

UNIVERSIDADE FEDERAL DE UBERLÂNDIA
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NATÁLIA SILVA DA TRINDADE

**EVIDÊNCIAS DA PROTEÍNA DA HETEROCROMATINA 1 (HP1) NO CICLO
DE VIDA E SUA INTERAÇÃO COM PROTEÍNAS NUCLEARES EM
CERCÁRIAS DE *Schistosoma mansoni***

PATOS DE MINAS – MG
JANEIRO DE 2020

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Dissertação de Mestrado
apresentada ao Programa de Pós-
graduação em Biotecnologia como
requisito parcial para obtenção do
título de Mestre em Biotecnologia.

Orientadora: Profa. Dra. Enyara Rezende Moraes

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Reuniu-se na Sala 110, Campus Patos de Minas, da Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Biotecnologia, assim composta: Professores Doutores: Enyara Rezende de Moraes, orientadora da candidata; Andressa Barban do Patrocínio, Universidade de São Paulo; e Lizandra Guidi Magalhães, Universidade de Franca.

Iniciando os trabalhos a presidente da mesa, Enyara Rezende de Moraes, apresentou a Comissão Examinadora e a candidata, agradeceu a presença do público, e concedeu a discente a palavra para a exposição do seu trabalho. A duração da apresentação da discente e o tempo de arguição e resposta foram conforme as normas do Programa.

A seguir o senhor(a) presidente concedeu a palavra, pela ordem sucessivamente, aos(às) examinadores(as), que passaram a arguir o(a) candidato(a). Ultimada a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu o resultado final, considerando o(a) candidato(a):

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Nada mais havendo a tratar foram encerrados os trabalhos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.



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DEDICATÓRIA

Dedico esta dissertação aos meus pais, por me apoiarem, incentivarem e demonstrarem todo o amor e apoio em todos os momentos.

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RESUMO

A esquistossomose é uma doença causada por parasitas do gênero *Schistosoma*. Estima-se que 240 milhões de pessoas no mundo estejam infectadas pelo parasita e cerca de 700 milhões de pessoas estejam em risco de infecção. As modificações pós-traducionais das histonas são mecanismos epigenéticos, associados a estados silenciados ou ativos da cromatina, regulando a expressão gênica no nível transcricional. Embora sejam importantes as modificações pós-traducionais das histonas, estudos demonstraram que os co-reguladores da transcrição também são importantes para a regulação da expressão gênica do parasita. A Proteína da Heterocromatina 1 (HP1) é um conhecido co-regulador conservado na evolução e sua importância já foi descrita em mamíferos, leveduras e protozoários, mas até o momento em *Schistosoma mansoni* essa proteína ainda não foi descrita. Neste trabalho, SmHP1 foi identificada no banco de dados do parasita e sua estrutura primária de aminoácidos é conservada evolutivamente. A expressão do mRNA do SmHP1 foi analisada durante o ciclo de vida, e foi evidenciada uma maior expressão na cercária quando comparada com outras fases, no entanto, a expressão da proteína não é alterada em todas as etapas testadas. Além disso, a análise por Western blotting mostrou que, no ciclo de vida do parasita a SmHP1 pode formar dímeros de 56kDa em solução. A imunoprecipitação usando o anticorpo anti-HP1 seguido por espectrometria de massas mostrou que vários alvos proteicos envolvidos na regulação da transcrição podem interagir com a SmHP1 no estágio das cercárias, mas os papéis na manutenção dos estados repressivos da transcrição ainda não estão elucidados.

Palavras-chave: *Schistosoma mansoni*. HP1. Epigenética. Bioinformática.

ABSTRACT

Schistosomiasis is a disease caused by parasites of the genus *Schistosoma*. It is estimated that 240 million people worldwide are infected with the parasite and about 700 million people are at risk of infection. As post-translational modifications of the stories are epigenetic mechanisms, associated with muted or active chromatin states, regulating genetic expression at the transcriptional level. Although they are as important as the post-translational modifications of the stories, studies have shown that co-regulators of transcription are also important for the application of the parasite's genetic expression. Heterochromatin Protein 1 (HP1) is a known co-regulator conserved in evolution and its importance has already been detected in mammals, yeasts and protozoa, but so far in *Schistosoma mansoni* that has not yet been used. In this work, SmHP1 was not identified in the parasite's database and its primary amino acid structure is conserved evolutionarily. An expression of the SmHP1 mRNA was analyzed during the life cycle and a greater enclosed expression was evidenced when compared to other phases, however, an expression of the protein is not altered in all tested steps. In addition, a Western blotting analysis showed that, in the parasite's life cycle, SmHP1 can form 56kDa dimers in solution. An immunoprecipitation using the anti-HP1 antibody followed by mass spectrometry has shown that several tests of transcription of substances activated by SmHP1 are not being performed, but the maintenance roles of transcription repressants are not yet elucidated.

Keywords: *Schistosoma mansoni*. HP1. Epigenetics. Bioinformatics.

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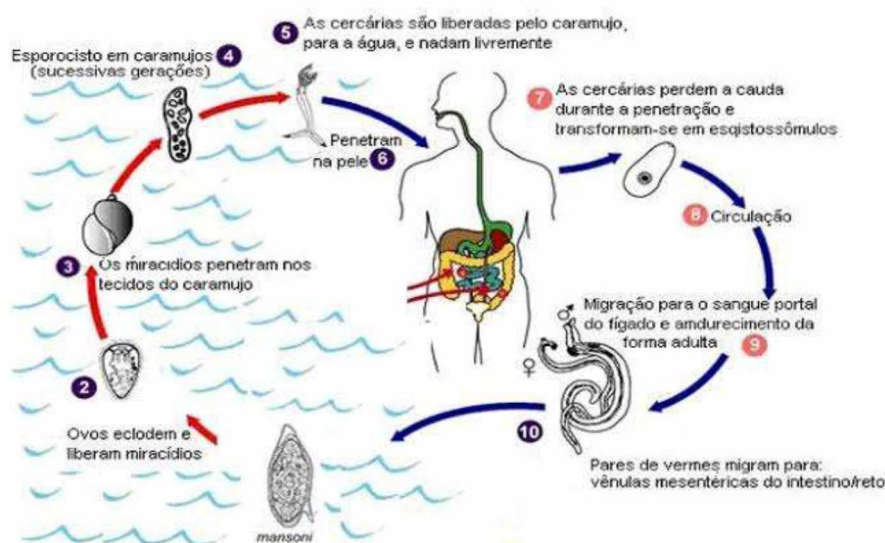
CAPÍTULO 1

1 INTRODUÇÃO

A esquistossomose é uma doença predominante nas áreas tropicais e subtropicais do planeta, causada pelo parasita do gênero *Schistosoma*. Segundo a Organização Mundial da Saúde (2017), 220,8 milhões de pessoas de pessoas necessitaram de tratamento preventivo para a esquistossomose em 2017. De acordo com dados do Ministério da Saúde do Brasil, entre 2008 e 2016 foram registradas 2.275 internações por esquistossomose e 4.473 óbitos (BRASIL, 2018). Estima-se que no Brasil cerca de 1,5 milhões de pessoas vivem em áreas sob o risco de contrair a doença (MINISTÉRIO DA SAÚDE, 2017). Os estados mais afetados pela doença se encontram nas regiões Nordeste e Sudeste, devido à presença dos hospedeiros intermediários nessas localidades (BRASIL, 2018).

Como todos os trematódeos, o ciclo de vida do *S. mansoni* (Figura 1) inclui hospedeiro intermediário e definitivo. Quando os ovos expelidos através das fezes de um ser humano infectado entram em contato com a água, eclodem para liberar as larvas ciliadas de vida livre, conhecidas como miracídeos. Estes infectam o caramujo, o hospedeiro intermediário, e sofrem uma transformação no desenvolvimento. Nesse momento, os miracídeos evoluem para o próximo de estágio, as cercarias que são liberadas no meio aquático e ficam livres para penetrar ativamente através da pele do hospedeiro definitivo, o homem. Dessa forma, esses parasitas entram na circulação sanguínea, migram como esquistossômulos e após cerca de 20 dias já se encontram no sistema porta-hepático. No mesentério e no sistema porta-hepático os vermes adultos machos e fêmeas se encontram pareados, maturam sexualmente, iniciando oviposição. Os ovos atravessam o intestino ou a bexiga e são liberados do hospedeiro através das fezes, e o ciclo é reiniciado em um novo hospedeiro ou até mesmo no mesmo hospedeiro através das reinfecções (WENDT; COLLINS III, 2016).

Figura 1 – Ciclo Biológico do *Schistosoma mansoni*



Adaptado de CDC, 2019

Devido a essas extensas modificações em sua morfologia durante seu ciclo de vida, acredita-se que alterações reversíveis no epigenoma do parasita podem regular os processos de desenvolvimento, diferenciação e coordenar as mudanças morfológicas marcantes que ocorrem. Diferentes perfis de expressão gênica são programados nas células e ao longo dos estágios do ciclo de vida eucariota. Os mecanismos epigenéticos desempenham um papel central na regulação gênica programada e incluem um grande número de modificações pós-traducionais de histonas, bem como a metilação do DNA genômico e modificações pós-traducionais de outras proteínas além das histonas. Essas modificações em histonas, em organismos eucariotos, envolvem vários processos baseados em cromatina, tendo um impacto significativo nos perfis de expressão gênica e fenótipos (CABEZAS-CRUZ et al., 2014).

Modificações de histonas estão associadas a estruturas de cromatina transcricionalmente ativas ou repressivas, controlando a expressão gênica no nível transcricional. A metilação da histona H3 na lisina 9 (H3K9me3) é uma marca da heterocromatina relacionada ao silenciamento dos genes, em contraste, a acetilação da mesma posição é uma marca da eucromatina e facilita a transcrição. As modificações na histona H3 são muito ricas em regiões teloméricas, pericentroméricas e ricas em repetição, e acredita-se que seja responsável pelo estado de repressão (RIU et al., 2007;

SUZUKI et al., 2016). Em mamíferos, a histona H3 é acetilada nas lisinas K14, K18 e K9, enquanto que histona H4, acetilada nas lisinas K5 e K8 (MISHIMA et al., 2013).

Os co-reguladores transcricionais são necessários para manter um estado silenciado e/ou ativado, de expressão gênica. Esses podem formar complexos proteicos, que recrutam proteínas que se ligam ao DNA da cromatina e silenciam ou ativam determinadas porções do DNA. Em *S. mansoni*, algumas evidências mostram que as modificações pós-traducionais de histonas sozinhas, não são capazes de regular a expressão gênica do parasita. Portanto, são necessários outros co-reguladores de expressão, como os fatores de transcrição e remodeladores de cromatina que participam desse processo e inclusive formam complexos de proteínas que regulam a transcrição. Mas apesar de os co-reguladores de histonas serem importantes no desenvolvimento do parasita, ainda temos pouco conhecimento sobre sua atividade (COSSEAU et al., 2017; ROQUIS et al., 2015).

Neste contexto, a proteína da heterocromatina 1 é um co-regulador conservado durante a evolução e de suma importância para manter estruturas silenciadas ou heterocromáticas, através da ligação nas histonas com marcas do tipo H3K9me3, e ainda não existe nenhuma evidência do seu papel de co-regulador transcricional em *S. mansoni* (WATANABE et al., 2018).

1.1 Objetivos

Objetivo Geral

Identificar e caracterizar *in silico* e *in vivo* a proteína da heterocromatina 1 (HP1) em *S. mansoni*.

Objetivos específicos

- Analisar a expressão do transcrito *SmHP1* identificado nas diferentes fases do ciclo de vida de *S. mansoni*.
- Analisar a expressão proteica nas diferentes fases do ciclo.
- Identificar alvos proteicos envolvidos na regulação da transcrição que podem interagir com o HP1 no estágio das cercarias.

1.2 Justificativa

A esquistossomose é uma doença parasitária causada por helmintos do gênero *Schistosoma* que atinge mais de 200 milhões de pessoas no mundo. Dentre os mais de 78 países relatados com a ocorrência desta patologia, o Brasil é o país sul-americano maior incidência de esquistossomose. A espécie mais comum capaz de se reproduzir e disseminar a doença no Brasil é o *S. mansoni*, uma vez que a presença do hospedeiro intermediário suscetível à infecção foram encontrados no local, o *Biomphalaria glabrata* (MAEDA et al., 2018). De acordo com dados disponibilizados pelo Ministério da Saúde e pelo Programa de Controle da Esquistossomose (PCE), em 2017, por volta de 14 mil casos da doença foram descritos, sendo encontrados, principalmente, nas regiões nordeste e sudeste do país (MINISTÉRIO DA SAÚDE, 2017).

Neste contexto, considerando o complexo ciclo de vida do parasito, a transcrição gênica na passagem de cercária para esquistossômulo parece ser altamente regulada, pois além de apresentar vários genes diferencialmente expressos durante todo o desenvolvimento do esquistossômulo, em cercárias essa regulação parece ainda ser mais elaborada, pois neste estágio o nível transcricional é baixo, sendo um estágio quase transcionalmente silenciado. Em outros organismos, diversos relatos apontam para a proteína da heterocromatina (HP1), como a responsável por recrutar proteínas para formar e sustentar estados silenciados (BEAR; MCKERROW, 2017; WILLBANKS et al., 2016).

Alguns coreguladores foram descritos em *S. mansoni* como importantes fatores na regulação gênica, bem como várias enzimas modificadoras de histonas e tem sido sugerido que a regulação da transcrição gênica nos esquistossomos pode exigir a ação dessas enzimas (COSSEAU et al, 2009). Além disso, alguns desses coreguladores tem sido descritos durante o estágio de vida de esquistossomos, o que sugere uma relação com a ativação da transcrição (MACIEL et al., 2008)

A sequência predita da proteína da heterocromatina (HP1) está anotada no genoma do parasita sob o número de acesso Smp_179650. Considerando a importância dessa proteína como coregulador transcricional e seu papel na regulação da transcrição gênica faz-se necessário o estudo desta para ampliar o conhecimento a respeito da biologia do parasita.

2 REFERENCIAL TEÓRICO

2.1 Esquistossomose

No hospedeiro intermediário ocorre a multiplicação do parasito. Essa interação exige respostas adaptativas bem complexas e para que seja possível o desenvolvimento do *S. mansoni* no hospedeiro intermediário é necessário que fatores ambientais sejam propícios, além do comportamento biológico dos miracídios, dos mecanismos intrínsecos de defesa dos caramujos e dos mecanismos adaptativos do parasito ao organismo do molusco (KNIGHT et al, 2016).

Ao atingirem o meio aquático, os ovos maduros sofrem influência da baixa osmolaridade do meio, de estímulos luminosos e da temperatura ambiente. Essas mudanças intensificam os movimentos da larva e provoca a ruptura transversal da casca do ovo, ocorrendo então, a eclosão do miracídio (PEARCE; MACDONALD, 2002).

O miracídio é uma larva que apresenta uma intensa atividade natatória. Após sua eclosão, o tempo de vida de um miracídio é 8 horas. A presença de substâncias do molusco na água estimula os movimentos dos miracídios, facilitando a infecção. O processo de penetração faz com que ocorram perdas de estruturas pela larva. Após 72 horas, a larva é denominada esporocito primário e dobra de tamanho. Sob condições adequadas, o esporocisto secundário desenvolve-se e apresenta uma musculatura associada à formação de espinhos. Essa musculatura tem um papel importante na motilidade e na capacidade de migração intratecidual das larvas. Um único miracídio pode gerar 300 mil cercarias. A formação das cercarias até sua liberação no meio aquático pode demorar 27 a 30 dias. As cercarias apresentam duas ventosas que serve para fixar-se na pele do hospedeiro definitivo (CARVALHO et al, 2018).

A cercária penetra na pele ou mucosa, após a penetração o parasito atinge vários ciclos na circulação sistêmica. Ao tocar a pele do hospedeiro, as cercarias fixam fortemente, sendo estimuladas pelo calor e por sinais de L-arginina, e permanecem em contato duradouro com o hospedeiro, em reposta a lipídios de superfície e calor. Seguem então, gradientes de temperatura e de concentração de arginina em busca de sítios adequados para realizar a penetração nas camadas mais profundas da pele. Ocorre mudança de cercaria para esquistossômulo de pele, esquistossômulo pulmonar, verme imaturo e verme adulto macho ou fêmea. (CARVALHO et al., 2018).

Entre um e três horas após a penetração das cercarias na pele do hospedeiro, ocorre uma série de adaptações bioquímicas e morfológicas específicas que preparam o

parasito para sobreviver ao novo ambiente. Poucas dessas mudanças estão associadas à alteração na expressão gênica, exceto para um aparente aumento na síntese proteica. A membrana plasmática do helminto absorve uma variedade de proteínas do hospedeiro mascarando o estado de reconhecimento dos esquistossomos pelo sistema imunológico. A saída dos esquistossômulos da derme pode ocorrer pelo sistema nervoso venoso, capilares venosos dilatados ou por linfáticos. Após a forma de esquistossômulos, os vermes evoluem para a fase adulta. As fêmeas maduras passam a depositar ovos, que ao saírem do hospedeiro, através das fezes, liberam miracídeos, que infectam o hospedeiro intermediário, mantendo o ciclo biológico (GIERA et al., 2018).

Para que o ciclo de vida complexo do parasita seja completado é necessário adaptações aos diferentes ambientes e hospedeiros. Estudos indicam que as mudanças epigenéticas estejam envolvidas nessas adaptações e possibilita alterar os fenótipos em vários pontos-chaves do ciclo de vida e de evitar as defesas do hospedeiro. As alterações globais nos epialelos ou alterações no número relativo de células com diferentes estruturas de cromatina no indivíduo podem ser refletidas pelas alterações epigenéticas ocorridas durante o desenvolvimento de parasitas multicelulares (ROQUIS et al., 2018).

Um estudo verificou que a estrutura da cromatina muda nas diferentes fases do ciclo de vida. Nos estágios de cercaria e verme adulto continham a trimetilação na lisina 4 da histona H3 (H3K4me3) e trimetilação na lisina 27 da histona H3 (H3K27me3). No entanto, nas cercarias essas marcas podem aparecer simultaneamente e podem estar associadas ao estado de transcrição inativo das cercarias. Quando a marca repressiva H3K27me3 foi removida nos esquistossomos ocorreu a ativação da transcrição (ROQUIS et al., 2015).

2.2 Epigenética

No contexto da regulação da expressão por modificação das histonas e do DNA, algumas descobertas importantes nos últimos anos foram realizadas em *S. mansoni*. Foram descritas proteínas que são capazes de atuar na regulação da transcrição modificando as histonas e metilando o DNA. As histonas H1, H2A, H2B, H3 e H4 estão preditas no banco de dados do parasito e as proteínas *SmCBP1* e *SmCBP2* apresentam-se diferencialmente expressas durante o ciclo e modificam principalmente histonas H4 (GEYER et al 2011).

As histonas desacetilases (HDAC) formam uma família de enzimas conservadas que controlam a expressão gênica por meio da remoção de resíduos de acetil dehistonase e são proteínas que estão sob crescente investigação como alvos terapêuticos, notadamente em câncer e doenças parasitárias. Os mRNAs de *SmHDAC1*, 3 e 8 são expressos em todos os estágios do ciclo de vida do esquistossomo, sugerindo ser altamente regulada (OGER et al., 2008).

A marca H3K4me3 está associada a promotores e locais de início de transcrição de genes transcricionais e é geralmente considerada um ativador da transcrição e em *S. mansoni*. Está preferencialmente distribuída em locais de início da transcrição de genes, similar a metazoários (SOARES et al., 2017). Na análise do epigenoma esta marca é estável, no entanto, quando se considera apenas os locais de início da transcrição ocorre uma redução significativa em cercarias, o que caracteriza uma cromatina em estado silenciado, mas pronta para o início da transcrição (poised) (COSSEAU et al., 2017; ROQUIS et al., 2015). Cercarias e adultos contêm marcas características da cromatina, H3K4me3 e H3K27me3, no entanto, em cercarias essas marcas estão presentes simultaneamente, sendo que essas marcas bivalentes estão associadas ao estado de transcrição inativa, e assim que a marca repressiva H3K27me3 é removida e a transcrição é ativada em esquistossômulos (ROQUIS et al., 2018).

As mudanças na cromatina durante o ciclo de vida indicam que a metilação de histonas exerce importante papel no desenvolvimento (ROQUIS et al, 2015 e 2018). A monometilação na lisina 20 da histona H4 (H4K20me1) está associada ao desenvolvimento e diferenciação em fêmeas adultas (ROQUIS et al., 2018). Além disso, tanto essa marca quanto a H3K27me3, estão relacionadas a maior parte do tempo a cercarias fêmeas e compreendem genes relacionados ao desenvolvimento e regulação da transcrição como revisado em Liu e colaboradores (LIU, 2016).

Os co-reguladores transcricionais são necessários para manter um estado silenciado e/ou ativado, de expressão gênica. Esses co-reguladores podem formar complexos de proteínas, que recrutam proteínas que se ligam ao DNA da cromatina e silenciam ou ativam determinadas porções do DNA. Em *S. mansoni*, algumas evidências mostram que as modificações pós-traducionais de histonas sozinhas, não são capazes de regular a expressão gênica do parasita, portanto são necessários outros co-reguladores de expressão, como os fatores de transcrição e remodeladores de cromatina que participam desse processo e inclusive formam complexos de proteínas que regulam a transcrição. Mas apesar de os co-reguladores de histonas serem importantes no

desenvolvimento do parasita, ainda temos poucas evidências bioquímicas da sua atividade. Neste sentido, em *S. mansoni* foi identificado o fator de transcrição *SmYB1*, homólogo às proteínas ligantes de Y-box (YBPs) ao motivo CCAAT no DNA dos promotores dos genes, e também ao RNA regulando a expressão gênica (VALADÃO et al., 2002). Outro estudo muito elegante, através de experimentos de interações in vitro mostrou que a arginina metiltransferase (PRMT1), é capaz de interagir fisicamente com o coativador da transcrição SRC-1 e também com receptores nucleares, formando um complexo de proteínas que se ligam aos promotores dos genes (MANSURE et al., 2005). Em *S. mansoni* também foi caracterizado o gene *SmZF1* que codifica um receptor nuclear homólogo ao membro da família gênica FTZ-F1, que são proteínas como motivos dedos de zinco que são muito conservadas em eucariotos em geral e estão relacionadas com regulação transcricional (GHALEB et al., 2008; WANG et al., 2008). Através de imunohistoquímica, foi demonstrado que a proteína se localiza no núcleo de vermes adultos, cercarias e esquistossômulos e após transfecção do gene *SmZF-1* em células COS-7, a proteína *SmZF-1* foi capaz de se ligar ao DNA e ativar a transcrição. Além do mais, pode ter função sexo específica, pois a proteína não foi detectada em parasitas fêmeas, embora o mRNA foi detectado em ambos os estágios (DRUMMOND et al., 2009; DE MENDONÇA et al., 2002; FANTAPPIÉ et al., 2008). Outro achado interessante foi a identificação no parasita de uma proteína homóloga de *Drosophila*, GCN5, que contém o domínio histona acetiltransferase. Essa proteína acetila as histonas H3 e H2A, com efeitos na remodelação da cromatina e se ligando a receptores nucleares. Assim, GCN5 de *S. mansoni* pode atuar como cofator na regulação gênica, relacionando a acetilação de histonas e o controle da transcrição gênica (MACIEL et al., 2004).

2.3 Proteína HP1

HP1 é um coregulador e está envolvida no estabelecimento e manutenção de estruturas de cromatina de ordem superior, reconhece e se liga especificamente a histona H3K9 e H3K27 que geram marcas distintas na heterocromatina. Além disso, é um componente estável de telômeros e possui três diferentes funções: formação e silenciamento dos genes da heterocromatina, cobertura e silenciamento de telômeros e regulação positiva de expressão gênica (ESKELAND et al., 2007; WANG et al., 2016)

Esta proteína foi identificada pela primeira vez em *Drosophila* como supressor de efeitos de variação de posição (PEV) no silenciamento do gene da heterocromatina. A família HP1 está envolvida no estabelecimento e manutenção de estruturas de cromatina de ordem superior. O HP1 reconhece e se liga especificamente a histona H3K9 e H3K27 que geram marcas distintas na heterocromatina. Além disso, é um componente estável de telômeros e possui três diferentes funções: formação e silenciamento do gene da heterocromatina, cobertura e silenciamento de telômeros e regulação positiva de expressão gênica. Recentemente, HP1 γ e H3K9me3 de mamífero foram encontrados desempenhando um papel inesperado na ativação transcrição dependente da associação com alongamento da RNA Polimerase II (MICHAELA et al., 2010). Em *Arabidopsis* a metilação das histonas pode regular a metilação de DNA através da proteína HP1 (RANDO, 2012; WANG; PATEL, 2011).

As proteínas HP1 consistem em dois domínios altamente conservados, um cromodomínio N-terminal (CD) necessário para a ligação da cromatina e um domínio cromoshadow C-terminal (CSD) que medeia a interação proteína-proteína. Estes dois são separados por uma região de comprimento variável que participa em diversas interações incluindo ligação com histona H1, ligação não específica de DNA e RNA, bem como ligação de cromatina (PIACENTINI et al, 2003).

O CD de HP1 reconhece e se liga especificamente à histona metilada H3K9. Essa interação é importante para o recrutamento de HP1 para regiões heterocromáticas do genoma. Além disso, a perda da metiltransferase H3K9 resulta na deslocalização do HP1. Como esta modificação é encontrada principalmente na cromatina silenciada, o HP1 tem sido considerado uma proteína silenciadora (STEWART; AASLAND, 1995).

O CSD é necessário para a dimerização do HP1 e interação com muitas proteínas que possuem um motivo penta-peptídico conservado PxVxL. O CSD de HP1 tem sido implicado numa ampla variedade de interações proteína-proteína com fatores que incluem componentes de cromatina, reguladores de transcrição, modificadores de cromatina, fatores relacionados com o ciclo de replicação celular, e proteínas nucleares. Suspeita-se que o CSD possa ser responsável pelo direcionamento contínuo de HP1 à heterocromatina na ausência de H3K9me (BRASHER et al., 2000).

Apesar do avanço do conhecimento de proteínas relacionadas ao silenciamento gênico em *S. mansoni*, como por exemplo, as proteínas que participam de vias de silenciamento pós-transcricional (KRAUTZ-PETERSON, SKELLY, 2008; GOMES et al., 2009), e vias de modificação pós-traducional que putativamente controlam a

expressão gênica no parasita, ainda pouco se conhece sobre os reguladores transcricionais que participam da manutenção do silenciamento de genes como a proteína HP1 neste parasito.

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CAPÍTULO 2

Evidences for Heterochromatin protein 1 (HP1) in life cycle and candidates for interaction with nuclear proteins in *Schistosoma mansoni* cercariae

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Abstract

Schistosomiasis is a disease caused by *Schistosoma* parasites. It is estimated that 240 million people in the world are infected by the parasite and about 700 million people are living in risk of infection. It is well-known that is necessary two hosts for parasite's life cycle completion, a vertebrate and an invertebrate host. Moreover, due to extreme changes in its morphology during life cycle, some studies have shown that reversible alterations on the epigenome is necessary for adaptation in different hosts and environments. Posttranslational histones modifications are epigenetic mechanisms, that are associated with silent or active states of chromatin, regulating gene expression at the transcriptional level. Although importance of histones PTMs, several studies have shown that transcriptional co-regulators are also important for regulation of gene

expression. The Heterochromatin Protein 1 (HP1) is well-known conserved co-regulator in the evolution and its importance were already described in mammals, yeast and protozoa, such as *Plasmodium falciparum*, but until date, none were described about the existence of this protein and its roles as transcriptional co-regulator in *Schistosoma mansoni*. In this work we have shown that HP1 is annotated in the parasite databank and its primary amino acids structure is conserved through organisms. SmHP1 mRNA expression were analyzed during life cycle, and it was evidenced a higher expression in cercaria when compared with other stages, however, the expression of protein is not altered in all tested stages. Moreover, Western blotting analysis has shown that in the parasite life cycle HP1 can form dimers of 56kDa in solution. Immunoprecipitation using anti-HP1 antibody followed by mass spectrometry, showed that several protein targets involved in transcriptional regulation can interact with HP1 in cercariae stage, but the roles in maintenance of repressive transcriptional states are not still elucidated.

Introduction

Schistosomiasis is the second parasitic disease in the tropical and subtropical areas of the world, caused by the parasite *Schistosoma mansoni* (Pinto-Almeida et al 2016). According to the World Health Organization (WHO), 240 million people worldwide are infected, and more than 700 million people live in endemic areas. It is estimated that in Brazil about 1.5 million people live in areas at risk of contracting the disease (Ministry Of Health, 2017). Being a trematode, the *S. mansoni* life cycle includes an intermediate and definitive host. Due to extensive changes in its morphology during its life cycle, and passage through two different hosts, it is believed that reversible changes in the epigenome of the parasite, may regulate the processes of

development, differentiation and coordinate the marked morphological changes that occur.

Epigenetic mechanisms are associated with reversible regulation of gene expression in various organisms. In *S. mansoni*, histone methylation marks H3K27me3 and H3K4me3 are associated with parasite life cycle progression (Roquis et al 2015, 2018), but there are still few reports related to expression and function of co-regulators in *S. mansoni* related to regulation of expression in the cycle. An important point in the development of *S. mansoni*, is concerned with transcriptional regulation in cercariae. The cercariae stage is transcriptionally silent, with silencing mechanisms regulated by epigenetic modifications (Dillon et al 2006, Roquis et al 2015). Although the epigenetic marks are well-known in cercariae silencing processes until now nothing is elucidated about other proteins, such as HP1 (Heterochromatin Protein 1) in cercariae transcriptional silencing and heterochromatin formation.

Transcriptional co-regulators are required to maintain a repressed and/or activated state of gene expression. These can form protein complexes that recruit proteins that bind to chromatin DNA and silence or activate certain portions of the DNA. In *S. mansoni* the transcription factor SMYB1 was identified, homologous to the Y-box binding proteins (YBPs) that binds to the CCAAT motif in the gene DNA promoters, and also regulating mRNA gene expression (Valadão et al., 2002). Another study using *in vitro* interaction experiments showed that arginine methyltransferase (PRMT1) can physically interact with the SRC-1 transcription coactivator, forming a complex of promoter-binding proteins in the genes (Mansure et al., 2005). Other study characterized the gene SmZF1 that encodes a nuclear receptor homologous to the member of the FTZ-F1 gene family, which are zinc finger motif proteins that are very conserved in eukaryotes in general and are related to transcriptional regulation (Ghaleb

et al. al., 2008; Wang et al., 2008). Through immunohistochemistry, it has been shown that the protein is localized in the nucleus of adult worms, cercariae and schistosomes and after transfection of the SmZF-1 gene into COS-7 cells, it was found that SmZF-1 protein was able to bind to DNA and activate the transcription. Moreover, it may have a sex-specific function because the protein was not detected in female parasites, although mRNA was detected in both stages (Drummond et al, 2009; De Mendonça et al., 2002; Fantappi  et al., 2008). Another interesting finding was the parasite identification of a homologous *Drosophila protein*, GCN5, which contains the histone acetyltransferase domain. This protein acetylates histones H3 and H2A, with effects on chromatin remodeling and binding to nuclear receptors. Thus, *S. mansoni* GCN5 can act as a cofactor in gene regulation, relating histone acetylation and gene transcription control (Maciel et al., 2004).

In this context, HP1 is a co-regulator conserved during evolution and of paramount importance for maintaining silenced or heterochromatic structures by binding to H3K9me3 histones marks, and there is yet no evidence of its role as co-regulator *in S. mansoni*. HP1 is involved in the establishment and maintenance of higher-order chromatin structures, specifically recognizes and binds to histone H3K9 and H3K27 that generate distinct marks on heterochromatin. Besides, it is a stable component of telomeres and has three different functions: heterochromatin gene formation and silencing, telomer coverage and silencing, and positive regulation of gene expression (Eskeland et al., 2007; Wang et al., 2016).

In parasites such as *Plasmodium falciparum*, PfHP1 protein is related to gene expression control mechanisms. PfHP1 specifically recognizes H3K9me2 and H3K9me3 histone modifications by chromodomain and the affinity of this protein appears to be higher for trimethylated peptide than for di-methylated peptide,

suggesting that the degree of methylation in histone H3K9 may lead to the formation of heterochromatin structures. (Perez-Toledo et al., 2009). Due to its tertiary structure, PfHP1 can form dimers in solution, which is consistent with the formation and maintenance of heterochromatin, like its orthologs in *Schizosaccharomyces pombe*, *Drosophila melanogaster* and *Homo sapiens* (Pérez-Toledo et al. , 2009).

For this, in this work we were attempted to characterize for the first time, the amino acid primary structure of SmHP1 sequence deposited in the databank and the similarity of SmHP1 sequence to orthologous. Specific oligonucleotides using the sequence Smp_179650 was designed for evaluation of mRNA expression in the life cycle using qRT-PCR. Protein expression in the stages was also evaluated by Western blotting. Immunoprecipitation assay was done using the anti-HP1 antibody in the nuclear cercarial protein extract followed by mass-spectrometry analysis, to identify possible HP1 nuclear interactors in cercariae stage.

Materials and Methods

Retrieving of SmHP1 amino acids sequences and phylogenetic analysis

The amino acids SmHP1 sequence was annotated in the *S. mansoni* databank (<https://schistodb.net/schisto/>) under access number Smp_179650. Orthologous amino acid sequences were chosen after extensive literary research and retrieved sequences from NCBI databank (<http://www.ncbi.nlm.nih.gov/>). The predicted deposited in the *Schistosoma mansoni* (SchistoDB) parasite database (<http://schistodb.net/schisto/>) (Berriman et al., 2009; Protasio, 2012) was used to search for putative HP1 proteins. Afterward, the BLASTp (Basic Local Alignment Search Tool - Protein) tool present on the site (<http://www.ncbi.nlm.nih.gov/blast>) (Johson et al., 2008) was used for the

search of orthologous organisms and identity and e-value determination. Conserved domains were analyzed by the databank *Conserved domain database* (CDD) (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) (Marchler-Bauer et al., 2015). For the conservation analyses of domains, active sites and structure evaluation, putative protein sequence, we used the multiple alignment by ClustalX 2.1 and ClustalW (Larkin et al., 2007). The proteins sequences logos were originated from WebLogo 2.8.2 (<http://weblogo.berkeley.edu/logo.cgi>) (Crooks et al., 2004).

The conserved protein domains were recovered separately to verify, through multiple alignment, the presence of important amino acid residues. In addition, the PFAM family protein database (<http://pfam.xfam.org/>) (Finn et al., 2016) was used to find conserved domains and their putative functions.

The phylogenetic analysis was performed using MEGAX program and the neighbour-joining method for putative proteins (Kumar et al., 2018; Saitou; Nei, 1987). The putative proteins evolutionary distance was calculated using the Jones-Taylor-Thornton (JTT) model (Jones; Thornton, 1992). A consensus tree was obtained using a bootstrap of 1000 replicates for the proteins, representing the evolutionary history of the analysed sequence group.

Parasites

The Belo Horizonte (BH) strain of the *S. mansoni* biological cycle is routinely maintained in the Department of Animal Biology of the Institute of Biology of the State University of Campinas, by passing Swiss female mice and mollusks of the species *Biomphalaria glabrata*. The cercariae obtention was performed by exposing 30 infected snails to artificial light for 1 hour. After the period of light exposure, the cercariae were collected and mice infection was performed by immersion of the animal tail in the

solution containing cercariae. After 7 weeks we obtained adult worms from mice perfusion and parasite's eggs from feces was obtained for snail infection for life cycle maintenance. Schistosomula was obtained from mechanically transformed cercariae (Harrop & Wilson 2003). Briefly, cercariae were collected and maintained in 50 ml Falcon tubes and kept for 30 minutes on ice. Then centrifuged at 2000rpm, 4 ° C for 2 minutes. The supernatant was discarded, and the pellet was washed with penicillin-streptomycin RPMI medium. Then the material was homogenized on vortex for one and a half minutes to break the tails and waited 10 minutes to decant the schistosomula. The tails were removed from the medium by culture medium by washing in RPMI medium and were transferred to a culture bottle and kept for 3 hours in the CO₂ environment. Afterward, tails were removed and the schistosomula was transferred to culture bottles with M-169 medium, the final volume of 15ml and maintained in CO₂ environment for 24h and 3 days. The experiments were done in biological triplicate for qRT-PCR and duplicate for Western blotting. Ethical approval commission of Institute of Biology of University of Campinas for animal maintenance is CEUA protocol (4799-1/2018).

Nuclear protein extraction and western blotting

Parasites were prepared according to previously described (Pereira et al., 2018). Briefly, samples were homogenized in 1 ml of lysis buffer (1x PBS Saline Phosphate Buffer, 0.5% Triton X-100, 0.02% NaN₃) with 1x protease inhibitor (Mini Inhibitor Cocktail, Complete Roche) was added to the parasites. Further were homogenized in dounce glass potter to separate nucleus and cytoplasm. It was then centrifuged at 2000rpm for 15 minutes at 4 ° C. Supernatant was removed and the nuclear pellet was resuspended in 1x lysis buffer. The supernatant solution was sonicated for 10 minutes, with intervals of 5 seconds on and off, with amplitude 20%. The parasites were then centrifuged for 20 minutes at 13000xg at 4 ° C and the supernatant transferred to a clean tube and stored at -80 ° C until use, as well as the pellet. Protein content was determined using Bradford assay reagent (Biorad). Western blotting was performed using 1:500 anti-HP1 antibody human recombinant Anti-HP1 alpha antibody [EPR5777] (Abcam), and 1:1000 Histone H3 (Anti-Histone H3 antibody [EPR16987]) as the loading control

RNA extraction and quantitative real-time PCR

Total RNA from cercariae, adult worms, schistosomula 24h and 3 days were extracted by Trizol reagent (Sigma) following manufacturer instructions. Briefly, 750µl of Trizol was added to the pellet of parasites and homogenized with the polytron. To the mixture was added 200 µl chloroform and homogenized for 1 minute, incubated at room temperature for 15 minutes and centrifuged for 15 minutes at 4 ° C at 12000 rpm. The aqueous phase was transferred to a new tube and 500 µl of 2-propanol added. Precipitation occurred on ice for 1 hour. Sample was centrifuged for 45 minutes at 12000 rpm at 4 ° C and discarded the supernatant. The pellet was washed with 75% EtOH and centrifuged for 5 minutes at 12000 rpm at 4 ° C. The pellet was air-dried for 2 minutes and resuspended in 20 µl RNase free water. Then the sample was heated for

15 minutes at 56 ° C in dry bath. The sample was quantified in the 260nm range in Nanodrop (Thermofisher). Total RNA was treated with DNaseI (Sigma) following manufacturer instructions. Reverse transcriptase was followed by the protocol of iSCRIPT kit (Biorad). Real-time PCR was performed using iTAQ Sybr green kit (Biorad), following manufacturer instructions.

Immunoprecipitation and mass spectrometry sample preparation

Immunoprecipitation was performed using Abcam protocol (www.abcam.com). Briefly, cercariae lysates in biological triplicate were prepared with the addition of 1mL of lysis buffer (20mM Tris-HCl pH8,0; 137mM NaCl; 1%Triton x-100; 2mM EDTA) and homogenized in electric potter, and then was maintained in constant agitation in an orbital shaker for 2h/4°C. After that sample was centrifuged for 20 min at 12000 rpm at 4°C and supernatant was recovered. The lysate was pre-cleared, and immunoprecipitation was performed with 1:50 anti-HP1 antibody for 12h at 4°C under gentle agitation. After that, it was added 100 µL of Protein A-sepharose slurry (Sigma) and incubated under agitation for 4h at 4°C. Beads were washed in lysis buffer and eluted in 1x SDS loading buffer and proceeded electrophoresis in SDS gel 12%. Seven bands were excised from the gel (Fractions 1 to 7) and digested in-gel with trypsin sequencing grade (Promega V5111).

Mass spectrometry and data analysis

The dried samples were dissolved in 10 µL of 1% acetonitrile solution and each was chromatographed in the Waters Acquity UPLC I-class by three times with a gradient of 2 - 90% acetonitrile in 45 minutes using 0.1% formic acid as modifier. The raw were acquired in the Waters Xevo TQ-S mass spectrometer in survey mode with masses for

parent peaks ranging from 200 - 1800 and for child peaks 200 - 1600 with cone voltage of 50 V and collision energy of 20 eV. The files were converted to Mzxml format using the masswolf software and the analysis was performed on the LabKey server platform with the Comet peptide search engine, where the FASTA protein sequence (www.uniprot.org/schistosoma), trypsin digestion. Carbamidomethyl were inserted as fixed modification and oxidation of methionine and tryptophan were inserted as variable modifications.

Results

HP1: alignment and analysis of conserved protein domains and phylogenetic tree

To verify the conservation of HP1 protein between organisms, a local alignment of the deposited sequence (Smp_179650) on Clustal X2 was performed. Domain alignment, presence, and conservation were used to select the 17 proteins that underlie this study. In bold is highlighted the *S. mansoni* HP1 protein (SmHP1), in addition to the two conserved domains between species (Figure 1).

The two conserved domains, chromo and chromoshadow, were found in the studied organisms and presented important conserved amino acid residues in certain positions. Threonine at position 51 of the chromodomain and the hydrophobic amino acids tyrosine, valine and tryptophan in the chromoshadow domain that forms the hydrophobic groove responsible for the formation of hetero or homodimers (Figure 2). Protein identification sequence was confirmed based on the presence of the sequence on data bank and essential protein domains, thus a phylogenetic analysis was performed with the amino acid sequence of the domains. The sequences of the *S. mansoni* domains

were aligned with orthologous species previously selected by Clustal X2 alignment (Figure 2). It was possible to verify the separation of the two domains into separate clades (Figure 3).

SmHP1 mRNA and protein expression and dimer/monomer formation

Specific oligonucleotides were designed for mRNA expression evaluation in life cycle. We investigated mRNA expression in cercariae, 24h schistosomula, 3 days schistosomula and adult worms (Figure 4). Our results have shown that mRNA for SmHP1 is more expressed in cercariae and the expression is statistically significant reduced from 24h schistosomula to adult worms, suggesting that expression in cercariae should be important for heterochromatin formation in maintenance of transcriptional silent state of cercariae parasite. Due its tertiary structure, it was previously described that HP1 can form dimers and/or monomers (Pérez-Toledo et al., 2009). The analysis of Western blotting using anti -HP1 antibody was also performed to test of the formation of dimers and monomers. For this, we extracted proteins and recovered both supernatant, that is supposed to contain soluble proteins, and the pellet that contains insoluble part of the proteins from extract. HP1 dimers are supposed to be formed when protein is soluble in the heterochromatin context. In the figure 5A, in left panel we show the expression of a band of HP1 proteins with a size of 56 kDa in cercariae, 24h and 3 days schistosomula and adult worms, suggesting the formation of a dimer of HP1 in solution. In the right panel, we show the result of the protein expression at the pellet, showing HP1 protein as a monomer form with a size of 28 kDa. The difference of the size of the protein bands suggest the ability of dimer formation in solution. In the middle of the panel we show anti H3 antibody as a constitutive loading. The expression

of the dimer form concerning H3 expression, in the life cycle is evidenced in the figure 5B, where is shown a non-statistically significant expression in the life cycle, suggesting that protein expression is not altered in the tested stages.

Putative HP1 protein interactors in cercariae

To address the question that raised if HP1 in *S. mansoni*, especially in transcriptional silent cercariae, can interact with nuclear proteins, we performed immunoprecipitation experiments with anti-HP1 antibody followed by mass spectrometry analysis. The search on Uniprot databank has shown that several proteins with PepProphet score between 0.5 and 1.0 (Keller et al 2002), are related to nuclear localization (Supplementary Tables 1 to 7). Some of them are suggested to play nuclear role as regulating gene expression as transcription factor with zinc finger motif (A0A3Q0KTQ1). Other protein identified as putative interactor is Cir_N domain-containing protein (G4V992) is related to present activity as transcriptional corepressor (Supplementary Table 1). Other interesting target is the Helicase (A0A3Q0KR71) that presents several roles as ATPase activity, ATP binding, histone binding, chromatin remodeling, regulation of transcription, DNA-templated. The GO component at Uniprot database indicates that Helicase is related to SWI/SNF complex (GO:0016514) of chromatin remodeling ATP-dependent (Supplemental Table 2/3). It is well described in *S. mansoni* chromatin biology that histone enzymes are key players into gene regulation in life cycle (Roquis et al 2015, Padalino et al 2018, Cabezas-Cruz et al 2014). In this work, we found some histone modifying enzymes such as the putative histone-lysine n-methyltransferase, setb1(A0A3Q0KPQ6) (Supplemental Table 3), the Histone-lysine N-methyltransferase (G4VJB5), characterized as histone-lysine N-methyltransferase

activity and metal ion binding (GO:0018024) (Supplemental Table 4). Another interesting target identified is the putative CCAAT-binding transcription factor (A0A3Q0KTK8) (Supplemental Table 4/6). This transcription factor binds to CCAAT motif, previously characterized as important for gene regulation in *S. mansoni* (Valadão et al 2002).

Discussion

The result of Clustal X2 alignment corroborates with the literature and shows high conservation of domains between species and variable regions linking the domains, both of which are responsible for essential protein functions. Since each domain has an essential function, the amino acids present in each domain are expected to play important roles. Given the conservation of protein during evolution, many functions are maintained among different organisms. It was previously reported that phosphorylation on threonine 51 (Thr51) in the chromodomain of the HP1 β is involved in DNA repair mechanism, by constraining the direction of HP1 to DNA repair sites mediated by suppression by casein kinase 2 activity (LeRoy et al., 2009).

Even considering that there is only one copy of the protein in *S. mansoni* we verified the conservation of Thr51, suggesting conservation of function. It is interesting to note, that function of regulatory chromatin interacting protein Ku70 and its repressive activity in transcriptional stretching areas is strongly dependent of HP1 γ phosphorylation in serine 83 (Ser83) (Lomberg et al, 2006). In concomitance with phosphorylation at Ser 83, in the next Ser95 residue, phosphorylation occurs at higher levels suggesting that this mark is important for establishing of HP1 γ occupancy in heterochromatin (LeRoy et al., 2009). Although we have no data about the importance

of Ser95, the residue is conserved in *S. mansoni* protein (Figure 2). To ensure that domain conservation exists between different species, we infer a phylogenetic analysis with domain sequences (Figure 3). In *S. mansoni* we found just only one copy of HP1 gene presenting the both chromodomain and chromoshadow(CSD), that probably may play a role in maintenance of repressive states on heterochromatin. In addition, it is possible to observe a separation between the same phyla, corroborating with the tree of life.

We performed Western blotting using anti-HP1 antibody to determine expression in the life cycle and to evaluate the possibility of dimer/monomer formation in solution. The HP1 protein has a molecular weight of approximately 28 kDa, however, this protein can form dimers (Trembecka-Lucas; Dobrucki, 2012) through the chromoshadow domain. Thus, when in dimer form the molecular weight is double, approximately 56 kDa. Dimer formation is important for targeting the protein in the absence of H3K9, and is important for recruiting other proteins that aid in proper functioning in chromatin context (Machida et al, 2018). Further, experiments involving mutation of isoleucine amino acid impairs dimer formation, HP1 α dinucleosome complexes formation and binding in H3K9me3 histone tail, reinforcing the importance of sequence conservation to function of HP1 protein (Machida et al, 2018).

Several interesting targets were detected by immunoprecipitation with HP1 antibody followed by mass spectrometry analysis in cercariae nuclear extract. This experiment was performed to determine which nuclear proteins were intended to interact with HP1 to form complexes to regulate gene expression, heterochromatin formation and repressed transcriptional state in cercariae stage. From analysis of our results listed in Supplemental tables 1 to 7, we found that HP1 in cercariae is suggested to interact with Helicase (A0A3Q0KR71) that GO analysis has shown that presents a

SWI-SNF domain. Other study has shown that *in vitro* studies using recombinant histones and human HP1 were shown that HP1 chromoshadow domain interacts with histones H3 in the entry/exit of nucleosome in contact with SWI-SNF catalytic subunit, inhibiting chromatin remodeling by presence of HP1, favoring HP1-mediated repression (Lavigne et al., 2009). Other interesting target supposed to interact with HP1 in cercariae is the protein Cir_N domain-containing protein (G4V992). The CIR protein is the well-recognized protein CBF1 interacting co-repressor (CIR) family of DNA binding transcription factors that play a central role in Notch signaling (Zhang et al., 2001). Besides, it was reported that CIR binds to GTGGGAA motifs in DNA and acts as a repressor linking to the histone deacetylase complex (Hsieh et al., 1999). Moreover, we found that HP1 in cercariae may be associated with Histone N-Methyltransferases. It well-recognized that tri-methylation of histone H3 in lysine 9 is important for HP1 recruitment to certain genome regions permitting gene expression regulation and chromatin package (Sims et al., 2003; Schotta et al., 2004). Finally, we found that CCAAT-binding transcription factor (A0A3Q0KTK8), was immunoprecipitated together with HP1 in cercariae. Other studies have shown that CCAAT/enhancer-binding protein alpha (C/EBP α) interacts directly with HP1 α (Demarco et al., 2006; Sun et al., 2010; Siegel et al., 2012). Moreover it was early reported in *S. mansoni*, that the protein Y-box binding protein (SMYB1) interacts with CCAAT motif and can bind to RNA and DNA (Valadao et al 2002). Although, immunoprecipitation and mass spectrometry enable an identification of cercariae transcriptional partners for regulating the chromatin package and transcriptional repression states, further studies are necessary to elucidate the roles of DNA motifs and chromatin binding proteins for formation of stable protein complexes *in vitro* and *in vivo* that play a role in regulating gene expression and establishment of repressive transcriptional complexes in *S.*

mansoni life cycle. Taken together, our results have shown sequence conservation, expression and dimer formation in life cycle and candidates for interacting partners of SmHP1 in cercariae nuclear extract.

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Author contributions:

FJC, ERM and MSG: designed study.

NST, GGC, GFC, TCSC, FJC performed research

APM, GPL, VF: performed mass spectrometry analysis

SMA: coordinated *Schistosoma* life cycle

FJC, NST: analyzed results

FJC, NST, ERM: wrote the manuscript

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List of Figures and Tables

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Mmu – *Mus musculus*, Rno – *Rattus norvegicus*, Hsa – *Homo sapiens*, Sma – *Schistosoma mansoni*, Dme – *Drosophila melanogaster*, Egr – *Echinococcus granulosus*, Nle – *Neodiprion lecontei*, Cfl – *Copidosoma floridanum*, Cca – *Ceratina calcarata*, Far – *Fopius arisanus*, Ovi – *Opisthorchis viverrini*

Figure 3 – Phylogenetic tree showing the division of binding conserved domains to DNA (chromodomain) and to chromatin (chromoshadow)

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Figure 5 – (A) Western blotting in the life cycle evidencing dimer formation of soluble protein extract (56 kDa dimer) and monomer form in the protein pellet (28kDa). (B) Densitometry of dimer in the tested stages: cercariae, 24h schistosomula (24h somula), 3 days schistosomula (3 days somula) and adult worms.

Supplemental Tables

Supplemental Table 1 – List of proteins of fraction 1 immunoprecipitated with anti-HP1 antibody and identified with mass spectrometry

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Supplemental Table 4 – List of proteins of fraction 4 immunoprecipitated with anti-HP1 antibody and identified with mass spectrometry

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Supplemental Table 7 – List of proteins of fraction 7 immunoprecipitated with anti-HP1 antibody and identified with mass spectrometry

Figures

Figure1

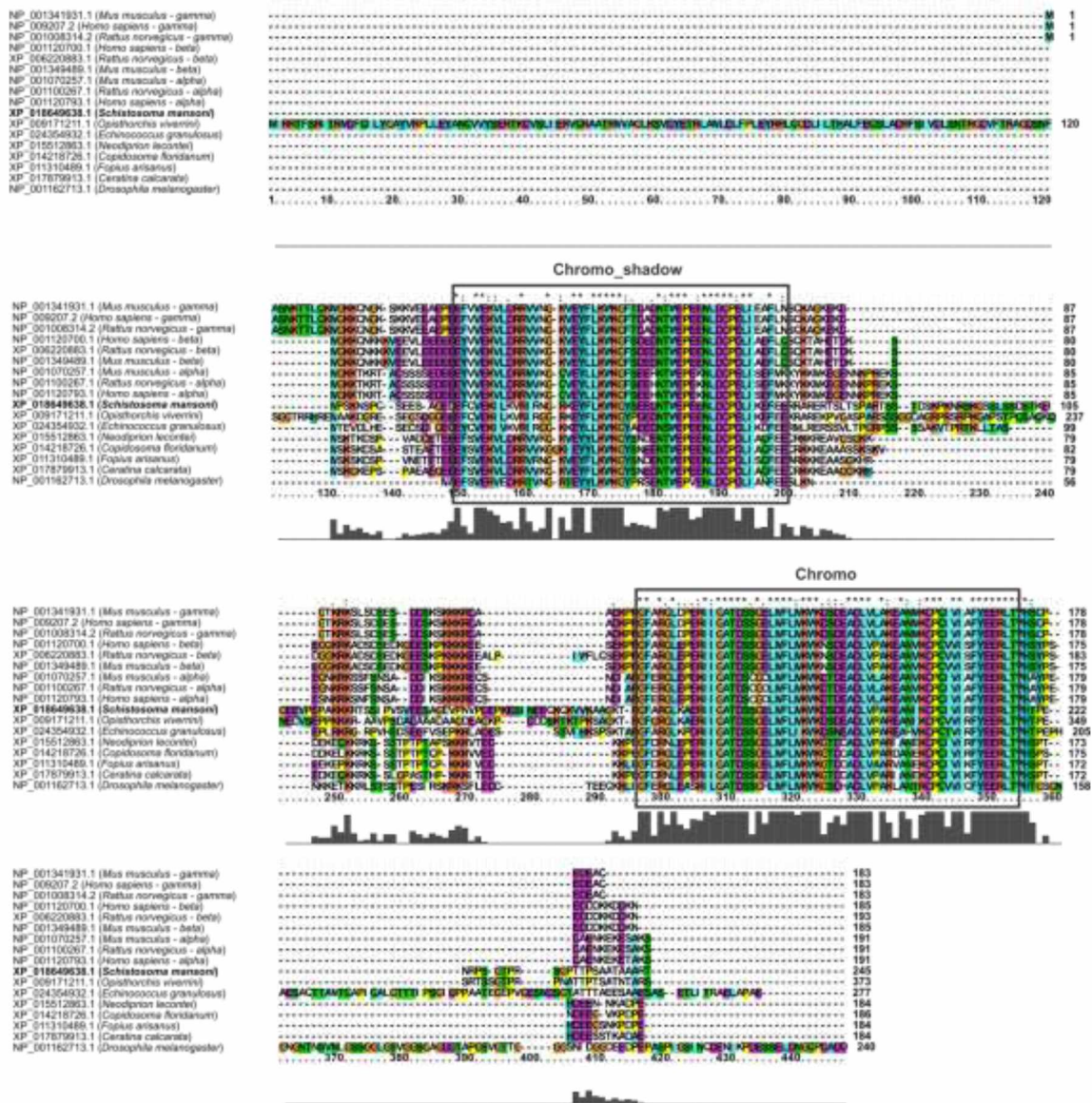
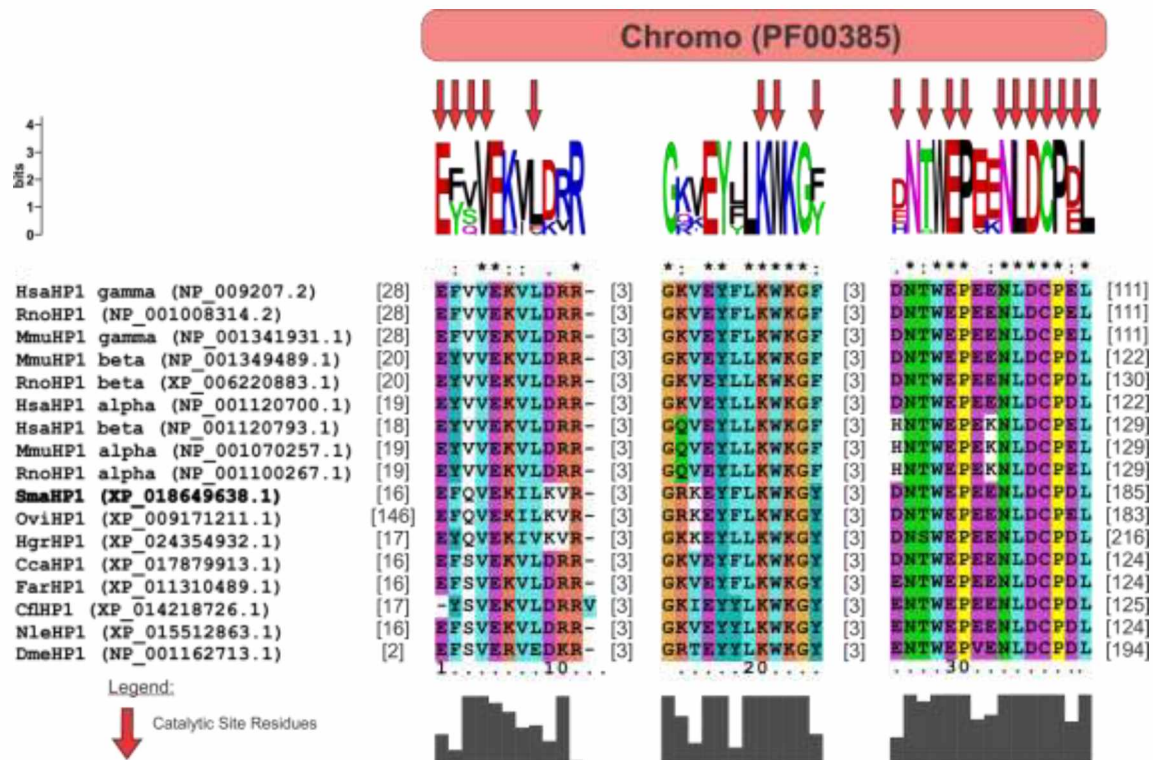


Figure 2

A



B

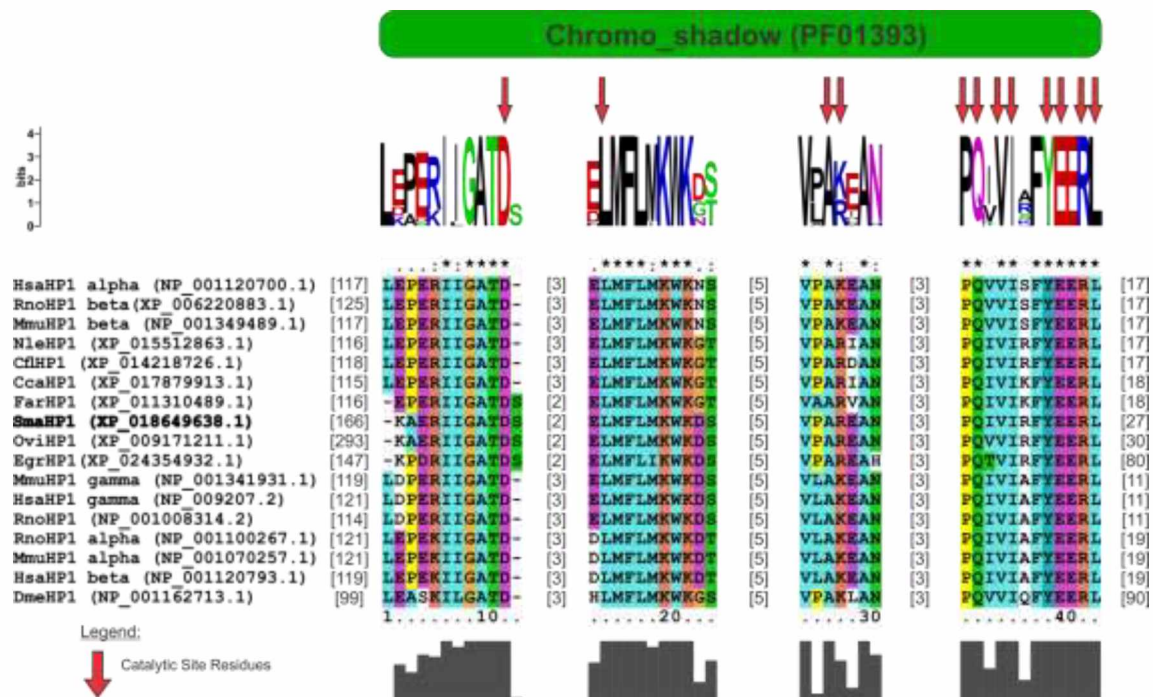


Figure 3

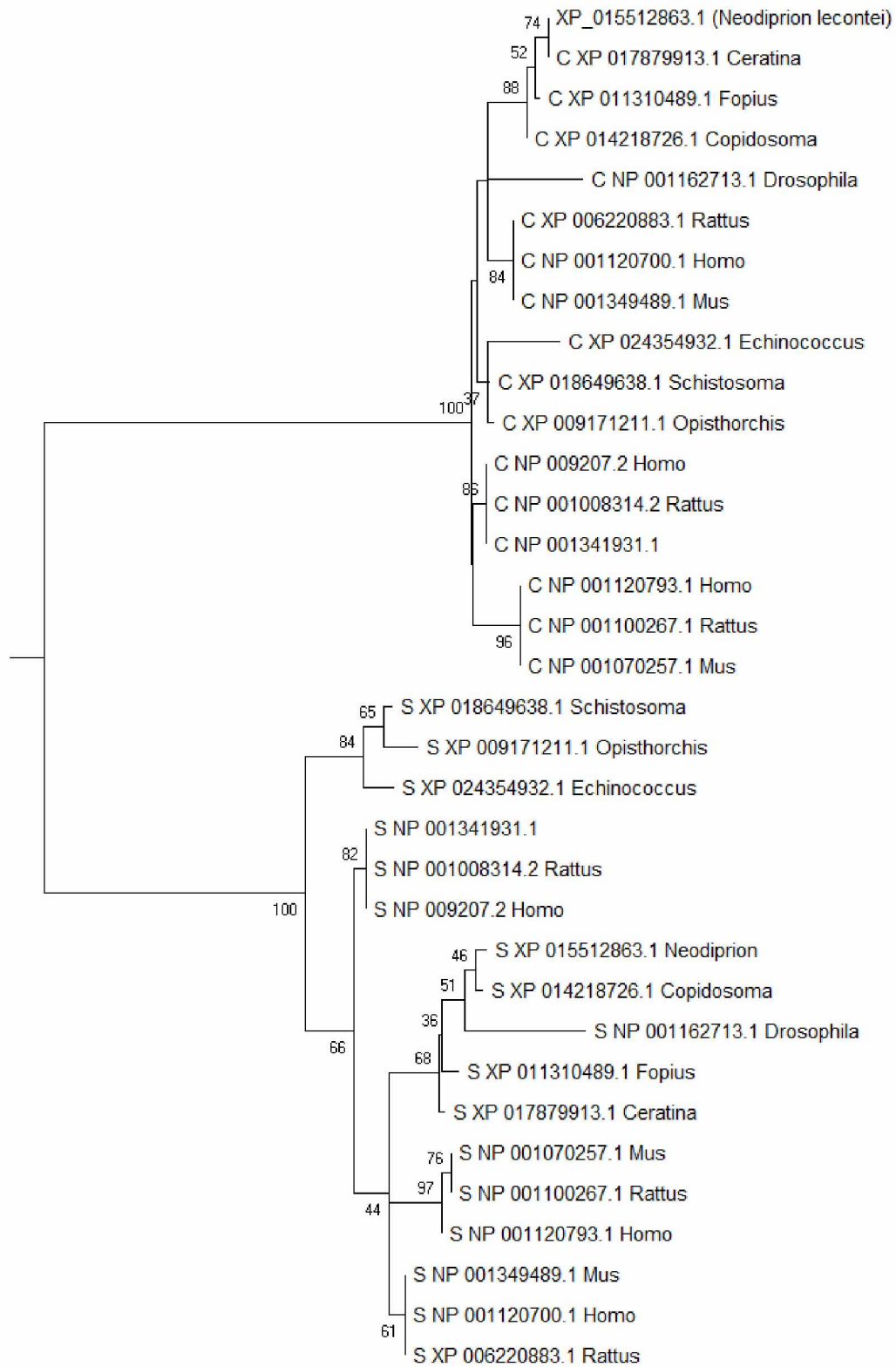


Figure 4

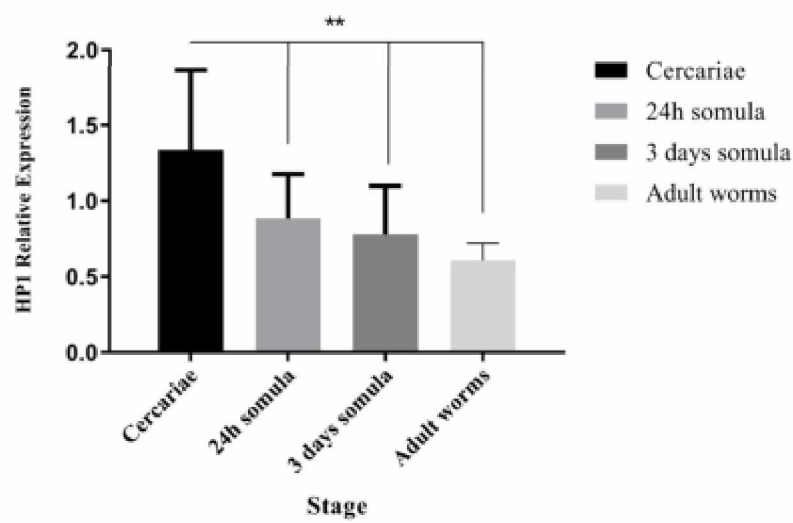
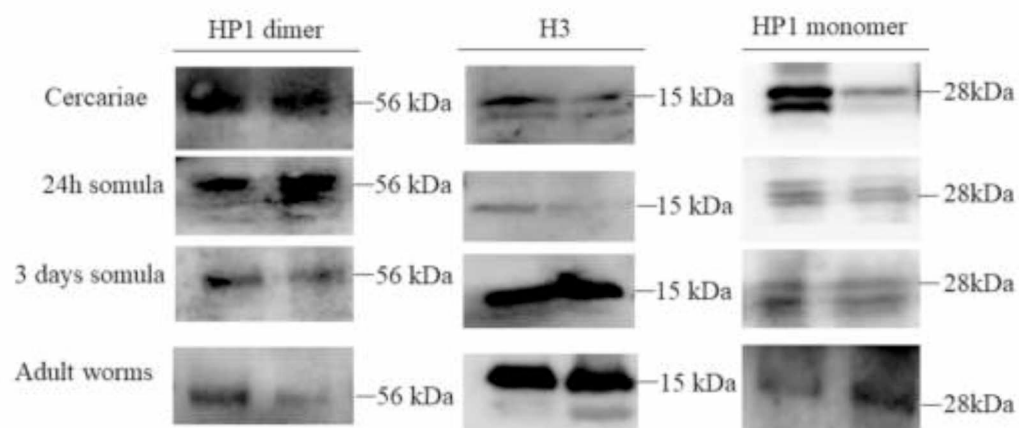
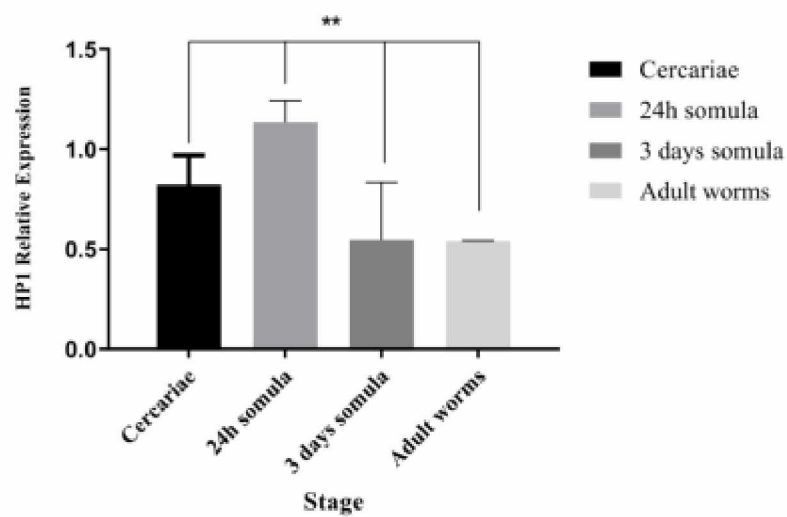


Figure 5

A



B



CONCLUSÃO

As análises demonstraram que a proteína HP1 está presente no parasito *S. mansoni*, nas diferentes fases do seu ciclo de vida, apesar de haver uma diminuição da expressão do mRNA de cercária até verme adulto.

A função exata que HP1 exerce no parasito não está completamente elucidada, mas os resultados apresentados sugerem que ela é um importante corregulador transcricional necessário nas diferentes etapas do ciclo de vida, sendo necessária na etapa de vida cercaria. Assim, mais estudos são necessários para compreender o papel dessa proteína durante o ciclo e sua correlação com o desenvolvimento do parasito e o estabelecimento da esquistossomose.

ANEXO 1 - Normas para publicação na revista “Parasites and Vectors”

Preparing main manuscript text

Quick points:

- Use double line spacing
- Include line and page numbering
- Use SI units: Please ensure that all special characters used are embedded in the text, otherwise they will be lost during conversion to PDF
- Do not use page breaks in your manuscript

Title page

The title page should:

- present a title that includes, if appropriate, the study design
- list the full names and institutional addresses for all authors
 - if a collaboration group should be listed as an author, please list the Group name as an author. If you would like the names of the individual members of the Group to be searchable through their individual PubMed records, please include this information in the “Acknowledgements” section in accordance with the instructions below
- indicate the corresponding author

Abstract

The Abstract should not exceed 350 words. Please minimize the use of abbreviations and do not cite references in the abstract. The abstract must include the following separate sections:

- Background: the context and purpose of the study
- Methods: how the study was performed and statistical tests used
- Results: the main findings
- Conclusions: a brief summary and potential implications

Keywords

Three to ten keywords representing the main content of the article.

Background

The Background section should explain the background to the study, its aims, a summary of the existing literature and why this study was necessary.

Methods

The methods section should include:

- the aim, design and setting of the study
- the characteristics of participants or description of materials
- a clear description of all processes, interventions and comparisons. Generic names should generally be used. When proprietary brands are used in research, include the brand names in parentheses
- the type of statistical analysis used, including a power calculation if appropriate

Results

This should include the findings of the study including, if appropriate, results of statistical analysis which must be included either in the text or as tables and figures.

Discussion

For research articles this section should discuss the implications of the findings in context of existing research and highlight limitations of the study. For study protocols and methodology manuscripts this section should include a discussion of any practical or operational issues involved in performing the study and any issues not covered in other sections.

Conclusions

This should state clearly the main conclusions and provide an explanation of the importance and relevance of the study to the field.

List of abbreviations

If abbreviations are used in the text they should be defined in the text at first use, and a list of abbreviations can be provided.

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The following word processor file formats are acceptable for the main manuscript document:

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- Rich text format (RTF)
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- Visiting the [English language tutorial](#) which covers the common mistakes when writing in English.
- Asking a colleague who is a native English speaker to review your manuscript for clarity.

Reference

Vancouver style

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When preparing figures, please follow the formatting instructions below.

- Figures should be numbered in the order they are first mentioned in the text, and uploaded in this order. Multi-panel figures (those with parts a, b, c, d etc.) should be submitted as a single composite file that contains all parts of the figure.
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- Figure titles (max 15 words) and legends (max 300 words) should be provided in the main manuscript, not in the graphic file.
- Figure keys should be incorporated into the graphic, not into the legend of the figure.
- Each figure should be closely cropped to minimize the amount of white space surrounding the illustration. Cropping figures improves accuracy when placing the figure in combination with other elements when the accepted manuscript is prepared for publication on our site. For more information on individual figure file formats, see our detailed instructions.
- Individual figure files should not exceed 10 MB. If a suitable format is chosen, this file size is adequate for extremely high quality figures.

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- Microsoft Word (suitable for diagrams and/or images, figures must be a single page)
- PowerPoint (suitable for diagrams and/or images, figures must be a single page)
- TIFF (suitable for images)
- JPEG (suitable for photographic images, less suitable for graphical images)

- PNG (suitable for images)
- BMP (suitable for images)
- CDX (ChemDraw - suitable for molecular structures)

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- width of 170 mm for full page width figure
- maximum height of 225 mm for figure and legend
- image resolution of approximately 300 dpi (dots per inch) at the final size

Figures should be designed such that all information, including text, is legible at these dimensions. All lines should be wider than 0.25 pt when constrained to standard figure widths. All fonts must be embedded.

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If additional material is provided, please list the following information in a separate section of the manuscript text:

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- File format including the correct file extension for example .pdf, .xls, .txt, .pptx (including name and a URL of an appropriate viewer if format is unusual)
- Title of data
- Description of data

Additional files should be named "Additional file 1" and so on and should be referenced explicitly by file name within the body of the article, e.g. 'An additional movie file shows this in more detail [see Additional file 1]'.