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Protein kinase A signalling in *Schistosoma mansoni* cercariae and schistosomules

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Abstract

Cyclic AMP (cAMP)-dependent protein kinase/protein kinase A (PKA) regulates multiple processes in eukaryotes by phosphorylating diverse cellular substrates, including metabolic and signalling enzymes, ion channels and transcription factors. Here we provide insight into PKA signalling in cercariae and 24 h in vitro cultured somules of the blood parasite, *Schistosoma mansoni*, which causes human intestinal schistosomiasis. Functional mapping of activated PKA using anti-phospho PKA antibodies and confocal laser scanning microscopy revealed activated PKA in the central and peripheral nervous system, oral-tip sensory papillae, oesophagus and excretory system of intact cercariae. Cultured 24 h somules, which biologically represent the skin-resident stage of the parasite, exhibited similar activation patterns in oesophageal and nerve tissues but also displayed striking activation at the tegument and activation in a region resembling the germinal 'stem' cell cluster. The adenylyl cyclase activator, forskolin, stimulated somule PKA activation and produced a hyperkinesia phenotype. The biogenic amines, serotonin and dopamine known to be present in skin also induced PKA activation in somules, whereas neuropeptide Y (NPY) or [Leu^{31},Pro^{34}]-NPY attenuated PKA activation. However, NPY did not block the forskolin-induced somule hyperkinesia. Bioinformatic investigation of potential protein associations revealed 193 medium confidence and 59 high confidence PKA interacting partners in *S. mansoni*, many of which possess putative PKA phosphorylation sites. These data provide valuable insight into the intricacies of PKA signalling in *S. mansoni* and a framework for further physiological investigations into the roles of PKA in schistosomes, particularly in the context of interactions between the parasite and the host.

Keywords: Cyclic AMP (cAMP)-dependent protein kinase/protein kinase A; Cercariae; Schistosomule; Neuropeptide Y; Dopamine; Serotonin (5-HT); STRING

1. Introduction

The human blood parasite *Schistosoma mansoni* possess ~252 protein kinases (Berriman et al., 2009; Andrade et al., 2011), however their functional roles and mechanisms of action are not well understood, particularly in the context of host-parasite interactions. Within the eukaryotic protein kinase super-family, cyclic-AMP (cAMP)-dependent protein kinase/protein kinase A (PKA) is one of the best characterized (Pidoux and Taske, 2007). Regulation of PKA activity in humans is achieved through mechanisms including the non-covalent coupling of catalytic (C) subunits and regulatory (R) subunits to form a tetrameric holoenzyme, phosphorylation of residues in the C subunit, and compartmentalization by A-kinase-anchoring proteins (AKAPs) (Cauthron et al., 1998; Nolen et al., 2004; Kim et al., 2007; Romano et al., 2009). Ligand/G-protein coupled receptor (GPCR) interaction and subsequent activation of adenylyl cyclase produces cAMP that binds R subunits causing a conformational change in the holoenzyme that unleashes the C subunits. Phosphorylation of a threonine residue (Thr197) within the C activation loop by phosphoinositide-dependent protein kinase 1 (PDK1) or by another C subunit is crucial to enzyme activation, whereas phosphorylation on Ser338 in the C-terminal tail supports PKA processing/maturation (Cauthron et al., 1998; Cheng et al., 1998; Romano et al., 2009; Keshwani et al., 2012). When activated, PKA phosphorylates serine/threonine residues in defined substrate proteins that possess the consensus motif (K/R)(K/R)X(S*/T*). In humans >1000 putative PKA substrates exist (Keshwani et al., 2012; Imamura et al., 2014) that include transcription factors (Sands and Palmer, 2008), metabolic enzymes and signalling proteins (Bornfeldt and Krebs, 1999; Natarajan et al., 2006; Bachmann et al., 2013). Thus, PKA controls a plethora of biological functions (Shabb, 2001; Gold et al., 2013). PKA has been identified as a potential drug target in *S. mansoni* (Swierczewski and Davies, 2009) and is highly conserved between the three main species of schistosome (*S. mansoni*, *Schistosoma japonicum* and *Schistosoma*

haematobium) that cause human schistosomiasis (Swierczewski and Davies, 2010a), a disease that results from eggs released by mature female worms becoming trapped in host tissues (Walker, 2011). Human schistosomiasis is endemic in 76 developing countries with ~230 million people infected and ~0.75 billion at risk (Steinmann et al., 2006; Colley et al., 2014).

When schistosome cercariae locate their definitive host they attach to and penetrate the skin, shed their tails and rapidly transform into schistosomulae (somules) (Walker, 2011). The somules then navigate within the epidermis before they cross the stratum basal (Curwen and Wilson, 2003; Grabe and Haas, 2004), enter the dermal vasculature, migrate within the blood stream and further develop. As the parasite tunnels through the skin significant cellular damage, apoptosis (Hansell et al., 2008) and inflammatory reactions ensue (Mountford and Trottein, 2004). Concurrently, the parasite undergoes physiological and biochemical developmental changes that enable it to circumvent the immune responses and survive (Gobert et al., 2010; Parker-Manuel et al., 2011). Molecular signalling from the host to the parasite likely plays an important part in the behaviour and survival of the parasite during skin penetration and migration, but such interactions are not well understood.

Recently, we characterised PKA activation in adult male and female *S. mansoni* and discovered that PKA plays an important role in neuromuscular communication in these worms (de Saram et al., 2013). In the current paper we provide valuable insights into the precise locations of functionally activated PKA in intact cercariae and 24 h in vitro cultured somules that model the skin stage of the parasite and identify putative interacting partners of this kinase. Further, we demonstrate that human neurotransmitters that are known to be present in the skin can differentially modulate PKA activation within these early stage somules, opening the possibility such host molecules could 'switch' PKA signalling 'on' and 'off' in the parasite during skin invasion.

2. Materials and methods

2.1. Parasite material

Biomphalaria glabrata snails infected with *S. mansoni* (Strain: NMRI) were provided by the NIAID Schistosomiasis Resource Center of the Biomedical Research Institute (Rockville, MD, USA). When patent, snails were placed under a light source and emergent cercariae collected. Cercariae were then either immediately fixed for immunohistochemistry or were mechanically transformed into somules using an adaptation of various published methods (Ramalho-Pinto et al., 1974; Keiser, 2010; Milligan and Jolly, 2011; Tucker et al., 2013). Collected cercariae were transferred to 15 ml Falcon tubes, placed on ice for 15 min and pelleted at 100 *g* for 5 min. All but ~1 ml of supernatant was discarded and Eagles Basal Medium (BME) containing antibiotics/antimycotics (Sigma, UK) added to ~4 ml; tubes were mixed to re-suspend cercariae and placed at 37°C to encourage cercarial movement. The cercariae were then vortexed for 5 min. To remove the detached tails Hanks Basal Salt Solution (HBSS) was added to a total volume of ~7 ml and tubes placed on ice for 7 min and re-centrifuged for 2 min; this process was then repeated. Supernatant was then removed, warmed BME added, and the suspension swirled in a high-walled glass Petri dish to concentrate 'heads' into the center of the dish. The 'heads' were then collected, enumerated, transferred to individual wells of a 24 well culture plate (Nunc; ~1000 'heads'/1 ml of BME containing antibiotics/antimycotics), and incubated in 5% CO_2 at 37°C.

2.2. Pharmacological assays, protein extraction and SDS-PAGE/western blotting

Somules (~1000), cultured in BME for 24 h from initial transformation, were exposed to the following compounds for increasing durations: forskolin (50 μM; Calbiochem, UK); dopamine or serotonin (5-hydroxytryptamine; 5-HT) (each at 1 μM, 10

μM or 25 μM; Sigma); and NPY or (Leu^{31}Pro^{34})-NPY (each at 1 μM, 10 μM or 25 μM; Tocris, R&D Systems, UK). At each time point, somules were transferred immediately to microfuge tubes on ice for 5 min and pulse centrifuged. Pelleted somules were then lysed in SDS-PAGE sample buffer (Pierce, UK, Thermo Fisher Scientific, UK) and samples heated to 90°C for 5 min. Protein extracts were obtained from cercariae by lysing pelleted cercariae in a similar manner. Samples were then either electrophoresed immediately or were stored at -20°C, in which case HALT protease/phosphatase inhibitors (Pierce) were added. SDS-PAGE/western blotting were carried out using 10% Precise Precast gels (Pierce) as previously described (Ressurreição et al., 2011a, b). Briefly, electrophoresed proteins were semi-dry transferred to nitrocellulose membranes, stained with Ponceau S (Sigma), blocked in 1% BSA (Sigma) for 1 h, then incubated in either anti-phospho PKA-C (Thr197) or anti-phospho PKA substrate motif antibodies (each 1:1000 in tween tris-buffered saline (TTBS) containing 1% BSA; Cell Signalling Technology (CST), New England Biolabs, UK) overnight at 4°C on a rocking platform. For detection, blots were incubated for 2 h in horse-radish peroxidase (HRP)-conjugated secondary antibodies (1:3000 in TTBS; CST) and immunoreactive bands visualized using West Pico chemiluminescence substrate (Pierce) and a GeneGnome CCD chemiluminescence imaging system (Syngene, UK). After stripping blots with Restore Western Blot Stripping Buffer (Pierce), HRP-conjugated anti-actin antibodies (1:3000 in TTBS; Santa Cruz Biotechnology, UK) were used to assess protein loading differences; GeneTools (Syngene) was used to quantify band intensities and phosphorylation levels were normalized against differences in signal between samples.

2.3. Immunohistochemistry

Cercariae or 24 h in vitro cultured somules were fixed in acetone on ice and stored at 4°C. They were then briefly washed in PBS, further permeabilized in 0.3% Triton X-100 in PBS for 1 h, washed twice each for 15 min and blocked for 2 h in 10% goat serum. After two further 10 min washes, samples were incubated in either anti-phospho PKA-C (Thr197) or anti-phospho PKA substrate motif antibodies (1:50 in 1% BSA in BS) for 3 days at 4°C. Parasites were then washed three times for 30 min each in PBS before incubating in AlexaFluor 488 secondary antibodies (1:500 in PBS; Invitrogen, UK) and 2 µg/ml of rhodamine phalloidin for 2 days at 4°C followed by two 30 min washes in PBS. Control parasites were prepared in a similar fashion but without primary antibodies. Parasites were mounted onto silane prep slides (Sigma), covered with Vectashield (Vecta Laboratories, UK), sealed with clear nail polish and visualized on a Leica TCS SP2 AOBS confocal laser-scanning microscope using 40x or 63x oil immersion objectives.

2.4. Somule movement analysis

The effect of either forskolin (50 µM), NPY or [Leu^{31}Pro^{34}]-NPY (each at 10 µM) on the movement of 24 h in vitro-cultured somules was assessed. With forskolin, 30 s movies were captured at 0 min (control) and at various time points thereafter over a 30 min period using a Canon EOS 1100D camera attached to a binocular dissecting microscope. For NPY or [Leu^{31}Pro^{34}]-NPY, somules were incubated with either peptide for 2 h and then exposed to forskolin for 10 min at which time 30 s movies were captured. Movies were then visualized through captured frames using ImageJ for Windows (http://rsbweb.nih.gov/ij/), and the number of gross muscular contractions that each somule made at each time point determined; a gross muscular contraction was defined as when a somule extended in length (by ~20% or more) and contracted. In addition, the custom ImageJ plugin, wrMTrck (http://www.phage.dk/plugins) was used to determine the

177 average speed of movement of the somules (contractions; Length/Time (pixels/s)
178 parameter) at each time point following exposure to forskolin.
179
180 *2.5. Bioinformatics*
181 The protein sequence for the *Homo sapiens* NPY Y1 receptor (NPY1R; **NCBI**
182 **P25929.1**) was retrieved from National Centre Biotechnology Information (NCBI), USA
183 (http://www.ncbi.nlm.nih.gov/protein) and a Basic Local Alignment Search Tool (BLAST)
184 search performed against *S. mansoni* protein sequences held within GeneDB
185 (http://genedb.org/Homepage) (Logan-Klumpler et al., 2012). Uniprot Align
186 (http://www.uniprot.org/align/) was used to generate a pairwise alignment of *H. sapiens*
187 and *S. mansoni* sequences and the seven transmembrane spanning regions were
188 predicted using TMHMM (http://www.cbs.dtu.dk/services/TMHMM/).
189 Potential interactions between *S. mansoni* PKA and other *S. mansoni* proteins
190 using Smp_152330 as the input sequence were predicted using Search Tool for Retrieval
191 of Interacting Genes (STRING; version 10) (von Mering et al., 2005; Szklarczyk et al.,
192 2011) in 'protein' mode. Interaction clusters were visualized using the KMEANS algorithm.
193 Generation of specific interaction maps was achieved using Gene Ontology (GO)
194 assignments within STRING. Sequences of the identified proteins were then submitted to
195 KinasePhos (http://kinasephos.mbc.nctu.edu.tw) (Huang et al., 2005), limiting
196 phosphorylation to Ser and Thr residues with PKA as kinase and with 100% prediction
197 specificity. Phosphosite analysis was also performed using pkaPS
198 (http://mendel.imp.ac.at/pkaPS/) (Neuberger et al., 2007) limited to 'only good hits'.
199 Putative phosphorylated peptides were then submitted to Seq2Logo to generate a
200 probability weighted Kullback-Leibler (with hobohm1 clustering) representation of the
201 phosphorylated sequences (http://www.cbs.dtu.dk/biotools/Seq2Logo/) (Thomsen and
202 Nielsen, 2012).

2.6. Statistical analysis

Where appropriate raw data were subjected to ANOVA using Fisher's multiple comparison post-hoc test with Minitab 15.

3. Results

3.1. PKA activation in S. mansoni cercariae and somules, and effects on somule muscular activity

Previously, we employed 'smart' phospho-specific PKA (Thr197) antibodies to map phosphorylated (activated) PKA in adult *S. mansoni* (de Saram et al., 2013). These antibodies recognize only the activated form of PKA in *S. mansoni* because the sequence (RVKGRTWTLCGT) including/surrounding this activation loop residue is conserved between *S. mansoni* and humans, and because phosphorylation at this residue is crucial for PKA maturation, optimal conformation and catalytic activity (Yonemoto et al., 1997; Kim et al., 2007; Walker et al., 2014). Treatment of western blots of *S. mansoni* lysates with lambda phosphatase leads to complete loss of immunoreactivity, demonstrating the phospho-specificity of these antibodies towards *S. mansoni* PKA (de Saram et al., 2013). Here, these antibodies detected an immunoreactive band at approximately 42 kDa in cercariae and 24 h in vitro cultured somules, with an additional band sometimes observed at ~40 kDa in the latter life stage (Fig. 1A), which likely represents an additional PKA-C, or splice variant thereof, as seen with adult worms (de Saram et al., 2013). This double phosphorylated PKA banding was not, however, always observed in 24 h cultured somules. Given the extreme differences in niches experienced by cercariae (freshwater, ambient temperature) and somules (human tissue, 37°C) we hypothesised that PKA might phosphorylate different downstream substrates and attempted to visually resolve potential differences using anti-phospho PKA substrate motif antibodies that detect PKA-preferred

proteins containing a Ser/Thr residue with Arg at the -3 position. The overall banding pattern of PKA-preferred phosphorylated substrates was, however, broadly similar between these distinct life stages (Fig. 1A). Somules were then exposed to the adenylyl cyclase activator, forskolin (50 μM), to ascertain whether PKA could be activated beyond the basal levels observed, and a significant, ~65 – 70 %, stimulation of PKA phosphorylation (activation) was seen at 10 and 30 min ($P \leq 0.001$) (Fig. 1B). In addition, it was apparent that forskolin induced a highly contractile hyperkinesia phenotype. Thus, 24 h somules were exposed to forskolin and movies captured (example Supplementary Movies S1, S2). Visual analysis of movie frames in ImageJ revealed that forskolin significantly enhanced somule contractions as early as 5 min ($P \leq 0.01$) when the mean number of contractions increased from 1.5/30 s in controls to 4.7/30 s in exposed somules ($P \leq 0.01$; Fig. 1C). Thereafter, mean contractions peaked to 17.7/30 s at 20 min ($P \leq 0.001$) and declined slightly at 30 min (Fig. 1C). Further analysis of somules with the ImageJ wrMTrck plugin revealed that the mean average speed of somule movement increased from 10.9 pixels/s in the untreated controls to 13.7 and 13.9 pixels/s at 1 and 5 min, respectively, peaking at 27.3 pixels/s at 10 min ($P \leq 0.01$) and declining thereafter (to 16.0 pixels/s at 30 min). Thus, forskolin induces a transient increase in both number and speed of contractions with maximal effects observed at 10 – 20 min.

3.2. In situ distribution of activated PKA, and PKA-preferred substrates, within S. mansoni cercariae and somules

Activated PKA was next localized within intact cercariae and 24 h somules to 'functionally map' the kinase within the parasite. PKA substrates were also mapped using anti-phospho PKA substrate motif antibodies. In all cases and across multiple experiments, negative control cercariae and somules displayed minimal background staining (e.g. Fig. 2A). In contrast, labelling of cercariae with anti-phospho PKA (Thr197)

antibodies and analysis of image projections/individual confocal z-sections revealed activated PKA associated with the CNS including the cephalic ganglia and longitudinal and ventral nerve cords (Fig. 2B, C); the nerve net of the acetabulum (Fig. 2B) and the sensory papillae at the oral tip also displayed activated PKA (Fig. 2D). In addition, activated PKA was seen associated with the oesophagus, excretory duct and nephridiopore (Fig. 2B - E), and deep scanning revealed activated PKA associated with the protonephridial tubules in the head region (Fig. 2C) that joined the excretory duct at the head-tail junction (Fig. 2E). Cercariae labelled with anti-phospho PKA substrate motif antibodies displayed broadly similar immunoreactivity to those stained with anti-phospho PKA (Thr197) antibodies (Fig. 2F - I); however, in addition, striking immounoreactivity was seen in the anterior cone (oral sucker; Fig. 2F), the tail muscle was clearly labelled (Fig. 2G, I), and the excretory system was particularly well defined (Fig. 2G-I). This somewhat broader immunoreactivity is presumably due to the anti-phospho PKA substrate motif antibodies detecting many more targets than the anti-phospho PKA antibodies (Fig. 1A), resulting in increased sensitivity.

While the cephalic ganglia and acetabular region of 24 h in vitro cultured somules also displayed activated PKA (Fig. 3A, B), other nervous tissue was less well stained compared with cercariae (Fig. 2B). In addition, there was striking activation of PKA at the somule tegument, particularly anteriorly (Fig. 3B, C, G - I), with considerable activation also evident in the sub-tegument regions revealed by deep scanning (Fig 2B, C). Moreover, PKA activation was evident along the length of oesophagus/rudimentary gut (Fig. 3C) and was seen in the area where a population of totipotent stem cells (Wang et al., 2013), also known as germinal cells, are located (Fig. 3B, and for individual z-sections, Fig. 3D-F). Putative PKA-preferred substrates were phosphorylated in regions including the tegument/sub-tegument (Fig. 3J, K), gland ducts, anterior cone, cephalic ganglia, acetabulum including the acetabular musculature (Fig. 3K, L), flame cells and network of protonephridial tubules (Fig. 3J, K, M).

3.3. PKA activation is stimulated by 5-HT and dopamine, and attenuated by NPY or [Leu^{31}Pro^{34}]-NPY in S. mansoni somules

The biogenic amines (BAs) 5-HT and dopamine exist in the nervous system/other tissues of *S. mansoni* and affect somule motility (El-shehabi et al., 2012; Ribeiro et al., 2012; Patocka et al., 2014). Receptors for these BAs also exist (Taman and Ribeiro, 2009; El-shehabi et al., 2012; Ribeiro et al., 2012; Patocka et al., 2014) and a transport system is intact enabling BA inactivation/recycling and possible transport/uptake of host 5-HT/dopamine across the tegument (Ribeiro and Patocka, 2013). Therefore, given the tegumental and neural localization of activated PKA in somules and the effects of forskolin on somule movement observed here, we hypothesized that these BAs might modulate PKA activation in somules. In vitro cultured somules were exposed to 5-HT and dopamine at increasing doses (5 μM, 10 μM and 25 μM) and PKA phosphorylation increased noticeably at ≥10 μM for either BA (data not shown). Somules were thus exposed to 10 μM 5-HT or dopamine for increasing durations, with a forskolin positive control. For 5-HT this concentration is approximately 10 times greater than the concentration present in the blood (Weiss et al., 2005) and, although blood levels of dopamine are lower, they can reach the low μM range in dopamine-producing nerve tissues (Zeng and Jose, 2011). Whereas increased PKA phosphorylation was apparent after 10 min BA exposure, digital analysis of blots revealed activation was only enhanced significantly for both BAs at 30 min, with 77% and 55% increases seen for 5-HT and dopamine, respectively ($P \leq 0.05$) (Fig. 4).

Schistosomes also express neuropeptide F (NPF) that is structurally similar to vertebrate NPYs, with a C-terminal Arg-X-Arg-Phe-amide motif resembling that of vertebrate NPY family members (Arg-X-Arg-Phe/Tyr-amide) and conserved tyrosyls at positions 10 and 17 (Humphries et al., 2004; McVeigh et al., 2009). Because porcine NPY

(as well as *S. mansoni* NPF) supressed cAMP accumulation in schistosome homogenates (Humphries et al., 2004), we searched the *S. mansoni* sequences in GeneDB for an NPY receptor-like protein with similarity to the *H. sapiens* NPY1R protein sequence using BLAST. This search revealed a putative *S. mansoni* NPY receptor (**Smp_118040**; GenBank: **AAQ57211.1**) (Fig. 5) with a predicted molecular mass of ~57 kDa, annotated electronically as a neuropeptide receptor of the rhodopsin-like 7-transmembrane GPCR family. Pair-wise alignment of human NPY1R with **Smp_118040** revealed the conservation of residues identified in humans as being important to ligand (NPY agonist, or antagonist) binding and receptor activation by NPY (Du et al., 1997) (Fig. 5). Interestingly, transcriptomic data available at GeneDB reveal that the expression of this receptor is strikingly upregulated in the 3 h and 24 h somule compared with cercariae or adult worms (relative normalized reads: cercariae <0.1; 3 h somule > 0.8; 24 h somule 1.0; adult worm < 0.05). Given these findings, we explored whether human NPY could modulate phosphorylation (activation) of *S. mansoni* PKA when applied exogenously to intact 24 h somules. To test this, we employed NPY which targets several NPY receptor subtypes in humans, and a modified [$Leu^{31}Pro^{34}$]-NPY which is a potent Y1-selective receptor agonist (Fuhlendorff et al., 1990). Initially, somules were exposed to 5, 10 or 25 µM [$Leu^{31}Pro^{34}$]-NPY for 1 h and proteins processed for western blotting. Results were somewhat variable but, often, apparently reduced phosphorylation was observed at 1 h with 10 or 25 µM [$Leu^{31}Pro^{34}$]-NPY (data not shown). Therefore, somules were exposed to 10 µM [$Leu^{31}Pro^{34}$]-NPY or NPY for increasing durations. Analysis of blots revealed a time-dependent reduction of mean phosphorylation levels with either peptide, with [$Leu^{31}Pro^{34}$]-NPY attenuating activation by 33, 36, and 41% at 30, 60 and 120 min ($P \leq 0.05$; $P \leq 0.01$ at 60 and 120 min), and NPY by 36 and 45% at 60 and 120 min ($P \leq 0.05$), respectively (Fig. 6). Although an apparent increase in phosphorylation was observed after 5 min exposure,

this was not consistent across all blots and analysis revealed no significant change at this time point (Fig. 6).

Next we tested the ability of [Leu^{31}Pro^{34}]-NPY or NPY to block forskolin-induced somule movement by pre-incubating somules in these neuropeptides prior to the addition of forskolin. Despite the apparent inhibitory effects of [Leu^{31}Pro^{34}]-NPY or NPY on PKA activation (Fig. 6), no discernable affect on somule motility was observed (data not shown).

3.4. Network analysis of S. mansoni proteins reveals 59 'high confidence' PKA interacting partners

Finally, to discover potential interacting partners of PKA in *S. mansoni*, we interrogated the STRING database with **Smp_152330**, a PKA recently shown to be expressed in *S. mansoni* somules (Sotillo et al., 2015). The medium confidence (STRING Global Score ≥ 0.40) 'hit' list comprised 193 putative interacting proteins (Supplementary Table S1). Further analysis revealed 59 high confidence interactions (STRING Global Score ≥ 0.70), which were next constrained to eight interaction clusters using the KMEANS algorithm to visualize proteins that 'clustered' together (Fig. 7A). Next, a predictive interaction map was generated, with five KMEANS clusters, for the GO Biological Process 'Signal Transduction' ($P = 1.03e^{-8}$) which was superimposed onto PKA (**Smp_152330**) (Fig. 7B). Putative interactions between PKA and 18 other proteins were retrieved including interactions with nine cAMP/cGMP-specific 3,5-cyclic phosphodiesterases, three adenylate/guanylate cyclases, a Ral GTPase and a Ras GTP exchange factor (Son of Sevenless) important in monomeric g-protein signalling, a serine/threonine kinase (extracellular signal-regulated kinase (ERK)), β-catenin, a peptidase and a hepatocyte nuclear factor (Fig. 7B; with identifiers in Supplementary

Table S1). Finally, the Biological Process 'Regulation of Protein Phosphorylation' mapped five putative cAMP-dependent protein kinase regulatory sub-units onto PKA (Fig. 7C).

The amino acid sequences of proteins from the 'high confidence' list were then screened for possible PKA phosphorylation sites using the computational phosphorylation prediction tool KinasePhos. Of the 59 proteins, 41 were predicted to possess one or more potential PKA phosphorylation sites with 104 sites (32 Ser/72 Thr) predicted in total (Supplementary Table S2); motif analysis using Seq2Logo revealed the preponderance of the canonical PKA phosphorylation motif (K/R)(K/R)X(S*/T*) (Fig. 7D). Furthermore, 63 of these phosphorylation sites were also predicted to be PKA phosphorylation sites using an alternative prediction tool, pkaPS (Supplementary Table S2). However, of the two tools, pkaPS predicted many more potential sites (551) overall amongst the 59 proteins, including in 12 of the 18 proteins predicted by KinasePhos to have no PKA phosphorylation sites (data not shown).

4. Discussion

PKA signalling/function has been characterised in many eukaryotes, including a number of parasites (Abel et al., 2001; Bao et al., 2008; Kurokawa et al., 2011). In schistosomes, the PKA-C subunit is 99% identical (at amino acid level) between *S. mansoni*, *S. japonicum* and *S. haematobium* (Swierczewski and Davies, 2010a) and seems essential for survival (Swierczewski and Davies, 2009, 2010b) and motor activity (de Saram et al., 2013), indicating that it might be a viable drug target. In the current study we characterized PKA signalling in cercariae and in 24 h in vitro cultured somules that biologically represent the 'skin-resident' stage of the parasite (Protasio et al., 2013), and have evaluated effects of host molecules on somule PKA activation. Moreover, we have computationally identified a number of putative PKA interacting partners.

Functional mapping of phosphorylated PKA within cercariae revealed the activated kinase associated with the central and peripheral nervous systems including the nerve net of the acetabulum and sensory papillae at the oral tip. Activation within the nervous system of somules agrees with that recently found in adult worms (de Saram et al., 2013), whereas activation at the oral tip suggests an involvement of PKA in sensory perception during host detection and, possibly, invasion. Cercariae of *S. mansoni* are attracted/respond to a range of host skin molecules including fatty acids (Shiff and Graczyk, 1994; Haas et al., 2008; Haeberlein and Haas, 2008) and it is plausible that PKA mediates cercarial adaptive responses to such compounds warranting further investigation. In this context, we have recently discovered that the skin molecule linoleic acid activates ERK and protein kinase C in *S. mansoni* cercariae concurrently with acetabular gland release (Ressurreição et al., 2015). Activated PKA was also found associated with the protonephridial system and excretory ducts and, although PKA-mediated regulation of excretory processes has not been not reported in flatworms, PKA does participate in transepithelial ion transport in insect malpighian tubules (Tiburcy et al., 2013).

While activated PKA also localized to the CNS of 24 h somules, striking activation was seen at the tegument suggesting the possibility that this kinase mediates host-parasite interactions. During schistosome migration through the skin, damage to host tissue occurs (Hansell et al., 2008) and the schistosome develops biochemically and physiologically into a competent endoparasite. The skin, richly innervated with sympathetic nerves, is an active neuroendocrine organ and the presence of several neuropeptides/neurotransmitters/other bioactive molecules such as 5-HT and dopamine is due to local synthesis and active transport from blood and release from immune cells/nerve endings (Slominski et al., 2002; Zmijewski and Slominski, 2011). 5-HT is also released during skin tissue damage where it helps sustain homeostasis (Mann and

Oakley, 2013). Thus, given the tegumental localization of activated PKA in 24 h somules, we examined whether host dopamine and 5-HT might modulate PKA activation in somules and demonstrated enhanced activation after exposure to these BAs. *Schistosoma mansoni* also expresses 5-HT and dopamine receptors such as Sm5HTR and SmGPR-3, respectively (Ribeiro et al., 2012; Patocka et al., 2014), and although these two receptors localize largely to nerves and sub-tegumental tissues of somules, other receptors likely exist that could bind these BAs and, similar to the histamine receptor (El-Shehabi et al., 2009), may be expressed in the somule tegument. NPY is also expressed in skin and regulates cutaneous wound healing, particularly during the inflammatory and proliferation/migration phase (Chéret et al., 2013). A BLASTp search of the *S. mansoni* genome revealed a putative NPY receptor and alignment with human NPYR1 revealed that many of the proposed NPY interaction sites are conserved in the *S. mansoni* protein including Gln219 (Gln203 in *S. mansoni*), the site thought to be critical for receptor activation (Du et al., 1997). Thus, the finding that expression of a putative NPY receptor is greatly upregulated in 3 h and 24 h somules and that exogenous NPY or (Leu^{31}Pro^{34})-NPY downregulate PKA activation in 24 h somules might be important for the physiological response of the parasite during skin invasion and migration. Curiously, 5-HT and dopamine have opposing effects on *S. mansoni* somule motility in vitro, such that 5-HT is myoexcitatory whereas dopamine is inhibitory (El-Shehabi et al., 2012; Patocka et al., 2014) and relaxes body wall muscles; this is despite their apparently similar effects on PKA activation seen in the current research. Therefore, because PKA activation by forskolin increases somule motility, 5-HT and dopamine likely differentially influence pathways as well as PKA that are coupled to the motile response; indeed, such specificity in PKA signalling is well documented in eukaryotes (Taskén and Aandahl, 2004; Pidoux and Taske, 2007). Furthermore, NPY did not markedly affect forskolin-induced somule motility despite it being able to block forskolin induced cAMP production in *S. mansoni*

434 homogenates (Humphries et al., 2004). While this could be due to the activation of
435 adenylyl cyclase by forskolin being more potent than GPCR-mediated NPY inhibition of
436 adenylyl cyclase, further research is required to study the effects of NPY on intact
437 schistosomes. Taken together, however, an apparent differential regulation by the BAs
438 dopamine/5-HT and NPY has been demonstrated here in vitro. The full physiological
439 significance of such host ligand-mediated activation/deactivation of PKA in somules and
440 whether such modulation occurs in vivo requires further investigation, and it is plausible
441 that responses could be involved in the regulation of multiple processes in addition to
442 movement during host skin invasion and migration. Additionally, the importance of PKA
443 activation in cells that resemble the germinal/stem cells of somules warrants further
444 investigation, particularly as PKA is a possible drug target in schistosomes (Swierczewski
445 and Davies, 2009).

446 To obtain an overview of potential PKA protein associations in schistosomes we
447 took **Smp_152330**, recently shown to be expressed in *S. mansoni* somules (Sotillo et al.,
448 2015), as a 'model' PKA and interrogated the STRING database. This process mapped
449 193 medium confidence and 59 high confidence PKA interacting proteins, many of which
450 possessed one or more potential PKA phosphorylation sites as determined using
451 KinasePhos. Putative high-confidence interacting proteins such as troponin
452 (**Smp_018250.1**), heat shock protein (**Smp_072330.2**), cAMP-dependent protein kinase
453 type II-alpha regulatory subunit (**Smp_079010**) and calmodulin (**Smp_026560.2**) were
454 also recently detected in the tegument fraction of somules using proteomic approaches
455 (Sotillo et al., 2015). Moreover, the high confidence interacting protein dataset included
456 four heat shock/DnaJ-like proteins/factors, four protein kinases, nine leucine-rich repeat
457 containing proteins, three Shoc-2 proteins, and other signalling proteins such as TGF-β
458 family member, and a Ras GTP exchange factor and Ras suppressor protein 1 that would
459 be important to ERK signalling in the parasite (Ressurreição et al., 2014). Thus PKA

potentially interacts with a wide range of molecules that drive diverse functions in *S. mansoni*. Although STRING associations are largely derived from predictions or from transferring associations/interactions between organisms ('interlog' transfer) (von Mering et al., 2005; Szklarczyk et al., 2011) it provides a snapshot of possible interactions with associated probabilistic confidence scores. Thus, while it is premature to discuss individual interacting partners in terms of their possible functional significance in schistosomes, as they remain putative, the high confidence interactions described here provide a framework for developing hypotheses and designing experiments to answer important questions concerning PKA/interacting partner function in this parasite, perhaps at different life stages and in the context of host-parasite interactions. Such elucidation of signalling processes and interactions, and protein networks in schistosomes should help identify important proteins or 'nodes' that might be targeted in future anti-schistosome therapies.

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694 **Figure Legends**
695

696 **Fig. 1.** Detection of phosphorylated (activated) protein kinase A (PKA) and PKA-preferred
697 substrate proteins in *Schistosoma mansoni* cercariae and somules, and effect of PKA
698 activation on somule movement. (A) Protein extracts of cercariae or somules cultured for
699 24 h, (~1000 of each) were processed for western blotting with anti-phospho PKA (Thr197)
700 antibodies (Ab) or anti-phospho PKA substrate motif antibodies. Results are representative
701 of two independent experiments. (B) Twenty-four h cultured somules (~1000 per
702 treatment) were exposed to forskolin (50 µM) for increasing durations before processing
703 for western blotting with anti-phospho PKA antibodies; anti-actin antibodies were used to
704 assess protein loading between samples. Immunoreactive bands were analysed with
705 GeneTools and mean relative change (graph; $n = 8$, ± S.D.; normalised for actin) in PKA
706 phosphorylation calculated relative to the phosphorylation levels of untreated controls that
707 were assigned a value of 1 (dotted line). (C) Twenty-four h somules were incubated in
708 forskolin (50 µM) for increasing durations (0 – 30 min) and movies captured for 30 s at
709 time points shown; values represent mean number of somule contractions in 30 s at each
710 time point (± S.D.; $n = 60$ from three biological replicates). ** $P \leq 0.01$ and *** $P \leq 0.001$
711 compared with control values.
712

713 **Fig. 2.** In situ distribution of phosphorylated (activated) protein kinase A (PKA) and PKA-
714 preferred substrates in *Schistosoma mansoni* cercariae. Intact cercariae were fixed and
715 stained with anti-phospho PKA (Thr197) or anti-phospho PKA substrate motif antibodies
716 (Ab) followed by Alexa Fluor 488 secondary antibodies (green); D is an overlay of
717 activated PKA with F-actin stained by rhodamine phalloidin (red). Images show z-axis
718 projections in maximum pixel brightness mode. (A) Negative control cercaria incubated
719 without primary antibodies but with Alexa Fluor (488) secondary antibodies. (B) Regions of
720 activated PKA in a whole cercaria; (C, D, E) detailed scanning of activated PKA in the

721 head, oral tip and head/tail junction, respectively. (F, G/H, I) Phosphorylated PKA-
722 preferred substrates revealed in regions of the head, tail, and head/tail junction,
723 respectively. The region in F highlighted with the dotted line shows the anterior cone (oral
724 sucker). Bar = 20 µm.
725
726 **Fig. 3.** In situ distribution of phosphorylated (activated) protein kinase A (PKA) and PKA-
727 preferred substrates in *Schistosoma mansoni* 24 h in vitro cultured somules. Intact
728 somules were fixed and stained with anti-phospho PKA (Thr197) or anti-phospho PKA
729 substrate motif antibodies (Ab) followed by Alexa Fluor 488 secondary antibodies (green);
730 H and I show F-actin stained by rhodamine phalloidin (red) and phosphorylated PKA/F-
731 actin overlay, respectively. Images show z-axis projections in maximum pixel brightness
732 mode unless stated otherwise. (A) Regions of activated PKA in whole somules; (B, C)
733 deep scanning revealing activated PKA within somules; (D - F) serial optical sections
734 through the region resembling germinal cells (dashed boxes); and (G - I) surface scanning
735 revealing PKA activation at the somule tegument. (J - M) Phosphorylated PKA-preferred
736 substrates revealed by (J) partial scanning of whole somule, (K) single scan through
737 somule, and (L and M) deep scanning of mid and posterior regions of somule,
738 respectively. Bar = 20 µm.
739
740 **Fig. 4.** Exogenous human serotonin (5-hydroxytryptamine; 5-HT) or dopamine stimulates
741 protein kinase A (PKA) activation in 24 h in vitro cultured *Schistosoma mansoni* somules.
742 Somules (~1000 per treatment) were treated with forskolin (50 µM; positive control) for 30
743 min, 5-HT or dopamine (10 µM each) for 10 or 30 min, or were left untreated (0 min,
744 control) and proteins extracted and processed for western blotting with anti-phospho PKA
745 (Thr197) antibodies. Anti-actin antibodies were used to assess protein loading between
746 samples. Immunoreactive bands were analysed with GeneTools and mean relative change

747 (graph; $n = 4$, ± S.D.; normalised for actin) in PKA phosphorylation calculated relative to
748 the phosphorylation levels of untreated controls that were assigned a value of 1 (dotted
749 line). *$P \leq 0.05$ and **$P \leq 0.01$ compared with control values.
750
751 **Fig. 5.** Sequence alignment and analysis of *Schistosoma mansoni* neuropeptide receptor.
752 The *S. mansoni* putative neuropeptide receptor (NPR) sequence (GeneDB: **Smp_118040**)
753 was aligned with that for the *Homo sapiens* NPY Y1 receptor (NPY1R; NCBI: **P25929.1**)
754 using Uniprot Align. The seven transmembrane spanning regions (predicted using
755 TMHMM) are highlighted with red and green lines for human NPY1R and *S. mansoni*
756 NPR, respectively. Amino acid residues identified as being important for NPY1R ligand
757 (NPY agonist, or antagonist) binding that are conserved in *S. mansoni* NPR are
758 highlighted with boxes: blue, residues within 5Å from ligand; orange, residues beyond 5Å
759 from the ligand; and green, proposed interaction necessary for receptor activation (Du et
760 al., 1997).
761
762 **Fig. 6.** Exogenous human neuropeptide Y (NPY) or modified (Leu^{31},Pro^{34})-NPY suppress
763 protein kinase A (PKA) activation in 24 h in vitro cultured *Schistosoma mansoni* somules.
764 Somules (~1000 per treatment) were treated with 10 µM NPY or (Leu^{31},Pro^{34})-NPY for
765 increasing durations and proteins processed for western blotting with anti-phospho PKA
766 (Thr197) antibodies. Anti-actin antibodies were used to assess protein loading between
767 samples. Immunoreactive bands were analysed with GeneTools and mean relative change
768 (graph; $n = 3$ for NPY and $n = 5$ for (Leu^{31},Pro^{34})-NPY, ± S.D.; normalised for actin) in PKA
769 phosphorylation calculated relative to the phosphorylation levels of untreated controls that
770 were assigned a value of 1 (dotted line). *$P \leq 0.05$ and **$P \leq 0.01$ compared with control
771 values for each peptide.
772

773 **Fig. 7.** Network and phosphorylation analysis of putative protein kinase A (PKA)
774 interacting partners. Using an *Schistosoma mansoni* PKA sequence (**Smp_152330**; 351
775 amino acids, cAMP-dependent protein kinase catalytic subunit) shown by proteomics to be
776 present in somules (Sotillo et al., 2015), putative interacting partners were identified by
777 interrogating the STRING database. (A) High confidence (STRING Global Score ≥ 0.70)
778 interaction map in evidence mode with eight interaction clusters (each of different colour)
779 defined using the KMEANS algorithm; inter-cluster edges are shown with dashed lines,
780 interacting proteins with high Global Scores appear in the same cluster (colour).
781 **Smp_152330**, coloured blue, appears towards the network centre. (B) Interaction map of
782 proteins associated with the Gene Ontology (GO) Biological Process, 'Signal Transduction'
783 and mapped onto **Smp_152330**; five interaction clusters were defined with KMEANS. (C)
784 Proteins associated with GO term 'Regulation of Protein Phosphorylation' and mapped
785 onto **Smp_152330**. (D) Motif analysis generated by Seq2Logo (probability Weighted
786 Kullback-Leibler method) of putative PKA phosphorylation sites identified using
787 KinasePhos (Supplementary Table S2) in the 59 high-confidence STRING interacting
788 sequences.
789
790 **Supplementary Movie S1.** Movement of *Schistosoma mansoni* 24 h in vitro cultured
791 somules.
792
793 **Supplementary Movie S2.** Movement of *Schistosoma mansoni* 24 h in vitro cultured
794 somules exposed to forskolin (50 μM) for 10 min.
795
796
797
798

Figure 1

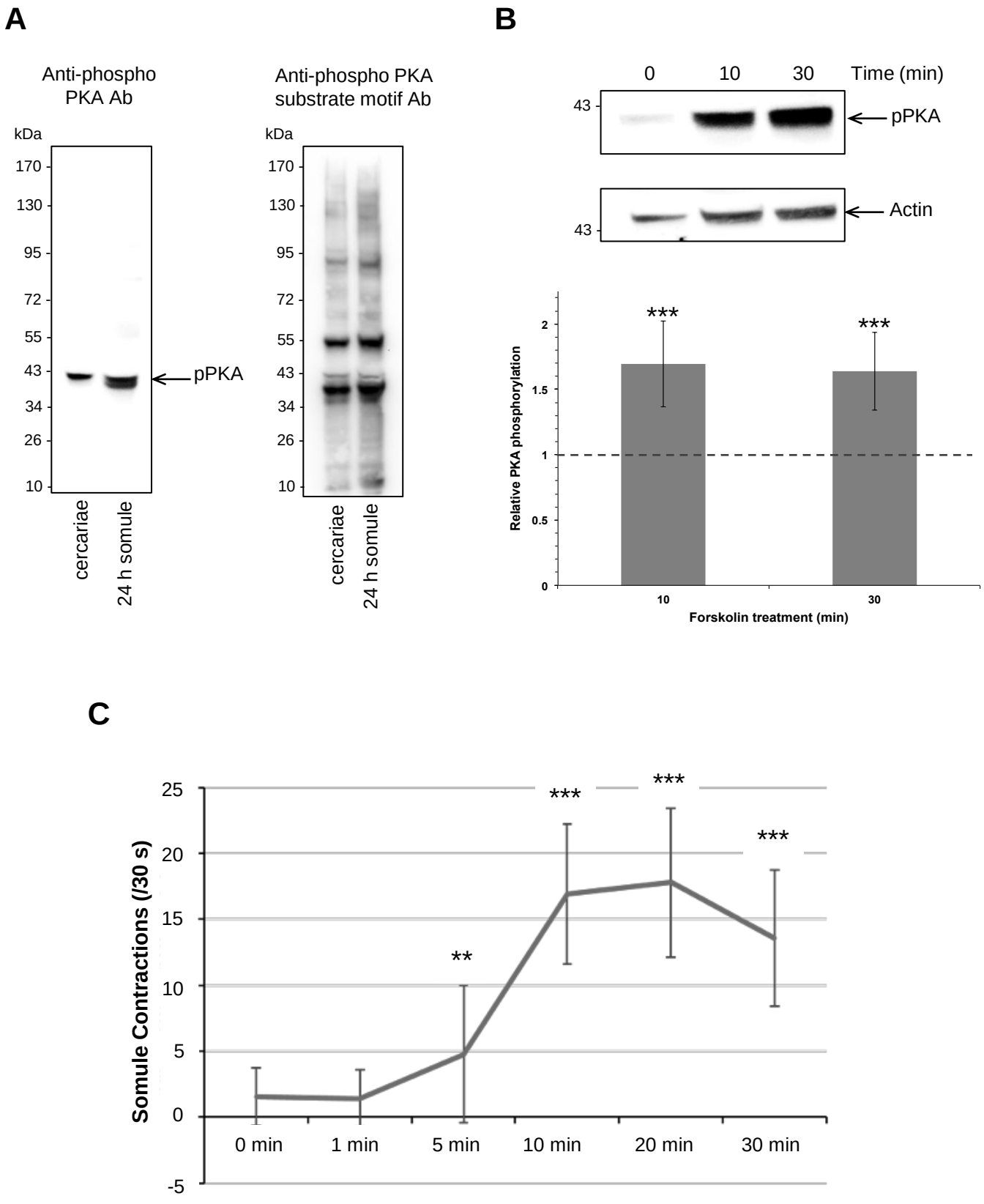


Fig. 1

Figure 2

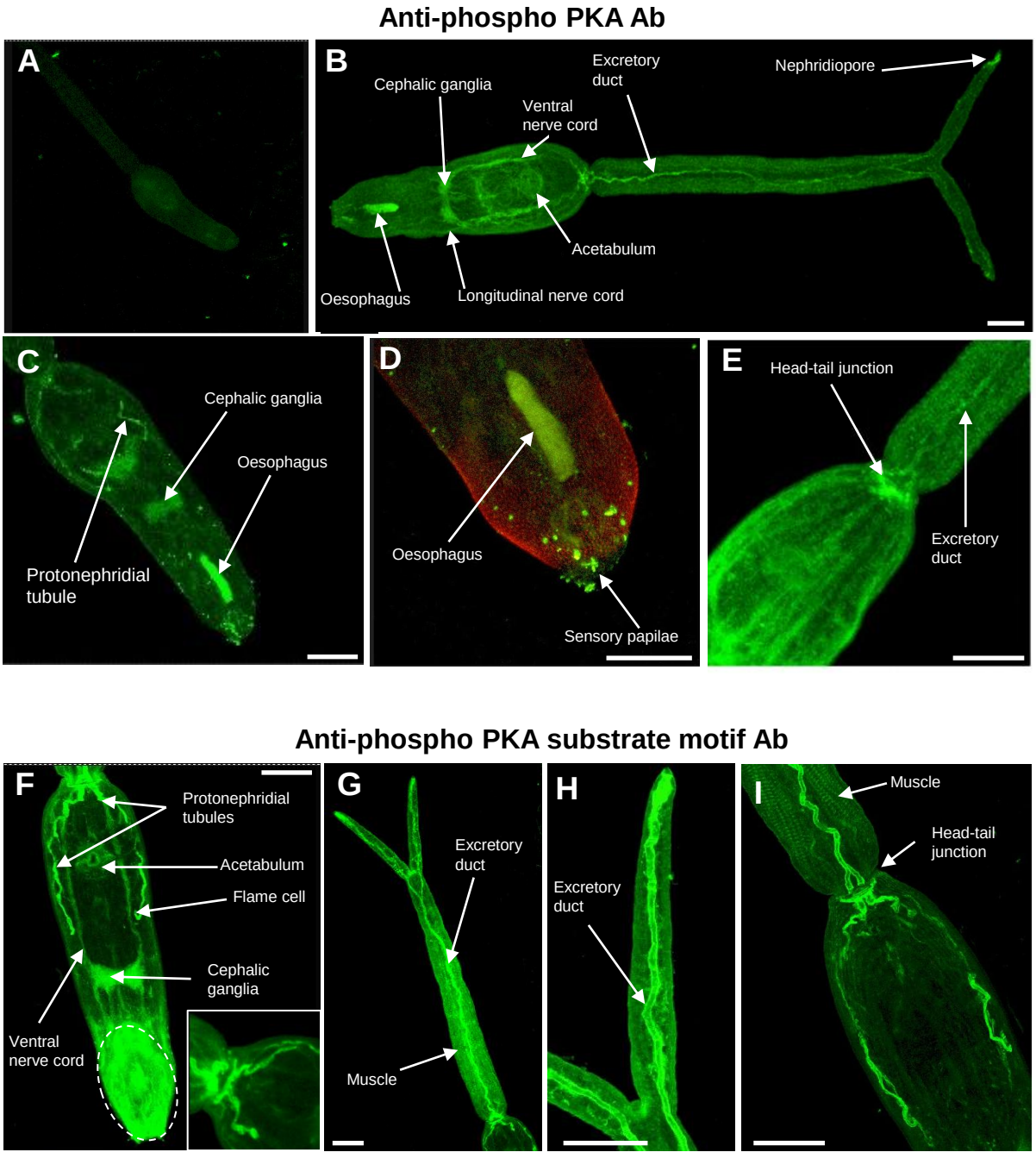


Fig. 2

Figure 3

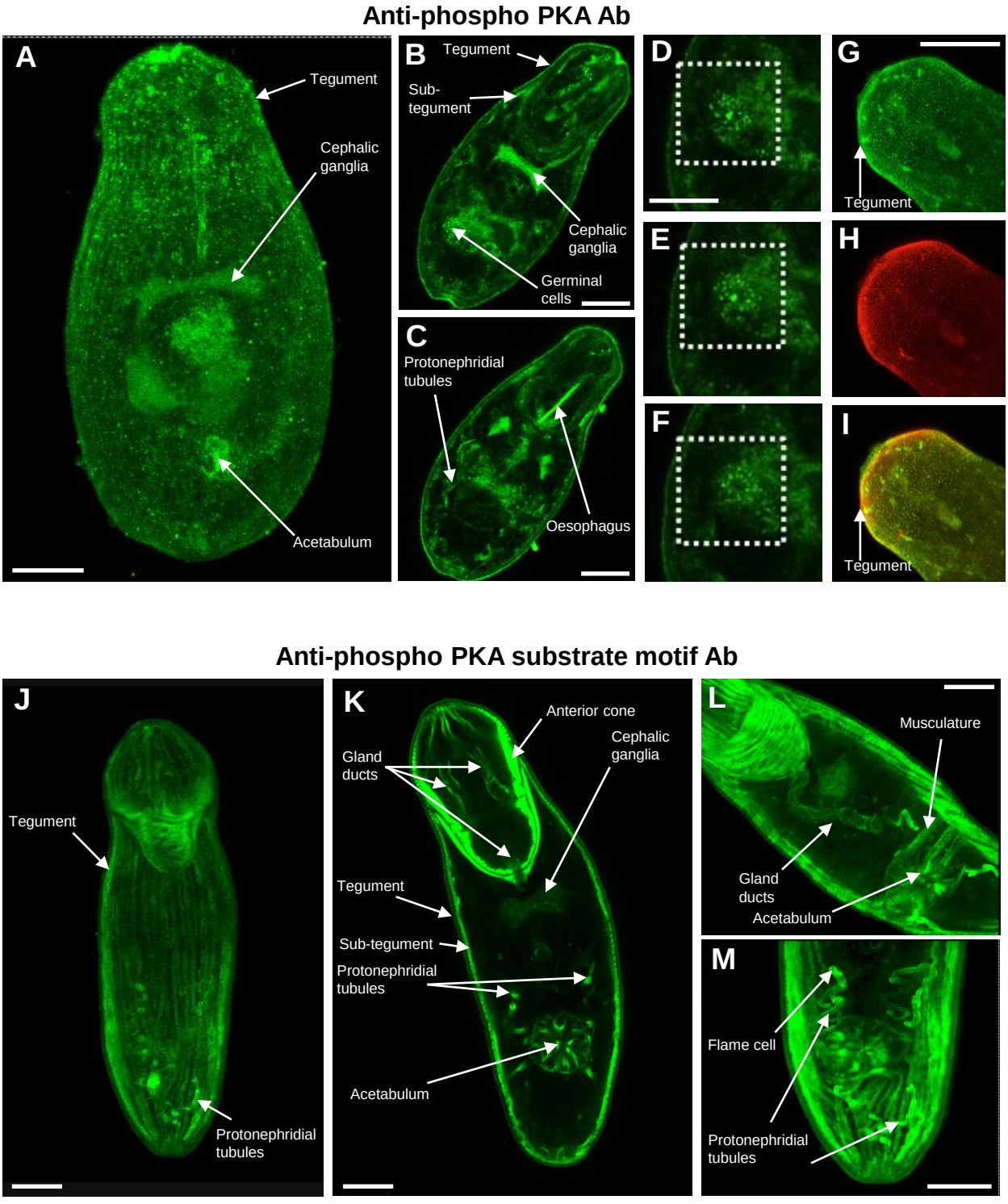


Fig. 3

Figure 4

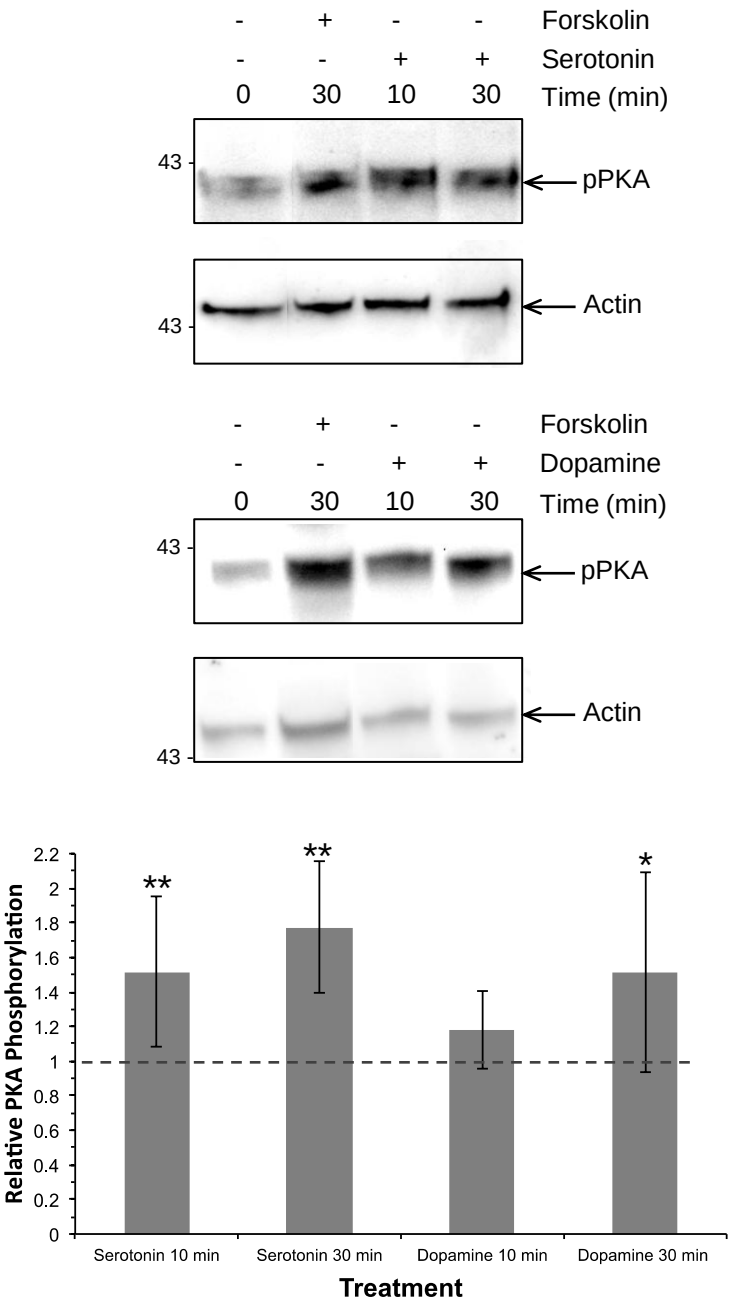


Fig. 4

Figure 5

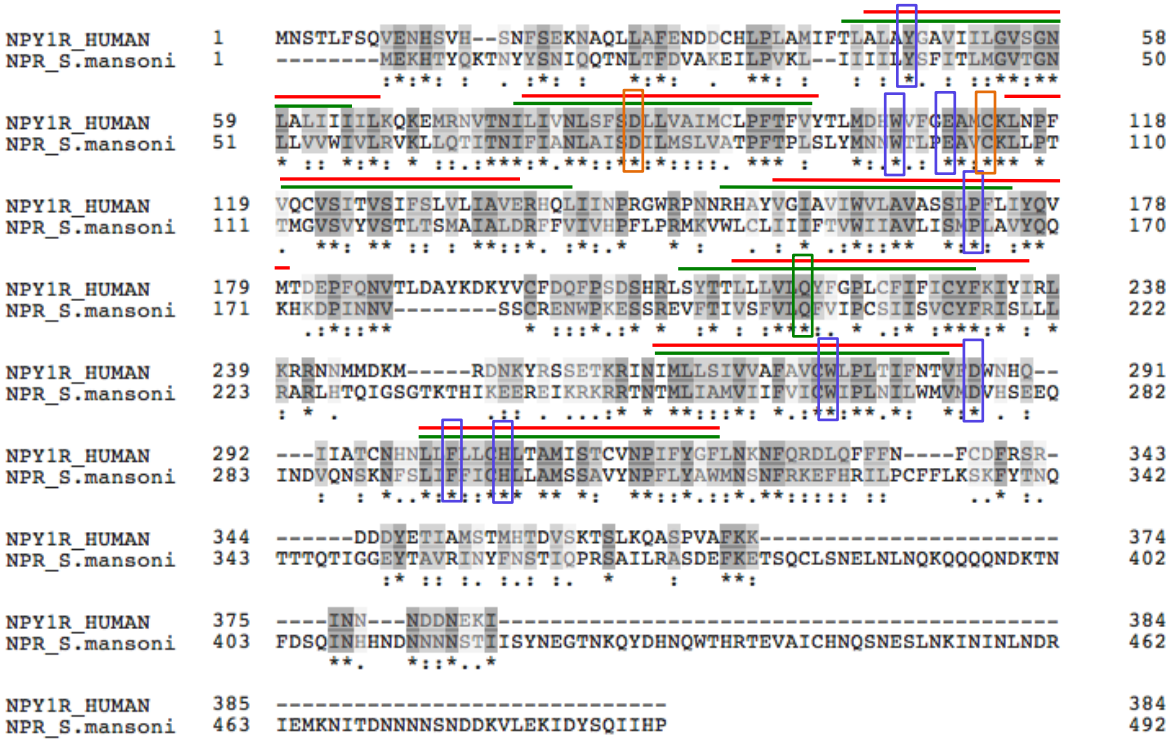


Fig. 5

Figure 6

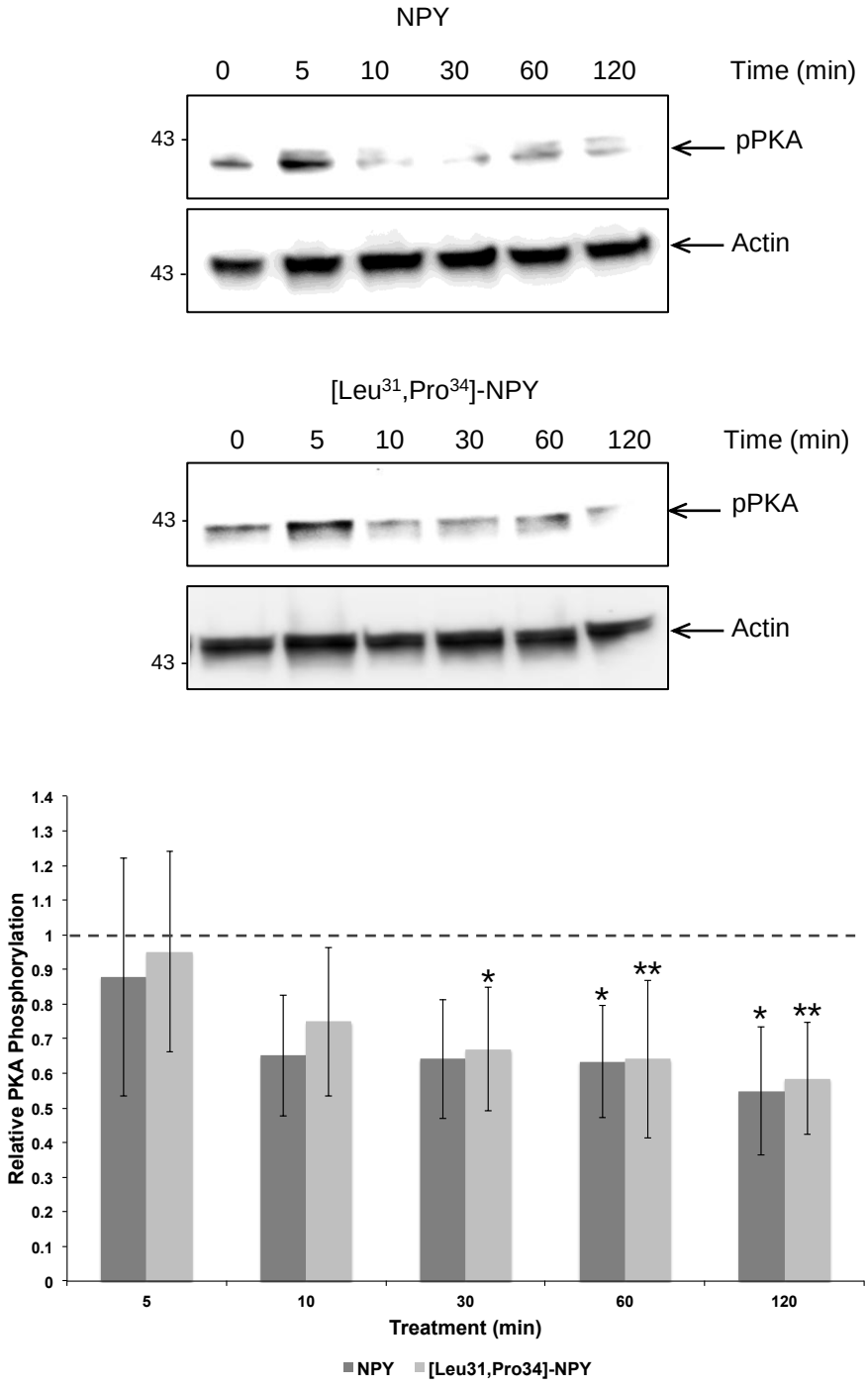


Fig. 6

Figure 7

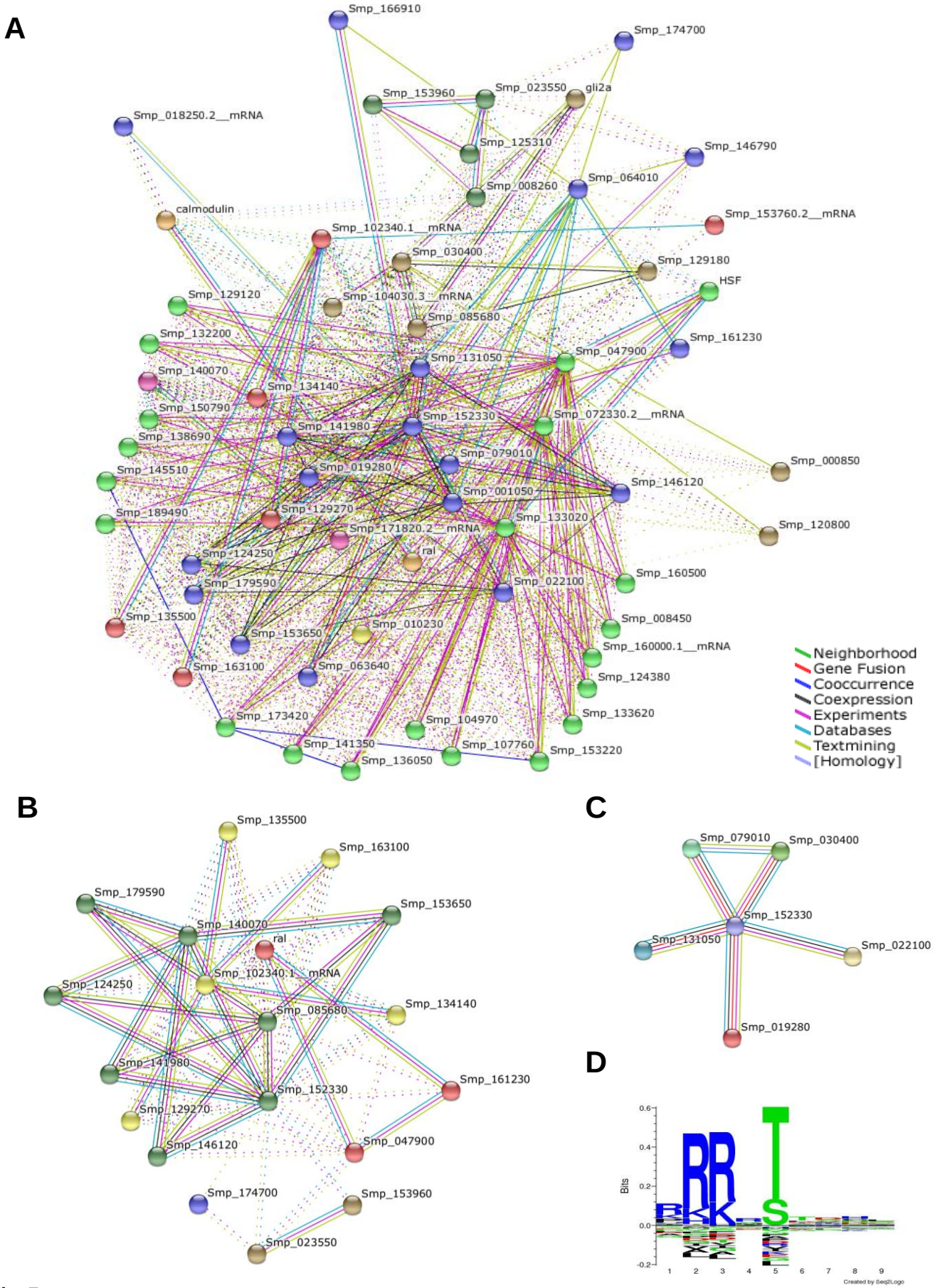
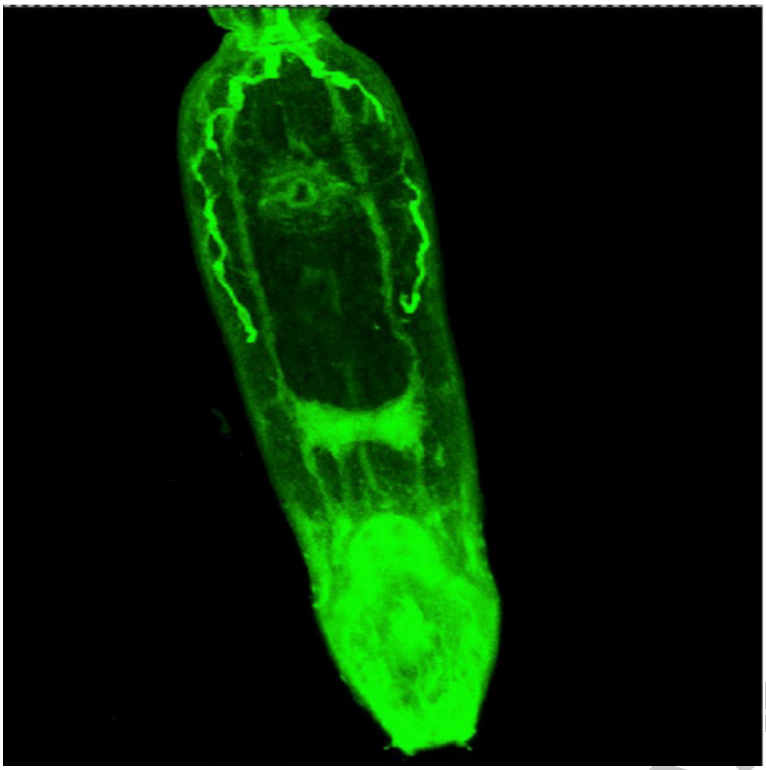


Fig. 7

Highlights

- Activated protein kinase A (PKA) mapped to nervous/excretory systems of *Schistosoma mansoni* cercariae
- *Schistosoma mansoni* somules also displayed striking PKA activation at the tegument
- Activation of PKA resulted in a hyperkinesia phenotype in somules
- Serotonin/dopamine stimulated, whereas neuropeptide Y attenuated, PKA activity
- In silico analysis revealed 59 high confidence putative PKA-interacting proteins.



Graphical abstract