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**Validação por PCR quantitativo da variação do nível de
expressão de RNAs longos não-codificadores de proteínas
entre os diferentes estágios do ciclo de vida do parasita
*Schistosoma mansoni***

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SUMÁRIO

	Pág.
LISTA DE ABREVIATURAS	4
RESUMO	5
1. INTRODUÇÃO	6
2. OBJETIVOS E JUSTIFICATIVA.....	9
3. MATERIAIS E MÉTODOS	10
4. RESULTADOS E DISCUSSÃO	15
6. CONCLUSÃO	20
7. BIBLIOGRAFIA	21
8. ANEXOS	25

LISTA DE ABREVIATURAS

PC gene	<i>Protein Coding Gene</i> (gene codificador de proteína)
ncRNA	<i>non coding RNA</i>
lncRNA	<i>long non coding RNA</i>
lincRNA	<i>Long intervening non-coding RNA</i>
RT-qPCR (Reverse Transcription-qPCR)	<i>Reverse Transcription Quantitative Polymerase Chain Reaction</i>
SmLINC	<i>Schistosoma mansoni lincRNA</i>
NGS	<i>Next Generation Sequencing</i>
Smp	<i>Schistosoma mansoni protein coding gene</i>
CT	<i>Cycle Threshold</i>
<i>S. mansoni</i>	<i>Schistosoma mansoni</i>
h	horas
mM / μ M	concentração em mili molar e micro molar respectivamente
3S / 24S	esquistossômulo mantido em cultivo por 3 e 24 horas respectivamente
cDNA	DNA da fita molde (RNA) complementar; produto da transcrição reversa usando RNA alvo como molde
ng	nano gramas = 10^{-9} gramas
μ L	micro Litros = 10^{-6} Litros
RNA-Seq	<i>RNA Sequencing</i>

RESUMO

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Palavras-chave: [*Reverse Transcription-qPCR*, *Schistosoma mansoni*, lincRNA]

INTRODUÇÃO: A esquistossomose, causada pelo parasita *Schistosoma mansoni*, é a mais prevalente das doenças tropicais negligenciadas, perdendo apenas para a malária. Recentemente, por estudos bioinformáticos, o nosso grupo verificou a presença de 7029 lincRNAs e 402 lncRNAs antissenso (long non-coding RNA antissenso) em 88 ensaios de transcriptoma (RNA-Seq), em diversos estágios evolutivos do parasita (Vasconcelos et al., 2017). É a primeira vez que foram descobertos, em larga escala, lincRNAs com potencial funcional no parasita *Schistosoma mansoni*.

OBJETIVO: Foram encontrados 181 lincRNAs possuindo as quatro evidências de serem transcritos funcionais. Assim, o trabalho desenvolvido aqui se foca em validar por *Reverse Transcription-qPCR* os achados das análises dos dados de RNA-Seq por meio de ferramentas de bioinformática, ou seja, validar a existência e expressão diferencial dos lincRNAs em diferentes estágios do ciclo de vida de *Schistosoma mansoni* e apontar suas possíveis funções exercidas.

MATERIAIS E MÉTODOS: Todos os parasitas foram obtidos da cepa BH de *S. mansoni* mantidos nos hospedeiros definitivo e intermediário, respectivamente: hamster-sírio (*Mesocricetus auratus*) e o caramujo da espécie *Biomphalaria glabrata*. Para obtenção de vermes adultos os hamsters foram perfundidos após sete semanas de infecção com a respectiva coleta dos vermes pela veia porta hepática, enquanto para a obtenção de esquistossômulos, as cercárias foram transformadas mecanicamente (Basch, 1981) com a perda de suas caudas. A extração de RNA foi realizada com a trituração dos diferentes estágios do parasita, seguido do protocolo de extração de RNA da Qiagen Mini/Micro RNeasy Kit, com adaptações. Foram escolhidos e desenhados pares de primers para 26 dos 181 lincRNAs verificados como contendo as quatro características que os evidenciam como funcionais, por critério de serem os 26 lincRNAs mais correlacionados com genes codificadores de proteínas, segundo uma abordagem realizada por Vasconcelos et al., 2017. A transcrição reversa das amostras foi realizada com 100 ng de RNA total, com quatro réplicas biológicas obtidas para cada estágio de vida do parasita. Os dados gerados pelo *Reverse Transcription-qPCR* foram normalizados em relação à expressão do gene Smp_092920, e os p-valores foram determinados por *one-way analysis of variance* (ANOVA) e teste de Tukey.

RESULTADOS: O resultado dos ensaios de RT-qPCR foi analisado pela comparação dos CT, segundo o método de Livak e Schmittgen, 2001. Foram detectados 16 de 26 lincRNAs testados em *Reverse Transcription-qPCR*, dentre os quais oito foram validados com expressão diferencial coerente com o estágio

esperado, ou seja, o nível de expressão do lincRNA foi maior no ensaio de RT-qPCR no mesmo estágio do parasita encontrado nas análises de RNA-Seq realizadas previamente.

CONCLUSÃO: Foram detectados por RT-qPCR 16 lincRNAs em *S. mansoni*, dentre os quais oito foram validados como mais expressos no estágio de vida do parasita previsto por ensaios de RNA-Seq. Por possuírem grandes evidências de serem transcritos e estarem correlacionados à expressão de PC genes, os lincRNAs parecem participar de importantes processos vitais para o parasita como, por exemplo, modular as transformações de estágio de vida do mesmo.

1. INTRODUÇÃO

A esquistossomose, causada pelo parasita *Schistosoma mansoni*, é a mais prevalente das doenças tropicais negligenciadas, perdendo apenas para a malária. Dados epidemiológicos levantados pela WHO (2017) apontam que a patologia afeta mais de 200 milhões de pessoas no mundo, distribuídas em 78 países, e ocorrendo cerca de 200 mil mortes anualmente. Assim, tanto pelo elevado número de ambientes com água não tratada e ausência de saneamento básico, quanto pela falta de informação da população sob risco, a esquistossomose ainda é muito recorrente no mundo.

O parasita trematódeo *Schistosoma mansoni*, diferente de outros trematódeos, se desenvolve de forma dióica (macho e fêmea) ao longo dos diferentes estágios de evolução do parasita. A manutenção do ciclo de vida do parasita é complexa e envolve diversos estágios de desenvolvimento, um hospedeiro intermediário (caramujo) e o hospedeiro definitivo (mamíferos/homem).

A patologia está relacionada à numerosa liberação de ovos pela fêmea na circulação mesentério-hepática do hospedeiro, provocando reações imunes crônicas conforme o ovo atinge a circulação sistêmica. Os sintomas variam de dores abdominais, diarreia, inchaços provocados pela obstrução dos vasos abdominais até fibroses hepáticas, provocadas pela formação de granulomas em resposta aos antígenos dos ovos do parasita, que se alojam nas veias do fígado (Carvalho, O. S. et al., 2008).

Parte dos ovos produzidos pelo parasita saem da circulação, atravessam a parede do intestino, caem nas fezes, e quando atingem a água doce de rios e lagos os ovos eclodem, dando origem a uma forma denominada miracídio, que

infecta caramujos aquáticos do gênero *Biomphalaria*. Neste hospedeiro intermediário, ocorre o desenvolvimento da forma infectante no homem, a cercária, a qual abandona o caramujo e permanece em meio aquático.

O início do ciclo no homem começa com a penetração, na pele do homem, do estágio larval infectante (cercária). As cercárias penetram ativamente na pele exposta do homem, em contato com ambientes aquáticos não tratados, e durante o processo de penetração perdem sua cauda para dar origem ao esquistossômulo. O esquistossômulo circula passivamente pela corrente sanguínea do hospedeiro, em direção aos pulmões, até chegar no coração, por onde são bombeados para a circulação sistêmica do homem e atingem a veia porta-hepática. Os esquistossômulos se alimentam de plasma sanguíneo e amadurecem sexualmente para sua forma dióica adulta: macho ou fêmea, os quais se pareiam e migram para o plexo mesentérico superior do intestino. O casal macho-fêmea produz diariamente cerca de 300-3000 ovos, parte dos quais é levada pela circulação até o fígado, onde causa a destruição hepática, e outra parte atravessa a parede dos vasos e a parede do intestino, e é eliminada nas fezes do hospedeiro completando o ciclo de vida (Carvalho, O. S. et al., 2008).

Estratégias de Sequenciamento de Nova Geração (“*Next Generation Sequencing*”, NGS), como RNA-Seq, estão sendo utilizadas amplamente para as descobertas médicas do funcionamento complexo do genoma dos seres vivos, principalmente de eucariotos, gerando uma imensa quantidade de dados (Goodwin, Sara et al. 2016). Devido à sensibilidade da técnica e a era do “*Big Data*”, muitos transcritos biológicos, que até então eram desconhecidos, acabaram sendo descobertos por métodos bioinformáticos, tais como a variedade de RNAs não-codificadores de proteína “*non-protein-coding RNAs*” (ncRNAs) (Cech, Thomas R. et al., 2014).

Apesar do nome, os ncRNAs são funcionais e estão se revelando como os grandes responsáveis em número e importância pela complexa regulação da expressão gênica na maior variedade de organismos, particularmente dos eucariotos (Cech, Thomas R. et al., 2014).

Dentre os ncRNAs, existem os RNAs longos intergênicos não-codificadores “lincRNAs” (*long intervening non-coding RNA*), os quais são definidos como sequências de 200 nucleotídeos ou mais que não possuem

potencial de codificar uma proteína, que não se sobrepõem ao locus de um gene codificador de proteína “*Protein Coding gene*” (PC) e que podem atuar como *enhancers* ou se ligando a complexos proteicos com funções epigenéticas.

Recentemente, por estudos bioinformáticos, o nosso grupo verificou a presença de 7029 lincRNAs e 402 lncRNAs antissenso (long non-coding RNA antissenso) em 88 ensaios de transcriptoma (RNA-Seq, seja em dados públicos ou gerados pelo nosso grupo) de *Schistosoma mansoni*, em diversos estágios evolutivos do parasita (Vasconcelos et al., 2017). É a primeira vez que foram descobertos, em larga escala, lincRNAs com potencial funcional no parasita *Schistosoma mansoni*.

Para obtenção de um conjunto de lincRNAs com maior chance de representação de transcritos funcionais, os critérios utilizados para encontrar um lincRNA nos ensaios de RNA-Seq foram as quatro características a seguir (não necessariamente todas essas características precisam coexistir em um mesmo lincRNA):

- Marca epigenética de H3K4me3 (trimetilação da lisina 4 da histona H3), que já foi amplamente estudada como evidência de início de transcrição de um gene (Barski A. et al. 2007);
- conservação de pelo menos um éxon do lincRNA entre outras espécies de *Schistosoma* : *S. haematobium* e *S. japonicum*), através do *score* gerado pelo programa PhastCons, evidenciando um possível lincRNA funcional ao longo da evolução do parasita;
- o lincRNA achado deve ser diferencialmente expresso na comparação entre os cinco estágios evolutivos do parasita (cercária, esquistossômulos transformados mecanicamente e cultivados in vitro por 3h (3S) ou 24h (24S), verme adulto macho e fêmea);
- correlação positiva ou negativa com a expressão de seus PC vizinhos no genoma (dois mais próximos *upstream* e *downstream*) através de uma correlação de Pearson.

A figura 1, abaixo, mostra o número de lincRNAs encontrados que possuem cada uma das características listadas acima (figura obtida de Vasconcelos et al., 2017):

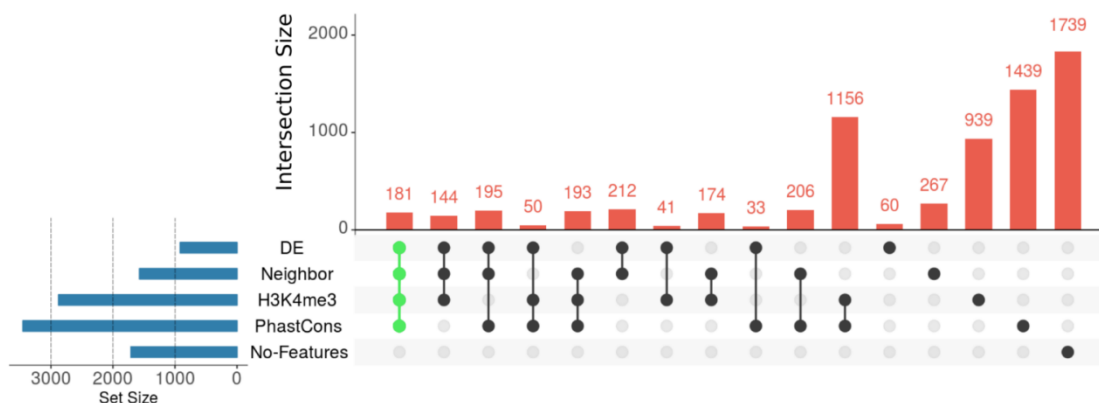


Figura 1: Gráfico representando os lincRNAs encontrados em *S. mansoni* a partir das análises de dados de RNA-Seq (extraído de Vasconcelos et al., 2017). O gráfico acima mostra: o número de lincRNAs detectados como sendo diferencialmente expressos entre os cinco estágios de vida do parasita (DE), como correlacionados com a expressão de seus respectivos PC vizinhos (*Neighbor*), como possuindo marca epigenética de início de transcrição no primeiro exon (H3K4me3) e como sendo conservado entre outras espécies de *Schistosoma spp.* (*PhastCons*). 181 lincRNAs possuem as quatro evidências citadas. A análise dos dados de RNA-Seq evidencia a existência dos lincRNAs e sugere possível funcionalidade.

2. OBJETIVO(S) E JUSTIFICATIVA

Como mostrado na figura 1, 181 lincRNAs foram encontrados possuindo as quatro evidências de serem transcritos funcionais. Assim, o trabalho desenvolvido aqui se foca em validar por *Reverse Transcription*-qPCR os dados de RNA-Seq obtidos por meio de ferramentas de bioinformática, ou seja, validar por RT-qPCR a existência e expressão diferencial dos lincRNAs em diferentes estágios do ciclo de vida de *Schistosoma mansoni* e apontar para possíveis funções exercidas.

Schistosoma mansoni foi escolhido como alvo de estudos devido à ausência de anotações sobre lincRNAs e suas possíveis funções nos diversos e complexos estágios de vida do parasita. Um estudo aprofundado poderia explicar em nível molecular como se dá a transformação complexa de vida desse

eucarioto. Além disso, pode revelar novos alvos farmacológicos em prol do combate à patologia.

3.MATERIAIS E MÉTODOS

3.1 - Obtenção dos diferentes estágios de *S. mansoni*

Todo os parasitas foram obtidos da cepa *BH* de *S. mansoni* mantidos nos hospedeiros definitivo e intermediário, respectivamente: hamster-sírio (*Mesocricetus auratus*) e o caramujo da espécie *Biomphalaria glabrata*.

As cercárias foram obtidas pela infecção dos caramujos com sete a dez miracídios cada. Após 35 dias, os caramujos foram deixados por duas horas sob iluminação para induzir a liberação de cercárias. Em seguida, as cercárias foram suspensas em água em tubos Falcon e resfriadas com gelo. Após 30 minutos, foram centrifugadas, lavadas novamente com água e coletadas em um eppendorf com RNAlater (Ambion) até a extração de seu RNA.

Os esquistossômulos foram obtidos pela transformação mecânica das cercárias (Basch, 1981), e depois pela separação (por decantação) dos esquistossômulos e das caudas de cercária. As cercárias foram suspensas em 15 mL de meio M169 (Vitrocell) contendo penicilina/estreptomicina, anfotericina e pressionadas com uma agulha 23G para a separação da cauda e do esquistossômulo. Essas duas partes foram decantadas, o sobrenadante (caudas) foi descartado e o sedimento (esquistossômulo) foi ressuspensionado em 7 mL do meio M169 preparado com antibióticos. O procedimento foi refeito até se verificar não mais que 1% de cauda restante da suspensão. Então, os esquistossômulos foram mantidos sob 37°C e 5% CO₂ por 3h (3S) e 24h (24S) nesse meio de cultura adicionado de 2% de soro fetal bovino, 1 µM serotonina, 0.5 µM hipoxantina, 1 µM hidrocortisona e 0.2 µM tri-iodotironina. Os esquistossômulos 3S e 24S foram lavados três vezes com PBS e armazenados com RNAlater (Ambion) até a extração de seu RNA.

Os vermes adultos foram coletados da perfusão da veia porta hepática sete semanas após hamster-sírios serem infectados com cerca de 250 cercárias cada. Após a perfusão, os vermes adultos pareados foram mantidos por 3h sob 37°C e 5% CO₂ em meio Advanced RPMI Medium 1640 (Gibco) suplementado

de 10% de soro fetal bovino inativado por calor, 12 mM HEPES ácido 2-[4-(2-hidroxietil)-piperazin-1-il]-etanosulfônico pH 7.4, e 1% penicilina/estreptomicina, anfotericina. Após 3h, os vermes foram lavados três vezes com PBS e mantidos em RNAlater (Ambion) em um eppendorf. Antes de serem extraídos, os casais foram separados manualmente e os RNAs de machos ou fêmeas foram extraídos separadamente.

3.2 - Extração de RNA, quantificação e verificação de integridade

O RNA das cercárias e dos esquistossômulos (3S e 24S) foi extraído seguindo o protocolo baseado em Roquis et al., 2015. Cerca de 25 mil cercárias ou esquistossômulos foram triturados usando *beads* de vidro refrigerados em nitrogênio líquido por cerca de cinco minutos. Então, o RNA foi extraído utilizando o RNeasy Micro Kit (Qiagen) e seguindo o respectivo protocolo, exceto pela adição do dobro da quantidade recomendada de DNase I, que foi mantida agindo por 45 minutos.

Os vermes adultos macho e fêmea foram triturados em *potters* de vidro com pistilos, suspendidos em tampão Qiagen RLT. O RNA foi extraído seguindo o RNeasy Mini Kit (Qiagen) e o protocolo respectivo, exceto pela adição do dobro da quantidade recomendada de DNase I, que foi mantida agindo por 45 minutos. Todas as amostras obtidas (quatro réplicas biológicas para cada estágio) de RNA foram quantificadas por fluorescência utilizando o Qubit RNA HS Assay Kit (Thermo Fisher Scientific) e a integridade dos RNAs foi verificada por eletroforese capilar utilizando o Agilent RNA 6000 Pico Kit (Agilent Technologies) no equipamento 2100 Bioanalyzer Instrument (Agilent Technologies).

3.3 - Escolha dos lincRNAs e desenho dos primers

Foram escolhidos e desenhados pares de *primers* para 26 dos 181 lincRNAs verificados como contendo as quatro características que os evidenciam como funcionais (vide 1 - Introdução). Esses 26 lincRNAs foram selecionados por critério de sua expressão estar mais correlacionada com a expressão dos genes codificadores de proteínas vizinhos no genoma, segundo uma abordagem realizada por Vasconcelos et al., que correlaciona PC genes com lincRNAs como uma hipótese para atribuir funcionalidade a esses lincRNAs.

Os *primers* para o qPCR foram desenhados utilizando a ferramenta *online* Primer 3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) e verificados pela sua qualidade com os cálculos realizados pelo OligoAnalyzer 3.1 (<https://www.idtdna.com/calc/alyzer>).

As tabelas a seguir contém os lincRNAs (SmLINC) e os respectivos pares de *primers* desenhados (tabela 1). Além destes, encontram-se os *primers* dos PC genes (Smps) que foram escolhidos e utilizados como controle positivo (tabela 2), ou seja, genes codificadores de proteínas cujos níveis de expressão são sabidamente aumentados entre os estágios analisados.

Tabela 1 : Lista dos 26 lincRNAs e seus respectivos pares de *primers* utilizados nas reações de RT-qPCR para validação da expressão diferencial entre estágios de *Schistosoma mansoni*

ID do lincRNA	Primer	Sequência
SmLINC00001-IBu	SmLINC00001-IBu-F	CACATGGGTTTAGATGATTGAAG
SmLINC00001-IBu	SmLINC00001-IBu-R	AACCGAATCGTTTATCACCTG
SmLINC00133-IBu	SmLINC00133-IBu-exon2-F	TCACGGATTGCATTCATGTT
SmLINC00133-IBu	SmLINC00133-IBu-exon2-R	TCAATTTACTGGAACAGCACTCA
SmLINC00282-IBu	SmLINC000282-IBu-F	GAGCTGTTGTTGCTTCAAAGG
SmLINC00282-IBu	SmLINC000282-IBu-R	GCCAAGACTTTCCATGTCAAT
SmLINC01084-IBu	SmLINC01084-IBu-F	CGGCGTTTGTGAGGATGTAA

SmLINC01084-IBu	SmLINC01084-IBu-R	TTCATTTGCGGAATAACAAAG
SmLINC01122-IBu	SmLINC01122-IBu-F	TTGTTGGAGAAAAGTTAAACCAG
SmLINC01122-IBu	SmLINC01122-IBu-R	GTGCCCCGTAACACACAGATG
SmLINC01431-IBu	SmLINC01431-IBu-F	CGTTGATGACTCAAAGCCAAA
SmLINC01431-IBu	SmLINC01431-IBu-R	TCTCATATTGGAGGAAGTTGATG
SmLINC02394-IBu	SmLINC02394-IBu-F	AACGTCTGATCAATCTTTGGAA
SmLINC02394-IBu	SmLINC02394-IBu-R	TCATTGTCAATTATGGTCATCCA
SmLINC02630-IBu	SmLINC02630-IBu-F	CTGTTCACCGCAATTACAGAA
SmLINC02630-IBu	SmLINC02630-IBu-R	ATGGATAGCGGCTAGAGATGG
SmLINC03930-IBu	SmLINC03930-IBu-F	CATTGAAAGAATCGAATGATTTGTA
SmLINC03930-IBu	SmLINC03930-IBu-R	CAAGTGGATAGCCTGTTGGTT
SmLINC04271-IBu	SmLINC04271-IBu-F	CCAACTATTTCCAAACACTCAACA
SmLINC04271-IBu	SmLINC04271-IBu-R	CGAATCGTGTGCGATGAAATC
SmLINC04521-IBu	SmLINC04521-IBu-F	CTATTTTTTTGAATAGTGTCAAG
SmLINC04521-IBu	SmLINC04521-IBu-R	CATTTCTTGGGATTGCAGGT
SmLINC05284-IBu	SmLINC05284-IBu-F	CTGGTGGTAGTTACGATGATGAA
SmLINC05284-IBu	SmLINC05284-IBu-R	CTCGACCAAACCATCCATCT
SmLINC05716-IBu	SmLINC05716-IBu-F	CGAACAAGATCGTACTTGATTGAC
SmLINC05716-IBu	SmLINC05716-IBu-R	CCATTTCTTGGCGTAATGTGT
SmLINC05720-IBu	SmLINC05720-IBu-F	CCTAAATTAGAATTTTCATGAATGGAT
SmLINC05720-IBu	SmLINC05720-IBu-R	AATATTTATCTAAAGAACGAAGCCTT T
SmLINC06024-IBu	SmLINC06024-IBu-F	TTGCTGGACAAAGTAAATGGAA
SmLINC06024-IBu	SmLINC06024-IBu-R	CGAGAATGAGGAACGAACTGA

SmLINC06535-IBu	SmLINC06535-IBu-F	CCAAGATTACAAGGACGGAAG
SmLINC06535-IBu	SmLINC06535-IBu-R	TTTCACTGGTCTGTTGTATCGAA

Tabela 2: *Protein-coding genes* e seus respectivos pares de *primers* utilizados como controle positivo de qPCR para os testes de expressão diferencial em cinco estágios do *Schistosoma mansoni*

Descrição do gene	Primer	Sequência	ID Smp gene
Metalloprotease	Smp_044250-MP-PM-2011-F	CGTCTGGATGCTCTCCTTAAGTC	Smp_044250
Metalloprotease	Smp_044250-MP-PM-2011-R	TTCTGAGAACTGGACAGACAAACG	Smp_044250
Lactate Dehydrogenase	Smp_033040-LD-PM 2011-F	CGAAAAGCCTGAACAAAACTAGTAA	Smp_033040
Lactate Dehydrogenase	Smp_033040-LD-PM-2011-R	TGTGTCATTATATCAACTGGATTTGT G	Smp_033040
WNT5	WNT5_Smp_145140_F	CAGCATTTGGTCCGGTAACT	Smp_145140
WNT5	WNT5_Smp_145140_R	CGAGCTTCTTTACAACTTCTTGC	Smp_145140
Serotonin Receptor	5HTR_Smp_126730_F	AAGTGCAGCTGGTTACAACACT	Smp_126730
Serotonin Receptor	5HTR_Smp_126730_R	TGGATTGAGAAAAGTGTTCACG	Smp_126730
EggShell protein	EggShell_Smp_000430_F	GCCTACAGACAATCAGTACGGTAA	Smp_000430
EggShell protein	EggShell_Smp_000430_R	GACGTTGGTTGGTAATTGGAA	Smp_000430
Trematode eggshell protein	TrEgg_Smp_000390_F	AAAGAAGCACGGAATGCAAC	Smp_000390
Trematode eggshell protein	TrEgg_Smp_000390_R	CTTTCCACTGGTTTGCATCA	Smp_000390

3.4 - Validação por RT-qPCR

A transcrição reversa (RT) das amostras foi realizada com 100 ng de RNA total, utilizando o kit SuperScript IV First-Strand Synthesis System (Life Technologies) e *primers* hexâmeros randômicos, em volume final de 20 µL. A fita de DNA complementar (cDNA) foi obtida e o meio foi diluído dez vezes em água. Os ensaios de qPCR foram realizados usando 2,5 µL de cada cDNA diluído, num volume total de 10 µL, contendo 1X LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics) e 800 nM de cada *primer* no equipamento LightCycler 480 System (Roche Diagnostics). Cada *Reverse Transcription*-qPCR foi corrido com três replicatas técnicas.

Foram escolhidos doze genes candidatos como gene normalizador (*Housekeeping gene*) de acordo com a menor variação de expressão nos cinco estágios nos ensaios de RNA-Seq analisados. As ferramentas BestKeeper, NormFinder and GeNorm foram utilizadas para escolher como o gene normalizador para os ensaios de qPCR deste trabalho o gene que menos varia sua expressão entre os estágios estudados. Segundo as análises, o Smp_092920 foi o gene que se comportou como melhor normalizador, e ele foi então utilizado como referência nos ensaios de validação dos lincRNAs.

Os dados gerados pelo *Reverse Transcription*-qPCR foram normalizados em relação à expressão do gene Smp_092920, e os p-valores foram determinados por *one-way analysis of variance* (ANOVA) e teste de Tukey.

5- RESULTADOS E DISCUSSÃO

O resultado dos ensaios de RT-qPCR foi analisado pela comparação dos CT, segundo o método de Livak e Schmittgen, 2001. Foram detectados 16 de 26 lincRNAs testados em Reverse Transcription qPCR, dentre os quais oito foram validados com expressão diferencial coerente com o estágio esperado, ou seja, o nível de expressão do lincRNA foi maior no ensaio de RT-qPCR no mesmo estágio do parasita encontrado nas análises de RNA-Seq realizadas previamente. As figuras 2 e 3 abaixo mostram quais foram os *protein coding genes* testados e os lincRNAs detectados respectivamente por RT-qPCR (figuras extraídas de - Vasconcelos et al., 2017):

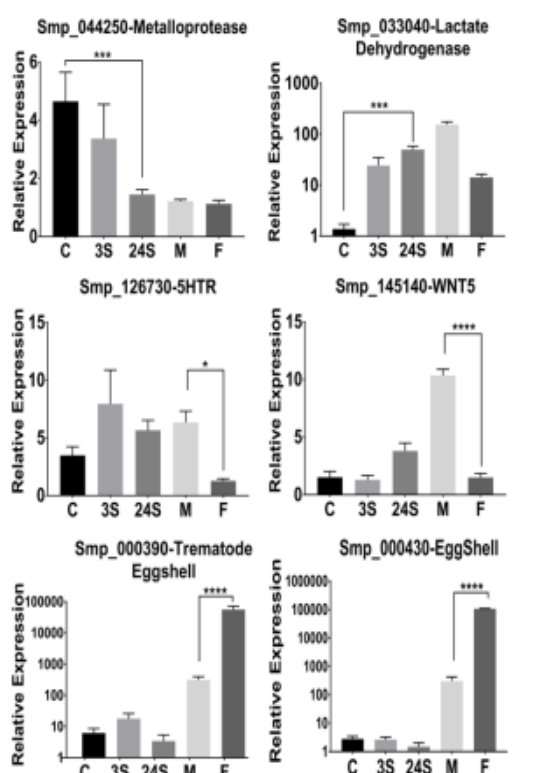


Figura 2: *Protein coding genes* testados como controle positivo por ensaios de Reverse Transcription qPCR (Vasconcelos et al., 2017). Os gráficos indicam no eixo horizontal o estágio do parasita dentre os cinco estágios estudados (C - Cercária, 3S - Esquistossômulo 3h, 24S - Esquistossômulo 24h, M - adulto macho, F - adulto fêmea); o eixo vertical indica a expressão relativa dos PC genes (Smp) encontrada nos ensaios de RT-qPCR. Todos os Smps mostrados estão com expressão diferencial (validados) conforme os dados de RNA-Seq prévios para os cinco estágios.

Os dados de expressão diferencial obtidos para os controle positivos apresentados são todos coerentes com os achados anteriores para as análises de RNA-Seq previamente estudadas nos cinco estágios: Smp_044250 (*Metalloprotease*) foi confirmada com expressão aumentada em cercária em relação ao esquistossômulo, Smp_033040 (*Lactate dehydrogenase*) está com expressão aumentada em esquistossômulo em relação à cercária, Smp_126730-5HTR e Smp_145140-WNT5, confirmados com expressão aumentada em vermes adultos macho em relação à adultos fêmea, Smp_000390-Trematode Eggshell e Smp_000430-EggShell, confirmados com expressão aumentada em vermes adultos fêmea em relação à adultos macho.

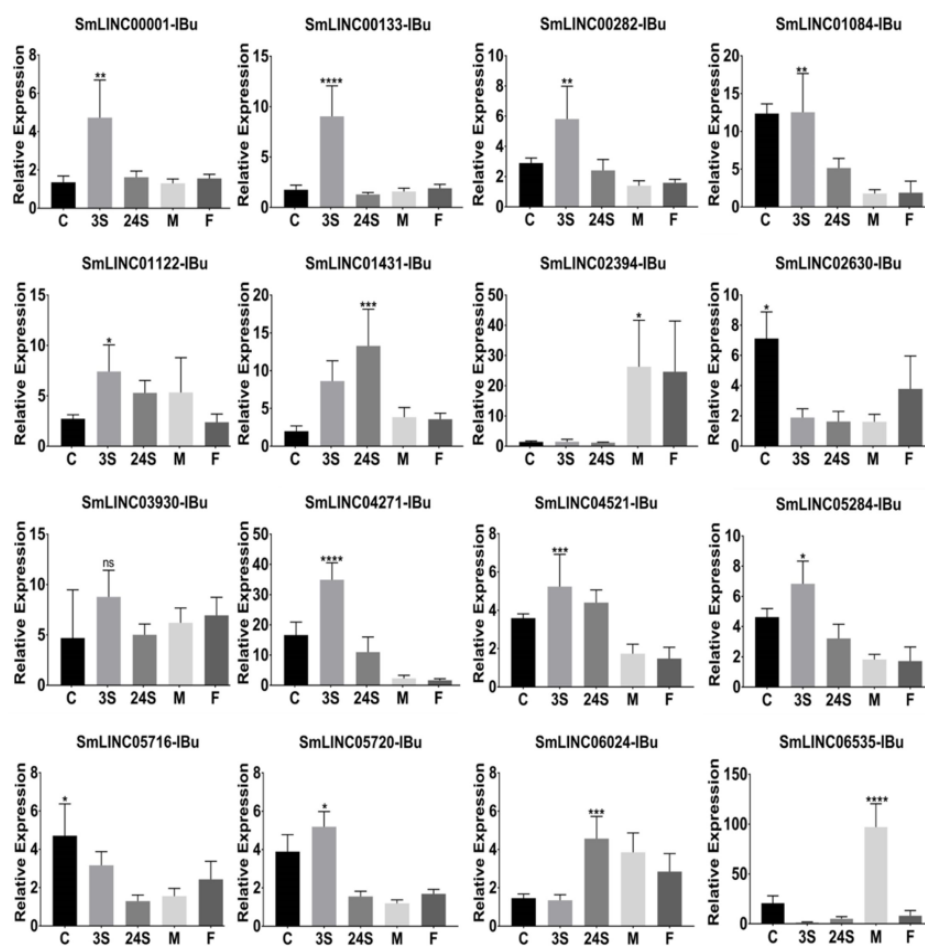


Figura 3: lincRNAs detectados por ensaios de *Reverse Transcription*-qPCR (Vasconcelos et al., 2017). Os gráficos indicam no eixo horizontal o estágio do parasita dentre os cinco estágios estudados (C - Cercária, 3S - Esquistossômulo 3h, 24S - Esquistossômulo 24h, M - adulto macho, F - adulto fêmea); o eixo vertical indica a expressão relativa dos lincRNAs encontrada nos ensaios de RT-qPCR. 26 lincRNAs foram escolhidos dentre 181 lincRNAs que possuíam as quatro evidências de lincRNAs funcionais (marca epigenética H3K4me3, DE nos cinco estágios, expressão correlacionada com os PC genes vizinhos, conservação de pelo menos um éxon com outras espécies de *Schistosoma*). Dos 26 lincRNAs escolhidos, 16 lincRNAs foram detectados nos ensaios de RT-qPCR, sendo oito validados e confirmando seu maior nível de expressão no mesmo estágio encontrado previamente por RNA-Seq.

Os oito lincRNAs que tiveram o estágio de maior expressão validado, ou seja o nível de expressão variava entre os estágios e o estágio de maior expressão no RNA-seq coincidiu com o estágio de maior expressão no RT-qPCR, foram: SmLINC00133-IBu, SmLINC00282-IBu, SmLINC02394-IBu, SmLINC02630-IBu, SmLINC03930-IBu, SmLINC04271-IBu, SmLINC05284-IBu e SmLINC05720-IBu.

Para os demais 8 lincRNAs, confirmamos que havia níveis de expressão diferentes para cada lincRNA entre os estágios, mas o estágio de maior expressão no RT-qPCR diferiu do estágio visto no RNA-seq. Dos 16 genes detectados por RT-qPCR, dois foram mais expressos em cercária (SmLINC026300IBu e SmLINC05716), dez em esquistossômulo 3h (SmLINC00001-IBu, SmLINC00133-IBu, SmLINC00282-IBu, SmLINC01084-IBu, SmLINC01122-IBu, SmLINC03930-IBu, SmLINC04271-IBu, SmLINC04521-IBu, SmLINC05284-IBu, SmLINC05720-IBu), dois mais expressos em esquistossômulo 24h (SmLINC01431-IBu, SmLINC06024-IBu) e dois em adulto macho (SmLINC02394-IBu, SmLINC06535-IBu).

Para o restante dos lincRNAs que não foram detectados por RT-qPCR, pode-se pensar na possibilidade do par de *primers* ser ineficiente para anelar na região de interesse, pela formação de *hairpins* ou dímeros de *primer*. Isso se deve ao fato que o genoma do *Schistosoma mansoni* é conservado em algumas regiões, com grandes repetições exônicas de nucleotídeos A e T e alguns cálculos realizados pelo Primer 3 e OligoAnalyzer já indicavam provável reestruturação tridimensional do *primer*, que não se pôde evitar para aquele determinado lincRNA alvo.

Para alguns *primers*, por exemplo, não se conseguiu desenhar um tal que sua sequência abrangesse dois éxons do lincRNA, o que validaria apenas uma determinada isoforma do gene, devido à correspondência da sequência desenhada com outra região do genoma que não a de interesse - realizando uma busca com Blat no “*Schistosoma mansoni* Genome Browser”. Além disso, alguns *primers* possuíam mais de 22 nucleotídeos, pois a grande sequência de A e T abaixa a temperatura de *melting* desse *primer* (por formação de ligações mais fracas que entre C e G); somado a isso, um produto amplificado mais longo que os produtos de demais *primers* desenhados pode atrasar e/ou impedir a reação em cadeia da Taq-polimerase adequadamente e dificultar uma comparação entre CTs.

Por serem escolhidos entre os 181 lincRNAs previamente citados, esses 16 lincRNAs, especificamente os oito com estágio de maior expressão validado, apresentam expressão diferencial em cinco diferentes estágios evolutivos do parasita *S. mansoni*. Como já citado, uma das funções já descritas dos lincRNAs

é regular a expressão dos PC genes. Assim, os lincRNAs encontrados e validados aqui em *S. mansoni* podem estar regulando a diferenciação vital do parasita entre os seus diversos estágios evolutivos por meio, por exemplo, da regulação da expressão de PC genes vizinhos, num mecanismo em *cis*, como já foi descrito em outros estudos com outras espécies animais (Quinn et al., 2016 e Mondal et al., 2010).

Uma análise também realizada por Vasconcelos et al., 2017 correlaciona os 181 lincRNAs de acordo com a sua expressão com PC genes através da correlação de Pearson $r > 0,8$ ou $r < -0,8$ e correspondente p-valor $< 0,05$, ou seja, estatisticamente significativo. Essa abordagem de buscar co-expressão entre lincRNAs e PC genes já foi utilizada previamente na literatura (16, 17, 18 e 22). Para os 181 lincRNAs, foram encontrados 2359 PC genes correlacionados de acordo com os critérios acima, formando 68.625 pares SmLINC-Smp.

Pelas abordagens realizadas para relacionar lincRNAs com PC genes de *S. mansoni* (Vasconcelos et al., 2017) segundo os termos GO (Gene Ontology) mais enriquecidos, prevalecem as categorias: “*RNA-dependent DNA replication*”, “*DNA integration*” e “*Monovalent inorganic cation transmembrane transporter activity*”. Logo, os SmLINCs podem estar regulando genes codificadores de proteína cujas funções estão relacionadas a essas categorias, e estas podem ser essenciais para a transformação complexa dos estágios de vida do parasita.

O primeiro genoma do *S. mansoni* foi publicado em 2009 e melhorado em 2012 com técnicas de NGS (Protasio et al., 2012). Nesse genoma, 380 Mb e aproximadamente 12.000 PC genes (Smps) foram mapeados. De todo o genoma do parasita, estima-se que a fração de genes codificadores de proteína seja de apenas 4,5 % (Vasconcelos et al., 2017). O restante do DNA, como já estudado para outros seres vivos, poderia ser codificador de RNAs regulatórios que participariam em diversos processos pós-transcricionais que sabidamente ocorrem em *S. mansoni*, tais como o *splicing* alternativo de mRNAs, regulações epigenéticas de modulação da cromatina, entre outros (6, 10, 15, 21, 24).

Assim, cerca de 95% do genoma do *S. mansoni* não corresponde a genes codificadores de proteínas, e um estudo mais aprofundado pode ajudar a entender a complexidade do organismo. Agora sabemos que existe uma parcela do genoma de *S. mansoni* que é transcrita em lincRNAs, que estes possuem

expressão regulada entre os diferentes estágios do parasita e que poderiam ser vitais ao patógeno.

Considerando os lincRNAs detectados, pode-se apontar alguns Smps como possivelmente regulados quanto à sua expressão gênica ao longo dos estágios do animal segundo as abordagens de correlação Smp-SmLINC (PC gene-lincRNA).

O SmLINC04271-IBu, por exemplo, está correlacionado à proteína *lamin* ou *Class V intermediate filaments* (Smp_159900), que são proteínas que estruturam o núcleo da célula animal e inclusive regulam a transcrição gênica a nível célula-tecido específico (Burke et al., 2012). O gene codificador de proteína Smp_159900 poderia então ter papel fundamental para a diferenciação entre estágios do animal *Schistosoma mansoni* através da regulação estrutural do núcleo, e podendo ser expresso de diferentes formas pela sua regulação de expressão através de um lincRNA corregulador, como o SmLINC04271-IBu. O Smp_081720 (*Transmembrane protein 231*) é uma proteína que age como uma barreira estrutural entre a camada ciliar e a membrana plasmática e tem importância na ciliogênese (UniProtKB). Uma vez que os miracídios - um dos estágios de vida do parasita - são ciliados, poder-se-ia atribuir alguma funcionalidade a essa proteína e à sua regulação por lincRNAs como importante para a constituição do miracídio.

Através de abordagens realizadas por Vasconcelos et al., foi possível destacar, por exemplo, o SmLINC01637-IBu, um lincRNA que aparece negativamente correlacionado com proteínas relacionadas à membrana e atividades secretórias, como por exemplo: *fibrillin 2* (Smp_001100), *transmembrane protein 26* (Smp_026670), *transmembrane protein 231* (Smp_081720), *fras1 related extracellular matrix protein* (Smp_149390), *protocadherin 9* (Smp_151620), *surface membrane antigen* (Smp_195180) and *saposin B domain containing protein* (isoformas de Smp_016490.1 e .2), que já foi apontada em estudos como candidata a alvo de vacinas. Também aparece correlacionado positivamente com o Smp_122830 (*ataxin 2*).

6- CONCLUSÃO

Desde que o genoma do *S. mansoni* foi anotado, é sabido que apenas 4,5% corresponde a genes codificadores de proteína. Até recentemente, a porção do DNA que não codificava proteína era considerado “DNA lixo”, mas hoje se sabe que a maior parte do DNA codifica para transcritos de RNA que não são traduzidos em proteína. Ainda assim, estes RNAs parecem possuir papel essencial na regulação da expressão gênica, de forma a participar da complexidade da expressão fenotípica de um organismo. Neste sentido, segundo estudos da literatura, conforme a complexidade do organismo aumenta, o número de transcritos não-PC aumenta também (Mattick et al., 2006 e Dunham et al., 2012). Foram detectados por RT-qPCR 16 lincRNAs em *S. mansoni*, dentre os quais oito foram validados como mais expressos no estágio de vida do parasita previsto por ensaios de RNA-Seq. Por possuírem grandes evidências de serem transcritos e estarem correlacionados à expressão de PC genes, os lincRNAs parecem participar de importantes processos vitais para o parasita tais como, por exemplo, modular as transformações de estágio de vida do mesmo. Estudos que visam silenciar ou aumentar a expressão desses lincRNAs (*in vivo* e *in vitro*) no parasita poderiam confirmar e também revelar outras funcionalidades para os SmLINC.

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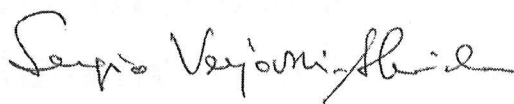
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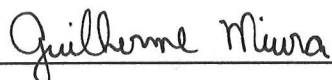
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**Validação por PCR quantitativo da variação do nível de
expressão de RNAs longos não-codificadores de proteínas
entre os diferentes estágios do ciclo de vida do parasita
*Schistosoma mansoni***

Trabalho de Conclusão do Curso de Farmácia-Bioquímica da Faculdade de
Ciências Farmacêuticas da Universidade de São Paulo.



12 de abril de 2018



Data e assinatura do aluno(a)

Data e assinatura do orientador(a)

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The *Schistosoma mansoni* genome encodes thousands of long non-coding RNAs predicted to be functional at different parasite life-cycle stages

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Next Generation Sequencing (NGS) strategies, like RNA-Seq, have revealed the transcription of a wide variety of long non-coding RNAs (lncRNAs) in the genomes of several organisms. In the present work we assessed the lncRNAs complement of *Schistosoma mansoni*, the blood fluke that causes schistosomiasis, ranked among the most prevalent parasitic diseases worldwide. We focused on the long intergenic/intervening ncRNAs (lincRNAs), hidden within the large amount of information obtained through RNA-Seq in *S. mansoni* (88 libraries). Our computational pipeline identified 7029 canonically-spliced putative lincRNA genes on 2596 genomic loci (at an average 2.7 isoforms per lincRNA locus), as well as 402 spliced lncRNAs that are antisense to protein-coding (PC) genes. Hundreds of lincRNAs showed traits for being functional, such as the presence of epigenetic marks at their transcription start sites, evolutionary conservation among other schistosome species and differential expression across five different life-cycle stages of the parasite. Real-time qPCR has confirmed the differential life-cycle stage expression of a set of selected lincRNAs. We have built PC gene and lincRNA co-expression networks, unraveling key biological processes where lincRNAs might be involved during parasite development. This is the first report of a large-scale identification and structural annotation of lncRNAs in the *S. mansoni* genome.

Over the past ten years, the advent of Next Generation Sequencing (NGS) strategies like RNA-Seq have revealed the transcription of a wide variety of non-protein-coding RNAs (ncRNAs) in the genomes of eukaryote organisms. Early on this age, it had become quite evident that life in the eukaryotic cells is orchestrated by complex regulatory networks in which ncRNA molecules appear to be the most numerous and main signaling effectors on many vital reactions¹. Within this large class of ncRNA genes, long non-coding RNAs (lncRNAs) have been conventionally defined as being over 200 nt-long transcripts that have no protein-coding (PC) potential². lncRNAs seem to display a number of regulatory functions and many of them now have a well-characterized role as epigenetic modulators^{3–5}.

There are several subtypes of lncRNAs, which are classified by their architecture and/or location onto the genome rather than by their function. The following are the terminologies established for the already known lncRNAs' subtypes: (i) antisense lncRNAs, which overlap the exons of PC genes on the opposite strand; (ii) sense intronic lncRNAs, which reside exclusively into PC gene introns; (iii) large intergenic/intervening non-coding RNAs (lincRNAs), which do not overlap PC genes and may act either in *cis*, like enhancer RNAs (eRNAs), or in *trans*, by binding to chromatin modifier protein complexes^{2,3,6,7}.

Despite sharing similar general traits with their PC gene counterparts such as being transcribed by RNA polymerase II, following the same chromatin state rules, often 5'-capped, spliced and polyadenylated², it is known

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in vertebrates that lncRNAs exhibit several peculiar and distinct features when compared with mRNAs, such as: (i) rapid evolution across species from the same clade^{8,9}; (ii) both low and transient expression, being most of the times specimen-, tissue-, and/or cell-specific^{6,7,10–12}; (iii) reduced splicing efficiency¹⁰; (iv) high propensity to *cis*-regulatory action immediately following or during their own transcription^{2,13}; (v) participation in *trans*-acting mechanisms when associated to polycomb repressive complex 2 (PRC2), for instance^{3,14,15}; (vi) formation of RNA-DNA triplex structures that may mediate both *cis*- and *trans*-actions^{14,16–18}; (vii) more enriched in the nucleus than in the cytoplasm⁶.

Schistosoma mansoni was chosen by us as a target organism to unravel the class of lincRNA genes because of both the lack of lncRNAs annotation on its genome and its global health importance as a neglected tropical disease-causing agent. It is a flatworm endemic in both Africa and South America continents that causes an infectious and parasitic disease known as schistosomiasis that affects, together with other schistosome species, over 250 million people worldwide¹⁹. Besides its public health impact, this parasite is also an interesting model for studies on the Molecular Parasitology field because of its complex life cycle and, consequently, the drastic gene expression changes it faces during development. From the nearly 12,000 PC genes already mapped onto *Schistosoma* spp. genomes^{20–22}, there are about 2,000 stage-specific differentially expressed (DE) ones among *S. mansoni* cercariae (the human infective larval stage), schistosomula (the first stage in a mammal host) and mature mixed adults residing in the mesenteric veins²¹, as well as between separate male and female adult forms of *S. haematobium* and their respective eggs²².

Up to date only one single study has been devoted to a large-scale mapping of ncRNA genes in schistosomes²³. However, the authors have only adopted homology-based annotation through the use of bioinformatics tools in order to screen for structured RNAs on a comparison between *S. mansoni* and *S. japonicum* genomes²³. No lncRNAs were reported on that investigation. On the other hand, our group has already pointed to the existence of lncRNAs in the *S. mansoni* transcriptome, by using both microarray and low-coverage RNA-Seq approaches^{24,25}.

Recently, several new bioinformatics tools have been developed with the aim of capturing novel lncRNAs mapped to the genomes^{26–29}. However, they are mostly geared towards applying the machine learning techniques to the well-curated human and/or mouse transcriptome mapping annotations. For invertebrates, *Caenorhabditis elegans* (a nematode free-living worm) and *Plasmodium falciparum* (a protozoan parasite) have already had their transcriptomes screened for lncRNAs by independent groups who established their own *ad-hoc* pipelines for analyzing RNA-Seq data^{30,31}. They have unraveled ~1,000 transcripts among antisense lncRNAs and lincRNAs for each of the above two organisms.

In the present work, we thoroughly screened the *S. mansoni* genome and transcriptome and we present a novel repertoire of spliced lncRNA genes, with a main focus on the lincRNAs subtype. We first describe our computational pipeline aimed at retrieving lncRNAs from dozens of raw large-scale RNA-Seq samples (either public data available at the NCBI-SRA repository or data generated by us). Subsequently, we report their location and architecture onto the genome, the evidence for being transcribed, and their evolutionary conservation among other species. Finally, we analyze the lincRNAs putative functionality by building co-expression networks that associate them to their PC gene counterparts that are co-expressed across five different parasite developmental stages. This is the first study that provides a holistic systems overview that merges *S. mansoni* lincRNAs content with what is encoded into proteins and raises hypotheses about the lincRNAs functionality through the construction of co-expression networks.

Results

A computational pipeline is able to retrieve thousands of lncRNAs from *S. mansoni* RNA-Seq samples. Our *ad-hoc* computational pipeline (Fig. 1) was designed based on two distinct transcriptome assembly approaches: (i) a *de novo* method using Trinity³² (Fig. 1, path A), and (ii) a reference genome/annotation-based method, using Tophat2 (ref. 33), Cufflinks and Cuffmerge³⁴ (the Tuxedo suite) (Fig. 1, path B). As suggested by Ulitsky⁹, the use of different assembly algorithms increases the chance of detecting the lncRNAs complement of a cell or organism (more details on the Methods section). Table 1 shows the number of transcripts obtained with each assembly algorithm.

We have established a series of more stringent filtering steps in our pipeline (red border rectangles on Fig. 1) compared with the previously described ones for other invertebrates³¹, and we used a greater number of RNA-Seq samples as input ($n = 88$, see Methods). The first filtering step was the removal of PC-overlapping transcripts when rescuing lincRNAs, or the removal of both intervening and sense PC-overlapping transcripts when rescuing the antisense lncRNAs. As an example, Table 1 shows the number of lincRNA transcripts that remain at this step and at each of the further ones in our pipeline. The subsequent steps included the removal of repeats (low complexity and/or transposable elements), rRNAs, monoexonic transcripts, and spliced transcripts presenting all introns with less than 30 nt each and/or not showing canonical splice donor/acceptor (GT/AG) sites. Further, we removed transcripts presenting ORFs greater than 25 amino acids (aa) and covering 25% or more of the transcript lengths, as well as transcripts assigned as having coding potential probability by either CPC³⁵ or TransDecoder³⁶ algorithms, and, finally, transcripts containing Pfam and/or PANTHER domains as detected by InterProScan³⁷ (see Methods).

At the end of the filtering pipeline we obtained two datasets: one with 7029 lincRNA isoforms (Table 1) located on 2596 genomic loci (at an average of 2.7 lincRNA isoforms per each lincRNA genomic locus), and another dataset with 402 lncRNAs that are antisense to 268 PC genes. The transcript sequences are on the Supplementary File S1. On Supplementary Table S1 we provide the genomic location and architecture of each lncRNA for the two datasets. We decided to focus our analyses on the lincRNAs gene set due to the greater amount retrieved, and also because the lincRNAs arise from PC-independent novel gene loci. For a better visualization of the lincRNA loci and architecture, we have created a track for them on the *S. mansoni* UCSC-like genome browser (<http://schistosoma.usp.br>), which we recently implemented and reported²⁴. On Fig. 2 we selected three lincRNA loci to

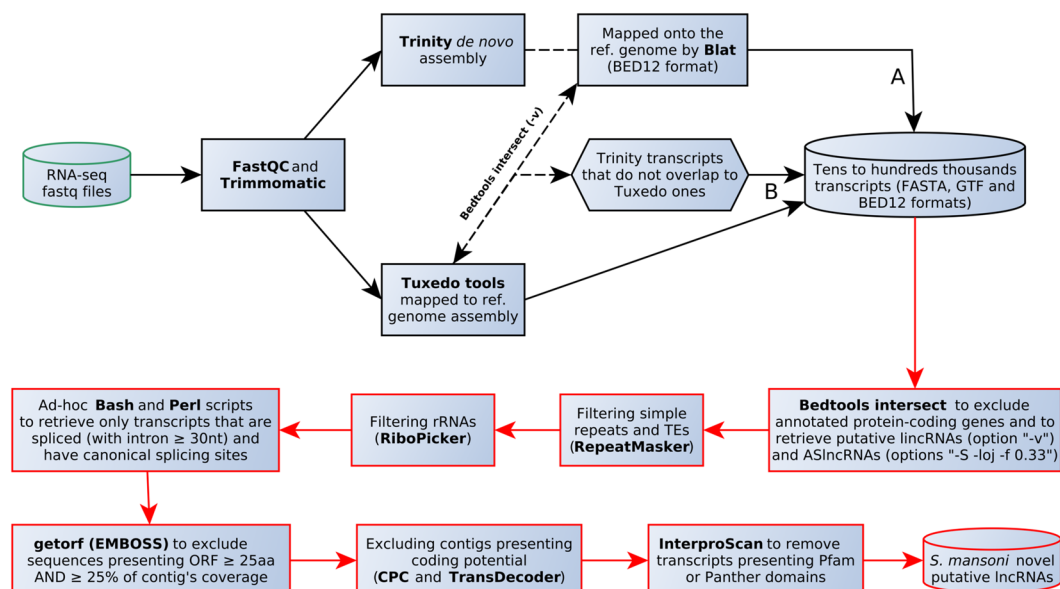


Figure 1. Computational workflow for rescuing and identifying novel putative lincRNAs from *S. mansoni* high-throughput transcriptomic data. Path A depicts the use of a *de novo* assembly method, whereas path B comprises reference-guided assembly. The red section is built in one single PERL script for the automation of tasks. On the bedtools intersect step (first red rectangle), one can choose whether the pipeline retrieves antisense lincRNAs or lincRNAs.

Filtering Steps	Number of transcripts for each indicated dataset and assembly tool that remain after the indicated filtering step			
	trin_strA*	trin_strB*	trin_sra*	tux_all*
Total assembled transcripts	245,237	230,961	389,266	83,821
Remove annotated protein-coding genes	54,927	48,269	79,684	10,531
Remove repeats	43,132	36,440	61,820	7928
Remove rRNAs	43,123	36,421	61,810	7928
Remove monoexonic transcripts	16,499	13,779	15,324	4677
Remove transcripts with all introns < 30 bp	11,026	9398	7347	4642
Remove transcripts with non-canonical splice sites	7355	6294	2207	4347
Remove transcripts with ORFs ≥ 25 aa (AND ≥ 25% transcript length)	2495	2032	628	2767
Remove transcripts with coding potential (CPC and TransDecoder)	2432	1985	613	2250
Remove transcripts with protein domains (Interproscan)	2407	1956	604	2062
Total <i>S. mansoni</i> novel putative lincRNAs (sum of the four datasets)	7029			

Table 1. Summary of *S. mansoni* lincRNAs mining steps and their respective number of filtered transcripts after the execution of the pipeline from Fig. 1. *Two different assembly methods (Trinity *de novo* “trin” and Tuxedo tools genome-guided “tux”) and four datasets (strA, strB, sra and all) were used as starting point for lincRNAs identification (see Methods for more details).

be viewed as genome browser pictures, where one can see different tracks on each of three different loci, showing evidence for transcription and evolutionary conservation of these novel lincRNA genes. Such evidence will be described further on this section.

Because there is no gold standard *in silico* protocol for detecting lincRNAs in ancient parasite genomes/transcriptomes, we ran a sensitivity test of our pipeline, namely trying to reproduce the list of previously identified lincRNAs from *Plasmodium falciparum*³⁰, and we successfully recovered 53% of the *P. falciparum* lincRNAs. We believe the 53% sensitivity rate reflects the more stringent filtering steps that we have deployed on our pipeline compared with the ones from Broadbent *et al.*³⁰. We expect that low levels of false positive lincRNAs detection may be achieved in our *S. mansoni* data analysis due to our stringency choices.

A great variety of lincRNA transcript isoforms was detected. In order to check whether different assembly tools and datasets used as input in our pipeline (see Table 1) yielded similar transcripts, and to calculate the degree of redundant transcripts obtained, we performed a blastn³⁸ alignment of the total 7029 lincRNA sequences against themselves. This all against all comparison has revealed only 15 identical pairwise alignments

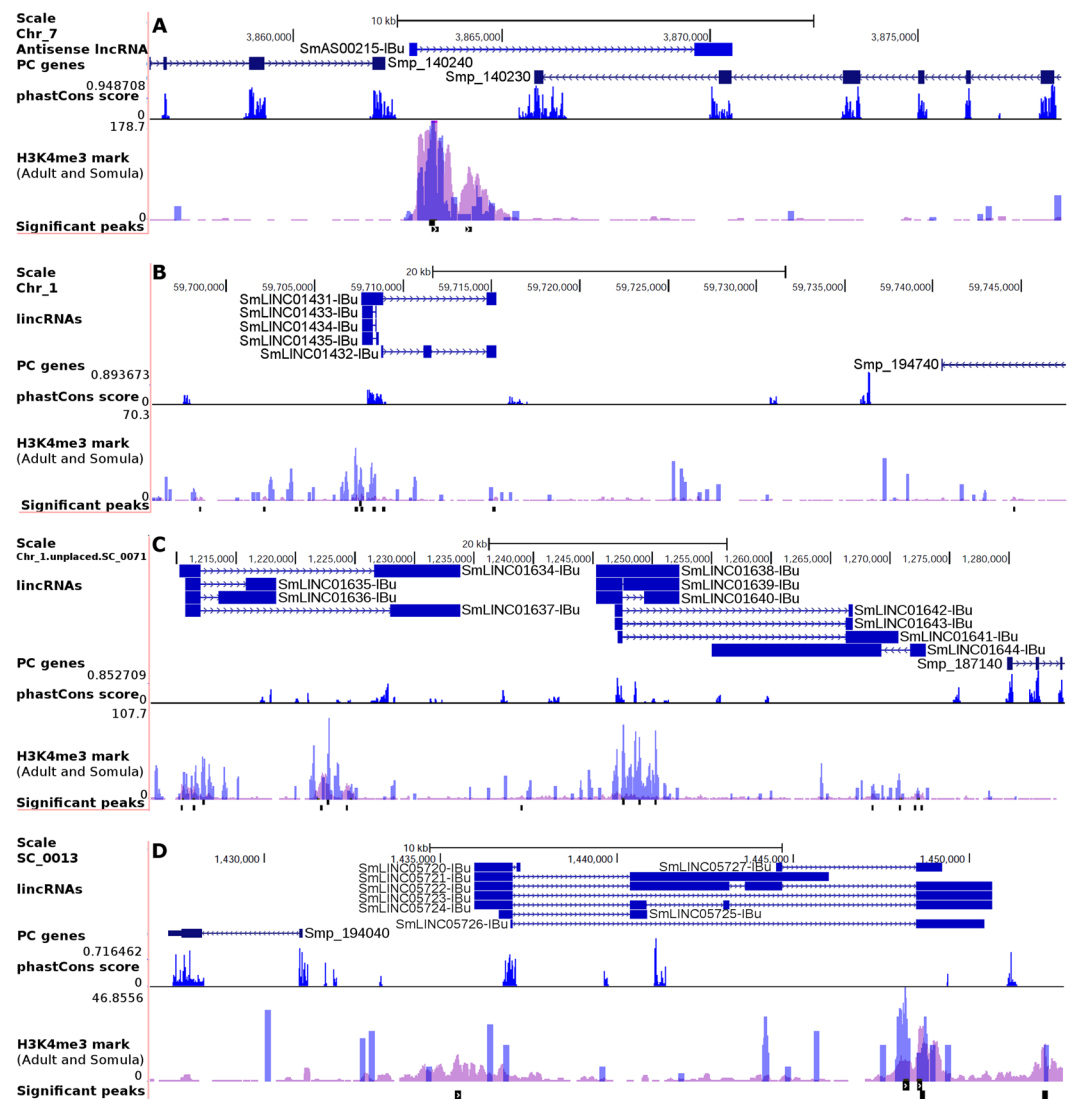


Figure 2. Novel *S. mansoni* spliced lncRNA gene loci. All lncRNA isoforms depicted on the figure display both evolutionary conservation (phastCons score) among other schistosome species (*S. haematobium* and *S. japonicum*) and epigenetic marks (H3K4me3) that correspond to transcription initiation. **(A)** LncRNA antisense to a 78 kDa centrosomal protein gene (Smp_140230). The PC genes' "tail to tail" orientation indicates that the H3K4me3 marks are exclusive to the lncRNA gene. **(B)** LncRNA locus upstream of a G protein coupled receptor gene (Smp_194740) of which only the last exon is shown. **(C)** LncRNA loci upstream of a cathepsin L proteinase gene (Smp_187140). **(D)** LncRNA loci downstream a hypothetical protein gene (Smp_194040). Both protein-coding and lncRNA gene IDs are searchable on the *S. mansoni* UCSC-like genome browser which our group has deployed (<http://schistosoma.usp.br>) and previously reported²⁴.

(100% identity and 100% coverage of both query and subject sequences) encompassing 21 lincRNAs (0.3% redundancy) (Supplementary Table S2). Excluding the requirement of complete identity and coverage, and computing the pairs in which one transcript is completely contained within a longer transcript isoform (i.e. lincRNA queries completely covered by the subject lincRNA with 100% identity and with partial subject coverage) the number of pairs was increased to 281 (4%). This reflects the presence of quite similar isoforms in our assembled transcripts dataset.

Further agreeing with the existence of multiple isoforms of each lincRNA per locus, we found that, keeping the 100% identity threshold but decreasing the requirement of the extent of query coverage (qcov), the all against all comparison has identified approximately similar numbers of lincRNA queries being aligned to the subject at the different qcov ranges: 1422 ($90\% \leq qcov < 100\%$), 1310 ($80\% \leq qcov < 90\%$), 1304 ($70\% \leq qcov < 80\%$), 1327 ($60\% \leq qcov < 70\%$), 1221 ($50\% \leq qcov < 60\%$) (Supplementary Table S2).

In summary, sorting all these lincRNA query lists and eliminating the redundancy, we obtained 4212 (60%) lincRNAs that share 100% identity on at least half of their length with other lincRNAs in the dataset. This result indicates that different transcriptome assembly approaches plus different samples used as input lead to the detection of distinct lincRNAs transcript isoforms. These isoforms mapped to 2596 unique genomic loci, as seen above.

Finding different lncRNA sets when using different RNA-Seq datasets and assembly tools was already reported when analyzing sets of different individuals, tissues and even at the single-cell level^{8–10,12}. We therefore decided to keep in our downstream analyses all the 7029 *S. mansoni* putative lincRNAs (hereafter called SmLINC) as *bona fide* representatives of the complement of expressed *S. mansoni* lincRNAs.

We analyzed the density of lincRNAs mapped to the long, assembled chromosomes and found that the average density was 1.86 lincRNAs per 100 kb of genomic sequence (range 1.31 to 2.30) (Table S3), with no higher density in any particular chromosome. A linear correlation between chromosome size and number of lincRNAs per chromosome was obtained (coefficient of determination $R^2 = 0.97$) (Table S3). A higher density of lincRNAs in certain chromosomes was found in vertebrates such as in the mouse, where there is a higher density of lincRNAs in chromosomes 2, 4 and 11 (ref. 39); this pattern was not observed in *S. mansoni*.

***S. mansoni* putative lincRNAs (SmLINC) display evidence of being functional genes.** We have selected four features to scrutinize our *S. mansoni* putative lincRNAs dataset in the search for evidence of their possible functional role: (i) presence of histone H3 lysine 4 trimethylation (H3K4me3) on the transcription start site (TSS) as an epigenetic mark for transcriptional activation; (ii) evolutionary conservation among two other schistosomes; (iii) differential expression across five parasite developmental stages; and (iv) positive or negative expression correlation to their PC gene neighbors.

H3K4me3 is a very well studied epigenetic mark present on the promoter region of actively transcribed genes⁴⁰. Moreover, it is already known that such mark is not exclusive to PC genes, being also present on active lncRNA loci². Therefore, we screened the lincRNAs' TSS surrounding regions for the presence of significant H3K4me3 peaks in the ChIP-Seq data for both somula and adult forms of the parasite (publicly available data on SRA-NCBI^{41,42}). Forty percent of SmLINC (2878/7029) showed the H3K4me3 mark on their TSS surrounding regions. In addition, the pattern of distance between the lincRNAs TSS and the H3K4me3 closest peak (Fig. 3A) was quite similar to the pattern observed for PC genes (Fig. 3B).

Regarding the evolutionary conservation, we computed the phastCons scores⁴³ assigned at the single-base level, after whole genomes multiple alignments had been obtained with the MULTIZ-TBA package of tools⁴⁴. As already shown for the human genome⁴⁵, phastCons scores are able to distinguish between features of high selective constraint (PC genes) and the ones of weak conservation (lncRNAs, ancestral repeats and desert regions), and even within the latter group it is still possible to differentiate genomic elements by their phastCons profile. After aligning three schistosomes' genomes (*S. mansoni*, *S. haematobium* and *S. japonicum*), we obtained phastCons scores for about 56.9 Mb of genome sequence (see Methods), on which 3453 SmLINC were mapped and presented scored/conserved bases on at least one of their exons.

Table 2 presents an overview of three different *S. mansoni* genomic features (PC genes, lincRNAs and desert regions with neither annotation nor transcript evidence) and their phastCons evolutionary conservation scores' content. As one can see by this rough summary, lincRNAs are more similar to desert regions than to PC genes, corroborating what was already seen in both human and mouse studies^{6,46}. By performing an empirical cumulative distribution function (ECDF) analysis of normalized phastCons scores per block together with a Kolmogorov-Smirnov (KS) test, we were able to distinguish a significantly different evolutionary conservation between SmLINC and desert regions (800 bp) in the *S. mansoni* genome, being the former higher than the latter (KS one-sided p-value = 4.2e-06, Fig. 3C). As a comparison, we calculated the phastCons scores for human chr1 annotated lincRNAs compared with desert regions (400 bp) on the same chromosome, using the 20 mammals phastCons scored bases provided by the UCSC genome browser (<https://genome.ucsc.edu>) for the human hg38 genome sequence assembly. Similar ECDF profiles were obtained (Fig. 3D, KS one-sided p-value = 2.6e-07). These results are indicative that almost half of the SmLINC reported herein (3453/7029) are under some sort of selective evolutionary pressure in the *Schistosoma* genus.

Differential expression along parasite development is another important trait that may characterize lincRNAs as transcripts that are required during the life cycle. We performed a one-way ANOVA-like analysis comparing somula (both 3 h and 24 h) and adult worms (male and female) against cercariae (see Methods for details). First, we identified 2450 differentially expressed (DE) PC genes (Smgs) (Fig. 3E), a number close to what was identified by others comparing several developmental stages on both *S. mansoni*²¹ and *S. haematobium*²². Next, 916 SmLINC were identified as DE on at least one developmental stage (p-value < 0.01) (Fig. 3F). The complete list of DE genes and their respective log₂(fold-change), p-value and adjusted p-value are provided as a spreadsheet on Supplementary Table S4. We observed a lower frequency of DE lincRNAs (916/7029 = 13%) compared with DE PC genes (2450/11,844 = 20.7%). We believe that we might have missed some lowly expressed lincRNAs on our final DE list (having TPM < 1 on all analyzed life cycle stages), since lincRNAs have lower expression levels compared with PCs (Supplementary Fig. S1) and because we have established for the ANOVA-like DE analysis a cutoff TPM ≥ 1 on at least one life cycle stage (three replicates). Similar to what can be seen for DE PC genes (Fig. 3E), DE lincRNAs also display stage-specific up-regulation patterns (Fig. 3F): adult-specific up-regulated genes can be seen on the heatmap's top half, some somula-specific up-regulated lincRNAs are depicted at the middle portion of the heatmap, and cercariae up-regulated lincRNAs (i.e., somula and adults down-regulated lincRNAs) are observed at the bottom half of the heatmap (Fig. 3F). This result suggests that the lincRNA genes described herein might be important for the parasite development.

The fourth and last evidence sought by us was whether there is an expression correlation between SmLINC and their PC gene neighbors in the genome, in comparison with control pairs of the SmLINC and randomly-selected PC genes. First, we picked four PC gene neighbors (the first two upstream and two downstream) for each SmLINC (when it was possible, see below) and computed the pairwise (SmLINC-PC gene) Pearson expression correlation coefficient (r) (using TPM expression values for each gene from the same five developmental stages described above for the DE one-way ANOVA-like analysis, Fig. 3E,F). Next, we compared

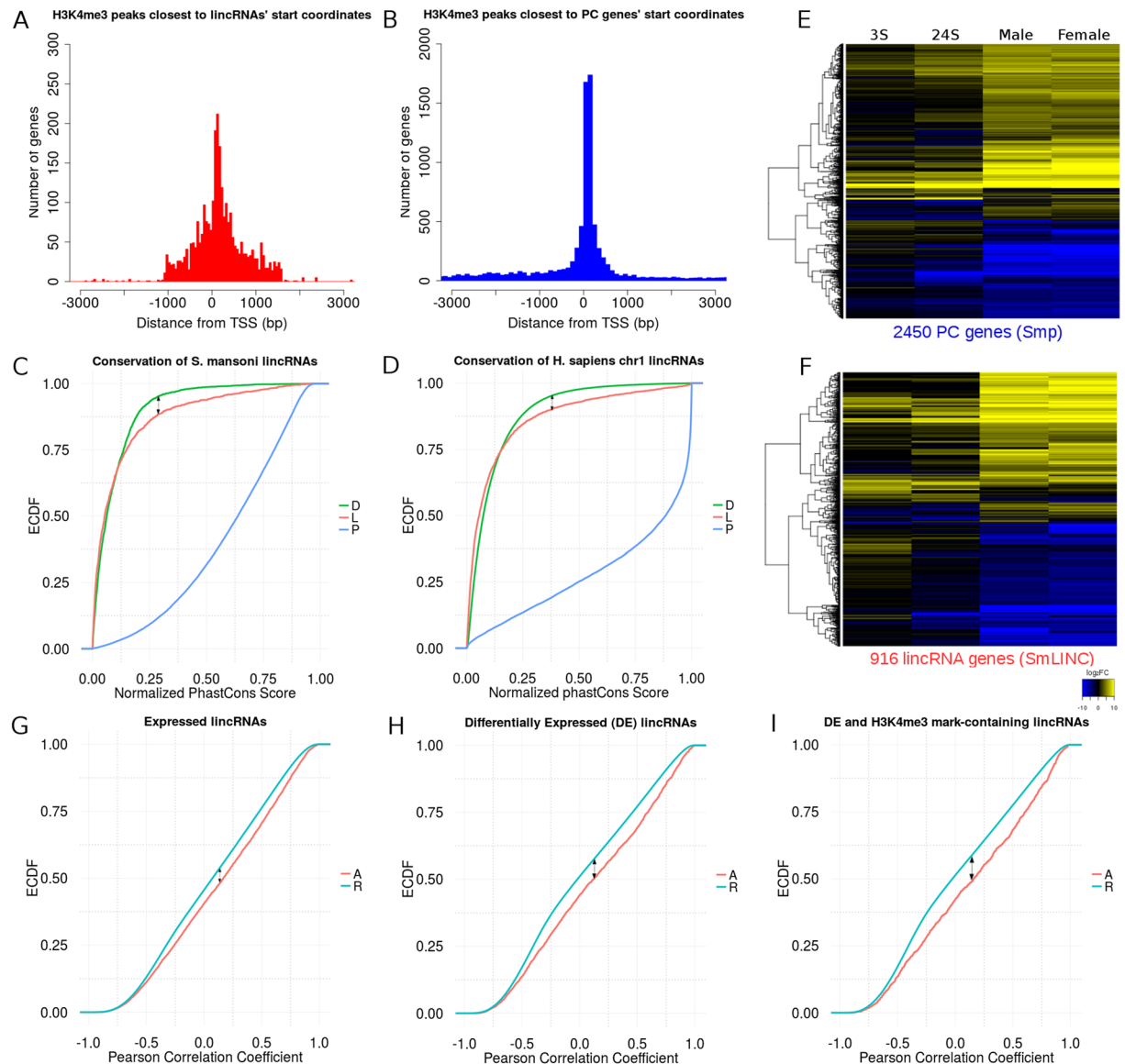


Figure 3. *S. mansoni* lincRNAs display four different traits that may characterize them as functional genes. (A,B) transcriptional activation epigenetic mark (H3K4me3) surrounding the TSS on both lincRNAs (red) and PC genes (blue). (C) An empirical cumulative distribution function (ECDF) showed that lincRNAs' (red) evolutionary conservation scores (phastCons) among three schistosome species are significantly different from genomic deserts (green) (arrow: one-sided KS-test p-value = 4.25×10^{-6}) and from PC genes (blue). (D) As a control, lincRNAs from human chromosome 1 (red) were compared with genomic deserts (green) and with PC genes (blue) from the same chromosome, and they display a phastCons score (comparison among 20 mammals provided by UCSC Table Browser for human genome) ECDF pattern similar to the one observed for *S. mansoni* (arrow: one-sided KS-test p-value = 2.67×10^{-7}). (E,F) Heatmap of 2450 PC genes (E) and 916 lincRNA transcripts (F) that were detected as differentially expressed with one-way ANOVA-like analysis comparing RNA-seq samples from somula 3 h (3S), 24 h (24S) and adults (male and female) against cercariae (the average expression of biological triplicates for each life cycle stage was used, and an adjusted p-value threshold of 0.01 was employed). For each gene (lines), the expression log-ratio between the indicated life cycle stage and cercariae was obtained (columns), and it was colored according to the scale indicated at the bottom of the panel F. (G–I) LincRNAs tend to be co-expressed with their PC gene neighbors (A curves, red) rather than with a set of 1000 randomly picked PC genes (R curves, cyan) during parasite development (same five life cycle stages assessed on E and F panels). Pearson correlation values' ECDF for 1,572 expressed lincRNAs (TPM ≥ 1) that showed either $r > 0.5$ or $r < -0.5$ with their actual PC gene neighbors (G), for 893 differentially expressed lincRNAs (TPM ≥ 1) correlated with their actual PC gene neighbors (H) and for 402 DE lincRNAs (TPM ≥ 1) that also have H3K4me3 marks at their TSSs and are correlated with their actual PC gene neighbors (I) (arrows: KS-test p-value $< 1 \times 10^{-12}$ for each of the three distributions).

Genomic Features	# blocks (or exons for gene features)	# phastCons score-containing bases	# phastCons score-containing blocks	# blocks with no score
Protein-coding	69,809	14,247,623	57,974 (83%)	11,835 (17%)
lincRNAs	6,246	559,217	1,567 (25%)	4,679 (75%)
Desert regions	4,134	842,215	1,336 (32.3%)	2,798 (67.7%)
Deserts 800 bp	14,838	842,215	2,503 (16.8%)	12,335 (83.2%)

Table 2. Summary of conserved features identified by phastCons on the *S. mansoni* genome. The scores are related to conservation on three genomes (*S. mansoni*, *S. haematobium* and *S. japonicum*). Protein-coding represents 11,844 PC genes (Smps) annotated from the latest genome version. LincRNAs are the 7029 assembled transcripts from the current study that do not overlap to Smps. Desert regions are the *S. mansoni* genomic loci with neither gene annotation nor transcript signals from the 88 RNA-Seq samples analyzed herein. These desert regions were split onto 800 bp-long blocks to mimic the mean length of lincRNAs' exons.

the expression correlation coefficients (r) against the ones from a negative control consisting of 1,000 times bootstrapped random PC genes paired to the same SmLINC set.

We started the analysis with 2039 expressed lincRNAs that had TPM ≥ 1 (on all three replicates of at least one life cycle stage), yielding 7269 SmLINC-neighbor_PC gene pairs (some lincRNAs may not have four PC gene neighbors because they can either be located on a scaffold bearing quite few (or no) PC genes or be at the extremities of assembled chromosomes/scaffolds). After comparing the ECDF profiles of all r -values computed for the actual 7269 SmLINC-neighbor_PC gene pairs against a negative control consisting of r -values for 7,269,000 pairs of SmLINC-randomly_selected_Smps, we observed a significant difference on the two distribution curves (KS one-sided p -value = $5.6e-25$, Fig. 3G). It is noteworthy that 1572/2039 SmLINC-neighbor_PC gene pairs (77%) showed significant expression correlation (r) p -values < 0.05 , whereas in the negative control only 2,547,966/7,269,000 SmLINC-randomly_selected_Smp pairs (35%) had r p -values < 0.05 .

Next, we reduced the set of lincRNAs in the analysis by keeping only the 893 lincRNAs that are significantly differentially expressed (DE) across the stages within the set of 2039 lincRNAs with TPM ≥ 1 . For these 893 DE lincRNAs there were 3167 SmLINC-neighbor_PC gene pairs, which were compared with 3,167,000 SmLINC-randomly_selected_Smp bootstrapped pairs. The difference between their ECDF curves was also significant (KS one-sided p -value = $6.8e-20$, Fig. 3H) and notoriously greater than the difference in the previous ECDF plot (Fig. 3G). Seven hundred thirty two out of the 893 SmLINC-neighbor_PC gene pairs (81.9%) showed significant expression correlation (r) p -values (< 0.05), whereas 1,206,153/3,167,000 SmLINC-randomly_selected_Smp pairs (38%) had the same correlation significance.

Finally, filtering the 2039 SmLINC by both being DE and having the H3K4me3 mark at their TSS-surrounding genomic region, we obtained 402 lincRNAs and 1406 SmLINC-neighbor_PC gene pairs. Their ECDF plot showed the greatest difference between the curves (KS one-sided p -value = $1.5e-13$, Fig. 3I); in this set, a total of 325/402 SmLINC-neighbor_PC gene pairs (80.8%) had expression correlation (r) p -values < 0.05 , while this same feature was seen for only 532,925/1,406,000 random pairs (37.3%) in the negative control.

The above results indicate that it is possible that SmLINC may play a *cis*-acting role regulating their PC gene neighbors, as already known for vertebrates^{2,5}. In addition, as we decreased the lincRNAs' set by keeping the ones displaying more evidence for being active, we observed an increase in the difference between the correlation of actual neighbors versus random pairs (Fig. 3GHI), suggesting that a set of lincRNAs is more prone to show a co-expression pattern with their PC gene neighbors.

An intersection diagram displaying the number of *S. mansoni* lincRNAs having from one to four of the analyzed traits that may characterize them as functional genes was obtained for the 7029 lincRNAs (Fig. 4), showing that only 1739 lincRNAs (25%) could not be associated with these traits. We decided to focus our downstream analyses on the main intersection group of 181 lincRNA genes (Fig. 4). The genes that were displayed as genome browser images on Fig. 2 are part of that main intersection set. The complete list of the 181 robust SmLINC is provided as supplementary file (Supplementary File S2). Each of them can be accessed through our online genome browser (<http://schistosoma.usp.br>).

RT-qPCR assays confirm the SmLINC's differential expression across distinct developmental stages.

Sixteen out of the 181 robust SmLINC's set from the UpSet intersection diagram on Fig. 4 were selected for individual assessment of their expression levels on cercariae (C), somula 3 h (3S) and somula 24 h (24S) after mechanical transformation, male and female stages/forms. First, in order to confirm that the RNA samples herein used for RT-qPCR contained previously known stage-specific/enriched transcripts, we measured the expression levels of six protein-coding genes as positive controls (Supplementary Fig. S2). In the RT-qPCR assays (Supplementary Fig. S2), all six selected control PC transcripts corroborated the up-regulation pattern at the life cycle stage that had already been reported in the literature: Smp_044250 (Metalloprotease), shown as up-regulated in cercariae compared with somula⁴⁷; Smp_033040 (Lactate dehydrogenase), up-regulated in somula compared with cercariae⁴⁷; Smp_126730-5HTR and Smp_145140-WNT5, assessed as up-regulated in adult male compared with female²⁴; Smp_000390-Trematode Eggshell and Smp_000430-EggShell, up-regulated in adult female compared with male²⁴.

We then performed RT-qPCR to assess the expression levels of sixteen selected lincRNAs across the five *S. mansoni* stages studied. The primers used are listed on Supplementary Table S5. It was possible to detect differential expression of lincRNAs on all the four life stages: two more highly expressed in cercariae (SmLINC02630-1Bu

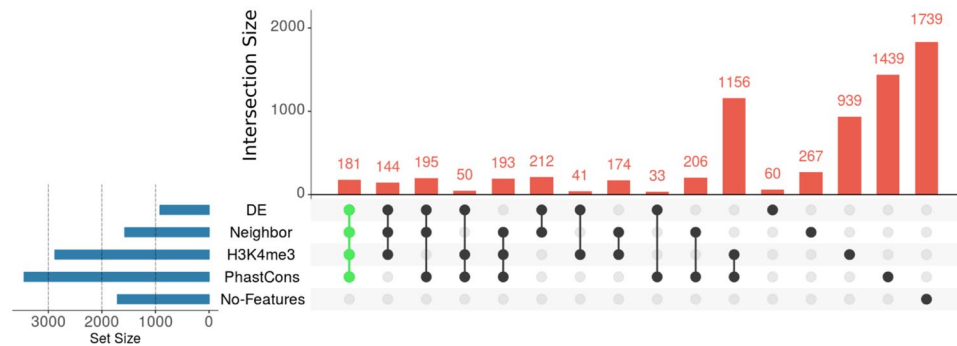


Figure 4. Hundreds of SmLINC s share two or more traits that may characterize them as functional genes. The UpSet intersection diagram shows the number of lincRNAs that were detected as being differentially expressed across five life cycle stages (DE), as having their expression correlated with the expression of protein-coding gene neighbors (Neighbor), as having histone active transcription marks at their TSSs (H3K4me3) and as being conserved among *Schistosoma spp.* (PhastCons). The 181 lincRNAs that share all traits were selected for further investigation using systems approaches.

and SmLINC05716-IBu), ten in somula 3 h (SmLINC00001-IBu, SmLINC00133-IBu, SmLINC00282-IBu, SmLINC01084-IBu, SmLINC01122-IBu, SmLINC03930-IBu, SmLINC04271-IBu, SmLINC04521-IBu, SmLINC05284-IBu and SmLINC05720-IBu), two more highly expressed in somula 24 h (SmLINC01431-IBu and SmLINC06024-IBu) and two in male adults (SmLINC02394-IBu and SmLINC06535-IBu) (Fig. 5). Half of the genes showed an expression pattern concordant to those measured by RNA-Seq analysis (SmLINC02630-IBu, SmLINC00133-IBu, SmLINC00282-IBu, SmLINC03930-IBu, SmLINC04271-IBu, SmLINC05284-IBu, SmLINC05720-IBu and SmLINC02394-IBu). It is noteworthy that some SmLINC s from the other half, for which the qPCR did not corroborate the RNA-Seq stage of higher expression, had very low RNA-Seq read counts on all stages (SmLINC01084-IBu, SmLINC01122-IBu and SmLINC01431), which in turn may diminish the detection of differential expression of some lincRNAs by the high-throughput approach.

Co-expression networks (lincRNAs-PC gene) raise hypotheses about lincRNAs' functionality.

A very often-adopted approach to hypothesize about lincRNAs' function is the investigation of their co-expression patterns along with PC genes^{12, 45, 48, 49}. We analyzed expression data for five different developmental stages of *S. mansoni* (15 RNA-Seq samples) in order to identify DE genes (previous topic), and we have selected a robust list of 181 SmLINC s that have strong evidence for being regulated genes. We therefore decided to perform an expression correlation analysis of these 181 lincRNAs against all DE PC genes (from the one way ANOVA-like heatmap on Fig. 3E) using read-counting values for each gene from the 15 RNA-Seq samples (biological triplicates for each life cycle stage). Among the correlated genes, we kept only gene pairs (lincRNA-PC gene) presenting $r > 0.8$ (classified as positively correlated) or $r < -0.8$ (negatively correlated). A co-expression network was built relying on that correlation information (Fig. 6A). It contains 181 lincRNAs (red nodes), 2359 PC genes (blue nodes), and 68,625 correlated pairs (edges), among which 92% (63,156/68,625) are for positive correlations (cyan edges) and 8% (5469/68,625) for negative ones (gray edges). As a negative control, a random network was generated with the same number of edges (68,625), 181 and 2359 randomly-picked lincRNAs and PC genes, respectively, in order to give support for the actual network significance. In this negative control dataset, we obtained 4788 edges that had either $r \geq 0.8$ or $r \leq -0.8$ out of 68,625 total edges (6.9%). Therefore, we could estimate a false discovery rate of 6.9% (4788/68,625), or a precision of ~ 93% for the correlations displayed on the actual network (see details on the Methods section).

A gene enrichment analysis for those 2359 PC genes was performed relying on the gene ontology (GO) terms assigned to them (see Methods). Sixteen GO categories comprise enriched genes with a significant hypergeometric test adjusted p-value < 0.01 (Fig. 6B). They are distributed into 10 cellular components (CC), 3 biological processes (BP) and 3 molecular functions (MF). Two terms from both BP and MF are highlighted: RNA-dependent DNA replication/RNA-directed DNA polymerase activity and G-protein coupled receptor (GPCR) signaling pathway/GPCR activity. The former represents genes involved in the transposition of retroelements, the major class of transposable elements (TEs) in *S. mansoni* genome, which is about 47% comprised by repeats⁵⁰. TEs are also an important source for the birth of new lincRNAs within a genome⁹. The latter GO category is related to signal transduction via GPCR, which was already cited as a potential target in the context of drug discovery in *S. mansoni*⁵¹. In addition, a recent study that built lincRNA-mRNA co-expression networks from venous congestion-subject human endothelial cells has identified GPCR as a potential pathway dynamically regulated by lincRNAs⁴⁸.

Filtering the network by topological proximity between SmLINC-PC gene pairs. Since it is known that lincRNAs may act by regulating their flanking-PC gene neighbors (as already mentioned herein), and that they may form triplex structures (dsDNA-RNA) anchoring on different genomic loci and recruiting chromatin remodelers such as PRC2 (refs 13, 14), we idealized a two-step filtering method in order to build a co-expression network where the lincRNA-PC gene expression correlations would be linked to the topological proximity of the pair on the chromatin. The first step was to filter each gene pair (lincRNA-PC gene) by a pre-defined correlation coefficient (r)

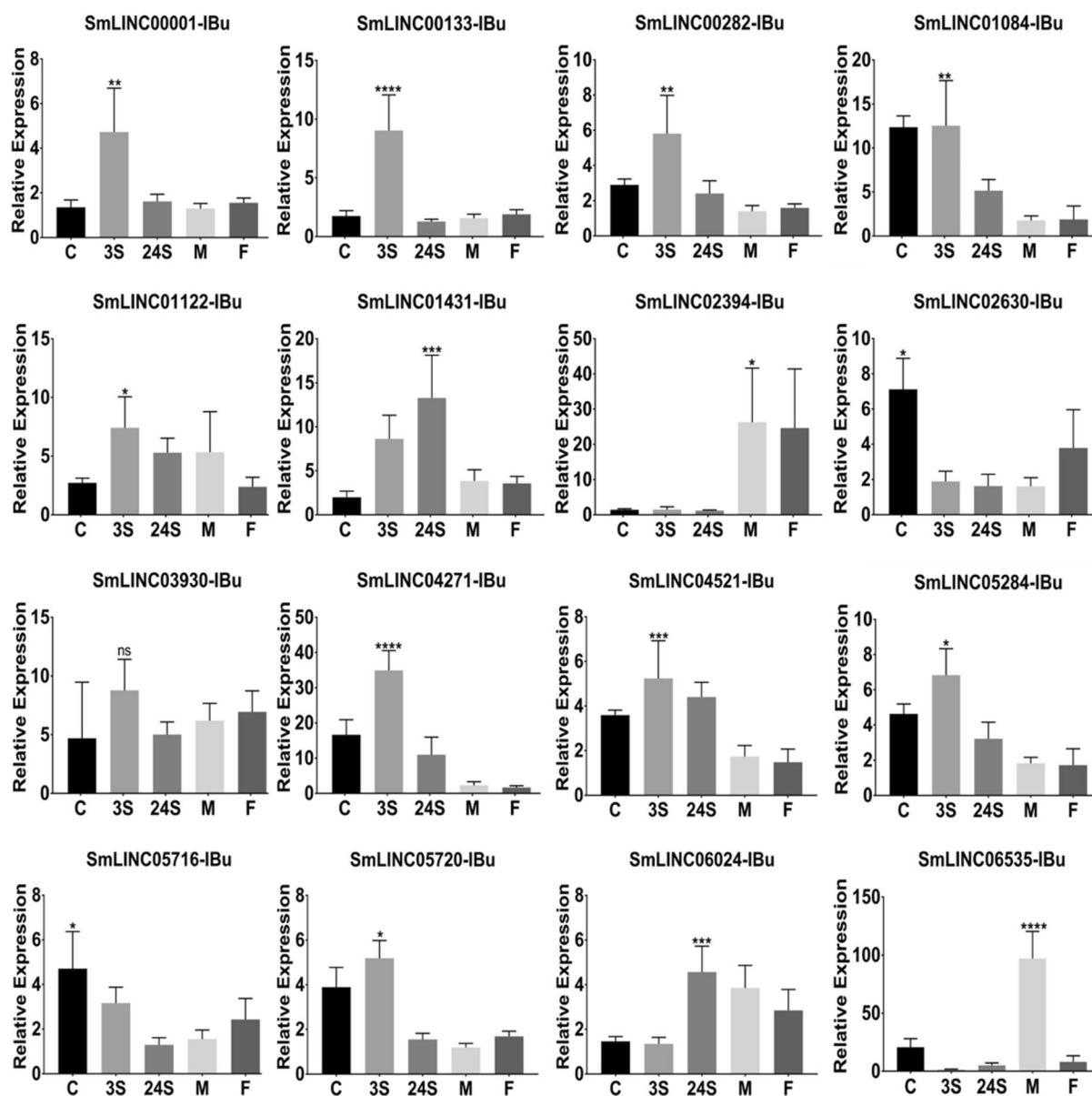


Figure 5. Confirmation by RT-qPCR of the differential expression of selected SmLincRNAs across the parasite life cycle stages. Sixteen SmLincRNAs were selected for validation by RT-qPCR at the parasite stages, namely cercariae (C), schistosomula after 3 hours of mechanical transformation (3S), schistosomula after 24 hours of mechanical transformation (24S), adult male and female (x axis). All sixteen lincRNA loci are among the 181 lincRNAs loci shown at the main intersection in Fig. 4. For each lincRNA plot, the individual sample with the lowest normalized expression value across all stages was chosen and arbitrarily set to 1. The expression values of the same lincRNA for all the other samples are represented as the relative expression compared with the lowest one (y axis). Bars represent standard deviation of the mean from four biological replicates for each stage. Three technical replicates were performed for each of the four biological replicates per stage. The Smp_092920 was used for internal normalization as the reference gene among the parasite stages (see Methods). The ANOVA Tukey test was used to calculate the statistical significance of the expression differences among the parasite forms (*p-value ≤ 0.05 ; **p-value ≤ 0.01 ; ***p-value ≤ 0.001 ; ****p-value ≤ 0.0001). For clarity purposes, it is shown only the highest p-value representation for the stage in which the lincRNA was detected with the highest expression.

threshold. Starting with the same input used for building the network on Fig. 6A, we have now decreased the cutoff to $r \geq 0.5$ or $r \leq -0.5$. The second step, which we will hereafter call topological filtering step (TFS), was aimed at keeping only the SmLINC-PC gene neighbors and/or the pairs presenting a triplex structure predicted by the triplexator algorithm⁵² (see Methods for details). After those two rounds of gene pairs filtering, we obtained a network containing 326 nodes (89 lincRNAs and 237 PC genes) that comprised 62.5% positively correlated pairs (204/326 edges) and 37.5% negatively correlated ones (122/326 edges) (Supplementary Fig. S3).

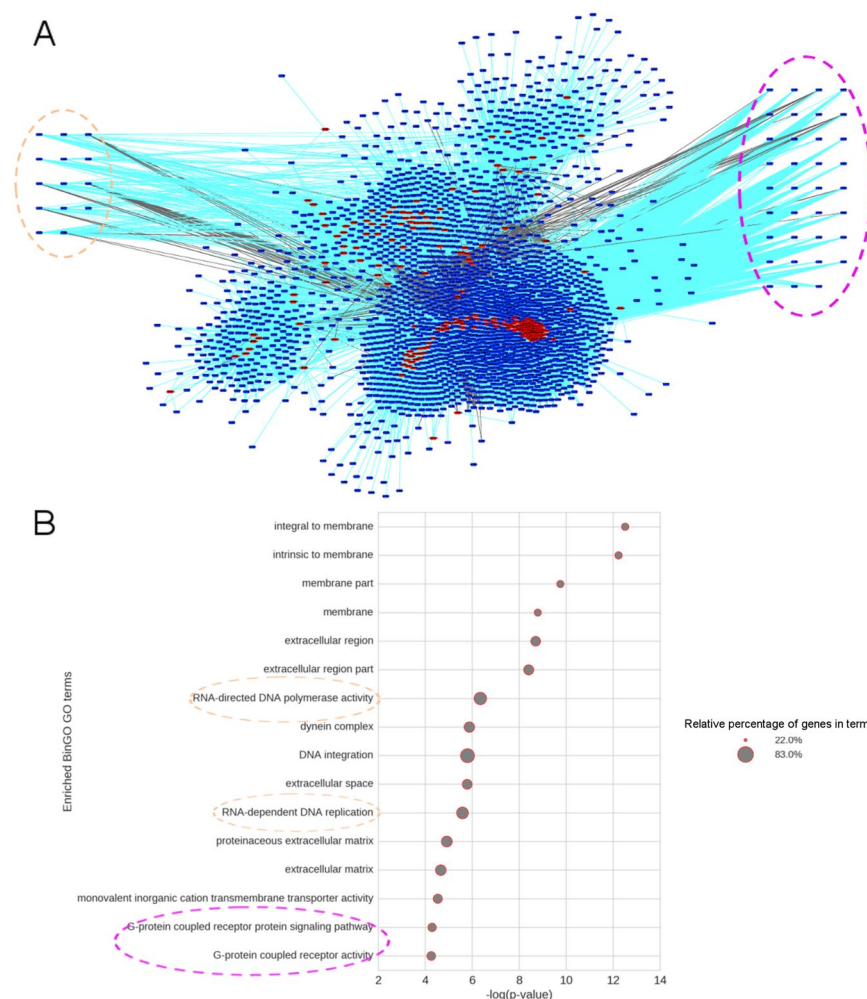


Figure 6. Co-expression gene network (lincRNA-PC gene) pinpoints the processes in which lincRNAs may act. **(A)** The 181 robust lincRNAs (red nodes; lincRNAs from the main intersection in the UpSet Intersection Diagram on Fig. 4) were used as bait for catching 2359 PC genes (blue nodes) whose expression levels across five developmental stages are either positively ($r \geq 0.8$, cyan edges) or negatively ($r \leq -0.8$, gray edges) correlated with the expression levels of the lincRNAs. The network contains 68,625 edges among which 63,156 (92%) are positive correlations and 5469 (8%) negative ones. **(B)** A GO gene-enrichment analysis on the PC genes' list revealed 16 significantly enriched GO terms ($-\log(p\text{-value})$ on the x-axis), and among them there were membrane-related terms, as well as retrotransposons activity (orange dashed circles) and GPCR-associated pathways (purple dashed circles). The PC genes from the dash-circled GO categories were manually searched and clustered on the peripheral region from the network in (A), allowing one to see that both positive and negative correlations between lincRNA and PC genes are present on those processes.

We have also calculated the betweenness centrality (BC) score in order to identify important nodes within the network⁵³. Roughly, BC measurement reflects the ratio of the number of shortest paths between two nodes (x, z) that pass through a node y by the number of total shortest paths between x and z , inferring, therefore, the propensity node y has to be a hub. The network layout that we show displays the node size proportional to the BC in each subnetwork (Fig. 7A and Supplementary Fig. S3). As examples, three subnetworks were selected and the expression profile across the five developmental stages of each target SmLINC and the correlated PC genes comprising each subnetwork have been displayed (Fig. 7B).

We performed a k-means clustering analysis on all the 326 genes present in the entire network, being able to detect that 70% of the genes (230/326) grouped into clusters that had an expression pattern similar to what was observed for the majority of the genes composing the SmLINC-hubs' subclusters depicted on Fig. 7B, that is, an up-regulation profile from cercariae to adult forms (Supplementary Fig. S4). It appeared to be concurrent to what was recently reported about cercariae transcriptional poised state⁴².

Another co-expression network building approach highlights PC gene hubs and their correlated lincRNAs. A second network building method, where we captured the most DE PC genes-correlated lincRNAs, was idealized. First we selected 307 PC genes (Smgs) that were highly differentially expressed among the five life cycle stages on focus ($\log_2FC \geq 10$ on at least one stage), and then we rescued all the other genes

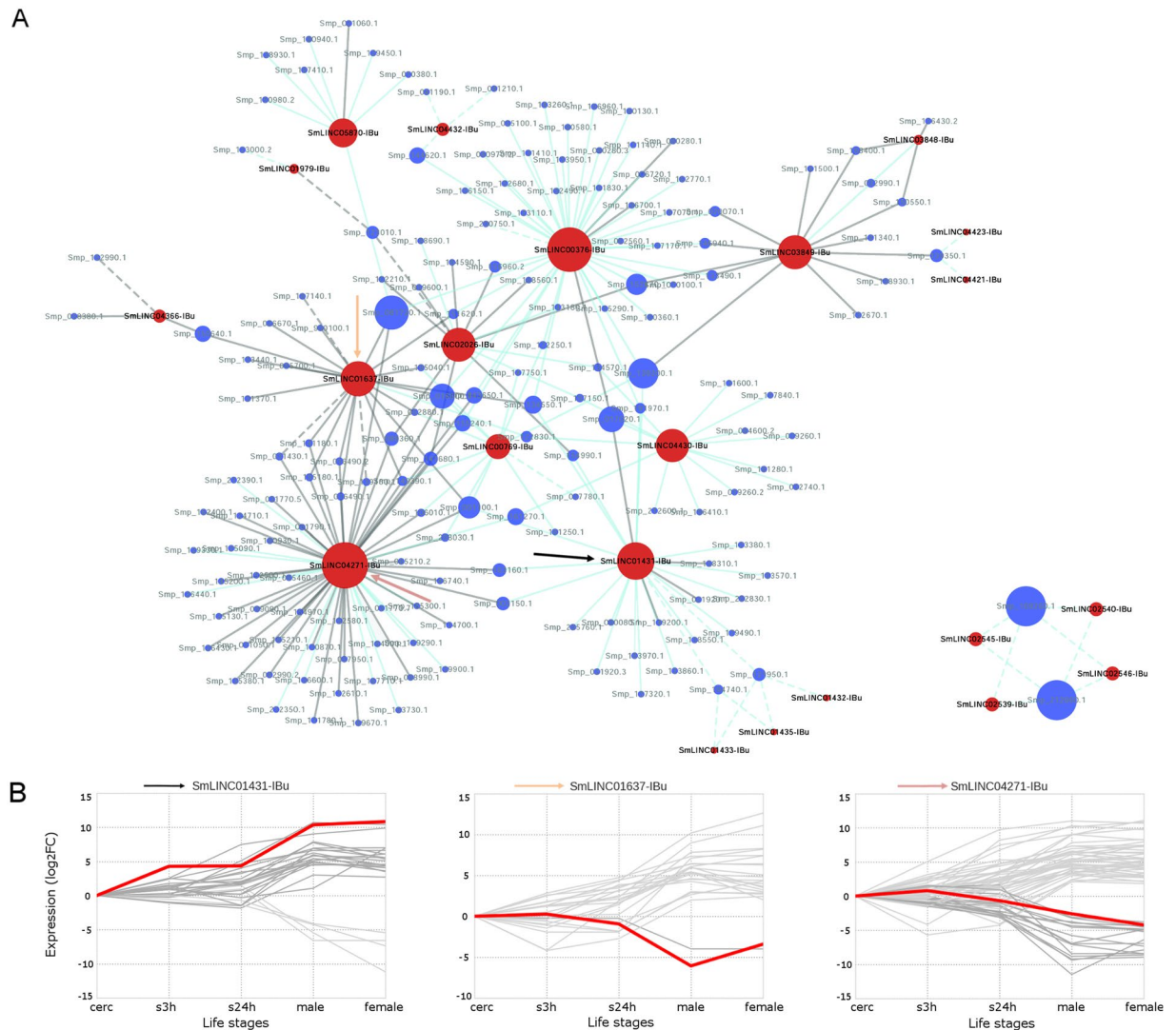


Figure 7. Topological filtering step (TFS) approach decreases the number of nodes and improves the visual inspection of lincRNAs' subclusters. **(A)** Eighty nine out of the 181 robust lincRNAs from Fig. 4 passed at least one of the two TFS rules: (i) have a PC gene neighbor (Smp) as one of its correlated mates (putative *cis*-acting mechanism), and (ii) have an *in silico* computed probability to form a triplex structure (RNA-DNA) on its PC gene mate locus (putative *trans*-acting mechanism). The “Network Analyzer” function from cytoscape was used in order to obtain betweenness centrality score for each node, meaning the bigger the node, the higher propensity to be a hub. The 22 SmLINC correlated with multiple PC genes are shown here. The entire network is shown on Supplementary Fig. S3 and it contains 326 nodes (89 SmLINC and 237 Smps) with 62.5% positively correlated pairs (204/326, $r \geq 0.5$, cyan edges) and 37.5% negatively correlated ones (122/326, $r \leq -0.5$, gray edges). Dashed edges represent neighbor genes. **(B)** Gene expression patterns on three subnetworks that were manually selected by highlighting three SmLINC hubs, marked by arrows in (A) and named at the top of each panel in (B). Co-expression kinetics of the SmLINC (red line), positively correlated Smps (dark gray lines) and negatively correlated Smps (light gray lines) expressed across the five life cycle stages: cercariae (cerc), somula 3 h (s3h), somula 24 h (s24h), male and female.

(Smps + SmLINC) that presented a very stringent expression correlation (coefficient $r \geq 0.9$ or $r \leq -0.9$) with the 307 pre-chosen PC genes. A network with 2965 nodes (750 lincRNAs + 2215 PCs) and 52,913 edges (51,807 positive r and 1106 negative r) was generated by this approach (Supplementary Fig. S5 panel I). Due to their highest BC scores, three PC genes were detected as the most important nodes in the network: Smp_055340.2, Smp_038870.2 and Smp_036270.4. The first one (Smp_055340.2), with the highest BC value (~ 0.15 , Supplementary Fig. S5 panel II), encodes the protein Lin-9, a nuclear tumor-suppressing agent in mammal cells⁵⁴ that acts inhibiting DNA synthesis (G1/S transition). The second (Smp_038870.2) and third (Smp_036270.4) ones encode a NADH ubiquinone oxidoreductase and a splicing factor arginine/serine rich splicing factor 4, respectively. The expression patterns of SmLINC that are either positively or negatively correlated with those three PC gene hubs along parasite differentiation are depicted on Supplementary Fig. S5 panel III.

Similarly to what was seen on the gene enrichment analysis for the 2359 SmPs correlated with the 181 robust SmLNCs (Fig. 6A, B), the same assay performed on the different set of 2215 PC genes from this second network has also revealed “RNA-dependent DNA replication” and “DNA integration” as significantly enriched GO biological processes, as well as “Monovalent inorganic cation transmembrane transporter activity” as significantly enriched GO molecular functions (Supplementary Fig. S5 panel IV). Membrane and extracellular components’ terms were also significant in the gene enrichment.

Again, as a negative control we built a random network containing 2215 and 750 randomly-selected PC genes and lincRNAs, respectively, and the same number of edges present on the actual network. In this negative control, we retrieved 1550 out of 52,913 edges with $r \geq 0.9$ or $r \leq -0.9$, estimating a false discovery rate on the original network of 2.9%, or a precision of ~97%.

These results are indicative that SmLINC-Smp co-expression network construction may be considered as a reliable initial approach to hypothesize and direct further investigations on *S. mansoni* lincRNAs’ regulatory pathways.

Discussion

Long non-coding RNAs are striking molecules acting on a variety of biological processes within the cell, mostly related to regulation of gene expression^{2–4, 7, 8, 12, 14, 45}. Due to this broad landscape of content and functionality and despite the increasing efforts to unravel novel lincRNAs (mainly in higher eukaryotes), as well as their respective roles, the hitherto understanding of these molecules action on the diverse intracellular regulatory pathways still corresponds to a small tip of a huge iceberg. Regarding lincRNAs from more ancient important organisms, such as parasites, this tip is even smaller and few studies are devoted to unravel such genes.

The *S. mansoni* genome, which had its first version published in 2009 (ref. 51), and improved with the use of a NGS strategy in 2012 (ref. 21), has a size of about 380 Mb and nearly 12,000 PC genes (SmPs) already mapped/annotated. These static data pose the even greater challenge of understanding the molecular dynamics responsible for the peculiar features of the parasite biology. Post-transcriptional control of gene expression events, such as modulation of mRNA alternative splicing and silencing by RNAi, as well as epigenetic events such as chromatin remodeling by histone modifications and the consequent epigenetic mechanisms to activate transcription, are present in schistosomes^{41, 42, 55–57}. As in other higher eukaryotes, it is believed that a variety of lincRNAs may mediate such reactions in the parasites.

Regulatory RNAs appear to occur in all forms of life on the earth and it has been described that the protein-coding fraction of genomes decreases according to the complexity of organisms, ranging from 80–95% in prokaryotes to only a minor proportion in mammals (~1.22% in humans) (refs 58 and 59). On a brief screening on the latest version of *S. mansoni* genome and its current predicted proteome²¹, we detected a protein-coding DNA content of about 4.5%. Thus, it is apparent that *S. mansoni* has a considerable “free space” on the non-protein-coding portion of the genome which can be a source of hundreds to thousands lincRNAs that may act as regulatory and adaptive elements on the strenuous environmental changes this parasite experiences in order to complete its life cycle.

Due to the weak evolutionary constraint regarding lincRNAs’ sequence conservation^{8, 9}, conventional local alignment search tools, commonly used for identifying homology evidence between PC genes from quite divergent organisms, are not the ideal ones for any kind of non-coding RNA sequence analyses⁶⁰. With the advent of RNA-Seq, it is now possible to screen a whole transcriptome, on a high-throughput manner, in order to catch such transcripts. Both GENCODE⁶¹ and FANTOM⁶² consortia made use of the NGS strategy providing a great volume of input data to their computational pipelines aimed to assemble, distinguish/classify and annotate the maximum number of transcripts in humans and mice, respectively.

In the present study, we relied on tens of RNA-Seq samples ($n = 88$) from different developmental stages (Supplementary Table S6) and we built a transcriptomics/bioinformatics computational pipeline (Fig. 1) that successfully retrieved over 7,000 *S. mansoni* multiexonic lincRNA transcripts (402 antisense to PC genes and 7029 lincRNAs) (Supplementary File S1 and Supplementary Table S1). It is important to note that our datasets do not represent the entire lincRNAs’ complement from the *S. mansoni* transcriptome, since we ruled out monoexonic transcripts in order to reduce the chance of artifacts⁹.

The conservative RNA-Seq assembly and filtering approach used here has shown 53% sensitivity when applied to the *P. falciparum* lincRNAs dataset³⁰, as described in the Results. Therefore, it indicates that we are probably missing some *S. mansoni* lincRNA loci (most probably monoexonic ones), however we are certainly decreasing our false-positive rate by the stringent selection criteria established in our pipeline, which gives us a lower-boundary for the lincRNAs complement in our target *S. mansoni* organism.

The search for evidence of being active genes revealed only few hundreds lincRNAs (181) that have a combination of the four investigated traits: presence of H3K4me3 marks on their TSS-surrounding genomic regions, *Schistosoma* spp. phastCons conservation score assigned to at least one exon, differential expression across five developmental stages/forms (cercariae, somula 3 h, somula 24 h, male and female) and a significant either positive or negative expression correlation with their PC gene neighbors (Fig. 3). Sixteen out of those 181 lincRNAs had their expression individually quantified by us across the five parasite life stages/forms through RT-qPCR. It is noteworthy that, while some lincRNAs were found to be upregulated in adults (such as SmLINC02394-IBu and SmLINC06535-IBu, Fig. 5), most of the lincRNAs showed upregulation in schistosomula. This might indicate an importance of lincRNAs on regulating some processes involved in the rapid adaptation of schistosomula after transition from the free-living larvae to the early mammal parasitic stage, such as the worm body remodeling and defense against the host immune system.

We were guided by studies on mice and humans that make use of co-expression approaches on the attempt of hypothesizing lincRNAs’ function^{12, 45, 49}. After clustering lincRNA-PC genes by either positive or negative expression correlation (roughly mimicking either activation/stabilization or inhibition/destabilization, respectively)

across a set of different samples, co-expression networks were built and/or gene enrichment analyses were performed in order to acquire a holistic systems overview of potential lincRNAs' roles. Two approaches were used for the networks construction that basically differ only on their initial dataset (list of genes) used for rescuing the additional positively or negatively correlated genes along parasite development. The first approach started with a list of 181 robust lincRNA candidates used for capturing their correlated Smp protein-coding genes (Figs 6 and 7), whereas the second one began with a list of 307 highly DE PC genes that were used for capturing their correlated genes in general (both SmLINC and other Smps) (Supplementary Fig. S5). One interesting feature displayed by these two different network construction approaches was the presence of common GO categories of enriched PC genes: "RNA-dependent DNA replication", "DNA integration", "Monovalent inorganic cation transmembrane transporter activity" (Fig. 6B and Supplementary Fig. S5 panel IV), indicating that SmLINC might be regulating such processes.

Both topological filtering step (TFS) and betweenness centrality (BC) measurement allowed us to highlight important SmLINC (Fig. 7 and Supplementary Figs S3 and S5). For instance, SmLINC01637-IBu and SmLINC04271-IBu (orange and pink arrows on Fig. 7) appeared to be interesting candidates for functional studies. The former (SmLINC01637-IBu), while positively correlated to only one PC gene (ataxin 2 - Smp_122830), is negatively correlated to several mRNAs coding for membrane and/or secreted proteins, such as: fibrillin 2 (Smp_001100), solute carrier family 1 (Smp_016600), sodium dependent glucose transporter 1 (Smp_139150), transmembrane protein 26 (Smp_026670), transmembrane protein 231 (Smp_081720), frs1 related extracellular matrix protein (Smp_149390), protocadherin 9 (Smp_151620), surface membrane antigen (Smp_195180) and saposin B domain containing protein (Smp_016490.1 and 0.2), which has already been investigated as a vaccine candidate⁶³. Whereas the latter (SmLINC04271-IBu) displays a mix of positively and negatively correlated mRNAs: more than half (8/15) on the first set ($r \geq 0.5$) code for positively correlated hypothetical proteins (and noteworthy, the set includes a gene coding for the transcription factor forkhead box protein P1 - Smp_212350); and on the second set ($r \leq -0.5$) there are 35% (15/43) and 39.5% (17/43) negatively correlated mRNAs coding for hypothetical proteins and membrane/secreted proteins, respectively, of which several are shared with SmLINC01637-IBu as negatively correlated (Smp_001100, Smp_016490.1, Smp_016490.2, Smp_016600, Smp_031430, Smp_081720, Smp_139150, Smp_141180, Smp_149390 and Smp_195180). Other interesting mRNAs are also listed on the $r \leq -0.5$ set from SmLINC04271-IBu, such as SPARC protein (Smp_171780), PDZ domain-containing protein GIPC3 (Smp_170870), fasciclin domain-containing protein (Smp_141680), transmembrane protein 145 (Smp_125130), MEG-5 (Smp_152580) and a couple of transcription factors (HNF 4 - Smp_174700 and engrailed 2C - Smp_145200).

This is the first time an approach for rescuing lncRNAs plus another for proposing their possible functionality through co-expression networks' construction are reported in a single study on a parasite's genome/transcriptome landscape. Adding to what was already seen for *P. falciparum* regarding lncRNAs^{30, 64}, the data presented herein reinforce that those transcripts are expressed and may play a role on the biology of neglected tropical disease-causing agents. Thus, parasites' lncRNAs and their pathways of action should start being considered as possible new therapeutic targets on future investigations.

Conclusions

The established computational pipeline appears to be a robust tool for the identification of thousands of multiexonic *S. mansoni* lncRNAs and might be applicable to any other organism. Hundreds of the putative lincRNAs display evolutionary conservation within the *Schistosoma* genus, transcriptional activation epigenetic mark (H3K4me3) at their TSSs, differential expression across five developmental stages and expression correlation with their protein-coding gene neighbors. In addition, RT-qPCR assays for 16 SmLINC have confirmed that they undergo differential expression along the parasite development.

The construction of co-expression networks allows a holistic systems overview that helps us to decipher the role of these intriguing molecules on *S. mansoni* biology. We believe that the networks built and disclosed herein have now paved the way, as an important initial source, for investigations on *S. mansoni* regulatory pathways involving lincRNAs and their correlated PC genes. Functional assays are necessary to characterize individual SmLINC as regulatory elements of their PC gene counterparts within the same co-expression subcluster.

Methods

Full methods are available on-line in the Supplementary Materials section.

Ethics statement. All protocols involving animals were conducted in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA), and the protocol/experiments have been approved by the Ethics Committee for Animal Experimentation of Instituto Butantan (CEUAIB Protocol number 1777050816).

Genomic and transcriptomic data. The *Schistosoma* genomes analyzed herein were downloaded from the Wellcome Trust Sanger Institute ftp site for *S. mansoni* (http://ftp.sanger.ac.uk/pub/pathogens/Schistosoma_mansoni/Latest_assembly_annotation_others/Schistosoma_mansoni_v5.2.fa) and from wormbase ftp for both *S. haematobium* (http://ftp.ebi.ac.uk/pub/databases/wormbase/parasite/releases/WBPS8/species/schistosoma_haematobium/PRJNA78265/schistosoma_haematobium.PRJNA78265.WBPS8.genomic.fa.gz) and *S. japonicum* (http://ftp.ebi.ac.uk/pub/databases/wormbase/parasite/releases/WBPS8/species/schistosoma_japonicum/PRJEA34885/schistosoma_japonicum.PRJEA34885.WBPS8.genomic.fa.gz). The most recent genomic annotation of nearly 12,000 PC genes (Smps) was used²¹.

We have used 88 *S. mansoni* RNA-Seq libraries as input for our computational pipeline (more details on the next topic): 52 from public studies already deposited in the Sequence Read Archive (SRA) NCBI database, which encompass several developmental stages (Supplementary Table S6), and 36 in-house separate male (Accession numbers SAMN06221530-SAMN06221541 and SAMN06221554-SAMN06221559) and female libraries (Accession numbers SAMN06221542-SAMN06221553 and SAMN06221560-SAMN06221565).

Assembly of RNA-Seq reads. Trinity *de novo* assembly³² was applied to three different RNA-Seq datasets: (1) trin_strA dataset, comprised of ~300 million raw paired-end reads (SRA accession SAMN06221530-SAMN06221553) obtained in house from 24 samples of adult worm couples exposed *in vitro* to TH65 (compound 131) (ref. 65) or to vehicle (controls); (2) trin_strB dataset, comprised of ~300 million raw paired-end reads (SRA accession SAMN06221554-SAMN06221565), again obtained in house from 12 samples of adult worm couples exposed *in vitro* to GSK343 (ref. 66) or to vehicle (controls); (3) trin_sra dataset, which encompasses 52 RNA-Seq libraries (Supplementary Table S6) (~2 billion raw reads) from *S. mansoni* at several different life cycle stages (both untreated samples or samples treated with different compounds), which were downloaded from the SRA-NCBI public repository. A second assembly approach using the Tuxedo tools (Tophat2 (ref. 33), Cufflinks and Cuffmerge³⁴) was applied to a subset of the above RNA-Seq libraries that included 44 out of the 52 SRA RNA-Seq libraries comprised exclusively of control untreated samples, plus 12 in house-derived libraries from both strA and strB comprised exclusively of control samples (total of ~2.2 billion raw reads). Each of those 56 samples (44 + 12) was used as an independent input file for both tophat2 and cufflinks, and then all 56 independently assembled transcript sets were merged onto a single non-redundant gtf file through cuffmerge execution. See further details in the Supplementary Materials section.

Retrieving long non-coding RNAs from the assembled transcripts' dataset. After having all the assembled transcripts mapped to the reference genome (bed12 and gtf formats for *de novo* and genome-guided assemblies, respectively), a series of filtering steps was applied as described below, aimed at both removing unwanted protein-coding transcripts and rescuing putative lncRNAs from the entire transcriptome datasets (red rectangles section from the pipeline depicted on Fig. 1). The tools that were used and their respective parameters were all placed in one single PERL script and adapted for automation; the script can be downloaded from <https://github.com/eltonjrv/Smansoni.lncRNAs>. See further details in the Supplementary Materials section.

Searching for evidence supporting *S. mansoni* lincRNAs as functional genes. *H3K4me3 marks.* In order to search for the presence of histone H3 lysine 4 trimethylation (H3K4me3) on the transcription start site (TSS) of *S. mansoni* genes as an epigenetic mark for transcriptional activation, we relied on four ChIP-Seq assays publicly available at the SRA-NCBI database: SRR1107840 and SRR2530135 were obtained from adult worms, whereas SRR2120359 and SRR2120360 from schistosomula parasites. Those data were all generated by the same group and are part of three different publications^{42, 67, 68}. See further details in the Supplementary Materials section.

Evolutionary conservation by whole genomes' comparison. In order to mask both repeats and low complexity regions present within *Schistosoma spp.* genomes, we ran RepeatMasker (<http://repeatmasker.org>) (-e crossmatch -pa 20 -q -xsmall -gff -norna -lib RepBasePerpignanSma52.fasta) on each of the three whole genomes compared herein. Pairwise alignments of *S. mansoni* repeat-masked genome against *S. haematobium* and *S. japonicum* ones were performed using lastz algorithm (<https://www.bx.psu.edu/~rsharris/lastz/>), an improved version of blastz⁶⁹. See further details in the Supplementary Materials section.

Differential Expression and correlation analyses across five developmental stages. Fifteen RNA-Seq libraries from five different developmental stages of *S. mansoni* (biological triplicate each) were selected for investigation of gene expression profile of both PC and lncRNA genes: cercariae (ERR022872, ERR022877 and ERR022878), somula 3 h (ERR022874, ERR022876 and ERR022879), somula 24 h (ERR022880, ERR022881 and ERR022882), male (SAMN06221530, SAMN06221531 and SAMN06221532) and female (SAMN06221542, SAMN06221543 and SAMN06221544) adults. See further details in the Supplementary Materials section.

Co-expression network construction and analyses. Based on the general gene expression correlation analysis among *S. mansoni* five developmental stages (described on the topic just above), we were able to retrieve lncRNA-PC gene pairs that are either positively or negatively correlated by selecting arbitrary *r* thresholds (see *r* cutoffs established by us further in this topic). We used Unix/Shell tools on the output of the ad-hoc correlation's R script in order to prepare the simple interaction formats (.sif) to feed Cytoscape software⁷⁰ for both network visualization and further analyses within the network. For each correlated genes' pair we assigned a "pos" or "neg" edge name regarding whether the correlation between the genes is positive or negative, respectively. See further details in the Supplementary Materials section.

Statistical analyses and charts plotting. Statistical analyses and charts plotting were performed within the R environment (version 3.3.2) with limma, edgeR, gplots and ggplot2 libraries loaded. Gene expression line charts were generated using matplotlib in Python. The intersection diagram in the UpSet format⁷¹ was plotted using Intervene (<https://asntech.shinyapps.io/intervene/>) (doi:10.1101/109728).

Parasite materials. All parasite material was from a BH isolate of *S. mansoni* maintained by passage through golden hamster (*Mesocricetus auratus*) and *Biomphalaria glabrata* snails. See further details in the Supplementary Materials section.

RNA extraction, quantification and quality assessment. Total RNA from cercariae (C) and schistosomula (3S and 24S) was extracted using a protocol based on Roquis *et al.*, 2015 (ref. 42). Briefly, 25,000 cercariae or schistosomula were ground with glass beads in liquid nitrogen for 5 minutes. Then the Qiagen RNeasy Micro Kit (Cat number 74004) was used for RNA extraction and purification according to the manufacturer's instructions, except for the DNase I treatment: the amount of DNase I was doubled and the time of treatment was increased to 45 minutes. Male or female worms were first disrupted in Qiagen RLT buffer using glass potters and pestles. RNA from males and females was then extracted and purified using the Qiagen RNeasy Mini Kit (Cat number 74104), according to the manufacturer's instructions, except for the DNase I treatment, which was the same used for cercariae and schistosomula RNA extraction. All the RNA samples were quantified using the Qubit RNA HS Assay Kit (Q32852, Thermo Fisher Scientific) and the integrity of RNAs was verified using the Agilent RNA 6000 Pico Kit (5067-1513 Agilent Technologies) in a 2100 Bioanalyzer Instrument (Agilent Technologies). Four biological replicates were assessed for each life cycle stage.

RT-qPCR assays. The reverse transcription (RT) reaction was performed with 100 ng of each total RNA sample using the SuperScript IV First-Strand Synthesis System (18091050, Life Technologies) and random hexamer primers in a 20 µL final volume. The obtained complementary DNAs (cDNAs) were diluted 10 times in water and quantitative PCR was performed using 2.5 µL of each diluted cDNA in a total volume of 10 µL containing 1X LightCycler 480 SYBR Green I Master Mix (04707516001, Roche Diagnostics) and 800 nM of each primer in a LightCycler 480 System (Roche Diagnostics). Primers for selected transcripts (Supplementary Table S5) were designed using the Primer 3 tool (http://biotools.umassmed.edu/bioapps/primer3_www.cgi), and each real-time qPCR was run in three technical replicates. The results were analyzed by comparative Ct method⁷². The reference gene Smp_092920 was chosen from twelve genes that showed no differential expression in the RNA-Seq data along the five stages. Data from the RT-qPCR expression values of the twelve genes across the five stages were analyzed with RefFinder⁷³ (<http://150.216.56.64/referencegene.php>) using three tools (BestKeeper, NormFinder and GeNorm) in order to choose the most stable gene for qPCR. Real-time data were normalized according to the expression level of the Smp_092920 reference gene, and p-values were determined by one-way analysis of variance (ANOVA) and Tukey post-hoc tests.

Data Availability. The RNA-seq datasets generated during the current study are available in the Sequence Read Archive (SRA) NCBI repository under Accession numbers SAMN06221530-SAMN06221541, SAMN06221554-SAMN06221559, SAMN06221542-SAMN06221553 and SAMN06221560-SAMN06221565. A GTF file with the *S. mansoni* lincRNAs identified with our pipeline can be downloaded from <http://verjolab.usp.br/tracks/schMan/schMan1/>. All other public RNA-seq data analyzed during the current study are from the SRA repository and their Accession numbers are listed in Supplementary Table S6.

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Author Contributions

E.J.R.V. and S.V.-A. conceived the project, analyzed the data and wrote the manuscript. E.J.R.V. designed and carried out all the *in silico* experiments encompassing pipeline development (programming), data curation/annotation, systems approach and statistical analyses, as well as wrote the original draft of the manuscript. LfDS guided on statistical analyses and performed part of data visualization. D.S.P. gave support on computational tasks, genome browser administration and performed part of data visualization. M.S.A., A.S.A.P. and G.L.M. designed and carried out the RT-qPCR assays for the validation/confirmation of lincRNAs' expression. M.S.A. wrote the methodology for the *in vitro* experiments. All authors reviewed the manuscript.

Additional Information

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Supplementary Methods and Figures for

The *Schistosoma mansoni* genome encodes thousands of long non-coding RNAs predicted to be functional at different parasite life-cycle stages

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Index

Supplementary Methods	page 2
Fig. S1	page 9
Fig. S2	page 10
Fig. S3	page 11
Fig. S4	page 12
Fig. S5– panel I	page 13
Fig. S5 – panels II, III, IV	page 14
Fig. S6	page 15
Supplementary References	page 16

Supplementary Methods

Assembly of RNA-Seq reads

Trinity *de novo* assembly¹ was applied to three different RNA-Seq datasets: (1) trin_strA dataset, comprised of ~ 300 million raw paired-end reads (SRA accession SAMN06221530-SAMN06221553) obtained in house from 24 samples of adult worm couples exposed *in vitro* to TH65 (ref. 2) or to vehicle (controls); (2) trin_strB dataset, comprised of ~ 300 million raw paired-end reads (SRA accession SAMN06221554-SAMN06221565), again obtained in house from 12 samples of adult worm couples exposed *in vitro* to GSK343 (ref. 3) or to vehicle (controls); (3) trin_sra dataset, which encompasses 52 RNA-Seq libraries (Supplementary Table S5) (~ 2 billion raw reads) from *S. mansoni* at several different life cycle stages (both untreated samples or samples treated with different compounds), which were downloaded from the SRA-NCBI public repository. A second assembly approach using the Tuxedo tools (Tophat2 (ref. 4), Cufflinks and Cuffmerge⁵) was applied to a subset of the above RNA-Seq libraries that included 44 out of the 52 SRA RNA-Seq libraries comprised exclusively of control untreated samples, plus 12 in house-derived libraries from both strA and strB comprised exclusively of control samples (total of ~ 2.2 billion raw reads). Each of those 56 samples (44 + 12) was used as an independent input file for both tophat2 and cufflinks, and then all 56 independently assembled transcript sets were merged onto a single non-redundant gtf file through cuffmerge execution.

After checking the libraries' quality with FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and trimming adapters and low quality bases (phred score < 20) with Trimmomatic⁶, the following parameters were used for both *de novo* and genome-guided assemblies:

- *de novo* (Trinity transcript assembler):

Trinity v2.2.0 parameters for in house libraries: --CPU 32 --max_memory 216G --SS_lib_type RF --seqType fq --min_contig_length 200;

Trinity v2.2.0 parameters for SRA libraries: --CPU 32 --max_memory 216G --seqType fq --single --run_as_paired --min_contig_length 200;

Blat⁷ parameters (trinity contigs against *S. mansoni* genome): -stepSize=5 -t=dna -q=rna;

pslToBed script from kentUtils (<https://github.com/ENCODE-DCC/kentUtils/tree/master/src/hg/pslToBed>) was run for converting psl Blat output to bed12 format.

- Genome-guided (Tuxedo tools):

Tophat2 v2.1.1 parameters: --min-intron-length 10 --max-intron-length 30000 --library-type [fr-firststrand or fr-unstranded] --b2-very-sensitive --GTF SMPs-withUTRs-5.2-woSm-wParent-NEW.gff --num-threads 26 Schistosoma_mansoni_v5.2;

Cufflinks v2.2.1 parameters: -p 20 -g SMPs-withUTRs-5.2-woSm-wParent-NEW.gff --multi-read-correct --frag-bias-correct Schistosoma_mansoni_v5.2.fa --min-intron-length 10;

Cuffmerge (an executable of the Cufflinks package) parameters: -p 20 -o cuffmergeOUT -g SMPs-withUTRs-5.2-woSm-wParent-NEW.gff -s Schistosoma_mansoni_v5.2-woSm.fa transcripts-GTF.fofn.

Retrieving long non-coding RNAs from the assembled transcripts' dataset

After having all the assembled transcripts mapped to the reference genome (bed12 and gtf formats for *de novo* and genome-guided assemblies, respectively), a series of filtering steps was applied as described below, aimed at both removing unwanted protein-coding transcripts and rescuing putative lncRNAs from the entire transcriptome datasets (red rectangles section from the pipeline depicted on Fig. 1). The following tools and their respective parameters were all placed in one single PERL script and adapted for automation; the script can be provided upon request.

Bedtools v2.25.0 (ref. 8) parameters: intersect -v (for rescuing lincRNAs) or -S -loj -f 0.33 (for rescuing anti-sense lncRNAs) -b SMPs-withUTRs-5.2.bed;

RepeatMasker version open-3.1.5 (<http://repeatmasker.org>) parameters: -s -lib RepBasePerpignanSma52-plusDNAtranspNCBI.fasta -x -gff -gc 35 -pa 12;

Ribopicker v0.4.3 (ref. 9) parameters: -i 70 -c 50 -dbs rrnadb;

Shell (bash) tools and an ad-hoc PERL script were executed in order to retrieve transcripts that have at least one intron greater than 30 nt and canonical splicing sites on all junctions;

Getorf (an executable from the EMBOSS v6.6.0.0 package) (ref. 10) parameters: -noreverse -minsize 75 -find 0;

CPC v0.9-r2 (ref. 11) and TransDecoder v2.0.1 (<http://transdecoder.github.io/>) were run using default parameters;

Interproscan v5.19 (ref. 12) parameters: -appl Panther,Pfam -t n -goterms -iprlookup.

At the end of this mining batch pipeline, we obtained a general dataset of *S. mansoni* putative lncRNAs.

Searching for evidence supporting *S. mansoni* lincRNAs as functional genes

H3K4me3 marks

In order to search for the presence of histone H3 lysine 4 trimethylation (H3K4me3) on the transcription start site (TSS) of *S. mansoni* genes as an epigenetic mark for transcriptional activation, we relied on four ChIP-Seq assays publicly available at the SRA-NCBI database: SRR1107840 and SRR2530135 were obtained from adult worms, whereas SRR2120359 and SRR2120360 on schistosomula forms. Those data were all generated by the same group and are part of three different publications¹³⁻¹⁵.

We adopted the same computational pipeline described by Anderson et al., 2015 for mapping those marks on *S. mansoni* genome¹⁶ and statistically assigning the significant peaks¹⁷. Bedtools⁸ (window -c -l 1000 -r 1500 -sw) was used for capturing genes (both PC genes and lincRNAs) that have at least one significant peak on their flanking regions (1 kb upstream and 1.5 kb downstream the TSS).

Evolutionary conservation by whole genomes' comparison

In order to mask both repeats and low complexity regions present within *Schistosoma* spp. genomes, we ran RepeatMasker (<http://repeatmasker.org>) (-e crossmatch -pa 20 -q -xsmall -gff -norna -lib RepBasePerpignanSma52.fasta) on each of the three whole genomes compared herein. Pairwise alignments of *S. mansoni* repeat-masked genome against *S. haematobium* and *S. japonicum* ones were performed using lastz algorithm (<https://www.bx.psu.edu/~rsharris/lastz/>), an improved version of blastz¹⁸. The following commands were used to obtain pairwise genome alignments (Sman vs Shae and Sman vs Sjav) on a maf format.

```
$ blastzWrapper Smansoni Shaematobium Y=9000 H=0 >Smansoni.Shaematobium.lav
$ lav2maf Smansoni.Shaematobium.lav Smansoni Shaematobium
>Smansoni.Shaematobium.maf
$ maf_sort Smansoni.Shaematobium.maf Smansoni > Smansoni.Shaematobium.orig.maf
$ blastzWrapper Smansoni Sjaponicum Y=9000 H=0 >Smansoni.Sjaponicum.lav
$ lav2maf Smansoni.Sjaponicum.lav Smansoni Sjaponicum >Smansoni.Sjaponicum.maf
$ maf_sort Smansoni.Sjaponicum.maf Smansoni > Smansoni.Sjaponicum.orig.maf
```

The multiple alignment arrangement was generated by running TBA¹⁹ (tba E=Smansoni "((Smansoni Shaematobium) Sjaponicum)" *.maf schistos_pub-tba.maf) in order to get a single maf file containing all the aligned genomic stretches from the three species that are used as input for phastCons algorithm²⁰ (phastCons --target-coverage 0.25 --expected-length 12 --estimate-trees treeSchistos_pub --msa-format MAF Chr_1-RandomSample.maf init.mod --no-post-probs). Noteworthy that, before running phastCons, a phylogenetic tree model had to be constructed with phyloFit²¹ (phyloFit --tree "((Smansoni,Shaematobium),Sjaponicum)" --msa-format MAF --out-root init schistos_pub-tba-cleaned.maf).

Differential Expression and correlation analyses across five developmental stages

Fifteen RNA-Seq libraries from five different developmental stages of *S. mansoni* (biological triplicate each) were selected for investigation of gene expression profile of both PC and lncRNA genes: cercariae (ERR022872, ERR022877 and ERR022878), somula 3h (ERR022874, ERR022876 and ERR022879), somula 24h (ERR022880, ERR022881 and ERR022882), male (SAMN06221530, SAMN06221531 and SAMN06221532) and female (SAMN06221542, SAMN06221543 and SAMN06221544) adults.

Read counts and “transcripts per million” normalized counts (TPM) were assessed by running kallisto²² (-t 8 -b 50) against an indexed reference transcriptome file that comprised all 11,876 already annotated PC genes (Smp) and the 7029 putative lincRNAs identified herein. In order to avoid bias due to very low expression levels some genes may have (mainly lincRNAs), we decided to select only the genes that showed $TPM \geq 1$ on all the three replicates of at least one life cycle stage. We retrieved 10,482 Smps and 2091 lincRNAs that served as input for the one-way ANOVA-like method from the edgeR suite²³ (following the default parameters described on the edgeR user guide, last revised on Jun/30/2016), which were used for detecting differentially expressed genes across the five parasite forms (adjusted p-value < 0.01). Cercariae were set as the intercept group. A multi-dimensional scaling plot was generated by edgeR before performing the one-way ANOVA-like in order to check the samples convergence/divergence (Supplementary Fig. S6).

Besides the differential expression analysis, we have also performed Pearson Correlation Coefficient (r) assessment on the genes having $TPM \geq 1$ (10,482 Smps and 2091 lincRNAs = 12,573 genes) using the TPM values for each gene from the 15 libraries in the following order: cercariae, somula 3h, somula 24h, male and female (biological triplicate each). An R script was

written to automatically assign r to all possible gene pairs' combination within the list. The output of that script was used as the main source for further analyses involving expression correlation, such as the screening for correlation between lincRNA-PC gene neighbors and the co-expression network construction (described below).

Co-expression network construction and analyses

Based on the general gene expression correlation analysis among *S. mansoni* five developmental stages (described on the topic just above), we were able to retrieve lincRNA-PC gene pairs that are either positively or negatively correlated by selecting arbitrary r thresholds (see r cutoffs established by us further in this topic). We used Unix/Shell tools on the output of the ad-hoc correlation's R script in order to prepare the simple interaction formats (.sif) to feed Cytoscape software²⁴ for both network visualization and further analyses within the network. For each correlated genes' pair we assigned a "pos" or "neg" edge name regarding whether the correlation between the genes is positive or negative, respectively.

We idealized two approaches for the co-expression network construction. (i) The first one was based on the selection of the 181 lincRNAs (red nodes) that shared all the four traits for being functional (H3K4me3 mark at their TSSs, phastCons evolutionary conservation score, differentially expressed across the five stages and with expression correlated with that of its PC gene neighbor) and then rescuing their positively (cyan blue edges) or negatively (gray edges) correlated PC gene counterparts (blue nodes). At first we chose an r cutoff of $r \geq 0.8$ or $r \leq -0.8$, which retrieved 2359 PC genes (see Fig. 6 and Results section). Subsequently, we established a two-steps filtering method in order to rescue lincRNA-PC gene pairs that might be topologically close to each other, which we called topological filtering step (TFS). For the TFS we decreased the r threshold to $r \geq 0.5$ or $r \leq -0.5$ (first step) and then filtered the pairs by obeying one of the following two rules: the lincRNA and the PC genes are either neighbors (considering the first two PC genes upstream and two downstream of each lincRNA) or the lincRNA may form a putative triplex structure on their co-expressed PC loci within the genome (the entire PC locus plus 1 kb upstream of its TSS). The triplex structure predictions were obtained by running triplexator v1.3.2 (ref. 25) (-mf -dl). Applying that topological filtering strategy we obtained a much smaller network with 89 out of the 181 lincRNAs and 237 Smgs (see Fig. 7 and Results section). (ii) The second approach for a co-expression network construction was based on the selection of 319 highly differentially expressed PC genes (yellow nodes), which showed a

$\log_2FC \geq 10$ on at least one out of the five developmental stages, and then rescuing their positively ($r \geq 0.9$, cyan blue edges) or negatively ($r \leq -0.9$, gray edges) correlated lincRNAs (red nodes) and/or other PC genes (blue nodes). Three hundred and seven out of the 319 selected PC genes were correlated with at least one lincRNA, so they were the genes used to build the network. This network contained 2215 PC genes and 750 lincRNAs (see Supplementary Fig. S5 and Results section).

The “Network Analyzer” tool from Cytoscape was used in order to calculate the betweenness centrality (BC) values²⁶ for each node on the networks built herein (see Results for more details).

To further test the significance of the networks built on both approaches described above, we adopted the same method used by Necseulea et al., 2014 (ref. 27). We randomly-picked expressed lincRNAs and PC genes ($TPM \geq 1$ on at least one out of the five developmental stages on focus) in order to build a random network presenting the same architecture of the actual one, that is, same number of lincRNAs, PC genes and edges (keeping the same number of edges that connect each lincRNA to their PC gene counterparts on the actual network). This strategy showed us a false discovery rate less than 7% for both network construction approaches, indicating that our construction methods have a precision (positive predictive value) greater than 93%.

The hypergeometric test for Gene Ontology (GO) gene enrichment analyses on the PC genes present on the co-expression networks was performed by BinGO²⁸, a Cytoscape plugin, using an adjusted p-value cutoff of 0.01.

Parasite materials

All parasite material was from a BH isolate of *S. mansoni* maintained by passage through golden hamster (*Mesocricetus auratus*) and *Biomphalaria glabrata* snails. Cercariae were collected from snails infected with 10 miracidia each. Thirty-five days after infection, the snails were placed in the dark in water and then illuminated for two hours to induce shedding. The emerging cercariae were cooled on ice for 30 minutes to prevent swimming and collected by centrifugation, washed with water once and then stored in RNeasy lysis buffer (Qiagen) until RNA extraction. Schistosomula were obtained by mechanical transformation of cercariae and separation of their bodies as previously described²⁹, with some modifications. Briefly, cercariae

were collected as described above and then suspended in 15 ml of M169 medium (Vitrocell, cat number 00464) containing penicillin/streptomycin, amphotericin (Vitrocell, cat number 00148). Mechanical transformation was performed by passing the cercariae ten times through a 23G needle. In order to separate schistosomula from the tails, the tail-rich supernatant was decanted and the sedimented bodies resuspended in a further 7 ml of M169 medium. The procedure was repeated until less than 1% of tails remained. The newly transformed schistosomula were maintained for 3h or 24h in M169 medium (Vitrocell, cat number 00464) supplemented with penicillin/streptomycin, amphotericin, gentamicin (Vitrocell, cat number 00148), 2% fetal bovine serum, 1 μ M serotonin, 0.5 μ M hypoxanthine, 1 μ M hydrocortisone and 0.2 μ M triiodothyronine at 37°C and 5% CO₂. Schistosomula cultivated for 3h (3S) or for 24h (24S) were washed 3 times with PBS and stored in RNAlater (Ambion) until RNA extraction.

Adult *S. mansoni* worms were recovered by perfusion of golden hamsters that had been infected with 200-300 cercariae, 7 weeks previously. Approximately 200 *S. mansoni* (BH strain) adult worm pairs were freshly obtained through the periportal perfusion of hamster, as previously described¹⁶. After perfusion, the adult worm pairs were kept for 3 hours at 37°C and 5% CO₂ in Advanced RPMI Medium 1640 (Gibco, #12633–012) supplemented with 10% heat-inactivated calf serum (freshly added), 12 mM HEPES (4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid) pH 7.4, and 1% penicillin/streptomycin, amphotericin (Vitrocell, cat number 00148). After 3h of incubation, the adult worm pairs were washed 3 times with PBS and stored in RNAlater (Ambion) for further RNA extraction. Right before RNA extraction, adult worm pairs were manually separated in RNAlater (Ambion) using tweezers in order to obtain male- and female-only RNA samples.

Supplementary Figures

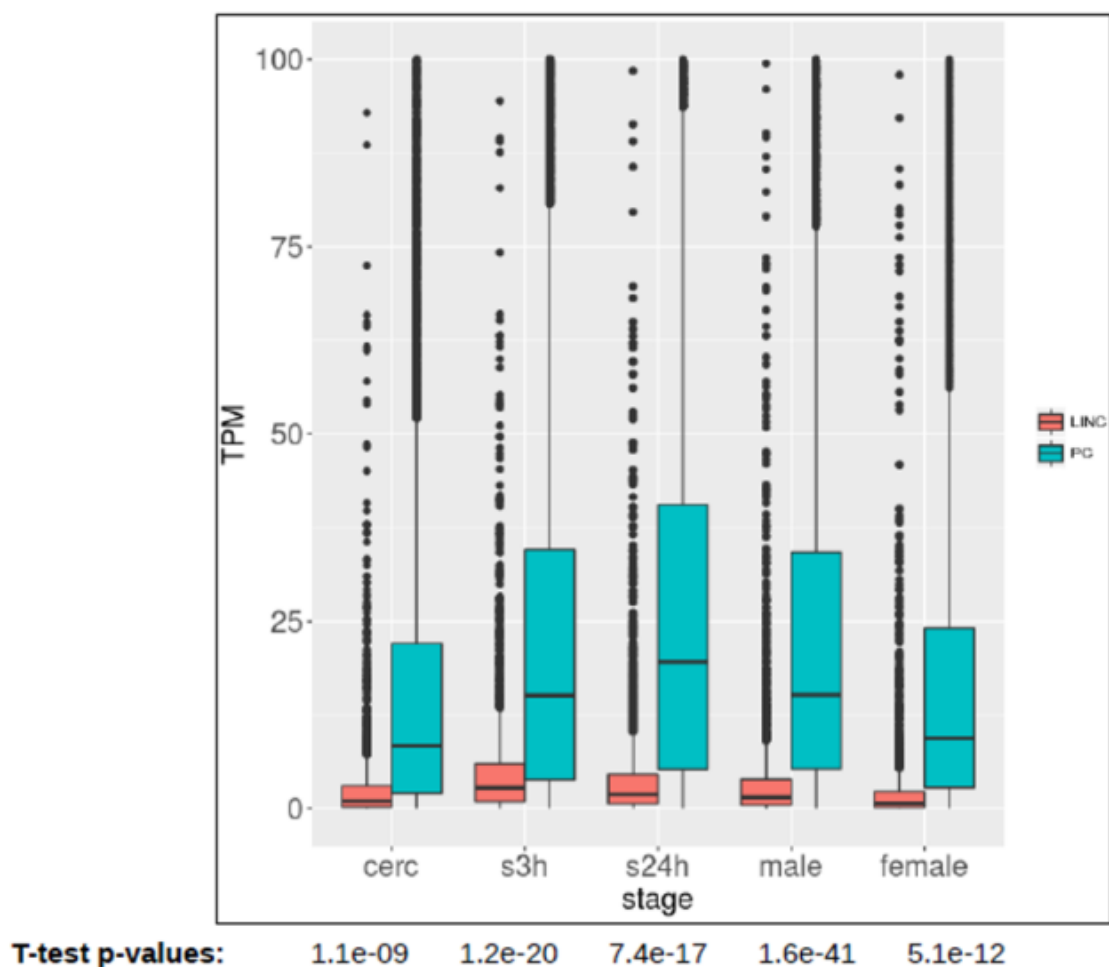


Figure S1. SmLINC genes have significantly lower expression when compared to protein-coding Smp genes on five different developmental stages. The SmLINC genes lists (red) and the protein-coding Smp genes lists (cyan) were filtered by $\text{TPM} \geq 1$. Y axis: normalized expression levels (transcripts per million, TPM). X axis: cercariae (cerc), somula 3h (s3h), somula 24h (s24h), male and female forms of the parasite. t-test p-values $< 1e-08$.

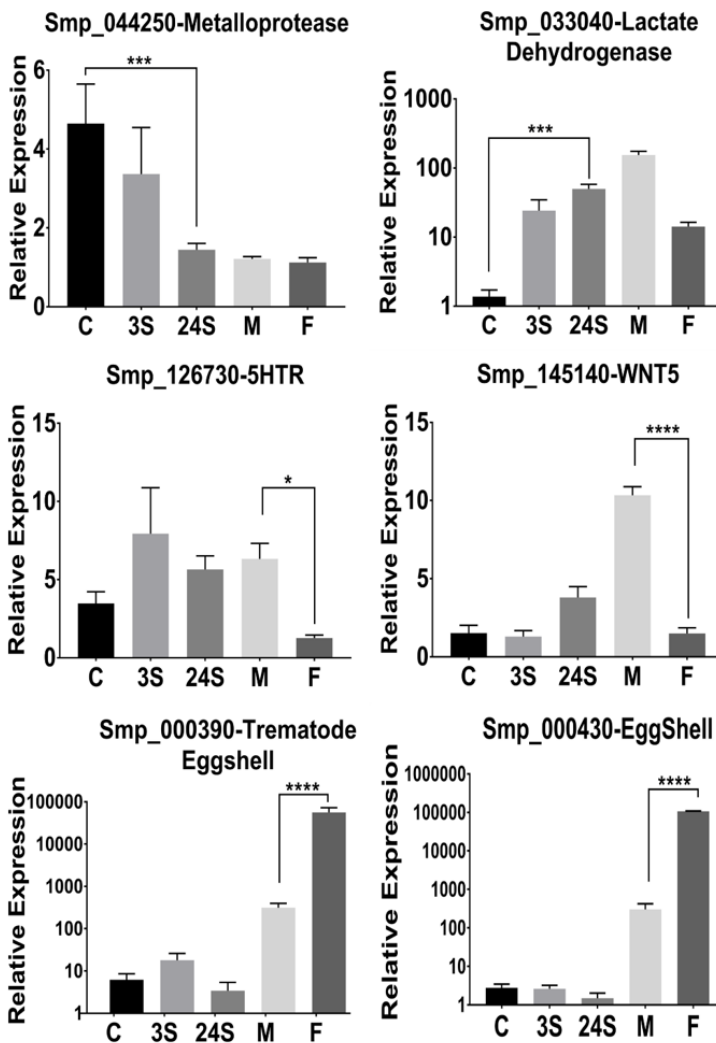


Figure S2. Confirmation by RT-qPCR of the differential expression of six protein-coding genes selected as positive controls. Smp_044250 (Metalloprotease), shown as up-regulated in cercariae compared with somula³⁰; Smp_033040 (Lactate dehydrogenase), up-regulated in somula compared with cercariae³⁰; Smp_126730-5HTR and Smp_145140-WNT5, assessed as up-regulated in adult male compared with female¹⁶; Smp_000390-Trematode Eggshell and Smp_000430-EggShell, up-regulated in adult female compared to male¹⁶. RT-qPCR assays were carried out on five parasite stages/forms, namely cercariae (C), schistosomula after 3 hours of mechanical transformation (3S), schistosomula after 24 hours of mechanical transformation (24S), adult male and female. The relative expression measurements, as well as the number of replicates and statistical analyses were the same as described in Fig 5.

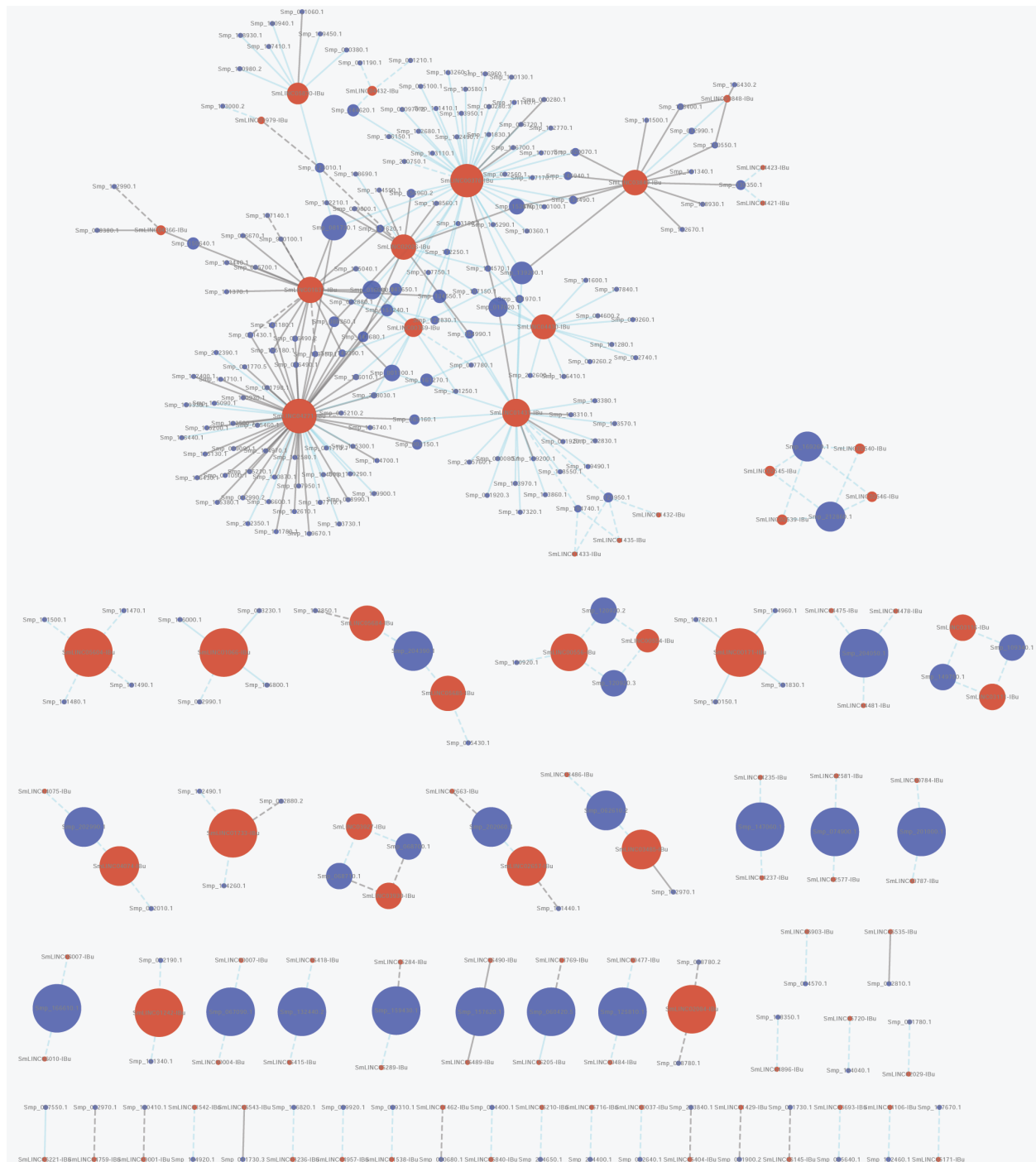


Figure S3. Complete view of the lincRNA-PC gene co-expression network built by the Topological Filtering Step (TFS) approach. This is the entire network from Fig 7A. The “Network Analyzer” function from cytoscape was used in order to obtain betweenness centrality score for each node, meaning the bigger the node, the higher propensity to be a hub. The network contains 89 SmLINC nodes (red) and 237 PC gene (Smp) nodes (blue), with 62.5% of positively correlated pairs (204 / 326, $r \geq 0.5$, cyan edges) and 37.5% of negatively correlated ones (122 / 326, $r \leq -0.5$, gray edges). Dashed edges represent neighbor genes.

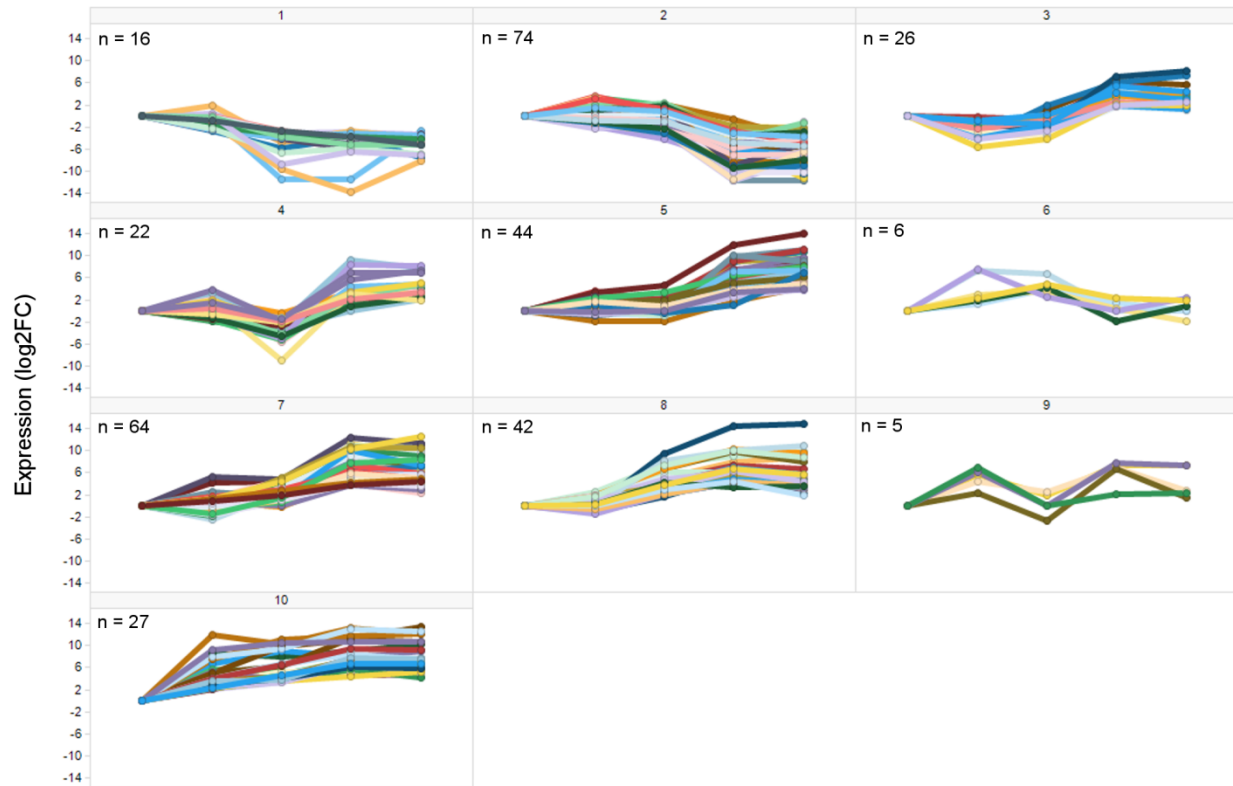


Figure S4. K-means analysis on all the 326 genes present in the entire network from Fig C. We performed the K-means analysis with 10 sets, being able to detect that 230/326 (70%) of the genes were grouped into clusters that displayed an up-regulation pattern from cercariae to adult forms (clusters 3, 4, 5, 7, 8, 9 and 10).

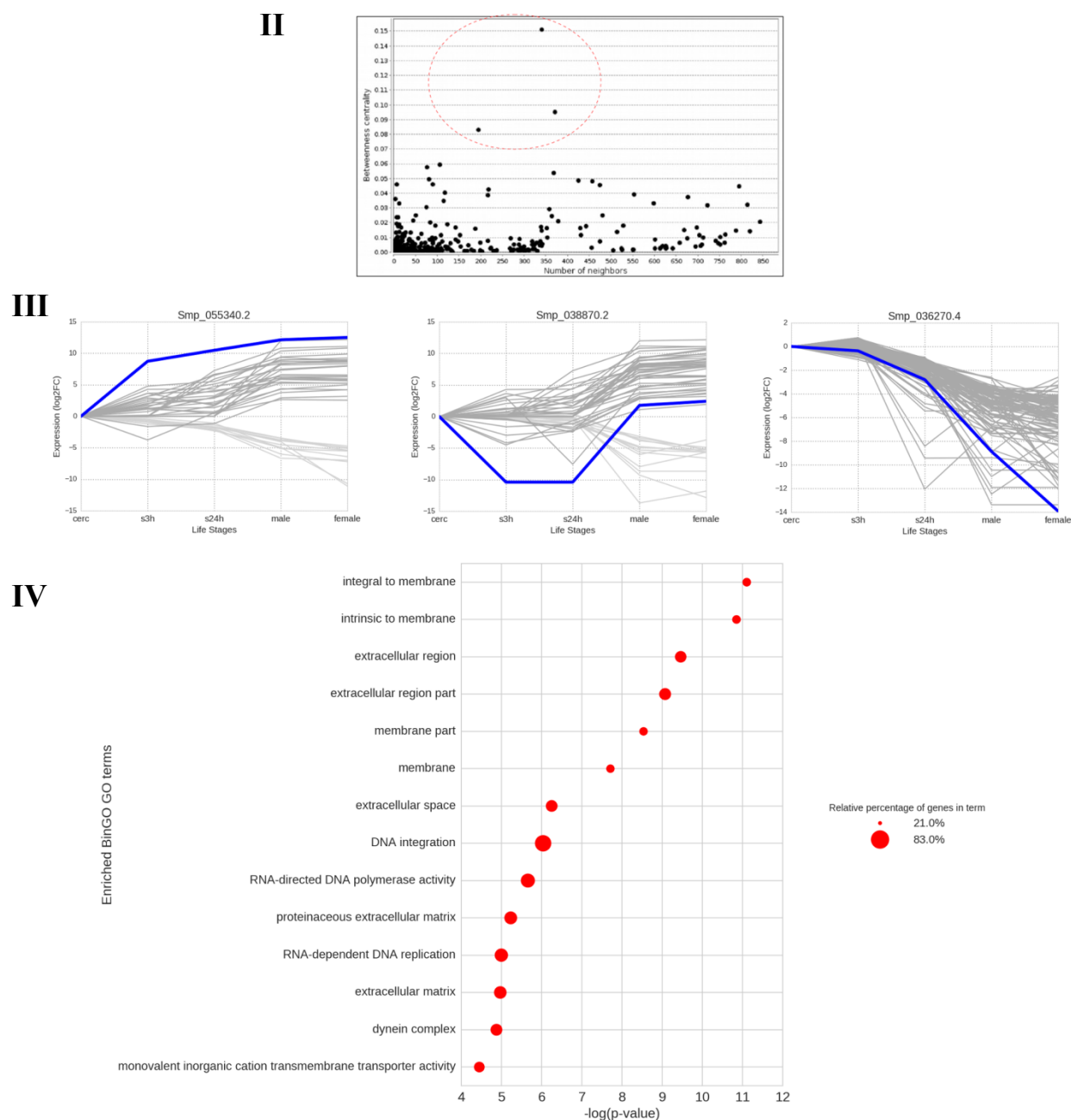


Figure S5. A second co-expression network construction approach highlights PC gene hubs and their correlated lincRNAs. (II) Betweenness centrality score calculation revealed three PC genes as the most important nodes in the network: Smp_055340.2, Smp_038870.2 and Smp_036270.4. (III) Co-expression kinetics on each subcluster from the three SmPs described above (blue lines), displaying both positively (dark gray lines) and negatively (light gray lines) correlated SmLINC across five life cycle stages: cercariae (cerc), somula 3h (s3h), somula 24h (s24h), male and female. (IV) Gene enrichment analysis on the 2215 PC genes in the network revealed significant GO terms, some of them identical to the ones depicted on Fig 6B from the 181 robust lincRNAs' network.

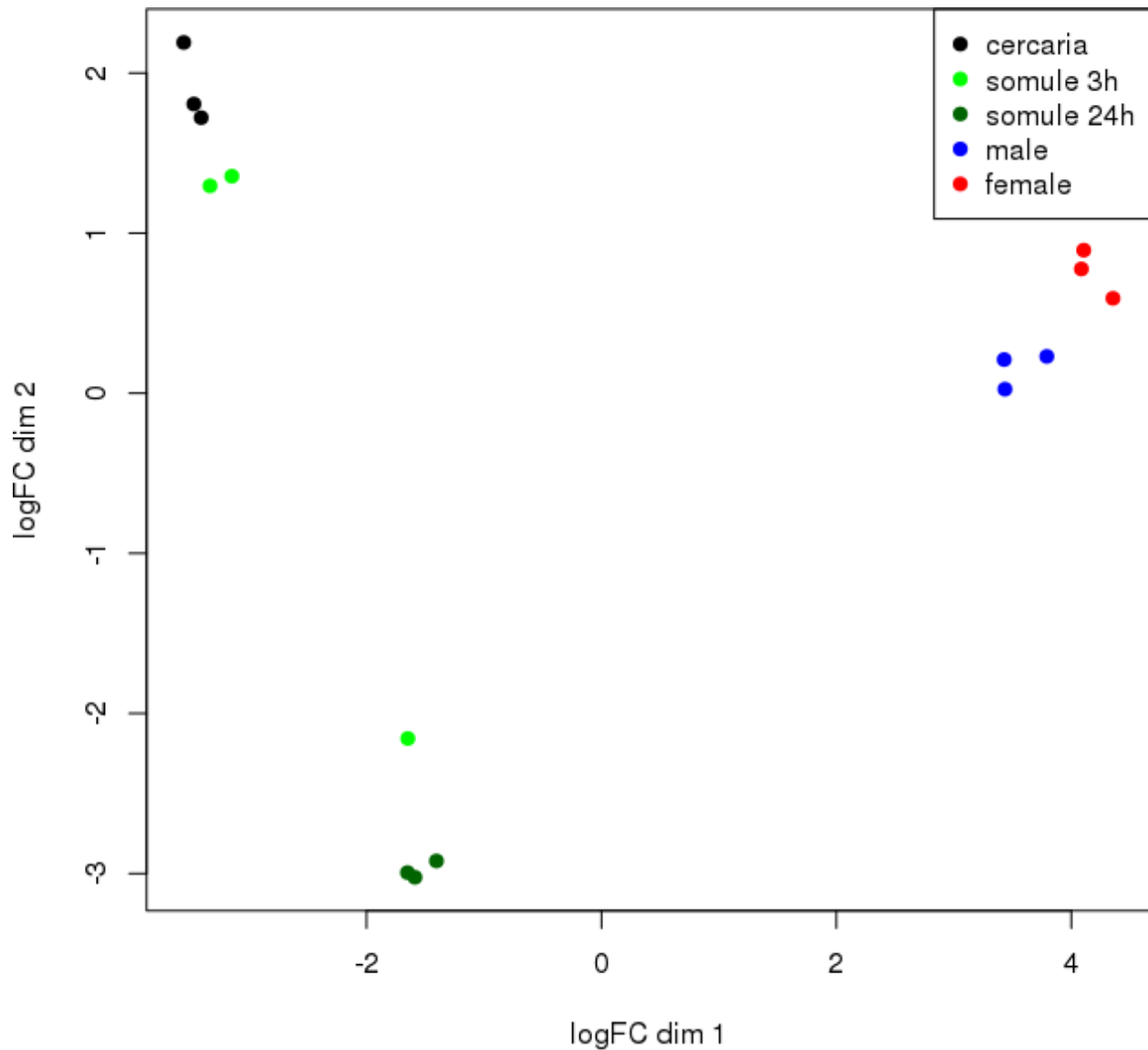


Figure S6. EdgeR multi-dimensional scaling plot (PCA-like) on five different developmental stages comprising 15 RNA-Seq samples of *S. mansoni* (biological triplicates for each stage). Cercariae (ERR022872, ERR022877 and ERR022878), somula 3h (ERR022874, ERR022876 and ERR022879), somula 24h (ERR022880, ERR022881 and ERR022882), male (SAMN06221530, SAMN06221531 and SAMN06221532) and female (SAMN06221542, SAMN06221543 and SAMN06221544) adults.

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