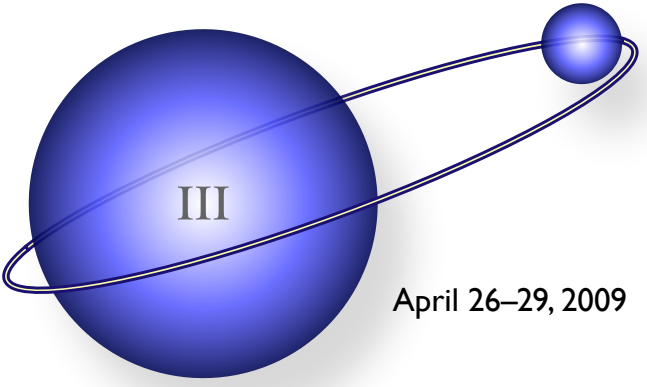
Kinetoplastid Molecular Cell Biology

An International Conference

Marine Biological Laboratory · Woods Hole · MA · USA



April 26–29, 2009

KMCBM 2009 Acknowledgements

Third Kinetoplastid Molecular Cell Biology Meeting, April 26–29, 2009

Organized by George A. M. Cross (The Rockefeller University, New York, USA)

Hosted by the Marine Biological Laboratory, Woods Hole, Massachusetts, USA

The organizer wishes to thank:

The Program Committee:

Vivian Bellofatto (University of Medicine and Dentistry of New Jersey, Newark, USA)
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Eileen McDonald, Director Housing, Conferences & Dining Services & her staff- Judith Taylor for Conferencing; Deborah Deering, Suzanne Livingstone, Cindy MacDonald, Darren Salo, Barbara Stackhouse for Housing; Cheryl Greene & Sodexho Dining Services staff; Noreen McNamara & Housekeeping staff.

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KMCBM 2009 Program

Sunday, April 26

02:00 – 05:00	Arrival, Registration and POSTER SECTION A set–up*
05:00 – 06:30	Dinner
07:00 – 09:00	Session I: Cell Biology (chair: Jay Bangs)
09:00 – 11:00	Mixer

* Registration tables will be located in Swope Building, 5 North Street. Please contact the reception desk inside Swope for registration after this period. All talks will be in Lillie.

* POSTER SECTION A: please put up your posters as soon as possible after arrival. You must remove them by 11:00 pm on Monday to allow SECTION B Posters to go up.

Monday, April 27

07:00 – 08:30	Breakfast
08:45 – 12:00	Session II: Biochemistry (chair: Buddy Ullman)
12:00 – 01:30	Lunch
01:30 – 04:30	Session III: Genetics (chair: Marc Ouellette)
05:00 – 06:30	Dinner
06:30 – 09:00	POSTER PRESENTATIONS Section A
09:00 – 11:00	Mixer

Tuesday, April 28

07:00 – 08:30	Breakfast
08:45 – 11:30	Session IV: Nuclear Structure and Function (chair: Christian Tschudi)
12:00 – 01:30	Lunch
01:30 – 04:15	Session V: Kinetoplast and Mitochondrion (chair: Michele Klingbeil)
05:00 – 06:30	Dinner
06:30 – 09:00	POSTER PRESENTATIONS Section B
09:00 – 11:00	Mixer

Wednesday, April 29

07:00 – 08:30	Breakfast
08:45 – 11:00	Session VI: Gene Expression (chair: Piet Borst)
11:00	Lunch. Lunch boxes will be available for those who would like them (numbers will be determined on TUESDAY)

ROOMS MUST BE VACATED BY 10:00 am ON WEDNESDAY

TALK SESSIONS: I - VI

April 26 7:00p.m. Chair - Jay Bangs

Session I: Cell Biology

1A Dynamics of flagellum formation in trypanosomes. *Buisson, J., Blisnick, T., Julkowska, D., Rotureau, B.,* ***Bastin, P.***

1B How do procyclic and bloodstream form *T. brucei* trypomastigotes divide? Some answers and implications. *Vaughan, S., Wheeler, R., Palmer, R.,* ***Gull, K.***

1C Structure-function analysis of flagellar proteins identifies viable bloodstream-form motility mutants in T. brucei. ***Hill, K.****, Ralston, K., Kisalu, N., Kabututu, Z., Melehani, J., Thayer, M.*

1D Structure and function of the *Leishmania* amastigote flagellum. ***Gluenz, E.****, Smith, A., Höög, J., Dawe, H., Heah, C., Shaw, M., Gull, K.*

1E A glycosomal DxDxT phosphatase, *Tb*PIP39, is a substrate of *Tb*PTP1 in the *T.brucei* differentiation signalling pathway. ***Szoor, B.****, Ruberto, I., Burchmore, R., Matthews, K.*

1F NDR protein kinases as drug targets in *Trypanosoma brucei*. *Ma, J., Grimaldi, R., Cleghorn, L., Benz, C., Stockdale, C., Woodland, A., Wyatt, P., Frearson, J.,* ***Hammarton, T.***

1G Eradication of animal and human infective African trypanosomes by TLF gene therapy. *Thomson, R., Molina-Portela, P.,* ***Raper, J.***

April 27 8:45 a.m. Chair - Buddy Ullman

Session II: Biochemistry

2A *Trypanosoma brucei* adaptor protein-3 (AP-3) β subunit is required for acidocalcisome biogenesis and endocytosis. *Rohloff, P., Fang, J., SantAnna, C., Wellems, D.,* ***Docampo, R.***

2B Developmentally Regulated Sphingolipid Synthesis in African Trypanosomes. *Sevova, E., Sutterwala, S., Hsu, F., Schwartz, K., Zhang, K., Key, P., Goren, M., Fox, B., Turk, J., Beverley, S.,* ***Bangs, J.***

2C The Achilles Heel of the Purine Salvage Pathway of *Leishmania donovani*. ***Boitz, J.****, Ullman, B.*

2D Systems analysis of metabolism in *Leishmania major:* elucidation of high-priority drug targets. ***Chavali, A.****, Papin, J.*

Coffee Break

2E Pterin Metabolism in African Trypanosomes. ***Ong, H.****, Wyllie, S., Fairlamb, A.*

2F Characterization of potassium channels in *Trypanosoma cruzi*. ***Jimenez, V.****, Wagner, J., Docampo, R.*

2G Transporters of S-Adenosyl-methionine in and out of *Leishmania* cells. ***Dridi, L.****, Ahmed Ouameur, A., Vergnes, B., Roy, G., Ouellette, M.*

2H Polyamine Transport in *Trypanosoma cruzi*. ***Hasne, M.****, Ullman, B.*

2I Dissection of the Fe-S cluster assembly in *Trypanosoma brucei*. ***Lukes, J.****, Long, S., Vavrova, Z., Paris, Z., Changmai, P., Wen, Y.*

2J Unusual inactivation of a MAP kinase from *Leishmania mexicana* by dual- and tyrosine phosphorylation in the TXY motif. *Melzer, I., Bleicher, N., Rosenqvist, H., Jensen, O.,* ***Wiese, M.***

April 27 1:30 p.m. Chair - Marc Ouellette

Session III: Genetics and Epigenetics

3A Application of second generation sequencing to parasite genomes. ***Hertz-Fowler, C.***

3B Four histone variants mark the boundaries of polycistronic transcription units in *Trypanosoma brucei*. ***Siegel, N.****, Hekstra, D., Kemp, L., Figueiredo, L., Lowell, J., Fenyo, D., Wang, X., Dewell, S., Cross, G.*

3C The enrichment of base J at polycistronic transcriptional termination sites in the genome of trypanosoma brucei is regulated by JBP1. ***Cliffe, L.****, Kieft, R., Marshall, M., Siegel, N., Cross, G., Sabatini, R.*

3D The phenotype of J-binding protein 2 (JBP2) knock-out in *Leishmania*. ***Vainio, S.****, Jan, S., van Luenen, H., Borst, P.*

Coffee Break

3E TbISWI binds ubiquitously in the *T. brucei* genome and is involved in repression of transcriptionally silent regions. ***Kushwaha, M.****, Stanne, T., Wand, M., Rudenko, G.*

3F Illuminating the endogenous small interfering RNA pathway in Trypanosoma brucei by deep sequencing. ***Franklin, J.****, Shi, H., Ullu, E., Tschdui, C.*

3G A novel component of the RNAi pathway in *Trypanosoma brucei* is required for loading siRNAs into Argonaute. ***Barnes, R.****, Shi, H., Tschudi, C., Ullu, E.*

3H RNAi in *Leishmania braziliensis*. ***Beverley, S.****, Lye, L., Owens, K., Notton, T., Shi, H., Vieira, A., Turco, S., Tschudi, C., Ullu, E.*

3I A genetic analysis of pathogenesis in Trypanosoma brucei. *Morrison, L., McLellan, S., Sweeney, L., Chan, C., Tait, A., MacLeod, A.,* ***Turner, M.***

April 28 8:45 a.m. Chair - Christian Tschudi

Session IV: Nuclear Structure and Function

4A Cohesin-mediated inheritance of monoallelic VSG expression in *Trypanosoma brucei*. *Landeira, D., Bart, J., Van Tyne, D.,* ***Navarro, M.***

4B Nuclear organization in Trypanosomatids. *Casanova, M., Blaineau, C., Crobu, L., Pages, M.,* ***Bastien, P.***

4C The Aurora-like Kinase in Trypanosoma brucei Plays Critical Roles of Promoting Metaphase-Anaphase Transition and Initiating Cyokinesis. ***Li, Z.****, Umeyama, T., Wang, C.*

4D Asymmetric chromosomal distribution leads to constitutive mosaicism in *Leishmania*. ***Sterkers, Y.****, Lachaud, L., Pages, M., Bastien, P.*

Coffee Break

4E Genome wide adaptable amplification in *Leishmania*. ***Ubeda, J.****, Raymond, F., Corbeil, J., Ouellette, M.*

4F A single endonuclease-generated DNA break induces antigenic switching in Trypanosoma brucei. ***Boothroyd, C.****, Dreesen, O., Leonova, T., Figueiredo, L., Cross, G., Papavasiliou, F.*

4G HAT3 and SIR2rp1 control RAD51-dependent DNA repair in African trypanosomes. ***Glover, L.****, Horn, D.*

4H Evolutionary changes in the *Leishmania* cap4-binding eIF4F complex as reflected by the eIF4E-eIF4G interactions. ***Shapira, M.****, Yoffe, Y., Zinoviev, A., Leger, M., Zuberek, J., Darzynkiewicz, E., Wagner, G.*

April 28 1:30 p.m. Chair - Michele Klingbeil

Session V: Kinetoplast and Mitochondrion

5A RNA Editing in kinetoplastids: Physical and functional interactions. ***Read, L.**, Ammerman, M., Fisk, J., Presnyak, V., Foda, B.*

5B Structure of the Core Editing Complex (L-Complex) Involved in Uridine Insertion/Deletion RNA Editing in Trypanosomatid Mitochondria. ***Simpson, L.**, Li, F., Hui, W., Atanasov, I., Rogers, K., Guo, Q., Osato, D., Falick, A., Zhou, H., Ge, P.*

5C How uridylylation is shaping mitochondrial transcriptome in T. brucei. ***Aphasizhev, R.**, Etheridge, R., Aphasizheva, I.*

5D *Trypanosoma brucei* Tob55 functions as a protein translocator on mitochondrial outer membrane. ***Sharma, S.**, Singha, U., Hamilton, V., Duncan, M., Chaudhuri, M.*

Coffee Break

5E A double inducible system to study mitochondrial tRNA import in living *Trypanosoma brucei*. ***Tschopp, F.**, Mascha, P., Andre, S.*

5F The unexpected effects of ethidium bromide on Trypanosoma brucei. ***Roy Chowdhury, A.**, Wang, J., Yildirir, G., Liu, B., Shapiro, T., Englund, P.*

5G Mitochondrion of kinetoplastid parasites as a chemotherapeutic target. ***Yang, G.**, Siqueira-Neto, J., Jang, J., Moon, S., Cechetto, J., Genovesio, A., Christophe, T., Freitas-Junior, L.*

5H Discovery of the First Drug-like Inhibitors of an Essential RNA Editing Ligase in *Trypanosoma brucei*. ***Amaro, R.**, Schnaufer, A., Interthal, H., Hol, W., Stuart, K., McCammon, J.*

April 29 8:45 a.m. Chair - Piet Borst

Session VI: Gene Expression

6A Trans-splicing regulation – spliced leader RNA silencing, and spliced leader RNA discard – two processes that control the level of SL RNA. ***Michaeli, S.***, *Goldshmidt, H., Shaked, H., Gupta, S., Tkacz, I.*

6B The pseudo-CTD of RNAPII is essential for productive RNA synthesis in *Trypanosoma brucei* . ***Das, A.***, *Bellofatto, V.*

6C A novel, TFIIH-associated, multi-subunit transcription factor is essential for SL RNA gene transcription in *Trypanosoma brucei*. ***Lee, J.***, *Panigrahi, A., Günzl, A.*

6D A novel strategy for the identification of RNA binding proteins reveals putative regulators of EP procyclin expression. ***Schimanski, B.***, *Heller, M., Roditi, I.*

6E Differential Trypanosome Surface Coat Regulation by a CCCH Protein That Co-Associates with *procyclin* mRNA cis-Elements. ***Walrad, P.***, *Acosta-Serrano, A., Matthews, K.*

6F Cold-inducible PKA-like kinase of *Trypanosoma brucei*. ***Bachmaier, S.***, *Brenndorfer, M., Kramer, S., Pepperl, J., Boshart, M.*

6G Inhibition of initiation of translation by degron-mediated depletion of eIF2 alpha and the effect on mRNA metabolism. *Brown, E., Kramer, S., Sunter, J., Webb, H.,* ***Carrington, M.***

6H PUF9 regulates transcript stability over the cell cycle in *Trypanosoma brucei*. ***Archer, S.***, *Luu, V., Queiroz, R., Brems, S., Clayton, C.*

WEB SESSION

1A TriTrypDB: A Functional Genomics Database for Kinetoplastid Parasites. *Berriman, M.,* ***Brunk, B.****, Carrington, M., Gardner, M.,* ***Harb, O.****,* ***Hertz-Fowler, C.****, Kissinger, J., Myler, P.,* ***Roos, D.****, Smith, D., Stoeckert, C.*

POSTER SESSIONS: A-B

A SERIES: April 27 6:30 p.m.

B SERIES: April 28 6:30 p.m.

200A Functional characterization of the Leishmania folate/biopterin transporters (FBT) family. ***Ahmed Ouameur, A.***, *Dridi, L., Ouellette, M.*

201A The SUMOylation system in Trypanosoma cruzi. *Bayona, J., Cazzulo, J.,* ***Alvarez, V.***

202A Regulated expression of the surface virulence factor lipophosphoglycan (LPG) of *Leishmania major* using a conditional destabilized protein-fusion regulatory system. *Madeira da Silva, L., Owens, K., Murta, S.,* ***Anderson, B.****, Beverley, S.*

203A The pseudo-CTD of RNAPII is essential for productive RNA synthesis in *Trypanosoma brucei*. ***Das, A.****, Bellofatto, V.*

204A Investigation of fucosylation in *Trypanosoma brucei*. ***Bandini, G.****, Guther, L., Ferguson, M.*

205A Identification of a new flagellar protein in *Trypanosoma brucei* : a role in microtubule stabilisation?. *Dacheux, D., Landrein, N., Thonnus, M.,* ***Robinson, D.****,* ***Bonhivers, M.***

206A AcetylCoA synthetase : the link between glucose/amino acid degradation and lipid biosynthesis in procyclic trypanosomes. *Riviere, L., Moreau, P., Hahn, M., Allman, S., Biran, M., Plazolles, N., Franconi, J., Boshart, M.,* ***Bringaud, F.***

207A Role of thiomodifications in tRNA import in *Trypanosoma brucei*. ***Bruske, E.****, Schneider, A.*

208A Characterization of zinc-iron transporter members and of a putative ferric reductase in *Leishmania infantum*. ***Carvalho, S.****, Cruz, T., Costa, V., Tomas, A.*

209A Bioluminescence: A new light on *in vivo* trypanosome modeling. ***Claes, F.****, Vodnala, S., Van Reet, N., Boucher, N., Lunden-Miguel, H., Goddeeris, B., Buscher, P., Rottenberg, M.*

210A Role of the PERK eIF2-alpha kinase and eIF2-alpha phosphorylation in the differentiation process of *Leishmania* within macrophages. ***Cloutier, S.****, Chow, C., Dumas, C., Papadopoulou, B.*

211A Trypanosome Mitochondrial DNA Polymerase IC Behaves as a Stable Component of the Tripartite Attachment Complex. ***Concepcion, J.***, *Laitinen, K., Klingbeil, M.*

212A Iron overload favours the elimination of *Leishmania infantum* from mouse tissues through the production of reactive oxygen and nitrogen species. ***Costa, S.***, *Pereira, S., Teixeira, C., Tomás, A., Appelberg, R., Gomes, M.*

213A Functional Studies of the Multiple Mitochondrial DNA Polymerases in *Trypanosoma brucei* using Chimeric Gene Silencing. ***De, A.***, *Bruhn, D., Mozeleski, B., Klingbeil, M.*

214A The function of the mitochondrion in bloodstream form trypanosomes. ***Dean, S.***, *Schnaufer, A.*

215A Characterization of Leishmania donovani amastigote specific protein and its role in virulence. ***Dey, R.***, *Nakhasi, H., Duncan, R.*

216A A Comparative Genome-wide Study of ncRNAs in Trypansomatids. ***Doniger, T.***, *Wachtel, C., Katz, R., Michaeli, S., Unger, R.*

217A Biomarkers of attenuation in the Leishmania centrin deleted cell line-measures of safety in a live vaccine candidate. ***Duncan, R.***, *Dey, R., Tomioka, K., Hairston, H., Selvapandiyan, A., Nakhasi, H.*

218A Protein palmitoylation in trypanosomes - from a global perspective to a specific role in protein targeting to the flagellar membrane. ***Emmer, B.***, *Souther, C., Engman, D.*

219A Transcriptome remodeling during the growth curve of procyclic *Trypanosoma brucei*. *Fernandez-Moya, S.,* ***Estevez, A.***

220A Genomic distribution and developmental changes of DNA methylation in the parasite *Trypanosoma cruzi* . *Respuela, P.,* ***Ferella, M.***, *Aslund, L.*

221A Active VSG Expression Site is organized in a uniquely open chromatin structure. ***Figueiredo, L.***, *Cross, G.*

222A A tripanosomal sirtuin homologue is involved in mitochondria regulation. *García-Perez, A., del Castillo, T., Morales, S.,* ***Garcia-Salcedo, J.***

223A A genome-wide RNAi screening to identify human host factors necessary for *Trypanosoma cruzi* infection. ***Giardini, M.***, *Genovesio, A., Dossin, F., Kwon, Y., Kim, N., Choi, S., Kim, H., Jung, S., Emans, N., Freitas-Junior, L.*

224A Trypanosome flagellar RNAi mutants: location-to-function for novel proteins and the role of calmodulin in PFR assembly. *Collingridge, P., Shaw, M., Gull, K.,* ***Ginger, M.***

225A Exploiting the drug-activating properties of a novel trypanosomal nitroreductase. ***Hall, B.***, *Wu, X., Hu, L., Bot, C., Wilkinson, S.*

226A Oleate Desaturase as a DNA Vaccine for Chagas Disease. ***Hernandez, A.***, *Maldonado, R.*

227A Role of a MAP2 kinase in virulence in *Trypanosoma brucei*. ***Jensen, B.***, *Kifer, C., Parsons, M.*

228A CMF70 is a novel subunit of the dynein regulatory complex in *Trypanosoma brucei*. ***Kabututu, Z.***, *Thayer, M., Melehani, J., Hill, K.*

229A Centromere-associated topoisomerase activity in Trypanosoma brucei. ***Kelly, J.***, *Obado, S., Bot, C., Martin, T.*

230A Sequences From The Haptoglobin / Hemoglobin Receptor Untranslated Region Play An Important Role In Human Serum Resistance in *Trypanosoma brucei brucei*. ***Kieft, R.***, *Hajduk, S.*

231A DHH1 is central to the correct expression of many developmentally regulated genes in *Trypanosoma brucei*. ***Kramer, S.***, *Queiroz, R., Ellis, L., Webb, H., Sunter, J., Kiefer, C., Hoheisel, J., Clayton, C., Carrington, M.*

232A Ubiquitin-mediated internalisation and degradation among the invariant surface glycoprotein family in Trypanosoma brucei. ***Leung, K.***, *Chung, W., Field, M.*

233A Maintenance of the flagellar pocket by an essential CCCH zinc finger protein in procyclic form *T.brucei*. ***Ling, A.***, *Trotter, J., Hendriks, E.*

234A *Trypanosoma brucei* Mitochondrial DNA polymerase complexes. ***Luo, J.***, *Concepción, J., Klingbeil, M.*

235A Assessing stumpy formation and stumpy-specific gene expression using bloodstream reporter lines in *Trypanosoma brucei*. ***MacGregor, P.**, Dean, S., Matthews, K.*

236A The transcriptome of two life cycle stages of the human infective parasite, Trypanosoma brucei gambiense revealed by Digital SAGE. ***MacLeod, A.**, Veitch, N.*

237A Alba-domain proteins post-transcriptionally regulate GPEET expression in *T. brucei*. ***Mani, J.**, Schimanski, B., Guettinger, A., Heller, M., Roditi, I.*

238A Determinants for membrane targeting of Trypanosoma cruzi Flagellar Calcium Binding Protein (FCaBP). ***Maric, D.**, McGwire, B., Buchanan, K., Olson, C., Maric, D.*

239A MudPIT Proteomic Analysis of the T. brucei Flagellar Skeleton Reveals Many Novel Proteins. ***Melehani, J.**, Kabututu, Z., Jonsson, Z., Wohlschlegel, J., Hill, K.*

240A Investigating the oxidative folding pathway in Trypanosoma brucei. ***Millerick, M.**, Nic a Bhaird, N., Rubotham, J.*

241A Screening approaches for identification of *Trypanosoma brucei* hexokinase 1 inhibitors. ***Morris, M.**, Lyda, T., Dodson, H., Sharlow, E., Leimgruber, S., Lazo, J., Morris, J.*

242A Lymphocyte surface targets of *Trypanosoma cruzi trans*-sialidase. ***Muia, R.**, Yu, H., Hellman, U., Chen, X., Bertozzi, C., Campetella, O.*

243A *VSG* expression in bloodstream *Trypanosoma brucei* depends on an essential CITFA-interacting protein of 17 kDa. ***Nguyen, T.**, Nguyen, B., Lee, J., Panigrahi, A., Günzl, A.*

244A Ca^{2+} inhibition reveals mechanistic and kinetic aspects of the trypanosomal RNA editing machinery. ***Niemann, M.**, Goringer, U.*

245A Mitochondrial binding complex 1 (MRB1) in *Trypanosoma brucei*: its composition and function in RNA metabolism. ***Novotna, L.**, Hashimi, H., Cicova, Z., Wen, Y., Hanzalkova, L., Lukes, J.*

246A Kinetoplastid adenylosuccinate lyase as drug target. ***Opperdoes, F.**, Montovani, M., Verplaetse, E., Pedrosa, A., Sturm, N., Cruz, A., Campbell, D., Thiemann, O.*

247A Sphingosine Kinase is Essential for Proper Cell Cycle Progression and Cytokinesis in *Trypanosoma brucei*. ***Pasternack, D.***, *Fridberg, A., Olson, C., Engman, D.*

248A Transcriptome analysis of differentiating trypanosomes reveals the existence of multiple post-transcriptional regulons. ***Queiroz, R.***, *Hoheisel, J., Clayton, C.*

249A Do actin filaments exist in *Trypanosoma brucei*? ***Rubotham, J.***, *Hanrahan, O., Nolan, D.*

250A Deadenylation in *Trypanosoma brucei*. ***Schwede, A.***, *Carrington, M., Clayton, C.*

251A Functional Characterization of *Trypanosoma cruzi* Small Sub-Unit (ó1) Clathrin-Associated Adaptor Protein-Like *(APClaTc)*. ***Serna, C.***, *Ulisses de Carvalho, T., Shpak, M., Miranda, M., Maldonado, R.*

252A Homology-independent functional annotation of *Trypanosoma brucei* genome. ***Shateri Najafabadi, H.***, *Salavati, R.*

253A Perturbation of phosphatidylethanolamine synthesis affects mitochondrial morphology and cell cycle progression in procyclic form *Trypanosoma brucei*. ***Signorell, A.***, *Gluenz, E., Rettig, J., Schneider, A., Shaw, M., Gull, K., Bütikofer, P.*

254A Lipid biosynthesis in *Trypanosoma brucei*- a source of novel therapeutic targets. ***Smith, T.***, *Major, L., Young, S., Gibellini, F., Lilley, A.*

255A A New RNA Binding Module: Evolving Multi-substrate Specificity in Editing Deaminases. ***Spears, J.***, *Ragone, F., Wohlgamuth-Benedum, J., Howard, C., Alfonzo, J.*

256A Growth and microscopic analyses of knockdowns of a putative inner arm dynein in *Trypanosoma brucei*.. ***Springer, A.***, *Kinzel, K.*

257A Active VSG Expression Sites are Devoid of Histone H3 in *Trypanosoma brucei*. ***Stanne, T.***, *Rudenko, G.*

258A New diagnostic tools for Human African Trypanosomiasis. ***Sullivan, L.***, *Ferguson, M.*

259A Identification and Characterization of *Trypanosoma brucei* Decapping Enzyme. ***Takagi, Y.**, Ho, K.*

260A Identification of dynein regulatory complex subunits via PTP tagging in *Trypanosoma brucei*. ***Thayer, M.**, Hill, K.*

261A Intracellular trafficking of potential virulence factors in *Leishmania major*. ***Tonn, D.**, Coombs, G., Mottram, J.*

262A Casein kinase 1 isoform 2 is essential for bloodstream form *Trypanosoma brucei*. ***Urbaniak, M.***

263A Blocking the Synthesis of Variant Surface Glycoprotein in *Trypanosoma brucei* Causes a Global Translation Arrest. ***Vasileva, N.**, Smith, T., Gluenz, E., Terry, S., Portman, N., Kramer, S., Carrington, M., Gull, K., Rudenko, G.*

264A Acetyl-CoA Carboxylase: Potential Hot Spots for Controlling Fatty Acid Synthesis in *Trypanosoma brucei*? ***Vigueira, P.**, Paul, K.*

265A TbPIF8, related to a yeast mitochondrial DNA helicase, is required for trypanosome viability and kDNA organization. ***Wang, J.**, Liu, B., Jensen, R., Englund, P.*

266A Role of the *Trypanosoma brucei* RNA-binding protein RBP10 in bloodstream form energy metabolism. ***Wurst, M.**, Queiroz, R.*

267A New Tools for Controlling Constitutive and Inducible Transgene Expression in *Leishmania donovani*. ***Yates, P.**, Ullman, B.*

268A Processing and subcellular localization of *Leishmania major* metacaspase. ***Zalila, H.**, Gonzalez, I., Delgado, M., Desponds, C., Mottram, J., Fasel, N.*

269A The role of the initial acyltransferase *Lm*DAT in glycerolipid metabolism, metacyclogenesis, and autophagy of *Leishmania*. *Zufferey, R., Al-Ani, G.*

200B An elongator orthologue regulates RNA Pol I transcription in trypanosomes. ***Alsford, S.**, Horn, D.*

201B Spliceosomal proteomics revealed several new candidate SL *trans* splicing factors in *Trypanosoma brucei*. ***Ambrosio, D.**, Lee, J., Panigrahi, A., Nguyen, T., Cicarelli, R., Günzl, A.*

202B Essential components of the guide RNA binding complex. ***Aphasizheva, I.**, Etheridge, R., Aphasizhev, R.*

203B Activity of indenoisoquinolines against African Trypanosomes. ***Bakshi, R.**, Sang, D., Morrell, A., Cushman, M., Shapiro, T.*

204B Transcription profile around Strand Switch Regions (SSRs) in *T.brucei*. *Banday, M., Das, A., Bellofatto, V.*

205B Role of cargo and retrograde transport motor proteins in the construction of the trypanosome flagellum. ***Blisnick, T.**, Buisson, J., Cayet, N., Absalon, S., Bastin, P.*

206B Exploiting Trypanosmal Nitroreductase as a Drug Target. ***Bot, C.**, Hall, B., Wilkinson, S.*

207B *Trypanosoma brucei* Mitochondrial DNA Polymerase IB plays an essential role in leading and lagging strand minicircle replication. ***Bruhn, D.**, De, A., Mozeleski, B., Klingbeil, M.*

208B Definition of residues that confer substrate specificity in *Leishmania mexicana* sugar transporters. *Naula, C., Logan, F., Barrett, M.*, ***Burchmore, R.***

209B Global gene expression analysis of *Trypanosoma cruzi* under hyperosmotic stress. ***Alvarez, V.**, Li, Z., De Gaudenzi, J., Frasch, A., Cazzulo, J., Roberto, D.*

210B Knockout of acyl carrier protein in T. brucei causes kDNA loss. ***Clayton, A.**, Guler, J., Lindsay, M., Gluenz, E., Gull, K., Smith, T., Englund, P.*

211B Gene disruption of a Leucine rich repeats (LRR) protein involved in antimonials resistance in *Leishmania* spp. ***Coelho, A.**, Ouellette, M.*

212B Over-expression of the histone methyltransferase DOT1B disturbes cell cycle progression in *Trypanosoma brucei*. ***Corman, A.**, Boshart, M., Janzen, C.*

213B The mitochondrial elongation factor Tu of *Trypanosoma brucei* contains an insertion essential for function. ***Cristodero, M.**, Schneider, A.*

214B Characterization of multiple homologues of the cap binding protein (eIF4E) from *Trypanosoma brucei* . *Freire, E., Dhalia, R., Moura, D., da Costa Lima, T., Lima, R., Reis, C., Figueiredo, R., Standart, N., Carrington, M.,* ***de Melo Neto, O.***

215B Comparative expression profiling of *Leishmania* amastigotes. ***Depledge, D.****, Evans, K., Ivens, A., Maroof, A., Aziz, N., Kaye, P., Smith, D.*

216B Regulation of Gene Expression of Two Nearly Identical *Trypanosoma brucei* hexokinases, TbHK1 and TbHK2. ***Dodson, H.****, Sayce, A., Morris, M., Morris, J.*

217B Characterization of a nuclear Pumilio domain protein in *Trypanosoma brucei*. ***Droll, D.****, Archer, S., Fenn, K., Delhi, P., Matthews, K., Clayton, C.*

218B Stage-regulated mRNA decay in the protozoan parasite *Leishmania*. ***Dupe, A.****, Haile, S., Smith, M., Papadopoulou, B.*

219B VSG expression is monitored via a conserved stem-loop in the 3'UTR of VSG genes. *Batram, C., Jones, N., Hamacher, K., Günzel, M.,* ***Engstler, M.***

220B The Formation and Function of Long (A/U) Tails of *T. brucei* Mitochondrial mRNAs: An Emerging Role for **P**entatrico**P**eptide **R**epeat (PPR) Proteins. ***Etheridge, R.****, Aphasizheva, I., Aphasizhev, R.*

221B Functional characterization of the RNA-binding protein *Tb*RBP33. ***Fernandez-Moya, S.****, Estevez, A.*

222B The Role of a CTD-Interacting Protein in RNA Polymerase II-Associated Transcription in Trypanosoma brucei. ***Garcia, S.****, Bellofatto, V.*

223B Editing and modification of Kinetoplastid threonyl-tRNA. ***Gaston, K.****, Krivos, K., Rubio, M., Limbach, P., Alfonzo, J.*

224B The ethanolamine branch of the Kennedy pathway is essential in bloodstream *Trypanosoma brucei* . ***Gibellini, F.****, Hunter, W., Smith, T.*

225B Structural Requirements for the Attachment of a Rare Ethanolamine Modification to Eukaryotic Elongation Factor 1A. ***Greganova, E.**, Heller, M., Butikofer, P.*

226B The N-terminal signal peptide from human haptoglobin related protein exhibits potent and specific toxicity towards bloodstream form *Trypanosoma brucei*. ***Harrington, J.**, Johnson, T., Hajduk, S.*

227B Electron tomography of the Trypanosoma brucei flagella connector. ***Hoog, J.**, Lacomble, S., Hoenger, A., McIntosh, R., Gull, K.*

228B Dynamic mRNA Expression analysis of cells undergoing synchronous life-cycle differentiation in *Trypanosoma brucei*. ***Kabani, S.**, Ross, A., Ivens, A., Ghazal, P., Matthews, K.*

229B Identification of the KREPA4 RNA binding domain and elucidation of its biochemical role in RNA editing. ***Kala, S.**, Salavati, R.*

230B Characterisation of trans-sialidase genes from *Trypanosoma congolense*. *Gbem, T., Koliwer-Brandl, H., Dietz, F.,* ***Kelm, S.***

231B Roles for T. brucei Topo3α-Rmi1 pathway in antigenic variation. ***Kim, H.**, Cross, G.*

232B Structural Studies of the Interaction Between the Translation Initiation Factors 4e And 4g from Leishmania Major. ***Leger, M.**, Arthanari, H., Shapira, M., Wagner, G.*

233B RAP1 is Essential for Silencing Telomeric *Variant Surface Glycoprotein* Genes in Procyclic *Trypanosoma brucei*. *Pandya, U.,* ***Li, B.***

234B Social Motility and Cell-Cell Communication in African trypanosomes. ***Lopez, M.**, Oberholzer, M., McLelland, B., Hill, K.*

235B Identification of a C-terminal Flagellar Targeting Sequence in *Trypanosoma brucei* Hexokinase. ***Lyda, T.**, Dodson, H., Coley, A., Morris, M., Michels, P., Morris, J.*

236B Trafficking and Secretion of the *Leishmania* HASPB Protein. ***MacLean, L.**, Stark, M., OToole, P., Smith, D.*

237B Subcellular Localization and Complex Association Differentiate *T. brucei* REAP-1 from 45 kDa Protein. *Briggs, J.,* ***Habura, A.****, Madison-Antenucci, S.*

238B A general platform for homology-independent analysis of parasite genome sequences. ***Mao, Y.****, Shateri Najafabadi, H., Salavati, R.*

239B In vivo/in vitro characterization of the two thymidine hydroxylases involved in the synthesis of the modified DNA base J in the genome of *Trypanosoma brucei*. ***Marshall, M.****, Southern, T., Cliffe, L., Kieft, R., Birkeland, S., Sabatini, R.*

240B Trypanocidal Action of AN3520: A Novel Oxaborole Compound Showing Efficacy in Mouse Models for African Trypanosomiasis. ***Mercer, L.****, Bowling, T., Jacobs, R., Orr, M., Chen, D., Sligar, J., Jenks, M., Noe, A., Bacchi, C., Yarlett, N., Freund, Y., Jarnagin, K., Plattner, J., Don, R., Nare, B.*

241B A Common mechanism of cell death induced by anti-*Leishmania* drugs. ***Moreira, W.****, Ouellette, M.*

242B Fluorescence-based reporter substrate for monitoring RNA editing in Trypanosomatid pathogens. ***Moshiri, H.****, Salavati, R.*

243B Gene disruptions indicate an essential function for gamma glutamyl cysteine synthetase in *Leishmania infantum*. ***Mukherjee, A.****, Roy, G., Ouellette, M.*

244B Genes of the actin cytoskeleton of Trypanosoma brucei. ***Nicabhaird, N.****, Rubotham, J., ORourke, J., Nolan, D.*

245B A life cycle stage specific membrane glycoprotein involved in flagellar adhesion in T. brucei. *Nolan, D., Dooley, C., Perez Morga, D.,* ***Nolan, D.***

246B Environmental sensing and social motility of African trypanosomes. ***Oberholzer, M.****, Lopez, M., Mclelland, B., Hill, K.*

247B The role of Argonaute-like PIWI protein in *Leishmania* . ***Padmanabhan, P.****, Rochette, A., Dumas, C., Papadopoulou, B.*

248B Characterization of two *N*-Myristoylated Trypanosome Orthologues of Proteins Implicated in Human Genetic Disorders. ***Price, H.**, Curwen, R., Hodgkinson, M., Stark, M., Smith, D.*

249B Hesperadin inhibits Aurora kinase-1 and blocks mitotic progression in bloodstream forms of *Trypanosoma brucei*. *Jetton, N., Rothberg, K., Hubbard, J., Wise, J., Li, Y., Ball, H.,* ***Ruben, L.***

250B RNA editing as a drug target in trypanosomes: complementary approaches for the identification of inhibitors of the essential enzyme REL1. ***Schnaufer, A.**, Lewis Ernst, N., Colangelo-Lillis, J., Hol, W., Stuart, K., McCammon, J., Amaro, R.*

251B Functional analysis of trypanosomatid-specific RNA editing ligases in the presence and absence of their interacting protein partners. ***Sen, R.**, Salavati, R.*

252B Streamlined architecture and GPI-dependent trafficking in the early secretory pathway of African trypanosomes. ***Sevova, E.**, Peck, R., Bangs, J.*

253B kDNA replication initiation is regulated through a redox signaling pathway. *Dotan, S., Milman, N., Yaffe, N., Kapeller, I., Kapach, G.,* ***Shlomai, J.***

254B A bacterial type mitochondrial porin in Trypanosoma brucei is essential for its energy production and cell survival. ***Singha, U.**, Sharma, S., Duncan, M., Hamilton, V., Chaudhuri, M.*

255B Identification of Amastigote-Specific Protein Kinases in *Leishmania Donovani* by Activity-Based Proteome Analysis. *Schmidt-Arras, D., Leclercq, O.,* ***Späth, G.***

256B Role of myosin in bloodstream form of *T. brucei*. ***Spitznagel, D.**, Nic a Bhaird, N., Nolan, D.*

257B Interacting partners of *Leishmania major* metacaspase. ***Sprissler, C.**, Gonzalez, I.*

258B An Unusual Protein Trafficking Pathway in African Trypanosomes Leads to Infectivity in Humans. ***Stephens, N.**, Hajduk, S.*

259B Development of the next generation of vectors. ***Sunter, J.**, Reed, J., Kramer, S., Wickstead, B., Carrington, M.*

260B Investigation of the role of Vitamin C in *Trypanosoma cruzi*. ***Taylor, M.***, *Logan, F., Wilkinson, S., Kelly, J.*

261B New prospects regarding the classical tryparedoxin/peroxiredoxin pathway operating within the *Leishmania infantum* mitochondrion. *Castro, H., Romao, S.,* ***Tomas, A.***

262B Identification of proteins localized to the contractile vacuole of *Trypanosoma cruzi*. ***Ulrich, P.***, *Park, M., Docampo, R.*

263B The Role of Poly(A)-Specific Ribonuclease in mRNA Turnover in *Trypanosoma brucei*. ***Utter, C.***, *Bellofatto, V.*

264B High-throughput screening of a drug library for *Leishmania major* the causative agent of cutaneous leishmaniasis. ***Vasquez, M.***, *Beverley, S., Lema, C., Dimmock, J., Maldonado, R.*

265B Structure of the C-terminal domain of Transcription Factor IIB from *Trypanosoma brucei*. *Syed Ibrahim, B., Kanneganti, N., Rieckhof, G., Das, A., Laurents, D., Palenchar, J., Bellofatto, V.,* ***Wah, D.***

266B Investigating mechanisms of diamidine resistance in *Leishmania* . ***Wildridge, D.***, *Scheltema, R., Kamleh, A., Watson, D., Burchmore, R., Barrett, M.*

267B RAP1 Is Essential for Silencing Telomeric *Variant Surface Glycoprotein* Genes in *Trypanosoma brucei*. ***Yang, X.***, *Figueiredo, L., Espinal, A., Okubo, E., Li, B.*

268B Cyclosporin A treatment leads to stage-specific effects on *Leishmania donovani* morphology, proliferation, and viability. ***Yau, W.***, *Blisnick, T., Morales, M., Schmidt-Arras, D., Bastin, P., Späth, G.*

269B A mitochondrial RNase D family exoribonuclease from *Trypanosoma brucei* impacts both mRNAs and gRNAs. ***Zimmer, S.***, *McEvoy, S., Menon, S., Read, L.*

ABSTRACTS: SESSION I

Cell Biology

April 26 7:00p.m.

Chair(s) - Jay Bangs

Dynamics of flagellum formation in trypanosomes

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The flagellum is an essential organelle for trypanosomes as it is involved in motility, morphogenesis, adhesion to tsetse fly tissues and possibly sensing of the environment. This complex organelle is made from a basal body and contains two structural elements (the axoneme and the paraflagellar rod), a matrix and is wrapped by the flagellar membrane. It is constructed by the action of intraflagellar transport (IFT) where a kinesin motor transports a complex of IFT proteins towards the tip of the organelle (anterograde movement) that is then recycled to the base by a dynein motor (retrograde). We have identified 20 proteins involved in IFT either as motors or as cargos. All the proteins investigated so far are localised to the basal body and to the flagellum matrix. Examination of fluorescent IFT motors or cargos in live cells revealed both anterograde and retrograde motility, the first one being the most striking. Kymograph analysis demonstrated the fastest rates of IFT in any organism investigated so far. Two different populations of anterograde IFT tracks were identified in the same cells, in contrast to retrograde transport that appears more homogenous. FRAP (fluorescence recovery after photobleaching) analysis revealed that 40-60 % of the IFT material is in the flagellum and continuously exchanges with the basal body pool. It also allowed a clear discrimination between anterograde and retrograde transport. To identify partners of the IFT machinery, we have purified intact flagella by mechanical shearing and used proteomics to analyse the content of the matrix. Finally, Trypanosomatids provide excellent model organisms to study IFT regulation as they naturally modulate the length of their flagellum during their life cycle.

How do procyclic and bloodstream form *T. brucei* trypomastigotes divide? Some answers and implications

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RNAi technologies in *Trypanosoma brucei* have led to a rapidly increasing set of mutant descriptions being reported as cell cycle or cytokinesis phenotypes and that these often vary between procyclic and bloodstream forms. Given this, we were struck by the absence of any satisfactory, detailed comparison of normal cytokinesis and morphogenetic processes in these forms. We have undertaken the first detailed, comparative description and have employed videomicroscopy of living cells with immunofluoresence, transmission and scanning electron microscopy. We describe the process of furrow ingression and cytokinesis and show that the cleavage plane forms along an asymmetrical plane in both life cycle stages, but the extent and positioning of this asymmetry differs. Asymmetry is also seen in the shape of the two daughter posterior ends and there is a cytoplasmic bridge connection between them, but we note differences in this connection between life cycle forms. There are also distinct differences in the flagellar connector, in spatial positioning and segregation of the kinetoplasts and nuclei and also in the end stage division processes and re-entry to the next cell cycle in the two forms. These descriptions have implications for interpretation of cell morphogenesis and division phenotypes in both cell types.

Structure-function analysis of flagellar proteins identifies viable bloodstream-form motility mutants in T. brucei

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The dynein regulatory complex (DRC) is required for flagellar motility in the protists *Chlamydomonas reinhardtii* and *Trypanosoma brucei*. We recently demonstrated a DRC requirement for cilium motility in vertebrates and revealed an unexpected mechanism for cilium-dependent fluid dynamics in inner ear development, showing fluid vortices generated by motile cilia guide otolith assembly atop specific cells of the sensory epithelium (*Nature* **457**, 205-9). To further understand function of axonemal dynein regulatory systems, we exploited the experimental tractability of *T. brucei*. We report identification of a novel DRC subunit, designated DRC-2, which is tightly associated with the DRC subunit trypanin. To move beyond gene/protein identification and more deeply into functional analysis, we developed an RNAi-based system for structure-function studies of dynein-regulatory proteins. The system employs inducible knockdown of an endogenous gene, with simultaneous induction of an ectopic copy harboring specific point mutations. Using this system we identified amino acids required for function of the dynein regulatory light chain LC1 in procyclic and bloodstream trypanosomes. Notably, bloodstream LC1 mutants are immotile, yet viable, demonstrating that normal motility is not essential in this life-cycle stage. To summarize, we have established a system for facile structure-function studies of flagellar proteins, identified amino acids necessary for dynein motor function and revealed that the connection between motility and viability in bloodstream-stage trypanosomes is more complex than might be interpreted from knockdown studies alone. These studies open new avenues for dissecting mechanisms of flagellar motility and provide an important step in efforts to exploit the potential of the flagellum as a target for therapeutic intervention in African sleeping sickness.

Structure and function of the *Leishmania* amastigote flagellum

Eva Gluenz*, Amy E. Smith, Johanna Höög, Helen Dawe, Charmain Heah, Mike Shaw, Keith Gull

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Eukaryotic flagella and cilia are complex macromolecular structures that perform motility and sensory functions. The single flagellum of the trypanosome is a multifunctional organelle used for locomotion, cell morphogenesis, attachment to insect vector epithelial cells and, most likely, sensory functions. *Leishmania* promastigotes have a motile flagellum with a canonical 9+2 microtubule array and associated components critical for motility such as dynein arms and radial spokes and a paraflagellar rod. The amastigotes, which are often wrongly described as being 'aflagellate', possess a small flagellum that transverses the flagellar pocket, protrudes only a short distance from the pocket and is thought to be non-motile. Why then do amastigotes retain a short flagellum at all? To address this question, we have used electron microscopy (EM) and 3D electron tomography to analyse and model the structure of *Leishmania mexicana* amastigote flagella at high resolution. We found that the amastigote axoneme architecture differs markedly from the promastigote. Amastigotes have an unusual 'collapsed' arrangement of nine microtubule doublets, which are associated only with outer- but not inner dynein arms. Further EM analysis showed that the 'collapsed' microtubule arrangement structure is strikingly similar to the arrangement of microtubules in the sensory, primary cilia of mammalian cells. Other aspects of the biology of amastigotes suggest the flagellum is a critical multi-functional organelle. We postulate that the amastigote flagellum acts in a number of roles as a sensory organelle with important functions in the intracellular stage of the *Leishmania* life-cycle.

A glycosomal DxDxT phosphatase, *Tb*PIP39, is a substrate of *Tb*PTP1 in the *T.brucei* differentiation signalling pathway

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In preparation for transmission from the bloodstream, trypanosomes generate division-arrested stumpy forms. These forms are pre-adapted for differentiation and rapidly respond to external signals when ingested by tsetse flies. Recently, we discovered that a protein tyrosine phosphatase (*Tb*PTP1) holds stumpy forms in division arrest until they enter the tsetse fly, at which point *Tb*PTP1 is inactivated and cell differentiation occurs (Szoor et al., *J Cell Biol*. 2006). Now, we have used 'substrate-trapping' mutants of *Tb*PTP1 to select its substrates from stumpy-cell extracts. One molecule was consistently isolated by this approach and identified by mass spectrometry as a 39kDa protein, named *Tb*PIP39. *Tb*PIP39 is a DxDxT phosphatase, a member of an unusual class of protein phosphatases with diverse roles in stress signaling and transcriptional control. *Tb*PIP39 is already present in stumpy cells but is up-regulated by cold shock (i.e. at 20^{o}C) and during differentiation to procyclic forms. Consistent with its being a substrate of *Tb*PTP1 *in vivo*, *Tb*PIP39 has a single conserved C-terminal tyrosine phosphorylation site and can be dephosphoryated by recombinant *Tb*PTP1 *in vitro*. Moreover, *Tb*PIP39 is dephosphorylated (and inactive) when *Tb*PTP1 is active in stumpy forms but becomes tyrosine-phosphorylated (and activated) when *Tb*PTP1 is inactivated during differentiation to procyclic forms. Intriguing, these interactions are also modulated *in vitro* by the differentiation stimuli, citrate and cis-aconitate. Combined these findings suggest that *Tb*PIP39 acts downstream from *Tb*PTP1 on the differentiation signalling-pathway. Interestingly, *Tb*PIP39 has a peroxisomal localisation signal and can be localised to the glycosomes. We propose this localisation plays an important role in the regulation of differentiation, with *Tb*PIP39 acting as a glycosomal signalling molecule.

NDR protein kinases as drug targets in *Trypanosoma brucei*

Jiang Tao Ma[1], Raffaella Grimaldi[2], Laura Cleghorn[2], Corinna Benz[1], Christopher Stockdale[1], Andrew Woodland[2], Paul Wyatt[2], Julie Frearson[2], Tansy C. Hammarton[*,1]

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Cytokinesis in *Trypanosoma brucei* is easily perturbed with disastrous consequences for the parasite. It occurs via the ingression of a unidirectional cleavage furrow along the long axis of the cell, which differs from the actomyosin contractile ring employed by mammalian cells. Hence, regulators of cytokinesis may have potential as drug targets in trypanosomes. *T. brucei* possesses two NDR (nuclear DBF2-related) protein kinases, PK50 and PK53. NDR kinases function in a variety of eukaryotic cellular processes including mitotic exit, cytokinesis and morphogenesis, and are activated by the binding of a MOB protein partner. In *T. brucei*, MOB1 is essential for cytokinesis furrow ingression, and has previously been shown to bind to PK50. We now show that depletion of PK50 in bloodstream stage trypanosomes prevents cells from initiating cytokinesis, while PK53 depletion inhibits furrow ingression. These kinases also appear to be essential for cell division in procyclic trypanosomes. Since PK50 and PK53 are essential cell cycle regulators, a focused kinase inhibitor library of $>$4000 compounds based on $>$150 scaffolds was screened with active recombinant PK50 and PK53. A number of compounds, which inhibit PK50 and/or PK53, with IC50 values ¡1 μM, were identified. The most potent inhibitors of each enzyme are based on different scaffolds, indicating selective inhibition of individual NDR protein kinases is possible. Counter-screening revealed that they do not inhibit the majority of human kinases tested. These compounds also inhibit trypanosome proliferation in culture, but without causing significant defects in cell cycle progression, arguing that despite their relative specificity against the NDR kinases in vitro, they hit other cellular targets in vivo.

Eradication of animal and human infective African trypanosomes by TLF gene therapy

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A sub-fraction of human high-density lipoprotein (HDL), which we term trypanosome lytic factor (TLF), kills African trypanosomes. TLF is only made by higher primates; human, gorilla, baboon, sooty mangabey and mandrill. Haptoglobin related protein (Hpr) and apolipoprotein L-I (apoL-I), are the unique components of TLF. We have created transgenic mice by hydrodynamic gene delivery that express functional TLF, which allows dissection of each component's contribution. We have used our transgenic mice to show that only apoL-I reconstituted into HDL is lytic, although Hpr can enhance activity by increasing uptake of TLF efficiently killing *T. b. brucei*, *T. congolense* and *T. evansi*. Human TLF cannot kill *T. b. rhodesiense* due to the serum resistance-associated protein (SRA). In contrast, the TLF of baboons are able to kill human serum resistant parasites despite the presence of SRA. We have purified baboon TLF, identified the proteins, cloned the genes and reconstituted the baboon TLF by expressing the key components in transgenic mice. The transgenic baboon-TLF-mice are resistant to animal infective *T. b. brucei*, *T. congolense* and resistant to human infective *T. b. rhodesiense*. Through the creation of chimeric human-baboon proteins in our transgenic mice, we have delineated a novel domain required for resistance to human infective trypanosomes.

ABSTRACTS: SESSION II

Biochemistry

April 27 8:45 a.m.

Chair(s) - Buddy Ullman

Trypanosoma brucei adaptor protein-3 (AP-3) β subunit is required for acidocalcisome biogenesis and endocytosis.

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Acidocalcisomes are acidic calcium- and phosphorus-storage organelles found in a diverse range of organisms including trypanosomatdis in which they were first defined. (Docampo et al., *Nat. Rev. Microbiol.* 3, 251-61, 2005). Targeting of membrane proteins to the acidocalcisomes of *Leishmania major*, but not the biogenesis of the organelle itself or the growth of the parasite in vitro, was affected by deletion of the δ subunit of the adaptor protein 3 complex (AP-3) (Besteiro et al., *J Cell Sci.* 121, 561-70, 2008). To investigate whether transport of proteins to acidocalcisomes of *Trypanosoma brucei* is linked to the expression of AP-3 function, we investigated the effects of ablation of its β subunit by RNA interference (RNAi). In contrast to the results reported in *L. major*, knock down of this subunit led to a dramatic disappearance of acidocalcisomes from both procyclic and bloodstream forms, as revealed by immmunofluorescence and electron microscopy assays, and by the decrease in their total calcium, pyrophosphate, and polyphosphate content, as well as defects in cytokinesis, growth, and susceptibility to osmotic stress. These changes were accompanied by an increase in steady-state levels of procyclin in procyclic forms and a decrease in receptor-mediated endocytosis in bloodstream forms. In conclusion, AP-3β is essential for the biogenesis of acidocalcisomes, and is also involved in receptor-mediated endocytosis and in maintaining steady-state levels of membrane proteins in *T. brucei*.

Developmentally Regulated Sphingolipid Synthesis in African Trypanosomes

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Sphingolipids, essential components of eukaryotic membranes, play critical roles in cellular metabolism, signaling, and recognition. Sphingolipid synthesis begins in the ER with formation of ceramide, which following transfer to the Golgi, is modified to complex phosphosphingolipids by the addition of polar head groups. The major phosphosphingolipid in mammals is sphingomyelin, but many unicellular eukaryotes, including fungi and kinetoplastid protozoa, are thought to synthesize exclusively inositol phosphorylceramide (IPC). Here we characterize sphingolipids from *Trypanosoma brucei*, and a trypanosome sphingolipid synthase gene family (*TbSLS1-4*) orthologous to *Leishmania* IPC synthase. ESI mass spectrometry indicates that procyclic trypanosomes contain IPC, and also sphingomyelin, while surprisingly bloodstream stage parasites contain sphingomyelin and ethanolamine phosphorylceramide (EPC), but no detectable IPC. In vivo fluorescent ceramide labeling confirmed stage specific biosynthesis of both sphingomyelin and IPC. Expression of *TbSLS4* in *Leishmania* resulted in production of sphingomyelin and EPC, as judged by both ESI-MS and in vivo labeling, indicating that *TbSLS4* has bi-functional synthase activity - a novel finding for SLS enzymes. We have used liposome-supplemented cell-free synthesis to confirm the enzymatic specificity of TbSLS4, and will extend this strategy to TbSLS1-3, as well as SLSs from other kinetoplastid parasites. Pan-specific RNAi silencing of *TbSLS1-4* in bloodstream trypanosomes led to rapid (8 hr) growth arrest and eventual cell death (24 hr). Ceramide levels were also rapidly elevated (3-fold) suggesting growth arrest via downstream effect(s) of this potent intracellular messenger, rather than disruption of membrane structural integrity. Thus de novo synthesis of phosphosphingolipids is essential in trypanosome, unlike *Leishmania*.

The Achilles Heel of the Purine Salvage Pathway of *Leishmania donovani*

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Mutational analysis of the purine acquisition pathway of *Leishmania donovani* has revealed that all exogenous purines are salvaged to the nucleotide level through the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and/or xanthine phosphoribosyltransferase (XPRT) enzymes. Specifically, a *hgprt xprt* genotype exhibits a conditionally lethal phenotype in promastigotes that can only be stably maintained in the presence of adenine or adenosine and deoxycoformycin (dCF), an inhibitor of adenine deaminase (AAH), and this double knockout is essentially avirulent in mice. However, a small number of δ *hgprt xprt* parasites persist, a process that is associated with a specific amplification of the adenine phosphoribosyltransferase (APRT) gene. These findings lead to a model of purine salvage in *Leishmania* in which adenylosuccinate synthetase (ADSS), as well as adenylosuccinate lyase, are essential enzymes for which a genetic defect cannot be overcome by *APRT* amplification. To test this hypothesis, we created a *adss* null mutant by double targeted gene replacement. Among all the naturally occurring purines, the *adss* knockout is only capable of growth in adenine or adenosine, but only when dCF was present in the culture medium. No other purines supported growth of the *adss* mutant. The conditionally lethal phenotype of the *adss* mutation and the functional role of ADSS in the purine salvage pathway predict that ADSS is an essential protein and that targeting the enzyme with inhibitory drugs would not trigger persistence mechanisms that could lead to drug resistance.

Systems analysis of metabolism in *Leishmania major:* elucidation of high-priority drug targets

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As part of a strategy based on model-driven biological discovery, systems biology can be used to gain valuable insight into parasitic processes and associated disease mechanisms. To be able to characterize phenotypic properties of an organism, it is necessary to understand its intracellular network of interactions that are hard-coded into its genome. Whether questions are related to the synthesis of critical intermediates required for growth of a pathogen or those pertaining to the interference of host cellular components by pathogen virulence factors, computational reconstructions provide a robust framework for performing perturbation analysis and generating testable hypotheses (e.g., quantitative prediction of growth rates and byproduct secretion profiles; endpoint of adaptive evolution; gene essentiality). Computational modeling facilitates the analysis of biological systems on the genome-scale and predictive models of biochemical networks can be built using available genomic, proteomic and metabolomic data. Recently, we published a metabolic network reconstruction of *Leishmania major*. This network accounted for the function of 560 genes, 1112 reactions and 1101 metabolites with 8 unique sub-cellular localizations. We hypothesized a comprehensive set of lethal single and double gene deletions, some of which were validated using available literature with approximately 70% accuracy. We also generated hypothetical annotations to dozens of previously uncharacterized genes in the *L. major* genome and proposed a minimal medium for growth. This reconstruction and the associated network analyses of *L. major* was the first of its kind for a protozoan. Subsequently, we identified known drugs for the growth-reducing and essential metabolic genes predicted by the model. This analysis has resulted in a list of high-priority targets against *L. major* with their potential effector drugs.

Pterin Metabolism in African Trypanosomes

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Trypanosomatids, such as Crithidia and Leishmania spp., have previously been shown to be pterin auxotrophs. In other organisms, tetrahydropterins are essential cofactors in a number of enzymatic reactions including the hydroxylation of aromatic amino acids and the biosynthesis of nitric oxide. However, the functions of pterins in trypanosomatids are not known. Here we describe key differences in pterin metabolism between the African trypanosome (Trypanosoma brucei) and the extensively studied Leishmania parasite. Progress towards elucidating the possible roles of pterins within trypanosomatids will also be discussed.

Characterization of potassium channels in *Trypanosoma cruzi*

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Potassium channels are members of one of the largest and most diverse family of membrane proteins. In *T. cruzi*, K^+ channels are important players on plasma membrane potential regulation, pH homeostasis, and osmoregulation. Trypomastigotes are particularly sensitive to changes in extracellular potassium concentration while epimastigotes and amastigotes are less responsive to this ion (VanderHeyden and Docampo, Mol. Biochem. Parasitol. 105, 237-51, 2000). We found sequences encoding for putative voltage-dependent potassium channels (TcKv) and calcium-activated potassium channels (TcCAKC) in the *T. cruzi* database. The His-tagged recombinant proteins were expressed in *E. coli*, purified and used to obtain specific antibodies. TcKv was localized by IFA, near or in the plasma membrane of trypomastigotes with a clear defined punctate pattern. In epimastigotes, TcKv is also membrane-related, but some intracellular labelling can also be observed. These results were confirmed by immuno-electron microscopy. TcCAKC immunolocalization was also performed and the protein showed a peripheral distribution in the parasites, suggesting an association with the plasma membrane. Interestingly, in trypomastigotes this channel is partially co-localized with flagellar-calcium binding protein and calmodulin, which could be involved in channel activity regulation. TcKv and TcCAKC were expressed in *Saccharomyces cerevisiae* mutants deficient in potassium transporters and channels, and protein expression was verified by immunofluorescence and western-blot analysis. In both cases, overexpressed *T. cruzi* K^+ channels complemented the deficient transport system and restored the normal cell phenotype, indicating the functionality of these proteins. Electrophysiological experiments are currently under way in order to establish the biophysical properties and functional roles that these channels can play in *T. cruzi* physiology.

Transporters of S-Adenosyl-methionine in and out of *Leishmania* cells

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S-Adenosyl-methionine (SAM, also known as AdoMet) is an important biological methyl donor. It is also the precursor of aminopropyl groups utilized in polyamine biosynthesis and also involved in the glutathione and trypanothione synthesis in trypanosomatids by the trans-sulfuration pathway of cysteine. The methionine adenosyltransferase is an important enzyme for the metabolic synthesis of SAM in *Leishmania*, but the parasite is also able to acquire SAM by active transport from the outside. Sinefungin (SF), a nucleoside antibiotic produced by *Streptomyces griseolus* is structurally related to AdoMet and very effective against strains of *Leishmania*. To identify the SAM transporter, we have generated in vitro SF resistant *Leishmania major LV39* strain. We have observed in this mutant a loss of SAM accumulation related to SF resistance. Resistance to SF was correlated with gene rearrangement of some members of the folate/biopterin transporter. One of these genes SAMT, was found by gene transfection, gene inactivation and transport studies to be the main SAM transporter. Intriguingly, not all *Leishmania* strains are SF sensitive and this does not appear to be related to SNPs in SAMT but to gene expression level. To further study SAM metabolism and transport, we used functional cloning using SF selection and we have identified one cosmid conferring SF resistance. This cosmid codes for two ABCG transporters and ABCG transfectants exhibited a decrease of accumulation of SAM. This suggests that ABCG proteins may be involved in the efflux of SAM. This study, has allowed the characterization of the first SAM importer and exporter in parasites.

Polyamine Transport in *Trypanosoma cruzi*

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As part of an ongoing effort to study nutrient acquisition in kinetoplastids via specific permeases, we have been interested in identifying polyamine transporters in *Trypanosoma cruzi*. The synthesis of putrescine and spermidine de novo does not occur in *T. cruzi* that lack both ornithine decarboxylase and arginine decarboxylase. Consequently *T. cruzi* survival depends on its ability to acquire putrescine from its environment. A polyamine transporter, TcPOT1.1, was identified, cloned and characterized in the protozoan parasite *Trypanosoma cruzi*. The functional characterization of TcPOT1.1 was accomplished by over-expression of the transporter in *T. cruzi* epimastigotes and revealed a high affinity transporter for putrescine (Km: 158±18 nM and Vmax: 6.3±1 pmol/1e^{8}cells/s) and cadaverine (Km: 1.14±0.01 μ M and Vmax of 60±10 pmol/1e^{8}cells/s). TcPOT1.1 did not appear to transport spermidine or spermine in this background. TcPOT1.1 activity and localization was also found to be regulated with putrescine availability. The transporter, localized at the cytostome of the parasite when putrescine is readily available, displayed the ability to traffic from to the entirety of the plasma membrane upon putrescine scarcity.

Dissection of the Fe-S cluster assembly in *Trypanosoma brucei*

Julius Lukes*, Shaojun Long, Zuzana Vavrova, Zdenek Paris, Piya Changmai, Yan-Ze Wen

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Iron-sulfur (Fe-S) clusters are small inorganic cofactors that are omnipresent in prokaryotic and eukaryotic cells. Biogenesis of Fe-S clusters involves the participation of numerous proteins and in a typical eukaryotic cell is composed of the mitochondrial ISC assembly and the cytosolic CIA machinery, several components of which are highly conserved. We are investigating the Fe-S cluster assembly in the procyclic stage of *T. brucei*. To this end we have knocked down almost a dozen of proteins involved in the Fe-S cluster assembly and will present the analysis of obtained phenotypes, most of which are eventually lethal. We show that the ablation of some components disrupts only the synthesis of mitochondrial Fe-S clusters, while cytosolic clusters are still assembled. Moreover, we will show that thanks to their conservation, some components of the Fe-S cluster machinery in T. brucei can be replaced with homologues from distantly related eukaryotes.

Unusual inactivation of a MAP kinase from *Leishmania mexicana* by dual- and tyrosine phosphorylation in the TXY motif

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The mitogen-activated protein (MAP) kinase LmxMPK1 is essential for the proliferation of *Leishmania mexicana* amastigotes. Recombinant LmxMPK1 showed autophosphorylation and phosphorylated myelin basic protein without preceding activation by a MAP kinase kinase. Highest enzymatic activity was observed above 34C and at pH7.4 in the presence of Mn^{2+}. Whereas phosphorylation of threonine 176 of the TDY motif in the phosphorylation lip of the recombinant kinase could not be detected in mass spectrometry analyses, tyrosine 178 of the TDY motif was found to be autophosphorylated in an intramolecular process with a K_M value for ATP of 14.7 μM. Prolonged autophosphorylationof the recombinant kinase led to a decrease in the kinetics of substrate phosphorylation. We corroborated this inhibitory effect of tyrosine autophosphorylation by *in vitro* and *in vivo* analyses of LmxMPK1 TDY motif mutants demonstrating that phosphorylation on tyrosine 178 leads to an inactivated enzyme irrespective of the phosphorylation state of threonine 176. However, single threonine phosphorylation is essential for the *in vivo* activation of the kinase. Therefore, unlike classical MAP kinases, LmxMPK1 is regulated by a novel mechanism with highest activity in the presence of phosphothreonine and inhibition in the presence of phosphotyrosine only and in the dually phosphorylated state.

ABSTRACTS: SESSION III

Genetics and Epigenetics

April 27 1:30 p.m.

Chair(s) - Marc Ouellette

Application of second generation sequencing to parasite genomes

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The challenge of providing access to and integrating sequence data with biological knowledge is immense. GeneDB (http://www.genedb.org/) provides a single point of entry for sequence and annotation of eight kinetoplastid organisms. The resource combines data from finished and ongoing projects with computational predictions and manual curation, aiming to provide up-to-date, high quality and consistent datasets. The web presentation will focus on how: GeneDB displays primary annotation (e.g. predicted peptide properties and protein domains, gene ontology annotations, similarity information, database cross-references) and integrates these with expression, phenotype, localisation and orthologue data. Coding sequences can be viewed in the genomic context via the genome browsers Artemis and Gbrowse. Sequence updates and curation are handled within GeneDB. The majority of available trypanosomatid genomes are still actively being finished and frequent sequence updates are coupled with ongoing curation. Development of a new website, extension of existing annotation tools and curation infrastructure underpins the curation process and supports remote annotation. GeneDB and EuPathDB interact to provide integrated and concurrent resources, supporting user choice of interface and data display. Whilst GeneDB will continue to offer a live window on annotation and curation as it is produced, researchers are now encouraged to visit TriTrypDB to download versioned snapshots of the kinetoplastid genomes for use in publications.

Four histone variants mark the boundaries of polycistronic transcription units in *Trypanosoma brucei*

Nicolai Siegel[1], Doeke R. Hekstra[2], Louise E. Kemp[3], Luisa M. Figueiredo[1], Joanna E. Lowell[4], David Fenyo[5], Xuning Wang[6], Scott Dewell[7], George A. M. Cross[*,1]

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Unusually for a eukaryote, genes transcribed by RNA polymerase II (pol II) in *Trypanosoma brucei* are arranged in polycistronic transcription units. With one exception, no pol II promoter motifs have been identified, and how transcription is initiated remains an enigma. *T. brucei* has four histone variants: H2AZ, H2BV, H3V and H4V. Using ChIP-seq to examine the genome-wide distribution of chromatin components, we show that histones H4K10ac, H2AZ, H2BV and the bromodomain factor BDF3 are enriched up to 300-fold at probable pol II transcription start sites (TSSs). We also show that nucleosomes containing H2AZ and H2BV are less stable than canonical nucleosomes. Our analysis also identifies more than 60 unexpected TSS candidates and reveals the presence of long guanine-runs at all TSSs. Apparently unique to trypanosomes, additional histone variants H3V and H4V are enriched at pol II transcription termination sites. Our findings suggest that histone modifications and histone variants play crucial roles in transcription initiation and termination in trypanosomes and that destabilization of nucleosomes by histone variants is an evolutionarily ancient and general mechanism of transcription initiation, demonstrated in an organism in which no general pol II transcription factors have been identified.

The enrichment of base J at polycistronic transcriptional termination sites in the genome of trypanosoma brucei is regulated by JBP1

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Base J is an unusual DNA modification implicated in the regulation of antigenic variation in African trypanosomes. JBP2 and JBP1 represent thymidine-hydroxylases (TH) involved in J-biosynthesis. Based on our previous analysis of JBP function; JBP2 is a chromatin remodeling protein that induces de novo J synthesis whereas JBP1 is a J-DNA binding protein that propagates J-synthesis. The deletion of both enzymes from the bloodstream-form trypanosome generates a J-null cell line, confirming the importance of each in J-biosynthesis. Surprisingly, both JBP2 and JBP1 were capable of stimulating de novo J-synthesis when re-expressed in the J-null. We localized the JBP1 and JBP2 stimulated J by performing anti-J (IP) and DNA sequencing using solexa (Illumina) high throughput sequencing. This quantitative, non biased genome wide analysis revealed an enrichment of base J at polycistronic transcriptional termination sites throughout the genome, along with Histone variant 3 (H3V), in both WT and Null + JBP1 cells. Whilst JBP2 stimulated J-synthesis within the telomeric environment, it failed to stimulate J at the genome internal sites. The J that is stimulated by JBP1 is lost over time unless WT JBP2 (but not SWI/SNF mutant) is co-expressed. This suggests an epigenetic event inhibiting the maintenance of J by JBP1, which is relieved by JBP2. These results indicate that two THs are needed to provide both the specific localization and stability of J-biosynthesis. The co-localisation of J with H3V as well as its function at chromosome internal sites (ie chromatin structure and transcription termination) is currently under investigation.

The phenotype of J-binding protein 2 (JBP2) knock-out in *Leishmania*

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The genomic DNA of kinetoplastid parasites contains a hypermodified base, beta-D-glucosylhydroxymethyluracil or base J [1]. Base J localizes to repetitive elements in the genome [2]; in *Leishmania*, it is found almost exclusively in the telomeres [3]. To unravel the function of base J, we seek to target proteins involved in its synthesis. We have identified two proteins, JBP1 and JBP2, that are likely to be the enzymes catalyzing the first step in J biosynthesis [4,5]. JBP1 is essential in *Leishmania* [6], but JBP2 turned out to be dispensable [5]. The JBP2-/- cells gradually lose J, eventually reaching a ~8-fold reduction in comparison to WT, and display a strong synthetic lethal phenotype upon treatment with bromodeoxyuridine (BrdU; a thymidine analogue that lowers J-levels in kinetoplastids [7]). This phenotype is accompanied with increased cell death but not with specific cell cycle blocks, telomere attrition or loss of genome integrity. To gain more insight into the underlying mechanisms, we performed a genomic cosmid library screen to discover genes capable of rescuing the BrdU-sensitivity of the JBP2-/- cells. As expected, the most commonly occurring cosmid among the rescue colonies contained JBP2, but in addition, a few other cosmids were found back multiple times. We are in the process of cloning and testing individual candidate genes from these cosmids. References: [1] Gommers-Ampt JH, et al. Cell 1993;75:1129-36. [2] Van Leeuwen F, et al. MBP 2000;109:133-45. [3] Genest PA, et al. NAR 2007;35:2116-24. [4] Yu Z, et al. NAR 2007;35:2107-15. [5] Vainio S, et al. MBP 2009;164:157-61. [6] Genest PA, et al. NAR 2005;33:1699-709. [7] Van Leeuwen F, et al. MCB 1998;18:5643-51.

TbISWI binds ubiquitously in the *T. brucei* genome and is involved in repression of transcriptionally silent regions

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TbISWI, a member of the ISWI family of SWI2/SNF2-related chromatin-remodelling proteins, is involved in VSG expression site (ES) downregulation in both bloodstream form (BF) and procyclic form (PF) *T. brucei*. Here, we use chromatin immunoprecipitation (ChIP) on myc-tagged TbISWI cell lines to probe for the genomic loci it binds. We find that TbISWI binds the minichromosome repeats as well as the 50-bp and the telomeric repeats in both BF and PF *T. brucei*. Interestingly, TbISIWI is enriched in the CR147 centromeric repeats in BF *T. brucei* only. There is no significant difference in TbISIWI binding to an active versus a silent ES in BF *T. brucei*. Although TbISIWI is depleted on the ES promoter, there is little variation in its distribution along the length of an ES in both BF and PF *T. brucei*. TbISWI is present on other transcriptionally silent areas of the genome including the chromosome-internal silent VSG arrays and the rDNA spacers. TbISWI, like its yeast counterparts, appears to bind throughout the *T. brucei* genome and could be influencing the transcription of a broad range of genomic loci. We monitored the effect of RNAi-mediated TbISWI depletion on the expression of an eGFP gene, inserted either with or without an ES promoter, into the transcriptionally silent minichromosomes and the chromosome-internal silent VSG arrays in both BF and PF *T. brucei* life cycle stages. We find that TbISWI depletion causes derepression of the ES promoter driven reporter gene in all these genomic contexts, however, no promoter independent fortuitous initiation of transcription was observed.

Illuminating the endogenous small interfering RNA pathway in Trypanosoma brucei by deep sequencing

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At the core of the RNA interference (RNAi) pathway in the ancient parasitic eukaryote Trypanosoma brucei is a single Argonaute protein, TbAGO1, with an established role in the destruction of potentially harmful retroposon transcripts. Recent evidence from higher eukaryotes suggests that, in addition to transposons, a variety of genomic sequences with the potential to produce double-stranded RNA are sources for small interfering RNAs (siRNAs). To test whether such endogenous siRNAs are present in T. brucei, we affinity purified TbAGO1 from procyclic- and bloodstream-form trypanosomes and subjected the bound RNAs to Illumina high-throughput sequencing. We obtained a total of 2.7 million procyclic and 1.2 million bloodstream sequences able to be mapped to the genome. Confirming our earlier observations, the majority of reads (about 75%) in both libraries originated from retroposons. Nevertheless, we also considerably expanded the repertoire of trypanosome siRNAs to encompass a family of 147 bp satellite-like repeats (CIR147 for chromosomal internal repeats and a putative centromeric region), many of the regions where RNA polymerase II (Pol II) transcription converges, large inverted repeats and a few pseudogenes. Notably, our data indicate that putative centromeric regions, excluding the CIR147 repeats, are not a significant source for endogenous siRNAs. Furthermore, the relative representation of different classes of endogenous siRNAs differs substantially between procyclic and bloodstream trypanosomes. For instance, SLACS retroposons contribute 20% of bloodstream-form siRNAs, but only 8% in procyclic parasites. Taken together, our data suggest that endogenous RNAi targets may be as evolutionarily old as the mechanism itself.

A novel component of the RNAi pathway in *Trypanosoma brucei* is required for loading siRNAs into Argonaute

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Our functional studies, supported by comparative genomic analysis, identified five factors required for RNA interference (RNAi) in *Trypanosoma brucei*, namely a member of the Argonaute protein family (*Tb*AGO1), two Dicer-like enzymes (*Tb*DCL1 and *Tb*DCL2), *Tb*570, the siRNA-AGO1 loading factor, and *Tb*1509, a protein still in search of a function. These five factors are likely to constitute the core RNAi machinery. The only recognizable domain in *Tb*570 is a 3'-5' exonuclease domain, also present in the *Neurospora* QIP protein (qde2/AGO-interacting protein). We know that *Tb*570 is central to the RNAi pathway, since KO cells resulted in RNAi deficiency, as assessed by transfection of double-stranded RNA, and in the accumulation of long retroposon transcripts. However, we found that in 570KO cells the level of *Tb*AGO1 was reduced approximately 20-fold. This is to our knowledge the only example of an AGO protein whose accumulation is affected by a specific factor. Both RNAi deficiency and the low level of AGO1 were restored to wild-type levels by reintroduction of a *Tb*570-GFP fusion construct. *Tb*570-GFP localizes to the cytoplasm and the nucleus and is present in large complexes with an apparent MW greater that 500 kDa. Notwithstanding the low AGO1 levels, retroposon-derived siRNAs accumulated in 570KO cells. However, the majority of the siRNAs were not found in a complex with AGO1. Furthermore, whereas siRNAs in wild-type and 570 complementation cells appeared to be mostly single-stranded, siRNAs in 570KO cells were present in duplex form. Thus, our results are consistent with a model whereby *Tb*570 is required for loading double-stranded siRNAs into AGO1 and to stabilize AGO1.

RNAi in *Leishmania braziliensis*

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RNA interference (RNAi) is a powerful tool well suited for genetic analysis in trypanosomatids, where diploidy, gene families and genetic crossing pose experimental challenges. Previously we reported that *L. braziliensis* shows good RNAi activity when tested using GFP reporters. We have extended these studies to additional reporters and chromosomal genes. While strong down regulation of target mRNAs was seen, a range of phenotypic effects ranging from weak to strong were observed. Little or no effects on LPG synthesis were seen despite strong down regulation of several LPG gene transcripts. In contrast, RNAi of two *L. braziliensis* genes required for paraflagellar rod formation *PFR1* and *PFR2* gave strong phenotypes including loss of the paraflagellar rod and swimming defects, comparable to null mutants described previously in *L. mexicana*. These findings are similar to those seen in other creatures and suggest that RNAi will be comparably useful as a genetic tool in *L. braziliensis*. Further studies suggest that RNAi activity may occur in several isolates of the *Leishmania* subgenus *Viannia* including *L. panamensis* and *L. guyanensis*, but has been lost in the lineage leading to the higher *Leishmania* including *L. tarentolae*, *L. mexicana*, *L. donovani* and *L. major*. This suggests the possibility that loss of the RNAi pathway may be related to differences in the biology of *Viannia* species relative to other *Leishmania*, including infectivity, drug resistance mediated by circular gene amplification, and RNA viruses. To probe the forces impacting upon the retention or loss of RNAi in *Leishmania* evolution, we have generated an informative *Argonaute* null mutant (*ago1*$^{-}$) of *L. braziliensis*, and a complemented derivative, whose properties will be described.

A genetic analysis of pathogenesis in Trypanosoma brucei

Liam Morrison, Sarah McLellan, Lindsay Sweeney, Chi Chan, Andy Tait, Annette MacLeod, Mike Turner*

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We have investigated the extent to which variation amongst strains of T. brucei determines pathogenesis in mice and then investigated the underlying mechanisms. We compared the pathology in an inbred mouse strain infected with either of two strains of trypanosome. Infections with one strain, 927, cause greater anaemia than infections with the other, 247, that cause greater splenomegaly and more reticulocytosis. We then investigated the inheritance of these traits in F1 progeny of a genetic cross of the two trypanosome strains. Quantitative Trait Locus (QTL) analysis showed that the pathology phenotypes were partly heritable and candidate loci could be identified in our previously described genetic map. Major loci for splenomegaly and hepatomegaly were identified on the same chromosome along with other QTLs for anaemia and reticulocytosis. Clinical pathology 'readouts' are expected to be the products of multiple mechanistic pathways and so to elucidate potential mechanisms that determine differences in pathogenesis in infections with the two strains we undertook a microarray analysis of host gene expression, comparing spleens from mice infected with each of the two trypanosome strains. Approximately 60 % of transcripts up- or down-regulated (at p¡0.01) are common to infections with both strains and the remaining 40 % are regulated in a strain-specific manner. Ingenuity Pathway analysis identified 13 significantly differentially regulated canonical pathways. We conclude that pathology in the host is partly parasite driven in a strain-specific and heritable manner.

ABSTRACTS: SESSION IV

Nuclear Structure and Function

April 28 8:45 a.m.

Chair(s) - Christian Tschudi

Cohesin-mediated inheritance of monoallelic VSG expression in *Trypanosoma brucei*

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Antigenic variation in *Trypanosoma brucei* allows the parasite to elude the host immune response. This is achieved because only one out of ~20 subtelomeric Variant Surface Glycoprotein-Expression Sites (VSG-ES) is transcribed at a given time and maintained active throughout many generations. VSG-ES transcription is mediated by RNA polymerase I (pol I), which is found in a discrete nuclear site named the Expression Site Body (ESB). We here investigate how the VSG-ES active transcriptional state is passed on to the next generation. During S-phase, G2-phase and early mitosis the active VSG-ES locus remains associated with the single ESB. Upon mitosis, the active VSG-ES exhibits a delay in the separation of sister chromatids relative to control loci. Such a delay is dependent on the cohesin complex, as the knockdown of cohesin subunits resulted in premature separation of sister chromatids of the active VSG-ES. Importantly, cohesin depletion also prompted transcriptional switching from the active to previously inactive VSG-ESs. These data suggest that in addition to maintaining sister chromatid cohesion until mitosis, the cohesin complex plays an essential role in the correct epigenetic inheritance of the active transcriptional VSG-ES state.

Nuclear organization in Trypanosomatids

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Mitosis in Trypanosomatids displays original or intriguing features such as persistence of the nuclear membrane and of the nucleolus, absence of centrosomes and an insufficient number of kinetochores. Our objectives are to understand the architecture and functioning of their nucleus during the cell cycle. A preliminary step to this ambitious goal is to establish the subcellular localization of proteins involved in basic molecular processes associated to nuclear activity, and to correlate these data with mitotic spindle and chromosomal dynamics. The results show a high level of structuration of the nucleoplasm, associated to original features of Trypanosomatids, as well as to specific differences between Leishmania major and Trypanosoma brucei that might have relevant consequences upon their genetics. Thus, the ca. 200 nuclear pores are distributed as a discrete number of 15-20 clusters. These alternate with dense chromatin structures where we found chromosome telomeres, located at the nuclear periphery. More internally, the nucleole appears as a second structuring element: (i) it contains proteins with a discrete localization; (ii) the centromeres (solely identified in T. brucei) were found located by FISH in its periphery and associated with spastin (also only present in T. brucei), a protein that might be associated with kinetochores. The presence of non-coding sequences in this region classically known as transcriptionnally hyperactive is intriguing (”functional patchwork”). Moreover, the chromosomes appear in a radiating position, with centromeres at the nucleolar periphery and telomeres at the nuclear periphery. Finally, the location of enzymes responsible for chromatin condensation as well as of microtubule associated proteins will be discussed in the light of their possible implication in mitosis and of novel genetic data.

The Aurora-like Kinase in Trypanosoma brucei Plays Critical Roles of Promoting Metaphase-Anaphase Transition and Initiating Cyokinesis

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The unusual chromosomal passenger complex (CPC) in *T. brucei* consists of an Aurora-like kinase (AUK1) and two novel proteins CPC1 and CPC2 and bears little resemblance to the CPC in yeast or metazoans. It moves from chromosomes to the spindle midzone during metaphase-anaphase transition, and then displays a unique trans-localization to the dorsal side of the cell to initiate cytokinesis by gliding from the anterior to the posterior end of the cell along the apparent flagellar attachment zones to divide the cell into two (Li et al. (2008) PLoS ONE 3: e3814). To envision the potential role of AUK1 in driving this unusual process, the procyclic cells expressing fused CPC1-enhanced yellow fluorescence protein were synchronized by hydroxyurea and released in late S-phase. An inhibitor of AUK1 VX-680 with an IC50 of 190 nM on the enzyme and 10 M on cell growth was added to the cells at different times after the release. The progression of cell cycle thereafter was monitored with FACScan and fluorescence microscopy. From 0 to 2 hours, addition of VX-680 arrested the cells in metaphase, suggesting that AUK1 is essential for the metaphase-anaphase transition as shown in other eukaryotes. When the cells reached anaphase 3 hours after the release, however, VX-680 addition stopped the CPC trans-localization from the spindle midzone, dispersed and distributed the CPC back to the two segregating nuclei and blocked cytokinesis. Addition of VX-680 after 4 hours showed no effect on the cell cycle progression. This unusual phenomenon, recorded by time-lapse videos, demonstrates the uniquely AUK1-mediated mechanism of cytokinetic initiation that could provide an important model for in-depth understanding of cytokinesis.

Asymmetric chromosomal distribution leads to constitutive mosaicism in *Leishmania*

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Leishmania is generally considered as diploid but aneuploidy exists, particularly chromosome 1 is known to be triploid in *L. major* Friedlin strain. In this work, we analyzed by fluorescence in situ hybridization (FISH) the actual number of homologues for seven chromosomes in individuals cells of the *L. major* Friedlin strain, of two other strains of *L. major* and of eight clones derived from them. Our results show that (i) aneuploidy affects most of the studied chromosomes. (ii) Ploidy is not even for a given chromosome in a given strain: cells are either disomic or trisomic for that chromosome (Chr 1, Chr 5 and Chr 17), or the repartition is monosomic/disomic (Chr 2). These data show constitutive intra-strain ploidy heterogeneity, or mosaicism, in *Leishmania*. (iii) Proportions of monosomy/disomy or disomy/trisomy may vary for a given chromosome from a strain to another. (iv) In sibling clones, these proportions were generally, but not always, similar to those observed in the parental strain, showing a certain stability of aneusomy. We hypothesized that the mechanism leading to aneuploidy might be of epigenetic nature. In order to further elucidate the genetic mechanisms involved in mosaicism, we studied the distribution of chromosomes in dividing cells. We observed asymmetric chromosome distribution between both divided nuclei for a number of chromosomes. For Chr 5, asymmetric chromosome distribution was seen in as much as 50% of dividing cells. The whole of these data strongly suggest that mis-replication and/or mis-segregation may be the general rule in *Leishmania*. These original data should modify the conception of the biology of these divergent eukaryotes.

Genome wide adaptable amplification in *Leishmania*

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DNA amplification is found in various organisms and occurs when the enhanced expression of the amplified genes is advantageous. In *Leishmania* DNA amplification is mainly known as a drug resistance mechanism and many studies pinpoint the importance of homologous recombination of repeated sequences (RS) for DNA amplification. Using a bioinformatic approach we analyzed the *Leishmania* genome to identify non-coding RS and all related amplifiable locus. A total of 5500 RS were highlighted scattered through the 36 chromosomes of *Leishmania*. A large majority of the genes of the *Leishmania* genome could thus potentially be amplified extra-chromosomally through these RS. We report here that stochastic homologous recombination between these RS occurs randomly in *Leishmania* parasites at a rate estimated to ¡10-8 and provide to *Leishmania* populations a bank of amplicons. Indeed at the population scale and with a sensitive PCR strategy we showed that up to 80% of the potential amplicons are detected in a single in-vitro culture. A similar situation may also occur in *T. brucei*. This random amplification of different genomic loci at the population level may represent a mechanism of adaptation to environmental stresses. To test this we induced stresses and we monitored by qPCRs the increase of targeted amplicons. We showed that the cells containing an amplicon providing an advantage under a specific stress are positively selected and ultimately form a new (sub)population. This subpopulation reverts to the initial population when stresses are removed. Effective gene amplification and clonal selection for increased copy number and expression of specific genes under selective conditions may have evolved as a strategy to compensate for the lack of transcriptional control in *Leishmania*.

A single endonuclease-generated DNA break induces antigenic switching in Trypanosoma brucei

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Trypanosoma brucei is the causative agent of African Sleeping Sickness in humans and one of the causes of Nagana in cattle. This protozoan parasite evades the host immune system by antigenic variation, a periodic switching of its variant surface glycoprotein (VSG) coat. VSG switching is thought to occur predominantly through gene conversion, a form of homologous recombination initiated by a DNA lesion that is used by other pathogens (e.g. *Candida albicans*, *Borrelia sp.* and *Neisseria gonorrhoeae*) to generate surface protein diversity, and by B lymphocytes of the vertebrate immune system to generate antibody diversity. Very little is known about the molecular mechanism of VSG switching in *T. brucei*. Here we demonstrate that the introduction of a DNA double-strand break (DSB) within the ~70-bp repeats upstream of the transcribed VSG increases switching *in vitro* ~250-fold, producing switched clones with a frequency and features similar to those generated early in an infection. We were also able to detect spontaneous DSBs within the 70-bp repeats upstream of the actively transcribed VSG, suggesting that a DSB is a natural intermediate of VSG gene conversion and that VSG switching is the result of the resolution of this DSB by break-induced replication.

HAT3 and SIR2rp1 control RAD51-dependent DNA repair in African trypanosomes

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Antigenic variation in *T. brucei* depends upon Variant Surface Glycoprotein recombination and RAD51 recombinase activity. Covalent post-translational chromatin modifications have important roles in recombination and repair in other organisms but the mechanisms remain only partially characterised. We are currently assessing the role of histone acetylation in DNA repair in *T. brucei*. Among eleven putative histone acetyltransferases (HATs) and deacetylases (DACs), several are dispensable for growth. We used an inducible meganuclease system to introduce site-specific DNA double-strand breaks (DSB) and to assess the dispensable enzymes for roles in DNA repair. This revealed defects in *hat3* (histone H4-K4 acetyltransferase) and *sir2rp1* (histone deacetylase) null cells. Further analysis revealed *hat3*-defects in DNA resection and RAD51 assembly. In contrast, the *sir2rp1* strains displayed defects in ssDNA processing and RAD51 disassembly. Neither protein was required for efficient single-strand annealing, a RAD51-independent repair mechanism. These results indicate a role for histone acetylation and deacetylation (possibly involving H4-K4) in RAD51-dependent DNA repair in trypanosomes.

Evolutionary changes in the *Leishmania* cap4-binding eIF4F complex as reflected by the eIF4E-eIF4G interactions

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Most translation initiation events in eukaryotes involve the assembly of an eIF4F complex over the 5' cap of mRNAs. Complex formation is based on the interaction between the cap-binding protein eIF4E and the scaffold-protein eIF4G. The four *Leishmania* eIF4E orthologues (LeishF4E-1 through -4) are structurally diverged from their higher eukaryote counterparts, possibly since they have evolved to bind the cap-4 structure. This evolutionary diversity is further emphasized by their inability to functionally complement the mutated yeast eIF4E, unlike the *Drosophila* and *Arabidopsis* orthologues. Based on biophysical and biochemical assays, LeishIF4E-1 and LeishIF4E-4 are most likely the basal factors involved in translation initiation. Evolutionary changes are also reflected in the structure of the eIF4G orthologue of *Leishmania* that we characterized (LeishIf4G-3) from a pool of candidates. LeishIF4G-3 is smaller than the mammalian eIF4G, but contains a conserved MIF4G domain, interacts directly with LeishIF4E-1 and LeishIF4E-4 but not with the mammalian eIF4E, copurifies with these factors over m7GTP-Sepharose and comigrates with them over sucrose gradients. In higher eukaryotes the eIF4E-eIF4G interaction is mediated by the N-terminus of eIF4G that contains a conserved peptide signature [Y(X4)Lϕ], where X is any aa and ϕ is a hydrophobic residue. Although the N-terminus of LeishIF4G-3 is much shorter, we identified by yeast-two hybrid assays a parallel eIF4E binding peptide and verified its binding by NMR spectroscopy. However, this binding motif varies extensively from the parallel peptide in higher eukaryotes, as indicated by site-directed mutagenesis. In view of these diversities, the characterization of the parasite eIF4E-eIF4G interaction may serve as a novel target for inhibiting Leishmaniasis and provide an important insight for future drug discovery.

ABSTRACTS: SESSION V

Kinetoplast and Mitochondrion

April 28 1:30 p.m.

Chair(s) - Michele Klingbeil

RNA Editing in kinetoplastids: Physical and functional interactions

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Kinetoplastid RNA editing involves insertion and deletion of uridines to produce translatable mitochondrial RNAs. A multiprotein editosome catalyzes the enzymatic steps of editing. In addition, numerous dynamically associated factors appear to be required to facilitate macromolecular interactions during editing. TbRGG2 is an essential editing factor in both procyclic and bloodstream *Trypanosoma brucei*. The protein transiently interacts with the editosome and with other factors implicated in mitochondrial RNA metabolism. RNAi-mediated depletion of TbRGG2 causes a dramatic inhibition of editing of all pan-edited RNAs, while leaving minimally editing RNAs unaffected. TbRGG2-depleted cells accumulate RNAs that are edited at their 3' ends, but unedited at their 5' ends. This indicates that editing initiates correctly in the absence of TbRGG2, but that the 3' to 5' progression of editing is severely compromised. Accordingly, core editosomes are not disrupted upon TbRGG2 depletion. To determine the step of editing impacted by TbRGG2, we carried out detailed sequence analysis of partially edited RNAs (RNAs in the process of being edited) in TbRGG2 RNAi cells. We observe a decrease in junction lengths and a correlation between editing pause sites and the 3' ends of known gRNA sequences in TbRGG2-depleted cells, consistent with a model in which TbRGG2 facilitates sequential gRNA usage during editing. Biochemical properties of the protein support this model. In vitro, TbRGG2 exhibits gRNA and mRNA binding. Moreover, the protein displays both RNA annealing and RNA unwinding activities, which are separable and can be attributed to distinct domains of the protein. TbRGG2 is the first described editing processivity factor, and likely acts through its capacity to modulate RNA-RNA interactions.

Structure of the Core Editing Complex (L-Complex) Involved in Uridine Insertion/Deletion RNA Editing in Trypanosomatid Mitochondria

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Uridine insertion/deletion RNA editing is a novel form of post-transcriptional RNA processing that occurs in mitochondria of kinetoplastid protists. Although the basic mechanism of editing is understood, there is no structural information available with which to understand the process in molecular detail. We have carried out three-dimensional structural analyses of the core editing complex or L-complex from Leishmania tarentolae mitochondria. The complex was isolated using the TAP procedure. The purified material sedimented at 20-25S, migrated in a blue native gel at 1 MDa and exhibited both pre-cleaved and full cycle gRNA-mediated U-insertion and U-deletion in vitro activities. The structural homogeneity of the purified L-complex sample was subjected to structural analysis by electron tomography. Three-dimensional structural comparisons of individual particles in the tomograms revealed that a majority of the complexes have a similar shape of a slender triangle. A small portion of the particles exhibited an additional density protruding from the central region. Single particle reconstruction of L-complex particles at a resolution of $\sim$15 Å showed a very similar triangular structure with a dimension of 200x140x80 Å with a central ring and several channels, which might have implications in RNA editing. This structure should prove useful for a detailed analysis of the editing reaction. body

How uridylylation is shaping mitochondrial transcriptome in T. brucei

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Developmentally-regulated expression of the mitochondrial genome in Trypanosoma brucei is controlled primarily at the post-transcriptional level. RNA processing begins with a nucleolytic partitioning of multicistronic transcripts into ribosomal RNAs and pre-messenger RNAs. All pre-mRNAs are subjected to polyadenylation and many are further modified by extensive uridine insertion/deletion RNA editing, which is directed by guide RNAs. Ribosomal RNAs and guide RNAs are post-transcriptionally uridylylated (10-15 Us); never-edited and fully-edited mRNAs are marked by A/U heteropolymers (100-200 nt). We scrutinized the roles of RNA Editing TUTase 1 (RET1) in the formation and regulation of the mitochondrial transcriptome. The effects of RET1 repression and overexpression on the processing and stability of mitochondrial RNAs have been investigated. In addition, we analyzed protein factors that may modulate RET1 activity. The conclusions are: 1) RET1- catalyzed uridylylation stabilizes short RNAs ultimately affecting RNA-directed processes of RNA editing and, possibly, nucleolytic partitioning of multi-cistronic transcripts; 2) U-addition directly controls the stability and, therefore, the abundance of ribosomal RNAs; 3) The balance of adenosine and uracil residues in A/U tails determines the half-life of translatable mRNAs regardless of their processing history. Possible mechanisms of these processes will be discussed. We postulate the existence of a new class of short RNAs, termed processing guide RNAs (pgRNA). These RNAs are predicted to be shorter than 30 nucleotides, uridylylated, and to act in nucleolytic processing of maxicircle- and minicircle-encoded RNA precursors.

Trypanosoma brucei Tob55 functions as a protein translocator on mitochondrial outer membrane

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Tob55 is the major component of the TOB complex that is responsible for the assembly of beta barrel proteins on mitochondrial outer membrane (OM). A Tob55 homolog is identified in *Trypanosoma brucei*, a parasitic protozoan, which causes African trypanosomiasis. *T. brucei* Tob55 (TbTob55) possesses a predicted beta barrel structure and showed 17-22% identity and 30-38% similarity to Tob55 from other species. TbTob55 knock-down decreased the level of several mitochondrial proteins in the OM, inner membrane (IM), intermembrane space (IMS), and matrix indicating that TbTob55 is crucial for mitochondrial protein biogenesis. TbTob55 depletion decreased and its over expression increased the level of the matured complex of mitochondrial porin suggesting that TbTob55 is involved in beta barrel protein assembly on OM. Furthermore, *in vitro* import of the cytochrome oxidase subunit IV (COIV) is drastically reduced in TbTob55 knock-down mitochondria. Preincubation of isolated mitochondria from *T. brucei* with anti-Tob55 antibody blocked the import of this nuclear encoded mitochondrial protein, showing that TbTob55 is directly involved in mitochondrial preprotein import. These results strongly suggest that TbTob55 is a protein translocator of OM. Various searches in trypanosome genome database have failed to identify either a homolog of Tom40 or any other compenents of the TOM complex. Thus, it is likely that TbTob55 is the protein translocator on the OM in *T. brucei*.

A double inducible system to study mitochondrial tRNA import in living *Trypanosoma brucei*

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The mitochondrial genome of the kinetoplastid *Trypanosoma brucei* does not encode any tRNA genes. Thus, in order for mitochondrial translation to occur, all mitochondrial tRNAs have to be imported from the cytosol. The observed extent of import ranges for 1 - 12% of the total cellular complement depending on the tRNA species. While all mitochondrial tRNAs derive from cytosolic ones, there are two tRNA ($tRNA^{Met}$ and the $tRNA^{Sec}$) that are cytosol-specific. The composition of the tRNA import machineries in the inner and the outer mitochondrial membranes is still unknown. Thus, we have established an *in vivo* system that permits us to identify tRNA import factors. It consists of a cell line that allows separate induction of RNAi by tetracycline and expression of a tagged tRNA by IPTG at any desired time point. This double inducible system allows us to follow import of a newly synthesized tagged tRNA during RNAi-mediated knockdown of any putative mitochondrial import factor. Preliminary results showed that ablation of the trypanosomal Tim17, a component of the inner membrane protein translocation machinery, as well as ablation of an unidentified protein necessary for mitochondrial matrix protein import (unpublished data), abolished import of the newly synthesized tRNA. Those results indicate that some components of the protein import machinery are, directly or indirectly, required for mitochondrial tRNA import in *Trypanosoma brucei*.

The unexpected effects of ethidium bromide on Trypanosoma brucei

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Ethidium bromide (EB) has been used to treat bovine trypanosomiasis in Africa for a half-century. EB causes kDNA loss in BSFs and is a poison for mito-topoisomerase II. We now report unexpected effects of EB on kDNA. EB should intercalate within network minicircles and free minicircle replication intermediates. Those that were covalently-closed would develop positive supertwists that would be removed by an endogenous topo, thereby reducing the minicircle linking number. Following DNA isolation and EB extraction, the minicircles would then develop negative supertwists. As shown by gel electrophoresis and other methods, this occurred with covalently-closed free-minicircle replication intermediates. The minicircles average linking number was reduced to a level so low that it could not be compensated by supertwisting and some sequences were forced to flip into left handed Z-DNA. These extreme effects on free minicircle topology explain our observation that EB abruptly stops kDNA replication. We found a totally different situation with network minicircles. Surprisingly, EM demonstrated that EB does not supertwist network minicircles, even though they reside in the same compartment as the free minicircles. One probable reason is that networks are bound to proteins that effectively prevent helix unwinding and, consequently, permit little EB binding. In support of this hypothesis, we found that one such protein, p19, which localizes to the kinetoplast, prevents EB binding to networks in vitro. Since we now know that kDNA is essential for wild type BSF viability, these results provide a mechanism for EB toxicity to BSFs. However, there must be other mechanisms because akinetoplastic BSFs are also killed by EB.

Mitochondrion of kinetoplastid parasites as a chemotherapeutic target

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Kinetoplast is the mitochondria of flagellated protozoa of the order Kinetoplastida, showing unique structure and function and known to be essential in survival of these parasites. The kinetoplast DNA (kDNA) consists of few dozen maxicircles, encoding typical mitochondrial proteins and ribosomal RNA and several thousands minicircles, encoding guide RNA molecules that function in the edition of maxicircles mRNA transcripts. We designed a high-throughput/high-content assay for *Leishmania donovani* intramacrophagic amastigote as a primary screening for selection of compounds that kill *L. donovani* in a unspecified manner, and a kDNA-targeted assay in *L. donovani* promastigote as a secondary screening in order to identify, among the leishmanicidal compounds from the primary screening, those acting through a kDNA replication-dependent mechanism. Giving the uniqueness of kinetoplast replication (as compared to host mitochondria), this approach provides substantial advantages, increasing potential of finding parasite-specific drugs. In primary screening, human macrophage THP-1 infected by *L.donovani* metacyclic form were cultured in 384-well plates. After drug exposure, cells and parasites were fixed and DNA was stained for reading in the automated confocal microscopy. Amphotericin B was used as positive control for drug activity. Automated image analysis used in-house built algorithm taking into account parameters such as infection ratio, cell numbers, and parasite numbers in order to evaluate infection of macrophages by amastigotes. Currently, we are about to finish 200,000 drug-like small compounds from chemically diverse library in the primary screening, and selected hits will be screened in kinetoplast targeted assay, which is under development and will be based on high-content screening and analysis of a BrdU replication assay. Sponsored by IPK, DNDi, GTZ on behalf of the German Government.

Discovery of the First Drug-like Inhibitors of an Essential RNA Editing Ligase in *Trypanosoma brucei*

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Trypanosomatid RNA editing is a unique process and essential for these organisms. It therefore represents a promising new drug target for a group of protozoa that includes the causative agents for African sleeping sickness and other devastating tropical and subtropical diseases. Here, we present the first drug-like inhibitors of a key enzyme in the editing machinery, RNA editing ligase 1 (REL1). These inhibitors were identified through a novel strategy employing molecular dynamics to account for protein flexibility. A virtual screen of the REL1 crystal structure against the NCI Diversity Set was performed using AutoDock4. The top 30 compounds, predicted to interact with REL1's ATP binding pocket, were further refined using the relaxed complex scheme (RCS), which redocks the compounds to receptor structures extracted from an explicitly solvated molecular dynamics trajectory. The resulting reordering of the ligands and filtering based on drug-like properties resulted in an initial recommended set of eight ligands, two of which exhibited micromolar activity against REL1. A subsequent hierarchical similarity search with the most active compound over the full NCI database and RCS rescoring resulted in an additional set of six ligands, two of which were confirmed as REL1 inhibitors with IC50 values of $\sim$1 μM. Tests of the three most promising compounds against the most closely related bacteriophage T4 RNA ligase 2 as well as against human DNA ligase IIIβ indicate a considerable degree of selectivity for RNA ligases. These compounds are promising scaffolds for future drug design and discovery efforts against these important pathogens.

ABSTRACTS: SESSION VI

Gene Expression

April 29 8:45 a.m.

Chair(s) - Piet Borst

Trans-splicing regulation – spliced leader RNA silencing, and spliced leader RNA discard – two processes that control the level of SL RNA

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Trans-splicing is regulated at different levels; transcription of the SL RNA, biogenesis of the SL RNP and its utilization. SL RNA silencing (SLS) was first observed in cells depleted for the SRP receptor (Lustig et al., EMBO rep. 2007). Here we show that SLS is induced also by depletion of ER translocation factors SEC63 and SEC61. SLS elicits a specific shut-off of SL RNA transcription due to the failure of the SL RNA transcription factor tSNAP42 to bind to its cognate promoter (Lustig et al., 2007). We now find that SLS is induced under ER stress by inducers of the unfolded protein response (UPR) in other eukaryotes. SLS is a death pathway that is induced upon persistent ER stress and has all the hallmarks of programmed cell death (PCD). The PCD is associated with perturbation in calcium homeostasis. Proteome and transcriptome analyses are in progress to identify the signaling pathway leading to SLS. SL RNA biogenesis is also a highly regulated process. Here we describe a novel process that we term SL RNA discard (SLD) that removes defective SL RNA from the nucleus, exports it to the cytoplasm and then excretes it from the cell. SLD is induced in cells depleted for Sm proteins. The particle (SL RNP-C) was purified and proteins such as actin, helicase, and ATPase were identified in the complex. Live cell imaging and SEM microscopy were used to follow the secretion of the complex from the cell. SLD is therefore the first mechanism described to date that removes defective RNA not by degradation, but by sequestration and excretion from the cell.

The pseudo-CTD of RNAPII is essential for productive RNA synthesis in *Trypanosoma brucei*

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The RNA polymerase II (RNAPII) machinery is complex and only studied extensively in metazoa and yeast. In these organisms, the carboxy-terminal domain (CTD) of the largest subunit of RNAPII is composed of heptapeptide repeat sequences and is essential for gene expression. The CTD orchestrates myriad transcriptional and co-transcriptional processes that are accomplished in part by a dynamic set of modifications. Differentially phosphorylated CTD signals release of enzyme from the preinitiation complex at transcriptional promoters, ensures subsequent elongation and coordinates messenger RNA (mRNA) 5' capping, processing, polyadenylation and termination. Despite this strict structural and functional conservation of CTD found in many organisms, a surprising number of eukaryotes lack a heptapeptide repeat containing RNAPII and yet produce 5' capped, processed and polyadenylated mRNAs. To investigate how this non-heptapeptide CTD, called pseudo-CTD, contributes to RNAPII machinery we explored this phenomenon in trypanosomes. African trypanosome, *Trypanosoma brucei* contains an ~285 amino acids long pseudo-CTD, rich in serine and proline, that follows the highly conserved A through H domains characteristic of the largest subunit (RPB1) of all eukaryotic RNAPII. We learned that RNAPII lacking the CTD altogether or containing only 95 amino acids, fails to support cell viability. In contrast, RNAPII with 186 amino acids long CTD maintains cellular growth. Moreover we observed that RNAPII with pseudo-CTD truncations resulted in abortive transcription initiation that was more pronounced in the case of the shorter pseudo-CTD. These data reveal that a highly divergent CTD plays as important a role in gene expression as a heptapeptide-repeat containing CTD in RNAPII transcription.

A novel, TFIIH-associated, multi-subunit transcription factor is essential for SL RNA gene transcription in *Trypanosoma brucei*

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In eukaryotes, general transcription factors (GTFs) including TFIIH and TFIIE form pre-initiation complexes with RNA polymerase (pol) II at all core promoters. Only recently, extremely divergent GTF orthologues were identified in trypanosomatids and shown to be essential for transcription of spliced leader (SL) RNA genes (*SLRNAs*) which encode the SL donor in *trans* splicing of all nuclear pre-mRNA. Since SL RNA is consumed in the process, parasite survival requires a continuously high SL RNA synthesis rate. We have characterized an active form of *Trypanosoma brucei* TFIIH and shown that it is essential for *SLRNA* transcription. TbTFIIH harbors orthologues of all seven core subunits but apparently lacks the cyclin-activating kinase (CAK) sub-complex which, in other eukaryotes, is involved in RNA pol II phosphorylation; instead, TbTFIIH contains two additional core subunits which have no sequence resemblance to other GTFs. Currently, we are investigating the possibility that these subunits represent orthologues of the TFIIE alpha/beta ubunits. Furthermore, we noted co-purification/sedimentation of several minor proteins with TFIIH. Since they could be CAK components, we identified one of them by mass spectrometry. Subsequent PTP tagging and purification combined with a sedimentation analysis revealed a protein complex of nine subunits all of which were annotated as *hypotheticals* and none of which resembled a kinase. Moreover, only seven of these subunits are present in leishmanias and we were unable to detect a transcription factor motif in these proteins or find sequence similarities outside of trypanosomatids. Nevertheless, by analyzing one of the subunits, we showed *in vivo* and *in vitro* that this factor is essential for parasite viability, localizes to the nucleus, and is indispensible for *SLRNA* transcription.

A novel strategy for the identification of RNA binding proteins reveals putative regulators of EP procyclin expression

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The insect forms of *Trypanosoma brucei* are densely covered with procyclins. In the early phase of tsetse fly infection, both EP and GPEET are expressed whereas the latter is downregulated as development progresses. The regulation of these genes occurs at the posttransriptional level involving elements in the 3'UTR of the mRNAs. We have recently identified members of a protein family that interact with a loop region in the 3'UTR of GPEET mRNA by standard biochemical purification methods. For the isolation of EP regulating factors, a novel strategy was developed. The method combines the specific interaction of the bacteriophage coat protein MS2 with a particular RNA stem-loop motif and the advantages of tandem affinity purification (TAP). We have generated stable cell lines for inducible expression of TAP tagged MS2-PTP to purify GFP reporter mRNAs containing a MS2 binding site at the 5' terminus and the 3'UTR of EP1. This reporter mRNA can be isolated via TAP in a sequence-specific manner in order to identifiy copurified RNA-associated proteins by mass spectrometric analysis. In addition to highly abundant proteins which are known to be common contaminants in TAP procedures, we were able to identify several proteins with annotated RNA binding activity. Two proteins were chosen for further analysis. One of them shows sequence specific interaction with EP1 mRNA whereas the other one interacts with all procyclin mRNAs. Currently, we are analyzing their subcellular localization, interacting proteins and the effect on the expression of procyclins after downregulation by RNA interference.

Differential Trypanosome Surface Coat Regulation by a CCCH Protein That Co-Associates with *procyclin* mRNA cis-Elements

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The genome of *Trypanosoma brucei* is regulated almost entirely at the post-transcriptional level. Although *procyclin* mRNA cis-regulatory sequences have provided the paradigm for post-transcriptional control in kinetoplastid parasites, trans-acting regulators of *procyclin* mRNAs have remained elusive despite intensive effort. Here we identify the developmental regulator, TbZFP3, a CCCH-class RNA binding protein, as an isoform-specific regulator of Procyclin surface coat expression in trypanosomes. We demonstrate (i) that endogenous TbZFP3 shows sequence-specific co-precipitation of *EP1* and *GPEET*, but not *EP2* and *EP3 procyclin* mRNA isoforms, (ii) that ectopic overexpression of TbZFP3 does not perturb the mRNA abundance of *procyclin* transcripts, but rather that (iii) their protein expression is regulated in an isoform-specific manner, as evidenced by mass spectrometric analysis of the Procyclin expression signature in the transgenic cell lines. Hence, the TbZFP3 mRNA-protein complex (TbZFP3mRNP) is a long-sought trans-regulator of differential surface protein expression in trypanosomes. To investigate other targets of the TbZFP3mRNP, specific RNA-precipitation coupled with Solexa analysis of selected transcripts has been used, this revealing significant enrichment of several transcript classes. Interestingly, *in silico* analysis of the 3'UTRs of these transcripts demonstrates the presence of significantly enriched motifs that are shared with the selected *procyclin* isoforms. Combined with an ongoing analysis of protein components of the TbZFP3mRNP, our studies are dissecting the pathway of gene expression control mediated by a novel group of RNA binding proteins, for the first time linking specific mRNA targets to the gene expression machinery in trypanosomes.

Cold-inducible PKA-like kinase of *Trypanosoma brucei*

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Cyclic AMP is one of the most important second messengers in many organisms and the abundance of cyclases and phosphodiesterases in *T. brucei* suggests the presence of cyclic nucleotide signaling. Yet, the major target for cAMP in other organisms, the cAMP-dependent protein kinase (PKA) does not bind cAMP, and alternative targets were not identified. Using reverse genetic manipulation of intracellular cAMP concentration and a transgenic kinase reporter assay we demonstrated *in vivo* that PKA-like kinase is not regulated by cAMP. However, this kinase is specifically activated in the bloodstream stage by cold shock, an established differentiation trigger. Structural differences in the regulatory subunit PKAR provide a unique example of evolution of a PKA-family kinase that has acquired an alternative activation mechanism. To investigate the functional role and respective signaling pathway downstream of PKA-like kinase we use a combination of phosphoproteomics and phospho-specific antibodies that recognize the highly conserved target phosphorylation sequence RXXS*/T*. Several substrates phosphorylated by PKA-like kinase *in vivo* immediately after cold shock were identified.

Inhibition of initiation of translation by degron-mediated depletion of eIF2 alpha and the effect on mRNA metabolism

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The relationship between mRNA translation and decay remains enigmatic. Translation elongation inhibitors such as cycloheximide, which causes ribosomes to arrest, and puromycin, which causes ribosome release, often have different effects on mRNA stability. For example, in procyclic trypanosomes *GPI-PLC* mRNA is stabilised by cycloheximide but not by puromycin. This difference may result from the effect of the inhibitors on P-bodies, cycloheximide causes them to disappear whereas puromycin causes them to increase. Here, translation was blocked without the use of inhibitors by making a cell line with a conditional mutation in the gene encoding eIF2alpha. The cell line contained a single *eIF2A* gene encoding eIF2alpha fused to a degron derived from human FKBP12; the fusion protein was stable in the presence of Shld-1 (a non-active rapamycin derivative) but unstable in the absence of Shld-1. In the presence Shld-1 the cells grew normally; removal of Shld-1 caused a cessation of proliferation after 4 h, and a depletion of protein to ¡10% within 16 h. Polysomes were greatly reduced. The eIF2alpha depletion was repeated in reporter cell lines expressing fluorescent stress granules or P-body markers. Depletion caused an increase in the number of P-bodies, but did not cause the appearance of stress granules. Unexpectedly, P-bodies remained sensitive to cycloheximide after eIF2alpha depletion. These data provide evidence that the action of cycloheximide on P-bodies may not require translation initiation providing an explanation for the effect of cycloheximide of the stability of some mRNAs. In conclusion, the use of a degron-based conditional mutant was successful and has been used to answer questions about the relationship between mRNA translation and sites of decay.

PUF9 regulates transcript stability over the cell cycle in *Trypanosoma brucei*

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In kinetoplastids, regulation of gene expression is almost entirely post-transcriptional, leading to interest in RNA-binding proteins as important regulators of gene expression for differentiation, the cell cycle, and other processes. One widespread family of eukaryotic RNA-binding proteins consists of the Pumilio/Fem-3 binding factor (PUF) domain proteins, of which up to 12 members are encoded in the *Trypanosoma brucei* genome. *T. brucei* PUF9 possesses 4-6 repeats of the PUF RNA-binding domain, and RNAi against the protein results in a mild growth phenotype and an accumulation of G2-phase cells. Four transcripts co-precipitate with an affinity-tagged version of the PUF9 protein when expressed in bloodstream-form cells, and three of these are strongly downregulated when PUF9 is knocked down by RNAi. One of these transcripts, encoding kDNA ligase alpha, has previously been shown to be regulated in the cell cycle in *Crithidia fasciculata*, prompting us to investigate cell-cycle regulation in *T. brucei*. All three PUF9-regulated transcripts were indeed cell-cycle regulated, peaking in late S-phase, while RNAi against PUF9 abolishes regulation. We have identified 3'UTR motifs that appear to play a role in regulation and are also analyzing other co-regulated transcripts.

ABSTRACTS: WEB SESSION

TriTrypDB: A Functional Genomics Database for Kinetoplastid Parasites

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TriTrypDB.org is an integrated database providing access to genome-scale datasets for kinetoplastid parasites, and supporting a variety of complex queries driven by research and development needs. TriTrypDB represents a joint undertaking on behalf of the Kinetoplastid scientific community utilizing computational infrastructure developed by EuPathDB, integrating genome annotation and analyses from GeneDB and elsewhere, along with a wide variety of functional genomics datasets made available by members of the global research community, often pre-publication. These resources have been made possible by support from the Bill & Melinda Gates Foundation, the NIH (US), and the Wellcome Trust (UK). Currently, TriTrypDB integrates datasets from *Leishmania braziliensis*, *L. infantum*, *L. major*, *Trypanosoma brucei* and *T. cruzi*. Users may examine individual genes or chromosomal spans in their genomic context, including syntenic alignments with other kinetoplastida. Data within TriTrypDB can be interrogated based on text terms, gene IDs, GO/EC numbers, chromosomal location, BLAST, sequence features (length/MW, gene type and structure, signal sequences, Pfam domains, user-defined motifs), predicted protein structure, epitopes, orthology, and functional information (proteomics and transcriptomics, etc.). User queries are stored, allowing future access and integrated searches. Tools permit users to combine queries such as "Find *L. major* kinases with expression evidence and no human orthologs in any kinetoplastid parasite". User Comments may be added to any gene page, enhancing available annotation. Such comments are immediately searchable via the text

search and are forwarded to the annotation team for integration. Representatives from EuPathDB and GeneDB will be available throughout the meeting at the booth outside the cafeteria to provide demonstrations, answer questions, gather feedback, arrange to accept your data and discuss new datasets.

ABSTRACTS: POSTER SESSIONS

A SERIES: April 27 6:30 p.m.

C SERIES: April 28 6:30 p.m.

Functional characterization of the Leishmania folate/biopterin transporters (FBT) family

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The genome of *Leishmania infantum* contains 14 members of the folate/biopterin transporter (FBT) family. Other members of this family are found in trypanosomes, apicomplexa parasites, higher plants and cyanobacteria. *Leishmania* parasites are folate and pterin auxotrophs and thus rely entirely on membrane transporters to mediate the uptake of these molecules from their host cells. Previous work has led to the characterization of three FBT members in *Leishmania*, two folate transporters (FT1 and FT5) and one biopterin transporter (BT1). FT1, the main folate transporter, is a plasma membrane protein with 14 putative transmembrane domains with the N- and C-termini experimentally shown to be in the cytoplasm. All FBT members, except one as determined by GFP-tagged versions, are located in the plasma membrane. We have investigated the role of the most conserved charged amino acids of FBTs on the transport kinetics of FT1 by site-directed mutagenesis. Some of these conserved amino acids appear to be essential for the transport or the binding of the substrate. Folic acid and S-adenosylmethionine (SAM) are the main metabolic carbon donor in the cells. Interestingly, a methotrexate-mutant with several rearrangements in the FBT genes was cross-resistant to sinefugin (SF), a structural analogue of SAM. Gene transfection, gene inactivation and transport studies indicated that the main SAM plasma membrane transporter of *Leishmania* is part of the FBT family. Despite their structural similarities, this study highlights the functional diversity of the FBT members and their importance in the biology of the parasite.

The SUMOylation system in Trypanosoma cruzi

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SUMOylation is a post-translational modification present in eukaryotic organisms that involves the covalent attachment of the ubiquitin-like protein SUMO to other cellular proteins. Unlike ubiquitination, which targets proteins for degradation, SUMOylation participates in a number of cellular processes such as nuclear transport and transcriptional regulation. The SUMO conjugation pathway is biochemically similar to the ubiquitin conjugation pathway. SUMO is initially synthesized as inactive precursor that needs to be processed at the C-termini by specific proteases to expose the glycine carboxylate that is the site of substrate conjugation. The processed SUMO is then activated by E1 enzyme, transferred to the E2 conjugating enzyme and finally ligated to a substrate with the aid of an E3 enzyme. In order to determine if the SUMO conjugation system operates in *T. cruzi*, western blot analysis of total extracts from the different forms of the parasite was performed. Anti-TcSUMO polyclonal antibodies revealed the presence of free-SUMO protein as well as several bands within the range of about 40 kDa to 100 kDa corresponding to potentially sumoylated targets. In vivo processing of SUMO precursor was studied by transfecting epimastigotes with TcSUMO with an N-terminal 6xHis-HA and a C-terminal Flag tags. Western blot analysis showed that TcSUMO is processed at the C-terminus and is conjugated to several target proteins. The SUMOylation pattern was also observed when epimastigotes were transfected with mature SUMO already exposing the Gly residue. Indirect immunofluorescence studies performed with anti-TcSUMO antibodies suggests that SUMO targets are predominantly nuclear proteins. In order to identify the identity of SUMOylated proteins a proteomic approach based on tandem affinity purification scheme is being developed.

Regulated expression of the surface virulence factor lipophosphoglycan (LPG) of *Leishmania major* using a conditional destabilized protein-fusion regulatory system

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Surface glycoconjugates play important roles in the infectious cycle of *Leishmania major*, including the abundant lipophosphoglycan (LPG) implicated in parasite survival in the sand fly vector and the initial stages of establishment in the mammalian host macrophage. We describe a system for inducible expression of LPG, applying a novel protein based system that allows controlled degradation of a key LPG biosynthetic enzyme, UDP-galactopyranose mutase (UGM). This methodology relies on a mutated FK506 binding protein (FKBP) destabilizing domain ('dd') fused to the protein of interest; in the absence of rapamycin analogs such as Shld1, the 'dd' domain is destabilized leading to proteasomal degradation, whereas drug treatment confers stabilization. Tests in *L. major* using 'dd' fusions to a panel of reporters and cellular proteins confirmed its functionality, with a high degree of regulation and low background, and we established the kinetics of protein activation and/or loss. Two inexpensive and widely available ligands, FK506 and rapamycin, functioned similar to Shld1 without effect on *Leishmania* growth or differentiation. We generated parasites lacking UGM through deletion of the GLF gene and substitution with a ddGLF fusion construct, either as chromosomal knock-ins or through episomal complementation; these showed little or no LPG expression in the absence of inducer, while in its presence high levels of LPG were rapidly attained. Complement lysis tests confirmed the correct integrity of the *Leishmania* LPG coat. These data suggest that the 'dd' approach has great promise in the study of LPG and other pathways relevant to parasite survival and virulence.

The pseudo-CTD of RNAPII is essential for productive RNA synthesis in *Trypanosoma brucei*

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The RNA polymerase II (RNAPII) machinery is complex and only studied extensively in metazoans and yeast. In these organisms, the carboxy-terminal domain (CTD) of the largest subunit of RNAPII is composed of heptapeptide repeat sequences and is essential for gene expression. The CTD orchestrates myriad transcriptional and co-transcriptional processes that are accomplished in part by a dynamic set of modifications. Differentially phosphorylated CTD signals release of enzyme from the preinitiation complex at transcriptional promoters, ensures subsequent elongation and coordinates messenger RNA (mRNA) 5' capping, processing, polyadenylation and termination. Despite this strict structural and functional conservation of CTD found in many organisms, a surprising number of eukaryotes lack a heptapeptide repeat containing RNAPII and yet produce 5' capped, processed and polyadenylated mRNAs. To investigate how this non-heptapeptide CTD, called pseudo-CTD (YCTD), contributes to RNAPII machinery we explored this phenomenon in trypanosomes, which are unicellular eukaryotes that parasitize birds, fish and mammals. African trypanosome, *Trypanosoma brucei* contains an $\sim$285 amino acids long YCTD, rich in serine and proline, that follows the highly conserved A through H domains characteristic of the largest subunit (RPB1) of all eukaryotic RNAPII. We learned that RNAPII lacking the YCTD altogether or containing only 95 amino acids, fails to support cell viability. In contrast, RNAPII with 186 amino acids long YCTD maintains cellular growth. Moreover we observed that RNAPII with YCTD truncations resulted in abortive transcription initiation that was more pronounced in the case of the shorter YCTD. These data reveal that a highly divergent CTD plays as important a role in gene expression as a heptapeptide-repeat containing CTD in RNAPII transcription.

Investigation of fucosylation in *Trypanosoma brucei*

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Trypanosoma brucei is the extracellular protozoan parasite responsible for African sleeping sickness. The lysosomal and endosomal compartments of this organism are rich in glycoconjugates that play a very important role in its virulence, infectivity and also survival. An interesting angle in glycobiology is to focus on the building blocks of the glycan chains, which are the monosaccharides. The source of monosaccharides in the glycans are sugar nucleotides. The sugar nucleotides pools in *T. brucei* has been previously analyzed using a LC-ES-MS/MS approach. GDP-fucose was amongst those detected [1]. Interestingly, there was no prior knowledge of any fucose-containing glycoconjugates in this organism. Furthermore, null mutants for GDP-mannose dehydratase, one of the enzymes involved in the *de novo* synthesis of GDP-fucose, revealed this sugar nucleotide to be essential for the parasite cell growth [2]. The fucosylation process in *T. brucei* still needs to be investigated. Several approaches have been utilized to try to identify the fucose-containing glycoconjugates: labelling with GDP-[^{3}H]fucose in cell free-system, metabolic radio-labelling, detection with fucose-specific lectins (i.e. AAL, UEA-1) and mass spectrometry. The results of these experiments will be discussed here. One putative fucosyltransferase has been identified in *T. brucei*. It shows homology to the CAZy GT11 family, which comprises alpha1,2-fucosyltransferases. This protein is expressed in both proliferative forms of the parasite. Results from the trials for the recombinant expression and characterization of this enzyme will be analyzed here. [1]. Turnock D.C., Ferguson M.A.J., *Eukaryotic Cell* 2007, 6:1450-1463 [2]. Turnock D.C.et al., *J. Biol. Chem.* 2007, 282:28853-28863

Identification of a new flagellar protein in *Trypanosoma brucei* : a role in microtubule stabilisation?

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Cilia and flagella are widespread eukaryotic organelles that are conserved from protists to mammals. Their functions are involved in cell motility, organelle segregation, extracellular fluid movement and sensory reception. We have characterised a novel cytoskeleton protein from *T. brucei* that we have named TbSL. Initially identified and characterised by our group as a flagella marker, TbSL has also been identified in the *T. brucei* flagellome (1). We show here using RNAi knockdown studies that TbSL is a flagellar protein involved in motility. We have identified TbSL orthologs that apparently are present only in flagellated or ciliated organisms from ciliated/flagellated protozoan to mammals. Further, BLAST mediated analysis of the TbSL primary sequence allowed us to identify a new group of putative STOP proteins. STOP proteins are calmodulin-regulated microtubule associated proteins involved in the stabilisation of cold- or nocodazole-resistant microtubules in superior eukaryotes (2). TbSL appears to belong to a family of STOP-Like proteins and our data suggest that these STOP-Like proteins might be involved in the stabilisation of specific microtubules such as the microtubules of the axoneme. The identification of TbSL and TbSL orthologs opens new avenues in the search for proteins involved *T. brucei* and kinetoplastisd flagella function and in ciliary related diseases. (1) Broadhead et al. (2006) Flagellar motility is required for the viability of the bloodstream trypanosome. Nature. 440:224 (2) Bosc C et al. (2003) STOP proteins. Biochemistry. 42:12125

AcetylCoA synthetase : the link between glucose/amino acid degradation and lipid biosynthesis in procyclic trypanosomes

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AcetylCoA synthetase : the link between glucose/amino acid degradation and lipid biosynthesis in procyclic trypanosomes Acetyl-CoA produced in mitochondria from carbohydrate or amino acid catabolism needs to reach the cytosol to initiate *de novo* synthesis of fatty acids. All eukaryotes analyzed so far use the citrate/malate shuttle to transfer acetyl group equivalents from the mitochondrial matrix to the cytosol. We have investigate how this acetyl group transfer occurs in the procyclic life cycle stage of *Trypanosoma brucei*. Deletion of the potential citrate lyase gene, a critical cytosolic enzyme of the citrate/malate shuttle, has no effect on *de novo* biosynthesis of fatty acids and sterols from ^{14}C-labeled glucose, indicating that another route is used for acetyl group transfer. Since acetate is produced from acetyl-CoA in the mitochondrion of this parasite, we considered genes encoding cytosolic enzymes producing acetyl-CoA from acetate. We identified an acetyl-CoA synthetase gene encoding a cytosolic enzyme (AceCS), which is essential for cell viability of the procyclic trypanosomes. Interestingly, induction of RNAi against AceCS results in a 10- to 20-fold reduction of ^{14}C-radiolabeled glucose or acetate incorporation in *de novo* synthesized fatty acids or sterols. We have demonstrated that the essential cytosolic enzyme AceCS of *T. brucei* is responsible for activation of acetate into acetyl-CoA to feed *de novo* biosynthesis of lipids. Therefore, we identify the procyclic form of *T. brucei* as the first eukaryotic organism where acetate instead of citrate is used to transfer acetyl groups from the mitochondria to the cytosol.

Role of thiomodifications in tRNA import in *Trypanosoma brucei*

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In *T. brucei* essentially all tRNAs have a dual localisation and are in part imported into the mitochondrion whose genome does not encode any tRNAs. Interestingly, the extent of tRNA import is highly variable and specific for a given tRNA species. It ranges from 1 to 12% of the total cellular content. This raises the question of how the extent of import is regulated? It has been suggested that a cytosol-specific thiomodification in the anticodon wobble nucleotide of trypanosomatid $tRNA^{Glu}$ and $tRNA^{Gln}$ prevents their import and by doing so regulates the extent of their mitochondrial localization (Kaneko, T. *et al. EMBO J.* **22**, 657-667 (2003)). A prediction of that model is that the lack of the thiomodification should enhance import of the two tRNAs. To test this prediction RNAi cell lines allowing inducible ablation of enzymes essential for the thiomodification of tRNAs were produced. In these cell lines an up to 10-fold accumulation of non-thiomodified $tRNA^{Glu}$ and $tRNA^{Gln}$ was observed. However, even though non-thiomodified tRNAs accumulated in the cytosol this did not lead to an increased mitochondrial import of the molecules. Moreover, *in vitro* experiments showed that both thiomodified and non-thiomodified $tRNA^{Glu}$ were imported into mitochondria with comparable efficiency. In summary, these results show that the thiomodification in the $tRNA^{Glu}$ and $tRNA^{Gln}$ is not acting as an antiderminant for tRNA import. Further studies comparing the kinetics of tRNA thiomodification and tRNA import are in progress and may reveal why *in vivo* thiomodified $tRNA^{Glu}$ and $tRNA^{Gln}$ are restricted to the cytosol.

Characterization of zinc-iron transporter members and of a putative ferric reductase in *Leishmania infantum*

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In order to survive and replicate in the phagolysosome harsh environment, *Leishmania* amastigotes must acquire certain nutrients, amongst which are iron and zinc. We are looking for the *Leishmania infantum* proteins involved in these processes. Mining the parasite genome for potential candidates, we selected three proteins with homology to members of the zinc-iron family and one reductase for further analysis. The study of these proteins involves the following aspects: expression and localization in the parasite and particularly in amastigotes, demonstration of the capacity to transport or to reduce the metal and analysis of their involvement in infection. The results obtained so far will be presented. Briefly, one of the zinc-iron members (*Li*IZT1), which is expressed mainly in the early stages of the parasite growth, locates to a region of the cell that likely corresponds to the flagellar pocket in both promastigotes and amastigotes. In the case of the vector stage, *Li*IZT1 is also found in the cellular membrane. It is not yet clear which metal does *Li*IZT1 transport. On the contrary, *Li*IZT3 was found to transport zinc. As for the reductase (*Li*FRed), the protein was found in internal membranes of promastigote overexpressing cells and is downregulated by iron excess suggesting that it functions as a ferric reductase *in vivo*.

Bioluminescence: A new light on *in vivo* trypanosome modeling

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Current *in vivo* trypanosome models can not monitor the spread of parasites in different tissues; except when *ex vivo* studies are included. Monitoring parasite spread using real-time imaging provides a fast method to evaluate parasite distribution especially in immunoprivileged locations and allows a significant reduction in the number of animals required. Here, we generated *Trypanosoma brucei* recombinants, expressing *Renilla* luciferase, allowing us to conduct real-time bioluminescent *in vivo* trypanosome studies. In vitro luciferase activity measurements confirmed the uptake of the coelenterazine substrate from live parasites and light emission. We further validated the use of *Renilla* luciferase-tagged trypanosomes for real-time bioluminescent *in vivo* analysis. Interestingly, a preferential testis tropism was observed with both the monomorphic and pleomorphic recombinants. This is of importance when considering trypanocidal drug development, since parasites might be protected from many drugs by the blood-testis barrier. This hypothesis was supported by our final study of the efficacy of treatment with trypanocidal drugs in *T. brucei*-infected mice. We showed that parasites located in the testis, as compared to the abdominal cavity, were not readily cleared by the drugs.

Role of the PERK eIF2-alpha kinase and eIF2-alpha phosphorylation in the differentiation process of *Leishmania* within macrophages

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During the infection of the mammalian host, *Leishmania* encounters drastic environmental changes, including a high temperature shift and acidic pH within the phagolysosome of macrophages. These conditions trigger rapid morphological and biochemical changes that allow infective promastigotes to differentiate into amastigote forms, adapt and survive within the phagolysosome. In other eukaryotes, the response to stress or to diverse environmental stimuli predominantly involves the phosphorylation of the translation initiation factor eIF2-alpha by four distinct kinases leading to a decrease in protein synthesis and growth suppression. In *Leishmania*, eIF2-alpha is constitutively expressed in both life stages of the parasite. However, when *Leishmania* are grown as axenic amastigotes under conditions mimicking macrophage growth, eIF2-alpha becomes phosphorylated at Thr166. EIF2-alpha phosphorylation coincides with downregulation of global mRNA translation. We have generated a *Leishmania* mutant lacking one allele of the PERK eIF2-alpha kinase gene homolog encoding an endoplasmic reticulum (ER) kinase regulating the response to ER stress. PERK is an essential gene in *Leishmania*. We showed that the PERK heterozygous mutant exhibits a significant delay in the differentiation process, especially within the first hours following entry of the parasite into macrophages. Furthermore, we demonstrated that a PERK dominant negative mutant that is defective in eIF2-alpha phosphorylation exhibited a more pronounced delay in differentiation. These findings demonstrate for the first time in protozoan parasites that an eIF2-alpha kinase can act as a key player during the parasite's differentiation in the mammalian host and that there is probably a link between eIF2-alpha phosphorylation and adaptation of *Leishmania* to the harsh environment of the macrophages. Proteomic and transcriptomic analyses are now underway to better characterize these PERK mutants.

Trypanosome Mitochondrial DNA Polymerase IC Behaves as a Stable Component of the Tripartite Attachment Complex

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The *Trypanosoma brucei* mitochondrial genome is a network containing thousands of catenated DNA molecules (minicircles and maxicircles) called kinetoplast DNA (kDNA). Multiple proteins with specific roles at different stages of kDNA replication surround this condensed network. POLIC is one of three essential *T. brucei* mitochondrial DNA polymerases required for parasite growth and maintenance of the kDNA network. Additionally, POLIC is an active DNA polymerase, contains two putative transmembrane domains, and localizes to the kinetoflagellar zone, the region between the kDNA disk and the basal body where minicircle replication intermediates are detected. This region also contains a transmembrane filament system called the Tripartite Attachment Complex (TAC) that connects the kDNA to the flagellar basal body. The TAC is present throughout the cell cycle, plays a role in kDNA segregation and is insensitive to extraction by non-ionic detergents. Only two TAC components have been identified, mitochondrial p166 and AEP-1. RNAi demonstrated that p166 plays an important role in kDNA network segregation, but not in kDNA replication. Based on pilot purifications, we hypothesized that POLIC was the first membrane-associated replicative DNA polymerase in any organism and pursued characterization of this novel association. Using alkaline carbonate extraction, POLIC behaves as a membrane protein partitioning with the mitochondrial membrane protein alternative oxidase (TAO) while lipoamide dehydrogenase (matrix marker) remains soluble. Surprisingly, POLIC mainly associated with the pellet fraction following Tritox-X100 extraction while TAO was completely solubilized. POLIC partitioning was unaffected by DNase I treatment suggesting an association with the TAC. We are currently isolating kDNA-flagellar complexes to confirm that POLIC stably associates with the TAC and provide evidence of the first TAC-associated replication protein.

Iron overload favours the elimination of *Leishmania infantum* from mouse tissues through the production of reactive oxygen and nitrogen species

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We have previously determined that iron overload decreases the growth of *Leishmania infantum* in the liver and spleen of a susceptible mouse strain. The purpose of this work was to investigate if the inhibitory effect of iron overload on the growth of this protozoan is dependent on the production of reactive oxygen and nitrogen species. To achieve this goal, we evaluated the effect of iron overload on the expression of iNOS in *L. infantum*-infected tissues. Additionally, we used mice genetically deficient in the p47phox subunit of NADPH oxidase (p47phox knock-out) or in the inducible nitric oxide synthase (iNOS knock-out) to study the course of infection. Control and knock-out mice were treated with iron-dextran (10mg) or saline solution and 10 days later were infected with *L.infantum*. Mice were sacrificed 15 days after infection and the parasite load was determined in the liver and spleen. Iron overload tended to increase the expression of iNOS. Iron treatment decreased *L.infantum* growth in control mice but not in p47phox- or iNOS-knock-out mice, indicating that the mechanism through which iron exerts its inhibitory effect is dependent on the production of reactive oxygen and nitrogen species by the host cell. Iron overload may increase the host capacity to control *L.infantum* growth, through the stimulation of the production of reactive oxygen and nitrogen species, potent antimicrobial mechanisms to which *Leishmania* are susceptible.

Functional Studies of the Multiple Mitochondrial DNA Polymerases in *Trypanosoma brucei* using Chimeric Gene Silencing

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The unique mitochondrial DNA of Trypanosomatids is a catenated network of minicircles and maxicircles called kinetoplast DNA (kDNA). The network is essential for survival, and replication involves release of covalently closed minicircles, replication as free molecules, and reattachment of progeny to the network. Single-gene silencing experiments revealed that three of the six *Trypanosoma brucei* mitochondrial DNA polymerases (POLIB, POLIC and POLID) are essential for parasite growth and maintenance of the kDNA network. How *T. brucei* uses three polymerases to replicate the maxicircles and minicircles is unknown. Analysis of free minicircle replication intermediates in single-gene silencing experiments suggested non-redundant, cooperative roles for the essential DNA polymerases. To test this hypothesis we used chimeric stemloop RNAi to knockdown the polymerases in different combinations (IB/IC, ID/IB, ID/IC and IB/IC/ID) to evaluate cooperative roles in kDNA replication. Targeted transcripts were knocked down >85%, resulting in growth inhibition and kDNA network loss similar to single-gene silencing experiments. Within 4 days, dual silencing of IB/IC and ID/IC led to 50% and 60% loss of kDNA, respectively. Dual silencing of ID/IB, however, resulted in a rapid and complete loss of kDNA compared to single or dual-gene silencing. Simultaneous ablation of ID and IB reduced both minicircles and maxicircles by 80% within 4 days and caused a 3-fold increase in abundance of all free minicircle species within 48 hours. Although ongoing analysis of both minicircle and maxicircle replication intermediates will provide additional information on how these polymerases are working together to replicate the kDNA network, our current findings highlight the utility of chimeric-gene silencing as a tool to study the complex mechanism of kDNA replication.

The function of the mitochondrion in bloodstream form trypanosomes

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The bloodstream form trypanosome does not use mitochondrial oxidative or substrate phosphorylation for energy generation, instead relying exclusively upon glycolysis for ATP production. Nonetheless, the mitochondrion and mitochondrial gene expression are essential in these forms. Recent work (Schnaufer *et al.*, 2005) has shown that ATP synthase is essential for the maintenance of mitochondrial membrane potential and that dyskinetoplastid (dk) trypanosomes that lack mitochondrial DNA appear to maintain their mitochondrial membrane potential through the action of a mutated ATP synthase. Work in our laboratory now uses transgenic trypanosome cell lines and next-generation sequencing techniques to determine the exact role of ATP synthase mutations in the dk phenomenon and to idenify other potential mechanisms that allow dk trypanosomes to survive without mitochondrial gene expression. Preliminary results from these studies will be presented.

Characterization of Leishmania donovani amastigote specific protein and its role in virulence

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Leishmania are vector-borne pathogens which invade the macrophage in the vertebrate host. In an effort to generate an attenuated L. donovani parasite that could be evaluated as a vaccine candidate, we have characterized an amastigote specific 27kDa protein encoding gene (p27), which was identified by gene expression analysis on a Leishmania donovani genomic microarray. Northern blot analysis confirmed the stage specific expression of the gene coding for p27 in axenic amastigotes. Antibodies raised against the recombinant protein confirmed abundant expression in the amastigote stage. Analysis of the deduced amino acid sequence revealed motifs similar to 1) a mitochondrial targeting signal at the N-terminus, 2) a transmembrane helix in the center of the protein, 3) a putative tyrosine kinase domain on the C-terminal end. Colocalization of p27 antigen using p27 specific antibody with the mitotracker dye revealed its possible mitochondrial localization. Biochemical analysis of mitochondrial fraction revealed that it's a membrane bound protein. Further analysis suggests that p27 protein is a component of Cytochrome C Oxidase (COX) complex. A point mutated form of p27 disrupts the COX activity. Moreover p27 null mutant and parasites over-expressing a point mutated p27 show defects in virulence in infecting human macrophages. The p27 null mutant shows reduced survival inside the BALB/c mice. Immunization of mice with p27 null mutant parasite protected them from virulent challenge as indicated by significantly reduced parasite burden both in liver and spleen. These results clearly suggesting that p27 null mutant parasite could be used as a live attenuated vaccine for leishmaniasis.

A Comparative Genome-wide Study of ncRNAs in Trypansomatids

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Recent experimental and computational studies have provided evidence for multitudes of non-coding RNA (ncRNA) transcripts in a wide range of eukaryotic genomes. ncRNAs are emerging as key players in multiple layers of cellular regulation. With the availability of many whole genome sequences, comparative analysis has become a powerful tool to identify ncRNA. We undertake a systematic genome-wide in silico screen to search for novel ncRNAs in the genome of *Trypanosoma brucei* by comparative genomics. The entire *Trypanosoma brucei* genome was divided up into a window size of 100 bps with a sliding window of 50bps. These sequences were searched for similarity against the other Trypansomatid genomes using FASTA. Scripts written in PERL/bio-PERL has been used to post-process the FASTA results. Sequence alignments were further analyzed if they fit the following criteria: 25 bps or longer, an e-value less than or equal to 0.01, and percent identity equal or greater than 60%. Fasta matches were than mapped back to the *T. brucei* genome. A total of 8877 and 15,141 sequences were found to be conserved in six genomes and at least four genomes, respectively. Almost one third of the known ncRNA was found in six genomes, and about half were found in four genomes. Annotated sequences were then filtered out. Thus, yielding at total of 57 conserved unannotated sequences in six genomes and 126 in at least four genomes. Among this collection we identified tRNA-sec, previously annotated incorrectly in the GeneDB *T. brucei* genome annotation. Many of the predicted ncRNAs were validated experimentally and categorized to their families.

Biomarkers of attenuation in the Leishmania centrin deleted cell line-measures of safety in a live vaccine candidate

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Biomarkers of the attenuated phenotype are needed to develop live attenuated parasites into safe *Leishmania* vaccines. The centrin-1 gene deleted strain of *Leishmania donovani* (*LdCEN1*$^{-/-}$) shows promise as a vaccine candidate. To identify genes whose expression patterns are indicators of attenuation, the *LdCEN1*$^{-/-}$ line was compared to wild type by gene expression microarray. Two genes, one coding for a 27kDa protein (p27) and another coding for putative Argininosuccinate Synthase (AS) have such expression patterns. Both genes are expressed more in the amastigote stage than in the promastigote stage of wild type cells; however they are down-regulated in the *LdCEN1*$^{-/-}$ amastigote cells. Western blots indicated that the AS protein level is also reduced in the *LdCEN1*$^{-/-}$ amastigotes, while the p27 protein level is not reduced even when its mRNA level has diminished. Northern and Western blot analysis with these two biomarkers showed that *LdCEN1*$^{-/-}$ parasites recovered after five weeks of infection in mice had the same expression pattern as they had prior to infection. Therefore, both the mRNA and/or the protein levels of these two genes could be used as biomarkers of attenuation to monitor the safety of the *LdCEN1*$^{-/-}$ cell line as it is developed as a potential vaccine.

Protein palmitoylation in trypanosomes - from a global perspective to a specific role in protein targeting to the flagellar membrane

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In eukaryotic cells, the sorting of many proteins to specific membrane domains is regulated by the covalent attachment of palmitic acid. Protein palmitoylation is catalyzed by palmitoyl acyltransferases (PATs), which share an enzymatic DHHC-cysteine-rich domain. Through acyl-biotin exchange chemistry of African trypanosomes, we have uncovered the first cellular palmitoyl-proteome of a protozoan cell. The proteins identified include several whose orthologues were similarly identified in yeast and rat neuronal palmitoyl-proteomes, as well as many kinetoplastid-specific hypothetical proteins. Several enzymes with putative signaling functions, such as protein kinases, phosphatases, and cyclic nucleotide phosphodiesterases, were also identified. To investigate the function and regulation of protein palmitoylation, we engineered individual RNAi mutants for each of the twelve DHHC-CRD genes in *T. brucei*. Despite the lethality of pan-PAT inhibition with a chemical inhibitor, none of the PAT mutants exhibited a growth deficit *in vitro*, suggesting either enzymatic redundancy and/or non-canonical PAT catalysis of essential palmitoyl-proteins. However, an *in vivo* attenuation in a mouse model of infection was demonstrated for several PAT mutants, including TbPAT7, which we previously identified as the enzyme responsible for calflagin palmitoylation and trafficking to the flagellar, rather than pellicular, membrane. Inhibition of either TbPAT7 or calflagins themselves renders parasites susceptible to the early host response, as first wave parasitemias fall to undetectable levels before subsequent proliferation of a population expressing an alternative VSG. Finally, epitope tagging of TbPAT7 shows it, like its substrates, to localize to the flagellar membrane. We therefore present a model whereby palmitoylation of calflagin at the flagellar membrane restricts its lateral diffusion out of this organelle, possibly via an association with lipid raft microdomains and intraflagellar transport particles.

Transcriptome remodeling during the growth curve of procyclic *Trypanosoma brucei*

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We have compared, using microarray hybridizations, the transcriptome of procyclic *T. brucei* cells harvested at the beginning of logarithmic growth with that of trypanosomes grown until stationary phase. Our results indicate that there are about 80 transcripts whose abundance is altered at least 2.5-fold when cells enter stationary phase. The determination of the half-lives of some selected up- and down-regulated transcripts indicates that the observed changes in mRNA abundance are due, at least in part, to changes in the degradation rates. These results could be useful to understand how trypanosomes control gene expression in order to adapt to different extracellular environments.

Genomic distribution and developmental changes of DNA methylation in the parasite *Trypanosoma cruzi*

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In most organisms epigenetic mechanisms have emerged as major regulatory machineries of cellular processes. As such, DNA methylation plays important role in regulating gene expression, development and replication. In higher eukaryotes, cytosine methylation is the most common DNA modification, but seems to be present to a much lower extent, or even undetectable, in several protozoans (1). This epigenetic modification appears to be present in trypanosomes (2,3), however, the targets and functions of DNA methylation in *Trypanosoma cruzi*, the etiological agent of Chagas disease, are unknown. Here, we report the detection and genomic distribution of 5-methyl cytosine (m5C) in *T. cruzi* DNA determined by methyl DNA immunoprecipitation (MeDIP). Among the identified methylated genomic loci were repetitive retrotransposons or pseudogenes, suggesting a role for DNA methylation in silencing of gene expression. DNA methylation was also detected at intergenic regions in proximity to transcription start sites, as well as in regions of transcriptional termination. Immunostaining and immunoprecipitation results demonstrated that DNA methylation is particular abundant in *T. cruzi* kinetoplast DNA. DNA methylation might also be involved in *T. cruzi* differentiation as variable amount of methylation was detected in non-replicative compared to replicative stages of the parasite. Further studies will reveal whether DNA methylation has any function in *T. cruzi*. Such epigenetic modifications might provide means for development of new and effective drugs.
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Active VSG Expression Site is organized in a uniquely open chromatin structure

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In most eukaryotes, RNA polymerase I is dedicated to transcribing ribosomal RNA genes. It has become evident, recently, that rDNA is subject to sophisticated epigenetic regulation, which depends on the balance between activating and silencing complexes. Both of these remodel chromatin and interact with histone-modifying enzymes and non-coding RNAs to establish a euchromatic or heterochromatic state at a rDNA unit. In *Trypanosoma brucei*, Pol I can also transcribe other genes, including those encoding Variant Surface Glycoproteins (VSG). Although a few chromatin-related factors are important for *VSG* gene regulation, the differences between the euchromatic and heterochromatic structures at the active and silent VSG expression sites (ES) is unclear. By chromatin immunoprecipitation, we show that the active ES contains 4-10 fold less Histone H3 than silent ESs, which suggests fewer nucleosomes at the active site. Using a fractionation method that differentiates DNA by the tightness of its association with nucleosomes, we observed that chromatin at silent ESs is about 10 times more compact than at the active ES. Micrococcal nuclease digestion confirmed that the active ES is essentially devoid of regularly spaced nucleosomes. Thus, we conclude that there are major structural differences between the chromatin of active and silent ESs, with the active ES being more open. These differences may be involved in marking and propagating the epigenetic state of each ES from one generation to the next, a hallmark of antigenic variation. Moreover, an open chromatin structure at the active ES may facilitate the introduction of double-strand breaks that promote VSG switching by gene conversion.

A tripanosomal sirtuin homologue is involved in mitochondria regulation

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Homologues of the *Sacharomyces cerevisiae* Sir2 protein, the sirtuin family of NAD+-dependent protein deacetylase/mono-ADP-ribosyl-transferase enzymes are emerging key players in nuclear and cytosolic signaling but also in mitochondrial regulation and aging. *T. brucei* genome contains three situins, one of them, Tbsir2rp1, is nuclear, the other two, Tbsir2rp2 and Tbsir2rp3, are mitochondrial. We are investigating the function of Tbsir2rp2 in regulating energy metabolism. We show that trypanosome Tbsir2rp2 is a soluble mitochondrial protein that interacts with the glutamate deshydrogenase and likely ribosylates the ADP/ATP translocase. Functional studies using Tbsir2rp2 RNAi cell lines as well as enzymatic inhibitors will be presented and discussed.

A genome-wide RNAi screening to identify human host factors necessary for *Trypanosoma cruzi* infection

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The protozoan parasite *Trypanosoma cruzi* is the etiologic agent of Chagas' disease. Current chemotherapy provides unsatisfactory results and suffers from considerable side effects, making urgent the development of new efficient drugs. Recent studies have been gradually clarifying the host cellular events during the infection process by *T. cruzi*, but although several host determinants of the entry have been uncovered, the molecular requirements for intracellular growth and parasite persistence could not be satisfactorily determined. With this aim, we performed a genome-wide RNAi screening to identify human host factors required during *T. cruzi* infection. We used a microarray technology where spots containing siRNAs covering the entire human genome are printed onto 7 glass slides. Human cells are seeded over these spots and reverse transfected with the siRNAs, silencing the corresponding host genes. After 24 hours, the cells are infected by *T. cruzi* tissue culture trypomastigotes. The screening procedure was reproduced 6 times, creating more than 163,000 experiments (including controls). In our hit selection system, we used an in-house built highly specific selection algorithm that employed several independent criteria, and a novel multidimensional visual profiling was used to distinguish direct effects on parasite/host interactions from indirect, pleiotropic or false positive readouts. Presently, all hits are being carefully analyzed and organized in order to provide to the scientific community a comprehensive list of the human proteins that are important for each step of the infective process. The identification of these proteins can expose the necessities of the pathogen and hence indicate potential targets for anti-parasitic therapies.

Trypanosome flagellar RNAi mutants: location-to-function for novel proteins and the role of calmodulin in PFR assembly

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With the availability of tractable reverse genetics tools for and several hundred candidate flagellum proteins identified, *Trypanosoma brucei* is a popular model for studies of flagellar biology. Insight into the biochemistry of the iconic 9+2 microtubule axoneme is of fundamental interest, and many aspects of human health are recently known to be dependent upon the assembly of functional flagella. However, the exquisite sensitivity of bloodstream trypanosomes to perturbations in flagellum function identifies flagellar motility as an intriguing drug target for sleeping sickness. As part of our ongoing interest in probing the function of putative metabolic proteins in the trypanosome flagellum we have combined comparative bioinformatics with RNAi-mediated ablation of prominent axonemal sub-structures as approaches to defining location for novel proteins. Here, we will illustrate how loss of radial spokes from the axoneme unmasks the substructure of the central pair microtubules and points towards identification of a divergent (i.e. not readily recognisable by sequence analysis alone) trypanosome member of the CPC1 adenylate kinase-related protein family. As part of our comparative analysis of flagellar RNAi mutants we also looked at calmodulin, a conserved component in both the central pair apparatus and radial spokes of many flagellate eukaryotes. In trypanosome flagella, calmodulin is additionally present within the paraflagellar rod (PFR). We report here that RNAi against calmodulin has no discernable effect on axoneme sub-structure, but results in the total failure of PFR assembly, and flagellar detachment. This detachment is sufficient to explain why a PFR is needed by *T. brucei*, informs on the function of the glycoprotein FLA1, and suggests one reason why endosymbiont-bearing trypanosomatids might have retained a relic PFR.

Exploiting the drug-activating properties of a novel trypanosomal nitroreductase

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The nitroheterocyclic compounds nifurtimox and benznidazole function as pro-drugs and must undergo enzyme-mediated activation to have cytotoxic effects. The key step involves reduction of the nitro-group, reactions catalyzed by type I nitroreductases (NTRs). Crucially, type I NTRs are associated with bacteria and absent from most eukaryotes, with trypanosomes being a major exception. Here we exploit this difference by evaluating the trypanocidal activity of a library of nitroaryl phosphoramide mustards against *T. brucei*. Biochemical screening against purified enzyme revealed that a subset of halogenated compounds were effective substrates for TbNTR, having apparent $K_{\mathrm{cat}}/K_{\mathrm{M}}$ values approximately 100 times greater than that of nifurtimox. When tested in vitro against bloodstream form *T. brucei*, cytotoxicity mirrored the enzyme activity. The IC50 values of the most effective substrates were <5 μM with the most potent compounds having IC50s <10 nM. Alteration of cellular NTR levels confirmed that this enzyme plays a key role in parasite killing: *T. brucei* NTR heterozygous lines displayed resistance to the compounds while parasites over expressing NTR showed hypersensitivity. We also evaluated the therapeutic index of those mustards with highest trypanocidal activity against mammalian THP-1 cells. In most cases, the relative toxicity of these newly identified compounds was lower than that of the existing nifurtimox therapy. We conclude that halogenated nitroaryl phosphoramide mustards are a promising novel class of anti-trypanosomal drug and their efficacy validates the strategy of specifically targeting NTR activity to develop new therapeutics.

Oleate Desaturase as a DNA Vaccine for Chagas Disease

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More than 11 million individuals are affected in Latin America by Chagas disease. Also, this is an emerging disease in the U.S. Currently; there is no vaccine available for Chagas disease. Oleate desaturase (OD_{Tc}) is a transmembrane enzyme that is expressed in all stages of *T. cruzi's* life cycle. Based on computational analysis we proposed to use OD_{Tc} as antigen for a DNA vaccine against this disease. In this research we made a construct with an OD_{Tc} C-terminal region ($^{252-417}OD_{Tc}$) in pcDNA3. The adjuvants used in this experiment were á-galactosyl ceramide (PBS-57) and Galá 1-3Galâ 1-4GlcNAc-BSA (trisaccharide), and the immunization route was nasal. After the third immunization 10 μl of blood were taken from the tail of the animals to prepare antiserum. However, after the fourth immunization the Western Blot will be repeated. In case that we do not obtain better results other immunization routs will be tested as well as the complete OD_{Tc} ORF.

Role of a MAP2 kinase in virulence in *Trypanosoma brucei*

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Trypanosoma brucei encounters a number of different environments that it must sense and respond to accordingly. Key molecules involved in sensing and responding to changing environments are protein kinases, such as those participating in the MAP kinase signaling cascade. In an effort to determine role that these kinases play within *T. brucei* we have started to examine the role of the MAP kinase kinases (MKKs). We have generated strains individually deleted for two of these genes, Tb927.3.4860 (MKK1) and Tb10.70.1800 (MKK2). These knockout strains show no obvious defect in growth *in vitro*, or in their ability to adapt to a number of stresses. Mice infected with either the wild-type or the MKK1 knockout strain showed similar patterns of infection, with a near-lethal parasitemia by four to five days post infection. In contrast, the parasitemia of mice infected with the strain deleted for MKK2, was delayed by one day, and then dropped to undetectable levels by day five post infection. The mice were not able to eliminate the infection as evidenced by successive waves of low-level parasitemia. Increasing the inoculum of MKK2 deletion parasites yielded a first wave of parasitemia similar to that seen for the low dose of the wild-type strain, but once again the mice were able to control the parasitemia. This evidence indicates that the defect in MKK2 knockout parasites is not solely in establishing an infection. The phenotype was partially rescued when MKK2 was expressed exogenously. Overall the data support the hypothesis that deletion of MKK2 sensitizes *T. brucei* to *in vivo* host defenses.

CMF70 is a novel subunit of the dynein regulatory complex in *Trypanosoma brucei*

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Flagellar motility is central to pathogenesis and development in the protozoan parasite *Trypanosoma brucei*. The dynein regulatory complex (DRC) plays an important role in the flagellar motility of *T. brucei* by transmitting regulatory signals from the central pair microtubules via radial spokes to the dynein arms. Of the estimated seven polypeptides thought to comprise the DRC, trypanin is the only identified subunit. Here we provide evidence that CMF70, a conserved coiled coil protein identified through comparative genomics for proteins of motile flagella, is a novel subunit of the DRC in *T. brucei*. Fluorescence localization and biochemical fractionation of epitope-tagged CMF70 confirm a flagellar distribution for this protein. CMF70 and trypanin co-sediment on a sucrose density gradient and co-immunoprecipitate in *T. brucei* flagellar skeletons. RNAi knockdown of trypanin expression in *T. brucei* cells disrupts the association of CMF70 with the axoneme, further supporting that both components are part of the same complex. CMF70-depleted *T. brucei* cells display a range of motility-related phenotypes including a slow swimming, sedimentation phenotype and growth defect, as seen in other motility mutants. Loss-of-function DRC mutants act as extragenic suppressors of paralyzed flagella in central pair mutants. Ongoing functional studies will determine if suppression of flagellar paralysis without restoration of the defective central pair structure is achieved in CMF70-depleted PF16 central pair mutants. Taken together, our results indicate that CMF70 is a novel subunit required for normal DRC-mediated regulation of flagellar motility in *T. brucei* and provide a molecular basis for identifying the remaining DRC components.

Centromere-associated topoisomerase activity in Trypanosoma brucei

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Topoisomerase-II accumulates at active centromeres during prometaphase, where it resolves the DNA catenations that represent the last physical link between sister chromatids. Previously, using approaches including etoposide-mediated topoisomerase-II cleavage, we mapped centromeric domains in trypanosomes, organisms in which the mechanisms involved in chromosome segregation are poorly understood. In *Trypanosoma brucei*, the organisation of centromeric DNA is more complex than suggested by the genome project. Here, we show that in bloodstream form *T. brucei*, RNAi-mediated depletion of topoisomerase-IIalpha, but not topoisomerase-IIbeta, results in the abolition of centromere-localised activity and is lethal. Both phenotypes can be rescued by expression of the corresponding enzyme from *Trypanosoma cruzi*. Therefore processes which govern centromere-specific topoisomerase-II accumulation/activation have been conserved within trypanosomes, despite the long evolutionary separation of these species and differences in the organisation of centromeric DNA. The variable carboxyl terminal domain of topoisomerase-II is known to have a major role in regulating biological function. We therefore generated *T. brucei* lines expressing *T. cruzi* topoisomerase-II truncated at the carboxyl terminus and examined activity at centromeres after the RNAi-mediated depletion of endogenous enzyme. A region necessary for nuclear localisation was delineated to 6 residues. The carboxyl terminal domain also contains two putative sumoylation motifs. However, in contrast to other organisms, sumoylation of topoisomerase-II does not appear to be required for centromere-specific activity in trypanosomes.

Sequences From The Haptoglobin / Hemoglobin Receptor Untranslated Region Play An Important Role In Human Serum Resistance in *Trypanosoma brucei brucei*

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The animal pathogen *Trypanosoma brucei brucei* has been shown to be sensitive to a subclass of human High Density Lipoproteins (HDL), named the trypanosome lytic factor (TLF). Sensitivity towards TLF is demonstrated by cell lysis due to Haptoglobin / Hemoglobin (Hp/Hb) receptor mediated uptake and trafficking of TLF to the lysosome. The human pathogen *Trypanosoma brucei rhodesiense* is resistant to TLF because of the expression of a serum resistance associated protein (SRA), which binds TLF and prevents TLF mediated lysosomal membrane break down. We have generated a TLF resistant *T.b.brucei* cell line *in vitro*, which is able to withstand high concentrations of TLF. The initial observation correlating with the change in the TLF resistance phenotype is an expression site (ES) recombination event. Continuous culturing in the absence of TLF has resulted in subpopulations of TLF resistant and TLF sensitive cells that outgrew the original TLF resistant cells. In these cells, RT PCR and FACS analysis has not revealed a direct correlation between ES recombination or switching events and TLF resistance. However, all cell lines show a clear correlation with the presence or absence of Hp/Hb receptor mRNA and sensitivity or resistance to TLF. Both TLF sensitive and resistant cell lines retain an unaltered genomic copy of the Hp/Hb receptor gene. TLF sensitivity can be restored in TLF resistant cells by expressing an ectopic copy of the Hp/Hb receptor with 5' actin and 3' tubulin untranslated regions (UTR). We propose that the endogenous Hp/Hb receptor UTR sequences play an important role in stabilization of the mRNA and therefore sensitivity to TLF in *T.b.brucei*.

DHH1 is central to the correct expression of many developmentally regulated genes in *Trypanosoma brucei*

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The RNA helicase DHH1 was identified in an RNAi screen for genes involved in developmental regulation of gene expression. Expression of a dominant negative dhh1 E182Q mutant in procyclic trypanosomes caused rapid growth arrest, a reduction in polysomes, a small decrease in total mRNA and increased P-bodies. This phenotype is consistent with a general reduction in translation. A microarray experiment was used to screen mRNAs specifically affected by dhh1 E182Q expression. The mRNAs identified were nearly all from developmentally regulated genes. The up-regulated mRNAs were almost exclusively expressed at higher levels in BSFs than in PCFs (10 out of 12 tested). In contrast, the down-regulated genes were enriched for mRNAs expressed at higher levels in PCFs than BSFs (6 out of 7 tested). The effect of dhh1 expression on the mRNAs was apparent within 4 hours of dhh1 E182Q expression, and resulted from changes in half-lives. In the case of one gene, *ISG75*, there was an increase in protein resulting from translation of the up-regulated mRNA. Thus, while the expression of the mutant dhh1 causes the majority of mRNAs to move from polysomes, consistent with the general repressor of translation function identified in yeast, one group of mainly developmentally regulated genes is excluded and is stabilized (possibly by maintainance in polysomes) and another is destabilized. These data provide strong evidence that DHH1 is involved in regulating developmental expression of multiple genes and also suggests that, unlike previously assumed, both active stabilization and destabilization of mRNAs might contribute to regulate differential mRNA levels during development.

Ubiquitin-mediated internalisation and degradation among the invariant surface glycoprotein family in Trypanosoma brucei

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The invariant surface glycoprotein (ISG) 65 and ISG75 families are exclusively expressed in the *Trypanosoma brucei* bloodstream form and are among the most abundant *trans*-membrane domain (TMD) proteins. Since their discovery over 15 years ago, very little has been uncovered concerning ISG function. While classical endocytic signals such as tyrosine-based and dileucine-based motifs are absent, ISG65 family proteins possess three highly conserved lysine residues within their cytoplasmic domains and ISG75 family proteins possess five. These are potential sites for modification by ubiquitin, which can act as a signal for endocytosis and/or protein degradation. Using a reporter protein BiPNHA, containing the TMD and cytoplasmic domain of either ISG65 or ISG75, we demonstrate that the cytoplasmic domain of ISG65 and ISG75 undergo modification by multiple ubiquitin moieties in a lysine-dependent manner, with more ubiquitin conjugates found for ISG75. More importantly, deletion of all cytoplasmic lysines abolished ubiquitylation and almost completely inhibits protein degradation and internalisation of the ISG75 reporter, while the ISG65 lysine null mutant leads to a similar result, albeit less significant than for ISG75. The discovery of a ubiquitin-mediated endocytosis pathway implicated a role for the Endosomal Sorting Complex Required for Transport (ESCRT) which is involved in the sorting of ubiquitylated cargo. Using both comparative genomics and functional analysis, we find that the ESCRT machinery is evolutionarily conserved in the kinetoplastids as well as in a spectrum of divergent eukaryotes and that it is functionally conserved in *T. brucei*. Thus trafficking and protein turnover of ISG65 and ISG75 are mediated by signals within their cytoplasmic domain *via* a ubiquitin-dependent pathway.

Maintenance of the flagellar pocket by an essential CCCH zinc finger protein in procyclic form *T.brucei*

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In trypanosomes, the flagellar pocket is the exclusive site for endo/exocytosis, and its biogenesis and maintenance is essential for cell viability. This work focuses on two CCCH zinc finger proteins, TbZC3H20 and TbZC3H21, which are hypothesised to regulate gene expression via mRNA stability, and consequently control normal cell functions. Overexpression of TbZC3H20 in insect procyclic form cells causes a severe growth defect. Immunofluorescence and electron microscopy showed that TbZC3H20 overexpression causes the flagellar pocket to enlarge, a phenotype previously referred to as BigEye. Additionally, quantitative-PCR data shows that clathrin and β-tubulin mRNAs are downregulated as a result of TbZC3H20 overexpression. Other studies have shown that downregulation of clathrin by RNAi results in BigEye, an increase in the expression of a cysteine-rich acidic transmembrane (CRAM) protein and a defect in endocytosis, all of which are shown to occur when TbZC3H20 is overexpressed in procyclic cells. Our data indicate that perturbing the expression of TbZC3H20 influences an arm of the endocytic pathway in procyclic *T. brucei*. Downregulation of TbZC3H20 or 21 by RNAi results in compromised growth, and null mutants could not be created, indicating that both are essential in procyclic parasites. Microarray data from downregulating TbZC3H20 has shown mRNA downregulation of META1, a protein of unknown function that is expressed 3 times higher in procyclic than bloodstream cells. A *Leishmania major* META1 is known to localise in vacuoles around the flagellar pocket. Collectively, the data suggests that the tight regulation of TbZC3H20 is required for maintaining the flagellar pocket, and perturbing TbZC3H20 expression has profound effects on endocytosis, cell architecture and cell viability.

Trypanosoma brucei Mitochondrial DNA polymerase complexes

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Kinetoplast DNA (kDNA), the unique mitochondrial DNA of Trypanosomatids, is a catenated network of minicircles and maxicircles. RNA interference indicated that three of the six DNA polymerases (POLIB, IC, and ID) in *Trypanosoma brucei* are essential and have non-redundant roles in kDNA replication. POLIB and POLIC localize to where minicircle replication intermediates are detected. However, it is unclear how *T. brucei* utilizes three DNA polymerases to replicate the kDNA network. One possible mechanism may be different asymmetric replication fork complexes for the minicircle and maxicircle templates. To test this hypothesis, we are systematically purifying three mitochondrial Pol complexes to clarify whether the Pols associate within a putative replisome and to identify interacting proteins. To date, no mitochondrial replisome has been isolated from any organism. For this study, we fused the PTP (ProtA-TEV-ProtC) tag to the C-terminus of POLIB, POLIC and POLID in individual cell lines for tandem affinity purification. The non-ionic detergent NP-40 (0.25%) provides optimal solubilization of the three PTP-tagged pols. Mass spectrometry analyses indicated that the major component in each of the three independent purifications was the tagged protein. Tb 927.3.5590 (a conserved hypothetical protein) was co-purified with POLIB-PTP and has been detected in the mitochondrial proteome project. These data suggests that the majority of the PTP-tagged polymerases are not present in a stable complex. Immunofluorescence indicated that the PTP tag did not disrupt endogenous localization of the Pols. POLIB-PTP and POLIC-PTP localized near the kDNA and POLID localized throughout the mitochondrial matrix. Future PTP purifications in hydroxyurea-synchronized cells may reveal temporal replication protein associations and provide additional insight into the complex replication mechanism of kDNA.

Assessing stumpy formation and stumpy-specific gene expression using bloodstream reporter lines in *Trypanosoma brucei*

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During the bloodstream stage of the *Trypanosoma brucei* lifecycle, the parasite exists in two different states: the proliferative slender form and the non-proliferative, transmissible, stumpy form. The transition from the slender to stumpy form is important in infection dynamics and contributes to ordered antigenic variation. The study of the differentiation between these two forms, however, has been hindered by the lack of non-mitochondrial molecular markers for the stumpy life stage. PAD1 is a recently identified stumpy-specific surface protein. To monitor stumpy formation *in vivo* and *in vitro* reporter cell lines which couple the chloramphenicol acetyltransferase (CAT) reporter gene to the *PAD1* 3'UTR have been created in monomorphic and pleomorphic cells. The pleomorphic cell line shows an increase in CAT expression upon differentiation to the stumpy life stage. The monomorphic cell line, when treated with compounds reported to induce the slender to stumpy transition, shows an increase in CAT expression. These cell lines are being used to study the dynamics of the slender to stumpy transition during a pleomorphic infection as well as to evaluate the biological relevance of compounds reported to induce stumpy formation. Further, the identification of any instability elements in the *PAD1* mRNA may contribute to the understanding of stumpy specific post-transcriptional control. Therefore a series of reporter constructs have been generated in which CAT is attached to a deletion series of the *PAD1* 3'UTR and activity measured in monomorphic cells. The full length 3'UTR causes repression of CAT expression in monomorphs, yet this is alleviated to varying degrees with progressive deletions. Further mutations are being created to identify the precise regulatory elements involved.

The transcriptome of two life cycle stages of the human infective parasite, Trypanosoma brucei gambiense revealed by Digital SAGE

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Next generation sequencing promises to revolutionize expression profiling. By combining serial analysis of gene expression (SAGE) technology with massively parallel sequencing, an unprecedented level of sensitivity is achievable which is orders of magnitude greater than that offered by microarray technology or affordable by RNAseq. Here we have combined Illumina (Solexa) sequencing technology with SAGE to analyse the transcriptome of two different life cycle stages of the evolutionary ancient protozoan parasite that is the causative agent of African sleeping sickness, *Trypanosoma brucei gambiense*. The implications of this work are discussed.

Alba-domain proteins post-transcriptionally regulate GPEET expression in *T. brucei*

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Modulation of mRNA stability and translation represent the main means of regulating gene expression in trypanosomatids. We have identified a novel protein complex involved in post-transcriptional control of GPEET, a member of the procyclin family of *T. brucei*. The GPEET mRNA harbors several *cis* regulatory elements in its 3' UTR, of which the so-called glycerol responsive element (GRE) was previously shown to affect GPEET mRNA abundance. Four proteins containing Alba domains (TbAlba1-4) were isolated based on their affinity for the GRE in a gel retardation assay. Members of the Alba superfamily bind chromosomal DNA in *Archaea* and are (possibly) components of RNAse P in higher eukaryotes. Knockdown of Alba1, 2 or 3 caused a reduction in specific complexes in gel retardation assays, and a decrease in GPEET mRNA, with the most pronounced effect being observed for Alba1. Alba proteins in *T. brucei* are constituents of cytoplasmic ribonucleoprotein particles and colocalize to granules together with poly(A) RNA when the parasites are deprived of glucose. Tandem affinity purification using tagged Alba proteins revealed interactions between the four proteins, suggesting that they are part of one or more protein complexes. In addition, several components of the translation machinery, including elongation factors and ribosomal proteins, copurified with Alba2. Proteins containing Alba domains are also encoded in the genomes of *T. cruzi*, *Leishmania* and *Plasmodium*. Taken together, these findings suggest new functions for members of the Alba superfamily in regulating mRNAs and open up avenues of research that might have implications for gene regulation in many other organisms.

Determinants for membrane targeting of Trypanosoma cruzi Flagellar Calcium Binding Protein (FCaBP)

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The flagellum of Trypanosoma cruzi is a multifunctional organelle that plays critical roles in motility, chemotaxis, cell signaling, and host cell invasion. The unique lipid composition of flagellar membrane is responsible for localization of some dually acylated proteins. One such protein is the 24 kDa flagellar calcium-binding protein (FCaBP). Studies of this highly immunogenic and abundant protein, and the related calflagin proteins T. brucei, have illuminated unique mechanisms of protein trafficking to the flagellar membrane. We previously demonstrated that the N-terminal 24 amino acids of FCaBP are necessary and sufficient for flagellar localization of this protein. Dual acylation with myristate and palmitate is also required. However, since other dually acylated proteins are not targeted exclusively to membranes, acylation cannot be the sole determinant for membrane targeting. Our data indicate that the membrane targeting of FCaBP is abolished by deletion of amino acids 13-24 suggesting that a specific property of this domain plays a contributing role in flagellar targeting. Our data indicate that a cluster of positively charged residues within this domain act in conjunction with acyl groups to anchor the protein to membranes. In a parallel line of investigation we examined the role of calcium in flagellar targeting of FCaBP and found out that, while calcium is required for flagellar targeting of FCaBP, the binding of calcium by FCaBP is not essential. This suggests that a calcium-dependent association of FCaBP with a binding partner and/or dimerization are also required. An FCaBP binding partner has been identified that exhibits calcium-dependent binding association. Current studies employ Leishmania as a living test tube for fine dissection of the molecular requirements for flagellar targeting.

MudPIT Proteomic Analysis of the T. brucei Flagellar Skeleton Reveals Many Novel Proteins

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The flagellum is a single organelle that drives motility of the African trypanosome, *Trypanosoma brucei*, the causative agent of African sleeping sickness. RNA interference (RNAi)-induced depletion of individual proteins within the flagellum disrupts flagellum substructures, interferes with the generation of a productive motor force and disrupts cell division. In the bloodstream life cycle state, this often results in lethality. Attachment of the trypanosome flagellum to Tsetse fly host tissues as well as social motility studies *in vitro* suggest that the flagellum also plays a role in sensing and communication with the surrounding environment. However, the exact mechanism by which the flagellum influences these processes is unclear. We have taken a proteomic approach to identify proteins within the detergent-insoluble fraction of the flagellum, referred to as the flagellar skeleton, which includes the axoneme, basal body and flagellum attachment zones structures. The current inventory of flagellar proteins in *T. brucei* is incomplete, since previous proteomic analyses employed flagellar skeletons extracted with high salt concentrations that disrupt flagellum ultrasturcture. Here we report that improvements on flagellar skeleton preparation preserve flagellum substructures, yielding a preparation that closely resembles the flagellum in intact cells. MudPIT proteomic analysis of the flagellar skeleton resulted in the identification of 867 proteins, including 467 proteins not identified in previous datasets. This dataset is referred to as the T. brucei flagellar skeleton proteome (TbFSP). Comparison to earlier flagellar proteomic datasets from *T. brucei* shows that significant variability exists in the number and identity of proteins and that our proteomic analysis encompasses this variability. Evaluation of flagellar localization and investigation of function are underway for many interesting targets.

Investigating the oxidative folding pathway in Trypanosoma brucei

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The formation of correct disulphide bridges during protein folding, frequently referred to as the oxidative folding pathway, is important in all cells. African trypanosomes are well suited for investigation of this pathway. Firstly, there are only a limited number constituents in this pathway compared to mammalian cells. For example, the genome of T. brucei appears to encode only five putative protein disulphide isomerases (TbPDIs) and a single endoplasmic reticulum oxidase 1 (TbEro 1). In addition there is an obvious endogenous substrate for the pathway in bloodstream forms, namely the variable surface glycoprotein (VSG). Despite extensive variation in primary structure, all VSGs fold into the same tertiary structure which is underpinned by a series of conserved disulphide bonds. We have observed that elements of the oxidative pathway appear to be up-regulated in bloodstream forms and evidence will be presented that indicates that these proteins are essential in bloodstream forms.

Screening approaches for identification of *Trypanosoma brucei* hexokinase 1 inhibitors

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In *Trypanosoma brucei*, glycolysis is an essential pathway that is initiated when hexokinase (HK) transfers the γ-phosphoryl of ATP to a hexose. Here, we describe three approaches to identify novel inhibitors *T. brucei* HK1 (TbHK1). First, we screened HK inhibitors identified in other systems for activity against TbHK1. Using this approach, we identified the bioflavonoid quercetin (QCN) as a potent inhibitor of TbHK1 (IC50 $\sim$35 μM). QCN also inhibited TbHK activity in cell lysates and was toxic to both bloodstream (BSF) and procyclic (PF) form parasites with LD_{50}s of $\sim$7.5 μM and $\sim$35 μM, respectively. Genetic manipulation to increase or deplete cellular TbHK1, respectively, protected or sensitized the parasites to QCN. We have also completed two library screens to identify TbHK1 inhibitors. The first screen of a library of 1280 pharmaceutically active compounds (LOPAC) was used to validate the assay developed for high throughput screening (HTS) of the recombinant enzyme. This screen yielded 12 primary hits (a 0.94% hit rate) that inhibited TbHK1 >50% at 10 μM. Of note, one compound, myricetin, differs structurally from QCN by a single hydroxyl group. The screen also revealed 8 compounds that activated the enzyme at 10 μM. Last, we have completed an HTS of 220,233 unique compounds, which yielded 239 compounds as primary hits (>50% inhibition at 10 μM) for a 0.1% hit rate. The average Z-factor for the screen was 0.80 +/- 0.14. IC_{50} determinations have resolved this list to 14 compounds with IC_{50}s less than QCN <35 μM). LD_{50} determination for cultured BSF parasites is underway, and structure-function relationships explored. (Research funded in part by NIH R03-MH082340 and U54-MH07441).

Lymphocyte surface targets of *Trypanosoma cruzi trans*-sialidase

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The *trans*-sialidase (TS) from *Trypanosoma cruzi* is a virulence factor that is able to transfer alpha(2,3) sialyl residues between glycoconjugates. It is shed by trypomastigotes which is responsible to apoptotic events in thymus, spleen and ganglia during the acute phase of the infection. The apoptosis is induced by TS-mediated sialylation of cell surface molecules. Identification of such molecules is then important to understand the pathogenic mechanisms involved. In this work we assayed the use of a bio-orthogonal pair group (azide/phosphineFLAG) to label the acceptor molecules of the sialyl residue transferred by TS. Metabolic enzymes involved in glycoprotein biosynthesis tolerate the introduction of an azido group in their nucleotide-sugar substrates. To test if TS accepts N-Azidoacetyl sialic acid (SiaNAz), Jurkat cells fed with the precursor N-Azidoacetyl mannosamine were used. TS hydrolyzed SiaNAz and transferred it to an exogenously added glycoprotein. By using SiaNAz-lactose as donor, the trypomastigote cell surface was labelled by the endogenous or exogenously added TS and the pattern of acceptor molecules was compatible with the expected mucins, thus validating the approach. Then, the surface of thymocytes and Jurkat cells was labelled using SiaNAz-lactose as donor and a discrete band pattern was observed. Several sialyl residue acceptors were identified by MALDI-TOF assay.

VSG expression in bloodstream *Trypanosoma brucei* depends on an essential CITFA-interacting protein of 17 kDa

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The unique multifunctional RNA polymerase I of *Trypanosoma brucei* transcribes ribosomal gene units and units encoding the parasite's major antigens variant surface glycoprotein (VSG) and procyclin. Antigenic variation of the dense VSG cell surface coat is the parasite's means to evade the mammalian immune system but, as shown by Dr. Gloria Rudenko and co-workers, *VSG* expression itself is essential for parasite viability in the bloodstream form (BF) because its silencing caused rapid cell-cycle arrest *in vitro* and effective clearance of parasites from infected mice. Recently, we characterized the general class I transcription factor A (CITFA) which is absolutely required for cell viability and *VSG* transcription. The factor binds specifically to class I promoters and consists of six trypanosomatid-specific subunits and the dynein light chain DYNLL1. Since dynein light chains have been implicated in factor sequestration, we have formulated the hypothesis that the position-dependence of class I transcription is mediated through the DYNLL1 subunit of CITFA. We have now identified a protein of 17 kDa which localizes to the nucleolus [and prelim-wise to the ESB], interacts with DYNLL1 and co-sediments with purified CITFA. In BFs, RNA interference of p17 targeting its 3' UTR results in rapid cessation of cell growth and affects the levels of rRNA and *VSG* mRNA. This effect is specific to *p17* silencing because the cells can be rescued by expressing *p17* with a different 3' UTR. Interestingly, the *p17* knockdown leads to a concomitant reduction of *CITFA-2* mRNA but not of other mRNAs confirming the functional association between p17 and CITFA. Our current efforts are directed towards unraveling the specific role of p17 in class I transcription.

Ca^{2+} inhibition reveals mechanistic and kinetic aspects of the trypanosomal RNA editing machinery

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RNA editing in African trypanosomes is a process that is characterized by the insertion and/or deletion of uridylates into otherwise incomplete mitochondrial (mt) transcripts. The individual reaction steps are executed by sub-cellular machines, called editosomes. Editosomes are high molecular mass protein complexes that orchestrate the individual steps of the catalytic cascade. The majority of the mtRNAs is incompletely edited at steady-state conditions *in vivo*. In addition, mis- and alternatively edited RNAs have been found as well as chimeric gRNA/mRNA side products. Is this an attestation of the poor accuracy of the editosomal machinery *in vivo*? Additionally, *in vitro* RNA editing assays show multiple reaction byproducts. Does this reflect the situation in the cell? Or is this effect attributed to suboptimal conditions *in vitro*? Here we show that editosomes possess both higher accuracy and catalytic activity in the absence of Ca^{2+}-ions *in vitro*. Ca^{2+}-ions abolish the insertion of uridylates by inhibiting the TUTase with an $IC_{50}[Ca^{2+}]$ of $\sim$0.4mM. In U deletion-type editing, Ca^{2+}-ions affect individual reaction steps differentially: exoUase $IC_{50}[Ca^{2+}]$ = 20$\pm$5mM; ligase $IC_{50}[Ca^{2+}]$ = 30mM. However, generation of edited product is to $>$70% reduced at Ca^{2+}-concentrations $>$5mM. These results are congruent with the inhibition of the 3' nucleotidyl phosphatase by Ca^{2+}-ions. Furthermore, we provide a kinetic analysis of the U-insertion/deletion editing reaction *in vitro*. The data indicate that individual catalytic steps of the reaction cascade initially compete for substrate binding and processing. This supports the concept that editosomes provide a reaction platform for substrate binding and individual processing steps.

Mitochondrial binding complex 1 (MRB1) in *Trypanosoma brucei*: its composition and function in RNA metabolism

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The mitochondrial RNA binding complex 1 (MRB1) is comprised of 14 proteins, which are associated with the poly(U) binding protein TbRGG1 in an RNA-mediated manner. This complex was shown to have essential functions in RNA metabolism of the procyclic and bloodstream stages of *T. brucei*. Some subunits of the MRB1 complex have motifs involved in RNA metabolism and protein-protein interactions, whereas other subunits have no known motifs. Several laboratories have recently reported different but overlapping compositions of MRB1. Here we address this issue by comparing the phenotypes of various RNAi knockdowns for putative MRB1 subunits in the procyclic and bloodstream forms. To further determine the structural composition of the MRB1 complex, glycerol gradients of purified mitochondria of various knockdowns were analyzed using antibodies against the GAP1 subunit of this complex.

Kinetoplastid adenylosuccinate lyase as drug target

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Kinetoplastid parasites are completely dependent on purine recycling and the scavenging of host purines due to the lack of a *de novo* biosynthetic pathway. We have characterized the *Leishmania major* Friedlin adenylosuccinate lyase (LmjASL) and shown by RNA interference in *Trypanosoma brucei* cells the importance of this salvage enzyme for parasite survival. The ASL gene is transcribed as a 2060 nucleotide-long mRNA from a single copy gene located in *Leishmania* on chromosome 4, defined by restriction analysis, pulse field gel electrophoresis and genome survey. Immunolocalization and cell fractionation showed that the enzyme is present in both the glycosome and the cytoplasm. A phylogenetic reconstruction of homologous ASL sequences indicated that the parasite enzyme belongs to a different class than the mammalian enzyme and may be the result of a lateral gene transfer event from a bacterium. The recombinant ASL from *Leishmania* (rLmjASL) exhibited kinetic properties which were significantly different from the human enzyme with a pH optimum at pH 8.5 and with a Km of 9 $\pm$ 1 microM, and kinetic efficiency (Kcat/Vm) of 3.3 x 10e6 M-1 s-1, for the adenylosuccinate (ADS) substrate. Contrary to the human enzyme it shows no activity when SAICAR is used as a substrate, indicating that it has lost its activity in the de novo pathway. We suggest that kinetoplastid ASL may serve as a potential target for drug development.

Sphingosine Kinase is Essential for Proper Cell Cycle Progression and Cytokinesis in *Trypanosoma brucei*

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Sphingolipids are second messengers that regulate growth, proliferation, and apoptosis in a wide variety of eukaryotic cells. Sphingosine kinase (SPHK), a key enzyme in the signaling process, phosphorylates sphingosine to produce sphingosine-1-phosphate (S1P), a pro-proliferative lipid. In higher organisms, S1P promotes extracellular signal-regulated kinase (ERK)-mediated proliferation, which requires the activation of growth factor receptors. S1P-mediated signaling is unexplored in *Trypanosoma brucei*, a protozoan parasite and causal agent of African sleeping sickness. A putative SPHK ortholog that harbors a conserved sphingosine kinase domain has been identified in the *T. brucei* genome. Genetic and pharmacological inhibition of *T. brucei* SPHK (TbSPHK) indicates that TbSPHK is essential for parasite viability. Depletion of TbSPHK by RNA interference results in cell cycle and cytokinesis defects, suggesting that TbSPHK plays a role in *T. brucei* proliferation. TbSPHK-depleted cells exhibit enhanced tubulin tyrosination and elongated and/or branched posterior end morphology, indicating that TbSPHK plays a role in cytoskeleton remodeling and acts as a negative regulator of tubulin polymerization. Serum growth factors and ERK family members of mitogen-activated protein kinases are known to play a role in *T. brucei* proliferation; however, the signal transduction pathways involved in this growth regulation are unexplored. Because trypanosomes lack obvious orthologs of eukaryotic S1P and growth factor receptors, it is likely that the mechanisms of TbSPHK activation and S1P-mediated signaling are divergent. We hypothesize that S1P acts as a positive regulator of ERK-mediated proliferation in which novel signal transduction pathways are utilized. The goal of this study is to characterize the function of TbSPHK and to elucidate the novel mechanisms of TbSPHK/S1P-mediated signaling in *T. brucei*.

Transcriptome analysis of differentiating trypanosomes reveals the existence of multiple post-transcriptional regulons

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We used microarrays to analyse differentiation of pleomorphic EATRO1125 *Trypanosoma brucei brucei in vitro*. 1113 mRNAs showed reproducible differences in expression. We clustered them into classes based on their regulation patterns. We found striking co-regulation of genes in particular functional classes. The mRNAs encoding procyclins and META were co-regulated; so were mRNAs encoding different subunits of mitochondrial enzymes, and those for several glycosomal enzymes. The most dramatic discovery was that 28 mRNAs encoding ribosomal proteins showed a coordinate and transient decrease after the onset of differentiation; flagellar protein mRNAs followed, with a 12-hour lag. Intriguingly, mRNAs encoding some RNA-binding proteins showed co-regulation with their mRNA targets. These results provide strong support for the concept of post-transcriptional regulons in trypanosomes.

Do actin filaments exist in *Trypanosoma brucei*?

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The polymeric status of actin remains open in *T. brucei* as direct evidence for the presence of F-actin in these parasites is lacking since no obvious microfilament structures have been observed in electron micrographs and universal F-actin binding agents such as the phallotoxins do not bind to actin from trypanosomes or indeed other protozoan parasites. Nevertheless, indirect data suggest that actin can be organized into filaments at least in bloodstream forms of *T. brucei*. For example, the distribution of actin is highly polarized in bloodstream forms and co-localizes with the endocytic pathway, whereas the protein appears to be uniformly distributed in procyclic forms. Moreover loss of actin compromises endocytic traffic in the former cells. Finally, bloodstream but not procyclic forms are extremely sensitive to inhibitors of actin polymerization such as latrunculin. Since F-actin cannot be visualized directly in trypanosomes using conventional approaches, we approached the problem from an alternative direction: to localize the G-actin pool. This approach was feasible because DNase I binds tightly and specifically to G-actin from trypanosomes. Alexa labeled DNaseI and confocal microscopy was employed to investigate the distribution of G-actin in trypanosomes and the pattern was compared with that of the total pool as observed with anti actin antibodies. Evidence will be presented which indicates that G-actin is the predominant form of actin in procyclic but not in bloodstream forms. We propose that the bulk of actin in bloodstream forms may be organized into short, dynamic filaments as appears to be the case in other protozoan parasites.

Deadenylation in *Trypanosoma brucei*

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Deadenylation, the shortening of the poly(A) tail at the 3' mRNA end, to less than a threshold length is usually the rate-limiting step in mRNA degradation. Three deadenylases have been described in eukaryotes so far: the CCR4/CAF1/NOT complex, the PAN2/PAN3 deadenylase and PARN. Trypanosome cell extracts possess deadenylase activity but the proteins had not been identified. Two different deadenylases were identified: the *Tb*CAF1/NOT complex and the PAN2 homologue, are required for normal cell growth. Interestingly, *Tb*CAF1 is the only catalytic subunit of the *Tb*CAF1/NOT complex and possesses deadenylase activity *in vivo* and *in vitro*. *Tb*CAF1 depletion strongly inhibited the degradation of constitutively expressed mRNAs whereas at the same time the decay of some developmentally regulated, unstable mRNAs was only partially inhibited. This leads to the hypothesis that deadenylation by *Tb*CAF1 is the rate-limiting step in the degradation of stable mRNAs, while several pathways are involved in the degradation of some unstable mRNAs. One of the degradation intermediates of an unstable reporter mRNA with a secondary structure slowing exonuclease degradation was a polyadenylated 3' UTR intermediate, which could come from a pathway that does not require prior deadenylation. *Tb*CAF1 depletion stabilised both, the polyadenylated full-length reporter and the polyadenylated 3' UTR degradation intermediate. *Tb*PAN2 is mainly localised in the cytosol. *Tb*PAN2 depletion resulted in a weak deadenylation defect. *Tb*PAN2 seems to play a role in the degradation of rapidly degraded transcripts (including a developmentally regulated mRNA) but not in the degradation of more stable transcripts. We conclude that there are at least two different deadenylases in trypanosomes which seem to have different functions.

Functional Characterization of *Trypanosoma cruzi* Small Sub-Unit (ó1) Clathrin-Associated Adaptor Protein-Like *(APClaTc)*

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Clathrin-associated adaptor proteins (AP) are large heterotetrameric structures, located between the membrane and the clathrin lattice. The adaptor protein complexes have been shown to be involved in intracellular trafficking. Two AP complexes are known, AP1 and AP2. These complexes are responsible for the clathrin recruitment and fulfill a role as the sorting component in the clathrin-dependent traffic pathway recognizing membrane proteins targeted for internalization. In mammalian cells, the AP1 complex is located in the *trans* -Golgi network and is composed of two large sub-units (gamma and beta-1), a medium chain (micro-1) and a small sub-unit (sigma-1). In this study we analyze the role of the small sub-unit AP-19-like protein ($APCla_{\mathrm{Tc}}$) in *Trypanosoma cruzi*, the causative agent of Chagas' disease. In this regard, we performed functional assays, electronic microscopy and phylogenetic analysis. Epimastigotes overexpressing AP-19 fussed to GFP ($APCla_{\mathrm{Tc}}$-GFP) showed a great extent of co-localization with Texas red-labeled transferrin. In addition, the immunogold electron micrographs, from all stages of the parasite's life cycle, showed the localization of $APCla_{\mathrm{Tc}}$ in the Golgi and other membrane structures. The co-immunoprecipitation experiments showed the interaction of $APCla_{\mathrm{Tc}}$ and clathrin coated proteins, strongly suggesting the involvement of $APCla_{\mathrm{Tc}}$ in membrane protein trafficking. The analysis of the ortholog tree demonstrates that clathrin assembly protein $APCLa_{\mathrm{Tc}}$ is evolutionarily conserved across the eukaryota, as is confirmed by its presence in most eukaryotic genomes assayed, and by the fact that its phylogeny closely mirrors eukaryotic phylogenies that have been obtained from ribosomal RNAs and combined data methods. The paralog phylogeny demonstrates that the gene family to which the $APCla_{\mathrm{Tc}}$ belongs has undergone extensive duplication events.

Homology-independent functional annotation of *Trypanosoma brucei* genome

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The genomes of trypanosomatids contain many trypanosomatid-specific genes whose functions cannot be determined using homology-dependent annotation methods. This is while a very limited set of homology-independent computational methods are available for predicting gene function, all of which require additional data beyond genome sequences. Here, we present two novel homology-independent methods to directly infer biological functions of genes solely based on their sequences. The first method is based on the premise that coexpressed genes have similar synonymous codon usages. Using rigorous statistical analysis, we have shown that codon usage may regulate the expression pattern of proteins in a wide range of organisms, and have provided experimental data supporting this hypothesis. Knowing the high correlation between expression pattern and gene function, we have developed a method for prediction of function based on codon usage. The second method uses a set of function-specific regulatory motifs in 5' and 3' untranslated regions in order to predict gene function. We have shown that the combination of these two methods provides a powerful homology-independent annotation tool. Applying these methods to the genome sequence of *Trypanosoma brucei*, we have been able to predict the functions of many hypothetical proteins. When applicable, these predictions are usually corroborated by predicted interactome of *T. brucei* and/or other sequence features of genes.

Perturbation of phosphatidylethanolamine synthesis affects mitochondrial morphology and cell cycle progression in procyclic form *Trypanosoma brucei*

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Phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are the two major constituents of eukaryotic cell membranes. In the protist *Trypanosoma brucei*, PE and PC are synthesized exclusively via the Kennedy pathway. To determine which organelles or processes are most sensitive to a disruption of normal phospholipid levels, the cellular consequences of a decrease in the levels of PE or PC, respectively, were studied following RNAi knockdown of four enzymes of the Kennedy pathway. RNAi against ethanolamine-phosphate cytidylyltransferase (ET) disrupted mitochondrial morphology and ultrastructure. At the electron microscope level, the mitochondria showed alterations of inner mitochondrial membrane morphology, defined by a loss of disk-like cristae. Despite the structural changes in the mitochondrion, the cells maintained oxidative phosphorylation. Our results indicate that the inner membrane morphology of *T. brucei* procyclic forms is highly sensitive to a decrease of PE levels, as a change in the ultrastructure of the mitochondrion is the earliest phenotype observed after RNAi knockdown of ET. Interference with phospholipid synthesis also impaired normal cell cycle progression. ET RNAi led to an accumulation of multinucleate cells. By contrast, RNAi against choline-/ethanolamine phosphotransferase, which affected PC as well as PE levels, caused a cell division phenotype characterised by non-division of the nucleus and production of zoids.

Lipid biosynthesis in *Trypanosoma brucei*- a source of novel therapeutic targets

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An overview of phospholipids biosynthesis will be given, along with highlighting key areas the group are focusing on to genetically and chemically validate drug targets in the fight against African sleeping sickness. Two key enzymes will be covered 1) Cytidine triphosphate synthase- CTP is a central metabolite not only in nucleic acid synthesis but also for all glycolipid and phospholipid de novo biosynthesis. 2) Neutral sphingomyelinase- an enzyme involved in recycling a specific pool of sphingomylein involved in protein (including VSG) exocytosis.
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A New RNA Binding Module: Evolving Multi-substrate Specificity in Editing Deaminases

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RNAs from all three domains of life–eukarya, bacteria, and archaea–can act as substrates for numerous posttranscriptional modifications, including tRNA editing. Of particular interest is the deamination of adenosine to inosine that occurs at the first or wobble position of the anticodon in tRNAs. Given the ability of inosine to base pair with cytosine, uridine and adenosine, this editing event allows a single tRNA to decode multiple codons. In eukaryotes, adenosine deaminases acting on tRNA (ADATs) are heterodimeric enzymes (comprised of subunits ADAT2 and ADAT3) responsible for conversion of adenosine to inosine. Here, we show through systematic single amino acid substitutions to active site residues of ADAT2 that the RNA binding domain is not within the active site, which is in contrast to the bacterial system. We further demonstrate that mutations to a region rich in positively charged residues at the c-terminus of ADAT2 impairs tRNA binding, which defines a potential tRNA binding site on the enzyme that is distal from the active site. We have termed this region the KR-domain, which is reminiscent of the R-domain found in ADAR3. The identification of this KR-domain provides the first clues for the basis of substrate recognition for this group of eukaryotic enzymes and has implications in the evolution of new substrate specificities among eukaryotic deaminases.

Growth and microscopic analyses of knockdowns of a putative inner arm dynein in *Trypanosoma brucei.*

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The trypanosome flagellum is an important organelle that controls motility and is a key player in cell polarity and cell division. While some features of the flagellum are unique to trypanosomes, the axoneme is highly conserved among eukaryotes. Many axonemal components have yet to be characterized in trypanosomes, including the dyneins of the inner arm. These are complex and heterogeneous structures, and the functions of many of the components are not well understood. We are taking advantage of the molecular and genetic tools available in *T brucei* to begin to characterize some of these components. We have identified a *T brucei* gene predicted to encode the inner arm dynein alpha heavy chain. This gene, TbDNAH10, has amino acid identity to *Chlamydomonas reinhardtii* DhcI and 44% to human DNAH10, the respective inner arm dynein alpha heavy chains of these organisms. In addition, phylogenetic analyses suggest that TbDNAH10 clusters with this class of dyneins. Procyclic *T brucei* cells in which TbDNAH10 was silenced are immotile, cell growth slows dramatically and cells are found in large clusters that can be disrupted by agitation. Microscopic analysis shows that the flagella of cells in these silenced strains appear detached and tangled, small clumps of cells that appear entangled are visible. DAPI analyses show normal nuclear and kinetoplast staining suggesting that mitosis is unaffected. The inner arm dyneins in other organisms have a role in proper control of motility and the coordination of flagellar beat. Studies to characterize the roles of individual components of the flagellum will help define the mechanism of this coordination.

Active VSG Expression Sites are Devoid of Histone H3 in *Trypanosoma brucei*

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In eukaryotes DNA is wrapped around an octamer of core histone proteins, which together form nucleosomes- the basic unit of chromatin. Here we use chromatin immunoprecipitation (ChIP) and qPCR to show that one of these core histones, histone H3, is not uniformly distributed throughout the *Trypanosoma brucei* genome. Two bloodstream form *T. brucei* cell lines containing marker genes in silent and active VSG expression sites (ES), as well as a procyclic form cell line were used in our analyses. In both life cycle stages, the rDNA promoter and the genes within the rDNA transcription unit contained significantly less histone H3 compared with the non-transcribed rDNA spacer. This finding is consistent with studies in yeast, where chromatin structure differs in silent and active rDNA genes, the latter being devoid of nucleosomes. To investigate whether nucleosome occupancy is inversely proportional to the rate of transcription at other highly transcribed regions in the genome, we looked at VSG and procyclin transcription units which encode the major cell-surface proteins of bloodstream and procyclic form trypanosomes, respectively. The average histone H3 content on all 20 ES promoters was lower compared to control non-transcribed regions in bloodstream form, but was comparable in procyclic *T. brucei* where all ESs are silent. In addition, we determined that the actively transcribed ES in bloodstream form *T. brucei* is completely devoid of histone H3 extending from the ES promoter to the telomeric VSG gene. Interestingly, histone H3 was less abundant on the procyclin promoter and in the procyclin genes in both life cycle stages. This lack of histone H3 may be a common feature of highly-transcribed regions in *T. brucei*.

New diagnostic tools for Human African Trypanosomiasis

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Current diagnostic tests for Human African Trypanosomiasis (HAT) have variable success throughout the endemic area, which spans over 30 countries. Diagnosis is crucial for the correct drugs to be administered; however, the current diagnostic tests need to be updated and improved. African Trypanosomiasis is caused by the protozoan parasite, *Trypanosoma brucei*, which evades the host immune system by switching its Variant Surface Glycoprotein (VSG) coat. GPI-specific Phospholipase C (GPI-PLC) cleaves the VSG coat from the plasma membrane, simultaneously creating a Cross-Reacting Determinant (CRD) present on the soluble VSGs (sVSGs) product. An ELISA assay was developed using immobilised sVSG221 and anti-sVSG117 antibodies. This assay can detect any heterologous sVSG by competition between the anti-CRD antibodies in the anti-VSG serum and the CRD epitope on the immobilised sVSG221. This assay will be used to screen for CRD epitope-containing sVSGs in patient sera. In addition to this approach, the main bulk of our research will be to discover new biomarkers. For this work, trypanosomal antigens will be isolated by affinity purification using antibodies obtained from infected sera. The antigens will then be processed and analysed by mass spectrometry. The use of new methodologies including Label-Free Quantitation will be explored.

Identification and Characterization of *Trypanosoma brucei* Decapping Enzyme

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In kinetoplastid protozoan, protein coding genes are transcribed polycistronically, and thus the level of gene expression is controlled largely by post-transcriptionally. NUDIX hydrolase is a major protein family that is responsible for eukaryotic mRNA decapping, which leads to rapid degradation of the message from 5' end. Among several putative NUDIX proteins in *Trypanosoma brucei* genome, we have identified TbDcp2 as a *T.brucei* decapping enzyme. Recombinant TbDcp2 can hydrolyze m7Gpp from 43 mer m7Gppp-terminated RNA. TbDcp2 is also capable of removing Gpp from Gppp-RNA. TbDcp2 decapping activity progressively decline as capped RNA was shortened down to 13 nucleotides. Includion of dinucleotide cap analogues, such as m7GpppG, m7GpppA, GpppG and GpppA, did not inhibit decapping of 43 mer RNA. Down regulation of TbDcp2 by RNAi showed prolonged half-life of mRNA, but did not inhibit cell growth. The effect of TbDcp2 overexpression in *T.brucei* is currently under investigation to understand the role of this enzyme in mRNA turnover.

Identification of dynein regulatory complex subunits via PTP tagging in *Trypanosoma brucei*

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Trypanosoma brucei is the causative agent of African trypanosomiasis and possesses a single flagellum with a canonical 9 + 2 axoneme that is central to parasite development in the insect vector and pathogenesis in the mammalian host. The flagellum is responsible for motility of the parasite and flagellar beating results from the sliding of outer doublet microtubules relative to one another driven by ATP-dependent dynein translocation. This requires the coordinate spatial and temporal regulation of thousands of dynein motors. The Dynein Regulatory Complex (DRC) is a reversible inhibitor of dynein and an essential complex for normal motility. Only one confirmed DRC subunit, trypanin, has been identified to date. The work presented here seeks to purify the intact DRC from *T. brucei* using a modified tandem affinity purification (TAP) tag, termed ProtC-TEV-ProtA (PTP). We have used an in allele integration vector to fuse the C-terminal coding sequence of trypanin to the PTP tag. Transfected cells have been fractionated to isolate the axoneme and extracted to solubilize the DRC. The extracted DRC will be incubated with IgG Sepharose, facilitating binding of endogenous PTP tagged trypanin. Bound proteins will be released with AcTEV protease, bound to an anti-protein C affinity matrix and finally eluted with EGTA elution buffer. Proteins will then be identified using Multidimensional Protein Identification Technology (MudPIT) analysis. We will conduct genetic tests for DRC function and subunits will be selected for further analysis. Dynein regulation is a fundamental and unresolved question in eukaryotic biology. The work presented here will substantially address this question, thereby advancing our understanding of dynein regulation and trypanosome pathogenesis.

Intracellular trafficking of potential virulence factors in *Leishmania major*

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Leishmania resides inside mammalian macrophages from where it is thought to manipulate the host immune system by releasing virulence factors. Such factors are trafficked through the cell, from the site of synthesis to the flagellar pocket, the only site of endocytosis and exocytosis in *Leishmania*. The cysteine peptidase CPB has been shown to be released by the parasite and act as a virulence factor. CPB is released through the flagellar pocket while being trafficked to the lysosome or through direct secretion. We are interested to discover if other peptidases are secreted and whether they also act as virulence factors. To identify secreted peptidases, we have been investigating the intracellular location of several candidates, including a Bem46-like serine peptidase of the Clan SC, family S9. The S9 serine peptidase family includes oligopeptidase B, which is a known trypanosomatid virulence factor. Bem46 is located close to the flagellar pocket, but is not apparently secreted. *L. major* Bem46-like null mutants have been generated and their phenotype will be presented. Apart from secretion of virulence factors, rapid protein turnover in the lysosome is also important for differentiation and thus infectivity of *Leishmania*. To investigate lysosome structure and function in *L. major*, two potential lysosomal membrane proteins, a LAMP-like protein and a CLN3-like protein, have been identified and their location and role explored through generation of transgenic parasites.

Casein kinase 1 isoform 2 is essential for bloodstream form *Trypanosoma brucei*

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Protein kinases (PKs), key mediators of growth and cell signalling, are one of the major drug target families being tackled by the pharmaceutical industry. The *T. brucei* genome encodes 176 putative PKs, which are in theory attractive targets for anti-trypanosomal drug discovery, given the possibility of 'piggy-back' drug discovery. The serine/threonine protein kinase casein kinase I family (CK1s) plays an important role in eukaryotic signalling pathways, and their substrates include key regulatory protein involved in cell differentiation, proliferation, chromosome segregation and circadian rhythms. Essential CK1s are attractive targets for anti-trypanosomal drug discovery as CK1s are monomeric, constitutively active and usually co-factor independent, simplifying assay development, and there are several high resolution structures of CK1s with ATP or inhibitors bound to aid rational drug design. The *Leishmania major* CK1 isoform 2 (LmCK1.2, LmjF35.1010) has been implicated as an essential enzyme through studies using immobilized or radiolabelled inhibitors. The same compounds were also shown to be cytotoxic to *T. brucei*, implying that the homologous *T. brucei* enzyme (TbCK1.2) may also be essential. Induction of RNA interference targeted against CK1 isoform 2 (TbCK1.2, Tb927.5.800) in bloodstream form *Trypanosoma brucei in vitro* results in rapid cessation of growth, gross morphological changes, multinucleation and ultimately cell death. A null mutant of the highly homologous CK1 isoform 1 (Tb927.5.790) in bloodstream form *T. brucei* displays no growth or morphological phenotype *in vitro*. A truncated form of TbCK1.2 expressed in *E. coli* as a GST fusion produces catalytically active recombinant protein, facilitating screening for small molecule inhibitors.

Blocking the Synthesis of Variant Surface Glycoprotein in *Trypanosoma brucei* Causes a Global Translation Arrest

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The surface of the unicellular parasite *Trypanosoma brucei* is covered with a homogeneous layer of variant surface glycoprotein (VSG). Earlier we have shown that blocking VSG synthesis by inducing VSG RNAi results in a precise pre-cytokinesis cell cycle arrest with the accumulation of 2N2K cells. Here we show that the induction of VSG221 RNAi triggers a global block in translation. This observed translation arrest is not due to a decrease in levels of total mRNA, but is caused by a decrease in polysomes. The increase in the pool of free mRNA is correlated with a dissociation of the ribosomes from the endoplasmic reticulum. The VSG221 RNAi phenotype is not caused by siRNA toxicity as the VSG221 RNAi-induced cell cycle arrest is alleviated if a second different VSG (VSG117), which is not recognised by the VSG221 RNAi, is inserted downstream of the active VSG Expression Site promoter. Further, we show that it is possible to knock-out the telomeric VSG221 leaving the cells completely reliant on the second complementing VSG117 gene. Transiently transfecting cells with anti-VSG221 morpholino oligonucleotides allows us to specifically block translation of VSG221 mRNA without degrading it. This results in a pre-cytokinesis cell cycle arrest similar to that induced by VSG221 RNAi. These experiments demonstrate that the VSG RNAi-induced cell cycle arrest is due to blocking VSG221 protein synthesis rather than a consequence of ablating the abundant VSG221 transcript.

Acetyl-CoA Carboxylase: Potential Hot Spots for Controlling Fatty Acid Synthesis in *Trypanosoma brucei*?

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In lieu of the conventional fatty acid synthesis (FAS) pathway, *Trypanosoma brucei* uses an unusual elongase (ELO) pathway as the predominant mechanism of FAS. The ELO pathway is likely essential in both bloodstream (BSF) and procyclic forms (PCF), and appears to be modulated in response to the availability of exogenous lipids. Acetyl-CoA carboxylase (ACC) catalyzes the conversion of acetyl-CoA to malonyl-CoA, the two carbon donor in the ELO pathway. Malonyl-CoA decarboxylase (MCD) can convert malonyl-CoA back to acetyl-CoA. We hypothesize these two enzymes play a role in the regulation of the ELO pathway by controlling the availability of malonyl-CoA. To test our hypothesis, we generated ACC RNAi mutant cell lines that reduced ACC enzymatic activity (87% in BSF and 93% in PCF). Total ELO activity is reduced upon the knock down of ACC. Induction of ACC RNAi in PCF parasites grown in normal media caused no growth defect. However, ACC knock down in low lipid media resulted in a 36% reduction in growth rate. We have also generated MCD RNAi and MCD over-expressing cell lines. Initial studies of both cell lines showed unchanged parasite growth rates in both normal and low-lipid media. The *T. brucei* ACC protein sequence has no known targeting motifs and is predicted to be a cytosolic protein. Immunofluorescence microscopy of myc-tagged ACC shows a cytoplasmic punctuate pattern. ACC does not co-localize with known markers for the glycosomes, mitochondrion, endoplasmic reticulum, or lipid droplets. Subcellular fractionation of myc-tagged and native ACC demonstrates that ACC is cytosolic and not associated with membranes. These localization studies indicate a novel cytoplasmic localization pattern for ACC.

TbPIF8, related to a yeast mitochondrial DNA helicase, is required for trypanosome viability and kDNA organization

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In investigating T. brucei kDNA replication, we studied 8 proteins homologous to the yeast mitochondrial DNA helicase, ScPIF1. We found that six of them localize to the mitochondrion and at least 3 of these are essential for viability. One of the essential proteins, TbPIF8, localizes to the kinetoplast disk. However, since it lacks some conserved helicase domains, this protein may not have helicase activity. Nonetheless, RNAi of TbPIF8 caused growth arrest at day 5 and shrinking of the kinetoplast. Surprisingly, when we assessed the kDNA level during the course of RNAi by Southern blotting, we found little change in levels of either minicircles or maxicircles. We reconciled these apparently contradictory results by fluorescence microscopy of DAPI-stained networks isolated from RNAi cells. We found that many networks became not only smaller in surface area but also brighter. This result suggested that RNAi caused condensation of the network, possibly without large changes in the minicircle copy number. EM of isolated networks confirmed this finding and raised the possibility that condensation was due to increasing the number of interlocked neighbors of each mincircle. These findings suggest that TbPIF8 may control network topology by antagonizing the action of kDNA network condensing factors. For example, p19, a basic protein that localizes in the kDNA disk, condenses kDNA in vitro. It is likely that depletion of p19 by RNAi would make the network looser. In contrast, RNAi knockdown of TbPIF8 makes the network more condensed. During kDNA loosening or condensation, a topoisomerase II would be needed to change the number of interlocked neighbors of each minicircle. How this occurs is under investigation.

Role of the *Trypanosoma brucei* RNA-binding protein RBP10 in bloodstream form energy metabolism

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The energy metabolism of *Trypanosoma brucei* shows strong developmental regulation. In the bloodsteam form (BS), ATP is generated by glycolysis. In the procyclic form (PC), additional pathways are present. *T. brucei* RBP10 is a cytoplasmic RNA-binding protein containing one RNA recognition motif (RRM). RBP10 is found only in BS trypanosomes. RNAi targeting *RBP10* was lethal in BS, but had no effect in PC. In BS *RBP10* RNAi, 100 mRNAs were significantly decreased; strikingly, the most strongly affected transcripts included many encoding glycolytic enzymes such as PGKC, and proteins involved in glucose metabolism, e.g. the hexose transporter THT1. *VSG* mRNA was decreased, while *EP* procyclin mRNA increased. It is unclear which of these effects are a direct result of RBP10 depletion, and which are secondary to metabolic disturbance. Many developmentally-regulated mRNAs were not affected (for example, alternative oxidase mRNA) and the results were not just due to growth arrest. Overexpression of myc-tagged RBP10 in PC caused a severe growth effect and up-regulation of the endogenous *RBP10* mRNA and RBP10 protein. *PGKC* and *THT1* mRNAs increased, while PC-specific *PGKB* mRNA decreased. We are currently assaying the effect of RBP10 expression on the PC transcriptome, and determining which portions of the protein are required for the regulatory effects. So far, our results suggest that RBP10 may be a key regulator for expression of proteins required for bloodstream-form energy metabolism.

New Tools for Controlling Constitutive and Inducible Transgene Expression in *Leishmania donovani*

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A variety of vectors exist for episomal gene expression in *Leishmania* that exploit the propensity of nearly any transfected circular DNA molecule to be transcribed and replicated under appropriate selective conditions. While these standard vectors have proven invaluable tools for genetic manipulation of *Leishmania*, they have limitations. For example, the wide variation in both episome copy number and expression levels within the transfected population has made it impossible to select a vector that will express a transgene at a predetermined level in all cells. Moreover, the instability of standard vectors in the absence of antibiotic selection restricts their usefulness for experiments in animal models where selection cannot be maintained. To overcome these limitations, we have designed a novel set of low copy number vectors that allow uniform expression throughout the transfected population and are retained in the absence of selection both in vitro and following passage in mice. Stability and copy number control are conferred by a 550 base pair (bp) fragment containing the *L. donovani* ribosomal RNA promoter and 200 bp of adjacent downstream sequence. A series of six vectors covering a 450-fold range of defined expression levels (from very high to very low) was created using different combinations of 5'- and 3'-untranslated regions to modulate pre-mRNA processing efficiency and mRNA stability, respectively. These vectors have extensive multiple cloning sites, and versions are available for producing N- or C- terminal GFP fusions. In addition, a useful inducible expression system has been generated by combining these vectors with the commercially available ProteoTuner system which allows direct modulation of transgenic protein levels by the membrane-permeable small molecule Shield-1.

Processing and subcellular localization of *Leishmania major* metacaspase

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Metacaspases are cysteine peptidases that could play a role similar to caspases in the cell death pathway of plants, fungi and protozoa. *Leishmania major* expresses a single metacaspase gene (LmjMCA). Structurally, LmjMCA comprises an N-terminal mitochondrial localization sequence (MLS), a central domain with the catalytic dyad histidine/cysteine described for caspases and a C-terminal proline-rich sequence. LmjMCA has been shown to be autoprocessed and its catalytic domain active against defined substrates having arginine in the P1 position. In this study, we investigated the activation by processing of LmjMCA and its cellular localization. Processing of LmjMCA occurs in every stage of the life cycle of the parasite. It can be induced by stress conditions such as heat shock, H_2O_2 or by environmental conditions. Using tagged MCA polypeptides and site directed mutagenesis of potential cleavage sites, we determined the sites which are important in the processing of LmjMCA catalytic domain. Although LmjMCA polypeptide precursor form harbors a functional MLS, we demonstrated that LmjMCA is mainly localized in the cytoplasm due to an amino acid sequence blocking its transport into the mitochondrion. We also found that in stress conditions, overexpression of LmjMCA enhanced sensitivity of parasites to reactive oxygen species likely by disrupting the mitochondrion. These results suggest that LmjMCA processing and differential localization could be essential in its interactions with other polypeptides and its role in physiological and stress situations.

The role of the initial acyltransferase *Lm*DAT in glycerolipid metabolism, metacyclogenesis, and autophagy of *Leishmania*

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Leishmania species are arthropod-born protozoan parasites that cause important diseases in human, collectively termed leishmaniasis, which affects at least twelve million patients worldwide. Because of the lack of effective vaccine and adequate pharmacological agents, more efficacious drugs are urgently needed for the treatment and prevention of this parasitic disease. The ability of the parasite to generate new membranes, composed mostly of ester and ether glycerolipids, is essential for their active multiplication in order to cause disease. Furthermore, the *Leishmania* cell surface is made of glycerolipid-derived lipoglycoconjugates that play a pivotal role for the parasite development in the insect vector and in the mammalian host. Last, glycerolipid-based drugs are inhibiting *Leishmania* growth in vitro and in vivo, suggesting that the glycerolipid biosynthetic pathway may offer a novel target for chemotherapeutic intervention. All together these observations support the notion that genes/enzymes involved in the biosynthesis of glycerolipids are likely to be important for parasite virulence. The focus of our research is to identify and characterize the molecular machinery involved in *Leishmania* glycerolipid biosynthesis, with an eye toward drug development. One key enzyme of the glycerolipid biosynthetic pathway *Lm*DAT, an unusual dihydroxyacetonephosphate acyltransferase, will be described. Data related to its role in glycerolipid metabolism, metacyclogenesis, and autophagy will be presented.

An elongator orthologue regulates RNA Pol I transcription in trypanosomes

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In the African trypanosome, constitutive polycistronic RNA polymerase (RNAP) II transcription is widespread, while RNAP-I is bi-functional, directing transcription of ribosomal RNA (*rRNA*) in the nucleolus and mono-telomeric transcription of a variant surface glycoprotein (*VSG*) gene in an extranucleolar expression site body (ESB). The putative catalytic component of the RNAP II elongator in yeast and human cells is a histone acetyltransferase (ELP3), but the protein also functions in other pathways. Trypanosomatid genomes are unique in encoding two distinct ELP3 orthologues. In *T. brucei*, both genes were dispensable but *elp3b* null cells displayed a specific increase in resistance to the transcription elongation inhibitor 6-azauracil (6-AU) suggesting that ELP3b has a negative effect on elongation. GFP-tagged ELP3b complemented the 6-AU phenotype and localised to the nucleolus indicating a role in RNAP-I transcription. Consistent with these findings, transcription assays revealed an increase in RNAP-I transcription in *elp3b* null strains. Our results reveal a transcription regulatory role for ELP3b and the emergence of distinct orthologues that discriminate among RNA polymerases.

Spliceosomal proteomics revealed several new candidate SL *trans* splicing factors in *Trypanosoma brucei*

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In trypanosomatid parasites, SL *trans* splicing and polyadenylation resolve individual mRNAs from polycistronic precursor RNA. Since SL *trans* splicing does not occur in mammalian and insect hosts of trypanosomatids and since the latter organisms have diverged very early in evolution from the main eukaryotic lineage, we hypothesize that the trypanosomatid spliceosome harbors unique proteins and/or highly divergent orthologues of known proteins which are essential this process. A commonality of the spliceosomal snRNPs U1, U2, U4/U6 and U5 as well as of the SL RNP is a complex of seven proteins called Sm. Fusing the PTP-tag C-terminally to *T. brucei* SmD1, which is shared among all spliceosomal snRNPs, we tandem affinity-purified components of the spliceosome and identified 47 proteins by LC/MS/MS. Except for three LSm proteins, this set contained all known spliceosomal snRNP proteins of trypanosomes and 21 proteins lacking a specific annotation. The latter were analyzed bioinformatically and revealed several highly divergent orthologues of known splicing factors including the U1-specific U1A protein which was hypothesized to not exist in trypanosomatids. RNA analyses of epitope-mediated protein pull down assays as well as immunofluorescence microscopy confirmed the identity of several of the newly identified snRNP proteins. We also found a novel U5-specific factor and showed that it is essential for cell growth and *cis* and *trans* splicing. Interestingly, this protein was detected in both nucleus and cytoplasm whereas the other factors localized exclusively to the nucleus suggesting that the novel U5 factor has an important role beyond the splicing process itself. Currently, we are analyzing snRNP-association and functional relevance in cell growth and in RNA splicing of those proteins which remain un-annotated.

Essential components of the guide RNA binding complex

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In mitochondria of Trypanosomes, insertion and deletion of uridines occurs, often on a massive scale, to create functional messenger RNAs. The editing machinery is a dynamic system, in which multi-protein complexes undergo transient interactions in the course of gRNA processing, gRNA-mRNA recognition and the cascade of nucleolytic and phosphoryl transfer reactions. Identification of RNA binding proteins that interact with the gRNAs, facilitate editing reactions or contribute to mRNA stability is critical for understanding the editing process. We have identified a guide RNA binding complex, termed GRBC, which is essential for the guide RNA stabilization and function in the editing process. The core of GRBC is composed of subunits 1 and 2, which form a stable alfa-betta heterotetramer. Approximately a dozen proteins associate with this core particle by protein-protein and RNA-mediated interactions. RNAi repression of GRBC1 or 2 led to accelerated gRNA decay thereby inhibiting mRNA editing. GRBC1/2 complex was co-expressed in bacteria and purified to homogeneity. In vitro studies demonstrated that the GRBC1/2 heterotetramer forms a single, discrete complex with gRNA. RNA binding is accomplished mostly by the GRBC2 subunit and does not require oligo(U) tail. Collectively, our data suggest that gRNAs are stabilized by direct binding to GRBC1/2 particle prior to uridylylation by RET1 TUTase. The GRBC interactome overlaps with editing and polyadenylation complexes but its depletion does not affect the 20S editosome activity or mRNA polyadenylation. Further investigation of GRBC subunits revealed specific factors that may contribute to gRNA-mRNA hybrid definition and post-editing gRNA displacement.

Activity of indenoisoquinolines against African Trypanosomes

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African trypanosomiasis (sleeping sickness), caused by protozoan *Trypanosoma brucei* species, is a debilitating disease that is lethal if untreated. Available drugs are antiquated, toxic and compromised by emerging resistance. The indenoisoquinolines are a class of non-camptothecin topoisomerase IB poisons that are under development as anti-cancer agents. We tested a variety of indenoisoquinolines for their ability to kill *T. brucei*. Indenoisoquinolines proved trypanocidal at submicromolar concentrations *in vitro*. Structure-activity analysis yielded motifs that enhanced potency including alkylamino substitutions on N6, methoxy groups on C2 and C3, and a methylenedioxy bridge between C8 and C9. Detailed analysis of eight water-soluble indenoisoquinolines demonstrated that in trypanosomes the compounds inhibited DNA synthesis and acted as topoisomerase poisons. Testing these compounds on L1210 mouse leukemia cells revealed that all eight were more effective against trypanosomes than against mammalian cells. In preliminary *in vivo* experiments one compound delayed parasitemia and extended survival in mice subjected to a lethal trypanosome challenge. The indenoisoquinolines provide a promising lead for developing drugs against sleeping sickness.

Transcription profile around Strand Switch Regions (SSRs) in *T.brucei*

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Many protein-coding genes in trypanosomes are transcribed as polycistronic pre-mRNAs and mature mRNA are generated by addition of SL RNA and 3'end processing. The majority of these polycistronic genes are arranged as divergent head-to-tail arrays radiating away from regions of non-coding DNA termed Strand Switch Regions (SSRs). In these SSRs, the direction of transcription switches from one strand of DNA to the other. Studies in *Leishmania major* have indicated that transcription initiates bidirectionally from these regions (P.Myler laboratory). In addition the SSRs harbors modified histones, which are associated with actively transcribed genes (L.Aslund laboratory). Since our knowledge of RNA Pol II transcription initiation in *T.brucei* is limited due to the lack of any known RNA Pol II promoter, SSRs make an interesting study. To investigate the role of SSRs in RNA Pol II transcription we have chosen to study two representative SSRs, one on chromosome 3, and the other on chromosome 7. This analysis will include enzyme based reporter assays to test promoter activity of regions of SSRs, and transcription profile using nascent transcription analysis. Recent work will be presented.

Role of cargo and retrograde transport motor proteins in the construction of the trypanosome flagellum

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The trypanosome flagellum is constructed by intraflagellar transport (IFT), a dynamic process where flagellum precursors are transported to the tip of the organelle by a kinesin motor and a complex of cargo proteins that are recycled to the base by the action of a dynein motor associated to a different set of cargo proteins. The dynein complex is usually composed of a single heavy chain termed DHC1b that has motor activity and of two intermediate chains called XBX1 (or D1bLIC) and FAP133. Curiously, trypanosomes possess two distinct genes encoding DHC1b and DHC1bis that possess the signature of IFT dyneins but only share 37 % identity. In contrast, XBX1 and FAP133 exist as single copy genes, exactly as genes encoding IFT cargo proteins. Both dyneins are present at the basal body and in the flagellum matrix where spinning disk microscopy analysis revealed IFT motility in anterograde and retrograde directions. Scanning and transmission electron microscopy revealed that inhibition of DHC1b or DHC1bis resulted in formation of short dilated flagella, filled with IFT material, demonstrating that they are not redundant and both involved in retrograde transport. The same phenotype was obtained upon knock-down of XBX1 or FAP133 expression, or of 5 different IFT cargoes. This can be explained if kinesin and IFT cargo proteins enter the flagellar compartment but fail to be recycled due to the absence of the dynein motor. This was confirmed by immunofluorescence that showed large amounts of IFT cargo proteins in the short flagella. However, dynein heavy chain(s) did not show this behaviour and were able to cycle back to the basal body in the absence of cargo.

Exploiting Trypanosmal Nitroreductase as a Drug Target

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Nitroheterocyclic drugs such as nifurtimox and benznidazole have been used for over forty years against Chagas disease. However, their use is problematic due to toxic side-effects and emerging resistance. Despite this, plans to extend the use of nifurtimox in co-therapy with eflornithine against African Sleeping Sickness are being evaluated. Both nifurtimox and benznidazole function as pro-drugs and are activated in trypanosomes by a type I nitroreductase (NTR). Here we exploit this mechanism to analyse a library of nitrofuran based compounds for efficacy and mode of action against both *Trypanosoma brucei* and *Trypanosoma cruzi*. When screened against wild type bloodstream form *T. brucei*, half of the compounds displayed significant trypanocidal activity (IC50 values ¡1 uM). Their effects against parasites with altered NTR levels were also evaluated: heterozygous lines displayed resistance to the nitrofurans and conversely, over expressing lines showed hypersensitivity. No obvious structure-activity relationship was observed in this study. To investigate the efficacy of these drugs against *T. cruzi*, we have developed a luciferase based reporter system to facilitate high throughput compound screening against the clinically relevant mammalian stage. Data regarding this evaluation will be presented. Together this information will provide a valuable resource in the design of the next generation of NTR activatible, trypanocidal nitroheterocyclic drugs.

Trypanosoma brucei Mitochondrial DNA Polymerase IB plays an essential role in leading and lagging strand minicircle replication

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Kinetoplast DNA (kDNA) of Trypansomatids is a catenated network composed of minicircles and maxicircles that is essential for parasite survival. Maintenance of the kDNA network requires numerous proteins for the release, replication and reattachment of minicircles. Despite the identification of several new kDNA associated proteins, characterization of a protein working at the minicircle replication fork remains elusive. To fill this gap in knowledge we investigated the cellular role of POLIB, one of six *Trypanosoma brucei* mitochondrial DNA polymerases. POLIB localizes where minicircle replication intermediates are detected and contains amino acid residues critical for proofreading exonuclease and DNA polymerase activities suggesting it is an ideal candidate for the minicircle replicative DNA polymerase. Using inducible stemloop RNAi, POLIB silencing resulted in 90% reduction of *POLIB* mRNA, growth inhibition, and progressive loss of the kDNA network. Within 48 hours of POLIB ablation, minicircle and maxicircle abundance decreased by 40%. Analysis of free minicircle replication intermediates revealed that newly replicated free minicircle progeny (nicked/gapped species) declined to almost undetectable levels while unreplicated, covalently closed molecules were most abundant. Additionally, a heterogenous population of free minicircles migrating similarly to incompletely replicated, multiply gapped minicircles accumulated during POLIB RNAi. As evident from two-dimensional gel electrophoresis, leading and lagging strand minicircle progeny similarly declined during silencing, indicating POLIB plays a role in replication of both strands. Furthermore, in the second dimension the heterogenous minicircle population migrated as a distinct and previously unreported population of free minicircles. Characterization of this new free minicircle species is ongoing. These data indicate that POLIB plays a critical role at the core of the minicircle replication machinery.

Definition of residues that confer substrate specificity in *Leishmania mexicana* sugar transporters

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Sugars, the major energy source for many organisms, must be transported across biological membranes. Glucose is the most abundant sugar in human plasma, and in many other biological systems, and has been the primary focus of sugar transporter studies in eukaryotes. We have previously cloned and characterised a family of glucose transporters from the protozoan parasite *Leishmania mexicana*. These transporters, called LmGT1, LmGT2 and LmGT3, are homologous to the well-characterised GLUT family of mammalian glucose transporters. We have demonstrated that LmGT proteins are important for parasite viability. Here we show that one of these transporters, LmGT2, is also an effective carrier of the pentose sugar ribose. A comparative reverse genetic analysis has shown that a pair of threonine residues, located respectively in the loops joining transmembrane helices 3 and 4, and transmembrane helices 7 & 8, appear to act as part of a filter that prevents ribose approaching the exofacial substrate binding pocket. When these are substituted by alanine residues as found in LmGT2 the LmGT3 permease acquires robust ribose-permease activity. The fact that these residues are located near the extracellular face of the transporter molecule supports recent suggestions that substrate recognition and substrate binding may not be a single step process in this group of transporters.

Global gene expression analysis of *Trypanosoma cruzi* under hyperosmotic stress

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Osmoregulation is essential for *Trypanosoma cruzi* since it is subjected to a variety of osmotic stresses during its life cycle. When epimastigotes were subjected to hyperosmotic stress (from 300 to 650 mOsm, by addition of sorbitol) they shrank dramatically within a few minutes, and did not regain their normal volume at least during the following 2 h. However they adapted well to these conditions, being virtually indistinguishable in terms of motility from control cells maintained in isosmotic buffer. Treatment of the cells with low concentrations of HgCl2, a known inhibitor of *T. cruzi* aquaporin 1 (TcAQP1), reduced the intensity of shrinking while there was a direct correlation between levels of shrinking and TcAQP1 overexpression, suggesting that aquaporin could be mediating water efflux during osmotic challenge. Hyperosmotic stress also resulted in a great increase in the size of the contractile vacuole. A genome-wide transcriptional analysis of *T. cruzi* epimastigotes submitted to hyperosmotic stress resulted in up-regulation and down-regulation of the expression of a number of genes. Several of these changes were confirmed by northern and western blot analyses. Sequence analysis of gene transcripts allowed the identification of different RNA motifs, with stem-loop structures of 35-50 nt, preferentially localized in the 3'-UTRs. These motifs are enriched in the experimental dataset compared with the entire transcriptome: Up-m was found in 85% of up-regulated mRNAs (24 of 28) against 21% of the *T. cruzi* Reference Sequence (RefSeq) database; Down-m was present in 50% (15 of 30) of the down-regulated genes, and in 3% of the transcripts in the database. The lists of the Up-m- and Down-m-containing transcripts include trans-sialidases and mucins, among other proteins.

Knockout of acyl carrier protein in T. brucei causes kDNA loss

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While studying mitochondrial fatty acid synthesis (FAS) in T. brucei BSFs, we discovered that conditional knockout of acyl carrier protein (ACP, a component of the FAS machinery) caused changes in phospholipid content. Surprisingly, it also caused a loss of kDNA as observed by microscopy of DAPI-stained cells. kDNA loss is frequently caused by knockdown of a replication enzyme. However, we found using Southern analysis that ACP depletion caused little change in total minicircle or maxicircle levels or in free minicircle replication intermediates. These results indicate that kDNA loss is not due to a deficiency in replication but instead may be caused by a kDNA segregation defect. Normally, kDNA replicates to produce a double-size network that divides into two equal-size daughter networks. During defective segregation, a double-size network can divide asymmetrically to produce daughters differing in size or it may not divide at all. In this case, one progeny cell acquires a double-size network and the other receives no kDNA. Direct evidence for a segregation defect following ACP depletion came from observations of large networks either by DAPI-staining or EM. We also observed that nearly 20% of dividing kDNAs were doing so asymmetrically. kDNA segregation is mediated by the tripartite attachment complex (TAC), a transmembrane filament system linking the kDNA to the flagellar basal body. One TAC component is a specialized segment of the mitochondrial inner and outer membranes. Since mitochondrial FAS produces fatty acids for phospholipids in the mitochondrial membranes, we speculate that ACP depletion alters the lipid composition of the TAC-associated membranes. This change could affect TAC structure and cause the defective kDNA segregation.

Gene disruption of a Leucine rich repeats (LRR) protein involved in antimonials resistance in *Leishmania* spp

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Pentavalent antimonials (SbV) are the main drugs for the control of leishmaniasis, a parasitic disease with an estimated 500,000 new cases each year. Resistance to this class of drug is now prevalent in several endemic areas. Recently, we isolated a novel resistance protein, LinJ34.0570, which belongs to the superfamily of leucine-rich repeat (LRR) whose parasites overexpressing this LRR protein were resistant to antimonials (Genest *et al*., 2008). This gene was re-isolated in a functional cloning screen while selecting for resistance to antimonials. Here, we generated double knockout parasites for this gene by Loss of Heterozigoty (LOH) and we found that *Leishmania major* promastigote parasites are more sensitive to SbIII . This phenotype is reverted in the add back mutant. Our next goal is to verify the role of this gene in intracellular amastigotes, the form responsible for the disease in humans. These data indicate that this gene has an important role in the mechanism of resistance to antimonials.

Over-expression of the histone methyltransferase DOT1B disturbes cell cycle progression in *Trypanosoma brucei*

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Trypanosoma brucei has a complex parasitic life cycle in different hosts. Many cellular functions, including cell cycle control are carefully coordinated and adapted in order to survive in different host environments. Although many cell cycle regulators are conserved in trypanosomes, substantial divergence of their functions and other regulatory mechanisms seem to have evolved. We observed cell cycle associated changes in chromatin structure mediated by methylation of histone H3K76 by two members of the DOT1 histone methyltransferase family (DOT1A and DOT1B). Di-methylation of H3K76 by DOT1A is only detectable during mitosis and cytokinesis. RNAi-mediated depletion of DOT1A causes severe cell cycle defects and generates a cell population with a potential haploid DNA content (Janzen et al., 2006). To further investigate the possible role of these methyltransferases in cell cycle regulation, we analyzed the effects of over-expression of DOT1B, which is responsible for tri-methylation of H3K76. DOT1B over-expression is lethal and generates a substantial population of zoids suggesting that the parasites are forced into premature cytokinesis without completion of karyokinesis. This is an unexpected observation considering that histone phosphorylation (eg. H3S10p) but not histone methylation is involved in cell cycle regulation in other eukaryotes. We will discuss our hypothesis that coordinated regulation of DOT1A and DOT1B activity and hence histone methylation influence cell cycle progression in trypanosomes.

The mitochondrial elongation factor Tu of *Trypanosoma brucei* contains an insertion essential for function

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The mitochondrial genome of *Trypanosoma brucei* does not encode any tRNAs. This lack is compensated for by import of a small fraction of nucleus-encoded cytosolic tRNAs. As a consequence the mitochondrial (and therefore bacterial-type) translation machinery has to work exclusively with imported eukaryotic type tRNAs. During mitochondrial translation aminoacylated tRNAs are targeted to the ribosome by the bacterial-type elongation factor Tu (EFTu), a small GTPase. In contrast to other systems trypanosomal EFTu has to recognize imported tRNAs of eukaryotic origin. Analysis of the *T. brucei* EFTu sequence showed that it contains a trypanosomatid-specific insertion close to the C-terminus. This insertion is similar to a sequence located in the same relative position of the cytosol-specific eukaryotic elongation factor 1a (eEF1a). Here we show that RNAi-mediated depletion of EFTu causes a growth arrest in procyclic and bloodstream *T. brucei* cells. Moreover, *in vivo* complementation studies demonstrate that the trypanosomatid-specific insertion is required for correct function of the protein, and that it can be replaced by the corresponding region of *T. brucei* eEF1a. However, the insertion is not required for binding of trypanosomal EFTu to its GTP-GDP exchange factor EFTs suggesting that its absence does not interfere with correct EFTu folding.

Characterization of multiple homologues of the cap binding protein (eIF4E) from *Trypanosoma brucei*

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The initiation stage of protein synthesis is a likely target for events regulating gene expression in the trypanosomatids. This is highlighted by the identification in several species of multiple homologues of the translation factor eIF4E, the cap binding protein. Here we describe work aimed at the characterization of four eIF4E homologues from *Trypanosoma brucei* (*Tb*EIF4E1-4). First, their genes were cloned and the corresponding recombinant proteins produced and used to raise polyclonal sera. Northern and western blots were then performed using total RNA and protein extracts from both the procyclic and bloodstream stages of the parasite. All four mRNAs were found to be expressed constitutively although the *Tb*EIF4E2 message seems to be less abundant than the others. *Tb*EIF4E1, 3 and 4 proteins were detected in protein extracts from both stages with *Tb*EIF4E3 and 4 being more abundant than *Tb*EIF4E1. Next, through cap-binding assays, using ^{35}S labelled proteins, *Tb*EIF4E1, 2 and 4 were found to be able to bind the 7-methyl-GTP sepharose resin. Subcellular localization experiments were then carried out and whilst *Tb*EIF4E3-4 were found to be strictly in the cytoplasm, *Tb*EIF4E1 was also found in the nucleus but seemed to localize preferentially to the posterior end of the cell. Through RNA interference experiments, only *Tb*EIF4E3 was found to be strictly required for cellular growth in both procyclic and bloodstream forms whilst *Tb*EIF4E1 and 4 were also required for survival of bloodstream cells. Finally, pull down and immunoprecipitation assays, investigating the interaction between *Tb*EIF4E3 and selected *T. brucei* homologues of its binding partner eIF4G, have uncovered a specific interaction between *Tb*EIF4E3 and *Tb*EIF4G4 implying a relevant role for both proteins in translation.

Comparative expression profiling of *Leishmania* amastigotes

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Genome sequencing of three species of *Leishmania* (*L. major*, *L. infantum* and *L. braziliensis*) that cause a spectrum of disease phenotypes in the host has revealed a highly conserved gene content and synteny across the genus, with only a small number of differentially-distributed genes present *(Peacock et al. (2007) Nature Genetics 39, 839)* . We have used custom microarrays (representative of a target gene set covering ~10% of the genomes) to confirm that all of these differentially-distributed genes are expressed in intracellular amastigotes with only a few regulated at the RNA level. In the first study of gene expression during the *L. braziliensis* life cycle, we show that only ~9% of the target genes analysed are regulated in their RNA expression, a figure consistent with that observed in other *Leishmania* species. Comparison of the amastigote expression profiles generated for each species confirms that *Leishmania* transcriptomes undergo little regulation, although a number of genes conserved across the genus are differently regulated between the species. These findings support the hypothesis that *Leishmania* amastigotes are pre-adapted for intracellular survival and undergo little dynamic modulation of gene expression at the RNA level. In addition, the immune competence of the host has little influence on parasite gene expression, at least in *L. major*. Comparing amastigotes derived from either wild type (BALB/c) or immunologically-compromised ($Rag2^{-/-}c^{-/-}$) mice has shown that while parasite dissemination from the site of infection is enhanced in the $Rag2^{-/-}c^{-/-}$ background, parasite RNA expression profiles remain unperturbed.

Regulation of Gene Expression of Two Nearly Identical *Trypanosoma brucei* hexokinases, TbHK1 and TbHK2

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Trypanosoma brucei expresses two nearly identical hexokinases (TbHK1 and TbHK2) in both procyclic form (PF) and bloodstream form (BSF) parasites. The TbHKs share 98.5% identity at the nucleotide level from the 5'UTR to the ORF, however, major sequence differences are found in the 3'UTRs. Using a reporter gene system, we have found that the two TbHKs are differentially expressed in the BSF and PF in good agreement with steady state mRNA abundance. TbHK1 expression is nearly twice that of TbHK2 in PF parasites, while in BSF parasites, expression levels for the two genes are more similar, with TbHK2 expression 15% lower than TbHK1. The 3'UTRs for both genes contain putative K-box and CAAC elements. In *Drosophila*, these elements bind microRNAs and function as negative expression regulators. To determine if the putative K-box/CAAC elements are involved in differential regulation of expression of TbHKs, we generated deletions and point mutations in the TbHK1 3'UTR and assessed their impact on reporter expression. Notably, a point mutation in the K-box led to a 6-fold increase in expression of the reporter when compared to the authentic 3'UTR. Downstream and anti-parallel to the TbHK1 K-box/CAAC motif, there is a DNA sequence that would yield a RNA that is highly complementary to the K-box/CAAC motif. Transfection of *in vitro* transcribed RNA from this region restored regulation of the point mutant K-box/CAAC construct, suggesting a possible role for the small RNA in K-box/CAAC regulation.

Characterization of a nuclear Pumilio domain protein in *Trypanosoma brucei*

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Proteins of the PUF family contain Pumilio RNA binding domains. They have been found in all eukaryotes so far examined. Some of them bind to the 3'-untranslated regions of mRNAs, repressing translation and destabilising the bound mRNA. Others are known to be involved in ribosomal RNA maturation. The genome of *T. brucei* has eleven open reading frames containing pumilio repeats. A previous phylogenetic analysis, concentrating on the Pumilio domains alone, grouped PUF7 and PUF8 together with yeast Nop9 and Puf6, and with human KIAA0020, all of which are found in the nucleolus. Consistent with this, we have found that epitope-tagged *Tb*PUF7 is located in the nucleolus. Tandem affinity purification of tagged *Tb*PUF7 revealed binding to a cyclophilin-like protein, *Tb*NCP1, which is found throughout the nucleus, but no associated RNA could be detected. The PUF7-NCP1 interaction was verified by bidirectional co- immunoprecipitation. RNAi targeting PUF7 caused a mild growth defect, but we could find no defect in the processing of rRNA or 7SL RNA.

Stage-regulated mRNA decay in the protozoan parasite *Leishmania*

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Leishmania life cycle alternates between developmental forms residing within the insect vector (promastigotes) and the mammalian host (amastigotes). In *Leishmania*, nearly all control of gene expression is posttranscriptional and involves sequences in the 3′-untranslated regions (3′UTRs) of mRNAs. Little is known as to how these *cis*-acting elements regulate mRNA turnover and translation rates in *Leishmania*. Using the amastin mRNA-an amastigote-specific transcript-as a model, we showed that a U-rich element (URE) within its 3′UTR significantly accounts for developmental gene regulation. Destabilizing AU-rich or URE elements have also been associated with rapid turnover of developmentally regulated transcripts in *Trypanosoma* species. RNase-H-RNA blot analysis revealed that rapid turnover of the amastin mRNA in promastigotes is initiated without a prior shortening of the poly(A) tail. This is in contrast to the stable amastin mRNA in amastigotes and to reporter RNAs lacking the URE, which, similarly to most eukaryotic mRNAs are degraded through a progressive deadenylation. In our attempt to identify *trans*-acting factor(s) involved in the rapid decay of amastin mRNA in promastigotes, we carried out UV-crosslinking assays. The target URE RNA and control RNAs were mixed with several fractions derived from ammonium sulfate protein prefractionation of *Leishmania* lysates and a ∼25 kDa protein was detected in the 60%-70% ammonium sulfate fraction to bind specifically the URE RNA. RNA affinity tagged-RNA with the D8 RNA-tag that binds Sephadex beads and RNA affinity chromatography combined to mass spectrometry has now been completed. MS/MS analysis allowed the identification of promising RNA-binding protein candidates. We are currently validating the MS/MS results. The identification of trans-acting factors would shed light into the mechanistic aspects of mRNA decay in *Leishmania*.

VSG expression is monitored via a conserved stem-loop in the 3'UTR of VSG genes

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¡p align=justify¿ The expression of variant surface glycoprotein (VSG) is vital for bloodstream stage *Trypanosoma brucei*. VSG RNAi causes a rapid and precise cell cycle arrest (Sheader *et al.*, 2005). It has been proposed that the declining amount of VSG protein triggers this phenotype. However, VSGs are relatively stable proteins (half-life ca 30 h) and, hence, it is difficult to envisage how trypanosomes would recognize minute changes in VSG concentration. We propose that the amount of VSG mRNA is being controlled rather than the VSG protein itself. Two conserved motifs within the VSG 3'UTR are required and sufficient for monitoring VSG expression levels. In the presence of an ectopic VSG gene the native VSG gene can be knocked-out or knocked-down, but only if the rescue copy contains the VSG 3'UTR including the conserved 8mer and 16mer. In the absence of these conserved motifs, even high-level expression of an ectopic VSG cannot rescue the cell cycle arrest. Mutation analyses have shown that both motifs form a single stem-loop. Any interference with the stem-loop structure leads to complete loss of function. The VSG open reading frame is not involved, neither is the genomic context of the VSG gene. We propose that via the 8/16mer motif the cellular concentration of VSG mRNAs is continuously monitored by an as yet unknown, labile factor. In the event of an expression site shut down, the rapidly declining amount of VSG mRNA could act as most upstream signal for activation of a new expression site. To our knowledge this would be the first example of a control of gene expression by mRNA counting.

The Formation and Function of Long (A/U) Tails of *T. brucei* Mitochondrial mRNAs: An Emerging Role for PentatricoPeptide Repeat (PPR) Proteins

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Expression of the *T. brucei* mitochondrial genome requires the coordination of several RNA processing steps in order to generate mature mRNAs. Initial partitioning of multicistronic precursors is followed by KPAP1-catalyzed addition of a short poly(A) tail to pre-mRNAs. To generate translatable messages, however, many of these transcripts must undergo U-insertion/deletion RNA editing. Completion of the editing process triggers the extension of the short A-tail into a long (A/U) heteropolymer (100-200 nucleotides) structure. This work focuses on the long tail's synthesis and possible function in translation. Analysis of the KPAP1 polyadenylation complex revealed the presence of several **P**entatrico**P**eptide **R**epeat (PPR) proteins. Members of this family have been identified as sequence-specific RNA binding proteins in organelles of plants and other organisms. Two proteins, PPR1 and PPR21 appeared to be nearly stoichiometric with the catalytic subunit of the polyadenylation complex. An RNAi knockdown of PPR1 resulted in growth inhibition and loss of long-tail structures while knockdown of PPR21 had no discernible phenotype. Proteomics of the affinity-purified complexes revealed that PPR1 and PPR21 form a heterodimer which interacts with polyadenylation, Guide RNA Binding (GRBC), editing, and translation machineries. The PPR1-PPR21 heterodimer has been co-expressed and purified from bacteria. Biochemical characterization of this PPR heterodimer will be discussed. Sedimentation analysis of mitochondrial ribosomal RNA-containing particles demonstrated a co-sedimentation of the assembled monosomes and mRNAs bearing a long A/U tail. Taken together these data are the first demonstration of a PPR heterodimer and suggests a functional role for mRNA long-tails in ribosome association and translation.

Functional characterization of the RNA-binding protein *Tb*RBP33

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*Tb*RBP33 is a putative RNA-binding protein that has been identified by affinity chromatography using a U-rich RNA element as bait. It is 318 amino acids long and has a predicted molecular mass of 34 kDa. Its sequence reveals a canonical RNA recognition motif (RRM) and a nuclear localization signal. The carboxyl half has no similarity to any known protein outside the Kinetoplastids. Antibodies raised against *Tb*RBP33 recognize a single protein of the expected size in bloodstream and procyclic trypanosome extracts. The protein seems to be located mainly in the nucleus, as judged by immunofluorescence assays. To study the function of *Tb*RBP33, the protein has been depleted *in vivo* using RNA interference in bloodstream and procyclic trypanosomes. *Tb*RBP33 ablation results in cell death after 48 h of tetracycline induction in both life forms. We present preliminary evidence suggesting that *Tb*RBP33 is a ribonuclease, whose properties and role in RNA metabolism are currently being investigated.

The Role of a CTD-Interacting Protein in RNA Polymerase II-Associated Transcription in Trypanosoma brucei

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Trypanosomes exhibit many unique transcriptional characteristics, including the non-repetitive C-terminal domain (CTD) of the largest subunit of RNA polymerase (RNAP) II. The repetitive CTD of RNAP II is pivotal in many co-transcriptional processes in well-studied organisms. Therefore, we predict that the non-repetitive CTD of trypanosome RNAP II interacts with trypanosome-specific proteins to achieve gene expression. To identify CTD-interacting proteins, we performed a yeast two-hybrid screen and identified four candidate *Trypanosoma brucei* proteins. One such protein, A10 is a novel protein of unknown function. We hypothesize that the interaction between A10 and the CTD occurs in the *T. brucei* cell nucleus and supports RNAP II-mediated transcription. We have generated a cell line with a tagged A10 and are currently using an immunofluorescence assay to determine the localization of A10 in the cell. We will then assess the interaction between A10 and RNAP II *in vivo* by determining whether A10 and RNAP II co-immunoprecipitate from nuclear extracts. The result of this work may implicate A10 as the first trypanosome-specific protein to be involved in RNAP II-mediated transcription.

Editing and modification of Kinetoplastid threonyl-tRNA

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The ethanolamine branch of the Kennedy pathway is essential in bloodstream *Trypanosoma brucei*

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Phosphatidylethanolamine (GPEtn), a major component of the trypanosome membrane phospholipids, is synthesised *de novo* from ethanolamine through the Kennedy pathway. Because membrane phospholipids determine membrane fluidity and the surface charge of cell surfaces, ethanolamine utilization and GPEtn biosynthesis control a variety of cellular processes, including vesicle formation and intracellular trafficking, membrane fusion events, and mitochondrial function. GPEtn is also the donor of the ethanolamine-phosphate capping of the glycosylphosphatidylinositol (GPI) anchor. The GPI anchor is required for membrane attachment of cell surface proteins and this is particularly important for the bloodstream form of *Trypanosoma brucei*, which relies on a dense coat of GPI-anchored variant surface glycoprotein (VSG) to circumvent the attack of the host immune system. Disruption of GPEtn biosynthetic pathways in *T. brucei* is likely to severely impair the parasite homeostasis and thus, the constituent enzymes may represent novel targets for chemotherapy. In our study we use biochemical and molecular-genetic approaches to characterise the Kennedy pathway in bloodstream *T. brucei* and establish its suitability as a drug target.

Structural Requirements for the Attachment of a Rare Ethanolamine Modification to Eukaryotic Elongation Factor 1A

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Apart from its pivotal role during polypeptide synthesis, eukaryotic elongation factor 1A (eEF1A) is involved in many other cellular processes. Remarkably, although eEF1A contains multiple post-translational modifications, their roles have remained unknown. One modification involves the attachment of ethanolamine-phosphoglycerol (EPG) to two glutamate residues that are conserved between mammals and plants. Recently, we showed that EPG is also present in eEF1A of *Trypanosoma brucei*. However, in contrast to mammalian and plant eEF1A, *T. brucei* eEF1A contains a single EPG modification only. To determine a minimal structural motif required for EPG attachment, we expressed a set of truncated versions of HA-tagged eEF1A in *T. brucei* procyclic forms. The presence of the EPG moiety was analyzed by *in vivo* labeling of *T. brucei* eEF1A with [^{3}H]-ethanolamine, and by mass spectrometric analyses of the tryptic peptides containing the EPG modification site. Deletions of domains I or II and truncations in domain III of the full length protein revealed that an 80 amino acid peptide is sufficient for the attachment of the EPG moiety. In addition, to define the sequence requirements for EPG attachment, we introduced a series of amino acid point mutations in *T. brucei* eEF1A at, or around, the conserved modification site glu^{362}. As expected, the substitution of glu^{362}by alanine, glutamine or aspartate abolished EPG attachment. In contrast, several point mutations of conserved amino acids around glu^{362} did not affect EPG addition. Finally, since *Saccharomyces cerevisiae* eEF1A was reported not to contain the EPG modification, we are currently studying the expression of yeast eEF1A, or modified constructs of it, in *T. brucei*.

The N-terminal signal peptide from human haptoglobin related protein exhibits potent and specific toxicity towards bloodstream form *Trypanosoma brucei*

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Haptoglobin related protein (Hpr) is a component of the trypanolytic human high density lipoprotein, termed trypanosome lytic factor (TLF). Unusual for secreted proteins, Hpr retains its N-terminal 19 amino acid signal peptide. Native, delipidated Hpr has been shown to be cytotoxic to *Trypanosoma brucei*. We show here that trypanosome killing by Hpr is dependent upon the presence of the highly hydrophobic signal peptide and that a synthetic peptide corresponding to the signal sequence is sufficient for killing. Indeed, the synthetic Hpr signal peptide specifically kills bloodstream but not procyclic form *T. brucei*, is not inhibited by the serum resistance associated protein (responsible for evasion of TLF killing in *T. b. rhodesiense*), and appears to act at the surface of the trypanosome. *In vitro* studies with model liposomes suggests that bloodstream form *T. brucei* are uniquely susceptible to killing by the peptide due to the acyl chain composition of their cellular membrane. Due to the VSG coat, bloodstream form *T. brucei* have a high content of relatively short myristoyl acyl chains (14 carbon, saturated), whereas the signal peptide resistant procylic form *T. brucei* contain a larger content of palmitoyl (16 carbon, saturated) and stearoyl (18 carbon, saturated) chains, providing greater van der Waals interactions within the bilayer and possibly inhibiting signal peptide penetration. Preliminary data shows that the Hpr signal peptide is not toxic towards mammalian cells, thus it may provide a novel therapeutic for treatment of African trypanosomiasis.

Electron tomography of the Trypanosoma brucei flagella connector

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We are undertaking full cell electron tomography (ET) reconstructions of high pressure frozen (HPF) *T. brucei* bloodstream and procyclic cells. These reconstructions of complete cells will reveal the ultrastructural details of cytoskeletal events at key cell cycle stages. Initially, we have focused on the flagella connector (FC), a mobile transmembrane junction that connects the extending new flagellum tip to the side of the old flagellum in procyclic form parasites. Electron microscopy of chemically fixed cells revealed the FC as a tripartite structure consisting of an extracellular domain and two internal plates inside each flagellum. The electron dense plates connect the axonemes to the internal face of the respective membranes in this area. ET reconstructions and 3D models of this chemically-fixed material show that this tripartite structure is positioned in register to the two surrounding axonemes. However, in HPF cells the view is more complex; both the extra cellular structure and the axonemal internal plate of the old flagellum are thinner and the material is more uniformly distributed. The internal plate of the new flagellum is less observable because of the higher density of preserved material. We conclude that the three regions in the FC that interconnect become more evident when using chemical fixation. However, it is likely that the more diffuse structure we see in HPF cells is the more native state of this structure. A previously undetected electron dense cap in the new growing flagellum tip is present in the HPF cells. The axonemal microtubule cohort coalesces in this growing flagella tip and the doublet microtubules extending the furthest into the tip end in the electron dense cap.

Dynamic mRNA Expression analysis of cells undergoing synchronous life-cycle differentiation in *Trypanosoma brucei*

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The gene expression profiles of bloodstream and procyclic forms of African trypanosomes have been previously analysed by microarray hybridization. However, such studies have used parasite populations grown in vitro or monomorphic cell lines. Such assays have ignored stumpy forms and have not tracked changes in gene expression profiles as these parasites differentiate between bloodstream and procyclic forms. In this study we have used AnTat1.1 cells capable of generating stumpy forms and exploited the ability of such cells to undergo synchronous differentiation to procyclic forms to assay the dynamic changes in gene expression during parasite development. In total, 5 biological replicates representing the differentiation of 5 matched parasite populations derived from individual mouse infections were assayed, with RNAs being derived at key biological time points during the time course of their synchronous differentiation to procyclic forms (i.e. at 0h, 1h, 6h, 18h and 48h after differentiation of stumpy forms was induced by the addition of cis-aconitate). Importantly, the context of these mRNA profiles was established by assaying the coincident cellular events in each population (surface antigen exchange, morphological restructuring, cell cycle re-entry), thereby linking the observed gene expression changes to the well-established framework of trypanosome differentiation. Using stringent statistical analysis and validation of the derived profiles against experimentally-predicted gene-expression changes, we have established a reliable profile of regulated genes during this life cycle transition. Specific analysis of those genes regulated in the stumpy-form of the life cycle reveal genes important in cell cycle control, RNA metabolism and ion transport. We have begun initial characterisation of some of these genes to assess their role in differentiation.

Identification of the KREPA4 RNA binding domain and elucidation of its biochemical role in RNA editing

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Mitochondrial mRNAs in trypanosomes undergo RNA editing by insertion and deletion of uridylates (Us) to produce mature functional mRNAs. The editing process is carried out by a multi-protein complex, the editosome that has not yet been fully characterized. KREPA4 (Kinetoplastid RNA Editing Protein A4) is an essential protein component of the editosome. It is potentially associated with both insertion and deletion editing subcomplexes and may play a role in the stability and perhaps assembly of the editosome. Structural prediction and compositional analysis of KREPA4 has identified a conserved oligonucleotide binding fold (OB fold) at the C-terminal, and two low compositional complexity regions (LCRs) at the N-terminal of KREPA4. Concurrent with these predictions, recombinant KREPA4 has been shown to bind to synthetic guide RNA in vitro, specifically the gRNA 3'-(U)-tail. Here we report the RNA binding domain of KREPA4 by analyzing the RNA binding activities of the full length and truncated versions of this protein. Our results suggest that the RNA binding activity of KREPA4 is mainly localized to the OB fold at the C-terminal of the protein, with additional contribution from the LCRs which might further stabilize the interaction. We also show that KREPA4 has RNA annealing activity as it is able to stimulate gRNA/pre-mRNA hybrid formation.

Characterisation of trans-sialidase genes from *Trypanosoma congolense*

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Like other African trypanosomes, *T. congolense* express trans-sialidases (TS) in the procyclic form in the insect vector (1), where the sialylation appears to improve the survival rate dramatically (2). Based on a partial sequence (3) and an open reading frame (TS1) in the WTSI data base, we amplified DNA sequences encoding for seven proteins with overall 83% amino acid identity. Several of the resulting amino acid changes were found in the predicted active site of the enzyme. Further database searches confirmed the presence of these TS1 gene copies in the *T. congolense* genome and provided evidence for at least 5 additional TS-like genes having only 50-60% amino acid identity with TS1. All these TS-like sequences were clearly more closely related to each other than to the *T. brucei* TS genes (4). Four of the putative TS were expressed as recombinant proteins, isolated and assayed for trans-sialylation activity. Three of the purified proteins desialylated fetuin only in the presence of lactose and produced 2,3-sialyllactose, clearly demonstrating their TS activity. In summary, our observations demonstrated that the heterogeneity of TS in *T. congolense* is somewhat more complex than in *T. brucei*. Of particular interest are the 7 almost identical copies of one gene with different enzymatic activities raising the question of what their functions might be. 1) Engstler *et al.* (1993), *Mol. Biochem. Parasitol.* 61: 1-14 2) Nagamune *et al.* (2004), *J. Exp. Med.* 199:1445-1450 3) Tiralongo *et al.* (2003), *Biol. Chem.* 384: 1203-1213 4) Montagna *et al.* (2002), *Eur. J. Biochem.* 269: 2941-2950

Roles for T. brucei Topo3α-Rmi1 pathway in antigenic variation

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Homologous recombination (HR) mediates one of the major mechanisms of antigenic variation in T. brucei. It has been shown that essential components for HR, including RAD51 and BRCA2, play critical roles. The molecular mechanism of antigenic variation however, remains a mystery. Duplicative-gene conversion occurs predominantly, while crossover is tightly suppressed during recombination-mediated switching. Suppression of crossover is intriguing because, in principle, the outcome of VSG gene conversion (VSG GC) holds no apparent advantage over crossover, as re-expressing old VSG, either exchanged or duplicated, will be lethal. A RecQ family helicase, Sgs1 is one of the major factors that regulate spontaneous crossovers in yeast. The Sgs1 pathway includes Top3 (a type IA topoisomerase) and Rmi1 (RecQ-mediated genome instability), and suppresses crossover by 'dissolving' double Holliday junction (dHJ) intermediates. To understand how trypanosomes employ recombination factors to control antigenic variation, we took advantage of two potential members of T. brucei Sgs1 pathway, TbTOPO3α and TbRMI1. With a new switching-reporter strain in which we could measure switching frequency and score different switching mechanisms easily, we discovered that TOPO3α deficiency increased switching frequency by 5-20-fold and that Topo3α is required to specifically control VSG GC and crossover, but not ES GC. We found that 70-bp repeat units are important for VSG GC. Finally, Topo3α physically interacts with Rmi1, and RMI1 deficiency mimicked topo3α mutation. Topo3α-Rmi1 may be required to remove recombination intermediates arising between the active ES and potential VSG donors, thereby acting to control antigenic switching. Our results suggest critical regulatory roles for the T. brucei Sgs1 pathway, and provide a cornerstone to further understand the molecular mechanism of recombination-mediated switching.

Structural Studies of the Interaction Between the Translation Initiation Factors 4e And 4g from Leishmania Major

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Eukaryotic translational initiation requires that the 4E factor (eIF4E) binds to the cap structure located at the 5 end of messenger RNAs (mRNA). The 4G factor (eIF4G) serves as a scaffold protein that mediates recruitment of the small ribosomal subunit at the cap structure. Four eIF4E homologues were found in the parasite Leishmania major among which two (LIF4E-1, LIF4E-4) were identified as reasonable candidates to adopt the function of eIF4E, while a putative homologue of eIF4G (LIF4G 3) has also been found in L. major. Our aim is to develop specific leishmaniasis inhibitors by perturbing the interaction between LIF4E-1 and LIF4G-3. We use NMR spectroscopy to pursue structure characterization of LIF4E-1 and validated by titration experiments the binding of LIF4G-3 minimal interaction domain to LIF4E. Finally, the LIF4G-3 minimal interaction domain will be used in fluorescence-polarization assays to screen for inhibitors that prevent the interaction of LIF4E-1 and LIF4G-3 from combinatorial-libraries available at the Institute for Chemistry and Chemical Biology.

RAP1 is Essential for Silencing Telomeric *Variant Surface Glycoprotein* Genes in Procyclic *Trypanosoma brucei*

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Trypanosoma brucei has a large family of genes encoding the Variant Surface Glycoprotein (VSG), a surface protein present on bloodstream form cells. However, *VSG* is exclusively expressed from subtelomeric *VSG* expression sites (ES) in a strictly monoallelic fashion at the bloodstream form stage and is completely silent at the procyclic form stage when procyclins are produced as surface molecules. Therefore regulation of *VSG* expression is important for *T. brucei* pathogenesis and differentiation. Telomeres are the nucleoprotein complex at the end of linear chromosomes. In *T. brucei*, telomeres form a specialized chromatin structure that can suppress nearby RNA Pol I transcribed genes and have been proposed to play an important role in *VSG* silencing. Indeed, we have recently identified tbRAP1 as an intrinsic component of the *T. brucei* telomere complex and have shown that tbRAP1 plays a critical role in ES silencing in bloodstream form cells, demonstrating for the first time that the telomere structure is important for *VSG* silencing. To explore tbRAP1's function at the procyclic stage, we depleted tbRAP1 protein by RNAi and found that this again led to derepression of subtelomeric ES *VSG* genes, indicating that the telomere structure is also essential for *VSG* silencing at the procyclic stage.

Social Motility and Cell-Cell Communication in African trypanosomes

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Trypanosoma brucei is the causative agent of African sleeping sickness. T. brucei exhibits a biphasic lifecycle in which motility is thought to play an important role. Within its insect vector, the parasite must complete a series of migrations required for its maturation into a mamailian-infectious form. Within the mammalian host, mortality is associated with migration of the parasite into the central nervous system. The means by which these parasites navigate through their host environments are not yet known. Through studying trypanosome motility on semisolid agarose plates, we have discovered that procyclic T. brucei cells exhibit highly coordinated social behaviors. This behavior, termed social motility, is characterized by the active assembly of motile parasites into large groups, which are able to seek out satellite colonies and initiate a polarized movement in the direction of that colony resulting in the merger of the two colonies. In addition, cells engaged in social motility are able to move en masse from the source of inoculation, resulting in the formation of multicellular rafts stretched out across the agarose plate. We hypothesize that this ability of trypanosomes to coordinate movement in response to external cues might facilitate tropism of the population to specific host tissues. We are currently employing various tools to identify the signals and genes involved in social motility. The ability to direct migration in response to external stimuli is a fundamental property of living cells. Identifying the mechanisms governing social motility in trypanosomes should shed light not just on how trypanosomes migrate within their hosts, but also on how other microbial pathogens and non-pathogenic eukaryotic cells communicate and engage in complex social behaviors.

Identification of a C-terminal Flagellar Targeting Sequence in *Trypanosoma brucei* Hexokinase

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Trypanosoma brucei expresses two hexokinases (TbHK1 and TbHK2) that are 98% identical in both bloodstream form and procyclic form parasites. While both proteins harbor a PTS2 N-terminal signal sequence that directs them to glycosomes, immunofluorescence studies using anti-TbHK2 antibody revealed additional flagellar localization. This localization is not without precedent, as glycolytic enzymes, including HKs, have been found associated with flagella in other organisms, including *Chlamydomonas* and mice. Kinetoplastid metabolic enzymes have also been found proximal to the flagellum. These include adenylate kinase isoforms and a *Leishmania* HK that localizes to an extra-glycosomal compartment in the flagellar pocket. Comparison of the TbHK protein sequences with known flagellar proteins revealed that the TbHKs harbor a sequence similar to a described flagellar targeting sequence that could direct the protein to the flagellum. This sequence is modeled to be exposed on a surface face of the proteins and available for protein-protein interaction. Analysis of live cells expressing GFP with an N-terminal PTS2 targeting sequence and a C-terminal portion of TbHK2 harboring the putative targeting sequence directs fluorescence partially to the flagellum. Mutation of the putative targeting sequence disrupts flagellar targeting, supporting that the sequence is important for localization.

Trafficking and Secretion of the *Leishmania* HASPB Protein

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The *Leishmania*-specific hydrophilic acylated surface (HASP) proteins are exclusively expressed in infective stages of Old World *Leishmania* species and show both inter- and intra-specific variation in their repetitive domains. Sera from leishmaniasis patients recognise recombinant HASPB with high specificity, while this protein also induces long-term protection at low doses, in the absence of adjuvant, in the mouse model of visceral disease. This promising vaccine candidate is currently entering phase I clinical trials. The HASPs are dually acylated by the N-terminal addition of myristate and palmitate, co- and post-translational modifications essential for trafficking to the plasma membrane (Denny et al. 2000). Using N-terminal HASPB-GFP reporter constructs, we have confirmed that HASPB transport requires *N*-myristoylation to reach the Golgi and subsequent palmitoylation to target the plasma membrane. To further investigate HASPB presentation at the cell membrane and to quantify its exposure on the external surface of infective *Leishmania*, we have developed live cell FACS and microscopy methods, utilising confocal, transmission and scanning electron microscopy. These approaches have confirmed that HASPB can be translocated across the plasma membrane and shed from the parasite surface. FACS analysis of live cells during *in vitro* differentiation has allowed quantitation of HASPB exposure on the extracellular surface, a process correlating with entry into stationary growth phase. Further analysis of this unusual pathway for protein trafficking may help further elucidate the functions of these unusual *Leishmania* proteins during intracellular infection.

Subcellular Localization and Complex Association Differentiate *T. brucei* REAP-1 from 45 kDa Protein

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Within the mitochondrion of kinetoplastids, genes are modified by RNA editing. Alteration of pre-mRNA is carried out through the coordinated activities of a number of proteins constituting the core editing complex(es), plus additional proteins that are not stably associated with the core. We previously identified REAP-1, an RNA binding protein with the ability to recognize unedited RNAs. We have compared the sequences of the *REAP-1* gene cloned from *Trypanosoma brucei* 667 cells with the sequences from the 927 cell line and the human pathogen *T. b. gambiense*. Surprisingly, the open reading frame could be extended at the 5' end to encode a 65-kDa protein. Reexamination of the sequence confirmed the larger open reading frame in both *T. brucei* cell lines and in *T. b. gambiense*. We determined that monoclonal antibodies, which identified the *REAP-1* cDNA from an expression library, also react with a second, distinct 45-kDa protein. The 45-kDa protein is the primary protein recognized in cell lysates. We show that the 45-kDa protein sediments with a 35-40S complex while REAP-1 remains near the top of glycerol gradients, indicating that it is not found in a protein complex. Immunofluorescent microscopy for the 45-kDa protein and REAP-1/GFP fusion protein shows distinctive mitochondrial staining patterns for both proteins. However, cell fractionation, digitonin solubilization and proteinase K sensitivity indicate only the 45-kDa protein is protected by the mitochondrial membrane. Immunoaffinity purification and mass spectrometry to identify the 45-kDa protein are underway.

A general platform for homology-independent analysis of parasite genome sequences

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A common approach to infer the function of uncharacterized genes is to compare them with their known homologues. However, in the case of species such as *Trypanosoma brucei*, where a large portion of its genome is specific to its class, homology-based predictions are of little use. Our lab aims to develop a set of homology-independent computational genome analysis tools that will be available in several user-friendly formats including a web-interface and stand-alone software packages for the most common operating systems. Currently two homology-independent computational platforms are under implementation. The first one predicts the function of a protein using the codon usage of its coding sequence, based on the observation that proteins of the same function have similar codon usages. The second package is based on an algorithm for function prediction based on a set of function-specific 3' and 5' UTR regulatory motifs. Each of the modules of the web-interface can be used on its own to create valuable data. For example, our computational methods provide information about functional and/or physical association with other proteins, likely regulatory mechanisms, and likely stage of expression of genes. It is our goal to design a reliable, efficient and user-friendly pipeline that complements the recently launched TriTrypDB. This pipeline will give the researchers the option to modify the communications among the modules if desired, and to feed the pipeline with their own data instead of automatically-generated data. We plan to use this pipeline to compile a catalogue of computationally predicted functional data for all available parasite genomes, providing researchers with one of the most comprehensive databases specialized in parasite genomics.

In vivo/in vitro characterization of the two thymidine hydroxylases involved in the synthesis of the modified DNA base J in the genome of *Trypanosoma brucei*

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Beta-D-glucosyl hydroxymethyluracil, also known as base J, is a modified thymidine residue found in the genome of African trypanosomes and implicated in the regulation of antigenic variation. The base is synthesized in a two-step pathway. Initially, a thymidine residue in DNA is hydroxylated by a thymidine-hydroxylase enzyme (TH). This intermediate (HOMedU) is then glucosylated to form base J. There are two proteins proposed to be involved in the first step of the pathway. JBP2 stimulates de-novo site-specific J-synthesis, whereas JBP1 binds base J in DNA acting as a J-maintenance factor. Previous analysis has indicated that both JBP1 and JBP2 contain a putative thymidine hydroxylase domain related to the family of Fe2+/2-oxoglutarate-dependent hydroxylases. Although mutations in the TH domain of JBP1 kill its ability to function, comparative analysis in JBP2 has not been carried out. Here we show that mutation of key residues in the TH domain of JBP2 ablate its ability to induce de-novo J-synthesis. While the individual JBP1 and JBP2 KO have reduced J levels, the deletion of both from the trypanosome genome generates a cell line that completely lacks base J. Reintroduction of JBP2 in the J-null stimulates the formation of HOMedU *in vivo*. In order to explore TH activity in vitro, we have expressed JBP1 and JBP2 in E. coli. Anti-HOMedU IP analysis indicates that IPTG induced expression of JBP1 or JBP2 leads to HOMedU synthesis in the E. coli genome. JBP catalyzed HOMedU synthesis in E. coli is stimulated by Fe2+in a concentration-dependent manner. Thus, JBP1 and JBP2 are the two TH enzymes related to the Fe2+/2-oxoglutarate-dependent hydroxylase family involved in J-synthesis.

Trypanocidal Action of AN3520: A Novel Oxaborole Compound Showing Efficacy in Mouse Models for African Trypanosomiasis

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Human African Trypanosomiasis (HAT) is a fatal disease caused by *Trypanosoma brucei ssp.* There is a need for new treatment for HAT because current treatments are costly, difficult to administer and frequently toxic. A collaborative drug discovery effort by SCYNEXIS, Anacor, DND*i* and Pace University has identified boron-containing compounds as promising leads for development of new, safe and effective treatments for HAT. AN3520 is a lead oxaborole compound that demonstrates potent activity against *T. brucei brucei* (IC_{50} ~100 nM) *in vitro* and exhibits efficacy against both the acute and chronic CNS stages of HAT in mouse models. The precise mechanism of action for AN3520 is currently unknown. Exposure of *T. b. brucei* to AN3520 leads to significant changes in shape, reduction in cellular size and detached flagella at the time of death. Assessment of AN3520 time-dose-response kinetics on *T. b. brucei in vitro* shows parasite killing beginning within 9 hours of exposure, as indicated by the parasites reduced ability to generate ATP. Parasite commitment to death occurs with similar kinetics even when AN3520 is washed out after 3 hours of exposure. Fluorescently tagged analogues of AN3520 have been synthesized and used to study the sub-cellular distribution of oxaboroles within the parasite. In addition, an analogue of AN3520 has been immobilized on an agarose matrix for use in affinity capture of parasite target proteins which will be identified by mass spectrometry and data base searches. All of these studies could lead to a better understanding of the mechanism through which oxaboroles exert their trypanocidal effects and also provide valuable tools for use in our lead optimization program.

A Common mechanism of cell death induced by anti-*Leishmania* drugs

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Anti-*Leishmania* treatment relies on chemotherapy with antimonial derivatives as the main therapeutics, and amphotericin B or miltefosine becoming more frequently used. Antimony has no known specific cellular target identified so far. It is associated with oxidative stress generation, mitochondrial failure and apoptotic cell death. Amphotericin B targets ergosterols containing membranes. It is associated with mitochondrial failure and apoptotic cell death. Miltefosine is a phosphocholine analog that interacts with membrane lipids. Its action also implies mitochondrial failure leading to cell death. In brief, the mode of action of these anti-*Leishmania* drugs remains elusive and poorly understood. In this study we used *Leishmania infantum* wild-type cells and three corresponding mutants, Sb2000.1 (resistant to trivalent antimony SbIII) Mf200.5 (resistant to miltefosine) and Aö1000.1 (resistant to amphotericin B) in order to clarify the common mechanism of cell death induced by these drugs. Our results pinpointed commonalities in the mode of action of these drugs. Indeed, wild-type cells in contact with any of the three drugs accumulated reactive oxygen species (ROS) within their mitochondria. This was not observed in the drug resistant mutants. Furthermore, pre-incubating the cells with an iron chelator or anti-oxidants prevented the accumulation of ROS within the mitochondria. The three drug resistant mutants contained less NAD(P)H reducing equivalents compare to the susceptible cells. This was correlated with changes in Krebs cycle's enzymatic activities. We also confirmed the apoptotic cell death induced by these three drugs by controlling the mitochondrial depolarization and the DNA laddering. These results can be incorporated into a unifying model where drugs with different cellular targets do nonetheless share similarities in their mechanism of cell death and of resistance.

Fluorescence-based reporter substrate for monitoring RNA editing in Trypanosomatid pathogens

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Mitochondrial gene expression in trypanosomatid pathogens requires extensive post transcriptional modification called RNA editing. This unique molecular mechanism, catalyzed by a multiprotein complex (the editosome), generates translatable transcripts for essential components of parasite respiratory complex. How editosome proteins are assembled and perform RNA editing is not fully understood. Also, previous studies have shown that editosome proteins are essential for parasite survival, which makes editosome a potential target for drug discovery. Currently, researchers use radiolabeled based assays to monitor RNA editing process. However, these assays are not suitable for high throughput screening of editosome inhibitors, have low detection limits, and cannot monitor RNA editing in real time. Here we present a homogenous fluorescence-based RNA editing reporter assay that is based on a hammerhead ribozyme activity. Ribozyme structure was remodelled by adding or removing uridylates in its conserved catalytic core to make an inactivate ribozyme. In the presence of the editosome, inactive ribozyme is edited to an active ribozyme. Consequently, hammerhead ribozyme activity can be measured by cleaving its fluorescently labelled substrate. We show that higher sensitivity is achieved using fluorescent based assay than conventional radio-labelled assay. Moreover, we can use this assay for rapid screening and identification of chemical compounds for their ability to selectively inhibit editosome assembly and/or function in trypanosomatids.

Gene disruptions indicate an essential function for gamma glutamyl cysteine synthetase in *Leishmania infantum*

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Gamma glutamyl cysteine synthetase which catalyzes the ATP dependent ligation of L-Cysteine and L-Glutamate to produce gamma glutamyl cysteine has been demonstrated to be the rate limiting enzyme in the biosynthesis of glutathione in mammalian cells and of trypanothione in *Leishmania*. We tried to decipher the importance of *GSH1* gene encoding for this enzyme in *Leishmania* by attempting to generate *GSH1* null mutants by gene disruption in *L. infantum*. Removal of even a single wild type allelic copy of *GSH1*, invariably led to the generation of an extra copy of *GSH1*, maintaining two intact WT alleles. However, by first supplementing the WT parasites with a rescue plasmid and then with two rounds of inactivation at the chromosomal locus, we succeeded in obtaining a null mutant at the chromosomal locus, thus indicating the essentiality of this gene in Leishmania. Parasites with one intact *GSH1* allele at the chromosomal locus lost the plasmid but not the double knock out, when grown in the absence of antibiotic, again indicating its essentiality. Heterozygous mutants with one allele inactivated transcribed less *GSH1* mRNA and synthesized less glutathione and trypanothione than WT. Mutants grew like WT in culture but were more susceptible to handle oxidative stresses *in vitro* as promastigotes. Significantly, these mutants showed attenuated infectivity and a decreased survival inside macrophages which had the capacity to produce reactive oxygen species. These mutants as intracellular parasites showed a significant decreased survival in the presence of antimony (SbV) compared to control cells. All phenotypes were reverted in the add back mutant, thus proving the importance of this enzyme in dealing with oxidants including the action of antimonials.

Genes of the actin cytoskeleton of Trypanosoma brucei

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Actin is a ubiquitous, highly conserved cytoskeletal protein which is involved in cell movement, intracellular traffic and cell division. Actin is expressed at similar levels but is organized differently in the bloodstream and procyclic forms. Ablation of actin by RNAi has shown that it is an essential protein in bloodstream but not in procyclic forms. Indirect evidence shows that filamentous actin is present only in bloodstream forms and that inhibition of its formation leads to cell death. Since the levels of actin are the same in both life forms, the differential regulation of actin function may occur at the level of nucleation/depolymerization carried out by actin binding and regulatory proteins. Screening of the genome of *T. brucei.* identified 22 proteins that are likely to be involved in the regulation of the actin cytoskeleton. We have begun a systematic analysis to investigate the expression, localization and function of these proteins. Transcriptional analysis by qRTPCR shows that a number of these proteins are upregulated in the bloodstream form. We have, to date, carried out RNAi knockdown on several of these proteins in bloodstream and procyclic forms. In procyclic cells the knockdown of these proteins had no effect on growth or morphology whereas in bloodstream forms differential effects ranging from no observable phenotype to cell death were observed.

A life cycle stage specific membrane glycoprotein involved in flagellar adhesion in T. brucei

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Flagellar attachment is a striking morphological feature of the African trypanosome Trypanosoma brucei. Here we describe and characterize in detail the first example of a life cycle stage specific membrane protein involved in flagellar attachment. The protein has no similarity with previously described proteins and is expressed only in bloodstream forms. The protein exhibits extensive N-glycosylated and locates to the flagellar attachment zone. Ablation of the mRNA through RNAi leads to detachment of the new but not the old flagellum with a concomitant failure of cytokinesis. Flagellar detachment is obvious within 6 h induction of the dsRNA and the new flagellum is often completely detached as it emerges from its own flagellar pocket. In other cases the loss leads to detachment mid way during the synthesis of the new flagellum leading to a partially attached flagellum at the posterior end of the cell. Within 12 h most cells possess multiple detached flagellae along side a single attached flagellum. These results suggest that the protein is not shared or exchanged between the two attachment zones. In other respects the detached fllagellae appear normal, they beat even faster than normal although directional motion is lost,appear to be approximately the same lenght and they possess an apparently normal axoneme and paraflagellar rod structure. However, ultrastructural and immunofluorescence analysis indicates that the flagellar attachment zone appears to disrupted in these cells. The significance of these results will be discussed.

Environmental sensing and social motility of African trypanosomes

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The impact of cell-cell communication and a multicellular lifestyle on the physiology and pathogenesis of bacterial pathogens is now well-established, but this paradigm has not been applied to parasitic protozoa. We have discovered a novel aspect of trypanosome biology, demonstrating that these parasites communicate with one another and assemble into multicellular communities on agarose surfaces. These colonies grow through active recruitment of nearby cells and migrate en masse, requiring coordinate motility of hundreds to thousands of cells. Trypanosome communities exhibit features similar to that described for bacteria that undergo swarming/social motility, suggesting cells in each community are able to sense nearby communities and adjust their behavior in response. Motility mutants are incapable of social motility, demonstrating that normal motility is required and that this behavior is an active process. Social motility of trypanosomes indicates that these parasites are able to sense each other, to communicate and to coordinate their movement. These behaviors reveal a level of communication and coordination not previously recognized in trypanosomes and are expected to impact interactions with their vertebrate and invertebrate hosts. Social motility further provides a defined in vitro assay for analyzing cell-cell communication and environmental sensing in trypanosomes. Currently we are investigating the cell surface receptors and downstream signalling pathways underlying social motility. Various flagellar signalling pathways crucial for environmental sensing in a variety of cell types like sperm or epithelial cells are present in the *T. brucei* flagellum. This suggests that the flagellum provides a sensing platform and might host key proteins involved in social motility of trypanosomes.

The role of Argonaute-like PIWI protein in *Leishmania*

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Leishmania species such as *L. infantum* and *L. major* lack RNAi machinery. However, their genome codes for an Argonaute-like protein with a PIWI domain that is homologous to the RNase H-like catalytic domain in Piwi proteins present in *Drosophila*, zebrafish, and mice that are required for germline development and associate with 24-30 nucleotide RNAs termed piRNAs. PIWI proteins have also been associated with transposon suppression, and the *Leishmania* Piwi homolog is may be not foreign to the widespread distribution of SIDER retroposon elements in this parasite. To address the role of this protein in *Leishmania*, we have generated a *piwi* -/- mutant by homologous recombination both in *L. infantum* and *L. major*. The *piwi* -/- mutant grows normally in culture media. However, this mutant demonstrated lower infection rates in mouse bone marrow macrophages. BALB/c mice infected with the *piwi* -/- mutant showed an important delay in footpad lesion development. To better understand the cause(s) of the delayed infection in mice, we carried out DNA microarray analysis to compare expression profiling between WT and *piwi* -/- mutant *Leishmania*. The whole genome microarray analysis revealed two striking observations in the *piwi* -/- mutant. First, there was a downregulation of several membrane-bound proteins, including GP63, which plays a role in *Leishmania* pathogenesis. Second, all the histone genes were upregulated. As histones are involved in chromatin modification, we are investigating the possibility that *piwi* plays a role in epigenetic control in *Leishmania*, similarly to what has been reported in higher eukaryotes. Experiments are also underway to assess whether *piwi* gene inactivation affects SIDER retroposon-mediated control or gene expression within highly repetitive genomic loci.

Characterization of two *N*-Myristoylated Trypanosome Orthologues of Proteins Implicated in Human Genetic Disorders

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The enzyme myristoyl-CoA: protein *N*-myristoyltransferase (NMT) catalyses the *N*-myristoylation of target proteins and is a promising drug target in *Trypanosoma brucei* and *Leishmania major* . In an ongoing study to define the essential downstream targets of NMT, we have identified over 60 putative *N*-myristoylated proteins encoded in the *T. brucei* genome, including ARF1 and ARL1 which are both essential for parasite viability. Current targets for functional analysis are two *N*-myristoylated trypanosome orthologues of proteins which have been implicated in human genetic disorders with possible links to ciliagenesis. The small GTPase ARL6 is implicated in the multisystemic human disorder Bardet-Biedl syndrome but its functions are largely unknown. ARL6 orthologues are only found in organisms with flagella or cilia. However, the *T. brucei* orthologue of this protein does not predominantly localise to the flagellum but to a punctate pattern throughout the cell. The nucleotide binding properties and binding partners of TbARL6 have been investigated in detail using fluorescence spectroscopy and Tandem affinity Purification (TAP). TbDIP13 is the *T. brucei* orthologue of a *Chlamydomonas* basal body protein implicated in flagellum biosynthesis. The human orthologue of this protein is NA14, identified as an autoantigen in serum taken from a patient with Sjögren's syndrome. Despite the clinical relevance of the NA14 protein, little is known about its precise function and orthologues are not found in the model organisms *S. cerevisiae*, *C. elegans* and *Drosophila*. Modulation of TbDIP13 expression in bloodstream form *T. brucei* causes extensive disruption of normal cell morphology; the functional consequences of these changes are under study, using imaging and proteomics-based approaches.

Hesperadin inhibits Aurora kinase-1 and blocks mitotic progression in bloodstream forms of *Trypanosoma brucei*

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Aurora kinase family members coordinate a range of events associated with mitosis and cytokinesis. Anti-cancer therapies are currently being developed against them. Here, we evaluate whether Aurora kinase-1 (TbAUK1) from pathogenic *Trypanosoma brucei* might be targeted in anti-parasitic therapies as well. Conditional knockdown of TbAUK1 within infected mice demonstrated its essential contribution to infection. An in vitro kinase assay was developed which used recombinant trypanosome histone H3 (rTbH3) as a substrate. Tandem MS identified a novel phosphorylation site in the carboxyl-tail of rTbH3. Hesperadin, an inhibitor of human Aurora B, prevented the phosphorylation of substrate with IC50 of 40 nM. Growth of cultured bloodstream forms (BF) was also sensitive to Hesperadin (IC50 of 50 nM). Hesperadin blocked nuclear division and cytokinesis, but not other aspects of the cell cycle. Consequently, growth arrested cells accumulated multiple kinetoplasts, flagella and nucleoli; similar to the effects of RNAi-dependent knockdown of TbAUK1 in cultured BF cells. Molecular models predicted high affinity binding of Hesperadin to both conserved and novel sites in TbAUK1. Collectively, these data demonstrate that cell cycle progression is essential for infections with *T. brucei*, and that parasite Aurora kinases can be targeted with small-molecule inhibitors.

RNA editing as a drug target in trypanosomes: complementary approaches for the identification of inhibitors of the essential enzyme REL1

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Posttranscriptional RNA editing by uridine insertion and removal is essential for mitochondrial gene expression in trypanosomatids but absent from the host and therefore a potentially powerful novel drug target. The editing process is catalyzed by a multiprotein complex, the editosome, and involves a series of enzymatic steps. A key catalyst is RNA editing ligase 1 (REL1), an essential enzyme in bloodstream and insect stage *Trypanosoma brucei*. To identify REL1 inhibitors that might serve as leads for the development of new anti-trypanosomal drugs we are pursuing two complementary approaches. A biochemical high-throughput screen (HTS) is under development that will enable us to rapidly screen large compound libraries using recombinant enzyme. Preliminary experiments with an assay based on fluorescence-resonance energy transfer (FRET) are very encouraging and we are currently validating whether this assay satisfies criteria for a HTS campaign. A parallel virtual screening approach incorporates molecular dynamics simulations of the REL1 structure. An initial screen of the National Cancer Institute (NCI) diversity set was followed by a hierarchical similarity search with the most active compound over the full NCI database. Top compounds were tested for their effects on REL1 activity in biochemical assays. Three drug-like compounds were confirmed to inhibit REL1 with IC_{50} values around 1 micromolar. Tests of these compounds against the most closely related bacteriophage T4 RNA ligase 2, as well as against human DNA ligase IIIbeta indicated a considerable degree of selectivity for RNA ligases. These studies as well as preliminary tests of anti-parasite activity will be presented.

Functional analysis of trypanosomatid-specific RNA editing ligases in the presence and absence of their interacting protein partners

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Trypanosomatid mitochondrial mRNAs are remodeled through RNA editing to make usable messenger RNAs for components of an essential energy generating system. This process is catalyzed by a multiprotein complex (editosome) through the insertion and deletion of uridylate (U) residues in pre-mRNA. This study is an in-vitro comparison of two closely related editosome Kinetoplastid RNA Editing Ligase 1 (KREL1) and 2 (KREL2) in the presence and absence of their interacting partners KREPA2 and KREPA1, respectively. The role of KREPA1/A2 proteins is important, as they seem to provide the Oligonucleotide/Oligosaccharide Binding (OB) fold in trans for KREL1 and KREL 2 proteins and hence play an important role in substrate recognition. Full length recombinant proteins are used to analyze structural interaction of KREL1 with KREPA2 and KREL2 with KREPA1, using co-immunoprecipitation assay. For functional interactions, an adenylation assay is performed to inspect the adenylation rate of KREL1 and KREL2 in the presence and absence of their interacting partners. Preliminary results show a two fold increase in KREL1 pull down in the presence of KREPA2 while its adenylation is increased six fold in the presence of KREPA2 compared to KREL1 alone. On the other hand, KREL2 adenylation is increased by two folds in the presence of its interacting partner KREPA2. These results suggest a significant increase in adenylation activity at least in the case of KREL1 in the presence of its interacting partner and provide clear evidence for the role of OB fold in the adenylation activity. Furthermore, it highlights the unique structural and functional features of the related RNA editing ligases in trypanosomatids.

Streamlined architecture and GPI-dependent trafficking in the early secretory pathway of African trypanosomes

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Trypanosoma brucei devotes ~10% of its protein synthesis to GPI-anchored VSG, which is rapidly transported (t1/2 ~15 min) to the cell surface. The GPI anchor is critical for efficient export of VSG from the ER, giving rate enhancements of 3-5 fold relative to non-anchored VSG reporters. COPII vesicles mediate export of newly synthesized proteins from the ER, and contain three core components: Sar1, Sec23/Sec24, and Sec13/Sec31. The Sec23/Sec24 heterodimer binds and selects nascent cargo into budding vesicles. *T. brucei* has two orthologues of the Sec23/Sec24 subunits (TbSec23.1/TbSec23.2; and TbSec24.1/TbSec24.2). RNAi against each subunit is lethal, but little effect is seen on the rates of transport of the soluble and membrane lysosomal markers, trypanopain and p67. Strikingly, VSG trafficking is severely delayed (3-5 fold) following knockdown of either TbSec23.2 or TbSec24.1. Pull-down analyses revealed that these two components form an obligate physical pair, as do the TbSec23.1/TbSec24.2 subunits. IFA indicates that all four subunits colocalize to one or two ER Exit Sites (ERES) that align with the FAZ:associated ER, closely juxtaposed to corresponding Golgi clusters. Interestingly in bloodstream trypanosomes, TbGRASP defines an intermediate zone between the ERES and Golgi, in contrast to procyclic parasites where TbGRASP localizes precisely with the Golgi [He et. al. (2004) JCB 165:313], suggesting a primary role in tethering these organelles together. Studies are underway to investigate the effect of TbGRASP silencing on Golgi architecture and protein trafficking in bloodstream trypanosomes. Our results suggest a cargo selective mechanism for VSG loading into COPII vesicles, and a remarkable degree of streamlining in the early secretory pathway, all presumably designed to maximize efficiency of VSG transport.

kDNA replication initiation is regulated through a redox signaling pathway

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Silencing of the universal minicircles sequence binding protein (UMSBP) by RNAi has previously revealed its function in kDNA minicircles replication initiation and in the segregation of the replicated kDNA network. Regulation of UMSBP interactions with the minicircle replication origin is mediated via the cell cycle control of the protein redox state. Enzymes reconstitution analyses revealed that an enzymatic mechanism, consisting of the trypanosomal major redox-regulating pathway, can catalyze the direct reversible reduction and oxidation of UMSBP and thereby may function as a redox-based molecular switch in the regulation of minicircles replication initiation. Protein affinity chromatography and yeast two hybrid analyses, revealing protein-protein interactions between UMSBP and both tryparedoxin and tryparedoxin peroxidase are in accord with this notion. A previous model, proposing that binding of UMSBP to the origin sequence is regulated via the redox-mediated interconversions of active (reduced) UMSBP monomers and inactive (oxidized) UMSBP oligomers, has been challenged both *in vivo* and *in vitro*, revealing that the two redox-driven processes are independent and that regulation of UMSBP binding to the replication origin is rather mediated through the reversible generation of intra-molecular disulfide bonds. Kinetics analyses of UMSBP interactions with the origin sequence demonstrated that redox affects the pre-initiation loading of free UMSBP onto the origin sequence, but not its release from the nucleoprotein complex, suggesting that redox signaling may control the generation of the origin-associated initiation complex. This is corroborated by protein affinity chromatography, co-immunoprecipitation and yeast two hybrid analyses, which revealed high affinity interactions between UMSBP and the kDNA replication proteins SSE1 and pol beta, as well as with the kDNA associated proteins KAP3 and KAP4.

A bacterial type mitochondrial porin in Trypanosoma brucei is essential for its energy production and cell survival

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Mitochondrial porin in Trypanosoma brucei, a unicellular parasitic protozoa and the causative agent of African trypanosomiasis possesses a â-barrel structure with 8 predicted â-strands and the C-terminal á-helix similar to bacterial outer membrane porin OmpA. T. brucei Porin (TbPorin) expression is developmentally regulated. TbPorin is present as a monomer as well as oligomer on mitochondrial outer membrane (OM). In spite of its distinct structure, TbPorin function is similar to other eukaryotic porins. It is essential for cell growth. TbPorin RNAi decreased the steady-state level of mitochondrial ATP, as well as ATP production upon addition of ADP and substrates to isolated mitochondria. Furthermore, a decrease in the level of mitochondrial porin reduced cellular respiration via trypanosome alternative oxidase (TAO), which is not coupled with oxidative phosphorylation, but increased the capacity of the cytochrome-dependent pathway (CP). Due to TbPorin RNAi, the expression level of TAO decreased, while the level of the cytochrome oxidase subunit 4 (COIV) and cytochrome c1 increased. A blue-native gel electrophoresis showed an increased level of complex III and complex IV due to porin RNAi. Together, these data revealed that TbPorin knock-down reduced the mitochondrial ATP level, which in turn increased the CP capacity in an attempt to compensate mitochondrial energy crisis. However, a simultaneous decrease in the substrate level phosphorylation due to TbPorin RNAi caused growth inhibition in T. brucei. We also found that the expression of TAO and CP proteins are coordinately regulated in T. brucei according to mitochondrial energy demand

Identification of Amastigote-Specific Protein Kinases in *Leishmania Donovani* by Activity-Based Proteome Analysis

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The FP7-LEISHDRUG consortium (www.leishdrug.org) uses a highly interdisciplinary approach to reveal *Leishmania* signaling molecules associated with amastigote virulence. Our major aim is to exploit the *Leishmania* kinome for anti-parasitic drug development using target-based strategies. We screened the *Leishmania* proteome for appropriate target kinases, showing amastigote phospho-transferase activity, using in-gel kinase assay (IGKA) in combination with 2D electrophoresis. We observed multiple radioactive signals in a 1D-IGKA, which were sensitive to inhibition by Staurosporine and Genistein, indicating the presence of abundant kinase activities in *Leishmania*. Compared to promastigotes, amastigote extracts showed substantially increased signal intensities of 120kD, 50kD and 30kD bands, and novel robust phosphorylation activities were detected in the 50-70kD as well as in the low molecular range. These data are consistent with our previous findings showing increased phosphoprotein abundance in amastigotes. In order to increase resolution and allow for the identification of protein kinases we adapted the in-gel kinase assay on 2D gels. We used either total amastigote extracts or phosphoproteins enriched by immobilized metal affinity chromatography (IMAC). In the former case parasites were lysed directly in a low salt IEF buffer, as TCA precipitation interfered with kinase renaturation. Protein extracts were subjected to isoelectric focusing on pH 4-7 IEF strips prior to separation by SDS-PAGE and subsequent in-gel kinase assay. Our current efforts are focused on differentiating between auto- and substrate kinase activities employing amastigote-specific phosphoproteins and phosphopeptides previously identified by phosphoproteomics. The use of 2D-IGKA may allow us to identify atypical parasite protein kinases which escaped previous bio-informatic kinase screens and are more suitable for anti-leishmanial drug development.

Role of myosin in bloodstream form of *T. brucei*

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A functional actin cytoskeleton involves the molecular motor myosin. Genome analysis reveals the presence of only two genes that potentially code for myosin motors in *T. brucei*. This number is considerably less than found in mammalian cells, where ~15 classes are present and myosins represent a large superfamily of proteins. The precise function of many of these motor proteins remains unknown. The two myosins in *T. brucei* can be differentiated by their tail regions. The encoding genes are Tb927.4.3380, which is a class I myosin, and Tb11.01.7990, which might be a class VII myosin. Class I myosins have been implicated in vesicle transport, therefore we focused on the role of this protein in *T. brucei*. Analysis of the putative protein revealed a motor domain (Pfam score: 7.4e-293) and a tail domain (Pfam score: 2.4e-07). The tail region also contains a weak hit for a zinc binding domain possibly involved in endosomal targeting. Expression analysis using antibodies and qRT-PCR indicated upregulation of this myosin in the bloodstream form versus the procyclic form. The role of was investigated using RNAi. Loss of the protein produced severe effects on growth and morphology in bloodstream but not in procyclic forms. The results of functional and localization studies will be presented and discussed.

Interacting partners of *Leishmania major* metacaspase

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Unicellular organisms such as *Leishmania* undergo cell death (CD) with characteristic features of programmed cell death. CD has been well characterized in mammalian cells where caspases play an essential role. In *L. major*, the genome code for a single metacaspase (LmjMCA) which represents a distant orthologue of caspases. LmjMCA has little sequence identity with caspases but shares the conserved catalytic His/Cys-dyad and similar secondary structure. Contrary to aspartate substrate specificity of caspases, LmjMCA possesses a arginine/lysine substrate specificity. Structually, LmjMCA is composed of an N-terminal mitochondrial localization sequence, a caspase-like catalytic domain with the dyad His147/Cys202, and a C-terminal proline-rich domain. In this study we were interested in analyzing the functional role of LmjMCA by investigating potential interaction partners and substrates using a yeast-two-hybrid interaction assay. Firstly we observed that LmjMCA could interact with itself like caspases do. This interaction is mediated mainly by the C-terminal sequence. Interestingly this self-interaction induced flocculation of yeast cells. Using cDNA libraries from different life cycle stages of the parasite, we observed also that two proteins interacted with the catalytic domain suggesting that they could be substrates of LmjMCA. Finally several other proteins interacted with the full length form but not with the catalytic domain indicating that the C-terminal sequence might be responsible for those interactions. Thus far, we confirmed the interaction of two of those proteins by co-immunoprecipitation. These proteins are known to be involved in cell death pathways in other organisms.

An Unusual Protein Trafficking Pathway in African Trypanosomes Leads to Infectivity in Humans

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Trypanosoma brucei brucei is unable to infect humans due to its susceptibility to a minor subclass of high-density lipoproteins (HDL) found in human serum, called trypanosome lytic factor (TLF). Unlike *T. b. brucei, T. b. rhodesiense* causes acute human trypanosomiasis and evades TLF-mediated killing through the expression of the serum resistance associated (SRA) gene. SRA is closely related to the variant surface glycoproteins (VSG), which are the major coat proteins of *T. brucei*. VSGs contain several key features involved in the trafficking and cell surface localization of the mature protein. These features include an N-terminal signal peptide, and a GPI-anchor addition site, which are also common to the transferrin receptor (TfR) of *T. brucei*, another VSG-like protein. Both VSGs and TfR are resident cell surface proteins that are continually endocytosed into endosomes, trafficked to the lysosome and recycled to the cell surface. The signal peptide and GPI-anchor, in particular, are critical to the targeting and surface localization of these membrane-bound proteins. Like VSGs and TfR, we have confirmed that SRA is a GPI-anchored protein, however, SRA appears to localize only transiently to cell surface. We have shown that SRA is then endocytosed into endosomes, and is ultimately trafficked to the lysosome. We have also shown that TLF colocalizes with SRA in both intracellular compartments. This trafficking pathway of SRA therefore conveniently allows its intersection of the toxin's intracellular route as it is transported to the lysosome. This unusual trafficking and localization of a GPI-anchored protein facilitates its interaction with TLF, which is critical to SRA-mediated inhibition of trypanosome lysis.

Development of the next generation of vectors

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Currently there are a few vector systems available, which allow inducible expression, such as pLEW and and pDEX. Both vectors rely upon the *EP* promoter under the control of the tet operator to drive expression of the gene of interest. There are some differences, pLEW uses a disabled T7 promoter to drive the expression of the antibiotic resistance gene and is targeted to the non-transcribed ribosomal spacer whereas pDEX uses a rRNA promoter for expression of its resistance gene and is targeted to the 177 repeats in the minichromosomes. However there are problems with these vectors, pLEW can only be used in cell lines expressing the T7 RNA polymerase and the levels of expression from both are lower than would be expected from an *EP* promoter. A modular vector system has been designed where the gene of interest and the antibiotic resistance gene can be readily shuttled in and out of the vector. The major alteration over previous vectors is to position the vector backbone between the two promoters that drive the antibiotic resistance gene and the gene of interest. The new vector is used in conjunction with the tetracycline repressor expressed from the procyclin locus. Initially expression levels of an eYFP tagged protein were incredibly high when induced but the vector was not silent when uninduced. Mutations and deletions were made in the *EP* promoter to reduce the level of expression, creating a series of vectors that can give a range of expression levels that are mostly fully inducible.

Investigation of the role of Vitamin C in *Trypanosoma cruzi*

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Vitamin C (ascorbate) functions widely in eukaryotes as an antioxidant and enzyme co-factor. It is an essential dietary component of humans, who lack the last enzyme in the biosynthetic pathway. *T. cruzi* possesses an ER-localised ascorbate-dependent peroxidase (APX), an enzyme absent from *T. brucei*. APX is expressed throughout the life cycle and plays an important role in protecting *T. cruzi* against hydrogen peroxide, but not organic peroxides. Deletion of APX is achievable only in the presence of an ectopic copy. However the episome is readily lost in the absence of drug selection and APX null mutants can be generated. These parasites are capable of traversing the entire life-cycle and infecting Vero cells. They show a reduced resistance to exogenous H_2O_2, but otherwise display no obvious phenotype. In *T. cruzi* , the ascorbate biosynthetic enzyme is a glycosomal FMN-dependent galactonolactone oxidase (GAL). *T. cruzi* appears to lack an ascorbate uptake capacity and the GAL genes are not readily deleted except in the presence of an ectopic copy [pTEX-GAL]. In contrast, GAL is dispensable in bloodstream-form *T. brucei* parasites which are also deficient in ascorbate uptake. We are currently testing the *T. cruzi* GAL -/- [pTEX-GAL] cells to determine if the ectopic copy is retained in the absence of drug selection or whether vitamin C deficient null mutants can be generated. The latest results will be presented.

New prospects regarding the classical tryparedoxin/peroxiredoxin pathway operating within the *Leishmania infantum* mitochondrion

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In *Leishmania* spp., as well as in other trypanosomatids, 2-cysteine peroxiredoxins (Pxns) are chief components of the hydroperoxide (ROOH) reducing machinery. In biochemical assays, Pxns eliminate ROOH at expenses of reducing equivalents provided by tryparedoxins (TXNs), members of the thioredoxin superfamily. In *Leishmania infantum* the occurrence of mitochondrial Pxn and TXN enzymes (*Li*mTXNPx and *Li*TXN2, respectively) suggests that, within this organelle, ROOH reduction proceeds via the classical TXN/Pxn pathway. To assess the essentiality of both enzymes for parasite survival, we have produced *Li*mTXNPx and *Li*TXN2 knockouts by homologous recombination (*Li*mTXNPx-/- and of *Li*TXN2-/-, respectively). As promastigotes, both mutants are morphologically similar to wild type cells. In addition, *Li*mTXNPx-/- mutants display identical susceptibility to exogenous sources of peroxides (H2O2, t-bOOH, ONOO-) as wild type promastigotes. Interestingly, however,*Li*mTXNPx-/- and *Li*TXN2-/- display different phenotypes when tested in *in vivo* infections. Using Balb/c mice as model, we observed that *Li*mTXNPx depletion impairs parasite infectivity in comparison to wild type parasites or to *Li*mTXNPx-/- mutants with restored *Li*mTXNPx expression. As for *Li*TXN2, abrogation of this enzyme does not influence the outcome of parasite infection. Together these observations indicate that (i) *Li*mTXNPx is critical for *L. infantum* invasion and/or persistence within the mammalian host, and (ii) *Li*mTXNPx function in the amastigote stage is independent of *Li*TXN2. Because experimental evidence points towards *Li*TXN2 being the only TXN molecule sharing the same subcellular compartmentalization as *Li*mTXNPx, these data challenge the classical view regarding TXN/Pxn pathways. We are now looking for *Li*mTXNPx partners which, in the context of drug design, might be amenable for specific inhibition. FCT: SFRH/BPD/20610/2004; POCI/SAU-IMI/59560/2004.

Identification of proteins localized to the contractile vacuole of *Trypanosoma cruzi*

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The contractile vacuole (CV) is an osmoregulatory organelle which controls the intracellular water balance by accumulating and expelling excess water from the cell. *Trypanosoma cruzi* is the etiologic agent of Chagas' disease and survives a wide range of osmotic conditions in its life cycle as it moves among the insect gut, the bloodstream, phagolysosomes, and host cell cytosol. We hypothesize that the CV mediates volume regulation in each of these environments, particularly through fusion with acidocalcisomes. Proteomic analysis of CV-enriched fractions from epimastigotes identified a total of 1695 proteins (false discovery rate 1%) of which ~60% are annotated as hypothetical. Transport-related proteins were represented by at least 55 members. Additionally, 39 unique disperse gene family 1 (DGF-1) proteins were also identified. We expressed GFP-fusions of 3 proteins (TcSNARE2-1, TcSNARE2-2, and TcVATPase subunit B) with known CV localizations in Paramecium and Dictyostelium to validate our proteomic dataset. All 3 localized to the contractile vacuole of *T. cruzi* epimastigotes. We are focusing on improving predictions of CV-specific proteins and validation of the cellular locations of additional proteins. We anticipate that comparison of the CV proteome with our existing acidocalcisomal proteomics databases (*T. cruzi* and *Trypanosoma brucei*) will identify organellar interactions that facilitate volume regulation in *T. cruzi*.

The Role of Poly(A)-Specific Ribonuclease in mRNA Turnover in *Trypanosoma brucei*

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In trypanosomes most protein-encoding genes are transcribed as part of long polycistronic pre-mRNAs that are subsequently processed into mature monocistronic mRNAs. Each of the open reading frames of the original pre-mRNA can exhibit different steady state levels, suggesting a reliance on post-transcriptional regulation in these organisms. Therefore, our laboratory is defining the enzymatic processes of mRNA turnover in trypanosomatids to better understand the mechanisms of their regulation of gene expression. In eukaryotes, mRNA degradation usually initiates by shortening of the poly(A) tail by one or more deadenylases. We have identified three potential orthologues of mammalian poly(A)-specific ribonuclease (PARN) in *Trypanosoma brucei*; however, the specific functions of each of these proteins is unknown. Biochemical assays have shown that two of these proteins have deadenylase activity *in vitro*. In order to determine the role of PARN on gene regulation *in vivo*, these proteins have been both overexpressed and depleted by RNAi in both the insect form and bloodstream form stages of the parasite. Microarray analysis was then used to examine the effects on steady stage mRNA levels.

High-throughput screening of a drug library for *Leishmania major* the causative agent of cutaneous leishmaniasis

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The protozoan trypanosome Leishmania major is an obligate intracellular parasite that is the causative agent for cutaneous leishmaniasis. This disease is endemic is many regions such as Asia, Africa, South America, Latin America, and with the constant deployment of U.S. troops to endemic areas it has now become a major concern for the U.S. as well. Therapeutic treatment for this disease is very limited with only antimony-containing compounds and fungicides being used as the primary treatment. However, these drugs can be toxic to patients and there has been a rise in frequent treatment failures as a result of drug resistance. This has led to a higher demand for the availability of newly synthesized therapeutic drugs. High-throughput drug screening provides a starting point for drug design and for understanding the interaction of a compound with its target site. In this study, this method is being used to perform experiments on L. major (Freidlin V1) promastigotes and LLC-MK2 mammalian cells using the Dimmock library. Viability of L. major is rapidly analyzed utilizing the bioluminescent activity of the LUC gene that has been integrated into the parasite's ribosomal SSU RNA locus. Cell proliferation and cytotoxicity of the mammalian cells is analyzed using alamarBlue®. Analysis of the results showed that 3 of the 126 drugs tested provided sufficient parasiticidal effects with the absence of harmful cytotoxicity to mammalian cells, showing potential for use as chemotherapeutic agents against Leishmaniasis. These 3 drugs are currently being researched in detail and will soon be tested in vivo.

Structure of the C-terminal domain of Transcription Factor IIB from *Trypanosoma brucei*

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In metazoans, Transcription Factor IIB (TFIIB) plays a key role in RNA polymerase II (RNAP-II) transcription initiation. In particular, TFIIB binds specifically to TATA-box binding protein (TBP) and the DNA promoter through its C-terminal domain, and, using its N-terminal domain, recruits RNAP-II and positions it directly at the transcription start site. The highly divergent trypanosome TFIIB (tTFIIB) has been shown to be essential for SL RNA gene transcription and cell viability, interacting with tTBP, tRNAP-II, and the SL RNA gene promoter region. Here we describe the 2.3 Å ngstrom resolution structure of the C-terminal domain of tTFIIB from *Trypanosoma brucei* (tTFIIBc). The structure consists of two helical modules followed by a 32 amino acid extended region that is disordered. The helical modules are similar in overall structure to metazoan TFIIBc, but motifs involved in tTBP binding and site-specific DNA recognition are divergent. Moreover, the 32 amino acid extension is absent from metazoan TFIIB, suggesting that tTFIIB may make additional protein-protein or protein-DNA interactions through the extension. Docking of tTFIIBc in place of TFIIBc in the human TFIIBc/TBP/DNA complex structure reveals three regions in tTFIIBc that may be involved in DNA or tTBP binding. Alanine substitutions of amino acids in each of these regions reduce or abolish the activity of full-length tTFIIB by *in vitro* transcription. Our tTFIIBc structure provides a foundation from which to study assembly of RNAP-II-dependent complexes in trypanosomes.

¡B¿ Investigating mechanisms of diamidine resistance in *Leishmania* ¡B¿

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Leishmania parasites can acquire resistance to pentamidine through serial exposure to sub-lethal drug concentrations. The mechanisms through which pentamidine acts, and by which resistance develops are incompletely understood. We have generated a pentamidine resistant *Leishmania mexicana* line (LmPentR) and initiated a comparative phenotypic comparison with the isogenic pentamidine sensitive progenitor (LmPentS). The LmPentR displayed a 12-fold resistance to pentamidine compared with LmPentS, in addition to 5-fold cross resistance to DB75, a related diamidine compound. Cell were not however, cross resistant to arylimidamines - a novel class of drug with potent leishmaniacidal activity. Resistance was stable for several weeks in the absence of drug pressure indicating the observed resistance has a genetic basis. We are investigating the differences between these cell lines using proteomic and metabolomic approaches. Difference gel electrophoresis (DiGE) has highlighted a small subset of proteins whose expression is significantly and reproducibly changed in the LmPentR cell line. Using NMR and high resolution mass spectrometry we are also investigating changes at the metabolic level. These approaches should provide a greater understanding of the mode of action of pentamidine and the mechanism(s) behind resistance.

RAP1 Is Essential for Silencing Telomeric *Variant Surface Glycoprotein* Genes in *Trypanosoma brucei*

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Trypanosoma brucei is a protozoan parasite that causes African trypanosomiasis in humans. In mammalian host, *T. brucei* regularly switch its surface antigen, Variant Surface Glycoprotein (VSG), to evade host immune elimination. To maximize the efficiency of antigenic variation, *T. brucei* expresses *VSG* genes in a strictly monoallelic fashion and exclusively from one of 15 nearly identical subtelomeric *VSG* Expression Sites (ESs), but it is unclear how this important virulence mechanism is enforced. Telomere position effect (TPE), an epigenetic phenomenon, has been proposed to play a critical role in *VSG* regulation, yet no telomeric protein was identified whose disruption led to *VSG* derepression. We have recently identified tbRAP1 as an intrinsic component of the *T. brucei* telomere complex and a major regulator for silencing ESs. Knockdown of tbRAP1 led to derepression of all *VSGs* in silent ESs, but not *VSGs* located elsewhere, and resulted in stronger derepression of genes located within 10 kb from telomeres than genes located further upstream. This graduated silencing pattern suggests that telomere integrity plays a key role in tbRAP1-dependent silencing and *VSG* regulation.

Cyclosporin A treatment leads to stage-specific effects on *Leishmania donovani* morphology, proliferation, and viability

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We previously identified *L. donovani* cyclophilin-40 (LdCyP40) as amastigote-specific phosphoprotein utilizing a phosphoproteomic approach. LdCyP40 belongs to the cyclophilin (CyP) protein family characterized by the binding of the polypeptide inhibitor cyclosporin A (CsA). The presence of 17 highly conserved CyP-like proteins in the *L. major* genome, and the amastigote-specific phosphorylation of LdCyP40, primed us to investigate the effects of CsA on parasites in culture. CsA treatment of *L. donovani* promastigotes resulted in a dose-dependent inhibition of cell growth with a nearly 50% reduction in parasite numbers 48h after treatment with 15 μM inhibitor. This defect was independent form cell death but due to a significant slow down in proliferation and cell cycle progression. The growth phenotype was associated with a striking change in promastigote morphology reminiscent to amastigote differentiation, including reduction of flagellum length and transition from spindle to oval cell shape. However, CsA treatment had no effect on stage-specific gene expression as judged by the maintenance of surface LPG and the lack of expression of amastigote-specific A2 protein. In contrast to promastigotes, CsA treatment was highly toxic to axenic amastigotes, indicating stage-specific functions of CsA target proteins. Our data implicate *Leishmania* CyPs in parasite differentiation and identify members of this protein family as potential drug targets.

A mitochondrial RNase D family exoribonuclease from *Trypanosoma brucei* impacts both mRNAs and gRNAs

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Ribonucleases are expected to impact kinetoplastid mitochondrial gene expression at multiple levels. This includes regulating the stability of mature mRNAs, gRNAs, and rRNAs as well as removing non-functional maturation by-products. Moreover, 3' end maturation of mRNAs, rRNAs, gRNAs likely involves both endo- and exoribonucleases. Here, we report characterization of a putative exoribonuclease of the ribonuclease D family from *Trypanosoma brucei*, which we term TbRND. TbRND has been assigned to the mitochondrial proteome by mass spectrometry studies. It exhibits a unique domain structure for RND family enzymes, possessing a C-terminal zinc knuckle that may confer RNA binding specificity. RNAi studies indicate that TbRND is essential for procyclic form growth. Cells depleted of TbRND exhibit a modest stabilization of a subset of mRNAs, suggesting that TbRND is not exclusively responsible for mRNA turnover. To determine whether TbRND functions in the previously reported decay of non-adenylated edited and never-edited RNAs, we created dual knockdown of TbRND and kPAP1, the major mitochondrial poly(A) polymerase. Again, we observed stabilization of a subset of mRNAs, but TbRND depletion did not restore the levels of all destabilized, deadenylated RNAs, indicating specificity in mRNA target selection by TbRND. Interestingly, the total gRNA population in TbRND-depleted cells was reproducibly slightly extended by 2-3 nt. Thus, TbRND may function in gRNA processing, potentially acting on precursor minicircle transcripts or maintaining the strict length of a gRNA's non-coded U tail. Future experiments will include analysis of 3' end sequences of those mRNAs and gRNAs stabilized upon TbRND depletion and determination of the sequence specificity of both recombinant enzyme and myc-his-TAP tagged enzyme isolated from trypanosomes.

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