TALK SESSIONS: I - VII

April 8 7:00p.m. Chair - Isabel Roditi

Session I: Cell Biology I

1A Leishmania surface molecules: virulence factors all? ***Smith, D.***

1B *Leishmania* metacaspases: role in life and death? ***Castanys-Munoz, E.****, Coombs, G., Mottram, J.*

1C *Leishmania* RNA Virus controls the severity of mucocutaneous leishmaniasis. *Ives, A., Ronet, C., Zangger, H., Lye, L., Hickerson, S., Beverley, S., Masina, S.,* ***Fasel, N.***

1D Sphingolipid Degradation in *Leishmania* Virulence. ***Zhang, O.****, Xu, W., Zhang, K.*

1E A human genome-wide RNA interference screen identifies critical host cell regulators of *Trypanosoma cruzi* infection. ***Caradonna, K.****, Engel, J., Burleigh, B.*

1F The regulation of the sub-cellular localisation of GPI-PLC. ***Sunter, J.****, Webb, H., Carrington, M.*

1G *Trypanosoma brucei* Rab7 regulates lysosomal delivery of endocytosed but not newly synthesized proteins. ***Silverman, J.****, Bangs, J.*

April 9 8:45 a.m. Chair - Michael Ferguson

Session II: Drug Development

2A Drug Discovery for Neglected Diseases - Challenges, Opportunities and Lessons Learned. ***Fairlamb, A.***

2B Identification of a novel lead against African sleeping sickness, exploratory chemistry and preliminary target identification studies. ***Jones, D.***, *Patterson, S., Stojanovski, L., Smith, V., Cleghorn, L., Gilbert, I., Read, K., Frearson, J., Fairlamb, A.*

2C Post-translational modifications as drug targets in tryps. ***Ferguson, M.***

2D Discovery and Optimization of a Novel Oxaborole-6-Carboxamide to Treat Late-stage Nervous System Human African Trypanosomsiasis. ***Nare, B.***, *Wring, S., Bacchi, C., Brun, R., Plattner, J., Beaudet, B., Bowling, T., Chen, D., Freund, Y., Gaukel, E., Jenks, M., Keiser, M., Mercer, L., Noe, A., Orr, M., Parham, R., Randolph, R., Rewerts, C., Sligar, J., Yarlett, N., Don, R., Jacobs, R.*

Coffee Break

2E Cyclic nucleotide-specific phosphodiesterases: new drug targets for old diseases? *Seebeck, T., Sterk, G., Tenor, H., Gould, M., Luginbuehl, E., Kunz, S.*, ***De Koning, H.***

2F Target Repurposing: Identification of trypanosomal TOR/PI3K as a therapeutic approach. ***Pollastri, M.***, *Diaz-Gonzalez, R., Kuhlmann, M., Galan-Rodriguez, C., Madeira da Silva, L., Karver, C., Beverley, S., Rodriguez, A., Navarro, M.*

2G Identifying new targets for drug development against Trypanosomatids. ***Monnerat, S.***, *Cestari, I., Merritt, C., Parsons, M., Stuart, K.*

2H Novel drug uptake and potential resistance mechanisms in African trypanosomes: Suramin. ***Alsford, S.***, *Baker, N., Leung, K., Field, M., Horn, D.*

2I Distinct drug-resistant *T. cruzi* clones can arise independently in a single population undergoing benznidazole-selection. *Mejia, A., Hall, B., Taylor, M., Wilkinson, S., Triana, O.*, ***Kelly, J.***

April 9 1:45 p.m. Chair - Torsten Ochsenreiter

Session III: RNA

3A RNA interference libraries for high throughput phenotyping and drug-resistance studies in the African trypanosome. ***Horn, D.***, *Turner, D., Eckert, S., Obado, S., Sanchez-Flores, A., Glover, L., Berriman, M., Hertz-Fowler, C., Baker, N., Alsford, S.*

3B The role of RNA interference in the virulence of *Leishmania braziliensis* . ***Beverley, S.***, *Notton, T., Hickerson, S., Lye, L., Owens, K., Anderson, B., Atayde, V., Ullu, E., Haydock, A., Myler, P., Kruvand, E.*

3C Modification of siRNAs by HEN1 methyltransferase reveals diversification of the RNAi mechanism between *T. brucei* and *L. braziliensis*. *Shi, H.,* ***Barnes, R.***, *Atayde, V., Tschudi, C., Ullu, E.*

3D Alternative trans-splicing determines the localization of the dually targeted IleRS of *T. brucei*. ***Rettig, J.***, *Ochsenreiter, T., Schneider, A.*

Coffee Break

3E RNA-binding proteins orchestrating *T. brucei* gene expression during differentiation in tsetse flies: the case of RBP6. ***Kolev, N.***, *Franklin, J., Savage, A., Aksoy, S., Ullu, E., Tschudi, C.*

3F The Role of RBP42, a newly identified RNA binding protein, in Trypanosoma brucei. *Das, A.,* ***Bellofatto, V.***

3G Global and gene-specific analysis of the targets of the post-transcriptional gene regulator *Tb*ZFP3. ***Walrad, P.***, *Matthews, K.*

3H The small RNome of Trypanosoma brucei:-novel RNAs and deciphering the role of parasite specific snoRNAs and their maturation by differential polyadenylation. *Gupta, S., Doniger, T., Ullu, E.,* ***Shulamit, M.***

April 10 8:45 a.m. Chair - Christine Clayton

Session IV: Gene Expression

4A Base J: history, distribution, biosynthesis and function. ***Borst, P.***, *Vainio, S., Jan, S., Tripathi, P., Genest, P., Ter Riet, B., Wong, I., Beverley, S., Carol, F., Myler, P., Velds, A., Kerkhoven, R., Nieuwland, M., Heidebrecht, T., Perrakis, A., Luenen, H.*

4B Base J is required for proper termination of RNA polymerase II transcription. ***van Luenen, H.***, *Farris, C., Tripathi, P., Jan, S., Velds, A., Kerkhoven, R., Nieuwland, M., Haydock, A., Ramasamy, G., Myler, P., Borst, P.*

4C Epigenetic regulation of gene expression and virulence in Trypanosoma cruzi. ***Ekanayake, D.***, *Minning, T., Weatherly, B., Gunasekera, K., Nilsson, D., Tarleton, R., Ochsenreiterc, T., Sabatini, R.*

4D Transcriptional Regulation of an RNA Polymerase II Transcription Unit in *Trypanosoma brucei brucei*. ***Kieft, R.***, *Hajduk, S.*

Coffee Break

4E The FACT chromatin remodelling complex affects chromatin structure at promoter regions of silent VSG expression sites. ***Denninger, V.***, *Rudenko, G.*

4F *Trypanosoma brucei* Tin2 Suppresses VSG Switching Rate. ***Jehi, S.***, *Li, B.*

4G Loss-of-silencing (LOS) screen in Trypanosoma brucei: Roles of DNA replication and RNA surveillance in VSG silencing. ***Kim, H.***, *Park, S., Günzl, A., Cross, G.*

4H In *Trypanosoma brucei*, high promoter occupancy of CITFA distinguishes the active from an inactive *VSG* expression site. ***Nguyen, T.***, *Lee, J., Nguyen, B., Park, S., Günzl, A.*

4I Stage-specific pathways for translation initiation in *Leishmania* - novel components and unique regulatory models. *Zinoviev, A.*, ***Shapira, M.***

April 11 8:45 a.m. Chair - Marilyn Parsons

Session V: Biochemistry

5A The structure of the Haptoglobin Haemoglobin Receptor and the fate of Apoliprotein L-1. *Higgins, M., Reed, J.,* ***Carrington, M.***

5B The Mechanism of Trypanosome Lytic Factor-1: A Role For Lipid Peroxidation. ***Harrington, J.****, Styer, A., Widener, J., Hajduk, S.*

5C Getting ahead of African trypanosomiasis: Baboons lead the way. ***Thomson, R.****, Canon, C., Higgins, M., Carrington, M., Raper, J.*

5D Adaptive responses to Purine stress in *Leishmania*. ***Carter, N.****, Yates, P., Galagan, S., Wilmarth, P., David, L., Fulwiler, A., Boitz, J., Landfear, S., Ullman, B.*

Coffee Break

5E Proteomics gives insight into signaling and sensory mechanisms of the *T. brucei* flagellum membrane and flagellum matrix. ***Oberholzer, M.****, Langousis, G., Nguyen, H., Jonsson, Z., Shimogawa, M., Saada, E., Kabututu, Z., Wohlschlegel, J., Hill, K.*

5F Molecular and Functional Characterization of Antimony Resistance in *Leishmania amazonensis*. ***Monte Neto, R.****, Coelho, A., Raymond, F., Légaré, D., Corbeil, J., Melo, M., Frézard, F., Ouellette, M.*

5G Whole Genome Sequencing and Comparative Genomic Hybridization of Leishmania and their Application to Antimony Resistance. ***Mukherjee, A.****, Boisvert, S., Mukhopadhyay, R., Corbeil, J., Ouellette, M.*

5H *Trypanosoma brucei* Isopentenyl Diphosphate Isomerase - the crystal structure of a validated drug target. ***MAJOR, L.****, SMITH, T.*

5I Lipid metabolism in *Trypanosoma brucei*: a plethora of drug targets. ***SMITH, T.****, Young, S., Major, L., Denton, H., Lilley, A., Williams, C.*

April 13 1:45 p.m. Chair - Derrick Robinson

Session VI: Cell Biology II

6A The complexities of cell division in *Trypanosoma brucei*. *May, S., Peacock, L., Gibson, W., Tetley, L.,* ***Hammarton, T.***

6B Identification and functional characterisation of CRK12:CYC9, a novel CDK-cyclin complex in *Trypanosoma brucei* . ***Costa, C.****, Monnerat, S., Forkert, A., Benz, C., Mottram, J., Novo, C., Hammarton, T.*

6C Polo like kinase is necessary for FAZ duplication and basal body segregation in *T. brucei*. *Ikeda, K.,* ***de Graffenried, C.***

6D The kinetoplast duplication cycle in *Trypanosoma brucei*: integration of DNA replication with cell morphogenesis. ***Gluenz, E.****, Povelones, M., Englund, P., Gull, K.*

Coffee Break

6E Trypanosome motion represents an adaptation to the environment of the vertebrate bloodstream. *Heddergott, N.,* ***Krueger, T.****, Wei, A., Stellamans, E., Uppaluri, S., Pfohl, T., Engstler, M.*

6F Beyond just counting Ks and Ns; high throughput image analysis of trypanosomatid cell organisation. ***Wheeler, R.****, Gluenz, E., Gull, K.*

6G Monitoring stumpy formation in *Trypanosoma brucei*. ***MacGregor, P.****, Matthews, K.*

6H Novel TOR kinase regulates proliferative slender to quiescent stumpy form differentiation in Trypanosoma brucei. ***Barquilla Panadero, A.****, Diaz, R., Saldivia, M., Bart, J., Vidal, I., Navarro, M.*

April 12 8:45 a.m. Chair - Keith Gull

Session VII: Nucleus & Kinetoplast

7A Architecture and function of the trypanosome nuclear envelope revealed using high resolution interactome mapping. ***Obado, S.***, *DeGrasse, J., DuBois, K., Field, M., Chait, B., Rout, M.*

7B Epigenetic regulation at the nuclear periphery mediated by a nonmetazoan lamin analogue. ***Field, M.***, *duBois, K., Alsford, S., Buisson, J., Swiderski, M., Bart, J., Ratushny, A., Wan, Y., Bastin, P., Barry, D., Navarro, M., Horn, D., Aitchison, J., Rout, M.*

7C Identification of ORC1/CDC6-interacting factors in Trypanosoma brucei reveals critical features of Origin Recognition Complex architecture. ***Tiengwe, C.***, *Marcello, L., Farr, H., Barry, D., Bell, S., McCulloch, R.*

7D The CMG complex in trypanosomes regulates DNA replication and interacts with the origin recognition complex consisting of two Orc1-like proteins. *Dang, H.,* ***Li, Z.***

7E The life dyskinetoplastic: how single point mutations allow *Trypanosoma brucei* survive complete loss of mitochondrial DNA. ***Gould, M.***, *Dean, S., Schnaufer, A.*

7F PPR Proteins Stimulate mRNA Adenylation/Uridylation to Activate Mitochondrial Translation in Trypanosomes. ***Aphasizheva, I.***, *Maslov, D., Wang, X., Huang, L., Aphasizhev, R.*

7G Endonuclease Associations with Three Distinct Editosomes in *Trypanosoma brucei*. ***Carnes, J.***, *Zelaya Soares, C., Wickham, C., Stuart, K.*

7H gRNA Metabolism in Trypanosome Mitochondria Involves a Novel Endonuclease. *Madina, B., Gokulan, K., Wohlschlegel, J., Vashisht, A., Liang, Y., Ji, X., Sze, S.,* ***Cruz-Reyes, J.***

7I Yeast two hybrid analysis of protein-protein interactions in the MRB1 complex of *T. brucei* identifies a core complex. ***Ammerman, M.***, *Hashimi, H., Downey, K., Tomasello, D., Fisk, J., King, T., Handzliková, L., Faktorová, D., Lukes, J., Read, L.*

POSTER SESSIONS: A-C

A SERIES: April 9 6:30 p.m.

B SERIES: April 10 6:30 p.m.

C SERIES: April 11 6:30 p.m.

200A A compensatory mutant of *Leishmania major* lacking ether lipids exhibits normal growth and neomycin resistance. ***Bibis, S.**, Zhu, T., Dhalladoo, S., Zufferey, R.*

201A The essential F_oF_1-ATPase complex in *Trypanosoma brucei* can be specifically inhibited by the inhibitory peptide $TbIF_1$. *Panicucci, B., Subrtova, K.,* ***Zikova, A.***

202A The ATG12-ATG5 conjugation pathway and autophagy in *Leishmania*. ***Williams, R.**, Mottram, J., Coombs, G.*

203A Chemical and peptide inhibitors of the *Leishmania major* cysteine synthase. ***Westrop, G.**, Clark, R., Ramos, T., Mackay, S., Müller, S., Coombs, G.*

204A TbKAP6, an essential small basic protein, is required for kDNA condensation and replication in *Trypanosoma brucei*. ***Wang, J.**, Pappas-Brown, V., Englund, P., Jensen, R.*

205A Stage-Specific Requirements for Acetyl-CoA Carboxylase in *Trypanosoma brucei*. ***Vigueira, P.**, Paul, K.*

206A *Trypanosoma brucei* BRCA2: BRC-mediated RAD51 interaction and genome stability. ***Trenaman, A.**, Hartley, C., McCulloch, R.*

207A Moonlighting functions for mitochondrial proteins in the African trypanosomes. ***Sykes, S.**, Zhu, X., Orlando, R., Hajduk, S.*

208A TbRGG3 is a mitochondrial methylprotein that affects mRNA stabilization and interacts with MRB1 components. ***Sudhakaran, J.**, Fisk, J., Downey, K., Ammerman, M., Presnyak, V., Qu, J., Read, L.*

209A Functional characterization of the trypanothione biosynthetic pathway in *Leishmania infantum*. ***Sousa, A.**, Jaeger, T., Flohé, L., Tomás, A., Castro, H.*

210A A Global Functional Atlas of *Trypanosoma brucei* Genome. ***Shateri Najafabadi, H.**, Macpherson, C., Mehta, V., Salavati, R.*

211A *Trypanosoma brucei* Sec16 - Size Matters. ***Sealey, M.**, Warren, G.*

212A The Telomere Terminal Structure in *Trypanosoma brucei*. ***Sandhu, R.**, Li, B.*

213A Targeting of receptor-type adenylate cyclases to flagellum membrane sub-domains in procyclic-form *Trypanosoma brucei*. ***Saada, E.**, Kabututu, Z., Lopez, M., Langousis, G., Oberholzer, M., Riestra, A., Hill, K.*

214A Comparative Genomics of Four Leishmania Species Reveals Few Gene Differences And Large Structural Changes. ***Rogers, M.**, Hilley, J., Wilkes, J., Dickens, N., Bates, P., Depledge, D., Her, Y., Imamura, H., Berriman, M., Smith, D., Hertz-Fowler, C., Mottram, J.*

215A Pyrimidine transport and resistance to 5-fluorouracil in Leishmania. ***Ritt, J.**, Ouellette, M.*

216A Protein-protein interaction network of post-transcriptional gene expression regulation in *Trypanosoma cruzi*. ***Probst, C.**, Preti, H., Pavoni, D., Marchini, F., Vidal, N., Krieger, M.*

217A Using comparative proteomics to identify life-cycle regulated proteins in *Trypanosoma brucei*. ***Portman, N.**, Gull, K.*

218A RNA polymerase I of *Trypanosoma brucei* functions independently of RPB7. ***Park, S.**, Nguyen, T., Kirkham, J., Günzl, A.*

219A Characterization of a putative trypanosome-specific transcription factor. ***Palenchar, J.**, Miller, R., Ogorzalek, P., Whitecavage, K., Sing, A.*

220A Polyamine Biosynthesis in *Leishmania donovani*. ***Olenyik, T.**, Gilroy, C., Roberts, S., Ullman, B.*

221A Identification of genes regulating social behavior in *Trypanosoma brucei*. ***Nguyen, H.**, Sandhu, J., Nguyen, D., Lopez, M., Oberholzer, M., Hill, K.*

222A Development of an aptamer based assay to detect parasite biomarker in blood for the diagnosis of Chagas disease. ***Nagarkatti, R.**, Fortes de Araujo, F., Debrabant, A.*

223A Naphthalene-based RNA editing inhibitor blocks RNA editing activities and Editosome assembly in *Trypanosoma Brucei*. ***Moshiri, H.**, Acoca, S., KALA, S., Shateri Najafabadi, H., Hogues, H., Purisima, E., Salavati, R.*

224A Genome-wide binding and functions of ORC1/CDC6, a nuclear DNA replication factor in *Trypanosoma brucei*
. *Tiengwe, C., Marcello, L., Farr, H., Barry, J., Bell, S.,* ***McCulloch, R.***

225A The role of XRNA in transcriptome-wide mRNA degradation in *Trypanosoma brucei*. ***Manful, T.****, Fadda, A., Clayton, C.*

226A Mitochondrial DNA Polymerases POLIB and POLID Cooperate in Minicricle Replication. ***Luo, J.****, Bruhn, D., Klingbeil, M.*

227A A novel platelet-activating factor molecular species synthesized by the human parasite Trypanosoma cruzi. ***Lopes, F.****, Marques, A., Gomes, M., Vieira, D., Souto-Padrón, T., Lopes, A., Almeida, I.*

228A Nucleotide Sugar Transport Inhibition In *Trypanosoma brucei* Alters Surface Glycoproteins: Implications For Pathogenesis. ***Liu, L.****, Xu, Y., Burleigh, B., Bangs, J., Hirschberg, C.*

229A A putative ion channel is required for flagellum attachment in Trypanosoma brucei. ***Langousis, G.****, Oberholzer, M., Hill, K.*

230A Kinetoplastid flagellate *Phytomonas* survives without heme. ***Koreny, L.****, Kovarova, J., Sobotka, R., Gnipova, A., Horvath, A., Obornik, M., Lukes, J.*

231A A stringent model for studying the function of N-linked glycans. ***Jones, N.****, Carrington, M., Engstler, M.*

232A RNA editing in trypanosomes: why is RNA editing ligase 1 (REL1) essential, whereas REL2 is not? ***Jeacock, L.****, Spencer, M., Lin, T., Stuart, K., Schnaufer, A.*

233A The role of small G proteins associated to IFT in trypanosomes. ***Huet, D.****, Blisnick, T., Bastin, P.*

234A Characterisation of *Trypanosoma brucei* Nucleoporins. ***Holden, J.****, DuBois, K., Rout, M., Field, M.*

235A High Throughput RNA-Seq analysis reveals differences in mRNA abundance and Splice Leader site location between different lifecycle stages of several *Leishmania* species. ***Haydock, A.****, Ramasamy, G., Sivam, D., Mittra, B., Akopyants, N., Anderson, B., Lahav, T., van Luenen, H., Andrews, N., Zilberstein, D., Beverley, S., Borst, P., Myler, P.*

236A Lateral mobility of the VSG implies a crowding threshold on the trypanosome cell surface. ***Hartel, A.**, Abuillan, W., Schubert, T., Tanaka, M., Jones, N., Engstler, M.*

237A Dynamics and interactions of *Trypanosoma brucei* antigenic variation. ***Hall, J.**, Barry, D.*

238A Identification of an essential *myo*-inositol transporter in *Trypanosoma brucei*. ***Gonzalez-Salgado, A.**, Steinmann, M., Greganova, E., Mäser, P., Sigel, E., Bütikofer, P.*

239A Biochemical roles of Leishmania Infantum RAD51 and BRCA2 in Homologous Recombination. ***Genois, M.**, Coulombe, Y., Buisson, R., Roy, G., Ouellette, M., Masson, J.*

240A The flagellar pocket collar: A lord of the rings? ***Florimond, C.**, Sahin, A., Landrein, N., Bonhivers, M., Dacheux, D., Robinson, D.*

241A The role of CAF130 in the process of deadenylation and mRNA turnover in *Trypanosoma brucei*. ***Färber, V.**, Schwede, A., Clayton, C.*

242A A unique homolog of Tim50 in *Trypanosoma brucei*. ***Duncan, M.**, Chaudhuri, M.*

243A Regulation of Expression of *Trypanosoma brucei* Hexokinase 1 Mediated by Polyadenylation Site. ***Dodson, H.**, Morris, M., Morris, J.*

244A The VSG repertoire of *Trypanosoma brucei* Lister 427. ***Cross, G.**, Wickstead, B.*

245A Dynamic Localization of Mitochondrial DNA Polymerases in *Trypanosoma brucei*. ***Concepcion, J.**, Luo, J., Klingbeil, M.*

246A Metabolic network analysis-directed assessment of FDA-approved drugs for a neglected tropical disease. ***Chavali, A.**, Pearson, R., Papin, J.*

247A Import of cytoplasmic RNA-binding proteins into the nucleus of *Trypanosoma cruzi* . ***Cassola, A.**, Read, L., Frasch, A.*

248A Composition and Trypanosome Killing by Trypanosome Lytic Factor-2. ***Bullard, W.**, Kieft, R., Hajduk, S.*

249A The LEXSY Technology - a valuable tool in *Kinetoplastida*. ***BREITLING, R.***

250A Regulation of NDR kinase activity in *Trypanosoma brucei*. ***Benz, C.**, Stockdale, C., Hammarton, T.*

251A Identification of drug uptake and resistance mechanisms using genome-wide RNAi screens in African trypanosomes. ***Baker, N.**, Alsford, S., Glover, L., Horn, D.*

252A Characterization of the histone variants H2AV and H2BV in *Leishmania major*. ***Anderson, B.**, Wong, I., Beverley, S.*

253A RNAi knockdown studies reveal that intraflagellar transport (IFT) genes are essential in *Leishmania braziliensis* promastigotes. ***Lye, L.**, Marcus, J., Acino, E., Beverley, S.*

200B Oligo(U) tails stabilize selected mitochondrial mRNAs and appear to be substrates for TbRND. ***Zimmer, S.**, McEvoy, S., Read, L.*

201B Metacaspase: a cysteine protease inducing cell death in *Leishmania major*. ***Zalila, H.**, Gonzalez, I., Calderon, L., Servis, C., Fasel, N.*

202B Phosphoproteomics analysis of *Leishmania mexicana*. *Rosenqvist, H., Jensen, O.,* ***Wiese, M.***

203B A survey of kinetoplastid genomes. ***Warren, W.***

204B A Sensitive Method for Structural Characterization of N-glycans in Trypanosomes. ***Wadum, M.**, Kelner, A., Ferguson, M.*

205B Late stage division differences between procyclic and bloodstream T. brucei trypomastigotes. ***Vaughan, S.**, Wheeler, R., Palmer, R., Gull, K.*

206B Pleiotropic effects caused by overexpression of the non- coding RNA ODD3 in *Leishmania*. ***toledo, j.**, Vasconcelos, E., Nogueira, K., Cruz, A.*

207B The structural basis of localizing polo-like kinase to the flagellar attachment zone in *Trypanosoma brucei*. ***Sun, L.**, Wang, C.*

208B Functional analysis of a novel and essential subunit of the F_oF_1-ATP synthase in *Trypanosoma brucei*. ***Subrtova, K.**, Panicucci, B., Zikova, A.*

209B Molecular characterization of the *Trypanosoma cruzi* specific RNA binding protein TcRBP40 and its associated mRNAs. ***Guerra-Slompo, E.**, Probst, C., Pavoni, D., Goldenberg, S., Krieger, M., Dallagiovanna, B.*

210B Comparative genomics and functional analysis of proteins involved in mRNA nucleocytoplasmatic export in trypanosomes. ***Serpeloni, M.**, Vidal, N., Moraes, C., Muniz, J., Fragoso, S., Kessler, R., da Rocha, W., Yamada-Ogatta, S., Goldenberg, S., Motta, M., Hoffmann, F., Freitas-Junior, L., Ávila, A.*

211B The effect of loss of disulphide bridges in the VSG N-terminal domain on *Trypanosoma brucei* bloodstream form cells. ***Schwede, A.**, Jones, N., Engstler, M., Carrington, M.*

212B CMF22, a candidate calmodulin-binding protein, localizes to the flagellar cytoskeleton and is essential for forward motility in African trypanosomes. ***Sandhu, J.**, Nguyen, H., Hill, K.*

213B Elucidation of the role of the *Leishmania mexicana A600* gene locus in amastigote growth. ***Rosamont-Ursulet, M.**, Murray, A., Lynn, M., McMaster, R.*

214B Druggable DUBs in Trypanosomes. ***Rodenko, B.**, De Jong, A., Berkers, C., Burchmore, R., Ovaa, H.*

215B TbRACK1 is a Signal Scaffold That is Present on Cytoplasmic Free Ribosomes but is Absent From Er-Bound Ribosomes. ***Regmi, S.**, Rothberg, K., Hubbard, J., Pandarinath1, V., Nett, I., Ferguson, M., Ruben, L.*

216B *Trypanosoma brucei* ARL6 (BBS3) is a vesicle-associated tubulin binding protein required for flagellum extension. ***Price, H.**, Hodgkinson, M., Smith, B., Carrington, M., Stark, M., Smith, D.*

217B TbLpn and its role in phospholipid biosynthesis in *Trypanosoma brucei*. ***Wiemer, J.**, Pelletier, M.*

218B *Trypanosoma brucei* Mitochondrial Respiratome: Composition and organization in procyclic form. ***Panigrahi, A.***, *Acestor, N., Zíková, A., Dalley, R., Anupama, A., Stuart, K.*

219B Identification of downstream targets of *N*-myristoyltransferase in *Leishmania donovani*. ***Paape, D.***, *Wright, M., Price, H., Tate, E., Smith, D.*

220B Protein Phosphorylation in *Trypanosoma brucei* Mitochondria. *Cirovic, O., Schranz-Zumkehr, J.,* ***Ochsenreiter, T.***

221B Mechanism of action of Trypanosome Lytic Factor against Leishmania. ***Nelson, M.***, *Samanovic, M., Raper, J.*

222B Functional analysis of LmxMPK2, a MAP kinase essential for *Leishmania mexicana* amastigotes. ***Munro, L.***, *MacDonald, A., Schmetz, C., Wiese, M.*

223B Regulating gene expression in transmissible stumpy forms of *Trypanosoma brucei*. ***Monk, S.***, *Matthews, K.*

224B Differential phosphorylation of LmxMPK1 - teasing out a meaning from *in vivo* and *in vitro* observations. ***McAleer, P.***, *Rosenqvist, H., Melzer, I., Bleicher, N., Jensen, O., Wiese, M.*

225B Screening of metal-based Azole derivatives with antiparasitic activity on Trypanosoma Cruzi and Leishmania Major. *Carreon, T., Vasquez, M., Sanchez-Delgado, R.,* ***Maldonado, R.***

226B Fe-S cluster assembly in procyclic and bloodstream stages of Trypanosoma brucei. ***Lukes, J.***, *Changmai, P., Vavrova, Z., Basu, S., Skalicky, T., Kovarova, J., Long, S., Paris, Z.*

227B Stimulation of *Leishmania chagasi* infectivity by intrinsic platelet-activating factor. *Vieira, D., Gazos-Lopes, F., Gomes, M., Souto-Padron, T., Lima, A., Almeida, I.,* ***Lopes, A.***

228B The *Trypanosoma brucei* CDP-DAG and PI Synthases: Essential Enzymes in Multiple Cellular Locations. ***Lilley, A.***, *Smith, T.*

229B Vacuolar transport chaperone 4 (VTC4) is an acidocalcisomal polyphosphate synthase in *Trypanosoma brucei* and *T. cruzi*. ***Lander, N.***, *Ulrich, P., Miranda, K., Docampo, R.*

230B Flagellar functions investigated in a paraflagellar rod mutant. *Marande, W., Travers, A., Demonchy, R., Bastin, P.,* ***Kohl, L.***

231B Extra-Glycosomal Localization of *Trypanosoma brucei* Hexokinase 2. ***Joice, A.****, Lyda, T., Sayce, A., Verplaetse, E., Michels, P., Morris, J.*

232B A neutral amino acid permease in Leishmania regulates metacyclogenesis and cell volume by building large pool of proline. ***Inbar, E.****, Schlisselberg, D., Grotemeyer, M., Rentsch, D., Zilberstein, D.*

233B Identification of acidocalcisome proteins in *Trypanosoma brucei* by a novel purification method and *in situ* tagging. ***Huang, G.****, Moreno, S., Docampo, R.*

234B Nucleolar changes associated to growth rate conditions in *Trypanosoma cruzi* epimastigotes. ***Hernandez, R.****, Nepomuceno-Mejía, T., Guerrero-Sánchez, E., López-Villaseñor, I., Cevallos, A., Jiménez-García, L.*

235B Genetic Analysis of the *T. cruzi* TcPOT1.1 diamine transporter. ***Hasne, M.****, Soysa, R., Ullman, B.*

236B Membrane Rigidification in African Trypanosomes: An Unusual Mechanism of Peptide Cytotoxicity. ***Harrington, J.****, Scelsi, C., Hajduk, S.*

237B Comparative Transcriptomics of *T. brucei* and *T. cruzi* Insect and Blood Form Life Stages Using Spliced Leader Trapping. ***Gunasekera, K.****, Nilsson, D., Ekanayake, D., Sabatini, R., Tarleton, R., Ochsenreiter, T.*

238B Antigenic variation in *T. brucei*: The importance of DNA break location and RAD51. ***Glover, L.****, Alsford, S., Horn, D.*

239B Deregulation of DOT1 family histone methyltransferases causes replication defects in *Trypanosoma brucei*. ***Gassen, A.****, Schandry, N., Kaufmann, D., Boshart, M., Janzen, C.*

240B Multiple Protein Arginine Methyltransferases and Classes of Methylproteins in *Trypanosoma brucei*. ***Fisk, J.****, Li, J., Qu, J., Read, L.*

241B Repeat arrays associated with centromeric domains on the mega-chromosomes of *T. brucei* are much larger than predicted. ***Echeverry, M.***, *Obado, S., Bot, C., Taylor, M., Kelly, J.*

242B Involvement of the CCCH zinc finger protein *Tb*ZC3H11 in stress response. ***Droll, D.***, *Stewart, M., Clayton, C.*

243B Development of a dual reporter system to identify regulatory cis-acting elements in untranslated regions of *Trypanosoma cruzi* mRNAs. ***Araujo, P.***, *Burle-Caldas, G., da Silva, R., Bartholomeu, D., DaRocha, W., Teixeira, S.*

244B Comparative study of the leishmanicidal activity of imidazoquine derivatives of primaquine against *Leishmania infantum*. ***Costa, S.***, *Vale, N., Matos, J., Moreira, R., Gomes, P., Gomes, M.*

245B Whole Genome sequencing and functional studies of miltefosine-resistant *Leishmania major* mutants. ***Coelho, A.***, *Boisvert, S., Corbeil, J., Ouellette, M.*

246B *Trypanosoma brucei rhodesiense* contains two copies of the Serum Resistance Associated Protein gene. ***Chandler, C.***, *Kieft, R., Hajduk, S.*

247B *Leishmania infantum* IZT3 is a zinc transporter that is tightly regulated by zinc status. ***Carvalho, S.***, *Silva, R., Shawki, A., Mackenzie, B., Castro, H., Eide, D., Costa, V., Tomás, A.*

248B The nonsense mediated decay factor, TbNMD3, regulates *PAG* transcripts in a process involving CRM1-mediated nuclear export. ***Buehlmann, M.***, *Matthews, K.*

249B The Purine Salvage Pathway of *Leishmania donovani* . ***Boitz, J.***, *Ullman, B.*

250B A feedback loop controls VSG expression site activity. ***Batram, C.***, *Jones, N., Engstler, M.*

251B Analyzing the functional role of a divergent XPB helicase paralog. ***Badjatia, N.***, *Lee, J., Nguyen, T., Pease, J., Günzl, A.*

252B Trafficking of a novel family of type I transmembrane proteins in *Trypanosoma brucei*. ***Allison, H.***, *Field, M.*

200C Proteomic and phosphoproteomic analysis of Leishmania donovani development. ***Zilberstein, D.***, *Gheradini, P., Smith, D., Borchers, C., Tsigankov, P.*

201C Antitumor Quinol PMX464 is a Cytocidal Anti-Trypanosomal Inhibitor Targeting Trypanothione Metabolism. ***Wyllie, S.***, *Konig, J., Fairlamb, A.*

202C The trypanosome-specific N-terminus of RPA2 is required for nuclear localization and also RNA polymerase I function. *Daniels, J., Gull, K.,* ***Wickstead, B.***

203C Dissecting molecular interactions in a novel preribosomal particle in *T. brucei*. ***Wang, L.***, *Williams, N.*

204C Building a flagellum: two tales of cytoplasmic assembly and flagellar incorporation. ***VINCENSINI, L.***, *Blisnick, T., Bastin, P.*

205C The phosphoproteome of *Trypanosoma brucei*. ***Urbaniak, M.***, *Martin, D., Ferguson, M.*

206C TBMLP1: A Putative Endosoma Iron Channel in *Trypanosoma Brucei*. ***Taylor, M.***, *McLatchie, A., Kelly, J.*

207C Identification and validation of diagnostic antigens for Human African Trypanosomiasis. ***Sullivan, L.***, *Carrington, M., Ferguson, M.*

208C *Leishmania donovani* receptor-cargo protein complexes form in the cytosol and dock at glycosomal protein Peroxin14, modulating its conformation. ***Strasser, R.***, *Pilar, A., Jardim, A.*

209C A Novel protein translocase of mitochondrial inner membrane in Trypanosoma brucei. ***Singha, U.***, *Chaudhuri, M.*

210C A tRNA Import Complex in the Mitochondria of *Trypanosoma brucei*. ***Seidman, D.***, *Gerbasi, V., Golden, D., Hajduk, S.*

211C RNA editing as a drug target in trypanosomes: development of a high throughput screening assay for RNA editing ligase 1. *Hall, L.,* ***Schnaufer, A.***

212C The flagellar pocket collar: a marriage of rings. ***Sahin, A.***, *Florimond, C., Landrein, N., Bonhivers, M., Dacheux, D., Robinson, D.*

213C Lipoamide dehydrogenase is essential for both bloodstream and procyclic *Trypanosoma brucei*. ***Roldán, A.**, Comini, M., Fernández, G., Krauth-Siegel, R.*

214C The role of *L. Mexicana* Arginase in host infection. ***Roberts, S.**, Riley, E., Gilroy, C., Olenyik, T., Gaur, U., Wilson, M., Ullman, B.*

215C Regulation of Trypanosoma brucei Acetyl-CoA Carboxylase, the key enzyme for initiation of fatty acid synthesis. ***Ray, S.**, **Paul, K.**, Vigueira, P.*

216C Histone H1 plays a role in silencing VSG expression sites in *Trypanosoma brucei*. ***Povelones, M.**, Dembek, M., Rudenko, G.*

217C Characterization of Major Surface Proteases of *Trypanosoma rangeli*. ***Pedrosa, A.**, Ferreira, K., Calixto, P., Lages-Silva, E., Ramirez, L.*

218C *Trypanosoma Brucei* RAP1: A key Regulartor for*VSG* Expression. ***PANDYA, U.**, LI, B.*

219C Tsetse Midgut Serine Protease Inhibitors (SERPINS) Protect African Trypanosomes from the Action of Serum Complement. *Ooi, C., Lehane, M., Acosta-Serrano, A.*

220C Towards the Mitochondrial Outer Membrane Proteome of *T. brucei*. ***Niemann, M.**, Schneider, A.*

221C Functional analysis of the HMGB protein TDP1 in *Trypanosoma brucei* . ***Narayanan, M.**, Rudenko, G.*

222C Molecular analysis of pentamidine transporters in *Trypanosoma brucei*. ***Munday, J.**, Teka, I., Eze, A., Burchmore, R., Barrett, M., de Koning, H.*

223C Iron-y of fate: Role of Iron in differentiation of *Leishmania amazonensis*. ***Mittra, B.**, Huynh, C., Andrews, N.*

224C The A600.1 protein is localized in the mitochondria of *Leishmania mexicana*. ***Marr, A.**, Abraham, T., Mcmaster, R.*

225C Development and optimization of RNAi tools for *Leishmania* (Viannia). ***Lye, L.**, Owens, K., Notton, T., Beverley, S.*

226C SUMOylation in the VSG Expression site Chromatin. ***López-Farfán, D.**, Vidal, I., Navarro, M.*

227C TrypanoCyc: a community effort towards the development of a metabolic pathway database for Trypanosoma brucei. ***Logan-Klumpler, F.**, Cottret, L., Vinson, F., Bringaud, F., Boshart, M., Butikofer, P., Berriman, M., Carrington, M., Chukualim, B., De Koning, H., Ferguson, M., Ginger, M., Hertz-Fowler, C., Maeser, P., Michels, P., Nolan, D., Opperdoes, F., Oullette, M., Phillips, M., Roos, D., Smith, T., Tielens, A., Taylor, M., Van Hellemond, J., Jourdan, F., Barrett, M.*

228C Global gene expression changes in *Trypanosoma cruzi* under nutritional and hyperosmotic stresses. ***Li, Z.**, Alvarez, V., Docampo, R.*

229C Inhibition of trans-splicing causes an unexpected cytoplasmic response. ***Kramer, S.**, Marnef, A., Carrington, M.*

230C The Quest for Endonucleases Causing Instability of Procyclin mRNAs. ***Knüsel, S.**, Roditi, I.*

231C Characterization of the membrane kinases of *Trypanosoma brucei*. ***Jensen, B.**, Flaspohler, J., Parsons, M.*

232C Antigenic variation in *T. brucei*: Simultaneous VSG expression from two telomeres and a search for a VSG negative regulatory sequence. ***Hutchinson, S.**, Alsford, S., Horn, D.*

233C The affect of Telomere length on antigenic variation in *Trypanosoma Brucei*. ***Hovel-Miner, G.**, Boothroyd, C., Dreesen, O., Cross, G., Papavasiliou, N.*

234C RNA interference of enzymes involved in parasite antioxidant defense indicates the novel antitrypanosomal compound **OSU-40** acts through generation of reactive oxygen species. ***He, S.**, Drew, M., Werbovetz, K.*

235C A putative mitochondrial calcium/proton antiporter is essential for both life stages of *Trypanosoma brucei brucei* and the dyskinetoplastic *Trypanosoma brucei evansi*. ***Hashimi, H.**, McDonald, L., Lukes, J.*

236C Differential Preference of TAO Presequence Cleavage Site in Two Developmental Forms of *Trypanosoma brucei*. ***Hamilton, V.**, Singha, U., Chaudhuri, M.*

237C Chemical validation of GSK3 in bloodstream form *Trypanosoma brucei*. ***Grimaldi, R.**, Urich, R., Sienkiewicz, N., Frearson, J., Wyatt, P., Fairlamb, A.*

238C Purification and biochemical properties of *T. brucei* PKA-like kinase. ***GITHURE, G.**, BADEL, A., WILD, N., HERBERG, F., BOSHART, M.*

239C The Role of Poly(A)-Specific Ribonuclease in Stage-Specific mRNA Turnover in Trypanosoma brucei. ***Garcia, S.**, Utter, C., Bellofatto, V.*

240C Insights into the function of the RNA-binding proteins RBP33 and DRBD3 in *Trypanosoma brucei*. ***Fernandez-Moya, S.**, Kramer, S., Carrington, M., Estevez, A.*

241C Tbp27, a novel component of the cytochrome c oxidase complex in *Trypanosoma brucei* procyclic forms. *Dey, R., Debrabant, A., Nakhasi, H.,* ***Duncan, R.***

242C Investigating the roles of HASPB and SHERP during *Leishmania* metacyclogenesis in the sandfly. ***Doehl, J.**, Sádlová, J., Volf, P., Smith, D.*

243C The *Leishmania donovani* glycosomal protein peroxin 14 hydrophobic region is necessary for membrane association. ***Cyr, N.**, Jardim, A.*

244C *In vitro* analysis of *Trypanosoma brucei gambiense* adaptation to different mammal sera. ***Cordon-Obras, C.**, Vidal, I., Cano, J., Benito, A., Navarro, M., Bart, J.*

245C Towards the elucidation of the host-pathogen interactome of *Trypanosoma cruzi*.. ***Choi, J.**, Fernandes, M., Sheng, Z., El-Sayed, N.*

246C Identifying new drug targets against African Trypanosomiasis. ***Cestari, I.**, Monnerat, S., Merritt, C., Stuart, K.*

247C Human ApoL-I Variants in African Trypanosomiasis. ***Canon, C.**, Thomson, R., Raper, J.*

248C Mapping the differentiation block in pleomorphic DOT1B-deficient trypanosomes. ***Bucerius, F.**, Boshart, M., Janzen, C.*

249C Repurposing human phosphodiesterase inhibitors as a route to developing anti-trypanosomal drugs. ***Bland, N.**, Gustafson, A., Wang, C., Ochiana, S., Pollastri, M., Campbell, R.*

250C Functional characterization of Pseudo-CTD of RNA polymerase II of Trypanosoma brucei. ***Banday, M.**, Das, A., Bellofatto, V.*

251C Screening for downstream effectors of cAMP and PKA-like kinases in *T. brucei*. ***Bachmaier, S.**, Krumbholz, C., Kramer, S., Githure, G., Salmon, D., Israel, L., Imhof, A., Pays, E., Boshart, M.*

252C Defining the *T. brucei* mitochondrial DNA-binding proteome to discover novel proteins involved in kDNA replication, organization and maintenance. ***Allary, M.**, Acestor, N., Panigrahi, A., Yildirir, G., Englund, P., Jensen, R.*

ABSTRACTS: SESSION I

Cell Biology

April 8 7:00p.m.

Chair(s) - Isabel Roditi

Leishmania surface molecules: virulence factors all?

Deborah F. Smith

Biology, University of York, York, UK

Leishmania surface molecules: virulence factors all?

Leishmania metacaspases: role in life and death?

Esther Castanys-Munoz[*,1], Graham H. Coombs[2], Jeremy C. Mottram[1]

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Metacaspases are cysteine peptidases present in plants, fungi and protozoan parasites, but absent from mammals. They are related to caspases, showing conservation of the catalytic histidine/cysteine residues. They were thought to be responsible for the caspase-like activities found in those organisms, but it still remains controversial whether metacaspases might have a similar role to caspases. Despite sharing some common substrates, such as Tudor nuclease, metacaspases show specificity for arginine/lysine residues, instead of the aspartic acid specificity of caspases. Furthermore, there is no convincing evidence that metacaspases play a role in PCD in parasitic protozoa. *Leishmania major* expresses a single metacaspase, which we described as being essential, with a role in cell cycle control (Ambit et. al, 2008, Cell Death Diff. 15, 113-122). To improve our understanding of *Leishmania* metacaspases, we identified a single metacaspase gene in *L. mexicana*. Using Nucleofector^{TM} transfection, we generated *LmxMCA* null-mutants. Over-expressor and re-expressor cell lines were also generated, which allowed us to assess the protein role in the parasite cell and life cycle. When cell death was induced with H_20_2 or leishmanicidal drugs (miltefosine, camptothecin), no differences were observed in the number of cells undergoing apoptosis (assessed by PS exposure) between wild-type and *LmexMCA* null-mutants, which would question a role for LmxMCA in *L. mexicana* cell death. However, in vitro infectivity results indicate that null-mutants replicate better in macrophages (double the number of parasites/cell in null-mutants compared to wild-type), even in the presence of miltefosine or nitric oxide, and provide experimental evidence of the increased infectivity of *LmxMCA* null-mutants in mice. These data provide new evidence for metacaspases having cell functions other than cell death.

Leishmania RNA Virus controls the severity of mucocutaneous leishmaniasis

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In Latin America leishmaniasis caused by the *Leishmania* Viannia (*L.Viannia*) subgenus is endemic causing cutaneous (CL) and mucocutaneous (MCL) leishmaniasis. Clinical MCL involves parasitic dissemination to the nasopharyngeal areas of the face leading to destructive metastatic secondary lesions and hyper-inflammatory immune responses. Approximately, 5-10% of individuals asymptomatic or with resolved CL lesions can develop MCL, which is associated with persistent immune responses showing pro-inflammatory mediators. In addition to parasite-derived virulence factors, host genetics and immune status appear to influence MCL development. Hamsters infected with *L.Viannia* parasites isolated from human MCL lesions reproduce the metastatic phenotype with primary and secondary lesion development. Using this model, clones derived from metastasizing *L.guyanensis* strains were characterized as being metastatic (L.g.M+), or non-metastatic (L.g.M-) after infection, depending on their ability to reproducibly develop secondary metastatic lesions. Here we show that metastasizing parasites have a high *Leishmania* RNA Virus-1 (LRV1) burden that is recognized by the host Toll-Like receptor 3 (TLR3) to induce pro-inflammatory cytokines and chemokines. Paradoxically, these TLR3-mediated immune responses rendered mice more susceptible to infection and the animals developed an increased footpad swelling and parasitemia. Our work showed that recognition of LRV1 within metastasizing L.g. parasites by the host promoted inflammation, and subverted the immune response to infection to promote parasite persistence. We hypothesize that LRV1 dsRNA is released from dead parasites, unable to survive within the host macrophage. These results open the doorway to better diagnosis of risk for MCL disease and facilitate the development of new, and more efficient treatment regimes.

Sphingolipid Degradation in *Leishmania* Virulence

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Leishmania parasites alternate between flagellated promastigotes residing in sandflies and nonflagellated amastigotes inside the phagolysosome of macrophages. While inositol phosphoryceramide (IPC) constitutes the dominant species of sphingolipids (SLs) in promastigotes, amastigotes of *Leishmania* contain both host-derived SLs such as sphingomyelin as well as the parasite-specific IPC. Functions of these SLs in *Leishmania* infection are not well understood. In *L. major*, a single ISCL (**I**nositol phospho**S**phingolipid phospholipase **C**-**L**ike) protein is responsible for the degradation of both sphingomyelin (the SMase activity) and IPC (the IPCase activity). As revealed by quantitative RT-PCR and western-blot analyses, ISCL is preferentially expressed during the virulent stage. This enzyme co-localizes with mitochondria in promastigotes but is secreted during the amastigote stage. Gene deletion study showed that *ISCL* is not only important for cell morphology and acid resistance in promastigotes, but also vital for the survival and growth of amastigotes inside host cells. Interestingly, introduction of a sole IPCase (CnISC1p) reversed the cell shape and acid resistance defects in the ISCL-null mutant (*iscl*), but failed to restore its virulence in mammals. In contrast, a sole SMase (BcSMase) did not complement the promastigote defects but fully restored the virulence of *iscl*. Therefore, SL degradation plays distinct roles in *Leishmania* at different stages: while the IPCase activity is required for the resistance to acid shock in promastigotes, the turnover of host SM is essential for amastigote survival in the mammalian host. Further studies will elucidate the mechanism by which SL degradation contributes to *Leishmania* growth and pathogenesis.

A human genome-wide RNA interference screen identifies critical host cell regulators of *Trypanosoma cruzi* infection

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Establishment of intracellular infection in mammalian host cells by *Trypanosoma cruzi* is a multi-step process that serves to target extracellular parasites to the host cell cytoplasm where replication occurs. While significant progress has been made over the past 20 years in our understanding of the molecular and cellular basis for host cell invasion by *T. cruzi* trypomastigotes, we have little information regarding the biology of the intracellular amastigote stage of *T. cruzi*. In particular, the contribution of host cell pathways/processes toward the growth and survival of this obligate intracellular parasite is largely unknown. To identify critical host cell regulators of the *T. cruzi* infection process, a genome-wide RNA interference screen was conducted in HeLa cells where knockdown of host cell gene expression was achieved by transfecting HeLa cells with an arrayed Dharmacon siGENOME siRNA library containing 26,136 siRNA pools in 384-well plates. Transfected cells were infected with *T. cruzi*-β-galactosidase and the impact of gene knockdown on parasite infection was assessed by measuring relative intracellular parasite abundance (BetaGlo) in a multiplex assay system that also determined host cell viability (CellTiter-Fluor) at 72 hours post-infection (hpi). Hits from the primary screens were screened in secondary assays conducted at 18 hpi and 72 hpi to identify host cell genes preferentially associated with regulation of trypomastigote invasion/pre-replication events or with intracellular amastigote growth. Considering these data in the context of existing transcriptional data sets, we present an integrated view of critical host cell pathways and cellular networks that support aspects of intracellular *T. cruzi* infection, with a focus on those that support amastigote replication.

The regulation of the sub-cellular localisation of GPI-PLC

Jack Sunter, Helena Webb, Mark Carrington*

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GPI-PLC catalyses the release of the VSG from the plasma membrane of bloodstream form trypanosomes by hydrolysing the phosphoglycerol bond in the GPI anchor. The VSG is not shed at a significant rate in live cells; this is thought to be the result of the separation of GPI-PLC and the VSG. GPI-PLC is found predominantly on the flagella membrane; a three-cysteine motif has been shown to be palmitoylated in Xenopus oocytes and mutation of all three residues affected the ability of GPI-PLC to access the VSG substrate. Here, the relationship between sub-cellular localisation and access to the VSG substrate has been investigated through expression of GPI-PLC mutants in a null background. When all the cysteines within the motif are mutated to serines, GPI-PLC is cytoplasmic and unable to release the VSG coat on hypotonic lysis, yet it is still enzymically active. The localisation of GPI-PLC to the membrane appears to be essential for access to the VSG on hypotonic lysis. The correct pattern of modifications of the cysteine motif is required for successful trafficking of GPI-PLC: as the number of cysteines is reduced from three to one GPI-PLC becomes localised to the endosomal system. However, acylation of the cysteine residues is not sufficient as there is a further localisation signal close to the C-terminus that is necessary for the localisation of GPI-PLC to the flagella membrane. Mutation of a single proline results in the failure to concentrate on the flagella membrane. The concentration on the flagella membrane requires a functional flagella pocket collar but does not require flagella attachment.

Trypanosoma brucei Rab7 regulates lysosomal delivery of endocytosed but not newly synthesized proteins

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We have previously demonstrated that the terminal lysosome is the default destination for secretory cargo in bloodstream form *Trypanosoma brucei*. To examine the role of the late endosome in this process, we now study the small GTPase TbRab7. In mammalian cells Rab7 is localized in the late endosome and is critical for lysosomal delivery of both endocytosed cargo and newly synthesized endogenous proteins. Rab7 function has not been investigated in *T. brucei* but it has been localized to the late endosome (Engstler et al., J Cell Sci. 2004). RNAi results in growth arrest (24 hr) and eventual death indicating that TbRab7 is essential. Ablation does not affect the rate of endocytosis, but lysosomal delivery of cargo, including transferrin, tomato lectin (TL) and trypanolytic factor (TLF) is blocked. TL but not TLF binding was reduced, suggesting that steady state distribution of flagellar pocket receptors are differently affected by TbRab7 silencing. Killing by TLF is greatly reduced confirming that the lysosomal environment is critical to the function of this innate immune factor. Unexpectedly, TbRab7 silencing has no effect on normal trafficking of the endogenous lysosomal proteins TbCatL and p67, suggesting that lysosomal delivery of endocytosed and endogenous proteins occur via different routes and/or are regulated differently, unlike in mammalian cells. Trafficking of the default reporters p67ΔTM and VSGΔGPI was also unaffected. Finally, preliminary data using a conditional knockout approach reveal that TbRab7 is non-essential in the procyclic insect form, although growth rate is reduced 40%. Further studies will investigate the role of TbRab7 in and the importance of lysosomal delivery of endocytic cargo in procyclic cells.

ABSTRACTS: SESSION II

Drug Development

April 9 8:45 a.m.

Chair(s) - Michael Ferguson

Drug Discovery for Neglected Diseases - Challenges, Opportunities and Lessons Learned

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Better, safer and more effective drugs are required for the treatment of many neglected tropical diseases. This talk will provide an overview of recent developments in my laboratory in collaboration with the Drug Discovery Unit to meet the unmet medical needs in the treatment of late stage human African trypanosomiasis. The strengths and weaknesses of target-based versus cell-based (phenotypic) screening will be illustrated with reference to studies on key enzyme targets in folate / pterin metabolism and enzyme targets in trypanothione metabolism; and from quinols and opioid agonists identified by phenotypic screening.

Identification of a novel lead against African sleeping sickness, exploratory chemistry and preliminary target identification studies

Deuan C. Jones*, Stephen Patterson, Laste Stojanovski, Victoria C. Smith, Laura AT Cleghorn, Ian H. Gilbert, Kevin D. Read, Julie A. Frearson, Alan H. Fairlamb

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As part of the early-stage drug discovery process we are engaged in identifying new drug targets and leads in *Trypanosoma brucei*, the causative agent of African sleeping sickness. Sleeping sickness results in an estimated 30,000 deaths per annum and related veterinary diseases have massive economic impact. Existing treatments are hampered by difficulties in administration, cost, duration of treatment, toxicity and resistance. *In vitro* phenotypic screening of a library of pharmacologically active compounds identified 33 compounds with nanomolar EC_{50} values against bloodstream-form *T. brucei* cells. These inhibitors of *T. brucei* growth included compound classes with activity against protein kinases, topoisomerases, tubulin, as well as DNA and energy metabolism. The novel inhibitor (60 nM) with greatest selectivity in reference to human MRC5 cells (270-fold) was (+)-U50488. Surprisingly (-)-U50488, a known CNS-active k-opioid receptor agonist was $>$70-fold less active, as were several u- and k-opioid antagonists. Although (+)-U50488 was well tolerated by the oral route and displayed good pharmaceutical properties, including high brain penetration, the compound was not curative in the mouse model of infection. Nonetheless, the divergence of antinociceptive and antitrypanosomal activity appears to present a novel mode of antitrypanosomal action. The antitrypanosomal activity of the compound is highly specific to bloodstream-form *T. brucei*, and the structure-activity relationship has been the subject of an exploratory chemistry programme. In order to identify molecular targets of (+)-U50488, we have generated a resistant cell-line and produced chemical analogues amenable to affinity purification techniques.

Post-translational modifications as drug targets in tryps

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Post-translational modifications (PTMs) like phosphorylation, glycosylation and lipidation, make interesting drug targets. They tend to have pleiotropic effects (i.e. multiple downstream targets) such that inhibition of PTMs tends to be 'cidal' rather than 'static' because energy metabolism and protein synthesis continue as normal but the newly synthesized proteins are fatally damaged goods. Examples of the identification and characterization of potential drug targets in protein N-glycosylation, sugar nucleotide biosynthesis and protein N-myristoylation will be discussed.

Discovery and Optimization of a Novel Oxaborole-6-Carboxamide to Treat Late-stage Nervous System Human African Trypanosomsiasis

Bakela Nare[*,1], Stephen Wring[1], Cyrus Bacchi[2], Reto Brun[3], Jacob Plattner[4], Beth Beaudet[1], Tana Bowling[1], Daitao Chen[1], Yvonne Freund[4], Eric Gaukel[1], Matthew Jenks[1], Marcel Keiser[3], Luke Mercer[1], Andy Noe[1], Matt Orr[1], Robin Parham[1], Ryan Randolph[1], Cindy Rewerts[1], Jessica Sligar[1], Nigel Yarlett[2], Robert Don[5], Robert Jacobs[1]

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Human African trypanosomiasis (HAT) represents a significant public health problem in sub-Saharan Africa affecting hundreds of thousands of individuals. An urgent need exists for the discovery and development of new, safe, and effective drugs to treat HAT, as existing therapies have poor safety profiles, difficult treatment regimens, limited effectiveness, and a high cost of goods. From a collaborative discovery effort between SCYNEXIS, Anacor Pharmaceuticals, Pace University, and DNDi, we have identified a novel class of small boron-containing compounds, the oxaboroles. An optimized lead from this class, SCYX-7158, is active *in vitro* against relevant strains of *Trypanosoma brucei*, including *T. b. rhodesiense*, and *T. b. gambiense*. The compound is safe and effective against all disease stages in mouse models. Physicochemical and *in vitro* ADME properties of SCYX-7158 are consistent with the compound being orally available, metabolically stable, readily CNS permeable and with low risk for drug-drug interactions. SCYX-7158 is effective orally at doses as low as 12.5 mg/kg (QD per 7 days) against CNS disease in mice. *In vivo* pharmacokinetic characterization of SCYX-7158 shows that the compound is highly bioavailable in rodents and non-human primates, has low plasma clearance, a 24-hr elimination half-life, and a volume of distribution that indicates good tissue penetration. Brain exposure of SCYX-7158 in rodents is high, with CSUB¿max¡/SUB¿ higher than 10 μg/mL and AUC_{0-24hr} greater than 100 $\mu g^*hr/mL$ following a 25 mg/kg oral dose. Furthermore, SCYX-7158 readily distributes into the CSF to achieve therapeutically-relevant concentrations. Based on these

properties, which promise lower rates of recrudescence than with current standard of care, SCYX-7158 has been progressed into preclinical evaluation for treatment of stage 2 HAT.

Cyclic nucleotide-specific phosphodiesterases: new drug targets for old diseases?

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All kinetoplastid genomes analyzed to date contain a similar set of four different cyclic nucleotide specific phosphodiesterases (PDEs), PDEA through PDED. The gene for PDEB has undergone duplication before kinetoplastid radiation and is present in two tandem copies that code for strongly conserved but functionally distinct PDEs. Earlier experiments in T. brucei have demonstrated that functional inactivation of TbrPDEB1 and TbrPDEB2 by RNAi leads to lysis of bloodstream trypanosomes in culture, and to the rapid elimination of ongoing infections in a mouse model. Pharmacological inhibition of these PDEs might represent an attractive strategy to develop a new generation of trypanocidal drugs. The catalytic domains of trypanosome and human PDEs are structurally highly conserved, as demonstrated by X-ray crystallography. This extensive structural conservation between human and parasite PDEs will allow the exploitation of current technology and expertise already developed for human PDE inhibitors. High-throughput screening of a proprietary compound library (400,000 compounds) produced several chemical classes of inhibitors. One of the hits, the tetrahydrophthalazinone Cpd A is presented in this study. It causes a rapid, sustained elevation of intracellular cAMP, cell proliferation is inhibited immediately, and cell lysis occurs within 2 - 3 days. Cpd A does not directly affect DNA synthesis or mitosis, but prevents cytokinesis, resulting in multinuclear, multikinetoplastid, multiflagellar cells that eventually lyse. Beyond the pharmacological validation of trypanosomal PDEs as drug targets, our findings highlight the general potential of targeting enzymes that are highly conserved between host and parasite.

Target Repurposing: Identification of trypanosomal TOR/PI3K as a therapeutic approach

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The repurposing of known pharmaceuticals for treatment of trypanosomal infections can speed up the process of new drug discovery. The trypanosomatids *Trypanosoma brucei, Trypanosoma cruzi,* and *Leishmania major* possess multiple target of rapamycin (TOR) enzymes that are essential for cell growth and virulence. The mammalian homolog, mTOR, has been the focus of many industrial drug discovery programs for the treatment of cancer. Thus, there is a rich medicinal chemistry history associated with mTOR with many details regarding optimal chemical matter. We describe the profiling of established inhibitors of the kinase domain of the mammalian Target of Rapamycin (mTOR) and human phosphoinositide-3-kinases (PI3Ks) against cultures of *T. brucei, Trypanosoma cruzi,* and *Leishmania sp.* and in *in vivo* models of infection. From this profiling we have identified three highly potent inhibitors of parasite growth that represent excellent starting points for drug discovery. Our findings include NVP-BEZ235, a Phase II clinical candidate for oncology, is a sub-nanomolar inhibitor of *T. brucei gambiense* and *rhodesiense* growth that also displays *in vivo* efficacy.

Identifying new targets for drug development against Trypanosomatids

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Human African Trypanosomiasis, Chagas'disease and Leishmaniasis, caused by related kinetoplastid pathogens, are responsible for extensive suffering, death and economic burden in developing countries. The few drugs approved to treat these diseases were developed generations ago and are inadequate, toxic and the parasites are developing drug resistance. The Trypanosomatid Drug Discovery Consortium (TDDC) is a collaborative group that links investigators at public and private institutions who have committed themselves to the discovery and development of new anti-trypanosomatid drugs. Scientists at Seattle BioMed, as part of the TDDC, are involved in the identification and validation of new drug targets following a system-based approach. 65 putative enzymes, most of which belong to the phosphatase, kinase or tRNA synthetase families or to the GPI pathway, were assessed for essentiality by RNAi in the bloodstream form of *Trypanosoma brucei*. 15 enzymes showed a growth defect upon RNAi induction *in vitro*. They include several tRNA synthetases, a peptidyl-prolyl cis-trans isomerase, a phosphatase, a kinase, an adenylosuccinate synthetase and a fructose bisphosphatase. *In vivo* essentiality and assay development are currently being explored.

Novel drug uptake and potential resistance mechanisms in African trypanosomes: Suramin

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A better understanding of drug uptake and potential mechanisms of clinical resistance will facilitate the design, application and assessment of more effective therapies. We have carried out loss-of-function screens for drug resistance mechanisms in African trypansomes; all five drugs in clinical use against Human African Trypanosomiasis have been screened using genome-scale RNA interference libraries. The approach was validated by the identification of the nitro pro-drug activator, NTR, and the eflornithine transporter, AAT6 (1). Several novel genes have now been identified, and characterization of suramin uptake will be described; although suramin treatment failures have been reported, no molecular mechanism of clinical or experimental resistance has been documented. Suramin uptake appears to be via receptor mediated endocytosis, and data will be presented to demonstrate roles for an invariant surface glycoprotein, an endosomal membrane channel and the ubiquitin pathway. (1) Baker, Alsford & Horn (2011) Genome-wide RNAi screens in African trypanosomes identify the nifurtimox activator NTR and the eflornithine transporter AAT6. *Molecular & Biochemical Parasitology* **176** 55-57.

Distinct drug-resistant *T. cruzi* clones can arise independently in a single population undergoing benznidazole-selection

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Benznidazole and nifurtimox are the front line drugs used to treat *Trypanosoma cruzi* infections. Both are pro-drugs and are activated within the parasite by a mitochondrial type I nitroreductase (TcNTR). Loss or disruption of a single copy of this gene is sufficient to cause cross-resistance. Here, we demonstrate that distinct cross-resistant clones can arise independently within a single population undergoing benznidazole treatment *in vitro*. Following selection of benznidazole-resistant parasites, CHEFE analysis of DNA from both clonal and polyclonal populations showed that all cell lines examined had lost one of the chromosomes containing the *TcNTR* gene (the smaller homologue in each case). Sequence analysis of the remaining *TcNTR* allele revealed the presence of three distinct mutant genes in different resistant clones, each arising from missense mutations. Recombinant expression studies showed that, unlike the enzyme from the parental sensitive strain, those from the resistants were unable to reduce benznidazole. This correlated with a loss FMN-binding capacity. The drug-resistant phenotype could be reversed by transfection with an active copy of the *TcNTR* gene. These results confirm TcNTR as a central player in the development of resistance to the therapeutic drugs used against Chagas disease. Worryingly, they also demonstrate that *T. cruzi* has an innate propensity to undergo genetic changes that can readily lead to drug-resistance.

ABSTRACTS: SESSION III

RNA

April 9 1:45 p.m.

Chair(s) - Torsten Ochsenreiter

RNA interference libraries for high throughput phenotyping and drug-resistance studies in the African trypanosome

David Horn[*,1], Daniel J. Turner[2], Sabine Eckert[2], Samson O. Obado[1], Alejandro Sanchez-Flores[2], Lucy Glover[1], Matthew Berriman[2], Christiane Hertz-Fowler[2], Nicola Baker[1], Sam Alsford[1]

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A high throughput phenotyping approach termed RNA interference (RNAi) target sequencing, or RIT-seq will be described that, using Illumina sequencing, maps fitness-costs associated with RNAi. We scored the abundance of >90,000 integrated RNAi targets recovered from trypanosome libraries before and after induction of RNAi. Data were generated for 7,435 protein coding sequences, >99% of a non-redundant set in the *Trypanosoma brucei* genome. Analysis of bloodstream and insect life-cycle stages and differentiated libraries revealed genome-scale knockdown profiles of growth and development, linking thousands of previously uncharacterised and 'hypothetical' genes to essential functions. Genes underlying prominent features of trypanosome biology are highlighted, including the constitutive emphasis on post-transcriptional gene expression control, the importance of flagellar motility and glycolysis in the bloodstream, and of carboxylic acid metabolism and phosphorylation during differentiation from the bloodstream to the insect-stage. Lipid metabolism and integral membrane proteins are implicated in differentiation and insect-stage growth respectively. RIT-seq represents a versatile new tool for genome-scale functional analyses and the current data-set provides much needed genetic validation to identify new drug-targets. We have also used a related approach to identify drug-uptake and resistance mechanisms and this work will also be briefly described.

The role of RNA interference in the virulence of *Leishmania braziliensis*

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RNAi has been lost at least twice in trypanosomatids, once in the lineage leading to *Trypanosoma cruzi* following its divergence from the African trypanosome branch, and in *Leishmania* following the divergence of the subgenus *Viannia*. To probe the forces impacting upon the retention or loss of RNAi in *Leishmania* evolution, we generated an *argonaute* null mutant (*ago1*-) of *L. braziliensis*, and a complemented derivative. As expected, *ago1*- mutants showed loss of RNAi activity in tests with Luciferase or GFP-based RNAi activity reporters, while *ago1*-/+*AGO1* lines show WT RNAi. *ago1*- parasites showed elevated levels of transcripts arising from transposable element families as well. The *ago1*- promastigotes grew at WT rates with no apparent cytological abnormalities, and to WT stationary phase densities, where they differentiated to metacyclics normally. Expression profiling using microarrays or RNA-seq showed little difference in protein-coding gene expression between WT or *ago1*- in either promastigote stage. We used several mouse models and bioluminescent imaging to study disease pathology and host response *in vivo*. Remarkably, *ago1*- parasites were much less virulent than WT or *ago1*-/+*AGO1* lines, as defined by the time required to induce lesion pathology or metastasis. Strikingly, the phenotypic changes seen in mouse infections by WT vs. *ago1*- parasites show parallels with those seen in mucocutaneous vs. cutaneous leishmaniasis caused by species of *Viannia* vs. *Leishmania*.

Modification of siRNAs by HEN1 methyltransferase reveals diversification of the RNAi mechanism between *T. brucei* and *L. braziliensis*

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Central to the RNAi interference (RNAi) pathway is the assembly of a ribonucleoprotein complex consisting of an Argonaute (AGO) endonuclease and a small interfering RNA (siRNA) that guides target transcript degradation. Biogenesis of the mature RNP begins in *Trypanosoma brucei* with cleavage of long dsRNA into duplex 24-26 nt siRNAs by one of two Dicer enzymes, followed by the action of RIF4, a 3'-5' exonuclease that processes duplex siRNA to single-stranded molecules and loads guide siRNAs into *Tb*AGO1. Mature siRNAs bear a modified 3' terminus. Here, we report that a HEN1-like methyltransferase is responsible for this modification. In its absence, siRNAs are no longer modified and their average size is reduced (17-26 nt compared to 24-26 nt), consistent with nucleolytic nibbling of unmodified siRNAs, and with 3' modification protecting siRNAs as in other systems. Since duplex siRNAs, which accumulate in RIF4-null cells, are unmodified, HEN1 probably acts concomitantly with or after RIF4. Intriguingly, we found that in HEN1-null cells all size classes of siRNAs are associated with *Tb*AGO1. Since only modest RNAi downregulation is observed in HEN1-null cells, shorter siRNAs might guide target RNA cleavage. Surprisingly, *Leishmania (Viannia) braziliensis* siRNAs are unmodified, and the *L.V. braziliensis* genome does not appear to code for HEN1, revealing a fundamental difference between *Trypanosoma* and *Leishmania* RNAi mechanisms. Modification of guide siRNA has been proposed to safeguard the 3' end, which in certain AGOs may be dislodged from the PAZ domain during target cleavage, becoming accessible to nucleotydyltransferases and exonucleases. We propose that *Tb*AGO1 and *Lb*AGO1 have different catalytic properties, a theory supported by the finding that *Lb*AGO does not complement *Tb*AGO1 deficiency.

Alternative trans-splicing determines the localization of the dually targeted IleRS of *T. brucei*.

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A full set of aminoacyl-tRNA-synthetases (aaRS) is essential for all translationally active compartments. In eukaryotic cells all mitochondrial aaRSs are nuclear encoded, synthesized in the cytosol and finally imported into mitochondria. In *T. brucei* only the LysRS, AspRS and TrpRS are encoded by two nuclear genes, all remaining aaRSs derive from single genes and are dually localized to the mitochondria and the cytosol. Bioinformatic analysis of the IleRS ORF from *T. brucei* predicts a mitochondrial targeting sequence. However, how the dual localization of the IleRS is achieved is unknown. Genome wide analyses of alternative splicing uncovered two splice acceptor sites for the IleRS mRNA. This lead to the hypothesis that alternative splicing may regulate the localization of the IleRS in that the longer mRNA, which codes for the presequence-containing IleRS, is imported into mitochondria whereas the shorter version would give raise to a IleRS that remains in the cytosol. In line with this idea two mRNAs were detected on Northernblots. Moreover, epitope-tagging followed by cell fractionation detected a long and a short IleRS variant that were found exclusively in the mitochondrial and the cytosolic fraction, respectively. Finally, RNAi targeting the ORF resulted in the ablation of both IleRS variants, whereas if the RNAi was targeting the 5'-UTR that is only present in the long IleRS mRNA only the mitochondrial variant of the IleRS was affected. These results strongly suggest that alternative transsplicing regulates the localization of the trypanosomal IleRSs.

RNA-binding proteins orchestrating *T. brucei* gene expression during differentiation in tsetse flies: the case of RBP6

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RNA-binding proteins have been implicated as important regulators of gene expression in trypanosomes. However, direct evidence is sparse, in particular during the developmental cycle in the fly vector. To determine mRNA abundance profiles for the *T. brucei* transcriptome during life cycle stages in the insect vector, we performed RNA-seq analysis of infected tsetse tissues. Midgut, proventriculus and salivary gland datasets will be discussed. Proventriculus trypanosomes showed high mRNA levels for the RNA-binding protein RBP6, suggesting a possible function during differentiation into epimastigotes. To investigate the role of RBP6 in RNA metabolism and regulation of gene expression, we studied its behavior in procyclic form cultures. GFP-tagged RBP6 displayed a localization pattern characteristic for cytoplasmic mRNPs, implying a role in mRNA stability or regulation of translation. Systematic Evolution of Ligand by EXponential enrichment (SELEX) was performed with recombinant RBP6 and yielded the consensus RNA-binding site 5'-CU(U/A)AU(G/A)-3'. This sequence was found present in the 3' untranslated region of a large number of transcripts enriched in an RBP6 immunoprecipitate, e.g. the mRNAs from the BARP genes with high abundance in salivary glands. To test whether RBP6 affects the stability of its target transcripts, we performed RNA-seq transcriptome analysis of procyclic cell lines inducibly expressing RBP6. Surprisingly, transcripts that are putative targets of RBP6 showed no change in abundance upon induction of RBP6 expression. Our results highlight the combinatorial mode of action of RNA-binding proteins in post-transcriptional regulation of gene expression in trypanosomes and suggest that RBP6 does not affect the stability of its mRNA targets. One possible mechanism for its action is enhancement of translation for bound mRNAs during differentiation to epimastigotes.

The Role of RBP42, a newly identified RNA binding protein, in Trypanosoma brucei

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Post transcriptional control modulated by RNA binding proteins plays a pivotal role in controlling gene expression in trypanosomes. However, we are just beginning to understand the set of RNA binding proteins, and how they function, in these parasites. We have identified an RNA binding protein, RBP42, which was not recognizable by homolog searches of the trypanosome genomes. RBP42 is essential for procyclic T. brucei parasite survival, localizes to both the nuclear periphery, in a punctate manner, and to the cytoplasm. RBP42 appears to function in mRNA metabolism, as it is tightly associated with polysomes in cell fractionation studies. Finally, cells depleted of RBP42 appear to have a cytokinesis defect. We have found that RBP42 binds to cellular mRNAs in vivo. These experiments were done by irradiating cells with ultra-violet light, capturing polyA-containing RNA-protein complexes (polyA-RNPs) using oligo-d(T) affinity chromatography, and detecting RBP42-RNA complexes using specific anti-RBP42 antibodies on Western Blots. Our goal now is to determine the global population of cellular mRNAs that are bound by RBP42 and how these interactions contribute to cellular metabolism. To this end, we have used RNA-CLIP methodologies to purify the crosslinked RNA using RBP42 specific antibody. Initial sequencing of 50 representative cDNA clones from the RNA-CLIP material indicates that RBP42 binds a diverse, but limited, set of mRNA. In all cases RBP42 interacts within the coding region of mRNAs, likely at a consensus CC(A/G)C(A/G) sequence.

Global and gene-specific analysis of the targets of the post-transcriptional gene regulator *Tb*ZFP3

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*Tb*ZFP3 is a verified trans-acting post-transcriptional regulator of Procyclin surface antigen expression in Trypanosoma brucei. To provide additional insight into the function(s) of the *Tb*ZFP3 regulatory complex during trypanosome differentiation, a global analysis of the transcripts associated with this mRNP was carried out by RNA immunoprecipitation (RIP) from procyclic forms. The association of the selected transcripts with the *Tb*ZFP3 mRNP was then verified and their quantitative enrichment with respect to unselected mRNA determined by SOLEXA sequencing, gene tags being aligned with ORF and 3'UTR data available from the tritrypdb.org website. Validating our previous findings, both *gpeet* and *ep1* procyclin mRNAs displayed enrichment demonstrating their consistent association with *Tb*ZFP3, while *ep2* procyclin mRNA was not selected. Interestingly, the *Tb*ZFP3-selected transcripts appeared to consist of many that are enriched in the transmissible stumpy form. Quantitative RT-PCRs validated the association of a set of the enriched transcripts with *Tb*ZFP3mRNP, whereas both qRT-PCR and Northern blot analysis demonstrated that *Tb*ZFP3 also regulates these target transcripts. Specifically, *Tb*ZFP3 overexpression increased the abundance of each mRNA, this being dependent upon the integrity of its CCCH predicted RNA-binding domain. Moreover, analysing reporter constructs revealed that this regulation is mediated through signals in the 3'UTR of the target transcripts. Unlike *gpeet* and *ep1* targets, however, increased levels of *Tb*ZFP3 do not detectably alter protein expression of tested selected transcripts in procyclic forms. Apparently, the association of different transcripts with *Tb*ZFP3 can generate diverse and target-specific effects on regulated expression. We suggest that this might be determined by a combination of mRNA sequence and *Tb*ZFP3mRNP diversity.

The small RNome of Trypanosoma brucei:-novel RNAs and deciphering the role of parasite specific snoRNAs and their maturation by differential polyadenylation

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The discovery of small non-coding RNAs present from bacteria to man has completely changed our understanding of how gene expression is regulated. Relatively little is known about the repertoire of these RNAs in trypanosomes. In this study, we employed the power of deep sequencing of RNA (RNA-seq) to examine the repertoire of ncRNAs RNP particles of Trypanosoma brucei. In addition to the known ∼200 small RNAs, a large repertoire of novel ncRNA and 142 C/D and H/ACA snoRNAs was revealed. The unique processing of pre-rRNA in these parasites is still poorly understood. Here, we identified highly abundant and trypanosome-unique snoRNAs that carry out conventional processing events as well as snoRNAs that mediate trypanosome-specific cleavages to produce the large subunit rRNA with its associated small rRNAs. Additionally, we found that polyadenylation is essential for processing of snoRNAs from polycistronic precursors, and that the most abundant snoRNA species require the action of two poly(A) polymerases for their maturation. A model for the concerted action of PAP1 and PAP2 together with the nuclear exosome and the MTR4 helicase will be presented. We will present data showing how using a battery of approaches, including cell fractionation, in situ hybridization, association in RNP complexes and interaction with other cellular RNAs, we elucidate the function of novel ncRNAs that do not belong to conventional RNA families. Finally, we have discovered that the abundance of certain ncRNAs varies between procyclic and bloodstream forms, suggesting that ncRNAs may contribute to gene regulation during the complex parasite's life cycle. This is the first study to provide a whole-genome analysis of the large repertoire of small RNPs in trypanosomes

ABSTRACTS: SESSION IV

Gene Expression

April 10 8:45 a.m.

Chair(s) - Christine Clayton

Base J: history, distribution, biosynthesis and function

Piet Borst*[,1], Saara Vainio[1], Sabrina Jan[1], Pankaj Tripathi[1], Paul-Andre Genest[1], Bas Ter Riet[1], Iris Wong[2], Stephen M. Beverley[2], Farris Carol[3], Peter J. Myler[4], Arno Velds[1], Ron M. Kerkhoven[1], Marja Nieuwland[1], Tatjana Heidebrecht[1], Anastassis Perrakis[1], Henri G.A.M. Van Luenen[1]

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Base J (glucosyl-hydroxymethyluracil) replaces about 1% of T in the nuclear DNA of kinetoplastid flagellates, such as *Leishmania*. The first step in J biosynthesis, the hydroxylation of selected T-residues, is catalyzed by two enzymes, J-Binding Protein 1 (JBP1) and JBP2[1]. JBP1 is essential in *L. tarentolae*[2]. In the absence of JBP2 *L. tarentolae* becomes hypersensitive to growth in BrdU[3]. We have shown that this is associated with loss of the intra-chromosomal J that marks transcriptional termination sites and with massive read-through of the normal RNA polymerase II stops and cell death. We have now found that the loss of J in *JBP2*$^{-/-}$ *L. tarentolae* and associated pathological effects are reversed by the introduction of an ectopic copy of histone H3, and exacerbated by an ectopic copy of the gene for histone variant H3V. H3V is preferentially associated with J-containing chromatin in *T. brucei*[4] and experiments are underway to verify that this is also the case in *Leishmania*. We speculate that JBP2 is primarily required to introduce J into H3V-containing nucleosomes and that JBP1 is unable to do so efficiently. If H3V is displaced by H3, JBP1 can take over from JBP2 and restore normal J levels resulting in normal transcriptional termination. We are doing the experiments to test this speculation. References (1) Borst P and Sabatini R. Ann Rev Microbiol 2008;62:235-51 (2) Genest PA et al. Nucleic Acids Res 2005;33:1699-709 (3) Vainio S et al., J. Mol Biochem Parasitol 2009;164:157-61 (4) Cliffe LJ et al., Nucleic Acids Res 2010;38:3923-35

Base J is required for proper termination of RNA polymerase II transcription

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Genomic DNA of trypanosomatids contains a hypermodified base, beta-D-hydroxymethyluracil or J, that replaces a fraction of thymines (Gommers-Ampt et al., Cell, 1993). In *Leishmania*, base J is found almost exclusively at telomeres (Genest et al., Nuc. Acids Res. 2007). The approximately 1%, chromosomal internal J has now been mapped by the isolation of DNA fragments containing J using anti-J DNA antibodies or a protein that specifically binds J DNA (JBP1, Cross et al. EMBO J. 1999) followed by high throughput sequence analysis of the isolated fragments. We find that J is located at the transcription termination region between convergent protein-coding gene clusters in *L. major* and *L. tarentolae*. J is also found upstream of all presumed internal transcription initiation sites within protein-coding gene clusters, which are marked by acetylated histone H3 (Thomas et al., BMC Genomics, 2009), and at some transcription initiation sites between divergent protein-coding gene clusters, especially those with a relative long distance between transcription starts. Finally, J is found flanking the rRNA repeat cluster, and upstream of the mini-exon locus. Reducing J in the genome of *L. tarentolae* results in massive read-through at transcription termination sites. The read-through leads to a genome wide alteration of transcript levels, which provides a plausible explanation why *Leishmania* parasites die when J is lost. The specific localization of J and the read-through observed when J is reduced strongly suggests that J is required to terminate RNA polymerase II transcription at specific locations in the genome. This conclusion is supported by read-through transcription at the only divergent strand-switch region in wild type *L. major* and *L. tarentolae* that does not contain base J.

Epigenetic regulation of gene expression and virulence in Trypanosoma cruzi

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The protein coding genes of Trypanosoma cruzi are arranged into large polycistronic transcription units (PTU) transcribed by polymerase II. Consistent with our work in T. brucei and Leishmania, we have localized the glycosylated thymine DNA base (base J) at regions flanking PTUs throughout the T. cruzi genome, implicating its role in regulating Pol II transcription initiation/termination. To investigate this further, we generated mutant T.cruzi cell lines with 20-fold and 8-fold reduced levels of base J following the deletion of the J-biosynthesis enzymes (JBP1 and JBP2, respectively). Both microarray and whole-genome transcriptome analysis indicate genome-wide changes in gene expression following the loss of J at the PTU flanks. These changes include activation and increased expression of surface proteins involved in virulence. Accordingly, these mutants display significant changes in parasite invasion and egress. In vivo 3H-uridine incorporation, mRNA stability and nuclear run-on analysis indicate that changes in gene expression are due to an increased Pol II transcription rate throughout the PTU with no effect on termination. Analysis of nucleosome abundance and specific modified histones demonstrate both the formation of open chromatin and increased levels of AcH3/AcH4 at promoter regions following the loss of J. In contrast, no changes in chromatin structure are detected at termination regions. The differential affects of base J on structure of chromatin at different regions of the genome may explain the differential affects of base J on Pol II transcription initiation verses termination. These studies not only provide a function for the novel modified DNA base but also one possible mechanism of epigenetic regulation of transcription in T. cruzi.

Transcriptional Regulation of an RNA Polymerase II Transcription Unit in *Trypanosoma brucei brucei*

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The animal pathogen *Trypanosoma brucei brucei* is sensitive to a subclass of human high-density lipoproteins named the trypanosome lytic factor (TLF-1). Susceptibility to TLF-1 requires trypanosome binding and uptake, mediated by a high affinity haptoglobin/hemoglobin (Hp/Hb) receptor and subsequent trafficking of TLF-1 to the lysosome. *In vitro* growth of *T. b. brucei* 427 in the presence of TLF-1 selected for a cell line that lacks expression of Hp/Hb receptor and is completely resistant to killing by TLF-1. Here we show that loss of Hp/Hb receptor expression in these cells is not regulated at a post-transcriptional level. Stability of ectopically expressed Hp/Hb receptor mRNA, with endogenous 5' and 3' untranslated regions (UTR's), was unchanged in cells that do not show expression of the endogenous Hp/Hb receptor. In contrast, we observe that all neighboring genes present in the divergent poly transcription unit (PTU) are not expressed. Nuclear run-on analysis showed that transcription throughout this divergent PTU is sensitive to α-amanitin, indicative of transcription by RNA polymerase II and that TLF-1 resistant cells lack transcription throughout the entire PTU. Furthermore, we have not observed any mutations in the divergent promoter region, nor have we detected any epigenetic changes in chromatin structure associated with the change in transcriptional state. We postulate that the lack of bidirectional transcription within the PTU in TLF-1 resistant cells maybe caused by premature termination or elongation defects. To our knowledge, this is the first example of regulated gene expression in African trypanosomes at the level of polymerase II transcription initiation/ elongation and may prove to be a new mechanism for the regulation of gene expression in african trypanosomes.

The FACT chromatin remodelling complex affects chromatin structure at promoter regions of silent VSG expression sites

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Bloodstream form *Trypanosoma brucei* is covered with a dense coat of Variant Surface Glycoprotein (VSG), which is expressed from one of about 15 telomeric VSG expression sites (ESs). We have recently shown a link between chromatin structure and the monoallelic transcription of VSG ESs. We have also demonstrated that chromatin remodelling proteins and complexes like ISWI, NLP and FACT (FAcilitates Chromatin Transcription) are involved in downregulation of VSG ESs. Previously we have reported that depletion of Spt16, the large subunit of the FACT chromatin remodelling complex, results in a cell cycle specific derepression of silent VSG ESs in G2/M phase. Spt16 binds preferentially to the promoter region of silent VSG ESs, but is depleted from the active ES. Tandem affinity purification (TAP) of Spt16 revealed that the FACT complex in *T. brucei* is composed of two subunits, Spt16 and Pob3. Depletion of Pob3 also results in derepression of silent VSG ESs. Using chromatin immunoprecipitation (ChIP), we show that knockdown of Spt16 leads to a decrease of the histones H2A and H3 immediately downstream of the promoter region of silent VSG ESs. Additionally, we observe an increase in sensitivity to MNase digestion specifically at this locus. In contrast, MNase sensitivity of the telomeres of both silent and active VSG ESs does not change, suggesting additional control mechanisms maintaining these genomic regions in their respective activation states. Depletion of Spt16 has no effect on MNase sensitivity of other actively transcribed loci. Interestingly, the nucleosome distribution at pol II transcribed genes appears to be more similar to that of silent VSG expression sites rather than highly transcribed pol I transcription units.

Trypanosoma brucei Tin2 Suppresses VSG Switching Rate

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Trypanosoma brucei, the causative protozoan parasite for Human African Trypanosomiasis, evades the host immune response by regularly switching its surface antigen - Variant Surface Glycoproteins (VSG). VSGs are exclusively expressed in a monoallelic manner from VSG expression sites (ESs) located at subtelomeric loci. Telomeres are nucleoprotein complexes located at the ends of linear chromosomes. They often form a heterochromatin structure that affects subtelomeric gene expression. In fact, we have found that telomeres are important for subtelomeric VSG silencing, as depletion of a telomere protein RAP1 led to derepression of ES-linked VSG genes. In addition, telomeres protect the chromosome ends from illegitimate DNA processes including degradation, repair and recombination. Because homologous recombination-mediated gene conversion is one of several important mechanisms for VSG switching, it is possible that the telomere structure also influences VSG switching. We have now identified another telomere protein, the *T. brucei* Tin2 homolog, which interacts with the duplex TTAGGG repeat binding factor, TRF, directly. Depletion of Tin2 led to growth arrest but does not seem to affect the silencing of subtelomeric VSG genes. However, using a cell line carrying both a positive and a negative selective marker in the active ES, we found that a temporary depletion of Tin2 led to an increased VSG switching rate, indicating for the first time that the telomere structure is also important for the regulation of VSG switching. In addition, we also found that depletion of Tin2 caused a decrease in TRF protein level without affecting its mRNA amount, indicating that Tin2 is important for TRF protein stability.

Loss-of-silencing (LOS) screen in Trypanosoma brucei: Roles of DNA replication and RNA surveillance in VSG silencing

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Monoallelic VSG expression is a complicated cellular process involving many players and crosstalk between multiple pathways. The complexity suggests that the best way to uncover the molecular mechanism of VSG expression are large-scale genetic screens, which the trypanosome field has been slow to adopt due to technical difficulties. We have performed large-scale mariner transposon-mediated random targeting. The goal of the screen was to identify genes that affect VSG silencing and characterize their roles in monoallelic VSG expression. A silent ES was targeted with a triple-reporter cassette containing puromycin-resistance, luciferase, and emerald-GFP genes, adjacent to an ES-promoter in the procyclic form. Random transposition was induced by transfecting cells with a donor plasmid containing a hygromycin-resistance gene flanked by mariner inverted repeats (Leal S, Acosta-Serrano A, Morris JC, Cross GAM (2004) J Biol Chem 279:28979-88). Cells can only become resistant to hygromycin once the donor cassette is transposed into a transcribed chromosomal orientation. Transpositions that caused the loss of VSG silencing (LOS) were selected with puromycin and tested for luciferase activity, which allows quantification of expression levels of each clone. 75 clones were isolated and they will be referred to as 'los' mutants. Transposon target sites in 27 los clones were mapped. LOS1 interacts with a replication factor. LOS2 appears to be a novel protein and contains a nucleic acid binding domain. We will present LOS screening strategies and preliminary results from LOS1 and LOS2 analyses including the isolation of LOS1 complexes by tandem affinity purification. Our working model of how these proteins might control VSG expression will be discussed.

In *Trypanosoma brucei*, high promoter occupancy of CITFA distinguishes the active from an inactive *VSG* expression site

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Trypanosoma brucei has a multifunctional RNA polymerase I that transcribes ribosomal gene units (*RRNA*) and units encoding variant surface glycoprotein (VSG) and procyclin. In bloodstream forms of the parasite, only one *VSG* gene, drawn from a large repertoire and located at a telomeric expression site (ES), is transcribed in a nuclear compartment termed expression site body. Since trypanosomes harbor ~15 ESs, only one ES is expressed at a time while the others are transcriptionally silenced. De-repression of silent ESs by interference with heterochromatin formation activated silent ESs only partially suggesting that a dedicated activating mechanism is responsible for the extremely high expression level of active *VSG*. We previously characterized the class I transcription factor A (CITFA) which is essential for all RNA pol I transcription and consists of six trypanosomatid-specific subunits (CITFA-1 to -6) and a dynein light chain. We now identified an additional CITFA subunit (CITFA-7) and showed *in vivo* and *in vitro* that this subunit is essential for parasite viability and RNA pol I transcription. Surprisingly, *CITFA-7* expression silencing led to rapid co-depletion of CITFA-2 and CITFA-6 indicating that CITFA-7 is essential for factor integrity. Immunofluorescence microscopy localized CITFA-7 in the nucleolus and ChIP experiments demonstrated that it cross-linked to *RRNA* and *VSG* ES promoters. Most interestingly, using a cell line in which an active and an inactive ES are differently tagged, we discovered that CITFA-7 was predominantly associated with the promoter of the active *VSG* ES. Switching the active site led to a reversal of the occupancy pattern demonstrating that CITFA occupancy is a *VSG* activating mechanism. A model outlining how this might work will be presented.

Stage-specific pathways for translation initiation in *Leishmania* - novel components and unique regulatory models

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The translation machinery in higher eukaryotes is extremely sensitive to environmental stresses, resulting in the replacement of the common cap-dependent translation initiation by cap-independent pathways. We have characterized the various components of the translation initiation machinery in *Leishmani*a, and show that similar to higher eukaryotes, the cap-4 binding complex is temperature sensitive, suggesting that amastigotes must adopt alternative pathways for translation initiation. Trypanosomatids express four paralogs of the cap-binding translation initiation factor eIF4E, all are diverged from their eukaryote counterparts and none of them can genetically complement the yeast eIF4E. LeishIF4E-4 was marked as the conventional translation initiation factor in promastigotes, and anchors the typical cap-4 binding complex. Indeed, this complex is thermosensitive, as it disassembles in amastigotes and upon exposure to mammalian-like temperatures. In addition, expression of all eIF4E paralogs decreases in amastigotes, except for LeishIF4E-1 which is the only isoform that maintains its expression in the mammalian life-form. LeishIF4E-1 is associated with cap-independent translation, since it does not interact with any MIF4G protein at any temperature, but it does pull-down a multitude of other translation factors. We identified a novel and non-conserved 4E-interacting protein (Leish4E-IP) which is unique to *Leishmania* and other trypanosomatids. Leish4E-IP specifically binds LeishIF4E-1 in promastigotes but not in amastigotes, thus it appears to be a translation regulator that is possibly involved in switching between translation initiation pathways. In accordance, we describe a novel model that explains how translation in amastigotes is directed by regulatory elements in the downstream UTR, which melt at elevated temperatures.

ABSTRACTS: SESSION V

Kinetoplast and Mitochondrion

April 11 8:45 a.m.

Chair(s) - Marilyn Parsons

The structure of the Haptoglobin Haemoglobin Receptor and the fate of Apoliprotein L-1

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The uptake of host haptoglobin haemoglobin (HpHb) complexes by a specific receptor provides some of the haem required by bloodstream form trypanosomes. The HpHb receptor (HpHbR) is exploited by the primate-specific innate immunity factor TLF1, a high density lipoprotein particle that contains both a ligand for the HpHbR and the toxin apolipoprotein L1. Endocytosis of TLF1 results in loss of lysosome membrane integrity and thus cell death. Here, the structure of the HpHb receptor has been determined by X-ray crystallography to 1.6 Angstroms. The receptor is an elongated three helical bundle with an axis as long or longer than the VSG. The binding site for HpHb is at one end of the molecule and the putative GPI-anchor site at the other. Two of the helices in the HpHbR are reminiscent of the coiled coil present in VSG N-terminal domains and it is possible that receptors such as HpHbR represent the evolutionary precursors of the VSG. Any receptor on the bloodstream form trypanosome cell surface has to be able to bind its ligand in the context of the variant surface glycoprotein coat and this imposes two apparently opposing constraints on the structure of a receptor, the requirements to: (i) be shielded by the VSG monolayer and (ii) access a large ligand, such as TLF1, that cannot penetrate the VSG coat. The dimensions of the HpHbR indicate that it almost certainly protrudes above the VSG coat. Furthermore, structural predictions for other trypanosome cell surface proteins suggest that the three helical bundle may be a common motif.

The Mechanism of Trypanosome Lytic Factor-1: A Role For Lipid Peroxidation

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Trypanosome lytic factor-1 (TLF-1) is a subset of high-density lipoproteins (HDL) that provides innate immunity against many African trypanosomes. This complex macromolecule consists of a phospholipid monolayer containing embedded apolipoproteins and a hydrophobic core of cholesterol and triglycerides. Haptoglobin related protein (Hpr) and apolipoprotein L-1 (apoL-1) distinguish TLF-1 from non-trypanolytic HDL. Hemoglobin bound to Hpr has been shown to be necessary for receptor-mediated uptake of TLF-1. This complex has also been proposed to play a role in the generation of oxygen radicals and lipid peroxidation. Despite detection of lipid peroxides and the ability of iron chelators and H2O2 depletion to inhibit TLF-1 killing, it has been proposed that apoL-1 alone mediates killing via the formation of ion channels. Here we present evidence for the direct involvement of heme iron in killing and lipid peroxidation within the TLF particle. We propose a model in which TLF-1 lipids are peroxidated prior to apoL-1 mediated targeting to the lysosomal membrane where fusion or transfer of lipids results in the propagation of lipid peroxidation, causing breakdown of the lysosomal bilayer and subsequent cell death.

Getting ahead of African trypanosomiasis: Baboons lead the way

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Some primate species, including our own, cannot be infected with most African trypanosome species due to a trypanolytic factor in our blood. The causative agent of east-African sleeping sickness - *Trypanosoma brucei rhodesiense* is, by definition, resistant to the human trypanolytic factor, which was identified as apolipoprotein L-I (apoL-I). Resistance is conferred by the production of a single trypanosome protein, termed SRA, which binds and neutralizes apoL-I. Nevertheless, it appeared that the sera of some old-world monkey species contained a cryptic lytic-factor that could evade neutralization by SRA and lyse human-infective *T. b. rhodesiense*. We have identified this factor as a divergent apoL-I ortholog, which is common amongst the old-world monkey species that have trypanolytic sera. Using a transgenic mouse model and *in vitro* binding assays, we show that four monkey-specific lysines cooperate to abolish binding to SRA, without compromising trypanolysis. In addition, we found that the appropriate positioning of a single lysine (K4) could reduce SRA binding sufficiently to provide full protection against a human-infective trypanosome infection. A newly discovered deletion in the human apoL-I gene, which was propagated by a selective sweep in sub-Saharan Africa, provides a partially protective imitation of K4 - at a substantial cost to the host.

Adaptive responses to Purine stress in *Leishmania*

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Leishmania are unable to synthesize purines *de novo* and are obligatory scavengers of host purines. While the components of the purine salvage pathway have been characterized at a molecular level, little is known about their regulation in response to purine stress. As a first step to understanding the adaptive mechanisms to changes in the purine milieu, extracellular purines were removed from the growth medium of *L. donovani* promastigotes. Withdrawal of purines led to an acute increase in key purine transporters and salvage enzymes. This augmentation was not due to an increase in mRNA abundance or stability. Removal of purines also led to demonstrable morphological changes and cessation of growth, with purine-starved parasites entering into apparent quiescence after one doubling. Since regulation appears post-transcriptional, we are employing shotgun proteomic approaches to gain insight into the networks and pathways involved in adaptation to purine stress. Preliminary experiments using a label-free MudPIT approach on parasites starved for purine for 24 h revealed some 152 proteins out of the 3944 that could be surveyed that were significantly altered in abundance. Of the 70 proteins upregulated, were constituents of the purine salvage pathway, including the LdNT2 and LdNT3 purine transporters, as well as cell signaling components. Of the 82 proteins downregulated, were components involved in protein translation, RNA and DNA metabolism, and chromosomal segregation. In conjunction with these studies, branch-point mutants of the purine salvage pathway are also being evaluated to determine whether adenylate, or guanylate nucleotides, or both, are important for sensing alterations in the extracellular purine environment.

Proteomics gives insight into signaling and sensory mechanisms of the *T. brucei* flagellum membrane and flagellum matrix

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Recognition of the vertebrate cilium/flagellum as a sensory organelle has profoundly impacted our understanding of human development and physiology. Increasing evidence indicates that the kinetoplastid flagellum is also a sensory platform. However, the sensory and signaling capacity of kinetoplastid flagella is largely unexplored on the molecular level. This stems from a lack of convenient in vitro assays for sensing and a dearth of knowledge of surface-exposed flagellum membrane proteins. We therefore developed an approach for purifying intact, membrane-enclosed flagella from the mammalian-infectious form of *T. brucei*. By combining flagellum purification with affinity purification of surface-exposed proteins and multidimensional protein identification technology (MudPIT) we conducted a proteomic analysis of the flagellum membrane and an independent analysis of the flagellum matrix. The flagellum membrane proteome includes 116 proteins exhibiting great structural and functional diversity and encompasses nearly all characterized *T. brucei* flagellum membrane and flagellum pocket proteins. Prominent among the membrane dataset are receptor-like and channel-like proteins, which are largely kinetoplastid-specific. Conversely, the flagellar matrix contains many conserved components of signaling pathways found in flagella of other organisms. Immunolocalization identified proteins located in discrete flagellum sub-domains, e.g. the flagellum attachment zone, the flagellum pocket or the flagellum tip, indicating functional specialization of different domains of the flagellum membrane. RNAi against proteins identified provides insight into molecular mechanisms of flagellum attachment and signaling mechanisms that control flagellar motility. Moreover, RNAi and pharmacological inhibition of flagellar signaling pathways identified proteins required for social motility (Oberholzer and Lopez et al. 2010). Our combined studies indicate that the trypanosome flagellum membrane contains a diverse and parasite-specific cohort of proteins suited for sensation of the environment.

Molecular and Functional Characterization of Antimony Resistance in *Leishmania amazonensis*

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Drug resistance is a major problem in leishmaniasis chemotherapy. RNA expression profiling using DNA microarrays is a suitable approach to study simultaneous events leading to a drug-resistance phenotype. Genomic analysis has been performed primarily with Old World *Leishmania* species and here we investigate molecular and functional alterations in antimony (Sb^{III}) resistance in the New World specie *L. amazonensis*. Promastigote forms of *L. amazonensis* were selected for resistance to antimony by step-wise drug pressure. Gene expression of highly resistant mutants was studied using DNA microarrays, a technique useful to study resistance mechanisms in *Leishmania*. RNA expression profiling of antimony-resistant *L. amazonensis* revealed the overexpression of genes involved in drug resistance including the ABC transporter MRPA and several genes related to thiol metabolism as trypanothione synthase. The MRPA overexpression was validated by quantitative real-time RT-RTPCR and further analysis revealed that this increased expression correlated to gene amplification as part of extrachromosomal linear amplicons in some mutants and as part of supernumerary chromosomes in other mutants. The expression of several additional genes encoding hypothetical proteins but also nucleobase and glucose transporter encoding genes were found to be modulated. The intracellular thiol levels were found enhanced in four independent *L. amazonensis* resistant mutants and functional analyses revealed the overexpression of genes associated with the glutathione metabolism. Additionally, a reduced uptake of Sb^{III} was observed in the mutants when compared to its parental sensitive wild-type strain. Furthermore, energy dependent-efflux was evidenced in some, but not all mutants. Mechanisms classically found in Old World antimony resistant *Leishmania* were highlighted in New

World antimony-resistant *L. amazonensis*. These studies were useful to the identification of resistance molecular markers.

Whole Genome Sequencing and Comparative Genomic Hybridization of Leishmania and their Application to Antimony Resistance

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Antimonials are still the mainstay of treatment against Leishmaniasis but now resistance is a severe threat. The identification of genetic changes that confer drug resistance or other phenotypic changes in pathogens can help optimize treatment strategies. We carried out short read whole genome sequencing and Comparative Genomic Hybridization (CGH) of three independent resistant mutants of antimony. Copy number variation was detected consistently in both sequencing and CGH and was a major attribute to antimony resistance. The copy number of *AQP1*, (*LmjF31.0020*), a gene encoding for aquaglyceroporin which facilitates uptake of trivalent metalloids, was decreased in all the resistant lines and we unraveled the mechanism by Southern blot hybridizations and discovered that chromosome 31 is polyploid (about seven copies) in this wild type strain of *L. major* but upon induction of SbIII resistance, all the three mutants decrease the copy number of the subtelomeric region of chromosome 31 from seven to two, keeping the rest of the chromosome in seven copies suggesting that there appears to be a break on at least five copies of the chromosome 31 thus deleting the subtelomeric region of the chromosome, *AQP1* being the second subtelomeric gene. The sequence that generated this break is currently under investigation. Transfection of *AQP1* into resistant mutants rendered them hypersensitive to SbIII but not to WT levels. Another interesting modulation in SbIII resistance revealed by CGH and whole genome sequencing and confirmed by Southern blot analysis was an intrachromosomal amplification of a subtelomeric region on chromosome 34. The is a novel form of amplification in Leishmania and its role in SbIII resistance is being studied by gene overexpression

Trypanosoma brucei Isopentenyl Diphosphate Isomerase - the crystal structure of a validated drug target

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Isopentenyl Diphosphate Isomerase (IDI) catalyses a critical step in the *de novo* biosynthesis of isoprenoids, the isomerisation of isopentenyl diphosphate to dimethylallyl diphosphate. There are two evolutionarily distinct classes of IDI enzymes[1], class I enzymes are dependent upon Mg^{2+} or Mn^{2+} for catalysis[2] , while type II enzymes are dependent upon riboflavin (FMN) and NAD(P)H and Mg^{2+}[3]. Kinetoplastids have a type II IDI protein, while human IDI is a type I protein. Structural and mechanistic differences between the human and kinetoplastid IDI enzymes should facilitate the design of selective inhibitors. A TbIDI conditional knock-out demonstrated that TbIDI is an essential gene. A potent inhibitor of TbIDI has been characterised *in vitro* and used for chemical validation. To assist our future drug discovery efforts we have solved a 2.6 Å resolution crystal structure of recombinantly expressed TbIDI in complex with FMN, in the presence of inhibitor. The diversity of human and kinetoplastid IDI proteins, the essentiality of IDI and structural information about the TbIDI protein makes TbIDI an appealing drug target against *T. brucei* , the causative agent of African sleeping sickness. References 1) Kaneda *et al*., (2001) PNAS, 98, 932-937. 2) Durbecq *et al*., (2001) EMBO J, 20, 1530-1537. 3) Yamashita *et al*., (2004) Eur J Biochem, 271, 1087-1093. This work is funded by the Wellcome Trust.

Lipid metabolism in *Trypanosoma brucei*: a plethora of drug targets

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The membranes of *Trypanosoma brucei* contain a complex array of phospholipids that are *de novo* synthesized from precursors obtained either directly from the host, or as catabolised endocytosed lipids. An update on our current understanding of lipid metabolism in this ancient divergent eukaryote will be given. Numerous molecular species of all of the major subclasses of glycerophospholipids have been identified including phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol as well as phosphatidic acid, phosphatidylglycerol and cardolipin. Sphingolipids, such as sphingomyelin, inositol phosphoceramide and ethanolamine phosphoceramide are also present. Insights into the lack of inter-connection between phospholipid pathways highlight the possibilities for drug targets and therapeutic intervention in this area. Plans for the formation of a lipidomic database to aid future biochemical phenotyping of either genetically or chemically manipulated trypanosomatids will be discussed. This research is supported by the Wellcome Trust.

ABSTRACTS: SESSION VI

Cell Biology II

April 13 1:45 p.m.

Chair(s) - Derrick Robinson

The complexities of cell division in *Trypanosoma brucei*

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AIR9 is an important cytoskeleton-associated protein in *Arabidopsis thaliana* with roles in cytokinesis and cross wall maturation, and has homologues in land plants and excavate protists, including trypanosomatids. Here we show that the *Trypanosoma brucei* orthologue, TbAIR9, which lacks the N-terminal microtubule-binding domain present in *A. thaliana* AIR9, is also a cytoskeleton-associated protein that colocalises with the subpellicular microtubules, and is essential for normal proliferation of trypanosomes in vitro. Depletion of TbAIR9 from procyclic trypanosomes resulted in an increase in cell length, most likely due to increased microtubule extension at the posterior end of the cell. Additionally, a dramatic migration of the nucleus towards the posterior pole of the cell occurred, such that the nucleus was positioned posterior to the kinetoplast, leading to defects in cytokinesis and the generation of aberrant progeny. In contrast, in bloodstream trypanosomes, depletion of TbAIR9 had little effect on nucleus positioning, but resulted in aberrant cleavage furrow placement, and the generation of non-equivalent daughter cells following cytokinesis. We suggest that TbAIR9 may act to integrate molecular signals determining the spatial organisation of organelles and the cleavage furrow with the microtubule cytoskeleton. Our data not only provide the first molecular insight into the control of nucleus positioning in trypanosomes, but also emphasise differences in cell division between two life cycle stages of *T. brucei*.

Identification and functional characterisation of CRK12:CYC9, a novel CDK-cyclin complex in *Trypanosoma brucei*

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Cyclin dependent kinases (CDKs), such as Cdc2 in yeast, regulate progression through the cell cycle in eukaryotes and are activated by the binding of a cyclin partner. *Trypanosoma brucei* possesses a family of Cdc2-related kinases (CRK1-4 and CRK6-12) and ten cyclins (CYC2-11). Previously, CRK3 has been shown to interact with CYC2 and CYC6 in vivo to regulate progression through the G1/S and G2/M transitions, respectively. However, the cyclin partners of the other CRKs remain unknown. Here we report the identification of a new CRK:cyclin complex and characterise its function. Using a yeast two hybrid assay, tandem affinity purification and co-immunoprecipitation, we show that CRK12 interacts with CYC9. Immunoprecipitated CRK12 autophosphorylates, suggesting the CRK12:CYC9 complex is active *in vivo*, but does not trans-phosphorylate generic kinase substrates. Depletion of CRK12 or CYC9 via RNA interference is lethal in bloodstream stage *T. brucei* and hence CRK12:CYC9 may have potential as a novel drug target for trypanosomiasis. However, the mechanisms leading to cell death appear to differ following depletion of kinase and cyclin. CRK12 depletion has little effect on the cell cycle, but results in enlarged flagellar pockets and defects in endocytosis, while depletion of CYC9 results in inhibition of furrow ingression during cytokinesis. The different RNAi phenotypes obtained may suggest that CYC9 and/or CRK12 interact with other CRK or cyclin partners, respectively during the cell cycle. Work is in progress to address this possibility.

Polo like kinase is necessary for FAZ duplication and basal body segregation in *T. brucei*

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A bilobe structure marked by centrins positions the Golgi in the protozoan parasite, *Trypanosoma brucei*. This structure must itself duplicate during the cell cycle for Golgi inheritance to proceed normally. The duplication of the bilobe structure is dependent on the single polo-like kinase (TbPLK) homolog in *T. brucei*. Depletion of TbPLK leads to malformed bilobe structures and an increase in the number of dispersed Golgi. During the cell cycle, TbPLK is initially localized to the basal bodies, then progresses to the bilobe as the structure is duplicating. Once bilobe duplication is complete, TbPLK migrates along the growing tip of the new flagellum attachment zone (FAZ), a structure that neighbors the bilobe and is responsible for adhering the flagellum to the cell body. The FAZ is comprised of two distinct elements: a protein filament that begins at the flagellar pocket and a quartet of microtubules that originates at the probasal body. TbPLK depletion causes defects in the assembly of both FAZ elements, which in turn lead to detached flagella. This detachment has a strong effect on basal body biogenesis. In TbPLK depleted cells the basal body manages to duplicate and nucleate a new flagellum, but the newly formed structures are not able to separate. This lack of separation may occur because the flagellum is not attached and cannot drive basal body separation. TbPLK depletion causes defects in the duplication of multiple cytoskeletal elements, which disrupt flagellar adhesion and cell division.

The kinetoplast duplication cycle in *Trypanosoma brucei*: integration of DNA replication with cell morphogenesis

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Trypanosoma brucei mitochondrial DNA (kDNA) is organised in a network of concatenated DNA maxi- and minicircles. The kDNA network forms a compact nucleoid of defined shape and position within the cell, which is faithfully duplicated once per cell cycle. In this study we define for the first time the complete kinetoplast duplication cycle in *T. brucei* procyclic forms based on three-dimensional reconstructions from serial-section electron micrographs. This structural model was enhanced by analyses of the replication process of DNA maxi- and minicircles. We show that kinetoplast S-phase occurs concurrent with the re-positioning of the new basal body from the anterior to the posterior side of the old flagellum. This emphasises the role of basal body segregation in kinetoplast division and suggests a possible mechanism for driving the rotational movement of the kinetoplast during minicircle replication. Fluorescence *in situ* hybridisation with minicircle and maxicircle-specific probes showed that maxicircle DNA is stretched out between segregated minicircle networks. Cells that over-replicate their maxicircles due to over-expression of the TbPIF2 helicase have longer and thicker maxicircle threads than wild type cells, which persist longer in the cell cycle. We conclude that unlinking of maxicircles marks the final stage in kinetoplast segregation. These new data and their integration with earlier studies provide novel insights into the dependencies between dynamic remodelling of the procyclic cytoskeleton and inheritance of the *T. brucei* mitochondrial genome.

Trypanosome motion represents an adaptation to the environment of the vertebrate bloodstream

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Blood is a remarkable habitat: it is highly viscous, contains a dense packaging of cells and is constantly flowing at velocities varying over three orders of magnitude. Only few pathogens endure the harsh physical conditions within the vertebrate bloodstream and can prosper despite being constantly attacked by host antibodies. African trypanosomes are strictly extracellular blood parasites, which evade immune response through antigenic variation and incessant motility. We show that in cell culture trypanosomes are pulled forward by the unidirectional beat of the single flagellum, which leads to a stepwise rotation of the parasite. So far, however, it is unclear how the flagellates move in blood. Here, we demonstrate that the mode and dynamics of trypanosome locomotion are a trait of life within a crowded environment: (1) Using high-speed light and fluorescence microscopy as well as ordered micro-pillar arrays, we show that parasite motility is adapted to the density of cells in blood. The presence of particles with the shape, size and spacing of blood cells is required and sufficient for trypanosomes to reach maximum forward swimming velocity. (2) Furthermore, we report conditions prompting *T. brucei* wild type cells to swim backwards by reversal of the flagellar beat. This could facilitate trypanosome movement through physical barriers, such as host tissues or the blood-brain barrier, in a trial and error manner without getting stuck. This provides a further example of how the microenvironment controls the swimming behavior of cells.

Beyond just counting Ks and Ns; high throughput image analysis of trypanosomatid cell organisation

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The precise morphology and replication of trypanosomatids facilitates an approach using automated morphometric analysis for investigation of their cell cycle, life cycle stage differentiation, mutants and chemical insults. High throughput image analysis has the capability to accelerate morphological measurement and extract more quantitative data from micrographs. However image analysis of trypanosomatid morphology is complicated by the presence of two, often closely apposed, DNA containing organelles; the kinetoplast and the nucleus. Accurate identification and analysis of these organelles is central to determining cell cycle stage of a cell which is in turn key for understanding the aberrations caused by a drug or mutation. We addressed the difficulties of automated identification of kinetoplasts and nuclei by taking advantage of the different sequence binding biases of different fluorescent DNA stains. We have successfully used colour deconvolution to separate the signal from kinetoplast and nuclear DNA in fluorescence microscopy images. This produces two new images of the same field of cells, one with only kinetoplasts and one with only nuclei. These images are amenable to automated analysis and can also simplify manual analysis of complex phenotypes where kinetoplast and nuclear complement and structure are perturbed. Using our approach to staining, image processing and automated analysis we can analyse around 20000 cells per hour. We have correlated these automated approaches with manual analysis of *Trypanosoma brucei* and *Leishmania mexicana* revealing distinct advantages in speed and precision.

Monitoring stumpy formation in *Trypanosoma brucei*

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During the bloodstream stage of the *Trypanosoma brucei* lifecycle, the parasite exists as the proliferative slender-form or the non-proliferative, transmissible, stumpy-form. The transition from the slender to stumpy-form is stimulated by a density-dependent mechanism and is important in infection dynamics, ordered antigenic variation and disease transmissibility. The study of the differentiation between these forms, however, has been hindered by a lack of molecular markers for the stumpy life-stage. The transporter protein PAD1 is a stumpy-specific surface protein and functional marker of transmission competence. We have determined how PAD1 expression is restricted to stumpy forms, identifying repressive elements in the 3'UTR that prevent premature expression of PAD1 in slender forms. Further, we have created monomorphic and pleomorphic transgenic parasite lines that report stumpy formation in quantitative assays *in vitro*. The pleomorphic reporter cell line has been shown to increase reporter gene expression upon differentiation to the stumpy life stage *in vivo* as well as respond to cell density *in vitro*, indicative of appropriate reporter expression for analysis of stumpy formation. However, thus far, we have not observed a response to parasite conditioned medium, expected to contain the elusive stumpy induction factor. Finally, using a monomorphic cell line, over 6000 kinase inhibitors have been screened for their ability to induce stumpy-like formation in a high-throughput screening programme. Over 190 positive candidate compounds have been identified during primary screening, many of which have been validated in titration assays. These offer two opportunities: firstly, compounds able to induce stumpy formation may have therapeutic potential and, secondly, identification of the targets of stumpy-inducing compounds provides tools to dissect the stumpy induction pathway.

Novel TOR kinase regulates proliferative slender to quiescent stumpy form differentiation in Trypanosoma brucei

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African trypanosomes are protozoan parasites with a complex digenetic life cycle that alternates between the tsetse fly vector and the mammalian host, a cycle characterized by multiple cell-type differentiation processes. In the bloodstream, the proliferative 'slender' form develops into the quiescent 'stumpy' form, preventing the exhaustion of the mammal host and pre-adapting the parasite for life in the tsetse fly. The signalling pathways controlling the developmental switch between the two forms remain unknown. In *Trypanosoma brucei*, the two Target of Rapamycin (TOR) kinases TbTOR1 and TbTOR2 are found in the complexes TbTORC1 and TbTORC2, and mediate temporal and spatial control of cell growth. Surprisingly, two additional TOR kinases are encoded in the *T. brucei* genome. Here we show that the novel TbTOR4 kinase functions as a negative regulator of the cell differentiation process from the slender to the stumpy bloodstream form in the life cycle of *T. brucei*. TbTOR4 associates with TbLST8 and forms a novel multiprotein complex (TbTORC4), functionally and biochemically distinct from TbTORC1 and TbTORC2. Interestingly, TbTOR4 expression is developmentally downregulated in the stumpy form. We found TbTOR4 expression to be post-transcriptionally regulated by the cAMP second messenger, whose production is triggered upon high parasitemia. These findings suggest that TbTOR4 coordinates the developmental switch from slender to stumpy bloodstream form in response to cAMP levels and indicate a new complexity in TOR signaling in eukaryotes.

ABSTRACTS: SESSION VII

Nucleus & Kinetoplast

April 12 8:45 a.m.

Chair(s) - Keith Gull

Architecture and function of the trypanosome nuclear envelope revealed using high resolution interactome mapping

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The nuclear pore complex (NPC) is a macromolecular assembly embedded within the nuclear envelope (NE) that mediates transport between the nucleus and cytoplasm. Both the NPC and NE serve as structural platforms from which intranuclear compartments and the nuclear lamina are organized, playing a key regulatory role in gene expression through influencing nuclear architecture and acting as a point for the control for various nuclear functions. Each NPC is a large (∼50 MDa in yeast) cylindrical octagonally symmetric complex comprised of ∼480 components (termed nucleoporins or Nups) built from a common set of ∼30 different proteins. Currently, detailed compositional and structural information of NPCs is only available in yeast and metazoa, which are closely related. We previously identified 22 Nups in *Trypanosoma brucei* (TbNups) using a proteomics based approach, and tagged each ORF genomically with GFP. Further understanding of NPC/NE interactions within the trypanosome genome, and hence uncovering precise functions, requires a detailed description of the NPC/NE protein interactome. Using GFP as an affinity handle, we are currently dissecting the interactions formed by these proteins in an effort to build a structural map of the NPC/NE. Using a novel cryogrinding approach, we have affinity isolated core scaffold sub-complexes that form the inner and outer ring of the NPC. We demonstrate a highly efficient method for isolation of trypanosome protein complexes, finding several novel nucleoporins that represent trypanosome-specific subunits. These results are already shedding new light on the evolution of the NPC and the nuclear architecture of the last common eukaryotic ancestor.

Epigenetic regulation at the nuclear periphery mediated by a non-metazoan lamin analogue

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Divergent eukaryotic lineages employ many distinct transcriptional mechanisms, but a unifying feature is genome segregation into transcriptionally active euchromatin and transcriptionally repressed heterochromatin. In metazoa, lamin proteins preserve nuclear integrity and higher order heterochromatin organization at the nuclear periphery, but as lamin homologues are restricted to metazoans, and absent from yeast, the molecular basis for maintaining such nuclear organization in other lineages remains undefined. We demonstrate that NUP-1, a large coiled-coil protein, forms an essential scaffold at the inner face of the nuclear envelope of trypanosomes, a highly divergent eukaryote. NUP-1 directs positional organization of nuclear pore complexes and most significantly, epigenetically organizes chromatin so as to maintain the silenced state of developmentally regulated genes at the nuclear periphery. We conclude, therefore, that unlike many forms of transcriptional control, lamina-mediated sequestration of gene cohorts at the nuclear periphery is conserved across eukaryotes. Furthermore, NUP-1 is a major organizer of chromatin structure for the regulation of VSG gene expression.

Identification of ORC1/CDC6-interacting factors in Trypanosoma brucei reveals critical features of Origin Recognition Complex architecture

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The mechanism and machinery of DNA replication initiation is well-conserved and in most eukaryotes, the origin recognition complex (Orc1-Orc6; ORC) and Cdc6 are recruited sequentially to replication origins, and Cdt1 then loads the replicative helicase complex (MCM2-7) to form a pre-replication complex. In Trypanosoma brucei and related trypanosomatids, comparative genome analysis revealed that the pre-replication complex consist of a single protein that is homologous to both Orc1 and Cdc6, called ORC1/CDC6. The structural similarity of this factor to Orc1/Cdc6 in archaea suggests that trypanosomatid parasites may be archaeal-like, lacking further ORC subunits. In a search for TbORC1/CDC6-interacting factors, we have identified one protein (Tb927.10.13380) that acts as a component of the T. brucei pre-replicative machinery, and bioinformatics suggest that this is a previously unidentified orthologue of Orc4. We also identified two other proteins (Tb927.10.7980; Tb09.10.3120) that may also act in DNA replication, but whose identities are unclear. Taken together, we propose that TbORC contains at least two components, TbORC1/CDC6 and TbORC4, and it is more analogous to the eukaryotic model than previously thought, though still substantially diverged. Whether the two additional proteins represent highly diverged components of TbORC, or whether they provide trypanosomatid-specific activities is currently unclear. We also show that TbORC1/CDC6 does not directly interact with the TbMCM helicase (for which orthologues of all subunits can be identified), consistent with other eukaryotes and contrary to reports in some archaeal species. Moreover, MCM subunits in T. brucei form at least one subcomplex (TbMCM2/4/6/7) homologous to that previously observed for human, yeast, Drosophila, Xenopus and mouse MCM proteins.

The CMG complex in trypanosomes regulates DNA replication and interacts with the origin recognition complex consisting of two Orc1-like proteins

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Accurate DNA replication requires a complex interplay of many regulatory proteins at replication origins. The CMG (Cdc45/Mcm2-7/GINS) complex is recruited by Cdc6 and Cdt1 onto replication origins bound by the heterohexameric origin recognition complex (ORC) and functions as the replicative helicase. *Trypanosoma brucei* appears to possess an archaea-like ORC consisting of a single Orc1/Cdc6-like protein. However, unlike in archaea, components of the eukaryote-like CMG complex are identified in trypanosomes, but whether these proteins form an active helicase complex, associate with ORC, and regulate DNA replication remains unknown. Through GST pull-down and yeast two-hybrid assays, interactions among Cdc45, Mcm2-7 and GINS proteins were detected, suggesting the formation of the CMG complex. In vitro helicase activity assay showed that Mcm2-7 helicase activity is activated by association with Cdc45 and GINS proteins. Mcm2-7 and GINS proteins are confined to the nucleus throughout the cell cycle, and localization of individual CMG proteins is not inter-dependent, suggesting that they are not targeted to the nucleus together as a complex and that the machinery for preventing re-replication through nuclear export of Mcm2-7 is not operating in trypanosomes. With the exception of Mcm4, Mcm6 and Psf1, knockdown of individual CMG genes inhibits DNA replication. Finally, we identified a novel Orc1-like protein, Orc1b, that forms the ORC with Orc1/Cdc6. This unusual ORC appears to form the pre-replicative complex (pre-RC) with Mcm2-7 through direct interactions with Mcm3. All together, we have identified in trypanosomes the Cdc45/Mcm2-7/GINS complex as the replicative helicase for DNA replication, the pre-RC consisting of Mcm2-7, Orc1/Cdc6 and Orc1b for replication licensing, and a simple ORC comprising two Orc1-like proteins, Orc1/Cdc6 and Orc1b, for origin recognition.

The life dyskinetoplastic: how single point mutations allow *Trypanosoma brucei* survive complete loss of mitochondrial DNA

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In trypanosomes, mitochondrial DNA takes the form of the kinetoplast - the defining feature of the order *Kinetoplastida* - replication and expression of which is normally essential in procyclic and bloodstream form *Trypanosoma brucei*. However, viable dyskinetoplastic (DK) bloodstream forms have been induced in the lab, as well as DK (sub)species, *T. evansi* and *T. equiperdum*, identified in the wild. Polymorphisms in the ATP synthase gamma gene have been located in both lab and naturally occurring DK strains (1, 2) and we present the functional testing of these mutations with regard to the ability to compensate for the loss of the kinetoplast. We have generated and characterised transgenic *T. brucei* strain 427 bloodstream forms expressing the various ATP synthase gamma polymorphisms. Our data unequivocally demonstrate that a single amino acid substitution in this protein is necessary and sufficient for survival of kinetoplast loss. We also provide evidence as to the biochemical mechanism of the DK adaptation. A number of anti-trypanosomatid drugs are known to accumulate in the parasite mitochondrion where they bind to kinetoplast DNA, and a long-standing question in the field is whether this property is related to their mode of action. The isogenic character of the kinetoplast-dependent and -independent strains of *T. brucei* that we have generated makes them ideal tools to resolve this question and we will present preliminary data in this regard. (1) Schnaufer, A et al (2005) EMBO J 24: 4029-4040 (2) Lai, D-H et al (2008) PNAS 105: 1999-2004

PPR Proteins Stimulate mRNA Adenylation/Uridylation to Activate Mitochondrial Translation in Trypanosomes

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The majority of trypanosomal mitochondrial pre-mRNAs undergo massive uridine insertion/deletion editing which creates open reading frames. Although the pre-editing addition of short 3' A-tails is known to stabilize transcripts during and after the editing, the processing event committing the fully-edited mRNAs to translation remained unknown. Here we show that a heterodimer of pentatricopeptide repeat-containing (PPR) proteins, termed kinetoplast polyadenylation/ uridylation factors (KPAFs) 1 and 2, induces the post-editing addition of A/U-heteropolymers by KPAP1 poly(A) polymerase and RET1 terminal uridyltransferase. Edited transcripts bearing 200-300 nucleotide-long A/U-tails, but not short A-tails, were enriched in translating ribosomal complexes and affinity-purified ribosomal particles. KPAF1 repression led to a selective loss of A/U-tailed mRNAs and concomitant inhibition of mitochondrial protein synthesis. These results establish A/U extensions as the defining cis-elements of translation-competent mRNAs. Furthermore, we demonstrate that A/U-tailed mRNA preferentially interacts with the small ribosomal subunit, whereas edited substrates and complexes bind to the large ribosomal subunit. Based on physical interactions and functional coupling between mRNA editing and 3'-modification complexes and translation apparatus, we propose an integrated model of mitochondrial gene expression in trypanosomes.

Endonuclease Associations with Three Distinct Editosomes in *Trypanosoma brucei*

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Three distinct editosomes, typified by mutually exclusive KREN1, KREN2 or KREN3 endonucleases, are essential for mitochondrial RNA editing in *Trypanosoma brucei*. The three editosomes differ in substrate endoribonucleolytic cleavage specificity, which may reflect the vast number of editing sites that need insertion or deletion of uridine nucleotides (Us). Each editosome requires the single RNase III domain in each of endonuclease for catalysis. Studies reported here show that the editing endonucleases do not form homodimeric domains, and may therefore function as intermolecular heterodimers, perhaps with KREPB4 and/or KREPB5. Editosomes isolated via TAP-tag fused to KREPB6, KREPB7 or KREPB8 have a common set of 12 proteins. In addition, KREN3 is only found in KREPB6 editosomes, KREN2 is only found in KREPB7 editosomes, and KREN1 is only found in KREPB8 editosomes. These are the same associations previously found in editosomes isolated via the TAP tagged endonucleases KREN1, KREN2, or KREN3. Furthermore, TAP-tagged KREPB6, KREPB7, and KREPB8 complexes isolated from cells in which expression of their respective endonuclease was knocked down were disrupted and lacked the heterotrimeric insertion subcomplex (KRET2, KREPA1, and KREL2). These results and published data suggest that KREPB6, KREPB7 and KREPB8 associate with the deletion subcomplex while the KREN1, KREN2 and KREN3 endonucleases associate with insertion subcomplex.

gRNA Metabolism in Trypanosome Mitochondria Involves a Novel Endonuclease

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The mitochondrial genome of kinetoplastids is an unprecedented DNA structure of catenated maxicircles and minicircles. Maxicircles represent the typical mitochondrial genome encoding components of respiratory complexes and ribosomes. However, most mRNAs need extensive editing by U-insertion and U-deletion, which is directed by hundreds of small guide RNAs (gRNAs). These small transcripts must be generated from long polycistronic precursors (pre-gRNAs), which contain multiple gRNA genes. However, the relevant nucleases that process pre-gRNAs into individual gRNAs are unknown. Here, we report the identification and functional characterization of an endonuclease, mRPN1 (mitochondrial RNA precursor-processing endonuclease 1), which is required for efficient pre-gRNA maturation. Secondary structure-modeling and multi-sequence alignments revealed a nuclease domain of mRPN1, which is also found in eukaryotic Dicer and Drosha, and bacterial RNase III enzymes. Repression of mRPN1 inhibits the growth of trypanosomes in culture, and results in loss of gRNAs and accumulation of pre-gRNAs. Isolated recombinant mRPN1 specifically cleaves synthetic double-strand RNAs in vitro, and this activity requires Mg2+ and a conserved catalytic carboxylate. Finally, native mRPN1 in trypanosomes associates with two other proteins in a discrete complex. We will discuss these data as well as our ongoing progress in the characterization of protein-protein interactions and enzymatic activity of native mRPN1 complexes, cleavage determinants and a crystal of mRPN1. These studies offer mechanistic insights on an outstanding process in trypanosomal RNA biology.

Yeast two hybrid analysis of protein-protein interactions in the MRB1 complex of *T. brucei* identifies a core complex

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Uridine insertion/deletion RNA editing in kinetoplastid mitochondria is catalyzed by a 20S editosome. Additional non-editosome proteins are required for efficient editing *in vivo*. The multiprotein Mitochondrial RNA Binding complex (MRB1; aka GRBC) was independently purified by three groups in 2008, and shown to contain components essential for efficient editing as well as gRNA and mRNA stability. The MRB1 complex purifications contained both common and unique subunits, and diverse MRB1 functions suggest the complex is composed of subcomplexes with RNA-dependent and independent interactions. To gain insight into the organization of the MRB1 complex and component subcomplexes, we performed a comprehensive yeast two hybrid analysis of all proteins identified in the three MRB1 purifications and additional proteins that apparently associate with MRB1. We also used *in vitro* protein-protein binding assays, *in vivo* pulldowns, and glycerol gradient sedimentation to analyze direct and RNA-mediated interactions. Our data identify a core complex comprised of five proteins and maintained by numerous direct interactions. RNAi of one of the uncharacterized core proteins results in substantial depletion of all edited RNAs and accumulation pre-edited RNAs, indicating that the core plays a fundamental role in RNA editing. Our data are consistent with a model in which the MRB1 core interacts with numerous additional subcomplexes by protein-protein and RNA-enhanced or RNA-dependent interactions. Analyses of mitochondrial RNA metabolism in cells depleted of additional MRB1 components by our lab and others indicate functions for MRB1 subcomplexes in RNA editing progression, mRNA stabilization, polyadenylation, and potentially protein modification. These findings elucidate the architecture of the MRB1 complex and suggest mechanisms by which MRB1 coordinates multiple roles in RNA biogenesis and editing.

ABSTRACTS: POSTER SESSIONS

A SERIES: April 9 6:30 p.m.

B SERIES: April 10 6:30 p.m.

C SERIES: April 11 6:30 p.m.

A compensatory mutant of *Leishmania major* lacking ether lipids exhibits normal growth and neomycin resistance

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Ether glycerolipid biosynthesis in *Leishmania major* initiates with the acylation of dihydroxyacetonephosphate by the glycosomal dihydroxyacetonephosphate acyltransferase *Lm*DAT. We previously reported that a null mutant of *Lm*DAT is severely affected in logarithmic growth, survival during the stationary phase, and in virulence in mice. In addition, it lacks all ether glycerolipids, produced altered forms of the ether-lipid based virulence factors lipophosphoglycan and increased levels of GPI-anchored protein gp63. Here, we describe the characterization of a compensatory mutant of a null strain of *Lm*DAT. Similarly to the null mutant, the revertant strain formed altered forms of lipophosphoglcyan and increased levels of gp63, and was avirulent in mice infection. In contrast, the revertant grew normally but still exhibited poor survival during stationary phase. Agarose gel analysis of its genomic DNA failed to detect any amplified DNA. Surprisingly, the revertant was more resistant to aminoglycoside based antibiotic neomycin, while resistance to blasticidin and hygromycin was similar to the null mutant.

The essential F_oF_1-ATPase complex in *Trypanosoma brucei* can be specifically inhibited by the inhibitory peptide $TbIF_1$

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The F_oF_1-ATP synthase plays an essential role in *Trypanosoma brucei*, an important pathogen of humans and livestock that alternates between an insect vector and a mammalian host. Interestingly, the function of the mitochondrial (mt) F_oF_1-ATP synthase differs between the insect and mammalian stages, producing ATP in the former while consuming ATP to maintain the mt membrane potential in the latter. This indispensable activity of the F_oF_1-ATPase complex can be specifically inhibited by the natural inhibitory peptide, $TbIF_1$. When we over-express TbIF1 *in vivo*, only the F_oF_1-ATPase activity is inhibited, leading to the death of the infectious stage of the parasite due to a measured decrease in the mt membrane potential. In addition, we purified the recombinant $TbIF_1$, which specifically inhibits the F_1-ATPase activity *in vitro*. This ability to inhibit an essential activity of the parasite but not the host F_oF_1-ATP synthase, which utilizes the conventional activity of this complex to create ATP, makes the *T. brucei* F_oF_1-ATPase an attractive drug target. Therefore, we are also attempting to further characterize the functional organization of this complex. Specifically, we are interested in the 14 novel subunits previously identified, one of which is Tb7760. We have demonstrated that Tb7760 is essential in the mammalian stage and the growth inhibition is caused by a sudden decrease of the mt membrane potential. The structural integrity of the F_oF_1-ATPase is not affected, but its sensitivity to oligomycin is significantly decreased. Thus, it seems that this membrane-bound subunit Tb7760 is important for proton translocation across the mt inner membrane. These results are expanding our knowledge about this intriguing complex and may open new opportunities for drug development.

The ATG12-ATG5 conjugation pathway and autophagy in *Leishmania*

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Macroautophagy is a catabolic process involved in the development of organisms. The main molecular players involved in autophagosome biogenesis, and identified in yeast, are from two pathways: ATG8 lipidation and ATG12-ATG5 conjugation. Reported analyses on the genomes of trypanosomatids suggested a secondary loss of the ATG12-ATG5 conjugation pathway. Nevertheless, we identified genes potentially encoding the pathway's components and have now used genetic and biochemical approaches to validate the existence of this conjugation pathway in *Leishmania*. We have generated ATG5-deficient mutant lines and used this to assess the role of ATG5 in autophagosome biogenesis, the parasite's development and its ability to withstand stress. The findings show that ATG5 is key, and acts in concert with the ATG8 lipidation pathway to form autophagosomes. ATG5-deficient lines were unable to form autophagosomes, incapable of an efficient differentiation and replication and exhibited abnormal morphology. The potential for targeting ATG proteins as a means of interfering with the parasite's autophagic pathway and development will be discussed.

Chemical and peptide inhibitors of the *Leishmania major* cysteine synthase

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Leishmania has two pathways for cysteine biosynthesis, a *de novo* pathway involving serine acetyltransferase (SAT) and cysteine synthase (CS) and the reverse trans-sulfuration pathway that converts homocysteine to cysteine. CS is found in bacteria, plants and some protozoa but is absent from mammals and is therefore a potential drug target. Chemical inhibitors of the *Leishmania major* CS (LmjCS) were obtained by virtual screening using *Arabidopsis* CS as a model. 25 compounds identified in the first round were tested in enzyme assays and one of these showed 50% inhibition of LmjCS at 100 uM. A secondary screen for compounds with the same pharmacaphore identified 5 additional inhibitors, all with lower activity (2 to 4 fold) than the original compound. In plants and bacteria SAT and CS associate reversibly to form a complex in which SAT is active but CS is strongly inhibited. Short peptides corresponding to the C-terminus of SAT bind to the active site pocket of CS and inhibit enzyme activity. The *Leishmania* SAT and CS also form a complex when co-expressed as recombinant proteins in *E. coli*. We show that a peptide (DYVI) from the *Arabidopsis* SAT is a competitive inhibitor of LmjCS. Surprisingly, the equivalent peptide from *Leishmania* SAT was a very weak inhibitor although a longer peptide had higher activity. Chemical analogues of SAT peptides could be tested as inhibitors of LmjCS and be potential leads in the search for new antileishmanial agents.

TbKAP6, an essential small basic protein, is required for kDNA condensation and replication in *Trypanosoma brucei*

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kDNA, the mitochondrial genome in *T. brucei*, is a network structure composed of several thousand minicircles interlocked with a few dozen maxicircles. Networks are condensed *in vivo* into a small, disk-like structure within the mitochondrion. Several small basic proteins that condensed kDNA, called KAPs (kDNA-associated proteins), have been indentified in *C. fasiculata* and *T. cruzi*. By searching the *T. brucei* genome for genes of similar proteins with mitochondrial targeting signals, we discovered a 21 kDa protein (pI, 11.3), which we have named TbKAP6. RNAi of TbKAP6 caused a severe cell growth defect and kDNA loss. DAPI staining or EM showed RNAi causes network shrinking and disorganization. We examined kDNA replication in TbKAP6-depleted cells and found that covalently-closed free minicircles (replication precursors) and nicked/gapped species (replication products) rapidly disappeared. Similar analyses indicated a defect in maxicircle replication. Although minicircle replication occurs in the kinetoflagellar zone (the region between the kDNA disk and the double membrane near the flagellar basal body), immunofluorescence studies showed that TbKAP6 is positioned on the opposite face of the kDNA disk. This localization suggests that the requirement for TbKAP6 in minicircle replication may be indirect. We speculate that TbKAP6 plays a critical role in kDNA condensation, which is required for other kinetoplast activities, such as DNA replication and segregation. Supporting this view, we find that recombinant TbKAP6 avidly condenses kDNA *in vitro*.

Stage-Specific Requirements for Acetyl-CoA Carboxylase in *Trypanosoma brucei*

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Trypanosoma brucei possesses two fatty acid synthesis pathways: a major de novo synthesis pathway in the ER and a mitochondrial pathway. The 2-carbon donor for both pathways is malonyl-CoA, which is synthesized from acetyl-CoA by Acetyl-CoA Carboxylase (ACC). ACC is cytoplasmic and appears to be distributed throughout the cell in numerous puncta distinct from glycosomes, mitochondrion, endoplasmic reticulum, and lipid droplets. ACC is active in both bloodstream and procyclic forms. Reduction of ACC activity (87% in BSF and 93% in PCF) by RNA interference (RNAi) resulted in a stage-specific phenotype. In procyclic forms, ACC RNAi resulted in 40-70% reduction in fatty acid synthesis and a 64% reduction in growth in low lipid media. This slow-growth phenotype is partially rescued by the addition of stearate to the media. In bloodstream forms, ACC RNAi resulted in a minor 17% decrease in fatty acid synthesis and no growth defect in culture, even in low lipid media. However, ACC RNAi did attenuate virulence in a mouse model of infection. Thus, the requirement for ACC in *T. brucei* is dependent upon the growth environment in two different life cycle stages. As ACC is a promising drug target, we have used two known inhibitors for further characterization. *T. brucei* ACC activity in lysate is sensitive to inhibition by the herbicide Haloxyfop (EC_{50} of 67.6 μM), despite possessing amino acid residues reported to confer resistance in sensitive plant ACCs. The ACC activity of lysate is also inhibited by the green tea catechin, epigallocatechin gallate (EGCG). Inhibition by EGCG is dependent upon the presence of phosphatase inhibitors, suggesting ACC's activity is controlled in part by phosphorylation.

Trypanosoma brucei BRCA2: BRC-mediated RAD51 interaction and genome stability

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BRCA2 is a key factor in homologous recombination, interacting with Rad51 via conserved BRC repeats. The purpose of this interaction appears to be to mediate Rad51-directed homologous strand exchange, and BRCA2 mutants display loss of genome integrity in mammals, nematodes and a fungus. *T. brucei* BRCA2 displays a striking, species-specific expansion in BRC repeat number, which appears not to be needed for the activation of intact *Variant Surface Glycoprotein (VSG)* genes during antigenic variation. Bloodstream stage *T. brucei brca2-/-* mutants of strain Lister 427, examined after prolonged growth, display chromosomal rearrangements resulting from the loss of genetic material from the megabase chromosomes, including copies of at least one *VSG*. To test the hypothesis that the BRC repeat expansion in BRCA2 may be due to the necessity to maintain the *VSG* subtelomere archive, and to further investigate the molecular basis of chromosomal rearrangements, *brca2-/-* mutants were made in procyclic form *T. brucei* of strain TREU927, for which much of the *VSG* archive has been assembled from the genome sequence. Such *brca2-/-* mutants are deficient in the repair of DNA damage, but no evidence for chromosomal rearrangements, including loss of *VSGs* or other genes, could be detected. It is therefore possible that *T. brucei* BRCA2 acts in a bloodstream stage-specific process that is suppressed in procyclic form cells. To examine this further, we are testing *VSG* stability in procyclic form *brca2-/-* mutants in Lister 427 *T. brucei*. In addition, we have made BRCA2 variants, in both life cycle stages, possessing BRC repeat numbers varying from three to twelve, and will discuss how these function in DNA repair and in RAD51 subcellular dynamics.

Moonlighting functions for mitochondrial proteins in the African trypanosomes

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Alternative editing of the cytochrome c oxidase III pre-mRNA in *Trypanosoma brucei* produces a novel mitochondrial membrane protein, alternatively edited protein-1 (AEP-1). This protein is part of the tripartite attachment complex (TAC) and is necessary for maintenance of the kinetoplast DNA (kDNA) network. AEP-1 also assembles into smaller subcomplexes within the mitochondrial membrane, which we purified by anion exchange chromatography and analyzed for subunit composition using ESI-ion trap-tandem mass spectrometry. Sequencing revealed AEP-1, thus providing the first direct evidence for a protein encoded by an alternatively edited mRNA. In addition, we identified the dihydrolipoyl succinyltransferase (E2) subunit of the Krebs cycle α-ketoglutarate dehydrogenase as a component of the AEP-1 complex. The generation of ProtC-TEV-ProtA (PTP) tagged E2 cell lines for bloodstream and procyclic developmental stages revealed association of this protein with the mitochondrial matrix, membrane and the TAC, suggesting that E2 may function in kDNA positioning, structure and replication. Bloodstream *T. brucei*, which lack a functional Krebs cycle, were deficient in α-ketoglutarate dehydrogenase activity but RNAi analysis of the E2 component resulted in abnormal cell growth and kDNA structure in these cells. These data suggest that kDNA maintenance has been a powerful selection for the evolution of bifunctional proteins in *T. brucei* mitochondria.

TbRGG3 is a mitochondrial methylprotein that affects mRNA stabilization and interacts with MRB1 components

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The MRB1 (Mitochondrial RNA Binding 1) complex is an ill-defined multi-protein complex in the mitochondria of Trypanosoma brucei that functions in RNA editing, gRNA stabilization, and mRNA stabilization. The reported composition of MRB1 varies between groups, but comprises $>$ 20 proteins, including TbRGG3 (Tb927.3.1820), which was reported by one group. TbRGG3 is a glycine-arginine-rich (GAR) protein, suggestive of RNA binding ability and methylation on arginine residues. To characterize the function of TbRGG3 in T. brucei, we generated procyclic form (PF) TbRGG3-depleted cells using tetracycline-inducible RNA interference (RNAi). TbRGG3-depleted cells exhibit impaired growth starting at day 6 post-induction, demonstrating that the protein is required for optimal PF growth. Analysis of the abundance of various mitochondrial mRNAs upon TbRGG3 depletion by quantitative real-time PCR implicates a role for TbRGG3 in the stabilization of A6 and ND7 extensively edited mRNAs and MURF1 and COI never-edited mRNAs. This effect on stability was confirmed by full-gene RT-PCR experiments that demonstrate a concomitant decrease both in the pre-edited and edited populations of A6 and ND7 mRNAs. Recombinant TbRGG3 exhibits both RNA annealing activity and relatively nonspecific RNA binding ability. Yeast two-hybrid assays are consistent with a direct interaction between TbRGG3 and at least two MRB1 components. Mass spectrometry of purified mitochondrial extracts identified 13 methylated arginine residues on TbRGG3, 11 of which are within the GAR region. Collectively, these data indicate that TbRGG3 is an essential methylprotein in PF T. brucei that plays a role in the stabilization of specific mitochondrial mRNAs. Future studies will evaluate the requirement for arginine methylation in TbRGG3 function and also aim to characterize this protein in bloodstream form T. brucei.

Functional characterization of the trypanothione biosynthetic pathway in *Leishmania infantum*

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Trypanothione is the trypanosomatids' major and unique thiol. Owing to its unique character, trypanothione biosynthesis is regarded as one potential target for therapeutic intervention. Synthesis occurs in two steps, wherein two molecules of glutathione are sequentially added to spermidine. The enzyme trypanothione synthetase (TRYS) catalyzes both conjugation steps and was found essential in *Trypanosoma brucei*. In parasites such as *Leishmania infantum* and *T. cruzi*, which harbor an additional enzyme (glutathionylspermidine synthetase or GSPS) capable of driving the first step of synthesis, the relevance of TRYS and GSPS remains unknown. The present study aims at characterizing the *TRYS* and *GSPS* genes of *L. infantum* at the functional level. To that end, we are employing classical gene replacement techniques to generate *trys-/-* and *gsps-/-* homozygous promastigotes. Concerning *TRYS*, attempts to delete both gene alleles resulted in transgenic parasites carrying the drug selection genes used to target both *TRYS loci*, which, nevertheless, preserved the *TRYS* ORF, an observation that per se is indicative of TRYS essentiality. Elimination of both *TRYS* alleles was only possible in promastigotes carrying an episomal copy of the gene. These mutants are currently being tested for their ability to maintain episomal *TRYS*, both as promastigotes and as amastigotes, in the absence of the selective drug, as this will provide a definitive proof of TRYS essentiality. In parallel we are also testing the leishmanicidal activity of N^5-substituted paullones that specifically inhibit TRYS *in vitro* (IC_{50} between 31 and 235 nM). One of these compounds (FS-554) exhibited a promising activity against *L. infantum* amastigotes in monolayers of murine peritoneal macrophages (IC_{50} below 20 μM). Supported by FCT, FCG and CM0801.

A Global Functional Atlas of *Trypanosoma brucei* Genome

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The evolutionary distance between trypanosomatids and well-studied model organisms has imposed many obstacles on the challenging task of functional characterization of trypanosomatid genomes. About half of trypanosomatid genes have no obvious homologs in available non-trypanosomatid genomes, making it impossible to predict their functions based on homology. To address this issue, we have constructed a functional interaction network of *Trypanosoma brucei* genes using an integrated genomics-proteomics approach. In the first step, we compiled a compendium of 70 gene expression profiling experiments, including 39 microarray experiments from our lab and 31 publicly available microarray/RNA-Seq datasets from other labs. Our microarray experiments included profiling of 23 different RNAi clones, 11 different chemical treatments, and five independent cultures of procyclic form *T. brucei* cells. This compendium was used to construct a co-expression network of *T. brucei* genes. Also, we created a low-resolution complex co-membership network of T. brucei proteins by resolving protein complexes using native separation techniques and identifying the proteins of different fractions using mass spectrometry. By combining this complex co-membership network and the co-expression network, and also considering the codon usage content and phylogenetic profiles of *T. brucei* genes, we have been able to create a high-quality functional interaction network which has led us to predict the functions of several hundred previously uncharacterized *T. brucei* genes. These predictions include assignment of uncharacterized genes to several essential biological processes and pathways. This great leap towards comprehensive functional annotation of *T. brucei* genome leads us to a better understanding of the biology of trypanosomatids, and also provides new potential targets for treatment of their respective diseases.

Trypanosoma brucei Sec16 - Size Matters

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The Trypanosome cell has a highly organized and polarized architecture, in which positions, sizes and shapes of its intracellular organelles are relevant for their cellular functions. However, how the cell regulates the size of the intracellular structures is poorly understood. Newly synthesized secretory proteins are exported from the ER at a specialized region known as the ER exit site (ERES) where COPII transport vesicles are produced. We have identified a putative Sec16 in *Trypanosoma brucei*: a large peripheral endoplasmic reticulum (ER) membrane protein, which is involved in the generation of COPII transport vesicles. Sec16 has also been proposed to be involved in the biogenesis of the ER exit site (ERES), acting as a key player in the early steps of the assembly process. Here, we show by epifluorescence microscopy that TbSec16 localizes to the ERES. We also demonstrate that TbSec16 is involved in the regulation of the size of the ERES. Overexpression leads to an enlarger ERES and this effect seems in turn to make a bigger Golgi. Conversely, knockdown of TbSec16 expression decreases the size of the ERES and the Golgi. Interestingly, the protein levels of Golgi stack proteins and other COPII proteins localized to the ERES do not appear to change by western blotting. Based on these observations, we propose that TbSec16 might be involved in controlling the size of ERES and Golgi, two intracellular structures positioned on the protein transport pathway.

The Telomere Terminal Structure in *Trypanosoma brucei*

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Telomeres are specialized nucleoprotein complexes at the end of linear chromosomes. They are essential for chromosome stability and genome integrity. In most eukaryotes, telomere DNA consists of simple repetitive TG-rich sequences, and there is a single-stranded 3' G-rich overhang at the very end of the telomere. This telomere G-overhang structure is essential for telomere maintenance: in the presence of telomerase, G-overhang serves as a substrate; in the absence of telomerase, G-overhang can mediate efficient break-induced-repair (BIR) of chromosome ends, an alternative mechanism for telomere maintenance and an important pathway for telomere recombination. Telomere recombination is particularly important for *Trypanosoma brucei*, a protozoan parasite and the causative agent of Human African Trypanosomiasis. *T. brucei* evades the host's immune attack by regularly changing its variant surface glycoprotein (VSG), and homologous recombination is one of several important mechanisms for VSG switching. Therefore, abnormal telomere recombination may affect VSG switching efficiency. So far, the telomere terminal structure in *T. brucei* is poorly understood, and the only available native in-gel hybridization analysis is not sensitive enough to reveal the details of this structure. To better characterize the *T. brucei* telomere G-overhang structure, we adopted a ligation-mediated primer extension assay. This assay allowed us to determine both the length and the terminal nucleotide of the telomere G-overhang, and we observed that the majority of *T. brucei* telomeres have very short G-overhangs that end in 5' TTAGGG 3'. Such TTAGGG-ending G-overhangs are greatly diminished in cells lacking TERT, the telomerase protein component, and in cells depleted of tbTRF, the duplex telomere DNA binding factor.

Targeting of receptor-type adenylate cyclases to flagellum membrane sub-domains in procyclic-form *Trypanosoma brucei*

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The kinetoplastid flagellum is recognized for its motility functions, but increasing evidence indicates the flagellum functions in signaling and that flagellar membrane proteins present an important host-parasite interface. In other organisms, flagellum-mediated signaling requires dedicated trafficking of transmembrane receptors to the flagellar membrane, which has a protein composition distinct from the rest of the cell surface. Despite advances in identifying molecular machinery for flagellum protein targeting, little is known about signals that direct proteins to the flagellum membrane and/or flagellum membrane sub-domains. Proteomic analysis of the flagellar membrane from procyclic-form *T.brucei* identified a subset of surface-exposed receptor-type adenylate cyclases (ACs) [1], that are encoded by a multigene family and postulated to function in signaling. Immunofluorescence localization revealed distinct localizations for different AC paralogs. One group localized along the full flagellar length, while another group specifically localized to the flagellum tip. The tip contacts the tsetse salivary gland epithelium at the onset of differentiation to mammalian-infectious forms. Distinct AC localizations indicate specialization of flagellum membrane subdomains, involving differential targeting. We employed truncation mutants and chimeric proteins to define flagellar targeting and tip targeting sequences. We identified a 45 amino acid segment within the intracellular domain that is necessary for flagellum membrane targeting and, further, dictates tip-specific localization. Site-directed mutagenesis of individual residues is underway to more precisely define the minimal targeting motifs for flagellum and flagellum tip localization. Studies on these differentially localized receptors will advance understanding of principles that govern protein targeting to the flagellar membrane as well as provide insight into *T.brucei* host-sensing mechanisms, both of which are poorly understood but fundamentally important features of trypanosome biology. Funding:NIH,BWF,SNF
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Comparative Genomics of Four Leishmania Species Reveals Few Gene Differences And Large Structural Changes

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Using a combination of traditional and second generation sequencing technologies, we have generated a reference genome for Leishmania mexicana and refined the existing reference genomes for L. major, L. infantum and L. braziliensis. This has allowed for new orthologue comparisons to be performed across these four genomes, leading to the identification of unexpectedly low numbers of species-specific orthologue clusters in each of these (2, 14, 19 and 67 respectively). We have also used this data to predict allelic variation between species, and find that L. major Friedlin and L. infantum JPCM5 have a surprisingly low number of predicted heterozygous sites compared to L. braziliensis and L. mexicana. We use short read coverage to infer ploidy and gene copy numbers in each of these four genomes. In contrast to the minor differences in gene content, large copy number variations (CNV) exists between species, with 204 multi-copy genes in L. major and 306 in L. mexicana. Chromosome copy number also varied significantly between species, with 9 supernumerary chromosomes in L. infantum, 4 in L. mexicana, 2 in L. braziliensis and one in L. major. A significant bias was shown to exist against multi-copy genes on supernumerary chromosomes, indicating that duplication events may occur more frequently on disomic chromosomes. Taken together, our data demonstrates that while there is little variation in unique gene content across Leishmania species, large-scale genetic heterogeneity can result through gene amplification on disomic chromosomes and variation in supernumerary chromosomes. Increased gene copy number on multivalent chromosomes may contribute to alterations in gene expression in response to environmental conditions in the host, providing a genetic basis for disease tropism.

Pyrimidine transport and resistance to 5-fluorouracil in Leishmania

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Little is known about pyrimidine nucleobase transporter in *Leishmania*. In order to identify the gene(s) coding for this activity and to understand better pyrimidine metabolism, we generated a panel of five independent mutants resistant to 5-fluorouracil (5-FU), an anti-cancer drug also having strong anti-leishmanial activity. We found that the resistance phenotype of a subset of our mutants could be explained by the amplification of the gene encoding the dihydrofolate reductase-thymidylate synthase (*dhfr-ts*), the cellular target of 5-FU. The resistance conferred by the amplification of the *dhfr-ts* gene was not stable however, as the mutants lost the amplicon after 30 passages in the absence of drug pressure. Interestingly, the mutants not harbouring an amplification of *dhfr-ts* were defective in the uptake of labelled uracil. In contrast to the amplification of *dhfr-ts*, the resistance of these transport deficient mutants was stable after 30 passages without 5-FU. Competitive transport experiments with different inhibitors confirmed that the transport defect was specific to uracil. The transport defect did not involved the equilibrate nucleoside transporters (ENT) as no mutations or gene rearrangements could be observed for any of these genes. In order to identify the gene encoding the uracil transporter, we performed functional cloning experiments and looked for clones in which the 5-FU sensitivity was restored in transport deficient mutants. One cosmid was shown to sensitize resistant cells to 5-FU, although this does not seem to be by increased transport. This is further studied and additional functional cloning experiments are underway to isolate the putative transporter.

Protein-protein interaction network of post-transcriptional gene expression regulation in *Trypanosoma cruzi*

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Trypanosoma cruzi is a protozoan causing Chagas isease. One of its major characteristics is post-transcriptional gene expression regulation. As RNA-binding proteins are extremely important for its control, we are conducting several *T. cruzi* large scale analyses, including RNA-seq and mass spectrometry; these results are providing important clues about *T. cruzi* biology, but their integration will provide a clearer picture. However, integration is not trivial and a possible way to improve it is creating a large map of protein-protein interaction (PPI). Aiming to create an initial map of RNA-binding PPI, we have constructed a *T. cruzi* ORFeome, comprising ∼8,000 proteins representing almost the whole non-repetitive *T. cruzi* proteome. We have selected ∼500 proteins (RNA-binding or constituent of interacting functional complexes, as ribosome, splicing or decay machinery, etc) for yeast two-hybrid (Y2H) analyses using selective media, as HIS-, URA- and lacZ, obtaining, based in two distinct stringency criteria, 75 and 29 interactions, of 57 and 19 proteins, respectively. Some of these interactions were also identified in other interactome datasets, but only for a small fraction. Although it raises some concerns about false positives, we hypothesize that, due to its large evolutionary distance from other model organisms whose interactome is well studied, and to the specificity of its post-transcriptional gene expression regulation, comparison of interactome datasets based on orthologs is of low power to detect true interactions. We are currently performing in vivo analysis of selected interactions to assess the specificity of our results. Besides that, we are integrating several large scale datasets over the interaction network, and increasing the interactome coverage.

Using comparative proteomics to identify life-cycle regulated proteins in *Trypanosoma brucei*

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The life cycle of the African trypanosome, *Trypanosoma brucei*, is characterised by a transition between insect and mammalian hosts, very different environments that present the parasite with very different challenges. These challenges are met by the expression of life-cycle stage-specific cohorts of proteins which function in systems such as metabolism, immune evasion and cytoskeletal organisation. Here we employ a gel-based comparative proteomic technique, DiGE, to identify cytoskeletal proteins that are expressed differentially in mammalian bloodstream and procyclic insect form trypomastigotes. From this analysis we identify a pair of novel, related proteins, one of which is expressed in the procyclic form and the other in the bloodstream form. We show that these proteins, SAP-P and SAP-B, localise to the subpellicular corset of microtubules and are essential for correct cell division only in their respective life cycle stages. These newly identified proteins join a growing cohort of cytoskeletal components involved in cell division such as CAP5.5 that are present in the genome in at least two forms with life-cycle stage-specific expression and function.

RNA polymerase I of *Trypanosoma brucei* functions independently of RPB7

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Trypanosoma brucei has a multifunctional RNA polymerase (pol) I that transcribes ribosomal gene units (*RRNA*) and units encoding its major cell surface proteins variant surface glycoprotein (VSG) and procyclin. Previous analysis of tandem affinity-purified, transcriptionally active RNA pol I identified ten subunits including an apparently trypanosomatid-specific protein termed RPA31. Another ortholog, RPB12, was identified *in silico*. No orthologs of the yeast subunit doublet RPA43/RPA14 have been identified yet. Instead, a recent report presented evidence that RPB7, the RNA pol II paralog of RPA43, is an RNA pol I subunit and essential for *VSG* transcription (Penate *et al*., 2009, EMBO Rep. 10:252-257). Since we did not detect RPB7 in active RNA pol I preparations, we revisited RPB7's role in RNA pol I transcription. Firstly, we did reciprocal co-immunoprecipitations of RPB7 and the RNA pol I-specific subunit RPB6z and found that, if at all, RPB7 interacted with RPB6z to less than 1%. Secondly, ChIP experiments showed that RPB6z - but not RPB7 - cross-linked to the *VSG* expression site (ES) promoter and the 18S rRNA coding region. Interestingly, RPB7 did crosslink to a low extent to the *RRNA* promoter but so did the RNA pol II-specific subunit RPB9 suggesting that RNA pol II is recruited to this promoter. Finally, immunodepletion of RPB7 from bloodstream form extract strongly inhibited RNA pol II transcription, as expected, but did not affect *RRNA* or *VSG* ES promoter transcription in the same reactions. Since ChIP and *in vitro* transcription reactions avoided secondary effects of protein expression inhibition by *RPB7* silencing, our results demonstrate that RPB7 is not a functional part of RNA pol I as previously published.

Characterization of a putative trypanosome-specific transcription factor

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Trypanosoma brucei, the parasite responsible for causing African Sleeping Sickness, has many unusual biochemical features and processes, including RNA Polymerase II (RNAPII)-dependent gene transcription. We have identified a trypanosome-specific protein that is associated with the RNAPII transcriptional machinery: TFIIB Associated Factor, 49kDa (*Tb*TAF49). While this protein contains motifs associated with known transcription factors, the protein is unique to trypanosomes. We are characterizing the role of *Tb*TAF49 in the parasites. RNA interference studies, verified through Western blot analysis, reveal that *Tb*TAF49 is essential for parasite growth. Using biotinylated DNA probes and trypanosome nuclear extract, which contains *Tb*TAF49, we have found that *Tb*TAF49 interacts tightly with DNA. Five unique DNA sequences were queried for interaction with *Tb*TAF49: the trypanosome U6 and Spliced Leader gene promoter regions, internal regions from the *Tb*TAF49 and *bla* genes, and unrelated lambda phage DNA. When the interaction was challenged with either high salt (up to 1.0M KCl) or non-ionic detergent (up to 0.2% TWEEN20), *Tb*TAF49 still interacted with all DNA sequences tested. Thus, *Tb*TAF49 appears to bind DNA in a non-specific fashion.

Polyamine Biosynthesis in *Leishmania donovani*

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Significant differences exist between the polyamine biosynthetic pathways of *Leishmania* and their mammalian hosts, reinforcing this pathway as a potential therapeutic target for leishmaniasis. The polyamine pathway of *Leishmania* consists of four enzymes: arginase (ARG), ornithine decarboxylase (ODC), spermidine synthase (SPDSYN), and S-adenosylmethionine decarboxylase (ADOMETDC). To characterize this pathway in detail, we employed a targeted gene replacement strategy to create a series of null mutants, each lacking one of the four genes in the pathway. Each of the genetic lesions creates a conditional lethal mutation that can be bypassed by media supplementation with downstream metabolites *in vitro*, illustrating that an intact polyamine biosynthetic pathway is essential for promastigote survival and growth. Moreover, the Δldarg, Δldodc, Δdadometdc, and Δldspdsyn mutants are all markedly compromised in their abilities to establish infections in mice. The extents of the virulence deficits, however, vary considerably with the nature of the genetic lesion, which we hypothesize is attributable to the differential availability of amino acid and/or polyamine pools in the mammalian phagolysosome. Furthermore, we have shown that oral administration of putrescine to Δldodc-infected mice can partially restore the virulence defect of Δldodc parasites, whereas oral administration of DFMO, an ODC inhibitor, to mice inoculated with wild-type L. donovani results in a partial decrease in parasite burdens, indicating that the polyamine biosynthetic machinery of *L. donovani* can be nutritionally or pharmacologically targeted. Ongoing studies with the Δldodc mutant include infectivity experiments in Syrian Golden hamsters, a more robust model for visceral leishmaniasis, and preliminary results indicate that these parasites are unable to establish an infection, further validating polyamine enzymes, especially LdODC, as promising therapeutic targets.

Identification of genes regulating social behavior in *Trypanosoma brucei*

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Surface-induced social behaviors are well documented in many microorganisms and provide a variety functionalities such as defense, tissue colonization, navigation, host-pathogen interaction and genetic diversity. Within the tsetse fly vector, *Trypanosoma brucei* remains in constant contact with host tissue surfaces, but the impact of surface exposure is unknown. Recent advances have shown that procyclic-stage *T. brucei* engages in social motility upon contact with semisolid surfaces [1]. The findings reveal that these parasites can sense, communicate and coordinate their movements in response to extracellular signals. Social motility represents a novel aspect of trypanosome behavior but how trypanosomes communicate and the signaling mechanisms employed are unknown. We are using two strategies to understand the mechanisms underlying social motility. The first is a candidate gene approach in which specific genes are targeted. RNAi and pharmacological inhibitor studies have implicated the cAMP pathway in the regulation of social motility (see also the presentation by Oberholzer at this meeting). To identify effectors, we have targeted 14 proteins with putative cNMP-binding domains and are currently using RNAi to individually test these genes for a role in social behavior. The second strategy is an unbiased approach in which an RNAi library (Englund lab) is utilized as part of a forward genetics screen to identify genes that regulate social motility. With the combined targeted and unbiased approach, we expect to elucidate mechanisms underlying trypanosome social behavior, as well as dissect signaling pathways employed by eukaryotic pathogens for host-pathogen communication. 1. Oberholzer et al. (2010) *PLoS Pathogens*, doi: 10.1371/journal.ppat.1000739 Funding: NIH Training Grant in Cell & Mol. Biol, BWF

Development of an aptamer based assay to detect parasite biomarker in blood for the diagnosis of Chagas disease

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The blood borne pathogen, *Trypanosoma cruzi* is the etiological agent of Chagas disease in humans. Unlike the chronic phase, parasites can be easily detected in blood of infected individuals by microscopy or PCR based methods during the acute phase of the disease. To overcome the difficulty of detecting parasites directly, diagnostic assays detect host anti-*T. cruzi* antibodies as a surrogate marker for infection. However, these assays are not reliable during the initial window period, or to follow cure after drug treatment due to the persistence of parasite specific antibodies. The parasites secrete various antigens in the blood and these have been collectively termed as *T. cruzi* Excreted Secreted Antigens (TESA). We utilized *in-vitro* RNA SELEX methods to develop TESA aptamers (short nucleic acid molecules) with the goal of utilizing them as specific ligands in detection assays. The TESA SELEX was performed using *T. cruzi* trypomastigote infected NIH 3T3 cell culture supernatant. Based on their binding activity to TESA, 4 aptamers were selected for further analysis. These aptamers were tested in an enzyme linked oligonucleotide assay for binding to serum obtained from *T. cruzi* infected mice. Aptamer L44 (AptL44) demonstrated a consistent and strong binding to infected mouse serum. This interaction was specific as a scrambled aptamer did not bind to either infected or uninfected mouse serum. AptL44 was also able to detect TESA in chronically infected mice. Further analysis of the binding properties of AptL44 and the identification and purification of its target are being carried out. This is the first demonstration of an aptamer based assay that detects a parasite biomarker for the diagnosis of Chagas disease.

Naphthalene-based RNA editing inhibitor blocks RNA editing activities and Editosome assembly in *Trypanosoma Brucei*

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RNA editing, catalyzed by the multi-protein editosome complex, is an essential step for the expression of most mitochondrial genes in trypanosomatid pathogens. It has been shown previously that *Trypanosoma brucei* RNA editing ligase 1 (*Tb*REL1), a core catalytic component of editosome, is essential in mammalian life stage of these parasitic pathogens. Because of availability of its crystal structure and absence from human, the adenylylation domain of *Tb*REL1 has recently become the focus of several studies for designing inhibitors that target its adenylylation pocket. Here, we have studied new and existing inhibitors of *Tb*REL1 in order to better understand their mechanism of action. We found that these compounds are moderate to weak inhibitors of adenylylation of *Tb*REL1, and in fact enhance adenylylation at higher concentrations of protein. Nevertheless, they can efficiently block deadenylylation of *Tb*REL1 in editosome and, consequently, result in inhibition of the ligation step of RNA editing. Further experiments directly showed that the studied compounds inhibit the interaction of editosome with substrate RNA. This was supported by the observation that not only the ligation activity of *Tb*REL1, but also the activities of other editosome proteins such as endoribonuclease, terminal RNA uridylyltransferase (TUTase), and U-specific exoribonuclease (ExoUase), all of which requiring the interaction of editosome with the substrate RNA, are efficiently inhibited by these compounds. In addition, we found that these compounds can interfere with integrity and/or assembly of editosome complex, opening the exciting possibility of using them to study the mechanism of assembly of the editosome components.

Genome-wide binding and functions of ORC1/CDC6, a nuclear DNA replication factor in *Trypanosoma brucei*

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DNA replication is central to the propagation of life and initiates by the designation of genome sequences as origins. Despite considerable progress in understanding mitochondrial DNA replication in kinetoplastid parasites, little is known about nuclear DNA replication. The mechanism and machinery of DNA replication initiation is well-conserved amongst characterised eukaryotes: a six-protein origin recognition complex (ORC; Orc1-Orc6), Cdc6 and Cdt1 are recruited sequentially to DNA and, once bound, load the replicative helicase complex (Mcm2-7) to form a pre-replicative complex at origins. Orc1 and Cdc6 are related in sequence, indicative of derivation from a common ancestor, and such an ancestral protein is found in present-day archaea and, remarkably, in *Trypanosoma brucei* (named TbORC1/CDC6), related trypanosomatids and some other protists. We have used RNAi to examine TbORC1/CDC6 function, and our findings in procyclic form cells are in agreement with work from other labs, suggesting an essential nuclear replication function, while we see a distinct and more severe phenotype in bloodstream form cells. Using chromatin immunoprecipitation of TbORC1/CDC6, coupled with microarray hybridisation, we have mapped the binding sites of TbORC1/CDC6 genome-wide, revealing relatively sparse binding in the conserved core of the *T. brucei* chromosomes and higher-density binding in the more variant subtelomeres, perhaps consistent with origin and non-origin functions. Analysis of gene expression following RNAi suggests that TbORC1/CDC6 knockdown can be associated with increased mRNA abundance of *Variant Surface Glycoprotein* genes and of a number of genes in the so-called 'strand switches' separating directional gene clusters. We suggest that these data show that replication and transcription have functional overlaps within trypanosomes, and TbORC1 has silencing functions, as in other organisms.

The role of XRNA in transcriptome-wide mRNA degradation in *Trypanosoma brucei*

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In eukaryotes, most mRNAs are degraded by initial 3'-5'exonucleolytic digestion of the poly(A) tail. After deadenylation, there is rapid degradation of the remaining mRNA, either by 3'-5' exonucleases, or by decapping and 5'-3' exonucleolytic degradation by XRN1. We found that in *Trypanosoma brucei*, the *Saccharomyces cerevisiae* XRN1 homologue, XRNA, was important for the degradation of two highly unstable developmentally regulated mRNAs (Li et al., 2006). By using an RNA circularization and sequencing technique, we showed that depletion of XRNA caused accumulation of mRNA that had been degraded at both the 5' and 3' ends (NAR 2009 37(16): 5511-5528). We have now measured transcriptome-wide mRNA degradation and the role of XRNA in mRNA decay by high-throughput sequencing of total RNA. In bloodstream-form trypanosomes, mRNA half-lives range between 7 min and several hours, with 90% of transcripts having half-lives of less than 30 min. Transcripts encoding proteins with coordinated functions tend to have similar half-lives; those encoding ribosomal proteins and glycolytic enzymes were among the most stable. Our results showed that mRNA stability and gene copy number are major determinants of mRNA abundance, but other processes also contribute. For most mRNAs, poly(A) shortening precedes the decay of the body of the transcript. There is no correlation between mRNA half-lives and the lengths of ORFs, 5'-untranslated regions or 3'-untranslated regions. XRNA depletion had only minor effects on steady-state mRNA abundance but caused increases in overall mRNA half-lives; the greatest effects were seen on short-lived mRNAs.

Mitochondrial DNA Polymerases POLIB and POLID Cooperate in Minicricle Replication

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Kinetoplast DNA (kDNA) of *Trypanosoma brucei* and related protist parasites is a catenated network composed of minicircles and maxicircles that is essential for parasite survival. Network replication involves release of covalently closed minicircle monomers, theta structure replication, and reattachment of progeny minicircles to the network periphery. At least 6 mitochondrial DNA polymerases are required for kDNA transactions. Three of these (POLIB, POLIC and POLID) are essential for parasite growth and perform non-redundant roles in kDNA maintenance. Previously, we showed that individually silencing POLIB or POLID perturbed minicircle replication and resulted in the accumulation of a covalently closed dimeric minicircle species known as fraction U. Since fraction U is produced only when silencing a subset of kDNA replication proteins (also including mitochondrial topoisomerase II and PIF2 helicase), this unique phenotype suggests that POLIB and POLID perform non-redundant roles but cooperate in minicircle replication. We tested this hypothesis by simultaneously silencing POLID and POLIB. A >90% reduction in the cellular levels of both proteins by RNAi resulted in earlier growth inhibition and more rapid loss of kDNA networks than seen when silencing either polymerase alone. Additionally, dual gene silencing resulted in a near-complete inhibition of minicircle replication, which was never achieved with single-gene silencing. Our data strongly suggest that POLIB and POLID cooperate during minicircle replication. We are currently examining the functional interaction of POLIB and POLID in vivo by coimmunoprecipitation.

A novel platelet-activating factor molecular species synthesized by the human parasite Trypanosoma cruzi

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Trypanosoma cruzi is the causal agent of Chagas disease. Platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a potent lipid autacoid that functions as an intercellular mediator and can also serve as an intracellular messenger through a specific receptor. Previous data from our group suggested that T. cruzi synthesizes a PAF-like phospholipid that modulates the parasite's differentiation and infectivity. Here, we describe the purification and molecular characterization of PAF species from complex lipid mixtures of all five life-cycle stages of T. cruzi. Using solid-phase extraction (SPE) followed by electrospray ionization tandem mass-spectrometry (ESI-MS/MS), we identified three species of PAF, namely, the common C16:0-PAF, the rare C16:1-PAF, and the novel C16:2-PAF. C16:0 PAF was present in very low or untraceable amounts, whereas C16:1- and C16:2-PAF species were found in much higher concentrations. Quantification of C16:1- and C16:2-PAF by ESI/MS revealed that these PAF species were very abundant in mammalian host-dwelling trypomastigote, and intracellular and extracellular amastigote stages. We also observed that highly infective trypomastigotes had their ability to invade mouse macrophages dramatically reduced when the parasites were pre-treated with PAF-acetylhydrolase (PAF-AH), an enzyme that converts PAF to the biologically inactive lyso-PAF, as well with WEB 2086, a classic competitive PAF antagonist that binds specifically to PAF receptor. The trypomastigote infectivity was fully restored two hours after removal of PAF-AH from the incubation medium, suggesting that parasite was able to reestablish its PAF levels. The

presence of a novel PAF species indicates that T. cruzi may possess a non-canonical PAF biosynthetic pathway that could be further explored for potential drug targeting applications. Acknowledgments - NIH (1R01AI070655, 3R01AI070655-04S1, 5S06GM08012-39, 2G12RR008124-16A1, 2G12RR008124-16A1S1), CNPq, FAPERJ, and INCTEM (Brazil).

Nucleotide Sugar Transport Inhibition In *Trypanosoma brucei* Alters Surface Glycoproteins: Implications For Pathogenesis

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Trypanosoma brucei is the pathogenic agent of African trypanosomiasis. Sugars of surface glycoproteins play pivotal roles in parasite infectivity. A key step in glycosylation is the transport of nucleotide sugars, by nucleotide sugar transporters, from the cytosol into the lumen of the Golgi apparatus where they serve as sugar substrates in glycosylation reactions. Mutations in the above transporters have been shown to result in developmental diseases as well as impaired cell walls of yeast and pathogenic fungi. We have identified four nucleotide sugar transporters in *T. brucei* by using in vitro biochemical transport assays and phenotypic correction of mutants including *Kluyveromyces lactis* defective in UDP-*N*-acetylglucosamine transport, MDCK cells defective in UDP-galactose transport and *S. cerevisiae* defective in GDP-mannose transport. TbNST1 and TbNST2 transport UDP-galactose, TbNST3 transports GDP-mannose and TbNST4 transports UDP-*N*-acetylgalactosamine, UDP-*N*-acetylglucosamine and GDP-mannose. Inhibition of TbNST4 gene expression by RNAi resulted in underglycosylated procyclins isolated from biotinylated surface proteins. In addition, immunofluorescence staining showed significant reduction of procyclin at the cell surface from RNAi-induced cells as compared with uninduced cells suggesting a transport defect. We are currently generating RNAi cell lines of *T. brucei* bloodstream form to determine the parasite's virulence in a mouse model.

A putative ion channel is required for flagellum attachment in Trypanosoma brucei

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Trypanosoma brucei is the etiological agent of sleeping sickness, which causes significant human mortality and limits economic development in sub-Saharan Africa. Critical to trypanosome development and disease pathogenesis is the parasite's single flagellum which drives cell motility and participates in host-pathogen interactions, immune evasion and cell morphogenesis. A unique and defining feature of *T. brucei* cell architecture is the flagellum attachment zone (FAZ), a region of membrane-cytoskeleton linkages that provide for lateral attachment of the flagellum to the cell. Disruption of the FAZ results in flagellum detachment and is lethal. Only a handful of proteins have been localized to the FAZ, some of which function in maintenance of attachment and others that participate in surface remodeling. However, the nature of the attachment architecture and the involved components remain enigmatic. We discovered that Goldengate, a putative calcium channel, localizes to the FAZ region and is required for flagellum attachment. Goldengate is a massive 304 kDa multi-spanning membrane protein with orthologs in many eukaryotes. We *in situ* tagged Goldengate in both procyclic and bloodstream form parasites and showed FAZ localization and membrane partitioning by immunofluorescence and biochemical fractionation respectively. Flagellum-detachment experiments suggested that Goldengate resides on the flagellar side of the FAZ. Surprisingly, RNA-interference knockdown of Goldengate results in flagellar detachment and is lethal in both life stages. Precise localization of Goldengate by immuno-electron microscopy and structure-function experiments are underway to distinguish between ion channel functions and structural functions in flagellum attachment.

Kinetoplastid flagellate *Phytomonas* survives without heme

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Heme is essential for fundamental cellular processes such as electron transport in the respiratory chain, oxidative stress response or redox reactions in various metabolic pathways. Parasitic kinetoplastids represent a rare example of organisms that depend on oxidative metabolism but are heme auxotrophs. The media for their *in vitro* cultivation has to be supplemented with heme. However, we found that heme is dispensable for the survival of *Phytomonas*, the serious yet understudied parasite of plants, as even one-year long continuous cultivation in a heme-free medium had no impact on cell growth. Furthermore, we were not able to detect any traces of heme in this culture using very sensitive assays. Together with the lack of heme biosynthesis genes in two recently sequenced *Phytomonas* genomes, these findings clearly indicate that it can survive without heme. In order to find out how this is actually possible, we carefully examined the putative functions of heme in *Phytomonas serpens*. We found that heme does not play any important role in the mitochondrial respiratory chain. Supplementation with heme does not improve resistance against oxidative stress in *Phytomonas*, which corresponds with the absence of heme-peroxidases in the genome. While it seems that for desaturation of fatty acids, *P. serpens* uses an alternative electron donor, heme is required for the 14-demethylation of lanosterol to produce ergosterol. However, we found that *P. serpens* grown in the absence of heme can utilize 14-methyl-sterols in its membranes. It is therefore apparent that this flagellate has unique metabolic properties bypassing the requirement for heme. To our knowledge, this is the first example of a eukaryote totally lacking heme.

A stringent model for studying the function of N-linked glycans

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N-linked glycosylation of proteins plays an essential role in a variety of cellular processes, such as protein folding, protein stability, routing to the cell surface, cell adhesion and signaling. A function in the formation of a protein barrier and under conditions of molecular crowding has also been hypothesized. However, despite extensive studies no concise picture of N-linked glycosylation exists to date. The cell surface of the unicellular African trypanosome is covered by a dense coat of at least 95% of a single protein, the variant surface glycoprotein (VSG). Antigenic variation leads to the expression of a new VSG and thus the formation of a new surface coat. All known functional VSGs are modified by the addition of at least one N-linked glycan, but addition of two or three N-linked glycans is also common. This unique surface, densely packed with near to only one type of glycosylated surface protein, coupled with the inherent variability of its protein within a similar structural context makes the VSG of African trypanosomes an ideal and novel model for probing the function of N-linked glycans. As a proof-of-concept we have generated glycosylation mutants of VSGs, containing different numbers of N-linked glycans. Transgenic trypanosomes devoid of any N-glycan in their surface coat could be generated for only one of these proteins, suggesting that N-glycosylation fulfills different functions even in the same type of protein.

RNA editing in trypanosomes: why is RNA editing ligase 1 (REL1) essential, whereas REL2 is not?

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Uridylyl (U) insertion/deletion RNA editing in *Trypanosoma brucei* is governed by multiprotein complexes called the editosomes. The final step in each round of this post-transcriptional process is that of religating the edited mRNA fragments. The ~20S core editosomes (RECC) contain two RNA editing ligases, REL1 and REL2, and these enzymes have been localized to different RECC subcomplexes: REL1 and REL2 are closely associated with the U removal enzyme REX2 and the U addition enzyme RET2, respectively. While REL1 is clearly essential for RNA editing, REL2 knockdown by RNAi has not resulted in a detectable phenotype. To explain these findings, alternative scenarios have been suggested: (a) REL2 is not functional *in vivo*; (b) REL1 can function in both insertion and deletion editing, whereas REL2 can only function in insertion editing; (c) REL1 has an additional role in repairing mRNAs cleaved at incorrect sites. To further investigate the respective functions of the two RELs we use a combination of genetic complementation with chimeric ligase enzymes and analysis of the editing intermediates that accumulate *in vivo* after REL1 knockdown. Expression of a chimeric ligase protein where the REL1 C-terminal protein interaction domain had been fused with the REL2 catalytic domain resulted in incorporation into RECCs, but did not rescue the growth defect caused by knockdown of endogenous REL1. This indicates that specific catalytic properties of REL1 rather than its position within the deletion subcomplex make it an essential ligase. We are also in the process of developing a deep sequencing strategy to comprehensively identify the *in vivo* substrates of REL1. Preliminary data on this will be presented.

The role of small G proteins associated to IFT in trypanosomes

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The *Trypanosoma brucei* flagellum possesses a canonical 9+2 axoneme structure made of nine outer doublets of microtubules and a central pair. This organelle is present throughout the life cycle and is essential for the survival of the parasite in both the insect and mammal host. During the life cycle, the flagellum undergoes dramatic size variations (from 5 to 30μm) (**Rotureau et al., 2010**). The organelle length is regulated by intraflagellar transport (IFT), a mechanism used in the vast majority of cilia and flagella in eukaryotic cells. Although the role of basic IFT components has been well established, still little is known about IFT regulation. Small GTPases are known for being key regulators of many trafficking processes thanks to their rapid cycle of activation/deactivation, making them attractive candidates for the IFT regulation. Two RAB-like proteins termed **RABL4** (IFT27) and **RABL5** (IFT22) have been identified in *Trypanosoma brucei*. We used different approaches in order to localize and to determine the role of RABL4 and RABL5 during flagellum formation in the trypanosome. First, GFP fusion experiments showed that the proteins are localized in the cell body, at the basal body and within the flagellum, like all IFT proteins studied so far in trypanosomes. Bi-directional movement of GFP fusion protein was observed in the flagellum. RNAi silencing demonstrated an essential role in flagellar assembly and revealed that RABL4 and RABL5 play distinct roles in IFT regulation. The localization of RABL4 in different IFT mutants is currently being determined, as well as in the different stages within the tse-tse fly.

Characterisation of *Trypanosoma brucei* Nucleoporins

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The nuclear pore complex (NPC) is the sole site for molecular exchange between the cytoplasm and the nucleus. Nucleoporins that make up the *Trypanosoma brucei* NPC share a low sequence similarity to their metazoan homologs, despite being both structurally and architecturally similar to their yeast and vertebrate counterparts. In higher eukaryotes, various nucleoporins are involved in gene expression, telomere maintenance and cell cycle events. We aim to functionally characterize a selection of nucleoporins to see if they fulfill similar roles and possibly influence gene expression in trypanosomes. The majority of trypanosome nucleoporins display an exclusively punctate distribution around the nuclear envelope. However, two nucleoporins TbNup92 and TbNup53b that form part of the nuclear basket and FG-repeat group respectively, demonstrate strikingly different locations during the cell cycle. TbNup92, which corresponds to the mammalian Tpr and yeast Mlp2 nucleoporins, migrates from the NPC to the spindle pole body in mitotic cells. TbNup53b, on the other hand, localises at the NPC throughout the cell cycle, and during interphase this protein forms a lattice structure throughout the nucleus as well as a distinct puncta at the nucleolar periphery. RNAi analysis of these two nucleoporins was carried out to deduce their function. Preliminary results show that both TbNup92 and TbNup53b knockdown cells show defects in proliferation and morphology. Further, in knockdown cells, telomeres often fail to aggregate at the metaphase plate which coincides with apparent loss of the mitotic spindle. Other consequences of down-regulation of these proteins are under study.

High Throughput RNA-Seq analysis reveals differences in mRNA abundance and Splice Leader site location between different lifecycle stages of several *Leishmania* species

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We have analyzed the transcriptomes of *Leishmania major* Friedlin V1(LmjF), *L. donovani* (LdoS), *L. tarentolae* (LtaU), *L. amazonensis* (LamP) and *L. braziliensis* M2903 (LbrM), which represent all three forms (cutaneous, visceral and mucocutaneous) of leishmaniasis. Several approaches were utilized to construct different Next Generation Sequencing (NGS) libraries, including oligo(dT)-priming for first strand cDNA synthesis (to map the 3' end of mRNAs) and Splice Leader (SL) priming for second strand cDNA synthesis (to map the 5' end of mRNAs). The latter was particularly effective, since it could be performed on total RNA and provides data indicating both SL site location and abundance of each mRNA species. In LmjF, we mapped the SL and polyA sites for >98% and 85% of the mRNAs, respectively, and identified changes in transcription abundance and SL site location between the procyclic, metacyclic and amastigote stages. In LdoS, we mapped SL sites for >99% of protein coding genes and showed that >2000 mRNAs changed abundance by more than 2-fold during axenic promastigotes-to-amastigote differentiation. In addition, several hundred genes showed changes in SL site location during differentiation. The studies in LtaU, LamP and LbrM were focused on changes in mRNA abundance due to specific gene deletions or changes in culture conditions. Comparison of SL site location and mRNA abundance between the five *Leishmania* species are currently underway.

Lateral mobility of the VSG implies a crowding threshold on the trypanosome cell surface

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The lateral mobility of a membrane protein is an essential determinant of the protein's function. Factors such as protein glycosylation, membrane anchoring, molecular crowding, or the influence of the extracellular environment have been proposed to influence the diffusion of membrane proteins. However, a systematic study that integrates these different aspects is still missing, and contradictory results exist. Here, we focus on a systematic analysis of the contribution of the extracellular domain of a protein to the diffusion of GPI-anchored proteins. The cell surface of the African trypanosome presents us with an ideal model as it is covered by a dense protein layer containing more than 95% of one member of the variant surface glycoprotein (VSG) family. Diffusion measurements were performed on immobilized *Trypanosoma brucei* cells as well as on HPLC purified VSGs in a solid supported lipid bilayer (SSLB) system. A number of different membrane form VSGs and their glycosylation mutants were purified and the secondary structure, dimerization and presence of an intact GPI anchor were analyzed and verified. The diffusion coefficients and mobile fractions measured *in vivo* and using the SSLB system suggest that the VSG coat on the trypanosome surface is as tightly packed as physically possible. Any further increase in protein crowding should result in a dramatic reduction of lateral mobility of VSG, which would interfere with VSG recycling and hence, with the viability of the parasite.

Dynamics and interactions of *Trypanosoma brucei* antigenic variation

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Antigenic variation—a process in which frequent changes to key parasite antigens allows an infecting population to sequentially escape adaptive immunity—is employed by a diverse range of pathogens, allowing them to evade the sophisticated defences of their hosts. It seems to be particularly important to African trypanosomes, which are constantly exposed to immune effectors for weeks, months and even years in mammalian blood and extracellular fluid. African trypanosome antigenic variation is mediated by a dense, uniform coat of variable surface glycoprotein (VSG). The coat covers the entire parasite surface, shielding invariant antigens, and is highly immunogenic. By switching to express a different *VSG* gene, parasites can escape existing specific immune responses. Although the vast majority (>95%) of the ~2000 silent *VSG* genes in the *Trypanosoma brucei brucei* genome are damaged by fragmentation, frameshifts and stop codons, these can be repaired by segmental gene conversion, in which silent donors combine to generate complete 'mosaic' *VSG*s. Other pathogens, including *Babesia*, *Anaplasma*, *Borrelia* and *Neisseria* use segmental gene conversion to generate antigens during infection. But thus far, identified mosaic *VSG*s retain high levels of identity with one another and their silent donors. Is trypanosome mosaic *VSG* construction also a source of antigenic diversity? To address this, and follow antigenic variation in vivo, *VSG*s were sampled longitudinally from chronic mouse infections and analysed to see how expressed *VSG*s correspond with silent copies. Many expressed *VSG*s were identifiable mosaics, and there is evidence of similar mosaics forming independently in different infections. Tb11.09.0005-like ('Green') mosaics were the most prevalent, persistant and diverse set, and were selected for detailed serological and structural analyses.

Identification of an essential *myo*-inositol transporter in *Trypanosoma brucei*

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The cyclic polyol, *myo*-inositol, is an important osmolyte and an essential component for the production of inositol phosphates and inositol phospholipids in all cells. It has previously been reported that *Trypanosoma brucei* bloodstream forms generate inositol de novo from glucose 6-phosphate and that this pathway is essential for normal growth of parasites in culture. We now show that the parasite's need for *myo*-inositol cannot be fulfilled by endogenous production alone but depends on uptake of extracellular *myo*-inositol. We describe the identification and characterization of a novel *myo*-inositol transporter in *T. brucei* which belongs to the hexose transporter superfamily but is a singleton gene and clearly distinct from the *T. brucei* glucose transporters. RNAi-mediated down-regulation of this transporter in procyclic forms completely blocks [^{3}H]-labelled *myo*-inositol uptake and inhibits cell growth. Similar results are also obtained in bloodstream forms. The addition of glucose, mannose or sorbitol shows no effect on [^{3}H]*myo*-inositol uptake, indicating that the transporter is specific for *myo*-inositol. In addition, we show that down-regulation of the transporter changes the phospholipid composition of *T. brucei* procyclic forms by blocking the synthesis of phosphatidylinositol and inositolphosphorylceramide. Expression of the *T. brucei* transporter in *Xenopus laevis* oocytes and characterization of the uptake of *myo*-inositol using two-electrode voltage clamp techniques reveals an electrogenic symport with protons. This is the first report showing that *myo*-inositol uptake into *T. brucei* parasites is essential

Biochemical roles of Leishmania Infantum RAD51 and BRCA2 in Homologous Recombination

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Mutations in BRCA1 or BRCA2 (BReast CAncer) genes lead to approximately 10% of familial breast cancers as well as increasing the risk of ovarian cancer. An hallmark of these cancers is genomic instability, characterized by an increase in chromosomal breaks and translocations. In normal cells, this is suppressed by homologous recombination which allows the faithful repair of DNA double-strand breaks. The homologous recombination enzymes are stimulated by protein "mediators" such as the tumor suppressor gene BRCA2. The large size of the human BRCA2 protein (384 kDa) poses a great technical challenge for biochemical analyses. Thus, we purified a BRCA2 homolog form Leishmania infantum (Li) which is three times smaller (125 kDa), to better understand the function of BRCA2 and RAD51 in genomic stability. The advantage of using this parasite is that Leishmania amplify or delete its genome stochastically by homologous recombination from repeated sequences distributed throughout the genome. The genes encoding LiRad51 and LiBrca2 were cloned into vectors allowing the expression of recombinant proteins in insect cells. After several optimizations, we succeded to purify the two proteins. We show that both LiRad51 and LiBrca2 interact together and bind DNA. One key step in homologous recombination is the invasion of a resected DNA double-strand break in an undamaged template to form a D-loop structure. Remarkably, LiBrca2 stimulates LiRad51 strand invasion into a homologous sequence. Our data show that LiBrca2 catalyses a vital step of homologous recombination. Further in vivo analyses will support this conclusion.

The flagellar pocket collar: A lord of the rings?

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Trypanosomiasis is a Sub-Saharan Neglected Tropical Disease, caused by *Trypanosoma brucei*, a mono-flagellate protozoan parasite. The *T. brucei* flagellum originates from a cytoplasmic basal body then grows, to emerge from the cell, by traversing an unusual and essential structure called the Flagellar Pocket (FP). The FP is an invagination of the pellicular membrane at the base of the flagellum. This structure is essential for the survival of the parasite, because it is the unique site of endo- and exocytosis. The Flagellar Pocket Collar (FPC) is a cytoskeletal component of the FP, and is located at the neck of the FP where it maintains a ring/horseshoe structure around the proximal base of the flagellum. The FPC contains numerous uncharacterised proteins, including the first protein identified as FPC component called BILBO1. BILBO1 is essential for FP biogenesis and cytokinesis of the parasite (Bonhivers M. et al, PLoS Biol. 2008 May 6;6(5):e105). A two-hybrid *T. brucei* genomic screen was done (Hybrigenics) using BILBO1 as bait and revealed a number of potential BILBO1 partners. In the work presented here we have identified a BILBO1 binding protein that localizes to the FPC. We have named this protein FPC4. We have identified how BILBO1 interacts with FPC4 and we have preliminary data that functionally characterises it with other BILBO proteins (See poster by A. Sahin). GFP tagging illustrates that FPC4 forms a ring-like structure in the FPC but additionally, we have characterised the effects of the GFP fusion protein over-expression on cell growth and morphology in *T. brucei*. We are now carrying out functional analysis using RNAi knockdown.

The role of CAF130 in the process of deadenylation and mRNA turnover in *Trypanosoma brucei*

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In the protozoan parasite *T. brucei*, gene expression is mainly controlled at the post-transcriptional level. The parasite and other studied eukaryotes are known to have two main cytosolic mRNA degradation pathways and both start with deadenylation of the mRNA. It has been demonstrated that deadenylation is the major determinant of mRNA degradation. The CAF-NOT deadenylation complex in *T. brucei* consists of CAF1, NOT1, NOT2, NOT5, DHH1 and a possible homologue of Caf130. NOT1 acts as a scaffold to which the remaining subunits attach themselves. The catalytic subunit CAF1 has a deadenylation activity and is conserved in all eukaryotes. The functions of the other subunits are elusive. My first experiments focused on the putative homologue CAF130, since it is very divergent to its orthologue in higher eukaryotes, and it is only half the size of CAF130 in many other organisms. With co-immunoprecipitation experiments, I could verify that CAF130 is part of the CAF-NOT complex. Moreover, glycerol gradient experiments showed that CAF130 runs with CAF1 and NOT1. By fluorescent microscopy I could demonstrate that CAF130 is located in the cytoplasm. RNAi targeting *caf130* caused a strong proliferation defect, indicating that it has an essential role in the parasite. If the protein level of CAF130 declined below $\asymp$40% proliferation was inhibited. RNA degradation assays revealed that CAF130 is important for mRNA degradation and mRNA deadenylation. After RNAi the steady-state levels and stabilities of studied mRNAs were increased and their deadenylation was affected. These data demonstrate that CAF130 plays an important role in the CAF-NOT complex - not shown in any other organism so far.

A unique homolog of Tim50 in *Trypanosoma brucei*

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Similar to other eukaryotes, *T. brucei* imports a majority of mitochondrial proteins from the cytosol. However, mitochondrial protein import machinery in *T. brucei* is poorly understood. In eukaryotes, proteins are imported into mitochondria *via* multi-protein complexes located on mitochondrial outer and inner membranes known as TOM and TIM, respectively. In *T. brucei*, only a homolog of Tim17 has been characterized so far. Therefore it is critical to identify other trypanosome proteins involved in this process. We found a putative homolog of Tim50 in *T. brucei*. In fungi, Tim50 functions primarily as the receptor for pre-proteins during their translocation from TOM to TIM. *T. brucei* Tim50 (TbTim50) showed 17% identity and 30% similarity to Tim50 from other eukaryotes. Similar to Tim50 proteins from fungi and mammals, TbTim50 possesses a mitochondrial targeting signal at the N-terminus and the NL1 interacting factor-like phosphatase domain at the C-terminus of the protein. However, in contrast to Tim50 proteins in other eukaryotes, the single transmembrane region of TbTim50 is located at the C-terminal domain instead of the N-terminal domain, suggesting the topology of this protein is unique in *T. brucei*. Co-immunoprecipitation analysis revealed that TbTim50 interacts with TbTim17 *in vivo*. Depletion of TbTim50 by RNAi reduced cell growth and inhibited import of proteins into mitochondria. Together, these results revealed that *T. brucei* possesses a unique homolog of Tim50 that associates with Tim17 and is involved in the import of nuclear encoded mitochondrial proteins.

Regulation of Expression of *Trypanosoma brucei* Hexokinase 1 Mediated by Polyadenylation Site

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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. Recently, a deep sequencing effort published by Siegel et al. (2010), identified polyadenylation sites for 5,948 genes, with many genes containing more than one polyadenylation site. Seven polyadenylation sites were identified in *TbHK1* resulting in 3'UTR lengths of: 1026, 1034, 1086, 1166, 1331, 1339 and 1352. Interestingly, *TbHK2* only contains one polyadenylation site resulting in a 353nt 3'UTR. To determine if 3'UTR length impacts transcript level or protein expression in response to environmental cues, each length was cloned downstream of a CAT reporter gene and transiently transfected into both BSF and PF parasites. Probing for the reporter gene using qRT-PCR, we found that steady state mRNA levels of the seven TbHK1 3'UTR lengths are plastic with respect to carbon source availability. The transcript of two of the UTR lengths (1086 and 1352) remained unchanged regardless of the carbon source, while other UTR lengths exhibited up to a 9-fold increase in transcript level dependent on the carbon source in the media. Expression studies also revealed differences in expression levels of TbHK1, dependent on the UTR length and carbon source available. The variation in steady state mRNA level and differences in protein expression together suggest choice of polyadenylation site may serve as a gene expression regulatory mechanism in *T.brucei*.

The VSG repertoire of *Trypanosoma brucei* Lister 427

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DNA was sequenced using Illumina and 454 technologies and assembled de novo. 2,732 (2,527 ¿ 249 amino acids) ORFs encoding complete or partial VSG sequences were evaluated and annotated. 308 sequences appear to be truly complete VSGs, another 46 are complete except for an atypical GPI site, and a further 171 are either missing a few N-terminal amino acids or have a slightly shortened or extended C-terminus. The rest are partial sequences, of which 954 include a probable N-terminus and 350 an identifiable C-terminus. Chromosomal locations are being evaluated. Sequences of 525 predicted complete and almost complete non-pseudogenic VSGs were compared to the complete VSGs in the TREU927 and *T. brucei gambiense* Dal972 assemblies (138 and 28 sequences, respectively). Clustering using a metric based on BLASTp score provides a fast and robust method to type N- and C-terminal domains into classes, and identified several TREU927 sequences that have been mis-classified. The same VSG classes are found in *T. gambiense*, both *T. brucei* strains, and in *T. evansi* and *T. equiperdum*, and there is no apparent bias to the sequence classes in VSG expression sites. Closely similar sequences have similar patterns of cysteine residues, but this pattern alone does not define a VSG class. Clusters formed by N- and C-domains are not fully congruent, but there is a highly significant bias in the distribution of types (p¡10^{-6}), showing that recombination has not efficiently mixed the domains. Moreover, the presence of numerous orthologous sequences with >90% sequence identity among the strains shows that VSGs can be stable over significant timescales, or that there has been recent genetic exchange among the subspecies.

Dynamic Localization of Mitochondrial DNA Polymerases in *Trypanosoma brucei*

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Trypanosoma brucei kinetoplast DNA (kDNA) network replication is a highly coordinated process that occurs once per cell cycle nearly in parallel with nuclear S phase. Proteins involved in replicating the maxicircles and minicircles localize mainly to discrete sites; the kinetoflagellar zone (KFZ) and antipodal sites. Minicircles replicate as free molecules by a topoisomerase-mediated release and reattachment mechanism and undergo Okazaki fragment processing and gap repairing at the antipodal sites. Remarkably, six mitochondrial DNA polymerases are required for kDNA transactions. Two of these, POLIB and POLIC, have essential roles in kDNA replication and localize to the KFZ. A third, POLID, was also found essential for kDNA replication via RNAi silencing. However, POLID localized throughout the mitochondrial matrix suggesting it must redistribute to the kDNA in order to perform its role in replication. Here we demonstrate that POLID has a dynamic localization that is linked to kDNA S phase. POLID was distributed throughout the mitochondrial matrix in 1K or 2K cells that lacked TdT labeling at the antipodal sites. However, in 25% of an unsynchronized population, POLID was detected as discrete foci near the kDNA network. 43% of TdT positive cells contained two POLID foci that colocalized with the antipodal sites, and 6% had an additional labeling at the midzone of the kDNA network. This third focus was observed in cells with a bilobed kinetoplast. Additionally, we demonstrated that POLID is stable following cyclohexamide treatment, suggesting redistribution is not regulated by the recently identified HslVU proteolytic breakdown mechanism. These data suggest that POLID is spatially and temporally available to perform its essential role in kDNA replication by redistributing to the kDNA disk.

Metabolic network analysis-directed assessment of FDA-approved drugs for a neglected tropical disease

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Systems biology holds promise as a new approach to drug target identification and drug discovery against neglected tropical diseases (NTDs). Genome-scale metabolic reconstructions, assembled from annotated genomes and a vast array of bioinformatics and biochemical resources, provide a framework for the interrogation of human pathogens and serve as a platform for generation of future experimental hypotheses. Computational metabolic networks have been reconstructed for several pathogenic organisms, including *Leishmania major*, an agent of cutaneous leishmaniasis. Analysis of pathogen intracellular processes can be used to predict critical protein targets that when perturbed singly or in combination have adverse effects on growth. A direct metabolic network-driven method is proposed that generates a set of high-priority *L. major* targets that are in turn associated with a select number of Food and Drug Administration (FDA)-approved drugs. Selection criteria are applied on both *L. major* targets (e.g. *in silico* gene essentiality) and drugs (e.g. toxicity) to rationally focus on a subset of low-toxic FDA-approved drugs. This network-driven approach identified 15 *L. major* genes as high-priority targets and 240 FDA-approved drugs that are candidate antileishmanials. A small number of these drugs were evaluated *in vitro* against *L. major* promastigotes, and results were compared to previous literature findings and existing high-throughput screens. Synthetic lethality predictions from the metabolic network were simulated to identify additional drugs and to arrive at superadditive drug combinations. Selection of high-priority double-drug combinations might provide for an alternate avenue for drug discovery against leishmaniasis. By integrating publicly available resources using a simple and efficient method, the proposed approach offers significant implications to future drug discovery and drug repurposing strategies against leishmaniasis and other NTDs.

Import of cytoplasmic RNA-binding proteins into the nucleus of *Trypanosoma cruzi*

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In trypanosomes, cytoplasmic RNA-binding proteins (RBPs) play an essential role in regulating gene expression post-transcriptionally. This is achieved by core cytoplasmic post-transcriptional mechanisms such as mRNA degradation, silencing and translation efficiency. Our lab has previously characterized the TcRBP family of RBPs in *T. cruzi*, which is composed by six cytoplasmic members containing a conserved RNA-recognition motif (RRM). From the TcRBP family, TcUBP1 has been characterized extensively. This protein was shown to be involved in the cytoplasmic regulation of the levels of cohorts of mRNAs, and is a core component of P body-like mRNA granules induced by starvation stress. Recently, we found that all the members of the TcRBP family accumulate in the nucleus of *T. cruzi* parasites treated with arsenite. The characterization of TcUBP1 nuclear accumulation led to the identification of the RRM as the motif involved in nuclear import. Mutant versions of TcUBP1 with defective RRMs can not accumulate in the nucleus under arsenite treatment. This RRM is also responsible for the normal cytoplasmic localization of the protein. The nucleocytoplasmic nature of TcUBP1, together with its arsenite-induced nuclear colocalization with mRNA suggests that this protein might be binding to its target transcripts in the nucleus, which are later exported together to the cytoplasm. With the objective of interfering with the normal nucleocytoplasmic shuttling of TcUBP1, we performed affinity chromatography with the immobilized TcUBP1-RRM coupled to mass spectrometry to identify possible nuclear importers of this RBP. Only one protein, TcP22, appeared as a strong candidate for this function. We are characterizing the TcUBP1-RRM/TcP22 interaction, which could lead to interfere with TcUBP1 function in an RNAi-defective parasite.

Composition and Trypanosome Killing by Trypanosome Lytic Factor-2

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Humans are resistant to infection by the cattle pathogen *Trypanosoma brucei brucei*. This innate immunity is dependent on the activity of two primate specific protein complexes present in normal human serum, named trypanosome lytic factor (TLF) 1 and 2. The well-characterized TLF-1 particle is a 500-kDa subclass of human high density lipoprotein (HDL) that contains the primate specific proteins haptoglobin-related protein (Hpr), apolipoprotein L-1 (apo-L1) and apolipoprotein A-1 (apo-A1). TLF-1 killing of trypanosomes occurs through haptoglobin/hemoglobin (Hp/Hb) receptor mediated uptake and vesicular trafficking to the lysosome where finally lysosomal swelling and lysosomal membrane breakdown results in cell death. The characterization of TLF-2 is incomplete and its mechanism of uptake and killing has not been studied. Initial studies by the Raper laboratory (1999) showed that TLF-2 was a high molecular weight serum protein complex, largely devoid of lipids. A partial proteomic analysis of the 1 MDa protein complex, revealed that it contained Hpr, Apo-A1, and immunoglobulin M (IgM). Here we have extended the analysis of the TLF-2 particle and show that it also contains apo-L1. Trypanosome killing by TLF-2 appears not to be mediated through Hp/Hb receptor mediated uptake of the particle since a mutant cell line, which does not express the Hp/Hb receptor is resistant to TLF-1 killing but is susceptible to TLF-2. Our initial studies on the mechanim of TLF-2 killing of *T. b. brucei* suggests that its uptake is independent of the Hp/Hb receptor and killing is not stimulated upon hemoglobin addition. We are currently completing the proteomic analysis of TLF-2 by LC MS/MS and investigating the mechanism of TLF-2 uptake and killing of *T. b. brucei*.

The LEXSY Technology - a valuable tool in *Kinetoplastida*

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We have engineered the non-pathogenic parasite of lizard *Leishmania tarentolae* into a flexible and efficient protein expression platform called LEXSY. This biosafety 1 host system can be used for expression of target proteins of other kinetoplastida, making use of the close relationship of cellular machineries including protein folding and modification. On the other hand the LEXSY vectors are functionally in other *Leishmania* species and were used successfully in *L. amazonensis, L. donovani, L. infantum, L. major, L. mexicana*. and also in the plant parasite *Phytomonas serpens*. Two principal LEXSY architectures yield constitutive or inducible expression. The constitutive version is based on integration of expression cassettes into the 18S rRNA gene (*ssu*) for transcription by the strong RNA polymerase I of the host. Four alternative antibiotic selection markers permit integration of up to four expression cassettes into the *ssu* locus, useful e.g. for expression of multisubunit proteins. The inducible version employs T7 RNA Polymerase and TET repressor, expressed from the *ssu* locus of an engineered host. Target gene cassettes under control of a T7 promoter TET operator assembly can be integrated into ß-tubulin or ornithine decarboxylase loci. Alternatively, expression cassettes can be positioned on linear or circular episomes with telomeric ends. The use of a fusion of the *bleo* resistance and *cherry* fluorescence genes provides a tool for selection and screening. The LEXSY toolbox has recently been complemented by a cell-free version for expression of recombinant proteins.

Regulation of NDR kinase activity in *Trypanosoma brucei*

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NDR (nuclear DBF2-related) kinases regulate diverse and important cellular processes and are highly conserved from lower eukaryotes to humans. Phosphorylation of conserved Ser/Thr residues is important for NDR activity. Binding of a MOB partner protein is known to activate most NDR kinases, leading to autophosphorylation on a T-loop serine, while STE20-like kinases (SLKs) phosphorylate a threonine in a hydrophobic motif at the C-terminus of the NDR kinase, leading to further activation. Additionally, SLKs phosphorylate MOB proteins, increasing their affinity for NDR kinases. *Trypanosoma brucei* has two NDR kinases, PK50 and PK53, and two MOB proteins, MOB1A and MOB1B, all of which we have previously shown are essential for cytokinesis in the bloodstream stage of the parasite. However, the trypanosome NDR kinases are active in the absence of MOB proteins and do not appear to interact with the MOB proteins *in vitro* or *in vivo*, suggesting that PK50/PK53 activity is regulated in an unusual way. Here we investigate whether two *T. brucei* SLKs, SLK1 and SLK2, are also essential for cytokinesis, whether they phosphorylate PK50, PK53 or the MOB proteins and whether they affect the activity of the NDR kinases. Additionally, we investigate whether phosphosites previously identified on PK50 and PK53 are important for the activity or function of the NDR kinases.

Identification of drug uptake and resistance mechanisms using genome-wide RNAi screens in African trypanosomes

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To be effective, therapeutic compounds must typically enter target cells and, in some cases, must be concentrated or modified. Thus, uptake and activation mechanisms often form the basis of selectivity against infectious agents. Loss-of-function screens can be used to identify proteins involved in drug uptake and metabolism and may also identify clinically relevant potential resistance mechanisms. We have used a genome-scale RNA interference (RNAi) library to identify loss-of-function resistance mechanisms in bloodstream-form *Trypanosoma brucei*. A screen for resistance to nifurtimox identified loss of nitroreductase (NTR) pro-drug activator function while a screen for resistance to the amino-acid analogue, eflornithine, identified loss of amino-acid transporter (AAT6) function [1]. Further screening using other drugs has implicated other genes in resistance and some of these results will be presented. [1] Baker N, Alsford S and Horn D. (2010) Genome-wide RNAi screens in African trypanosomes identify the nifurtimox activator NTR and the eflornithine transporter AAT6. Mol Biochem Parasitol. 2011 176: 55-7.

Characterization of the histone variants H2AV and H2BV in *Leishmania major*

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Transcription of polycistronic gene clusters (PCGs) by RNA polymerase II can be initiated bidirectionally at diverging strand switch regions (SSRs), where two PCGs are arranged in a head-to-head fashion on opposite strands. In *T. brucei*, the histone variants H2A.Z and H2BV are enriched at probable sites of transcription initiation, indicative of epigenetic regulation of transcription initiation. To study the roles of these variant histones in *Leishmania* gene expression and development, particularly during metacyclogenesis where the parasite downregulates all types of transcription and shows increased heterochromatin levels, we generated antisera to the *Leishmania major* histone variants H2AV and H2BV. The variant to core histone ratios (H2AV/H2A or H2BV/H2B) did not change significantly during metacyclogenesis. Transfection of wild type *L. major* with a multi-copy episomal expression vector containing *H2AV* caused a slight growth defect in logarithmic-phase parasites, but no defect was observed in wild type parasites transfected with a multi-copy episomal expression vector containing *H2BV*. Metacyclogenesis occurs normally under both conditions. Attempts to generate *H2AV* -/- or *H2BV* -/- parasites have been unsuccessful in *L. major*. Heterozygotes for both genes were generated successfully, and the replacement of the second chromosomal allele in the presence of an ectopic copy will be performed to validate the essentiality of both histone variants.

RNAi knockdown studies reveal that intraflagellar transport (IFT) genes are essential in *Leishmania braziliensis* promastigotes

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The trypanosomatid flagellum is required for motility and contributes to sensing of environmental conditions. Other key roles include organization of the flagellar pocket, the primary site of endo- and exocytosis, and in cell division and morphogenesis. While the *Leishmania* amastigote is immotile, it retains a short flagellum, perhaps reflecting flagellar roles unrelated to motility. It has been suggested that the amastigote flagellum may function as a conduit for the introduction of parasite proteins and glycoconjugates into the infected host cell, for which there is increasing evidence in *Leishmania*. While many *Leishmania* species lack RNAi pathway genes and activity, species within the subgenus *Viannia* retain both. We showed that this could be used for functional genetic testing, one example being the generation of strong phenotypic knockdowns of the paraflagellar rod (PFR) (Lye et al, *PLoS Pathogens* 2010). Using RNAi we have initiated studies further exploring the role of the *Leishmania* flagellum. We focused first on the intraflagellar transport (IFT) pathway, which mediates transport of cargo in both anterograde and retrograde directions required for proper flagellar assembly, and targeted four IFT genes (*IFT88, IFT122, IFT140* and *IFT172*) using RNAi constructs expressing long stem-loop (StL) dsRNA triggers. While control gene StL construct transfections of promastigotes were successful (*PFR1, PFR2, LUC* (Luciferase)), we were unable to recover *IFT* StL transfectants. In contrast, transfections into an RNAi-deficient *ago1*- knockout *L. braziliensis* with both control and *IFT* StL constructs were comparably successful. We conclude that the IFT pathway is essential in *L. braziliensis* promastigotes.

Oligo(U) tails stabilize selected mitochondrial mRNAs and appear to be substrates for TbRND

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The mitochondrial mRNA population in kinetoplastids is impacted by posttranscriptional RNA processing, editing, and turnover. One compelling feature of mitochondrially-encoded RNAs in *Trypanosoma brucei* is their short non-encoded adenosine/uridine 3' nucleotide tails. The ratio of the two nucleotides comprising these tails is transcript-specific and thus may be a regulator of turnover. In this study, we address the potential regulatory role of short 3' tails and the enzymes forming and degrading them. The poly(A)polymerase kPAP1 adds adenosines to the 3' ends of mRNAs, and kPAP1 depletion has previously been shown to destabilize edited RNAs and stabilize selected pre-edited RNAs. Here we show by circular RT-PCR that 3' tails on pre-edited RPS12 RNAs, which are normally adenosine-rich, become nearly-homopolymeric uridine tails when kPAP1 is depleted. This change in tail composition correlates with increased stability of pre-edited RPS12 RNA. In contrast, for never-edited ND4 RNA, kPAP1 depletion simply causes the nearly-homopolymeric adenosine tails to disappear, and these RNAs are modestly destabilized. Newly-revealed homopolymeric uridine tails on pre-edited RPS12 RNA may be substrates for the uridine-specific exoribonuclease TbRND, previously shown to be active only on small, noncoding guide RNAs. Indeed, uridylated pre-edited RPS12 RNAs are further stabilized by kPAP1/TbRND co-depletion. Several other mitochondrial RNAs, including pre-edited, never-edited, and precursor RNAs, are similarly stabilized in kPAP1/TbRND-depleted cells. Studies are underway to correlate changes in transcript abundance with changes in 3' tail composition in kPAP1/TbRND co-depleted cells. Together, our data show that 3' uridine tails, previously only shown to be destabilizing elements, can serve as stabilizing elements on selected mitochondrial RNAs. The exoribonuclease TbRND contributes to the decay of these uridylated mRNAs.

Metacaspase: a cysteine protease inducing cell death in *Leishmania major*

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Despite a lot of controversy during the last decade, there is increasing experimental evidence that cell death (CD) is genetically programmed in lower eukaryotes. Although it has been deeply studied in yeast cells where several effectors have been identified, the molecular mechanisms of CD in trypanosomatids are less understood. In the CD proteolytic cascade of plants and protozoa, caspases are likely replaced by metacaspases that are cysteine peptidases recognizing arginines or lysines in P1-position. Metacaspases have been found to control cell death in plants. The human protozoan parasite *Leishmania major* expresses a single metacaspase (LmjMCA) harboring a central domain with the catalytic dyad histidine and cysteine as found in caspases. Metacaspase could therefore be one of the executioners of the death pathway in *Leishmania*. In this work we showed that, in stress conditions, LmjMCA precursor forms were extensively processed into soluble forms containing the catalytic domain and this domain was sufficient to enhance sensitivity of parasites to hydrogen peroxide by impairing the mitochondrion function. We tested different lengths of LmjMCA catalytic domain and found that the overexpression of the polypeptide corresponding to amino acids 136-218 was sufficient to sensitize *L. major* mitochondria to oxidative stress. These findings were used to synthesize an 83aa long peptide corresponding to the minimal metacaspase catalytic domain (aa136-218). This peptide has been characterized as having the specific metacaspase activity *in vitro* and is currently used to investigate its activity on possible target proteins, which have been identified in a yeast two-hybrid screen. Identifying proteins involved in the metacaspase signaling pathway will shed light on the understanding of CD in *Leishmania* and in lower eukaryotes.

Phosphoproteomics analysis of *Leishmania mexicana*

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Protein phosphorylation is known to affect cellular function and homeostasis. We have analysed the *Leishmania mexicana* phosphoproteome using different preparative and mass spectrometry approaches comparing promastigotes, axenic amastigotes and lesion-derived amastigotes. We have currently identified 5127 peptides with potential phosphorylation sites and patterns, almost 2000 of which being validated. As mediators of protein phosphorylation we focused on the analysis of phosphorylated residues in protein kinases and phosphatases. We were able to detect 424 non-redundant phosphorylation sites in 97 protein kinases and 24 protein phosphatases. Initial quantification of phosphorylated peptides using selected reaction monitoring (SRM) of TiO_2-enriched whole cell lysates resulted in the identification of life stage-specific phosphorylations, but also significant alterations in phosphorylation abundance likely having an effect on the phosphorylation state of cellular proteins in pro- and amastigotes.

A survey of kinetoplastid genomes

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Deciphering the genetic code for a species through de novo assembly is still the critical enabler for designing experiments that define molecular pathways. Through the establishment of a consortium, we have collected DNA resources of many species/strains of Leishmania and Trypanosoma to sequence, assemble and characterize their genomes using next-generation sequencing. At present we have generated genome assemblies of T.cruzi Esmeraldo, T. cruzi JR cl4, L. major SD 75, L. braziliensis M2903, and Crithidia fasciculata. A metric used to assess assembly contiguity is N50 contig and supercontig length, for which half of the assembled genome is the reported length or higher. For T. cruzi Esmeraldo, T. cruzi JR cl4, L. major SD 75, L. braziliensis M2903, and Crithidia fasciculata, we observe N50 contig lengths of 2 kb, 3.9 kb, 89.4 kb, 17.7 kb and 6.5 kb, respectively. This observed genome contiguity of each assembly reflects the previously known repetitive properties of each, trypanosomes being the most repetitive in structure. Our estimates of genome repeat content using for T.cruzi Esmeraldo, L. major, and C. fasciculata were 40%, 11% and 26%, respectively. A further test of assembly quality is gene coverage. Using BLAT, we have aligned conserved genes with no detected paralogs for L. major (n=5055) and T. cruzi (n=5302) to the T. cruzi JR cl4 and L. major SD 75 assemblies at an alignment length threshold of 95% and nucleotide identity of 90%. Using these criteria, we observe gene coverage rates of 84% and 99% for T. cruzi JR cl4 and L. major SD 75, respectively. We will report our progress on additional Leishmania and Trypanosoma genomes and their basic properties.

A Sensitive Method for Structural Characterization of N-glycans in Trypanosomes

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Trypanosomes contain an elaborate surface glycocalyx that plays important roles in the parasites virulence and survival in its hosts. Based on the repertoire of glycosidic linkages present in the *T. brucei* surface glycoconjugates, a minimum of 38 glycosyltransferase activities have been proposed present. Only a few of these activities have been assigned to specific genes/enzymes, however candidate genes have been proposed. One way of assigning function to genes suspected of playing a role in glycosylation is to analyze the glycoconjugate composition and structure in knock-out cells lacking expression of the potential enzymes of interest. However, current methods, such as HPLC and MS analysis of lectin-binding glycans, may prove insufficiently sensitive to detect the effects of knocking out glycosyltransferases involved in peripheral branching and extension of the extremely large poly-N-acetyllactosamine N-glycan structures found in *T. brucei*. To address this current weakness we are developing a sensitive method, derived from the Smith degradation, for detection of changes in N-glycan structures. Based on the known proportions of the β1-3 and β1-6 inter N-acetyllactosamine glycosidic linkages and β1-3/β1-6 branch points the nature of the Smith fragments that will be produced from wild-type parasites can be predicted. Converse, structural definition and stoichiometric quantification can be deduced from the nature of isotope labeled and permethylated Smith fragments following analysis by MS/MS. Importantly, while minor changes in N-glycan glycosidic linkages may barely change in monosaccharide composition and lectin-binding potential the composition of Smith fragments changes substantially, illustrating the sensitivity of the proposed strategy to minor structural changes. Here we compare results of this method obtained from experiments with different glycosylation phenotypes in *T. brucei*.

Late stage division differences between procyclic and bloodstream T. brucei trypomastigotes

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As part of an extensive detailed, comparative analysis of cell division in bloodstream and procyclic *T. brucei* trypomastigotes we have demonstrated some intriguing differences in late stage events. Both cell types exhibit a characteristic longitudinal folding of the dividing cell. There appears to be a fundamental difference in the progress of the cytokinesis furrow. In procyclic cells there appears to be 5 characteristic stages; fold generation, cleft formation, furrow ingression and then furrow progression to the doublet cell prior to final abscission. In bloodstream forms we cannot find evidence for the same type of cleft formation. We have previously provided extensive evidence in the procyclic form for a flagella connector - a mobile, transmembrane junction linking the tip of the new flagellum to the side of the old flagellum. This study has confirmed that we cannot detect a canonical flagella connector in the bloodstream form. However, we can now provide evidence that the tip of the growing bloodstream flagellum is intimately associated with a small groove in the cell body adjacent to the old flagellum. Serial section analysis of this area shows that in the bloodstream form (in contrast to the procyclic form) the underlying microtubule cytoskeleton is disrupted and replaced by a cloud of electron dense, sub-plasma membrane associated filaments. These structural descriptions add more detail to our understanding of the divergent manner in which these two cell types accomplish cytokinesis.

Pleiotropic effects caused by overexpression of the non- coding RNA ODD3 in *Leishmania*

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Sequencing of a *Leishmania* cDNA library previously described revealed several genes carrying odd features when compared to usual protein coding genes. An example is the ODD3, a regulatory non-coding RNA candidate. This work shows the pleiotropic effects caused by ODD3 overexpression. This mutant exhibit ploidy and morphological changes and grows poorly in axenic culture. The proteome analysis revealed 69 spots up-regulated and 53 down-regulated in ODD3 overexpressor. The differentially expressed were clustered according to Kegg and GO biological process and the metabolic pathways were mapped in using LeishCyc. Our results indicate that the expression of ODD3 is rigorously controlled and a subtle increment of this gene disturbs metabolic pathways related to proteolysis, cellular redox state, purine and pyrimidine metabolism, TCA cycle, fatty acid beta-oxidation, aerobic/anaerobic respiration, metabolite biosynthesis and protein dimerization. The precise molecular mechanism it was not revealed yet but two putative mRNA targets of ODD3 were identified by RT-PCR and predicted by BLASTN. We are hypothesizing that this regulatory RNA is base pairing with the target(s) mRNA to control their expression. The absence of the classic RNAi pathway in *Leishmania major* indicates that a distinct machinery must be in place to allow ODD3 to interact with its target and play its role.

The structural basis of localizing polo-like kinase to the flagellar attachment zone in *Trypanosoma brucei*

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The polo-like kinase in *Trypanosoma brucei* (TbPlk) has many unique features. Unlike all the other polo-like kinases known to localize in the nucleus and controlling both mitosis and cytokinesis, TbPlk localizes to the flagella attachment zone (FAZ) and regulates only cytokinesis in *T. brucei*. TbPlk was, however, found capable of complementing all the Plk (Cdc5) functions in *Saccharomyces cerevisiae*, whereas Cdc5 tagged with an enhanced yellow fluorescence protein (EYFP) was found localized exclusively in the FAZ of *T. brucei*. These observations suggest that TbPlk has acquired all the multiple functions of Cdc5. Its peculiar localization and limited function in *T. brucei* are probably attributed to the particular environment in the protozoan cell. In the present study, we investigated the structural basis of TbPlk localization to the FAZ by over-expressing TbPlk mutants tagged with EYFP in *T. brucei* and monitor its subcellular location with fluorescence microscopy. It was found that TbPLK activity is not required for FAZ localization. But the two polo box domains PBD1 and PBD2 in TbPlk are apparently playing important roles in localizing TbPlk, which becomes distributed in the cytoplasm and nucleus when either PBD1 alone or both PDBs are deleted. When the linker region between the two PBDs is deleted, the mutant protein localizes only in the cytoplasm. There is thus apparently specific binding(s) between the PBDs and certain component(s) in FAZ that determines the FAZ localization of TbPlk. The identity of the FAZ component(s) is currently under investigation.

Functional analysis of a novel and essential subunit of the F_oF_1-ATP synthase in *Trypanosoma brucei*

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The *Trypanosoma brucei* F_oF_1-ATP synthase is an essential multisubunit enzyme that synthesizes ATP during the parasite's insect stage, but then reverses its function as it hydrolyzes ATP to maintain the mitochondrial (mt) membrane potential in the infectious mammalian stage. The composition of the *T. brucei* F_oF_1-ATP synthase is unique compared to the well conserved F_oF_1-ATP synthases found in metazoan, fungal and plant mitochondria because it contains up to 14 novel subunits in addition to the conserved core subunits. Importantly, these subunits do not possess any obvious homology outside of Kinetoplastida. This observation raises key questions regarding the function of these subunits within the parasite's complex and the mitochondria. RNAi silencing of one of these novel subunits, Tb7760, demonstrated that it is essential for the infective stage of the parasite, even though the structural integrity of the F_oF_1-ATPase complex stays intact. A significant decrease of the mt membrane potential precedes the growth inhibition of these cells, indicating that the F_oF_1-ATPase is not functioning properly. However, *in vitro* F_oF_1-ATPase assays revealed an increase in the ATP hydrolysis of the Tb7760 knockdown cells, suggesting that the decrease in the mt membrane potential is due to the failure of the F_oF_1-ATPase complex to translocate protons. Combined with our preliminary results that Tb7760 is localized to the mitochondrion membrane, these data suggest that Tb7760 participates in the the proton flux through the inner mitochondrial membrane.

Molecular characterization of the *Trypanosoma cruzi* specific RNA binding protein TcRBP40 and its associated mRNAs

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Trypanosoma cruzi is the causative agent of Chagas disease, a neglected disorder that affects millions of people in the Americas. It relies mostly upon post-transcriptional regulation to control stage specific gene expression. RNA binding proteins (RBPs) associate with functionally related mRNAs forming ribonucleoprotein complexes that define post-transcriptional operons. The RNA Recognition Motif (RRM) is the most common and ancient family of RBPs that mediate this post-transcriptional control. This family of RBPs has been identified in trypanosomatid parasites and only a few of them have been functionally characterized. In *T. cruzi*, members of this protein family are involved in the regulation of mRNA stability in the different parasite forms. We described the functional characterization of TcRBP40, a *T. cruzi* specific RBP, and its associated mRNAs. We used a modified version of the recombinant RIP-CHIP assay to identify the mRNAs with which it associates and in vivo immunoprecipitation and TAP-tag assays to confirm these results. TcRBP40 binds to an AG-rich sequence in the 3'UTR of the associated mRNAs, which were found to encode mainly putative transmembrane proteins. These mRNAs are upregulated in the infective metacyclic trypomastigote forms in an inverse pattern when compared to TcRBP40 expression. Surprisingly, in epimastigotes, TcRBP40 is expressed in the cytoplasm but is concentrated in the reservosomes, a *T. cruzi* specific organelle. This cellular localization in epimastigote reservosomes suggests a putative new function for this parasite organelle.

Comparative genomics and functional analysis of proteins involved in mRNA nucleocytoplasmatic export in trypanosomes

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In eukaryotes, RNA is exported by specialized pathways. The nuclear export of miRNAs, rRNAs, snRNAs, and tRNAs follow the RanGTP-exportin model, but the export of mRNAs doesn't follow this model. The conservation of RNA export pathways in early eukaryotes and the complexity of nucleocytoplasmic RNA export in the last eukaryotic common ancestor (LECA) remain unknown. We aimed to reconstruct the evolutionary history of RNA export pathways, screening an array of eukaryotic genomes for the presence of homologs involved in RNA export in metazoa and fungi. The results indicate that several key proteins involved in different RNA export pathways are conserved across most eukaryotes, inferring that orthologs were highly likely to have been present in the LECA. The mRNA export pathway is the less conserved, suggesting that innovations appeared later in the evolution of eukaryotes. We determined that the most conserved protein across all eukaryotes was Sub2/UAP56, a component of a mRNA transcription/export complex and decided to investigate the function of this protein in trypanosomatid protozoa. Immunocytochemistry analysis showed that *T. cruzi* Sub2 localizes in foci all over the nuclei, excluding nucleolus, and BrUTP incorporation assays showed that TcSub2 colocalizes with RNA pol II transcription sites and not with SL transcription sites, suggesting that TcSub2 participates in mRNA transcription/export. The double knockout of the Sub2 gene is lethal in *T. cruzi* and RNA interference assays demonstrated that it is an essential

protein in *T. brucei*. The knockdown of TbSub2 causes mRNA accumulation in the nucleus and decreasing of translation levels, confirming that Sub2 is probably a component of mRNA transcription/export pathway in trypanosomes.

The effect of loss of disulphide bridges in the VSG N-terminal domain on *Trypanosoma brucei* bloodstream form cells

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Bloodstream form trypanosomes maintain a densely packed VSG coat. Here, the production of functional VSG has been manipulated and the phenotype partially characterised. The starting cell line expressed VSG121 (MITat1.6) and ectopic copies of wild type or mutant VSG221 (MITat1.2) genes were targeted to the VSG expression site to produce trypanosomes expressing two VSGs. Co-expression of wild type VSG221 resulted in a decrease in cell doubling time from 11 to 9 h in culture. The trypanosomes grew normally in mice suggesting a fully functional VSG coat. The N-terminal domain of VSG221 contains two disulphide bridges. Three mutant VSG221 transgenes were made missing one, the other, or both disulphide bridges, and co-expressed from the VSG121 expression site. Steady state expression levels of the mutant VSGs were reduced compared to wild type. However, pulse chase experiments showed that the mutant VSG221s are synthesised at the same rate but are turned over within 4h after synthesis. BiP, PDI-1 and PDI-2 levels were increased in cells expressing mutant VSG221 compared to wild type VSG221. Hence, there is a response to unstable VSGs in bloodstream form cells. The reduced stability of the VSG had a profound effect on cell doubling time, the mutations led to very slow growth with the population doubling every 24h. The VSG221/VSG121 cysteine mutants do not show a specific cell cycle arrest and cannot grow to high cell densities. Future experiments aim to find out where the mutant VSG221 is degraded - in the ER or when the VSG is recycled.

CMF22, a candidate calmodulin-binding protein, localizes to the flagellar cytoskeleton and is essential for forward motility in African trypanosomes

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Motility of the eukaryotic flagellum provides propulsion for many pathogenic protozoa and is essential for normal developmental and physiological processes in vertebrates. In virtually all flagella, calcium has been implicated as a secondary messenger in signal transduction pathways that modulate motility in response to external cues. Though a number of calcium-binding calmodulins have been localized to flagellar axonemes, the molecular mechanisms underlying motility regulation by intraflagellar calcium are poorly understood. Here we have examined the role of component of motile flagella 22 (CMF22), a putative calmodulin-binding protein, in the flagellum of *Trypanosoma brucei*. Comparative genomics identified CMF22 as being broadly and uniquely conserved in organisms with motile flagella. Presence of a predicted IQ-calmodulin binding motif suggested CMF22 might be an effector of Ca++ on motility. Immunoflourescence microscopy and fractionation localized CMF22 to the flagellar cytoskeleton. RNAi-mediated knockdown of CMF22 results in defective forward motility, as revealed by video microscopy and defective social motility. Biochemical and functional analyses of CMF22 are underway to determine its precise localization within the axoneme, the significance of the IQ-motif in calmodulin binding, and the unique contribution to motility modulation. Results from these studies will provide insights into how the flagellar signaling network is regulated in response to calcium. Funding: Arnold and Mabel Beckman Scholars Program, NIH-NIAD, NIH Training Grant in Cell & Molecular Biol.

Elucidation of the role of the *Leishmania mexicana A600* gene locus in amastigote growth

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After transmission to the mammalian host via the bite of an infected sandfly, the protozoan parasite *Leishmania* infects macrophages where it differentiates and replicates as amastigotes. Under this form, the parasite can evade the immune system and persists for a long time, resulting in the chronic symptoms of the disease. Research in our lab has been focused on the study of genes that are specifically expressed in *Leishmania* amastigotes. It is believed that those genes are involved in the pathogenesis of leishmaniasis, either by playing a role in parasite survival and/or in the modulation of the host immune response. Elucidating their functions may allow the identification of new strategies to control leishmaniasis. We have identified a novel family of four tandemly linked genes in *Leishmania mexicana*, the *A600* locus. Members of this gene family are preferentially expressed in amastigotes. Targeted deletion of this locus has shown that those genes are necessary for the successful replication of amastigotes; the mutant parasites fail to proliferate beyond five days, both in culture and in macrophages. Re-expression of the *A600.1* gene in the $A600^{-/-}$ amastigotes partially rescues their proliferation defect. To investigate further the function of the *A600* genes, we are currently investigating the interacting partners of the A600 proteins and the signalling pathways in which they may be involved. Experiments are underway to isolate A600.1 interacting proteins in promastigotes and amastigotes, using a GST pull-down assay approach. LC-MS/MS will then be performed to analyse the isolated proteins.

Druggable DUBs in Trypanosomes

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Exploiting biochemical differences between host and parasite is generally seen as a safe pharmacological option to treat infections. Ubiquitination and deubiquitination of proteins plays a central role in protein degradation, trafficking, DNA repair and epigenetic events. Kinetoplasts have a functional ubiquitin-proteasome system and compared to mammalian ubiquitin (Ub), kinetoplastid Ub differs at several amino acid residues: *T. brucei* (T14A, P19A, D52E, S57A), *Leishmania* (T14A, D52E) and *T. cruzi* (T14A, P19S, S57A). Homolog searches of the parasite genomes have indicated putative enzymes with ubiquitin and ubiquitin-like protease activity. In mammals >90 deubiquitinating enzymes (DUBs) have been characterized, but none in kinetoplastids to date. We aim to identify and characterize parasite specific DUBs as novel therapeutic targets. We have developed activity based probes to visualize trypanosomal DUB activity and to allow for mapping of kinetoplastid DUBs by proteomic analysis. The druggability of trypanosomal DUBs is under investigation by forward chemical genetic screening using a DUB-targeted small molecule library followed by activity based protein profiling. Thus, at the NKI Robotics and Screening Centre we have screened a collection of >350 DUB inhibitors and analogs for trypanocidal activity. Active compounds are currently being profiled for parasite DUB inhibitory activity. This project aims to deliver new drug-target combinations for the treatment of diseases inflicted by kinetoplastid parasites.

TbRACK1 is a Signal Scaffold That is Present on Cytoplasmic Free Ribosomes but is Absent From Er-Bound Ribosomes

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RACK1 is a core protein of the 40S ribosome. Its binding is stabilized by interactions with the 18S rRNA and adjacent proteins. As a WD repeat scaffold, it also associates with a wide range of kinases, phosphatases, ion channels, transcription factors, RNA-binding proteins, translation factors and proteasome targets. To account for its many activities, some amount of RACK1 is presumed to be free in the cytoplasm. Here we investigate whether TbRACK1, once bound to ribosomes is ever released. We report that TbRACK1 is found on monosomes and translating polysomes, but not on microsomal ribosomes. Co-localizations identified two distinct cellular regions that contained BiP but lacked TbRACK1. The observation was confirmed with western blots of microsomal fractions. A tagged construct of TbRACK1 exhibited the same behavior. Thr10, Ser18 and Thr24 were identified as endogenous phosphorylation sites by MS/MS. When all three residues were mutated to Asp, the corresponding TbRACK1 never associated with ribosomes. Conversely, when all three residues were mutated to Ala, TbRACK1 was associated with cytoplasmic and ER-bound ribosomes. No single mutation was sufficient to affect ribosome binding. The 3XAla mutant cell lines exhibited a slower growth rate, suggesting that the constant association of TbRACK1 with microsomal ribosomes was deleterious. T. cruzi RACK1 lacks phosphorylatable residues at positions corresponding to T10, S18 and T24. Nonetheless, it dissociated from microsomal ribosomes. Collectively these data indicate that release of RACK1 from ER-bound ribosomes is a shared phenomenon of kinetoplastid parasites, although the mechanism of release may vary with different species. This is the first study to indicate that free cytoplasmic ribosomes may be biochemically distinct from those bound to ER.

Trypanosoma brucei ARL6 (BBS3) is a vesicle-associated tubulin binding protein required for flagellum extension

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The small GTPase Arl6, implicated in the ciliopathic human genetic disorder Bardet-Biedl syndrome, acts at primary cilia in recruitment of the octomeric BBSome complex which mediates activation of Rab8 and facilitates the export of excess signalling proteins from the cilium. We have characterised Arl6 in bloodstream form *Trypanosoma brucei*, which require motility for viability and express orthologues of the BBSome subunits but not Rab8. TbARL6 is associated with electron-dense coated vesicles throughout the cell body, requiring both *N*-myristoylation and nucleotide binding for correct localisation. Modulation of expression by RNA interference does not prevent motility but causes a significant reduction in growth rate and flagellum length. Tubulin has been identified as an ARL6 interacting partner, suggesting that ARL6 may act as an anchor between vesicles and cytoplasmic microtubules. We provide evidence that the interaction between ARL6 and the BBSome is conserved in unicellular eukaryotes. Overexpression of BBS1 leads to translocation of endogenous ARL6 to the site of the exogenous BBS1 at the flagellar pocket. Furthermore, a combination of BBS1 overexpression and ARL6 RNAi has a synergistic inhibitory effect on cell growth. Our findings indicate that the trypanosome BBSome contributes to flagellum extension by a Rab8-independent mechanism. Work is now in progress to characterise BBS1 and its interacting partners in *T. brucei* and *Leishmania major*.

TbLpn and its role in phospholipid biosynthesis in *Trypanosoma brucei*

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African sleeping sickness is a vector-borne devastating disease caused by the parasitic protozoan *Trypanosoma brucei*. Of great importance is the fact that, as opposed to other parasitic organisms, trypanosomes synthesize phospholipids *de novo*. This makes the trypanosome phospholipids biosynthesis machinery a very attractive target for new drug design. We recently identified TbLpn, a protein homologous to yeast and human lipin, a phosphatidate phosphatase involved in membrane biogenesis, energy metabolism, and adipose tissue development. In yeast and mammals, lipin catalyzes the dephosphorylation of phosphatidic acid (PA) to diacylglycerol (DAG) which, in turn, is used for the synthesis of phospholipids. Careful examination of the predicted amino acid sequence of TbLpn has revealed the presence of two conserved domains characteristic of the lipin family of protein, as well as two aspartic acid residues essential for lipin enzymatic activity. This clearly suggests that TbLpn represents a functional homologue of lipin proteins. The aim of this research was to characterize the *in vivo* enzymatic activity of *T. brucei* TbLpn. As TbLpn was shown by Western hybridization to be localized to the cytosol, native TbLpn was immunoprecipitated from *T. brucei* cytosolic extract and is being assayed for its ability to carry out dephosphorylation of phosphatidic acid using a colorimetric assay. We anticipate that the immunopurified TbLpn will result in a concentration-dependent release of phosphate from PA.

Trypanosoma brucei Mitochondrial Respiratome: Composition and organization in procyclic form

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The mitochondrial respiratory chain is comprised of five different protein complexes (I-V), which are embedded in the inner membrane. In this study, the respiratory complexes I, II and III were affinity purified from *Trypanosoma brucei* procyclic form cells and their composition was determined by mass spectrometry. The results along with those that we previously reported for complexes IV and V showed that the respiratome of Trypanosoma is divergent since many of its proteins are unique to this group of organisms. The studies also identified two mitochondrial subunit proteins of respiratory complex IV that are encoded by edited RNAs. Proteomics data from analyses of complexes purified using numerous tagged component proteins in each of the five complexes were used to generate the first predicted protein-protein interaction network of the *Trypanosoma brucei* respiratory chain. These results provide the first comprehensive insight into the unique composition of the respiratory complexes in *Trypanosoma brucei*, an early diverged eukaryotic pathogen.

Identification of downstream targets of *N*-myristoyltransferase in *Leishmania donovani*

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Myristoyl-CoA:protein *N*-myristoyltransferase (NMT) catalyses the covalent attachment of C14-myristate to the N-terminal glycine residue of a subset of eukaryotic proteins that function in multiple cellular processes. These include vesicular protein trafficking, signal transduction and protein-protein interactions important for transient or permanent localisation of proteins within cells. NMT activity is essential for the viability of all eukaryotic cell types tested to date, including bloodstream stages of ¡/¿Trypanosoma brucei¡/I¿ and insect stages of *Leishmania* species. A recent study has identified high-affinity NMT-specific inhibitors that inhibit protein *N*-myristoylation in trypanosomes, leading to rapid parasite killing, both *in vitro* and *in vivo*, and are able to cure acute trypanosomiasis in mice (Frearson *et al*., 2010, Nature 464, 728). Bioinformatic analysis allows identification of predicted *N*-myristoylated proteins (which number ~60 in kinetoplastids; Mills *et al*., 2007, MBP 152,22) and some of these have been validated experimentally (e.g. ARF1, ARL1, HASPB, PPEF). The algorithms used exclude potential post-translational myristoylation, however, as reported to occur during apoptotic cell death. Here, we describe an *in vivo* labelling technique based on click chemistry that is being used to isolate *N*-myristoylated proteins in *L. donovani* intracellular amastigotes, which can then be identified by mass spectrometry. These methods will generate a more comprehensive analysis of *N*-myristoylated proteins in *Leishmania* species, allowing us to better understand the downstream effects of small molecule NMT inhibitors on the regulation of signalling processes and vesicular transport in these cells.

Protein Phosphorylation in *Trypanosoma brucei* Mitochondria

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Trypanosoma brucei, contains a single mitochondrial organelle which is developmentally regulated during the life cycle of the parasite. While we have some understanding of the regulation at the level of transcript abundance little is known about the effect of posttranslational modifications. The steadily increasing number of reported mitochondrial protein kinases (PK), phosphatases and phosphoproteins in other systems suggest that reversible phosphorylation of proteins might play an important role in regulation of mitochondrial functions. However, tittle is known about mitochondrial PKs and their substrates in *T. brucei*. Using bioinformatic analysis of the genome of *T. brucei* we predicted 35 putative mitochondrial kinases. Using fluorescence microscopy and GFP-tagging we confirmed the predicted mitochondrial localization of six putative kinases in procyclic form of *T. brucei*. We established an in vitro kinase labeling assay, which in combination with kinase inhibitors confirmed the activity of protein kinases in the mitochondrion of *T. brucei*. Subsequently we analyzed the effect that RNA interference of five of the putative kinases had on growth of the bloodstream and/or procyclic form parasite.

Mechanism of action of Trypanosome Lytic Factor against Leishmania

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Trypanosome Lytic Factor (TLF) is approximately one percent of high density lipoprotein and provides innate immune defense by protecting against most species of African Trypanosomes. The protein is activated in low pH, thus indicating potential action against pathogens localizing to acidic environments. TLF has been shown to protect against Leishmania, a kinetoplastid parasite which replicates in the phagolysosomes of macrophages. TLF acts to opsonize the parasite for uptake by macrophages, where it is activated in the acidified parasitophorous vacuole. TLF is able to reduce parasite load by forming pores in promastigote-stage Leishmania inside the phagolysosome without activating the macrophage. The parasite is no longer susceptible to TLF, however, once it transforms to the amastigote stage inside the mammalian host. We show that these pores are formed directly in the plasma membrane of the parasite at a low pH such as that within the macrophage phagolysosome, resulting in dissipation of membrane potential and swelling of the parasite due to osmotic influx of water. Using FACS analysis, we demonstrate the difference in susceptibility of metacyclic promastigotes vs. amastigotes may be due to their ability to bind TLF. Binding of TLF to metacyclics and not amastigotes is likely due to differences in the composition of the glycocalyx. We are currently testing this hypothesis with mutant parasites which do not synthesize lipophosphoglycan or proteophosphoglycan. In conclusion, TLF should be considered a mechanism in innate immunity as the transformation of Leishmania into a stage unsusceptible to TLF indicates the adaptive pressure TLF exerts on pathogens.

Functional analysis of LmxMPK2, a MAP kinase essential for *Leishmania mexicana* amastigotes

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LmxMPK2 is a mitogen-activated protein kinase homologue of *Leishmania mexicana* that is expressed in promastigotes and amastigotes. Homozygous gene knock out mutants were unable to cause lesions in infected Balb/c mice whereas genomic add-backs caused the disease indicating that the kinase is essential in amastigotes representing a suitable drug target. Phenotypic analysis of promastigote null mutants revealed morphological alterations with cells showing multiple flagella, kinetoplasts and nuclei, lobed cell bodies, spiked posterior ends and division furrow ingression from the posterior end. Further, phenotypic analysis and localisation studies are currently under way. Recombinant expression of LmxMPK2 resulted in an active enzyme already phosphorylated on tyrosine residues. Co-expression with phosphatases (lambda-phosphatase or human PTP1B) led to a dephosphorylated kinase which retained the ability of auto- and substrate phosphorylation.

Regulating gene expression in transmissible stumpy forms of *Trypanosoma brucei*

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The transmission of *Trypanosoma brucei* depends upon the development in the bloodstream of stumpy forms from non-transmissible slender forms. Although slender and stumpy forms share the same environment of the mammalian bloodstream, they show differential gene expression. Many mRNAs are downregulated in stumpy forms and translation is generally repressed, however, a small subset escape this repression and are upregulated, presumably as an adaptation for transmission. To understand the basis of this, regulatory sequences within the 3'UTR of a major stumpy-enriched transcript (an ESAG9 gene) have been characterised. This has identified the signals responsible for gene silencing in slender forms and gene activation when cells develop to stumpy-like forms in response to 8-pCPT-2'-O-Me-cAMP. Complementing these studies, another transcript is being investigated which has been found to produce two transcripts through trans-splicing at different sites. These transcripts, which exhibit some differential abundance between lifecycle stages, would generate a long and short form (from an internal ATG) of the encoded protein. The encoded proteins are both predicted to contain a UBA/TS-N (ubiquitin associated) domain, however, the longer form of the protein is also predicted to contain a transmembrane helix and a cleavable signal peptide missing from the short protein form, suggesting a different localisation. This reveals a possible functional consequence of alternative splicing in distinct developmental forms.

Differential phosphorylation of LmxMPK1 - teasing out a meaning from *in vivo* and *in vitro* observations

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The *Leishmania mexicana* MAP kinase homologue, LmxMPK1, is essential for the proliferation of amastigotes and hence establishment of an infection in the mammalian host. LmxMPK1 showed phosphorylation of the TDY phosphorylation lip motif threonine 176 and tyrosine 178 or tyrosine alone in promastigotes, whereas in amastigotes we could only detect phosphorylation of the tyrosine. Moreover, threonine 224 was found phosphorylated in both life stages. Recombinantly expressed LmxMPK1, also only showed phosphorylation of tyrosine 178 and threonine 224. In kinase assays using recombinant protein further tyrosine phosphorylation could be observed. To ensure a replicable starting point for all kinase assays we co-expressed LmxMPK1 with one of three phosphatases: the dual-specificity Lambda-phosphatase, human Protein-Tyrosine Phosphatase-1β and a novel *Leishmania* protein tyrosine phosphatase homologue, LmxPTP. This resulted in differential dephosphorylation of LmxMPK1, affecting but not ablating the ability of the kinase to autophosphorylate and phosphorylate myelin basic protein (MBP). Hence, phosphorylation of further residues like threonine 224 has a modulating effect on kinase activity. As phosphorylation of the TXY motif in the phosphorylation lip of kinases from higher eukaryotes is central for kinase function we are investigating (1) the *in vivo* effect of mutations in the TDY motif displaying non-phosphorylatable or phosphoamino acid mimicking residues using GFP-tagging and expression in the deletion mutant background and (2) the activity of the kinase mutants following affinity purification.

Screening of metal-based Azole derivatives with antiparasitic activity on Trypanosoma Cruzi and Leishmania Major

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The trypanosomatids *Leishmania major* and *Trypanosoma cruzi* are bloodborne pathogens of medical importance that affect millions of people worldwide. Owing to increasing global migration, deployment to endemic regions, lack of screening in blood banks, and climatic changes leishmaniasis and Chagas disease have become a growing health concern in the United States and Europe. The sterol metabolism had has been validated as drug target for these trypanosomatids, also it is known that azole compounds inhibit the sterol biosynthesis pathway. In this study, we assayed the antiparasitic activity of a novel series of metal-based compounds containing clotrimazole or ketoconazole and ruthenium. A total of 14 new compounds were tested in a concentration ranging from 30 μM to 234 nM. The viability of *T. cruzi* epimastigote forms was analyzed using alamarBlue®. In the case of *L. major* , transgenic promastigotes (Friedlin clone V1) expressing firefly luciferase were used to determine the compounds' activity. Both parasites were incubated for 72 hr with the drugs and all the experiments were performed in triplicate. The most potent trypanocidal compounds were AM163 and AM161 (86% and 78% mortality at 468 nM, respectively), followed by AM160 and AM103 (both given 91% mortality at 937 nM). For *L. major* the most efficient derivatives were AM162 and AM161 (84% and 80% mortality at 243 nM). To determine the clearance of parasites in infected cells, in vitro experiments using high content imaging are planned. Supported by the NIH/NIGMS/MORE grant R25GM069621-02 (to RASD) and NIH/NIGMS/SCORE grant 1SC1GM089558-01A1 (to RAM); and the NIH Research Initiative for Scientific Enhancement (RISE) program.

Fe-S cluster assembly in procyclic and bloodstream stages of Trypanosoma brucei

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Iron-sulfur (Fe-S) clusters are small inorganic co-factors, the assembly of which is essential for every extant prokaryotic and eukaryotic cell. In a typical eukaryotic cell they are indispensable for the function of more than 100 proteins, their assembly being composed of the conserved mitochondrial (ISC) and cytosolic (CIA) machineries. To date, we have knocked down more than a dozen conserved components of both pathways in Trypanosoma brucei and analyzed the ensuing phenotypes. Ablation of the ISC components disrupts assembly of the mitochondrial and/or cytosolic clusters, while the down-regulation of the CIA pathway impacts only the cytosolic co-factors. The Fe-S cluster assembly in the procyclic cells is quite similar to that in other eukaryotes, such as yeast and human, while the same pathway in the bloodstream stage seems to have some unique features. Our current understanding of the Fe-S cluster assembly in trypanosomes will be discussed as well as insights into the assembly in human cells, obtained thru the studies of T. brucei.

Stimulation of *Leishmania chagasi* infectivity by intrinsic platelet-activating factor

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PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, exhibits potent biological activity and is synthesized by a wide variety of cells, including neutrophils, platelets, macrophages, and lymphocytes. Previous data from our group showed some physiological roles related to cell differentiation induced by PAF in trypanosomatids and suggested that *Trypanosoma cruzi* synthesizes a PAF-like phospholipid that modulates the parasite's differentiation and infectivity. Here we describe the purification and molecular characterization of PAF species from complex lipid mixtures of *Leishmania chagasi* promastigotes. Using solid-phase extraction (SPE) followed by electrospray ionization tandem mass-spectrometry (ESI-MS/MS), we identified three species of PAF, namely, the common C16:0-PAF, the rare C16:1-PAF, and the novel C16:2-PAF. C16:0 PAF was present in very low amounts, whereas C16:1- and C16:2-PAF species were found in much higher concentrations. We also show that a mixture of these three PAF species induced the aggregation of rabbit platelets and stimulated mouse macrophage infection by *L. chagasi*, when promastigotes were kept for 5 days in the presence of *L. chagasi*-PAF species, before interacting with the macrophages, with the involvement of the protein kinases PKA, PKC and CK2. *L. chagasi*-PAF species induced an inhibition of NO production by these macrophages. *L. chagasi*-PAF species also stimulated *L. chagasi* metallo and cysteine proteinases, as well as PKA, PKC and CK2 activities. All *L. chagasi*-PAF species effects here described were abrogated by WEB 2086, a classic antagonist of PAF that binds specifically to PAF-receptor. These data suggest that *L. chagasi* contains the components of an autocrine PAF ligand-receptor system that modulates *L. chagasi* infectivity towards mouse macrophages.

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The *Trypanosoma brucei* CDP-DAG and PI Synthases: Essential Enzymes in Multiple Cellular Locations

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Lipid metabolism in *Trypanosoma brucei* differs in several ways from the human host, so may present novel drug targets. *T. brucei* do not utilise intact phospholipids from their host but *de novo* synthesise them. The nucleolipid cytidine diphosphate diacylglycerol (CDP-DAG) is central in phospholipid synthesis. It is synthesised from phosphatidic acid and CTP via the membrane bound enzyme CDP-DAG synthase (CDS) and is the precursor to several other important cellular phospholipids including phosphatidylinositol (PI), which is synthesised by phosphatidylinositol synthase (PIS). Only one gene in the *T. brucei* genome is predicted to encode CDS, whereas other trypanosomes have two, one prokaryotic-like and one of a eukaryotic origin. By RNA interference, we have found CDS to be essential in both bloodstream and procyclic *T. brucei*. We have previously shown that PIS is essential to the bloodstream form parasite, where it was found in both the Golgi and the ER, with Golgi synthesised PI being destined for bulk cellular phospholipid, and ER synthesised PI being destined for incorporation into GPI anchors. Preliminary results indicate that CDS is also found in more than one cellular location in both bloodstream and procyclic form *T. brucei*. However, we now show that in procyclics PIS is only found in the Golgi. This work is funded by BBSRC and Wellcome Trust.

Vacuolar transport chaperone 4 (VTC4) is an acidocalcisomal polyphosphate synthase in *Trypanosoma brucei* and *T. cruzi*

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Polyphosphate (poly P) is a polymer of inorganic phosphate found in both prokaryotes and eukaryotes. Poly P typically accumulates in acidic, calcium-rich organelles known as acidocalcisomes. Poly P synthesis was unclear until recent work demonstrated that vacuolar transport chaperone 4 (VTC4) catalyzes its synthesis in yeast (Hothorn et al., Science 324, 513-6, 2009). *Trypanosoma brucei* possesses a VTC4 homologue (TbVTC4, Tb11.01.4040) which we detected in a proteomic analysis of *T. brucei* acidocalcisomes. *Trypanosoma cruzi* also possesses a VTC4 homologue (TcVTC4, Tc00.1047053511127.100). Localization studies using antibodies against TbVTC4 revealed its colocalization with the vacuolar proton pyrophosphatase (VP1), a marker for acidocalcisomes, in both parasites. Both proteins were expressed in bacteria and shown to have poly P synthase activity. Ablation of *TbVTC4* expression by RNA interference (RNAi) in procyclic forms (PCF) led to decreased growth and poly P content, and increased pyrophosphate content, while overexpression of this gene led to the opposite results. Electron microscopy of PCF in which *TbVTC4* has been ablated by RNAi exhibited 2-fold reduction in acidocalcisome number, but remaining acidocalcisomes have greatly increased volume relative to non-induced control cells. Furthermore, we obtained conditional double knockout mutants in both *T. brucei* PCF and bloodstream forms (BSF). Phenotypic characterization of BSF mutants indicated that *TbVTC4* gene ablation produced a decrease in parasite growth rate while gene overexpression did not have any effect on growth. Our data suggests that TbVTC4 and TcVTC4 are essential enzymes involved in poly P synthesis. Since VTC4 is absent in vertebrates, this enzyme could be a potential drug target.

Flagellar functions investigated in a paraflagellar rod mutant

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The flagellum of trypanosomes is built, like most eukaryotes, using the IntraFlagellar Transport (IFT) machinery. It contains a supplementary structure, the ParaFlagellar Rod (PFR) that is connected to the classical 9+2 axoneme via microtubule doublets 4 and 7. PFR assembly is dependent on a specific kinesin motor, KIF9B whose deletion does not modify axoneme construction but leads to severe misorganisation of the PFR structure: along the same flagellum, some regions display excessive amounts of PFR material, others show nude axonemes. Since the presence of the PFR is proposed to be responsible for the restricted localisation of IFT particles, we decided to analyse intraflagellar transport in the KIF9BRNAi mutant. Both anterograde and retrograde transport were examined by live cell imaging using various GFP::IFT proteins. Data were completed by immunofluorescence analysis. Due to the accumulation of PFR material, the KIF9BRNAi mutant displays detached flagella, allowing their isolation by mechanical shearing. The composition of such purified flagella was analysed by mass-spectrometry, revealing novel matrix proteins that are currently under investigation. We also analysed the colony forming behaviour of the KIF9B mutant on semi-solid agarose plates.

Extra-Glycosomal Localization of *Trypanosoma brucei* Hexokinase 2

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Trypanosoma brucei hexokinases (TbHKs) are targeted to glycosomes by an N-terminal peroxisomal targeting sequence (PTS2). Digitonin fractionation of blood-stream form parasites revealed an unexpected partitioning of TbHK in non-glycosomal fractions. Further analysis using immunofluorescence with a TbHK2-specific antibody revealed co-localized staining with the axoneme and basal bodies. In order to begin to elucidate the mechanisms of the extra-glycosomal localization, TbHK2 truncations tagged with hemagglutinin were episomally expressed in the parasite. These studies suggest a potential flagellar targeting sequence between amino acids 250 and 440. Unexpected localization of glycolytic enzymes (in particular HKs) is not unprecedented. In *Leishmania donovani*, HK has been found to be a hemoglobin receptor in the flagellar pocket (Krishnamurthy et al., JBC, 2005), while in mammalian sperm HK is attached to the sheath around the axoneme (Travis et al., MBC, 1998). What is the function of TbHK2 that is associated with the flagellum? We have found that procyclic form parasites lacking TbHK2 have defects in their response to environmental change, as well as altered social motility. These observations suggest that TbHK2 localizes to the flagellum to play a role in environmental signaling.

A neutral amino acid permease in Leishmania regulates metacyclogenesis and cell volume by building large pool of proline

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Proline is a versatile amino acid with roles in osmoregulation, stress responses and metabolism. Unlike all other organisms, *Leishmania* as well as all members of the Trypanosomatid family maintain a large cellular pool of proline that, together with alanine, serve as alternative carbon source as well as reservoir of organic osmolytes. We cloned and characterized a new neutral amino acid transporter, LdAAP24 that translocates proline and alanine across the *L. donovani* plasma membrane. By knocking out the gene that encodes for LdAAP24 we showed that this transporter fulfills multiple functions: it is the sole supplier for the intracellular pool of proline and contributes to the alanine pool; it is essential for cell volume regulation after osmotic stress; and it regulates transport and homeostasis of glutamate, arginine and glycine, none of which are its substrates. Interestingly, ∆ldaap24 mutants transiently express *sherp* and *meta1*, both markers of metacyclogenesis, at late log rather than late stationary phase. In addition, they change to metacyclic-like morphology at mid log, but resume oval shape at stationary phase. Finally, ∆ldaap24 are significantly more susceptible to oxidative stress than wild type. We hypothesize that absence of cellular proline trigger metacyclogenesis and thus, LdAAP24 that is responsible for the cellular pool of proline plays a key role in *Leishmania* development inside its vector.

Identification of acidocalcisome proteins in *Trypanosoma brucei* by a novel purification method and *in situ* tagging

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Acidocalcisomes are acidic calcium-containing organelles widely distributed from bacteria to humans (Patel and Docampo, *Trends Cell Biol.* 20, 277-86, 2010). Phosphorus is stored mainly as pyrophosphate and polyphosphate (poly P) and acidity is maintained through the activity of two proton pumps, a vacuolar H^+-pyrophosphatase (TbVP1) and a vacuolar H^+-ATPase (TbV-ATPase). There is physiological evidence for the subcellular localization of the V-ATPase in *Trypanosoma brucei* acidocalcisomes but no immunolocalization studies have yet been done. In this work we describe a new method using two iodixanol density gradients for purification of the acidocalcisome from *T. brucei* procyclic (PCF) and bloodstream (BSF) forms. Pyrophosphatase assays indicated that the isolated organelle was greatly enriched compared with the large organelle fraction. Assays for other organelles generally indicated no enrichment in the acidocalcisome fraction. Through epitope tagging of the endogenous loci, we have demonstrated that TbVTC1 (vacuolar transporter chaperone 1), subunits beta and delta of *T. brucei* adaptor complex-3 (AP-3), and the *a* subunit of the V-ATPase localize to acidocalcisomes of both PCF and BSF. Subunit *a* of TbV-ATPase was able to complement yeast deficient in this subunit demonstrating its functionality. RNA interference of these proteins resulted in a growth defect in both PCF and BSF.

Nucleolar changes associated to growth rate conditions in *Trypanosoma cruzi* epimastigotes

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Our group is interested in rRNA and ribosome biogenesis in the parasitic protozoan *Trypanosoma cruzi*. Epimastigotes represent an extracellular replicative stage of *T. cruzi* and can be cultured in axenic media. The growth curve of epimastigotes allows assessment of potential differences in the nucleoli of cells undergoing growth-rate transitions. To establish cellular parameters for studying ribosome biogenesis in T. cruzi, a morphometric analysis of the nucleoli of cultured cells in the exponential and stationary phases was conducted. Electron micrograph-based measurements of nuclear sections from independent cells demonstrated that the nucleolar area is over twofold higher in exponentially growing cells, as compared with epimastigotes in the stationary phase. The granular component of the nucleoli of actively growing cells was the main structural element. Granules of this type (presumably ribosomes) appeared abundantly in the cytoplasm of actively growing cells. To address the potential need of protein synthesis to maintain the nucleolus architecture, parasites treated with Cycloheximide were analyzed as well. Our findings show that this drug correlates with the reduction of the nucleolus' apparent size without an important disruption of its architecture. Our results provide a firm basis for the establishment of an experimental model to study the organization of the nucleolus during the growth and development of *T. cruzi*.

Genetic Analysis of the *T. cruzi* TcPOT1.1 diamine transporter

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Trypanosoma cruzi cannot synthesize putrescine *de novo*, and therefore, polyamine transport plays an indispensable nutritional function for this single cell eukaryote. We have previously identified a high affinity putrescine-cadaverine transporter, TcPOT1.1, in *T. cruzi* that accounts for the capacity of the parasite to transport these diamines (Hasne, M. P. and Ullman, B. (2010) Mol Microbiol, 76:78-91). The *TcPOT1.1* locus consists of two alleles, *TcPOT1.1* and *TcPOT1.2*, both of which encode functional diamine transporters. A Δ*tcpot1.1*/Δ*tcpot1.2* null mutant has been constructed that lacks putrescine transport capability. To identify amino acids responsible for TcPOT1.1 ligand binding, we constructed a homology model of the *T. cruzi* transporter using the high resolution crystal structure of the AdiC prokaryotic arginine-agmatine antiporter. This homology model allowed for the mapping of residues participating in the TcPOT1.1 permeation pathway and highlighted amino acids forming the putative binding pocket of the diamine transporter. The homology model was then experimentally tested by site-directed mutagenesis of residues conjectured to be important in the permeation pathway by assessing the capacities of these *tcpot1.1* mutants to transport putrescine and to target propertly to the parasite cell surface. Most of the *tcpot1.1* mutants displayed profoundly affected transport capabilities without affecting their cellular localization. These findings initiate a mechanistic understanding of a *T. cruzi* transporter that serves an indispensable nutritional function for the parasite and may consequently be amenable to therapeutic exploitation

Membrane Rigidification in African Trypanosomes: An Unusual Mechanism of Peptide Cytotoxicity

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African trypanosomes cause sleeping sickness in humans and Nagana in cattle. The bloodstream developmental forms in mammalian hosts are uniquely susceptible to killing by hydrophobic peptides. Previously we have shown that specificity of hydrophobic peptides is due to the unusually fluid plasma membrane of African bloodstream form trypanosomes. Here we define the characteristics of hydrophobic trypanocidal peptides and show that the mechanism of killing is rigidification of the outer leaflet of the plasma membrane. Trypanocidal hydrophobic peptides can be highly heterogenous in primary structure, but require a central hydrophobic region of sufficient length with a positively charged residue at the N-termini. Anisotropic probes that report the rigidity at different depths in the plasma membrane reveal that both the internal acyl chain and the interfacial region are rigidified by hydrophobic trypanolytic peptides. Rigidification of the internal region is not sufficient for cell killing. We suggest that the plasma membrane is a novel target for pharmaceutical agents to treat human and animal African trypanosomiasis. Additionally the mechanism of peptide cytotoxicity reveals a phenotype, membrane fluidity, that is so far unique to BSF African trypanosomes and unexplored in terms of molecular and biochemical regulatory mechanisms.

Comparative Transcriptomics of *T. brucei* and *T. cruzi* Insect and Blood Form Life Stages Using Spliced Leader Trapping

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Using a recently developed method to map 5' splice sites and the expression profile of the corresponding gene we have now determined the splicing and expression profile of three life stages of *Trypanosoma brucei* and four life stages of *Trypanosoma cruzi*. We have identified sequence tags for more than 8000 and 9000 genes in *T. brucei* and *T. cruzi*, respectively. Analysis of the major splice acceptor sites indicated that AG is the predominant splice acceptor dinucleotide in both species. However, *T. cruzi* seemed to be more promiscuous in the choice of the acceptor dinucleotide. For the first time we can now identify the precise 5' UTR length of the majority of transcripts from both genomes. The median 5' UTR length on a per transcript level ranged from 47nt (*T.brucei*) to 59 nt (*T.cruzi*) excluding the 39nt of the spliced leader sequence. The number of genes spliced exclusively downstream of the predicted initiator ATG was very similar in the two transcriptomes. We identified a set of more than 500 genes in the *T. cruzi* genome that are specifically upregulated in either the amastigote or the trypomastigote stage indicating their importance for invasion or egrass. Using the combined information from recently published high throughput sequencing studies we could identify more than 600 novel transcripts in the *T. cruzi* transcriptome, 52 of which did not contain any coding potential. A preliminary dataset is available at www.splicer.unibe.ch.

Antigenic variation in *T. brucei*: The importance of DNA break location and RAD51

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Antigenic variation in African trypanosomes requires the monoallelic expression and switching of variant surface glycoproteins (*VSG*) genes that are flanked by a repetitive '70-bp' sequence and a telomere. Replacement of the active gene with a copy of a second telomeric *VSG* can occur via one-sided recombination reactions known as break-induced replication (BIR) while the majority of *VSGs* must utilize a two-sided 'cassette-conversion' mechanism. The 70-bp sequence commonly provides homology for either reaction and RAD51-dependent and independent mechanisms make a contribution. We detect natural breaks flanking both active and silent telomeric *VSGs*. All survivors switch *VSG* when an artificial break is introduced adjacent to the active 70-bp sequence. In contrast, a break between the active *VSG* and telomere often generates heterogeneous populations of *VSG* expressers. A 'control' break adjacent to the distal promoter typically fails to trigger switching. Thus, cells with a 70 bp-proximal break remain viable only if a switch rapidly restores *VSG* expression while a telomere-proximal break appears to provide a window of opportunity for further cell division, with daughter cells repairing the telomere with or without switching. This is possible because of the absence of a telomere break checkpoint in *T. brucei*. We show that single-stranded DNA is generated adjacent to these breaks but that RAD51 repair foci are not detected. We are currently determining the contribution of RAD51 to these switching events.

Deregulation of DOT1 family histone methyltransferases causes replication defects in *Trypanosoma brucei*

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Cell cycle progression requires careful regulation to ensure accurate distribution of genetic material to daughter cells. Although many cell cycle regulators are conserved in trypanosomes, additional regulatory mechanisms seem to have evolved. We are interested in the function of posttranslational histone modification, in particular methylation of histone H3 on lysine 76 (H3K76me), during cell cycle progression. Monomethylation and di-methylation of H3K76 are mediated by the conserved histone methyltransferase DOT1A. Interestingly, H3K76me1 peaks in G2-phase cells while H3K76me2 is mainly detectable during mitosis and cytokinesis, suggesting a function in cell cycle progression. To test this hypothesis, we manipulated H3K76 methylation levels by RNAi-mediated depletion of DOT1A as well as over-expression of DOT1B, which is responsible for tri-methylation of H3K76. Depletion of DOT1A decreases H3K76me1 and H3K76me2 and abolishes replication of nuclear DNA. Interestingly, this replication block does not prevent karyokinesis, generating a population with a 1C nuclear DNA content. DOT1B over-expression also decreases H3K76me1 and H3K76me2 but generates a population of cells with aneuploid nuclei as well as enucleated cells. Detailed analysis revealed that DOT1B over-expression causes continuous nuclear replication, which generates nuclei with increasing DNA content. We suggest that accurate H3K76 methylation levels are essential for proper cell cycle progression and that premature *de novo* methylation of H3K76 causes continuous replication of nuclear DNA in *T. brucei.*

Multiple Protein Arginine Methyltransferases and Classes of Methylproteins in *Trypanosoma brucei*

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Arginine methylation is a common posttranslational modification in eukaryotes that is catalyzed by a family of protein arginine methyltransferases (PRMTs). In yeast and humans, arginine methylation functions in many processes including chromatin remodeling, RNA processing, and DNA repair. The genome of *Trypanosoma brucei* (and those of *T. cruzi* and *Leishmania major*) encodes five putative PRMTs, one of which (TbPRMT7) is unique to kinetoplastids. With regard to types of methylarginine products, *in vitro* methylation assays define three different classes of PRMTs in *T. brucei*. RNAi-mediated depletion of TbPRMT6 results in a modest growth defect and abberant cytokinesis in both bloodstream and procyclic stages, TbPRMT1 depletion causes a growth defect in bloodstreams, and knockdown of the other PRMTs has no effect on growth. These results, along with the growth defect of TbPRMT1/7 dual knockdowns in proyclic forms, suggest redundancy among PRMTs. Immunoblotting of subcellular fractions with anti-methylarginine antibodies revealed many unique arginine methylproteins in the cytosol, nucleus, and mitochondrion of *T. brucei*. We are currently employing combined ETD/CID mass spectrometry to characterize the arginine methylproteome of procyclic form *T. brucei*. We have identified diverse classes of arginine methyproteins including multiple RNA binding proteins (RBPs) and RNA modifying enzymes, metabolic enzymes, DNA replication and repair proteins, and structural components. Interestingly, some of these proteins exhibit methylation on the typical glycine-arginine-rich motif, but many are methylated at noncanonical sites, greatly expanding the potential targets of methylation in eukaryotes. We currently are interested in determining the roles of protein arginine methylation in the function of RBPs, particularly as it applies to gene regulation and mitochondrial function.

Repeat arrays associated with centromeric domains on the mega-chromosomes of *T. brucei* are much larger than predicted

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The locations of centromeric loci in trypanosome chromosomes have been inferred from biochemical mapping and by delineation of regions required for mitotic stability. In the *Trypanosoma brucei* mega-chromosomes, these domains are situated between directional gene clusters, in regions containing degenerate retroelements and arrays of AT-rich repeats. Here, we show that these repeat arrays, which occur once per chromosome, are much larger than predicted in the *T. brucei* draft genome sequence. Our approach involved the use of long range restriction mapping and pulsed field gel electrophoresis to more accurately delineate these regions in chromosomes 1-8. The experimental results show that the AT-rich repeat regions range from 50 - 120 kb, and are therefore considerably larger than originally reported. This implies that *T. brucei* centromeric domains are typical of regional centromeres in higher eukaryotes and are composed predominantly of high copy-number sequence elements that stretch over extensive sections of chromosomal DNA. There is a general agreement that the location of centromeric regions is determined epigenetically. However, most of the functionally conserved, centromeric- associated proteins present in other eukaryotes appear to be absent in trypanosomatids. The physical mapping of these regions should provide a more robust framework to facilitate analysis of centromeric chromatin organization and help to formulate hypotheses to explain how these atypical centromeres might function.

Involvement of the CCCH zinc finger protein *Tb*ZC3H11 in stress response

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Kinetoplastids such as *Trypanosoma brucei* mainly rely on posttranscriptional mechanisms of gene regulation. Members of the CCCH zinc finger RNA binding protein family have been shown to influence mRNA levels and location in other organisms. One example is mammalian tristetraproline (TTP) that destabilizes AU-rich element containing mRNAs by recruiting them to P-bodies. Only a few of the numerous trypanosomal CCCH proteins have been studied so far. Three have been shown to affect surface coat expression and differentiation. The trypanosomal ZC3H11 protein contains a single CCCH domain. RNAi revealed that it is essential in bloodstream form trypanosomes. The *in situ* -tagged protein accumulates upon inhibition of the proteasome in both life stages, suggesting that the protein is unstable. An accumulation was also observed upon heat shock of procyclic cells. To get a clue for possible target transcripts of ZC3H11 a microarray analysis comparing total RNA from ZC3H11-depleted and wildtype cells was performed. Unexpectedly, among the few significantly reduced transcripts was *HSP70* mRNA. By immunoprecipitation of ectopically expressed ZC3H11, bound *HSP70* transcript could be obtained, suggesting a direct binding. These results suggest an involvement of ZC3H11 in the regulation of heat shock response in *Trypanosoma brucei*.

Development of a dual reporter system to identify regulatory cis-acting elements in untranslated regions of *Trypanosoma cruzi* mRNAs

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In trypanosomatids, transcription is polycistronic and gene expression control occurs mainly at the post-transcriptional level. To investigate the role of sequences present in the 3'UTR of stage-specific mRNAs of *Trypanosoma cruzi*, we generated a new vector, named pTcDUALuc, containing the firefly and Renilla luciferase reporter genes. To test this vector, sequences derived from the 3'UTR plus intergenic regions of the alpha tubulin gene, which is up-regulated in epimastigotes, and amastin, which is up-regulated in amastigotes, were inserted downstream from the firefly reporter gene and luciferase activity was compared in transient and stable transfected parasites. As expected, increased luciferase activity was detected in epimastigotes transiently transfected with pTcDUALuc containing tubulin sequences. Using stable transfected cell lines that were allowed to differentiate into amastigotes, we observed increased luciferase activity and mRNA levels in amastigotes transfected with pTcDUALuc containing amastin sequences. We also showed that the spliced leader sequence and poly-A tail were inserted in the predicted sites of the firefly luciferase mRNA and that deletions in the alpha tubulin 3'UTR resulted in decreased luciferase expression because it affects polyadenylation. In contrast to the constructs containing 3'UTR sequences derived from tubulin and amastin genes, the presence of the 3'UTR from a trans-sialidase gene, whose expression is higher in trypomastigotes, resulted in increased luciferase activity in trypomastigotes without a corresponding increase in luciferase mRNA levels.

Comparative study of the leishmanicidal activity of imidazoquine derivatives of primaquine against *Leishmania infantum*

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Since no reliable vaccine has been developed so far, the management of leishmaniasis relies exclusively on chemotherapy. Given the fact that the standard treatments are toxic, and parasite resistance has been reported, new anti-leishmanial drugs are urgently needed. Based on the anti-protozoal activity of 8-aminoquinolines, we evaluated the leishmanicidal activity of several peptidomimetic imidazolidin-4-one derivatives of the 8-aminoquinoline primaquine. Firstly, a screening of the most efficient compounds at inhibiting the multiplication of *L.infantum* promastigotes was performed. Then, cytotoxicity and killing efficacy of the most active compounds was assessed on infected bone marrow derived macrophages. Primaquine, BD4-506 and BD4-511 derivatives, had similar inhibitory effects on the proliferation of *L.infantum* promastigotes. Nevertheless, BD4-506 was more efficient than primaquine and BD4-511 at eliminating intramacrophagic *L.infantum*, without causing toxicity to the host cell. These preliminary results reveal the potential usefulness of the imidazoquine BD4-506 to treat *L.infantum*. Further studies will comprise the understanding of the leishmanicidal mechanism and the *in vivo* efficacy of this compound.

Whole Genome sequencing and functional studies of miltefosine-resistant *Leishmania major* mutants

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Miltefosine (MF), a drug that can be taken orally, has been used in the chemotherapy of visceral leishmaniasis. Since both the mechanism of action of MF and its target(s) are unknown, we generated four independent mutants of *Leishmania major* highly resistant to MF. These respective mutants were characterized and two of them were submitted to next generation whole genome sequencing. Our goal was to study copy number variation and to identify potential gene mutations associated with MF resistance. Read depth analysis revealed the absence of amplifications / deletions in the genome of these two mutants. Analysis of the genome sequence revealed mutations in a P-type plasma membrane ATPase involved in phospholipid translocation, a protein already described and associated to MF resistance in *Leishmania*. Interestingly, several different mutations were found in this ATPase and the role of these mutations in resistance was validated experimentally. Sequence analysis revealed also mutations in pyridoxal kinase and alpha-adaptin like protein in several independent mutants and their role in resistance are being evaluated experimentally.

Trypanosoma brucei rhodesiense contains two copies of the Serum Resistance Associated Protein gene

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Trypanosoma brucei brucei cannot infect humans because of susceptibility to a minor subclass of a high-density lipoprotein (HDL) called the trypanosome lytic factor (TLF-1). *Trypanosoma brucei rhodesiense*, the causative agent of acute human sleeping sickness, has evolved resistance to TLF-1 killing through expression of the serum resistance associated protein (SRA). Previous studies have indicated a single copy of the SRA gene, which is located exclusively in a truncated Bloodstream Expression Site (BES) (Xong et al., 1998). Here we report the discovery of a second copy of the SRA gene in the *T. b. rhodesiense* strain KETRI 2482. Consistent with previous findings, one copy is located within a BES whereas the second copy is located on a different chromosome. Transcriptional analysis of isogenic KETRI 2482 lines revealed that one copy of SRA is transcribed by RNA polymerase I while the second copy is transcribed by RNA polymerase II, independently of the expressed variant surface glycoprotein (VSG). Metacyclic variant surface glycoprotein (mVSG) gene expression occurs exclusively from a mono-cistronic Metacyclic Expression Site (MES) and expression of the mVSG can last up to 7 days post infection. Once fully differentiated into the bloodstream form, trypanosomes express their VSG from one of the polycistronic RNA polymerase I transcribed BES's. Expression of the SRA containing BES allows protection to TLF-1 killing. We postulate that expression of the second SRA copy could confer TLF-1 resistance to cells expressing a non-SRA containing BES and / or allow metacyclic form *T. b. rhodesiense* to resist TLF-1 killing while still expressing a MES.

Leishmania infantum IZT3 is a zinc transporter that is tightly regulated by zinc status

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The ZIP family comprises proteins involved in the transport of divalent metals in several organisms. Among three putative ZIP family members in *Leishmania infantum*, we have identified IZT3 (Iron/Zinc Transporter #3) as a protein involved in the acquisition of zinc since (1) expression of IZT3 in a yeast strain defective in zinc acquisition systems rescued the growth in zinc-limiting medium; and (2) expression of IZT3 in RNA-injected Xenopus laevis oocytes modestly stimulated by up to 16-fold the uptake of 2μM $^{65}Zn^{2+}$. We tested whether IZT3 expression in *L. infantum* is regulated according to zinc availability and found that IZT3 expression was induced in the presence of the metal chelator EDTA but decreased following the addition of zinc (1μM). Copper and cadmium also induced IZT3 expression, probably due to competition with zinc. We found that, whereas the protein was present at the surface of the parasite under zinc-limiting conditions, IZT3 levels decreased by approximately 80% after 24 hours of zinc supplementation and membrane localization was lost. IZT3 mRNA levels increased 3-fold when parasites were grown in low-zinc medium and decreased by around 60% within one hour following zinc addition, indicating that regulation of IZT3 expression is mediated largely at the level of mRNA stability. We observed no IZT3 mRNA down-regulation in response to zinc when we inhibited protein synthesis by the addition of cycloheximide, implicating a labile protein in the destabilization of the mRNA when zinc is available. Together, our data suggest that IZT3 is a zinc transporter - the first identified in *Leishmania* - and that IZT3 expression is tightly regulated by zinc status.

The nonsense mediated decay factor, TbNMD3, regulates *PAG* transcripts in a process involving CRM1-mediated nuclear export

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Nonsense-mediated decay (NMD) is an evolutionarily conserved, translation-dependent mRNA degradation pathway targeting mRNAs with a stop codon in a poor context for translation termination. Although the existence of NMD in trypanosomes has not been demonstrated, the *T.brucei* genome has homologues of NMD factors, such as *TbNMD1*/Tb927.5.2140, *TbNMD2*/Tb11.02.5270, and *TbNMD3*/Tb927.7.970. In addition to NMD1 and NMD2, which interact with NMD3 in a complex, nuclear pre-60S particles also bind NMD3, which recruits the export receptor Crm1 in *S. cerevisiae* assisting nuclear export. During functional analysis of the NMD factors, we noticed significant upregulation of some stage-regulated transcripts, particularly *PAG* transcripts, upon *NMD3* depletion. *PAG* transcripts are co-transcribed with the *EP1/EP2 procyclin* genes. *NMD3* ablation also disrupted 60S rRNA processing, as expected. To investigate why *PAG* transcripts are altered in abundance we assayed the consequences of disrupted transcription, translation, trans-splicing and nuclear export. We found that sinefungin counteracts the effect of *NMD3* ablation, whereas leptomycinB reproduces the same phenotype. This suggests that TbNMD3-mediated transcript regulation may involve trans-splicing and CRM1-dependent nuclear export efficiency Analysis of the pool of RNAs perturbed by *NMD3* ablation via RNA sequencing is currently being pursued to understand in detail how NMD3 is contributing to the abundance and processing of particular mRNAs in trypanosomes.

The Purine Salvage Pathway of *Leishmania donovani*

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An intricate genetic dissection of the purine salvage pathway of *Leishmania donovani* has revealed that this parasite can fulfill its nutritional needs by funneling any naturally occurring host purine into hypoxanthine, guanine, or xanthine. These purines are then incorporated into the parasite nucleotide pool through either hypoxanthine-guanine phosphoribosyltransferase (HGPRT) or xanthine phosphoribosyltransferase (XPRT). Through the isolation and characterization of a conditionally lethal Δ*hgprt*Δ*xprt* line, we have established that either HGPRT or XPRT is indispensable for purine salvage by *L. donovani*. The products of these two enzymes, IMP, GMP, and XMP, are then funneled to AMP, which is synthesized from IMP through adenylosuccinate sythetase (ADSS) and adenylosuccinate lyase (ASL). Our model of purine salvage, therefore, predicts that ADSS and ASL are essential enzymes for *L. donovani* survival and replication. To test this hypothesis we generated Δ*adss* and Δ*asl* knockouts using targeted gene replacement. Whereas wild type parasites can salvage any purine nucleobase or nucleoside as a purine source, Δ*adss* and Δ*asl* parasites, as expected, are only capable of growing in adenine or adenosine in the presence of deoxycoformycin (dCF), a potent inhibitor of adenine aminohydrolase (AAH). Thus, the lesions in the Δ*adss* and Δ*asl* cells are conditionally lethal mutations, and no other purine source is able to support the growth of these mutants. Both Δ*adss* and Δ*asl* knockouts are profoundly compromised in their ability to infect macrophages. Interestingly, however, only Δ*asl* parasites exhibit a significant reduced parasitemia in the mouse virulence model. Collectively, these data suggest that ADSS and ASL are essential enzymes to the parasite and that host purines are incorporated into the parasite nucleotide pool via HGPRT and/or XPRT.

A feedback loop controls VSG expression site activity

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In bloodstream form *Trypanosoma brucei*, high-level expression of the variant surface glycoprotein (VSG) is essential for parasite survival, *in vivo* as well as *in vitro*. Only one VSG gene is expressed at a time in a monoallelic fashion from one of 15 telomeric expression sites (ES). Switching VSG expression, by means of activation of a different ES, provides an effective mechanism to evade the host immune response. Although it has become clear that chromatin remodeling plays a major role in ES regulation, little is known about the mechanisms ensuring monoallelic expression of *vsg*. Here, we provide evidence for the existence of a feedback mechanism, in which the level of VSG expression itself regulates expression site activity. Any increase in the amount of VSG mRNA immediately triggers events leading to temporary ES silencing, most probably by chromatin remodeling. Our results suggest that monoallelic expression of ESs underlies continuous monitoring of the VSG mRNA level, most likely by recognition of the 16mer, a conserved sequence motif within the 3'UTR , which is the only common denominator of all VSG genes.

Analyzing the functional role of a divergent XPB helicase paralog

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TFIIH is a basal factor for initiation of RNA polymerase II transcription and, in higher eukaryotes, also essential for DNA repair and cell cycle control. Recently, the *Trypanosoma brucei* TFIIH core complex, consisting of DNA helicases *Xeroderma pigmentosum* B (XPB) and XPD and orthologs of p62, p52, p44, p34 and TFB5, was shown to be required for transcription of spliced leader (SL) RNA genes. While there are two divergent XPB helicases in trypanosomatid genomes, only the larger variant was detected in XPD-purified TFIIH. Since we are unaware that XPB helicases function independently of TFIIH, we began a functional analysis of the smaller variant (XPB-S) in *T. brucei*. XPB-S was dispensable for SL RNA gene transcription in vitro and, accordingly, immunofluorescent microscopy showed that XPB-S, though being nuclear, does not co-localize to the putative sites of SL RNA gene transcription as other basal transcription factors. Since RNAi-mediated expression silencing of XPB-S was not lethal, we performed a knockout of the gene and found that the KO cells exhibit a growth defect with a ~two-fold higher doubling time than wild-type cells. Since trypanosome TFIIH lacks a kinase that could be involved in cell cycle control, we speculated that XPB-S functions specifically in nucleotide excision repair. Accordingly, XPB-S KO cells exhibited a considerably higher sensitivity than wild-type cells to exposure of UV light and of the DNA-damaging compound Cisplatin. Interestingly, C- or N-terminally tagged XPB-S did not stably assemble into a TFIIH complex. Since this may be a consequence of the tag, we are currently raising a polyclonal anti-XPB-S antibody to analyze whether XPB-S defines a DNA repair-specific TFIIH or functions independently.

Trafficking of a novel family of type I transmembrane proteins in *Trypanosoma brucei*

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Endocytosis of surface molecules from the *Trypanosoma brucei* plasma membrane is both exclusively clathrin-dependent and dynamin-independent, and non-concentrative in both life-cycle stages. Extensive studies on the major surface type I *trans*-membrane proteins, invariant surface glycoproteins 65 and 75 (ISG65/75), revealed highly conserved lysine residues in the cytoplasmic region, demonstrated to be involved in ubiquitin-mediated trafficking. Here, we have analysed the trafficking constraints and signals required for targeting of a novel family of type I *trans*-membrane domain proteins distinct from ISG65/75. This *T. b. brucei* and *T. b. gambiense*-specific family are predicted to have short, lysine-rich cytoplasmic domains and are grouped into three distinct subfamilies (A, B and C). Members of each subfamily are expressed in both the major life-cycle stages, as shown by quantitative real-time PCR (qRT PCR), while treatment of cells with cycloheximide reveals a comparatively rapid turnover. Trafficking was examined by generating chimeric proteins containing the C-terminal portion of representative proteins from the two larger subfamilies (A and B) (encompassing the 23 C-terminal residues of the extracellular domain plus the *trans*-membrane and cytoplasmic domains) fused to the N-terminal domain of BiP (BiPN). Immunofluorescence analysis of these constructs, together with epitope-tagged versions of the full length proteins, indicates that subfamilies A and B are intracellular proteins, probably associated with the endoplasmic reticulum. RNA interference (RNAi) indicates the families are required for robust growth and normal cellular proliferation. The signals regulating the targeting of these novel proteins will be investigated and approaches will be taken to carry out functional analysis.

Proteomic and phosphoproteomic analysis of Leishmania donovani development

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During infection, Leishmania promastigotes undergo rapid differentiation to amastigotes that proliferate in phagolysosomes of mammalian macrophages. This process is mimicked in vitro by shifting cultured promastigotes to a lysosome-like environment. Time course analyses showed that promastigotes differentiation into amastigotes is a regulated process that involves ordered changes in morphology, protein and gene expression. Our goal is to uncover the signaling pathway(s) and the cellular processes that initiate differentiation of Leishmania inside its host. The axenic differentiation model and a high-coverage, comparative proteomic methodology was used to analyze in detail protein and phosphoprotein expression changes throughout the differentiation process. Proteins from cell lysates were trypsinized, enriched for P-peptides using titanium-dioxide columns and then subjected to isobaric tagging followed by tandem mass-spectrometry (iTRAQ/LC-MS/MS). These studies showed that during differentiation parasites retool their metabolism for life in the new host environment. We found that Phases I (signal perception) and III (morphological change) were associated with more protein phosphorylation, while phases II (aggregation) and IV (amastigotes maturation) showed greater de-phosphorylation. The majority of the identified phosphorylated residues are stage specific, appearing exclusively in either promastigotes or amastigotes. A number of novel phosphorylation motifs that might represent parasite-specific phosphorylation sites have been identified. The analyses identified several protein-kinases and phosphatases as well as a few proteins of particular interest such as eIF2á, transcription factor like-protein and Nucleolar RNA helicase II that we suggest as potential regulators of differentiation. This work constitutes the first time course phosphoproteomic analysis of parasite differentiation.

Antitumor Quinol PMX464 is a Cytocidal Anti-Trypanosomal Inhibitor Targeting Trypanothione Metabolism

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Better drugs are urgently needed for the treatment of African sleeping sickness. We tested a series of promising anticancer agents belonging to the 4-substituted 4-hydroxycyclohexa-2,5-dienones class ('quinols') and identified several with potent trypanocidal activity (EC50 ¡ 100 nM). In mammalian cells, quinols are proposed to inhibit the thioredoxin / thioredoxin reductase system, which is absent from trypanosomes. Studies with the prototypical 4-benzothiazole-substituted quinol, PMX464, established that PMX464 is rapidly cytocidal, similar to the arsenical drug, melarsen oxide. Cell lysis by PMX464 was accelerated by addition of sub-lethal concentrations of glucose oxidase implicating oxidant defences in the mechanism of action. Whole cells treated with PMX464 showed a loss of trypanothione (T(SH)2), a unique dithiol in trypanosomes, and tryparedoxin peroxidase (TryP), a 2-Cys peroxiredoxin similar to mammalian thioredoxin peroxidase. Enzyme assays revealed that T(SH)2, TryP and another tryparedoxin-dependent peroxidase (TDPX) were inhibited in a time- and concentration-dependent manner. The inhibitory activities of various quinol analogues against these targets showed a good correlation with growth inhibition of T. brucei. The monothiols glutathione and L-cysteine bound in a 2:1 ratio with PMX464 with Kd values of 6 and 27 nM, respectively, whereas T(SH)2 bound more tightly in a 1:1 ratio with a Kd value of 430 nM. Overexpression of trypanothione synthetase in T. brucei decreased sensitivity to PMX464 indicating that the key metabolite T(SH)2 is a target for quinols. Thus, the quinol pharmacophore represents a novel lead structure for the development of a new drug against African sleeping sickness.

The trypanosome-specific N-terminus of RPA2 is required for nuclear localization and also RNA polymerase I function

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African trypanosomes are the only known organisms to use RNA polymerase I (polI) in the transcription not only of ribosomal RNA, but also of protein-coding genes. These genes include the major surface glycoproteins of both procyclic form and bloodstream form cells, and their expression requires the coupling of polI transcription to the mRNA processing machinery. Several trypanosome polI subunits vary compared to their homologues elsewhere, and the question arises as to how these variations relate to polI function. In particular, are there subunit differences that relate solely to transcription of protein-coding genes? A clear example of a trypanosomatid-specific subunit variation is the N-terminal extension found on the second-largest subunit of polI, RPA2. We have asked if this extension is necessary for polI function in trypanosomes and if it plays a general role or acts solely in the bloodstream-form expression site body (ESB). N-terminal truncation of RPA2 leads to nuclear exclusion in both bloodstream-form and procyclic cells and a growth defect in bloodstream-form cells which phenocopies loss of an allele. The N-terminus does not act as a general nuclear localization signal (NLS), however, and it fails to accumulate unrelated proteins in the nucleus. An ectopic NLS is sufficient to reinstate nuclear localization of truncated RPA2, but does not restore function. Moreover, NLS-tagged, truncated RPA2 has a different sub-nuclear distribution to full-length protein. We conclude that the RPA2 N-terminal extension is required for nuclear import and also polI function within the nucleus via a trypanosome-specific mechanism. However, it does not have a role exclusive to the expression of protein-coding genes in the ESB.

Dissecting molecular interactions in a novel preribosomal particle in *T. brucei*

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5S rRNA is essential for eukaryotic ribosome functions, and the ribosomal protein L5 alone can stabilize and transport 5S rRNA to the nucleolus for ribosomal assembly. Our laboratory has previously identified two novel trypanosome-specific 5S rRNA binding proteins, P34 and P37. These proteins form a complex with L5 and 5S rRNA in the nucleoplasm and nucleolus. The absence of P34 and P37 leads to a decrease in 5S rRNA levels. Since a number of residues involved in interactions with 5S rRNA are not conserved in *T. brucei* L5, we hypothesize that L5 maybe be deficient in its binding and subsequent stabilization of 5S rRNA and that P34 and P37 complement the defects in these functions. We are using the FRET (fluorescence resonance energy transfer) to determine the associations between these molecules using cerulean and eYFP tagged proteins. Initial FRET experiments *in vitro*indicate that P34 and L5 have associate in the absence of 5S rRNA and their fluorescent tags have moved into the range required for energy transfer to occur. We set up a titration in which the acceptor-tagged eYFP-P34 was added to its donor-tagged cerulean-L5 while the emission spectra were monitored. This steady-state binding assay was applied in multi-well plate format, and resulted in measurement of a Kd=0.6007 $\pm$0.027 μM for eYFP-P34 and cerulean-L5. Hence, the FRET-based binding assay is suitable for studying the binding between trypanosome proteins L5 and P34. Since this preribosomal particle is unique and plays an essential role in the viability and ribosomal biogenesis in trypanosomes, these studies will allow us to define a strategic target for chemical intervention.

Building a flagellum: two tales of cytoplasmic assembly and flagellar incorporation

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The flagellum of Trypanosoma brucei is present throughout its life and cell cycle, and is essential for parasite survival. This complex organelle is composed of over 300 proteins, whose mode of assembly remains poorly understood. Flagellar proteins are synthesized in the cytoplasm, and are then imported to the flagellar compartment, where they are transported along the axoneme by intraflagellar transport (IFT). Previous work investigated the contribution of ODA7, a protein containing leucine-rich repeats, to flagellum architecture and beating (1). RNAi silencing of ODA7 leads to striking motility defects, as parasites twist but fail to swim forward. Transmission electron microscopy analyses revealed the absence of a significant percentage of outer dynein arms. Strikingly, we found the protein to be exclusively cytosolic and not associated with the flagellum. Cell fractionation demonstrated that TbODA7 silencing does not affect the total amount of DNAI1, but leads to a shift to the detergent soluble fraction. Dynein arm components are therefore properly synthesised but fail to assemble and remain in the cytoplasm. We are currently investigating the dynamics of axonemal components, from their assembly in the cytoplasm to their incorporation and maintenance in the flagellum. Using the spinning disk fluorescence microscopy technology, we are investigating the polarity, speed and rate of assembly of dynein arm components within the flagellum, assessing the role of ODA7 and the contribution of IFT. We are taking advantage of the fact that trypanosomes maintain their old flagellum while assembling a new one to compare situations of maintenance versus assembly. (1) Duquesnoy et al. Am J Hum Genet 85(6),890(2009).

The phosphoproteome of *Trypanosoma brucei*

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Reversible phosphorylation of proteins is an important post-translation modification with a central role in cell signalling in eukaryotes. The genome of *Trypanosoma brucei* contains 182 putative protein kinases and 24 putative protein phosphatases, and there is growing evidence of the important biological role that reversible phosphorylation plays in *T. brucei*. Phosphoproteomics, the global measurement of phosphorylation state of the proteome, is a rapidly developing technique that has the potential to provide valuable biological insights. A recent phosphoproteomics study identified 491 phosphoproteins in the cytosolic fraction of bloodstream from *T. brucei*, with phosphorylation of serine, threonine and tyrosine residues observed. Here, we examine the phosphorylation of detergent solubilised whole cell lysates of procyclic form and bloodstream form *T. brucei*, and the development of quantitative methods.

TBMLP1: A Putative Endosoma Iron Channel in *Trypanosoma Brucei*

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Iron is an essential nutrient in all trypanosomatids analyzed. Bloodstream form *T. brucei* derive iron from the host carrier protein transferrin. Transferrin is endocytosed after binding to the ESAG6/7 heterodimer in the flagellar pocket. Iron is subsequently released from transferrin in the late endosome/lysosome compartment after acidification. However the pathway by which iron is then transported into the cytosol from the endosomal system has not been resolved.Here, we report the identification of an orthologue of the human endosomal iron-release channel Mucolipin 1, which may perform this role in *T. brucei*. TbMLP1 (Mucolipin-like protein 1) is a six transmembrane domain protein which we show is confined to the endolysosomal system. TbMLP1 mRNA is constitutively expressed in bloodstream and procyclic forms.RNA interference mediated knockdown of the TbMLP1 gene resulted in a slight growth retardation which became apparent between 3 and 6 days after induction, consistent with previous reports that trypanosomes have an internal iron store. However, when exposed to the iron chelators deferroxamine and salicylhydroxamic acid, the induced cells have a severe growth defect in comparison to the uninduced controls and are hypersensitive. Thus TbMLP1 appears to play a central role in facilitating iron transport into the cytoplasm of the parasite, an essential function for parasite survival and replication.

Identification and validation of diagnostic antigens for Human African Trypanosomiasis

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Human African Trypanosomiasis (HAT) is still a prevalent disease in many parts in Sub-Saharan Africa. HAT is a parasitic infection caused by two species, *Trypanosoma brucei gambiense* and *T. b. rhodesiense*. Clinical diagnosis is not sufficient as symptoms from other endemic diseases are similar, e.g. malaria. In addition, some patients do not show any symptoms until the parasites have invaded the brain; ninety percent of all cases are diagnosed in the second stage. Current diagnostic tests require lab based equipment, trained personnel and have varying degrees of sensitivity and specificity. New diagnostic tests are needed. Our aim is to find and validate diagnostic antigens for *T. brucei* species for use in a lateral flow test. Over twenty potential diagnostic antigens were identified by immunoprecipitation experiments using purified antibodies from infected and control sera. Antigens were cloned, expressed and purified from E. coli. To validate the recombinant antigens, a chemiluminescent ELISA was developed and used to screen 170 clinically assessed human sera against a number of recombinant antigens. The sera comprised *T. b. gambiense* and *T. b. rhodesiense* infection sera from first and second stage patients, with matched human control sera. The results show diagnostic antigens that are able to discriminate between infection and control serum.

Leishmania donovani receptor-cargo protein complexes form in the cytosol and dock at glycosomal protein Peroxin14, modulating its conformation

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The glycosomes of *Leishmania donovani* are unique microbody organelles that compartmentalize a variety of metabolic pathways essential for parasite survival. Trafficking and import of newly synthesized proteins to the glycosome is dependent on the receptor proteins peroxin 5 (LdPEX5) and peroxin 7 (LdPEX7), and the docking protein peroxin 14 (LdPEX14), a peripheral membrane protein anchored to the surface of the glycosomal membrane. To understand the molecular events required for the sorting and trafficking of nascent polypeptides to the glycosome we employed a variety of techniques to examine the structure of native LdPEX5, LdPEX7, and LdPEX14. Biochemical analysis revealed that in the cytosol of *L. donovani*, LdPEX5 and LdPEX7 form heteromeric complexes that are loaded with various PTS1 and PTS2 cargo proteins. These structurally diverse arrays of heteromeric complexes are responsible for trafficking of proteins into the glycosome via LdPEX14, the glycosomal docking protein. At the glycosome surface these receptor-cargo complexes interact with LdPEX14. Investigation of the effects of receptor-cargo complex binding to LdPEX14 was done by investigating the native protein at the glycosomal surface. Via proteolytic digestion, extraction experiments, and native protein electrophoresis, it was found that binding of this trafficking complex triggers conformational changes in LdPEX14 that facilitate the import of proteins across the membrane of the organelle.

A Novel protein translocase of mitochondrial inner membrane in Trypanosoma brucei

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Import of nuclear-encoded proteins is known to be critical for mitochondrial function however mitochondrial protein translocation machinery in the kinetoplastid parasites, like *Trypanosoma brucei*, has been poorly characterized. In fungi and higher eukaryotes, there are two translocases of the mitochondrial inner membrane (TIM), TIM23-17 and TIM22-54. These multiprotein complexes have distinct specificity for two types of targeting signals containing proteins. The N-terminal targeting signal containing proteins are transported via TIM23-17 and TIM22-54 translocates mitochondrial carrier proteins that do not have N-terminal signal but thought to have internal signals. The only protein translocator of T. *brucei* mitochondrion that has been characterized so far is TbTim17. However homologs of Tim23 and Tim22 have not been found in the parasite genome. The TbTim17 is an essential protein for both the procyclic and bloodstream form of T. *brucei* and is critical for mitochondrial protein biogenesis. The TbTim17 is present in the mitochondrial membrane protein complex of about 1100 kDa, which is much larger than the TIMs found in other eukaryotes. Depletion of TbTim17 impairs the import of cytochrome oxidase subunit IV (COIV), an N-terminal signal-containing protein, and the ADP/ATP carrier (AAC) into mitochondria of the procyclic forms. Antibody inhibition studies also indicate the involvement of Tim17 for import of both proteins. The data collected so far suggest that T. *brucei* possesses a single mega alton sized TIM complex responsible for import of different targeting signal containing proteins.

RNA editing as a drug target in trypanosomes: development of a high throughput screening assay for RNA editing ligase 1

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RNA editing is essential for mitochondrial gene expression in all trypanosomatids but absent from the host and therefore a potentially powerful drug target[1]. The process is catalyzed by multiprotein complexes, the editosomes, and involves several enzymatic steps. A key enzyme in the process is RNA editing ligase 1 (REL1)[1]. The high-resolution crystal structure of *Trypanosoma brucei* REL1 revealed a deep pocket that serves to bind and orient ATP and offers numerous opportunities for specific interactions with small molecules[2]. Using a virtual drug screening strategy we had previously identified compounds that inhibit *Tb*REL1 with single-digit micromolar IC_{50} values[3,4]. A limiting factor in our efforts to discover potent and specific REL1 inhibitors has been the lack of a fast and economical enzymatic assay. Here we describe the development of a novel, fluorescence-based REL1 activity assay, compatible with high throughput screening (HTS) strategies. In this assay, 5' and 3' RNA substrates are labeled with donor and acceptor fluorophores, respectively. Guide RNA-mediated ligase activity results in a covalent linkage between the two fluorophores and therefore denaturation-resistant fluorescence resonance energy transfer (FRET). This approach has the advantage that all components can be pre-mixed and the reaction started by adding enzyme (mix-and-measure). Denaturation is achieved by heat or chemically. The assay is quantitative and robust, tolerates DMSO up to 5%. and produces a signal-to-background ratio $>$10. We determined a Z' factor of 0.6 in the 96-well format, indicating very good performance and suitability for HTS. [1] Schnaufer *et al.* (2001). Science 291(5511):2159-62. [2] Deng *et al.* (2004). JMB 343(3):601-13. [3] Amaro *et al.* (2008). PNAS USA 105(45):17278-83. [4] Durrant *et al.* (2010). PLoS NTD 4(8):e803.

The flagellar pocket collar: a marriage of rings

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Trypanosoma brucei, the causative agent of Sleeping sickness, is a parasitic protozoan, belonging to the kinetoplastida order of protists. The kinetoplastida have numerous important and essential single copy organelles. One of these is the Flagellar Pocket (FP), which is an invagination of the pellicular plasma membrane. This is an important organelle because it is the unique site of endo- and exocytosis and by consequence the FP is involved in mechanisms that permit parasite survival such as recycling of surface glycoproteins. However, the mechanisms underlying the biogenesis of FP are not yet elucidated. The FP presents several distinct structural sub-domains. One of them is the Flagellar Pocket Collar (FPC), which is found at the neck of the FP. The protein BILBO1 was the first characterized protein of the FPC and forms a ring structure along or within the FPC. BILBO1 is essential for FP biogenesis and cell survival (Bonhivers M. et al, PLoS Biol. 2008 May 6;6(5):e105). Screening of a *Trypanosoma brucei* genomic library using BILBO1 as bait (Hybrigenics) has identified numerous partners. Here, we focus on FPC3. By using a yeast two-hybrid system, we show that FPC3 is a partner of BILBO1 and FPC4, another FPC protein (see poster by C. Florimond). We have characterized the location of FPC3 by overexpression of GFP-FPC3 and show that it forms a ring structure that overlaps with BILBO1. We have identified and present here the domains of BILBO1 that interact with FPC3. Also, we have analyzed the growth and morphology phenotypes after overexpression of FPC3 and carried out functional studies by knock down of FPC3 using RNA interference.

Lipoamide dehydrogenase is essential for both bloodstream and procyclic *Trypanosoma brucei*

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In nearly all eukaryotes, lipoamide dehydrogenase (LipDH) is a subunit of four mitochondrial multienzyme complexes. The enzyme is involved in the energy metabolism, the degradation of branched-chain amino acids as well as the generation of methylene-tetrahydrofolate needed for DNA synthesis. Procyclic parasites multiplying in the tsetse fly, show intra-mitochondrial production of ATP. In contrast, bloodstream African trypanosomes which multiply in the mammalian host rely solely on glycolysis for energy supply. Their rudimentary mitochondrion lacks pyruvate dehydrogenase and 2-ketoglutarate dehydrogenase, nonetheless, the parasites contain LipDH. However, the physiological - if any - significance of the flavoenzyme in the infective form of *Typanosoma brucei* remained elusive. RNA-interference or the deletion of both *lipdh* alleles resulted in bloodstream cells with an absolute requirement for exogenous thymidine. In its absence, LipDH-deficient parasites showed severely altered morphology and cell cycle distribution. Most probably, LipDH is essential as a component of the glycine cleavage complex generating methylene-tetrahydrofolate for dTMP synthesis. Our data further revealed that degradation of branched-chain amino acids takes place but is dispensable. In procyclic, parasites with their fully elaborated mitochondrion, LipDH-mRNA depletion caused an even stronger proliferation defect that was not reversed by thymidine, suggesting that the primary effect might not be on the glycine cleavage complex. The thymidine concentrations needed to rescue the proliferation of bloodstream *lipdh*-/- parasites in culture were orders of magnitude higher than those in human or mouse serum. As expected, *lipdh*-/- trypanosomes were unable to survive or sustain an infection *in vivo*. These findings constitute the first prerequisites for the validation of LipDH as a putative future target molecule of drugs against African sleeping sickness.

The role of *L. Mexicana* Arginase in host infection

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Arginase catalyzes the conversion of arginine to ornithine, the first step of the polyamine biosynthetic pathway in *Leishmania*. The analysis of arginase gene deletion mutants revealed that the enzyme, located in the glycosome, is essential for promastigote proliferation in the absence of exogenous sources of ornithine or putrescine. Infectivity studies in macrophages and mice demonstrated that the Δ*arg L. mexicana* are able to salvage ornithine and/or putrescine from the host. However, compared to wild type parasites the infectivity of Δ*arg* parasites is reduced, which appears to correlate with an increased ability of the host macrophage to synthesize the potent antimicrobial nitric oxide. The mammalian host synthesizes nitric oxide from arginine, a crucial metabolite that can alternatively be converted to ornithine by the host arginase. It has been shown by several laboratories that an increased activity of host arginase I correlates with increased parasitemia and that inhibition of host arginase reduces parasite numbers in infected mice. Thus, although inhibition of parasite arginase alone is not a viable strategy, dual inhibition of host and parasite arginase may be a novel therapeutic approach. We have performed a comparative characterization of the *L. mexicana* and host recombinant arginases and a preliminary inhibitor screen. Of 37 compounds tested, several inhibited both enzymes potently while others showed selective efficacy *in vitro*. Furthermore, we are investigating the importance of the glycosomal localization for the function of arginase in promastigotes and in intracellular parasites *in vivo*.

Regulation of Trypanosoma brucei Acetyl-CoA Carboxylase, the key enzyme for initiation of fatty acid synthesis

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As GPI-anchored proteins are implicated in antigenic variation, fatty acid synthesis (FAS) could represent a novel target for therapeutic intervention. *T. brucei* can synthesize its own fatty acids by an unconventional FAS pathway. A key substrate for FAS is malonyl-CoA, which is synthesized from acetyl-CoA by Acetyl-CoA Carboxylase (ACC), and thus, ACC likely serves as one control point for regulating FAS. Specifically, we hypothesize that ACC is regulated in response to environmental lipids. Quantification of ACC protein showed increased ACC when the cells were grown in low lipid media, suggesting regulation of ACC at the level of mRNA processing, stability, and/or translation. As ACC is regulated by phosphorylation in other systems, and bioinformatic phospho-prediction showed multiple high-scoring phosphorylation sites (≥0.95), we assessed directly whether ACC is phosphorylated. We observed a phosphorylated band corresponding to ACC after metabolic labeling with [32P]orthophosphate of ACC-myc cells followed by immunoprecipitation of ACC-myc and autoradiography and SA-HRP blotting. [32P]phospho-labeling of ACC-myc cells grown in low, normal, and high lipid media demonstrated a 500% increase in phosphorylation of ACC-myc in high lipid media and an 80% reduction in low lipid media. In addition, ACC enzyme activity was higher in cells grown in low lipids than high lipids. Taken together, this data is consistent with our model that T. brucei ACC is dynamically regulated in response to the environmental lipid supply. Further, ACC appears to be phosphorylated under conditions when the environmental supply of lipids is abundant and FAS therefore should be down-regulated. Currently, we are examining the direct effect of phosphorylation on ACC activity.

Histone H1 plays a role in silencing VSG expression sites in *Trypanosoma brucei*

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The chromatin of trypanosomes is unusual in that it is less condensed than that of higher eukaryotes. It also does not form 30 nm fibers, structures that are typically established by the linker histone H1. Kinetoplastid organisms have atypical histone H1 proteins that lack the globular domain thought to interact with nucleosomes. We have investigated the role of these truncated histone H1 proteins in *Trypanosoma brucei*. *T. brucei* contains five histone H1 genes, which are very similar in sequence. The properties of these H1 proteins are distinct from those of core histones, as H1 is more easily dissociated from chromatin. *T. brucei* histone H1 proteins localize to the nucleus, but appear relatively absent from the nucleolus. In chromatin immunoprecipitation (ChIP) experiments, histone H1 is depleted from areas transcribed by RNA polymerase I (pol I), such as the active VSG expression site. In contrast, H1 is abundant in non-transcribed regions and, surprisingly, in regions transcribed by RNA pol II. To determine the function of histone H1 in trypanosomes, we used RNAi to block histone H1 synthesis in both procyclic and bloodstream form cells. Although induction of histone H1 RNAi results in only a slight growth defect, micrococcal nuclease sensitivity assays indicate global changes in chromatin structure. In addition, depletion of histone H1 results in 6-8 fold derepression of silent VSG expression sites. This result is consistent with changes in MNase sensitivity in regions downstream of the expression site promoter after blocking synthesis of H1. These data suggest that the unusual histone H1 proteins in *T. brucei* play a role in higher order chromatin structure and contribute to VSG expression site control.

Characterization of Major Surface Proteases of *Trypanosoma rangeli*

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Trypanosoma rangeli is a non-pathogenic protozoa which is capable of infecting humans. Parasite populations can be divided in two groups, based on the presence (KP1+) or absence (KP1-) of KP1 kDNA minicircles. Major Surface Proteases (MSP or gp63) are glycoproteins present in the cytoplasmic membrane of trypanosomatids. Recently, we have described by Genome Survey Sequence (GSS) analysis that *T. rangeli* possesses MSP sequences. Thus, the aims of this work are: (i) generate and analyze T. rangeli MSP sequences; (ii) investigate the genomic organization of these sequences and (iii) determine the expression of MSP transcripts in *T. rangeli*. Twelve T. rangeli MSP sequences were rescued from a GSS library. Sequences had 38% to 61% of sequence identity with *T. cruzi* MSP proteins. The HEMAHALG motif, previously associated with the catalytic site of Leishmania MSPs, was found in two of these sequences. Primers were designed in order to amplify a fragment encompassing the predicted catalytic site of the protease, which was used as a probe in Southern blot hybridizations. These experiments revealed that *T. rangeli* possesses multiple copies of MSP genes, however, no clear differences were observed between the two parasite groups regarding MSP sequences. MSP expression was investigated by reverse-transcriptase PCR using the primers described above. Total RNA samples were obtained from epimastigote forms of *T. rangeli* and *T. cruzi* strains. The specific 212-bp amplicon was detected in all *T. rangeli* strains. No specific amplification was observed in the *T. cruzi* strains. Information generated from the characterization of the MSP gene family in a non-pathogenic trypanosome can be useful for the understanding of the mechanisms that determine pathogenicity in other trypanosomatids.

¡B¿*Trypanosoma Brucei* RAP1: A key Regulartor for*VSG* Expression¡B¿

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Trypanosoma brucei uses antigenic variation to evade host immune defense. In mammalian host, bloodstream form (BF) *T. brucei* regularly switches its surface antigen, Variant Surface Glycoprotein (VSG), which is exclusively transcribed from the VSG Expression Sites (ESs) located at the subtelomeric loci. Monoallelic expression of *VSG* from only one ES ensures the effectiveness of antigenic variation. While in the mid-gut of its insect host, procyclic form (PF) *T. brucei* expresses procyclins as its surface molecules and all *VSGs* are silent. *VSG* expression is therefore dynamically regulated and is essential for *T. brucei* pathogenesis and normal development. Telomeres are nucleoprotein complexes located at ends of linear chromosomes. They maintain chromosome stability and are essential for genome integrity. In addition, telomeres often form a specialized chromatin structure that influences transcription of genes located nearby. In fact, we have shown recently that tbRAP1, an integral component of the *T. brucei* telomeric complex, is required for normal subtelomeric *VSG* silencing in both BF and PF cells. We now have found that depletion of tbRAP1 led to less tightly packed chromatin at the de-repressed ESs compared to silent ones. Furthermore, in bloodstream form cells, tbRAP1 preferentially associates more with silent ES marked telomeres compared to the active ES marked telomere. Thus it is likely that tbRAP1 acts similarly to its yeast homolog and mediates *VSG* silencing through modulation of local chromatin structure.

Tsetse Midgut Serine Protease Inhibitors (SERPINS) Protect African Trypanosomes from the Action of Serum Complement

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Trypanosoma brucei alternate its life cycle between a vertebrate host and the tsetse. When an infected bloodmeal is ingested by the fly, short-stumpy bloodstream form (BSF) trypanosomes quickly transform into the procyclic form (PCF) within the midgut. While BSF survive in the mammal using antigenic variation and are partially resistant to serum complement (SC), PCF are highly susceptible to the action of SC. This observation is puzzling considering that tsetse flies feed every two days and therefore, PCF trypanosomes should be in close contact with a fresh source of SC after every bloodmeal. Thus, African trypanosomes must have developed ways to avoid the lethal effect of SC in order to establish a midgut infection. Here we show that PCF form *Trypanosoma brucei* exploit a series of tsetse fly Serine Protease Inhibitors (SERPINS) to escape vertebrate SC. *In vitro* assays showed that both cultured and midgut extracted procyclics display a similar pattern of mortality when exposed to SC, which is reverted in the presence of cobra venom factor (specifically inhibits SC). SC is quickly ($\sim$1h) inactivated within the fly midgut, though this does not eliminate SC killing as a barrier to trypanosome establishment within the fly as transforming BSFs acquire SC susceptibility quite rapidly. RNAi of several *Glossina m. morsitans* serpin-like genes significantly diminished midgut infections, strongly suggesting that tsetse serpins may inactivate SC within the midgut. Furthermore, recombinant serpins inhibit serine proteases in vitro and are able to suppress SC-mediated killing of PCFs both *in vitro* and *in vivo*. Taken together, these results suggest that establishing procyclic trypanosomes exploit anti-complement serpin molecules within the tsetse to achieve fly infection.

¡B¿Towards the Mitochondrial Outer Membrane Proteome of *T. brucei*

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The outer mitochondrial membrane (OM) of African trypanosomes is unchartered territory. Compared to the inner mitochondrial membrane (IM), the OM is rather devoid of proteins. Nonetheless, a multitude of trafficking and routing processes are orchestrated by protein complexes residing in this barrier, e.g. protein import, metabolite exchange or lipid metabolism to name only a few. Of special interest is the question as to how tRNAs translocate across the OM, given that the mitochondrial genome of *T. brucei* lacks tRNA genes and all tRNAs have to be imported from the cytosol. The transport process is suggested to rely on protein factors, possibly involving components of the protein import machinery. Since the components of the TOM complex also remain elusive, we decided to undertake an attempt at raising a protein inventory of exclusively the OM. To that end, a method to isolate OM-vesicles from mitochondria of *T. brucei* was established. To verify, whether candidate proteins really reside in the OM, a subtractive MS-analysis is employed. The distinction of ER-membrane residents vs. genuine OM proteins proves particularly challenging in that context. Gauging from other systems (yeast, potato) the overall number of proteins located on the OM is 50 ¡ x ¡ 100, thus it is feasible to test all unknown protein factors in tRNA and protein *in vivo* import studies established in our lab.

Functional analysis of the HMGB protein TDP1 in *Trypanosoma brucei*

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The high mobility group B (HMGB) protein family constitutes a major abundant class of non-histone chromatin associated DNA-binding proteins which play a role in chromatin architecture in a wide range of eukaryotes. In *T. brucei*, the HMGB protein TDP1, which contains two HMG boxes and one DEK C terminal DNA-binding domain, was first identified as binding to VSG expression site promoter oligomer sequences. We report that TDP1 is an essential nuclear protein which could be involved in facilitating transcription. We find that blocking TDP1 synthesis using RNAi mediated knock-down results in an approximately 40% reduction in transcription of genes transcribed by RNA polymerase I. Using chromatin immunoprecipitation (ChIP), we find that TDP1 appears to be enriched on the active VSG expression site in bloodstream form trypanosomes, as well as on the promoter regions of certain pol I, II and III transcribed loci of both bloodstream form and insect form cells. Additionally, we show that the relative proportion of TDP1 on the procyclin promoter compared with the upstream spacer and downstream EP1 genes is greater in insect form compared with bloodstream form trypanosomes. Lastly, we performed tandem affinity purification (TAP) on TDP1 followed by mass spectrometry and found that TDP1 interacts with the core histones. These results indicate that TDP1 is an architectural chromatin protein important for transcription control in ¡i¿ T. brucei¡/i¿.

Molecular analysis of pentamidine transporters in *Trypanosoma brucei*

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Three transporters of pentamidine are known in *Trypanosoma brucei*: the AT-1/P2 transporter, the high affinity pentamidine transporter (HAPT1) and the low affinity pentamidine transporter (LAPT1) [1]. Whilst the gene for the AT-1/P2 transporter was identified in the 1990s [2], the genes for HAPT1 and LAPT1 are still to be elucidated. We are investigating the gene(s) encoding HAPT1 in *T. brucei*. Candidate genes were identified through analysis of sequence homology to AT-1/P2 and our investigation focused on the gene most phylogenetically similar to AT-1/P2, designated AT-E [3]. Sequence analysis revealed two alleles for this gene, with expression data indicating that only one allele encoded a fully functioning transporter. To investigate the function of AT-E several avenues have been completed. RNA interference (RNAi) was used to assess impact on transporter activity, along with expression of AT-E in pentamidine resistant lines to determine whether this returned sensitivity to pentamidine and expression of AT-E in a yeast background to evaluate pentamidine uptake. Finally, null mutants for AT-E are being produced and their characteristics investigated. An updated model for pentamidine uptake in *T. brucei* will be presented. [1] de Koning, H.P. (2001) Molecular Pharmacology 59, 586-592 [2] Maser, P., Sutterlin, C., Kralli, A., and Kaminsky, R. (1999) Science 285, 242-244 [3] deKoning, H.P., Bridges, D.J., and Burchmore, R.J.S. (2005) FEMS Microbiology Reviews 29, 987-1020

Iron-y of fate: Role of Iron in differentiation of *Leishmania amazonensis*

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Trypanosomatid parasites of the genus *Leishmania* alternate between mammalian and insect hosts during their life cycle as amastigote and promastigote forms respectively. The stage-specific differentiation process involves drastic phenotypic and metabolic changes orchestrated at post-transcriptional and translational levels, in response to environmental cues like temperature and pH. Earlier, the Andrews laboratory reported that availability of iron plays a critical role in the *Leishmania* life-cycle, as expression of the iron transporter LIT1 is crucial for growth of *L.amazonensis* inside macrophages. *LIT1*-/- parasites failed to establish *in vitro* infections of macrophages and of BALB/c mice *in vivo*. To examine the role of iron in *Leishmania* virulence, currently we have formulated a promastigote growth media specifically depleted in iron and found that expression of *LIT1* and several other genes associated with iron homeostasis are upregulated in absence of iron. We also found that iron depleted condition slows growth rate of wild type *L. amazonensis* and promotes development of aflagellate parasite forms, similar to that observed during *in vitro* differentiation of promastigote to amastigote forms, and express amastigote stage-specific markers. *LIT1* null mutant parasite growth is not affected by iron depletion; cells continue to grow at normal rates but suddenly crash as the culture reaches stationery phase concentrations. No change in cell morphology was observed for *LIT1* null mutants suggesting that the ability to import iron could play an important role in stage-specific differentiation. We are currently exploring the molecular mechanism that responds to the availability of iron in the environment and directs the fate of *Leishmania* cells during differentiation.

The A600.1 protein is localized in the mitochondria of *Leishmania mexicana*

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The leishmaniasis are a spectrum of globally widespread diseases that range from skin lesions to disfigurement and fatal systemic infection. The *Leishmania* parasite has a dimorphic life cycle: promastigotes are transmitted by a bite of an infected female sandfly, and are transformed into amastigotes residing in macrophages of the human host. Differentiation of promastigotes to the amastigote stage involves significant morphological and biochemical changes; however, most genes of *Leishmania* parasites are constitutively expressed in both life stages. An exception is the A600-gene family that includes genes specifically expressed in the amastigote form that is present in several *Leishmania* species and is essential for survival in macrophages. Given their stage-specific expression and potential role in virulence, an A600-deficient mutant might have a potential role as a vaccine candidate. In *L. mexicana* the A600-family comprises four homologous genes of unknown function, namely *A600.1, A600.2, A600.3*, and *A600.4*. Towards the elucidation of the function of A600 proteins, the aim of this study was to determine the subcellular localization of the gene product of *A600.1* in *L. mexicana*. We describe the construction of pAM1, a transfer vector for expressing target proteins in-fusion with GFP separated by a linker fragment. Using pAM1, we generated an *A600.1- gfp* construct that was transfected into *L. mexicana* wild type cells. Expression of *A600.1-gfp* in *L. mexicana* was confirmed by flow cytometry and immunofluorescence microscopy. Furthermore, we observe co-localization of A600.1-GFP with MitoTracker Red CMX Ros, indicating that A600.1 is localized in the mitochondria or the mitochondrial membrane of the *Leishmania* parasite. Studies are ongoing to determine the function of the A600 gene family in *L. mexicana*.

Development and optimization of RNAi tools for *Leishmania* (Viannia)

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RNA interference (RNAi) is a powerful tool well suited for genetic analysis in trypanosomatids. We previously demonstrated that *Leishmania braziliensis* shows good RNAi activity, enabling functional genetic tests (Lye *et al.*, *PLoS Pathogens* 2010). As stem-loop (StL) constructs generally give stronger knockdown phenotypes we have focused on these. First we investigated the effect of stem-length at the hypoxanthine-guanine phosphoribosyltransferase (*HGPRT*) locus. We found that reductions in HGPRT protein levels increased linearly as a function of stem length, from 3-fold for a 495 nt stem to 11-fold for a 1005 nt stem. This suggests that when not compromised by off-target effects, bigger stems are better. Secondly we developed vectors facilitating generation of StL constructs using Gateway (Invitrogen®) technology. Following introduction of a 'stem' into a donor plasmid by TA cloning, StL constructs can be generated efficiently in a single step using our pIR-GW vectors. Lastly, since independent RNAi transfectants can show a range of phenotypic strengths, we incorporated a counterselection that facilitates recovery of clonal lines with stronger and more uniform RNAi phenotypes. We introduced an adenosine phosphoribosyl transferase (*APRT*) StL construct and showed that this rendered parasites resistant to 4-aminopyrazolopyrimidine (APP), which is metabolized by APRT to a cytotoxic form. APRT-StL transfectants showing enhanced RNAi activity should express less APRT and show elevated resistance to APP. When tested simultaneously with a Luciferase StL RNAi reporter, selection for APP resistance resulted in up to 7-fold less Luciferase (stronger RNAi). We are now testing whether this approach will increase the efficiency of RNAi against endogenous *L. braziliensis* genes.

SUMOylation in the VSG Expression site Chromatin

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In *Trypanosoma brucei* the variant surface glycoprotein (VSG) gene is transcribed by RNA polymerase I (RNAPI) instead of RNAPII. We previously reported that transcription mediated by RNAPI in trypanosomes requires TbRPB7, a dissociable subunit of the RNAPII complex (Peñate, *et al*., 2009). We have investigated the TbRPA1 and TbRPB7 occupancy profile along the active VSG-expression site (ES) by Chromatin IP. ChIP analysis by qPCR showed that TbRPB7 associates in vivo with the VSG-ES, similar to TbRPA1. To identify TbRPB7-interacting proteins, a two hybrid assay was performed. This approach detected several putative interacting proteins, including a protein with a conserved E3 SUMO ligase domain (MIZ/SP-RING), named TbSIZ. Depletion of TbSIZ reduced the nuclear signal of SUMO-conjugated proteins analysed by IF using an anti-SUMO antibody. The reporter activity of a luciferase gene inserted downstream of the active VSG promoter decreased upon TbSIZ RNAi, without significant changes in a RNAPII-driven reporter activity. Next, we determined the occupancy of sumoylated proteins within the VSG-ES chromatin by ChIP using an anti-SUMO antibody. Sumoylated proteins were detected along the active VSG transcription unit but not in silent VSGs. Sumoylation of chromatin-associated proteins was not significant at the ribosomal DNA, the SL promoter, the tubulin or the U2 loci, suggesting this is a distinct feature of the VSG-ES. Interestingly, conserved sequences upstream of VSG-ES promoters are highly enriched in sumoylated proteins, suggesting that sumoylation is involved in the regulation of the VSG-ES.

TrypanoCyc: a community effort towards the development of a metabolic pathway database for Trypanosoma brucei

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Linking biochemical data to the reference genome for *Trypanosoma brucei*, the aetiological agent of Human African Trypanosomiasis, is important for comparative genomic and metabolomic studies and for investigating *T. brucei* biology and the disease it causes. TrypanoCyc is the metabolic pathway database for *T. brucei*, and will be an invaluable resource for detailed analyses of the metabolic network in

this organism, as well as cross species comparisons with other kinetoplastids. The TrypanoCyc database was initially built from the genome sequence of Trypanosoma brucei, using a collaborative web platform (TrypAnnot), based on an annotation published by GeneDB at the Wellcome Trust Sanger Institute. The pathway-tools software that generated the initial automatic genome-based reconstruction indicate the presence of a pathway if just a few enzymes associated with the classical pathway are noted. Furthermore, trypanosome-specific pathways were only included if deposited in the Metacyc repository. Therefore, post-construction manual curation is essential to generate an accurate depiction of the metabolome. This has been a collaborative effort, and involved removing errors, correcting automated predictions, and adding information from the literature. Initially, pathways were annotated according to their presence, sub-cellular localisation and stage-specific expression. A further round of annotating focused on individual enzymes. Ongoing curation will be based on public sources, literature searches, and results of experimental and bioinformatics studies. These metadata will allow users to generate tailor-made metabolic networks (e.g. metabolic network taking place in procyclic's mitochondria). All genes in TrypanoCyc are linked to the corresponding entry in GeneDB (Wellcome Trust Sanger Institute) and TriTrypDB (EuPathDB). The current stage of the annotation process is available on the internet at www.metexplore.fr/trypnets.

Global gene expression changes in *Trypanosoma cruzi* under nutritional and hyperosmotic stresses

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Trypanosoma cruzi epimastigotes are subjected to nutritional and osmotic stresses during their development in the insect vector. There is a very considerable increase in osmolarity in the lower digestive tract of the insect. Osmolarity increases slightly from the feces to the urine, from 320 to 410 mosmol.Kg^{-1}, but there is a very strong increase in the yellow rectal content, up to 1,000 mosmol.Kg-1 [Kollien and Schaub., *Parasitol. Today* 161, 381-7, 2000]. Starvation conditions are also predominant in the rectum and simulation of these conditions (transfer of epimastigotes into triatomine artificial urine (TAU) supplemented with amino acids, bicarbonate, and glucose, TAU3AAG) has been used to increase metacyclogenesis in vitro [Contreras et al., *Mol. Biochem. Parasitol*. 161, 315, 1985]. Here we report global gene expression analyses under amino acid starvation conditions using DNA microarrays that show up-regulation of genes encoding proteins involved in amino acid transport and mobilization, suggesting a response to amino acid starvation. A genome-wide transcriptional analysis of *T. cruzi* epimastigotes submitted to hyperosmotic stress (800 mOsm) resulted in up-regulation and down-regulation of the expression of a number of genes, which depend on the incubation conditions (culture medium or starvation buffer). Several of these changes were confirmed by northern blot analyses. These results reveal that despite the lack of gene regulation at the level of transcription initiation, there is extensive regulation of mRNA abundance associated with exposure of epimastigotes to different environments. *T. cruzi* reveals a notable plasticity in its adaptation to different environments and transcript abundance appears to be an important level of gene expression regulation.

Inhibition of trans-splicing causes an unexpected cytoplasmic response

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In yeast, a small fraction of unspliced mRNAs leak into the cytoplasm; this fraction can be enhanced in the absence of functional nuclear retention proteins (Mlp1, Pml1, Esc1, Pml39). Cytoplasmic unspliced mRNAs can enter translation and are degraded by cytoplasmic decay pathways. Trypanosomes process mRNAs by trans-splicing and small amounts of incompletely spliced oligocistronic RNAs are constitutively present. Nuclear retention mechanisms for incompletely spliced RNAs in trypanosomes are unknown. The finding that a fraction of the unspliced RNAs were detectable in cytoplasmic fractions prompted us to investigate the cytoplasmic response to inhibition of trans-splicing. We observed the formation of a novel type of cytoplasmic granule close to the periphery of the nucleus. The granules contain all proteins identified in P-bodies so far, as well as PABP2 and the trypanosome homologue to the RNA helicase VASA, but no proteins from early RNA processing pathways were detected. RNA was not detectable by oligos antisense to the miniexon, poly(A) or tubulin mRNA. The granules differ from P-bodies in several ways: (i) they are dependent on active transcription, (ii) they are not dissipated by cycloheximide, (iii) they form in cells depleted of the core P-body component SCD6 and (iv) they do contain additional proteins. It is possible that the nuclear periphery granules are involved in quality control of newly transcribed mRNAs, aiming to prevent translational entry of unspliced mRNAs. They resemble perinuclear germ granules in their location, their dependency on transcription and the presence of VASA: Germ granules have been suggested to determine the fate of newly transcribed RNAs in *C. elegans*.

The Quest for Endonucleases Causing Instability of Procyclin mRNAs

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EP and GPEET procyclin are the major surface proteins of procyclic forms of *T. brucei*. Expression of the two proteins is differentially regulated - GPEET is expressed exclusively by early procyclic forms while EP is expressed by both early and late procyclic forms in tsetse. Trypanosomatids are (in)famous for their reliance on post-transcriptional regulation and for the great complexity this can assume. EP and GPEET share three elements in their 3' UTRs that regulate mRNA stability and translation. An element known as the 26mer destabilises procyclin mRNAs in bloodstream forms, and to a lesser degree in procyclic forms. An additional element, known as the glycerol-responsive element (GRE), is unique to GPEET. Although GPEET is transcribed in late procyclic forms, the GRE acts as an RNA destabilising element at this stage. We are attempting to identify the endonuclease(s) cleaving the procyclin 3' UTRs by two approaches. First, we are trying to purify them from trypanosome extracts, and we have established an assay to detect nucleolytic activity in the different fractions. As a second approach, we are targeting selected candidate nucleases by RNAi and monitoring their influence on EP and GPEET expression. Several RNAi clones have been generated and are currently being analysed.

Characterization of the membrane kinases of *Trypanosoma brucei*

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Unlike mammalian cells, *Trypanosoma brucei* possesses few predicted transmembrane protein kinases. Bioinformatic analysis revealed nine such predicted proteins, representing less than 10% of the kinome. The predicted structures for seven of the kinases suggest that most have only small domains or hairpins on the opposite side of the membrane from the kinase domains, raising the question of whether the trans-facing regions are capable of interacting with ligands or other proteins or if transmembrane domains serve primarily to anchor the kinase to a specific site. The seven kinases studied by our group thus far localized to diverse structures within the parasite, indicating that they have varied cellular functions. One modulates the biogenesis of lipid droplets, organelles that function in intracellular lipid homeostasis. Four kinases localized to the endoplasmic reticulum. A sixth kinase showed developmentally-regulated localization, with the tagged protein lysosomal in procyclic cells, but residing in the ER and a number of small vesicles in bloodstream forms. The seventh kinase resides in the flagellar membrane and pocket. We had no difficulty expressing this kinase in bloodform cells but could only detect the exogenously expressed kinase in approximately 0.01%of procyclic cells suggesting that its expression may be developmentally regulated at the protein level. Another kinase also localized to the flagellar pocket by other workers (Moraes et al 2007). Unlike most eukaryotic transmembrane kinases which are present on the plasma membrane, the majority of T. brucei membrane kinases are present on intracellular organelles and thus likely function in intracellular regulatory processes. However, the two protein kinases localized to the flagellar pocket and/or flagellum may be positioned to interact with the host milieu.

Antigenic variation in *T. brucei*: Simultaneous VSG expression from two telomeres and a search for a VSG negative regulatory sequence

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The DNA sequences and mechanisms that maintain monoallelic VSG expression remain largely mysterious. We are using artificial mini expression sites (aESs) to explore the mechanisms underlying the primary negative and positive controls. We find that aESs are efficiently silenced at a chromosome-internal locus yet competent for expression at a telomere. Telomeric aESs can escape the silencing mechanism and be co-expressed with the native active ES. They can also be subject to silencing or, when active, can silence the previously active ES in trans. Thus, aESs display the key properties of native ESs in relation to expression, silencing and allelic exclusion. Our current favoured hypothesis, different in some ways from other models (see [1]), invokes positive control by the telomere and negative control by a VSG-associated sequence; other factors could reinforce silencing once established. aESs must now be examined in different genomic contexts and the system should allow us to identify the putative VSG-associated negative regulatory sequence. [1]: Horn D, McCulloch R. (2010) Molecular mechanisms underlying the control of antigenic variation in African trypanosomes. Curr Opin Microbiol. 6:700-5.

The affect of Telomere length on antigenic variation in *Trypanosoma Brucei*

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The ends of chromosomes are inherently fragile. Telomeres are structures of DNA and protein, maintained by the telomerase enzyme (TERT), that protect chromosome ends from damage. Many genomes are organized such that essential genes reside near the centromere, whereas those that can tolerate diversification are closer to the telomere. An ideal example is the African trypanosome, *Trypanosoma brucei*. Expression of variant surface glycoprotein (VSG) is essential for *T. brucei* growth and survival, yet this coat is highly immunogenic. Therefore, persistent infection in the host is dependant upon the ability of trypanosomes to vary their VSG expression, a process termed antigenic switching. The bloodstream form VSG expression sites (ES) are located in dedicated subtelomeric regions, in which the VSG encoding gene is the most proximal element to the telomeric repeats. VSG switching occurs primarily via duplicative gene conversion, an event that is likely precipitated by the formation of a DNA break. Furthermore, natural isolates of *T. brucei* have shorter telomeres and higher frequencies of antigenic switching than laboratory adapted strains. Based on these observations, we hypothesized that VSG switching is mechanistically linked to telomere length. Specifically, we proposed that telomeric shortening at the active ES results in increased DNA fragility and a correlative increase in VSG switching frequency. To address this hypothesis, we quantified the switching frequency of wild-type as well as congenic TERT-/- isolates of various telomere lengths. We will report our findings with regard to switch frequencies, the types of switch events precipitated by telomere breakage, and the nature of DNA breakage within the active expression site.

RNA interference of enzymes involved in parasite antioxidant defense indicates the novel antitrypanosomal compound OSU-40 acts through generation of reactive oxygen species

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Dihydroquinoline derivative **OSU-40** (1-benzyl-1,2-dihydro-2,2,4-trimethylquinolin-6-yl acetate) was synthesized in previous study as a drug candidate against human African trypanosomiasis. **OSU-40** displayed a potency [IC_{50} = 0.014 micromolar, selectivity index (SI) = 1700] close to that of melarsoprol (IC_{50} = 0.008 micromolar, SI = 1000) against *T. b. rhodesiense* in vitro. A dose-dependent increase in intra-parasitic reactive oxygen species (ROS) was detected when parasites were treated with **OSU-40** using ROS detecting reagent, CM-H_2DCFDA. Several lines of evidence support the hypothesis that **OSU-40** kills trypanosomes through the generation of ROS. Inducible RNA interference (RNAi) was utilized to down-regulate key enzymes in parasite antioxidant defense, including trypanothione synthetase (TryS) and superoxide dismutases (SODA and SODB). Following induction of RNAi, TryS RNAi-induced cells showed 4-fold reduced mRNA level, 1.4-fold lower growth rate and up to 5-fold higher sensitivity towards **OSU-40**; SODA RNAi-induced cells showed 6-fold reduced mRNA level with no effects on growth rate and up to 2-fold higher sensitivity towards **OSU-40**; SODB RNAi-induced cells showed 1.5-fold lower growth rate and up to 4-fold higher sensitivity towards **OSU-40**. Lower expression of key parasite antioxidant enzymes were thus associated with increases in sensitivity to **OSU-40**, consistent with the hypothesis that **OSU-40** acts through ROS generation. We are currently confirming the RNAi mediated reduction of TryS protein levels by western blotting. Additionally we have utilized electron spin resonance (ESR) spectroscopy to directly measure free radical formation after incubation of *T. brucei* with **OSU-40**. A dose dependant formation of free radicals was detected in as little as 30 minutes post treatment. Taken together, these studies strongly suggest that **OSU-40** kills African trypanosomes through generation of ROS.

A putative mitochondrial calcium/proton antiporter is essential for both life stages of *Trypanosoma brucei brucei* and the dyskinetoplastic *Trypanosoma brucei evansi*

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Calcium cations (Ca^{2+}) notably play a role in cell signaling events that occur in the cytoplasm. The capacity of vertebrate mitochondria to uptake Ca^{2+} has also been established for almost half a century ago, stimulating ATP production in the organelle, presumably in response to these signals. In addition, the increase of the cation in the cytoplasm is mitigated by its rapid uptake into the mitochondrial matrix, which acts as a Ca^{2+} sink. The mitochondrion of trypanosomatids has also been shown to have this Ca^{2+}-uptake activity as well in a membrane-potential dependent manner, which potentially links the organelle to signaling events and Ca^{2+} homeostasis of these protists. Here, we describe the functional analysis of the *Trypanosoma brucei* ortholog of the leucine zipper-, EF-hand transmembrane protein 1 (Letm1) by RNAi. The deletion of the human homolog has been implicated in the genetic disease, Wolf-Hirschhorn syndrome, which counts seizures, growth and mental retardation among its symptoms. Recently, it has also been shown to have a role in Ca^{2+}/H+ antiport across the mitochondrial inner membrane. We show that the silencing of Letm1 in both *T. b. brucei* life stages dramatically affects growth, perhaps due to the exhibited mitochondrial swelling. In addition, membrane potential is collapsed. RNAi silencing in the dyskinetoplastic *T. b. evansi* also results in growth inhibition, an interesting observation given that this strain lacks a conventional proton gradient across the inner membrane.

Differential Preference of TAO Presequence Cleavage Site in Two Developmental Forms of *Trypanosoma brucei*

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The Trypanosome Alternative Oxidase (TAO) is a nuclear-encoded protein that functions as the sole terminal oxidase in the bloodstream (BS) form of *Trypanosoma brucei*. TAO possesses a predicted N-terminal mitochondrial targeting signal (MTS) of 24 amino acids. *In vitro* import assays of the full-length and N-terminal deletion mutants of TAO have revealed that processing of the protein is modulated differentially in two developmental forms of *T. brucei*. Deletion of the first ten amino acids (Δ10 TAO) had no affect on the import of the protein in either procyclic (PS) or bloodstream (BS) mitochondria. Contrarily, deletion of the first twenty amino acids (Δ20 TAO) hampered import in PS mitochondria. This same mutation still had no affect on import in BS mitochondria. In addition, deletion of the first ten amino acids from the N-terminus of TAO altered the requirement for mitochondrial membrane potential in BS mitochondria. Interestingly, TAO was processed to a higher molecular weight protein in BS mitochondria than in PS. However, Δ10 and Δ20 TAO were processed at a site downstream than that for the full length TAO in the BS mitochondria generating the same molecular weight protein in mitochondria from both developmental forms. Immunoprecipitation and mass spectrometry analysis are currently being conducted to determine the cleavage site of the TAO presequence in BS and PS mitochondria *in vivo*. It is possible that TAO may possess more than one cleavage site with differential preferences in the two developmental forms.

Chemical validation of GSK3 in bloodstream form *Trypanosoma brucei*

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There is an urgent need for drugs with novel mechanisms of action against human African Trypanosomiasis. Protein kinases have been largely investigated as drug targets for a variety of human diseases with many kinase inhibitors either under development or already in the clinic. A homologue of glycogen synthase kinase 3 (*Tb*GSK3 short; Tb927.10.13780) has been proposed as a potential drug target in *Trypanosoma brucei* [1]. Here we report mode of action studies on DDD85893, an ATP-competitive inhibitor that has nanomolar potency against recombinant *Tb*GSK3 short and low micromolar potency against the bloodstream form of *T. brucei*. The enzymatic and cellular activities for this compound series show a good correlation. At the cellular level, DDD85893 lacks toxicity against human MRC-5 cells. DDD85893 also showed selectivity for human GSK3beta against a panel of mammalian protein kinases. This compound has also been shown to act on target by both chemical proteomics and genetic methods. Using the Kinobeads technology [2], *Tb*GSK3 short has been identified as a DDD85893 binder from a *T. brucei* lysate with affinity in the nanomolar range. The sensitivity of this inhibitor was increased against mutant single knock-out cell lines and reduced against TbGSK3 short over-expressing cell lines. Thus, DDD85893 represents a useful chemical tool for elucidating the role of *Tb*GSK3 in *T. brucei*. [1] Ojo et al.(2008) Antimicrobial Agents and Chemotherapy [2] Bantscheff et al. (2007) Nature Biotechnology

Purification and biochemical properties of *T. brucei* PKA-like kinase

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PKA-like kinases are unusual as they are not activated by the second messenger cAMP (see poster by S. Bachmaier at this meeting). Comparison of their kinetic properties and subunit interaction *in vitro* with established data for numerous eukaryotic PKAs will shed light on the evolution of this kinase subfamily and its parasite-specific properties and functions. Heterologous expression in *E. coli*, the Baculovirus expression system (BVES) and the LEXSY *Leishmania tarentolae* expression system has been optimized to obtain soluble recombinant subunits of PKA-like kinase. The catalytic subunits PKAC1 and PKAC3 purified from the BVES had specific activities of 0,05 U/mg and 20-30 U/mg, respectively, using kemptide as substrate. PKAC3 is comparable to mammalian PKA with respect to specific activity and kinetic properties (k_M for ATP and substrate), whereas PKAC1 seems to lack full activity when produced in the BVES. The PKAR subunit is highly expressed in all three expression systems, yet it does not interact with the recombinant catalytic subunits. In contrast, the holoenzyme complex can be readily purified from *T. brucei* cells. BVES-produced PKAC3 forms functional holoenzyme complexes with hetreologous mammalian RI and RII regulatory subunits. Coexpression of *T. brucei* R and C in the BVES and in mammalian cells did not result in holoenzyme formation. Our data suggest that kinetoplastid-specific modifications of PKAR or chaperones are essential for biogenesis of a functional PKA-like kinase holoenzyme.

The Role of Poly(A)-Specific Ribonuclease in Stage-Specific 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 open reading frame of the original pre-mRNA can exhibit different steady state levels, indicating that post-transcriptional regulation is important for gene expression. Deadenylation is often the rate-limiting event in regulating the turnover of cellular mRNAs in eukaryotes. Removal of the poly(A) tail initiates mRNA degradation by one of several decay pathways, including 5' to 3' exonuclease decay and 3' to 5' exosome-mediated decay. Poly(A)-specific ribonuclease (PARN) is a key deadenylase involved in regulating gene expression in mammals, Xenopus oocytes, and higher plants. Trypanosomatids possess three different PARN genes, PARN-1, -2, and -3. Here, we show that *Trypanosoma brucei* PARN-1 is an active deadenylase in vitro. Microarray data indicates that overexpression of PARN-1 down regulates specific RNAs. mRNAs affected by PARN-1 overexpression include a family of mRNAs encoding stage-specific coat proteins. qRT-PCR of these stage-specific mRNAs confirms that PARN-1 overexpression increases the decay rate of these messages. Taken together, these data suggest that PARN-1 is a deadenylase that regulates stage-specific protein expression.

Insights into the function of the RNA-binding proteins RBP33 and DRBD3 in *Trypanosoma brucei*

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*Tb*RBP33 and *Tb*DRBD3 were identified in our lab by affinity chromatography using a U-rich RNA element as bait. Both proteins are essential for viability in boodstream and procyclic trypanosomes. *Tb*RBP33 is mainly nuclear and binds to a subset of mRNAs enriched in transcripts encoding protein kinases. *Tb*DRBD3 locates to the cytoplasm but is not homogeneously distributed. It is found in a complex with poly(A)-binding proteins and ribosomal proteins, among others. Both the intracellular localization and the composition of the complex are altered upon nutritional and oxidative stresses. These results are helping us to gain insight into the role of *Tb*RBP33 and *Tb*DRBD3 in the regulation of gene expression in trypanosomes.

Tbp27, a novel component of the cytochrome c oxidase complex in *Trypanosoma brucei* procyclic forms

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A trypanosomatid specific mitochondrial inner membrane protein, Ldp27, is essential in the survival of *Leishmania* amastigotes in host cells. The presence of Ldp27 correlates with increased cytochrome c oxidase (COX) activity. One of the proteins identified in a recent analysis of the components of the COX complex in the closely related, *T. brucei*, is the homologue of p27 (Tbp27). We demonstrated that Tbp27 is localized to the mitochondria as in *Leishmania*. The expression level of Tbp27 is substantially higher in the procyclic form than the blood stream form, correlating with the known higher COX activity in the insect stage of *T. brucei*. The higher p27 expression in the insect stage of *T. brucei*, in contrast to the lower expression in the insect stage of *Leishmania*, must reflect the difference of the nutritional environment of the tsetse fly vector compared to the gut of the sandfly where *Leishmania* resides. RNA inhibition mediated knockdown of Tbp27 in procyclic cells eventually stops growth compared to un-induced cells. Depletion of Tbp27 destabilized the COX complex indicated by the reduction in COX IV and COX VI components after RNAi, unlike in *Leishmania* where loss of p27 does not destabilize the other components of the COX complex. A functional effect of RNAi depletion of Tbp27 is further demonstrated by the loss of mitochondrial membrane potential indicated by FACS analysis of TMRE stained cells. The data suggest that the p27 protein may play a role in enhancing COX activity in the procyclic form of *T. brucei* as seen in the amastigote stage of *Leishmania*.

Investigating the roles of HASPB and SHERP during *Leishmania* metacyclogenesis in the sandfly

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Leishmania is transmitted to a mammalian host by sandfly bite. After uptake of intracellular amastigotes during blood feeding, *L. (Leishmania) spp.* develop within the sandfly midgut, a process termed metacyclogenesis, to generate mammalian infective metacyclic promastigotes. Recently, we have used null and complemented parasite mutants to show that the LmcDNA16 locus (~11.5Kb on chromosome 23 of *L. major*) is essential for completion of metacyclogenesis in the insect vector (Sadlova *et al.*, 2010, Cellular Microbiology 12:1765). This locus contains 2 classes of genes (the HASPs and SHERPs) coding for proteins of unknown function. Studies using the null locus mutant complemented by over-expression of each gene individually suggested that HASPB plays a major role in this process. To investigate further, individual replacement mutants for HASPB and SHERP, designed to support expression of each gene at the correct developmental stage and at wild type level, were generated and characterised. SHERP replacement lines expressed the protein product at a barely detectable level, while HASPB lines did express the protein product at the correct developmental stage, but did not appear to transport it to the cell surface as in wild type parasites. Neither of the clone types were compromised in their development in *in vitro* culture, but when used to infect the sandfly vector, development of the HASPB lines rarely passed the nectomonad stage, while the stomodeal valve remained largely uncolonized (indicative of defective parasite transmission). These data suggest that HASPB cannot recover metacyclogenesis on its own *in vivo*.

The *Leishmania donovani* glycosomal protein peroxin 14 hydrophobic region is necessary for membrane association

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Leishmania and other kinetoplastids segregate several metabolic pathways into a specialized organelle named the glycosome. Proteins destine for the glycosome contain a PTS1 or PTS2 targeting signal sequence that is recognized by the receptors peroxin 5 (LdPEX5) and peroxin 7 (LdPEX7), respectively. These receptors traffic newly synthesized proteins to the surface of the glycosome where they dock to peroxin 14 (LdPEX14), an event that facilitates translocation of proteins across the glycosomal membrane. Genetic studies have demonstrated that the glycosome biogenesis machinery is essential for parasite viability and represents a potential therapeutic target. *L. donovani PEX14* (LdPEX14) is crucial for the translocation of PTS1 and PTS2 proteins across the glycosomal membrane. LdPEX14 has been shown to be a peripheral membrane protein that in solution adopts an oligomeric structure that is maintained by both a hydrophobic and coiled-coil domain. Structural and biophysical analysis of LdPEX14 showed that the binding of LdPEX5 causes a dramatic conformational change in the LdPEX14 complex, which is accompanied by the reorganization of the hydrophobic segment. Here we show, using liposomes mimicking the glycosomal membrane composition that LdPEX14 binds to this membrane only when acidic phospholipids are incorporated into the lipid bilayer. Membrane-associated LdPEX14 recruits the PTS1-LdPEX5 and PTS2-LPEX7 complex to liposomes. Further investigations indicate that LdPEX14 penetrates the phospholipid bilayer, and is protected from limited proteolysis. Leakage experiments with carboxyfluorescein-loaded liposomes showed that insertion of the hydrophobic region caused release of this fluorescent dye. Deletion of the LdPEX14 hydrophobic segment completely abrogated the membrane binding activity. These studies suggest that LdPEX14 may be involved in formation of a transmembrane pore through which glycosomal matrix proteins are imported.

In vitro analysis of *Trypanosoma brucei gambiense* adaptation to different mammal sera

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Trypanosoma brucei gambiense is the main etiologic agent of African sleeping sickness. Whilst it is widely accepted to be an anthroponotic disease, recent data strongly suggest an epidemiological role of fauna in infection maintenance. We have recently reported the occurrence of *T. b. gambiense* in goats and sheep blood obtained in Equatorial Guinea. Despite long-standing control strategies, complete elimination of human cases was never accomplished, suggesting the presence of other possible reservoirs for *T. b. gambiense*. To address whether *T. b. gambiense* is able to proliferate in different reservoirs, we analyzed, under controlled *in vitro* conditions, the growth of the parasite in several animal sera such as cattle, swine, sheep and goat, and compared with growth in human serum. We show that *T. b. gambiense* (ELIANE strain), is able to grow in vitro in all different mammal sera tested, although the growth dynamic varied between animal sera. We developed mAb against the expressed VSG, which allow us to investigate by flow-cytometry and immunofluorescence whether serum adaptation is associated with VSG switching. Antigenic analysis of sera-adapted cell lines consistently showed the occurrence of different VSG variants, suggesting that bloodstream expression-site associated factors may confer selective advantages to growth in different host sera. We are currently analysing the sequences of the BES promoter region, ESAG2, ESAG6/7 and VSGs of these adapted cell lines. Importantly, VSG switching is not associated with the loss of human serum resistance, either at population or at clonal level, confirming this is a stable feature. These findings suggest that *T. b. gambiense* retains the capacity to infect humans after being maintained in local fauna.

Towards the elucidation of the host-pathogen interactome of *Trypanosoma cruzi*.

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We have established a robust high-throughput platform for screening protein-protein interactions between trypanosomatid and human proteins. In order to investigate a possible role for *T. cruzi* mucin-associated surface proteins (MASPs) at the host-pathogen interface, we have included MASPs as baits in our initial screens against the human proteome. MASPs are members of a multigenic family recently identified during the genomic sequencing. This family contains around 1,400 members, consisting of approximately 6% of the diploid genome. Highly conserved N- and C-terminal domains, which encode a signal peptide and GPI-anchor addition site respectively, and a hypervariable central region, characterize MASP. Members of this family are predominantly expressed in trypomastigotes. Yeast two-hybrid screens identified a few human interacting partner proteins including Snapin, a member of the SNARE complex proteins, which may have a role in a calcium-dependent exocytosis. The MASP-Snapin interaction was further validated using *in vivo* co-Affinity Purification and *in vitro* pull-down assays. Immunofluorescence assays showed Snapin is recruited to the parasite surface during invasion. Colocalization study indicates that Snapin is associated with late endosomes and lysosomes. Supporting our initial hypothesis, Snapin depletion using siRNAs in HeLa cells and Snapin knockout (KO) in Mouse Embryonic Fibroblast (MEF) cells significantly inhibited *T. cruzi* invasion, suggesting the participation of Snapin in this process. In addition, lysosomes display aberrant morphology in Snapin KO MEF cells and the parasites are not able to recruit lysosomes efficiently when compared to wild-type cells. The link between *T. cruzi* MASP and human Snapin in the host cell invasion and survival remains under investigation, however we have launched large-scale screens against the human proteome with a subset of trypanosomatid proteins.

Identifying new drug targets against African Trypanosomiasis

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Sleeping sickness, a parasitic disease caused by *Trypanosoma brucei spp.*, affects 60 million people in Africa. Current drug therapies are inadequate, hard to administer and have increasing drug-resistance problems. Hence, there is an urgent need for new drugs. Currently, there are only few drug targets known in *T. brucei*. To identify and validate new targets for drug discovery we are systematically analyzing 200 genes coding for enzymes involved in different biochemical and signaling pathways by RNAi knockdown or gene knockout. Of 65 genes analyzed, 15 were found to be essential for *in vitro* growth of *T. brucei* bloodstream form. Of these, 7 are involved in translation/transcription, 3 are involved in carbohydrate metabolism, and 5 are kinases or phosphatases. Since protein translation has been a successful drug target in many microorganisms, and since aminoacyl-tRNA synthetases (AARS) play an important role in this process, we decided to further investigate AARS. *T. brucei* has 26 genes encoding AARS, which are responsible for charging a specific amino acid to a cognate tRNA. RNAi against 5 AARS resulted in growth inhibition of *T. brucei* bloodstream form *in vitro*. Furthermore, RNAi against isoleucyl-tRNA synthetase rendered parasites incapable of infecting mice, indicating that AARS are an attractive drug target. For further validation of AARS as a drug target, recombinant proteins were produced and a fluorescence polarization-based assay was developed to measure AARS activity. The National Cancer Institute drug database will be used for screening for compounds that can effectively inhibit recombinant AARS and parasite growth. Overall, this work contributes toward a systematic analysis of *T. brucei* enzymes to identify and validate new targets against African Trypanosomiasis.

Human ApoL-I Variants in African Trypanosomiasis

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Humans and some primates are able to resist most species of African trypanosomes due to an innate trypanosome lytic factor (TLF). The species *Trypanosoma brucei rhodesiense* is able to evade lysis by encoding a serum resistant associated protein (SRA), which is able to bind and effectively neutralize the activity of apoL-I. It has been shown that baboons effectively resist infection by *T.b. rhodesiense* due to the change of one specific lysine on the C-terminal end of apoL-I that prevents the binding and neutralization of apoL-I by SRA. We have found a 6 bp deletion in the C-terminal end of apoL-I in the genome of some pygmies and Bantus in Africa, which causes a frameshift mutation that results in the generation of the lysine in the same position present in the baboon apoL-I. Two other point mutations have been discovered in African genomes which may help provide resistance to SRA. Hydrodynamic gene delivery of these various mutations in mice has shown, partial protection from human infective parasites and both kidney and liver damage when compared with the most prevalent human apoL-I sequence. The data show that these mutations, though contributing to resisting trypanosomes,may be toxic to cells and tissues.

Mapping the differentiation block in pleomorphic DOT1B-deficient trypanosomes

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The transition of African trypanosomes from bloodstream (BSF) to procyclic forms (PCF) involves dramatic changes in morphology and cellular functions in order to adapt to the new environment. Developmental differentiation in trypanosomes has been studied intensively and many molecular and cellular markers are available to examine this process. Although changes in chromatin structure, nuclear architecture and posttranslational histone modifications correlated with differentiation were reported many years ago, so far only few functional studies have aimed to unravel the function of chromatin structure during stage differentiation. We showed previously that deletion of the histone methyltransferase DOT1B results in an inability of BSF to differentiate to PCF. The strain used in these initial experiments is culture adapted and very convenient for reverse genetics, but initiation of differentiation is inherently inefficient and asynchronous. The differentiation process can be investigated properly in pleomorphic strains that behave like field strains of *T. brucei*. Using several markers, we could show that development to short stumpy forms and some early events during short stumpy to procyclic form differentiation are not impaired in DOT1B pleomorphic trypanosomes. However, later into differentiation, DOT1B-deficient cells undergo growth arrest and die after several days. FACS analysis showed that growth inhibition is accompanied by the appearance of cells with abnormal DNA content suggesting that nuclear integrity is disturbed. Dissection of this DOT1B phenotype should tell us more about the specific function of this histone methyltransferase during the process of differentiation in trypanosomes.

Repurposing human phosphodiesterase inhibitors as a route to developing anti-trypanosomal drugs

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One tactic for the development of new therapeutics is the repurposing of existing drug discovery programs for human enzymes to target homologous parasite proteins. Not only does it begin with a known druggable target, but such programs have produced compounds that are well characterized biochemically and pharmacologically. We employ this approach to the development of anti-trypanosomal drugs, based on inhibitors of phosphodiesterases (PDEs). PDEs are important regulators of the secondary messenger cAMP and, in humans, are the targets of highly selective drugs treating disorders such as erectile dysfunction and chronic obstructive pulmonary disorder. Double RNAi knockdown of the *Trypanosoma brucei* phosphodiesterases PDEB1 and PDEB2 in bloodstream forms are unable to divide correctly and ultimately die. We have repurposed the PDE inhibitors piclamilast, sildenafil and tadalafil as a starting point for the development of therapeutics for trypanosomiasis. We will disclose analogues with improved potency against parasite enzymes and evidence of structure activity relationships that may be relevant to the development of selective drugs. Our most potent compounds belong to the piclamilast series and show micromolar IC_{50} values with both PDEB1 and PDEB2. Additionally, we will describe these compounds' trypanocidal activity and their effects on cAMP levels.

Functional characterization of Pseudo-CTD of RNA polymerase II of Trypanosoma brucei

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The carboxyl-terminal end of all eukaryotic RNA polymerase II enzymes consists of a well recognizable domain called the Carboxy Terminal Domain (CTD). In yeast and mammals, the RNA polymerase II CTD contains tandem heptapeptide repeats, consisting of YSPTSPS, which orchestrate the essential co-transcriptional functions associated with mRNA maturation, including transcription initiation, elongation, capping, splicing and polyadenylation. These repeats undergo specific phosphorylation events, which generate a phospho-CTD code that regulates these co-transcriptional processes. In Trypanosoma brucei, RNA polymerase II lacks tandem repeats in its carboxyl terminus, replacing them with a non-repeated sequence termed as pseudo-CTD. The T.brucei pseudo-CTD is essential for viability of the organism and contains what seems to be a dynamic set of phosphoserine amino acids. Our hypothesis is that the pseudo-CTD contains a CTD-code, and by cracking this code we will understand the basic components of co-transcriptional events in these evolutionarily diverse organisms. We have established a versatile experimental setup in which cells are depleted of endogenous RPB1 (by tetracycline-regulated RNAi-mediated knock-down) and simultaneously supplied with an ectopic and tagged copy of RNAi-resistant RPB1. Using this system, we are testing RPB1 proteins that have multiple serine to alanine substitutions. Our study so far has indicated that specific sets of serine residues are indeed essential for trypanosome cell viability. Currently we are assessing which functions of RNA polymerase II are disrupted by these mutations.

Screening for downstream effectors of cAMP and PKA-like kinases in *T. brucei*

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Adenylate cyclases, phosphodiesterases and cAMP-dependent protein kinases are major components of the cAMP signaling pathway, which is highly conserved in many organisms. The abundance of these proteins in *T. brucei* suggests the presence of cyclic nucleotide signaling. However, the *T. brucei* PKA-like kinases do not react to cAMP. Manipulation of the intracellular cAMP concentration by RNAi against the ESAG4 adenylate cyclase family or the phosphodiesterases PDEB1 and PDEB2 do not influence PKA activity in an *in vivo* reporter assay. Instead, the *T. brucei* PKA-like kinases have acquired an alternative activation mechanism, induction by a drop in ambient temperature (cold shock), an established differentiation trigger in the parasite's life cycle. No homologues of the other known mammalian cAMP-responsive downstream effectors, Epac or cyclic nucleotide gated (CNG) channels, have been identified in the parasite. However, knockdown of the ESAG4 family by RNAi shows a severe growth and cytokinesis phenotype. Thus, the role of cyclic nucleotide signaling in *T. brucei* remains unknown. Currently we focus on the identification of downstream effectors of cAMP as well as PKA-like kinases. To identify substrates of PKA-like kinases we use complementary phosphoproteomic methods. Enrichment of soluble substrates was achieved by IP with a phospho-specific antibody that recognizes the highly conserved PKA target phosphorylation sequence RXXS*/T*. Phosphopeptides of the insoluble fraction were enriched by TiO2 extraction. Enriched proteins from both fractions were analysed by MS and RXXS*/T* containing phosphopeptides were identified.

Defining the *T. brucei* mitochondrial DNA-binding proteome to discover novel proteins involved in kDNA replication, organization and maintenance

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The mitochondrial genome of *Trypanosoma brucei*, kinetoplast DNA (kDNA), consists of a few thousand minicircles and a handful of maxicircles all interlocked in a single giant network. The kDNA is condensed into a disk-shape structure and connected to the flagellar basal body via a system of filaments. Replication, segregation, maintenance, and intracellular organization of kDNA requires many proteins, but the identity of many remains elusive. Here, we used a proteomics strategy to discover new kDNA-associated proteins. We fractionated soluble proteins from isolated mitochondria on a DNA affinity column and analyzed binding proteins by mass spectrometry. Among the proteins identified, we here report a previously-uncharacterized, small basic protein (Genbank accession no. XM_818029) sharing homology with H1 linker histone from insects and plants, and histone-like protein H-NS from proteobacteria. We named this protein TbKBP17. Interestingly, TbKBP17 is not conserved in *Leishmania* and *T. cruzi*. By endogenous myc-tagging, TbKBP17 was detected in the mitochondrion, where it localizes on the kDNA disk at all stages of the cell cycle. Although knock-down of TbKBP17 mRNA by stem-loop RNAi had little effect on kDNA, TbKBP17 overexpression caused rapid kDNA loss (about 2/3 of the cells had no or a very small kDNA within 4 days after induction). TbKBP17 overexpression also led to kDNA segregation defects in some cells, as shown by the presence of asymmetrically-dividing kinetoplasts and threads of kDNA stretched out between segregated networks. Studies are underway to elucidate the function of TbKBP17.

WEB PRESENTATIONS

1 TriTrypDB: A Functional Genomics Database for Kinetoplastid Parasites. ***Brunk, B.***, ***Harb, O.***, ***Logan-Klumpler, F.***, ***Rogers, M.***, *Representing GeneDB and EuPathDB, *.*

TriTrypDB: A Functional Genomics Database for Kinetoplastid Parasites

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TriTrypDB (http://tritrypdb.org) provides a one-stop Omics resource for the kinetoplastid research community, integrating genomes, annotation and functional data at a single site. TriTrypDB exploits the computational infrastructure developed for EuPathDB, integrating genomes, with annotation and analyses from GeneDB and elsewhere, and a wide variety of functional genomics datasets made available by members of the global research community, often pre-publication. These resources are supported by the Bill & Melinda Gates Foundation, the NIH (US), and the Wellcome Trust (UK). Since its first release in 2009, TriTrypDB has expanded to include genomic data from >15 kinetoplastida, along with numerous functional datasets (ie. RNA-seq, microarray and proteomics), and improved/updated annotation. Annotation has been spearheaded by GeneDB, adding/updating hundreds of annotations based on publications, TriTrypDB User Comments, and manual curation efforts. TriTrypDB allows users to examine individual genes or chromosomal spans, including syntenic alignments with other kinetoplastida. Data within TriTrypDB may be interrogated based on text terms, gene IDs, GO/EC numbers, chromosomal location, BLAST, sequence features (length/MW, gene type/structure, signal sequences, Pfam domains, user-defined motifs), predicted protein structure, epitopes, orthology, and functional information (proteomics, transcriptomics, etc.). Set operations (union, intersect and minus) allow multiple searches to be combined to refine results as a Search Strategy that can be stored under personal profiles, and shared with collaborators. New features include the ability to combine sequence-based queries with gene-based queries (e.g.

find genes flanking transcriptional strand-switch regions). Representatives from EuPathDB and GeneDB will be available throughout the meeting at the booth outside the cafeteria to provide demonstrations, answer questions, gather feedback, solicit User Comments to enhance available annotation, and discuss the availability of new datasets.

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