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Sperm Flagellar 1 Binds Actin in Intestinal Epithelial Cells and Contributes to Formation of Filopodia and Lamellipodia

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Abstract

Background & Aims: Sperm flagellar 1 (SPEF1, also called CLAMP) is a microtubule-associated protein that regulates microtubule dynamics and planar cell polarity in multi-ciliated cells. We investigated the localization and function of SPEF1, or CLAMP, in human intestinal epithelia cells (IECs).

Methods: We performed studies with SKCO-15 and human intestinal enteroids established from biopsies from different intestinal segments (duodenal, jejunum, ileal, and colon) of a single donor. Enteroids were induced to differentiation after incubation with growth factors. The distribution of endogenous CLAMP in IECs was analyzed by immunofluorescence microscopy using total internal reflection fluorescence-ground state depletion and confocal microscopy. CLAMP localization was followed over the course of intestinal epithelial cell polarization as cells progressed from flat to compact, confluent monolayers. Protein interactions with endogenous CLAMP were determined in SKCO-15 cells using proximity ligation assays and co-immunoprecipitation. CLAMP was knocked down in SKCO-15 monolayers using small hairpin RNAs and cells were analyzed by immunoblot and immunofluorescence microscopy. The impact of CLAMP knockdown in migrating SKCO-15 cells was assessed using scratch-wound assays.

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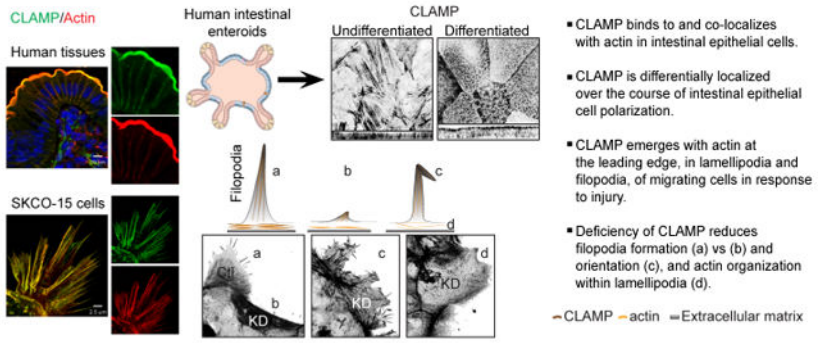
Results: CLAMP bound to actin and apical junctional complex proteins but not microtubules in IECs. In silico analysis predicted the CH domain of CLAMP to contain conserved amino acids required for actin binding. During IEC polarization, CLAMP distribution changed from primarily basal stress fibers and cytoplasm in undifferentiated cells to apical membranes and microvilli in differentiated monolayers. CLAMP accumulated in lamellipodia and filopodia at the leading edge of migrating cells in association with actin. CLAMP knockdown reduced the number of filopodia, perturbed filopodia polarity, and altered the organization of actin filaments within lamellipodia.

Conclusions: CLAMP is an actin-binding protein, rather than a microtubule-binding protein, in IECs. CLAMP distribution changes during intestinal epithelial cell polarization, regulates the formation of filopodia, and appears to assist in the organization of actin bundles within lamellipodia of migrating IECs. Studies are needed to define the CLAMP domains that interact with actin and whether its loss from IECs affects intestinal function.

Graphical Abstract



Keywords

TIRF-GSD; AJC; cytoskeleton; migration

Introduction

Sperm flagellar 1 (SPEF1, also called CLAMP and referred to as such in the remainder of the paper) was first detected in flagella of developing spermatids and mature spermatozoa.[1] The N-terminal domain of CLAMP has a calponin-homology (CH) domain and its C-terminus contains a coiled-coil domain, regions present in many cytoskeletal and motor proteins.[1-4] CLAMP, calponin-homology and microtubule-associated protein, has been reported to be a microtubule-associated protein (MAP). Immunogold electron microscopy revealed CLAMP presence, albeit sparse, along microtubule bundles of pillar cells in the organ of Corti.[2] Recombinant CLAMP co-localizes with β- and γ-tubulin protecting against cold-induced microtubule depolymerization.[2,5,6] In mammalian cilia, CLAMP stabilizes microtubules enabling the assembly and function of central apparatus formation.[6] In the embryonic epidermis of *Xenopus leavis*, exogenously expressed CLAMP localizes to motile cilia.[7] During the intercalation process of the skin of *Xenopus*, CLAMP binds to Par polarity complex components, positioning CLAMP apically where it accumulates with stable acetylated microtubules at the leading edge of cells.[5] CLAMP was recently reported to be a

novel regulator of planar cell polarity (PCP), promoting cell-cell communication and signaling important for PCP asymmetry in *Xenopus* skin.[8]

In intestinal epithelial cells (IECs) and other biological systems, the interplay between cytoskeletal structures such as microtubules, actin microfilaments, and intermediate filaments determines cell morphology and facilitates motility, cell-cell adhesion, cell polarization, vesicular transport, and other cellular functions. Cytoskeletal elements associate with the apical junction complex (AJC) and regulate intestinal epithelial integrity and function.[9-14] Although CLAMP has been shown to be involved in regulating microtubule dynamics and PCP, the expression and localization of this protein in intestinal epithelia has not been examined. In the present study, we define the localization and participation of CLAMP in human intestinal epithelia during homeostasis and injury to begin to understand its contribution to intestinal epithelial physiology.

Materials and methods

Tissue culture

SKCO-15 and Cos-7 epithelial cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (Gibco), 100 U/I penicillin and 100 U/I streptomycin (Gibco) at 5% CO_2. Cos-7 cells were supplemented with 4 mM glutamine (Gibco) and 1 mM Sodium Pyruvate (Gibco). Human intestinal enteroids (HIEs) established from intestinal biopsies obtained from different intestinal segments (duodenal, jejunum, ileal and colon) of a single donor (104) were grown as 3-dimensional (3D) cultures in Matrigel and maintained as previously reported.[15,16] HIE monolayers were generated from by seeding single cell suspensions onto Transwells.[17] Briefly, 3D HIEs free of Matrigel were trypsinized, passed through a cell strainer and suspended in complete medium with growth factors [CMGF (+)] containing the ROCK inhibitor Y-27632 (10 μM, Sigma). Cell suspensions (100 ml) were seeded onto Transwells precoated with collagen IV and 600 μl of CMGF (+) medium containing 10 μM Y-27632 was added to the lower compartment of the well. HIE monolayers were established after 24 hours and these undifferentiated monolayers were incubated an additional 24 hours in fresh CMGF+ media and then harvested for immunostaining. Enteroids were induced to differentiate after several days in CMGF+ media minus Wnt3A, SB202190, and nicotinamide as well as a 50% reduction in the concentration of Noggin and R-spondin. Differentiation media was replaced every 24 hours. Transepithelial electrical resistance of undifferentiated and differentiated monolayers was measured before preparing them for immunostaining.

To study the localization of CLAMP during the polarization process, SKCO-15 cells were plated on Transwells and allowed to progress from short and flat cells (24 hours post-plating) to tall and compact cells (72 hours post-plating). Monolayers were processed for immunofluorescence microscopy at several time points.

Antibodies

Primary antibodies used were: CLAMP (sc-85485/sc-374099, Santa Cruz), tubulin (11H10; 9F3, Cell signaling; T5168, Sigma; sc-5274, Santa Cruz), Clasp2 (ab95373, Abcam), actin (A5316, Sigma; ab-8226, Abcam; MCA5775GA, Bio-rad), myc (C-395, Sigma; sc-9E10, Santa Cruz), ZO-1 (61-7300, Invitrogen), occludin (331500, Invitrogen), cingulin (sc-365264, Santa Cruz), E-cadherin (610181, BD transduction), p120 (sc-23873, Santa Cruz), FAK (sc-557, Santa Cruz), paxillin (05-417, Millipore), vinculin (V9131, Sigma), GAPDH (NB600-502, Novus), phalloidin (A12380, Alexa Fluor 647 and 532-conjugated, Life technologies), rabbit-IgG isotype (02-6102, ThermoFisher), and mouse-IgG isotype (sc-2025, Santa Cruz). Secondary antibody used for immunofluorescence was Alexa Fluor (Life Technologies).

Bacteria culture

EPEC 0127:H6 E2348/69 was grown at 37°C in Luria broth overnight. EPEC infections were performed as previously reported.[18]

Transfections

In order to knockout CLAMP, SKCO-15 monolayers were co-transfected with CRISPR technology against human CLAMP gene (CLAMP HDR Plasmid (h): sc-411818-HDR; CLAMP CRISPR/Cas9 KO Plasmid (h): sc-411818, Santa Cruz). To generate knockdown cells of CLAMP, three shRNA vectors were obtained from VectorBuilder. The interferent sequences (shRNA#2: GTACTGAAGAGGCTGAACTTT, shRNA#3: TCGTCCTCCAGATCGCTGAAA and shRNA#8: CAAAGCAGTGTCCAACACCTT) were cloned into mammalian shRNA knockdown PiggyBac vectors (pPB-[shRNA]-EGFP). A scramble sequence (CCTAAGGTTAAGTCGCCCTCG) was used as a nonspecific control. Transfection reagents: UltraCruz (Santa Cruz), Continuum (Gemini Bio-products) and Gene Juice (Novagen) were used according to manufacturer's instructions. For stable CRISPR transfection, cells were selected with puromycin for two weeks and the expression of the transgene was determined by immunofluorescence. Depletion of endogenous CLAMP or detection of the recombinant protein was evaluated by immunofluorescence microscopy and immunoblots. Full-length mouse myc-CLAMP plasmid (pCMVmyc- CLAMP) was kindly provided by Dr Gerard W. Dougherty (Laboratory of Heymut Omra, University Hospital Münster, Department of General Paediatrics, Germany).[2]

Immunofluorescence

SKCO-15 cells and HIEs were plated on glass coverslips or Transwells. For immunostaining, the samples were washed three times with cold PBS then fixed in prechilled (−70°C) methanol by incubating them for 10 minutes at −20°C or with PFA 4% and permeabilized with Triton X-100 (0.1%) in PBS. Samples were blocked with 5% BSA and stored at 4°C until stained. Paraffin embedded human colon biopsies were obtained from the NIH Digestive Disease Research Core Center, Washington University, St. Louis, MO. Samples were processed as previously reported.[18] For cold treatment, cells were processed as described by Glotfelty et al.[19] For Proximity Ligation Assays (PLA), SKCO-15 cells were

plated on glass coverslips, fixed, and processed for Duolink® PLA (Millipore Sigma) fluorescence protocol according to manufacturer's instructions.

For Total Internal Reflection Fluorescence-Ground State Depletion (TIRF-GSD) microscopy, SKCO-15 cells were grown on collagen-coated Mat-Tek glass-bottom Petri dishes and prepared for imaging as described.[20] Briefly, cells were washed with 37°C DPBS (Corning 21-030-CV), fixed with PFA 4% in PEM buffer (80 mM PIPES, 5 mM EGTA, 2 mM $MgCl_2$) for 10 minutes at 37°C, washed with PBS, and permeabilized using 0.5% Triton X-100 in PBS for 10 minutes at 37°C. Monolayers were blocked overnight at 4°C using 1% BSA in PBS.

Cells were incubated overnight at 4°C in primary antibody diluted 1:50 – 1:100 in 1% BSA in PBS, then with secondary antibodies for 1-2 hours at room temperature in the dark. Monolayers were washed with PBS and prepared for imaging. For TIRF-GSD microscopy, OxEA buffer [50 mM MEA, 3% OxyFluor ™ (Oxyrase, Inc.) enzyme, 20% DL-lactate in PBS at pH 7.4] was added to cells to promote efficient fluorophore blinking.[21] All solutions used for fixation and staining were filtered.

Immunoprecipitation

SKCO-15 cells were plated in 100-mm dishes, rinsed with cold PBS, and incubated for 15 minutes with immunoprecipitation buffer (150 mM NaCl; 20 mM Tris-HCl pH 7.5; 1% NP40; 1.0 mM EDTA and protease inhibitors). Cell lysates were sonicated and centrifuged at 13,000 rpm for 15 minutes at 4°C and the soluble fraction was immunoprecipitated using Dynabeads™ Protein G (10003D, Invitrogen) according to manufacturer's instructions.

Immunoblotting

For protein detection, cells were processed and analyzed by immunoblot as previously reported.[18]

Scratch wound assays

SKCO-15 cells were grown to confluence on glass coverslips then a scratch was made using a 10 μl tip. Samples were processed for immunofluorescence.

In silico analysis

Phyre2 and Pymol software tools were used to predict the homology and structure of human CLAMP.

Imaging

Confocal images were processed and analyzed using Leica TCS SPE DMI 4000B (LAS X software) microscope. A Leica TIRF-GSD microscope equipped with an Andor iXon Ultra 888 camera and lasers of wavelengths 405nm, 488 nm, 532 nm, and 642 nm was used. Approximately 50,000-100,000 single molecule localization frames were acquired per channel with 14.67 ms exposure, averaging 50-150 localizations per frame. Images were processed using Leica Acquisition Software X (LASX). All images were processed using Adobe Photoshop and ImageJ software.

Results

Endogenous CLAMP does not co-localize with microtubules in intestinal epithelial cells.

Published studies demonstrate that CLAMP plays an important role in microtubule stabilization. We therefore questioned whether CLAMP associates with microtubules in intestinal epithelia. Interestingly, endogenous CLAMP does not co-localize with tubulin in SKCO-15 cells, human intestinal enteroids (HIEs) or human colonic epithelial tissues (Figure 1A). To further investigate the role of CLAMP in microtubule organization, SKCO-15 monolayers were exposed to cold (4°C) to disassemble microtubule networks then immuno-detection of CLAMP, tubulin, and Clasp2 was analyzed. CLAMP localized to cell-cell contacts as in control cells despite microtubule disruption (Figure 1B). In contrast, Clasp2, a microtubule binding protein that participates in microtubule stabilization, was disrupted as were microtubules following cold exposure (Figure 1B). Similar results were obtained in MDCK I monolayers (data not shown). These data demonstrate that endogenous CLAMP does not interact with tubulin in intestinal epithelia.

CLAMP harbors conserved amino acids in the CH domain of cytoskeletal proteins important for binding actin.

As previously reported, CLAMP contains a CH domain in its N-terminal region and a coiled-coil domain in the C-terminus (Figure 2A). Phyre2[22] multiple comparison analysis shows that full-length CLAMP shares homology with the cytoskeletal proteins MACF1/ACF7, α-parvin, nesprin-2, β-spectrin, plectin, MICAL 1/2/3, smoothelin, utrophin, ehbp1, α-actinin 3/4, filamin B/C, dystrophin, dystonin, and Spef2 (Figure 2A and Supplementary Table 1). Our analysis predicts that CLAMP has a type 2 CH domain and harbors 11 conserved amino acids (L13, L15, W16, N28, D35, G36, I43, P48, L80, L104, L07) present in the CH region of cytoskeletal proteins (Figure 2A). The residues L13, L15, and W16 within the CH domain of CLAMP are conserved in actin binding site 3 (ABS3) of some cytoskeletal proteins (Figure 2A).[3,23] The CH domain architecture is important for protein-protein interaction and functionality. To obtain structural insight into the CH domain of CLAMP, conformational analysis was performed. Ribbon diagram prediction shows that the putative spatial conformation of the CH domain of CLAMP consists of six a-helices similar to the CH2 domain of α-actinin[24] (Figure 2B). The hydrophobic amino acid tryptophan (W11) located in α-helix A of the CH1 and CH2 domains of spectrin and fimbrin proteins plays an important role in CH structure.[25] Interestingly, this amino acid is conserved in α-helix A of the CH domain of CLAMP (W16) (Figure 2B). These findings suggest that CLAMP could directly interact with actin through these conserved residues or through interaction with actin-binding proteins.

Endogenous CLAMP co-localizes with and binds to actin in cultured and native intestinal epithelia.

To determine if endogenous CLAMP co-localizes with actin, cultured SKCO-15 cells, HIEs and native human colonic tissues were examined using immunofluorescence microscopy. Co-immunofluorescence staining of CLAMP and actin reveals that indeed, these two proteins co-localize at cell-cell contacts of SKCO-15 and HIEs, and at the apical and lateral cell membranes of human colonic tissues (Figure 2C-E). To determine the proximity of

CLAMP to actin, we performed PLA, which indicates the closeness of two proteins (<40 nm).[26,27] PLA demonstrates close proximity between CLAMP and actin but not tubulin (Supplementary Figure 1A). To determine the specificity of this assay, the PLA signal of each primary antibody used separately or in the absence of primary antibodies was evaluated. PLA quantification shows that the signal from CLAMP-tubulin is not significantly different from that of cells incubated with CLAMP antibody alone (Supplementary Figure 1A and B). The binding between CLAMP and actin was assessed by co-immunoprecipitation from SKCO-15 and cell extracts were probed for actin or tubulin by immunoblotting. Interestingly, CLAMP interacts with actin but not tubulin (Figure 2F). To confirm CLAMP-actin binding, endogenous actin was immunoprecipitated from SKCO-15 cell lysates and immunoblotted for endogenous CLAMP. Again, CLAMP co-immunoprecipitated with actin but not tubulin (Figure 2G, upper panel). Cell lysates were probed to confirm the presence of tubulin and actin (Figure 2G, bottom panel). These data indicate that endogenous CLAMP co-localizes and associates with actin but not tubulin in IECs.

In concordance with previous studies, we confirmed that ectopic myc-CLAMP decorated the microtubule networks of Cos-7 cells (Supplementary Figure 1C). SKCO-15 cells were transfected with myc-CLAMP and expression of the transgene was confirmed by immunoblot 24, 48, and 72 hours post-transfection (Supplementary Figure 1D). However, endogenous CLAMP co-localizes with actin, but not microtubules in transfected Cos-7, SKCO-15 (Supplementary Figure 1E), and MDCK I cells (data not shown). To confirm the association of myc-CLAMP-tubulin, co-immunoprecipitation studies were performed and show that ectopic myc-CLAMP indeed associates with tubulin but not actin (Supplementary Figure 1F). These results suggest that over-expression of CLAMP may drive microtubule localization.

In view of the close association of CLAMP with actin, SKCO-15 monolayers were infected with the enteropathogenic *Escherichia coli* (EPEC), which intimately attaches to and effaces the microvilli of host IECs and reorganizes the actin cytoskeleton to form actin-rich pedestals. As predicted based on our data showing its interaction with actin, CLAMP is recruited to actin pedestals underlying attached EPEC (Figure 2 H). The interaction of CLAMP and actin was examined in more detail using TIRF-GSD microscopy, which detects fluorophore molecules in proximity as close as 20 nm. As seen in Figure 2 I, CLAMP perfectly aligns along individual actin filaments, as well as at the cell edge and within lamellipodia of sparse SKCO-15 cells. Together, these data confirm the close association of CLAMP with actin and suggest that this complex is involved in intestinal epithelial cytoskeletal dynamics.

CLAMP distribution changes over the course of intestinal epithelial cell polarization.

CLAMP has been demonstrated to interact with the Par polarity complex and regulates PCP in the *Xenopus* ciliated epithelia.[5,8] We therefore investigated the distribution of CLAMP during the polarization process of IECs. To address this question, CLAMP and actin localization was analyzed as SKCO-15 cells progressed from flat and sparse to tall and compact over time. At 24 hours post-plating, CLAMP is present in the cell cytoplasm, at

cell-cell contacts, and in stress fibers at the basal surface of flat migrating cells, co-localizing with actin (Figure 3A). By 48 hours post-plating, CLAMP has largely cleared from the cytoplasm and moved away from stress fibers, settling primarily in the apical membrane (Figure 3A). In fully polarized monolayers, at 72 hours, the amount of CLAMP associated with stress fibers is further reduced and is almost exclusively localized to the apical membrane (Figure 3A). The distribution of CLAMP was also determined in HIEs under undifferentiated and differentiated conditions (Figure 3B-E). Transepithelial electrical resistance measurements indicated the degree of differentiation (data not shown). Similar to SKCO-15 cells, undifferentiated HIEs show CLAMP localized at the cell-cell contacts, in the cytoplasm, and in stress fibers at the basal surface (Figure 3B-E, upper panels). As HIEs reach differentiation, CLAMP accumulates mainly at the brush border of duodenum, jejunum, and ileum (Figure 3B-D, lower panels). CLAMP is also observed at the cell-cell contacts and within cytoplasm (Figure 3B-D, lower panels). In colonic HIEs, CLAMP localizes to cell-cell contacts and within the cytoplasm but less in microvilli, which are much less robust in colonocytes than enterocytes (Figure 3E, lower panels). Together these data show that CLAMP migrates to different areas of the cell during the polarization process ultimately localizing primarily to the apical membrane where it interacts with actin in microvilli and at sites of cell-cell contact.

CLAMP localizes to cell-cell contacts and interacts with adhesion proteins.

We next investigated CLAMP localization to tight junctions (TJ) and adherens junctions (AJ), sites crucial to polarized epithelia. Co-staining for CLAMP and TJ proteins in SKCO-15 cells demonstrates its co-localization with ZO-1, occludin, and cingulin (Figure 4A). CLAMP is also present in the lateral membrane associating with the AJ proteins E-cadherin and p-120-catenin (Figure 4B). PLA indicates that CLAMP closely interacts with ZO-1 and E-cadherin, although its association with E-cadherin is stronger as compared to ZO-1 (Figure 4C). In duodenal and jejunal HIEs, CLAMP co-localizes with ZO-1 (Figure 4D). Interestingly, CLAMP interaction with ZO-1 is less robust in the ileum than in the duodenum or jejunum (Figure 4D). Co-immunoprecipitation studies in SKCO-15 cells reveal that CLAMP forms a complex with ZO-1 and cingulin, but not occludin; it also interacts with E-cadherin (Figure 4E). The presence of CLAMP in immunoprecipitates of ZO-1 and E-cadherin, but not occludin, confirmed these interactions (Figure 4F). Images of focal adhesions show that CLAMP weakly co-localizes and interacts with vinculin but not with paxillin or focal adhesion kinase (FAK) (Supplementary Figure 2A and B). Together, these results suggest that CLAMP participates in mediating TJ, AJ, and adhesion dynamics, crucial steps in cell polarization.

CLAMP contributes to lamellipodia and filopodia formation.

We next explored the localization of endogenous CLAMP at the leading edge of migrating intestinal epithelial cells using scratch wound assays. Confluent SKCO-15 monolayers were wounded by scraping, then CLAMP and actin were imaged 30 minutes later using immunofluorescence microscopy as cells migrated to heal the wound. CLAMP and actin co-localize at the leading edge of migrating cells within lamellipodia and filopodia (Figure 5A, top and bottom panels, respectively). CLAMP is also present at the leading edge and in lamellipodia of undifferentiated migrating duodenal HIEs, co-localizing with actin (Figure

5B). In an attempt to gain insight into the physiological function of CLAMP, we knocked out CLAMP in SKCO-15 cells using CRISPR technology. Surprisingly, CLAMP-deficient cells failed to survive (Supplementary Figure 3A). These results indicate that CLAMP is crucial for cell survival.

In order to reduce the endogenous expression of CLAMP without affecting cell viability, three different short hairpin RNAs were designed. The expression level of CLAMP was determined by immunoblot analysis. Knockdown cells show a significant reduction of endogenous CLAMP (sh#2: 33.0%±3.0%; sh#3: 47.0%±11.0% and sh#8: 42.0%±10.0%) as compared to control (sh-Scramble-EGFP) monolayers (Supplementary Figure 3B). We assessed the impact of CLAMP knockdown on migrating cells. Transfected SKCO-15 monolayers were scratched and processed 4 hours later for immunofluorescence microscopy. Control sh-Scramble-EGFP monolayers exhibit CLAMP localization throughout the cell, lamellipodia and the entire length of filopodia co-localizing with actin (Figure 5C, panel a). Interestingly, down-regulation of CLAMP (sh-CLAMP-EGFP) resulted in a number of different phenotypes. First, there was a significant reduction in the number of filopodia (Figure 5C, panel b). In addition, filopodia of sh-CLAMP-EGFP cells failed to maintain a forward direction demonstrating random directionality (Figure 5C, panel c) whereas filopodia of other cells appeared collapsed upon themselves consistent with little to no tensile strength (Figure 5C, panel d). Down-regulation of CLAMP also impacted the ability of cells to form lamellipodia (Figure 5C, panel e). In sh-CLAMP-EGFP cells, actin appears as single disorganized filaments rather than bundles at the leading edge of the cell (Figure 5C, panel e). Quantitative analysis of filopodia was obtained from confocal images of 20-30 transfected cells from each condition. As shown in Figure 5D, sh-CLAMP-EGFP cells possessed significantly fewer filopodia per cell (sh#2: 6.0±0.7; sh#3: 5.0±1.4 and sh#8: 3.0±0.8) than control cells (Scramble: 30.0±4.0). Filopodia misorientation was quantified as well. The sh-CLAMP-EGFP cells exhibit a higher percentage of cells with misoriented filopodia (sh#2: 45.0%±7.0%; sh#3: 55.0%±7.0% and sh#8: 29.0%±6.0%) and disorganized actin filaments (sh#2: 56.0%±9.0%; sh#3: 72.0%±16.0% and sh#8: 93.0%±9.0%) versus control cells (Scramble: 11.0%±5.0% and 13.0%±5.0%), respectively (Figure 5E). These findings suggest that CLAMP is involved in filopodia and lamellipodia formation and participates in the structure and dynamics of the actin cytoskeleton, crucial steps during cell migration.

Discussion

The maintenance of intestinal integrity and physiology has been widely studied in many biological systems. The present study provides evidence that CLAMP is an actin binding protein and its expression and distribution are important for cell survival and may impact intestinal epithelial cell migration.

Previous studies demonstrated that CLAMP plays a role in microtubule dynamics.[2,5,6] However, we found that endogenous CLAMP does not bind to tubulin in IECs and exerts no impact on the stability of microtubule networks; only ectopically expressed CLAMP decorates microtubules in epithelial cells. In agreement with our observations, Mitchell group detected that CLAMP weakly associates with microtubule networks but strongly

associates at cell-cell contacts, thus proposing that different pools of CLAMP exist in *Xenopus* cells to exert different functions.[8]

Using multiple approaches including confocal and TIRF-GSD microscopy, co-IP, and PLA, we demonstrate that endogenous CLAMP binds to actin and localizes to actin-based cell structures including microvilli, cell-cell contacts, and stress fibers of epithelial cells. In agreement with our results, Dougherty et al[2] observed by electron microscopy that endogenous CLAMP localizes near the apex and base of mouse pillar cells where it co-stains with actin. These structures contain actin microfilaments, tropomyosin, spectrin, and microtubules that crosslink with the actin cytoskeleton.[28-30] CLAMP is also present in the flagella of mouse spermatozoa,[1] localizing to areas that contain cytoskeletal and signaling proteins.[31] Interestingly, in mouse testis, CLAMP does not bind directly to actin, despite the presence of a CH domain.[1] These controversial results led us to perform a detailed analysis of the sequence of CLAMP.

Single CH domains have been identified in a large number of cytoskeletal and signaling proteins. CLAMP contains a single CH domain that shares similarity with the CH2 domain of actin-binding proteins. These proteins have been reported to interact with actin through ABS1-3. The CH domain of CLAMP contains amino acids (L13, L15, and W16) within ABS3 that are putative actin-binding surface residues. Interestingly, the W16 residue, conserved in the CH domain of CLAMP, has been reported to regulate binding to actin and the spatial structure of the CH domains of filamin, spectrin and fimbrin.[25,32,33] The direct association of single CH domain proteins with actin is controversial. Gimona et al[34] demonstrated that the CH region of calponin is not sufficient to bind actin and localize this protein to stress fibers in fibroblasts, suggesting that this domain may cooperate with other molecules to mediate actin-binding. Despite these data, we do not discount the notion that CLAMP could directly interact with actin or through other actin-binding partners. Further biochemical analysis is necessary to determine the complete functionality of the CLAMP CH domain. Our data demonstrate however that endogenous CLAMP preferentially binds to actin and only over-expression of this protein drives it to a secondary binding partner, microtubules. Further studies are needed to determine the biological functions of the actin and microtubule binding regions present in the CLAMP sequence.

The subcellular distribution of CLAMP appears to be important during cell migration and polarization. For example, CLAMP has differential distribution during spermatid maturation in mouse testis.[1] As spermatids mature, CLAMP localizes towards the lumen of these cells.[1] In mouse tracheal epithelial cells, CLAMP is expressed at a time that coincides with multicilia formation and is enriched at the ciliary tip during multiciliogenesis in ependymal cells.[6] During the intercalation process of *Xenopus*, CLAMP binds to the Par polarity complex positioning apically to stabilize microtubules at the leading edge.[5] CLAMP was shown to localize to cell-cell contacts in *Xenopus* cells.[8] Here, we show that endogenous CLAMP co-localizes and interacts with ZO-1, cingulin, E-cadherin, p-120-catenin and vinculin in intestinal epithelia. Furthermore, the distribution of CLAMP in SKCO-15 cells and HIEs depends on the degree of cell polarization, indicating that CLAMP migrates through different cell compartments finally positioning at cell-cell contacts and microvilli. Although the molecular mechanism for the final destination of CLAMP is still unknown, we

speculate that CLAMP may participate in positioning actin and other proteins to the apical plasma membrane of IECs.

Furthermore, our work supports the notion that CLAMP is crucial for intestinal epithelial cell survival. Depletion of CLAMP by CRISPR in SKCO-15 cells caused a dramatic change in cell morphology and cell death. In the *Xenopus* model, both high dose CLAMP morpholino and CRISPR caused early embryonic lethality, thus the function of CLAMP was explored using low dose morpholino, leading to only partial depletion.[8] Down-regulation of CLAMP in *Xenopus* ciliated epithelia results in the loss of cilial polarity and orientation, demonstrated by asymmetric accumulation of Prickle-2 (Pk2), Disheveled (Dvl1), and atypical cadherin Celsr1, transmembrane proteins that contribute to PCP.[8] Using three different sh-CLAMP-EGFP RNAs we were able to partially down-regulate the endogenous expression of CLAMP in SKCO-15 monolayers. Interestingly, we found that CLAMP is crucial for filopodia and lamellipodia assembly and that CLAMP down-regulation leads to a loss of filopodia orientation. Knockdown of CLAMP resulted in a reduced number of filopodia as well as an altered pattern of actin filaments at the leading edge of migrating cells suggesting that CLAMP is required to reorganize actin bundles in response to the signaling of cell injury. Recently it was reported that CLAMP deficiency reduces the number of cilia and alters the ciliary beat pattern from planar to rotational in multiciliated ependymal cells as well as microtubule stability crucial for assembly of the central pair (CP) cilia apparatus.[6] Together, these findings suggest that the localization and expression of CLAMP play an important role in modulating cell migration, microtubule stabilization, cell-cell communication and cell polarity.

Actin bundling proteins associate with actin filaments and are present in cellular protrusions such as brush border microvilli, lamellipodia, filopodia, microspikes, and sterocilia among others.[35,36] Our findings demonstrate that CLAMP is localized in actin-based structures and functions as an important mediator in the assembly and maintenance of filopodia, which are required to establish cell-cell contact and cell motility.[35] CLAMP appears to be required for reorganization of the actin cytoskeleton during cell migration associated with wound healing. We therefore speculate that CLAMP may function as an actin bundling protein; however, further analysis is needed to address this possibility.

In summary, CLAMP is crucial for cell survival, moves throughout the cell polarization process, and is involved in lamellipodia and filopodia formation in migrating cells. Association of CLAMP with AJC proteins suggests that it may also contribute to TJ barrier formation and function in IECs. The present study shows that while CLAMP may associate with microtubules in other cell types and under specific conditions, its primary binding partner in intestinal epithelia is actin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

AJ	adherens junctions
EPEC	enteropathogenic *E. coli*
HIEs	human intestinal enteroids
IECs	intestinal epithelial cells
TJ	tight junctions

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Author names in bold designated shared co-first authorship.

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What you need to know:

BACKGROUND AND CONTEXT: Sperm flagellar 1 (SPEF1, also called CLAMP) regulates microtubule dynamics and planar cell polarity in multi-ciliated cells. We investigated the localization and function of CLAMP in human intestinal epithelia cells (IECs).

NEW FINDINGS: CLAMP is an actin-binding protein, rather than a microtubule-binding protein, in IECs. CLAMP participates in the formation of filopodia and assists in the organization of actin bundles within lamellipodia of migrating IECs.

LIMITATIONS: These studies were performed in cultured cells. Studies in animals are needed to determine its function in intestinal homeostasis.

IMPACT: Studies of this actin-binding protein in IECs could provide information on the movement and function of these cells.

Lay Summary: We identified a protein that may participate in intestinal epithelial cell development and movement.

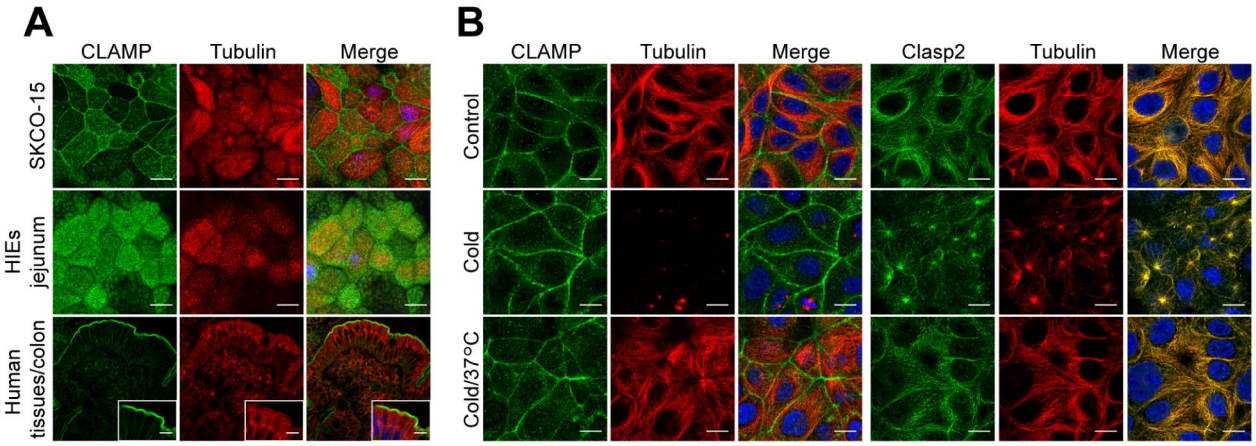

Figure 1.

Endogenous CLAMP does not co-localize with microtubules. (A) SKCO-15, HIEs, and human colonic tissues were stained for endogenous CLAMP (green) and tubulin (red). CLAMP did not co-localize with tubulin in any of these tissues. (B) SKCO-15 monolayers were treated or not with cold to destabilize microtubules then incubated at 37°C to allow reassembly of the microtubule networks. Co-localization of CLAMP and tubulin was not seen in any of these conditions. As a positive control, co-staining of tubulin with the MAP Clasp2 was performed. Complete co-localization of Clasp2 with tubulin was apparent. A representative confocal image is shown from at least three independent experiments plated in duplicate. Nuclei were stained with Hoechst in all images. Scale bar, 10 μm.

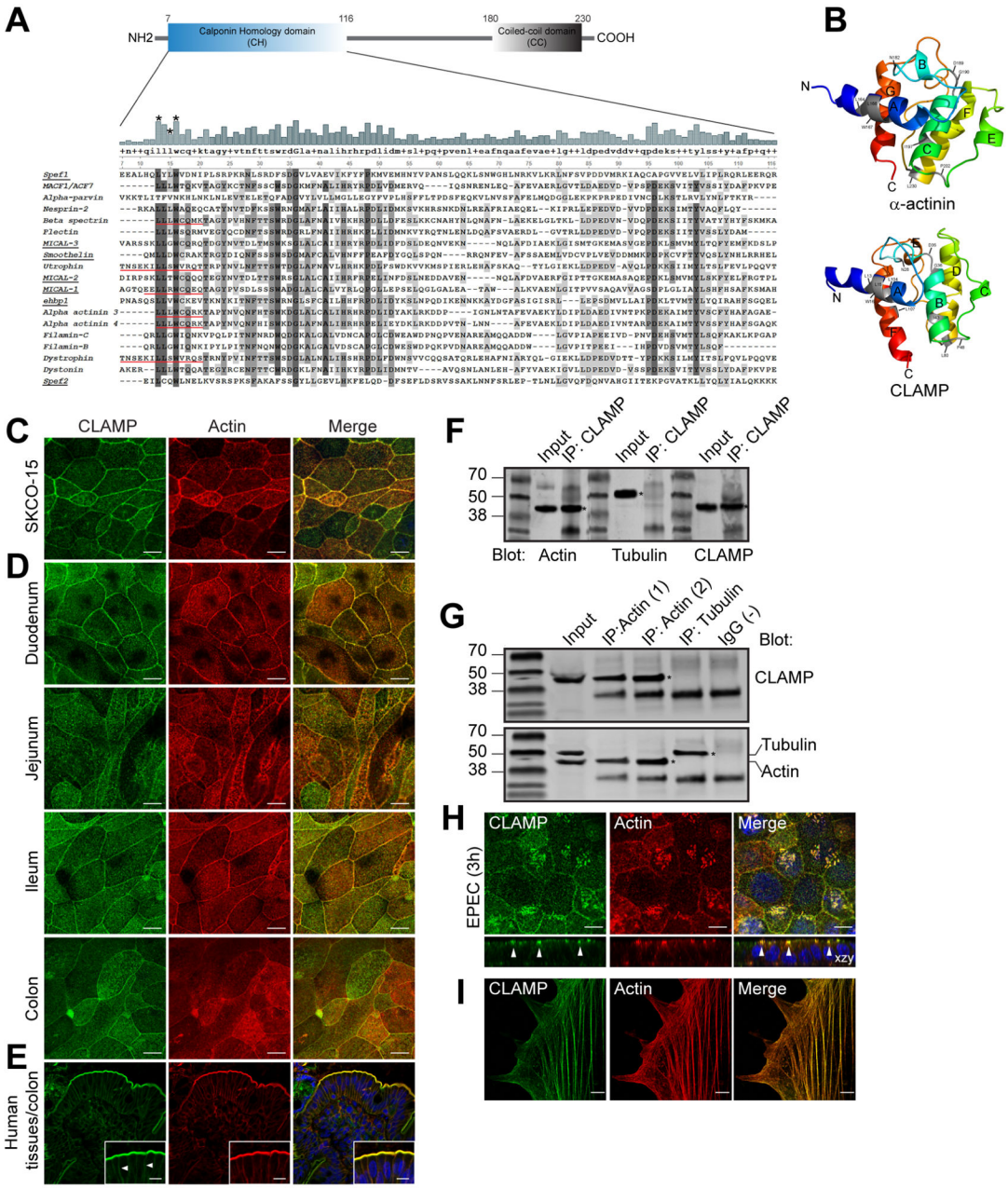

Figure 2.

The CH domain of CLAMP is similar to many actin-binding proteins, CLAMP co-localizes and interacts with actin in cultured and native intestinal epithelia. (A) CLAMP is a 236 amino acid protein with a CH domain in the N-terminal region (7-116 residues) and a coiled-coil domain (180-230 residues) in the C-terminus. The CH domain of CLAMP contains amino acids that are conserved in the CH domain of several cytoskeletal proteins (grey gradient color) and putative actin binding surface residues (*) reported in the ABS3 (red underline) of some cytoskeletal proteins. Single CH domain containing proteins are underlined. (B) Ribbon diagram of the putative 3D structure of the CH domain of α-actinin and CLAMP indicate the spatial distribution of six α-helices of both proteins. Conserved

amino acids are labeled in 3D structures. (C-E) SKCO-15 cells, HIEs, and human colonic tissues were stained for endogenous CLAMP (green) and actin (red). (C and D) Localization to cell-cell contacts and apical membranes of SKCO-15 and HIEs is seen. (E) In human colonic tissues, CLAMP is prominent at the apical membrane and is also seen within the basolateral membrane (inset, arrowheads). Scale bar, 10 μm. (F and G) SKCO-15 cell lysates were immunoprecipitated for endogenous CLAMP, actin or tubulin, then immunoblot analysis was performed to confirm these interactions. (F) Co-immunoprecipitation of CLAMP with actin, but not tubulin, is observed. Probe for CLAMP was positive as expected. (G) Immunoprecipitation using two different actin antibodies, (1) and (2), was positive for CLAMP (upper panel, *), however those for tubulin were negative. Detection of tubulin and actin in the cell lysate was confirmed (bottom panel, *). IgG was used as a negative control. (H) SKCO-15 cells were plated on Transwells and infected with EPEC. Endogenous CLAMP (green) and actin (red) were immunodetected. CLAMP is recruited away from the cell-cell contacts of IECs to EPEC-induced actin pedestals (arrowheads). Scale bar, 10 μm. (I) TIRF-GSD microscopy reveals that CLAMP (green) associates with individual actin (red) filaments and localizes to the leading edge and in lamellipodia of sparse migrating SKCO-15 cells. Scale bar, 2.5 μm.

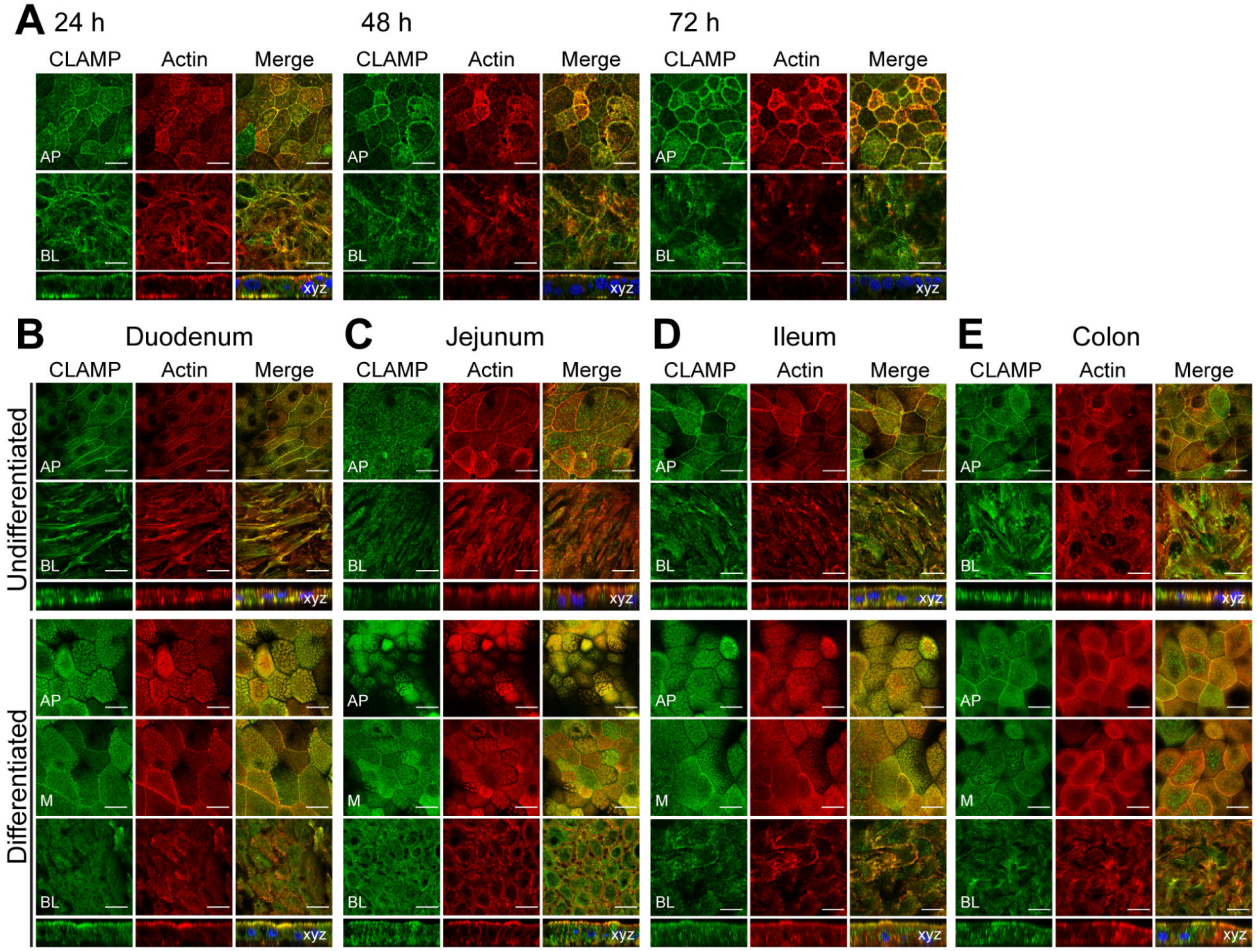

Figure 3.

CLAMP localization changes during intestinal epithelial cell polarization. (A-E) SKCO-15 and HIEs were plated on Transwells and immuno-detection of CLAMP (green) and actin (red) was evaluated. (A) SKCO-15 cells after 24 hours post-plating show CLAMP present throughout the cell, at apical and basolateral membranes and within the cytoplasm. As monolayers start to become polarized, CLAMP moves from the cytoplasm and the basal membrane becoming more prominent at the apical domain. By 72 hours, CLAMP is almost exclusively localized to the apical membrane. CLAMP interaction with actin is seen at all time points. (B-E) Confocal images of different segments of HIEs before and after enterocyte differentiation. CLAMP is present at the cell-cell contacts and stress fibers at the basal surface of undifferentiated HIEs. CLAMP is observed at the brush border, cell-cell contacts and cytoplasm of differentiated HIEs. Apical membrane (AP), basolateral membrane (BL), plane of cell-cell contacts (M). Z-projected images are shown. Scale bar, 10 μm.

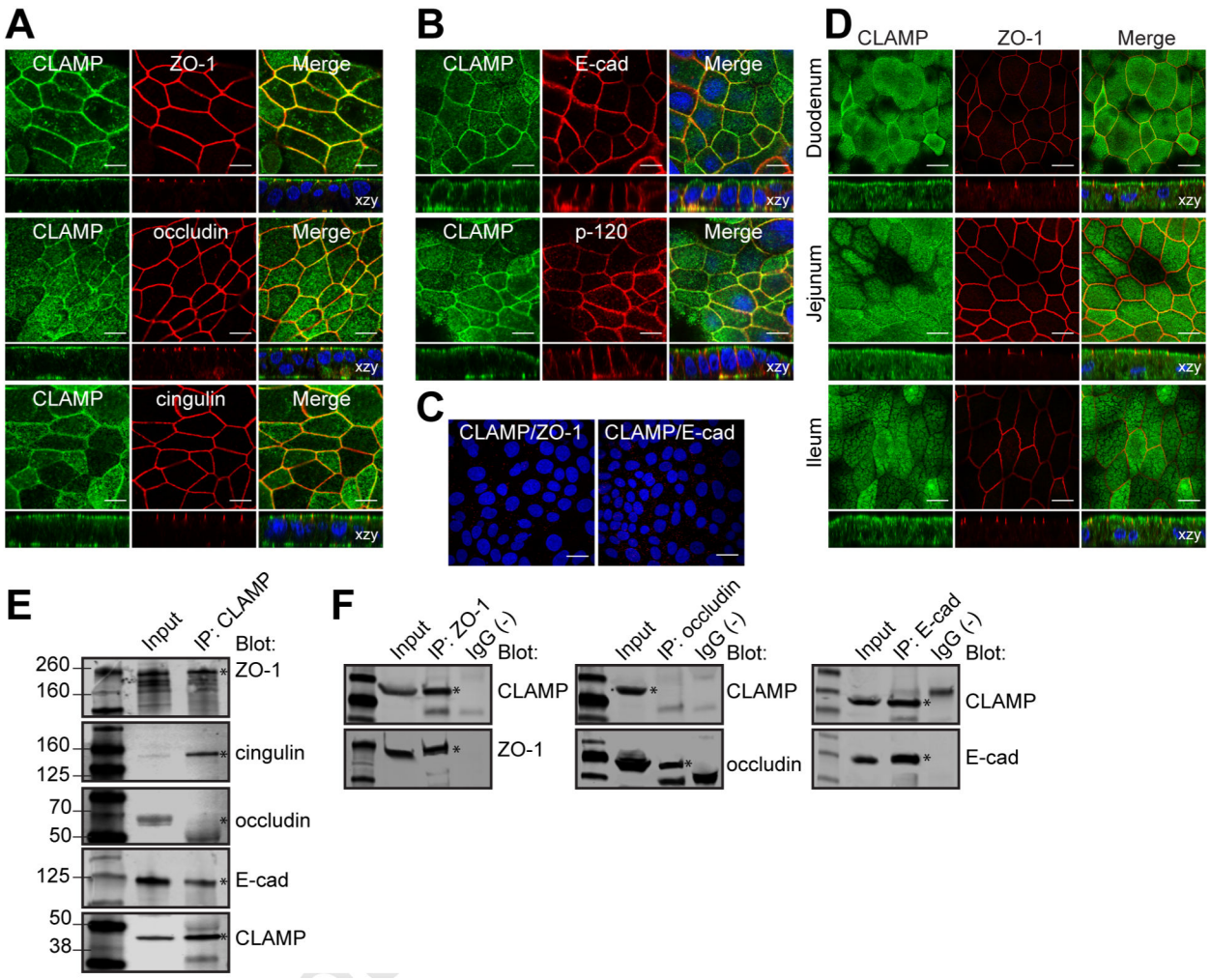

Figure 4.

CLAMP co-localizes and interacts with AJC proteins. (A-D) Immuno-detection of CLAMP, TJ, and AJ proteins was determined in SKCO-15 and HIEs cells by immunofluorescence and PLA. (A and B) In SKCO-15 monolayers, CLAMP localizes to cell-cell contacts in association with TJ proteins (ZO-1, occludin, and cingulin) and at the lateral membrane with AJ proteins (E-cadherin and p-120-catenin). (C) Positive PLA signal for ZO-1 and E-cadherin confirms their interaction with CLAMP. (D) Duodenal and jejunal segments from differentiated HIEs exhibit co-localization of CLAMP with ZO-1, whereas ZO-1 co-localization in the ileum appears to be less. Scale bar, 10 μm. (E) CLAMP was immunoprecipitated from SKCO-15 cells and immunoblots against TJ and AJ proteins were performed. CLAMP interacts with ZO-1, cingulin and E-cadherin, but not occludin. (F) Immunoprecipitation of ZO-1 and E-cadherin, but not occludin, confirmed the presence of CLAMP. Immuno-detection of ZO-1, occludin, and E-cadherin in cell lysates is shown.

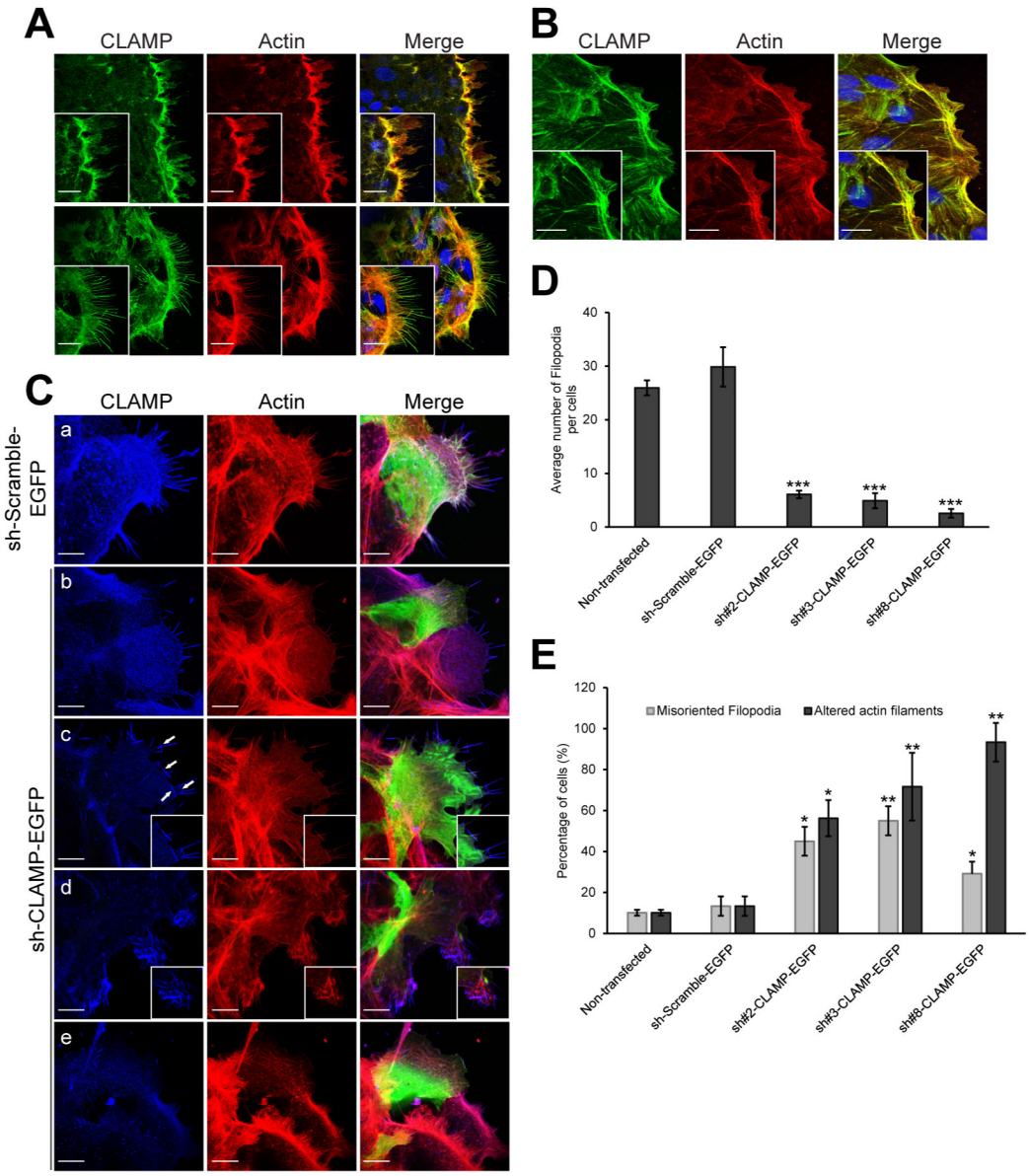

Figure 5.

CLAMP participates in lamellipodia and filopodia formation at the leading edge of cells migrating in response to intestinal epithelial cell injury. (A) SKCO-15 cells were plated on glass cover slips, then scratch-wound assays were performed and samples imaged after 30 minutes. (A) Immuno-detection shows CLAMP (green) accumulates with actin (red) at the leading edge, lamellipodia and filopodia (inset) of migrating cells. (B) Undifferentiated HIEs from duodenum confirm CLAMP and actin localization at the leading edge and in lamellipodia of migrating cells. (C) SKCO-15 monolayers transfected with sh-Scramble-EGFP or sh-CLAMP-EGFP were scratched then examined by immunofluorescence after 4 hours. Transfected cells are positive for GFP, endogenous CLAMP is labeled blue, and actin red. Down-regulation of CLAMP results in reduction of the number of filopodia (panel b), alteration in the orientation of filopodia (panels c and d, inset/arrows), and the lack of

lamellipodia formation at the leading edge as well as altered actin filaments in migrating cells (panel e). (D-E) The number of filopodia as well as the pattern of orientation and altered actin filaments in transfected cells were quantified. (D) There is a significant reduction in the number of filopodia in sh-CLAMP-EGFP cells compared to non-transfected or sh-Scramble-EGFP conditions (P=0.004, n=30). (E) There is a significantly greater number of cells with misoriented filopodia and altered actin filaments in sh-CLAMP-EGFP compared to control conditions (P=0.001, n=30). The results shown are the mean ± SEM of two independent experiments performed in triplicate. P values were calculated by ANOVA Tukey's Multiple Comparison Test.