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Integrin-linked kinase and ELMO2 modulate recycling endosomes in keratinocytes

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ABSTRACT

The formation of tight cell-cell junctions is essential in the epidermis for its barrier properties. In this tissue, keratinocytes follow a differentiation program tightly associated with their movement from the innermost basal to the outer suprabasal layers, and with changes in their cell-cell adhesion profile. Intercellular adhesion in keratinocytes is mediated through cell-cell contacts, including E-cadherin-based adherens junctions. Although the mechanisms that mediate E-cadherin delivery to the plasma membrane have been widely studied in simple epithelia, this process is less well understood in the stratified epidermis. In this study, we have investigated the role of Engulfment and Cell Motility 2 (ELMO2) and integrin-linked kinase (ILK) in the positioning of E-cadherin-containing recycling endosomes during establishment of cell-cell contacts in differentiating keratinocytes. We now show that induction of keratinocyte differentiation by Ca^{2+} is accompanied by localization of ELMO2 and ILK to Rab4- and Rab11a-containing recycling endosomes. The positioning of long-loop Rab11a-positive endosomes at areas adjacent to cell-cell contacts is disrupted in ELMO2- or ILK-deficient keratinocytes, and is associated with impaired localization of E-cadherin to cell borders. Our studies show a previously unrecognized role for ELMO2 and ILK in modulation of endosomal positioning, which may play key roles in epidermal sheet maintenance and permeability barrier function.

1. Introduction

The epidermis is a complex stratified epithelium mainly composed of keratinocytes at various stages of differentiation. It is derived from surface ectoderm cells attached to a basement membrane, which adopt a keratinocyte fate towards mid-gestation and then form the innermost epidermal basal layer (reviewed in [1]). At this time, hair follicles, which will act as a reservoir for keratinocyte stem cells, also begin to form. Subsequently, the epidermis begins to stratify, and as stratification progresses, keratinocytes begin a terminal differentiation program. In this manner, interfollicular epidermal stem cells and their transit-amplifying progeny populate the basal layer. When the latter cells begin to differentiate, they are pushed outward, forming the spinous and the granular layers. In the uppermost epidermal layers, cells lose their nuclei, become highly cross-linked and undergo a form of programmed cell death, thus giving rise to the cornified envelope.

Critical components for epidermal function are cell-cell adhesions. Three types of intercellular adhesions are found in the epidermis: adherens junctions, tight junctions and desmosomes [1]. They each have different localization patterns and fulfill distinct functional roles. Basal keratinocytes are polarized, and assemble E-cadherin-containing adherens junctions and desmosomes laterally and apically, unlike simple epithelia, which lack adherens junctions on the apical aspect. Suprabasal keratinocytes assemble these junctions all around the cell membrane, and those cells in the granular layer also contain tight junctions assembled in a polarized manner, which serve to impede loss of fluids and electrolytes. Due to differences in architecture between the epidermis and simple epithelia, the mechanisms that control formation of intercellular junctions in the latter are not identical to those in the epidermis. Although cell-cell junctions have been fairly well characterized in simple epithelia, the mechanisms that govern their formation and maintenance in the epidermis are less well understood.

Keratinocyte cell-cell junctions collectively integrate the actin and microtubule cytoskeletons, thus contributing to epidermal mechanical strength [1]. An important scaffold protein that modulates both F-actin and microtubule dynamics is ILK. Targeted inactivation of *Ilk* in mouse epidermis has revealed key roles for this protein in hair follicle morphogenesis, keratinocyte phagocytic

Abbreviations: ANOVA, analysis of variance; EGTA, ethylene glycol tetraacetic acid; ELMO2, Engulfment and Cell Motility 2; ERI, ELMO2-RhoG-ILK; GAPDH, glyceraldehyde 3-phosphodehydrogenase; GFP, green fluorescent protein; ILK, integrin-linked kinase; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride.

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capacity and permeability barrier function [2–4]. In undifferentiated keratinocytes, ILK can form tripartite complexes containing ELMO2 and RhoG. These species mediate acquisition of front-rear polarity in response to epidermal growth factor and promote forward migration [5,6]. As scaffolding proteins that interact with numerous other factors, ILK and ELMO also participate in several other cellular processes, such as regulation of intracellular vesicular movements. Specifically, ILK-deficient epidermal keratinocytes exhibit impaired delivery of caveolae to the plasma membrane as a result of microtubule abnormalities [7], whereas the *C. elegans* orthologue of mammalian ELMO proteins, CED-12, is involved in recycling endosome trafficking in intestinal epithelial cells. In this organism, loss of ELMO results in intracellular accumulation of recycling cargo and disruption of endosome morphology and trafficking through mechanisms that involve altered Rab GTPase regulation [8]. ILK and ELMO2 complexes are also found in keratinocytes induced to differentiate by culture in the presence of ≥1.0 mM extracellular Ca^{2+}. In these cells, ILK-ELMO2 species contribute to the maintenance of microtubule stability, and their disruption is associated with increased microtubule catastrophe [5,6]. In this study, we report that ILK and ELMO2 also localize to Rab4- and Rab11a-containing endosomes in differentiating keratinocytes, and that ILK or ELMO2 loss of function alters the intracellular positioning of Rab11a-positive long-loop recycling endosomes in these cells. Our findings have potential important implications for the formation of keratinocyte epithelial sheets, because this process involves recycling endosome delivery of cargo proteins, such as E-cadherin, to cell-cell junctions.

2. Materials and methods

2.1. Mouse strains

All animal experiments were approved by the University of Western Ontario Animal Care Committee (Protocol No. 2015-021), in accordance with regulations and guidelines from the Canadian Council on Animal Care. The strains used were CD-1, and mice with epidermis-restricted inactivation of *Ilk* alleles (*K14Cre;Ilk$^{f/+}$* or *K14Cre;Ilk$^{f/f}$*) previously described [2,9].

2.2. Antibodies, plasmids and reagents

The antibodies used and their sources are: Mouse anti-E-cadherin (610181) and mouse anti-ILK (611802, BD Transduction Laboratories, San Jose, CA), rabbit anti-ELMO2 (ab181234) and mouse anti-GAPDH (ab9484, Abcam, Cambridge, UK), mouse anti-FLAG-M2 (F1804, Sigma, St. Louis, MO), Alexa Fluor-conjugated goat anti-mouse and goat anti-rabbit IgG were purchased from Molecular Probes (ThermoFisher Scientific, Carlsbad, CA). Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibodies were purchased from Jackson ImmunoResearch Laboratories (West Groove, PA). The plasmids encoding V5- and mCherry-tagged ILK proteins, and FLAG- and GFP-tagged ELMO2 proteins have been described [10–13]. Plasmids encoding canine GFP-tagged Rab proteins (Rab4a, Rab5a, Rab7a and Rab11a) were a kind gift from Drs. John Di Guglielmo and Robert Lodge, and have been described [14]. Cholera toxin (100) and insulin (16634) were from List Biological (Campbell, CA, USA) and Invitrogen (Carlsbad, CA, USA), respectively. Polyethyleneimine (PEI), 25 kDa linear (23966) was from Polysciences (Warrington, PA). Chelex 100 resin was purchased from Biorad (Mississauga, ON, Canada). Lipofectamine 2000 transfection reagent (1166827) was purchased from ThermoFisher Scientific (Carlsbad, CA), Ca^{2+}-free EMEM was from Lonza (06-174G, Rockland, ME), Chelex resin was from Bio-Rad (142-2832, Hercules, CA). Rat tail Collagen Type I was from BD Biosciences (354236, Mississauga, Canada). All other reagents were purchased from Sigma (St. Louis, MO).

2.3. Cell culture and transfections

Primary mouse keratinocytes were isolated from 2-d-old CD-1 mice or from 4-d-old *K14Cre;Ilk$^{f/+}$* or *K14Cre;Ilk$^{f/f}$* animals. These cells and spontaneously immortalized mouse keratinocyte iKT cells were cultured in Ca^{2+}-free EMEM supplemented with growth additives and 8% Chelex-treated FBS, as described [6,11,15,16]. Keratinocyte differentiation was induced by culture in growth medium containing 1 mM $CaCl_2$ ("High Ca^{2+} medium"). All experiments were conducted when keratinocytes were 70–80% confluent, and between 3 and 5 days after isolation. For experiments in which Ca^{2+} was substituted by other divalent cations, Low Ca^{2+} medium was supplemented with 1 mM $MgCl_2$ or 1 mM $MnCl_2$. Where indicated, the culture medium contained 0.25 mM EGTA or 0.25 mM EGTA plus 1 mM Ca^{2+}. For Ca^{2+} withdrawal experiments, cells were first cultured in High Ca^{2+} medium for 16 h, washed thrice with Ca^{2+}-free PBS (pH 7.6), and subsequently cultured in Low Ca^{2+} medium for intervals indicated in individual experiments. Transient plasmid transfections were conducted with PEI, as described [17]. For experiments using siRNA, cells were transfected with Lipofectamine 2000 and siRNA (30 nM) in serum-, additive and Ca^{2+}-free EMEM for 4 h, following the manufacturer's instructions. The medium was then replaced with Low Ca^{2+} growth medium, and the cells were cultured for 72 h prior to using for experiments. The siRNAs used were: *Silencer* Negative Control #1 (AM4611), and *Silencer* Pre-designed siRNAs targeting ELMO2 (AM16708; siRNA ID# 86210, 86119 and 86028), purchased from Life Technologies-ThermoFisher Scientific (Carlsbad, CA).

2.4. Immunoblot analysis

Keratinocytes were cultured in High Ca^{2+} medium for intervals indicated in individual experiments prior to harvesting and lysis in Buffer A (1% Triton X-100, 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM NaF, 2 mM Na_3VO_4, 1 mM PMSF, 2 μg/ml each aprotinin, leupeptin, and pepstatin) for 30 min at 4 °C. Lysates were centrifuged (20, 800 ×*g*, 10 min, 4 °C), and supernatants were used for further analysis. Samples containing 50 μg total cellular protein each were resolved by SDS-PAGE and analyzed by immunoblot, using antibodies indicated in individual experiments.

2.5. Microscopy and image analysis

For microscopy studies, CD-1 cells were plated onto 12-mm glass coverslips coated with poly-L-lysine and rat tail Collagen Type I (50 μg/ml), whereas ILK^{+}, ILK^{KO} and iKT cells (ELMO2-expressing and ELMO2^{KD}) were plated onto glass coverslips coated with poly-L-lysine and rat tail Collagen Type I (50 μg/ml) and laminin 332 matrix, and processed for microscopy as described [3,11,18,19]. Fluorescence photomicrographs were obtained with a Leica DMIRBE fluorescence microscope equipped with an Orca-ER digital camera (Hamamatsu Photonics, Hamamatsu City, Japan), using Volocity 6.1.1 software (Improvision-PerkinElmer, Waltham, MA). Confocal analysis was conducted at the Biotron Integrated Microscopy Facility, University of Western Ontario. Cells were imaged with a Plan-Apochromat 63×/1.40 Oil DIC M27 objective mounted on an inverted Zeiss LSM 510 DUO scanning laser confocal microscope. Images were acquired and analyzed with Zen 2009 SP1 software (Zeiss, Göttingen, Germany). Measurement of vesicle sizes was performed with ZEN 2007 SP1 software (Zeiss). The vesicles scored were consistently circular in shape. Therefore, the formula πr^2, where r is the radius, was used to obtain cross sectional areas. Statistical significance was set to $P < 0.05$ (*t*-test, unpaired).

For live-cell imaging studies, keratinocytes were seeded on 35-mm μ-Dish culture plates (81156, Ibidi USA Inc., Madison WI) coated with rat tail Collagen Type I (50 μg/ml) and laminin 332 matrix. Cells were transiently transfected with vectors encoding GFP-Rab11a, and

cultured in Low Ca^{2+} medium for 16 h. The medium was replaced with High Ca^{2+} medium, and the position of Rab11a-positive vesicles in live cells was determined at timed intervals thereafter. Time-lapse videomicroscopy imaging was conducted at the Functional Proteomics Facility of the Department of Biochemistry at The University of Western Ontario. Cells were imaged with a Plan-Apochromat VC 100×/1.4 Oil DIC N2 objective mounted on a Nikon Eclipse Ti inverted microscope. Video images were acquired with NIC-Elements Imaging Software version 4.20 (Nikon Instruments Inc., Melville, NY), and analyzed with Fiji software [20]. The results shown in all figures are representative of 3–8 experiments, each conducted in triplicate and with independent cell isolates.

3. Results

3.1. Ca^{2+}-dependent changes in subcellular localization of ILK and ELMO2 in keratinocytes

ILK plays key roles in various keratinocyte responses to Ca^{2+} during differentiation, including changes in F-actin and microtubule organization, activation of RhoA and generation of intracellular Ca^{2+} currents [4,6] Differentiation in keratinocytes is also accompanied by changes in ILK subcellular localization. Specifically, whereas ILK can be found throughout the cytoplasm and at focal adhesions in undifferentiated cells, it distributes along cell-cell junctions in keratinocytes induced to differentiate by culture in medium containing 1 mM Ca^{2+} ("High Ca^{2+}" medium) [10]. To better understand this phenomenon, we began by examining ILK subcellular localization in differentiating keratinocytes as a function of time. Cells expressing exogenous ILK were cultured in either Low Ca^{2+} medium (~0.05 mM Ca^{2+}) or were induced to differentiate by culture in High Ca^{2+} medium. We observed that ILK showed a diffuse cytoplasmic distribution, and was also enriched in structures adjacent and perpendicular to the plasma membrane in a pattern consistent with localization to focal adhesions in undifferentiated keratinocytes, in agreement with previous reports (Supplemental Fig. S1) [18]. Culturing cells in High Ca^{2+} medium for up to 4 h did not disrupt this pattern of localization. After 8 h in High Ca^{2+} medium, ILK remained cytoplasmic, but was no longer evident at focal adhesions (Supplemental Fig. S1). Close inspection of keratinocytes cultured in High Ca^{2+} medium for 16 h revealed that ILK localized to areas adjacent to cell-cell contacts and, in 25%–40% of cells we were also able to detect ILK in cytoplasmic vesicles, some of them adjacent to cell-cell contacts, without detectable changes in total ILK protein levels (Fig. 1A, Supplemental Figs. S1 and S3). This pattern persisted in cells cultured in High Ca^{2+} medium for up to 48 h, our last time of analysis.

ELMO2 associates with ILK in keratinocytes [11]. Similar to ILK, confocal microscopy analysis revealed that ELMO2 localized predominantly to the cytoplasm in undifferentiated cells. However, after 16 h–48 h of culture in High Ca^{2+} medium, ELMO2 was also observed in intracellular vesicles, a fraction of which aligned along cell borders, without detectable changes in cellular ELMO2 levels (Supplemental Figs. S1 and S3). Further, when we expressed mCherry-tagged ILK together

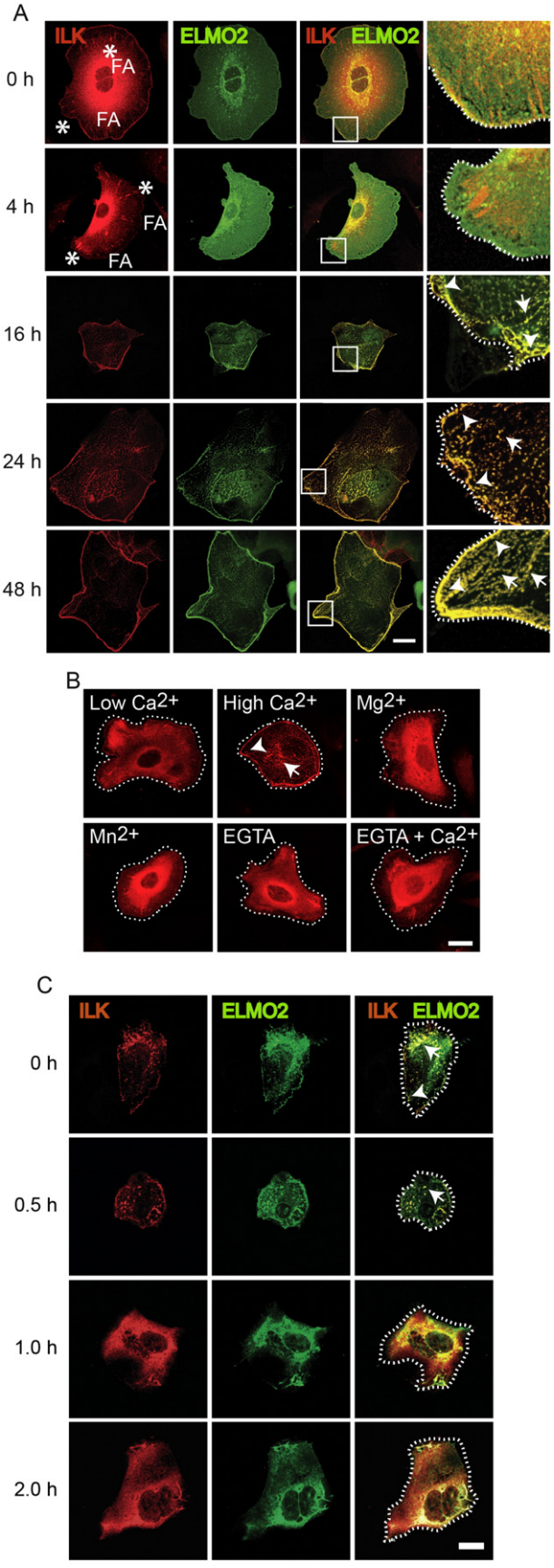


Fig. 1. Intracellular vesicles containing ILK and ELMO2 form in differentiated keratinocytes. (A) Confluent undifferentiated primary keratinocytes cultured in Low Ca^{2+} medium were transfected with vectors encoding mCherry-tagged ILK and GFP-tagged ELMO2. Twenty-four hours later, the cells were cultured in High Ca^{2+} medium and processed for confocal microscopy at the indicated times. Boxed areas are shown at higher magnification in the accompanying micrographs to the right. (B) Confluent cultures of primary keratinocytes were transfected with a vector encoding mCherry-tagged ILK, and 16 h later were cultured in Low Ca^{2+} medium, High Ca^{2+} medium, Low Ca^{2+} medium supplemented with 1.0 mM Mg^{2+}, 1 mM Mn^{2+} or 0.25 mM EGTA, or High Ca^{2+} medium supplemented with 0.25 mM EGTA. Sixteen hours later, the cells were processed for confocal microscopy. (C) Keratinocytes were transfected and cultured in High Ca^{2+} medium for 16 h, as in (A). Cells were rinsed and cultured in Low Ca^{2+} medium for the indicated times prior to processing for microscopy. All images represent individual transfected cells that were surrounded by non-transfected keratinocytes. Dashed lines represent the plasma membrane of transfected cells. Asterisks show ILK enrichment at areas with patterns consistent with focal adhesions (FA). Arrows show cytoplasmic vesicles away from the membrane, and arrowheads show vesicles adjacent to the membrane at cell-cell contacts. Bar, 20 μm.

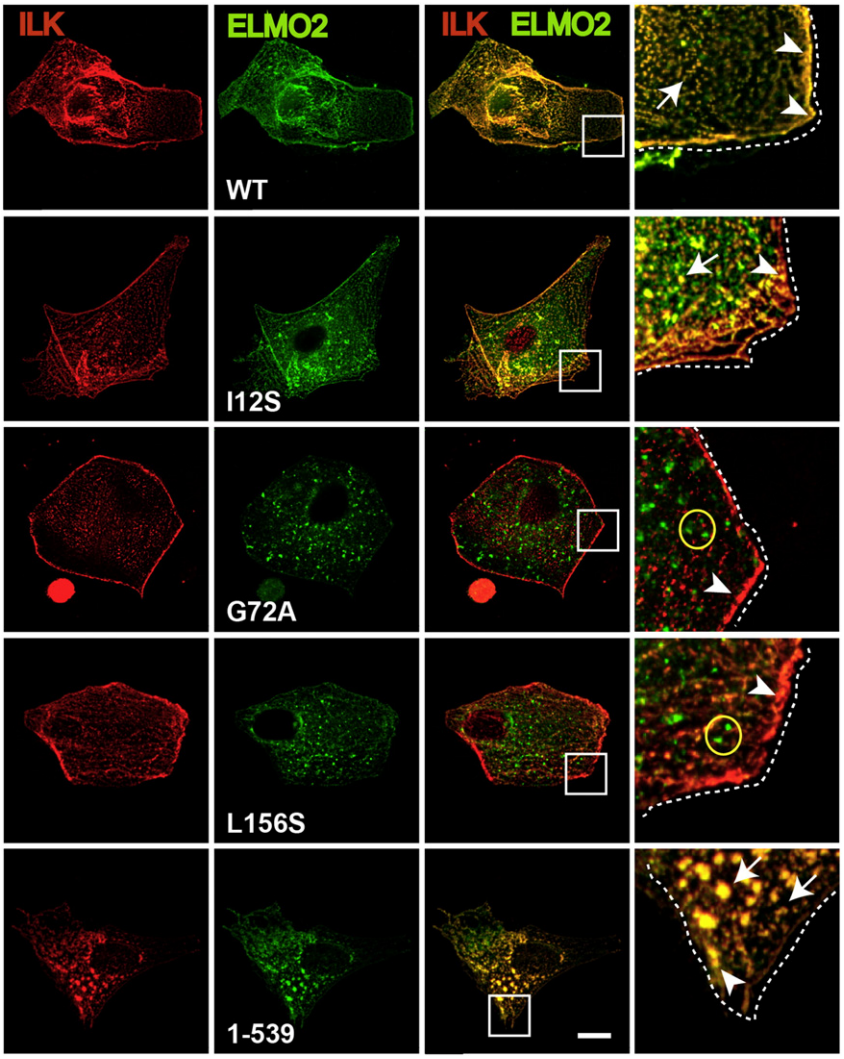


Fig. 2. ILK localization to endosomal vesicles requires its interactions with ELMO2, and ELMO2 also regulates vesicle size. Confluent undifferentiated keratinocytes were transfected with vectors encoding mCherry-tagged ILK together with GFP-tagged wild type (WT) or the indicated ELMO2 mutants. Sixteen hours post-transfection, the cells were cultured for 16 additional hours in High Ca^{2+} medium, and processed for confocal microscopy. Boxed areas are shown at higher magnification in the accompanying micrographs at right. All images represent individual transfected cells that were surrounded by non-transfected keratinocytes. Dashed lines in higher magnification images indicate the plasma membrane of transfected cells. Yellow circles indicate cytoplasmic vesicles positive for only ILK or ELMO proteins. For vesicles showing co-localization of ILK and ELMO2, arrows indicate cytoplasmic vesicles away from the membrane, and arrowheads show vesicles adjacent to the membrane at cell-cell contacts. Note the enlarged vesicles containing both ILK and ELMO2 1-539, relative to vesicles containing all other ELMO2 forms expressed. Bar, 20 μm.

with GFP-tagged ELMO2, we observed their colocalization in these vesicles, within a similar timing of appearance as the individually expressed proteins, after 16 h of culture in High Ca^{2+} medium (Fig. 1A).

We next examined whether the changes in ILK subcellular localization were specifically dependent on increased concentrations of extracellular Ca^{2+}. Several divalent cations modulate keratinocyte function, but do not induce differentiation. For example, changes in extracellular concentrations of Mg^{2+} and Mn^{2+} can enhance keratinocyte attachment and/or motility due to modulation of integrin ligation and expression, respectively [21–23]. To determine if other divalent cations can also modulate the intracellular distribution of ILK, keratinocytes were cultured in medium containing 1 mM Mg^{2+} or 1 mM Mn^{2+} instead of 1 mM Ca^{2+}. Addition of Mg^{2+} or Mn^{2+} to the culture medium did not appreciably alter keratinocyte morphology or ILK subcellular distribution relative to cells cultured in Low Ca^{2+} medium (Fig. 1B). Further, ILK did not redistribute to either cell borders or vesicles in cells cultured in High Ca^{2+} medium in the presence of the Ca^{2+} chelator EGTA (Fig. 1B). To determine whether high extracellular Ca^{2+} concentrations are required for the maintenance of ILK/ELMO2-containing vesicles, we complemented these studies with Ca^{2+} withdrawal experiments. Keratinocytes expressing ILK and ELMO2 were cultured in High Ca^{2+} medium for 16 h. Then, the culture medium was replaced with Low Ca^{2+} medium for up to 2 h (Fig. 1C). Thirty minutes after Ca^{2+} withdrawal, vesicles with ILK and ELMO2 immunoreactivity were still apparent, although these two proteins appeared less evident at cell borders (Fig. 1B). One hour after Ca^{2+} withdrawal, ILK and ELMO2 exhibited a cytoplasmic distribution, and they were no longer detectable in vesicles. Rather, ILK and ELMO2 co-localized to membrane ruffles in a manner reminiscent to their distribution in undifferentiated keratinocytes (Fig. 1C) [11]. Together, these observations indicate that keratinocyte differentiation promotes localization of ILK and ELMO2 to intracellular vesicles through processes that require extracellular Ca^{2+}.

3.2. Recruitment of ILK by ELMO2 to endosomal vesicles

Complexes containing ELMO2, RhoG, and ILK ("ERI" complexes) regulate microtubule dynamics in differentiated keratinocytes [6]. In ERI species, ELMO2 acts as a bridge between ILK and RhoG, and the

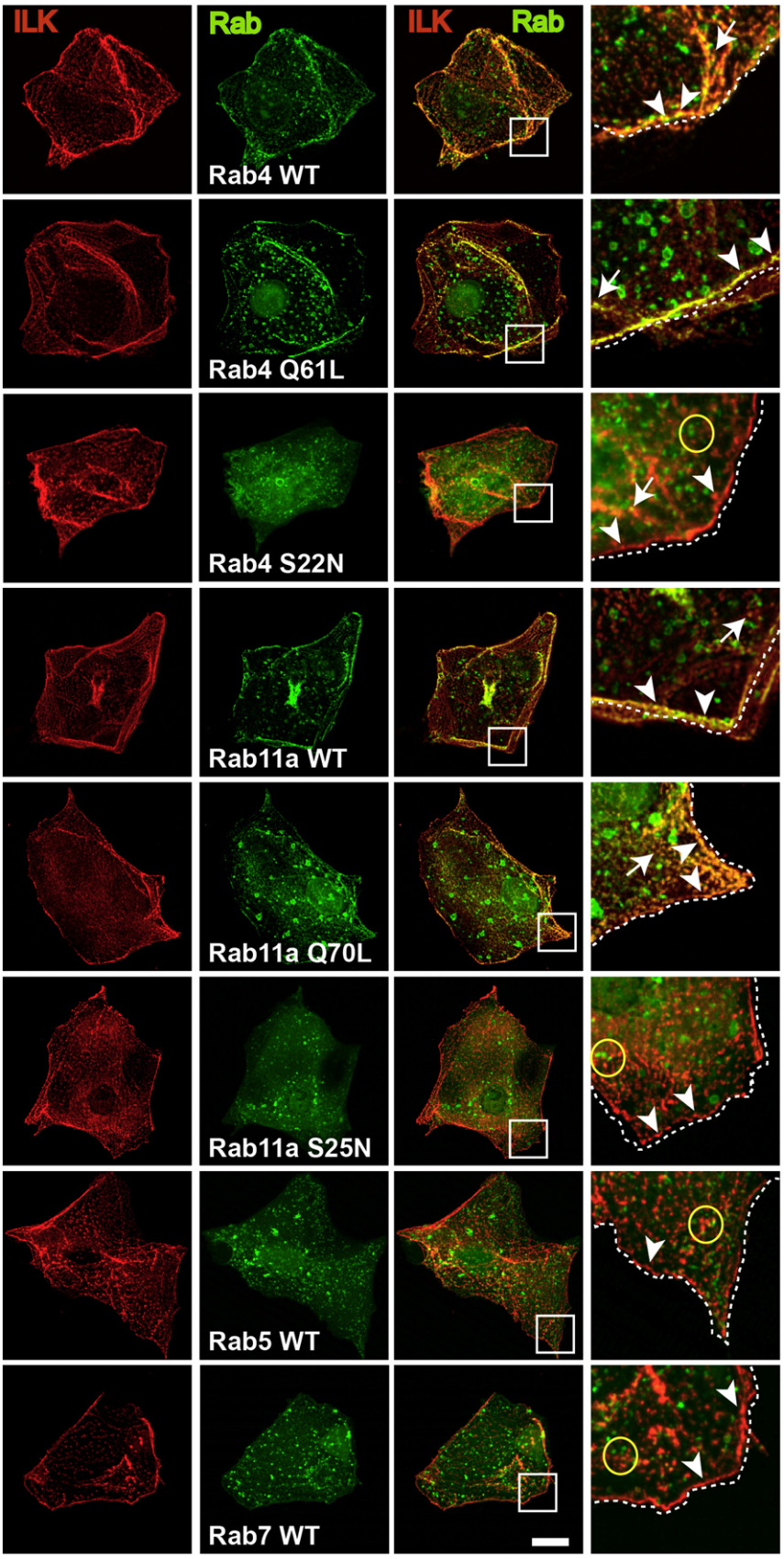


Fig. 3. ILK localizes to recycling endosomes. Confluent keratinocytes cultured in Low Ca^{2+} medium were transfected with vectors encoding mCherry-tagged ILK together with the indicated wild type (WT) or mutant GFP-tagged Rab proteins. Sixteen hours post-transfection, the culture medium was replaced with High Ca^{2+} medium, and 16 h later cells were processed for confocal microscopy. Boxed areas are shown at higher magnification in the accompanying micrographs at right, and dashed lines represent the plasma membrane of transfected cells. Yellow circles indicate cytoplasmic vesicles positive for only ILK or Rab proteins. For vesicles showing co-localization of ILK and Rab4 or Rab11a, arrows indicate cytoplasmic vesicles away from the membrane, and arrowheads show vesicles adjacent to the membrane at cell-cell contacts. The images represent individual transfected cells that were in contact with neighbouring keratinocytes. Bar, 20 μm.

latter can serve to target the complex to areas adjacent to the plasma membrane [12]. To determine if RhoG is implicated in the vesicular localization of ILK and ELMO2, we exogenously expressed in keratinocytes ILK and a mutant ELMO2 protein (I12S) that binds ILK, but does not bind RhoG. ELMO2 I12S was detectable in intracellular vesicles and along cell borders, where it co-localized with ILK, similar to wild type ELMO2 (Fig. 2). Thus, interactions between ELMO2 and RhoG are not required for Ca^{2+}-induced recruitment of ELMO2 and ILK to intracellular vesicles.

We next investigated if interactions between ELMO2 and ILK are necessary for their joint presence in intracellular vesicles. To this end, we expressed ILK together with ELMO2 G72A, (which binds neither RhoG nor ILK) or ELMO2 L156S (which does not bind ILK). We observed that ILK was not detected in the vast majority of vesicles containing either ELMO2 G72A or ELMO2 L156S (Fig. 2). Thus, ELMO2 appears to recruit ILK to endosomal vesicles in differentiating keratinocytes.

The C-terminus of ELMO2 mediates interactions with several proteins, including Dock1 [24]. To determine whether the C-terminus of ELMO2 is important for localization to cell borders and vesicles in differentiated keratinocytes, we analyzed the distribution of jointly expressed ILK and an ELMO2 C-terminus deletion (ELMO2 1-539), and observed their co-localization in intracellular vesicles (Fig. 2), indicating that an intact N-terminal half of ELMO2 is sufficient for recruitment of ILK to these structures. Significantly, those vesicles that contained ELMO2 1-539 were substantially enlarged relative to those observed with wild type ELMO2 (Fig. 2). Specifically, the average cross sectional area of ILK/ELMO2 1-539 vesicles was 7.1 ± 1.0 μm, almost six-fold greater than that in vesicles containing ILK and wild type ELMO2, which was 1.2 ± 0.1 μm. Moreover, ELMO2 1-539 did not localize as prominently to cell borders as wild type ELMO2 and ILK also displayed reduced localization to cell-cell junctions in the presence of ELMO2 1-539 (Fig. 2). The single ELMO orthologue expressed in *Caenorhabditis elegans* has been implicated in the regulation of recycling endosome formation and trafficking, and the observed alterations in cells expressing ELMO2 1-539 suggest that ELMO2 may also be involved in the regulation of endosomal trafficking in keratinocytes.

3.3. Localization of ELMO2 and ILK to recycling endosomes

Endocytic organelles regulate a variety of cellular functions, including protein and particle internalization from the cell surface, recycling of integrins, receptors and adhesion molecules, as well as protein degradation [25]. For example, integrins and various receptors can be internalized to early endosomes and recycled to the plasma membrane through two distinct pathways. In the short-loop recycling pathway, Rab4 regulates the delivery of early endosomal cargo back to the membrane. Alternatively, cargo can traffic from early endosomes to perinuclear recycling compartments, and be delivered back to the membrane through long-loop pathways regulated by Rab11a [26]. To further investigate a potential role of ILK-ELMO2 complexes in trafficking, we first investigated their presence in Rab4- or Rab11a-positive recycling endosomes. Expression of wild type, GFP-tagged Rab4 in keratinocytes cultured in High Ca^{2+} medium for 16 h labelled numerous vesicles throughout the cytoplasm and occasionally associated with cell borders (Supplemental Fig. S2). Both exogenous ILK and ELMO2 were found in a subset of Rab4-containing vesicles, and their presence appeared to be somewhat enriched at regions adjacent to the cell membrane (Figs. 3, 4). To address the relationship between Rab4 activation and presence of ILK and ELMO2 in these endosomes, we co-expressed ILK or ELMO2 with constitutively active Rab4 Q61L or dominant negative Rab4 S22N mutant proteins. The co-localization patterns of ILK and ELMO2 with Rab4 Q61L were indistinguishable from those observed with the wild type protein. In contrast, few vesicles labelled by Rab4 S22N localized to cell borders or contained either ILK or ELMO2 (Figs. 3, 4). Similarly, ILK and ELMO 2 associated with wild type Rab11a and the constitutively active form Rab11a Q70L, but not the inactive Rab11a S25N mutant (Figs. 3, 4).

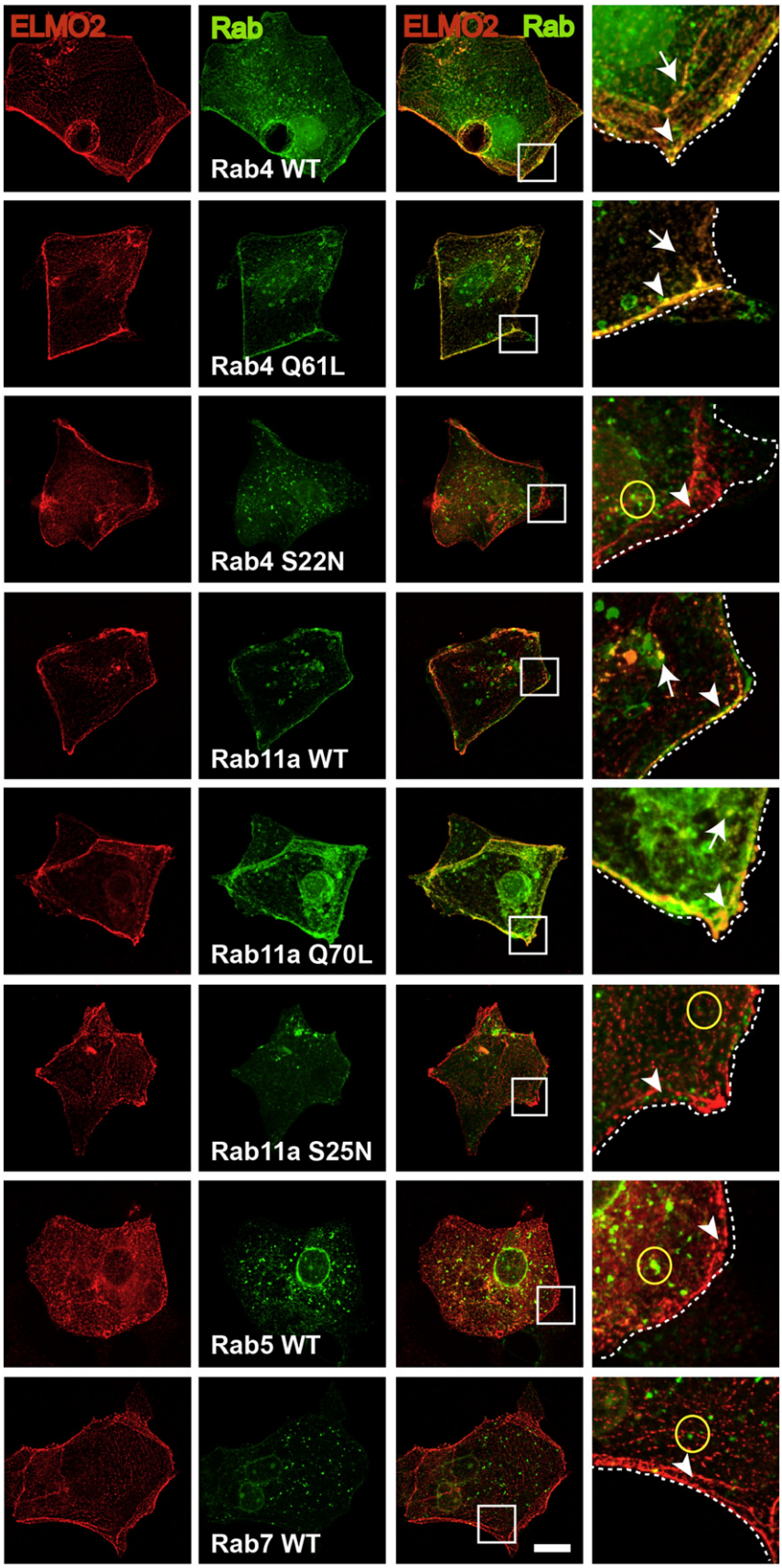
In keratinocytes, uptake of *S. aureus* requires ILK, and internalized bacteria can be found in Rab5-positive early endosomes/phagosomes and in Rab7-positive phagolysosomes [19]. We expressed GFP-tagged Rab5 or Rab7, and did not observe their co-localization with ILK- or ELMO2-containing vesicles (Figs. 3, 4). Expression of these Rab proteins had no detectable effect on the localization of ILK or ELMO2, as they were still found in intracellular vesicles as well as along cell borders. Together, our observations are consistent with the concept that ILK and ELMO2 are present in endosomes integrated into both short- and long-loop recycling pathways, and likely associated with active Rab4 or Rab11a.

3.4. Regulation of Rab11a and E-cadherin trafficking by ILK

Among the various layers of the epidermis, the lowermost basal layer is composed of undifferentiated keratinocytes. Basal keratinocytes exhibit apical-basal polarity. Specifically, focal contacts and hemidesmosomes, which mediate cell adhesion to the extracellular matrix, are found on the basal zone, whereas adherens junctions localize to lateral and apical surfaces, linking neighbouring cells [1]. Keratinocytes cultured in Low Ca^{2+} medium are undifferentiated and proliferative, similar to basal cells, and express E-cadherin and other adherens junctions proteins. However, E-cadherin is mainly found stored in intracellular vesicles in keratinocytes cultured in Low Ca^{2+} medium, which do not form adherens junctions [10]. Increases in extracellular Ca^{2+} to 1 mM induce, within minutes, several signaling cascades, including those linked to activation of the Ca^{2+}-sensing receptor CaR, RhoA, phospholipases and protein kinase C [27]. CaR and activated RhoA contribute to the activation of downstream processes involved in the subsequent delivery of E-cadherin to the plasma membrane and formation of adherens junctions [4].

Within 1 h of culture in High Ca^{2+} medium, keratinocytes produce cell extensions that result in numerous but transient contacts with neighbouring cells, as these extensions frequently retract [28,29]. Occasionally, an intercellular contact becomes a point of stable adhesion, giving rise to an elongated filopodium-like projection. At this stage, the formation of additional cell extensions and contact points ensues at sites adjacent to the first stable contact, giving rise to structures containing E-cadherin and resembling adhesion zippers, which function as intermediates during epithelial sheet formation in epidermal cultures [28,29]. This process is essential to bring membranes of adjacent cells together as a first step in the formation of stable cell-cell contacts, and is not generally observed in confluent immortalized simple-epithelium cell lines, which have been described to partially crawl over, rather than to extend filopodia towards one another [28,29]. These “adhesion zippers” become prevalent in keratinocytes between 1 h and 5 h after switching to culture in High Ca^{2+} medium. In more confluent monolayers this stage is reached in a shorter time period than in sparser cultures. The formation of “adhesion zippers” occurs coordinately with the development of apical-basal polarity and assembly of E-cadherin-containing adherens junctions in these cells [28,29]. Thus, whereas focal contacts can be found on the basal plane of the monolayer, intercellular junctions assemble apically. At the same time, a process of stratification also ensues on the apical regions, giving rise to a sheet of interconnected epidermal cells. In this stratified arrangement, E-cadherin immunoreactivity associated with adherens junctions is observed all along cell borders around 8–12 h after culture in High Ca^{2+} medium, giving the cultures a honeycomb appearance. Through this polarized arrangement, intercellular adhesion, mainly achieved through the formation of cortical actin fibers and alternating adherens junctions and desmosomes, generates a functional seal between the membranes of adjacent cells, creating a barrier at the apical surface of the cultures that resembles the upper layers of the epidermis [28,29].

Thus, culture in High Ca^{2+} medium activates myriad processes in keratinocytes that culminate in terminal differentiation, entry into quiescence, upregulation of differentiation markers, and formation of an epithelial sheet in which the cell membranes of adjacent cells are essentially sealed mainly as a result of the formation of desmosomes, adherens and tight junctions.



In keratinocytes and other epithelial cells, E-cadherin trafficking can occur via Rab4 short- and Rab11a long-loop pathways [26]. Given that ILK and ELMO2 can also be found in recycling endosomes, we next examined if E-cadherin is present in ILK- and ELMO2-containing vesicles. E-cadherin immunoreactivity was observed at cell borders and in intracellular vesicles in keratinocytes cultured in High Ca^{2+} medium for 16 h (Fig. 5). Further, a subset of vesicles containing E-cadherin was also positive for ILK and ELMO2, suggesting that the latter two proteins may be involved in the trafficking of endosomes containing E-cadherin.

We next analyzed the time dependence of changes in Rab11a-containing recycling endosomes and in E-cadherin intracellular localization patterns following culture in High Ca^{2+} medium. We additionally evaluated the role of ILK in these processes. For these experiments, we compared primary keratinocytes isolated from mice with epidermis restricted *Ilk* gene inactivation (hereafter termed ILK^{KO} cells) with cells from ILK-expressing littermates (hereafter termed ILK^{+} cells) [9]. Rab11a-positive endosomes were observed predominantly in perinuclear regions in ILK^{+} cells cultured in Low Ca^{2+} medium ($t = 0$ h, Fig. 6), as well as 2 h after switching the cells to High Ca^{2+} medium (Supplemental Fig. S5). However, we began to detect Rab11a-positive endosomes to areas adjacent to cell-cell contacts after 6 h of culture in High Ca^{2+} medium, and by 16–24 h they were clearly visible all along cell borders (Fig. 6 and Supplemental Fig. S5). This pattern closely mirrored changes in E-cadherin subcellular localization (Supplemental Fig. S5, ILK^{+} cells). Importantly, E-cadherin immunoreactivity was readily observed in association with a subset of Rab11a-positive endosomes in close proximity to cell-cell contacts after 16 h and 24 h of culture in High Ca^{2+} medium (Fig. 6 and Supplemental Fig. S5), suggesting the possibility that delivery of E-cadherin to the cell membrane in response to Ca^{2+} may occur through various pathways, including long-loop Rab11a-mediated vesicle movements.

Cultured ILK^{KO} keratinocytes exhibit deficits in spreading, as well as microtubule stability and growth [6]. Analysis of Rab11a-positive endosomes in ILK^{KO} cells cultured in Low Ca^{2+} medium revealed that they concentrated in perinuclear areas, and generally failed to reach the cell periphery (Fig. 6). We observed E-cadherin immunoreactivity in a subset of Rab11a-positive vesicles, as well as in other vesicles distributed throughout the cytoplasm (Fig. 6). Notably, ILK^{KO} keratinocytes continued to show Rab11a- and E-cadherin-containing vesicles in perinuclear regions, rather than localized to areas adjacent to cell-cell contacts, even after 16–24 h of culture in High Ca^{2+} medium (Fig. 6 and Supplemental Fig. S5). Significantly, the characteristic honeycomb appearance of the cultures, visible through E-cadherin immunoreactivity in ILK-expressing cells, was absent in ILK^{KO} keratinocytes (e.g. Supplemental Fig. S5, 24 h micrographs).

To better understand the positioning of Rab11a-containing recycling endosomes in keratinocytes, we used time-lapse videomicroscopy to visualize GFP-tagged Rab11a movements during Ca^{2+}-induced differentiation in ILK^{+} keratinocytes (Supplemental Videos 1 and 2, and Fig. 7). Because primary keratinocytes exhibited susceptibility to phototoxicity after repeated imaging events, we recorded GFP-Rab11a in cells incubated in High Ca^{2+} medium for 7 h–16 h. We initially observed abundant GFP-Rab11a-positive vesicles in perinuclear areas, and by 10 h they were clearly visualized concentrated as groups in discrete regions at the cell membrane that were adjacent to neighbouring cells. Rab11a-positive vesicles appeared to remain concentrated in such discrete regions for as long as 6–7 h. Similar experiments were conducted with ILK^{KO} keratinocytes. Initial experiments did not show evidence of

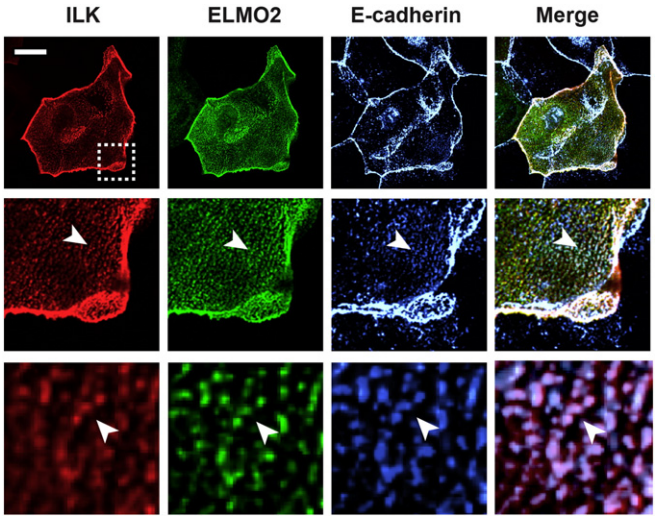


Fig. 5. E-cadherin localizes to ELMO2/ILK-positive endosomes. Confluent keratinocytes were cotransfected with vectors encoding mCherry-tagged ILK and GFP-tagged ELMO2, and cultured in Low Ca^{2+} medium for 16 h, followed by culture in High Ca^{2+} medium for an additional 16-h period. The cells were then processed for confocal microscopy to detect exogenous ILK and ELMO2, as well as endogenous E-cadherin. The images represent individual transfected cells that were in contact with neighbouring keratinocytes. Boxed areas are shown at higher magnification in the accompanying micrographs underneath. Arrowheads indicate examples of intracellular vesicles where ILK, ELMO2 and E-cadherin are jointly detected. Bar, 25 μm.

Rab11a-containing vesicle translocation to cell borders within the first 16 h of incubation in High Ca^{2+} medium (data not shown). Therefore, and to avoid cell death due to phototoxicity, we extended our analysis and generated time-lapse images of cells that had been cultured in the presence of 1 mM Ca^{2+} for 16-through-24 h (Supplemental Videos 3 and 4, and Fig. 8). We found prominent Rab11a-containing vesicles in these cells, but the vesicles were rarely observed at areas adjacent to the cell membrane. Together, the data suggest that the intracellular positioning of Rab11a/E-cadherin-positive endosomes is modulated by Ca^{2+} in differentiating keratinocytes through mechanisms that also require ILK, although ILK may not be essential for the formation of E-cadherin-containing endosomes per se.

3.5. Regulation of Rab11a and E-cadherin endosomes by ELMO2

The presence of enlarged vesicles we had observed in keratinocytes expressing the ELMO2 1-539 truncation mutant prompted us to extend our studies to further investigate the role of ELMO2 in E-cadherin endosomal trafficking, using RNAi to downregulate ELMO2. For these experiments, we used iKT keratinocytes, because their viability was unaffected by the transfection procedure. iKT cells are a spontaneously immortalized mouse epidermal keratinocyte line we previously generated, and respond to culture in High Ca^{2+} medium in a manner undistinguishable from primary keratinocytes [6]. In these studies, we transfected the cells with control or ELMO2-targeting siRNAs, followed 48 h later by a second transfection with a vector encoding GFP-tagged Rab11a. Twenty-four hours after the second transfection, the cells were cultured in High Ca^{2+} medium, and the subcellular distribution of GFP-Rab11a and endogenous E-cadherin was assessed in fixed specimens at timed intervals thereafter. Similar to our findings in primary

Fig. 4. ELMO2 localizes to recycling endosomes. Confluent keratinocytes cultured in Low Ca^{2+} medium were transfected with vectors encoding FLAG-tagged ELMO2 together with the indicated wild type (WT) or mutant GFP-tagged Rab proteins. Sixteen hours post-transfection, the culture medium was replaced with High Ca^{2+} medium, and 16 h later cells were processed for confocal microscopy. Boxed areas are shown at higher magnification in the accompanying micrographs at right, and dashed lines represent the plasma membrane of transfected cells. Yellow circles indicate cytoplasmic vesicles positive for only ELMO2 or Rab proteins. For vesicles showing co-localization of ELMO2 and Rab4 or Rab11a, arrows indicate cytoplasmic vesicles away from the membrane, and arrowheads show vesicles adjacent to the membrane at cell-cell contacts. The images represent individual transfected cells that were in contact with neighbouring keratinocytes. Bar, 20 μm.

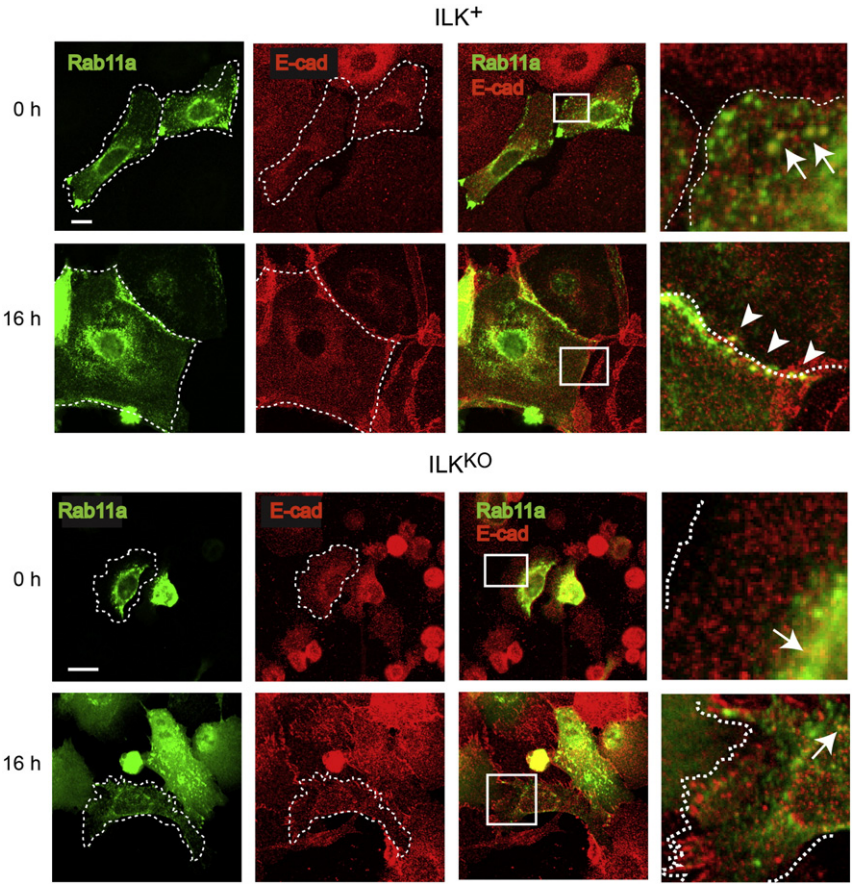


Fig. 6. Rab11a/E-cadherin-containing recycling endosomes do not localize to cell borders in ILK^{KO} keratinocytes. ILK-expressing (ILK^{+}) or ILK-deficient (ILK^{KO}) keratinocytes were cultured in Low Ca^{2+} medium and transfected with a vector encoding GFP-tagged Rab11a. Sixteen hours post-transfection, the cells were switched to High Ca^{2+} medium, and processed for confocal microscopy at the indicated times thereafter, using anti-E-cadherin antibodies and GFP-associated fluorescence. The plane of imaging shown represents regions towards the apical cell surface. Dashed lines outline the membrane of transfected cells. Boxed areas are shown at higher magnification in the accompanying micrographs at right. Examples of vesicles positive for both Rab11a and E-cadherin adjacent to cell-cell contacts or in cytoplasmic regions are shown, respectively, with arrowheads and arrows. Bar, 20 μm.

mouse keratinocytes, both GFP-Rab11a and E-cadherin immunoreactivity were visualized at regions of cell-cell contacts in ELMO2^{+} iKT cells transfected with a negative control siRNA, starting within 6 h after culture in High Ca^{2+} medium (Fig. 9 and Supplemental Fig. S6).

To generate ELMO2-deficient iKT cells, we tested several independent ELMO2 siRNAs, and were able to induce substantial decreases in ELMO2 protein levels with one of them (siRNA 86210; Supplemental Fig. S4). Keratinocytes treated with this ELMO2 siRNA (ELMO2^{KD} cells) did not show any appreciable defects in adhesion, spreading or viability when cultured in Low Ca^{2+} medium. Under these conditions, Rab11a-positive vesicles were prominent in perinuclear regions. Significantly, we also noted enlarged cross-sectional areas in a fraction of Rab11a-positive endosomes (Fig. 9). In ELMO2^{KD} cells cultured in High Ca^{2+} medium, we did not observe substantial enrichment of Rab11a-containing endosomes at areas adjacent to the cell membrane even after 24 h, although enlarged perinuclear vesicles were readily observed (Fig. 9 and Supplemental Fig. S6). Thus, ELMO2 deficiency may alter normal Rab11a-containing long-loop endosome trafficking. Similarly, in ELMO2^{KD} cells, E-cadherin failed to concentrate at the cell membrane, and the typical honeycomb arrangement of E-cadherin immunoreactivity observed in normal cells as early as 8 h after culture in High Ca^{2+} was not evident in ELMO2^{KD} keratinocytes, even after 24 h (Fig. 9 and Supplemental Fig. S6). As mentioned above, the formation of cell-cell contacts in differentiating keratinocytes occurs somewhat faster in more confluent cultures, but this process is completed within approximately 8 h of culture in High Ca^{2+} medium, irrespective of cell density. Significantly, the characteristic honeycomb appearance of the cultures, visible through E-cadherin immunoreactivity in ELMO2-expressing cells, was absent in ELMO2^{KD} keratinocytes (e.g. Supplemental Fig. S6, 24 h micrographs). Because ELMO2^{KD} cells were about 90% confluent in these experiments, similar to ELMO2-expressing keratinocytes, we conclude that defects in long-loop recycling endosome movements may contribute to the significant abnormalities in E-cadherin delivery to cell borders observed in ELMO2-deficient differentiating keratinocytes.

4. Discussion

Our study underscores ILK and ELMO2 as critically important regulators of proper keratinocyte differentiation and formation of epithelial sheets. These two proteins are necessary for normal E-cadherin delivery to the plasma membrane in response to induction of differentiation by Ca^{2+}. Importantly, our work uncovers a hitherto unrecognized mechanism whereby ILK and ELMO2 modulate the positioning of long-loop recycling endosomes containing E-cadherin in these cells.

ILK plays pleiotropic roles in the epidermis, from maintenance of tissue integrity to establishment of keratinocyte polarity. In particular, apical-basal polarity in hair follicle cells and basal interfollicular keratinocytes is severely altered in the absence of ILK, through mechanisms that appear to involve abnormal extracellular matrix deposition [2]. In polarized simple epithelia, apical microtubules participate in and promote clustering of E-cadherin and adherens junction maintenance, through processes modulated by integrins [30]. Epidermal keratinocytes are maintained in the undifferentiated state by culture in Low Ca^{2+} medium, and express integrins and E-cadherin, but adherens junctions do not form because the latter protein does not

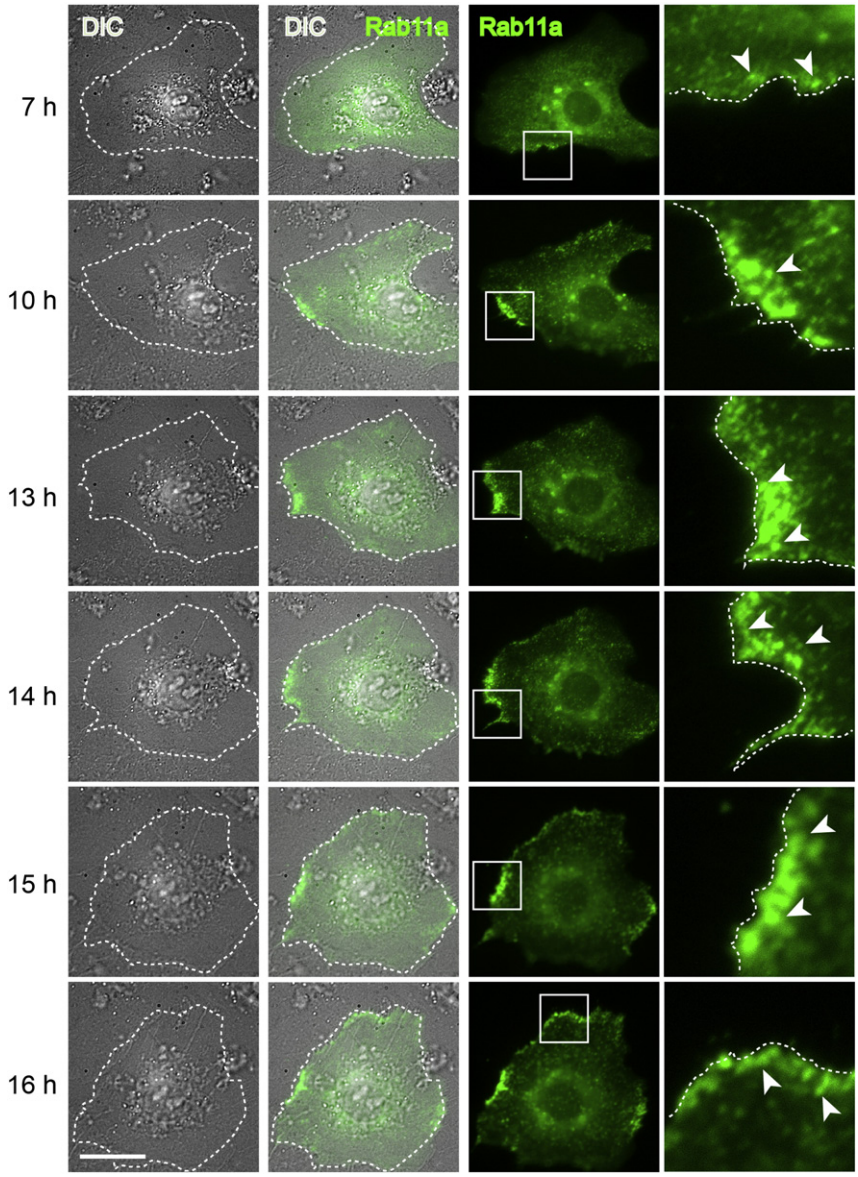


Fig. 7. Rab11a-containing endosomes are found at areas adjacent to cell-cell contacts in differentiating ILK^{+} keratinocytes. ILK-expressing (ILK^{+}) keratinocytes were cultured in Low Ca^{2+} medium and transfected with a vector encoding GFP-tagged Rab11. Sixteen hours post-transfection, the cells were switched to High Ca^{2+} medium. Seven hours later, DIC and GFP fluorescence images of live cells were acquired by time-lapse videomicroscopy. The micrographs show images of a transfected cell in a keratinocyte epithelial sheet, acquired at the indicated times of culture in High Ca^{2+} medium. Boxed areas are shown at higher magnification in the accompanying micrographs at right. Dashed lines show the borders of the imaged cell, and arrowheads indicate Rab11a-positive endosomes adjacent to the cell membrane. Bar, 20 μm.

form homotypic contacts bridging adjacent cells. In undifferentiated keratinocytes, ILK stabilizes microtubules at the cell cortex through processes that involve binding to and recruitment of IQGAP1. Disruption of these interactions through *Intb1* or *Ilk* gene inactivation prevents fusion of caveolae and delivery of their cargo to the basal aspect of the plasma membrane [7]. Significantly, ILK is not found in caveolin 1-positive vesicles, further confirming the notion that its modulation of caveolar traffic occurs indirectly.

The differentiation of keratinocytes by culture in High Ca^{2+} medium is accompanied by downregulation of β1 integrins and assembly of cell--cell junctions [6,29] Under these conditions, ILK also participates in the modulation of microtubule stability. In differentiated keratinocytes, multiprotein complexes containing ELMO2, RhoG and ILK function as signaling platforms to stabilize microtubules [6]. In vivo, the significance of ILK in these processes is emphasized by the abnormal formation and distribution of adherens and tight junctions in the differentiated keratinocytes of the spinous and granular layers, which is accompanied by deficits in epidermal permeability barrier properties [4].

Both ELMO and ILK are scaffolds that act as hubs for a variety of signaling pathways. Three ELMO proteins that share high homology have been described in mammals. They all bind members of the Dock family and activate Rac1 in a variety of cell types [31]. Few properties specific to ELMO2 have been described, including its ability to interact with ILK [11]. Indeed, ELMO2 can recruit ILK to lamellipodia, where it also binds to active RhoG. This ternary complex promotes lamellipodia formation, front-rear polarity and directional migration in undifferentiated keratinocytes [11,12]. Our studies now show that ELMO2 also has unique functions among mammalian ELMO homologues associated with its interactions with ILK and modulation of Rab11a-positive endosomes.

The single ELMO orthologue expressed in *Drosophila melanogaster* was recently identified in a genome-wide screen as a protein required for the formation cadherin-based intercellular adhesions [32]. In mammalian MDCK kidney epithelial cells, ELMO2, but not ELMO1 or ELMO3, has been implicated in the assembly of E-cadherin mediated adherens junctions [24]. When MDCK cells are cultured in medium with low

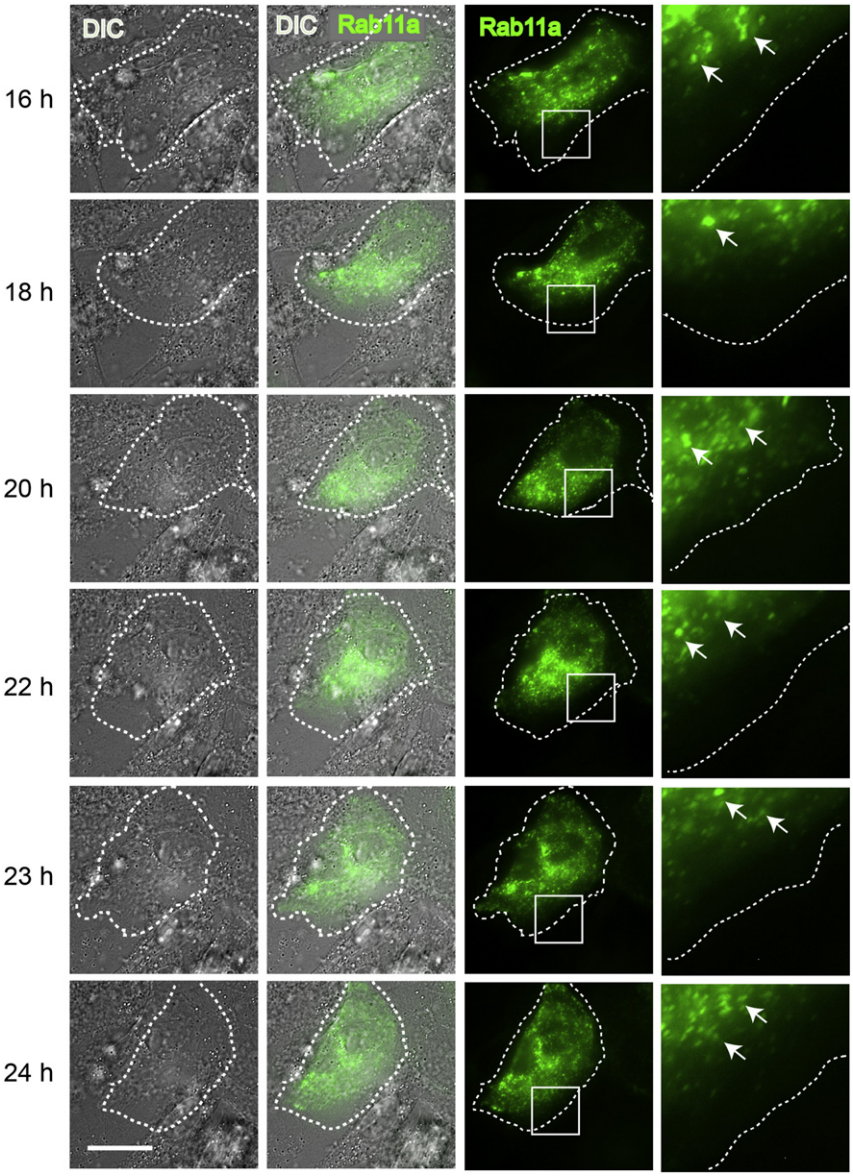


Fig. 8. Rab11a-containing endosomes do not appreciably localize to cell borders in differentiating ILK^{KO} keratinocytes. ILK-deficient (ILK^{KO}) keratinocytes were cultured in Low Ca^{2+} medium and transfected with a vector encoding GFP-tagged Rab11. Sixteen hours later, DIC and GFP fluorescence images of live cells were acquired by time-lapse videomicroscopy. The micrographs show images of a transfected cell, acquired at the indicated times of culture in High Ca^{2+} medium. Boxed areas are shown at higher magnification in the accompanying micrographs at right. Dashed lines show the borders of the imaged cell, and arrows indicate Rab11a-positive endosomes found in the cytoplasm, but away from the cell membrane. Bar, 20 μm.

Ca^{2+} concentrations, E-cadherin adherens junctions are absent. Culture of these cells in 1.8 mM Ca^{2+} results in formation of adherens junctions by 5 h. In this system, ELMO2 is recruited to initial cell-cell contacts, where it plays key roles in modulating actin reorganization and Rho GTPase activation, necessary for the formation of stable E-cadherin intercellular junctions. Significantly, once adherens junctions mature in MDCK cells, ELMO2 no longer associates with them, but rather is found throughout the cytoplasm. In this system, the absence of ELMO2 slightly delays adherens junction formation, by about 2.5 h [24], suggesting the presence of alternative and/or additional pathways that mediate the formation of these structures. In cultured differentiating keratinocytes, adherens junction assembly and generation of sealed cell-cell borders normally occurs within 4 h–8 h of Ca^{2+} addition, depending on the confluence of the cell monolayer. In these cells, Ca^{2+} induces initial formation of filopodial membrane extensions containing F-actin and E-cadherin between adjacent cells, which progress to form interdigitated structures that ultimately seal cell borders [10,29]. In the absence of detectable ELMO2 in differentiating keratinocytes, we observed E-cadherin immunoreactivity in numerous cytoplasmic vesicles. Moreover, the presence of E-cadherin at cell borders only appeared to occur uniformly after about 16 h of culture in High Ca^{2+} medium. This is approximately a 10-h delay relative to ELMO2-expressing keratinocytes. Significantly, the positioning of Rab11a-containing recycling endosomes to areas adjacent to cell borders was also substantially impaired in the absence of ELMO2. Under these conditions, enlarged Rab11a-positive vesicles were observed in perinuclear regions, suggestive of impaired trafficking. In our experiments, not all E-cadherin-containing vesicles were positive for Rab11a, suggesting that E-cadherin may be delivered to the plasma membrane through other pathways, in addition to Rab11a long-loop recycling endosomes. The activation of these additional pathways may be responsible, at least in part, for the observation that cell-cell contacts are eventually observed, even in the absence of ELMO2. Thus, our studies now implicate a novel role for ELMO2 in intracellular positioning of recycling endosomal vesicles in epidermal keratinocytes. Consistent with these properties, the *C. elegans* ELMO orthologue CED-12 also

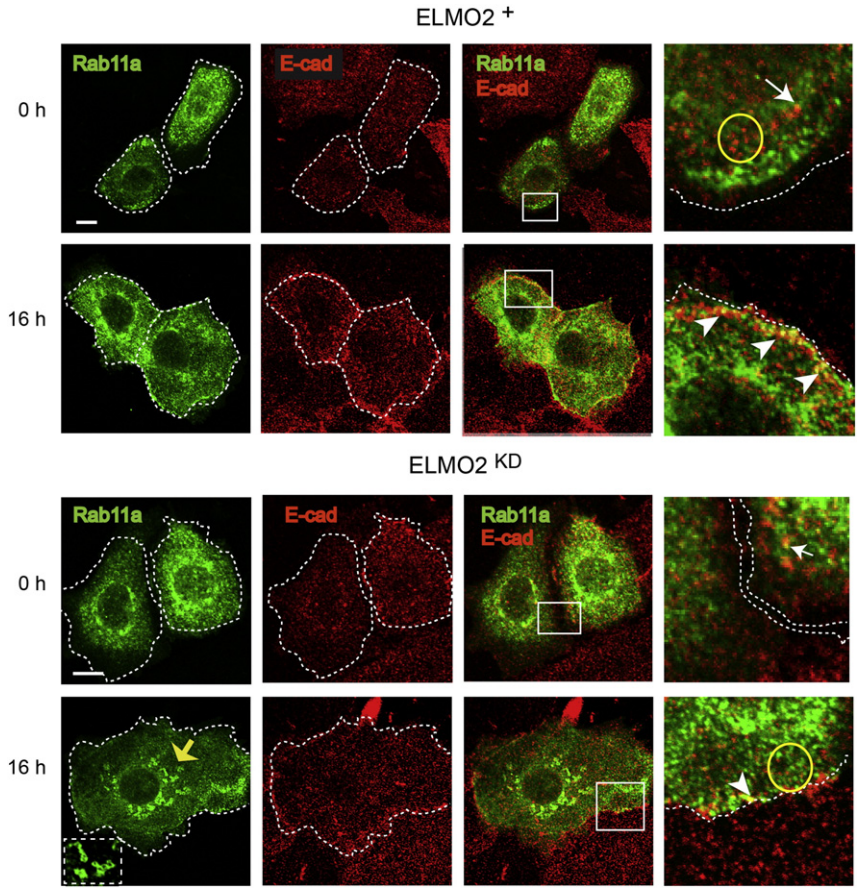


Fig. 9. ELMO2 is required for normal positioning of Rab11a/E-cadherin-containing recycling endosomes to cell borders in differentiating keratinocytes. iKT cells were cultured in Low Ca^{2+} medium and transfected with a negative control siRNA ($ELMO2^{+}$), or siRNA targeting ELMO2 (siRNA 210; $ELMO2^{KD}$). Seventy-two hours later, the cells were transfected with a vector encoding GFP-tagged Rab11a. Sixteen hours following the second transfection, the cells were cultured in High Ca^{2+} medium, and processed for confocal microscopy at the indicated times thereafter, using anti-E-cadherin antibodies and GFP-associated fluorescence. The plane of imaging shown represents regions towards the apical cell surface. Dashed lines outline the cell membrane. Boxed areas are shown at higher magnification in the accompanying micrographs at right. Yellow circles indicate cytoplasmic vesicles positive for only E-cadherin or Rab11a. Examples of vesicles positive for both Rab11a and E-cadherin adjacent to cell-cell contacts or in cytoplasmic regions are shown, respectively, with arrowheads and arrows. The yellow arrow in the $ELMO2^{KD}$ cell imaged at 16 h indicates a region shown in the inset containing abnormal and enlarged Rab11a-positive endosomes. Bar, 20 μm.

regulates recycling endosome trafficking, and its absence results in cargo trapping in these compartments [8]. Whether other mammalian ELMO orthologues participate in these processes will be an important area for future research.

Endocytic trafficking plays central roles in epithelial cells. For example, the distribution of membrane proteins in migrating cells is organized in a front-rear orientation through endocytic pathways [33]. This form of movement and polarization is relevant for cutaneous injury repair, during which keratinocytes move forward on a temporary matrix covering the wound. In vivo, ILK is necessary for the migration of activated keratinocyte stem cells out of the hair follicle bulge and upwards towards the wound edge [34]. ILK and ELMO2 are involved in the development of front-rear polarity in cultured keratinocytes [12], and some of their actions may involve modulation of recycling endosomes.

Endocytic traffic and recycling also contribute to the establishment of apical-basal polarity and formation of tight epithelial sheets. This process has been extensively studied in renal epithelial cells and other simple epithelia. In those cells, Rab11a is a well-established regulator of exocytic and recycling processes [30]. In particular, Rab11a localizes to apical recycling endosomes, regulating the trafficking and sorting of E-cadherin to apical cell surfaces, through mechanisms that involve complex formation with motor proteins and bidirectional transport along microtubule tracks [35]. Protein trafficking in epidermal keratinocytes, which form a stratified squamous epithelium, is less well characterized, although evidence for its importance in the epidermis has begun to emerge. For example, the cadherin Celsr1, involved in the control of planar cell polarity, was recently shown to undergo endocytosis during keratinocyte mitosis, followed by recycling back to the plasma membrane [36]. Similarly, β4 integrin is constitutively endocytosed and delivered to Rab11a-positive recycling endosomes, and this cycle is essential to maintain hemidesmosome stability in undifferentiated keratinocytes and epidermal integrity [37]. Equally scant is the understanding of how Rab11a is regulated in keratinocytes. We now show that Rab11a-positive vesicles respond to increases in extracellular Ca^{2+}, positioning themselves to areas adjacent to the plasma membrane. Rab11a has been detected in apical regions of differentiated keratinocytes, from the mid-spinous layer to the upper granular layers [38]. These epidermal layers also contain epidermal lamellar granules filled with structural proteins, lipids and proteases. Lamellar granules fuse with the plasma membrane and release their contents, contributing to the generation of the epidermal permeability barrier. Because abnormalities in lamellar granule proteins are associated with severe skin diseases [38], it will be important to determine if Rab11a, ILK and/or ELMO2 modulate exocytosis of keratinocyte secretory proteins. Equally important will be to develop deeper insight into the regulation and integration of the different Rab11a functions that participate in cellular signal transduction cascades in the epidermis.

In summary, we have demonstrated that ILK and ELMO2 modulate positioning of Rab11a-E-cadherin-containing endosomes to areas adjacent to cell-cell contacts in response to Ca^{2+}-induced keratinocyte differentiation. Whether these two proteins also regulate other

endosomal pathways to establish cell polarity will be an important area for future research.

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Conflict of interest

The authors declare no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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