Trypanosoma brucei centrin5 is enriched in the flagellum and interacts with other centrins in a calcium-dependent manner

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Centrins are a member of the EF-hand calmodulin (CaM) superfamily, which are highly conserved in eukaryotic cells. Centrins were initially identified in unicellular green algae [1], as the essential components of basal bodies-associated and Ca\textsuperscript{2+}-sensitive contractile fibers. Subsequently, homologous centrin proteins were observed in the microtubule-organizing center [2–5]. The functions of centrins were diverse in the cellular processes, including spindle pole body duplication [4], cellular morphogenesis [6], nucleotide excision repair [7], mRNA export [8], and protein degradation [9]. In green algae, the flagellar contraction is regulated by contractile fibers containing centrin and the contraction depending on the increase in intracellular concentration of Ca\textsuperscript{2+} ions [1,10], suggesting that centrin is responsible for Ca\textsuperscript{2+}-dependent cell motility. Target Sfi1 in yeast and its homologous protein (hSfi1) in humans were identified to interact centrins and responsible for SPB duplication [11,12]. In humans, two centrins (HsCentrin1 and HsCentrin2) with high sequence identity are involved in the centrosome/basal body segregation, ciliary beating, and mRNA and protein export [2,3,13–15]. HsCentrin3 is involved in centrosome/basal body duplication [16,17].

The overall topology of centrins or centrin/target peptide complexes reported by NMR spectroscopy or X-ray crystallography [6,11,18–21] is highly conserved and similar to CaM and troponin (TnC). The structure of centrin generally contains four EF-hands comprised of seven to eight \textalpha-helices. The structure of centrin can be divided into two independent domains, N-terminal domain (NTD) and C-terminal domain (CTD). Each

Abbreviations
CaM, calmodulin; CTD, C-terminal domain; DAPI, 4',6-diamidino-2-phenylindole; ITC, isothermal titration calorimetry; NTD, N-terminal domain; RT-PCR, reverse transcription-PCR; TnC, troponin.
TbCentrin5 interacts with Ca\textsuperscript{2+} ions and other TbCentrins

Domain contains a pair of EF-hands. The two domains are linked by a loop or a long \( \alpha \)-helix and form a dumbbell structure. In all structures of centrins except \textit{Mus musculus} Centrin1 (MmCentrin1) [19] and \textit{Chlamydomonas reinhardtii} centrin (CrCentrin) [22], the two EF-hands of CTD instead of all the four EF-hands can be saturated by Ca\textsuperscript{2+} ions. The highly conserved CTD shows a higher affinity with Ca\textsuperscript{2+} ions and other partners [11,23,24]. Meanwhile, NTD containing unconserved and unstructured 20–25 residues at the front shows a lower affinity with Ca\textsuperscript{2+} ions and its partners [11,20]. Compared with CaM and TnC, the extended 20–25 residues in the NTD may have specialized biological function that is metal ion-dependent during self-assembly of centrin [25,26].

In \textit{Trypanosoma brucei} (\textit{T. brucei}), a protozoan parasite that causes sleeping sickness in humans and nagana in cattle in sub-Saharan Africa, five centrin isoforms have been identified [27,28]. TbCentrin1 (Tb927.04.2260) and TbCentrin2 (Tb927.08.1080) are localized to the basal body and are essential for basal body duplication [27]. TbCentrin3 (Tb927.10.8710) is localized in the flagellum and is required for the flagellar motility [29]. TbCentrin4 (Tb927.07.3410) is localized to both the basal bodies and the bilobe structure and is involved in organelle segregation and the coordination between karyokinesis and cytokinesis [30,31]. The role of centrin5 of \textit{T. brucei} (TbCentrin5; Tb927.11.13900) is still unknown.

Here, we investigated the localization of TbCentrin5 in \textit{T. brucei} and identified the interactions between TbCentrin5 and Ca\textsuperscript{2+} ions. We further identified that TbCentrin5 is able to interact with other TbCentrins and the interactions are Ca\textsuperscript{2+}-dependent. The work will provide a basis for better understanding of the biological functions of TbCentrin5.

Materials and methods

Phylogenetic analyses of centrin protein sequences

The centrin protein sequences of \textit{T. brucei} were obtained from TriTrypDB (https://tritrypdb.org/tritrypdb/). All other centrin protein sequences were obtained from NCBI protein database (https://www.ncbi.nlm.nih.gov/protein) and UniProt (https://www.uniprot.org/). Sequences were aligned using \textsc{Clustalx} [32] with default alignment parameters. The sequence trees were reconstructed with Neighbor-Joining algorithm using \textsc{megabyte} [33]. Bootstrap analysis (> 70, based on 500 replicates) provided a confidence measure for the detected relationships of branches in the phylogenetic tree.

Cell culture

The wild-type procyclic Lister 427 strain was cultivated at 28 °C in Cunningham’s medium supplemented with 10% FBS. The procyclic 29-13 strain [34] was cultivated at 28 °C in Cunningham’s medium containing 10% FBS, supplemented with 15 µg·mL\(^{-1}\) G418 and 50 µg·mL\(^{-1}\) hygromycin.

RNA interference

RNA interference (RNAi) of TbCentrin5 was performed using the RNAi vector pZJM. Recombinant pZJM vector containing segment (nucleotide number 121–420) of TbCentrin5 was linearized and electroporated into \textit{T. brucei} procyclic forms from 427 strain. The transfection by electroporation was carried out as follows: Briefly, 10\(^6\) cells were harvested and washed twice with cytomyx buffer (120 mM KCl, 0.15 mM CaCl\(_2\), 10 mM K\(_2\)HPO\(_4\), 10 mM KH\(_2\)PO\(_4\), 25 mM Heps, 2 mM EGTA, 5 mM MgCl\(_2\), 2 mM ATP, 5 mM glutathione, pH 7.6) and suspended in 0.45 mL of cytomyx buffer containing 30 µg of the linearized vectors. Electroporation was carried out in a 2-mm cuvette (Bio-Rad, Berkeley, CA, USA) using the Gene Pulser (BTX ECM630, Holliston, MA, USA) with parameters set as follows: 2.0 kV voltages, 25 µF capacitance, and 200 Ω resistance. The transfected cells were immediately transferred into 10 mL of fresh Cunningham’s medium. Transfectants were selected with 10 µg·mL\(^{-1}\) zeocin and were induced with 1.0 µg·mL\(^{-1}\) tetracycline.

In situ epitope tagging of endogenous proteins

For \textit{in situ} tagging of TbCentrin5, the cDNA segment (nucleotide number 121–558) was cloned into pC-EYFP-NEO vector [35]. The recombinant vectors were linearized and electroporated into \textit{T. brucei} procyclic forms from 427 strain. The transfection conditions were the same described as above. Successful transfectants were selected under 2.5 µg·mL\(^{-1}\) G418. Expression of TbCentrin5-EYFP fusion protein was verified by western blotting.

Immunofluorescence microscopy

Cells stably expressing TbCentrin5-EYFP were harvested and washed twice with PBS. Resuspended cells were fixed with 4% paraformaldehyde and washed with PBS. Fixed cells were settled on the coverslip at room temperature for 30 min. The cells were stained with 4 µM DAPI and examined with an inverted microscope (Model IX73; Olympus, Tokyo, Japan). Images were analyzed by \textsc{ImageJ} (NIH, Bethesda, MD, USA).

Protein expression and purification

Full-length gene encoding TbCentrin5 was amplified by PCR from the genomic DNA of \textit{T. brucei} and cloned into
a modifier vector pET-28a (+) (Novagen, Darmstadt, Germany), which provided a cut site of TEV protease at the N terminus to remove His-tag. The recombinant vector was transformed into Escherichia coli BL21 (DE3). The transformed cells were cultured in LB at 37 °C until OD₆₀₀ reached 0.8 and induced by 0.5 mM IPTG at 16 °C for 20 h. The cells were harvested and purified in lysis buffer containing 20 mM Tris and 500 mM NaCl at pH 7.0. The eluted protein was digested by TEV protease and further purified by gel filtration column Sephadex G-75 (GE Healthcare, Chicago, IL, USA).

Structure modeling

The 3D structure of TbCentrin5 or the complex of TbCentrin5 and Ca²⁺ ions was carried out using SWISS-MODEL based on homology modeling techniques [36]. The evolutionarily related protein structures were searched as templates based on the amino acid sequence of TbCentrin5. More than five hundred templates were searched and estimated by Global model quality estimate (GMQE) [37] and quaternary structure quality estimate (QSQE) [38]. Top-ranked templates were selected as templates for building model automatically. The model was finally evaluated and optimized by pairwise distance constraints that represented ensemble information from all template structures found.

Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) measurements were performed on iTC200 (GE Healthcare) at 16 °C to investigate the Ca²⁺-binding capacity and the interactions between TbCentrin5 and TbCentrin1/2/3/4. Samples of TbCentrin5 titrated with Ca²⁺ ions were mixed with EGTA to remove potential Ca²⁺ ions and then further purified by gel filtration column Sephadex G-75 (GE Healthcare). TbCentrin5 and calcium ions were equilibrated in the same buffer containing 20 mM Tris/HCl (pH 7.0) and 100 mM NaCl. 0.1 mM TbCentrin5 in cell was titrated against 2.0 mM CaCl₂ from syringe. Two microliter of CaCl₂ was injected into 0.2 mL TbCentrin5 at 120 s intervals. Samples of TbCentrin1/2/3/4 titrated with TbCentrin5 were purified and equilibrated as above expect mixed with EGTA or not. Fifty micromolar TbCentrin1/2/3/4 in the cell was titrated against 0.5 mM TbCentrin5 from the syringe with the same sample volume and rotate speed. The data collected were analyzed by MICROCAL LLC ITC software (MicroCal, Malvern, UK).

Sedimentation assay

Sedimentation assay was carried out according to the procedures published previously [29]. In brief, the wild-type 29-13 cell line, the noninduced control cells, and TbCentrin5 RNAi cells after tetracycline induction for 3 days were each suspended to ~ 5×10⁶ cells·mL⁻¹ in fresh medium. Each cell line was cultured in two cuvettes and incubated at 26 °C and was measured by optical density (600 nm) every 2 h. To monitor the OD₆₀₀, one cuvette was resuspended for monitoring cell growth while the other cuvette was not disturbed for monitoring sedimentation. The change in the OD₆₀₀ (ΔOD₆₀₀) was calculated by subtracting the OD₆₀₀ of the resuspended sample from that of the undisturbed sample. The experiment was repeated three times.

Results and Discussion

Phylogenetic analysis of TbCentrin5

To further understand the position of TbCentrin5 in evolution and its relationship with other centrins that have been identified in other organisms, a phylogenetic analysis was performed using a Neighbor-Joining method (Fig. 1A). Because the sequences of centrins are conserved, Neighbor-Joining method was selected. The presence of sequences of centrins from animal, fungi, and protist in this evolutionary tree indicates that those centrins come possibly from an ancestral protein in eukaryotic evolution. In the evolutionary tree, centrins from T. brucei are on different branches with centrins from higher animal, but closer to centrins from fungi and other protest, especially to centrins from Leishmania major (L. major) which belong to the same family of Trypanosomatidae as T. brucei. TbCentrin5 and TbCentrin2 were on utterly different branches with other centrins but same as LmCentrin4 and LmCentrin3, suggesting that TbCentrin5 and TbCentrin2 are far from other centrins but closer to LmCentrin4 and LmCentrin3. The phylogenetic analysis indicated the position of TbCentrin5 and its relationship with other centrins, which is helpful for us to determine the location of Trypanosoma genera in the evolution.

TbCentrin5 is localized throughout the cytosol and nucleus and enriched in the flagellum

To determine the subcellular localization of TbCentrin5, TbLa was endogenously tagged with EYFP at the C terminus. The level of TbCentrin5-EYFP fusion protein was examined by western blot with GFP probe, which indicated the successful expression of TbCentrin5-EYFP in vivo (Fig. 1B). Fluorescence microscopy demonstrated that TbCentrin5-EYFP appeared to spread throughout the cell and was enriched in flagellum (Fig. 1C). Intriguingly, the localization of TbCentrin5 in nucleus changed through the cell cycle. In the early stage (1N1K) of the cell cycle, TbCentrin5 was slightly
distributed in nucleus. As the cell cycle progressed (1N2K and 2N2K), the distribution of TbCentrin5 in nucleus became stronger. The results suggested that TbCentrin5 might be involved in the karyokinesis.

Sequence analysis of TbCentrin5

The sequence of TbCentrin5 was aligned with other centrins from T. brucei, L. major and Homo sapiens using CLUSTALW2 and ESPRIPT 3.0 [32,39]. TbCentrin5 shares about 29%, 33%, 27%, 31%, 38%, and 45% sequence identity with TbCentrin1-4, HsCentrin1, and LmCentrin2, respectively (Fig. 2). The result of sequence alignment also verified the credibility of the evolutionary tree in Fig. 1A. Although the structures of centrins are conserved [6,11,18–21], the sequences of centrins show diversity. Especially, the sequences of the first ~25 residues at the N terminus show diverse length and very low similarity. In contrast with CaM and TnC, the extended ~25 amino acids at the N

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**Fig. 1.** Characterization of TbCentrin5. (A) Evolutionary tree of centrins. Numbers on branches represent bootstrap support values. Hs, *Human sapiens*; Mm, *Mus musculus*; Cr, *Chlamydomonas reinhardtii*; Tg, *Toxoplasma gondii*; Eo, *Euplotes octocarinatus*; Sd, *Scherffelia dubia*; Sc, *Saccharomyces cerevisiae*; Tt, *Tetrahymena thermophila*; Lm, *Leishmania donovani*; Lm, *Leishmania major*; Tb, *Trypanosoma brucei*; (B) The expression of TbCentrin5-EYFP examined by western blot with anti-GFP probe. The levels of α-tubulin served as the loading control. (C) The subcellular localization of TbCentrin5. The localization of TbCentrin5-EYFP (green) was examined in paraformaldehyde-fixed intact cells. Cells were stained DAPI for DNA (blue), small blue dots are kinetoplasts, and large blue structures are nuclei. 1N1K, 1N2K, and 2N2K cells were tabulated, respectively. Scale bars: 5 μm.

**Fig. 2.** Multiple sequence alignments of TbCentrin5 with other centrins. Tb, *Trypanosoma brucei*; Hs, *Human sapiens*; Lm, *Leishmania major*. Identical residues are shaded in red box, and conserved residues are colored in red.
terminus of centrins display diversity in length and sequence, indicating that centrins may have more versatile functions than CaM and TnC.

**Structure modeling of TbCentrin5**

To better understand the functions of TbCentrin5, the model of TbCentrin5 was built using SWISS-MODEL [36], which builds model based on homology modeling techniques. Because the first 25 amino acids of TbCentrin5 show low conservation, the sequence of TbCentrin5 without the first 25 amino acids was placed for modeling. A total of 4015 templates were found from template library extracted from the PDB [40] to match the sequence of TbCentrin5, and five templates were selected for modeling. The quality of model built by SWISS-MODEL is rapidly reduced when sequence identity is below ~30% and is reliable when sequence identity is more than ~40% [41,42]. Finally, MmCentrin1 (PDB: 5d43), HsCentrin2 (PDB: 2ggm), SdCentrin (PDB: 3kf9), and CrCentrin (PDB: 3qrx) with respective sequence identity of 43%, 42%, 41%, and 39% were selected to build model (Fig. 3). The scores of the model estimated by GMQE and QSQE were 0.64 and −1.23, respectively. In addition, the analysis of Ramachandran plots showed that 96% of the residues are in the most favored region (Fig. 4). The evaluation indicated that the model is of high quality and reliable. The structure model of TbCentrin5 is comprised of four EF-hands containing seven α-helices. Interestingly, a very short α-helix was formed in the loop of EF-hand IV because the loop is longer than that in general EF-hand. TbCentrin5 is divided into NTD and CTD by the long α4 and adopts a shape like a dumbbell.

**Purification of TbCentrin5**

Centrin5 of *T. brucei* was expressed and purified as described above. The elution volume of TbCentrin5 from Superdex G-75 column is approximate 66 mL, which corresponds to a molecular weight about 36 kDa (Fig. 5A). The calculated molecular weight of TbCentrin5 containing 186 amino acids is about 20 kDa. In addition, SDS/PAGE indicated no

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**Fig. 3.** Structure model of TbCentrin5. The model was built by SWISS-MODEL based on homology modeling techniques.
disulfide bond formation of the purified TbCentrin5 proteins, which suggested TbCentrin5 was expressed and purified as a homodimer.

**TbCentrin5 binds to Ca\(^{2+}\) ions with a high affinity**

As an important second messenger, Ca\(^{2+}\) ion is involved in many biological regulation processes [43]. ITC was performed to investigate the Ca\(^{2+}\)-binding property of TbCentrin5 (Fig. 5B). The result showed that TbCentrin5 (pretreated with EDTA) binds to Ca\(^{2+}\) ions with a high affinity \((K_d = 4.8 \mu M)\) in an exothermic mode and one molecule of TbCentrin5 is able to bind two Ca\(^{2+}\) ions \((N = 2)\).

The Ca\(^{2+}\)-binding mode of TbCentrin5 is similar to that of TbCentrin4 [21], HsCentrin2 [20,44], and ScCdc31 [11] where one centrin molecule binds two Ca\(^{2+}\) ions. In other Ca\(^{2+}\)-binding modes, one centrin molecule binds more than two Ca\(^{2+}\) ions [19,22]. Because of the higher binding affinity of CTD of centrins compared with NTD, CTD was preferentially saturated with Ca\(^{2+}\) ions [11,20,23,24]. Therefore, the Ca\(^{2+}\)-binding site of TbCentrin5 should be located in the CTD containing EF-hand III and EF-hand IV. The structure model of TbCentrin5 bound by Ca\(^{2+}\) ions was built using SWISS-MODEL (Fig. 5C). As a Ca\(^{2+}\)-binding motif, EF-hand contains the specific amino acid sequences

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**Fig. 4.** The model of TbCentrin5 was evaluated by Ramachandran plots.
**Fig. 5.** The Ca\(^{2+}\) binding of TbCentrin5. (A) Gel filtration of Superdex G-75 column and SDS/PAGE analysis of TbCentrin5. (B) Saturated titration of TbCentrin5 with Ca\(^{2+}\) ions was measured by ITC. (C) The model of TbCentrin5 bound by Ca\(^{2+}\) ions. Ca\(^{2+}\)-binding site at the loop of EF-hand III (D) and EF-hand IV (E). Cyan-colored sticks represent the residues which interact with Ca\(^{2+}\) ions, red represents oxygen atom, blue represents nitrogen atom. Magenta lines represent coordinated bonds.

**Table 1.** The sequence preference of the loop in EF-hand. bb, backbone; sc, side chain.

| EF-hand loop position | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 |
|----------------------|----|----|----|----|----|----|----|----|----|----|----|----|
| Coordinated ligand   | X  | Y  | SC | SC | Z  | -Y | -X | -Z |
| Most common          | Asp100% | Lys29% | Asp100% | Gly56% | Gly56% | Thr23% | Ile68% | Asp76% | Phe23% | Gly56% | Gly96% |
| Other frequently observed | Ala | Asn | Lys | Ser | Phe | Val | Ser | Tyr | Asp | Lys | Ala | Val |
| Glu | Arg | Asn | Lys | Leu | Thr | Ala | Lys | Ala | Glu | Thr | Ala |
| Thr | Val | Ile | Ser | Glu | Arg | Glu | Tyr | Arg | Glu | Arg | Glu |

*The ligand typically provided by a water molecule that is hydrogen-bonded to the side chain of the residues at position 9.*
that provide carboxyl oxygen atoms for coordinating Ca\(^{2+}\) ions. The residues (Asp117, Tyr123, Asp128) in the loop of EF-hand III may provide five coordinated bonds for Ca\(^{2+}\) ions binding (Fig. 5D). The conformation of EF-hands may change due to the binding of Ca\(^{2+}\) ions to EF-hands, resulting in the change of the direction of Lys121, which may also provide a coordinated bond for Ca\(^{2+}\) ions binding. The loop of EF-hand IV in TbCentrin5 is longer than its counterparts in other centrins (Fig. 2), resulting in the change of the location of the residues that provide coordinated bonds for Ca\(^{2+}\) ions binding. The residues (Pro154, Asp162, Thr170, Glu175) in the loop of EF-hand IV may provide six coordinated bonds for Ca\(^{2+}\) ions binding (Fig. 5E). The residues in the EF-hand III and EF-hand IV have the ability to provide sufficient coordinated bonds for Ca\(^{2+}\) ions binding, indicating TbCentrin5 has a high Ca\(^{2+}\)-binding affinity and forms a stable complex with Ca\(^{2+}\) ions.
TbCentrin5 interacts with other centrins depending on Ca\(^{2+}\) ions binding

In *T. brucei*, five centrin isoforms have been identified [27,28]. We have previously reported that TbCentrin4 interacts with TbCentrin1, TbCentrin2, and TbCentrin5 [21]. To investigate the interactions between TbCentrin5 and other TbCentrins and further enrich the interaction network of centrins from *T. brucei*, GST-pull down assay and ITC were performed.

GST-fused TbCentrin1-4 and TbCentrin5-HA were expressed and used in GST-pull down assay (Fig. 6A). The results showed that TbCentrin5 interacts with TbCentrin1, TbCentrin3, and TbCentrin4 but not TbCentrin2.

To further investigate the binding affinity of TbCentrin5 with TbCentrin1, TbCentrin3, and TbCentrin4, ITC analysis of TbCentrin1-4 titrated with TbCentrin5 was carried out (Fig. 6B). The results verified the interactions between TbCentrin5 and TbCentrin1, 3, 4. The dissociation constants (\(K_{d}\) values) of TbCentrin5 binding to TbCentrin1, TbCentrin3, and TbCentrin4 are 105, 52, and 12 \(\mu\)M, respectively.

In the above experiments, EGTA was not added to remove the remaining Ca\(^{2+}\) ions in TbCentrins. In the protein expression in LB culture medium and purification procedures, Ca\(^{2+}\) ions may be copurified with TbCentrins. Therefore, it is necessary to ensure complete removal of Ca\(^{2+}\) ions by treatment of EGTA to investigate the effect of Ca\(^{2+}\) ions on the interactions between TbCentrin5 and other TbCentrins. ITC analysis of TbCentrin1, 2, 3, 4 titrated with TbCentrin5 was then performed without Ca\(^{2+}\) ions. In the absence of Ca\(^{2+}\) ions, TbCentrin5 is not able to interact with TbCentrin1, 2, 3, 4 (Fig. 6C). The results indicated that TbCentrin5 interacts with TbCentrin1, 3, 4 depending on Ca\(^{2+}\) ions binding. Binding to Ca\(^{2+}\) ions might induce the local conformational change of TbCentrin5, which results in the exposure of more hydrophobic region of TbCentrin5 to interact with other TbCentrins. Owing to these interactions, TbCentrin5 is able to form different complexes with other TbCentrins depending on cellular Ca\(^{2+}\) ions to participate in the relevant biological processes.

Depletion of TbCentrin5 does not compromise the cell motility

To investigate whether depletion of TbCentrin5 impacts cell growth, RNAi targeting on TbCentrin5 was performed in procyclic 29-13 cell line. Quantitative reverse transcription-PCR (RT-PCR) monitored that the mRNA level of TbCentrin5 in the RNAi cells decreased by \(~80\%\) compared with that in the noninduced control cells after tetracycline induction for 2 days (Fig. 7A). The result demonstrated that depletion of TbCentrin5 does not inhibit the cell growth significantly (Fig. 7A).

TbCentrin5 has the same flagellum localization as TbCentrin3, and the effect of TbCentrin5 RNAi on cell growth is also similar to that of TbCentrin3 RNAi [29]. It was reported that knockdown of TbCentrin3 compromised the cell motility [29]. Therefore, the impact of knockdown of TbCentrin5 on the cell motility was also investigated. Sedimentation assay of cells after TbCentrin5 RNAi was performed. Monitored OD\(_{600}\) values among wild-type 29-13 cell line, the noninduced control cells, and TbCentrin5 RNAi cells showed no significant distinction (Fig. 7B). Under light microscopy, cells after the deficiency of TbCentrin5 did not display any unusual phenotype such as spinning and tumbling or losing motility compared with the noninduced control cells. The results indicated that...
the depletion of TbCentrin5 does not compromise the cell motility.

Conclusion
In conclusion, we identified that TbCentrin5 is localized at the cytosol and nucleus and enriched in the flagellum. We further identified that TbCentrin5 binds Ca$^{2+}$ ions with a high affinity and built the model of TbCentrin5 bound by Ca$^{2+}$ ions. Besides, we demonstrated that TbCentrin5 interacts with TbCentrin1, TbCentrin3, and TbCentrin4 depending on Ca$^{2+}$ ions binding, suggesting TbCentrin5 might be able to form different complexes with other TbCentrins depending on cellular Ca$^{2+}$ ions to participate in the relevant biological processes. Our study will provide a basic information for better understanding the biological functions of TbCentrin5.

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Conflict of interest
The authors declare no conflicts of interest.

Author contributions
FS and XT designed the research. FS, XY, YD, and HM performed the experiments. FS, XY, and XT analyzed the data. FS and XT wrote the paper. XY, YD, and HM discussed and gave suggestions on the manuscript.

References
1 Salisbury JL, Baron A, Surek B and Melkonian M (1984) Striated flagellar roots: isolation and partial characterization of a calcium-modulated contractile organelle. J Cell Biol 99, 962–970.
2 Errabolu R, Sanders MA and Salisbury JL (1994) Cloning of a cDNA encoding human centrin, an EF-hand protein of centrosomes and mitotic spindle poles. J Cell Sci 107, 9–16.
3 Lee VD and Huang B (1993) Molecular cloning and centrosomal localization of human caltractin. Proc Natl Acad Sci USA 90, 11039–11043.
4 Baum P, Furlong C and Byers B (1986) Yeast gene required for spindle pole body duplication: homology of its product with Ca$^{2+}$-binding proteins. Proc Natl Acad Sci USA 83, 5512–5516.
5 Wolfrum U (1992) Cytoskeletal elements in arthropod sensilla and mammalian photoreceptors. Biol Cell 76, 373–381.
6 Ivanovska I and Rose MD (2001) Fine structure analysis of the yeast centrin, Cdc31p, identifies residues specific for cell morphology and spindle pole body duplication. Genetics 157, 503–518.
7 Araki M, Masutani C, Takemura M, Uchida A, Sugasawa K, Kondoh J, Ohkuma Y and Hanaoka F (2001) Centrosome protein centrin 2/caltractin 1 is part of the xeroderma pigmentosum group C complex that initiates global genome nucleotide excision repair. J Biol Chem 276, 18665–18672.
8 Fischer T, Rodríguez-Navarro S, Pereira G, Rácz A, Schiebel E and Hurt E (2004) Yeast centrin Cdc31 is linked to the nuclear mRNA export machinery. Nat Cell Biol 6, 840–848.
9 Chen L and Madura K (2008) Centrin/Cdc31 is a novel regulator of protein degradation. Mol Cell Biol 28, 1829–1840.
10 Huang B, Mengersen A and Lee VD (1988) Molecular cloning of cDNA for caltractin, a basal body-associated Ca$^{2+}$-binding protein: homology in its protein sequence with calmodulin and the yeast CDC31 gene product. J Cell Biol 107, 133–140.
11 Li S, Sandercock AM, Conduit P, Robinson CV, Williams RL and Kilmartin JV (2006) Structural role of Sfi1p-centrin filaments in budding yeast spindle pole body duplication. J Cell Biol 173, 867–877.
12 Salisbury JL (2004) Centrosomes: Sfi1p and centrin unravel a structural riddle. Curr Biol 14, R27–R29.
13 Salisbury JL, Suino KM, Busby R and Springett M (2002) Centrin-2 is required for centriole duplication in mammalian cells. Curr Biol 12, 1287–1292.
14 Laoukili J, Perret E, Middendorp S, Houcine O, Guennou C, Marano F, Bornens M and Tournier F (2000) Differential expression and cellular distribution of centrin isoforms during human ciliated cell differentiation in vitro. J Cell Sci 113(Pt 8), 1355–1364.
15 Resendes KK, Rasala BA and Forbes DJ (2008) Centrin 2 localizes to the vertebrate nuclear pore and plays a role in mRNA and protein export. Mol Cell Biol 28, 1755–1769.
16 Middendorp S, Paolletti A, Schiebel E and Bornens M (1997) Identification of a new mammalian centrin gene, more closely related to Saccharomyces cerevisiae CDC31 gene. Proc Natl Acad Sci USA 94, 9141–9146.
17 Middendorp S, Küntziger T, Abraham Y, Holmes S, Bordes N, Paintrand M, Paolletti A and Bornens M (2000) A role for centrin 3 in centrosome reproduction. J Cell Biol 148, 405–416.
18 Popescu A, Miron S, Blouquit Y, Duchamblon P, Christova P and Craescu CT (2003) Xeroderma pigmentosum group C protein possesses a high affinity
binding site to human centrin 2 and calmodulin. J Biol Chem 278, 40252–40261.

Kim SY, Kim DS, Hong JE and Park JH (2017) Crystal Structure of Wild-Type Centrin 1 from Mus musculus Occupied by Ca2+. Biochemistry (Mosc) 82, 1129–1139.

Thompson JR, Ryan ZC, Salisbury JL and Kumar R (2006) The structure of the human centrin 2-xeroderma pigmentosum group C protein complex. J Biol Chem 281, 18746–18752.

Shan F, Ye K, Zhang J, Liao S, Zhang X, Xu C and Tu X (2018) Solution structure of TbCentrin4 from Trypanosoma brucei and its interactions with Ca2+ and other centrins. Biochem J 475, 3763–3778.

Sosa Ldel V, Alfaro E, Santiago J, Narváez D, Rosado MC, Rodríguez A, Gómez Á, Schreiter ER and Pastrana-Ríos B (2011) The structure, molecular dynamics, and energetics of centrin-melittin complex. Proteins 79, 3132–3143.

Vonderfecht T, Stemml-Wolf AJ, Hendersott M, Giddings TH Jr, Meehl JB and Winey M (2011) The two domains of centrin have distinct basal body functions in Tetrahymena. Mol Cell 22, 2221–2234.

Radu L, Durussel I, Assaïr L, Blouquit Y, Miron S, Cox JA and Craescu CT (2010) Scherffelia dobia centrin exhibits a specific mechanism for Ca2+ -controlled target binding. Biochemistry 49, 4383–4394.

Duan L, Zhao Y-Q, Wang Z-J, Li G-T, Liang A-H and Yang B-S (2008) Lutetium(III)-dependent self-assembly binding site to human centrin 2 and calmodulin. J Biol Chem 278, 40252–40261.

Kim SY, Kim DS, Hong JE and Park JH (2017) Crystal Structure of Wild-Type Centrin 1 from Mus musculus Occupied by Ca2+. Biochemistry (Mosc) 82, 1129–1139.

Thompson JR, Ryan ZC, Salisbury JL and Kumar R (2006) The structure of the human centrin 2-xeroderma pigmentosum group C protein complex. J Biol Chem 281, 18746–18752.

Shan F, Ye K, Zhang J, Liao S, Zhang X, Xu C and Tu X (2018) Solution structure of TbCentrin4 from Trypanosoma brucei and its interactions with Ca2+ and other centrins. Biochem J 475, 3763–3778.

Sosa Ldel V, Alfaro E, Santiago J, Narváez D, Rosado MC, Rodríguez A, Gómez Á, Schreiter ER and Pastrana-Ríos B (2011) The structure, molecular dynamics, and energetics of centrin-melittin complex. Proteins 79, 3132–3143.

Vonderfecht T, Stemml-Wolf AJ, Hendersott M, Giddings TH Jr, Meehl JB and Winey M (2011) The two domains of centrin have distinct basal body functions in Tetrahymena. Mol Cell 22, 2221–2234.

Radu L, Durussel I, Assaïr L, Blouquit Y, Miron S, Cox JA and Craescu CT (2010) Scherffelia dobia centrin exhibits a specific mechanism for Ca2+ -controlled target binding. Biochemistry 49, 4383–4394.

Duan L, Zhao Y-Q, Wang Z-J, Li G-T, Liang A-H and Yang B-S (2008) Lutetium(III)-dependent self-assembly study of ciliate Euplotes octocarinatus centrin. J Inorg Biochem 102, 268–277.

Tourbez M, Firanescu C, Yang A, Unipan L, Duchampon P, Blouquit Y and Craescu CT (2004) Calcium-dependent self-assembly of human centrin 2. J Biol Chem 279, 47672–47680.

He CY, Pypaert M and Warren G (2005) Golgi duplication in Trypanosoma brucei requires Centrin2. Science 310, 1196–1198.

Berriman M, Ghedin E, Hertz-Fowler C, Blandin G, Renaud H, Bartholomeu DC, Lennard NJ, Caler E, Hamlin NE, Haas B, et al. (2005) The genome of the African trypanosome Trypanosoma brucei. Science 309, 416–422.

Wei Y, Hu H, Lun Z-R and Li Z (2014) Centrin3 in trypanosomes maintains the stability of a flagellar inner-arm dynein for cell motility. Nat Commun 5, 4060.

Shi J, Franklin JB, Yelink J, Ebersberger I, Warren G and He CY (2008) Centrin4 coordinates cell and nuclear division in T. brucei. J Cell Sci 121, 3062–3070.

Selvapandian A, Kumar P, Morris JC, Salisbury JL, Wang CC and Nakhasi HL (2007) Centrin1 is required for organelle segregation and cytokinesis in Trypanosoma brucei. Mol Biol Cell 18, 3290–3301.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGahtigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, et al. (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947–2948.

Kumar S, Stecher G and Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol 33, 1870–1874.

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

Schimanski B, Nguyen TN and Gunzl A (2005) Highly efficient tandem affinity purification of trypanosome protein complexes based on a novel epitope combination. Eukaryot Cell 4, 1942–1950.

Waterhouse A, Bertoni M, Bienert S, Studer G, Tauriel G, Guimieny R, Heer FT, de Beer TAP, Rempfer C, Bordoli L, et al. (2018) SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic Acids Res 46, W296–w303.

Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Schmidt T, Kiefer F, Gallo Cassarino T, Bertoni M, Bordoli L, et al. (2014) SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. Nucleic Acids Res 42, W252–258.

Bertoni M, Kiefer F, Biasini M, Bordoli L and Schwede T (2017) Modeling protein quaternary structure of homo- and hetero-oligomers beyond binary interactions by homology. Sci Rep 7, 10480.

Gouet P, Courcelle E, Stuart DI and Métoz F (1999) ESPriet: analysis of multiple sequence alignments in PostScript. Bioinformatics (Oxford, England) 15, 305–308.

Westbrook J, Feng Z, Chen L, Yang H and Berman HM (2003) The Protein Data Bank and structural genomics. Nucleic Acids Res 31, 489–491.

Arnold K, Bordoli L, Kopp J and Schwede T (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22, 195–201.

Bordoli L, Kiefer F, Arnold K, Benkert P, Battey J and Schwede T (2009) Protein structure homology modelling using SWISS-MODEL workspace. Nat Protoc 4, 1–13.

Sanz JM, Grecu D and Assaïr L (2016) Ca2+ signaling and target binding regulations: calmodulin and centrin in vitro and in vivo. Bioenergetics: Open Access, 05, 1000144.

Charbonnier JB, Renaud E, Miron S, Le Du MH, Blouquit Y, Duchampon P, Christova P, Shosheva A, Rose T, Angulo JF, et al. (2007) Structural, thermodynamic, and cellular characterization of human centrin 2 interaction with xeroderma pigmentosum group C protein. J Mol Biol 373, 1032–1046.

Gifford JL, Walsh MP and Vogel HJ (2007) Structures and metal-ion-binding properties of the Ca2+-binding helix-loop-helix EF-hand motifs. Biochem J 405, 199–221.