

ORIGINAL RESEARCH ARTICLE

MICROTUBULES REGULATE BRUSH BORDER FORMATION[†]

Running head: Microtubules regulate actin organization at the brush border

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ABSTRACT

Most epithelial cells contain apical membrane structures associated to bundles of actin filaments, which constitute the brush border. Whereas microtubule participation in the maintenance of the brush border identity has been characterized, their contribution to *de novo* microvilli organization remained elusive. Hereby, using a cell model of individual enterocyte polarization, we found that nocodazole induced microtubule depolymerization prevented the *de novo* brush border formation. Microtubule participation in brush border actin organization was confirmed in polarized kidney tubule MDCK cells. We also found that centrosome, but not Golgi derived microtubules, were essential for the initial stages of brush border development. During this process, microtubule plus ends acquired an early asymmetric orientation towards the apical membrane, which clearly differs from their predominant basal orientation in mature epithelia. In addition, overexpression of the microtubule plus ends associated protein CLIP170, which regulate actin nucleation in different cell contexts, facilitated brush border formation. In combination, the present results support the participation of centrosomal microtubule plus ends in the activation of the polarized actin organization associated to brush border formation, unveiling a novel mechanism of microtubule regulation of epithelial polarity. This article is protected by copyright. All rights reserved

Keywords: brush border; epithelial polarity; microtubules; actin; MTOC

INTRODUCTION

Epithelial cells coating hollow organs contain single apical poles rich in cylindrical membrane protrusions (microvilli), which constitute the brush border. The best characterized brush borders are those developed by enterocytes and kidney tubule epithelial cells. Brush borders associated membrane protrusions increase the cell surface available for the digestion and absorption of lumen nutrients in enterocytes, or for the differential absorption of ultrafiltrate components during urine formation in kidney tubule epithelial cells. At the core of these protrusions are parallel bundles of actin filaments, which are connected to the membrane by the ezrin-radixin-moesin proteins (Sauvanet et al., 2015). These actin structures provide the force to generate the membrane protrusion (Nambiar et al., 2010) and constitute tracks for myosin dependent transport (Crawley et al., 2014).

The brush border is also supported by another cytoskeletal network, microtubules, which are intrinsically polarized structures with slow-growing minus ends, usually connected to the microtubules organizing centers (MTOCs), and fast-growing plus ends, associated to plus-end binding proteins (+TIPs) that act as regulators of microtubule dynamic instability. Microtubule participation in the maintenance of epithelial polarity and, more specifically, enterocyte brush border organization has been well documented and attributed to their conventional role as polarized tracks that can direct the traffic of membrane components to the already developed apical membrane (Achler et al., 1989; Gilbert et al., 1991; Müsch, 2004). Furthermore, microtubules have been implicated in two additional events essential for epithelial polarity: the formation of adherent junctions (Stehbens et al., 2006), and the specification of the apico-basal axis induced by cell-extracellular matrix interaction (Akhtar & Streuli, 2013). In contrast to the characterization of microtubule and actin cytoskeletons functional interaction during the development of polarity in other systems (Rodriguez et al., 2003), microtubule participation in the polarized actin nucleation during the *de novo* formation of epithelial brush border has not been addressed so far.

Ls174T-W4 cells are derived from the Ls174T colon epithelial cell line, whose polarization capacity has been restored by overexpression and stabilization of the liver kinase b1 (Lkb1) activity to produce an enterocyte-like phenotype (Baas et al., 2004). Lkb1 is a kinase which signals upstream AMPK and AMPK related kinases, acting in different pathways involved in

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the definition of epithelial cell polarity, and behaving, in some contexts, as a tumor suppressor (Partanen et al., 2013). Upon induction of Lkb1 cytosolic activity, Ls174T-W4 cells promptly reorganize their actin cytoskeleton as a cap at the top or at one side of the cell, independently of cell-cell or cell-extracellular matrix interaction. Those actin accumulations associate with microvilli-structures, as demonstrated by cryo-immuno electron microscopy (Baas et al., 2004). Furthermore, these structures contain typical brush border markers and exclude basolateral markers (Baas et al., 2004; Gloerich et al., 2012). The fact that Ls174T-W4 polarizes as single cells within 6h of induction of Lkb1 activity make them an appealing model for studying the involvement of microtubules in the early stages of brush border development, independently of their participation on epithelial polarity signaling downstream cell-cell or cell-extracellular matrix interactions. In this study, we evaluated the participation of microtubules in *de novo* brush border formation, and characterized the role of the Golgi apparatus and the centrosome as MTOCs in this process. We found that microtubules are essential for actin organization at the brush border in epithelial cells, process which mainly comprises the centrosome as MTOC, and provided evidence that microtubule plus ends associated proteins might be involved in those events.

MATERIALS AND METHODS

Cell Culture

Ls174T-W4 cells were genetically engineered to restore stable LKB1 expression levels and to generate doxycycline-induced STRAD α expression that leads to the cytosolic stabilization and activation of LKB1 (Baas et al., 2004). Ls174T-W4 cell line was generously provided to us by Hans Clevers. The Ls174T-W4 line stably expressing Utrophin Calponin Homology Domain-EGFP as a probe for F-actin was generated in the Tyska laboratory. MDCK cells were obtained from Keith Mostov lab. Cells were grown on plastic dishes in DMEM with 4.5 g of glucose/L, supplemented with 10% fetal bovine serum and antibiotics.

Ice Recovery Assay

Ls174T-W4 cells were seeded 24h before the experiment, incubated on ice 40 min and allowed to recover at room temperature for different periods. For analysis of Golgi and centrosome nucleation of microtubules, cells were recovered for 7 min and immediately

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treated 45 sec with extraction buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl₂, 0.1% Saponin, pH 6.9, supplement with 0.25 nM nocodazole and 0.25 nM paclitaxel). Cells were then fixed and stained as described in Immunofluorescence Confocal Microscopy

Reduction of AKAP350 expression by RNA interference

In order to reduce AKAP350 expression in Ls174T-W4 cells we proceeded as we described (Tonucci et al., 2015). Briefly, constructs were made by annealing and ligating appropriate oligonucleotides containing the AKAP350 mRNA specific sequence (5'-AAATCCCTTGCCAGCACATGA-3') or its scrambled control, into the AgeI and EcoRI cloning sites of pLKO.1-puro vector (details at <http://www.addgene.org>). These were sequenced and used to co-transfect human embryonic kidney 293 FT cells with Virapower lentiviral packaging mix (Invitrogen, Carlsbad, CA, USA). Cells were allowed to produce virus for 24 h. Media containing virus were collected and used to directly transduce LS174T-W4 cells overnight. The cells were allowed to recover for 24 h and subject to puromycin selection (5 µg/ml) for two weeks. Silencing was confirmed by Western blot and immunofluorescence.

Cell Transfection

The plasmids coding for the AKAP350(3643-3908) (AKAP350CTD) and the AKAP350(1-1229) (AKAP350NTD) domains fused to GFP were kindly donated by JR Goldenring (Vanderbilt University). The EB3-cherry construct was prepared as we described (Efimov *et al.*, 2008) and CLIP170-GFP and CLIP170 Δ head-GFP were a gift from A Akhmanova (Komarova *et al.*, 2002). 2×10^6 LS174T-W4 cells were transfected with 4 µg of DNA using Amaxa Nucleofector™ 2b program X-001, as described in the manufacturer protocol.

Immunoblotting

Protein expression by immunoblotting was analyzed as previously described (Larocca et al., 2004). Briefly, cells were washed with cold PBS, scraped and pelleted at 200 g for 5 min at 4°C. Pelleted cells were resuspended in Triton X-100 1% in PBS pH 7.4 with protease and phosphatase inhibitors, and subjected to two freeze–thaw cycles. Lysates were centrifuged at 1000 g for 5 min, and the clear supernatants were conserved. Total protein concentrations

were measured according to Lowry et al. (1951). Solubilized membranes were heated for 10 min at 70°C in sample buffer (20 mM Tris-HCl, pH 8.5, 1% SDS, 400 µM DTT, 10% glycerol). Samples containing equal amounts of protein were subjected to SDS 4% or 12% polyacrylamide gel electrophoresis. The proteins in the gel were transferred to polyvinyl difluoride membranes. Blots were blocked with 5% non-fat milk in PBS with 0.3% Tween-20. Membranes were probed with mouse monoclonal antibodies against AKAP350 (Schmidt et al., 1999) or α -tubulin (1:5000, Sigma-T9026) or with rabbit polyclonal antibodies against calreticulin (1:2000, Sigma-C4606), or phospho-ezrin (1:500, Cell Signalling Technology-mAb3726). The blots were washed and incubated with the corresponding horseradish-peroxidase-conjugated secondary antibodies. Bands were detected by using chemiluminescence reaction (Pierce, Thermo Scientific) after exposure to Kodak XAR film. Bands were quantified using the ImageJ program. In preparing the figures, brightness and contrast were adjusted in order to improve visualization.

Immunofluorescence Confocal Microscopy

The cells grown on glass coverslips were washed with PBS and fixed with 4% paraformaldehyde or 1% glutaraldehyde at room temperature, or in 100% methanol at -20 °C. Fixed cells were permeabilized/blocked with 0.3% Triton X-100/bovine serum albumin 1%/PBS, pH 7.4 for 10 min. Then, they were incubated with antibodies rabbit anti-GM130 (Abcam-EP892Y, 1:300), anti- γ -tubulin (Sigma-T5192, 1:250) or phospho-ezrin (1:500, Cell Signalling Technology-mAb3726) and mouse anti- γ -tubulin (Sigma-T6557, 1:250) or anti- α -tubulin (Sigma-T9026, 1:300). The coverslips were washed, incubated with the secondary fluorescent conjugated antibodies or phalloidin-Alexa 555 (Molecular probes-A34055, 1:200) for actin staining and with 4',6-diamidino-2-phenylindole (DAPI) for nuclear staining and mounted with ProLong. Fluorescence localization was detected by confocal laser microscopy (Nikon C1SiR with inverted microscope Nikon TE200). Serial optical 0.3 µm thick sections were collected in the Z axis. For structural illumination superresolution microscopy a Delta OMX microscope was used and optical 0.1 µm thick sections were collected in the Z axis. Live imaging Microscopy was performed using a Yokogawa QLC-100/CSU-10 spinning disk head, attached to a Nikon TE2000E microscope as described (Efimov et al., 2007).

Annexin V/propidium iodide assay

Apoptotic cells were detected as we have previously described (Ferretti et al, 2016). Briefly, cells were detached from the petri dishes by trypsinization, gently homogenized in the culture medium/PBS, harvested (5 min, 400 g) and carefully re-suspended in the appropriate buffer. Apoptotic externalization of phosphatidylserine and cell death was assessed by staining with Annexin V-FITC and propidium iodide (Sigma Chemical Co.) coupled to flow cytometric analysis (Cell Sorter BD FACSAria II, BD Biosciences), following the manufacturers' instructions. Green and red fluorescence intensities detected in non-stained cells were used to set the thresholds for each channel.

Cell treatments

In order to interfere with microtubules function, Ls174T-W4 cells were treated with nocodazole (17 μ M) for 30 min and then activated with doxycycline (1 μ g/ml) for different periods in the presence of both drugs. To analyse microtubule participation in the organization of the brush border associated actin in MDCK cells, cells were grown on transwells containing 0.4 μ m pore size polycarbonate membrane inserts (Corning Inc.) for 7 days, and cell polarization verified by immunofluorescence. Polarized cells were preincubated with nocodazole (33 μ M) on ice for 1 h to induce loss of unstable microtubules, or in regular media, and then treated with cytochalasin D (20 μ M) for 1 h. Inserts were washed three times with PBS, and then incubated in media containing nocodazole 33 μ M for 2h (Cytochalasin washout + Nocodazole) or in regular media (Cytochalasin washout), respectively. Additional groups of cells were fixed without any treatment (Control) and immediately after cytochalasin D treatment (Cytochalasin). In order to evaluate the role of Golgi derived microtubules on brush border formation, Ls174T-W4 cells were treated with nocodazole, and activated with doxycycline 30 min after the initiation of the treatment. BFA (5 μ g/ml) was added to the media after 4 h of activation to one group of cells. After 30 min of BFA treatment, nocodazole was removed, and cells were incubated in nocodazole free media, in the presence or absence of BFA for 2 additional hours. In order to assess the role of centrosome-microtubules on brush border, the same protocol, in absence of BFA, was applied to Ls174T-W4 cells expressing the AKAP350CTD domain.

Live imaging Microscopy

Confocal stacks were taken by a Yokogawa QLC-100/CSU-10 spinning disk head (Visitec assembled by Vashaw) attached to a Nikon TE2000E microscope using a CFI PLAN APO VC 100× oil lens, NA 1.4, with or without 1.5× intermediate magnification, and a back-illuminated EM-CCD camera Cascade 512B (Photometrics) driven by IPLab software (Scanalytics). A krypton-argon laser (75 mW 488/568; Melles Griot) with AOTF was used for two-color excitation. Custom double dichroic mirror and filters (Chroma) in a filter wheel (Ludl) were used in the emission light path. Z steps (0.2 μm) were driven by a Nikon built-in Z motor. Live cells plated on MatTech glass bottom dishes were maintained at 37°C by heated stage (Warner Instruments) on a Nikon TE2000E inverted microscope equipped with a PerfectFocus automated focusing device. Single-plane confocal video sequences were taken as described for confocal stacks. A similar setup with a Pinkel triple-filter set (Semrock) was used for nearly simultaneous two-color wide-field imaging

Analysis of brush border formation and microtubules, centrosome and Golgi reorientation

In Ls174T-W4 cells, brush borders were identified as structures with high actin staining, which were specifically located at the top or at one side of the cell. Microtubule directionality and Golgi and centrosome orientation were determined in cells whose brush borders were located at one side of the cell. Golgi and centrosome orientation were determined considering them apical if the majority was located in a 120° sector emerging from the centre of the nucleus and facing the brush border. The percentage of cells with Golgi or centrosome polarization was calculated by dividing the number of cells with the organelle oriented towards the brush border by the number of total cells for each condition. Microtubule directionality at the brush border pole was determined by using an ImageJ plugin. In polarized MDCK cells, we determined that the brush border correspond to the top 2 μm of the cells in the actin channel. To analyse actin organization at the brush border in MDCK cells, we quantified the phalloidin staining present at the top 2 μm of the cells in the z-sections of the corresponding channel and related it to the total cell fluorescence measured in the same section. At least 120 randomly selected cells per experiment were analyzed.

Statistical Analysis

Data are expressed as mean \pm s.e.m. Paired Student's t test was used for comparison between groups and non-parametric Mann-Whitney test was used for comparisons within each experiment. $p < 0.05$ was considered statistically significant.

RESULTS

1. Ls174T-W4 polarized cells organize their microtubule cytoskeleton as columnar epithelial cells

We first characterize the microtubule organization in polarized Ls174T-W4 cells. Differentiated epithelial cells have a characteristic vertical organization, with microtubule minus ends located below the apical membrane and their plus ends pointing the basal membrane. These vertical nets are complemented with a subapical mesh of microtubules with random orientation (Müsch, 2004). In order to evaluate microtubule arrangement in Ls174T-W4 polarized cells, cells were activated 6 h with doxycycline, to induce brush border formation, and immunostained for actin and microtubule identification (Figure 1A,B). Images obtained by confocal microscopy indicated the presence of transcellular apico-basal arrays of microtubules (Figure 1A). In order to get more detail on microtubule directionality at the brush border, we analyzed the staining by structural illumination microscopy (Figure 1B). We observed at least two populations of microtubules: those which organized sub apically, with random distribution (arrowheads) and those which were oriented perpendicular to the brush border (arrows). Consistently, our quantitative analysis of microtubule directionality at the brush border pole indicated the presence of a microtubule population with a Gaussian distribution centered at 90° with respect to the tangent to the brush border (Figure 1B). The centrosomes and the Golgi apparatus, are the best characterized MTOCs in animal cells (Bornens, 2012; Sanders & Kaverina, 2015). We examined the positioning of both organelles in Ls174T-W4 polarized cells dual stained for γ -tubulin and GM130, which were used as centrosome and Golgi markers, respectively. We found that most of Ls174T-W4 polarized cells showed apical centrosome and Golgi localization (Figure 1C), what resembles centrosome and Golgi localization in epithelial differentiated cells.

2. Microtubules are essential for de novo brush border formation

We evaluated the impact of nocodazole-induced microtubule disruption on brush border development. Our results showed that Ls174T-W4 cells subjected to nocodazole treatment were unable to form brush borders (Figure 2A). Previous studies demonstrated that ezrin gets phosphorylated at the T567 (pEzrin) and that pEzrin accumulates at the brush border upon induction of Ls174T-W4 cells (Gloerich et al., 2012; ten Klooster et al., 2009). Therefore, we analyzed the effect of microtubule disruption on pEzrin levels and distribution in Ls174T-W4 activated cells. The analysis of pEzrin levels by western blot in control and in nocodazole treated cells confirmed that induction of cell differentiation lead to augmented levels of pEzrin and indicated that this increase was not affected by the presence of Nocodazole (Figure 2B). We further analyzed pEzrin distribution by immunofluorescence confocal microscopy. In accordance to the results shown in Figure 2B, the analysis of pEzrin total levels by quantitative confocal microscopy showed that nocodazole treatment elicited no effect on the total levels of pEzrin. On the other hand, pEzrin polarized distribution was severely affected by nocodazole treatment. These results confirm that microtubules participated in the polarized organization of the brush border in Ls174T-W4 cells (Figure 2B). Nocodazole, as well as other drugs that interfere with microtubule dynamics, can induce apoptotic cell death (Mollinedo and Gajate, 2003). We analyzed apoptosis levels in Ls174T-W4 cells subjected to Nocodazole treatment, in the same conditions used above, and found a subtle but significant increase in apoptotic death (Supplemental Figure 1A). In order to evaluate if the inhibition of brush border development induced by microtubule disruption was related to the induction of apoptosis, we analyzed the effect of a different pro-apoptotic agent on brush border formation. Metformin is a drug which, in the conditions of treatment assessed, induced levels of apoptotic cell death comparable to Nocodazole treatment (Supplemental Figure 1A). Metformin treatment did not induce any evident effect on brush border formation (Supplemental Figure 1B), what indicates that, at the levels elicited by the Nocodazole treatment used in these study, apoptosis itself does not affect this process.

To analyze if our observations regarding the dependence of brush border formation on microtubule integrity were extensive to other epithelial cells, we analyzed the impact of the impairment in microtubule polymerization on apical actin organization in MDCK cells, a very well characterized model of kidney tubule epithelial cells. MDCK cells were grown as

monolayers on filters for 7 days, as described in Materials and methods. In these conditions, as soon as 3 days after cell seeding, MDCK cells develop brush borders associated to rods of actin filaments, which organize perpendicular to the apical surface (Hyman, Shmuel, & Altschuler, 2006). Polarized cells were subjected to microtubule depolymerization by incubation on ice in the presence of nocodazole, and then treated with cytochalasin D in conditions that do not perturb tight junction integrity (Van Itallie et al., 2009). Cytochalasin D was washed, and cells were further incubated in the presence of nocodazole, to evaluate actin recovery in this condition. A similar protocol applied to cells which were not exposed to nocodazole was used to analyze actin reorganization in the presence of intact microtubules. Cells were immunostained to visualize actin and microtubule cytoskeleton. Actin organization at the apical pole was analyzed in the z-sections of the images obtained by confocal microscopy. Before any treatment, cells showed its characteristic apical actin organization (Figure 3, first column). As expected, after cytochalasin treatment, most actin was redistributed to aggregates, with the exception of that associated to cellular junctions (Figure 3, second column). After two hours of recovery in cytochalasin free media, cells with intact microtubules could partially reconstitute their actin cytoskeleton at the brush borders (Figure 3, third column). Nevertheless, in the presence of nocodazole, even though most actin aggregates disappeared, the apical actin did not recover (Figure 3, fourth column). These results confirm that microtubules have a central role in the regulation of actin organization at the brush border in epithelial cells.

3. Centrosomal, but not Golgi derived microtubules, regulate brush border development

We first characterized the ability of the centrosome and the Golgi apparatus to nucleate microtubules in Ls174T-W4 cells, by performing ice recovery assays as we have previously described (Grimaldi et al., 2013). Similarly to what we observed in RPE1 cells, after incubation on ice for 40 min, polymerized microtubules were hardly observed in Ls174T-W4 cells (Supplemental Figure 1A). We further analyzed the appearance of microtubules after different periods of incubation at room temperature, and found that after 7 min incubation individual-newly formed microtubules were already evident (Supplemental Figure 1B). At this time lapse, centrosomal microtubules average length was around 2 μm , and, consequently, could still be differentiated from Golgi derived microtubules. We analyzed the presence of

Golgi and centrosome derived microtubules after 7 min of rewarming, as we described (Grimaldi et al., 2013). Briefly, centrosomal microtubules were recognized as radial arrays of microtubules associated to γ -tubulin spots, while Golgi derived microtubules were identified as asymmetric arrays originated from the GM130 labeled organelle. We identified that both the centrosome (Supplemental Figure 1C) and the Golgi apparatus (Supplemental Figure 1D) nucleated microtubules in Ls174T-W4 cells.

Previous studies indicate that Golgi derived microtubules direct apical actin organization in hepatocytes (Mattaloni et al., 2012). We analyzed the role of Golgi microtubules, and the participation of the Golgi itself, in brush border formation in Ls174T-W4 cells. Microtubule nucleation at the Golgi and at the centrosome requires the presence of γ -tubulin and its associated proteins, which form the γ -tubulin ring complex (γ -TURC). AKAP350 is an A-kinase anchoring protein which participates in the recruitment of the γ -TURC to both organelles (Rivero et al., 2009; Takahashi, 2002). Nevertheless, the effect of AKAP350 depletion on microtubule nucleation at each organelle is cell type dependent (Larocca et al., 2006; Oddoux et al., 2013; Ori-McKenney et al., 2012; Rivero et al., 2009). We first characterized the impact of AKAP350 depletion on microtubule nucleation at the centrosome and at the Golgi apparatus in Ls174T-W4 cells. We generated Ls174T-W4 cells with stable decrease in AKAP350 expression (AKAP350KD), as described in materials and methods. AKAP350 levels in control and AKAP350KD cells were checked by western blot (Figure 4A). Similarly to what was shown in other epithelial derived cells, in Ls174T-W4 cells the decrease in AKAP350 expression did not affect the overall nucleation of microtubules at the centrosome, but severely impaired the formation of microtubules at the Golgi apparatus (Figure 4B). Hence, we analyzed the “de novo” brush border formation in AKAP350KD cells. We found that, after 6h of induction of cell polarization, the percentage of cells that developed brush border was not modified by AKAP350 depletion (Figure 4C). These experiments also showed that the Golgi apparatus did not polarize towards the brush border in AKAP350KD cells, but got fragmented and dispersed (Figure 4C), similarly to what was described in other cells (Larocca et al., 2004; Rivero et al., 2009). On the other hand, the polarized localization of the centrosome was not affected (Figure 4C).

We further analyzed brush border formation in cells with Golgi dysfunction using two additional strategies. 1. We subjected cells to Brefeldin A treatment in conditions that induced Golgi unstacking and fragmentation (Figure 4D), as previously described (Fujiwara, Oda, & Ikehara, 1989). 2. We prepared LS174T-W4 cells with transient expression of AKAP350(1-1029) (AKAP350NTD) domain, which contains a strong Golgi targeting domain and, by acting as a dominant negative AKAP350 mutant, inhibits microtubule nucleation at this organelle (Hurtado et al., 2011). We studied *de novo* brush border formation in both conditions and, in accordance to our observations in AKAP350KD cells, Golgi dysfunction did not affect brush border development in any case (Figure 4D, E).

Thereafter, we evaluated the participation of centrosome-derived microtubules in the development of actin rich brush borders in LS174T-W4 cells. AKAP350 and pericentrin share an evolutionary conserved domain, which mediates their targeting to the centrosome: the pericentrin-AKAP350 centrosomal targeting (PACT) domain (Gillingham & Munro, 2000). Expression of the PACT domain inhibits centrosomal nucleation of microtubules, by displacing pericentrin and AKAP350 from the centrosome (Gillingham & Munro, 2000). We prepared cells with transient expression of the AKAP350 domain comprising the PACT domain (AKAP350CTD), and verified that AKAP350CTD expression inhibited microtubule nucleation at the centrosome, but did not affect microtubule nucleation at the Golgi apparatus (Figure 5A). The analysis of *de novo* brush border formation in these cells indicated that polarized actin organization was significantly decreased in AKAP350CTD cells (-25%, $p < 0.05$). In order to further evaluate the role of newly formed centrosomal microtubules on brush border formation, we treated control and AKAP350CTD cells with nocodazole to depolymerize microtubules during the first 4 h of activation with doxycycline, washed the drug to induce microtubule polymerization and evaluated brush border formation after two hour of activation in nocodazole free media. The effect of the inhibition of centrosomal nucleation of microtubules on polarized actin nucleation in that condition was dramatic (Figure 5B), comparable to the effect elicited by nocodazole induced microtubule disruption (Figure 2A). Therefore, the perturbation of centrosomal nucleation of microtubules led to a significant defect in brush border formation, what make a clear contrast with the insensitivity of Ls174T-W4 cell polarization to the impairment of the Golgi nucleation of microtubules (Figure 4).

4. Microtubule plus ends have an early apical orientation and condition apical actin organization

In order to get mechanistic information, we studied microtubule dynamics during brush border formation. We performed live imaging experiments in Ls174T-W4 cells stably expressing the actin marker Utrophin-GFP and transiently expressing the plus end marker EB3-Cherry. Our results showed that, during Ls174T-W4 cell polarization, there was a clear plus end microtubule propagation towards the apical pole, which was synchronized with polarized actin organization (Figure 6A and Movie 1). Quantification of the fluorescence associated to EB3 in the brush border pole showed a clear increase during the first 120 min of cell induction to polarize, which contrasted with the decrease of EB3 levels in the other sections of the cells (Figure 6B). We hypothesized that the presence of microtubule plus ends at the cell cortex could provide a signaling platform to regulate the initial events in brush border development, as it occurs in other models of cell polarity (Rodriguez et al., 2003). The +TIP “cytoplasmic linker protein” (CLIP) 170, one of the best characterized plus ends associated proteins, can regulate actin nucleation in “in vitro” systems (Henty-Ridilla et al., 2016). We prepared Ls174T-W4 cells with transient overexpression of CLIP170 and evaluated the effect of the increase in this protein levels on brush border formation. Our results showed that overexpression of CLIP170 induced a clear increase in brush border formation (Figure 7A). CLIP170 amino terminal domain mediates its interaction with microtubule plus ends, but is dispensable for CLIP170 dependent induction of actin elongation (Henty-Ridilla et al., 2016). To further evaluate if microtubules were implicated in CLIP170 induced apical actin organization, we analyzed brush border formation in cells with transient expression of a CLIP170 mutant lacking this amino-terminal domain: CLIP170(348-end) (CLIP170 Δ head). As expected, CLIP170 Δ head shows homogenous cytosolic localization, which clearly differs from CLIP170 “comet-like” distribution (Figure 7, insets). We found that CLIP170 Δ head cells did not increase, but, on the contrary, diminished brush border actin organization (Figure 7B). These results showed that CLIP170 Δ head behaves as a dominant negative mutant of CLIP170 in this particular process, thus indicating that CLIP170 interaction with microtubule plus ends is necessary for its stimulatory effect on brush border actin organization.

DISCUSSION

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The implication of microtubules in the development and maintenance of epithelial polarity have been thoroughly studied from different perspectives. On this regard, microtubules have been implicated in apical traffic of membrane components, which ensures the stability of the apical pole of columnar epithelial cells (Müsch, 2004). Furthermore, microtubule plus ends associated proteins are involved in the positioning of E-cadherin during the formation of adherent junctions (Stehbens et al., 2006), and in the regulation of the apico-basal axis orientation downstream integrin interaction with the extracellular matrix (Akhtar & Streuli, 2013). On the other hand, microtubules functional interaction with the actin cytoskeleton is essential during symmetry breaking events in processes as diverse as integrin mediated phagocytosis (Lewkowicz et al., 2008), directional cell migration (Waterman-Storer & Salmon, 1999), and guided extension of developing axons (Buck & Zheng, 2002). In spite of the relevance of apical actin organization in brush border development and, therefore, in epithelial morphology and function, the role of microtubules in the modulation of apical actin organization has not been addressed so far, most probably due to the lack of proper models. In the present study we analyzed the role of microtubules in the development of actin rich brush borders. Our results demonstrated that: 1. Microtubules are essential for actin nucleation at the brush border; 2. Centrosomes are the main MTOC associated with this process; 3. Microtubule plus ends orient apically early during brush border development, and its associated protein CLIP170 enhances brush border formation.

We first characterized microtubule organization in Ls174T-W4 polarized cells. Previous studies reported that there were no changes in microtubule cytoskeleton organization after Ls174T-W4 cells activation (Baas et al., 2004). Since microtubule reorganization in mature enterocytes might be not as conspicuous as actin remodeling, we performed a quantitative analysis making focus on two issues: 1. Microtubule orientation; 2. MTOCs localization. Our results showed that, in polarized cells, there are transcellular arrays of microtubules. The analysis of individual microtubules orientation showed that there is a population of microtubules which orient perpendicular to the brush border, where another group of microtubules located directly below the brush border exhibit random orientation. Additionally, we found that most of the cells have apical orientation of the Golgi apparatus and the centrosomes. This arrangement of the microtubule cytoskeleton resembles the actual microtubule organization in mature epithelial cells, as opposed to the radial arrays of

microtubules with centrally located centrosome and Golgi apparatus characteristic of non-polarized cells. These results confirm the fitness of LS174T-W4 cells as a polarized epithelial cell model for studies related to cytoskeleton functions.

We demonstrated that nocodazole induced microtubule depolymerization severely impairs brush border development in Ls174T-W4 cells. A previous study showed that microtubule disruption induced by the same drug promotes brush border formation in long term cultures of HT29 cells which, otherwise, do not polarize (Cohen et al., 1999). The differences between this study and our results are most probably due to differences inherent to the cell models. To explore if our findings were a peculiarity of Ls174T-W4 cells, we analyzed the effect of nocodazole treatment on brush border organization using MDCK cells, a very well characterized model of columnar epithelial cell polarity. As noted before, microtubules regulate the maturation of adherent junctions, which is a very early event during the development of epithelial polarity (Stehbens et al., 2006). In order to evaluate microtubule effect on actin nucleation at the brush border independently of that process, we analyzed actin organization at the apical pole on cells which had already established adherent junctions. Our results showed that, in control conditions, MDCK cells subjected to cytochalasin D treatment could partially reconstitute actin organization at the brush border after two hours of incubation in cytochalasin free media. On the contrary, cells whose microtubule integrity was disrupted were not able to recover their typical apical actin organization. Therefore, we can conclude that actin organization at the brush border in epithelial cells depends on microtubule integrity, what we found to be the most outstanding result that emerges from our study.

Hepatocytes are epithelial hepatic cells which, in contrast to most epithelial cells, develop multiple apical poles. Our previous studies demonstrated that Golgi derived microtubules participate in apical (canalicular) actin nucleation in hepatocytes (Mattaloni et al., 2012). Therefore, we expected that the interference with Golgi microtubule nucleation would inhibit brush border formation in Ls174T-W4 cells. Using three different strategies we demonstrated that Golgi derived microtubules were not necessary for the development of the apical pole in LS174T-W4 cells, evidencing a differential impact of Golgi microtubules on the development of both types of epithelial polarity. Moreover, these results further suggest that vesicle trafficking from the Golgi apparatus is not essential for the initial stages of brush border formation. On the other hand, impairment of microtubule nucleation at the centrosome

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severely inhibited brush border formation, thus implicating this particular group of microtubules as main actors in columnar epithelial cell differentiation.

Regarding how microtubules modulate actin organization during brush border development, our results demonstrate that, immediately after induction of cell polarization, there is a remarkable enrichment in microtubule plus ends at the pole where the brush border is to be developed. Microtubule plus ends behave as signaling platforms by recruiting several regulatory proteins. As a matter of fact, microtubule plus ends associated proteins are highly relevant in the specification of cell asymmetry in processes such as cell migration (Etienne-Manneville, 2013), mitotic spindle orientation (Johnston et al., 2013) and neuronal development (van de Willige et al., 2016). A recent study demonstrated that CLIP170 accelerates formin-mediated actin elongation. CLIP170 associates to microtubule (+) ends by interacting with the +TIP EB1 (Dixit et al., 2009). Moreover, EB1 sequentially recruits CLIP170 and the formin mDia to microtubules (+) ends, and this complex can markedly enhance the rate of actin nucleation (Henty-Ridilla et al., 2016). Our results show that CLIP170, but not its microtubule binding deficient mutant CLIP170 Δ head, enhances brush border actin organization, thus implicating microtubule plus end associated CLIP170 in actin organization at the apical pole of epithelial cells during brush border formation.

In conclusion, our studies demonstrate that microtubules act in the early stages of *de novo* brush border organization in epithelial cells. We show that centrosome, but not Golgi-derived microtubules are essential for intestinal Ls174T-W4 cell polarization. In addition, we show that, in these cells, microtubule plus ends have an early apical orientation and that the +TIP CLIP170 stimulates brush border formation. The backbone of the pathways regulating the initial events during the establishment of epithelial polarity is highly conserved in epithelial cells with simple polarity. Hence, it is likely that the participation of centrosomal microtubules and their plus end associated protein CLIP170 in the organization of the apical actin cytoskeleton is a general feature in the development of columnar epithelial polarity.

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FIGURE LEGENDS

Figure 1. Ls174T-W4 polarized cells organize their microtubule cytoskeleton as typical columnar epithelial cells. (A,B) Ls174T-W4 cells stably expressing Utrophin-GFP (UtrGFP) as actin marker (red) were activated with doxycycline for 6h and fixed with glutaraldehyde. Cells were stained with anti α -tubulin antibody for microtubule visualization (green) and analyzed by confocal microscopy (A) or structural illumination microscopy (B). (B) Arrowheads indicate microtubules with random distribution; arrows, microtubules oriented perpendicular to the brush border. Microtubule directionality towards the brush border was analyzed using an ImageJ plugin. The chart shows microtubule distribution according to their direction, corresponding to the cell shown in the image. These are typical results, representative of 10 different cells. (C) Cells were stained with anti-GM130 (blue) and anti- γ -tubulin (γ -tub, red) antibodies for Golgi and centrosome visualization, respectively. Bars show the percentage of cells which contains the Golgi apparatus or the centrosomes oriented towards the brush border and are representative of three independent experiments. At least 80 cells were analyzed in each experiment. Scale bars, 2.5 μ m. * p <0.05.

Figure 2. Microtubules are essential for de novo brush border formation in Ls174T-W4 cells. Cells were treated with nocodazole 17 μ M for 30 min previous to and during activation with doxycycline. (A) Images show actin staining in control cells and in cells pretreated with nocodazole. Bars represent the percentage of control and nocodazole treated cells which developed brush border in the presence or absence of doxycycline, for at least 80 cells counted in three independent experiments. (B) Typical western blot showing pEzrin expression in control and nocodazole treated cells subjected to induction with doxycycline. α -tubulin was used as loading control. Bars show the densitometric analysis of the western blot. (C) The images show pEzrin (green) and actin (red) localization in activated cells subjected or not to nocodazole treatment. Bars represent total cell pEzrin-specific fluorescence intensity for 30 cells. Scale bars, 10 μ m (A) or 5 μ m (C). * p <0.05.

Figure 3. Microtubules play a central role in actin organization at the brush border in MDCK cells. MDCK cells were seeded on filters at confluence and grown for 7 days. One group of cells was fixed to analyze actin localization at the brush border in control conditions.

In order to induce microtubule depolymerization, cells were pretreated with nocodazole 33 μM on ice during 60 min and maintained with nocodazole 33 μM throughout the experiment. Non-treated and nocodazole treated cells were exposed to cytochalasin D 20 μM for 60 min. One group of cytochalasin D treated cells was fixed after this treatment (Cytochalasin) to verify the effect of cytochalasin D treatment on actin integrity at the brush border. The other groups of cells treated with cytochalasin D, in the absence or presence of nocodazole, were washed with PBS and then incubated in cytochalasin free media for 2 h (Cytochalasin washout and Cytochalasin washout + nocodazole, respectively). Cells were fixed and stained with anti α -tubulin, phalloidin, and DAPI, and analyzed by confocal microscopy. The figure shows three-dimensional reconstructions of F-actin (red), nuclear (blue) and microtubule (green) staining representative of each condition (first row) and orthogonal views of merged channels (second row) or actin staining (third row) corresponding to the same fields. Bars represent the F-actin present at the top 2 μm of the cell, which corresponds to the brush border area, expressed as percentage of total F-actin, for at least 120 cells, representative of three independent experiments. Scale bars, 10 μm . * $p < 0.05$.

Figure 4. The impairment in Golgi function does not affect brush border development.

(A) Western blot showing AKAP350 expression in control and AKAP350KD cells. Calreticulin was used as loading control. Molecular weight markers are indicated. (B) Control and AKAP350KD cells were subjected to ice recovery assays, and microtubule nucleation at centrosomes and Golgi apparatus, quantified. Images show centrosome (red, first row) or Golgi (red, second row) derived microtubules (green) 7 min after cell rewarming. The inset images show views of microtubule nucleation sites (boxed areas). (C) Images show the organization of the actin cytoskeleton (green), the Golgi apparatus (blue) and the centrosomes (red) in AKAP350KD activated cells. Bars represent the percentage of cells that developed brush borders and, among these, the percentage of cells that had polarized localization of the centrosomes or Golgi apparatus. (D) Cells were treated as indicated in the schema. As positive control, a group of cells received nocodazole treatment the whole period (Noc). Images show actin (red) and Golgi (green) organization in BFA treated cells. Bars represent the fraction of activated cells which developed brush borders. (E) The RGB image shows brush border development (red) in a cell which expressed AKAP350NTD (green), and in a control cell. The

grey scale image shows the channel corresponding to AKAP350 staining. Bars represent the fraction of activated cells which developed brush border. Data are representative of 40 (B) or 80 (C,D,E) cells. Scale bars, 5 μm . * $p < 0.05$.

Figure 5. Centrosome-derived microtubules are essential for actin organization at the brush border. Cells were transfected with AKAP350CTD fused to GFP (AKAP350CTD), or with the empty plasmid (control). (A) Cells were subjected to ice recovery assays. Images show centrosome (red, first row) or Golgi (red, second row) derived microtubules (green) in control cells and in cells expressing AKAP350CTD (grey, arrowhead). The inset images show views of centrosome or Golgi microtubule nucleation sites (boxed areas). (B) AKAP350CTD and control cells were treated with nocodazole, activated with doxycycline, and brush border formation analyzed 2 h after nocodazole removal. Images show AKAP350CTD-GFP expression (arrowhead, white), actin (red) and microtubule (green) organization. Bars represent the fraction of cells that developed brush borders. Data are representative of 80 cells. Scale bars, 5 μm . * $p < 0.05$.

Figure 6. Microtubule plus ends have an early apical orientation during de novo brush border formation. UtrGFP Ls174T-W4 cells were transiently transfected with EB3-Cherry. Cells were activated with doxycycline, and visualized by live imaging. Frames were obtained every 30 seconds. (A) Images show actin (red) and EB3 (green) distribution. (B) EB3 accumulation in the anterior pole (BB), measured in a 120° angle towards the brush border, or media EB3 accumulation in the other 120° sections of the cell (other) were analyzed using ImageJ tools. Data are representative of three independent experiments. Scale bar, 5 μm .

Figure 7. CLIP170 promotes, whereas CLIP170 Δ head inhibits, actin organization at the brush border. Ls174T-W4 cells were transfected with CLIP170 (A) or CLIP170 Δ head (B) fused to GFP. Cells were activated with doxycycline, and brush border formation analyzed 4 h after activation. Images show F-actin staining (red) and CLIP170 (A) or CLIP170 Δ head (B) (green) expression. The inset images show magnified views of CLIP170 (A) or CLIP170 Δ head (B) expression (boxed areas). Bars represent the fraction of transfected or non-transfected (control) cells which developed brush borders representative of, at least, 80 cells counted per experiment. Stars indicate transfected cells. Scale bars, 20 μm . * $p < 0.05$.

Figure 1

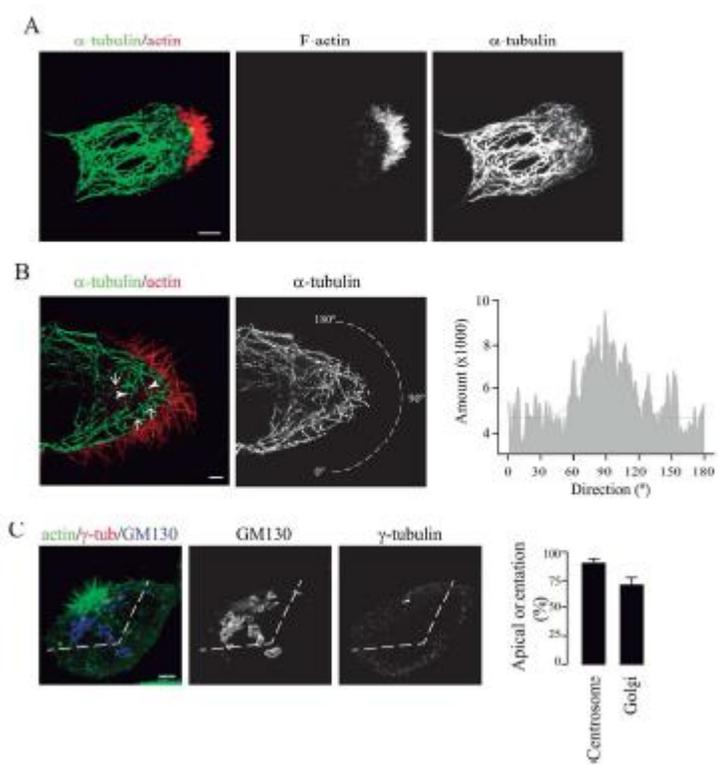


Figure 2

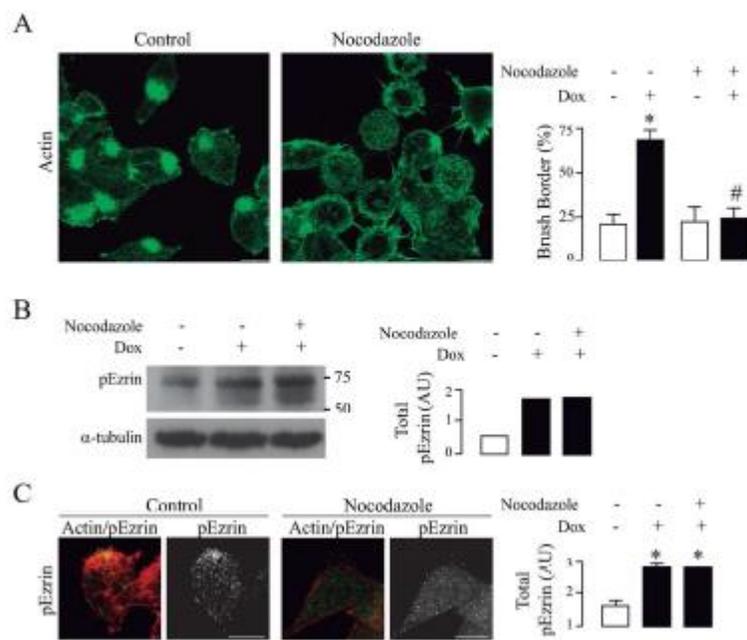


Figure 3

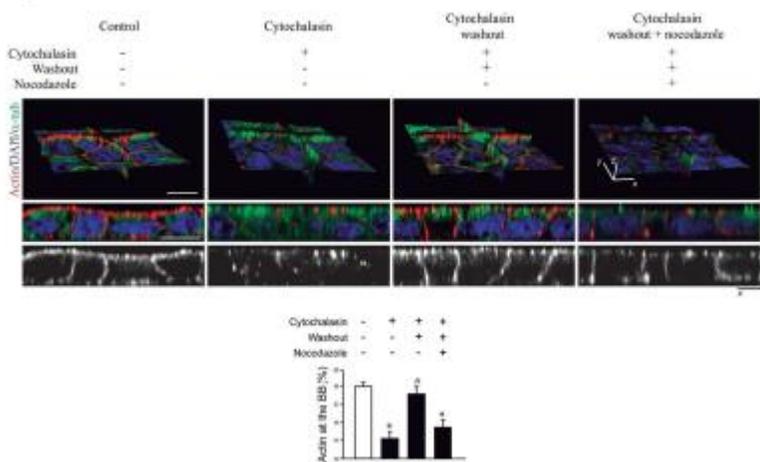


Figure 4

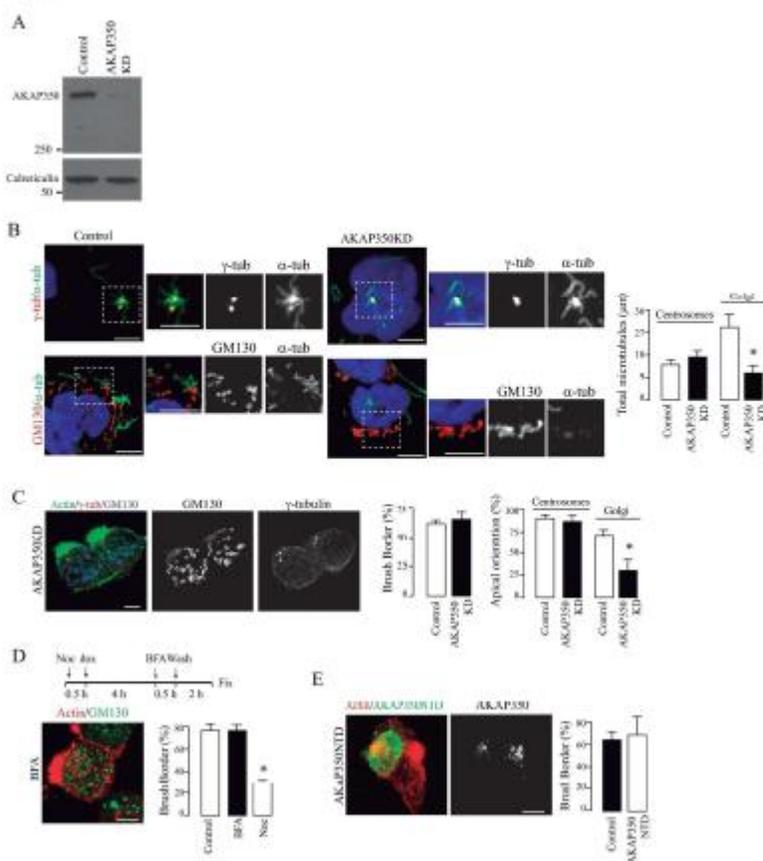


Figure 5

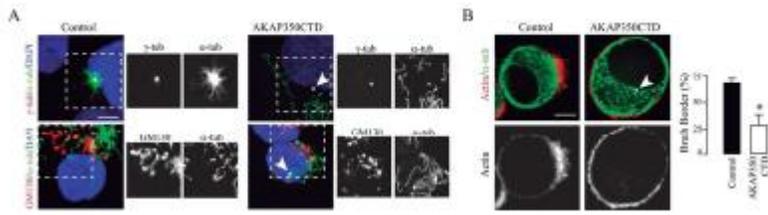


Figure 6

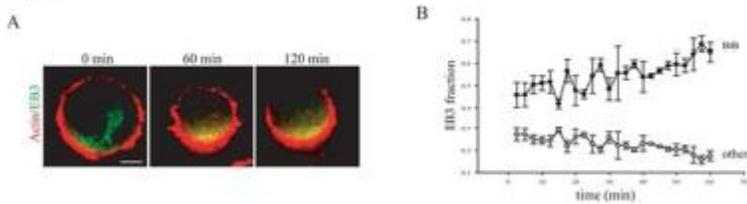


Figure 7

