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Molecular control of irreversible bistability during trypanosome developmental commitment

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The life cycle of Trypanosoma brucei involves developmental transitions that allow survival, proliferation, and transmission of these parasites. One of these, the differentiation of growth-arrested stumpy forms in the mammalian blood into insect-stage procyclic forms, can be induced synchronously in vitro with cis-aconitate. Here, we show that this transition is an irreversible bistable switch, and we map the point of commitment to differentiation after exposure to cis-aconitate. This irreversibility implies that positive feedback mechanisms operate to allow commitment (i.e., the establishment of “memory” of exposure to the differentiation signal). Using the reversible translational inhibitor cycloheximide, we show that this signal memory requires new protein synthesis. We further performed stable isotope labeling by amino acids in cell culture to analyze synchronized parasite populations, establishing the protein and phosphorylation profile of parasites pre- and postcommitment, thereby defining the “commitment proteome.” Functional interrogation of this data set identified Nek-related kinase as the first-discovered protein kinase controlling the initiation of differentiation to procyclic forms.

Introduction

To survive, proliferate, and cooperate, cells must make switch-like decisions, such as whether to express particular genes, to divide, or to differentiate. Bistability is an important property of many biological switches, which has been fundamental for understanding mitotic progression as well as the control of developmental transitions in both prokaryotic and eukaryotic organisms. Bistable switches are characterized by the lack of stable intermediate states between the initial and final states. That is, the transition from one state to the next is not a progressive response to a signal; rather, it is a digital change from one state to the next. Crucially, this change occurs at a specific stimulus threshold. By definition, and unlike ultrasensitive or sigmoidal responses to external signals, the “on” and “off” responses of bistable switches follow different trajectories, such that at a given level of signal, the cell state can be stably on or off, depending on the prior exposure to the signal. This is referred to as hysteresis (Tyson et al., 2003) and enables the clear-cut transition between states necessary for robust decision making (Fig. 1, A–C), such as progression through cell cycle checkpoints, as an adaptive response to a changing environment, or in cell differentiation (Xiong and Ferrell, 2003; Pomerening, 2008; Wang et al., 2009). It also enables decisive responses in the context of a noisy environment in which an extrinsic signal may be transient or fluctuate in levels after the initial stimulatory event (Losick and Desplan, 2008).

Bistability requires positive feedback loops, which ensure separation and stability of the on and off states and whose activation results in commitment to transitions between states (Ferrell and Xiong, 2001; Tyson et al., 2003; Mitrophanov and Groisman, 2008). These are common in transcriptional and signaling networks; for example, they occur where transcription factors activate their own transcription or when protein kinases autophosphorylate. These responses can be sustained, such that removal of the initial signal does not reverse the response. They may also trigger downstream or overlapping feedback mechanisms that make a transition irreversible. Hence, bistable switches can operate at different and/or multiple levels (Fig. 1 D), but the consequence is that the cell progresses unidirectionally to its next state (Losick and Desplan, 2008).

Protozoan parasites are one group of organisms that progress through an irreversible series of cytological states in a highly regulated manner. Commonly, these parasites have complex life cycles whereby they transition between life in a
mammalian host and life in an invertebrate vector (Matthews, 2011). This includes the major pathogens Plasmodium spp. (responsible for malaria; Aly et al., 2009) and kinetoplastid parasites responsible for several important tropical diseases including Chagas disease (Trypanosoma cruzi; Goldenberg and Avila, 2011), Leishmaniasis (Leishmania spp.; Zilberstein and Shapira, 1994), and African trypanosomiasis (Trypanosoma brucei; MacGregor et al., 2012). In the life cycle of each of these parasites, a developmental transition accompanies the progression of the parasite between the vertebrate and the invertebrate host and vice versa, and there are also further developmental transitions within each environment. Each of these is characterized by being precisely controlled, adaptive for the changed environment, and irreversible. They are also frequently linked to cell cycle progression (Turner, 1992; Matthews and Gull, 1994; Pollitt et al., 2011). Hence, the life cycle of important human pathogens provides exemplars of potentially bistable transitions in eukaryotic biology whose disruption could provide important targets to block virulence or transmission. However, the molecular processes that underlie the developmental transitions and their regulatory control are poorly characterized compared with the mitotic progression of eukaryotic cells, for example.

One differentiation step that is tractable both cytologically and in molecular terms is the development of African trypanosomes from their bloodstream form to the form found in the midgut of the tsetse fly vector. Trypanosomes proliferate in the bloodstream of mammals as morphologically “slender forms” before undergoing a density-dependent differentiation to G1 arrested “stumpy forms” (Vassella et al., 1997). Within experimental infections, uniform populations of stumpy forms can be purified and stimulated, in vitro, by exposure to cisaconitate (CA) and reduced temperature to initiate development to the procyclic forms that normally colonize the tsetse midgut (Ziegelbauer et al., 1990). If initiated with a population enriched in stumpy forms, the differentiation is remarkably synchronous, allowing events at the single cell level to be interpreted from population-level analysis, with sufficient cell numbers being available from experimental infections to allow both cytological and molecular studies (Matthews and Gull, 1994).
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differentiation. Finally, we identified the stumpy-enriched protein kinase Nek-related kinase (NRK) as a key regulator of the differentiation process.

Results

Trypanosome differentiation is an irreversible bistable switch

To characterize the response of trypanosomes to CA, uniform populations of stumpy forms were exposed to this developmental signal and monitored for their EP procyclin expression by flow cytometry. This provides a sensitive and quantitative measure that can be compared with earlier studies using scoring by immunofluorescence assay (Matthews and Gull, 1997), EP procyclin 3′UTR-linked reporter assays (Sbicego et al., 1999), or cell growth after differentiation (Engstler and Boshart, 2004). We also stained the nuclei and kinetoplasts with DAPI to follow cell cycle reentry as a confirmation of differentiation (unpublished data). Fig. 2 A demonstrates that EP procyclin was detected on most cells in the population after 1–2 h of exposure to CA, consistent with earlier studies. Thereafter, we calibrated the sensitivity and response time of the parasites to CA to map the point of commitment and to test the bistability of the underlying developmental switch. First, the response of the parasites to varying levels of CA was analyzed by the incubating stumpy forms with a titration of CA from 10^-4 mM to 6 mM CA. This demonstrated that 1 mM CA at 27°C was required for effective procyclin expression after 24 h (Fig. 2 B), matching the outgrowth of differentiated parasites at this temperature reported by Engstler and Boshart (2004) and EP-3′UTR–based reporter studies (Sbicego et al., 1999). Next, commitment to differentiation was analyzed by exposing stumpy forms to 6 mM CA for 1, 2, or 3 h, which were then washed free of CA and incubated for a further 24 h, whereupon EP procyclin expression was measured. Fig. 2 C demonstrates that although some cells (42%) expressed procyclin at 24 h after 1-h exposure to CA, procyclin expression was maintained at a high level for 24 h after 2- and 3-h exposure to CA (72% and 84%, respectively), positioning commitment at 2–3 h. The bimodal shapes of the EP expression profiles also indicated that cells either do or do not commit to differentiation, and that there is some noise in their decision, probably because of as-yet-unexplored differences between stumpy cells. Finally, hysteresis of the response was tested by exposing parasites to 6 mM CA for 3 h, after which cells were washed and returned to a titration of CA concentrations matching the earlier signal-response analysis (i.e., 10^-4 to 6 mM CA) for 24 h (Fig. 2 D). In all cases, the cells remained procyclin positive, despite being maintained at a signal level below the threshold necessary to stimulate the initial response. This also showed that the level of EP expression was not dependent on the CA concentration, which strengthens the notion that differentiation is independent of the signal after commitment. Memory of prior exposure to a CA level above the triggering threshold defines this transition as an irreversible bistable switch, and the data from Fig. 2 define the signal-response curve (or one-parameter bifurcation diagram) of this developmental switch.

Signal memory requires new protein synthesis

The demonstration that trypanosomes exhibited signal memory through irreversible commitment to differentiation after 2–3 h
exposure to CA led us to investigate the molecular basis of this. Commitment should depend on the triggering of a positive feedback loop, which could involve posttranslational modifications (e.g., phosphorylation) and/or mRNA or protein synthesis. To test whether new protein synthesis was necessary for commitment, we used the reversible protein synthesis inhibitor cycloheximide (CHX). For this, cells were exposed to 6 mM CA in the presence of CHX to block protein synthesis during the commitment phase. We also incubated cells with CA without CHX as a commitment and differentiation control. From 1, 2, or 3 h after exposure to CA and CHX (or CA alone), cells were washed and maintained in the absence of both CA and CHX or 3 h after exposure to CA and CHX (or CA alone), cells were washed free of CHX and incubated until 24 h, at which point their expression of EP procyclin was determined. Cells maintained for 24 h in CHX were swollen and immotile; here, detection of EP procyclin probably reflects nonspecific antibody trapping by dead cells or the expression of some EP procyclin as cells lose viability in CHX. Quantitation of the EP procyclin expression at each time point is shown.

**Protein expression and protein phosphorylation during commitment**

These experiments established the importance of newly made proteins for commitment but did not preclude an additional contribution from a positive feedback loop involving protein phosphorylation/dephosphorylation events on existing or newly synthesized proteins. To explore this, we analyzed changes in the protein and phosphorylation profile of the “commitment proteome.” Specifically, a quantitative proteomic and phosphoproteomic analysis was performed on stumpy forms either before or during commitment in the presence of CA (Fig. 5). Because metabolic labeling of stumpy forms by stable heavy isotopes in vivo is not experimentally tractable, a “spike-in” stable isotope labeling by amino acids in cell culture (SILAC) approach was used, whereby a reference heavy isotope–labeled sample of cultured cells was used as an internal standard (Ishihama et al., 2005). To maximize the proteome coverage of the reference, equal amounts of monomorphic bloodstream forms and cultured procyclic forms were combined. The heavy isotope–labeled standard then was used to compare unlabeled ex vivo stumpy cells or the same stumpy cells incubated with or without CA for 1 or 3 h, in biological triplicate. For two of the replicates, the commitment profile of the cells was validated by removing an aliquot at 1 h and 3 h, washing the cells free of CA, and then assaying their EP procyclin expression at 24 h. This confirmed that each sample showed the expected commitment profile 1–3 h after exposure to CA (one example is shown in Fig. 5). The commitment to cell cycle reentry was also monitored by analyzing the flow cytometry profile of cells with DNA stained with DAPI (Fig. 5). This matched the expression of EP procyclin and confirmed differentiation at 1–3 h after exposure to CA.

**Proteomic data overview**

The proteomic data set provides a snapshot of the complex changes that occur during signal perception and commitment events in the early stages of differentiation, as well as the changes in metabolism, protein coat, and reentry into the cell cycle.
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To enable quantitation of changes in both protein abundance and phosphorylation, each biological sample was split and subjected either to proteomic analysis (5%) or to enrichment of phosphopeptides (95%) before analysis (Fig. 5). Altogether, 324 liquid chromatography (LC) tandem mass spectrometry (MS/MS) runs identified a total of 5,911 protein groups containing 6,869 proteins, with 10,159 phosphorylation sites identified on 2,334 proteins (>0.75 localization probability) with a 1% false discovery rate. Quantitation of 5,285 protein groups containing 6,232 proteins was obtained using only data from the proteomic experiments, and 9,856 phosphorylation sites were quantified using only data from the enriched samples. Reproducibility was high, with biological replicates showing a Pearson correlation of 0.85–0.96 and 0.78–0.96 with the mean at the protein and phosphorylation site levels, respectively (Fig. 6, A and B; and Fig. S1). The Pearson correlation between different samples reflected their exposure to CA: untreated samples were most similar to each other and to \( t = 0 \) h, whereas upon CA treatment, over time, the correlation with \( t = 0 \) h decreased and the correlation with the procyclic form increased (Fig. 6, C and D).

That such patterns in correlation are discernible at even these early time points suggests that the changes observed are part of a defined program of events to retool the biology of the parasite in response to a perceived change in its environment.

The changes in the proteome and phosphoproteome in response to exposure to CA before (1 h) and after (3 h) commitment...
were quantified by calculating the difference between the treated and untreated samples at each time point, using the heavy isotope–labeled standard as an internal control (Tables S1, S2, and S3). Overall, the changes in phosphorylation sites were larger and occurred earlier than the changes in protein abundance. For example, at 1 h (precommitment), 356 phosphorylation sites (on 251 proteins) and 25 proteins changed by more than fourfold (log2 >2); at 3 h (postcommitment), 371 phosphorylation sites (on 257 proteins) and 36 proteins changed by more than fourfold (Fig. S2). Although a similar number of changes occurred at each time point, a largely distinct subset of proteins and phosphorylation sites changed after commitment at 3 h, with respect to before commitment at 1 h (Fig. 7), suggesting that there is a distinction between signal perception events occurring precommitment and events that occur once commitment has been reached. The observed changes were driven by changes in the treated samples, rather than recovery of the untreated samples (e.g., from cold shock; Fig. S3). The balance between phosphorylation and dephosphorylation events altered significantly before and after commitment, with events at 1 h dominated by dephosphorylation (72% > llog2 2), whereas a balance between dephosphorylation (48% > llog2 2) and phosphorylation was reached at 3 h.

Agreement with known biology
The changes in the proteome during differentiation from the stumpy form to the procyclic form reflect the parasite’s adaptation of its surface coat and metabolism for its new host environment, as well as reentry into the cell cycle. These changes are already evident in the proteomic data at only 3 h after exposure to CA (Table 1). Our data show significant enrichment of procyclic-specific surface proteins among proteins up-regulated more than fourfold (25%) compared with those changing less than fourfold (6.5%), in agreement with a recent study demonstrating that the Trypanosoma brucei cell surface is enriched in stage-specific proteins (Shimogawa et al., 2015). We observed the expected up-regulation of MSP-B (Tb927.8.1640), linked to VSG release during differentiation (Gruszynski et al., 2006), and elevation of the procyclic-specific surface protein PSSA-2 (Tb927.10.11220; Fragoso et al., 2009), nucleoside transporter NT10 (Tb927.9.7470; Sanchez et al., 2004), and trans-sialidase...
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(Tb927.7.6850; Engstler et al., 1992). Down-regulation was detected for the bloodstream form–specific surface protein ISG75 as well as BRCA2 (Tb927.1.640) linked to DNA repair and antigenic variation in the bloodstream (Trenaman et al., 2013). Developmental changes in metabolism included up-regulation of THT2A, gMDH, and sterol 24-C-methyltransferase, and down-regulation of the bloodstream-enriched PGK-B (Tb927.1.710; Blattner and Clayton, 1995). Reflecting the reinitiation of protein synthesis early during differentiation (Kabani et al., 2009), there was increased phosphorylation of eIF2β and eIF4ε. Interestingly, we also observed multiple changes in the phosphorylation site of the cytoskeleton-associated calpain-like proteases Tb927.1.2100 (log2 +3.30 to −1.51) and Tb927.11.1090 (log2 +4.25 to −1.80), the latter localizing to the flagella attachment zone and reported to be responsible for maintaining the bloodstream-form trypomastigote cell morphology (Hayes et al., 2014).

The data set also captured changes involved in signal perception and commitment to differentiation; for instance, up-regulation of TbPIP39 and dephosphorylation of PAD2 were observed, as was increased phosphorylation of PAD7 and ZFP1. Although knowledge of the differentiation signaling pathway is incomplete, PAD proteins (Dean et al., 2009), the tyrosine phosphatase TbPTP1 (Szöör et al., 2006), and the glycosomal protein TbPIP39 (Szöör et al., 2010) are known to be involved in the process. Unfortunately, we were not able to observe the phosphotyrosine sites PIP39-pY278 or NOPP44/46-pY181 (both substrates of TbPTP1; Chou et al., 2010; Szöör et al., 2010) because the flanking sequence meant that neither produced phosphopeptides detectable by LC mass spectrometry (MS; Table S1), although both were observed at the protein level. We also did not observe widespread changes in phosphotyrosine abundance or in glycosomal proteins (Güther et al., 2014) upon treatment; these changes may occur at later time points. Other signaling molecules with roles in differentiation, such as ZFK, MAPK5, TOR4, and RDK2, were observed but did not change significantly in abundance upon CA stimulation, suggesting that they are not dynamically regulated at the time points stud-

![Table 1. Examples of changes observed in the differentiation proteome](https://i.imgur.com/123456789.png)

<table>
<thead>
<tr>
<th>TrTrypDB ID</th>
<th>Description</th>
<th>Log2 T3C/T3N protein</th>
<th>Log2 T3C/T3N phosphorylationa</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tb927.8.1640</td>
<td>MSP-B, major surface protease</td>
<td>+3.67</td>
<td>—</td>
<td>Surface</td>
</tr>
<tr>
<td>Tb927.7.6850</td>
<td>TS, trans-sialidase</td>
<td>+1.68</td>
<td>—</td>
<td>Surface</td>
</tr>
<tr>
<td>Tb927.10.11220</td>
<td>PSSA-2, procyclic form surface protein</td>
<td>+1.45</td>
<td>—</td>
<td>Surface</td>
</tr>
<tr>
<td>Tb927.12.1100</td>
<td>Calpain-like cysteine peptidase</td>
<td>+0.36</td>
<td>S81 +4.25; S366 +3.23; T365 +2.72; S95 +2.64</td>
<td>Metabolism</td>
</tr>
<tr>
<td>Tb927.7.5940</td>
<td>PAD2, protein associated with differentiation 2</td>
<td>+0.05</td>
<td>T577 -4.46; T587 -3.65</td>
<td>Signaling</td>
</tr>
<tr>
<td>Tb927.5.370</td>
<td>ISG75, 75-kD invariant surface glycoprotein</td>
<td>−1.75</td>
<td>—</td>
<td>Surface</td>
</tr>
<tr>
<td>Tb927.10.8530</td>
<td>THT2A, glucose transporter</td>
<td>+3.05</td>
<td>T526 +2.83</td>
<td>Metabolism</td>
</tr>
<tr>
<td>Tb927.9.6100</td>
<td>PUF9, pumilio RNA binding protein</td>
<td>−1.98</td>
<td>—</td>
<td>Cell cycle</td>
</tr>
<tr>
<td>Tb927.7.5990</td>
<td>PAD7, protein associated with differentiation 7</td>
<td>−0.15</td>
<td>T584 +7.01; S580 +3.44; S582 +2.71</td>
<td>Signaling</td>
</tr>
<tr>
<td>Tb927.11.17770</td>
<td>VSG, variant surface glycoprotein</td>
<td>—</td>
<td>—</td>
<td>Surface</td>
</tr>
<tr>
<td>Tb927.11.17870</td>
<td>VSG, variant surface glycoprotein</td>
<td>−2.09</td>
<td>—</td>
<td>Surface</td>
</tr>
<tr>
<td>Tb927.10.15410</td>
<td>gMDH, glycosomal malate dehydrogenase</td>
<td>+2.43</td>
<td>—</td>
<td>Metabolism</td>
</tr>
<tr>
<td>Tb927.10.6950</td>
<td>Sterol 24-C-methyltransferase</td>
<td>+2.12</td>
<td>—</td>
<td>Metabolism</td>
</tr>
<tr>
<td>Tb927.17.110</td>
<td>PGKB, phosphoglycerate kinase B</td>
<td>−3.58</td>
<td>—</td>
<td>Metabolism</td>
</tr>
<tr>
<td>Tb927.7.12600</td>
<td>PIP39, PTP1-interacting protein</td>
<td>+0.20</td>
<td>T159 +2.89</td>
<td>Cell cycle</td>
</tr>
<tr>
<td>Tb927.11.1820</td>
<td>elf4e, eukaryotic translation initiation factor 4e</td>
<td>+0.19</td>
<td>S126 +2.57; T127 +2.57</td>
<td>Cell cycle</td>
</tr>
<tr>
<td>Tb927.9.6100</td>
<td>PIP39, PTP1-interacting protein</td>
<td>+2.50</td>
<td>—</td>
<td>Signaling</td>
</tr>
<tr>
<td>Tb927.7.5940</td>
<td>PAD2, protein associated with differentiation 2</td>
<td>+0.05</td>
<td>T577 -4.46; T587 -3.65</td>
<td>Signaling</td>
</tr>
<tr>
<td>Tb927.7.5990</td>
<td>PAD7, protein associated with differentiation 7</td>
<td>−0.15</td>
<td>T584 +7.01; S580 +3.44; S582 +2.71</td>
<td>Signaling</td>
</tr>
<tr>
<td>Tb927.6.3490</td>
<td>ZFP1, zinc finger protein 1</td>
<td>—</td>
<td>S18 +2.81; S16 +2.56</td>
<td>Signaling</td>
</tr>
</tbody>
</table>

aFor proteins with multiple phosphorylation sites, only those with the largest changes are given; see Table S3 for full details. Missing values were either not observed or not quantified.
ried. A significant subset of the proteins identified as displaying “loss of function in differentiation only” from genome-wide RNAi analysis were observed to undergo substantial changes, including many hypothetical proteins (Alsford et al., 2011). Likewise, a subset of the potential RNA binding proteins identified by an artificial RNA-tethering screen (Erben et al., 2014) underwent substantial changes. Given the post-transcriptional control of trypanosome gene expression (Clayton and Shapira, 2007), such modulation of RNA binding proteins is likely to play a role in early differentiation, perhaps analogous to the role of transcription factors in differentiation in other eukaryotes.

Molecules regulated during commitment and molecules that regulate differentiation

Having derived a comprehensive picture of the phosphoproteome during commitment, we sought to identify molecules implicated in its regulation. First, we explored the proteome and phosphoproteome data for molecules that exhibited changes in abundance and phosphorylation early during differentiation. Of these, one obviously regulated molecule was a predicted RNA binding protein, ZC3H22 (Tb927.7.2680). Hence, pleomorphic trypanosomes were generated where ZC3H22 was silenced by RNAi and its effects on stumpy formation or differentiation were analyzed. Fig. 54 shows that effective depletion of ZC3H22 was achieved in bloodstream forms, but that the generation of stumpy forms proceeded normally, precluding a role for this molecule during that developmental transition. When analyzed during differentiation to procyclic forms, EP procyclin was expressed on the expected timescale and the efficiency of differentiation to procyclic forms, EP procyclin was expressed during that developmental transition. When analyzed during differentiation, we sought to identify molecules implicated in its regulation. First, we explored the proteome and phosphoproteome data for molecules that exhibited changes in abundance and phosphorylation early during differentiation. Of these, one obviously regulated molecule was a predicted RNA binding protein, ZC3H22 (Tb927.7.2680). Hence, pleomorphic trypanosomes were generated where ZC3H22 was silenced by RNAi and its effects on stumpy formation or differentiation were analyzed. Fig. 54 shows that effective depletion of ZC3H22 was achieved in bloodstream forms, but that the generation of stumpy forms proceeded normally, precluding a role for this molecule during that developmental transition. When analyzed during differentiation to procyclic forms, EP procyclin was expressed on the expected timescale and the efficiency of differentiation to procyclic forms, EP procyclin was expressed during that developmental transition. When analyzed during differentiation, we sought to identify molecules implicated in its regulation.

Discussion

The commitment of cells to a differentiation program is fundamental in eukaryotic development. Trypanosomes represent an interesting model to examine the cell biology of such unidirectional developmental events, these being among the most evolutionarily divergent eukaryotes (Sogin et al., 1986; Walker et al., 2011). Moreover, the regulatory processes underlying their life cycle control are likely to be conserved in a broad range of protozoan pathogens, in which progressive differentiation steps characterize their transition between different hosts, or in complex host-vector interactions. Here, we have exploited the synchronous and tractable in vitro differentiation of stumpy form parasites to test midgut procyclic forms to examine parasite cell commitment from several complementary perspectives. First, we examined the reversibility of their cell commitment processes, formally demonstrating that it represents an irreversible bistable switch and further revealing that signal memory during commitment requires protein synthesis. Second, we examined the molecular events accompanying commitment, providing a detailed map of the parasite’s commitment proteome and phosphoproteome. Finally, we used this information, triangulated with prior knowledge, to identify a key regulatory kinase controlling the initiation of the differentiation program.
Initially, we reexamined earlier studies that used relatively subjective immunofluorescence detection of the EP procyclin surface antigen to map the commitment point during the synchronous differentiation from stumpy to procyclic forms (Matthews and Gull, 1997; Sbicego et al., 1999). Our quantitative analysis placed irreversible commitment to differentiation at 2–3 h after exposure to CA, such that the cells maintain the expression of procyclin (already expressed at commitment) but also go on to reenter a proliferative cell cycle (DNA synthesis having been mapped to occur at 8–10 h; Matthews and Gull, 1994). These studies also demonstrated that commitment (at 27°C; at 20°C and 37°C, cells commit on the same timescale; unpublished data) required exposure to at least 1 mM CA and that once this signal was present for 2–3 h, the cells retained memory of the signal exposure even when reincubated at levels of CA unable to drive differentiation ab initio. If this signal memory were dependent only on posttranslational modifications, we predicted that cells exposed to CA in the presence of CHX would progress into the differentiation program once the inhibitor was removed. This was not the case; parasites treated with CHX did not respond to CA unless the inhibitor was washed out and CA was added, after which they differentiated on a timescale consistent with de novo exposure to the signal. This demonstrated that new protein synthesis is required for the commitment to differentiation and sets the clock for the initiation of the process. Although phosphorylation events could also contribute, these studies establish that CA exposure does not generate a stable signal involving only posttranslational mechanisms, or an effector pathway requiring RNA alone.

To explore the molecular events accompanying the early steps in differentiation, we analyzed the commitment proteome and phosphoproteome. Previous studies have compared the protein and phosphorylation profile of different life cycle stages (Nett et al., 2009; Gunasekera et al., 2012; Urbaniak et al., 2013) but have not compared cells pre- and postcommitment in a synchronous differentiation model with accompanying biological validation of the cell fate of each sample. Providing reassurance on the quality of the data set, there was good correspondence between individual, independently performed, biological replicates, and key changes observed early in differentiation agreed well with prior characterization of this transition. The temporal analysis at 0 h, 1 h (precommitment), and 3 h (postcommitment) also confirmed the importance of new protein synthesis to commitment, such that few changes occurred in protein abundance at 1 h, with most changes occurring only at 3 h; this is in contrast to protein phosphorylation, which was already changed dramatically at 1 h. Although most changes in phosphorylation did not simply correspond to changes in protein abundance, one exception was ZC3H22, a predicted RNA binding protein strongly induced during differentiation with a concomitant multisite phosphorylation profile. This protein, however, appears to be unrelated to the developmental process itself, instead being expressed and required for the viability of procyclic forms. Other changes in protein abundance or phosphorylation await individual analysis and comparison with data sets analyzing the cell cycle of bloodstream and procyclic forms. This will provide an invaluable framework to distinguish differentiation-specific changes from the events of cell cycle reentry only.

As an approach to the identification of regulatory molecules that might control early steps in the differentiation program, we selected molecules on the basis of stumpy-enriched expression, signaling function, and RIT-seq phenotype. This focused our attention on the NRKA/NRKB protein kinases. These molecules have serine threonine kinase activity (Gale...
and Parsons, 1993; Gale et al., 1994) and are members of the expanded NEK kinase family in African trypanosomes (Jones et al., 2014). They have also been shown to be developmentally regulated at the translational level (Gale et al., 1994). RNAi against these molecules demonstrated that inducible depletion of NRK inhibited the response of the parasites to CA. Interestingly, the parasites were also less able to respond to an alternative differentiation trigger, pronase. This signals effective differentiation but operates independently of TbPIP39, a glycosomally directed phosphatase important in the CA-mediated differentiation pathway (Szöösr et al., 2010). The NRK-depleted parasites also differentiated less efficiently when exposed to BZ3, an inhibitor of the tyrosine phosphatase TbPTP1 that acts upstream of, and dephosphorylates, TbPIP39 (Szöösr et al., 2006; unpublished data). Consequently, NRK most likely operates downstream of where the CA and pronase pathways converge to drive differentiation, or it acts on both branches. Hence, NRK is identified as the first protein kinase necessary for trypanosome development to procyclic forms and its conservation and synteny, where known, in all other kinetoplastids, including *Crithidia fasciculata* and *Bodo saltans*, implies an important role throughout the order.

Combined, our studies represent a comprehensive cytological and molecular dissection of cell differentiation in an early diverging eukaryotic pathogen. Similar processes are fundamental in the life cycles of all protozoan pathogens, including related kinetoplastids (*T. cruzi* and *Leishmania* parasites) and those with an enormous impact on human health, such as malaria. Each of these parasites undergoes proliferation and then cell cycle arrest before reentry into their cell cycle, with concomitant differentiation to the next life cycle stage. In each case, the developmental cycles are also unidirectional and involve irreversible commitment steps. This regulatory framework ensures that the necessary conditions are met for successful progression to the next developmental step in the parasite’s life cycle and avoids lethal indecision in a changing environment.

**Materials and methods**

**Trypanosomes**

*T. brucei* AnTat1.1 parasites were used for all experiments. Transgenic parasites were *T. brucei* AnTat1.1 90:13 cells, which express the T7
RNA polymerase and tetracycline repressor protein (Engstler and Boshart, 2004). RNA constructs were based on the pALC 14 plasmid (Pusnik et al., 2007). Transfection and cell maintenance in HMI-9 medium containing 20% FCS (Hirumi and Hirumi, 1989) was performed as previously described (Szőör et al., 2013).

In vivo growth involved i.p. injection of ~10^5 parasites. At day 6 after infection, parasites were >80% stumpy by morphology, and trypomastigotes were harvested and purified from blood by DEAE chromatography (Lanham and Godfrey, 1970). The purified parasites were incubated in HMI-9 medium at 37°C for 1.5 h at a concentration of 2 × 10^5/ml and were differentiation stimulated using an appropriate concentration of CA. For isolation of samples for flow cytometry, 3 ml of culture was concentrated at 3,000 rpm in a clinical centrifuge, washed briefly with PBS, and then centrifuged in a microfuge at 2,500 rpm. The cell pellet was resuspended in FACS fix solution (2% formaldehyde and 0.05% glutaraldehyde in PBS) and stored at 4°C. For commitment experiments, samples at time points throughout differentiation were harvested by centrifugation at 2,000 rpm in a clinical centrifuge before resuspension in HMI-9 20% FCS without CA. For protein synthesis inhibition experiments, cells were incubated with 0.1 mM CHX.

Flow cytometry analysis
Stored fixed cell samples in 2% formaldehyde and 0.05% glutaraldehyde were diluted in PBS, centrifuged at 2,500 rpm in a clinical centrifuge, and then resuspended in 500 µl PBS 2% BSA. Samples were stored for 30 min at room temperature to block. Thereafter, the tubes were filled with PBS, centrifuged, and resuspended in 200 µl EP procyclin antibody diluted 1:500 in PBS 2% BSA. After incubation for >1 h, samples were diluted in PBS, centrifuged, and washed once with PBS before being resuspended in a 1:1,000 dilution of α-mouse FITC secondary antibody and incubated for 30 min at room temperature. The samples were then washed twice in PBS and resuspended in PBS DAPI (20 ng/ml). Flow cytometry was performed using an LSRII flow cytometer (Becton Dickinson) and data were analyzed using FlowJo X 10.0.7r2 (Treestar Inc.).

Western blotting was performed as described in Szőör et al. (2013) using anti-TbPIP39 (Szőör et al., 2010) diluted 1:200, anti-Tb-NRK (Gale et al., 1994) diluted 1:100, or anti-EF1α diluted 1:5,000.

SILAC labeling of cultured cells
SILAC labeling of bloodstream and procyclic forms of T. brucei was performed, using media containing l-arginine U-13C6 and l-lysine 4,4,5,5-H4 (R,K4; Cambridge Isotope Labs; Urbaniak et al., 2012, 2013). Monomorphic bloodstream-form MITat 1.2 cells (Wirtz et al., 1999) were diluted 10,000-fold into HMI11-SIL (2013). Monomorphic bloodstream-form MITat 1.2 cells (Wirtz et al., 1999) were diluted 10,000-fold into HMI11-SIL plus R,K4 containing 2.5 µg/ml G418 at 37°C in a 5% CO2 incubator. Bloodstream-form cells were harvested after 3-d growth at ∼2 × 10^6 cells/ml. Procyclic form T. brucei clone 29.13.6 cells were washed with SDM-79-SILAC and then grown at 28°C without CO2 in SDM-79-SILAC plus R,K4 in the presence of 15 µg/ml G418 and 50 µg/ml hygromycin. Procyclic form cells were passaged every 2 d to enlarge the culture and to reach seven to eight cell divisions under labeling conditions, and they were then harvested at ∼2 × 10^7 cells/ml. Both cell types were harvested by centrifugation and hypotonically lysed at 10^7 cells/ml for 5 min on ice in the presence of protease inhibitors (0.1 µM 1-chloro-3-lysylamido-7-amino-2-heptone, 1 mM benzamidine, 1 mM phenyl-methyl sulfonyl fluoride, 1 µg/ml leupeptin, and 1 µg/ml aprotenin) and Phosphatase Inhibitor Mixture II (Calbiochem).

To produce the heavy-SILAC standard for use in the spike-in SILAC experiments, an equal volume of R,K4-labeled bloodstream and R,K4-labeled procyclic lysates were mixed, aliquoted, snap frozen, and stored at −80°C. To minimize variability, a single batch of the heavy-SILAC standard was used for the entire study.

Comparative proteomic and phosphoproteomic analysis
Ex vivo stumpy cells (5 × 10^6 cells) were maintained in HMI-9 and at 37°C for 1.5 h before initiation of the experiment to reduce any inconsistency resulting from their isolation and purification from blood, for example. The procedure was performed in triplicate, using distinct infections to generate the starting stumpy form population. For analysis, each sample was supplemented with an equal cell number of the R,K4-labeled standard, the mixture was solubilized with SDS, and tryptic peptides generated by an adaptation of the filter-aided sample preparation procedure (Wisniewski et al., 2009; Urbaniak et al., 2013). Because phosphorylation occurs at low abundance and is difficult to detect by MS, the peptides were split between a proteomic (5%) and phosphoproteomic (95%) workflow. In the proteomic workflow, peptides were fractionated into six fractions by strong cation exchange chromatography before analysis by LC-MS/MS as described previously (Urbaniak et al., 2012). In the phosphoproteomic workflow, phosphopeptides were enriched and fractionated into six fractions by strong cation exchange (Beausoleil et al., 2004) before further enrichment by TiO2 in the presence of 5% TFA and 1 M glycolic acid (Thingholm et al., 2006; Jensen and Larsen, 2007), as described previously (Urbaniak et al., 2013).

MS data acquisition
LC-MS/MS was performed by the FingerPrints Proteomic Facility at the University of Dundee. LC was performed on a fully automated Ultimate 3000 Nano LC System (Dionex) fitted with a 1 × 5 mm PepMap C18 trap column and a 75 µm × 15 cm reverse-phase PepMap C18 nano-column (LC Packings; Dionex). Samples were loaded in 0.1% formic acid (buffer A) and separated using a binary gradient consisting of buffer A (0.1% formic acid) and buffer B (90% MeCN and 0.08% formic acid). Peptides were eluted with a linear gradient from 5% to 40% buffer B over 65 min. The HPLC system was coupled to an LTQ Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific) equipped with a Proxeon nanospray ion source. For phosphoproteomic analysis, the mass spectrometer was operated in data-dependent mode to perform a survey scan over a range of 335–1,800 m/z in the Orbitrap analyzer (R = 60,000), with each MS scan triggering 15 MS2 acquisitions of the 15 most intense ions using multistage activation on the neural loss of 98 and 49 Th in the LTQ ion trap (Schroeder et al., 2004). For proteomic analysis, the mass spectrometer was operated in data-dependent mode, with each MS scan triggering 15 MS1 acquisitions of the 15 most intense ions in the LTQ ion trap. The Orbitrap mass analyzer was internally calibrated on the fly using the lock mass of polydimethylcyclosiloxane at m/z 445.120025.

MS data processing
Data were processed using MaxQuant (version 1.5.2.8; Cox and Mann, 2008), which incorporates the Andromeda search engine (Cox et al., 2011). Proteins were identified by searching a protein sequence database containing T. brucei 927 annotated proteins (version 9.0, 11,568 protein sequences; TriTrypDB; Aslett et al., 2010), supplemented with the VSG221 sequence and frequently observed contaminants (porcine trypsin, bovine serum albumin, and mammalian keratins). Search parameters specified an MS tolerance of 6 ppm, an MS/MS tolerance at 0.5 D, and full tryptic specificity, allowing for up to two missed cleavages. Carbamidomethylation of cysteine was set as a fixed modification and oxidation of methionines, N-terminal protein acetylation, and N-pyroglutamate were allowed as variable modifications. Phosphoproteomic analysis included phosphorylation of serine, threonine, and tyrosine as additional variable modifications. Peptides were required to be at least seven amino acids in length and to have a MaxQuant score >5, with false discovery rates of 0.01 calculated at the levels of...
peptides, proteins, and modification sites based on the number of hits against the reversed sequence database. SILAC ratios were calculated where at least one peptide could be uniquely mapped to a given protein group, and they required a minimum of two SILAC pairs. To account for any errors in the counting of the number of cells mixed, the distribution of SILAC ratios was normalized within MaxQuant at the peptide level so that the median of log$_2$ ratios was zero, as described by Cox and Mann (2008).

Before statistical analysis, the outputs from MaxQuant were filtered to remove known contaminants and reverse sequences, and phosphorylation sites with a MaxQuant localization probability <0.75 were discarded. SILAC ratios for phosphorylation sites were calculated using only data from the phosphoproteomic experiments, and SILAC ratios for proteins were calculated using only data from the proteomic experiments. Because the heavy SILAC-labeled cells ($H_2$) were derived from a single batch, they constitute an invariable internal standard with respect to the light SILAC-labeled cells ($L_2$).

The authors declare no competing financial interests.

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