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Scribble is required for normal epithelial cell–cell contacts and lumen morphogenesis in the mammalian lung

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ABSTRACT

During lung development, proper epithelial cell arrangements are critical for the formation of an arborized network of tubes. Each tube requires a lumen, the diameter of which must be tightly regulated to enable optimal lung function. Lung branching and lumen morphogenesis require close epithelial cell–cell contacts that are maintained as a result of adherens junctions, tight junctions and by intact apical–basal (A/B) polarity. However, the molecular mechanisms that maintain epithelial cohesion and lumen diameter in the mammalian lung are unknown. Here we show that Scribble, a protein implicated in planar cell polarity (PCP) signalling, is necessary for normal lung morphogenesis. Lungs of the *Scrib* mouse mutant *Circletail* (*Crc*) are abnormally shaped with fewer airways, and these airways often lack a visible, 'open' lumen. Mechanistically we show that *Scrib* genetically interacts with the core PCP gene *Vangl2* in the developing lung and that the distribution of PCP pathway proteins and Rho mediated cytoskeletal modification is perturbed in $Scrib^{Crc/Crc}$ lungs. However A/B polarity, which is disrupted in *Drosophila Scrib* mutants, is largely unaffected. Notably, we find that Scrib mediates functions not attributed to other PCP proteins in the lung. Specifically, Scrib localises to both adherens and tight junctions of lung epithelia and knockdown of Scrib in lung explants and organotypic cultures leads to reduced cohesion of lung epithelial cells. Live imaging of Scrib knockdown lungs shows that Scrib does not affect bud bifurcation, as previously shown for the PCP protein Celsr1, but is required to maintain epithelial cohesion. To understand the mechanism leading to reduced cell–cell association, we show that Scrib associates with β-catenin in embryonic lung and the sub-cellular distribution of adherens and tight junction proteins is perturbed in mutant lung epithelia. Our data reveal that *Scrib* is required for normal lung epithelial organisation and lumen morphogenesis by maintaining cell–cell contacts. Thus we reveal novel and important roles for *Scrib* in lung development operating via the PCP pathway, and in regulating junctional complexes and cell cohesion.

Introduction

Lung organogenesis involves the formation of a network of epithelial tubes with an extensive surface area to support postnatal respiration. New tubes are formed by budding of groups of polarised epithelial cells from an existing tube (Andrew and Ewald, 2009; Hogan and Kolodziej, 2002; Nelson, 2003). In the mouse, the spatial pattern of lung branches is remarkably stereotypical and is generated by three modes of local branching, named domain branching and planar and orthogonal bifurcation (Metzger et al., 2008).

Establishment and maintenance of a central lumen within each epithelial tube is a key step in tubulogenesis that allows efficient transport of liquids or gases (Andrew and Ewald, 2009; Chung and Andrew, 2008; Paul et al., 2003). Moreover, lumen diameter must be carefully regulated to facilitate optimal organ

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function (Datta et al., 2011). Current understanding of the molecular mechanisms of mammalian lumen morphogenesis is limited, yet disrupted lumen diameter is a feature of many human diseases such as polycystic kidney disease, hypertension and ischemic injury. In the lung, understanding the mechanisms used to establish and maintain lumen size may be important for treatment of cystadenomatoid malformations, pulmonary hypertension and even asthma, in which narrowing of the upper airways occurs.

Preserving sufficient lumen diameter requires maintenance of close contacts between epithelial cells through adherens junctions and tight junctions (Chung and Andrew, 2008; Lubarsky and Krasnow, 2003; Martin-Belmonte and Mostov, 2008). Formation of these junctional complexes is underpinned by the establishment of A/B polarity, characterised by similarly aligned cells with their basal sides immediately adjacent to the basement membrane and their apical sides adjacent to the lumen (Martin-Belmonte and Mostov, 2008; Martin-Belmonte et al., 2008). In lung branching morphogenesis, lumina are not formed *de novo*, but instead, new tubes arise from clefting or budding of existing tubes containing polarised epithelial cells so that the lumen of the new bud/branch is continuous with the lumen of the existing branch (Andrew and Ewald, 2009; Chung and Andrew, 2008; Hogan and Kolodziej, 2002). Initially, the lumen has a narrow diameter and this subsequently widens as the tube matures to its optimal size (C.D. unpublished observations). Although it is known that establishment of ion channels and secretion of fluid into the luminal space *in utero* play a role in regulating lung lumen diameter (Wilson et al., 2007), epithelial cells must first establish and preserve A/B polarity, undergoing considerable dynamic cell shape changes, mediated by the cytoskeleton, in order to adopt the morphology necessary to encompass a lumen. Moreover, it is essential that strong cell–cell interactions be maintained, to preserve the luminal space (Andrew and Ewald, 2009).

Scribble is a large cytoplasmic protein containing multiple domains including 4 PDZ domains (Bilder and Perrimon, 2000; Nakagawa and Huibregtse, 2000; Nakagawa et al., 2004). In *Drosophila*, Scrib is initially located at the basolateral membranes of epithelial cells and later in development becomes more restricted to septate junctions (Bilder and Perrimon, 2000). In mammalian cells *in vitro*, Scrib is observed at the plasma membrane where it has been shown to influence certain adherens and tight junction proteins including E-cadherin, β-catenin, ZO-1 and ZO-2 (Ivanov et al., 2010a; Metais et al., 2005; Navarro et al., 2005; Qin et al., 2005; Yoshihara et al., 2011). However these studies have reported divergent data concerning the interaction of Scrib with junctional proteins and to date, the mechanism is still unclear. It is notable that mice have only one *Scrib* gene, in contrast to many of the major apical–basal and planar polarity proteins which are represented by multiple family members.

Scribble acts as a tumour suppressor (Etienne-Manneville, 2009): *Drosophila Scrib* null mutants exhibit disorganization of epithelial tissues, leading to neoplastic growth and multilayering of epithelial cells (Bilder et al., 2000; Bilder and Perrimon, 2000) and *SCRIB* expression is decreased in a number of human cancers (Gardiol et al., 2006; Ivanov et al., 2010a; Navarro et al., 2005; Pearson et al., 2011; Thomas et al., 2005). Related to its tumour suppressor role, *Scrib* has been shown to play a part in maintaining contacts between epithelial cells (Dow et al., 2007; Qin et al., 2005) and in regulating the assembly of tight junctions in intestinal epithelium (Ivanov et al., 2010a).

Drosophila Scrib is required to maintain A/B polarity as part of a polarity protein complex, along with lethal giant larvae (Lgl) and discs large (Dlg); knockdown of Scrib disrupts *Drosophila* A/B polarity (Humbert et al., 2008). In contrast, most mammalian investigations have shown that *Scrib* operates within the PCP pathway, to regulate planar cell polarity (Montcouquiol and Kelley, 2003; Montcouquiol et al., 2003; Murdoch et al., 2003; Vandenberg and Sassoon, 2009; Wansleeben et al., 2010). In addition, *Scrib* has previously been shown to genetically interact with *Vangl2*; double heterozygotes exhibit defects such as craniorachischisis and disrupted stereociliary bundle orientation that are indicative of planar polarity pathway defects (Montcouquiol et al., 2003; Murdoch et al., 2001). Interestingly, a recent study revealed that *Scrib* does play a role in establishing PCP in *Drosophila*, in addition to its well-characterized role in A/B polarity (Courbard et al., 2009), and one study demonstrated mild A/B polarity defects in mammary epithelial cells (Courbard et al., 2009; Zhan et al., 2008). In fact, *Drosophila* studies show that PCP and A/B polarity pathways are closely linked at the molecular level (Courbard et al., 2009; Djiane et al., 2005) and it may be that many epithelial tissues require both A/B polarisation and planar polarisation for optimal organisation and function.

Given the known functions of *Scrib* in cell polarity and epithelial organisation along with our previous studies showing the importance of PCP proteins in lung development, we investigated lung morphogenesis in the *Scrib* mouse mutant *Circletail*. Here we show that $Scrib^{Crc/Crc}$ lungs are irregularly shaped and contain fewer epithelial branches. Branches are comprised of disorganised epithelial cells with a narrow lumen diameter or, frequently, no lumen at all. Molecular analysis reveals no overt disruption to A/B polarity but significant perturbation of the actin–myosin cytoskeleton. Moreover, there are reduced levels of active RhoA and altered localisation of the PCP proteins Vangl2 and Celsr1, consistent with Scrib operating within the PCP pathway during lung development. We also show a genetic interaction between *Scrib* and the core PCP gene *Vangl2* in embryonic lung. Additionally, our studies reveal unique roles for Scrib that have not been attributed to other previously studied PCP genes in lung development. Time-lapse imaging of lung branching morphogenesis in the presence of Scrib antisense morpholinos reveals reduced cohesion between epithelial cells. Moreover, *in vivo*, Scrib interacts with the adherens protein β-catenin in lung tissue. Further functional studies show mislocalisation of some tight and adherens junction proteins in $Scrib^{Crc/Crc}$ lungs. These defects in epithelial tubulogenesis are mimicked *in vitro*, where Scrib knockdown in organotypic cultures results in cysts comprised of disordered cells, small or absent lumina and disrupted subcellular localisation of β-catenin, ZO-2 and ZO-1. Our data reveal the importance of *Scrib* function during normal mammalian lung tubulogenesis, particularly in sustaining lumen diameter.

Materials and methods

Mouse strains and genotyping

$Scrib^{Crc}$ mice, originally described in Rachel et al.(2000) were maintained on a C3H/HeH background. $Scrib^{Crc}$ mice carry a single base insertion (Murdoch et al., 2003) and were genotyped by PCR amplification of flanking SNPs at 74.88 and 76 Mb (primer sequences available on request) with an annealing temperature of 62 °C and 38 cycles, followed by pyrosequencing. Using limb morphology as an indicator of developmental age, we found no evidence of developmental delay in homozygous mutant embryos compared to wildtype.

Morphometric analysis

Transverse sections of E14.5 or E18.5 left lung lobes stained with H&E were used to measure the width and number of airways. Sections were obtained from equivalent levels along

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