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The Role of RNA Interference in the Control of Leishmania RNA virus 1 Infection

Erin Acino Brettmann
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The Role of RNA Interference in the Control of

*Leishmania RNA virus 1* Infection

By

Erin Acino Brettmann

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

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Erin Brettmann
ABSTRACT OF THE DISSERTATION

The Role of RNA Interference in the Control of

*Leishmania RNA virus 1* Infection

By

Erin Acino Brettmann

Doctor of Philosophy in Biology and Biomedical Sciences

Molecular Cell Biology

Washington University in St. Louis, 2017

Professor Stephen M Beverley, Chair

The presence of *Leishmania RNA virus 1* (LRV1) in parasites of the *Leishmania* (*Viannia*) subgenus increases the virulence of the parasite in mouse models of leishmaniasis and is correlated with treatment failure, relapse, and the development of mucocutaneous disease in humans. LRV1 is not shed or infectious; rather, the infection is persistent, and as yet it is unknown how the parasite controls virus levels. Many eukaryotic organisms use RNA interference (RNAi) to limit virus replication, and *Leishmania* (*Viannia*) parasites have an active RNAi pathway. To determine whether *Leishmania* are capable of using RNAi to control LRV1, we sequenced sRNAs from LRV1-containing *L. braziliensis* and *L. guyanensis* and found that these cells have abundant LRV1-derived sRNAs. Further, I targeted LRV1 using an RNAi transgene in these species, which resulted in a loss of virus. Together, these data suggest that RNAi can limit LRV1 replication. In contrast, knockout of the RNAi effector protein gene *Argonaute1* resulted in only a small increase in LRV1 levels, as opposed to the expected dramatic increase. While we did not find evidence of a role for *Dicer1/2* or *Piwi* in control of
LRV1, we cannot rule out that such a role exists. These studies suggest that RNAi may play a role in control of LRV1, but that other mechanisms may contribute more or be redundant. In addition to these studies, I also developed a new genetic tool for the manipulation of *Leishmania* in the laboratory. These “popout constructs” use GFP expression to facilitate the removal of the construct after it has been integrated into the parasite genome, and will allow short-term expression of genes and RNAi transgenes in *Leishmania (Viannia)* species. Finally, I present investigations into the effect of RNAi transgenes on parasite biology and virulence. I found that the presence of an RNAi transgene impairs knockdown of an unrelated target, results in an accumulation of stable dsRNA and transposable element transcripts, and may increase parasite virulence. These findings suggest that caution is warranted when using these constructs.
Chapter 1: Introduction
Preface

The first draft of this chapter was written by EAB. Comments from SMB were incorporated into the final version, presented here.
1.1 – Leishmaniasis: a global health threat

Leishmaniasis is a group of diseases caused by the protozoan parasite *Leishmania* and spread by sand flies that occurs in tropical and sub-tropical regions around the world (1), with nearly a billion (2) to 1.7 billion (1) people at risk for infection. Official figures compiled by the World Health Organization total nearly 300,000 new infections every year and 20,000-40,000 deaths (3). Underreporting is rampant, however, and true case numbers are likely to be much higher (3, 4). Further, large numbers of asymptomatic infections go unrecognized and untreated (5, 6). While strategies such as indoor spraying and insecticide-treated bed nets show promise in limiting cases of leishmaniasis (7, 8), they require a high rate of compliance (9), and resistance of the sand flies to insecticides is increasing (10). Situations of mass displacement, such as the civil war in Sudan and current Syrian refugee crisis, frequently result in outbreaks of leishmaniasis (11), sometimes with huge loss of life (12). Infections are likely to increase in the future as urban sprawl and deforestation increase contact of people to infected sand flies (13–15). Further, climate change is likely to increase the range of the sand fly vector and reservoir hosts into areas that previously had no known transmission (16), including the United States (17). Indeed, while cases are still rare, there are increasing reports of autochthonous transmission of leishmaniasis in Texas and Oklahoma (18).

Leishmaniasis has three main disease phenotypes, which correlate largely with infecting species. In the relatively mild cutaneous disease (CL), ulcerating skin lesions ultimately heal, leaving significant scarring. In mucocutaneous disease (MCL), parasites metastasize to and cause destruction of the mucous membranes of the nose, throat, and mouth (19). MCL can occur simultaneously with a cutaneous lesion, or can present years to decades later, even after apparently-successful treatment of the cutaneous lesion (20). In both CL and MCL, the resulting
disfigurement can have substantial social repercussions (21). In visceral disease, parasites metastasize to the lymphatic and vascular systems and cause hepatosplenomegaly, anemia, and ultimately death when left untreated (22).

While attempts to create a vaccine to prevent leishmaniasis are underway (23, 24), there is not one currently available. Chemotherapies for the treatment of leishmaniasis leave much to be desired: they are expensive, cannot be delivered orally, have significant toxicities, and/or require cold storage, all of which serve to limit their usefulness in the healthcare settings where they are most needed (reviewed in (25–27)). Additionally, more severe cases of MCL appear to be refractory to standard treatment (20), and recurrence is common (28). As metastasis of parasites from the site of the sand fly bite is required for the progression to MCL, it is possible that prevention of metastasis could improve cure rates and prevent the necessity of further or more intense treatment regimens.

1.2 – Leishmania life cycle and infection of the mammalian host

*Leishmania* parasites are digenetic, with cycles of replication in two hosts: the sand fly midgut and the mammalian macrophage. When a sand fly bites an infected mammal, parasites are ingested with the bloodmeal and differentiate into the replicative procyclic promastigote stage (reviewed in (29, 30)). Over time, these differentiate into non-dividing, infectious metacyclic promastigotes. When the infected sand fly bites another mammal, the metacyclic promastigotes are regurgitated into the wound (31). They are phagocytosed by endocytic immune cells such as macrophages, neutrophils, and dendritic cells, where they differentiate into the amastigote stage, replicate within the phagolysosome, and infect neighboring cells (32, 33).

The mammalian host immune response to *Leishmania* infection plays a large role in disease progression and parasite clearance. For example, increasing levels of TNF-α and IFN-γ
production are correlated with increasing disease severity. Patients with subclinical infections have very low IFN-γ and TNF-α production compared to patients with CL disease (34), while patients with MCL have elevated levels of these cytokines compared to patients with CL. Modulation of this response could serve as a therapeutic tool for the treatment of leishmaniasis or the prevention of disease progression to MCL. One parasite factor that modulates the host immune response is *Leishmania RNA virus*, which will be explored in further detail through this work.

### 1.3 – Viruses of microbes – *Leishmania RNA virus and the Totiviridae*

*Leishmania* parasites can be infected with a virus of the *Totiviridae* family, *Leishmania RNA virus* (LRV) (35). Within the *Leishmaniavirus* genus, LRV1 occurs in New World parasites of the *Leishmania (Viannia)* subgenus (36–38), including *L. braziliensis* and *L. guyanensis*, while LRV2 occurs in Old World *Leishmania (Leishmania)* parasites (39–41), including *L. major* and *L. aethiopica*. This work will focus on LRV1 infecting *L. braziliensis* and *L. guyanensis*.

LRV1 consists of a dsRNA genome (42) approximately 5.3 kb in length that encodes only two proteins, a capsid protein and an RNA-dependent RNA polymerase (RDRP), in overlapping open reading frames (43). It is located in the parasite cytoplasm (35) and assembles into ~30nm viral particles that can be purified by sucrose (35) or CsCl (44) gradient. A related virus from the family *Totiviridae* found in *Saccharomyces cerevisiae*, L-A virus, is the type species of the *Totiviridae* family, and has been characterized much more extensively than has LRV. Due to the similarities between the two, it is assumed that the replication cycle of LRV matches that of L-A (reviewed in (45)) (Figure 1). Where direct evidence exists for LRV, I have noted it. Briefly, the RDRP transcribes a plus-strand transcript from the dsRNA genome
contained within the capsid and extrudes it into the cytoplasm (42, 46), where its genes are translated by host translational machinery. This single transcript encodes both the capsid and RDRP, and the RDRP is likely translated via frameshift as a fusion protein (39, 43, 47). Finally, the plus-strand transcript is encapsidated (48, 49) and used by the RDRP as a template for synthesis of the minus-strand (42), regenerating the dsRNA genome. Importantly, the dsRNA genome remains enclosed within and protected by the protein capsid, and only (+)-strand mRNA is exposed to the contents of the cytoplasm.

Like most viruses of the Totiviridae family, LRV is not shed in culture, nor are viral particles infectious (48). Only a select few viruses of this family, such as Giardiavirus (50) and piscine myocarditis virus (51) have extracellular routes of infection. Fungal totiviruses, such as L-A, can be transmitted by cytoplasmic fusion, as during mating (52). Leishmania genetic exchange can occur at low frequency during sand fly passage (53, 54). Crosses done in collaboration with David Sacks between L. major strains 5-ASKH (containing LRV2) and Sd resulted in hybrid progeny containing LRV2, suggesting that LRV2 can likewise be transmitted by mating (Owens, Lye, Inbar, Akopyants, Sacks, Beverley, unpublished data). Attempts to super-infect parasites with LRV1 or to introduce LRV1 into parasites not naturally containing the virus, however, have not produced persistent virus infections (48, 55). Prior to this work, there had been one report in the literature of loss of LRV1 from an infected L. guyanensis strain (56); however, the mechanism by which this occurred is unclear, and it could not be replicated by the same or other laboratories. Because LRV1 cannot be introduced into uninfected parasites, and cannot be reliably “cured” from strains that contain it, research on its biological effects has relied on either non-isogenic, naturally-occurring isolates or on the single isogenic L. guyanensis strain that arose cryptically. The field would benefit from a reliable method with which to
generate LRV1+ lines from an LRV-negative strain, or LRV-negative lines from a strain containing LRV1. I address this topic in Chapter 2 of this dissertation.

1.4 – The role of LRV1 in parasite biology & virulence

Despite its long history in the literature, very little was known about the effect of LRV1 on parasite biology. Recently, it has become clear that LRV affects the pathogenesis of *Leishmania*, and therefore has large implications for human disease. Much of the mechanistic work has been done in mouse models of infection using the single isogenic pair of *L. guyanensis* lines. Experiments were either *in vitro* infections of murine bone marrow-derived macrophages or *in vivo* injection of parasites into hind footpad of mice.

*L. guyanensis* parasites that contain LRV1 produce larger lesions with higher parasite burdens than do LR1-negative strains in WT C57BL/6 mice (57). Similarly, LRV1+ parasites induce higher pathology and parasite burden in IFN-γ knockout mice, and metastasize earlier in the course of infection than do LRV1-negative parasites (58). LRV1+ parasites also stimulate the release of a number of proinflammatory cytokines, including TNF-α, CXCL-10, IL-6 (57), and IL-17A (58). Recent work by Eren et al. found that the presence of LRV1 induced the expression of miR155, which led to phosphorylation of Akt and promoted the survival of macrophages (59). The induction of cytokine release, miR155 expression, and Akt phosphorylation all depended on Toll-like receptor 3 (TLR3) in the mouse (57–59), which senses dsRNA. Induction of miR155 appears to occur via a separate downstream pathway than the LRV1-induced hyperinflammatory response, however, as ΔmiR155 macrophages display a cytokine profile similar to that of WT macrophages upon infection with LRV1+ parasites (59). These findings provide direct mechanistic links between LRV1 and increased virulence of the LRV1-containing parasites.
Research into the implications of LRV on human disease relies on correlative associations in clinical samples. The impact of LRV1 on the progression of disease to MCL is not black-and-white, as many factors, both in the parasite and in the human host, contribute to metastasis (60). In some regions, MCL is relatively common, but LRV is absent (61, 62); in these cases, MCL is clearly triggered by other factors. Surveys in other regions have given conflicting results, with one finding no correlation between the presence of LRV1 and MCL (63), and another finding LRV1 in a higher percentage of cases of MCL than cases of CL (64). Consistently, though, studies have found that the presence of LRV1 correlates with treatment failure (63, 65) and relapse (65). Some of these correlative studies show similar immune responses to LRV1 in patients as were seen in mice. In one study, patients infected with LRV1+ \textit{L. guyanensis} had higher levels of IL-17A and lower IFN-\(\gamma\) in lesion tissue than patients infected with LRV1-negative \textit{L. guyanensis}, as occurred in mice (59). In another, patients infected with LRV1+ \textit{L. guyanensis} had higher levels of intralesional cytokines (65), including IL-6 and CXCL-10.

While the role of LRV1 in promoting MCL disease in humans is unclear, the evidence from mouse studies supporting LRV1’s effect on parasite virulence is strong, and has real-world implications for the treatment of leishmaniasis. There are a number of open questions on the subject, however, that could improve patient treatment. First, we need a diagnostic tool that can identify LRV1 in patient samples at high accuracy and low cost. This would enable us to identify patients that might be at high risk of treatment failure, relapse, or metastasis. Second, we need to understand the mechanisms by which LRV1 is maintained within LRV1. By understanding these mechanisms, we may be able to develop ways to eliminate the virus from the infection and improve treatment outcomes. Third, we need drug treatment regimens that are optimized for
infections containing LRV1. This would decrease the incidence of treatment failure and relapse, and improve quality of life for affected patients. This work focuses on the second need, understanding the mechanisms by which LRV1 is maintained, and uses as its basis an antiviral mechanism common within eukaryotic life, RNA interference (RNAi).

1.5 – RNA interference – RNA-based gene regulation

The RNAi pathway is an RNA-directed posttranscriptional regulation mechanism that is ubiquitous across eukaryotic life, with only isolated instances of loss. It can be divided into three main branches: the short interfering RNA (siRNA)-, micro RNA (miRNA)-, and piwi-interacting RNA (piRNA)-mediated pathways. Each pathway uses a characteristic small RNA to direct the regulation of another RNA. The piRNA pathway is found in the germline of multicellular animals and primarily restricts the mobilization of transposable elements (66). The miRNA pathway is found in both plants and animals, and regulates the translation of mRNAs (67). The siRNA pathway is the most evolutionarily widespread pathway, and uses dsRNA to trigger the cleavage and degradation of a complementary ssRNA (68). The remainder of this work will focus on the siRNA-mediated RNAi pathway, and all references to “RNAi” will refer to this mechanism, unless otherwise specified. siRNA-mediated RNAi begins with the production of siRNAs from long dsRNA by the RNaseIII protein Dicer, which bind to the RNaseH nuclease protein Argonaute (Figure 2). Perfectly-complementary base pairing between the siRNA and a target RNA causes Argonaute to cleave the target RNA, triggering its degradation.

1.6 – RNAi as an antiviral defense

Many organisms, including fungi (69), plants (70), and insects (71), use RNAi as an antiviral defense pathway. Upon infection with an RNA virus, Dicer cleaves viral dsRNA into
viral siRNAs (vsiRNAs) which are loaded onto Argonaute. Base pairing between the vsiRNAs and the viral mRNA guides Argonaute to the transcript, which is cleaved and subsequently degraded. RNAi is well-suited to the task of defense against viruses for two main reasons: 1) it is sequence independent – any dsRNA will trigger a response, and mutation of the virus does not enable it to evade the RNAi pathway; and 2) it is specific – vsiRNAs only target perfectly complementary sequences for degradation, leaving host genes unaffected. Mutations in RNAi pathway genes in these organisms result in increased susceptibility to viruses, virus titers, and/or lethality (72–75), demonstrating their importance in the control of viruses. The role of RNAi in the control of viruses in mammalian somatic cells is not settled; some studies have found RNAi to not be antiviral in mammalian somatic cells (76), while others have found evidence suggesting mammalian RNAi may serve an antiviral function (77–79).

In response to the pressure by the RNAi pathway, most, if not all, RNA viruses infecting organisms with an antiviral RNAi pathway encode an RNA Silencing Suppressor (RSS). These suppressors operate through diverse mechanisms: some bind to viral RNA or siRNA, preventing cleavage by Dicer or Argonaute (80, 81); others directly interact with and inhibit RNAi pathway proteins (82, 83); other viruses produce high levels of subgenomic transcripts that suppress RNAi (84).

1.7 – RNAi in Leishmania and the trypanosomatids

RNAi is highly conserved throughout eukaryotic life, with only infrequent instances of loss. Some of these losses occurred in the trypanosomatid lineage, and include Trypansosoma cruzi and the Leishmania (Leishmania) subgenus of parasites. Other trypanosomatids, including T. brucei and Leishmania (Viannia) species, have active RNAi pathways (85, 86) (Figure 3). RNAi was first identified within the trypanosomatid lineage by the Ullu and Tschudi laboratories
when the introduction of an inverted repeat containing sequence from the alpha-tubulin transcript resulted in a “FAT” cell phenotype and marked reduction in alpha-tubulin mRNA (86). They identified a protein in the *T. brucei* genome with the PAZ and Piwi domains indicative of Argonaute, which they showed to bind siRNAs and to be required for RNAi, and which they named Argonaute1 (*AGO1*) (87). It was soon clear that *T. cruzi* and *L. major* did not have functional RNAi pathways (88, 89), and these organisms also lacked the *AGO1* gene (90). Further database mining from the Ullu and Tschudi labs identified two RNaseIII domain genes present in *T. brucei* but not *T. cruzi* or *L. major* and showed that these genes encoded Dicer proteins (91, 92). Peacock et al. identified an *AGO1* and *Dicer* gene in the completed *L. braziliensis* genome sequence (93), and our lab showed that *L. braziliensis* and *L. guyanensis* both have functional RNAi pathways (85). A final attempt to identify new RNAi pathway genes in trypanosomatids by comparative genomics returned only the three previously-identified components and two additional genes, RNA Interference Factor (RIF) 4 and RIF5, an exonuclease required for loading of siRNAs onto Argonaute and a Dicer 1 cofactor, respectively (94). Interestingly, while *AGO1*, *Dicer1*, *Dicer2*, *RIF4*, and *RIF5* are found only in RNAi-proficient species, a second Argonaute protein containing a readily-identifiable PIWI domain and a highly divergent PAZ domain (95) is also present in the RNAi-null *T. cruzi* and *L. (Leishmania)* species (90). This protein was termed “Piwi” because it was initially thought to lack the PAZ domain (90). It is evolutionarily distinct from the mammalian Piwi protein (95), and its role in the RNAi pathway is unclear. It is not essential in the RNAi-null *L. major* (96), but attempts in our lab to knock *PIWI* out in *L. braziliensis* were unsuccessful (Tsang, Anderson, and Beverley, unpublished data). If this result can be shown to be meaningful (rather than technical),
this suggests that Piwi may serve an additional, essential function in RNAi-competent species. The distribution of known RNAi components in trypanosomatids is summarized in Table 1.

Attempts in the Beverley lab to reconstitute the RNAi pathway in *L. major* by introduction of the missing pathway components were unsuccessful (Lye and Beverley, unpublished data). While technical issues may contribute to this, it also suggests that more pathway machinery has yet to be identified. It is possible that there are additional RNAi components that serve dual functions in RNAi-proficient species. These proteins would still be present in RNAi-null species, but have lost their RNAi functionality, such as may be the case with Piwi. In trypanosomatids, RNAi appears to function in the silencing of active transposable elements and repeats (97–99) which are not present in *Leishmania (Leishmania)* species (93). The lack of active transposable elements in *Leishmania (Leishmania)* species likely removed the selective pressure maintaining the activity of the RNAi pathway in these species. Until now, the role of the parasite RNAi pathway in control of viruses has not been investigated.

1.8 – Toolkit for the study of *Leishmania* biology and virulence

*Leishmania* is a genetically-tractable organism amenable to many manipulations both *in vitro* and *in vivo*. Procyclic promastigote parasites can be grown in suspension in the laboratory using media supplemented with components for which they are auxotrophic. The *L. guyanensis* and *L. braziliensis* strains used in this work have doubling times of 7-8 hours and reach densities of $10^7 – 10^8$ cells/mL in culture, enabling large amounts of cells to be obtained in a short period of time and small culture volume.

The Beverley lab pioneered the transfection and genetic manipulation of *Leishmania* parasites (100, 101). *Leishmania* can be plated on semisolid media (i.e. agarose plates) to obtain clonal lines, and antibiotics are available for selection of desired mutants. Experiments in this
work utilize the following drug/gene pairs: nourseothricin/SAT, hygromycin B/HYG, blasticidin S/BSD, and puromycin/PAC. Because of these capabilities, a number of genetic manipulations are possible, and will be used in this work.

*Leishmania* parasites do not appear to repair double-stranded break via non-homologous repair to any significant extent (102); instead, homologous recombination is the default DNA repair pathway (103). The field has taken advantage of this feature to stably integrate transfected linear DNA into the parasite genome using regions of homology ranging from 250-1,000 bp. This method allows for two manipulations that will be used extensively in this work: allelic replacement to generate gene knockouts and ectopic expression from the ribosomal small subunit (SSU) locus.

Non-essential genes can be readily knocked out by replacement with gene knockout cassettes (101), which are targeted to the appropriate gene using regions of homology 5’ and 3’ of the gene of interest (GOI). The regions of homology flank a drug resistance gene (Figure 4A), allowing the desired replacement mutants to be selected. *Leishmania* are “diploid-ish” – most chromosomes are disomic, but cells display substantial aneuploidy of individual chromosomesome, leading to trisomic and even tetrasomic tetrasomic chromosomes (104, 105). Most gene knockouts require two rounds of replacement with separate drug resistance genes, but some genes require further replacements. In addition, since gene regulation occurs post-transcriptionally in *Leishmania* (106, 107), entire chromosomes can be duplicated in response to stress, such as antibiotic pressure (108, 109). Some attempts at gene knockout result in chromosome or whole genome amplification (110–112), which can be a sign that the GOI is essential. Even for non-essential genes, there are factors that can make proper targeting a
challenge, including poorly assembled flanking regions and flanking regions containing repeats. These factors come into play in this work, and will be addressed in Appendix A.

Many species of cultured promastigotes can be transfected with circular plasmid DNAs (episomes), which are maintained extrachromosomally (100, 113, 114) and drive expression of exogenous genes. This is particularly useful for tests of essentiality when attempts to replace a gene fail to yield viable null mutants. In these cases, an episome encoding GFP and the gene to be knocked out (GOI) is transfected into cells under selection conferred by a drug resistance cassette (Figure 4B). The GOI is expressed from the episome while the chromosomal alleles are replaced by homologous recombination, and once a chromosomal null mutant is obtained the drug selection maintaining the episome is removed. The cells are cultured for a time to allow the episome to be lost and then sorted on the basis of GFP expression. If the GOI is not essential, this process will yield viable GFP-dim clones that have lost the episome; if the GOI is essential any GFP-dim cells will not survive or will still contain low levels of episome (115).

The parasites used in this work, *L. braziliensis* and *L. guyanensis*, do not support the maintenance of episomes, likely due to the presence of an active RNAi pathway (85). In these species, a gene is assumed to be essential if repeated attempts to knock it out fail, but succeed when the gene is integrated into (and expressed from) the ribosomal small subunit locus. While this assumption is sound, it has two major drawbacks. First, transfection efficiency is low and homologous recombination is a relatively rare event, and therefore it is possible that too few cells were examined to obtain the desired mutant. Second, there have been instances where genes appeared to be essential by this criteria, but the episome segregation test revealed them to be not essential (116, 117). Therefore, it would be advantageous to have an analogous system for the
Viannia subgenus species to facilitate genetic manipulations, which will be discussed further in Chapter Four.

Another tool that utilizes homologous recombination is the pIR expression system, in which a construct is integrated into the ribosomal SSU locus, which is transcribed at high levels by RNA polymerase I. These constructs contain a drug resistance gene for selection of mutants, sequences enabling proper splicing and translation of genes, and two cloning sites that can each carry a gene to be expressed (Figure 4C). This system was originally developed for the expression of genes, such as luciferase, at high levels, with little clone-to-clone variation. More recently, it is also used to drive transgenic RNAi in Leishmania (Viannia) species.

As described above, species of the Viannia subgenus have an active RNAi pathway that can be harnessed to knock down parasite genes (85). To do this, sequence from the GOI is cloned as an inverted repeat into one of the expression sites of an IR vector (Figure 4D). The sequence is transcribed at high levels by RNA polymerase I, and it is believed that the inverted repeat folds into a long hairpin structure (“stem-loop,” StL). Dicer cleaves this dsRNA StL into siRNAs, which are incorporated into Argonaute and guide cleavage and degradation of the GOI’s transcript. Reduction in target mRNA scales approximately linearly with the length of the sequence in the stem (Lye, unpublished data), but the stem requires a minimum length of approximately 250 nt for appreciable reductions. The caveat of knockdown using StL vectors, as with all RNAi experiments, is that reductions in mRNA do not always generate equivalent (or any) reductions in protein level and therefore do not always result in an observable phenotype.

After the desired mutant parasites have been generated, the effects of the manipulation on parasite virulence can be investigated by infection of laboratory mice. To achieve infection, luciferase-expressing procyclic promastigote parasites are grown to stationary phase in vitro,
which causes them to differentiate into metacyclic promastigotes (118, 119), and injected into the hind footpad of mice. The mice are monitored over the course of infection for luciferase activity, which correlates with the number of parasites present at the site of infection, and the thickness of the footpad, which is a measure of the host immune response. The *L. guyanensis* parasites used in this work cause a self-limiting infection in wild-type C57B6 mice that does not metastasize and ultimately resolves. In contrast, the same parasites in IFN-γ knockout mice cause chronic infections with a relatively high rate of metastasis (58).

### 1.9 – Aims and Scope

LRV1 plays a substantial role in the virulence of *Leishmania* parasites, and a deeper understanding of the mechanisms by which it is maintained and controlled within *Leishmania* could provide targets for the improvement of patient treatment outcomes. As LRV1 is a dsRNA virus and *Leishmania (Viannia)* species have active RNAi pathways, it is logical to suspect that the RNAi plays a major role in control of LRV1. The primary aim of this work is to investigate the relationship between LRV1 and the *Leishmania* RNAi pathway and determine to what extent it functions in control of LRV1.

For RNAi to serve an antiviral purpose, siRNAs must be generated from LRV1 sequence and competent for the targeting and degradation of the LRV1 transcript. In Chapter 2, I investigated whether siRNAs with LRV1 sequence can reduce LRV1 levels in both *L. braziliensis* and *L. guyanensis*, and show that LRV1 can indeed be targeted and eliminated by transgenic RNAi, suggesting that the parasite RNAi pathway may well be antiviral. Further, targeting by RNAi provides a mechanism by which LRV1-negative lines can be generated at will from *Leishmania* isolates. In Chapter 3 I disrupted the *L. guyanensis* RNAi pathway by knocking out Argonaute 1 (*AGO1*) and show that, contrary to predictions, there was little change in the
level of LRV1, suggesting that *AGO1*-dependent RNAi is not the major mechanism by which LRV1 levels are regulated in *Leishmania*. In Appendix A I describe experiments to map the region immediately 3’ of *Argonaute 1* in *L. guyanensis*, facilitating the experiments described in Chapter 3. In Appendix B, I describe a protocol that I developed to isolate genomic DNA from small numbers of *Leishmania* cells, facilitating the validation of transfectants in situations where transfection efficiency was predicted to be particularly low. In Appendices C and D, I describe preliminary attempts to investigate the roles of additional RNAi pathway components and their roles in the control of LRV1.

Investigation of gene function in *Leishmania (Viannia)* species is made more difficult by the inability of these species to support extrachromosomal circular DNAs (85), which are used in *Leishmania (Leishmania)* species in tests of essentiality. In Chapter 4 I present a plasmid system analogous to the above-described episome sort, which I term “pop-outs.” Further, I harness the pop-out system to investigate whether transgenic RNAi has previously-unrecognized consequences to *Leishmania* biology that should be considered during further use of transgenic RNAi. Finally, in Appendix E, I describe experiments investigating the knockdown of flagellar genes in *Leishmania braziliensis*, which occupied my earliest work in the laboratory.

The experiments described in this dissertation add to our understanding of the interactions between *Leishmania* parasites and the RNAi pathway, as well as to our understanding of the possible interactions between viruses and their hosts, more generally. Further, they provide new tools for the future investigations of *Leishmania* biology and gene function.
1.10 – Table Legends

Table 1-1: Summary of known RNAi components in the trypanosomatid parasites.

Components present in a given species are denoted by a “+” while absent components are denoted by a “-“. *L. major* is representative of *Leishmania* subgenus species, while *L. braziliensis* is representative of *Viannia* subgenus species, as indicated in Figure 3.

1.11 – Figure Legends

Figure 1-1: Replication cycle of LRV1.

The dsRNA genome is enclosed within the protein capsid (circle), where the RDRP (arrow shape) synthesizes a plus-strand transcript. The transcript is extruded into the cytoplasm, where it is translated into protein. The transcript in encapsidated, and the RDRP generates a minus strand, yielding dsRNA. Figure modified from (45).

Figure 1-2: Biogenesis of siRNAs.

Long dsRNA is cleaved by Dicer into duplex siRNAs. The siRNA duplex is loaded onto Argonaute, and the passenger strand is ejected. Base pairing between the siRNA and a ssRNA target causes cleavage of the target by Argonaute and subsequent degradation. Figure from (120).

Figure 1-3: Presence and absence of RNAi and LRV1 in select species of *Leishmania* and *Trypanosoma*.

Species are marked with a + when RNAi or LRV1 is consistently present and a – when consistently absent. +/- indicates that isolated occurrences have been identified. For LRV, the species present is indicated.

Figure 1-4: Schematics of constructs used in the genetic manipulation of *Leishmania* parasites.

A) Gene of interest (GOI) knockout by homologous recombination. An allele is replaced
with a gene conferring resistance to a selective antibiotic (here, \( HYG^R \) confers resistance to hygromycin B).

B) Episomes encode a GOI, GFP, and resistance to a selective antibiotic. When cells containing the episome are grown without selection, a portion of cells lose the episome, and can be recovered by single cell sorting on GFP expression.

C) IR constructs integrate into the 18S rRNA small subunit (SSU) locus by homologous recombination. Two sites (A and B) are available for cloning of genes to be expressed. Splice acceptor (SA) sequences enable proper trans-splicing of transcripts. A drug resistance cassette (here, mediating resistance to hygromycin B) enables positive selection of transfectants.

D) Stem-loop (StL) constructs use the pIR backbone to drive RNAi knockdown of a GOI. Sequence from the GOI is cloned into an inverted repeat in the A cloning site using Gateway technology (Invitrogen, Thermo Fisher).

1.12 – References


63. Adaui V et al. (2016) Association of the endobiont doubled-stranded RNA virus LRV1 with treatment failure of human leishmaniasis caused by Leishmania braziliensis in Peru.


85. Lye L-F et al. (2010) Retention and loss of RNA interference pathways in trypanosomatid...


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<th></th>
<th><em>T. brucei</em></th>
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<th><em>L. braziliensis</em></th>
<th><em>L. major</em></th>
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<td>Piwi</td>
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1) Transcript synthesized

2) Proteins translated

3) Transcript encapsidated

4) Minus-strand synthesized

RDRP
Figure 1-2

dsRNA precursor

Dicer

Cleavage

siRNA duplex

Loading

AGO1

Release of passenger strand

Target mRNA

Target degradation
Figure 1-3

<table>
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<th></th>
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<th>LRV</th>
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<td>−</td>
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<td><em>L. braziliensis</em></td>
<td>+</td>
<td>+ (LRV1)</td>
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<td><em>Trypanosoma brucei</em></td>
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</tr>
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</table>
GoI

5' flank  GoI  3' flank

5' flank  HYG^R  3' flank

5' flank  HYG^R  3' flank

**Figure 1-4**

A

B

C

D

18S ribosome small subunit (SSU)

5' SSU

SA  SA  SA

HYG^R

3' SSU

Episome retained

Episome lost

Grow without drug

Sort for GFP expression

GFP fluorescence

# of cells
Chapter 2: Tilting the balance between RNA interference and replication eradicates *Leishmania RNA virus 1* and mitigates the inflammatory response
Preface

EAB designed and performed the majority of experiments, analyzed data, created figures, and wrote the manuscript. SMB supervised the work, assisted in experiment design and data analysis and assisted with manuscript preparation. HZ and CR performed macrophage infection experiments under the supervision of NF. NF and FMK contributed to editing the manuscript. NSA and DMO performed RNA sequencing experiments, and JS and SMB analyzed the data. KLO, L-FL, FMK, and SMH assisted with experiments.

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2.1 – Abstract

Many *Leishmania (Viannia)* parasites harbor the double-stranded RNA virus *Leishmania RNA virus 1* (LRV1), which has been associated with increased disease severity in animal models and humans, and drug treatment failures in humans. Remarkably, LRV1 survives in the presence of an active RNAi pathway, which in many organisms controls RNA viruses. We found significant levels (0.4-2.5%) of small RNAs derived from LRV1 in both *L. braziliensis* and *L. guyanensis*, mapping across both strands and with properties consistent with Dicer-mediated cleavage of the dsRNA genome. LRV1 lacks cis or trans-acting RNAi inhibitory activities, suggesting that virus retention must be maintained by a balance between RNAi activity and LRV1 replication. To tilt this towards elimination, we targeted LRV1 using long-hairpin/stem-loop constructs similar to those effective against chromosomal genes. LRV1 was completely eliminated, at high efficiency, accompanied by a massive overproduction of LRV1-specific siRNAs, representing as much as 87% of the total. For both *L. braziliensis* and *L. guyanensis*, RNAi-derived LRV1-negative lines were no longer able to induce a Toll-like receptor 3-dependent hyper-inflammatory cytokine response in infected macrophages. This is the first demonstration of a role for LRV1 in *L. braziliensis* virulence in vitro, the *Leishmania* species responsible for the vast majority of mucocutaneous leishmaniasis cases. These findings establish the first targeted method for elimination of LRV1, and potentially of other *Leishmania* viruses, which will facilitate mechanistic dissection of the role of LRV1-mediated virulence. Moreover, our data establish a third paradigm for RNAi-viral relationships in evolution, one of balance rather than elimination.
2.2 – Introduction

*Leishmania* is a genus of early-diverging protozoan parasites that cause leishmaniasis in many regions of the world, with an estimated 12 million symptomatic cases, at least 120 million asymptomatic cases, and nearly 1.7 billion at risk (1-5). The disease has three predominant clinical manifestations, ranging from the relatively mild cutaneous form to mucocutaneous disease, where parasites metastasize to and cause destruction of mucous membranes of the nose, mouth, and throat, and fatal visceral disease. Disease phenotypes segregate primarily with the infecting species; however, it is not fully understood which parasite factors affect severity and disease manifestations.

One recently identified parasite factor contributing to disease severity in *L. guyanensis* is the RNA virus *Leishmaniavirus* (6, 7). This virus is a member of the *Totiviridae* family, and consists of a single-segmented dsRNA genome that encodes only a capsid protein and an RNA-dependent RNA polymerase (RDRP) (8, 9). It is most frequently found (as LRV1) in New World parasite species in the subgenus *Viannia* such as *L. braziliensis* (*Lbr*) and *L. guyanensis* (*Lgy*), which cause both cutaneous and mucocutaneous disease (6), but it has also been found sporadically in Old World subgenus *Leishmania* species (as LRV2) (10, 11). Like most totiviruses, LRV1 is neither shed nor infectious, and thus can be viewed as a long-term evolutionary endosymbiont whose activities on the mammalian host arise indirectly through the parasite, rather than by direct infection of the mammalian host by the virus (6). Previous work has shown that mice infected with LRV1-bearing strains of *Lgy* exhibit greater footpad swelling and higher parasitemia than mice infected with LRV1-negative *Lgy* (7). Similarly, macrophages infected *in vitro* with LRV1+ *Lgy* or LRV2+ *L. aethiopica* release higher levels of cytokines, phenotypes that were dependent on Toll-like receptor 3 (7, 10). The assignment of the LRV1
specificity of these phenotypes benefited greatly from the availability of a single isogenic LRV1-free line of Lgy (12). Importantly, recent studies have shown that disease severity is increased in patients infected with LRV1+ Lgy, relative to LRV1-negative parasites (13).

In humans, Lbr is associated with cutaneous leishmaniasis, as well as the larger share of the more debilitating mucocutaneous leishmaniasis (MCL) (14, 15). While in some studies LRV1 was not correlated with MCL (16, 17), in others there was a strong association (6, 18, 19). Recent studies show that LRV1 in Lbr and Lgy clinical isolates correlates with drug treatment failure (16, 20). Thus, while other parasite or host factors may play a significant role in the development of MCL (21, 22), current data support a role for LRV1 in exacerbating the pathogenesis of human leishmaniasis caused by Lbr and Lgy. A similar role in pathogenicity has been proposed for the Trichomonas vaginalis totiviruses (23). In contrast, endobiont viruses in other systems more often impair the host or have no known effect on disease. Hypoviruses of Cryphonectria parasitica are associated with decreased virulence of their fungal host whereas the L-A totivirus of Saccharomyces cerevisiae is not thought to affect pathogenicity, instead contributing to intermicrobial competition (24-27).

Research into the role of LRV1 in Lbr disease is hampered by the fact that animal models are less well developed than for other Leishmania (28) and by the absence of isogenic lines bearing or lacking LRV1. Since reverse genetic systems for Totiviridae do not exist and attempts to stably transfer LRV1 have proven unsuccessful (29), we asked whether RNA-interference (RNAi) could be used to generate LRV1-free isogenic isolates. Unlike Old World Leishmania, species of the Viannia subgenus, including Lbr and Lgy, retain an active endogenous RNAi pathway (30). The RNAi pathway converts double-stranded RNA into siRNAs, which trigger the degradation of an mRNA with complementary sequence (31). Importantly, the RNAi pathway
acts as a defense against RNA viruses in plants and some animals, leading to great reductions or complete elimination (32, 33). Further, introduction of RNAi pathway proteins from Saccharomyces castellii into the naturally RNAi-null S. cerevisiae resulted in greatly decreased levels of persistently-infecting L-A totivirus (26). In mammals, siRNA-mediated RNAi activity appears to play a smaller direct role in antiviral responses in adult mice (34, 35), although evidence of a direct response has been found in embryonic stem cells and young animals (36, 37).

Here we explore further the interactions of the RNAi pathway with LRV1 in both Lbr and Lgy, and show first that LRV1 is indeed seen by the endogenous RNAi pathway, as judged by the presence of significant levels of antiviral sRNAs. Thus and different than other systems, RNAi and viral replication appear to be balanced. However, by increased siRNA expression RNAi could be used to efficiently eliminate the virus. Importantly, these LRV1 negative transfectants recapitulate the in vitro macrophage cytokine release defect seen in naturally-occurring LRV1-negative lines, suggesting that the engineered LRV1-negative isogenic lines will be valuable in studying the role of LRV1-mediated biology and virulence.

2.3 – Results

2.3.1 – Naturally abundant siRNAs directed against LRV1 of L. braziliensis and L. guyanensis

Previous siRNA studies in Leishmania analyzed RNAs using a tagged Argonaute inserted into an ago1- knockout of Lbr M2903, which lacks LRV1 (9, 29, 38, 39). Because the lines bearing LRV1 studied here had not been similarly modified, we sequenced total small RNAs (sRNAs) as an alternative. Lbr siRNAs bear a 5’-P and 3’-OH, reflecting their origin through the action of cellular Dicer nucleases (39), and we used these properties to make siRNA-focused sRNA (<42 nt) libraries for next-generation sequencing (Table S1). For Lgy we chose the
established LRV1+ *Lgy* M4147 strain (7), and three different *Lbr* shown to bear LRV1 by PCR and/or anti-dsRNA antibody tests (40).

For sRNAs from *Lbr* M2903 mapping to the *Lbr* reference genome, read length displayed a biphasic distribution, with a major peak centered around 23 nt (20-26 nt, 77.9% of total mapped reads) and a minor one around 33 nt (30-36nt, 9.4% of total mapped reads) (Fig. 1A, Table S1,S2). The 33 nt peak reads mapped primarily to structural RNA loci (62% of mapped reads; Table S2) similar to a sRNA class described in many eukaryotes including trypanosomes and *Leishmania* lacking the RNAi pathway (41-44). In contrast, reads from the 23 nt peak showed properties similar to AGO1-bound siRNAs (39), including their size and the presence of 1-2 untemplated nucleotides at the 3’ end in about 21% of the reads (Fig. 1A; Table S1). The 3’ untemplated bases likely arise from the action of cellular terminal transferases, as *Leishmania* sp. lack the HEN1 methyltransferase that normally blocks their action (39). When both AGO1-bound siRNAs and the 23 nt sRNA peak reads were mapped to the *Lbr* genome their distributions were very similar, with the vast majority mapping to transposable elements (Figs. 1B, S2; Table S2) (39). We concluded that the 23 nt peak sRNAs (23 nt sRNAs) provides a reasonable proxy for siRNAs.

The properties of sRNAs from the LRV1-bearing *Lgy* M4147 and *Lbr* LEM2700, LEM2780 and LEM3874 mapping to the *Lgy* or *Lbr* reference genomes were similar to those of *Lbr* M2903, including the 23 and 33 nt sRNA peaks, genomic mappings, and the presence and level of 3’ nt extensions in the 23nt sRNAs (Figs. 1, S1; Tables S1 & S2). Importantly, a substantial fraction of sRNA reads obtained from the LRV1+ *Lgy* and *Lbr* lines mapped to the LRV1 genomes, ranging from 0.4-2.5% of the 23nt mapped reads (Fig. 1B, Table S1). Unlike those aligned to the nuclear genome, LRV1-mapped reads showed a single size distribution
centered around 23 nt (Fig. 1A), with about 20% again showing short 3’ extensions (Table S1), typical of \textit{Lbr} siRNAs and 23 nt sRNAs (39). LRV1-mapping 23 nt sRNAs showed no consistent strand- or region-specific biases in all four strains (Fig. S2), suggesting that they likely originated from the action of DICERs on the viral dsRNA genome.

We previously showed that LRV1 does not encode a \textit{trans}-acting inhibitor of RNAi activity (30), and the presence of high levels of LRV1-directed sRNAs similarly suggests that it does not encode a strong \textit{cis}-acting inhibitor. Importantly, the levels of 23 nt sRNAs mapping to LRV1s were in the same range as siRNAs mapping to an efficiently silenced Luciferase reporter (0.4–2.5% vs. 0.8% targeted by long hairpin/stem loop transgene) (30, 39). Thus, LRV1 is able to persist in the face of a significant RNAi response, as judged by 23 nt sRNA levels.

\subsection*{LRV1 can be efficiently targeted by transgenic RNAi}

These data are consistent with a model where RNAi activity and LRV1 replication has achieved a ‘balance’ between viral synthesis and degradation, which might be shifted by increasing or decreasing RNAi activity. With an eye towards virus elimination, we focused on increasing LRV1-targeting siRNA levels through the use of transgenic RNAi methods developed previously (30), in which long hairpin RNA is expressed at high levels from a stem-loop (StL) construct containing LRV1 sequences integrated into the ribosomal RNA locus (Fig. 2A). We targeted regions of LRV1 from the capsid or RDRP ORFs (\textit{Lgy} M4147, \textit{Lbr} LEM2700 and LEM2780), or a region that spanned them (\textit{Lbr} LEM3874), ranging in length from 794 to 1,143 bp (Fig. 2B & Table S3); since the two viral genes reside within the same RNA segment, targeting either should lead to degradation of the entire LRV1 RNA. Since LRV1 sequences diverge substantially between parasite strains (69-90% nt identity), ‘stems’ specific for each species/strain were used. To assess non-specific effects, we integrated an StL construct for an
AT-rich GFP (GFP65 StL), which efficiently silences expression of GFP65 (30). The untransfected parental lines served as LRV1+ controls, and Lbr M2903 or Lgy M4147/HYG (12) served as LRV1-negative controls.

To screen for loss of LRV1, StL transfectants were analyzed by flow cytometry of fixed, permeabilized cells using an antibody raised against the Lgy M4147 LRV1 capsid (45), which cross reacts with Lbr LRV1. For both Lgy M4147 (Fig. 3, top) and Lbr LEM2780 (Fig. 3, bottom), there was a clear separation in capsid staining between the LRV1-positive (red) and LRV1-negative controls (green). While control GFP65 StL lines (purple) had capsid protein levels similar to WT, capsid protein was undetectable in LRV1-targeted StL lines (Fig. 3, light & dark blue), indistinguishable from the LRV1-negative control. This was observed whether the capsid or RDRP was targeted (Fig. 3). Similar results were obtained with LRV1 StL transfectants from Lbr LEM2700 and Lbr LEM3874 (data not shown). In support of the flow cytometry data, western blot analysis with an anti-capsid antibody showed high LRV1 levels in the Lgy parental line and GFP65 StL transfectants, while capsid protein was undetectable in the capsid StL transfectants (Fig. S3).

2.3.3 – StL constructs result in high levels of siRNAs mapping to the LRV1 stem

Despite the insensitivity of LRV1 to ‘natural’ levels of RNAi, as judged by the abundance of 23nt sRNAs, introduction of StL constructs targeting LRV1 resulted in great reduction in LRV1 levels. To understand the basis for this reduction, we analyzed 23 nt sRNA peak reads mapping to the nuclear and LRV1 genomes, for one LRV1 StL transfectant of each species (Fig. 4). Remarkably, the percentage of total 23 nt sRNAs mapping to LRV1 had increased greatly from that seen in the WT parent, from 2.5% to 86.7% for Lgy and from 1.8% to 73.0% for Lbr LEM3874 (Figs. 4A, S1B). Concomitantly, the percentages of 23 nt sRNAs
mapping to the nuclear genome was proportionately reduced, with some variability amongst loci and/or lines (for example, rRNA reads were unchanged in both species, while tRNA reads decreased in *Lgy*; Figs. 4A, S1B). While we did not measure the absolute levels of sRNAs, previous studies show these are tightly controlled by the level of Argonaute 1 and thus are unlikely to differ significantly (39). Essentially all LRV1-mapping sRNAs in LRV1 StL lines now mapped only to the RNAi-targeted ‘stem’ region (Fig. 4B, dark grey), as expected since LRV1 had been eliminated (below). This also argues against the occurrence of ‘transitive’ siRNA formation (46, 47).

The levels of LRV1 23 nt sRNAs (76-87%) in LRV1 StL-transfectants were much greater than seen with siRNAs mapping to the LUC ORF/stem targeted using the same StL transfection construct (0.8%) (39). To rule out the possibility that this arose from reliance on 23nt sRNAs, we analyzed these from a line bearing the LUC StL RNAi reporter used in the siRNA studies (IR2-LUCStL(b)-LUC(a)). For this, 1.14% of the 23nt sRNA peak reads mapped to the LUC ORF/stem, suggesting that use of 23nt sRNAs vs siRNAs did not significantly impact quantitation. To assess the target-specific effects, we compared the results here with those obtained in other studies, quantitating 23 nt peak sRNAs after RNAi StL targeting of a panel of 10 chromosomal genes. For these, 1.5-34% of 23 nt sRNAs mapped to the RNAi-targeted gene, compared to less than 0.02% basally (unpublished data). Thus, the StL-bearing IR vectors generate a high but variable level of sRNAs for all genes tested, with the LUC reporter being at the low end and LRV1 at the high end. This may reflect the fact that while the LRV1 target is typically eliminated by RNAi (Fig. 3 and below), chromosomal RNAi targets continuously transcribe mRNAs. In other organisms, studies have shown that the presence of a cognate target facilitates the turnover of sRNAs; thus, the absence of LRV1 target may lead to higher levels of
siRNAs (48, 49). Future studies may address the factors contributing to the differences in sRNA levels amongst genes and to the very high steady-state levels of LRV1-directed 23 nt sRNAs seen here.

2.3.4 – Complete virus elimination following RNAi of LRV1

RNAi-mediated LRV1 knockdown would be most useful as a tool if it resulted in a complete elimination of LRV1. To achieve a sensitivity beyond that of flow cytometry (~20 fold) or western blotting (~100 fold), we validated a sensitive quantitative RT-PCR assay (qRT-PCR) for LRV1, using strain- and LRV1-specific primers to amplify a region located outside the ‘stem’ regions (Table S4; Fig. 2B). Since the melting temperatures of PCR amplicons are sequence- and length-dependent, comparison of dissociation (melt) curves facilitated discrimination between specific and non-specific amplification.

Because LRV1 copy number was estimated to be ~100/cell (50), a cutoff for classification as LRV1-negative was set at 10^4-fold below WT. Analysis of Lbr qPCR data by the ΔΔC\text{t} method (51) showed that most LRV1 StL transfectants had LRV1 RNA levels more than 10^5-fold lower than WT (Figs. 5A, S5A,B). Raw C\text{t} values for LRV1 StL lines with LRV1-specific primers were indistinguishable from mock cDNA preparations, and ΔC\text{t} values were indistinguishable from those of negative controls. Melt curves show that products seen at C\text{t} arose from non-specific amplification (Figs. 5A, S5A,B; white bars). As expected for control GFP65 StL lines, LRV1 RNA levels were similar to those in WT (Figs. 5A, S5A,B; black bars).

Similar results were obtained with RNAi of LRV1 in Lgy M4147, with most transfectants showing reductions below the 10^4-fold cutoff (Fig. 5B). However, low levels of LRV1 remained in two lines where the RDRP was targeted, approximately 300- to 500-fold less than the parent line (Fig. 5B, black bars); here melt curve analysis suggested these products were LRV1-specific
Alternate primers targeting other regions across the virus gave similar results (data not shown), suggesting the presence of intact LRV1. We hypothesized that this was due to heterogeneity in viral load, with most but not all cells lacking LRV1. In support of this, we generated and showed that all clonal lines arising from one of the “weakly positive” lines were negative for LRV1 by flow cytometry (data not shown) and satisfied the $10^4$-fold cutoff by qPCR (Fig. S5C). The occasionally incomplete LRV1 elimination is consistent with our prior observation that RNAi was somewhat less efficient in Lgy than in Lbr (30). Nonetheless, even for “weakly positive” Lgy transfectants, RNAi was sufficiently efficient for the ready isolation of LRV1-negative lines (Fig. 3, top; 5B; S3).

2.3.5 – LRV1 knockdowns induce less cytokine production in in vitro macrophage infection assays

Previous reports showed that LRV1+ Lgy stimulated the TLR3-dependent release of higher levels of cytokines from bone marrow-derived macrophages (BMDMs) than LRV1-negative strains (7). The availability of defined RNAi-derived LRV1-negative lines now allowed tests of this in Lbr for the first time as well as confirmation of prior results obtained with a single isogenic LRV1- Lgy. Briefly, BMDMs were infected in vitro with LRV1 StL and GFP65 StL Lbr and Lgy transfectants, as well as positive and negative control lines, and the levels of two cytokines known to be induced by LRV1 (TNF-α and IL-6) (7, 10) were measured.

Capsid StL and RDRP StL LRV1-negative lines of both Lbr and Lgy induced significantly lower levels of cytokine production than did the LRV1-positive lines (both parental and GFP65 STL) (Fig. 6, Fig. S5). Additionally, when macrophages from TLR3-deficient mice were infected with Lbr LEM2700, the LRV1-positive parasites no longer elicited higher levels of cytokine release (Fig. S5). Of note, all Lgy LRV1 StL lines induced background levels of
cytokine release, including the two lines that retained low levels of LRV1 (Fig. 5B & 6B, Fig. S5), consistent with the observation that high levels of LRV1 were necessary for cytokine stimulation (7, 10).

2.4 – Discussion

In this study we have characterized the endogenous RNAi response in *Leishmania* bearing the dsRNA virus LRV1, and used these insights to generate virus-negative lines that facilitate the study of the role of LRV1 in parasite biology and host-parasite interactions.

*Leishmania LRV1 and the endogenous RNAi pathway*

We identified two populations of sRNA in *Lbr* and *Lgy*. The less abundant 33 nt sRNAs mapped primarily to genes encoding structural RNAs (Table S2), as seen in other organisms including trypanosomatids (41-44). In contrast, the more abundant 23 nt sRNA fraction exhibited properties similar to authentic, AGO1-bound *Lbr* siRNAs (39), including size, the presence of 3’ untemplated bases at the same frequency (~20%), and mapping primarily to transposable elements and repetitive sequences (Fig 1; Tables S1 & S2). Only 23 nt sRNA reads mapped to the LRV1 dsRNA genome (Fig. 1A), and these also bore 3’ nucleotide extensions at the same frequency, again consistent with an origin via the RNAi pathway (Table S1). Importantly, the levels of 23 nt sRNAs mapping to LRV1 constituted a substantial fraction of total aligned 23nt sRNAs (Fig 1B, Table S1), comparable to those targeting an efficiently-silenced LUC reporter gene (30, 39). Thus, LRV1 can persist in the face of RNAi pressure that gives rise to sRNA levels comparable to that which efficiently silences a chromosomal target gene.

In other organisms, sRNA/siRNA levels provide a gauge of RNAi pathway recognition and targeting of viruses: when RNAi controls virus replication, as in plants, fungi, and insects
(26, 32, 33), high levels of siRNAs accompany viral infections, leading to eradication of the virus. In mammals, quantitatively fewer siRNAs are present, which do not effectively control virus levels, at least in adult somatic tissues (34, 36, 37). In contrast, high levels of siRNA-like 23 nt sRNAs in *Leishmania* suggest an attack on LRV1 by the RNAi pathway, but the virus persists. While many viruses encode *trans*-acting RNAi suppressors mediating their survival (52), this seems unlikely for LRV1. There is no obvious coding potential for this in the compact LRV1 genome, our studies here suggest there is no strong *cis*-acting inhibitory activity, and we showed previously that a luciferase reporter was equally silenced in the LRV1+ and LRV1-negative *Lgy* studied here (30). This suggests a third model where LRV1 is targeted strongly by the RNAi pathway, but the RNAi-mediated degradation is ‘balanced’ by virus replication or other factors. We are currently working to identify which component(s) of the RNAi machinery mediate this balance. While the slicer activity of Argonaute is perhaps the most likely agent, previous studies examining the role of RNAi in control of viruses frequently raise the possibility of Dicer-mediated control as well (53-55). It is likely that the sequestration of the LRV1 dsRNA genome within the capsid may also contribute by limiting the exposure of the LRV1 dsRNA to the RNAi machinery and other degradative pathways. In yeast, *SKI* genes act to prevent deleterious effects of L-A viruses towards its fungal host through alterations in mRNA degradation and/or surveillance (27), and homologous genes for several of these are evident in the *Leishmania* genome.

In other organisms, persistent viruses can also be maintained in the face of an active RNAi pathway, but at considerably reduced levels (26, 56). Over evolutionary time, this strong pressure likely accounts for the inverse relationship in fungi between virus levels and the activity and/or presence of the RNAi pathway, especially when associated with a selective advantage for
viral retention, as seen with the yeast killer factors which are dependent on the L-A virus (26, 57). Similarly, in *Leishmania* we had originally proposed that RNAi pressure would be sufficiently strong as to in some cases provide a driving force for loss of RNAi, in order to maintain LRV1-dependent increases in pathogenicity (30). Given the greater ability of LRV1 to survive in the presence of an active RNAi pathway, our data suggest that the magnitude of this effect may be considerably less than envisioned. However, even small pressure could prove a significant force towards down-regulating pathways impacting on LRV1 levels during evolution.

**RNAi as a tool for generating LRV1-negative lines for biology**

Following the predictions of the ‘balance’ hypothesis, we aimed to increase activity against LRV1 through the increased synthesis of siRNAs targeting LRV1. This proved quite successful; the fraction of 23 nt sRNAs targeting LRV1 rose dramatically in lines expressing StL constructs targeting LRV1 (Figs. 1B & 4A). Correspondingly, the fraction of 23 nt sRNAs mapping to the *Leishmania* genome dropped proportionately, most of which again mapped to TEs and repeats (Fig. 4A). Importantly, LRV1 levels were dramatically reduced for all LRV1 StL transfectants, and in most cases the virus eliminated, as judged by protein and RNA methods (Figs. 3, 5, S3, S5). Targeting of either the capsid or RDRP gene eliminated LRV1, as was expected given that both are encoded by the same RNA (Fig. 2A). Only in *Lgy* were some transfectants found that retained low levels of LRV1, which could reflect less RNAi activity in this species, as was seen with reporter genes (30). However, most transfectants had completely lost LRV1.

Viral infection has been reported for *Giardiavirus* (58), and stable viral transfer for several fungal Totiviruses (59). However, *de novo* infection and stable viral transfer have been unsuccessful with *Lgy* (29), and reverse genetic systems have yet to be reported for any
Totivirus. Therefore, the ability to reproducibly mediate viral cure by RNAi is of great value for biological studies of LRV1. Previous work used an LRV1-negative Lgy which was obtained following transfection with an episomal Leishmania vector expressing resistance to hygromycin B, followed by a long period of growth under selection (12); however, this method seems to have been successful only once. Neither have we succeeded with several ‘stress-related’ treatments that have proven effective in curing mycoviruses, such as yeast L-A (60) (F.M. Kuhlmann and S.M. Beverley, unpublished data). Our studies establish RNAi as a viable strategy for cure of LRV1 and perhaps other viruses in RNAi-competent Leishmania species.

LRV1+ but not LRV1-negative Lgy induce a ‘hyperinflammatory’ cytokine response in infections of BMDMs in vitro, which is TLR3-dependent <sup>2</sup>(Ives et al. 2011)(6, 7). Infectivity tests of mouse BMDMs in vitro showed that RNAi-generated LRV1-negative Lgy lines likewise failed to induce a substantial cytokine response, as shown for two cytokines (TNF-α and IL-6) known to be diagnostic for an LRV1-driven innate immune response. Interestingly, this occurred with RNAi-derived lines where LRV1 loss was substantial but incomplete (RDRP StL c3 & 4; 500- and 300-fold below parental levels, respectively; Fig 5B, 6B, S7), consistent with data from natural Lgy showing low LRV1 levels (7). Thus, a partial reduction in LRV1 levels is sufficient to ameliorate LRV1-dependent virulence, which may facilitate future efforts targeting LRV1 in human disease. Importantly, the continued presence of the integrated StL constructs appeared to have no ‘off target’ effect in the BMDM infections, despite the high levels of transgene-derived 23 nt sRNAs present in these lines; the LRV1 StL “cured” lines induced the release of cytokines at a level similar to that of StL-negative, LRV1-negative controls (Fig. 4), and control GFP65 StL lines that maintained LRV1 induced the
release of cytokines at a level similar to the StL-negative, LRV1+ parent (Fig. 3, 5, 6). Future studies will assess whether this also pertains to other cell types or host infections.

**LRV1-dependent virulence in Leishmania braziliensis**

Previous studies of LRV1-dependent virulence focused primarily on *Lgy*; however, in humans, *Lbr* is associated with the larger share of MCL (14, 15). Our studies extend the generality of LRV1-dependent virulence to *Lbr*, as LRV1+ *Lbr* likewise induce strong TLR3-dependent cytokine responses. These findings are especially important in light of published work on the association of LRV1 with MCL, with mixed results depending on the geographic region and methods used (6, 16-19). Our data show that in *in vitro* infections, LRV1 contributes strongly to the pro-inflammatory phenotype associated with elevated pathogenicity, as seen in *Lgy*. This suggests that in human infections it may be informative to seek for correlations between LRV1 and the severity of CL in *Lbr* infections in future studies. Indeed, recent studies show that LRV1 in *Lbr* clinical isolates correlates with drug treatment failure (16), as was also seen in *Lgy* (20). Thus, while other parasite or host factors may play a significant role in the development of MCL (21, 22), current data now bolstered by our studies of isogenic LRV1+/negative lines support a role for LRV1 in severity of human leishmaniasis caused by *Lbr*.

**2.5 – Materials and Methods**

*Parasites and in vitro culture*

*Lbr* LEM2700 (MHOM/BO/90/AN), LEM2780 (MHOM/BO/90/CS) and LEM3874 (MHOM/BO/99/IMT252 n°3) were from Patrick Bastien (Université de Montpellier), *Lbr* M2903 (MHOM/BR/75/M2903) was from Diane McMahon Pratt (Yale School of Public
Health), and *Lgy* M4147 (MHOM/BR/78/M4147) and its derivative *Lgy* M4147/HYG was from Jean Patterson (Southwest Foundation for Biomedical Research, San Antonio, Texas). Prior to introduction of StL constructs, parasites were transfected with the linear SSU-targeting SwaI fragment from B6367 pIR2SAT-LUC(B) (30), and clonal lines were derived, validated, and used. The luciferase-expressing clone of *Lbr* LEM2780 contained only LRV1-*Lbr*LEM2780(b).

Parasites were grown in fresh Schneider’s Insect Medium supplemented with 10% heat-inactivated fetal bovine serum, 100 μM adenine, 10 μg/mL hemin, 2 μg/mL bipterin, 2 mM L-glutamine, 500 units/ml penicillin and 50 μg/mL streptomycin, and selective drugs as indicated below.

**RNAi Stem-loop Constructs**

Regions of interest from LRV1 were screened using the RNAit target selection tool to ensure that there was no homologous sequence in the parasite genome (61), amplified from cDNA by PCR using KlenTaq-LA polymerase, and cloned into the pCR8/GW/TOPO cloning vector (Thermo Fisher Scientific, Waltham, Massachusetts) using the protocol recommended by the manufacturer and a 20 min ligation. The ‘stem’ segments and PCR primer sequences can be found in Table S3. The ‘stems’ were transferred from the pCR8/GW/TOPO donor vector to the pIR2HYG-GW(A) (B6365) destination vector (which contains sequence from the parasite rRNA locus to enable integration into the genome and inverted LR recombinase sites for the generation of inverted repeat through Gateway© technology) using LR Clonase II (Thermo Fisher) in an overnight reaction at room temperature. Reactions were terminated by incubating with proteinase K for 1 hour at 37°C. Constructs were verified by restriction digest.
Transfections

Stable transfections were performed as previously described (30, 62). Clonal lines were obtained by plating on semisolid media with 50 µg/mL hygromycin B. After colonies formed, cells were grown to stationary phase in 1 mL media and passaged thereafter in 10 mL media with 30 µg/mL hygromycin B.

RNA preparation and quantitative real-time PCR (qPCR)

Total RNA was prepared from log-phase cells dissolved in Trizol reagent (Thermo Fisher) at 3 x 10^8 cells/mL using the Direct-zol kit (Zymo Research, Irvine, California) and eluted in 50 µL of nuclease-free water. The RNA was DNaseI-treated (Thermo Fisher) in a 200 µL reaction using the provided buffer and 20 Units of enzyme for 1 hour at 37 °C, purified using the RNA Clean & Concentrator - 25 kit (Zymo Research), and eluted in 50 µL of nuclease-free water. Reverse transcription was performed using the Superscript III first-strand synthesis kit (Thermo Fisher) according to the manufacturer instructions in a 20 µL reaction containing 0.25 µg purified RNA. Control reactions contained the same amount of RNA but lacked reverse transcriptase enzyme. For qRT-PCR, primers were designed to amplify ~100 bp regions of the LRV1 genome that lie outside the stem regions (Table S4). qPCR reactions were performed with cDNA templates in 20 µL total reaction volume using the Power SYBR Green Master Mix (Thermo Fisher), 5 µL of ten-fold diluted cDNA, and final primer concentrations of 0.2 µM. Reactions were run on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Thermo Fisher). PCR amplification conditions were as follows: 50 °C for 2 min and 95 °C for 10 sec followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. PCR products were confirmed to be specific by melt curve analysis. All experiments were performed in triplicate. Amplification of KMP-11 was used as an internal control to normalize parallel reactions.
Small RNA (sRNA) sequencing

sRNA libraries were generated from total RNA as described (39); briefly, a primer (5'-rApppATCTCGTATGCGCTTTCTCAGCTTD/ddC for all samples except Lgy M4147, which used primer rApppTGGAATTCTCGGGTGCCAAGG/ddC) was ligated first to the 3' end using truncated mutant T4 RNA Ligase (New England Biolabs), and then a second riboprimer (5'-GUUCAGAGUUUCACAGUCCCGACGAGC to the 5’ end with T4 RNA Ligase. cDNA was generated using reverse transcriptase and primer 5'-CAAGCAGAAGACGGCATACGA, and then PCR was performed with this in conjunction with primer 5'-AATGATACGGCGACCACGACAGGTTCTACAGTCCGA. Products corresponding to inserts of 10-50 nt were purified, and taken for sequencing with Illumina HiSeq2500 technology. Sequences have been deposited in the NCBI Short Read Archive (accession SRP082553).

Bioinformatic analysis of sRNAs

The 5′ and 3′ adapter sequences were removed from the sRNA reads, those less than 15 nt removed, and the trimmed reads were mapped to homologous LRV1 or Leishmania genomes (Lbr M2904 (63) or a draft Lgy M4147 genome (Bioproject PRJEB82; accession CALQ0100001 – CALQ01004013)) using Novoalign software [http://www.novocraft.com]; parameters were set as -F ILMFQ; -H; -g 40; -x 6; -R 5; -r; and -e 1000). A random strategy was employed to align reads mapping to multiple regions and hard clipping of low coverage bases at 3’ end was performed. sRNA abundance was assessed directly, or after ‘collapsing’ to remove duplicate reads using algorithms within the fastx toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html ). To annotate transposable or repeated elements, we used RepeatMasker ( [http://www.repeatmasker.org]) to identify known elements.
and/or BLAST to identify regions corresponding to *Leishmania* specific elements (SLACS, TAS, and TATE (63)). The annotations were collected in .bed file format for further use. Coverage was calculated by counting the number of reads that align to each strand of the LRV1 genome.

**LRV1 sequences**

From the sRNA sequences we assembled whole or partial LRV1 contigs, which were confirmed and completed by PCR amplification and sequencing. The sequences for LRV1-*Lbr*LEM2700, LRV1-*Lbr*LEM2780(a) and (b), LRV1-*Lbr*LEM3874, and a revision of the LRV1-*Lgy*M4147 (formerly LRV1-4; (64)) genome sequences were deposited in GenBank (accession numbers KX808483-KX808487).

**LRV1 capsid flow cytometry.**

The development and optimization of this protocol will be described elsewhere (F.M. Kuhlmann et al. in preparation). Briefly, 1 x 10^7 cells were fixed at room temperature (RT) using 2% paraformaldehyde (Thermo Fisher) in PBS for 2 min, and then then incubated in blocking/permeabilization buffer (BPB) (10% normal goat serum(Vector Laboratories) and 0.2% Triton X-100 in PBS) for 30 min, at RT. Anti-*Lgy* LRV1 capsid antibody (45) was added (1:20,000 dilution) and incubated at RT for 1 hr. After two washes with PBS, cells were resuspended in 200 μl BPB with Alexa488-labeled goat-anti-rabbit antibody (Thermo Fisher) (1:2,000 dilution) and incubated 1 hr at RT. After two additional washes with PBS, cells were subjected to flow cytometry and the data analyzed using CellQuest© software (BD Bioscience).

**Western blot, macrophage infections and cytokine assays.**

After an initial wash with PBS, 5x10^7 parasites were resuspended in 100 μL of 1x PBS. 1x10^7 cells (20 μL) were lysed with 7 μL of 4x Laemmli’s gel sample buffer. After heating for 5 min at
95 °C, cell lysates were loaded and separated on a 10% polyacrylamide denaturing gel, transferred to a nitrocellulose membrane and visualized by Ponceau Red staining. The membrane was blocked for 1h in 5% powdered milk diluted in TBS + 0.05% Tween20, incubated overnight at 4 °C with the g018d53 anti-capsid polyclonal antibody (1:5000 in 1% milk TBS-Tween20), washed 4x 15 min at RT, incubated for 1h with an anti-rabbit IgG antibody coupled to peroxidase (Promega) (1:2500 in 1% milk TBS-Tween20), washed again 4x and finally revealed by ECL chemiluminescence (Amersham). Infections of BL6 mouse BMDM and cytokine assays were performed as previously described (7, 10).

Statement identifying institutional and/or licensing committee approving animal experiments.
Animal handling and experimental procedures were undertaken with strict adherence to ethical guidelines relevant in both host countries. These are set out by the SFVO and under inspection by the Department of Security and Environment of the State of Vaud, Switzerland. Experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the United States National Institutes of Health. Animal studies were approved by the Animal Studies Committee at Washington University (protocol #20090086) in accordance with the Office of Laboratory Animal Welfare's guidelines and the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.6 – Acknowledgments
This work was supported in part by NIGMS Cell and Molecular Biology Training Grant GM: 007067 and the Monsanto Excellence Fund for Graduate Fellowships (EAB), NIH grants RO1AI029646 and R56AI099364 (SMB), grants FNRS N° 3100A0-116665/1 and IZ70Z0-131421 (NF), and the Division of Infectious Diseases (FMK). We thank D.E. Dobson for
comments on this manuscript, Florence Prevel for excellent technical support, Jean Patterson (Southwest Foundation for Biomedical Research, San Antonio, Texas) for providing Lgy M4147 and anti-capsid antisera, P. Bastien (U. Montpellier, Montpellier FR) for Lbr strains, and S P. Calderon-Copete for use of draft Lgy genome. Next-generation sequencing was performed at the Washington University School of Medicine, Dept. of Genetics Genome Technology Access Center (partially supported by grants NCI Cancer Center Support P30 CA91842 and NCRR ICTS/CTSA UL1 TR000448).

2.7 – Figure Legends

Figure 2-1: Properties of Lbr siRNAs and sRNAs from Lbr and Lgy.

A) Distributions of read lengths of siRNAs or sRNAs mapping to Leishmania genomes or LRV1s. Shown are 1) AGO1-bound siRNAs (black, solid) or sRNAs (black, dashed) from WT Lbr M2903 mapping to the Lbr genome, 2) Lgy M4147 sRNAs mapped to the Lg genome (blue, solid) or LRV1-LgyM4147 (blue, dashed), and 3) Lbr LEM2780 sRNAs mapped to the Lbr genome (green, solid) or LRV1-LbrLEM2780 (green, dashed).

B) Percentage of 23 nt sRNA reads (20-26nt) mapping to transposable elements (TEs, white), rRNA (red), tRNAs (black), genomic repeat regions (yellow), LRV1 (purple), and other Leishmania genomic regions (other, gray).

Figure 2-2: RNAi constructs for LRV1 elimination.

A) Schematic of an RNAi “stem-loop” (StL) construct. Each construct includes an inverted repeated sequence containing 800-1200 bp of the target gene (gene of interest, GOI) and a hygromycin drug resistance marker (HYG^r). The construct is flanked with sequence of the small subunit ribosomal RNA gene, which allows it to integrate into this
locus, where it is transcribed at high levels. Splice acceptor (SA) signals within the construct allow for polyadenylylation and processing.

B) Schematic showing LRV1 genome organization and regions targeted for RNAi StL constructions (thick bars) from Lbr LEM2700, LEM2780, and LEM3874, and Lgy M4147 targeted by RNAi (white, capsid; gray, RDRP). The locations of qPCR amplicons for quantification of LRV1 levels are shown (thin black bars).

Figure 2-3: Loss of LRV1 induced by RNAi

Anti-capsid flow cytometry analysis of LRV1-knockdown lines in Lgy M4147 and Lbr LEM2780 (top and bottom panels respectively). LRV1 capsid protein levels are unchanged in GFP65 StL lines, while LRV1 StL lines have undetectable capsid protein. Red, parent lines; purple, GFP65 StLs (off target control); green, LRV1-negative controls; light blue, Capsid StL; dark blue, RDRP StL.

Figure 2-4. Overexpression of LRV1-mapping 23 nt sRNAs in LRV1 StL transfectants.

A) Genomic mapping of 23 nt sRNA reads from sRNA sequencing of parental or capsid StL Lgy M4147 (left) or capsid-RDRP StL Lbr LEM3874 (right) mapping to transposable elements (TEs, white), rRNA (red), tRNAs (black), genomic repeat regions (yellow), LRV1 (purple), and other Leishmania genomic regions (other, gray). B) LRV1 mapping of 23 nt sRNA reads from LRV1StL lines described in panel A (Lgy M4147, top; Lbr LEM3874, bottom). Light gray trace indicates parental read distributions; dark gray trace indicates LRV1 StL read distributions. The dark box indicates the region targeted by the StL stems.
Figure 2-5: The LRV1 genome is completely lost in most LRV1-StL transfectants

qPCR analysis of LRV1 RNA levels in LRV1 StL transfectant clones of Lbr LEM2700 (A) and Lgy M4147 (B), along with positive and negative controls (+ and – respectively) and control GFP65 StL transfectants. White bars denote a non-specific qPCR product, while black bars denote an LRV1-specific amplicons (melt curve analysis). Dashed line indicates cutoff for designating a clone as LRV1-negative. Error bars are the standard deviation of three technical replicates for each line.

Figure 2-6: LRV1 elimination results in decreased release of cytokines from infected macrophages.

TNF-α or IL-6 levels were quantified 24h after infection of macrophages with Lbr LEM2780 (A) or Lg M4147 (B) parent, GFP65 knockdown control, or LRV1-StL transfectants. In both studies the LRV1- control was Lgy M4147. For A, results are averages of two-three technical replicates for two clones of each line. For B, results are the averages of two technical replicates for three to six clones of each line. NS, not significant; ** p < 0.01; *** p < 0.0001 by t-test.

2.8 – References


60. Fink GR & Styles CA (1972) Curing of a killer factor in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* 69(10):2846-2849.


2.9 – Supplemental Table Legends

Supplemental Table 2-1: 23 nt siRNA analysis to Leishmania genome and LRV1.

For Lbr, Lbr M2904 reference genome was used, and for Lgy, a M4147 draft genome (in preparation) was used. References for viral genomes are sequences reported in this work.

Supplemental Table 2-2: Distributions of reads mapped to Lbr and Lgy genomes for Ago1-bound siRNAs, 23 nt (20-26 nt) and 33 nt (30-36 nt ) sRNAs.

Supplemental Table 2-3: Primer sequences used to amplify regions of LRV1 for cloning into stem-loop constructs.

Supplemental Table 2-4: Primer sequences used to measure LRV1 RNA levels by qPCR.

2.10 – Supplemental Figure Legends

Supplemental Figure 2-1: Properties of Lbr siRNAs and 23 nt sRNAs from Lbr and Lgy.

This figure shows mapping of the indicated small RNAs after ‘collapsing’ the data to remove duplicate reads. Shown are the percentages of 23 nt sRNA reads (20-26nt) mapping to transposable elements (TEs, white), rRNA (red), tRNAs (black), genomic repeat regions (yellow), LRV1 (purple), and other Leishmania genomic regions (other, gray). A) As in Figure 1B, mappings in WT parent lines. B) As in Figure 4A, comparing
parental lines with capsid StL Lgy M4147 (left) or capsid-RDRP StL Lbr LEM3874 (right).

Supplemental Figure 2-2: Mapping of 23nt sRNA reads (20-26 nt) from the respective parasite lines to LRV1-LbrLEM2700 (A), LRV1-LbrLEM2780(b) (B), LRV1-LbrLEM3874 (C), and LRV1-LgyM4147 (D).

Reads mapping to the positive strand, (light gray); negative strand, (dark gray).

Supplemental Figure 2-3: Capsid protein is lost in Lgy M4147 capsid StL transfectants.

Three GFP65 StL control clones and six Capsid StL clones were evaluated. Top panel: Western blot analysis was performed using g018d53 anti-capsid polyclonal antibody (35). The arrow marks the location of the capsid protein band. Bottom panel: Ponceau S stain of protein gel.

Supplemental Figure 2-4: qPCR analysis of LRV1 RNA levels in LRV1 StL clones of L. braziliensis strain LEM2780 (A), L. braziliensis strain LEM3874 (B), and re-cloned L. guyanensis M4147 RDRP StL c3 (C).

White bars denote a non-specific product; black bars denote an LRV1-specific product (melt curve analysis). Dashed line indicates cutoff for designating a clone as LRV1-negative. Error bars are the standard deviation of three technical replicates for each line.

Supplemental Figure 2-5: Infection of macrophages by Lbr LEM2700 (A) and Lgy M4147 (B).

TNF-α or IL-6 levels were quantified 24h after infection of macrophages with Lbr (A) or Lgy (B) parasites. NI, not infected; LRV1+ or LRV1-, infected with Lgy M4147 LRV1+ or LRV1-negative cells; GFP65 StL transfectants; and RDRP StL or capsid StL transfectants. A) Results are the averages of two technical replicates of two clones per
line. Dark gray bars, experiment performed using WT macrophages; light gray bars, experiment performed using TLR3 knockout macrophages. B) Results are the averages of two technical replicates for each representative clone indicated. Lines found to be LRV1+ by qPCR are denoted by black bars; white bars are lines found to be LRV1-negative by qPCR.
Figure 2-1

A

B

Ago1-bound siRNAs

small RNAs (20-26 nt)

LbrM2903 siRNAs

LbrM2903 sRNAs

LgyM4147 to genome

LgyM4147 to virus

LbrLEM2780 to genome

LbrLEM2780 to virus

Read Length

Percent of reads

15
20
25
30

TEs

tRNA

rRNA

repeats

LRV1

other

Fraction of total

0% 0% 0.4% 1.2% 1.8% 2.5%

LRV1: 0% 0% 0.4% 1.2% 1.8% 2.5%

TE (78.9%)

TE (80.0%)

TE (76.2%)

TE (72.6%)

TE (67.3%)

TE (70.6%)
Figure 2-2

A

\[ P_{rRNA} \]

\[ \text{SSU rDNA} \]

\[ \text{stem} \]

\[ \text{stem} \]

\[ \text{loop} \]

\[ \text{GOI} \]

\[ \text{SA} \]

\[ \text{GOI} \]

\[ \text{SA} \]

\[ \text{SA} \]

\[ \text{SSU} \]

\[ \text{SSU} \]

B

LRV1 genomic organization

\[ \text{Capsid} \]

\[ \text{RDRP} \]

\[ \text{Lbr LEM2700} \]

\[ \text{Lbr LEM2780} \]

\[ \text{Lbr LEM3874} \]

\[ \text{Lgy M4147} \]
Figure 2-3

![Graph showing the expression of various viral proteins in different cell lines. The x-axis represents the capsid protein level, and the y-axis represents the log of the number of cells. The graph compares parental and LRV1 viral proteins in Lbr and LEM2780 cell lines.]
Figure 2-4

A

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B

Log_{10} coverage vs LRV1 genomic position
Figure 2-5

A Lbr LEM2700

B Lgy M4147
Figure 2-6

A Lbr LEM2780

B Lgy M4147

TNF-α (pg/mL)

IL-6 (pg/mL)

NI LRV1 WT GFP StL RDRP StL Capsid StL

NI LRV1 WT GFP StL RDRP StL Capsid StL

60 120 180

10^1 10^2

0 20 40 60

10^1 10^2

0 10 20 30

10^1 10^2

0 10 20 30
## Supplemental Table 2-1

### A. Total Reads.

<table>
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<th>Sample</th>
<th>Total reads (raw)</th>
<th>Total Trimmed Reads aligned to the <em>Leish.</em> genomes + viruses (Percent total)</th>
<th>Aligned 33 nt peak reads to <em>Leishmania</em> (% alignable reads)</th>
<th>Aligned 23 nt peak reads to <em>Leishmania</em> (% alignable reads)</th>
<th>Aligned 23 nt peak reads to LRV1 (% alignable reads)</th>
<th>Percent with 3' extension (<em>Leishmania</em>)</th>
<th>Percent with 3' extension (LRV1)</th>
<th>Genome-mapping reads 3' extension base A-T-C-G (%)</th>
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<tr>
<td><em>Lbr</em> M2903&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29,391,347</td>
<td>19,447,509 (66.2%)</td>
<td>1,827,623 (9.40 %)</td>
<td>15,147,603 (77.9 %)</td>
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<td><em>Lbr</em> LEM2780&lt;sup&gt;b&lt;/sup&gt;</td>
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### B. Collapsed Reads

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<th>Total reads (raw)</th>
<th>Total Trimmed Reads aligned to the <em>Leish.</em> genomes + viruses</th>
<th>Aligned 33 nt peak reads to <em>Leishmania</em> (% alignable reads)</th>
<th>Aligned 23 nt peak reads to <em>Leishmania</em> (% alignable reads)</th>
<th>Aligned 23 nt peak reads to LRV1 (% 23 nt reads)</th>
<th>Percent with 3' extension (<em>Leishmania</em>)</th>
<th>Percent with 3' extension (LRV1)</th>
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<sup>a</sup> Lbr M2903 SSU:IR2-LUCSR.  
<sup>b</sup> The sum of reads mapping to LRV1-LbrLEM2780(a) and (b) are shown, which map quantitatively to similar levels.
**Supplemental Table 2-2**

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Transposable elements, repeats and structural RNAs were classified as defined in the Methods and by Atayede *et al* (26)
### Supplemental Table 2-3

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### Supplemental Table 2-4

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Supplemental Figure 2-1

A

Ago1-bound siRNAs

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<td>TE (78.0%)</td>
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Fraction of total

- TEs
- rRNA
- tRNA
- repeats
- LRV1
- other

LRV1: 0% 2.2% 5.2% 6.5% 10.7%

LRV1: Repeats (43.2%)

LRV1: TE (30.5%)

B

Lgr M4147

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<td>LRV1 (61.2%)</td>
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Fraction of total

- TEs
- rRNA
- tRNA
- repeats
- LRV1
- other

LRV1: 10.7% 61.2% 6.5% 33.9%

LRV1: Repeats (43.2%)

LRV1: TE (39.0%)

LRV1: LRV1 (33.9%)
Supplemental Figure 2-2

A

B

C

D

LRV1 genomic position

LRV1 genomic position

LRV1 genomic position

LRV1 genomic position

Log₂(coverage)
Supplemental Figure 2-3

_Lgy M4147_

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anti-CP

Ponceau staining
Supplemental Figure 2-4

A. *Lbr* LEM2780

B. *Lbr* LEM3874

C. *Lgy* M4147 RDRPStL c3 re-cloned
Supplemental Figure 2-5

**Lbr LEM2700**

- TNF-α (pg/mL)
- IL-6 (pg/mL)

**Lgy M4147**

- TNF-α (pg/mL)
- IL-6 (pg/mL)

Dark gray: WT macrophages
Light gray bars: TLR3 KO macrophages

Black bars: LRV1+
White bars: LRV1−
Chapter 3: Knockout of *Leishmania guyanensis* Argonaute1 has little effect on LRV1 levels
Preface

The majority of experiments in this chapter were designed, performed, and analyzed by EAB. SMB supervised the work and contributed to study design. Suzanne Hickerson assisted with mouse virulence experiments. The first draft of this chapter was written by EAB and the final version presented here incorporates comments from SMB. This chapter is being prepared for publication.
3.1 – Abstract

Many isolates of *Leishmania (Viannia)* parasites harbor the dsRNA virus *Leishmania RNA Virus 1 (LRV1)*, which is associated with increased disease severity in animal models and humans, and with drug resistance and treatment failure in humans. Interestingly, LRV1 survives in the face of an active RNAi pathway, which many organisms use as an antiviral mechanism. To determine whether the *Leishmania* RNAi pathway functions in control of LRV1, we knocked out the *Argonaute1 (AGO1)* gene from the LRV1-containing *L. guyanensis* strain M4147. Loss of AGO1 resulted in a 3.8-fold increase in transposable element transcripts and loss of transgene-driven RNAi, indicating that the mutant parasites are RNAi-deficient. While in many organisms loss of RNAi results in dramatic increases in virus levels, there was only a 35% increase in LRV1 RNA after loss of AGO1. Virulence experiments in a mouse model of infection indicate that, while loss of AGO1 had no effect on virulence in the absence of LRV1, Δ*ago1* lines with LRV1 had 2-fold higher parasitemia and induced lesions 25% larger than WT at the peak of infection. These experiments suggest that AGO1-dependent RNAi is likely not the main mechanism by which LRV1 levels are controlled, and further experiments are needed to identify antiviral pathways in *Leishmania*. Further, they suggest that even small changes in LRV1 level can result in changes in virulence.
3.2 – Introduction

*Leishmania* are single-celled eukaryotes spread by sand flies that cause the disease leishmaniasis. These parasites are endemic to tropical and sub-tropical regions around the world, with an estimated 1-1.5 million infections and 20,000-40,000 deaths per year (1), many of which go unreported. *Leishmania* parasites can harbor a dsRNA virus, *Leishmania RNA Virus* (LRV) (2, 3). This genus of viruses has been found in both New World and Old World *Leishmania* species (4–6), but is most frequently reported as the virus species LRV1 in *Leishmania (Viannia)* subgenus parasites, including *L. braziliensis* and *L. guyanensis*. Infection with LRV1 is persistent, and virus particles are not infectious (7); rather, the virus is transmitted vertically through cell division, and virus strains have in fact co-evolved with their parasite hosts (8). The presence of LRV1 has been shown to increase parasite virulence in mice (9), and to correlate with treatment failure (10, 11), disease recurrence (11, 12), and the development of mucocutaneous disease (13, 14) in humans. Because of the potential for LRV1 to contribute to disease severity, a greater understanding of the interaction between parasite and virus, including mechanisms by which the virus is maintained and controlled, would be beneficial. Disruption or exploitation of these interactions could provide therapeutic benefit in leishmaniasis cases where LRV1 is present.

In many organisms, including insects, fungi, and plants, the RNA interference (RNAi) pathway is known to be antiviral (15–17). RNAi is a conserved pathway found throughout eukaryotes that uses dsRNA to degrade a complementary ssRNA. The RNaseIII family protein Dicer cleaves long dsRNA into short interfering RNAs (siRNAs), which are loaded into the RNA-Induced Silencing Complex (RISC), which contains an Argonaute family protein. Perfect complementarity between the siRNA and a target ssRNA results in cleavage of the target by
Argonaute, and ultimately degradation of the target RNA. RNA viruses have dsRNA stages of their replication cycles, either in the form of a dsRNA genome or replication intermediates, which can serve as a substrate for Dicer. The resulting siRNAs can then target the viral mRNA for degradation. In organisms with antiviral RNAi, mutations to Dicer and/or Argonaute result in increased virus titers and pathology upon virus infection (18–20). Similarly, introduction of Dicer and Argonaute from Saccharomyces castellii into the RNAi-null S. cerevisiae results in the loss of L-A virus infection in the majority of cells (21).

The role of RNAi in control of viruses in protozoa, however, has not been extensively studied. While many Leishmania species have lost the genes required for RNAi, Viannia subgenus parasites have retained an active RNAi pathway (22). Previous work from our lab found small RNAs with LRV1 sequence in LRV1-infected L. guyanensis and L. braziliensis parasites (23), and we further showed that LRV1 is eradicated from L. braziliensis and L. guyanensis parasites when additional pressure is placed on the virus using transgene-driven RNAi (23). Together, these results suggest that the LRV1 genome serves as a substrate for the RNAi pathway to generate siRNAs; further, it suggests that the LRV1 transcript is a target for the RNAi pathway, resulting in siRNA-directed cleavage by AGO1. This RNAi-mediated downregulation of LRV1 levels could be a mechanism by which Leishmania (Viannia) parasites control LRV1 replication.

To further probe the role of the RNAi pathway in LRV1 infection, we knocked out Argonaute 1 (AGO1), which previous work from our lab and others has shown to be required for RNAi activity (22, 24), from LRV1-containing L. guyanensis. Ago1-deficient parasites were viable and lacked active RNAi. These parasites exhibited a small but significant increase in LRV1 levels; concurrently, Δago1 parasites were more virulent in mice when LRV1 was present,
but not in its absence. Therefore, Argonaute 1-dependent RNAi is unlikely to be the primary method of control of LRV1 in *Leishmania guyanensis* parasites.

### 3.3 – Results

#### 3.3.1 – Generation of Δago1 parasites

We began by knocking out the *AGO1* gene in a luciferase (LUC)-expressing clone of *L. guyanensis (Lgy)* strain M4147 (hereafter referred to as “WT”) by successive homologous recombination, replacing each copy of *AGO1* with an antibiotic resistance gene (Figure 1A). Confirmation of proper integrations by PCR is shown in Figure 1B & C. Three double replacement clones were obtained that lacked detectable *AGO1* sequence by PCR in isolated genomic DNA (Figure 2A). Two clones were selected for further experimentation. Both Δago1 clones lacked detectable AGO1 protein by Western blot using an anti-AGO1 antibody raised against *L. braziliensis* AGO1 (Figure 2B, lanes 2 and 5).

To complement the knockout, we integrated an N-terminal Ty1-tagged *LgyAGO1* into the 18S ribosomal RNA locus of *L. guyanensis Δago1* cells. This tag has been used successfully with *LbrAGO1* in *L. braziliensis* and results in functional protein (24). Complemented lines (+AGO1c) expressed AGO1 protein near wild-type levels (Figure 2B, lanes 3, 4, 6, 7). As genes are transcribed at high levels from the 18S rRNA locus (25, 26), one might expect AGO1 protein levels to be increased above WT. Instead, this is evidence of the post-transcriptional gene regulation that is common in *Leishmania* parasites (27, 28). It also suggests that *AGO1* cannot be easily over-expressed in *L. guyanensis*. 
3.3.2 – Δago1 parasites are insensitive to RNAi driven by a transgene

Previous work has shown that loss of AGO1 in L. braziliensis and the related parasite species Trypanosoma brucei results in a loss of RNAi activity (24, 29). In Leishmania (Viannia) species, transgene-driven RNAi is achieved by engineering an inverted repeat that contains sequence of the gene to be knocked down (stem-loop, StL) and integrating it into the small subunit (18S) ribosomal RNA locus of the parasite genome (22). To verify the loss of transgene-driven RNAi activity in the Δago1 lines, we transfected a StL construct targeting the luciferase ORF (LUCStL), which was present in the WT prior to knockout of AGO1. In WT parasites, luciferase activity of the LUCStL-transfected line was 97% lower than that of the untransfected luciferase-expressing line (Figure 3A). This is less than the 300-fold reduction in luciferase activity we have previously shown in L. braziliensis, but in line with results in L. guyanensis that showed a 96% reduction in luciferase activity (22). This further supports our previous conclusions that the RNAi pathway is somewhat less efficient in L. guyanensis than in L. braziliensis. In contrast, luciferase activity was unchanged in Δago1 parasites (Figure 3A), indicating that transgene-driven RNAi activity is lost in these lines.

3.3.3 – Δago1 parasites accumulate transposable element RNA

Previous work in T. brucei (29) and L. braziliensis (24) showed that loss of AGO1 results in the loss of siRNAs derived from transposable elements (TEs) and increased TE transcript levels. To confirm loss of RNAi activity on endogenous targets in our L. guyanensis AGO1 knockout, we evaluated SLACS and TATE transcript levels by sequencing and qPCR.

We depleted ribosomal RNA from total RNA isolated from WT, Δago1, and +AGO1c parasites and subjected these to Illumina sequencing. The fraction of reads mapping to two control genes, glucose-6-phosphate dehydrogenase and the large subunit of RNA polymerase II,
were the same across the three genotypes. The proportion of reads mapping to SLACS was not affected by the loss of AGO1, but the proportion of reads mapping to TATE significantly increased in the Δago1 line and returned to the level of the WT line upon complementation (Figure 3B). To further confirm these findings, we prepared cDNA from total RNA for the WT line, Δago1, and +AGO1c and performed qPCR using primers specific for SLACS and TATE. As with RNAseq, SLACS levels were unaffected by the loss of AGO1, but TATE levels more than tripled in the Δago1 samples compared to the WT line and returned to the level of the WT line with complementation (Figure 3C). Reasons for the discrepancy between SLACS and TATE will be explored in the Discussion.

Because the Δago1 parasites lack AGO1 proteins, have increased transposon levels, and are insensitive to transgene-driven RNAi, we conclude that they are functionally RNAi-deficient. Likewise, because AGO1 protein is expressed at WT levels and TATE RNA returns to WT levels in the +AGO1c complemented line, we conclude that the complemented line is RNAi-proficient.

3.3.4 – Knockout of AGO1 did not deregulate other Leishmania genes

Previous work in L. braziliensis and L. guyanensis found that very few siRNAs originate from protein-coding genes (23, 24), suggesting that RNAi does not regulate these RNAs. We compared the number of RNAseq reads mapping to Leishmania genes for WT and Δago1 lines to determine if any were affected by the loss of AGO1. In this comparison, any data points that diverge from the best fit line are differentially expressed between the two samples. Because the RNA we sequenced was depleted of rRNA, we anticipated that levels would be somewhat variable; therefore, we omitted rRNA genes from our analysis. Further, we omitted LUC from the analysis due to its high expression (data not shown). As expected, AGO1 levels were
dramatically reduced in the Δago1 cells (Figure 4, arrow). While a number of TE elements were more highly expressed in Δago1 cells (Figure 4, red dots), no other gene appeared to be dysregulated in the absence of RNAi (Figure 4, blue dots). For reference, the highly expressed genes in both samples are alpha-tubulin. These data are preliminary, and more clones are currently being sequenced in order to allow statistical analysis of differential expression. The lack of an effect on global gene expression levels following knockout of AGO1 further confirms that RNAi is not involved in the regulation of these loci.

3.3.5 – Minimal change in LRV1 levels on loss of AGO1

In other organisms, mutation of RNAi pathway components results in increases in virus titer by up to 1,000-fold (18, 30–34). If the RNAi pathway plays a role in controlling LRV1 levels, then loss of AGO1 should result in an increase in LRV1 levels. To determine whether this occurred, we analyzed capsid protein level by flow cytometry using a polyclonal antibody raised against the capsid protein found in L. guyanensis strain M4147 (5). The WT line, Δago1, and +AGO1c parasites were indistinguishable from one another (Figure 5a), indicating that a loss of RNAi activity did not result in increased translation of viral protein.

To determine whether LRV1 RNA levels were likewise unchanged, we performed qPCR using primers specific to LRV1. We found that the Δago1 parasites had ~35% higher LRV1 RNA levels than did the WT line, and that this increase was eliminated in the complemented lines (Figure 5b). While the increase in LRV1 levels observed in the knockout was statistically significant, such a small effect is unlikely to indicate a major mechanism of virus control.
3.3.6 – LRV1 capsid knockdown transgene does not reduce LRV1 levels in Δago1 parasites

Previous work from our lab has shown that LRV1 can be eliminated from RNAi-competent *Leishmania* by stable integration into the parasite genome of a stem-loop containing sequence from LRV1 (23). We proposed that this occurred through massive overproduction of siRNAs targeting LRV1. If this were the case, then LRV1 StL constructs should not reduce LRV1 levels in RNAi-deficient Δago1 parasites. Indeed, integration of the CapsidStL construct did not reduce LRV1 protein levels as assessed by flow cytometry, or RNA levels as assessed by qPCR (Figure 6). Instead, Δago1 CapsidStL lines had ~33% higher LRV1 RNA than the WT (RNAi-competent) line, similar to levels seen for the Δago1 alone (Figure 6b).

3.3.7 – Virulence of Δago1 parasites is elevated compared to WT

In the LRV1-negative *L. braziliensis* strain M2903, Δago1 parasites have decreased virulence and metastasis in mouse footpad infections (Hickerson & Beverley, unpublished data). We predicted that loss of AGO1 in *L. guyanensis* would, in the absence of LRV1, result in a similar virulence defect, but that the elevated levels of LRV1 in the Δago1 line would reduce this loss of virulence or even make this line more virulent that the WT line. To untangle the respective contributions of RNAi and LRV1 to virulence, we generated LRV1-negative clones of the WT, Δago1, and +AGO1c lines using the small molecule 2’C-methyladenosine (2CMA). Previous work in the lab has shown that short-term treatment with a low dose of 2CMA leads to a heterogeneous population of cells, some of which have lost LRV1 (35). This population can be sub-cloned to generate isogenic lines that bear and lack LRV1. We injected 2CMA-treated parasites of each genotype, both LRV1+ and LRV1-negative clones, into the hind footpad of C57B/6 mice and monitored infection by measuring lesion size (a measure of pathology) and luciferase activity (a measure of parasitemia).
As expected, LRV1-negative clones of each line were less virulent than were the LRV1+ clones (Figure 7). Somewhat to our surprise, there was no significant difference between the Δago1 line and either the WT or complemented line in the absence of LRV1. In contrast, in the presence of LRV1, the Δago1 line was more virulent than either the WT or complemented line at the peak of infection, with 25% higher footpad swelling and ~2-fold higher parasitemia in the Δago1 parasites than the WT.

Previous work has shown that infection of IFN-γ−/− deficient mice with *L. guyanensis* results in a chronic infection and a tendency for parasites to metastasize to the tail and/or uninjected foot (36). To determine whether the loss of AGO1 (and increase in LRV1) affected metastasis, we injected the same 2CMA-treated lines used above into IFN-γ−/− mice. A number of mice developed severe infections and had to be sacrificed before metastases could develop (Figure 8A). Of the remaining mice, infection with parasites containing LRV1 was more likely to lead to tail metastasis than infection with LRV1-negative parasites (Figure 8B). In the LRV1-negative lines, loss of AGO1 did not appear to affect the development of metastases, similar to the virulence data in WT mice. In contrast, infections with Δago1 parasites were more likely to metastasize than WT in the presence of LRV1. This data is preliminary, and these experiments will have to be repeated with a lower inoculum to reduce mouse mortality and obtain high-confidence data.

### 3.4 – Discussion

In our experiments, knockout of AGO1 from *L. guyanensis* parasites resulted in ablation of the RNAi pathway, as occurred in *L. braziliensis*. In the knockout parasites, introduction of a stem-loop targeting the luciferase ORF had no effect on luciferase activity, while in the WT line the same construct results in a greater than 95% reduction in luciferase activity (Figure 2A).
Likewise, levels of the TATE element transcript tripled, indicating that control of endogenous targets was disrupted (Figure 2B & C).

Interestingly, SLACS levels did not appear to change with loss of AGO1 in these experiments, whereas they were clearly affected by loss of AGO1 in *L. braziliensis* (24). It is unclear why this should occur. It is not likely due to the level of expression, as there was not a large discrepancy in the proportion of RNAseq reads mapping to SLACS compared with TATE (there were 2-fold more TATE-mapping reads) (Figure 2B), and reads mapped to each TE at levels higher than any of the housekeeping genes that we examined. Sequencing of sRNAs in *L. guyanensis* revealed that a large proportion of sRNAs originate from SLACS (23), so presumably they should be capable of repressing SLACS transcripts. Instead, it may be an artifact of the methods used, as neither RNAseq nor qPCR can differentiate between large degradation products and full transcripts. We have previously shown that qPCR over-estimates the level of an RNA undergoing knockdown (22) compared with the level of expression determined by Northern blot. It is possible that our analysis overestimated the level of full transcript in WT and +AGO1c lines; further work would be required to confirm this.

Because of the substantial effect of RNAi on virus levels in other systems, we hypothesized that loss of AGO1 would result in a large increase in LRV1 levels, to the point that it could have been lethal. Not only were Δago1 parasites viable, the increase in LRV1 RNA was much smaller than expected, only 35% over the level in the WT line (Figure 3). Further, the parasites do not appear to accumulate increasing levels of LRV1 RNA during continued culture, indicating that the 35% increase represents a new “set point” level of LRV1. While this increase (and subsequent decrease upon complementation) was statistically significant, this small of an effect does not support a substantial role for Ago1-dependent RNAi in the control of LRV1. It
does not, however, rule out a role for AGO1 in control of LRV1, either. We hypothesized that loss of AGO1 could result in uncontrolled virus replication, resulting in loss of parasite viability. If this occurred, only parasites that had developed a compensatory mechanism for controlling LRV1 levels would survive to be evaluated; perhaps the clones analyzed in this work have such a compensatory mechanism. Alternatively, small changes can have large effects over long timescales. It is possible that this small increase in LRV1 is too mild a stress to measure in a laboratory setting, but is substantial enough to support the maintenance of LRV1 over millions of year of evolution.

In previous work, we showed that the introduction of the CapsidStL construct into the WT line resulted in a loss of LRV1 (23). As transgene-driven RNAi was ablated in the Δago1 line we expected that the StL construct would be non-functional, and indeed introduction of the CapsidStL construct into the Δago1 line did not affect LRV1 levels (Figure 4). These results confirm the AGO1-dependency of the CapsidStL and suggest that the interaction between LRV1 and the RNAi pathway functions differently in the presence of the StL construct than in its absence. Our previous work showed that the CapsidStL construct results in massive over-production of LRV1-mapping sRNAs (23); perhaps there is a maximum level of RNAi pressure that LRV1 is able to withstand, and the levels of anti-LRV1 siRNAs produced under normal conditions do not meet this threshold.

While Δago1 L. braziliensis parasites had decreased virulence in the mouse model of infection, this did not occur in LRV1-negative L. guyanensis; instead, the Δago1 line was not significantly different from the WT line (Figure 5A & B). There are two possibilities why this may occur. First, it appears that RNAi is somewhat less efficient in L. guyanensis than in L. braziliensis (22). It is possible that this less-efficient RNAi pathway has less of an effect on
virulence than the more active *L. braziliensis* pathway. Second, this may suggest that RNAi regulates a virulence-associated endogenous target in *L. braziliensis* but not in *L. guyanensis*.

In contrast, loss of AGO1 in the presence of LRV1 resulted in an increase in virulence (Figure 5C & D). This is likely due to the increased levels of virus in LRV1+ Δago1 parasites compared to WT. Previous reports found that the presence of LRV1 significantly increased the virulence of *L. guyanensis* parasites in mouse models of infection (9). Additionally, previous work in strains of *L. aethiopica* containing LRV2 showed that the inflammatory response in macrophages was correlated with viral load (6). The previous studies relied on different strains with varying LRV1 burdens, and some of the observed differences in virulence could be due to parasite genetic factors. In our experiments, we examined two isogenic lines with different levels of LRV1; to our knowledge, this is the first experiment of its type. As relatively small increases in LRV1 resulted in observable increases in virulence, these results suggest that it is possible to improve patient outcomes by reducing the level of LRV1 even partially in patient infections.

Many RNA viruses infecting plants and insects encode Viral Suppressors of RNAi (VSRs) to evade their hosts’ antiviral RNAi pathways (reviewed in Szittya and Burgyan 2013; Bronkhorst and Van Rij 2014). LRV1 does not appear to have the coding capacity for such a mechanism, and certainly there is no *trans*-acting VSR, as *L. guyanensis* parasites with LRV1 are equally capable of downregulating luciferase by RNAi as an LRV1-negative isogenic line (22). Additionally, the ability of the LRV1 StL to ablate LRV1 suggested that there was no *cis*-acting VSR; however, the StL generated massive amounts of LRV1-targeting siRNAs, which could overwhelm any VSR present (23). It is possible that LRV1 encodes a *cis*-acting VSR capable of evading the RNAi pathway at natural levels of pressure. If this were the case, loss of AGO1 would have little effect on LRV1 level.
Alternatively, it is possible that anti-LRV1 siRNAs are bound to another Argonaute protein than AGO1. Trypanosomatids have a second Argonaute, termed “Piwi” (so named because the PAZ domain has a highly divergent sequence and is apparent only by structural homology (38), leaving only an identifiable Piwi domain), whose function is unknown. Perhaps anti-LRV1 sRNAs preferentially bind Piwi rather than AGO1, and RNAi control of LRV1 occurs through Piwi, with only incidental regulation via AGO1. Future experiments will be needed to determine whether this is the case; however, efforts to knock out Piwi in Viannia subgenus parasites have thus far been unsuccessful, and RNAi knockdown of Piwi did not result in a decrease in Piwi RNA levels (Appendix C).

It is also possible that RNAi control of LRV1 occurs at the level of Dicer. Perhaps the LRV1-derived sRNAs, while capable of eliciting downregulation of LRV1 at high concentrations, don’t efficiently direct cleavage of the LRV1 transcript at physiological levels; rather, cleavage of the LRV1 dsRNA genome by Dicer is sufficient to inhibit LRV1 replication and maintain sustainable virus levels. There are some reports in the literature in which loss of Dicer impacted virus levels more strongly than loss of Argonaute (18, 39, 40). Finally, it is possible that neither cleavage of the LRV1 dsRNA genome by Dicer, nor cleavage of the LRV1 transcript by AGO1 plays a substantial role in control of LRV1 levels. If this were the case, some other unknown mechanism would be required to maintain LRV1 at sustainable levels.

3.5 – Materials and Methods

Parasite strains and cell culture

L. guyanensis strain M4147 (MHOM/BR/78/M4147) was obtained from Jean Patterson (Southwest Foundation for Biomedical Research, San Antonio, Texas) and transfected with Swal-linearized B6367 pIR2SAT-LUC(B). Clonal lines were derived, and clone 3 was used in
further experiments. Parasites were grown in fresh Schneider’s Insect Medium supplemented with 10% heat-inactivated fetal bovine serum, 100 μM adenine, $5 \times 10^{-5}$% hemin, 2 μg/mL biop terin, 2 mM L-glutamine, 500 units/ml penicillin and 50 μg/mL streptomycin.

Selective agents used were nourseothricin sulfate (Gold Biotechnology), blasticidin S HCl (Fisher Scientific), puromycin dihydrochloride (Sigma-Aldrich), and hygromy cing B (Hygro99, Gold Biotechnology).

**Constructs and transfections**

$1 \times 10^8$ cells were resuspended in 0.5 mL cytomix buffer (120 mM KCl, 150 μM CaCl$_2$, 10 mM K$_2$HPO$_4$, 25 mM Hepes, 2 mM EDTA and 5 mM MgCl$_2$, pH 7.6 using KOH), mixed with 10 μg linearized DNA, and electroporated twice in a 4mm gap cuvette at 1400 V and 25 μF, waiting 10s between zaps. Cells were placed in 10 mL Schneider’s Medium without selection and allowed to recover overnight.

To generate Δago1 parasites, the blasticidin and puromycin resistance genes were fused between 823bp of sequence at the 5’ flank and 281bp of sequence at the 3’ flank of the AGO1 gene. These constructs were used to successively replace the two alleles of AGO1. Transfected cells were plated on semisolid media containing 100 μg/mL nourseothricin and either 10 μg/mL blasticidin or 20 μg/mL puromycin. Colonies were picked into 1 mL Schneider’s Medium without selection, expanded to 5 mL Schneider’s Medium with 50 μg/mL nourseothricin and 5 μg/mL of either blasticidin or puromycin, and passaged thusly.

To generate the complemented +AGO1c line, we tagged the N-terminus of the AGO1 gene with the Ty1 epitope (EVHTNQDPLD) and cloned this ORF into a modified pIR1HYG vector that replaces the BglII cloning site with a PacI site [B7397 pIR1HYG(B-PacI)]. We linearized this DNA with SwaI and transfected it into Δago1 parasites. Clones were selected on
semisolid media containing 50 μg/mL nourseothricin and 150 μg/mL hygromycin B and passaged in liquid Schneider’s Medium containing 25 μg/mL nourseothricin, 2.5 μg/mL blasticidin, 2.5 μg/mL puromycin, and 50 μg/mL hygromycin B.

B7066 pIR2HYG-LRV1_LgyM4147_CapsidStL(A) and B6486 pIR2HYG-LUCStL(A) were linearized with SwaI and transfected into Δago1 parasites. Control LUCStL lines were generated by transfection of B6486 into L. guyanensis M4147 LUC c3. Clonal lines were selected by limiting dilution in 96-well plates. Transfected cells were allowed to recover overnight and diluted to 10^5 cells/mL in Schneider’s Medium containing 50 μg/mL nourseothricin and 75 μg/mL hygromycin B. 200 μL of cells were seeded into 96-well plates. Wells that survived selection were expanded and passaged in Schneider’s Medium containing 25 μg/mL nourseothricin and 50 μg/mL hygromycin B, with the addition of 2.5 μg/mL blasticidin and 2.5 μg/mL puromycin for the Δago1 transfections. Control CapsidStL lines were described previously (23).

*Treatment with 2’C-methyladenosine (2CMA)*

Parasites were seeded at 1x10^5 cells/mL in Schneider’s medium lacking supplemental adenine and containing 10 μM 2CMA. After 5 cell doublings, cultures were pelleted at 3,000 rpm and resuspended in fresh Schneider’s medium containing adenine without 2CMA. After 48 hours, cells were plated on semisolid media. Colonies were picked into 1 mL Schneider’s medium, expanded to 5 mL with appropriate selective antibiotics, and evaluated for LRV1 levels using flow cytometry.
**Western blot**

Mouse BB2 antibody was obtained from Sigma-Aldrich. Rabbit anti-H2A (41) and anti-AGO1 antibodies were produced by Proteintech. Secondary antibodies used were IRDye800CW goat anti-mouse and IRDye680RD goat anti-rabbit (LI-COR Biosciences).

5 x 10^7 cells from mid-log culture were resuspended in 100 μL 1x Laemmli buffer (62.5 μM Tris, pH6.8; 2% SDS; 10% glycerol; 2.5% 2-mercaptoethanol; 0.001% bromophenol blue) and heated at 95°C for 5 min. Cell lysates were stored at -20°C. Lysates were run on polyacrylamide (4% stacking, 10% resolving) gels at 200V and proteins were transferred to nitrocellulose membranes at 60V for 2 hours at 4°C. Membranes were blocked overnight at 4°C in Odyssey blocking buffer (LI-COR Biosciences). Primary antibodies were incubated at room temperature for 2 hours at the following dilutions: α-BB2, 1:00; α-H2A, 1:10,000; α-Ago1, 1:1,000. All primary antibody dilutions were made in Odyssey blocking buffer. Membranes were washed 4 times with PBS-T (0.1% Tween-20 in PBS) for 10 min per wash. Secondary antibodies were diluted 1:10,000 in Odyssey blocking buffer containing 0.2% Tween-20 and membranes were incubated for 1 hour at room temperature in the dark. All subsequent steps occurred in the dark. Membranes were washed again 4 times with PBS-T for 10 minutes/wash and twice with PBS (no Tween-20). Membranes were scanned using the LI-COR Odyssey Infrared Imaging System.

**Luciferase activity assay**

10^6 log phase cells in 200 μL of Schneider’s Medium were added to a 96-well plate (Black plate, Corning Incorporated, NY, U.S.A.). D-luciferin (Biosynth AG) was added to a final concentration of 150 μg/mL and plates were incubated for 1 min. Plates were imaged using a In Vivo Imaging System (IVIS) photomager (Perkin Elmer), and luciferase activity quantified as
photons/s. Each sample was run in duplicate. Three clones each of the WT line and two knockout clones transfected with empty vector and the LUCStL construct were evaluated.

**Flow cytometry**

Flow cytometry using the anti-capsid antibody (5) was performed as described previously (23).

**RNA preparation**

2.5-3 x 10^8 log phase promastigotes were spun down and resuspended in 1 mL Trizol reagent (Ambion, Thermo Fisher Scientific), and 250 μL of dissolved cells were used for each preparation of total RNA. RNA was isolated using the RNA miniprep kit (Zymo Research), DNaseI-treated in a 200 μL reaction using 20 Units of enzyme and the supplied buffer (Ambion, Thermo Fisher Scientific) for 1 hour at 37°C. Enzyme was removed using RNA Clean & Concentrator - 25 kit (Zymo Research) and eluted in 50 μL nuclease-free water. RNA concentrations were obtained using the Qubit RNA Broad-Range assay (Thermo Fisher Scientific) and RNAs were stored at -80°C.

**RNA sequencing and bioinformatics analysis**

RiboZero RNA libraries were generated from total RNA as described (42). Briefly, sample integrity was assessed by Agilent Bioanalyzer 2100 (Santa Clara, CA). rRNA was depleted from 1 mg input RNA with the Ribo-Zero™ rRNA Removal Kit (“RiboZero”) from EpiCentre (an Illumina company, Madison, WI). RiboZero-depleted RNA was chemically fragmented to generate fragments ranging from 200-600 nt in length, then made into cDNA with Superscript III (Life Technologies, Thermo Fisher) and random hexamers followed by a second strand reaction. cDNA was then end-repaired, A-tailed, and standard Illumina adapters were
ligated on. Libraries were amplified with primers to incorporate a unique index to each sample. Equal masses of each library were pooled and sequenced on an Illumina HiSeq2500 platform, with 2 × 100 base pair paired end reads (Illumina Inc., San Diego, CA).

Data were analyzed using CLC Genomics v9.5.3. Briefly, 5’ and 3’ adaptors were removed and trimmed reads were mapped to annotated genes and transposable elements from the L. guyanensis M4147 genome, as well as to LRV1 genes (KX808487) using default RNA-Seq parameters. Reads mapping to multiple locations were aligned randomly.

**cDNA synthesis and qPCR**

cDNA was synthesized by reverse transcription using the Superscript III First Strand Synthesis kit (Thermo Fisher Scientific) according to manufacturer instructions in a 20 μL reaction containing 0.25 μg purified total RNA. Control reactions contained an equal amount of RNA but lacked reverse transcriptase enzyme.

Primers used for qPCR can be found in Table 1. Annotated SLACS elements were aligned to identify consensus regions in common among multiple elements, and primers were designed to amplify a 97 bp region of this consensus sequence. These primers bind to 7/15 annotated elements, which are responsible for 61,740/88,075 (70.0%) of total SLACS-mapping sequencing reads. Similarly, TATE elements were aligned to identify common sequences, and primers were designed to amplify 106 bp of this consensus sequence. These primers bind to 15/53 annotated elements, which are responsible for 210,037/298,077 (70.5%) of TATE-mapping sequencing reads. LRV1-specific primers were designed to amplify an approximately 100 bp region of the LRV1 genome. Control primers were designed to amplify approximately 150 bp of the KMP-11 5’ UTR.
qPCR reactions were performed in MicroAmp Optical 96-well plates or MicroAmp Fast Optical 96-well plates (Thermo Fisher Scientific) using 20 μL reactions. Each reaction contained 5 μL of ten-fold diluted cDNA, 10 μL of 2x Power SYBER Green Master Mix (Applied Biosystems, Thermo Fisher Scientific), and primers to a final concentration of 0.2 μM each. Reactions were run on the ABI PRISM 7000 Sequence Detection System or ABI ViiA 7 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). PCR amplifications were as follows: 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. PCR products were confirmed to be specific by melt curve analysis. +RT reactions were performed in triplicate and –RT control reaction in duplicate.

Serial 4-fold dilutions of cDNA were used to generate primer efficiency curves for each primer set on each plate, and relative SLACS, TATE, and LRV1 level was determined using the Pfaffl method (43). For the SLACS and TATE experiments, three biological replicates of the WT line, two biological replicates of each of two Δago1 clones, and four +AGO1c clones were averaged and statistical significance determined by ANOVA followed by the Tukey post hoc test. For the LRV1 experiment, four biological replicates of the WT line (L. guyanensis M4147 LUC c3), two biological replicates of each of two ago1- clones, and 12 clones of the Ty1-tagged complemented line were averaged and statistical significance determined by ANOVA followed by the Tukey post hoc test.

Statement on Institutional and Licensing Committee Approval of Animal Experiments

Animal handling and experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (44) of the US National Institutes of Health. Animal studies were approved by the Animal Studies Committee at Washington University (protocol 20090086) in accordance with the Office of Laboratory Animal
Welfare’s guidelines and the Association for Assessment and Accreditation of Laboratory Animal Care International.

**Mouse infection**

Female C57/B6 mice were obtained from Jackson Laboratories. IFN-γ−/− mice were obtained from Dr. Herbert Virgin (Washington University School of Medicine, St. Louis, MO). Parasites were grown to day 2 stationary phase and collected at 1x10^6 cells/50 μL DMEM. Mice were injected subcutaneously in the left hind footpad with 1x10^6 parasites using a 30 gauge needle. Luciferase activity was monitored weekly by imaging using the IVIS (Perkin Elmer). Briefly, mice were injected intra-peritoneally with 150 mg/kg D-luciferin, potassium salt (Gold Biotechnology) 10 minutes before imaging. Five minutes before imaging they were anaesthetized with isoflurane, and anesthesia was continued throughout the procedure. Emitted photons were quantified using Living Image v2.60.1 software (Perkin Elmer). Lesion size was measured weekly using calipers.

**3.6 – Acknowledgments**

Thanks to Jean Patterson for providing parasite strains and the anti-capsid antibody. This work was supported in part by NIGMS Cell and Molecular Biology Training Grant GM: 007067 and the Monsanto Excellence Fund for Graduate Fellowships (EB) and NIH grants RO1AI029646 and R56AI099364 (SMB).

**3.7 – Table Legends**

Table 3-1: Primers used in qPCR analysis of SLACS, TATE, and LRV1 RNA levels.
3.8 – Figure Legends

Figure 3-1: Confirmation of AGO1 replacement by PCR.

A) Schematic of replacement of AGO1 by drug resistance cassettes.

B) Schematic of PCR primer binding sites for the confirmation of integration. For each reaction, one primer binds within the drug resistance gene; the other binds to a region of the flanking region outside the targeting construct. Expected PCR product size is indicated.

C) Integration PCR products confirming replacement. Left, replacement with BSDR to generate the heterozygote; right, replacement with PACR to generate the knockout. NTC, no-template control.

Figure 3-2: Replacement of AGO1 alleles results in Δago1 parasites.

A) PCR failed to amplify the AGO1 gene in L. guyanensis knockout clones 15, O, and R, as well as L. braziliensis strain M2093 Δago1 (Lbr Δago1), but not in the L. guyanensis AGO1 heterozygote. NTC, no template control.

B) AGO1 protein was undetectable by Western blot in Δago1 clones and present at WT levels in +AGO1c complemented lines. Loading control, α-Histone H2A antibody.

Figure 3-3: Δago1 parasites are functionally RNAi-deficient.

* p<0.05; **** p<0.0001.

A) Δago1 parasites transfected with a transgenic RNAi construct targeting luciferase (LUCStL) have no reduction in luciferase activity compared to empty vector control.

B) Fraction of RNAseq reads mapping to SLACS and TATE transposable elements, as well as to Argonaute and two housekeeping genes, glucose-6-phosphate dehydrogenase
(G6PD) and the large subunit of RNA polymerase II (RNAPII-LS). Reads mapped at similar frequencies to SLACS and the two housekeeping genes between the WT, Δago1, and +AGO1c lines. TATE-mapping reads were significantly higher in the Δago1 line.

C) Relative level of SLACS and TATE transposable element RNA by qPCR. SLACS levels were similar between the lines, while TATE levels were increased in Δago1.

Figure 3-4: Comparison of global gene expression between WT and Δago1 parasites.

Average read counts of two biological replicates are plotted for each genotype. Red, TE genes; blue, all other genes. rRNA genes and LUC were removed from the analysis. The location of the AGO1 data point is indicated.

Figure 3-5: Knockout of AGO1 results in small increases in LRV1.

A) Flow cytometry of WT line (red), Δago1 (green), +AGO1c (purple), and LRV1-negative (black) parasites using an anti-capsid antibody. Traces are representative of 3-4 clones/biological replicates.

B) LRV1 RNA levels determined by qPCR. Averages and standard deviations are of 4-12 biological replicates/ clones. * p<0.05

Figure 3-6: The CapsidStL transgenic RNAi construct is non-functional in Δago1 parasites.

A) Flow cytometry of WT line (red), Δago1 (green), Δago1 + CapsidStL (purple), LRV1-negative (black), and WT + CapsidStL (blue) parasites using the anti-capsid antibody. Traces are representative of 2-6 clones/biological replicates.

B) LRV1 RNA levels determined by qPCR. Averages and standard deviations are of 2-6 biological replicates (WT) or independent clones (Δago1 CapsidStL). **** p<0.0001.
Figure 3-7: Virulence of Δago1 parasites in the presence and absence of LRV1.

Infections in WT mice with Δago1 parasites have similar virulence in the absence of LRV1 (top panel), while Δago1 parasites are more virulent in the presence of LRV1 (bottom panel). Parasitology was evaluated by luciferase activity (panels A & C) and pathology by lesion size (panels B & D). Averages and standard deviations are of 3 experiments with two clones per group, 4 mice per clone. #, significance of Δago1 vs WT; +, significance of WT vs +AGO1c; *, significance of Δago1 vs +AGO1c. * p<0.05; ** p<0.01; **** p<0.0001.

Figure 3-8: LRV1+ parasites are more likely to metastasize than LRV1-negative parasites in IFN-γ−/− mice.

A) Mouse survival curve of lines evaluated.

B) Number of tail metastases per mouse.

3.9 – References


## Table 3-1

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Figure 3-1

A

\[
\begin{align*}
\text{AGO1} \\
\text{AGO1} \\
\downarrow \\
\text{AGO1} \\
\text{BSD}^R \\
\downarrow \\
\text{BSD}^R \\
\text{PAC}^R
\end{align*}
\]

B

\[
\begin{align*}
\text{AGO1} & \quad +/\quad \Delta \text{ago1} \\
\text{M} & \quad 15 & \quad O & \quad R & \quad \text{AGO1}^{+/+} & \quad \text{NTC} & \quad \text{M}
\end{align*}
\]

C

\[
\begin{align*}
\text{BSD}^R \text{ replacement} \\
\text{PAC}^R \text{ replacement}
\end{align*}
\]

\[
\begin{align*}
\text{5' integration} & \quad \text{1 kb} \quad 850 \text{ bp} \\
\text{3' integration} & \quad 650 \text{ bp} \quad 500 \text{ bp}
\end{align*}
\]
Figure 3-2

A

Δago1

15 O R Lbr Δago1 NTC Lgy het

5' integration of ORF

Primers internal to ORF

B

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<td>Δ</td>
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Figure 3-3

A. Luciferase activity

B. Fraction of mapped reads

C. Relative RNA
Figure 3-4

![Graph showing WT read count (x1000) vs. AGO1 read count (x1000)]
Figure 3-5

A

B

![Graph A](image)

![Graph B](image)

# of cells

Relative LRV1 RNA

Parent

Δago1

+AGO1c

10^1

10^2

10^3

Capsid protein

0.0

0.5

1.0

1.5

2.0

Parent

Δago1

+AGO1c

* *
Figure 3-6

A

B

LRV1- Capsid StL
Δago1
Δago1 Capsid StL

# of cells

10^1 10^2 10^3

Capsid protein

Relative LRV1 RNA

****

Parent
Δago1 Capsid StL
Figure 3-8

A

![Graph showing percent survival over days post infection for different groups: LRV1+, LRV1- WT, LRV1- Δ ago1, LRV1+ WT, LRV1+ Δ ago1, LRV1+ +AGO1c, and LRV1- +AGO1c.](image)

B

![Bar graph showing the number of metastases per mouse for LRV1+ and LRV1- groups: WT, Δ ago1, and +AGO1c.](image)
Chapter 4: Popping DNA out of the *Leishmania* genome – a control for mutants with dominant phenotypes
Preface

The experiments in this chapter were designed, performed, and analyzed by EAB under the supervision of SMB. The first draft of the chapter was written by EAB, and the final version presented here incorporates comments from SMB.
4.1 – Abstract

Here we present a method for short-term expression of a gene or RNAi transgene in *Leishmania* (Viannia) parasites. We show that parasites transfected with these constructs display a mutant phenotype, and revert to a wild-type phenotype following removal of the construct. This method provides a tool to determine the essentiality of genes, which could not previously be sufficiently proven in *L. (Viannia)* species.
4.2 – Introduction

The process of culturing cell lines in the laboratory leads to mutations and gene expression alterations that affect cell growth and biology (1–4). If these changes occur during the course of planned genetic manipulations, they can confound interpretation of the results of the experiment. To combat this, one must take steps to separate effects of the planned manipulations from culture artifacts. For example, when genes are knocked out, it is standard procedure to complement the mutant by expression of the deleted gene. If the complemented line reverts to a WT phenotype, one can be reasonably certain that any phenotype observed in the mutant is due to the loss of the gene under investigation.

Similar controls are less common during the expression of dominant genes, including the expression of dominant negative mutants or heterologous genes. Typically, multiple independent mutants are evaluated to rule out random mutations. This, however, does not control for compensatory mutations.

Many Leishmania species, such as L. major, L. mexicana, and L. donovani, support the replication of circular extrachromosomal elements called episomes (5–8). In these species, short-term expression of genes can be achieved by transfection of an episome that confers resistance to a selective agent, and that can then be lost after removal of selective drug pressure. By expressing dominant genes from an episome, one can confirm that an observed phenotype is gene-specific by selecting for cells that have lost the episome and confirming that they have a WT phenotype.

These episomes, however, do not work well in Leishmania (Viannia) species (9), possibly because transcription from these episomes occurs from both strands (10). This likely results in long dsRNA that enters the RNAi pathway, which is present in Viannia species, but not
Leishmania (Leishmania) species (9, 11). As a result, any genes expressed from the episome, including those conferring resistance to a selection agent, are likely silenced. Because of this, genes must be stably integrated into the parasite genome to attain expression, which is effectively a permanent modification.

Here we describe an analogous method of gene expression for use in Leishmania. “Popout” constructs integrate into the parasite genome and confer GFP expression, allowing cells to be rapidly scored for presence or absence of the construct. We show that popout constructs carrying an RNAi transgene targeting the paraflagellar rod 2 (PFR2) gene trigger downregulation of PFR2, and that loss of the construct returns cells to a wild-type phenotype.

4.3 – Results

4.3.1 – Construction of a removable expression construct

We had three criteria for the design of our expression construct: 1) stable integration into the parasite genome; 2) a propensity for spontaneous loss; and 3) a rapid and facile method to identify parasites that had lost the construct. To accomplish this, we cloned the GC-rich GFP+ ORF (12) into the “B” cloning site of pIR3 and pIR3-GW(A) plasmids (Figure 1A). pIR3 plasmids are derivatives of pIR1 (11), and replace the L. pifanoi CYS2 intergenic region of IR1 with the L. braziliensis alpha-tubulin intergenic region. These plasmids integrate into the 18S small subunit ribosomal RNA array by homologous recombination, where they are transcribed at high levels (13). The pIR3-GW(A) plasmid uses Gateway technology (Invitrogen, Thermo Fisher Scientific) to facilitate cloning of an inverted repeat (stem-loop, StL). These StL constructs are used to trigger transgene-driven RNAi in Leishmania (Viannia) species (9, 14).

In principle, we expected these constructs to behave in a manner analogous to the pXG episome (15, 16) (Figure 1B). Briefly, integration of a pIR3-GFP+(B) construct into the
Leishmania genome would cause expression of three genes: one conferring resistance to selection antibiotic; GFP; and the desired gene or StL. Transfectants could be selected either by culturing with the selective antibiotic or by single-cell sorting for GFP-positive cells. Removal of drug selection would allow the integrated DNA to be lost by gene conversion – “popped out” – at which time cells would no longer express GFP, the antibiotic resistance gene, or the transgene or StL. Single-cell sorting for GFP-dim cells would allow recovery of clonal populations that no longer contain the construct. Due to the ability of these constructs to be readily lost, we have termed them “pop-out constructs.”

4.3.2 – Knockdown of paraflagellar rod 2 (PFR2) using a popout construct efficiently downregulates PFR2 expression

To test the functionality of pop-out constructs, we targeted the paraflagellar rod 2 (PFR2) gene by RNAi in WT L. braziliensis strain M2903 using a pop-out StL construct. We previously showed that knockdown of this gene results in loss of the paraflagellar rod structure and defective motility (9), similar to deletion mutants in L. mexicana (17, 18). We integrated the PFR2StL(A)-GFP+(B) construct or a GFP-only control into the L. braziliensis genome and obtained clonal lines by drug selection on semisolid media. Transfectants expressed GFP at high levels, and cell pellets were visibly green. PFR2StL(A)-GFP+(B) clones had a ~80% reduction in PFR2 RNA compared to cells expressing GFP alone (Figure 2).

4.3.3 – Removal of PFR2 StL returns cells to WT phenotype in L. braziliensis

We withdrew drug selection from the PFR2StL(A)-GFP+(B) and the GFP-only control lines to enable cells to lose the integrated DNA. A substantial GFP-negative population arose within two passages without selection, or roughly 12 cell doublings (Figure 3). We single-cell
sorted for GFP-positive and GFP-negative cells to obtain clonal lines with the constructs integrated and popped out, respectively. Hereafter, clones with an integrated construct are labeled “SSU:Construct” (ex, SSU:GFP+(B)); clones with a construct popped out are labeled “PO:Construct (ex, PO:GFP+(B)).

Cell survival following sorting was markedly higher for GFP-negative clones than for GFP-positive clones (78.8 ± 7.6% vs. 37.1 ± 8.9%, p<0.0001). After the cultures grew out we tested clones for GFP expression; 37 of 38 clones had the expected level of GFP expression. A single GFP-low clone expressed high levels of GFP; this clone was likely a result of imperfect sorting.

We next performed PCR on genomic DNA from the GFP-positive SSU:PFR2StL(A)-GFP+(B) and GFP-negative PO:PFR2StL(A)-GFP+(B) clones to confirm the presence/absence of the StL construct. We were able to amplify PCR products confirming both 5’ and 3’ integration of the PFR2StL(A)-GFP+(B) construct for six of eight SSU:PFR2StL(A)-GFP+(B) clones (Figure 4A). Unexpectedly, we amplified the 5’ and 3’ integration products from six of nine popped-out PO:PFR2StL(A)-GFP+(B) clones, as well (Figure 3B). This suggested that, even though the clones did not express GFP, they still retained portions of the StL construct. The three PO:PFR2StL(A)-GFP+(B) clones that were confirmed to have lost the construct had PFR2 mRNA levels similar to that of SSU:GFP+(B) and PO:GFP+(B) clones (Figure 4B).

Finally, we functionally evaluated the SSU:PFR2StL(A)-GFP+(B) and PO:PFR2StL(A)-GFP+(B) clones for loss of PFR2 protein. We expected that, since PFR2 RNA levels rebounded following pop-out, protein levels would as well. In this situation, PO:PFR2StL(A)-GFP+(B) lines would have a WT phenotype. EM imaging of SSU:PFR2StL(A)-GFP+(B) cells revealed a lack of paraflagellar rod structure, which was clearly visible in both SSU:GFP+(B) and
PO: PFR2StL(A)-GFP+(B) cells (Figure 5, top). Finally, substantially fewer cells swam normally in the SSU: PFR2StL(A)-GFP+(B) line than either the SSU: GFP+(B) or PO: PFR2StL(A)-GFP+(B) line; the SSU: GFP+(B) line and PO: PFR2StL(A)-GFP+(B) line had comparable proportions of cells swimming normally (Figure 5, bottom). These results are in agreement with our previous knockdown of PFR2 (9). Further, they support the use of popout constructs to return cells to a WT phenotype.

4.4 – Discussion

In our tests of the popout system using the PFR2StL(A)-GFP+(B) construct, we successfully showed that 1) StL constructs using this system are functional in the downregulation of their target; 2) popout constructs can be lost from the Leishmania genome; and 3) following popout, expression from the construct ceases, and cells become phenotypically wild-type.

There are a few considerations to keep in mind when performing these experiments. First, cell survival was lower following single-cell sorting for integrated (GFP-expressing) cells than for popped-out (GFP-negative) cells. This may have occurred because GFP-expressing cells were sorted into culture media containing selective antibiotic to preserve integration of the construct; GFP-negative cells were sorted into culture media lacking selection. Survival of GFP-expressing cells could likely be increased by sorting into culture media lacking antibiotic and resuming selection once cell density has increased.

Second, a large fraction of the GFP-negative cells analyzed retained portions of the StL construct. This demonstrates the importance of thoroughly validating any clones obtained; fortunately, the validation is easily accomplished with a simple PCR reaction. The presence of portions of the construct but absence of GFP expression suggests that GFP expression may be deleterious. It is possible that high levels of GFP act as negative selection, and cells that eject the
GFP ORF have a selective advantage. Continued culture with selective antibiotic maintained high levels of GFP expression in the vast majority of cells, suggesting that loss of GFP and drug resistance occurs simultaneously.

There are a number of publications of transgenic *Leishmania* parasite lines expressing GFP from the 18S rRNA locus (19–22). In most instances, the authors report stable expression of GFP, even after prolonged removal of drug selection. However, their data show small populations of GFP-negative cells within “GFP-expressing” populations (less than 1% to more than 10% of total cells). In our hands, cells cultured with selection had proportions of GFP-negative cells in this range (Figure 3). In contrast, up to 40% of cells lost GFP expression after we removed selection. Of these, the majority (two thirds) of cells tested retained portions of the construct integrated into the parasite genome. It is possible that, when expressed from our constructs, GFP expression was less stable in the absence of drug selection. The other groups used different expression vectors in their experiments, with different intergenic regions driving trans-splicing and polyadenylation. As gene regulation in *Leishmania* primarily occurs post-transcriptionally and relies heavily on untranslated regions (reviewed in (23–25)), this could affect the steady-state level of protein expression.

Popout constructs have many potential uses. For experiments utilizing StL constructs, popout of the construct would confirm that any observed phenotype is due to the presence of the construct rather than due to culture artifacts or compensatory mutations. Additionally, popout constructs driving expression of genes could be of particular use. For example, they could drive the expression of Cas9 until gene editing is successfully completed, at which time the Cas9 construct could be popped out, eliminating any off-target effects due to the presence of the nuclease.
Further, since *Leishmania (Viannia)* species appear unable to support the replication of episomes (9), popout constructs could enable functional tests of essentiality. In other *Leishmania* species, essentiality can be confirmed using an episome sort method, wherein a gene is expressed from an episome that also carries the GFP ORF during replacement of chromosomal alleles (15, 16, 26). After a chromosomal null mutant is obtained, selection maintaining the episome is removed, and cells are sorted on the basis of GFP expression. If GFP-dim cells survive, and have lost resistance to the selective agent, then the episome is no longer present and the gene is not essential. If, however, few GFP-dim cells survive, and those that do are still resistant to the selective agent, then the episome is still present, and the gene is likely essential.

Because of the inability to perform these experiments in *Viannia* subgenus species, genes must be presumed essential if knockout or knockdown fails to yield viable mutants. This presumption is not always accurate; there have been instances in which genes presumed essential by this criterion were successfully knocked out using the episome sort protocol (16, 27). Now, the gene to be knocked out can be cloned into the popout construct and transfected into parasites. The construct would integrate, and the gene would be expressed ectopically while the alleles from the native locus are replaced. After a chromosomal knockout is obtained, the ectopic copy would be popped out. This procedure would facilitate the generation of “difficult” mutants or rule out technical hurdles in the attempted generation of those mutants.

While this system was designed with *Leishmania (Viannia)* species in mind, it should work in any *Leishmania* species, including those for which the episome sort works well. For experiments requiring high expression of a gene, or little inter-clone variation in expression (13), the integrating popout constructs are likely a better choice, as episomes copy number can vary from cell to cell (5, 6).
4.5 – Materials and Methods

Parasite strains and cell culture

*L. braziliensis* strain M2903 (MHOM/BR/75/M2903) was obtained from Diane McMahon-Pratt (Yale University). Parasites were grown in fresh Schneider’s Insect Medium supplemented with 10% heat-inactivated fetal bovine serum, 100 μM adenine, 5 x 10^-5% hemin, 2 μg/mL bioppterin, 2mM L-glutamine, 500 units/ml penicillin and 50 μg/mL streptomycin.

Constructs and transfections

The GFP+ ORF was amplified by PCR using primers B6857 and B6858 (5’ – ATCGATAAGATCTCCACCATGGTGAGCAAGGGCGAG / 5’ – ATCGATAAGATCTTTACTTGTACAGCTCGTCCATGC), which flank the ORF with Clai and BglII sites, A-tailed, and cloned into pGem-T (Promega). The ORF was released from B7379 pGEM-GFP+ with BglII and ligated into the BglII site of B7089 pIR3HYG to generate pIR3HYG-GFP+(B). The GFP+ ORF was released from B7379 pGEM-GFP+ with Clai and ligated into the Clai site of B7381 pIR3HYG-GW(A) to generate B7405 pIR3HYG-GW(A)-GFP+(B). A 718 bp region of *PFR2* was amplified with primers B3515 and B3517 (5’ – CGCTAGTCTAGATTACGCTACGCAGAAAGAAG / 5’ – CGCTAGTCTAGATCCGTCCTCCACCTCCTCCGCG) and cloned into pCR8/GW/TOPO (Invitrogen, Thermo Fisher Scientific) according to the manufacturer instructions. The *PFR2* sequence was transferred to pIR3HYG-GW(A)-GFP+(B) using LR Clonase II (Invitrogen, Thermo Fisher Scientific) in an overnight reaction at room temperature (RT); the reaction was terminated by digestion with proteinase K for 1 hr at 37°C.

Transfections were performed as described previously (9, 11). Following transfection, cells were plated on semisolid media containing 15 μg/mL hygromycin B (Gold
Biotechnologies). Colonies were picked and grown to stationary phase in 1 mL of media and passaged thereafter in 5 mL of media containing 10 µg/mL hygromycin B.

**RNA and cDNA preparation and qPCR**

2.5-3x10⁸ cells were pelleted and dissolved in 1mL Trizol reagent (Ambion, Thermo Fisher Scientific). 250 µL of dissolved cells were used to isolate total RNA with the RNA miniprep kit (Zymo Research). RNAs were digested with 20 Units of DNaseI (Ambion, Thermo Fisher Scientific) in a 200 µL reaction using the supplied buffer and purified using the RNA Clean & Concentrator kit (Zymo Research) and eluted in 50 µL of nuclease-free water.

cDNA was synthesized by random hexamer-primed reverse transcription using the Superscript III First-Strand Synthesis kit (Thermo Fisher Scientific) in 20 µL reactions containing 0.25 µg RNA. Control reactions contained an equal amount of RNA but omitted the reverse transcriptase enzyme.

qPCR reactions were performed using 5 µL of ten-fold diluted cDNA in a 20 µL reaction containing 10 µL of 2x Power SYBR reaction mix (Applied Biosystems, Thermo Fisher Scientific) and primers (Table 2)to a concentration of 0.2 µM each in MicroAmp Optical 96-well plates (Thermo Fisher Scientific). Reactions were run on the ABI PRISM 7000 Sequence Detection System. PCR amplifications were as follows: 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. PCR products were confirmed to be specific by melt curve analysis. +RT reactions were performed in triplicate and –RT control reactions in duplicate.

On each plate, 5-fold dilution series were performed for each primer set to calculate primer efficiencies, and relative *PFR2* level calculated using the Pfaffl method (28) using amplification of *KMP-11* as an internal control. The data are averages and standard deviations of
3-6 independent clones, and significance was calculated by t-test (Figure 2B) or ANOVA followed by the Tukey post hoc test (Figure 2D).

**Removal of StL construct, flow cytometry, and cell sorting**

Cells containing a StL construct were split to $10^5$ cells/mL in Schneider’s medium lacking selection and GFP expression monitored by flow cytometry. Parallel cultures under drug selection were used as a comparison. When a GFP-negative population appeared in the cultures lacking selection, clonal lines were obtained by single-cell sorting.

Log-phase cells were resuspended in phosphate-buffered saline, passed through a CellTrics 50 µm filter (Partec) to remove clumps, and single cells were recovered on the basis of GFP expression using a Beckman Coulter MoFlo cell sorter. Individual cells were placed into wells of a 96-well plate containing 200 µL of Schneider’s Insect Medium containing no selective antibiotic (GFP-negative cells) or 10 µg/mL hygromycin B (GFP-positive cells) and incubated at 27 °C for 10 days before parasite growth was scored. Wells were expanded to 5 mL with or without antibiotic, as appropriate, and passaged thusly.

**Genomic DNA preparation and PCR**

$10^8$ cells were pelleted and washed with phosphate buffered saline, resuspended in 0.5 mL TELT lysis buffer (50 mM Tris HCl, pH 8.0; 62.5 mM EDTA, pH 9.0; 2.5M LiCl; 4% v/v Triton X-100), incubated 5 min at RT, and extracted twice with an equal volume of phenol:chloroform (1:1). Genomic DNA was ethanol precipitated and resuspended in TE containing 20 µg/mL RNase A.
Genomic DNA was ten-fold diluted and 1 µL used in a 25 µL PCR reaction using KlenTaq-LA polymerase. Primers used were: 5’ integration, B2618 and B3515 (5’ – ACATCAGACGTAATCTGCCGC / 5’ – CGCTAGTCTAGATTACGCTACGCAGAAAGAGAAG); 3’ integration, B3517 and B2619 (5’ – CGCTAGTCTAGAGCCGTCCTCCACCTCCTCCGCG / 5’ – CGACTTTTGTTCCTCTCTATTG). PCR products were run on 1% agarose gel and visualized with ethidium bromide staining.

Transmission electron microscopy

For ultrastructural analyses, samples were fixed in 2% paraformaldehyde/2.5% glutaraldehyde (Polysciences Inc., Warrington, PA) in 100 mM sodium cacodylate buffer, pH 7.2 for 1 hr at room temperature. Samples were washed in sodium cacodylate buffer and postfixed in 1% osmium tetroxide (Polysciences Inc.) for 1 hr. Samples were then rinsed extensively in dH₂O prior to en bloc staining with 1% aqueous uranyl acetate (Ted Pella Inc., Redding, CA) for 1 hr. Following several rinses in dH₂O, samples were dehydrated in a graded series of ethanol and embedded in Eponate 12 resin (Ted Pella Inc.). Sections of 95 nm were cut with a Leica Ultracut UCT ultramicrotome (Leica Microsystems Inc., Bannockburn, IL), stained with uranyl acetate and lead citrate, and viewed on a JEOL 1200 EX transmission electron microscope (JEOL USA Inc., Peabody, MA) equipped with an AMT 8 megapixel digital camera and AMT Image Capture Engine V602 software (Advanced Microscopy Techniques, Woburn, MA).
**Swimming assay**

Log-phase cell culture was placed in a hemocytometer and 150 cells were visually scored on the basis of swimming ability. A focal plane between the bottom of the cell and coverslip was used to avoid counted cells adhering to either surface as immotile. Non-adjacent fields of view were examined to minimize re-counting of motile cells. Cells were scored as immotile if flagellar beating resulted in tumbling or in lack of forward motion.

4.6 – Acknowledgements

Thanks to W. Beatty and B. Anthony for carrying out the EM studies. Single-cell sorting was performed with the assistance of the staff of the Flow Cytometry Core at the Siteman Cancer Center. This work was supported in part by NIGMS Cell and Molecular Biology Training Grant GM: 007067 and the Monsanto Excellence Fund for Graduate Fellowships (EB) and NIH grants RO1AI029646 (SMB).

4.7 – Table Legends

Table 4-1: Primers used in qPCR analysis of *PFR2* levels.

4.8 – Figure Legends

Figure 4-1: The popout construct system.

A) Schematic of IR3-*GFP*+(B) and IR3-GW(A)-*GFP*+(B) constructs carrying resistance to hygromycin B (*HYG*®). Constructs carry a sequence from the 18S rRNA locus that enables integration into the parasite genome by homologous recombination and splice acceptor sites (SA) that permit proper *trans*-splicing and polyadenylation of transcribed RNA. The *GFP*+ ORF is inserted into the B cloning site, while the A site remains available for cloning of a gene of interest (GOI) or an RNAi transgene targeting the GOI.
B) Workflow for the use of popout constructs.

Figure 4-2: qPCR analysis of *PFR2* levels following integration of the *PFR2StL(A)-GFP+(B)* construct.

The *PFR2StL(A)-GFP+(B)* construct efficiently suppresses *PFR2* expression while integrated. **** p≤0.0001.

Figure 4-3: Withdrawal of drug selection from cells containing an integrated *GFP+(B)* or *PFR2StL(A)-GFP+(B)* construct allows for the accumulation of a GFP-negative population.

Cells were grown for two passages (~12 cell doublings) with or without hygromycin B selection and analyzed by flow cytometry. Shown is one representative clone out of three examined for each construct.

Figure 4-4: Confirmation of the loss of *PFR2StL(A)-GFP+(B)* following popout.

A) PCR amplification across the integration site after sorting for GFP expression. Six of nine GFP-negative clones amplified both integration PCR products, indicating that the *PFR2StL(A)-GFP+(B)* construct was still present despite the lack of GFP expression.

B) qPCR analysis of *PFR2* levels following popout of *PFR2StL(A)-GFP+(B)* and control *GFP+(B)* constructs. *** p<0.001; **** p≤0.0001; all other comparisons NS.

Figure 5: SSU:*PFR2StL(A)-GFP+(B)* parasites lack a paraflagellar rod structure and are defective in swimming.

Transverse (top) and longitudinal (middle) sections of flagella imaged by transmission electron microscopy of SSU:*GFP+(B)* control, SSU:*PFR2StL(A)-GFP+(B)*, and PO:*PFR2StL(A)-GFP+(B)* cells. The paraflagellar rod structure is indicated (PFR). Bottom: fraction of promastigote cells that swim normally in culture.
4.7 – References


11. Robinson K a, Beverley SM (2003) Improvements in transfection efficiency and tests of


<table>
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<td>B7412</td>
<td>5’-GGTGACGATGCGGGGATTACC</td>
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<td>B3638</td>
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<td>B3639</td>
<td>5’-TGGATTGCATCGTGCAAGGCTGTG</td>
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Figure 4-1

A

IR3HYG-GFP+(B)  18S rRNA  A  GOI  B  GFP+  HYGR  18S rRNA

IR3HYG-GW(A)-GFP+(B)  18S rRNA  A  IOG  GOI  B  GFP+  HYGR  18S rRNA

B

Transfect construct and select transfectants with drug or GFP expression

Perform required validations and/or manipulations

Remove drug selection and continue culturing

Single-cell sort on the basis of GFP expression

Sensitive to drug?

YES

NO

Construct popped out  Construct integrated

Construct integrated
Figure 4-2
Figure 4-3

![Graph showing GFP fluorescence](image)

- GFP+(B) + drug
- GFP+(B) - drug
- PFR2StL(A)-GFP+(B) + drug
- PFR2StL(A)-GFP+(B) - drug

# of cells vs. GFP fluorescence

144
Figure 4-4

A

SSU: PFR2StL-GFP PO: PFR2StL-GFP

5' integration 3' integration

B

Relative PFR2 RNA

Integrated or popped out: SSU: PO: SSU: PO:

Construct: GFP+ PFR2StL-GFP+
**Figure 4-5**

<table>
<thead>
<tr>
<th>SSU:GFP</th>
<th>SSU:PFR2StL-GFP</th>
<th>PO:PFR2StL-GFP</th>
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</tbody>
</table>

Fraction of cells swimming normally: 141/150, 9/150, 147/150
Chapter 5: Conclusions and Future Directions
Preface

The first draft of this chapter was written by EAB. The final version presented here incorporates comments from SMB.
5.1 – Project Goals

The presence of Leishmania virus 1 (LRV1) profoundly affects the severity of disease caused by Leishmania (Viannia) parasites (1, 2) and correlates with chronic disease, treatment failure, and relapse (3–5). Little is known, however, about the mechanisms by which LRV1 copy number is controlled within the parasite. Disruption of these mechanisms could augment treatments for leishmaniasis that occurs in the presence of LRV1, with potential for improved patient outcomes. Until relatively recently, virus infection of protozoa had been a curiosity with little known relevance to the biology of the host organism. As such, little work has been done to investigate antiviral mechanisms in these organisms. One way in which the parasite may regulate the replication of LRV1 is through the RNAi pathway, which serves an antiviral function in many eukaryotic organisms. The bulk of this work investigates the interaction between LRV1 and the RNAi pathway of L. guyanensis and L. braziliensis parasites. These experiments are described in Chapters 2 and 3, and in Appendices C and D. In the course of the completion of this work, I also developed tools for working with Leishmania in the laboratory (described in Chapter 4 and Appendix B) and identified complications in our understanding of Leishmania biology (described in Appendices A, E, and F). In this chapter, I will discuss the broader conclusions of this work, and propose future directions.

5.2 – LRV1 is not immune to RNAi pressure, but Argonaute 1-dependent RNAi plays little role in control of LRV1

In Chapter 2, we investigated whether LRV1 is processed by the RNAi pathway by sequencing small RNAs (sRNAs) from infected cell lines. We found that substantial proportions of the sRNA pool mapped to LRV1, similar to levels seen with an efficiently-silenced luciferase knockdown reporter. This reporter elicits a 30-fold reduction in luciferase (LUC) activity in L.
and a 300-fold reduction in *L. braziliensis* (Chapter 3 and ref. 6), so this level of LRV1-derived sRNA could be evidence of substantial targeting of LRV1 by the RNAi pathway. When we knocked out *AGO1* in *L. guyanensis*, however, we observed only small effects on LRV1 levels (Chapter 3). All RNAi in *Leishmania* is believed to occur through the activity of *AGO1* (7, 8); therefore, if RNAi played a role in control of LRV1, we expected to see a much larger effect.

This apparent contradiction could be explained if the LRV1-derived sRNAs are not true siRNAs, and do not associate with AGO1 to direct cleavage of the LRV1 transcript. I do not think this scenario is likely. While we did not definitively prove that the LRV1-derived sRNAs are true siRNAs, we showed that the sRNA pool that we sequenced resembles siRNAs in size and chemical composition. Specifically, both sRNA and siRNA lengths ranged from 20-26 nt with a mode of 23 nt. Both contained untemplated bases at the 3’ end. Finally, the sRNAs map to genomic loci in the same proportions as do AGO1-bound siRNAs. Therefore, there is no reason to suspect that the LRV1-derived sRNAs would not be bioactive and capable of reducing LRV1 gene expression, thereby reducing LRV1 levels. Loss of AGO1 is known to destabilize the siRNAs that bind to it (7, 8), so we could use the Δago1 line to confirm that the sRNAs derived from LRV1 are true siRNAs. Specifically, we plan to evaluate the level of LRV1-derived sRNAs by Northern blot in the WT and Δago1 lines of *L. guyanensis*. If the levels of LRV1-derived sRNAs fall in the Δago1 line, it would suggest that they are stabilized by AGO1 and are therefore siRNAs.

I consider it more likely that a combination of transcript abundance and protection within the protein capsid is responsible for the relative insensitivity of LRV1 to AGO1-dependent RNAi. As described in the introduction (Chapter 1), LRV1 replicates by transcribing a plus-
strand and extruding it into the cytoplasm, where it is translated and encapsidated. After encapsidation, the minus-strand is synthesized, regenerating the dsRNA genome. Because of this, some fraction of the LRV1 transcript is enclosed within the capsid and therefore is inaccessible to cytoplasmic complexes, such as AGO1-siRNA and ribosomes. Work in *T. brucei* showed that siRNAs co-sediment with actively translating ribosomes (9), so exclusion of ribosomes likely would further prevent the transcript from encountering AGO1. The dynamics of transcript encapsidation have not been investigated. It is unknown how long a transcript is exposed to the cytoplasm (and AGO1), or what fraction of transcripts become encapsidated. If unprotected LRV1 transcripts are relatively rare, then encounters between them and AGO1 proteins loaded with anti-LRV1 siRNAs would be relatively infrequent. Further studies into the dynamics of encapsidation would be required to determine whether this is a plausible mechanism for the evasion of AGO1-dependent RNAi.

We further showed in Chapter 2 that introduction of an RNAi transgene (stem-loop construct, StL) targeting LRV1 resulted in total loss of the virus from cells. Concomitantly, the proportion of sRNAs derived from LRV1 increased dramatically. This is consistent with the above hypothesis that the LRV1 transcript is protected from cleavage by AGO1 within the capsid. The increase in LRV1-targeting siRNAs would increase the likelihood that a newly-synthesized transcript encounters a molecule of AGO1 loaded with an anti-LRV1 siRNA before it is encapsidated. As the degradation of free transcript increased, translation of LRV1 protein would decrease. As the level of protein translation decreased, fewer transcripts would be protected, and more would be cleaved by AGO1. Eventually, there would be insufficient transcript levels to maintain LRV1. By this hypothesis, loss of AGO1 should lead to substantial increases in LRV1 level. Without the cleavage of free transcript by AGO1, more protein should
be translated and more transcripts should be encapsidated, leading to even higher levels of transcription and translation. Alternative mechanisms for limiting translation of the LRV1 transcript will be discussed later in this chapter.

It remains possible that cleavage of the LRV1 dsRNA genome by Dicer, or of the transcript by Piwi, plays a substantial role in control of LRV1. I was unable to probe this conclusively in this work due to technical difficulties (Appendix C), but the advent of CRISPR technology will likely make the generation of these mutants much simpler. By generating single and double mutants of the two Dicer genes, we can determine whether Dicer cleavage of the LRV1 genome reduces virus replication. Further, while we previously were unable to generate Piwi-null mutants in L. braziliensis, the increased efficiency of CRISPR may facilitate this in the future.

5.3 – An alternative mechanism for control of LRV1

If further work reveals that control of LRV1 does not occur primarily via the RNAi pathway, LRV1 could be maintained at sustainable levels through a balance of replication and degradation. There are two main features of LRV1 would contribute to this balance:

First, the LRV1 transcript is not capped or trans-spliced. This presents two hurdles to virus replication. First, the transcript cannot efficiently recruit ribosomes for translation of its gene products. The 5’ UTR of the LRV1 transcript contains an internal ribosome entry site (IRES) that is about 5% as efficient as a trans-spliced transcript at recruiting ribosomes (10), resulting in less translation and therefor slower replication. Second, the lack of a cap structure exposes the LRV1 transcript to degradation by RNases. There are six XRN family 5’-3’ exoribonuclease homologs in Leishmania, one of which is known to play a role in degrading mRNA(11, 12). It is reasonable to suspect that one or more of these may degrade LRV1
unencapsidated transcripts.

Second, in addition to the possible degradation of LRV1 transcripts by exoribonucleases, cleavage of the LRV1 transcript by a mechanism other than AGO1 is known to occur. Prior work in LRV1 from the Patterson lab described a “short transcript” 320 nt in length that was produced by \textit{in vitro} transcription from sucrose gradient-purified virions, and that was also present in living cells (13). This short transcript is generated via site-specific cleavage of the 5’ UTR of the LRV1 transcript by the viral capsid protein (14, 15). This would limit the accumulation of full-length dsRNA genome, as any dsRNA generated using cleaved transcript as a template would be missing a portion of its sequence. Further, the LRV1 IRES loses substantial activity with the deletion of just 120 nt (10) from the 5’ end of the transcript. Therefore, this cleavage could serve as a double hit – reduction in full length genome, and reduction in protein translation. At the time of its discovery, this cleavage activity was hypothesized to act as a check on virus replication (14).

I favor the hypothesis that LRV1 is controlled by a balance of replication and degradation not involving RNAi. This hypothesis is attractive because it would be effective independent of the species of \textit{Leishmania}. RNAi is active only in the \textit{Viannia} subgenus species; if RNAi played a substantial role in the control of LRV1, then a separate mechanism would likely be required for the control of LRV2 in \textit{L. major} and other species of the \textit{Leishmania} subgenus. In addition, this hypothesis addresses the lack of impact of AGO1 on control of LRV1. As the number of LRV1 virions rises following loss of AGO1, so too would the cleavage of the transcript, limiting LRV1 accumulation.

This hypothesis also does not rule out a role for RNAi in control of LRV1, as the two mechanisms may be somewhat redundant in \textit{Viannia} subgenus species. When both mechanisms
are present, loss of one may have minimal impact on virus levels. In this scenario, loss of AGO1 has only a small impact on LRV1 levels while the virus is capable of self-regulation. If the transcript cleavage activity were ablated, loss of AGO1 may increase LRV1 levels to a much greater extent. The RNA sequence required for cleavage of the LRV1 transcript has been mapped. One could, in principle, mutate this sequence to investigate whether the cleavage activity restricts virus replication and whether this activity and RNAi are redundant. However, in the absence of a reverse genetics system in LRV1, these hypotheses remain difficult to test.

5.4 – LRV1 may be unstable in some Leishmania strains

In Appendix C, I showed that LRV1 was unstable in the WT line of L. guyanensis M4147. When I examined this line for capsid protein levels by flow cytometry, I observed a heterogeneous distribution with a sizeable fraction of cells stained similarly to an LRV1-negative control. When this line was cloned (whether following transfection or not), many clones (15/42, 35.7%) of clones were uniformly LRV1-negative; these presumably arose from LRV1-negative cells within the original population. Of the clones that contained LRV1, a 17/42 (40.5%) displayed heterogeneity in LRV1 level similar to the parent/uncloned population. Even one of the clones that initially appeared homogeneously LRV1+ became heterogeneous over time in culture. This may have occurred in other LRV1+ clones were they cultured long enough, but I did not systematically test this. In contrast, the lab has worked extensively with a LUC-expressing clone of this strain which has a uniform, high level of LRV1 capsid staining.

The instability of LRV1 in the WT line was not apparent until now because previous work with WT examined LRV1 on a population level. For example, dsRNA bands were visualized from total RNA, LRV1 sequence was amplified by PCR, capsid protein was visualized by Western blot, or virions were purified from cell culture (16–19). Because many of
the cells in the population contain LRV1, these experiments identified the strain as LRV1+ but could not detect LRV1-negative cells in the population. The flow cytometry assay was developed by Matt Kuhlmann in our lab (20), but we were exclusively using the LUC-expressing clone and had never analyzed the WT.

This observation does explain a lingering discrepancy in the field, however. The Patterson lab identified an LRV1-negative isogenic clone of *L. guyanensis* M4147 that arose in culture (21). This clone was isolated following transfection with a plasmid conferring resistance to hygromycin B, and they hypothesized that drug selection mediated the loss of LRV1. We were unable to replicate this; however, we were using other parasite strains or the LUC-expressing clone. It is possible that their LRV1-negative clone originated as an LRV1-negative cell in the WT population prior to plating, as occurred in my *DCL* knockout experiments.

This heterogeneity in LRV1 level has not been observed in any other line we’ve evaluated by flow cytometry, which includes at least three strains of *L. braziliensis*. It is unclear what makes LRV1 stable in these lines, but unstable in *L. guyanensis* M4147. It is unlikely to be caused by the difference in species, as LRV1 is stable in the LUC-expressing clone of *L. guyanensis* M4147. In addition, it is unclear why the LRV1 is stable in the LUC-expressing clone, while it was unstable in the clones and transfectants of WT that I obtained. One possible explanation for this is a difference in the way clones were obtained. The LUC-expressing clone was isolated by plating on agar plates. At the time I was performing these experiments, agar did not support growth of *Viannia* subgenus species, and I obtained clones by limiting dilution. This strain is not “clumpy,” but I cannot rule out that my clones were not, in fact, mixed populations. If that were the case, the clones that appeared LRV1+ contained small numbers of LRV1-negative parasites, which then increased in proportion during culture. We would likewise expect
apparently LRV1-negative clones to develop LRV1+ populations during culture; I did not cultivate LRV1-negative clones long enough to observe this.

5.5 – StL constructs may not be biologically neutral

In the course of this work, I and others in the lab began to notice alterations in *Leishmania* biology in the presence of StL constructs. These included the presence of stable dsRNA derived from the transcribed inverted repeat, defects in the knockdown of additional StL construct targets, and possibly augmented virulence. In Appendix E, I documented experiments designed specifically to investigate these observations. I also identified an increase in the level of TATE transposable elements (TEs) in cells containing a StL construct. This data do not provide irrefutable evidence that StL constructs are problematic; rather, they suggest that caution is warranted during their use. Further, as RNAi appears to be more efficient and to play a role in virulence in *L. braziliensis*, the presence of a StL construct in this species could be more problematic than in *L. guyanensis*.

It is interesting to note that the effects of the presence of StL constructs are similar to those observed after the knockout of *AGO1*. In both cases, levels of TATE RNA rose and knockdown of a luciferase reporter fell, though the magnitudes of these effects were greater in the Δ*ago1* lines. This leads me to believe that a StL construct functions as a dominant negative on the RNAi pathway – its presence depletes the ability of the RNAi pathway to knock down other targets.

Additionally, in both the Δ*ago1* and StL lines, LRV1 levels increased. In Chapter 2 I documented that the introduction of a StL targeting the LRV1 Capsid or RDRP, but not a control GFPStL, resulted in loss of LRV1. In fact, LRV1 levels in the GFPStL lines were 20-100% higher than WT, depending on the strain examined. At the time, we assumed this was the noise
of the assay; however, the small impact of the loss of AGO1, coupled with the observation that the GFPStL affected knockdown of LUC, caused me to rethink this assumption. In L. guyanensis M4147, LRV1 levels rose 35% upon loss of AGO1 and approximately 50% in the presence of the GFPStL. The data from the two experiments were analyzed differently, and the StL experiment examined fewer biological replicates. Because of this, the magnitudes of the two experiments cannot be directly compared, but the data suggest that the effect is similar.

Together, this supports the hypothesis that AGO1-dependent RNAi has some function, albeit small, in reducing LRV1 levels in Leishmania (Viannia) species. The presence of the GFPStL impairs the regulation of other RNAi targets, and in its presence, LRV1 levels increased. If RNAi were not involved in control of LRV1, then the presence of the GFPStL would not have affected LRV1 levels.

5.6 – New tools for the study of Leishmania

During the course of my experiments, I required tools for working with Leishmania that did not yet exist. It was through these needs that the work described in Chapter 4 and Appendix B arose.

During the RNaseIII experiments detailed in Appendix B, my transfections yielded many colonies, but initial screens did not identify any successful transfectants. Because of this, I needed a less cumbersome method to enable me to evaluate relatively large numbers of clones. The existing protocol required 1-2 weeks in culture and 11 mL of medium for each clone screened; by reducing these requirements I could save substantial time and materials. The “quick, crude gDNA prep” protocol that I developed reduces the media requirement by 10 mL and the time requirement by up to a week in culture. This allowed me to save approximately 300 mL of media on this experiment, and perhaps more importantly, allowed me to quickly conclude
that the experiment was unlikely to succeed. If this protocol becomes widely used in the lab for integrations that are predicted to be rare, the cumulative time and materials savings could be substantial.

I developed the crude gDNA prep protocol to a stage where it was functional in my hands, but there are aspects of the protocol that could be optimized. For example, the crude gDNA isolated in my protocol is not reliably stable beyond one day; increasing the stability of the gDNA in storage would increase its utility. One way to do this would be to incorporate a phenol:chloroform extraction, but the volumes are likely to small for this to be feasible. Increasing the volume would require either using more culture volume (more material and more time) or diluting the gDNA, which would necessitate adding an ethanol precipitation step. Instead, my protocol should be viewed as preliminary screening to identify promising clones. These can then be grown to a larger culture volume to prepare “clean,” stable gDNA.

The popout constructs detailed in Chapter 4 and used in Appendix E arose out of our concern that the presence of a StL construct may not be neutral beyond its capacity to downregulate a target. We intended for LRV1StL parasites to be used to further probe the role of LRV1 in parasite biology and virulence because of a lack of isogenic lines. In order for this to be possible, however, parasite biology could not be altered by the LRV1StL construct. I developed the popout system both to 1) test the effect of the presence of the StL; and 2) remove the LRV1StL from parasites to create truly isogenic lines. This system works in principle: the StL construct is functional, and one can easily obtain cells that have lost it. However, there are signs that care must be taken with these lines.

First, a very large percentage (up to 40%) of *L. braziliensis PFR2StL(A)-GFP+(B) cells lost GFP expression when selection was withdrawn, but a sizeable fraction (6/9) of these retained
fragments of the StL construct integrated into the parasite genome. I suspect that the very high level of GFP expression selects for the loss of GFP by any means possible. I did not observe this phenomenon with the *L. guyanensis CapsidStL(A)-GFP+(B)* lines. In order for GFP-expressing *L. braziliensis* cells to be on the scale of the x-axis, I have to reduce the sensitivity of the flow cytometer approximately five-fold from settings I use to analyze *L. guyanensis* cells (data not shown). At the same time, less than 5% of *L. guyanensis* cells were GFP-negative following two passages without selection, and none of these contained the stable dsRNA that is characteristic of an integrated StL construct. This suggests that GFP is expressed less strongly in *L. guyanensis* than *L. braziliensis*, which may have helped the full construct remain integrated.

Second, a fraction of the *L. guyanensis CapsidStL(A)-GFP+(B)* clones had elevated doubling times in culture and were avirulent in mice. While *Leishmania* cultured for long periods of time often lose virulence, we have not typically observed this in *Viannia* subgenus species, such as *L. guyanensis*. Many groups have found that high levels of GFP expression do not affect parasite virulence (22–24). Rather, it is possible that perturbations induced by single-cell sorting contributed to the loss of virulence. It may be wise to systematically test whether clones obtained by cell sorting are more likely to lose virulence compared to clones obtained by limiting dilutions or plating. Regardless, special attention should be paid to characteristics such as doubling time to minimize the possibility of infecting mice with “dud” clones.

5.7 – Conclusions

Through this work, I investigated the role of the RNAi pathway in control of LRV1 in *Leishmania (Viannia)* parasites. I found that parasites containing LRV1 produce abundant virus-derived sRNAs, and that LRV1 can be eliminated by RNAi targeting. In contrast, endogenous RNAi has little effect on virus levels. Additional studies investigating the roles of other RNAi
pathway components were inconclusive. These experiments have deepened our understanding of parasite-virus interactions, and provided a starting point for further work on this topic. Further, I developed new tools for the study of *Leishmania (Viannia)* parasites, including a protocol for the isolation of genomic DNA from small numbers of parasites and a construct that facilitates removal of transfected DNA integrated into the parasite genome. Finally, I determined that increased caution may be warranted during RNAi experiments, as the StL construct may disrupt global RNAi within the cell. This knowledge will aid the study of *Leishmania (Viannia)* parasites going forward, and facilitate a wide range of investigations.

### 5.8 – References


17. Widmer G, Keenan MC, Patterson JL (1990) RNA polymerase activity is associated with


Appendix A: Chromosome 11/AGOL locus architecture
Preface

The experiments in this appendix were designed, performed, and analyzed by EAB under the guidance of SMB. The first draft was written by EAB and the final version presented here incorporates comments from SMB.
A1 – Introduction

Previous work in the lab generated a \textit{\textDelta ago1} parasite line in \textit{L. braziliensis} strain M2903. This line was functionally RNAi-deficient, both as assessed by RNAi reporters (Notton, Lye, and Beverley, unpublished data) and in terms of endogenous knockdown of transposable elements (1); ectopic expression of the \textit{AGO1} gene restored RNAi function. Together, these data suggested that the \textit{AGO1} gene was replaced as anticipated. However, during generation of this parasite line, the lab was unable to confirm proper integration of the knockout constructs at the 3’ end into the \textit{AGO1} locus. This suggested that the assembly of this genomic region was incorrect.

Further, when I was cloning constructs to knock out \textit{AGO1} in \textit{L. guyanensis}, I discovered that there was an approximately 800 nt sequence just 3’ of the \textit{AGO1} ORF that was repeated in two other loci on chromosome 11. It is likely that the length of the repeat caused an error in assembly of these regions. Because I would need to confirm the integration of my knockout constructs into the \textit{AGO1} locus, I set about determining which sequence was immediately 3’ of the \textit{AGO1} gene and confirming the integration of the \textit{\textDelta ago1} line of \textit{L. braziliensis}.

A2 – Results

A2.1 – The \textit{L. braziliensis} and \textit{L. guyanensis} genome assemblies are improperly assembled

To determine whether genes on chromosome 11 in \textit{L. braziliensis} M2903 and \textit{L. guyanensis} M4147 were arranged as annotated in the \textit{L. braziliensis} M2904 assembly, I used PCR to amplify across each individual repeat. I designed primers to bind to unique sites 5’ and 3’ of each repeat, and set up reactions containing each possible primer pair. The general scheme of primer binding is shown in Figure 1. For both species, PCR products of the expected size were obtained for the following primer combinations: a/d; c/b; and e/f (Figure 2). Other primer
combinations did not give PCR products of the expected size (data not shown). This suggests that the loci surrounding these intergenic regions are laid out as in Figure 3.

*The AGO1 replacement cassettes are properly integrated in the Δago1 line of L. braziliensis*

I next used PCR amplification to confirm the integration of the constructs used to knock out AGO1 in *L. braziliensis* strain M2903. I paired forward primers that hybridize to the drug resistance genes used in the AGO1 replacements with the proper reverse primer (primer d) that hybridizes to unique sequence 3’ of the repeat (Figure 4A). These PCR reactions gave products of the expected size (Figure 4B), indicating that the knockout constructs were properly integrated into the correct genomic locus in this parasite line.

A2.2 – *PacBio sequencing of L. guyanensis confirms chromosome 11 gene arrangement*

Since this work was completed, the *L. guyanensis* genome was sequenced on the PacBio platform. PacBio sequencing generates very long reads, which facilitates the assembly of repetitive elements. While the PacBio assembly does not precisely match my predicted arrangement of genes (compare Figure 3 with Figure 5, bottom), it is consistent with the results of my primer amplification experiments. Each of the regions of mis-assembly aligns to regions of the *L. braziliensis* assembly that contained sequencing gaps or the large repeat. These situations are known to contribute to difficulties in generating assemblies (2, 3). I identified other mis-assembly due to sequencing gaps and gene families along the chromosome, as well. More surprisingly, chromosome 11 in the PacBio assembly contains a 90.7 kb sequence at the 5’ end that is annotated in the *L. braziliensis* assembly as chromosome 19. This sequence contains 31 genes, none of which are annotated as part of *L. guyanensis* chromosome 19 in the PacBio assembly. This may be a sequencing artifact, or it may represent a true difference between the
two species, such as a chromosomal translocation. Further experiments will be required to differentiate between the two possibilities.

**A3 – Discussion**

Results from PCR amplification suggest that the presence of the 800nt repeat in chromosome 11 caused errors in the *L. braziliensis* strain M2904 genome assembly. Broadly, this particular mis-assembly is unlikely to dramatically impair study of these organisms, as it affects only a handful of loci. In the instance of the study of *AGO1*, however, it proved to be a stumbling block. Fortunately, the existing Δ*ago1* line of *L. braziliensis* had the anticipated and proper replacement of *AGO1* with drug resistance genes. Further, I was able to use this new data in the successful generation of the Δ*ago1* line in *L. guyanensis* (see Chapter 3).

Whole genome sequencing of *L. guyanensis* M4147 using the PacBio platform revealed that there were additional errors in the *L. braziliensis* M2904 assembly. These errors were due to sequencing gaps, and to the presence of gene families. Chromosome 11 is not likely to be the only instance of these types of sequencing errors in the *L braziliensis* M2904 genome, so it’s reasonable to assume that similar errors occur throughout the genome. Because the majority of genes on chromosome 11 were arranged as expected, however, the *L. braziliensis* M2904 assembly is likely sufficient in most cases for planning genetic manipulations of *Leishmania (Viannia)* parasites. In cases where the *L. braziliensis* assembly is not sufficient, or cases where extra certainty is desired, a PacBio assembly such as the *L. guyanensis* M4147 assembly will provide a more accurate view of the parasite genome.
A4 – Materials and Methods

Chromosome 11 sequences

The *L. braziliensis* strain M2904 genome assembly (Genbank accession ID: GCA_000002845.2) was used to identify the repeat regions on chromosome 11 by BLAST search and to design primers to these regions. The entire intergenic regions containing the repeats were extracted and labeled Intergenic Regions 1 (IR1), 2 (IR2), and 3 (IR3). The chromosomal locations of these regions are listed in Table 1.

Primers

Oligonucleotide primers were designed to hybridize to unique regions 5’ and 3’ of each repeat region. Primer sequences can be found in Table 2, along with the locations they bind according to the annotated genome. Also included in the table are primers that bind to the drug resistance ORFs used in the knockout of AGO1 from *L. braziliensis*.

PCR analysis of gDNA

Genomic DNA (gDNA) was isolated from the WT and Δago1 lines of *L. braziliensis* strain M2903 and from *L. guyanensis* strain M4147. Briefly, 10^8 parasites were lysed in TELT buffer (50mM Tris HCl, pH 8.0/62.5 mM EDTA, pH 9.0/2.5M LiCl/4% Triton X-100) and protein was removed by extraction twice with phenol:chloroform:isoamyl alcohol (25:24:1). Nucleic acids were concentrated by ethanol precipitation and RNA removed by digestion with 20 μg/mL RNaseA.

Purified gDNA was used as the PCR template at a final dilution of 1:500 in PCR reactions containing:1x KTLA buffer; 200 μM dNTPs; 400 μM each primer; 1M betaine; and
Klen-Taq LA polymerase to a final dilution of 1:500 in a 50 μL reaction. PCR products were analyzed by agarose gel electrophoresis.

**A5 – Table Legends**

Table A1: Chromosome 11 intergenic regions containing the approximately 800 nt repeat.

The open reading frames (ORFs) annotated to be either side of each intergenic regions are specified, as well as the nucleotide positions of the intergenic regions.

Table A2: Primers used to decipher the genomic architecture of chromosome 11.

Primer orientation, sequence, and binding location on chromosome 11 are specified.

**A6 – Figure Legends**

Figure A1: Regions of chromosome 11 surrounding 800nt repeats.

Repeats are depicted as black boxes labeled “R”, as annotated in the *L. braziliensis* M2904 assembly. Primer binding sites are indicated (a-f).

Figure A2: PCR products obtained for *L. guyanensis* M4147 and *L. braziliensis* M2903 WT parasites.

PCR products using genomic DNA from *L. guyanensis* (left) and *L. braziliensis* (right), indicating successful amplification across the repeated region.

Figure A3: Inferred genomic arrangement surrounding repeated regions on chromosome 11.

Repeats are depicted as black boxes. Primers that successfully amplified a PCR product of the expected size are indicated (a-f).

Figure A4: Confirmation of proper integration of *AGO1* knockout cassettes in *L. braziliensis* M2903.
A) Primer combinations for the confirmation of AGO1 replacements. Forward primers bind within the drug resistance cassette, and were paired with reverse primer d.

B) PCR products of the expected size were amplified from the Δago1 line of L. braziliensis M2903, confirming the proper integration of the replacement constructs used to generate the line.

Figure A5: Arrangement of genes on chromosome 11 surrounding repeat regions according to the L. guyanensis PacBio genome assembly.

Errors in the L. braziliensis M2904 assembly of chromosome 11 (top) occur around regions containing sequencing gaps (gray boxes) and the repeat (black boxes). Bottom, chromosome 11 as ordered in the L. guyanensis PacBio assembly. In the PacBio assembly, gray boxes do not denote sequencing gaps, but rather regions where sequencing gaps occurred in the L. braziliensis assembly (for reference). Vertical lines between the two assemblies indicate how regions within the chromosome differ. Primer pairs that amplified the expected product are shown as arrows connected by solid lines.

A7 – References


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<th>ORF annotated 3’ of IR</th>
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Figure 1

A

Primer combinations:

a+b  c+b  e+b
a+d  c+d  e+d
a+f  c+f  e+f
Figure 2

*L. guyanensis* M4147

Expected bands:
- 4912/4923: 1621 bp

*L. braziliensis* M2903

Expected bands:
- 5080/4911: 1941 bp
- 5081/4922: 2700 bp
Figure 3
Figure 4

A

B

Expected bands:
HYG\textsuperscript{R}: 1904 bp
BSD\textsuperscript{R}: 2101 bp
Figure 5
Appendix B – Development of a quick, crude *Leishmania* gDNA prep protocol
Preface
The protocol presented here was developed by EAB under the supervision of SMB. The first draft of this appendix was written by EAB and the final version presented here incorporates comments from SMB.
**B1 – Introduction**

The standard protocol for molecular validation of transfectants involves purification of genomic DNA (gDNA) from parasites followed by PCR amplification across the integration site. Previously, to obtain sufficient cells for gDNA purification, colonies were picked from semisolid agar plates, cells grown in 1 mL medium without selection to high parasite density (3-7 days), expanded to 10 mL with selective antibiotic and grown to high parasite density (2-5 days), and finally pelleted and the gDNA purified. In total, it requires 1-2 weeks of growth in culture, and 11 mL of medium for each clone to be evaluated. When large numbers of colonies must be screened, this constitutes a large commitment of resources and time.

To reduce the consumption of medium and the time required to screen clones, I developed a protocol to quickly isolate gDNA of sufficient quality to use as a PCR template from small numbers of *Leishmania* cells, as little as 200 μL of dense culture (2 x 10^6 – 2 x 10^7 cells, depending on species), with a minimal hands-on time commitment. This allows us to screen large numbers of clones at the 1 mL stage, and only expand promising clones to 10 mL for further analysis. It has a high rate of success – in my largest experiment using this method, I prepared gDNA from 24 clones, and a positive control PCR product was amplified in all 24 reactions (Figure 1).

There is undoubtedly room in the protocol for optimization, but it is sufficient for the purpose it was developed for.

**B2 – Protocol**

1) Grow cells to high density (late-log phase)

2) Pellet desired number of cells and wash with 0.5 volumes PBS.
3) Resuspend cells in 0.2 volumes lysis buffer (1% Triton X-100 in PBS containing 50 μg/mL proteinase K).

4) Incubate at 37 degrees C for 1 hour.

5) Heat-inactivate the proteinase K at 95 °C for 10 min.

6) Pellet any cell debris in table-top microcentrifuge at full speed for 5 min.

Use 2 μL of the supernatant as template in a 50 μL PCR reaction.

Crude gDNA samples can be stored at -20 °C for use later that day, but do not give reliable amplification after overnight storage.

**B3 – Figure Legend**

Figure B1: Positive control PCR product was amplified from crude genomic DNA preparations from 24 of 24 transfectants.

The WT line contained IR2SAT-LUC(B) integrated into the 18S rRNA small subunit locus, and primers bound either side of the site of integration. M, dsDNA ladder (the 1kb band is indicated); (+), positive control gDNA prepared via a “clean” protocol using Triton X-100/LiCl lysis followed by phenol:chloroform extraction; (-) no-template negative control.
Figure B1
Appendix C – Disruption of Dicers and Piwi
Preface

The experiments described in this appendix were designed, performed, and analyzed by EAB. SMB supervised the work and contributed to study design. The first draft was written by EAB, and the final version presented here incorporates comments from SMB.
In Chapter 3, I hypothesized that the parasite RNAi pathway played a role in containing replication of LRV1 to levels that the cell could sustain, permitting persistent infection. Loss of Argonaute 1 (AGO1) did not produce the expected dramatic increase in LRV1 levels, suggesting that if a substantial antiviral role for RNAi exists in *Leishmania*, it likely is AGO1 independent (Chapter 3). To determine whether any other components of the RNAi pathway play a role in control of LRV1, I attempted to deplete the levels of these other components by gene knockout and/or RNAi knockdown. These components are illustrated in Figure 1.

There remains a second Argonaute family protein in *Leishmania* whose function is unknown (1). Previous work in the Beverley lab suggested that this protein (known as Piwi) is localized to the mitochondrion, and efforts to knock it out in *L. braziliensis* M2903 were unsuccessful (Tsang, Anderson, and Beverley, unpublished data). It is unclear what role, if any, RNAi might play in mitochondrial function. However, RNAi knockdown of *PIWI* impaired concurrent knockdown of luciferase (Lye and Beverley, unpublished data), an effect similar to what was seen with knockdown of *AGO1* (2). While this may suggest that Piwi plays a role in the RNAi pathway, other possibilities not involving RNAi have not been eliminated and may be more likely. Nonetheless, it is possible that Piwi has a more substantial role than AGO1 in defense against viruses.

Alternatively, experiments in other organisms suggest that Dicer can, in some cases, play a role equal to or larger than Argonaute in defense against viruses (3–5). *Leishmania* (Viannia) parasites encode two Dicer proteins, and work in *Trypanosoma brucei* suggests that the functions of these proteins have specialized to some extent (6). Perhaps inactivation of the LRV1 genome
through cleavage by one or both Dicer is sufficient to limit LRV1 replication. In this situation, the resulting siRNAs may have minimal impact on LRV1 levels.

In this appendix, I present studies investigating the role of Piwi and Dicer proteins in the control of LRV1 in *L. guyanensis*. I first attempted to generate *Dicer1 (DCL1)* and *Dicer2 (DCL2)* null mutants; however, technical hurdles resulted in the abandonment of this approach. RNAi knockdown of *DCL1, DCL2*, and *PIWI* did not affect LRV1 levels; however, it is unlikely that knockdown resulted in sufficient loss of function for an effect to be visible. No conclusions could be drawn from this work on the role of Dicer proteins or Piwi in control of LRV1.

**C2 – Results**

**C2.1 – LRV1 is unstable in WT *L. guyanensis* M4147**

We first attempted to knock out the *DCL1* and *DCL2* genes individually by homologous recombination, replacing each allele with a drug resistance gene. We performed the initial rounds of replacement for each of *DCL1* and *DCL2* in WT *L. guyanensis* M4147 and obtained four heterozygous mutant clones for *DCL1* (two with a *PAC*<sup>R</sup> replacement and two with a *HYG*<sup>R</sup> replacement) and one heterozygous mutant clone for *DCL2* (*BSD*<sup>R</sup> replacement). Each heterozygous mutant clone had the expected PCR products confirming proper integration of the knockout cassettes (Figure 1). When I performed flow cytometry using the anti-capsid antibody, however, the WT parent and mutant clones were either LRV1-negative or a mixed population of LRV1-positive and LRV1-negative cells (Figure 2A). In an attempt to obtain homogeneous LRV1+ populations, I re-cloned the WT parent and two heterogeneous *DCL1* replacement clones (one PAC<sup>R</sup> replacement and one HYG<sup>R</sup> replacement) by limiting dilution. I evaluated LRV1 levels in 8-12 clones per genotype by flow cytometry (Figure 2B). The majority of clones were
either LRV1-negative or mixed populations, but a few clones appeared to be approximately homogeneous LRV1-positive.

**C2.2 – DCL1 double replacement clones retain a copy of the DCL1 ORF**

I performed a second round of replacement in a *DCL1 PAC*<sup>+</sup> replacement subclone that appeared to be LRV1-positive (clone 2.6, Figure 2B) and obtained six double replacement clones. Transfectants were evaluated by drug resistance and PCR tests for the presence of the *DCL1* ORF and proper 3’ integration of the replacement cassette. Five of six clones were properly integrated at the 3’ end, but all six retained a copy of *DCL1* (Figure 3). Chromosome 23, where *DCL1* is located, is predicted by sequencing read depth to be trisomic (Shaik and Beverley, unpublished data). The presence of a third copy of the *DCL1* ORF suggests that the gene is present in at least three copies, requiring a third round of replacement in order to generate a null mutant.

**C2.3 – Apparently-homogeneous lines develop substantial LRV1-negative populations over time**

When I evaluated the double replacement clones for LRV1 level by flow cytometry, the parent heterozygote that had previously appeared LRV1-positive (Figure 2B) was now heterogeneous (Figure 4). This suggested that either a small LRV1-negative population had been present previously or that LRV1 is intrinsically unstable in this line. Two double replacement clones were LRV1-negative, one was heterogeneous, and three were approximately LRV1-positive (Figure 4). Because the double replacement clones are not null mutants and the parent heterozygote developed a substantial LRV1-negative population, the LRV1-negative double replacement clones likely arose from LRV1-negative parent cells.

**C2.4 – Knockdown of PIWI and DCL1/2 was inefficient and did not affect LRV1 level**
Because of the instability of LRV1 in the WT *L. guyanensis*, I switched to a clone that had been transfected with a construct that confers luciferase expression and resistance to nourseothricin (*Sat^R*). Previous work in the lab has demonstrated this clone to harbor a single LRV1-positive population, and transfectants similarly do not show instability in LRV1 levels (7, 8). *Leishmania* have a limited number of available selection markers, which would make generating a *DCL1/2* double null mutant challenging in this genetic background. Because of this, I used RNAi-of-RNAi to reduce levels of *DCL1*, *DCL2*, and *PIWI*. The lab has previously used this approach to investigate the role of *AGO1* in the RNAi pathway (2).

Integration of *DCL1-* , *DCL2-* , and *PIWI*-targeting stem-loop (StL) constructs reduced mRNA levels of their targets by 0-55% (Figure 5). The *PIWI*StL construct failed to reduce *PIWI* mRNA levels at all, while the *DCL2*StL construct halved *DCL2* mRNA levels, and knockdown of *DCL1* was intermediate between the two. Analysis of LRV1 level in knockdown lines by flow cytometry showed no change in LRV1 levels upon integration of the StL constructs (Figure 6).

**C3 – Discussion**

_Heterogeneity in LRV1 level in the WT *L. guyanensis* M4147_

*L. guyanensis* M4147 WT, unexpectedly, was a mixed population of LRV1-positive and LRV1-negative cells. It is unlikely that this represents contamination with another strain or species of LRV1-negative parasites, as transfectants and clones of this line exhibited a wide range of LRV1 levels: LRV1-negative, heterogeneous, and LRV1-positive clones were readily obtained (Figure 2A and data not shown). If the LRV1-negative populations of the original WT line were merely the result of contamination or a mixed isolate, we would expect to obtain clones and transfectants that were LRV1-positive and LRV1-negative. We would not expect to obtain clones resembling the original line, with both LRV1-positive and LRV1-negative populations.
Further, when we re-cloned and subsequently transfected a heterozygous \textit{Dcl1} mutant, some of the resulting clonal lines displayed heterogeneity similar to the WT line (Figure 2B and Figure 4). For the observed heterogeneity to persist through three cloning steps makes contamination unlikely to be the cause by parsimony. In addition, a clone that initially appeared to be LRV1-positive developed substantial LRV1-negative populations over time in culture (compare Figure 2B, green trace with Figure 4, red trace). This suggests that the cell-to-cell heterogeneity in LRV1 level stems from an inherent instability in LRV1.

Because of this apparent instability of LRV1, the WT line of \textit{L. guyanensis} M4147 is not a good candidate for further work exploring effects on LRV1 levels, whether in regard to the role of RNAi or other applications, including screening for antiviral compounds. It is unknown why LUC c3, which is derived from the WT line, has stable levels of LRV1, but this line is a better candidate for further study. Alternatively, \textit{L. guyanensis} M4147 obtained from other sources may have stable, uniform LRV1 presence.

\textit{Investigations into the role of Dicers and Piwi were inconclusive}

Two rounds of replacement generated multiple clones with two copies of \textit{Dcl1} replaced by drug resistance genes; however all of these retained a copy of \textit{Dcl1}. Aneuploidy can signal that a gene is essential in \textit{Leishmania} (9–11); however, it is unlikely that \textit{Dcl1} is essential in \textit{L. guyanensis}, as \textit{Dcl1} mutants were viable in the related organism \textit{Trypanosoma brucei} (6). In addition, \textit{L. guyanensis} parasites are viable after knockout of \textit{Argonaute1}, which ablates the parasite RNAi pathway (Chapter 3). Instead, it is more likely that chromosome 23 is trisomic, as predicted. Therefore, three rounds of replacement will be required to obtain a Dcl1KO line.

Previous work in \textit{T. brucei} found that the two Dicer proteins have some overlap in the substrates from which they produce siRNAs (6, 12). For example, loss of either \textit{Dcl1} or \textit{Dcl2}
affects the accumulation of siRNAs derived from the CIR147 repeat element and from chromosomal inverted repeats (12). As Dcl1 protein is predicted to localize to the cytoplasm (13), we might expect this protein to play a predominant role in control of LRV1, which also localizes to the cytoplasm (14). We cannot rule out, however, that the presence of Dcl2 will compensate for loss of Dcl1. It may therefore be necessary to generate a double knockout in order to observe an effect on LRV1 levels, which would require five rounds of replacement and five different drug resistance cassettes. There are only six drug resistance cassettes commonly used in *Leishmania*, all of which would be required to generate a double knockout in the LUC c3 background (which already contains a $SAT^R$ cassette). In order to complement the mutant, the double knockout line would have to be cultured without selection for a time in the hope that gene conversion would replace one of the drug resistance genes with another, freeing it to be used to complement the knockout. This protracted approach is beyond the scope of this thesis.

To circumvent this hurdle, I changed tactics to knocking down *Dcl1, Dcl2*, and *Piwi* by RNAi. This RNAi-of-RNAi approach is somewhat counter-intuitive, as the activity of the target is required for knockdown. Because of this, we expect less knockdown that would ordinarily be achieved with other, non-RNAi genes. It has been successfully used, however, in the establishment of *AGO1* in the *Leishmania* RNAi pathway (2) and of *Dcl1* in the *Trypanosoma* RNAi pathway (13).

RNA levels fell by 55% at best (*Dcl2*) and, at worst, were unchanged (*Piwi*) following knockdown (Figure 5). This is likely to be an underestimate of knockdown at the RNA level, as the lab has previously documented the presence of large degradation intermediates by Northern blot (2). These degradation products would be amplified by PCR, even though they would not be competent for translation. A better estimate of knockdown could be achieved by Northern blot,
but more relevant would be Western blot analysis of knockdown at the protein level. Previous work in the lab showed that efficient knockdown at the RNA level did not guarantee equivalent reductions in protein level (2). Since Dcl1, Dcl2, and Piwi are predicted to play a role in the RNAi pathway, and are therefore required for knockdown of Dcl1, Dcl2, and Piwi, it is unlikely that protein levels would be dramatically reduced.

Regardless of the precise level of knockdown attained, LRV1 levels were unchanged following knockdown of each gene (Figure 6). This could be due to some combination of insufficient knockdown, redundancy between Dicer proteins, and/or a lack of involvement of the proteins in control of LRV1. Concurrent knockdown of both Dcl genes could circumvent the issue of redundancy, but more effective may be a combination knockout-knockdown approach, wherein, for example, Dcl1 is knocked out and Dcl2 is simultaneously knocked down. In the absence of the redundant protein, knockdown may confer sufficient reductions in RNAi to reveal a role for Dcl proteins in control of RNAi. Since this approach would still confer only a partial phenotype, however, any lack of effect on LRV1 levels would not be meaningful.

Alternately, CRISPR technology could be harnessed to delete all copies of a gene in a single transfection, using a single drug resistance marker. If this becomes routinely available in Leishmania, the double knockout could be generated rapidly and complemented to confirm any observed phenotypes.

C4 – Materials and Methods

Parasite strains and in vitro culture

L. guyanensis M4147 (MHOM/BR/78/M4147) was obtained from Jean Patterson (Texas Biomedical Research Institute, San Antonio, Texas). I transfected this line with the linear 18S rRNA-targeting SwaI fragment from B6367 pIR2SAT-LUC(B) (30), and clonal lines were
derived and validated. Either the WT parental line or the luciferase-expressing clone 3 was used, as indicated. Parasites were grown in fresh Schneider’s Insect Medium supplemented with 10% heat-inactivated fetal bovine serum, 100 μM adenine, 10 μg/mL hemin, 2 μg/mL biopterin, 2 mM L-glutamine, 500 units/ml penicillin and 50 μg/mL streptomycin, and selective drugs as indicated below.

**Constructs**

To knock out DCL1 and DCL2, genes conferring resistance to blasticidin S HCl (BSD\(^R\)), puromycin (PAC\(^R\)), and nourseothricin (SAT\(^R\)), hygromycin B (HYG\(^R\)), as well as DNA regions flanking the DCL1 and DCL2 ORFs were amplified by PCR (from B6173 pIR1BSD, B6176 pIR1PAC, B6351 pIR2SAT, B6441 pIR2HYG, and *L. guyanensis* M4147 WT genomic DNA, respectively) using Phusion polymerase and the primers found in Table 1. PCR products were gel-purified and used as templates in fusion PCR reactions. Reactions contained the 5’ flanking region forward primer, 3’ flanking region reverse primer, and 5µL each of 5’ flanking region, drug resistance ORF, and 3’ flanking region PCR products. Fusion PCR products were gel-purified, ligated into pCR-Blunt using the Zero-Blunt PCR Cloning Kit (Thermo Fisher Scientific), and used to transform TOP10 chemically competent cells. Transformants were selected on 100 µg/mL kanamycin, colonies picked, and DNAs confirmed by restriction digest and sequencing.

StL constructs to knock down DCL1, DCL2, and PIWI genes were similar to other RNAi constructs described in this document and elsewhere (7). “Stems” comprised of 600-900 bp regions of each ORF were amplified by PCR from *L. guyanensis* M4147 WT genomic DNA using the primers listed in Table 1 and KlenTaq-LA polymerase. Products were gel purified using the Qiaquick Gel Extraction kit (Qiagen), and ligated into pCR8/GW/TOPO (Thermo
Fisher) in a 10 min reaction at room temperature (RT). Stems were transferred into pIR3HYG-GW(A) using LR Clonase II (Thermo Fisher) in overnight reactions at RT. Reactions were quenched by addition of proteinase K and incubation at 37°C for 1 hour. Constructs were confirmed by restriction digest.

**Transfections**

Stable transfections were performed as previously described (2, 7, 15). Clonal lines were obtained either by plating on semisolid media with 150 µg/mL hygromycin B (RNAi knockdowns) or by limiting dilution in liquid media with 5 µg/mL puromycin, 50 µg/mL nourseothricin, 5 µg/mL blasticidin S HCl, or 75 µg/mL hygromycin B, as appropriate (knockouts). Transfectants were grown to stationary phase in 1 mL media and passaged thereafter in 10 mL media with selective drugs, as appropriate for the construct. When the luciferase-expressing line was used, plates and liquid media also contained 50 µg/mL or 25 µg/mL nourseothricin, respectively.

**Flow cytometry**

Flow cytometry using the anti-capsid antibody (16) was performed as described previously (7).

**RNA preparation and qPCR**

RNA was prepared as described previously (7). qPCR primers were designed to amplify ~100 bp regions of the appropriate ORF that lie outside the regions targeted by the StL construct (Table 2). qPCR reactions were performed with cDNA templates in 20 µL total reaction volume using the Power SYBR Green Master Mix (Thermo Fisher), 5 µL of ten-fold diluted cDNA, and final primer concentrations of 0.2 µM. Reactions were run on the ABI PRISM 7000 Sequence
Detection System (Applied Biosystems, Thermo Fisher). PCR amplification conditions were as follows: 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. PCR products were confirmed to be specific by melt curve analysis. Amplification of *KMP-11* was used to normalize parallel reactions. +RT reaction were performed in triplicate and –RT reactions in duplicate. A 10-fold dilution series was used to calculate primer efficiency and relative RNA calculated using the Pfaffl method (17).

**C5 – Table Legends**

Table C1: Primers used in the cloning of constructs to knock out Dicer 1 and 2 and to knock down Dicer 1, Dicer 2, and Piwi.

Restriction sites to enable release of the knockout cassette from the plasmid are underlined. Linker regions enabling fusion PCR are in bold.

Table C2: Primers used for qPCR of Dicer 1, Dicer 2, and Piwi.

**C6 – Figure Legends**

Figure C1: Schematic of RNAi pathway components Dicer1, Dicer2, Ago1, and Piwi in *L. guyanensis*.

Figure C2: Confirmation of *DCL1* (A) and *DCL2* (B) single replacement by PCR. Primers amplify across the site of integration.

Figure C3: LRV1 levels by flow cytometry using an anti-capsid antibody.

A) Substantial variability in LRV1 level is present in the WT and single replacement clones following transfection. Shown are representative clones. Black, LRV1-negative control; red, WT; blue, heterogeneous *DCL1+/-* clone; green, LRV1-negative *DCL1+/-* clone.
B) Re-cloning of a DCL1+- clone generates sub-clones with substantial variability in level of LRV1. Representative clones are shown. Black, LRV1-negative control; purple; LRV1-negative subclone; green, LRV1-positive sub-clone; blue, heterogeneous sub-clone.

Figure C4: Confirmation PCR reactions following transfection of HYG\textsuperscript{R} replacement construct into PAC\textsuperscript{R} replacement of DCL1.

Left, amplification across site of integration. Right, amplification of DCL1 ORF. NTC, no-template control reaction. HYG\textsuperscript{R} single replacement was used as a positive control (+).

Figure C5: DCL1 double replacement clones display substantial variability in level of LRV1 as assessed by flow cytometry.

Black, LRV1-negative control; red, single replacement parent; green, LRV1-positive clone; purple, LRV1-negative clone; blue, heterogeneous clone.

Figure C6: qPCR analysis of DCL1 (A), DCL2 (B), and PIWI (C) mRNA levels after knockdown relative to untransfected cells.

Averages and standard deviations are of two biological replicate of the parent line and six independent clones of each knockdown.

Figure C7: Knockdown of DCL1, DCL2, and PIWI has no effect on LRV1 protein levels as assessed by flow cytometry.

Representative clones are shown. Black, LRV1-negative control; red, untransfected parent line; green, Dcl1StL; blue, Dcl2StL; purple, PiwiStL.
C7 – References


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Figure C1
Figure C2

A

\[ \text{DCL1-HYG}^R \quad \text{DCL1-PAC}^R \]

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Figure C3

A
LRV1-Dcl1+/− c1
WT
Dcl1+/− c2

B
LRV1-Dcl+/− c2.7
Dcl1+/− c2.6

Capsid protein

Number of cells

% of cells
### Figure C4

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Figure C6

A  

B  

C  

Relative Dcl1 RNA  

Relative Dcl2 RNA  

Relative Piwi RNA  

Parent  

DCL1  

StL  

Parent  

DCL2  

StL  

Parent  

PIWI  

StL  

0  

0.3  

0.6  

0.9  

1.2  

1.5  

0  

0.3  

0.6  

0.9  

1.2  

1.5  

0  

0.3  

0.6  

0.9  

1.2  

1.5
Figure C7

![Graph showing cell distribution with peaks labeled Parent, Dcl1 StL, Dcl2 StL, and PivriStL.](image-url)
Appendix D: Expression of bacterial RNaseIII
Preface

The experiments in this appendix were designed and performed by EAB under the guidance of SMB. The first draft was written by EAB and the final version presented here incorporates comments from SMB.
D1 – Introduction

We previously showed that a substantial number of sRNAs are generated from LRV1 in *Leishmania* parasites (1). Loss of Argonaute1, however, did not elicit the expected increase in LRV1 level (this work, Chapter 3). This suggested that Argonaute1 is not involved substantially in control of LRV1. The presence of sRNAs derived from LRV1 implied that Dicer proteins cleaved the LRV1 dsRNA genome; perhaps this cleavage was sufficient to control LRV1 replication without contribution from Argonaute. To test this, I attempted to over-express RNaseIII in LRV1+ *L. guyanensis*.

In most organisms, Dicer proteins contain two RNaseIII domains, a dsRNA binding domain (dsRBD), a helicase domain, a Piwi/Argonaute/Zwille (PAZ) domain, and a DUF283 domain (2, 3). Work on the Dicer proteins of the relative parasite *Trypanosoma brucei*, however, has identified only the two RNaseIII domains (4, 5). In other systems, bacterial RNaseIII proteins have shown promise in degradation of RNA viruses. Langenberg *et al.* found that expression of *E. coli* RNaseIII in *Nicotiana tabacum* conferred increased resistance to a number of plant viruses, but also resulted in stunting (6). Lee *et al.* found that expression of *E. coli* RNaseIII in *Bombyx mori* resulted in an enhancement of RNAi activity, but also resulted in nonspecific RNA degradation (7). Together, these results suggest that bacterial RNaseIII proteins degrade viral dsRNA and/or generate sRNAs capable of entering the RNAi pathway, but also have the potential to be detrimental to cells.

It is not known which *Leishmania* Dicer protein, if either, is responsible for control of LRV1, whether control of LRV1 requires the combined action of both proteins, or whether Dicer proteins can be overexpressed in *Leishmania*; therefore, I chose to express bacterial RNaseIII proteins from *Escherichia coli* and *Staphylococcus aureus* in *L. guyanensis*. In theory, this
should increase the cleavage of the LRV1 dsRNA genome, potentially resulting in loss of the virus. I was unable, however, to obtain viable RNaseIII-expressing parasites.

**D2 – Results**

*D2.1 – Heterologous expression of bacterial RNaseIII proteins is likely toxic to Leishmania*

I transfected SwaI-linearized IR3HYG(empty), IR3HYG-Ecoli_RNaseIII(B), and IR3HYG-SAureus_RNaseIII(B) into a luciferase-expressing clone of *L. guyanensis* strain M4147. Transfection of the empty vector positive control yielded 4-10 times more colonies than transfection of the RNaseIII-bearing DNA (Table 1). This disparity in the number of colonies obtained can signal that the gene product being delivered is toxic. PCR reactions to confirm integration at the 3’ end of the construct yielded a product of the expected size (Figure 1A). I performed PCR reactions on genomic DNA to confirm integration at the 5’ end using a forward primer binding outside the targeting region and a reverse primer binding to the RNaseIII ORF; these reactions did not yield any product, even after optimization of reaction conditions (data not shown). To confirm that the RNaseIII ORF was present in the cells, I attempted to amplify the RNaseIII ORF from six clones of each RNaseIII construct. However, the expected PCR product did not amplify from any sample (data not shown). I repeated the transfection, and again, the positive control DNA yielded 10-fold higher numbers of colonies (Table 1). I did not attempt the integration PCR reactions, but again, none of the RNaseIII-transfected colonies examined contained the RNaseIII ORF by PCR (Figure 1B & C, data not shown), but control PCR reactions amplified a fragment of the luciferase construct in all DNAs. In total, I screened 18 colonies transfected with the *E. coli* gene and 34 colonies transfected with the *S. aureus* gene.
**D3 – Discussion**

This experiment attempted to increase RNaseIII activity in LRV1-positive *L. guyanensis* parasites through the heterologous expression of bacterial RNaseIII genes. I was unable, however, to recover viable cells with the RNaseIII ORF integrated, which suggests that it is toxic when expressed at the levels obtained by integration into the parasite 18S rRNA locus.

The colonies that arose following transfection may have integrated the RNaseIII construct and ejected the RNaseIII ORF by rearrangement; in this situation, the cells would have carried the drug resistance gene. Indeed, PCR reactions to confirm 3’ integration use a forward primer binding to the *HYG* gene, and generated a product of the expected size. This suggests that these clones may have contained the HYGR gene and therefore been transfectants, but ejected the RNaseIII ORF. My lack of success in amplifying a 5’ integration PCR product using primers that binds to the RNaseIII ORF supports this conclusion. I could have further confirmed this by attempting to amplify the *HYG* gene, but since none of the clones contained the RNaseIII ORF, I did not feel a need to.

If this experiment were attempted again, more success might be had by integrating RNaseIII into a lower-expression locus, such as the alpha-tubulin locus. Additionally, this experiment would benefit from an inducible system, in which transcription could be initiated only when desired and the level of transcription better controlled.

**D4 – Acknowledgements**

Thanks to Jean Patterson (Southwest Foundation for Biomedical Research) for providing parasite strains and to Gautam Dantas (Washington University School of Medicine) for providing *S. aureus* genomic DNA. Thanks to Nicole McAllister, who performed the cloning of the RNaseIII constructs.
**D5 – Materials and Methods**

*Parasites and in vitro culture*

*Leishmania guyanensis* strain M4147 was obtained from Jean Patterson (Southwest Foundation for Biomedical Research, San Antonio, Texas). This line was transfected with the linear 18S rRNA-targeting SwaI fragment from B6367 pIR2SAT-LUC(B), and clonal lines were derived, validated, and used in these experiments. Parasites were grown in fresh Schneider’s Insect Medium supplemented with 10% heat-inactivated fetal bovine serum, 100 μM adenine, 10 μg/mL hemin, 2 μg/mL biopterin, 2 mM L-glutamine, 500 units/ml penicillin and 50 μg/mL streptomycin, and selective drugs as indicated below.

*RNaseIII constructs*

*Staphylococcus aureus* genomic DNA was obtained from Dr. Gautam Dantas (Washington University School of Medicine) and *Escherichia coli* genomic DNA was isolated from strain K12. Briefly, bacterial cultures were pelleted, resuspended in lysis buffer (0.6% SDS and 0.12 mg/mL proteinase K in TE), and incubated at 37°C for 1 hour. DNA was extracted with phenol:chloroform (1:1), ethanol precipitated, and the pellet dissolved in TE. RNaseIII genes were amplified from bacterial genomic DNA using Phusion polymerase and the primers listed in Table 2 and ligated into the BglII site of pIR3HYG. RNaseIII ORFs were confirmed by sequencing.

*Transfection*

Stable transfections were performed as previously described(8, 9). After overnight recovery, clonal lines were obtained by spreading % of the transfected cells per plate on semisolid media with 50 μg/mL nourseothricin and 150 μg/mL hygromycin B. Colonies were
picked into 1 mL media without selection and passaged thereafter in 10 mL media with 50 µg/mL hygromycin B.

Leishmania genomic DNA isolation and PCR

200 µL dense culture was pelleted, washed with phosphate buffered saline, and resuspended in 40 µL lysis buffer (1% Triton X-100 in PBS containing 50 µg/mL proteinase K). Samples were incubated at 37 °C for 1 hour and heat-inactivated at 95 °C for 10 min. 2 µL of genomic DNA was used in 50 µL PCR reactions. DNA was pooled (3 samples/pool) and PCR performed to amplify the RNaseIII ORF using the primers in Table 2. Genomic DNA from each sample was assayed for quality by amplification of a control PCR product.

D6 – Table Legends

Table D1: Colonies per µg of DNA obtained from transfection with positive control IR3YG and RNaseIII constructs.

The number of plates used in each attempt is given in parentheses. For each attempt, transfected cells were allowed to recover overnight, and ⅙ of each transfection was spread per plate.

Table D2: Primers used for cloning of RNaseIII genes from E. coli and S. aureus.

BglII sites indicated in bold; 6x His tag indicated in italics; Kozak sequence indicated by underlining.
D7 – Figure Legends

Figure D1: Confirmation of transfectants by PCR amplification of genomic DNA.

A) PCR reactions to confirm 3’ integration of RNaseIII constructs. The forward primer binds to the HYG\textsuperscript{R} ORF and the reverse primer binds 3’ of the targeting region. 12 of 12 clones amplified a product of the expected size.

B) In \textit{S. aureus} RNaseIII-transfected cells, a control PCR reaction amplified a portion of the luciferase construct from all genomic DNAs tested.

C) Genomic DNA from clones transfected with the \textit{S. aureus} RNaseIII construct was pooled (3 clones/pool) and PCR performed to amplify the RNaseIII ORF. Plasmid DNA was used as a positive control (+).

D8 – References


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Figure D1

A

\[ \begin{array}{c}
18S\text{ rRNA} \quad \text{RNase III} \quad \text{HYG} \quad 18S\text{ rRNA} \\
\hline
E\text{ coli RNaseIII} & S\text{ aureus RNaseIII} & M
\end{array} \]

B

Individual \textit{S. aureus} gDNAs

Luciferase integration

C

\[ \begin{array}{c}
M \quad \text{S. aureus gDNA pools (3)} \quad M + \\
\hline
\text{RNaseIII} & \text{ORF} \end{array} \]
Appendix E – Effects of StL constructs on *Leishmania* biology & virulence
Preface

The experiments presented in this appendix were designed, performed, and analyzed by EAB under the guidance of SMB. The first draft was written by EAB, and the final version presented here incorporates comments from SMB.
E1 – Introduction

Evidence in the laboratory has accumulated over the last few years to suggest that stem-loop (StL) constructs may not be biologically neutral. In this Appendix I will detail experiments that I have conducted to determine whether the presence of a StL construct integrated into the parasite genome alters *Leishmania* biology and virulence. While these results are not conclusive, they suggest that caution is warranted when StL constructs are used.

E2 – Results

E2.1 – The presence of a StL construct interferes with the effectiveness of a second StL

Previous work in the lab found that the presence of LRV1 had no effect on the efficiency of the RNAi pathway (1), but this relied on a single LRV1-negative isogenic line. We decided to use our LRV1-targeting StL constructs to confirm this finding using multiple independent LRV1-negative clones. We transfected *CapsidStL* and *RNA-dependent RNA polymerase* (RDRP) StL constructs into a clone of *L. braziliensis* strain LEM2780 containing an integrated luciferase “self-reporter” (LUCSR) construct. This construct contains the luciferase (LUC) ORF and a luciferase-targeting StL and integrates into the 18S rRNA locus; *L. braziliensis* parasites transfected with this construct have LUC activity more than 100-fold below that of parasites transfected with the LUC ORF alone. The resulting lines were LRV1-negative as assessed by flow cytometry using the anti-capsid antibody (Figure 1).

We then evaluated the lines for LUC activity relative to LUC-expressing, LRV1-negative LRV1 StL lines (described in Chapter 2), as well as to untransfected LUC and LUCSR parasites. In untransfected cells, the LUC line had 143x higher luciferase activity than the LUCSR line; in the LRV1 StL parasites, the LUC line had 9x higher luciferase activity compared to the LUCSR line (Figure 2A). To control for non-specific effects of the stem-loop, we also transfected a *GFP*
targeting StL construct into the parental LUCSR line and compared LUC activity with LUC-expressing cells carrying the same \textit{GFP}\textsubscript{StL} (2). The untransfected LUC cells again had 141x higher luciferase activity than the untransfected LUCSR cells. The LUC \textit{GFP}\textsubscript{StL} cells had 15x higher luciferase activity than LUCSR \textit{GFP}\textsubscript{StL} cells (Figure 2B). This suggests that the reduction in knockdown of LUC was not specifically caused by knockdown of LRV1, but rather nonspecifically by the introduction of a second StL construct.

\textit{E2.2 – Removal of StL targeting Leishmania RNA virus 1 (LRV1) in \textit{L. guyanensis} does not rescue presence of LRV1}

I previously described a construct system that enabled integrated IR vectors to be easily removed from the parasite genome (see Chapter 4), termed “popout constructs.” These constructs use 18S rRNA sequence to integrate into the parasite genome by homologous recombination and carry two genes to enable selection: a drug resistance gene and green fluorescent protein (\textit{GFP}). After culturing cells without positive selection, a proportion of the cells “pop out” the integrated construct and can be selected by their lack of GFP expression.

In Chapter 2, we described LRV1StL-generated LRV1-negative lines as “isogenic.” Ideally, a true isogenic line would no longer contain the RNAi construct. Therefore, we created a popout version of the \textit{Capsid} StL construct targeting LRV1 from \textit{L. guyanensis} strain M4147. In addition to being truly isogenic, these lines would give us an opportunity to investigate the effects of the StL construct on \textit{Leishmania} biology.

I transfected linearized IR3HYG-\textit{CapsidStL}(A)-\textit{GFP}+(B) and IR3HYG-\textit{GFP}+(B) into a luciferase-expressing clone of \textit{L. guyanensis} strain M4147 (hereafter, WT). As an LRV1-negative control, I also transfected IR3HYG-\textit{GFP}+(B) into an LRV1-negative line that arose spontaneously in culture (M4147/\textit{HYG}) (3). After transfection and subsequent popout, integrated
clones had GFP expression 300-fold above that of popped out clones (data not shown). In addition, popped out clones were sensitive to hygromycin B, while integrated clones were resistant. Parasites with the \textit{CapsidStL(A)-GFP+(B)} construct integrated (hereafter, SSU:\textit{CapsidStL(A)-GFP+(B)}) and parasites with the \textit{CapsidStL(A)-GFP+(B)} construct popped out (hereafter, PO:\textit{CapsidStL(A)-GFP+(B)}) were LRV1-negative by RT-PCR (Figure 3). WT cells transfected with the \textit{GFP+(B)} construct contained LRV1, indicating that expression of GFP did not induce loss of LRV1 (Figure 3). As PO:CapsidStL-GFP clones are LRV1-negative and no longer contain the StL construct, they represent truly isogenic LRV1-negative lines.

\textit{E2.3 – Integrated StL constructs mobilize transposable elements}

The results of the LUC activity assay in \textit{L. braziliensis} LEM2780 above (Section E2.1) suggested that the presence of the StL construct non-specifically interferes with the efficiency of a second StL construct. As the RNAi pathway in \textit{Leishmania} likely plays a role in the control of transposable elements (TEs) \cite{2, 4}, integration of StL constructs could also result in mobilization of TEs and a subsequent loss of genome stability. To assess whether this occurred in StL lines, we sequenced RNA from \textit{L. guyanensis} SSU:\textit{CapsidStL(A)-GFP+(B)}, PO:\textit{CapsidStL(A)-GFP+(B)}, and control SSU:\textit{GFP+(B)} parasites (Figure 4A), and mapped the reads to the \textit{L. guyanensis} and LRV1 genomes. The three lines had a similar proportion of reads mapping to the housekeeping genes \textit{glucose-6-phosphate dehydrogenase} and \textit{RNA polymerase II large subunit}. Likewise, reads mapped at a similar proportion to SLACS elements. In contrast, reads mapped to TATE elements at twice the frequency in SSU:\textit{CapsidStL(A)-GFP+(B)} as in PO:\textit{CapsidStL(A)-GFP+(B)} or the SSU:\textit{GFP+(B)} control. In SSU:\textit{GFP+(B)}, reads mapped to the entire length of the LRV1 genome, whereas in SSU:\textit{CapsidStL(A)-GFP+(B)}, reads mapped exclusively to the
region targeted by the StL construct (Figure 4B). Only 51 of 12,801,644 reads mapped to LRV1 in PO: TesidStL(A)-GFP+(B); these are likely noise.

We also sequenced small RNAs (sRNAs) isolated from total RNA from these lines and mapped the 20-26 nt fraction to the L. guyanensis and LRV1 genomes. We have previously showed that the 20-26 nt fraction of sRNAs are a good proxy for siRNAs (2). Reads mapped to SLACS and TATE elements at similar proportions between PO: TesidStL(A)-GFP+(B) and the SSU: GFP+(B) control (43.7 and 37.8% vs 39.8 and 33.9% of mapped reads for SLACS and TATE, respectively, in PO: TesidStL(A)-GFP+(B) vs the SSU: GFP+(B) control) (Figure 4C). In contrast, sRNA reads from SSU: TesidStL(A)-GFP+(B) mapped to SLACS and TATE elements at a frequency ~10% that of the PO: TesidStL(A)-GFP+(B) or SSU: GFP+(B) controls. Simultaneously, frequencies of LRV1-mapping reads increased from 4.0% of mapped reads in the SSU: GFP+(B) control to 86.2% in the SSU: TesidStL(A)-GFP+(B) line. These reads mapped exclusively to the region targeted by the StL construct (Figure 4D), similar to what was seen previously with LRV1 StL constructs (2). Likewise, the few LRV1-mapping reads in PO: TesidStL(A)-GFP+(B) mapped to the stem region.

The results of RNA and sRNA sequencing suggested that TATE elements were indeed mobilized in CapsidStL-containing lines. To confirm, we evaluated lines for SLACS and TATE transcript levels by qPCR (Figure 5). As was seen with sequencing, SLACS levels were similar across the lines, while TATE levels were approximately doubled in SSU: TesidStL(A)-GFP+(B) compared to PO: TesidStL(A)-GFP+(B) or the SSU: GFP+(B) control.

E2.4 – Integrated StL constructs produce stable dsRNA

Other members of the laboratory have identified stable dsRNA in parasites transfected with StL constructs targeting viral RNAs. These dsRNA species were reverse transcribed,
cloned, and found to contain sequence from the inverted repeat of the StL construct (Akopyants, Lye, and Beverley, unpublished data). To examine this, I digested total RNA from parasites transfected with the $\text{CapsidStL(A)}$-GFP+(B) and control GFP+(B) constructs with S1 nuclease to determine if this stable dsRNA was present in integrated, but not popped out, StL lines. A clear LRV1 band was present in the LRV1+ SSU:GFP+(B) and PO:GFP+(B) controls and absent from SSU:$\text{CapsidStL(A)}$-GFP+(B), PO:$\text{CapsidStL(A)}$-GFP+(B), and LRV1-negative lines (Figure 6, white arrowhead). Both clones of SSU:$\text{CapsidStL(A)}$-GFP+(B) had visible dsRNA, which was not present in either of the PO:$\text{CapsidStL(A)}$-GFP+(B) clones (Figure 6, black arrow).

**E2.5 – The presence of a StL construct may affect parasite virulence**

It is thought that LRV1 increases parasite virulence by acting as a source of dsRNA, which is detected by TLR3 (5, 6). In support of this, the addition of LRV1 dsRNA to macrophages in vitro results in a release of cytokines similar to that observed when the macrophages are infected with $L.\text{guyanensis}$ parasites containing LRV1 (5). It is possible that the stable dsRNA present in StL lines could serve as a source of dsRNA, similarly affecting parasite virulence. To test this, we injected the $\text{CapsidStL(A)}$-GFP+(B) popout lines and controls into the hind footpad of C57/B6 mice and monitored the infections for luciferase activity (a measure of parasite numbers) and lesion size (a measure of disease pathology).

At least one clone each of SSU:GFP+(B), PO:GFP+(B), and SSU:$\text{CapsidStL(A)}$-GFP+(B) failed to induce pathology or accumulate any substantial number of parasites (data not shown). These clones also had elevated doubling times in culture (data not shown). I concluded that they were exhibiting culture-induced loss of virulence, and omitted them from further analysis (7, 8). As expected, LRV1+ controls were significantly more virulent than LRV1-
negative controls, regardless of the presence or absence of GFP expression (Figure 7). Unexpectedly, both SSU:CapsidStL(A)-GFP+(B) and PO:CapsidStL(A)-GFP+(B) parasites displayed virulence phenotypes intermediate between the LRV1+ and LRV1-negative controls (Figure 7). I had expected the PO:CapsidStL(A)-GFP+(B) parasites to behave similarly to LRV1-negative PO:GFP+(B) controls, as neither group contains LRV1 or expresses GFP. Possible reasons for this will be explored in the Discussion.

**E3 – Discussion**

In these experiments, introduction of a StL construct targeting either GFP or LRV1 into *L. braziliensis* LEM2780 interfered with the knockdown of luciferase. Additionally, integration of an LRV1StL popout construct into *L. guyanensis* resulted in increased levels of transposable element RNA and an accumulation of stable dsRNA. Both of these phenotypes returned to WT after the construct was popped out. Finally, the presence of the LRV1StL popout construct in *L. guyanensis* may affect parasite virulence, but the data were not conclusive.

The use of popout CapsidStL-GFP+(B) constructs confirms that targeting of LRV1 by RNAi results in a complete loss of virus. In previous work (see Chapter 2), the StL construct was irreversibly integrated into the *Leishmania* genome. We were unable to amplify LRV1 sequence from cDNA or detect capsid protein by flow cytometry in these lines; however, we could not rule out that a very low level of LRV1 persisted in these cells, continually knocked down to undetectable levels. With the removal of the popout StL construct, LRV1 levels did not rebound, confirming that the virus is eradicated from these cells.

One caveat to these results is that none of the StL constructs used here have a complementary target in the cell. Transfection of a StL targeting LRV1 results in loss of the virus, at which time there is no longer any viral transcript present for base paring with anti-LRV1
siRNAs. Similarly, the control GFPStL was transfected into a cell line that does not express GFP; therefore, siRNAs produced from that construct do not have a target. There are conflicting reports in the literature regarding the effect of the presence of target RNA on sRNA stability. On one hand, Chatterjee and Großhans found that the presence of a cognate target stabilized the let-7 miRNA-AGO complex in *C. elegans* lysate and protected the miRNA from degradation (9). Conversely, De et al. found that the presence of a target RNA promoted the release of a miRNA from human AGO2 (10). If target binding does in fact promote siRNA turnover and degradation in *Leishmania*, then the absence of a target could result in an accumulation of AGO1 bound to StL-derived siRNAs at the expense of other siRNAs. More research will be required to confirm or refute this hypothesis. Further, more research will be required to extend the effects observed here to StL constructs targeting endogenous *Leishmania* genes. For these genes, continuing transcription of the target gene ensures the presence of the target of the StL-derived siRNAs.

Little conclusive results can be drawn from the investigation of the effect of the StL construct on parasite virulence. I considered the PO:*CapsidStL*(A)-*GFP*+(B) parasites to be a negative control before the start of the experiment, and expected them to be indistinguishable from the LRV1-negative PO:*GFP*+(B) cells. However, they displayed a virulence phenotype intermediate between the LRV1+ and LRV1-negative controls. It is possible that some lingering effect of the StL construct persisted in the PO:*CapsidStL*(A)-*GFP*+(B) parasites, but I do not think this is likely. There were at least 80 cell doublings that separated SSU:*CapsidStL*(A)-*GFP*+(B) and PO:*CapsidStL*(A)-*GFP*+(B) lines. I would anticipate this would be more than enough time for any StL-derived siRNAs or stable dsRNA to be lost by dilution. Indeed, no stable dsRNA was visible in PO:*CapsidStL*(A)-*GFP*+(B) lines following digestion with S1 nuclease, and very few sRNA reads mapped to LRV1.
Rather, I think it is more likely that these results were due to a technical artifact. A number of the clones I injected failed to produce a lesion; it is possible that the LRV1-negative controls were similarly avirulent. Because I was expecting low virulence from these lines, I did not flag them as problematic. Without confidence in the negative control, I cannot draw any conclusions. In order to determine whether the StL construct results in increased virulence, new integrated and popped out clones should be obtained and evaluated. If these do not demonstrate culture loss of virulence, then we can have confidence in the results.

The presence of a StL construct in Leishmania parasites appears to impair knockdown of other RNAi targets, lead to the accumulation of stable dsRNA, and may affect virulence of the parasite. Much of this data is preliminary, and more work will be necessary to attain a high confidence in these results. For example, I only sequenced RNA from one clone of each line – I will need to sequence additional clones in order to confirm these results. However, together the results suggest that caution is warranted when StL constructs are used.

**E4 – Materials and Methods**

*Parasite strains and cell culture*

*L. braziliensis* LEM2780 (MHOM/BO/90/CS) was from Patrick Bastien (Université de Montpellier, Montpellier, France); *L. braziliensis* M2903 (MHOM/BR/75/M2903) was from Diane McMahon Pratt (Yale School of Public Health, New Haven, CT); and *L. guyanensis* M4147 (MHOM/BR/78/M4147) and its derivative *L. guyanensis* M4147/HYG were from Jean Patterson (Southwest Foundation for Biomedical Research, San Antonio, Texas). *L. braziliensis* LEM2780, *L. guyanensis* M4147, and *L. guyanensis* M4147/HYG were previously transfected with Swal-linearized B6367 pIR2SAT-\textit{LUC}(B), and clonal lines were used in these experiments.
*L. braziliensis* LEM2780 was also separately transfected with Swal-linearized B6386 pIR2SAT-LUC(B)-LUCStL(A), and clone 71 was used in this work.

Parasites were grown in fresh Schneider’s Insect Medium supplemented with 10% heat-inactivated fetal bovine serum, 100 μM adenine, 5 × 10^{-5} % hemin, 2 μg/mL biopterin, 2mM L-glutamine, 500 units/ml penicillin and 50 μg/mL streptomycin. Selective agents used were nourseothricin sulfate (Gold Biotechnology) and hygromycin B (Hygro99, Gold Biotechnology).

**Constructs and transfections**

B7061 pIR2HYG-LRV1_Lbr2780_CapsidStL(A) and B7062 pIR2HYG-LRV1_Lbr2780_RDRPStL(A) were described previously (2). To generate the popout CapsidStL construct used in *L. guyanensis*, Gateway cloning reactions were performed between B7059 pCR8-LRV1_LgyM4147_Capsid and B7405 pIR3HYG-GW(A)-GFP+(B) using LR Clonase II (Thermo Fisher Scientific) in an overnight reaction at room temperature (RT); the reaction was terminated by digestion with proteinase K for 1 hr at 37 ℃. This yielded B7416 pIR3HYG-LRV1_LgyM4147_CapsidStL(A)-GFP+(B).

Transfections were performed as previously described (1, 11). For *L. braziliensis* transfections, cells were plated on semisolid media containing 15 μg/mL hygromycin B. Colonies were picked and grown to stationary phase in 1 mL of media and passaged thereafter in 5 mL of media containing 10 μg/mL (*L. braziliensis*) hygromycin B. For *L. guyanensis*, transfected cells were diluted to 10^5 cells/mL in Schneider’s media containing 50 μg/mL nourseothricin and 75 μg/mL hygromycin B. They were diluted 1:10 daily for three days and GFP-expressing cells were obtained by single-cell sorting.
Flow cytometry

Flow cytometry using the anti-capsid antibody (12) was performed as previously described (2, 13).

For single-cell sorting, log-phase cells were resuspended in phosphate-buffered saline, passed through a CellTrics 50 μm filter (Partec) to remove clumps, and single cells were recovered on the basis of GFP expression using a Beckman Coulter MoFlo cell sorter. Individual cells were placed into wells of a 96-well plate containing 200 μL of Schneider’s Insect Medium and incubated at 27 ℃ for 8 days before parasite growth was scored. Wells were expanded to 5 mL with selection and passaged thusly.

Luciferase activity assay

10^6 log phase cells in 200 μL of Schneider’s Medium were added to a 96-well plate (Black plate, Corning Incorporated, NY, U.S.A.). D-luciferin (Biosynth AG) was added to a final concentration of 150 μg/mL and plates were incubated for 1 min. Plates were imaged using a In Vivo Imaging System (IVIS) photoimager (Perkin Elmer), and luciferase activity quantified as photons/s. Each sample was run in duplicate.

StL construct popout

Cells containing a popout StL construct were split to 10^5 cells/mL in Schneider’s medium lacking selection and GFP expression monitored by flow cytometry. Parallel cultures under drug selection were used as a comparison. When a GFP-negative population appeared in the cultures lacking selection, clonal lines were obtained by single-cell sorting.

Log-phase cells were resuspended in phosphate-buffered saline, passed through a CellTrics 50 μm filter (Partec) to remove clumps, and single cells were recovered on the basis of
GFP expression using a Beckman Coulter MoFlo cell sorter. Individual cells were placed into wells of a 96-well plate containing 200 µL of Schneider’s Insect Medium containing no selective antibiotic (GFP-negative cells) or 10 µg/mL hygromycin B (GFP-positive cells) and incubated at 27 ºC for 10 days before parasite growth was scored. Wells were expanded to 5 mL with or without antibiotic, as appropriate, and passaged thusly.

**RNA preparation and cDNA synthesis**

2.5-3x10^8 cells were pelleted and dissolved in 1mL Trizol reagent (Ambion, Thermo Fisher Scientific). 250 µL of dissolved cells were used to isolate total RNA with the RNA miniprep kit (Zymo Research). RNAs were digested with 20 Units of DNaseI (Ambion, Thermo Fisher Scientific) in a 200 µL reaction using the supplied buffer and purified using the RNA Clean & Concentrator kit (Zymo Research) and eluted in 50 µL of nuclease-free water.

cDNA was synthesized by random hexamer-primed reverse transcription using the Superscript III First-Strand Synthesis kit (Thermo Fisher Scientific) in 20 µL reactions containing 0.25 µg RNA. Control reactions contained an equal amount of RNA but omitted the reverse transcriptase enzyme.

**RT-PCR and qPCR**

RT-PCR reactions were performed using 1 µL of ten-fold diluted cDNA in 25 µL reactions to amplify 94 bp of LRV1 or ~400 bp of beta-tubulin sequence. Primer sequences are located in Table 1. PCR products were run on 2% agarose gels and visualized using ethidium bromide.

qPCR reactions were performed using 5 µL of ten-fold diluted cDNA in a 20 µL reaction containing 10 µL of 2x Power SYBR reaction mix (Applied Biosystems, Thermo Fisher Scientific).
Scientific) and primers to a concentration of 0.2 µM each in MicroAmp Optical 96-well plates (Thermo Fisher Scientific). Primer sequences are located in Table 1. Reactions were run on the ABI PRISM 7000 Sequence Detection System. PCR amplifications were as follows: 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. PCR products were confirmed to be specific by melt curve analysis. +RT reactions were performed in triplicate and –RT control reactions in duplicate.

On each plate, 5-fold dilution series were performed for each primer set to calculate primer efficiencies, and relative RNA level calculated using the Pfaffl method (14) using amplification of KMP-11 as an internal control. The data are averages and standard deviations of 2-3 independent clones, and significance was calculated by ANOVA followed by the Tukey post hoc test.

RNA sequencing and bioinformatics analysis

RiboZero RNA libraries were generated from total RNA as described (15). Briefly, sample integrity was assessed by Agilent Bioanalyzer 2100 (Santa Clara, CA). rRNA was depleted from 1 mg input RNA with the Ribo-Zero™ rRNA Removal Kit (“RiboZero”) from EpiCentre (an Illumina company, Madison, WI). RiboZero-depleted RNA was chemically fragmented to generate fragments ranging from 200-600 nt in length, then made into cDNA with Superscript III (Life Technologies, Thermo Fisher) and random hexamers followed by a second strand reaction. cDNA was then end-repaired, A-tailed, and standard Illumina adapters were ligated on. Libraries were amplified with primers to incorporate a unique index to each sample. Equal masses of each library were pooled and sequenced on an Illumina HiSeq2500 platform, with 2 × 100 base pair paired end reads (Illumina Inc., San Diego, CA).
Data were analyzed using CLC Genomics v9.5.3. Briefly, 5’ and 3’ adaptors were removed and trimmed reads were mapped to annotated genes and transposable elements from the *L. guyanensis* M4147 genome, as well as to LRV1 genes (KX808487) using default RNA-Seq parameters. Reads mapping to multiple locations were aligned randomly.

*S1 nuclease digestion*

15 µg of total RNA was digested with S1 nuclease in a 20 µL reaction containing 1x reaction buffer, 0.6M NaCl, and enzyme at a final dilution of 1:400. Samples were heated to 65 °C for 5 min prior to addition of the enzyme, then incubated at 37 °C for one hour.

*Statement on Institutional and Licensing Committee Approval of Animal Experiments*

Animal handling and experiments were carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* (16) of the US National Institutes of Health. Animal studies were approved by the Animal Studies Committee at Washington University (protocol 20090086) in accordance with the Office of Laboratory Animal Welfare’s guidelines and the Association for Assessment and Accreditation of Laboratory Animal Care International.

*Mouse infections*

Female C57/B6 mice were obtained from Jackson Laboratories. Parasites were grown to day 2 stationary phase and collected at 1x10^6 cells/50 μL DMEM. Mice were injected subcutaneously in the left hind footpad with 1x10^6 parasites using a 30 gauge needle. Luciferase activity was monitored weekly by imaging using the IVIS (Perkin Elmer). Briefly, mice were injected intra-peritoneally with 150 mg/kg D-luciferin, potassium salt (Gold Biotechnology) 10 minutes before imaging. Five minutes before imaging they were anaesthetized with isofluorane,
and anesthesia was continued throughout the procedure. Emitted photons were quantified using Living Image v2.60.1 software (Perkin Elmer). Lesion size was measured weekly using calipers. IFN-γ−/− mice were monitored for the development of metastases, and any located on the uninjected foot were measured using calipers.

E5 – Table Legends

Table E1: RT-PCR and qPCR primer sequences.

E6 – Figure Legends

Figure E1: Integration of a CapsidStL or RDRPStL construct into L. braziliensis LEM2780 previously transfected with a LUCSR construct results in loss of LRV1. Transfectants (blue, CapsidStL; green, RDRPStL) were evaluated by flow cytometry using an anti-capsid antibody. WT L. braziliensis LEM2780 (red) and L. braziliensis M2903 (black) were used as positive and negative controls, respectively.

Figure E2: Integration of a second StL construct impairs knockdown of luciferase. The luciferase activity of WT, untransfected luciferase-expressing and LUCSR, and luciferase-expressing and LUCSR parasites carrying a second StL were measured. A) Parasites transfected with an LRV1-targeting StL. Averages and standard deviations are of six to seven clones of the LRV1StL lines. B) Parasites transfected with a GFP-targeting StL. Averages and standard deviations are of three to five clones.

Figure E3: LRV1 is eliminated by transfection of CapsidStL(A)-GFP+(B) into L. guyanensis. cDNA from two clones of each of the indicated lines was used as template for the PCR amplification of a fragment of LRV1 (top) or β-tubulin (bottom).
Figure E4: The levels of RNAseq and sRNAseq reads mapping to TEs is altered in SSU:CapsidStL(A)-GFP+(B) parasites.

A) RNA was sequenced for the indicated lines and mapped to the L. guyanensis M4147 and LRV1 genomes. The levels of housekeeping genes were not affected by the presence of the StL construct, but levels of TATE elements increased.

B) Reads mapping to LRV1 mapped to the entire viral genome in SSU:GFP+(B) parasites, but only to the stem region in SSU:CapsidStL(A)-GFP+(B) parasites.

C) sRNAs were sequenced for the indicated lines and mapped to the L. guyanensis M4147 and LRV1 genomes. Reads mapping to SLACS and TATE fell 10-fold in SSU:CapsidStL(A)-GFP+(B) parasites. Levels of LRV1-mapping reads increased 20-fold in SSU:CapsidStL(A)-GFP+(B) parasites and fell 400-fold in PO:CapsidStL(A)-GFP+(B) parasites compared to SSU:GFP+(B).

D) sRNA reads mapping to LRV1 mapped to the entire viral genome in SSU:GFP+(B) parasites, but only to the stem region in SSU:CapsidStL(A)-GFP+(B) parasites.

Figure E5: Lines with an integrated CapsidStL(A)-GFP+(B) construct have elevated levels of TATE RNA.

cDNA from the indicated lines was used as template in qPCR reactions with SLACS- and TATE-specific primers. Averages and standard deviations are of two clones of SSU:GFP+(B) and three clones each of SSU:CapsidStL(A)-GFP+(B) and PO:CapsidStL(A)-GFP+(B). ** p<0.01; all other comparisons NS.

Figure E6: Lines with an integrated StL construct accumulate stable dsRNA.

15 µg of total RNA from two clones of each of the indicated lines was digested with S1 nuclease, the reaction run on a 1.8% agarose gel and stained with ethidium bromide.
LRV1 genome is indicated by a white arrowhead; stable StL-dependent dsRNA is indicated by a black arrow.

**Figure E7:** Tests of the effect of StL constructs on parasite virulence were inconclusive.

A) Luciferase activity of lines containing integrated *GFP*+(B) or *CapsidStL*(A)-*GFP*+(B) constructs.

B) Lesion size of lines containing integrated *GFP*+(B) or *CapsidStL*(A)-*GFP*+(B) constructs.

C) Luciferase activity of lines containing popped out *GFP*+(B) or *CapsidStL*(A)-*GFP*+(B) constructs.

D) Lesion size of lines containing popped out *GFP*+(B) or *CapsidStL*(A)-*GFP*+(B) constructs. Plotted are the geometric means and standard deviations of luciferase activity and the arithmetic means and standard deviations of lesion size for two independent experiments. Significance was determined by two-way ANOVA.

**E7 – References**


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Figure E1

![Graph showing capsid protein distribution for different strains.](image-path)
Figure E2

A

B

Luciferase activity

\begin{align*}
\text{WT} & \quad \text{LUC} & \quad \text{LUC} & \quad \text{LVRI} & \quad \text{StL} \\
\text{Parent} & \quad \text{LUC} & \quad \text{LUC} & \quad \text{LUC} & \quad \text{StL} \\
\text{WT} & \quad \text{LUC} & \quad \text{LUC} & \quad \text{LUC} & \quad \text{GFP} & \quad \text{StL}
\end{align*}
### Figure E3

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**LRV1**

β-tubulin
Figure E4

A

B

C

D
Figure E5
Figure E6
Appendix F – Knockdown of IFT genes
Preface

The experiments described here were conducted as part of a rotation project and will be incorporated into a future larger publication. I performed experiments designed by Stephen M Beverley and Lon-Fye Lye. L-FL assisted in data analysis. I wrote the first draft of this Appendix, and the final version here incorporates comments by SMB.
**F1 – Introduction**

The flagellar pocket is the sole site of endo- and exocytosis in trypanosomatids such as *Leishmania* (1, 2), and components of sensory pathways such cyclic nucleotide (3, 4) and calcium signaling (5) are localized to the flagellum. Additionally, the axoneme of the amastigote flagellum exhibits a microtubule architecture more similar a pattern characteristic of sensory cilia than of the promastigote flagellum and other motile flagella (6). Finally, Gluenz *et al.* observed that the *L. mexicana* amastigote flagellar tip was frequently in close contact with the parasitophorous vacuole membrane (6). Because of these observations, it has been hypothesized that the flagellum of *Leishmania* could serve an important role in host-parasite interactions.

The *Leishmania* flagellum is maintained by the collective action of Intraflagellar Transport (IFT), the mechanism by which flagellar components are shuttled from the cell body to the tip of the flagellum (anterograde) and back (retrograde). To investigate the role of the *Leishmania* flagellum in parasite biology and virulence, we attempted to disrupt its assembly by knocking down IFT in in a clone of *L. braziliensis* M2903 adapted for *in vitro* differentiation into the amastigote life cycle stage. Inducible RNAi knockdown of IFT in *T. brucei* disrupted flagellum formation, reduced cell body size, and induced cell cycle arrest (7). There are structural differences between *Leishmania* and *Trypanosoma* flagella, however, that decrease the likelihood that IFT disruption in *Leishmania* will be lethal. The *Trypanosoma* flagellum is attached along the length of the cell body, whereas the *Leishmania* flagellum protrudes freely from the anterior tip (8). The *Trypanosoma* flagellum plays crucial roles in defining cell polarity and directing organelle segregation and cell size during mitosis (9). While there is evidence that the mitochondrion-kinetoplast-basal body-flagellum complex in *Leishmania* also plays a role in directing organelle segregation(10), the different morphology of *Leishmania* during cell division
makes it unlikely that the flagellum plays a crucial role in directing cell division (10). Further, there appears to be some species-specific variation among Leishmania in the timing of flagellar replication during cell division (10–12), lessening the likelihood that the flagellum serves a crucial role in cell division.

In our experiments, RNAi knockdown of IFT genes in L. braziliensis failed to generate viable mutants, whereas transfection of the same knockdown constructs in a Δago1 line yielded many viable transfectants. Later work in the lab pursued this project further, and the combined results paint a more complete picture of the role of IFT and the parasite flagellum in biology and virulence.

**F2 – Results**

We transfected constructs into L. braziliensis strain M2903 (SA2) to knock down four different IFT genes: IFT88, IFT122, IFT140, and IFT172. Two genes, IFT88 and IFT172, belonged to the anterograde pathway, moving cargo from the base of the flagellum to the tip, while the other two, IFT122 and IFT140, belonged to the retrograde pathway, returning cargo from the tip of the flagellum to the cell body. In parallel, we transfected the following positive controls: 1) a construct that enabled luciferase expression (“LUC”); a construct that simultaneously expressed and knocked down luciferase (“LUCSR”); and constructs that knocked down the paraflagellar rod proteins (“PFR1StL” and “PFR2StL”), which are known to be not essential for viability (13, 14). Positive control transfections yielded viable transfectants (Table 1) with the expected phenotypes. Namely, LUC-transfected cells had high luciferase activity, while LUCSR-transfected cells had activity levels 130-fold lower (Figure 1). PFR1StL- and PFR2StL-transfected cells were defective in swimming (data not shown). In contrast, no colonies grew for cells transfected with any IFT StL construct (Table 1).
To confirm that this inability to recover transfectants was RNAi-dependent, we transfected *IFT122*StL, *IFT140*StL, and *IFT172*StL, as well as control LUC and LUCSR constructs, into an Argonaute1 knockout mutant (Δago1) of *L. braziliensis* M2903. Viable transfectants were recovered for each construct (Table 2).

**F3 – Discussion**

While positive control transfections into *L. braziliensis* M2903 (SA2) were successful, we were unable to recover viable transfectants after knockdown of IFT genes. This suggested that parasites were not viable in the absence of these gene products. This was not entirely unexpected, as IFT knockdown cells in *T. brucei* are likewise inviable. The essentiality of these genes was confirmed by transfection of the IFT knockdown constructs into Δago1 cells, which have a non-functional RNAi pathway, and therefore are insensitive to StL constructs. In this cell line, we easily obtained transfectants containing the IFTStL constructs, suggesting that the lethality seen in the SA2 line required an active RNAi pathway, and therefore was due to knockdown. Because knockdown of four separate genes gave an identical phenotype, this was unlikely to be due to off-target effects, but rather to loss of IFT, specifically.

Later research in the lab, however, showed this not to be the case. Tiffanie Fowlkes demonstrated in her dissertation that IFT140 could be knocked out in *L. donovani* parasites if an ectopic copy was first provided episomally. After replacement of both chromosomal copies with drug resistance cassettes, the antibiotic maintaining selection of the episome was removed, allowing it to be segregated out. Using this technique, she showed that Δift140 promastigote cells were viable, lacked flagella, and accumulated vesicles in the flagellar pocket. In addition, *in vitro* differentiation of Δift140 cells was impaired and the lines were avirulent in a mouse model of visceral leishmaniasis (15). An analogous essentiality test for RNAi experiments would require
temporary ectopic expression of the IFT target, recoded to be resistant to knockdown by the StL construct, such as from a popout construct (see Chapter 4). After integration of the IFTStL construct, the RNAi-resistant ectopic copy could be removed, and if the gene is essential, no viable cells would be obtained; if not essential, IFT knockdown cells would be obtained.

Further, Lon-Fye (George) Lye in the lab has been able to recover parasites with some IFT genes knocked down in *L. braziliensis* by using shorter “stems” in the StL constructs, which reduces knockdown efficiency (unpublished data). This approach yielded mutants with shortened flagella and vesicle accumulation, similar to the Δift140 mutant in *L. donovani*.

While somewhat unusual, it is not unprecedented for a gene at first to appear essential in *Leishmania*, only to prove otherwise using a “gentler” approach such as an episome sort. Efforts to knock out the hexose transporter GT2 from *L. mexicana* required the transient overexpression of a suppressor gene, but after Δgt2 mutants were obtained, the suppressor was no longer required for viability(16). Similarly, work in our lab investigating *LPG4A* failed to yield viable mutants without the expression of the gene from an episome; after knockout of chromosomal copies of the gene, episomal expression was no longer required for viability (Guo and Beverley, unpublished data).

**F4 – Acknowledgements**

Thanks also to Joseph Marcus, who performed the cloning of the IFTStL constructs, and to Lon-Fye (George) Lye, who followed up on the transfections into Δago1 cells.
F5 – Materials and Methods

Parasites and in vitro culture

A variant of *L. braziliensis* strain M2903 adapted to differentiate in culture was obtained from the laboratory of S.C. Alfieri(17) and plated to generate clonal lines; clone SA2 was used in the following experiments. The Δago1 line of *L. braziliensis* was described previously(18). Parasites were grown in fresh Schneider’s Insect Medium supplemented with 10% heat-inactivated fetal bovine serum, 100 μM adenine, 10 μg/mL hemin, 2 μg/mL biopterin, 2 mM L-glutamine, 500 units/ml penicillin and 50 μg/mL streptomycin, and selective drugs as indicated below.

Stem-loop constructs

Stem-loop constructs were constructed as described previously (19). Specifically, regions of each IFT gene to be knocked down were amplified by PCR using the primers listed in Table 3 to ultimately generate B6613 pIR2HYG-IFT88StL(A), B6664 pIR2SAT-IFT122StL(A), B6666 pIR2SAT-IFT140StL(A), and B6674 pIR2HYG-IFT172StL(A). B6386 pIR2SAT-LUCStL(A)-LUC(B), B6282 pIR1SAT-PFR2StL(A), and B6294 pIR1SAT-PFR1StL(A) were described previously(14).

Transfection

Stable transfections were performed as previously described(14, 20). After overnight recovery, ⅙ of the transfected cells were spread per plate on semisolid media with 100 μg/mL nourseothricin or 30 μg/mL hygromycin B. Transfectants were grown to stationary phase in 1 mL media without selection and passaged thereafter in 10 mL media with 100 μg/mL nourseothricin or 30 μg/mL hygromycin B.
**Luciferase activity assay**

10^6 log phase cells in 200 μL of Schneider’s Medium were added to a 96-well plate (Black plate, Corning Incorporated, NY, U.S.A.). D-luciferin (Biosynth AG) was added to a final concentration of 150 μg/mL and plates were incubated for 1 min. Plates were imaged using an In Vivo Imaging System (IVIS) photoimager (Perkin Elmer), and luciferase activity quantified as photons/s. Each sample was run in duplicate. One clone of WT, three clones of LUC-transfected, and eight clones of LUCSR-transfected cells were evaluated.

**F6 – Table Legends**

Table F1: Colonies per µg of DNA obtained from transfection of StL constructs into WT *L. braziliensis* strain M2903 (SA2).

Also indicated are the number of plates scored, each of which represents 1/6 the total number of cells from a transfection. The IFT172StL transfection was attempted twice.

Table F2: Colonies per µg of DNA obtained from transfection of StL constructs into Δago1 *L. braziliensis* M2903.

Indicated in parentheses are the number of plates scored, each of which represents 1/6 the total number of cells from a transfection.

Table F3: Primers used in the cloning of StL constructs for the knockdown of IFT genes.

XbaI site underlined.

**F7 – Figure Legends**

Figure F1: Cells transfected with the positive control LUC and LUCSR constructs displayed the expected luciferase activity.

Error bars are standard deviation.
F8 – References


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