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RETRACTION

Retraction: Eya1 controls cell polarity, spindle orientation, cell fate and Notch signaling in distal embryonic lung epithelium. Development doi: 10.1242/dev.058479

Ahmed HK El-Hashash, Gianluca Turcatel, Denise Al Alam, Sue Buckley, Hiroshi Tokumitsu, Saverio Bellusci and David Warburton

The journal is retracting 'Eya1 controls cell polarity, spindle orientation, cell fate and Notch signalling in distal embryonic lung epithelium' by Ahmed HK El-Hashash, Gianluca Turcatel, Denise Al Alam, Sue Buckley, Hiroshi Tokumitsu, Saverio Bellusci and David Warburton (2011). *Development* **138**, 1395-1407 (doi: 10.1242/dev.058479).

This Retraction updates and replaces the Publisher's Note (doi: 10.1242/dev.148718) relating to the above-referenced article. Development 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.

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PUBLISHER'S NOTE

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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Editor in Chief, Development (dev@biologists.com)

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

El-Hashash, A. H., Al Alam, D., Turcatel, G., Bellusci, S. and Warburton, D. (2011). Eyes absent 1 (Eya1) is a critical coordinator of epithelial, mesenchymal and vascular morphogenesis in the mammalian lung. *Dev. Biol.* **350**, 112-126.

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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 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 a proximodistal axis (Cardoso, 2000; Warburton et al., 2000; Warburton, 2008; Metzger et al., 2008). In order to function effectively, the epithelial surface must form a selectively permeable monolayer where cell-cell contact provides important spatial cues that are required to generate cell polarity/communication (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

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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$^{-/-}$, *Spc-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*$^{+/-}$*Spc-rtTA*$^{+/-}$*tet(o) Cre*$^{+/-}$ mice were generated by intercrossing *Eya1*$^{+/-}$ mice with *Spc-rtta*$^{+/-}$*tet(o) Cre*$^{+/+}$ mouse strain previously generated in our laboratory. The resulting *Eya1*$^{+/-}$*Spc-rtTA*$^{+/-}$*tet(o) Cre*$^{+/-}$ mouse males were intercrossed with *NICD;Eya1*$^{+/-}$ females to increase Notch1 activity in the distal epithelium of mutant lungs by generating *NICD-Eya1*$^{-/-}$*; Spc-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 e[xpression] than *Eya1*$^{-/-}$ littermates, were generated at expected Mendelian r[atios] 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 inst[ructions] and standard protocols as described previously (Tefft et al., 20[02]; 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); [cells] cultured for 24 hours. The cells were lysed in RIPA [buffer], centrifuged [and] the supernatant containing ~1 mg protein was pr[e]cleared by incubation with rabbit IgG and protein A/G agarose, th[en] centrifuged. The cleared supernatant was immunoprecipitated with [2] µg Ey[a1] antibody followed by overnight incubation with protein A/G [sep]harose, then [washing] before re-suspension in electrophoresis sample buffer. The immunopre[cipitate] was loaded onto Tris-glycine gel, with a ly[sates] of AEC2 as a positive control, and the non-specific proteins preci[pitated] by rabbit IgG as a negative control. The separated proteins wer[e] transferred [to] immobilon, and probed overnight with a polarity protein [antibody]. Fl[uo]rescence intensity/protein quantification were produced b[y] densitome[try] analysis [with] the Image J software as described (Carraro [et al.,] 2009; [Shigeoka et al., 2007]).

Cell culture/transfection and in vitro [phosphatase] assay

Transfection of epithelial cells [with] siRNAs or [wild]-type expression/mutant (D323A) vectors [and] phosphatase assays were performed following standard pro[cedures] as [described] previously (Carraro et al., 2009; Cook et al., 2009; [Dutil et al., 1998]). For siRNA experiments, there is [no] change in [results] of blank controls or lipofectamine controls, and their d[ata] are not p[resented]. The knockdown/overexpression efficiency was analyzed [by] western blot/immunostaining of targeted protein. In addition, we used an [expression] vector encoding a VP16 fusion protein, and the transfection effic[iency] was further monitored by fluorescence staining using anti-VP16 antibody. aPKCζ inhibitor was used at a concentration of 50 µmol/l, at which it is effective without displaying cytotoxicity [as reported in [cell] systems (Davies et al., 2000; Buteau et al., 2001)].

Quantification of LGN/NuMA/Insc localization and spindle orientation and statistical analysis

Mitotic cells and polarity [localization] were identified by phospho-histone3/pericentrin staining. Quantification of LGN/NuMA/Insc localization and spindle orientation was performed as described previously (Lechler and Fuchs, 2005). Statistical analysis was performed as described previously (Carraro et al., 2009).

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 (Fo[ucherousse 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 (Os[orio] et al., 2009; Wang C. et al., 2009). In this study, E[14.5] 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 7±5.6%, respectively; $P<0.05$), suggesting that *Eya1* deletion changes cell

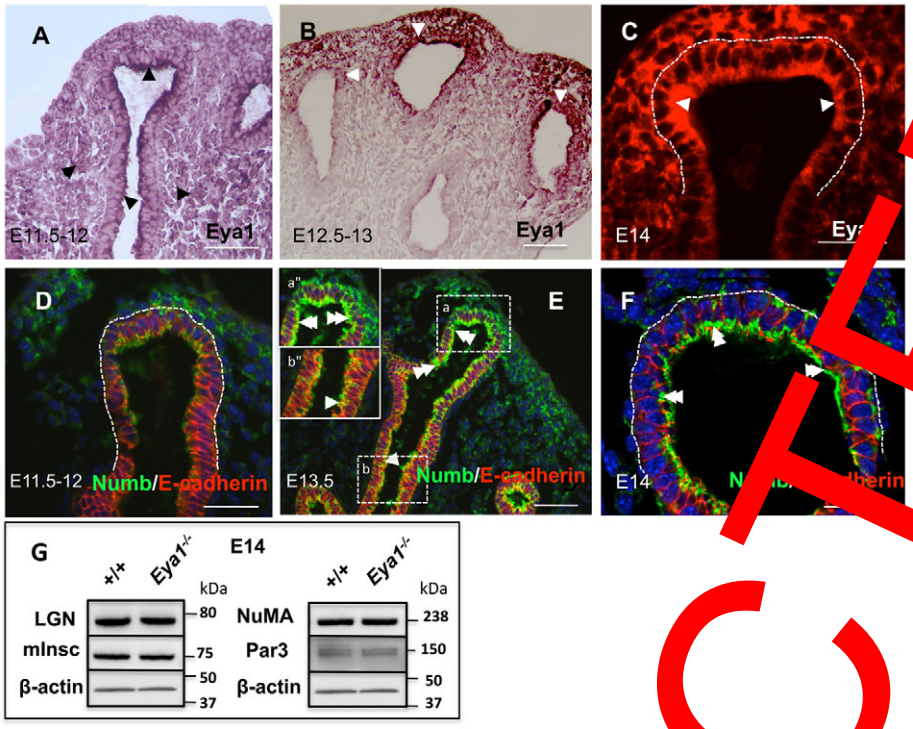

Fig. 1. Eya1 and polarity proteins are e… the lung. (**A-C**) Antibody …aining shows …lespread expression of …ya1 in both lung …ithelium and mesenchyme at E1…-E12.0 (arrowheads), and strong polariz… Eya1 signals in the …l epithelium …12.5-E14.0 (B,C; arrow…). (**D**…) Immunofluorescence shows very … Numb expression at E11.5-E12.0 distal epithelium (D), and strong …larized Numb signals in the distal (inset a″ …n E and F; …uble arrowheads) rather than proximal …thelium (inset b″ in E; arrowhe…) from E13-E13.5. (**G**) Western …lot sh…s no apparent changes in the …on of polarity proteins in E14 *Eya1*$^{-/-}$ lungs. Scale bars: 50 μm.

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 …nome Informatics) proteins (see Fig. S1 in the supplementary ma…al).

To facilitate quantification of cells dividing perpendicu… versus laterally, we stained E14 *Eya1*$^{-/-}$ distal epithelium fo… centrosomes with anti-pericentrin antibody. Then, mitotic cells were quantified based on centrosome orientation … to the basement membrane in order to distinguish pa…el/late… from perpendicular spindle alignments in mitotic cells. Centroso…s were oriented at 0±30° to the basement memb…ne were …ssed as parallel; those that were oriented at 90±…ere …ssed as perpendicular. In *Eya1*$^{-/-}$ distal epithelium, mos… divisions (87±3.0%) seemed to occur parallel/l…al to the …ent membrane, while about 12±5% mitotic …s had an alignment t…t appeared perpendicular in contrast …ild-type cells where perpendicular alignments were abund… (…P).

LGN, Insc and NuMA control spindle orient…on, and Numb regulates the …l fate of lung epithelial cells in vitro d…ending on Eya1 phosphatase activity

Next, we addressed whether m…he LGN…sc and N…IA functions in the regulation of spindle …entation…re conse…d in the lung epithelium, using gene-specific …A…the MLE…5 lung epithelial cell line. MLE-15 cells were used …study because of their intense expression of di…t polarity p…s and progenitor/differentiation cell mar…s. As…other epithelial cells (Lechler and Fuchs, 2005), LGN, …MA, In… and Par3 …d a mitosis-specific polarized distribut…n in …cells, often localizing asymmetrically to…e cell c…ex with one of the spindle poles positioned directly …which indicates a perpendicular alignment of the spindle (se… S2A,B,J,K,L in the supplementary material). Knock-down of *Insc*, …3 or *Numa1* function caused obvious mitotic defects, as judged by the misoriented and disrupted …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 …with the function of different polarity proteins in vitro (see …g. 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 …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/Insc/NuMA/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

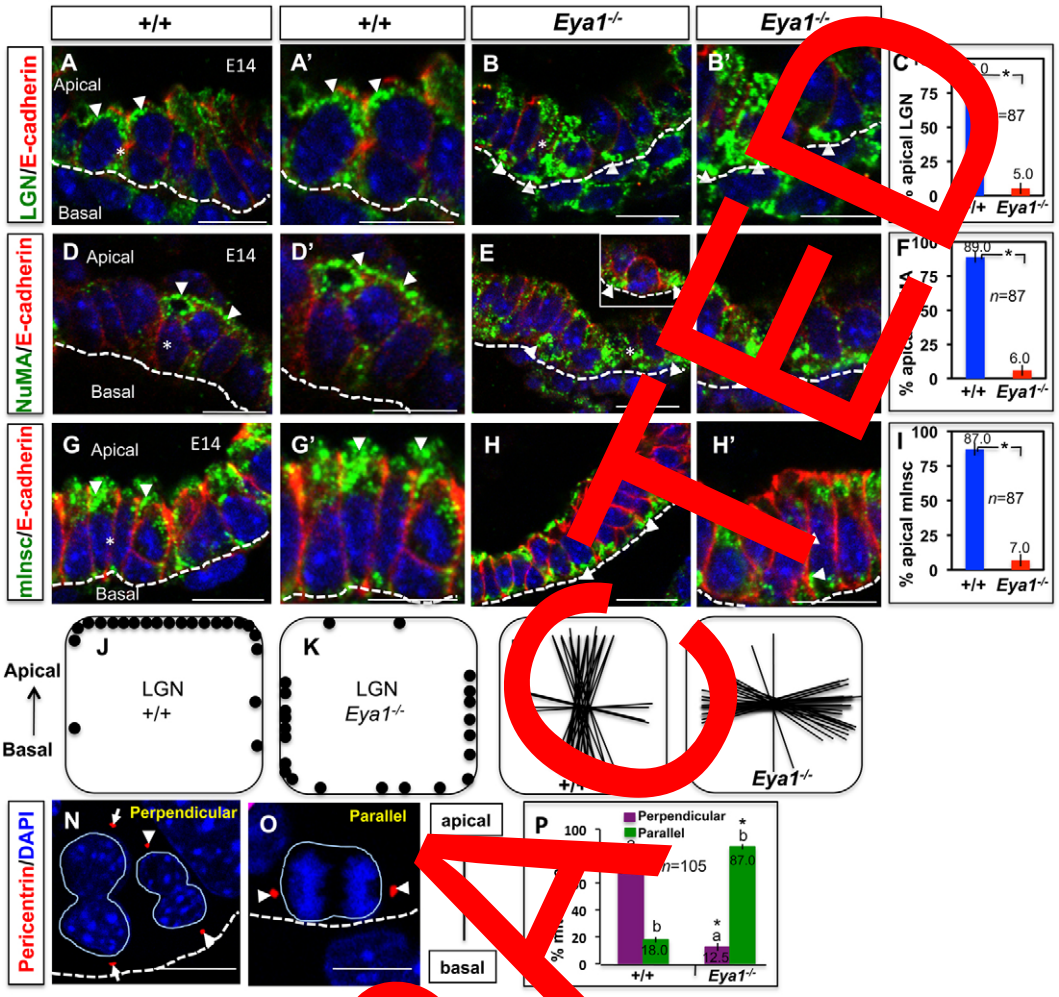

Fig. 2. *Eya1* deletion causes mislocalization of spindle-regulatory proteins, and increases parallel spindle alignments in mitotic distal epithelium. (**A**,**A′**,**B**,**B′**,**D**,**D′**,**E**,**E′**,**G**,**G′**,**H**,**H′**) Immunofluorescence and confocal microscopic antibodies shows that LGN, NuMA and Insc specifically localize to apical cell sides of wild-type distal epithelial cells (A,A′,D,D′,G,G′; arrowheads) and have a diffuse, basolateral or basal localization in *Eya1*$^{-/-}$ distal epithelial cells (B,B′,E,E′,H,H′; arrowheads). Broken line represents the collagen IV-stained basement membrane. A′,B′,D′,E′,G′ are electronic magnifications from areas marked with asterisks in A,B,D,E,G, respectively. (**C**,**F**,**I**) Quantification of mitotic distal epithelial cells with apical localization of LGN, NuMA or Insc for the experiments shown in A-H′. This is expressed as a percentage of all mitotic distal epithelial cells. *Significantly different from control ($P<0.05$; Student's *t*-test). Error bars indicate s.e.m. (**J**,**K**) Schematic representation of LGN localization in wild-type (J) or *Eya1*$^{-/-}$ (K) distal epithelial cells. Each dot represents the centre of an LGN crescent in a single mitotic cell. (**L**,**M**) Schematic representation of spindle orientation in E14 wild-type (L) and *Eya1*$^{-/-}$ (M) distal epithelium. Each line represents the spindle axis of a single late mitotic cell. (**N**,**O**) Examples of distal epithelial mitotic cells that divide perpendicularly, as represented by the perpendicular orientation of pericentrin-stained centrosomes (arrowheads/arrows) relative to the basement membrane (broken line; N), and others that have their centrosomes aligned parallel to the basement membrane (O; arrowheads). (**P**) Quantitation of the spindle orientations, which is expressed as a percentage of all divisions in the distal epithelium, of the experiments shown in N,O for E14 wild-type/*Eya1*$^{-/-}$ lungs. Mitotic cells are quantified based on centrosome orientation relative to the basement membrane in order to distinguish parallel from perpendicular spindle alignments.Bars carrying the same letter (a,b) are significantly different from one another (*$P<0.05$; Student's *t*-test). Data are mean±s.e.m. Scale bars: 50 μm.

Numb knockdown, as indicated by increased signal/fluorescence intensity for the Notch target *Hes1*/*Hes5* and increased number of Hes1-positive cells (Fig. 3J). This suggests a conserved function for Numb in controlling cell fates via 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

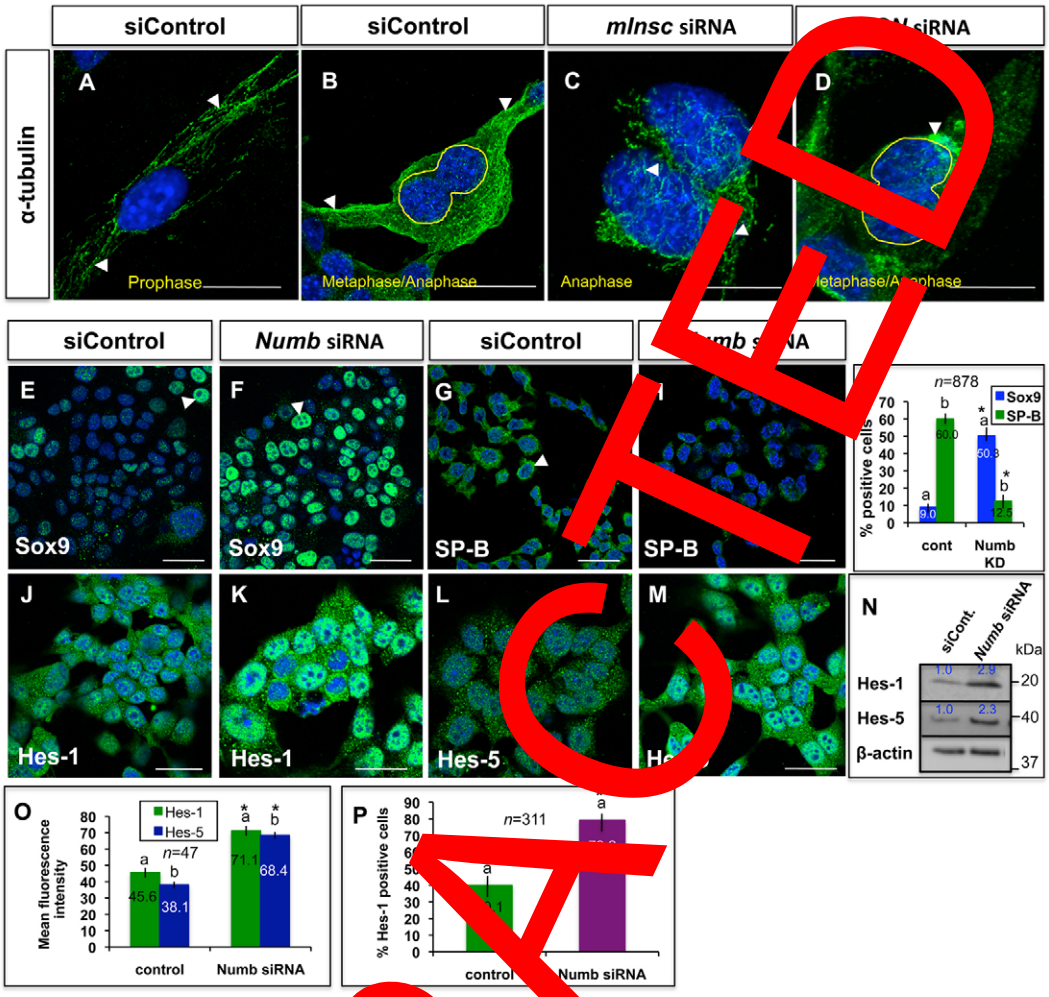

Fig. 3. Functions of polarity proteins in lung epithelium in vitro. (**A**,**B**) Immunocytochemistry with α-tubulin antibody shows well-organized and oriented spindle fibers (arrowheads) in MLE15 cells during (A) prophase (B) mitosis. (**C**,**D**) Spindle fibers are disorganized/disoriented (arrowheads) in mitotic MLE15 cells after *Insc* or *G*-*m2* knockdown. (**E-M**) Immunocytochemistry shows that MLE15-positive cells (arrowheads) for Sox9 (F), Hes-1 (K) or Hes-5 (M) increase with strong nuclear staining, while SP-B-positive cells (H) decrease after *Numb* knockdown. (**I**) Quantitation of Sox9- or SP-B-positive cells, which is expressed as a percentage of all counted MLE15 cells, of the experiments shown in E-H. Bars carrying the same letter (a,b) in I, O or P are significantly different from another (*P<0.05; Student's t-test). Data are mean±s.e.m. (**N**) Western blot of the experiments shown in J-M. (**O**) Means fluorescence intensity of Hes-1 or Hes-5 staining for experiments showing in J-M. (**P**) Quantitation of Hes-1-positive cells, which is expressed as a percentage of all counted MLE15 cells, of the experiments shown in J-K. In O,P, Error bars indicate s.e.m. Scale bars: 50 μm.

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,F). Conversely, Numb staining markedly increased, Numb was diffused and localized at both apical and basal cell poles in *Eya1*$^{-/-}$ distal epithelial cells (Fig. 4B,G).

Furthermore, closer inspection revealed that Numb staining is consistently concentrated in 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 daughters in 84±6% of *Eya1*$^{-/-}$ 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 *Eya1*$^{-/-}$ 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 *Eya1* 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 *Eya1*$^{-/-}$ 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 *Eya1*$^{-/-}$ lungs. Indeed, phosphorylated Numb increased in E14-

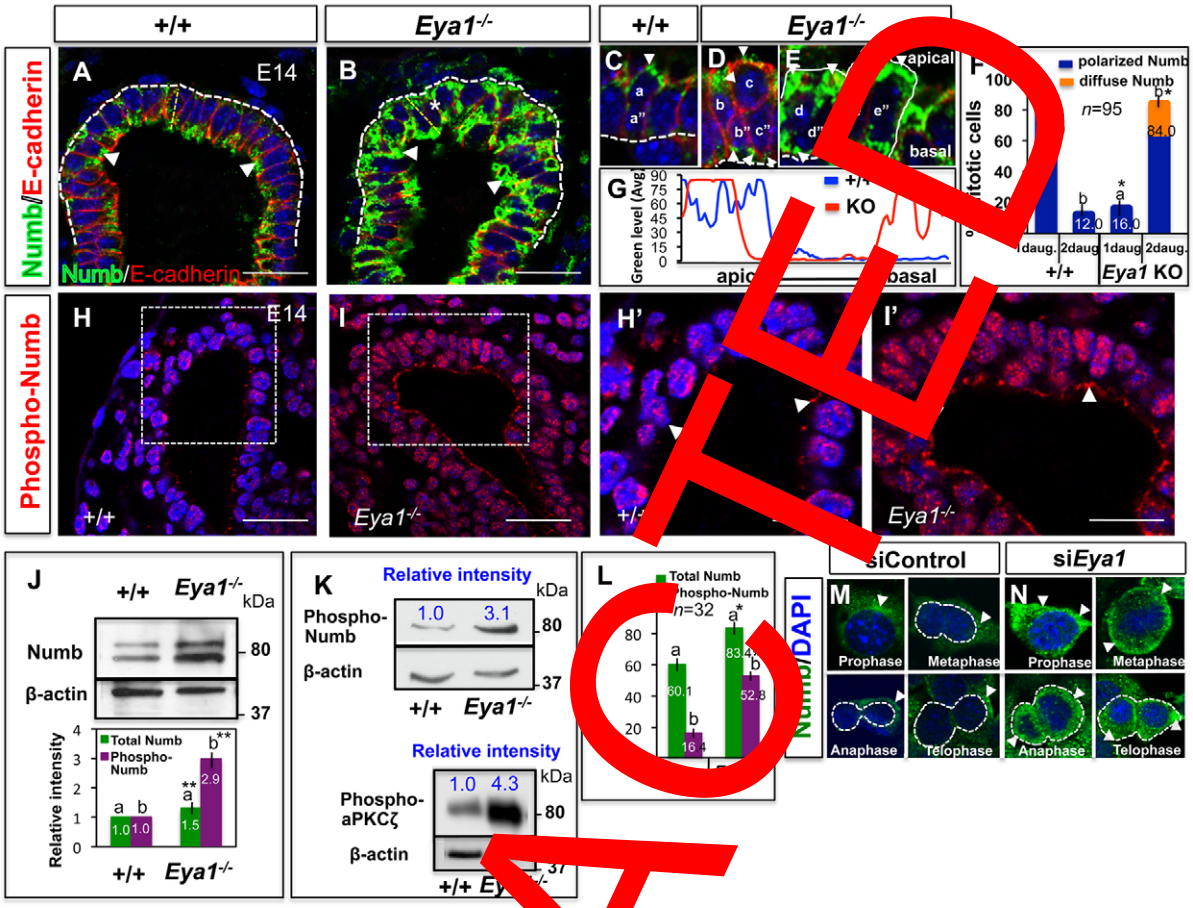

Fig. 4. Eya1 is required for polarized apical localization and pho… …ylation of Numb in distal epithelial cells. (**A**,**B**) Immunofluorescence for Numb shows preferential Numb localization to the apical side of dista… …ithelial cells in wild-type lungs (A; arrowheads). (**B**) Increased Numb expression but loss of its asymmetric localization in *Eya1*… …epithelium… …owheads). (**C-E**) High magnification of wild-type/mutant mitotic distal epithelial cells at anaphase/telophase shows tha… …umb l…lizes asymme…ically, and is inherited only by one daughter cell in wild-type lungs (a in C). a″ is a daughter cell that does not inherit Nu… …o. (**D**,**E**) … …mb (arrow…ads) fails to localize asymmetrically and is inherited by both daughter cells (b,b″,c,c″ in D and d,d″,e,e″ in E) in *Eya1*$^{-/-}$ mit… …c distal e… …cells with planer/parallel (D) or perpendicular (E) divisions (E, which represents the area marked with an asterisk in B)… …ative to t… …collagen IV-stained basement membrane (thick white broken lines in A-E). (**F**) Quantification of late mitotic distal epithelial p… …itors… …th 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… …nificantly different from one another (*P<0.05; Student's t-test). Data are mean±s.e.m. (**G**) Morphometric analysis of Nu… …b signal intens… …tribution of selected areas (thin yellow broken lines in A,B) shows loss of polarized/asymmetric localization of Numb, … …ich is distributed at b… … apical and basal sides of *Eya1*$^{-/-}$ distal epithelium. (**H-I′**) Immunostaining with specific Ser295 phospho-Numb antib… …shows increased Numb phosphorylation in E14 *Eya1*$^{-/-}$ distal epithelium (I,I′; arrowheads: staining in the cytoplasmic side of cell membrane an… …clei) compared with control lungs (H,H′; arrowheads). (H′,I′) High magnification of boxed areas in panels H,I, respectively. (**J**,**K**) Western … …ts of E14… …with anti-Numb (J), anti-Ser-295 phospho-Numb or anti-tyrosine phosphorylated aPKCζ antibody (K) show increased Numb/aPK… …phosphorylation… …*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 *Ey…*$^{-/-}$ 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 (arrow…ds) segregated asymmetrically and was inherited by one daughter cell in anaphase/telophase. Upon *Eya1* knockdown, Numb segregated to …oth daug…ers (N; arrowheads). Scale bars: 50 μm.

E14.5 *Eya1*$^{-/-}$ lungs (Fig. … …oreover, …hospho-Numb immunoreactivity using phospho-… …b… …(S…-295) antibody increased in vivo (Fig. 4H-K) and was m… …at the cell cortex and in the nuclei of *Eya1*… …distal epithelium (Fig. 4H-I′). Furthermore, Fig. 4L compares … …e mean fl… …rescence intensity of phospho:total Numb… …f wild-ty… …as *Eya1*$^{-/-}$ distal epithelium, showing that the …hospho:t… …l Numb was markedly altered between wild-type a… …l… …epithelium.

Similarly, a polarized N… …ignal localized to one side of the cell was detected in MLE-15 cel… …ulture (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 decreased in number while differentiated cell number increased in *Eya1*$^{-/-}$ embryonic lung epithelium. In

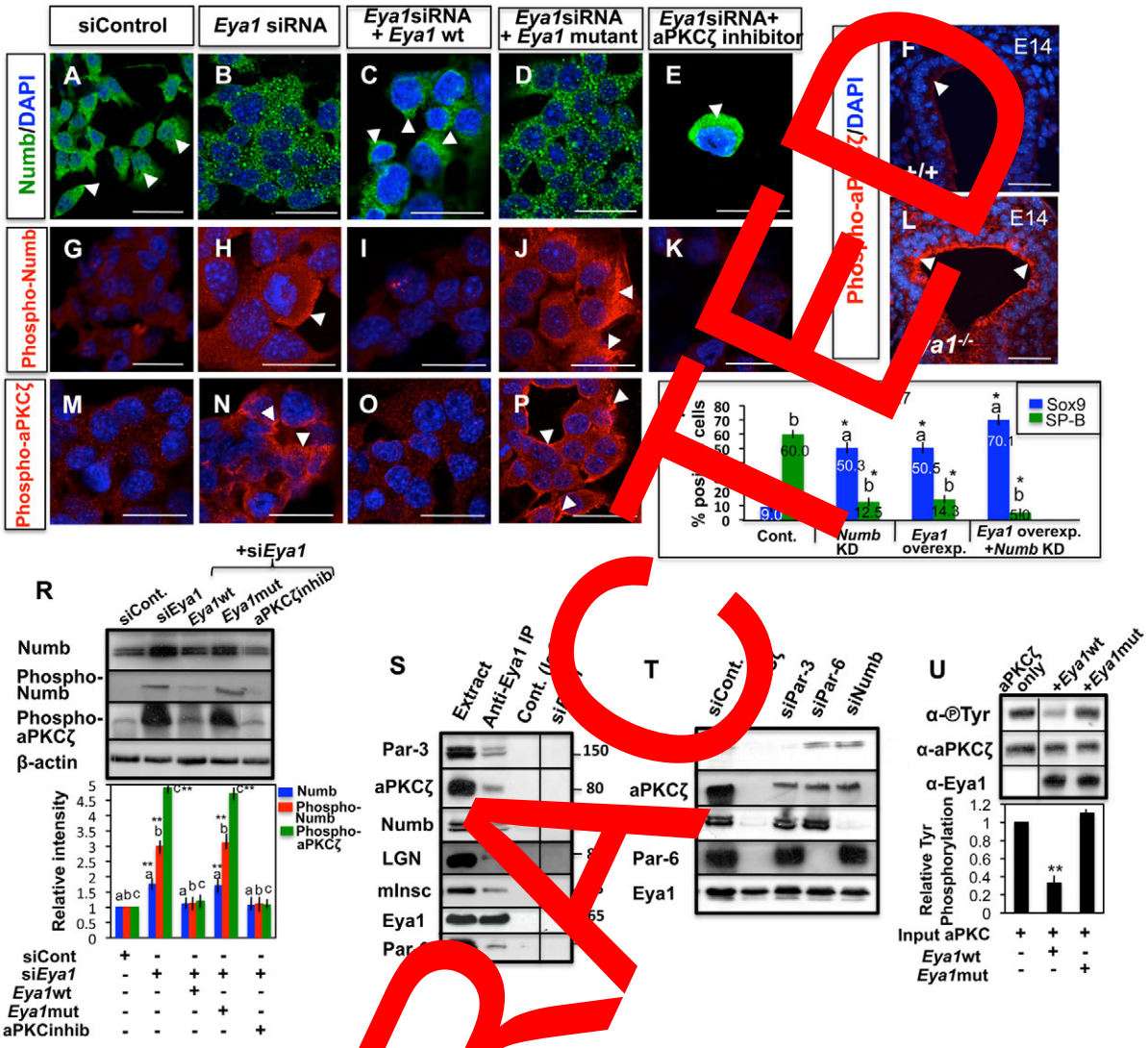

Fig. 5. Eya1 regulates aPKCζ and Numb phosp[illegible]tion. (**A**,**B**,**G**,**H**,**M**,**N**) Antibody staining of MLE15 cells shows changes of Numb distribution/Ser295 phosphorylation (B,H; arrowheads) a[illegible]ζ tyrosine phosphorylation (N; arrowheads) after *Eya1* knockdown compared with control cells. Arrowheads in A indicate polarize[illegible]Numb staining[illegible]**L**,**O**,**P**) Rescue of endogenous Eya1 function by co-transfection of murine siRNA and murine wild-type or enzymatically inactiv[illegible]utant *Eya1* construct[illegible]8 hours) in MLE15 cells reveals that Numb and aPKCζ distribution/phosphorylation (arrowheads) a[illegible]ependent on Eya1 phosphatase activity. (**E**,**K**) Inhibition of aPKCζ in *Eya1* siRNA-transfected MLE15 cells rescues Numb distribution/Ser295 phospho[illegible]rrowhead). (**F**,**L**) Increased aPKCζ tyrosine phosphorylation (arrowheads) in *Eya1*$^{-/-}$ distal lung epithelium. (**Q**) Quantitation of Sox9- or S[illegible]-positive[illegible]which is expressed as a percentage of all counted MLE15 cells, after interfering with the function of Numb and/or Eya1. Bars carr[illegible]g the same lette[illegible]are significantly different from the control of the same protein (*P<0.05; ANOVA-Dunnett test). Data are mean±s.e.m. (**R**) Western blot of Numb, phospho-Numb or phospho-aPKCζ for experiments showing in A-E,G-K,M-P. Bar graphs represent quantified western[illegible]t signals (mean±s.e.m.). Bars carrying the same letter (a,b,c) are significantly different from the control of the same protein (**P<0.001; ANOVA-[illegible]nnett test). (**S**) Endogenous Eya1 was immunoprecipitated from AEC2 cells with a specific Eya1 antibody and western blotting was performed w[illegible]t antibod[illegible]specific to different polarity proteins. Anti-Eya1IP of *Eya1* siRNA-transfected cells was used as a control. (**T**) siRNA knockdown of endoge[illegible]s aPKCζ[illegible]different[illegible]arity proteins in epithelial cells (48 hours) and subsequent IP for Eya1 and western blot for different polarity proteins. (**U**) In[illegible]o phosp[illegible]tase assay[illegible]ng immunopurified wild-type Eya1 or enzymatically inactivated mutant protein (Eya1 D323A) and aPKCζ protein. Grap[illegible]s quantifie[illegible]western blot signals normalized to input (n=3; mean±s.e.m., **P<0.001). Scale bars: 50 μm.

addition, *Eya1* overex[illegible]ssio[illegible]n MLE15 cells increases Sox9-positive progenitors c[illegible]ls, but d[illegible]reases SP-B differentiated cells (El-Hashash et al., 2[illegible]1) (Fig.[illegible]g. S3 in the supplementary material), similar[illegible]*Numb* k[illegible]ockdown effects (Fig. 3E-I). We therefore examined[illegible]he[illegible]the magnitude of Eya1 effects in balancing proliferation/di[illegible]iation of lung epithelial cells is changed in a *Numb* knockdown c[illegible]ound 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 (60.0±4.0% versus 12-14±3.0%, respectively; P<0.05) following, respectively, *Numb* knockdown or *Eya1* overexpression in MLE-15 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.

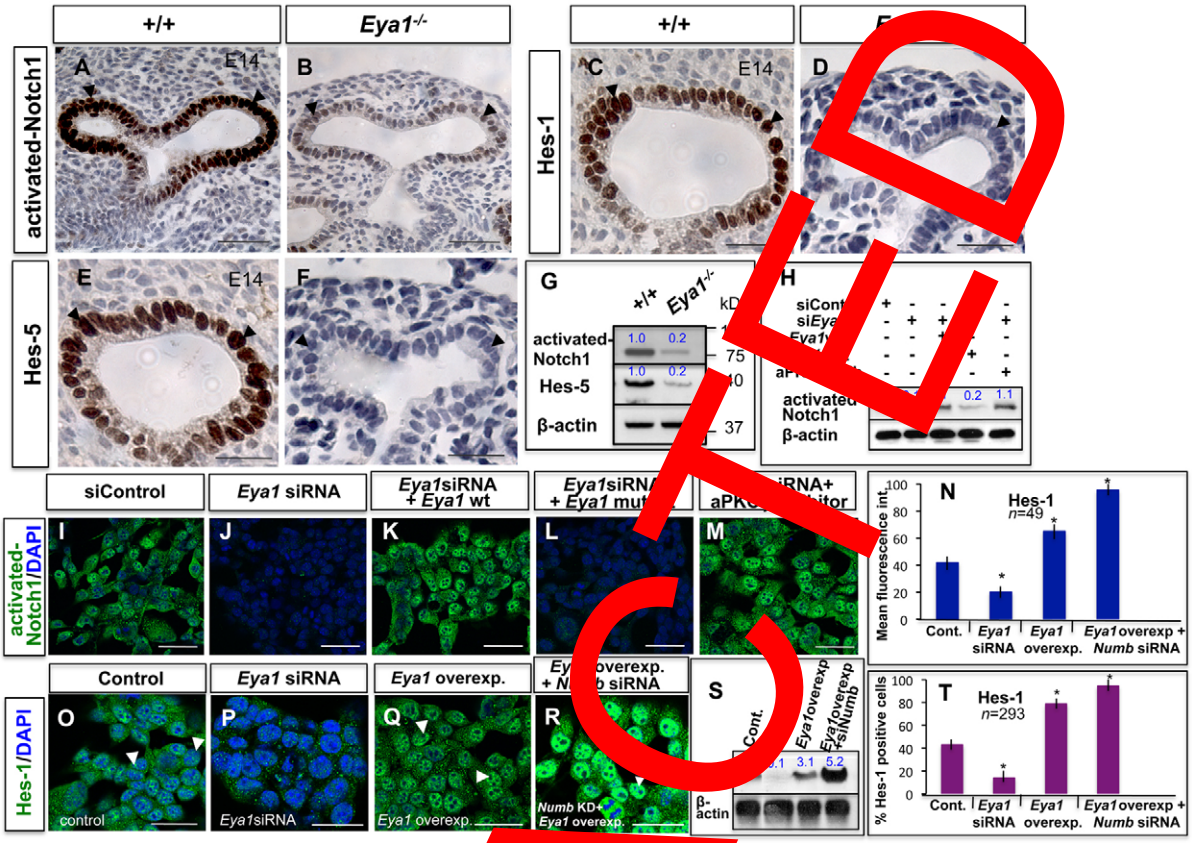

Fig. 6. Inhibition of Notch signaling in *Eya1*$^{-/-}$ lung distal epithelium. (A-F) Immunohistochemistry with specific antibodies shows reduced staining of activated-Notch1 (B), Hes1 (D) and Hes5 (F) in E14 *Eya1*$^{-/-}$ distal epithelium (arrowheads) compared with control lungs (A,C,E; arrowheads). (**G**) Western blots show reduction of activated-Notch1 and Hes-5 in E14 *Eya1*$^{-/-}$ lungs. (**H**) Western blot of activated-Notch1 for experiments shown in I-M. Blue numbers in G,H,S represent relative band intensity. (**I**,**J**) Immunocytochemistry shows reduced activated-Notch1 expression in MLE-15 after *Eya1* knockdown. (**K**,**L**) Rescue of endogenous Eya1 function by co-transfection of murine siRNA and murine wild-type or enzymatically inactive mutant *Eya1* constructs for 48 hours in MLE15 cells reveals that Notch1 signaling/activity is dependent on Eya1 phosphatase activity. (**M**) Inhibition of aPKCζ in *Eya1*siRNA-transfected MLE15 cells rescues activated-Notch1 expression. (**N**) Mean fluorescence intensity of Hes1 staining for experiments showing in O-R. *Significantly different from control (P<0.05; ANOVA-Dunnett test). Error bars indicate s.e.m. (**O-Q**) Immunocytochemistry of MLE-15 cells shows decreased Hes1 expression after *Eya1* knockdown (O,P), but increased Hes-1 expression upon *Eya1* overexpression (Q; arrowheads). (**R**) Hes1-positive cells with strong nuclear staining further increase after co-transfection of *Numb* siRNA and wild-type *Eya1* expression vector in MLE-15 cells (arrowheads). (**S**) Western blot of Hes1 for experiments showing in O-R. (**T**) Quantitation of Hes1-positive cells, which is expressed as a percentage of all cells of MLE15 cells, of the experiments shown in O-R. *Significantly different from control (P<0.05; ANOVA-Dunnett test). Data are mean±s.e.m. Scale bars: 50 μm.

Eya1 is essential for atypical protein kinase Cζ (aPKCζ) phosphorylation

Eya1 has well-known tyrosine phosphatase activities (Li et al., 2003). As Numb phosphorylation is increased in *Eya1*$^{-/-}$ lungs on Ser295 residue, which is phosphorylated by aPKCζ leading to Numb asymmetric localization (Smith et al., 2007), we therefore tested whether tyrosine phosphorylated aPKCζ is a direct substrate for Eya1 phosphatase. aPKCζ activity that is probed by a tyrosine phosphorylated aPKCζ 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 siRNAs, into these *Eya1*siRNA-transfected cells led to near control level of phospho-aPKCζ, 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 aPKCζ activity/phosphorylation in *Eya1*$^{-/-}$ lungs. This conclusion was confirmed by inhibiting aPKCζ activity in *Eya1*siRNA-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 aPKCζ by co-immunoprecipitation. The endogenous aPKCζ 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 aPKCζ and other polarity proteins in AEC2 cell lysate (Fig. 5S). To determine whether Eya1 binds to aPKCζ-Par-Numb/polarity protein complex by binding to aPKCζ, we performed Eya1/aPKCζ co-immunoprecipitation studies and analyzed other polarity proteins in cells treated with *aPKCζ*siRNA. Indeed, co-immunoprecipitation of Eya1, Numb, Par/polarity proteins was not observed after *aPKCζ* knockdown, but was observed after knocking down Numb or other polarity proteins (Fig. 5T).

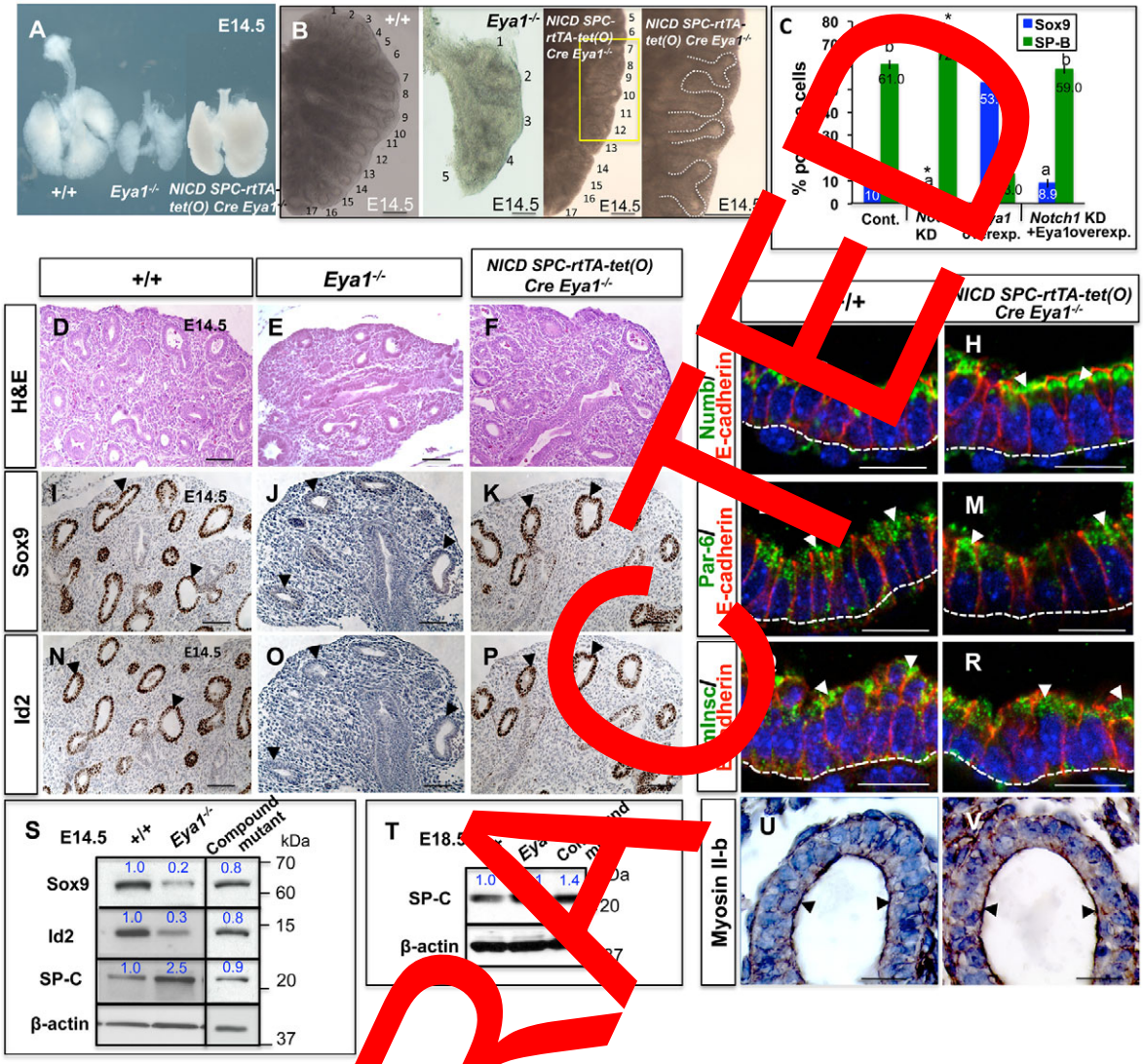

Fig. 7. Genetic activation of Notch signaling in *Eya1*$^{-/-}$ lungs partially rescues epithelial progenitor defects and branching phenotype. (**A**,**B**,**D-F**) External appearance (A,B) and histological analysis (D-F) of control versus *Eya1*$^{-/-}$ lungs show reduced epithelial branching and size of *Eya1*$^{-/-}$ lungs, which are restored in *NICD; Spc-rtTA*$^{+/-}$*-tet(O) Cre*$^{+/-}$*Eya1*$^{-/-}$ compound mutant lungs (A,B,F). The last panel in B is high magnification of the yellow boxed area. (**C**) Quantitation of Sox9- or SP-B-positive cells, which is expressed as a percentage of all counted MLE15 cells, after interfering with the function of Notch1 and Eya1. *Bars carrying the same letter (a,b) are significantly different from the control of the same protein (P<0.05; ANOVA-Dunnett test). Data are mean±s.e.m. (**G**,**H**,**L**,**M**,**Q**,**R**,**U**,**V**) Specific antibody staining shows similar polarized localization of polarity proteins (arrowheads) between compound mutant and wild-type lung distal epithelium. Broken lines represent the collagen IV-stained basement membranes. (**I-K**,**N-P**) Immunohistochemistry of adjacent sections shows reduced expression of progenitor markers Sox9 and Id2 in E14.5 *Eya1*$^{-/-}$ distal epithelium compared with control lungs (arrowheads). (K,P) Sox9/Id2 expression is substantially rescued in *NICD; Spc-rtTA*$^{+/-}$*-tet(O) Cre*$^{+/-}$*Eya1*$^{-/-}$ lungs (arrowheads). (**S**,**T**) Western blots show changes of the expression of Sox9, Id2 and SP-C between wild-type, *Eya1*$^{-/-}$ and compound mutant lungs. Blue numbers represent relative band intensity. Scale bars: 50 μm.

To further determine whether aPKC 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; Guo et al., 1996; Guo 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 *Eya1*siRNA-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. 3J,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 (Tsao 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) (El-Hashash 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 epithelial branching and expression of distal epithelial progenitor markers compared with lungs of *Eya1*$^{-/-}$ littermates (Fig. 7A-F,I-K, 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 epithelial differentiation markers (Fig. 7S,T; Fig. S3 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* knockdown background in MLE-15 cells. As shown in Fig. 7C, *Notch1* knockdown reduced Sox9-positive progenitors (60%; 10±3.0% versus 4.2±3.0%), but increased SP-B-positive differentiated cells (61.0±% 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.

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 proteins 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 Eya 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 cells during inner ear development (Zou et al., 2008). Here, we 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, 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 (Yu 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 regulation of cell polarity/spindle orientation, as well as aPKCζ/Numb phosphorylation (discussed below). In mammalian epithelium, the Par3/6 proteins localize predominantly to apically adherens junctions and bind to aPKCζ, Insc and LGN. This binding is crucial for the establishment of epithelial polarity and control for apical-basal/perpendicular spindle orientation (Macara, 2004; Suzuki and Ohno, 2006; Siller and Doe, 2009). Thus, the proper localization of aPKCζ-Par3/6-LGN-Insc polarity complex is crucial for cell polarization (Ohno, 2001). Our findings that Eya1 may bind to aPKCζ 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 determine their future fates. Thus, Notch signaling promotes progenitor cell identity at the expense of differentiated cell phenotypes (Jadhav et al., 2006; Mizutani et al., 2007). Differences in the Notch activities 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; Tsao 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* knockout/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 aPKCζ phosphorylation, which is essential for Numb phosphorylation and asymmetric localization/segregation (Dho et al., 2006; Smith et al., 2007), as reported for other phosphatases (Nunbhakdi-Craig et al., 2002). Indeed, aPKCζ-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*$^{-/-}$ lung (Figs 4-7; see Fig. S3 in the supplementary material). Indeed, Numb in[stance] in daughter cells acts to inhibit Notch signaling (Chapman [et] al., 2006). Consistent with our results, *Eya1* abrogation inhibits N[otch] signaling during sensory progenitor development in mammalia[n] inner ear (Zou et al., 2008), whereas high levels of *Eya1* inhibit neuronal differentiation, but expand the pool [of] [pro]genitive neuronal progenitors (Schlosser et al., 2008). [Our] findi[ngs] that genetically increasing Notch activity in *Eya1*$^{-/-}$ [lu]ngs subs[tan]ti[ally] rescues the abnormal lung epithelial phenotyp[e] in vivo (F[ig.] 7; see Fig. S3 in the supplementary material) provid[es] [strong] ev[idence] that Eya1 is indeed required for controlling Notch signa[l] [ac]tivity to ensure appropriate self-renewal/differe[n]tiation of lu[ng] [dis]tal epithelium. Whether Eya1 directly or i[ndi]rectly regulates Not[ch] signaling will be the subjects of future [stud]y.

In our future studies, we plan to [use] a [con]ditional knockout approach to delete *Eya1* specifically from [the] epithelial compartment to further investigate its specific functional [ro]les in epithelial cell development. N[ev]ertheless, the *Eya1* mutants reported herein provide a new [mo]use model for congenital lung hypoplasia/malformations and help [us] to understand the mechanisms that control lung [ep]ithelial [mo]rphogen[esis].

Does distal lung epithe[lium] [pro]vide asymmetrically? Does Eya1 p[hos]phat[ase] control asymmetric division[s] in the lung?

Recent reports sugg[est] that [un]differentiated lung epithelial progenitors undergo [mu]ltiple d[ivi]sion-linked cell fate decisions [symmetric and asy[m]metric c[ell] [div]ision (ACD)] that lead to an apparently homo[ge]neous e[x]pansion of the progenitor cell population (Rawlins, [E]. L. et al., 2008). No reports about ACD in the embryonic lung have [ap]peared as yet to our knowledge, but our study provides some evide[nce] 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 apical[ly] [en]riched Insc, LGN, NuMA and Par proteins, with mitotic spindles [ali]gned perpendicular to the basement membrane a[nd] a characteristic [a]symmetric segregation/inheritance of Numb (Figs 1, 2; see Fig. S[1] in the supplementary material). Indeed, a [st]rong correlation exis[ts] between ACD and the apical localization of th[e] polarity proteins, perpendicular alignment of mi[to]tic spindles an[d] [a]symmetric Numb segregation in *Drosophila*/[m]ammalian epithelium (Cayouette and Raff, 2002; Cayouette and [Ra]ff, 200[3]; Haydar et al., 2003; Noctor et al., 2004; Lechler and [Fu]chs, 200[5]). In this r[e]gard, our data suggest a crucial role for Ey[a1] in cont[ro]lling ACD, similar to other phosphatases (Wang et al., 2[006]; [Ka]wa et al., 2009), because *Eya1* abrogation perturbe[s] the organi[za]tion of polarity proteins and spindle orienta[tion], as well as Num[b] [se]gregation in distal embryonic lung epithe[lium], providing a conceptual framework for future mech[anistic] studies in this area.

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Competing inter[est] statement

The authors declare[] competing financial interests.

[Su]pplementary [ma]terial

[Sup]plementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.058479/-/DC1

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