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ORIGINAL PAPER

WCB is a C2 Domain Protein Defining the Plasma Membrane — Sub-Pellicular Microtubule Corset of Kinetoplastid Parasites

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WCB is a protein that locates between the inner face of the plasma membrane and the sub-pellicular corset of microtubules in *Trypanosoma brucei*. We provide the molecular identity of WCB and bioinformatic analysis suggests that it possesses a C2 domain implicated in membrane/protein interactions and a highly charged region possessing characteristics of a putative tubulin-binding domain. Functional analyses via RNA interference (RNAi) depletion show that WCB is essential for cell morphogenesis. Depletion results in gross abnormalities in cell shape, mainly at the cytoskeletal/plasma membrane dynamic posterior end of the trypanosome. Failures in cytokinesis and zoid production are also evident. Furthermore, electron microscopy reveals that RNAi-induced trypanosomes lose local plasma membrane to microtubule corset integrity.

Key words: C2 domain; Kinetoplastida; microtubules; plasma membrane; *T. brucei*; trypanosomes.

Introduction

The shape and form of many protists are defined by a complex internal cytoskeleton lying directly underneath the plasma membrane. In trypanosomes this structure takes the form of a sub-pellicular corset of microtubules. The sub-pellicular microtubules cover the entire inner surface of the plasma membrane necessitating all membrane traffic, to and from the plasma membrane, to occur at the flagellar pocket (for reviews see Field and Carrington 2004; Gull 2003; Landfear and Ignatushchenko 2001). It is only this flagellar pocket region of the plasma membrane that is devoid of microtubules. The microtubule array is cross-linked together and is present throughout the full cell cycle with new microtubules being added and the array being inherited in a semi-conservative manner by the two daughter cells (Sherwin and Gull 1989a, 1989b). This form of a sub-pellicular corset of microtubules is a characteristic feature of both free living and parasitic kinetoplastid protozoa. Whilst the biochemistry and structural organisation of the sub-pellicular microtubules themselves have been studied in detail (Gull et al. 1986; Sasse and Gull 1988; Schneider et al. 1987; Sherwin and Gull 1989a, 1989b) we know little about the proteins that might act either as microtubule—microtubule or microtubule—plasma membrane linkers to orchestrate and regulate cytoskeletal events. A few proteins such as MARPs, CAP 15 and CAP17 have been implicated as sub-pellicular array microtubule

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proteins (Hemphill et al. 1991, 1992; Vedrenne et al. 2002).

We have previously characterised a monoclonal antibody WCB-1 (whole cell body-1) which defined a protein that was highly phosphorylated and intimately associated with the sub-pellicular microtubule corset (Woods et al. 1992). The WCB protein was located on the plasma membrane-facing side of the sub-pellicular cage by electron microscope immunogold studies (Woods et al. 1992). WCB is tightly bound to the detergent insoluble cytoskeleton but only on the sub-pellicular corset—it is not found on the flagellar or spindle microtubules. We now reveal the molecular identity of the WCB protein, using the original monoclonal antibody to screen an expression library. WCB exhibits both an N-terminal region C2 domain normally found in proteins which interact with membranes (Davletov and Sudhof 1993) and a repetitive, charged C-terminal region which has characteristics of a microtubule-binding domain. We have studied the function of WCB using inducible RNAi.

Results

The expression library was screened with the well-characterised WCB-1 monoclonal antibody (Woods et al. 1992) and four positive clones were finally sequenced, which all identified the same protein. The resulting sequence was used to search the GeneDB *Trypanosoma brucei* genome database and identified a protein (Tb927.7.3550) with homologs conserved in *Trypanosoma cruzi* and *Leishmania major.* Synteny of this gene was conserved between the three kinetoplastids. Homologs were also detected in the other kinetoplastid protozoa with genome sequence in GeneDB but no convincing homologs were detected in any other organisms. The Tb927.7.3550 predicted protein is 1241aa in length with a predicted mass of 138.1 kD and a P.I. of 5.3. We have termed this protein WCB. Bioinformatic analysis of WCB revealed it to possess a C2 domain close to the N-terminus (aa130—210). C2 domains are often Ca^{2+}- dependent membrane targeting domains found in many proteins with a role in signal transduction or membrane trafficking (Davletov and Sudhof 1993). The C-terminus of WCB is dominated by a region of repetitive, charged motifs (Fig. 1A). There are five "repeats" of 32 amino acids built around a central KSAED sequence. The repeats are imperfect and contain many acidic and basic amino acids — 270 residues out of 580 are charged in this region, compared to 193 charged residues out of 661 in the remaining sequence. The overall PI of this domain is 6.37. Whilst not showing complete conservation of sequence, this area fits a general pattern of basic and acidic residue repeats seen amongst many other microtubule-associated proteins in different organisms (Felgner et al. 1997; Goode et al. 1997; Noble et al. 1989). Eleven other C2 domain containing proteins were identified from GeneDB and alignments prepared. No significant homology existed between the 12 genes and the C2 domains were variously located, suggesting that these C2 domain proteins do not form a common family but present as individual proteins with a common membrane targeting motif. Finally, bioinformatic analysis revealed a large number of potential phosphorylation sites for a number of kinases within the WCB protein.

WCB is expressed in both procyclic and bloodstream cells as judged by Western blotting (data not shown). The function of WCB was studied using an RNAi system in procyclic cells since we know most about WCB localisation and biochemistry in these cells (Woods et al. 1992). Non-induced trypanosome populations maintained a normal growth profile over 72 h. However, after 24 h the induced population exhibited a slight reduction in growth which became more extreme with time such that between the 48 h and 72 h passages little growth occurred (Fig. 1B). Morphological examination of the induced cell population showed that at 24 h some cells exhibited a rather enlarged and rounded posterior end. After 48 h this phenotype was more extreme and cells exhibited a fattened posterior end. Zoids (non-nucleated cytoplasts with a kinetoplast, 1K0N) were present and clumped cells were visible in the culture. After 72 h very large clumps were observable in flasks by the naked eye. These clumps of cells were easily dispersed by a few moments shaking such that individual cells were observed by microscopy. Western blot analysis using the WCB-1 monoclonal antibody along with L8C4 anti PFR monoclonal antibody (as a loading control) (Kohl et al. 1999) showed that RNAi was extremely effective at reducing WCB protein expression. There was a large reduction in the level of WCB at 24 h and further reduction through the 72 h period used for phenotype analysis (Fig. 1C).

Counts of kinetoplast (K) and nuclear (N) numbers in the cells provided information on progression of cell cycle events under depletion of WCB protein. Even at 24 h, major changes had

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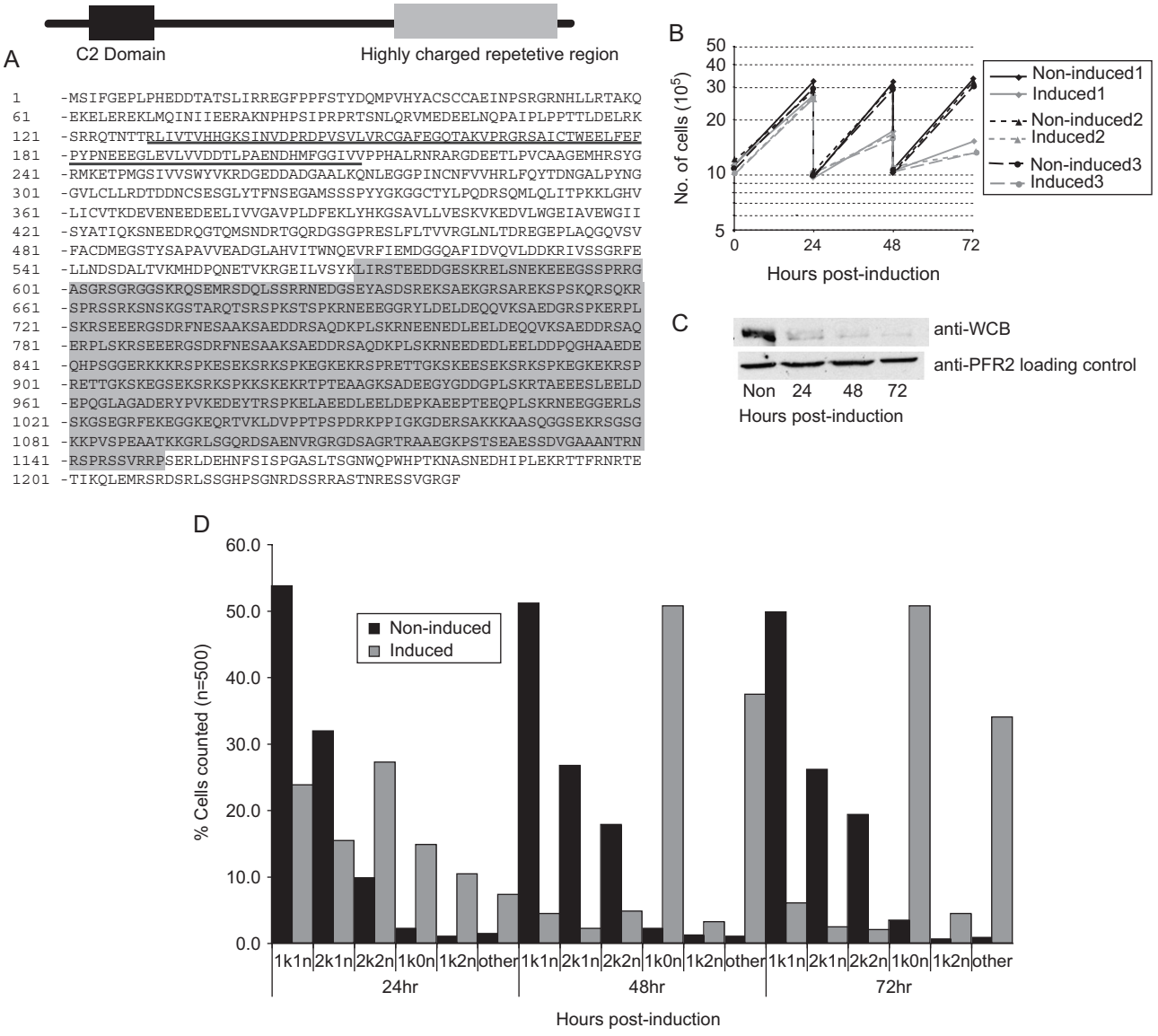

Figure 1. Bioinformatic and phenotypic analyses of WCB. **A**: Diagram showing domain positioning and the deduced amino acid sequence of Tb927.7.3550. In the amino acid sequence, the N-terminal C2 domain is underlined and the repetitive, highly charged C-terminal domain is boxed in grey. **B**: Three independent growth curves of non-induced and induced WCB RNAi cells are shown (labelled non-induced/induced 1,2,3). Decrease in growth rate after induction becomes more pronounced over time. **C**: Western blot of non-induced and induced WCB RNAi cells showing a marked drop in WCB expression after induction. Anti-PFR2 (L8C4) antibody is used as a loading control. **D**: Kinetoplast (K) and nuclear (N) counts of non-induced and induced WCB RNAi cells showing a marked increase in abnormal cells after induction. Bars labelled "Other" represent multinucleate, multi-kinetoplast cells.

occurred to the K/N ratios in the population in contrast to controls (Fig. 1D). There was a significant drop in the number of 1KIN and 2KIN cells (to 22% and 15%, respectively). The 2K2N cell population had risen to be the most abundant sub-population (28%) with an increase in the number of abnormal 1K2N cells and zoids (1K0N). These latter abnormal cell types, together with the large rise in the 2K2N population, strongly suggested that depletion of WCB delays, inhibits or induces mistakes in cytokinesis. After 48 h induction very few normal cells were present.

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The population consisted almost entirely of zoids (1K0N cytoplasts) and multinucleated/multikinetoplast (51% and 38%, respectively) cells (Fig. 1D). This pattern was maintained at 72 h.

In normal and RNAi non-induced trypanosomes the WCB-1 monoclonal detects WCB across the whole of the cell body but not the flagellum (Woods et al. 1992) (Fig. 2). On RNAi induction WCB disappeared from the posterior end of the cell with the immunofluorescence signal often being retained at the anterior portion. At 48 h cells are rather rounded at the posterior end. Some, such as that seen in Fig. 2B, appear to be 1K2N cells (presumably having lost 1K in a zoid that has cleaved off). Flagella and their associated cytoplasmic flagellar attachment zone (FAZ) filament, defined by the monoclonal antibody L6B3 (Kohl et al. 1999), continued to be formed even in the absence of effective new WCB synthesis. Fig. 2D shows a very aberrant multinucleated,

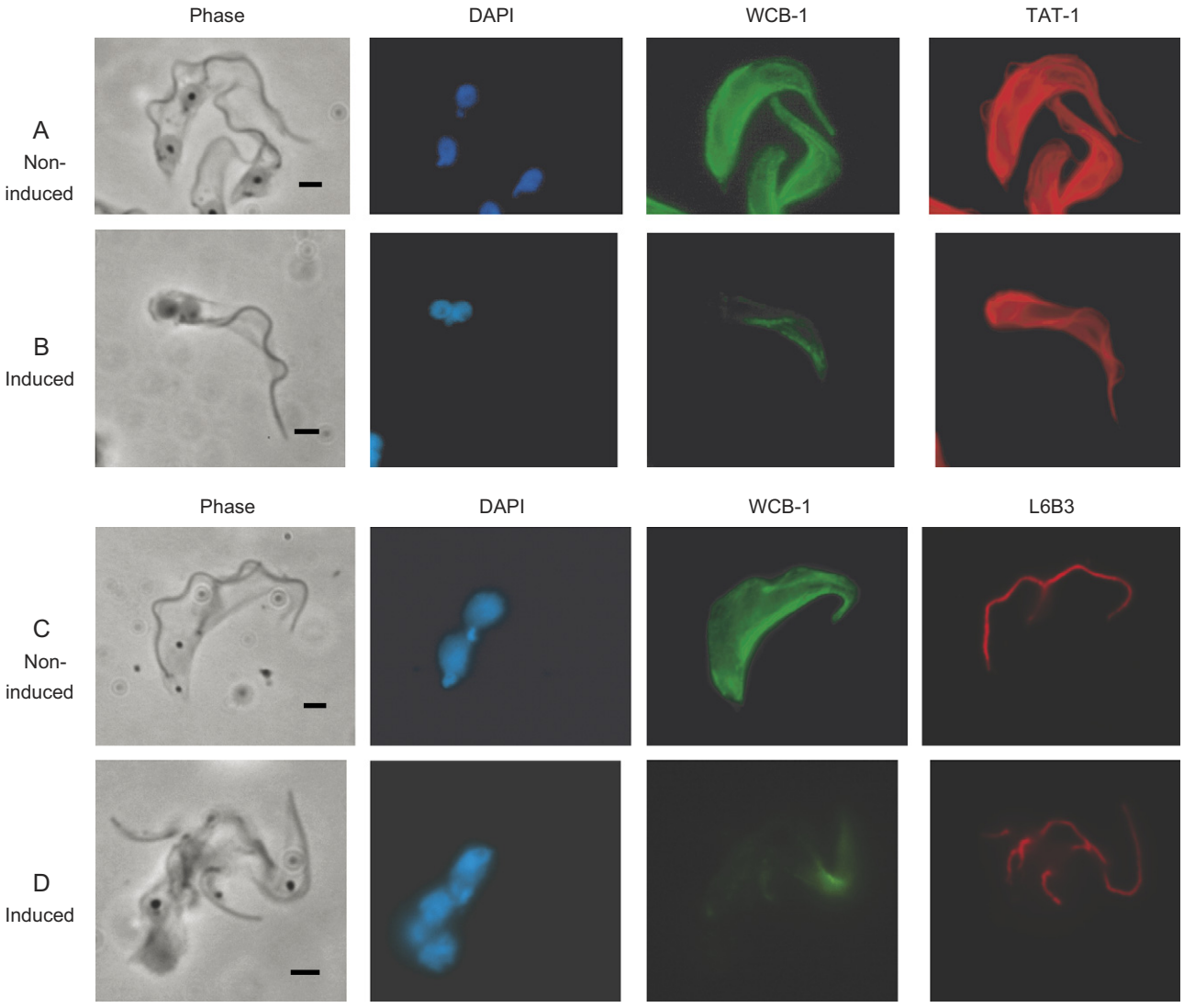

Figure 2. WCB depletion occurs after induction whilst other proteins are unaffected. **A**: Non-induced WCB RNAi cells labelled with WCB-1 and TAT-1 (anti-alpha tubulin) MAbs. **B**: Induced (24 h post-induction) WCB RNAi cells labelled with WCB-1 and TAT-1 MAbs. WCB-1 labelling disappears after induction, initially from the posterior end of the cell, although TAT-1 labelling shows the microtubule network remains intact. **C**: Non-induced WCB RNAi cells labelled with WCB-1 and L6B3 (anti FAZ) MAbs. **D**: Induced (48 h post-induction) WCB RNAi cells labelled with WCB-1 and L6B3 MAbs. Despite gross abnormalities in the induced cell population, flagella and corresponding flagellum attachment zones are still produced. Scale bar = 2 μM.

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multi-kinetoplastid cell which has the WCB signal still at what we presume is the original anterior end. However, even though extremely abnormal, it clearly possesses multiple flagella and multiple FAZ filaments. Staining with the anti α-tubulin TAT-1 monoclonal antibody (Woods et al. 1989) revealed that although WCB is missing initially at the posterior ends of cells and then more extensively, the microtubule network seemed to be present throughout (Fig. 2B).

We next examined the ultrastructural effects of RNAi-induced WCB protein depletion on the structure of the trypanosome. Thin section electron micrographs of non-induced trypanosomes revealed them to have normal ultrastructure with a sub-pellicular corset of evenly spaced microtubules when viewed in both glancing section and cross-section (Fig. 3A and C). In RNAi-induced trypanosomes, the sub-pellicular corset of microtubules was present and relatively normal. In thin section electron micrographs this was best seen in glancing sections where sweeps of the array are visible (Fig. 3B and D). However, more general aspects of the plasma membrane—microtubule structure appeared abnormal. First, cells exhibited examples of flagella where the flagellar axoneme together with a paraflagellar rod (PFR) and flagellar membrane were directly surrounded by closely associated flagellar pocket membrane (Fig. 3F). Second, induced cells exhibited many examples where plasma membrane containing cytoplasm was blebbing from the cell surface. Examples of this are shown in Fig. 3G—K. It appeared that the normally close connection between the sub-pellicular microtubule corset and membrane had been locally loosened. We analysed the culture supernatant of these induced cells for the presence of the surface protein procyclin by Western blotting, but could detect none, suggesting that these blebs are not released en masse into the medium. Measurements of cell peripheries from a number of micrographs of non-induced and induced cells were carried out and the average number of blebs per 10 μm was calculated. For non-induced cells, this figure was 0.2 ($\pm$0.14) and for induced cells the figure was 5.7 ($\pm$1.1). Micrographs of other mutant cell lines generated in our laboratory (Dawe et al. 2005; Moreira-Leite et al. 2001) were studied to see if this blebbing phenomenon was a general feature of dying cells with cytokinesis defects, but blebs were not observed in these cases.

In order to assess the general integrity of the sub-pellicular cytoskeleton we viewed cytoskeletal preparations both by thin section electron microscopy and by whole mount negative stain electron microscopy. Thin sections of cytoskeletons of non-induced trypanosomes revealed the normal pattern of flagella profiles with cytoskeletal sub-pellicular 'cages' often surrounding the nuclear remnant. Glancing sections of the sub-pellicular array of non-induced cells usually revealed the microtubules to be evenly spaced (Fig. 4A, B, D). However, cytoskeletons of RNAi-induced trypanosomes revealed a significant loss of overall integrity. Sub-pellicular corsets of microtubules appeared less well organised after detergent treatment and glancing sections revealed rather uneven spacing (Fig. 4C, E).

Finally, we examined whole mount, negatively stained cytoskeletons. Cytoskeletons from non-induced cells showed a normal appearance with examples of 1K1N and 2K2N cells seen in Fig. 5A, B. Zoids were apparent in the induced population and zoid cytoskeletons often exhibited a looser organisation of microtubules at a 'swollen' posterior end (Fig. 5C), although a more normal microtubule structure is visible at the anterior end of the cell, with ordered microtubules running parallel to the flagellum. Examples of cytoskeletons of multiflagellated and multinucleated trypanosomes are shown in Fig. 5D, E. Both of these micrographs exhibit the characteristic features of these WCB RNAi-induced cells and cytoskeletons. The sub-pellicular microtubule cytoskeleton in the main body of the cell at the swollen posterior end is disorganised. Under the influence of detergent this end of the cell appears to have been catastrophically lysed by the preparation procedure with the inter-microtubule organisation being extremely disorganised. However, even in these cells the anterior end organisation appears much more normal, with ordered microtubule cytoskeletal arrays running alongside the emerging flagella (arrows in Fig. 5D, E). In Figure 5D, another example of a zoid can be seen (arrowhead). This cell is defined as a zoid and not a continuation of the large multiflagellated and multinucleated cell because the ends of two flagella are clearly seen (white arrows) emerging from the large cell — the flagellum of the zoid cytoplast is not attached to this cell.

Discussion

WCB was named after the original observation that the WCB-1 monoclonal antibody recognized the 'whole cell body' cytoskeleton of *T. brucei* but not the flagellum. Our previous immunolocalisation

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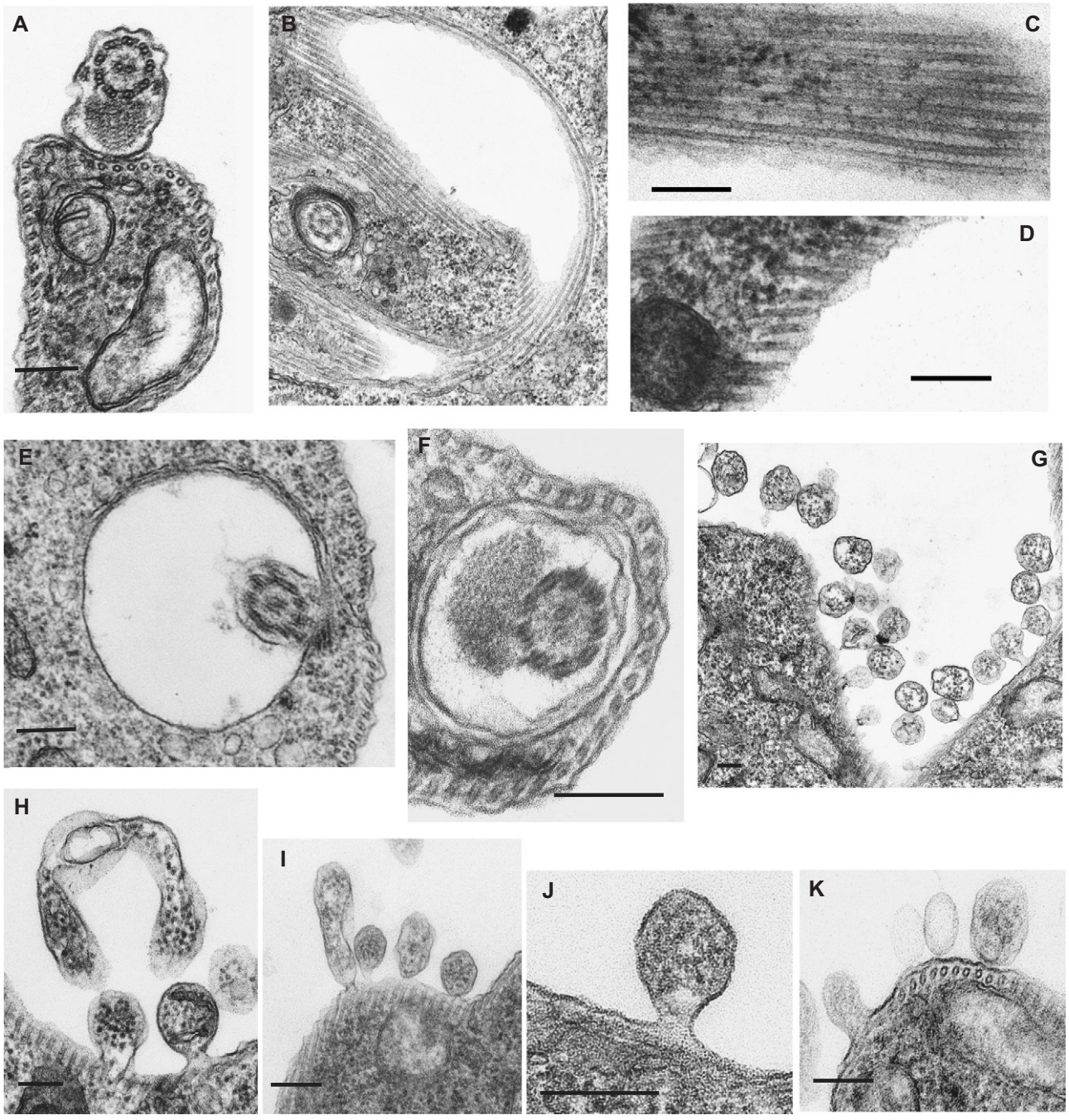

Figure 3. Thin section TEMs of non-induced and induced (48 h post-induction) WCB RNAi cells, with induced cells showing ultrastructural abnormalities. **A** and **C**: Non-induced cells showing regularly spaced microtubules. **B** and **D**: Induced cells showing that although microtubules are present and relatively normal, some abnormalities in their alignment and spacing occur. **E**: Non-induced cell showing a normal flagellum in a flagellum pocket. **F**: Induced cell with an aberrant flagellum containing a paraflagellar rod structure within a flagellum pocket. **G**—**K**: Plasma membrane blebs away, suggesting loss of integrity of the plasma membrane from the sub-pellicular microtubule corset. Scale bar = 0.2 μM.

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studies at the electron microscope level (Woods et al. 1992) revealed WCB to be located on the plasma membrane-facing side of the sub-pellicular microtubule corset. It is tightly associated with the microtubule cytoskeleton. This location implicated WCB in either directly or indirectly linking the microtubules to the plasma membrane (Woods et al. 1992). Our present demonstration of the molecular identity of WCB is intriguing in this respect. First, the C-terminus of the protein contains a repetitive region with a high percentage of interspersed basic and acidic residues (K, R, E, D). Tubulin-binding domains in microtubule-associated proteins are rather diverse but this region is reminiscent of the characterised highly repetitive KKE motifs in MAP1B and the repetitive KK-containing motifs of Tau in mammalian cells (Goode et al. 1997; Noble et al. 1989). Second, bioinformatic analysis suggests that WCB protein possesses a C2 domain near the N-terminus. This domain was originally identified as a conserved Ca^{2+}-dependent domain in classical protein kinase C (PKC) protein and is important in lipid and membrane binding (for review see Cho and Stahelin 2006). To date a large number of C2 domain containing proteins have been identified and they exhibit diverse involvements in signal transduction or membrane trafficking. Alpha, beta and gamma PKCs have C2 domains that are Ca^{2+}-dependent and possess key aspartic acid residues involved in the Ca^{2+}-binding and regulation. However, non-classical PKCs and many other proteins possess C2 domains that do not bind Ca^{2+} and in these the key aspartic acid residues are missing (Cho and Stahelin 2006). WCB appears to fall into this Ca^{2+}-independent group of C2 domain proteins. Some of these Ca^{2+}-independent C2 domains have been reported to bind membrane but many of them may also be involved in protein—protein interactions. There is recent evidence that the C2 domain can bind multiple targets such as multiple lipids or lipids/proteins combinations and can operate in molecular scaffolds at the membrane (Cho and Stahelin 2006). Future work beyond this study may resolve whether this WCB C2 domain is mediating plasma membrane interaction directly

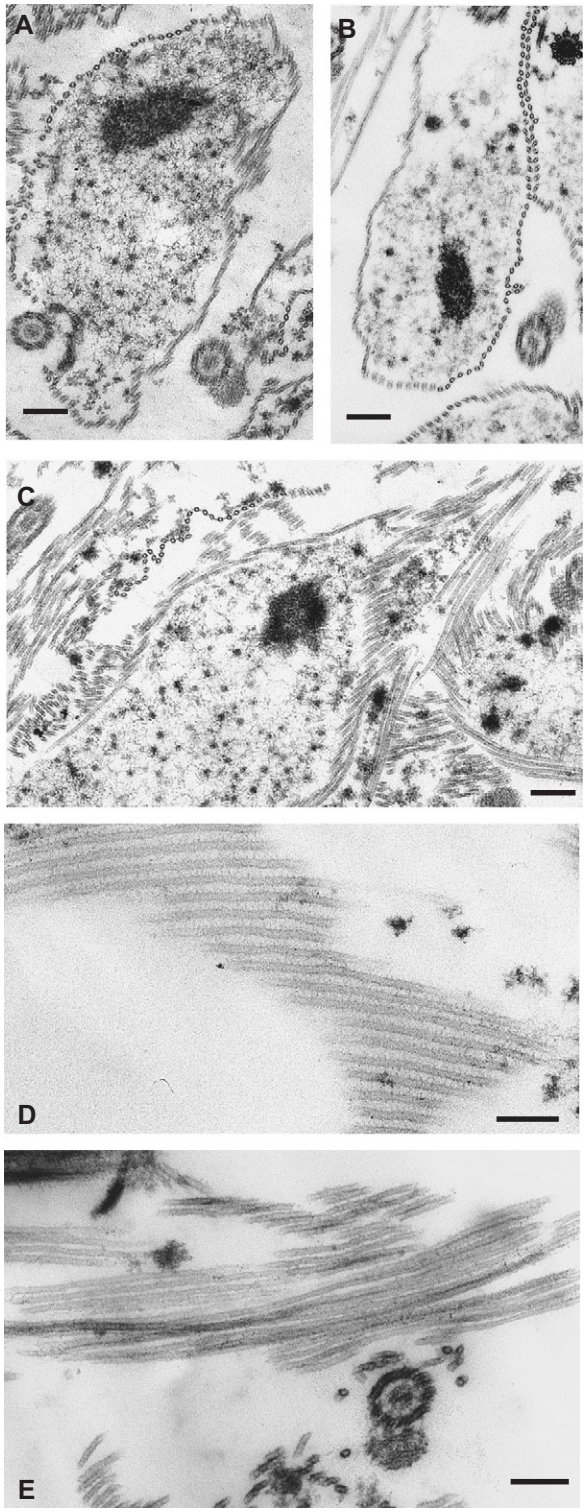

Figure 4. Comparison of thin-section electron micrographs of cytoskeletal preparations of non-induced and induced WCB RNAi cells shows how induced cells lose overall integrity. **A**, **B** and **D**: Non-induced cells with regularly spaced and ordered microtubules. **C** and **E**: Induced cells (48 h post-induction) with much looser associations between microtubules and more random spacing. Scale bar = 0.2 μM.

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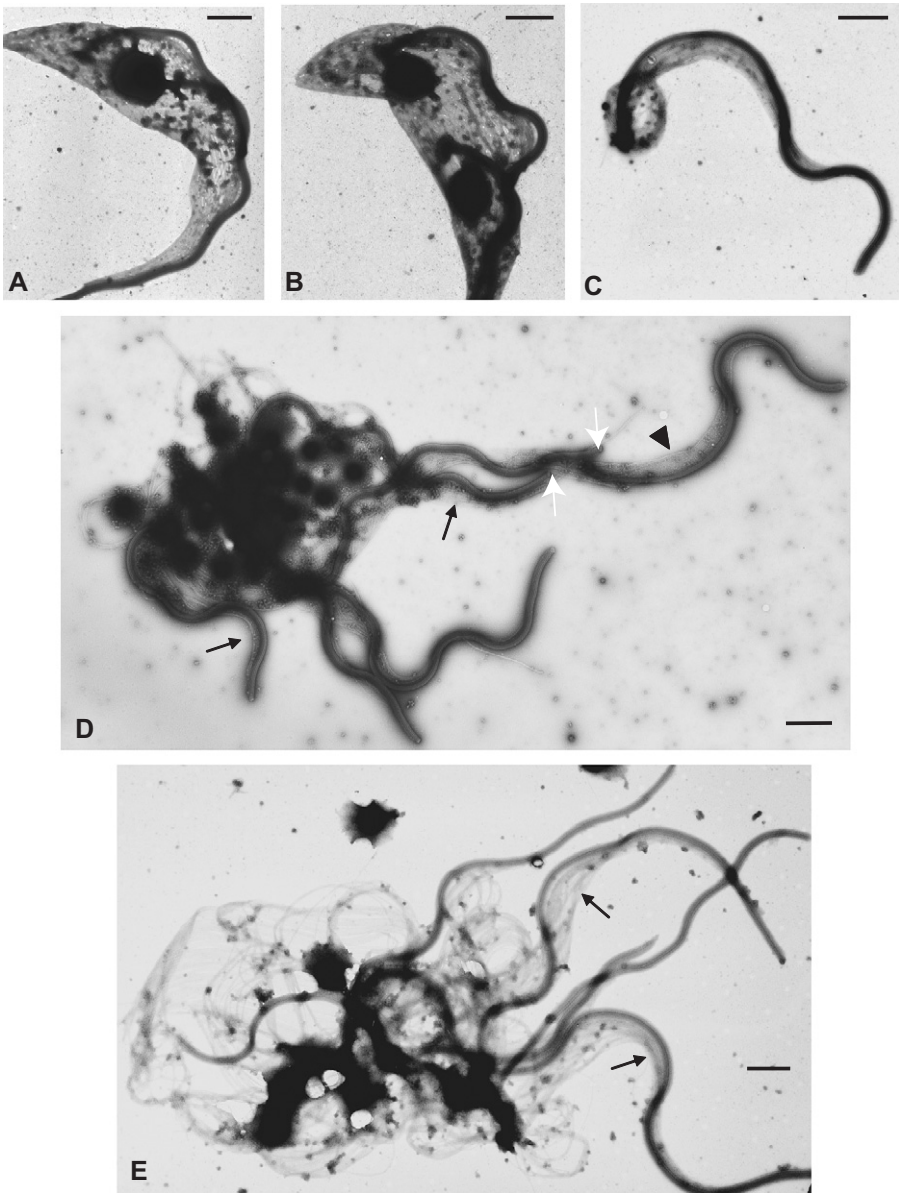

Figure 5. Whole mount negatively stained cytoskeletons of induced WCB RNAi cells reveal a swollen posterior end with a disorganized microtubule cytoskeleton. **A** and **B**: Non-induced trypanosomes at the 1K1N and 2K2N stage (respectively) of the cell cycle. **C**: Zoid with a rounded posterior end, but more organised microtubules at the anterior end are produced following induction (72 h post-induction). **D** and **E**: Induction (72 h post-induction) also produces multinucleated, multi-kinetoplast cells with disorganised sub-pellicular microtubules at the swollen posterior ends. In comparison to the posterior end of the cells, microtubules at the anterior ends of the large abnormal cells, where the flagella emerge from the cell body, are much more organised (arrows). The large, abnormal cell in **D** lies over a zoid (arrow head): the ends of two flagella emerging from the large cell are clearly visible (white arrows) and do not adjoin the flagellum of the zoid cell. Scale bar = 2 μM.

through lipid interaction or via interaction with unknown proteins or a combination of both. Plant cells also exhibit interphase bundles of cytoplasmic microtubules linked to the inner face of the plasma membrane. Interestingly, a C2 domain protein has also been implicated in transient binding both to microtubules and the plasma membrane in tobacco cells (Gardiner et al. 2001).

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Other than the C2 domain, this 90-kD protein shows no primary sequence homology to WCB but has been shown to possess phospholipase D activity. In a similar manner WCB may participate directly or indirectly in linker functions and possess signalling or regulatory properties. The cytoskeletal proteins CAP15 and CAP17 have been shown to localise to the sub-pellicular microtubule corset of *T. brucei* but, unlike WCB, mainly to the less dynamic anterior end (Vedrenne et al. 2002). It is unclear whether either of these proteins link between microtubules or between microtubules and the membrane. However, Vedrenne et al. (2002) conjecture that an additional 20 amino acid hydrophobic domain on CAP17 might indicate a membrane interaction.

Our functional analysis reveals that upon RNAi-mediated protein depletion the major effect is that the cells have increasing difficulty in completing an effective cytokinesis. This results in a dramatic increase in 2K2N cells and in the production of zoids (1K0N) which are indicators of cytokinetic mistakes. Examination of these abnormal cells revealed that the major aberrations in shape and form occur at the posterior end of the cell. The multinucleated cells apparently perform many attempts to cleave by constructing new flagella, new flagellar attachment zone filaments and relatively coherent anterior poles. However, these anterior pole initiations of cleavage do not progress through to the full posterior zone resulting in large multinucleated monstrous cells with many individual anterior ends each associated with a small group of microtubules, FAZ filament and flagellum. This phenotype is understandable when one appreciates that the major site of new microtubule assembly is at the posterior end of cells where the plus ends are located (Robinson et al. 1995). Thus, it is at this growing posterior end that the major effects would be seen of depletion of a protein such as WCB that interacts with the microtubules at the inner face of the plasma membrane. Although there is a 'ballooning' of this end of the cell in RNAi depletion, microtubules are still present and are still orientated together. The basic microtubule—microtubule cross-bridging appears still to exist. What is almost impossible to discern by electron microscopy is if there is some rather general dislocation of the coordination and integration of the membrane—microtubule interaction. The normal microtubule cytoskeleton of trypanosomes involves both microtubule—microtubule connections and microtubule—membrane cross bridges. Local disruption of the geometry of the latter could, we presume, produce concomitant affects on the former. The phenotype of 'ballooning' of the posterior end and blebbing of the cell surface suggests a local loss of microtubule corset to plasma membrane-inner face connection in the absence of adequate WCB. Overexpression studies of the cytoskeletal proteins CAP15 and CAP17 similarly led to the production of zoids, multinucleated cells and failures in cytokinesis (Vedrenne et al. 2002).

One of the major characteristics of the kinetoplastid protozoa, both free-living and pathogenic, is their stable sub-pellicular microtubule corset. This is a case of the extreme biology of trypanosomes. Whilst many other organisms have individual microtubules that interact with the plasma membrane and some, such as the land plants and protists, have groups of sub-plasma membrane microtubules, few have a complete corset that is present throughout the cell cycle. Our initial bioinformatic analysis suggests that WCB is involved in a trypanosome-specific example of this phenomenon. Identification of WCB represents a glimpse into a likely multi-protein macromolecular structural and regulatory complex (possibly including other proteins such as CAP15, CAP17 and MARPs) involved in this corset/inner plasma membrane face milieu.

Methods

Expression library screening: A λgt11 expression library was screened according to standard protocols (Sambrook and Russell 2001) and filters were exposed to a 1/10 dilution of WCB-1 antibody (Woods et al. 1992) in Tris-buffered saline (TBS)/0.05% Tween-20/1% milk at room temp for 1 h. Following washes in TBS/0.05% Tween-20 the filters were exposed to 1/20,000 dilution of anti-mouse alkaline phosphatase-conjugated secondary antibody (Sigma) in TBS/0.05% Tween-20/1% milk at room temperature for 1 h. Following washes in TBS/0.05% Tween-20, the filters were exposed to 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium developing solution (BioRad) for approximately 20 min.
Positive plaques were identified by dark purple spots on the filter and were stabbed out from plates. The stab was mashed up in 500 μl SM (100 mM NaCl, 10 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris-Cl (pH 7.5), 0.01% (w/v) gelatin solution) buffer. Plates of neat and 10^{-1} to 10^{-4} dilutions of this preparation were re-screened. Again, a positive plaque was stabbed out from the corresponding plate and re-screened. Finally, four positive plaques were selected, resuspended in SM buffer and amplified by PCR using T3/T7 standard primers. Resultant products were sequenced and a GeneDB (http://www.genedb.org/) search identified a conserved hypothetical protein, Tb927.7.3550.

Constructs and trypanosome transfection: A 581 base pair fragment (from 899—1480bp) of Tb927.7.3550 was amplified by PCR by specific primers incorporating XhoI and BamHI sites onto the forward and reverse primers,

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respectively (WCB for CCGCTCGAGGTGGTGTGCTGTGTCTGCTT WCBrev: CGCGGATCCCTGGCGCAGAGTATGTTGAA). The resultant product was cloned into the p2T7-177 inducible RNAi vector (Wickstead et al. 2002) using the XhoI and BamHI sites. Procyclic *T. brucei* 29—13 cells (Wirtz et al. (1999)) were transfected using standard protocols and selected using 5 $\mu g\,ml^{-1}$ phleomycin.

Culture and induction of trypanosomes: Cells were cultured in SDM-79 (Brun and Schönenberger 1979) medium, supplemented with 10% fetal calf serum and 5 $\mu g\,ml^{-1}$ phleomycin. Prior to induction, phleomycin was removed from the culture for three days. For RNAi, cells were induced with 1 $\mu g\,ml^{-1}$ doxycycline. For growth curves, cells were counted and passaged to a density of 1×10^6 cells ml^{-1} into two flasks; one induced with doxycycline as above, one non-induced. After 24 h, cells were counted again, passaged back to 1×10^6 cells ml^{-1} into new flasks, with or without drug. Counts were repeated for both flasks at 48 and 72 h post-induction. Counts were repeated on three separate inductions for consistency.

Immunolocalisation studies: For DNA staining, trypanosome cells at zero, 24, 48 and 72 h post-induction of RNAi were settled onto glass slides and fixed in 4% formaldehyde in phosphate buffered saline (PBS). They were stained with 1 $\mu g\,ml^{-1}$ 4,6-diamide-2-phenylindole (DAPI) and mounted in Vectashield (Vector Laboratories).

For immunofluorescence, trypanosome cells at zero, 24, 48 and 72 h post-induction of RNAi were settled onto glass slides and were extracted with 1% Igepal CA-630 (Sigma) in PEM (0.1 M PIPES, 2 mM EGTA, 1 mM $MgSO_4$ pH 6.9) for three minutes before being fixed in 4% formaldehyde in PBS. Cells were single labelled with WCB-1 (Woods et al. 1992), TAT-1 (Woods et al. 1989) and L6B3 (Kohl et al. 1999) and double labelled with WCB-1/TAT1 and WCB-1/L6B3. Secondary antibodies were FITC-conjugated anti-mouse IgG (Sigma), TRITC-conjugated anti-mouse IgG (Sigma) and Rhodamine-conjugated anti-mouse IgM (Chemicon International).

Cells were then stained with 1 $\mu g\,ml^{-1}$ DAPI and mounted in Vectashield. Slides were examined on a Leica Leitz DMRBE microscope using a 100×1.4NA oil immersion lens. Images were captured on a CCD camera controlled by IPLab software (Universal Imaging) and processed in Adobe Photoshop.

Western blotting: Trypanosome cells at zero, 24, 48 and 72 h post-induction of RNAi were washed in PBS-containing protease inhibitors and were resuspended in a volume of boiling Laemmli sample buffer to give a concentration of 2×10^5 cells per μl. Samples were run on 10% acrylamide gels and were then transferred by wet transfer onto nitrocellulose at 120 mA for 16 h. Western blotting was carried out according to standard protocols (Sambrook and Russell 2001) using a 1/10 dilution of WCB-1 antibody (Woods et al. 1992) with a 1/200 dilution of anti-PFR2 antibody L8C4 (Kohl et al. 1999), as a loading control, in TBS/0.05% tween/1% milk. Secondary antibody was peroxidase-conjugated anti-mouse IgG (Sigma), diluted 1:20,000 as before. Membranes were covered with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer), exposed to Kodak X-OMAT LS film and developed in a Kodak X-OMAT 2000 developer.

Preparation of cells for thin-section TEM: Trypanosome cells were fixed at zero, 24, 48 and 72 h post-induction of RNAi in 2.5% glutaraldehyde in tissue culture medium. Cytoskeletons were generated by washing the cells (at 0, 24, 48 and 72 h post-induction of RNAi) with 1% Igepal-CA630 (Sigma) in PEM for five minutes. The cells were pelleted and fixed in 2.5% glutaraldehyde, 2% paraformaldehyde and 0.1% picric acid in 100 mM phosphate (pH 6.5) for 2 h at 4°C followed by 1% osmium tetroxide in 100 mM phosphate buffer (pH 6.5) for 1 h at 4°C. In both cases, the fixed material was stained en bloc with 2% aqueous uranyl acetate for 2 h at 4°C. The material was dehydrated through a graded series of acetone and propylene oxide and was then embedded in Epon resin for sectioning.

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