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SHORT COMMUNICATION

Mitochondrial membrane potential-based genome-wide RNAi screen of *Trypanosoma brucei*

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Abstract We have screened the *Trypanosoma brucei* genome-wide RNAi library by staining the procyclics with the dye JC-1 followed by sorting the differentially stained cells by flow cytometry. This allowed us to highly enrich for cells in which mitochondrial membrane potential was decreased. We have further validated a subset of selected clones by a reverse approach in which we showed that cloning the selected genomic regions into another RNAi vector also results in a drop in mitochondrial membrane potential.

Introduction

Trypanosoma brucei is an important model organism for several reasons: (1) together with related kinetoplastid flagellates, it causes numerous serious diseases of man, animals, and plants; (2) its genome has been sequenced; (3) it is the best studied representative of the Excavata, one of the six recognized eukaryotic superdomains; and (4) it is amenable to all main methods of forward and reverse genetics (Motyka and Englund 2004). The function of a substantial number of the estimated 8,131 predicted open reading frames in the genome of *T. brucei* (Subramaniam et al. 2006) has already been established, and functional analysis of many more is under way.

Trypanosomes belong to organisms in which RNAi was demonstrated for the first time (Ngo et al. 1998). Ever since, the RNAi technology became critical for generation of knock-down trypanosomes for one or more target genes in which specific phenotypic effects can be studied. This approach is much faster than knocking target genes out by homologous recombination. Currently, several RNAi vectors are available, which use either a single or two opposing tetracycline-inducible T7 or PARP promoter(s) (Wang et al. 2000; Wickstead et al. 2002). However, even with the extensive application of RNAi, functional analysis remains a tedious process. This is true especially for genes that have no homology outside of Trypanosomatidae, which represent a substantial fraction of all predicted open reading frames. Since deficiency for many of these genes is lethal, it is often a challenge to identify the primary cause of lethality. One attempt to accelerate research in this area was the generation of RNAi knock-down cell lines for all genes encoded on chromosome I of *T. brucei* (Subramaniam et al. 2006). Another approach aimed for high throughput screening of trypanosome genes was based on a genomic RNAi library, in fact the first of its kind in any organism (Morris et al. 2002).

RNAi library used in this study was created by the insertion of random DNA fragments, 660 bp on average, into the pZJMα vector equipped with opposing T7 promoters, from which a strong transcription can be induced by the addition of tetracycline into the cultivation medium (Motyka and Englund 2004; Englund et al. 2005). The authors estimated that the entire *T. brucei* genome is contained about six times in this library. They also suggested that their RNAi library will be particularly useful for easy-to-detect phenotypes or for the study of processes involving a large number of genes, while identification of a single protein was considered to be potentially difficult (Motyka and Englund 2004). So far, several assays were used to fish genes such as loss of

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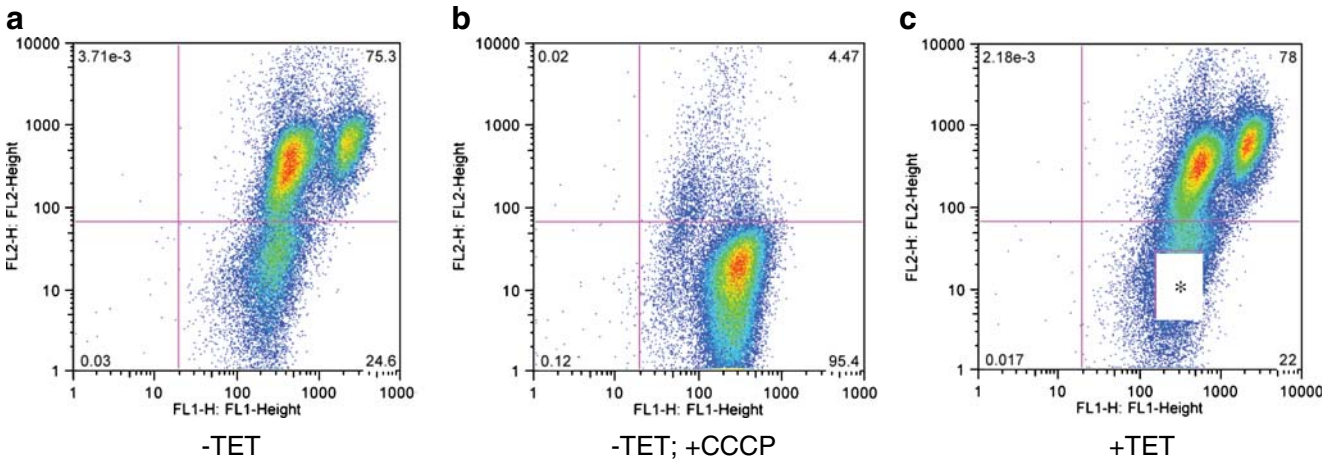

Fig. 1 Result of FACS analysis after staining with JC-1. Mitochondrial membrane potential was measured in the non-induced RNAi library (*−TET*) that was either untreated (**a**) or treated (**b**) with mitochondrial uncoupling agent CCCP (*−TET; +CCCP*). This allowed to define the area containing cells with decreased or disrupted mitochondrial membrane potential. From the tetracycline-induced RNAi library (*+TET*), a subpopulation of cells with affected mitochondrial membrane potential was gated (*white rectangle with a star*) and subsequently sorted out (**c**)

binding to concanavalin A, a lectin that binds procyclin (Morris et al. 2002). The *T. brucei* RNAi library was also very efficiently used to identify candidate proteins involved in maintenance and replication of the kinetoplast DNA network (Zhao et al. 2008).

By screening RNAi library in the procyclic cells, we have attempted to identify genes whose function is associated with mitochondrial physiology, in particular mitochondrial membrane potential. It has been shown that in the mitochondrion of the procyclic *T. brucei*, respiratory complexes are composed of many subunits that lack homologues in other organisms (Morales et al. 2009; Zíková et al. 2009). By screening for altered mitochondrial membrane potential, we aimed to identify novel subunits of respiratory complexes as well as other proteins required to uphold this potential.

In the initial experiment, 1 ml of the non-induced RNAi library was stained with the dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolyl carbocyanine iodide; 2 μM final concentration) for 20 min at 27°C. The advantage of using JC-1 to other mitotrackers is that the vitality of stained cells is not compromised by this dye. The stained cells were then subjected to flow cytometry, using the fluorescence-activated cell sorter (FACSort Vantage, BD, Franklin Lakes, NJ, USA), resulting in a pattern of distribution shown in Fig. 1a. In parallel, the same fraction of the library was treated for 5 min at 27°C with carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) at final concentration 50 μM, which is a very potent mitochondrial uncoupling agent. Cell sorting of the CCCP-treated cells showed a shift in the distribution, defining an area occupied by cells with low

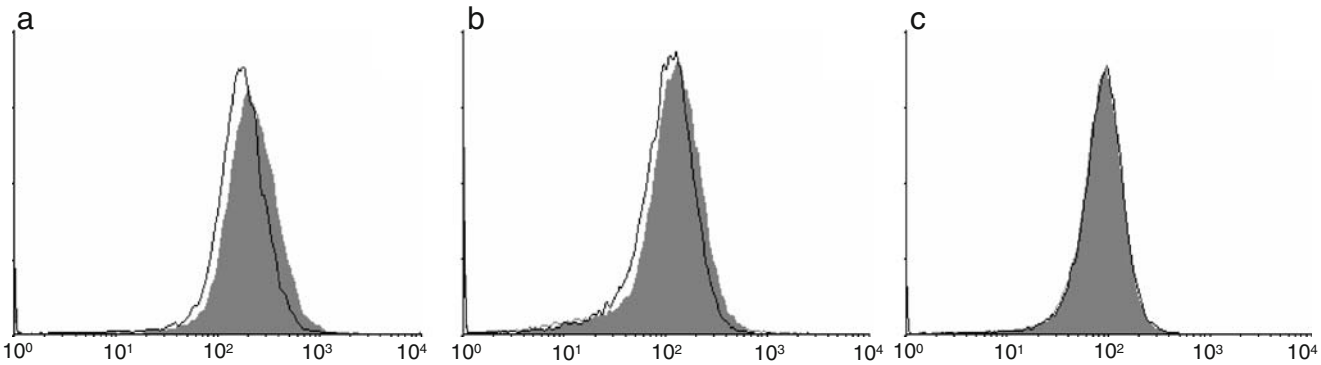

Fig. 2 Measurement of mitochondrial membrane potential using TMRE. **a** Non-induced and RNAi-induced cell line containing fragment of the gene encoding telomerase-associated protein in the pZJM vector isolated from the RNAi library. **b** Non-induced and RNAi-induced cell line containing the same genomic fragment in the p2T7-177 vector. **c** Cell line showing no difference in membrane potential upon RNAi induction. *Gray-filled* membrane potential of non-induced cells; *black line* membrane potential of RNAi-induced cells 6 days of induction. *x*-axis refers to log fluorescence of TMRE; *y*-axis depicts number of events in histograms

membrane potential (Fig. 1b). Finally, 1 μg/ml of tetracycline was added to 20 ml of the library, which represents approximately 2×10^8 cells, and RNAi was allowed to proceed for 6 days. Then, the cells were screened for mitochondrial membrane potential (Fig. 1c), and a region known from the control experiments to contain cells with decreased potential was gated and used for cell sorting. Cells were sorted either directly into a 96-well plate or pooled into a cuvette. After 2 to 3 weeks, 75 clones were expanded into larger volume and used for further experiments or stored in liquid nitrogen. RNAi knock-downs of respiratory complexes III (Rieske protein) and IV (cytochrome *c* oxidase subunit 4), previously described to be essential for the maintenance of mitochondrial membrane potential (Horváth et al. 2005), were used as positive controls.

As mitochondrial membrane potential was used for the initial screen, in 20 randomly selected clones RNAi was triggered by the addition of 1 μg/ml tetracycline in order to determine whether they are still responsive to RNAi. Six days upon induction, the uptake of tetramethylrhodamine ethyl ester (TMRE) into the organelle was measured as described elsewhere (Horváth et al. 2005). In 12 out of these 20 clones, a shift of the peak testified to the impaired maintenance of mitochondrial membrane potential (Fig. 2a). In all cases, the shift was shown to be highly reproducible, proving the validity of the used selection approach. However, in all cases the shift was not as dramatic as in our previous study (Horváth et al. 2005), suggesting that the selected target genes likely do not encode subunit of respiratory chain.

In the following step, inserts from the pZJM vector were PCR amplified from clones, in which membrane potential was altered upon RNAi induction. Three randomly chosen clones were sequenced in their entirety. One of them contained a fragment of the telomerase-associated protein Tb927.7.830, another one was identified as an unlikely hypothetical protein Tb09.160.1350, while the last fragment was a part of the intergenic region located between genes Tb927.5.2010 and Tb927.5.2020. Since neither of the genes nor the intergenic region have so far been associated with the mitochondrion, additional experimentation will be needed to shed light on their possible function in membrane potential maintenance.

In order to rule out possible improper integration of the pZJM vector or other effects responsible for altered membrane potential, we decided to test two of the sequenced inserts by a direct approach. For that purpose, primers were designed to PCR amplify from the genomic DNA of the *T. brucei* 29-13 strain the first and third region described above. Since the unlikely hypothetical proteins were in many cases shown not to be expressed (Panigrahi et al. 2009), we excluded this protein from further testing. Both were cloned into the p2T7-177 vector (Wickstead et al. 2002), a widely used RNAi vector, which allows a tightly regulated expression in both the procyclic and bloodstream stages of *T. brucei* (Hashimi et al. 2009). The insert-containing p2T7-177 vectors were upon linearization by *Not*I electroporated into the *T. brucei* 29-13 procyclics, and phleomycin-resistant cell lines were obtained as described elsewhere (Hashimi et al. 2009). In these lines, RNAi was induced and membrane potential was measured using TMRE as described above. Reassuringly, after 6 days of induction, cells transfected with the p2T7-177 vector containing part of the telomerase-associated protein showed the same decrease in membrane potential (Fig. 2b) as the cell line mined from the RNAi library that carried the same genomic fragment in the pZJM vector. However, the other tested region did not trigger measurable changes of membrane potential upon integration into the p2T7-177 vector (Fig. 2c).

It was shown that occasionally the pZJM vector becomes integrated into unexpected location, resulting in complex effects on downstream genes (Motyka et al. 2004). However, a similar situation can be excluded in both our cases. None of our sequences contain *Not*I within the cloned fragment, and in the case of the telomerase-associated gene, two different RNAi vectors showed the same outcome. At present, we have no straightforward explanation that would associate this protein with changes in mitochondrial membrane potential.

All in all, we have demonstrated by reverse and forward approaches that our set of 75 *T. brucei* clones is highly enriched for cells containing genomic fragments in the pZJM vector, RNAi against which results in decrease or disruption of mitochondrial membrane potential. These cells are available for the research community.

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