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Trypanosome Signaling—Quorum Sensing

Keith R. Matthews

Institute for Immunology and Infection Research, Ashworth Laboratories, School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3FL, United Kingdom;
email: keith.matthews@ed.ac.uk

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Trypanosoma brucei, trypanosomiasis, quorum sensing, coinfection, differentiation, transmission

Abstract

African trypanosomes are responsible for important diseases of humans and animals in sub-Saharan Africa. The best-studied species is *Trypanosoma brucei*, which is characterized by development in the mammalian host between morphologically slender and stumpy forms. The latter are adapted for transmission by the parasite's vector, the tsetse fly. The development of stumpy forms is driven by density-dependent quorum sensing (QS), the molecular basis for which is now coming to light. In this review, I discuss the historical context and biological features of trypanosome QS and how it contributes to the parasite's infection dynamics within its mammalian host. Also, I discuss how QS can be lost in different trypanosome species, such as *T. brucei evansi* and *T. brucei equiperdum*, or modulated when parasites find themselves competing with others of different genotypes or of different trypanosome species in the same host. Finally, I consider the potential to exploit trypanosome QS therapeutically.

Contents

1. BACKGROUND

African trypanosomes are single-celled protozoan parasites that live an extracellular lifestyle both in their mammalian host and in their tsetse fly vector (105). *Trypanosoma brucei brucei* and *Trypanosoma congolense* are restricted to sub-Saharan Africa because their transmission by tsetse flies is obligatory. *Trypanosoma vivax*, in contrast, has spread outside sub-Saharan Africa because it can also be transmitted mechanically (i.e., without cyclical development) by other biting flies (39, 65). Close relatives of *T. brucei brucei* can also be transmitted either mechanically between mammals (*T. brucei evansi*, causing the disease surra) or venereally between horses, mules, and donkeys (*T. brucei equiperdum*, causing dourine) (2, 21, 22).

T. brucei brucei is broadly infective across mammals but cannot infect humans and some primates. This is due to the serum of these resistant species containing the trypanolytic factor ApoL1, a component of high-density lipoprotein. When taken up via the haptoglobin-related protein receptor, ApoL1 leads to trypanosome lysis (106). Two subspecies of *T. brucei* have evolved mechanisms to escape ApoL1-mediated killing and can therefore infect humans: *T. brucei rhodesiense*, via SRA (serum resistance associated) (110), and *T. brucei gambiense*, via TgsGP and reduced ApoL1 uptake (25, 100). Currently, 97% of human cases of trypanosomiasis are due to *T. brucei gambiense*, which causes chronic infection, and are focused in West Africa. *T. brucei rhodesiense* causes a more rapid disease progression and is focused in East Africa. Human cases of trypanosomiasis worldwide have declined in recent years to around 2,000 per annum (46), generating the hope of disease elimination, although this optimism is tempered by the recent discovery of asymptomatic carriers (24).

More important than human disease is the indirect impact of trypanosomes on livestock health and hence economic development. African animal trypanosomiasis (AAT) caused by *T. brucei brucei*, *T. congolense*, and *T. vivax* has an estimated multibillion-dollar annual cost to agricultural productivity in Africa, exacerbating poverty and its health impacts for human populations (39). Each of the species that contributes to AAT exhibits interesting and distinct biology, and previous assumptions that paradigms established in *T. brucei* would be readily transferable to *T. congolense* and *T. vivax* have not been borne out. In part this reflects the different life cycles of each species (78) (**Figure 1*a***). *T. brucei* and *T. congolense* initially establish themselves in the gut of the tsetse fly, but

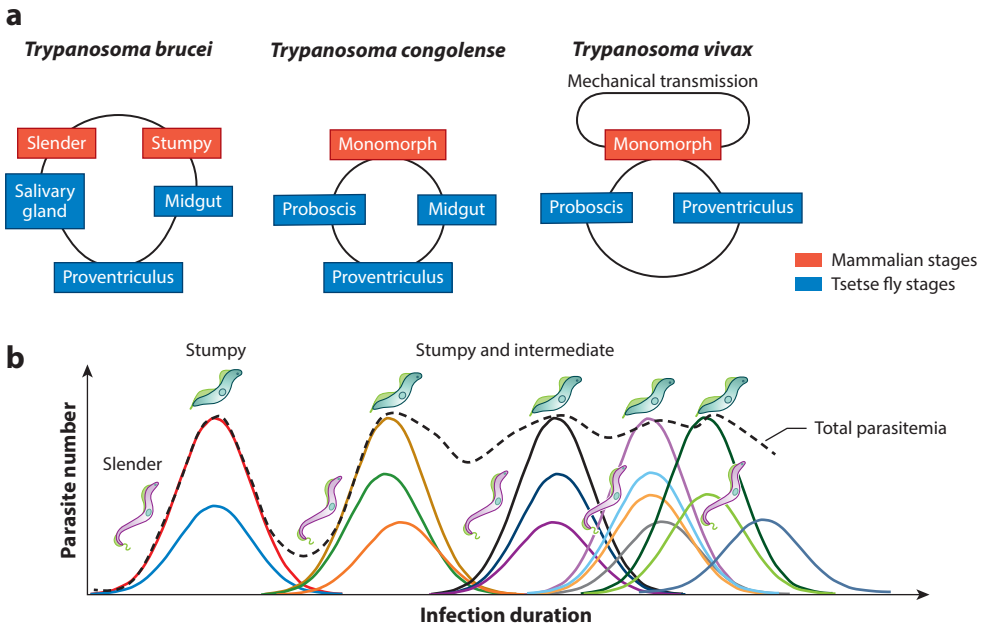

Figure 1

(*a*) Life cycle of different African trypanosome species. *Trypanosoma brucei* ssp. (including *T. brucei brucei*, *T. brucei rhodesiense*, and *T. brucei gambiense*) are pleomorphic in their mammalian host, exhibiting proliferative slender and G1/G0-arrested, transmissible, stumpy forms. These compartmentalize in the blood, adipose tissue, and skin and, later in infection, the central nervous system. Upon uptake in a tsetse fly blood meal, the stumpy forms differentiate to procyclic forms in the fly midgut. These then migrate to the proventriculus and then salivary glands, where epimastigotes and then infective metacyclic forms develop. *Trypanosoma congolense* does not exhibit morphological heterogeneity in the mammalian host and is predominantly present in the blood and microvasculature. In the tsetse fly, it initially develops as procyclic forms in the midgut, and then it migrates to the proboscis, where it develops infective metacyclic forms. *Trypanosoma vivax*, like *T. congolense*, is monomorphic in the mammalian host, and parasites are largely maintained in the circulation. *T. vivax* can be transmitted mechanically without tsetse fly involvement, or it can be transmitted by tsetse flies, attaching in the mouthparts of the fly and undergoing full development in that location. (*b*) The infection profile of *T. brucei*, representing chronic infection. The early wave of parasitemia is dominated by one or a small number of antigenic variants (each antigenically distinct type is represented by a colored line), with the ascending parasitemia highly enriched for proliferative slender forms. As parasite numbers increase in the first wave, intermediate forms and then stumpy forms accumulate, almost reaching homogeneity. In subsequent parasitemic waves, the antigenic complexity of the infection increases and the relative ratio of stumpy/intermediate forms to slender forms fluctuates, although stumpy and intermediate forms usually predominate. This balance sustains transmissibility, whereas the slender forms in the population give rise to new antigenic variants. The total parasitemia throughout the infection is represented by a dashed curve.

T. brucei then matures in the salivary glands whereas *T. congolense* matures in the proboscis. The *T. vivax* life cycle is significantly abbreviated, with parasites entering the tsetse fly and then adhering in the mouthparts of the fly during further maturation to infective forms. This review focuses on the developmental biology of *T. brucei*, which perhaps exhibits the most extreme adaptations with respect to transmission biology of all the African trypanosome species. However comparative features and regulatory mechanisms are also discussed in relation to *T. congolense*, as well as the loss of developmental complexity exhibited by *T. vivax*, *T. brucei evansi*, and *T. brucei equiperdum*, which has allowed these parasites to spread beyond Africa.

2. LIFE IN THE MAMMALIAN HOST

2.1. Antigenic Variation

As an extracellular parasite, *T. brucei* lives exposed to the host humoral immune response and sustains prolonged infections through its extreme and sophisticated capacity for immune evasion via antigenic variation (32, 90). The entire parasite surface is enshrouded in a densely packed and uniform forest of protein composed of a single protein type—the variant surface glycoprotein (VSG). *VSG* genes are transcribed by RNA polymerase I at telomeric *VSG* expression sites that also comprise several cotranscribed expression site–associated genes (*ESAG*s) (42). The genome contains approximately 20–25 expression sites, each able to transcribe a single *VSG* gene, although only one is ever active at a time (40). Tsetse fly salivary gland metacyclic forms generated as a preadaptation for the infection of a new mammalian host express *VSG* genes from a distinct subset of shorter expression sites encoding few or no *ESAG*s (9).

The *VSG* gene repertoire is extensive, making up approximately 20% of the genome (15). In addition to *VSG* genes in expression sites, there are extensive arrays of silent *VSG* genes in subtelomeric clusters, and also a further pool of genes on the trypanosome's 50- to 150-kb minichromosomes. *VSG* genes can only be expressed within an active expression site, and the majority of silent genes elsewhere in the genome are pseudogenes (58). As a consequence, changing the expressed *VSG* gene requires silent genes to be relocated to an expression site and, if pseudogenes, to be recombined with other VSG genes and gene fragments to create functional mosaics.

The mechanism of *VSG* gene activation contributes to the infection dynamics of the parasite in vivo (64). Early on, expression site–resident *VSG* genes are preferentially expressed, and this is followed by expression of intact genes that can relocate from subtelomeric arrays or silent expression sites to the active expression site through gene conversion. Later in infection, once the available easily activated *VSG* genes have been expressed and antibodies raised against their antigen type, parasites expressing functional mosaic *VSG* types can appear. These require within–coding region recombination, such that loose lineages of related genes are expressed (50). This differs from early in the infection, when switches between *VSG* genes are coding region–independent because flanking repeats initiate gene conversion events. During the course of infection, the complexity of expressed VSG types increases—early on only one or a few variants may dominate at any one point, but later many variants can coexist within an infected host (68) (**Figure 1*b***).

Interestingly, frequent *VSG* mosaic gene assembly appears to be a characteristic of *T. brucei* that is not shared with *T. congolense* or *T. vivax*, where the prevalence of *VSG* pseudogenes is low (44, 86). Moreover, the structure of expression sites in *T. congolense* differs from that in *T. brucei* (1), with distinct flanking repeat sequences and *ESAG*s. *T. vivax* lacks the repeat elements that contribute to *VSG* gene rearrangement in *T. brucei*, and the *VSG* repertoire appears more limited (at least in terms of antigenic diversity) and conserved between isolates from diverse geographic locations (86). This is consistent with the apparent absence of mosaic gene formation in *T. vivax* and the classical observations that animals are able to self-cure from infection, potentially through exhaustion of the available antigen repertoire (8).

2.2. Developmental Biology

A second significant and potentially dominant contributor to the infection profile of African trypanosomes in their mammalian host concerns their production of transmission stages. *T. brucei* proliferates in mammalian hosts as slender forms characterized by acicular morphology, with a central ovoid nucleus and terminal kinetoplast (mitochondrial genome) positioned at the extreme posterior end of the cell. The flagellum wraps in a left-handed helix along the length of the cell, is closely tethered via the flagellar membrane, and often extends several microns beyond

the anterior limit of the cell body (105). Parasites in this form are highly mobile and move with a spiral path that can be directional but also shows occasional tumbling and altered trajectory (41). In this form, the parasites replicate by longitudinal binary fission approximately every six hours (13, 107). Metabolically, the parasites are characterized by their reliance on the glycolysis of blood glucose; the parasite's single mitochondrion is tubular, lacks cristae, and does not have an operational electron transport chain (92).

During each wave of parasitemia, *T. brucei* slender forms transition via ill-defined intermediate forms to morphologically stumpy forms that are arrested in G1/G0 phase (59, 111). These are characterized by their broader morphology, pronounced undulating membrane between the flagellum and cell body, and variable positioning of the cell nucleus and kinetoplast. The flagellar pocket (the site of endocytosis and exocytosis for the trypanosome cell) is often significantly enlarged. This expansion can displace the kinetoplast and may be related to the endocytosis of VSG-bound antibody transported by hydrodynamic forces generated by trypanosome motility (37). The nucleus, rounder than in slender forms, is often positioned closer to the cell posterior and can even become fully terminal in so-called posteronuclear stumpy forms. The stumpy forms show less vibrant motility and do not show directional movement. With respect to metabolism, stumpy forms can utilize α-ketoglutarate and mitochondrial activity increases, reflected in a more elaborate mitochondrial structure (34).

The generation of stumpy forms aids parasite transmission, and unlike slender forms, these forms express regulatory molecules required for trypanosome development in the fly. Further, stumpy forms are more resistant to pH stress, proteases (70), and antibody-mediated lysis (62), and they resist the alternative pathway of complement through their expression of a factor H receptor (54). This receptor is not present on slender forms, and its expression contributes to the transmissibility of stumpy forms in the tsetse fly gut. Stumpy forms also express two members of a family of PAD (protein associated with differentiation) membrane transporter proteins, PAD1 and PAD2, these being involved in the environmental sensing of the parasites as they are taken up in a blood meal (31). Specifically, PAD1 and PAD2 can transport citrate, a carboxylate in blood that stimulates differentiation of the parasites to their tsetse fly midgut form, procyclics. Reduced temperature in the fly upregulates the expression of PAD proteins, rendering parasites sensitive to blood citrate and stimulating onward development. Signal perception upon entering the fly gut involves a phosphatase signaling cascade (93, 95), with regulatory components of development initially concentrated at a specialized site close to the flagellar pocket of stumpy forms, the stumpy regulatory nexus (STuRN) (75, 94).

As with antigenic variation, development of *T. congolense* and *T. vivax* during infection is different from that of *T. brucei* (**Figure 1*a***). Neither *T. congolense* nor *T. vivax* generates a morphologically stumpy form, but both show G1/G0 arrest at high density, similar to *T. brucei* stumpy forms (85, 89). Furthermore, both species have orthologs of the genes in *T. brucei* required for the development of stumpy forms, and in at least one case, a *T. congolense* ortholog can restore stumpy formation in a *T. brucei* null mutant, demonstrating functional equivalence. This suggests that *T. congolense*, at least, has a cryptic transmission stage (cryptostumpy). Gene expression analysis through the first wave of parasitemia of *T. congolense* (i.e., from low to high parasite density) reveals less-dramatic changes than those seen with *T. brucei*, where strong regulation of mRNAs involved in glycolysis and cell cycle progression, in particular, is observed (88). In contrast, *T. congolense* appears to have more subtle regulation, with the exception of transferrin receptor mRNAs and at least one surface protein family. Indeed, the transcriptome of these parasites appears more similar to those of tsetse fly stages, whether in the early or peak stages of the parasitemia. As with their capacity for antigenic variation, therefore, *T. brucei* slender forms appear to represent an atypical extreme, whereas intermediate and stumpy forms are more similar to *T. congolense* with respect to

their preadaptation for life in the tsetse fly. Consequently, although the morphological development of stumpy forms is the obvious distinction between *T. brucei* and other African trypanosomes, in fact it may be the slender form that is the developmental anomaly.

2.3. Compartmentation

African trypanosome species have long been known differ in their compartmentation in mammalian hosts. For the most part, experimental infections have focused on *T. brucei* parasites in the bloodstream of rodents. However, *T. brucei* also concentrates in the adipose tissue (97) and skin (23, 26). Slender and stumpy forms are detected in both niches, although their relationship to the equivalent morphologies in the bloodstream are not fully known and metabolic adaptation appears to occur (87). Similarly, their capacity for antigenic variation and the flux of variants between the bloodstream and adipose and skin compartments are under exploration (60). With respect to transmission potential, the presence of skin parasites has obvious relevance, particularly where parasitemia is undetectable.

Unlike *T. brucei*, *T. congolense* adheres in the blood vessels of infected hosts, particularly the microvasculature (5), but has not been shown to occupy adipose tissue. Some isolates can also bind to red blood cells (6). *T. vivax*, in contrast, is predominantly a blood-circulating parasite, although it can be detected in other tissues, including those of the skin and gonads (87).

3. SOCIAL INTERACTIONS IN THE MAMMALIAN HOST

3.1. Evidence for Community Action

The polymorphism of trypanosomes in their mammalian host was quantitated by Bruce et al. (20) and interpreted by Robertson (76) in 1912. Bruce and colleagues highlighted the morphological heterogeneity of trypanosomes in a bloodstream infection and described the presence of *T. brucei* long and slender and short and stumpy forms. After monitoring changes in the type and proportion of morphological forms during the parasitemia, Robertson concluded that only stumpy forms were responsible for continuing establishment in the tsetse fly. This view was supported in 1960 by Wijers & Willett (108), with contradictory evidence prior to this (3, 29, 101) likely due to the permissive nature of recently emerged flies. Critically, Robertson (76, p. 528) also speculated on the nature of the factors controlling the trypanosome's infection profile, predicting both antibody-mediated lysis ("liberation in the serum of protective substances") and parasite-mediated density sensing ("self-conditioned processes within the body of the parasite"). Further, she considered the infection profile would result from both components in "a very complex aggregate of interacting circumstances which we have at present practically no knowledge" (76, p. 528). Slightly over 100 years later, this incredible insight is being unraveled in molecular detail.

Early descriptions of a parasite density-dependent mechanism controlling trypanosome development came from Seed & Sechelski (83, 84). They proposed the existence of a stumpy inducing factor whose accumulation in vivo promoted development, this being different in different host backgrounds (83). The same authors also achieved passive transfer of this activity through inoculation of serum from infected animals (84). With the development of methods to culture developmentally competent trypanosomes on semisolid media, further evidence of a stumpy inducing factor released by parasites and generating density-dependent growth control of the parasites was provided (102). Importantly, the effects of parasite-conditioned culture media were shown to reflect physiological differentiation of the parasites seen in vivo, with density-dependent cell cycle arrest, morphological change, NADH diaphorase activity, and enhanced capacity for development into procyclic forms (tsetse fly midgut forms) (74). These responses all reflected the hallmarks of stumpy forms and supported the existence of a released stumpy induction factor (SIF) (104)

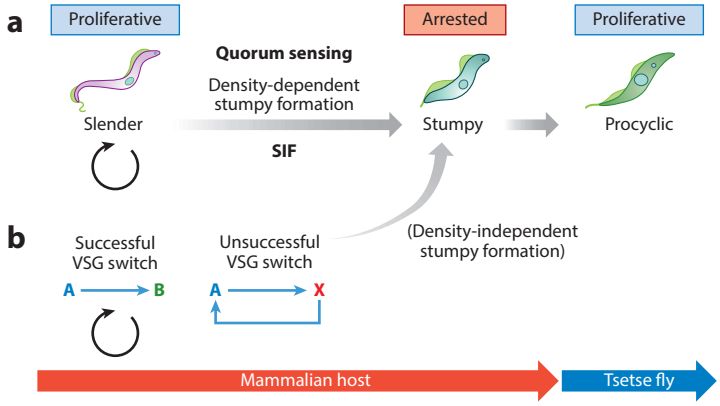

Figure 2

Mechanisms of stumpy induction. Stumpy formation apparently operates through two distinct processes. (*a*) The first is most dominant, particularly in early infection, and involves density-dependent signaling between parasites in the population. This results from the accumulation (locally or systemically) of the quorum-sensing signal: stumpy induction factor (SIF). This signal stimulates proliferative slender forms to arrest in their cell cycle and differentiate to morphologically distinct, transmission-adapted, stumpy forms. These differentiate efficiently to tsetse fly midgut procyclic forms when ingested in a tsetse fly bloodmeal. (*b*) The second mechanism involves a density-independent response as parasites undergo antigen type switching. If the switch involves activation of an expression site without successful generation of a new antigen type, parasites can arrest and potentially reactivate the previously expressed variant surface glycoprotein (VSG). Alternatively, they may differentiate to stumpy forms. This phenomenon is likely to operate more frequently later in infections, when successful antigenic switches are less probable.

consistent with earlier mathematical modeling of the infection dynamics (82). Critically, these in vivo and in vitro studies did not require a role for the immune system in promoting stumpy formation, and parasite development occurs in both immunocompetent and immunocompromised rodent models (4). Nonetheless, it appears that the host environment can shape the kinetics of the developmental progression of trypanosomes, with the density at which differentiation occurs dependent on the host species or mouse strain (55–57). Mathematical modeling also provides a better fit to experimental data when both density-dependent differentiation and immune control are considered in combination (52, 99). A further interaction between parasite development and the host concerns changes in the activity of expression sites during antigenic variation of the parasite. Specifically, parasites that fail to successfully activate a new VSG may initially enter reversible G1 arrest before commitment to stumpy formation. This developmental progression might provide parasites with an escape mechanism if switching to a new variant is unsuccessful, as might occur more frequently later in infections as mosaic genes are generated (11, 112). In combination, these observations suggest development in the mammalian host is dominated by parasite density but potentially modulated by host factors and the parasite's immune evasion mechanism (**Figure 2**).

A density-dependent model of trypanosome differentiation controlled by a parasite-derived soluble entity is closely aligned with long-established features of quorum sensing (QS) among social microbes, i.e., unicellular organisms that exhibit coordinated group behaviors for selective benefit (69). In gram-negative bacteria, small-molecule autoinducers allow communication between cells (72, 91), whereas in gram-positive bacteria oligopeptides are common messages. In each case intricate control feedback loops generate sophisticated and responsive signaling systems able to regulate the coordinated production of enzymes, mating factors, toxins, etc. to promote cooperation or competition within and between species and in different environments.

3.2. Molecular Control of Quorum Sensing

The purification of SIF from parasite cultures has proved challenging. Activity-guided analysis was initially hampered by the absence of a quantifiable and rapid bioassay for the development of stumpy forms (74), but this limitation was overcome as molecular markers specific for the stumpy forms were identified, allowing regulatory sequence motifs to be adapted for reporter cell line generation (51). Nonetheless, such assays remained very challenging for the identification of SIF because active fractions inevitably inhibit cell growth as a component of the developmental response such that careful integration of reporter activity and cell number was necessary. Further, whether active SIF comprised a single or multiple factors was unclear, and nonphysiological reporter activation can occur where parasites are exposed to metabolic stress. Consequently, impressive and intense effort in a number of laboratories ultimately did not identify physiologically verifiable SIF activity.

More recently an experimentally supported model for density-dependent stumpy induction has emerged with strong parallels with the QS response of gram-positive bacteria. Specifically, study of a member of the membrane-spanning GPR89 protein family fortuitously provided evidence for its role in stumpy formation (77). This molecule is located on the surface of slender cells but not stumpy cells. Further, when overexpressed, the molecule promotes differentiation to stumpy forms in developmentally competent parasites, but not in laboratory-selected cell lines that have lost this capacity through serial passage in culture (monomorphs). GPR89 family proteins were originally characterized as orphan G protein–coupled receptor (GPCR) proteins, with two representatives in plants (GTG1 and GTG2) being responsive to abscisic acid (71). More detailed analysis indicates that plant GTG1 and GTG2 are not GPCRs (96), and moreover, trypanosomes lack the components of a GPCR signaling pathway, a deficit characteristic of all excavate eukaryotes (16). Instead, in silico modeling predicted similarity between *T. brucei* GPR89 (*Tb*GPR89) and the structures of several characterized oligopeptide transporters. Interestingly, these molecules—which are well conserved from bacteria to mammals—are missing in African trypanosomes despite their presence in other kinetoplastid parasites. This raises the possibility that *Tb*GPR89 replaces this function or unifies oligopeptide signaling to prevent input and signal interference through more than one transport system. Supporting a role for oligopeptide transport in stumpy formation, expression of a bacterial oligopeptide transporter in slender form parasites provokes premature stumpy formation, as does exposure of trypanosomes to complex oligopeptide sources or chemically synthesized dipeptide or tripeptide mixtures (77).

Given that oligopeptides can promote stumpy formation, how can this be used in density sensing by the parasite in vivo? The answer to this question was provided by earlier studies showing that parasites release active peptidases into the bloodstream of hosts, through either secretion or lysis of parasites (10, 66). These have the potential to generate oligopeptides in the environment in proportion to the number of parasites, providing a proxy for parasite density. Accordingly, engineering trypanosomes to ectopically overexpress peptidases enhances differentiation of not only the expressing parasites but also a coinfecting reporter line, demonstrating the generation of a paracrine signal (77).

All of these experiments provide support for the idea that oligopeptides, generated by parasite-released peptidases, act as SIF (**Figure 3**). Definitive evidence that this is the only signal for QS remains lacking because *Tb*GPR89 is apparently essential for bloodstream parasites, preventing its deletion and consequent elimination of stumpy formation. However, it is clear that oligopeptide signaling operates through the physiological SIF signaling pathway, because parasites that are unresponsive to SIF do not respond to oligopeptides or ectopic *Tb*GPR89 expression (112). Furthermore, the use of oligopeptides as the major or sole contributor to

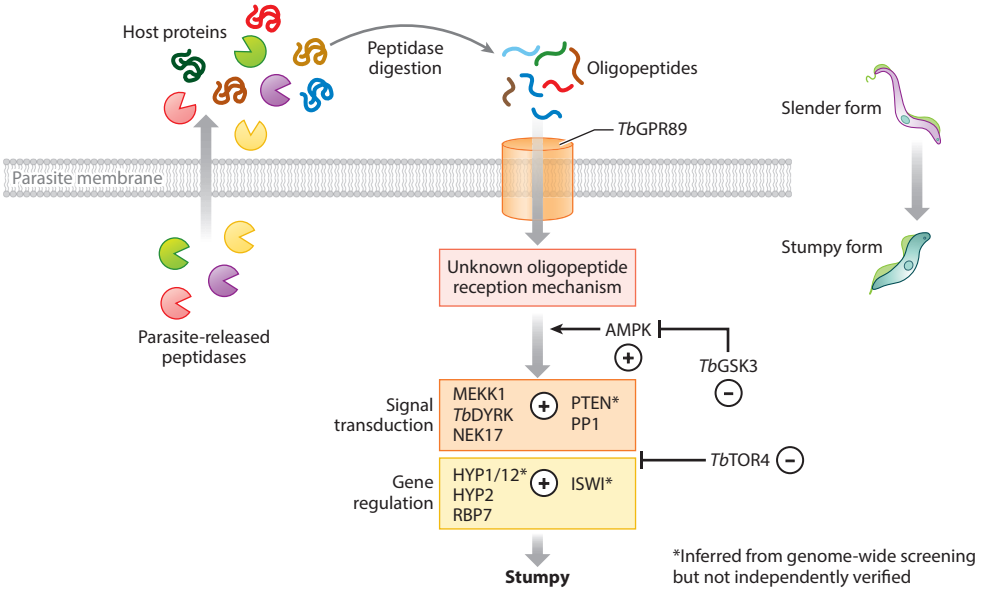

Figure 3

The quorum-sensing (QS) signaling pathway. Trypanosomes release peptidases into their environment during their infection of mammalian hosts. This degrades external polypeptides to oligopeptides, which are then transported by the trypanosome surface transporter protein *Tb*GPR89. In the parasite the QS signaling pathway is activated, some components of which have been identified by a genome-wide RNA interference screen. The pathway includes unidentified molecules that receive and/or process the signal and also signal transducing protein kinases and phosphatases. Ultimately gene-regulatory molecules are stimulated to enact differentiation from slender to stumpy forms. Positive regulation is exerted through AMPK-containing protein complexes, whereas negative regulation of the pathway operates via *Tb*GSK3 (an inhibitor of AMPKα1) and *Tb*TOR4 (which operates independently of, and apparently downstream from, AMPKα1). The pathway structure may be branched because ectopic overexpression of some components can compensate for the deletion of other components but not all. Hence, there are dependency relationships between pathway components that are not easily interpreted by a linear regulatory cascade, and their relative ordering is not clear. Many of the identified components do not have recognizable motifs that would allow their function or interactions to be predicted.

QS signaling is consistent with the host environment contributing to differentiation kinetics. Hence, hosts where oligopeptides can reach higher levels will show lower parasitemia (because stumpy formation will be accelerated), and hosts with lower levels of oligopeptides will have greater parasitemia. Similarly, tissue-resident parasites may experience a local concentration of peptidases and oligopeptides that differs from that of circulating blood parasites. The impact of the environment on the response to density-sensing signals for trypanosomes in different hosts and body compartments is analogous to that for *Pseudomonas aeruginosa* in suspension culture or biofilms where QS signals are subjected to flow (36).

3.3. Transduction of the Trypanosome Quorum-Sensing Signal

The first regulators of stumpy formation identified in *T. brucei* were two protein kinases, a MAP kinase (35) and a protein kinase with a Phox and FYVE zinc finger domain (103), that promote stumpy formation when deleted, implicating them as negative regulators of the process. Similarly, silencing of an unusual component of the target of rapamycin (TOR) complex identified in

T. brucei, *Tb*TOR4 (7), induced laboratory-adapted cell lines unresponsive to the physiological QS signal to become stumpy in vivo. This result implicated the TOR signaling pathway as an inhibitor of stumpy formation, similar to the TOR-mediated nutrient-sensing mechanisms that operate in many eukaryotes in response to ATP/AMP balance. Supporting this possibility, AMPK, a sensor of cellular energy levels, promotes the generation of stumpy forms (63, 80). One isoform in particular, AMPKα1, drives stumpy formation through its activation by phosphorylation—this being potentially stimulated by the transient elevation of mitochondrial reactive oxygen species (80). There may be redundancy between AMPKα1 and AMPKα2, since depletion of both by RNA interference (RNAi) is needed to prevent AMP-mediated growth inhibition and AMPKα2 but not AMPKα1 was identified in the screen for activators of stumpy formation described below.

Positive regulators of the signal transduction pathway controlling the QS of trypanosomes in their mammalian host have been identified using a genome-wide gene-silencing approach to select parasites resistant to the QS signal (63). Such screening approaches have until recently relied on the use of laboratory-adapted parasite lines that have lost the capacity for stumpy formation in vivo. However, work by Vassella et al. (104) established that these adapted lines can undergo many aspects of stumpy formation in vitro when exposed to cell-permeable cAMP analogs, a response mediated through AMP as a breakdown product of cAMP (48). Exploiting this, a parasite library capable of tetracycline-inducible RNAi silencing of most genes in the genome was selected with cell-permeable cAMP or AMP analogs (63). Resistant outgrowing cell lines were then subjected to deep sequencing to identify the genes whose silencing rendered parasites resistant to the imposed signal. This approach provided a list of around 30 genes potentially involved in the transduction of the QS signal. These included genes encoding molecules associated with purine salvage, likely selected on the basis of their capacity to rebalance AMP levels within the parasites rather than a function in SIF signal transduction. However, other molecules were more explicitly linked to a signal transduction cascade, including the aforementioned AMPKα2, several protein kinases, protein phosphatases, and predicted RNA-binding proteins that had been shown in independent screens to be able to regulate mRNA or protein levels (38). As expected, no transcription factors were identified—in trypanosomes, genes are predominantly regulated posttranscriptionally.

Several of the molecules identified have been explored further or have interesting functions. For example, silencing the expression of the genes for the small predicted RNA-binding proteins RBP7A/B reduces differentiation to stumpy forms, whereas the process is accelerated when the genes are ectopically overexpressed. The genes for these proteins are so designated because they are organized as two tandemly arranged, highly similar genes, *RBP7A* and *RBP7B*, that cannot be discriminated by RNAi. The predicted RNA-binding proteins have a single RRM domain and are only 116 amino acids in length, similar to RBP6 (239 amino acids), a key regulator of developmental events within the tsetse fly (47). It is unclear whether RBP7A and RBP7B interact physically or functionally or have independent roles. Their modes of action and targets are also currently unclear. Other potential gene regulators identified in the genome-wide screen include HYP1 and HYP2, proteins that have no conserved recognizable domains but have been shown experimentally to regulate a reporter gene when artificially tethered to the 3′ UTR (untranslated region) (38).

Of the protein kinases identified, one was of particular interest. This molecule, originally named YAK, was renamed *Tb*DYRK after closer phylogenetic analysis revealed it to be an unusual member of the DYRK family of kinases (28). Mutational and functional analysis of its catalytic activity revealed that the molecule exists in an unusual preactivated state, with motif diversity over the normally conserved DFG triad (the sequence being DFS in trypanosomes) and an unusual HxY configuration in the activation loop. Both sequence features are rare in other eukaryotic

kinases but enriched in trypanosomatids, suggesting a distinct regulatory paradigm in these parasites. Interestingly, phosphoproteomic analysis of predicted targets of this kinase indicated that *Tb*DYRK can inhibit molecules that maintain the slender form state and activate molecules that promote stumpy formation, pointing to a pivotal position in development.

The interactions between identified components of the QS signaling pathway have also been explored by an epistasis approach whereby the ability of some components to compensate for the deletion of other components provides information on their dependency relationships and pathway structure (61). Although complicated by potential autoregulation of components of the pathway, the inability to perturb some pathway components, and the induction of phenotypes apparently unrelated to differentiation (i.e., cell growth, morphology, and cell cycle phenotypes), these studies indicated that the pathway seemed to be branched. This conclusion was unexpected and counterintuitive. If ablation of each component individually prevents differentiation, how can another component on a distinct branch compensate for its loss? Possible answers involve feedback regulation in the pathway or the requirement for two signal inputs to regulate differentiation, both of which must be present unless overstimulation through only one branch compensates for the absence of one signal. Signaling pathways in eukaryotic cells are rarely linear, and it appears the trypanosome QS pathway will likewise require considerable further analysis of its molecular interactions to understand how the parasite effectively responds to the signal.

As well as these complexities, there remains an important black box in the transduction of the QS pathway. Oligopeptides are known to stimulate stumpy formation and to be transported via *Tb*GPR89. However, we do not know how the oligopeptide signal links to the signal transduction pathway described above. Further, we do not know the specificity of the signal and which particular oligopeptide sequences stimulate differentiation and why. These are important questions that remain to be unraveled.

4. MONOMORPHISM AND THE LOSS OF QUORUM SENSING

The mechanistic basis of trypanosome QS entails release of peptidases and the generation of extracellular oligopeptides and their transport to activate the intracellular signaling pathway. As with bacterial QS, this kind of intercellular molecular communication generates the potential for signal interference, competition, or cooperation between different strains or species (17) and the potential for the selection of cheats, which exploit shared public goods (18). In trypanosomes, the competing selective forces center around the transmission advantage of generating stumpy forms versus the virulence advantage of remaining as slender forms, with the resulting transmission disadvantage of having fewer stumpy forms potentially being compensated for by increased capacity for mechanical transmission by biting flies.

Most familiar to experimental trypanosome researchers are laboratory-adapted monomorphs. After long-term culture passage, or rapid serial passage between rodent hosts without tsetse fly transmission, these parasites have no (or reduced) ability to generate arrested stumpy forms (98), creating a growth advantage that is rapidly selected. In the field, in contrast, the advantage of generating stumpy forms for tsetse fly transmission provides a counterselection that sustains pleomorphism. Monomorphism most likely results from the emergence of parasites that cannot respond to the external SIF signal rather than mutants that do not produce the signal. This is because the selection operates in the context of a population that is producing the signal, such that only signal-blind individuals have a selective advantage, at least at the outset (53). In bacterial populations, the ability to generate a QS signal may also be eventually lost if there is a fitness cost to its production. Whether the same is true in trypanosomes is unclear because the dependency on peptidase secretion and oligopeptide uptake in slender forms has not been fully

investigated. In a systematic analysis of serine peptidases produced by trypanosomes, most could be depleted by RNAi without a disadvantage for monomorphic parasites in culture, suggesting they either are not essential in vitro or exhibit substantial redundancy (67). With respect to oligopeptide uptake, *Tb*GPR89 does appear to be required for the viability of slender forms, suggesting an essential role (77). However, the basis of this requirement needs exploration, since it is unclear whether it is nutritionally necessary to take up oligopeptides in a rich bloodstream environment.

Monomorphism has also arisen many times in the field, generating the parasite subspecies *T. brucei evansi* and *T. brucei equiperdum*. *T. brucei evansi* causes the disease surra, whereas *T. brucei equiperdum* is responsible for the equine venereal disease dourine (22). Neither subspecies generates stumpy forms, with transmission by tsetse flies having been lost in favor of mechanical transmission by biting flies or transmission during equine coitus. These alternative modes of transmission have allowed these species to escape the tsetse fly belt of sub-Saharan Africa, with infections detected in Asia, South America, and Europe as well as Africa. A characteristic feature of both *T. brucei evansi* and *T. brucei equiperdum* is their loss of their mitochondrial genome, the kinetoplast (81). However, this loss does not cause monomorphism—parasites engineered to express a mutant F0 ATPase that is permissive for kinetoplast loss remain able to generate stumpy forms, although these stumpy forms have reduced lifespans (34). Rather, it appears that the selection for increasing monomorphism allows *T. brucei* to bypass cyclical transmission by tsetse files by increasing their overall parasitemia (33). For *T. brucei evansi*, this increases the probability of transmission by biting flies such as tabanids and *Stomoxys*, further reducing selection for retention of pleomorphism. Secondarily, after loss of cyclical development, the necessity to retain the mitochondrial genome is reduced, and with appropriate mutation(s) to sustain a mitochondrial membrane potential (30), the kinetoplast is reduced (dyskinetoplasty) or lost (akinetoplasty). For *T. brucei equiperdum*, altered tropism to favor concentration in the gonadal region might promote venereal transmission, again reducing dependency on pleomorphism or mitochondrial genome maintenance. These selective steps have occurred on multiple occasions for *T. brucei evansi*, with characterized phylotypes showing closer similarity to *T. brucei brucei* isolates than to each other (27). Hence, monomorphism has emerged on multiple occasions in nature, and with the decline of tsetse flies in some regions of sub-Saharan African due to vector control or environmental change (49), the selective pressure to be released from dependence on this vector may be increasing. Indeed, given the genomic similarity between *T. brucei brucei*, *T. brucei evansi*, and *T. brucei equiperdum* and the secondary loss of the kinetoplast, it is possible there is a constant but unrecognized emergence of trypanosomes that exhibit a spectrum of reduced pleomorphism at certain geographical sites. These trypanosomes might be at different points on the journey to becoming recognizable (i.e., with depleted kinetoplast DNA) *T. brucei evansi* or *T. brucei equiperdum* with the potential to explain the variable virulence profiles between *T. brucei* isolates circulating in the field. An important consideration is whether *T. brucei rhodesiense* can undergo equivalent selection, threatening the emergence of human-infective trypanosomes competent for mechanical transmission with unrestricted dissemination (45). Although there is no current direct evidence for this, human infections with *T. brucei evansi* have been reported outside the tsetse fly belt, including in some patients whose serum retains ApoL1-mediated trypanolytic activity (55).

The molecular basis of the loss of pleomorphism in *T. brucei* isolates is unknown but likely relates to defects in the QS signaling pathway. These defects are undefined, and it is unclear whether all monomorphs arise through the same selective trajectory or multiple independent pathways have been exploited. Nonetheless, understanding the key limitations and selective bottlenecks in this process is important in order to predict disease virulence and the consequences of imposing tsetse fly control in regions where parasites are prevalent.

5. COINFECTION AND ITS EVOLUTIONARY CONSEQUENCES FOR QUORUM SENSING

The molecular mechanisms of stumpy formation are conserved not only in *T. brucei* subspecies but also in *T. congolense* and *T. vivax*, despite their inability to make morphologically stumpy forms (89). Furthermore, there is evidence that trypanosome species share QS signals, allowing interaction in coinfections. For example, superinfection with a *T. brucei* population in a host already infected with *T. congolense* promotes premature differentiation to stumpy forms, which is dependent upon integrity of the QS signaling pathway (89). If sustained in a chronic infection or through multiple hosts, this interaction has the potential to select for reduced sensitivity to a shared QS signal, providing a fitness advantage that allows some parasites to increase their relative numbers in a coinfection (**Figure 4**). Indeed, if coinfection is common, the sensitivity to the QS signal may become optimally adapted to growth and transmission in the context of competing parasites. Such competition dynamics can generate unpredictable outcomes when parasites variably exist in mixed infections or monoinfections over time, or when they infect different hosts. An explicit example of this is *T. brucei rhodesiense*, a human-infective subspecies that probably spends most time in livestock and game animals. In this setting, they frequently infect their host at the same time as *T. congolense*,

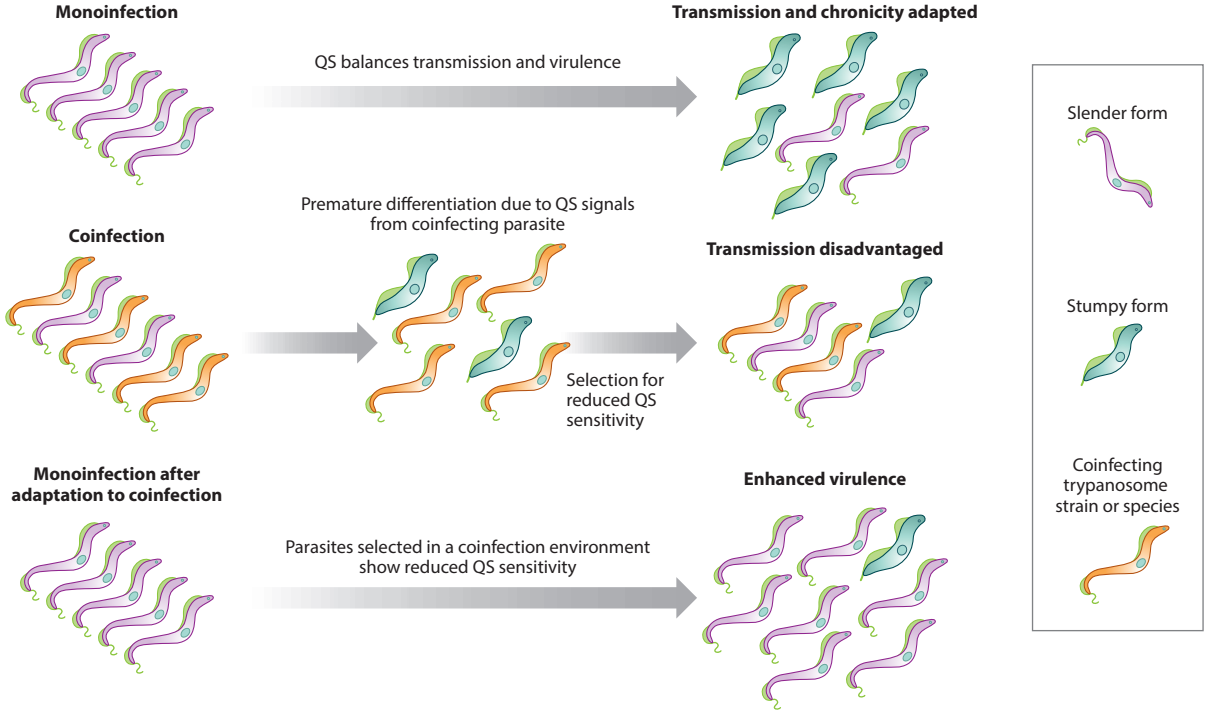

Figure 4

The consequences of coinfection for virulence and transmission phenotypes. In parasites adapted to monoinfection, the production of and response to the quorum-sensing (QS) signal are adapted to generate stumpy forms at a parasite density that sustains infection and ensures transmission. However, with coinfection, shared QS signals can lead to premature differentiation to arrested stumpy forms, restricting the capacity of the parasites for transmission (due to low prevalence) and limiting their fitness in infections with competing parasite genotypes. This can lead to selection for reduced sensitivity to the QS signal, thereby restoring the transmission/virulence balance. However, if coinfection-adapted parasites subsequently infect hosts in a monoinfection (e.g., where competing parasites are removed by therapeutic drugs or are not viable due to the presence of serum trypanolytic factors), the infection virulence may be significantly elevated due to reduced responsiveness to the QS signal.

T. vivax, or *T. brucei brucei*, whereupon they may be selected for reduced sensitivity to the QS signals of the coinfecting parasites. However, if transmitted to humans, only *T. brucei rhodesiense* can survive, establishing a monoinfection with potentially higher virulence due to reduced QS sensitivity. Similar selective outcomes might result where coinfecting parasites exhibit different sensitivities to therapeutic drugs such that a subset of the coinfecting parasites is removed and drug-resistant parasites increase in virulence. The consequences of this dynamic situation cannot easily be predicted when parasites enter human populations or where drug treatment has variable efficacy for different members of the infecting population.

The coexistence of different trypanosome species in some hosts might also provide clues to the different biologies they exhibit. The relative prevalences of different trypanosome species vary by geographical location, but usually *T. vivax* and *T. congolense* are most prevalent, with *T. brucei* being relatively rare. Hence, *T. brucei* is most likely to encounter competition, and selection for the developmental extremes of antigenically diverse slender forms and transmission-enhanced stumpy forms has perhaps allowed *T. brucei* to sustain itself in a competitive environment with more prevalent trypanosome species. Furthermore, the ability to generate mosaic *VSG*s through frequent recombination has also allowed the evolution of *SRA* (110) and *TgsGP* (14), both apparently VSG derived, providing *T. brucei* with the flexibility to escape competition with other trypanosome species by infecting hosts with trypanolytic factors in their serum. Finally, the differing tropism shown by different trypanosome species might also be an adaptation for coinfection, with *T. brucei* tissue specialization allowing avoidance of *T. congolense* populations restricted to the blood vessels. Through local avoidance, the different species might ensure that they are predominantly exposed to their own QS signal and thereby optimize their own transmission responses without signal interference from genetically distinct competing trypanosome parasites.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

The phenomenon of QS in African trypanosomes has long been recognized, but only recently has it been understood in molecular detail. This understanding has provided tools for dissecting the process in detail and its impact on the biology and pathology of the parasite. Many questions remain outstanding or have been newly posed by recent discoveries (see the section titled Future Issues). Although these are critical biological questions, a bigger question with respect to disease control relates to whether the phenomenon of trypanosome QS can be exploited for therapeutic advantage. Bacterial QS systems have been proposed as a route to control microbial pathogens through QS interference or by manipulating the intercellular communications between bacterial communities to reduce their persistence, viability, or pathogenicity (19, 109). Could something similar be applied in trypanosomes? In principle, chemical mimetics of SIF could induce premature differentiation of trypanosomes, reducing their number below the threshold needed to sustain transmission (and simultaneously reducing potential virulence). Indeed, the use of small peptides to promote differentiation is one therapeutic route because peptidomimetic-based drugs are a well-established therapeutic strategy for a large spectrum of diseases, from cardiovascular disease to metabolic disorder and cancer, including those that target hormonal receptors and GPCRs (12, 73). Despite this, caution is necessary because promoting premature differentiation might select reduced sensitivity to the QS signal. This might lead to more virulent parasites, potentially adapted for mechanical transmission, worsening outcomes for individual patients and disease spread. Clearly, close consideration is needed of the potential impact of interfering with trypanosomes' communication networks in their host. This requires detailed molecular insight into the function and redundancy of molecular components of the signaling pathway and modeling of the impact of their perturbation for parasite infection and evolution.

Finally, this review has focused on trypanosome interactions in their mammalian host. However, these are not their only point of community action. Social motility also occurs in the tsetse fly gut, and this is excellently reviewed elsewhere (43, 79). Is the sociality of African trypanosomes unique in this respect, and do other kinetoplastid parasites exercise social distancing? At present this is unclear, but certainly orthologs of many of the key molecular components of the QS pathway are conserved in both *Trypanosoma cruzi* and *Leishmania* spp. Like *T. brucei*, these parasites show exquisite sensitivity to their external environment, and it will be intriguing to discover whether they too communicate as a group to coordinate their development or other behaviors.

FUTURE ISSUES

1. What are the exact specificity of oligopeptide signals and their contribution to trypanosome quorum sensing (QS)?
2. How are oligopeptide signals perceived by the parasite intracellularly to initiate the QS response?
3. How do antigenic variation and QS interact to shape the parasites' infection dynamic in both acute and chronic infection scenarios?
4. Do coinfections generate evolutionarily selective forces that shape the importance or magnitude of QS?
5. What impact does the host species have on the QS process, and does this vary in different body compartments?

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