# The C-terminal tail of tetraspaninproteinsregulates their intracellular distribution in the parasite*Trichomonasvaginalis*

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## ABSTRACT

The parasite *Trichomonasvaginalis* is the causative agent of trichomoniasis, a prevalent sexually transmitted infection. Here, we report the cellular analysis of the *T. vaginalis*tetraspanin family (TvTSPs). This family of membrane proteins has been implicated in cell adhesion, migration and proliferation in vertebrates. Here we report that the expression of several members of the family is up-regulated upon contact with vaginal ectocervical cells (VECs). We demonstrate that most

TvTSPs are localized on the surface and intracellular vesicles and that theC-terminal intracellular tails of surface TvTSPs are necessary for proper localization. Analyses of full length TvTSP8 and a mutant that lacks the C-terminal tail indicates that surface localized TvTSP8 is involved in parasite aggregation, suggesting a role for this protein in parasite: parasite interaction.

## INTRODUCTION

Sexually transmitted infections (STI) and their sequelae are among the top five reasons that people in developing countries seek medical treatment (Cudmore *et al.*, 2010). The World Health Organization (WHO) estimates that there are about 499 million cases of curable STI annually and 276 million are of trichomoniasis, making infection with *Trichomonasvaginalis* the most common non-viral STI in the world(WHO, 2008).

*T.vaginalis* is anextracellular parasite that lives in the urogenital tract in men and women.Although infections may be asymptomatic, when symptoms do occur they are generally mild, manifesting as general irritation,vaginitis, urethritis, and prostatitis.In recent years, this pathogen has been recognized to have significant implications for global public health. The infection has been associated with serious consequences, such as adversepregnancy outcomes and preterm birth, infertility, pelvic inflammatory disease(Swygard *et al.*, 2004, Fichorova, 2009) and predisposition to cervical and prostatecancer(Gander *et al.*, 2009, Stark *et al.*, 2009, Sutcliffe *et al.*, 2009, Twu *et al.*, 2014). Importantly,trichomoniasis has also emerged as a cofactor in human immunodeficiency virus (HIV) transmission and acquisition(McClelland *et al.*, 2007, Van Der Pol *et al.*, 2008). These severe complications and high incidence of infection underscore the need to identify new drug targets and to advance vaccine development.

Understanding the mechanisms by which *T. vaginalis* colonizes the host is central to developing strategies to prevent infection. Despite the prevalence of trichomoniasis, the underlying biochemical processes that lead to pathogenesis are poorly defined (Fiori *et al.*, 1999, Hirt *et al.*, 2011, Ryan *et al.*, 2011). As an extracellular organism, surface proteins are likely to play important roles in the initial adherence to mucosal tissue as well as the long-term survival of the pathogen on mucosal surfaces.A family of cell surface proteins that have yet to be fully examined in *T. vaginalis* but are good candidates for playing important roles in parasite biology are the tetraspanins(TvTSPs). These cell surface proteins are involved in the coordination of

intracellular and intercellular processes, including signal transduction, cell proliferation, adhesion, migration, cell fusion and host-parasiteinteractions by functioning as organizers of multimolecular membrane complexes, termed tetraspanin-enriched microdomains(Hemler, 2003, Pols *et al.*, 2009).The extensive spectrum of biological functions in which TSPs involvement has been implicated is compatible with their wide distribution in multiple cell types and organisms, and indicates their functional importance. However, although TSPs are expressed in a wide range of organisms, the only sequenced parasitic protist genomes that contain TSP genes are those of *T. vaginalis* and *Entamoebahistolytica*. This suggests that TSPs may be specifically involved in the pathogenesis of a subgroup of extracellular mucosal parasites.

The common tetraspaninstructure consists of four transmembrane (TM) domains,two extracellular loops and two short cytoplasmic tails.Distinct TSPs structural domains are associated with specific functions. In mammalian cells, a 'transmembrane linker' model for TSPs has been proposed (Hemler, 1998). In this model, extracellular domains link to integrins, whereas cytoplasmic domains link to intracellular signaling enzymes such as phosphatidylinositol 4-kinase and PKC (Hemler, 1998, Yauch *et al.*, 2000, Zhang *et al.*, 2001). Whereas the functional importance of tetraspanin large extracellular loops (EC2) is well recognized, the C-terminal tails have received less attention. A mutation in the CD151 C-terminal tail markedly altered α6integrin-dependent cell spreading, cellular cable formation functions, and adhesion strengthening (Zhang *et al.*, 2002, Lammerding *et al.*, 2003)and the YRSL sequence was found to be critical for intracellular trafficking and function (Liu *et al.*, 2007). As in other cells, our previous work (de Miguel *et al.*, 2012) indicated that a 16-amino-acid C-terminal intracellular tail of *T. vaginalis* TSP6 is necessary and sufficient for flagellar localization and protein redistribution. However, essentially nothing is known about the function and biochemistry of the C-terminal tails of the other members of the TvTSP family.

Here we have characterized the complete family of *T. vaginalis* TSPs and analyzed the role of their C-terminal tail in protein localization. Interestingly, using TvTSP8 mutant parasites, we found that TvTSP8 is involved in parasite aggregation suggesting a role for this protein inparasite: parasite communication.

## RESULTS

### *T. vaginalis*contains eight predicted Tetraspanin family members

Tetraspanins are transmembrane proteins that are defined by their similarity in size (20–30 kDa protein core), topology (small and large outer loops, short N-terminal and C-terminal tails, four transmembrane domains) and shared structural features (Fig. 1A). Bioinformatics analysis retrieve 39 sequences from the *T. vaginalis* genome (http://www.trichdb.org) that are predicted to be related to the TSP family(Table 2.S). Of these,only twelve possess the characteristic four typical transmembrane domains and ~~the tetraspanin domain~~ the key residues of a member of the tetraspanin familiy, PF00335, in Pfam data base (http://pfam.xfam.org/)~~FAM~~(Table 2.S). Our previous results analyzed two different TSPs (TvTSP1 and TvTSP6) (de Miguel *et al.*, 2012, Twu, 2013) that belong to the same paralog group (OG5_142422) (Table 2.S). Based on the importance of the previous analyzed genes, we now decided to characterize the complete OG5_142422 group. Of these, two sequences (TVAG_552380 and TVAG_485160) are predicted to have only one transmembrane domain and do not share any of the classical structural features of the TSPs family, hence,are not considered bonafide TSP genes. Additionally, TVAG_458280 (TvTSP4) and TVAG_050030 sequences are identical. Although we could not identify the tetraspanin ~~PF00335~~ domain ~~in~~ with the ~~PFAM~~ Pfam domain searcher for TvTSP7 (TVAG_446110), we decided to include it in our analysis due to the presence of CCG, four transmembrane domains and signal peptide. Therefore, our experimental analysis focused on eight putative *T. vaginalis*TSP family members (Fig. 1.B.).The similarity between these eight analyzed genes vary substantially with the most similar sharing 60.7% identity and 74.4% similarity in primary amino acid sequence (TvTSP2 and TvTSP5). The most divergent of the eight TvTSP genes are 23.2% similar and 12.1% identical (TvTSP3 and TvTSP7).

All the analyzedTvTSPs contain a C-terminal tail that is short and non-conserved among members of the family(Fig. 1B).The larger extracellular loop contains the CCG motif, a hallmark of the tetraspanin family (Seigneuret et al., 2001). Notably, the signature CCG classical motif in the large extracellular loop (LEL) is replaced by CCS in TvTSP6 (Fig. 1.B). Additionally, the classical asparagine (N)residue contained in the first transmembrane domain (ref berditchvsky 2013 p47) is conserved in all analyzed TvTSPs with the exception of TvTSP7 and the glutamine (Q) or Glutamic acid (E) residues from the third and fourth transmembrane domain(ref

berditchvsky 2013 p47), are also conserved in most of TvTSPs analyzed (Fig 1.B). Finally, the length of the EC1 loop is in the range of what was previously described for other TSPs (Fig. 1.A) (ref libro 2013 p47), ranging from 13 to 19 amino. Differing, the EC2 loop generally contains 76–131 amino acids (ref), however, the EC2 of TvTSPs range from 48 to 71 amino acids.

To explore phylogenetic relationship among tetraspanins in different species, a phylogenetictree was constructed including tetraspanins from *D. melanogaster*, *C. elegans, Homo sapiens*, *Entamoeba histolytica,* among others. We observed that TvTSPs cluster all together derived probably from local duplications. Interestingly, the TvTSPs grouped closer to TSPs from chordates than the correspondent in amoebas; as was predicted.

**Most TSPs members analyzed localize to the cell surface of*T. vaginalis***

Our previous analyses have shown that two members of the TvTSPfamily (TvTSP1, and TvTSP6)reside on the surface (plasma membrane and flagella) and internally on membranes of intracellular vesicles (de Miguel *et al.*, 2012, Twu *et al.*, 2013). To examine the subcellular localization of additional members of the *T. vaginalis*TSP family, their genes were cloned and expressed under the control of the α-SCS promoter as a C-terminally haemagglutinin (HA)-tagged fusionprotein in *T. vaginalis*strain B7RC2. We then determined the localization of the tagged protein in transfected cells using immunofluorescence assays. TvTSP2, TvTSP3, TvTSP5 and TvTSP8show a strong signal on the plasma membrane(Fig. 3), as previously described for TvTSP1 and TvTSP6 (21, 25), Unlike that observed for TvTSP2 and TvTSP3, TvTSP5 and TvTSP8 were also found to localize strongly to intracellular vesicles (Fig. 3).In contrast,TvTSP4 signal was observed around the nuclei(Fig.3). Repeated attempts to express TvTSP7 as a recombinant HA-tagged protein were not successful (data not shown); therefore no further analyses of this gene were pursued.

**TvTSPs, with the exception of TvTSP2, are up-regulated upon binding of the parasite to host cells**

*T. vaginalis*is an extracellular parasite thatadheres to vaginal epithelial cells to colonize its human host.Several observations suggest that Trichomonas surface proteins are involved in attachment to host cells (Alderete *et al.*, 1985, Crouch *et al.*, 1999).In this context, the kinetics of expression of the surface localizedmembers of the family was examined by qPCRduring theexposure to VECs. With the exception of TvTSP2, all the TvTSPswere found to be up-

regulated upon contact with VECs (Fig.4). Interestingly, TvTSP1 is the most abundant member of the family(de Miguel *et al.*, 2010) and the most up-regulated one (Fig.4), increasing ~ 25-fold at 4 h. TvTSP8 expression is also greatly increasedto ~13-fold at 2 h.Interestingly, the peak of expression for all up-regulatedTSPs is around 2-4 h after exposure to VECs. Since *in vitro* attachment of the parasites is mostly completed within ~20 min of exposure (Okumura *et al.*, 2008), the observed pattern of up-regulation suggests that TvTSPsmay play a role in events occurring downstream of adherence.

**The C-terminal cytoplasmic tail of surfaceTvTSPs is required for properlocalization**

Targeting of transmembrane proteins to different compartments of the endocytic and late (post-Golgi) secretory pathways is largely dependent upon sorting signals contained within the cytosolic domains of the proteins (Bonifacino *et al.*, 1999). The C-terminal cytoplasmictailsof mammalian TSPs have been shown to play critical roles in determiningthe functional consequences of ligand binding (Zhang *et al.*, 2002, Latysheva *et al.*, 2006).Additionally, our previous work demonstrated that a 16-amino-acid C-terminal intracellular tail of TvTSP6 is necessary and sufficient for flagellar localization and protein redistribution when the parasite is in contact with VECs(de Miguel *et al.*, 2012). Based on these data, we asked whether the localization of TvTSPs is generally dependent upon information contained within its C-terminal tail. To test this possibility, we generated C-terminal deletion constructs of the surface TSPs family members of *T. vaginalis*, which are identical to full length except for lacking the C-terminal most amino acids listed in Fig. 4.When truncated versions of TvTSP1, TvTSP2, TvTSP3, TvTSP5 and TvTSP8 arestably expressed in *T. vaginalis*a dramaticchange in localization is observedby immunofluorescence microscopy employing an anti-HA tag antibody(Fig.5). TvTSP5ΔCthas a subcellular localization around the nuclei; clearly different to the fulllength version of TSP5. Interestingly, TvTSP2ΔCt, TvTSP3ΔCt, and TvTSP8ΔCtshowan intense intracellular vesicles localization pattern with a faint signal on the plasma membrane (Fig.5).The pattern of TvTSP1ΔCt intracellular vesicles appears to differ relative to the full length protein; however not as dramatically as the other TvTSPΔCt proteins(Fig.5). These datademonstrate that the cytoplasmic tail is necessary for membrane targeting suggesting it contains plasma membrane sorting information.

Given the evidence for reduced signal on the surface and the large size of intracellular vesicles of TvTSPΔCt parasites, we hypothesized that this could be the result of the retention of the protein in vesicles in transit to, or endocyted from, the cell surface and that retention of TSPs could be leading to enlarged vesicles. Cells expressing TvTSP8ΔCt appear to contain particularly large intracellular vesicles by IFA (Fig. 5). Hence, we quantified the amount and size of intracellular vesicles in parasites transfected with TvEpNEO, TvTSP8 and TvTSP8ΔCt using Transmission Electron Microscopy (TEM).The results obtained indicate that no differences in quantity and size frequency of intracellular vesicles size was observed in the three analyzed cell lines (Fig. 2.S.), suggesting that these large intracellular vesicles are normally present in *T. vaginalis.*

**High host cell adherence capacity and exogenous expression of full length TvTSP8 correlates with parasite: parasite aggregation**

We have previously noted that a *T. vaginalis* strain (MSA 1132) with a high capacity to adhere to host cells appeared to aggregate more than the poorly adherent T1 strain(Lustig *et al.*, 2013). As shown in Fig. 6.A, we confirm and extend upon these data by showing thathighly adherent strains B7268 and SD7 strains form clumps in cell culture in contrast to poorly adherent strains T1 and G3. To test whether surface-localized TvTSPs might contribute to parasite aggregation, we examined whether parasites expressing full length TSPs would promote the ability to aggregate of a strain that normally do not form clumps (Fig. 6.B). Unexpectedly, we found that only expression of TvTSP8FL, but not the other TvTSPs, promoted aggregation (Fig.6.B,C). Based on this observation, we examined whether expressing TvTSP8FL, TvTSP8ΔCt and TvEpNEO would promote the ability of non-clumping strain to aggregate. Interestingly, we found expression of surface localized TvTSP8FL but not TvTSP8ΔCt and TvEpNEO promoted aggregation (Fig.7.A,B). These results suggest a role for surface-localized TvTSP8 in parasite aggregation and cell:cell communication. Hence, we determined the expression levels of endogeneous TvTSP8 in the four strains analyzed in Fig. 5A and found that the 2 strains that aggregate have 8 and 10-fold higher levels of TvTSP8 mRNA (Fig. 7.C). A greater abundance ofmRNA expression observed in highly adherent versusless adherent parasites is consistent with an involvement of TvTSP8 in parasite: parasite interaction.

## DISCUSSION

Here, we described the presence of at least 12 TSPs genes in the *T. vaginalis* genome and characterize 8 of them. We found that TvTSPs possess the classical tetraspaninhallmarks, such as four TMs, a small EC1, a large EC2,the tetraspanin signature CCG and conserved cysteines, Interestingly, the signature CCG classical motif in the large extracellular loop (LEL) is replaced by CCS in TvTSP6. Although this feature alone will disqualify it from been called a classical tetraspanin in mammalian cells, sequences lacking the CCG motif are also present in other protozoasuch as *Trypanosoma brucei*(berditchvsky 2013 p 187) ora free-living amoeba such as *Dictyostelium discoideum*but structural analysis justified their identity_(Shengfeng Huang genomics 2005). These results suggest that the tetraspanin signature CCG is not actually100% conserved.

Here, we have also demonstrated that the *Trichomonasvaginalis*TSPs proteins (TvTSPs) generally encode sorting signals in their C-terminal cytoplasmic tails that are important for their correct localization. Usually, membrane proteins are endowed with sorting signals that specify their sites of functional residence (Hunziker *et al.*, 1994, Matter *et al.*, 1994). Presumably, sorting signals are recognized by components of a cell's sorting machinery which act upon the targeting instructions they encode. Here we show that a relatively small truncation of the C-terminal tail of TvTSPs has a major effect on protein localization. TSPs in mammalian systems have been shown behave as membrane scaffolds interacting with a range of extracellular proteins which regulate outside in signaling (Hemler, 2005). As the relocation of TvTSPs is likely to alter its interactions with external protein partners, this could, in turn, alter the ability of TvTSPs to direct signaling and cytoskeletal regulatoryevents such as adhesion strengthening, cell migration, invasion and/or motility.In this sense, it has been shown that the binding between humanSITAC (a PDZ protein) and L6A (Tetraspanin protein) depends on the C-terminal tail of the latter and this binding is required for the precise colocalization of both proteins in the same subcellular compartment (Borrell-Pages *et al.*, 2000). Additionally, the C-terminal tail ofCD63 binds to AP-3 adaptor subunit α3 (Rous *et al.*, 2002)and to a PDZ domain in syntenin-1 (Latysheva *et al.*, 2006), which affects CD63 distribution and trafficking. In concordance, several *Trichomonasvaginalis*TSPs have putative binding motif for proteins containing a PDZ domain (Tonikian *et al.*, 2008)in their C-terminal tail. In general, PDZ domains bind to the very C-

terminus of the cytoplasmic domain of transmembrane proteins and may have a dynamic role in different compartments of the secretory pathway(Fanning *et al.*, 1999)(Borrell-Pages *et al.*, 2000).Taking into account these observations and our results, it is logical to speculate that the change in localization of truncated versions of TvTSPs could be attributed, in some cases, to a disruption of the interaction with PDZ domain containing proteins. However, alternative possibilities are also plausible.

Many tetraspanins are expressed at the plasma membraneand also in the intracellular membranes ofexocytoticand endocytotic vesicles and thus are likely to be involvedin the trafficking of these vesicles both into and outof the cell (Monk *et al.*, 2012).A well-characterized example is CD63, which has been linked to the redistribution of other proteins into secretorycellular compartments(Monk *et al.*, 2012).Using mutagenic analysis, we determinedthat the C-terminal tails of surface TSPs are involved in protein distribution.Specifically, immunofluorescence analysis of permeabilizedcells transfected with TvTSP2, TvTSP3, TVTSP6 (de Miguel *et al.*, 2012)and TvTSP8 truncated versionsshowed accumulation of tagged proteinsin intracellular vesicles. This steady-state distribution of these mutants could be interpreted as the accumulation in different compartments of the secretory pathway, of molecules in transit to, or endocyted from, the cell surface. These results suggest a general role for the C-terminal tail of surface TSPs in determining the proper localization of these proteins.

The change in intracellular localizationis manifested in differences in the ability of parasites to aggregate or clump in TvTSP8 transfected parasites.Whereas over-expression of the surface-localized full-length TvTSP8 promoted increased aggregation, over-expression of the TvTSP8 C-terminal tail mutant (TvTSP8ΔCt) did not. We propose this difference results from TvTSP8ΔCthaving a dominant negative effect on endogenousTvTSP8, similar to that observed upon the deletionof the C-terminal tail of the TSP protein CD151(Zhang *et al.*, 2002) and TvTSP6 (de Miguel *et al.*, 2012). This might be explained by the mutant TvTSP8ΔCt shifting the stoichiometric balance of endogenouswild-type TvTSP8 via the formation of oligomericcomplexes between the two proteins. Thedifference in the amount of TSP8 on the surface of these parasites would then be translated in a reduction in parasite clump formation. The formation of *T. vaginalis* clumps has been noted by several investigators,however, the importance of clumping and the molecular mechanisms behind this phenomenon still remain

puzzling. As previously reported (Lustig *et al.*, 2013) and shown here, clumping is a distinct adhesivephenotype, exhibited by some but not allparasitestrains that correlates with the capacity of the strain to adhere to host cells.Our data indicate that the formation of clumps of*T. vaginalis*may be mediated, at least in part, by TSP8. In concordance with our results, it has been shown that the mammalian tetraspaninn CD9 promotes homotypic cell–cell aggregation and microvilli formation, whereas the C-terminal tail mutant of CD9 did not (Wang *et al.*, 2011). We postulate that the increased clumping capacity of TvTSP8transfected parasites could be attributed, at least in part, due to the increase of TSP8 on the cell surface of these parasites.However, this isonly one of several possible explanations and much additional work will be required to define themechanism underlying the observed parasite clumping in TvTSP8 transfected parasites. The association of clumping with parasite adherence is interesting in the light of emerging evidence suggestingthat aggregation may have a role in the pathology of Trichomonas.Defining the biochemical properties required for adhesive phenotypes of *T. vaginalis*may therefore help us to understand how the parasite colonizes the urogenital tract and how to prevent or treat infections.

## EXPERIMENTAL PROCEDURES

### Parasites, cell culture and media

The *T. vaginalis* strain B7RC2 (PA strain, ATCC 50167) was cultured in Diamond's Trypticase-yeast extract-maltose (TYM) medium supplemented with 10% horse serum and 10 U/ml penicillin/10 ug/ml streptomycin (Invitrogen). Parasites were grown at 37°C and passaged daily. 100 ug/mL G418 (Invitrogen) was added to culture of the TvTSPs-HA transfectants.

The human cervical ectocervical cell line Ect1 E6/E7 (ectocervical, ATCC CRL-2614), referred to as VECs, were grown as previously described (Fichorova *et al.*, 1997) in keratinocyte-SFM complemented with provided recombinant protein supplements, penicillin, streptomycin and cultured at 37°C/5% $CO_2$

### Sequence analysis

Database searches and sequence comparisons were performed using blastn, blastx, and BLAST two-sequence programs (www.ncbi.nlm.nih.gov/BLAST). TSPs predicted sequences were

collected by searching the *T. vaginalis* genome. Multiple alignments of the amino acid sequences were performed using ClustalW in the BioEdit program.

**Phylogenetic analysis**

Tetraspanin protein sequences from different organism were retrieved from NCBI data base (http://www.ncbi.nlm.nih.gov), AmoebaDB (www.amoebaDB.org) and *T. vaginalis* genome (www. trichDB.org). The repeated sequences were removed and the alignmentof 45 TSPs full length protein sequences was performed using MUSCLE algorithm with Guidence (http://guidance.tau.ac.il/). Visualization of the obtained alignment was performed with MEGA5 and the phylogenetic tree was generated using UPGMA method. Bootstrap analysis with 1000 replicates was performed to test the significance of nodes.

**Plasmid construction and exogenous protein expression in *T. vaginalis***

The TvTSP2, TvTSP2ΔCt, TvTSP3, TvTSP3ΔCt, TvTSP4, TvTSP4ΔCt, TvTSP5, TvTSP5ΔCt, TvTSP7, TvTSP7ΔCt, TvTSP8 and TvTSP8ΔCt constructs were generated using primers with NdeI and KpnI restriction sites engineered into the 5′- and 3′-primers respectively (Supplementary Table 1).

PCR fragments were generated using standard procedures and the resulting fragments were then cloned into the Master-Neo-$(HA)_2$ plasmid (Delgadillo *et al.*, 1997) to generate constructs to transfect into *T. vaginalis*. Electroporation of *T. vaginalis* strain B7RC2 was carried out as described previously (Delgadillo *et al.*, 1997) with 50 µg of circular plasmid DNA. Transfectants were selected with 100 mg/ml G418 (Sigma).

**Immunolocalization experiments**

Parasites in the absence of host cells were incubated at 37°C on glass coverslips as previously described (de Miguel *et al.*, 2010) for 4 hours. The parasites were then fixed and permeabilized in cold methanol for 10 min. The cells were washed and blocked with 5% FBS in PBS for 30 min, incubated with a 1:500 dilution of anti-HA primary antibody (Covance, Emeryville, CA, USA) diluted in PBS plus 2%FBS, washed and then incubated with a 1:5000 dilution of Alexa Fluor conjugated secondary antibody (Molecular Probes). The coverslips were mounted onto microscope slips using ProLong Gold antifade reagent with 4′, 6′-diamidino-2-phenylindole

(Invitrogen). All observations were performed on a Nikon E600 epifluorescence microscope. Adobe Photoshop (Adobe Systems) was used for image processing.

**Quantitative PCR (qPCR)**

For exposure to host cells, ~$10^7$ wild type B7RC2 parasites were incubated with vaginal epithelial cells (VECs) for various times as indicated. Unattached parasites were removed and RNA was subsequently prepared from attached parasites and VECs scraped from the plate. Total RNA was treated with amplification grade DNase I (Invitrogen) and reverse transcribed using SuperScript III reverse transcriptase and oligo(dT) primers (Invitrogen). Real-time PCRs were performed using Brilliant SYBR Green qPCR Master Mix (Stratagene), a 150–450 nM concentration of each primer, and 200–500 ng of cDNA in a 20 µl reaction volume using an Eppendorf Mastercycler and realplex v.1.5 (Eppendorf). Parallel reactions performed without reverse transcriptase were included as negative controls. Using data from the exponential phase of the qPCR, threshold cycle (CT) base lines were set according to Eppendorf Mastercycler protocols. Data from different samples were interpolated from standard curves established for each primer set and then normalized against two different housekeeping genes: tubulin and Hmp24. Every experimental and standard curve sample was tested in duplicate in three independent experiments.

**Electron microscopy**

Parasites were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. Cells were post-fixed in 1% OsO4 and 0.8% potassium ferricyanide, dehydrated in acetone series and infiltrated in Epon. Polymerization was carried out for 72 h. Thin sections were collected on copper grids, stained with uranyl acetate and lead citrate, and examined with a Tecnai G2 Spirit (FEI) transmission electron microscope.

**Parasite Aggregation**

Parasite aggregation was analyzed in anaerobic conditions when parasites reach a concentration of $10^6$ parasites/ml using a Nikon E600 epifluorescence microscope with a magnification of 10X. Adobe Photoshop (Adobe Systems) was used for image processing.

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**REFERENCES**

Alderete, J.F. and Garza, G.E. (1985). Specific nature of Trichomonas vaginalis parasitism of host cell surfaces. *Infect Immun***50,** 701-708.

Bonifacino, J.S. and Dell'Angelica, E.C. (1999). Molecular bases for the recognition of tyrosine-based sorting signals. *The Journal of cell biology***145,** 923-926.

Borrell-Pages, M., Fernandez-Larrea, J., Borroto, A., Rojo, F., Baselga, J. and Arribas, J. (2000). The carboxy-terminal cysteine of the tetraspanin L6 antigen is required for its interaction with SITAC, a novel PDZ protein. *Molecular biology of the cell***11,** 4217-4225.

Crouch, M.L. and Alderete, J.F. (1999). Trichomonas vaginalis interactions with fibronectin and laminin. *Microbiology***145 ( Pt 10),** 2835-2843.

Cudmore, S.L. and Garber, G.E. (2010). Prevention or treatment: the benefits of Trichomonas vaginalis vaccine. *J Infect Public Health***3,** 47-53.

de Miguel, N., Lustig, G., Twu, O., Chattopadhyay, A., Wohlschlegel, J.A. and Johnson, P.J. (2010). Proteome analysis of the surface of Trichomonas vaginalis reveals novel proteins and strain-dependent differential expression. *Mol Cell Proteomics***9,** 1554-1566.

de Miguel, N., Riestra, A. and Johnson, P.J. (2012). Reversible association of tetraspanin with Trichomonas vaginalis flagella upon adherence to host cells. *Cell Microbiol***14,** 1797-1807.

Delgadillo, M.G., Liston, D.R., Niazi, K. and Johnson, P.J. (1997). Transient and selectable transformation of the parasitic protist Trichomonas vaginalis. *Proceedings of the National Academy of Sciences of the United States of America***94,** 4716-4720.

Fanning, A.S. and Anderson, J.M. (1999). PDZ domains: fundamental building blocks in the organization of protein complexes at the plasma membrane. *The Journal of clinical investigation***103,** 767-772.

Fichorova, R.N. (2009). Impact of T. vaginalis infection on innate immune responses and reproductive outcome. *J Reprod Immunol***83,** 185-189.

Fichorova, R.N., Rheinwald, J.G. and Anderson, D.J. (1997). Generation of papillomavirus-immortalized cell lines from normal human ectocervical, endocervical, and vaginal epithelium that maintain expression of tissue-specific differentiation proteins. *Biology of reproduction***57,** 847-855.

Fiori, P.L., Rappelli, P. and Addis, M.F. (1999). The flagellated parasite Trichomonas vaginalis: new insights into cytopathogenicity mechanisms. *Microbes and infection / Institut Pasteur***1,** 149-156.
Gander, S., Scholten, V., Osswald, I., Sutton, M. and van Wylick, R. (2009). Cervical dysplasia and associated risk factors in a juvenile detainee population. *Journal of pediatric and adolescent gynecology***22,** 351-355.
Hemler, M.E. (1998). Integrin associated proteins. *Curr Opin Cell Biol***10,** 578-585.
Hemler, M.E. (2003). Tetraspanin proteins mediate cellular penetration, invasion, and fusion events and define a novel type of membrane microdomain. *Annual review of cell and developmental biology***19,** 397-422.
Hemler, M.E. (2005). Tetraspanin functions and associated microdomains. *Nature Reviews Molecular Cell Biology***6,** 801-811.
Hirt, R.P., de Miguel, N., Nakjang, S., Dessi, D., Liu, Y.C., Diaz, N.*, et al.* (2011). Trichomonas vaginalis pathobiology new insights from the genome sequence. *Advances in parasitology***77,** 87-140.
Hunziker, W. and Fumey, C. (1994). A di-leucine motif mediates endocytosis and basolateral sorting of macrophage IgG Fc receptors in MDCK cells. *The EMBO journal***13,** 2963-2969.
Lammerding, J., Kazarov, A.R., Huang, H., Lee, R.T. and Hemler, M.E. (2003). Tetraspanin CD151 regulates alpha6beta1 integrin adhesion strengthening. *Proc Natl Acad Sci U S A***100,** 7616-7621.
Latysheva, N., Muratov, G., Rajesh, S., Padgett, M., Hotchin, N.A., Overduin, M. and Berditchevski, F. (2006). Syntenin-1 is a new component of tetraspanin-enriched microdomains: mechanisms and consequences of the interaction of syntenin-1 with CD63. *Mol Cell Biol***26,** 7707-7718.
Liu, L., He, B., Liu, W.M., Zhou, D., Cox, J.V. and Zhang, X.A. (2007). Tetraspanin CD151 promotes cell migration by regulating integrin trafficking. *The Journal of biological chemistry***282,** 31631-31642.
Lustig, G., Ryan, C.M., Secor, W.E. and Johnson, P.J. (2013). Trichomonas vaginalis contact-dependent cytolysis of epithelial cells. *Infect Immun***81,** 1411-1419.
Matter, K., Yamamoto, E.M. and Mellman, I. (1994). Structural requirements and sequence motifs for polarized sorting and endocytosis of LDL and Fc receptors in MDCK cells. *The Journal of cell biology***126,** 991-1004.
McClelland, R.S., Sangare, L., Hassan, W.M., Lavreys, L., Mandaliya, K., Kiarie, J.*, et al.* (2007). Infection with Trichomonas vaginalis increases the risk of HIV-1 acquisition. *The Journal of infectious diseases***195,** 698-702.
Monk, P.N. and Partridge, L.J. (2012). Tetraspanins: gateways for infection. *Infectious disorders drug targets***12,** 4-17.
Okumura, C.Y.M., Baum, L.G. and Johnson, P.J. (2008). Galectin-1 on cervical epithelial cells is a receptor for the sexually transmitted human parasite Trichomonas vaginalis. *Cellular Microbiology***10,** 2078-2090.
Pols, M.S. and Klumperman, J. (2009). Trafficking and function of the tetraspanin CD63. *Exp Cell Res***315,** 1584-1592.
Rous, B.A., Reaves, B.J., Ihrke, G., Briggs, J.A., Gray, S.R., Stephens, D.J.*, et al.* (2002). Role of adaptor complex AP-3 in targeting wild-type and mutated CD63 to lysosomes. *Molecular biology of the cell***13,** 1071-1082.
Ryan, C.M., de Miguel, N. and Johnson, P.J. (2011). Trichomonas vaginalis: current understanding of host-parasite interactions. *Essays in biochemistry***51,** 161-175.

Stark, J.R., Judson, G., Alderete, J.F., Mundodi, V., Kucknoor, A.S., Giovannucci, E.L., *et al.* (2009). Prospective study of Trichomonas vaginalis infection and prostate cancer incidence and mortality: Physicians' Health Study. *Journal of the National Cancer Institute* **101,** 1406-1411.

Sutcliffe, S., Alderete, J.F., Till, C., Goodman, P.J., Hsing, A.W., Zenilman, J.M., *et al.* (2009). Trichomonosis and subsequent risk of prostate cancer in the Prostate Cancer Prevention Trial. *International journal of cancer. Journal international du cancer* **124,** 2082-2087.

Swygard, H., Miller, W.C., Kaydos-Daniels, S.C., Cohen, M.S., Leone, P.A., Hobbs, M.M. and Sena, A.C. (2004). Targeted screening for Trichomonas vaginalis with culture using a two-step method in women presenting for STD evaluation. *Sexually transmitted diseases* **31,** 659-664.

Tonikian, R., Zhang, Y., Sazinsky, S.L., Currell, B., Yeh, J.H., Reva, B., *et al.* (2008). A specificity map for the PDZ domain family. *PLoS biology* **6,** e239.

Twu, O., de Miguel, N., Lustig, G., Stevens, G.C., Vashisht, A.A., Wohlschlegel, J.A. and Johnson, P.J. (2013). Trichomonas vaginalis exosomes deliver cargo to host cells and mediate hostratioparasite interactions. *PLoS pathogens* **9,** e1003482.

Twu, O., Dessi, D., Vu, A., Mercer, F., Stevens, G.C., de Miguel, N., *et al.*(2014). Trichomonas vaginalis homolog of macrophage migration inhibitory factor induces prostate cell growth, invasiveness, and inflammatory responses. *Proceedings of the National Academy of Sciences of the United States of America* **111,** 8179-8184.

Twu, O.d.M., N.; Lustig, G.; Stevens, G.C.; Vashisht, A.A.; Wohlschlegel, J.A.; Johnson P.J. (2013). Trichomonas vaginalis Exosomes Deliver Cargo to Host Cells and Mediate Host:Parasite Interactions. *PLoS Pathog*.

Van Der Pol, B., Kwok, C., Pierre-Louis, B., Rinaldi, A., Salata, R.A., Chen, P.L., *et al.* (2008). Trichomonas vaginalis infection and human immunodeficiency virus acquisition in African women. *The Journal of infectious diseases* **197,** 548-554.

Wang, H.X., Kolesnikova, T.V., Denison, C., Gygi, S.P. and Hemler, M.E. (2011). The C-terminal tail of tetraspanin protein CD9 contributes to its function and molecular organization. *Journal of cell science* **124,** 2702-2710.

WHO (2008) Global incidence and prevalence of selected curable sexually transmitted infection. W.H. Organization (ed.). Geneva, Switzerland, World Health Organization.

Yauch, R.L. and Hemler, M.E. (2000). Specific interactions among transmembrane 4 superfamily (TM4SF) proteins and phosphoinositide 4-kinase. *Biochem J* **351 Pt 3,** 629-637.

Zhang, X.A., Bontrager, A.L. and Hemler, M.E. (2001). Transmembrane-4 superfamily proteins associate with activated protein kinase C (PKC) and link PKC to specific beta(1) integrins. *J Biol Chem* **276,** 25005-25013.

Zhang, X.A., Kazarov, A.R., Yang, X., Bontrager, A.L., Stipp, C.S. and Hemler, M.E. (2002). Function of the tetraspanin CD151-alpha6beta1 integrin complex during cellular morphogenesis. *Mol Biol Cell* **13,** 1-11.

## FIGURE LEGENDS

Fig. 1. A. The classical predicted structure of TSPs is shown. EC1 and EC2 = extracellular domains; cytoplasmic NH2 and C-terminal tails (COOH) are depicted. B. Sequence analysis of TvTSPs. The alignment includes the open reading frame of the eight predicted TvTSPs: TSP1 (TVAG_019180), TSP2 (TVAG_485170), TSP3 (TVAG_280860), TSP4 (TVAG_050030), TSP5 (TVAG_485140), TSP6 (TVAG_460770), TSP7 (TVAG_446110) and TSP8 (TVAG_008950). Black shading indicates amino acid residues that are conserved in six or more TvTSPs. The transmembrane domains (TM), extracellular loop 1 (EC1), extracellular loop 2 (EC2), the N-terminal (NT) and C-terminal (CT) tails are underlined. Residues that are typically conserved among TSPs are listed above the alignment.

Fig. 2. Phylogenetic analysis of tetraspanin based on amino acid sequences. Numerical values at nodes correspond to support values. Abbreviations used: Af, *Aptenodytes forster*; Am, *Astyanax mexicanus*; Bas, *Balaenoptera acutorostrata scammoni*; Bt, *Bos taurus*; Ce, *Caenorhabditis elegans*; Cc, *Condylura cristata*; Dr, *Danio rerio*; DdAX4, *Dictyostelium discoideum* AX4 strain; Dm, *Drosophila melanogaster*; Eh, *Entamoeba histolytica*; Gg, Gallus gallus; Hl, Haliaeetus leucocephalus; Hs, *Homo sapiens*; Lc, *Latimeria chalumnae*; Mz, Maylandia zebra; Mm, *Mus musculus*; Nv, *Nasonia vitripennis*; On, *Oreochromis niloticus*; Ph, *Pantholops hodgsonii*; Sp, *Stegastes partitus*; Tv, *Trichomonas vaginalis*; Xt, *Xenopus tropicalis*.TvTPS are marked with a red square.

Fig. 3. Subcellular localization of TvTSPs in *T. vaginalis* transfectants. Cells exogenously expressing full length TvTSPs (TvTSPFL) with a C-terminal haemagglutinin (HA) tag were stained for immunofluorescence microscopy using a mouse anti-HA antibody. The nucleus (blue) was also stained with DAPI. This localization was observed in more than one hundred parasites in three independent experiments.

Fig. 4. Expression analysis of endogenous TvTSPs upon exposure to host cells. B7RC2 parasites were exposed to VECs and the kinetics of TvTSPs expression was analyzed by qPCR. Data are expressed as fold increase compared with time 0 min ± the standard deviation of the mean. With the exception of TvTSP2, all surface TvTSPs mRNA levels are up-regulated in contact with VECs. Generally, an increase in expression is observed after at 2 – 4 h of exposure to VECs. Two different housekeeping genes were used to analyze expression (Tubulin and Hmp24) Data from

tubulin is presented here, however, similar results were obtained with Hmp24 gene.All experiments were carried out in duplicates in three independent experiments.

Fig. 5. Subcellular localization of TvTSPFL and TvTSPsΔCt. Cells exogenously expressing TvTSPs full length (TSPFL) and truncated versions (TSPΔCT) with a C-terminal haemagglutinin (HA) tag were stained for immunofluorescence microscopy using a mouse anti-HA antibody. The nucleus (blue) was also stained with DAPI. The short amino acid sequence of the deleted C-terminal tails are listed. Note the change in localization when C-terminal tails are deleted. The change in localization was observed in more than one hundred parasites in three independent experiments.

Fig. 6. Parasite aggregation. A. Highly adherent *T. vaginalis* strains form larger aggregates than poorly adherent strains. Strains B7268 and SD7 (top) which are significantly more adherence to host cells (24) form large aggregates, unlike poorly adherence strains T1 and G3 (24) (lower panel). Ability to form clumps was assessed by light microscopy (10X magnification). B. Ability to form clumps of different TSPs full length (TSPsFL) transfected parasites. C. Quantification of clumps formation of TSPsFL transfected parasites. Fifty fields were counted in triplicate in threeindependent experiments. A clump was defined as an aggregate of ~10 or more parasites. A representative experiment is shown.

Fig. 7. Aggregation of TvTSP8 transfected parasites and expression of endogenous TSP8 in strains with different clump formation capacity. A. TvTSP8 and TvTSP8ΔCt differentially affect parasite aggregation. Ability to form clumps was assessed by light microscopy (10X magnification) for TvEpNeo (upper panel), TvTSP8 (middle panel) and TvTSP8ΔCt (lower panel) transfected parasites. B. Quantification of parasite aggregation of TvTSP8, TvTSP8ΔCt and EpNEO cells. Fifty field were counted in triplicate in three independent experiments. A clump was defined as an aggregate of ~10 or more parasites. C. Expression analysis of TvTSP8. mRNA expression levels of TvTSP8 in strains T1, G3, B7268 and SD7 were analyzed by qPCR. Data are expressed as fold increase compared with G3 strain ± the standard deviation of the mean. All data points were collected in duplicate in three independent experiments.

**Fig 1.S**. Transmission Electron Microscopy (TEM) analysis of TvEpNEO, TvTSP8 and TvTSP8ΔCt parasites. The quantification of the obtained images indicated no differences in quantity and size frequency of intracellular vesicles. A representative image was shown.