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Novel regulators of social motility in the African trypanosome, Trypanosoma brucei

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Novel regulators of social motility in the African trypanosome, *Trypanosoma brucei*

A thesis submitted in partial satisfaction

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Biochemistry, Molecular and Structural Biology

by

Shahriyar Jahanbakhsh

2016

ABSTRACT OF THE THESIS

Novel regulators of social motility in the

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Shahriyar Jahanbakhsh

Master of Science in Biochemistry, Molecular and Structural Biology

University of California, Los Angeles, 2016

Professor Kent L. Hill, Co-Chair

Professor Louis-Serge Bouchard, Co-Chair

Trypanosoma brucei is the causative agent of sleeping sickness, a major threat to economic stability and public health across sub-Saharan Africa. This protozoan parasite features a complex digenetic life cycle that alternates between the tsetse fly (*Glossina* spp.) vector and the mammalian host. Infections in both the fly and vector are characterized by intimate contact with tissue surfaces. Recent studies have shown that when cultivated on semisolid agarose surfaces, *T. brucei* engages in social behavior, termed social motility, which manifests as parasites assembling into groups that move collectively to form symmetrical arrays of radial projections. Herein we describe two

novel modulators of social motility in *T. brucei*, the zinc finger protein ZC3H34 and the prolyl isomerase cyclophilin A (CyPA). Using RNA interference, we show that knockdown of either ZC3H34 or CyPA delays social motility. The delay in social motility observed with CyPA knockdown is not due to decreased propulsive motility, although ZC3H34-depleted parasites do show reduced propulsive motility. Our study provides new insights into the biochemistry of trypanosome social behavior and identifies potential targets for chemical inhibition of vector-to-host parasite transmission.

The thesis of Shahriyar Jahanbakhsh is approved.

James Gober

Louis-Serge Bouchard, Committee Co-Chair

Kent L. Hill, Committee Co-Chair

University of California, Los Angeles

2016

DEDICATION

I dedicate this work to Justin Thayer,

the brilliant educator and scientist who profoundly

shaped my course in science.

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The RNAi cell lines described in sections (I)(A)(i)-(iii) and (II)(A)(i) of Chapter 3 were generated and characterized in collaboration with Alexandra Stream.

CHAPTER 1

Background and Introduction

Social behavior is ubiquitous among microbes[1]. Multicellular groups of interacting microbes possess emergent properties not possible in isolated contexts. The biochemical mechanisms underlying bacterial social behavior and the consequences of this behavior have been extensively studied. Among the best characterized social phenomena are quorum sensing[2-5], biofilm formation[2-4], and social motility[2-4,6]. These cooperative behaviors provide important advantages to participants. Quorum sensing enables populations of bacteria to coordinately regulate gene expression and efficiently utilize exoenzymes and other extracellular "public goods"[2-5]. Biofilms confer protection against predation, phagocytosis, and antimicrobial compounds and facilitate nutrient acquisition and genetic exchange[2-4]. In addition, the coordinated movement that occurs during social motility has been shown to enhance nutrient uptake, facilitate colonization of and adaptation to differing physiological and ecological environments, protect against macrophages and antibiotics, and promote toxin secretion[2-4,6,7]. The secondary messenger cyclic dimeric GMP (c-di-GMP) has been shown to have important roles in many of these social behaviors and is a key regulator of biofilm formation, swarming behavior, virulence, and cell cycle progression in bacteria[8].

Social behaviors have also been described for eukaryotic microorganisms. Perhaps the best recognized is the starvation response in the slime mold *Dictyostelium discoideum*, which initiates an oscillatory cyclic AMP (cAMP) relay signal that results in

the aggregation of individual cells into an organized, multicellular fruiting body[9,10]. Additional examples include flocculin-mediated self-recognition and biofilm-like cooperation in *Saccharomyces cerevisiae*[11] and biofilm formation in the fungus *Candida albicans*[12]. Despite well-documented instances of social behaviors among bacteria and many eukarya, however, the existence of, and mechanisms underlying such behaviors among protozoan parasites remain largely unexplored[13,14].

The protozoan parasite *Trypanosoma brucei* is the causative agent of sleeping sickness in humans and Nagana in cattle and other mammals. *T. brucei* is endemic to sub-Saharan Africa, where it poses a major threat to public health and agriculture in 36 countries[15]. All three subspecies of *T. brucei*, *Trypanosoma brucei* (*T. b.*) *gambiense*, *T. b. rhodesiense*, and *T. b. brucei*, are able to infect non-primate mammals, whereas only *T. b. gambiense* and *T. b. rhodesiense* are human-infective. *T. brucei* is transmitted to mammals through the bite of an infected tsetse fly (*Glossina* spp.) vector. Thus, the lifecycle of *T. brucei* is digenetic, alternating between infection of the mammalian host and transmission through an insect vector[15,16].

T. brucei transitions through many distinct morphological states during the course of its lifecycle. After ingestion, short-stumpy bloodstream trypomastigotes differentiate into procyclic form trypomastigotes in the tsetse fly midgut[17]. To be capable of re-infecting the mammalian host, these cells must then undergo a series of ordered developmental changes and directional migrations from the midgut through the proventriculus, foregut, proboscis, and into the salivary gland ducts[17]. Throughout this process, the parasites are in intimate contact with fly tissue epithelia, colonizing and migrating along these epithelial surfaces. Surface-induced changes in organism

behavior and motility are known to occur among diverse groups of bacteria and protists[10-12,18-23], raising the intriguing possibility that surface contact may also induce and modulate parasite behavior in *T. brucei*.

In support of this hypothesis, recent work discovered that when cultivated on semisolid agarose surfaces, procyclic form (insect midgut stage) trypanosomes form communities that undergo polarized coordinated movements outward from the site of inoculation.[24] These coordinated movements yield symmetrical arrays of radial projections reminiscent of those formed by *Pseudomonas aeruginosa*[18,19], *Myxococcus xanthus*[22], and *Paenibacillus dendritiformis*[23] during swarming. This form of coordinated group movement was termed social motility, by analogy to social motility in bacteria. Coordinated movement of groups of cells requires signaling, as individuals cells in the group must respond to external cues and to each other. Notably, knockdown of several *T. brucei* receptor-type adenylate cyclases (AC1, combined AC1/AC2, and AC6) was found to increase the number and density of radial projections advancing from the site of inoculation, whereas knockdown or chemical inhibition of the cAMP-specific phosphodiesterase PDEB1 abolished social motility[25,26]. These results indicate that social motility is controlled by a signaling pathway that modulates cAMP levels within the trypanosome flagellum[25,26]. The architecture of this signaling pathway shows parallels to that of the c-di-GMP signaling pathway that modulates swarming motility and biofilm formation in bacteria[26].

A growing body of evidence indicates that social motility is physiologically relevant in the *Glossina* vector. Foremost, social motility is a feature of “early” procyclic form cells, a specific *T. brucei* developmental stage found in the fly midgut during the

first week of infection; only cells staining positive for GPEET, a glycophosphatidylinositol (GPI)-anchored surface procyclin and a marker for early procyclic forms, exhibit social motility[27]. Proteomic analyses have identified a number of other proteins differentially expressed between early and late procyclic forms, although whether or not these proteins function as regulators of social motility in *T. brucei* has not been established[27]. As further evidence of a physiological role for social motility, null mutants lacking *Rft1*, a protein required for translocation of lipid-linked oligosaccharides across the ER membrane[28], display attenuated social motility and a four-fold decrease in efficiency in establishing fly midgut infections[29]. Despite this recent progress, however, additional genes involved in the control of social motility in *T. brucei* have yet to be identified. Given that social behavior and cell-cell communication depend on signaling systems, the relevance of social motility in *T. brucei* extends beyond parasite transmission through the tsetse fly to include opportunities for studying signal transduction, which represents a critical yet poorly understood aspect of parasite biology[14].

Herein we describe two novel genes involved in regulation of social motility in *T. brucei*. A prior transcriptome-wide RNA-seq screen revealed that certain genes are differentially upregulated in trypanosomes cultivated on semisolid agarose compared to parasites cultured in suspension. We use RNA interference (RNAi) to probe these candidate genes for a potential functional role in social motility. We demonstrate that stable knockdown of a zinc finger protein, ZC3H34, or of cyclophilin A delays the onset of social motility. We further show that the social motility phenotype of CyPA-depleted cells is not the result of defective propulsive motility. The phenotype of ZC3H34-depleted cells is correlated with decreased propulsive motility in suspension culture,

though a causal connection between these phenotypes has not been established. Our work elucidates new regulators of social motility in *T. brucei* and identifies potential targets for chemical inhibition of parasite transmission.

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CHAPTER 2

Materials and Methods

Cell culture. Procyclic form 29–13 cells[1], which stably express T7 RNA polymerase and the tetracycline repressor, were cultured in Cunningham's semi-defined medium (SM) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 15 µg/mL G418 (Gibco), and 50 µg/mL hygromycin B (Gibco), as described previously[2]. RNAi lines were cultured in media also containing 10 µg/mL blasticidin (Gibco). Cells were passaged to maintain early- to mid-logarithmic growth. Cultures were maintained at 28°C and 5% CO_2.

Generation of RNAi lines. All gene sequences were obtained from the TriTrypDB kinetoplastid parasite genome database[3]. RNAi target regions were selected using the TrypanoFAN RNAit algorithm[4]. These regions were PCR-amplified and cloned into the p2T7-177 RNAi vector[5]. The primers used to amplify and clone the genes of interest are listed in Supplemental Table 1. The resulting constructs were linearized with *Not*I and stably transfected into 29–13 cells using electroporation, as described previously[6,7]. Transfectants were selected by addition of 10 µg/mL blasticidin to the culture medium. Clonal lines were obtained *via* limiting dilution.

Growth assays. RNAi was induced by addition of 1 µg/mL tetracycline. Cells were passaged to an initial concentration of 5×10^5/mL (early logarithmic phase). Cells density was monitored daily or twice daily using a Z1 Coulter Particle Counter (Beckman Coulter). Cells were passaged back to a density of 5×10^5/mL every ~24 h.

Cumulative cell densities at time points beyond 24 h after the assay startpoint were estimated as: $(\rho_t^* \times \rho_{t-1})/(\rho_{t-2})$, where ρ_t^* is the measured density, ρ_{t-1} is the cumulative cell density at the most recent prior time point, and ρ_{t-2} is the cumulative cell density at the second most recent prior time point (or post-dilution density [i.e. $\rho_{t-2} = 5 \times 10^5$/mL], if the cells were passaged).

RT-qPCR. RNAi lines were incubated in suspension culture for 72 h in presence and absence of 1 µg/mL tetracycline. Following this period, total RNA was isolated from mid-logarithmic cells using an RNeasy Mini Kit (Qiagen) and treated with amplification grade DNase (Thermo Fisher). cDNA was then synthesized from 2 µg total RNA using SuperScript II reverse transcriptase (Thermo Fisher) and oligo (dT) primers to select for the poly(A)$^+$ RNA fraction. qPCR primers were designed using NCBI Primer-BLAST[8] and the sequences are listed in Supplemental Table 2. qPCR was performed in duplicate for both induced (with tetracycline) and uninduced (without tetracycline) conditions, and on 1 to 3 independent RNA preparations. Samples were analyzed using a CFX Connect Real-Time PCR Detection System (Bio-Rad). Fluorescence values were normalized against values from two genes (PFR2 and TERT) abundantly expressed in both procyclic and bloodstream form *T. brucei*. Relative gene expression between the induced and uninduced conditions was quantified using the $2^{-\Delta\Delta C_T}$ method[9].

Social motility assays. Cultivation on semisolid agarose plates was performed essentially as described in Oberholzer *et al*.[2] A sterile solution of 4% (w/v) agarose (SeaPlaque GTG Agarose, Lonza) was diluted into prewarmed (42°C for 20 min) SM supplemented with 15 µg/mL G418 and 50 µg/mL hygromycin B to a final concentration of 0.4%. Ethanol and methanol were added to a final concentration of 1%, and

tetracycline to a final concentration of 1 µg/mL (addition of tetracycline was omitted for control plates). The resulting solution was poured in aliquots of 11.5 mL into Petri dishes (85 mm × 15 mm) and allowed to cool, with the Petri dishes uncovered, for 1 h in a laminar flow hood at room temperature. RNAi lines were cultured in suspension at mid-logarithmic phase (with and without 1 µg/mL tetracycline) for 72 h prior to plating on agarose. For inoculation onto the plates, 5 µL of cells from a suspension culture at a density of 2×10^7/mL were added to the center of the agarose surface. The plates were incubated at 28°C and 3.5% CO_2 and imaged at 24 h intervals post-inoculation.

Radial projection formation was quantified using ImageJ[10]. Given the considerable heterogeneity in projection length, only those projections at or extending past a defined radial distance from the site of inoculation were included in the count. These threshold radii were set as 7.5 mm, 10.8 mm, 19.9 mm, and 24.9 mm for time points 48 h, 72 h, 96 h, and 120 h post-inoculation, respectively, for both ZC3H34 and CyPA.

Motility traces. RNAi lines were cultured in suspension (with and without 1 µg/mL tetracycline) for 72 h prior to analysis. Logarithmic-phase cells were diluted to 2×10^6/mL and observed in polyglutamate-coated slide chambers[11]. Cells were viewed under dark-field illumination on a Zeiss Axioskop II compound microscope using a 10× objective. Approximately 30 s of video from separate regions on each slide was captured. Motility traces were generated using MetaMorph software (Molecular Devices). Cells that could not be tracked for the full 30 s, as a result of leaving the focal plane or field of view, were excluded from the analysis.

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CHAPTER 3

Results

We sought to identify and characterize additional regulators of social motility in *T. brucei*. To this end, we assayed social motility and propulsive motility characteristics in cell lines stably expressing dsRNA against the transcripts of selected candidate genes. A previous RNA-seq screen (Figure 1) had identified 2,141 genes differentially regulated ($p < 0.01$) in trypanosomes cultured on semisolid agarose versus in suspension (21.8 percent of total hits). Of these, 1,240 genes were transcribed above 50 RPKM (reads per kilobase per million reads mapped[1]) in both suspension- and agarose-cultured trypanosomes. Our candidate genes were among a 22-member subset of these 1,240 genes for which $RPKM_{plate}/RPKM_{suspension} \geq 2$.

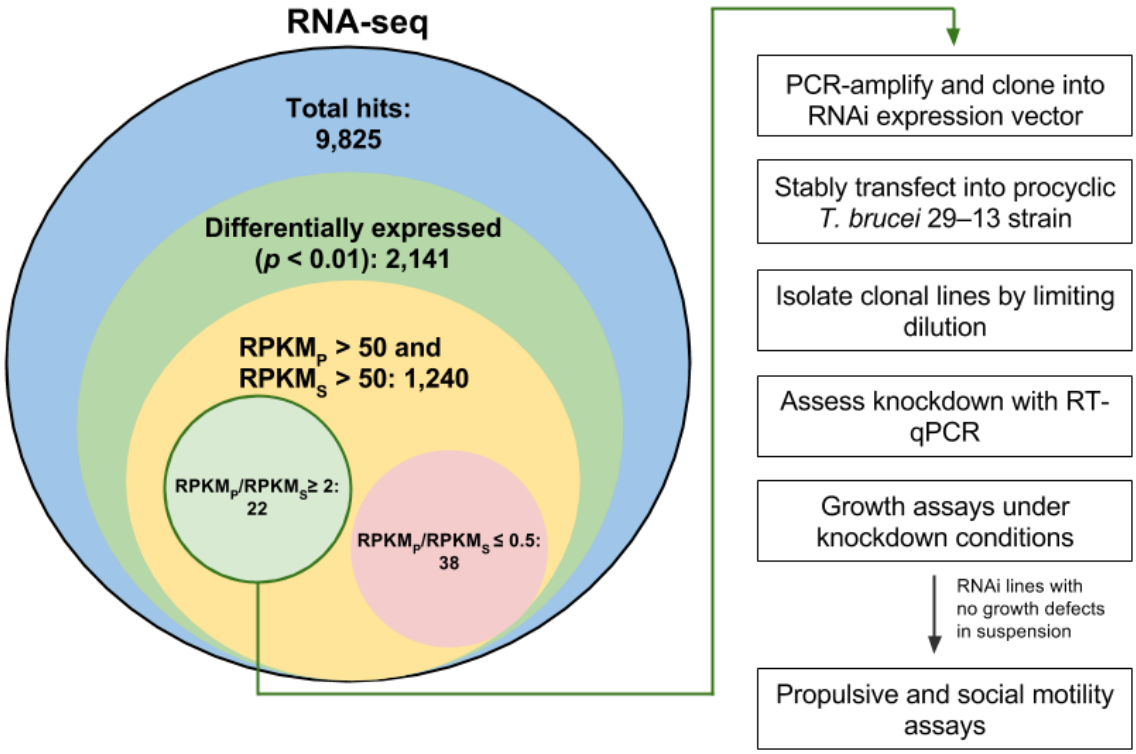


Figure 1: Summary of RNA-seq screen of suspension- and semisolid agarose-cultivated *T. brucei* and experimental workflow. The subscripts P and S denote plate and suspension culture RPKM values, respectively.

I. Genes essential for viability *in vitro*.

A. *Translation elongation factor-1β, histone H3, universal minicircle sequence-binding protein 2, S-adenosylhomocysteine hydrolase, and RNA-binding protein 11 are essential for viability.*

Of the 22 genes found to be upregulated 2-fold or more in trypanosomes cultivated on semisolid agarose, seven genes with physiologically distinct functions were selected for analysis for a possible role in social motility (Table 1). We began by generating transfected *T. b. brucei* cell lines harboring tetracycline-inducible RNAi constructs against these seven candidate genes: translation elongation factor-1β (EF-1β, GeneDB accession Tb927.10.5840), histone H3 (H3, Tb927.1.2510), universal minicircle sequence-binding protein 2 (UMSBP2, Tb927.10.6060), *S*-adenosylhomocysteine hydrolase (SAHH, Tb927.11.9590), RNA-binding protein 11 (RBP11, Tb927.8.4450), zinc finger protein ZC3H34 (ZC3H34, Tb927.10.12330), and cyclophilin A (CyPA, Tb927.11.880). In all cases, knockdown of the target gene was verified using reverse transcription–quantitative PCR (Figure 2).

GeneDB accession	Gene name	Major functions
Tb927.10.5840	Elongation factor-1β	Protein elongation during translation
Tb927.1.2510	Histone H3	Transcription regulation; cell cycle control; VSG expression
Tb927.10.6060	Universal minicircle sequence-binding protein 2	Kinetoplast DNA replication; mitosis regulation
Tb927.11.9590	*S*-adenosylhomocysteine hydrolase	Methionine metabolism
Tb927.8.4450	RNA-binding protein 11	Possibly involved in rRNA processing
Tb927.10.12330	Zinc finger protein ZC3H34	RNA processing, export, and stability
Tb927.11.880	Cyclophilin A	Protein folding; RNA processing; immunomodulation; cell-cell communication; chemotaxis

Table 1: List of candidate genes investigated in this study and their known major functions.

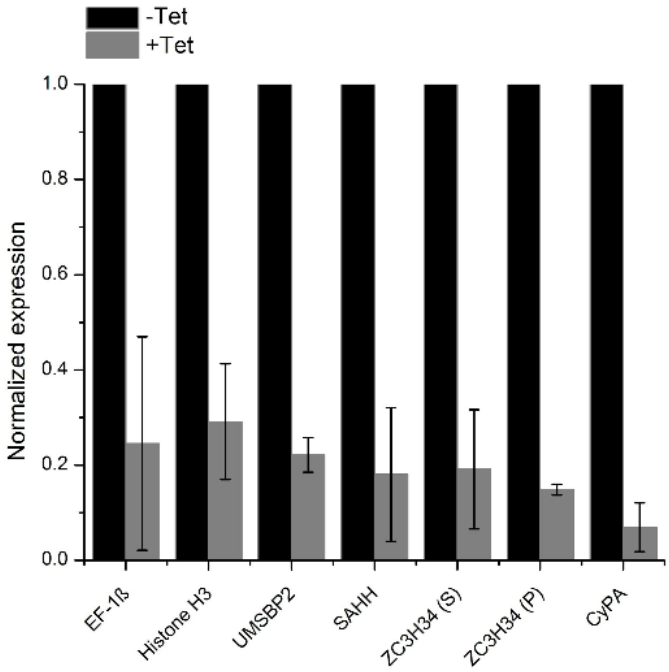


Figure 2: Knockdown of candidate social motility genes. RT-qPCR analysis of transcript levels in cell lines induced for RNAi normalized to levels in uninduced controls. For ZC3H34, (S) denotes expression levels in cells in suspension and (P) denotes expression levels in cells obtained from radial projections on semisolid

agarose. Expression levels for all other genes are for cells grown in suspension. Normalized transcript expression levels, expressed as mean ± standard deviation, for RNAi-induced cells are: 24.53% ± 22.45% (EF-1β), 29.14% ± 12.18% (Histone H3), 24.14% ± 3.64% (UMSBP2), 18.02% ± 14.03% (SAHH), 19.13% ± 12.52% [ZC3H34 (S)], 14.86% ± 1.13% [ZC3H34 (P)], and 6.92% ± 5.12% (CyPA). *n*=3 biological replicates for CyPA, 2 for ZC3H34 (S) and ZC3H34 (P), and 1 for the other genes. Two technical replicates were performed for all genes. For genes for which only a single biological replicate was performed, error bars indicate standard deviation of technical replicates. Otherwise, error bars indicate standard deviation of biological replicates.

i. *Translation elongation factor-1β*

Peptide elongation during protein biosynthesis in eukaryotes is a GTP-dependent process promoted by the elongation factor-1 (EF-1) complex (comprised of three subunits, α, β, and γ) and elongation factor (EF-2). The elongation step involves binding of aminoacyl-tRNA to the ribosomal A site (catalyzed by EF-1α), formation of the amide bond, GTP hydrolysis and ejection of EF-1α•GDP, peptide bond formation (catalyzed by the peptidyl transferase center in the large (60S) ribosomal subunit), and subsequent translocation of the newly-formed peptidyl-tRNA to the P site (catalyzed by EF-2). The guanine nucleotide exchange factor EF-1β regenerates the GTP-bound EF-1α necessary for each elongation cycle and is therefore a key regulator of protein elongation[2]. The γ subunit not essential for viability in yeast[3] and is thought to anchor the EF-1βγ complex to the endoplasmic reticulum[4], a major site of protein synthesis. In addition to its conserved role in translation, the tetrameric EF-1 holocomplex ($[\alpha\beta(\gamma_2)]_4$)

exhibits trypanothione *S*-transferase and peroxidase activities in *Leishmania*[5,6], another kinetoplastid parasite.

We first evaluated the effect of EF-1β depletion on cell viability. RT-qPCR confirmed knockdown of EF-1β transcript expression to 24.5 ± 22.5 percent (mean ± standard deviation) of levels in uninduced controls (Figure 2). EF-1β knockdown lines displayed severe growth defects beginning ~24 h after tetracycline induction (Figure 3A). The lethality of EF-1β knockdown in procyclic form *T. brucei* is consistent with the observation that this factor is also essential for growth in *S. cerevisiae*[7]*.* Given the lethal phenotype observed with EF-1β depletion, this gene was not explored further.

ii. *Histone H3*

We next evaluated the effect of histone H3 depletion on cell viability. H3 was one of several histone variants identified in our RNA-seq analysis as being highly upregulated in *T. brucei* cultured on semisolid agarose. Histone H3 is one of four core histone proteins that comprise the nucleosome, the fundamental unit of DNA organization in eukaryotes[8]. The N-terminal tail of histone H3 is subject to extensive post-translational modifications that regulate gene expression. The *T. brucei* histone H3 lacks the serine 10 residue found in other eukaryotes. Phosphorylation of this residue is required for chromosome condensation and segregation in other eukaryotes, and the absence of Ser10 in *T. brucei* histone H3 may be functionally significant, as mitotic chromosome condensation does not occur in trypanosomatids[9]. Furthermore, deletion of the H3 lysine 76 dimethyltransferase Dot1A results in premature progression through mitosis without DNA replication, and H3K76 trimethylation is essential for the developmental transition from the bloodstream to procyclic forms[10].

In addition to its roles in cell cycle control, histone H3 is a key regulator of antigenic variation in *T. brucei*. H3 depletion in bloodstream form *T. brucei* de-represses variable surface glycoprotein (VSG) genes found adjacent to telomeres at polycistronic expression sites (ESs)[11]. Moreover, several of the chromatin-associated factors required for maintenance of VSG silencing at ESs act on histone H3, including the chromatin remodeler ISWI[12], the H3K76 methyltransferase Dot1B[13], the DAC3 histone deacetylase[14], and the FACT chromatin-remodeling factor[15].

Knockdown of histone H3 transcript expression in procyclic stage *T. brucei* to 29.1 ± 12.2 percent relative to uninduced controls (Figure 2) produced a rapid and severe growth defect (Figure 3B), consistent with the essential nature of the core histone proteins in all eukaryotes[16]. Due to this lethal phenotype, histone H3 was not investigated further for a potential role in social motility.

iii. *Universal minicircle sequence-binding protein 2*

We proceeded to evaluate the effect of UMSBP2 depletion on cell viability. A distinctive feature of *T. brucei* and other kinetoplastid protozoans is the organization of the cell's mitochondrial DNA into a unique catenated DNA network called kinetoplast DNA (kDNA). This kDNA network consists of several thousand topologically-linked DNA minicircles located within the cell's single mitochondrion. Replication of kDNA minicircles initiates at a single-stranded dodecamer, termed the universal minicircle sequence (UMS, 5'-GGGGTTGGTGTA-3') that is conserved in all trypanosomatid species. The UMS-binding protein (UMSBP) specifically recognizes and binds this origin-associated sequence. In addition to binding ssDNA, UMSBP also binds ssRNA, but is unable to bind double-stranded or quadruplex DNA structures[17].

The *T. brucei* genome encodes two UMSBP orthologs, UMSBP1 and UMSBP2, which contain five and seven CCHC-type zinc fingers, respectively[18]. Silencing of UMSBP2 yields significant changes in the cell's dimensions and inhibits nuclear mitosis. Combined knockdown of UMSBP1 and UMSBP2 arrests growth, inhibits minicircle replication initiation, causes abnormal cell ploidy and morphology, and impairs the segregation of the kDNA network and the flagellar basal body[18].

Consistent with the results of Milman *et al*.[18], knockdown of UMSBP2 expression to 22.1 ± 3.64 percent of transcript levels in uninduced controls (Figure 2) resulted in a moderate growth defect that became apparent ~40 h post-induction (Figure 3C). Given the confirmed essential role of UMSBP2 in maintenance of the kDNA network[18], this gene was not examined further for a potential role in social motility.

iv. S-*adenosylhomocysteine hydrolase*

We then evaluated the effect of *S*-adenosylhomocysteine (SAHH) depletion on cell viability. SAHH catalyzes the conversion of *S*-adenosylhomocysteine (SAH) to homocysteine and adenosine. SAH is a toxic byproduct of methyl group transfer from *S*-adenosylmethionine (SAM), and a critical feature of SAM metabolism is the need to rapidly remove SAH. The toxicity of SAH arises from its potent inhibition of methylation reactions mediated by SAM[19]. In *T. brucei* treated with ^{35}S-methionine, SAH levels do not exceed 10 percent of the total soluble ^{35}S incorporated, even in the presence of a small molecule inhibitor of ornithine decarboxylase that diverts SAM from the polyamine synthesis pathway to use in methylation events[20]. This non-accumulation of SAH under conditions of increased transmethylation activity indicates that SAHH is highly active in

T. brucei. Given its critical role in SAM metabolism, SAHH has emerged as a target for antitrypanosomal agents[21].

Knockdown of SAHH transcript expression to 18.0 ± 14.0 percent of levels in uninduced controls (Figure 2) produced a rapid and severe growth defect (Figure 3D), consistent with the effects of SAHH depletion or inhibition in yeast[22], *Arabidopsis*[23], zebrafish[24], and mammals[25,26]. Due to the lethal phenotype observed following induction of RNAi, SAHH was not investigated further for a potential role in social motility.

v. *RNA-binding protein 11*

We next assessed the effect of RNA-binding protein 11 (RBP11) knockdown on cell viability. A whole-cell stable isotope labeling by amino acids in culture (SILAC) proteomic analysis revealed a 2.7-fold increase in abundance of RBP11 in procyclic form *T. brucei* over bloodstream form cells[27]. The function of this putative RNA-binding protein is not known, although the domain architecture suggests involvement in rRNA processing[28].

Depletion of RBP11 produced a rapid and severe growth defect (Figure 3E). Our result contradicts that reported by Wurst *et al.*[28], who observed no growth defect upon RNAi of RBP11. Wurst *et al*. did not assess knockdown, however, and this discrepancy in growth phenotype may be attributed to poor knockdown of RBP11 in Wurst *et al*. Our qPCR analysis yielded an expression value for RBP11 of 141.7 ± 77.1 percent relative to uninduced control cells (n=1 biological replicate). This result is inconsistent with the drastic decline in growth observed in the presence of tetracycline, indicating that the qPCR result may be in error and that additional replicates should be performed. In any

case, given the lethality observed upon induction of RNAi, RBP11 was not investigated for a possible role in social motility.

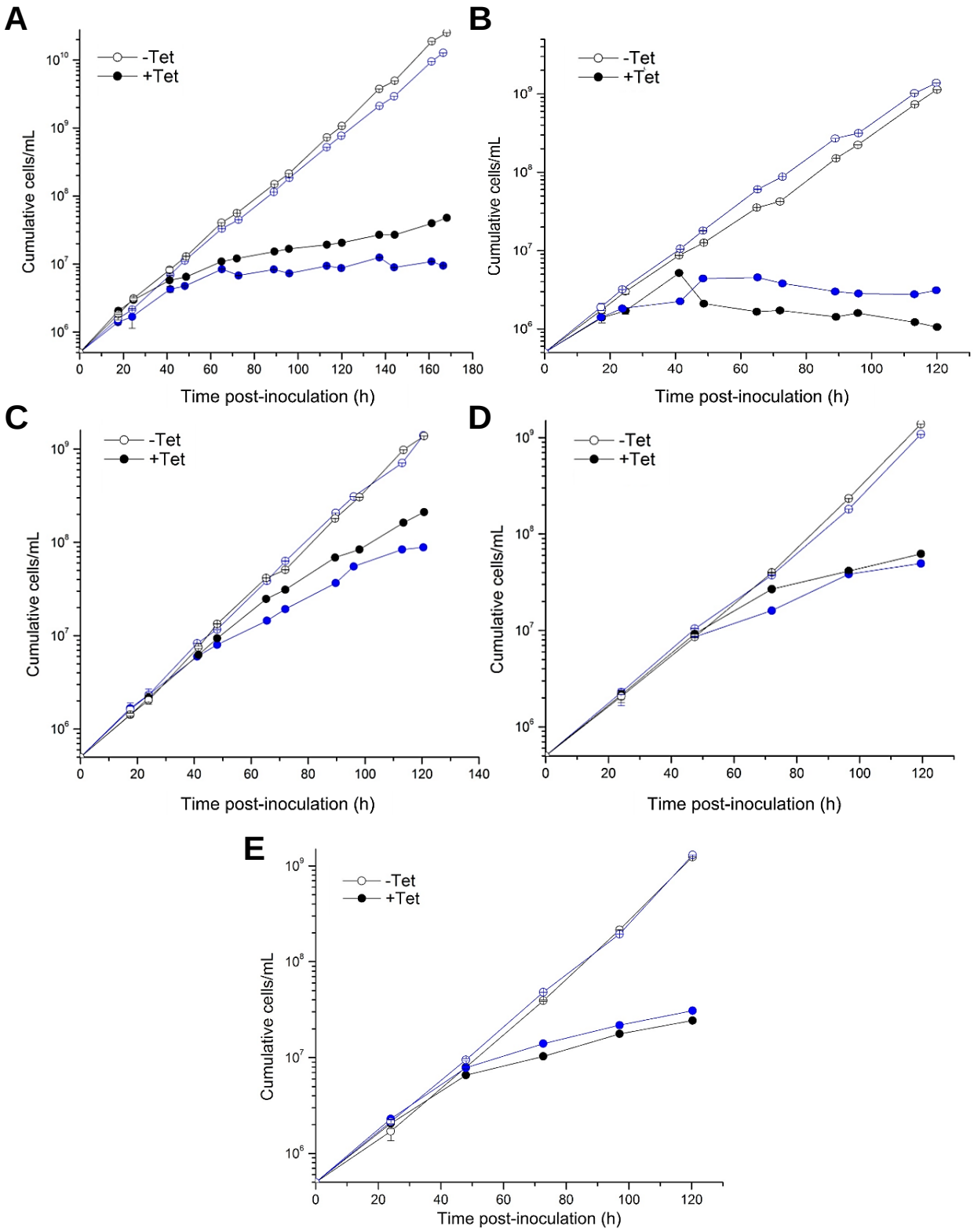


Figure 3: Knockdown of EF-1β, Histone H3, UMSBP2, SAHH, and RBP11 yields severe growth defects. Growth curves for (A) EF-1β, (B) Histone H3, (C) UMSBP2, (D) SAHH, and (E) RBP11 RNAi lines. Each color corresponds to an independent clonal line. Closed and open symbols indicate presence and absence of 1 μg/mL tetracycline, respectively. Error bars are ± 1 standard deviation. Three technical replicates were performed per data point. Tet, tetracycline.

II. Genes dispensable for viability *in vitro*.

A. *Zinc finger protein ZC3H34 and cyclophilin A knockdown lines maintain logarithmic growth.*

i. *Zinc finger protein ZC3H34*

The genome of *T. brucei* is organized into polycistronic gene clusters containing multiple genes that are co-transcribed from a single promoter[29]. Unlike operons in prokaryotes, these co-transcribed genes are not functionally related. Because of this genome arrangement, most gene regulation in *T. brucei* occurs at the post-transcriptional (RNA processing, stability, and translation) and post-translational levels[29]. *T. brucei* ZC3H34 is a CCCH-type zinc finger protein with a molecular weight of 22.4 kDa and a single C–X_7–C–X_5–C–X_3–H finger motif, where X is any amino acid[30]. Most of the characterized CCCH-type zinc finger proteins bind to RNA and are associated with RNA metabolism, including RNA cleavage, RNA degradation, RNA polyadenylation, or RNA export[30], although DNA-binding CCCH motifs have been reported[31-33]. ZC3H34 has been shown to increase the stability of target transcripts in *T.*

brucei; transcript abundance increased 20.9-fold when ZC3H34 was attached to the UTR of an mRNA reporter in a “tethering” assay[34].

Knockdown of ZC3H34 to 19.1 ± 12.5 percent of expression levels in uninduced controls (Figure 2) did not affect viability, as these cell lines maintained logarithmic growth throughout the time course of the assay (Figure 4A).

ii. *Cyclophilin A*

The cyclophilins are a family of evolutionarily well-conserved proteins that are present in all prokaryotes and eukaryotes. Cyclophilins possess peptidyl-prolyl isomerase activity, catalyzing the *cis-trans* isomerization of peptide bonds preceding proline residues and thereby facilitating protein folding[35]. In addition to their role as molecular chaperones, cyclophilins participate in a diverse array of other biological processes, including protein trafficking and maturation[36], receptor complex stabilization[37], receptor signaling[38], RNA processing[39], detoxification of reactive oxygen species[40], immune response[41], spliceosome assembly[42], miRNA activity[43], RNA-induced silencing complex (RISC) assembly[44], and chemotaxis[45,46].

Cyclophilin A (CyPA) is the most abundantly expressed cyclophilin, comprising 0.1 to 0.6 percent of total cytosolic protein content[47]. In *T. brucei*, CyPA (MW = 18.7 kDa) is 1.65 times more abundant in bloodstream form than in procyclic form cells[27], and 1.55 times more abundant in the intact flagellum of procyclic form cells than in the cellular debris fraction[48]. Consistent with these proteomic analyses, CyPA localizes to the cytoplasm and also along the flagellum in immunofluorescence micrographs, although the staining is not uniform throughout the flagellum, indicating differences in distribution and density[49].

Proteins involved in folding and degradation are major components of the *T. brucei* secretome, and CyPA was recently shown to be among the proteins secreted by *T. brucei*[50]. Moreover, antisera obtained from cattle immunized with cell lysate from *T. congolense* reacted strongly with an 18 kDa band on a western blot of trypanosome whole-cell lysate as well as with recombinant CyPA[49]. CyPA is a modulator of the immune system of mammalian hosts[51] and is known to stimulate macrophages[52]. Moreover, extracellular CyPA has a potent chemotactic effect on leukocytes[45], monocytes[46], and lymphocytes[46]. Finally, CyPA has been implicated in the intracellular replication cycle of the kinetoplastid parasite *Leishmania*, as CyPA siRNA interference or sequestration by cyclosporin A reduced parasite burden in murine macrophages[53]. Taken together, these observations raise the intriguing possibility that CyPA is important to the pathogenesis African trypanosomiasis. CyPA's role as a paracrine factor capable of mediating intercellular communication also makes it an especially promising candidate regulator of trypanosome social motility. `

Knockdown of CyPA expression to 6.9 ± 5.1 percent of levels in uninduced controls (Figure 2) did not affect viability, as these cell lines maintained logarithmic growth throughout the time course of the assay (Figure 4B), consistent with the dispensability of cyclophilins for viability in yeast[54] and mammals[55].

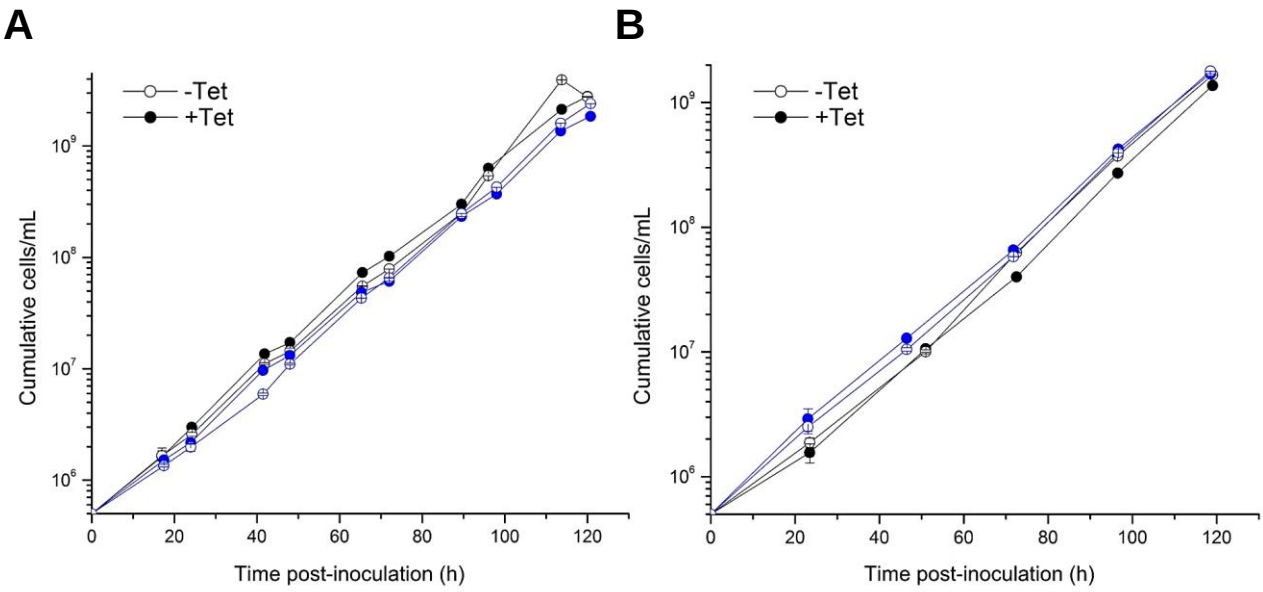


Figure 4: Growth curves for ZC3H34 and CyPA knockdown lines. (A) ZC3H34 and (B) CyPA knockdown lines maintain logarithmic growth following induction of RNAi. Each color corresponds to an independent clonal line. Abbreviations and symbols same as in Figure 3. Three technical replicates were performed per data point.

The ZC3H34 and CyPA RNAi lines were selected for further analysis to evaluate the effect of knockdown of these genes on social and propulsive motility.

III. ZC3H34 and CyPA are important for social motility.

A. *ZC3H34 knockdown lines are delayed in social motility and show reduced propulsive motility.*

Having identified those candidate genes not essential for viability, we next sought to evaluate whether any of these genes function as regulators of social motility. To this end, we assessed the motility behavior of the ZC3H34 and CyPA knockdown lines on semisolid agarose using the social motility assay of Oberholzer *et al*.[56] Cells were cultured in suspension in the presence and absence of tetracycline for 72 h and

subsequently inoculated on corresponding plates ± tetracycline. The motility of individual cells obtained from the same suspension culture was also analyzed.

To evaluate the effect of ZC3H34 knockdown on social motility, we first assessed the number of radial projections formed by ZC3H34-depleted cells and non-depleted controls at fixed time points post-inoculation on semisolid agarose. Figure 5A shows representative images of projection formation by RNAi-induced (+ tetracycline) and uninduced (- tetracycline) control groups over the time course of the assay. Knockdown of ZC3H34 significantly delayed the onset of social motility. Cells depleted of ZC3H34 had formed significantly fewer radial projections at 2, 3, and 4 days post-inoculation on semisolid agarose compared with uninduced controls at the same time points (Figure 5B). Moreover, the projections that did form in the presence of tetracycline were less extensive than those formed by controls, only occasionally reaching the edge of the plate (Figure 5A). qPCR analysis verified that formation of radial projections on the + tetracycline plates was not the result of poor transcript knockdown (Figure 2).

Cells that lack propulsive (directional) motility are unable to perform social motility[56]. We therefore examined the propulsive motility of individual cells in suspension culture to better understand the basis for the delayed social motility phenotype. Of note, ZC3H34-depleted cell lines also displayed significantly reduced propulsive motility in suspension compared to uninduced controls, as measured by total distance traveled (Figure 6A) and mean-square displacement (Figure 6B). Whether the observed delay in social motility onset represents a *bona fide* defect in social motility or is the result of slower single-cell motility is unclear (see Discussion).

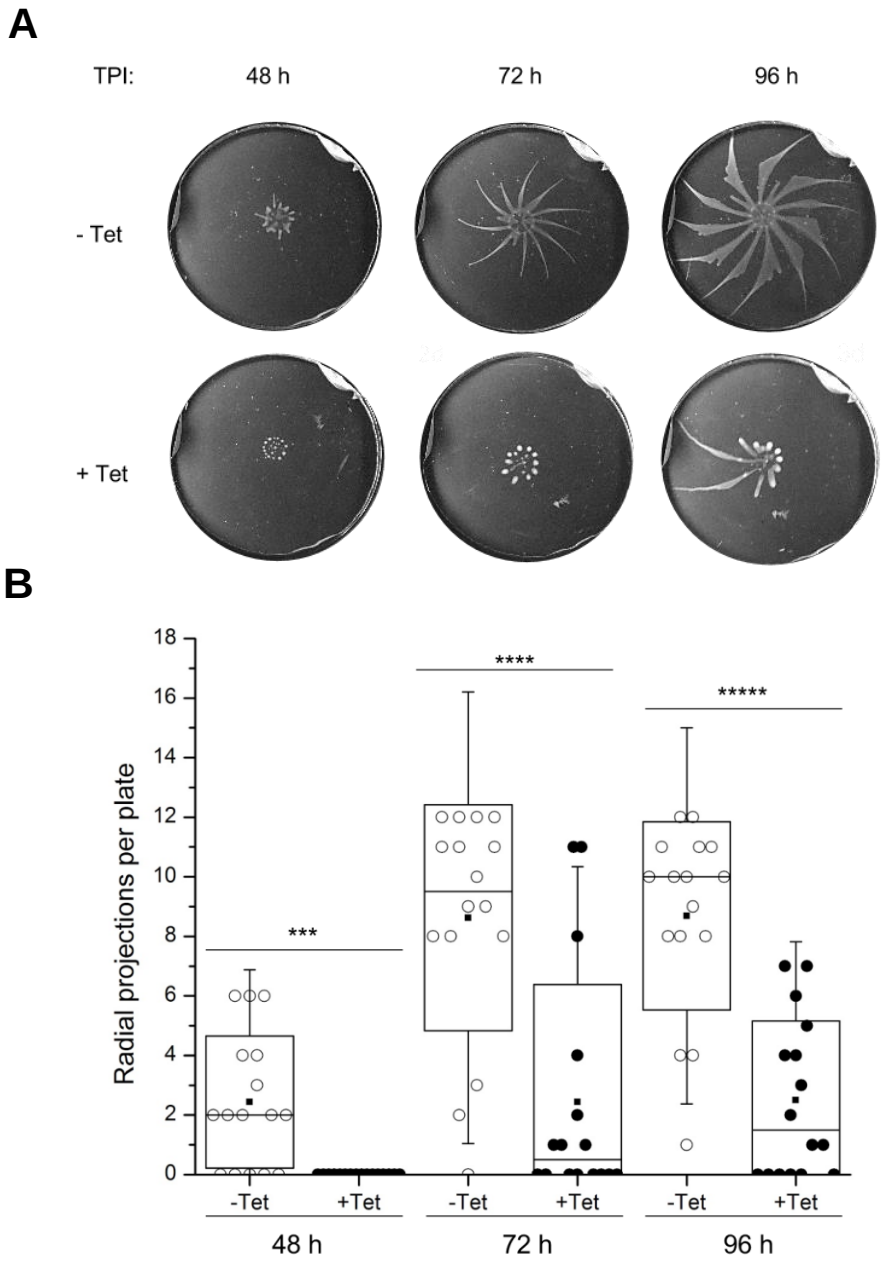


Figure 5: Knockdown of ZC3H34 delays social motility. (A) Representative images of radial projections on semisolid agarose formed by uninduced, ZC3H34-expressing control cells (upper panel) and cells depleted of ZC3H34 (lower panel) at the indicated times post-inoculation (TPI). (B) Distribution of number of radial projections per plate at the indicated times point post-inoculation. Vertical axis indicates number of projections extending ≥ 7.5 mm, 10.8 mm, and 19.9 mm from the inoculation site for time points 48

h, 72 h, and 96 h post-inoculation, respectively. Boxes indicate the 25th, 50th, and 75th percentiles. Closed squares (▪) indicate the mean. Error bars are + 2 standard deviations. *n*=16 plates for each time/condition. ***$p < 0.001$; ****$p < 0.0001$, *****$p < 0.00001$ (two-tailed Student's *t*-test).

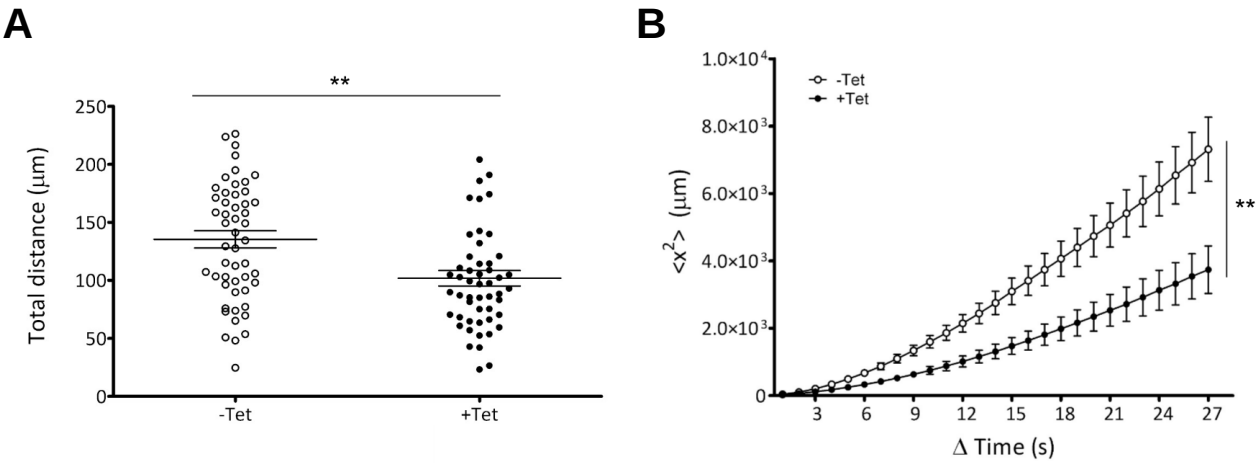


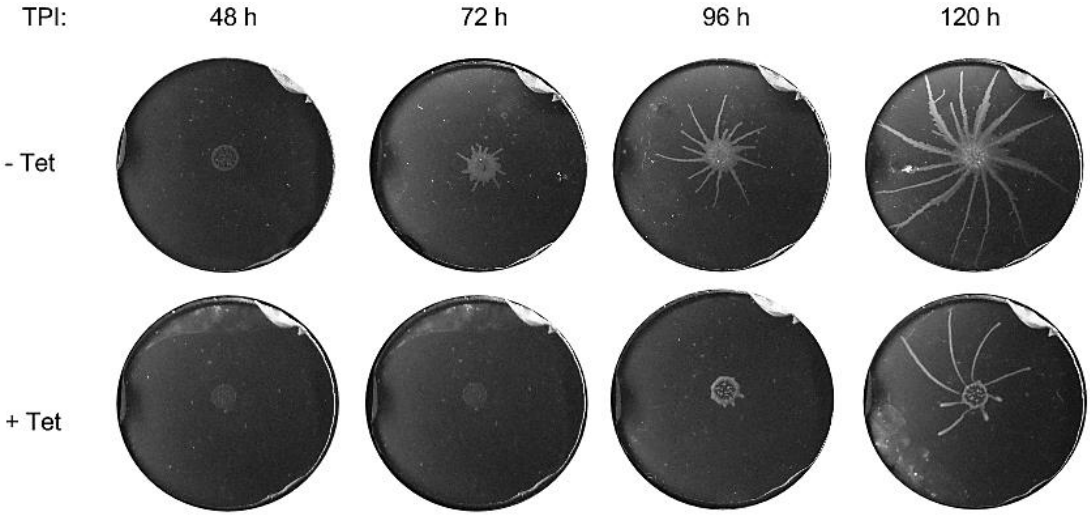
Figure 6: ZC3H34 knockdown decreases propulsive motility. (A) Total distance traveled and (B) mean-square displacement ($<x^2>$) of ZC3H34 RNAi knockdown lines in presence and absence of 1 µg/mL tetracycline. Center line in (A) is the mean. Error bars are ± 1 SEM. *n*=52 cells tracked per condition. **$p < 0.01$ (two-tailed Student's *t*-test).

B. *Knockdown of CyPA delays social motility, and this delay is not attributable to a defect in propulsive motility*.

We evaluated the effect of CyPA knockdown on social motility in a manner analogous to our assessment of ZC3H34 knockdown. Figure 7A shows representative images of projection formation by RNAi-induced and uninduced control groups over the time course of the assay. As with ZC3H34 depletion, knockdown of CyPA significantly delayed the onset of social motility. Cells depleted of CyPA had formed significantly fewer radial projections at 3, 4, and 5 days post-inoculation on semisolid agarose

compared with uninduced controls at the same time points (Figure 7B). This delay was not attributable to decreased propulsive motility, as the total distance traveled (Figure 8A) and mean-square displacement (Figure 8B) of CyPA knockdown cells was slightly or even significantly greater than CyPA-expressing controls. Interestingly, the number of radial projections formed by CyPA-depleted parasites increased rapidly between days 4 and 5, as reflected by the 10^5-fold decrease in *p*-value.

A



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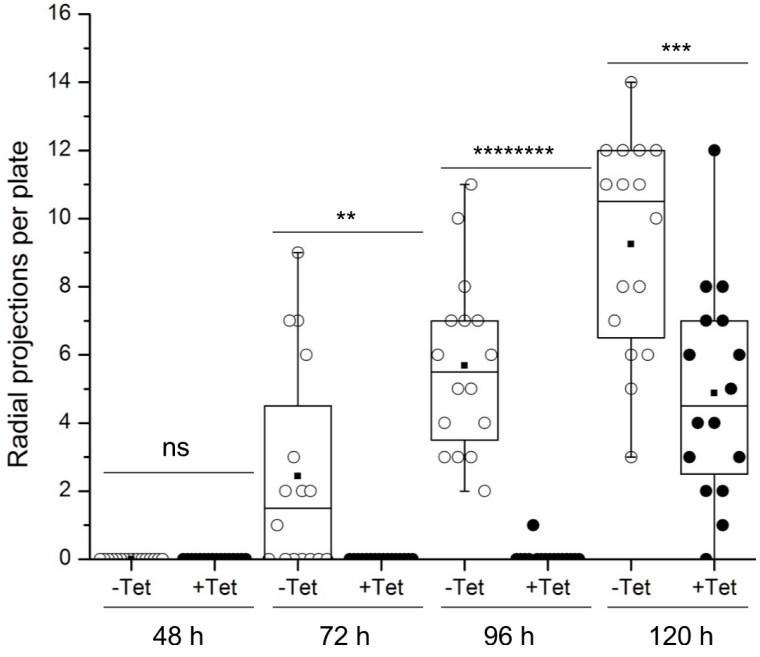

Figure 7: Knockdown of CyPA delays social motility. (A) Representative images of radial projections on semisolid agarose formed by uninduced, CyPA-expressing control cells (upper panel) and cells depleted of CyPA (lower panel) at the indicated times post-inoculation (TPI). (B) Distribution of number of radial projections per plate at the indicated time points post-inoculation. Graph and box plot elements same as in Figure 5; radial projections at 120 h are those extending ≥ 24.9 mm from site of inoculation. *n*=16 plates for each time/condition. ns, $p > 0.05$; **$p < 0.01$; ***$p < 0.001$; ********$p < 0.00000001$ (two-tailed Student's *t*-test).

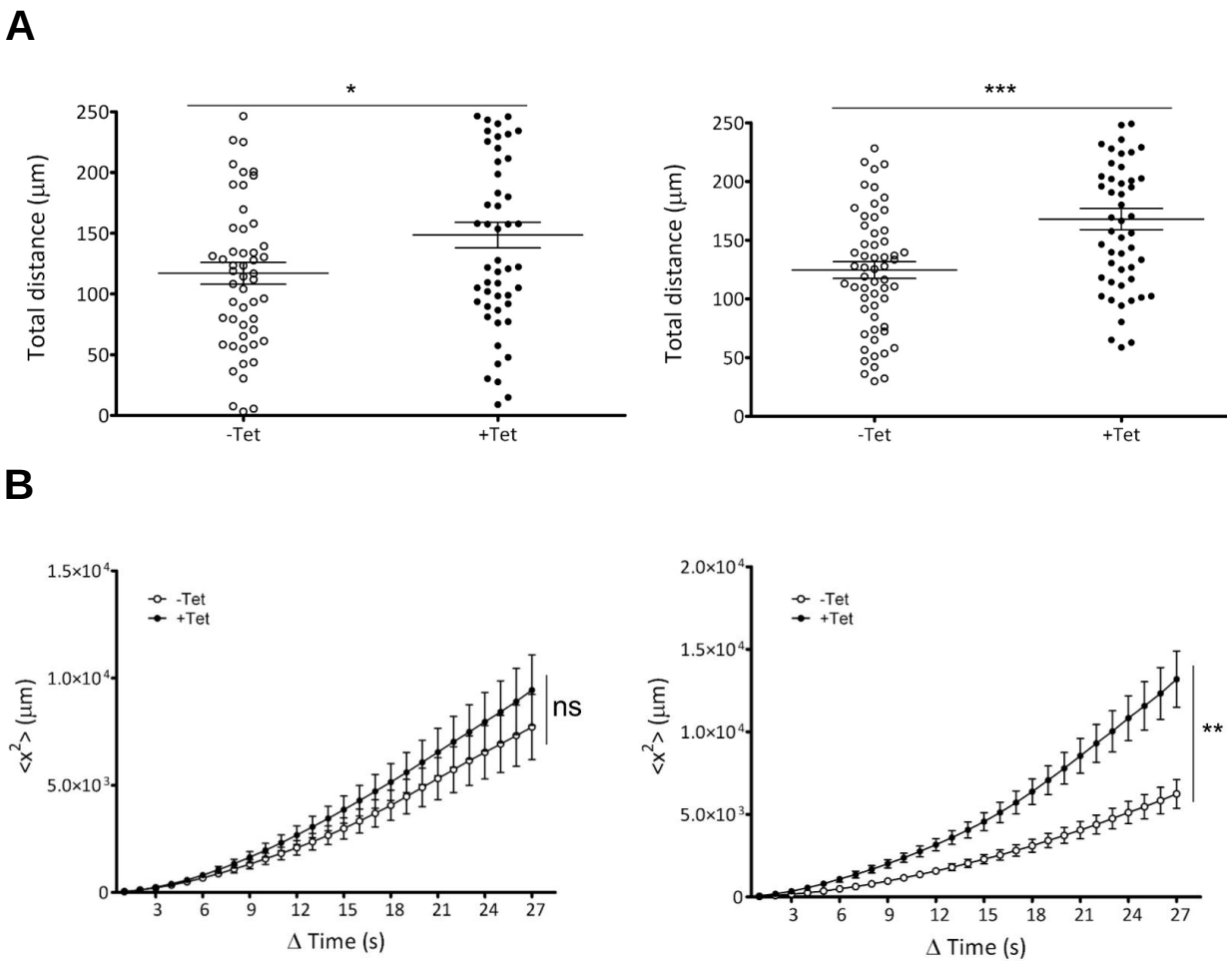

Figure 8: Depletion of CyPA does not reduce general propulsive motility. (A) Total distance traveled and (B) mean-square displacement (<x^2>) of CyPA RNAi knockdown

lines in presence and absence of 1 μg/mL tetracycline. Two biological replicates were performed, and each column shows results from a given biological replicate. Plot elements same as in Figure 6. n=52 cells tracked per condition per replicate. ns, $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (two-tailed Student's t-test).

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CHAPTER 4

Discussion

African trypanosomiasis ranks among the three most neglected tropical diseases[1]. Despite the considerable medical and economic burden of this disease, only four drugs are available for the treatment of sleeping sickness, and no new treatments have been developed in over a quarter century[2]. Moreover, existing therapeutics are toxic, increasingly ineffective against early-stage, hemolymphatic infection, and limited in their ability to treat late-stage, central nervous system infection[2].

Social motility in *Trypanosoma brucei* was first described in 2010[3], and was recently selected as one of the seven most influential discoveries in parasitology of the past decade by the editors of *PLoS Pathogens*[4], a leading microbial pathogenesis journal. Numerous studies since then have shown that social motility is controlled by cAMP signaling in the trypanosome flagellum[5,6] and have linked social motility to establishment of tsetse fly midgut infection and vector-to-host transmission[7]. Despite this progress, many genetic modulators of social motility have yet to be discovered, and the mechanisms underlying this phenomenon remain poorly understood. Biochemical studies of genes that control social motility in *T. brucei* have been limited to a few flagellar adenylate cyclases[5,6], a flagellar phosphodiesterase[6], and the ER membrane protein *Rft1*[7]. Additional regulators of social motility were unknown prior to this work.

Although direct evidence implicating social motility in the transmission of *T. brucei* from vector to mammalian host is lacking, the observation that *Rft1*$^{-/-}$ cell lines

defective in social motility are also impaired in their ability to establish midgut infection in the *Glossina* vector[7] supports the idea that social motility may play a role in promoting trypanosome colonization and infection of tsetse flies. Thus, chemical inhibition of regulators of social motility is a potentially promising approach for blocking the transmission of trypanosomiasis. More broadly, social motility requires both intra- and intercellular signaling events, including the ability of cells to alter their movement in response to external stimuli. Identification and characterization of the genes required for social motility in *T. brucei* will therefore also advance our understanding of parasite signaling, an important yet highly understudied aspect of parasite biology.

The present work identifies two novel modulators of social motility in *T. brucei*, the zinc finger RNA-binding protein ZC3H34 and cyclophilin A. Knockdown of ZC3H34 delayed social motility, although the ZC3H34 knockdown parasites also exhibited reduced propulsive motility of individual cells in liquid culture. A complete block of directional motility causes a complete block of social motility[3]. Thus, it is possible that the delayed social motility phenotype of ZC3H34 knockdowns is due to reduced propulsive motility. However, *T. brucei* depleted of the AC4 receptor-type adenylate cyclase show a decrease in propulsive motility comparable to that observed with ZC3H34 knockdowns (~35 μm decrease in total distance traveled relative to non-depleted controls) but do not exhibit any apparent differences in social motility compared to wild type controls[5]. It therefore remains to be determined whether reduced propulsive motility is the cause of the delayed social motility seen with the ZC3H34 knockdowns. A direct parallel time course analysis of radial projection formation by ZC3H34- and AC4-depleted cells will help distinguish between these possibilities.

Additional work is needed to define the mechanism by which ZC3H34 influences social motility. ZC3H34 is an RNA-binding protein that associates with DRDB3[8], another RNA-binding protein that functions in stabilization and transport of mRNAs[9]. As noted in Chapter 3, gene expression in *T. brucei* is regulated almost exclusively at the post-transcriptional level by RNA-binding proteins[10]. This makes ZC3H34 a particularly interesting candidate, as it could function in controlling the expression of other genes involved in social motility. The RNA substrates of ZC3H34 are unknown. Epitope tagging of ZC3H34 followed by RNA-immunoprecipitation sequencing (RIP-seq) would provide a means to identify the RNA targets of ZC3H34. Subsequent analysis of social motility phenotype upon knockdown or overexpression of the identified ZC3H34 target will clarify the mechanism through which ZC3H34 regulates social motility. Given the predicted role of ZC3H34 as an RNA-binding protein and its ability to stabilize RNA transcripts when fused to the UTR of an mRNA reporter[11,12], we speculate that ZC3H34 may function in stabilizing the transcripts of genes involved in social motility and/or individual cell motility.

As with ZC3H34 depletion, knockdown of cyclophilin A resulted in a delayed social motility phenotype. Unlike ZC3H34, however, this delay in social motility was not accompanied by reduced motility of individual cells. In fact, CyPA knockdown lines were found to have slightly or even significantly elevated propulsive motility compared to non-depleted controls (Figure 8). Cyclophilin A is secreted by *T. brucei*[13,14] and has been shown to elicit immunosuppression in an *in vitro* model of T-cell proliferation[15]. Moreover, CyPA stimulates the migration of eosinophils and neutrophils at nanomolar concentrations[16], and also elicits monocyte chemotaxis[17]. Our observation that CyPA

knockdown lines are defective in social motility, combined with CyPA's possible role as a cytokine-like protein involved in interactions with infected species and previous work implicating social motility in tsetse fly infection[7], suggest that CyPA might be important to parasite transmission. Experiments that assess the ability of CyPA (and ZC3H34) null mutants to infect the tsetse fly are needed to determine whether the social motility defects observed *in vitro* are indeed physiologically relevant.

The other five genes analyzed in this study were not evaluated for a potential role in social motility owing to the lethality observed in suspension culture after tetracycline-induced knockdown of transcript expression. The finding that a gene upregulated in trypanosomes cultured on semisolid agarose is essential for viability does not exclude a possible role for that gene in social motility, and the genetic system used in this study did not allow for control over dsRNA expression levels. Thus, low-level knockdown of the genes whose depletion resulted in lethality might yield detectable differences in social motility phenotype while maintaining cell viability. Conveniently, an inducible expression system that enables precise control over expression of both genes and dsRNAs in *T. brucei* was recently developed by Sunter[18]. A more practical and perhaps more informative approach, however, would be to assay social motility in parasites overexpressing these genes. Future studies should also explore the potential functional relevance to social motility of the remaining genes among the list of 22 candidates upregulated in trypanosomes cultivated on semisolid agarose using a workflow similar to that employed in this study. Overexpression of the 38 genes found to be downregulated in *T. brucei* plated on semisolid agarose followed by assays of social motility will also prove insightful.

In summary, elucidating and characterizing regulators of social motility in *Trypanosoma brucei* not only has the potential to alleviate the burden of African trypanosomiasis by informing the development of chemical inhibitors of vector-to-host parasite transmission, but will also advance our understanding of signaling pathways in protozoan parasites and improve the relevance of *T. brucei* as a model organism for the study of eukaryotic signaling in general.

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SUPPLEMENTAL INFORMATION

Supplemental Table 1: Primers used for generation of RNAi amplicons. Restriction sites are italicized.

GeneDB Accession (Gene Description)	Primer Sequence
Tb927.10.12330 (ZC3H34)	FWD: ATAT*TCTAGA*ACCCGCCTCAACAGTATCAC REV: ATAT*AAGCTT*CACATCATGTTTTCCATCGG
Tb927.11.880 (CyPA)	FWD: AGTCGA*TCTAGA*ATGTGAGCATTGCAGGTCAG REV: AGTCGA*CTCGAG*TAGTCGGTGTTCGTCTGTGC
Tb927.10.5840 (EF-1β)	FWD: ATAT*TCTAGA*TAAAGGAAATCAACGGTCGC REV: ATAT*AAGCTT*TGTGGTCACCCCACAGTAGA
Tb927.1.2510 (Histone H3)	FWD: ATAT*TCTAGA*AGGCCTCAAAGGGTTCTGAT REV: ATAT*AAGCTT*TTGTCTCAACCCTGACCCTC
Tb927.10.6060 (UMSBP2)	FWD: ATAT*TCTAGA*TTGAAACGTCTCCAACCCTC REV: ATAT*AAGCTT*CTTTTCCATCCCTCCTCTCC
Tb927.11.9590 (SAHH)	FWD: AGTCGA*TCTAGA*CTCGTGCAACATCTTCTCCA REV: AGTCGA*AAGCTT*TATCCACAGACACATGCGGT
Tb927.8.4450 (RBP11)	FWD: ATAT*TCTAGA*GGAGTGACGACTTTGGTGGT REV: ATAT*AAGCTT*GCGGCTATGGGATTCTTGTA

Supplemental Table 2: Primers used for qPCR quantification of gene products.

GeneDB Accession (Gene Description)	Primer Sequence
Tb927.10.12330 (ZC3H34)	FWD: GCTGCCGAATCCCCCTAATG REV: ATGCAAGAGGACGGTCGAGA
Tb927.11.880 (CyPA)	FWD: ATGGACGTCGTCAAGGCAAT REV: TAGTCGGTGTTCGTCTGTGC
Tb927.10.5840 (EF-1β)	FWD: GTCGTTTGGAATGTGGCGAG REV: TTTACGGTTGAGGAGGCTGC

Tb927.1.2510 (Histone H3)	FWD: ACCACAACTCTCAAACCAAGCA REV: ACCCTTTGAGGCCTTCTTGC
Tb927.10.6060 (UMSBP2)	FWD: CTTTCGCCATAAAGCGGTGC REV: CACTACGACCACCACCCAC
Tb927.11.9590 (SAHH)	FWD: GTTTTCGTAACCGCACGCTG REV: GCGTATCGGTAACACCCTCC
Tb927.8.4450 (RBP11)	FWD: ATGCAAACGTGTCAATGCGT REV: AGGAGAAACTCCCTACGGCT
Tb427.08.5010 (PFR2)	FWD: GAAGTTGAAGGTGTTGTGAGTCC REV: CCTCCAGCGTGATATCTGTTACC
Tb927.11.10190 (TERT)	FWD: GAGCGTGTGACTTCCGAAGG REV: AGGAACTGTCACGGAGTTTGC