Foxj1 is required for apical localization of ezrin in airway epithelial cells

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Summary

Establishment and maintenance of epithelial cell polarity depend on cytoskeletal organization and protein trafficking to polarized cortical membranes. ERM (ezrin, radixin, moesin) family members link polarized proteins with cytoskeletal actin. Although ERMs are often considered to be functionally similar, we found that, in airway epithelial cells, apical localization of ERMs depend on cell differentiation and is independently regulated. Moesin was present in the apical membrane of all undifferentiated epithelial cells. However, in differentiated cells, ezrin and moesin were selectively localized to apical membranes of ciliated airway cells and were absent from secretory cells. To identify regulatory proteins required for selective ERM trafficking, we evaluated airway epithelial cells lacking Foxj1, an F-box factor that directs programs required for cilia formation at the apical membrane. Interestingly, Foxj1 expression was also required for localization of apical ezrin, but not moesin. Additionally, membrane-cytoskeletal and threonine-phosphorylated ezrin were decreased in Foxj1-null cells, consistent with absent apical ezrin. Although apical moesin expression was present in null cells, it could not compensate for ezrin because ERM-associated EBP50 and the β_2 adrenergic receptor failed to localize apically in the absence of Foxj1. These findings indicate that Foxj1 regulates ERM proteins differentially to selectively direct the apical localization of ezrin for the organization of multi-protein complexes in apical membranes of airway epithelial cells.

Key words: Cytoskeleton, Differentiation, Lung, Cilia, Mouse

Introduction

Epithelial cell differentiation requires a highly regulated sequence of molecular events to establish polarized domains with specialized functions. Establishment of epithelial cell polarity is initially directed by cell adhesion molecules, followed by organization of the cytoskeleton and sorting of proteins to basolateral and apical compartments (Yeaman et al., 1999). Protein clustering at the cortical membranes occurs, in part, through binding to amino acid sequences called PDZ domains located on sub-membrane scaffolding proteins (Fanning and Anderson, 1999). Genetic studies in *Drosophila* and *Caenorhabditis elegans* reveal that PDZ-domain-containing proteins polarize to apical and basolateral compartments to mediate epithelial cell differentiation for signaling and vectoral transport (Bredt, 1998; Knust, 2000). PDZ-domain proteins are held polarized and fixed to the cytoskeleton by members of the Band 4.1 family (Fanning and Anderson, 1999; Knust, 2000). These proteins, including the ERM proteins ezrin, radixin and moesin, perform structural and regulatory functions at polarized domains (reviewed in Mangeat et al., 1999; Bretscher et al., 2002). Thus, identification of factors regulating PDZ-domain and ERM-protein localization is crucial for understanding establishment and maintenance of cell polarity and differentiation.

ERM family members are closely related proteins expressed in varied combinations in different cell types to act as linkers between the cortical membrane and actin cytoskeleton. Patterns of expression of the three most conserved ERMs (ezrin, radixin and moesin) are similar in epithelial cell lines, cultured fibroblasts, leukocytes and neurons, occurring at projections such as microvilli, ruffled membranes, uropods or growth cones (Bretscher, 1983; Takeuchi et al., 1994; Bonilha et al., 1999; Parlato et al., 2000). When polarized at these sites, ERMs maintain an activated conformation linking cortical membrane proteins and cytoskeletal actin through conserved N- and C-terminal domains termed N- and C-ERM-association domains (N- and C-ERMADs), respectively (Algrain et al., 1993; Turunen et al., 1994; Gary and Bretscher, 1995). The N-ERMAD can bind directly to plasma membrane proteins in the case of ICAM-1, 2, 3, CD43 and CD44 or, indirectly, through binding to PDZ-domain proteins such as EBP50 (ERM-binding phosphoprotein 50, also called NHERF) and E3KARP (Reczek et al., 1997; Yun et al., 1997). In turn, the PDZ domains in EBP50 provide docking sites for transmembrane proteins including the cystic fibrosis transmembrane conductance regulator (CFTR), β_2 adrenergic receptor (β_2AR), and the NHE3 exchanger (Reczek et al., 1997; Hall et al., 1998; Yonemura et al., 1998; Shenolikar and Weinman, 2001). Additionally, ERMs function as A-kinase-anchoring proteins (AKAPs) by binding molecules that confer transmembrane protein regulation (Dransfield et al., 1997; Yun et al., 1997; Sun et al., 2000). Thus, polarized ERM proteins play a central role

in organizing and regulating specialized apical membrane proteins.

Accumulating information indicates that polarized ERMs are derived from a large cytoplasmic pool of dormant ERM proteins present in a folded conformation favoring N-ERMAD and C-ERMAD self-association, masking cortical-membrane- and F-actin-binding sites (Gary and Bretscher, 1995; Magendantz et al., 1995; Gautreau et al., 2000; Pearson et al., 2000). Following an activation signal, phosphorylation of a conserved threonine in the C-ERMAD (T567 in ezrin, T564 in radixin, and T558 in moesin) is associated with protein unfolding to reveal cortical-membrane- and F-actin-binding domains (Matsui et al., 1998; Shaw et al., 1998; Nakamura et al., 1999; Gautreau et al., 2000). Evidence supporting that threonine phosophorylation regulates ERM activation and retention at cortical membranes includes the association of unfolded ERMs with biochemical detection of phosphorylated ERM and immunofluorescent localization of phosphorylated ERM in actin-rich membrane structures (Shaw et al., 1998; Matsui et al., 1998; Hayashi et al., 1999; Nakamura et al., 1999; Gautreau et al., 2000). Kinase(s) responsible for this function are not well defined and in vivo specificity for regulation of different ERMs is not known, but several kinases have been shown to be capable of in vitro ERM threonine phosphorylation (Matsui et al., 1998; Pietromonaco et al., 1998; Jeon et al., 2002). Additional studies show Rho-mediated signal transduction and phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2) also play roles in ERM activation but regulatory pathways are not fully identified (Oshiro et al., 1998; Shaw et al., 1998; Matsui et al., 1999; Nakamura et al., 1999; Yonemura et al., 2002).

Differentiation of the airway epithelium is highly regulated to generate ciliated and secretory cells with unique apical membrane functions. We and others have shown that forkhead box (F-box) transcription factor Foxj1 (previously HFH-4) is expressed in ciliated epithelial cells and is required for differentiation of ciliated cells (Chen et al., 1998; Blatt et al., 1999; Tichelaar et al., 1999; Brody et al., 2000). Foxj1 deficient mice fail to develop cilia (Chen et al., 1998; Brody et al., 2000). Although cilia precursors are present within the apical cell compartment, there is a failure of basal bodies to dock at the apical membrane and form cilia (Brody et al., 2000). To determine whether Foxj1 has a more global function in directing programs for localization of apical membrane proteins, we evaluated the expression of ERM proteins in a primary airway epithelial-cell culture model in which differentiation can be modulated, and in Foxj1-deficient mice. We found that in highly differentiated epithelial cells, apical ezrin and moesin are expressed only in ciliated cells, but that Foxj1 is specifically required for apical localization of ezrin but not moesin. The ciliated cells also selectively expressed a complex of apical membrane proteins containing ERM-associated EBP50 and β_2 AR. Importantly, the Foxj1-null cells lacking apical ezrin also lacked these proteins at the apical membrane. These observations indicate that moesin cannot compensate for ezrin and that Foxj1 is necessary for localization of ezrin and crucial apical protein complexes.

Materials and Methods

Antibodies

Primary antibodies and dilutions or concentrations used were: mouse anti-ezrin (1 μg/ml^{-1}, clone 3C12, Neomarkers, Fremont, CA); rabbit anti-ezrin (1:500, Upstate Biotech, Lake Placid, NY); mouse anti-β-tubulin-IV (1:250; BioGenex, San Ramon, CA); rabbit and goat anti-mouse Clara cell secretory protein (1:500; CCSP; kindly provided by S. Reynolds, University of Pittsburgh, Pittsburgh, PA); β-catenin (1 μg/ml^{-1}, Chemicon); α-actin (0.5 μg/ml^{-1}; Chemicon, Temecula, CA), rabbit anti-Foxj1 (1:500) (Blatt et al., 1999); rabbit anti-phosphothreonine ERM (1:500, Cell Signaling, Beverly, MA); rabbit anti-moesin (1 μg/ml^{-1}, Upstate Biotech); rabbit anti-moesin (Franck et al., 1993; kindly provided by A. Bretscher, Cornell University, Ithaca, NY); rabbit anti-EBP50 (1 μg/ml^{-1}, Affinity Bioreagents, Golden, CO); rabbit β_2AR (1:200, Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-E-cadherin (1:500, Santa Cruz Biotechnology); and mouse anti-Na^+K^+-ATPase (H1-α1, 1 μg/ml^{-1} kindly provided by R. Lubman, University of Southern California, Los Angeles, CA).

Immunohistochemistry

Mouse tissues were fixed with 4% paraformaldeyde for 1 hour at room temperature and subjected to antigen retrieval as described previously (Blatt et al., 1999). Cells on supported membranes were fixed with 4% paraformaldehyde in PBS, pH 7.4 for 10 minutes at 25°C, and processed for immunodetection as described previously (You et al., 2002). Fixed samples were incubated for 2 hours at 25°C or 18 hours at 4°C with isotype-matched control antibody or primary antibody. Antibody binding was detected using fluorescein isothiocyanate (FITC)- or indocarbocyanine (CY3)-labeled secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). No detectable staining was observed for isotype matched control antibodies. Membranes were mounted on slides with media (Vectashield, Vector, Burlingame, CA) containing 4′,6-diamidino-2-phenylindole (DAPI) to stain intracellular DNA. Microscopy was performed using a Zeiss laser scanning system with LSM-510 software (Zeiss, Thornwood, NY) and an Olympus BX51 (Melville, NY) for reflected fluorescent with a CCD camera interfaced with MagniFire software (Olympus) for image acquisition. Images were composed using Photoshop and Illustrator software (Adobe Systems, San Jose, CA).

Mouse tracheal epithelial cell isolation and culture

Mouse tracheal epithelial (MTE) cells were harvested and grown on supported membranes under air-liquid interface (ALI) conditions as described (You et al., 2002). Before culturing, purified cells were greater than 99% cytokeratin expressing epithelial cells when immunostained. MTE cells were cultured on semi-permeable membranes (Transwell, Corning-Costar, Corning, NY). Media was maintained in upper and lower chambers until the transmembrane resistance was greater than 1000 Ω cm^2, indicating tight junction formation. Media was then removed from the upper chamber to establish ALI.

Cell lysis and subcellular fractionation

Total cell lysates were resuspended in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate) containing protease [1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml^{-1} leupeptin, 10 μg/ml^{-1} aprotonin] and phosphatase inhibitors (10 mM NaF, 1 mM orthovanadate, 2 mM sodium pyrophosphate). Cell partition into detergent-soluble and -insoluble fractions was performed as previously described (Algrain et al., 1993). Briefly, MTE cells growing on Transwell membranes were incubated in 120 μl/cm^2 MES exaction buffer [50 mM 2-(*N*-morpholino)] ethane sulfonic acid, 5 mM $MgCl_2$, 3 mM EGTA, 0.5% Triton X-100, protease and phosphatase inhibitors) for 40 seconds at 25°C. The detergent-soluble fraction was precipitated in 85% prechilled acetone for 4 to 18 hours

at –20°C recovered by centrifugation at 300 ***g*** for 10 minutes at 4°C. The remaining insoluble material was removed with cold PBS containing protease and phosphatase inhibitors, and recovered by centrifugation. The detergent-soluble fraction and detergent-insoluble pellets were resuspended in RIPA buffer.

In some studies, subcellular components were separated as described to obtain cytosol, cell membrane and cytoskeletal fractions (Parlato et al., 2000). Briefly, cells were resuspended in hypotonic solution (10 mM HEPES, pH 6.9, 10 mM KCl and protease inhibitors), homogenized and cleared of nuclei by centrifugation. The remaining supernatant was centrifuged at 100,000 ***g*** for 30 minutes at 4°C. The resulting supernatant was reserved as the cytosolic fraction. The pellet was resuspended in detergent containing NTENT buffer (500 mM NaCl, 10 mM Tris-HCl, pH 7.2, 1 mM EDTA, 1% Triton X-100, and protease inhibitors) and then centrifuged at 18,000 ***g*** for 30 minutes at 4°C. The resulting supernatant contained the membrane fraction and the pellet (resuspended in NTENT buffer) the cytoskeleton fraction.

Immunoblot analysis

Protein concentrations were estimated by using Bio-Rad protein assay reagent (Hercules, CA) and equal amounts resuspended in sample buffer prior to separation by 7.5% SDS-PAGE. Protein was transferred to PVDF paper (Millipore, Bedford, MA) and blocked with 5% milk and 0.2% Tween-20 for 1 hour at 25°C or overnight at 4°C. Primary antibody was incubated in blocking solution for 2 hours at 25°C. Horseradish-peroxidase-labeled secondary antibody binding was detected by enhanced chemiluminescence (ECL, Amersham Pharmacia). The relative difference in protein expression was quantified using Molecular Analyst software (Bio-Rad, Hercules, CA) based on signal density. The mean values and standard deviation from independent experiments were compared by Student's *t* test.

Adenovirus transfer to airway epithelial cells

Mouse *Foxj1* cDNA (Brody et al., 1997) was subcloned into adenovirus shuttle vector Ad5RSVknpa and used to generate a replication-deficient adenovirus vector (AdFoxj1) by the University of Iowa Gene Transfer Vector Core as previously described (Welsh et al., 1995). MTE cells were pre-treated with 6 mM EGTA for 1 hour to allow access to basolateral adenovirus receptors (Wang et al., 2000). AdFoxj1 was then incubated using 20 infectious particles per cell on the apical aspect of ALI day 0 MTE cells for 90 minutes.

Phosphothreonine ERM detection

To detect threonine phosphorylated ERM, cultured MTE cells were incubated in ice-cold 10% trichloroacetic acid (TCA) for 15 minutes, then washed with ice-cold PBS containing 20 mM glycine (G-PBS) as described (Hayashi et al., 1999). Remaining cellular material was resuspended in G-PBS and collected by centrifugation at 300 ***g*** for 10 minutes for 4°C. The pellet was resuspended in RIPA buffer and rotated 30 minutes at 4°C. The sample was subjected to centrifugation at 12,000 ***g*** for 10 minutes at 4°C and the supernatant removed for immunoblot analysis. Alternatively, cells were incubated in media containing 40 nM calyculin A (calycA) (Upstate Biotechnology) for 10 minutes at 37°C, then washed and resuspended in RIPA buffer containing 1 μM calycA as modified from a previously described method (Gautreau et al., 2000).

Results

Ezrin is expressed in the apical domain of ciliated airway epithelial cells in vivo

Immunostaining of adult mouse lung demonstrated that ezrin expression was localized within the apical membrane of a subpopulation of airway epithelial cells (Fig. 1A,B). Dual localization of ezrin with β-tubulin-IV, a protein expressed in cilia, indicated that apical ezrin was expressed in ciliated cells but absent from non-ciliated cells (Fig. 1A,B). In the mouse airway, most non-ciliated epithelial cells are secretory cells expressing Clara cell secretory protein (CCSP) (Van Winkle et al., 1996; Look et al., 2001). Simultaneous immunostaining of mouse lung tissue for CCSP and ezrin confirmed that ezrin was not expressed in the apical membrane of cells that expressed CCSP (Fig. 1C). Thus, ezrin expression is specifically localized to the apical membrane of ciliated airway epithelial cells in the lung, suggesting that ERM localization is related to cell differentiation.

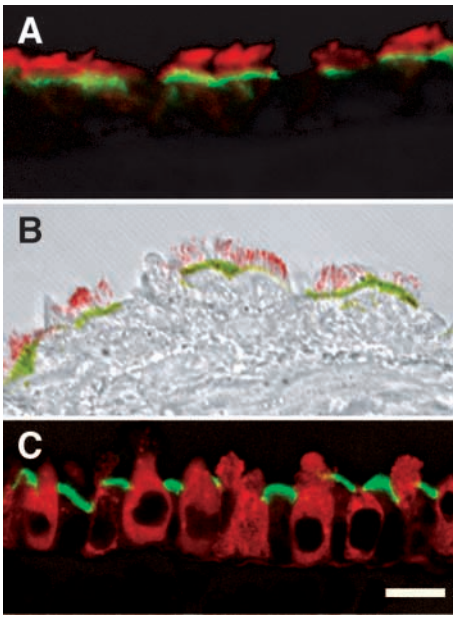

Fig. 1. Ezrin is expressed in the apical domain of ciliated airway epithelial cells in vivo. (A) Mouse lungs were immunostained with anti-ezrin (clone 3C12, green) and β-tubulin-IV (red) antibodies to identify cilia. (B) Ezrin localization detected as in A highlighted by confocal microscopy with diffusion interference contrast. (C) Mouse lungs were immunostained with anti-ezrin (clone 3C12, green) and anti-Clara cell secretory protein (CCSP) antibody (red). Tissue sections (6 μm) were incubated with primary antibodies and detected using fluorescent-labeled secondary antibodies. Representative sections are shown. Bars 10 μm (A,C); 7 μm (B).

Ezrin localization at the apical membrane immediately precedes maturation in a population of highly differentiated epithelial cells

We used a primary culture system of MTE cells that recapitulates many features of in vivo airway epithelial cell differentiation to assess changes in ezrin expression (You et al., 2002). In this system, cells purified from mouse tracheas form tight junctions and microvilli, but not cilia, before differentiation. Exposure to ALI conditions results in differentiation to ciliated and non-ciliated secretory cells by ALI day 7. Initial evaluation of ezrin expression at ALI day 7 revealed apical ezrin in about a third of the top layer of cells

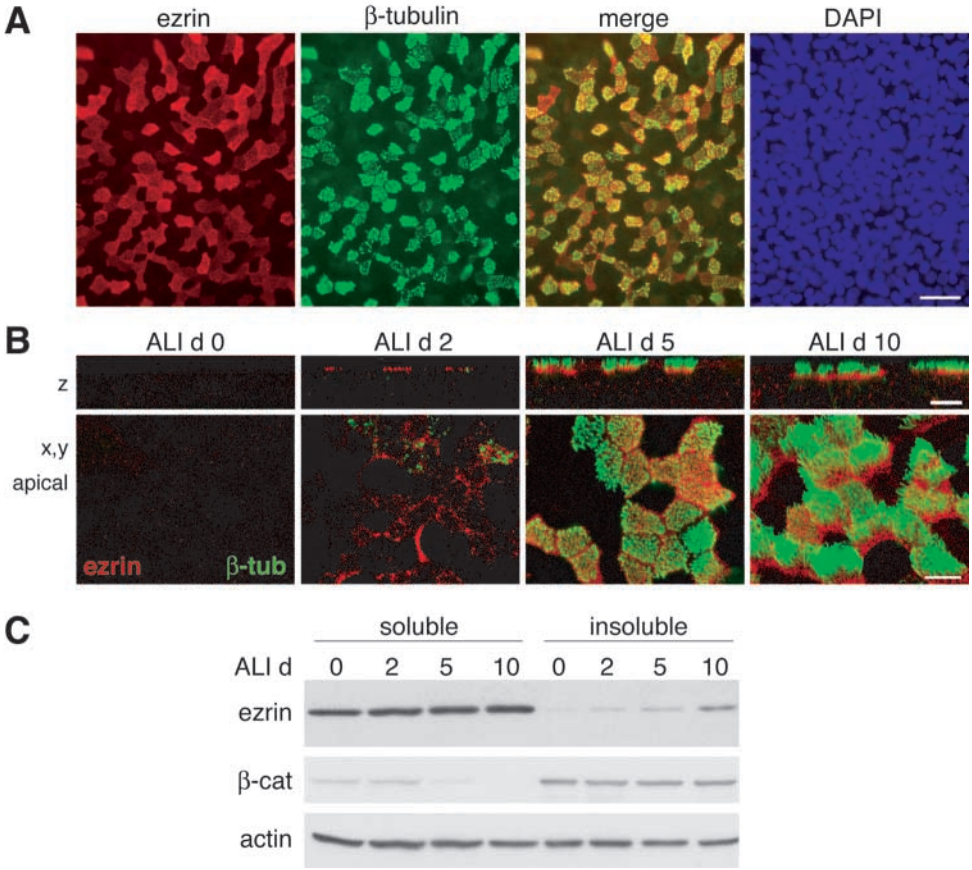

Fig. 2. Ezrin localization at the apical membrane immediately precedes maturation in a population of highly differentiated epithelial cells. (A) Primary culture MTE wild-type cells differentiated for 7 days on semipermeable, supported membranes under ALI conditions were immunostained for ezrin (red) and β-tubulin-IV (green) expressed in cilia then imaged by immunofluorescent microscopy and merged. Nuclei were detected in the same field by DAPI staining. Bar, 10 μm. (B) Primary culture MTE cells grown and immunostained as in A were harvested on the indicated days (d) and imaged by confocal microscopy at the level of the apical membrane (*x*,*y*). Images were reconstructed to generate *z*-axis images. Bar, 10 μm. (C) MTE cells cultured as in A were partitioned into detergent-soluble and -insoluble fractions as described (Algrain et al., 1993) at indicated days and 10 μg of protein was subjected to immunoblot analysis for detection of ezrin (clone 3C12), β-catenin (β-cat) and actin. Representative data from four independent preparations are shown.

(Fig. 2A). Ezrin was expressed in ciliated cells and, similarly, cilia (identified by β-tubulin-IV expression) were present only in cells expressing ezrin. Because ezrin has been implicated in the determination of apical polarity, we next simultaneously tracked the expression of ezrin and the appearance of cilia (an indication that the cell is highly polarized) during differentiation (Fig. 2B). At ALI day 0, confocal microscopy showed no cilia and no ezrin was detected. At ALI day 2, ezrin appeared as a ring-like pattern around the apical membrane. By ALI day 5, ezrin was present as a dense band at the apical membrane beneath thick clumps of cilia (Fig. 2B, in *z*-axis reconstructed images). Here, ezrin developed a characteristic pattern of diffuse expression across the apical surface (Fig. 2B, *x*,*y* images). A similar pattern was present at ALI day 10, when apical ezrin was expressed in more cells, consistent with continued differentiation towards the ciliated cell phenotype. These studies show that apical ezrin localization precedes ciliated cell differentiation and is highly concentrated within the apical compartment characteristic of ERMs (Bonilha et al., 1999; Bretscher, 1983; Berryman et al., 1993; Takeuchi et al., 1994).

Protein blot analysis of biochemically fractioned cells has previously shown that much of ezrin is present within the cytoplasm, although this large pool is not detected by immunohistochemistry (Algrain et al., 1993; Shaw et al., 1998; Yonemura et al., 2002). To characterize further the change in localization of ezrin from the cytosol to the membrane-cytoskeletal fraction during differentiation, we carried out cell fractionation based on detergent solubility of protein complexes in MTE cells as described by others (Algrain et al., 1993; Crepaldi et al., 1997). As expected, immunoblot analysis of MTE cells during differentiation showed abundant ezrin in the soluble (cytosol) fractions at all times (Fig. 1C). By contrast, little ezrin was detected in the insoluble (membrane-cytoskeletal) fraction at ALI day 0. After ALI day 0, there was increased insoluble ezrin, consistent with increased apical ezrin detected during differentiation by immunofluorescence. As an additional control for cell differentiation and purity of fractionation, we also found concurrent changes in the expression of cell-lateral-junction protein β-catenin as it moved from the cytosol to the lateral membrane. This was reflected by a decrease in soluble β-catenin during differentiation and is consistent with the behavior of this protein in MDCK cells (Hinck et al., 1994). Together, these in vivo and in vitro findings demonstrate that apical-membrane ezrin localization in the airway epithelium is restricted to ciliated cells and highly regulated during differentiation.

Apical ezrin is developmentally regulated and dependent on Foxj1 expression

We have previously shown that expression of Foxj1 precedes the appearance of cilia, is restricted to the nuclei of ciliated cells and is required for docking ciliary basal bodies at the apical membrane (Brody et al., 2000). To determine whether Foxj1 regulates programs for the apical localization of ezrin, we evaluated the relationship between the expression of Foxj1 and apical ezrin during lung development (Fig. 3A). In the

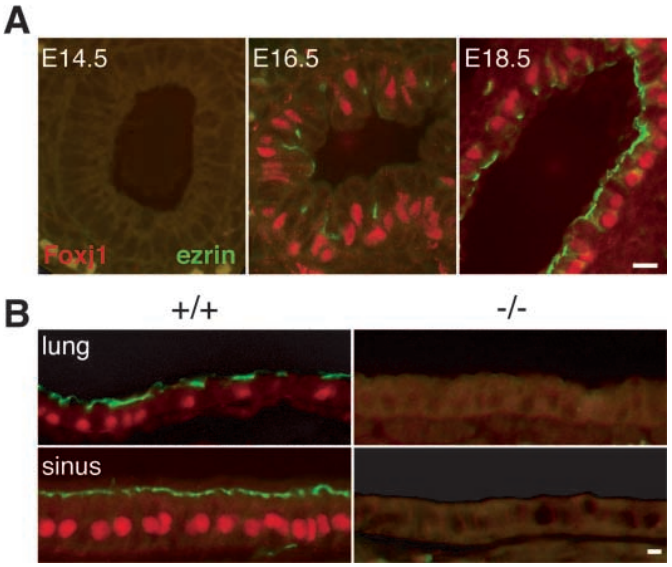

Fig. 3. Apical ezrin is developmentally regulated and dependent on Foxj1 expression. (A) Lung sections from indicated mouse embryonic day (E) were immunostained for Foxj1 (red) and ezrin (clone 3C12, green). At E14.5, auto-fluorescense is present in red blood cells at the bottom edge. Bar, 10 μm. (B) Tissue sections from lung or sinus of wild-type (+/+) or Foxj1-null (–/–) mouse were immunostained for Foxj1 (red) and ezrin (green) as in A. Representative sections are shown. Bar, 30 μm.

mouse lung, the onset of Foxj1 protein production is at mouse embryonic day 15.5 (E15.5) (Blatt et al., 1999). Apical membrane expression of ezrin was initially detected at E16.5 but only in cells expressing Foxj1. During subsequent epithelial cell differentiation in the developing lung, apical ezrin was temporally related to expression of Foxj1. By E18.5, the expression of apical ezrin was more extensive but remained limited to Foxj1-expressing cells. This pattern persisted in the adult lung airway and paranasal sinuses of the upper airway (another site of ciliated epithelium) (Fig. 3B, left). To determine directly whether Foxj1 is required for apical ezrin expression, we examined lung and sinus from Foxj1-null mice. In each case, apical ezrin was absent (Fig. 3B, right). These observations indicate that, in ciliated cells, apical ezrin localization depends on the expression of Foxj1.

Foxj1 regulates apical localization of ezrin in primary culture airway epithelial cells

To establish a more certain role for Foxj1 in mediating apical ezrin localization, we performed studies using primary culture MTE cells from wild-type and Foxj1-null mice. As in vivo, the loss of apical ezrin expression was also observed in MTE cells from Foxj1-null mice (Fig. 4A, middle). Reconstitution of Foxj1 in these cells using a Foxj1-expressing adenovirus vector resulted in the appearance of apical ezrin and rescued the cilia-deficient phenotype (Fig. 4A, right). Immunoblot analysis of primary culture MTE cells separated into cytosol and membrane-cytoskeletal fractions using two different methods (Algrain et al., 1993; Parlato et al., 2000) demonstrated that membrane-cytoskeletal-associated ezrin was markedly decreased in Foxj1-null MTE cells (Fig. 4B,C). These findings substantiate a requirement for Foxj1 in apical membrane localization of ezrin in ciliated cells.

Ezrin threonine phosphorylation is decreased in Foxj1-null airway epithelial cells

Threonine phosphorylation is required for maintaining cortical membrane localization of ERM proteins (Shaw et al., 1998; Nakamura et al., 1999; Gautreau et al., 2000). To determine whether threonine phosphorylation is regulated by Foxj1, wild-type and Foxj1-null MTE cells were fixed with TCA or calycA to inhibit phosphatase degradation of threonine-phosphorylated residues (Gautreau et al., 2000; Hayashi et al., 1999) and then subjected to immunoblot analysis using a threonine phospho-ERM-specific antibody. This assay showed markedly decreased ezrin threonine phosphorylation in the Foxj1-null MTE cells compared to the wild type samples, consistent with finding absent apical ezrin (Fig. 5A,B). Despite the decrease in ezrin threonine phosphorylation, there was no difference in the total amount of ezrin in wild type- and Foxj1-null samples. Phosphorylated radixin was not detected. By comparison, threonine phospho-moesin was less abundant than threonine phospho-ezrin and no significant change in the level of threonine-phosphorylated moesin was observed in Foxj1-null compared with wild-type-samples. The presence of threonine phosphorylated moesin in MTE cells led us to determine whether moesin localization also depends on Foxj1. Like ezrin, wild-type and Foxj1-null MTE cells contained similar amounts of total moesin (Fig. 5A). However, in contrast to ezrin, moesin abundance in the detergent-insoluble fraction was not altered in the absence of Foxj1 (Fig. 5C). These data suggest that ezrin and moesin are regulated differently during airway-epithelial-cell differentiation and that moesin expression is Foxj1 independent.

Apical expression of moesin is independent of Foxj1 expression in airway epithelial cells

To specifically assess differences in ezrin and moesin expression in wild-type and Foxj1-null cells, we used immunoflurorescence to evaluate the localization of moesin in wild-type MTE cells during differentiation (Fig. 6A). At ALI day 0, moesin expression was detected in apical and basolateral membranes of all cells. At ALI day 5, apical moesin localized with apical ezrin (the cell population associated with Foxj1 expression and cilia). By ALI day 10, apical moesin expression was heterogeneous: present in the apical membrane of cells with apical ezrin and in some cells without apical ezrin, and absent from other cells. A similar pattern of apical moesin was present in Foxj1-null cells (Fig. 6A). Further analysis of moesin expression was performed by immunostaining in vivo wild-type airway epithelium. This revealed a pattern of moesin expression similar to in vitro staining. Approximately half of the airway cells expressed both apical membrane ezrin and moesin (consistent with the location and numbers of ciliated cells in the airway), few cells expressed only moesin, and the remainder of cells expressed neither apical ezrin nor moesin (Fig. 6B). In summary, during early differentiation moesin is more widely expressed than ezrin but, later, it is expressed with ezrin in the apical membrane of ciliated cells, thus indicating that these two ERMs are regulated differently.

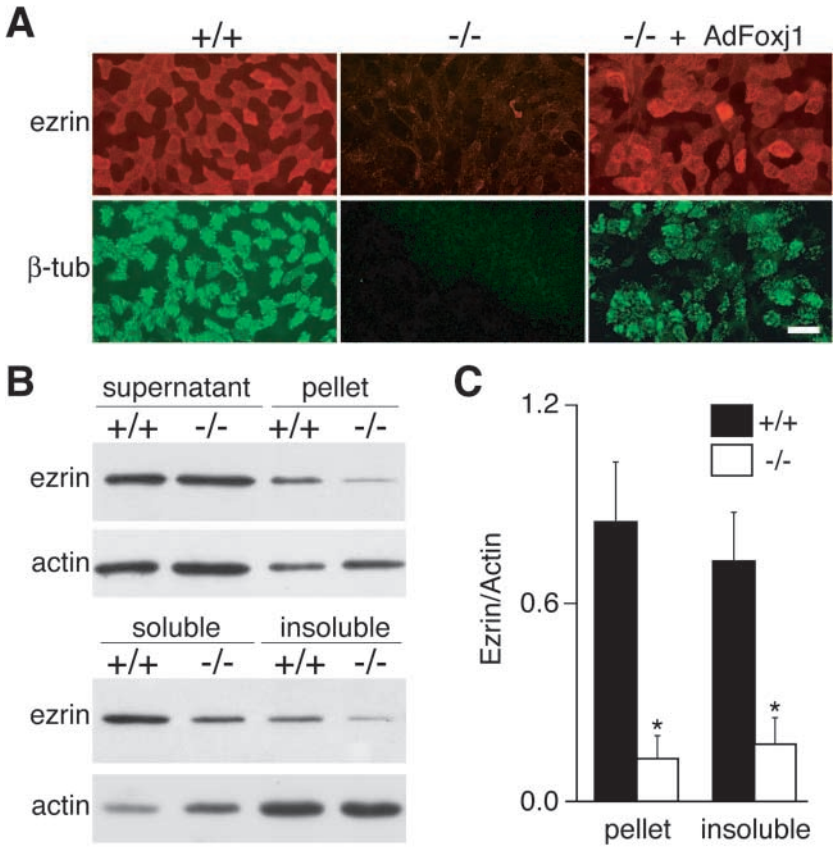

Fig. 4. Foxj1 regulates apical localization of ezrin in primary culture airway epithelial cells. (A) Wild-type (+/+) or Foxj1-null (–/–) MTE cells were cultured under ALI conditions and at ALI day 14 were immunostained for expression of ezrin (red) and β-tubulin-IV (green) as in Fig. 2. Null MTE cells (right) were transfected with an adenovirus vector expressing Foxj1 or control GFP (not shown) at ALI day 0, then harvested at ALI day 14. Identical fields were imaged for each genotype and condition. Bar, 30 μm. (B) Primary culture MTE cells were collected at ALI day 14. Cells analyzed in the upper panel were separated by centrifugation into supernatant (cytosol portion) and pellet (membrane-cytoskeleton portion) as described (Parlato et al., 2000) and 2 μg of protein analyzed per lane. Samples in the lower panel were separated by Triton X-100 detergent solubility into soluble fraction (cytosol portion) and insoluble fractions (membrane-cytoskeleton portion) as described (Algrain et al., 1993). Proteins (4 μg) were subjected to immunoblot analysis for detection of ezrin (clone 3C12) and actin. (C) Relative protein expression ratio of ezrin to actin in the membrane-cytoskeletal portion determined by densitometry from immunoblots as in B. Data represent the mean and standard deviation of expression from three independent preparations. The asterisk indicates a difference in means (P<0.05).

To further characterize the phenotype of the ezrin-moesin negative cells, we examined apical ERM expression in the secretory-airway-cell population identified by expression of CCSP. In primary culture MTE cells, we found that CCSP-expressing cells did not express apical membrane moesin (Fig. 6C). Examination of apical moesin and CCSP in the airway epithelium of wild-type and Foxj1-null mice in vivo verified this pattern (Fig. 6C, right). Regardless of genotype, CCSP and moesin were expressed in mutually independent populations of cells. Taken together, these observations indicate that ERM proteins ezrin and moesin are co-localized in the apical membrane of ciliated cells but absent from the apical membrane of CCSP-expressing secretory cells, suggesting that they have specific roles in ciliated cells.

Apical membrane moesin cannot compensate for ezrin in Foxj1-null cells

Overlapping patterns of ERM expression and the lack of phenotypic abnormality in the moesin knock-out mouse suggested that ERMs can compensate for one another (Doi et al., 1999). We thus examined the expression and localization of proteins that bind ezrin in the Foxj1-null cells to determine whether moesin can compensate for ezrin in airway epithelial cells. Ezrin binds EBP50 and localizes to the apical membrane but it is unknown whether EBP50 can localize to the apical membrane in the absence of apical ezrin (Short et al., 1998; Shenolikar and Weinman, 2001). As anticipated, we found that EBP50 was expressed in the apical membrane of wild-type cells, where it co-localized with ezrin. Moreover, EBP50 was present only in cells expressing ezrin (Fig. 7A, left). EBP50 contains a PDZ domain that binds transmembrane proteins expressed in the airway, including β_2AR, in a complex containing ezrin (Hall et al., 1998). We found that β_2AR was expressed apically in wild-type cells and at various levels, but was present only in cells that expressed apical ezrin (Fig. 7A, right). Confocal microscopy confirmed that both ezrin-associated proteins were expressed apically in wild type MTE cells (Fig. 7B). However, neither EBP50 nor β_2AR was expressed in the apical membrane of Foxj1-null MTE cells. The findings suggest that these proteins depend on ezrin for proper localization at the apical membrane and that apical moesin cannot substitute for this function. Both EBP50 and β_2AR were present in equal amounts in protein blot analysis of cells from wild-type and Foxj1-null mice, consistent with the predicted defect in protein localization in the Foxj1-null cells (data not shown). Finally, we found that in vivo, as in vitro, ezrin-dependent proteins EBP50 and β_2AR were not expressed in the apical membrane of the Foxj1-null cells (Fig. 7C). By contrast, basolateral proteins were intact, as demonstrated by normally polarized localization of proteins β-catenin and Na^+K^+-ATPase in Foxj1-null airway cells. Thus, Foxj1-dependent mechanisms are essential for the regulation of apical ezrin and subsequently required for the organization of crucial apical membrane protein complexes in airway epithelial cells.

Discussion

ERM family members are structural and regulatory proteins

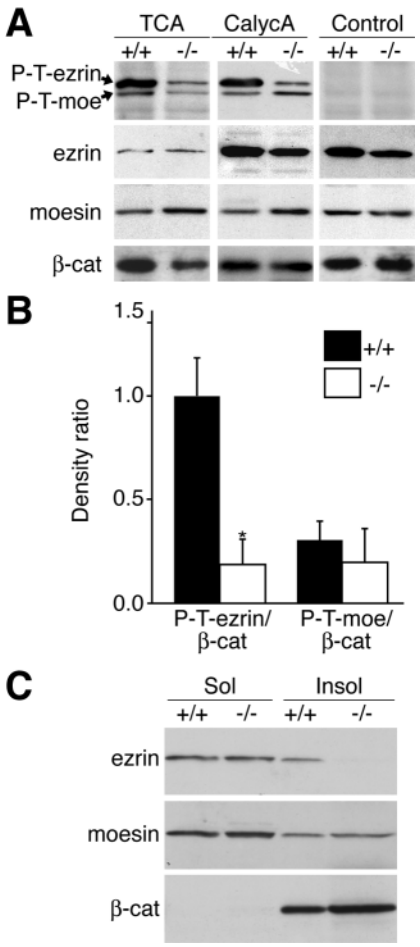

Fig. 5. Decreased ezrin threonine phosphorylation in Foxj1-null airway epithelial cells. (A) Wild-type (+/+) and Foxj1-null (–/–) primary MTE cells cultured as in Fig. 2 and analyzed at ALI day 14 were immediately fixed in TCA, calycA or control buffer then 5 μg of protein was subjected to immunoblot analysis with phosphothreonine (P-T) ERM-specific and other indicated antibodies. Phosphothreonine ezrin (P-T-ezrin) and moesin (P-T-moe) are indicated by arrows. Ezrin (clone 3C12) and moesin (Upstate Biotech) were detected in whole cell lysates. (B) P-T-ezrin and P-T-moesin expressed as a ratio of β-catenin (β-cat) determined by densitometry from immunoblots as in A. Data represents the mean and standard deviation of expression from four independent preparations. The asterisk indicates a difference in means ($P<0.05$). (C) Expression of moesin in the detergent-insoluble fraction of primary culture MTE cells from wild-type or Foxj1-null cells from ALI day 14. Fractions were prepared and 5 μg of protein per lane were analyzed as in Fig. 2C.

linking polarized membrane proteins with F-actin. Because these family members contain highly conserved domains that mediate protein binding, the function and regulation of ERMs is often generalized. However, in vivo, cell-specific patterns of ERM expression suggest that these proteins must be individually regulated in different cell types. This report is one of the first to identify a factor that independently regulates ERM protein localization in a cell-differentiation-dependent fashion. Here, we identify the F-box transcription factor Foxj1 is a crucial member of a pathway specifically required in vivo and in vitro for apical membrane localization of ezrin, but not moesin, in ciliated airway epithelial cells. Furthermore, we show that Foxj1 and ezrin are required for the assembly of an apical membrane protein complex with crucial epithelial cell functions (Hall et al., 1998; Naren et al., 2003; Taouil et al., 2003).

Several observations support the finding that Foxj1 is specifically required for ezrin function in airway epithelial cells. First, Foxj1 expression is temporally related to the expression of apical ezrin in airway epithelial cells of developing lung and co-expression persists in the adult airway. Second, in vivo and in vitro, airway epithelial cells from Foxj1-null mice do not express apical membrane ezrin but can revert to the wild-type phenotype when complemented with exogenous Foxj1. Third, the amount of ezrin, but not moesin, in the membrane-cytoskeletal cell fraction is decreased in Foxj1-null airway cells compared with the wild type. Fourth, ezrin threonine phosphorylation, associated with localization of ezrin at the cortical membrane, is decreased in Foxj1-null cells compared with wild type cells. Finally, ezrin-associated proteins EBP50 and β_2AR, which form an apical membrane complex with ezrin in wild-type cells, fail to apically localize in the Foxj1-null airway cells. Thus, by affecting ezrin localization, Foxj1 has a central role in regulating the apical membrane organization of ciliated airway epithelial cells.

Cell-specific ezrin expression in airway cells

We previously found that Foxj1 expression was restricted to ciliated epithelial cells of the ependyma, testis, oviduct and airway. Localization of apical ezrin in ciliated airway epithelial cells is, in agreement with prior reports of ezrin expression in the apical membrane of epithelial cells, lining the airways and ciliated nasal epithelial cells (Berryman et al., 1993; Brezillon et al., 1997; Mohler et al., 1999; Laoukili et al., 2001; Castillon et al., 2002). Our use of multiple airway cell markers in vivo and in vitro (primary cell culture), and high-resolution imaging now confirms specific expression in the ciliated cell of the airway. These reports and our findings are in contrast to a recent study showing that ezrin was expressed specifically in the apical membrane of CCSP-expressing cells (Kulaksiz et al., 2002). The lack of apical ERM detection in secretory cells might be a limitation of our assay or, alternatively, it might be advantageous for the apical membrane of secretory cells to be unencumbered by a dense network of actin and ERMs. Instead, the localization of ezrin indicates a unique function in ciliated cells that cannot be met by other ERM proteins and probably involves specific interaction with proteins regulated by ezrin at the apical membrane. Although ERM proteins have been implicated in microvillius formation, there are also roles in the regulation and localization of apical proteins in epithelia of liver, kidney and lung (Yun et al., 1997; Sun et al., 2000; Kikuchi et al., 2002). In lung epithelial cells, this function includes the binding and activation of EBP50, β_2AR, and CFTR (Sun et al., 2000; Naren et al., 2003; Taouil et al., 2003). Low levels of CFTR expression in the mouse airways excluded immunolocalization in our studies (Rochelle et al., 2000). However, relevant to cystic fibrosis pathogenesis, the presence of CFTR in the ezrin-based apical complex, and the absence of ezrin and moesin in Clara cells suggests that the ciliated cell has a central role in the airway for controlling salt and water. This is consistent with the localization of CFTR in ciliated

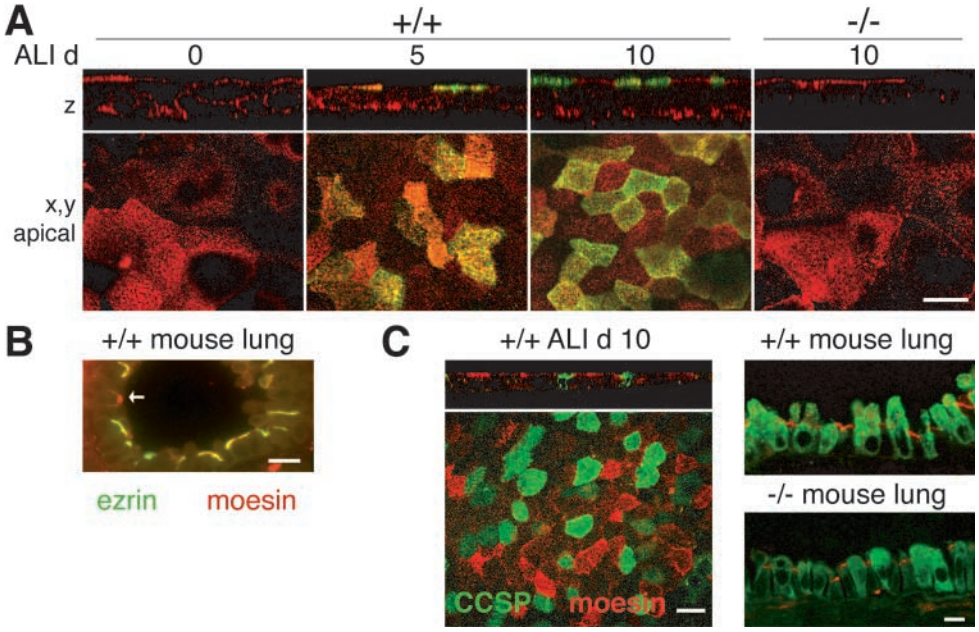

Fig. 6. Apical expression of moesin is independent of Foxj1 expression in airway epithelial cells. (A) Wild-type (+/+) and Foxj1-null (–/–) MTE cells were cultured and immunostained for expression of ezrin (clone 3C12; green) and moesin (red) at indicated day (d) as in Fig. 2B. Confocal microscopy images were reconstructed to generate z-axis images. Bar, 10 μm. (B) Mouse lung cells were immunostained for moesin (Franck et al., 1993) (red) and ezrin (green). Arrow indicates a rare cell expressing only apical moesin. Bar, 10 μm. (C) Wild-type MTE cells from ALI day 10 (left) or mouse lung cells from wild-type and Foxj1-null mice (right) were immunostained for expression of moesin (red) and CCSP (green). Bar, 10 μm.

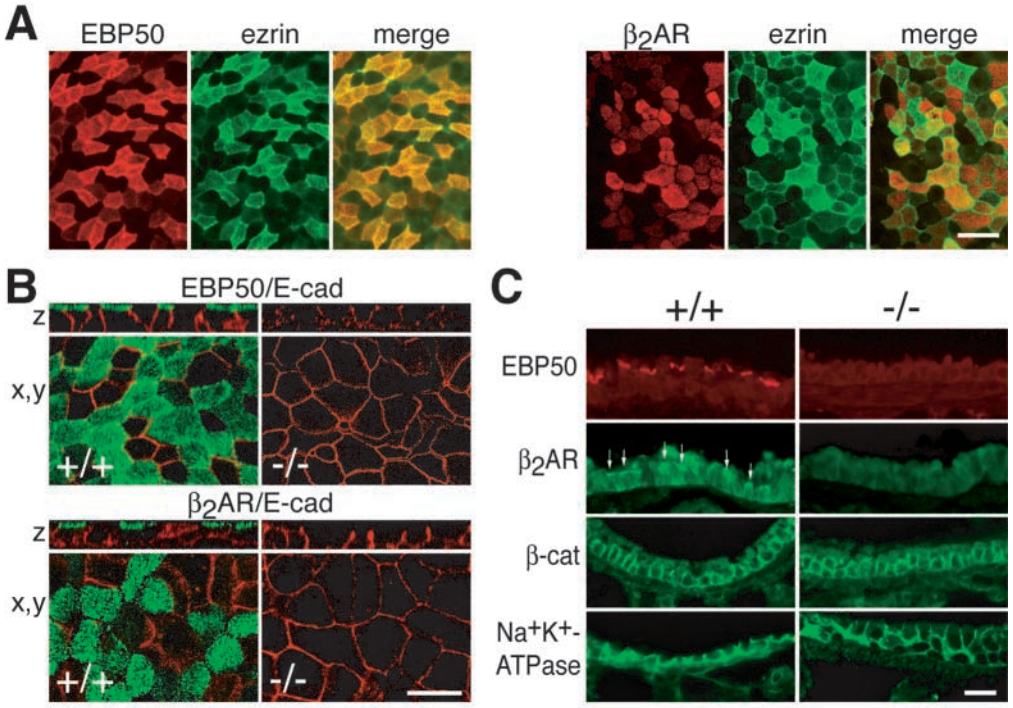

Fig. 7. Apical membrane moesin cannot compensate for ezrin in Foxj1-null cells. (A) MTE cells were grown to ALI day 10 as in Fig. 2, then immunostained for expression of either EBP50 (red) or β_2AR (red) together with ezrin (green). Immunofluorescent microscopy images focused on the apical membrane of cells in identical fields analyzed for expression of ezrin, EBP50, or β_2AR then merged. Bar, 10 μm. (B) MTE cells from wild-type (+/+) and Foxj1-null (–/–) mice cultured as in A, immunostained for expression of either EBP50 or β_2AR (both green) together with lateral cell junction membrane protein E-cadherin (E-cad, red) and imaged by confocal microscopy at the level of the apical membrane (*x*,*y*). Images were reconstructed to generate *z*-axis images. Bar, 10 μm. (C) Tissue sections from wild-type and Foxj1-null mouse lungs were immunostained for expression of indicated apical ezrin-dependent and basolateral protein as in Fig. 1. Arrows mark apical expression of β_2AR. Bar, 10 μm.

human cells previously reported (Brezillon et al., 1997) and implies a need for Foxj1-expressing cells to maintain CFTR function in the airway.

Apical expression of moesin is Foxj1 independent

The differential regulation of ezrin and moesin expression in airway epithelial cells supports the specificity of Foxj1 regulation of ezrin. This is in contrast to similar localization and regulation of ezrin and moesin observed in T cells (Allenspach et al., 2001; Delon et al., 2001). Although moesin has the capacity to bind EBP50 in vitro (Reczek et al., 1997), expression during MTE cell differentiation in apical and basolateral membranes suggests a broader function in the airway. For example, it is possible that moesin is widely expressed in the apical membrane to initiate the formation of microvilli in all cells. Thus, the persistence of moesin in Foxj1-null cells could account for the presence of microvilli in these

cells (Brody et al., 2000). Further evaluation of the different roles of ERM proteins will be important for understanding cell morphology and the regulation of proteins linked to ERMs.

Ezrin and ciliogenesis

The precise function of Foxj1 in the ezrin activation pathway is unknown. Although a putative DNA binding sequence for Foxj1 has been determined, in vivo gene targets of Foxj1 are not established (Lim et al., 1997). The absence of cilia in the Foxj1-null mouse suggested that one Foxj1 target is a protein in a program of ciliogenesis, although biochemical details of a ciliogenesis pathway are not known and few putative regulatory molecules for Foxj1 binding have been identified. The finding that Foxj1 is also required for apical ezrin localization suggests that Foxj1 directs either a common or a unique factor(s) that is required for ciliogenesis and apical ezrin localization. Within this scheme, it is possible that because apical ezrin localization precedes the appearance of cilia (Fig. 2B), final steps of ciliogenesis depend on the physical presence of apical ezrin to bind cilia structures. Studies are in progress to elucidate these potential mechanisms. Alternatively, it is possible that factors required to unfold dormant ezrin or to maintain ezrin at the apical membrane are also required to move basal bodies to the apical membrane.

Candidates for Foxj1-mediated ezrin activation

The movement and maintenance of ERMs at the cortical membrane is correlated with threonine phosphorylation and is consistent with finding decreased (but not absent) threonine-phosphorylated ezrin in the Foxj1-null cells (Fig. 5A) (Matsui et al., 1998; Nakamura et al., 1999). The specific kinase directing this event is not known but in vitro analysis indicates that threonine phosphorylation might be carried out by the Rho kinase ROCK (Matsui et al., 1998; Jeon et al., 2002) or protein kinase C-θ (PKC-θ) (Pietromonaco et al., 1998). Our immunoblot analysis of each of these proteins shows similar amounts in wild-type and Foxj1-null cells (T.H. and S.L.B., unpublished), but kinase activity might be controlled by proteins regulated by Foxj1. In each case, regulation must be ERM specific because moesin localization is not affected by the absence of Foxj1. The total absence of apical ezrin detection in Foxj1-null cells suggests that ezrin is inactive and remains in the cytosol. Thus, evidence indicating that threonine phosphorylation is insufficient for apical localization and that PtdIns(4,5)P_2 is sufficient to switch ezrin to the active state points to additional candidate targets in a Foxj1 pathway (Matsui et al., 1999; Shaw et al., 1998; Barret et al., 2000; Tran Quang et al., 2000). For example, cells transfected with plasmids containing mutations in ezrin at putative PtdIns(4,5)P_2 binding sites altered localization of ezrin at the cortical membrane and decreased ezrin present in detergent-insoluble cell fractions, similar to our finding in the Foxj1-null cells (Barret et al., 2000). These observations suggest that a defect in PtdIns(4,5)P_2 activation and/or localization might play a role in the Foxj1-null phenotype.

In summary, lack of apical membrane expression of ezrin in the Foxj1-deficient ciliated cell identifies Foxj1 as one of the first factors to regulate a single ERM in a cell-specific fashion. Unlike Hox proteins that have conserved roles, a unifying role for F-box proteins has not been identified. Other F-box family members also have a role in the establishment of cell polarity. Recently, the *Drosophila* F-box protein Jumeaux was shown to regulate apical localization of Inscuteable in neuronal cell differentiation and the *Drosophila forkhead* gene was shown to regulate the apical morphology of salivary glands (Mach et al., 1996; Cheah et al., 2000). Identification of specific targets regulated by these F-box proteins as well as Foxj1, will be important for revealing the molecules required to determine and maintain polarized proteins during epithelial cell differentiation.

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