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ORIGINAL PAPER

Uncovering Notch pathway in the parasitic flatworm *Schistosoma mansoni*

Lizandra G. Magalhães[1,2] · Enyara R. Morais[2,3] · Carla B. Machado[2] · Matheus S. Gomes[3] · Fernanda J. Cabral[2] · Julia M. Souza[1] · Cláudia S. Soares[2] · Renata G. Sá[4] · William Castro-Borges[4] · Vanderlei Rodrigues[2]

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Abstract Several signaling molecules that govern development in higher animals have been identified in the parasite *Schistosoma mansoni*, including the transforming growth factor β, protein tyrosine kinases, nuclear hormone receptors, among others. The Notch pathway is a highly conserved signaling mechanism which is involved in a wide variety of developmental processes including embryogenesis and oogenesis in worms and flies. Here we aimed to provide the molecular reconstitution of the Notch pathway in *S. mansoni* using the available transcriptome and genome databases. Our results also revealed the presence of the transcripts coded for *SmNotch*, *SmSu(H)*, *SmHes*, and the gamma-secretase complex (*SmNicastrin*, *SmAph-1*, and *SmPen-2*), throughout all the life stages analyzed. Besides, it was observed that the viability and separation of adult worm pairs were not affected by treatment with N-[N(3,5)-difluorophenacetyl)-L-Alanyl]-S-phenylglycine t-butyl ester (DAPT), a Notch pathway inhibitor. Moreover, DAPT treatment decreased the production of phenotypically normal eggs and arrested their development in culture. Our results also showed a significant decrease in *SmHes* transcript levels in both adult worms and eggs treated with DAPT. These results provide, for the first time, functional validation of the Notch pathway in *S. mansoni* and suggest its involvement in parasite oogenesis and embryogenesis. Given the complexity of the Notch pathway, further experiments shall highlight the full repertoire of Notch-mediated cellular processes throughout the *S. mansoni* life cycle.

Keywords Gamma-secretase complex · Notch pathway · *Schistosoma mansoni*

✉ Lizandra G. Magalhães
lizandra.magalhaes@unifran.edu.br

[1] Present address: Núcleo de Pesquisa em Ciências Exatas e Tecnológicas, Universidade de Franca, FrancaAvenida, Dr Armando Salles de Oliveira, 201 Franca, SP, Brazil

[2] Departamento de Bioquímica e Imunologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil

[3] Instituto de Genética e Bioquímica, Universidade Federal de Uberlândia, Patos de Minas, MG, Brazil

[4] Departamento de Ciências Biológicas, Instituto de Ciências Exatas e Biológicas, Universidade Federal de Ouro Preto, Ouro Preto, MG, Brazil

Introduction

Schistosoma mansoni is a metazoan parasite that belongs to the lophotrochozoan phylum Platyhelminthes and it is one of the three major parasitic pathogens causing schistosomiasis, a neglected tropical disease that affects millions of people in 76 countries (Rollinson et al. 2013). *S. mansoni* has a complex life cycle depending on two hosts and various life stages to complete its developmental cycle (King 2009). *S. mansoni* eggs constitute an important phase of the life cycle since they are released to the environment in human feces, a critical step during disease transmission. Also, inside the human host, eggs trapped in intestines and liver are primarily the leading cause of morbidity and mortality associated to schistosomiasis (Gryseels 2012).

"Omic" studies in *S. mansoni* have elucidated signaling pathways that could be involved in parasite development and differentiation (Verjovski-Almeida et al. 2003; Berriman et al. 2009). In particular, from mammals to the simplest metazoans, the Notch pathway is a highly conserved signaling mechanism involved in a wide variety of developmental processes including embryogenesis, oogenesis, and cell fate determination (Moskowitz and Rothman 1996; Greenwald 1998; Larkin et al. 1999; Artavanis-Tsakonas et al. 1999; Priess 2005).

Both the Notch receptor and its ligands are transmembrane proteins (Kopan and Ilagan 2009). Ligand binding promotes two consecutive proteolytic events of the Notch receptor: the first cleavage is dependent on a metalloprotease and the second performed by the gamma-secretase enzymatic complex which contains Presenilin, Nicastrin, Pen-2 (presenilin enhancer 2), and Aph1 (anterior pharynx-defective 1) (Kopan and Ilagan 2009; Ogura et al. 2006). The latter cleavage releases the Notch intracellular domain (NICD), which then translocates to the nucleus and cooperates with the DNA-binding protein Suppressor of Hairless (Su(H)) and its co-activator to promote transcription of target genes, such as hairy and enhancer of split (Hes) gene families (Oellers et al. 1994; Chen et al. 1997; Kopan and Ilagan 2009). N-[N(3,5)-difluorophenacetyl)-L-Alanyl]-S-phenylglycine t-butyl ester (DAPT) is an inhibitor of the gamma-secretase complex and previous studies have suggested that this drug efficiently mimics the developmental defects caused by Notch mutations (Geling et al. 2002; Micchelli et al. 2003; Münder et al. 2010; Wang et al. 2014).

The biological roles performed by the Notch pathway in *S. mansoni* remain to be determined. In this context, our group first cloned and evaluated by qRT-PCR a cDNA coding for *SmPresenilin* a putative Notch protease. Their transcript expression in the *S. mansoni* stages was the first indicator of the Notch signaling in the parasitic flatworm *S. mansoni* (Magalhães et al. 2009). Here, we have attempted to reconstitute the Notch signaling pathway in *S. mansoni* using bioinformatic approaches. We also showed by qRT-PCR the transcripts expression of other Notch pathway components during the parasite's life cycle, and that DAPT affects the *S. mansoni* eggs production and eggs development in vitro.

Materials and methods

Molecular reconstitution of the Notch signaling pathway in *S. mansoni*

Sequences encoding putative components of the Notch pathway were retrieved from the *S. mansoni* genome database (Gene DB, available at http://www.genedb.org/genedb/smansoni/). This represents a compilation of clustered ESTs and gene predictions from the parasite's transcriptome and genome initiatives (Verjovski-Almeida et al. 2003; Berriman et al. 2009; Logan-Klumpler et al. 2012; Protasio et al. 2012). Orthologue protein sequences from *Homo sapiens*, *Drosophila melanogaster*, and *Caenorhabditis elegans* were retrieved from KEGG pathway database (Kanehisa and Goto 2000) and used as query sequences with which stand-alone BlastP searches were carried out against the schistosome protein database. The Pfam database was used to search for conserved domains in the putative *S. mansoni* proteins (Finn et al. 2014).

Parasites

The *S. mansoni* LE strain (Luis Evangelista) is routinely maintained by serial passages through *Biomphalaria glabrata* snails and BALB/c mice. Infected snails were induced to shed cercariae by exposing the snails to artificial illumination in a 26 °C water bath for 1 h. Schistosomula were obtained by mechanical transformation of cercariae (Harrop and Wilson 1993) and cultured for 24 h at 37 °C in a humid atmosphere containing 5 % CO_2 in RPMI 1640 medium (Gibco) buffered with 20 mM HEPES pH 75, and supplemented with penicillin (100 UI mL^{-1}), streptomycin (100 μg mL^{-1}), and 10 % bovine fetal serum (Gibco). Adult worm pairs were recovered from mice, under aseptic conditions, by perfusion of the livers and mesenteric veins on the 56th day after infection with cercariae (Smithers and Terry 1965). Eggs were obtained by trypsinization of the livers and recovered through sieving as described by Ashton et al (2001). All experiments were authorized by the Ethical Committee for Animal Care the University of São Paulo, in agreement with the national accepted principles for laboratory animal use and care.

Evaluation of viability

After hepatic perfusion of infected mice, one of adult worm pair was transferred to each well of a 24-well culture plate containing RPMI 1640 medium (Gibco), supplemented with penicillin (100 UI mL^{-1}), streptomycin (100 μg mL^{-1}), and 10 % bovine fetal serum (Gibco) and maintained at 37 °C in 5 % CO_2, for 24 h. A stock solution of DAPT (Sigma-Aldrich) at 100 mM was then prepared in dimethyl sulfide (DMSO) and added to the culture medium to final concentrations of 5, 10, 20, and 30 μM. Fresh medium and DAPT were replaced every 24 h. Parasites were kept for 120 h under treatment and the viability was examined at 24, 72, and 120 h using the colorimetric assay 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), as described by Comley et al. (1989). Negative control worms were incubated in RPMI 1640 medium containing or not 01 % DMSO. Positive control worms were treated with praziquantel (PZQ) at 1.6 μM. For each assay, 12 adult worms pairs were evaluated. Three independent biological replicates were performed.

An additional criterion for viability was supported by microscopic observation of *S. mansoni* adult worms with an emphasis on changes in parasite motility and the occurrence of death based on standard procedures for compound screening at WHO-TDR (Ramirez et al. 2007). The adult worm pairs were incubated during 120 h the same conditions described before and monitored using an inverted microscope each 24 h. The phenotypic changes were scored using a viability scale of 0–3: (3 = totally active, 2 = slow activity, 1 = minimal activity, 0 = worm's death—death was defined as the absence of movement observed for at least 2 min of examination). After the last

observation period, the culture medium was removed, and fresh culture medium without DAPT was added, and motility was re-examined for up to 24 h. Additionally, the separation of couples adult worms was examined. The experiment was repeated three times and evaluated ten pairs of adult worms in each experiment. For negative control groups, adult worm pairs were incubated in RMPI 1640 medium or RPMI 1640 medium with 01 % DMSO. For positive control group, couples of adult worms were incubated with PZQ (1.6 μM).

Evaluation of egg production and egg development

For evaluation egg production, one adult worm pair was transferred to each well of a 24-well culture plate containing the same culture medium described before, and DAPT previously dissolved in DMSO was added to the culture medium to final concentrations of 5, 10, 20, and 30 μM. After 120 h, egg production was examined by visual inspection under an inverted microscope evaluated using an inverted microscope.

For evaluation of DAPT on *S. mansoni* egg development, two adult worm pairs were transferred to each well and cultured as above during 48 h for egg laying. After this period, the worms were removed, and DAPT added at the equivalent concentrations described before to the medium containing eggs. Eggs were maintained at 37 °C in a humid atmosphere containing 5 % CO_2. After 120 h of culture, eggs were examined microscopically and scored as either developed or undeveloped based on the presence or absence of a miracidium as described by Michaels and Prata (1968). Eggs were photographed using a camera (AxionCam ERc5s Zeiss) coupled to a microscope (Zeiss). Control adult worms or eggs were treated with 01 % DMSO in RPMI 1640 medium. These experiments were carried out in quadruplicate and repeated three times.

RNA preparation and expression analysis by quantitative RT-PCR

S. mansoni total RNAs (adult worm pairs; female and male adult worms separated manually, after perfusion; eggs; cercariae; and schistosomula) were isolated using a combination of the Trizol reagent (Invitrogen) for extraction and the PureLink™ Micro-to-Midi Total RNA Purification System (Invitrogen) for purification. For cDNA synthesis, 1 μg of total RNA was treated with 4 U of DNAase I (Promega) and used as template to synthesize cDNA using an oligodT primer from the ThermoScript™ RT-PCR System (Invitrogen) following the manufacturer's protocol. Gene-specific primers for *SmNotch* (Smp_105360), *SmSu(H)* (Smp_145970), *SmHes* (Smp_152190), *SmAph-1* (Smp_050110), *SmPen-2* (Sm04062), *SmNicastrin* (Smp_167240), and *Smα-tubulin* (M80214) (Webster et al. 1992) were designed using the program Vector NTI (Invitrogen) (Table 1). To confirm primer specificities, the PCR products were sequenced on the ABI 3100 automated sequencer (Applied Biosystems) using the Dye terminator kit.

Reactions were performed in triplicate and carried out using 7500 real-time PCR system (Applied Biosystems). The total reaction volume was 10 μL with 200 nM of each primer, 5 μL of SYBR green PCR (Applied Biosystems), and 1 μL of cDNA as template (or water as a negative control). The PCR efficiency (E) was determined for both primer sets by plotting cycle thresholds from a 10-fold serial dilution of cDNA and by inputting the slope in the equation $E = 10^{(-1/\text{slope})}$. For expression analyses considering different life cycle stages, the quantification of transcripts relative to α-tubulin was calculated according to the $2^{-\Delta Ct}$ method. For expression analyses related to inhibition experiments, adult worms and eggs were separately cultured with DAPT at 5 and 10 μM during 24 h RNA extractions, and quantitative PCR was performed as described before. Expression analyses were calculated according to the $2^{-\Delta\Delta CT}$ method (Livac and Schmittgen 2001) using the α-tubulin transcript as a reference for constitutive expression. Both analyses were performed using the Applied Biosystems 7500 software.

Statistical analyses

The statistical analyses were performed using the Graphpad Prism (version 50) software. One-way analysis of variance followed by determination of the significant differences between control and DAPT-treated groups (Dunnet pairwise comparison) was used for statistical analyses of viability, egg production, and development. One-way analysis of variance Kruskal-Wallis followed by Dunn's test was used for statistical analyses related to differential expression of transcripts.

Results

The *S. mansoni* genome encodes highly conserved members of the Notch signaling pathway

We performed an in silico analysis using proteins sequences from *H. sapiens*, *D. melanogaster*, and *C. elegans* as queries to identify putative Notch pathway sequences in *S. mansoni* genome database. We were able to identify putative components of the Notch pathway in *S. mansoni* including the following: the Notch receptor, Suppressor of Hairless (Su(H)) (transcriptional factor), Jagged/Serrate ligands, co-repressors (Smart and Groucho), and the Skip co-activator. Proteases involved with the cleavage of Notch receptor were identified including the gamma-secretase complex (Presenilin, Nicastrin/Aph-2, Aph-1, and Pen-2), Furin convertase, and the metalloproteases (Adam 17 and Kuzbanian). We also retrieved four putative proteins involved in regulation of the Notch pathway named Notchless, Numb, Dishevelled, and WWP1. Also, the Hairy

Table 1 Oligonucleotide primers used in the study

Gene	GeneDB number	Primers	Domain in amplification region
SmNotch	Smp_105360	F 5′ ATGGAGATGGTGTTTGTCAGTCACA 3′ R 5′GCACATATTTCCTTCGGTTGTTTTA3′	*Notch* (PF00066)
SmSu(H)	Smp_145970	F 5′CAATTCTACGAGATATGCGAGTGAA 3′ R 5′CGCACTAAACTAATTGGCACCTCTA 3′	*TIG* (PF01833)
SmHes	Smp_152190	F 5′TGGAACTCAAACAAGTTGGAAA 3′ R 5′AAAGGCAGTTTCACAAGAAAGA 3′	*HLH* (PF00010)
SmAph-1	Smp_050110	F 5′GGGGGAGTTGGCTGTACTCT 3′ R 5′CAACCAGCTGTGGTCTTGAA 3′	*Aph-1* (PF06105)
SmPen-2	Sm04062	F 5′ATGGATATAAAACACCTTA 3′ R 5′TTACAGTCTACCAGGTGGTAT 3′	*Pen* (PF10251)
SmNicastrin	Smp_167240	F 5′TATGACCGTCCACCTTTCCT 3′ R 5′GGCCCAGTGGTAGGTAGGTT 3′	NI
Smα-tubulin	M80214[a]	F 5′ GAAATGCTTGTTGGGAGTTG 3′ R 5′ TTATCACTTGGCATCTGTCC 3′	*Tubulina* (PF00091)

F sequence forward, *R* sequence reverse, *NI* not identified

[a] Webster et al. (1992)

and Enhancer of Split (Hes), a transcriptional factor regulated by the Notch pathway, was identified (Fig. 1).

Full-length *SmNotch* nucleotide sequences obtained from *S. mansoni* genome database Smp_105360 and Smp_140800 are 1130 and 2001 amino acids long, respectively. In both sequences, distinct repeated domains coding for the Epidermal Growth Factor (EGF), Notch or NOD, and Ankyrin (AKN) can be found (Fig. 2).

Transcripts coding for components of the Notch pathway are presence during the parasite's life cycle

We next aimed to investigate, by qRT-PCR, the relative levels of some central Notch components throughout different larvae and adult stages of the parasite's life cycle. The selected transcripts coded for *SmNotch*, *SmSu(H)*, *SmHes*, and the gamma-secretase complex (*SmNicastrin*, *SmAph-1*, and *SmPen-2*), all putatively participating at distinct events of the Notch signaling pathway. Our results revealed the presence of the investigated transcripts throughout all the analyzed stages (Fig. 3). However, in the schistosomula stage, a significant down-regulation of *SmHes* expression was observed compared to its levels in eggs. *SmNotch*, *SmSu(H)*, *SmHes*, and *SmAph-1* transcripts in the cercariae were also significantly down-regulated compared to their respective levels in eggs. In contrast, *SmNicastrin* and *SmPen-2* transcripts did not exhibit difference of expression for the investigated stages.

The gamma-secretase inhibitor DAPT neither affects the viability or induces separation of *S. mansoni* adult worms

As the activation of genes regulated by the Notch pathway depends on the cleavage of the Notch receptor by gamma-secretase complex (Kopan and Ilagan 2009), we aimed to investigate whether DAPT would promote separation or compromise viability of paired adult worms. Incubation of adult worms with DAPT at 5, 10, and 20 μM for 24 h, no alteration in the viability of the female and male worms was observed (Fig. 4). After 72 and 120 h of incubation, a significant decrease in the viability was observed at concentrations of 20 and 30 μM. Also, DAPT at higher than 10 μM induced separation of adult worms after 24 h (data not shown). Adult worms in the negative control groups (RPMI 1640 medium alone or in combination with 01 % DMSO) exhibited normal viability and those in the positive control group (PZQ at 1.6 μM and heat-killed) showed no viability (100 % death) (Fig. 4).

A differential viability after exposure to DAPT was supported by the microscopic observation of couples *S. mansoni* adult worms. The viability was examined with an emphasis on changes in the parasites motor activity and the occurrence of death based on standard procedures for compound screening at WHO-TDR (Ramirez et al. 2007). DAPT at concentrations 5, 10, and 20 μM for 24 h, no alteration in the motor activity of the adult worms was observed. However, at 72 and 120 h, at concentrations 20 and 30 μM, a significant decrease in the motor activity was observed. On the other hand, *S. mansoni* worms in the negative control groups (RPMI 1640 medium only or RPMI 1640 medium plus 01 % DMSO) showed normal motility, whereas *S. mansoni* worms in the positive control (PZQ at 1.6 μM) had no motility at all time (data not shown).

The gamma-secretase inhibitor DAPT reduces the production of phenotypically normal eggs in vitro

Although DAPT at 5 and 10 μM did not induce parasite separation, we observed a significant decrease of 49 and 74 %, respectively, in egg output when compared to output from the

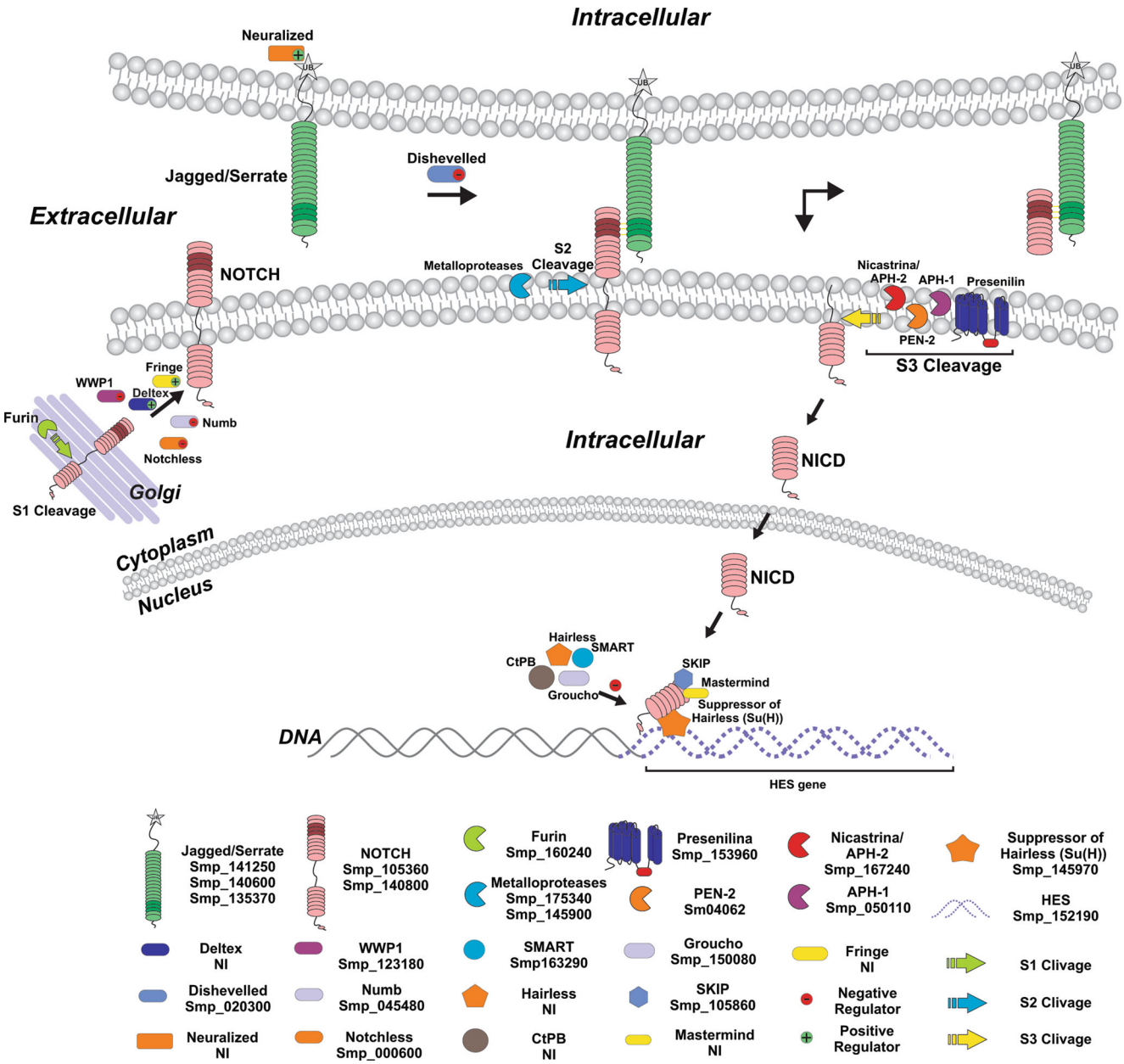

Fig. 1 Schematic representation of Notch pathway homology searches in the *S mansoni* genome database (http://www.genedb.org/genedb/smansoni/) revealed sequences for most of proteins in the pathway. The number of sequences present in *S mansoni* is indicated below, NI sequence not identified in the parasite

negative group (Fig. 5a). Additionally, at 20 and 30 μM, we observed a significant decreased of the 85 and 95 % in egg output. Microscopic examination of eggs laid by DAPT-treated worms revealed morphological defects, such as decreased size, unusual shapes, and loss of the lateral spine (Fig. 5b). These were in marked contrast to those from the control group which were normal in appearance.

To verify the effect of DAPT on *SmNotch* and *SmHes* transcripts expression, paired adult worms were incubated or not with DAPT at 5 and 10 μM, followed by RNA extraction and qRT-PCR analyses. We found that DAPT treatment while maintaining *SmNotch* transcript levels (Fig. 5c) was able to significantly decrease the expression of *SmHes* (Fig. 5d).

The gamma-secretase inhibitor DAPT arrests *S. mansoni* egg development in vitro

To verify whether DAPT affected *S. mansoni* embryo development, eggs were cultured in the presence of DAPT at 5, 10, 20, and 30 μM. The eggs produced by adult worm pairs during the first 2 days of in vitro culture developed during 5 days, with a typical progression of development through

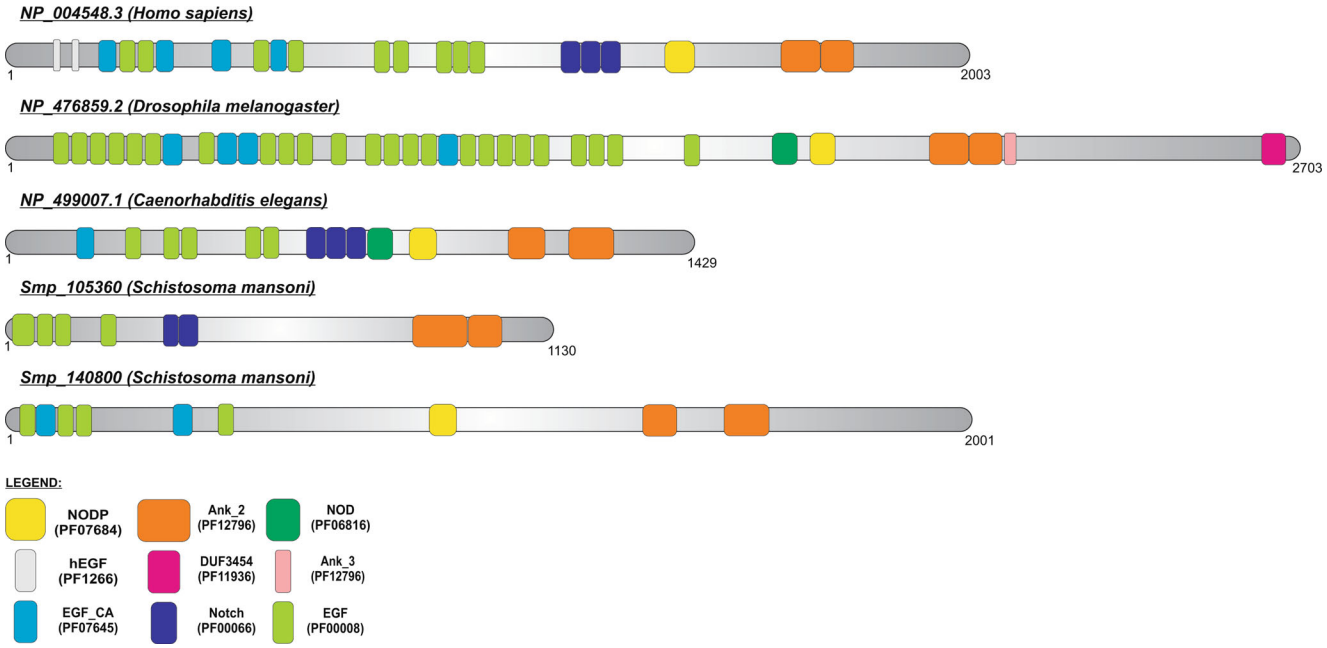

Fig. 2 Schematic comparison of the protein structures of *H. sapiens* Notch4 (NP_0045483), *D. melanogaster* (NP_ 4768592), *C. elegans* GLP-1 (NP_4990071), *S. mansoni* (Smp_105360), and *S. mansoni* (Smp_140800). Abbreviations: *EGF*, EGF-like repeats; *EGF-CA*, calcium-binding EGF domain; *hEGF*, human growth factor-like EGF; *Notch*, Lin-12/Notch repeats; *NOD and NODP*, Notch family; *ANK*, ankyrin repeats; *DUF*, domain of unknown function

six distinct stages as described previously by Michaels and Prata (1968) and Freitas et al. (2007) (Fig. 6a). Microscopic examination of eggs incubated with DAPT at 5, 10, and 20 μM showed a significant decrease in egg development when compared to those from the negative group (developed based on the presence of a miracidium) (Fig. 6b, c).

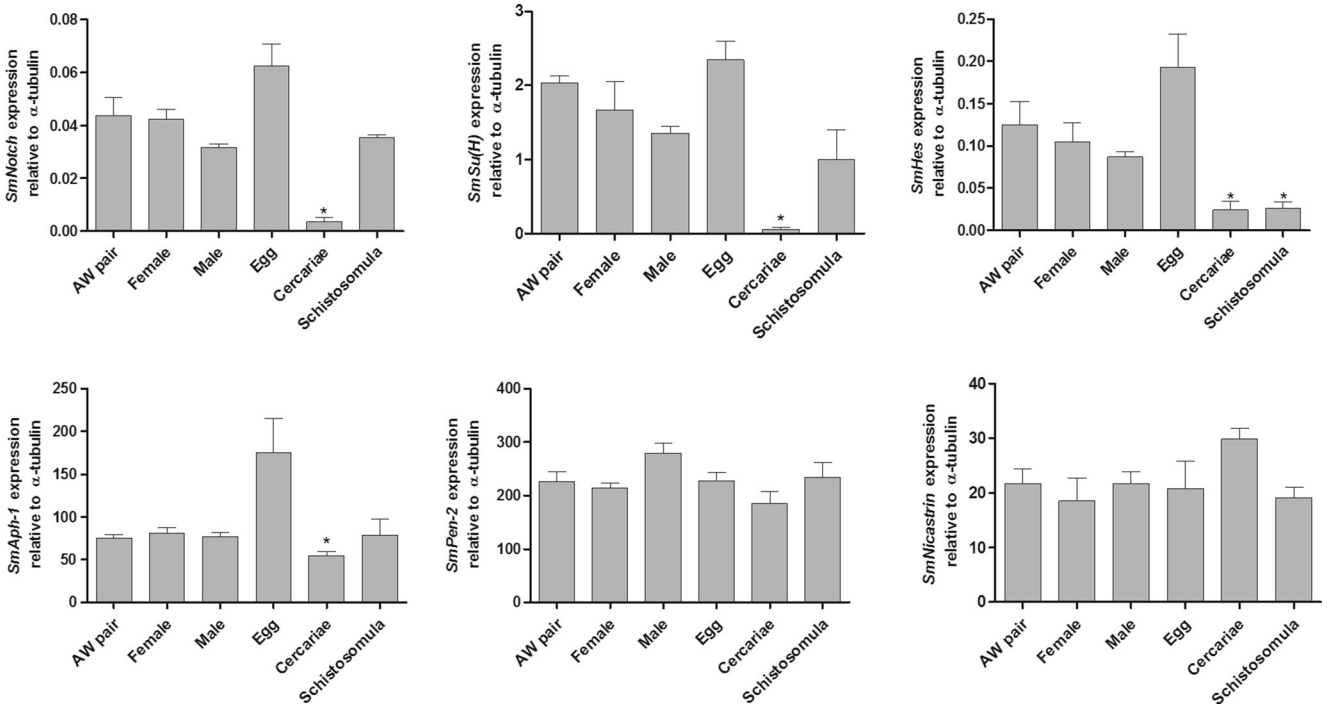

Fig. 3 Expression profile of Notch pathway components in various stages of the *S. mansoni* life cycle. mRNA expression levels were measured, based on three replicates obtained from paired adult worm, male and female adult worms separated manually, after perfusion, cercariae and schistosomula using quantitative PCR. Expression levels were calibrated according to the comparative $2^{-\Delta Ct}$ method, using the constitutively expressed *Smα-tubulin* as an endogenous control. $*p < 005$ indicates statistically different expression levels in relative to the egg stage

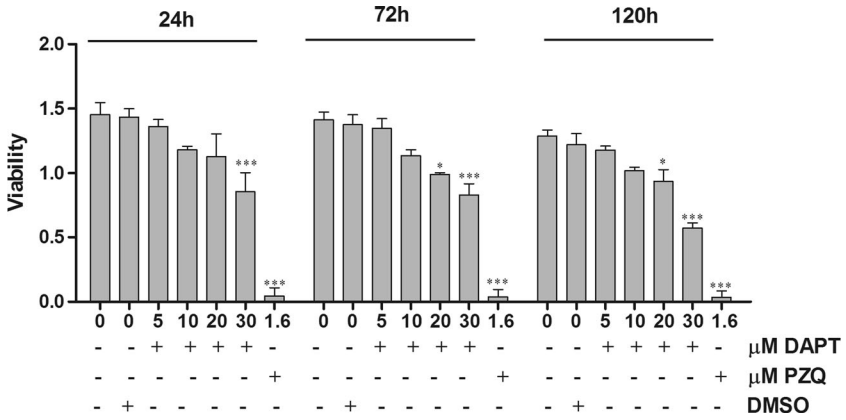

Fig. 4 Effect of gamma-secretase inhibitor (DAPT) on the viability of the *S. mansoni* adult worms in vitro. Pairs of adult worms were treated with DAPT at different concentrations during 24, 72, or 120 h and the viability was measured by MTT assay at 550 nm. The negative controls worms were incubated in RPMI 1640 medium or 01 % DMSO in a RPMI 1640 medium and the positive control worms were treated with praziquantel (PZQ) at 1.6 μM. $*p < 005$, $***p < 0001$ indicate statistically different in relation the negative control group (worms incubated with RPMI 1640 medium plus 01 % DMSO)

Additionally, we observed an arrest in egg development at stage 1 or 2 in 5 days of culture (Fig. 6b). The negative control eggs (cultured in RPMI plus 01 % DMSO) demonstrated normal development (Fig. 6b, c). A decreased transcription of *SmHes* was observed in eggs incubated with DAPT during 5 days when compared to negative control, and that does not coincide with reduced expression of *SmNotch* (Fig. 6d, e).

Discussion

The development and maintenance of multicellular organisms are triggered by a number of signaling pathways that interpret and transmit signals to activate the transcription of several genes, resulting in cell differentiation (Kestler et al. 2008; Yamamoto et al. 2014). The Notch pathway was firstly identified in *D.*

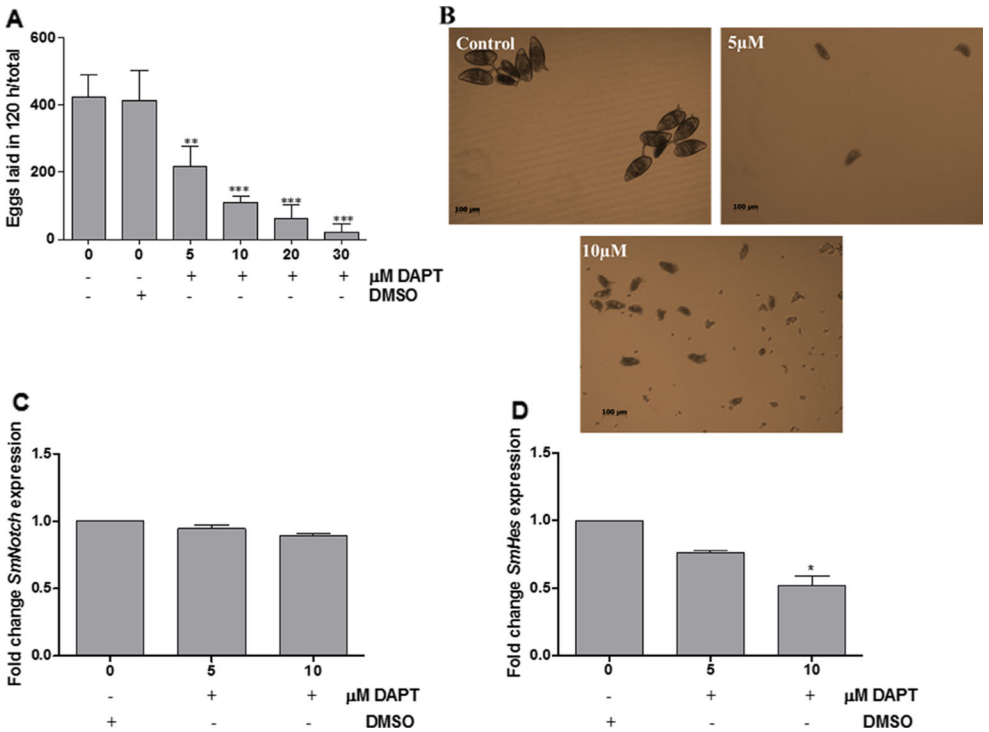

Fig. 5 Effect of gamma-secretase inhibitor (DAPT) on production of eggs for *S. mansoni* adult worms pairs in vitro. **a** Pairs of adult worms were treated with DAPT during 120 h and the number of eggs was monitored using an inverted microscope. **b** Eggs produced during the treatment with DAPT were photographed. **c** qRT-PCR analyses of *SmNotch* and *SmHes.* Expression of *S. mansoni* α-tubulin was used as the endogenous control to calculate relative expression levels, and expression levels were normalized to negative control group (worms incubated with 01 % DMSO + RPMI 1640 medium). $*p < 005$, $**p < 001$, $***p < 0001$ indicate statistically different in relation the negative control group (worms incubated with RPMI 1640 medium plus 01 % DMSO)

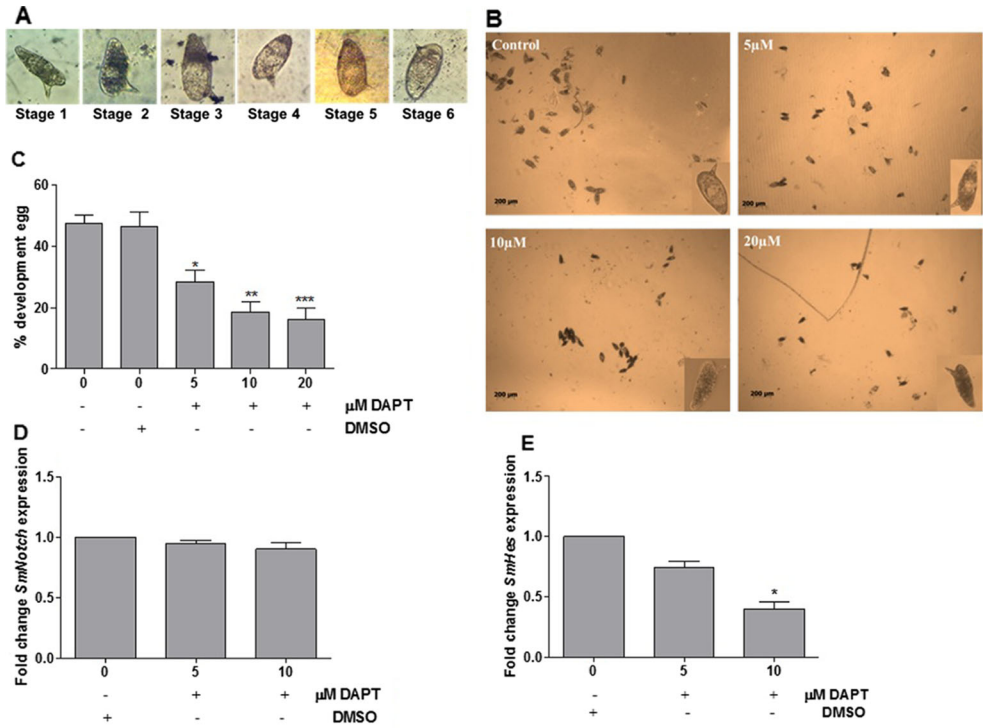

Fig. 6 Effect of gamma-secretase inhibitor (DAPT) on egg development in vitro. **a** Percentage of eggs developed. After treatment with DAPT, the eggs were microscopically examined and scored as developed or undeveloped, based on the presence or absence of the miracidium. **b** Immature eggs produced by adult parasites treated with DAPT failed to develop into miracidia. **c** qRT-PCR analyses of *SmNotch* and *SmHes.* Expression of *S. mansoni* α-tubulin was used as the endogenous control to calculate relative expression levels, and expression levels were normalized to negative control group (worms incubated with 01 % DMSO + RPMI 1640 medium). $*p < 005$, $**p < 001$, $***p < 0001$ indicate statistically different in relation the negative control group (eggs incubated with RPMI 1640 medium plus 01 % DMSO)

melanogaster and *C. elegans* and several studies have shown its importance in oogenesis and embryogenesis of these organisms (Xu et al. 1992; Larkin et al. 1996; Moskowitz and Rothman. 1996; Hansen et al. 2004). Several other Notch-mediated functions are described to trigger development (Lai 2004). The identification and function analysis of Notch pathway in *S. mansoni* have not been explored, and given the importance of parasite eggs to disease progression and transmission, understanding schistosome development, pairing, and sexual maturation is of upmost importance (Walker 2011).

In this study, an in silico analysis of the Notch pathway was conducted through bioinformatic mining of the *S. mansoni* database at GeneDB. This analysis revealed the presence of several putative sequences coding for components of the Notch pathway in *S. mansoni.* Berriman et al (2009) described eight predicted sequences for the Notch receptor (Smp_153090, Smp_173560, Smp_050520, Smp_135370, Smp_040110, Smp_122750, Smp_105360, Smp_140800). However, we found that only Smp_105360 and Smp_140800 exhibited the typical architectures of Notch receptors found in other organisms. These included a putative extracellular region containing repeats of the EGF and Notch/Lin-12 (NRL) domains, followed by a transmembrane region and then the intracellular portion bearing repeated ankirin (ANK) domains (Kopan and Ilagan 2009). It has been reported that Notch receptors in inferior invertebrates such as *C. elegans* and *Hydra vulgari* have a short extracellular region when compared to that found in other insect and vertebrate species (Rudel and Kimble 2001; Käsbauer et al. 2007; Kopan and Ilagan 2009).

We next aimed to evaluate the expression of some components of the Notch pathway in *S. mansoni* paired adults, male, female, eggs, cercariae, and schistosomula. The qRT-PCR analyses demonstrated that transcripts coding for *SmNotch*, *SmSu(H)*, *SmHes*, *SmNicastrin*, *SmPen-2*, and *SmAph-1* were expressed in all investigated stages. However, *SmNotch*, *SmSu(H)*, *SmHes*, and *SmAph-1* transcripts in the cercariae were significantly down-regulated compared to their respective levels in eggs, suggesting that this components can be involved with the development of parasite inside of vertebrate host.

In attempt to provide a functional validation of the Notch pathway in *S. mansoni*, we used the presenilin inhibitor DAPT to investigate its effect on egg production and development. The effect of DAPT, a compound known to mimic efficiently loss-of-function mutations of Notch, has been described in previous reports for *D. melanogaster*, *H. vulgaris*, and *Danio rerio*, among others (Cheng et al. 2003; Geling et al. 2002; Micchelli et al. 2003). However, it is worth emphasizing that as a primary target of DAPT, the gamma-secretase complex can act on other proteins such as Erb-4, nectin-1, amyloid precursor protein, cadherin, nectin-1, and low density lipoprotein receptor

(Kopan and Ilagan 2004). The latter three protein targets can be found in the *S. mansoni* database under accession numbers Sm01633, Smp_151620, and Sm07396, respectively.

Our results demonstrated that DAPT inhibited egg production and arrested their development in culture. In addition, a decreased transcript expression of *SmHes* was observed. Other experiments from our group have shown that after incubation of adult parasites with curcumin, a proposed regulator of Notch pathway (Shehzad and Lee 2013), a decreased expression of Notch receptor gene (Smp_105360) is observed in parallel to reduced egg production and impaired embryo development (Morais et al. 2013; Magalhães et al. 2009). A down-regulation of Notch transcript in neoblast-like cells of *S. mansoni* after exposed to varying doses of gamma-irradiation was described (Collins et al. 2013). Other studies have demonstrated that Notch pathway target genes such as *Hes* are down-regulated by DAPT, with no significant effects on the transcript levels of the Notch receptor (Chen et al. 2013; You et al. 2013).

The Notch signaling pathway is involved in oogenesis, spermatogenesis, and embryogenesis in *D. melanogaster* (Xu et al. 1992; Larkin et al. 1996, 1999), *C. elegans* (Hansen et al. 2004), and other organisms (Feng et al. 2014; Xu et al. 1992) and it showed that Notch receptor is expressed in germinal and somatic cells and alterations on Notch receptor caused inhibition of oocyte development in *D. melanogaster*. In other study, it was demonstrated that SEL-12 deletion gene (a Presenilin homolog) caused a decrease in egg laying by absence of Notch/lin-12 pathway in *C. elegans* (Jarriault and Greenwald 2002). In *C. elegans*, upon egg fertilization there is activation of Notch/Lin-12 pathway, leading to recurrent events in the development and differentiation of embryonic cell lineages (Shelton and Bowerman 1996; Moskowitz and Rothman 1996). In *S. mansoni*, embryonic development has been proposed to occur in ten distinct stages (Jurberg et al. 2009). Two pre-embryonic stages occur inside the female worm and these are characterized by the release of mature oocytes from the female ovary until its fertilization followed by migration of the zygote through the ootype, where the eggshell is formed, to the uterus. In the external environment, the completion of the eight remaining stages within the eggshell culminates with the formation of the embryo (miracidia). It is important to mention that the Notch pathway may interact with other signaling pathways to influence differentiation, proliferation, survival, and cell migration (Hurlbut et al. 2007). Many signaling molecules involved in reproductive development and embryogenesis have been identified in *S. mansoni* (Beckmann et al. 2010; You et al. 2011). Examples are components of the transforming growth factor β (TGF-β), protein tyrosine kinases (PTKs), nuclear hormone receptors, among others (Knobloch et al. 2007; Beckmann et al. 2010).

In summary, here we identified several putative components of the Notch pathway in *S. mansoni* and we found that the gamma-secretase inhibitor DAPT reduced the production of phenotypically normal eggs and causing arrested egg development. We also propose that such arrest is possibly linked to a decreased expression of *SmHes*. Future studies shall clarify the full amplitude of effects mediated by the Notch signaling pathway in *S. mansoni* adding valuable information on parasite's biology.

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