

Research brief

# Perturbation of local endogenous expression by insertion of Pol I expression constructs into the genome of *Trypanosoma brucei*

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## Abstract

Manipulation of gene expression is a common tool for the elucidation of biological function. Here we investigated the effects of over-expression in trypanosomes of a small GTPase, TbRABX1, using 2D gel electrophoresis and mass-spectrometry. An over-expression construct was targeting to the tubulin locus of chromosome I for stable integration and expression. Unexpectedly we observed alterations to the expression of gene products, i.e., tubulin, from surrounding regions of the genome; this effect was shown to be general and not dependent on the identity of the ectopic gene being expressed. These data suggest that local perturbation of the genome by insertion of DNA constructs can have wider impacts on gene expression, which need to be monitored.

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Determining the function of a gene product usually requires manipulation, in some fashion, of the host cell or organism. Ectopic overexpression of gene products is a widely used strategy, and more recently analysis by proteomic approaches has been applied to the investigation of global patterns of protein expression and hence function in the context of overexpression. For parasitic protozoa, proteomics is useful for determining the expression profiles of distinct life stages (Florens et al., 2002), for the investigation of developmental changes (Drummel-Smith et al., 2004; Nugent et al., 2004), or in the study of the influence of specific manipulations of the genome/proteome. Here we report the influence of overexpression of a small GTPase of the Rab family, TbRABX1 (Ackers et al., 2005; Field et al., 1999). We consider a major influence on tubulin levels. We consider this unexpected result is most likely due to the location of the ectopic expression construct and demonstrates perturbation of the surrounding endogenous Pol II-transcribed genes from a Pol I-driven expression system.

TbRABX1 is a constitutively expressed *Trypanosoma brucei* Rab protein that localises to the endoplasmic reticulum-Golgi intermediate compartment (Field et al., 1999). The TbRABX1 gene resides next to the open reading frame (ORF)<sup>1</sup> for TbRABX2 (Field and Field, 1997), a Rab protein localised to the Golgi complex (Field et al., 2000). TbRABX1 has no clear orthologue in higher eukaryote genomes

despite being well conserved amongst the kinetoplastida (Ackers et al., 2005). Overexpression of TbRABX1 results in elaboration of ER-like membrane elements and the formation of vacuolar structures within the cytoplasm (Field et al., 1999) suggesting that TbRABX1 could have an important role in membrane biogenesis in the early trypanosome secretory pathway. Ablation of TbRABX1 expression in bloodstream form trypanosomes does not generate a trafficking defect for the variant surface glycoprotein, the major surface protein (Dhir et al., 2004). Hence the function of TbRABX1 is not known, but is likely kinetoplastida specific. We considered that the somewhat impressive effect of TbRABX1 overexpression on ER membranes, coupled with the lack of other functional data, made the TbRABX1 overexpressor line an excellent candidate for proteomic analysis.

The TbRABX1 overexpressor cell line, together with parental 427 procyclics were grown at 27 °C in SDM79 using non-vented tissue culture flasks. Cells were sub-cultured when they had passed a density of  $2 \times 10^7$  cells/ml. Aliquots of these cultures ( $\sim 4 \times 10^7$  cells) were analysed by 2D-gel electrophoresis. Cultures were centrifuged (10 min, 800g, 4 °C) and resuspended in 10 ml TS buffer (25 mM sorbitol, 10 mM Tris-HCl, pH 7.3). Cells were again centrifuged and resuspended in 1 ml of TS and then pelleted in a microcentrifuge (7000 rpm, 2 min). The supernatant was carefully removed (without disturbing the parasite pellet) and immediately 14 µl of 25× protease inhibitors (Roche minicocktail dissolved in 1990 µl H<sub>2</sub>O, 10 µl pepstatin at 5 mg/ml) were added. Parasites were resuspended in 336 µl rehydration buffer (9 M urea, 2 M thiourea, 2% Chaps, 65 mM DTT, and 0.5% IPG buffer, plus a trace of bromophenol blue) using a pipette,

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<sup>1</sup> Abbreviation used: ORF, open reading frame.

before gently vortexing for 30 s. Samples were left at room temperature for 1 h, vortexing for 30 s every 15 min. Lysates were then centrifuged in a microcentrifuge (10 min, 14,000g, 4 °C) to remove insoluble debris before loading onto an IPG strip. Isoelectric focusing was performed using an IPGphor (Amersham) according to the manufacturer's instructions. Cycle settings were; rehydration (12 h, 20 °C), step 1: 500 V for 500 V h, step 2: 1000 V for 1000 V h, and step 3: 8000 V for 100,000 V h. Focused strips were prepared for the 2D by equilibration in 1.5 M Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, trace of bromophenol blue, and 0.1% DDT followed by the same buffer but replacing DTT with 0.1% iodoacetamide. Strips were then briefly washed in Laemmli running buffer, before placing onto a 10% 18 cm × 20 cm Acrylamide gel (2.5.2). Gels were run for 16 h at 70 V and 16 mA. On completion, the gel was removed and fixed (40% methanol/10% acetic acid) and silver stained. Gels were then scanned and examined (Fig. 1).

About 500 proteins were resolved and visualised by this method (Fig. 1A). A number of specific alterations were observed, and we focused attention on a selection of these that were abundant, well resolved and reproducible in all experiments. The majority of these fell within the regions highlighted in Fig. 1A as region of interest (ROI)

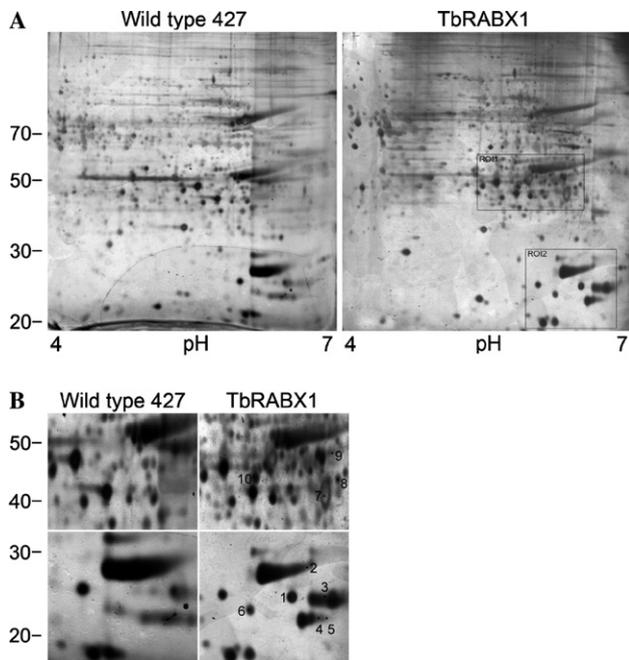


Fig. 1. 2D protein electrophoretic separations of wild type 427 procyclic trypanosomes and procyclics overexpressing TbRABX1. (A) Parasites ( $4 \times 10^7$ /sample) were centrifuged, washed and resuspended in rehydration buffer in the presence of protease inhibitors. Lysates were cleared by centrifugation at top speed in a microcentrifuge before loading onto an IPG strip (pH 4–7). Proteins were separated by isoelectric focusing and then by SDS-PAGE. Gels were silver stained and scanned. Lysates were analysed on multiple occasions (at least three times) to confirm reproducibility. Two regions of interest (ROI1 and ROI2) containing multiple changes to the proteome profiles between the two cell lines are shown boxed. The approximate pH gradient is shown. (B) Enlargements of the regions ROI1 (top) and ROI2 (bottom) for wild type and TbRABX1 overexpressors. The locations of the ten most prominent and reproducible protein spots taken for mass spectrometric analysis are indicated by numbers and asterisk. Both panels represent a pI range of  $\sim 4$ –5 U and the 40–50 kDa (ROI1) and 20–30 kDa (ROI2) mass range.

1 or 2 (Fig. 1B). We chose 10 spots as a representative sample and subjected these to analysis using a Q-TOF mass spectrometer. Data indicated that, with the exception of spot 6, the assignment was suggestive or conclusive of either  $\alpha$ - or  $\beta$ -tubulin.

Tubulin is a highly abundant protein and is a common contaminant in proteomic analysis. The strong features in the 2D-gel images at  $\sim 50$  kDa and pH 6 are derived from tubulin, and whilst it was possible that the analysis had simply identified tubulin contamination in the regions of the gel analysed, for this to be the case for all of the spots appeared unlikely. A second possibility we considered was that elaboration of ER membranes by TbRABX1 overexpression, which in other systems are intimately connected with the microtubule cytoskeleton, could result in upregulation of tubulin. A third possibility was that the expression construct, pXS219, was in some manner causing the increase in tubulin expression. This construct is integrated into the tubulin array on chromosome 1, and contains a procyclin RNA polymerase I-dependent promoter element designed to achieve high expression levels of the gene of interest.

We resolved these possibilities by analysis of the TbRABX1 cell line, together with two further cell lines overexpressing TbRABX2 and TbBiPN, a fragment of the ER chaperone BiP, which is secreted from the cell efficiently (Bangs et al., 1996), by 1D SDS-PAGE followed by Western analysis using monoclonal antibodies to  $\alpha$ - and  $\beta$ -tubulin (Fig. 2). Western blots with  $\alpha$ -tubulin clearly indicated that both TbRABX1 and TbRABX2 cell lines had increased expression, and also detected small amounts of lower molecular weight immunoreactivity, likely due to degradation, whilst both TbRABX1 and TbBiPN showed a clear increase in  $\beta$ -tubulin expression.

These data clearly eliminate the possibility that the detection of increased tubulin by the 2D-analysis was the result of contamination. Comparison of TbRABX1 and TbRABX2 also ruled out a specific involvement in the elaboration of ER as this does not occur in the

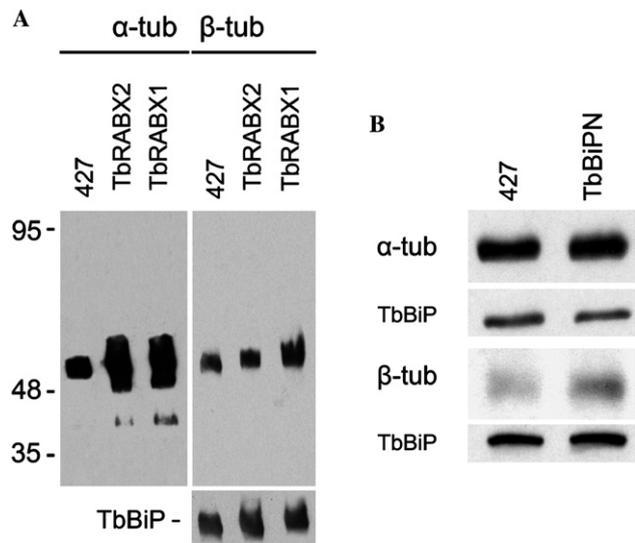


Fig. 2. Western blot analysis of tubulin expression in various procyclic lines. (A) Western blot analysis of  $\alpha$ - and  $\beta$ -tubulin expression in wild type (427) and TbRABX1 and TbRABX2 overexpressing procyclic stage trypanosomes. Top panels show lysates probed with antibodies against  $\alpha$ - or  $\beta$ -tubulin (left and right, respectively). A loading control using TbBiP shows equivalent loading of each lysate. Molecular weights are indicated at left of each panel in kDa. (B) Western blot analysis of  $\alpha$ - and  $\beta$ -tubulin expression in wild type (427) and TbBiPN overexpressing procyclic stage trypanosomes. The effect on the expression of tubulin is less dramatic than for TbRABX1, but by densitometry a 1.4- and 1.9-fold increase in expression of  $\alpha$ - and  $\beta$ -tubulin, respectively, was found in the TbBiPN cells.

Table 1

A summary of the proteome differences detected between 427 procyclics and the TbRABX1 overexpressor

Spot	Highest scoring protein	Score	Coverage %
1	Mixed spectra; probable tubulin	126.8	6.0
2	$\alpha$ -Tubulin	443.7	11.5
3	$\beta$ -Tubulin	348.6	7.5
4	Mixed spectra; probable tubulin	211.0	4.0
5	$\alpha$ -Tubulin	567.3	8.2
6	40S ribosomal protein S12	950.0	29.4
7	$\beta$ -Tubulin	195.9	29.9
8	$\beta$ -Tubulin	1706.2	21.0
9	Mixed spectra; probable tubulin	167.5	5.7
10	Mixed spectra; probable tubulin	170.3	6.5

Protein spots, corresponding to those designated in Fig. 1B, were cored, destained, digested with trypsin and prepared for analysis by mass spectrometry. Peptide fingerprints obtained on a Q-TOF mass spectrometer were searched against non-redundant databases and the closest matches identified. With the exception of spot 6, all others appear to be derived from trypanosome  $\alpha$  or  $\beta$ -tubulin. Scores were calculated using MOWSE (Pappin et al., 1993).

TbRABX2 overexpressor (Field et al., 1999), whilst increased tubulin observed with TbBiPN indicates that the effect is not Rab-specific. As BiPN is translocated into the ER lumen (Bangs et al., 1996) it is unlikely that an increase in tubulin abundance is the result of interactions at the protein level (see Table 1).

The most likely explanation is that the presence of a strong Pol I promoter within the tubulin array results in increased transcription from the endogenous locus due to the polycistronic nature of transcription in trypanosomatids. It is not clear if this is due to transcriptional read through or a localised alteration in chromatin structure that facilitates Pol II-driven transcription. We note that *cis*-activation of transcription from the procyclic promoter has been previously observed, although in these examples the influence on a second ectopic construct, also transcribed by Pol I, was reported rather than on the endogenous regions surrounding the insertion site (Qi et al., 1996; Urmenyi and Van der Ploeg, 1995; Zomerdijk et al., 1993). This is the first time, to our knowledge, that an influence on protein expression at an endogenous Pol II-transcribed genomic location has been described. We presume that the precise differences in the effects of TbRABX1, TbRABX2, and TbBiPN on tubulin abundance are due to the nature of the transformation event, and may reflect different integration positions or copy number within the tubulin cluster on chromosome I.

Regardless of the precise mechanism, the data indicate that integration of an expression construct within a protein-coding region of the trypanosome genome may effect transcription and protein levels from the surrounding ORFs. The differences between the TbRABX1 and TbRABX2 cell lines, and also a large number of others that we have generated using this technology (see Pal et al., 2003 for several examples) do not appear to preclude the generation of a specific and interpretable phenotype. However, in some circumstances, such effects may be critical, and care should be taken in analysis of overexpression.

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