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Motility and sensory functions of the *Trypanosoma brucei* flagellum

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Microbiology, Immunology & Molecular Genetics

By

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ABSTRACT OF THE DISSERTATION

Motility and sensory functions of the Trypanosoma brucei flagellum

by

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Doctor of Philosophy in Microbiology, Immunology & Molecular Genetics
University of California, Los Angeles 2014
Professor Kent L. Hill, Chair

Eukaryotic flagella, also known as cilia, are emblematic features of most extant eukaryotic cells. Cilia have important motility and sensory roles for cellular and organismal physiology. A prime example is the flagellum of Trypanosoma brucei, the unicellular causative agent of sleeping sickness in Africa. The T. brucei flagellum harbors a canonical eukaryotic axoneme and provides cell propulsion, dictates cell morphogenesis as well as constitutes a critical host pathogen interface.

Flagellar motility results from the coordinated activity of axonemal dyneins. While the mechanism, by which thousands of dynein motors are spatiotemporally controlled, is enigmatic, motility requires the Nexin Dynein Regulatory Complex (NDRC). The NDRC is a massive axonemal protein complex, present in virtually all flagellated eukaryotes, that reversibly inhibits dyneins. NDRC has important roles in human and trypanosome biology yet its exact composition and mode of action are unknown. We employed quantitative proteomics as well as a candidate approach to illuminate the T. brucei NDRC subunit composition. We discovered 14 proteins that are consistently reduced in
axonemes of NDRC mutants and are thus prime NDRC subunit candidates. Of these candidates, only two were specific to the trypanosome lineage while the remainder had clear orthologs in diverse eukaryotes. Our results support the existence of an NDRC core consisting of phylogenetically conserved subunits that operate in unison with lineage specific subunits. Moreover, NDRC is likely a calcium-regulated hub given the extensive representation of cognate domains among its subunits.

The trypanosome flagellum remains attached to the cell body for most of its length at the flagellum attachment zone (FAZ). FAZ has important structural and morphogenetic roles that dictate the shape and size of the trypanosome cell. Nevertheless, the protein repertoire of the FAZ has remained elusive. We focused on FS179, an uncharacterized membrane protein recently detected in our *T. brucei* flagellar membrane proteome. We showed that this putative calcium channel localizes to a subdomain of the flagellar membrane along the FAZ. Interestingly, FS179 mediates flagellum attachment and is required for cell shape and parasite viability. Our findings thus reveal a novel component of the trypanosome FAZ and shed light on flagellum roles in cell morphogenesis.

The trypanosome flagellar membrane is the interface with the extracellular milieu and its proteins have vital roles in host-pathogen interactions. This is exemplified by the flagellar pocket, a membrane invagination at the base of the flagellum where all endocytosis and uptake of host factors takes place. During efforts to understand flagellar membrane functions, we studied BBS proteins, subunits of the BBSome complex that controls cilium localization for certain membrane proteins in other eukaryotes. We show that *T. brucei* BBS proteins assemble into a BBSome-like complex
and localize to the flagellar pocket membrane and vesicles therein. Genetic knockouts of BBS genes further reveal that BBSome is dispensable for flagellum biogenesis yet is essential for proper uptake of host transferrin. Our results support a model whereby *T. brucei* BBSome functions as a clathrin adaptor facilitating endocytosis of a subset of flagellar pocket membrane proteins such as the transferrin receptor. In summary, our results highlight the importance of *T. brucei* as a model system to study conserved and parasite specific flagellar functions, illuminate novel regulators of axonemal motility and expand the roles of a cilium gatekeeper to endocytosis.
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2014
DEDICATION

to my Sigma Orionis
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Conferences

Publications


Chapter 1

Introduction
Cilia are important eukaryotic organelles

Cilia, synonymous with eukaryotic flagella, are prominent features of eukaryotic cells [1]. Uniquely among organelles, these cylindrical structures are anchored near the cell surface and protrude into the extracellular environment. Cilia consist of a specialized membrane that is distinct from the rest of the cell surface and cloaks the microtubule-based axoneme at the core of the organelle [2]. The axoneme emanates from the basal body, a modified centriole, while the space between the axoneme and the membrane is termed matrix. Matrix is topologically contiguous with the cytoplasm but cilia are populated by organelle-specific proteins by virtue of a diffusion barrier at their base [3]. Cilia are nearly ubiquitous among eukaryotes and structural, proteomic and functional data suggest that cilia were already present at the dawn of eukaryotes and the last eukaryotic common ancestor (LECA) [1,6,7]. Cilia and most of their components don’t have prokaryotic counterparts and are thus considered eukaryotic innovations, yet their origin remains enigmatic. A bacterial [8] or viral endosymbiosis [9] event have been proposed as giving rise to cilia but these scenarios are now considered unlikely [10]. Instead, an autogenous origin from diversification of pre-existing features, such as the microtubule cytoskeleton and the protocoatomer module, is currently the prevailing model for cilia origin [5,6]. The functions of cilia in LECA are uncertain but probably involved phagotrophy-related roles of motility and/or sensing [4,5,6]. Cilia were conserved during the course of eukaryotic evolution and are now found in most extant lineages with notable exceptions being the majority of fungi, land plants and amoebas [1]. Conservation is readily apparent in the structure of the axoneme. Axonemes consist of 9 peripheral microtubule doublets that surround a central doublet (9+2 axoneme) or
just 9 peripheral microtubule doublets (9+0 axoneme), although non-canonical architectures have also been observed [11,12,13]. The basal body comprises 9 triple microtubules that anchor the axoneme via the transition zone. The axonemal structural conservation reflects common building blocks that are typically encoded only by organisms that build cilia and this phylogenetic profile has been successfully used to discover novel ciliary proteins [14,15]. Interestingly, bona fide ciliary proteins can also function outside the cilium such as intraflagellar transport proteins operating at the lymphocytes’ immune synapse [16] as well as ciliary proteins found in plant cells [17]. Moreover, ciliary structures can be harnessed for novel functions with prominent examples being the striated fiber and conoid complex in apicomplexans [18,19].

Cilia are multifunctional organelles and typically provide motility that is harnessed for cell locomotion and movement of extracellular fluids or even of other cells [20,21]. Moreover, cilia are signaling platforms that allow cells to sense, integrate and respond to extracellular cues [22-25]. These motility and sensory functions are paramount for development and physiology of multicellular organisms as well as protists [26,27]. In humans for instance, ciliary motility is required for sperm propulsion, mucus clearance and the fluid flow that determines body asymmetry in embryos [28]. Moreover, sensory cilia are required for vision, smell and hearing and also orchestrate important developmental pathways such as Hedgehog signaling, Wnt signaling, epidermal growth factor and Notch signaling. [29,30,31]. In mammals, these sensory capabilities are typically displayed by non-motile primary cilia with 9+0 architecture, although motile 9+2 cilia exhibit sensory abilities too [22,32]. Given the widespread occurrence of cilia as well as their multiple roles, it comes as little surprise that defective cilia lead to a range
of inherited diseases in humans. These disorders are collectively called ciliopathies and manifest through a plethora of clinical symptoms that include retinal degeneration, renal abnormalities, obesity, mental retardation and skeletal dysplasias [28,33,34,35].

To fulfill their roles, cilia rely on organelle-specific proteins that need to be trafficked from the cytoplasm since cilia are devoid of ribosomes. Trafficking mechanisms need to negotiate the diffusion barrier at the base of cilia in order to deliver soluble, cytoskeletal and membrane proteins to the organelle [3,36]. The best characterized such mechanism is intraflagellar transport (IFT), first described in the flagella of the green algae *Chlamydomonas reinhardtii*, a process essential for axoneme biogenesis in virtually all ciliated organisms [37]. IFT is mediated by a cohort of proteins that assemble into complexes and mediate transport of axonemal building blocks to the ciliary tip (anterograde transport). Moreover, IFT operates from the tip to the base of cilia (retrograde transport) to recycle proteins and to drive signaling pathways. IFT takes place on the outer surface of peripheral axonemal microtubule doublets and requires kinesin and dynein motors for anterograde and retrograde transport respectively [38]. In addition to IFT, many eukaryotes rely on BBSome for trafficking of certain membrane proteins into and out of the cilium [3]. BBSome is a protein complex of conserved proteins that are specific to ciliated species [39]. In humans, defects in BBSome lead to Bardet Biedl Syndrome (BBS), a multiple organ disorder that is caused by defective ciliary functions [40]. In some cases BBSome interacts with IFT to traffic in unison along the axoneme. The exact mechanism via which BBSome and IFT proteins recognize their cargo and transverse the diffusion barrier at the base of cilia remains enigmatic [3,36].
Recent findings have shed light on the nature of the ciliary diffusion barrier [41,42]. While it is unknown whether there is a universal diffusion barrier or there are lineage specific variations, studies have suggested a structure analogous to the nuclear pore complex. This ciliary pore complex (CPC) contains nucleoporins and operates across a Ran GTP gradient, reminiscent of protein transport to the nucleus [43,44]. Moreover, structures consisting of ciliopathy proteins or septins assemble at the proximal end of the basal body and the transition zone (region between axoneme proper and basal body) to function as a ciliary gate [45,46,50,51,52]. Collectively, these studies indicate that the ciliary gate is required for cilium biogenesis and while small proteins can diffuse freely into the cilium, larger ones are excluded and need to negotiate the diffusion barrier via a trafficking mechanism [47-49].

In addition to multicellular organisms, a plethora of unicellular eukaryotes rely on flagella to move, interact with their environment and complete their life cycle. Some species such as fungi or land plants produce flagellated cells only during their sexual cycle yet others are characterized by the constant presence of flagellum. A prime example is Trypanosoma brucei, the single cell pathogen that causes human African trypanosomiasis or sleeping sickness [53]. T. brucei are vermiform cells that are characterized by a single flagellum with a central role in the biology of the parasite [27,54]. For instance, the relative position of the base of the flagellum in respect to the nucleus is used to define parasite morphotypes such as trypomastigotes (flagellum emanates posterior to the nucleus) and epimastigotes (flagellum emanates anterior to the nucleus). Furthermore, the flagellum provides motility, governs cell morphogenesis and is a critical host-pathogen interface. T. brucei belongs to Kinetoplastids, a divergent
group of eukaryotes that are characterized by a network of concatenated mitochondrial DNA circles, termed the kinetoplast, at the base of the flagellum [55]. *T. brucei* and related kinetoplastids such as *Trypanosoma cruzi* and *Leishmania* species are successful parasites, capable of infecting a wide range of animals. These pathogens are of great medical and economic importance since they cause lethal human diseases and limit economic growth in some of the poorest regions of our planet [56-58]. *T. brucei* in particular affects vast regions of sub Saharan Africa where it is transmitted to humans and other mammals by endemic tsetse flies of the genus *Glossina*. *T. brucei* have been afflicting humans for centuries and are thought to have left a mark on the human genome [59]. We currently recognize two human infectious *T. brucei* subspecies, namely *T. brucei gambiense* and *T. brucei rhodesiense*, that cause 50,000-70,000 new cases annually [60]. *T. brucei gambiense* is responsible for the majority (around 97%) of reported cases, causes a chronic form of the disease that takes couple of years to manifest and is found in West Africa. On the other hand, *T. brucei rhodesiense* causes acute sleeping sickness within weeks or months in East Africa and contributes only 3% of the cases. A third subspecies, *T. brucei brucei* is not human infectious but can infect non-primates and causes Nagana in cattle, a similar wasting disease that causes economic losses in the order of billions of dollars [61].

Sleeping sickness is characterized by two stages [62]. In the early hemolymphatic stage, parasites are found in the bloodstream and lymph and cause fever, headache, fever, lymphadenopathy and in some cases hepatosplenomegaly. The late neurological stage ensues when trypanosomes cross the blood brain barrier and invade the central nervous system. Symptoms then include sleep disturbances with
reversal of sleep/wake cycle, sensory disorders, motor abnormalities and psychiatric features. Failure to treat the disease leads to coma and is almost always fatal [63,64]. Type of treatment depends on the *T. brucei* subspecies and the stage of the disease with suramin and melarsoprol being the drugs of choice for *T. brucei rhodesiense* early and late stage respectively. *T. brucei gambiense* infections are treated with pentamidin at the early stage and melarsoprol or nifurtimox–eflornithine combination therapy (NECT) at the late stage [65]. Unfortunately, current drug treatments are not optimal since they can be toxic, difficult to administer while cases of drug resistance have also been reported. For example, melarsoprol is an arsenic-based compound that can cause lethal encephalopathic syndrome in up to 5% of patients. Moreover, there is no vaccine available and the immune evasion mechanisms employed by the parasite makes the development of one an unlikely prospect [66]. Despite the fact that the socioeconomic features of the disease have hindered therapeutics development, the discovery of novel trypanotoxic lead compounds and testing of new drugs in clinical trials, provide optimism for the combat against one of the world’s most neglected diseases [67].

In spite of their unicellular nature, *T. brucei* parasites can undergo dramatic developmental transitions and have a complex life cycle that entails forms adapted to the mammalian bloodstream as well tsetse fly tissues (Fig. 1) [27]. During a tsetse fly bite, cell cycle arrested metacyclic *T. brucei* are delivered to the mammalian host, differentiate into proliferating long slender forms and populate the bloodstream. *T. brucei* don’t invade host cells and remain extracellular under the constant attack of the host immune system. Parasites have evolved sophisticated mechanisms to withstand and evade both the innate and adaptive host immune responses [68]. A well studied case of
host-pathogen interactions during innate immunity is apolipoprotein L1 (ApoL1), a serum protein which leads to trypanosome cell death [60]. Upon uptake and drop of pH, ApoL1 is inserted into endosomal membranes and traffics to the lysosome, where it creates pores in the membrane that lead to cell lysis. This protein is encoded by many primates and confers resistance to *T.brucei brucei* parasites. However, the human infectious subspecies *T.b. gambiense* and *T.b. rhodesiense* are immune to ApoL1 via two distinct mechanisms [60]. *T.b. rhodesiense* encodes the serum resistance-associated (SRA) protein that interacts with ApoL1 and inactivates it while *T.b. gambiense* relies on reduced ApoL1 uptake and on *T.b. gambiense* specific glycoprotein (TgsGP) that stiffens the endosomal membranes and inhibits insertion of ApoL1. Moreover, trypanosomes employ antigenic variation of the variant surface glycoprotein (VSG) to thwart adaptive immunity. VSG is a GPI-anchored protein that covers the entire *T.brucei* cell surface and is highly immunogenic [69]. *T.brucei* encodes hundreds of different VSG alleles yet it only expresses one at a given time. During cell division, few cells of the population will switch to a new VSG allele and these parasites will survive and repopulate the bloodstream upon immune destruction of parasites with the initial VSG [70]. Antigenic variation coupled with immunosuppresion, allows trypanosomes to reside indefinitely in the bloodstream and makes the development of vaccines unlikely [71]. Long slender trypanosomes can also invade extravascular tissues such as CNS, an event that marks the onset of disease’s late stage [72]. It is unclear whether invasion of CNS and other tissues is beneficial to the parasite or the features of trypanosome developmental forms in these compartments. To prepare for transmission to tsetse fly vector, long slender trypanosomes at high cell densities
differentiate to short stumpy form via a quorum sensing-like mechanism [73]. This mechanism involves a mysterious stumpy induction factor (SIF) that elicits a response through recently characterized downstream pathways [74]. The stumpy form is cell cycle arrested and will survive the transition to tsetse fly midgut upon a bloodmeal where it will differentiate to procyclic form. Procyclic trypanosomes re-enter the cell cycle and embark on an epic journey through many different tsetse tissues that is accompanied by emergence of distinct trypanosome developmental forms such as the short epimastigotes [78,79]. Short epimastigotes will eventually arrive to the salivary gland of the fly and attach to epithelia cells using their flagella [75]. There, they replicate and complete the life cycle via an asymmetric division that produces metacyclics that are pre-adapted for survival in the mammalian bloodstream [75-77].

The *T. brucei* flagellum is a constant feature of the diverse morphotypes the parasite adopts during its life cycle. The flagellum is also present throughout the cell cycle, is essential for viability and provides the sole means for *T. brucei* propulsion as well as it orchestrates cell morphogenesis and host pathogen interactions [27]. Parasite motility is auger-like and this in fact spurred Gruby, one of the first to study trypanosomes, to name the genus *Trypanosoma* combining the Greek words for auger (trypanon) and body (soma) [80]. The *T. brucei* flagellum emerges from the posterior end of the cell and continues towards the anterior end (Fig. 2). Contrary to flagella of other eukaryotes, the parasite flagellum remains laterally attached for most of its length to the cell body and only the flagellum tip extends free [54]. Flagellum proper begins at the basal body, the barrel-like structure that nucleates the axoneme. Basal body consists of 9 peripheral microtubule triplets and is connected to the kinetoplast via the tripartite
attachment complex [81]. Anterior to the basal body lies the transition zone of 9 peripheral microtubule doublets and then the axoneme proper begins with 9 peripheral microtubule doublets surrounding a central pair. Filaments of unknown composition and function connect the transition zone microtubules to the membrane and form structures termed as ciliary necklace and collarette in the interior and exterior face of the membrane respectively [82,83]. While the basal body lies entirely in the cytoplasm, transition zone and the neighboring part of the axoneme are within a balloon-like invagination of the plasma membrane, known as flagellar pocket. The flagellar pocket is the only site of endocytosis and exocytosis since it resides in a gap of the subpellicular array of microtubules that subtend the rest of cell just beneath the plasma membrane [84]. The flagellar pocket membrane is a specialized domain of the cell surface with unique proteome and a critical host-pathogen interface since it is required for uptake of trypanotoxic ApoL1 as well as growth factors such as host transferrin [84]. The axoneme exits the pocket at the flagellar pocket collar, a cytoskeletal structure with mostly unknown subunits that is required for flagellar pocket biogenesis [85]. Anterior to the collar, the flagellum is entirely in the extracellular space and its membrane has a composition distinct from the rest of the cell surface [86]. A notable example is the proteins along the flagellum attachment zone (FAZ), the specialized structures of flagellum and cell body that enable lateral attachment of the flagellum [87]. Apart from the opposing flagellar and cell body membranes and their respective proteins, FAZ in the cell body includes the cytoskeletal FAZ filament, a microtubule quartet with reverse orientation when compared to the rest of subpellicular microtubules and a specialized part of the endoplasmic reticulum, the FAZ endoplasmic reticulum (FER). We might not
fully understand the underlying mechanisms but FAZ is of paramount importance for the parasite since it dictates cell shape and size by transmitting morphogenetic information from the flagellum to the cell body [87].

**Flagellum structure and assembly**

The core of the trypanosome flagellum is the axoneme, a nanomachine that employs dynein motors to provide motility for the parasite [88]. The *T. brucei* axoneme is canonical with 9 peripheral microtubule doublets and one central pair (Fig. 3) [89,90]. The peripheral doublets consist of two distinct microtubules, called A and B. A is a complete microtubule comprising 13 protofilaments while B is incomplete with only 10 and is fused to the side of A. Microtubule doublets provide the scaffold for the assembly of outer arm and inner arm dyneins as well as dynein regulators such as the nexin-dynein regulatory complex (NDRC), radial spokes (RS), modifier of inner arms (MIA) complex and calmodulin and spoke associated (CSC) complex [91-93]. Axoneme architecture is conserved between eukaryotes and it exhibits a repeating unit of about 96nm with species-specific variations in the arrangement of the aforementioned protein complexes [94,95,98]. In *T. brucei*, the repeating unit contains 4 outer arm dyneins, 3 radial spokes as well as inner arm dyneins and NDRC of unknown stoichiometry [90]. MIA and CSC subunits are encoded in the *T. brucei* genome but a higher resolution axoneme structure is needed to observe them *in situ*. Of note, each outer arm dynein has two heavy chains and the central pair has fixed orientation relative to outer doublets [96,97].
Flagellum motility is generated by ATP-induced structural changes of dynein motors. Axonemal dyneins on the A tubule of peripheral doublets harness ATP energy to change conformation and reversibly attach and “walk” on the B tubule of the neighboring doublet [99,100]. Dynein activity should thus cause doublet sliding. However this is not the case since microtubule doublets are connected to each other by NDRC and dynein activity results in doublet bending [101]. Localized bending can be harnessed for productive flagellum beating only if thousand of dynein motors are regulated along the length and the circumference of the axoneme [102]. The explicit mechanisms for spatio-temporal regulation of axonemal dyneins are enigmatic but are thought to involve protein complexes that control dynein activity. The NDRC is possibly the best characterized such complex. NDRC is massive (>1MDa) and present in almost all motile axonemes where it functions as a reversible inhibitor of dyneins in concert with central pair and radial spokes [91,103]. The exact composition of NDRC is unknown with studies in Chlamydomonas suggesting at least 11 subunits [104-106]. Of these, some are broadly conserved while others are lineage specific. In T.brucei, NDRC consists of trypanin and component of motile flagella 70 (CMF70), along with homology-inferred candidate subunits such as CMF40, CMF46, CMF22 and trypanin-related protein (TRP) [107-109].

Similar to virtually all eukaryotic flagella, T.brucei axoneme assembly depends on IFT and live imaging of the process has provided mechanistic insights into this poorly understood phenomenon [110,111]. For example, there are two pools of IFT particles at the base of the flagellum with only one actively engaging in IFT. Moreover, two populations of anterograde IFT particles were observed, each one moving at a different
speed [112]. Notably, new flagellum assembles using the pre-existing flagellum as a cytotoxic template and a disorganized axoneme is observed at the flagellum tip during its biogenesis [113,114]. *T. brucei* also encodes all eight BBSome subunits and there is indirect evidence linking trypanosome BBSome proteins to flagellum assembly [115]. In addition to the axoneme, the *T. brucei* flagellum contains another cytoskeletal element called paraflagellar rod (PFR) found only in kinetoplastids and related organisms [116]. PFR is a massive lattice-like filament that emerges anterior to the flagellar pocket collar and runs alongside the axoneme connecting to doublets 4 and 7. PFR is formed by dozens of proteins, comprises 3 substructures that are distal, intermediate and proximal to the axoneme-PFR interface and is required for flagellar motility [117,118]. The mechanism via which PFR influences motility remains enigmatic but PFR has been proposed to act as a biomechanical spring that absorbs and transmits energy produced by flagellum beating [90]. Moreover, proteins predicted to operate in calcium or cyclic AMP–dependent pathways localize to the PFR. Given the importance of these molecules in regulating flagellar motility, it is an attractive hypothesis that PFR is a regulatory nexus for calcium and cyclic AMP signaling pathways [119]. Assembly of this extra-axonemal structure remains enigmatic but it depends on axoneme assembly and a specific kinesin while depletion of abundant PFR components such as PFR1, PFR2 and calmodulin leads to catastrophic effects on the entire PFR structure [117,120,121].
Flagellum functions

Parasite motility

Early studies of trypanosome motility indicated that motility is driven by a beat that starts at the flagellum tip and propagates towards the posterior end of the cell [122]. Uncommon among other cilia [123], tip-to-base flagellum beat is characteristic of *T. brucei* and related species and results into parasite propulsion with the flagellum tip leading. Of note, beat reversal is also observed and that results in tumbling and backwards parasite movement [124]. Regulatory mechanisms of flagellum beat remain elusive. An attractive hypothesis based on studies of the related *Crithidia* is that direction of flagellum beat is governed by calcium with tip-to-base occurring at low concentrations and base-to-tip at higher calcium concentrations [125]. A critical feature of *T. brucei* motility is the attachment of the flagellum to the cell body that allows the flagellar wave to be efficiently harnessed for parasite motility and causes the cell to rotate while it moves. Helical movement is thought to be advantageous in viscous environments [126] that constitute the *T. brucei* niche and was recently revisited using state of the art technology. High speed video microscopy of *T. brucei* motility has been interpreted in two distinct ways, giving rise to a plane-rotational and a bihelical mode of parasite motility. The plane-rotational model claims that the flagellum tip beat is planar yet it results in rotational cell motility due to flagellum attachment to cell body. Moreover, parasites rotate uniformly in a left hand helix when viewed towards the anterior end [127]. On the other hand, the bihelical model postulates that trypanosomes alternate between left hand and right hand rotations during cell movement [128]. It remains to be seen whether the different models mirror alternative modes of propulsion used by
*T. brucei* cells during its multifaceted life cycle. Such a discovery wouldn’t be unprecedented since it is now accepted that *T. brucei* motility is influenced by the environment. A clear example is that motility of long slender trypanosomes is tuned to the mammalian bloodstream since parasite velocity and propulsive movement are maximized in media with blood-like viscosity and in chambers with pillars that recapitulate blood cells on size and density [127]. Interestingly, the use of optical traps have recently revealed the biophysical properties of trypanosome motility and indicated that it has low power efficiency [129]. An explanation put forward is that the extra power is needed for immune evasion via the hydrodynamic flow antibody clearance. This intriguing model proposes that parasite fast propulsive motility is required during mammalian infection to sweep host antibodies bound on surface proteins towards the flagellar pocket in the posterior end of the cell [130]. *T. brucei* moves with the flagellum tip leading and this mechanism thus concentrates host antibodies in the endocytic organelle of the parasite where they can be taken up and destroyed. As a result, trypanosomes can survive in low antibody titers and escape lysis by complement and other host humoral responses. Despite compelling *in vitro* data, the hydrodynamic clearance model was recently challenged by the infection properties of a *T. brucei* motility mutant. In this study, a trypanosome outer arm dynein mutant with defective propulsive motility was able to mount a wildtype-like infection in a mouse model [131]. Additional experiments are thus needed to explore the significance of propulsive motility for immune evasion *in vivo*.

Propulsive motility is also thought to be essential for tissue penetration during mammalian infection. Trypanosomes are mostly in the bloodstream but they can also
reside in extravascular tissues. It is currently unclear whether tissue invasion provides benefits to parasites [72]. Nevertheless, it is presumed that presence in immuno-privileged spaces such as CNS could favor parasite persistence during infection. Invasion of CNS demarcates the lethal stage of sleeping sickness and limits therapeutic options since most drugs cannot cross the blood brain barrier (BBB) [132]. Two different routes for CNS invasion have been nominated. In the direct invasion model, trypanosomes penetrate tight junctions of the BBB at brain microvessels to enter the brain [133]. In the second model, parasites enter the cerebrospinal fluid at the choroid plexus and from there they eventually invade the brain [134]. The host-pathogen interplay during invasion is partially known and the advent of an in vitro BBB model promises to illuminate more mechanistic details [135].

Forward motility is finally thought to be required for the parasite's life cycle in the tsetse vector. Trypanosomes’ migration from the midgut to the salivary gland of the fly equals several tsetse body lengths and entails ordered movement through the peritrophic space to the proventriculus and then the salivary glands [79]. These events are critical since trypanosomes differentiate into metacyclic mammalian infectious forms only in the salivary glands [75]. Given the sheer distance and the tissue barriers trypanosomes have to negotiate, it comes as little surprise that active parasite motility is postulated to be essential for migration. Indeed, recent studies showed that dynein motility mutants are unable to progress and fulfill midgut exodus, precluding thus emergence of mammalian transmissible trypanosomes [136]. Moreover, social motility might be required for the initial steps of parasite migration [137]. In summary, propulsive
motility is absolutely essential for parasite development, transmission and pathogenesis.

Flagellum in cell morphogenesis and cell division

Original descriptions classified trypanosome morphotypes based on relative size and position of the flagellum. It is now clear that changes in flagellum are not byproducts of other processes but that flagellum biogenesis indeed governs cell morphogenesis and directs cell division [87,138].

Early in the *T. brucei* cell cycle, assembly of the new flagellum starts (Fig. 4). Strikingly, assembly path is determined by the existing flagellum in a dramatic example of “structural inheritance” [113]. In procyclics, this take place via the flagella connector, a mobile molecular cord between the tip of the new flagellum and the side of the old flagellum [139]. Composition and functional details of the flagella connector remain elusive. Interestingly, as a possible adaptation for immune evasion, bloodstream form parasites lack flagella connector. Instead, the tip of the new flagellum is sinked within a “groove” of the plasma membrane while following the path of the existing flagellum [140]. The flagellum controls segregation of other organelles too, with the best example being the disk-like mitochondrial DNA known as the kinetoplast. Kinetoplasts are physically connected to the basal body and thus trypanosomes employ basal body duplication and segregation to achieve faithful kinetoplast distribution in progeny cells [141]. Basal body segregation requires microtubules but how it is coordinated with assembly of the new flagellum remains unclear [142]. In addition to controlling organelle inheritance, flagellum biogenesis is directly linked to cell size and shape. This control
takes place by transmission of morphogenetic information from the flagellum to the cell body via the attached flagellum at the FAZ [143]. Indeed, axoneme assembly controls FAZ filament elongation and this in turn dictates cell length since cytokinesis ensues at the anterior end of the new FAZ filament [110,138,144,145]. Mutants thus with shorter FAZ filament are overall shorter cells. Moreover, cell lines with detached flagella are typically unable to build the FAZ filament and are inviable [146,147], although exceptions have been observed [148]. Combined, these studies suggest that axoneme assembly is coupled to FAZ filament assembly via the FAZ junctions while the FAZ filament governs cell shape and size by dictating the cytokinesis plane [149,150]. In summary, trypanosomes, in a rather unique manner among eukaryotes, rely heavily on the flagellum for cell morphogenesis and cell division which becomes thus a critical regulator of parasite development, transmission and pathogenesis.

**Flagellum in host-pathogen interactions**

The *T. brucei* flagellum has emerged as a crucial host-parasite interface (Fig. 5). In this capacity the flagellum boasts a plethora of virulence factors and exhibits direct interactions with host as well as parasite structures [27]. This comes as no surprise since eukaryotic flagella typically constitute a nexus for signaling and sensory pathways and integrate many cellular responses to external stimuli [23-26]. While the assignment of important functions to a tiny hair-like organelle like the flagellum might be counterintuitive, it is thought to have occurred due to the greater sensitivity and control that the flagellum compartment provides and *T. brucei* is no exception.
A classic example of *T. brucei* host-pathogen interactions is the flagellum-mediated attachment of the parasite to the tsetse fly salivary gland. Attachment occurs between extensive flagellar membrane outgrowths that interlock with tsetse epithelial microvilli and form intimate contacts [75,76]. Parasite and host factors that participate in the attachment as well as the importance of attachment for the parasite’s life cycle remain a mystery. An attractive hypothesis is that attachment triggers signaling systems within the flagellum to initiate differentiation to mammalian infectious forms [151] or to modulate tsetse fly feeding behavior and thus promote trypanosome transmission to a mammal [152].

Strikingly, the *T. brucei* flagellum also mediates trypanosome cell-cell interactions during the sexual cycle of the parasite. Mating takes place in the salivary gland of the tsetse between specialized gamete-like cells that engage in flagellum-flagellum interactions prior to fusion [153]. Flagellum adherence prior to mating is also observed in other flagellated protists such as *Chlamydomonas* [154]. It remains to be seen whether flagellar homotypic interactions serve only to physically stabilize moving cells before mating or if they initiate a signaling cascade that culminates into fusion and genetic exchange. Finally, a plethora of virulence factors localize to the trypanosome flagellum [155-157]. Examples include membrane associated proteins with enigmatic functions such as calflagins, GPI-PLC and metacaspase 4 as well as the adenylate cyclase ESAG4. The latter converts ATP to cAMP upon phagocytosis by host macrophages. The rise of cAMP will eventually activate host protein kinase A which in turn will inhibit production of the trypanotoxic tumor necrosis factor (TNF) by myeloid...
cells [158]. These events promote thus group fitness and allow trypanosomes to thwart early innate host immunity.

More broadly, the *T. brucei* flagellum has emerged as an excellent model to study structure and function of eukaryotic flagella. The trypanosome flagellum is one of the best characterized eukaryotic cilia and has provided valuable insights into conserved and parasite specific features of flagellum biology [159,160]. Importantly, the existence of a powerful molecular toolbox promises to maintain *T. brucei* as the model of choice for fundamental questions regarding flagellar assembly and function [161,162]. With the importance of cilia in mammalian physiology being firmly established, studies on *T. brucei* could spur the next paradigm shift, namely the contribution of cilia to the biology of the panoply of flagellated human pathogens [27].

The overarching aim of this thesis has been to illuminate motility and sensory functions of the *T. brucei* flagellum. Flagellar motility is a complex phenomenon orchestrated by an elaborate biological machine; I thus focused on NDRC, a critical regulator of dynein motors that is a universal feature of motile axonemes, as described in Chapter 2. The importance of NDRC has been demonstrated in humans, green algae and trypanosomes, its exact composition however remains an enigma. NDRC is tightly associated with the axoneme and that makes biochemical purification and subsequent protein identification challenging. As an alternative approach we decided to take advantage of the fact that characterized NDRC mutants assemble defective NDRC where more than the cognate proteins are missing. To this end, I employed quantitative proteomics to disclose proteins that are less abundant in axonemes of known and candidate NDRC *T. brucei* mutants. This approach revealed around a dozen proteins
that are consistently depleted in NDRC mutant axonemes. Most of these candidate NDRC subunits have clear orthologs in other ciliated eukaryotes and have been linked to NDRC in other organisms. Interestingly, we detected four novel proteins that haven’t been associated with NDRC previously with three of them being kinetoplastid-specific. Intriguingly, many of the *T. brucei* NDRC candidates exhibit calcium-related domains, providing further support that NDRC is a calcium-responsive hub that integrates mechano-chemical signals to coordinate robust dynein activity.

Sensory stimuli are generally perceived by flagellum membrane proteins at the interface of the cell and the external milieu. The protein composition of the *T. brucei* flagellum membrane was mostly unknown so we undertook a proteomic analysis to characterize the organelle’s membrane and matrix proteome. I then validated few of the candidate proteins with the most interesting one being FS179, a putative calcium channel, detailed in Chapter 3. Indeed, FS179 localizes to the flagellar membrane along the FAZ in both bloodstream form and procyclic parasites. Surprisingly, FS179 is required for flagellum attachment and its depletion is lethal for *T. brucei*. These findings shed light on the enigmatic trypanosome FAZ and provide an attractive drug target for fighting sleeping sickness.

In addition to the candidate approach, we opted to disrupt trafficking mechanisms that control protein targeting and localization to the flagellum. Since depletion of IFT is lethal in *T. brucei*, we instead chose to ablate BBSome proteins, components of a conserved complex that enters the cilium and controls the dynamic localization of flagellar membrane proteins, as described in Chapter 4. To our surprise, we detected *T. brucei* BBSome proteins only at the base of the flagellum, in association with the
flagellar pocket membrane and neighboring vesicles. As judged by biochemical characterization, *T. brucei* BBSome proteins are indeed part of a complex with properties reminiscent of BBSome from other species. Furthermore, study of genetic knockouts suggests that BBSome is required for endocytosis of certain membrane proteins while being dispensable for flagellum assembly and parasite viability. Of note, BBSome mutants are also attenuated in a mouse model of infection, demonstrating the importance of membrane trafficking for virulence in this divergent eukaryotic pathogen. Our results thus illuminated a novel function that could provide a novel paradigm for BBSome function.
Figure 1. *T. brucei* life cycle. (A) Parasites infect mammals via the bite of a tsetse fly. Long slender form proliferates in bloodstream and can also invade the central nervous system (CNS). Short stumpy form is produced at high parasitemias and is pre-adapted for transmission to tsetse fly. There it will differentiate into procyclic form and establish
infection in the midgut. After a long journey through tsetse tissues and an asymmetric division, short epimastigotes arrive to the salivary gland and attach to the epithelium. There, they will differentiate into mammalian infectious forms, termed metacyclics. (B) Detailed view of the trypanosome migration in the tsetse fly. Parasites start at the midgut and end up in the salivary gland. Flagellar motility is required at least for midgut exodus. Metacyclics of salivary gland get released into the mammalian host upon a bloodmeal. (C) Trypanosomes can cross the blood brain barrier presumably relying on their flagellar motility. Reproduced with permission from [27].
Figure 2. Overview of the *T. brucei* flagellum. Reproduced with permission from [27].
Figure 3. Structure of the *T. brucei* flagellum. The upper panel depicts the axoneme repeating unit at longitudinal view. The lower panel depicts a cross-section of the
flagellum skeleton containing the axoneme and PFR. Reproduced with permission from [27].

Figure 4. Overview of *T. brucei* cell cycle. Emphasis is given on the flagellum and its morphogenetic roles. Upper panel: symmetric cell division for procyclic cells. Lower panel: asymmetric cell division of mesocyclics. Reproduced with permission from [27].
Figure 5. The *T. brucei* flagellum as a host-pathogen interface. Reproduced with permission from [27].
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Chapter 2

On the composition of the *T. brucei* NDRC
Summary

In this chapter I describe approaches to elucidate the subunit composition of the nexin dynein regulatory complex (NDRC). NDRC is a phylogenetically conserved, megadalton axonemal complex that is crucial for dynein regulation and hence productive flagellar motility. Activity of NDRC is critical for organismal physiology as shown by studies in trypanosomes, green algae and humans but the exact mechanism by which NDRC acts remains elusive. To this end, we sought to decipher the protein composition of T. brucei NDRC using quantitative proteomics of NDRC mutants. Our results indicate a broadly conserved NDRC core that incorporates lineage specific proteins to perform calcium-mediated regulation of axonemal motility.

Introduction

Eukaryotic flagella, also known as cilia, are microtubule-based organelles on the cell surface that protrude into the extracellular environment [1]. Cilia are widely conserved among eukaryotes and have a plethora of motility and sensory roles [2,3]. These roles are crucial since defects in cilia have dire effects on cellular and organismal physiology. For instance, defective ciliary motility in mammals can lead to hydrocephalus, male infertility, chronic respiratory disease and situs inversus [4]. Motile cilia typically harbor the iconic 9+2 axoneme, although exceptions do exist with 9+0 architecture [5]. Motile 9+2 axonemes contain nine peripheral microtubule doublets that comprise a partial B tubule connected to a whole A tubule. These outer doublets encircle a central pair of microtubules and are defined by the presence of dynein motors [6]. Axonemal dyneins are organized in two distinct complexes, namely outer dynein arms (ODA) and inner
dynein arms (IDA), which generate the force required for cilium beating. Both dynein arms are assembled on the A tubule and reversibly attach on the B tubule of the neighboring doublet; ODAs are thought to dictate the frequency of the beat while IDAs control the waveform [7,8]. Dyneins are minus end motors and thus “walk” on the B tubule surface towards the base of the axoneme. Dynein movement is a well orchestrated series of conformational changes that are controlled by ATP binding and hydrolysis which should eventually result in microtubule sliding [9,10]. This is not the case though since outer doublets are held in place by inter-doublet nexin links that convert dynein walk into axonemal bending [11,12]. Bending can be converted to productive flagellum beating if regulated in time and space along the length of the axoneme. How this is achieved remains elusive since it requires spatiotemporal coordination of thousands of dyneins; three theoretical models have been offered as holistic explanations of axonemal dynein regulation [13,14]. The sliding control model and the curvature control model propose that dynein activity control results from the linear arrangement of dyneins and flagellum bending respectively. The third model invokes mechanical and chemical signals from a series of axonemal complexes to govern dynein action [15-17]. These complexes include central pair (CP) projections, radial spokes (RS), the calmodulin spoke complex (CSC), the modifier of inner arms (MIA) complex and the nexin dynein regulatory complex (NDRC) [18-21]. NDRC was initially described as dynein regulatory complex (DRC) till the realization that it is the same entity as nexin links and thus renamed NDRC [22-26]. Indeed, high resolution tomography of *Chlamydomonas* axonemes demonstrated that DRC mutants lack nexin links. NDRC is thus the conspicuous structure that spans the distance between A and B
tubules of neighboring doublets and is estimated to amass approximately 1.5 MDa [22,44]. NDRC genes were originally characterized in Chlamydomonas mutant screens as extragenic suppressors of central pair and radial spokes mutants [26]. CP and RS null mutants have paralyzed flagella and mutations in NDRC genes restored flagellar motility to some degree. These studies were later combined with protein profiling in two-dimensional gel electrophoresis to detect seven proteins that were missing in NDRC mutants. The identity of these proteins was revealed decades later with the advent of the Chlamydomonas genomic sequence [27]. Recently, immunoprecipitation of known NDRC subunits coupled with quantitative proteomics identified four more NDRC subunits while other studies in Chlamydomonas and mammals suggest few more candidate subunits [28-31]. Thus far, the Chlamydomonas NDRC composition indicates the presence of core subunits that are broadly conserved among eukaryotes with motile cilia as well as lineage specific proteins [28]. Intriguingly, calcium-responsive domains are well-represented in NDRC subunits providing thus a mechanistic link between calcium-mediated regulation of flagellum motility and axonemal structures [14]. Despite all the insights from studies in Chlamydomonas, the exact NDRC composition and mode of action remain elusive. Illuminating NDRC subunits’ identity is thus an important first step in efforts to characterize the mechanism of action of this critical dynein regulator. Indeed, mutations in human NDRC proteins lead to inherited disorders such as primary ciliary dyskinesia [29-31]. This ciliopathy has pleiotropic symptoms that include respiratory infections, hearing loss and male infertility and arise due to defective ciliary motility in different tissues. NDRC has also critical functions in the causative agent of sleeping sickness, Trypanosoma brucei [32]. The T. brucei NDRC has partially
been characterized with known subunits being trypanin, trypanin-related protein, 
component of motile flagella 70 (CMF70) and CMF22 [33-35]. NDRC action is pivotal for 
the parasite since it is required for normal flagellum beat and hence propulsive motility. 
Interestingly, depletion of NDRC proteins results in cell death in bloodstream form 
parasites, consistent with knockdown phenotypes of other axonemal proteins in this life 
cycle stage [36]. NDRC has been notoriously difficult to purify biochemically so we 
sought to determine the NDRC composition in *T. brucei* using stable isotope labeling by 
amino acids in cell culture (SILAC) quantitative proteomics [36]. To this end, we 
compared abundance of axonemal proteins between wildtype and NDRC mutants. Our 
results are consistent with the predicted NDRC composition since our top hits were the 
conserved NDRC subunits previously characterized as NDRC 1-5 and NDRC 7-11 in 
*Chlamydomonas*. Moreover, four novel proteins were consistently less abundant in 
NDRC mutants and thus comprise new NDRC candidate subunits. Three of these 
proteins are trypanosome-specific, providing further support to the idea that lineage-
specific proteins are recruited to a conserved NDRC core. Further, the widespread 
presence of calcium-responsive elements in the putative subunits suggests that NDRC 
is a central and ancient hub of dynein regulation by virtue that links a classic secondary 
messenger to flagellar motility.

**Results**

Genetic and biochemical studies in *Chlamydomonas* flagella have indicated the 
existence of at least eleven NDRC subunits (Table 1). Ten of these proteins have clear 
orthologs in *T. brucei* and humans and are therefore prime candidates for NDRC
components. To illuminate the trypanosome NDRC, we decided to focus on DRC1, a critical factor for NDRC assembly. Despite being known for decades as a gene product that is part of NDRC, DRC1 actual protein sequence was only recently uncovered [29]. Combined structural and biochemical data suggest that DRC1 localizes to the base of NDRC and that it’s essential for assembly of a major part of the complex [22].

The *T. brucei* DRC1 ortholog was unexplored so I decided to characterize it and take advantage of its importance for NDRC assembly in order to shed light on trypanosome NDRC. Initially we tagged DRC1 at the C-terminus with either 3xHA or His-Biotin acceptor-His (HBH) epitopes. The resulting TbDRC1-HA fusion protein has the expected size (around 80 kDa) and localizes as expected to the trypanosome axoneme as judged by biochemical fractionation and immunofluorescence (Fig. 2). Further, TbDRC1 is required for normal flagellar motility. Depletion of TbDRC1 results in slight growth defect and an increased sedimentation rate (Fig. 3), both results being consistent with a role in flagellar motility [37]. Finally, we showed that TbDRC1 is required for stable association of trypanin with the axoneme since upon TbDRC1 depletion, trypanin becomes soluble at high salt extractions (Fig. 4). Moreover, trypanin is recovered in the bound fraction of TbDRC1-HBH immunoprecipitation, indicating that trypanin and TbDRC1 physically interact, consistent with the axonemal localization of TbDRC1-HBH (Fig. 4). Immunoprecipitation of TbDRC1-HBH was performed using NaI [29] and that prompted us to optimize this extraction and couple it to proteomics approaches to uncover novel NDRC subunits. Consistent with *Chlamydomonas* data, we begin to see efficient TbDRC1-HA extraction at 0.4M NaI while 0.6M extracts more of TbNDRC but the complex seems to disintegrate, at least partially (Fig 6). We opted
for 0.45-0.5M NaI as an acceptable tradeoff between extraction efficiency and integrity of the complex (Fig 6). The TbDRC1-containing entity we detect in BN-PAGE is likely the actual TbNDRC since known NDRC subunits are found in particles of similar size (Fig 7). We then used NaI-extracted TbDRC1 for immunoprecipitation followed by shotgun proteomics. In preliminary data we could detect most predicted NDRC subunits and a few novel putative ones (Table 2), suggesting that this approach could yield valuable insights into TbNDRC composition. More specifically, we could detect most predicted TbDRC subunits, namely DRC1, 2, 3, 4, 5, 7, 11 within the top 20 hits by number of spectra. Strikingly, seven TbDRC proteins (DRC1, 2, 3, 7, 11 and two TbDRC4 paralogs) were among the top nine hits. Of the other recovered hits, I note the orthologs of *Chlamydomonas* FAP61, FAP91, FAP251 and FAP264. The first three (FAP61, 91, 251) were recently found to be subunits of the calmodulin spoke complex (CSC) in *Chlamydomonas* axonemes [21]. CSC is located at the base of NDRC, in close proximity to the predicted localization of DRC1. On the other hand, FAP264 hasn’t been linked to axonemal complexes before and thus represents a novel NDRC candidate subunit.

We then turned to quantitative SILAC proteomics to further characterize the TbNDRC components. SILAC is a powerful approach that allows detection of even small changes in protein abundance between samples. We reasoned that we could use this approach and compare axonemal protein profiles of NDRC mutants to wildtype. Given the fact that NDRC mutants assemble a compromised and/or partial complex, this approach could disclose novel NDRC subunits. We chose to study DRC1, CMF70 (DRC2) and trypanin (DRC4) RNAi mutants since they are all predicted to result in
severe defects in NDRC assembly. We isolated axonemes and performed extractions either with 1M salt (high salt) or 150mM salt (low salt). In wildtype axonemes, both treatments don’t disrupt the NDRC which remains insoluble and attached to the outer doublets. In total we did three SILAC comparisons, two of high salt and one of low salt extractions. Intriguingly, the least abundant proteins in NDRC mutants consistently are predicted NDRC proteins, validating thus our approach. Moreover, four uncharacterized proteins are consistently less abundant in these mutants, rendering them novel NDRC candidates (Table 6). Notably, two of these novel candidates are kinetoplastid-specific, highlighting thus the lineage-specific architecture of NDRC.

Discussion
Motility is the archetypical function of eukaryotic flagella. Powered by axonemal dyneins, flagellar motility is required for extracellular liquid movement as well as cell propulsion [38]. These functions are indispensable for human development and physiology since aberrant flagellar motility results in diseases with diverse clinical manifestations. Collectively called ciliopathies, these syndromes have revealed the ubiquitous presence of cilia in human tissues and provided hints at ciliary functions [3]. For example, defective ciliary motility is the underlying cause of primary ciliary dyskinesia (PCD) with symptoms ranging from abnormal mucociliary clearance in lungs till organ patterning defects such as situs inversus [4]. Respiratory cilia are responsible for the former while situs inversus is caused by deficient embryonic nodal flow controlled by nodal cilia. Moreover, flagellar motility is essential for fertilization since male gametes rely on their single flagellum for translocation while female fallopian tubes
are also lined with cilia that mediate cell and liquid propulsion. Further, defects in ependymal flow in the brain ventricles causes hydrocephaly [39]. In addition to mammalian physiology, flagellar motility is crucial for a vast array of other species that rely on flagella to feed, mate and move. Examples include sponges and other basal animals as well as phylogenetically diverse microbes [40-43]. Studies of microbial flagella belonging to the green algae Chlamydomonas and the kinetoplastid parasite T. brucei have long proven valuable in understanding the mechanisms of flagellar motility across eukaryotes. A classic example was the discovery and characterization of NDRC in Chlamydomonas. NDRC was initially thought to be two discrete entities, the nexin links and DRC. Nexin links were the conspicuous bridge-like structures, observed in micrographs, which connect adjacent outer microtubule doublets [44]. Nexin links were thought to inhibit doublet sliding during axonemal dynein activity yet their composition was a total mystery [11]. On the other hand, DRC was defined as suppressor extragenic mutations that rescued paralyzed flagella of central pair and radial spoke mutants. These mutations were mapped to seven genes that function as reversible inhibitors of axonemal dyneins, prompting their naming as subunits of the dynein regulatory complex [23-26]. The realization that DRC and nexin links are the same entity came only recently with the completion of a high resolution structure of Chlamydomonas axoneme. It is now evident that DRC is the only structure that spans the gap between outer doublets and thus corresponds to the nexin link, hence NDRC [22]. NDRC is located at an ideal position and has extensive links to other axonemal complexes such as radial spokes and dynein arms. Genetic and biochemical evidence suggests that NDRC operates in the same pathway as central pair complex and radial spoke to control
dynein activity, by specifically functioning as a reversible dynein inhibitor [28]. The importance of NDRC in axonemal motility is highlighted by its broad conservation among eukaryotic lineages. Indeed, NDRC is even present in organisms missing other important modules of axonemal motility such as *Thalassiosira* and *Physcomitrella* which lack CP, RS, IDA and ODA respectively [45]. NDRC is thus a critical regulator of axonemal motility, encoded by virtually all eukaryotes with motile cilia. However, the exact mechanism of NDRC in the context of flagellar motility remains arcane. As a first step to elucidate the mechanistic details of NDRC we sought to characterize its composition in *T. brucei*. At the beginning of our studies, the only known NDRC subunits in any eukaryote were DRC2 and DRC4. We thus decided to study TbCMF9, TbCMF76 and TbCMF76b, three related proteins that their knockdown results in the *T. brucei* axoneme losing its 9+2 arrangement [37]. The resulting outer doublet spacing defects were suggestive of aberrant nexin links and thus made these conserved proteins prime candidates for NDRC components. We aimed to determine the axonemal localization of TbCMF9,76,76b at high resolution using immuno cryo-electron tomography (cryoET) since this had never been performed previously for NDRC proteins. Of note, *Chlamydomonas* NDRC proteins have been indirectly localized to subdomains of NDRC by correlating absent NDRC sub-structures with missing proteins in NDRC mutants. To this end, we epitope tagged TbCMF9,76,76b and verified their axonemal localization (Sup. Fig.1), however immuno-cryoET wasn’t successful (data not shown). We attribute this failure to antibody inability to reach the cognate epitope due to the densely packed nature of axonemal complexes. Due to the insoluble nature of NDRC, we then attempted to purify it from a cytoplasmic, soluble state. We reasoned
that NDRC could behave like other megadalton axonemal complexes such as ODA that get assembled in the cytoplasm and then travel with IFT particles in the flagellum where they get incorporated in the axoneme. At steady state, NDRC is not present in biochemically relevant amounts in a soluble state so we opted to increase this by depleting IFT and inducing its accumulation at the base of the flagellum. We thus performed RNAi against IFT88, a conserved anterograde IFT-B protein and checked DRC4 levels in detergent soluble fractions (Sup. Fig.2). We didn’t observe a dramatic increase in the levels of soluble DRC4 and didn’t pursue this strategy further, although we might revisit this approach targeting different IFT proteins for knockdown that don’t result in cell death as fast as IFT88 [46]. It would be worthwhile also to check the size of the complex, if any, that DRC4 is part of by blue native electrophoresis. Finally, we undertook another candidate approach, this time studying the *T. brucei* orthologs of the mammalian CCDC39 and CCDD40 proteins. These broadly conserved coiled coil domain proteins were recently proposed to comprise novel NDRC subunits since their absence results in defective assembly of NDRC and IDA in mammals [30,31]. We used epitope tagged versions of TbCCDC39 and TbCCDC40 to show that they fractionate with the flagellum skeleton (Sup Fig 3). Interestingly, RNAi depletion of TbCCDC39 results in major morphogenetic defects and is lethal yet we didn’t observe DRC4 losing its axonomal association as seen in mammals. Moreover, TbCCDC39 and TbCCDC40 weren’t reduced in axonemes of DRC mutants as judged by SILAC proteomics and they didn’t co-immunoprecipitate with DRC1. I propose that TbCCDC39 and TbCCDC40 localize to the unknown densities of NDRC which assemble independent of DRC1, DRC2 and DRC4 [22]. Alternatively, TbCCDC39 and TbCCDC40 may localize to
different axonemal structures and their absence has a catastrophic and pleiotropic effect on axoneme biogenesis including defective NDRC assembly.

Pioneering studies in Chlamydomonas have revealed the presence of eleven NDRC subunits, termed DRC1-11 (Table 1) [27-29]. Five more proteins (FAP61, FAP206, FAP230, FAP252, spot 11) are considered putative NDRC components, with FAP261 however recently identified as member of CSC, a protein complex adjacent to NDRC [21]. Interestingly, DRC6 as well as FAP206, FAP230, FAP252 are not conserved outside green algae or related organisms suggesting that NDRC has lineage specific additions to a broadly conserved core. T. brucei encodes all the conserved NDRC subunits (Table 1); we reasoned that studying TbDRC1 would lead to a better characterization of TbNDRC composition since DRC1 is crucial for assembly of a large NDRC substructure and is complementary to the DRC4-mediated NDRC substructure assembly [22]. We first verified that the predicted TbDRC1 is indeed a NDRC subunit. We achieved that by showing that TbDRC1 is axonemal (Fig. 2), is required for flagellar motility (Fig. 3), interacts with trypanin-DRC4 and is critical for DRC4 association with the axoneme (Fig 4). We then used SILAC proteomics to uncover differences in axonemal protein abundance between wildtype and DRC mutants using DRC1, DRC2 and DRC4 RNAi knockdowns for a comprehensive approach. We performed three rounds of SILAC with the first two using high salt extraction and the third one being a low salt. High salt extraction of the axoneme is known to solubilize dyneins yet NDRC is left intact. We thus performed high salt extraction to make the sample proteomic analysis less complex and disrupt unstable mutant NDRC assemblies. Low salt extraction was performed as a control to exclude the scenario that there are NDRC
subunits sensitive to high salt extractions. Our combined results argue against that scenario and instead favor high salt extractions that result in less complex samples and more striking differences in levels of NDRC subunits. Indeed, TbDRC proteins were consistently among the least abundant ones in axonemes of NDRC mutants. DRC1 and DRC2 mutants gave a similar pattern that was distinct from the DRC4 mutant, consistent with results from *Chlamydomonas* that predict DRC1 and DRC2 in close approximation at the base of NDRC while DRC4 localizes to a different substructure. Our SILAC results further support the assignment of TbDRC8, TbDRC9 and TbDRC10 since for these three proteins sequence similarities to other organisms were relatively low and could lead to erroneous annotations. Further, SILAC data suggest that both of TbDRC4 paralogs, trypanin (TPN) and trypanin-related protein (TRP), are part of NDRC. It is possible that TPN and TRP are subunits of different NDRC particles, in a scenario that postulates heterogeneity in NDRC composition. However, taking into account that TPN and TRP depend on each other for protein stability (Hill, unpublished data), I deem as most likely that TbNDRC composition is homogeneous and contains both TbDRC4 paralogs.

Perhaps the most important finding of our SILAC approach was the identification of four proteins that followed the pattern of known NDRC subunits (Table 6). These proteins (SILAC candidates TbDRC-SC 1-4) are thus bona fide NDRC candidate subunits with TbDRC-SC2 and TbDRC-SC3 being lineage specific; TbDRC-SC3 harbors a putative calcium binding EF-hand domain. TbDRC-SC1 and TbDRC-SC4 contain leucine-rich repeats (LRR) and have orthologs in other species. It is possible that TbDRC-SC1 and TbDRC-SC4 are divergent paralogs since TbDRC-SC4
sequence similarity to other species is restricted to the LLR. Interestingly, the 
*Chlamydomonas* ortholog to TbDRC-SC1 is FAP264, a predicted axonemal protein that
is broadly conserved in eukaryotes with motile cilia [45]. Our SILAC data are further
supported by TbDRC1-interacting proteins as detected in TbDRC1 immunoprecipitation
(Table 2) where the top hits are other TbDRC proteins. Moreover, we epitope tagged
the predicted TbDRC proteins as well the four SILAC candidates and verified axonemal
localization by immunofluorescence for most of them (Fig 7). The TbNDRC composition
is consistent with the *Chlamydomonas* one. Most NDRC subunits don’t exhibit
informative domains and they are characterized by protein-protein interaction modules
such as coiled coils (DRC1, DRC2, DRC7) and leucine rich repeats (DRC3, DRC5,
DRC-SC1, DRC-SC4). The presence however of proteins with calcium-related signaling
domains (DRC8, DRC9, DRC10, DRC11, TbDRC-SC3) advocates that NDRC is a
crucial hub that integrates calcium signals to control dyneins and therefore axonemal
motility.

In summary, we coupled quantitative proteomics and biochemical purification to
illuminate the composition of *T. brucei* NDRC (Table 7). Our results indicate that broadly
and highly conserved proteins (such as DRC1, DRC2, DRC3, DRC4, DRC5, DRC7,
DRC11, TbDRC-SC1) form the NDRC core while proteins like DRC9 and DRC10 can
fluctuate in sequence space with only domains being conserved. Moreover, NDRC
seems to be amenable to lineage-specific additions (TbDRC-SC2, TbDRC-SC3 as well
as DRC6, FAP206, FAP230, FAP252 in *Chlamydomonas*) that could theoretically fine
tune the characteristics of flagellum beat. Our studies thus constitute an important
advance in understanding a critical regulator of axonemal functions, demonstrate at the
molecular level the importance of calcium in flagellar motility and pave the way for mechanistic insights into the significance of ciliary motility in human health and disease as well as the life cycle of flagellated human pathogens.

**Materials and methods**

*T. brucei* cell lines. Cultivation, transfection and RNAi induction of trypanosomes in culture were done as described previously [47]. Parasites were maintained in SM medium supplemented with appropriate selection drugs. Epitope tag constructs were created by cloning parts of ORF and 3'UTR in suitable vectors using Kpn-Xho and Bam-Sac1 sites respectively [48]. RNAi constructs were created by cloning ORF or UTR segments in suitable vectors using Xba and HindIII. To amplify DRC1 ORF and UTR segments for 3xHA tagging we used gatggtaccGAAACAACAGGCACCGTTTATTGC (OFR-For), gatctcgagGGTATGGGCACGGTTTCCA (OFR-Rev), gatggatccGGTTAACTGGACAGTGCCATGC (UTR-For), gatgagctcCACACGAAACGTGTTAACATGCG (UTR-Rev), respectively. DRC1-HBH (His-Biotin acceptor domain-His) was created by subcloning HBH into DRC1-HA plasmid using Gibson assembly and amplicon of gttggaaaccctgccccataacctcgagAGCGGCAGCCAGCCAGCCAGCCAG (HBH-For) and aatcaggacatcgttaagggtacctcGATGGATGATGTTGGTGTAACGCG (HBH-Rev). To amplify DRC1 ORF for RNAi knockdown we used aacatctagaCGTTTCCCGCTACGCAGGCAGGCAGGCAGGCAGGCAGGCAG (OFR-For), and acataagcttCGTCCTACTAACGGCGCTTCT (OFR-Rev). DRC1-HA-RNAi line was created by transfecting RNAi construct into DRC1-HA line. To amplify CMF9 ORF and
UTR for creating CMF9-HA line we used AGGACCTCAAGCGGCAGGCCA (ORF-For),
CGACCTTAATGCTCCTCTGAACGCTTCA (ORF-Rev), GCATCGGTACGGAGGGACGC
(UTR-For), CAGCGATCAGCAGGGCCGGCAA (UTR-Rev). To amplify CMF76 ORF and
UTR for creating CMF76-HA line we used GCCAAAAAGACGAGGGAGGATGCG (ORF-
For), CTTACCCTCCTCTCCCGGTGTCGATC (ORF-Rev),
GCACACTACTGTGAAGGAGGATGATTCTC (UTR-For),
CTACTCCGACGAGGCACCGA (UTR-Rev). To amplify CMF76b ORF and UTR for
creating CMF76b-HA line we used AACTTCAAGCCGAGGGATGCG (ORF-For),
cctgaggggccccggtaccCGAGTTAGCGCAAAACCACCC (ORF-Rev),
ATCcactagttttagccgccccgcaCTTTTGCCTCTGCTTCTTTACATCGTCG (UTR-For),
AGAATGGCCGCCTACGCAGAGA (UTR-Rev). To amplify TbCCDC39 ORF and UTR for
creating TbCCDC39-HA line we used gatggattaccCgcgtgacg (ORF-For),
gatgtcagtttcataactacaaaaagactggccagc (ORF-Rev),
Gatgtcagtttcataactacaaaaagactggccagc (UTR-For), gatggagcgtgacgtgcgaatgacgacgtgcg
(UTR-Rev). To amplify TbCCDC40 ORF and UTR for creating TbCCDC40-HA line we
used gatggattaccTGGGAGAGGAAAATGGCTTGGCT (ORF-For),
gatggcccccGTCAGAGATGATGCTATATCAGAAAG (ORF-Rev),
gatgtcagtttcataactacaaaaagactggccagc (UTR-For),
gatgtcagtttcataactacaaaaagactggccagc (UTR-Rev). To amplify TbCCDC39 ORF for
RNAi knockdown we used catctagaGCACTGAAAGATTGCACGATGACTAC (ORF-
For) and cataagcttATCTCCGCGAGAAGATCTGATTGTC (ORF-Rev).
**Sedimentation assay.** Sedimentation assay was performed as described previously [33]. Briefly, procyclic cells were washed with fresh medium and resuspended at 5\times10^6 cells/ml in 1ml aliquots in cuvettes. OD600 was measured every 2h for 8h in total. 3 samples were left undisturbed and 3 samples were resuspended prior to measurements to account for cell growth. The latter measurement was substracted from the former to get Delta OD600.

**Biochemical fractionations and immunofluorescence.**

A two-step fractionation was performed as described previously [34]. Briefly, cells were extracted with PEME buffer (100 mM piperazine-\(N,N'\)-bis(2-ethanesulfonic acid), pH 6.9, 2 mM EGTA, 0.1 mM EDTA, 1 mM MgSO4, protease inhibitors cocktail) containing 1% NP40. Pellet fraction was subsequently extracted in 1000 PMN buffer [10 mM NaPO4 pH 7.4, 1 mM MgCl2, 1000 mM NaCl]. Cell lysates and detergent fractionations were then analyzed by western blotting. Cytoskeleton immunofluorescence (IF) was performed by attaching cells on poly-lysine slides for 20 min, followed by extraction in PEME-1%NP40 for 10 min and methanol fixation 5 min at -20 degrees. Primary antibody dilutions were as follows: monoclonal anti-HA antibody (HA 11.1 Covance) 1:3000 for western blot and 1:100 for IF, monoclonal anti-Trypanin antibody (mAb 37.2) 1:5000, monoclonal anti-Tubulin antibody E7 supernatant 1:10000 (Hybridoma Bank, University of Iowa), anti-PFR2 1:1000. Blue native PAGE was performed according to manufacturer’s instructions (Invitrogen). Initial sample was NaI extracts of axonemal proteins. Axonemes were purified using a modified one step purification. Cells were extracted in 25 mM HEPES pH 7.4, 3 mM MgCl2, 0.5% NP40, protease inhibitor
cocktail, 150 mM NaCl and 1x Turbo DNase (Invitrogen) for 15 min at room temperature. CaCl2 was then added to final concentration of 1 mM and samples kept on ice to depolymerize subpellicular cytoskeleton. Axonemes were pelleted and extracted using 25 mM HEPES pH 7.4, 3 mM MgCl2, 0.5% NP40, protease inhibitor cocktail, 0.4-0.6 M NaI.

SILAC proteomics
*T. brucei* DRC mutants were grown in SM medium complemented with dialyzed serum (Gibco) and mixed prior to axoneme purification with wildtype cells grown in SM medium complemented with dialyzed serum and 13C arginine and lysine. DRC mutants were DRC1-HA-RNAi, DRC2-RNAi, DRC4-RNAi. Axonemes were purified using a modified one step purification. Cells were extracted in 25 mM HEPES pH 7.4, 3 mM MgCl2, 0.5% NP40, protease inhibitor cocktail, 150 mM NaCl and 1x Turbo DNase (Invitrogen) for 15 min at room temperature. CaCl2 was then added to final concentration of 1 mM and samples kept on ice to depolymerize subpellicular cytoskeleton. Axonemes were pelleted and extracted using 25 mM HEPES pH 7.4, 3 mM MgCl2, 0.5% NP40, protease inhibitor cocktail, 150 mM or 1000 mM NaCl. Pelleted axonemes were then subjected to trypsinazation and shotgun MudPIT proteomics.

**Immunoprecipitation**
Axonemes were purified using a one step purification. Cells were extracted in 25 mM HEPES pH 7.4, 3 mM MgCl2, 0.5% NP40, protease inhibitor cocktail, 150 mM NaCl and 1x Turbo DNase (Invitrogen) for 15 min at room temperature. CaCl2 was then added to
final concentration of 1 mM and samples kept on ice to depolymerize subpellicular
cytoskeleton. Axonemes were pelleted and extracted using 25 mM HEPES pH 7.4, 3
mM MgCl2, 0.5% NP40, protease inhibitor cocktail, 0.4-0.5 M NaI. NaI extracts were
dialyzed by column centrifugation and incubated with agarose resin to preclear for 1h at
4 degrees. Pre-cleared lysate was then incubated with streptavidin beads for 2h at 4
degrees, and washed extensively. Beads then were subjected to trypsinazation and
shotgun MudPIT proteomics.
Figure 1. NDRC axonemal localization in *Chlamydomonas reinhardtii*. NDRC is the main structure protruding from A tubule to neighboring B tubule. The putative localization of NDRC subunits is indicated. $A_t$ is alpha microtubule, $B_t$ is beta microtubule, RS is radial spoke, IDA is inner dynein arm, ODA is outer dynein arm. Densities that haven’t been assigned to proteins are labeled as unknown. Figure adapted with permission from [27]
Figure 2. DRC1 localizes to the axoneme. (A) Western blot on cell lysates to check expression of DRC1-HA (expected size is 80.3 kDa). WT lysate is negative control. Tubulin serves as loading control. (B) Immunofluorescence of *T. brucei* cytoskeletons shows axonemal localization for DRC1-HA. DCR1-HA is in green, PFR in red. Arrows point to proximal end of DRC1-HA staining. Parental line was used as negative control.
Figure 3. DRC1 is required for flagellar motility. (A) Western blot for DRC1-HA showing RNAi knockdown at the protein level. WT and DRC1-HA are negative and positive controls respectively. Equal loading was verified with tubulin blot. (B) Sedimentation assay for DRC1-His-Biotin acceptor-His (HBH) in single knockout (SKO) background. Wildtype (WT), DRC1-SKO and DRC-RNAi were used as controls.
Figure 4. DRC1 interacts with DRC4-trypanin and is required for trypanin association with the axoneme. (A) TbDRC1-HBH localizes to the axoneme. Parental line was used as negative control. (B) Trypanin is recovered in the bound fraction of DRC1-HBH immunoprecipitation. (C) In the absence of DRC1, Trypanin is less stably associated with the axoneme since it becomes soluble (S2) upon 1M NaCl treatment. Lysate is L, Supernatant 1 is S1, Pellet 1 is P1, Supernatant 2 is S2, Pellet 2 is P2. S1, P1, S2, P2 correspond to cytoplasmic, cytoskeleton, subpellicular cytoskeleton and flagellum skeleton proteins respectively.
Figure 5. NaI extraction of TbDRC proteins. TPN is trypanin, TRP is trypanin-related protein.
Figure 6. Optimization of NaI extraction. (A) DRC1-HA extraction with different concentrations of NaI for 5 and 15 min. (B) DRC1-HA extraction for 20 min with different concentrations of NaI. (C) DRC1-HA extraction with 0.45M NaI for different time periods.
Figure 7. Immunofluorescence for epitope tagged TbDRC proteins (A-L). PFR is flagellum marker. Arrows point to proximal end of DRC1-HA staining. Parental lines were used as negative controls for Immunofluorescence and didn’t show any staining (not shown). Figure continues on next page.
Supplemental Figure 1. (A) Western blot for epitope tagged TbCMF9, TbCMF76 and TbCMF76b. Wildtype (WT) serves as negative control. (B) Immunofluorescence for epitope tagged TbCMF9, TbCMF76 and TbCMF76b. Wildtype (WT) serves as negative control.
Supplemental Figure 2. Western blot of detergent extracted IFT88 RNAi cells. L is Lysate, S1 is Supernatant, P1 is Pellet after 1% NP40 treatment. Time (hours) of RNAi induction (0-72h) is indicated.
Supplemental Figure 3. (A) TbCCDRC39 and TbCCDC40 fractionate with the flagellum skeleton. Lysate is L, Supernatant 1 is S1, Pellet 1 is P1, Supernatant 2 is S2, Pellet 2 is P2. S1, P1, S2, P2 correspond to cytoplasmic, cytoskeleton, subpellicular cytoskeleton and flagellum skeleton proteins respectively. (B) Trypanin stays associated with the cytoskeleton during TbCCDRC39 RNAi, L is Lysate, S is Supernatant, P is Pellet of 1% NP40 extraction.
Table 1. Predicted NDRC subunits in *T. brucei* and *H. sapiens* based on homology to *Chlamydomonas* NDRC proteins.

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<th><em>C. reinhardtii</em></th>
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Table 2. Top 20 hits in DRC1-HBH immunoprecipitation. Proteins are in descending order by number of spectra. TbDRC proteins are highlighted.

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Table 3. The 20 least abundant proteins in DRC1-RNAi versus WT axonemes. TbDRC proteins are highlighted.

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Table 4. The 20 least abundant proteins in DRC2-RNAi versus WT axonemes. TbDRC proteins are highlighted.

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Table 5. The 20 least abundant proteins in DRC4-RNAi versus WT axonemes. TbDRC proteins are highlighted.

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<th>Tritryp ID</th>
<th>Ratio</th>
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Table 6. NDRC-SILAC candidates. Ratios correspond to two rounds of high salt and one round of low salt extractions. ND is not detected. NR is no ratio. Highlighted IDs indicates detection of protein in DRC1-HBH immunoprecipitation.

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<th>DRC1-RNAi ratios</th>
<th>DRC2-RNAi ratios</th>
<th>DRC4-RNAi ratios</th>
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Table 7. Putative NDRC subunits in *T. brucei*. IP refers to proteins that co-immunoprecipitate with DRC1. Axonemal localization refers to immunofluorescence data.

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References


Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M.


44. Proc Natl Acad Sci U S A. 1963 Nov;50:1002-10. STUDIES ON THE PROTEIN COMPONENTS OF CILIA FROM TETRAHYMENA PYRIFORMIS. GIBBONS IR.


Chapter 3

FS179 is required for flagellum attachment in *Trypanosoma brucei*
Summary
This chapter describes efforts to functionally characterize FS179, a putative calcium channel that was discovered in our bloodstream form *T. brucei* flagellum membrane proteome. Immunofluorescence in both bloodstream and procyclic parasites demonstrate that FS179 localizes to the flagellar membrane of the Flagellum Attachment Zone (FAZ). Interestingly, gene knockdown studies indicate that FS179 is required for flagellum attachment to the cell body and that upon its depletion, cells lose morpogenetic axes, fail to divide properly and die off eventually. I provide an updated model of FAZ proteins and their interactions and discuss possible roles for calcium influx in the flagellar FAZ.

Introduction
The cilium, equivalent to eukaryotic flagellum, is a fundamental and broadly conserved organelle. Although most widely known for motility, the cilium is now recognized as a major signaling center that senses and transduces a variety of chemical and mechanical signals [1-5]. Cilium motility and sensory functions are both essential and disruption of these activities causes a broad spectrum of diseases in vertebrates [6,7]. Cilia are also prominent among parasitic protozoa that affect more than 500 million people worldwide, including some of the poorest regions on the planet [8]. Studies of ciliation biology thus have the potential to directly impact understanding of fundamental biology as well as inherited and infectious human diseases.
*T. brucei* is a protozoan parasite that causes African trypanosomiasis, a devastating disease afflicting humans and other mammals in sub-Saharan Africa. The human disease, sleeping sickness, is one of the world’s most neglected [9,10]. *T. brucei* is transmitted by a tsetse fly vector and survives extracellularly at all stages of infection through morphological and physiological adaptations [11]. Vital to trypanosome motility, transmission and disease pathogenesis is the parasite’s single flagellum [12]. In the tsetse, parasite migration from the midgut to the salivary gland is required for differentiation into mammalian-infectious forms, which is initiated by attachment of the flagellum to the salivary gland epithelium [13]. In the human host, parasite penetration of the blood brain barrier is a critical and defining step in pathogenesis [14]. The flagellum also promotes immune evasion [15] and is suggested to mediate environmental sensing, although this intriguing idea remains to be tested [16]. Lastly, the *T. brucei* flagellum is a crucial morphogenetic nexus that dictates cell shape and size [17,18]. To fulfill this rather unique role among ciliated eukaryotes, the trypanosome flagellum controls assembly of flagellum-associated structures such as the flagellum attachment zone (FAZ) [19,20]. FAZ corresponds to structures that allow flagellum-cell body attachment and contains membrane as well as cytoskeletal proteins on both the flagellum and cell body side. Conspicuous among FAZ structures is the FAZ filament, a cytoskeletal structure in the cell body side of mostly unknown composition [21,22]. FAZ biogenesis is intrinsically linked to flagellum duplication which is one of the first recognizable events during the cell cycle. The new flagellum emanates from a nascent basal body and is built alongside the existing one with the flagellum tip extending towards the anterior pole of the cell [18]. Division occurs when the new
flagellum reaches a specific length. Early studies implicated the flagellum and the FAZ as critical regulators of the cytokinesis plane [19]. Recent studies have validated these observations and provided a mechanistic framework of how flagellum assembly governs cell morphology through the control of FAZ assembly. For instance, depletion of IFT proteins ablates axoneme biogenesis and results in a concomitant failure to assemble the FAZ filament and cell death eventually [20]. A similar inability to assemble the FAZ filament and cell death is observed upon knockdown of FAZ membrane or cytoskeletal proteins [21-26]. Moreover, knockdown of FAZ proteins typically results in flagellum being detached from the cell body while axoneme assembly is unperturbed. Flagellum attachment thus has been hypothesized to be essential for parasite viability. This was recently challenged by studies of Fla1BP, a flagellar membrane FAZ protein, depletion of which resulted in clear flagellum detachment yet no growth defect [27]. Interestingly, Fla1BP mutants’ cellular architecture is compromised with basal body losing the typical posterior to nucleus positioning. This morphogenetic defect has been observed in other FAZ mutants too [23]. Fla1BP mutants manage to assemble a short FAZ filament that is nevertheless longer than the ones assembled in inviable FAZ mutants [24]. In all cases examined so far, cell length correlates with FAZ filament length better than with flagellum length [22,24]. This result is put into context by the finding that Polo-like and Aurora B-like kinases dynamically localize to the anterior tip of the new FAZ filament and control cytokinesis [28,29]. Collectively, these studies suggest that axoneme assembly is linked to FAZ filament assembly via the membrane junctions of the FAZ. In turn, FAZ filament dictates cytokinesis plane and thus cell size and shape [12].
Despite the wealth of structural information on FAZ membrane junctions [30,31], relatively few proteins have been localized to this discrete cell surface domain [25-27]. We serendipitously discovered FS179 as a new member of this compartment while validating our flagellum surface proteome [32]. FS179 is a putative calcium channel that localizes to the flagellar side of the FAZ and is required for flagellum attachment and cell viability. Our studies thus offer a new target for therapeutic intervention and illuminate the molecular composition of an enigmatic yet essential trypanosome-specific cellular structure.

Results

Identification of FS179 in the *T. brucei* flagellar surface proteome

Given the importance of the flagellum membrane as a dominant host-parasite interface we sought to determine the trypanosome flagellar surface proteome. To this end, we applied membrane-impermeant reactive biotin to live trypanosomes that had undergone FLA1 RNAi knockdown [32]. FLA1 depletion results in flagellum detachment and renders flagellum purification possible. We then detergent extracted enriched flagella, isolated biotin fusions with streptavidin beads and identified proteins with shotgun proteomics. Our data reveal a dynamic proteome, well suited for host-pathogen interactions [32]. During validation, I focused on a putative calcium channel, FS179 (Tb927.10.2880 in Tritryp database). This massive protein consists of 2693aa and is predicted to be a voltage-gated calcium channel. Indeed, previous bioinformatic analysis has revealed that FS179 contains most of the crucial conserved residues in both the pore region and the voltage sensor (Figure 1) [33]. For example, FS179
contains a QESE motif in lieu of EEEE in the calcium selectivity pore (Fig 1B) as well as regularly spaced basic residues in the voltage sensor domain (Fig 1D) [33]. Although biochemical characterization of FS179 would be needed, primary sequence analysis predicts a calcium or non-selective cation channel. In order to validate the flagellum localization of FS179, I epitope tagged one allele *in situ* with triple hemagglutinin tag (HA) at the C-terminus of the protein [oberholzer plasmid paper]. I verified expression of the fusion protein with western blot (Fig. 2). FS179-HA runs as expected (approximately 300 kDa) and is entirely soluble upon treatment with 1% NP40, indicating it doesn’t associate with cytoskeleton (Fig. 2).

**FS179 is a novel member of the flagellar FAZ**

I next asked whether FS179 localizes to the flagellum. Using immunofluorescence assay (IFA), I showed that FS179-HA localizes to the FAZ (Fig 3). This localization was seen for both bloodstream form (BSF) and procyclic (PCF) parasites and is typical of FAZ proteins (Table 1) since it is along the attachment zone between flagellum and cell body but doesn’t extend to the flagellum tip. Flagellar and cell body membranes at the FAZ are in close apposition [31] and thus assigning an exact localization for FS179-HA was challenging. To overcome this, I created a FS179-HA in FLA1 RNAi line. FLA1 is another FAZ protein that is required for flagellum attachment [25]. Upon FLA1 RNAi knockdown, flagella detach and FS179-HA exhibits flagellum localization (Fig 4A). FS179-HA costaining with paraflagellar rod (PFR), a flagellum cytoskeleton protein, showed that FS179-HA and PFR don’t colocalize extensively (Fig 4B) since FS179-HA is typically found on the cell body side of the PFR. Taken together, these results
suggest that FS179-HA localizes to the flagellar membrane of the FAZ. This localization is not on the entire circumference of the flagellar membrane but specifically on the side that faces the cell body. Thus, FS179-HA localizes to a specific subdomain of the flagellar FAZ membrane. I then performed IFA on various C-terminus deletion mutants in an effort to characterize cis elements that dictate this intriguing localization.

Membrane topology predictions indicated that FS179 has an extensive C-terminus tail of around 350 residues. I thus constructed deletions of 50, 100, 150 and 200 residues at the C-terminus (∆C lines) and determined mutant FS179-HA localization. While deletion of up to 150 residues doesn’t have an effect on FS179-HA localization (Fig 5A), the ∆C200 line didn’t exhibit the expected FAZ localization (Fig 5A) and was characterized by lower steady state FS179-HA levels (Fig 5B). This result is indicative of a requirement of the FS179 200 C-terminus residues for protein stability and/or trafficking to FAZ.

**FS179 is required for flagellum attachment**

I then interrogated FS179 function by RNAi knockdown. To this end, I targeted an open reading frame (ORF) segment in the FS179-HA line; this allowed me to monitor RNAi efficiency by western anti-HA blot. Interestingly, upon FS179 RNAi knockdown, I observed flagellum detachment in both life cycle stages (Fig 6A). This phenotype was eventually lethal since cells lost morphogenetic axes and failed to complete proper cell division and was accompanied by a drastic reduction in FS179-HA levels (Fig. 6B). Onset of phenotype occurred within 24 hours in BSF parasites and while cells kept duplicating nuclei and other organelles, they were unable to divide. In PCF, lethality
occurred by 72h and cells seemed to lose the typical trypomastigote (basal body posterior to nucleus) morphology and adopt a epimastigote-like (basal body anterior to nucleus) architecture (Fig 6A). The lethal FS179 RNAi phenotype could be further utilized to probe FS179 function. For this, we could express fusion or mutant versions of FS179 in a knockdown background and determine whether they can rescue the ensuing phenotype. This approach requires that knockdown won’t affect introduced FS179 versions but only deplete endogenous FS179 protein. As a proof of principle, I introduced FS179-HA in a cell line with FS179 3' untranslated region (UTR) knockdown. Since FS179-HA has a different 3'UTR, it shouldn’t be down-regulated by RNAi which should only target the endogenous FS179 transcript. Indeed upon induction (Fig 7A), FS179-HA, which is constitutively expressed (Fig 7B), rescues the lethal RNAi phenotype suggesting that HA tag doesn’t interfere with FS179 function and validating the approach for performing FS179 structure-function analysis.

**Discussion**

The *T. brucei* flagellum is now recognized as a major morphogenetic hub for the unicellular parasite [17]. In contrast to other ciliated eukaryotes, the trypanosome flagellum emerges and remains attached to the cell body for most of its length with only the flagellum tip extending free. Flagellum attachment is mediated by the FAZ, an intricate super-structure that entails membrane and cytoskeleton elements [30,31]. FAZ functions in a bridge-like fashion to connect a vibrating flagellum to the cell body. In addition to a structural role, FAZ orchestrates cell division by transmitting morphogenetic information from the flagellum to the cell body where the cell division
machinery is [18]. Despite the importance of FAZ for *T. brucei* and potential therapeutic targets, FAZ composition remains unclear with only a few subunits known (Table 1). We serendipitously discovered a novel FAZ component, FS179, during validation of our flagellar surface proteome [32]. FS179 is a polytopic membrane protein with up to 24 transmembrane segments; it is predicted to be a voltage-gated calcium channel based on conserved motifs in both the ion selectivity loop and the voltage sensor (Fig. 3) [33]. An alternative would be that FS179 is a sodium channel or a non-specific cation channel. We attempted to determine the ion specificity of FS179 by electrophysiology in *Xenopus* oocytes. We managed to produce *in vitro* FS179 mRNA having 5’ and 3’ UTRs from highly expressed *Xenopus* genes and injected it into oocytes. However, we failed to detect any current even in the presence of mammalian auxiliary channel subunits (data not shown). We speculate that codon usage bias or defective plasma membrane targeting may have led to this result. In any case, calcium selectivity is the most likely scenario given the conservation of pore region residues [33].

In order to study FS179, we tagged it at the C terminus with a triple HA epitope [34]. Epitope tagging in *T. brucei* is typically *in situ* given the high rate of homologous recombination and unlike ectopic transgenes, it is assumed to be subject to endogenous regulation [35]. FS179-HA exhibited the expected size on SDS electrophoresis and was entirely extracted by 1% NP40, indicating that it doesn’t interact stably with the cytoskeleton (Fig 2). Localization by IFA demonstrated that FS179-HA is a FAZ member. On the posterior end, FS179-HA is found in the flagellar pocket while on the other end it reaches the anterior tip of the FAZ (Fig 3). Given this typical FAZ localization, it is challenging to assign FS179 to the flagellum membrane or
the cell body membrane since they are in close apposition. To resolve this, we employed the FLA1 knockdown mutant that RNAi induction results in flagellum detachment. Upon FLA1 RNAi, FS179-HA localizes to the detached flagellum and interestingly it is now present till the flagellum tip (Fig 4). We interpret this as evidence that FS179 localizes to the flagellar membrane and that it is constrained in FAZ by a cell body membrane protein. Interestingly, absence of colocalization with PFR suggests that FS179-HA doesn’t localize around the entire flagellum membrane but rather it is concentrated on the FAZ side, consistent with FS179 interacting with a membrane protein on the cell body side of the FAZ (Fig 4). We performed deletion analysis as a first step of probing FS179 interactions and targeting to FAZ. Interestingly, while the C-terminal 150 residues are dispensable, deletion of 200 residues at the C-terminus has a dramatic effect on steady state protein levels and FS179 becomes barely detected by IFA with signal being mostly cytoplasmic/endosomal (Fig 5). It is unclear whether a targeting signal exists within C-terminal residues 200-150 and the inability to traffic to FAZ results in increased protein turnover. Alternatively, deletion of 200 residues could result in deleterious structural changes and protein instability or it could mediate an important protein-protein interaction. Finally, we used RNAi to test the importance of FS179 in parasite biology. Depletion of FS179 results in flagellum detachment and is eventually lethal (Fig 6). The phenotype is most dramatic in bloodstream form parasites; lethality ensues within 24 hours by a catastrophic uncoupling between mitosis and cytokinesis. Indeed, BSF cells keep on duplicating nuclei and other organelles yet they cannot initiate cytokinesis. Procyclic trypanosomes seem to survive a little longer (typically 72 hours post induction) and they exhibit a striking loss of cellular architecture
with the basal body and kinetoplast moving anterior and cells adopting an epimastigote or promastigote morphology. This phenotype is characteristic of FAZ mutants [22,23] and reflects the inability to assemble a full length FAZ filament. The lethal phenotype of FS179 depletion gives us a tool for structure-function analysis of this protein. Indeed, we can express any desired mutant of FS179 as an RNAi-immune version in the knockdown background and determine whether it rescues or not. As proof of principle we expressed RNAi-immune FS179-HA in FS179-RNAi and showed that the fusion protein can substitute for the most part the endogenous one (Fig 7). This further validates our previous results and allows us to mechanistically dissect this massive ion channel.

In summary, we provided evidence that a putative calcium channel is required for flagellum attachment and trypanosome viability. It remains to be seen whether FS179 has a strict structural role in the FAZ or if its channel activity is important. Of note, FS179 is in a strategic position to control calcium influx into the flagellum. For instance, ClpGM6, an essential flagellum FAZ cytoskeletal protein could be calcium-regulated [23]. Moreover, PFR and NDRC harbor a plethora of calcium-responsive proteins, such as calmodulins [12,36] and FS179 could thus regulate flagellum beating too. Remarkably, GPI-PLC has a similar to FS179 localization and is known to be regulated by calcium [37]. FS179 could thus regulate calcium influx that triggers transition from bloodstream to procyclic form or control this virulence factor [38]. For future experiments, we plan to check localization of a series of FAZ markers in FS179 knockdown to better understand the function of this protein. Given the lethal phenotype and morphological changes upon FS179 depletion, I predict that FS179 is crucial for
FAZ filament assembly. Since FS179 is on the flagellar membrane this implies that it interacts with a cell body membrane protein that dictates FAZ filament biogenesis. We will attempt to disclose any FS179-interacting proteins using proximity-dependent biotin identification (BioID) [39,40] and immunoprecipitation followed by mass spectrometry. Finally, we also plan to perform electrophysiology in mammalian cells using a FS179-GFP fusion to verify expression and localization in order to fully characterize this intriguing ion channel.

**Materials and methods**

*T. brucei* cell lines. Cultivation, transfection and RNAi induction of trypanosomes in culture were done as described previously [35]. Procyclics and bloodstream form cells were maintained in SM and HMI-9 medium respectively with appropriate selection drugs. Epitope tag constructs were created by cloning parts of ORF and 3’UTR in suitable vectors using Kpn-Xho and Bam-Sac1 sites respectively [34]. RNAi constructs were created by cloning ORF or UTR segments in suitable vectors using Xba and HindIII. To amplify FS179 ORF and UTR segments for 3xHA tagging we used

* gatggtaccGCGGAGAACGCCGCCTCTTTTTCC (ORF-For),
* gactctcagCTCTGCTGCTGTAGGCTCCCTGTC (ORF-Rev),
* GtgggatccCATCGATATTATCTCAAGCTATGCGT (UTR-For),
* GtgggatccCATCGATATTATCTCAAGCTATGCGT (UTR-Rev). To amplify FS179 ORF and UTR segments for RNAi constructs we used cataagctttcattgcgtcatattttgctta (ORF-For),
* cataagctttcattgcgtcatattttgctta (ORF-For),
* tctactacttgtatcactatggtctctct (UTR-For),
* cccacatggtctgcttgatcactatggtctctct (UTR-For). To amplify FS179 ORF for deletion constructs
we amplified suitable ORF segments and cloned them in FS179 epitope tagging constructs that already had FS179 UTR cloned. To this end, we use
gatggtaccTCGCTCCGTGCACTGCAAC (ORF-For Delta 50),
gatctcgagAACTCGCTGCGAATTCGAGCGCA (ORF-Rev Delta 50),
gatggtaccCACTCGTGCTGCACACACAC (ORF-For Delta 100),
gatctcgagCCGTTTCTACGTTTCTATCG (ORF-Rev Delta 100),
gatggtaccAACTGGAGCCCGTCGCTTCG (ORF-For Delta 150),
gatctcgagCGCTTCACTGGCACCTTTTCG (ORF-Rev Delta 150),
gatggtaccTCCCTTGCCAACGTGACGCT (ORF-For Delta 200),
gatctcgagAGCACGACCCTCGTGCCAAAG (ORF-Rev Delta 200).

Biochemical fractionations and immunofluorescence.
Cells were extracted with PEME buffer (100 mM piperazine-\(N,N^{\prime}\)-bis(2-ethanesulfonic acid), pH 6.9, 2 mM EGTA, 0.1 mM EDTA, 1 mM MgSO4, protease inhibitors cocktail) containing 1% NP40. Cell lysates and detergent fractionations were analyzed by western blotting as previously described. Whole cell immunofluorescence (IF) was performed by fixing cells with 0.1% PFA in solution on ice for 5 min. Cells were then left to dry on coverslip and permeabilized with methanol 10 min at -20 degrees. Cells were rehydrated with PBS for 15 min and blocked with PBS-20% serum for 1h. Mouse monoclonal anti-HA (Covance) was used at 1:200 and Alexa-conjugated secondaries at 1:1500.
Figure 1. FS179 is a putative voltage-gated calcium channel. (A) Cartoon representation of a polytopic calcium channel with the pore region highlighted in red. (B) Alignment of known and putative calcium channels in the pore region. FS179 is underlined. (C) Cartoon representation of a polytopic calcium channel with the voltage sensor highlighted in red. (D) Alignment of known and putative calcium channels in the voltage sensor. FS179 is underlined. Adapted and reproduced with permission [33].
Figure 2. FS179-HA is detergent soluble. Lys is total protein lysate, Sup and Pel correspond to supernatant and pellet after 1% NP40 extraction. BSF is bloodstream form, PCF is procyclic and WT is wildtype *T. brucei*. Tubulin blot serves as fractionation control.

Figure 3. FS179-HA localizes to the FAZ. Arrows point to the anterior end of FS179-HA staining which is distinct from the flagellum tip. DNA is visualized with DAPI. BSF is bloodstream form and PCF is procyclic *T. brucei*
**Figure 4.** FS179-HA localizes to a subdomain of the flagellar FAZ membrane. (A) Arrows indicate detached flagella of FLA1 knockdown bloodstream form *T. brucei*. DNA is stained with DAPI. (B) FS179-HA and PFR costaining in bloodstream form *T. brucei*. DNA is stained with DAPI.
**Figure 5.** Deletion analysis suggests that FS179 C-terminal 200aa are important for protein stability or FAZ targeting. (A) Localization of Delta C-terminal FS179-HA in bloodstream form *T. brucei*. (B) Western blot for Delta C-terminal 200 (three different clones). FL is full length FS179-HA.
Figure 6. FS179 RNAi knockdown results in flagellum detachment and is lethal in both life cycle stages. (A) Flagellum detachment in RNAi mutants. (B) Western blot for FS179-HA indicates strong knockdown. U denotes uninduced, I denotes 72 hour RNAi induction. –C and +C are negative and positive western controls respectively. 1 and 2 are different clonal lines. PCF is procyclic form, BSF is bloodstream form trypanosomes.
Figure 7. FS179-HA rescues the lethal phenotype of FS179 knockdown. (A) In vitro growth curve for FS179 UTR knockdown compared to FS179-HA rescue in bloodstream form. (B) Immunofluorescence demonstrates FS179-HA localizes to FAZ in the absence of wildtype protein in bloodstream form.
Table 1. FAZ associated proteins in *T. brucei*

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References


Chapter 4

The *T. brucei* BBSome regulates endocytosis
**Summary**

This chapter describes efforts to characterize protein trafficking mechanisms to the *T. brucei* flagellum membrane, a critical host-pathogen interface. To this end, I focused on BBSome, a broadly conserved octameric protein complex that regulates protein targeting to the cilium. We first demonstrated that *T. brucei* Bardet Biedl Syndrome (BBS) proteins assemble into a BBSome-like complex by glycerol gradient sedimentation, blue native gel electrophoresis and immunoprecipitation. Using immunofluorescence we showed that the *T. brucei* BBSome localizes to the base of the flagellum, in association with membranes and vesicles. Furthermore, we employed genetic knockouts of select BBS genes and uptake assays to provide evidence that BBSome regulates endocytosis at the trypanosome flagellar pocket, a function essential for parasite virulence. Our results are thus consistent with flagellum-related functions for this conserved protein complex and uncover a novel endocytic role with exciting ramifications for cilium biology.

**Introduction**

Cilia and flagella are monophyletic organelles, present in most extant eukaryotes [1]. The widespread structural and molecular conservation of cilia suggests that they were already present during the origins of eukaryotes [2]. Cilia don’t have any prokaryotic counterparts and are thus considered a eukaryotic innovation that arose by duplication and divergence of preexisting modules [3]. Structurally, cilia resemble hair-like cellular protrusions into the extracellular environment. The core of cilia is the microtubule-based axoneme that emanates from a modified centriole near the cell surface [4]. The
axoneme is surrounded by the ciliary membrane which is contiguous yet biochemically distinct from the plasma membrane [5]. Similarly, the base of the cilium is physically continuous with the cytoplasm yet proteins and other molecules do not enter freely the cilium matrix due to a diffusion barrier [6-11]. Cilia are thus bona fide organelles even though they are not entirely encircled by a membrane. As with all organelles that are devoid of ribosomes, cilia rely on specialized trafficking mechanisms that deliver proteins by overcoming the diffusion barrier [12-15]. The exact mechanistic details of trafficking to and from the cilium remain unclear. However, intriguing insights have been offered by studies of intraflagellar transport (IFT). IFT is the bi-redirectional movement of protein cargo along the axoneme via kinesin and dynein motors [16]. Kinesins are used for anterograde movement from the base of the cilium to the tip while cytoplasmic dyneins catalyze the opposite (retrograde) movement. IFT relies on protein complexes comprising IFT proteins that facilitate movement of proteins by functioning as adaptors between cargo and motors. IFT delivers axonemal building blocks at the tip of the cilium where IFT particle re-structuring occurs to allow retrograde movement to begin [17].

IFT is a virtually universal feature of cilia since only the male gametes of *Drosophila* and *Plasmodium* build axonemes in an IFT-independent way [18,19]. A similar phylogenetic profile is exhibited by another cilium-specific protein complex, the BBSome, which consists of eight Bardet Biedl Syndrome (BBS) proteins [20,21]. First described more than a century ago, BBS is a human inherited disorder that exhibits a diverse constellation of symptoms. Clinical manifestations include retinal degeneration, obesity, hypogonadism, kidney failure and cognitive impairment [22]. This diverse set of symptoms was puzzling for decades till the realization that BBS proteins function within
cilia [23]. Indeed, defects in cilia structure and function can explain the pleiotropic symptoms of BBS and that was one of the first examples of a ciliopathy [24]. BBS is caused by lesions in any of nineteen genes, BBS1-19, yet this number is probably an underestimation since not all cases can be matched to a known BBS gene [25-28]. Of these 19 genes, most are metazoan specific while eight encode for proteins that are BBSome subunits. BBSome proteins (BBS1, BBS2, BBS4, BBS5, BBS7, BBS8, BBS9) are broadly conserved among ciliated eukaryotes with the exception of such members of land plants, fungi and Apicomplexa [34]. BBSome proteins are dominated by tetratricopeptide repeat (TPR) and β-propeller domains that are known to function in protein-protein interactions [20,29]. Based on the presence of these domains, BBSome proteins are thought to have evolved from protocoatamer factors via IFT proteins [30]. This scenario links BBSome to vesicular coats such as COPI, COPII and clathrin-adaptin modules [3,31]. Consistent with this, BBSome has been shown to form a planar coat on liposomes in vitro, although a similar observation hasn’t been made yet in vivo [29]. Notably, recruitment to the liposome requires the small GTPase Arl6 (BBS3). While other BBS proteins function at the ciliary transition zone or as chaperones to mediate assembly of BBSome [32,33], BBSome itself is believed to interact with IFT and traffic within cilia proper to control localization of membrane proteins [35-39]. This is supported by the observation that certain proteins fail to reach cilia in BBSome mutants. These are mostly G protein coupled receptors (GPCR) and include somatostatin receptor 3, melanin concentrating hormone receptor and neuropeptide Y receptor [14]. Intriguingly, in the absence of BBSome some proteins accumulate aberrantly in cilia [40,41]. Consistent with a BBSome role in retrograde dynein-powered IFT, recent studies
suggest that BBSome mediates IFT particle restructuring at the base and tip of nematode cilia [37]. In a striking analogy, zebrafish BBSome proteins are required for dynein-mediated melanosome trafficking, in the only known BBSome function outside cilia [42]. Collectively, these studies indicate that BBSome is a conserved protein complex that governs cilium localization for certain membrane proteins. However, the exact mechanism remains unclear; it has been postulated that BBSome allows membrane proteins to traverse laterally the ciliary diffusion barrier. Another model suggests that BBSome mediates endocytosis of proteins that then get sorted to a cilium-bound endosome and finally reach the cilium [14,15].

Despite extensive studies of BBSome biology in metazoan and microbes [43], BBSome remains mostly unexplored in *T. brucei* [44]. Trypanosomes encode all 8 BBSome subunits and there is indirect link between BBSome and flagellum length control. Interestingly, BBSome is required for infectivity in the related parasitic *Leishmania* [45]. We sought to determine whether the *T. brucei* BBSome regulates protein trafficking to the flagellum and whether it has an effect on parasite virulence. We first showed that *T. brucei* BBSome proteins indeed assemble into a BBSome-like complex which localizes to the flagellar pocket membrane and endocytic vesicles. Strikingly, we provide evidence that *T. brucei* BBSome interacts with clathrin and is required for endocytosis of certain membrane proteins as well as parasite virulence in a mouse model of infection. Our studies thus identify a novel function for a conserved module and open new avenues for illuminating cilium biology.
Results

Despite the importance of the flagellum in the biology of *T. brucei*, the significance and functions of the trypanosome BBSome remained enigmatic. Having a long standing interest in the flagellar membrane capabilities as a host-pathogen interface, we set out to determine whether *T. brucei* BBS proteins assemble into a BBSome and if they govern trafficking of proteins to and from the flagellum.

Given the absence of antibodies against endogenous TbBBS proteins, we epitope tagged BBS1, BBS4, BBS5 and BBS7 with triple hemagglutinin (HA) tag at the C-terminus. All four proteins were expressed at the expected size (Fig. 1). We then employed three approaches to determine whether TbBBS proteins assemble into a complex. First, we performed Blue Native (BN) electrophoresis on detergent soluble samples, having determined that TbBBS proteins become entirely soluble with as little as 0.5% NP40 (data not shown). BN electrophoresis indicated that tagged TbBBS proteins run as part of a ~650 kDa complex (Fig. 2). We then employed glycerol gradient centrifugation and observed a similar result; tagged TbBBS4 and TbBBS5 behave as members of a 14.5S complex (Fig. 2). Both results are consistent with data from other organisms. To formally demonstrate the TbBBS are part of the same complex, we purified BBS4 and detected co-immunoprecipitating proteins via shotgun proteomics. BBS4 was epitope tagged at the C-terminus with a dual tag that allows tandem affinity purification. The tag contained a protein C epitope and two protein A epitopes separated by a TEV (Tobacco Etch Virus) cleavage site. In addition to tandem purification (Fig 3), we also performed single step immunoprecipitations (IP) targeting either protein C or protein A epitopes (data not shown). All three purifications gave
similar results with TbBBSome proteins being consistently the most abundant proteins recovered. As expected, tandem purification gave the least number of contaminants and interestingly it was the only IP that allowed detection of the 10kDa BBSome-associating protein (TbBBS18) (Table 1). Taken together, our biochemical and proteomics approaches indicate that TbBB proteins assemble into a BBSome-like complex.

We then asked where the TbBBSome localizes. In other systems, BBSome is mostly at the base of the cilium with only a small fraction travelling inside the organelle via IFT trains [36]. We performed immunofluorescence and detected TbBB proteins at the base of the flagellum, distal to the basal body (Fig. 4). To achieve higher resolution, we turned to immuno electron microscopy (EM). To our surprise, we detected TbBB associating with the flagellar pocket membrane and vesicles therein. This was the first time that BBSome was shown to interact in vivo with membranes and supports previous in vitro studies with liposomes [29]. Intrigued by the immunoEM localization we tested whether purified TbBBSome interacts with phosphoinositides or other lipids using PIP strips and observed binding chiefly to phosphatidylinositol 4-phosphate (PI(4)P) and phosphatidylinositol 5-phosphate (PI(5)P) and secondarily to phosphatidylinositol 3-phosphate (PI(3)P) (Fig. 6). Having established the localization and subunit composition of TbBBSome, we sought to illuminate its function. To this end, we performed genetic knockouts of select TbBBS genes by replacing both alleles with drug markers. TbBB knockouts (KOs) were viable in culture with only a slight growth defect as compared to wildtype (Fig 7). Interestingly, TbBBSome was differentially affected in distinct knockouts. For instance, we couldn’t detect TbBBSome in BBS1 or BBS9 KOs while a reduced in size complex was observed in BBS5 KO (Fig 8). Ablation of the TbBBSome
could lead to defects in flagellum biogenesis but we didn’t observe any conspicuous alteration in the flagellar pocket region (Fig. 9) or the flagellum proper (Fig. 10). We did observe however a slight motility defect for BBS1 and BBS9 KOs in a sedimentation assay (Fig. 11).

In our endeavor to understand TbBBSome function, we looked more carefully at the IP proteomics data and discovered that clathrin heavy chain (CHC) was a putative BBS4 interacting protein. Indeed, CHC was specific to BBS4 tandem IP and was also enriched compared to control in one step protein C IP (data not shown). We verified this interaction by repeating the IP and performing western blot for CHC as well as for ESAG7 (subunit of the \textit{T. brucei} transferrin receptor) which showed a similar pattern. Indeed, our results suggest that TbBBSome interacts with clathrin and ESAG7 (Fig. 12), consistent with our immunoEM data. Naturally, we next posed the question whether TbBBSome is required for endocytic phenomena involving transferrin receptor (TfR) at the flagellar pocket. To answer this, we performed a series of endocytosis assays with a plethora of markers. Initially, we compared transferrin uptake between WT and TbBBS-KOs and observed that all TbBBS mutants had defects in uptake with TbBBS7 and TbBBS9 being the most affected (Fig. 13). The uptake defect was not simply due to defective transferrin binding since that remained unchanged (Fig. 14). Moreover, defective transferrin uptake couldn’t be attributed to lower levels of CHC or TfR (Fig. 15) or deficiency in general endocytosis since that remained unchanged too (Fig. 16). Furthermore, we checked general receptor mediated endocytosis using the lectin concavalin A (conA) that binds to glycosylated proteins in the flagellar pocket. Surprisingly, we observed increased uptake for BBS1 and BBS9 KOs (Fig. 17) which
could be partially explained by increased binding of conA (Fig. 18). We also checked surface clearance of the Variant Surface Glycoprotein (VSG) as another proxy for endocytosis and observed slight defects for BBS4, BBS5, BBS7, BBS9 KOs in intermediate time points (Fig. 19). Finally, we used the TbBBS mutants in a mouse model of infection. Strikingly, TbBBS1 and TbBBS9 have clear infectivity (Fig. 20) and virulence (Fig. 21) defects. The attenuated parasites sometimes fall below detection limit in the mouse bloodstream after observed parasitemia, a phenomenon rarely seen, if ever, with wildtype trypanosomes (Fig. 21). Taken together, our results suggest that TbBBSome is a virulence factor that mediates critical protein trafficking at the flagellar pocket.

**Discussion**

The *T. brucei* flagellum membrane has emerged as a crucial scaffold for assembling important host-parasite modules [46]. For example, *T. brucei* flagellum comes into direct contact with the tsetse fly salivary gland and mediates attachment via yet unknown molecules. Moreover, in the bloodstream form parasite, the flagellar membrane is populated by a plethora of virulence factors that modulate the host immune system. Examples include metacaspase 4, GPI-PLC, calflagins and ESAG4. The latter is an adenylate cyclase that produces cyclic AMP (cAMP) upon trypanosome lysis inside a host macrophage [47]. The produced cAMP then activates host protein kinase A to downregulate trypanotoxic tumor necrosis factor alpha production. As an outcome, trypanosomes control host innate immunity at early stages of infection. Given the importance of flagellar membrane proteins for host-parasite interactions, we determined
a flagellum membrane proteome from bloodstream form trypanosomes and showed that it constitutes as dynamic and suitable interface with the host [48]. Interestingly, the flagellar membrane is not uniform as revealed by subdomain-specific protein localization. Having elucidated the identity of the flagellar membrane resident proteins, we then sought to study their function in terms of host-parasite interactions. Instead of targeting individual proteins, we decided to ablate trafficking mechanisms that control protein localization to the flagellar membrane. Flagella are separated by the rest of the cell by a diffusion barrier at their base and that implies the existence of systems that mediate entry into the flagellum [15]. The best characterized such module is intraflagellar transport (IFT). IFT is a bidirectional transport system that utilizes kinesin and dynein motors to transport axonemal components into and out of flagella in most ciliated species, including *T. brucei* [16]. Another critical regulator is BBSome. BBSome was originally described as heptameric complex of highly conserved proteins and recently a novel conserved 10kDa subunit was described. Studies in organisms as diverse as vertebrates, nematodes, green algae, ciliates and kinetoplastids imply that BBSome is generally not required for flagellum formation but rather it controls trafficking of certain flagellar membrane proteins. This provides us with a stellar opportunity to determine the role of *T. brucei* flagellar membrane proteome at the host-parasite interface. That was not previously feasible since IFT depletion in *T. brucei* is lethal [49]. Our first approach was to determine whether TbBBS proteins form a BBSome complex in bloodstream form parasites. To answer this, we used in situ tagging to integrate an HA epitope tag into the endogenous loci of select TbBBS genes. Proteins of the expected size were identified by western blotting (Fig. 1) and run as high molecular
weight complexes in blue native gels (Fig. 2). Moreover, TbBBS4 and TbBBS5 co-sediment in glycerol gradients as a large ~14.5S complex (Fig. 2). Both results are consistent with the 12-14S and 300-800kDa BBSome entity found in algae and mammals [20,36]. To test whether BBS proteins are physically associated in the same complex, we introduced a PTP affinity tag onto BBS4 and performed purifications using two independent resins [50]. Proteins co-purifying with BBS4-PTP were identified by MudPIT proteomics in collaboration with Dr. James Wohlschlegel. Purifications from wild type cells were run in parallel and detected proteins were used to define a set of proteins that specifically co-purified with BBS4-PTP. The only proteins that co-purified with BBS4-PTP in both samples were the seven TbBBS proteins (TbBBS1, TbBBS2, TbBBS4, TbBBS5, TbBBS7, TbBBS8, TbBBS9) and these were commonly the most abundant hits (data not shown). We further validated this by tandem affinity purification of BBS4-PTP (Fig. 3). Again, we detected all seven TbBBS proteins as well as the eighth BBSome subunit TbBBS18 (Table 1). Therefore, T. brucei BBS proteins BBS1, 2, 4, 5, 7, 8, 9 and 18 assemble into a stable BBSome complex in bloodstream form trypanosomes with properties resembling BBSomes from other systems.

To address the TbBBSome localization, we performed immunofluorescence (IF) and immunogold EM. IF showed that TbBBS proteins localize to the base of the flagellum, distal to the basal body (Fig. 4). This pattern is somewhat different from BBSome localization in other species where BBSome also enters the flagellum proper. It is possible that this reflects trypanosome-specific BBSome features or that simply the flagellum levels of BBSome are below the level of IF detection. We then employed immunogold EM to provide a more precise localization. Our results show that TbBBS
proteins localize to membranes and vesicles at the flagellar pocket near the base of the flagellum (Fig. 5). This location is consistent with IF data and with a BBSome role in controlling flagellum membrane protein trafficking. I note this was the first report of immunogold EM for BBSome in any organism and provides in vivo support for the observation that BBSome coats liposomes in vitro. Spurred by the vesicle localization we then determined whether TbBBSome binds lipid moieties using PIP strips containing an array of purified lipids. Indeed, TbBBSome interacts with phosphatidylinositol 4-phosphate [PI(4)P] and phosphatidylinositol 5-phosphate [PI(5)P] as well as phosphatidylinositol 3-phosphate [PI(3)P] at a lesser extent (Fig. 6). This pattern partially overlaps with BBSome lipid binding capacity in mammals and poses the question whether these phosphatidylinositol species decorate vesicles and regulate trafficking in T. brucei.

In order to investigate TbBBSome function, we generated double knockouts (DKO) of select TbBBS genes (BBS1, 4, 5, 7, 9) by ablating both alleles in bloodstream form parasites. TbBBS DKO didn’t exhibit any dramatic decrease in fitness as shown by in vitro growth curve and sedimentation assay. Indeed, TbBBS knockouts have a slight growth defect (Fig. 7) while TbBBS1-DKO and TbBBS9-DKO exhibit a minor motility defect (Fig. 11). Nevertheless, TbBBS-DKOs grow significantly faster than a previous characterized motility mutant (BLD) which can nonetheless infect mice as wildtype parasites [51]. Moreover, TbBBS-DKOs don’t show any major ultrastuctural defects in the flagellum when examined by TEM cross-sections (Fig. 10). We postulate that the minor motility defect might be attributed to secondary effects of BBSome loss and a not a requirement in motility per se. BBSome could regulate trafficking of ion
channels or ion pumps important for regulating flagellum motility. For example calcium is a well established regulator of axoneme motility in multiple species. Interestingly, a BBS4-PTP-specific interacting protein is a putative calcium pump; perhaps BBSome loss results in aberrant trafficking of this pump and hence influences flagellar motility indirectly.

We also checked ultrastructure of the flagellar pocket region in TbBBS-DKOs (Fig. 9). We didn’t observe any major difference when compared to wildtype cells like vesicle accumulation. The latter has been observed in BBSome mutants of retina photoreceptor cells and *Leishmania* parasites, however the molecular mechanism remains enigmatic [45,52]. It remains to be seen if the vesicle accumulation in BBSome mutants is a peculiarity of the mentioned cell types or whether TbBBSome has divergent functions. Further, we checked whether BBSome is affected in the different TbBBS-DKOs by introducing epitope-tagged TbBBS4 or TbBBS5 and checking their incorporation in a complex by blue native gel. Interestingly, we saw that there is no TbBBS4 or TbBBS5-containing BBSome complex in TbBBS1-DKO and TbBBS9-DKO (Fig. 8). In contrast, TbBBS4 in TbBBS5-DKO is part of BBSome, albeit BBSome is smaller in size (Fig. 8). Our results are in agreement with previous studies in mammals that posit the existence of core and peripheral BBSome subunits [32]. Indeed, these studies predict that BBS4 and BBS5 are in the periphery of the complex and their absence has minimal effects on the rest of complex while BBS1, BBS7, BSBS2 and BBS9 are core subunits whose depletion has catastrophic effects for BBSome.
TbBBSome localization at the flagellar pocket membrane and vesicles as well as the detection by mass spectrometry of clathrin and ESAG7 (transferrin receptor subunit) in BBS4-PTP interacting proteins led us to test whether TbBBSome is involved in endocytic phenomena. We first verified that BBS4-PTP can immunoprecipitate clathrin heavy chain (CHC) and ESAG7 by western blot (Fig. 12). We then gauged transferrin uptake in TbBBS-DKOs (Fig. 13). Interestingly, all mutants showed decreased transferrin uptake when compared to wildtype parasites with the most severely affected being TbBBS7-DKO and TbBBS9-DKO. The decrease in uptake wasn’t due to defective binding of transferrin to the transferrin receptor in conditions that block endocytosis. Indeed all mutants showed similar transferrin binding to wildtype cells (Fig. 14). This result is also supported by our trypanosome surface quantitative proteomics where transferrin receptor is slightly more abundant in the TbBBS-DKOs (data not shown). Moreover, the steady-state levels of both clathrin and transferrin receptor seem unchanged in the TbBBS mutants (Fig. 15). Given the observation that fluid flow endocytosis of dextran remains unperturbed in TbBBS mutants (Fig. 16), we postulate that the TbBBSome is essential for efficient endocytosis of a specific subset of membrane proteins such as the transferrin receptor. Defects in transferrin uptake in T. brucei have been reported before for endocytic mutants but they are commonly observed in non-viable mutants [53-56].

We extended our uptake assays by using the lectin concavalin A (con A) which binds to glycosylated proteins in the trypanosome flagellar pocket. To our surprise, TbBBS1-DKO and TbBBS9-DKO showed increased uptake of con A while the rest TbBBS mutants were indistinguishable from wildtype (Fig. 17). Moreover, all TbBBS
mutants exhibited increased binding of con A in non-endocytic conditions (Fig. 18). Further, our preliminary results for VSG clearance indicate that TbBBS mutants (except for TbBBS1-DKO) show a slight decrease in VSG removal from the cell surface (Fig. 19). This is somewhat unexpected since dextran uptake wasn’t affected in TbBBS mutants while con A uptake was either increased or not affected. VSG clearance is a technically challenging assay and I thus speculate that non biological variability influenced our results.

We finally checked virulence of TbBBS mutants in a mouse model of infection. Our studies indicate that loss of the TbBBSome results in attenuation since TbBBS1-DKO and TbBBS9-DKO show defects in their ability to mount (Fig. 20) and to maintain an infection (Fig. 21). Indeed, parasitemia was detected in less mice overall when using TbBBS mutants with severely affected BBSome. Moreover, in the infected animals, trypanosomes were controlled better and that resulted in multiple parasitemic waves or even clearance of infection in few cases. This is a rare phenomenon since we used the highly virulent 427 strain that almost invariably mounts a lethal parasitemia [51]. In mice with recurring parasitemic waves, we observed VSG switching (data not shown) indicating that the classic *T. brucei* immune evasion mechanism still operates. Taken together, our results suggest that the *T. brucei* BBS proteins assemble into a BBSome that operates at the flagellar pocket membrane, a critical host-parasite interface, and comprises a virulence factor. The TbBBSome binds specific phosphatidylinositol(s) possibly via the predicted lipid binding domain of BBS5 and might recruit clathrin heavy chain via putative clathrin boxes in TbBBS proteins (data not shown). TbBBS proteins are of very low abundance and we deemed unlikely that they would be required for
general endocytosis. Indeed, our uptake assays collectively suggest that TbBBSome mediates uptake of few membrane proteins such as the transferrin receptor. It is likely that TbBBSome is also required for uptake of a subset of glycosylated proteins of the flagellar pocket; this prediction stems from the observation that at least some of the TbBBS mutants don’t show increased uptake of con A, as expected by the increased con A binding in non-endocytic conditions. Remarkably, our IP data suggest that TbBBSome interacts with ubiquitin; it is possible that TbBBSome is part of this conserved mechanism for membrane protein turnover [57]. The increased presence of glycosylated proteins on the trypanosome cell surface further indicates that TbBBSome is essential for trafficking fidelity at the unique endocytic region of the cell. Proper endocytic activity has been shown previously to be essential for parasite infectivity [58,59] and is corroborated by the absence of any conspicuous ultrastructural change in the mutant flagella. Moreover, this is not the first time the BBSome has been implicated in endo/exocytic trafficking. Although originally postulated to serve as an IFT adaptor that allows proteins to enter the cilium, a series of reports now indicate that the main function of BBSome might be to remove proteins from the cilium or periciliary space. For example, dopamine receptor in mammals and phospholipase D in *Chlamydomonas* accumulate in cilia of BBSome mutants [40,41]. Further, components of Hedgehog signaling don’t traffic out of the cilium in the absence of BBSome [39]. It has been suggested that the inability of certain proteins to reach the cilium in BBSome mutants reflects secondary effects of BBSome loss and not a direct requirement for BBSome-mediated trafficking to the cilium [40]. In *C. elegans*, BBSome is thought to be required for exocytosis at the periciliary compartment [60] yet its absence could also lead to
hypersecretion [61]. Taken together, these studies in diverse systems could be interpreted in a framework that postulates BBSome to govern endocytosis at the periciliary base. Our model predicts that BBSome cycles through the cilium on IFT particles and removes cognate proteins by endocytosis at the ciliary pocket. Moreover, BBSome could be a bona fide regulator of protein entry into cilium by mediating endocytosis at the ciliary pocket and targeting of vesicles to an endosome destined for the cilium. The architecture of BBSome proteins and the phylogenetic relationship to IFT and protocoatomer further support the scenario that BBSome is an ancient endocytic adaptor that was recruited for ciliary functions. Continuing studies in the model flagellate T. brucei will likely elucidate more conserved BBSome functions as well as provide novel therapeutic targets for this deadly parasite.

Materials and methods

T. brucei cell lines. Cultivation, transfection and genetic knockouts of trypanosomes in culture were done as described previously [62]. Parasites were maintained in HMI-9 medium supplemented with appropriate selection drugs. Epitope tag constructs were created by cloning parts of ORF and 3’UTR in suitable vectors using Kpn-Xho and Bam-Sac1 sites respectively [63]. To amplify BBS1 ORF and UTR segments for 3xHA tagging we used gatgtaccCGATAcGGGCGCGAAGATGC (OFR-For), gatctcgagTGAATTGTCTTCTATGAATCCAGCGTCAG (OFR-Rev), gatggatccAGGTGGACAACTGATCGAACGT (UTR-For), gatgagctcTCGAGCTAGAGCTCCATACCCT (UTR-Rev). To amplify BBS4 ORF and UTR segments for 3xHA tagging we used gatgtaccCGTCCGACTCTAGGGCAACCA
(OFR-For), gatctcgagTTTGCCTAGCTTTTGCCGTAAGGA (OFR-Rev),
gatggatccTGAACGTAGGTGACTGCAAGTACAG (UTR-For),
gatgagctcGCTTTTCGTTTCCCCCTATCCCT (UTR-Rev). To amplify BBS4 ORF for
PTP tagging we used gatggggcccGCACCTCGCCTATTAGTTTGC (ORF-For) and
gatgcgcccgcTTGCGTAGCTTTGCGTAAGGAG (ORF-Rev). To amplify BBS5 ORF
and UTR segments for 3xHA tagging we used
gatgggtaccACGCCTAAAGGGCGTGAGCA (OFR-For),
gatctcgagTGCACGACATTCCAAATCCTGCA (OFR-Rev),
gatggatccGTCGGGGAAAGTGGAAAGAGAG (UTR-For),
gatgagctcACACCTCGTGGAATTTGATGTCGT (UTR-Rev). To amplify BBS7 ORF
and UTR segments for 3xHA tagging we used
atcgatggtaccGCTGCGGAACACATGTACG (5UTR-ORF),
cagatctcgagACAACGAAAAATACCGCAGCTC (5UTR-REV),
cacgatggatccGCTGAGGTATGTGTGGCTGG (3UTR-For),
gtgatgagctcAGGGGTAAAACAAGGAGAGGAAGAGA (3UTR-Rev). For genetic
knockouts we amplified segments of 5'UTR and 3'UTR and cloned in suitable vector
using Not1-BamH1 and Apa1 respectively. To amplify BBS1 5'UTR and 3'UTR
segments for knockout we used
TTCCATATGTCCGGAGAACACCATGTC (5UTR-ORF),
ACCGCTCGAGATAAAACGCTAATAATAACAATC (5UTR-REV),
CGCGGATCCAGGTGGACAACTGATCGAAC (3UTR-ORF),
GCTCTAGAAAAGTCTTTTGCCGCGTGC (3UTR-REV). To amplify BBS4 5'UTR and
3'UTR segments for knockout we used
ATATGCGGCGCTGTACACATATGTTCCGCGATTTTCTAGTTCCTCACCAC (5UTR-FOR),
ATATATACACTAGTCTCGGAGACCTATTGAGGGTTAGGGTTAGGGAAAGTCC (5UTR-REV),
ATATGGGGCCGGATCCACATGGGACTGGGAAAGCTACAGAGTGAAATCG (3UTR-FOR), ATATGGGGCCCTCTAGACGTCCACGCGCTTGTCCTCCTC (3UTR-REV).
To amplify BBS5 5'UTR and 3'UTR segments for knockout we used
gatgcggccgctGCGGGGAAACACGACACGA (5UTR-FOR),
gatggatccTGTTGTACACACAGCCGGAGAC (5UTR-REV),
gatcaaggccccacgctgtaaGTCGGGGGAAAGTGGAAAGAGAG (3UTR-FOR),
gatcaaggccccccgctgactggaCCATTCCAGCTCTAAACGGCCA (3UTR-REV). To amplify
BBS7 5'UTR and 3'UTR segments for knockout we used
acgtctgcggccgcaATCAGGAGTAGTCATCCTGCTTTATCT (5UTR-FOR),
tctcttgatccCCTCAGCTCCTCTCCCCTTC (5UTR-REV),
ccctttctaaagaaaaatagtsgaGCTGGAGGTATGGTGCTGG (3UTR-FOR),
agggaacaaaaagctggtaagccgsccgcgctgAACATGTGCGGGGTGTCCTCTCT (3UTR-REV).
To amplify BBS9 5'UTR and 3'UTR segments for knockout we used
gatgcggccgcaAGATAGTGAGGTGAAAGAGGCTGAG (5UTR-FOR),
gatggatccGGCTCGTGAAGCGCTATTATAACAG (5UTR-REV),
gatcaaggccccGTATAATGACGCTACCACCTGCGTTG (3UTR-FOR),
gatcaaggccccccgctgATATATACACGTGCGGTGAAGCAACG (3UTR-REV).
**Sedimentation assay.** Sedimentation assay was performed as described previously [64]. Briefly, procyclic cells were washed with fresh medium and resuspended at 2.5x10^6 cells/ml in 1ml aliquots in cuvettes. OD600 was measured every 2h for 8h in total. 3 samples were left undisturbed and 3 samples were resuspended prior to measurements to account for cell growth. The latter measurement was subtracted from the former to get Delta OD600.

**Biochemical fractionations and immunofluorescence**

Cells were extracted with PEME buffer (100 mM piperazine-N,N'-bis(2-ethanesulfonic acid), pH 6.9, 2 mM EGTA, 0.1 mM EDTA, 1 mM MgSO4, protease inhibitors cocktail) containing 1% NP40. Cell lysates and detergent fractionations were analyzed by western blotting or subjected to Blue Native gel electrophoresis according to manufacturer’s instructions (Invitrogen). Whole cell immunofluorescence (IF) was performed by fixing cells with 3% PFA in solution on ice for 5 min. Cells were then left to dry on coverslip and permeabilized with methanol 10 min at -20 degrees. Cells were rehydrated with PBS for 15 min and blocked with PBS- 20% serum for 1h. Mouse monoclonal anti-HA (Covance) was used at 1:200 and Alexa-conjugated secondaries at 1:1500. Surface SILAC proteomics was performed as described previously [65].

**Immunoprecipitation**

Tandem affinity purification of BBS4-PTP was performed as described previously [50]. Briefly, cells were extracted using 20 mM Tris-HCl pH 7.7, 3 mM MgCl2, 0.5% NP40, protease inhibitor cocktail, 150 mM KCl, 150 mM Sucrose, 20mM potassium glutamate.
Extracts were precleared with agarose resin for 1h at 4 degrees and then incubated with IgG resin for 2h at 4 degrees. After 6 washes, TEV protease was added and mixture was incubated overnight at 4 degrees. Following day, flowthrough was collected and incubated with protein C affinity matrix for 2 hours at 4 degrees. After 6 washes, bound proteins were eluted with 5 mM Tris-HCl pH 7.7, 5 mM EDTA, 10 mM EGTA and subjected to MudPit proteomics, SDS-PAGE or incubated with pre-blocked PIP strip according to manufacturer’s instructions.

**Uptake assays.**

Transferrin, concavalin A and dextran uptake was performed as previously described [66]. Briefly, cells were washed and incubated in HMI9-1% BSA for 30 min. Fluorescent substrates were then added (Transferrin-Alexa 594, concavalin A-Alexa 594, Dextran-FITC, Invitrogen) and samples were taken at regular time intervals. Cells were washed, fixed with 4% PFA and subjected to flow cytometry.
Figure 1. Expression of epitope tagged TbBBS proteins. Wildtype (WT) lysate was used as negative control. Expected sizes: BBS1-HA (69 kDa), BBS4-HA (55 kDa), BBS5-HA (44 kDa), BBS7-HA (86 kDa). Tubulin blot served as loading control.
Figure 2. TbBBS proteins are part of a BBSome-like complex (A) Blue Native electrophoresis. B) Glycerol gradient centrifugation. Sedimentation markers are shown in the bottom.
Figure 3. Tandem affinity purification of the TbBBSome. (A) Sypro ruby stain for tandem affinity BBSome purification. I is Input, E is eluate. Expected sizes for TbBBSome proteins are shown on the right. Wildtype was used as control (ctrl). (B) Western blot for tandem affinity BBSome purification. I is Input, E is eluate. Note the reduction in size.
due to TEV protease cleavage. Expected sizes for BBS4-PTP before and after cleavage are 70 kDa and 55 kDa respectively. Wildtype was used as control.

Figure 4. TbBBS proteins localize to the base of flagellum. BBS-HA proteins are stained in green. YL1/2 is a basal body marker in red. DNA is stained with DAPI in blue.
Figure 5. ImmunoEM of TbBBS. TbBBS proteins localize to flagellar pocket membrane and vesicles.
Figure 6. TbBBSome interacts with lipids. Western blot for BBS4-PTP on PIP strip.

TbBBSome interacts with Phosphatidylinositol 3-phosphate [PI(3)P], Phosphatidylinositol 4-phosphate [PI(4)P] and Phosphatidylinositol 5-phosphate [PI(5)P]. Wildtype (WT) was used as negative control.
Figure 7. TbBBS proteins are dispensable for in vitro growth. Growth curve of select TbBBS double knockouts (DKO) alongside a motility mutant (BLD+Tet).
Figure 8. TbBBS proteins are required for BBSome assembly. (A) Western blot for BBS4-HA and BBS5-HA in TbBBS mutants. (B) Blue native gel for BBS4-HA and BBS5-HA in TbBBS mutants.
Figure 9. Flagellar pocket ultrastructure in TbBBS mutants
Figure 10. Flagellum ultrastructure in TbBBS mutants
Figure 11. Sedimentation assay for TbBBS mutants. TbBBS1 and TbBBS9 mutants show increased sedimentation (unpaired t test). N=6.
Figure 12. TbBBSome interacts with clathrin and Transferrin receptor. (A) Western blot for Clathrin Heavy Chain (CHC) for BBS4-PTP immunoprecipitation (IP) fractions Input (I) and Eluate (E). Wildtype (WT) was used as negative control. CHC expected size (191 kDa). (B) Western blot for ESAG7 (subunit of the T. brucei Transferrin receptor) for BBS4-PTP immunoprecipitation (IP) fractions Input (I) and Eluate (E). Wildtype (WT) was used as negative control. ESAG7 expected size (38 kDa).
Figure 13. Transferrin uptake in TbBBS mutants. All TbBBSome mutants exhibit lower uptake (unpaired t test). Minimum n=5

* p<0.05
Figure 14. Transferrin binding in TbBBS mutants. There is no significant difference (ns) when compared to wildtype (WT) (unpaired t test). N=5
Figure 15. Clathrin Heavy Chain (CHC) and ESAG7 levels are not reduced in BBS KOs.
Figure 16. Dextran uptake in TbBBS mutants. There is no significance difference (ns) when compared to wildtype (WT) (unpaired t test). N=5
Figure 17. Concavalin A uptake in TbBBS mutants. TbBBS1 and TbBBS9 knockouts show increased uptake (unpaired t test). Minimum n=4
Figure 18. Concavalin A binding in TbBBS mutants. All TbBBS knockouts show increased binding (unpaired t test). N=3
Figure 19. VSG clearance in TbBBS mutants. There is no significant difference at 10 min. All TbBBS mutants, except for TbBBS1, show decreased Variant Surface
Glycoprotein (VSG) clearance off the parasite surface at 5 min (unpaired t test).
Minimum n=3.

Figure 20. TbBBS mutants exhibit reduced infectivity in a mouse model. Bars correspond to % infectivity rate for two different Multiplicity of Infection (MOIs). Asterisks indicate significant difference when compared wildtype (WT) (unpaired t test).
Figure 21. TbBBS mutants exhibit reduced virulence in a mouse model. Kaplan Meier graph of mice survival at multiplicity of infection (MOI) 1000. Asterisks indicate significant difference when compared to wildtype (WT).

Table 1. The eight TbBBSome proteins are the most-abundant proteins specific to tandem affinity-purified BBS4-PTP as determined by NSAF (label free quantitation)

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Chapter 5

Conclusions
The eukaryotic flagellum, also known as cilium, is now recognized as a motility and sensory organelle with diverse functions. Cilia are eukaryotic innovations and do not exhibit structural or functional equivalents in the prokaryotic world. It is unclear how this tiny organelle became a master regulator of a plethora of processes but it probably relates to its deep phylogenetic emergence and the unique biochemical environment it provides. The cilium is prominent in multicellular organisms where it orchestrates developmental pathways but also in unicellular microbes where it mediates motility, mating and environmental sensing. Cilium is also now recognized as a virulence factor in pathogens since it constitutes a critical host pathogen interface. A prime example is *Trypanosoma brucei*, the causative agent of sleeping sickness in Africa, where the flagellum is an essential and multifunctional organelle. *T. brucei* has also emerged as an excellent model system to study the biology of the eukaryotic flagellum. We took advantage of the *T. brucei* molecular toolbox and provide novel insights into the biology of the parasite but also new conceptual advances for the eukaryotic cilium.

First, we illuminated the composition of NDRC, a universal regulator of axonemal motility. This is only the second organism in which the NDRC has been systematically explored and its subunit composition defined. NDRC composition is not an academic curiosity since defective NDRC subunits result in aberrant ciliary motility and cause human disease. Our future experiments will include further validation of the NDRC candidate subunits. For instance, we plan to check whether their localization and axoneme association is compromised in NDRC mutants. Moreover, we would like to explore the TbCCDC39 and TbCCDC40 functions and localization and further verify their NDRC association. The stage is now set for understanding axonemal motility at the
molecular level. This will probably include state of the art microscopy coupled with mutant analysis as well as in vitro reconstitution of axonemal motility.

Second, our studies identified a novel FAZ component in the form of a massive ion channel. FS179 is a putative calcium channel that is required for flagellum attachment and parasite viability. Remarkably, FAZ resembles ciliary structures of retina photoreceptor cells that are also regulated by calcium. We plan to determine FS179 interacting proteins by BioID and standard immunoprecipitation to further define the FAZ network. FAZ proteins could be promising drug targets given their essential nature for the parasite but could also provide new paradigms for molecular interactions given the extreme forces they have to withstand in order to mediate flagellum attachment.

Finally, we explored the functions of a conserved cilium regulator in a divergent eukaryote. We provide evidence for the first time that BBSome can associate with membranes and vesicles in vivo, as previously predicted by in vitro approaches and protein domain composition. Moreover, we showed that TbBBSome interacts with clathrin, bridging thus the endocytic machinery with specific cargoes. Of note, T. brucei lacks AP-2, a conserved clathrin adaptor and TbBBSome thus could well be one of the many modules compensating for that absence. We now plan to define the TbBBSome cargoes and explore the connection if any with the ubiquitin pathway. Finally, given our success in purifying the complex, we would like to determine its structure at near atomic resolution using cryo electron microscopy techniques. In summary, the T. brucei flagellum is at exciting cross roads of cilium and pathogen biology and our continuing studies promise to illuminate human inherited and infectious maladies.