A coiled-coil- and C2-domain-containing protein is required for FAZ assembly and cell morphology in Trypanosoma brucei

Qing Zhou¹, Binghai Liu², Ying Sun¹,² and Cynthia Y. He¹,²,*

¹Department of Biological Sciences, National University of Singapore, Singapore 117543
²NUS Centre for Bioimaging Sciences, National University of Singapore, Singapore 117546

*Author for correspondence (E-mail: dbshyc@nus.edu.sg)

Accepted 27 July 2011
Journal of Cell Science 124, 3848–3858
© 2011. Published by The Company of Biologists Ltd
doi: 10.1242/jcs.087676

Summary

Trypanosoma brucei, a flagellated protozoan parasite causing human sleeping sickness, relies on a subpellicular microtubule array for maintenance of cell morphology. The flagellum is attached to the cell body through a poorly understood flagellum attachment zone (FAZ), and regulates cell morphogenesis using an unknown mechanism. Here we identified a new FAZ component, CC2D, which contains coiled-coil motifs followed by a C-terminal C2 domain. T. brucei CC2D is present on the FAZ filament, FAZ-juxtaposed ER membrane and the basal bodies. Depletion of CC2D inhibits the assembly of a new FAZ filament, forming a FAZ stub with a relatively fixed size at the base of a detached, but otherwise normal, flagellum. Inhibition of new FAZ formation perturbs subpellicular microtubule organization and generates short daughter cells. The cell length shows a strong linear correlation with FAZ length, in both control cells and in cells with inhibited FAZ assembly. Together, our data support a direct function of FAZ assembly in determining new daughter cell length by regulating subpellicular microtubule synthesis.

Key words: Trypanosoma brucei, Flagellum attachment zone (FAZ), Bi-lobed structure, Flagellum, Basal bodies, Cell morphology

Introduction

Trypanosoma brucei is a protozoan parasite containing a number of single-copy organelles encaged in a subpellicular microtubule array, all have to be duplicated and partitioned equally into the daughter cells during the cell cycle (Vaughan and Gull, 2008). A single, membrane-bound flagellum plays crucial roles in cell motility, coordinated organelle segregation, cell division and morphogenesis (Ralston and Hill, 2008; Ralston et al., 2009; Vaughan, 2010). Normal function of the T. brucei flagellum relies on its attachment to the cell body through a flagellum attachment zone (FAZ). Ultrastructurally, the FAZ is characterized by an electron-dense filament structure subtending the flagellum and a set of four microtubules in close association with smooth ER membranes (Sherwin and Gull, 1989a; Vickerman 1962; Vickerman, 1969).

In procyclic T. brucei, new FAZ filament assembles together with the elongation of the new flagellum during the cell cycle (Kohl et al., 1999), always posterior to the old structures. Cell division initiates at the anterior tip of the new FAZ. With the cleavage furrow ingressing posteriorly, the duplicated organelles are partitioned into two daughter cells, one inherits the old, more anteriorly located FAZ and flagellum and the other inherits the newly synthesized, more posteriorly located FAZ and flagellum.

Two FAZ components have been characterized in T. brucei. Fla1 is a surface glycoprotein homologous to GP72, which is found at the FAZ in Trypanosoma cruzi (Haynes et al., 1996; Nozaki et al., 1996). T. cruzi GP72-null mutants are viable in culture, although flagella are detached, and cells exhibit drastic morphological changes (Cooper et al., 1993; Rocha et al., 2006).

RNAi depletion of Fla1 in T. brucei leads to flagellum detachment, cytokinetic arrest and cell death (LaCount et al., 2002). Morphological changes in Fla1 RNAi cells are also noted, and are probably due to the accumulation of multiple nuclei in these nondividing cells. T. brucei FAZ1 is a component of the FAZ filament (Kohl et al., 1999; Vaughan et al., 2008). Depletion of FAZ1 results in partial detachment of the new flagellum and cytokinetic defects that lead to unequal segregation of nuclei and production of anucleated daughter cells. However, new FAZ assembly was not completely inhibited in FAZ1 RNAi cells (Vaughan et al., 2008).

Little is known about how new FAZ assembly is regulated in duplicating cells. Although new FAZ assembly initiates early in the cell cycle and is independent of new flagellum formation, its elongation requires continuous flagellum assembly (Kohl et al., 1999; Kohl et al., 2003). Inhibited flagellum formation in intraflagellar transport mutants results in truncated FAZ (Absalon et al., 2007; Kohl et al., 2003). Furthermore, our previous work suggests a possible link between the FAZ filament and the Golgi-adjacent bi-lobed structure, which plays a crucial role in organelle biogenesis and cell division (Morriswood et al., 2009; Shi et al., 2008; Zhou et al., 2010). The centrally located bi-lobe overlaps with the posterior tip of the FAZ and duplication of the bi-lobe is immediately followed by formation of a new FAZ. Inhibition of bi-lobe duplication also inhibits new FAZ formation (Zhou et al., 2010).

In this study, we report a new FAZ marker CC2D that is present on the basal bodies and FAZ in procyclic T. brucei. Depletion of CC2D inhibited the elongation of new FAZ.
filament, forming a FAZ stub that colocalized with the newly duplicated bi-lobed structure. Defective new FAZ assembly inhibited basal body segregation and subpellicular microtubule synthesis, producing daughter cells with detached flagellum, shorter cell length and changed polarity. Together, our data support a direct role of FAZ in controlling cell morphology by regulating subpellicular microtubule synthesis and organization.

Results

**T. brucei** CC2D

In a previous comparative proteomic study that compared the protein compositions between isolated flagella and flagellar complex (which contains flagella and associated cytoskeletal structures) (Zhou et al., 2010), one candidate protein (GeneID: Tb927.4.2080) was found associated with the flagellum or the FAZ when transiently overexpressed as a fusion to yellow fluorescent protein (YFP) (supplementary material Fig. S1). This protein was also found in the flagellar proteome, and considered an integral component of the flagellum (Broadhead et al., 2006). Although conserved among trypanosomatids, a similarity search did not identify any significant homolog to this protein in other eukaryotic organisms (Broadhead et al., 2006; Zhou et al., 2010). Further bioinformatic analyses predicted four coiled coils in the N-terminal region and a C2 domain near the C-terminus. This protein was therefore named CC2D, standing for coiled-coil- and C2-domain-containing protein. Proteins with similar domain organization have also been found in other organisms, and the human CC2D2A protein has been located to the cilia basal bodies and been implicated in ciliogenesis and human ciliary diseases (Doherty, 2009; Gorden et al., 2008; Mougou-Zerelli et al., 2009; Noor et al., 2008; Talllila et al., 2008).

To find out the exact intracellular localization of CC2D in *T. brucei*, a cell line stably expressing YFP- or Ty-tagged CC2D from one endogenous allele was generated by homologous replacement. At all stages of the cell cycle, endogenously expressed YFP–CC2D colocalized precisely with FAZ1, a FAZ filament marker (Kohl et al., 1999) (supplementary material Fig. S2A–C), and did not colocalize with the paraxflagellar rod (PFR) that was used as a marker for the flagellum (supplementary material Fig. S2D–F). FAZ1-labeled FAZ was previously shown to partially overlap with the bi-lobed structure marked with Centrin4, a small EF-hand-containing protein found on both the basal bodies and the bi-lobe in *T. brucei* (Shi et al., 2008). Similar overlap with the bi-lobe was also observed for Ty–CC2D, with the posterior tip of CC2D-labeling overlapping with the Centrin4-labeled bi-lobe (supplementary material Fig. S2J–L, arrows). Interestingly, unlike the endogenously expressed YFP–CC2D, the endogenously expressed Ty–CC2D was also consistently found present on the basal bodies marked with the YL1/2 antibody (Kilmartin et al., 1982) (supplementary material Fig. S2G–I, filled arrowheads). Centrin4 and CC2D, however, appeared to label different parts on the basal bodies (supplementary material Fig. S2I, open and filled arrowheads).

To further characterize CC2D, the N-terminal region of CC2D (1–480 aa), which contains the coiled-coil motifs, was fused with 6 x His-tag and expressed in *E. coli*. Purified protein was used to generate a polyclonal antibody in rabbits. Affinity-purified anti-CC2D recognized a single band at ~100 kDa in control wild-type cells. An additional ~130 kDa band was detected in cells expressing YFP–CC2D endogenously and this same band can be recognized by a polyclonal anti-GFP antibody (supplementary material Fig. S3). The anti-CC2D antibody was then used in immunofluorescence analysis on methanol-fixed *T. brucei* (Fig. 1). At all stages of the cell cycle, CC2D labeling colocalized with the FAZ filament (Fig. 1A–C), subtending but not completely overlapping with the flagellum (Fig. 1D–F). Additional labeling on the basal bodies was also observed (arrows, Fig. 1G–I), similarly to endogenously expressed Ty–CC2D (supplementary material Fig. S2G–I). The presence of a YFP tag at the N-terminus of CC2D, might therefore hamper its basal body targeting because most cells expressing endogenously replaced YFP–CC2D did not show detectable basal body labeling (supplementary material Fig. S2A–F).

CC2D is present at the basal bodies and the FAZ

To examine CC2D localization at the ultrastructural level, immuno-gold labeling was performed on cryo-sections of YTat1.1 cells using anti-CC2D antibodies. Gold particles were predominantly found associated with cytoplasmic membrane structures near the attached flagellum along the cell body (Fig. 2A,B). The close association between CC2D and the FAZ filament was verified by double labeling for CC2D and L3B2 (Fig. 2C,D), which labels FAZ1 in the FAZ filament (Vaughan et al., 2008).

Smooth-ER membranes have long been observed to be in close contact with the FAZ. (Sherwin and Gull, 1989a; Vickerman, 1969). To verify the ER nature of the CC2D-labeled membrane, double labeling was performed on cryo-sections of Ty–CC2D-expressing cells (cf. supplementary material Fig. S2) using anti-Ty to label Ty–CC2D and a polyclonal antibody against BiP, a luminal marker for the ER (Bangs et al., 1993). Whereas BiP was found on ER structures dispersed throughout the cell, including the nuclear envelope, it colocalized with CC2D only at the FAZ-ER (Fig. 2E,F).

Notably, CC2D and FAZ1 were both readily detected at the FAZ-ER region that nucleates the single ER exit site and single Golgi complex (Fig. 2D) (Sevova and Bangs, 2009), where the bi-lobed structure was previously mapped (He et al., 2005; Shi et al., 2008). This result was therefore consistent with the partial overlap between the bi-lobe and the FAZ previously observed by light microscopy (supplementary material Fig. S2J–L) (Shi et al., 2008; Morriswood et al., 2009; Zhou et al., 2010).

The presence of CC2D on the basal bodies and its association with the FAZ filament were further confirmed by immuno-gold labeling of detergent and salt-extracted YTat1.1 cells (see the Materials and Methods). As shown in Fig. 3A,B, CC2D was present on both mature and pro-basal bodies. In addition, CC2D labeled an electron-dense filament-like structure that initiated at the nuclear envelope, it colocalized with CC2D only at the FAZ-ER (Fig. 3E,F).

CC2D is essential for cell proliferation

To characterize the biological functions of CC2D, the protein was depleted using an inducible RNAi system (Djikeng et al., 2004). CC2D depletion was monitored by immunofluorescence assays and immunoblots using anti-CC2D over the course of induction (Fig. 4A,B). The CC2D expression level was reduced more than 90% by 48 hours post induction and was barely detectable at 72 hours post induction.
Fig. 1. **CC2D is present on the FAZ and basal bodies at all stages of the cell cycle.** Methanol-fixed YTat1.1 cells were labeled with anti-CC2D (green), DAPI for DNA (blue) and L3B2 for FAZ (red; A–C), or anti-PAR for PFR (red; D–F) or YL1/2 for basal bodies (red; G–I). All fluorescence images were merged with corresponding DIC. In addition to FAZ labeling, CC2D is also found at the basal bodies marked by YL1/2 (arrow). In *T. brucei*, kinetoplast (small blue structures) divides before the division of the nucleus (large blue structures). The number of kinetoplasts and nuclei in a cell therefore provides a reliable marker for cell cycle stages. Cells representing different cell cycle stages (1K1N, 2K1N and 2K2N) are shown for all labeling conditions. Scale bar: 5 μm.

Fig. 2. **CC2D localizes to the FAZ-associated ER.** Cryo sections of YTat1.1 (A–D) or cells with stable, endogenous Ty–CC2D expression (E,F) were processed for immuno-gold labeling with specified antibodies. CC2D is found present on membrane structures near the attached flagellum (A,B). CC2D (smaller gold particles) colocalized with L3B2-labeled FAZ1 (larger gold particles) (C,D). Note that the colocalization is readily observed at the bi-lobe region adjacent to the ER exit site and the Golgi (D). Double labeling with BiP (small gold particles) reveals specific labeling of CC2D (large gold particles) at the FAZ-associated ER, but not at other ER membranes such as the nuclear envelope (E,F). Scale bars: 200 nm. Fla, flagellum; Nuc, nucleus.

1K1N  2K1N  2K2N

| A | B | C |
|---|---|---|
| DAPI | anti-CC2D | DAPI | L3B2 | anti-CC2D |

| D | E | F |
|---|---|---|
| DAPI | anti-PAR | DAPI | YL1/2 | anti-CC2D |

| G | H | I |
|---|---|---|
| DAPI | YL1/2 | anti-CC2D |
The growth rate of CC2D RNAi cells was also measured every 24 hours after RNAi induction (Fig. 4C). Although the uninduced control cells replicated steadily with a doubling time of $10.4 \pm 0.3$ hours throughout the course of the experiments, CC2D RNAi cells grew at a similar rate (doubling time $11.6 \pm 1.0$ hours) until 24 hours post induction, and the duplication slowed and then stopped entirely after 48 hours post induction. Growth arrest beyond 24 hours post induction was accompanied by a rapid increase of multinucleated cells to $50\%$ at 48 hours and $75\%$ at 72 hours (Fig. 4A,D), suggesting a gross perturbation of cell division in the late induction stage. Therefore, to examine the primary effects of CC2D depletion, we focused on phenotypes exhibited at 24 hours post RNAi induction.

**CC2D depletion inhibits basal body segregation**

In *T. brucei*, the basal bodies play a crucial role in co-ordinating organelle positioning and segregation during the cell cycle. The mature basal body seeds the growth of the flagellum. The pro-basal body, initially constructed anterior to the mature basal body, migrates to the posterior side of the mature basal body upon maturation, thus allowing the construction of a new flagellum posterior to the old flagellum (Lacomble et al., 2010). The basal bodies also physically link to the kinetoplast (aggregated mitochondrial DNA) and drive kinetoplast division during the cell cycle (Ogbadoyi et al., 2003; Robinson and Gull, 1991). After kinetoplast division, the nucleus divides in a 'closed' mitosis. One daughter nucleus migrates posteriorly into the space between the segregated kinetoplasts, forming a typical KNKN organization in post-mitotic cells (Fig. 5A,D) (Robinson et al., 1995).

In CC2D RNAi cells, a significant increase of 1K2N cells was observed at 24 hours post induction (Fig. 4D), suggesting an inhibition of kinetoplast division. Consistent with this explanation,
majority of the 1K2N cells (>70%) contained duplicated basal bodies and flagella that remained unsegregated (Fig. 5B). The 2K2N cells showed a transient, slight increase at 24 hours post induction (Fig. 4D). Microscopic analyses revealed a marked decrease of cells with normal KNKN organization and an increase of cells with NKKN organization (Fig. 5C,D), further supporting a defect in basal body segregation in CC2D RNAi cells. Interestingly, in CC2D RNAi cells containing two flagella, the new, more posteriorly located flagellum was detached from cell body whereas the old flagellum remained attached (Fig. 5B,C).

**CC2D depletion inhibits assembly of the FAZ filament**

Though basal bodies and flagellum duplicated normally in CC2D RNAi cells, flagellum attachment was clearly disrupted in the CC2D RNAi cells (compare Fig. 5B,C with 5A). In the control population, cells with detached flagella were only occasionally observed (<1%). In CC2D-depleted cells, ~40% of cells had at least one detached flagellum at 24 hours post induction.

To examine whether CC2D depletion affected the assembly of the FAZ, CC2D RNAi cells at 24 hours post induction were fixed with methanol at −20°C and stained for FAZ1. FAZ1 labeling was found along attached flagellum in all control and some CC2D RNAi cells, overlapping only at its posterior tip with the Centrin4-labeled bi-lobe (Fig. 6A,B,G). However, only a small stub of FAZ1 staining ~1.4 μm in length was observed at the base of the detached flagellum in CC2D RNAi cells. Double-labeling experiments with Centrin4 suggested partial overlapping of the FAZ1 stub with the bi-lobed structure (Fig. 6F,G).

The distribution of FAZ1 and the FAZ filament formation in CC2D RNAi cells was further examined by immuno-scanning electron microscopy of detergent- and salt-extracted cells (see the Materials and Methods). In control cells, the FAZ filament could be observed as a structure similar to a string of beads, heavily labeled with L3B2 (Fig. 6C,D,E). In CC2D RNAi cells extracted using the same conditions, the FAZ filament was no longer observed, although the FAZ microtubule quartet appeared intact (Fig. 6H,I,J). Surprisingly, no FAZ1 labeling was found at the base of the flagellum where the FAZ stub was observed by immunofluorescence assays shown in Fig. 6C,D. This might be due to the detergent and salt extraction conditions used in sample preparation, and the FAZ stub might be more sensitive to these extraction conditions than the FAZ filament. Supporting these possibilities, immunofluorescence assays of detergent- and salt-extracted CC2D RNAi cells did not reveal detectable FAZ1 labeling at the base of detached flagella, whereas FAZ1 at the FAZ filament was consistently found subtending attached flagella (data not shown).

Depletion of CC2D did not affect the elongation of the detached flagella. The average length of detached flagella in CC2D-depleted cells was 14.6 ± 3.0 μm (n=250), which is similar to that of control cells (16.6 ± 2.7 μm; n=250) (see Table 1). Transmission electron microscopic analyses did not reveal any obvious morphological changes in flagellum or flagellar pocket organization in CC2D RNAi cells as compared with the control (supplementary material Fig. S4). Moreover, the detached flagella were capable of beating, although they lacked co-ordinated movement with the cell bodies as observed in normal cells (supplementary material Movies 1, 2).

**FAZ and cell size**

At 24 hours post CC2D RNAi induction, 1K1N cells accounted for ~70% of the population (Fig. 4D). Closer examination of these 1K1N cells revealed greater variation in cell size and morphology compared with the control 1K1N cells (Fig. 7A,B). Although all control 1K1N cells contained attached flagellum and had a relatively uniform cell length, 1K1N cells in the CC2D RNAi population appeared to form two distinct categories: ~60% had attached flagella, exhibited clear cell polarity with a stumpy posterior tip and a pointed anterior tip, and the kinetoplast always positioned at the posterior side of the nucleus; the other ~40% had detached flagella and appeared shorter in cell length. The polarity of the latter category was also changed. In some of these cells, the kinetoplast located anteriorly to the nucleus; whereas in others, it was not possible to distinguish the posterior and anterior tips based on the cell shape (Fig. 7B).

To further analyze these 1K1N cells, control and CC2D RNAi cells were fixed and double-labeled for DNA and FAZ, or flagellum. Flagellum length, FAZ length and cell size were then quantified in 100 randomly selected 1K1N cells containing a single flagellum in both control and CC2D RNAi populations (Fig. 7D). In control 1K1N cells, cell length correlated with both flagellum length (r² = 0.74) and FAZ length (r² = 0.81), which is consistent with previous observations (Kohl et al., 2003;
Rotureau et al., 2011). At 24 hours post CC2D RNAi induction, and with the emergence of cells containing detached flagella, the linear correlation between cell length and flagellum length decreased ($r^2=0.63$), whereas the linear correlation between cell length and FAZ length slightly increased ($r^2=0.88$), indicating a stronger linear correlation between FAZ length and cell length in both control and CC2D RNAi cells. Statistical analyses of measurements shown in Fig. 7C and 7D are listed in Table 1. Remarkably, in the CC2D RNAi population, 1K1N cells with attached flagellum showed normal cell size (20.9 ± 2.6 μm), normal flagellum length (17.9 ± 2.4 μm) and normal FAZ length (15.4 ± 4.1 μm), comparable to those of the control 1K1N cells (20.5 ± 2.6 μm, 16.9 ± 2.5 μm and 15.4 ± 3.4 μm, respectively). However, 1K1N cells with detached flagella contained only a short FAZ1 stub 1.4 ± 1.1 μm in length (cf. Fig. 6F; Table 1). Despite well-developed flagella with an average length of 14.2 ± 2.1 μm (supplementary material Fig. S4, Movies 1 and 2), these cells (9.3 ± 2.7 μm) were significantly shorter than

| Table 1. Measurements of 1K1N cells in control and CC2D RNAi populations 24 hours post induction* |
|---------------------------------------------------------------|
| Control 1K1N cells | Attached flagellum | Detached flagellum |
|---------------------|--------------------|---------------------|
| Cell length (μm)    | 20.5 ± 2.6         | 20.9 ± 2.6          | 9.3 ± 2.7 |
| Flagellum length (μm)| 16.9 ± 2.5        | 17.9 ± 2.4          | 14.2 ± 2.1 |
| FAZ length (μm)     | 15.4 ± 3.4         | 15.4 ± 4.1          | 1.4 ± 1.1 |

*100 1K1N cells each containing a single flagellum were randomly selected from control and CC2D RNAi populations (see Fig. 7C,D). At 24 hours post CC2D RNAi induction, ~60% of 1K1N cells had an attached flagellum and ~40% of 1K1N cells had a detached flagellum.
control or CC2D RNAi 1K1N cells with attached flagella. These results suggest that FAZ length directly affects cell length.

**CC2D RNAi disrupts anterior subpellicular microtubule synthesis and organization in cells with detached flagella**

Dividing 2K2N cells with cytokinetic furrow ingression already initiated were often observed in both control and CC2D RNAi cells at 24 hours post induction (Fig. 8A, B). However, the forming posterior daughter that contains the new flagellum appeared much shorter in CC2D RNAi cells than in the control, especially the anterior region marked by the brackets (Fig. 8A, B). Although division of control 2K2N cells produced two daughter cells similar in size, division of the CC2D-RNAi 2K2N cells produced two different 1K1N daughter cells, one with the old, attached flagellum and of normal cell length, and the other a shorter sibling containing the new, detached flagellum (Fig. 8C).

The subpellicular microtubules were also examined by scanning electron microscopy of cells extracted with cold 1% Triton X-100. In control cells with attached flagella, subpellicular microtubules crosslinked to form tightly bundled arrays in both 1K1N cells (Fig. 8F) and growing new daughters (Fig. 8G). By contrast, in CC2D RNAi cells containing detached flagella extracted and imaged under the same conditions, the anterior subpellicular microtubules appeared shorter and less organized (Fig. 8H, I). However, little difference in subpellicular microtubule organization was observed in the posterior part of the cells.

**Discussion**

The *T. brucei* flagellum and its attachment to the cell body are crucial for cell motility, cell division and cell morphogenesis (Ralston and Hill, 2008; Ralston et al., 2009; Vaughan, 2010). Although the exact mechanism of how flagellum assembly regulates cell morphology remains unclear, of particular interest is the function of the flagellum attachment zone (FAZ), a specialized cellular structure found in trypanosomatids (Sherwin and Gull, 1989a; Vickerman, 1962; Vickerman, 1969). In addition to mediating flagellum attachment to the cell body, the FAZ is also required for basal body segregation (Kohl et al., 2003) and might define the cell division axis (Li et al., 2009; Robinson et al., 1995). Little is known about the biochemical composition and assembly of the complex FAZ structure. Discovery of CC2D at the FAZ and inhibition of FAZ assembly upon CC2D depletion thus provided the opportunity to directly dissect FAZ biogenesis and functions in *T. brucei*.

CC2D localized primarily to the FAZ-associated ER in intact cells, but remained tightly associated with FAZ1-labeled FAZ filament in detergent-extracted cells, suggesting that CC2D might
act as a linker between the FAZ filament and the FAZ-ER. The ER membrane association might be mediated by the C2 domain present at the C-terminus of CC2D (Cho, 2001; Rizo and Sudhof, 1998). At least one other C2-domain-containing protein, WCB, has been characterized in T. brucei, and it is involved in interaction between the plasma membrane and subpellicular microtubules (Baines and Gull, 2008). How CC2D associates with FAZ filament is not clear, but this is perhaps attributed to the coiled coils that are generally implicated in protein oligomerization and protein–protein interactions (Burkhard et al., 2001; Strauss and Keller, 2008). It is important to note that in addition to FAZ, CC2D was also present on the basal bodies. This was confirmed by Ty tagging of the endogenous allele, as well as specific antibody targeted to the endogenous CC2D using both immunofluorescence and immunoelectron microscopy methods. It is not clear what the specific function of CC2D is at the basal bodies. Also, it is not clear whether CC2D at the basal bodies is associated with the ER membrane. Interestingly, the human CC2D2A protein with a similar arrangement of coiled coils followed with a C2 domain, is a member of a multi-protein complex that anchors the basal bodies (or adjacent transition zone) to the membranes, thus establishing a ciliary gate that restricts certain components from entering the cilia (Williams et al., 2011).

The primary effects of CC2D RNAi were the inhibition of new FAZ elongation and basal body segregation. New, full-length flagella could still be assembled from the new basal bodies. Although detached from the cell body because of the lack of a new FAZ, they showed no apparent defects in structural organization and were capable of beating. The flagellar pocket region also appeared normal in CC2D RNAi cells. Previous reports suggested that a new flagellum and a new FAZ play important roles in flagellar pocket organization, basal body positioning and segregation (Absalon et al., 2008; Kohl et al., 2003; Lacomble et al., 2010). Whereas elongation of the new flagellum, with its distal end anchored to the old flagellum, pushes the new basal body away to the posterior end of the cell, the new FAZ might restrain the segregation and hold the new basal body in place. In cells with depleted BILBO-1, a flagellar pocket collar component, new FAZ assembly is completely inhibited and the new basal body migrates far into the posterior end (Bonhivers et al., 2008). In CC2D RNAi cells, although new FAZ elongation was inhibited, a short FAZ stub was still present and might thus function in positioning the new basal body to the posterior end of the old basal bodies, limiting the segregation of the new basal body from the old. Similar phenotypes are also observed in various flagellar mutants that lead to FAZ elongation defects (Absalon et al., 2007; Dawe et al., 2005; Li and Wang 2008).

The formation of the FAZ stub perhaps represents the initial FAZ nucleation, which occurs before and independently of new flagellum assembly (Kohl et al., 1999; Kohl et al., 2003). Interestingly, FAZ1 in the FAZ stub was more sensitive to detergent and salt extraction than FAZ1 in the FAZ filament, suggesting a difference in FAZ1 organization or its association with cytoskeletal components in the FAZ stub. A similar...
accumulation of FAZ filament components in short structures near the flagellar pocket was also observed in T. cruzi GP72-null mutants (Rocha et al., 2006). The nucleation of FAZ is likely linked to the bi-lobe because mutant cells lacking bi-lobe duplication cannot assemble new FAZ structures (Zhou et al., 2010). Although ultrastructurally uncharacterized, the bi-lobe locates near the ER exit site and the Golgi in the neck region where FAZ filament initiates and FAZ microtubules join the subpellicular microtubule array (He et al., 2005; Lacomble et al., 2009; Rocha et al., 2006). Its close association with membranous organelles and cytoskeletal structures is similar to the organization of the FAZ complex. New FAZ assembly might therefore represent a co-ordinated membrane and cytoskeleton outgrowth from the new bi-lobe region.

Division of CC2D RNAi cells at 24 hours post induction produced two distinct types of daughter cells, one with intact FAZ, attached flagellum and normal cell length, and the other with detached flagellum, a short FAZ stub and short cell length. It has been shown previously that flagellum length strongly correlates with T. brucei cell size in cell culture or during development (Kohl et al., 2003; Rotureau et al., 2011). Using the CC2D RNAi mutants that specifically inhibited FAZ elongation but not flagellum elongation, we showed an even stronger correlation between FAZ length and cell size. Inhibition of new FAZ assembly led to inhibited subpellicular microtubule synthesis, resulting in a shorter microtubule array in the anterior part of cells with detached flagella. Although subpellicular microtubule integrity and spacing might not be drastically perturbed as suggested by previous work where flagellum growth or flagellum attachment is compromised (Kohl et al., 2003; Stephan et al., 2007), the subpellicular microtubules appeared less organized. A few parasite proteins have been implicated in crosslinking the subpellicular microtubules (Balaban et al., 1989; Detmer et al., 1997). It would be interesting to further examine how these proteins are regulated by CC2D and FAZ assembly. Our data support a function of the FAZ complex in regulating subpellicular microtubule organization, mainly in the anterior part of the cells. Because continuous flagellum elongation is important for complete FAZ assembly (Kohl et al., 2003), the flagellum might thus regulate cell size by regulating the FAZ complex assembly.

In summary, we describe CC2D as a component of basal bodies and the FAZ in T. brucei. Depletion of CC2D inhibited FAZ filament assembly but not new flagellum biogenesis. Inhibition of new FAZ elongation directly affected cell morphogenesis, possibly by perturbing subpellicular microtubule synthesis and organization.

Materials and Methods

Cell lines and cultivation conditions

Procycol form YTat1.1 cell line (Ruben et al., 1983) was maintained in Cunningham’s medium supplemented with 15% heat-inactivated fetal bovine serum (Hyclone) at 28°C. Procycol form 29.13 cell line (Wirtz et al., 1999) was maintained in Cunningham’s medium containing 15% heat-inactivated, tetracycline-free fetal bovine serum (Clontech) in the presence of 15 µg/ml G418 and 50 µg/ml hygromycin at 28°C. Growth assays were performed and doubling time calculated as previously described (Zhou et al., 2010). The cells were maintained at exponential growth phase by dilution with fresh medium.

Plasmid construction and transfection

For fusion protein expression, coding sequences for full-length CC2D (TBr27.4.2080) were PCR-amplified from T. brucei genomic DNA, digested by the corresponding restriction enzymes, and then cloned into a pXS2-based vector that contains YFP coding sequence (Bangs et al., 1993; He et al., 2004). For endogenous replacement, 500 bp of 5’-untranslated region and 500 bp of the 5’-end coding sequence of CC2D were PCR-amplified and then cloned separately into a modified pCR4Blunt-TOPO vector flanking a YFP- or Ty-tagging cassette (Morriswood et al., 2009). For RNAi studies, the RNAi program (Redmond et al., 2003) was used for the identification of suitable RNAI target. A 538 bp length of CC2D coding sequence (nt 1005–1542) was amplified and cloned into the pZJM vector (Wang et al., 2008). For transient transfection, 50 µg of plasmid DNA were introduced into YTat1.1 cells by electroporation. For stable transfection, 15 µg of linearized DNA were transfected into YTat1.1 or 29.13 cells by electroporation. Stable transfectants were then selected with appropriate antibiotics and clones obtained by limiting serial dilution.

Anti-CC2D antibody

The N-terminal 480 amino acids that contain the coiled-coil domains were fused to 6× His tag in pET30a+ vector (Invitrogen), expressed and purified from E. coli using Ni-NTA resin under denaturing conditions. The purified protein was used for polyclonal antibody production in rabbits.

Immunofluorescence microscopy

Intact or extracted cells were settled onto coverslips by centrifugation, fixed and permeabilized in methanol at −20°C for 15 minutes and then rehydrated in PBS, pH 7.2, for 10 minutes. For labeling with the anti-BiP antibody, the cells were first fixed with 4% paraformaldehyde for 5 minutes and then fixed and permeabilized with methanol at −20°C for an additional 20 minutes. All coverslips were blocked with 5% BSA in PBS for 45 minutes, and then prepared for antibody labeling. YL1/2 (1:2000) (AbCam) L3B2 (1:25) (Kohl et al., 1999), anti-PAR (1:3000) (Ismach et al., 1989), anti-Centrin4 (1:500) (Shi et al., 2008) and anti-BiP (1:800) (Bings et al., 1993) antibodies were used to label basal bodies, FAZ1 at FAZ filament, paragflagellar rod, bi-lobe (and basal bodies) and ER, respectively. The ratios in immunopurposes indicate the dilutions used for immunofluorescence assays. All fluorescent secondary antibodies (Invitrogen) were used at 1:3000, and no non-specific labeling was observed when the cells were labeled with these secondary antibodies alone (data not shown). Images were acquired using a Zeiss Axios Observer Z1 fluorescence microscope with a 63× NA 1.4 objective and a CoolSNAP HQ2 CCD camera (Photometrics) or a Zeiss LSM510 Meta confocal microscope with an EC Plan-Neofluar 100× NA 1.3 objective. The images were processed with ImageJ (National Institutes of Health) and Adobe Photoshop CS2 version 9.0.

Electron microscopy

For transmission electron microscopy, cells were harvested, washed and fixed with 2.5% glutaraldehyde in PBS, pH 7.2. The cells were then post-fixed with 1% OsO4 for 1 hour, dehydrated in serial concentrations of ethanol and embedded in Spurr’s resin. Ultrathin sections were stained with uranyl acetate and lead citrate and observed using an Tecnai 12 electron microscope (FEI).

For high-pressure freezing and freeze substitution, 10 ml of cells (1 × 10^6 cells/ml) were concentrated by centrifugation at 3000 r.p.m. for 7 minutes. The supernatant was gently removed, and the cell paste was transferred into a specimen holder and frozen at 2100 bar using a high-pressure freezer HPF compact 01 (Tecnai Biomedical International). The frozen samples were transferred into 1.5 ml tubes containing freeze substitution solution (1% O2O3, and 0.05% uranyl acetate in acetone) in liquid nitrogen. The samples were then placed in a Leica EM AF S2 (Leica Microsystems) preset at −90°C. The temperature was gradually raised as follows: −90°C for 48 hours, −60°C for 24 hours and −30°C for 18 hours. After slowly warming to 0°C over 4 hours, samples were kept at room temperature for 15 minutes and then washed three times with pure acetone. Samples were infiltrated with LX112 resin (Ladd Research Industries, Williston VT), polymerized at 4°C for 24 hours followed by 60°C for 48 hours. 90 nm sections were stained with 2% uranyl acetate followed by lead citrate. The samples were viewed using a Tecnai T12 transmission electron microscope (FEI).

For immuno-cryoEM, the cells were fixed with 4% paraformaldehyde and 0.2% glutaraldehyde in 100 mM phosphate buffer (pH 7.2) for 1 hour at 4°C. Samples were then embedded in 10% gelatin and observed with an Tecnai T12 transmission electron microscope (FEI).

For immuno-cryoEM, the cells were fixed with 4% paraformaldehyde and 0.2% glutaraldehyde in 100 mM phosphate buffer (pH 7.2) for 1 hour at 4°C. Samples were then embedded in 10% gelatin and infiltrated overnight with 2.3 M sucrose, 20% polyvinyl pyrrolidone in PIPEs with MgCl2 at 4°C. Samples were then trimmed, frozen in liquid nitrogen, and sectioned with a Leica Ultracut UCT cryo-ultramicrotome (Leica Microsystems, Bannockburn, IL). Sections were probed with the indicated primary antibodies followed by the appropriate secondary antibodies conjugated to 12 nm and/or 18 nm colloidal gold (Jackson ImmunoResearch Laboratories, West Grove, PA). A titration of the primary antibodies was performed to optimize antibody dilution that produced little or no labeling in other parts of the cells (Fig. 2A). Sections were then washed in PIPEs buffer followed by a rinse with water, stained with 0.3% uranyl acetate, 2% methyl cellulose and observed with a JEOL 1200 EX electron microscope (JEOL, Peabody, MA).

Immuno-gold labeling of detergent and salt-extracted cytoskeleton was performed using the method previously described by Robinson et al. (1991) with minor modifications. Briefly, ~1 × 10^7 cells were harvested, washed with PBS, pH 7.2, and attached to carbon formvar-coated grids for 30 minutes. The grids (with the cell side down) were then transferred to a 200 µl drop of PEM.
buffer (100 mM PIPES, 2 mM EGTA, 1 mM MgSO4, pH 6.9) containing 1% NP-40 and 1 M NaCl and extracted for 5 minutes at room temperature. The extraction conditions partially depolymerized the subpellicular microtubules, but maintained the integrity of the flagellum and FAZ. Extracted cytosomelets were fixed with 2.5% glutaraldehyde in PEM buffer for 15 minutes, washed with PEM buffer and neutralized with 100 mM glycine in PBS for 5 minutes. The grids with attached, extracted cytosomelets were then blocked with 1% BSA in PBS, incubated by primary antibodies followed by appropriate secondary antibodies conjugated to 10 nm or 15 nm colloidal gold particles (EMD). A titration of primary antibodies was always performed to optimize primary antibody dilution for least non-specific labeling. All secondary antibodies were used at 1:100 dilution, which produced no detectable labeling when used alone (data not shown). After secondary antibody labeling, the grids were washed three times with PEM buffer, fixed again with 2.5% glutaraldehyde in PEM buffer, washed with ddH2O and briefly stained with 1% uranyl acetate in ddH2O. After a brief air-dry, the grids were observed and imaged using a Tecnai 12 electron microscope (FEI).

For scanning electron microscopy, cells were attached to coverslips and extracted with ice-cold 1% Triton X-100 for 10 minutes (which preserved the overall shape of the parasite and the integrity of the subpellicular microtubules). Extracted cells were then fixed with 4% paraformaldehyde and 2.5% glutaraldehyde for 30 minutes and post-fixed with 1% OsO4 for 1 hour. Following ddH2O washes and critical-point drying, the sample was coated with gold using an ion sputter (JEOL) and viewed using a Helios NanoLab DualBeam electron microscope (FEI).

For immunoscanning electron microscopy, cells attached to coverslips were extracted with 1% NP-40 and 1 M NaCl in PEM buffer for 5 minutes at room temperature. Extracted cells were then fixed with 4% paraformaldehyde and 2.5% glutaraldehyde for 30 minutes and neutralized with 100 mM glycine for 5 minutes. After blocking with 1% BSA in PBS, the cells were incubated with L382 (1:25) followed by goat-anti-rabbit IgG conjugated to 40 nm colloidal gold using an ion sputter (JEOL) and viewed using a Helios NanoLab DualBeam electron microscope (FEI). An electron micrograph of a cell stained with L382 (1:25) followed by goat-anti-rabbit IgG conjugated to 40 nm colloidal gold is shown in Figure 2A.

Acknowledgements

We wish to thank Wendy Beatty and Xuezi Ouyang for help with immuno-cryoEM and TEM, respectively. We also thank all lab members for help with preparation of the manuscript.

Funding

This work was supported by the Singapore National Research Foundation. C.Y.H. is a Singapore National Research Foundation Fellow.

Supplementary material available online at

http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.087676/-/DC1

References

Abelson, S., Kohl, L., Brechta, C., Blinsnick, T., Toutiouris, G., Rusconi, F., Cosson, J., Bonhivers, M., Robinson, D. and Bastin, P. (2007). Basal body positioning is controlled by flagellation formation in Trypanosoma brucei. PLoS ONE 2, e437.

Abelson, S., Blinsnick, T., Bonhivers, M., Kohl, L., Cayet, N., Toutiouris, G., Buison, J., Robinson, D. and Bastin, P. (2008). Flagellar elongation is required for correct structure, orientation and function of the flagellar pocket in Trypanosoma brucei. J. Cell Sci. 121, 3704-3716.

Baines, A. and Gull, K. (2008). WCB is a C2 domain protein defining the plasma membrane - subpellicular microtubule corset of kinetoplastid parasites. Proteins 79, 115-125.

Balaban, N., Waitthaka, H. K., Ngour, A. R. and Goldman, R. (1989). Isolation of a subpellicular microtubule protein from Trypanosoma brucei that mediates microtubule crosslinking of microtubules. Cell Motil Cytoskeleton 14, 393-400.

Bangs, J. D., Uyetake, L., Brickman, M. J., Balber, A. E. and Boothroyd, J. C. (1993). Molecular cloning and cellular localization of a BIPA homologue in Trypanosoma brucei. Divergent ER retention signals in a lower eukaryote. J. Cell Sci. 105, 1101-1113.

Bonhivers, M., Nowachi, S., Landrein, N. and Robinson, D. (2008). Biogenesis of the trypanosome endo-exocytotic organelle is cytoskeleton mediated. PLoS Biol. 6, e68.

Broadhead, R., Dawe, H. R., Farr, H., Griffiths, S., Hart, S. R., Portman, N., Shaw, M. K., Gier, M. L., Gaskell, S. J., McKeon, P. G. et al. (2006). Flagellar motility is required for the viability of the bloodstream trypanosome. Nature 440, 224-227.

Burkhart, P., Stetefeld, J. and Strelof, S. V. (2001). Coiled coils: a highly versatile protein folding motif. Trends Cell Biol. 11, 82-88.
Robinson, D. R., Sherwin, T., Ploubidou, A., Byard, E. H. and Gull, K. (1995). Microtubule polarity and dynamics in the control of organelle positioning, segregation, and cytokinesis in the trypanosome cell cycle. J. Cell Biol. 128, 1163-1172.

Rocha, G. M., Brandao, B. A., Mortara, R. A., Attias, M., de Souza, W. and Carvalho, T. M. (2006). The flagellar attachment zone of Trypanosoma cruzi epimastigote forms. J. Struct. Biol. 154, 89-99.

Rothureau, B., Subota, I. and Bastin, P. (2011). Molecular bases of cytoskeleton plasticity during the Trypanosoma brucei parasite cycle. Cell Microbiol. 13, 705-716.

Ruben, L., Egwuaga, C. and Patton, C. L. (1983). African trypanosomes contain calmodulin which is distinct from host calmodulin. Biochim. Biophys. Acta 758, 104-113.

Sevova, E. S. and Bangs, J. D. (2009). Streamlined architecture and glycosylphosphatidylinositol-dependent trafficking in the early secretory pathway of African trypanosomes. Mol. Biol. Cell 20, 4739-4750.

Sherwin, T. and Gull, K. (1989a). The cell division cycle of Trypanosoma brucei: timing of event markers and cytoskeletal modulations. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 323, 573-588.

Sherwin, T. and Gull, K. (1989b). Visualization of detyrosination along single microtubules reveals novel mechanisms of assembly during cytoskeletal duplication in trypanosomes. Cell 57, 211-221.

Sherwin, T., Schneider, A., Sasse, R., Seebeck, T. and Gull, K. (1987). Distinct localization and cell cycle dependence of COOH terminally tyrosinolated alphatubulin in the microtubules of Trypanosoma brucei brucei. J. Cell Biol. 104, 434-446.

Shi, J., Franklin, J. B., Yelinek, J. T., Ebersberger, I., Warren, G. and He, C. Y. (2008). Centrin4 coordinates cell and nuclear division in Trypanosoma brucei. J. Cell Sci. 121, 3062-3070.

Stephan, A., Vaughan, S., Shaw, M. K., Gull, K. and McKeen, P. G. (2007). An essential quality control mechanism at the eukaryotic basal body prior to intrflagellar transport. Traffic 8, 1323-1330.

Strauss, H. M. and Keller, S. (2008). Pharmacological interference with protein-protein interactions mediated by coiled-coil motifs. Handb. Exp. Pharmacol. 186, 461-482.

Tallila, J., Jakkula, E., Peltonen, L., Salonen, R. and Kestila, M. (2008). Identification of CC2D2A as a Meckel syndrome gene adds an important piece to the ciliopathy puzzle. Am. J. Hum. Genet. 82, 1361-1367.

Vaughan, S. (2010). Assembly of the flagellum and its role in cell morphogenesis in Trypanosoma brucei. Curr. Opin. Microbiol. 13, 453-458.

Vaughan, S. and Gull, K. (2008). The structural mechanics of cell division in Trypanosoma brucei. Biochem Soc. Trans. 36, 421-424.

Vaughan, S., Kohl, L., Ngai, L., Wheeler, R. J. and Gull, K. (2008). A repetitive protein essential for the flagellum attachment zone filament structure and function in Trypanosoma brucei. Protist. 159, 127-136.

Vickerman, K. (1962). Patterns of cellular organization in Limax amoebae. An electron microscope study. Exp. Cell Res. 26, 493-519.

Vickerman, K. (1969). On the surface coat and flagellar adhesion in trypanosomes. J. Cell Sci. 5, 163-193.

Wang, Z., Morris, J. C., Drew, M. E. and Englund, P. T. (2000). Inhibition of Trypanosoma brucei gene expression by RNA interference using an integratable vector with opposing T7 promoters. J. Biol. Chem. 275, 40174-40179.

Williams, C. L., Li, C., Kida, K., Inglis, P. N., Mohan, S., Semene, L., Bialas, N. J., Stupay, R. M., Chen, N., Blacque, O. E. et al. (2011). MKS and NPHP modules cooperate to establish basal body/transition zone membrane associations and ciliary gate function during ciliogenesis. J. Cell Biol. 192, 1023-1041.

Wirtz, E., Leal, S., Ochatt, C. and Cross, G. A. (1999). A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in Trypanosoma brucei. Mol. Biochem. Parasitol. 99, 89-101.

Zhou, Q., Gheiratmand, L., Chen, Y., Lim, T. K., Zhang, J., Li, S., Xia, N., Liu, B., Lin, Q. and He, C. Y. (2010). A comparative proteomic analysis reveals a new bi-lobe protein required for bi-lobe duplication and cell division in Trypanosoma brucei. PLoS ONE 5, e9660.