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RESEARCH ARTICLE

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Persistence of *Trypanosoma brucei* as early procyclic forms and social motility are dependent on glycosylphosphatidylinositol transamidase

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Abstract

Glycosylphosphatidylinositol (GPI)-linked molecules are surface-exposed membrane components that influence the infectivity, virulence and transmission of many eukaryotic pathogens. Procyclic (insect midgut) forms of *Trypanosoma brucei* do not require GPI-anchored proteins for growth in suspension culture. Deletion of *Tb*GPI8, and inactivation of the GPI:protein transamidase complex, is tolerated by cultured procyclic forms. Using a conditional knockout, we show *Tb*GPI8 is required for social motility (SoMo). This collective migration by cultured early procyclic forms has been linked to colonization of the tsetse fly digestive tract. The SoMo-negative phenotype was observed after a lag phase with respect to loss of *Tb*GPI8 and correlated with an unexpectedly slow loss of procyclins, the major GPI-anchored proteins. Procyclins are not essential for SoMo, however, suggesting a requirement for at least one other GPI-anchored protein. Loss of *Tb*GPI8 initiates the transition from early to late procyclic forms; this effect was observed in a subpopulation in suspension culture, and was more pronounced when cells were cultured on SoMo plates. Our results indicate two, potentially interlinked, scenarios that may explain the previously reported failure of *Tb*GPI8 deletion mutants to establish a midgut infection in the tsetse fly: interference with stage-specific gene expression and absence of SoMo.

KEYWORDS
differentiation, GPI anchor, GPI8, PIG-K, social motility, trypanosomes

1 | INTRODUCTION

As the primary interface between a cell and its environment, surface components of the plasma membrane mediate direct contact, perception of external cues and release of signalling molecules, while acting as a barrier that allows binding, uptake and secretion of diverse classes of substances. For pathogenic organisms, the functional integrity and composition of the cell surface impacts their virulence, infectivity and transmission (recently reviewed in de Castro Neto et al., 2021). GPI-anchored glycoproteins and glycoconjugates are abundantly expressed by many parasitic protozoa and these surface molecules perform functions that are crucial for host colonization, adaptation to environmental changes and evasion of the host immune response (reviewed in Aresta-Branco et al., 2019;

Franco et al., 2012; Macleod et al., 2020; Sacks et al., 2000; Vassella et al., 2009).

Trypanosoma brucei spp. are protozoan pathogens that cause fatal diseases in humans and livestock in sub-Saharan Africa. They are transmitted between mammals by tsetse flies (*Glossina* species) which are the definitive host. Throughout its life cycle, *T. brucei* resides extracellularly in both its hosts. Bloodstream forms in the mammal and procyclic forms in the tsetse midgut are covered by several million molecules of GPI-anchored proteins. In the former, the major species is the variant surface glycoprotein which is key to escaping the mammalian immune response by antigenic variation (reviewed in Aresta-Branco et al., 2019). In procyclic forms, the procyclins, with their characteristic pentapeptide (GPEET) and dipeptide (EP) repeats constitute the dominant species (Acosta-Serrano et al., 1999; Bütikofer et al., 1997; Clayton & Mowatt, 1989; Ferguson et al., 1993; Mowatt et al., 1989). GPEET-positive trypanosomes are categorized as early procyclic forms, as expression of GPEET procyclin is limited to the first few days of a midgut infection, whereas EP procyclin is expressed by both early and late procyclic forms (Naguleswaran et al., 2021; Vassella et al., 2000). A limited number of other GPI-anchored proteins that are expressed by procyclic forms have been identified: major surface protease B (MSP-B), which is required for shedding of the VSG coat during differentiation (Gruszynski et al., 2003, 2006), a trans-sialidase, which transfers sialic acid to GPI anchors (Engstler et al., 1993; Pontes de Carvalho et al., 1993) and the receptor for mammalian factor H (Macleod et al., 2020). Other GPI proteins are predicted to be expressed at this stage of the life cycle, but have not been characterized (Savage et al., 2012).

In *T. brucei*, GPI anchor synthesis is essential in bloodstream forms, but not in procyclic culture forms (Nagamune et al., 2000). Infection studies with three null mutants from the GPI biosynthetic pathway (*Tb*GPI12, *Tb*GPI10 and *Tb*GPI8) illustrated, however, that GPI-containing molecules are required for efficient colonization of the fly midgut (Güther et al., 2006; Lillico et al., 2003; Nagamune et al., 2000, 2004), the first organ the parasite encounters when entering the insect host. Impaired infectivity was attributed to the reduced activity of GPI-anchored trans-sialidases (Nagamune et al., 2004) and was proposed to result from a lack of efficient defense against anti-microbial responses by the tsetse fly or inadequate response to the host environment (Lillico et al., 2003; Nagamune et al., 2000). Null mutants of *Tb*GPI12 (PIG-L in mammals) cannot de-acetylate the GPI precursor GlcNAc-PI to GlcN-PI, while *Tb*GPI10 null mutants (PIG-B in mammals) cannot catalyze the addition of the third mannose to the Man_2-GlcN-PI core structure (reviewed in Hong & Kinoshita, 2009). *Tb*GPI8 is the orthologue of yeast Gpi8p and mammalian PIG-K (also known as GPI8), which is crucial for catalysis of the transamidation reaction during transfer of the GPI anchor to the precursor protein. The *T. brucei* GPI transamidase complex consists of the three conserved subunits *Tb*GPI8, *Tb*GPI16 (orthologous to PIG-T and Gpi16p), *Tb*GAA1 (orthologous to GAA1 and Gaa1p) and the two trypanosomatid-specific proteins *Tb*TTA1 and *Tb*TTA2 (Nagamune et al., 2003). *Tb*TTA1 and *Tb*TTA2 potentially replace PIG-S or Gpi17p and PIG-U or Cdc91p as the mammalian or yeast counterparts, respectively (Nagamune et al., 2003). In all three GPI mutants, the surface is devoid of EP and GPEET procyclins (Güther et al., 2006; Lillico et al., 2003; Nagamune et al., 2004). As also seen with a procyclin null mutant (Vassella et al., 2003), deletion of *Tb*GPI8 (Lillico et al., 2003), *Tb*GPI10 (Nagamune et al., 2004) or *Tb*GPI2 (Jenni et al., 2021) in procyclic forms causes the accumulation of free (non-protein-linked) GPIs on the surface. This was not the case in *Tb*GPI12 null mutants, which did not express either procyclins or free GPIs on their surface (Güther et al., 2006). Midgut infections with *Tb*GPI12 deletion mutants were severely reduced, suggesting that GPI structures, whether protein-bound or free, are required for colonization of the tsetse midgut (Güther et al., 2006).

Gene deletions that negatively impact the initial phases of fly infection have been recently linked to defects in social motility (SoMo), a form of collective migration exhibited by procyclic forms on semi-solid surfaces (Imhof et al., 2014; Oberholzer et al., 2010). An Rft1 deletion mutant that displayed reduced protein *N*-glycosylation and GPI glycosylation of procyclins (Gottier et al., 2017; Jelk et al., 2013) was impaired in performing SoMo and in colonizing the tsetse midgut (Imhof et al., 2015). Procyclins are not required for SoMo, however (Imhof et al., 2014), and a null mutant showed only a modest decrease in its ability to establish midgut infections (Vassella et al., 2009). This may be because, in the absence of procyclins, the null mutants export free GPIs to the surface (Vassella et al., 2003, 2009). Membrane-associated enzymes that regulate cyclic AMP signalling, such as adenylate cyclases and the flagellar phosphodiesterase B1, were found to be crucial for SoMo (Lopez et al., 2015; Oberholzer et al., 2015). Detailed analyses of fly infections with a PDEB1 null mutant suggested that the SoMo defect correlates with failure to colonize a specific compartment of the midgut, the ectoperitrophic space (Shaw et al., 2019). Notably, the early procyclic form, but not the late procyclic form is SoMo-competent (Imhof et al., 2014). This finding is in line with the hypothesis that SoMo reflects the initial colonization of the midgut by procyclic forms and not later translocations or migrations required for transmission to the mammalian host.

To determine whether GPI-anchored proteins other than procyclins play a role in SoMo (Imhof et al., 2015; Shaw et al., 2019), we generated a conditional knockout of *Tb*GPI8 and derivatives of it that expressed green fluorescent protein (GFP) as a reporter for GPEET. Here we show that cells lacking transamidase activity are not able to perform SoMo. Intriguingly, cells in which *Tb*GPI8 is repressed down-regulate GFP expression on plates, indicating that they are transitioning from early to late procyclic forms. Down-regulation of GFP also occurs in liquid medium, but only within a subpopulation. Taken together, this suggests that one or more GPI-anchored proteins functions as an environmental sensor, determining whether trypanosomes maintain their differentiation state as early procyclic forms or progress to the next stage of their life cycle.

2 | RESULTS AND DISCUSSION

2.1 | Functional validation of a conditional *Tb*GPI8 KO in procyclic forms

Procyclic form null mutants have been described previously by two groups (Lillico et al., 2003; Ohishi et al., 2003). However, we were unable to generate *Tb*GPI8 knockout (KO) procyclic forms in several different attempts, including a CRISPR/Cas9-based system with a cell line that constitutively expresses the required machinery (Shaw et al., 2019). We therefore employed a conditional KO approach by first generating a tetracycline-inducible *Tb*GPI8 overexpression cell line (GPI8-cOE). The two endogenous copies of *Tb*GPI8 were subsequently deleted using a transient CRISPR/Cas9 transfection system (Shaw et al., 2020), giving rise to GPI8-cKO. The insertion of the inducible copy and deletion of the endogenous copies were previously confirmed by PCR genotyping (Shaw et al., 2020).

It has been shown that GPI anchor precursors and free surface GPIs accumulate in *Tb*GPI8 null mutants (Lillico et al., 2003; Nagamune et al., 2004). We therefore monitored specific GPI anchor-containing molecules enriched via differential extraction after labelling with [^{3}H]-ethanolamine (Vassella et al., 2003). GPI-anchored precursors and free GPI anchors were highly enriched in the absence of tetracycline in the GPI8-cKO, but were barely detectable in the parental GPI8-cOE cell line (Figure 1a,b). Expressing the ectopic copy of *Tb*GPI8 in GPI8-cKO reduced the amount of precursors and free GPIs, although not to the level of GPI8-cOE. To examine the kinetics of expression and repression, protein levels of *Tb*GPI8 were assessed by Western blotting using a monoclonal antibody (Nagamune et al., 2003). Over-expression of the *Tb*GPI8 ectopic copy was observed in the presence of tetracycline in both GPI8-cOE and GPI8-cKO (Figure 1c). Depletion of ectopic *Tb*GPI8 was apparent on day 2 after removal of tetracycline. In the GPI8-cKO cell line, we could still detect a band at the size of *Tb*GPI8 as late as day 6. However, these data do not distinguish between cross reaction with another protein or low levels of ectopic *Tb*GPI8 expressed in the absence of tetracycline. To monitor whether *TbGPI8* mRNA was still being produced, a Northern blot was performed (Figure 1d). No mRNA could be detected on days 6 and 12 after tetracycline removal, excluding *Tb*GPI8 expression due to leakiness of the tetracycline repressor. Two possible interpretations of these data are that *Tb*GPI8 protein is unusually stable or that there is indeed cross-reactivity by the antibody. The former is unlikely, given that the cells were dividing and residual *Tb*GPI8 would be diluted out. In support of the latter, additional cross-reactive bands were observed, for example a band at ~40 kDa (Figure 1c).

As reported previously (Lillico et al., 2003; Ohishi et al., 2003), we also found that procyclic forms are able to grow in vitro in the absence of *Tb*GPI8 (Figure 1e). At day 0 of the time course, cells were washed and split between two flasks to monitor growth in the presence or absence of tetracycline. GPI8-cKO cells displayed a reduced rate of growth starting between day 5 and day 8 after removal of tetracycline from the culture medium. A representative analysis is shown (n = 3), where growth of GPI8-cOE and GPI8-cKO was assessed in parallel (Figure 1e). The parental GPI8-cOE did not change its growth rate when the inducible copy of *Tb*GPI8 was switched on or off: the average population doubling time (PDT) over a period of 12 days was 11.8 hr ± 0.4 or 11.6 hr ± 0.7, respectively, in the presence or absence of tetracycline. GPI8-cKO grew more slowly than GPI8-cOE, even when the inducible copy was expressed, with an average PDT of 13.5 hr ± 0.3 from day 1 to day 12. This growth defect was more pronounced when ectopic *Tb*GPI8 was depleted from GPI8-cKO. In this case the PDT increased from 14.1 hr ± 0.3 (averaged over the first 6 days) to 18.2 hr ± 0.6 between days 7 and 12 (Figure 1e).

2.2 | Loss of GPI-anchored proteins in cells depleted of *Tb*GPI8

It has been estimated that there are 2–6 million copies of EP and GPEET procyclin per cell (Acosta-Serrano et al., 1999; Clayton & Mowatt, 1989; Ferguson et al., 1993). We found that over-expression of *Tb*GPI8 in GPI8-cOE had no effect on the amounts of procyclins detected by live cell flow cytometry and that approximately 90% of GPI8-cKO cells grown in the presence of tetracycline expressed both procyclins at similar levels as the parental GPI8-cOE cell line (Figure 2a). In contrast, EP and GPEET procyclins were lost from the surface of GPI8-cKO after depletion of *Tb*GPI8. A procyclin null mutant (Vassella et al., 2009) was included as a control for complete absence of procyclins. The loss of surface procyclins is consistent with a lack of transamidase activity.

The quantity of procyclins detected by flow cytometry could be an underestimate, since procyclins without a GPI anchor might not reach the surface. We therefore performed Western blots to assess the total amounts of EP procyclin and to gain information on possible posttranslational modifications (Figure 2b). Consistent with flow cytometry, induction of ectopic *Tb*GPI8 did not impact on the level of EP procyclin. The calculated molecular mass of EP procyclins before N- and C-terminal processing is ~14 kDa (Clayton & Mowatt, 1989; Roditi et al., 1987) and the fully modified anchor increases the apparent molecular mass to a diffuse band of 30–50 kDa (Bütikofer et al., 1997; Richardson et al., 1988; Treumann et al., 1997). Upon depletion of *Tb*GPI8, EP was detected at lower intensity and with a lower apparent molecular mass from day 4 onwards and was barely detectable by day 10. The weakly reactive bands migrating at ≤35 kDa suggest that some proteins had the core anchor, but were not fully modified (Figure 2b). A similar pattern was observed for GPEET procyclin, both in terms of staining intensity and shift to lower apparent molecular masses (Figure S2). Furthermore, flow cytometry of fixed cells illustrated that the loss of procyclins was synchronous at the population level (Figure 2c). We presume that the anti-EP-reactive bands observed by Western blotting in GPI8-cKO cells correspond to the non-GPI-linked EP precursors previously reported in *Tb*GPI10 (Nagamune et al., 2000) and *Tb*GPI12 (Güther et al., 2009) deletion mutants.

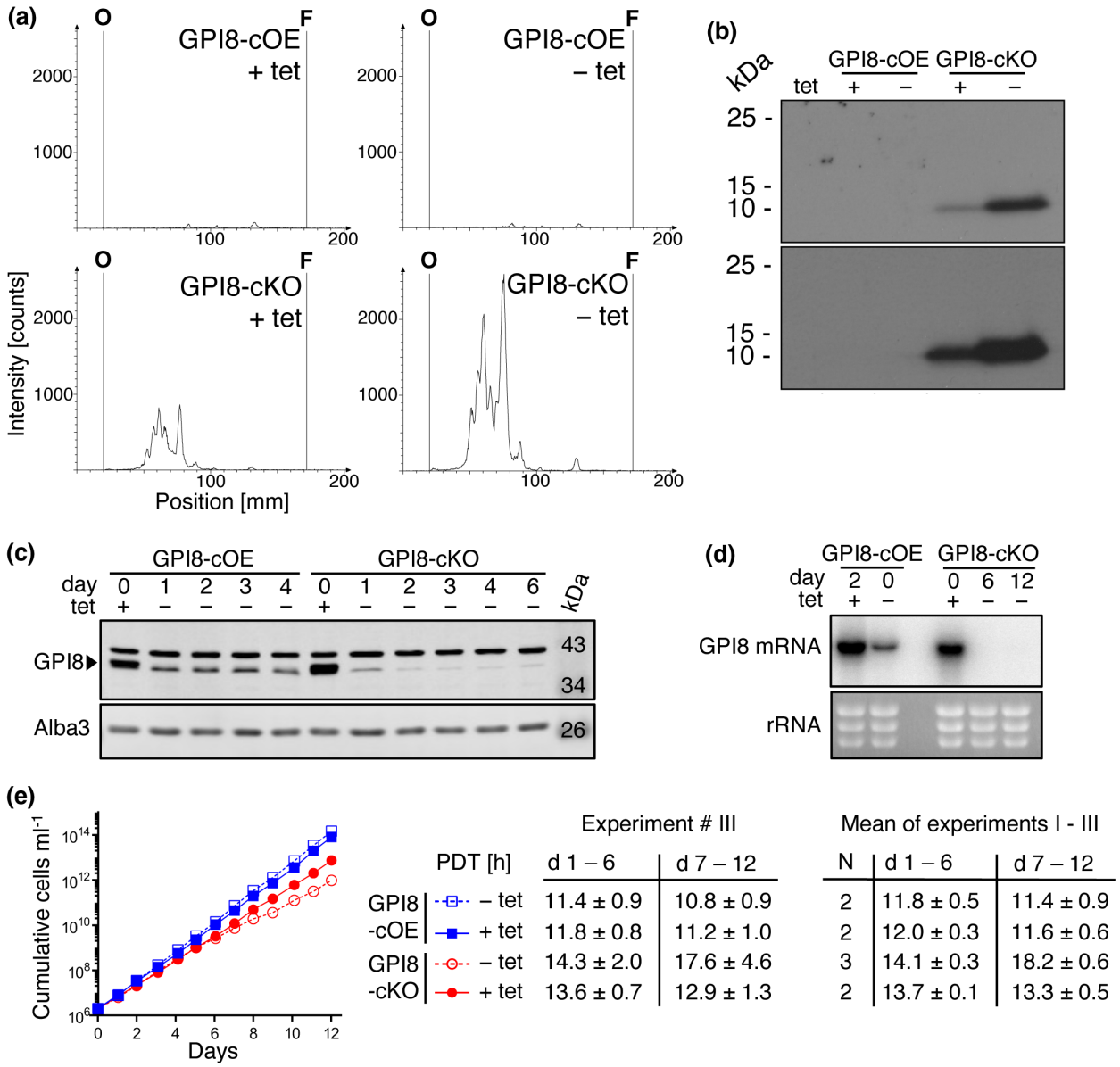

FIGURE 1 Characterisation of the GPI8 conditional knockout (GPI8-cKO) and overexpressor (GPI8-cOE). (a and b) Accumulation of free GPI anchors and precursors upon GPI8 depletion. Biosynthetic labeling of GPI moieties with [^{3}H]-ethanolamine. (a) GPI anchor precursors visualized by thin layer chromatography of the CMW_{but} fraction. The scales of the *x*-axes and *y*-axes are identical throughout. O: site of sample application; F: solvent front. (b) Autoradiograms after separation of the CMW_{aq} fraction by SDS-PAGE in the size range of free GPI anchors, after 4 days (top) or 11 days (bottom) exposure. (c) and (d) Samples were harvested at days indicated after removal of tetracycline (tet) during the time course depicted in (e). (c) Protein expression levels visualized by Western blotting, probing with anti-GPI8 antibody. For GPI8-cOE, an aliquot of the culture at day 8 + tet was washed to remove tet from medium and cultured in a separate flask for 4 days in the absence of tet. Loading control: anti-Alba3 antiserum. (d) Expression levels of GPI8 mRNA visualized by Northern blotting. The similarly sized endogenous and ectopic copies of GPI8 were detected simultaneously. Loading control: the three large cytoplasmic rRNAs were visualized by ethidium bromide staining after transfer to the membrane. See Figure S1 for quantification and information about transcript sizes. (e) Analysis of growth rates after removal of tet at day 0 (GPI8-cKO) or addition of tet at day 0 (GPI8-cOE). Population doubling time (PDT, mean ± *SD* in hours) is shown for time frames from day 1–6 and day 7–12. Centre panel: PDT of growth experiment # III depicted in the left panel, $n = 6$ data points per time frame. Right panel: PDT (mean ± *SD*) averaged over the independent growth experiments, $n = 2$ or 3 time courses. See Figure S1 for graphs and PDTs of the individual experiments

Alternatively, the faint bands migrating between 26–35 kDa might reflect remnants of stable, surface exposed GPI-linked EP species that are under-sialylated. Absence of *Tb*GPI8 or *Tb*GPI10 was previously shown to reduce the activity of GPI-anchored trans-sialidase to approximately 20%–30% of wild-type levels (Nagamune et al., 2004).

We also probed for another GPI-anchored protein, MSP-B. The results show that expression of ectopic *Tb*GPI8 caused a slight

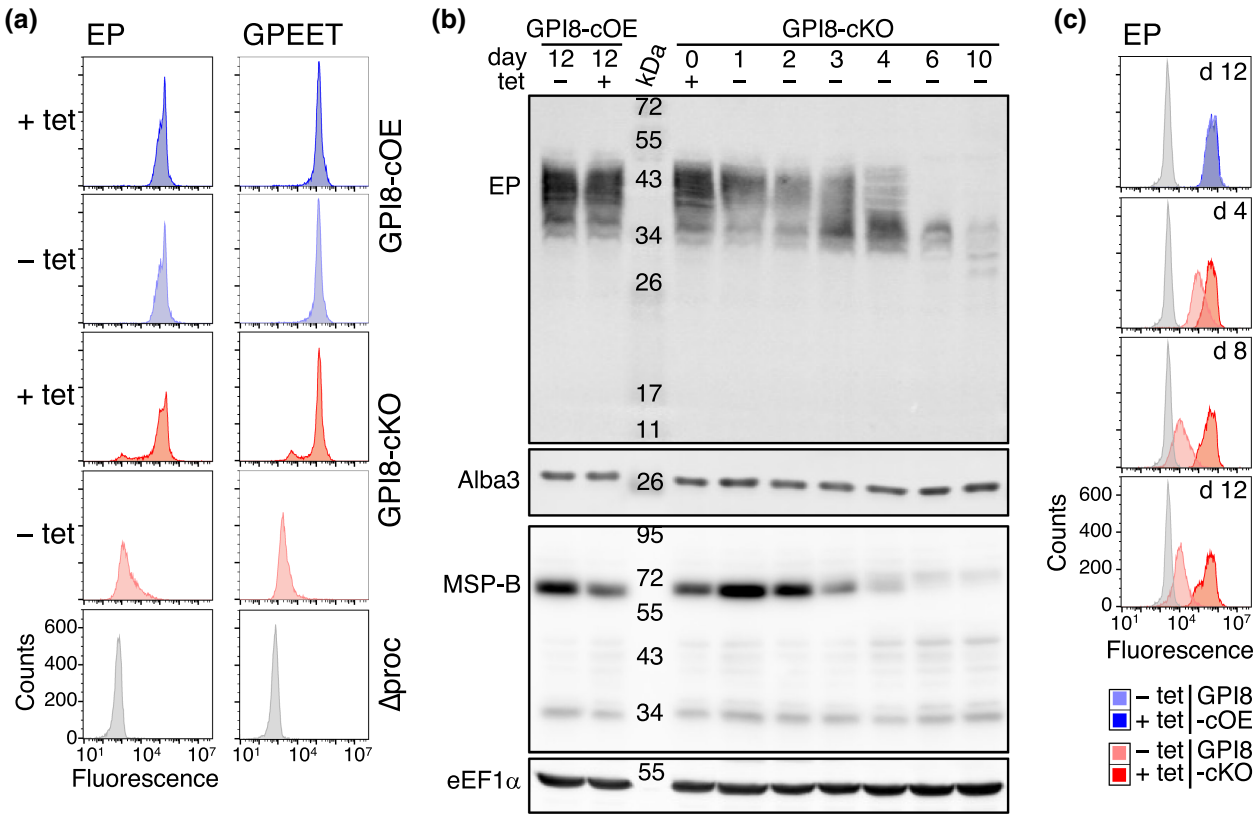

FIGURE 2 Loss of GPI-anchored proteins in cells depleted of GPI8. Samples were harvested at days indicated after removal of tet during the time course depicted in Figure 1e. (a) Flow cytometry of cells on day 9. Live cells were probed for surface expression of procyclins using anti-GPEET K1 antiserum (top panel) or anti-EP monoclonal antibody (bottom panel). Tet + or tet − refer to the addition (GPI8-cOE) or removal of tetracycline (GPI8-cKO) at day 0. A previously described procyclin null mutant cell line (Δproc, strain EATRO 1125 AnTat1.1) (Vassella et al., 2009) serves as a negative control. (b) Protein expression levels visualized by Western blotting. Two separate blots, using same cell lysates, were probed with anti-EP procyclin antibody (top panel) or anti-MSP-B antiserum (bottom panel). Loading controls: anti-Alba3 antiserum or anti-eEF1a antibody. (c) Flow cytometry of fixed cells. Expression of EP procyclin was assessed using anti-EP antibody, with all samples processed in parallel. GPI8-cOE − tet (light blue) or+ tet (blue), harvested at day 12. GPI8-cKO − tet (light red) or + tet (red) samples harvested at days 4, 8, and 12. For all histograms, the same sample of the procyclin null mutant Δproc (grey), harvested at day 12, is included as a negative control. The scales of the *x*-axes and *y*-axes on the fluorescence histograms are identical throughout

reduction in the steady-state levels of MSP-B in both GPI8-cOE and GPI8-cKO. Removal of tetracycline caused loss of MSP-B around day 4 and the appearance of a faint band with slightly higher apparent molecular mass, possibly representing a precursor (unprocessed pre-proenzyme) (Figure 2b).

2.3 | Social Motility (SoMo) is abolished in the absence of *Tb*GPI8

The primary aim of this study was to test whether GPI-anchored proteins are required for SoMo (Oberholzer et al., 2010). When early procyclic forms are applied onto the center of a plate, the cells proliferate, form protrusions and migrate outwards. The parasite communities can sense the environment and change their direction to avoid other communities inoculated onto the same plate. We first tested the ability of GPI8-cKO to perform SoMo. A representative experiment, which also included the parental GPI8-cOE, is shown (Figure 3; $n = 3$). In parallel, parasite growth was monitored in liquid culture. For the experiment shown in Figure 3a, GPI8-cKO was grown in the presence of tetracycline, while GPI8-cOE was grown without. On day 0, cells were washed and resuspended in medium without tetracycline, after which they were inoculated onto plates containing or lacking tetracycline. The result show that GPI8-cOE and GPI8-cKO formed protrusions both in the presence or absence of tetracycline in the plate (Figure 3a, left panel). However, while GPI8-cOE did not display noticeable differences in SoMo when grown in the presence or absence of tetracycline, GPI8-cKO grown in the absence of tetracycline formed protrusions more slowly than GPI8-cOE.

To assess whether *Tb*GPI8-depleted cells were still able to sense other communities and reorient their direction of migration, plates were inoculated with four different mutants: GPI8-cOE, GPI8-cKO, GPI8-c/sKO (a conditional single allele KO) and a control cell line harboring the same resistance genes as the GPI8-cKO. This setup also allowed for direct comparison of different cell lines grown on the same plate. The results illustrate the reduced outgrowth of GPI8-cKO compared to the other cell lines (Figure 3a, right panel, and Figure S3a). All four cell lines sensed the other communities,

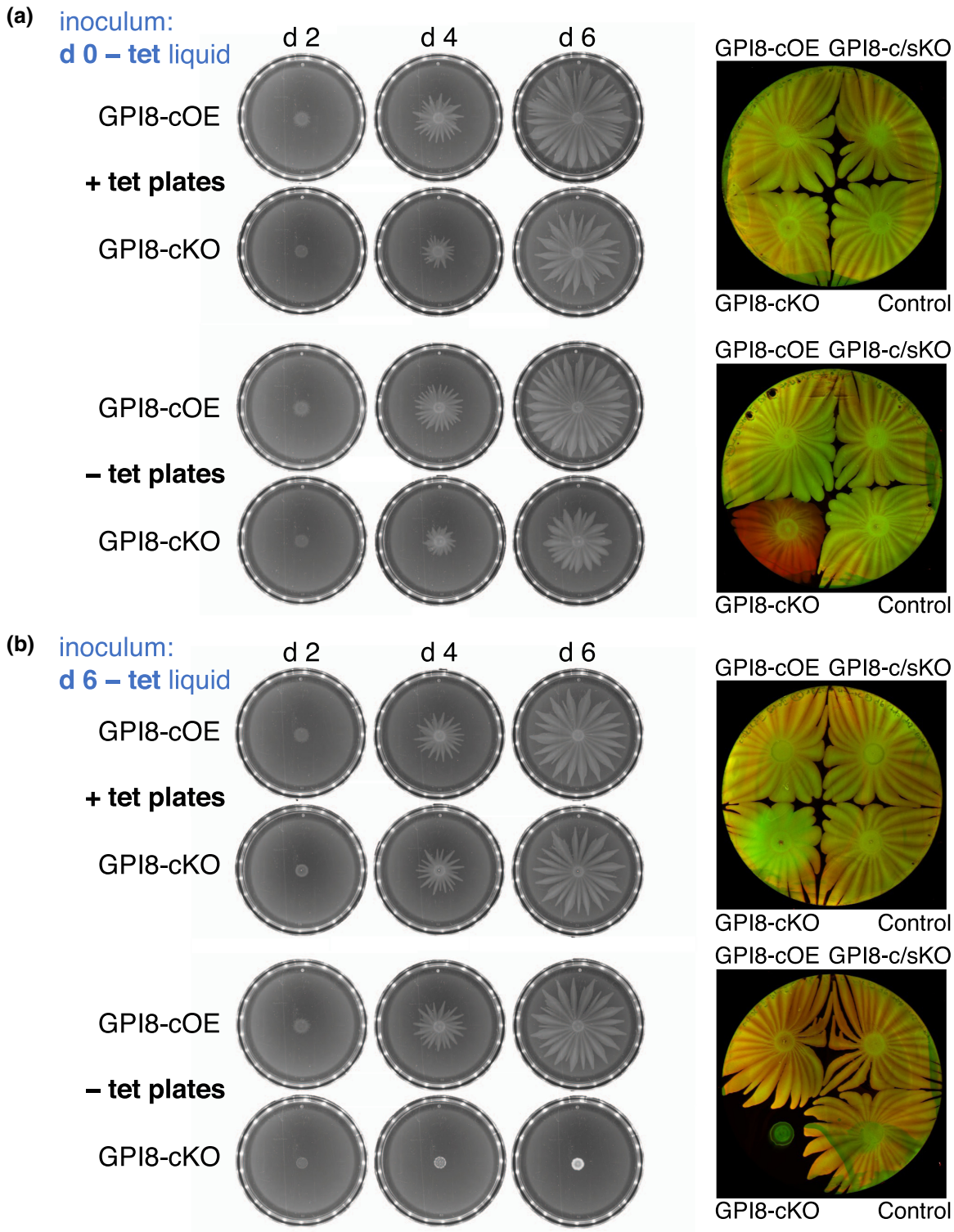

FIGURE 3 Growth on semi-solid medium is impaired upon sustained loss of GPI8. Social motility and community lift analyses performed with the GPI8-cOE, GPI8-cKO, GPI8-c/sKO (conditional semi KO), or control. The presence (+ tet, upper panels) or absence (− tet, lower panels) of tetracycline in the plate medium is indicated. Growth rates in liquid cultures used for inoculation of plates are shown in Figure 1e. Left panels: time course. Plates were photographed at days indicated post inoculation. Right panels: community lifts of plates at day 6 post inoculation. Plates were inoculated with four different cell lines to allow direct comparison of growth, sensing of other communities and detection of procyclins with anti-GPEET K1 (red) and anti-EP (green). See Figure S3 for single fluorescence channels for EP and GPEET and photographs of plates at day 4 and day 6 post inoculation. (a) Plates inoculated at day 0 of growth in liquid culture (− tet for GPI8-cOE, + tet for GPI8-cKO), after washing cells. (b) Plates inoculated from − tet cultures at day 6 of growth in liquid culture

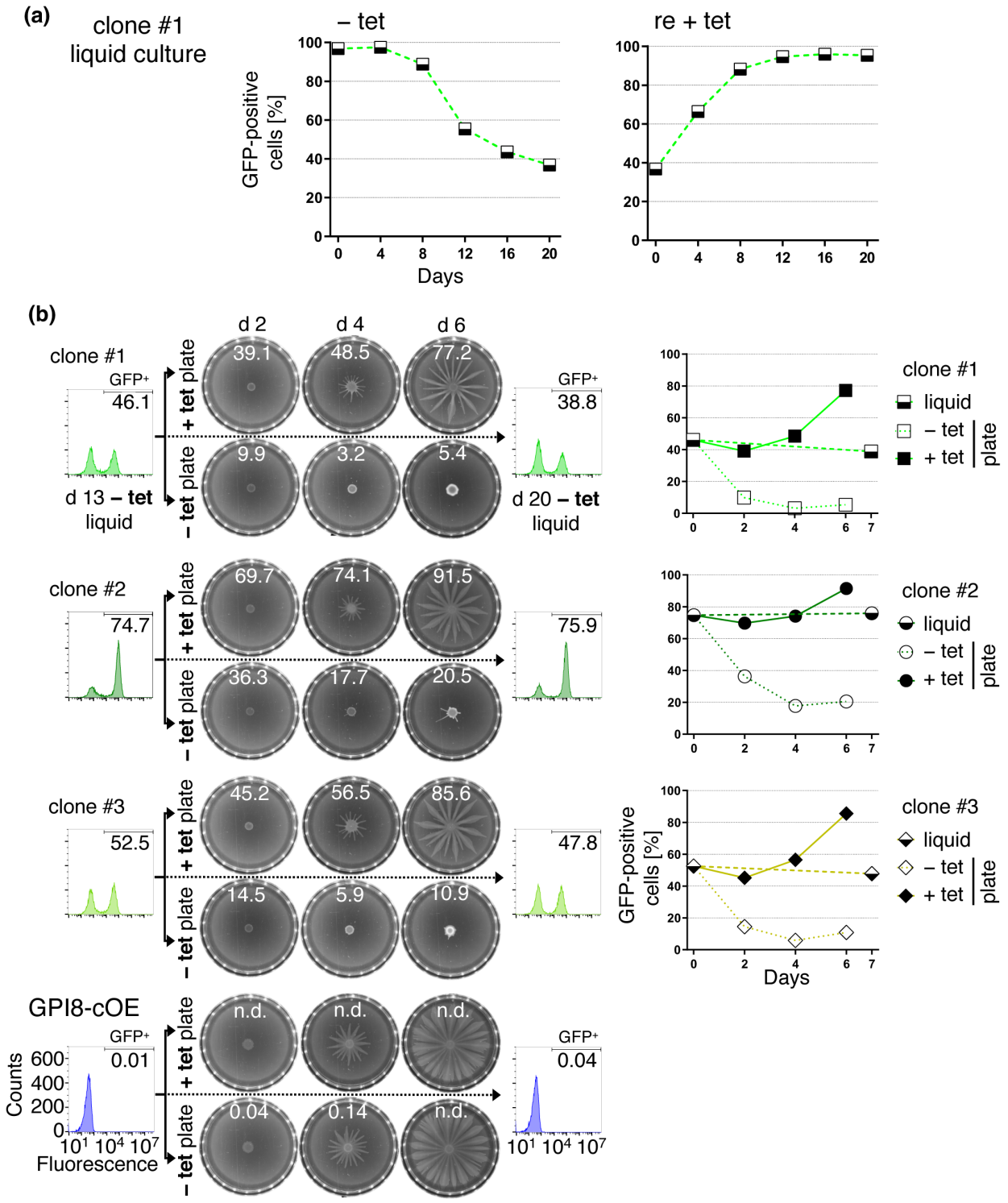

FIGURE 4 Legend on next page

indicating no defect in reacting to or in repelling other protrusions. Community lifts to detect EP and GPEET expression on day 6 after inoculation revealed that EP and GPEET expression was downregulated after depletion of *Tb*GPI8 compared to *Tb*GPI8-expressing cells. In an additional set of experiments, the four mutants were cultured for 6 days in the absence of tetracycline before being inoculated onto plates with or without the inducer (Figure 3b). We found that cells grown in the presence of tetracycline formed similar protrusions to those shown in Figure 3a. In contrast, GPI8-cKO cells no longer performed SoMo in the absence of tetracycline (Figure 3b, left panels). Nevertheless, they were still able to repel the other communities (Figure 3b, right panels, and Figure S3b). Notably, re-induction of *Tb*GPI8 in GPI8-cKO rescued the SoMo-negative phenotype (Figure 3b, upper panel).

FIGURE 4 GPI8 is required for maintenance as the early procyclic form. Flow cytometric analysis of live cells. The number of cells expressing GFP within the range indicated (GFP^+) are depicted as the percentage of total events recorded. Clones of GPI8-cKO+GFP constitutively express a GFP reporter for GPEET procyclin in the GPI8-cKO background. (a) Analysis of clone #1 during continuous growth in liquid culture. Graphs illustrating the kinetics of GFP expression (percentage of GFP-positive cells); values are taken from GFP histograms shown in Figure S4a. Samples were harvested at time points indicated after medium change. Left panel: days after removal of tet (− tet), initiated at day 15 of culture in the presence of tet. Right panel: days after re-addition of tet (re + tet), initiated at day 20 post tet removal. (b) Analysis of three clones after growth on SoMo plates for 2, 4, and 6 days. Plates with (+ tet) or without tet (− tet) were inoculated from liquid cultures at day 13 after tet removal. Left panels: Histograms illustrating the two populations of GFP-positive and GFP-negative cells on the day of plate inoculation (d 13 − tet liquid = d 0) and after 7 days (d 20 − tet liquid = d 7) of continuous growth in liquid culture. The scales of the *x*-axes and *y*-axes on the fluorescence histograms are identical throughout. Photographs of SoMo plates prior harvest for flow cytometry, at time points indicated after inoculation. Numbers on photographs indicate the percentage of GFP-positive cells of the respective cell communities. The non-fluorescent cell line GPI8-cOE (blue, analyzed in parallel) serves as a negative control. n.d., not determined. Right panels: percentage of GFP-positive cells of the three clones at time points indicated. Values are the same as depicted in the histograms or photographs shown in the left panels

2.4 | Persistence as early procyclic forms requires GPI-anchored proteins

SoMo is a property of a distinct developmental stage, the early procyclic form, which expresses GPEET procyclin (Imhof et al., 2014). However, expression of GPEET is not required for a cell to be early procyclic or SoMo-positive (Imhof et al., 2014). Cultured early and late procyclic forms display ≥2-fold differences in their expression of 73 genes other than GPEET, and one or more of these might be required for SoMo (Imhof et al., 2014; Naguleswaran et al., 2018). *Trypanosoma brucei brucei* Lister 427 (Bern) is consistently >87% GPEET-positive (Imhof et al., 2015) and frequently >95% (Shaw et al., 2019 and this study). Nevertheless, to rule out that perturbation of SoMo upon *Tb*GPI8 depletion is caused by differentiation into late procyclic forms, we performed Northern blots to assess the expression levels of mRNAs that discriminate between early and late procyclic forms (Imhof et al., 2014; Naguleswaran et al., 2018). In contrast to our expectations, we found that early procyclic form markers were down-regulated after depletion of *Tb*GPI8 (Figure S1b,c). In the GPI8-cKO cell line, *GPEET* mRNA was down-regulated 2-fold by day 12. Similarly, a 1.8-fold reduction in mRNA expression was observed for the adenylate cyclase AC330 (Tb927.5.330) on day 12. The decrease in early procyclic markers was accompanied by a 3.0-fold increase in EP mRNA, which is characteristic for late procyclic forms (Knüsel & Roditi, 2013). This blot also confirms the loss of endogenous and ectopic transcripts of *Tb*GPI8 in the GPI8-cKO cell line cultured in the absence of tetracycline (Figure S1b,c).

We previously found *GPEET* mRNA to be down-regulated more than 25-fold and *AC330* mRNA more than 9-fold when early procyclic forms differentiated to late procyclic forms (Imhof et al., 2014; Knüsel & Roditi, 2013). However, these values were obtained with a different isolate of *T. b. brucei* that can be induced to differentiate from early to late procyclic forms in vitro by changes in the composition of the culture medium (Knüsel & Roditi, 2013; Vassella et al., 2000). The modest reduction in early procyclic form markers observed in GPI8-cKO parasites in the present study might reflect partial down-regulation in the entire cell population. Alternatively, only a fraction of the population might transition to late procyclics, while the remainder is still in an early procyclic state. In the absence of transamidase activity, however, assessing the level of GPI-anchored GPEET protein is not feasible. We thus employed an alternative approach to determine the life-cycle stage of individual cells. A previously described GPEET reporter construct, in which cytoplasmic GFP is transcribed from a procyclin promoter, and under the control of *GPEET* mRNA untranslated regions, was stably introduced into the GPI8-cKO cell line (GPI8-cKO+GFP). This reporter mirrors expression of GPEET (Imhof et al., 2014; Schumann Burkard et al., 2013). Three clones were analyzed for expression of GFP in live cells by flow cytometry. Cells were maintained for 15 days in the presence of tetracycline and switched to medium without tetracycline to initiate depletion of *Tb*GPI8. Subsequently, the cells were cultured for 20 days in the absence of tetracycline, followed by re-induction of *Tb*GPI8 expression by adding tetracycline and further cultivation for 20 days. The fluorescence histograms of clone 1 were chosen to illustrate GFP expression levels (Figure 4a). The results show that upon depletion of *Tb*GPI8, the percentage of GFP-positive cells gradually decreased. On day 12 and day 20 after removal of tetracycline, 55% and 37% of cells, respectively, expressed the GFP reporter. Remarkably, re-induction of *Tb*GPI8 triggered the GFP-negative cells to re-express GFP. Starting from 37% GFP-positive cells on day 0, the fraction increased to 66% by day 4. From day 12 onwards, 95% of cells stably expressed GPEET (Figure 4a). The reversion of late to early procyclic forms is not unprecedented. Prolonged culture in medium without glycerol (Vassella et al., 2000) or perturbation of mitochondrial enzymes can also induce re-expression of GPEET in a subpopulation of cells (Vassella et al., 2004). The detection of distinct GFP-positive and GFP-negative populations indicates that loss of *Tb*GPI8 induces differentiation to late procyclic forms in a subpopulation of cells cultured in liquid medium. This explains the moderate down-regulation of *GPEET* and *AC330* mRNAs (Figure S1b,c). We have no explanation, however, why the absence of *Tb*GPI8 induced the transition to late procyclic forms in a subset of the population only. The finding that the ablation of GPI-anchored proteins results in differentiation to late procyclic forms, which leads to cell-cycle arrest for several days (Vassella et al., 2000), may explain why it was difficult to generate a knockout directly in a stock consisting predominantly of early procyclic forms.

2.5 | Growth on semi-solid medium drives the majority of GPI8-cKO cells to become late procyclic forms

Given that, in liquid culture, only a subpopulation transitioned to late procyclic forms after depletion of *Tb*GPI8, we explored whether growth on agarose had an influence on differentiation. For these experiments we employed flow cytometry to monitor the loss of GFP, i.e. transition to the GPEET-negative late procyclic forms, in cells cultured on SoMo plates. Prior to plating the cells, three clones of GPI8-cKO+GFP were grown in liquid culture for 13 days in the absence of tetracycline. Flow cytometry showed that the percentage of GFP-positive cells on the day of plate inoculation was 46%, 75% and 53%, respectively, in three separate clones (Figure 4b). Plates with or without tetracycline were inoculated with the four cell lines in technical replicates and monitored for 6 days. In parallel, cells were maintained in liquid culture in the absence of tetracycline. Every second day, cells were washed off the plates and GFP expression was analyzed by flow cytometry. The GPI8-cOE was used as a GFP-negative control. The results show that re-induction of *Tb*GPI8 caused re-expression of GFP in GPI8-cKO+GFP cells, mirroring the observation in liquid culture (see Figure 4a). By day 6, all three clones expressed GFP on plates containing tetracycline (77%, 92%, and 86%, respectively; Figure 4b), and were able to perform SoMo (Figure 4b). In contrast, in the absence of tetracycline, the GPI8-cKO+GFP cell lines were SoMo-negative or strongly impaired in forming protrusions. Intriguingly, the majority of cells that were still GFP-positive on the day of plate inoculation, now downregulated GFP on plates without tetracycline. By day 6, the initial percentage of cells expressing GFP dropped to 5%, 21%, and 11%, respectively, for the three GPI8-cKO+GFP clones (Figure 4b and Figure S4b). This markedly contrasts with the situation when cells were kept in liquid culture: in the period from days 13–20, the percentage of GFP-positive cells was much more stable (39%, 76% and 48%, respectively) (Figure 4b). We conclude that the microenvironment in semi-solid culture, compared to suspension culture, promotes the transition to late procyclic forms during depletion of *Tb*GPI8. It is possible that (a) the increase in cell density, (b) the contact to a solid substrate, (c) the altered uptake and secretion of molecules, and/or (d) the reduced diffusion rate within the matrix, resulting in nutrient/ion gradients on the culture plates, accelerate the differentiation process.

Although loss of *Tb*GPI8 drives the majority of cells to differentiate to the late procylic form, we suspect that the defect in SoMo cannot be explained solely by this finding. In our experience, cultures of a differentiation-competent strain that contain a minor fraction of early procyclic forms can still generate protrusions on SoMo plates, although they are thinner or take longer to form. We therefore performed experiments with defined mixtures of two cell lines, constitutively GPEET-positive Lister 427 Bern (427) (Cross & Manning, 1973; Ruepp et al., 1997) and constitutively GPEET-negative 29–13 Bern (29–13, a derivative of 427) (Wirtz et al., 1999). The percentage of GPEET-positive cells in liquid culture assessed by flow cytometry was 94.8% for 427 cells and 0.2% for 29–13 cells (Figure S6). Subsequently, SoMo plates were inoculated with 427 cells only, mixtures of 427 and 29–13 at ratios of 2:1 (resulting in 63% GPEET-positive cells) and 1:2 (resulting in 32% GPEET-positive cells), and 29–13 only (Figure S6). We observed uniform protrusions when the inoculum contained 427 early procyclic forms. SoMo still occurred with low percentages of GPEET-positive cells within an inoculum, but the protrusions formed were thinner (Figure S6). In contrast, no SoMo was observed if only 29–13 late procyclic forms were used (Figure S6). The three GPI8-cKO+GFP clones analyzed were SoMo-defective (forming no protrusions or only a few irregular and short projections), although the communities still contained a subpopulation of GPEET-positive cells on plates (see Figure 4b). This suggests that the defect in SoMo, upon depletion of *Tb*GPI8, cannot be attributed solely to the induced switch from early to late procyclic forms.

We recently reported that *T. brucei* procyclic forms lacking *Tb*GPI2 showed decreased viability on agarose plates (Jenni et al., 2021). To investigate whether *Tb*GPI8 was also required for survival on agarose plates, we determined the cell number and PDT in the presence and absence of tetracycline (Figure S5, upper panels). A community of GPI8-cKO, grown in the presence of tetracycline, reached 1.4×10^7 cells by day 4 and produced evenly spaced protrusions characteristic for this strain of *T. brucei*. In the absence of tetracycline, the conditional knockout grew more slowly for the first four days than cells in the presence of tetracycline, but then seemed to adapt and grew with a comparable PDT (Figure S5, upper panel). By day 6, this community contained 2.4×10^7 cells, yet it did not perform SoMo. Similar results were obtained with the three clones of GPI8-cKO+GFP (Figure S5, lower panels). When grown in the presence of tetracycline, pronounced protrusions were produced by day 4 ($1.6–1.7 \times 10^7$ cells). Once again, cells grown in the absence of tetracycline reached similar cell titers by day 6 ($1.8–2.1 \times 10^7$ cells), but did not perform SoMo or produced a few stunted and irregularly spaced protrusions only. It has been shown previously that, under defined conditions such as used here, the cell number is the determining factor for the onset of SoMo (Imhof et al., 2014). Thus, the cell number and the rate of growth on plates do not explain the conditional knockout's inability to perform SoMo.

3 | CONCLUSIONS

A conditional GPI8-cKO cell line allowed us to monitor the phenotypic responses to the loss of GPI transamidase activity over time. By switching the expression of *Tb*GPI8 "off" or "on" we induced the loss or synthesis of GPI-anchored proteins, respectively. We show for the first time that there is a requirement for *Tb*GPI8 and, consequently, GPI-anchored protein(s) in two processes examined in this study: developmental progression and SoMo of *T. brucei* procyclic forms. Firstly, in the absence of GPI-anchored proteins, transcripts encoding early procyclic form markers were down-regulated.

Unexpectedly, the transition from early to late procyclic forms in liquid culture, or at least the induction thereof, was only observed in a subpopulation of cells. This effect was more pronounced when parasites were cultured on SoMo plates, with the majority of cells downregulating the GPEET reporter in semi-solid cultures. Secondly, SoMo was abrogated upon depletion of GPI-anchored proteins. Instead of migrating outwards and forming protrusions, trypanosome communities grew and replicated at the site of inoculation. We postulate that several factors influence the behavior of *T. brucei* procyclic forms on semi-solid SoMo plates, including (a) the fluidity of the matrix, (b) parasite-derived lubricant(s), (c) signalling pathways that mediate the behavioral change in SoMo, (d) the metabolic and energetic state of a cell, and (e) interaction and putative communication between cells.

Our data suggest that the presence or absence of yet-to-be-identified GPI-linked protein(s) (Table S1) reversibly route the trypanosomes towards early or late procyclic forms and that at least one GPI-anchored protein, which is neither GPEET or EP procyclin, is required for a cell community to perform SoMo. By extension, we predict that defects in these two processes—maintenance as early procyclic forms and SoMo—may explain the reduced fly colonization reported for GPI mutants (Güther et al., 2006; Lillico et al., 2003; Nagamune et al., 2000, 2004). GPI-anchored receptors and enzymes have been identified in *T. brucei* (Gruszynski et al., 2006; Higgins et al., 2013; Macleod et al., 2020; Nagamune et al., 2004; Tiengwe et al., 2017). GPI-anchored proteins in other organisms can function as adhesion molecules (Coppi et al., 2011; Okumura et al., 2020; Rodrigues et al., 2019) or be cleaved for release into the environment (reviewed in Fujihara & Ikawa, 2016). These examples make them compelling candidates to regulate infectivity, developmental progression and collective behavior. A working model is presented in Figure 5. At present we can only speculate whether the defect in midgut infection reported for different GPI mutants is—either separately or in combination—due to (a) increased sensitivity to the fly's antimicrobial (immune) responses, (b) the reduction in or absence of surface enzyme activities (Gruszynski et al., 2006; LaCount et al., 2003; Nagamune et al., 2004), (c) interference with stage-specificity of gene expression, or (iv) defects in SoMo - with the latter two scenarios described herein.

4 | EXPERIMENTAL PROCEDURES

4.1 | Trypanosome isolates and general procedures for transfection and clonal selection

Procyclic forms of *T. b. brucei* were grown in SDM79 (9-04V01, Bioconcept) (Brun & Schönenberger, 1979) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Procyclic forms of *T. b. brucei* Lister 427 Bern (Cross & Manning, 1973; Ruepp et al., 1997), genetically manipulated derivatives thereof, and the 29–13 derivative (Wirtz et al., 1999) were used. Δproc is a procyclin null mutant (Vassella et al., 2009) initially derived from *T. b. brucei* EATRO1125, AnTat 1.1 bloodstream forms (Le Ray et al., 1977). Procyclic forms of Δproc were cultured in SDM79 supplemented with 10% FBS and 20 mM glycerol.

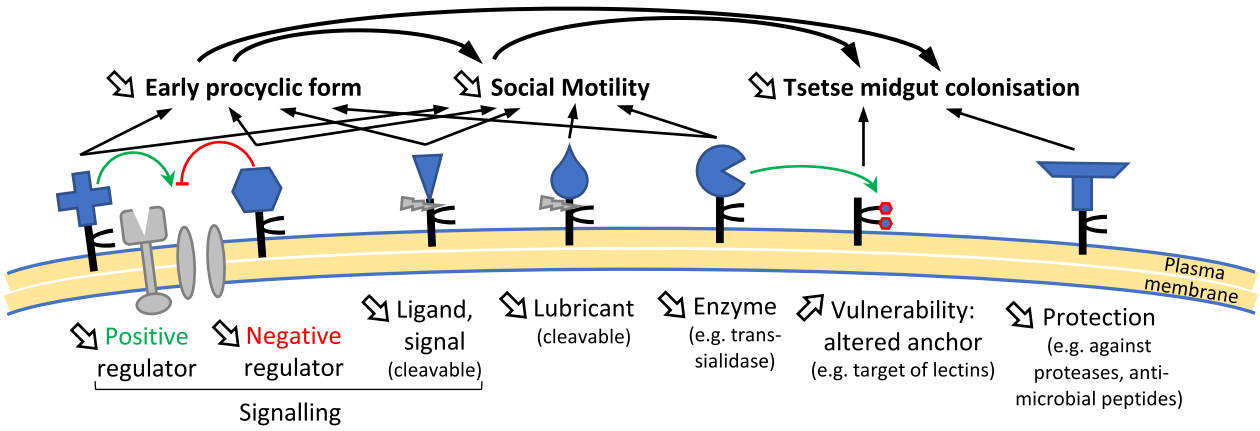

FIGURE 5 Working model - possible functions of GPI-anchored proteins and impact of TbGPI8 depletion on parasite behavior. Without *Tb*GPI8, target proteins cannot receive a GPI anchor and are not expressed on the cell surface. As yet unidentified GPI-anchored proteins (Table S1) may have the following functions that cause the phenotypes reported here: (a) Positive or negative regulators of signalling pathways (for example via a receptor or channel) that promote differentiation or induce SoMo. (b) One or more ligands that modulate these signalling pathways, as GPI-anchored proteins may be cleaved and released into the environment. (c) Glycoprotein(s), potentially cleavable, that act as surface lubricants and thus promote SoMo. In previous studies with different GPI-deficient parasites (Güther et al., 2006, 2009; Lillico et al., 2003; Nagamune et al., 2000, 2004), reduced fly infectivity was mainly attributed to (d) the lack of unidentified surface proteins or glycoconjugates that protect the parasite against proteases or antimicrobial peptides secreted by the fly, or (e) increased vulnerability caused by free GPI-anchors with altered side chains that might be the target of antimicrobial lectins. The reduced activity of a GPI-anchored trans-sialidase in such mutants, resulting in non-sialylated GPI side chains, was demonstrated to reduce fly infectivity (Nagamune et al., 2004). This example also illustrates that the proposed GPI-anchored protein may exert (f) an enzymatic function that might be required for SoMo or persistence as early procyclic form parasites. The presence or absence of EP and GPEET procyclins does not impact on these phenotypes (Imhof et al., 2014)

Stable transfections with plasmid-based constructs (pSmOx-nour, pLew100-cGPI8-phleo, or pCorleone-GFP/GPEET-puro) were performed as previously described (Burkard et al., 2007) using TbBSF transfection buffer (Schumann Burkard et al., 2011). Stable transformants were obtained by limiting dilution and selection in culture medium supplemented with the appropriate antibiotics (see below for concentrations used). Cells were incubated at 27°C and 2.5% CO_2. Knock out of endogenous GPI8 alleles was achieved after integration of an inducible ectopic copy of GPI8 (GPI8 conditional KO), using a transient CRISPR/Cas9 transfection system as described and validated in respective publication (Shaw et al., 2020). Final concentrations of antibiotics used for selection were as follows: 0.5 mg/ml Nourseothricin (Jena Bioscience AB-102L) for construct pSmOx-nour, 1.5 μg/ml Zeocin (Phleomycin, Life Technologies R250-01) for construct pLew100-cGPI8-phleo, 25 μg/ml Hygromycin B (Sigma H7772) for GPI8 KO allele 1, 15 μg/ml Geneticin (G418 Sulfate, Life Technologies 10131-035) for GPI8 KO allele 2, 1 μg/ml Puromycin dihydrochloride (Sigma P7255) for construct pCorleone-GFP/GPEET-puro.

4.2 | Generation of cell lines

The cell line 427/pSmOx was obtained by stable transfection of procyclic forms with pSmOx-nour to yield a tet-responsive cell line. The 427/pSmOx cell line was stably transfected with pLew100-cGPI8-phleo to generate the cell line GPI8-cOE, which allows for inducible over-expression of an ectopic, untagged (wild type) copy of *Tb*GPI8 in the presence of tetracycline. The GPI8-cOE cell line was used to create a GPI8 conditional KO. We obtained only one clone in which both endogenous copies of GPI8 had been deleted (GPI8-cKO). For some of the phenotypic analyses, we thus included a conditional semi KO cell line (GPI8-c/sKO), in which only one allele of GPI8 was deleted. An additional control cell line (Control) had both resistance cassettes correctly integrated and the ectopic copy could be regulated by tetracycline—an endogenous copy of GPI8 was still present, however, as determined by genotyping PCR (Shaw et al., 2020). Stable transfection of GPI8-cKO with pCorleone-GFP/GPEET-puro introduces a previously described GFP reporter for GPEET expression (Schumann Burkard et al., 2013), yielding GPI8-cKO+GFP.

4.3 | Plasmids and primers

The plasmid pSmOx-nour was generated from pSmOx (Poon et al., 2012) by replacing the selectable marker for puromycin resistance with the *SAT-1* streptothricin acetyltransferase (Joshi et al., 1995) which confers resistance to nourseothricin. The coding sequence of *SAT-1* flanked by synthetic restriction sites for EcoRI and NcoI was derived from the previously described plasmid pKOS (Ruepp et al., 1997). The plasmid pSmOx encodes the T7 RNA polymerase and the tetracycline repressor TetR (Wirtz & Clayton, 1995; Wirtz et al., 1998). Digestion with HindIII releases the construct for integration at the beginning of the tubulin gene array on chromosome 1 and constitutive expression by polycistronic transcription (Poon et al., 2012). The plasmid pLew100-cGPI8-phleo enables inducible overexpression of an ectopic, untagged copy of GPI8 in the presence of tetracycline (GPI8-cOE). The luciferase coding region in pLEW100 (Wirtz et al., 1999) was released by digestion with Hind III and Bam HI and replaced by GPI8. The coding sequence including the stop codon of *GPI8* flanked by synthetic restriction sites for HindIII and BamHI was amplified from EATRO1125 (AnTat1.1) genomic DNA.

Constructs and oligonucleotides used for knocking out GPI8 and for genotyping PCRs were described previously (Shaw et al., 2020). The GFP reporter construct for GPEET, pCorleone-GFP/GPEET has been described previously (Schumann Burkard et al., 2013). A version of this plasmid allowing selection with puromycin was linearized with SpeI for integration upstream of a procyclin locus on chromosome 6 or 10 (Fragoso et al., 2009) (Table 1).

4.4 | Conditions for in vitro culture

To allow for direct comparison between growth in liquid culture and on semi-solid surfaces (SoMo plates), trypanosomes were maintained in a humidified incubator at 27°C and 2.5% CO_2. Caps of liquid culture flasks were used in aeration position and SoMo plates were not wrapped with parafilm. The opto-mechanically treated culture flasks used are suitable for both suspension and adherent cells (TPP Techno Plastic Products). Cells for continuous liquid culture were maintained between 2.5×10^5 and 1.5×10^7 cells/ml by monitoring and diluting cultures daily or every other day. Cultures were diluted into the same 25 cm^2 flask and maintained in a vertical (5–7 ml) or horizontal (8–15 ml) position. The Δproc cell line was maintained between 5.0×10^5 and 1.0×10^7 cells/ml. Routinely, cell lines were kept in culture for less than one month. For time course experiments, cells were monitored and diluted daily for maintenance between 10^6 and 10^7 cells/ml. Growth rates are described as population doubling times (PDT) calculated as average ± standard deviation, with each day yielding one PDT value. Routinely, cells were harvested for analyses at a density between $6–12 \times 10^6$ cells/ml. Tetracycline was used at 1 μg/ml (10 mg/ml stock in 70% ethanol) to induce expression of the conditional copy of GPI8. To remove tet from culture medium, cells were pelleted at 1,500 × *g* for 10 min at room temperature, washed once in medium without tet and resuspended in the initial volume of medium without tet. The cell suspension was diluted appropriately into two fresh culture flasks and cultured in the presence and absence of tet, respectively.

4.5 | Social motility (SoMo) assay and community lifts

Plates containing medium and agarose were produced as described (Imhof et al., 2014). Directly after air-drying for 1 hr, the surface of

TABLE 1 Oligonucleotides for plasmid construction or PCR-based transfection constructs

Target[a]	Amplicon	Oligo name	Oligo sequence 5′–3′[b]	References
GPI8 cloning	ORF (960 bp)	GPI8 HindIII fwd	ccaagcttATGTTGCCCATGTTACTG	N/A
		GPI8 BamHI rev	taggatccCTAGAACAAATCGTAACGT	
SAT-1 cloning	ORF (525 bp)	SAT-EcoRI-for	tcgagaattcATGAAGATTTCGGTGATCCCTG	N/A
		SAT-NcoI-rev	ataccatggTTAGGCGTCATCCTGTGCTC	
GPI8 KO	5′ sgRNA template	GPI8 5′ sgRNA	gaaattaatacgactcactataggCGGTTGCA AAAAACGAATGCgttttagagctagaaatagc	Shaw et al. (2020)
	3′ sgRNA template	GPI8 3′ sgRNA	gaaattaatacgactcactataggGGTATGTC CCATCAGTTGGAgttttagagctagaaatagc	
	5′ repair construct	GPI8 upstream fwd	TTGGATCAGGCGCTTGCATATTTATTTCCAgt ataatgcagacctgctgc	
	3′ repair construct	GPI8 KO downstream rev	AGTTTCAGGAAGGAAGTTCGTTTTTCTCCTcc ggaaccactaccagaacc	

Abbreviation: N/A, not applicable.

[a]Gene identifiers. GPI8: Tb927.10.13860. SAT-1: Streptothricin acetyltransferase (Joshi et al., 1995).

[b]Target-specific sequences in upper case.

a plate was inoculated with 2×10^5 cells (5 μl of liquid culture concentrated to 4×10^7 cells/ml by centrifugation at 3,300 × *g* for 2 min at room temperature). Five to 10 min after inoculation, plates were transferred to the humidified incubator. The growth of trypanosome communities was documented with a digital camera as described (Imhof et al., 2014). In some cases, community lifts were performed to probe for procyclin protein expression as previously described (Imhof et al., 2014). After the trypanosome community was allowed to bind to a circular nitrocellulose membrane (Whatman Protran BA 85, 0.45 μm pore size, 82 mm diameter, GE Healthcare 10401116) for 10 min, the membrane was removed and air-dried for 15 min. After incubation in blocking buffer (5% w/v defatted dry milk in PBS pH 7.5 for at least 2 hr), membranes were rinsed twice in wash buffer (TBS-Tween20 0.05% pH, 7.4). The primary antibodies were rabbit α-GPEET-K1 antiserum (1:1,000, [Ruepp et al., 1997]) and mouse α-EP-247 mAb (1:2,500, clone TRBP1/247 [Richardson et al., 1988], Cedarlane CLP001A). The secondary antibodies (goat α-mouse IRDye 800CW and goat α-rabbit IRDye 680LT, LI-COR Biosciences) were used at dilutions of 1:10,000. Antibodies were diluted in blocking buffer. After incubation with antibodies, the membranes were washed three times for 5 min in wash buffer. After the final wash, membranes were rinsed in PBS, air-dried overnight in the dark and scanned on a LI-COR Odyssey Infrared Imager model 9120, using Odyssey Application Software, version 3.0.30.

4.6 | Flow cytometry

Flow cytometry was performed on live or fixed trypanosomes. Antibodies were diluted at the same concentration for analysis of live or fixed cells. Primary antibodies: rabbit α-GPEET-K1 antiserum (1:1,000, [Ruepp et al., 1997]) or mouse α-EP-247 mAb (1:500, clone TRBP1/247 [Richardson et al., 1988], Cedarlane CLP001A). Alexa Fluor 488-conjugated or Cy3-conjugated goat IgG secondary antibodies (Invitrogen, Thermo Fisher Scientific, 2 mg/ml) were diluted at 1:1,000. Flow cytometry of live trypanosomes was performed as described (Jenni et al., 2021). Parasites (4×10^6 cells) were harvested by centrifugation. All subsequent steps were performed at 4°C. The cells were resuspended in 200 μl medium containing the primary antibody and incubated with rotation for 30 min. After addition of 800 μl medium, cells were pelleted and washed once with 800 μl medium. After pelleting, the cells were resuspended, incubated with secondary antibodies and washed as described for the primary antibody. After resuspension in 1.6 ml medium, labeled and unstained control cells were subjected to flow cytometry as described for fixed trypanosomes. For analysis of fixed trypanosomes, cells were fixed in 2% (w/v) PFA/PBS at 4°C overnight at a concentration of 1×10^7 cells/ml and processed for flow cytometric analysis as previously described (Knüsel & Roditi, 2013; Roditi et al., 1989). For live and fixed cells, fluorescence of labelled and unstained control cells (no incubation with primary antibody) was quantified with a benchtop flow cytometer (ACEA NovoCyte, Agilent). To remove particles of subcellular size, a cut-off of 7.5×10^5 was applied to the forward scatter. A total of 10^4 events were recorded and analyzed using FlowJo software version 10.6.1_CL (BD Life Sciences) without gating. For analysis of cell communities grown on SoMo plates, cells were removed by two washes with 0.3–1 ml culture medium (depending on the size of the communities). Cells were counted and an appropriate dilution was analyzed by live-cell flow cytometry to quantify GFP expression as described above.

4.7 | Western blotting

After washing twice in cold PBS pH 7.5, trypanosomes were resuspended in PBS (5×10^5 cells/ml) and lysed by addition of an equal volume of 2× Laemmli sample buffer (120 mM Tris-HCl pH 6.8, 4% w/v SDS, 20% glycerol, 0.02% w/v bromophenol blue, 1.4 M β-mercaptoethanol freshly added), vortexing and heating for 3 min at 95°C. Lysate equivalent to 1.5 or 3×10^6 cells per lane was separated by SDS-PAGE (12% separating gel, 5% stacking gel) using a mini gel system. Transfer to

TABLE 2 Oligonucleotides used to generate probes for Northern blotting

Target[a]	Amplicon	Oligo name	Oligo sequence 5′–3′[b]	References
GPEET	Repeat GPEET[c]	MicroarrPROs	GCTATGACCATGGCGTGGGATTTGCC	Vassella et al. (2004)
		MicroarrPROas	GATTTCAGCGTTGCAGCACCAG	
EP	Repeat EP1[c]	As for GPEET	As for GPEET	
		As for GPEET	As for GPEET	
GPI8	ORF (960 bp)	GPI8 HindIII fwd	ccaagcttATGTTGCCCATGTTACTG	N/A
		GPI8 BamHI rev	taggatccCTAGAACAAATCGTAACGT	N/A
AC330	3′ UTR[d] (508 bp)	330RNAiBcl	tgatcaGGTTAACCATCACGAG	N/A
		330RNAiSal	gtcgacCGCACTTAAACAAAAAG	
18S rRNA	N/A	N/A	GTTCGTCTTGGTGCGGTCTA	Flück et al. (2003)

Abbreviation: N/A, not applicable.

[a]Gene identifiers. GPEET procyclin: Tb927.6.510. EP procyclins: Tb927.10.10260 (EP1), Tb927.10.10250 (EP2), Tb927.6.520 (EP3). GPI8: Tb927.10.13860. AC330: Tb927.5.330. 18S rDNA: Tb927.2.1452 (multicopy gene).

[b]Target-specific sequences in upper case.

[c]Plasmid template for PCR amplification: pBS-GPEET or pBS-EP1(EPα) (Ruepp et al., 1997).

[d]Partial 3′ UTR, distinct from Tb927.5.320/285b.

PVDF membrane (Immobilon-P 0.45 μm pore size, Merck Millipore IPVH00010) was performed by semi-dry blotting at 90 mA per blot for 1 hr (25 mM Tris-HCl pH 7.6, 190 mM glycine, 20% methanol) and assessed with Ponceau stain (0.1% w/v Ponceau S in 5% acetic acid). The membrane was blocked for at least 1 hr in blocking buffer (5% w/v defatted dry milk in TBS-Tween20 0.05% pH 7.4). Incubation with primary (overnight at 4°C) and secondary antibody (1 hr at room temperature) was performed in blocking buffer, followed by three washes in TBS-Tween. Blots wet with TBS-Tween were scanned on a LI-COR Odyssey Infrared Imager model 9120, using Odyssey Application Software, version 3.0.30. Membranes were stripped for 30 min at 50°C with rotation in freshly prepared stripping buffer (60 mM Tris-HCl pH 6.8, 2% w/v SDS, 10 mM β-mercaptoethanol).

Primary antibodies: mouse α-TbGPI8 mAb (1:800, 4 μg/ml, clone 7A2 [Nagamune et al., 2003]), rabbit α-GPEET-K1 antiserum (1:2,500 [Ruepp et al., 1997]), mouse α-EP-247 mAb (1:2,500, clone TRBP1/247 [Richardson et al., 1988], Cedarlane CLP001A), and sheep α-MSP-B (1:1,000, kind gift of John Donelson). Mouse α-eEF1α (1:10,000, clone CBP-KK1, Santa Cruz Biotechnology SC-21758) and rabbit α-Alba3 antigen-purified antiserum (1:500 [Mani et al., 2011]) served as loading controls. Secondary antibodies goat α-mouse IRDye 800CW and goat α-rabbit IRDye 680LT (LI-COR Biosciences) were diluted at 1:10,000, α-goat/sheep-HRP (10 mg/ml) at 1:2,500. MSP-B was detected via chemiluminescence (SuperSignal West Pico PLUS, Thermo Scientific 34580) and imaged using an Amersham Imager 600.

4.8 | RNA isolation and Northern blotting

RNA isolation and Northern blotting was performed according to procedures previously described (Knüsel & Roditi, 2013). Total RNA (10 μg per lane) was treated for 5 min at 50°C in denaturation solution (10 mM sodium phosphate buffer pH 6.8, 50% DMSO, 4% glyoxal) and resolved on a 1.4% agarose gel in 10 mM sodium phosphate buffer pH 6.8 for 3 hr at 80 V in the presence of 0.3 mg/ml ethidium bromide. After transfer to positively charged nylon 66 membrane (0.45 μm pore size, Roche 11417240001) by capillary blotting and UV crosslinking (twice 120 mJ/cm^2), the membrane was baked for 1 hr at 80°C under partial vacuum. Target RNAs were detected using [α-^{32}P]dCTP-labelled DNA probes generated using Megaprime DNA labelling system (GE Healthcare RPN1606) with gel-purified PCR product as template. Hybridization was performed overnight at 65°C in prewarmed hybridization solution (5 × SSC pH 7.2, 8 × Denhardts, 1% w/v SDS, 50 mM sodium phosphate buffer pH 6.8, 100 μg/ml sheared salmon sperm DNA), after blocking the membrane in hybridization solution for at least 2 hr at the hybridization temperature. For EP and GPEET probes, hybridization was performed at 60°C and the hybridization solution contained 50% v/v deionized formamide (Haenni et al., 2006). After hybridization, blots were washed three times in prewarmed wash solution (0.2 × SSC, 0.1% w/v SDS), at 5°C below hybridization temperature (T_{hyb}-5°C). Sample loading was assessed with 5′-[^{32}P]-labelled oligonucleotides to detect 18S rRNA: hybridization at 65°C and washing with 1 × SSC, 0.1% w/v SDS at T_{hyb}-5°C (Flück et al., 2003). Air-dried blots were exposed to Phosphorimager screen and scanned on a Typhoon FLA 7000 (GE Healthcare). Signal were quantified with ImageQuant TL version v8.1 (GE Healthcare) and processed in Fiji or Photoshop and normalized to 18S rRNA signal. Probes were stripped twice for 2 min in boiling stripping solution (0.1 × SSC, 0.5% w/v SDS), allowing the membrane to cool down between stripping repeats. Membranes were rinsed in H_2O, air-dried and stored at room temperature (Table 2).

4.9 | Radio-labelling with ethanolamine, extraction and detection of GPI-linked molecules

GPI precursors and GPI-anchored proteins were labeled and extracted as previously described (Bütikofer et al., 1997). Briefly, 50 ml

of trypanosome culture was incubated for 16–18 hr in the presence of 40 μCi of [^{3}H]-ethanolamine. 5×10^8 cells were harvested by centrifugation and washed twice with Tis-Buffered Saline (TBS; 140 mM NaCl, 10 mM Tris, pH 7.4). Phospholipids were removed by extraction with chloroform/methanol 2:1 (v/v). GPI precursors and free GPIs (Field et al., 1992; Vassella et al., 2003) were extracted three times with chloroform/methanol/water 10:10:3 (v/v/v; CMW fraction) and GPI-anchored proteins were extracted twice with 9% butan-1-ol in water (v/v; BuOH fraction). The CMW fractions were pooled and further partitioned between butan-1-ol and water, yielding fractions CMW_{but} and CMW_{aq} containing GPI precursors and free GPIs, respectively. The CMW_{aq} fraction was further purified by washing with butan-1-ol. All fractions were pooled and dried. CMW_{but} fractions were dissolved in 30 μl chloroform/methanol/water 10:10:3 (v/v/v).

CMW_{but} extracts containing GPI anchor precursors were spotted on Silica Gel 60 TLC plates (Sigma). The plates were developed using chloroform/methanol/water (10:10:3, v/v/v) as mobile phase. TLC traces were visualized using a Raytest Rita* radioactivity TLC analyzer (Berthold Technologies, Regensburg, Switzerland).

CMW_{aq} and BuOH extracts containing free GPIs and GPI-anchored proteins respectively were dissolved in 25 μl 1× loading buffer (15% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 2.5% (w/v) SDS, 50 mM Tris, 1 mM EDTA, 0.0025% (w/v) bromophenol blue) and subjected to SDS-PAGE. Gels were soaked in Amplify (GE Healthcare, Chicago, IL, USA), dried and exposed to films (Carestream Health Medical X-ray Blue, Rochester, NY, USA) at −70°C.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

SK, AJ, IR and PB conceived and designed experiments. SK, AJ and MB conducted experiments. SK, AJ, MB, PB and IR analyzed the data. SK and IR wrote the paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article.

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