Cyclic AMP Regulates Social Behavior in African Trypanosomes

Michael Oberholzer,[a] Edwin A. Saada,[a] Kent L. Hill[a,b]

Department of Microbiology, Immunology and Molecular Genetics[a] and Molecular Biology Institute,[b] University of California, Los Angeles, Los Angeles, California, USA

ABSTRACT The protozoan parasite *Trypanosoma brucei* engages in surface-induced social behavior, termed social motility, characterized by single cells assembling into multicellular groups that coordinate their movements in response to extracellular signals. Social motility requires sensing and responding to extracellular signals, but the underlying mechanisms are unknown. Here we report that *T. brucei* social motility depends on cyclic AMP (cAMP) signaling systems in the parasite's flagellum (synonymous with cilium). Pharmacological inhibition of cAMP-specific phosphodiesterase (PDE) completely blocks social motility without impacting the viability or motility of individual cells. Using a fluorescence resonance energy transfer (FRET)-based sensor to monitor cAMP dynamics in live cells, we demonstrate that this block in social motility correlates with an increase in intracellular cAMP levels. RNA interference (RNAi) knockdown of the flagellar PDEB1 phenocopies pharmacological PDE inhibition, demonstrating that PDEB1 is required for social motility. Using parasites expressing distinct fluorescent proteins to monitor individuals in a genetically heterogeneous community, we found that the social motility defect of PDEB1 knockdowns is complemented by wild-type parasites in *trans*. Therefore, PDEB1 knockdown cells are competent for social motility but appear to lack a necessary factor that can be provided by wild-type cells. The combined data demonstrate that the role of cyclic nucleotides in regulating microbial social behavior extends to African trypanosomes and provide an example of transcomplementation in parasitic protozoa.

IMPORTANCE In bacteria, studies of cell-cell communication and social behavior have profoundly influenced our understanding of microbial physiology, signaling, and pathogenesis. In contrast, mechanisms underlying social behavior in protozoan parasites are mostly unknown. Here we show that social behavior in the protozoan parasite *Trypanosoma brucei* is governed by cyclic-AMP signaling systems in the flagellum, with intriguing parallels to signaling systems that control bacterial social behavior. We also generated a *T. brucei* social behavior mutant and found that the mutant phenotype is complemented by wild-type cells grown in the same culture. Our findings open new avenues for dissecting social behavior and signaling in protozoan parasites and illustrate the capacity of these organisms to influence each other's behavior in mixed communities.

Address correspondence to Kent L. Hill, kenthill@mednet.ucla.edu.

Recognition of social behavior and cell-cell communication as ubiquitous among bacteria transformed our view of microbiology (1, 2). Examples of microbial social behavior are widespread and include assembly of biofilms and fruiting bodies, quorum sensing, and various forms of group motility across surfaces (1–6). Social behaviors enable bacteria to function as multicellular entities exhibiting emergent properties not evident in individual cells (1). Pathogenic bacteria and fungi exploit social behaviors to resist host immune defenses and antibiotics, to promote tissue colonization, and to exclude competing microbes from infection sites (7–9). As such, microbial cell-cell communication and social behavior are important for development and virulence, while the underlying mechanisms are potential targets for therapeutic intervention (10, 11).

Parasitic protozoa present a significant threat to global public health and agriculture and pose an economic burden in some of the world's most impoverished regions (12–16). The paradigm of social behavior can inform questions regarding parasite biology, transmission, and pathogenesis (17–20), but little is known about social behaviors and cell-cell interactions in these organisms. The protozoan parasite *Trypanosoma brucei* is transmitted by blood-sucking tsetse flies, causing sleeping sickness in humans and related diseases in wild and domestic animals throughout sub-Saharan Africa (21). Trypanosomes are typically considered individual cells but are capable of parasite-parasite communication and social behavior. In the mammalian host's bloodstream, for example, quorum sensing directs *T. brucei* development into "short stumpy" forms that are uniquely adapted for transmission through the tsetse fly (22, 23). Here, parasite-derived "stumpy induction factor" (SIF) accumulates in a cell density-dependent fashion and triggers cellular differentiation (22, 23). Procyclic (insect midgut stage) trypanosomes are also capable of social behavior. In this case, surface cultivation causes individual parasites to assemble into multicellular communities that engage in collective motility across the surface and modify their movements in response to signals from nearby parasites (24). This group behavior is termed social motility (SoMo) based on features shared with social motility and swarming motility in bacteria (4, 24).

In bacteria, social motility facilitates rapid surface colonization and promotes survival of bacterial populations in harsh environ-

ments (4, 5, 7). Specific features of transmission or pathogenesis that are reflected in *T. brucei* social motility are not yet known. However, recent work has shown that social motility is a property of a specific life cycle stage that occurs early during colonization of the fly midgut, consistent with the idea that social motility reflects parasite features relevant within the fly transmission stage (25). Moreover, the parasites are in constant contact with tissue surfaces in their natural environment, particularly in the tsetse fly and extravascular spaces in the mammalian host, and would benefit from functions provided by social motility in bacteria. More broadly, social motility presents a complex, group-level behavior that highlights the capacity of trypanosomes for cell-cell communication.

Social behaviors in microbes depend upon cell-cell communication and specific signaling systems in individuals within the population (1, 2, 4, 5). Quorum sensing and cyclic nucleotide signaling via the 2nd messenger cyclic-dimeric-GMP (c-di-GMP) have emerged as important regulators of surface-induced swarming motility in bacteria (26, 27). Quorum sensing and c-di-GMP also regulate biofilm formation, which is another surface-associated group behavior (27, 28). In eukaryotes, cyclic nucleotide signaling via cyclic AMP (cAMP) is critical for the surface motility of the social amoeba *Dictyostelium discoideum* (6).

T. brucei cAMP levels are controlled by receptor-type adenylate cyclases and cAMP-specific phosphodiesterases (PDEs) (29). *T. brucei* encodes five PDEs, PDEA, PDEB1, PDEB2, PDEC, and PDED (30, 31). PDEB1 and PDEB2 are essential for maintaining physiological levels of cAMP in *T. brucei* cells (32). *T. brucei* PDEB1 (TbPDEB1) is localized to the flagellum, while TbPDEB2 is distributed throughout the cell (32). Flagellum localization is of particular significance, because the eukaryotic flagellum is a signaling center that controls communication with the extracellular environment (33–35) and flagellar motility is required for social motility (24). TbPDEB1 and TbPDEB2 are essential in bloodstream trypanosomes *in vitro* and in an *in vivo* mouse infection model (32), making them targets for therapeutic intervention, and drug discovery efforts have led to development of potent and specific *T. brucei* PDE inhibitors (36–38). These inhibitors validate PDEs as drug targets and novel open avenues for dissecting cAMP signaling in trypanosomes with chemical genetics (39).

Given the widespread use of cyclic nucleotides for regulating surface-associated social behaviors in diverse microbes, we hypothesized that cAMP controls social motility in *T. brucei*. To test this hypothesis, we targeted TbPDEB1. We show that pharmacological or genetic inhibition of TbPDEB1 blocks social motility without affecting the viability or motility of individual cells. By employing a fluorescence resonance energy transfer (FRET) sensor for monitoring cAMP in live *T. brucei* cells, we showed that the block in social motility is directly correlated with a rise in intracellular cAMP levels. Finally, using fluorescent markers to monitor genetically distinct individuals in a mixed population, we demonstrated that the social motility defect of TbPDEB1 mutants can be complemented by wild-type (WT) cells in *trans*. Our findings reveal *T. brucei* social motility mechanisms, demonstrate that cyclic nucleotide regulation of microbial surface motility extends to parasitic protozoa, and reveal a novel form of cell-cell interaction in these parasites.

RESULTS

Intracellular cAMP levels regulate *T. brucei* social motility. To test the importance of intracellular cAMP dynamics for social motility, we applied a *T. brucei* PDE inhibitor, cpdA, that inhibits recombinant TbPDEB1 and TbPDEB2 *in vitro* and causes an increase in cellular cAMP concentrations when added to bloodstream-form *T. brucei* in culture (37). We tested cpdA at a range of concentrations, from 25nM to 1 μM, for its impact on social motility in procyclic *T. brucei*. When control cells are cultivated on soft agar, parasites assemble at the perimeter of the inoculation site and then move outward as groups, forming projections that radiate away from the center (Fig. 1) (24). cpdA showed dose-dependent inhibition of social motility, and the minimal concentration giving complete inhibition was 100 nM (Fig. 1a and c). Social motility requires active parasite motility (24, 40, 41). We therefore asked if cpdA affects motility of individual cells. We assessed motility using live-video microscopy combined with automated tracking of individual parasites treated with or without 100 nM cpdA in suspension culture. No difference was observed for cpdA-treated versus untreated cells (Fig. 1b). Treatment with 100 nM cpdA did not affect doubling time (Fig. 1d). Therefore, cpdA inhibits social motility in a dose-dependent fashion, with no impact on the proliferation or motility of individual cells.

The inhibitory effect of cpdA on *T. brucei* social motility is anticipated to be due to a rise in intracellular cAMP concentrations. CpdA has been demonstrated to increase intracellular cAMP concentrations in bloodstream-form *T. brucei* (37), but its impact on cAMP in procyclic parasites has not been assessed. We therefore tested this by applying a well-characterized fluorescence resonance energy transfer (FRET)-based sensor for cAMP, Epac1-camps (epac1camps), that has been used to monitor intracellular cAMP dynamics in a variety of cell types, including trypanosomes (38, 42, 43). When excited with a wavelength of 436 nm, epac1camps emits 2 distinct wavelengths, one in the cyan (488-nm) spectrum and one in the yellow (535-nm) spectrum, the latter of which is FRET dependent (see Fig. S2a in the supplemental material) (42). Binding of cAMP to the sensor causes a conformational change that reduces FRET, and the 488-nm/535-nm emission ratio therefore increases as a function of increasing intracellular cAMP concentrations (see Fig. S2a) (42). The dynamic range of epac1camps is 0.2 to 10 μM cAMP (42), consistent with the reported intracellular cAMP concentration for *T. brucei* (30). epac1camps was expressed using a tetracycline (tet)-inducible vector in procyclic *T. brucei*. Fluorescence microscopy analysis of live, immobilized epac1camps-expressing parasites revealed that epac1camps is evenly distributed throughout the cell, including the cytoplasm and flagellum (Fig. 1e, inset). epac1camps reporter parasites were then used in FRET assays (Fig. 1e and f; see also Fig. S2). When excited at 436 nm, epac1camps parasites showed 488-nm and 535-nm emission signals significantly higher than the background observed for WT controls without the reporter (see Fig. S2b and c). Addition of cpdA epac1camps parasites produced a dose-dependent increase in the 488-nm/535-nm emission ratio, which rose rapidly and plateaued within ~400 to 600 s, indicating a rapid and stable rise in cellular cAMP concentrations (Fig. 1e and f). The 488-nm/535-nm ratio did not change in control epac1camps parasites that did not receive cpdA. Therefore, inhibition of social motility by cpdA is directly correlated with a rise in intracellular cAMP concentrations.
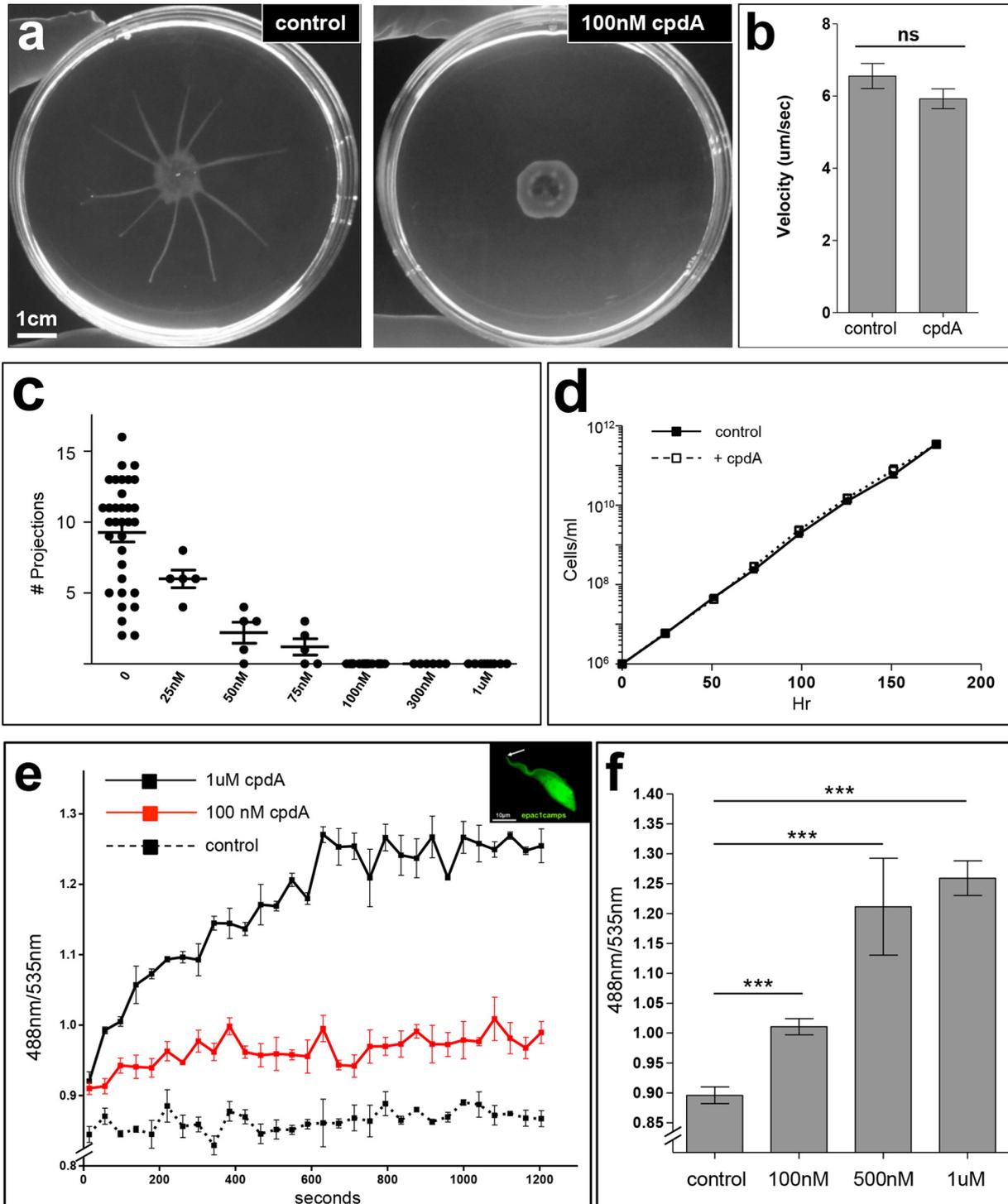
FIG 1 Pharmacological inhibition of *T. brucei* PDE blocks social motility and elevates cellular cAMP levels. (a) Social motility of cells treated with or without 100 nM cpdA as indicated. (b) Motility of individual cells in suspension cultures treated with or without 100 nM cpdA. (c) Chart showing the number of projections produced upon treatment with the indicated concentrations of cpdA (0 nM [$n = 33$]; 25 nM [$n = 5$]; 50 nm [$n = 5$]; 75 nm [$n = 5$]; 100 nM [$n = 27$]; 300 nm [$n = 6$]; 1 μM [$n = 9$]). (d) Growth curve for cells treated with or without 100 nM cpdA. (e) Fluorescence ratio (488 nm/535 nm) of epac1camps-expressing cells as a function of time following addition of cpdA at the indicated concentrations. The inset shows that the cAMP FRET sensor epac1camps is expressed throughout the parasite cell, including the flagellum (arrow). (f) Endpoint analysis of 488-nm/535-nm fluorescence ratio of epac1camps cells following 600 s of treatment with cpdA at the indicated concentration. Error bars show ± standard errors. ***, $P < 0.0001$; ns = not significant.

Previous studies suggested that cAMP mediates stumpy formation in BSF *T. brucei* because addition of cell-permeable cAMP induced stumpy differentiation (23). Subsequent studies showed that differentiation was not induced by hydrolysis-resistant cAMP, even when used at concentrations 20-fold to 200-fold higher than the hydrolyzable cAMP concentrations (44). Moreover, AMP and adenosine metabolic products were 100-fold to 1,000-fold more potent at inducing differentiation than was cAMP, indicating that cAMP downstream products were responsible for stumpy differentiation (44). We tested cAMP analogues for an effect on social motility. In contrast to what was observed for stumpy differentiation, hydrolysis-resistant cAMP inhibited social motility as well as or better than hydrolyzable cAMP, and the effect seen with either compound was equivalent to or more potent than that seen with AMP or adenosine (see Fig. S1 in the supplemental material). Notably, because cAMP added to *T. brucei* cells is unstable (44), the effective cAMP concentration is even lower than what is added. These results support the view that cAMP mediates inhibition of social motility.

Genetic inhibition of TbPDEB1 phenocopies the effect of the PDE inhibitor cpdA. Potent inhibition of TbPDEB1 and TbPDEB2 by cpdA has been demonstrated using recombinant proteins (37). However, there are five PDEs in *T. brucei* and selectivity of cpdA for any given PDE over the others has not been demonstrated. Likewise, as with any pharmacological treatment, there is potential for off-target effects that may produce a phenotype unrelated to inhibition of the drug's expected target. Therefore, to determine if the effect of cpdA on social motility is due to inhibition of a specific PDE, we used RNA interference (RNAi) to test the requirement for PDEB1 in social motility. We chose PDEB1 because of its flagellar localization (Fig. 2a) (45) and our goal of interrogating the role of flagellar cAMP signaling in social motility. Upon tet induction, PDEB1 mRNA levels were reduced to 11% of control parasite levels, while the abundance of PDEB2 mRNA was unaffected (Fig. 2b). Therefore, knockdown was potent and specific. PDEB1 knockdown did not affect the motility of individual cells and had only a modest effect on growth (Fig. 2c and d). When assayed for social motility, PDEB1 knockdown completely blocked social motility (Fig. 2e and f). Therefore, genetic ablation of PDEB1 expression phenocopied cpdA treatment, inhibiting social motility while not affecting the motility of individual cells. Social motility is observed in early procyclic but not late procyclic cells (25). Cells treated with cpdA or PDEB1 RNAi expressed the early procyclic marker GPEET procyclin (see Fig. S5 in the supplemental material), demonstrating that inhibition of social motility is not due to differentiation into late procyclics (25).

The social motility defect of TbPDEB1 knockdown parasites can be complemented by WT cells provided in *trans*. We explored potential mechanisms underlying the role of PDEB1 and cAMP in social motility. We hypothesize at least two alternative models. In model 1, genetic or pharmacological inhibition of PDEB1 leads to misregulation of cAMP signaling, which causes a cell-autonomous inhibition of social motility. In other words, PDEB1-deficient cells are incapable of social motility despite being motile as individuals. In model 2, misregulation of cAMP signaling disrupts an intercellular process that is required for social motility. For example, PDEB1-deficient cells may be competent for social motility but fail to engage in social motility because they lack something that could be provided by other cells. In bacteria,

for example, cells with swarming defects can be rescued by wild-type cells in mixed communities, owing to cell-cell transfer of outer membrane proteins important for social behavior (46). To discriminate between these two models, we engineered cell lines with different fluorescent markers to enable monitoring of individual genotypes within a genetically mixed community. We generated WT cells, trypanin knockdowns (47), and PDEB1 knockdowns, with each expressing red fluorescent protein (RFP) or green fluorescent protein (GFP). We then examined these cells in mixed communities. Expression of GFP or RFP did not affect social motility, as WT-RFP and WT-GFP cells are evenly distributed in a mixed social motility community (see Fig. S3d in the supplemental material). Trypanin is a subunit of the flagellar dynein regulatory complex that controls flagellar motility (48). Individual trypanin knockdown cells are incapable of propulsive motility (47) and are consequently defective in social motility (24). We found that the social motility defect of trypanin knockdowns is cell autonomous, as trypanin knockdown-GFP cells were unable to move into radial projections formed by wild-type–RFP cells in a mixed population (Fig. 3a to c and g). The RFP/GFP ratio of the mixed population rose rapidly as a function of the distance from the center, increasing 30-fold within a few millimeters (Fig. 3g). The inability of motility mutants to enter projections formed by wild-type cells demonstrates that parasites are not simply carried into a projection by bulk flow of other cells in the population. Mixing wild-type cells with PDEB1 knockdown cells gave a very different result (Fig. 3d to f and h). In this case, PDEB1 knockdowns, which are incapable of forming projections on their own (Fig. 2), moved into radial projections when cocultured with wild-type cells in a mixed population. Quantitation of relative levels of fluorescence intensity showed that the RFP/GFP ratio remained constant as the population moved outward (Fig. 3h). The result was independent of the status of the cell lines with respect to which harbored GFP versus RFP (see Fig. S4). The ability of the PDEB1 social motility defect to be complemented by wild-type cells in *trans* favors model 2, indicating that wild-type cells provide a critical factor that the PDEB1 mutants can respond to but cannot generate on their own.

DISCUSSION

cAMP regulates *T. brucei* social behavior. The molecular mechanisms underlying trypanosome social motility are unknown. Here we report that flagellar cAMP signaling systems function in regulation of *T. brucei* social motility. Our studies here and in recent work (49) provide the first dissection of the mechanisms underlying social motility in trypanosomes and provide new opportunities for investigating cAMP signaling in these pathogens.

Pharmacological inhibition of *T. brucei* cAMP-specific PDE with cpdA produces a dose-dependent increase in intracellular cAMP concentrations in live trypanosomes that is directly correlated with a dose-dependent block in social motility. These results indicate that PDE activity is required for social motility. Gene-specific knockdown of PDEB1 phenocopies the SoMo($-$) defect of cpdA, demonstrating that PDEB1 is specifically required for SoMo. This is in agreement with strong inhibition of recombinant PDEB1 by cpdA at low nanomolar concentrations (50% inhibitory concentration [IC_{50}] = 3.98 nM) (37). *T. brucei* encodes five PDEs, and it is possible that other PDEs may also participate in regulation of social motility, but our data indicate they are not able to substitute for PDEB1.
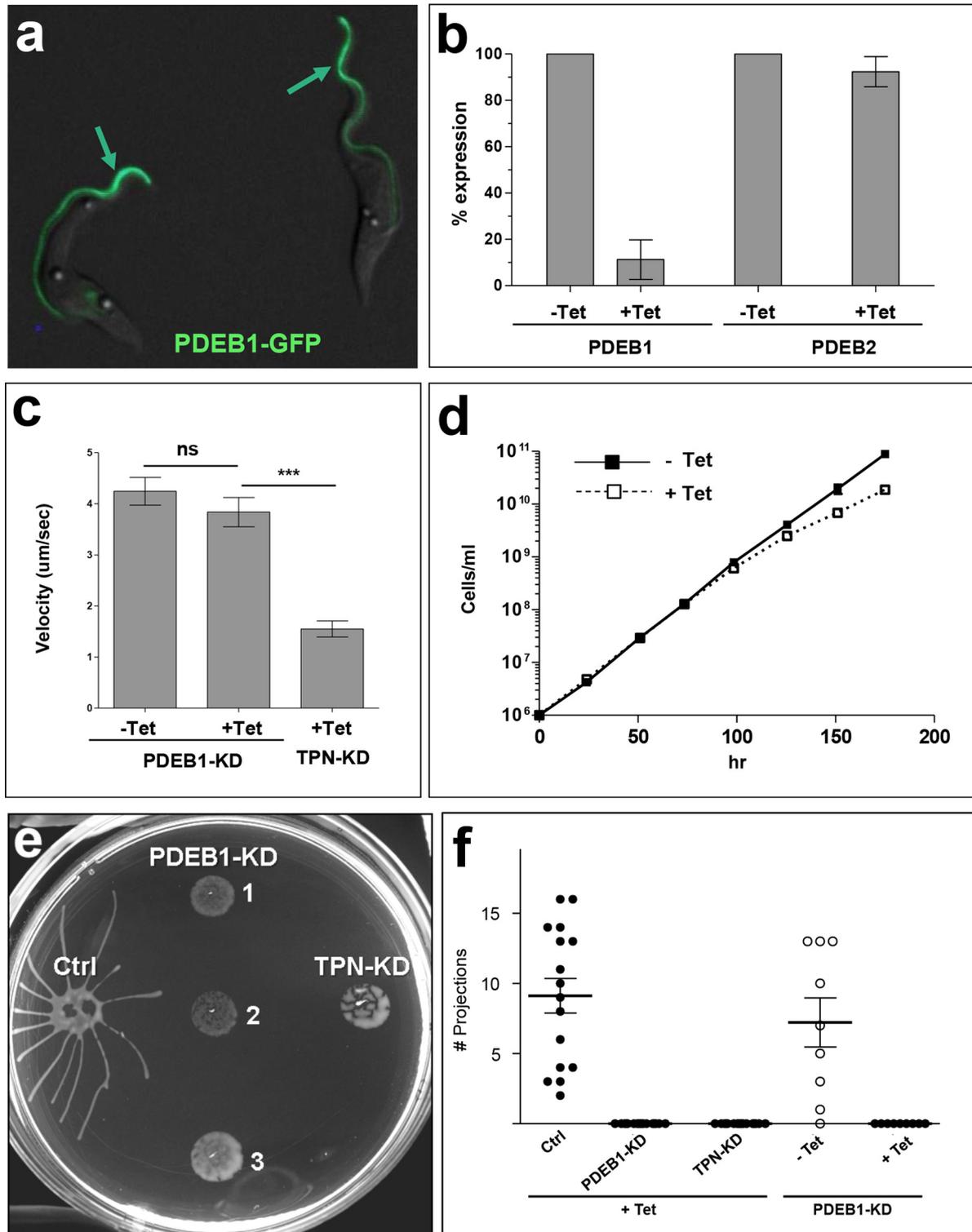
FIG 2 RNAi knockdown of PDEB1 blocks social motility without affecting motility of individual cells. (a) Fluorescence microscopy of procyclic cells expressing a PDEB1-GFP fusion protein, which is localized to the flagellum (arrows). (b) mRNA abundance for PDEB1 and PDEB2, as determined by qRT-PCR, in PDEB1 tetracycline (Tet)-inducible knockdown cells, maintained with or without tetracycline as indicated. Values are normalized to the −Tet expression level. (c) The cell motility of individual cells in suspension culture is shown for Tet-inducible PDEB1 knockdown cells (PDEB-KD), maintained with or without Tet as indicated. Trypanin knockdown (TPN-KD) motility mutants (47) were examined as a control. (d) Growth curve of PDEB1 Tet-inducible knockdown cells grown with or without Tet as indicated. (e) Social motility of 3 independent Tet-inducible PDEB1 knockdown clonal lines (clones 1, 2, and 3), control cells (Ctrl), and trypanin knockdown cells (TPN-KD). PDEB1-KD clone 1 was used for quantitative analysis as shown in panel f. (f) Quantitation of projections formed by control cells (Ctrl), PDEB1 knockdown cells (PDEB1-KD), or trypanin knockdown cells (TPN-KD), grown with or without tet as indicated. Error bars show ± standard errors. ***, $P < 0.0001$; ns = not significant.
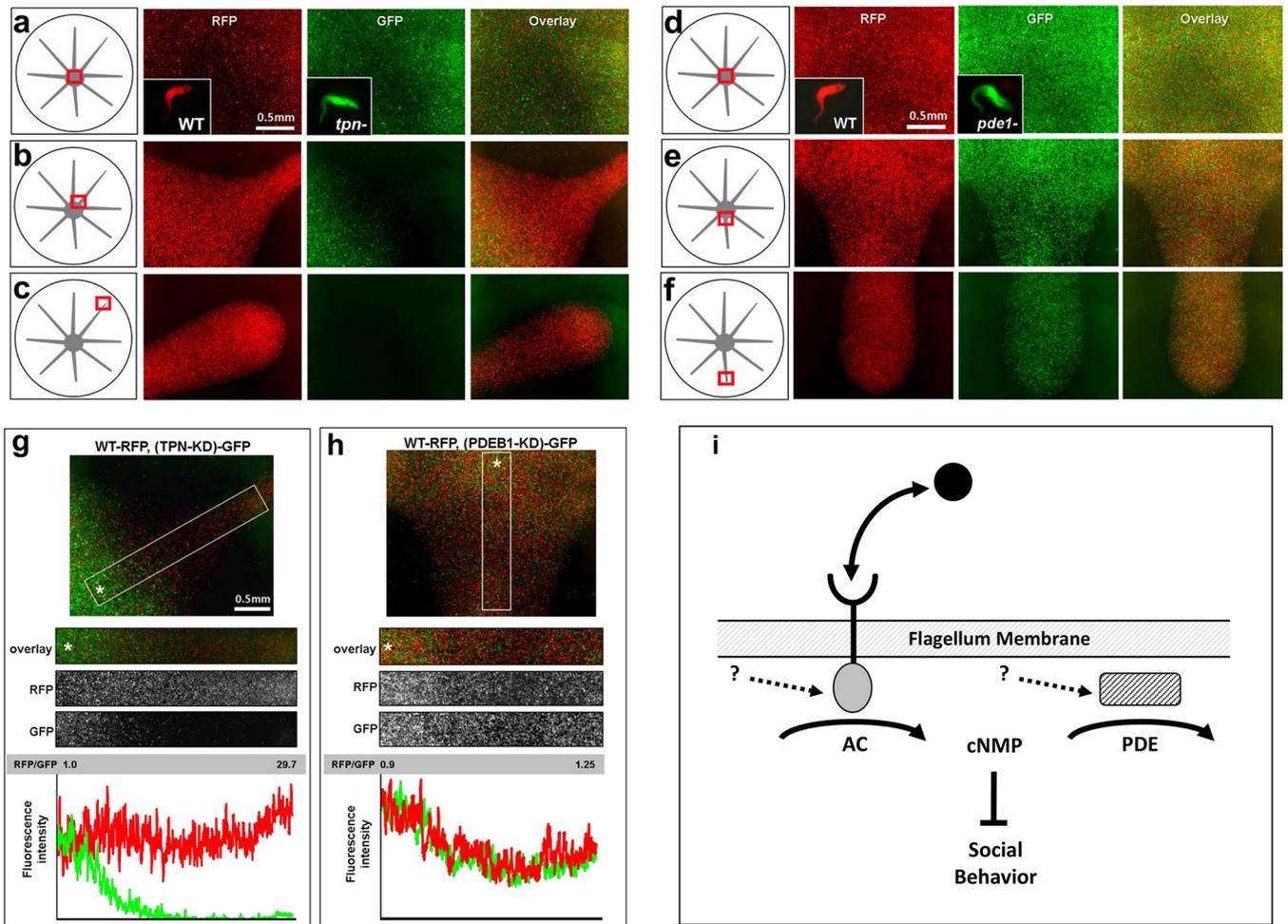
FIG 3 The SoMo(−) defect of PDEB1 knockdowns is complemented by wild-type cells provided in *trans*. (a to c) Social motility assays of mixed communities of wild-type–RFP and trypanin knockdown-GFP cells grown with tetracycline. (d to f) Social motility assays of mixed communities of wild-type–RFP and PDEB1 knockdown-GFP cells grown with tetracycline. Fluorescent images and merged images are shown at the right. The schematic illustrations on the left indicate where the fluorescent images were taken. Insets show representative fluorescent images of individual cells used for the analyses. (g and h) Quantitation of the relative levels of fluorescence of RFP and GFP at the indicated positions of projections formed by communities of wild-type–GFP parasites mixed with either trypanin knockdown-RFP (g) or PDEB1 knockdown-RFP cells (h). Merged and individual fluorescent channels are shown. (i) Generalized model for cyclic nucleotide regulation of social behavior. In *T. brucei*, receptor-type ACs (AC) in the flagellar membrane catalyze formation of cAMP (cNMP) and are responsive to extracellular ligands. cAMP attenuates social motility and is removed by flagellar phosphodiesterase (PDE). Loss of PDE elevates cAMP levels, blocking social motility, while loss of AC activity reduces cAMP levels, stimulating social motility. Dashed arrows indicate potential regulatory inputs. Similar systems operate in bacteria, except that the cyclic nucleotide produced is cyclic-di-GMP (26).

By exploiting a FRET-based cAMP sensor, we were able to directly visualize changes in intracellular cAMP concentrations following cpdA inhibition of PDE activity. Exact measurement of absolute intracellular cAMP levels is challenging. However, the cAMP concentration-dependent response of the epac1camps sensor has been titrated *in vitro*, allowing crude estimates of cAMP levels *in vivo* (42, 43). Based on titration of the epac1camps sensor *in vitro* (42, 43), the FRET ratio of ~0.9 in control *T. brucei* cells corresponds to a cAMP concentration below the 200 nM cAMP detection limit of the sensor. Addition of 100 nM cpdA increases cAMP levels to approximately 200 to 400 nM and completely blocks social motility, while addition of 500 nM or 1 μM cpdA increases cAMP levels to above 2 μM. These values reflect total cellular cAMP concentrations, and the changes within the flagellum are likely smaller. The combined pharmacological, gene knockdown, and FRET data indicate that flux through the cAMP

signaling pathway controls *T. brucei* social behavior. In addition, our findings provide the first demonstration, to our knowledge, of a specific function for an individual *T. brucei* phosphodiesterase, an enzyme that is the focus of current drug development efforts (36–38).

Several independent studies have implicated cAMP signaling as critical for *T. brucei* biology, development, and pathogenesis (22, 23, 32, 39, 50, 51), but the individual functions of adenylate cyclases (ACs) and PDEs are mostly unknown. Recent work provided an important advance by demonstrating a requirement for *T. brucei* bloodstream-specific ESAG4 adenylate cyclase function in host-parasite interaction and virulence in mice (51), and elegant genetic studies have identified candidate cAMP downstream effectors (22, 39). Notably, these studies focused exclusively on bloodstream parasites and even less is known regarding cAMP functions in procyclic forms. Recent studies identified procyclic-

specific adenylate cyclases, consistent with the suggestion that cAMP functions in parasite differentiation in the fly (52, 53), and at least two of these procyclic-specific ACs have been shown to regulate social motility (49). A primary contributor to the limited understanding of cAMP signaling in trypanosomes is the lack of convenient assays for cAMP function in live cells. Studies of surface-associated group behaviors have provided insights into cyclic nucleotide signaling in other microbes (26, 27, 54, 55). Thus, in addition to demonstrating a role for cAMP in social motility, our studies provide an important advance by establishing a convenient biological assay for dissecting cAMP signaling in *T. brucei*.

A microdomain model for flagellar cAMP signaling in *T. brucei*. cAMP signaling in eukaryotes is restricted to subcellular microdomains 50 to 100 nm in diameter (56–58). Compartmentalization is important for successful signal transduction, as it limits interference between pathways that use the same signal output, increases sensitivity through higher local cAMP concentrations, and enables transient activation and a rapid response to small changes in cAMP levels (56–58). PDE activity is critical for maintaining cAMP microdomains because it limits diffusion of cAMP to the immediate vicinity of synthesis (56–58). As such, only the proteins that sample these microdomains at the right time can transmit the cAMP-dependent signal. A microdomain model for cAMP operating within the trypanosome flagellum, as proposed by Oberholzer et al. (45), provides a potential explanation for the SoMo(−) phenotype observed upon PDEB1 knockdown or chemical inhibition. PDEB1 is localized throughout the flagellum (32). In separate work, we reported that a specific *T. brucei* adenylate cyclase, AC6, is localized to the tip of the flagellum and that the loss of AC6 results in hyperactivated social motility, i.e., in an effect opposite that of PDEB1 knockdown (49). The combined data are consistent with a model postulating that social motility is controlled by fluctuations of cAMP concentrations within a specific microdomain at the flagellum tip and that increased cAMP within this microdomain blocks social motility. In this model, PDEB1 is required to insulate the flagellum tip microdomain from cAMP originating elsewhere, for example, from other AC proteins in the flagellum (59). The diffusion coefficient of cAMP in olfactory cilia is estimated to be 2.7×10^{-6} cm$^2 \cdot$s^{-1} (60). Given a length of approximately 20 μm for the *T. brucei* flagellum, cAMP originating at any location would diffuse throughout the flagellum in less than 10 ms under unrestricted conditions. When PDEB1 is inhibited or knocked down, cAMP would be free to diffuse throughout the flagellum, thereby disrupting cAMP homeostasis and inhibiting social motility.

Conserved architecture of pathways that control social behavior in trypanosomes and bacteria. Cyclic nucleotide signaling regulates social behaviors in divergent microbes (6, 27, 61). A classic example is the social amoeba *Dictyostelium* sp., where cAMP functions as an extracellular chemoattractant and an intracellular signaling molecule that promotes development of multicellular fruiting bodies (55). A more directly comparable system is c-di-GMP regulation of bacterial swarming motility (26, 27, 62–64). In bacteria, c-di-GMP levels are controlled through the coordinate activity of diguanylate cyclases (dGCs) that synthesize the molecule and c-di-GMP-specific PDEs that degrade the molecule (54). Perturbation of either the PDE or dGC alters cellular c-di-GMP homeostasis and perturbs swarming motility (26, 27, 62–64). In *Pseudomonas aeruginosa*, knockout of the *bifA* gene, en-

coding a c-di-GMP-specific PDE, blocks swarming motility, owing to elevated intracellular c-di-GMP concentrations (62). Conversely, knockout of *sadC* or *roeA*, encoding dGCs, reduces cellular c-di-GMP concentrations and generates hyperswarmers (63, 64). The reciprocal effect of PDE and dGC mutants on bacterial swarming motility is analogous to what we observe for PDE and AC (49) mutants in *T. brucei* social motility. As such, our findings indicate a conserved architecture for the signaling pathways that control social behavior in trypanosomes and bacteria (Fig. 3i).

In *Pseudomonas* spp., it is postulated that c-di-GMP derived from specific dGCs, rather than total cellular levels of the molecule, controls swarming (64), and we suspect this may be analogous to the finding that only specific ACs influence *T. brucei* social motility (49). Trypanosomal ACs and PDEs contain known and suspected regulatory input domains, such as the GAF-A domain of PDEB1 and PDEB2 (65) and the periplasmic binding protein (PBP) domain of ACs (29, 65, 66). Thus, *T. brucei* PDE- and AC-mediated control of social motility may be regulated by endogenous molecules, as previously observed for PDEs and dGCs that control swarming motility in bacteria (26, 54).

PDE knockdowns are social motility competent but deficient in an intercellular process that promotes social motility. The genetic tractability of *T. brucei*, combined with the ease with which individual cells can be visualized, makes trypanosomes an excellent system for monitoring the behavior of individuals within a mixed population. Capitalizing on this, we found that the social motility defect of PDEB1 knockdowns can be complemented by WT cells provided in *trans*. To our knowledge, this is the first report of transcomplementation in parasitic protozoa, although such processes are well known in bacteria (46). Notably, individual fluorescent parasites in mixed communities retain either red or green fluorescence, indicating that complementation is not due to exchange of cytoplasmic material. We propose that PDEB1 knockdowns are competent for social motility but fail to produce something extracellularly that is necessary for social motility and that WT cells can provide this factor. The parasite-derived components responsible for transcomplementation of social motility remain unknown. These components might be specific proteins or small molecules, as seen, for example, in rescue of bacterial swarming mutants by outer membrane proteins transferred from wild-type cells in the same community (46, 67). Alternatively, they might be something that alters the physical environment, in analogy to the biosurfactants that promote swarming in bacteria (68). Trypanosomes are known to modify their environment by releasing proteins and uncharacterized low-molecular-weight factors as well as metabolic degradation products (69–71). Regardless of the mechanism, our findings reveal a form of cell-cell communication that was not previously recognized in these organisms.

MATERIALS AND METHODS

Cell culture. 2913 procyclic cells (72) were subjected to two rounds of enrichment by growth on semisolid agarose plates, isolating parasites from the tips of social motility projections (24), cloning by limiting dilution, and repeating. These cells, 2913^{MO2}, were used as controls (WT) for all experiments and were used as the parental line for all transfections. Cell culture, transfections, and isolation of clonal lines by limiting dilution were done as previously described (48).

PDEB1-GFP. The TbPDEB1 (Tb09.160.3590) open reading frame was PCR amplified from plasmid pCR-TbrPDEB1 (gift of T. Seebeck, Bern, Switzerland) using primers B1GAPRONEf and B1GAPRONEr

(primer sequences are provided below). The PCR product was cloned upstream of GFP in pG-eGFP-Blast (gift of Isabel Roditi, University of Bern) (73). The construct was verified by sequencing, linearized with SpeI, and transfected into 2913^{MO2} cells. Expression of the PDEB1-GFP fusion protein was determined by fluorescence microscopy using an Axioskop II microscope (Zeiss, Inc., Germany).

TbPDEB1 RNAi knockdown cells. A 279-bp fragment corresponding to bp 150 to bp 428 of TbPDEB1 (Tb09.160.3590) was subcloned from an RNAi knockdown construct previously published (32) into the p2T7-Ti-B vector (74) using HindIII and BamHI. The construct was verified by sequencing, linearized with NotI, and transfected into 2913^{MO2} cells, and clones were obtained by limiting dilution. Clone B1-1 was used for further analysis. Quantitative reverse transcription-PCR (qRT-PCR) was performed as previously described (75) using mRNA from cells at 72 h postinduction with or without 1 μg/ml tetracycline. Each sample was analyzed in duplicate using three independent RNA preparations. The TbPDEB1-specific primers were qRTPDEB1-f and qRTPDEB1-r. The TbPDEB2-specific primers were qRTPDEB2-f and qRTPDEB2-r (primer sequences are provided below). Values were normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Tb927.6.4280) and RPS23 (Tb10.70.7030) as described previously (75).

FRET reporter and RFP and GFP reporter cells. The epac1camps sensor was PCR amplified from plasmid pcDNA3-YFP-epac1-CFP (42) using primers EPACcamp-f and EPACcamp-r1 (primer sequences are provided below). The PCR product was cloned into pCRII Topo (Invitrogen), and the sequences were verified by sequencing. The epac1camps sequence was then subcloned into the HindIII and XbaI sites of pLew100 (72). The construct was linearized with NotI and transfected into 2913^{MO2} cells. epac1camps expression was detected following tet induction by fluorescence microscopy. RFP-expressing and GFP-expressing cells were obtained by transfection with SpeI-linearized pG-RFP-Blast and pG-eGFP-Blast (gift of Isabel Roditi, University of Bern) (73), and stable transfectants were selected using 10 μg/ml blasticidin and cloned by limiting dilution.

Primer sequences. The primers for generating PDEB1-GFP were B1GAPRONEf (5′-ATCTCGAGATGTTCATGAACAAGCCCTTTGG-3′) and B1GAPRONEr (5′-ATACCGGTAAACGAGTACTGCTGTTGTT GCC-3′). The XhoI and AgeI restriction sites are underlined.

The primers for qRT-PCR analysis of PDEB1 RNAi knockdown cells were as follows. The TbPDEB1-specific primers were qRTPDEB1-f (5′-T TCATGAACAAGCCCTTTGG-3′) and qRTPDEB1-r (5′-TGATAGCGA GCGAGGATTG-3′). The TbPDEB2-specific primers were qRTPDEB2-f (5′-CGGTGGTCGTCATCTGCTTG-3′) and qRTPDEB2-r (5′-GGAAT CATAAGGGGCGACCA-3′).

The primers for constructing FRET reporter cells were as follows: for the epac1camps sensor, EPACcamp-f (5′-TCACTATAGGGAGACCC<u>AA GCTT</u>-3′) and EPACcamp-r1 (5′-TA<u>ACTAGT</u>AGCGGGCGCTTACTT GTAC-3′). The HindIII and SpeI sites are underlined. The reverse primer deletes the internal NotI cloning site at the 3′ end of the cyan fluorescent protein (CFP) moiety.

FRET. epac1camps expression was induced for 24 to 48 h using 1 μg/ml tetracycline. Cells were washed once in culture medium and resuspended to 1.5 × 10^8 cells/ml in the same medium. A 100-μl volume of cells was added to each well of a 96-well black polystyrene Microplate (Greiner). An additional 100 μl of medium containing cpdA was added to each well to give final concentrations of 100 nM, 500 nM, and 1 μM cpdA. Several controls were performed for normalization of the reading. (i) epac1camps-expressing cells were treated with just dimethyl sulfoxide (DMSO), which is the solvent for cpdA. (ii) 2913^{MO2} parental cells, i.e., without epac1camps, were treated with cpdA. Immediately following addition of cpdA or DMSO, emission ratios were determined using a photospectrometer (FlexStation) and SoftMax Pro 4.8 software. Filter settings were as follows: excitation, 436 nm; emission, 480 nm (cutoff, 475 nm) or 535 nm (cutoff, 530 nm). The experiments at each time point (41-s inter-

vals) were performed in triplicate, and the results are reported ± standard deviations.

Motility traces and social motility assays. Motility trace experiments were done as previously described (76). Movies were exported at a resolution of 1 fps and analyzed using automatic tracking in MetaMorph software (Molecular Devices). For social motility assays, cells were diluted for three consecutive days to 3 × 10^6 cells/ml in suspension culture. A 4% (wt/vol) solution of SeaPlaque GTG agarose (Lonza) in MilliQ water was sterilized for 20 min at 250°C, evaporated water was replaced with sterile MilliQ water, and the stock solution was cooled to 70°C. Stock was diluted to 0.4% with prewarmed culture medium and then maintained at 37°C and supplemented with cpdA, DMSO, 1 μg/ml tetracycline, or methanol as indicated. Medium (13 ml) was poured into 100-mm-by-15-mm petri dishes (Fisherbrand), and plates were dried open for 1.5 h in a laminar flow hood. A total of 5 × 10^7 cells at 1.2 × 10^7 to 1.6 × 10^7 cell/ml were added to the agarose surface, and plates were sealed with Parafilm and incubated at 27°C and 5% CO$_2$.

For cAMP analogue experiments, social motility plates were supplemented with 8-Br-cAMP (Sigma) as cell-permeable cAMP; Rp-8-Br-cAMPS (Sigma) as cell-permeable, hydrolysis-resistant cAMP; 8-pCPT-2′-O-Me-Ado (BioLog, Germany) as cell-permeable adenosine; or 8-pCPT-2′-O-Me-5′-AMP (BioLog, Germany) as cell-permeable AMP; and assays were performed as described above, with plates kept in the dark. Concentrations to be used were determined by starting with 8-Br-cAMP at 150 μM, the concentration that induces stumpy formation in *T. brucei* (44), and titrating down to find the minimum concentration that inhibited social motility, which was 1 to 10 μM. The other compounds were then also tested at these concentrations. Inhibition of social motility was not due to inhibition of cell proliferation (not shown). AMP and adenosine gave inhibition when the concentration was raised to 20 μM (not shown), though this was likely due to nonspecific effects, as it is ~200-fold higher than the concentration (84 nM to 125 nM) that blocks proliferation and induces stumpy formation in bloodstream-form cells (44). In contrast, the concentration of externally added cAMP required to inhibit social motility is 10-fold to 100-fold lower than what is required to induce stumpy formation (44). Moreover, because cAMP analogues added to *T. brucei* cells are unstable (44), the effective cAMP concentration is actually much lower than what was added. It is recognized that cAMP analogues have potential off-target effects and may act as agonists or antagonists of cAMP effector proteins (44, 77), and we therefore consider these experiments to be an adjunct to those with the more specific pharmacological *T. brucei* PDE inhibitor cpdA (37) and gene-specific PDEB1 RNAi.

Colony lifts to assess GPEET expression. GPEET expression in cells on plates was assessed by colony lifts. Nitrocellulose membrane was incubated on the surface of social motility plates (day 4 postplating) for 5 min. The membrane was removed, air-dried, and stained with Ponceau S prior to imaging to visualize total protein. The membrane was washed in MilliQ water, blocked in phosphate-buffered saline (PBS) containing 5% powdered milk, and stained with anti-GPEET antibody (1:10,000) overnight at 4°C. The membrane was washed 3 times for 10 min in PBS–0.05% Tween 20 and stained with secondary antibodies coupled to horseradish peroxidase (HRP) (1:2,500) and washed as described above prior to development using an enhanced chemiluminescence (ECL) kit and exposure to film.

Social complementation assay. GFP-expressing cells and RFP-expressing cells were mixed in a 1:1 ratio and plated as described above for social motility assays. Fluorescence imaging of cells on plates was done 96 to 120 h postplating using an Axioskop II microscope (Zeiss, Inc., Jena, Germany) with a 2.56LD Plan NeoFluor objective and a GFP bandpass emission filter (41017; Chroma Technology) or an RFP bandpass emission filter (41007; Chroma Technology) on a Zeiss Axiovert microscope using an AxioCam camera. Pictures were processed using Adobe Photoshop, fluorescence intensities were determined using ImageJ (NIH), and values were plotted using GraphPad PRISM.

SUPPLEMENTAL MATERIAL

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