The cell biology of *Trypanosoma brucei* differentiation

Katelyn Fenn and **Keith R Matthews**
Institute of Immunology and Infection Research, School of Biological Sciences, University of Edinburgh, Ashworth Laboratories, King's Buildings, West Mains Road, Edinburgh EH9 3JT, UK

Abstract

Developmental events in the life-cycle of the sleeping sickness parasite comprise integrated changes in cell morphology, metabolism, gene expression and signalling pathways. In each case these processes differ from the eukaryotic norm. In the past three years, understanding of these developmental processes has progressed from a description of the cytological events of differentiation to a discovery of its underlying molecular controls. With an expanding set of reagents for the identification of distinct parasite life-cycle stages in the tsetse, trypanosome differentiation is being studied from the molecular to the organismal and population level. Interestingly, the new molecular discoveries provide insights into the biology of the parasite in the field.

Introduction

The transmission of the trypanosome (*Trypanosoma brucei*), causative agent of African sleeping-sickness, occurs upon the bite of tsetse flies. In the tsetse, parasite development takes 20–30 days to reach maturation [1], during which the trypanosome undergoes enormous and multiple changes in its biology before regenerating a form that is infective to mammalian hosts. The reacquisition of infectivity exactly correlates with the expression on the parasite of a dense surface coat, composed of a homogenous layer of variant surface glycoprotein (VSG). In mammalian hosts, this can be periodically changed in an extreme form of antigen variation [2]. This is required because trypanosomes live extracellularly in the bloodstream and undergo antigenic variation to stay ahead of the lytic immunoglobulin response raised to successive antigenic types. Often the cyclical waves of parasitaemia characteristic of textbook descriptions of trypanosome infections are entirely ascribed to antigenic variation. However, it has been proposed for nearly 100 years that a parasite-derived factor is also important in modulation of the parasitaemia [3] and this has been supported recently at the biochemical level [4–6] and by mathematical modelling [7••]. Indeed, it was always apparent that the bloodstream parasite population was not uniform and that the proportion of different forms changed during the parasitaemia. This is described as pleomorphism, with its two extremes being the morphologically 'slender' and 'stumpy' forms [8]. Robertson (1912) [3] was clear that only stumpy forms were capable of transmission to the tsetse fly, but the situation has become more complicated since then. Part of the reason for this is that laboratory lines were selected for growth in the bloodstream (and antigenic stability), without a corresponding pressure for the maintenance of transmissibility through the tsetse fly. The result was the selection of monomorphic lines, which are able to proliferate rapidly and without control in mammalian hosts but which no longer produce stumpy forms. The analysis of these lines has caused controversy as to the relative importance of slender and stumpy forms in the developmental biology of the

Corresponding authors: Fenn, Katelyn (k.fenn@ed.ac.uk) and Matthews, Keith R (keith.matthews@ed.ac.uk).

parasite since monomorphic cells, which resemble pleomorphic slender cells, remain able to differentiate to tsetse midgut (procyclic) forms *in vitro*.

Examining pleomorphism beyond morphology

The production of stumpy forms represents a form of quorum sensing. As parasite numbers increase through the proliferation of slender forms, a parasite-derived soluble factor accumulates which promotes cell-cycle arrest in G1/G0, mitochondrial elaboration and morphological transformation to the stumpy form [5,6] (Figure 1). This is accompanied by dysregulation of the monoallelic control of VSG gene expression sites [9]. The reason for this is unclear, though it may simply reflect the irreversibility of the slender–stumpy transition. Since stumpy cells are non-proliferative and are destined either for transmission or death, it may be of no consequence that the normal stringency of expression site control is lost in these forms.

To date, the external signal promoting stumpy formation, termed stumpy-induction factor (SIF) [5], has eluded purification though intracellular signalling pathway components that stimulate stumpy formation when perturbed have been identified. These include a PX-FVYVE family zinc finger kinase, ZFK [10] and a MAP kinase family member, TbMAPK5 [11•]. In both cases, genetic deletion in pleomorphic lines resulted in accelerated development to stumpy forms and reduced growth in culture and in mice. Importantly, monomorphic lines, unable to generate stumpy forms, did not exhibit a phenotype when either gene was deleted, supporting a role for these genes in development rather than effects on virulence, for example. Although SIF remains unidentified, a number of chemical factors that mimic its action have been described which might give some clue as to how the factor operates or is signalled in the cell. Among these, compounds linked to the cAMP pathway induce convincing progression to stumpy forms [5,12••]. Importantly, this has not only been measured on the basis of cell morphology but also on the ability of treated cells to undergo efficient differentiation to tsetse midgut procyclic forms *in vitro*, the hallmark of stumpy-forms generated *in vivo*. Originally, a cell-permeant cAMP analogue, 8-pCPTcAMP, was observed to induce stumpy formation, invoking the cyclic nucleotide-signalling pathway [5]. More recently, however, hydrolysable cAMP analogues have been used to inhibit proliferation and promote the development of morphologically stumpy forms, though this was in monomorphic populations naturally resistant to SIF [12••]. If verified in SIF-responsive pleomorphic lines, this would suggest that the cyclic nucleotide-signalling pathway were not itself responsible for SIF signalling, but rather the breakdown products of these. Treatment with troglitazone, a thiazolidinedione that acts on the peroxisome proliferating-activated receptor gamma in higher eukaryotes, also promotes morphological transformation of pleomorphic lines and causes cell growth arrest in monomorphic cells (although not at a specific cell-cycle position) [13]. Cell-cycle arrest and development to a stumpy morphology has also been observed with several other compounds or genetic perturbations that inhibit cell growth in bloodstream forms [14,15]. However, the precise relationship between cells described as 'stumpy-like' and the stumpy cells that arise in response to SIF *in vivo* or *in vitro* is not clear. This reflects the absence of molecular markers for this cell type, these being necessary to recognise activation of a bona fide developmental pathway rather than growth arrest, mitochondrial activation and morphological changes that can accompany cells under certain non-physiological conditions.

Two specific cytological characteristics of stumpy forms have been defined for the first time by Engstler and Boshart [16••]. It has long been known that differentiation from bloodstream forms to tsetse midgut forms could be reproduced *in vitro* by a reduction in temperature and exposure to citrate/*cis*-aconitate (CCA) [17,18]. However, the required concentrations of

these compounds appeared un-physiologically high for these molecules to be natural inducers of differentiation in the fly midgut [19]. Engstler and Boshart observed that when bloodstream forms were exposed to temperature reduction of >15 °C, then stumpy cells became hypersensitised to CCA, producing proliferative procyclic forms when treated with *cis*-aconitate in the low micromolar range. Although the relevance in flies is still to be validated, this is compatible with the reported concentration of CCA in the tsetse midgut (15 μM citrate; [20]); moreover the required temperature drop is consistent with tsetse feeding habits, where feeding at dusk exposes the ingested trypanosomes to cool night-time temperatures. Interestingly, it was also noted that bloodstream cells exposed to cold shock expressed EP procyclin (normally only detected on the surface of procyclic forms) but that this was trafficked to the cell surface in stumpy forms but not slender forms. Differential protein trafficking may be a key difference between these forms, which, through regulated surface access of a receptor, may explain the basis of stumpy form sensitivity to CCA (Figure 2).

The division-arrest of stumpy forms is central to their role in the biology of trypanosomes in their mammalian host. Unless stumpy forms arose during a parasitaemia, the uncontrolled growth of slender forms would rapidly kill the host, thereby limiting the parasite's transmission potential (reviewed in [21]). Cell-cycle arrest has also been proposed to be crucial in their transmission capacity [22]. This resulted from the observation that stumpy cells are uniformly arrested in G1/G0 [23] and initiate differentiation to procyclic forms *in vitro* synchronously and efficiently [5,22,24]. The molecular basis of the interrelationship between cell-cycle arrest and differentiation competence was investigated by analysis of a protein tyrosine phosphatase (TbPTP1) related to similar molecules with function in G0 in other systems [25••]. Transcript ablation of TbPTP1 by RNAi or pharmacological inhibition of the enzyme by a cell permeable PTP1B inhibitor induced spontaneous differentiation to procyclic forms, suggesting that this molecule normally inhibits this differentiation step [25••,26]. Although only a small proportion of cultured monomorphic bloodstream forms differentiated, uniformly arrested stumpy forms differentiated with nearly 100% efficiency when exposed to the inhibitor. This suggested a model in which cell-cycle arrest was required for differentiation and that in the bloodstream this was prevented by the activity of TbPTP1. Once in the fly, inactivation of this enzyme would allow differentiation of those cells competent to do so (Figure 2). Since differentiation can be stimulated by a number of alternative triggers including CCA, exposure of the parasite surface to limited protease treatment [19] or to reduced pH [27], it remains to be determined whether each pathway operates via the activity of TbPTP1 or whether multiple-independent pathways can act to initiate differentiation to procyclic forms.

Differentiation events in the tsetse fly: a model developmental system

Once stumpy forms initiate differentiation to procyclic forms they embark on a precisely regulated differentiation programme, the components of which occur in a consistent order and on a reproducible timescale *in vitro*. The major event is exchange of surface antigens between bloodstream and insect procyclic forms, that is, loss of the VSG and gain of procyclins. VSG is released from bloodstream forms by the combined action of a metalloendoprotease (MSP-B) and a glycophosphatidylinositol phospholipase C (GPI-PLC) activity [28]. MSP-B, which clips the VSG protein close to its C-terminus, is induced after the initiation of differentiation, and then remains expressed in procyclic forms. GPI-PLC, by contrast, is quickly downregulated during differentiation but gains access to the parasite surface (and hence its substrate) in the stumpy form. Other major events, each interlinked, fall broadly into three categories.

Gene expression

Gene expression changes are important in adaptation of trypanosomes from one life-cycle stage to the next, with microarray analysis suggesting that ~2% of genes show developmental regulation at the mRNA level [29]. This regulation is largely post-transcriptional because the trypanosome genome is organised into poly-cistronic transcription units where functionally unrelated genes are transcribed as a single unit and then processed into individual mRNAs. This means that protein levels are regulated through mRNA stability and degradation, coupled with translational efficiency and protein turnover. One common theme is that cycloheximide treatment increases the abundance of several stage-regulated transcripts, including *EP procyclin* [30], *MSP-B* [28], *GPI-PLC* [31] and several components of the cytochrome oxidase complex [32]. This effect does not appear to be mediated through signals in the 3′UTR [30,32], though these regions certainly do contain the dominant regulatory elements which govern the stage-specific expression of many genes [33].

A key ambition is the recognition of common motifs governing developmental gene expression [32]. One model for such analysis is the regulation of nuclear-encoded genes for components of the cytochrome oxidase (COX) complex. Trypanosomes are unusual in demonstrating developmental regulation of mitochondrial respiratory activity and require co-incident upregulation of COX subunits upon differentiation to procyclic forms. Reporter construct expression and bioinformatics analysis identified a dominant regulatory motif controlling *COX V* stage-regulated expression. This was similar to a '26mer' element identified in procyclin mRNAs that operates to negatively regulate gene expression in bloodstream forms. Genome-wide analysis using an oligomer-counting algorithm suggested that its central region (UAUUUUUU) was highly enriched in the 3′UTR of procyclic-enriched transcripts, implicating this motif as a widespread regulator of developmental gene expression. A second element, as yet functionally uncharacterised, is over-represented in the mRNAs for nuclear-encoded mitochondrial proteins and may represent a general *cis*-acting signal regulating such transcripts. Expression analysis of the bloodstream-specific *GPI-PLC* mRNA used derepression in procyclic forms of an antibiotic resistance gene linked to the *GPI-PLC* 3′UTR to map regulatory regions [34]. Selected mutants spontaneously deleted the last 800 bases of the 2300 base long 3′UTR suggesting presence of a negative control element in this region that operates in procyclic forms.

As further *cis*-acting sequence elements are identified within the genome there is a need to identify the *trans*-acting regulatory proteins recognising these signals. The *T. brucei* genome encodes an abundance of RNA-binding proteins (RBPs) [35–37] and linking these to their target sequence element will help to decipher the mechanisms involved in developmental gene regulation. Candidate molecules include small RNA-binding proteins presumed to act through modular interaction with other effector proteins. These include the *T. cruzi* UBP proteins [38], their *T. brucei* equivalents [39] and a group of CCCH zinc finger proteins, TbZFPs, one of which is required for kinetoplast repositioning during differentiation to procyclic forms [40]. Further members of this protein family associate with the translational apparatus in a stage-specific manner and enhance differentiation [41]. Interestingly, immunoprecipitation of one protein (TbZFP3) co-selects *EP procyclin* mRNA, suggesting that it may be positively regulating this transcript at the level of stability or translation.

In addition to *trans*-acting RNA-binding proteins other mechanisms may also regulate developmental gene expression. One mechanism, recently characterised in mammalian cells, is the action of the RNAi pathway on transcripts containing AU-rich elements [42]. However, at present there is no evidence for this in trypanosomes, with Argonaut mutants lacking a functional RNAi pathway and still exhibiting normal life-cycle progression and an absence of detectable changes in stage-regulated mRNAs [43]. Histone modification is

another potential regulatory mechanism for gene expression, with deletion of the DOT1B gene, responsible for trimethylation of H3K76, apparently causing defective differentiation to procyclic forms [44•]. Finally, gene expression may correlate with nuclear architecture. The monoallelleic expression characteristic of VSG expression site control is mediated through an expression site body proposed to associate with the active telomere at the nuclear periphery [45]. Upon differentiation to procyclic forms this subnuclear association appears to be lost, with telomere redistribution in the nucleus perhaps contributing to VSG gene silencing [46].

Signalling pathways

Accompanying changes in gene expression, various signalling components have been identified that regulate differentiation to procyclic forms. It can be difficult to distinguish signalling molecules that act on the differentiation process itself from those that simply regulate outgrowth and viability of the differentiated procyclic form population. Nonetheless, several protein kinases that act in a stage-specific manner have been identified, as have cell-cycle regulatory kinases that elicit developmental stage-specific effects [47–52]. Study of the complete complement of protein kinases encoded in the trypanosomatid genomes [53•] revealed an absence of tyrosine-specific kinases, though tyrosine phosphorylation patterns clearly change during life-cycle development [54], presumably through the action of dual specificity protein kinases. Analysis of the full complement of phosphatases encoded in the trypanosomatid genomes also reveals very few predicted tyrosine-specific phosphatases, with a corresponding increase in serine/threonine phosphatases (R Brenchley, H Tariq, H McElhinney, B Szoor, R Stevens, K Matthews, L Tabernero, unpublished data). Interestingly, a classic tyrosine phosphatase encoded in *Leishmania major* and *T. cruzi* is missing in *T. brucei*. Genetic ablation experiments in *L. major* [55] implicate this molecule as being important in the virulence of intracellular amastigote forms, perhaps explaining the absence of an orthologous gene in the exclusively extracellular *T. brucei*.

Metabolism and surface antigen regulation

During differentiation in the fly, trypanosomes exhibit complex changes in metabolism. The major differences between forms is that in bloodstream stages glycolysis is responsible for ATP synthesis, with mitochondrial activity being repressed except the activity of a plant-like alternative oxidase which maintains the redox balance of glycosomes [56]. In procyclic forms, by contrast, substrate level phosphorylation is responsible for ATP production in the presence of glucose, whereas in its absence, amino acids become the major carbon source, with ATP being generated via oxidative phosphorylation. These changes in mitochondrial activity are not only important in the metabolic activity of the cell but also in regulating the surface antigen expression. Thus, GPEET procyclin shows alternative expression depending on the activity of mitochondrial enzymes, with downregulation of the acetate, succinate-CoA transferase/succinyl-CoA synthetase (ASCT) pathway leading to expression of GPEET in late procyclic forms, this being reversible by drug inhibition of the alternative oxidase [57•].

Differentiation events in the tsetse fly

Although several components of the trypanosome life-cycle can be reproduced *in vitro*, analysis of events in the fly is also revealing. The best characterised is the expression profile of different procyclin isoforms. In culture, EP and GPEET procyclin (phosphorylated by a GPEET kinase restricted to the flagellar pocket; [58]) are expressed early, with GPEET procyclin being downregulated upon development of late procyclic forms. However, EP procyclin is also regulated. There are three isoforms of this molecule, EP1, EP2 and EP3.

Analysis of their expression in the fly has been monitored by mass spectrometry for protein expression [59,60] and by RTPCR analysis of mRNA expression using primers directed to regions of the 3′UTR that distinguish each type [61]. This revealed that EP1 and EP3 replace GPEET procyclin in late procyclic forms, and that EP2, which unlike EP1 and EP3 is not glycosylated, is not detectable at the protein level. After multiplication in the midgut/proventriculus, procyclic forms migrate toward the salivary glands, undergoing an asymmetric cell division generating a long epimastigote form and a short epimastigote form [62]. Only short forms are believed to be destined for attachment and proliferation in the fly salivary gland, where *EP procyclin* mRNA continues to be expressed although procyclin protein is not detectable on most parasites — this repression being due to sequences present in the procyclin coding region [61]. Instead of procyclins, a further surface coat protein, *b*rucei *a*lanine *r*ich *p*rotein (BARP), is expressed on epimastigotes though its function is unclear [63•]. Epimastigotes are believed to be the site of sexual exchange between trypanosome lines, with hybrids being detectable in the salivary gland after parasite lines tagged with distinct fluorescent reporters are transmitted through flies [64]. Finally, the parasites mature to metacyclic forms and reacquire the VSG coat [65]. Upon detachment from the salivary gland wall, these metacyclic forms are infectious for mammalian hosts, this being initiated when they are expelled during a tsetse blood meal.

Conclusions

There are still fundamental barriers to be overcome in the study of the trypanosome life-cycle. Among the most important is the identification of molecular markers for distinct life-cycle stages, particularly stumpy forms. The ambiguity of morphological descriptors of this life-cycle stage has, firstly, created controversy regarding the relative capacity of slender and stumpy form for differentiation to procyclic forms; secondly, hindered the development of effective bioassays for the generation of stumpy forms, an important component in systematic approaches to identify SIF biochemically and, thirdly, restricted accurate modelling of the dynamics of trypanosome profiles in the chronic infections characteristic of the disease in the field. A mathematical model was developed recently that effectively predicted the trypanosome parasitaemia in relation to the relative probabilities of antigen gene activation [7••]. However, such models can only be as good as the data upon which key assumptions are based. In consequence the contribution of the slender–stumpy transition to the developed models was, through necessity, based on data generated using relatively subjective morphological markers. Unambiguous markers are clearly needed to allow these transitions to be modelled quantitatively and, thereby, the balance between maintenance of the parasitaemia (as slender forms) and transmissibility of the parasite (as stumpy forms) to be understood.

Notwithstanding these issues, understanding the cell biology of differentiation in trypanosomes is moving from a descriptive era to one where molecular controls underlying several of the key transitions are becoming known. The identification of these regulators offers exciting prospects for analysing the pathways involved in differentiation control with the potential to identify routes to either block them or stimulate them inappropriately. Clearly, breaking the life-cycle offers new hope in blocking the cycle of infection for these important parasites.

Acknowledgments

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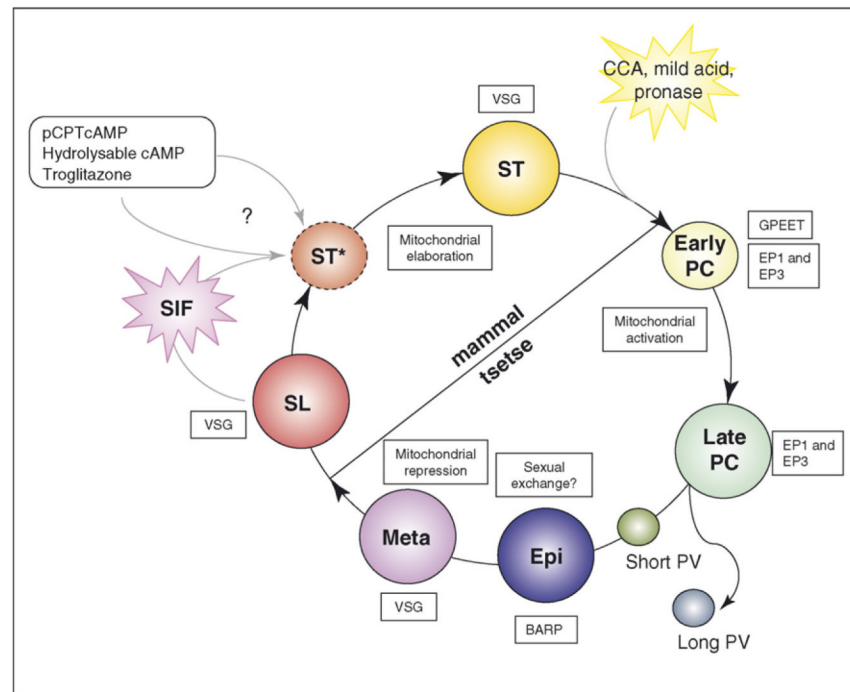
Figure 1. A schematic diagram of the life-cycle of *Trypanosoma brucei*.
Mammalian infection is initiated when metacyclic ('Meta') forms are inoculated by the bite of an infected tsetse fly. These develop to proliferative bloodstream slender (SL) forms that multiply in the blood to establish the infection. As numbers increase, a parasite-derived factor, stumpy induction factor (SIF), promotes division arrest and the generation of stumpy (ST) forms, commitment to this transition occurring while the parasite is still morphologically slender (this intermediate form has been termed Stumpy*; [25••]). The experimental induction of cells with stumpy characteristics can also be induced by various chemical treatments (8-pCPTcAMP, hydrolysable cAMP and troglitazone) although whether these act via the SIF signalling pathway is unclear. Once stumpy cells are up taken by tsetse flies they transform to procyclic (PC) forms, this being induced *in vitro* by exposure to citrate/*cis*-aconitate (CCA), mild acid or pronase treatment. Procyclic forms develop initially as early forms (which express GPEET procyclin), this being down regulated as they transform to late procyclic forms. Asymmetric division of proventricular (PV) forms generates a long and short form, the short form of which is believed to attach to the salivary gland and transform to proliferative epimastigote ('Epi') forms, which express BARP and are believed to be capable of sexual exchange. These eventually mature to detached metacyclic forms that express a VSG coat and are ready for transmission to a new mammalian host. Mitochondrial status is indicated inside the circle in boxes, surface antigen expression is indicated outside the circle.
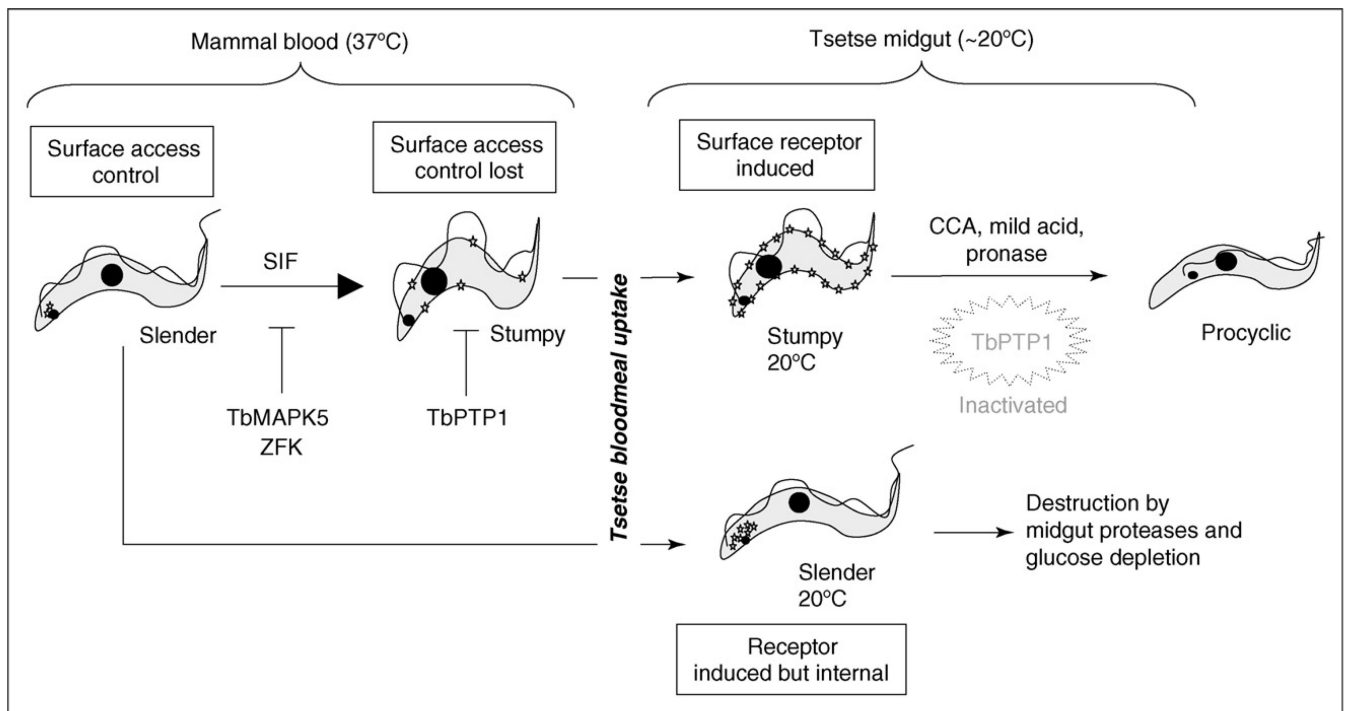
Figure 2. Cytological events leading from slender to procyclic forms.
Slender cells proliferate in the mammalian bloodstream and produce SIF, triggering the
density-dependent production of stumpy forms. This differentiation is inhibited by the action
of signalling pathway(s) involving TbMAPK5 and ZFK. Once stumpy forms are generated,
they are held poised for differentiation to procyclic forms by the action of TbPTP1 [25••].
These cells also express, at low level, a CCA receptor/transmitter for the signal to
differentiate, which has access to the cell surface (shown as a 'star'). Upon uptake by tsetse
flies, Engstler and Boshart [16••] propose that a reduction in temperature of >15 °C
promotes super-induction of the CCA receptor/transmitter that is trafficked to the stumpy
cell surface, sensitising these cells to the inductive signal. Slender cells, in contrast, do not
traffic the receptor/transmitter to the surface. Exposure to differentiation signals in the fly
inactivates TbPTP1 and initiates transformation to procyclic forms, this being induced by
CCA, proteolytic attack of the parasite surface or pH stress in the tsetse midgut and/or *in
vitro*.