27468

TBCCD1, A NEW CENTROSOMAL PROTEIN, IS REQUIRED FOR CENTROSOME AND GOLGI APPARATUS POSITIONING

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ABSTRACT

In animal cells the centrosome is actively positioned at the cell center in close association with the nucleus. The mechanisms responsible for this are not completely understood. Here we report the first characterization of human TBCCD1, a protein related to tubulin cofactor C. TBCCD1 localizes at the centrosome and at the spindle midzone, midbody and basal bodies of primary and motile cilia. Knockdown of TBCCD1 in RPE-1 cells caused the dissociation of the centrosome from the nucleus and disorganization of the Golgi apparatus. TBCCD1 depleted cells are larger, less efficient in primary cilia assembly and their migration is slower in wound-healing assays. However, the microtubule nucleating activity of the centrosome is not affected by TBCCD1 silencing. We propose that TBCCD1 is a key regulator of centrosome positioning and consequently of internal cell organization.

INTRODUCTION

The centrosome, the major microtubule (MT) organizing center in animal cells is a key organelle consisting of a pair of centrioles surrounded by the pericentriolar matrix (PCM). By nucleating and organizing the spatial distribution of MTs in interphase, the centrosome has been implicated in organelle positioning (*e.g.* Golgi apparatus, GA), cell migration, adhesion and polarity, while in mitosis it assists spindle pole formation (for review Luders and Stearns, 2007). The ability to nucleate the ciliary axoneme, becoming a basal body, constitutes another main centrosomal role. Usually, the centrosome is maintained at the cell center closely associated with the nucleus. This has been shown to be essential, for example, in the early development of *Caenorhabditis elegans* (Malone et al, 2003). The centrosome is repositioned to more peripheral sites during cell-state transitions such as wound healing, cell migration, and cell growth/differentiation (Yvon et al, 2002; de Anda et al, 2005).

Geometrical constraints imposed by the substratum play a crucial role in centrosome positioning and the cell's internal organization (Pouthas et al, 2008) but probably do not provide the sole answer to this question. MTs and forces exerted on them by actomyosin and dynein are also critical (Wittmann and Waterman-Storer, 2001; Burakov et al, 2003). A variety of data supports the existence of a physical link between the centrosome and the nuclear envelope being proteins such as Zyg-12 and Emerin implicated in it (Malone et al, 2003; Salpingidou et al, 2007). This interaction seems to be regulated by the p160ROCK Rho-associated kinase and the coordinate activity of Polo/Greatwall (Gwl) mitotic kinases (Chevrier et al, 2002; Archambault et al., 2007).

A dynamic nucleus-centrosome connection seems also to be important for directed cell migration, *e.g* in neurons (Higginbotham and Gleeson, 2007). Also, several studies reported centrosome reorientation towards the leading edge in migrating cells (Yvon et al, 2002). Nevertheless, this is not true for all cell types and is also substratum dependent leaving the relevance of this reorientation for cell migration still as a matter of debate (Yvon et al, 2002).

In recent years, tubulin cofactors (TBCA-E) which participate in the tubulin folding pathway, have emerged as proteins playing crucial roles, not always directly associated to their expected role in the pathway, but still related with the cytoskeleton. For example, TBCD is a centrosomal protein required for γ-TURC recruitment and mitotic spindle organization (Cunningham and Kahn, 2008). These data and the fact that specific TBC domains were identified in functionally related proteins, RP2 and E-like (Bartolini et al, 2002, 2005), raised the hypothesis that these could have evolved to deal with specific requirements of MT assembly and dynamics in specific cell types. This prompted us to search the human genome database for other proteins with TBC specific domains. During this process we found TBCCD1 (TBCC-domain containg 1), which is conserved through the phylogenetic tree (Stephan et al, 2007). TBCCD1 is related to TBCC which together with TBCD, acts as β-tubulin GTPase activating proteins (GAP) (Fontalba et al, 1993; Tian et al, 1999). TBCCD1 is also related to RP2, which functionally overlaps with TBCC (Bartolini et al, 2002) seeming to participate in tubulin quality control at the basal body of the *Trypanosoma* flagellum (Stephan et al, 2007).

Here we report the first characterization of human TBCCD1 and show that it is required for centrosome and GA perinuclear positioning in RPE-1 cells. We also show that

TBCCD1 silenced cells are less efficient in primary cilia assembly and affected in cell migration.

RESULTS AND DISCUSSION

TBCCD1 localizes at the centrosome and basal bodies

In addition to the TBCC domain, TBCC, RP2 and TBCCD1 possess a CARP domain, a feature of some actin interacting proteins (Fig S1A, B), being their aminoacid sequence identity low outside these domains**.**

By a yeast complementation assay we observed TBCCD1 to be unable to complement the yeast TBCC (CIN2) deletion (Fig S1C). This suggests that, unlike RP2, TBCCD1 does not functionally overlap with TBCC. This can be due to TBCCD1 lacking the arginine conserved in TBCC and RP2 that is crucial for their GAP activity towards tubulin (Veltel et al, 2008).

To study the sub-cellular localization of TBCCD1, a specific mouse polyclonal anti-TBCCD1 serum was raised (Fig S2A, B) and used to perform immunofluorescence analysis in HEK 293T, HeLa and RPE-1 cells. With this analysis we observed TBCCD1 to be localized at the cytoplasm and at the centrosome throughout the cell cycle (Fig 1A, B). Furthermore, using HeLa and RPE-1 cells expressing centrin-GFP we showed that TBCCD1 localizes at the PCM (Fig 1B, Fig S6A). TBCCD1 was also observed in the basal body of primary cilia (Fig 1A, Fig S4). Immunolocalization in mouse brain primary cultures showed TBCCD1 also in the basal bodies of motor cilia (Fig S3A). The localization data were supported by the expression of fluorescently tagged TBCCD1 (Fig

S4). Tagged-TBCCD1 accumulated at the centrosome during the cell cycle and also at the spindle midzone and the midbody. However, using the anti-serum, TBCCD1 was barely detected at the latter two sites, unless when HEK 293T cells were transfected with untagged TBCCD1 (Fig S2C) indicating that the endogenous TBCCD1 levels are too low to be detected or those localizations result from over-expression.

We also observed that TBCCD1 centrosomal localization is not affected by MT depolymerization with nocodazole (NC) (Fig S5A) indicating it to be an integral centrosome component. Also, its N-terminal domain (1-328 aa) is involved in centrosome targeting (Fig S5B). TBCCD1 was detected, with δ-tubulin, mainly in the nuclear fraction of RPE-1 cells (Fig 1C). Since centrosomes are known to fractionate with nuclei this observation also supports TBCCD1 centrosomal localization. Finally, RT-PCR analysis showed that *tbccd1* is transcribed in all mouse tissues tested (Fig S3B), whereas the protein was effectively detected in the brain and the testis, the two tissues where tubulin was more abundant. These results suggest that TBCCD1 may be post-transcriptionally regulated in the different tissues.

TBCCD1 silencing affects centrosome and Golgi positioning

To study the function of TBCCD1, the gene was silenced with a siRNA pool in RPE-1 cells. TBCCD1 silencing was confirmed by RT-PCR, western blot and immunolocalization analysis which showed decreased fluorescence intensity at the centrosome and the cytoplasm (Fig 2A, Fig S6A). 72h post-transfection the cells treated with the siRNA pool did not get confluent (Fig S7A). Flow cytometry analysis showed that at 48h post-transfection TBCCD1 silenced cells were larger than control cells (Fig

2Da), presented a 14% increase in the G1 peak and a corresponding decrease in the number of cells in S and G2/M phases (Fig 2Db and S7B) suggesting a cell cycle delay in G1. Using an antibody against γ-tubulin to visualize the centrosome, we observed that TBCCD1 depletion severely affected centrosome localization. Contrary to control cells where the centrosome is located in the cell center closely associated with the nucleus, in TBCCD1 depleted cells the centrosome was located away from it, often at the cell periphery (Fig 2Ba). Only 3.3±2.7% of the control population presented the centrosome more than 2 μm away from the nucleus, whereas in siRNA treated cells this value rose to 63.5±5.1 % (Fig 2Ca and b, Fig S6C).

The impact of centrosome displacement in the MT cytoskeleton was analysed by immunofluorescence showing that in TBCCD1 silenced cells MTs were still focused on the centrosome (Fig 2Bb). Next, we analysed MT recovery after depolymerisation with NC in TBCCD1 silenced cells. Similarly to control cells, the MT aster was already visible 5 minutes after NC washout (Fig S8) and MTs continued to grow from the centrosome. This showed that misplaced centrosomes were still able to nucleate MTs and that TBCCD1 is either not necessary for this centrosome function or its remaining levels are sufficient for it.

The ability of displaced centrosomes to assemble primary cilia was also tested. Since confluent cells are more efficient in primary cilia assembly, more cells were seeded before transfection to obtain a dense monolayer of siRNA treated cells. The cells were re-transfected 48h later and serum starved. TBCCD1 depleted cells, in similar density conditions as control cells, were able to assemble primary cilia even when centrosomes were far from the nucleus but the percentage of cells presenting cilia was lower

(30±5.9%) than in control cells (87±4.6%) (Fig 3A, Fig S6D). Thus, TBCCD1 depletion affects the effectiveness of RPE-1 cells to assemble primary cilia.

The MT network is responsible for the cell's internal organization being crucial for organizing and positioning the GA close to the nucleus (Rios and Bornens, 2003). As expected, using an antibody against the centrosome marker IFT88 and GA marker golgin 97 we observed that in control cells the GA has a perinuclear position and is organised around the centrosome (Fig 3B, S6E). However, in TBCCD1 silenced cells the GA was disorganized and appeared to follow the centrosome or be fragmented and spread in the cytoplasm (Fig 3B, S6E). A similar GA phenotype was observed during *Toxoplasma* host invasion, where the centrosome is displaced from its perinuclear position (Coppens et al, 2006).

MTs are important for GA biogenesis since its precursors are carried by motor proteins towards MT minus ends anchored at the centrosome (Rios and Bornens, 2003). Thus, the observed GA phenotype is probably explained by the displacement of the centrosome and the MT aster. With time this would lead to a displacement of the GA to the new centrosomal position. However, the centrosome might not adopt a fixed position after being uncoupled from the nucleus. To test this hypothesis we analysed non confluent RPE-1 cells expressing centrin-GFP by live imaging, in control and RNAi conditions. Control cells are highly motile and during their movement the centrosome is in close association with the nucleus without adopting a preferential localization in relation to it (Fig S9 and movie S1). In contrast, TBCCD1 depleted cells migrate slower and the centrosome tends to lag behind the nucleus coming close to it as the cell progresses and the tail retracts (Fig S9 and movie S2). In RNAi cells, although there is

not an increase in centrosome movement, as observed in p160ROCK silencing (Chevrier et al, 2002), its position relative to the nucleus changes over time, which could result in GA disorganization. However, a direct involvement of TBCCD1 in GA organization cannot be excluded although no apparent TBCCD1 localization at the GA was observed (Fig S6E). Moreover, the GA disorganization could be implicated in the lower efficiency of these cells to assemble primary cilia as GA proteins have been implicated in developmental problems due to defective cilium assembly (for review Barr, 2009).

It is well documented that centrosome and GA positioning is important for cell polarization and directed cell migration (Vinogradova et al., 2009). Given the phenotypes described above, we tested further if TBCCD1 depletion caused defects in directed cell migration by performing a wound healing assay. Thus, control and RNAi cell monolayers, obtained as described for cilia assembly, were wounded to trigger directional migration of cells toward the wound. By performing live imaging of wound closing we observed that RNAi cells were able to close the wound but were delayed compared with the controls (Fig 4A, movie S3 and S4), which is in accordance with the previous observations. The centrosome position relative to the nucleus was also analysed showing that in control cells there was not a preferential orientation of the centrosome (Fig 4B and C). However, in the RNAi population 48.1±1.7% of the cells presented the centrosome behind the nucleus (Fig 4B and C) which agrees with the observations made in migrating isolated cells.

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Here we describe a new human PCM component, TBCCD1, and show that its depletion in RPE-1 cells severely affects the centrosome position relative to the nucleus, with profound consequences on GA organization, cell shape and migration.

Many of the proteins that have been implicated in centrosome perinuclear positioning, like Zyg12, Emerin and Samp1 are nuclear envelope proteins (Malone et al, 2003; Salpingidou et al, 2007; Buch et al, 2009) constituting physical links between nucleoskeleton and centrosome/cytoskeleton. TBCCD1 cellular localization suggests that its involvement in centrosome nuclear association will be through a different mechanism. Since TBCCD1 contains a TBCC domain, we hypothesised that it could be a GAP for tubulin. This was not confirmed since it does not complement the yeast CIN2 deletion. However, the possibility that TBCCD1 is a GAP cannot be excluded. Similarly to what is observed for p160ROCK Rho-associated kinase (Chevrier et al, 2002) and the Polo-Gwl mitotic kinases (Archambault et al., 2007), it is conceivable that this TBCCD1 putative regulatory activity may be responsible for the described phenotypes. TBCCD1 also possesses a CARP domain, a characteristic of CAP proteins which regulate actin polymerization. Thus, an attractive hypothesis is that TBCCD1 could promote a cross talk between the centrosome/MT and actin cytoskeletons required for centrosome positioning.

Finally, during the reviewing of this manuscript Feldman and Marshall (2009) reported that *Chlamydomonas reinhardtii* TBCCD1 localises in centrioles and in the region between the two nucleus-centriole connectors (rhizoplasts). A TBCCD1 insertion mutant showed altered number of flagella and affected centriole linkage and positioning which leads to defects in spindle orientation. In spite of the differences between the two models,

both studies show TBCCD1 to be a centrosomal protein important for centrosome–nucleus connection.

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Methods

Cell lines and primary cultures

HEK 293T and HeLa-centrin-GFP cells were grown in DMEM whereas hTERT-RPE-1 and hTERT-RPE-1-centrin-GFP (gift from Khodjakov A) were grown in DMEM/F12 both supplemented with 10% fetal bovine serum. Mixed primary cell cultures were obtained from two-day-old murine dissociated cerebella.

Plasmid construction and transfection

Human *tbccd1* entire or truncated coding sequences were amplified by PCR from testis cDNA and cloned in: mammalian expression vectors (pIC111, pIC112 and pIC113, gifts from Cheesman I); yeast expression vector pRS413 (also used to clone *tbcc*) and bacterial expression vector pGEX-4T-1 (GE Healthcare). The human *tbcc* coding sequence was amplified from a brain cDNA and cloned in pRS413. Transfections with plasmids were done using Lipofectamine 2000 (Invitrogen).

RNA interference

RPE-1 cells (2×10^4 cells seeded in 12 well plates) were transfected with 100 nM of a pool of four siRNAs directed to TBCCD1 obtained from Dharmacon (ON-TARGETplus Duplex) and Ambion (Silencer® Select siRNAs) using Oligofectamine (Invitrogen). As negative controls, Silencer® Select Negative Control 2 siRNA (Ambion) or a nontarget fluorescent control siRNA (siGlo RISC-free siRNA, Dharmacon) were used.

For MT regrowth assays, MTs were depolymerised with NC (30 μM) for 40 minutes, then the cells were washed thoroughly with culture medium and placed in medium at

37ºC to allow MT repolymerisation.

To induce primary cilia assembly, the cells were seeded at a 2.5-fold higher density. 48 h post-transfection, the cells were re-transfected and serum starved (0.25% serum) for 24h. A similar strategy was used in wound healing assays except for serum starvation. 24h after the second transfection the cell monolayer was wounded with a micropipette tip. The cells were either live imaged or processed for immunofluorescence.

RT-PCR and western blot analysis

Total RNA samples from mouse tissues and cell lines were prepared using the RNeasy Mini kit (QIAGEN) followed by cDNA synthesis using Superscript II RT (Invitrogen).

Total protein extracts were prepared using as lysis buffer 50 mM HEPES pH8, 200 mM NaCl, 5 mM EDTA, 0.5% NP-40, with protease inhibitors. Cytoplasmic and nuclear extracts were prepared by lysing the cells with 50 mM HEPES pH8, 10 mM NaCl, 2 mM EDTA, 250 ml sucrose, 0.1% NP-40, with protease inhibitors followed by centrifugation at 3000 x g (10 min at 4ºC). The supernatant was collected as the cytosolic fraction. Nuclei were washed with buffer without NP-40 and ressuspended in 50 mM HEPES pH8, 400mM NaCl, 2 mM EDTA, glycerol 20% (v/v), with protease inhibitors. After incubation on ice (20 min), the samples were centrifuged at 10000 x g (10 min at 4ºC). The supernatant was collected as the nuclear fraction. Equal amounts of nuclear and cytosolic extracts were analyzed.

Antibody generation and immunofluorescence

Human TBCCD1 was expressed as a GST fusion in BL21 Rosetta DE3 cells and purified

from inclusion bodies using a standard protocol. The purified protein was used to immunize Balb/c mice.

For immunofluorescence, the cells were fixed with cold methanol (-20ºC, 10 min), blocked with BSA 3% (20 min), incubated with the primary antibodies (1h), washed and incubated with secondary antibodies (1h). Primary and secondary antibodies are listed in the supplementary data. The cells were analysed in a Deltavision System or in Leica DMRA2 and Zeiss LSM510 confocal microscopes. Images were analysed with ImageJ software.

Flow Cytometry analysis

Control and TBCCD1 silenced cells were collected, fixed with ethanol and stained with propidium iodide. Flow cytometry analysis was performed in a FACSCalibur system.

Legends

Fig 1 – TBCCD1 sub-cellular localization in human cells.

(A) Hek 293T cells were immunostained with antibodies against TBCCD1 and either IFT88 **(a-d)** or poly-glutamylated tubulin **(e)**. DNA was stained with DAPI. **(a)** interphase cell, **(b)** cell in metaphase, **(c)** cell in anaphase, **(d)** cell in telophase, **(e)** cell cycle arrested cell after serum starvation for 24 h. Arrowheads point to TBCCD1 at centrosomes **(a, d)**, constriction zone **(d)**, and basal body **(e)**. (**B)** Immunostaining of HeLa and RPE-1 interphase cells with anti-TBCCD1 showing PCM localization **(a,b). (c)** RPE-1 cell in anaphase**.** Scale bars = 5µm. **(C)** Western blot analysis of nuclear and cytosolic protein fractions (NF and CF) of RPE-1 cells shows that TBCCD1 is mainly present in the nuclear fraction. In A and B the panels are representative of 5 independent experiments, whereas in C are representative of 3.

Fig 2 - TBCCD1 knockdown in RPE-1 cells leads to loss of centrosome/nucleus association and cell cycle delay. (**A**) **(a)** RT-PCR analysis of *tbccd1*, *tbcc* and *rp2* expression in control and TBCCD1 siRNA treated cells. *Hprt* expression was used as an internal control. **(b)** Western blot analysis of TBCCD1 levels in control and TBCCD1 siRNA treated cells. TBCCD1 levels decreased ~65% in TBCCD1 silenced cells. β-tubulin was used as a loading control. **(B)** Control and TBCCD1 silenced cells immunostained with: (**a**) anti-γ-tubulin, (**b**) anti-α-tubulin and anti-IFT88, scale bar = 5 µm. DNA was stained with DAPI. Arrowheads point to centrosomes. (**C**) Nucleus edge-

centrosome measurement approach used with Image J software (**a**). (**b**) Mean percentage of cells (± SD) with a centrosome-nucleus distance >2 μm (n=total number of cells scored in 3 independent experiments). Statistical significance was calculated using the t-test. (**D**) Flow cytometry analyis of cell cycle (**b**) and size (**a**) in control and TBCCD1 depleted cells stained with propidium iodide. All the experiments were done using 72 post-transfection cells except for the flow cytometry analysis that was done at 48h.

Fig 3 – TBCCD1 silencing affects Golgi organization and primary cilia assembly

RPE-1 control and TBCCD1 silenced cells 72h post-transfection, were immunostained with antibodies against poly-glutamylated tubulin (**A**) or IFT88 and golgin97 (**B**). The graphics represent the percentage (±SD) of cells with cilia (**A**) and of cells with disorganized GA (n=total number of cells scored in 4 or 3 independent experiments, respectively) (**B**). Statistical significance was calculated using the t-test. Scale bar = 10 μm.

Fig 4 - TBCCD1 RPE-1 depleted cells are defective in directed cell migration.

(A) Control and TBCCD1 silenced cells were grown to confluence, wounded and imaged for 9h. Frames from 0, 196, 304 and 544 min are presented. Lines were used to limit the wound edges. Scale bar=50 μm. **(B)** Wounded control and RNAi cells were allowed to migrate for 1h and then were immunostained with anti-α-tubulin and anti-IFT88. Arrows point to centrosomes. DNA was stained with DAPI. Scale bar = 20 μm. **(C)** Graphic representation of centrosome positioning relative to the nucleus in cells at the wound

edge (n=total number of cells scored in 3 independent experiments). The differences between control and RNAi were statistically significant except for “over” (p=0.0075).

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