The flagellum-mitogen-activated protein kinase connection in Trypanosomatids: a key sensory role in parasite signalling and development?

Brice Rotureau, Miguel Morales, Philippe Bastin, Gerald F Späth

The flagellum-MAP kinase connection in Trypanosomatids: a key sensory role in parasite signaling and development?

Brice Rotureau[+^], Miguel A. Morales[*^], Philippe Bastin[+], Gerald F. Späth[*□]

Institut Pasteur, [*]Laboratory of Parasite Virulence and [+]Trypanosome Cell Biology Unit, and CNRS URA 2581, 25-28 rue du Dr. Roux, 75724 Paris Cedex 15, France

[□] corresponding author: gerald.spaeth@pasteur.fr

Tel : 01.40.61.38.58

Fax : 01.40.61.33.32

^These authors contributed equally to the manuscript

Running title: Flagellar sensing in Trypanosomatids

ABSTRACT

Trypanosomatid parasites are the causative agents of severe human diseases such as sleeping sickness, Chagas disease and leishmaniaseis. These micro-organisms are transmitted via different types of insect vectors and hence are confronted to changing environments during their infectious cycle in which they activate specific, and sometimes complex, patterns of differentiation. Several studies in *Trypanosoma brucei* and in different sub-species of *Leishmania* have shed light on the role of Mitogen Activated Protein (MAP) kinases in these processes. Surprisingly, several MAP kinases turned out to be involved in the control of flagellum length in the promastigote stage of *Leishmania*. Recently, a sensory function has been recognized for cilia and flagella in unicellular and multi-cellular eukaryotes. This review aims to stimulate discussions on the possibility that the Trypanosomatid flagellum could act as a sensory organ through the MAP kinase pathway, with the objective to encourage investigation of this new hypothesis through a series of proposed experimental approaches.

Environmental sensing defines the capacity of all organisms to detect and respond to changes in their local surroundings. This process is of pivotal importance for many parasitic microorganisms that need to adapt their physiology to resist and evade anti-microbial host activities. This review discusses mechanisms of environmental sensing of Trypanosomatids, a group of parasitic kinetoplastid protozoa, which cause major diseases in humans (Stuart *et al.*, 2008), including African sleeping sickness and South American Chagas disease, caused by species of the *Trypanosoma* genus, and various forms of leishmaniases caused by species of the *Leishmania* genus. Most of these parasites show a complex infectious cycle implicating various developmental stages that proliferate inside the insect vector (originally the primary host) and a secondary host (a vertebrate or a plant) (Figure 1). To differentiate into the next developmental stage, parasites must have evolved mechanisms to sense and respond to the microenvironment imposed by their different hosts in order to adapt their biology to extracellular or intracellular survival. This raises important questions on the nature of their sensory system, the kind of signals they detect, the activation they produce and the way they integrate this complex information. Understanding how these transitions are regulated and defined in space and time will lead to unique insights into mechanisms of Trypanosomatid developmental biology with relevance to virulence and pathogenesis, since parasite differentiation is a pre-requisite to the success of vector infection, vector-host transmission, and propagation in the host (Fenn *et al.*, 2007). Based on the observations that *Leishmania* MAP kinases are implicated in parasite environmental sensing and flagellar biogenesis, we propose here a new model in which the flagellum could act as a sensing organelle in trypanosomatid parasites. The objective of this review is less to summarize the current literature on Trypanosmatid MAP kinases and flagellar biology, which both have been reviewed recently (Wiese, 2007; Ralston and Hill, 2008), but beyond this to provide new

perspectives on how these two processes may be linked and how to approach this model experimentally.

TRYPANOSOMATID MAP KINASES:, MULTIPLE ROLES IN ENVIRONMENTAL SENSING, VIRULENCE AND FLAGELLAR LENGTH CONTROL

Stage differentiation is likely triggered by environmental signals, which are sensed and transduced through signalling networks of protein kinases and phosphatases. Genomic and proteomic studies of the membrane content in Trypanosomatids revealed the presence of group-specific sets of proteins involved in numerous signalling pathways well established in other organisms such as the cAMP cascade or the MAP kinases (Bridges *et al.*, 2008, El-Sayed *et al.*, 2005). The Trypanosomatid kinome lacks members of the receptor-linked or cytosolic tyrosine kinase families, but has an abundance of STE and CMGC family protein kinases, which include the members of the canonical MAP kinase cascade (MAPKS, MKS and MKKKs) involved in environmentally regulated cell cycle control, differentiation and the cellular response to various stress signals (Naula *et al.*, 2005). These two protein kinase families alone correspond to more than 40% of the conserved Trypanosomatid kinome (Parsons *et al.*, 2005), suggesting that kinases implicated in environmental sensing underwent considerable evolutionary expansion compared for example to humans, where STE and CMGC comprise only ca. 20% of the kinome. Conceivably, this expansion may reflect the particular requirements of Trypanosomatids to adapt to changes in the host environment during their infectious cycle. In *Leishmania major*, 17 MAPKs (MPK) and MAPK-like kinases have been identified (Parsons *et al.*, 2005). These are conserved in *T. brucei* and *T. cruzi* with two exceptions (MPK7 and MPK8) (for an exhaustive review, see Wiese, 2007).

The first MAPK identified and characterized in *T. brucei,* KFR1, is essential for the

procyclic insect stage and shows increased mRNA and protein abundance in the bloodstream form compared to the procyclic form (Hua *et al.*, 1994). Subsequent studies revealed that increased expression correlated with enhanced KFR1 activity, which was induced by host serum (Hua *et al.*, 1997). The *Leishmania* ortholog of KFR1, MPK1 (Table 1), is involved in intracellular parasite survival as revealed by the reduced parasite load of *L. mexicana* null mutant in macrophage infection experiments, as well as by reduced lesion formation in infected mice (Wiese, 1998).

A direct implication of MAPKs in *T. brucei* growth control and differentiation was revealed by gene targeting experiments. Null mutants of TbMAPK2, the orthologue of MPK4 in *Leishmania* (Table 1), proliferated normally in culture at the bloodstream stage but differentiated less efficiently into the procyclic insect stage, resulting in non-synchronous cell cycle arrest (Muller *et al.*, 2002). In contrast to *T. brucei*, attempts to generate a double knock-out of the MPK4 orthologue in *L. mexicana* were unsuccessful (Wang *et al.*, 2005), and null mutants were recovered only in the presence of an ectopic copy of the gene. Moreover, the parasites retained the episome with the transgene in culture and in infected mice in the absence of selection, suggesting an essential role of MPK4 at both life cycle stages. Genetic deletion of TbMAPK5, the orthologue of MPK5 in *Leishmania* (Table 1), did not interfere with proliferation of *T. brucei* at the procyclic stage neither in culture nor during the developmental program in the tsetse fly (Domenicali Pfister *et al.*, 2006). However, absence of TbMAPK5 resulted in low-rate infections in mice, accompanied by premature differentiation to the non-proliferative stumpy form. Together these results suggest that TbMAPK2 and TbMAPK5 may have complementary roles in the control of proliferation in procyclic and bloodstream *T. brucei* developmental stages, respectively.

TbMAPK4 is characterized by a 46 amino acid insertion with unknown function,

which is conserved in position and length, but divergent in sequence in the *Leishmania* ortholog MPK12 (Guttinger *et al.*, 2007). Null mutants of TbMAPK4 grew and differentiated normally, but showed increased sensitivity to elevated temperature. However, no evidence of association with chaperones could be detected. HA-tagged TbMAPK4 immunoprecipitated from transgenic procyclic trypanosomes cultured at 37°C showed increased kinase activity when compared with protein obtained from cultures at 27°C. Similar results were shown by purification of epitope-tagged recombinant MAPKs from transgenic *L. major* and *L. donovani* (Morales *et al.*, 2007). An increased phosphotransferase activity of recombinant MPK4, 7 and 10 was observed in response to environmental changes similar to those encountered during amastigote differentiation (pH 5.5 and 37°C) in *L. major*, and after development of axenic amastigotes in *L. donovani.* Moreover, western blot-based analysis of crude and affinity purified phosphoprotein extracts showed that the endogenous MPK10 was constitutively expressed in both pro- and amastigote stages, but phosphorylated only in the axenic amastigote stage and thus likely activated by upstream kinases of the MAPK cascade. Hence trypansomatid MAPKs seem to be functionally conserved and implicated in environmentally-regulated signal transduction similar to other non-parasitic eukaryotes, including yeast.

In *Leishmania*, genetic studies revealed a rather surprising role for some MPKs in flagellar morphogenesis. *L. mexicana* null mutants of the MAPKK homolog LmxMKK showed a reduction in flagellar length and lacked the paraflagellar rod in promastigotes (Wiese *et al.*, 2003), a phenotype that was reproduced in MPK3 null-mutants (Erdmann *et al.*, 2006). Phosphorylation of MPK3 by LmxMKK provided evidence that both protein kinases are likely organized in a hierarchical manner characteristic to the canonical MAPK cascades in other organisms (Erdmann *et al.*, 2006). MPK9 null-mutants on the other hand displayed elongated flagella compared with wild type promastigotes (Bengs *et al.*, 2005), and over-

expression of this kinase led to shortened flagella in some subpopulations, implicating MPK9 in flagellar length regulation. Unlike in the green algae *Chlamydomonas*, where multiple protein kinase families have been involved in flagellum length control, our knowledge on signalling molecules associated with the Trypanosomatid flagellum is very limited and restricted to MAPKs (Berman et al., 2003) (Wilson, Bradley, Tam see EndNote file).

Together these observations establish a link between the role of *Leishmania* MAP kinases in environmental sensing (Morales *et al.*, 2007), and an unexpected novel function in flagellar biogenesis. Given the extensive modification of Trypanosomatid flagellar morphology during environmentally-induced stage differentiation (Figure 1) and the function of flagella as sensory organs in many organisms, the question arises if this intricate relationship between Trypanosomatid flagella and MAP kinases extends to an integrated sensory function. The following chapters summarize published data that supports this hypothesis, and propose testable models on putative sensory mechanisms.

THE FLAGELLUM, A MULTIFUNCTIONAL ORGANELLE WITH PUTATIVE SENSORY FUNCTION

Trypanosomatid species possess a single flagellum, composed of the axoneme, a set of 9 microtubule doublets with a central pair (conserved in most eukaryotes) and of the paraflagellar rod (PFR), a crystalline-like structure that is unique to a sub-set of protists (Kohl *et al.*, 2005, Ralston *et al.*, 2008). The flagellum emerges from the flagellar pocket (FP), an invagination of the membrane resembling an inverted flask that is the exclusive site for endocytosis and exocytosis and as such forms an important interface of parasite/host interactions (Bonhivers *et al.*, 2008). During the infectious cycle, the flagellum shows variations in its length, point of emergence, and position, but is always attached to the cell

body at a differentiated region of the cytoskeleton, the flagellum attachment zone (FAZ). FAZ is composed of two structures: a filament and four modified subpellicular microtubules that are associated with the smooth endoplasmic reticulum (Sherwin *et al.*, 1989). Both FAZ structures initiate at the vicinity of the flagellar pocket and run parallel to the flagellum. These structures are easily recognizable by transmission electron microscopy in epimastigote or trypomastigote stages of *Trypanosoma* as the region of adhesion is long (Rocha *et al.*, 2006, Sherwin *et al.*, 1989) but also in *Leishmania*, where they are limited to a short region close to the flagellar pocket (Weise *et al.*, 2000). Recent analysis reveal that proteins involved in flagellar adhesion to the cell body of *T. brucei* are conserved in *Leishmania* (Kohl *et al.*, 2005, LaCount *et al.*, 2002, Vaughan *et al.*, 2008).

During the Trypanosomatid life cycle, spectacular changes in flagellum length have been described: from 20 μm to barely 1 μm in *Leishmania*, and from 10 to close to 40 μm in African trypanosomes. Studies of the *Chlamydomonas* flagellum and of sensory cilia of the nematode *Caenorhabditis elegans* revealed that the dynamics of the organelle is controlled by a process called intraflagellar transport (IFT) (Scholey, 2008), a bidirectional movement of particles between the flagellum membrane and the axoneme. It is operated by kinesin and dynein motors that move complexes of 15-18 proteins (Rosenbaum *et al.*, 2002), referred to as IFT rafts or IFT particles. In *Chlamydomonas*, these particles have been shown to transport axoneme precursors to the distal tip of the flagellum where they are assembled (Qin *et al.*, 2004). Inhibition of IFT by inactivation of any of the dynein/kinesin motors or any of the protein components of the IFT particle results in severe reduction of flagellum formation (Rosenbaum *et al.*, 2002). In *T. brucei* , this was first demonstrated upon RNAi silencing of the dynein motor or of the IFT88 protein (Kohl *et al.*, 2003) and subsequently of other IFT protein components (Absalon *et al.*, 2008b, Absalon *et al.*, 2007, Davidge *et al.*, 2006),

including 5 novel proteins termed PIFT (Absalon *et al.*, 2008b)(Adhiambo et al., Journal of Cell Science, in press, see EndNote file). Deletion of the dynein motor responsible for retrograde transport in the promastigote stage of *L. mexicana* leads to formation of a very small flagellum, without microtubules and PFR (Adhiambo *et al.*, 2005) reminiscent of the MKK and MPK3 null mutants in *L.mexicana* (Erdmann *et al.*, 2006). Remarkably, cell shape was drastically modified in both organisms, with a direct correlation between cell body size and flagellum length in trypanosomes (Kohl *et al.*, 2003) and with an evolution towards an oval-shaped morphology in *Leishmania* (Adhiambo *et al.*, 2005), revealing a role for the flagellum in the control of cell body shape and size. In conclusion, aside from its primary function in parasite motility, the flagellum is crucial for many aspects of Trypanosomatid biology, including flagellar pocket organization, exo- and endocytosis, cell size regulation, and cell division (Absalon *et al.*, 2008a).

Recently, cilia and flagella have emerged as critical sensing organs in unicellular or multicellular organisms (Singla *et al.*, 2006). They can be compared to cellular antennas with sensory function based on their defined positioning and orientation at the cell surface and to their high concentrations of sensing molecules (receptors and effectors), which are dynamically controlled by the action of intraflagellar transport (IFT) (Marszalek *et al.*, 2000, Scholey, 2008). Moreover, IFT has been shown to displace membrane proteins in primary cilia of mammalian cells (Kovacs *et al.*, 2008, Molla-Herman *et al.*, 2008), in cilia of sensory neurons in *C. elegans* (Qin *et al.*, 2005) or in flagella of the green algae *Chlamydomonas* (Wang *et al.*, 2005). IFT could therefore contribute to the sensory function of cilia and flagella by concentrating receptors but also by trafficking proteins upon receptor activation. IFT activity has been demonstrated in trypanosomes in both elongating and mature flagella (Absalon *et al.*, 2008b), suggesting it could contribute to other processes than organelle

assembly. We propose that the Trypanosomatid flagellum could fulfil a sensory role by concentrating receptors and effectors involved in environmental perception, and by exchanging these signalling molecules with the cell body through the dynamic action of IFT, which in turn may modulate flagellum behaviour itself.

MECHANISMS OF FLAGELLUM SIGNALLING

Flagellum signalling is likely to occur bi-directionally: from the cell body towards the flagellum in the case of flagellar biogenesis, and from the flagellum towards the cell body in the case of flagellum sensing (Figure 2). The first direction, regulation of flagellar dynamics through the MAP kinase pathway is well documented in *Leishmania*, *Chlamydomonas* and *C.elegans* (Berman *et al.*, 2003, Burghoorn *et al.*, 2007, Wiese, 2007) and most likely occurs at the level of IFT. At least three possibilities can be considered:

(1) Modulation of anterograde (kinesin-2) or retrograde (dynein) motor functions. Localization, activity and interactions of IFT components may be regulated directly or indirectly through phosphorylation by MAP kinases. In other eukaryotes, motor proteins as well as motor-associated proteins such the KAP3A sub-unit can be the targets of MAP kinases (Cuchillo-Ibanez *et al.*, 2008, Lukong *et al.*, 2008). Recently, an intriguing relationship between kinesin motors and the MAP kinase pathway has been established through their common interaction with scaffolding proteins. These proteins have dual function in kinesin cargo turnover and organizing the MAP kinase cascade (Horiuchi *et al.*, 2007). This opens the possibility that MAP kinases control IFT and kinesins via common interaction partners, and in return may implicate kinesins in regulation of MAPK trafficking and interactions. This hypothesis is supported by findings in *Drosophila* that null mutants of the Wnd-Hep-Bsk MAPK cascade show defects in axonal transport similar to kinesin-1 mutants (Horiuchi *et al.*,

2007). In addition, phosphoproteomic analysis of *Leishmania donovani* promastigotes and axenic amastigotes identified various isoforms of a C-terminal motor kinesin as stage-specific phosphoproteins, confirming that kinesin family members are kinase substrates in Trypanosomatids (Morales *et al.*, 2008).

(2) Control of flagellar gene expression. Construction of a flagellum requires a large amount of proteins (more than 300 for the cytoskeletal fraction (Broadhead *et al.*, 2006)) whose production must be coordinated both in time and in space. In all eukaryotes studied so far, this is controlled at the transcriptional level, with a burst in synthesis of mRNA corresponding to flagellar genes prior to flagellum assembly. Across many eukaryotic organisms, specific transcription factors such as FoxJ1 (Stubbs *et al.*, 2008, Yu *et al.*, 2008), RFX/DAF19 (Dubruille *et al.*, 2002, Swoboda *et al.*, 2000) or HFH-4 (Chen *et al.*, 1998) are controlling the expression of groups of flagellar genes and their inhibition results in improper flagellum formation. The situation is different in Trypanosomatids where gene expression is mostly controlled at the post-transcriptional level and recent data indicate that flagellar gene expression is no exception. Inhibition of flagellum formation upon RNAi knock-down of IFT gene expression results in a dramatic loss in the total amount of structural proteins from the axoneme and of the PFR, although their mRNA was not the RNAi target. This dramatic effect on protein abundance is not linked to mRNA reduction as the total amount of axoneme or PFR mRNA remains unchanged (Absalon *et al*., manuscript in preparation). MAP kinases could act on flagellar gene expression by phosphorylation of factors required for maturation of polycistronic transcripts into mature mRNA through trans-splicing, RNA binding proteins involved in transcript turnover, or factors that regulate the translation of flagellar mRNAs.

(3) Modification of IFT loading without a direct effect on motor proteins. An effect of MAP kinases could also occur at the level of IFT proteins themselves where phosphorylation

of one or more components could directly stimulate or interfere with the assembly of IFT particles. A large pool of IFT proteins is found at the basal body of the flagellum where proteins are not actively engaged in IFT. The abundance of IFT proteins analyzed in detail also appears identical at the basal bodies of mature or elongating flagella (Absalon *et al.*, 2008b). However, new subunits are mainly targeted to the new flagellum (Bastin *et al.*, 1999), suggesting that loading of IFT particles with flagellar components can discriminate between an elongating and a mature flagellum. Differential phosphorylation of IFT components or possibly of proteins involved in loading flagellar precursors to IFT particles at the base of the flagellum could provide a molecular explanation to this different "identity" of the two flagella.

The second direction of flagellar signalling, from the flagellum towards the cell body in the case of flagellar sensing, remains more elusive but several hypotheses deserve to be discussed that may provide useful working models for future investigations. A putative "antenna" function (Singla *et al.*, 2006) of the Trypanosomatid flagellum independent from its primary role in cell motility may rely on the below proposed mechanisms or various combinations thereof:

(1) Sensing through extra-axonemal structures. A first original aspect linking the flagellum to potential sensing functions is the presence of the PFR that is found from the point of emergence from the FP and runs parallel to the axoneme until the distal tip of the flagellum. In *Trypanosoma*, phosphodiesterase and adenylate kinase (two enzymes involved in the cyclic AMP pathway) are tightly associated to the PFR (Oberholzer *et al.*, 2007, Pullen *et al.*, 2004), suggesting this structure could act as a controller of sensory signals. These enzymes could participate to the production of metabolites that regulate the activity of the axoneme. This could provide an explanation for the phenotype of *Leishmania* (Maga *et al.*,

1999, Santrich *et al.*, 1997) or *Trypanosoma* (Bastin *et al.*, 1998) deprived of PFR that fail to swim properly despite the presence of a normal axoneme.

(2) Receptor-mediated signalling. The flagellum may be at the forefront of the possible direct responses a cell could produce to environmental signals: changing motility (direction, speed, orientation), altering its length (longer or shorter, with an effect on both flagellar length and cell body length) and attaching to substrates. These processes are relevant for example during parasites attachment to host tissues in insects. As they swim with their flagellum leading, the tip of this organelle is expected to make contact with an appropriate surface, as observed for example in *Leishmania*, where the interaction of surface lipophosphoglycan at the flagellar tip anchors promastigotes to the sandfly midgut epithelium via interaction with galectin (Kamhawi *et al.*, 2004). This kind of parasite/host surface interaction may extend beyond glycolipids and may implicate potentially promastigote surface proteins with signalling functions. This would result in activation of an effector that could translocate to the cell body, leading to production of proteins and lipids required for flagellum extension and development of the hemi-desmosome like structures typical of adhesion to host tissues (Kollien *et al.*, 1998, Tetley *et al.*, 1985). In such a situation (that remains to be demonstrated), the flagellum would perform both functions: sensor, by detecting the host tissue, and effector, by activating formation of the necessary structure to allow adhesion, and potentially down-stream effects on cellular processes such as RNA stability or protein translation.

(3) Trafficking of signalling molecules. In rat neurons, dynein- and microtubule-based transport was proven to be necessary for nerve growth factor tyrosine receptor kinase A signalling to Rap1 and MAPK1/2 (Wu *et al.*, 2007). The recently identified interplay between kinesin motors and members of the MAP kinase cascade through their interaction with

common scaffolding proteins (Horiuchi *et al.*, 2007) opens the possibility that IFT may regulate the transport and thus the local concentration of important signalling molecules. This may be of particular importance in the developmental transition of the *Leishmania* promastigote stage to the amastigote stage, which is characterized by a shorter flagellum that still emerges from the flagellar pocket but lacks the PFR (Kohl *et al.*, 2005). The main environmental signals known to induce amastigote differentiation (acidic pH and increased temperature) are the same that activate MAPK (Morales *et al.*, 2007, Zilberstein *et al.*, 1994), hence one could postulate that increased kinase activities may affect/suppress IFT by phosphorylation of motor or IFT proteins. Little is known about the regulation of IFT in general, but recent findings in *Leishmania* indicate two possible modes of action. First, interruption of the cycling of the small G protein *Ld*ARL-3A between a GDP- and a GTP-bound form leads to the reduction of the flagellum length(Sahin *et al.*, 2008). Second, a kinesin motor is able to trigger microtubule disassembly from the distal tip (Blaineau *et al.*, 2007). MAPK signalling may interfere with G protein functions or with kinesin, resulting in the characteristic shortening of the flagellum observed during the promastigote/amastigote differentiation..

INVESTIGATING FLAGELLAR SIGNALLING

The challenge now is to experimentally approach these hypotheses and investigate the putative sensing function of the Trypanosomatid flagellum. The experimental framework to respond to this question is in place with various MAP kinase mutants and flagellar mutants generated in *Leishmania* and *Trypanosoma,* respectively (Absalon *et al.*, 2008b, Bastin *et al.*, 1998, Kohl *et al.*, 2003, Wiese, 2007), and the accessibility of the parasite phosphoproteome for qualitative and quantitative assessment (Morales *et al.*, 2008). Comparative

phosphoproteomic analysis of these mutants through quantitative 2DE and LC-MS-MS analysis may be able to (i) establish specific kinase-substrate relationships between the *Leishmania* MAPKs implicated in flagellar length control, and proteins that control IFT in Trypanosomatids, and (ii) reveal changes in the phosphoproteome profile of flagellar mutants and thus establish a first experimental insight into flagellar sensing. This will require identification of the complete proteome of the membrane and matrix fractions from the flagellum of various stages of both parasites. At present, only the proteome from the cytoskeletal fraction of flagella purified from the procyclic stage of *T. brucei* has been determined. Nevertheless, it confirmed the flagellar localisation of candidate signalling enzymes such as phosphodiesterases and adenylate kinases (Broadhead *et al.*, 2006). These approaches may be complemented by cellular assays investigating (i) IFT activity in MAPK mutants through monitoring GFP::IFT fusion proteins by live video-microscopy, and (ii) MAP kinase activities in flagellar mutants, for example using transgenic approaches to obtain parasite recombinant kinases from these mutants, which will be tested by *in vitro* kinase assays (Morales *et al.*, 2007). Finally, recombinantly expressed IFT proteins combined with MAP kinase-enriched protein fractions (Wissing *et al.*, 2007), or recombinantly expressed MAP kinases combined with flagellar fractions may be employed to reveal specific interactions. This series of genetic, proteomic, cellular, and biochemical approaches should lead to an integrative view of the implication of the MAP kinases in flagellum biogenesis and its putative sensing functions.

Acknowledgements. We would like to thank Artur Scherf for discussion and support, and Geneviève Milon, Eric Prina, and Dirk Schmidt-Arras for critical reading of the manuscript. GFS and MAM were supported by the INSERM Avenir program. BR is funded by a post-

doctoral fellowship from a Fonds Dédié Sanofi-Aventis/Ministère de la Recherche 'Combattre les Maladies Parasitaires'.

Figure legends

Figure 1: *Infectious cycle of two of the major human infective Trypanosomatids.* For each phase of the cycle, Trypanosomatids develop highly specialized stages that are adapted for transmission and survival in the insect vector or the vertebrate host. These stages differentiate in response to environmental signals, and each stage is characterized by a distinct flagellar morphology.

Figure 2: *Model of the MAP kinase/flagellum interplay.* Regulation of flagellum dynamics through MAPKs, and conversely of MAPK signalling through flagellar sensing, may potentially rely on intra-flagellar transport (IFT), extra-axonemal structures such as the paraflagellar rod (PFR), or common interactions with scaffolding proteins. Modulation of these processes by host environmental factors may have profound effects on the parasite differentiation state.

Table 1: Overview of *L. major* and *T. brucei* MAP kinase orthologs discussed in this review. n.d., not determined.

Leishmania major			***Trypanosoma brucei***		
Name	**acc. no**	**Putative functions**	**Name**	**acc. no**	**Putative functions**
MPK1	LmjF36.6470	Essential for intracellular parasite survival, (Wiese, 1998).	KFR1	Tb10.6k15.2790	Essential for procyclic stage, (Hua *et al.*, 1994).
MPK3	LmjF10.0490	Null mutants display reduced flagella, (Erdmann *et al.*, 2006).	n.d.	Tb927.8.3550	n.d.
MPK4	LmjF19.1440	Unknown. Potentially essential for pro- and amastigote stages, (Wang et al., 2005).	TbMAPK2	Tb10.70.2070	Growth control and potential implication in differentiation, (Muller *et al.*, 2002).
MPK7	LmjF13.1640	Potential association with HSP70, (Morales *et al.*, 2007).	-	No ortholog	-
MPK12	LmjF30.0370	Not essential for for pro- or amastigote stage, (Wiese, 2007).	TbMAPK4	Tb10.61.0250	Implication in environmentally-regulated signalling, (Guttinger *et al.*, 2007).
MPK5	LmjF30.2910	Potential attenuation in virulence, (Wiese, 2007).	TbMAPK5	Tb927.6.4220	Control of proliferation in the bloodstream Stage, (Domenicali Pfister *et al.*, 2006).
MPK9	LmjF19.0180	Potential role in flagellar length regulation, (Bengs *et al.*, 2005).	n.d.	Tb10.61.1850	n.d.
MPK10	LmjF10.0200	Amastigote-specific phosphorylation and kinase activity, (Morales *et al*., 2007).	n.d.	Tb927.8.3770	n.d.

References

Absalon, S., Blisnick, T., Bonhivers, M., Kohl, L., Cayet, N., Toutirais, G.*, et al.* (2008a). Flagellum elongation is required for correct structure, orientation and function of the flagellar pocket in Trypanosoma brucei. *J Cell Sci* 121, 3704-3716.

Absalon, S., Blisnick, T., Kohl, L., Toutirais, G., Dore, G., Julkowska, D.*, et al.* (2008b). Intraflagellar transport and functional analysis of genes required for flagellum formation in trypanosomes. *Mol Biol Cell* 19, 929-944.

Absalon, S., Kohl, L., Branche, C., Blisnick, T., Toutirais, G., Rusconi, F.*, et al.* (2007). Basal body positioning is controlled by flagellum formation in Trypanosoma brucei. *PLoS ONE* 2, e437.

Adhiambo, C., Forney, J.D., Asai, D.J. and LeBowitz, J.H. (2005). The two cytoplasmic dynein-2 isoforms in Leishmania mexicana perform separate functions. *Mol Biochem Parasitol* 143, 216-225.

Bastin, P., Pullen, T.J., Sherwin, T. and Gull, K. (1999). Protein transport and flagellum assembly dynamics revealed by analysis of the paralysed trypanosome mutant snl-1. *J Cell Sci* 112 (Pt 21), 3769-3777.

Bastin, P., Sherwin, T. and Gull, K. (1998). Paraflagellar rod is vital for trypanosome motility. *Nature* 391, 548.

Bengs, F., Scholz, A., Kuhn, D. and Wiese, M. (2005). LmxMPK9, a mitogen-activated protein kinase homologue affects flagellar length in Leishmania mexicana. *Mol Microbiol* 55, 1606-1615.

Berman, S.A., Wilson, N.F., Haas, N.A. and Lefebvre, P.A. (2003). A novel MAP kinase regulates flagellar length in Chlamydomonas. *Curr Biol* 13, 1145-1149.

Blaineau, C., Tessier, M., Dubessay, P., Tasse, L., Crobu, L., Pages, M. and Bastien, P. (2007). A novel microtubule-depolymerizing kinesin involved in length control of a eukaryotic flagellum. *Curr Biol* 17, 778-782.

Bonhivers, M., Nowacki, S., Landrein, N. and Robinson, D.R. (2008). Biogenesis of the trypanosome endo-exocytotic organelle is cytoskeleton mediated. *PLoS Biol* 6, e105.

Branche, C., Kohl, L., Toutirais, G., Buisson, J., Cosson, J. and Bastin, P. (2006). Conserved and specific functions of axoneme components in trypanosome motility. *J Cell Sci* 119, 3443-3455.

Bridges, D.J., Pitt, A.R., Hanrahan, O., Brennan, K., Voorheis, H.P., Herzyk, P.*, et al.* (2008). Characterisation of the plasma membrane subproteome of bloodstream form Trypanosoma brucei. *Proteomics* 8, 83-99.

Broadhead, R., Dawe, H.R., Farr, H., Griffiths, S., Hart, S.R., Portman, N.*, et al.* (2006). Flagellar motility is required for the viability of the bloodstream trypanosome. *Nature* 440, 224-227.

Burghoorn, J., Dekkers, M.P., Rademakers, S., de Jong, T., Willemsen, R. and Jansen, G. (2007). Mutation of the MAP kinase DYF-5 affects docking and undocking of kinesin-2 motors and reduces their speed in the cilia of Caenorhabditis elegans. *Proc Natl Acad Sci U S A* 104, 7157-7162.

Chen, J., Knowles, H.J., Hebert, J.L. and Hackett, B.P. (1998). Mutation of the mouse hepatocyte nuclear factor/forkhead homologue 4 gene results in an absence of cilia and random left-right asymmetry. *J Clin Invest* 102, 1077-1082.

Cuchillo-Ibanez, I., Seereeram, A., Byers, H.L., Leung, K.Y., Ward, M.A., Anderton, B.H. and Hanger, D.P. (2008). Phosphorylation of tau regulates its axonal transport by controlling its binding to kinesin. *FASEB J* 22, 3186-3195.

Davidge, J.A., Chambers, E., Dickinson, H.A., Towers, K., Ginger, M.L., McKean, P.G. and Gull, K. (2006). Trypanosome IFT mutants provide insight into the motor location for mobility of the flagella connector and flagellar membrane formation. *J Cell Sci* 119, 3935-3943.

Domenicali Pfister, D., Burkard, G., Morand, S., Renggli, C.K., Roditi, I. and Vassella, E. (2006). A Mitogen-activated protein kinase controls differentiation of bloodstream forms of Trypanosoma brucei. *Eukaryot Cell* 5, 1126-1135.

Dubruille, R., Laurencon, A., Vandaele, C., Shishido, E., Coulon-Bublex, M., Swoboda, P*., et al.* (2002). Drosophila regulatory factor X is necessary for ciliated sensory neuron differentiation. *Development* 129, 5487-5498.

El-Sayed, N.M., Myler, P.J., Blandin, G., Berriman, M., Crabtree, J., Aggarwal, G*., et al.* (2005). Comparative genomics of Trypanosomatid parasitic protozoa. *Science* 309, 404-409.

Erdmann, M., Scholz, A., Melzer, I.M., Schmetz, C. and Wiese, M. (2006). Interacting protein kinases involved in the regulation of flagellar length. *Mol Biol Cell* 17, 2035-2045.

Fenn, K. and Matthews, K.R. (2007). The cell biology of Trypanosoma brucei differentiation. *Curr Opin Microbiol* 10, 539-546.

Guttinger, A., Schwab, C., Morand, S., Roditi, I. and Vassella, E. (2007). A mitogen-activated protein kinase of Trypanosoma brucei confers resistance to temperature stress. *Mol Biochem Parasitol* 153, 203-206.

Horiuchi, D., Collins, C.A., Bhat, P., Barkus, R.V., Diantonio, A. and Saxton, W.M. (2007). Control of a kinesin-cargo linkage mechanism by JNK pathway kinases. *Curr Biol* 17, 1313-1317.

Hua, S.B. and Wang, C.C. (1994). Differential accumulation of a protein kinase homolog in

Trypanosoma brucei. *J Cell Biochem* 54, 20-31.

Hua, S.B. and Wang, C.C. (1997). Interferon-gamma activation of a mitogen-activated protein kinase, KFR1, in the bloodstream form of Trypanosoma brucei. *J Biol Chem* 272, 10797-10803.

Kamhawi, S., Ramalho-Ortigao, M., Pham, V.M., Kumar, S., Lawyer, P.G., Turco, S.J.*, et al.* (2004). A role for insect galectins in parasite survival. *Cell* 119, 329-341.

Kohl, L. and Bastin, P. (2005). The flagellum of trypanosomes. *Int Rev Cytol* 244, 227-285.

Kohl, L., Robinson, D. and Bastin, P. (2003). Novel roles for the flagellum in cell morphogenesis and cytokinesis of trypanosomes. *EMBO J* 22, 5336-5346.

Kollien, A.H., Schmidt, J. and Schaub, G.A. (1998). Modes of association of Trypanosoma cruzi with the intestinal tract of the vector Triatoma infestans. *Acta Trop* 70, 127-141.

Kovacs, J.J., Whalen, E.J., Liu, R., Xiao, K., Kim, J., Chen, M.*, et al.* (2008). Beta-arrestin-mediated localization of smoothened to the primary cilium. *Science* 320, 1777-1781.

LaCount, D.J., Barrett, B. and Donelson, J.E. (2002). Trypanosoma brucei FLA1 is required for flagellum attachment and cytokinesis. *J Biol Chem* 277, 17580-17588.

Lukong, K.E. and Richard, S. (2008). Breast tumor kinase BRK requires kinesin-2 subunit KAP3A in modulation of cell migration. *Cell Signal* 20, 432-442.

Maga, J.A., Sherwin, T., Francis, S., Gull, K. and LeBowitz, J.H. (1999). Genetic dissection of the Leishmania paraflagellar rod, a unique flagellar cytoskeleton structure. *J Cell Sci* 112 (Pt 16), 2753-2763.

Marszalek, J.R., Liu, X., Roberts, E.A., Chui, D., Marth, J.D., Williams, D.S. and Goldstein, L.S. (2000). Genetic evidence for selective transport of opsin and arrestin by kinesin-II in mammalian photoreceptors. *Cell* 102, 175-187.

Molla-Herman, A., Boularan, C., Ghossoub, R., Scott, M.G., Burtey, A., Zarka, M.*, et al.*

(2008). Targeting of beta-arrestin2 to the centrosome and primary cilium: role in cell proliferation control. *PLoS ONE* 3, e3728.

Morales, M.A., Renaud, O., Faigle, W., Shorte, S.L. and Spath, G.F. (2007). Over-expression of Leishmania major MAP kinases reveals stage-specific induction of phosphotransferase activity. *Int J Parasitol* 37, 1187-1199.

Morales, M.A., Watanabe, R., Laurent, C., Lenormand, P., Rousselle, J.C., Namane, A. and Spath, G.F. (2008). Phosphoproteomic analysis of Leishmania donovani pro- and amastigote stages. *Proteomics* 8, 350-363.

Muller, I.B., Domenicali-Pfister, D., Roditi, I. and Vassella, E. (2002). Stage-specific requirement of a mitogen-activated protein kinase by Trypanosoma brucei. *Mol Biol Cell* 13, 3787-3799.

Naula, C., Parsons, M. and Mottram, J.C. (2005). Protein kinases as drug targets in trypanosomes and Leishmania. *Biochim Biophys Acta* 1754, 151-159.

Oberholzer, M., Marti, G., Baresic, M., Kunz, S., Hemphill, A. and Seebeck, T. (2007). The Trypanosoma brucei cAMP phosphodiesterases TbrPDEB1 and TbrPDEB2: flagellar enzymes that are essential for parasite virulence. *FASEB J* 21, 720-731.

Parsons, M., Worthey, E.A., Ward, P.N. and Mottram, J.C. (2005). Comparative analysis of the kinomes of three pathogenic Trypanosomatids: Leishmania major, Trypanosoma brucei and Trypanosoma cruzi. *BMC Genomics* 6, 127.

Pullen, T.J., Ginger, M.L., Gaskell, S.J. and Gull, K. (2004). Protein targeting of an unusual, evolutionarily conserved adenylate kinase to a eukaryotic flagellum. *Mol Biol Cell* 15, 3257-3265.

Qin, H., Burnette, D.T., Bae, Y.K., Forscher, P., Barr, M.M. and Rosenbaum, J.L. (2005). Intraflagellar transport is required for the vectorial movement of TRPV channels in the

ciliary membrane. *Curr Biol* 15, 1695-1699.

Qin, H., Diener, D.R., Geimer, S., Cole, D.G. and Rosenbaum, J.L. (2004). Intraflagellar transport (IFT) cargo: IFT transports flagellar precursors to the tip and turnover products to the cell body. *J Cell Biol* 164, 255-266.

Ralston, K.S. and Hill, K.L. (2008). The flagellum of Trypanosoma brucei: new tricks from an old dog. *Int J Parasitol* 38, 869-884.

Rocha, G.M., Brandao, B.A., Mortara, R.A., Attias, M., de Souza, W. and Carvalho, T.M. (2006). The flagellar attachment zone of Trypanosoma cruzi epimastigote forms. *J Struct Biol* 154, 89-99.

Rosenbaum, J.L. and Witman, G.B. (2002). Intraflagellar transport. *Nat Rev Mol Cell Biol* 3, 813-825.

Sahin, A., Espiau, B., Marchand, C. and Merlin, G. (2008). Flagellar length depends on LdARL-3A GTP/GDP unaltered cycling in Leishmania amazonensis. *Mol Biochem Parasitol* 157, 83-87.

Santrich, C., Moore, L., Sherwin, T., Bastin, P., Brokaw, C., Gull, K. and LeBowitz, J.H. (1997). A motility function for the paraflagellar rod of Leishmania parasites revealed by PFR-2 gene knockouts. *Mol Biochem Parasitol* 90, 95-109.

Scholey, J.M. (2008). Intraflagellar transport motors in cilia: moving along the cell's antenna. *J Cell Biol* 180, 23-29.

Sherwin, T. and Gull, K. (1989). The cell division cycle of Trypanosoma brucei brucei: timing of event markers and cytoskeletal modulations. *Philos Trans R Soc Lond B Biol Sci* 323, 573-588.

Singla, V. and Reiter, J.F. (2006). The primary cilium as the cell's antenna: signaling at a sensory organelle. *Science* 313, 629-633.

Stuart, K., Brun, R., Croft, S., Fairlamb, A., Gurtler, R.E., McKerrow, J.*, et al.* (2008). Kinetoplastids: related protozoan pathogens, different diseases. *J Clin Invest* 118, 1301-1310.

Stubbs, J.L., Oishi, I., Izpisua Belmonte, J.C. and Kintner, C. (2008). The forkhead protein Foxj1 specifies node-like cilia in Xenopus and zebrafish embryos. *Nat Genet* 40, 1454-1460.

Swoboda, P., Adler, H.T. and Thomas, J.H. (2000). The RFX-type transcription factor DAF-19 regulates sensory neuron cilium formation in C. elegans. *Mol Cell* 5, 411-421.

Tetley, L. and Vickerman, K. (1985). Differentiation in Trypanosoma brucei: host-parasite cell junctions and their persistence during acquisition of the variable antigen coat. *J Cell Sci* 74, 1-19.

Vaughan, S., Kohl, L., Ngai, I., Wheeler, R.J. and Gull, K. (2008). A repetitive protein essential for the flagellum attachment zone filament structure and function in Trypanosoma brucei. *Protist* 159, 127-136.

Wang, Q., Melzer, I.M., Kruse, M., Sander-Juelch, C. and Wiese, M. (2005). LmxMPK4, a mitogen-activated protein (MAP) kinase homologue essential for promastigotes and amastigotes of Leishmania mexicana. *Kinetoplastid Biol Dis* 4, 6.

Weise, F., Stierhof, Y.D., Kuhn, C., Wiese, M. and Overath, P. (2000). Distribution of GPI-anchored proteins in the protozoan parasite Leishmania, based on an improved ultrastructural description using high-pressure frozen cells. *J Cell Sci* 113 Pt 24, 4587-4603.

Wiese, M. (1998). A mitogen-activated protein (MAP) kinase homologue of Leishmania mexicana is essential for parasite survival in the infected host. *EMBO J* 17,

2619-2628.

Wiese, M. (2007). Leishmania MAP kinases--familiar proteins in an unusual context. *Int J Parasitol* 37, 1053-1062.

Wiese, M., Wang, Q. and Gorcke, I. (2003). Identification of mitogen-activated protein kinase homologues from Leishmania mexicana. *Int J Parasitol* 33, 1577-1587.

Wissing, J., Jansch, L., Nimtz, M., Dieterich, G., Hornberger, R., Keri, G.*, et al.* (2007). Proteomics analysis of protein kinases by target class-selective prefractionation and tandem mass spectrometry. *Mol Cell Proteomics* 6, 537-547.

Wu, C., Ramirez, A., Cui, B., Ding, J., Delcroix, J.D., Valletta, J.S.*, et al.* (2007). A functional dynein-microtubule network is required for NGF signaling through the Rap1/MAPK pathway. *Traffic* 8, 1503-1520.

Yu, X., Ng, C.P., Habacher, H. and Roy, S. (2008). Foxj1 transcription factors are master regulators of the motile ciliogenic program. *Nat Genet* 40, 1445-1453.

Zilberstein, D. and Shapira, M. (1994). The role of pH and temperature in the development of Leishmania parasites. *Annu Rev Microbiol* 48, 449-470.