Published in final edited form as:

Int J Parasitol. 2016 March ; 46(3): 205–212. doi:10.1016/j.ijpara.2015.11.004.

Technical advances in trigger-induced RNA interference gene silencing in the parasite *Entamoeba histolytica*

Mohamed I. Khalil^{a,b}, Bardees M. Foda^{a,c}, Susmitha Suresh^a, and Upinder Singh^{a,d,*}

^aDepartment of Internal Medicine, Division of Infectious Disease, Stanford University, Stanford, CA 94305, USA

^bDepartment of Molecular Biology, National Research Centre, El-Buhouth St., Cairo, Egypt

^cDepartment of Molecular Genetics and Enzymology, National Research Centre, El-Buhouth St., Cairo, Egypt

^dDepartment of Microbiology and Immunology, School of Medicine, Stanford University, Stanford, CA 94305, USA

Abstract

Entamoeba histolytica has a robust endogenous RNA interference (RNAi) pathway. There are abundant 27 nucleotide (nt) anti-sense small RNAs (AS sRNAs) that target genes for silencing and the genome encodes many genes involved in the RNAi pathway such as Argonaute proteins. Importantly, an *E. histolytica* gene with numerous AS sRNAs can function as a “trigger” to induce silencing of a gene that is fused to the trigger. Thus, the amebic RNAi pathway regulates gene expression relevant to amebic biology and has additionally been harnessed as a tool for genetic manipulation. In this study we have further improved the trigger-induced gene silencing method. We demonstrate that rather than using the full-length gene, a short portion of the coding region fused to a trigger is sufficient to induce silencing; the first 537 bp of the *E. histolytica* rhomboid gene (*EhROM1*) fused in-frame to the trigger was sufficient to silence *EhROM1*. We also demonstrated that the trigger method could silence two amebic genes concomitantly; fusion of the coding regions of *EhROM1* and transcription factor, *EhMyb*, in-frame to a trigger gene resulted in both genes being silenced. Alternatively, two genes can be silenced sequentially: *EhROM1*-silenced parasites with no drug selection plasmid were transfected with trigger-*EhMyb*, resulting in parasites with both *EhROM1* and *EhMyb* silenced. With all approaches tested, the trigger-mediated silencing was substantive and silencing was maintained despite loss of the G418 selectable marker. All gene silencing was associated with generation of AS sRNAs to the silenced gene. We tested the reversibility of the trigger system using inhibitors of histone modifications but found that the silencing was highly stable. This work represents a technical advance in the trigger

*Corresponding author. Dr. Upinder Singh, M.D., Division of Infectious Disease, Lane Building, Room 121, 300 Pasteur Drive, Stanford University School of Medicine, Stanford, California 94305, USA. Tel.: +1-650-723-4045; fax: +1-650-724-3892. usingh@stanford.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

gene silencing method in *E. histolytica*. Approaches that readily silence multiple genes add significantly to the genetic toolkit available to the ameba research community.

Graphical Abstract

Keywords

RNA interference; Small RNAs; Stable silencing; Protozoan parasite

1. Introduction

Entamoeba histolytica is a unicellular parasite that causes amebic colitis and systemic amebiasis of the liver, lung, heart and brain (Lohia, 2003). There are no precise data for the number of annual cases. However, the most recent published estimates are 50 million infections and 100,000 human deaths every year (World Health Organization (WHO), 1997). During the life cycle, the parasite alternates between two life stages, an encysted form and a pathogenic trophozoite. Transmission of the parasite occurs by the oral-fecal route through the ingestion of contaminated water or food. The cysts are shed in the feces of an infected host, ingested by a new host, and excyst in the small intestine with the emerging trophozoites colonizing the large intestine (Haque et al., 2003). The trophozoites can penetrate the intestinal mucous barrier, resulting in colitis. Rarely, the amebic trophozoites enter the blood stream and spread systemically, causing abscesses primarily in the liver and less frequently in the lung and brain. During host invasion and development the parasite actively modulates its gene expression profiles (Gilchrist et al., 2006; Ehrenkaufer et al., 2007b).

The RNA interference (RNAi) pathway is a crucial process for controlling gene expression in many organisms ranging from protozoan to human (Fire et al., 1998; Vance and Vaucheret, 2001; Ghildiyal and Zamore, 2009; Batista and Marques, 2011; Ketting, 2011). Small RNA (sRNA) populations are the key effectors in the RNAi pathway and associate with Argonaute proteins to confer gene silencing (Hutvagner and Simard, 2008; Ender and

Meister, 2010). The mechanism of the RNAi pathway is well studied in human and other eukaryotic systems. *Entamoeba histolytica* has a functioning RNAi pathway as the parasite encodes multiple RNAi related proteins and has an abundant population of anti-sense (AS) sRNAs which silence targeted genes (Zhang et al., 2008; Morf et al., 2013). RNAi gene silencing in ameba has been linked to heterochromatin formation (Zhang et al., 2011; Foda and Singh, 2015).

The endogenous RNAi pathway has recently been harnessed to develop a method for gene silencing (Morf et al., 2013; Pearson et al., 2013). The approach utilizes amebic genes with endogenous sRNAs mapping to them to function as a “trigger” for gene silencing. Fusion of a full-length coding region of a gene to a trigger region results in stable and permanent silencing of the fused gene. Using this approach, multiple amebic genes were silenced including *E. histolytica* Rhomboid protease 1 (*EhROM1*), a Myb transcription factor (*EhMyb*), and an H₂O₂ responsive transcription factor (Morf et al., 2013; Pearson et al., 2013). In all these situations, the trigger was fused to a full-length coding region of the gene of interest; silencing was observed and maintained despite loss of the trigger plasmid. Silencing was associated with the generation of AS sRNAs to the silenced gene and mediated by repressive histone modifications (di-methylation of histone H3) (Foda and Singh, 2015). Some genes, including those of the RNAi pathway, could not be silenced by this method despite generation of AS sRNAs to them (Pompey et al., 2014).

In this study, we optimized and improved the utility of the trigger-induced gene silencing method. We demonstrate that a portion of the gene fused to the trigger is sufficient to silence a gene. Additionally, we demonstrated that two genes could be silenced by fusing them both in-frame to the trigger in one silencing construct and that genes can be silenced sequentially. In all situations, the silencing was robust, was associated with the appearance of 27 nucleotide (nt) sRNAs to the silenced gene and was maintained despite loss of the trigger plasmid. These advances are important and add to the toolkit for genetic manipulation of an important human pathogen.

2. Materials and methods

2.1. Plasmids

Plasmid pKT-19T was used as the backbone for cloning of all trigger silencing constructs with expression in this vector driven by the cysteine synthase promoter (Morf et al., 2013). The pKT-19T-FL-Myb plasmid was obtained from Morf et al. (2013). The pKT-19T-537ROM1 plasmid was constructed by amplifying the fragment containing the first 132 bp of the 19-trigger (19T) ([EHI 197520](#)) and the first 537 bp of the ROM1 coding region ([EHI 197460](#)) from the pKT-19T-FL-ROM1 plasmid using the following primers: 19T-*NheI* primer (5' ACAGCTAGCATGTCCTTCA GCTCAACCAACT-3') and 537ROM1-*XhoI* primer (5'ACACTCGAGTTACATCCCAAGTCTTAA TTG-3'). This PCR product was cloned into the *NheI* and *XhoI* sites of the pKT plasmid, generating pKT-19T-537ROM1. The pKT-04T-537ROM1 plasmid was cloned using a similar strategy: the pKT-04T-FL-ROM1 plasmid was used to amplify the fragment containing the first 132 bp of the 04-Trigger (04T) ([EHI 048600](#)) and the first 537 bp of the ROM1 coding region ([EHI 197460](#)) with the following set of primers: 04T-*NheI* primer (5'-

ACAGCTAGCATGGAAAT TGAATTAACCCTC-3') and the 537ROM1-*XhoI* primer listed above. This PCR product was cloned into the *NheI* and *XhoI* sites of the pKT plasmid generating pKT-04T-537ROM1. The pKT-19T-537ROM1-FL-Myb plasmid was constructed by fusing the first 537 bp of ROM1 in-frame with the full-length (FL) Myb gene; the stop codon was added to the end of the fusion at the 3' of the Myb gene. The fragment contains the first 132 bp of 19T fused to the first 537 bp of ROM1 were amplified from pKT-19T-537ROM1 to remove the stop codon using the primers: 19T-*NheI* and 537ROM1-*AgeI* primer (5'-ACAACCGGTCATCCC AAGTCTTAATTG-3') and the PCR product cloned into the TOPO-TA2.1 cloning vector. The full-length Myb gene (**EHI 197980**) was amplified using the following pair of primers: FL-Myb-*AgeI* primer (5'-ACAACCGGTAGTGTCA CTCTTTCTTCTACT-3') and FL-Myb-*XhoI* primer (5'-ACACTCGAGTCAAATCAATGCTTGATT GAA-3'), and cloned into TOPO-TA2.1 cloning vector. The two newly generated TOPO vectors were digested using *AgeI* and *XhoI*, and the cleavage products were gel purified and ligated to form a TOPO-TA-19T-537ROM1-FL-Myb plasmid. This was then digested using *NheI* and *XhoI* restriction enzymes. The resultant 19T-537ROM1-FL-Myb was cloned into a pKT plasmid to generate the final pKT-19T-537ROM1-FL-Myb construct.

2.2. Parasite culture

Entamoeba histolytica trophozoites (HM-1:IMSS) were grown in TY media as described previously (Diamond et al., 1978). Stable cell lines harboring pKT-19T-537ROM1, pKT-04T-537ROM1 or pKT-19T-537ROM1-FL-Myb plasmids were independently generated by transfecting the *E. histolytica* HM-1:IMSS strain using a standard transfection protocol as previously described (Baxt et al., 2010). The stable transfected cells were selected and maintained using 6 µg/ml of Geneticin (G418, Thermo Fisher Scientific, USA). For sequential silencing, the pKT-19T-537ROM1 cell line was maintained without G418 drug selection, causing it to lose the pKT-19T-537ROM1 plasmid for at least 3 weeks. This parasite line was then transfected with the pKT-19T-FL-Myb construct and stably transfected cells were selected with 6 µg/ml of G418.

2.3. RNA isolation and reverse transcription PCR (RT-PCR)

Total RNA was collected from confluent T25 flasks using the mirVana kit (Ambion, Thermo Fisher Scientific, USA) according to the manufacturer's instructions. RNA was treated with an RNase-free DNase kit (Invitrogen, USA) to remove any residual DNA and reverse transcribed using an oligo dT primer and reverse transcription kit (Invitrogen). The negative control (-RT) was split away and was subjected to the same treatment but without the addition of reverse transcriptase. Genomic DNA served as a positive control and was isolated using the RNeasy Plant Mini Kit (Qiagen, Germany). The sRNA enriched from each of the stable cell lines was isolated using a mirVana kit (Ambion, Thermo Fisher Scientific, USA) following the manufacturer's instructions. Primers for RT-PCR were designed as follows:

ROM1 gene (**EHI 197460**): 5'-AGGCCTCATTCTCCACCACATAACAATA-3 and 5'-GAGCTCT TAATTGCATTTTCCAACATTG-3';

Myb gene (**EHI 197980**): 5'-ACAACCGGTAGTGTCACCTCTTTCTTCTACT-3' and 5'-ACACTC GAGTCAAATCAATGCTTGATTGAA-3';

EHI 199600: 5'-CCTTTAAGCCCAGCCTTTCT-3' and 5'-ACGTCATGCTGAATTTGCTG-3'

2.4. sRNA northern blot analysis

High resolution northern blots were done according to standard protocols (Zhang et al., 2008; Morf et al., 2013) using 75 µg of the sRNA enriched fraction. The probes used in the northern blot were as follows:

ROM1 probe: 5'-AGGCCTCATTCTCCACCACATAACAATA-3';

Myb probe: 5'-ACAACCGGTAGTGTCACCTCTTTCTTCTACT-3';

EHI 197520 probe: 5'-ACACCCGGGATGTCTTCAGCTCAACCAACT-3';

EHI 118130 probe: described in Zhang et al. (2008) and Morf et al. (2013).

2.5. Histone deacetylase (HDAC) inhibition

The HDAC inhibitors trichostatin A (TSA) and nicotinamide were obtained from Sigma (USA). *Entamoeba histolytica* trophozoites with a trigger-silenced ROM1 gene were grown for 24 h before treatment with the inhibitors. The inhibitors were used at the following concentrations: nicotinamide (2.5 mM, 5 mM, 10 mM or 20 mM) for 24 h and TSA (50 nM, 100 nM or 150 nM) for 48 h. After inhibitor treatment, cells were harvested, total RNA isolated (mirVana kit, Ambion) and cDNA synthesized (SuperScript II Reverse Transcriptase, Invitrogen). Cell growth was monitored at all the tested drug concentrations and confirmed the confluence and growth of the drug -treated cells.

3. Results

We have demonstrated that *E. histolytica* genes that have significant amounts of AS sRNAs mapping to them and have low mRNA levels can be used as “triggers” to silence amebic genes that are fused to them (Morf et al., 2013). These triggers were successfully used to down-regulate highly expressed genes including a gene involved in amebic virulence (ROM1) and Myb transcription factor. However, in these trigger constructs the full-length coding regions of these genes were used (Morf et al., 2013). In this paper, we sought to enhance the functionality of the trigger-based silencing approach. We now describe a method to successfully silence genes (i) using only a portion of the gene to be silenced fused to the trigger, (ii) fusing two genes to the trigger and causing both to be silenced, and (iii) using a sequential silencing approach.

3.1. A portion of the ROM1 gene fused to a trigger can induce gene silencing of the chromosomal ROM1 gene

The trigger-based approach has been used with full-length copies of the gene to be silenced fused to the trigger region. In order to determine if a truncated portion of the Trigger-gene fusion could efficiently function to silence the chromosomal copy of *EhROM1*, we fused 537 bp of ROM1 to the trigger. We used two genes as triggers: 132 bp of **EHI 197520**,

called 19T and 132 bp of **EHI 048600**, called 04T. The first 537 bp of the coding sequence of the ROM1 gene was fused to each trigger, resulting in 19T-537ROM1 and 04T-537ROM1 (Fig.1A). We did not have a specific rationale for using the first 537 bp of the coding sequence of the ROM1 gene, other than designing the construct to get stable transcript. Based on the mechanism of trigger-based gene silencing, we anticipate that any region in the coding sequence of the ROM1 gene should work efficiently to mediate silencing (Foda and Singh, 2015). Additionally, we designed a silencing construct using 132 bp of the **EHI 048600** trigger fused to the full-length ROM1 gene (04T-FL-ROM1). This full-length ROM1 cassette contains the entire coding sequence of the ROM1 gene. This trigger has been shown to mediate silencing of luciferase but has not previously been tested on highly expressed chromosomally encoded genes. Stable transfectants were generated using the trigger gene constructs and maintained at a G418 concentration of 6 µg/ml. RT-PCR analysis of independently transfected cells revealed that 537 bp of ROM1 worked effectively with both **EHI 197520** and **EHI 048600** triggers in strongly down-regulating the chromosomally encoded ROM1 gene (Fig.1B). Furthermore, we did not observe any difference in the extent of down-regulation of ROM1 transcript compared with the trigger full-length fusions. We previously showed that the silencing of a gene was associated with the generation of AS sRNA mapping to the silenced locus (Morf et al., 2013). In order to confirm if silenced genes were associated with generation of 27 nt AS sRNAs we used high resolution northern blot analysis to probe for ROM1 AS sRNA. We detected 27 nt sRNAs that were antisense to the ROM1 gene in all generated cell lines, showing that a portion of the gene was sufficient to generate AS sRNAs and this was linked to silencing the chromosomal copy of the gene (Fig. 1C). The down-regulation obtained using a portion of the gene was comparable to the silencing obtained using the full-length gene.

Entamoeba histolytica has developed an efficient epigenetic memory to maintain gene silencing, as it has been noted that silencing is maintained for years after removal of the trigger plasmid (Bracha et al., 2003; Morf et al., 2013; Foda and Singh, 2015). To test whether the persistence of silencing with a truncated portion of the gene had different dynamics, we released the cells from the G418 drug for at least 1 month and named these cell lines (04T-FL-ROM1^{No plasmid}, 04T-537ROM1^{No plasmid} and 19T-537ROM1^{No plasmid}). We confirmed the absence of the plasmid by testing genomic DNA from these cell lines by PCR (data not shown). We demonstrated ongoing and long-term gene silencing of ROM1 in all these cell lines together with the ongoing generation of AS sRNAs to the ROM1 gene (Fig. 2A, B). These data indicate that during ongoing gene silencing, the epigenetic memory of amebic cells may be induced successfully by trigger gene portion fusion, to keep the locus of the endogenous gene silenced. These results are comparable with the observation in trigger gene silencing obtained using the full-length genes. Thus, shorter portions of a gene can be used to mediate robust and stable long-term gene silencing. Using shorter portions of genes would help ease the laborious process of cloning and broaden the functionality of the trigger gene silencing approach.

3.2. Inducing concurrent silencing of ROM1 and Myb genes

We aimed to optimize the trigger system and silence more than one gene at a time. In order to do this, we used a two-gene fusion system to determine if we could concurrently silence

two genes that had previously been shown to be able to be silenced (ROM1 and Myb), using a single trigger construct. We constructed a silencing cassette containing 132 bp of **EHI 197520** trigger and the first 537 bp of the coding sequence of the ROM1 gene fused to the full-length Myb gene (567 bp) (19T-537ROM1-FL-Myb) (Fig. 3A). The construct was transfected and stable lines were generated and maintained at a G418 concentration of 6 µg/ml. RT-PCR analysis determined significant silencing of chromosomal copies of ROM1 and Myb genes, although the level of Myb silencing seemed less robust than the level of ROM1 silencing (Fig. 3B).

Next, we determined whether the silencing was associated with the formation of 27 nt AS sRNAs to both the ROM1 and Myb genes. A high resolution northern blot analysis demonstrated the generation of 27 nt AS sRNA to both ROM1 and Myb genes (Fig. 3C). Of note, the level of AS sRNA for the ROM1 gene seemed to be higher than that for the Myb gene. These results are consistent with the 5' bias of AS sRNAs in *E. histolytica*, since most sRNAs map to the 5' end of a gene (Morf et al., 2013). The silencing construct we designed had the ROM1 gene fused upstream to the Myb gene and hence the 5' bias of sRNA abundance would be towards the ROM1 gene and the results match the overall greater silencing of ROM1. These results indicate that we can readily use the trigger silencing approach to knock down two genes at the same time.

To test the persistence of silencing when two genes are silenced concurrently, we removed the G418 drug from the medium for at least 1 month and confirmed the absence of the plasmid using genomic DNA and PCR analysis (data not shown). We demonstrated long-term gene silencing of ROM1 and Myb in the plasmid-free cell line as well as the ongoing generation of AS sRNA for each gene (Fig. 4A, B). These data indicate that the two-gene fusion system can strongly and stably induce silencing of the two genes and the epigenetic memory is able to mediate long-term transcriptional silencing at both loci.

3.3. Sequential silencing of ROM1 and Myb genes

To enhance the feasibility of silencing two genes at one time, we were also interested in silencing ROM1 and Myb genes sequentially. In order to establish the sequential silencing, we used cells previously transfected with a construct containing 132 bp of **EHI 197520** fused to the first 537 bp of ROM1 (19T-537ROM1), which had been released from drug for at least 3 weeks. This cell line, which lacked plasmid, was then transfected with a silencing cassette including 132 bp of the **EHI 197520** gene fused to the full-length Myb gene (19T-FL-Myb construct). Stable transfectants were selected and RT-PCR analysis shows that the sequentially transfected parasites maintained silencing of the ROM1 gene and now also had down-regulation of the Myb gene (Fig. 5A). These results add to the ability to use the 19T-537ROM1 cell line without drug as a platform for silencing additional genes. Silencing genes in succession could serve a variety of purposes including the ability to compare the effects of silencing one gene with the effects of silencing two genes.

To examine the persistence of the long-term silencing in the sequential silencing experiment, G418 drug was removed for at least 3 weeks, RNA was extracted and RT-PCR carried out to determine the silencing of both *Eh*ROM1 and *Eh*Myb genes. RT-PCR showed that both ROM1 and Myb genes were silenced compared with the wild type strain (Fig. 5B).

3.4. Inhibition of histone deacetylation did not reverse the AS sRNA mediated trigger gene silencing

The role of epigenetics in regulating gene expression of *E. histolytica* has been previously suggested (Huguenin et al., 2010; Foda and Singh, 2015). Two histone epigenetic marks were shown to contribute to transcriptional gene regulation, H3K4Me2/3 for active genes and H3K27Me2 for silenced genes. The stable silencing of our generated cell lines lead us to ask whether the trigger gene silencing system could be reversed. To address this question, we treated stably silenced cells with HDAC inhibitors. In this manner, we used TSA which is a pan potent inhibitor affecting multiple classes of deacetylase (Johnstone and Licht, 2003; Rosato and Grant, 2003). Moreover, the effect of TSA on gene expression of *E. histolytica* cells has been previously reported with changes in mRNA levels of approximately 2% of tested transcripts as detected using genome-wide microarray analysis (Ehrenkauf et al., 2007a; Isakov et al., 2008). Here, we used different doses of TSA to treat cells with a trigger-silenced ROM1 gene but with the plasmid removed (Fig. 6A). We observed that 2 days of 50 nM treatment was the TSA dose that allowed cells to grow without killing them (data not shown). Consistent with the observation of persistent silencing, the level of ROM1 transcript did not revert to normal wild type levels even after TSA treatment (Fig. 6A). Moreover, cells with the silencing construct showed the same results. Additionally, we used another inhibitor, which is specific for class III histone deacetylases (Avalos et al., 2005). We tried a range of concentrations of nicotinamide at 2.5 mM, 5 mM, 10 mM or 20 mM for 48 h on trophozoites with silenced ROM1 and trigger plasmid removal, but did not observe significant differences after treatment (Fig. 6B). We also tested 24 h nicotinamide treatment using parasites that were still under plasmid selection pressure and contained the silencing construct but these treatments also did not show any increase in ROM1 transcript levels. These data suggest that the trigger-induced gene silencing is stable and non-reversible. We know that the TSA compound we used is active and impacts parasites, given that at higher concentrations it adversely affected parasite growth (Ehrenkauf et al., 2007a; Isakov et al., 2008). Similarly, with nicotinamide, we used levels that did not kill parasites but which impacted growth. Thus, we are confident that the drugs being used are functional and are used at biologically relevant concentrations.

4. Discussion

In the present study, we further optimized the trigger-induced gene silencing approach in *Entamoeba* in order to add to the genetic toolkit for ameba. We (i) demonstrated efficient silencing with only a portion of the coding sequence of the target gene, (ii) established a system for concurrently silencing multiple genes, and (iii) showed that silencing of multiple genes can occur efficiently in a sequential manner. Treating trigger-silenced parasites with deacetylation inhibitors confirmed the stability of the trigger-induced silencing.

These advancements to the trigger silencing methods are substantial progress which bring technical advantages. Using a portion of a gene instead of the whole coding sequence may be technically easier given the issues with construction of the silencing cassette. Ongoing silencing with only a portion of a gene, especially in the absence of drug selection, informed

us of the stability of the epigenetic memory, which is a specific determinant for long-term gene silencing. Furthermore, the ability to knock down two genes using the same silencing cassette or sequentially will also add to technical ease as it relates to studying gene families or pathways where multiple genes need to be genetically manipulated.

The transcriptional gene silencing process that has been described in the *E. histolytica* G3 strain (Mirelman et al., 2008) is dependent on an obvious change of histone methylation (H3K4) at the silenced gene loci and is associated with the presence of AS sRNAs to the silenced genes (Zhang et al., 2011). The long-term gene silencing after removal of the silencing plasmid, observed in the G3 strain as well as in our trigger silencing approach, suggests that epigenetic changes in genomic loci may play a crucial role in the ability of sRNAs to silence specific genomic loci in *E. histolytica*. The 537 bp of the coding sequence of ROM1 was enough to induce gene silencing, suggesting that most likely the structure of the generated mRNA influences the formation of the AS sRNA and gene silencing.

The trigger-induced gene silencing using the whole coding sequence of some *Entamoeba* genes such as Argonaute proteins and RdRP did not work efficiently and no silencing was achieved despite the generation of AS sRNAs (Pompey et al., 2014). Whether this inability to silence a gene is due to the essential nature of the gene or indicates some aspect of the chromosomal locus is not clear. Having methods for inducible trigger silencing would be very helpful to differentiate between these two possibilities and will form the basis of future efforts. Additionally, despite our efforts to use HDAC inhibitors to reverse the epigenetic silencing, we were not successful. The inability to reverse trigger-mediated gene silencing is reminiscent of the lack of success with a similar approach and the G3 method (Bracha et al., 2003; Mirelman et al., 2008).

Furthermore, we have analyzed the sRNA profile under different conditions (development, heat shock or oxidative stress) and found that the sRNA profiles do not change (Zhang et al., 2015). Thus, we have not identified an endogenously regulated trigger. Since we know that the trigger method is dependent on transcription of the trigger-gene fusion, a regulated promoter trigger silencing tool (such as a tet-regulated trigger gene) becomes of interest and would be useful to develop in the future as it will allow the study of important aspects such as virulence phenotypes and allow us to investigate the function of potentially essential genes. The trigger optimization efforts reported here build the technical tool kit for *Entamoeba* and will allow greater dissection of the parasite's biological processes.

Acknowledgments

We wish to thank all members of the Singh laboratory for useful discussions and technical help. This work was supported by the National Institutes of Health, USA (grant AI102277 to Singh).

References

- Avalos JL, Bever KM, Wolberger C. Mechanism of sirtuin inhibition by nicotinamide: altering the NAD(+) cosubstrate specificity of a Sir2 enzyme. *Mol Cell*. 2005; 17:855–868. [PubMed: 15780941]
- Batista TM, Marques JT. RNAi pathways in parasitic protists and worms. *J Proteomics*. 2011; 74:1504–1514. [PubMed: 21385631]

- Baxt LA, Rastew E, Bracha R, Mirelman D, Singh U. Downregulation of an *Entamoeba histolytica* rhomboid protease reveals roles in regulating parasite adhesion and phagocytosis. *Eukaryot Cell*. 2010; 9:1283–1293. [PubMed: 20581296]
- Bracha R, Nuchamowitz Y, Mirelman D. Transcriptional silencing of an amoebapore gene in *Entamoeba histolytica*: molecular analysis and effect on pathogenicity. *Eukaryot Cell*. 2003; 2:295–305. [PubMed: 12684379]
- Diamond LS, Harlow DR, Cunnick CC. A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. *Trans R Soc Trop Med Hyg*. 1978; 72:431–432. [PubMed: 212851]
- Ehrenkaufer GM, Eichinger DJ, Singh U. Trichostatin A effects on gene expression in the protozoan parasite *Entamoeba histolytica*. *BMC Genomics*. 2007a; 8:216. [PubMed: 17612405]
- Ehrenkaufer GM, Haque R, Hackney JA, Eichinger DJ, Singh U. Identification of developmentally regulated genes in *Entamoeba histolytica*: insights into mechanisms of stage conversion in a protozoan parasite. *Cell Microbiol*. 2007b; 9:1426–1444. [PubMed: 17250591]
- Ender C, Meister G. Argonaute proteins at a glance. *J Cell Sci*. 2010; 123:1819–1823. [PubMed: 20484662]
- Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*. 1998; 391:806–811. [PubMed: 9486653]
- Foda BM, Singh U. Dimethylated H3K27 is a repressive epigenetic histone mark in the protist *Entamoeba histolytica* and is significantly enriched in genes silenced via the RNAi pathway. *J Biol Chem*. 2015; 290:21114–21130. [PubMed: 26149683]
- Ghildiyal M, Zamore PD. Small silencing RNAs: an expanding universe. *Nat Rev Genet*. 2009; 10:94–108. [PubMed: 19148191]
- Gilchrist CA, Hout E, Traipaidze N, Fei Z, Crasta O, Asgharpour A, Evans C, Martino-Catt S, Baba DJ, Stroup S, Hamano S, Ehrenkaufer G, Okada M, Singh U, Nozaki T, Mann BJ, Petri WA Jr. Impact of intestinal colonization and invasion on the *Entamoeba histolytica* transcriptome. *Mol Biochem Parasitol*. 2006; 147:163–176. [PubMed: 16569449]
- Haque R, Huston CD, Hughes M, Hout E, Petri WA Jr. Amebiasis. *N Engl J Med*. 2003; 348:1565–1573. [PubMed: 12700377]
- Huguenin M, Bracha R, Chookajorn T, Mirelman D. Epigenetic transcriptional gene silencing in *Entamoeba histolytica*: insight into histone and chromatin modifications. *Parasitology*. 2010; 137:619–627. [PubMed: 19849886]
- Hutvagner G, Simard MJ. Argonaute proteins: key players in RNA silencing. *Nature reviews Mol Cell Biol*. 2008; 9:22–32.
- Isakov E, Siman-Tov R, Weber C, Guillen N, Ankri S. Trichostatin A regulates peroxiredoxin expression and virulence of the parasite *Entamoeba histolytica*. *Mol Biochem Parasitol*. 2008; 158:82–94. [PubMed: 18191469]
- Johnstone RW, Licht JD. Histone deacetylase inhibitors in cancer therapy: is transcription the primary target? *Cancer Cell*. 2003; 4:13–18. [PubMed: 12892709]
- Ketting RF. The many faces of RNAi. *Dev Cell*. 2011; 20:148–161. [PubMed: 21316584]
- Lohia A. The cell cycle of *Entamoeba histolytica*. *Mol Cell Biochem*. 2003; 253:217–222. [PubMed: 14619972]
- Mirelman D, Anbar M, Bracha R. Epigenetic transcriptional gene silencing in *Entamoeba histolytica*. *IUBMB life*. 2008; 60:598–604. [PubMed: 18493998]
- Morf L, Pearson RJ, Wang AS, Singh U. Robust gene silencing mediated by antisense small RNAs in the pathogenic protist *Entamoeba histolytica*. *Nucleic Acids Res*. 2013; 41:9424–9437. [PubMed: 23935116]
- Pearson RJ, Morf L, Singh U. Regulation of H₂O₂ stress-responsive genes through a novel transcription factor in the protozoan pathogen *Entamoeba histolytica*. *J Biol Chem*. 2013; 288:4462–4474. [PubMed: 23250742]
- Pompey JM, Morf L, Singh U. RNAi pathway genes are resistant to small RNA mediated gene silencing in the protozoan parasite *Entamoeba histolytica*. *PloS One*. 2014; 9:e106477. [PubMed: 25198343]

- Rosato RR, Grant S. Histone deacetylase inhibitors in cancer therapy. *Cancer Biol Ther.* 2003; 2:30–37. [PubMed: 12673114]
- Vance V, Vaucheret H. RNA silencing in plants--defense and counterdefense. *Science.* 2001; 292:2277–2280. [PubMed: 11423650]
- WHO. WHO/PAHO/UNESCO report. *Epidemiol Bull*; A consultation with experts on amoebiasis; Mexico City, Mexico. 28–29 January, 1997; 1997. p. 13-14.
- Zhang H, Alramini H, Tran V, Singh U. Nucleus-localized antisense small RNAs with 5'-polyphosphate termini regulate long term transcriptional gene silencing in *Entamoeba histolytica* G3 strain. *J Biol Chem.* 2011; 286:44467–44479. [PubMed: 22049083]
- Zhang H, Ehrenkaufer GM, Manna D, Hall N, Singh U. High Throughput Sequencing of *Entamoeba* 27nt Small RNA Population Reveals Role in Permanent Gene Silencing But No Effect on Regulating Gene Expression Changes during Stage Conversion, Oxidative, or Heat Shock Stress. *PLoS One.* 2015; 10:e0134481. [PubMed: 26248204]
- Zhang H, Ehrenkaufer GM, Pompey JM, Hackney JA, Singh U. Small RNAs with 5'-polyphosphate termini associate with a Piwi-related protein and regulate gene expression in the single-celled eukaryote *Entamoeba histolytica*. *PLoS Pathog.* 2008; 4:e1000219. [PubMed: 19043551]

Highlights

1. The RNA interference (RNAi) trigger-induced gene silencing functions using portions of an *Entamoeba histolytica* gene
2. *EhROM1* and *EhMyb* were silenced sequentially or in one step using a trigger silencing approach.
3. This optimization of RNAi trigger silencing will be useful for future genetic studies.

Fig. 1.

Trigger silencing of *Entamoeba histolytica* rhomboid protease 1 gene (*EhROM1*) using variable triggers and short portions of the gene coding regions. Fusion of triggers (**EHI 197520** or **EHI 048600** genes) to the first 537 bp of the ROM1 gene or to the entire ROM1 coding region induced silencing of the chromosomal copy of ROM1 in the presence of 6 µg/ml of G418. (A) Schematic depicts the silencing cassettes used in the transfection. (B) Reverse transcription PCR (RT-PCR) analysis of the *EhROM1* transcript level in wild type, 04T-FL-ROM1, 04T-537ROM1 and 19T-537ROM1 cell lines indicated that the level of ROM1 transcript is significantly decreased in all cell lines which contain the silencing cassette. Control PCR for the **EHI 199600** gene indicated equivalent loading in each lane. (C) Northern blot analysis shows the level of induced anti-sense (AS) small (s)RNA populations for the ROM1 gene in generated cell lines. **EHI 118130** AS sRNA serves as a loading control. gDNA, genomic DNA made from trophozoite cultures in our laboratory.

Fig. 2.

Long term silencing of the *Entamoeba histolytica* rhomboid protease 1 gene (*EhROM1*) gene despite removal of the trigger plasmid (T). (A) Reverse transcription PCR (RT-PCR) analysis of *EhROM1* in wild type, 04T-FL-ROM1, 04T-537ROM1 and 19T-537ROM1 cell lines shows that ROM1 silencing persists in cell lines after drug and trigger plasmid removal. **EHI 199600** transcript level serves as a loading control. (B) Northern blot analysis displays the continuous production of ROM1 anti-sense small RNAs (AS sRNA) in cell lines after drug removal. **EHI 118130** AS sRNA serves as a loading control. gDNA, genomic DNA, made from trophozoite cultures in our laboratory.

A

B

C

Fig. 3.

Silencing *Entamoeba histolytica* rhomboid protease 1 gene (*EhROM1*) and Myb (*EhMyb*) genes concurrently using a gene fusion trigger construct. (A) Schematic depicts a silencing cassette including 132 bp of **EHI 197520** trigger followed by the first 537 bp of the coding sequence of the ROM1 gene fused to the full-length (FL) coding sequence of the Myb gene. (B) Reverse transcription PCR (RT-PCR) analysis shows the expression levels of *EhROM1* and *EhMyb* in wild type cells and the generated 19T-537ROM1-FL-Myb cell line. Levels of ROM1 and Myb mRNA both decrease in the cell line with the silencing cassette.

EHI 199600 was used as the loading control. gDNA, genomic DNA, made from trophozoite cultures. (C) Northern blot analysis shows the generation of anti-sense small

RNA (AS sRNA) populations for both ROM1 and Myb in transfected cells. **EHI 118130**
As sRNA served as a loading control.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 4. Long-term silencing of the *Entamoeba histolytica* rhomboid protease 1 (*EhROM1*) and *EhMyb* genes despite removal of the trigger plasmid. (A) Reverse transcription PCR (RT-PCR) analysis of *EhROM1* and *EhMyb* transcript levels in wild type cells and the 19T-537ROM1-FL-Myb cell line indicating the down-regulation of both ROM1 and Myb expression in the cell lines that have lost the silencing cassette (no plasmid). **EHI_199600** was used as a loading control. gDNA, genomic DNA, made from trophozoite cultures. (B) Northern blot analysis confirms the continuous induction of both ROM1 and Myb anti-sense small RNA (AS sRNA) in the cell lines without silencing constructs. **EHI_118130** AS sRNA served as a loading control.

Fig. 5.

Sequential silencing of *Entamoeba histolytica* rhomboid protease 1 (*EhROM1*) and *EhMyb* genes using the trigger approach. Reverse transcription PCR (RT-PCR) analysis of *EhROM1* and *EhMyb* in wild type cells and the 19T-FL-Myb cell line shows that the level of ROM1 and Myb mRNA are reduced in the cell line with the silencing cassette.

EHI 199600 expression level served as a loading control (A) in the presence and (B) in the absence of 6 µg/ml of G418. gDNA, genomic DNA, made from trophozoite cultures.

Fig. 6.

Inhibition of deacetylation could not reverse gene silencing induced by the trigger system. *Entamoeba histolytica* trophozoites with long-term gene silencing were treated with histone deacetylase (HDAC) inhibitors at the indicated concentrations of trichostatin A (TSA) for 48 h or nicotinamide for 24 h. Reverse transcription PCR (RT-PCR) analysis of cells knocked down for ROM1 and treated with TSA (A) and nicotinamide (B) at the indicated concentrations. EHI_199600 expression level served as a loading control.