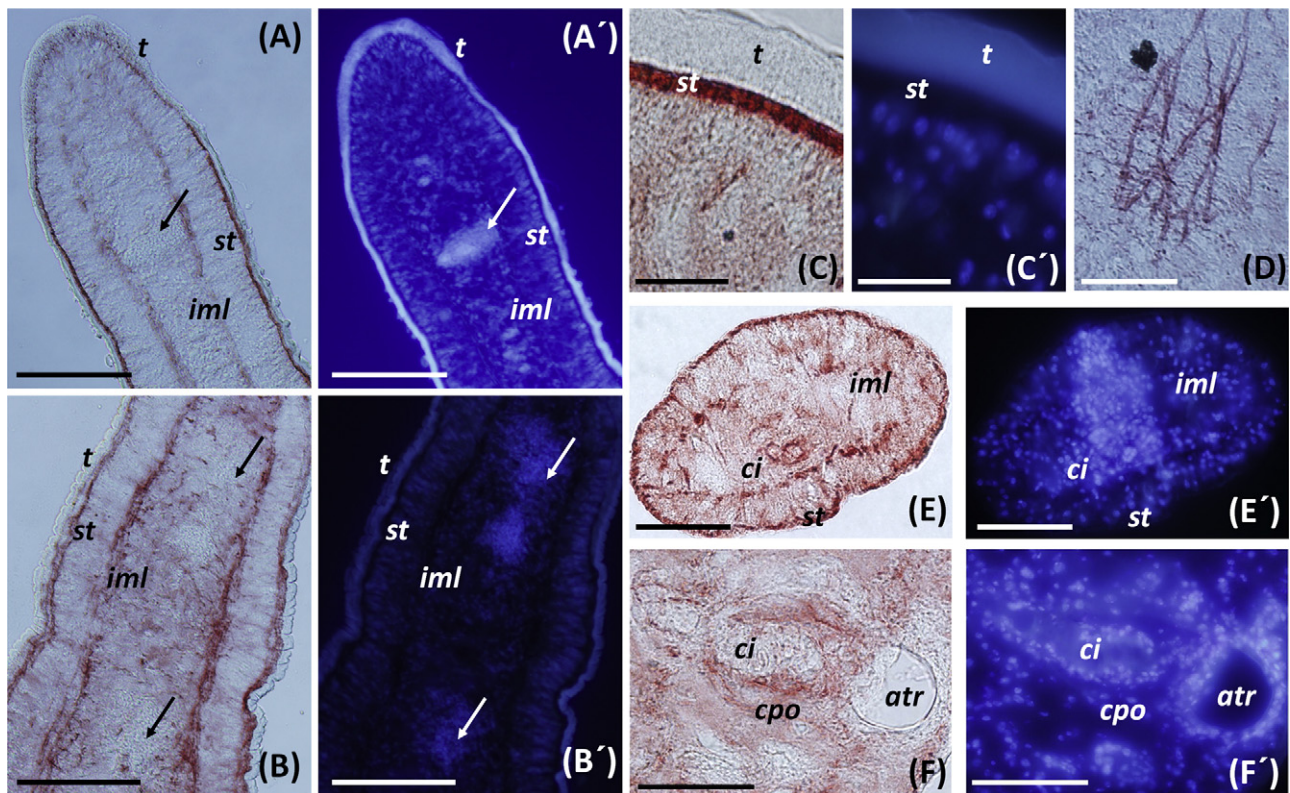


Fig. 6. Immunohistochemical staining of *M. corti* during different developmental stages of segmentation with anti-rEgTrp serum. (A). Longitudinal oblique section through early genital primordium (arrow). (B). Longitudinal section through late genital primordium (arrows); notice how the primordium is divided into anterior and posterior regions. (C) Detail of staining in the sub-tegumental muscle layer. (D) Detail of individual fibers in the inner muscle layer. (E) Transverse section through a developing cirrus. (F) Transverse section through developed cirrus and cirrus pouch. (A', B', C', E' and F') are paired fluorescence microscopy images of DAPI staining. Abbreviations are as in Figs. 4 and 5. Bars represent 100 μm (A, B, C, D, E, F) or 20 μm (A', B', C', D', E', F').

homo- or heterodimers may form. Dimer formation is not stable between HMW and LMW isoforms, therefore limiting the possible combinations [3].

4.2. Developmental distribution of F-actin and expression of HMW TPMs

From our results, it is clear that both phalloidin and HMW TPMs can be successfully used as markers for staining muscle fibers during the development of *M. corti*. Phalloidin staining was previously described in detail in *M. corti* tetrathyridia [34] but never for the later development of the reproductive ducts. Similar patterns of phalloidin staining have been described for the muscle layers, excretory system and reproductive ducts and gonads in *Diphyllbothrium dendriticum* [35], although in this species the development of the body muscular layers and muscle fibers of the reproductive organs is much more pronounced.

Phalloidin staining was considerably more sensitive than anti-HMW TPM immunohistochemistry, probably because it was coupled to a fluorophore while HMW TPMs were detected by a color developing enzymatic reaction. On the other hand, because other F-actin rich structures such as flame cells are strongly stained with phalloidin, it also is less specific for muscle fibers, while HMW TPM isoforms are almost exclusively expressed in muscle fibers, although a low level of expression was detected in the parenchyma. It is likely that non-muscular, LMW isoforms are localized to F-actin rich structures such as flame cells and in the cytoplasm of most cells, in which HMW isoforms are not found.

This and recent previous work [20] provide a detailed description of the development of the genital primordium into male and female genital primordia, and the later differentiation into the cirrus, cirrus pouch, and female ducts. The morphology of the cirrus and cirrus pouch was similar to previous descriptions, being weakly muscular and containing a small dilation (seminal vesicle) in the base of the cirrus [36]. The origin of both male and female primordia from a single original genital primordium was schematically proposed for *M. corti* by Thompson et al. [21] from observations of worms developing *in vitro* and *in vivo* (unlike the sequential formation of male and female genital primordia proposed by Barrett et al. [37]). Development of male and female genital ducts by division of one single mass of cells (named genital rudiment, somatic *anlagen* or somatic primordium, depending on the description) has also been demonstrated for the nematode *Cylindrotaenia diana* [38], the hymenolepidids *Hymenolepis diminuta*, *Diploposthe laevis* and *Diploposthe bifaria* [39,40] and the taeniid *E. granulosus* [41]. In these species, which possess lateral genital pores, this primordium grows from the center of the parenchyma towards the lateral margin of the body wall, and divides approximately dorso-ventrally into male and female primordia of the reproductive ducts. In *M. corti*, which has a dorsal genital pore, the primordium divides antero-posteriorly.

We have been unable to study the development of the ovaries and vitellaria in *M. corti*, because further development of the proglottids to maturation was not reproducibly reached under our *in vitro* culture conditions. The origin of cells forming the rudiments of the female gonads has been proposed to be in the cells of the genital primordium for *C. diana*, *Hymenolepis diminuta*, *D. laevis* and *D. bifaria* [38–40]. On the other hand, the origin of the cells of the testes primordia has been proposed to be from cells outside the main genital primordium [39,40] or from cells from the genital primordium [38]. In *M. corti*, testes primordia appear to the sides of the late genital primordium [20,21,37,42], and grow by *in situ* proliferation [20], but the origin of these cells is unknown. A detailed, experimentally determined fate map for the early cestode proglot-

tid in general, and for the genital primordium in particular, is lacking.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2010.11.009.

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