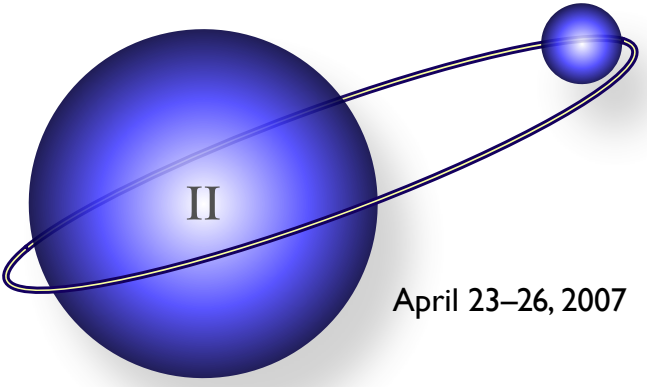
Kinetoplastid Molecular Cell Biology

An International Conference

Marine Biological Laboratory · Woods Hole · MA · USA



April 23–26, 2007

KMCBM 2007 Acknowledgements

Second Kinetoplastid Molecular Cell Biology Meeting, April 23–26, 2007

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

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

The organizer wishes to thank:

The Program Committee:

Vivian Bellofatto (University of Medicine and Dentistry of New Jersey, Newark, USA)
Markus Engstler (Darmstadt University of Technology, Darmstadt, Germany)
Michael A. J. Ferguson (University of Dundee, Dundee, Scotland)
Laurie Reed (State University of New York School of Medicine, Buffalo, USA)

The Staff at MBL:

Judith Taylor, Deb Deering and the Housing & Conferences Team for housing and registration, and the Housekeeping Staff. Chris Dematos for website design, web registration and abstract book preparation. Dave Space for payment website design and maintenance and all the staff in the Information Technology Department. Mike Baptiste for audio visual support. We also thank Cheryl Greene and Food Service Team of Sodexho at the MBL.

Cover Design: Markus Engstler

KMCBM 2007 Program

Monday, April 23

01:00 – 05:00	Arrival, Registration & Poster set–up*
05:00 – 06:45	Dinner
07:00 – 09:15	Session I: VSG Expression (chair: Ed Louis)
09:30 – 11:30	Mixer

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

PLEASE PUT UP YOUR POSTERS AS SOON AS POSSIBLE AFTER ARRIVAL

Tuesday, April 24

07:00 – 08:30	Breakfast
08:30 – 12:00	Session II: Cell Biology (chair: Jeremy Mottram)
12:00 – 01:30	Lunch
01:30 – 04:30	POSTER PRESENTATIONS Section A
05:30 – 07:00	Dinner
07:00 – 10:00	Session III: Biochemistry (chair: Deborah Smith)
10:00 – 11:30	Mixer

Wednesday, April 25

07:00 – 08:30	Breakfast
08:30 – 11:45	Session IV: Gene Expression (chair: Sergio Schenkman)
12:00 – 01:30	Lunch
01:30 – 03:30	Session V: Cell & Life Cycles (chair: Michael Boshart)
03:30 – 05:30	Free time
05:30 – 07:00	Dinner
07:00 – 10:000	POSTER PRESENTATIONS Section B
09:30 – 11:30	Mixer

Thursday, April 26

07:00 – 08:30	Breakfast
08:30 – 10:30	Session VI: Kinetoplast Structure & Expression (chair: Dan Ray)
11:00	Lunch. Lunch boxes will be available for those who would like them (numbers to be determined on Wednesday)

*** ROOMS MUST BE VACATED BY 10:00A.M. ON THURSDAY**

TALK SESSIONS: I - VI

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April 23 7:00 p.m. Chair(s) - Ed Louis

Session I: VSG Expression

1A Molecular basis of antigenic variation in trypanosomes. ***Cross, G.***

1B Structure and differential expression of the silent array *VSG* genes of *Trypanosoma brucei*. *Marcello, L.,* ***Verma, S.****, Barry, J.*

1C A novel protein complex is essential for *VSG* expression-site transcription in *Trypanosoma brucei*. *Brandenburg, J., Schimanski, B., Nogoceke, E., Nguyen, T., Padovan, J., Chait, B., Cross, G.,* ***Günzl, A.***

1D A novel ISWI is involved in VSG expression site downregulation in African trypanosomes. *Hughes, K., Wand, M., Foulston, L., Young, R., Harley, K., Terry, S., Ersfeld, K.,* ***Rudenko, G.***

1E Histone H3K76 methyltransferase DOT1B modulates monoallelic *VSG* expression in *T. brucei*. ***Figueiredo, L.****, Janzen, C., Cross, G.*

1F Telomeres control *VSG* expression site choice in African trypanosomes. ***Glover, L.****, Alsford, S., Kawahara, T., Horn, D.*

1G *T. brucei* RAP1 participate in the regulation of VSG expression from subtelomeric expression sites. ***Li, B.****, Espinal, A., Okubo, E., Figueiredo, L.*

1H A role for telomeric DNA glycosylation in the regulation of antigenic variation in *T. brucei*? ***Cliffe, L.****, Kieft, R., Sweeney, K., Birkeland, S., Sabatini, R.*

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Session II: Cell Biology

2A Current and future impact of postgenomic approaches in trypanosome cell biology. ***Field, M.***

2B Considerations of trypanosome swimming. *Heddergott, N., Stellamanns, E., Pfohl, T., Rosenhahn, A.,* ***Engstler, M.***

2C 3D ultrastructural architecture and morphogenesis of the flagellar pocket area of *Trypanosoma brucei*. ***Vaughan, S.***, *Gadleha, C., Lacomble, S., Shaw, M., Morphew, M., O'Toole, E., McIntosh, R., Gull, K.*

2D The cytoskeleton is essential for flagellar pocket biogenesis in procyclic trypanosomes. *Bonhivers, M., Nowacki, S., Landrein, N.,* ***Robinson, D.***

2E Centromere location in *Trypanosoma cruzi* and *Trypanosoma brucei*. ***Obado, S.***, *Bot, C., Kelly, J.*

Break or Coffee Break

2F Identification of host proteins that bind *Trypanosoma cruzi* alpha-galactosyl epitopes involved in host-cell invasion. *Ganiko, L., Nakayasu, E.,* ***Almeida, I.***

2G AP-3 adaptor is involved in the transport of membrane proteins to acidocalcisomes of *Leishmania*. ***Mottram, J.***, *Besteiro, S., Coombs, G.*

2H Modern methods, ancient organisms: the nuclear pore complex of the african trypanosome in an evolutionary context. ***DeGrasse, J.***, *Chait, B., Field, M., Rout, M.*

2I Hemoglobin is a necessary component of Trypanosome Lytic Factor. ***Widener, J.***, *Nielsen, M., Shiflett, A., Moestrup, S., Hajduk, S.*

2J Distinct roles of haptoglobin-related protein and apolipoprotein L-I in trypanolysis by human serum. *Vanhollebeke, B., Nielsen, M., Watanabe, Y., Truc, P., Vanhamme, L., Nakajima, K., Moestrup, S.,* ***Pays, E.***

2K Reconstitution of TLF function in transgenic mice reveals distinct contributions of each component. ***Raper, J.***, *Molina-Portela, P.*

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3A How trypanosomes make fatty acids: a new mechanism specialized for parasitism. ***Englund, P.***

3B Galactose Metabolism in *Trypanosoma brucei* . ***Urbaniak, M.****, McElroy, S., Frearson, J., Ferguson, M.*

3C *De novo* synthesis of *myo*-inositol in *Trypanosoma brucei* is a validated drug target. ***Smith, T.***

3D Restoration of virulence in a *Leishmania mexicana* glucose transporter mutant is associated with increased utilization of gluconeogenesis. ***Saunders, E.****, Ellis, M., De Souza, D., Likic, V., Landfear, S., McConville, M.*

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3E The antioxidative defence system of *Leishmania major*: two types of Tryparedoxin-dependent peroxidases. ***Konig, J.****, Fairlamb, A.*

3F Exploring iron-sulfur cluster assembly in *Trypanosoma brucei*. *Long, S., Jirku, M., Tachezy, J.,* ***Lukes, J.***

3G Linking ethanolamine to protein: biosynthetic pathway of a rare protein modification. ***Signorell, A.****, Bütikofer, P.*

3H Structure of a GPI-anchored type 1 VSG C-terminal domain of *Trypanosoma brucei*. ***Jones, N.****, Nietlispach, D., Sharma, R., Burke, D., Eyres, I., Mott, H., Carrington, M.*

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April 25 8:30 a.m. Chair(s) - Sergio Schenkman

Session IV: Gene Expression

4A The cadre of transcription factors that coordinate SL RNA expression in trypanosomes. ***Bellofatto, V.***

4B Characterizing the transcription pre-initiation complex at the SL RNA gene promoter of *Trypanosoma brucei*. ***Lee, J.***, *Nguyen, T., Günzl, A.*

4C SLS and SLD - novel mechanisms that regulate SL RNA in *Trypanosoma brucei*. *Lustig, Y., Shaked, H., Tkacz, I., Arvatz, G., Sheiner, L., Vagima, Y., Goldshmidt, H., Das, A., Bellofatto, V.,* ***Michaeli, S.***

4D RNA structural thermosensors and translation regulation in *Leishmania*. ***Shapira, M.***, *Ben David, M., Yoffe, Y., Gabdank, I., Barash, D.*

4E Development of a high-yield protein expression system based on *Leishmania tarentolae*. *Cirstea, I., Kushnir, S., Mureev, S., Breitling, R.,* ***Alexandrov, K.***

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4F RNAi is alive and well in *Leishmania braziliensis*. ***Beverley, S.***, *Owens, K., Lye, L., Shi, H., Tschudi, C., Ullu, E.*

4G Identification of a large family of short and extincted retroposons located in the 3-UTRs of *Leishmania major* mRNAs. ***Bringaud, F.***, *Müller, M., Cerqueira, G., El-Sayed, N., Papadopoulou, B., Ghedin, E.*

4H A large retroposon family is involved in the regulation of gene expression in *Leishmania*. ***Müller, M.***, *Rochette, A., Bringaud, F., Smith, M., Ghedin, E., Papadopoulou, B.*

4I Deadenylation of mRNA in *Trypanosoma brucei* . ***Schwede, A.***, *Stewart, M., Ellis, L., Carrington, M., Clayton, C.*

4J RNA granules in *Trypanosoma brucei*: unconventional heat-shock stress granules and minimal P-bodies. ***Kramer, S.***, *Ellis, L., Webb, H., Ramarao, R., Reed, J., Carrington, M.*

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Session V: Cell & Life Cycles

5A The biology of differentiation during the life-cycle of *Trypanosoma brucei*. ***Matthews, K.***

5B Identification and functional characterisation of a stumpy-specific protein implicated in citrate/cis aconitate dependent-differentiation in *Trypanosoma brucei*. ***Dean, S.***, *Marchetti, R., Kirk, K., Matthews, K.*

5C Visualizing genetic exchange in *Trypanosoma brucei* in the tsetse vector by fluorescence. ***Gibson, W.***, *Peacock, L., Ferris, V., Mick, B.*

5D Macroautophagy in *Leishmania major* is not required for metacyclic shrinkage but contributes to the metacyclogenesis. *Wang, C.*, ***Madeira, L.***, *Zhang, K., Beverley, S.*

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Session VI: Kinetoplast Structure & Expression

6A The Ins and Outs of RNA editing. ***Stuart, K.***

6B New views of kinetoplast structure. ***Gluenz, E.***, *Shaw, M., Gull, K.*

6C Functional role and regulation of universal minicircle sequence binding protein during kDNA replication and segregation. *Milman, N., Motyka, S., Englund, P., Robinson, D.,* ***Shlomai, J.***

6D Elongation factor 1a determines the specificity of mitochondrial tRNA import in *T. brucei*. ***Aeby, E.***, *Bouzaidi-Tiali, N., Charrière, F., Schneider, A.*

6E RBP16 stimulates RNA editing and annealing *in vitro* by a mechanism independent of high affinity RNA binding. ***Ammerman, M.***, *Read, L.*

6F Substrate recognition by trypanosome RNA editing complexes is governed by RNase III-like requirements. ***Hernandez, A.***, *Cifuentes-Rojas, C., Cruz-Reyes, J.*

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200B Proteomic analysis of *Trypanosoma brucei* mitochondrial membrane fractions. ***Acestor, N.***, *Panigrahi, A., Burnham, R., Zíková, A., Ogata, Y., Anupama, A., Myler, P., Stuart, K.*

201A Analysis of expression profile of folate transporters gene family in *Leishmania* wild-type and methotrexate-resistant cell lines. ***Ahmed Ouameur, A.***, *Girard, I., Légaré, D., Ouellette, M.*

201B Activation of the endocytic system as a component of adaptation to the mammalian host in the African trypanosome. ***Natesan, S.***, *Peacock, L., Matthews, K., Gibson, W., Field, M.*

202A Spliceosomal proteomics revealed several new candidate small nuclear RNP proteins in *Trypanosoma brucei*. ***Ambrosio, D.***, *Lee, J., Panigrahi, A., Nguyen, T., Cicarelli, R., Günzl, A.*

202B Novel RNA Uridylyltransferases and Non-canonical Poly(A) Polymerases of Trypanosomes. ***Aphasizhev, R.***

203A Characterization of a ligand-specificity determinant in a protozoan purine nucleoside transporter. ***Arendt, C.***, *Ullman, B.*

203B The LAMP-like protein p67 plays an essential role in the lysosome of African trypanosomes. *Peck, R., Shiflett, A.,* ***Tazeh, N.***, *Schwartz, K., Hajduk, S., Bangs, J.*

204A Bioinformatic analysis of *Trypanosoma brucei* ESAG5 reveals a relationship to a lipid-interactive protein superfamily. ***Barker, A.***, *Gluenz, E., Kelly, S., Wickstead, B., Gull, K.*

204B A developmentally-regulated VSG expression-site-associated gene in bloodstream forms of *Trypanosoma brucei*: a pre-adaptation for parasite transmission? ***Barnwell, E.***, *van Deursen, F., Acosta-Serrano, A., Matthews, K.*

205A The 14-3-3 proteins of *Trypanosoma brucei* . ***Benz, C.***, *Clayton, C.*

205B Purine salvage enzymes as infectivity and virulence determinants in *Leishmania donovani*. ***Boitz, J.***, *Ullman, B.*

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206A Evidence that centromere-targeting of topoisomerase-II has been conserved between trypanosomes. ***Bot, C.***, *Obado, S., Kelly, J.*

206B Characterization of the FKBP12 protein of *Trypanosoma brucei*. ***Brasseur, A.***, *Salmon, D., Etienne, P., Luc, V.*

207A Characterization of Adenylate Cyclases in *Trypanosoma brucei*. ***Bregy, P.***, *Seebeck, T.*

207B Cold shock regulation of EP1 surface protein in *Trypanosoma brucei*. ***Brenndoerfer, M.***, *Panzer, M., Muehl, B., Pepperl, J., Boshart, M.*

208A RNA editing-associated protein (REAP-1) and a related mitochondrially localized protein REAP-2. *Hans, J.,* ***Madison-Antenucci, S.***

208B Prediction of potential drug targets shared across kinetoplastid genomes. *McAllister, G., Coflan, B.,* ***Campbell, B.***

209A Reduced expression of ISG65 results in reduced parasitaemia. *Seisenberger, S., Voorheis, P.,* ***Carrington, M.***

209B Abrogation of *Leishmania infantum* mitochondrial peroxiredoxin impairs parasite infectivity. Function(s) for this enzyme? ***Castro, H.***, *Romao, S., Tomás, A.*

210A Structural and metabolic regulation of *Trypanosoma brucei* hexokinases. ***Chambers, J.***, *Morris, M., Smith, K., Morris, J.*

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206A Evidence that centromere-targeting of topoisomerase-II has been conserved between trypanosomes. ***Bot, C.***, *Obado, S., Kelly, J.*

206B Characterization of the FKBP12 protein of *Trypanosoma brucei*. ***Brasseur, A.***, *Salmon, D., Etienne, P., Luc, V.*

207A Characterization of Adenylate Cyclases in *Trypanosoma brucei*. ***Bregy, P.***, *Seebeck, T.*

207B Cold shock regulation of EP1 surface protein in *Trypanosoma brucei*. ***Brenndoerfer, M.***, *Panzer, M., Muehl, B., Pepperl, J., Boshart, M.*

208A RNA editing-associated protein (REAP-1) and a related mitochondrially localized protein REAP-2. *Hans, J.,* ***Madison-Antenucci, S.***

208B Prediction of potential drug targets shared across kinetoplastid genomes. *McAllister, G., Coflan, B.,* ***Campbell, B.***

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223B Protein kinase A catalytic subunit of *Trypanosoma cruzi* interacts and phosphorylates trans-sialidase. *huang, h.*, ***Bao, Y.**, Weiss, L.*

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248A *Leishmania infantum* glyoxalase II: enzyme kinetics, substrate specificity and comparison with its human counterpart. ***Sousa Silva, M.**, Barata, L., Ferreira, A., Romão, S., Tomás, A., Ponces Freire, A., Cordeiro, C.*

248B Functional role of alanine aminotransferase in *T. brucei*: implications of knockdown and knockout studies. ***Spitznagel, D.**, Henahan, G., Nolan, D.*

249A A molecular link between mitochondrial fatty acid synthesis and respiration in *Trypanosoma brucei*. ***Stephens, J.**, Protivinska, E., Lukes, J., Englund, P.*

249B Characterization of MSP protein in *Leishmania chagasi*. ***Storlie, P.**, Yao, C., Hsiao, C., Donelson, J., Wilson, M.*

250A A putative function in machrophage invasion for the *L. pifanoi* cysteine proteinase Lpcys2 C-terminal extension. *Villa, M., Chaves, S., Inchaustegui, D., Soares, M., Mendonça, S., Soong, L., Nakamura, T., Rossi-Bergman, B.,* ***Traub-Cseko, Y.***

250B Whole-Genome analysis of drug resistance in *Leishmania* parasites. ***Ubeda, J.**, Légaré, D., Corbeil, J., Rigault, P., Papadopoulou, B., Ouellette, M.*

251A The role of different deadenylases on mRNA stability in *Trypanosoma brucei*. ***Utter, C.**, Bellofatto, V.*

251B Characterization of putative transcription elongation factors in Kinetoplastidae. ***Uzureau, P.**, Walgraffe, D., Pays, E., Vanhamme, L.*

252A The J-binding proteins (JBP) 1 and 2 in *Leishmania*. ***Vainio, S.**, Genest, P., ter Riet, B., van Luenen, H., Papadopoulou, B., Borst, P.*

252B Functional studies of the origin recognition complex from *Trypanosoma brucei*. ***Vandoros, A.**, Mozeleski, B., Klingbeil, M.*

253A Characterization of mitochondrial NADH dehydrogenases in *Trypanosoma brucei* and *Phytomonas serpens*. *Cermakova, P.,* ***Verner, Z.**, Lukes, J., Horvath, A.*

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254A Towards a genetic screen for factors involved in regulating the developmental expression of the *Trypanosoma brucei GPI-PLC* gene. ***Webb, H.**, Ellis, L., Kramer, S., Ramarao, R., Reed, J., Carrington, M.*

254B A large-scale functional study of trypanosomal kinesins. ***Wickstead, B.**, Honeycutt, J., Kelly, S., Gull, K.*

255A The *T. brucei* mitochondrial desulfurase IscS is essential for tRNA thiolation: implications for tRNA editing and import. ***Wohlgamuth-Benedum, J.**, Lukes, J., Alfonzo, J.*

255B Chromatin and histone levels and modification in metacyclic *Leishmania*. ***Wong, I.**, Beverley, S.*

256A Pyrimidine biosynthesis deficiency in *Leishmania donovani*. ***Yates, P.**, Ullman, B.*

256B Analysis of three RNA editing endonuclease-associated zinc finger proteins. ***Zelaya Soares, C.**, Schnaufer, A., Stuart, K.*

257A Expression of *L.donovani infantum* genes in *L.major* and its effect on virulence. ***Zhang, W.**, Peacock, C., Matlashewski, G.*

257B Insights into the composition of several mitochondrial complexes in *Trypanosoma brucei* . ***Zikova, A.**, Burnham, R., Ogata, Y., Anupama, A., Acestor, N., Myler, P., Panigrahi, A., Stuart, K.*

258A Gene expression during *Leishmania donovani* differentiation. ***Zilberstein, D.**, Rosenzweig, D., Lahav, T., Smith, D., Saxena, A., Mandelboim, M., Michaeli, S., Myler, P., Olafson, R.*

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ABSTRACTS: SESSION I

VSG Expression

April 23 7:00 p.m.

Chair(s) - Ed Louis

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Structure and differential expression of the silent array *VSG* genes of *Trypanosoma brucei*

Lucio Marcello, Subhash Verma, J David Barry*

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Trypanosoma brucei evades acquired immunity through differential expression of its archive of silent variant surface glycoprotein (*VSG*) genes, many of which are present in subtelomeric tandem arrays. Analysis of 940 sequenced array *VSG*s (an estimated half to two thirds of the array set) revealed that 65 % are pseudogenes, 21 % are gene fragments, 9.5 % may encode atypical VSGs, and only 4.5 % are functional. Due to the abundance of pseudogenes, we hypothesized that the phenomenon of mosaic *VSG* formation, previously proposed to be a minor mode of switching in antigenic variation, is predominant. To test this, we sequenced *VSG* cDNAs isolated from mice infected with the genome strain and found that mosaics appear by 3 weeks of infection and predominate by 4 weeks. Examination of the genome database revealed that there was high identity (≥79%) between the silent genes donating to the mosaics. To measure the incidence of such high-identity matches in the array archive, we undertook pairwise comparisons between all full-length N-terminal encoding sequences. This revealed distinct high-identity groups within the 2 main N-terminal types, A and B, both groups comprising almost exclusively pairs or triplets. About 40 % of array *VSG*s are in high-identity subfamilies, estimated across the whole array set to comprise $\sim$ 180 pairs, $\sim$50 triplets and $\sim$10 quadruplets. Thus, the rapid evolution of the *VSG* archive appears to be balanced by retention of an extensive subfamily structure conducive to formation of expressed mosaic *VSG*s. We propose that formation of expressed mosaic *VSG*s is central to antigenic variation, both in chronic infections and in permitting reinfection of (partially) immune hosts in the field.

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1B

A novel protein complex is essential for *VSG* expression-site transcription in *Trypanosoma brucei*

Jens Brandenburg[1], Bernd Schimanski[1], Everson Nogoceke[2], Tu N. Nguyen[1], Júlio C. Padovan[3], Brian T. Chait[3], George A. M. Cross[2], Arthur Günzl[*,1]

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Antigenic variation of its dense cell-surface coat is crucial for the survival of *Trypanosoma brucei* in the mammalian bloodstream. Each cell's coat consists of $\sim 10^7$ identical variant surface glycoprotein (VSG) molecules, which are products of a single gene. The active *VSG* is transcribed at a very high rate and silencing of *VSG* expression causes rapid cell-cycle arrest *in vitro* and clearance of parasites from infected mice. RNA polymerase I is used for VSG mRNA synthesis: in other eukaryotes, this efficient enzyme exclusively transcribes the ribosomal RNA genes. Despite its relevance, our knowledge of the transcription machinery in *T. brucei* is limited to conserved RNA polymerase subunits. We employed EMSA to isolate *VSG* expression site (ES) promoter-binding proteins from a crude nuclear extract by conventional chromatography. By epitope-tagging four of the 17 identified proteins, we found one protein which specifically bound to the *VSG* ES and other class I promoters in a promoter pull-down assay. Silencing the expression of the protein was lethal to both procyclic and bloodstream trypanosomes and affected VSG mRNA abundance in the latter. *In vitro* transcription analysis then established by independent criteria that this protein is an essential general class I transcription factor. Moreover, epitope tagging and tandem affinity purification of the protein as well as a sedimentation analysis and mass spectrometric protein identification revealed a complex which consisted of six unique proteins specific to trypanosomatids and, surprisingly, a known motor subunit. Since we demonstrated that the protein complex is the functional entity that binds to the *VSG* ES promoter, we concluded that we have characterized a multi-subunit factor essential for *VSG* transcription.

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1C

A novel ISWI is involved in VSG expression site downregulation in African trypanosomes

Katie Hughes[1], Matthew Wand[1], Lucy Foulston[1], Rosanna Young[1], Kate Harley[1], Stephen Terry[1], Klaus Ersfeld[2], Gloria Rudenko[*,1]

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African trypanosomes express one of about twenty telomeric Variant Surface Glycoprotein (VSG) gene expression sites (ES) in a monoallelic fashion while multiplying in the mammalian bloodstream. In contrast, in insect form trypanosomes all ESs are silenced. We would like to understand how ES control operates. We screened for genes involved in ES silencing using flow cytometry and RNAi. We show that a novel member of the ISWI family of SWI2/ SNF2 related chromatin remodeling proteins (TbISWI) is involved in ES downregulation in *Trypanosoma brucei* . TbISWI has an atypical protein architecture for a member of the ISWI family, as it lacks characteristic SANT domains. Depletion of TbISWI by RNAi leads to 30-60 fold derepression of ESs in bloodstream form *T. brucei*, and 10-17 fold derepression in insect form *T. brucei*. We show that although blocking synthesis of TbISWI leads to derepression of silent VSG ES promoters, this does not lead to fully processive transcription of silent ESs, or an observable increase in ES activation rates. VSG ES activation in African trypanosomes therefore appears to involve multiple steps, whereby an increase in transcription from a silent ES promoter is necessary but not sufficient for full ES activation.

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1D

Histone H3K76 methyltransferase DOT1B modulates monoallelic *VSG* expression in *T. brucei*

Luisa M. Figueiredo[1], Christian J. Janzen[2], George AM Cross[*,1]

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Trypanosoma brucei escapes the immune system by periodically changing its Variant Surface Glycoprotein (VSG) coat. Among more than 1,000 *VSG* genes, only one is expressed at a time. Transcription occurs from one of ∼20 Expression Sites (ES) and switching among them is thought to be regulated epigenetically. *T. brucei* DOT1B is a non-essential methyltransferase responsible for the tri-methylation of histone H3 at lysine 76. In *Saccharomyces cerevisiae*, loss of *DOT1* reduces transcriptional silencing at telomeres and HM loci. To investigate if *DOT1B* is involved in *VSG* regulation, we measured *VSG* transcript levels by real-time PCR. In the absence of *DOT1B*, transcripts from several silent *VSG* were ∼10-fold more abundant than in wild-type cells. To study ES regulation, we inserted a *NEO* marker (which confers resistance to G418 drug) downstream of the silent *VSG1.13* ES (ES1.13). In wild-type cells, G418-selection resulted in the death of all cells expressing *VSG1.2* and the selection of cells that exclusively express *VSG1.13*. In contrast, in most $DOT1B^{-/-}$ G418-resistant clones, immunofluorescence analysis showed that more than 30% of the cells expressed VSG1.2 and VSG1.13 at the surface, suggesting that both ES were simultaneously active. FACS analysis of double-expressors showed that, whereas VSG1.2 expression remained as high as in wild-type VSG1.2 expressors, VSG1.13 expression was variegated and never reached levels as high as in wild-type VSG1.13 expressors. After 1-3 weeks of G418-selection, these intermediate double-expressors yielded a stable and homogeneous population of cells with a fully active ES1.13 and a silent ES1.2. In conclusion, our data show that in the absence of *DOT1B*, *VSG* transcription is partially deregulated and monoallelic expression is temporarily impaired.

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1E

Telomeres control *VSG* expression site choice in African trypanosomes

Lucy Glover, Sam Alsford, Taemi Kawahara, David Horn*

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Antigenic variation in *Trypanosoma brucei* requires monoallelic expression and occasional switching of the Variant Surface Glycoprotein (VSG) coat. The single active *VSG* gene is transcribed by RNA polymerase I at an extra-nucleolar focus known as the expression site body. It was recognised twenty-five years ago that the single expressed gene and many reversibly repressed genes are adjacent to telomeres but no role for the telomere has been demonstrated thus far. We used meganuclease cleavage to sever the active expression site telomere. Cells that heal the site *de novo* activate another *VSG* and reversibly repress the distal promoter indicating telomere-mediated positive control. To ask whether transcription at the active site negatively controls other sites we used the tetracycline repressor to blockade the promoter. Following transcription blockade, many cells assemble additional pol I foci and expression site switching frequency increases by up to 100,000-fold. Our results demonstrate that the telomere is a genetic positive control element for *VSG* allelic exclusion and that active site transcription negatively controls other loci.

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1F

T. brucei RAP1 participate in the regulation of VSG expression from subtelomeric expression sites

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Telomeres, the nucleoprotein complexes at linear chromosome ends, are essential for chromosome stability. Telomeres in *T. brucei* also form a heterochromatic structure and can repress nearby RNA polymerase I-mediated gene expression (1). Since *T. brucei* expresses VSG exclusively from subtelomeric loci at the bloodstream stage in an allelic-exclusion fashion, it has been hypothesized that telomeres are involved in regulation of VSG expression. However, there was no direct evidence to support this hypothesis. Previously, we have identified a TRF homologue in *T. brucei* that binds to duplex telomeric TTAGGG repeats and plays an essential role in telomere terminal structure maintenance (2). Recently, we have identified a tbTRF-interacting candidate through a yeast 2-hybrid screen. This novel protein has several domains with sequence similarities to telomeric Rap1 proteins and is named tbRap1. We have observed a partial co-localization of tbRap1 and tbTRF in *T. brucei* cells and confirmed that the central region of tbRap1 is essential for its interaction with tbTRF, suggesting that tbRap1 is a component of *T. brucei* telomere complex. Most interestingly, knocking-down tbRap1 protein level led to a partial de-repression of subtelomeric VSG genes located within silent ESs at the bloodstream stage. In addition, a reporter gene located $\sim$ 50 kb upstream of telomeres in a silent ES is also transiently de-repressed. These data indicate, for the first time, that telomeres indeed play an important role in regulation of surface antigen expression in *T. brucei*.

(1) L. Glover and D. Horn. 2006. EMBO Rep. 7: 93-97. (2) B. Li, A. Espinal, and G. A. M. Cross. 2005. Mol. Cell. Biol. 25: 5011-5021.

T. brucei RAP1 participate in the regulation of VSG expression from subtelomeric expression sites

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Telomeres, the nucleoprotein complexes at linear chromosome ends, are essential for chromosome stability. Telomeres in *T. brucei* also form a heterochromatic structure and can repress nearby RNA polymerase I-mediated gene expression (1). Since *T. brucei* expresses VSG exclusively from subtelomeric loci at the bloodstream stage in an allelic-exclusion fashion, it has been hypothesized that telomeres are involved in regulation of VSG expression. However, there was no direct evidence to support this hypothesis. Previously, we have identified a TRF homologue in *T. brucei* that binds to duplex telomeric TTAGGG repeats and plays an essential role in telomere terminal structure maintenance (2). Recently, we have identified a tbTRF-interacting candidate through a yeast 2-hybrid screen. This novel protein has several domains with sequence similarities to telomeric Rap1 proteins and is named tbRap1. We have observed a partial co-localization of tbRap1 and tbTRF in *T. brucei* cells and confirmed that the central region of tbRap1 is essential for its interaction with tbTRF, suggesting that tbRap1 is a component of *T. brucei* telomere complex. Most interestingly, knocking-down tbRap1 protein level led to a partial de-repression of subtelomeric VSG genes located within silent ESs at the bloodstream stage. In addition, a reporter gene located $\sim$ 50 kb upstream of telomeres in a silent ES is also transiently de-repressed. These data indicate, for the first time, that telomeres indeed play an important role in regulation of surface antigen expression in *T. brucei*.

(1) L. Glover and D. Horn. 2006. EMBO Rep. 7: 93-97. (2) B. Li, A. Espinal, and G. A. M. Cross. 2005. Mol. Cell. Biol. 25: 5011-5021.

1G

A role for telomeric DNA glycosylation in the regulation of antigenic variation in *T. brucei*?

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Trypanosome infection persists due to the ability of the parasite to evade host immunity. This is achieved through antigenic variation, whereby the parasite can switch the glycoprotein expressed on its surface. ?-D-glucosyl-hydroxymethyluracil (Base J) is an unusual DNA modification represented by the glycosylation of thymine residues. The localization of J within the silent telomeric Variant Surface Glycoprotein (VSG) expression sites has suggested its role in the regulation of antigenic variation. It has been shown that J is synthesized through a two-step reaction involving the key enzymes JBP1 and JBP2. JBP2 induces de novo J synthesis, whereas JBP1 propagates J synthesis. To address the function of base J, we have generated cell lines deficient in the machinery for J biosynthesis by deleting all four JBP alleles. Indeed, JBP1/JBP2 double knock out cells (J null) lack base J. Analysis of J null trypanosomes indicated an atypical morphology and apparent cell cycle defect. This defect is characterized by a significant increase in the number of cells remaining attached during cell division. Interestingly, J null trypanosomes display an enhanced rate of VSG switching. Furthermore, there exists a clear difference in the switch mechanism employed by J null cells in comparison with WT cells. J-null trypanosomes favor recombination through telomere exchange as the dominant mechanism for switching, unlike WT cells which primarily undergo antigenic variation by in situ switching. Our current model proposes base J to play an important role in the regulation of antigenic variation by the stabilization of repetitive sequences in trypanosome telomeres.

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1H

ABSTRACTS: SESSION II

Cell Biology

April 24 8:30 a.m.

Chair(s) - Jeremy Mottram

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Considerations of trypanosome swimming

Niko Heddergott[1], Eric Stellamanns[2], Thomas Pfohl[2], Axel Rosenhahn[3], Markus Engstler[*,1]

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Flagellar motility is essential for *Trypanosoma brucei* (Broadhead *et al.*, 2006). However, our knowledge about the swimming behavior of trypanosomes remains rudimentary, and we have no information on trypanosome motility in the most hostile natural habitat, the mammalian vasculature. Blood flow is at least 50-times faster than trypanosome motion and the width of capillaries approaches the diameter of trypanosomes. Thus, besides being constantly attacked by the host's immune system, trypanosomes have to cope with tremendous physical forces. Our study aims at measuring those forces. We have applied microscopic scattering methodology (digital in-line holography) to generate 3-dimensional models of trypanosome trajectories. Speed and mode of movement were measured under varying flow conditions using multi-channel microfluidics. The cellular elasticity and bending moment were determined in the presence of obstacles such as erythrocytes and in extreme velocity fields. Using laser tweezers fully mobile trypanosomes were optically trapped and forces acting on the trypanosome cell body were quantified *in situ*. Our results suggest that African trypanosomes are amazingly flexible and robust cells that withstand extreme physical forces. Furthermore, finite element simulations confirm that fluid flow at the surface of constantly swimming trypanosomes is not zero, as would be expected from laminar flow at low Reynolds numbers. In fact, trypanosomes successfully exploit this flow for survival in the mammalian host. The hydrodynamic forces resulting from directional motility very rapidly drag surface-bound immune complexes towards the flagellar pocket, where they are internalized by the highly efficient endocytosis machinery. This novel mechanism of membrane protein sorting protects trypanosomes from complement-mediated lysis and may explain why trypanosomes have to swim at all.

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2B

3D ultrastructural architecture and morphogenesis of the flagellar pocket area of *Trypanosoma brucei*

Sue Vaughan[1], Catarina Gadleha[1], Sylvain Lacomble[1], Michael Shaw[1], Mary Morphew[2], Eileen O'Toole[2], Richard McIntosh[2], Keith Gull[*,1]

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The flagellar pocket is a critical feature for pathogenicity of the kinetoplastid protozoa. In *T. brucei* it is the sole portal for the vesicular traffic involved in uptake and secretion. The flagellar pocket is defined by the exit point of the flagellum from the cell. Major questions arise as to how a new flagellar pocket forms, what cytoskeletal and membrane structures are involved in flagellar pocket morphogenesis and how correct spatial and temporal events are coordinated. We have used thin-section transmission electron microscopy, negatively stained detergent-extracted cytoskeletons and electron tomography to provide a description of the 3D architecture of flagellar pocket and associated structures including the basal bodies, flagellum, kinetoplast, microtubule quartet, FAZ filament and collar. The earliest morphological events during the cell division cycle occur with the duplication and spatial re-organisation of these structures. Our studies reveal new flagellar pocket formation occurring with invasion of the new flagellum into the existing pocket, connections between the flagella via the flagella connector and a major rotation of the new flagellum as new flagellar pocket morphogenesis occurs. Re-orientation of the two new probasal bodies from an orthogonal position at nucleation to a parallel position was also evident. Understanding the temporal and spatial organisation of these early events in the cell division cycle will be crucial to understanding new pocket formation and function as well as coordination with morphogenesis of the extensive microtubule cytoskeleton.

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2C

The cytoskeleton is essential for flagellar pocket biogenesis in procyclic trypanosomes

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In eukaryotes, exo/endocytosis requires vesicle trafficking that is functionally dependent on the actin cytoskeleton. In *Trypanosoma brucei*, the causative agent of African sleeping sickness, exo/endocytosis occurs exclusively through the flagellar pocket **(FP)**, an invagination of the pellicular membrane. The pocket is also responsible for sorting of proteins targeted to, or recycling from, the pellicular membrane. However, the mechanisms underlying FP biogenesis remain unknown. Here we show that a novel cytoskeletal protein BILBO1 localises to a discreet region of the flagellum cytoskeleton, the flagellar pocket collar **(FPC)** and is essential for cell survival. Remarkably, RNAi mediated ablation of BILBO1 prevents flagellar pocket biogenesis. We also show that the consequences of pocket ablation include the accumulation of protein trafficking vesicles that cannot be cleared by the cell. This traffic-associated phenotype is accompanied by, Golgi swelling and flagella repositioning. Ultimately, BILBO1 knockdown is lethal. These results indicate the presence of strict dependency relationships whereby a cytoskeletal framework is essential for the biogenesis of the endocytotic organelle. We provide the first evidence of the existence of an important interface between the cytoskeleton and the exo-endocytotic machinery in *T. brucei*. Detailed characterisation of the FPC will provide new insights into flagella positioning and FPC derived vesicle trafficking systems of kinetoplastids.

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2D

Centromere location in *Trypanosoma cruzi* and *Trypanosoma brucei*

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Centromeres are the chromosomal loci that facilitate segregation. They are the sites of assembly of the kinetochore, the protein complex that acts as an anchor for the microtubule spindles that separate sister chromatids and mediate their movement to daughter nuceli. In trypanosomes, the location of centromeric DNA could not be predicted from the genome sequence and genes encoding the functionally conserved core centromeric proteins were not identified. We have previously demonstrated using telomere associated chromosome fragmentation, that GC-rich transcriptional strand switch domains, composed predominantly of degenerate retrotransposons, are central features of the regions required for the mitotic stability of *Trypanosoma cruzi* chromosomes 1 and 3. Consistent with this, etoposide-mediated topoisomerase-II cleavage, a biochemical marker for active centromeres in other organisms, is concentrated at these domains. In the megabase-sized chromosomes of *Trypanosoma brucei*, topoisomerase-II activity is also focussed at single loci which encompass regions between directional gene clusters that contain transposable elements and additionally, AT-rich domains composed of repeated arrays that stretch over several kilobases. The *T. brucei* intermediate and minichromosomes do not exhibit site-specific accumulation of topoisomerase-II, suggesting that segregation of these atypical chromosomes might involve a centromere-independent mechanism. RNAi-mediated knockdown of topoisomerase-IIα in bloodstream form *T. brucei* results in the abolition of centromere-specific enzyme activity and is lethal within 48 hours. Both of these phenotypes can be rescued by expression of the *T. cruzi* enzyme. This suggests that the signals which govern centromere-specific localisation of topoisomerase-II have been conserved within the trypanosomes. These experiments therefore provide a system that can be used to explore the mechanisms by which topoisomerase-II is targeted to centromeres in these parasites.

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2E

Identification of host proteins that bind *Trypanosoma cruzi* alpha-galactosyl epitopes involved in host-cell invasion

Luciane Ganiko, Ernesto S. Nakayasu, Igor C. Almeida*

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We have recently shown that host cell-derived infective trypomastigote forms of T. cruzi secrete vesicles which are heavily α-galactosylated (TcαGalVes). TcαGalVes are potent virulence factors since they significantly enhance the invasion of host cells by the parasite via a mechanism dependent on Toll-like receptor 2 (TLR2) and on the expression of αGal epitopes on TcαGalVes. The present study focuses on the identification of host molecules capable to interact with αGal epitopes on TcαGalVes. By FACS analysis, we found that about 36% of RAW 264.7 macrophage cells specifically bind to Galα1-3Galβ1-4GlcNAc-biotin (αGal-LacNAc-biotin). For identification of the host αGal-binding proteins, a RAW 264.7 cell lysate was incubated with αGal-LacNAc-biotin and specifically bound proteins were isolated by affinity chromatography using streptavidin-agarose. Proteins were digested with trypsin and subjected to tandem LC-MS analysis. Annexins (A1, A2, A4, and A5), annexin-binding proteins (i.e., S100A10 and Calgizzarin), and galectin-3 (Gal-3) were consistently identified. By confocal microscopy, we observed that intracellular forms of T. cruzi are heavily coated by annexin A2 and S100A10, most likely from the host cells. Furthermore, we found that anti-annexin A2 and anti-Gal-3 antibody inhibited approx. 50-60% the host-cell invasion by the parasite. In conclusion, enhancement of host-cell invasion induced by TcαGalVes could involve cooperation of Gal-3 and annexin A2-S100A10 complex with TLR2, through an as-yet-unknown mechanism, currently under investigation. Supported by NIH/NCRR Grant No. 5G12RR008124.

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2F

AP-3 adaptor is involved in the transport of membrane proteins to acidocalcisomes of *Leishmania*

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Lysosomal function is crucial for the differentiation and infectivity of *Leishmania major* and an important virulence factor, the cysteine peptidase CPB, resides within the lysosomal compartment. To study lysosomal biogenesis, a *L. major* mutant deficient in the delta subunit of the adaptor protein 3 (AP-3 delta) complex was generated. The structure and proteolytic capacity of the lysosomal compartment were apparently unaffected in the AP-3-deficient mutant, however defects were identified in its acidocalcisomes. These are acidic organelles enriched in calcium and phosphorus, conserved from bacteria to eukaryotes, whose function remains enigmatic. The acidocalcisomes of the *L. major* mutant lacked membrane-bound proton pumps, were less acidic and devoid of polyphosphate, but contained a soluble pyrophosphatase. The mutant parasites were viable in vitro, but were unable to establish an infection in mice - indicating a role for AP-3 in determining, possibly through an acidocalcisome-related function, the virulence of the parasite. AP-3 transport function has been linked previously to lysosome-related organelles such as platelet dense granules, which appear to share several features with acidocalcisomes. Our findings implicating AP-3 in transport to acidocalcisomes thus provide evidence that acidocalcisomes resemble lysosome-related organelles and that they probably have conserved origins.

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Modern methods, ancient organisms: the nuclear pore complex of the african trypanosome in an evolutionary context

Jeffrey A. DeGrasse[*,1], Brian T. Chait[1], Mark C. Field[2], Michael P. Rout[1]

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Spanning the nuclear envelope (NE), the nuclear pore complex (NPC) facilitates transport of protein and nucleic acid molecules into and out of the nucleus. To build a generalized model of NPC architecture, to investigate further the mechanisms of nucleocytoplasmic transport and to understand the evolution of the NPC, we identified the protein constituents of highly enriched trypanosome NEs. We report the identification and classification of the nucleoporins (nups) comprising the trypanosome NPC. A subset of the candidate trypanosome nups were tagged at their C-termini. Restorative fluorescence imaging revealed a uniform and cell cycle independent distribution of NPCs around the NE. Overall, the data indicate that the architecture of the NPC (and so likely the transport mechanisms) is conserved across the eukaryotes and suggests that the modern NPC configuration has altered little during eukaryote evolution. Although the sequences are divergent, the dependence of nucleocytoplasmic transport on the natively disordered FG-repeat domains within the NPC is probably conserved within the trypanosomes. We also describe two trypanosome coiled coil proteins similar to yeast Mlp1 and Mlp2, with molecular weights about 50% of the yeast proteins. The Mlps are functionally implicated in the nuclear retention of unspliced mRNAs and telomere maintenance, and Mlp2 binds directly to the spindle pole body. Trypanosome Mlps localize to the NPC and, strikingly, TbMlp2 localizes to a region of the NE consistent with the spindle pole body during anaphase. This new NPC proteome identifies both well-conserved features and species-specific specializations; the functional importance of these will be discussed.

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2H

Hemoglobin is a necessary component of Trypanosome Lytic Factor

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Trypanosoma brucei brucei is unable to infect humans although the closely related parasite *Trypanosome brucei rhodeseinse* causes African sleeping sickness in humans. Humans are protected from *T.b brucei* infection because of an innate killing factor termed Trypanosome Lytic Factor (TLF). TLF is a minor subclass of human serum HDL that contains two lytic proteins, apolipoprotein L-1 (ApoL-1) and haptoglobin-related protein (Hpr). Proteomic analysis of TLF purified directly from plasma, with antibodies to Hpr or ApoL-1, revealed two interesting findings. First, the protein composition of TLF purified with either antibody was identical. Second, hemoglobin (Hb) was not present in the purified material although recent evidence shows that Hpr binds Hb. We investigated whether TLF was a Hb binding complex and if Hb was required for efficient *T.b. brucei* killing. Flow cytometry and fluorescence microscopy results showed that TLF binding was negligible in the absence of Hb, supporting the hypothesis that an interaction between Hpr and Hb is critical for binding and uptake of TLF and subsequent trypanosome lysis. Taken together, these data support a model in which both ApoL-1 and Hpr-Hb are critical for efficient trypanosome killing by TLF. Our studies also address directly the role of Hpr both as a ligand for the TLF receptor and as a toxin to *T. b. brucei*.

Hemoglobin is a necessary component of Trypanosome Lytic Factor

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21

Distinct roles of haptoglobin-related protein and apolipoprotein L-I in trypanolysis by human serum

Benoit Vanhollebeke[1], Marianne J. Nielsen[2], Yoshihisa Watanabe[3], Philippe Truc[4], Luc Vanhamme[5], Kazunori Nakajima[6], Soren K. Moestrup, Etienne Pays[*,1]

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Apolipoprotein L-I (apoL-I) is a human HDL component able to kill Trypanosoma brucei brucei by forming anion-selective pores in the lysosomal membrane of the parasite. Another HDL component, haptoglobin-related protein (Hpr), has been suggested as an additional toxin required for full trypanolytic activity of normal human serum (NHS). We recently reported the case of a human individual lacking apoL-I (apoL-I-/-HS) due to frameshift mutations in both apoL-I alleles (Vanhollebeke, B., Truc, P., Poelvoorde, P., Pays, A., Joshi, P.P., Katti, R., Jannin, J.G., & Pays, E. (2006) N. Engl. J. Med., 355, 2752-6). Here we show that this serum, devoid of any trypanolytic activity, exhibits normal concentrations of HDL-bound Hpr. Conversely, the serum of individuals with normal HDL-bound apoL-I but lacking Hpr and haptoglobin (Hp(r)-/-HS) due to gene deletion (anhaptoglobinemia) exhibited phenotypically normal but delayed trypanolytic activity. The trypanolytic properties of Hp(r)-/-HS were mimicked by free recombinant apoL-I, whereas recombinant Hpr did not affect trypanosomes. The lysis delay observed with either Hp(r)-/-HS or recombinant apoL-I could entirely be attributed to a defect in the uptake of the lytic components. Thus, apoL-I is responsible for the trypanolytic activity of NHS, while Hpr allows fast uptake of the carrier HDL particles presumably through their binding to an Hp/Hpr surface receptor of the parasite.

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2J

Reconstitution of TLF function in transgenic mice reveals distinct contributions of each component

Jayne Raper*, Pilar Molina-Portela

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Trypanosome lytic factor appears to be the major defense mechanism against trypanosomiasis because only two subspecies of trypanosomes that are resistant to lysis by TLF can infect humans. Two components haptoglobin-related protein and apolipoprotein L-I are instrumental in lytic activity, although their relative contributions have been controversial. These proteins circulate in 1% of high density lipoprotein particles in human blood. To resolve these controversies we have generated transgenic mice that express either Hpr or apoL-I or both proteins in order to evaluate their relative contributions *in vivo* in a physiologically relevant setting. We find that Tg-Hpr-apoL-I mice can resolve infections of the cattle pathogen *T. b. brucei*, but not of human infective *T. b. brucei* expressing the serum resistance associated gene, SRA. Tg-Hpr mice have no protection against these pathogens. As in human TLF, the transgene proteins are found exclusively in the circulating high density lipoproteins of the Tg-mice. Ex-vivo analysis of the Tg-HDLs reveals surprising differences in lytic activity based on their composition.

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2K

ABSTRACTS: SESSION III

Biochemistry

April 24 7:00 p.m.

Chair(s) - Deborah Smith

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Galactose Metabolism in *Trypanosoma brucei*

Michael D. Urbaniak*, Stuart McElroy, Julie A. Frearson, Michael A J Ferguson

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Bloodstream form *Trypanosoma brucei* contains many galactose-containing glycoproteins, yet unlike mammalian cells, the parasite hexose transporters are unable to uptake galactose directly. Instead, *T. brucei* obtains galactose *via* the enzyme UDP-glucose 4'-epimerase (GalE) that interconverts UDP-glucose and UDP-galactose. Construction of a conditional null mutant cell line has demonstrated that GalE is essential for *T. brucei*, validating it as a drug target [1]. Here, we describe the effect of galactose starvation on the parasite, and our attempts to discover inhibitors of GalE suitable for the treatment of African Sleeping Sickness. Using a combination of mass spectrometry, lectin binding and electron microscopy to we have shown that GalE is required to maintain the cellular levels of UDP-Gal, and that the decreased UDP-Gal levels lead to the loss of galactose from glycoproteins and changes in cell morphology and metabolism [2]. These data demonstrate that galactosylation of one or more glycoprotein, most likely in the lysosomal/endosomal system, is essential for the survival of bloodstream form *T. brucei*. We have developed a colourmetric coupled enzyme assay suitable for high-throughput screening, and a direct high-pH anion exchange chromatography secondary assay to confirm hits [3]. A carefully selected library of 62,000 compounds has been screened against *T. brucei* GalE in the Dundee Drug Discovery Unit, and hit series validated and potency tested using the direct assay. A hit-to-lead medicinal chemistry programme is underway to improve potency and selectivity. **References:** [1] Roper JR *et al.*, Proc. Natl. Acad. Sci. USA **2002**, 99:5884 - 5889 [2] Urbaniak MD *et al.*, Eukaryot. Cell **2006**, 5:1906-1913 [3] Urbaniak MD *et al.*, Bioorg. Med. Chem. Lett. **2006**, 5744-5747

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3B

De novo synthesis of *myo*-inositol in *Trypanosoma brucei* is a validated drug target

Terry K. Smith*

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The protozoan parasite *Trypanosoma brucei* avoids its hosts' innate immune system by the process of antigenic variation, involving switching of it's GPI-anchored variant surface glycoprotein (VSG). Previously the biosynthesis of GPI's has been genetically and chemically validated as a potential drug target against these parasites. Although phosphatidylinositol (PI) is a key component of GPI anchors little is known about *myo*-inositol metabolism or PI biosynthesis in these organisms. myo-Inositol is an ubiquitous six-carbon cyclitol which many cells are able to *de novo* synthesise as an alternative to uptake from extra-cellular sources. The rate-limiting step of the *de novo* synthesis is the isomerisation of glucose-6-phosphate to *myo*-inositol-3-phosphate, catalysed by an inositol-3-phosphate synthase (INO1). A conditional knockout of *TbINO1* clearly showed that it is an essential gene in bloodstream form *T.brucei*, surprisingly the effects of this deletion cannot be overcome by increased extra-cellular *myo*-inositol. The lack of change in PI levels in conditional knockout cells grown under non-permissive conditions, clearly showing that perturbation of growth is due to a specific lack of *de novo* synthesised myo-inositol and not a general inositol-less death. This suggests a distinction between *de novo* synthesised *myo*-inositol and that obtained from the extracellular environment. This is achieved by compartmentalisation of PI synthesis, with bulk cellular PI synthesis occurring in the golgi, whilst PI destined for the GPI synthetic pathway is synthesised in the ER. Recombinant expression of *T.brucei myo*-inositol-3-phosphate synthase has allowed substrate/inhibitor specificity studies. Another key discovery is the finding that the inositol-3-phosphate synthase can also isomerase fructose-6-phosphate to glucose-6-phosphate (phosphoglucose isomerase activity). This suggests possible regulatory relationships between glycolysis, *de novo myo*-inositol synthesis and other cellular processes.

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3C

Restoration of virulence in a *Leishmania mexicana* glucose transporter mutant is associated with increased utilization of gluconeogenesis

Eleanor Clare Saunders[*,1], Miriam A. Ellis[1], David P. De Souza[1], Vladimir A. Likic[2], Scott M. Landfear[3], Malcolm J. McConville[1]

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Leishmania spp are a parasitic protozoa that invade the mammalian host and proliferate within the mature phagolysosome of macrophages. This intracellular stage must scavenge all of their carbon sources from the phagolysosome, though the nature of these carbon sources remains unclear. A *L. mexicana* mutant, lacking all three known glucose transporters (LmKOGT), grows in culture but is unable to survive in macrophages. These studies indicate that glucose is critical for intracellular survival. We recently isolated an adapted strain of the mutant (LmKOGT^{v}) that has restored virulence in macrophages. LmKOGT^{v}, like LmKOGT, has a very low rate of glucose uptake capacity which indicates that the observed increase in virulence is not due to an augmented ability to scavenge glucose. Metabolic profiling methods (employing gas chromatography-mass spectrometry) combined with the use of ^{13}C-labeled glucose, glycerol and amino acids, indicated that the increased virulence of LmKOGT^{v} may be due to an up-regulation of gluconeogenesis. These studies indicate that intracellular stages of *L. mexicana* normally utilize a mixture of hexose and gluconeogenic substrates within the macrophage phagolysosome, but that up-regulation of gluconeogenesis, to compensate for loss of hexose uptake, can result in increased growth in macrophages.

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3D

The antioxidative defence system of *Leishmania major*: two types of Tryparedoxin-dependent peroxidases

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Most parasites including Leishmania parasites are more susceptible to reactive oxygen species (ROS) than their hosts. Mammalian cells have a battery of enzymatic systems for metabolising hydroperoxides: catalase; selenium- and sulphur-dependent glutathione peroxidases; glutathione-dependent 1-cys peroxiredoxins; and thioredoxin-dependent 2-cys peroxiredoxins. In contrast, leishmania lack catalase, selenium-dependent peroxidases, glutathione reductase and thioredoxin reductase. Instead, the entire antioxidant defence system is mediated via the unique dithiol trypanothione (N^1,N^8-bis(glutathionyl)spermidine) together with NADPH-dependent trypanothione reductase. Three types of peroxidases have been identified in parasites belonging to the family Trypanosomatidae: first, the classical tryparedoxin peroxidase, a 2-Cys peroxiredoxin, second, an ascorbate peroxidase, a membrane-bound haemoprotein, and third, a glutathione peroxidase-like protein. The latter one has been identified in *T. brucei* as a second type of tryparedoxin-dependent peroxidase. Previously it was demonstrated by RNA interference that both types of tryparedoxin peroxidases are essential for parasite survival. We have expressed the *L. major* encoded glutathione peroxidase-like protein in *E. coli* and demonstrated it to be a tryparedoxin dependent peroxidase. Glutathione-dependent reduction can be neglected. Kinetic analysis, a mechanistic study and expression profiles have been performed to elucidate the role in antioxidative defence in comparison to the classical tryparedoxin peroxidase.

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3E

Exploring iron-sulfur cluster assembly in *Trypanosoma brucei*

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Iron-sulfur (Fe-S) clusters are ancient and ubiquitous cofactors of proteins that are involved in a variety of biological functions, including enzyme catalysis, electron transport and gene expression. Formation of Fe-S clusters is mediated by a machinery of more than 10 proteins, from which cysteine desulfurase IscS, metallochaperone IscU, IscA, frataxin and ferredoxin are the key components. It seems that this function might be the only common denominator of all extant mitochondria and mitochondria-derived organelles. We have shown that TbIscS and TbIscU are functionally conserved and essential for *T. brucei*, and upon their depletion, the mitochondrion of procyclics is generally down-regulated resembling the organelle of the bloodstream stage. Knock-down of frataxin causes growth arrest and decrease of activities of marker Fe-S enzyme aconitase both in the mitochondrion and the cytosol. Functional analyses showed that only one of the two ferredoxins in the *T. brucei* genome is essential and its depletion results in the disruption of Fe-S assembly. Additional conserved component of the assembly, Isa1, is dispensable for the procyclic stage and causes only minor decrease of aconitase. All knock-downs are characterized by decrease of membrane potential, with the flow of electrons being (partially) redirected from the respiratory complexes to the alternative oxidase. Other consequences of down-regulation of these proteins are under study.

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3F

Linking ethanolamine to protein: biosynthetic pathway of a rare protein modification

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Biosynthetic labeling using [^{3}H]ethanolamine can be used to identify (novel) GPI-anchored proteins in cultured cells. Ethanolamine is taken up by *T. brucei* bloodstream or procyclic forms and incorporated into phosphatidylethanolamine (PE), which is the donor of the ethanolamine phosphate moiety linking the GPI anchor to protein. We have previously noted that, besides being incorporated into GPI-anchored proteins, [^{3}H]ethanolamine also labels a non-GPI-anchored protein of 49 kDa. A [^{3}H]ethanolamine-labeled protein with a similar molecular mass has also been described before in other organisms, including mammals and plants, and was identified as eukaryotic elongation factor 1A (eEF1A) carrying ethanolamine phosphoglycerol (EPG) residues attached to two glutamic acid side chains. Both EPG modification sites are highly conserved between different organisms. We now show using biochemical and mass spectrometric techniques that endogenous and HA-tagged eEF1A expressed in *T. brucei* procyclic forms is also modified with EPG; however, in *T. brucei* eEF1A only one of the two potential sites is used. Based on its chemical structure, we hypothesized that the EPG moiety is generated from PE. To test this, we interfered with the PE biosynthetic pathway in *T. brucei* procyclic forms by down-regulating all three enzymes involved in PE biosynthesis by the "Kennedy pathway". Our results show that reducing the expression of the enzymes using RNAi leads to an accumulation of the corresponding biosynthetic precursors, and a decrease in the amount of PE in these cells. Furthermore, reduction of PE biosynthesis results in a decreased incorporation of radioactivity into eEF1A, demonstrating that PE is a precursor of the EPG modification.

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Biosynthetic labeling using [^{3}H]ethanolamine can be used to identify (novel) GPI-anchored proteins in cultured cells. Ethanolamine is taken up by *T. brucei* bloodstream or procyclic forms and incorporated into phosphatidylethanolamine (PE), which is the donor of the ethanolamine phosphate moiety linking the GPI anchor to protein. We have previously noted that, besides being incorporated into GPI-anchored proteins, [^{3}H]ethanolamine also labels a non-GPI-anchored protein of 49 kDa. A [^{3}H]ethanolamine-labeled protein with a similar molecular mass has also been described before in other organisms, including mammals and plants, and was identified as eukaryotic elongation factor 1A (eEF1A) carrying ethanolamine phosphoglycerol (EPG) residues attached to two glutamic acid side chains. Both EPG modification sites are highly conserved between different organisms. We now show using biochemical and mass spectrometric techniques that endogenous and HA-tagged eEF1A expressed in *T. brucei* procyclic forms is also modified with EPG; however, in *T. brucei* eEF1A only one of the two potential sites is used. Based on its chemical structure, we hypothesized that the EPG moiety is generated from PE. To test this, we interfered with the PE biosynthetic pathway in *T. brucei* procyclic forms by down-regulating all three enzymes involved in PE biosynthesis by the "Kennedy pathway". Our results show that reducing the expression of the enzymes using RNAi leads to an accumulation of the corresponding biosynthetic precursors, and a decrease in the amount of PE in these cells. Furthermore, reduction of PE biosynthesis results in a decreased incorporation of radioactivity into eEF1A, demonstrating that PE is a precursor of the EPG modification.

3G

Structure of a GPI-anchored type 1 VSG C-terminal domain of *Trypanosoma brucei*

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The cell surface of bloodstream form African trypanosomes is covered by a monolayer composed of a single protein, the variant surface glycoprotein (VSG). The VSG acts as a protective barrier and is central to a system of antigenic variation necessary for a persistent infection. These two roles of the VSG impose opposite selection pressures on VSG sequences: divergence of sequence for antigenic variation and conservation of structure to maintain its protective function. The genome contains hundreds of VSG genes with highly divergent sequences but with conserved secondary and tertiary structure features. VSGs from *Trypanosoma brucei* have a large N-terminal domain and either one or two small C-terminal domains and are retained on the external face of the plasma membrane by a glycosylphosphatidylinositol-(GPI-)anchor, which is added to the C-terminal carboxyl group of the mature polypeptide. Here, we have determined the structures of two individual domains that together comprise a VSG C-terminal di-domain. The first domain has a structure similar to the single C-terminal domain of MITat1.2, whereas the second domain, although based on the same fold, is a minimised version missing several secondary structure features. The structure of the second domain includes the C-terminal residue that is attached to the GPI-anchor in the native protein and has been combined with the solution structure of the GPI-glycan to produce the first structure-based model of the GPI-anchored protein domain. The model suggests that the core glycan of the GPI-anchor lies in a groove on the surface of the second subdomain therefore leading to a close association between the GPI anchor and its proximal C-terminal subdomain.

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3H

An Adenosine to Inosine tRNA editing enzyme that can perform C to U deamination of DNA

Kirk W. Gaston[1], Mary Anne T. Rubio[1], Frank Ragone[1], Irena Pastar[2], Chistian J. Janzen[3], George A. M. Cross, F. Nina Papavasiliou[*], Juan D. Alfonzo[*,1]

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Editing of tRNAs is widespread in nature and either changes the decoding properties or restores the folding of a tRNA. Unlike the phylogenetically disperse adenosine (A) to inosine (I) editing (occurring in all domains of life), cytosine (C) to uridine (U) editing has only been previously described in organellar tRNAs. We have shown that cytoplasmic tRNAThr(AGU) undergoes two distinct editing events in the anticodon loop: C to U and A to I. Surprisingly, enzymes mediating tRNA deamination in bacteria and yeast contain conserved cytidine deaminase motifs, suggesting an evolutionary link between the two reactions. In trypanosomatids, the enzyme responsible for either reaction has not been identified. Here we show that all three tRNAThr isoacceptors in Leishmania tarentolae and Trypanosoma brucei undergo C to U editing in the nucleus. This represents the only known tRNA C to U editing event that occurs outside of mitochondria or chloroplasts. We also show that down-regulation of the Trypanosoma brucei tRNA editing enzyme by RNAi leads to a reduction in both C-to-U and A-to-I editing of tRNA in vivo. Surprisingly, in vitro, this enzyme can mediate A to I editing of tRNA and C to U deamination of ssDNA but not both in either substrate. The ability to utilize both DNA and RNA, provides a model for a multi-specificity editing enzyme. Notably, the ability of a single enzyme to perform two different deamination reactions also suggests that this enzyme still maintains specificities that would have been found in the ancestor deaminase, providing a first line of evidence for the evolution of editing deaminases.

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ABSTRACTS: SESSION IV

Gene Expression

April 25 8:30 a.m.

Chair(s) - Sergio Schenkman

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Characterizing the transcription pre-initiation complex at the SL RNA gene promoter of *Trypanosoma brucei*

Ju Huck Lee, Tu N Nguyen, Arthur Günzl*

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Trypanosomatid parasites share a gene expression mode which differs greatly from that of their human and insect hosts: protein coding genes are transcribed polycistronically and individual mRNAs are processed from precursors by spliced leader (SL) *trans* splicing and polyadenylation. In *trans* splicing, the SL RNA is consumed through a transfer of its 5'-terminal part to the 5'-end of mRNAs. Since all mRNAs are *trans* spliced, the parasites depend on strong and continuous SL RNA synthesis mediated by RNA polymerase II. In other eukaryotes, the conserved general transcription factors TFIIA, TFIIB, TBP/TFIID, TFIIE, TFIIF, and TFIIH form a pre-initiation complex (PIC), recruit RNA pol II to the correct transcription initiation site, separate the DNA strands, and mediate the polymerase's escape from the promoter. Until recently, the apparent lack of general transcription factors in trypanosomatid genomes suggested that in these organisms recruitment of RNA polymerase II to DNA occurs by a novel mechanism. However, recent studies identified extremely divergent orthologues of TFIIA and TFIIB which, in *T. brucei*, bind to the SL RNA gene (*SLRNA*) promoter together with the TBP homologue TRF4 and the small nuclear RNA-activating complex. TFIIB was indispensable for *SLRNA* transcription and, as its counterparts in other eukaryotes, interacted with both TRF4 and RNA polymerase II. Together, these results suggested that trypanosomes form a highly divergent PIC at the *SLRNA* promoter. We corroborated this hypothesis by demonstrating that *T. brucei* harbors a TFIIH which is required for *SLRNA* transcription. Our biochemical and functional analysis of this multi-subunit factor has thus far revealed an unusual subunit composition and a novel sub-complex structure.

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4B

SLS and SLD - novel mechanisms that regulate SL RNA in *Trypanosoma brucei*

Yaniv Lustig[1], Hadassa Shaked[1], Itai Tkacz[2], Gil Arvatz[3], Lilach Sheiner, Yaron Vagima, Hanoch Goldshmidt, Anish Das[4], Vivian Bellofatto, Shulamit Michaeli*

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In this study, we describe two novel mechanisms that regulate the production and fate of the spliced leader RNA (SL RNA) in *Trypanosoma brucei*. The first mechanism is induced under stress and leads to specific shut-off of SL RNA transcription. This mechanism that we termed SLS for SL RNA silencing was first discovered in cells silenced for the signal recognition particle (SRP) receptor, SRalpha. Silencing of SRalpha caused the accumulation of SRP on ribosomes which induced SLS. SLS operates due to failure of the SL RNA-specific transcription factor tSNAP42 to bind to its promoter. SL RNA reduction, in turn, abolished mRNA processing, and resulted in significant reduction of all mRNA tested. SLS was induced under variety of physiological stresses such as ER stress, pH and more and may function as a master regulator in trypanosomes. SLS is reminiscent but distinct from the unfolded protein response (UPR) and can potentially serve as a novel target for parasite eradication. In this study, we further provide evidence that SL RNA transcription and assembly with Sm proteins take place in an unique nuclear compartment. Under Sm depletion, SL RNA first accumulates in the nucleus and then migrates to the cytoplasm in a novel SL RNP-C complex. The complex was purified to homogeneity and several proteins were identified. We suggest that SL RNP-C represents a novel discard mechanism (SLD) to remove defective SL RNA from the nucleus.

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4C

RNA structural thermosensors and translation regulation in *Leishmania*

Michal Shapira*[,1], Miriam Ben David[1], Yael Yoffe[1], Idan Gabdank[2], Danny Barash[2]

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Translation control is mediated by 3' UTRs, through mechanisms that are still elusive. We present data indicating that structural changes in the 3' UTR of Hsp83 in *Leishmania* direct preferential translation at elevated temperatures. To examine these structural changes, the *in vitro* transcribed RNA region (1-472) was end-labelled and subjected to enzymatic probing of its secondary structure. The mapped positions were introduced as mFold constraints, resulting in a single computerized output. In this predicted structure, regions that were shown to be essential for preferential translation cluster on a discrete arm of the RNA. We further identified a single-stranded polypyrimidine-rich region in the Hsp83 3' UTR with sequence complementation to the ribosomal 18S RNA; this may possibly stabilize the ribosome in the translation initiation complex. The structure of this RNA region changed during a temperature switch, shown by RNaseH assays, suggesting that it functions as a thermosensing mechanism that controls translation. Thus the polypyrimidine tract in the 3' UTRs of *Leishmania* may affect the translation initiation complex via the circular mRNA model in an analogous manner to IRES-mediated translation initiation in 5' UTRs of higher eukaryotes. Translational regulation plays a key role in developmental gene expression in Leishmania. We previously identified the major components of the cap binding translation initiation complex, and determined their binding specificities to different cap structures. Here we report on developmental changes in expression and intracellular distribution of cap binding translation initiation factors (LeishIF4Es). These factors are currently examined in an experimental *in vitro* translation system.

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4D

Development of a high-yield protein expression system based on *Leishmania tarentolae*

Ion Cirstea[1], Susanna Kushnir[1], Sergei Mureev[1], Reinhard Breitling[2], Kirill Alexandrov[*,1]

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Production of functional eukaryotic proteins in recombinant form is a bottle-neck in various post-genomic applications and in life science in general. At least partially this is due to the problems associated with the use of endogenous RNA polymerase II for high-level transcription of heterologous genes in eukaryotic expression systems. To circumvent these problems we developed constitutive and an inducible protein expression systems based on the protozoan host Leishmania tarentolae. We created several expression architectures utilizing transcriptional activity of endogenous RNA polymerase I or heterologous T7 or T3 RNA polymerases. The expression cassettes can be either integrated in to the genome or maintained episomaly as linear or circular elements. The highest expression yields were achieved using the circular artificial chromosome (CAC) . We demonstrate that this element is stably inherited in L.tarentoale even without selection. Using enhanced green fluorescent protein (EGFP) as a model we obtained expression yields of ca. 300mg of recombinant protein per liter of L.tarentolae suspension culture. The system was successfully used for co-expression of two proteins using either two plasmids or a tricistronic vector. Further developments and applications of the system will be discussed.

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4E

RNAi is alive and well in *Leishmania braziliensis*

Stephen M. Beverley*[,1], Katherine Owens[1], Lon-Fye Lye[1], Huafang Shi[2], Christian Tschudi[3], Elisabetta Ullu[3]

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RNA interference (RNAi) is a powerful tool well suited for genetic analysis in trypanosomatids, where many genes occur in families and genetic crossing is impossible or difficult. While this has proven successful in *Trypanosoma brucei*, we showed previously that RNAi did not function in *L. major* or *L. donovani*, and the genomes of *L. major* and *L. infantum* lack the *AGO1* and *DICER* genes. However the *L. braziliensis* genome available on GeneDB (www.genedb.org) shows good homologs of both genes. Thus we tested whether RNAi was active in *L. braziliensis*. First we demonstrated the presence of siRNAs in *L. braziliensis*, from an endogenous retroelement (SLACS) or following expression of an AT-rich GFP hairpin transgene (GFP65-StL). These siRNAs were abundant and the same size as seen in *T. brucei* (24-26 nt). GFP-siRNAs correlated with expression of the GFP65-StL transgene and were not detectable in cells solely expressing a GFP65 mRNA. Then a GC-rich or an AT-rich GFP expression construct (GFP+ or GFP65) was introduced into WT *L. braziliensis* as well as the line expressing the GFP65-StL transgene. High levels of GFP protein and mRNA were evident in only the GFP+/GFP65-StL transfectants, which also lacked 'GFP+' siRNAs. In contrast, the GFP65/GFP65-StL transfectants showed greatly decreased mRNA levels and expressed GFP protein at levels ¡1% of the levels seen in control lines lacking the GFP65-StL transgene. These data confirm the specificity and activity of the RNAi pathway in *Leishmania braziliensis*. We are currently extending these observations to other genes and applications.

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4F

Identification of a large family of short and extincted retroposons located in the 3-UTRs of *Leishmania major* mRNAs

Frédéric Bringaud[*,1], Michaela Müller[2], Gustavo Coutinho Cerqueira[3], Najib M. A. El-Sayed[3], Barbara Papadopoulou[2], Elodie Ghedin[3]

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Transposable elements (TE) (retroposons, LTR retrotransposons and DNA transposons) are DNA sequences capable of moving from one chromosomal region to another. Retroposons constitute the most abundant TE described in the genome of *T. cruzi* (L1Tc, NARTc) and *T. brucei* (*ingi*, RIME) (∼3% of nuclear genome), while no potentially active TE have been characterized to date in *L. major*. TE are often considered as junk or parasitic DNA, however, this view tends to be replaced by a functionalist view supported by a rapidly increasing number of reports. Using the 79 bp signature, common to all trypanosomatid retroposons, as bait we identified in the *L. major* genome two new large families of small elements (∼550 bp), LmSIDER1 (785 copies) and LmSIDER2 (1073 copies) that fulfill all the characteristics of extinct trypanosomatid retroposons. SIDERs are ∼70-times more abundant in *L. major* compared to *T. brucei* and have been found almost exclusively within 3'-untranslated regions of *L. major* mRNAs in contrast to *T. brucei* where most retroposons have been mainly located outside of the polycistronic transcription units. LmSIDERs are ∼35-times more abundant than their extinct LmDIRE autonomous partners (1858 versus 52 copies per haploid genome), which together with the loss of the retrotransposition capacity of LmDIRE, makes these TE unable to propagate anymore. We showed that LmSIDERs fulfill important biological functions such as the regulation of gene expression, whereas *T. brucei* developed other mechanisms to maintain such a cellular function. This is the first example in eukaryotes of the domestication and expansion of a whole family of mobile elements involved in the maintenance of a critical cellular function.

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Transposable elements (TE) (retroposons, LTR retrotransposons and DNA transposons) are DNA sequences capable of moving from one chromosomal region to another. Retroposons constitute the most abundant TE described in the genome of *T. cruzi* (L1Tc, NARTc) and *T. brucei* (*ingi*, RIME) (∼3% of nuclear genome), while no potentially active TE have been characterized to date in *L. major*. TE are often considered as junk or parasitic DNA, however, this view tends to be replaced by a functionalist view supported by a rapidly increasing number of reports. Using the 79 bp signature, common to all trypanosomatid retroposons, as bait we identified in the *L. major* genome two new large families of small elements (∼550 bp), LmSIDER1 (785 copies) and LmSIDER2 (1073 copies) that fulfill all the characteristics of extinct trypanosomatid retroposons. SIDERs are ∼70-times more abundant in *L. major* compared to *T. brucei* and have been found almost exclusively within 3'-untranslated regions of *L. major* mRNAs in contrast to *T. brucei* where most retroposons have been mainly located outside of the polycistronic transcription units. LmSIDERs are ∼35-times more abundant than their extinct LmDIRE autonomous partners (1858 versus 52 copies per haploid genome), which together with the loss of the retrotransposition capacity of LmDIRE, makes these TE unable to propagate anymore. We showed that LmSIDERs fulfill important biological functions such as the regulation of gene expression, whereas *T. brucei* developed other mechanisms to maintain such a cellular function. This is the first example in eukaryotes of the domestication and expansion of a whole family of mobile elements involved in the maintenance of a critical cellular function.

4G

A large retroposon family is involved in the regulation of gene expression in *Leishmania*

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The recent completion of the *Leishmania major, T. brucei* and *T. cruzi* genomes has revealed the presence of LTR retrotransposons and retroposons, but no DNA transposons. We have recently identified in the *L. major* genome two new families of widespread extinct retroposons, LmSIDER1 (Short Interspersed DEgenerated Retroelements) and LmSIDER2 that are predominantly located within 3'-untranslated regions of *Leishmania* mRNAs. Using reporter gene assays, mRNA stability assays and polysome profiling studies we showed that members of the LmSIDER1 family act possibly as translation enhancers by facilitating the binding of mRNAs to highly translated polysomes, whereas LmSIDER2 elements act as negative regulators of gene expression by promoting destabilization of SIDER2-bearing mRNAs. Interestingly, several SIDER2-containing *Leishmania* transcripts are short lived. Experiments using two different translation inhibitors suggest that LmSIDER2-mediated mRNA destabilization involves a labile trans-acting protein factor. A large number of developmentally regulated *Leishmania* transcripts identified by a full-genome microarray analysis carry SIDER1 or SIDER2 elements in their 3'UTR, which suggests a potential link between stage-regulated gene expression and SIDER retroposons. Heat stress seems to trigger regulation by these two classes of retroposon elements. Regulation by SIDER elements could also be modulated to a different extent depending on the *Leishmania* species. The considerable expansion of SIDERs within 3'UTRs and their role in regulating gene expression support our conclusion that *Leishmania*, but not the other trypanosomatids, have recycled and probably expanded these elements to fulfill critical regulatory functions required for their specialized lifestyle. These important findings further emphasize the unique features of posttranscriptional regulation mechanisms in trypanosomatid protozoan parasites.

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4H

Deadenylation of mRNA in *Trypanosoma brucei*

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In Kinetoplastids, gene expression is almost exclusively controlled at the post-transcriptional level. Possible regulation points could be mRNA processing, export from the nucleus, degradation, and translation. Several enzymes responsible for mRNA degradation in trypanosomes have been studied: the exosome catalyses mRNA degradation in 3'-5' direction, XRNA degrades mRNA from the 5' to the 3'-end. Deadenylation - removal of the poly(A) tail - is however usually the first step in degradation. There are three deadenylases in mammals: PARN, the PAN2/PAN3 and the CCR4/CAF1/NOT complex. Putative homologues of all three deadenylases are encoded in the genome of *Trypanosoma brucei* . We could show by RNA interference that the TbPAN2/3 and the TbCCR4/CAF1/NOT complexes are important for normal growth. CAF1 is a highly conserved protein from protists, plants and humans and it is besides CCR4 one of the catalytic subunits of the eukaryotic CCR4/CAF1/NOT complex. The trypanosomal CAF1 is 46% identical with the human protein. Its depletion strongly inhibits decay of stable mRNAs made from housekeeping genes. Notably, however, the degradation of unstable developmentally regulated transcripts is only partially inhibited by CAF1 depletion. This supports the hypothesis that two different pathways are involved in the degradation of these transcripts: one is CAF1-dependent and the other requires XRNA. A bulk poly(A) tail assay shows that deadenylation is affected by CAF1 depletion *in vivo* ; *in vitro* experiments are on-going. We also identified CAF1-associated proteins, including homologues of known components of the CCR4/CAF1/NOT complex. Interestingly, so far it seems that the trypanosomal complex does not contain a CCR4 homologue.

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RNA granules in *Trypanosoma brucei*: unconventional heat-shock stress granules and minimal P-bodies

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Cytoplasmic RNA granules such as P-bodies and stress granules (SGs) have emerged as key players in regulating mRNA translation and decay. mRNA is degraded in P-bodies whereas SGs store mRNAs during stress. mRNAs shuttle between both granules and polysomes. Here, we report that P-bodies and SGs are present in trypanosomes but have different properties to those found in crown-group eukaryotes. SGs appear in trypanosomes during heat shock and azide treatment and disappear on reversing the stress. They localize to the periphery of the cell and contain all proteins located to SGs in other eukaryotes. We have studied the function of trypanosomal stress granules by assaying changes in mRNA stability, polysomes and translation during heat shock in the presence and absence of drugs that distinctly interfere with polysomes, translation and SG formation (cycloheximide and puromycin). Interestingly, our data indicate that trypanosomal SGs, unlike SGs in yeast or mammals, are involved in mRNA degradation rather than mRNA storage. Homologues to most classical P-body markers are either absent from the genome (DCP2) or do not localize to cytoplasmic granules in the absence of stress (PABP1-2, eIF4E1-4, Ccr4, XrnA). Thus, trypanosomal P-bodies might be divergent. However, two components, DHH1 and SCD6, do localize to cytoplasmic granules that possess all classical characteristics of P-bodies. Moreover, RNAi knock-down of either slightly increases stability of mRNAs, suggesting an involvement of P-bodies in mRNA degradation. Upon heat shock, the P-body components DHH1 and SCD6 colocalize with SGs. Interestingly, RNA binding mutants of DHH1 fail to localize to P-bodies but can still localize to SGs, suggesting different targeting mechanisms to the two types of granules.

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4J

ABSTRACTS: SESSION V

Cell & Life Cycles

April 25 1:30 p.m.

Chair(s) - Michael Boshart

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Identification and functional characterisation of a stumpy-specific protein implicated in citrate/cis aconitate dependent-differentiation in *Trypanosoma brucei*

Samuel D. Dean[1], Rosa Marchetti[2], Kiaran Kirk[2], Keith Matthews[*,1]

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[2] School of Biochemistry and Molecular Biology, Australian National University, Canberra, Australia
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Bloodstream trypanosomes initiate differentiation to procyclic forms in response to a citrate/cis aconitate (CCA) signal. We previously isolated a trypanosome line (defective in differentiation-clone 1; DiD1) unable to differentiate to procyclic forms (Tasker *et al*, 2000). Expression profiling of this line in comparison to the parental line by macroarray hybridisation identified a differentially-expressed transcript we named PAD1 (Protein Associated with Differentiation 1). PAD1 is a member of a closely-related gene cluster whose members show differential expression in the life-cycle, and bioinformatics analysis suggests that PAD1 may contribute to recognition of the CCA-differentiation signal. A number of additional lines of evidence support this. Firstly, PAD1 mRNA is highly expressed in differentiation-adapted stumpy cells but not in early slender cells. Secondly, PAD1 protein is located on the surface of bloodstream stumpy forms. Thirdly, gene knock-out of PAD1 compromises the capacity of bloodstream forms to differentiate in response to CCA. Finally, biochemical evidence indicates a specificity of this protein for CCA. These combined expression, cytological, reverse-genetic and biochemical data make PAD1 an excellent candidate for recognition of the signal to initiate differentiation in response to CCA. In addition, PAD1 provides the first surface marker protein identified that allows recognition of stumpy forms during their acquisition of competence to differentiate.

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5B

Visualizing genetic exchange in *Trypanosoma brucei* in the tsetse vector by fluorescence

Wendy Gibson[*,1], Lori Peacock[1], Vanessa Ferris[1], Bailey Mick[2]

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Trypanosoma brucei undergoes genetic exchange in its insect vector, the tsetse fly, by an unknown mechanism. Genetic crosses of red (mRFP) and green (GFP) parental trypanosomes produced progeny with red, green, yellow or no fluorescence. Genotypes of progeny clones were checked by microsatellites and PFGE; all progeny clones had non-parental genotypes, and inheritance of microsatellite alleles was consistent with Mendelian expectations for F1 progeny of the parental genotypes. However, while most progeny were diploid, a significant number were polyploid. Inheritance of kinetoplast DNA maxicircles was usually uniparental, although biparental inheritance was also observed; minicircle networks always showed biparental inheritance. Genetic exchange happened readily in this cross, as indicated by frequent occurrence of yellow trypanosomes in flies with mixed red and green salivary gland infections. The first yellow trypanosomes appeared in the salivary glands 13 days after infection of flies with bsf trypanosomes, implicating the epimastigote as the lifecycle stage that undergoes genetic exchange. Intermediate stages have so far proved elusive, possibly because they are rare or transient. In crosses of red and green clones of a single parental strain, occasional yellow trypanosomes were observed in the salivary glands with a mixed infection of red and green trypanosomes, but no hybrid genotypes were recovered after double drug selection. In contrast, the F1 progeny from the two-strain cross readily produced hybrids both in F1 crosses and backcrosses. Taken together, these results suggest that trypanosomes have the ability to distinguish individuals of the same or a different strain, but can mate with closely related individuals. A simple two-factor model to explain these results predicts that F1 progeny should be capable of intraclonal mating.

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5C

Macroautophagy in *Leishmania major* is not required for metacyclic shrinkage but contributes to the metacyclogenesis

Christine Wang, Luciana Madeira, Kai Zhang, Stephen M. Beverley*

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In the midgut of its sand fly vector, procyclic *Leishmania* parasites actively divide and differentiate into infective metacyclic forms. Metacyclogenesis involves a number of changes in parasite gene expression, morphology and structure. Previously we showed that the volume of metacyclics was about 10-fold less than procyclic promastigotes, a change we term shrinkage. There are several mechanisms that could underlie shrinkage, and we investigated the role of macroautophagy, a well characterized eukaryotic pathway for cell size control and other processes including differentiation. As a marker for autophagosome formation, we generated a GFP-ATG8 fusion reporter gene and showed that this specifically labeled autophagosomes soon after starvation; subsequently, it tracked to the multivesicular tubule/lysosome. Upon entry into stationary phase and initiation of metacyclogenesis, the GFP-ATG8 marker relocated from the cytosol to punctate autophagosome and then MVT locations as expected. Having confirmed the existence of a eukaryotic-like macroautophagy pathway, we then inactivated two key macroautophagy genes, *ATG7* and *ATG8*. In the *atg7-* mutant, the GFP-ATG8 reporter failed to traffic to autophagosomes upon starvation or differentiation, confirming that *atg7-* disrupted macroautophagy. Both *atg7-* and *atg8-* mutants showed similar phenotypes; the percentage of metacyclic formation declined 10-100 fold but was not abolished. However, 'shrinkage' seen within stationary phase or metacyclic parasites remained like WT, and upon inoculation into susceptible mice both mutants retained the ability to induce lesion pathology. All phenotypes were rescued by restoration of *ATG7* and *ATG8* expression. Therefore, our data suggests that macroautophagy contributes to metacyclogenesis in *L. major* but does not play an essential role, either in virulence or in parasite shrinkage.

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5D

Histone H4 K4 is cell-cycle regulated and acetylated by HAT3

T. Nicolai Siegel[1], Taemi Kawahara[2], Christian J. Janzen[3], David Horn[2], George A. M. Cross[*,1]

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Posttranslational histone modifications, in particular methylations, acetylations, and phosphorylations, have been studied intensively in several eukaryotes. It has been proposed that these modifications constitute a so-called 'histone code', which contains epigenetic information for transcription regulation. Deciphering this 'code' has been complicated by the large number of modifications and histone-modifying enzymes resulting in a high degree of redundancy. Given the prospect of a more limited number of histone-modifying enzymes, implying less redundancy, *Trypanosoma brucei* may represent an excellent organism to understand better the function of individual histone modifications and histone-modifying enzymes. Histone modifications also are likely to play a crucial role in regulating the expression of certain classes of genes in *T. brucei*. In this study, we characterized the role of acetylation on lysine (K) 4 of histone H4, the most highly acetylated site in *T. brucei* histones. We generated highly specific polyclonal antibodies to both the acetylated and unmodified K4, as well as a general H4 antibody. IF analysis revealed cell-cycle-dependent regulation of K4 acetylation and masking of the K4 epitope during G1. Western blots of FACS-sorted cells suggested that the G1-specific masking is due to a non-covalent interaction, probably by a binding protein. Finally, we showed that histone acetyltransferase 3 (HAT3) is responsible for K4 acetylation, but no deacetylase could be identified. Treatment of cells with the protein synthesis inhibitor cycloheximide led to an almost instantaneous loss of unmodified K4 sites. Given that HAT3 is found exclusively inside the nucleus, our findings suggest that newly synthesized histones are being imported into the nucleus with an unmodified H4K4, and acetylated rapidly, possibly irreversibly, once inside the nucleus.

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5E

Trypanosoma cruzi histone H4 is acetylated at specific lysines after DNA damage

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Histone tails provide sites for a variety of post-translational modifications that are involved in the control of gene expression, replication, chromatin assembly and DNA repair. In *Trypanosoma cruzi*, the agent of Chagas' disease, both histones and the control of gene expression are highly divergent compared to most eukaryotes. We have previously shown by mass spectrometry analysis that histone H4 lysine residues 4, 10, and 14 are acetylated. Antibodies against these acetylated lysines were produced and used here to show that lysines 4, 10 and 14 acetylations are maintained at different life stages of the parasite. As distinct transcriptional properties are characteristic of these stages, K4, K10 and K14 acetylations are not proportional to the transcriptional state. Acetylation is proportional to histone H4 amounts during the G1, S, G2 and mitosis indicating that they occur during parasite replication. Only, a modest increase in K10/K14 acetylation is observed during G2/mitosis. In contrast, these latter residues are largely acetylated 6 hours after gamma irradiation, while K4 acetylation is significantly reduced. Maximal K10/14 acetylation levels occur 48 hours after irradiation, decreasing when the parasite starts to replicate. No increase in acetylation occurs after hydrogen peroxide or ultraviolet exposure, suggesting that specific histone H4 acetylations occur during double strand break DNA repair in *T. cruzi*. Supported by FAPESP, CNPq.

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Histone tails provide sites for a variety of post-translational modifications that are involved in the control of gene expression, replication, chromatin assembly and DNA repair. In *Trypanosoma cruzi*, the agent of Chagas' disease, both histones and the control of gene expression are highly divergent compared to most eukaryotes. We have previously shown by mass spectrometry analysis that histone H4 lysine residues 4, 10, and 14 are acetylated. Antibodies against these acetylated lysines were produced and used here to show that lysines 4, 10 and 14 acetylations are maintained at different life stages of the parasite. As distinct transcriptional properties are characteristic of these stages, K4, K10 and K14 acetylations are not proportional to the transcriptional state. Acetylation is proportional to histone H4 amounts during the G1, S, G2 and mitosis indicating that they occur during parasite replication. Only, a modest increase in K10/K14 acetylation is observed during G2/mitosis. In contrast, these latter residues are largely acetylated 6 hours after gamma irradiation, while K4 acetylation is significantly reduced. Maximal K10/14 acetylation levels occur 48 hours after irradiation, decreasing when the parasite starts to replicate. No increase in acetylation occurs after hydrogen peroxide or ultraviolet exposure, suggesting that specific histone H4 acetylations occur during double strand break DNA repair in *T. cruzi*. Supported by FAPESP, CNPq.

5F

Tousled-like kinase in Trypanosoma brucei regulates spindle assembly and S-phase progression

C. C. Wang*, Ziyin Li, Stephane Gourgurchon

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The Tousled-like kinases are an evolutionarily conserved family of proteins implicated in DNA repair, DNA replication and mitosis among metazoan and plants. Their absence from the yeasts and other eukaryotic microbes postulated a specific role for them in the development of multi-cellular organisms. In this study, two closely related tousled-like kinase homologs TbTLK1 and 2 were identified in *Trypanosoma brucei,* a unicellular protozoan parasite. Only TbTLK1 plays an essential role in cell growth, whose depletion led to an enrichment of S-phase cells, defective spindle formation and aberrant chromosome segregation. Though both TbTLKs localize to the nucleus, only TbTLK1 also concentrates in the spindle poles during mitosis. Both TbTLKs are phosphorylated by the Aurora kinase (TbAUK1), can autophosphorylate and phosphorylate histone H3, the chromatin assembly factors TbAsf1A and TbAsf1B *in vitro,* but only TbTLK1 is autophosphorylated and capable of oligomerizing and interacting with TbAUK1, TbAsf1A and TbAsf1B *in vivo.* These discrepancies between the two TbTLKs are attributed to minor differences between their N- and C-terminal sequences. All together, TbTLK1 cooperates with Aurora kinase to regulate spindle assembly and chromosome segregation, and affects DNA replication and chromatin structure likely by regulating histone modification in trypanosomes.

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5G

ABSTRACTS: SESSION VI

Kinetoplast Structure & Expression

April 26 8:30 a.m.

Chair(s) - Dan Ray

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Kinetoplast Structure & Expression

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New views of kinetoplast structure

Eva Gluenz*, Michael K. Shaw, Keith Gull

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Kinetoplastid mitochondrial DNA (kDNA) is organised into a network of concatenated mini and maxicircles, forming a complex structure termed kinetoplast, positioned at the base of the flagellum, to which it is physically attached. To understand fully the mechanisms of kDNA replication, segregation, transcription and RNA editing there is a need to develop descriptions of kinetoplast nucleic acid and protein component locations at the electron microscopic level of resolution. Here we have used electron microscope cytochemistry to determine structural and functional domains involved in replication and segregation of the *Trypanosoma brucei* kinetoplast and to present new views of kinetoplast structure. We identified two distinct subdomains within the kinetoflagellar zone (KFZ) and show that the unilateral filaments are composed of distinct inner and outer filaments. Ethanolic phosphotungstic acid (E-PTA) and EDTA regressive staining show that basic proteins and DNA are major constituents of the inner unilateral filaments adjoining the kDNA disc. This evidence for an intimate connection of the unilateral filaments in the KFZ with DNA provides support for models of minicircle replication involving vectorial export of free minicircles into the KFZ. Unexpectedly however, the KFZ contains DNA throughout the cell cycle, suggesting that this domain is the site of other processes involving kDNA. Finally, we describe a hitherto unrecognised, intramitochondrial, filamentous structure rich in basic proteins that links the kDNA discs during their segregation and is maintained between them for an extended period of the cell cycle. We discuss our findings in light of current models of kinetoplast replication and function in different kinetoplastids.

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6B

Functional role and regulation of universal minicircle sequence binding protein during kDNA replication and segregation

Neta Milman[1], Shawn A. Motyka[2], Paul T. Englund[2], Derrick R. Robinson[3], Joseph Shlomai[*,1]

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Replication of kinetoplast DNA (kDNA) minicircles initiates at conserved origin sequences that are bound specifically by the universal minicircle sequence binding protein (UMSBP). Knocking down the two UMSBP encoding genes in *Trypanosoma brucei* (*TbUMSBP1* & *TbUMSBP2*) by RNAi, results in the cells growth arrest and a significant decreased rate of minicircles replication initiation, supporting a role for UMSBP in this process. Remarkably, these analyses have also suggested a novel, post replication, role for UMSBP in trypanosomes. Fluorescent microscopy analysis of *T. brucei* cells after RNAi induction of both *tbUMSBP* genes revealed the generation of giant kinetoplasts and significantly enlarged nuclei in these cells. Analysis of kDNA networks isolated from these cells revealed a dramatic increase in the networks size. Electron microscopy analysis demonstrated the generation of large kDNA networks that apparently failed to segregate. Interestingly, analyses of UMSBP activity in synchronized *Crithidia fasciculata* cell cultures showed that UMSBP activity, which is regulated *in vivo* through a cell cycle dependent control of the protein redox state, displays cycling of UMSBP activity with peaks during S phase and during M phase of the cell cycle. Searching for an enzymatic mechanism that may function in the redox mediated regulation of UMSBP, we have found that UMSBP reduction by tryparedoxin enabled its interaction with the replication origin. Recently, we have observed that a 2-Cys tryparedoxin peroxidase is capable of oxidizing UMSBP *in vitro*, inhibiting the binding of UMSBP to DNA, while tryparedoxin peroxidase is being reduced during this process. We have immunolocalized a 2 Cys tryparedoxin peroxidase that could potentially function in this reaction, at the kDNA region within the *C. fasciculata* kinetoplast.

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6C

Elongation factor 1a determines the specificity of mitochondrial tRNA import in *T. brucei*

Eric Aeby, Nabile Bouzaidi-Tiali, Fabien Charrière, André Schneider*

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The mitochondrial genome of many eukaryotes lacks a variable number of tRNA genes. In these cases, the missing tRNAs are compensated for by import of a small fraction of the corresponding cytosolic tRNAs. An intriguing situation is found in *Trypanosoma brucei*, which has lost all mitochondrial tRNA genes. Thus, all mitochondrial tRNAs derive from cytosolic tRNAs that are imported into mitochondria. Yet, the mechanism that determines the specificity of the process is unknown. The T-stem nucleotide pair 51:63 is the main localization determinant in tRNAs of *T. brucei*. In the cytosol-specific initiator $tRNA^{Met}$ this nucleotide pair is identical to the main antideterminant that prevents interaction with cytosolic elongation factor (eEF1a). Here we show that ablation of cytosolic eEF1a, but not of initiation factor 2, inhibits mitochondrial import of newly synthesized tRNAs well before translation or growth is affected. $tRNA^{Sec}$ is the only other cytosol-specific tRNA in *T. brucei*. It has its own elongation factor and does not bind eEF1a. However, a mutant of the $tRNA^{Sec}$ expected to bind to eEF1a is imported into mitochondria. This import requires eEF1a, since it is prevented after RNAi mediated ablation of eEF1a. In addition, if we block aminoacylation of the import competent $tRNA^{Sec}$ variant by replacing the discriminator nucleotide, the $tRNA^{Sec}$ variant cannot be imported anymore even in the presence of eEF1a. Only aminoacylated tRNAs can bind to eEF1a. Thus, for a tRNA to be imported into the mitochondrion of *T. brucei* it needs to bind eEF1a and it is this interaction that determines the import specificity.

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6D

RBP16 stimulates RNA editing and annealing *in vitro* by a mechanism independent of high affinity RNA binding

Michelle L. Ammerman, Laurie K. Read*

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RBP16 is an accessory factor that regulates editing of mitochondrial CYb mRNA *in vivo* and also stimulates editing *in vitro*. To understand the mechanism by which RBP16 modulates editing, we analyzed its ability to stimulate RNA annealing. We present evidence that RBP16 significantly stimulates the annealing of gRNAs to cognate pre-mRNAs *in vitro*. In addition, RBP16 also facilitates hybridization of partially complementary RNAs unrelated to the editing process. Although RBP16 binds gRNAs primarily through their oligo(U) tails, the presence of an oligo(U) stretch does not impact its ability to stimulate annealing of partially complementary RNAs. We further examined the activity of a RBP16 protein mutated in its cold shock domain (CSD) that is severely compromised in (CSD)-mediated RNA binding. The CSD mutant is capable of stimulating both RNA annealing and editing at levels comparable to the wild-type protein. These results demonstrate that the RNA annealing activity of RBP16 is independent of binding to the gRNA oligo(U) tail. Moreover, these data support a model in which RBP16 stimulates RNA editing activity by facilitating gRNA/pre-mRNA hybridization.

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6E

Substrate recognition by trypanosome RNA editing complexes is governed by RNase III-like requirements

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A unique post-transcriptional RNA editing process by uridine insertion/deletion mediates the maturation of primary pre-edited mRNAs (pre-mRNAs) in kinetoplastid mitochondria. The editing sites (ESs), specified by helical irregularities between pre-mRNAs and small complementary guide RNAs (gRNAs), are targeted by megadalton protein assemblies via unknown mechanisms of substrate recognition and site specificity. We recently reported the first observations of direct interactions of purified editing complexes from *T. brucei* with model ESs (1, 2). Such studies, using site-specific photo-reactive substrates, also showed that editing complexes exhibit a preferential recognition for pre-mRNA/gRNA hybrids, and that the association is primarily based on substrate secondary structure. We will present our initial identification of the RNA-crosslinking subunits in assembled complexes, including an RNase III-type endonuclease that cleaves ESs and initiates catalysis. Furthermore, our characterization of substrate features for editing complex docking defined a minimal model structure that support efficient association. We scored the editing complex enzyme/substrate interactions in competition analyses using our recently established electrophoretic mobility shift assay (EMSA), in parallel with photo-crosslinking and pre-mRNA cleavage assays. Importantly, common substrates and reaction conditions for all three assays yield complementary data, and the shift assay is allowing us to perform the first studies of assembled ribonucleoprotein complexes. Our current data support a working model in which RNase III-like requirements lay at the heart of the initial substrate recognition by trypanosome editing complexes. 1- Sacharidou. A., et al (2006). RNA 12(7) 1219-28. 2- Cifuentes-Rojas C., et al. (2007). J Biol Chem. 2007. 282:4265-76.

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6F

Novel protein produced by alternative mRNA editing in trypanosomes is involved in mitochondrial genome maintenance

Sedrick Anderson, Torsten Ochsenreiter, Stephen Hajduk*

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Trypanosomes use RNA editing to produce functional mitochondrial mRNAs. Precise insertion and deletion of hundreds of uridines is necessary to make full-length cytochrome c oxidase III (COXIII) mRNA. We show that COXIII mRNA can be alternatively edited by a mechanism using an alternative guide RNA to make a stable mRNA. This alternatively edited mRNA is translated to produce a unique protein (AEP-1) that fractionates with mitochondrial membranes and co-localizes with mitochondrial proteins. AEP-1 is identical to the C-terminus of COXIII but possesses a unique N-terminus. Alternative RNA editing represents a previously unknown mechanism generating protein diversity and thus, represents an important function for RNA editing. To address the function of this novel protein we first, fractionated trypanosomes expressing AEP-1 and found it was associated with a high molecular weight complex (∼500kDa) and was tightly bound to the base of the trypanosome flagellum. Three-dimensional reconstruction of the complex supports a role for AEP-1 within the tripartite attachment complex that facilitates the segregation of the trypanosome kinetoplast DNA during cell division. Second, to examine the role of AEP-1 in vivo we expressed the N-terminus of AEP-1 as a GFP-fusion protein in the nucleus and targeted it to the mitochondrion using a mitochondrial import sequence. Transfected cells show a dramatic growth defect and a significant increase in cells that have lost their kinetoplast DNA. Immunofluorescence microscopy shows that the ectopically expressed N-terminus of AEP-1 localizes to the kinetoplast and therefore might compete with the endogenous membrane bound AEP-1. Based on these data we propose a model in which AEP-1 is involved in segregation of mitochondrial DNA in trypanosomes during cell division.

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6G

ABSTRACTS: POSTER SESSIONS

A SERIES: April 24 1:30 p.m.

B SERIES: April 25 7:00 p.m.

ABSTRACTS: POSTER SESSIONS

A SERIES: April 24 1:30 p.m.

B SERIES: April 25 7:00 p.m.

A novel Rho-like GTPase of *Trypanosoma brucei* is required for nuclear stability and karyokinesis

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Abstract. Characterization of intracellular signaling pathways in trypanosomatids remains poor. We are investigating the functions of small GTPases that likely contribute to signal transduction. Rho GTPases are members of the Ras superfamily and are involved in signal transduction pathways, including maintenance of cell morphology and motility, cell cycle progression, and transcription activation. In *Trypanosoma brucei* there are no true members of the Ras or Rho subfamilies but divergent Ras- and Rho-like GTPases are present. In the present study, the highly divergent Rho GTPase, TbRHP, was characterized by localization and RNAi knockdown in both bloodstream and procyclic forms. In bloodstream form cells TbRHP-RNAi resulted in defects in nuclear division, cell-arrest at the G_2/M phase and generated cells with an enlarged nucleus. By contrast, in procyclic cells TbRHP-RNAi gave a multi-nucleated cell phenotype. However, the abnormal morphology of the nucleus in these cells cannot be explained by a defect in chromatin structure. Immunofluoresence studies localized the protein to distinct subcompartments of the nucleus, and showed that the amount of protein varied throughout the cell cycle, remaining in a constant ratio to the level of DNA in the cell. TbRHP also has an extremely short half-life. Phenotypically the effect of TbRHP knockdown is highly similar to that obtained for Aurora kinase (TbAUK) RNAi, and together with evidence for interaction between Aurora B and RhoA in higher eukaryotes, we suggest that TbRHP and TbAUK may function in the same pathway to regulate cytokinesis.

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200A

Proteomic analysis of *Trypanosoma brucei* mitochondrial membrane fractions

Nathalie Acestor, Aswini K. Panigrahi, Rachel Burnham, Alena Zíková, Yuko Ogata, Atashi Anupama, Peter J. Myler, Kenneth D. Stuart*

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T. brucei mitochondrion consists of four compartments, outer membrane, inter-membrane space, inner membrane and matrix; each harboring specific functions and structures. To obtain complete information on mitochondria-related functions, compositional analyses of the various sub-mitochondrial compartments are of high importance. We are undertaking experiments to identify the proteins present in different compartments of mitochondria and this study is focused on membranes. We used different lysis conditions and biochemical fractionation to enrich for mitochondrial membranes, and the proteins in these fractions were identified by LC-MS/MS analysis. We are also characterizing the composition of multi-protein complexes within mitochondrial membranes using affinity purification and mass spectrometry techniques. Analysis of inner-membrane enriched fraction identified 187 proteins of which about 50% are currently annotated as hypothetical proteins in GeneDB database. Sequence comparison with TMHMM database identified one or more trans-membrane domains in 57 proteins indicating they are localized to membranes. Ten of these proteins were tagged and analyzed and the results showed one of these (TAP041) is associated within a mitochondrial complex of unknown function that is composed of at least 10 proteins. The results also indicate the tagged protein is integrated into the mitochondrial inner membrane and the complex have sub-units specific to membrane and matrix fractions. The transgenic cell lines containing tagged proteins are explored to isolate different membrane fractions for further proteomic analyses. The data from these experiments will be presented and discussed.

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T. brucei mitochondrion consists of four compartments, outer membrane, inter-membrane space, inner membrane and matrix; each harboring specific functions and structures. To obtain complete information on mitochondria-related functions, compositional analyses of the various sub-mitochondrial compartments are of high importance. We are undertaking experiments to identify the proteins present in different compartments of mitochondria and this study is focused on membranes. We used different lysis conditions and biochemical fractionation to enrich for mitochondrial membranes, and the proteins in these fractions were identified by LC-MS/MS analysis. We are also characterizing the composition of multi-protein complexes within mitochondrial membranes using affinity purification and mass spectrometry techniques. Analysis of inner-membrane enriched fraction identified 187 proteins of which about 50% are currently annotated as hypothetical proteins in GeneDB database. Sequence comparison with TMHMM database identified one or more trans-membrane domains in 57 proteins indicating they are localized to membranes. Ten of these proteins were tagged and analyzed and the results showed one of these (TAP041) is associated within a mitochondrial complex of unknown function that is composed of at least 10 proteins. The results also indicate the tagged protein is integrated into the mitochondrial inner membrane and the complex have sub-units specific to membrane and matrix fractions. The transgenic cell lines containing tagged proteins are explored to isolate different membrane fractions for further proteomic analyses. The data from these experiments will be presented and discussed.

Analysis of expression profile of folate transporters gene family in *Leishmania* wild-type and methotrexate-resistant cell lines

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Folate transporters have been of considerable interest in *Leishmania* since these parasites lack the enzymes necessary for de novo folate synthesis and thus rely entirely on folate transport from their host cells. Three distinct folate transporters (BT1, FT1 and FT5) from different *Leishmania* species have been characterized and they define the BT/FT family. The completed *Leishmania* genome has revealed 11 other members that are part of this family. The expression pattern of these genes was assessed by a TaqMan real-time RT-PCR throughout the life stage and life cycle of *L. infantum*. Surprisingly, the expression of only FT1 (LinJ10.0700) was found to be maximal in the log phase of growth and this was more evident in the promastigote stage of the parasite. Interestingly, FT1 expression varied with folic acid concentration, suggesting that *Leishmania* can modulate FT RNA levels depending on substrate availability. Several genes were up-regulated in stationary phase, a life stage with no measurable folate accumulation. LinJ10.0680 is the most strongly up-regulated gene in stationary phase, and will be characterized to assess its function. At least two genes (LinJ10.0670, FT5) seem to be preferentially expressed in amastigote form. Stage specific gene expression is similar between *L. infantum* and *L. major*, suggesting the same FT gene regulation mechanisms in these parasites. In methotrexate *L. infantum* resistant mutants, the expression of several FT members was also modulated. FT1 expression was down regulated in two independent mutants and, interestingly, the expression of some genes was up-regulated. Cloning and expression of these genes is currently underway. This study highlights the multiplicity of FT genes in *Leishmania* and the strong regulation governing their expression.

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201A

Activation of the endocytic system as a component of adaptation to the mammalian host in the African trypanosome

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Immune evasion in African trypanosomes is mediated principally by antigenic variation, but rapid internalization/degradation of surface-bound immune factors likely contributes to longevity in the host bloodstream. Endocytosis is up-regulated ∼10-fold in bloodstream forms compared to procyclic forms and the surface coat is remodelled between the major life stages. Specifically variant surface glycoprotein (VSG) synthesis is repressed on entering the fly and only reactivated at the metacyclic stage in the salivary glands. We examined the expression of endocytic system markers throughout the trypanosome life cycle *in vivo* and monitored the alterations that occur during transition from bloodstream form to procyclics *in vitro*. Firstly, we find no evidence for altered expression of endocytic system markers in stumpy stages, and therefore the stumpy form likely has endocytic activity similar to rapidly dividing slender forms. Secondly, differentiation to procyclics *in vitro* is accompanied by rapid down-regulation of Rab11, with delayed decrease in levels of clathrin, suggesting two-phase modulation of endocytic and recycling systems. Thirdly, there is a rapid down-regulation of endocytic markers upon entering the insect host, and within 24 hours expression decreases to levels identical to procyclic stages in culture. Expression remains low throughout subsequent development in the fly, indicating that high levels of clathrin-mediated endocytic activity are probably not involved in combating insect defence mechanisms or in remodelling of the parasite. By contrast, expression of clathrin and Rab11 is upregulated in metacyclic forms, indicating that reemergence of mammalian infective forms is coupled to re-acquisition of a highly active endocytic system.

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201B

Spliceosomal proteomics revealed several new candidate small nuclear RNP proteins in *Trypanosoma brucei*

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In trypanosomatid parasites, spliced leader (SL) *trans* splicing and polyadenylation resolve individual mRNAs from polycistronic precursor RNA. Since SL *trans* splicing does not occur in mammalian and insect hosts of trypanosomatids and since this group of organisms has diverged very early in evolution from the main eukaryotic lineage, we hypothesize that the trypanosomatid spliceosome harbors unique proteins or highly divergent orthologues of known proteins which are essential for the *trans* splicing process. A commonality of the spliceosomal small nuclear ribonucleoprotein particles (snRNPs) U1, U2, U4/U6 and U5 as well as the SL RNP, is a complex of seven proteins called Sm or common proteins. By fusing the PTP tag (Protein A tandem domain, TEV protease cleavage site, Protein C epitope) C-terminally to the SmD1 orthologue of *Trypanosoma brucei*, which is shared by all spliceosomal snRNPs, we were able to tandem affinity-purify components of the spliceosome. 41 proteins which co-purified with SmD1-PTP were identified by liquid chromatography-tandem mass spectrometry; these included all Sm proteins, the known snRNP-specific proteins of *T. brucei* and 16 proteins which have been annotated as *conserved hypothetical*. We chose three proteins from the latter category for further analysis because they either harbored a relevant sequence motif such as an RRM binding domain or exhibited a remote similarity to the known SL RNP-specific proteins of *Ascaris*. Having epitope-tagged these proteins, we are now in the process of determining their snRNA binding partners and of analyzing their functional relevance in parasite growth and the *trans* splicing process.

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202A

Novel RNA Uridylyltransferases and Non-canonical Poly(A) Polymerases of Trypanosomes

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The terminal RNA uridylyltransferases (TUTases) are phylogenetically widespread enzymes that catalyze transfer of UMP residues to the 3′ hydroxyl group of RNA. Extensive uridine insertion/deletion editing in mitochondria of trypanosomatids requires 3′ uridylylation of guide RNAs by RNA editing TUTase 1 (RET1) and insertion of Us into messenger RNAs by RNA editing TUTase 2 (RET2). We have identified several new members of TUTase family playing key roles in mitochondrial mRNA processing. Minor Editosome Associated TUTase 1 (MEAT1) shares limited homology with RNA editing TUTases but lacks the middle domain found in RET1 and RET2. The UTP-specific recombinant enzyme exhibited guide-RNA depended insertion activity in vitro. In mitochondrial extracts, MEAT1 was present in unassociated and editosome-bound forms. The analysis of the affinity-purified MEAT1 complex revealed a particle that closely resembles the 20S editosome lacking the entire U-insertion sub-complex. RNA interference knockdown of MEAT1 expression produced growth inhibition phenotype in procyclic and bloodstream forms of T. brucei suggesting a supplementary function in U-insertion editing, which is essential for parasite's viability. Kinetoplastid Poly(A) Polymerases 1 (kPAP1) is a mitochondrial PAP closely related to editing TUTases. This enzyme is responsible for stabilization of translation-competent (non-edited and fully edited) mitochondrial mRNAs. Depletion of kPAP1 resulted in rapid loss of functional mRNA pool and cell death. The potential role of polyadenylation in regulation of mitochondrial genome expression will be discussed. Crystallographic analysis of trypanosomal TUTase 4 with bound UTP and RNA substrates generated a structural model in which a TUTase-like domain organization and active site are conserved among TUTases and TUTase-like poly(A) polymerases. These findings reconcile UTP/ATP specificities within TUTase protein family.

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202B

Characterization of a ligand-specificity determinant in a protozoan purine nucleoside transporter

Cassandra S. Arendt, Buddy Ullman*

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Parasitic protozoa are completely dependent upon salvage of purines from the host milieu to meet their essential nutritional needs for nucleotides. Uptake of purine nucleosides and nucleobases is performed by members of the equilibrative nucleoside transporter (ENT) family in these organisms. These transporters are also provide an entry point for purine-like anti-parasitic agents. ENTs are distinguished by the presence of eleven transmembrane segments connected mostly by short extramembranous loops. This structural arrangement suggests that the binding of ligand is likely to occur within an aqueous pore formed by the protein's membrane-spanning helices. High-affinity kinetoplastid ENTs typically have narrow ligand specificities; however, the structural basis for this specificity (i.e., the content and arrangement of amino acids in the ligand binding site) is unknown. An understanding of the shape of the ligand binding site and/or how ligand is contacted may inform rational design of drugs that could be transported by ENTs. In an attempt to locate ligand-binding amino acid residues within the structure of one such transporter - CfNT2, an inosine-guanosine transporter from *Crithidia fasciculata* - a genetic screen for gain-of-function mutants was performed in *Saccharomyces cerevisiae*. Mutation of lysine 155 (K155) of CfNT2 gave rise to transporters that were able to transport adenosine and its toxic analog tubercidin while simultaneously decreasing affinity for the natural ligand inosine. Studies using site-specific modifying reagents and purine analogs suggest that K155 is located in or near the ligand binding site and may be directly responsible for discrimination between 6-oxo and 6-amino purine nucleosides in the wild-type transporter.

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203A

The LAMP-like protein p67 plays an essential role in the lysosome of African trypanosomes

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p67 is a LAMP-like type I lysosomal transmembrane glycoprotein in African trypanosomes. There are two copies of the p67 gene on Chromosome V (Tb927.5.1810 & Tb927.5.1830) separated by a 60S ribosomal protein subunit gene (Tb927.5.1820). Therefore, in order to study p67 function an RNAi knockdown approach was employed to specifically ablate protein expression. Conditional induction of p67 dsRNA resulted in specific ∼90% reductions in de novo p67 synthesis in both mammalian bloodstream and procyclic insect stage parasites. There was minimal effect on expression of endogenous HSP70, nor did RNAi knock down of ornithine decarboxylase affect p67 expression. Procyclic cell growth was not affected by p67 knockdown, but bloodstream cell growth was severely retarded with extensive death apparent after 24 hours of induction. Biosynthetic lysosomal trafficking of residual p67, and of the soluble cathepsin-L-like lysosomal protease trypanopain, were unimpaired. Endocytosis of tomato lectin, a surrogate receptor-mediated cargo, was only mildly impaired (∼20%), but proper lysosomal targeting was unaffected. p67 ablation had dramatic effects on lysosomal morphology with gross enlargement and internal membrane profiles reminiscent of autophagic vacuoles. Surprisingly, ablation of p67 expression rendered bloodstream trypanosomes refractory to lysis by human trypanolytic factor, a lysosomally activated host innate immune mediator. Similar effects on lysosomal morphology and sensitivity to trypanolytic factor were also obtained by pharmacological agents known to neutralize lysosomal pH, i.e., chloroquine and bafilomycin A1. Efforts are under way to directly measure the effect of p67 depletion on lysosomal pH. Collectively these results suggest that p67 plays an essential role in maintenance of normal lysosomal structure and physiology in bloodstream-stage African trypanosomes.

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Bioinformatic analysis of *Trypanosoma brucei* ESAG5 reveals a relationship to a lipid-interactive protein superfamily

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Bloodstream Expression Sites (ESs) in *T. brucei* contain a series of Expression Site Associated Genes (ESAGs). Intriguingly, the smallest known ES contains only the *VSG, ESAGs 6* and *7* (encoding a heterodimeric transferrin receptor), *ESAG5* and *SRA* genes. The SRA protein is known to mediate human serum resistance. The function of ESAG5 is unknown. In the T. brucei genome, we have identified a family of *ESAG5*-like genes comprising 5 chromosome-internal copies in addition to those in ESs. While ES copies show high levels of sequence identity, non-telomeric genes are much more divergent. Comparison with other trypanosomatid genomes identifies orthologues of each chromosomal-internal gene in at least one other trypanosomatid organism. Further bioinformatic analysis predicts a glycosylated protein with either a signal peptide or transmembrane helix at the N-terminus. Though no homologues in other organisms could be identified by BLAST searches, more sensitive iterative searches suggest this gene family may be distantly related to the mammalian lipid binding/lipid transfer superfamily. This family includes Bactericidal/Permeability-Increasing Protein (BPI) and the Cholesterol Ester Transfer Protein (CETP) which share a similar three-dimensional fold, despite low sequence identity between family members and no conserved motifs. Two-dimensional structure analysis confirms a possible relationship between ESAG5 and BPI and indicates a possible role for ESAG5 in binding or metabolism of LPS or lipids, therefore implicating the ESAG5 protein in trypanosome pathogenicity. Further characterisation of the ESAG5 family has been carried out including examination of expression patterns and transfection-based studies to determine glycosylation status and cellular location.

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204A

A developmentally-regulated VSG expression-site-associated gene in bloodstream forms of *Trypanosoma brucei*: a pre-adaptation for parasite transmission?

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Trypanosoma brucei is a protozoan parasite that is the causative agent of sleeping sickness in sub-Saharan Africa. *T. brucei* has a complex life cycle involving passage between a mammalian host and the tsetse fly. The parasite evades the mammalian immune system via expression of Variant Surface Glycoproteins on the cell surface. VSG genes are expressed at telomeric expression sites and at these sites are a number of Expression Site Associated Genes (ESAGs). One unusual ESAG, ESAG9, is developmentally regulated: RNA for these genes accumulates during the transition from slender to stumpy cells in the mammalian bloodstream but protein is only expressed transiently during differentiation from the stumpy to procyclic forms in the tsetse fly. It is proposed that ESAG9 plays a role in establishment of trypanosomes in the tsetse fly midgut and this is being investigated via bioinformatics and functional analyses in vitro and in vivo. Using transgenic parasite lines that inducibly express different versions of ESAG9 in slender and procyclic forms, the capacity of ESAG9 expression to inactivate bloodmeal complement or tsetse lectins, or to promote establishment in the tsetse midgut, is being studied.

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204B

The 14-3-3 proteins of *Trypanosoma brucei*

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14-3-3 proteins are ubiquitously expressed eukaryotic proteins involved in the regulation of diverse cellular processes such as cell cycle control, trafficking, signal transduction pathways, and apoptosis. The two isoforms identified in *T. brucei* are essential in both bloodstream and procyclic life cycle stages. Microarray analysis identified several transcripts differentially expressed upon 14-3-3 depletion in bloodstream form cells. These transcripts encode kinases, histones, proteins involved in ubiquitination, trafficking and metabolic pathways. The function of two transcripts involved in ubiquitination, CFB1 and CFB2, was more closely analyzed by specific RNA interference and inducible overexpression. Depletion of CFB1 had a transient effect on growth and mitosis, whereas CFB2 depletion resulted in rapid growth arrest and cell death. Overexpression of both proteins was detrimental for cell viability. The effects of perturbations of CFB levels on the trypanosome cell cycle and cytokinesis will be presented. Putative 14-3-3 target proteins in both life stages were identified using the TAP-tagging approach. 14-3-3 interaction with one of these targets, a predicted protein containing a tandem zinc finger domain, was further investigated. This zinc finger protein only co-purifies with 14-3-3 in procyclic form cells and homozygous knock-outs in either monomorphic bloodstream forms or procyclics did not affect growth. We are currently investigating whether the ZFP is required for the differentiation of pleomorphic cells.

The 14-3-3 proteins of *Trypanosoma brucei*

Corinna Benz, Christine Clayton*

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205A

Purine salvage enzymes as infectivity and virulence determinants in *Leishmania donovani*

Jan M. Boitz*, Buddy Ullman

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Leishmania donovani express at least four enzymes capable of converting host purine nucleobases or nucleosides to the nucleotide level: 1) hypoxanthine-guanine phosphoribosyltransferase (HGPRT); 2) xanthine phosphoribosyltransferase (XPRT); 3) adenine phosphoribosyltransferase (APRT); and 4) adenosine kinase (AK). Mutational and gene replacement schemes in *Leishmania donovani* have demonstrated that the pathway is functionally redundant and that none of the four enzymes is essential by itself. Attempts to insert multiple mutations into a single cell has enabled the selection of double and triple mutants but no mutant deficient in both *HGPRT* and *XPRT*, lending indirect support to the hypothesis that either HGPRT or XPRT is essential for parasite survival. Recently, we confirmed this hypothesis through the construction of a conditionally lethal Δ*hgprt*/Δ*xprt* mutant strain of *L. donovani* that exhibits an absolute requirement for 2'-deoxycoformycin, an inhibitor of the leishmanial adenine aminohydrolase, and either adenine or adenosine as a purine source. Δ*hgprt*/Δ*xprt* parasites can proliferate as axenic amastigotes but cannot sustain an infection in any macrophage system tested, and mice infected with Δ*hgprt*/Δ*xprt* parasites harbor a parasite load at least 10,000-fold lower than mice infected with wild type *L. donovani* four weeks after inoculation. Thus, either HGPRT or XPRT activity is essential for purine acquisition, parasite viability and parasite infectivity, and the purine sources within the macrophage to which the parasites have access are HGPRT or XPRT substrates. These studies validate the conditional Δ*hgprt*/Δ*xprt L. donovani* mutant as a promising candidate for a live attenuated vaccine strategy to prevent visceral leishmaniasis.

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205B

Evidence that centromere-targeting of topoisomerase-II has been conserved between trypanosomes

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After replication, sister chromatids remain attached, partly through strand catenation at centromeres, as cells enter mitosis. In mammals, topoisomerase-II accumulation at centromeres during metaphase is a major regulator of sister chromatid cohesion. The decatenation activity of topoisomerase-II involves double-stranded DNA cleavage and religation to repair the lesion. Etoposide blocks re-ligation and induces DNA cleavage at topoisomerase-II binding sites. Etoposide-mediated topoisomerase-II DNA cleavage maps to active centromeric regions within human chromosomes. We have used this biochemical technique to map putative centromeric loci in *Trypanosoma brucei* and *Trypanosoma cruzi* to single sites on each chromosome that are located between directional gene clusters. In *T. cruzi* these sites coincide with chromosomal regions (¡70kb) that are required for mitotic stability. *T. brucei* contains two topoisomerase-II nuclear isoforms. RNAi-mediated knockdown of the α, but not the β transcript results in complete abolition of site-specific, etoposide-mediated chromosomal cleavage and is rapidly fatal in bloodstream forms. Constitutive expression of either the *T. cruzi* or the *L. major* topoisomerase-II in this background rescues the lethal phenotype, suggesting a conserved mechanism of centromeric targeting.

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Characterization of the FKBP12 protein of *Trypanosoma brucei*

Anas Brasseur*, Didier Salmon, Pays Etienne, Vanhamme Luc

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Trypanosoma brucei is a Kinetoplastid responsible for sleeping sickness in humans and nagana in cattle. Little is known on signal translation pathways in this parasite. In *Saccharomyces cerevisiae*, TOR (Target of Rapamycin) controls cell growth in response to nutrients. TOR is inhibited by rapamycin bound to its intracellular receptor FKBP12 (FK506 binding protein of 12 kDa). FKBP12 is the archetype of the FKBP family. The FKBP protein family is conserved in the majority of species; there are 4 members in *S.cerevisiae* and 8 in mammals. They display a peptidyl-prolyl cis-trans isomerase (rotamase) activity in vitro and are therefore likely to be involved in protein folding perhaps collaborating with chaperones. Some FKBP proteins play a role in parasite virulence (Tcmip in *T.cruzi*) and could therefore be the target of anti-parasitic drugs. FKBP proteins are the intracellular receptors of both rapamycin and FK506, two immunosuppressive agents. These ligand-receptor complexes inhibit TOR and calcineurin respectively. We identified 4 FKBP genes in the genome database of *Trypanosoma brucei*: FKBP12, FKBP11, FKBP21 and FKBP47. They are all expressed at the different stages of the parasitic cycle. Their knock-down by RNA interference has been performed. The knock-down of the FKBP12 yeast homolog is lethal in bloodstream forms due to a cytokinesis deficiency.

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206B

Characterization of Adenylate Cyclases in *Trypanosoma brucei*

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Cyclic AMP (cAMP) is an ubiquitous signalling molecule described in all organisms, from bacteria to mammals, but not in plants. Regulation of cAMP level within the cell is controlled by two enzymes: adenylate cyclases (AC) and phosphodiesterases (PDE). The cyclases convert ATP to cAMP and thereby activate signalling pathways which control various cellular effects, such as regulation of genetic, metabolic and physiological pathways. The PDEs hydrolyse cAMP to AMP and thereby down-regulate the cAMP signal. In trypanosomes, action and effects of cAMP signalling are only little investigated. Nevertheless, there is evidence that cAMP plays a role in differentiation of bloodstream form trypanosomes. When trypanosomes prepare to be taken up from the blood into the insect vector, parasites stop dividing and undergo a change in their shape from long slender to short stumpy form. One assumes that differentiation is triggered by a trypanosome-released molecule SIF (stumpy induction factor) which operates through the cAMP pathway. However, the underlying signalling cascade remains unknown. Here we describe a putative key player of cAMP signalling, the adenylate cyclase ESAG4. ESAG4 is expressed in bloodstream form trypanosomes exclusively and therefore might play an important role in triggering SIF-mediated differentiation process. So far one assumed that adenylate cyclases are located at the surface of the cell and activation is achieved through binding of an extracellular ligand and subsequent dimerization of the enzyme. Our current results suggest that ESAG4 is located intracellularly and hence, demand for an alternative model of AC activation.

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207A

Cold shock regulation of EP1 surface protein in *Trypanosoma brucei*

Martin Brenndoerfer, Marc Panzer, Bastian Muehl, Julia Pepperl, Michael Boshart*

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Bloodstream form trypanosomes (BSF) rapidly exchange their variant surface protein (VSG) coat to the procyclins EP and GPEET during differentiation to the procyclic stage (PCF). EP and GPEET expression is stage regulated >1000-fold at several levels. A temperature shift to 20 degrees Celsius (cold shock) alone is sufficient to increase EP expression in BSF. The *EP1* 3'UTR is necessary and sufficient for this BSF-specific induction. We present a comparative analysis of sequence elements required for cold induction and stage regulation. A set of 18 *EP1* 3'UTR mutations fused to a luciferase reporter was stably integrated in the genomic *EP* locus in BSF and PCF. Luciferase activity and *Luc* mRNA (TaqMan qPCR) were quantified for three conditions (BSF ± cold shock, PCF) in parallel. All mutants were conservative with respect to folding of the residual 3'UTR predicted by the mfold software, and correct splicing was verified by 3'RACE PCR. For accurate normalization of qPCR results, an optimal internal TaqMan reference probe was identified from a set of 7 candidate genes. From this systematic reanalysis of EP regulation we conclude that (1) The sequence requirements for cold inducibility and stage regulation are very similar if not identical; (2) The translational level of regulation is very important for cold inducibility and stage regulation; (3) A new reglatory element located in loop IV of the *EP1* 3'UTR was discoverd which acts independently from the elements investigated so far.

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207B

RNA editing-associated protein (REAP-1) and a related mitochondrially localized protein REAP-2

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Within the mitochondrion of *Trypanosoma brucei* genes are modified by RNA editing. We previously identified REAP-1, an RNA binding protein with the ability to recognize unedited RNAs and distinguish them from other mitochondrial RNAs. We have now compared sequences of the REAP-1 gene cloned from TREU 667 cells with the sequence found in the TREU 927 cell line sequenced in the genome project. Overall, there are five silent mutations, eleven amino acid substitutions, and a deletion of one of the 21 amino acid repeats that comprise the central third of the protein. Surprisingly, the open reading frame could be extended at the 5 end to encode a protein of 65 kDa. Reexamination of the sequence in TREU 667 confirmed the larger open reading frame. To determine if both start codons were utilized we identified the 3 spliced leader attachment site. Results show three separate spliced leader acceptor sites are utilized, all upstream of the larger open reading frame. We determined that monoclonal antibodies which identified the REAP-1 cDNA from a trypanosome expression library also react with a second distinct protein of 45 kDa. Furthermore, the second protein, which we call REAP-2, is the primary protein recognized when purified mitochondria or whole cell lysates are probed with the monoclonal antibody. We show that REAP-2 is localized to the mitochondrion, sediments with 35-40S complexes and is clearly distinct from REAP-1 as demonstrated by its absence in REAP-1 null mutants. REAP-1 sediments near the top of glycerol gradients indicating that it is not found in a large protein complex. Thus REAP-1 and REAP-2 are two distinct yet antigenically related proteins.

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208A

Prediction of potential drug targets shared across kinetoplastid genomes

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The neglected diseases most in need of new drug discovery are those caused by kinetoplastids. However, the low market value and high costs of drug discovery have limited work in this field. As a means to overcome some of these issues we have focused on identifying existing drug programs with known molecular targets that could have compound sets relevant for repositioning against neglected diseases. In an effort to prioritize candidate targets for repositioning we have analyzed genome sequences from four kinetoplastid parasites: *Trypanosoma brucei*, *T. cruzi*, *Leishmania infantum*, and *L. major*. Over a quarter of the known targets have homologs in at least one kinetoplastid and many have homologs in all four genomes. We propose that the resulting set of candidate targets is a good starting point for a functional validation campaign in *T. brucei* and a coordinated in vitro testing of target-associated compound sets against the different parasites. An advantage in this approach is that a focused investment in compounds and target-guided structure-activity elucidation can potentially impact one or more of these diseases. This model is commonly used in the drug discovery industry.

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208B

Reduced expression of ISG65 results in reduced parasitaemia

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Invariant surface glycoproteins (ISGs) are expressed on the cell surface of bloodstream form trypanosomes. Four ISGs have been identified biochemically and there are a further two ISGs identifiable in the genome. All are related type 1 membrane glycoproteins with a large extracellular domain of 300 to 450 amino acids and single transmembrane domain and a small cytoplasmic domain of 25 to 35 residues. The extracellular domain is structurally related to the N-terminal domain of the VSG. Analytical ultracentrifugation experiments indicated that the ISG64 extracellular domain is dimeric and elongated with the ratio of dimensions similar to a VSG N-terminal domain. ISGs are encoded by heterogenous multi-gene families. The structural homology to VSGs indicates that ISGs are probably dimers and the heterogeneity between members of individual ISG families is concentrated at the dimerisation interface. What is the function of ISGs? ISGs are expressed in bloodstream form trypanosomes but not in procyclic forms. Contrary to previous reports ISG65 and ISG75 are not located all over the cell surface but are concentrated in the endosome and on the cell surface in the region of the flagellum. Ablation of ISG65 expression has no effect on growth rate in culture but resulted in a tenfold reduction in parasitaemia in mice. The ISG65 knockdown trypanosomes were still able to undergo antigenic variation, as judged by relapsing parasitaemia but were either nutritionally compromised or had increased susceptibility to host antibody mediated defences.

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209A

Abrogation of *Leishmania infantum* mitochondrial peroxiredoxin impairs parasite infectivity. Function(s) for this enzyme?

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Leishmania infantum possesses one mitochondrial peroxiredoxin, *Li*mTXNPx, an enzyme previously reported to function as a peroxidase. Promastigotes depleted of this enzyme, *LimTXNPx*$^{-/-}$, have been produced in our lab by DNA recombination. These mutants are morphologically similar to, display the same growth rate and have identical susceptibility to exogenous sources of peroxides (H_2O_2, *t*-bOOH, SIN-1) as wild type parasites. Interestingly, however, when performing *in vivo* infection assays, we observed that depletion of the mitochondrial peroxiredoxin impairs parasite infectivity by week 2 and 4 post-infection. Furthermore, by week 8, balb/c mice infected with *LimTXNPx*$^{-/-}$ have almost undetectable levels of parasites in both their livers and spleens, in contrast to the high parasitemia levels of mice infected with wild type promastigotes. Looking for a rational for *LimTXNPx*$^{-/-}$ impaired infectivity, we are currently investigating whether *Li*mTXNPx might be implicated in *L. infantum* regulation of programmed cell death (PCD). This hypothesis was postulated with basis on previous reports stating that (i) mitochondrial peroxiredoxins of higher eukaryotes, and possibly also of *Leishmania donovani*, regulate PCD, and that (ii) PCD of *Leishmania* is an important mechanism for parasite establishment in the mammalian host. Understanding how *Li*mTXNPx affects parasite survival in the vertebrate host may provide us some answers about the mechanisms *L. infantum* makes use of to survive intracellularly.

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209B

Structural and metabolic regulation of *Trypanosoma brucei* hexokinases

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The African trypanosome expresses two 98% identical hexokinases, TbHK1 and TbHK2. HK activity is essential to *T. brucei* and distinct from mammalian HKs, making it an intriguing therapeutic target. Enzymatic studies on recombinant TbHK1 and TBHK2 have revealed that differences in the C-terminal tails lead to altered activity of the two proteins, with TbHK2 lacking detectable hexokinase activity. Mutational analyses have demonstrated that the tail residues N469, M466, F462, and D454 are essential for enzymatic activity. Molecular modeling of the C-terminal tails of TbHK1, TbHK2, and TbHK1 mutants suggest that the architecture of the tail is required for proper positioning of catalytic residue D214 by positioning of invariant S456. The C-terminal domain of yeast hexokinase has been shown to be important for dimerization of the enzyme. Authentic TbHK activity has been purified as a hexamer. To explore the oligomerization of TbHKs, recombinant proteins (TbHK1, TbHK2, and various mutants) have been subjected to gel filtration chromatography and results will be discussed. Based on RNAi and knockout studies, TbHK1 is likely to be responsible for the majority of cellular HK activity. Unlike most HKs, TbHK1 is not inhibited by its product, glucose-6-phosphate. We have investigated the impact of other metabolites on the catalytic function of TbHK1 and found that that free fatty acids (stearate, palmitate, myristate, luarate, and caprate) inhibit TbHK1, with IC_{50}s of 0.13 mM, 0.062 mM, 0.078 mM, 0.076 mM and 0.15 mM, respectively. Unlike a HK from *T. cruzi*, TbHK1 was not inhibited by acyl-CoAs. These observations suggest that TbHK1 is an unusual HK with distinct structural and metabolic regulators of catalysis.

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Trypanosoma vivax possesses a functional proline racemase

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Trypanosoma cruzi proline racemases (*Tc*PRAC) are the only eukaryotic proline racemases described so far. Beyond their enzymatic function as proteins capable of interconverting free D- and L-proline, their study revealed major biological functions for these proteins. Indeed, *TcPRAC* genes are essential and are implicated in parasite differentiation and infectivity to host cells. Furthermore, proline racemases passed the proof of concept as critical targets for drug development. The search of *TcPRAC* homologues was performed amongst trypanosomatids such as *T. brucei*, *T. congolense*, *T. vivax* and *Leishmania sp*. and led to the identification of a putative proline racemase in *T. vivax*. The corresponding gene was cloned and the produced recombinant protein presents a proline racemase activity towards L- and D-proline comparable to that of r*Tc*PRAC in terms of optimum conditions i.e., pH, buffer, temperature and substrate specificity. This enzyme is *bona fide* a proline racemase and is referred to as *Tv*PRAC. In addition, kinetic studies with L-proline revealed a K_M of 106 mM and of a V_{max} of 0.59 x 10^{-4} mol/s as compared to 29-75 mM and 0.53-2 x 10^{-4} mol/s respectively for the r*Tc*PRAC enzymes. Inhibition studies using the pyrrole-2 carboxylic acid (PAC) confirmed that it is a competitive inhibitor for both *Tc*PRAC and *Tv*PRAC with an apparent K_i of 6.7 and 25 microM, respectively. In this context, we postulate that therapeutic products based on *Tc*PRAC would be applicable to proline racemases from other pathogens should these enzymes be functional and essential. Finally, *Tv*PRAC biological relevance as well as the apparent deletion of the corresponding genes in *T. brucei*, *T. congolense* and *Leishmania* genomes will be discussed.

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210B

Ubiquitin-dependent endocytosis of ISG65 in *Trypanosoma brucei*

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Invariant surface glycoprotein 65 (ISG65) is a family of type I transmembrane proteins with structural similarity to variant surface glycoprotein (VSG), and are found on the surface of all bloodstream form *Trypanosoma brucei* cells regardless of the VSG being expressed. Although the function of ISG65 remains unknown, the cytoplasmic region of ISG65 family members are very highly conserved, with the presence of three cytosolically-disposed lysine residues in all family members. The trafficking of ISG65 was examined by generating a chimeric protein containing the *C*-terminal portion of ISG65 (encompassing the 23 *C*-terminal residues of the extracellular domain, the trans-membrane region and the cytoplasmic domain) fused to the N-terminal domain of BiP (BiPN). The *C*-terminus of ISG65 efficiently targeted the chimera to the same Rab5 and Rab11-positive endocytic compartments as native ISG65. Similarly, the BiPN fusion proteins and ISG65 exhibited very rapid turnover with similar half-lives. Immunoprecipitation and western blot analysis demonstrated that the lysine residues in the cytoplasmic region are covalently modified with ubiquitin, or its oligomers, which are generally involved in signaling for protein internalization, endosomal sorting and lysosomal degradation. The removal of all three lysine led to surface expression and stabilization of the chimeric proteins, likely due to the loss of functional ubiquitin acceptors. We suggest that ubiquitin acts as an endocytosis signal for the trans-membrane ISG65 protein family in trypanosomes.

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211A

Two RNA-binding proteins involved in ribosome biogenesis in *Trypanosoma brucei*

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The biogenesis of ribosomes is a complex process that involves the assembly of approximately 80 ribosomal proteins with four ribosomal RNAs to give rise to two mature ribosomal subunits: the large (60S) and the small (40S) subunits. Our laboratory has recently shown that in *Trypanosoma brucei*, two essential and closely-related RNA-binding proteins, p34 and p37, bind 5S rRNA specifically and play a role in its stabilization. Knockdown studies revealed that procyclic cells lacking p34 and p37 exhibit a decrease in the levels of 5S rRNA, as well as a decrease in overall translation. We have analyzed this phenomenon further by looking at the assembly of ribosomal subunits, ribosomes and polyribosomes. In cells lacking p34 and p37, there is a decrease in the levels of the 80S particle. This is accompanied by an accumulation in the levels of the 60S ribosomal subunits, of which 5S is normally a constituent. The 40S subunit is no longer seen as a distinct peak, being masked by an increase of lower-molecular weight components. In addition, we could observe a decrease in polysomes, consistent with our observation of a fall in overall protein synthesis. In wild-type cells, p34 and p37 are present in the fractions corresponding to 60S subunits, but not in mature ribosomes or polysomes. These results suggest that p34 and p37 act at the stage of incorporation of 5S to the 60 S subunit, and are released prior to the formation of the 80S particle. The detailed interactions between p34 and p37 and 5S rRNA and other components of the ribosome are being addressed.

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211B

Characterization of a mitochondrial monocysteine glutaredoxin from african trypanosomes

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Monothiol glutaredoxins (1Cys-Grxs) form a novel family of proteins that possess a single cysteine residue at their putative active sites. In yeast, they have been shown to be essential for iron-sulfur cluster biogenesis and for protection against oxidants. The genome of African trypanosomes encodes three putative 1Cys-Grxs. 1Cys-Grx1 and 2 are single domain monothiol glutaredoxins and harbor putative mitochondrial targeting sequences, while 1Cys-Grx 3 contains an additional N-terminal thioredoxin-like domain. Western blot analysis revealed that all three proteins are expressed in the different life stages of Trypanosoma brucei. Indirect immunofluorescence on procyclic and bloodstream cells overexpressing a myc-tagged version confirmed the mitochondrial compartmentalization of 1Cys-Grx1. Overexpression of 1Cys-Grx1 in both parasite forms did not yield any phenotype under optimal growth conditions. However, bloodstream parasites overexpressing 1Cys-Grx1 displayed a reduced growth rate when cultivated in the presence of the iron chelator deferoxamine, and an impaired resistance against hydrogen peroxide but, strikingly, not towards menadione (a generator of O2-). Recombinant 1Cys-Grx1 and 2 form mixed disulfides with tryparedoxin, indicating that this parasite specific multipurpose oxidoreductase is the physiological reductant of the proteins. Several attempts to down regulate 1Cys-Grx1 expression by dsRNAi in procyclic and bloodstream forms failed. 1Cys-Grx1-KO procyclic cell lines had integrated two marker cassettes but still retained a copy of the 1Cys-grx1 gene. Conditional null mutants were obtained in procyclic parasites but leakage did not allow a consistent depletion of the 1Cys-Grx1 extra-copy. Taken together, these data suggest the involvement of 1Cys-Grx1 in the iron and redox metabolism of trypanosomes. They point to an essential role of this protein and to the lack of functional redundancy among trypanosomatids 1Cys-Grxs.

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Identification and functional characterization of the putative RNA helicase MTR4 in *T. brucei*

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The exosome is a complex that degrades RNA molecules in the 3'-to-5' direction. It consists of 10-11 subunits with predicted or demonstrated exonuclease activity and is highly conserved throughout evolution. The nuclear exosome functions in the 3' trimming of stable RNAs, while the cytoplasmic exosome degrades mRNA molecules. The exosome also functions in several quality control pathways. So far, the mechanisms by which the specificity and mode of action of the complex are regulated have proved elusive. Factors involved probably include RNA binding proteins and helicases. Two putative RNA helicases, Mtr4p and Ski2p, are cofactors for yeast exosome activity. Ski2p assists the exosome in cytoplasmic mRNA degradation. Mtr4p participates in the biosynthesis of rRNA and has been implicated in a nuclear quality control mechanism involving the exosome. We have identified in trypanosomes a functional homologue of yeast Mtr4p, *Tb*MTR4. *Tb*MTR4 is found in the nucleus and is essential for parasite viability. After depletion of MTR4 by RNAi, trimming of the 7S precursor to the mature 5.8S rRNA was affected. Since depletion of exosome subunits had the same effect, it seems probable that *Tb*MTR4 is an exosome cofactor. *Tb*MTR4 RNAi also resulted in accumulation of polyadenylated rRNA precursors, an effect also observed after depletion of the exosome subunit RRP6. We hypothesize that *Tb*MTR4 is involved in a quality control mechanism that recognizes and degrades aberrant RNA molecules in the nucleus of *T. brucei*. This mechanism requires the activity of the exosome that would act in a MTR4- dependent manner. *Tb*MTR4 also interacts with several other proteins involved in RNA metabolism, suggesting a function of MTR4 in different RNA pathways.

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212B

Transcription Elongation Factor TFIIS in *Trypanosoma brucei*

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Transcription elongation potentially plays an important role in transcription of *Trypanosoma brucei* protein coding genes, which are organized in directional gene clusters, as well as in the mono-allelic expression of VSG. Therefore, we are interested in studying transcription elongation factors, such as TFIIS, in *T. brucei*. TFIIS helps RNA polymerase II to escape arrest and consists of the TFIIS domains I, II and III. We identified three homologues of TFIIS (TbTFIIS-1, TbTFIIS-2a, TbTFIIS-2b) and another protein with a domain similar to the TFIIS domain II (TbTDCP-1, TFIIS domain II containing protein 1) by iterative Hidden Markov Model based similarity searches. In TbTFIIS-1 and TbTFIIS-2a, TFIIS domains II and III are conserved, while a region similar to domain I was detected only in TbTFIIS-2a and TbTFIIS-2b. Additionally, TbTFIIS-2a and TbTFIIS-2b bear an N-terminal PWWP-domain, usually involved in chromatin targeting. Surprisingly, TbTFIIS-2b is a C-terminally truncated TFIIS, lacking both domains II and III, and thus might function as an inhibitor of TFIIS. The C-terminal domain of TbTDCP consists of a potential, unusual C4-zincfinger motif. All four proteins are expressed in procyclic *T. brucei*. They localize to heterogenously distributed nuclear foci. At least TbTFIIS-1, TbTFIIS-2a and TbTFIIS-2b are also expressed in bloodstream-form cells and show a similar nuclear distribution like in procyclic cells. The patterns of TbTDCP-1, TbTFIIS-1, TbTFIIS-2a and TbTFIIS-2b distribution may be different in individual cells. We propose that the TbTFIIS homologues have diversified functions that are important for regulatory steps in *T. brucei* transcription.

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Characterization of A7, a RPB4 associated protein, in *Trypanosoma brucei*

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RNA polymerase II, a large $\sim$ 550 kDa protein complex consisting of 12 subunits, plays the central role in eukaryotic mRNA biogenesis. Along with synthesizing the nascent RNA encoded by nuclear DNA, it co-ordinates the activities of numerous protein factors involved in the co-transcriptional processing of the precursor mRNA. Thus RNA polymerase II facilitates the formation of mature mRNA on many levels. In addition, recent studies in yeast indicate that one of the essential subunit of RNA polymerase II, RPB4, is a component of mRNA processing bodies (P bodies) within the cytoplasm; therefore RPB4 may be involved in 'cross-talk' between mRNA synthesis in the nucleus and mRNA degradation in the cytoplasm. In trypanosomatid organisms, mature mRNAs are produced by trans-splicing of a short spliced leader (SL) RNA upstream of each open reading frame within polycistronic precursor mRNAs. Both the SL RNA genes and the majority of the protein coding genes require RNA polymerase II. These observations underscore the importance of understanding RNA polymerase II's role in parasite survival. Recently we reported the purification of a transcriptionally active *Trypanosoma brucei* RNA polymerase II by affinity tagging the RPB4 subunit of the enzyme. This purification led to the discovery of a set of co-purified proteins. Using the RNAi knockdown strategy we found that one of the proteins, A7, is essential for parasite survival. Immuno-staining of A7 in procyclic parasites and fluorescence microscopy indicates that A7 localizes in speckles in the cytoplasm with increased concentration around the nucleus. The possible role of A7 in mRNA metabolism will be presented.

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Characterization of a putative immunoregulatory factor from *Trypanosoma brucei*

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Characterization of a putative immunoregulatory factor from *Trypanosoma brucei* African trypanosomes evade the mammalian host immune response by two major mechanisms, antigenic variation and immunomodulation. Regarding the second mechanism, they release factors acting on cells of the immune system including macrophages. A fraction released by *Trypanosoma brucei* in the culture medium was observed to induce arginase activity in peritoneal macrophages as opposed to the classical activation pathway monitored by NO production. Arginase activation antagonizes NO production thereby creating a favourable environment for the parasite development. A candidate active component of this fraction released by *T. brucei* was identified as a kinesin heavy chain (TbKHC). TbKHC was localised at the posterior end of the parasite. Functional invalidation of TbKHC did not affect trypanosome growth in vitro or their capacity to complete cell cycle. In mice, TbKHC absence resulted in a reduced number of parasites during the first peak of parasitemia and a prolonged survival of the mice. This situation contrasts with that obtained in immunodeficient SCID mice where no growth defect was observed. Those observations support the involvement of TbKHC in the interactions between trypanosomes and their host.

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Characterization of a putative immunoregulatory factor from *Trypanosoma brucei* African trypanosomes evade the mammalian host immune response by two major mechanisms, antigenic variation and immunomodulation. Regarding the second mechanism, they release factors acting on cells of the immune system including macrophages. A fraction released by *Trypanosoma brucei* in the culture medium was observed to induce arginase activity in peritoneal macrophages as opposed to the classical activation pathway monitored by NO production. Arginase activation antagonizes NO production thereby creating a favourable environment for the parasite development. A candidate active component of this fraction released by *T. brucei* was identified as a kinesin heavy chain (TbKHC). TbKHC was localised at the posterior end of the parasite. Functional invalidation of TbKHC did not affect trypanosome growth in vitro or their capacity to complete cell cycle. In mice, TbKHC absence resulted in a reduced number of parasites during the first peak of parasitemia and a prolonged survival of the mice. This situation contrasts with that obtained in immunodeficient SCID mice where no growth defect was observed. Those observations support the involvement of TbKHC in the interactions between trypanosomes and their host.

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Characterization of *Leishmania donovani* metacaspases and their association with parasite programmed cell death

Nancy Lee, Sreenivas Gannavaram, Angamuthu Selvapandiyam, Alain Debrabant*

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Metacaspases belong to the caspase-related clan CD cysteine protease family. They have been involved in the programmed cell death (PCD) pathway of plants and yeasts. Metacaspase genes can be found in the genomes of trypanosomatid parasites such as Leishmania and Trypanosoma however their involvement in PCD in these organisms remains to be demonstrated. We characterized two metacaspases of Leishmania donovani. These two proteins show 98% identity and contain a characteristic C-terminal proline-rich domain. An anti-LdMC antibody showed reactivity with a single ∼45 kDa protein band in both promastigote and axenic amastigote parasite whole cell lysates by western blot. Pulse-chase experiments suggest that LdMCs are not synthesized as pro-enzymes and immunofluorescence studies show that LdMCs are associated with the acidocalcisome compartments of L.donovani. Enzymatic assays of immunoprecipitated LdMCs from parasite lysates show that they cleave efficiently trypsin substrates and are unable to cleave caspase-specific substrates. Consistently, LdMCs are insensitive to caspase inhibitors and are efficiently inhibited by trypsin inhibitors such as leupeptin, antipain and TLCK. We showed that LdMC activity was induced in parasites treated with hydrogen peroxide, a known trigger of PCD in Leishmania, and parasites over-expressing metacaspases are more sensitive to hydrogen peroxide induced PCD. These findings suggest that Leishmania metacaspases are not responsible for the caspase-like activities reported in this organism and support a possible role for LdMCs as an effector molecule in Leishmania PCD.

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214B

Structure-Function analysis of the main folate transporter FT1 of *Leishmania*

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Leishmania is a pterin and folate auxotroph and has thus developed an extensive ability to transport and salvage these derivatives. Three transporters have so far been characterized; BT1, the only biopterin transporter of *Leishmania*, FT5 a high affinity, low capacity folate transporter and FT1 the main folate transporter of *Leishmania*. Analysis with topology software has predicted that the transporter FT1 contains 12 putative transmembrane domains. Epitope tagging with Histidine or haemagglutinin in differents cytosolic or external loops has been performed. Current results are consistent with the proposed topology with the C-terminal of the protein in the cytosol. A sequence alignment has revealed several conserved charged residues (D, E, K, R) among this family of transporters. To identify residues that play central roles in transport function, we have systematically substituted by site-directed mutagenesis all these charged residues located within predicted transmembrane domains. The production, localisation, and function of mutated FT1 proteins have been assessed. Some substitutions lead to decrease the protein stability. Most mutant proteins are still located in the plasma membrane. Substitution of some of these residues by uncharged amino acids resulted in loss of >95% transport activity. Further mutations at these residues are underway to understand the importance of the charge of these amino acid. These studies are contributing to our understanding of a novel class of membrane protein in *Leishmania*.

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215A

Biochemical and functional investigation of *Trypanosoma brucei* flagellar calcium-binding proteins

Brian Emmer*, Krista M. Toriello, Danijela Maric, Melvin D. Daniels, Kenneth V. Hyland, Cheryl L. Olson, David M. Engman

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The calflagins were discovered by Larry Ruben and colleagues over ten years ago as the predominant EF-hand calcium-binding proteins of T. brucei. A lack of homology to any proteins outside of kinetoplastids has hindered elucidation of their function(s) to date. We set out to characterize the biochemical properties and functional significance of these proteins. We found that the calflagins associate with the flagellar membrane in a manner that depends on dual acylation at their N-termini. Mutations abrogating acylation confer a cytoplasmic localization, while mutations that allow myristoylation but not palmitoylation lead to a pellicular membrane localization. Both modifications are necessary for the partitioning of calflagin into detergent-resistant membranes. To investigate the function of the calflagins, we generated a calflagin knockdown strain in both procyclic and bloodstream life cycle stages of T. brucei. Drug-inducible calflagin RNA interference allowed rapid and specific depletion of protein levels of all members of the calflagin family. These mutants were then analyzed by several assays to test whether calflagin was necessary for normal parasite viability and growth, morphology, directional motility, and virulence. Our results indicate that, despite their flagellar localization, calflagins are not required for normal parasite motility. Additionally, despite displaying a normal growth rate in cell culture under a variety of conditions, calflagin-deficient T. brucei demonstrate attenuated virulence in a susceptible mouse model of infection. Consistent with calflagin playing a specific role in the host, we found calflagin to be greater than 10-fold more highly expressed in the bloodstream form of the parasite than in the procyclic form. The implications of these findings and the direction of future investigations will be discussed.

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215B

Trafficking, evolution and the origin of intracellular transport - insights from parasite genome projects

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In membrane trafficking, the mechanisms ensuring vesicle fusion specificity remain to be fully elucidated. Early models proposed that specificity was encoded entirely by SNARE proteins; more recent models include contributions from Rab proteins, Syntaxin-binding (SM) proteins and tethering factors. Most information on membrane trafficking derives from an evolutionarily narrow sampling of model organisms. However, considering factors from a wider diversity of eukaryotes can provide both functional information on core systems and insight into the evolutionary history of the trafficking machinery. We have performed comparative genomic and phylogenetic analyses, when relevant, on multiple components of the vesicle transport system, including Rabs, SNAREs, tethering factors and coat protein systems. Our data support a highly complex last common eukaryotic ancestor and indicate that the basic architecture of the trafficking system is remarkably conserved and ancient, with members of the Rab family, SNARE proteins, SM proteins and tethering factors having originated before the diversification of extant eukaryotes. However, we also find considerable evidence for secondary loss as an important evolutionary driver, particularly in the endocytic system.

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Sphingolipid synthesis is required for viability and cytokinesis progression in *Trypanosoma brucei*

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Sphingolipids and sphingolipid metabolites are involved in a variety of cellular events, including membrane and protein trafficking, signal transduction, cell growth and cell cycle progression, and lipid raft formation. Sphingolipid synthesis is essential for viability in mammalian and yeast cells, but dispensable in *Leishmania* log-phase promastigotes and in amastigotes supplemented with ethanolamine. We examined the necessity for de novo sphingolipid biosynthesis in procyclic *Trypanosoma brucei* by downregulating serine palmitoyltransferase (SPT), the enzyme catalyzing the first step in sphingolipid biosynthesis, and by myriocin treatment. Sphingolipid reduction caused a dramatic block in cytokinesis; however, replication of DNA and organelles continued in affected cells, giving rise to a pleomorphic aneuploid population containing a number of cellular defects. Lipid inclusion bodies were frequently observed in the cytoplasm, suggesting that the secretory pathway may be disrupted. Also observed was the distention of the mitochondrial membrane, and less frequently, a disrupted Golgi apparatus. Features reminiscent of apoptosis, including an enlarged flagellar pocket, fragmentation of nuclear chromatin, and multilamellar structures within the mitochondrion and near the nucleus were seen at later timepoints of RNAi induction. However, neither the targeting of the lipid raft-associated protein calflagin to the flagellum nor its association with detergent-resistant membranes was affected in the sphingolipid-depleted procyclic cells, although calflagin's association with DRMs was disrupted in bloodstream forms. Our studies show that de novo sphingolipid biosynthesis is essential for cell viability and cycle progression, and suggest that sphingoid intermediates may be involved in this process. Sphingolipid synthesis inhibition may affect the secretory and endocytic pathways, but does not significantly affect the trafficking of flagellar membrane-associated proteins or lipid raft formation in procyclic *T. brucei*.

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216B

A membrane identifier protein for the flagellar pocket and recycling pathway in *Trypanosoma brucei*

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Endocytosis and recycling are vital aspects of membrane transport in African trypanosomes, and are focused on removal and delivery of membrane at the flagellar pocket. The conserved Rab5 and Rab11 GTPases mediate several of the steps required for this process. Rab11 is responsible for the recycling of VSG back to the flagellar pocket and is associated with disc/cup-shaped recycling endosomes. In the bloodstream form flux through this pathway is extensive, while expression of Rab11 is upregulated in this stage and essential. Additional roles have been assigned to Rab11 in different organisms, including participation in phosphatidylinositol 4-kinase-mediated signalling. Rab11 in mammals interacts with a family of coiled-coil containing proteins, FIPs, which participate in multiple cellular functions. To understand Rab11 function and regulation at a deeper level we have searched for Rab11-interacting proteins. Interrogation of the trypanosome genome failed to identify trypanosome FIP homologues, but a Saccharomyces cerevisiae two-hybrid screen did identify two proteins that interact with Rab11. One of these also interacts with Rab5, while the second is specific for the GTP-bound form of Rab11. Both contain extensive coiled-coil domains and a Rab-binding domain at the C-terminus, an architecture related to FIP1. Expression of HA-tagged demonstrates one of these Rab11 binding protein locates to the cytoplasmic face of the posterior side of the flagellar pocket, residing close to the Rab11-positive recycling endosomes. These data suggest that this protein provides a membrane marker for a subdomain of the flagellar pocket with a specialised function in fusion with recycling endosomes/vesicles.

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Trypanosome motility: flagellar and ciliary beating and a beat resonance hypothesis

Catarina Gadelha*, Bill Wickstead, Keith Gull

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The single flagellum of *Leishmania* and *Trypanosoma* parasites is becoming an increasingly attractive model for the analysis of flagellar function - driven largely by the abundance of genomic and proteomic information available for the organelle, the genetic manipulability of the organisms and the importance of motility for the parasite lifecycle. However, as yet, there is a paucity of published data on the beating of any genetically malleable trypanosomatid species. We undertook an in-depth analysis using high-speed videomicroscopy of the beating of free-swimming *Leishmania major* cells in comparison to *Crithidia* species (for which there is existing literature). In so doing, we describe a simple and generally-applicable technique to facilitate the quantitative analysis of free-swimming cells. Our analysis thoroughly defines the parameters of the expected tip-to-base symmetrical flagellar beat in these species. It also describes beat initiation from points other than the flagellum tip and a completely different, base-to-tip highly-asymmetric beat that represents a ciliary beat of trypanosomatid flagella. Moreover, detailed analysis of parameter interrelationships revealed an unexpected dependency of wavelength on oscillator length that forms the basis of a beat resonance hypothesis for the flagellar beat of these organisms.

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The role of CRK9 in *Trypanosoma brucei* cell cycle regulation

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Cell cycle regulation in *Trypanosoma brucei* is mediated by a variety of protein complexes, most notably the cyclin/CRK (cdc2 related kinase) complexes. Passage through the four stages of the cell cycle (G1, S, G2 and M phases) is regulated by CRK1-3, CYC2/cyclin E1, and CYC6/cyclin B2. Recently, two novel cyclins (CYC10, CYC11) and six new CRKs (CRK7-12) were identified using a bioinformatics approach (Parsons et al., 2005, Naula et al., 2005). RNAi depletion of these individual proteins from the procyclic-form cells showed that only CRK9 deficiency adversely affects cell growth. The primary structure of CRK9 lacks several residues conserved among CRK proteins, such as the PSTAIRE motif and the phenylalanine gatekeeper residue involved in cyclin binding and ATP recognition, respectively. It is thus possible that CRK9 may not be involved in cell cycle regulation. Flow cytometry analysis and microscopic examination suggest that CRK9 depleted cells were accumulated in the G2/M phase. But no anucleated cells, generally produced from a pre-mitotic block in the procyclic form, were observed. Furthermore, CRK9 RNAi also grossly deformed the majority of cells and turned their morphology into rounded shape. Preliminary immunofluorescence results suggest that flagellar morphogenesis and overall tubulin distribution in the cell are unaffected by CRK9 depletion. Rather, there appears to be defects in replication or segregation of the basal bodies, which may in turn cause the premitotic arrest and the rounded cell phenotype.

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Comparative study of methylglyoxal metabolism in the Trypanosomatids

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The Trypanosomatidae are the causative agents of Chagas disease, African sleeping sickness and Leishmaniasis. In the search for novel targets in these parasites for drug discovery, metabolic pathways which are either absent from, or significantly different to host pathways, are logical starting points. Several enzymes involved in trypanothione biosynthesis and its downstream metabolism, which have been genetically validated in the Trypanosomatidae, are examples of such pathways. In previous studies within our laboratory, a trypanothione-dependent glyoxalase I from L. major was characterised and found to efficiently use the endogenous toxic oxoaldehyde methylglyoxal as a substrate. However, the mechanism of methylglyoxal detoxification within trypanosomes is currently unknown. To further explore this question a putative glyoxalase I from T. cruzi was cloned and recombinantly expressed. The substrate specificity of this enzyme was examined with various thiol and oxoaldehyde substrates and the resulting kinetic constants determined. Glutathionylspermidine adducts were found to be the preferred subtrates for T. cruzi GLO1. In contrast BLAST searches of the T. brucei genome database failed to reveal a putative GLO1. Whether other pathways may be responsible for methylglyoxal metabolism within trypanosomatids is unclear. Trypanosomatid susceptibility to methylglyoxal was compared, and methylglyoxal was found to be differentially toxic to L. major, T. cruzi and T. brucei, when grown in the same media for comparison. In addition, methylglyoxal was found to be metabolised at different rates and to different end products within trypanosomatids. We hope that further investigation of the downstream metabolites of methylglyoxal will reveal the mode of oxoaldehyde metabolism within trypanosomes.

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Insights to the cytoskeletal architecture of *Trypanosoma brucei* in the context of mutant and phenotype analyses

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The development of RNAi technology has led to the easy production of mutants of T. brucei that lead to cellular phenotypes involving cytoskeletal, mitotic and and cell shape abnormalities. Understanding the individual characteristics of the phenotype in mutants and separating the important from the trivial appears to be more difficult. In this context we have been re-analysing a database of tens of thousands of electron micrographs of the normal T. brucei cell and, using this in combination with EM tomograms, have constructed detailed 3D images of the sub-pellicular corset of microtubules, the flagellum attachment zone filament, the flagella connector, the microtubule quartet, golgi and endoplasmic reticulum, the mitotic spindle and microtubule organising centres. We will illustrate examples of these areas of the cell and make comparisons with the effects of defined mutations in specific components revealing dependency relationships useful in analysing some phenotypes.

Insights to the cytoskeletal architecture of *Trypanosoma brucei* in the context of mutant and phenotype analyses

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Glycosomal location of Gal epimerase (TbGalE) is not essential for this *in vivo* validated drug target against *T.brucei*

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Human African sleeping sickness and the cattle disease Nagana are caused by the tse-tse fly transmitted protozoan parasite Trypanosoma brucei. These parasites present many galactose containing glycoconjugates, for instance the surface coat VSG, the transferrin receptor and the endosome/ lysosome/ flagellar pocket p67. Surprisingly, T.brucei is unable to take up galactose from the blood or culture media, and synthesizes galactose by interconversion of Glucose to Galactose by the enzyme UDP-Glc 4'-epimerase (GalE). We have previously generated Tetracycline responsive GalE conditional null mutants, and reported that GalE is essential for the parasite survival in culture media. In this study, we have generated a new version of a GalE conditional null mutant in which the Tet responsive ectopic copy is lacking the C-terminal PTS1 glycosome targetting motif (TKL). We have shown that this enzyme localizes in the cytosol, not in the glycosome, but it is still able to perform its function in the cell. We have also shown that both conditional null mutants only survive in mice under permissive conditions, providing in vivo validation of GalE as a drug target. This work is supported by Wellcome Trust Programme Grant 071463. Ademilson Panunto-Castello thanks FAPESP (2005/00479-8)/Brazil.

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Trypanosoma brucei Polo-like kinase is essential for basal body duplication, kDNA segregation and cytokinesis

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Polo-like kinases are conserved eukaryotic cell cycle regulators, which play multiple roles, particularly during mitosis. The function of *Trypanosoma brucei* Polo-like kinase (PLK) was investigated in procyclic and bloodstream form parasites. In procyclic trypanosomes, RNA interference (RNAi) of *PLK*, or overexpression of TY1-epitope tagged PLK (PLKty), but not overexpression of a kinase dead variant, resulted in the accumulation of cells that had divided their nucleus but not their kinetoplast (2N1K cells). Analysis of basal bodies and flagella in these cells suggested the defect in kinetoplast division arose because of an inhibition of basal body duplication, which occurred when PLK expression levels were altered. However, the 2N1K cells obtained by each approach were not equivalent. Following PLK depletion, the single kinetoplast was predominantly located between the two divided nuclei, while in cells overexpressing PLKty, the kinetoplast was mainly found at the posterior end of the cell, suggesting a role for PLK kinase activity in basal body and kinetoplast migration. *PLK* RNAi in bloodstream trypanosomes also delayed kinetoplast division, and was further observed to inhibit furrow ingression during cytokinesis. Notably, no additional roles were detected for trypanosome PLK in mitosis, setting this protein kinase apart from its counterparts in other eukaryotes.

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220A

BRCA2 in DNA damage response, recombination and antigenic variation in *Trypanosoma brucei*

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Trypanosoma brucei is an evolutionarily divergent eukaryotic parasite of mammals in sub-Saharan Africa and is transmitted by the tsetse fly vector. To evade the mammalian immune response, *T. brucei* utilises antigenic variation, which involves switching Variant Surface Glycoprotein (VSG) expression, primarily through recombination. We have shown that such *VSG* switching is a form of homologous recombination, as mutation of *RAD51* impairs the process. BRCA2 has emerged as a significant regulatory factor during RAD51-catalysed recombination. In humans, BRCA2 contains eight BRC repeats, six of which have been shown to bind RAD51. Similar repeats are present in BRCA2 from other organisms, though normally in smaller numbers. The *T. brucei* BRCA2 homologue appears exceptional in that it contains up to 12 BRC repeats. We hypothesise that this unusual BRCA2 organisation is due to the high levels of RAD51-directed recombination needed during antigenic variation. *T. brucei BRCA2* mutants have been generated and display impaired growth, sensitivity to induced DNA damage, impairment in the ability to form RAD51 foci, a reduced ability to recombine DNA constructs into their genome and a reduction in frequency of VSG switching, all of which are consistent with roles for BRCA2 in DNA repair and recombination. DNA content analysis of *T. brucei BRCA2* mutants reveals accumulation of cells with aberrant DNA content and increased numbers of cells undergoing cytokinesis without having completed nuclear division, phenotypes not observed in other *T. brucei* recombination mutants. This suggests that BRCA2 has a role in the regulation of cell division, with mutation causing impaired replication of *T. brucei* nuclear DNA, but without a cell cycle stall, leading to the accumulation of chromosomal aberrations.

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220B

The protein product of pan-edited ATP synthase subunit 6 is incorporated into Complex V in *Trypanosoma* species

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Throughout eukaryotes, the gene encoding subunit 6 (A6) of the ATP synthase (complex V) is maintained in mitochondrial genomes, presumably because of its high hydrophobicity since it is incorporated into the membrane-bound F_0 moiety. In *Trypanosoma* species, a mitochondrial transcript that undergoes extensive processing by RNA editing has low homology to A6 from other organisms. Complex V has an essential role in generation of ATP and maintaining mitochondrial membrane potential in insect and blood stage trypanosomes, respectively. The notion that the putative A6 subunit is required for assembly of the F_0 sub-complex is ostensibly challenged by the existence of naturally occurring dyskinetoplastic trypanosome species, which lack the DNA required for its expression. However, these cells have also escaped sensitivity to oligomycin, suggesting that this moiety has evolved compensatory modifications. To address whether the trypanosome A6 homolog is required for the F_0F_1 ATP synthase, a set of RNAi knockdown procyclic cell lines, targeting proteins involved in mitochondrial RNA metabolism, have been assayed for ATP synthase activities. RNAi silencing of mitochondrial RNA binding protein 1 (MRP1) down-regulates a subset of mRNAs for subunits of complexes I, III and IV of the respiratory chain, with the exception of complex V, and the affected complexes are subsequently disrupted. RNAi silencing of KREN1 and KREPA6, two components of the 20 S editosome, result in diminished RNA editing, affecting transcripts for all oxidative phosphorylation complexes, including A6. We exploit this difference among these cell lines to show that A6 is probably incorporated into complex V and its absence eliminates its ability to oligomerize. These results will also be compared with those from dyskinetoplastic and bloodform trypanosomes.

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Polyamine transport in *Leishmania* and *Trypanosoma cruzi*

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As part of an ongoing effort to study nutrient acquisition in kinetoplastids via specific permeases, we have been interested in identifying polyamine transporters in *Leishmania* and *Trypanosoma cruzi*. The synthesis of putrescine and spermidine de novo occurs in *Leishmania*, however, *T. cruzi* lacks both ornithine decarboxylase and arginine decarboxylase. Consequently *T.cruzi* survival depends on its ability to acquire putrescine from its environment. We have previously reported the characterization of LmPOT1, a polyamine transporter in *Leishmania*. The sequence of this permease has been used as a template to search the *T.cruzi* genome database, and this analysis identified five putative polyamine transporter genes in *T.cruzi* (*TcPOT1.1* to *TcPOT1.5*). The biochemical characterization and subcellular localization of these five transporters will illuminate the cellular functions of these proteins and their therapeutic potential.

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221B

The bloodstream form VSG expression site repertoire of *Trypanosoma brucei* strain 427

Christiane Hertz-Fowler*[,1], Luisa Figueiredo[2], Mike Quail[1], Marion Becker[3], Rosie Young[4], Andrew Jackson[1], Carol Churcher[1], George Cross[2], Dave Barry[5], Ed Louis[3], Gloria Rudenko[4], Matt Berriman[1]

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The surface of bloodstream-form *Trypanosoma brucei* is shielded by a variant surface glycoprotein (VSG) coat. The expressed *VSG* gene is transcribed monoallelically from one of ∼20 telomeric bloodstream-form VSG expression sites (BESs), which also embrace a diverse family of expression-site-associated genes (*ESAGs*) of largely unknown function. In order to better understand the organization and evolution of BESs, we have undertaken a systematic analysis of bloodstream ESs from *T. brucei* strain Lister 427. A BES telomere-specific library has previously been constructed and the resultant clones categorised into 17 BES groups (Becker et al., 2004). Nineteen of these clones have now been sequenced, assembled and annotated (http://www.genedb.org/), and *VSGs* encoded by some of these clones have been mapped to the *T. brucei* 427 genome. According to their respective promoter and *ESAG6* sequences, the clones represent 16 different BES classes and, with some notable exceptions, are remarkably conserved in their overall structure. Phylogenetic analyses are underway to assess relatedness amongst the expression sites, with a view to understand how the repertoire of BESs has evolved and been maintained over time. In complementary studies, 12 VSG expression sites have been tagged with selectable markers, 10 of which could be activated *in vitro*. Their *ESAG6* and *VSG* sequence matched the sequence of the telomeric clones, indicating consistency between the cloning and tagging studies. Taken together, the sequence and ability to 'switch on' individual sites represent a unique resource to not only elucidate host-parasite interaction but also to serve as a paradigm to investigate telomere function and regulation in systems beyond the trypanosomatids.

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Finding the missing link: functional genomics in *T. brucei* identifies conserved components of motile flagella, including a novel family of candidate nexin links

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The trypanosome flagellum is an essential and mutifunctional organelle. We previously demonstrated that flagellar motility in African trypanosomes is controlled by an evolutionarily-conserved dynein-regulatory-complex, DRC(1), that is essential in bloodstream-form trypanosomes, suggesting that motility itself might be essential(2). DRC subunits exhibit a distinctive phylogenic distribution, being uniquely conserved in organisms with motile flagella(2). To identify additional proteins necessary for flagellar motility, we employed comparative analysis of eukaryotic genomes to identify T. brucei genes with a similar phylogenetic distribution, i.e. absent in organisms that lack flagella or have only immotile flagella. We identified 50 genes that are conserved Components of Motile Flagella, TbCMF genes. This dataset includes several known flagellar genes and 30 novel genes that have never been characterized in any organism. Human homologues of 17 CMF genes map to loci associated with ciliary diseases. Several TbCMF genes are represented as gene families that are expanded in kinetoplastids relative to humans and other organisms. Functional analysis indicates that this does not simply reflect amplification of genes with redundant functions. We analyzed the 30 novel TbCMF genes and several others not previously investigated in T. brucei. RNAi of these 42 genes demonstrated a role in flagellar motility in the majority of cases. Motility phenotypes include paralyzed cells, tumbling cells and cells that move backward. Ultrastructural and motility analyses identified one family of novel TbCMF proteins that appear to function as part of the long-sought nexin links. This study therefore provides insight into trypanosome flagellar motility and identifies several new potential disease genes. 1. Ralston et al. Eukaryotic Cell 5, 696-711 (2006). 2. Ralston. & Hill. PLoS Pathogens 2, 873-882 (2006).

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222B

Trypanosoma brucei encodes a bifunctional capping enzyme essential for cap 4 formation on the spliced leader RNA

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The 5' end of kinetoplastid mRNA posseses a hypermethylated cap 4 structure, which is derived from standard m7GpppN (cap 0) with additional methylations at seven sites within the first four nucleosides on the spliced leader RNA. In addition to TbCe1 guanylyltrasferase and TbCmt1 (guanine N-7) methyltransferase, *Trypanosoma brucei* encodes a second cap 0 forming enzyme. TbCgm1 consists of a novel bifunctional capping enzyme with an amino-terminal guanylyltrasferase domain and a carboxyl-terminal methyltransferase domain. Recombinant TbCgm1 transfers the GMP to SL RNA via covalent enzyme-GMP intermediate, and methylates the guanine N-7 position of the GpppN-terminated RNA to form cap 0 structure. TbCGM1 is essential for parasite growth. Silencing of TbCGM1 by RNA interference increased the abundance of uncapped SL RNA and lead to accumulation of hypomethylated SL RNA. In contrast, silencing of TbCE1 and TbCMT1 did not affect parasite growth or SL RNA capping. We conclude that TbCgm1 specifically cap the SL RNA, and cap 0 is a prerequisite for subsequent methylation events leading to the formation of mature SL RNA.

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Protein kinase A catalytic subunit of *Trypanosoma cruzi* interacts and phosphorylates trans-sialidase

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Protein kinase A (PKA) has been suggested as a regulator of stage differentiation in T. cruzi. Using a yeast two-hybrid system we have begun to characterize the downstream substrates of PKA in T. cruzi. Twenty-three target plasmids interacting with BD- TcPKAc were obtained. Among the proteins identified as interacting with TcPKAc are several members of the trans-sialidase super family. The full length ORF of the transialidase genes were amplified by PCR and then subcloned into pAD-Gal4 vectors. Each pAD-Gal4 construct was transformed into yeast containing a TcPKAc bait construct and interactions were sought under high stringency conditions. Using this approach, we confirmed the interaction of each of these genes with TcPKAc. To further confirm these interactions, co-immunprecitation was performed of Triton-100X protein extracts from trypomastigotes using a trans-sialidase antibody. These experiments demonstrated that an anti-TcPKAc mAb was able to pull-down proteins recognized by a trans-sialidase antibody. In addition, trans-sialidase antibody was able to pull-down proteins detected by an antibody that recognized PKA specific phosphorylation (anti-phospho-(Ser/Thr) PKA substrate antibody). The proteins with phosphorylation had the same molecular weight as those identified by the trans-sialidase antibody suggesting that these proteins are, in fact, PKA substrates. In silico sequence analysis of trans-sialidase confirms the presence of sites that are likely to be modified by PKA. These studies have demonstrated a previously unknown interaction between the PKA pathway and these important trans-sialidase proteins in T. cruzi.

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Protein kinase A (PKA) has been suggested as a regulator of stage differentiation in T. cruzi. Using a yeast two-hybrid system we have begun to characterize the downstream substrates of PKA in T. cruzi. Twenty-three target plasmids interacting with BD- TcPKAc were obtained. Among the proteins identified as interacting with TcPKAc are several members of the trans-sialidase super family. The full length ORF of the transialidase genes were amplified by PCR and then subcloned into pAD-Gal4 vectors. Each pAD-Gal4 construct was transformed into yeast containing a TcPKAc bait construct and interactions were sought under high stringency conditions. Using this approach, we confirmed the interaction of each of these genes with TcPKAc. To further confirm these interactions, co-immunprecitation was performed of Triton-100X protein extracts from trypomastigotes using a trans-sialidase antibody. These experiments demonstrated that an anti-TcPKAc mAb was able to pull-down proteins recognized by a trans-sialidase antibody. In addition, trans-sialidase antibody was able to pull-down proteins detected by an antibody that recognized PKA specific phosphorylation (anti-phospho-(Ser/Thr) PKA substrate antibody). The proteins with phosphorylation had the same molecular weight as those identified by the trans-sialidase antibody suggesting that these proteins are, in fact, PKA substrates. In silico sequence analysis of trans-sialidase confirms the presence of sites that are likely to be modified by PKA. These studies have demonstrated a previously unknown interaction between the PKA pathway and these important trans-sialidase proteins in T. cruzi.

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Analysis of MAP2K function in *Trypanosoma brucei*

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T. brucei lacks many types of signaling molecules, its genome does encode for protein kinases involved in the MAP (mitogen activated protein kinase) signal transduction pathway. MAP signal cascades involve a series protein kinases-MAP kinase (MAPK), which is phosphorylated and activated by a MAP kinase kinase (MAP2K), which is regulated by a MAP kinase kinase kinase (MAP3K). MAPK cascades have been shown in other organisms to transduce both external signals, such as growth factors and pheromones, and internal signals including osmotic and oxidative stress. As varied as the inputs into the cascade, the outputs are just as varied, including cell growth (mitogen), cell cycle arrest (yeast mating pheromone) and increased synthesis of glycerol (osmotic stress). In trypanosomatids, MAPKs studied thus far regulate the disparate processes of differentiation and flagellar length. The *T. brucei* genome is predicted to encode ten MAPKs, four MAP2Ks, and fourteen MAP3Ks. We have chosen to concentrate our analysis on the functional analysis of the MAP2Ks, not only for their more manageable numbers, but also because their human homologues have been targets of drug discovery campaigns. Knockdown of the expression of one the MAP2Ks via RNAi in bloodstream forms led to severe decrease in cell growth and the formation of large multiflagellated cells. DNA analysis revealed an accumulation of G2 cells with improperly segregated nuclei and kinetoplasts. This defect in cell division represents a novel phenotype for the MAPK pathway.

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Stage regulation of genes encoding citric acid cycle enzymes in *Trypanosoma brucei*

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Adaptation to changing host environments requires rapid reprogramming of gene expression in the life cycle of *Trypanosoma brucei*. We investigate the molecular basis of coordinated induction of metabolic enzymes during bloodstream to procyclic differentiation. All citric acid cycle (TCA) activities are known to be very low or absent in the bloodstream stage and upregulated in the procyclic stage. The 5'- and 3'- UTRs of 9 genes encoding citric acid cycle (TCA) enzymes were mapped by RACE-PCR and comparatively analyzed by RNA secondary structure alignment algorithms. Regulation of endogenous aconitase in a pleomorphic strain was shown by labelled amino acid incorporation to be prodominantly at the level of protein biosynthesis, consistent with a minor change of mRNA. The apparently translational upregulation occured in bloodstream forms upon slender to stumpy differentiation. Further upregulation in procyclic trypanosomes is due to aconitase protein stability control as shown by pulse chase experiments. Reporter constructs with UTR replacements and mutations were targeted to the homologous aconitase locus of a monomorphic strain. Analysis of BSF and derived PCF by quantitative Western blotting and TaqMan qPCR assigned the regulation to both the 3'-UTR and the 5'-UTR of aconitase. Functional analysis of UTR sequences from other TCA enzymes is underway.

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224B

The mitochondrial DNA polymerase, Pol IB, is essential for minicircle DNA replication in *Trypanosoma brucei*

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A unique feature of *Trypanosoma brucei* and related parasites is their catenated mitochondrial DNA network of minicircles (thousands) and maxicircles (tens) called kinetoplast DNA (kDNA). Additionally, the single mitochondrion contains at least SIX DNA polymerases (Pol) for kDNA replication and repair processes whereas most other eukaryotes contain just one, pol gamma. RNA interference (RNAi) studies on four of these Pols (related to bacterial Pol I) indicated that three, PolIB, PolIC and Pol ID, are essential for parasite growth and kDNA maintenance. We hypothesize that the three pol I-like proteins serve non-redundant roles as replicative pols at the core of the kDNA synthetic machinery. To understand the specialized cellular role of Pol IB, we used inducible RNAi in procyclic cells. Pol IB silencing with a stemloop dsRNA resulted in >85% reduction of the Pol IB mRNA and growth inhibition after four days of RNAi induction. The induced cells showed a progressive loss of kDNA throughout the RNAi induction. Analysis of DAPI stained cells indicate that by day 8 of Pol IB silencing 37% do not contain kDNA, while 55% retain small kDNA networks. We further characterized the Pol IB silencing phenotype by examining the relative loss of minicircles vs. maxicircles. Minicircle abundance selectively decreased within 4 days of Pol IB silencing with subsequent decline in both minicircles and maxicircles. Additionally, covalently closed minicircle replication intermediates accumulated following 2 days of silencing indicating that Pol IB has a primary role in minicircle DNA replication. We are further analyzing minicircle replication intermediates using two dimensional gels with leading and lagging strand specific probes to clarify the role of Pol IB during minicircle replication.

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Genome-wide analysis of developmental regulation of trafficking systems in *Trypanosoma brucei*

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One of the major alterations between the proliferative life stages of *Trypanosoma brucei* is remodeling of the endosomal system; both endocytosis and recycling pathways are upregulated in the bloodstream form, while proportionally lysosomal delivery appears decreased when compared with the procyclic. These features suggest that specific functions are required of the transport system to support survival within the different hosts. Using a combination of sequence similarity, domain structure, GO and keyword annotation we identified ∼600 predicted genes with a putative role in trypanosome membrane trafficking. To monitor expression of this cohort of genes we developed a 70mer oligonucleotide microarray. We find that approximately 8% of the genes in the cohort are highly significantly differentially expressed in either the bloodstream or the insect stage, including previously characterised factors as well as many new gene products. In particular we find evidence for upregulation of both the AP3 and AP4 adaptin complexes in the insect stage. Further, to probe for interactions that may act as key regulatory networks of trafficking in trypanosomes we tested the response of the transcriptome to RNAi-mediated depletion or overexpression of defined components. Data indicate a lack of transcriptional flexibility in cells with altered expression levels of Rab5. Although *T. brucei* procyclic cells ectopically overexpressing Rab5A or Rab5B display an increase in both endocytic activity and in expression of certain proteins associated with endocytosis, transcriptome analysis shows no significant variation in steady-state mRNA levels for any of the 600 genes with a possible role in endocytosis.

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225B

Differential induction of apoptotic and non-apoptotic death of *Leishmania* by distinct classes of antimicrobial peptides

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We have recently reported that alpha- and theta-defensin-, magainin- and cathelicidin-type antimicrobial peptides (AMPs) can kill the pathogenic protozoan *Leishmania* and that the surface-protease (leishmanolysin) serves to protect parasites from the cytolytic effects of several AMPs. Comparative studies of a panel of AMPs have defined two distinct groups: those that induce non-apoptotic (class-I) and apoptotic (class-II) parasite killing based on their differential ability to induce: phosphatidyl serine exposure, loss of mitochondrial membrane potential and decreased ATP production, induction of caspase activity and DNA degradation. In addition class-II AMPs cause rapid influx of the vital stain SYTOX and increased intracellular Ca2+ while class-I AMPs cause a slow accumulation of SYTOX do not affect intracellular Ca2+ levels. Inhibitors of cysteine or caspase proteases diminish fast influx of SYTOX through the surface-membrane and DNA degradation does not ablate the annexin-V staining or the induction of apoptosis by class-II AMPs. This suggests that the changes in surface permeability in AMP-mediated apoptosis are related to the downstream events of intracellular cysteine/caspase activation or loss of ATP rather than to changes in surface membrane permeability. We are also testing the in vivo importance of AMPs in leishmanial disease. Mice lacking the cathelicidin-related AMP (CRAMP) develop markedly smaller cutaneous lesions than wildtype mice. In vitro *Leishmania* evade CRAMP-killing by leishmanolysin-mediated peptide degradation and together these results suggest that the interaction of parasites with CRAMP peptide fragments may directly or indirectly enhance parasite pathogenesis.

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Differentiation-induced up-regulation of SL RNA abundance in *Leishmania donovani*

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Leishmania donovani are intracellular parasitic protozoa that cause kala azar in humans. During infection the extracellular promastigotes undergo rapid differentiation to intracellular amastigotes that proliferate in phagolysosomes of mammalian macrophages. Little is known about the molecular mechanisms that regulate *Leishmania* differentiation. Recently, our laboratory initiated time course analysis of *L. donovani* differentiation. These studies showed that promastigotes differentiation into amastigotes is a regulated process that involves ordered changes in morphology, protein function and gene expression. In *Leishmania*, mRNAs are generated by *trans*-splicing using spliced leader RNA (SL RNA). Here we examined changes in *trans*-splicing and SL RNA transcription and abundance as a function of differentiation progression. We observed a burst in SL RNA transcription that was followed by a transient but significant increase in the steady state level of SL RNA in parasites cell nucleus. A small increase in *trans* splicing was also observed, but it was not sufficient to affect the accumulation of SL RNA. In parallel, SLA1 RNA, a polII transcripts that directs pseudouridayltion of SL RNA and is essential for *trans* splicing, increased during differentiation in a manner similar to that of the SL RNA. These changes coincided with the period in differentiation parasites underwent morphological change as well as major changes in mRNA abundance. Therefore, we conclude that *trans* splicing may play a role in regulating gene expression during *L. donovani* differentiation. This is the first report demonstrating that during Leishmania differentiation there is a change in *trans* splicing, SL and SLA1 RNA abundance.

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How did *Trypanosoma equiperdum* and *T. evansi* evolve from *T. brucei*?

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The present paradigm holds that *Trypanosoma equiperdum* and *T. evansi* evolved from *T. brucei* following partial and total loss of maxicircle kinetoplast (k) DNA, respectively. However, one of the studied strains of *T. equiperdum* contains entire maxicircle, the coding region of which differs from *T. brucei* in only a few nucleotides. Interestingly, this strain cannot differentiate into procyclics and the complexity of its minicircles seems to be lower than in *T. brucei*. All studied *T. equiperdum* and *T. evansi* strains are highly infectious to mice, are prone to kDNA loss upon injection of EtBr in the infected animals. Same as the *T. brucei* bloodstream stage, they contain only very weak NADH dehydrogenase activity, and no activity of complexes II, III and IV. Strong ATPase activity is present in the cell lysates of *T.equiperdum* and *T. evansi*, but by in-gel activity staining its pattern differs from that of *T. brucei*. Proteins involved in RNA editing and stability, such as mitochondrial RNA binding proteins 1 and 2, are present in the partially dyskinetoplastic flagellates, whereas the transcripts of a nuclear-encoded subunit of respiratory complex IV permanently transcribed in *T. brucei* are absent. Mitochondrial transcription and the extent of RNA editing in *T. equiperdum* and *T. evansi* are being investigated.

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Whole-genome expression profiling analysis of antimony resistance in the protozoan parasite *Leishmania infantum*

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The *Leishmania* parasite is the etiological agent of a range of diseases termed leishmaniasis against which the main drug treatment remains chemotherapy based on antimonial derivatives. Antimony efficacy is however greatly threatened by an increasing parasitic resistance in endemic areas. This resistance seems multifactorial and to involve many cellular processes including thiol metabolism and drug transport/sequestration. In order to study the mechanisms underlying antimony resistance, we performed whole-genome expression profiling analyses between an antimony-sensitive strain and an *in vitro* selected antimony-resistant mutant of *Leishmania infantum* using oligonucleotides DNA microarrays. Over 200 genes were found to be significantly modulated between the two phenotypes, most of which were modulated by a ratio between 1,5 and 2. Some of these genes are found alone in the genome whereas others are part of groups of modulated genes. Many metabolic pathways seem to be affected like purine and sugar metabolisms. *MRPA*, an ABC gene already known to be involved in antimony resistance, was found greatly overexpressed in the antimony-resistant mutant along with three other tandemly linked genes on chromosome 23. This four genes locus is flanqued by a 1,4 kb direct repeat sequence which involvement in the generation of an apparently new circular amplicon observed in the antimony-resistant mutant is now under investigation. The modulated expression of some genes has been confirmed by real-time RT-PCR and will be further studied by gene overexpression and gene deletion experiments.

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RNAi of an unusual protease HslVU in *Trypanosoma brucei* has a striking effect on kDNA segregation

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ATP-dependent protease complexes are present in all living organisms, including the 26S proteasome in eukaryotes, *Archaea*, and *Actinomycetes* and the HslVU protease in eubacteria. The two protease complexes are only distantly related. Their simultaneous presence in the same organism was recently suggested by genomic evidence among some primordial eukaryotes such as amoebozoa, plantae, chromoalveolata, rhizaria and excavata species, though the potential significance of this co-existence has not been explored. Here we report the identification and characterization of this unusual ATP requiring protease HsIVU in *T. brucei*; the genome encodes one HslV subunit (TbHslV) and two HslU subunits (TbHslU1 and TbHslU2). The genes are expressed in comparable levels in bloodstream and procyclic forms and, based on epitope tagging, the gene products are localized to the single mitochondrion, probably in the kinetoplast region. Depletion of TbHslVU by RNA interference led to defective kinetoplast division resulting in kinetoplasts of aberrant sizes and shapes as well as depressed cell growth. TbHslU1 and TbHslU2 are functionally redundant regulatory subunits, whereas TbHslV encodes a functional threonine protease. The most striking effect of TbHslVU knockdown appears to be on kinetoplast segregation, and we speculate that the protease complex may be involved in regulation of this process.

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ATP-dependent protease complexes are present in all living organisms, including the 26S proteasome in eukaryotes, *Archaea*, and *Actinomycetes* and the HslVU protease in eubacteria. The two protease complexes are only distantly related. Their simultaneous presence in the same organism was recently suggested by genomic evidence among some primordial eukaryotes such as amoebozoa, plantae, chromoalveolata, rhizaria and excavata species, though the potential significance of this co-existence has not been explored. Here we report the identification and characterization of this unusual ATP requiring protease HsIVU in *T. brucei*; the genome encodes one HslV subunit (TbHslV) and two HslU subunits (TbHslU1 and TbHslU2). The genes are expressed in comparable levels in bloodstream and procyclic forms and, based on epitope tagging, the gene products are localized to the single mitochondrion, probably in the kinetoplast region. Depletion of TbHslVU by RNA interference led to defective kinetoplast division resulting in kinetoplasts of aberrant sizes and shapes as well as depressed cell growth. TbHslU1 and TbHslU2 are functionally redundant regulatory subunits, whereas TbHslV encodes a functional threonine protease. The most striking effect of TbHslVU knockdown appears to be on kinetoplast segregation, and we speculate that the protease complex may be involved in regulation of this process.

ICP regulation of *Trypanosoma brucei* cysteine peptidases: influence on parasite traversal of the brain barrier

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ICP is a chagasin family natural tight-binding inhibitor of papain-like cysteine peptidases (CPs) found in protozoa and bacteria. We have generated *Trypanosoma brucei* bloodstream form *ICP*-deficient mutants (Δ*icp*) and detected a 4-fold increase in CP activity in Δ*icp* parasite lysates, which was restored to the levels of wild type by re-expression of the gene in the null mutant. Δ*icp* parasites reached higher parasitemia than wild type in infected mice, suggesting that ICP modulates parasite infectivity. Using a model of the human blood-brain barrier (BBB) consisting of human Brain Microvascular Endothelial Cells (BMEC), we have previously reported that bloodstream forms of *T. b. gambiense* cross the BBB at higher efficiency than *T. b. brucei* and that this ability may be due to differences in the levels of the cathepsin L-like CP, brucipain. We have found that Δ*icp* parasites traverse the BBB more efficiently than wild type, a phenotype that was reversed when parasites were pre-incubated with inhibitors to brucipain, but not with the inhibitor to cathepsin-B, CA074-Me. BBB crossing by wild type parasites was enhanced in the presence of secretion products derived from Δ*icp*, and this effect was countered by synthetic CP inhibitors. Δ*icp* induced phosphorylation of Akt in BMECs and parasite traversal was impaired when cells were pre-incubated with wortmannin or with an inhibitor to MEK, but not with inhibitors of ERK1/2. In contrast, BBB permeability and parasite traversal were enhanced by BMEC pre-treatment with inhibitors to adenylate cyclase. Taken together, these data suggest that CPs of *T. brucei* bloodstream form are endogenously regulated by ICP and drive trans-endothelial migration in vitro by engaging PI3-kinase dependent signaling pathways.

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LOK1, a *Trypanosoma brucei* protein discovered in an RNAi library screen, is involved in kinetoplast division

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During kinetoplast DNA (kDNA) network replication, the minicircle and maxicircle copy numbers increase, and when they have doubled, the network splits in two. Little is known about the kDNA division mechanism. By screening an RNAi library for cells that lost kDNA, we identified a novel 19 kDa highly basic protein, Lok1 (named for loss of kDNA), that seemed to be involved in kDNA division. Lok1 localizes to the mitochondrion and is essential for growth. RNAi causes gradual shrinking of the kDNA network, as measured by microscopy after DAPI-staining. We confirmed kDNA loss by Southern blotting, showing that both minicircles and maxicircles decline in parallel. RNAi also causes a decrease in free minicircle replication intermediates. The most interesting phenotype involved kDNA division, which we studied by DAPI-staining and fluorescence in situ hybridization (FISH) of intact cells and by EM of isolated networks. As reported previously and confirmed by our FISH of wild type cells, maxicircles concentrate at the center of dividing, dumbbell- shaped networks and on rare occasions form a short filament connecting sister networks. Surprisingly, RNAi cells have much longer maxicircle filaments connecting sister networks that have separated for larger distances. More surprisingly, kinetoplast segregation was usually asymmetric with one sister larger than the other. Our data indicate that segregation of sister maxicircles is a late or final step in kDNA division in both wild-type and Lok1 RNAi cells. Because these filaments are more prominent in RNAi cells, Lok1 may be involved in maxicircle segregation. Finally, our studies of several RNAi and knockout cell lines provide new evidence that network size contributes to the accuracy of kinetoplast division.

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Galactosylation of *Trypanosoma brucei* GPIs: evidence for the presence of two parallel pathways of GPI synthesis in insect forms

Yuk-Chien Liu, Alvaro Acosta-Serrano*

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The surface glycocalyx of procyclic *T. brucei* forms contains several millions of GPI-anchored procyclins and free GPIs. These GPIs can be modified with a large number of sialylated, *N*-acetyllactosamine (LacNAc) repeats, which are essential for the parasite survival in the fly midgut (1, 2). According to structural studies, LacNAc repeats are linked to the GPI glycan core via a linear chain of galactose residues, but the exact location (*i.e.*ER or Golgi) where these glycans are added is unknown. When membranes of procyclic trypanosomes were pulse-labelled with GDP-[^{3}H]Man (in the presence of an excess of UDP-Gal and UDP-GlcNAc) or UDP-[^{3}H]Gal alone, most GPI intermediates were galactosylated, starting with that containing only two Man residues. Each intermediate can contain up to two Gal residues. However, no GlcNAc was detected suggesting that the LacNAc repeats are not added in the ER. The latter finding was corroborated by labelling membranes from *GPI10-*/-cells (defective in the transfer of the third Man residue) and mass spectrometric analysis of free GPIs from the same mutant parasite. Taken together, our data suggest that the assembly of the procyclic GPI side chain begins early on during GPI biosynthesis, but that addition of the LacNAc repeats are likely to occur at the Golgi apparatus. The early transfer of Gal residues during biosynthesis might represent the divergent step during the formation of free GPIs and GPI anchor precursors at the ER. The latter is different to that described in blood stream forms, where GPI galactosylation occurs after the formation of mature precursors (3). **(1) Nagamune et al (2004) JEM; (2) Guther et al (2006) MBC; (3) Guther et al (2003) Biochem.**

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Vitamin C biosynthesis in *Trypanosoma cruzi*

Flora J. Logan*, Shane R. Wilkinson, Martin C. Taylor, Harparkash Kaur, John M. Kelly

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Humans lack the ability to synthesise vitamin C (ascorbate), due to the absence of gulonolactone oxidase, the last enzyme in the biosynthetic pathway in most other mammals. The corresponding enzyme in *Trypanosoma cruzi*, the causative agent of Chagas disease, may therefore represent a target for drug design. Here, we show that this enzyme (TcALO) can synthesise vitamin C using both L-galactono-γ-lactone and D-arabinono-γ-lactone as substrates, in reactions that obey Michaelis-Menten kinetics. It is 20-fold more active than the analogous enzyme from *Trypanosoma brucei*. TcALO requires flavin mononucleotide (FMN) for enzyme activity, and binding of this essential co-factor is by non-covalent linkage. In other flavoproteins, a histidine residue located within the amino terminal flavin-binding motif has been shown to be crucial for co-factor binding. We used site-directed mutagenesis to show that the corresponding residue in TcALO (K55) is not essential for this interaction. In contrast, we found that histidine (H447) and tryptophan (W448) residues, localised within a carboxyl terminal motif (HW*K) that is a feature of ascorbate-synthesising enzymes, are necessary for the FMN-linkage. The conserved lysine residue within this motif (K450) is not required for co-factor binding, but its substitution by glycine renders the protein completely inactive.

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Intracellular signaling involved with cell differentiation of *Trypanosoma cruzi* and *Herpetomonas* spp

Angela H. Lopes[*,1], Marta T. Gomes[1], Danielle P. Vieira[1], Fernando L. Dutra[1], Felipe A. Dias[1], Luciana T. Zimmermann[1], Thiago L. Alves e Silva[1], Georgia C. Atella[2], Mario AC Silva-Neto[2]

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Trypanosoma cruzi is the causative agent of Chagas disease. *Herpetomonas* spp are insect trypanosomatids that present three evolutive forms: promastigote, paramastigote and opisthomastigote. Platelet-activating factor (PAF) is a potent phospholipid mediator of several cellular functions in diverse biological and pathophysiological processes, such as cell differentiation, inflammation and allergy. Lysophosphatidylcholine (LPC) is a major bioactive compound of plasmatic lipoproteins like LDL. Intriguingly, our group recently shown a PAF-like activity in *T. cruzi* (Gomes et al., Int. J. Parasitol. 2006; 36:165-73), as well as the presence of LPC in the saliva and feces of *Rhodnius prolixus* (Golodne et al., J. Biol. Chem. 2003; 278:27766-71). PAF is known to enhance cell differentiation in *T. cruzi* and in *H. m. muscarum*. In the present work we show that PAF triggers cell differentiation of *T. cruzi*, clone Dm28c, from epimastigotes into trypomastigotes, through a cascade of signal transduction events, leading to an enhancement of cAMP activity, and inhibition of the expression of a metalloprotease and a cystein protease, involving protein kinase CK2 and protein kinase C (PKC). Also, here we demonstrate that the enhancement of cellular differentiation promoted by PAF and cAMP in *H. m. muscarum* is mediated by protein kinase A (PKA). An augmentation of PKA activity when *H. m. muscarum* parasites were treated with PAF, cAMP and PAF plus cAMP is demonstrated. We also show that LPC modulates some important signaling pathways involving PKC, PKA and CK2, which lead to cell differentiation of *H. samuelpessoai* and that phospholipase A2 probably rules this process by converting phosphatidylcholine into LPC. This work was supported by the Brazilian Agencies CNPq, FAPERJ, CAPES and PIBIC/CNPq/UFRJ.

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A *T. brucei* AMP-dependent kinase links nutrient sensing and surface molecule expression

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Trypanosoma brucei alternates between mammalian bloodstream form (BS) parasites and insect stage, procyclics (PF). The correct surface molecule expression at each of these life stages is important for parasite survival. PF parasites modulate surface molecule expression in response to changes in environmental glucose concentrations. The molecular basis of this regulation is unknown. The heterotrimeric AMP dependent protein kinase (AMPK) consists of a catalytic alpha subunit and two regulatory subunits (beta and gamma). In other systems, AMPK has been found to play a role in regulating multiple cellular processes after activation as a result in a change in the relative concentrations of AMP and ATP. Because the change in metabolism might alter the AMP:ATP ratio, we have hypothesized that Tb AMP-dependent protein kinase might play a role in linking nutrient sensing to surface molecule expression. Here, we describe the characterization of *T.brucei* homologs of AMPK (TbAMPK), with a particular focus on the role of the complex in nutrient sensing pathways. RNAi of subunits leads to altered surface molecule expression and changes in cellular hexokinase activity, further establishing the connection between metabolism and development. To study heterotrimeric complex formation, we have employed a bacterial co-expression system (developed for human AMPK studies, Neumann et. al., 2003) in which all three subunits are cloned in a single plasmid and expressed simultaneously in *E. coli*. Taken together, these studies suggest that TbAMPK is a key regulator of cellular responses to environmental conditions.

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Identification of histone modifications in the infectious bloodstream form of *Trypanosoma brucei*

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For most genes in Trypanosoma brucei, there is no apparent regulation of transcription initiation, so chromatin structure is expected to play a major role in regulating gene expression. The regulation of Variant Surface Glycoprotein gene expression is a particular case where chromatin modification has been experimentally implicated in regulation. No specific histone modifications have hitherto been identified, or correlated with the regulation of gene expression, in the mammalian-infectious forms of T. brucei. The tails of the four core histones of T. brucei are highly diverged from those of mammals and yeasts, so sites of potential modification cannot be reliably inferred, and no cross-species antibodies are available to map the modifications. We therefore undertook an extensive survey to identify posttranslational modifications by Edman degradation and mass spectrometry. Edman analysis showed that the N-terminal alanine of H2A, H2B, and H4 can be monomethylated. We found that the histone H4 N-terminus is heavily modified, while, in contrast to other organisms, the histone H2A and H2B N-termini had relatively few modifications. Histone H3 appears to have a number of modifications at the N-terminus, although we were unable to assign many of these to a specific amino acid. Therefore, we focused our efforts on uncovering modification-states of H4. Finally, we isolated histones from T. brucei lacking the Saccharomyces cerevisiae SIR2 histone deacetylase homologue, and compared the level of H4 acetylation in DTbsir2rp-1 and wild-type cells. No change in H4 acetylation was observed in DTbsir2rp-1 cells, indicating that TbSIR2RP-1 is probably not an H4 deacetylase.

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231B

Trypanosoma cruzi flagellar calcium-binding protein: investigation of function and requirements for localization

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In contrast to trafficking of proteins to many cellular organelles, which employs specific organelle targeting sequences, trafficking of proteins to the eukaryotic flagellum remains unclear. While the recently discovered process of intraflagellar transport (IFT) has been shown to mediate delivery of structural subunits to the flagellum, it is currently uncertain to what extend this mechanism applies to proteins of the flagellar membrane. Previous work on the 24-kDa flagellar calcium binding protein of T. cruzi (FCaBP) identified a 24 amino acid peptide at the N terminus that is both necessary and sufficient for flagellar localization. We have shown that this region of FCaBP is both myristoylated and palmitoylated, and that both modifications are required for flagellar membrane targeting and association with flagellar lipid rafts. However, proteins containing shorter truncations of this leader peptide failed to localize to the flagellum despite being properly acylated, suggesting that there might be requirements other than acylation for flagellar targeting. The studies presented here were initiated to distinguish between two possible models of interaction: (i) that N13-24 comprises a spacer that helps the acylated N-terminus to extend from the globular protein to effect maximal membrane association and (ii) that a peptide-peptide interaction between N13-24 and some binding partner contributes to localization. To distinguish between the two proposed models we have engineered proteins that contain various types of leader sequences, including homologous recoverin sequence, scrambled FCaBP sequence and sequence rich in glycine and proline residues, replacing the FCaBP residues 13-24. The results of study of these newly engineered proteins will be discussed.

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In contrast to trafficking of proteins to many cellular organelles, which employs specific organelle targeting sequences, trafficking of proteins to the eukaryotic flagellum remains unclear. While the recently discovered process of intraflagellar transport (IFT) has been shown to mediate delivery of structural subunits to the flagellum, it is currently uncertain to what extend this mechanism applies to proteins of the flagellar membrane. Previous work on the 24-kDa flagellar calcium binding protein of T. cruzi (FCaBP) identified a 24 amino acid peptide at the N terminus that is both necessary and sufficient for flagellar localization. We have shown that this region of FCaBP is both myristoylated and palmitoylated, and that both modifications are required for flagellar membrane targeting and association with flagellar lipid rafts. However, proteins containing shorter truncations of this leader peptide failed to localize to the flagellum despite being properly acylated, suggesting that there might be requirements other than acylation for flagellar targeting. The studies presented here were initiated to distinguish between two possible models of interaction: (i) that N13-24 comprises a spacer that helps the acylated N-terminus to extend from the globular protein to effect maximal membrane association and (ii) that a peptide-peptide interaction between N13-24 and some binding partner contributes to localization. To distinguish between the two proposed models we have engineered proteins that contain various types of leader sequences, including homologous recoverin sequence, scrambled FCaBP sequence and sequence rich in glycine and proline residues, replacing the FCaBP residues 13-24. The results of study of these newly engineered proteins will be discussed.

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Characterization of cyclic AMP export from *Trypanosoma brucei*

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Over the last two or three decades, our understanding of cAMP signalling in eukaryotes, mostly in mammalian cells and selected unicellular eukaryotes, has been expanded considerably. In contrast, the exploration of cAMP signalling in protozoan pathogens has only recently been initiated. Most investigations so far were done concerning the intracellular signalling, and processes such as cAMP formation by adenylate cyclases and cAMP hydrolysis by various phosphodiesterases have been studied. Even though cAMP signalling has traditionally been studied in the context of intracellular signal transduction, the capacity to export cAMP has been maintained in many (all?) eukaryotic cells. All currently available evidence indicates that this export represents more than just a way of disposing of excess cAMP. In several cell types and tissues, such as the kidney or in fat tissue, a cAMP-mediated, extracellular signalling pathway has been established. Moreover, in Dictyostelium discoideum, extracellular cAMP plays a critical role in mediating the developmental transition from the amoeboid to the multicellular form. In bacteria, cAMP export appears to be important for the regulation of intracellular cAMP concentration. In eukaryotes, cAMP export as a control mechanism for intracellular cAMP has largely been superseded by phosphodiesterase activity which allows a much faster regulation than export does. The aim of this project is to explore if *Trypanosoma brucei* is capable of exporting cAMP, and if so, if this extracellular cAMP exerts a physiological function. So far we were able to show that a large proportion of all cAMP synthesized by trypanosomes accumulates in the growth medium. We were able to identify one or possibly two different MRP-like ABC transporters that are involved in cAMP export.

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232B

Cell signalling in the african trypanosome

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The control of population size and differentiation status is essential for the survival of all organisms. Tight regulation of these processes is of particular importance for parasites. The African trypanosome, Trypanosoma brucei brucei, represents a highly successful parasite capable of surviving and thriving in the harsh environment of the blood and lymphatic liquids of vertebrate hosts and in the midgut and salivary glands of the tsetse fly vector. African trypanosomes exist in two stages in mammalian hosts, a long slender (LS) and a short stumpy (SS) stage. In the course of this work a putative transporter for the trypanosome stumpy inducing factor (SIF), also referred to as T-GIF, was identified. An Expression Site Associated Gene, ESAG10, was implicated in the long slender to short stumpy differentiation of bloodstream forms. In addition, a vesicle trafficking protein, the T. b. brucei ADP-ribosylation factor 1 (TbArf1), previously implicated in long slender differentiation by this group, was shown to interact with ESAG10. TbArf1 was shown to interact with cAMP as part of the differentiation process and a model of this LS to SS differentiation process is proposed.

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233A

Fibronectin binding and metallo- and cysteine proteolytic degradation by *Leishmania* contributes to parasite infectivity

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Infection by the vector-borne protozoan *Leishmania* occurs by the deposition of parasites within the skin of the mammalian host where they interact with extracellular matrix (ECM) and basement membrane proteins such as fibronectin (FN), leading to enhanced invasion. Here we show that both insect- and mammalian forms can bind directly to solid-phase and soluble FN and insect forms express a distinct surface protein of ~60 kDa which can bind both FN and laminin. Insect forms of multiple *Leishmania* species can also rapidly degrade FN using surface-localized and secreted surface-metalloprotease (leishmanolysin). FN degradation at the surface of amastigotes is leishmanolysin-dependent whereas both secreted leishmanolysin and cysteine protease B contribute to FN degradation extracellularly. The growth of intracellular *Leishmania* within macrophages exposed to parasite degraded FN was dramatically increased compared to those exposed to intact FN which correlates with decreased production of reactive oxygen intermediates. These findings show that both insect and mammalian forms of *Leishmania* bind to and proteolytically degrade FN at the parasite surface and distantly through secreted proteases which can enhance parasite infection.

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233B

A vacuolar transporter chaperone is involved in the biogenesis of acidocalcisomes in *Trypanosoma brucei*

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Acidocalcisomes play essential roles and have been conserved during evolution from bacteria to man. However, little is known about their biogenesis. Proteomic analysis of acidocalcisomes resulted in the identification of an homologous protein to the yeast vacuolar transporter chaperone 1 (Vtc1) in *Trypanosoma brucei* that is essential for acidocalcisome biogenesis and cell growth. *T. brucei Vtc1* (*TbVtc1*) encodes a 180 amino acid product with a predicted molecular mass of 19.8 kDa and a calculated isoelectric point of 8.8. The protein has 3 predicted transmembrane domains near the C-terminus. TbVtc1 is 75, 71, and 33% identical to the Vtc1's of *T. cruzi*, *L. major*, and *S. cerevisiae*, respectively. Immunofluorescence of a cell line expressing a TbVtc1 fused to GFP revealed its co-localization with the vacuolar H^+-pyrophosphatase, a marker for acidocalcisomes, what was confirmed by Western blot analysis of acidocalcisome fractions and immunogold electron microscopy using antibodies against a fragment of TbVtc1. TbVtc1 co-immunoprecipitated with the vacuolar H^+-ATPase, suggesting a similar chaperone role as occurs with its homologous protein in yeast. Knock down of TbVtc1 expression by RNA interference increased the size and absolute volume of acidocalcisomes, and induced marked gross morphological alterations–including multiple nuclei and flagella, as well as the appearance of large multilammelar vacuoles–followed by cell death. Ablation of TbVtc1 expression produced significant reduction in short and long chain polyphosphate content and alterations in intracellular Ca^{2+} and pH homeostasis, while overexpression of *TbVtc1* gene caused mild alterations in growth rate, but had no perceptible effect on morphology or intracellular pH and Ca^{2+}/ homeostasis. Altogether, these results provide support for an important role of TbVtc1 in the biogenesis of acidocalcisomes.

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RNAi analysis of cell-cycle regulators in *Trypanosoma brucei*

Severine Monnerat, Caroline Clucas, Tansy C. Hammarton, Jeremy C. Mottram*

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RNA interference is a powerful tool for the analysis of gene function in *Trypanosoma brucei* and has been extensively used to study cell cycle regulation in this parasite via a reverse genetic approach. However, while RNAi of trypanosome orthologues of eukaryotic cell cycle regulators has provided useful information about their function, a wider aim is to identify novel molecules involved in trypanosome cell cycle control. Therefore, using the RNAi plasmid library developed by Morris *et al.*, 2002 (***EMBO J***, 21, 4429-4438), we have developed a forward genetic RNAi screen to isolate cell cycle mutants. 236 independent procyclic form RNAi clones were analysed for defects in cell cycle progression. In a primary screen, growth was assessed and the RNAi vector insert was sequenced. 10% of the clones suffered a growth defect upon RNAi induction. Clones displaying a growth defect were analysed further in a secondary screen. DNA content and the number of nuclei/kinetoplasts per cell were analysed by flow cytometry and microscopy, in order to reject essential genes not involved in cell cycle control ($\sim$75% of clones with growth defects). Potential cell cycle candidate genes are currently being cloned into the p2T7^{ti} vector to allow confirmation of the RNAi phenotype by reverse genetics. Progress using these approaches will be discussed.

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234B

Phosphoproteomic analysis of *Leishmania* promastigote and amastigote life stages: from substrates to MAP kinases

Miguel A. Morales[1], Reiko Watanabe[1], Olivier Renaud[2], Abdelkader Namane[3], Gerald Spaeth[*,1]

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Leishmania has evolved mechanisms of environmental sensing that are able to distinguish between the physiology of vector and host during the infectious cycle and control the expression of *Leishmania* stage-specific virulence factors. Extracellular signals are translated into stage-specific gene expression by reversible protein phosphorylation that is controlled by a network of protein kinases and phosphatases. To gain insight into this signaling network we enriched by affinity chromatography and identified by MS/MS 46 distinct phosphoproteins that fall into five functional groups and are constitutively phosphorylated across the major life cycle stages of the parasite. Surprisingly, we found only few amastigote-specific phosphoproteins, including a cyclophilin A and the MAP kinase homolog LmaMPK10. MAP kinases are implicated in extra-intracellular signaling across all major eukaryotic species and may consequently be relevant for the development and the pathogenicity of the intracellular amastigote stage. Using a polyclonal antibody against LmaMPK10 we could validate our proteomics data and show that the endogenous kinase is constitutively expressed in *L.donovani* promastigotes and axenic amastigotes, but differentially phosphorylated only in the latter stage. Episomal expression of GFP-tagged LmaMPK10 revealed predominantly cytoplasmic localization and allowed us to investigate in situ phosphorylation and activity of the kinase using immuno-purified protein. Only little phosphotransferase activity was detected in LmaMPK10 isolated from *L.major* and *L.donovani* promastigotes, but it was significantly increased in pull-downs obtained from *L.major* promastigotes after exposure to pH5.5 and 34C, and from axenic *L.donovani* amastigotes. We demonstrated that enhanced activity correlated with increased GFP-LmaMPK phosphorylation. These results underscore the potential of proteomic and transgenic approaches to gain insight into signaling events during the *Leishmania* life cycle.

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The role of PTR1 in the resistance to oxidative stress in *Leishmania*

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Leishmania is a folate and pterin auxotroph and those compounds are essential co-factors for its growth. Reduced pterins have also been implicated in *Leishmania* metacyclogenesis. Pteridine reductase PTR1 is an enzyme of the folate pathway allowing the reduction of pterins and folates and capable of conferring resistance to the antifolate methotrexate in *Leishmania*. During its life cycle, the parasite experienced oxidative stresses when interacting with macrophages. Reduced pterins are known as scavengers of reactive oxygen and nitrogen species in several organisms. *L. tarentolae PTR1-/-* mutant is more sensitive to H_2O_2. *L. tarentolae* is a non human pathogen and to better investigate the role of PTR1 in resistance to oxidative and nitrosative stresses, we inactivated PTR1 in both *L. infantum* and *L. major* human pathogens. *PTR1-/-* mutants grew well in SDM-79 or M199 medium. They were both hypersensitive to methotrexate and their metacyclogenesis was altered. Both *PTR1-/-* mutants were found to be more sensitive to H_2O_2 and this phenotype was reverted by an episomal rescue or by reduced pterins supplementation. These results have been confirmed by measuring the oxidative stresses induced within the parasite using DCF-DA probe (dichlorofluorescein diacetate). Increased fluorescence (an indicator of more oxidants) was observed in the *PTR1-/-* mutants. Preliminary results with nitric oxide donors such as SNAP or DETA-Nonoate show that *PTR1-/-* mutants are slightly more sensitive to nitrosative stresses. While pterins are essential co-factors for growth, their exact role is unknown. This study has shown that they contribute to survival within hostile oxidative environments and their role during macrophage-parasite interactions is now underway.

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Thromboxane A_2 is a key regulator in *Trypanosoma cruzi* pathogenesis

Shankar Mukherjee, Anthony W. Ashton, Louis M. Weiss, Herbert B. Tanowitz*

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Trypanosoma cruzi, the etiological agent of Chagas' disease, causes vasculopathy and cardiomyopathy in humans and is associated with elevated levels of important vasoregulators such as nitric oxide and endothelin-1. Recently, we have observed that *T. cruzi* (Brazil strain) synthesizes thromboxane A_2 (TXA_2), a potent vasoconstrictor and an important regulator of immune response and inflammatory reactions. Physical chromatography and LC-MS-MS revealed that TXA_2 is the predominant eicosanoid present in all life stages of the parasite. In TXA_2 synthase null mice, parasite-derived TXA_2 can account for up to 90% of the circulating levels of TXA_2. TXA_2 receptor (TP) null mice, exhibited higher mortality and more severe cardiac pathology and parasitism (4-fold) than WT mice after infection. Infection of TXA_2 synthase null mice did not result in any increase in mortality, however, the cardiac pathology was reduced. TP expression on somatic cells, but not in immune cells, was the primary determinant of disease progression. The higher intracellular parasitism observed in TP-null endothelial cells was reduced upon restoration of TP expression by transfection of TP gene. Increased synthesis of TXA_2 is seen in the acute phase but also maintained to the chronic state. Since Chagas' disease is both a vasculopathy and a cardiomyopathy, the finding that these parasites can *de novo* synthesize TXA_2 is of immense importance and a deeper understanding of the role of TXA_2 may result in the development of new strategies for drug design for a disease with limited treatment options.

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Tyrosine phosphatases in *Leishmania* differentiation and virulence: a potential drug target

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Leishmania undergoes two main developmental stages during its life cycle, residing in the midgut of the sandfly as the extracellular flagellated promastigote and as obligate intracellular amastigotes multiplying in the phagolysosome of infected macrophages. The biochemical pathways enabling *Leishmania* to differentiate and survive in the mammalian host are poorly defined. We have therefore examined the role of protein tyrosine phosphorylation, which is essential in regulating cell function in higher eukaryotes. Using the completed *Leishmania* genome, we have identified and cloned a *Leishmania* protein tyrosine phosphatase gene (LPTP1) by virtue of its homology with the human protein tyrosine phosphatase 1B gene (hPTP1B) and confirmed its enzyme activity. We further demonstrate, by creating LPTP1 null mutants, that LPTP1 is necessary for survival as amastigotes in mice but it is dispensable for survival as promastigotes in culture. Human PTPs are actively pursued drug targets for a variety of diseases. The observations with the LPTP1 mutants in mice suggest it may also represent a drug target against the mammalian amastigote stage. However, *in silico* structure analysis of LPTP1 revealed a striking similarity with hPTP1B in the active site suggesting that, although this is an attractive drug target, it may be difficult to develop an inhibitor specific for the *Leishmania* LPTP1.

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Molecular basis of functional divergence in *Leishmania* monosaccharide transporters

Christina Naula[1], Flora Logan[2], Michael Barrett[1], Richard Burchmore[*,1]

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¡I¿Leishmania¡I/¿ can acquire monosaccharides by a high affinity transport system. ¡I¿L. mexicana¡I/¿ encodes three monosaccharide transporter genes, named ¡I¿Lm¡I/¿GT 1-3 after their initial characterisation as glucose transporters (Burchmore, 1998). Delta¡I¿Lm¡I/¿GT, a mutant lacking all three transporter genes, has reduced viability in the insect host, is unable to sustain infection in macrophages and is essentially avirulent in a murine model (Burchmore, 2003 and unpublished data). These observations indicate an essential and unanticipated role for monosaccharide transporters in the pathogenic form of ¡I¿Leishmania¡I/¿. By expressing individual ¡I¿Lm¡I/¿GT isoforms in the knockout background delta¡I¿Lm¡I/¿GT, we have previously shown that each ¡I¿Lm¡I/¿GT gene encodes a bona fide monosaccharide transporter. We have now focused on dissection of the functional differences between ¡I¿Lm¡I/¿GT2 and ¡I¿Lm¡I/¿GT3, which differ only at the N- and C-termini and at 15 amino acid residues between. We have shown that ¡I¿Lm¡I/¿GT2 is responsible for ribose uptake in ¡I¿L. mexicana¡I/¿, and is the first eukaryotic ribose transporter to have been identified at the molecular level. ¡I¿Lm¡I/¿GT3 does not transport ribose, though both proteins transport glucose with similar kinetics. To localise and identify divergent amino acid residues that are responsible for this difference in function, we have constructed a series of chimeras between ¡I¿Lm¡I/¿GT2 and ¡I¿Lm¡I/¿GT3. Assay of glucose and ribose transport competence in these mutants has enabled us to define domains that are important for substrate specificity.

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The phosphoproteome of bloodstream form *Trypanosoma brucei*

Isabelle R.E. Nett, David M.A. Martin, Douglas Lamont, Jon Barber, Angela Mehlert, Michael A.J. Ferguson*

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Cell signalling via reversible phosphorylation on serine/threonine and tyrosine residues has shown to regulate the vast majority of cellular activity in most eukaryotic cells (Cohen P., 2000). Although protein kinases and phosphatases have been predicted from bioinformatics analysis of the trypanosomatid genomes, little is known about their function and regulation in *Trypanosoma brucei* (*T. brucei*) and even less is known about the extent of protein phosphorylation in this organism. Classical biochemical approaches together with mass spectrometry analysis have so far revealed only seven precise phosphorylation sites in total, six on *T. brucei* GPEET-PARP (Mehlert A. et al., 1999) and one on *Trypanosoma cruzi* (*T. cruzi*) histone H1 (da Cunha J. P. et al., 2005). Using a gel-free peptide-based mass spectrometry approach in combination with phosphopeptide enrichment techniques, we performed a global analysis of the phosphoproteomes of bloodstream form *Trypanosoma brucei* cytosolic extracts. Our approach resulted in the identification of more than 3400 candidate phosphopeptides from 800 *T. brucei* proteins and detected 1860 phosphorylation sites. I.R.E. Nett is supported by a PhD studentship from the Wellcome Trust.

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237B

Purification of active RNA polymerase I reveals an essential and novel protein in *Trypanosoma brucei*

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RNA polymerase I of the protistan parasite *Trypanosoma brucei* has a unique role in transcribing the gene units coding for the major cell surface antigens variant surface glycoprotein and procyclin in addition to synthesis of rRNA like in other eukaryotes. Nine orthologues of known subunits have been identified to be part of the *T. brucei* RNA pol I complex but an active purified enzyme has been elusive. Here, we have epitope-tagged the specific subunit RPB6z and tandem affinity-purified RNA polymerase I from crude extract. The purified enzyme was active on both random-nicked DNA and on specific promoters and revealed three enriched protein bands with apparent sizes of 31, 29 and 27 kDa. The amino acid sequences of p31 and its trypanosomatid orthologues have no similarity to proteins of other eukaryotes nor do they contain a conserved sequence motif. Nevertheless, p31 cosedimented with purified RNA polymerase I, and silencing of p31 by RNA interference affected the abundance of rRNA and was lethal to procyclic trypanosomes. Moreover, extract of p31-silenced cells exhibited a specific defect in transcription of class I templates which was remedied by the addition of purified RNA polymerase I. An anti-p31 serum completely blocked RNA pol I-mediated transcription in the *in vitro* system identifying p31 as an RNA pol I subunit which is indispensable for promoter-dependent transcription. We therefore named this novel functional component of *T. brucei* RNA pol I TbRPA31.

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Loss of actin does not affect delivery of surface proteins in African trypanosomes

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Actin plays an essential role in endocytic traffic in bloodstream forms of African trypanosomes but the role of actin in exocytic traffic remains equivocal. Here we employ pulse chase labelling combined with surface biotinylation during the chase to demonstrate directly and quantitatively that loss of actin does not affect exocytic traffic in bloodstream and procyclic forms of *Tyrpanosoma brucei*.

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Study of bottlenecks in the trypanosome life cycle

Michael Oberle[1], Line Arni[2], Reto Brun[1], Isabel Roditi[*,2]

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Cyclical transmission of *T. brucei* by tsetse is a surprisingly inefficient process; flies can eradicate an infection from the midgut or stall the progress of the trypanosomes to the salivary glands. Even in a laboratory set-up, where the conditions for transmission are optimised, ∼50% of the flies that take an infective blood meal will establish midgut infections and ∼10% will produce mature infections in the salivary glands. Migration to the salivary glands occurs during a defined period, after which only a few migrating forms can be detected. At present we have no idea of the degree of diversity of trypanosomes in a mature infection, but hypothetically a single cell might be enough to colonise a gland. The extent to which a population is restricted during transmission has important implications for the acquisition and maintenance of traits such as human serum-resistance and drug resistance. To investigate population dynamics during transmission we recently initiated a project in which trypanosomes are tagged with sequences integrated into a ribosomal DNA spacer. These sequences are variable, giving each trypanosome a unique signature. Flies are infected with a mixture of tagged trypanosomes, and parasites are subsequently isolated from the midguts and salivary glands of individual flies at various time points after infection. The repertoire of tags is analysed by PCR and sequencing. By comparing data obtained from individual flies it should be possible to assess the magnitude of the bottlenecks during the establishment of midgut and salivary gland infections, respectively, and also to see if certain tags are consistently enriched during transmission.

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Purine nucleobase transport in *Leishmania major*: phenotypes of null mutants

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One distinctive feature of the biochemistry of parasitic protozoa is their absolute reliance upon the salvage of performed purines from their vertebrate and invertebrate hosts. *Leishmania major* expresses two nucleobase permeases, NT3 that is a high affinity transporter for purine nucleobases and NT4 that is a low affinity transporter for adenine. $nt3^{(-/-)}$ and $nt4^{(-/-)}$ null mutants were created by targeted gene replacement. $nt3^{(-/-)}$ promastigotes were unable to replicate in medium containing 10 μM hypoxanthine, guanine, or xanthine, and they replicated slowly in 10 μM adenine due to the residual low affinity uptake of that purine. $nt3^{(-/-)}$ mutants were also strongly impaired in their ability to grow in *Phlebotomus duboscqui* sandflies but were not impaired for growth inside murine macrophages. Notably, the NT3 transporter mediated the uptake of the anti-leishmanial drug allopurinol, an analogue of hypoxanthine, and the $nt3^{(-/-)}$ mutants were deficient in uptake of allopurinol and 10-20-fold resistant to this drug. Hypoxanthine uptake and expression of the NT3 permease were profoundly down regulated at the protein but not the mRNA level in stationary phase compared to logarithmic phase promastigotes. The $nt4^{(-/-)}$null mutant retained high affinity nucleobase transport function but was nonetheless quantitatively impaired in survival within murine bone marrow-derived macrophages. Extensive efforts to generate an $nt3^{(-/-)}/nt4^{(-/-)}$ dual null mutant were not successful, suggesting that one of the two nucleobase permeases must be retained for robust growth of the parasite. The phenotypes of these null mutants underscore the importance of purine nucleobase transporters in the *Leishmania* life cycle and pharmacology.

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Identification and characterization of species-specific genes that may influence *Leishmania* disease tropism

Samuel O. Oyola[1], Krystal J. Evans[1], Terry K. Smith[2], Christopher Peacock[3], Jeremy C. Mottram[4], Paul M. Kaye[1], Deborah F. Smith[*,1]

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Infection with different Leishmania species gives rise to a spectrum of disease in susceptible hosts. Thus L. infantum and L. major usually cause visceral and cutaneous leishmaniasis respectively while L. braziliensis is the causative agent of mucocutaneous leishmaniasis. Comparison of the genome sequences of these three species has identified species-specific genes which, we hypothesise, may directly influence disease outcome. We are currently characterizing a Leishmania orthologue of the bacterial gene encoding cyclopropane fatty acyl phospholipid synthase (CFAS), that is present in both the L. infantum and L. braziliensis genomes but not in L. major. These enzymes catalyze the cyclopropanation of unsaturated glycolipids in bacteria, a process important in maintenance of the bacterial plasma membrane. Cyclopropane modification of M. tuberculosis membrane glycolipids is associated with virulence and pathogen persistence in the host. We have generated L. infantum CFAS-knockout and complemented cell lines and compared their fatty acid content by GC/MS, identifying a potential CFAS substrate. The CFAS enzyme is dispersed in the cytoplasm during promastigote logarithmic growth but localizes in punctate structures when the parasites enter stationary phase, apparently co-localizing with the autophagosomal membrane protein marker, ATG8. We are further characterizing the mutant Leishmania parasites using in vivo infection models to determine the role of CFAS in the pathogenesis of visceral leishmaniasis.

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Functional analysis of RNA editing exonucleases in *Trypanosoma brucei*

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RNA editing in kinetoplastid protozoa transforms mitochondrial pre-mRNAs into functional mRNAs by the precise insertion and deletion of uridine residues (Us) as specified by guide RNAs (gRNAs). Editing is achieved by a coordinated series of enzymatic steps that cleave the pre-mRNA, add or remove Us, and re-ligate the RNA. The process is catalyzed by ~20S editosomes that we recently showed are heterogeneous with respect to the endonucleases and several other proteins. The editosome has two U-specific exonucleases, KREX1 and KREX2. KREX1 is only in KREN1 deletion endonuclease editosomes while KREX2 is in all editosomes in a subcomplex with KREPA2 and KREL1. Repression of KREX1 expression by RNAi in procyclic forms (PFs) reduced cell growth and editing *in vivo* and led to the concomitant reduction of KREN1, suggesting interaction between these proteins. No obvious change was detected in U removal *in vitro* however deletion endonuclease activity was reduced consistent with KREN1 reduction. Repression of KREX2 expression by RNAi in PFs did not inhibit cell growth or editing *in vivo* but did result in partial disruption of editosomes with a reduction of KREPA2 and KREL1, and significant reduction of U removal *in vitro*. Additionally, we generated a KREX2 null mutant in bloodstream forms that grows normally but has a dramatic reduction of KREPA2 and KREL1, similar to the results obtained with the KREX2 RNAi cells although more severe. Thus, KREX2 is not essential for parasite survival. Results of experiments further exploring the roles of these proteins will be presented.

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Trypanosoma brucei functional cellular and sub-cellular proteome

Aswini K. Panigrahi, Alena Zíková, Rachel Burnham, Yuko Ogata, Nathalie Acestor, Atashi Anupama, Peter J. Myler, Ken Stuart*

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Functions cannot be ascribed to majority of the predicted genes in trypanosomes and the validity of gene predictions have not been tested systematically. We are undertaking experiments to identify proteins by high throughput mass spectrometry in *T. brucei* bloodstream form (BF), procyclic form (PF) cells and PF mitochondrial vesicles. The data from these analyses will be presented. To date we have identified 3,660 proteins in *T. brucei* cells ∼50% of which are encoded by genes that are annotated as hypothetical proteins. Additionally ∼1% of these proteins are encoded by ORFs that are not annotated as genes in GeneDB database. We also used SILAC metabolic labeling and mass spectrometry analyses to determine the expression level of proteins between two cell stages and initial results indicate 8% of the proteins are up-regulated two fold or more in BF and 5.3% in PF. Our results showed in general the glycolytic enzymes are up-regulated in BF and ATP synthase in PF; however, some components of ribosome, ATP synthase complex and cytoskeleton etc. and numerous proteins with unknown function are also differentially expressed between the two life cycle stages. We also studied the composition of multi-protein complexes using mAb and TAP-tag affinity purification followed by mass spectrometry analysis. We attempted to generate 152 TAP-tagged proteins and have completed analyses of 116 tagged-proteins. We determined the composition of several complexes for the first time in T. brucei and identified a complex of unknown function i.e. designated as mt unknown-complex-A. These studies led to identification of a large proportion of proteins with unknown function in ATP synthase, cytochrome c oxidase, oxidoreductase, succinate dehydrogenase complexes and mitochondrial ribosomes.

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241A

Characterization of a novel Type II protein arginine methyltransferase in *Trypanosoma brucei*

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In mammals and yeast, protein arginine methyltransferases (PRMTs) regulate the function of many RG-rich RNA-binding proteins. PRMTs catalyze the formation of symmetric (Type I) or asymmetric (Type II) dimethylarginines, thereby modulating intermolecular interactions that impose their effects on cellular processes such as transcription, cell cycle regulation, DNA repair, and RNA processing. Since RNA-binding proteins constitute a large fraction of PRMT substrates, this posttranslational modification will likely play an important regulatory role in trypanosome gene expression. We have identified a type II PRMT in *T. brucei*, termed TbPRMT5, which displays broad substrate specificity and *in vitro* methyltransferase activity towards the RNA-editing accessory protein, RBP16. *In vivo* analysis indicates that TbPRMT5 is constitutively expressed in bloodstream and procyclic life stages of the parasite, and localizes to the cytoplasm. Tandem affinity purification of TbPRMT5 from procyclic form extracts, followed by LC-MS/MS analysis of co-purifying eluates, indicate that TbPRMT5 associates with an RG-rich putative ATP-dependent DEAD/H RNA helicase (Tb10.61.2130). TbPRMT5 also co-purified with two hypothetical proteins that are only present in the genomes of *T. cruzi* and *Leishmania major* kinetoplastids. We have disrupted TbPRMT5 expression by RNA interference in procyclic form parasites, and show that TbPRMT5 is not essential for growth. Analysis of TbPRMT5 disrupted cells indicates that, in direct contrast to PRMT5 function in mammalian cells, TbPRMT5 does not play a role in pre-mRNA splicing. Instead, TbPRMT5 appears to play a role in the expression and/or stability of a subset of mitochondrial transcripts.

Characterization of a novel Type II protein arginine methyltransferase in *Trypanosoma brucei*

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241B

T. brucei RNA binding proteins, p34 and p37, mediate NOPP44/46 cellular localization through the exportin 1 nuclear export pathway

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We have previously identified and characterized two novel nuclear RNA binding proteins, p34 and p37, which have been shown to interact with a family of nucleolar phosphoproteins, NOPP44/46, in *Trypanosoma brucei*. These proteins are nearly identical, the major difference being an 18 amino acid insert in the N-terminus of p37. In order to characterize the interaction between p34 and p37 and NOPP44/46, we have utilized an RNA interference cell line that specifically targets p34 and p37. Within these RNAi cells, we detected a dramatic increase in nuclear NOPP44/46 protein levels. We demonstrated that no change occurred in NOPP44/46 mRNA steady state levels or stability, nor was there a change in cellular protein levels. These results led us to investigate whether p34 and p37 regulate NOPP44/46 cellular localization. Examination of the p34 and p37 amino acid sequences revealed a leucine-rich nuclear export signal, which is known to interact with the nuclear export factor, exportin 1. Immune capture experiments using wild type procyclic nuclear extracts demonstrated that p34, p37, and NOPP44/46 associate with exportin 1. When performed using nuclear extracts prepared from p34/p37 RNAi cells, the association between NOPP4/46 and exportin 1 was no longer detected. Sequential immune capture experiments demonstrated that p34, p37, NOPP44/46, and exportin 1 exist in a common complex. Furthermore, specific inhibition of the exportin 1-mediated nuclear export using leptomycin B led to an increase in nuclear NOPP44/46 protein levels, indicating that they are exported from the nucleus via this pathway. Together, our results demonstrate that p34 and p37 regulate NOPP44/46 cellular localization by facilitating their association with exportin 1.

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Three new kinetoplast-associated proteins in *Trypanosoma brucei*

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DNA replication proteins have been observed in association with kinetoplast DNA (kDNA) in earlier studies in *Crithidia fasciculata* and *T. brucei*. Some replication proteins have been found at antipodal sites flanking the kDNA and others have been found at the flagellar face or surrounding the kDNA disk. Specific localization of some of these proteins has been shown to vary during the cell cycle. We have identified three new proteins in association with the kinetoplast DNA of *T. brucei*: a topoisomerase IA, an RNaseHIIC and a protein of unknown function P93. RNA interference (RNAi) of topoisomerase IA expression had no effect on cell growth whereas RNAi knockdown of RNaseHIIC and P93 expression resulted in a growth defect and shrinkage and loss of the kinetoplast DNA. For RNaseHIIC and P93 both minicircles and maxicircles are lost during RNAi. Lm P105, the *Leishmania major* ortholog of P93, shows antipodal localization of epitope tagged P105 that varies throughout the cell cycle. A possible role for RNaseHIIC in kDNA replication will be discussed.

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242B

The RACK1 homologue from *Trypanosoma brucei* is associated with cytoplasmic free ribosomes

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Receptor for Activated Protein Kinase C (RACK1) is a WD repeat protein that forms ternary complexes with a range of kinases, phosphatases and their substrates. A homologue of RACK1 (TRACK) has been identified in *Trypanosoma brucei*. We have previously demonstrated a role for TRACK in cytokinesis and also for infection in the mammalian host (Rothberg *et al*., JBC 281:9781; 2006). The RACK1 paradigm for signaling shifted when it was identified as a core 40S ribosomal subunit in organisms ranging from yeast to humans. RACK1 has been hypothesized to bring ribosomes to membrane sites for positional translation or to mediate PKC dependent phosphorylation of initiation factor eIF6. In trypanosomes, this could be especially important because gene regulation occurs post-transcriptionally. Surprisingly, cryo-EM studies of the 80S ribosome from *T. cruzi* failed to identify TRACK. Therefore, the following study was initiated to determine whether trypanosome ribosomes are fundamentally different and whether TRACK might play a role in translational regulation. When evaluated by western blotting we report that purified 80S monosomes contain TRACK. TRACK dissociated from the monosome after treatment with high salt or deoxycholate. During translation, TRACK also associated with polysomes. An AU1-epitope tagged TRACK was predominantly cytosolic and did not co-localize with the endoplasmic reticulum marker BiP. Differential cell fractionation failed to detect TRACK on ER fractions, although the ribosomal Po protein was detected. Taken as a whole, these data indicate that in *T. brucei*, TRACK is associated with free cytoplasmic ribosomes but not with ribosomes bound to the ER. We hypothesize that TRACK may recruit signal complexes to free ribosomes and modulate the expression of proteins potentially involved in cytokinesis.

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243A

Genome-wide analysis of stage- and species-specific gene expression in *Leishmania*

Annie Rochette, Frédéric Raymond, Jean-Michel Ubeda, Sébastien Boisvert, Jacques Corbeil, Marc Ouellette, Barbara Papadopoulou*

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Leishmania are unicellular parasites that exist in two developmental stages: free-living promastigotes in the alimentary tract of a sandfly vector and intracellular amastigotes residing in phagolysosomes of mammalian macrophages. These developmental stages display distinct morphologic and metabolic characteristics that must be the result of a highly regulated gene expression, which is central to parasite intracellular survival. Previous studies employed DNA microarrays using genomic fragments as probes. We report here the use of DNA oligonucleotide full-genome microarrays representing all annotated genes allowing the study of global mRNA expression profiles of both *Leishmania major* and *Leishmania infantum* species. Advanced statistical analyses identified a relatively low degree of differential mRNA expression. The comparison between *L. infantum* promastigotes and intracellular amastigotes revealed that 1.5% of the total genes are overexpressed in promastigotes and 2.7% are more expressed in amastigotes. The comparison between *L. major* promastigotes and lesion derived amastigotes showed that 4% of the genome is more expressed in promastigotes and 2.3% is predominantly expressed in amastigotes. Important differences in expression profiles were observed between *L. major* and *L. infantum*. These studies allowed the identification of novel genes or pathways that are regulated specifically in the intracellular amastigote form and depicted important differences in expression profiles between *Leishmania* species. The follow-up studies are expected to provide new insights about the intracellular development of this parasite and eventually lead to the identification of novel therapeutic targets.

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Sensitivity of glucose transporter null mutants of *Leishmania mexicana* to environmental stress

Dayana Rodriguez-Contreras*, Xiuhong Feng, Scott M. Landfear

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We have previously demonstrated that glucose transporter null mutants of *L. mexicana* (D*lmgt*) survive as promastigotes but exhibit dramatically reduced infectivity to macrophages and are unable to grow as culture-form amastigotes. *Leishmania* parasites are exposed to several stresses, including oxidative stress, nutrient limitation, reduced pH and increased temperature, when they enter macrophages. Increased sensitivity to one or several of these stress conditions could contribute to the inability of D*lmgt* to grow inside macrophages. To evaluate this hypothesis, we exposed wild type and D*lmgt* promastigotes to several stress conditions found inside macrophages, and have found that D*lmgt* parasites are more sensitive to oxidative stress but not to reduced pH, compared to wild type parasites. Significant reduction in D*lmgt* parasite viability was also observed upon nutrient limitation. Current experiments are addressing the question of whether D*lmgt* parasites are more sensitive to increased temperature.

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iTRAQ proteomic analysis of *Leishmania donovani* differentiation time-course

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The protozoan parasite *Leishmania donovani* leads a digenetic life-cycle, as a flagellated free-living promastigote in the sand-fly alimentary tract and an aflagellate, intraphagolysosomal amastigote in the host mammal macrophage. Promastigote to amastigote differentiation can be induced in-vitro by transferring promastigotes to a medium that mimicks phagolysosomal conditions. Little is known about the mechanisms driving this process. An important first step in addressing this problem is the establishment of a profile of the gene expression during differentiation. Since *Leishmania* gene regulation is practically entirely post-transcriptional, a proteomic approach was chosen. We used iTRAQ, a novel MS/MS based method that can compare multiple protein samples semi-quantitatively. Here we present results of differentiation time-course analysis. To date, 1433 proteins (17.3% proteome coverage) were identified at ≥95% confidence. Functional analysis of detected regulated proteins revealed significant changes in the proteins participating in energy metabolism as well as the parasite translation machinery. Enzymes of the glycolytic pathway down-regulated, while those of amino-acid catabolism, fatty acid beta-oxidation, citric acid cycle and cellular respiration up-regulated. In parallel, translation factors, ribosomal protein subunits and tRNA synthetases were coordinately down-regulated, suggesting a general decrease in translation. Detection of an increase in histones expression during late stages of differentiation may reflect transcriptional changes. All the changes appear to be coordinated and gradual. Post-translational modification changes (phosphorylation, acetylation and methylation) were detected throughout the differentiation time-course, while protein abundance changes were more prominent beyond the initial 10 hours post-differentiation signal, during the parasite morphological change.

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Functional analysis of ESAG4 adenylate cyclase in *T. b. brucei*

Didier Salmon[*,1], Carsten Krumbholz[2], Pierrick Uzureau[1], Michael Boshart[2], Etienne Pays[1]

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We investigated the role played by the adenylyl cyclases (AC) in cellular differentiation from LS to SS forms of the African trypanosome, in particular ESAG4, whose gene is located in VSG expression sites and is the only known stage-specific AC isoform specifically expressed in BF. Several complementary reverse genetics strategies have been applied in bloodstream stages of both monomorphic and pleomorphic strains: (1) expression of two different catalytically dead dominant negative (DN) mutants of ESAG4, (2) RNAi mediated repression in various ESAG4 dsRNA expressing cell lines (p2T7-ribo, p2T7-177, hairpin RNAi), (3) a targeted knock-out of ESAG4 in the active expression site. Inducible expression of an ESAG4 DN mutant in a monomorphic strain presented a transitory growth phenotype, whereas no growth phenotype was detected upon constitutive expression of an ESAG4 DN mutant in a pleomorphic strain. The latter clones had however drastically decreased AC activity. In contrast the ESAG4 knock-out in the active expression site had adenylyl cyclase activity comparable to wild-type. These results suggest that the dominant-negative ESAG4 versions affected not only ESAG4 itself but also other members of the GRESAG4 family. A preferential flagellar localization observed with dominant-negative GFP tagged ESAG4 mutants confirmed our published EM results showing that ESAG4 is primarily located on the flagellar membrane of the parasite. This supports the view that the flagellum serves sensory functions in kinetoplastids. Further analysis of the phenotypes of our ESAG4 mutant collection upon LS to SS and SS to PF differentiation is underway.

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The role of a replication control element appears to be developmentally modulated in *Trypanosoma brucei*

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Little is known about sequences that control nuclear DNA replication in *Trypanosoma brucei*. We have previously constructed and characterized a panel of autonomously replicating plasmids for this organism that exist at a copy number of 1-2 per cell and demonstrate substantial mitotic stability in the absence of selection. We are using these as model systems to define critical replication control elements in this organism. In the plasmid pEV-luc, a *GPEET* procyclin promoter drives the expression of firefly luciferase and neomycin phosphotransferase ($G418^R$). Autonomous replication of this plasmid is dependent on a $\sim$3.7 kb segment of *T. brucei*-derived DNA that we call the plasmid maintenance sequence (PMS) and, surprisingly, on the segment bearing the *GPEET*-promoter. Sequence analysis reveals that the PMS is made-up of 3 segments from disparate regions of the genome that were juxtaposed during the construction of this episome. The plasmid can initiate replication in the absence of any (or all) of the PMS segments, but becomes extremely inefficient with time. Sequence substitutions within the *GPEET*-promoter demonstrate that Domains I and IV are dispensable for autonomous replication. Mutations within Domain II delays the onset of replication but the plasmid replicates efficiently thereafter. However, a 10bp substitution within Domain III completely compromises this plasmids ability to replicate in procyclic *T. brucei*. Surprisingly, the same mutation has no effect in bloodstream forms suggesting that a class of replication control elements could be developmentally modulated.

The role of a replication control element appears to be developmentally modulated in *Trypanosoma brucei*

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Using Cre recombinase in *Trypanosoma brucei*: what marker problem?

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 We demonstrate that bacteriophage P1 Cre recombinase acts with great efficiency in bloodstream-form *Trypanosoma brucei*. We constructed a positive/negative marker cassette (LHTL) consisting of *loxP* elements flanking a fusion of a Hygromycin resistance gene (HYG) and *Herpes simplex* virus thymidine kinase (TK). A tet-inducible Cre expression construct (pLEW100-cre, kindly provided by John E. Donelson) driven by the GPEET promoter was stably integrated into an rRNA spacer locus. To validate the approach, we integrated the LHTL cassette into the ß-Tubulin locus and subsequently selected for gancyclovir resistance. When induced, Cre mediated excision of the LHTL cassette in nearly 50% of cells. By targeting LHTL to *PYR-F*, the homologue of the yeast *ura3* gene, and selecting for 5-fluoro-orotic acid resistance, we observed conversion to *PYR-F*$^{-/-}$ at frequencies of 10^{-5} to 10^{-6}, in agreement with the loss of heterozygosity reported for *Leishmania* but in contrast to the reported rate of $\sim 10^{-3}$ at the ALG12 locus in *T. brucei*. As extensions of the stably integrated Cre system, we examine the efficacy of transient transfection of Cre plasmids and application of purified Cre fused with HIV Tat for cellular uptake. Problems of Cre toxicity, presumably caused by non-specific recombination events at low levels of uninduced expression, are being addressed by employing the temperature-sensitive regulation of mRNA degradation conferred by the EP1 3'-UTR and by reducing overall Cre expression via specific mutations of the GPEET splice acceptor site. Our aim is to create a stable system to allow the limited repertoire of selectable drug markers to be recycled indefinitely.

Using Cre recombinase in *Trypanosoma brucei*: what marker problem?

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A type 4 phosphodiesterase and a phosphatidylinositol 3-kinase are involved in osmoregulation in *Trypanosoma cruzi*

Alejandra Cecilia Schoijet[1], Kildare Miranda[1], Guillermo Daniel Alonso[2], Hector Norberto Torres[2], Mirtha Maria Flawia[2], Roberto Docampo[*,1]

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During its development in different hosts, *T. cruzi* faces extreme fluctuations in osmolarity. Both insect and vertebrate stages of the parasite have been shown to possess a robust regulatory volume decrease (RVD) mechanism that reverse cell swelling under hypo-osmotic stress. Amino acid and K+ release account for 57% of the RVD of epimastigotes after hypo-osmotic stress. Volume recovery also involves aquaporin (TcAQP)-mediated water movement. In this work, we report the contribution of TcrPDEC2, a cAMP-specific phosphodiesterase and TcVps34, a class III phosphatidylinositol 3-kinase to osmoregulation and membrane trafficking in epimastigotes of *T. cruzi*. Over-expression of TcrPDEC2 or TcVps34 in epimastigotes caused differences in resistance of the parasites to hypo-osmotic stress when cells were incubated in the presence of type 4 PDE or PI3K inhibitors, respectively. Type 4 PDE inhibitors rolipram and etazolate induced an increase in volume recovery in wild type cells whereas PI3Ks inhibitors wortmannin and LY294,002 impaired this mechanism. No effects were observed in TcrPDEC2 and TcVps34 over-expressing cells. Immunofluorescence showed co-localization of TcrPDEC2 and calmodulin in the anterior portion of the parasite, near the contractile vacuole/flagellar pocket region. Structural analysis of TcVps34 over-expressing cells showed functional and large contractile vacuoles when compared to wild type cells. Kinetics of proton uptake in TcVps34 cells showed higher H^+-ATPase and lower H^+-PPase activities than in wild-type cells. Finally, endocytosis of FITC-transferrin was reduced in TcVps34 over-expressing cells. Taken together, these results suggest a potential role for TcrPDEC2 and TcVps34 in osmoregulation, vacuolar acidification, and membrane trafficking in *T cruzi*.

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246B

Chromosome number variation in virulent *Leishmania major*

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Leishmania are typically considered diploid, although exceptions have been seen in both *L. major* and *L. donovani*. Compiling knockout data from our lab and others, genetic evidence confirms disomy for at least 15 of the 36 chromosomes of *L. major*. However, we found in studies of the PTR1 locus on chromosome 23 in the *L. major* Friedin V1 line that it was present in 3 copies, in contrast to 2 copies in the *L. major* CC-1 line. Similarly, knockout studies of two loci on chromosome 31 suggested they were both present in 4 copies in *L. major* LV39cl5. These results suggested that aneuploidy may be more widespread and variable than previously appreciated. To probe this generally, we turned to comparative genome hybridization using *L. major* oligonucleotide microarrays. For calibration purposes we used comparisons between lines of known chromosome number as established through gene knockout and Southern blot analysis. Chromosome number differences were clearly evident in this analysis. The data showed that aneuploidy is widespread and variable amongst virulent *Leishmania major* lines. Some aneuploidies are shared in all strains, some are shared by a subset, and some are idiosyncratic. Chromosome 31 appears to be tetrasomic in all lines; interestingly this chromosome has high levels of *Leishmania*- and trypanosomatid-specific genes, and perhaps is less 'syntenic' with trypanosomes than other *Leishmania* chromosomes. It remains to be established whether these chromosome variations have relevance for the growth and pathogenesis of *Leishmania*, as has been found with pathogenic fungi. We thank the many members of the Beverley who contributed to chromosome counting by gene knockout results incorporated into this study.

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247A

The role of the ER quality control systems in export of variant surface glycoprotein in *Trypanosoma brucei*

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Variant surface glycoprotein (VSG), the dominant surface antigen of *Trypanosoma brucei* bloodstream form, is synthesized in the form of a nascent precursor and rapidly modified within the endoplasmic reticulum (ER) by N-glycosylation and glycosylphoshatidylinositol (GPI)-addition. The ER maintains chaperones, enzymes and other factors that assist protein folding, assembly and modification. Correctly folded proteins enter ER exit sites and are transported along the secretory pathway to their destination. Terminally misfolded proteins are recognized by quality control (QC) mechanisms and disposed by ER-associated degradation. To investigate the roles of QC mechanisms in export of VSG we used bioinformatic analysis and identified over thirty candidate gene products that are potentially involved in protein folding in the trypanosome ER. Using an inducible RNA interference approach we implicated a group of proteins from the calnexin/calreticulin cycle and also a group of hypothetical proteins with ER-localisation signatures in VSG QC. Investigation of the export kinetics of VSG and further detailed description of the chaperones involved in folding and ER export of VSG will allow comparison of QC systems between trypanosomes and higher eukaryotes as well as between VSG and other surface factors. There are deep evolutionary distances between trypanosomes and humans and examination of these systems will provide insight into the evolution of complex cellular functions. Elucidation of the QC mechanism in export of VSG will explore the possibility of exploiting aspects of VSG processing and trafficking as strategies for clinical intervention.

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247B

Leishmania infantum glyoxalase II: enzyme kinetics, substrate specificity and comparison with its human counterpart

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Trypanosomatids are parasitic protozoa responsible for serious human diseases. The functional replacement of glutathione by trypanothione is one of their most remarkable biochemical characteristics. Accordingly, trypanothione-dependent enzymes replace the glutathione-dependent ones [1]. The glyoxalase pathway, catalyzing the formation of D-lactate from methylglyoxal, is no exception [2-4]. Of the two enzymes involved in the glyoxalase pathway, glyoxalase II shows absolute specificity towards the trypanothione thioester [2, 3], making this enzyme an exceptional model to understand the molecular basis of trypanothione specificity. *L. infantum* glyoxalase II was over-expressed in *E. coli*, purified [5] and MALDI-TOF mass spectrometry studies indicated a molecular weight of 34.5 kDa for the His-tagged protein, in good agreement with the predicted value with His tag. LiGLO2 was kinetically characterized, and activity was assayed for glutathione-, trypanothione- and glutathionyl-spermidine-derived thioesters. Although S-D-lactoyl-trypanothione is the physiological substrate, the enzyme also catalyses the hydrolysis of S-D-lactoyl-glutathionyl-spermidine, revealing that the spermidine moiety is absolutely needed for the reaction to occur. Structural studies were carried out, providing us with a model of LiGLO2 to compare with its human homologue regarding substrate specificity. Work supported by POCTI/ESP/48272/2002, SFRH/BPD/28345/2006 and SFRH/BD/28691/2006. [1] S. Muller, E. Liebau, R.D. Walter, R.L. Krauth-Siegel (2003) Trends Parasitol 19, 320-328. [2] M. Sousa Silva, A.E. Ferreira, A.M. Tomas, C. Cordeiro, A. Ponces Freire (2005) Febs J 272, 2388-2398. [3] T. Irsch and R.L. Krauth-Siegel (2004) JBC 279, 22209-22217. [4] T.J. Vickers, N. Greig, A.H. Fairlamb (2004) PNAS USA 101, 13186-13191. [5] J. Trincão, M. Sousa Silva, L. Barata, C. Bonifácio, S. Carvalho, A.M. Tomás, A.E.N. Ferreira, C. Cordeiro, A. Ponces Freire, M.J. Romão (2006) Acta Crystal F62, 805-807.

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Functional role of alanine aminotransferase in *T. brucei*: implications of knockdown and knockout studies

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The function of alanine aminotransferase (TbAAT) is to convert glycolytic pyruvate into alanine. The cytosolic enzyme is expressed in high abundance in the BSF with a specific activity of ∼500nmol/min/mg. In the PCFs it is expressed with a specific activity of ∼300nmol/min/mg. RNA interference was carried out on both life forms. Incomplete knockdown of the enzyme led to residual activity of ∼20% in both life forms and no phenotype could be observed under normal growth conditions. In a stress situation, with lowered amount of FCS in the media, the BSF exhibited a slower growth rate. To probe these results further, an inducible gene knockout was carried out for TbAAT in PCF. The ablation of the gene had a severe growth effect on PCF proving the gene is essential. Due to leakiness of the rescue copy, normal growth was restored when the TbAAT activity increased to a level of ∼10%. In BSF a combination of RNAi and single allele knockout was performed. The activity of TbAAT was lowered to ∼5% but this had no effect on growth in normal conditions. A slower growth phenotype could be observed only under stress conditions. Therefore, the absence of TbAAT appears to be permissible in cultured BSF. This is an interesting result regarding its abundance within this cell type.

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A molecular link between mitochondrial fatty acid synthesis and respiration in *Trypanosoma brucei*

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Trypanosoma brucei employs a unique set of microsomal elongases to synthesize fatty acids de novo. In addition, this parasite utilizes a mitochondrial type II synthase to make the 8C acyl precursor for lipoic acid and longer fatty acyl chains up to 16C for use within the organelle. RNAi or knock-out of acyl carrier protein (ACP), a component of the mitochondrial synthase, suggests that this pathway is essential in both procyclic (PCF) and bloodstream form (BSF) trypanosomes (Stephens, J, et.al., JBC, 2007). Now we report that following RNAi of ACP, PCF respiration was reduced by 80%. In some fungi and mammals, ACP is an essential subunit of respiratory complex I. In RNAi trypanosomes, however, in-gel activity assays following blue-native PAGE and spectophotometric assays revealed only marginal decreases ($\sim$12%) in complex I activity. Instead, activities of respiratory complexes II, III, and IV were decreased by $\sim$90, 75, and 50%, respectively. To explain these decreases, we suggest that ACP RNAi reduces mitochondrial fatty acid synthesis that in turn inhibits synthesis or repair of phospholipids required for activity of these respiratory complexes. In support of this hypothesis, incorporation of radioactivity from [14C]pyruvate into [14C]cardiolipin (a mitochondrial phospholipid required for assembly of respiratory complexes) was decreased $\sim$80% following RNAi. Incorporation into other [14C]phospholipids, however, was reduced only by 30%.

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Characterization of MSP protein in *Leishmania chagasi*

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Leishmania chagasi is the causative agent of visceral leishmaniasis in South America. One of the most characterized virulence factors in *Leishmania spp* is the glycosylphosphatidylinositol (GPI) anchored major surface protease (MSP, also called GP63). The >18 tandem *MSP* genes in *L. chagasi* are classified according to unique sequences at their 3' untranslated ends. The five *MSPS* genes (*MSPS1*, *MSPS2*, etc.) express mRNA predominately in stationary phase promastigotes, the more than twelve *MSPL* genes express mRNA mainly in logarithmic phase cells, and the single *MSPC* gene is constitutively expressed throughout promastigote growth at much lower mRNA abundance. *MSPS* and *MSPL* genes encode a C-terminal GPI anchor addition signal, whereas the C-terminus of the *MSPC* gene product is more suggestive of a transmembrane region with a short cytoplasmic tail. Little is known about the expression of the different MSP protein isoforms because they have a very similar amino acid sequence. We are studying the protein products of *MSPS*, *MSPL*, and *MSPC* genes in *L. chagasi* using anti-peptide monoclonal antibodies (mAb) and myc-tagged recombinant MSP. Immunogenic peptides were generated to a divergent region on the C-terminal ends of the MSP sequences. From these, we generated a mAb designated as AbMSPS1L that specifically recognizes MSPS1 and MSPL1, and distinguishes these from a slower migrating MSPC. Further, we conclude that an MSPC isoform exists that is recognized when tagged with Myc that was not appreciated using polyclonal antisera available.

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A putative function in machrophage invasion for the *L. pifanoi* cysteine proteinase Lpcys2 C-terminal extension

Marcel M. Villa[1], Suzana Chaves[2], Diego AV Inchaustegui[3], Maurílio J. Soares[4], Sérgio Mendonça[5], Lynn Soong[3], Tania U. Nakamura[6], Bartira Rossi-Bergman[2], Yara M. Traub-Cseko[*,7]

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Cysteine proteinases have been implicated in many aspects of protozoan parasites pathogenesis. These hydrolases are normally found as zymogens, and in trypanosomatids some classes posess a long C-terminal extension (CTE), for which there is no assigned function. We have produced a polyclonal antibody specific to Lpcys2 CTE, the abundant lysosomal cysteine proteinase of *Leishmania pifanoi* amastigotes. Using this antibody, that also recognizes the CTE of *L. amazonensis*, in localization studies, we determined a high abundance of this domain in the lysosome and flagellar pocket of cultured promastigotes and even more abundantly in axenic amastigote forms of both parasite species. When we performed these immunolocalization studies in *Leishmania* that were exposed to macrophages, the labeling shifted to the surface of the parasite. This led us to examine a possible function for this molecule in macrophage infection. When macrophage infection was performed in the presence of anti-CTE antibody, we found a significant decrease in the number of intracellular parasites. This study suggests a role for leishmanial cysteine proteinases CTE in the establishment of an early infection in mammals.

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Whole-Genome analysis of drug resistance in *Leishmania* parasites

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With the recent completed genome of two *Leishmania* species, transcriptomic approaches are now feasible to understand the biology of this parasite. We report here the set up of a whole-genome microarray, carrying two 70 mer-oligonucleotides probes for each gene, and recognizing both *L. major* and *L. infantum* transcripts. We developed a useful on-line data base to normalize and conduct statistical tests. We describe our first study using this technology, a genomic approach analysis of drug resistance in *Leishmania sp.*. We analyzed and compared the genome expression of two mutants highly resistant to methotrexate (MTX), *L. major 60.4* and *L. infantum 20.5*. Surprisingly, their expression profiles were different, suggesting that *Leishmania* can adapt to the same drug by different mechanisms. Nevertheless, we highlighted some common resistance mechanisms. We observed an increased expression of two clusters of genes linked to *DHFR-TS* and in one mutant to *PTR1*, two key genes in MTX resistance. We observed that these increased expressions were related to the generation of large circular or linear extrachromosomal amplicons. The arrays were useful to delimit the boundaries of amplicons. Subsequent analysis revealed that these amplicons were generated by homologous recombination of direct or inverted repeated sequences flanking the amplified region. The modulation in expression not linked to amplified DNA of various genes were also observed, some specific to one mutant and others in both mutants. The role of some of them in MTX resistance is currently being investigated. This study demonstrate that DNA microarrays are useful tools to understand the adaptation of *Leishmania* to a drug.

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250B

The role of different deadenylases on mRNA stability in *Trypanosoma brucei*

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In ¡I¿Trypanosoma brucei¡I/¿, most protein-encoding genes are transcribed as part of long polycistronic pre-mRNAs that are subsequently processed into mature monocistronic mRNAs. Each of the open reading frames of the original pre-mRNA can exhibit differential steady state levels as mature messages, suggesting a reliance on post-transcriptional regulation in these organisms. Therefore, our laboratory is defining the enzymatic processes of mRNA turnover in trypanosomatids to better understand the mechanisms of their regulation of gene expression. In eukaryoytes, mRNA degradation usually initiates by shortening of the poly(A) tail by one or more deadenylases. We have identified three potential orthologues of mammalian poly(A)-ribonuclease (PARN) in ¡I¿Trypanosoma brucei¡I/¿; however, the specific functions of each of these proteins is unknown. To better understand the roles of different deadenylases in gene regulation in ¡I¿T. brucei¡I/¿, these proteins will be characterized for deadenylase activity and their roles on global mRNA stability. One of these proteins, PARN-1, has been shown to has been shown to have deadenylase activity in vitro, and has been knocked out by homologous recombination in procyclic parasites. PARN-1 is not an essential gene in procyclics, suggesting that this enzyme functions on only a subset of mRNAs or that decay enzymes can overlap in function. Northern analysis shows that each of the three PARN homologues are expressed in both procyclic and bloodstream form trypanosomes. Each of the PARN proteins will be depleted in procyclic and bloodstream form ¡I¿T. brucei¡I/¿ by RNAi, and microarray analysis will determine the effects of these proteins on global mRNA stability.

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Characterization of putative transcription elongation factors in Kinetoplastidae

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Kinetoplastidae display unique transcription features. The RNA polymerase II synthesizes polycictronic RNA that are processed by the polydenylation and trans-splicing machinery, to produce mature mRNA. The importance of the factors involved in the transcription elongation process is therefore emphasized in these organisms. The eukaryotic TFIIS transcription factor is involved in the rescue of paused RNA polymerase transcription complexes. Thus, the recruitment of this elongation factor stimulates the cleavage of nascent RNA, excision of nucleotides and restart of the polymerase. The *Trypanosoma brucei brucei* genome contains two TFIIS genes, which products share less than 10% identity. Phylogenetic analysis revealed a strong divergence between these TFIIS and their eukaryotic homologues. However, databases mining suggested that this duplication is restricted to the Trypanosomatidae. TbTFIIS1 only showed the canonical domains 2 and 3, which are necessary and sufficient for the TFIIS function in vitro. On the contrary, TbTFIIS2 was unusually long, presenting the 3 classical TFIIS domains plus a N terminal extension containing a PWWP domain. The latter was previously detected in eukaryotic nuclear proteins and could act as a protein-protein interaction domain. Both GFP tagged TbTFIIS localized as a heterogeneous punctuated pattern into the nucleoplasm of procyclic parasites. The generation of procyclic TbTFIIS1 or TbTFIIS2 knocked out parasites established that neither TFIIS1 nor TFIIS2 were essential for *T. brucei*. Noteworthy none of the drugs altering transcription elongation appeared to alter the growth of TFIIS knocked out parasites. However, the repetitive failure to generate double TFIIS knocked out parasites argued for the importance of the function encoded by these genes and the redundancy, at least partial of their role in transcription elongation.

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The J-binding proteins (JBP) 1 and 2 in *Leishmania*

Saara Vainio[1], Paul-André Genest[1], Bas ter Riet[1], Henri van Luenen[1], Barbara Papadopoulou[2], Piet Borst[*,1]

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The genomic DNA of trypanosomatids contains a hypermodified base, beeta-D-hydroxymethyluracil or J, that replaces a fraction of thymines (1). The distribution of J in the genome varies between parasite species; in *Leishmania*, it is found almost exclusively in telomeres (2). The function of J, as well as the details of its synthesis, remain elusive, but two proteins participating in the regulation of J levels have been identified (3, 4). These proteins are called J-binding protein (JBP) 1 and 2, and they share ∼40% homology, albeit only JBP1 binds to base J. Recently, we showed that these proteins contain features of thymidine hydroxylase (TH), and may thus be the enzymes catalysing the first step in the biosynthesis of J (5). We are at present setting up an *in vitro* assay to directly demonstrate the TH-activity of these proteins. *In vivo*, JBP1 has proven to be essential for *Leishmania* (6), but JBP2 -/- cell lines could readily be created. These cells grow normally but exhibit a 2 - 4 -fold reduction in J-levels, which can be rescued by ectopic expression of JBP2 but not JBP1. We are currently analysing whether the loss of JBP2 affects the telomere biology or the infectivity of the parasites. References: 1. Gommers-Ampt et al., (1993) Cell 75, 1129-1136. 2. Genest et al., (2007) Nucleic Acids Res, in press. 3. Cross et al., (1999) EMBO J 18, 6573-6581. 4. DiPaolo et al., (2005) Mol Cell 17, 441-451. 5. Yu et al., (2007) Nucleic Acids Res, in press 6. Genest et al., (2005) Nucleic Acids Res 33, 1699-1709.

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Functional studies of the origin recognition complex from *Trypanosoma brucei*

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Origin replication complex (ORC) is a critical component for eukaryotic and archaeal DNA replication initiation serving as a scaffold for the assembly of other key factors at origins of replication. Eukaryotic ORC is heterohexamic (Orc1-Orc6), whereas archaeal ORC is homohexameric (ORC1/Cdc6). Additionally, archaea contain a simplified version of eukaryotic initiation machinery that lacks major regulatory features, but are considered an alternative model to study replication initiation. Trypanosomes possess several unusual eukaryotic features; one example is the kinetoplast DNA (kDNA) network. Now, DNA replication initiation is emerging as another atypical trypanosome feature with a simplified repertoire of proteins, but still possessing a major regulator, Cdc45. Unlike other eukaryotic organisms, the Tri-Tryp genomes encode just a single subunit of ORC, ORC1. *Trypanosoma brucei* ORC1 has 24-26% amino acid identity with eukaryotic and archaeal ORC1/Cdc6 homologs. TbORC1 is notably smaller than eukaryotic ORC1s, contains a conserved AAA+ domain, but lacks a bromo-adjacent homology domain (BAH); similar to archaeal ORC1/Cdc6. To study the role of TbORC1, we used inducible RNAi in procyclic cells. Depletion of ORC1 mRNA by silencing with a stemloop dsRNA (∼98% reduction) resulted in growth arrest. DAPI staining of RNAi induced cells showed an accumulation of zoids that included a subset with multiple kDNA networks. These data indicate TbORC1 is essential for cell cycle progression likely as an initiator protein. Consistent with this role, ORC1-GFP localized to the nucleus mainly during S phase. Currently, we are using tandem affinity purification of PTP tagged ORC1 to identify other replication initiation proteins. Mass spectrometry analysis will further clarify the initiation machinery that is likely a mosaic of eukaryotic-like, archaeal-like and trypanosomatid-specific proteins.

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Characterization of mitochondrial NADH dehydrogenases in *Trypanosoma brucei* and *Phytomonas serpens*

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Complex I is the least characterized complex of the respiratory chain of the trypanosomatid flagellates. Spectrophotometric measurement of the NADH:ubiquinone 2 and NADH:ferricyanide dehydrogenase activities in the mitochondrial lysates of *Phytomonas serpens* and procyclic *Trypanosoma brucei* revealed that the sensitivity of these activities to rotenone, piericidin and diphenyl iodonium differed between the two species. NADH dehydrogenase activity was detected in both lysates by direct staining in the polyacrylamide gel after native electrophoresis. Two different high molecular weight activities were present in *P. serpens* but only one was found in procyclic *T. brucei*. Second dimension run as SDS-PAGE revealed the association of NADH dehydrogenase activities of *P. serpens* with distinct multisubunit complexes. One subunit present in NADH dehydrogenase complexes cross-reacted with the antibody against the genuine 39 kDa subunit of bovine complex I. Out of several subunits analyzed by mass spectrometry, one contained a domain characteristic for the LYR family, and seems to be a homologue of the B14 subunit of eukaryotic complex I. RNA interference against catalytic subunit of putative complex I in *T. brucei* did not reveal any observable phenotype.

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Interactions of the RNA-binding differentiation regulator, TbZFP3, with *ep procyclin* mRNA in *Trypanosoma brucei*

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The developmental regulation of gene expression is a key process during the trypanosome life cycle. Although this is largely controlled at the post-transcriptional level, the trans-acting RNA-binding proteins involved in controlling differentiation events are not well characterised. One group of proteins implicit in the control of several developmental events is the TbZFP family (comprising TbZFP1, 2 and 3). These proteins are characterized by their small size (¡150aa) and presence of a CCCH zinc finger RNA-binding domain. Interestingly, the proteins are capable of direct interaction in yeast and co-immunoprecipitate in procyclic forms, suggesting association in a complex. Furthermore the TbZFPs show a stage-specific association with the translational apparatus in procyclic but not bloodstream forms. RNA-immunoprecipitation using antibodies against TbZFP3 selected *ep procyclin* mRNA in procyclic-stage cells, suggesting it may be a target for this complex. To investigate this interaction we used reporter constructs provided with specific deletions of the *ep procyclin* 3'UTR. Also, to further examine whether ZFP3 has a potential role in the regulation of *ep procyclin*, we investigated the effect of knockdown and overexpression of ZFP3 protein on the levels of this transcript in procyclic cells. The results of these analyses will be presented. A. Paterou, **P. Walrad** et al., Identification and stage-specific association with the translational apparatus of TbZFP3, a CCCH protein that promotes trypanosome life-cycle development. *J. Biol. Chem.* **281** (2006), pp.39002-13.

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Towards a genetic screen for factors involved in regulating the developmental expression of the *Trypanosoma brucei GPI-PLC* gene

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The steady state level of *GPI-PLC* mRNA in *T. brucei* bloodstream forms is 50-fold greater than in procyclics. This differential expression results from a difference in the stability of the mature message. The necessary and sufficient cis-elements for procyclic instability are in the 2.3 kb 3'UTR. Regulation is very robust: a transgene containing a NEO open reading frame fused to a *GPI-PLC* 3'UTR confers G418 resistance in bloodstream trypanosomes but on differentiation into procyclics the transgene mRNA levels drop and ability to grow on G418 is lost. Such G418 sensitive procyclic cell lines have been the basis of two genetic screens. 1) Cis-acting elements. Spontaneous G418 resistant mutants arose at a frequency of 1 in 10^7 and invariably involved deletions or rearrangements which separated the NEO coding sequence from the distal 1.3 kb of the 3'UTR. No small deletions were generated. This outcome would be predicted if the 3'UTR harboured at least two sufficient instability elements at some distance apart. Such redundancy was confirmed by deletion analysis. 2) A Mariner transposon screen for trans-acting factors. The first screen generated 12 G418 resistant lines but in each case the transposon had hopped into the first kilobase of the transgene 3'UTR - a more likely event than a double hit in both alleles of a trans-acting factor (or a single hit followed by gene conversion). The screen is now being repeated with modified reporter cell lines containing two selectable transgenes. The second transgene has been introduced either as the second allele at the NEO-PLC3'UTR locus, to report on the occurrence of independent hits versus gene conversion, or at a distant genomic location.

Towards a genetic screen for factors involved in regulating the developmental expression of the *Trypanosoma brucei GPI-PLC* gene

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254A

A large-scale functional study of trypanosomal kinesins

Bill Wickstead*, Jared Honeycutt, Steven Kelly, Keith Gull

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One of the surprises emerging from the TriTryp sequencing effort was the identification of a great diversity of genes predicted to encode kinesin-like microtubule motors. The current version of the *Trypanosoma brucei* genome sequence contains 47 kinesin sequences - many more than found in budding yeast (6 kinesins), *Plasmodium* (9) or even humans (32). We have previously shown that within this diversity of kinesin sequences are members of 2 new, well-conserved, kinesin families which, on the basis of their distribution among eukaryotes, we predict to have as-yet uncharacterised flagellar functions. There are also 2 large kinetoplastid-specific kinesin groups for which little functional data is available. Surprisingly, trypanosomes lack several highly-conserved mitotic motors (BimC, CENP-E, chromokinesin, Kip3 and MLKP1), but possess a much-expanded kinesin-13 (MCAK) family - indicating that mitosis in trypanosomes must be different from the paradigm laid down in fungi. Here, we present the initial results of a large-scale investigation of kinesin localisation and function. This investigation uses some of a set of new vectors for tagging trypanosomal genes at their endogenous loci which cause minimal perturbation of protein levels and can be used in combination with RNAi. This study is designed to uncover some of the biology of the new kinesin families and of trypanosomal mitosis.

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254B

The *T. brucei* mitochondrial desulfurase IscS is essential for tRNA thiolation: implications for tRNA editing and import

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In Leishmania and Trypanosoma species, all mitochondrial tRNAs are transcribed in the nucleus and imported from the cytosol into the mitochondrion. The only tRNATrp encoded within the nucleus of these organisms contains a CCA anticodon that can decode the UGG codon in the cytosol, but is unable to decode the tryptophan codons in mitochondria. Trypanosomatids have solved this problem of UGA decoding by specifically changing the first position of the anticodon of tRNATrpCCA from cytidine (C34) to uridine (U34) by RNA editing, following import into the mitochondrion. In addition, this tRNA undergoes a number of posttranscriptional chemical modifications in the anticodon stem loop following mitochondrial import. Notably, position 33 (adjacent to the edited nucleotide) becomes thiolated but only in the edited tRNA species. We have hypothesized that thiolation of the normally unmodified U33 (97% of all the sequenced tRNAs from various organisms have an unmodified U33) to be an essential prerequisite for C to U editing of tRNATrp in trypanosomatids and thus important for cell viability. In E. coli, formation of s2U in tRNA is mediated by the cysteine desulfurase, IscS. In Trypanosoma brucei there are two IscS homologs, a cytosolic protein and a protein targeted to the mitochondria. Here we show by using RNAi that the mitochondria-targeted IscS is responsible for s2U formation in both cytosolic and mitochondial sulfur containing tRNAs. Since thiolation of tRNAs may play a critical role in both affecting tRNA import into the mitochondria as well as mitochondrial tRNA editing, our observations shed light into how this modification may regulate both processes.

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255A

Chromatin and histone levels and modification in metacyclic *Leishmania*

Iris L. K. Wong, Stephen M. Beverley*

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Packaging of genomic DNA together with core and linker histones and accessory proteins into chromatin profoundly influences gene expression and differentiation. Previously we reported that by EM staining, the chromatin of metacyclic promastigotes appeared to have a higher level of condensation than procyclics, and that the rate of transcription was globally decreased. This raised the possibility that as in higher eukaryotes, post-translational modification of histone and histone abundance in *Leishmania* may play important roles in regulation of gene expression by controlling changes in chromatin structure. We used quantitative electron microscopy of staining and morphology compared to reactivity with anti-DNA antibodies to establish that the dark-staining material was condensed heterochromatin, and that heterochromatin rises from 24% in procyclic promastigotes to 56% in metacyclics. Chromatin condensation during the differentiation in Leishmania was accompanied by the changes in histone modification and abundance. We generated antisera to the core histones and found that their relative levels were unchanged in development. However, increased levels of the variant H2A and variant H2B histones were seen in metacyclic stage. Similarly, H3K74 trimethylation levels were higher in metacyclic than procyclic parasites, whereas the H3K74 dimethylation level was lower in metacyclic than procyclic stage. Studies in progress are testing whether these differences play a causal role in *Leishmania* differentiation and/or chromatin condensation through genetic manipulations. We thank Christian Janzzen and George Cross for providing anti-methylated H3K74 antisera. Supported by a grant from the NIH.

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255B

Pyrimidine biosynthesis deficiency in *Leishmania donovani*

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Leishmania synthesize pyrimidine nucleotides via both *de novo* and salvage pathways. A single cosmid encoding all six enzymatic activities of the *de novo* pyrimidine biosynthetic pathway was isolated from a *L. donovani* genomic library using the *L. major* carbamoylphosphate synthetase (CPS) and dihydroorotate dehydrogenase (DHOD) genes as probes. The predicted amino acid sequences of each gene exhibited a high degree of homology to their counterparts from other trypanosomatids. To determine if pyrimidine biosynthesis is necessary for growth and virulence, a *CPS* gene knockout was created by double targeted gene replacement. Parasites deficient in CPS, the first step in the pyrimidine biosynthetic pathway, were auxotrophic for pyrimidines and required supplementation with exogenous pyrimidines for proliferation. Growth of the null mutant was supported by micromolar concentrations of uracil, uridine, deoxyuridine, cytidine, or deoxycytidine, millimolar concentrations of orotate, but not by millimolar amounts of cytosine, thymine or thymidine. These and other data suggest that salvaged pyrimidines are funneled through uracil and then incorporated into the parasite nucleotide pool via uracil phosphoribosyltransferase. To determine if *de novo* pyrimidine biosynthesis is essential for infectivity and virulence, we tested the infectivity of *cps* null mutants in a mouse model. Parasitemia of *cps* null cells in murine macrophages was equivalent to that of wild type cells, and liver and spleen parasite burdens of mice infected with the knockout were equivalent to those of wild type parasites at four weeks after inoculation. This intimates that the levels of exogenous pyrimidines encountered by the parasite *in vivo* are sufficient to maintain pyrimidine nucleotide pools and allow growth of the amastigote.

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256A

Analysis of three RNA editing endonuclease-associated zinc finger proteins

Carmen Zelaya Soares[1], Achim Schnaufer[2], Ken Stuart[*,1]

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Kinetoplastid RNA editing transforms cryptic mitochondrial pre-mRNAs into functional mRNAs by the insertion and deletion of uridine residues as specified by the sequence of short guide RNAs. Editing is catalyzed by the editosome, which contains the endonuclease, terminal uridylyl transferase (TUTase), exouridylylase (ExoUase) and ligase catalysts as well as proteins necessary for the coordination of these activities. Kinetoplastid RNA Editing Protein B6 (KREPB6, formerly MP49), KREPB7 (MP47), and KREPB8 (MP41) co-purify with the ∼20S editosome. Recent work using Tandem Affinity Purification (TAP)-tagged editing endonucleases showed that editosomes are compositionally heterogeneous, with B8 only found in KREN1 deletion endonuclease complexes and B7 in KREN2 insertion endonuclease complexes. Thus far, B6 had only been identified in KREPB5 TAP-tagged complexes in *Trypanosoma brucei*, although the *Leishmania* orthologue was identified in LtREL1 TAP-tagged complexes. All three proteins contain a conserved N-terminal U1-like C2H2 zinc finger, shown to be involved in protein-RNA and protein-protein interactions in other ribonucleoprotein complexes. Procyclic *T. brucei* cell lines were established for tetracycline inducible expression of TAP-tagged B6, B7 and B8. Western blots of the purified complexes probed with monoclonal antibodies confirm that the tagged proteins are associated with 20S editosomes. Mass spectrometry shows that TAP-tagged B7 and B8 reciprocally pull down N2 and N1 respectively. Importantly, B2 was pulled down by TAP-tagged B6. These results support the heterogeneous composition of the editosome architecture and suggest a specific role for B6, B7 and B8 in each type of 20S editosome. Full-round and precleaved editing assays are currently underway to more fully characterize these heterogeneous editing complexes. This work will extend our understanding of RNA editing, an essential process in kinetoplastids.

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256B

Expression of *L.donovani infantum* genes in *L.major* and its effect on virulence

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We have previously shown that expression of the *L.donovnai* specific *A2* gene in *L.major* increased its virulence in experimental visceral infection but reduced its lesion size in cutaneous infection in BALB/c mice, suggesting *Leishmania* species specific genes could play an important role in tropism and pathology of *Leishmania* infection. Comparison of recently completed *L.infantum* genome sequences with the *L.major* genome originally identified several *L.infantum* genes which were potentially absent or present as pseudogenes in *L.major*. To investigate whether these *L.infantum* genes are involved in the tropism of *Leishmania* infection, seven of these genes were cloned and crossing species transfected into *L.major*. The *L.major* transfectants were used (pooled or individually) to infect BALB/c mice by intravenous injection. After four to eight weeks selection of visceral infection in BALB/c mice, one of these *L.infantum* genes was shown to significantly increase liver and spleen parasite burden and cause splenomegaly. Further analysis of this *L.infantum* gene however revealed that it was indeed present in *L.major* and was flanked by two 384 bp repeated sequences and had 92% identity in amino acid sequence with the *L.major* ortholog. We are currently examining whether the polymorphism or overexpression of this gene altered *L.major*'s virulence. This study confirms that crossing species transfection can be an effective means to characterize hypothetical *Leishmania* virulence proteins.

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Insights into the composition of several mitochondrial complexes in *Trypanosoma brucei*

Alena Zikova, Rachel Burnham, Yuko Ogata, Atashi Anupama, Nathalie Acestor, Peter J. Myler, Aswini K. Panigrahi, Ken Stuart*

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Most cellular processes are carried out by multi-protein complexes. The identification and analyses of their components provide insights into how the ensemble of expressed proteins is organized into functional units. The TriTryp genomes have been sequenced though functions cannot yet be ascribed to the majority of the predicted genes. A useful approach for understanding the protein function is to determine the associations of proteins within complexes thus assigning their putative functions based on their interactions with known proteins. We used tandem-affinity purification and mass spectrometry in a high throughput approach to identify and characterize multi-protein complexes in *T. brucei* mitochondria. We have examined numerous tagged complexes including those involved in DNA/RNA/protein biosynthesis, oxidative phosphorylation and energy metabolism. For example, we analyzed the composition of mitochondrial ribosomes using 10 different TAP tagged proteins, identifying 77 proteins in the large and 58 proteins in the small ribosomal subunits. PHI-BLAST and PFAM search identified sequence homology for 35 of these proteins to LSU and SSU of eukaryotic mitochondrial/bacterial ribosomal proteins, most of these proteins are currently annotated as hypothetical. Interestingly, it seems that *T. brucei* mitochondrial ribosome contains most number of proteins and the smallest ribosomal RNAs compared to other organisms known to date. Similarly, respiratory complexes II, IV and V as well as oxidoreductase complex have been analyzed identifying 5, 32, 28 and 19 proteins respectively in these complexes, most of which are currently annotated as hypothetical proteins in GeneDB. We are also purifying these complexes from long slender bloodstream form, a stage in mammalian host with repressed mitochondrial functions. The data from these analyses will be presented and discussed.

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Gene expression during *Leishmania donovani* differentiation

Dan Zilberstein[*,1], Doron Rosenzweig[1], Tamar Lahav[1], Derek Smith[2], Alka Saxena[3], Michal Mandelboim[4], Shulamit Michaeli[4], Peter J. Myler[3], Robert W. Olafson[2]

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Differentiation of *Leishmania* promastigotes to amastigotes is a complex process, but most studies to date on *Leishmania* development have merely compared the two life stages, ignoring the molecular events that take place during the transition. We have examined temporal changes in gene expression during differentiation of *Leishmania donovani* using a host-free system. Transcriptome analysis, using genomic microarrays, indicated that ordered changes in mRNA abundance occurred during this process, with ∼5% of the transcription showing significant changes. In addition, a number of mRNAs were transiently up- or down-regulated during differentiation. In addition of number of non-protein-coding RNAs showed substantial changes in abundance. Examination of *trans* splicing and SL RNA abundance as a function of differentiation progression revealed a burst in SL RNA transcription a few hours after exposure to the differentiation signal that was followed by a transient but significant increase in the steady state level of SL RNA in parasites cell nucleus, as well as concomitant increase in trans splicing . High-coverage quantitative proteomics using amine-specific isotopic labeling (iTRAQ) was employed to determine protein expression profiling during differentiation. Of the 1433 proteins identified by these studies, ∼10% were up-regulated and 14% down-regulated by at least 2-fold during differentiation, with most changes occurring after the initial 10 hours following the differentiation signal. Functional analysis of these revealed coordinated changes in proteins involved in energy metabolism, DNA condensation and translation. Preliminary evidence suggests that posttranslational processes such as protein phosphorylation occur very soon after the differentiation signal and are primary to subsequent changes in mRNA and protein abundance. This work provides the first detailed insight into the time course of *Leishmania* differentiation.

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Differentiation of *Leishmania* promastigotes to amastigotes is a complex process, but most studies to date on *Leishmania* development have merely compared the two life stages, ignoring the molecular events that take place during the transition. We have examined temporal changes in gene expression during differentiation of *Leishmania donovani* using a host-free system. Transcriptome analysis, using genomic microarrays, indicated that ordered changes in mRNA abundance occurred during this process, with ∼5% of the transcription showing significant changes. In addition, a number of mRNAs were transiently up- or down-regulated during differentiation. In addition of number of non-protein-coding RNAs showed substantial changes in abundance. Examination of *trans* splicing and SL RNA abundance as a function of differentiation progression revealed a burst in SL RNA transcription a few hours after exposure to the differentiation signal that was followed by a transient but significant increase in the steady state level of SL RNA in parasites cell nucleus, as well as concomitant increase in trans splicing . High-coverage quantitative proteomics using amine-specific isotopic labeling (iTRAQ) was employed to determine protein expression profiling during differentiation. Of the 1433 proteins identified by these studies, ∼10% were up-regulated and 14% down-regulated by at least 2-fold during differentiation, with most changes occurring after the initial 10 hours following the differentiation signal. Functional analysis of these revealed coordinated changes in proteins involved in energy metabolism, DNA condensation and translation. Preliminary evidence suggests that posttranslational processes such as protein phosphorylation occur very soon after the differentiation signal and are primary to subsequent changes in mRNA and protein abundance. This work provides the first detailed insight into the time course of *Leishmania* differentiation.

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The role of *Leishmania* dihydroxyacetonephosphate acyltransferase in metacyclogenesis, lipid metabolism and virulence

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The ability of *Leishmania* parasites to generate new membranes is essential for their development and their pathogenesis. The initial step of synthesis for the major glycerolipids starts with the acylation at the *sn-1* position of glycerol-3-phosphate and dihydroxyacetonephosphate by glycerol-3-phosphate and dihydroxyacetonephosphate acyltransferases, respectively. While acylation of the precursor glycerol-3-phosphate is not essential for the synthesis of ester glycerolipids, glycerolipid production by acylation of dihydroxyacetonephosphate by the unique glycosomal dihydroxyacetonephosphate acyltransferase *Lm*DAT is important for growth and virulence. Interestingly, *Lm*DAT bears an intriguing large N-terminal extension that does not exhibit any similarity to any known proteins, and is absent in higher eukaryotic orthologs. We showed that truncated versions of *Lm*DAT lacking this extension lost their enzymatic activity. A null mutant of *LmDAT* produced altered, longer forms of the ether lipid based virulence factor lipophosphoglycan, which are not released in the media. In addition, the null mutant expressed higher levels of GPI-anchored proteins. Consistent with its poor survival during the stationary phase of growth, the null mutant lost its ability to differentiate into virulent metacyclics despite the fact that it still expressed metacyclic markers. Null mutant promastigotes and amastigotes were still able to cause disease in mice, but lesion formation was strongly delayed compared to wild type. All together, our results support an important role for the unique dihydroxyacetonephosphate acyltransferase *Lm*DAT in Leishmania glycerolipid metabolism and virulence.

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