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Cell Morphogenesis of *Trypanosoma brucei* Requires the Paralogous, Differentially Expressed Calpain-related Proteins CAP5.5 and CAP5.5V

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Proteins from the calpain super-family are involved in developmentally- and environmentally-regulated re-modelling of the eukaryotic cytoskeleton and the dynamic organisation of signal transduction cascades. In trypanosomatid parasites, calpain-related gene families are unusually large, but we have little insight into the functional roles played by these molecules during trypanosomatid lifecycles. Here we report that CAP5.5, a cytoskeletal calpain-related protein subject to strict stage-specific expression in the sleeping sickness parasite *Trypanosoma brucei*, is essential and required for correct cell morphogenesis of procyclic (tsetse mid-gut stage) *T. brucei*. Striking consequences of *CAP5.5* RNA interference are the loss of protein from the posterior cell-end, organelle mis-positioning giving rise to aberrant cytokinesis, and disorganisation of the sub-pellicular microtubules that define trypanosome cell shape. We further report that the stage-specificity of *CAP5.5* expression can be explained by the presence of a paralogue, *CAP5.5V*, which is required for cell morphogenesis in bloodstream *T. brucei*; RNAi against this paralogous protein results in a qualitatively similar phenotype to that described for procyclic *CAP5.5* RNAi mutants. By comparison to recently described phenotypes for other procyclic trypanosome RNAi mutants, likely functions for CAP5.5 and CAP5.5V are discussed.

Key words: calpain; cytoskeleton; microtubule; procyclic; trypomastigote; trypanosome.

Introduction

Cytoskeletal re-modelling is a critical feature in any cell division cycle. In eukaryotes, cytoskeletal re-modelling is also often central to cell motility and the dynamic organisation of signal transduction cascades. In animals, molecules that are often central to cytoskeletal organisation and signalling include calpains – a family of Ca^{2+}-regulated cysteine proteases – and calpain-related proteins (Franco and Huttenlocher 2005; Goll et al. 2003; Lebart and Benyamin 2006). Calpain-related proteins have been identified in fungi, protists, and plants too, where they are involved in developmental and environmentally-regulated processes (Croall and Ersfeld 2007;

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Denison et al. 1995; Futai et al. 1999; Li et al. 2004; Rosenthal 2004; Wang et al. 2003). However, calpain-related gene families in non-animal taxa are generally small, and often limited to a single gene (e.g. *DEK1* in *Arabidopsis thaliana* (Wang et al. 2003)). Thus, notwithstanding the challenges posed by the need for survival or growth within several distinctive environments in both mammalian host and insect vector, our report of unusually large calpain-related gene families in three rather different trypanosomatid parasites, *Trypanosoma brucei*, *T. cruzi*, and *Leishmania major* (Ersfeld et al. 2005), was both surprising and intriguing.

The trypanosomatids are a group of flagellate parasites and include several major human, livestock and plant pathogens. Most members of this parasite family undergo complex lifecycles, requiring differentiation into multiple, morphologically distinct forms in very different environments within a mammalian host and an insect vector. *Trypanosoma brucei*, the focus of this study, is the causal agent of human African sleeping sickness and livestock trypanosomiasis; it is transmitted between mammals by tsetse flies and seven morphologically distinct forms of the parasite are currently recognised (Sharma et al. 2008; Van Den Abbeele et al. 1999; Vickerman 1969, 1985). Of these different morphological forms two, the procyclic (tsetse mid-gut) and (pathogenic) long-slender bloodstream trypomastigote forms, can be cultured in vitro and subjected to genetic manipulation in the laboratory. Different trypanosomatid morphologies are classified according to several structural parameters, including the position of the nucleus and the kinetoplast (the name given to the unique and intricate mitochondrial genome structure in trypanosomatids) within the cell body (Gull 1999). However, trypanosome cell shape is always determined by the patterning of an elaborate, microtubule-based cytoskeleton (e.g. Gull 1999; Kohl et al. 2003; Matthews et al. 1995; Robinson et al. 1995; Sherwin and Gull 1989a, 1989b; Sherwin et al. 1987). This subpellicular cytoskeleton consists of a corset-like monolayer of evenly-spaced microtubules, in which neighbouring microtubules are cross-linked to one another and the plasma membrane by various microtubule-associated proteins (MAPs). During each cell division cycle new subpellicular microtubules are extended and intercalated between older microtubules assembled in previous cell cycles (Sherwin and Gull 1989b).

A number of MAPs have been identified from *T. brucei* and other trypanosomatids (Baines and Gull 2008; Hertz-Fowler et al. 2001; Vedrenne et al. 2002), but the molecular mechanisms which orchestrate cytoskeletal re-modelling during the cell cycle or differentiation, and the semi-conservative inheritance of subpellicular microtubules between the progeny at cytokinesis are largely unknown. In this context, we have very little insight into why expansion of the calpain-related gene family has occurred or of function in these important and evolutionarily divergent (Burki et al. 2008; Hampl et al. 2009) parasites. Intriguingly, the first biochemically identified *T. brucei* calpain-related protein, CAP5.5,[1] is a cytoskeletal protein in procyclic trypomastigotes which is evenly distributed across the sub-pellicular microbtuble corset in detergent-extracted cells (Hertz-Fowler et al. 2001). CAP5.5 is also subject to strict stage-specific regulation: protein is expressed in procyclic trypomastigotes, and classically provides a late stage marker for bloodstream-procyclic trypomastigote differentiation when detected using the anti-CAP5.5 monoclonal antibody (Matthews and Gull 1994). Interestingly, N-terminal myristoylation and palmitoylation of CAP5.5 suggests the protein interfaces with the plasma membrane, as well as subpellicular microtubules, but degeneracy within the putative active-site of the calpain-related domain raises uncertainty as to whether CAP5.5 is an active protease (Hertz-Fowler et al. 2001), and recombinant expression of native protein has been unsuccessful. Thus, several functions can be envisaged for CAP5.5: it could be a mere structural component of the procyclic cytoskeleton, it might be a protease required for cytoskeletal re-modelling, or perhaps the subpellicular microtubules provide a platform to facilitate organisation of a CAP5.5-dependent signalling cascade. Here, we report the results from RNAi experiments that selectively targeted either *CAP5.5* or a recently identified paralog, which we call *CAP5.5V* (*CAP5.5 variant*).

[1]CAP5.5 (Tb927.4.3950; encoding cytoskeletal-associated-protein 5.5) was previously named CALP1 by Hertz-Fowler et al. (2001), and then revised to TbCALP4.1CAP5.5 by Ersfeld et al. (2005). However, in the publicly available *T. brucei* genome sequence the original name of CAP5.5 is retained. Applying the nomenclature suggested by Ersfeld et al. (2005) CAP5.5V (Tb927.8.8330) would formally be recognised as TbCALP8.1-CAP5.5V.CAP5.5 (Tb927.4.3950; encoding cytoskeletal-associated-protein 5.5) was previously named CALP1 by Hertz-Fowler et al. (2001), and then revised to TbCALP4.1CAP5.5 by Ersfeld et al. (2005). However, in the publicly available *T. brucei* genome sequence the original name of CAP5.5 is retained. Applying the nomenclature suggested by Ersfeld et al. (2005) CAP5.5V (Tb927.8.8330) would formally be recognised as TbCALP8.1CAP5.5V.

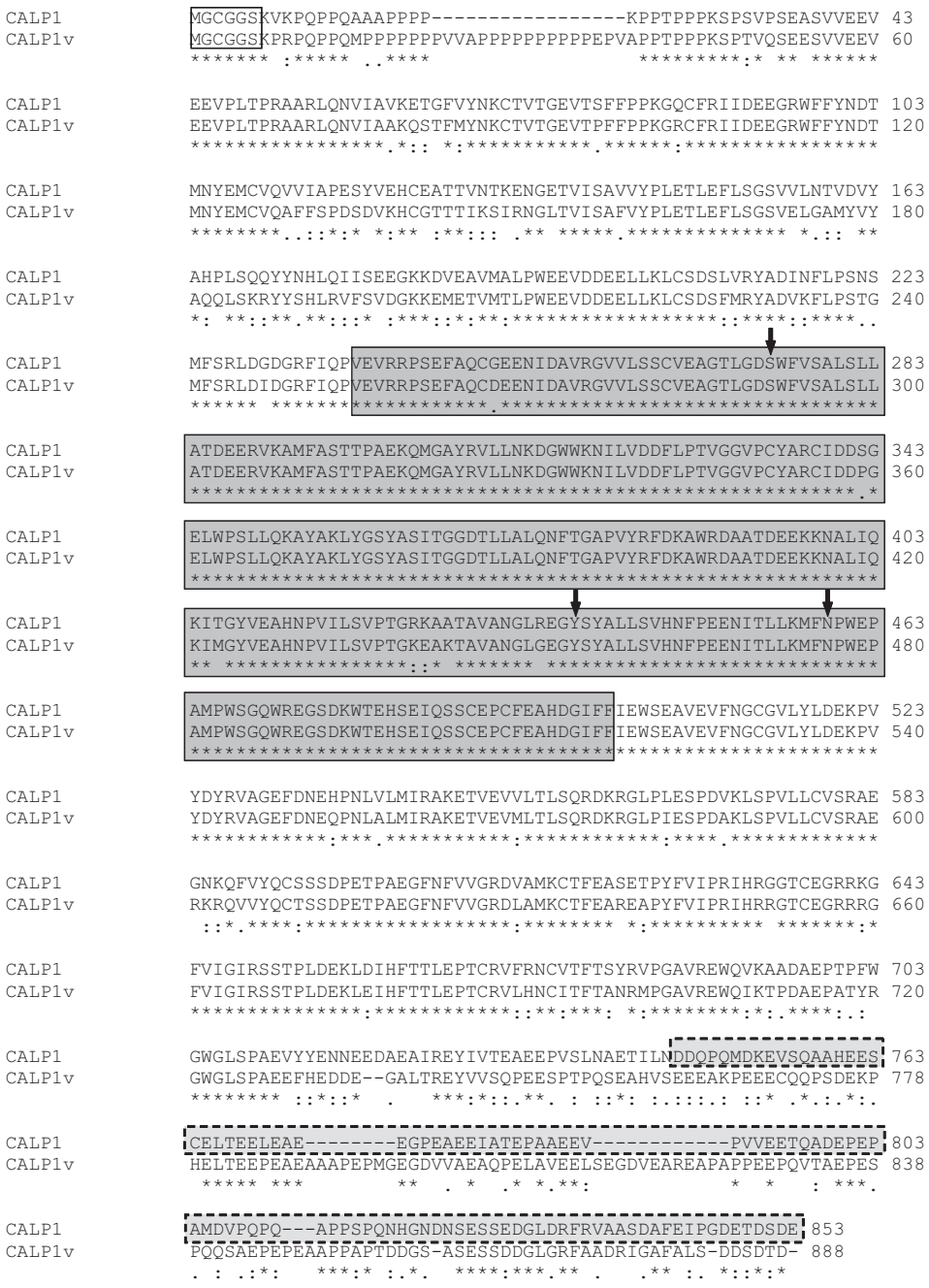

Figure 1. Amino acid sequence alignment between CAP5.5 and CAP5.5V. Positions of amino acid identity are marked with asterisks and an N-terminal dual acylation motif conserved in both proteins is boxed. The central calpain-related domain recognised from earlier studies of CAP5.5 (Hertz-Fowler et al. 2001) is boxed in grey; the C-terminal extension originally noted by Hertz-Fowler et al. (2001) to be present in CAP5.5 is highlighted by a dashed box and grey shading. Potential active site residues (which deviate from the classic "C-H-N" cysteine protease catalytic triad) are denoted by arrowheads. The DNA sequence identity between *CAP5.5* and *CAP5.5V* over the first 720 encoded amino acids of CAP5.5 is ~85%.

CAP5.5V lies within a 0.5 Mb region of *T. brucei* chromosome 4 that was duplicated and now forms a sub-telomeric arm on chromosome 8 (El-Sayed et al. 2005; Jackson 2007). At the DNA level, *CAP5.5* and *CAP5.5V* differ extensively only in the 3′ coding and 3′ untranslated regions; as

with many protein-coding genes in trypanosomes the 3′ untranslated region presumably contains the cis-regulatory elements responsible for controlling gene expression. Within the African trypanosome family (i.e. including the livestock pathogens *T. congolense* and *T. vivax*), the duplication of over seventy protein-coding genes from chromosome 4 appears specific to *T. brucei*, and represents a relatively recent evolutionary event for which the physiological consequences are unknown (Jackson 2007). Our analysis of these calpain-related paralogs reveals *CAP5.5* is essential for cell morphogenesis in procyclic *T. brucei*, whereas *CAP5.5V* is expressed and essential in bloodstream form trypanosomes. Immunocytochemistry of *CAP5.5* RNAi-induced procyclic cells using the CAP5.5 monoclonal antibody suggests cytoskeletal association of CAP5.5 at the posterior cell-end is highly dynamic during the cell division cycle, and reveals that loss of CAP5.5 from a cell's posterior end is followed by aberrant organelle segregation and cytokinesis. Ultimately, defective organisation of the subpellicular microtubules is observed. We discuss implications arising from these data.

Results

Stage-specific Expression of two Calpain-related Paralogs

The amino acid sequences of CAP5.5 and the paralog, CAP5.5V, from chromosome 8 are highly similar except for non-homologous C-terminal regions (Fig. 1). The 3′ untranslated regions of the genes which encode both proteins are also distinct. We exploited these differences in order to design gene-specific oligonucleotides for use in real-time RT-PCR analyses (Fig. 2). The analysis of *CAP5.5* expression, relative to that of γ-tubulin, revealed ~five-fold greater accumulation of mRNA in procyclic trypomastigotes than that observed in bloodstream cells, correlating with previous studies that revealed CAP5.5 to be a procyclic-specific protein (Hertz-Fowler et al. 2001). In contrast, the accumulation of *CAP5.5V* mRNA was ~nineteen-fold greater in bloodstream *T. brucei* relative to procyclic cells. The close similarity between CAP5.5 and CAP5.5V over their first seven hundred or so amino acids includes strict conservation of an N-terminal dual acylation motif and the presence of a proline-rich region immediately downstream of this myristoylation-palmitoylation signal; this similarity coupled to differential gene expression raised the possibility that the paralogous CAP5.5 and CAP5.5V provide similar functions in different life cycle stages.

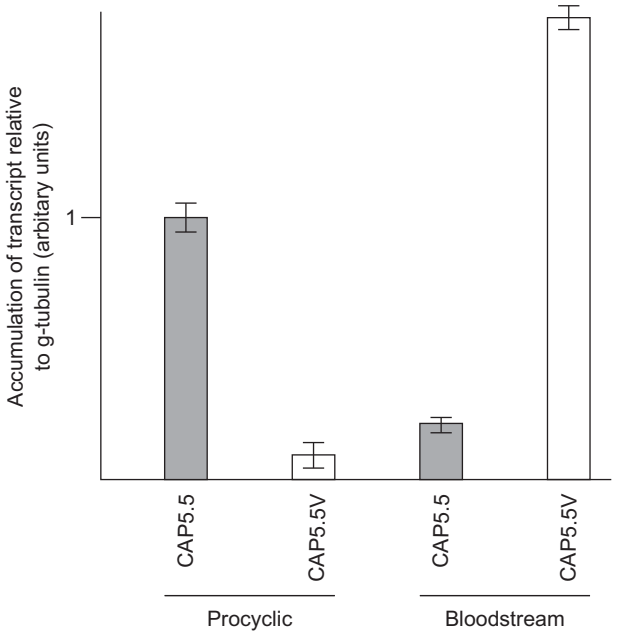

Figure 2. Quantitative real-time PCR analysis reveals the accumulation of *CAP5.5* and *CAP5.5V* transcripts in procyclic or bloodstream trypomastigotes, respectively. Accumulation of transcripts was determined by normalisation to γ-tubulin expression, and relative expression of *CAP5.5* in procyclic cells was arbitrarily set to 1. Values and errors are as described in the Methods. The same pattern of stage-specific expression was observed when transcript abundance was normalised against QM10 expression (not shown).

CAP5.5 is Essential in Procyclic *T. brucei*

To probe *CAP5.5* and *CAP5.5V* function we exploited differences between the 3′ coding regions and UTRs of both genes to design inducible RNAi constructs for gene-specific silencing. We first examined the RNAi phenotypes in procyclic trypomastigotes. RNAi against *CAP5.5*, but not *CAP5.5V*, resulted in loss of CAP5.5 protein and had a profound effect on both cell growth and, as discussed below, cell morphology (Figs 3 and 4). The lack of any discernable phenotype from the construct designed to silence *CAP5.5V* expression concurred with the stage-specificity of gene expression suggested by the RT-PCR analysis.

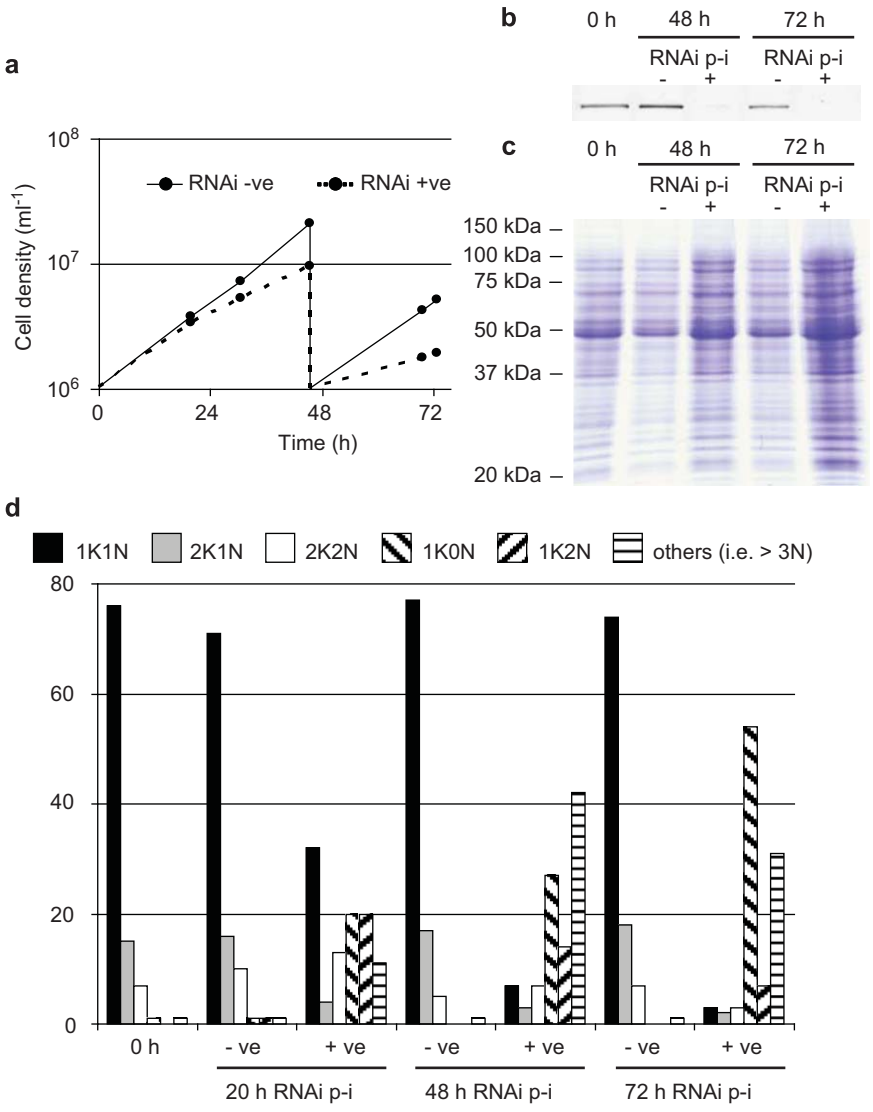

Figure 3. RNAi against *CAP5.5* affects growth and morphology of procyclic *T. brucei*. (**a**) A growth curve illustrates the effect of RNAi induction on cell density; neither culture (RNAi −ve and RNAi +ve) was allowed to reach stationary phase; both cultures were re-inocluated at a cell density of 10^6 cells ml^{-1} after 46 h RNAi induction. (**b**) Immunoblot anlaysis using the monoclonal antibody CAP5.5 reveals loss of CAP5.5 in RNAi-induced cells; 2×10^6 cell equivalents were loaded in each lane. (**c**) Loading control for the immunoblot analysis shown in (b) is provided by Coomassie blue staining of an equivalently loaded polyacrylamide gel – note how in the samples collected from RNAi-induced cells slightly more protein is loaded, but CAP5.5 protein is not readily detected in the immunoblot analysis. (**d**) The number of nuclei and kinetoplasts were counted in DAPI-stained populations of uninduced (−ve) and induced (+ve) cultures at 0, 24, 48, and 72 h post-induction of RNAi. One representative experiment from two performed is shown.

The temporal order of the key events in procyclic cell division cycle (e.g. the relative timing of kinetoplast replication, nuclear S-phase, kinetoplast division, mitosis, and various microtubule mediated events, such as new flagellum growth or cleavage furrow ingression) are well understood (Woodward and Gull 1990); key events relevant to this work are summarised in Figure 4a. Within asynchronous procyclic cultures the approximate position of individual cells within their cell division cycles is readily determined by 4,6-diamidino-2-phenylindole (DAPI) staining of the mitochondrial

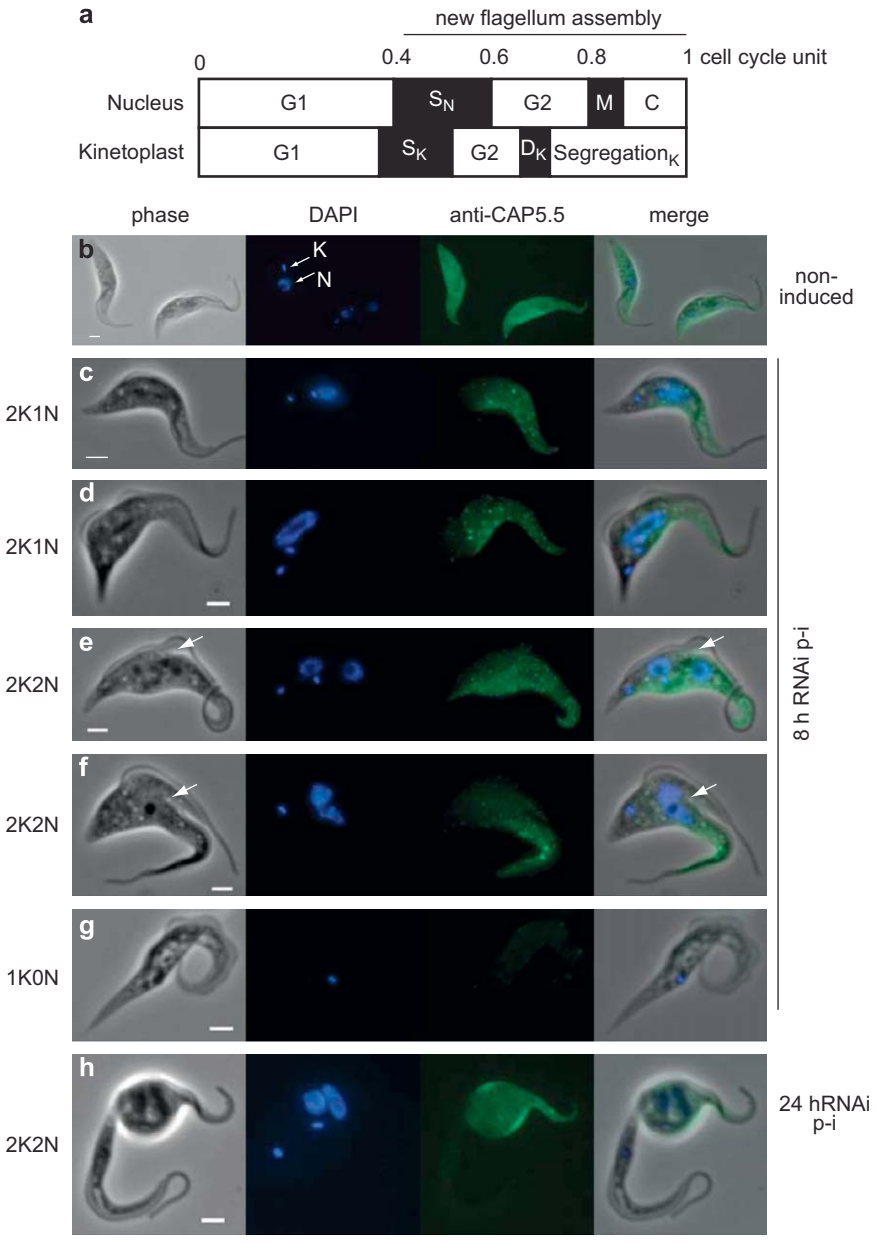

Figure 4. Loss of CAP5.5 from the posterior cell end results in aberrant cytokinesis. (**a**) Cartoon summary of key events in the cell cycle of procyclic *T. brucei* illustrates the timings of nuclear and kinetoplast DNA replication cycles (S_N, nuclear S-phase; M, mitosis; C, cytokinesis; S_k, kinetoplast S-phase; D_k, kinetoplast division). Mitochondrial DNA is physically attached to the flagellar basal bodies and kinetoplast segregation coincides with basal body segregation during new flagellum assembly (Ogbadoyi et al. 2003; Robinson and Gull 1991). (**b**) In wild type or non-induced *CAP5.5* RNAi mutants CAP5.5 is evenly distributed across the sub-pellicular microtubules of whole cells. Images of a 1K1N and a post-mitotic 2K2N cell are shown. (**c-h**) Loss of posterior end CAP5.5 correlates with aberrant cytokinesis. At 8 h post-induction of *CAP5.5* RNAi 2K1N (**c-d**) or 2K2N (**e-f**) cells exhibiting posterior end loss of CAP5.5 would have been at an earlier stage in the same cell cycle (either 1K1N or 2K1N, respectively) at the point when RNAi was induced. The ingression of a cleavage furrow (arrow) in the 2K2N cells evidences the aberrant positioning of nuclei following mitosis; predicted progeny of cytokinesis for these cells would include an anucleate zoid (e.g. panel **g**). (**h**) Aberrant organelle positioning and cytokinesis at 24 h post-induction of RNAi. Scale bars represent 2 μm.

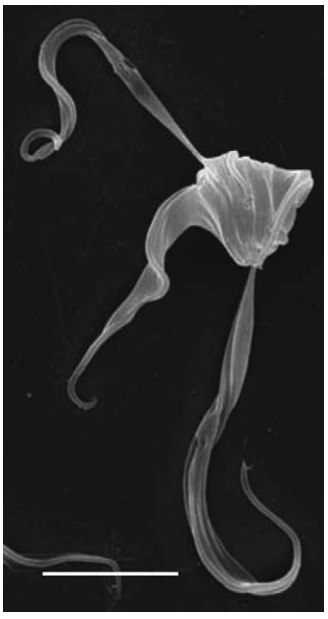

Figure 5. Zoid formation is likely at late time-points following *CAP5.5* RNAi induction. Cultures were fixed for SEM after 48 h induction of RNAi. In the cell shown, the relatively normal-looking anterior end contrasts vividly with an abnormal posterior end; abscission of the two attached daughter "cells" would yield two slender zoids. The scale bar represents 5 μm.

and nuclear DNA. Normally, 3 types of cell are evident, corresponding to 1K1N, 2K1N and 2K2N configurations in relative proportions similar to those indicated in Figure 3d. In such cells CAP5.5 protein is distributed evenly throughout the cell body but is absent from the flagellum (Fig. 4b; Hertz-Fowler et al. 2001; Matthews and Gull 1994). In cells induced for RNAi against *CAP5.5*, however, loss of CAP5.5 from the posterior end was evident after only 8 h post-induction (Fig. 4c–g). The cell cycle time for non-induced cultures was 11.1 h; thus, the cells shown in Figure 4c–f would all have been at an earlier stage in the same cell division cycle at the point when RNAi was induced (e.g. the 2K1N cells in Figure 4c and d would likely have been in G1 prior to RNAi induction). At points later in the cell cycle, loss of CAP5.5 from posterior cell ends correlated with the aberrant positioning of nuclei following mitosis (compare the 2K2N cells in Figure 4e and f with that shown in Figure 4b [ingression of cleavage furrows into the cells shown in 4e and 4f suggest mis-positioning of nuclei, whereas in a normal 2K2N cell (4b) the posterior nucleus sits between the divided and segregated kinetoplasts]). Despite

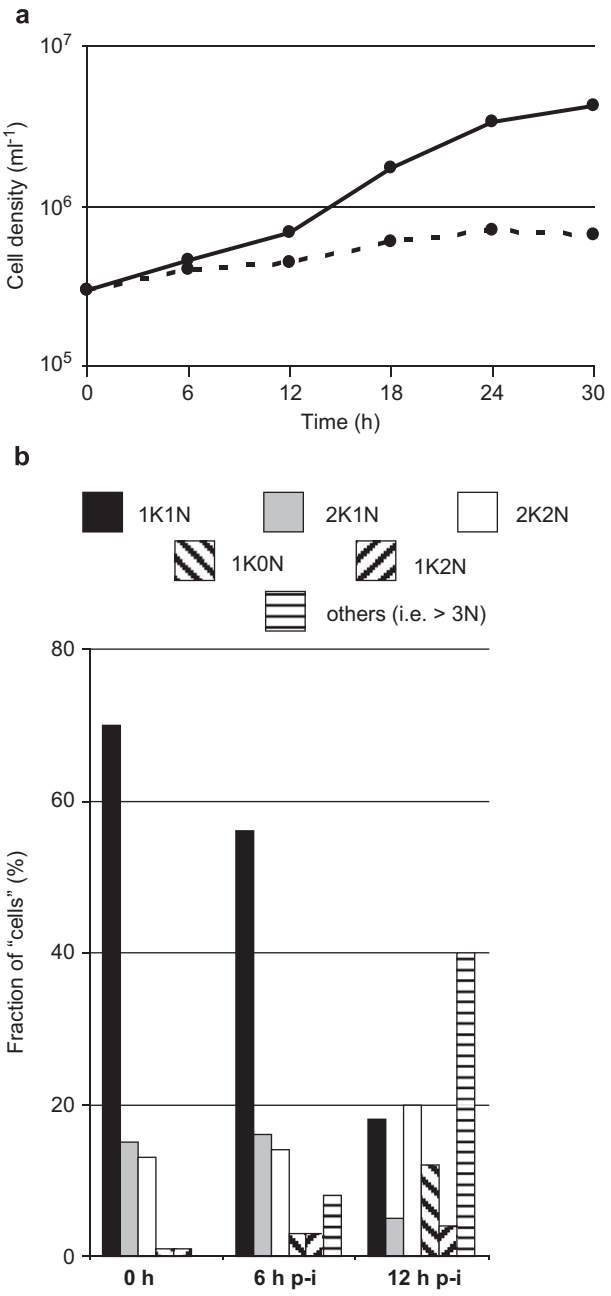

Figure 6. RNAi against *CAP5.5V* affects growth and morphology of bloodstream *T. brucei*. (**a**) The growth curve illustrates the effect of RNAi induction on cell density; cells were inoculated at an initial cell density of 3×10^5 cells ml^{-1}. (**b**) The number of nuclei and kinetoplasts were counted in DAPI-stained populations from induced cultures at 0, 6, and 12 h post-induction of RNAi. Data from one representative experiment of three performed is shown.

nuclear mis-positioning, cytokinesis still occurred in RNAi induced cells, giving rise to 1K2N and anucleate 1K0N progeny (Fig. 3d). In cultures

induced for 24 h aberrant cytokinesis events were readily observed (e.g. Fig. 4h), and the anucleate progeny of division, commonly known as "zoids", contained either little or no CAP5.5 protein when viewed by immunofluorescence using the CAP5.5 monoclonal antibody. As evidenced by the accumulation of multinucleate cells at later time-points, the 1K2N progeny of aberrant cell division were able to enter into further cell division cycles. Zoids also continued to accumulate as a function of time in RNAi-induced cultures, and were the most prevalent "cell" type by 72 h post-induction (Fig. 3d). Since "cell density" always increased between the 48 and 72 h time-points, albeit at a slower rate than in non-induced cultures, the distribution of "cell" types present in RNAi-induced cultures points towards the longevity of zoids (which presumably cannot replicate) and/or the ability of multinucleate cells to undergo cytokinesis and abscission perhaps producing, as suggested by image shown in Figure 5, multiple zoids.

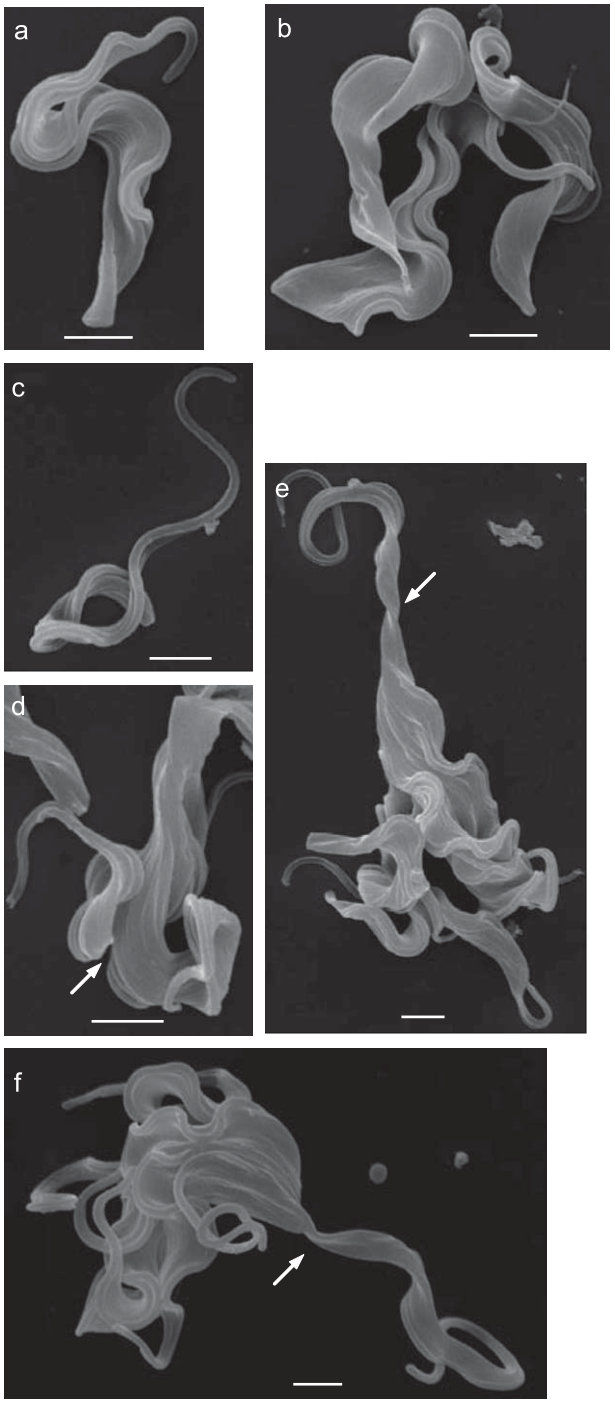

Figure 7. Representative SEM images of bloodstream *CAP5.5V* RNAi mutants. (**a-b**) Normal morphology of cells not induced for RNAi: (a) a single cell with two flagella (2F) is shown; (**b**) another 2F cell is evident in the centre of this panel, and is surrounded by two cells that each possess one flagellum (1F). (**c-f**) Aberrant cell morphologies after 15 h of *CAP5.5V* RNAi induction: (**c**) a likely example of a zoid; (**d**) an aberrant cleavage furrow (arrow) is evident in the cell shown; (**e-f**) two different examples of contorted multi-flagellate cells where abscission (arrow) is likely to produce a zoid. Scale bars represent 5 μm.

CAP5.5V is Essential in Bloodstream *T. brucei*

We used the same RNAi constructs for experiments with bloodstream trypomastigotes. Here, as expected from the expression studies, RNAi induction against *CAP5.5V*, but not *CAP5.5* yielded growth and morphology phenotypes. In cultures induced for *CAP5.5V* RNAi the growth rate declined markedly at ~6-8 h post-induction (Fig. 6a), and coincided with the onset of obvious morphological difficulties (Fig. 6b). Thus, taking into account the difference between the cell cycle times of the procyclic (11.1 h) and bloodstream (~8 h) cell lines, the kinetics of phenotype onset, relative to the cell cycle time, were similarly fast for *CAP5.5* RNAi in procyclic cells and *CAP5.5V* RNAi in bloodstream trypanosomes. As with the procyclic *CAP5.5* RNAi mutant, zoids and multinucleate cells accumulated in RNAi-induced cultures. This is a significant result given the patterns of organelle (kinetoplasts and nuclei) positioning prior to cell division are distinct in

procyclic and bloodstream trypomastigotes.[2] The contorted appearance of multinucleate, multi-flagellate cells that had been fixed in culture medium, spread, and air-dried onto slides meant it was difficult to ascertain numbers of nuclei and kinetoplasts with any confidence for samples collected after ~12 h post-induction. Slight increases in "cell" density of RNAi-induced cultures beyond the 12-15 h post-induction in all our experiments were consistent with a steady increase in the zoid population (to ~25-30% of "cell" types present; not shown), most probably as a result of abscission from multinucleate cells; support for this conclusion is provided by the representative scanning electron microscopy (SEM) images shown in Figure. 7.

There is a high level of identity between CAP5.5 and CAP5.5V along much of their length and we have now shown *CAP5.5V* to be essential in bloodstream trypanosomes. Thus, it is perhaps reasonable to ask why the anti-CAP5.5 monoclonal antibody provides such a good marker reagent for tracking the bloodstream-to-procyclic differentiation programme (Hertz-Fowler et al. 2001; Matthews and Gull 1994), but fails to show any cross-reactivity with the bloodstream-expressed CAP5.5V. The specificity of this reagent is readily explained, since the anti-CAP5.5 monoclonal was raised against a recombinant peptide that corresponded to the C-terminal domain, which is poorly conserved between CAP5.5 and CAP5.5V (Fig. 1).

Ultrastructural Analysis of RNAi Phenotypes

To study the effects of RNAi against either *CAP5.5* or *CAP5.5V* further, we also fixed cultures for thin section electron microscopy. For the procyclic *CAP5.5* RNAi mutant, we fixed cultures at 0, 18, and 48 h post-induction. Transverse sections of cells fixed at the 0 h time-point exhibited the characteristic, evenly spaced microtubule array that lies beneath the plasma membrane of a normal trypanosome cell (Fig. 8a), but by 18 h RNAi post-induction some defects in microtubule organisation were evident in a few cell sections, most notably in sections corresponding to posterior or flagellar pocket areas of cells (e.g. Fig. 8b). By 48 h many cell sections were characterised by multinucleate profiles and an abnormal shape, and ~15% of sections revealed some form of microtubule abnormality (Fig. 8c and d), most notably either aberrations in inter-microtubule spacing or the bundling of cytoplasmic microtubules beneath the plasma membrane. At this later time-point microtubule-related defects were observed in both the posterior region of cells, the flagellar pocket region, and, as judged by the width of the cell body, more anterior regions of cells, too. Apparent irregularities in anterior-end microtubule organisation potentially represented cross-sections through zoids or cells such as that shown in Figure 5, in which multiple, partially ingressed cleavage furrows are evident. The image in Figure 8d illustrates how defects in microtubule organisation correlated with difficulties in flagellar pocket morphogenesis: three cross-sections of flagella are evident in the two pocket profiles, including one transverse section where a paraflagellar rod (PFR) is abnormally present within the flagellar pocket (in normal cells the PFR is present and attached to the axoneme only from the point where the flagellum exits its flagellar pocket). The finger-like projection of the cell shown in Figure 8d reveals a longitudinal view of microtubules and provides evidence for a further abnormality, conceivably corresponding to an example of where cleavage furrow ingression and subsequent abscission would give rise to a zoid. Defects in microtubule organisation similar to those outlined above were also observed in the bloodstream *CAP5.5V* RNAi mutant (data not shown).

An RNAi Construct Simultaneously Targeting both *CAP5.5* and *CAP5.5V* Phenocopies Gene-specific RNAi Mutants

At the point when we started this work the presence of *CAP5.5V* was not evident in the publicly available *T. brucei* genome database. Thus, in our initial RNAi experiments we used an insert for dsRNA production which corresponded to base-pairs 122-1060 of *CAP5.5*, and was therefore highly similar to the N-terminal coding region of *CAP5.5V* (~85% identity). Indeed, use of this construct in either procyclic or bloodstream *T. brucei* yielded phenotypes which were highly similar to those seen for the *CAP5.5* procyclic RNAi mutant and the *CAP5.5V* bloodstream RNAi mutant, respectively (data not shown). However, these similarities in phenotype readout rule

[2]In contrast to procyclic trypomastigotes where one daughter kinetoplast sits between divided nuclei, in bloodstream trypomastigotes replicated and segregated kinetoplasts lie posterior to divided nuclei and are subject to a more limited movement apart during segregation.In contrast to procyclic trypomastigotes where one daughter kinetoplast sits between divided nuclei, in bloodstream trypomastigotes replicated and segregated kinetoplasts lie posterior to divided nuclei and are subject to a more limited movement apart during segregation.

against the possibility of off-target RNAi cross-talk for either of our gene-specific RNAi constructs.

Discussion

When viewed using conventional thin-section microscopy, the organisation of the subpellicular cytoskeleton in *T. brucei* shows few notable differences between bloodstream and procyclic trypomastigotes, but expression of the cytoskeletal calpain-related protein CAP5.5 is subject to strict stage-specific regulation. Thus, prior to the work presented here, a role in vector-parasite mediated signal transduction seemed a likely prediction for CAP5.5 function. Here, however, we have revealed that CAP5.5 is essential for correct morphogenetic patterning during the cell division cycle of procyclic trypomastigotes and for the organisation of the subpellicular microtubule corset. Our characterisation of the *CAP5.5* paralog *CAP5.5V* also provides a likely explanation for the stage-specificity of *CAP5.5* expression: the domain architecture, expression profile, and RNAi phenotype of the newly described CAP5.5V strongly suggest that paralogous genes provide analogous roles in cytoskeletal re-modelling for the two life cycle stages that can be cultured in vitro. Key issues to resolve in the future are how CAP5.5 and CAP5.5V contribute to cytoskeletal re-modelling, whether the function of either protein is dependent upon an intrinsic proteolytic activity, and if inhibition of CAP5.5V function might provide a realistic new drug target for sleeping sickness. Unfortunately, our efforts so far to develop recombinant expression of soluble protein have met with no success.

The earliest visible phenotype following *CAP5.5* RNAi induction in procyclic trypomastigotes is the loss of CAP5.5 from the posterior end of cells. It is

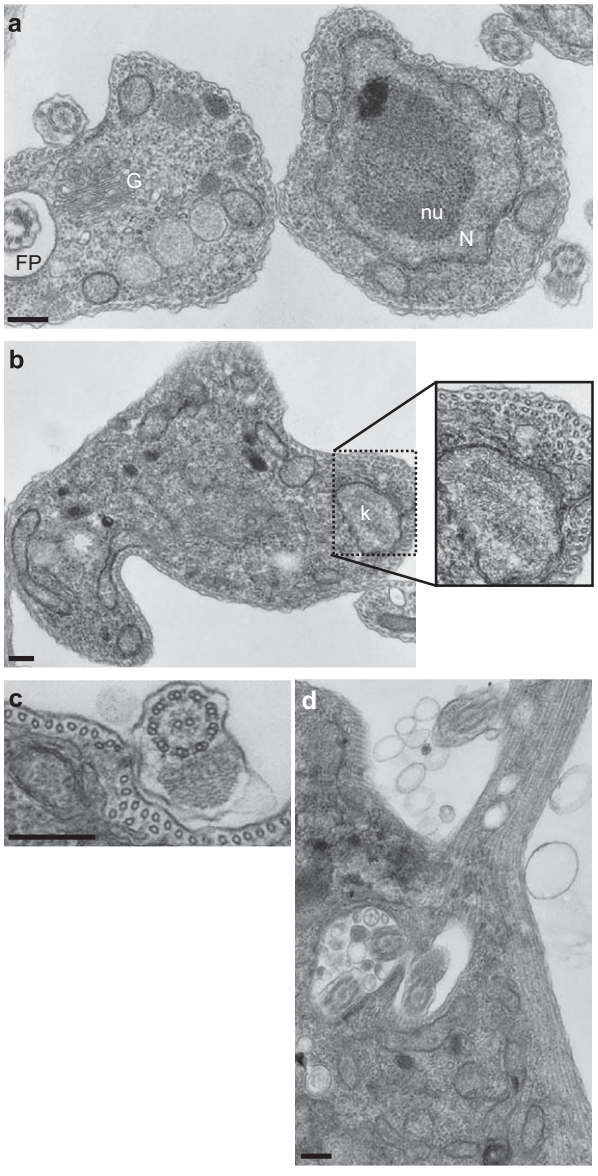

Figure 8. Defects in microtubule organisation following loss of *CAP5.5*. (**a**) Transverse section through two procyclic trypomastigotes not induced for RNAi. Profiles through the flagellar pocket (FP) and Golgi stacks (G) in the left-hand cell identify a transverse section through the posterior end of a cell; the nuclear (N) profile in the cell on the right identifies a likely transverse section through the midpoint of a cell body. In both sections an evenly spaced monolayer of micrtubules lies under the plasma membrane. nu, nucleolus. (**b**) Transverse section through a posterior cell end (as evidenced by a kinetoplast (k) profile) reveals an example of microtubule disorganisation at 18 h post-induction of RNAi. The boxed region is shown at higher magnification in the inset. (**c**) Examples of aberrant inter-microtubule spacing and additional microtubules lying beneath the sub-pellicular monolayer at 48 h post-induction of RNAi; the attached flagellum identifies this as a transverse section anterior to the flagellar pocket. (**d**) Difficulties in flagellar pocket biogenesis at 48 h post-induction of RNAi. Two flagellar cross-sections in the left-hand flagellar pocket profile identify an abnormality in flagellar pocket biogenesis, as does the sectioning of an axoneme plus PFR in the right-hand flagellar pocket profile. Scale bars represent 200 nm.

from the posterior cell end that procyclic cells elongate during the early stages of the cell cycle, and this involves both microtubule extension and the intercalation of new microtubules into the subpellicular microtubule array (Sherwin et al. 1987). Interestingly, loss of CAP5.5 from the posterior cytoskeleton by only 8 h post-induction of RNAi mirrors the initial RNAi phenotype of another trypanosome MAP, WCB (Baines and Gull 2008). In this context, the older observation (Sherwin and Gull 1989b) that inheritance of subpellicular microtubules at cytokinesis is semi-conservative is also significant: the near-immediate loss (i.e. within the same cell cycle in which RNAi was induced) of CAP5.5 from the posterior cytoskeleton implies that new microtubule growth and intercalation during cell elongation is accompanied by extensive re-modelling that sees CAP5.5, and presumably other MAPs, stripped entirely from the elongating subpellicular microtubule corset. However, since CAP5.5 is evenly distributed across the detergent-extracted cytoskeleton of normal (wild type) procyclic trypanosomes, irrespective of position in the cell cycle (Fig. 4b; Hertz-Fowler et al. 2001), our observations also point towards fast, dynamic kinetics for key events in cytoskeletal remodelling (removal of MAPs, microtubule growth and intercalation, addition of MAPs to a newly re-modelled subpellicular microtubule array).

The consequence of RNAi-induced loss of either CAP5.5 (this study) or WCB (Baines and Gull 2008) from procyclic posterior cell ends is an aberrant division, giving rise to the production of one anucleate zoid (1K0N) and a binucleate 1K2N cell. The distinctive phenomenon of zoid formation by procyclic *T. brucei* has been reported previously on various occasions and, for example, occurs as a consequence of (i) pharmacological inhibition of mitosis (using the anti-microtubule agent rhizoxin) (Ploubidou et al. 1999; Robinson et al. 1995); (ii) pharmacological inhibition of nuclear S phase (using aphidocolin as an inhibitor of DNA polymerase α) (Ploubidou et al. 1999); (iii) RNAi against various components involved in cell cycle control [e.g. the mitotic cyclin CYC6 (Hammarton et al. 2003)] or organelle segregation [e.g. centrin 4 (Shi et al. 2008)]; (iv) dominant-negative expression of mutant proteins interfering with cell cycle control/organelle segregation [e.g. cohesin (Gluenz et al. 2008)] or (v) the overexpression of the normally anterior-cytoskeleton associated proteins CAP15 and CAP17 (the accumulation of posterior cytoskeletal-associated CAP15 or CAP17 results in variable defects in organelle segregation prior to cytokinesis) (Vedrenne et al. 2002). We do not know whether following CAP5.5 or WCB RNAi zoid formation occurs directly as a result of aberrant cleavage furrow ingression into a defective subpellicular corset or if zoid formation is the inevitable consequence of incorrect organelle (nuclear) positioning in an aberrantly re-modelled cytoskeleton. However, further comparison between WCB and CAP5.5 RNAi phenotypes is informative. Thus, RNAi against WCB, but not CAP5.5 results in the "blebbing" of plasma membrane vesicles to the extracellular environment (Baines and Gull 2008), suggesting that an important property of WCB, but not CAP5.5, is to maintain local plasma-membrane-to-microtubule corset integrity. Our observation that WCB localisation is unaffected in cells following the induction of RNAi against *CAP5.5* is consistent with this hypothesis (data not shown). It is tempting to speculate that mutant-specificity of membrane blebbing is linked to the different motifs present in each protein which can mediate an interaction with the inner-leaflet of the plasma membrane: WCB contains an N-terminal phospholipid-binding C2-like domain, whereas CAP5.5 is subject to N-terminal acylation (Baines and Gull 2008; Hertz-Fowler et al. 2001). Another major significant difference between CAP5.5 and WCB RNAi phenotypes that points to different functional roles for each is that the milieu associated with intercalation of new microtubules into the subpellicular array is strongly affected in the CAP5.5 RNAi mutant. In neither WCB nor other RNAi mutants in which multinucleate cells accumulate as a consequence of cytoskeletal perturbations (e.g. Baines and Gull 2008; Davidge et al. 2006; Moreira-Leite et al. 2001), does the subpellicular microtubule corset lose its typical organisation in intact cells. In the absence of membrane "blebbing", the examples of microtubule bundling beneath the plasma membrane and aberrant inter-microtubule spacing described here are more likely to reflect a requirement for CAP5.5 to either break or form appropriate inter-microtubule cross-links, than to represent difficulties in cross-linked microtubules maintaining an association with the inner-leaflet of the plasma membrane. The relatively small number of sections through RNAi-induced cells ($\sim$15% in total) exhibiting microtubule abnormalities, could reflect the relatively small area of the cytoskeleton within which defects in microtubule organisation can occur (remembering, for instance, that CAP5.5 is not so readily lost from the anterior region of subpellicular microtubule cytoskeleton).

Alternatively, our data also are explained as a consequence of localised accumulation of numerous microtubule abnormalities at relatively few points on the cytoskeleton, with many more isolated examples of cytoskeletal abnormalities beneath the level of detection using conventional electron microscopy.

The determination of whether CAP5.5 or CAP5.5V are bona fide proteases is obviously crucial for understanding further the role(s) played by these proteins in trypanosome microtubule biology. For instance, the observation that tubulins and various MAPs provide in vitro substrates for "classic" mammalian calpains (Goll et al. 2003) could be used to provide support for the hypothesis that aberrant cytoskeletal organisation arises because the ability of new microtubules to invade the existing subpellicular array is lost following *CAP5.5* RNAi. However, the bioinformatics of the *T. brucei* calpain-related family argues against this possibility as CAP5.5 and CAP5.5V both lack the C-H-N catalytic triad which characterises the cysteine protease family. In these trypanosome proteins a S-Y-N triad is present. Notwithstanding the possibility that cytoskeleton-dependent conformational change may influence proteolytic activity[3] or the recent description of a cysteine protease family member with an atypical catalytic triad (Hsu et al. 2008), it is hard to envisage without further data how nucleophilic cleavage of a peptide bond could arise from either a Ser_{274}-Tyr_{437} or Ser_{291}-Tyr_{454} interaction within the putative degenerate catalytic triads of CAP5.5 and CAP5.5V, respectively.

While it is tempting to think of CAP5.5 as a potential protease, loss of catalytic activity may equally have been an important step in the functional evolution of this cytoskeletal protein. In mammals, Capn6 provides an example of an atypical calpain which lacks the essential Ca^{2+}-sensitive regulatory domain IV and a canonical catalytic triad (Tonami et al. 2007). Capn6 binds microtubules, and over-expression promotes formation of stable cytoplasmic microtubule bundles in fibroblasts. Coupled to the Capn6 RNAi phenotypes of actin re-organisation and membrane ruffling, the available data suggest Capn6 is a microtubule-stabilising protein involved in regulation of cytoskeletal organisation (Tonami et al. 2007). Given a dynamic association of some calpains with the cytoskeleton (Gil-Parrado et al. 2003), use of the atypical calpain Capn6 to stabilise a cytoskeletal organisation represents an innovative, if logical adaptation. However, cleavage in vitro of tubulin and other MAP substrates by mammalian calpains (Goll et al. 2003) suggests loss of proteolytic capacity would have been have a necessary early step in the evolution of capn6 as a microtubule-stabilising protein. A similar loss in proteolytic activity may have been a necessary step in the evolution of CAP5.5 function.

The evolutionary distance between trypanosomatids and animals is very large, but in both groups, and to the exclusion of most other major taxonomic groupings, sampled thus far, large calpain or calpain-related gene families are present. Since streamlining of functional complexity is a trait commonly associated with parasites, the presence of large calpain-related families in trypanosomatid parasites is particularly intriguing. The observations described here provide not only a strong indication that in *T. brucei* paralogous calpain-related proteins perform analogous roles in procyclic and bloodstream trypomastigotes, respectively, but identify a second example of related, but differentially expressed cytoskeleton-associated proteins in African trypanosomes – Bringaud and co-workers previously showed that CAP17 and CAP15 are differentially expressed in procyclic and bloodstream parasites (Vedrenne et al. 2002). It will no doubt be interesting to learn how many other cytoskeletal components are subject to stage-specific regulation in African trypanosomes and the reason(s) for this stage-specific expression. In the case of *CAP5.5* and *CAP5.5V*, one possibility is that the differential expression of paralogous genes provides a mechanism to fine tune absolute protein requirements in two morphologically distinct parasite forms.

Methods

Cell culture and plasmid construction: RNAi experiments were performed using the procyclic 29-13 and bloodstream 90-13 cell lines (Wirtz et al. 1999). Procyclic trypomastigotes were cultured in SDM-79 medium supplemented with 10% v/v heat-inactivated foetal calf serum (HIFCS), and bloodstream parasites were cultured in HMI-9 medium supplemented with 20% v/v HIFCS, as described previously (Brun and Schönenberger 1979; Hirumi and Hirumi 1989). Cell counts were made using either a Neubauer haemocytometer or a CASY1 cell counter (Schärfe System). Transfection of RNAi constructs was carried out using standard methods (McCulloch et al. 2004) and stable transformants were selected in the presence of 3 $\mu g\,ml^{-1}$ phleomycin. RNAi was induced by the addition of

[3]A key property of "classic" calpains is the dependence of protease activity upon Ca^{2+}-dependent conformational change (Hosfield et al. 1999).

doxycycline to a final concentration of 1 μg ml^{-1}; all cultures were maintained in the absence of selectable markers for 48 h prior to the start of an RNAi induction. For the expression analysis shown in Figure 2, RNA was isolated from "wild-type" 427 cell stocks cultured as described above.

For the RNAi experiments gene-specific inserts were cloned between the inverted T7 promoters of BamHI-HindIII digested P2T7^{Ti}-177 vector (Wickstead et al. 2002). To amplify a gene-specific insert from *CAP5.5* the primer combination used was GCAGGATCCACGAAAACAATGAAGAAGATG and GCA*AAGCTT*TTCCTTACAGTTCATTCATACAA (BamHI and HindIII are underlined and italicised, respectively). For amplification of a gene-specific insert from *CAP5.5V* the primer combination used was GCAGGATCCCAGAACCCG-CAACATACA and GCA*AAGCTT*GAAAACACCAACACCAA-CAC. Finally, the primer combination of TTAGGATCCAG-GAAGTTGAGGAGGTGC and CCG*AAGCTT*TAACCTTCCCT-GAGACCATTCG was used to amplify a *CAP5.5* gene-specific PCR product that mediated RNAi cross-talk against *CAP5.5* and *CAP5.5V* (an internal HindIII site meant that following restriction-digestion the resultant PCR product spanned bp 122-1060, rather than bp 122-1309 of the CAP5.5 open reading frame). Plasmids were linearised by digestion with NotI prior to transfection.

Expression analysis by real-time PCR: RNA was isolated from bloodstream and procyclic trypomastigotes using a High Pure RNA isolation kit (Roche) and cDNA was synthesised using an Omniscript reverse transcriptase kit (Qiagen), 2 μg RNA template and oligo dT primer (final concentration 1 μM) in a final volume of 20 μl. The accumulation of *CAP5.5* and *CAP5.5V* transcripts relative to that of either γ-tubulin or the ribosomal QM10 protein was then determined by quantitative real-time PCR as described previously (Dawe et al. 2005). For these PCR reactions 1 μl of cDNA product was used as the starting template in a final volume of 25 μl, which also included 12.5 μl of Brilliant©SYBRGreen QPCR master mix (Stratagene), gene-specific PCR primers (5 μM), and the Expand HighFidelityPlus polymerase (Roche). Two reverse transcriptase reactions were carried out per RNA sample and cDNA samples were analysed in duplicate for the PCR reactions. The values in Figure 2 represent relative ratios from the means of four individual values ± standard error. Probes used for amplification of cDNA corresponding to γ-tubulin or QM10 transcripts were described previously (McKean et al. 2003). Primer combinations used for the detection of calpain-related protein transcripts: *CAP5.5* GCCGGAAGCTGAGGA (forward primer) and TTCATCGGCTTGGGTCTCT (reverse primer); *CAP5.5V* GATGAGAAGCCGCACGA (forward primer) and GTTCCGGTTGCGCTTC (reverse primer).

Epifluoresence microscopy and immunoblotting: The CAP5.5 monoclonal antibody was used in immunofluoresence and immunoblotting as described previously (Hertz-Fowler et al. 2001). For cell imaging, live cells were settled onto poly-l-lysine coated slides, fixed with 3.7% para-formaldehyde (20 min), and permeabilised at -20 °C with methanol (2-5 min) prior to processing for immunofluoresence analysis. For accurate counts of cell type (nuclei and kinetoplast counts), cells were fixed in culture medium (by the addition of paraformaldehyde (3.7% v/v final concentration), spread onto slides, and then air-dried prior to processing. Nuclear and mitochondrial DNA was always stained using 6-diamidino-2-phenylindole (DAPI), and cells were imaged using a Zeiss Axioplan 2 microscope.

Electron microscopy: For transmission electron microscopy, cells were initially fixed by adding 1 ml of 25% glutaraldehyde to 9 ml of cells in culture media at ambient temperature. After ~5 min the cells were gently centrifuged (~500 g for 3 min) and then transferred into a buffered fixative consisting of 2.5% glutaraldehyde, 2% paraformadehyde and 0.1% picric acid in 100 mM phosphate (pH 7.0). Cells were pelleted in an Eppendorf and fixation continued for 2-24 h at 4 °C. Samples were post-fixed in 1% osmium in 100 mM phosphate (pH 7.0) for 1.5 h at 4 °C, en bloc stained with 2% aqueous uranyl acetate for 2 h at 4 °C in the dark, dehydrated and embedded in epoxy resin. Ultrathin (~70 nm thick) sections were double stained with uranyl acetate and lead citrate and examined in a FEI Tecnai 12 electron microscope.

For SEM, trypanosomes were washed in PBS and aliquots of live cells deposited on clean 13 mm coverslips. The cells were allowed ~10 mins to attach to the glass after which they were fixed with a mixture of 2.5% glutaraldehyde in 100 mM phosphate (pH 7.0). After fixation the samples were rinsed several times with distilled H_2O, dehydrated through a series of ethanols-water mixes and critically point-dried. After sputter-coating with gold the samples were examined in a JOEL JSM 6390 scanning electron microscope.

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