# Silencing of SmAgo2 increases abundance of retrotransposons in the parasitic flatworm *Schistosoma mansoni*

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## Abstract

Transposable elements (TEs) are mobile parts of the genome that can jump or self-replicate, posing a threat to the stability and integrity of the host genome. TEs are prevented from causing damage to the host genome by defense mechanisms such as epigenetic changes and through nuclear silencing of TE transcripts in an RNAi-like fashion. These pathways are well known in model organisms but very little is known about them in other species. Parasitic flatworms present an interesting opportunity to investigate evolutionary novelties in TE control because they lack canonical pathways identified in model organisms (such as the piRNA pathways, etc) but have conserved central players such as DICER and AGO (argonaute) enzymes. Notably, parasitic flatworm AGOs are phylogenetically distinct from classical AGOs, raising the question of whether they play special roles in these organisms. In this report, we investigate the role of one of these flatworm-specific AGOs, AGO2, in the parasitic flatworm *Schistosoma mansoni*. We show that transcript abundance of retrotransposable elements significantly increased upon silencing of SmAGO2. We further demonstrate that SmAGO2 protein is localised in the nucleus and the cytosol of adult worms. This is the first evidence of the presence of a nuclear silencing mechanism in schistosomes.

### Keywords

*Flatworm, retrotransposon, argonaute, Schistosoma, RNAi.*

## Introduction

Genome integrity is an essential aspect of the biology for all organisms. Active mobile genetic elements, also termed transposable elements (TEs), pose a threat to this integrity as they can insert themselves randomly in the host genome [1]. Organisms from plants to humans have developed elegant molecular mechanisms to regulate TE activity. These are broadly divided into epigenetics, base or histone modifications that occur at the locus where the TE is encoded, and silencing or more specifically nuclear silencing (NS), where transcripts arising from active TE genes are intercepted and degraded by 'host' mechanisms ultimately preventing their insertion in the 'host' genome.

These important control pathways are the subject of much research in model organisms and disease and, consequently, much is known about TE control and NS mechanisms in plants [2], fruit flies [3, 4], nematodes [5], mammals [6] and cancer [7, 8]. Notably, the mechanisms of NS vary greatly across the tree of life, indicating independent evolutionary origins [9, 10].

Small non-coding RNAs and long double stranded RNA (dsRNA) play a central role in most NS pathways. In animals, small RNAs (20-32 nucleotide) called piRNAs are generated from a long RNA precursor encoded in the host genome. They are expressed in the gonads and stem-cells and exported to the cytoplasm where they guide PIWI and argonaute (AGO) proteins towards the expressed TE to mediate silencing by enzymatic degradation. At the same time, the signal is amplified via a ping-pong mechanism producing a second generation of small RNAs. In some cases, PIWI loaded with small RNAs can also enter the nucleus and continue silencing. PIWI proteins guided by these small RNAs can cleave TEs in their RNA intermediate state. In other systems where piRNAs are absent, single stranded mRNAs from TEs are converted into dsRNA by an RNA-dependent RNA-polymerase (RDRP) and subsequently degraded by a DICER enzyme. Plants also have RDRP and DICER activity but the small RNAs produced, as well as targeting RNA species for degradation, drive DNA methylation and locus-specific formation of heterochromatin with the subsequent silencing of the targeted TE (reviewed in [2]).

Parasitic flatworms are a group of pathogens of clinical and veterinary relevance that cause some of the most prevalent and devastating infectious parasitic diseases affecting the poorest communities in the world [11, 12]. Ongoing work in the field of genomics has provided much insight into the genetic composition of these parasites. In particular, our current unprecedented knowledge of the genomic constitution of Schistosomes, blood flukes infecting $\sim$ 200 million people worldwide and responsible for $\sim$ 200,000 annual deaths, has revealed that TEs constitute a sizable proportion of the genome. Indeed, approximately 50% of the genome of all sequenced schistosome species, *S. haematobium* [13], *S. japonicum* [14] and *S. mansoni* [15] is covered by repetitive elements.

In *S. mansoni*, TEs account for almost 40% of the genome and some are transcriptionally active (Protasio *et al*, unpublished). Despite this fact, very little is known about how (and if) these parasites actively regulate TEs. Earlier studies revealed that schistosomes lack PIWI proteins [16] and RDRP activity while the presence or absence of targeted DNA methylation remains controversial [17, 18, 19, 20]. Bioinformatics and phylogenetic analyses of schistosomes genomes [16, 21] indicate that these parasites encode three AGO genes, namely AGO1 (SmAGO1, Smp_198380), AGO2 (SmAGO2, Smp_179320) and AGO3 (SmAGO3, Smp_102690) as well as a DICER (SmDCR1, Smp_169750) and a DICER-like gene (SmDCR2, Smp_033600). Phylogenetic studies revealed that SmAGO1 shares high homology to AGOs of other organisms where the primary function is to mediate the canonical RNA interference pathway in the cytosol. While SmAGO2 and SmAGO3 belong to a cluster of flatworm-specific AGO proteins (also called FLAGO), the presence of all the conserved domains that define an argonaute protein suggest that SmAGO2 and SmAGO3 have similar and possibly redundant functions.

Whole-mount *in situ* hybridization of SmAGO2 has located its expression in vitelline glands and testes of adult worms [22] as well as stem-cells [23]. In addition, Cai *et al.* [24] reported that immunoprecipitation of *S. japonimum* SjAGO2 yielded a significant enrichment of siRNAs with sequence identity to TEs. Taken together, these results suggest SmAGO2 may play a role in the maintenance of genome stability in highly proliferative cell lineages.

We hypothesise that flatworm-specific AGOs could have a role in nuclear silencing. Here we present evidence that SmAGO2 plays a role in regulating the abundance of retrotransposable elements in *S. mansoni*.

# Results

## Gene silencing of argonautes in *S. mansoni*.

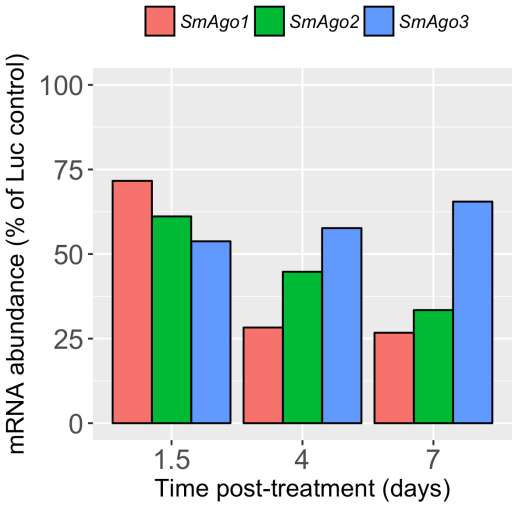


**Figure 1. Silencing of Argonaute genes in *S. mansoni*.** Time course experiment showing silencing of target genes, SmAgo1, SmAgo2 and SmAgo3 in adult *S. mansoni* males. Y-axis represents the percentage of transcripts measured by RT-qPCR in worms electroporated and incubated with dsRNA of the target genes with respect to the same target measured in the irrelevant dsRNA (firefly luciferase) control.

In order to determine an informative time point at which a successful knockdown of the target genes was achieved and apparent, a time course experiment for SmAgo1, SmAgo2 and SmAgo3 gene silencing was undertaken. Seven-week old adult *S. mansoni* males were transfected by square wave electroporation with dsRNA specific for SmAgo1, SmAgo2, SmAgo3, or an irrelevant control (firefly luciferase).

An RT-qPCR experiment confirmed the reduction of SmAgo1, SmAgo2 and SmAgo3 transcripts at 1.5, 4 and 7 days post electroporation **(Figure 1)**. We achieved more than 50% reduction for SmAgo1 and SmAgo2 transcripts at 4 and 7 days post treatment. Silencing of SmAgo3 was less efficient. Based on these results, we decided to focus on SmAgo1 and SmAgo2 and the effect of their silencing in the expression of retrotransposable elements.

## Increased abundance of retrotransposon transcripts following silencing of SmAgo2.

We wanted to investigate whether a reduction in the expression of SmAgo2 could have an effect in the abundance of retrotransposons transcripts. To investigate this possibility, we repeated the gene silencing experiment on SmAGO1 and SmAGO2 and we harvested the dsRNA-treated worms 4 days after electroporation.

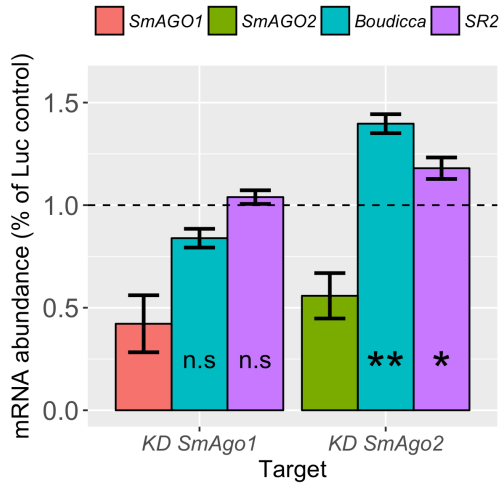


**Figure 2. Increase in retrotransposons transcripts in SmAgo2 knockdowns.** Relative abundance of retrotransposons Boudicca and SR2 in adult male *S. mansoni* worms upon silencing of SmAgo1 and SmAgo2 at 4 days after treatment. Error bars represent the standard error of the mean of two biological replicates. P-value ** $< 0.05$; * $< 0.1$; n.s., not significant.

We used real-time quantitative RT-qPCR to measure the abundance of transcripts from Boudicca [25] and SR2 [26] (LTR and non-LTR elements respectively), two well-described retroelements in the genomes of *S. mansoni* and *S .japonicum*. Our results show that there is a significant increase in abundance of both of the TEs assayed **(Figure 2)**.

The mean fold change increase in worms where SmAgo2 was silenced over control worms treated with an unrelated dsRNA was $1.4 \pm 0.05$ for Boudicca and $1.2 \pm 0.05$ for SR2. Both differences were significant, albeit with modest fold change increases (Boudicca: paired t-test p-value $< 0.05$; SR2: paired t-test p-value $< 0.1$; data collected from two biological replicates). No significant differences in abundance of retrotransposon transcripts were evident in SmAGO1 knockdowns.

These results suggested that SmAgo2, but not SmAgo1, has a significant effect in the abundance of retrotransposon transcripts.

## SmAGO2 protein is localised in the nucleus and cytoplasm in adult schistosomes.

We reasoned that if SmAGO2 plays a role in the silencing of retrotransposable elements, it would most likely do so from a location within the nucleus. To test this hypothesis, we used an antibody with reactivity against *S. japonicum* AGO2 [24] to map the subcellular localisation of SmAGO2 using immunohistochemistry **(Figure 3)** in 7 week-old male *S.mansoni* worms. We detected SmAGO2 protein either in the cell cytoplasm or in the cell nucleus but not in both locations within the same cell.

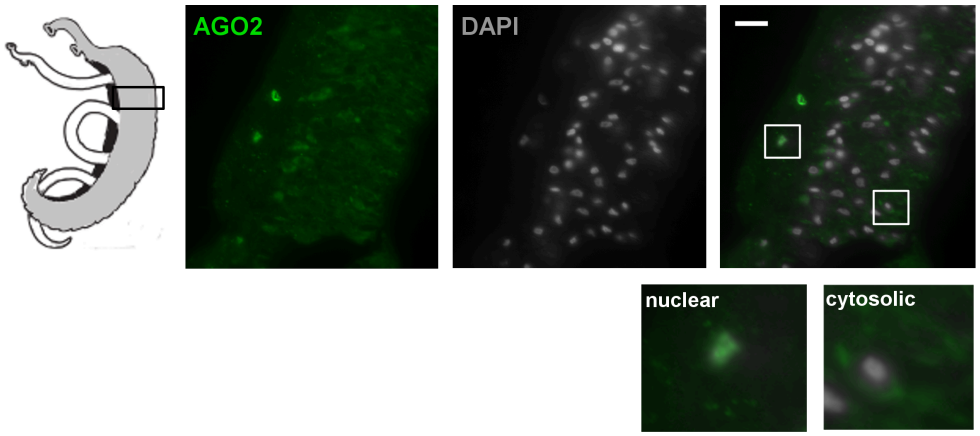


**Figure 3. The *S. mansoni* protein AGO2 is localised within the cytoplasm and the nucleus.** Using an antibody raised against SjAGO2 of *S. japonicum*, we were able to detect SmAGO2 protein in histological preparations of a male adult worm (black square, left). A section of the mid-body shows staining with SjAGO antibody (green) and DAPI (grey), which stains the cell nucleus. The merged image and the zoom-in boxes show that the antibody is detected in the nucleus and the cytosol. Scale bar represents 25 um.

# Discussion

Nuclear silencing (NS) is an essential cell function of all organisms. From bacteria to humans, NS controls the activity of transposable elements (TEs) in the cell nucleus. Pivotal to many NS pathways are the Argonaute (AGOs) proteins, which target nascent TEs for degradation in a sequence-specific fashion. Parasitic flatworms have a peculiar collection of what seem to be flatworm-specific AGOs or FLAGOs [21]. The roles of this phylogenetically distinct cluster is not yet known.

In this work, we used experimental approaches to further characterise the FLAGOs of *S. mansoni*. We chose *S. mansoni* because of its relatively simple life cycle maintenance but we believe that these important molecular pathways are likely to be shared among other members of the genera. We successfully silenced two of the *S. mansoni* AGOs, SmAGO1 and SmAGO2, and we further demonstrated that the reduced expression of the flatworm-specific SmAGO2 is associated with an increased expression of the two retrotransposable elements tested, namely Boudicca and SR2 **(Figure 2)**.

It is possible that FLAGOs have similar roles to those of the newly identified AGO in nematodes. In a recent report, Chow *et al.* [27]demonstrated that a specific nematode AGO, exWAGO (extracellular WAGO), is released inside vesicles and to the environment by *Heligmosomoides polygyrus bakeri*, a gastrointestinal nematode. The authors further report that exWAGO is associated with siRNAs with sequence identity to newly evolved TEs.

The localisation of SmAGO2 to the nucleus and the cytoplasm **(Figure 3)** may reflect a dual role for this protein. While in the nucleus, SmAGO2 could undertake nuclear silencing using TE-derived small RNAs [24] to target nascent TE transcripts, when shifted to the cytoplasm, SmAGO2 could be packed into vesicles ready to be deployed to

the environment, as is the case in *H. polygyrus bakeri* [27]. However, proteomics studies targeting extracellular vesicles have not detected SmAGO2 in samples from *S. mansoni* [28, 29, 30]. Further investigations will be required to underpin the extent of functions that can be attributed to these flatworm-specific genes.

# Materials and Methods

## Ethics Statement

Mouse infections to maintain the life cycle of NMRI strain of *Schistosoma mansoni* and collect adult worms were conducted under the Home Office Project Licence No. P77E8A062, and all protocols were approved by the Animal Welfare and Ethical Review Body (AWERB) of the Wellcome Sanger Institute. The AWERB is constituted as required by the UK Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012.

## Parasite material

The *S. mansoni* life cycle is maintained at the Wellcome Sanger Institute. Balb/c female mice were infected with 250 *S. mansoni* cercariae either intraperitoneally or percutaneously and adult worms were recovered 7 weeks post infection through portal perfusion. Parasites were washed extensively in DMEM and incubated in Adult Worm Media for 24 hours prior to either dsRNA treatment or fixation for immunofluorescence.

Adult Worm Media: DMEM (5.4 g/L D-Glucose, Sigma) supplemented with Antibiotic-Antimycotic (Thermo Fisher Scientific, UK) and 10% fetal calf serum.

## Double-stranded RNA (dsRNA) design and synthesis

Primers that incorporate the T7 promoter sequence at the 5'-end were designed to amplify a region of 500-800 bp of SmAGO2 and SmAGO3 (Supplementary Table 1). End-point PCR was performed using cDNA from *S. mansoni* adults as template, 1 uM final concentration of a mix of SmAGO2 or SmAGO3 forward and reverse primers (Sigma, UK) and 2x Qiagen Fast Cycling PCR master mix (Qiagen, cat. no. 203743) following manufacturer's instructions and 55°C annealing temperature. PCR products were checked for expected size and cloned into pCR-TOPO TA vector (Invitrogen, UK). Plasmid transformation and propagation was performed in heat-shock competent BL21 *Escherichia coli* cells [31]. Plasmids were then isolated using QIAprep Spin Miniprep (Qiagen, cat no 27104), sequenced and used as templates to generate PCR products. These were then used as templates for *in vitro* transcription using a MEGAscript T7 Transcription Kit (Invitrogen, cat no AM1334) according to manufacturer's instructions. DsRNA integrity check and quantification was performed in agarose gels plus ethidium bromide staining against a ladder of known concentration. dsRNA stocks were kept at -80°C in nuclease-free water at a concentration of 1ug/ul.

## Parasite transformation with dsRNA

Five to 8 *S. mansoni* adult males were transformed with 30 ug of dsRNA targeting either SmAGO1, SmAGO2, SmAGO3 or a control dsRNA targeting firefly luciferase. Parasites were placed in a 4 mm electroporation cuvette (BioRad, UK) containing 100 ul of simple media and dsRNA (30 ul at a concentration of 1 ug/ul) and incubated for 10 minutes in ice. Electroporation was performed with a single pulse at 125 V for 20 ms using a BTX Gemini X2 (BTX) immediately followed by addition of pre-warmed (37°C) full media. Parasites were transferred to 6-well plates and incubated at 37°C

and 5% $CO_2$. Cultures were checked daily and one-third of the culture media volume was replaced every other day. On the day of parasite collection, worms were transferred to a 1.5 ml centrifuge tube and washed extensively with simple media. After the final wash, all media was removed, 0.5 ml of Trizol reagent (Invitrogen, UK) were added to the tubes and these were kept at -80°C until RNA extraction.

Simple media: DMEM (5.4 g/L D-Glucose, Sigma) supplemented with Antibiotic-Antimycotic (Thermo Fisher Scientific, UK). Full media: Simple media supplemented with 10% fetal calf serum and 2 mM L-glutamine.

## RNA extraction and real-time quantitative PCR

RNA extraction was performed using a standard phenol-chloroform method (for full protocol see Trizol reagent user manual) where RNA was precipitated using 1 volume of isopropanol per volume of acquose phase extracted. RNA was resuspended in nuclease-free water, assayed for integrity and quantification of nucleic acids in an Agilent Bioanalyzer. Poly-A enrichment prior to cDNA synthesis was performed using a Dynabeads mRNA Purification Kit (Thermo Fisher Scientific, UK, cat no 61006) according to manufacturer instructions. Superscript III Reverse Transcriptase (Invitrogen, UK, cat no 10368252) was used for cDNA synthesis. Real time quantitative PCR was performed with KAPA SYBR FAST qPCR Master Mix (Sigma-Aldrich - Merck, UK, cat no KK4602) according to manufacturer's instructions in a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Applied Biosystems).

All primer sequences used in these experiments can be found in Supplementary Table 1. Raw Ct values from RT-qPCR experiments are available from Supplementary Table 2

## Statistical Analysis and visualisation

Student paired T-test implemented in R [32] was used to test the difference between sample means. Supplementary Table 3 shows the values used for these calculation as well as the p-values obtained. Figures 1 and 2 were prepared using the ggplot2 package [33] implemented in R. Figure 3 was assembled using the free software GIMP[34].

## Immunofluorescence and histology

Adult worms were relaxed in 0.6M of MgCl2 for 1 minute before fixation in 4% paraformaldehyde in PBSTx (1 x PBS + 0.3% Triton-X 100) for 4 hours at room temperature. They were rinsed in PBSTx (3 x 5 minutes, 2 x 1 hour washes) at room temperature and dehydrated in a stepwise ethanol series.

The dehydrated worms were cleared in histosol (National Diagnostics) for 20 minutes, and embedded in paraffin overnight. Paraffin blocks were sectioned at 8um using a Leica (RM2125 RTF) microtome. Consecutive sections were used to compare histology and immunofluorescence. For histological analysis, sections were stained with Masson's trichrome (MTC) [35]. For immunostaining of paraffin sections, slides were dewaxed in Histosol (2x5 min), then rehydrated through a descending ethanol series into PBS + 0.1% Triton (PBT, 2x5 min). Slides were blocked with 10% heat-inactivated sheep serum in PBT for 1 hour at room temperature in a humidified chamber. The primary antibody (see below) was diluted in block (10% heat-inactivated sheep serum in PBT) and applied to the slide, covered with parafilm, and incubated at 4°C for 48 hours. Slides were then rinsed in PBT (3x10min). A secondary antibody diluted in block solution were then applied to each slide, and slides were covered with parafilm and incubated in a humidified chamber, in the dark, at room temperature for 2 hours. Slides were rinsed in PBT 3 x 10 minutes, and then 4 x 1 hour prior to counterstaining with the nuclear marker 4',6-diamidino-2-phenylindole (DAPI) (1 ng/ml) and mounting in

Fluoromount G (Southern Biotech, Birmingham, AL). The primary antibody used was anti-*Schistosoma japonicum* AGO2 (clone 650-1-1-KM864-3-11E8) from Abcam lnc (NJ, US) with kind permission from Dr Pengfei Cai (QIMR, Australia). This was used diluted 1:200. An anti-mouse secondary antibody was used at 1:500 dilution. A negative control was obtained by omitting the primary antibody and no fluorescence signal was detected. Imaging was carried out using an epi-fluorescent Zeiss Axioscope microscope.

# Supplementary Tables

## Supplementary Table S1

Primer sequences [click here to view]

## Supplementary Table S2

Real Time quantitative PCR raw data [click here to view]

## Supplementary Table S3

Student T-test [click here to view]

# Declarations

## Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

## Conflict of interest

The authors declare that they have no competing interests

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## Authors' contributions

AVP - Conception and design of the work, data acquisition, analysis, and interpretation of data. Preparation of the manuscript. KAR - Data acquisition, analysis, and interpretation of data. GR - Provided materials and reagents and contributed to data interpretation. EAM - Provided materials and reagents.

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