Publisher’s Note: Eya1 controls cell polarity, spindle orientation, cell fate and Notch signaling in distal embryonic lung epithelium by El-Hashash et al. Development doi:10.1242/dev.058479

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This Publisher’s Note relates to the article ‘Eya1 controls cell polarity, spindle orientation, cell fate and Notch signaling in distal embryonic lung epithelium’ by El-Hashash et al. (doi:10.1242/dev.058479).

We have recently been made aware that several of the panels in Fig. 7 of this article may also appear in a paper from some of the same authors published in Developmental Biology (El-Hashash et al., 2011). We have contacted the authors to alert them to this alleged duplication to enable them to conduct further investigation. We are publishing this Note to inform readers of this situation.

This course of action follows the advice set out by COPE (The Committee on Publication Ethics), of which Development is a member.

Reference
Eya1 controls cell polarity, spindle orientation, cell fate and Notch signaling in distal embryonic lung epithelium

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SUMMARY

Cell polarity, mitotic spindle orientation and asymmetric division play a crucial role in the self-renewal/differentiation of epithelial cells, yet little is known about these processes and the molecular programs that control them in embryonic lung distal epithelium. Herein, we provide the first evidence that embryonic lung distal epithelium is polarized with characteristic perpendicular cell divisions. Consistent with these findings, spindle orientation-regulatory proteins Insc, LGN (Gpsm2) and NuMA, and the cell fate determinant Numb are asymmetrically localized in embryonic lung distal epithelium. Interfering with the function of these proteins in vitro randomizes spindle orientation and changes cell fate. We further show that Eya1 protein regulates cell polarity, spindle orientation and the localization of Numb, which inhibits Notch signaling. Hence, Eya1 promotes both perpendicular division as well as Numb asymmetric segregation to one daughter in mitotic distal lung epithelium, probably by controlling aPKCζ phosphorylation. Thus, epithelial cell polarity and mitotic spindle orientation are defective after interfering with Eya1 function in vivo or in vitro. In addition, in Eya1–/– lungs, perpendicular division is not maintained and Numb is segregated to both daughter cells in mitotic epithelial cells, leading to inactivation of Notch signaling. As Notch signaling promotes progenitor cell identity at the expense of differentiated cell phenotypes, we test whether genetic activation of Notch could rescue the Eya1–/– lung phenotype, which is characterized by loss of epithelial progenitors, increased epithelial differentiation but reduced branching. Indeed, genetic activation of Notch partially rescues Eya1–/– lung epithelial defects. These findings uncover novel functions for Eya1 as a crucial regulator of the complex behavior of distal embryonic lung epithelium.

KEY WORDS: Embryonic lung, Polarity, Eya1, Progenitor cells, Numb, Notch, Spindle orientation, Mouse

INTRODUCTION

The correct functioning of lung epithelium is essential to life. Mammalian lung development begins when two primary buds consisting of an inner epithelial layer surrounded by mesenchyme, arise from the laryngotracheal groove in the ventral foregut. These buds undergo stereotypic rounds of branching and outgrowth to give rise to a tree-like respiratory organ, which contains different specialized epithelial cell types organized along the proximodistal axis (Cardoso, 2000; Warburton et al., 2000; Warburton, 2008; Metzger et al., 2008). In order to function effectively, the alveolar surface must form a selectively permeable monolayer where cell-cell contact provides important spatial cues that are required to generate cell polarity/communications (Nelson, 2003a; Nelson, 2003b; Boitano et al., 2004).

Cell polarity, the asymmetry in distribution of cellular constituents within a single cell, is fundamental to cellular functions and essential for generating cell diversity. Epithelial cells have a characteristic apicobasal polarity, which is necessary for their function as barriers between different extracellular environments (Drubin and Nelson, 1996; Mostov et al., 2000). In epithelial cells, the axis of polarity that will determine the orientation of the apical-basal cell division plane is defined by the cell fate determinants (CFDs), e.g. Numb and Par proteins. Intrinsic CFDs are asymmetrically localized in dividing cells, and preferentially segregate into one of two sibling daughters in order to mediate asymmetric divisions (Betschinger and Knoblich, 2004).

The regulation of spindle orientation is often associated with cell polarity regulation in polarized cells in model organisms. The orientation and positioning of mitotic spindles, which determine the plane of cell division, are tightly regulated in polarized cells such as epithelial cells by intrinsic and extrinsic cues, e.g. cell polarity/geometry. Orientation of mitotic spindle and cell division axis can impact normal physiological processes, including epithelial tissue branching and differentiation (Betschinger and Knoblich, 2004). Despite their likely importance for lung branching, little is known about cell polarity and spindle orientation, and factors/mechanisms that regulate these processes are not well understood in the embryonic lung epithelium.

The Eyes Absent (Eya) proteins possess dual functions as both protein tyrosine phosphatases and transcriptional co-activators, and are involved in cell-fate determination and organ development (Jemc and Rebay, 2007). In mammals, Eya1-4 and sine oculis (Six) family genes exhibit synergistic genetic interactions to regulate the development of many organs (Xu et al., 1997a; Xu et al., 1997b; Ford et al., 1998; Coletta et al., 2004). Eya1–/– and Six1–/– mouse embryos have defects in the proliferation/survival of the precursor cells of multiple organs, and die at birth (Xu et al., 1999; Xu et al., 2002; Li et al., 2003; Zou et al., 2004). The phosphatase function of Eya1 switches Six1 function from repression to activation in the nucleus, causing transcriptional activation through recruitment of co-activators, which provides a mechanism for activation of specific gene targets, including those regulating precursor cell
proliferation/survival during organogenesis (Li et al., 2003). Although Eya1 transcriptional activity has been extensively characterized, little is known about the targets and functions of its phosphatase activity. Moreover, the physiological requirements for Eya1 phosphatase activity in the lung epithelium remain obscure.

Herein, we show that Eya1 is located in the distal epithelium, wherein it regulates cell polarity, spindle orientation, and both aPKCζ phosphorylation and Numb segregation. Interfering with Eya1 function in vivo or in vitro results in defective cell polarity, spindle disorientation and Numb segregation into both daughters, as well as inactivation of Notch signaling in embryonic lung epithelium. Furthermore, activation of Notch signaling in Eya1−/− distal epithelium partially rescues Eya1−/− embryonic lung epithelial defects.

MATERIALS AND METHODS

Animals

Eya1+/−, Spe-rtTA+/− and Notch1 conditional transgenic (NICD) mice, and their genotyping have been published (Xu et al., 1999; Xu et al., 2002; Perl et al., 2002; Yang et al., 2004). Wild-type littermates were used as controls. Conditional NICD:Eya1+/− female mice were generated by intercrossing Eya1+/− mice with NICD mouse strain. Eya1+/− Spe-rtTA+/− tet(o) Cre+/− mice were generated by intercrossing Eya1+/− mice with Spe-rtTA+/− tet(o) Cre+/− mouse strain previously generated in our laboratory. The resulting Eya1+/− Spe-rtTA+/− tet(o) Cre+/− mouse intercrosses were generated with NICD:Eya1+/− females to increase Notch1 activity in the distal epithelium of mutant lungs by generating NICD-Eya1−/−; Spe-rtTA+/− tet(o) Cre−/− mutant mice for analysis. Pregnant NICD:Eya1−/− females were maintained on doxycycline (DOX) containing food (Rodent diet with 0.0625% Doxycycline, Harlan) from E6.5 till sacrifice. 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.

Phenotype analyses, antibody staining, western blot and immunoprecipitation

Antibody staining on paraffin sections or fixed MLE-15 cells, western blot and immunoprecipitation were performed in triplicates using commercially available antibodies following the manufacturer’s instructions and standard protocols as described previously (Tefft et al., 2005; Tefft et al., 2002; Buckley et al., 2005; del Moral et al., 2006a; del Moral et al., 2006b). Briefly, for alveolar type-2 (AEC2) cells, cells were isolated from lavaged lungs using the method of Dobbs et al. (Dobbs et al., 1986), and cultured for 24 hours. The 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 followed by overnight incubation with protein A/G agarose, then washing before re-suspension in electrophoresis sample buffer. The immunoprecipitate was loaded onto Tris-glycine gel, with a lysates of AEC2 as a 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 a polarity protein antibody. Fluorescence intensity/protein control. The separated proteins were transferred to immobilon, and probed overnight with a polarity protein antibody. Fluorescence intensity/protein control. The separated proteins were transferred to immobilon, and probed overnight with a polarity protein antibody. Fluorescence intensity/protein control. The separated proteins were transferred to immobilon, and probed overnight with a polarity protein antibody. Fluorescence intensity/protein control. The separated proteins were transferred to immobilon, and probed overnight with a polarity protein antibody. Fluorescence intensity/protein control.

RESULTS

Eya1 is expressed in embryonic lung distal epithelium and controls both cell polarity and proper spindle orientation

Eya1 protein phosphatase is expressed in the nucleus and cytoplasm, where it functions as a cytoplasmic protein phosphatase (Fougnerousse et al., 2002; Xiong et al., 2009). Two lines of reasoning have led us to examine Eya1 functions in distal lung epithelial cell polarity. First, Eya1 has a polarized (mostly apical) expression pattern in the distal epithelial tips, particularly from E12.5-E13 (Fig. 1A,B,C), similar to polarity proteins Numb/LGN/Insc (Fig. 1E,F; Fig. 2A,G). Second, other members of the protein phosphatase family, e.g. protein phosphatase 2A, are crucial regulators of cell polarity, spindle orientation and cell fate in Drosophila neural epithelium (Ogawa et al., 2009; Wang C. et al., 2009). In this study, E14-E14.5 was used as the developmental stage of choice to analyze the behavior of distal epithelium because cell proliferation and expression of progenitor cell markers Sox9, Id2 and N-myc (Mycn – Mouse Genome Informatics) are relatively high. In addition, Eya1−/− early lung development is normal and Eya1−/− epithelial lung phenotype is evident at E14-E14.5, as discussed later.

The polarity proteins LGN (Gpsm2 – Mouse Genome Informatics), NuMA (Numa1 – Mouse Genome Informatics) and Insc regulate mitotic spindle orientation during epithelial morphogenesis (Siller and Doe, 2009; Zheng et al., 2010). Epithelial cells in interphase or undergoing lateral/planar divisions have a diffuse or basolateral localization of LGN, whereas cells undergoing perpendicular (i.e. apical-basal) divisions have LGN only at the apical cell side (Lechler and Fuchs, 2005). In wild-type lungs, an apical staining of anti-LGN labeling was seen at the cortex of most mitotic cells of distal epithelial tips (Fig. 2A,A′,J), which are highly mitotic (Bishop, 2004). In Eya1−/− distal epithelial tips, no apparent changes in LGN, NuMA, Par3 and Insc expression levels were observed (Fig. 1G), and most mitotic cells had a diffuse, basolateral or basal localization of LGN (Fig. 2B,B′,K). Closer inspection revealed that cells with an apical localization of LGN accounted for about 86±5.0% of all mitoses in wild-type tip cells, but in Eya1−/− distal epithelial tips, this number decreased markedly to about 5.0±4.0% (Fig. 2C; P<0.05). These quantified data are further presented in the diagrams in Fig. 2J,K, in which each dot represents the centre of an LGN localization in a mitotic cell. Concomitantly, and as shown in Fig. 2M, spindle orientations were overwhelmingly lateral in Eya1−/− (i.e. parallel to the basement membrane), as measured in mitotic cells at most distal epithelial tips in Eya1−/− compared with control lungs (Fig. 2L) and following methods described by Lechler and Fuchs (Lechler and Fuchs, 2005). Similarly, most Eya1−/− distal epithelial cells had a diffuse or basolateral localization of NuMA and Insc, which were apically localized in wild-type lungs (Fig. 2D-I; 87.0±6.0% versus 74±5.6%, respectively; P<0.05), suggesting that Eya1 deletion changes cell
polarity/spindle orientation and induces lateral (i.e. planar) cell divisions. Similarly, interfering with Eya1 functions disrupted asymmetric localization of Par, myosin IIb (Myh10 – Mouse Genome Informatics) and F-actin (Actg1 – Mouse Genome Informatics) proteins (see Fig. S1 in the supplementary material).

To facilitate quantification of cells dividing perpendicularly versus laterally, we stained E14 Eya1^{-/-} distal epithelium for centrosomes with anti-pericentrin antibody. Then, mitotic cells were quantified based on centrosome orientation relative to the basement membrane in order to distinguish parallel/lateral from perpendicular spindle alignments in mitotic cells. Centrosomes that were oriented at 0±30° to the basement membrane were classed as parallel; those that were oriented at 90±30° were classed as perpendicular. In Eya1^{-/-} distal epithelium, most cell divisions (87±3.0%) seemed to occur parallel/lateral to the basement membrane, while about 12±5% mitotic cells had an alignment that appeared perpendicular in contrast to wild-type cells where perpendicular spindle alignments were abundant (Fig. 2N-P).

**LGN, Insc and NuMA control spindle orientation, and Numb regulates the cell fate of lung epithelial cells in vitro depending on Eya1 phosphatase activity**

Next, we addressed whether murine LGN, Insc and NuMA functions in the regulation of spindle orientation are conserved in the lung epithelium, using gene-specific siRNA in the MLE-15 lung epithelial cell line. MLE-15 cells were used in this study because of their intense expression of different polar protein androgen/ differentiation cell markers. As in other epithelial cells (Lechler and Fuchs, 2005), LGN, NuMA, Insc and Par3 had a mitosis-specific polarized distribution in MLE15 cells, often localizing asymmetrically to the cell cortex with one of the spindle poles positioned directly below it, which indicates a perpendicular alignment of the spindle (see Fig. S2A,B,J,K,L in the supplementary material). Knock-down of Insc, Gpsm2 or Numa1 function caused obvious mitotic defects, as judged by the misoriented and disrupted morphology of mitotic spindles in transfected cells, compared with control-siRNA-transfected cells (Fig. 3A-D and data not shown). Conversely, Eya1 expression did not apparently change after interfering with the function of different polarity proteins in vitro (see Fig. S2Q-U in the supplementary material).

We next test Eya1 functions in controlling spindle-orientation-regulatory proteins in culture. Although polarization of LGN, NuMA and Insc in culture was more variable, it was observed in at least 60±7% and sometimes as many as 73±6% of mitotic MLE-15 cells (see Fig. S2I,M in the supplementary material). Upon Eya1 knockdown, LGN/Insc/NuMA/Par3 were seen at both apical and basal cell sides or were diffuse (see Fig. S2C,D,N-P in the supplementary material). Thus, the percent of cells with a polarized localization of LGN/NuMA/Insc greatly decreased upon Eya1 knockdown to about 6-8%. Rescuing Eya1 function by expressing wild-type murine Eya1 construct, not targeted by the siRNAs, into these siRNA-transfected cells rescued the polarized distribution of LGN/NuMA/Insc proteins (see Fig. S2I,M in the supplementary material), while a phosphatase-dead mutant Eya1 failed to rescue (examples are shown for LGN in Fig. S2A,C-H in the supplementary material). This suggests that the polarized localization of LGN/NuMA/Insc/Par, and hence proper spindle orientations are dependent on Eya1 phosphatase activity.

The polarity protein Numb is essential in maintaining vertebrate epithelial progenitors by allowing cells to choose progenitor over differentiation fates, and specifies cell fate by repressing Notch signaling (Petersen et al., 2004; Betschinger and Knoblich, 2004; Hutterer and Knoblich, 2005). We therefore investigated Numb functions in epithelial cell differentiation versus proliferation by staining MLE15 cells for SP-B (Sftpb – Mouse Genome Informatics) and Sox9, which are markers for epithelial differentiation and progenitor cells, respectively. As shown in Fig. 3E-I, the number of Sox9-positive cells increased fivefold (9.0±2.0% versus 50.3±5.0%, respectively; P<0.05), while SP-B-positive differentiated cells greatly decreased upon knockdown of Numb (60.0±4.0% versus 12.5±5.0%, respectively; P<0.05). Moreover, Notch signaling was activated upon
**Numb knockdown**, as indicated by increased signal/fluorescence intensity for the Notch target genes Hes1/Hes5, and increased number of Hes1-positive cells (Fig. 3J-P). This suggests a conserved function for Numb in controlling cell fates and Notch signaling in the lung epithelium.

**Eya1 deletion enhances Numb expression and phosphorylation, but inhibits its asymmetric localization**

Numb regulates cell polarity, and its phosphorylation/localization is controlled by apically localized Par proteins during the establishment of apical-basal polarity in mammalian epithelial cells, which is necessary to maintain Numb asymmetric segregation into one of the daughter cells and its function as a cell fate determinant (Smith et al., 2007; Wang Z. et al., 2009).

The disrupted cell polarity, mislocalized Par3/6 and increased lateral (planar) divisions in Eya1–/– mitotic distal epithelium (Fig. 2; see Fig. S1 in the supplementary material) raise the possibility that Numb segregation/functions are disrupted in these cells, which result in distribution of Numb equally to their two daughters at cytokinesis after Eya1 deletion. To test this possibility, we first examined Numb distribution in distal epithelial tips (Fig. 4). Numb concentrates in the cell-cortex area overlying one of the two spindle poles and is preferentially inherited by one of the two daughter
cells during asymmetric cell division (Knoblich et al., 1995). In wild-type lungs, Numb was asymmetrically distributed and highly concentrated at the apical side of distal epithelial cells with a little or no staining at the basal pole (Fig. 4A,G). Conversely, Numb staining markedly increased, but was diffused and localized at both apical and basal cell poles in \( \text{Eya}1^{-/-} \) distal epithelial cells (Fig. 4B,G).

Furthermore, closer inspection in mitotic cells revealed that Numb staining is consistently concentrated as a crescent at the apical pole of one (apical) daughter cell in 88±3% of wild-type distal epithelial tip cells (Fig. 4C,F). Conversely, Numb seemed to be inherited by both daughter cells in 84±6% of \( \text{Eya}1^{-/-} \) mitotic distal tip cells (Fig. 4D,F). This suggests that the more planar (parallel) a cell division is (Fig. 2M,P), the more likely it is to segregate Numb preferentially to both daughter cells in mitotic \( \text{Eya}1^{-/-} \) distal epithelial cells. This conclusion was further confirmed in mitotic MLE15 cells in vitro (Fig. 4M,N). Numb staining was cortical and started to be confined to one side of the cell at prophase, then localized asymmetrically in metaphase/anaphase, and was inherited by one daughter cell in anaphase/telophase in most mitotic cells (Fig. 4M). Upon \( \text{Eya}1 \) knockdown, Numb staining was diffuse in the cytoplasm at prophase and became cortical later in metaphase (Fig. 4N). Numb failed to localize asymmetrically in metaphase, and was inherited by both daughters in anaphase/telophase in most mitotic cells (Fig. 4N).

In mammalian epithelium, phosphorylation of phosphotyrosine-binding domain is essential for asymmetric localization of Numb to the cortical membrane (Dho et al., 2006; Smith et al., 2007). We therefore tested whether Numb phosphorylation changed in \( \text{Eya}1^{-/-} \) lungs. Numb proteins were detected as two bands, with the higher band representing the modified form of Numb (Rhyu et al., 1994). If Numb phosphorylation changes, the modified form of Numb, which is the putative phosphorylated form, will increase in \( \text{Eya}1^{-/-} \) lungs. Indeed, phosphorylated Numb increased in E14-
14.5 *Eya1*−/− lungs (Fig. 4J). Moreover, phospho-Numb immunoreactivity using phospho-Numb (Ser-295) antibody increased in vivo (Fig. 4H–I, K) and was highest at the cell cortex and in the nuclei of *Eya1*−/− distal epithelium cells (Fig. 4H–I, K) in *Eya1*−/− mitotic distal epithelium cells with planer/parallel (D) or perpendicular (E) divisions (E, which represents the area marked with an asterisk in B), relative to the collagen IV-stained basement membrane (thick white broken lines in A–E). (F) Quantification of late mitotic distal epithelial progenitors, with Numb inherited by one (1daug.) or both (2daug.) daughter cells in E14 wild-type or *Eya1*−/− lungs. Bars carrying the same letter (a, b) in F, L are significantly different from one another (*P<0.05; Student’s t-test). Data are mean±s.e.m. (G) Morphometric analysis of Numb signal intensity/distribution of selected areas (thin yellow broken lines in A, B) shows loss of polarized/asymmetric localization of Numb, which is distributed at both apical and basal sides of *Eya1*−/− distal epithelium. (H–I) Immunostaining with specific Ser295 phospho-Numb antibody shows increased Numb phosphorylation in E14 *Eya1*−/− distal epithelium (I, arrowheads; staining in the cytoplasmic side of cell membrane and in the nuclei) compared with control lungs (H, arrowheads). (H, I) High magnification of boxed areas in panels H, I, respectively. (J, K) Western blots of E14 lungs with anti-Numb (J), anti-Ser-295 phospho-Numb or anti-tyrosine phosphorylated aPKCζ antibody (K) show increased Numb/aPKCζ phosphorylation in *Eya1*−/− lungs. Bars in J represent quantified western blot signals (mean±s.e.m., **P<0.001). Blue numbers in K represent relative band intensity. (L) Mean fluorescence intensity of total Numb or phospho-Numb staining compared between wild-type and *Eya1*−/− distal epithelium for experiments showing in A, B and H–I. Error bars indicate s.e.m. (M, N) In control mitotic MLE15 cells, Numb (arrowheads) segregated asymmetrically and was inherited by one daughter cell in anaphase/telophase. Upon *Eya1* knockdown, Numb segregated to both daughters (N, arrowheads). Scale bars: 50 μm.

E14.5 *Eya1*−/− lungs (Fig. 4J). Moreover, phospho-Numb immunoreactivity using phospho-Numb (Ser-295) antibody increased in vivo (Fig. 4H–I, K) and was highest at the cell cortex and in the nuclei of *Eya1*−/− distal epithelium (Fig. 4H–I). Furthermore, Fig. 4L compares the mean fluorescence intensity of phospho:total Numb of wild-type versus *Eya1*−/− distal epithelium, showing that the phospho:total Numb was markedly altered between wild-type and *Eya1*−/− epithelium.

Similarly, a polarized Numb signal localized to one side of the cell was detected in MLE-15 cells in culture (Fig. 5A). Upon *Eya1* knockdown, Numb was not polarized, was localized uniformly to the cytoplasm/cell membrane as small puncta and exhibited increased Ser295 phosphorylation (Fig. 5B, H, R). In the rescue experiments, re-expression of wild-type *Eya1*, not targeted by the siRNAs, rescued the polarized distribution and phosphorylation level of Numb, whereas re-expression of the tyrosine-phosphatase-dead mutant *Eya1* did not (Fig. 5C, D, I, J, R). This suggests that Numb phosphorylation is Eya1 dependent.

Recently, we reported that Eya1 controls the balance between self-renewal and differentiation of distal epithelial cells, where progenitor cells greatly increased in number while differentiated cell number increased in *Eya1*−/− embryonic lung epithelium. In
addition, Eya1 overexpression in MLE15 cells increases Sox9-positive progenitors cells, but decreases SP-B differentiated cells (El-Hashash et al., 2011) (Fig. 7; see Fig. S3 in the supplementary material), similar to Numb knockdown effects (Fig. 3E-I). We therefore examined whether the magnitude of Eya1 effects in balancing proliferation/differentiation of lung epithelial cells is changed in a Numb knockdown background in vitro. As shown in Fig. 5Q, the number of Sox9-positive progenitors increased fivefold (9.0±5.0% versus 50±7.0%, *P<0.05), while SP-B-positive differentiated cells decreased (60±4.0% versus 12-14±3.0%, respectively; *P<0.05) following, respectively, Numb knockdown or Eya1 overexpression in MLE15 cells. Overexpression of Eya1, together with the knockdown of Numb in MLE15 cells led to a greater increase in the number of Sox9-positive cells (eightfold), and a more severe decrease in the number of SP-B-positive cells (45%; Fig. 5Q) compared with control cells.
Eya1 is essential for atypical protein kinase Cγ (aPKCγ) phosphorylation

Eya1 has well-known tyrosine phosphatase activities (Li et al., 2003). As Numb phosphorylation increased in Eya1−/− lungs after Eya1 knockdown, we therefore tested whether tyrosine phosphorylated aPKCγ is a direct substrate for Eya1 phosphatase. aPKCγ activity that is probed by a tyrosine phosphorylated aPKCγ antibody increased both in vivo at the cell cortex of Eya1−/− distal epithelium, similar to Numb (Fig. 4K; Fig. 5F,L), and after Eya1 knockdown in MLE15 cells in vitro (Fig. 5N,R). Rescuing Eya1 function by expressing wild-type murine Eya1 construct, not targeted by the siRNAs, into these Eya1 siRNA-transfected cells led to near-control level of phospho-aPKCγ, whereas re-expression of the tyrosine-phosphatase-dead mutant Eya1 did not (Fig. 5O,P,R). This suggests that increased Numb phosphorylation is likely to be due to the increased aPKCγ activity/phosphorylation in Eya1−/− lungs. This conclusion was confirmed by inhibiting aPKCγ activity in Eya1siRNA-transfected MLE15 cells, which rescued the polarized distribution and phosphorylation level of Numb (compare Fig. 5A,B,G,H with 5E,K,R).

We next assessed Eya1 phosphatase activity on aPKCγ by co-immunoprecipitation. The endogenous aPKCγ forms a complex with Par3/Par6/Numb, which binds to LGN/Insc/NuMA in epithelial cells (Lechler and Fuchs, 2005; Suzuki and Ohno, 2006; Nishimura and Kaibuchi, 2007). Expectedly, Eya1 co-immunoprecipitated aPKCγ and other polarity proteins in AEC2 cell lysate (Fig. 5S). To determine whether Eya1 binds to aPKCγ-Par-Numb/polarity protein complex by binding to aPKCγ, we performed Eya1/aPKCγ co-immunoprecipitation studies and analyzed other polarity proteins in cells treated with aPKCγsiRNA. Indeed, co-immunoprecipitation of Eya1, Numb, Par/polarity proteins was not observed after aPKCγ knockdown, but was observed after knocking down Numb or other polarity proteins (Fig. 5T).
To further determine whether aPKCζ tyrosine phosphorylation might be a target of Eya1 phosphatase activity, we performed an in vitro phosphatase assay, mixing aPKCζ protein with immunopurified HA-tagged Eya1. As shown in Fig. 5U, wild-type Eya1 significantly inhibited aPKCζ phosphotyrosine, while the phosphatase-inactive mutant protein had no significant effect.

**Inhibition of Notch signaling in Eya1−/− lung distal epithelium**

Numb functions as a negative regulator of Notch in mammals and *Drosophila* (French et al., 2002; Cayouette and Raff, 2002), and inactivated Notch1 signaling in MLE15 lung epithelial cells (Fig. 3J-P). As Eya1 controlled Numb segregation/function (Figs 4, 5), we next investigated whether Eya1 also regulates Notch signaling in distal lung epithelium.

Signals for activated (cleaved) Notch1 and for its downstream transcriptional targets Hes1 and Hes5 were strong in wild-type distal epithelium, but greatly decreased in Eya1−/− distal epithelium (Fig. 6A-F). This was also shown by immunoblot analysis (Fig. 6G). Similarly, activated Notch1 expression decreased after Eya1 knock-down in MLE-15 cells (Fig. 6I,J,H). Rescuing Eya1 function by expressing wild-type murine Eya1 construct, not targeted by the siRNAs, into these siRNA-transfected cells rescued the expression levels of activated-Notch1, while a phosphatase-dead mutant Eya1
failed to rescue (Fig. 6K,L,H). Interestingly, inhibition of aPKC activity in Eya1siRNA-transfected cells led to near-Eya1 wild-type transfected level of activated Notch1 (Fig. 6K,M,H).

To determine whether Numb is involved in Eya1 control of Notch signaling in the lung epithelium, we tested whether the magnitude of Eya1 effects on Notch activity in MLE15 cells changes in a Numb knockdown background. As shown in Fig. 6N,R,S,T, Hes1 nuclear signal levels and Hes1-positive cells greatly increased, and were higher than Hes1 signals/number after either Eya1 overexpression (Fig. 6Q,N,S,T) or Numb knockdown (Fig. S3I,K-N,P), carried out separately in MLE15 cells.

**Genetic activation of Notch signaling in Eya1+/− lungs partially rescues epithelial progenitor defects and branching phenotype**

Notch signaling promotes progenitor cell identity at the expense of differentiated cell phenotypes (Jadhav et al., 2006; Mizutani et al., 2007). It also controls cell fates in developing airways (Tsa et al., 2009), while Notch activation inhibits the differentiation of distal lung progenitors into alveolar cells (Guseh et al., 2009). Loss of epithelial progenitors from E14-E14.5, reduced epithelial branching/lung size and increased epithelial differentiation are major Eya1−/− lung phenotypes (Fig. 7A-E,I,J,N,O,S,T; see Fig. S3 in the supplementary material) (EI-Hasnash et al., 2011). We therefore tested the hypothesis that inactivation of Notch signaling causes the epithelial 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. No changes in lung phenotype or gene/protein expression were evident in controls: DOX-fed Spc-rtTa and Spc-rtTa-tet(O) Cre mice (data not shown).

**NICD; Spc-rtTA+/-tet(O) Cre+/-/Eya1−/− compound mutant lungs were comparable with doxycycline-untreated control lungs, albeit smaller in size (Fig. 7A).** Following induction with DOX feeding, they showed increased lung size and restoration of both epithelial branching and expression of distal epithelial progenitor markers compared with lungs of Eya1−/− littermates (Fig. 7A-F,I,J,N,P,S,T; see Fig. S3A-C in the supplementary material). Moreover, the polarized cortical localization of polarity proteins (Fig. 7G,H,L,M,Q,R,U,V) and the expression levels of distal epithelial differentiation markers (Fig. 7S,T; Fig. S3D-I in the supplementary material) were restored into the wild-type control range in compound mutant lungs versus Eya1−/− lungs, suggesting partial but substantial rescue of the Eya1−/− hypoplastic lung phenotype.

Finally, we examined whether the magnitude of Eya1 effects in balancing proliferation/differentiation of lung epithelial cells is blunted in a Notch1 knockout background in MLE-15 cells. As shown in Fig. 7C, Notch1 knockout reduced Sox9-positive progenitors (60%; 10±3.0% versus 4.2±3.0%), but increased SP-B-positive differentiated cells (61.0±3.0% versus 72±4.0%, respectively; P<0.05). By contrast, the number of Sox9-positive progenitors increased fivefold, while SP-B-positive cells greatly decreased (78%) upon Eya1 overexpression. These changes were blunted and the percentage of cells that are positive for Sox9 or SP-B was restored into the control range in cells co-transfected with Numb siRNA and wild-type Eya1 expression vector versus Eya1-overexpressing cells alone (Fig. 7C).

**DISCUSSION**

The function and growth of pulmonary epithelial cells lining the distal tubes/air sacs depend on their polarity, which its loss is involved in lung cancers, chronic obstructive pulmonary disease and disruption of lung epithelial differentiation (Matsui et al., 1999; Xu et al., 2006). Yet, cell polarity remains uncharacterized in lung epithelium. Herein, we demonstrate that distal lung epithelium, which represents the epithelial progenitor pool (Rawlins et al., 2009), is polarized with characteristic perpendicular divisions that are controlled by Eya1 phosphatase.

**Eya1 controls cell polarity and mitotic spindle orientation in embryonic distal lung epithelium**

Mammalian Eya1 protein phosphatase has been implicated in cell polarity, because progressive Eya1 depletion results in the loss of polarity in hair cells during inner ear development (Zou et al., 2008). Here, we have extended these observations to the lung to demonstrate that Eya1 is crucial for the maintenance of cell polarity and mitotic spindle orientation of distal epithelium. Eya1−/− distal epithelial cells exhibited a severe perturbation in the asymmetric localization and organization of several polarity proteins (Figs 2, 4 and 5; see Figs S1, S2 in the supplementary material). Similarly, members of protein phosphatase family are crucial regulators of cell polarity and spindle orientation in Drosophila epithelial cells (Wang et al., 2009; Ogawa et al., 2009).

How does Eya1 protein function to maintain cell polarity and control mitotic spindle orientation? From the present study, Eya1 appears to exert this effect by influencing multiple processes, including the apical cell localization of Par, Insc, LGN and NuMA proteins, which are evolutionarily conserved and essential for the formation of cell polarity/spindle orientation, as well as aPKC/Numb phosphophosphorylation (discussed below). In mammalian epithelium, the Par3/6 proteins localize predominantly to apically located tight junctions and bind to aPKCC. Insc and LGN. This binding is crucial for the establishment of epithelial polarity and for apical-basal/perpendicular spindle orientation (Macara, 2004; Suzuki and Ohno, 2006; Siller and Doe, 2009). Thus, the proper localization of aPKCC-Par3/6-LGN-Insc polarity complex is crucial for cell polarization (Ohno, 2001). Our findings that Eya1 may bind to aPKCC, and that Eya1 deletion causes mislocalization of Par/Insc/LGN, together with increased planar cell divisions at the expense of perpendicular/apical-basal division (Figs 2, 5; see Figs S1, S2 in the supplementary material), provide strong evidence that Eya1 is indeed required for controlling cell polarity and spindle orientation in the embryonic lung.

**Eya1 regulates Numb segregation and Notch signaling in distal lung epithelium**

Notch signaling is used for cell fate determination throughout the animal kingdom, and differences in Notch activity between two daughter cells can be specified by the asymmetric localization and inheritance of Numb, a negative regulator of the Notch pathway (Guo et al., 1996; Cayouette et al., 2001; Petersen et al., 2002; Shen et al., 2002). In the embryonic lung, Notch signaling controls cell fates in developing airways (Post et al., 2000; Tsa et al., 2008; Tsao et al., 2009), and Notch activation inhibits the differentiation of distal progenitors into alveolar cells (Guseh et al., 2009). Yet the role of asymmetric segregation of cell fate determinant/Notch inhibitor Numb during lung development, and the way the process might be regulated are still unknown.

Herein, the failure of polarized Numb localization after Eya1 knockdown (Figs 4, 5) supports our conclusion that one of the principal functions of Eya1 is the regulation of asymmetric
Numb localization/segregation in mitotic lung epithelium. This is further confirmed by our finding that Eya1 phosphatase controls aPKCC phosphorylation, which is essential for Numb phosphorylation and asymmetric localization/segregation (Dho et al., 2006; Smith et al., 2007), as reported for other phosphatases (Numbakdi-Craig et al., 2002). Indeed, aPKCC-dependent phosphorylation of Numb inhibits its cortical/polarized localization (Casanova, 2007). Increased Numb expression in Eya1–/– epithelium provides further evidence, because Numb localization is also inhibited upon overexpression of the protein, presumably as a result of saturation of the localization machinery (Rhyu et al., 1994). Upon overexpression, Numb is segregated into both daughter cells that then adopt the fate of the daughter that normally inherits Numb (Rhyu et al., 1994). Moreover, mislocalization and perturbation of Par3/6 and myosin IIb, together with inactivation of Notch signaling, in Eya1–/– lungs further support our hypothesis of Eya1 control of Numb segregation/expression, because myosin IIb and Par proteins regulate Numb asymmetric segregation/localization (Barros et al., 2003; Betschinger and Knoblich, 2004). Moreover, high levels of Notch activation cause a reduction in Numb protein levels (Chapman et al., 2006).

Furthermore, the lack of polarized Numb localization, and consequently loss of the difference in Numb levels between two daughter cells (both inherit Numb) may be responsible for the failure of Eya1–/– cells to upregulate Notch signaling pathway and hence to execute the epithelial progenitor cell self-renewal program at distal tips. This may explain enhanced epithelial differentiation and the great reduction of both Notch activity and expression of epithelial progenitor cell markers in the Eya1–/– lungs (Figs 4-7; see Fig. S3 in the supplementary material). Indeed, Numb inheritance in daughter cells acts to inhibit Notch signaling (Chapman et al., 2006). Consistent with our results, Eya1 abrogation inhibits Notch signaling during sensory progenitor development in mammalian inner ear (Zou et al., 2008), whereas high levels of Eya1 inhibit neuronal differentiation, but expand the pool of proliferative neuronal progenitors (Schlosser et al., 2008). Our findings that genetically increasing Notch activity in Eya1–/– lungs substantially rescues the abnormal lung epithelial phenotype in vivo (Fig. 7; see Fig. S3 in the supplementary material) provide strong evidence that Eya1 is indeed required for controlling Notch signaling activity to ensure appropriate self-renewal/differentiation of lung distal epithelium. Whether Eya1 directly or indirectly regulates Notch signaling will be the subject of future study.

In our future studies, we 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 congenital lung hypoplasia/malformations and help us to understand the mechanisms that control lung epithelial morphogenesis.

**Does distal lung epithelium divide asymmetrically? Does Eya1 phosphatase control asymmetric division in the lung?**

Recent reports suggest that undifferentiated lung epithelial progenitors undergo multiple division-linked cell fate decisions [symmetric and asymmetric cell division (ACD)] that lead to an apparently homogeneous expansion of the progenitor cell population (Rawlins, 2008; Lu et al., 2008). No reports about ACD in the embryonic lung have appeared as yet to our knowledge, but our study provides some evidence to suggest that distal lung epithelial cell populations that contain progenitor cells (Rawlins et al., 2009) divide asymmetrically. For example, most of the distal epithelial cells had apically localized Insc, LGN, NuMA and Par proteins, with mitotic spindles aligned perpendicular to the basement membrane and a characteristic asymmetric segregation/inheritance of Numb (Figs 1, 2; see Fig. S1 in the supplementary material). Indeed, a strict correlation exists between ACD and the apical localization of these polarity proteins, perpendicular alignment of mitotic spindles and asymmetric Numb segregation in *Drosophila/mammalian epithelium* (Cayouette and Raff, 2002; Cayouette and Raff, 2003; Haydar et al., 2003; Noctor et al., 2004; Lechler and Fuchs, 2005). In this regard, our data suggest a crucial role for Eya1 in controlling ACD, similar to other phosphatases (Wang et al., 2009; Ogawa et al., 2009), because Eya1 abrogation perturbed the organization of polarity proteins and spindle orientation, as well as Numb segregation in distal embryonic lung epithelium, providing a conceptual framework for future mechanistic studies in this area.


