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RETRACTION

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Ahmed H. K. El-Hashash, Gianluca Turcatel, Saaket Varma, Mohamed Berika, Denise Al Alam and David Warburton

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Journal of Cell Science is retracting this article at the request of the institution. The authors have been notified of this request.

Unfortunately, the journal has no further information on the reasons behind this retraction.

Eya1 protein phosphatase regulates tight junction formation in lung distal epithelium

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Summary

Little is known about the regulatory mechanisms underlying lung epithelial tight junction (TJ) assembly, which is inextricably linked to the preservation of epithelial polarity, and is highly coordinated by proteins that regulate epithelial cell polarity, such as aPKCζ. We recently reported that Eya1 phosphatase functions through aPKCζ–Notch1 signaling to control cell polarity in the lung epithelium. Here, we have extended these observations to TJ formation to demonstrate that Eya1 is crucial for the maintenance of TJ protein assembly in the lung epithelium, probably by controlling aPKCζ phosphorylation levels, aPKCζ-mediated TJ protein phosphorylation and Notch1–Cdc42 activity. Thus, TJs are disassembled after interfering with Eya1 function in vivo or during calcium-induced TJ assembly in vitro. These effects are reversed by reintroduction of wild-type Eya1 or partially inhibiting aPKCζ in *Eya1*siRNA cells. Moreover, genetic activation of Notch1 rescues $Eya1^{-/-}$ lung epithelial TJ defects. These findings uncover novel functions for the Eya1–aPKCζ–Notch1–Cdc42 pathway as a crucial regulatory mechanism of TJ assembly and polarity of the lung epithelium, providing a conceptual framework for future mechanistic and translational studies in this area.

Key words: Eya1, Tight junction, Lung, aPKCζ, Notch1

Introduction

In mammals, epithelial barrier integrity plays a pivotal role in maintaining the normal function of multiple organs, including lung and intestine (Niessen, 2007). Polarized epithelial cells have a series of intercellular junctions, including tight junctions (TJs) that are localized at the most apical side of cell–cell contact sites (Tsukita et al., 2001; Tsukita et al., 2008). In normal epithelial cells, TJs are the main components of paracellular permeability barriers in many epithelial cell types and composed of several transmembrane molecules, including claudin, occludin and zonula occludens-1 (ZO-1) (Tsukita et al., 2001; Tsukita et al., 2008; Niessen, 2007). Although the molecular structure of TJs is well documented, little is known about the basic regulatory mechanisms underlying TJ formation in many epithelial cell types.

In epithelia, the assembly and maintenance of TJs are inseparably linked to the preservation of polarity, and is highly coordinated by proteins that regulate epithelial cell polarity, including the aPKCζ/Par complex (Shin et al., 2006). The aPKCζ/Par polarity complex, together with several scaffolding/adhesion molecules, promotes TJ formation and designates the site of TJ assembly that defines the apical and basolateral membrane domains. These polarity proteins also maintain TJ structure by modulating targeted insertion of newly synthesized proteins to the junctional complex (Shin et al., 2006). We recently reported mechanisms of cell polarity control by Eya1 protein phosphatase that regulates aPKCζ activity and Par subcellular localization in the lung distal epithelium (El-Hashash et al., 2011a). However, little is known neither about the basic regulatory mechanisms underlying TJ and permeability barrier formation in the lung epithelium, nor how Eya1-controlled cell polarity affects TJ protein assembly, in particular this is still unknown in the lung distal epithelium.

The Eyes Absent (Eya) 1–4 protein tyrosine phosphatases are components of the conserved retinal determination pathway, which controls cell-fate determination in different organs and species. Eya contains phosphatase activity that is essential for regulation of precursor cell proliferation, directing cells to the repair instead of apoptosis pathway upon DNA damage, as well as mediating Eya cytoplasmic cellular functions (Li et al., 2003; Jemc and Rebay, 2007; Cook et al., 2009). $Eya1^{-/-}$ mouse embryos have defects in the proliferation of the precursors of multiple organs, and die at birth of respiratory failure (Xu et al., 1999; Xu et al., 2002; Zou et al., 2004; El-Hashash et al., 2011a; El-Hashash et al., 2011b). Yet the specific functional control of TJs by Eya1 is unknown, likewise how aPKCζ dephosphorylation events by Eya1 are involved in the structural regulation of TJs remains obscure in the lung epithelium.

In the present study, we attempted to characterize the functional role of Eya1 phosphatase in TJ and permeability barrier formation in embryonic lung epithelium. We show herein that Eya1 is colocalized with TJ proteins at TJs and is also essential for the maintenance of TJ protein assembly in the lung epithelium, probably by controlling aPKCζ phosphorylation levels, aPKCζ-mediated TJ protein

phosphorylation and Notch1–Cdc42 activity. Interfering with Eya1 function in vivo or in vitro results in defective TJ formation with inactivation of Notch1 and Cdc42 signaling in lung epithelial cells. Furthermore, activation of Notch1 signaling in *Eya1*$^{-/-}$ distal epithelium rescues *Eya1*$^{-/-}$ embryonic TJ assembly defects in vivo as well as in lung epithelial cells in vitro by restoring Cdc42 activity.

Results

Eya1 is expressed on the apical side of embryonic lung distal epithelial cells and its deletion causes TJ protein disassembly

We have recently reported Eya1 expression patterns and null mutant lung phenotype (El-Hashash et al., 2011b). Two lines of reasoning have led us to examine Eya1 functions in distal lung epithelial TJs. First, Eya1 has an apical expression pattern and is critical for establishing apico-basal polarity in the distal epithelial tip cells (supplementary material Fig. S1A) (El-Hashash et al., 2011a). This localization is similar to TJ proteins (supplementary material Fig. S1B–D; Fig. 1E), which are also essential for establishment and maintenance of apico-basal polarity (Ohno, 2001; Shin et al., 2006). Second, other members of the protein phosphatase (PP) family, e.g. PP2A/PP2B, are known to be crucial regulators of epithelial TJ assembly and function (Lum et al., 2001; Nunbhakdi-Craig et al., 2002).

Establishment of cell polarity and TJ protein assembly are closely related in the epithelium and disruption of TJs leads to loss of epithelial cell polarity (Yamanaka et al., 2001; Suzuki et al., 2001; Suzuki et al., 2002; Hirose et al., 2002). Since cell polarity is disrupted in *Eya1*$^{-/-}$ distal epithelium (El-Hashash et al., 2011), we first examined changes in TJs. In the *Eya1*$^{-/-}$ distal epithelium, epithelial cells were disorganized, and ZO-1, occludin and claudin1 had a diffuse staining pattern and failed to concentrate in the most apical part of lateral membranes, in contrast to wild-type (WT) control lungs (Fig. 1B,F,D,H). These results suggested that *Eya1* deficiency must affect the molecular assembly of epithelial TJs.

Examination of possible functional roles of Eya1 in TJ formation

In different types of epithelial cells grown in vitro, Ca^{2+} depletion from the culture medium results in disruption of intercellular junctions such as TJs; conversely, the formation of functional TJs can be triggered upon transferring cells cultured in low Ca^{2+} (LC) medium to normal Ca^{2+} (NC) medium (Gonzalez-Mariscal et al., 1990; Cereijido et al., 2000; Nunbhakdi-Craig et al., 2002).

To examine the possible functional roles of Eya1 phosphatase in the process of TJ formation, a Ca^{2+} switch assay was performed in MLE15 lung epithelial cells as described in Materials and Methods. Formation of TJs in MLE15 cells, which were used in this study because they are polarized and express endogenous Eya1 (El-Hashash et al., 2011a) as well as formed well-assembled TJs (Fig. 2A,E,I), is Ca^{2+}-dependent similar to other epithelial cell line such as Madin-Darby canine kidney (MDCK) cells (Cereijido et al., 2000; Nunbhakdi-Craig et al., 2002). Thus, depletion of Ca^{2+} from the culture medium resulted in disruption of TJs, as indicated by the failure of TJ proteins to concentrate in the most apical part of lateral cell membranes (Fig. 2B,F,J). Conversely, transferring MLE15 cells cultured in low Ca^{2+} (LC) medium to normal Ca^{2+} (NC) medium for 4 hours triggered the formation of TJs (Fig. 2C,D,G,H,K,L).

Next, we determined Eya1 behavior during Ca^{2+} switch experiments in MLE15 cells. Eya1 protein phosphatase is

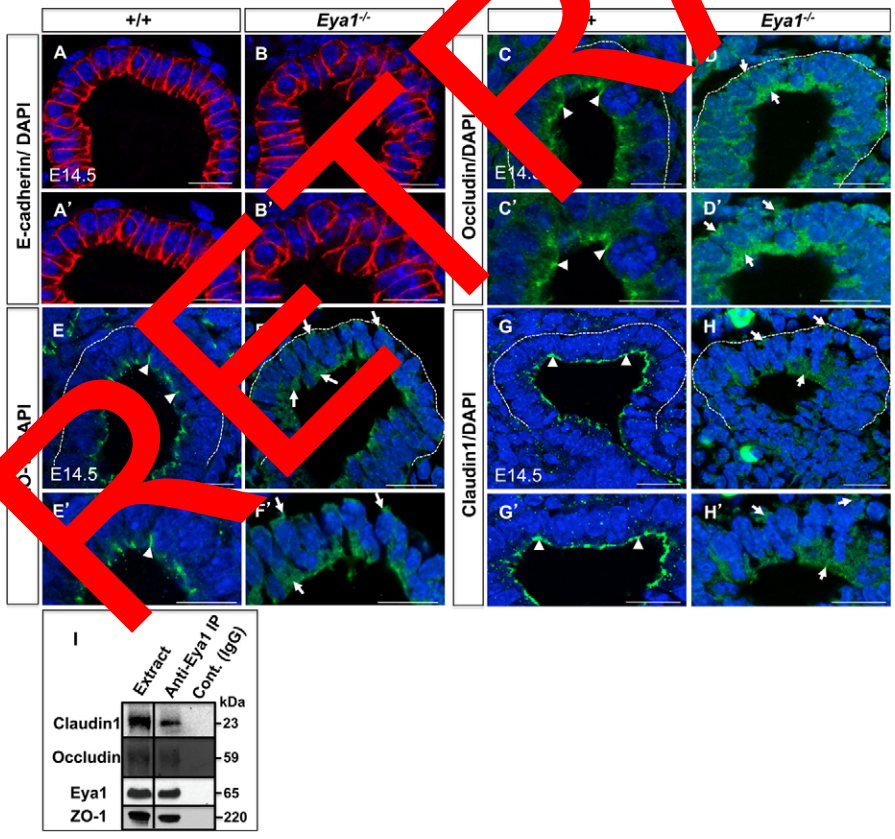

Fig. 1. *Eya1* deletion causes TJ protein disassembly in lung distal epithelium. (**A–H′**) Immunofluorescence with specific antibodies shows no apparent differences in E-cadherin expression in *Eya1*$^{-/-}$ versus WT epithelium (A,A′,B,B′). ZO-1 (E,E′), occludin (C,C′) and claudin1 (G,G′) specifically localize to the most apical part of lateral membranes of WT distal epithelial cells (arrowheads), but fail to concentrate apically in lateral membranes and localize at both the basal and apical sides in *Eya1*$^{-/-}$ epithelial cells (F,F′,D,D′,H,H′, arrows). Note disorganized distal epithelial tip cells in B,B′,H,H′ compared to A,A′,G,G′. The dashed line represents the collagen-IV-stained basement membrane. A′–H′ are electronic magnifications from epithelial tip areas in A–H respectively. Scale bars: 50 μm. (**I**) Eya1 co-immunoprecipitates TJ proteins in vivo. Endogenous Eya1 was immunoprecipitated from E14.5 lung cell lysates with a specific Eya1 antibody and western blotting was performed with antibodies specific to different TJ proteins.

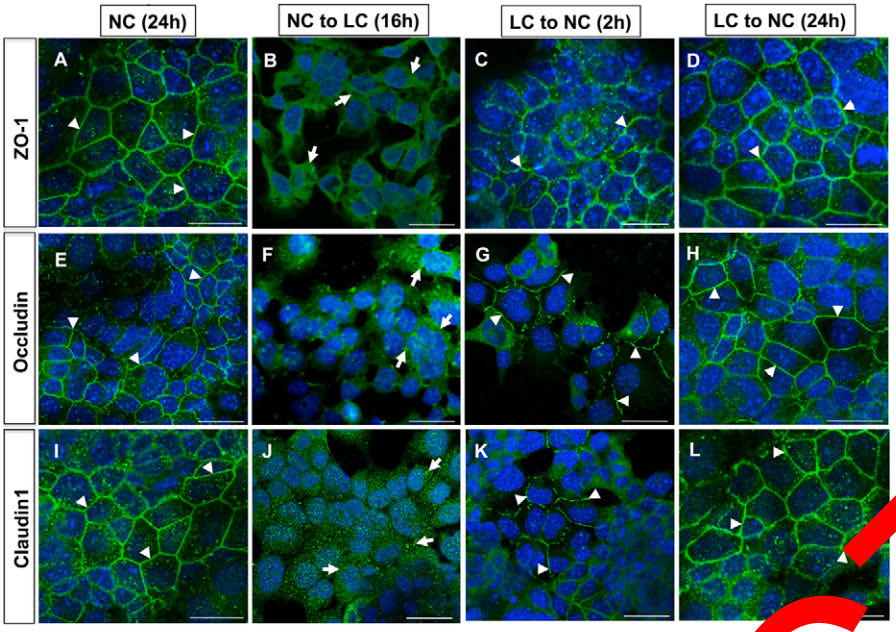

Fig. 2. Ca^{2+}-dependent membrane localization of TJ proteins in MLE15 cells. MLE15 cells grown in normal Ca^{2+} medium (NC) for 48 h (**A**,**E**,**I**) were Ca^{2+} starved overnight (NC to LC; **F**,**J**), then switched to LC medium for 2 h or 24 h (LC to NC; **C**,**D**,**G**,**H**,**K**,**L**) to induce TJ biogenesis, and then processed for immunofluorescence with specific TJ protein antibodies. Note belt-like pattern of membrane immunostaining for ZO-1, occludin and claudin1 at areas of intercellular contact in A,E,I,D,H,L (arrowheads), predominant cytosolic concentration of these proteins in B,F,J (arrows) and partial formation of TJs at areas of intercellular contact in C,G,K (arrowheads). Scale bars: 50 μm.

expressed in the cytoplasm, where it functions as a cytoplasmic protein phosphatase (Fougerousse et al., 2002; Xiong et al., 2009). The Eya1 expression domain was strongly visualized at the periphery of MLE15 cells, where it colocalized with TJ proteins (Fig. 3A–C,E–G; supplementary material Fig. S2). Ca^{2+}-deprived MLE15 cells showed an apparent disappearance of the peripheral membrane staining for Eya1 that then localized to the cytosol (compare supplementary material Fig. S2A,B with Fig. 3E). Interestingly, Ca^{2+} starvation of cells right before switching to NC medium in order to induce junction biogenesis resulted in gradual re-concentration of Eya1 protein at sites of cell–cell contact (supplementary material Fig. S2D), suggesting that Eya1 recruitment to regions of cell–cell contact is Ca^{2+} dependent.

Furthermore, the similarity in the expression pattern of Eya1 and TJ proteins in vivo/in vitro and the dependency of both TJ proteins and Eya1 membrane localization on the presence of Ca^{2+} suggested that Eya1 protein associates with TJ complexes. This conclusion was further confirmed by co-immunoprecipitation assays, which showed that Eya1 co-immunoprecipitated occludin, claudin1 and ZO-1 proteins from lung cell lysates in vivo (Fig. 1I), which further suggests that Eya1 interacts with the TJ protein complex.

Phosphorylation and membrane sorting of TJ proteins to the cell membrane are dependent on Eya1 phosphatase activity

Eya1 has well-known phosphatase activities (Li et al., 2003) and controls protein phosphorylation in the lung epithelium in vivo and in MLE15 cells in vitro (El-Hashash et al., 2011a). Serine phosphorylation is essential for the recruitment of cytoplasmic ZO-1, occludin and claudin1 to the membrane during Ca^{2+}-induced TJ biogenesis, while decreased serine phosphorylation of these TJ proteins leads to failure of their migration from the cytosol to cell periphery causing TJ disassembly (Stuart and Nigam, 1995; Farshori and Kachar, 1999; Nunbhakdi-Craig et al., 2002). Since Eya1 can colocalize with TJ proteins (Fig. 3A–C,E–G) that fail to translocate to the cell membrane of $Eya^{-/-}$ epithelial cells (Fig. 1), we next examined how changes in Eya1 activity influence TJ protein assembly and regulation. In polarized MLE15 cells potent inhibition of endogenous Eya1 activity is obtained by treating cells with specific *Eya1*siRNA, while increased levels of Eya1 activity are achieved after expression of wild-type *Eya1* construct (El-Hashash et al., 2011a) (data not shown). First, we tested whether TJ proteins could interact together and with Eya1 protein in MLE15 grown in vitro by co-immunoprecipitation assays. As shown in Fig. 3D, ZO-1 co-immunoprecipitated Eya1, claudin1 and occludin proteins from MLE15 cell lysates. This Eya1–ZO-1 protein association was Ca^{2+} dependent because it was lost in cells growing in LC medium (Fig. 3D). Similarly, disruption of TJs by long-term exposure to low calcium (20 hours or more, results in dissociation of TJ multiprotein complex (ZO-1–Occludin–Claudin1; Fig. 3D) in MLE15 cells, in agreement with the findings in other epithelial cell types (Sakakibara et al., 1997; Farshori and Kachar, 1999; Seth et al., 2007). Second, to assess whether changes in Eya1 activity affect the phosphorylation state of TJ proteins at mature TJs, TJ proteins were immunoprecipitated from polarized MLE15 (untreated) cells, or from those cells that underwent loss or gain of Eya1 function, then analyzed by immunoblotting with anti-phosphoserine antibody (Fig. 3H,I). Upon *Eya1* knockdown, TJ proteins ZO-1 and occludin exhibited a decrease of serine phosphorylation (Fig. 3H,I). In the rescue experiments, re-expression of wild-type *Eya1*, not targeted by the siRNAs, led to a near control level of phosphorylated TJ proteins ZO-1/occludin, whereas re-expression of the tyrosine-phosphatase-dead mutant *Eya1* did not (Fig. 3H,I). No apparent change in the expression levels of ZO-1 or occludin was evident in these experiments as determined by western blotting (Fig. 3H–J). Together, these data suggest that TJ protein phosphorylation and consequently translocation from the cytosol to cell periphery during junctional biogenesis are Eya1 phosphatase dependent.

Finally, to determine Eya1 activity on the subcellular localization of TJ proteins in a cellular context, the distribution of ZO-1, occludin, and claudin1 was compared by immunofluorescent staining during Ca^{2+} switch experiments

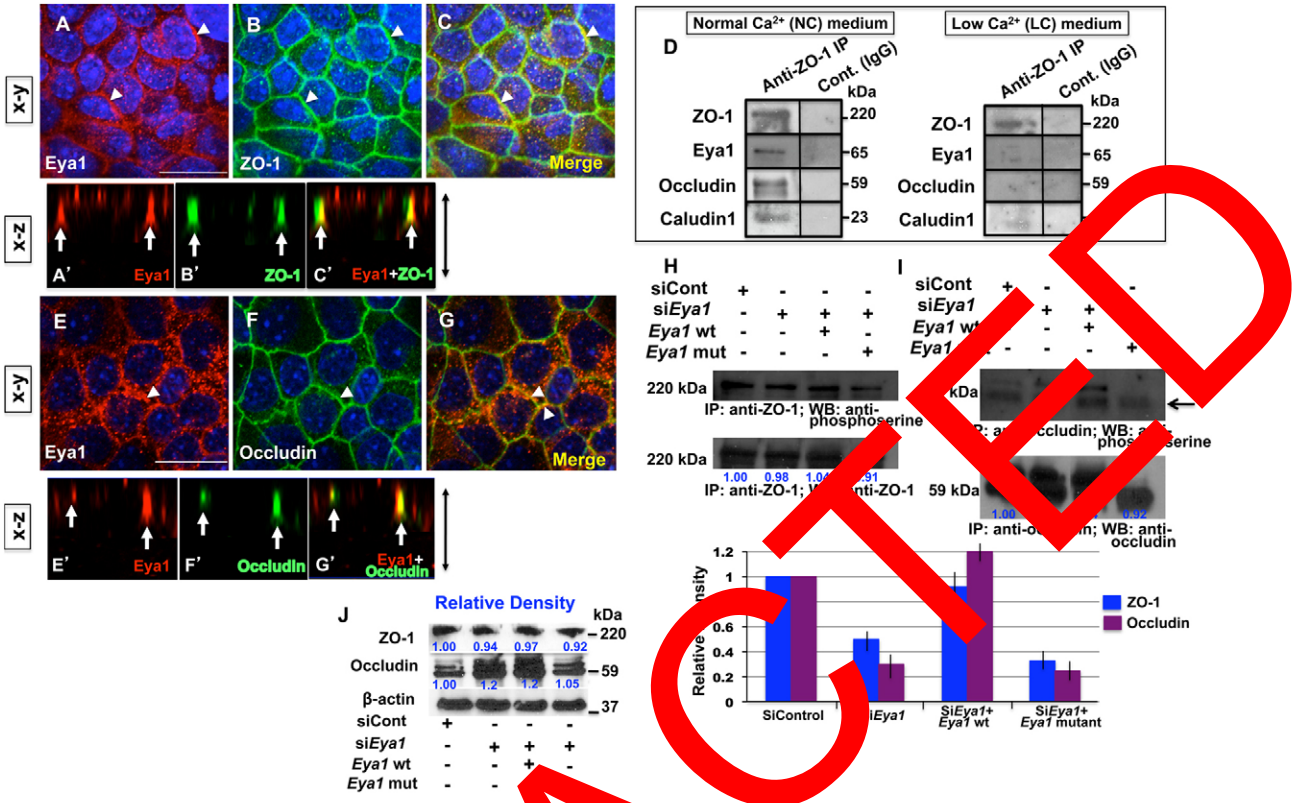

Fig. 3. Eya1 colocalizes with and co-immunoprecipitates as well as controls the phosphorylation state of TJ proteins in vitro. (**A–C′**,**E–G′**) Representative x–y sections and transverse x–z views obtained in polarized MLE15 cells grown in NC medium labeled with either Eya1 (A,E), ZO-1 (B) or occludin (F) antibodies. Note Eya1 and TJ protein colocalization at areas of intercellular contact (colocalization appears yellowish green in C and G; arrowheads and arrows in x–z images). Scale bars: 50 µm. Black double-headed arrows next to x–z images indicate the thickness of the epithelium (10 µm). (**D**) Endogenous ZO-1 was immunoprecipitated from MLE15 cells grown in NC medium for 3 days (left panel) or 2 days then in LC medium overnight (right panel) with a specific ZO-1 antibody, and western blotting was performed with antibodies specific to different TJ proteins or Eya1. (**H**,**I**) ZO-1 and occludin were immunoprecipitated from MLE15 cells grown for 3 days in NC medium from cells that had *Eya1* loss or gain of function. The samples were analyzed by SDS-PAGE and immunoblotting with anti-phosphoserine antibody, and the blots were reprobed with anti-ZO-1 or occludin antibodies. The arrow indicates the slower-migrating isoform of occludin (bottom band that was quantified by densitometry), which is more highly serine phosphorylated than the faster-migrating isoform (top band) (Sakakibara et al., 1997; Balakrishnan et al., 1998). The bar graph shows the relative band intensity of phosphorylated ZO-1 or occludin of cells under different treatments relative to control siRNA-treated cells. Error bars indicate s.d. ($n=2$). (**J**) Western blotting shows no apparent changes in the expression levels of ZO-1 and occludin proteins in the total cell extracts used for experiments shown in H,I. Equal loading was confirmed by reprobing for β-actin. Blue numbers are relative band intensity for ZO-1 (top row) or of occludin (bottom row) proteins in H,I,J.

performed in untransfected MLE15 cells (data not shown) or cells receiving either control siRNA or *Eya1*siRNA (Fig. 4). MLE15 cells were Ca^{2+} starved overnight to induce TJ downregulation, which results in redistribution of TJ proteins from the cell periphery to the cytosol. Then they were transferred to NC medium to induce TJ biogenesis. Previously, the Ca^{2+} switch initiates a rapid but partial sealing of TJ proteins from the cytosol to the membrane (e.g. 4A,B), and Eya1-based TJ stabilization, while resealing is achieved by >20 h after the Ca^{2+} switch (Fig. 4B), as reported in other epithelial cell types (Farshori and Kachar, 1999; Nunbhakdi-Craig et al., 2002). In control siRNA-transfected cells, 2 h after the Ca^{2+} switch a portion of total TJ proteins had already migrated to the cell periphery, but this redistribution was almost completely inhibited in *Eya1*siRNA-transfected cells (compare Fig. 4A,C,D with Fig. 4E,G,H). The failure of TJ protein migration to the cell periphery continued 24 h after the Ca^{2+} switch in *Eya1*siRNA-transfected cells (compare Fig. 4B with Fig. 4F). Rescuing Eya1 function by expressing wild-type murine Eya1 construct, which is not targeted by the siRNAs, into these siRNA depleted cells reversed this, permitting accumulation of TJ proteins at cell–cell contact sites (Fig. 4I–L), while a phosphatase-dead mutant Eya1 failed to rescue (Fig. 4M–P). These data reinforce the idea that Eya1 phosphatase activity is critical for triggering the initial sorting and translocation of TJ proteins from the cytosol to cell membrane/periphery during junctional biogenesis.

aPKCζ-dependent regulation of TJ proteins by Eya1

Phosphorylation of aPKCζ is critical for its activation, which is essential for serine phosphorylation of occludin, claudin1 and ZO-1 that is critical for their migration from the cytosol to the cell periphery in order to assemble TJs in vivo and during Ca^{2+}-induced TJ biogenesis (Stuart and Nigam, 1995; Farshori and Kachar, 1999; Yamanaka et al., 2001; Suzuki et al., 2001; Suzuki et al., 2002; Nunbhakdi-Craig et al., 2002). However, unregulated and prolonged activation of different PKC isoforms leads to dephosphorylation and failure of TJ protein migration during junctional biogenesis that results in TJ disassembly in different cell types (Clarke et al., 2000a; Clarke et al., 2000b; Song et al.,

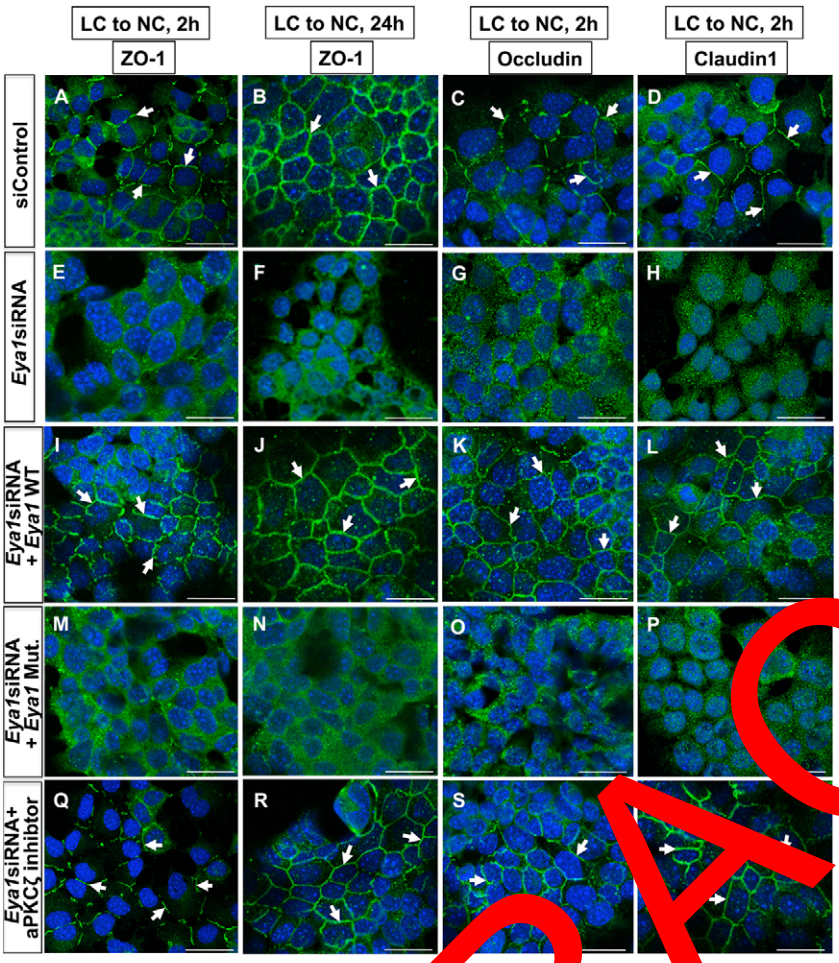

Fig. 4. Effects of deregulating Eya1 activity on the initial redistribution of TJ proteins during Ca^{2+}-induced TJ biogenesis. MLE15 cells grown in NC medium for 24 h were incubated overnight in LC medium then switched back for 2 h or 24 h to NC medium as indicated for each panel. (**A–D**) Immunofluorescence shows gradual TJ protein assembly/formation (arrows) between 2 h (A,C,D) and 24 h (B) in NC medium. (**E–H**) TJ protein assembly was inhibited after *Eya1* knockdown compared with control (**I–P**) Rescue of endogenous Eya1 function by co-transfection of murine siRNA and murine wild-type or enzymatically inactive mutant *Eya1* constructs into MLE15 cells reveals that TJ protein assembly (arrows) is dependent on Eya1 phosphatase activity. (**Q–T**) Partial inhibition of aPKCζ in *Eya1*siRNA-transfected MLE15 cells rescued TJ protein assembly (arrows). Scale bars: 50 μm.

2001). In addition, PKC has been shown to variably induce junction assembly and disassembly depending on the cell type, conditions of activation and PKC isozyme. Moreover, specific PKC isozymes can affect the same physiological function in either a similar or opposite (counter-regulatory) fashion. The pattern of selectivity for target proteins may reflect association of the particular isozyme with specific anchoring proteins or other protein–protein interactions (Feigin and Muthuswamy, 2009).

We recently reported that Eya1, which has well-known phosphatase activities (Li et al., 2003), may bind to and is able to partially dephosphorylate aPKCζ in vitro as well as regulating aPKCζ phosphorylation that strongly increases after *Eya1* knockout/knockdown in lung epithelial cells (El-Hashash et al., 2011a). We therefore tested the hypothesis that unregulated/increased phosphorylation of aPKCζ after *Eya1* knockout/knockdown causes reduction of TJ protein phosphorylation and thus TJ disassembly. As shown in Fig. 5A,B,E,F,I–M, increased aPKCζ phosphorylation by treating MLE15 lung epithelial cells with phosphatidic acid (PA), which physically binds to and is a physiological activator of aPKCζ (Limatola et al., 1994), results in decreased serine phosphorylation and disassembly of TJ proteins, compared to cells treated with vehicle (control). Interestingly, overexpression of Eya1 in PA-treated cells did not rescue TJ assembly defects (Fig. 5P,Q), suggesting that Eya1 does not act downstream of aPKCζ. Notably, Eya1 localization at TJs/cell periphery was reduced in PA-treated cells, with increased Eya1 localization to the cytoplasm (Fig. 5G,H), suggesting a role for aPKCζ activity levels in Eya1 subcellular localization.

Next, we investigated the aPKCζ functional role in Eya1-dependent TJ protein regulation by inhibiting aPKCζ activity on *Eya1*siRNA-induced disassembly of TJ proteins. Eya1 protein is sufficient to partially inhibit aPKCζ phosphorylation/activity in an in vitro phosphatase assay (El-Hashash et al., 2011a). Interestingly, partial inhibition of aPKCζ phosphorylation in *Eya1*siRNA-transfected MLE15 cells rescued the accumulation of TJ proteins at junctional areas and TJ formation (compare Fig. 4Q–T with Fig. 4E–H), suggesting that aPKCζ acts downstream of Eya1. This also suggests that aPKCζ phosphorylation state plays key roles in the Eya1 regulatory pathway that controls both TJ formation (current data) and its inextricably linked cell polarity (El-Hashash et al., 2011a), while the unregulated/prolonged increase of aPKCζ phosphorylation after *Eya1* knockdown/knockout may be the reason for the TJ defects noted herein.

Genetic activation of Notch1 signaling in *Eya1*$^{-/-}$ lungs rescues TJ formation defects as well as the distal epithelial architecture phenotype

Most recently, it has been shown that loss of Notch1 activity results in disruption of intestinal epithelial TJ formation and barrier function (Dahan et al., 2011). The Eya1–aPKCζ pathway controls Notch1 signaling activity, which not only genetically interacts with Eya1 but also is severely inhibited after *Eya1* knockdown/

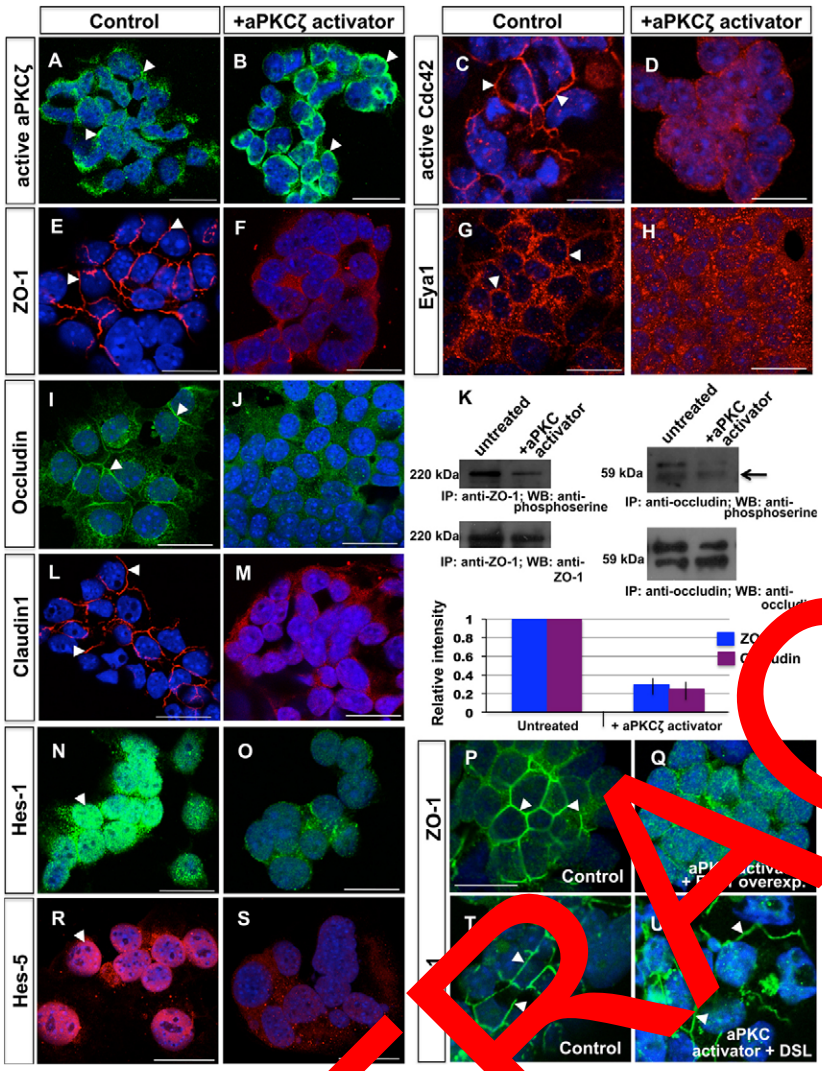

Fig. 5. Increased aPKCζ activity during Ca^{2+}-induced TJ biogenesis causes both disassembly and reduced phosphorylation of TJ proteins as well as inhibition of Notch1 signaling activity in lung epithelial cells. (**A–J,L–O,R–S**) Specific antibody staining of MLE15 cells treated with 300 μM phosphatidic acid (PA), which were grown in NC medium for 24 h and incubated overnight in LC medium containing EGTA, then switched back to NC for 24 h, show increased activity of aPKCζ at the cell periphery (B; arrowheads), but inhibition of TJ protein assembly at the cell periphery (F,J,M), reduced nuclear Hes-5 (S) expression (O) and decreased Cdc42 activity at cell–cell contacts (D) as well as reduced Eya1 localization at TJs/cell periphery (H) after Ca^{2+} switch, compared to cells treated with vehicle (control: A,C,E,G,I,L,N,R; arrowheads). (**K**) Immunoprecipitation of ZO-1 or occludin from the experiments shown in A–J,L–O,R–S. The samples were analyzed by SDS-PAGE and immunoblotting with anti-phosphoserine antibody, and then the blots were reprobed with anti-ZO-1 or occludin antibodies. Arrow indicates the slower-migrating isoform of occludin (bottom band that was quantified by densitometry), which is more highly serine phosphorylated than the faster-migrating isoform (top band) (Sakakibara et al., 1997; Gopalakrishnan et al., 1998). The bar graph shows the relative band intensity of PA-treated cells relative to untreated control cells. Error bars indicate s.d. (n=2). (**P,Q,T,U**) Notch1 signaling activity induced by the Notch agonist peptide DSL rescued TJ protein assembly in PA-treated MLE15 cells (T,U, arrowheads), whereas transfection with wild-type murine *Eya1* construct failed to rescue TJ assembly (P,Q). Scale bars: 50 μm.

knockout in the lung epithelium (Hashash et al., 2011a). We previously provided evidences that reduced Notch1 signaling activity after *Eya1* knockdown/knockout is due to increased aPKCζ activity (Hashash et al., 2011a), which was indeed sufficient to inhibit both Notch1 activity and TJ formation in lung epithelial cells (Fig. 5A–B,E,F,I,J,L–O,R,S), suggesting that aPKCζ functions upstream of Notch1 signaling during TJ assembly. This conclusion was further confirmed by induction of Notch1 activity signaling via the Notch agonist peptide DSL in PA-treated MLE15 cells that restored TJ protein assembly (Fig. 5T,U).

Therefore, we next determined the functional roles of Notch1 signaling in Eya1-dependent TJ formation. First, we addressed whether Notch1 function to regulate TJ formation is conserved in lung epithelium, using gene-specific siRNA in MLE-15 cells. Similar to *Eya1*siRNA-transfected cells (Fig. 4E–H), knockdown of Notch1 function caused clear TJ formation defects, as judged by the predominant cytosolic concentration of TJ proteins in transfected cells, compared with control cells (compare Fig. 6F–I with Fig. 6A–D). Moreover, the failure of TJ protein migration to the cell periphery continued 24 h after the Ca^{2+} switch in *Notch1*siRNA-transfected cells (Fig. 6G). The silencing efficiency of *Notch1*siRNA was determined by western blotting/immunocytochemistry (Fig. 6E), and the specificity of the siRNAs for *Notch1* was validated by use of multiple controls, in which nonspecific siRNAs displayed no apparent effect on Notch1 expression levels (supplementary material Fig. S3).

We then examined the effects of inhibiting Notch1 signaling on *Eya1* wild-type-induced redistribution of TJ proteins in *Eya1*siRNA-transfected cells. As shown in Fig. 4I–L, re-expression of wild-type Eya1, not targeted by the siRNAs, was sufficient alone to rescue TJ formation and promote the translocation of ZO-1, occludin, and claudin1 from the cytosol to the membrane in *Eya1*siRNA-transfected cells switched from LC to NC medium. However, this effect was blocked by stably knocking down *Notch1* expression in cells transfected with *Eya1*siRNA and wild-type *Eya1* (Fig. 6J–M). Interestingly, Eya1–TJ protein association was Notch1 independent because it was not apparently changed in cells transfected with *Notch1*siRNA, as assessed by immunoprecipitation assay (data not shown).

We next tested the hypothesis that inactivation of Notch1 signaling causes the epithelial TJ defects in $Eya1^{-/-}$ embryos by conditional genetic increase of *Notch1* levels in $Eya1^{-/-}$ lung epithelium, using *NICD*; *Spc-rtTA*$^{+/-}$*tet(O) Cre*$^{+/-}$*Eya1*$^{-/-}$ compound mutant mice (Fig. 6N–U). No changes in lung

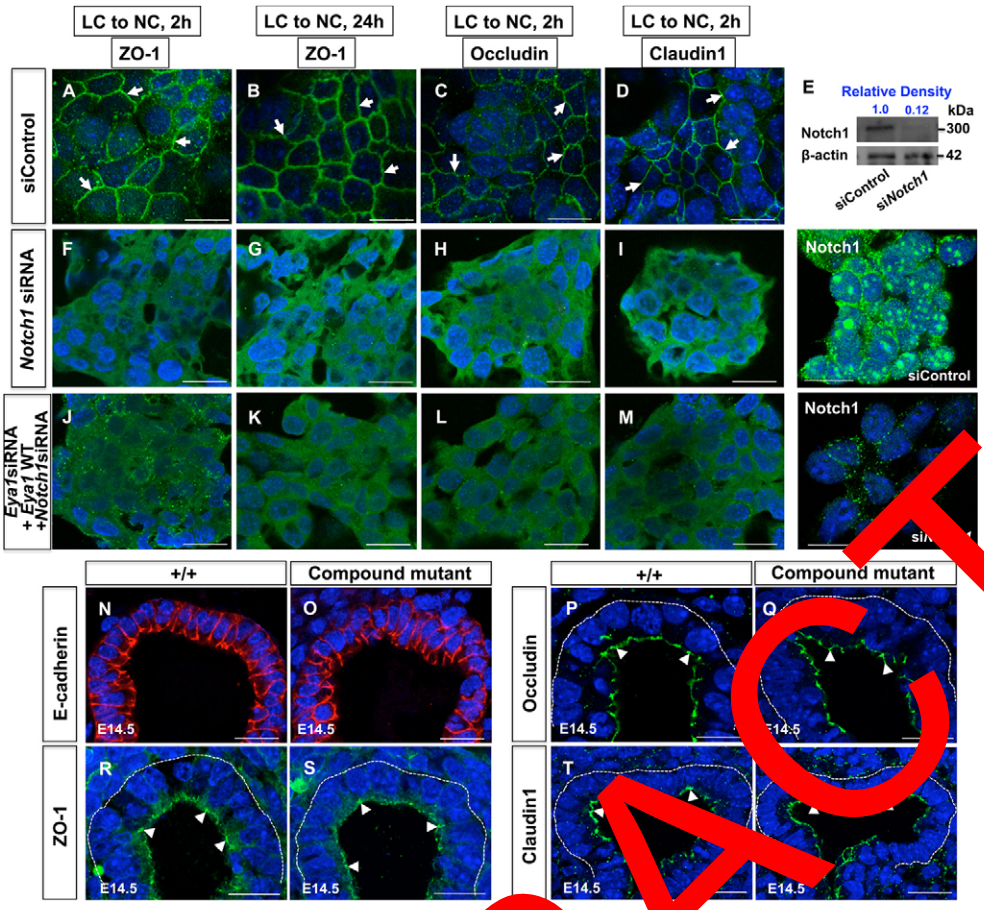

Fig. 6. Notch1-dependent regulation of TJ proteins by Eya1 during in vitro Ca^{2+}-induced TJ biogenesis and in vivo. (**A–D**,**F–M**) MLE15 cells grown in NC medium for 24 h were incubated overnight in LC medium then switched for 2 h or 24 h back to NC medium as indicated for each panel. (A–D) Immunofluorescence shows gradual TJ protein assembly/barrier formation (arrowheads) between 2 h (A,C) and 24 h (B,D) in NC medium. (F–I) TJ protein assembly is impaired after *Notch1* knocking down, compared with control cells. (J–M) Rescue of TJ protein assembly by co-transfection of murine *Eya1*siRNA and murine wild-type *Eya1* constructs was blocked by stably knocking down *Notch1* expression. (**E**) Western blot and immunocytochemistry for Notch1 expression show the silencing efficiency of *Notch1* siRNA in MLE15 growing for 3 days in NC medium. Blue numbers represent relative band intensity. (**N–U**) $Eya1^{-/-}$ TJ disassembly can be rescued by genetic activation of Notch1 in the lung epithelium in vivo. Both distal epithelial organization (E-cadherin staining) and apical cell concentration of ZO-1, occludin and claudin1 (arrowheads) are restored in *NICD*; *Spc-rtTA*$^{+/-}$*-tet(O) Cre*$^{+/-}$*Eya1*$^{-/-}$ compound mutant lungs. The broken line indicates the collagen-IV-stained basement membrane. Scale bars: 50 μm.

phenotype, distal epithelial architecture and TJ formations were evident in controls: DOX-fed *Spc-rtTA* and *Spc-rtTA-tet(O) Cre* mice (data not shown). As recently reported by us, *NICD*; *Spc-rtTA*$^{+/-}$*tet(O) Cre*$^{+/-}$*Eya1*$^{-/-}$ compound mutant lungs are comparable with doxycycline-untreated control lungs, and following induction with DOX feeding, they showed increased lung size and restoration of epithelial branching compared with lungs of $Eya1^{-/-}$ littermates (El-Hashash et al., 2011a). Moreover, the accumulation of TJ proteins at cell–cell contact sites was promoted and the distal epithelial cell organization was restored into the wild-type control image in compound mutant lungs versus $Eya1^{-/-}$ lungs (compare Fig. 6N–U with $Eya1^{-/-}$ phenotype in Fig. 1), suggesting substantial rescue of the $Eya1^{-/-}$ TJ defect phenotype. Together, these data suggest that Eya1-controlled TJ formation is Notch1 dependent.

Eya1–aPKCζ–Notch1 signaling is essential for the activity of GTPase Cdc42

To determine the possible mechanisms by which Eya1-controlled Notch1 signaling affects TJ formation, and how ectopic expression of NICD within distal tip progenitor cells rescues the TJ assembly defect in $Eya1^{-/-}$ lungs, we examined the activity of GTPase Cdc42. Two lines of reasoning led us to examine Cdc42 activity and its relationship with Eya1–Notch1 signaling in the lung. Firstly, similar to *Eya1*siRNA (Fig. 4E–H), substantial internalization and re-distribution of TJ proteins away from the cell membrane periphery and TJ disassembly were observed in Cdc42 mutant MDCK epithelial cells (Bruewer et al., 2004). Secondly, reduction of Cdc42 activity results in TJ disassembly and disruption of cell polarity (Schwamborn and Püschel, 2004), similar to $Eya1^{-/-}$ lung epithelium (El-Hashash et al., 2011a). In addition, both immunostaining and immunoprecipitation analyses showed reduced activated Cdc42 that localized to cell–cell contacts after *Eya1* knockout (Fig. 7A–C) or knocking down *Eya1* in MLE15 cells in culture (Fig. 7D,F,G), which was accompanied by both TJ protein disassembly (Fig. 7N–O,R–S,V–W) and loss of cell polarity (El-Hashash et al., 2011a), without apparent changes in Cdc42 expression levels (Fig. 7A′,B′,D′,J,K) in the lung epithelium. Immunoblotting showed that Cdc42 was present in equivalent amounts in the original extracts (Fig. 7D′), thus showing the specificity of the antibody used against the activated form of Cdc42 protein and confirming equal protein loading for the immunoprecipitation/immunoblotting experiment of active Cdc42 (Fig. 7D,D′). Interestingly, Cdc42 activity at cell periphery is reduced in PA-treated cells (Fig. 5C,D). Together, these data suggest that Cdc42 acts downstream of Eya1–aPKCζ signaling in the lung.

Next, to determine whether Cdc42 is involved in Eya1–aPKCζ–Notch1 signaling pathway that controls TJ assembly in the lung epithelium, we tested whether activation of Notch1 signaling is sufficient to rescue Cdcd42 activity and consequently TJ assembly defects in *Eya1* knockdown background MLE-15 cells. As shown in Fig. 7H,I,P,Q,T,U,X,X′, both Cdc42 activity and TJ assembly were reduced after *Notch1* knockdown, while induction of Notch1 activity signaling via the Notch agonist peptide DSL in *Eya1*siRNA-transfected lung epithelial cells

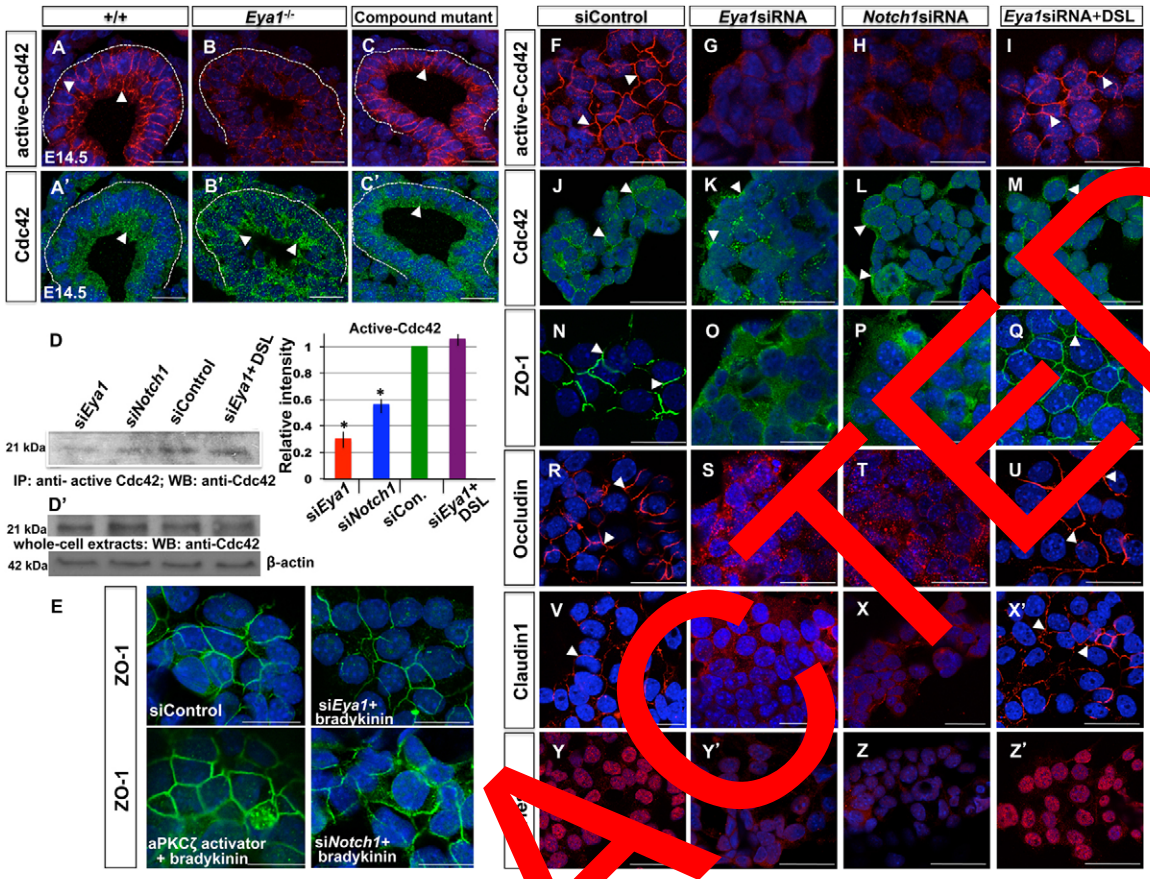

Fig. 7. Eya1–Notch1 signaling controls Cdc42 activity during epithelial morphogenesis. (**A–C**,**A′–C′**) Immunofluorescence for active Cdc42 shows decreased Cdc42 activity, but no apparent change in Cdc42 expression levels in E14.5 *Eya1*$^{-/-}$ distal epithelium compared with control lungs (arrowheads). Cdc42 activity is substantially rescued in *NICD*; *Spc-rtTA*$^{+/-}$-*tet(O)*-*Cre*$^{+/-}$*Eya1*$^{-/-}$ lungs (arrowheads). (**D**) Immunoprecipitation of active Cdc42 from the experiments shown in F–Z′. MLE15 cells grown for 3 days in NC medium underwent different loss or gain of function and then active Cdc42 was immunoprecipitated. The samples were analyzed by SDS-PAGE and immunoblotting with anti-Cdc42 antibody. The bar graphs show the relative band intensity from cells under different treatments relative to control siRNA-treated cells. Error bars indicate s.d. (n=2). (**D′**) Western blotting shows no apparent changes in the expression levels of Cdc42 protein from whole-cell extracts from the experiments showing in D. Equal loading was confirmed by reprobing for β-actin. (**E**) Immunofluorescence for ZO-1 in MLE15 cells transfected with *Eya1*siRNA or *Notch1*siRNA or treated with 300 μM PA, and grown in NC medium for 3 days before incubation with 200 nM bradykinin for 5 minutes in order to stimulate endogenous Cdc42 activity prior to fixation. Note the well-assembled TJs in bradykinin-treated cells. (**F–Z′**) Specific antibody staining of MLE15 cells grown for 3 days in NC medium shows reduction of active Cdc42 localized to cell–cell contacts (F–H; arrowheads), no apparent change in Cdc42 expression (J–L; arrowheads) and inhibition of both TJ protein assembly (N–P,R–T,V–X; arrowheads) and Hes-5 nuclear expression (Y–Z) after *Eya1* or *Notch1* knockdown compared with control cells. (I,M,Q,U,X′,Z′) Activation of Notch1 signaling by treatment of *Eya1*siRNA-transfected MLE15 with 100 nM DSL peptide restored Cdc42 activity and rescued TJ protein assembly (arrowheads). Scale bars: 50 μm.

restored both Cdc42 activity and TJ protein assembly. This was also shown in vivo after genetic activation of Notch1 signaling in *Eya1*$^{-/-}$ lung epithelium (Fig. 7A–C). Cells treated with control vehicle for DSL showed no apparent changes in Cdc42 activity/assembly (data not shown).

To further examine the requirement for Cdc42 downstream of Eya1–aPKCζ–Notch1 signaling, we stimulated endogenous Cdc42 activity by treating MLE15 cells with bradykinin, a known activator of Cdc42 (Kozma et al., 1995; Kim et al., 2000) after knocking down *Eya1* or *Notch1*, or treating cells with PA; separately. As shown in Fig. 7E, stimulation of endogenous Cdc42 restored TJ assembly in these cells. Cells treated with control vehicle for bradykinin or PA showed no apparent changes in TJ assembly (data not shown). From these experiments, we concluded that Cdc42 acts downstream of Eya1–aPKCζ–Notch1 signaling in the process of TJ protein assembly.

Cdc42 controls TJ protein trafficking downstream of Eya1–aPKCζ–Notch1 signaling

Since perturbation of Eya1–aPKCζ–Notch1 signaling alters TJ protein distribution in lung epithelial cells from a continuous cell border localization to intracellular puncta and cytoplasmic localization (Figs 4–7), we next determine whether Cdc42 functions downstream of Eya1–aPKCζ–Notch1 signaling to induce TJ protein trafficking from endosomes to the cell border. To evaluate changes of protein distribution, colocalization ICC for TJ proteins (ZO-1) and a marker of late endosomes was performed in MLE15 cells (Fig. 8). In control cells, immunoreactivity for TJ proteins was continuous at the cell borders, whereas knocking down *Eya1* or *Notch1* both disrupted TJ protein immunoreactivity at the cell border and increased intracellular punctate labeling, which colocalized with lysosome-associated membrane protein 1 (LAMP1), which is a marker of late endosomes and lysosomes

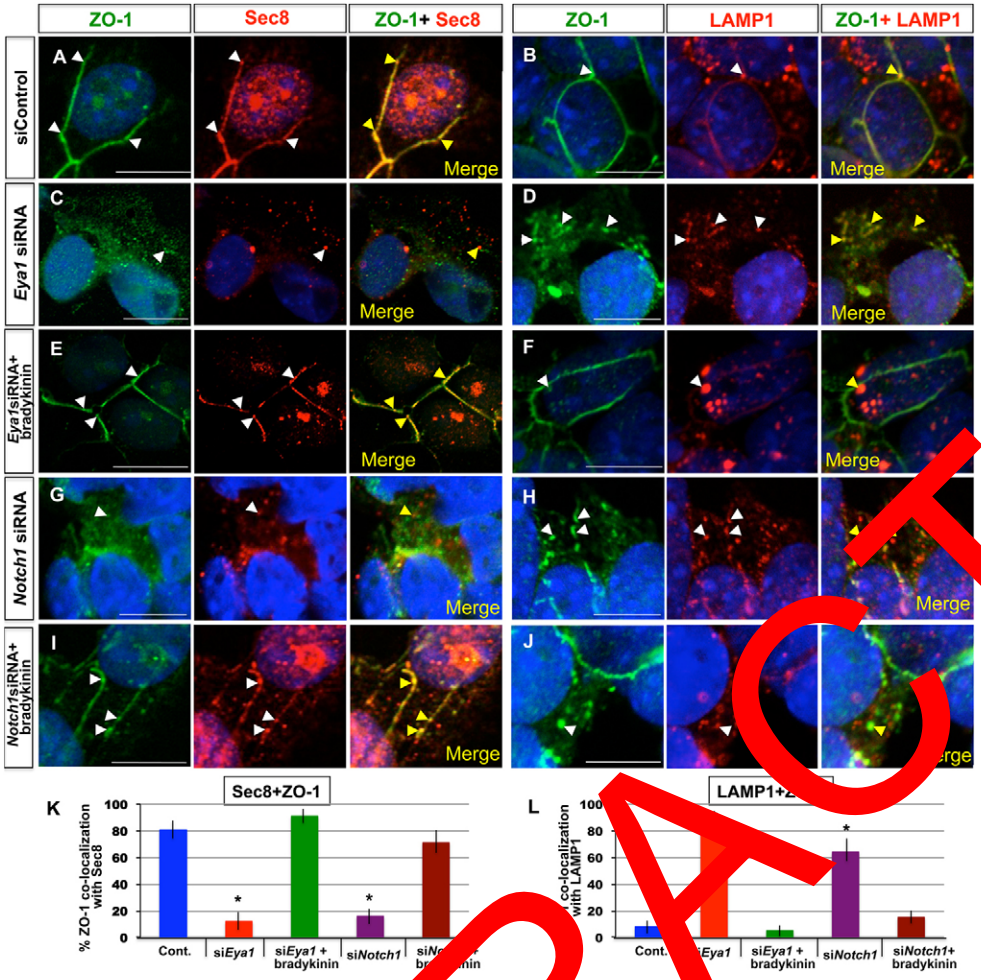

Fig. 8. Cdc42 a downstream of 1–aPKCζ–Notch signaling to induce protein trafficking ring TJ assemb (**A–J**) ble staining ZO-1 and e r traf ng protein Sec8 AMP1 ows th knocking d n *Eya1* o rupted the tinuous cell b der d ibuti ZO-1, and decreased the colo ion between 1 and the exocys 8 in ML cells (C,G; arrowhead ed to control cells (A; arrowheads). calization between ZO-1 and the endosomal marker LAMP1 reased in these cells (D,H; arrowheads), ared to control cells (B; arrowheads). (E,I) Treatment of *Eya1*siRNA- or *Notch1*siRNA-transfected cells with 200 nM of the Cdc42 activator bradykinin for 5 minutes prior to fixation restored colocalization of ZO-1 with both Sec8 and LAMP1 (arrowheads), and TJ assembly. Arrowheads in A–J indicate colocalization puncta. Scale bars: 50 μm. (**K**,**L**) Quantification of the colocalization shown in A–J ($n=4$). Error bars represent the s.d. *Significantly different from control (ANOVA, Dunnett's test; *$P<0.05$ versus control cells).

(Fig. 8B,D,H,L). Stimulation endogenous dc42 greatly reduced colocalization of t TJ ein ZO-1 wit AMP1 and restored TJ assembly in these cells (F 8F,J,L). Cells treated with control vehicle for br kinin showed n parent changes in ZO-1–LAMP1 coloca ion (data not shown). ese data suggest that Cdc42 function ownstream f Eya1–aPKCζ–Notch1 signaling to induce TJ pr s to traff rom late endosomes to the cell border.

During epith l rization, e targeting of membrane-associat and intr ular prote to cell junctions is controlled by t exo t pathw (Hsu al., 1999). To further determine w ether Cdc 2 is requi vesicular trafficking of TJ protein downstrea of Eya1–aPKCζ–Notch1 signaling, we next analyze localiz ins with trafficking protein Sec8, which is mber of the exocyst complex that is essential for vesicular traffi g in polarized cells (Lipschutz and Mostov, 2002). As shown ig. 8C,G,K), there was little colocalization between the exocys ec8 and ZO-1 in cells treated with *Eya1*siRNA or *Notch1*siRNA at cell periphery, compared to control cells (Fig. 8A,K). This colocalization was increased and restored to a near-control cell level after stimulation of endogenous Cdc42 in *Eya1*siRNA or *Notch1*siRNA MLE15 cells (Fig. 8E,I,K). These data further confirm the requirement of Cdc42 for TJ protein trafficking downstream of Eya1–aPKCζ–Notch1 signaling in the lung epithelium.

Discussion

The normal growth and functioning of the lung depends on the establishment and maintenance of a milieu in the alveolar space that is distinct from the composition of the sub-epithelial compartment. This process depends on the formation and proper functioning of TJs between adjacent cells making up the alveolar epithelial sheet, loss of which is involved in acute lung injury and acute respiratory distress syndrome (ARDS), between adjacent cells making up the epithelial sheet. Yet, very little is known about the basic regulatory mechanism(s) underlying permeability barrier formation and integrity of the lung epithelium. Herein, we uncovered what we believe to be a novel function for Eya1 phosphatase in controlling epithelial TJ formation and barrier integrity in the lung.

Using *Eya1* knockout and siRNA approaches, we found that Eya1 phosphatase is critical for both lung epithelial polarity (El-Hashash et al., 2011a) and the formation of TJs (this study). We also found severe TJ disassembly and cell polarity loss in *Eya1*siRNA-transfected cells, and significant rescue of TJ and polarity defects after reintroducing wild-type murine Eya1 back to *Eya1* RNAi lung epithelial cells (Fig. 4) (El-Hashash et al., 2011a). On the other hand Eya1 phosphatase did not appear to modulate the expression of TJ proteins ZO-1, occludin and claudin1 nor of polarity proteins aPKCζ, Par3 and Par6, (this

study and El-Hashash et al., 2011a). Similarly, members of the protein phosphatase family such as PP2A and PP2B were shown to function as crucial regulators of both epithelial TJ assembly/function and cell polarity (Lum et al., 2001; Nunbhakdi-Craig et al., 2002; Sontag and Sontag, 2006; Chabu and Doe, 2009).

How does Eya1 protein function to maintain TJ protein assembly and epithelial integrity? Based upon the data herein, Eya1 phosphatase appears to exert this effect by influencing multiple closely related processes. First, the function of Eya1 in cell polarization, which is inextricably linked to the preservation of TJ assembly (Shin et al., 2006), may help to explain its role in TJ protein assembly. Phosphorylated (active) aPKCζ, which is a target of Eya1 phosphatase activity in the lung epithelium in vivo/in vitro (El-Hashash et al., 2011a), has been shown to form a complex with Par proteins in the epithelium and to act as a critical regulator of both apico-basal polarity and TJ assembly in other epithelial cell types (Ohno, 2001; Hirai and Chida, 2003; Shin et al., 2006). Activated aPKCζ is essential for serine phosphorylation and recruitment of ZO-1, occludin and claudin1 to the cell periphery during junctional biogenesis, and thus is critical for TJ assembly (Ohno, 2001). Moreover, aPKCζ-mediated TJ protein phosphorylation is critical for Ca^{2+}-induced TJ assembly (Stuart and Nigam, 1995; Suzuki et al., 2001). Our data presented here suggest that aPKCζ also plays a regulatory role in lung epithelial TJ formation and could mediate the effects of Eya1 phosphatase in both TJ (this study) and cell polarity regulation (El-Hashash et al., 2011a). An attractive model is that Eya1 phosphatase acts to reduce and optimize aPKCζ phosphorylation (activity) to be optimal for proper epithelial TJ protein phosphorylation/assembly and cell polarity in the lung epithelium. Thus, *Eya1* deletion in vivo or in vitro causes unregulated and prolonged activation of aPKCζ that leads to reduction of the phosphorylation of ZO-1, occludin and claudin1, which results in failure of their migration and recruitment from the cytosol to the cell periphery during junctional biogenesis, leading to TJ disassembly and consequently loss of cell polarity. Indeed, unregulated and prolonged activation of different PKC isoforms leads to dephosphorylation and failure of migration of TJ proteins during junctional biogenesis that results in TJ disassembly and subsequent loss of cell polarity in different cell types (Clarke et al., 2000a; Clarke et al., 2000b; Song et al., 2001). This model might explain defects of both TJ formation and cell polarity after interfering with Eya1 function in vivo or in vitro (this work and El-Hashash et al., 2011a). Indeed, increased aPKCζ activity severely reduced TJ protein phosphorylation and assembly in the lung epithelium (Fig.). Our findings that Eya1 may bind to aPKCζ and that *Eya1* deletion causes a significant increase in aPKCζ phosphorylation (El-Hashash et al., 2011a), together with reduced TJ protein phosphorylation and both TJ and polarity defects that can be rescued by partial inhibition of aPKCζ phosphorylation (Figs 3, 4) (El-Hashash et al., 2011a), provide strong evidence for this model and support the concept that the Eya1–aPKCζ regulatory pathway is essential for controlling both TJ protein assembly and cell polarity. These findings also suggest that unregulated/prolonged increase of aPKCζ phosphorylation after *Eya1* knockdown/knockout may be the reason for TJ defects. Similarly, other protein phosphatase–PKC interactions have been previously well reported as mechanisms for TJ assembly and polarity regulation in both epithelium and endothelium of other organs (Lum et al., 2001; Nunbhakdi-Craig et al., 2002). Further studies are needed to identify the precise Eya1 binding partner(s) at the TJ, and to confirm the significance of this novel Eya1–aPKCζ regulatory pathway model of TJ formation in the lung epithelium.

The control of Notch1 signaling activity provides another mechanism for Eya1 phosphatase regulation of TJ formation in the lung epithelium. There is growing evidence that interaction between the Notch receptor and its ligand is essential for the formation of TJs. Thus, loss of Notch1 expression in the intestinal epithelium leads to disruption of TJ formation, decreased transepithelial resistance and dysregulated localization of TJ proteins (Dahan et al., 2011). Here, the reduction of *Notch1* by siRNA had a very similar effect on inhibiting TJ formation as reduction of *Eya1* in MLE15 epithelial cells (Figs 4, 6). In addition, the ability of *Notch1*siRNA to block the rescue of TJ and polarity defects after reintroducing wild-type Eya1 back to *Eya1*siRNA lung epithelial cells (Fig.) supports our conclusion that Notch1 signaling is a critical mediator of Eya1 phosphatase-regulated TJ formation and polarity establishment in the distal lung epithelium. This is further confirmed by our finding that Notch1 activity is dependent on Eya1 phosphatase activity and is severely inhibited after *Eya1* knockout/knockdown, and also that Eya1 phosphatase targets aPKCζ phosphorylation, which controls Notch1 signaling activity by regulating the function of the Notch inhibitor Numb (Chapman et al., 2006; Dho et al., 2006; Smith et al., 2007), in the lung epithelium in vivo/in vitro (El-Hashash et al., 2011a). Our findings that genetically increasing Notch1 activity in *Eya1*$^{-/-}$ lungs substantially rescues both epithelial TJ (Fig. 6) and polarity defects (El-Hashash et al., 2011a) provide strong evidence that Notch1 activity is critically involved in Eya1 phosphatase-regulated TJ and polarity formation. Whether Eya1 directly or indirectly acts through aPKCζ to regulate Notch signaling will be the subject of future study.

The mechanisms and external factors that regulate TJ assembly, cell polarity and spindle orientation in epithelial cells are poorly understood. However, the main players in this process in mammalian epithelial cells may be the same, including Cdc42 and aPKCζ–Par3/6 protein complex (Siller and Doe, 2009; Schwamborn and Püschel, 2004; Bruewer et al., 2004). TJ disassembly and paracellular permeability is enhanced in Cdc42 mutant cells. Thus, expression of dominant negative form of Cdc42 in MDCK cells induces substantial internalization and re-distribution of TJ proteins away from the cell membrane periphery (Bruewer et al., 2004). In addition, downregulation of Cdc42 activity inhibits the trafficking, and consequently localization of Par polarity complex, which are essential for both TJ assembly and establishment of cell polarity, to TJs (Schwamborn and Püschel, 2004). Herein, we have shown that Eya1–aPKCζ–Notch1 signaling positively controls Cdc42 activity, which is an essential regulator of TJ assembly, cell polarity and spindle orientation (Schwamborn and Püschel, 2004; Bruewer et al., 2004; Jaffe et al., 2008). These novel findings in the embryonic lung epithelium are consistent with recently reported data in other cell types showing that Notch1 signaling pathway interacts with Cdc42 signaling during the neural cell lineage specification phase (Endo et al., 2009); while the Eya tyrosine phosphatase activity positively regulates Cdc42 activity in breast cancer cells (Pandey et al., 2010). We also found that Cdc42 is required downstream of Eya1–aPKCζ–Notch1 signaling to induce TJ proteins to traffic from the cytoplasm to the cell border in order to form TJs (Fig. 8), and that activation of Notch1 signaling in *Eya1*siRNA-transfected lung epithelial cells restored Cdc42 activity, which was reduced after

Eya1 knockdown, and consequently TJ protein assembly (Fig. 7). This further supports our in vivo data that ectopic expression of NICD within distal tip progenitor cells rescued the TJ assembly defect in *Eya1*$^{-/-}$ lungs in vivo (this study) and also induces proper trafficking of Par polarity protein complex, and consequently rescues cell polarity/spindle orientation defects in *Eya1*$^{-/-}$ lung in vivo (El-Hashash et al., 2011a). Collectively, our finding that Notch1 signaling positively controls Cdc42 activity in lung epithelial cells could explain how genetic activation of Notch1 signaling within lung epithelial cells rescues both TJ assembly (this study) and cell polarity/spindle orientation (El-Hashash et al., 2011a) defects after *Eya1* knockout/knockdown, and further suggests Eya1–Notch1–Cdc42 as a novel regulatory pathway for these processes.

Several recent reports show that the permeability of TJs in epithelia is an important factor in several pulmonary diseases. Thus, alteration of TJ formation plays an important role in lung diseases such as COPD, asthma and ARDS (Mazzon and Cuzzocrea, 2007; Soini, 2011). In this regard, our data on Eya1 regulatory mechanisms controlling embryonic epithelial TJ formation and cell polarity suggest that influencing alveolar epithelial TJ formation by manipulating Eya1 phosphatase activity might identify future targets in the treatment of lung injury, providing a conceptual framework for future mechanistic and translational studies in this area.

Our future studies will focus on the determination of Eya1–aPKCζ–Notch1 functional and molecular interactions: the function of dephosphorylation and protein–protein interaction of Eya1 and aPKCζ and its effect on Notch signaling and on both TJ and polarity establishment in the lung. We also plan to use a conditional knockout approach to delete *Eya1* specifically from the epithelial compartment to further investigate its specific functional roles in epithelial cell development. Nonetheless, the *Eya1* mutants reported herein provide a new mouse model for lung epithelial TJ and polarity defects and will help us to understand the mechanisms that control lung epithelial morphogenesis.

Materials and Methods

Animals

Eya1$^{-/-}$, *Spc-rtTA*$^{+/-}$ and *Notch1* conditional transgenic (NICD) mice, and their genotyping have been published (Tefft et al., 2002; Xu et al., 2002; Tefft et al., 2002; Yang et al., 2004). Wild-type littermates were used as controls. Conditional *NICD;Eya1*$^{+/-}$ female mice were generated as described (El-Hashash et al., 2011a). Ten compound mutant embryos, which showed more increase of pulmonary Notch1 expression than *Eya1*$^{-/-}$ littermates, were generated at expected Mendelian ratios and examined at different stages.

Immunocytochemistry/immunohistochemistry and confocal microscopy

MLE15 cells were fixed with 4% PFA for 20 min. Lungs were fixed with 4% PFA overnight, embedded in paraffin and processed for antibody staining, following previously described standard methods (Tefft et al., 2002; Tefft et al., 2005; del Moral et al., 2006a; del Moral et al., 2006b; El-Hashash et al., 2011a; El-Hashash et al., 2011b). The primary antibodies used were: anti-Eya1 (1:50) and anti-Cdc42 (1:100; Abnova), anti-ZO-1, anti-occludin anti-claudin1 (1:100; Invitrogen), anti-E-cadherin (1:100; BD Biosciences), anti-Hes-1 (1:100; Santa Cruz), anti-Hes-5 (1:100; Millipore), anti-Notch1 (1:100; Cell Signaling), anti-active Cdc42 (1:100; NewEast Biosciences), anti-LAMP1 and anti-Sec8 (1:50; BD Biosciences). Immunofluorescence was performed using secondary antibodies conjugated to Alexa Fluor 488 or 568 (1:200; Invitrogen) and analyzed using a Zeiss confocal laser-scanning microscope as previously described (El-Hashash and Kimber, 2006).

Colocalization of TJ proteins and LAMP1 or Sec8 was evaluated using ImageJ Plugin. Briefly, after three areas were at random selected in each sample, colocalization of red and green signals was individually quantified and the ratio of colocalized/total TJ protein was averaged.

Phenotype analyses, western blotting and immunoprecipitation

Western blotting and immunoprecipitation were performed using ZO-1, occludin, claudin1, E-cadherin and Eya1 antibodies described before (Nunbhakdi-Craig et al., 2002; El-Hashash et al., 2011a), and standard protocols, as described (Tefft et al., 2002; Tefft et al., 2005; Buckley et al., 2005; del Moral et al., 2006a; del Moral et al., 2006b; El-Hashash et al., 2011a; El-Hashash et al., 2011b). Briefly, for immunoprecipitation, E14.5 lung or MLE15 (grown for 3 days in NC medium) cells were lysed in RIPA buffer, centrifuged and the supernatant containing ~1 mg protein was pre-cleared by incubation with rabbit IgG and protein A/G agarose, then centrifuged. The cleared supernatant was immunoprecipitated with 3 μg Eya1 antibody for lung lysates (ZO-1, occludin or active Cdc42 antibody for MLE15 cell lysates) followed by overnight incubation with protein A/G agarose, and then washing before re-suspension in electrophoresis sample buffer. The immunoprecipitate was loaded onto Tris-glycine gel, with a lysates of lung or MLE15 cells as positive control, and the non-specific proteins precipitated by rabbit IgG as a negative control. The separated proteins were transferred to Immobilon and probed overnight with an anti-phosphoserine, anti-TJ protein or anti Cdc42 antibodies as indicated in each figure. The specificity of Eya1 antibody was determined by western blotting where it produces a band that is specific for Eya1 protein (El-Hashash et al., 2011a). The specificity of different TJ protein/Cdc42 antibodies was determined by western blotting (this study).

Cell culture and transfection

MLE15 cells were grown in culture as described by Tefft et al. (Tefft et al., 2002). Transfection of epithelial cells with siRNA or *Eya1* wild-type expression/mutant (D323A) vectors were performed following standard procedures as described (Carraro et al., 2009; del Moral et al., 2009; Dutil et al., 1998; Dutil et al., 1998; El-Hashash et al., 2011a). For siRNA experiments, there was no change in cells of blank controls or Lipofectamine controls, and their data are not presented. The knockdown/overexpression efficiency was analyzed by western blotting and immunostaining of targeted protein. Also, we used an expression vector encoding a VP16 fusion protein, and the transfection efficiency was further monitored by fluorescence staining using anti-VP16 antibody. Experiments were performed in triplicates.

Calcium switch experiments

MLE15 cells were grown in culture as described (Tefft et al., 2002; El-Hashash et al., 2011b). To induce TJ disassembly, cells were grown at first in normal Ca^{2+} (NC) medium (DMEM/F12 + 10% FBS; 1.8 mM Ca^{2+}) for 24–48 h in order to get better cell growth and number. They were then incubated in low Ca^{2+} (LC) medium (Ca^{2+}-free DMEM containing 1% dialyzed FBS) overnight for prolonged arrest. For the Ca^{2+} switch, Ca^{2+}-starved cells were transferred to NC medium for 2 h or 24 h to initiate the reassembly of TJs, and the process was followed. Experiments were performed in triplicates.

Treatment with the Notch agonist, aPKCζ activator, aPKCζ inhibitor or Cdc42 activator

MLE15 cells were treated for 3 days in NC medium with the Notch agonist peptide Delta–Serrate–Lag2 (DSL; Biomatik R&D), which corresponds to the conserved domain of the Notch ligands and shown to be the minimal unit for binding and activation of Notch receptors (Dontu et al., 2004), at a concentration of 100 nM that effectively induces Notch signaling in different cell types (Dontu et al., 2004; Chen et al., 2006). For activation of aPKCζ, MLE15 cells first grew in NC medium for 24 h in order to get enough number of cells and then incubated overnight in LC medium, which was treated with 300 μM phosphatidic acid (PA; Sigma), then switched back to NC medium for 24 h. Exogenous PA acts as a specific physiological activator of aPKCζ in cells stimulated under conditions where intracellular Ca^{2+} is at (or has returned to) basal level (Limatola et al., 1994). For aPKCζ inhibition, the aPKCζ inhibitor was used from day 1 in culture at a concentration of 50 μmol/l (Promega), at which it is effective to partially inhibit aPKCζ without displaying cytotoxicity as reported both in MLE15 cells (El-Hashash et al., 2011a) and in different cell systems (Davies et al., 2000; Buteau et al., 2001). For stimulation of endogenous Cdc42 activity, MLE15 cells were grown in NC medium for 3 days before incubation with 200 nM bradykinin for 5 minutes as described in other cell types (Kozma et al., 1995; Kim et al., 2000).

Statistical and densitometry analyses

Statistical analysis was performed as described previously (El-Hashash et al., 2005; El-Hashash et al., 2011a; El-Hashash et al., 2011b). Protein quantification was produced by densitometry analysis with the Image J software as described (El-Hashash et al., 2011a).

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References

Bruewer, M., Hopkins, A. M., Hobert, M. E., Nusrat, A. and Madara, J. L. (2004). RhoA, Rac1, and Cdc42 exert distinct effects on epithelial barrier via selective structural and biochemical modulation of junctional proteins and F-actin. *Am. J. Physiol., Cell Physiol.* **287**, C327-C335.

Buckley, S., Barsky, L., Weinberg, K. and Warburton, D. (2005). In vivo inosine protects alveolar epithelial type 2 cells against hyperoxia-induced DNA damage through MAP kinase signaling. *Am. J. Physiol. Lung Cell Mol. Physiol.* **288**, L569-L575.

Buteau, J., Foisy, S., Rhodes, C. J., Carpenter, L., Biden, T. J. and Prentki, M. (2001). Protein kinase Czeta activation mediates glucagon-like peptide-1-induced pancreatic β-cell proliferation. *Diabetes* **50**, 2237-2243.

Carraro, G., El-Hashash, A., Guidolin, D., Tiozzo, C., Turcatel, G., Young, B. M., De Langhe, S. P., Bellusci, S., Shi, W., Parnigotto, P. P. et al. (2009). miR-17 family of microRNAs controls FGF10-mediated embryonic lung epithelial branching morphogenesis through MAPK14 and STAT3 regulation of E-Cadherin distribution. *Dev. Biol.* **333**, 238-250.

Cereijido, M., Shoshani, L. and Contreras, R. G. (2000). Molecular physiology and pathophysiology of tight junctions. I. Biogenesis of tight junctions and epithelial polarity. *Am. J. Physiol. Gastrointest. Liver Physiol.* **279**, G477-G482.

Chabu, C. and Doe, C. Q. (2009). Twins/PP2A regulates aPKC to control neuroblast cell polarity and self-renewal. *Dev. Biol.* **330**, 399-405.

Chapman, G., Liu, L., Sahlgren, C., Dahlqvist, U. and Lendahl, U. (2006). High levels of Notch signaling down-regulate Numb and Numblike. *J. Cell Biol.* **175**, 535-540.

Chen, J., Crabbe, A., Van Duppen, V. and Vankelecom, H. (2006). The notch signaling system is present in the postnatal pituitary: ... expression and regulatory activity in the newly discovered side population. *Mol. Endocrinol.* **20**, 3293-3307.

Clarke, H., Soler, A. P. and Mullin, J. M. (2000a). Protein kinase C activation leads to dephosphorylation of occludin and tight junction permeability increase in MDCK epithelial cell sheets. *J. Cell Sci.* **113**, 3187-3196.

Clarke, H., Ginanni, N., Laughlin, K. V., Smith, J. B., Petti... and Mullin, J. M. (2000b). The transient increase of tight junction permeability induced by bryostatin 1 correlates with rapid downregulation of protein kinase C-alpha. *Cell Res.* **261**, 239-249.

Cook, P. J., Ju, B. G., Telese, F., Wang, X., Glass, C. K. and Rosenfeld, M. G. (2009). Tyrosine dephosphorylation of H2AX modulates apoptosis and survival decisions. *Nature* **458**, 591-596.

Dahan, S., Rabinowitz, ... M., Martin, A. P., Berin, ... C., Unkeless, J. C. and Mayer, L. (2011). ... n-1 signaling regulates intestinal epithelial barrier function, through interacti... with CD4+ T..., in mice and humans. *Gastroenterology* **140**, 550-559.

Davies, S. P., Red... H., G...ano, M. and Cohen, P. (2000). Specificity and mechanism of action ... commonly us... protein kinase inhibitors. *Biochem. J.* **351**, 9...

del M... P., ...De Langh... P., Sala... G., Veltmaat, J. M., Tefft, D., Wang, K., ...rburton, ...and Bellusci... (20...). Differential role of FGF9 on epithelium and mesenchyme ... mouse embryon... ng. *Dev. Biol.* **293**, 77-89.

...el Moral, P... Sala, F. G., Tefft, D., Shi, W., Keshet, E., Bellusci, S. and ...Warbur... (... F-A signaling through Flk-1 is a critical facilitator of ...yonic lung epithelial to endothelial crosstalk and branching morphogenesis. *De... ol.* **290**, 177-188.

Dho, S... Trejo, J., Siderovski, D. P. and McGlade, C. J. (2006). Dynamic regulation of mammalian numb by G protein-coupled receptors and protein kinase C activation: ...tural determinants of numb association with the cortical membrane. *Mol. Biol. Cell* **17**, 4142-4155.

Dontu, G., Jackson, K. W., McNicholas, E., Kawamura, M. J., Abdallah, W. M. and Wicha, M. S. (2004). Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells. *Breast Cancer Res.* **6**, R605-R615.

Dutil, E. M., Keranen, L. M., DePaoli-Roach, A. A. and Newton, A. C. (1994). In vivo regulation of protein kinase C by trans-phosphorylation followed by autophosphorylation. *J. Biol. Chem.* **269**, 29359-29362.

Dutil, E. M., Toker, A. and Newton, A. C. (1998). Regulation of conventional protein kinase C isozymes by phosphoinositide-dependent kinase 1 (PDK-1). *Curr. Biol.* **8**, 1366-1375.

El-Hashash, A. H. and Kimber, S. J. (2006). PTHrP induces changes in cell cytoskeleton and E-cadherin and regulates Eph/Ephrin kinases and RhoGTPases in murine secondary trophoblast cells. *Dev. Biol.* **290**, 13-31.

El-Hashash, A. H., Esbrit, P. and Kimber, S. J. (2005). PTHrP promotes murine secondary trophoblast giant cell differentiation through induction of endocycle, upregulation of giant-cell-promoting transcription factors and suppression of other trophoblast cell types. *Differentiation* **73**, 154-174.

El-Hashash, A. H., Turcatel, G., Al Alam, D., Buckley, S., Tokumitsu, H., Bellusci, S. and Warburton, D. (2011a). Eya1 controls cell polarity, spi... ientation, cell fate and Notch signaling in distal embryonic lung epithel... ...nt **138**, 1395-1407.

El-Hashash, A. H., Al Alam, D., Turcatel, G., B...sci, S. and War...on, D. (2011b). Eyes absent 1 (Eya1) is a critical coordi... of epithelial, mesenc...al and vascular morphogenesis in the mammalian lung. ... *Biol.* **350**, 112-126.

Endo, M., Antonyak, M. A. and Cerione, R. A. (2009). ...dc42-mTOR signa...g pathway controls Hes5 expre...n in retinoic ...-dependent ...al differentiation. *J. Biol. Chem.* **284**, 5107...18.

Farshori, P. and Kachar, B. (1999). ...stribution and phosphory...n ...ccludin during opening and resealing of tig...unctions in c...ed epithelial ...*Membr. Biol.* **170**, 147-156.

Feigin, M. E. and Muthuswa... S. K...09). ...arity proteins ...ulate mammalian cell-cell junctions and can... pathogenes... *Opin. Cell ...* **21**, 694-700.

Fougerousse, F., Dura... M., Lopez, S... el, L., D...gnon, J., Thornton, C., Ozaki, H., Kaw...i, K., Barbet, P., B...mann, ...S. et al. (2002). Six and Eya expression ...g ...an somitogenesis a... ...D gene family activation. *J. Muscle Res. ... Motil.* ...255-264.

Gonzalez-Mariscal, L., Contre... R. G., Bolívar, J. J., Ponce, A., Chávez De Ramirez, B. and Cereijido, M. (...0). Role of calcium in tight junction formation betwe...l cells. *Am. J. Ph...* **259**, C978-C986.

Gopa...rishnan, S., Raman, N., Atkinso... J. and Marrs, J. A. (1998). Rho GTPase s...aling regulates tight junction assembly and protects tight junctions during ATP ...letion. *Am. J. Physiol.* **275**, C798-C809.

Hi... T. and Chida, K. (2...). Protein kinase Czeta (PKCzeta): activation mechanisms ... cellular functions. *J...ochem.* **133**, 1-7.

Hir... T., Izumi, Y., Na...hima, Y., Tamai-Nagai, Y., Kurihara, H., Sakai, T., Su... Y., Yamanak... ., Suzuki, A., Mizuno, K. et al. (2002). Involvement of ASIP... 3 in the ...otion of epithelial tight junction formation. *J. Cell Sci.* **115**, 2485-249...

... S. C., Hazuka, C. D., Foletti, D. L. and Scheller, R. H. (1999). Targeting vesicles ...fic sites on the plasma membrane: the role of the sec6/8 complex. *Trends Cell ...Biol.* ...153.

Jaffe, A. B., Kaji, N., Durgan, J. and Hall, A. (2008). Cdc42 controls spindle orientation to position the apical surface during epithelial morphogenesis. *J. Cell Biol.* **183**, 625-633.

Jemc, J. and Rebay, I. (2007). The eyes absent family of phosphotyrosine phosphatases: properties and roles in developmental regulation of transcription. *Annu. Rev. Biochem.* **76**, 513-538.

Kim, S. H., Li, Z. and Sacks, D. B. (2000). E-cadherin-mediated cell-cell attachment activates Cdc42. *J. Biol. Chem.* **275**, 36999-37005.

Kozma, R., Ahmed, S., Best, A. and Lim, L. (1995). The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. *Mol. Cell. Biol.* **15**, 1942-1952.

Limatola, C., Schaap, D., Moolenaar, W. H. and van Blitterswijk, W. J. (1994). Phosphatidic acid activation of protein kinase C-zeta overexpressed in COS cells: comparison with other protein kinase C isotypes and other acidic lipids. *Biochem. J.* **304**, 1001-1008.

Lipschutz, J. H. and Mostov, K. E. (2002). Exocytosis: the many masters of the exocyst. *Curr. Biol.* **12**, R212-R214.

Lum, H., Podolski, J., Gurnack, M., Schulz, I., Huang, F. and Holian, O. (2001). Protein phosphatase 2B inhibitor potentiates endothelial PKC activity and barrier dysfunction. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **281**, L546-L555.

Mazzon, E. and Cuzzocrea, S. (2007). Role of TNF-alpha in lung tight junction alteration in mouse model of acute lung inflammation. *Respir. Res.* **8**, 75.

Niessen, C. M. (2007). Tight junctions/adherens junctions: basic structure and function. *J. Invest. Dermatol.* **127**, 2525-2532.

Nunbhakdi-Craig, V., Machleidt, T., Ogris, E., Bellotto, D., White, C. L., 3rd and Sontag, E. (2002). Protein phosphatase 2A associates with and regulates atypical PKC and the epithelial tight junction complex. *J. Cell Biol.* **158**, 967-978.

Ohno, S. (2001). Intercellular junctions and cellular polarity: the PAR-aPKC complex, a conserved core cassette playing fundamental roles in cell polarity. *Curr. Opin. Cell Biol.* **13**, 641-648.

Pandey, R. N., Rani, R., Yeo, E. J., Spencer, M., Hu, S., Lang, R. A. and Hegde, R. S. (2010). The Eyes Absent phosphatase-transactivator proteins promote proliferation, transformation, migration, and invasion of tumor cells. *Oncogene* **29**, 3715-3722.

Perl, A. K., Wert, S. E., Nagy, A., Lobe, C. G. and Whitsett, J. A. (2002). Early restriction of peripheral and proximal cell lineages during formation of the lung. *Proc. Natl. Acad. Sci. USA* **99**, 10482-10487.

Sakakibara, A., Furuse, M., Saitou, M., Ando-Akatsuka, Y. and Tsukita, S. (1997). Possible involvement of phosphorylation of occludin in tight junction formation. *J. Cell Biol.* **137**, 1393-1401.

Schwamborn, J. C. and Püschel, A. W. (2004). The sequential activity of the GTPases Rap1B and Cdc42 determines neuronal polarity. *Nat. Neurosci.* **7**, 923-929.

Seth, A., Sheth, P., Elias, B. C. and Rao, R. (2007). Protein phosphatases 2A and 1 interact with occludin and negatively regulate the assembly of tight junctions in the CACO-2 cell monolayer. *J. Biol. Chem.* **282**, 11487-11498.

Shin, K., Fogg, V. C. and Margolis, B. (2006). Tight junctions and cell polarity. *Annu. Rev. Cell Dev. Biol.* **22**, 207-235.

Siller, K. H. and Doe, C. Q. (2009). Spindle orientation during asymmetric cell division. *Nat. Cell Biol.* **11**, 365-374.

Smith, C. A., Lau, K. M., Rahmani, Z., Dho, S. E., Brothers, G., She, Y. M., Berry, D. M., Bonneil, E., Thibault, P., Schweisguth, F. et al. (2007). aPKC-mediated phosphorylation regulates asymmetric membrane localization of the cell fate determinant Numb. *EMBO J.* **26**, 468-480.

Soini, Y. (2011). Claudins in lung diseases. *Respir. Res.* **12**, 70-79.

Song, J. C., Hanson, C. M., Tsai, V., Farokhzad, O. C., Lotz, M. and Matthews, J. B. (2001). Regulation of epithelial transport and barrier function by distinct protein kinase C isoforms. *Am. J. Physiol.* **281**, C649-C661.

Sontag, J. M. and Sontag, E. (2006). Regulation of cell adhesion by PP2A and SV40 small tumor antigen: an important link to cell transformation. *Cell. Mol. Life Sci.* **63**, 2979-2991.

Stuart, R. O. and Nigam, S. K. (1995). Regulated assembly of tight junctions by protein kinase C. *Proc. Natl. Acad. Sci. USA* **92**, 6072-6076.

Suzuki, A., Yamanaka, T., Hirose, T., Manabe, N., Mizuno, K., Shimizu, M., Akimoto, K., Izumi, Y., Ohnishi, T. and Ohno, S. (2001). Atypical protein kinase C is involved in the evolutionarily conserved par protein complex and plays a critical role in establishing epithelia-specific junctional structures. *J. Cell Biol.* **152**, 1183-1196.

Suzuki, A., Ishiyama, C., Hashiba, K., Shimizu, M., Ebnet, K. and Ohno, S. (2002). aPKC kinase activity is required for the asymmetric differentiation of the premature junctional complex during epithelial cell polarization. *J. Cell Sci.* **115**, 3565-3573.

Tefft, D., Lee, M., Smith, S., Crowe, D. L., Bellusci, S. and Warburton, D. (2002). mSprouty2 inhibits FGF10-activated MAP kinase by differentially binding to upstream target proteins. *Am. J. Physiol., Cell Physiol.* **283**, L700-L706.

Tefft, D., De Langhe, S. P., Del Moral, P. M., Sala, F., Shi, W., Bellusci, S. and Warburton, D. (2005). A novel function for the protein tyrosine phosphatase Shp2 during lung branching morphogenesis. *Dev. Biol.* **282**, 422-431.

Tsukita, S., Furuse, M. and Itoh, M. (2001). Multifunctional strands in tight junctions. *Nat. Rev. Mol. Cell Biol.* **2**, 285-293.

Tsukita, S., Yamazaki, Y., Katsuno, T., Tamura, A. and Tsukita, S. (2008). Tight junction-based epithelial microenvironment and cell proliferation. *Oncogene* **27**, 6930-6938.

Xiong, W., Dabbouseh, N. M. and Rebay, I. (2009). Interactions with the Abelson tyrosine kinase reveal compartmentalization of eyes absent function between the nucleus and cytoplasm. *Dev. Cell* **16**, 271-279.

Xu, P. X., Adams, J., Peters, H., Brown, M. C., Heaney, S. and Maas, R. (1999). Eya1-deficient mice lack ears and kidneys and show abnormal apoptosis of otic primordia. *Nat. Genet.* **23**, 113-117.

Xu, P. X., Zheng, W., Laclef, C., Maire, P., Maas, R. L., Peters, H. and Xu, X. (2002). Eya1 is required for the morphogenesis of mammalian thymus, parathyroid and thyroid. *Development* **129**, 3033-3044.

Yamanaka, T., Horikoshi, Y., Suzuki, A., Sugiyama, Y., Kitamura, K., Maniwa, R., Nagai, Y., Yamashita, A., Hirose, T., Ishikawa, H. et al. (2004). PAR-6 regulates aPKC activity in a novel way and mediates cell contact-induced formation of the epithelial junctional complex. *Genes Cells* **9**, 721-731.

Yang, X., Klein, R., Tian, X., Cheng, H. T., Kopan, R. and Shen, J. (2004). Notch activation induces apoptosis in neural progenitor cells through a p53-dependent pathway. *Dev. Biol.* **269**, 81-94.

Zou, D., Silvius, D., Fritzsch, B. and Xu, P. X. (2004). Eya1 and Six1 are essential for early steps of sensory neurogenesis in mammalian cranial placodes. *Development* **131**, 5561-5572.