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Knockout of the abundant *Trichomonas vaginalis* hydrogenosomal membrane protein *Tv*HMP23 increases hydrogenosome size but induces no compensatory up-regulation of paralogous copies

Xavier Pereira Brás [a,1], Verena Zimorski [a,1], Kathrin Bolte [b], Uwe-G. Maier [b,c], William F. Martin [a], Sven B. Gould [a,*]

[a] Institute of Molecular Evolution, Heinrich-Heine-Universität Düsseldorf, D-40225 Düsseldorf, Germany
[b] Laboratory for Cell Biology, Philipps-Universität Marburg, D-35032 Marburg, Germany
[c] LOEWE-Zentrum für Synthetische Mikrobiologie (SynMikro), Hans-Meerwein-Straße, D-35032 Marburg, Germany

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ABSTRACT

The *Trichomonas vaginalis* genome encodes up to 60 000 genes, many of which stem from genome duplication events. Paralogous copies thus accompany most *T. vaginalis* genes, a phenomenon that limits genetic manipulation. We characterized one of the parasite's most abundant hydrogenosomal membrane proteins, *Tv*HMP23, which is phylogenetically distinct from canonical metabolite carriers, and which localizes to the inner hydrogenosomal membrane as shown through sub-organellar fractionation and protease protection assays. Knockout of *Tv*hmp23 through insertion of the selectable neomycin marker led to a size increase of hydrogenosomes, the first knockout-induced phenotypes reported for *Trichomonas*, but no growth impairment. The transcriptional response of its four paralogous copies then analyzed revealed that they are not up-regulated, and hence do not compensate for the *Tv*hmp23 knockout.

1. Introduction

Trichomonas vaginalis is an anaerobic protist and the causative agent of trichomoniasis, a widespread sexually transmitted infection of the urogenital tract of humans, causing symptoms particularly in women where the parasite feeds on vaginal epithelial cells [13,15] in a low oxygen environment [12,17,44]. Like all trichomonads, *T. vaginalis* possesses hydrogenosomes, anaerobic relatives of mitochondria, that generate ATP via substrate level phosphorylation through the fermentative breakdown of pyruvate to acetate, CO_2, and H_2 [25,27]. Like oxygen-respiring mitochondria, hydrogenosomes are surrounded by two membranes. Their matrix is granular and electron-dense [3], and because they lack a genome and translation machinery, trichomonad hydrogenosomes must import all their proteins from the cytosol [5,37].

Reports investigating the molecular machineries behind protein import and metabolite exchange across hydrogenosomal membranes are scarce. Bioinformatic analyses identified homologs of Tom40, Sam50, Tim17/22/23, Tim44 and Pam18 in *T. vaginalis* [8,36]. Some of these components have since been localized to the hydrogenosomal membranes [9,20] and proteomic analysis focusing on hydrogenosomal membrane proteins (HMPs) have identified additional components of the translocases of the outer and inner mitochondrial membrane (TOMs and TIMs, respectively; [36]). Multiple paralogs of the core component TOM40 and uniquely modified small TIM chaperones were detected. Other HMPs characterized include HMP35, a potential pore forming protein of the porin family, whose exact function remains unknown [11]. The only other identified metabolite carriers of *Trichomonas* are five genes belonging to the otherwise much larger family of mitochondrial carrier proteins (MCF; [36]). One MCF protein, HMP31, was characterized in detail and phylogenetic analysis and heterologous studies in yeast mitochondria demonstrated the protein to be an ATP/ADP carrier [10,41].

Information about protein and substrate transporters in *Trichomonas* is still limited. Functional analysis of novel components is often hampered by the many paralogous copies of a gene that can be present due to genome duplication events [6]. The lack of tools to genetically manipulate *Trichomonas* further complicates matters. The knockout of a gene through homologous

* Corresponding author. Address: Institute of Molecular Evolution, Heinrich-Heine-Universität Düsseldorf, Universitätsstr. 1, D-40225 Düsseldorf, Germany. Fax: +49 211 81 13554.
E-mail address: gould@hhu.de (S.B. Gould).
[1] These two authors contributed equally to the manuscript.

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recombination has only been reported once, for a hydrogenosomal ferredoxin [22]. The knockout produced no detectable phenotype, most likely because at least six potentially redundant copies of ferredoxin were later found encoded when the genome was sequenced [6]. The knockdown technique using antisense RNA generally seems feasible in *Trichomonas*, as indicated by two separate reports [28,30]. These however also remain isolated, suggesting this technique, too, has its limits.

To revisit the possibility of insertional gene deletion in *T. vaginalis* and in particular to determine whether the knockout of a gene results in the transcriptional up-regulation of its paralogous copies, we focused on *Tv*HMP23 (TVAG_455090). This protein was found to be among the most abundant proteins of the hydrogenosomal membranes [36], its function however remains unknown. Through homologous recombination we successfully knocked out *Tv*hmp23 and characterize the first knockout-induced phenotype reported for the parasite.

2. Materials and methods

2.1. Culturing and cloning

T. vaginalis strain T1 (JH Tai, Institute of Biomedical Sciences, Taipei, Taiwan) was cultivated in TYM medium at 37 °C as described previously [14]. *Tv*HMP23 (TVAG_455090) was tagged with a dihemagglutinin-tag (HA-tag) at the amino- and at the carboxy-terminus independently into pTagVag2 [18]. The knockout of the gene was based on homologous recombination. We generated a construct, in which the resistance cassette (Neo^R, with *Kpn*I and *BamH*I restriction sites) was flanked by 1182 base pairs of the 5′ upstream (containing *Sac*II and *Kpn*I restriction sites) and 974 base pairs of the 3′ downstream sequence of the *hmp23* encoding locus (containing *BamH*I and *Apa*I restriction sites). The construct was ligated into the *Sac*II/*Apa*I restricted plasmid pα-SCSB-NEO [7]. All constructs were verified through sequencing before transfecting *T. vaginalis* T1 as described previously [7,22] with 50 μg plasmid DNA for localization studies and with 100 μg of *Sac*II and *Apa*I digested plasmid p*Tv*HMP23-NEO. The restriction of the latter plasmid was verified through gel analysis prior to transfection. A list of all primers used is found in the supplementary material (Table S1). Positive transformants were selected for two weeks before further analysis with 100 μg/ml G418 (Roth) in TYM medium for pTagVag2 transfected cells and with 80 μg/ml G418 for p*Tv*HMP23-NEO transfected cells. Cell density was determined using a hemocytometer Thoma New (Superior-Marienfeld). Counts were made of quadruple samples from each strain, and 12 ml of TYM medium (w/o G418) were initially inoculated with 20000 cells.

2.2. Localization analysis

Hydrogenosomes of *T. vaginalis* were isolated as described by Bradley and colleagues [4] with slight modifications [23,34]. All centrifugations were performed at 4 °C. *T. vaginalis* cells were lysed by grinding them in a mortar for 20 min on ice with glass beads (∅ 4–6 μm). Cell lysis was verified through microscopic examination. Glass beads were removed through centrifugation at 755×*g* for 10 min and crude cell lysate obtained from the supernatant. A cytosolic fraction was collected from the supernatant after subsequent centrifugation at 7500×*g* for 10 min of the initial lysate. Hydrogenosomes were separated from the remaining cell components by isopycnic centrifugation with 45% (v/v) Percoll. To separate hydrogenosomal membranes from the matrix, 2 mg of hydrogenosomes (wet paste) were incubated with 30 ml 0.1 M Na_2CO_3 pH 11.5 for 45 min on ice, including a 10 s shake every 10 min. The membrane fraction was then pelleted by ultracentrifugation at 208000×*g* for 1 h at 4 °C. To reduce cross contaminations the procedure was repeated for the two individual fractions. 10 ml of the resulting supernatant was precipitated with acetone/TCA (7.5:1) and resuspended in 70 μl resuspension buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS] and 10 μl concentrated Tris solution to neutralize the pH. The membrane pellet was washed with 100 μl ddH_2O and resuspended in 40 μl ddH_2O.

100 μg of each fraction collected were separated by a 12% SDS–PAGE and blotted onto nitrocellulose membranes (Hybond™-C Extra, GE Healthcare). Membranes were blocked for 1 h in TBS [20 mM Tris–HCl pH 7.4, 150 mM NaCl] containing 5% (w/v) dried milk powder and then incubated for 1 h at RT, or over night at 4 °C with mouse anti-HA monoclonal antibody (Sigma) diluted 1:5000 in blocking solution. After three TBS washes the membrane was incubated for 1 h at RT, or over night at 4 °C with goat anti-mouse horseradish peroxidase-conjugated antibody (ImmunoPure, Pierce) diluted 1:10000 in blocking solution. After three TBS washes signals were detected using 3 ml solution A [1.25 mM luminol (Sigma) in 0.1 M Tris–HCl pH 8.6], 300 μl solution B [6 mM para-hydroxycoumaric acid (Sigma) in DMSO], 0.9 μl 30% (v/v) H_2O_2 and Lumi-Film chemiluminescent detection films (Roche). Original blots were stripped as described previously [47] and reprobed with anti-ASCT [42] and treated as above, but using a goat anti-rabbit horseradish peroxidase-conjugated as secondary antibody. Stripping was performed with two 10 min incubations with mild stripping buffer [1.5% glycine, 0.1% (w/v) SDS, 1% (v/v) Tween 20; pH 2.2], 2 × 10 min washes with PBS [0.8% (w/v) NaCl, 0.02% (w/v) KCl, 0.144% Na_2HPO_4, 0.024% (w/v) KH_2PO_4; pH 7.4] and 2 × 5 min washes with TBST (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20). Protease protection assays were performed with freshly isolated hydrogenosomes following a previously established protocol [47].

Immunofluorescent images of the expressed HA-tagged proteins and ASCT (hydrogenosomal matrix marker) were performed using fixed *T. vaginalis* cells and mouse anti-hemagglutinin monoclonal antibody (Sigma–Aldrich) and rabbit anti-ASCT polyclonal antibody [42] as primary antibodies and secondary Alexa Fluor-488 donkey anti-mouse and Alexa Fluor-633 donkey anti-rabbit antibodies (Invitrogen) as described previously [47]. Images were obtained using a LSM 510 Meta confocal laser scanning microscope (Zeiss). For determination of potential transmembrane-spanning regions using TMHMM, HMMTOP and TMPred, the default settings were used.

2.3. Isoelectric focusing (IEF) and SDS–PAGE electrophoresis

IEF, equilibration and SDS–PAGE electrophoresis were performed as described by Pütz and colleagues [35]. IEF was run on the Ettan IPGphor 3 system (GE Healthcare) using an 18 cm immobilized pH gradient (IPG) (Immobiline DryStrip, GE Healthcare) with a linear gradient pH 6–11, followed by a standard 2nd dimension SDS–PAGE using a 12% gel. Protein spots were picked from gels and tryptic digested in-gel. Eluted protein spots were digested in-gel with trypsin (Promega) as described previously [19]. Peptides were sequenced through mass spectrometry at the BMFZ (HHU Düsseldorf) and a MASCOT search used to identify the proteins [33].

2.4. Transmission electron microscopy

T. vaginalis cells were pelleted at 1000×*g* for 10 min and washed three times with PBS [0.8% (w/v) NaCl, 0.02% (w/v) KCl, 0.144% Na_2HPO_4, 0.024% (w/v) KH_2PO_4; pH 7.4]. After fixation over night at 4 °C in fixation buffer [2.5% (v/v) glutaraldehyde in 0.1 M Na-cacodylate buffer pH 7.3] the cells were washed four times for 10 min with 0.1 M Na-cacodylate buffer pH 7.3. Post fixation was done within two hours incubation with 2% (w/v) osmiumtetroxide diluted in

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0.1 M Na-cacodylate buffer pH 7.3 containing 0.8% (w/v) potassium ferricyanide III. The cells were washed again four times like before and were then dehydrated by incubation with 50% (v/v), 70% (v/v), 80% (v/v), 90% (v/v) and absolute acetone (15 min each). Impregnation was done over night in 1:1 acetone–epon mixture. The samples were polymerized in pure epon within 48 h at 60 °C. Ultra thin sections of embedded samples were collected on formvar coated nickel grids (400 square mesh) and contrasted with subsequent incubation with saturated uranyl acetate solution and with 1% lead citrate for 5 min. Pictures were obtained using either the electron microscope JEOL 2100 TEM operated at 80 kV in combination with a fast-scan 2K × 2K CCD camera F214 (TVIPS, Gauting) or a Zeiss CEM 902 operated at 80 kV equipped with a wide-angle Dual Speed 2K CCD camera (TRS, Moorenweis Germany).

2.5. qPCR

Total RNA was isolated from *T. vaginalis* T1 wild type cells and Δ*Tv*HMP23 using TRIzol® (Invitrogen) and and DNA using DNAzol®, in both cases following the manufacture's protocol. Isolated RNA was additionally DNase-treated (Fermentas) and cDNA synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad) and each time using exactly 1 μg of total RNA. Primer functionality was tested using genomic DNA from *T. vaginalis* as a template. Each qPCR reaction had a volume of 10 μl, containing 1 μl cDNA, 5 μl 2 × Mastermix Power SYBR® Green (Applied Biosystems) and 100 nm of each primer (Table S1). We normalized against the expression of 40S ribosomal protein S5 (TVAG_163210) from wild type and Δ*Tv*HMP23 cells and used biological, as well as technical triplicates.

3. Results

3.1. The TvHMP23 gene family

The hydrogenosomal membrane protein *Tv*HMP23 (TVAG_455090) is predicted to have a molecular mass of 22.6 kDa and depending on the prediction program used, to contain a minimum of two membrane spanning regions (Table 1). Four paralogous copies are listed for the gene at TrichDB (orthologous group OG5_174446) and, like *Tv*HMP23, all are annotated as conserved hypothetical proteins without a known function. Their identities to *Tv*HMP23 vary from 24% to 39% (Table 1). Albeit the amount of predicted α-helical transmembrane domains varies between one and six for all members, only two potential membrane-spanning regions continuously receive high scores among all *Tv*HMP23 paralogs. One of these two transmembrane domains also represents the region with the highest sequence similarity (Fig. S1). A simple BLAST-based search retrieved no homologous proteins from the databases. A more detailed comparison with yeast proteins of the mitochondrial carrier family for example, and the known ATP/ADP carrier *Tv*HMP31 of *T. vaginalis*, demonstrates the paralogous group of *Tv*HMP23 clusters separately without any significant similarity to the proteins analyzed (Fig. S2).

3.2. TvHMP23 localizes to the inner hydrogenosomal membrane

*Tv*HMP23 does not harbor the characteristic hydrogenosomal targeting signal found at the N-terminus of approximately 250 hydrogenosomal proteins [5,6,47]. Two independent proteomic surveys of the hydrogenosome did however identify the protein to be hydrogenosomal [36,37]. To verify and furthermore determine the sub-organellar localization, we tagged the protein at both termini with a di-hemagglutinin (HA–) tag. The C-terminal tagged protein (*Tv*HMP23C) located exclusively to the hydrogenosomal membrane fraction, while the N-terminal tagged construct (*Tv*HMP23N) was found equally distributed between the membrane and matrix fraction (Fig. 1A). These results were confirmed through immunofluorescent localization, in which we observed *Tv*HMP23C to form a fluorescent ring around the matrix marker ASCT (acetate:succinate CoA-transferase), whereas *Tv*HMP23N was found to co-localize with ASCT also in a punctuate pattern typical for hydrogenosomal matrix proteins (Fig. 1B). Protease protection assays furthermore demonstrated that both *Tv*HMP23N and *Tv*HMP23C were not only associated with the hydrogenosomal organelle, but to have passed the outer hydrogenosomal membrane, as proteinase K added to the isolated organelle did not lead to degradation unless Triton X-100 was added (Fig. 1A).

3.3. Knockout of Tvhmp23 increases the size of hydrogenosomes

The knockout of *Tv*hmp23 was based on classic homologous recombination, for which a neomycin resistance cassette was flanked with 1182 base pairs of the 5′ upstream and 974 base pairs of the 3′ downstream sequence of the *Tv*hmp23 encoding locus. Only after reducing the amount of the selective drug G418 from the usually used 100 μg/ml down to 80 μg/ml for initial selection, resistant clones that expressed the Neo^R resistance gene under the control of the *Tv*hmp23 promoter were obtained. To verify the knockout of *Tv*hmp23 and correct genomic integration of the Neo^R resistance cassette, we performed a set of PCR-based controls. The forward primer to check for 5′ integration (HK5F) and the reverse primer to check for 3′ integration (HK3R) bind to areas adjacent to the affected locus and will not amplify a product unless the resistance cassette was integrated into the genome. The PCRs confirmed that the *Tv*hmp23 gene had been correctly replaced by Neo^R resistance cassette and that the construct had integrated into the genomic background as planned (Fig. 2A). Hydrogenosomes from both the wild type and the knockout line were then isolated to screen for potential differences among the protein contents of the two samples, using two-dimensional gel electrophoresis. As expected the spot for *Tv*HMP23 – as determined by protein sequencing – was missing (Fig. 2B). However apart from this difference, we did not notice any other significant and reproducible change.

Ultrastructural analysis using transmission electron microscopy did reveal a change. From cross-sections of Δ*Tv*HMP23 it was apparent that the hydrogenosomes had a larger diameter when compared to wild type cells (Fig. 3A). With an average cross-sectional area of

Table 1
*Tv*HMP23 and its paralogs. For *Tv*HMP23 (TVAG_455090) four paralogous copies are listed at TrichDB (orthologous group OG5_174446). Shown are the predicted masses (in kilodaltons), the isoelectric focusing points (pI, pH), sequence identities and *E*-values of the paralogs. Three transmembrane prediction programs (TMHMM, HMMTOP and TMPred) all predict a different amount of transmembrane domains for the individual genes.

TrichDB ID	Mass (kDa)	pI	Sequence	*E*-value	TMHMM	HMMTOP	TMPred
TVAG_455090	22.6	10.2	–	–	2	6	5
TVAG_485120	23.6	9.3	39%	1.00E-37	3	5	4
TVAG_077910	23.6	10.4	29%	1.30E-21	4	5	4
TVAG_423530	23.2	6.9	29%	3.10E-20	1	2	6
TVAG_087280	24.5	8.8	24%	2.00E-16	2	3	6

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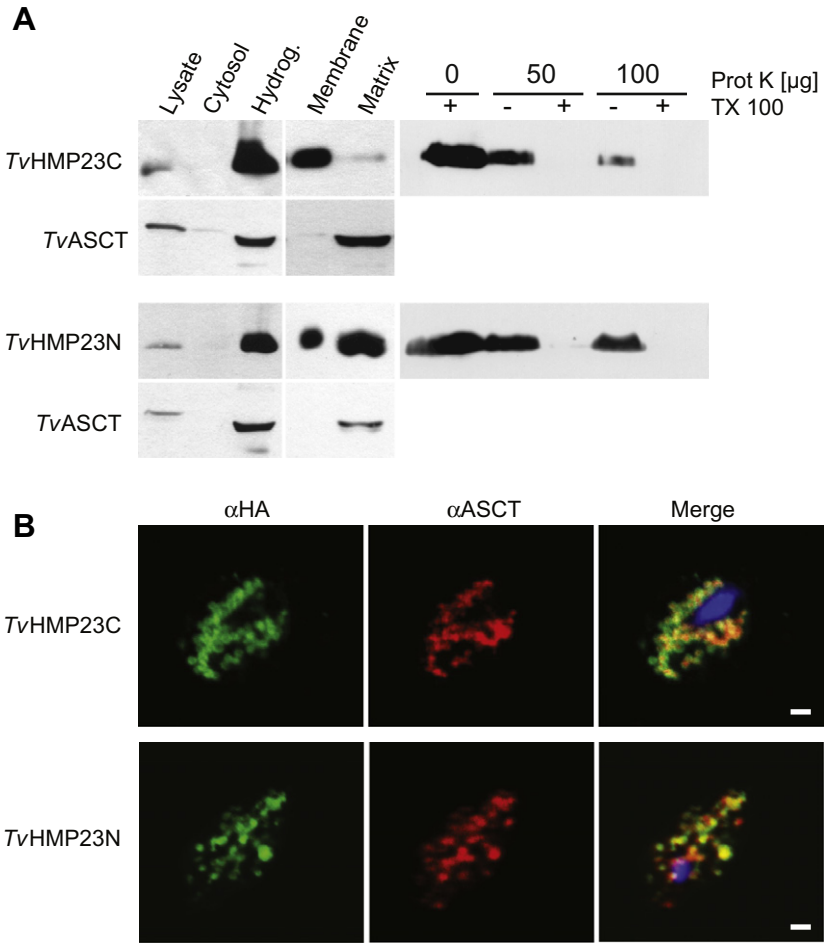

Fig. 1. *Tv*HMP23 localizes to the inner hydrogenosomal membrane. Both the N-terminal and C-terminal tagged *Tv*HMP23 (*Tv*HMP23N and *Tv*HMP23C, respectively) localize to the hydrogenosome, but whereas *Tv*HMP23C localized almost exclusively to the membrane fraction, *Tv*HMP23N was also found enriched in the matrix. As a control the blots were stripped and reprobed with an antibody against the hydrogenosomal marker protein acetate:succinate CoA transferase (ASCT). *Tv*HMP23N and *Tv*HMP23C are protected against Proteinase K (Prot K) and proteolysis only occurred, if Triton X-100 (TX 100) was additionally added. (B) Immunofluorescent co-localization of ASCT with *Tv*HMP23N and *Tv*HMP23C confirms the western blot result and shows only *Tv*HMP23C to form a ring around the matrix localized ASCT. Scale: 1 µm.

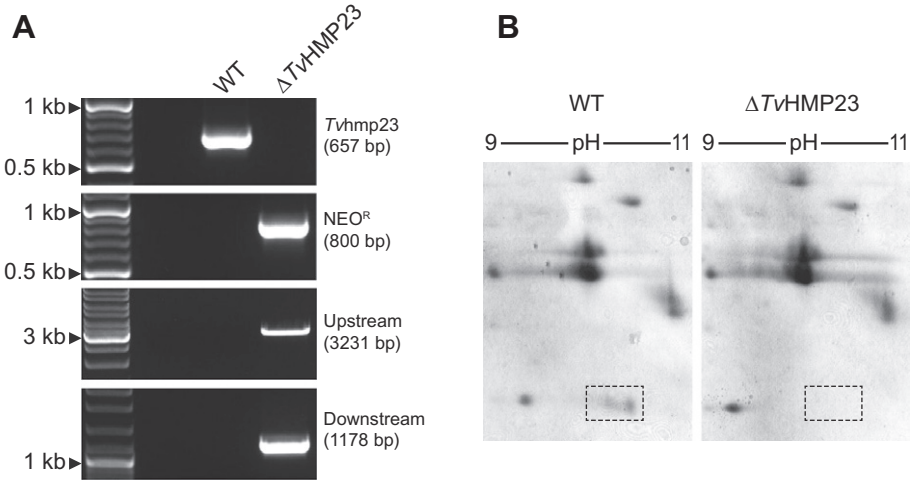

Fig. 2. Gene knockout of *Tv*hmp23. (A) Through PCR the *Tv*hmp23 gene was only detectable in the wild type genomic DNA (gDNA), while the resistance cassette (Neo^R) was only present in the knockout line. To further verify the insertion of the resistance construct into the correct genomic locus we amplified the upstream and downstream region of the *Tv*hmp23 gene using a specific set of primers, which demonstrated the correct replacement of the *Tv*hmp23 gene locus through the Neo^R cassette. Expected fragment sizes in parentheses. (B) Details of two-dimensional gel electrophoresis using protein extract of hydrogenosomes purified from wild type and knockout cells. A spot identified through protein sequencing to be *Tv*HMP23 (dotted square) was only detected in the wild type.

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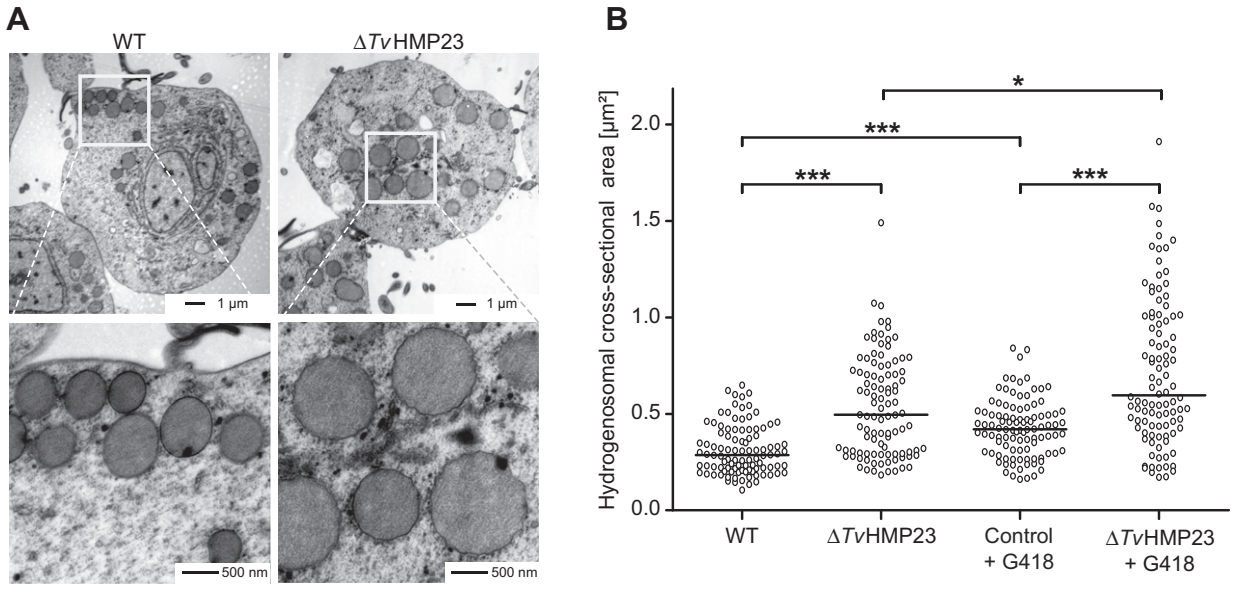

Fig. 3. Knockout of *Tv*hmp23 causes hydrogenosomal swelling. (A) Transmission electron micrograph of wild type *T. vaginalis* and Δ*Tv*HMP23 cross-sections. Regions of interest (indicated by a white frame) are shown as blow-ups below the original images of whole cells. (B) Scatter plot of cross-sectional areas (µm^2) from hydrogenosomes of the different *T. vaginalis* strains. Each circle represents one measured hydrogenosome. The deletion of *Tv*hmp23 results in a significant increase of the hydrogenosomal cross-sectional area [Kruskal–Wallis test [21] $P < 0{,}0001$; ***], although growth under G418 also causes an increase in size, but not significantly when comparing the knockout line grown in the absence and presence of G418 [Kruskal–Wallis test [21] $P < 0{,}01$; *].

0.50 µm^2, the hydrogenosomes of Δ*Tv*HMP23 were 72% larger than hydrogenosomes of the wild type cells, which had an average cross-sectional area of 0.29 µm^2 (Fig. 3B, Table S2). Assuming the organelle is a perfect round sphere that means the average volume of a hydrogenosome in Δ*Tv*HMP23 is 2.3 times that of a wild type hydrogenosome (0.266 vs. 0.117 µm^3). To exclude that the observed phenotype was induced through the selective drug G418 alone, as drug-induced size shifts were previously observed to occur [2], we cultured Δ*Tv*HMP23 in the absence of G418. As an additional control we analyzed the hydrogenosomes of cells resistant to G418 due to the transfection with a plasmid conveying resistance. We found that G418 does increase hydrogenosomal size in the controls by approximately 20–40%, but the further increase in size due to the knockout of *Tv*hmp23 in the wild type strain remains significant (Fig. 3B). Apart from the size difference we did not observe any obvious ultrastructural difference (Fig. 3A), and the knockout of *Tv*hmp23 had no effect on the duplication rate of the parasite (Fig. S3).

3.4. Expression of TvHMP23 paralogs is not up regulated in ΔTvHMP23

As mentioned above the orthologous group of *Tv*HMP23 contains four additional members. To test whether the knockout of *Tv*hmp23 might be compensated by the transcriptional up-regulation of the paralogous genes we performed reverse-transcriptase PCR (RT-PCR) and quantitative real-time PCR (qPCR). RT-PCR showed that only two of the paralogous copies (TVAG_423530 and TVAG_087280) were transcribed, while for the other two (TVAG_485120 and TVAG_077910) no transcript was detected, neither among RNA of wild type cells, nor among RNA isolated from Δ*Tv*HMP23 (Fig. 4A). The qPCR on the two paralogous copies TVAG_423530 and TVAG_087280 then revealed that their transcription level was only marginally, but not significantly (which is generally considered to be at least twofold), up-regulated in the Δ*Tv*HMP23 strain (Fig. 4B). The $\Delta\Delta C_t$ values were 1.09 ± 0.13 for TVAG_423530 and 1.29 ± 0.24 for TVAG_087280. These results support the two-dimensional gel electrophoresis results mentioned above (Fig. 2B), in which we did not observe a noticeable change in protein content among the Δ*Tv*HMP23 sample, also not within the area where TVAG_423530 and TVAG_087280 would be expected to migrate based on their predicted mass and isoelectric focusing point (Table 1).

4. Discussion

The genome of *T. vaginalis* is characterized by the majority of genes being present in multiple copies, with some gene families consisting of hundreds of members [6]. The potentially main protein translocator of the outer hydrogenosomal membrane TOM40 for example, a homolog of the yeast TOM40, is encoded by at least six paralogous copies, all of which are expressed and localize to the hydrogenosomal membranes [20,36,47]. The presence of many paralogs is why the only report that documents the knockout of a gene (ferredoxin) in *Trichomonas* so far, did not lead to a phenotypic effect [22]. Either the knockout of the hydrogenosomal ferredoxin had no effect, because the gene was not essential for viability – which appears unlikely considering its core function in energy metabolism – or its function was compensated by the minimum of six redundant ferredoxin copies present in the genome.

In comparison to the better-studied mitochondria of yeast for example, the biochemical complexity of hydrogenosomes is reduced [40]. Yet many hundred proteins, ions and metabolites still need to be exchanged between the *Trichomonas* cytosol and the hydrogenosomal matrix. Pyruvate, end product of the cytosolic glycolysis [26], and malate are imported into the hydrogenosomes as a source for the fermentative ATP-production. Exported end products of hydrogenosomal metabolism include H_2, CO_2, acetate and one mol of ATP per mol of pyruvate turned over. In addition to ATP synthesis, the assembly of FeS-clusters [38,39] and an amino acid and polyamine metabolism [6,24,46] are localized within the hydrogenosomal organelle. Little remains known about the membrane composition of the two hydrogenosomal membranes of *T. vaginalis*. Although a proteomic survey of the hydrogenosomal membrane fraction uncovered many new proteins [36], the function of the majority of hydrogenosomal membrane proteins remains unknown. Empirical evidence for the molecular function of

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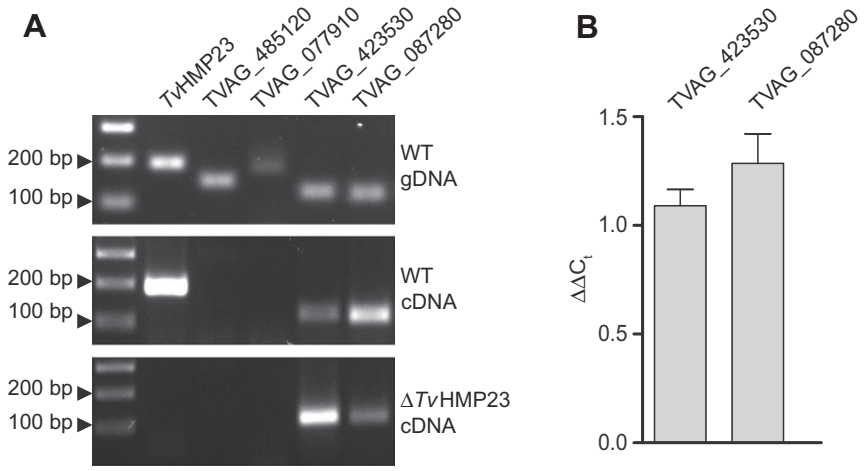

Fig. 4. Expression of Tvhmp23 and its paralogs. (A) *Tv*hmp23 and all four paralogs were amplifiable from genomic DNA (gDNA; primer control PCR). While TVAG_423530 and TVAG_087280 could be amplified also from RNA (cDNA) samples of the wild type and knockout line, the remaining two homologs could not. (B) Resulting $\Delta\Delta C_t$ values for TVAG_423530 and TVAG_087280 from the qPCR using cDNA of the Δ*Tv*HMP23 strain. Both genes were not significantly higher expressed in Δ*Tv*HMP23 cells when compared to the wild type level.

a hydrogenosomal membrane protein has only been obtained for a single ATP/ADP carrier protein (HMP31; [10,41]), while for a handful of others – mainly components of the protein import machinery – their predicted function is based on homology only.

*Tv*HMP23 might represent an extremely distant relative of the MCF family, which serves to translocate an unknown substrate. Protease protection assays suggest the protein to be located in the inner hydrogenosomal membrane as both tagged termini were protected from proteolysis (Fig. 1A). This is consistent with the prediction of α-helical and not β-sheet based transmembrane domains. Although α-helical-based transmembrane containing proteins were identified in both the inner and in the outer mitochondrial membrane [31], outer mitochondrial membrane proteins are usually embedded into the membrane through barrel forming β-sheets [45] and the aid of the SAM50 complex [1], which is encoded by *T. vaginalis*. However, significant sequence similarity to any known translocators of mitochondria could not be identified, and hence the function of *Tv*HMP23 remains unknown.

2The targeting of *Tv*HMP23 is intriguing, too. *Tv*HMP23 does not contain a canonical N-terminal targeting sequence as previously defined for more than 200 putative hydrogenosomal proteins [5,6,47]. This indicates *Tv*HMP23 contains an internal targeting motif, and which is in line with what is known for yeast metabolite carriers of the inner mitochondrial membrane [1]. Further, while the C-terminal tag did not disturb the integration into the inner membrane, N-terminal tagging of *Tv*HMP23 hampered correct targeting. The majority of *Tv*HMP23N was found to remain in the hydrogenosomal matrix (Fig. 1), which suggests N-terminal tagging in this case likely disturbs the correct recognition by the TIM, but not TOM complex.

We were able to knockout *Tv*hmp23 through homologous recombination, only the second time an insertional knockout has been reported for *T. vaginalis*. Successful gene replacement of *Tv*hmp23 through the resistance cassette was verified by PCR (Fig. 2A) and neither *Tv*hmp23 transcript (Fig. 4A) nor *Tv*HMP23 protein (Fig. 2B) was detected in the knockout strain. Knockout of one of the most highly abundant hydrogenosomal membrane proteins had no significant effect on the parasite's viability under the culture conditions tested, but led to a significant increase in hydrogenosomal size. Hydrogenosomes, which usually range between 200 to 500 nm in diameter, can reach up to 2 μm under stress conditions [3,29]. Albeit the loss of *Tv*HMP23 induces stress resulting in larger hydrogenosomes, it does not result in a growth deficit under the conditions tested (Fig. S3). This demonstrates an unknown mechanism must compensate for the loss of one of the most abundant hydrogenosomal membrane proteins.

The knockout of *Tv*HMP23 did not result in a significant up-regulation of the paralogous genes TVAG_087280 and TVAG_423530, for which expression evidence exists, and that would help to explain why a hydrogenosomal phenotype, but no growth deficit was observed. TVAG_087280 and TVAG_423530 might fulfill a similar role as *Tv*HMP23, albeit less efficiently. If then there is a fixed dependency in hydrogenosomes between the surface area and the amount of a certain membrane protein that can be present, the inability to up-regulate paralogous copies might require more membrane area to allow for more TVAG_087280 and TVAG_423530 to be present. Alternatively, metabolic reactions that increase the size of the hydrogenosomes could also compensate the knockout, something that in yeast was shown to occur [16,32,43]. Still, hydrogenosomal swelling could simply be the effect of accumulated metabolites or a change in osmotic pressure. As hydrogenosomal swelling was observed to occur as to compensate stress conditions induced through a variety of different drug treatments [2,3], this kind of phenotype does however suggest that it is linked to an altered metabolic process. In the present case, Δ*Tv*HMP23 hydrogenosomes were significantly larger than wild type hydrogenosomes (Fig 3).

Genome biology of *Trichomonas* is characterized by the several duplications of at least parts of the genome, with some gene families reaching many hundred paralogs per group [6]. Although the knockout of one gene within a paralogous group does not necessarily result in the transcriptional up-regulation of the paralogous copies, their basic transcription might suffice to, at least in parts, compensate the loss of a gene. This reflects the complex situation present in *Trichomonas* and shows that albeit reverse genetics remains a substantial challenge in this parasite, knockouts are generally feasible and can aid in the analysis of the molecular function of a protein in *T. vaginalis*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.03.001.

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