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Short communication

Expression site silencing and life-cycle progression appear normal in Argonaute1-deficient *Trypanosoma brucei*

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In recent years, one of the major breakthroughs in eukaryotic biology has been the discovery of the RNA interference (RNAi) pathway [1], a powerful gene silencing mechanism that operates both at the transcriptional and post-transcriptional levels. Originally discovered as a mechanism through which double-stranded RNA (dsRNA) triggers degradation of homologous transcripts, the RNAi pathway is essential for a variety of gene silencing phenomena [2], including heterochromatin formation, DNA and histone methylation, macronuclear DNA elimination in *Tetrahymena*, promoter silencing in plants, and translational control by micro RNAs. Furthermore, in certain organisms RNAi appears to provide or has been proposed to provide a genome defense mechanism to limit the spreading of mobile elements [3–9] and in plants it is a powerful antiviral response [10]. At the genetic level there are two proteins that are the universal hallmark of the RNAi pathway: Dicer, an RNAse III-related enzyme, and a member of the Argonaute protein family [2]. In the 'classical' RNAi pathway, which is triggered by long dsRNAs, Dicer [11] is the endonuclease that processes dsRNA into 20–30 nt small interfering RNAs (siRNAs), whereas an Argonaute family member provides the endonuclease activity or "Slicer" that cleaves transcripts after base-pairing with complementary siRNAs [12–16].

Trypanosoma brucei was one of the first organisms where the RNAi pathway was found [17]. In the bloodstream of mammals, trypanosomes proliferate as morphologically slender forms and, as the parasitemia increases, they normally develop into non-dividing stumpy forms, which are thought to be pre-adapted for survival in the insect vector (*Glossina* sspp., the Tsetse) [18]. The surface of bloodstream forms (BS) is covered by a dense coat of a single member of the Variant Surface Glycoprotein (VSG) family. Sequential expression of antigenically distinct VSGs, a phenomenon known as antigenic variation [19], allows the parasites to escape antibody killing. VSG expression occurs at a telomeric expression site (ES), of which there are approximately twenty, only one of which is transcribed at any time. Once trypanosomes are ingested by the Tsetse, they differentiate into procyclic forms, with the concomitant replacement of VSGs with another dense coat consisting of members of a small family of 'procyclins'. In procyclic trypanosomes, all ESs are silenced through a mechanism that is likely to involve remodeling of ES chromatin to a transcriptionally repressed state [20].

So far, studies of the biological significance of RNAi in trypanosomes have focused on cultured procyclics. We [21] and others [22] have shown that the *T. brucei* Argonaute1 (TbAGO1) gene is essential for RNAi. Furthermore, mutational analysis is consistent with TbAGO1 functioning as the trypanosome Slicer [23]. Ablation of the RNAi pathway by deletion of *TbAGO1* [21,22], or selection for RNAi-deficient trypanosomes [24], demonstrated that RNAi is not essential for survival of procyclics. However, RNAi deficiency is not without consequences, as ablation of *TbAGO1* leads to growth defects that,

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depending on the laboratory strain used, range from mild [24] to severe [22]. Chromosome segregation abnormalities have been proposed to be the cause of severe growth defects [22]. At the RNA level, *ago1*$^{-/-}$ and RNAi-deficient trypanosomes show augmented levels of retroposon transcripts [21,23] that are accounted for by a combination of transcriptional silencing and post-transcriptional mechanisms. Thus, one proposed function of RNAi in *T. brucei* procyclics is to attenuate retroposon transcripts and to reduce the potential for retrotransposition. RNAi may also play a role in chromosome segregation [22].

As the nuclear branch of the RNAi pathway is involved in heterochromatin formation and consequent gene silencing [28 and references therein], we reasoned that RNAi may contribute to ES regulation and to developmental progression in trypanosomes. To this end we investigated the consequences of deleting the RNAi pathway from BS trypanosomes. For these studies we chose two BS cell lines, namely the 'single-marker' cell line, a derivative of Lister 427 expressing T7 RNA polymerase and Tet repressor [25], and STIB247 [26]. Both cell lines have been adapted to grow *in vitro* and offer different advantages. Lister 427 has been widely used for studies of antigenic variation and several reagents are available to follow this process, but the current lines do not complete the developmental cycle in the Tsetse. STIB247 retains the ability to be transmitted through the Tsetse, and will undergo two developmental steps in the trypanosome life cycle in the laboratory: transition from proliferative slender to non-proliferative stumpy BS in mice and progression from BS to procyclic forms *in vitro*.

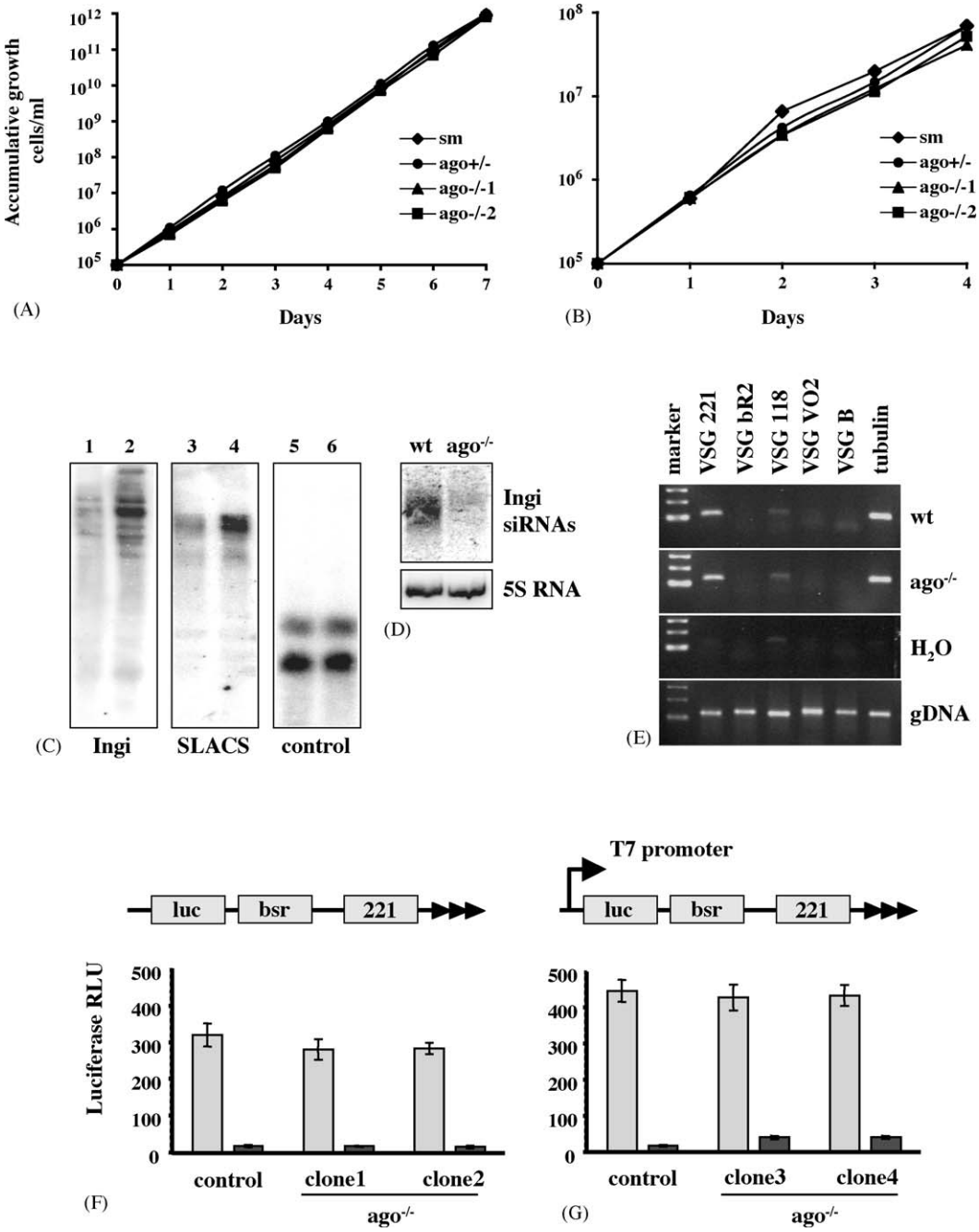
Both alleles of *AGO1* were deleted in Lister 427 and STIB247, by replacement with the same selectable markers used for generating *T. b. rhodesisense ago1*$^{-/-}$ procyclics [21]. The correct integration of the marker genes and the deletion of both alleles of *AGO1* were confirmed by Southern blotting (data not shown). Transfection of Lister 427 *ago1*$^{-/-}$ trypanosomes with α-tubulin dsRNA did not give rise to FAT cells [17], thus confirming that *AGO1* deletion resulted in RNAi deficiency, as previously reported for procyclic trypanosomes [21]. To determine whether ablation of the RNAi pathway affected cell proliferation, we monitored growth of *AGO1* null mutants for several days. Both Lister 427 and STIB247 *ago1*$^{-/-}$ were viable, morphologically indistinguishable from wild-type cells, and showed no significant growth phenotype *in vitro* (Fig. 1A and B, and data not shown).

To investigate whether ablation of *AGO1* in BS trypanosomes was associated with changes in the levels of high-molecular-weight retrotroposon transcripts and retroposon-derived siRNAs, RNA from Lister 427 *ago1*$^{-/-}$ cells was analyzed with probes specific for the ubiquitous *INGI* or the site-specific *SLACS* retroelements. We found that the level of high molecular weight *INGI* or *SLACS* transcripts increased 2–3 fold (Fig. 1C), whereas the accumulation of *INGI* siRNAs was significantly reduced (Fig. 1D), similar to the results obtained for procyclic trypanosomes [21,24].

We then examined whether the loss of *AGO1* affected mono-allelic *VSG* expression, by comparing the steady-state levels of several *VSG* RNAs in Lister 427 wild-type and *agoI*$^{-/-}$ trypanosomes, by RT-PCR. We chose specific primer pairs to detect transcripts of *VSG* 221 (single-copy gene, active ES), *VSG* bR2 (2 telomeric and 1 internal copy), *VSG* 118 (single-copy gene, inactive ES), *VSG* VO_2 (1 telomeric and 1 internal copy) or *VSG* B (1 telomeric and 2 internal copies). Genomic DNA from wild-type cells served as a positive control. As expected, mRNA of the active *VSG* 221 was abundant in both cell lines (Fig. 1E). In contrast, transcripts of the other *VSGs* were not detectable. The faint band with primers for *VSG* 118 represented unspecific amplification (possibly due to DNA contamination of the sample), because it was also visible in the water control. Thus, within the limits of our analysis, mono-allelic expression of *VSG* 221 was maintained in *ago1*$^{-/-}$ cells.

During differentiation of BS trypanosomes to procyclic forms *in vitro*, transcription of the active ES promoter is attenuated [27]. Although the events leading to ES silencing in procyclics are not understood, ES chromatin is probably remodeled during this process [20], resulting in a less accessible more compact structure. Given the present compelling evidence that the RNAi pathway plays a role in heterochromatin formation in various organisms [28], we decided to test whether Lister 427 *ago1*$^{-/-}$ cells were able to repress the *VSG* 221 ES during differentiation to procyclics *in vitro*. Following the previously employed strategy [20], we generated cells containing a luciferase reporter cassette without (Fig. 1F) or with (Fig. 1G) a T7 promoter immediately upstream of the active *VSG* 221 in Lister 427 wild-type and *ago1*$^{-/-}$ cells. Two independent *ago1*$^{-/-}$ clones for each reporter cell line were selected for analysis. Luciferase activity was measured in each BS clone and in corresponding procyclics after 10 days under differentiation conditions *in vitro*. Both wild-type and *ago1*$^{-/-}$ BS forms (light gray bars) showed robust luciferase activity, attributable to ES promoter-driven transcription, irrespective of whether or not the integrated reporter cassette was equipped with the T7 promoter (Fig. 1G and 1F, control). As reported previously, after 10 days of incubation under differentiation conditions, the procyclic cells (dark grey bars) derived from each of the two control reporter lines had efficiently down regulated luciferase expression (Fig. 1G and 1F, control). A similar extent of luciferase repression was observed in the differentiated *ago1*$^{-/-}$ reporter procyclics, independently of the absence (F) or presence (G) of the T7 promoter. Taken together, these observations indicate that *AGO1* is not required for mono-allelic ES regulation in BS nor for establishing the developmental compaction of ES chromatin in procyclics.

Lastly, we investigated the effect *AGO1* deletion on life cycle progression from long slender to stumpy forms in mice and from stumpy to procyclics *in vitro*. 1×10^6 STIB247 *ago1*$^{-/-}$ cells were injected into cyclophosphamide-immunocompromised Balb/C mice. After 6 days, 100 μl of infected blood was passaged into a second mouse treated with cyclophosphamide 24 h prior to infection. Blood smears were prepared over the following 9 days, and assayed by phase contrast microscopy for the presence of morphologically stumpy trypanosomes (Fig. 2A). On day 4, at least 90% of parasites were clearly slender in morphology (examples are indicated by arrowheads in Fig. 2A). As the infection progressed, an increase in the proportion of cells that were stumpy, or intermediate in morphology between slender and stumpy, was observed (arrows). At 9 days post-



infection, >80% of the cells were morphologically intermediate or stumpy, consistent with the kinetics in wild type cells [29] and our unpublished observations. These observations indicated that deletion of *AGO1* did not prevent formation of stumpy cells *in vivo*.

Stumpy cells are functionally defined by their capacity for efficient differentiation to procyclic forms. To assay differentiation, stumpy STIB247 *ago1*$^{-/-}$ cells were harvested from mice, incubated under differentiation conditions and periodically assayed for EP-procyclin expression by immunofluorescence. EP procyclin appeared rapidly after initiating differentiation (Fig. 2B and C). Prior to 2 h, few cells expressed EP procyclin, but approximately 80% of cells had activated procyclin expression by 4 h, increasing to 100% by 24 h. This profile of procyclin expression is characteristic of the differentiation of stumpy-enriched pleomorphic populations [30,31], and demonstrated that *AGO1* deletion had no detectable consequence for the ability of STIB247 cells to initiate differentiation to procyclic forms.

A second characteristic of differentiation is repositioning of the mitochondrial genome (the kinetoplast) from a posterior position in the BS cell to a location midway between the nucleus and cell posterior in procyclic forms [32]. This morphological restructuring was retained in the STIB247 *ago1*$^{-/-}$ cells (Fig. 1D). These results showed that two diagnostic events for differentiation to procyclic forms occurred normally in STIB247 *ago1*$^{-/-}$ cells.

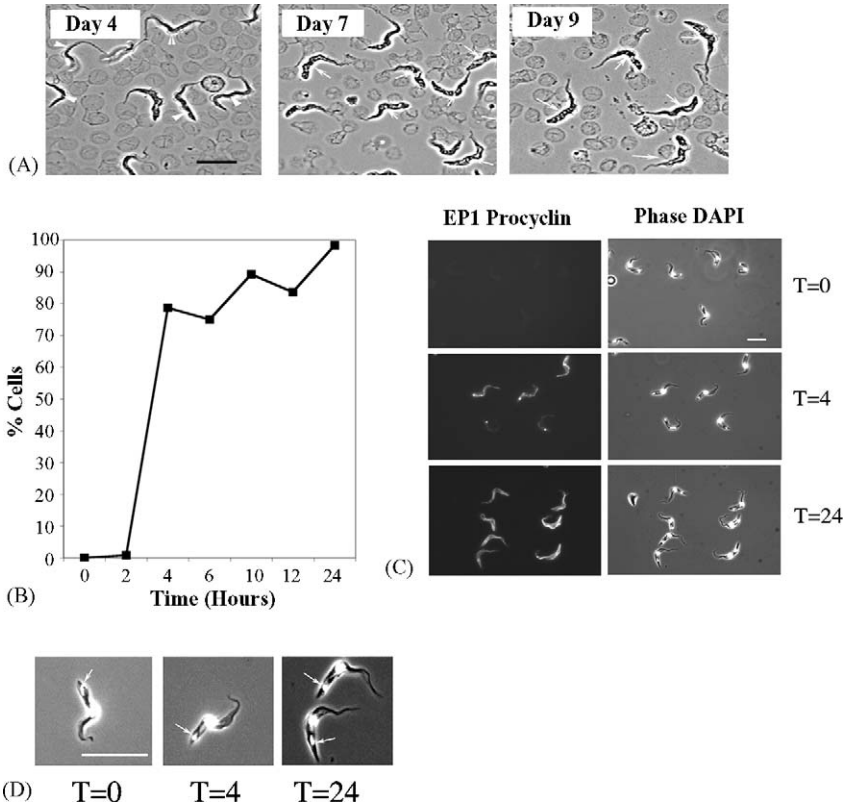


Fig. 2. Differentiation of STIB247 *ago1*$^{-/-}$ *in vivo* and *in vitro*. (A) Glutaraldehyde-fixed blood smears of *ago1*$^{-/-}$ cells at the indicated time points during the course of infection in Balb/c mice. Arrowheads indicate morphologically slender cells, which predominate early in the parasitaemia. Arrows indicate cells that are morphologically intermediate or stumpy, which accumulate during days 7–9 post infection. Bar = 20 μm. (B) Kinetics of EP procyclin appearance after the incubation of STIB247 *ago1*$^{-/-}$ BS in SDM-79 with 6 mM cis aconitate at 27 °C. The *ago1*$^{-/-}$ cells were harvested from a mouse 9 days post-infection, when the population was assessed by microscopy to be >80% intermediate or stumpy in morphology. Procyclin appearance was assessed by immunofluorescence in >250 cells at each time point after the initiation of differentiation. (C) Representative images of TbAGO1 null mutant cells isolated at the indicated time points after the induction of differentiation. The left panel shows the immunofluorescence images of cells stained for EP procyclin, the right panel shows phase contrast images of the same cells counterstained with DAPI to reveal the position of the kinetoplast and nucleus. (D) Higher resolution images showing kinetoplast repositioning away from the posterior end of the cells during stumpy to procyclic differentiation.

Fig. 1. *AGO1* is not essential in BS *T. brucei*. (A) Growth of *ago1*$^{-/-}$ Lister 427 'single marker' cell line [25]. Log-phase wild-type, *ago1*$^{+/-}$, and two *ago1*$^{-/-}$ clones were counted at 24-h intervals and then diluted 1:10. The dilution factor was used to calculate the cumulative cell growth. (B) Growth of *ago1*$^{-/-}$ STIB247. Wild-type, *ago1*$^{+/-}$ and *ago1*$^{-/-}$ clones were split daily to 1×10^5 parasites/ml. The data are presented as cumulative population growth. (C) Upregulation of *INGI* and *SLACS* retroposon transcripts in 427 *ago1*$^{-/-}$ cells. Total RNA from wild-type (lanes 1, 3 and 5) or from *ago1*$^{-/-}$ (lanes 2, 4 and 6) cells was fractionated on a formaldehyde-agarose gel, transferred to a nylon membrane and hybridized first to a *SLACS* radiolabeled DNA probe (nt 1512 to 2726 of SLACS). After stripping, the membrane was hybridized to an *INGI* radiolabeled DNA probe (nt 3421 to 4620 of INGI) and then to a calflagin probe as a control for RNA loading. (D) Northern blot analysis of small RNAs isolated from wild-type or *ago1*$^{-/-}$ 427 BS cells with a sense riboprobe derived from a portion of the *INGI* retroelement (nt 3421 to 4620 of INGI). Hybridization to 5S RNA was used as a control for RNA loading. Northern blotting of siRNAs and of larger transcripts was performed as previously described [21]. (E) ES silencing in AGO1-deficient Lister 427. RT-PCR with *VSG*-specific primers was performed on RNA isolated from wild-type (WT) and *AGO1*-deficient BS (*ago1*$^{-/-}$). Genomic DNA (gDNA) served as a positive control. Total RNA from 1×10^8 cells was isolated using RNA Stat-60 (Tel-Test), following the manufacturer's instructions. 10 μg total RNA was incubated with RQ1 RNAse-free DNAse (Promega), according to supplier's recommendations, followed by phenol/chloroform/isoamyl-alcohol extraction and ethanol precipitation. The precipitated RNA was dissolved in 10 μl DEPC-treated water and used for reverse transcription using the ProStar First Strand RT-PCR Kit (Stratagene) with the supplied random primers. PCR with primers specific for different VSG genes was performed with a Perkin-Elmer 9600 GeneAmp system. The sequences of primers used in the PCR reaction can be obtained upon request. (F) and (G) Developmental silencing of ES in *AGO1*-deficient Lister 427. The structures of the two luciferase cassettes are diagrammed at the top of each panel (not to scale). Telomeres are represented by filled arrow heads and the T7 RNA polymerase promoter is shown as an arrow. To generate the reporter, the blasticidin S deaminase open reading frame was PCR amplified from pHD309-BSD including the 3′ and 5′ UTRs. The PCR product was cloned into *NheI*-digested pMig39 [20]. The bleomycin cassette was removed by digesting with *SrfI* and *StuI*. Religation of the vector backbone generated the reporter plasmid CJ25A. The T7-promoter in C J25 A was removed by digesting with *SpeI* and *XhoI* followed by treatment with Klenow enzyme. Religation generated the reporter plasmid CJ24A. The final constructs were cut with *BstAPI* before transfection by standard methods [33]. The luciferase reporter cassettes minus (panel F) or plus (panel G) the T7 promoter were introduced at the *VSG* 221 active ES of wild-type (control) or *ago1*$^{-/-}$ cells. Two BS *ago*1$^{-/-}$ clones of each reporter cell line were selected for further analysis. Luciferase activity was monitored in BS (light gray bars) and 10 days post differentiation into procyclic-form trypanosomes (dark grey bars). BS forms were differentiated to procyclic forms as described previously with minor modifications [34]. Briefly, 2×10^7 cells were transferred into 5 ml depleted trypanosome medium (DTM) with 15% FCS and 3 mM citric acid intermediates at 27 °C without feeder cells. Procyclic cultures were established for 10 days prior to analysis. Luciferase assays were performed as previously described [35].

In summary, ablation of the RNAi pathway by deletion of *AGO1* did not cause detectable growth defects of two *T. brucei* BS strains. Thus, as in procyclics, *AGO1* is not essential for viability, at least under laboratory conditions. However, as the establishment of *AGO1* null lines is a long procedure we cannot discount the possibility that, during selection in culture, trypanosomes may activate alternate pathways to counteract the loss of *AGO1*. BS trypanosomes of STIB247 *ago1*$^{-/-}$ were also able to differentiate from long slender to stumpy forms during infection of mice, and the stumpy-enriched populations differentiated normally into procyclic forms when differentiation stimuli were applied. We conclude that if deletion of *AGO1* causes a defect at any of the steps we tested, it is most likely a minor one. However, it remains to be seen whether STIB247 *ago1*$^{-/-}$ BS forms retain the ability to infect the insect vector and complete the life cycle.

Lister 427 *ago1*$^{-/-}$ BS forms allowed us to analyze the role of *AGO1* in ES regulation. We did not detect any deregulation of mono-allelic *VSG* expression, suggesting that the loss of *AGO1* does not result in widespread ES activation. Furthermore, these mutant trypanosomes were able to attenuate ES expression during differentiation to procyclic forms, regardless of whether expression of the luciferase reporter was under the control of the endogenous ES promoter or the T7 promoter. These observations suggest that the developmental remodeling of ES chromatin is maintained in the absence of *AGO1*. However, to firmly conclude that the RNAi pathway is not involved at any steps in ES regulation, we need to analyze the rate of ES switching, which is technically challenging and will be addressed in future experiments.

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