

Investigating the role of Vangl2 in the postnatal lung

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Declaration of originality

I confirm that I conducted all the work presented in this thesis with the following exceptions: tissue processing and histological staining (H&E, Miller's elastin stain, and MSB) were performed by the Inflammation, Repair, and Development Section Histology service (National Heart and Lung Institute, Imperial College London), or at MRC Harwell (Oxfordshire). Lung function experiments were performed with the assistance of Simone Walker. Vangl2 immunostaining was conducted by Nina Gross. The MMP-9 ELISA was performed by Samia Akthar, and MMP-9 zymography by Robert Snelgrove. Scratch assays in the presence of Wnt5a were performed by Akmol Ali. Influenza intranasal dosing and mouse health checks were performed by James Harker.

Abstract

The non-canonical planar cell polarity (PCP) pathway is important for embryonic development of many organs including the lungs. However, comparatively little is known about the role of this pathway in adults, partly because homozygous mutations of many PCP genes result in neonatal lethality, preventing post-natal investigations. We studied *Looptail* (*Lp*) mice, which carry a dominant negative mutation in Van Gogh-like 2 (*Vangl2*), a core component of the PCP pathway. Importantly, heterozygous *Vangl2*^{*Lp*} mice are viable postnatally, allowing us to study the adult lung. To investigate the role of *Vangl2* in adult lungs, we used a combination of *Vangl2*^{*Lp*} mice alongside human *in vitro* studies.

We show that a single mutant copy of *Vangl2*^{*Lp*} is sufficient to perturb lung development, resulting in severe airspace enlargement and decreased adult lung function. Lungs of adult *Vangl2*^{*Lp/+*} mice exhibit phenotypes associated with tissue damage, such as abnormal elastin organisation, elevated levels of the elastin-cleaving enzyme *Mmp-12*, increased MMP-9 activity, and an altered macrophage population. Furthermore, disruption of PCP signalling in embryonic *Vangl2*^{*Lp*} lungs perturbed actin cytoskeleton organisation, and regulation of the actin interacting protein Cofilin. Directed cell migration after scratch injury was also impaired following *in vitro* VANGL2 knockdown. Moreover, activation of PCP signalling by Wnt5a promoted wound healing *in vitro*, thereby, indicating that PCP signalling can enhance cell migration in repair.

In this study, we identified a novel role for PCP signalling in the adult lung. *Vangl2*^{*Lp*} mutations perturb adult lung structure and function, and we propose that these defects are due to actin cytoskeleton driven cell migration and repair deficiencies. Moreover, our data indicates that manipulation of the PCP pathway may promote lung repair in injury and disease to provide a curative treatment for chronic lung diseases.

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Well Mother, you'll be pleased to know I'm finally done.

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Chapter 1: Introduction

1.1. Thesis introduction

Chronic lung diseases arise from a combination of environmental and genetic factors, and are an increasing burden on the healthcare system [1,2]. Although in some cases medical intervention is able to slow disease progression, therapies targeted to promote lung repair are currently lacking. Studies in a wide range of animal models and observations in humans have shown that lungs can regenerate following injurious insult, such as pneumonectomy [3–6]. Furthermore, the lungs contain resident tissue stem cell populations which are able to proliferate and differentiate in response to lung injury [7]. A number of signalling pathways which are essential for embryonic lung development, such as the Sonic hedgehog (Shh) pathway, have been shown to be essential for adult tissue homeostasis, and are dysregulated in lung disease [8–10].

Another signalling pathway involved in lung development and disease is the Wingless/Int (Wnt) signalling pathway [11–14]. Wnts signal through two main cascades; the canonical β -catenin dependent pathway which has been extensively researched in lung development and disease, and the non-canonical Wnt/ Planar cell polarity (PCP) signalling pathway which is the main focus of this thesis. The PCP pathway directs cytoskeletal reorganisation and is important for the development of a number of organs [14–16]. Van Gogh-like2 (*Vangl2*), a core protein essential for PCP signalling, has previously been shown to be important for embryonic lung development in murine models. Embryonic mice harbouring the *Looptail* (*Lp*) mutation in *Vangl2* show disrupted branching morphogenesis and abnormal tissue architecture resulting from perturbed actin cytoskeleton organisation [14]. However, the role of the PCP pathway in the adult lungs is completely unknown.

In this thesis I characterise the impact of the *Vangl2*^{Lp} mutation in adult mouse lungs by histology and testing lung function. I also investigate extracellular matrix components and immune cell populations in the lungs; all of which are essential for lung homeostasis. I elucidated the molecular mechanisms underlying the disrupted adult lung structure in *Vangl2*^{Lp/+} mice, identified in this study by assessing actin cytoskeleton organisation and regulation. Finally, I examine whether the *Vangl2*^{Lp} mutation increases susceptibility to injury by challenging adult mice with lipopolysaccharide (LPS) and influenza virus.

1.2. Overview of the lungs

The lungs are an essential respiratory organ located in the thoracic cavity and are required for gas exchange. Human lungs consist of five lobes, three on the right and two on the left [17]. Mouse lungs also consist of five individual lobes arranged in a different pattern, one on the left, and four right lobes called the cranial, medial, caudal, and accessory lobe. The lungs are a highly branched organ that starts at the trachea and then divides into the two main bronchi, one on either side of the lungs. The bronchi then divide and branch further to form small bronchioles, eventually terminating at the alveoli [18]. Each of these structures consists of a diverse population of epithelial, mesenchymal, and immune cells which are adapted

to allow the lungs to function efficiently, and are necessary for maintaining its function [19]. The main cell types in the lungs include alveolar type I (ATI) cells, alveolar type II (ATII) cells, club cells, goblet cells, capillary endothelial cells, fibroblasts, and alveolar macrophages (which will be discussed in detail in *Section 1.2.3*). During homeostasis in the adult lung, the epithelial cell populations are stable and have a slow rate of turn over [20], with experiments showing an estimated time of 4-6 weeks for renewal of the alveolar epithelial cells (AEC) [21].

Adult lung diseases are a major and growing concern worldwide. These diseases include chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF), bronchopulmonary dysplasia (BPD), asthma, and lung cancers which interfere with breathing, affect quality of life, and are frequently fatal [17,20,22]. Lung diseases can arise from numerous factors or combinations of factors such as genetics, pathogens, and environmental exposure [22]. Chronic lung diseases are predicted to become the third leading cause of death worldwide by 2020 [23], and are a major strain on the healthcare systems.

Chronic lung diseases have a wide variety of clinical symptoms that can result in respiratory failure and death. Chronic lung diseases such as IPF cause scarring of the lung tissue as a result of excessive extracellular matrix (ECM) deposition which prevents efficient gaseous exchange [20]. However, within the syndrome of COPD there is not only excessive ECM deposition in the airways with irregular scarring of the airspaces, but also destruction of lung parenchyma resulting in enlarged airspaces known as emphysema [22]. Diseases such as asthma result from reversible airway obstruction in response to a stimulus. Respiratory diseases present with symptoms of cough, breathlessness and chest tightness and may be progressive, resulting in lung failure and death [24].

Although chronic lung diseases present a major health risk and are widely studied, effective treatments are lacking. Treatments for many lung diseases slow disease progression but the damage cannot be reversed [23]. In extreme cases, lung transplantations are performed but this is technically challenging, limited by a shortage of donor organs, prone to rejection, and the majority of lung transplants fail after 10 years [7]. To this end, research in lung regeneration has been of great interest as treatment for chronic lung diseases. Although lungs classically have been thought to lack the potential to regenerate [7], it is now appreciated there exists a degree of plasticity, which remains poorly understood, illustrated by a case study following a pneumonectomy where the lung was able to regenerate and neo-alveolarisation observed [6].

Currently, relatively little is known about the regenerative ability of the lungs in humans. Studies in animal models, including mice and dogs, have also demonstrated the regenerative ability of the lungs [3–5]. These studies revealed that following pneumonectomy on one side of the lung, the remaining lobes responded with compensatory growth and induce proliferation of endothelial cells, epithelial cells, smooth muscle cells, and fibroblasts [3–5]. Thus, studies have begun to investigate the developmental processes

that occur in embryonic lung development to provide an insight into the mechanisms underlying lung regeneration [7]. One pathway which is known to promote repair is the Wingless/Int (Wnt) signalling pathway, with studies in murine and human models demonstrating that reactivation of canonical Wnt signalling can initiate the repair processes in adult lung [13].

Studies have identified multiple tissue stem cell populations in the lungs that have both proliferative and regenerative abilities [7] (see *Section 1.2.3* for a detailed description of the different cell types in the lung). The current dogma in lung biology, defines tissue stem cells as a population of cells which are able to proliferate and differentiate into other cells types found within the tissue [7,25,26]; in this thesis tissue stem cells are defined according to the current literature. In the lung parenchyma, cuboidal alveolar ATII cells are able to differentiate into flat, elongated ATI cells that constitute the majority of the lung alveolar surface area [7]. Proximal airway cells such as basal cells, secretory club cells, and variant club cells are able to proliferate following injury, and are therefore thought to be lung stem cells [7]. The lungs also contain vascular progenitor cells such as cardiopulmonary mesoderm progenitor (CPP) cells which contribute to a large proportion of cells in the pulmonary vasculature [7].

In addition to the proliferative potential of these stem cells, differentiated cells in the lungs also exhibit cellular plasticity. Studies have shown that following ablation of the basal stem cell population, Scgb1a1⁺ club cells that have not yet fully matured are able to dedifferentiate back into basal stem cells. These dedifferentiated cells adopt a similar morphology to basal stem cell and express basal stem cell markers such as CK5 and p63 [26]. Dedifferentiated basal stem cells are able to act as true stem cells: they are multipotent, giving rise to Scgb1a1⁺ secretory cell and FoxJ1⁺ ciliated cell, as well as being able to self-renew [26].

Taken together, these studies reveal that the lungs contain a large number of cells with stem cell-like properties, such as the ability to proliferate and differentiate. This suggests that lungs are able to regenerate after injury, which may be important in the search for curative treatments for chronic lung diseases.

1.2.1. Lung development and branching morphogenesis

Lung development is a highly complex process that utilises branching morphogenesis to form a complex tree-like structure. Reciprocal signalling between the epithelium and mesenchyme tightly regulates this process. The exact cellular and molecular mechanisms driving lung development are not fully understood, but studies have begun to elucidate some of the key pathways that are required. In both mice and humans, lung development starts in early embryonic development and completes postnatally. Lung development can be divided into five stages based on the histological appearance: embryonic, pseudoglandular,

canalicular, saccular, and alveolar (Figure 1.1). In both embryonic and postnatal lung development, cell migration plays an important role in forming the lung structure. This cell migration in turn relies on the cytoskeleton that provides the contractive forces to enable cells to move (see *Section 1.6.1*). Throughout lung development many signalling pathways including the Wingless/Int (Wnt), fibroblast growth factor (FGF), retinoic acid (RA), and sonic hedgehog (Shh) are crucial for normal lung development to regulate patterning and cell differentiation [19]; these will be discussed further in *Section 1.2.4*. Lung development in mice is well researched and this thesis will discuss murine lung development, and allude to human lung development where possible.

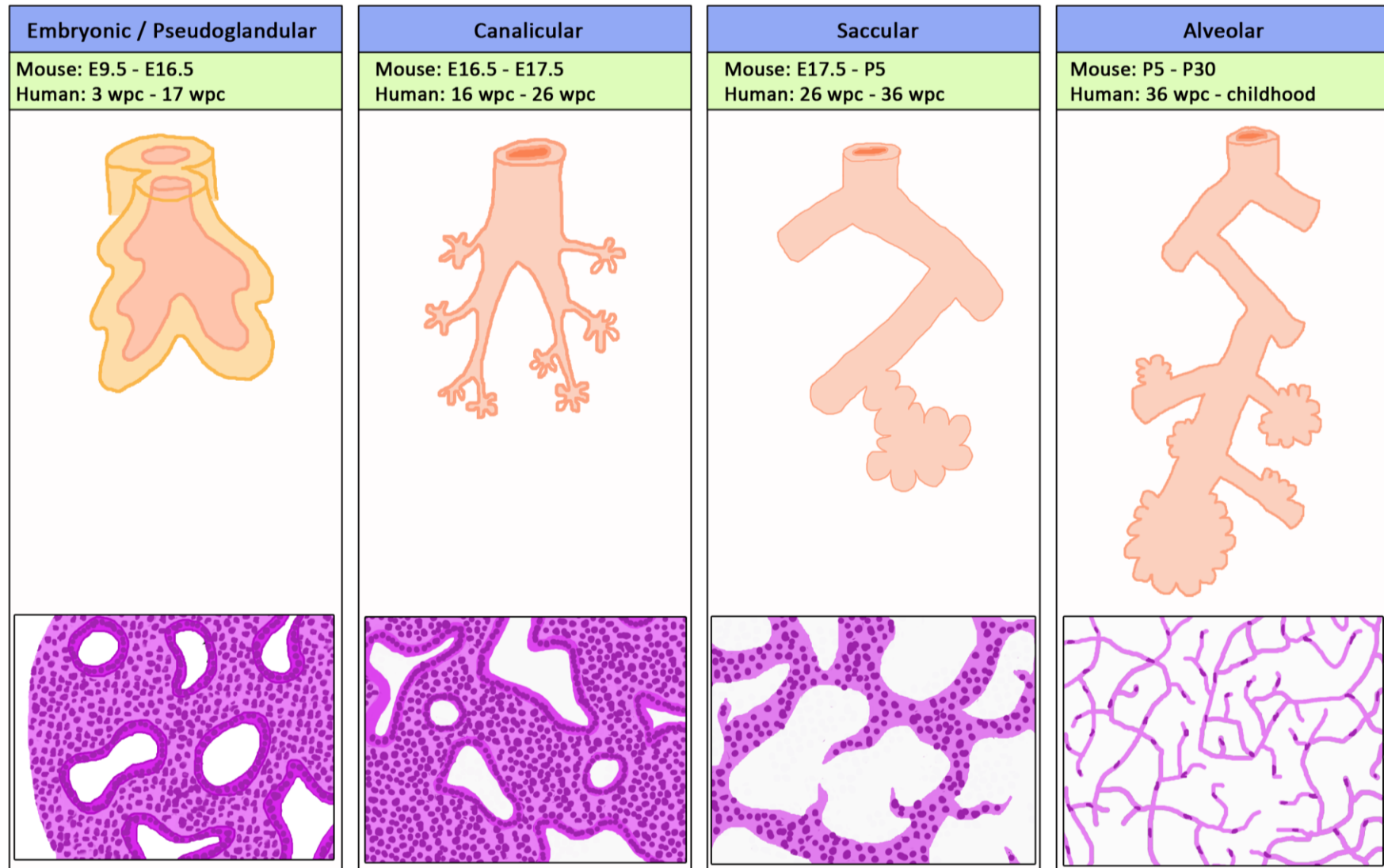


Figure 1.1. Stages of embryonic and post-natal lung development

As lung development occurs, the airways continue to branch and narrow producing numerous bronchioles that terminate in the distal air sacs (alveoli) (top panel). Histologically (bottom panel) the lung interstitium becomes thinner allowing airspaces to increase in preparation for gaseous exchange after birth. Abbreviations: Embryonic day (E), postnatal day (P), weeks post conception (wpc). (Adapted from Warburton *et al*, Seaborn *et al*, and Hameed *et al* [17,27,28])

1.2.1.1. The embryonic and pseudoglandular stage

Lung development begins with the embryonic stage at E9.5 in the mouse and it continues until E12 [19] (Figure 1.1). In humans, this approximately correlates to 3-7 weeks post conception (wpc). At the embryonic stage, lung development begins with the appearance of the primary lung bud from the anterior foregut; as such the lung epithelial cells are derived from the endodermal cells and surrounding connective tissue from the mesoderm. One of the earliest signal that is involved in this process is *Foxa2*, which is involved in closure of the gut tube, while *Gata-6* initiates the activation of lung development from the foregut endoderm [22]. The epithelial bud grows out into the surrounding mesoderm tissue and divides into the primordial left and right lung buds that contain the two main bronchi [22,29]. These initial buds then undergo branching morphogenesis to form the respiratory tree (primitive airways), invading the mesenchyme in response to localised Fgf10 signalling via fibroblast growth factor receptor 2 (Fgfr2) in the endoderm [30].

The pseudoglandular phase then starts at E12 and continues till E16.5 (5-17 wpc in humans) [17,19,22]. In the pseudoglandular stage, the airways undergo extensive branching and by the end of this stage, all of the bronchioles will have developed. At this stage, the airway epithelial cells are cuboidal in appearance [28]. Vascularisation of the pulmonary blood vessels also occurs alongside lung branching [31].

1.2.1.2. Branching morphogenesis

The main process occurring in the pseudoglandular stage is branching morphogenesis, during which all of the proximal airways are formed. In mice, lungs undergo 16 rounds of branching to establish the respiratory tree. Metzger *et al* used static wholemount images of immunostained lungs to map the patterns of branching. From this they reported that all lung branching occurs from repeated patterns of three distinct modes of branching: domain branching, planar bifurcation, and orthogonal bifurcation (Figure 1.2) [18].

Domain branching occurs first. In this type of branching, buds sprout from the stalk at set distances. Initially buds sprout along the lateral surface (referred to as L1, Figure 1.2) of the primary branch, and as this branch grows distally, further buds appear distal to L1. As the lung continues to grow, buds sprout along the distal, medial, and ventral surfaces of the primary stalk [18]. This establishes the main branch structure within the lung lobes. Following domain branching, the airways undergo planar or orthogonal bifurcation. In planar bifurcation, branching occurs within the same plane as the parent stalk, whilst orthogonal bifurcation occurs orthogonal to the parent stalk (Figure 1.2) [18]. Both planar and orthogonal bifurcation occurs at the tips of existing airways. To form the respiratory tree, the buds undergo elongation, cessation of the bud growth, followed by bifurcation at the tips; repetition of this process gives rise to the highly branched lung airway structure.

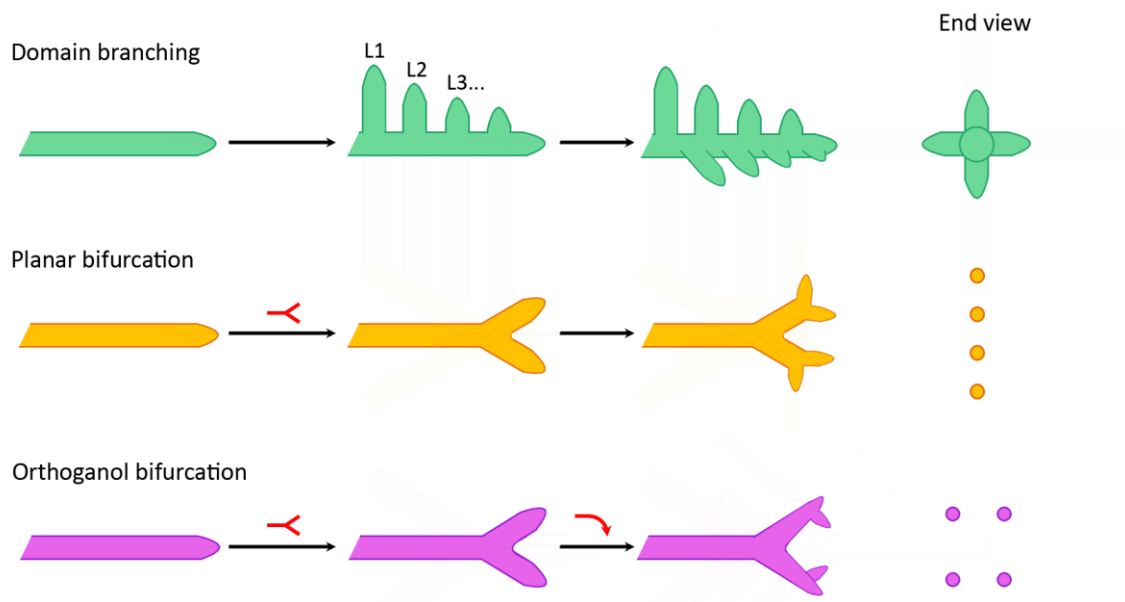


Figure 1.2. Pattern of lung branching in embryonic development

Branching initiates by domain branching, with buds appearing along the parental stalk with a medial-lateral and dorsal-ventral orientation. Following domain branching, planar and orthogonal branching occurs at the distal bud tips. In planar bifurcation, the branching occurs within the same plane as the parental stalk, whereas in orthogonal bifurcation branching occurs orthogonal to the parental stalk. (Adapted from Metzger *et al*, 2008 [18])

Another more recent study used an automated approach to quantify branching in the lung using optical projection tomography (OPT) to image the lungs, along with specially developed software 'Tree Surveyor' [32]. Here, they showed the pattern of lung branching broadly corresponded with Metzger *et al*. However, they found that the direction of bifurcation was not strictly in the planar and orthogonal orientation, as proposed by Metzger *et al*, and there was variability in the branching pattern between individuals, even among littermates [32].

1.2.1.3. Canalicular stage

The canalicular stage occurs between E16.5 to E17.5 in mice (16-26 wpc in humans) (Figure 1.1). During this stage, the respiratory tree continues to grow in length and diameter, with the distal lung developing at a faster rate. The terminal bronchioles divide and narrow, with the pulmonary acini forming at the distal tips of the bronchioles. A key process during this stage, is the extensive angiogenesis and vascularisation leading to the "canalization" of the lung parenchyma by capillaries in close proximity around the acini; allowing for later gas exchange to take place [17,33]. At this stage, the cuboidal shaped epithelial cells begin to differentiate into cell types found in the mature lung (see *Section 1.2.3*).

1.2.1.4. Saccular stage

The saccular phase occurs between E17.5 - post natal day (P)5 (26-36 wpc in humans), during which expansion of the air spaces takes place (Figure 1.1). At this stage, the lungs begin to mature and the cells differentiate to resemble the mature lung. The interstitium thins as a result of apoptosis, and the mesenchymal cells differentiate into specific cell types such as fibroblasts. Furthermore, the parenchymal epithelial ATII cells flatten and thin into ATI cells to form structures that allow for optimal gas exchange [17]. The cells also migrate and continue to septate resulting in the subdivision of the immature alveoli (sacculi) to increase the presumptive gaseous exchange surface area. Blood vessels also continue to develop in close proximity to the epithelium, surrounding the sacculi to support gas exchange. At this stage, the lungs are capable of supporting gas exchange through the blood-air barrier. Neonates are able to survive premature birth at this stage if the lungs are able to synthesis and secrete mature surfactant to reduce surface tension and prevent alveoli collapsing [17].

1.2.1.5. Alveolar stage

The final stage of lung development is the alveolar stage, which takes place postnatally in mice from P5 and continues until P30. In humans alveolarisation begins prenatally at 36 wpc and continues through childhood, until at least 7 years of age [22] (Figure 1.1). Recent studies have shown that alveolarisation may continue until adulthood [34,35]. Using non-invasive measurements in humans aged between 7-21 years, Narayanan *et al* showed that in addition to the lungs increasing in size, neoalveolarisation also occurred throughout adolescence [35]. In the alveolar phase, the alveoli mature to form thin walls that are optimised for gas exchange. At birth, the airways are fully established, however, the parenchyma is still immature [33]. The parenchymal epithelial cells continue to differentiate and thin, and become closely associated with capillaries to allow for optimal gas exchange. In addition to cell differentiation, there is also extensive cell migration as lungs undergo alveolarisation to form secondary septa (also referred to as secondary crests). The secondary septa arise from existing primary septa, which are formed in the saccular phase, by lifting off to form new tissue ridges. As such, the septation events form new alveoli, increasing the alveolar surface area available for gas exchange [33]. Another important process in this period is the increased production of mature surfactant by ATII cells, which is essential to reduce the surface tension at the air liquid interface to prevent the alveoli from collapsing [33].

1.2.2. Lung vascular development

In parallel to branching morphogenesis and pneumocyte development, the vascular system also develops in embryonic lung development, to allow for efficient gaseous exchange [17]. Vascular development starts around E10, in the pseudoglandular phase of lung development, with the presence of isolated “lakes”

containing haematopoietic cells [31]. In the saccular phase, the vasculature then continues to develop in close proximity to the acini as the lungs develop [17,33]. Murine studies have shown that perturbation of vascular development can result in disrupted lung architecture. Mice deficient for endothelial nitrogen oxide (NO) synthase (eNOS), a crucial enzyme for NO production and an important regulator of vascular growth, had impaired pulmonary angiogenesis correlating with reduced expression of vascular markers such as Foetal Liver Kinase 1 (*Flk1*), also known as Vascular Endothelial Growth Factor Receptor 2 (*Vegfr2*), and *Tie-2* in development [36]. Interestingly *eNOS*^{-/-} mice also have disrupted lung development with reduced airspace number and thickened septa [36].

1.2.3. Cells types in the mouse lung

The lung is comprised of a number of different cells types, the composition of which varies depending on the location of the airway and species [19]. The lungs begin with the cartilaginous airways that form the trachea and bronchi, the main function of which is to carry air through the lungs. These regions are lined by pseudostratified epithelium and consists of goblet cells, club cells (previously referred to as Clara cells), basal cells, and neuroendocrine cells [19,20]. The bronchi then branch to form bronchioles that are lined by simple columnar epithelium consisting of club cells, ciliated cells, and clusters of neuroendocrine cells. The most distal region of the lungs is the alveoli that form the gaseous exchange surface area and is comprised primarily of ATI cells and ATII cells.

The human lung structure is similar to the mouse lung but has some subtle differences. For example, in the human bronchioles, basal cells are also found in addition to the cell types in mice but at a lower frequency than the proximal airway [19]. The main difference in structure is the presence of respiratory bronchioles in humans which are found distal to the bronchioles, but are not found in mice; this is thought to be due to the difference in species size [37]. The respiratory bronchioles are lined by ciliated cells and club cells [19].

1.2.3.1. Club cells

Club cells are cuboidal cells, identified by their expression of Club Cell 10 kDa protein (CC10) [38], and are found throughout the respiratory airways. Club cells are able to act as resident stem cells that self-renew and differentiate into ciliated cells following epithelial injury in order to repair damaged epithelium [20,39], as well as mucus secreting cells in response to allergens [19]. Variant club cells are a class of naphthalene-resistant club cells identified by *Scgb1a1* expression, which is the gene for CC10, but not cytochrome p450 (*Cyp2f2*) [7]. Variant club cells are located at the bronchoalveolar duct junction and are able to differentiate into mature club cells and ATII cells to repair the epithelium following damage [19,20].

In addition, club cells are able to synthesise and secrete a wide range of proteins such as surfactant proteins (which will be discussed below), proteases, chemokines, and mucins that are essential for lung homeostasis [38].

1.2.3.2. Goblet cells

Goblet cells are columnar epithelial cells located in the large airways and are essential for secretion of mucus [40]. Mucus is formed by mucins, which are large highly glycosylated proteins comprised of amino acids and short carbohydrate chains (65-90 %) [41]. Mucus forms a chemical barrier for protection against environmental pathogens and provides lubrication in the lung [41]. Goblet cells increase secretion of mucins upon irritation by external stimuli such as dust and smoke, inflammatory mediators, or microbes [42]. Goblet cells are able to respond to airway injury; in murine models of allergic diseases, where house dust mite (HDM) is used to elicit an asthmatic phenotype, goblet cells proliferate and increase in number [43].

1.2.3.3. Ciliated cells

Ciliated cells are another prominent lung cell population in the bronchiole and trachea [44]. Ciliated cells are important for the removal of external particles and microbes trapped in mucus by co-ordinated beating during mucociliary clearance [40,44].

1.2.3.4. Bronchioalveolar stem cells

Bronchioalveolar stem cells (BASCs) are located in the bronchioalveolar duct junctions and act as stem cells for lung repair and regeneration. BASCs are able to differentiate into both club cells and ATII cells *in vitro*, and express both *Scgb1a1* and *Sftpc* which are markers for both of these cell types respectively [19,21,45]. Furthermore, these cells are more resistant to damage and increase their proliferative ability following lung injury [45], and represent an important stem cell population in the lung for promoting lung repair.

1.2.3.5. Basal cells

Basal cells are a population of epithelial cells that are adjacent to the basal lamina but are not in contact with the airway lumen. Basal cells are identifiable by their expression of keratin 5 (K5), K6 and K14, and K16; different subsets of basal cells are classified by their keratin expression [19]. Basal cells are an abundant stem cell population in the lung that show the greatest potential for differentiation. Lineage tracing

experiments have shown basal cells to be able to self-renew, as well as differentiate into club cells, ciliated cells, ATI cells, and AII cell [19].

1.2.3.6. Alveolar type I cells

Alveolar type I cells (ATI) cells are long, thin cells which cover 90-95 % of the total alveolar surface area to allow for efficient gas exchange between the alveoli and surrounding capillaries [20,21]. In the lungs, ATI cells were initially identified histologically as podoplanin (T1 α) positive cells, which in the lungs is specific for the ATI population [46]. In addition to enabling gas exchange in the lungs, ATI cells have been shown to be able to protect the lung from oxidative stress by the production of apolipoprotein E and transferrin [47]. ATI cells are typically difficult to isolate and culture, which has limited research of their full functional capability. However, more recently some labs have reported successful isolation and genetic characterisation of the ATI population [48,49].

1.2.3.7. Alveolar type II cells

Alveolar type II cells (AII) cells are cuboidal cells which cover less than 5 % the total lung alveolar surface area, but comprise approximately 60 % of the total alveolar epithelial cell (AEC) population [50]. AII cells play a key role in the maintenance of normal lung physiology, and are capable of self-replication and differentiation into ATI cells following injury [51,52]. AII cells also synthesise and secrete hydrophobic and hydrophilic surfactant proteins, which are a complex of protein and lipids (10 % and 90 % respectively), and are essential for maintaining the alveolar structure and providing immune protection [21].

Surfactant protein (SP)-C and SP-B are small hydrophobic apolipoproteins. They essential in the postnatal lungs for lowering the lung surface tension at the air-liquid interface (ALI) to prevent the alveoli from collapsing, and allow them to inflate easily [21,53]. In human SP-B is vital for respiratory function, and lack of SP-B production is fatal during infancy [54]. SP-C mutations have also been found in a number of human lung diseases, including interstitial lung disease [55]. In mice, knockout of *SP-C* is not fatal and lung morphology is not disrupted [56].

SP-A and SP-D are involved in host defence by mediating cellular phagocytosis by alveolar macrophages [21,57]. In addition to mediating innate immune responses, SP-A and SP-D can themselves bind pathogens and inflict damage to microbial membranes [57]. SP-D is crucial for normal lung homeostasis, with mice lacking SP-D developing emphysema and increased inflammation in the lungs [58].

1.2.3.8. Lung vasculature cells

The lung is a highly vascularised organ in order to allow for efficient gas exchange between blood and air [59]. All blood vessels contain a sheet of simple squamous endothelial cells surrounded by the basal lamina. Blood vessels found in the capillary bed surrounding the alveoli are composed of only the endothelial lining and basal lamina. The closely associated thin capillary and ATI structures are essential to allow for efficient gas exchange [60]. In larger blood vessels the endothelial cells are surrounded by connective tissue and smooth muscle [60].

Pericytes are a specialised subset of mesenchymal cells that are closely associated with endothelial cells in the microvasculature, such as in the pre-capillary artery, capillary, and post-capillary venules [61]. Pericytes are essential for controlling blood flow by regulating dilation and constriction of blood vessels. Moreover, pericytes are able to act as mesenchymal stem cells (MSC) and show pluripotency; they are able to differentiate into other cells types such as fibroblasts upon injury [62,63]. In chronic lung diseases such as IPF, pericytes form a significant part of the fibroblast population that contribute to the pulmonary fibrosis observed in IPF patients [61].

1.2.3.9. Fibroblasts

Mesenchymal cells such as fibroblasts and lipofibroblasts also play an important role in lung homeostasis and disease. Fibroblasts are interstitial cells located within the connective tissue, and are characteristically spindle shaped. Their main function is the production of ECM proteins such as collagen and elastin which provide structural support to cells [64]. In diseases such as IPF or following epithelial injury the number of fibroblasts increase due to rapid proliferation, resulting in increased deposition of ECM proteins [65].

Like fibroblasts, lipofibroblasts are also interstitial cells but contain a large lipid vacuole. Lung lipofibroblasts are located at the base of newly formed septa, and are often located close to ATII and endothelial cells [66]. Thus, lipofibroblasts are thought to act as shuttles for lipids from the blood to ATII cells. A study by Torday *et al* showed that lipofibroblasts were able to mediate transfer of triglycerides to neighbouring ATII cells for surfactant production [67].

1.2.3.10. Lung macrophages

In addition to the structural lung cell types, immune cells are also crucial for maintenance of lung homeostasis. The lungs contain two populations of macrophages categorised by their location. Alveolar macrophages (AMs), which are located in the alveolar space, and interstitial macrophages (IMs) in the alveolar walls [68]; these different macrophages populations are thought to have different functions in the lung. AMs are primarily involved in immunosurveillance and removal of microorganisms and environmental

particles, thus they provide the first line of defence against pathogens [68]. Historically, the lower lungs were thought to be sterile, however recent work has demonstrated that the lungs contain a microbiome. Thus, the AM population is crucial for regulating the microbiome to avoid infection from opportunistic pathogens [69]. IMs are thought to be involved in regulation of tissue structure by controlling inflammation and tissue fibrosis.

In addition to immunosurveillance, macrophages have been shown to be crucial in the development of many organs, including the lung, pancreas, and breast [70–73]. In the lungs, the AM population appear early during development from the yolk sac, around E8 which is prior to haematopoiesis [74,75]. These macrophages increase in number in embryonic lung development, and are localised at the budding tips of the lung [73]. In organs such as the breast which also develop through branching morphogenesis, macrophages are also localised to the terminal ends of the budding ducts, and macrophage depletion results in fewer and disorganised ducts [76]. As lung development continues, the number of macrophages increases, and in adults form a stable, self-maintaining population that are capable of proliferating to renew. As such, AMs are not derived from blood monocyte in adults unless extensive injury occurs causing depletion of the resident AM population [77]. As well as during development, macrophages have also been shown to be essential in tissue repair and regeneration in other organs such as the kidney and liver [78,79]. Models of regeneration in the axolotl have shown that depletion of macrophages inhibits limb regeneration [80], demonstrating the crucial role of macrophages in non-immunological processes.

1.2.4. Key mediators of lung development

In embryogenesis, many signalling pathways play a vital role in the initiation, maintenance, and control of lung development. Furthermore, the interaction between the mesenchymal and epithelial cells is crucial in various processes including branching morphogenesis. Some of the key genes involved in this process are discussed below.

1.2.4.1. Wnt 2/2b

Wingless/Int 2 (*Wnt2*) and *Wnt2b* are two of the earliest genes expressed to initiate lung development. *Wnt2* and *Wnt2b* are a part of the Wnt glycoprotein family that act in a β -catenin dependent manner, and are essential in embryonic lung development. Both *Wnt2* and *Wnt2b* are expressed by mesodermal cells surrounding the ventral surface of the anterior foregut at embryonic day (E)9-10.5, the earliest stages of lung development [11]. *Wnt2* is then strongly expressed in the mesenchyme of the distal airways between E12.5 and E18.5; *Wnt2* knockout mice (*Wnt2*^{-/-}) die shortly after birth and display significant lung hypoplasia in embryonic development [11]. *Wnt2b* is expressed in the distal mesenchyme at lower levels between

E12.4-E14.5, after which *Wnt2b* expression is lost [11]; *Wnt2b*^{-/-} knockout mice do not show any lung defects as *Wnt2* is capable of compensating for its loss [11]. Double knockout (DKO) mice for both *Wnt2* and *Wnt2b* failed to develop lungs, and did not express thyroid specific transcription factor-1 (*TTF-1*) which is the earliest marker of the lung endoderm [11]. Through this study, Goss *et al* showed that β -catenin dependent Wnt signalling by *Wnt2* and *Wnt2b* is essential for defining the lung endoderm progenitor cells in the foregut.

1.2.4.2. Thyroid specific transcription factor-1

TTF-1, also referred to as NK2 homeobox 1 (*Nkx2.1*), is a homeodomain containing transcription factor, and is one of the first genes required for lung development and differentiation [81]. *TTF-1* is a highly conserved protein, with 98 % similarity between humans and mice, and has an orthologue in *Drosophila*, highlighting its importance in development. Expression of *TTF-1* is found in the epithelial cells of the lungs, thyroid and certain parts of the brain [82–84]. In the lungs, it is involved in the separation of the trachea from the foregut, from which the lungs originate [17]. In mice, *TTF-1* is expressed in both the proximal and distal lung in early development, and is detectable in all lung epithelial cells from E10 [85]. In late gestation at E18, *TTF-1* expression is also found in the distal lung in ATII cells [85,86]. In adult mice, *TTF-1* expression becomes restricted to ATII cells and subsets of non-ciliated bronchiolar cells [85].

Knockout of *TTF-1* severely impairs lung development inhibiting distal branching, resulting in death at birth due to respiratory failure [87,88]. In *TTF-1*^{-/-} mice, tracheal separation from the gut tube is impaired and remains as a single lumen, although in wild type (WT) mice the oesophagus and trachea separate into two distinct lumens [89]. Furthermore, the trachea is shortened compared with WT mice [89]. Perturbation of *TTF-1* signalling inhibits branching morphogenesis, resulting in a reduced number of lung buds [87,88]. Mild overexpression of *TTF-1* caused an increase in ATII cell proliferation, whereas strong overexpression resulted in pathological disease including severe pulmonary inflammation, fibrosis, and respiratory failure [17]. Thus, *TTF-1* expression needs to be tightly regulated during development. *TTF-1* expression is also important in driving the expression of genes which are key for lung function, such as surfactant proteins in ATII cells, and club cell secretory protein (CC10) in club cells [81].

1.2.4.3. Fibroblast growth factor 10

Fibroblast growth factor (Fgf) signalling is another key pathway required for development and homeostasis of many adult organs, including the limbs and lungs [30,90]. Fgf10 is one of the key Fgfs that is essential for early lung development [30], and is highly conserved throughout evolution. The amino acid sequence of Fgf10 shows over 90 % homology between mice, rats, and humans; it also shares similarity (37 %) to the

Drosophila gene *Branchless (bnl)* which is the only Fgf orthologue in *Drosophila*. Mutations in *bnl* result in branching deficiencies similar to those observed in *Fgf10*^{-/-} mice [30]. *Fgf10* is expressed by the mesenchymal cells in the lungs [20], under the regulation of Wnt signalling, and acts on Fgf receptor 2b (Fgfr2b) which is highly expressed on epithelial cells in development [20,30]. In the lung, Fgf10 acts as a chemoattractant for the lung epithelium to drive branching morphogenesis [91,92]. Thus, Fgf10 is retained locally by binding to the heparan sulphate basement membrane protein, causing Fgf10 to act only over short distances at the tips of the branching airways [20]. Fgf10 signalling induces expression of *Sox9*, whilst repressing expression of *Sox2*, preventing differentiation of cells into proximal airway cells [20]. Thus, as the lungs continue to branch, the proximal cells that are not under the influence of Fgf10 are able to differentiate.

The generation of *Fgf10* null mice has demonstrated the crucial role of *Fgf10* in lung development, where homozygous *Fgf10* knockout (*Fgf10*^{-/-}) mice die at birth as they lack the formation of lungs. Heterozygous (*Fgf10*^{+/-}) knockout mice however continue to develop normally [30,90]. *Fgf10*^{-/-} mice form a distinguishable trachea, although shortened, in which the epithelium differentiates into a simple columnar pseudostratified layer. However, further lung development is halted, and fails to divide to form the main bronchi resulting in these mice being born without lungs [30]. These mice also do not express Sonic Hedgehog (*Shh*) or bone morphogenetic protein (*Bmp*) [90] both of which are key regulators of lung development.

1.2.4.4. Retinoic acid

Retinoic acid (RA), the biologically active metabolite of vitamin A, is important for the development of many organs including the lungs [93]. RA is synthesised from retinol, via a two-step oxidation process [94]. Initially, retinol is metabolised into retinaldehyde by retinol dehydrogenases, which is then metabolised into its active form RA by retinaldehyde dehydrogenases (RALDH). RA mediates its action in the cell by binding to an RA receptor (RAR). RARs form heterodimers with retinoid X receptor (RXR), which then translocates to the nucleus where it binds to DNA at RA response elements (RARE). The binding of RAR to RARE mediates transcriptional changes that either promote or repress gene transcription.

RA deprivation during organogenesis results in defective lung development [93,95,96]; similar phenotypes were also observed in mice with RAR mutations [97]. In the lungs, RA is secreted by mesenchymal cells where there is high RALDH levels, but is not present in the epithelial cells [96]. RA is then able to mediate signalling in both mesenchymal and epithelial cells [96]. Prominent RA signalling is found in both cell types in early lung development at E9.5 and is localised to regions with low branching activity; thereby establishing a complimentary pattern of activity to Fgf10 signalling [96]. RA activity then

diminishes, and is lost in the mesenchyme by E11 followed closely by the loss of RA signalling in the epithelium at E11.5 [96].

1.2.4.5. Sonic hedgehog

The Hedgehog (Hh) signalling pathway is an evolutionarily conserved pathway. In *Drosophila*, Hh is essential for patterning and cell fate determination in organs such as the brain, eyes, wings, and limbs. Hh is a secreted morphogen which mediates its action in a concentration dependent manner [98]. In vertebrates, there are three Hh homologues: *Sonic Hedgehog (Shh)*, *Indian Hedgehog (Ihh)*, and *Desert Hedgehog (Dhh)*. Both *Ihh* and *Dhh* show a restricted expression pattern to specific cell type, and only *Shh*, which is the most widely expressed, is found in the lungs. Shh signalling is mediated by Shh binding to the Patched receptor, which releases its inhibitory effect on membrane localisation of the G-protein coupled seven pass transmembrane protein Smoothened (Smo). The accumulation of Smo at the plasma membrane prevents the degradation of Gli, allowing it to accumulate and enter the nucleus to regulate gene transcription [98].

In lung development, Shh plays an essential role in mediating interaction between the epithelium and mesenchyme. Expression of *Shh* begins very early in the lungs at E10 and is maintained throughout embryonic development [98]. *Shh* is expressed throughout the lung epithelium initially, but proximal expression is later reduced, with higher levels of expression in the distal lung and is important for secondary branching [17,98]. After the canalicular phase at E16.5, *Shh* is expressed in a subset of non-ciliated cells in the proximal airways and is maintained until birth, where upon its expression ceases [98].

Shh^{-/-} mice display disrupted lung development forming only a single lobed lung and fails to branch, resulting in cyst like sacs [99,100]. Although these mice form trachea they are malformed [98]. Furthermore, mesenchymal cell differentiation is defective and smooth muscle cells do not develop around the proximal airways. However, differentiation in proximal epithelial cells is unaffected in these mice [98–100]. Overexpression of *Shh* causes respiratory failure as a result of alveolar hypoplasia and the increased proliferation of cells in both the epithelium and mesenchyme [101].

1.2.4.6. Transforming growth factor β

Transforming Growth Factor β (TGF- β) signalling is mediated by binding of the secreted ligand to the TGF- β receptor, a serine-threonine receptor kinase. Initially, TGF- β binds to the dimeric type II receptors, which in turn recruit type I receptors resulting in the phosphorylation of Smad2 and Smad3 that are cytoplasmic signalling molecules. Smad2 and Smad3 then activate Smad4 which translocates to the nucleus to mediate gene transcription [102].

There are three isoforms of TGF- β , which show differing patterns of expression in the lungs, and play unique, non-redundant roles. *TGF- β 1* is expressed in distal mesenchymal cells at the epithelial branching points [17]. *TGF- β 1*^{-/-} mice develop normally, however, they die in early adulthood from aggressive pulmonary inflammation [103]. Overexpression of *TGF- β 1* also disrupts lung development by inhibiting branching morphogenesis. Conversely, TGF- β 2 is localised to epithelial cells in the distal lung, with *TGF- β 2*^{-/-} mice developing severe lung dysplasia, and cardiac defects resulting in embryonic death at E14.5 [17,104]. *TGF- β 3* also localises to mesenchymal cells in the proximal lung. *TGF- β 3*^{-/-} mice die perinatally from breathing difficulties [105,106].

1.2.5. Development pathways in adult disease

Although the majority of chronic adult lung diseases have been thought to be driven by inflammatory processes, there is growing interest in the role of developmental pathways in adult lung disease [22]. Many studies have shown strong links between perturbed developmental signalling pathways and adult lung diseases [7,13,107–109]. In development, tight regulation of signalling pathways is essential, as under or over activation can result in abnormal development. Many of these pathways are then turned off in adult homeostasis, or have limited activity. However, in disease states these pathways may become reactivated [22]. For example, in lung cancer Fgf and Wnt signalling pathways become reactivated and are markers of poor prognosis. A number of important developmental pathways have also been shown to play a major role in COPD and IPF. Wnt signalling has come to the fore recently as a developmental pathway that is aberrantly activated in lung disease; this is discussed in detail below (Section 1.3).

The Shh signalling pathway is another key developmental pathway associated with chronic lung disease. During homeostasis in the adult lung, *Shh* is expressed in epithelial club cells in the proximal airway and parenchymal ATII cell [8]. Complementary to this, active Hh signalling is present in mesenchymal tissue surrounding the proximal airways and blood vessels, with some activity in the parenchymal interstitium [8,110]. Peng *et al* showed that Hh signalling is constitutively active in the adult lung to maintain the lung mesenchymal cells in a quiescent state [8]. Following naphthalene injury to epithelial cells, Hh activity is reduced and Hh responsive cells proliferate. Three months after naphthalene injury resolution occurs, with Hh signalling returning to homeostatic levels [8]. However, studies of fibrotic lung disease models, such as in bleomycin and fluorescein isothiocyanate (FITC) induced injury, there is increased *Shh* expression, which is predominantly in fibroblasts and myofibroblasts [110,111]. Furthermore, Shh stimulation increases survival of fibroblast; thus, fibroblasts are able to maintain the fibrotic state of the lung, leading to worsened fibrosis [110]. Although, Hh signalling can become activated or inhibited in response to injury, these differences may be due to the model of injury used in the respective studies. Following a single dose of naphthalene injury the lungs are able to regenerate and reepithelialise the lungs [112], whereas

bleomycin injury causes extensive fibrosis [113]. Thus, the difference in Hh signalling responses may differ due to the complex epithelial-mesenchymal cell interaction occurring in disease. It is possible that the early injury response may inhibit Hh signalling to promote reepithelialisation, but in extensive fibrosis, Hh signalling becomes aberrantly activated. Complementary to this, genome wide association studies (GWAS) have shown decreased mRNA and protein levels of hedgehog-interacting protein (*HHIP*), which inhibits Hh signalling, in COPD patients, correlating with decreased lung function [9,10]. Similarly studies in asthma patients have found a correlation with the *HHIP* locus and decreased lung function [114].

Aberrant FGF signalling has also been associated with chronic lung disease. Analysis of lung function in patients with *FGF10* haploinsufficiency, caused by the loss of one functional *FGF10* allele, showed reduced lung function such as forced expiratory volume 1 (FEV₁) similar to COPD patients [115]. Polymorphisms in other members of the FGF family, such as *FGF7*, have also been associated with COPD susceptibility in patients. An rs10519225 single nucleotide polymorphism (SNP) in *FGF7* was identified to correlate with a higher risk of COPD in Chinese Han cohort of patients [116]. In an independent study, Brehm *et al* identified two other SNPs in *FGF7* in the Costa Rican cohort and Lovelace Smokers Cohort that occurred in COPD patients [117]. Furthermore, increased expression of *FGF7* in patients correlated with worsened FEV₁ lung function. *FGF7* expression was also increased in COPD patients compared with control subject, although this change was not significant [117].

Together, these studies suggest an important role for developmental pathways in adult lung diseases, where perturbed developmental signalling pathways can lead to reduced lung function in adults. Thus, understanding these developmental pathways in more detail, may allow for manipulation of the lungs own repair processes and provide a therapeutic treatment for chronic lung diseases.

1.3. Wingless/Int pathway

The Wingless/Int (Wnt) signalling pathway is highly conserved throughout evolution, and is important in the development of many organs. Wnt signalling is initiated by the binding of secreted Wnt glycoproteins to the Frizzled (Fz) cell surface receptors. Wnts can initiate three independent pathways with differing cellular responses: the canonical Wnt/ β -catenin dependent pathway, non-canonical Wnt/Planar cell polarity pathway (PCP), and non-canonical Wnt/Calcium (Wnt/Ca⁺⁺) pathway. Although, these pathways share some upstream components, they have different downstream proteins and effect different biological responses [118–120].

1.3.1. Shared components of Wnt signalling pathways

1.3.1.1. Wingless/Int ligands

Wnts are glycoproteins that are roughly 40 kDa in size, of which there are 19 identified members in vertebrates. Wnts ligands are highly modified post-transcriptionally by the addition of mono-unsaturated fatty acids (palmitic acid) on conserved serine residues. Typically Wnt2, Wnt7b, and Wnt8 are considered to initiate the canonical pathway, whereas Wnt5a and Wnt11 are thought to stimulate the non-canonical pathway; however, a number of Wnts have been shown to be capable of activating both canonical and non-canonical signalling [121]. In fact, the branch of signalling activated by a particular Wnt is not definitive, rather it depends on the precise cellular context [122]. Typically, activation of the canonical Wnt/ β -catenin pathway is assessed by upregulation of genes such as *Axin2* and *Naked Cuticle Homolog 1* (*Nkd1*), which serve as key read-outs of active canonical signalling [13]. In contrast, specific activation of the non-canonical pathways is less easily determined. PCP signalling is often studied by observing changes in cytoskeletal organisation, such as the uniform orientation primary cilia and sensory hairs [123,124].

Wnts were initially discovered as proto-oncogenes, then called *Integration1* (*Int-1*) located on chromosome 15, responsible for driving mammary tumours following induction by the mouse mammary tumour virus (MMTV) [125]. *Int-1* is highly conserved across species, with 99 % homology between mice and humans [126]. The *Drosophila* orthologue of *Int-1*, *wingless* (*wg*), had previously been identified as a developmental regulator [126], highlighting *Int1/Wingless* as one of the first genes shown to be involved in both development and cancer [125]. The *Int1/Wingless* family was later renamed Wnt following the identification of other genes that have high homology to the *Int* gene [126]. In addition to breast cancer, Wnts have also been shown to be involved in a number of diseases including diabetes, heart defects, neural tube defects, and other types of cancers such as prostate cancer and glioblastoma [126,127].

In embryonic development, the Wnt ligands play an indispensable role in many cellular processes such as body axis patterning, cell fate specification, cell proliferation, cell migration, and adhesion. Additionally Wnts are able to regulate gene transcription of other Wnt signalling components such as *Fz*, *Axin2*, and *Dickkopf* (*Dkk*) for auto-regulation [13].

1.3.1.2. Frizzled receptor

Frizzleds (*Fz*) are key receptors in all Wnt signalling pathways. *Fz* was initially discovered in *Drosophila* where *Fz* mutants displayed disrupted polarity of epidermal cells [128]. In vertebrates, there are ten homologues of *Fz*, whereas in *Drosophila* there are four *Fz* genes [128]. The *Fz* receptors are seven-pass transmembrane G-protein coupled receptors that can vary in length between 500 to 700 amino acids. They contain an N-terminal extracellular domain with a cysteine-rich domain (CRD) that is used for interaction

with the Wnt ligands, and a hydrophilic linker region which can range from 40-100 amino acids [129]. The CRD region of Fz contains ten highly conserved cysteine residues which form disulphide bonds [128]. Adjacent to this there are seven hydrophobic transmembrane domains connected by three intracellular and three extracellular loops. The C-terminal end is located intracellularly and contains a highly conserved KXXXXW motif which can interact with Postsynaptic density 95/ Discs Large/ Zonula occludens-1 (PDZ) containing proteins such as Dvl to mediate downstream signalling [128].

1.3.1.3. Dishevelled

Dishevelled (Dvl), like the Fz receptor, is common to all branches of the Wnt signalling. Dvl is a key scaffold protein for Wnt signalling, and was initially identified in *Drosophila* [118]. In mice and humans there are three homologues of Dvl: Dvl1, Dvl2, and Dvl3 [118]. Dvl is a 700 amino acid protein and contains high homology between each homologue [118]. The high homology and expression pattern results in some functional redundancy in Dvl; mice which were either double knockout or even trans-heterozygote have more severe phenotype than a single knockout [118,130,131]. In *Dvl2*^{-/-} mice, 90 % have vertebral and rib malformations, but *Dvl1*^{-/-};*Dvl2*^{-/-} mice have a more severe phenotype [131]. Furthermore, neural tube defects (NTDs) are only found in a small proportion of *Dvl2*^{-/-} mice (2-3 %), but *Dvl1*^{-/-};*Dvl2*^{-/-} double knockout mice all had NTDs [131]. Rescue experiments have further demonstrated functional redundancy among Dvls. Homozygous *Dvl3* knockout in mice is lethal, and mice die at birth due to heart defects. However, overexpression of either *Dvl1* or *Dvl2* in the *Dvl3*^{-/-} is able to rescue the lethal phenotype [130]. Furthermore, the *Dvl2*^{-/-} mutation is lethal in 50 % of mice [131]; however, overexpression of *Dvl3* is able to compensate of *Dvl2* loss and rescue this phenotype, resulting in 96 % survival of *Dvl2*^{-/-} mice when overexpressing *Dvl3* mice [130]. Together, these studies showed that Dvls have functional redundancy and are able to compensate for each other.

Dvl contains many highly conserved regions that are essential for mediating protein-protein interactions and phosphorylation. The N-terminus contains the Dishevelled Axin (DIX) domain. This region spans roughly 80 amino acids and is important for promoting Wnt signalling [118]. The DIX region has also been shown to be important for mediating polymerisation to other Dvls [118]. Following the DIX domain is a region of positively charged (basic) amino acids that are an important phosphorylation site for casein kinase II (CK2) and Protease activated receptor 1 (PAR1). Phosphorylation of the basic region mediates interaction with other proteins such as Nkd together with the PDZ domain which proceeds this region [118]. The PDZ domain is a 90 amino acid region through which Dvls can interact with a wide variety of other PDZ binding domain containing proteins. The Fz receptors contain PDZ domains enabling direct interaction with Dvls to mediate downstream Wnt signalling [118].

1.3.2. Canonical Wnt signalling

The canonical pathway is the most extensively studied Wnt signalling pathway. In mice, canonical Wnt signalling (Figure 1.3) is initiated by binding of Wnts to the Fz receptors, and its co-receptor lipoprotein receptor related protein 5/6 (LRP 5/6) on the cell surface. In the absence of Wnt ligands, referred to as the “off-state”, Axin, adenomatosis polyposis coli (APC), protein phosphatase 2A (PP2A), glycogen synthase kinase 3 (GSK3), and casein kinase 1 α (CK1 α) form a destruction complex. In this complex, Axin acts as a scaffold for interaction with β -catenin [132]. Axin then mediates the phosphorylation of β -catenin by GSK3 and CK1 α , creating sites for ubiquitin ligase to bind and ubiquitinate β -catenin, targeting it for destruction by the proteasomes; thereby decreasing its cytosolic levels [124,132].

In the “on-state”, β -catenin is stabilised and can accumulate in the cytoplasm. Upon Wnt binding to the Fz receptor and LRP5/6, Dvl is recruited to the plasma membrane and is phosphorylated. This in turn promotes translocation of Axin, a key regulator of the destruction complex, to the cell membrane where it interacts with the C-terminal tail of LRP5/6. This binding to LRP5/6 is promoted by phosphorylation of LRP5/6 by CK1 and GSK3, which are a part of the destruction complex. Activated Dvl inhibits GSK3 phosphorylation of β -catenin, thereby preventing it from being targeted for destruction [124]. This mediates the release of β -catenin from the destruction complex, promoting its cytoplasmic accumulation. β -catenin then translocates to the nucleus, although how this translocation is mediated is unknown, as β -catenin itself does not contain any nuclear targeting sequence, nor does it rely on importin proteins for transport into the nucleus. Once in the nucleus, β -catenin binds to transcriptional regulators, the best characterised of which is the T Cell Factor/lymphoid enhancer factor-1 (TCF/LEF) DNA binding transcription factor family, to promote gene transcription [122,124]. Canonical Wnt signalling has been shown to regulate genes involved in processes such as cell proliferation, differentiation and tissue remodelling, including *cyclin D1*, *c-Myc*, *Vegfr*, and matrix metalloproteinases (MMP) such as *Mmp-2*, *Mmp-3* and *Mmp-9* [122]. In addition, TCF/ β -catenin is also able to regulate the transcription of canonical Wnt signalling components such as *Axin2* [13], and regulators of canonical Wnt signalling such Dickkopf (*Dkk*), which is a Wnt antagonist, to mediate the control of Wnt signalling [127].

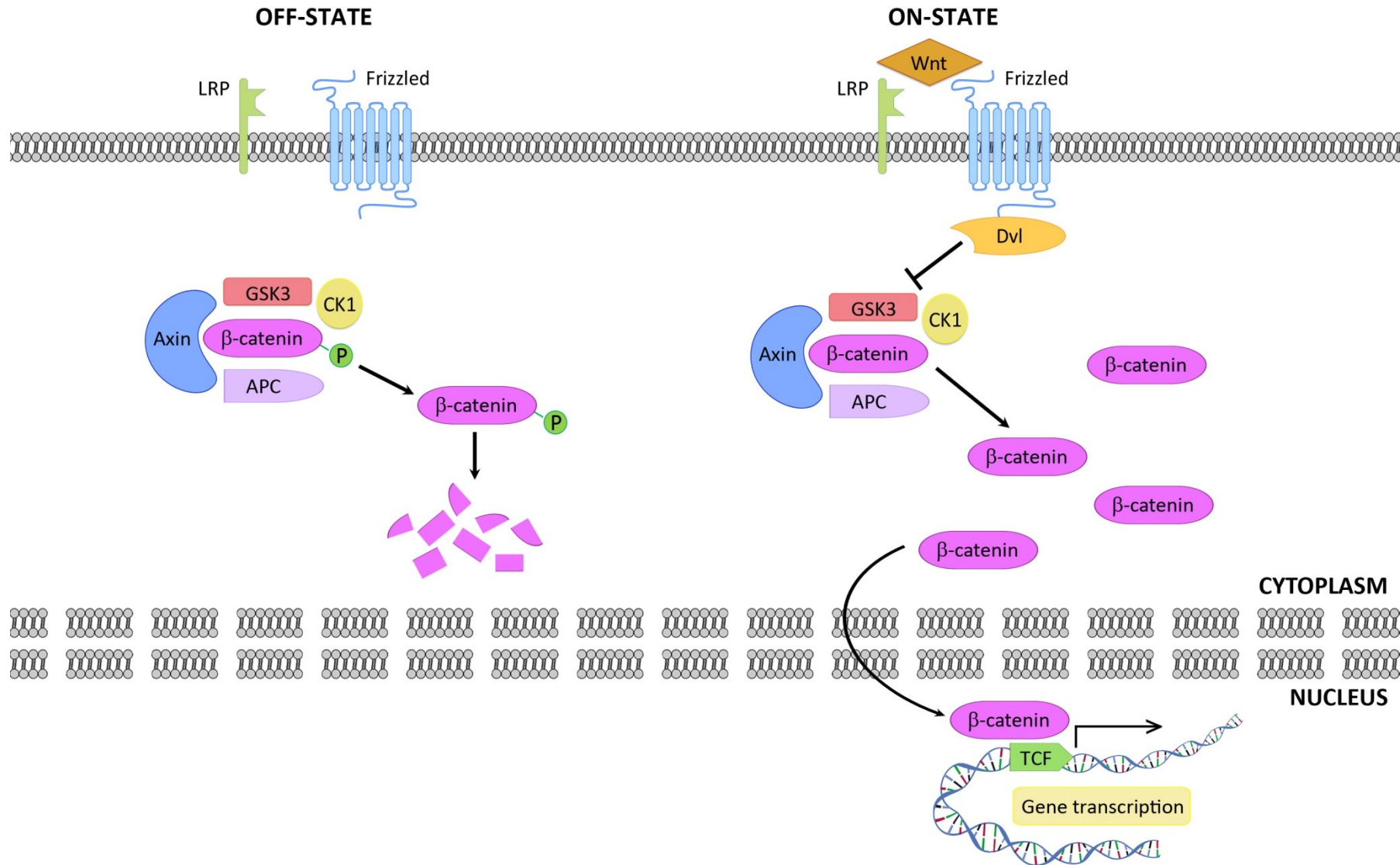


Figure 1.3. The canonical PCP signalling pathway

In the “off-state”, β -catenin is phosphorylated by the destruction complex (Axin, GSK3, CK1, and APC), which targets it for ubiquitination, resulting in β -catenin degradation by proteasomes. In the “on-state”, the destruction complex is inhibited, resulting in accumulation of cytoplasmic β -catenin, which then translocates into the nucleus and in conjunction with the LEF/TCF transcription factor mediates gene transcription driving cellular processes such as proliferation and differentiation.

1.3.2.1. Canonical Wnt signalling in development

Canonical Wnt signalling is an important pathway in development. Following initiation of lung development, Wnt/ β -catenin signalling is maintained throughout development in the embryonic lungs, with strong activity in the distal regions of branching airways [133]. Canonical Wnt signalling reporter mice such as *TOPGAL* [134], *BATGAL* [135], and *Axin2^{LacZ}* [136] have been used to study localisation of active Wnt signalling in lung development. The TOPGAL and BATGAL reporter mice lines contain a TCF/LEF promoter to drive β -galactosidase gene expression, whereas the *Axin2^{LacZ}* reporter has a *LacZ* knock-in into the endogenous *Axin2* site [134–136]. Therefore, β -galactosidase is expressed when canonical Wnt signalling is active. Thus, these reporter mice are ideal for assessing regions in which canonical Wnt signalling is active. However, the expression pattern of β -galactosidase in the lungs differs significantly between the three reporter mice [137]. In the lungs of TOPGAL mice, β -galactosidase is expressed throughout development in epithelial cells of the proximal and distal lungs. *Axin2^{LacZ}* mice also maintain β -galactosidase expression throughout development but express β -galactosidase in both the epithelium with low levels expression in mesenchymal cells [137]. In contrast, BATGAL mice express β -galactosidase in epithelial cells in early development at E11.5. β -galactosidase then reduces drastically and is undetectable at E18.5 [137]. The difference in the expression may result from the method of reporter insertion into the genome. Both the TOPGAL and BATGAL rely on random insertion of the reporter into the genome [134,135], therefore, the location of the insertion may alter the transcriptional activity. In contrast, the *Axin2^{LacZ}* reporter has the *LacZ* gene at the *Axin2* locus which is transcribed when canonical Wnt signalling is active, and so may provide a more robust model [136].

The high canonical Wnt activity that is maintained throughout lung development shows the importance of this signalling pathway. A number of Wnts have been shown to be essential in lung development. As mentioned previously, induction of canonical signalling by Wnt2 and Wnt2b is crucial for defining lung endodermal progenitor cells [11]. *Wnt4* expression also occurs very early and appears along the lateral surface of the gut tube at E8.25 in the mesoderm of the region where the lungs will originate [12]. When the lungs bud at E8.75, Wnt4 is expressed in cells adjacent to epithelial cells which express TTF-1 [12]. In mice, Wnt4 is required for patterning of the tracheal cartilage rings; *Wnt4^{-/-}* mutant mice had smaller tracheas which had incomplete cartilage rings [12]. Furthermore, *Wnt4^{-/-}* mice had reduced branching and smaller lungs arising as a result of reduced proliferation [12]. Wnt7b is another essential Wnt that is also expressed early in lung development at E9.5 [12]. *Wnt7^{-/-}* mutation is lethal at birth in mice; lungs of *Wnt7^{-/-}* mice were smaller and had perturbed distal branching compared with wildtype littermates [138]. Together, these studies show an essential role for Wnts throughout embryonic lung development.

However, other studies have shown that promotion of canonical Wnt signalling in the lungs, such as by Wnt3a, resulted in reduced branching as a result of reduced proliferation [133]. These results were corroborated by promoting canonical Wnt signalling using lithium chloride (LiCl), which inhibits GSK3 β

mediated β -catenin destruction; LiCl stimulation in lung explants cultures also inhibited lung branching [133]. In addition, Dean *et al* further demonstrated that inhibition of canonical Wnt signalling up-regulates *Fgf10* expression in the lacrimal gland which also develops by branching morphogenesis, similar to the lung [133].

In addition to its role in branching, Wnt/ β -catenin signalling is crucial for cell fate determination in the distal lung. Mucenski *et al* showed that deletion of *Ctnnb1*, the β -catenin gene, did not alter proximal epithelial cell differentiation. However, these mutant mice die at birth due to respiratory failure. In the distal lung, *Ctnnb1* deletion resulted in perturbation of normal sacculle development, inhibited squamous cell differentiation, and disrupted vascular development [139]. In a subsequent study, they showed that overexpression of β -catenin in mice displayed air space enlargement and epithelial cell dysplasia. β -catenin overexpression also disrupted cell fate determination as ATII cell were found in the bronchioles of these mice [140].

1.3.2.2. Canonical Wnt signalling in disease

As well as being important in development, perturbation of canonical Wnt signalling has been associated with adult lung disease in both humans and mice. In an experimentally induced IPF model using bleomycin treatment, Königshoff *et al* observed upregulation of the canonical Wnt signalling pathway genes, *Wnt10a*, *Wnt-1 inducible signalling protein 1 (Wisp1)*, and *Tcf4*. Furthermore, ATII cells isolated from bleomycin treatment mice had increased expression of canonical Wnt signalling components such as *Wnt1*, *Wnt2*, *Wnt7b*, *Tcf*, and *Gsk3b* [108].

Similar to data generated from mouse models, patients with IPF have increased *WISP1* expression in ATII cells, which drives cell proliferation [108]. Furthermore, the Wnt co-activators, *Lrp5* and *Lrp6*, were elevated in IPF patients and was associated with increased disease severity [141]. However, it is worth noting that elevated levels of Dickkopf1 (*Dkk1*), a potent canonical Wnt signalling antagonist, was found in IPF patient which is contradictory to the evidence presented above [141]. Thus, indicating that both increased or decreased Wnt signalling can drive IPF, and other genetic or environmental factors may determine disease progression.

In diseases such as emphysema, canonical Wnt signalling has been shown to be reduced. Kneidinger *et al* reported reduced expression of canonical Wnt target genes such as *Axin1*, and *Axin2* in emphysema patients compared with healthy controls [107]. Furthermore, activation of canonical Wnt signalling in mouse models and samples from patients with emphysema was able to rescue the remodelling phenotype, indicating a potential therapeutic mechanism to target chronic lung diseases [13,107]. The difference in regulation of Wnt signalling in IPF and COPD may lie in the mechanisms of the disease. Where

the over activation of Wnt signalling may lead to fibrotic disease by activating mesenchymal cells, whereas in emphysema the lack of Wnt signalling may not to activate efficient repair mechanisms.

However, the precise role of Wnt signalling in chronic lung disease remains controversial. In contrast to the finding of Kneidinger *et al*, studies by Baarsma *et al* have shown that canonical Wnt signalling is increased in fibroblasts isolated from patients with COPD [107,142]. In another study, Heijink *et al* showed that WNT4 levels were increased in bronchial epithelial cells isolated from COPD patients [109]. The differences seen between these studies may be due to differences in the methodology and models used in each study. In their respective studies, Kneidinger *et al* used the whole parenchymal tissue which will contain a mixed population of cells, whereas Baarsma *et al* studied isolated mesenchymal cells, and Heijink *et al* used isolated bronchial epithelial cells [107,109,142]. Thus, canonical Wnt signalling may be activated differently in epithelial and mesenchymal cells and elicit differing responses, resulting in the difference between these studies. Furthermore, the cell microenvironment may play a role in determining the response to Wnt signalling and the cellular outcome, therefore, cells isolated from different regions of the lungs, such as the parenchyma and bronchioles, may respond differently.

1.3.3. Non-canonical Wnt signalling

Non-canonical signalling can be divided into two main cascades; the planar cell polarity (PCP) pathway (discussed in Section 1.4.2), and the calcium pathway (Wnt/Ca⁺⁺). The Wnt/Ca⁺⁺ pathway uses intracellular calcium as a second messenger to elicit a cellular responses. Binding of non-canonical Wnts such as Wnt5 and Wnt11 to the Fz receptor and co-receptor induces recruitment of Dvl [143]. In turn, Dvl recruits and activates phospholipase C (PLC) via a trimeric G protein. PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) in the plasma membrane into intracellular inositol trisphosphate (IP3) and diacylglycerol (DAG) which is maintained at the membrane. IP3 then binds to Ca⁺⁺ channel receptors, in the endoplasmic reticulum to promote Ca⁺⁺ release. Ca⁺⁺ then activates protein kinase C (PKC) and calcium-calmodulin dependent kinase II (CAMK2); DAG also activates PKC [144]. The activation of PKC mediates many cellular responses such as gene transcription, cell adhesion, and migration. Activation of the Wnt/Ca⁺⁺ pathway has also been shown to inhibit canonical Wnt signalling [118].

Wnt/Ca⁺⁺ signalling has been shown to be involved in developmental processes such as body plan specification [119]. Loss of *Wnt5a* in zebrafish results in hyperdorsalisation, which is the process by which dorsal cells arise; *Wnt5a* overexpression which increases Ca⁺⁺ release however, had a hyperventralisation phenotype and also showed defects in cell movement [143]. However, the role of Wnt/Ca⁺⁺ signalling in murine lung development has yet to be studied.

1.3.4. Interactions between canonical and non-canonical pathways

Studies have suggested that the non-canonical and canonical Wnt signalling pathways can act in an antagonistic manner in some cases [145]. Overexpression of non-canonical Wnts such as Wnt5a in *Xenopus* antagonises the action of the canonical Wnt1 in development [146]. Torres *et al*, showed that Wnt1 induces expression of *gooseoid* (*gsc*) in the *Xenopus* ventral marginal zone, which was inhibited by Wnt5a [146]. However, Wnt5a was not able to antagonise *gsc* induction when β -catenin or a kinase-dead GSK3 was overexpressed [146]. In mammals, overexpression of Wnt5a antagonises TCF transcription factors which are required to mediate β -catenin signalling to regulate gene expression [145]. Conversely, Wnt5a removal in zebrafish promotes canonical Wnt signalling by stabilising β -catenin, resulting in increased expression of *bozozok* [143], a downstream target of canonical Wnt signalling in zebrafish [147]. Together, these studies indicate that canonical and non-canonical Wnt signalling are able to antagonise each other, although the molecular mechanisms underlying this interaction have yet to be elucidated. However, this antagonism does not seem to apply to all cases as shown by Torres *et al* where Wnt5a was not able to antagonise β -catenin activity in *Xenopus* [146]. Therefore, canonical and non-canonical antagonism may occur in a cell context dependent manner; however, further extensive studies are required to validate this.

1.4. The PCP pathway

In contrast to the many studies of the canonical Wnt pathway in both lung development and disease to date, very few studies have focused on the Planar Cell Polarity (PCP) pathway [13,107]. PCP was first studied in *Drosophila*, and was later found to be important in other organisms as well. The term planar cell polarity defines the axis that is orthogonal to apical/basal axis in individual cells, such as the orientation of the primary cilium in a cell and hair follicles in skin cells [148]. Planar or tissue polarity refers to the same axis as PCP, but at the level of the whole tissue such as the uniform orientation of hair cells in the wings of *Drosophila*. PCP signalling refers specifically to the signalling pathways induced by Wnt ligands.

Studies have identified two groups of proteins that are capable of regulating planar polarity: the Fat-Dachsous (Ft-Ds) pathway and the PCP pathway. Both of these pathways are essential in embryonic development and are vital for the definition of polarity across the apical surface. However, the interaction between the Ft-Ds and PCP pathways is not fully understood. The original hypothesis for how polarity is defined comes from the three-tiered hierarchy model [149], there are however, conflicting data within this model. The model suggests that the Ft-Ds pathway functions upstream of the core PCP pathway [150,151], and complimentary expression of Ds and Fj across the epithelium leads to a graded activity of Ft, creating global polarity. The graded Ft activity is then converted at the sub-cellular level to mediate polarity of PCP components. The PCP components are then able to mediate polarised activity of tissue specific effectors to mediate cellular function [149]. Although this is an attractive model and holds true in some respects, not all evidence supports this mechanism as other studies have shown that both pathways operate in parallel

[152,153]. In *Drosophila ft* mutants, the wing hair orientation, which is used as a marker of polarity, is misaligned and form swirls, whereas in wild type flies wing hairs are aligned in parallel. Despite wing hairs being disorientated in the *ft* mutants, PCP components are still asymmetrically localised at the cell membranes [150]. This suggests that although global polarity plays a role in providing directional cues to orientate PCP components, it is not essential for the asymmetrical localisation at cell membranes. Additionally, other studies have shown that the PCP defects observed in *ft* mutants can be rescued by a truncated form of Ft which is unable to bind to Ds, and should not be able to mediate polarity [154]. This work indicates that the extracellular domain of Ft which mediates binding to Ds is not necessary for its activity, and other directional cues may be required for patterning [154]. Currently there is currently no uniform consensus on the hierarchy of two PCP pathways, and it may depend on the cellular context.

1.4.1. Fat-Dachsous planar polarity

The Ft-Ds system has been studied widely in *Drosophila*, and is known to function in mammals. The Ft-Ds pathway consists of three key components, Fat (Ft), Dachsous (Ds), and the transmembrane golgi kinase Four Jointed (Fj). Ft and Ds are large atypical cadherins which localise to the cell membrane and are binding partners for each other across neighbouring cells [155]. Fj is a kinase which phosphorylates both Ft and Ds on the extracellular domains to mediate polarity [156]. Fj mediated phosphorylation of Ft increases its affinity for Ds, however, Ds phosphorylation decreases its affinity for Ft [156,157]. Unlike the PCP pathway, Ft-Ds polarity is not strictly established by the asymmetric localisation of its components, but relies on the expression levels and strength of the intercellular cadherin interaction based on its graded activity to mediate polarity [152]. Taking the *Drosophila* wings as an example, the proximal end has high Ds expression that decreases distally, whereas Fj expression is high at the distal end and declines proximally. Ft expression levels are similar throughout the cell [158]. The gradient of Fj and Ds expression results in a graded Ft-Ds intercellular interaction which is important for establishing polarity across the tissue (Figure 1.4) [158].

In mammals, there are four homologues of Ft (Ft1-4), and two homologues of Ds (Ds1 and Ds2). There is only one mammalian Fj orthologue, referred to as Four jointed box 1 (Fjx1) [159]. The function of the Ft-Ds system is conserved from *Drosophila* to mice. In *Drosophila*, *ff* expression is regulated by *fat* [151], similarly, mammalian *Fjx1* is also regulated by *Fat*; thus, in *Fat4^{-/-}* mice there is increased expression of *Fjx1* [160]. In mammalian embryogenesis, the Ft-Ds system plays an essential role in the development of organs that undergo branching morphogenesis including kidney and lungs. Ft and Ds expression is also observed in many other organs such as the heart, intestine, and skeleton [161].

Studies in *Fat4^{-/-}* mice that show PCP defects such as disrupted orientation of hair cells in the cochlea, and show kidney defects leading to cystic kidney disease (CKD) [160]. *Fat4^{-/-}* mice have smaller kidneys compared with WT mice, and show disrupted tissue architecture such as cystic dilations of tubules,

reduced and wider tubules in the Loop of Henle, and expansion of intraglomerular space of the nephrons [160]. These disruptions however, were not caused by disruption in cell fate determination, proliferation, or apoptosis [160]. Furthermore, *Fat4* was found to genetically interact with the PCP gene Van Gogh-like 2 (*Vangl2*) in the kidney; *Fat4*^{-/-} mice harbouring a single allele loss of *Vangl2* had increased severity of cyst formation in the kidney compared with *Fat4*^{-/-} or *Vangl2*^{+/-} alone [160]. However, *Vangl2* localisation was unaffected by *Fat4*^{-/-} loss. This shows that the Ft-Ds and PCP pathways work in parallel in a redundant fashion in cyst formation [160].

In an independent study, Mao *et al* showed that *Ds1*^{-/-} also had smaller kidneys with CKD; however, the number of cysts in *Ds1*^{-/-} mice was less than *Fat4*^{-/-} mice [161]. In contrast to the study by Saburi *et al* [160], Mao *et al* observed increased apoptosis and proliferation in both *Ds1*^{-/-} and *Fat4*^{-/-} mutant kidneys [161]. In addition to kidney defects, both *Ds1*^{-/-} and *Fat4*^{-/-} mice had smaller lungs compared with wildtype littermates [161]. Although lung defects have been noted in these mice, detailed studies of the Ft-Ds pathway have yet to be performed in the lungs.

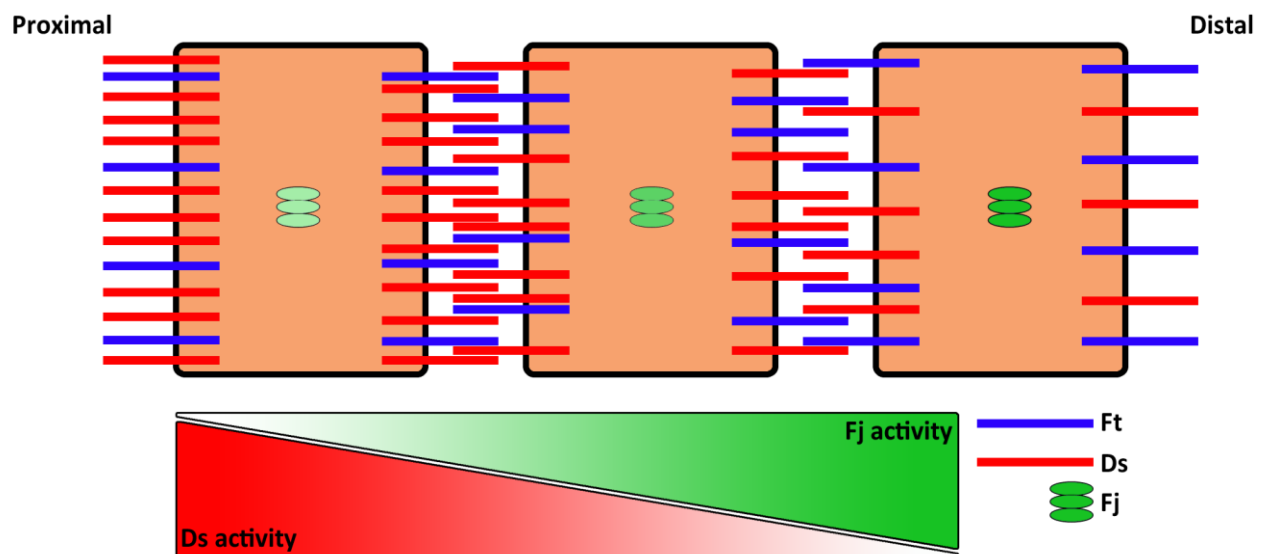


Figure 1.4. Fat-Dachsous signalling

Schematic of Ft and Ds interaction across the epithelium; Ft expression is unchanged across the tissue. Ds and Fj expression varies across the tissue, with a complementary pattern of expression. Regions of high Ds expression have low Fj activity, and vice versa. The graded activity of Ds and Fj mediates Ft activity that establishes planar polarity. Abbreviation: Fat (Ft), Dachsous (Ds), Four jointed (Fj). (Adapted from Lawrence *et al* [152])

1.4.2. The PCP pathway

PCP signalling is a highly evolutionarily conserved pathway from *Drosophila* to vertebrates. However, vertebrate PCP signalling is more complex than the *Drosophila* system owing to the presence of multiple homologues of many of its components. The function of PCP signalling is to mediate cytoskeletal reorganisation for processes such as directed cell migration. Components of the PCP signalling pathway can be grouped into two categories: the core and effector PCP proteins. The term “core PCP protein” refers to proteins that are present in all cells in PCP signalling, such as Fz and Dvl (which have been discussed previously in *Section 1.3.1*), Van Gogh-like 2 (Vangl2), and Cadherin EFG LAG seven pass G-type receptor (Celsr). PCP associated proteins are those that are involved in PCP signalling and are present some but not all cells, such as Scribble (Scrib), Prickle-like protein (Pk), Fuzzy, and Inturned (In) [148,149,162].

Initiation of the PCP signalling pathway (Figure 1.5) is mediated by binding of non-canonical Wnts to the Fz receptor, which mediates the recruitment of Dvl to the plasma membrane via interaction with its PDZ domain. Dvl in turn mediates recruitment of the Formin homology adaptor protein Dishevelled associated activator of morphogenesis (Daam). This interaction is mediated by the DAD region of Daam, and the DEP and PDZ domain of Dvl, promoting the activation of Daam [118]. Daam in turn recruits and activates RhoA, a member of the small GTPase family, via the weak-similarity Rho guanine nucleotide exchange factor (WGEF). RhoA then phosphorylates and activates Rho associated protein kinase (ROCK), which in turn is able to regulate the actin cytoskeleton. This is necessary for processes such as cell migration and contraction [123], and is of particular interest in our study.

Dvl has also been shown to recruit another small GTPase, Rac1 (Figure 1.5); however, this pathway is less well investigated. Rac1 mediates activation of c-Jun N-terminal kinase (JNK), which phosphorylates and activates its downstream target c-Jun [163]. c-Jun, in conjunction with c-Fos, forms an early response transcription factor which is able to mediate gene transcription, however detailed studies of these genes are yet to be published [123]. The JNK pathway is also able to regulate cell movement, and has been shown to mediate convergent extension (CE) movement in gastrulation in *Xenopus* [163]. *Xenopus* with JNK knockdown at the 4-cell embryo stage had a shortened anterior-posterior axis and showed incomplete CE; however, control *Xenopus* developed normally and underwent CE [163].

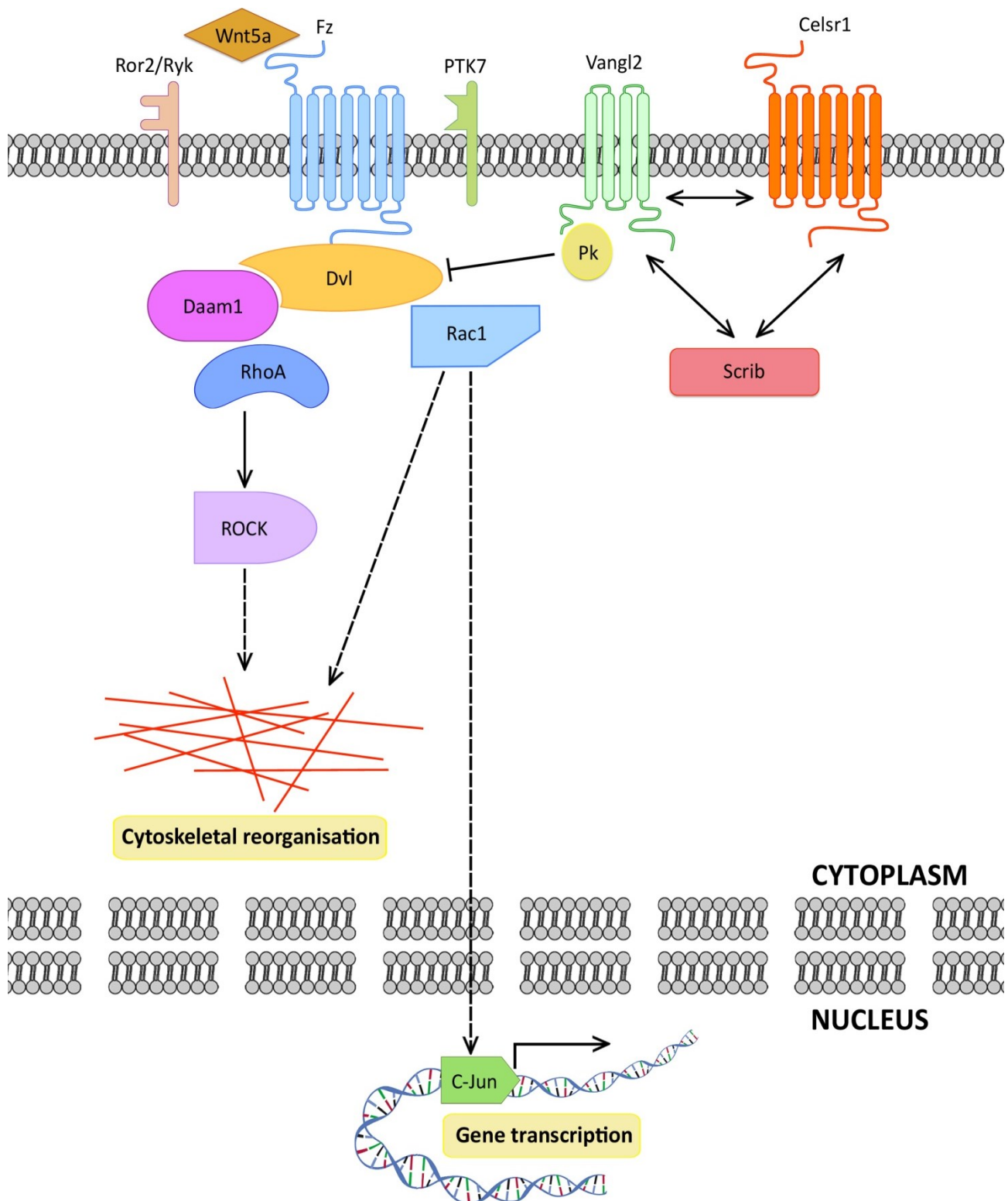


Figure 1.5. The non-canonical PCP pathway

The PCP pathway utilises some components of the canonical PCP pathway, for example, it is initiated by Wnt binding to a Fz receptor and the subsequent recruitment of Dvl to the plasma membrane. Dvl in turn recruits cytoplasmic proteins such as RhoGTPases to promote cytoskeletal reorganisation, and gene transcription. Abbreviations: Frizzled (Fz), Protein tyrosine kinase (PTK), Van Gogh-like 2 (Vangl2), Prickle (Pk), Cadherin EGF LAG seven-pass G-type receptor 1 (Celsr1), Dishevelled (Dvl), Dvl associated activator of morphogenesis (Daam), Scribble (Scrib).

The core PCP proteins, Vangl2 and Celsr1, as well as the PCP effector protein Scrib are known to have a fundamental role in PCP signalling, which is evident from many studies of PCP mutants. In *Drosophila* models, PCP defects can be assessed by disorientated hair polarity and the localisation of wing hairs which occur in the centre of the cell rather than at the distal end [164]. In *Drosophila* models with mutations in Van Gogh (*Vang*), the *Vangl* orthologue, wing hairs were disoriented and formed swirls instead of the aligned pattern seen in WTs [165]. Similarly mutations in *Drosophila Flamingo* (*Fmi*), the *Celsr* orthologue, also resulted in disorientated wing hair [166]. Mouse models of *Vangl* and *Celsr* mutations recapitulated the PCP defects seen in *Drosophila*. Mice with mutations in *Vangl2*, *Celsr1*, and the PCP effector protein *Scrib* all had disrupted polarisation of hair in the cochlea [16,167]. Together, these studies showed an essential role of Vangl2, Celsr1, and Scrib for PCP; however, it is still relatively unknown how these components interact with each other. Domain analysis of Vangl2 has shown that its PDZ binding domain (PDZBD) and the PDZ domain on Dvl are able to interact directly [168]. Furthermore, Vangl2 and Dvl are able to inhibit membrane accumulation of each other in the same compartment, thereby resulting in asymmetric localisation of the proteins. Pk which is associated with Vangl2 has also been shown to directly interact with Dvl and prevent its membrane localisation (Figure 1.5) [169].

Studies have shown that the main way in which PCP components establish polarity is by the asymmetrically localisation of core proteins to different sides of cells [170]. Most detailed studies of PCP have been performed in *Drosophila* epidermis, and relatively little is known of how polarity is established in vertebrates. In *Drosophila*, Fz and Dvl localise to the distal edge of cells in the tissue, whereas Vang and Pk localise to the proximal edge [170]. Fmi localises to both the distal and proximal edge (Figure 1.6) [170,171]. In the mouse, similar localisation of the vertebrate orthologues are found in tissues such as the airway epithelium suggesting that the pattern of asymmetrical localisation is conserved from *Drosophila* [171]. The mechanism of how core PCP proteins become asymmetrically localised remains poorly understood, although various mechanisms have been suggested. One mechanism by which polarity is thought to arise is by feedback loops where interaction at neighbouring cells recruits the PCP components. For example in *Drosophila*, Fz and Vang are able to recruit each other to the intercellular junction of a neighbouring cell [172,173] (Figure 1.6). However, within the cell there is mutual antagonism to prevent the cortical localisation of the other complex on one side of the cell (Figure 1.6) [174].

A mechanism by which the asymmetrical localisation of PCP components can arise is by targeted transport to the plasma membrane. Guo *et al* showed that a C-terminal YYXXF motif of Vangl2 is required for interaction with AP-1, a Golgi localised clathrin adaptor complex, and Arfrp1, a GTP-binding protein, and is essential for the targeted transport of Vangl2 to the proximal edge from the Trans-Golgi network [175,176]. Another independent study also showed the YXXF motif, in particular the phenylalanine (F) residue, is essential for plasma membrane targeting [176]. Interestingly the Arfrp1 and AP-1 complex is also required for membrane localisation of protein tyrosine kinase 7 (PTK7), another component of the PCP

pathway, which harbours conserved YVDL (tyrosine sorting motif) residues. However, this complex was not necessary for transport of Fz6 which is localised to the opposing side of the cell [175]. These studies show that a number of key PCP components contain membrane-targeting motifs that promote asymmetrical localisation.

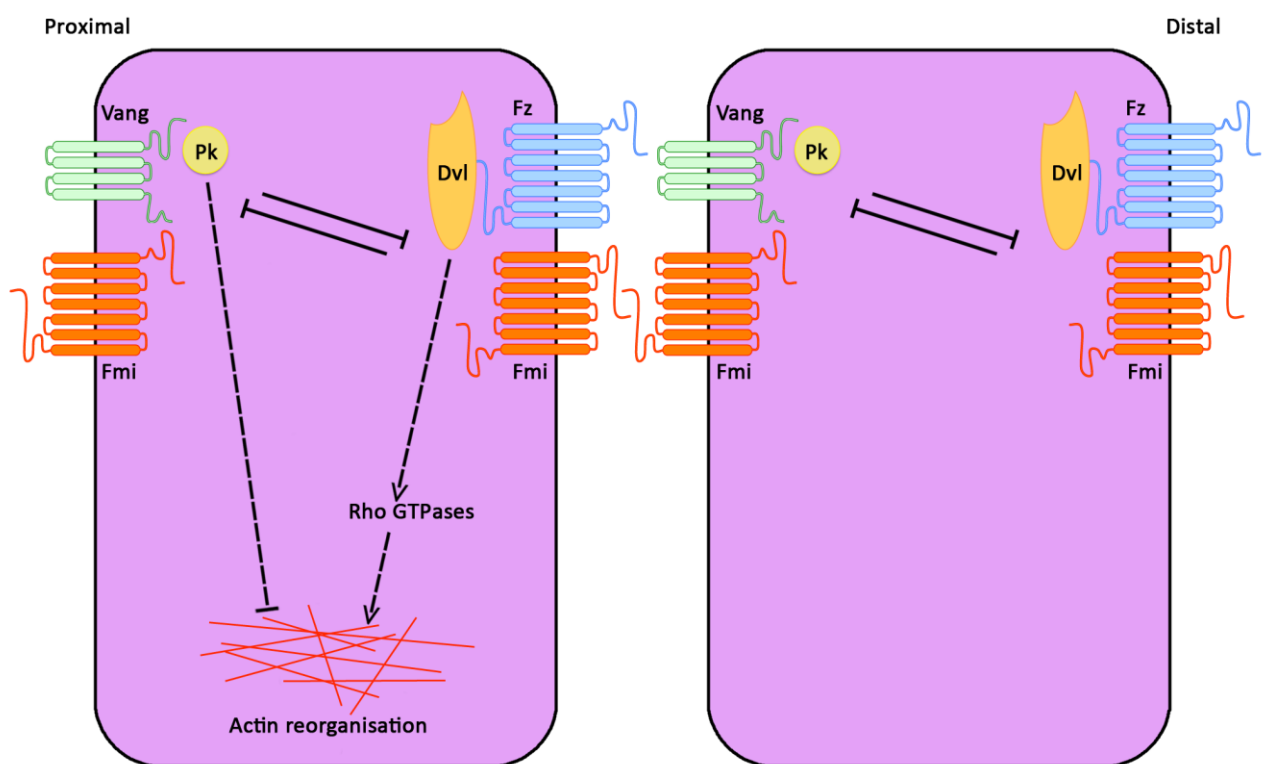


Figure 1.6. PCP components are asymmetrically localised in cells

PCP proteins have polarised localisation with the Fz, Dvl, and Fmi complex at the distal edge and the Vang, Pk, and Fmi complex at the proximal edge. Asymmetrical localisation of the PCP components can be driven by the recruitment and interaction of components across the intercellular junctions of neighbouring cells, or by mutual antagonism of proteins within the cell. (Adapted from Yang & Mlodzik [171])

1.4.3. Mammalian PCP genes

A number of important PCP genes have been associated with development and disease in mammals, such as Vangl2 (which will be discussed in detail in *Section 1.5*), Celsr, Scrib, and Daam to name a few.

1.4.3.1. Cadherin EFG LAG seven pass G-type receptor

The Cadherin EFG LAG seven pass G-type receptor (Celsr) family of proteins are atypical seven pass transmembrane cadherins. In mammals, there are three homologues (Celsr1-3), which are related to the *Drosophila* orthologue *Fmi* [177,178]. Celsr proteins contain a very large extracellular N-terminal domain with many highly conserved regions. At the N-terminus, there are nine cadherin repeats which are used for homophilic interaction between Celsr proteins on neighbouring cells to allow for intercellular signalling [179]. Following this are EGF-like domains and laminin-G domains [179,180].

In *Drosophila*, *Fmi* is thought to act upstream to regulate the localisation of other PCP proteins [181]. Similarly, mice harbouring the *Crash* (*Crsh*) mutation in *Celsr1* (*Celsr1^{Crsh}*) have disrupted Vangl2 localisation. However, *Vangl2* mutation did not result in disrupted Celsr1 localisation, providing evidence that in the mammalian PCP system Celsr may also work upstream to regulate distribution of other PCP proteins [14].

All three mammalian Celsrs are essential in brain development, showing differing patterns of localisation [180], and mutations lead to developmental defects. Analysis of expression pattern of all Celsr showed that *Celsr3* was specific to the brain, however, both *Celsr1* and *Celsr2* are expressed in the lungs and kidney in addition to the brain [180]. Yates *et al* have demonstrated using *Celsr1^{Crsh}* mice that Celsr1 plays an essential role in lung development. Mice with the *Celsr1^{Crsh}* mutation have a smaller lung size, with defective branching morphogenesis in all three modes of lung branching [14]. *Celsr^{Crsh}* mutant mice also had fewer airways in the lung which were narrower than WT mice, and in some case the airway lumina were absent [14]. At E18.5, the lungs were hypoplastic and lacked septation. The airway epithelial cells were also disorganised and randomly orientated [14]. This study showed an essential role for *Celsr1* in lung development in mice [14].

1.4.3.2. Scribble

Scribble (Scrib) is a large cytoplasmic scaffold protein which not only has roles in the PCP pathway but also acts as a tumour suppressor [182,183]. Scrib is part of the Lucine rich repeat (LRR) and PDZ (LAP) family of proteins, and contains 16 LRR and 4 PDZ domains. Like other LAP proteins Scrib is highly conserved throughout evolution [184]. In both *Drosophila* and vertebrates, only one homologue of Scrib has been identified. Scrib was initially discovered in *Drosophila*, where it was shown to aid in defining apical-basal

polarity. Apical-basal polarity occurs perpendicular to the epithelial sheet and is important in the formation of the lumen, with the basal surface of the cell in contact with the ECM proteins of the underlying basal lamina, and the apical surface on the luminal side [184]. Cell-cell junctions (discussed further in *Section 1.6.2*) play an important role in regulating diffusion of proteins and compounds across apical and basal surfaces by physically segregating the two surfaces. As such, strong Scrib localisation is found at the cell membrane and cell junctions with some cytoplasmic staining [185].

Although the proteins that are involved in establishing the apical-basal polarity are known, the exact molecular mechanisms driving the establishment of apical-basal polarity is not fully understood. Both the Partitioning defective (Par) complex and Crumbs complex are localised to the apical surface at tight junctions, whereas, the Scrib complex, which is composed of Scrib and two other tumour suppressor proteins Lethal giant larvae (Lgl) and Disc large (Dlg), is localised toward the basal end [184,186]. The majority of the work on Scrib has concentrated on its role in establishing *Drosophila* apical-basal polarity. Mammalian studies however, have shown Scrib to interact with junction complexes such as at adherens and tight junction [184]. There have been strong implications for the role of Scrib in the PCP pathway in mammals, and *Scrib* mutant mice display inner ear defects such as disorientated stereociliary bundles in mouse cochlea, which is a common phenotype in mammalian PCP defects [187]. This is further supported by evidence showing genetic interaction of Vangl2 with Scrib via its PDZBD [184]. Additionally, mice which harbour the *Circletail* (*Crc*) mutation in *Scrib* (*Scrib^{Crc}*), which disrupts its function [15], have altered localisation of both Vangl2 and Celsr1 [188].

Recent studies have also shown an important role for Scrib in mammalian lung development. Mice harbouring the *Scrib^{Crc}* mutation have smaller misshapen lungs that histologically had fewer open airway lumen in embryonic development [188]. Furthermore, *Scrib^{Crc}* mice have disrupted branching morphogenesis resulting in the formation of fewer lung buds and epithelial tubes which were narrower compared with WT littermates [188].

1.4.3.3. Dvl associated activator of morphogenesis

Dishevelled associated activator of morphogenesis (Daam) is a cytosolic protein family, which has two homologues in mammals (Daam1 and Daam2). Both Daam1 and Daam2 are highly conserved throughout evolution, and play a role in cell polarity. Daams are scaffold proteins related to the Formin Homology (FH) family of proteins of actin nucleators. Daams contain two highly conserved FH domains, sharing roughly 69 % homology [189,190], which it uses for interaction with other proteins to promote actin polymerisation. At the N-terminus, Daams are able to interact with Rho-GTPases which are essential in regulating actin polymerisation.

Daam1 has shown to be important in the non-canonical Wnt signalling pathway in vertebrate gastrulation via direct interaction with Dvl [191]. Whereas Daam2 has been shown to mediate patterning in the spinal cord in development via the canonical Wnt signalling pathway [192]. Both Daam1 and Daam2 are expressed in the lung, however, they show different subcellular localisation [193]. Daam1 is localised primarily in the cytoplasm with some plasma membrane localisation. Whereas, Daam2 shows strong nuclear localisation with some cytoplasmic staining [185].

Recent studies in humans have linked *DAAM* mutations with chronic lung disease. In a study comparing mRNA and microRNA expression in COPD patients, both *DAAM1* and *DAAM2* expression was reduced in patients with COPD [194]. Furthermore, in children harbouring a rs2395730 SNP in *DAAM2* and had been exposed to cigarette smoke had reduced lung function compared with those with the SNP but had no cigarette smoke exposure [195]. Additionally, in patients with COPD, *DAAM2* SNP also showed an association with lung function, as measured by the forced vital capacity (FVC) and forced expiratory volume (FEV), however this interaction did not show statistical significance [196].

1.5. Van Gogh-like

Van Gogh (Vang), a core PCP protein, was initially identified in *Drosophila* where its disruption was found to cause PCP defects such as misaligned wing hair orientation [165]. The mammalian orthologue of Vang, Van Gogh-like (Vangl), is also essential for PCP signalling. In mammals, there are two homologues: Vangl1 and Vangl2. Both Vangl1 and Vangl2 are highly conserved and show 67 % and 74 % homology respectively among vertebrates [168]; in this thesis we focus primarily on Vangl2. Human VANGL2 has been shown to have 89 % similarity with mouse Vangl2 in nucleotide sequence, and 99 % similarity in amino acid sequence [197]. hVANGL2 also shares 57 % identity with *Drosophila* Vang [198].

Vangl2 is a 521 amino acid protein with an intracellular N-terminal domain, four transmembrane domains, and a highly conserved intracellular C-terminal domain [197,199]. The sequence of the transmembrane domains and C-terminal tail are highly conserved throughout evolution. The C-terminal domain contains a conserved PDZBD, which interacts with PDZ domain containing proteins, and a membrane targeting motif which is essential for Vangl2 membrane localisation which is its site of action (Figure 1.7) [200].

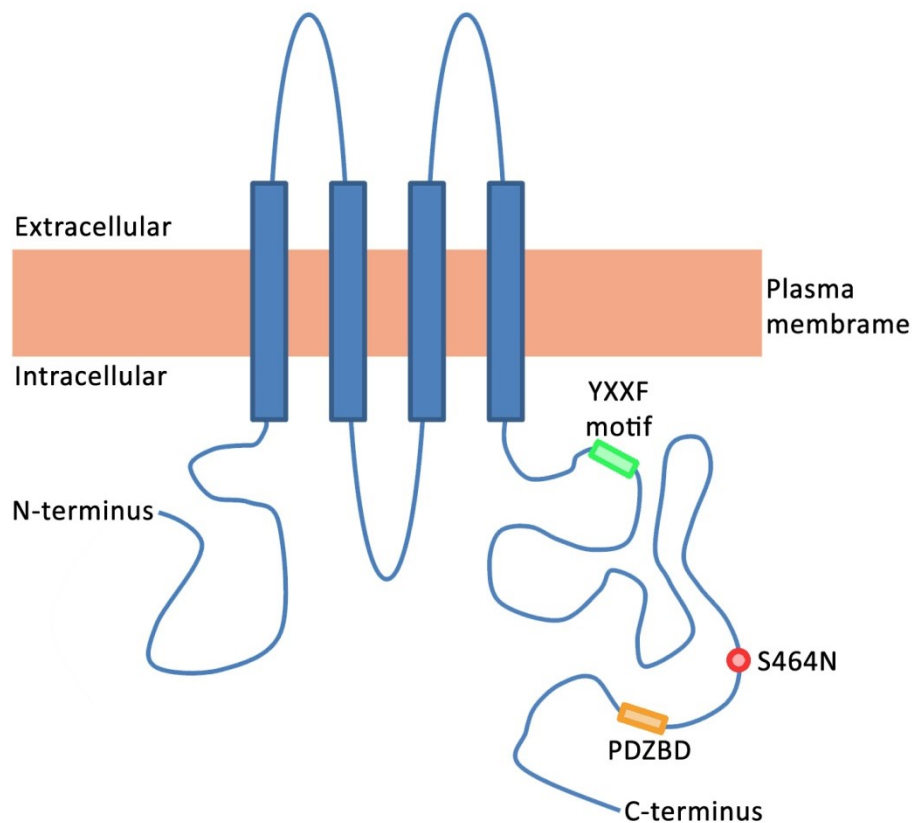


Figure 1.7. Protein structure of Vangl2 showing key domains

Vangl2 structure showing the intracellular N-terminal domain, four transmembrane domains, and the C-terminal tail. The C-terminal domain contains a conserved YXXF motif required for membrane targeting, PDZBD for protein-protein interaction, and the position of the S464N amino acid change responsible for the *Looptail* phenotype. Abbreviations: PDZ binding domain (PDZBD).

1.5.1. Mammalian *Looptail* mutation (*Vangl2^{Lp}*)

In mice, *Vangl2* was initially named *Looptail protein 1 (Lpp1)*, and was identified as the gene responsible for causing the *Looptail (Lp)* mutation [197]. The *Vangl2^{Lp}* mutation is a spontaneous mutation first reported by Strong and Hollander in 1949 [201]. Homozygous *Vangl2^{Lp}* mice die at birth from craniorachischisis. This severe neural tube defect (NTD) arises from failure of closure 1, which is essential for the closure of the whole spinal cord and up to the hindbrain [202]. However, mice carrying a heterozygous *Vangl2^{Lp}* mutation (*Vangl2^{Lp/+}*) survive but frequently exhibit some adult phenotypes including tail looping due to mild NTD, inner ear polarity defects, and head bobbing behaviour [187,201]; imperforate vagina is also found in 50 % of female mice [201].

The *Vangl2^{Lp}* mutation is the most widely studied *Vangl2* mutation. This point mutation causes a guanine to adenine nucleotide substitution at position 1841, located in exon 8 of *Vangl2* resulting in an amino acid change at position 464 from serine to asparagine (S464N) [197]. This mutation occurs in one of

the highly conserved regions of Vangl2 protein [168]. The *Vangl2^{Lp}* mutation is a dominant negative mutation, which severely disrupts PCP signalling. Primarily, the *Vangl2^{Lp}* mutation results in reduced stability of the Vangl2 protein, thereby reducing the half-life of the mutant protein compared with the WT isoform, resulting in its increased degradation [167,203,204]. Some studies have reported that the increased rate of protein degradation results in no detectable levels of Vangl2 by immunostaining in homozygous *Vangl2^{Lp}* mutant mice [14,200]. Furthermore, the *Vangl2^{Lp}* mutation disrupts Vangl2 protein localisation to the membrane, and increased its cytoplasmic retention [167,200]. Disruption of Vangl2 membrane localisation has been suggested to arise from disrupted interaction with Sec24b, a cargo sorting protein necessary for transport from the endoplasmic reticulum (ER). *In vitro* studies have shown that the *Vangl2^{Lp}* mutation perturbs Vangl2 interaction with Sec24b, thereby disrupting membrane localisation [205]. Mice with *Sec24b* mutations showed a similar defect in Vangl2 membrane localisation [206].

The dominant negative nature of the *Vangl2^{Lp}* mutation is of great advantage for studying PCP defects for several reasons. Firstly, the *Vangl2^{Lp}* mutation causes more severe phenotypes than those found in *Vangl2* knockout (KO) mice [167]. The severity of the *Vangl2^{Lp}* mutation is thought to arise from disruption of other members of the PCP pathway in addition to Vangl2. In particular, the *Vangl2^{Lp}* mutation disrupts Vangl1 localisation to the cell membrane, resulting in increased cytoplasmic retention [167]. Vangl1 and Vangl2 are highly homologous and show a similar pattern of protein localisation, indicating that Vangl1 may be able to compensate for Vangl2. Consistent with this, double homozygous *Vangl1* and *Vangl2* KO mice have a more severe phenotype than single homozygous KOs [207,208]. Therefore, in *Vangl2^{Lp}* mutants, where Vangl1 localisation is disrupted, it is unable to compensate for the loss of Vangl2 resulting in a more severe phenotype. Similarly, the *Vangl2^{Lp}* mutant protein also disrupts localisation of the WT Vangl2 protein [167]. Thus, as the *Vangl2^{Lp}* mutation is a dominant negative mutation, in *Vangl2^{Lp/+}* mice the WT protein is not as capable of compensating for the single mutant protein, thereby resulting in more severe Vangl2 disruption than in *Vangl2* KO mice. In addition to disrupting the Vangl homologues, the *Vangl2^{Lp}* mutation also disrupts localisation of key PCP proteins such as Pk2 [167] and Fz3 [200]. Moreover, the *Vangl2^{Lp}* mutation disrupts Vangl2 interaction with other PCP proteins. WT Vangl2 is able to interact directly with all three Dvls via their PDZ domains [168]. However, the mutant *Vangl2^{Lp}* isoform is unable to interact with all three Dvls, and so disrupts downstream PCP signalling [168]. As the *Vangl2^{Lp}* mutation not only affects Vangl2 function but other PCP proteins as well, it provides a powerful tool for studying PCP pathway disruption.

1.5.2. Role of Vangl2 in development

Although Vangl2 has been shown to be essential for embryonic development of the neural tube, cochlea, heart outflow tract, kidney, and lungs [14,167,197,201,209,210] and is widely expressed throughout

embryonic development, expression is not detectable in many adult tissues. For example, Vangl2 is not detected in adult brain [197]. Similarly in the kidney, Vangl2 is necessary for embryonic development and is highly expressed, but Vangl2 protein is not detected in adult kidney homeostasis, although *Vangl2* transcript is detectable [211]. Interestingly however, Vangl2 transcript increases in the adult kidney following injury [211].

1.5.2.1. Vangl2 in neural tube development

The role of Vangl2 in the neural tube has been extensively studied as NTDs were one of the most noticeable defects observed when the *Vangl2*^{Lp} mice was first characterised [201]. NTDs span a range of malformations that can arise due to incomplete closure of the neural tube. In mice there are three types of neural tube closures: closure 1, closure 2, and closure 3 [212]. Closure 1 extends from the hindbrain and cervical spine; failure of closure 1 results in the most severe NTD, craniorachischisis, where the whole spinal cord remains open, and is the defect caused by the homozygous *Vangl2*^{Lp} mutation and is lethal at birth [202,212]. Closure 2 arises from between future forebrain and midbrain, and closure 3 occurs at the most rostral end of the forebrain [212,213]; failure of both closure 2 and closure 3 results in anencephaly and is also lethal at birth [212,213]. Spina bifida is caused by failure of the caudal part of the spinal cord to close, but is not lethal and can be treated postnatally [212]. Ybot-Gonzalez *et al* demonstrated that neural tube closure occurs by convergent extension (discussed further in Section 1.6.4), however this is impaired by the *Vangl2*^{Lp} mutation [214]. Studies of other PCP gene mutants such as in *Celsr*, *Scrib*, and *Ptk7* have also shown severe NTDs arising from failure of closure 1, demonstrating an important role for PCP genes in neural tube closure [212].

1.5.2.2. Vangl2 in kidney development

The kidneys, like the lungs, are organs that contain a branched network of epithelial tubes. The PCP pathway plays a critical role in embryonic development, and core components such as Vangl2 and Celsr1 are expressed in the kidney [209]. In embryonic development, both *Vangl2*^{Lp} and kidney specific *Vangl2* KO mice have defects in kidney development [209,211]. In *Vangl2*^{Lp} mutant mice embryonic kidneys have a reduced number of ureteric buds which were shorter and wider compared with WT littermates [209]. Furthermore, at E17.5 both *Vangl2*^{Lp} and *Vangl2* KO mice had an increased proportion of immature glomeruli compared with controls [211]; immature glomeruli were classed as those with one or two capillary loop, and mature glomeruli as those with three or more loops. At E17.5 *Vangl2* KO mice had a smaller glomerular area and reduced podocyte number per glomerulus [211]; a similar phenotype was also observed in *Vangl2*^{Lp} mutant mice at this stage [211]. At E18.5, *Vangl2*^{Lp} mice have smaller kidneys with a lower length:width ratio compared with WT littermates [215]. In mice, kidney development continues for

two weeks postnatally. At the end of postnatal development at P14, *Vangl2* KO mice continued to have a reduced glomerular area and podocyte number per glomerulus compared with controls [211]. However, at P14 glomerular development had caught up in *Vangl2* KO mice, and all glomeruli in both *Vangl2* KO and control mice were classed as mature [211]. However, this difference did not reduce glomerular function in either adult *Vangl2*^{Lp/+} or *Vangl2* KO mice when assessed up to 12 months of age [209,211,215]. Interestingly, *Vangl2* KO mice have increased susceptibility to injury in adult kidney compared with WT mice, which resulted in an increase in the morphological defects and progression of chronic disease in *Vangl2* KO animals [211].

1.5.2.3. Vangl2 in lung development

Very few studies have looked at the non-canonical PCP pathway in lung development. However, recently Yates *et al* showed an important role for Vangl2 in embryonic lung development. Homozygous *Vangl2*^{Lp} mice have smaller lungs with misshapen lobes [14]. Microscopically, tissue architecture was grossly disrupted throughout development, with fewer and narrower airways, as well as disrupted organisation of epithelial cells [14]. At E18.5, during the saccular phase of lung development in mice, the interstitium thins and form sacculi which are the immature alveoli [17]. Furthermore, septation of sacculi occurs to subdivide the immature alveoli to increase the surface area [17]. However, in *Vangl2*^{Lp} mice the epithelium was disorganised and had thickened interstitium [14]. Moreover, the *Vangl2*^{Lp} lungs had reduced septation events in the sacculi, and also had reduced airway number compared with WT as a result of branching defects [14]. Although airway branching was perturbed, the mutant lungs were able to respond to branching signals such as Fgf10 [14], which is one of key drivers of branching in development [30]. In *Vangl2*^{Lp} lung epithelial cells, phospho-ERK1/2 was upregulated when stimulated by Fgf10, demonstrating that *Vangl2*^{Lp} mutant lungs are still able to induce Fgf10 signalling, however are not able to undergo branching in response to the signal [14]. Although *Vangl2*^{Lp} mice have severely altered epithelial organisation and branching defects, no defects in cell differentiation, proliferation, or apoptosis were observed [14].

As discussed previously, PCP signalling is crucial for cytoskeletal reorganisation in processes such as cell migration. In *Vangl2*^{Lp} mutant lungs, the actin cytoskeleton is disorganised with some focal enrichment; this corresponded with altered Myosin II localisation [14]. Myosin II is an actin cytoskeleton interacting protein which is phosphorylated and activated by Rho Kinase to facilitate cell migration [216]. The Rho kinase pathway is directly downstream of PCP signalling, which recruits Rho kinase to mediate cytoskeletal reorganisation (Figure 1.5). Thus, disruption of Rho signalling will alter cell migration as cytoskeletal regulation is perturbed. In line with this, Yates *et al* demonstrated that the phenotypic changes occurring in the *Vangl2*^{Lp} lung were mimicked by Rho inhibition [14]. As such, Vangl2 plays a crucial role in lung

embryonic development to regulate cytoskeletal dynamics to promote branching morphogenesis, which becomes disrupted by the *Vangl2^{Lp}* mutation. However, the role of the *Vangl2^{Lp}* mutation in the adult lung has yet to be published.

1.6. Key cellular functions of the PCP pathway

1.6.1. The cell cytoskeleton

One of the key roles of the PCP pathway is the regulation of cytoskeletal reorganisation, with disrupted PCP signalling leading to perturbed cytoskeletal organisation [14,188,209,217,218]. The cytoskeleton is a spatially organised filamentous network present in all eukaryotic cells. Cytoskeletal proteins comprise a large proportion of the cell's dry mass, and form an integral part of the cell. The cytoskeleton has a wide range of functions, including: structural support, cell migration, intracellular trafficking, chromosome segregation and cytokinesis [219]. The cytoskeleton is comprised of three main types of filaments: microtubules, intermediate filaments, and microfilaments (more commonly referred to as actin filaments) [220]. Each type of filament is comprised of repeated subunits enabling rapid assembly and disassembly during dynamic processes such as cell migration.

1.6.1.1. Microtubules

Microtubules are the largest type of filament at 25 nm in width [220,221]. They serve a wide variety of functions in the cell such as in maintenance of cell shape and are key structural units in cilia and flagella. In addition to their structural roles, microtubules provide a structure for dynein and kinesin to travel along during organelle and vesicle transport and are also essential in mitosis for the formation of mitotic spindles [220].

Microtubules are polarised, hollow cylinders that form rigid tubular structures. Microtubules are formed from repeating tubulin subunits. Each tubulin subunit is formed by a covalently linked α -tubulin and β -tubulin monomer [221]. The initiation of microtubule assembly occurs by the head to tail attachment of tubulin subunits, where the α -tubulin of one tubulin subunit interacts with the β -tubulin of another subunit. The continued polymerisation of tubulin in a head to tail orientation results in the formation of protofilaments. 13 protofilaments then join in parallel to form a hollow tube with α -tubulin subunits interacting with other α -tubulin subunits; similarly the β -subunits also interact with each other [220,221]. The high level of contact between each subunit provides strength to the unit, resulting in a rigid structure. The structured lattice prevents tubulin from depolymerising in the middle of the structure, thus depolymerisation and polymerisation can only occur at the microtubule ends [220].

Studies have shown that the PCP pathway can regulate microtubule organisation in motile cilia. For example, the airway epithelium is well organised, with cilia orientated in the proximal direction (towards the mouth) [217]. The PCP pathway is essential for the orientated direction of the ciliary basal bodies which give rise to the motile cilia, thus asymmetric distribution of PCP proteins such as Vangl1 and Fz6 is established prior to the appearance of basal bodies [217]. However, in mice with PCP defects, such as the *Vangl1* KO and *Vangl2*^{Lp} mice, the cilia organisation is disrupted and its orientation is not polarised [217]. Studies in *Drosophila* have also demonstrated that microtubules are essential for transport of PCP protein and asymmetrical localisation [218]. Shimada *et al* showed that in the *Drosophila* epithelium, Fz moved in a defined direction towards the distal cell surface [218]; however, mutation to Fz, such as the Fz^{P278L}, resulted in less consistent pathways to the cell surface and had disrupted localisation at the cell membrane [218]. The transport of Fz and Fmi to the distal cell boundary was found to occur by transport in vesicles along the proximal-distal polarised microtubules. Thus, disruption of microtubule organisation using drugs perturbed transport of Fz6 and Fmi to the cell membrane in *Drosophila* wing and increased cytoplasmic retention [218].

1.6.1.2. Intermediate filaments

Intermediate filaments are rope like filaments which are roughly 10 nm in width and are necessary for structural integrity to support the cell membrane, as well as for relaying signals to the nucleus from surface receptors [220]. There are six types of intermediate filament subunits, characterised by their sequence homology, which are encoded by 70 genes [222]. Within the cell, intermediate filament networks are found throughout the cytoplasm and in the outer nuclear membrane [223]. These two networks are joined by plectin and inner and outer nuclear membrane proteins [222]. Unlike the microtubules and actin filaments that contain only one type of protein subunit, intermediate filaments contain various types of subunits. Furthermore, intermediate filaments also lack polarity with no distinct fast and slow growing ends, thereby forming flexible fibrous structures with no polar ends [222]. To date, studies have not shown any association between intermediate filaments and PCP, potentially due to intermediate filaments being the only non-polarised component of the cytoskeleton.

1.6.1.3. Actin filaments

Actin filaments are the smallest cytoskeletal structures and are 6 nm in diameter [220], and are formed by the polymerisation of globular (G)-actin into filamentous (F)-actin. Actin monomers are small globular proteins of 42 kDa with intrinsic ATPase activity, which can undergo self-assembly [224]. Rotation of the monomers during polymerisation causes the filaments to form helical structures, similar to the DNA double helix [220]. Actin is a highly conserved protein in eukaryotic cells, and in mammals there are six isoforms

which differ in their amino acid sequence but share high homology: α -skeletal-actin, α -cardiac-actin, α -smooth-actin, and γ -smooth-actin are expressed primarily in skeletal, cardiac, and smooth muscle cells, whereas the β -cyto- and γ -cyto-actin are expressed ubiquitously [225]. The varying isoforms and plasticity enable the actin filaments to have a large range of cellular function, including migration, junction formation, cell shape, cell division, chromatin remodelling, endocytosis, and vesicle trafficking [226].

Actin polymerisation occurs in three phases: nucleation, elongation, and steady state. It is initiated by nucleation of an individual G-actin monomer in a head-tail orientation with two actin monomers to form a stable “actin nucleus” [220]. The actin nuclei, although able to assemble spontaneously, are unstable and require actin nucleators such as formins and the actin related protein (Arp) 2/3 complex to stabilise their structure [224]. Unlike intermediate filaments, actin subunits polymerise to form polarised filaments with barbed (plus) ends, and pointed (minus) ends [220]. The barbed end is the fast growing region where polymerisation primarily occurs, whereas the pointed end is the slow growing end of the actin filament [220]. During the elongation phase, polymerisation of actin monomers continues at the barbed ends, and at the steady state, actin filaments reach dynamic equilibrium where the rate of polymerisation at the barbed end is equal to the depolymerisation at the pointed end. The state of assembly and disassembly is referred to as “treadmilling”, and is an important process in directed cell migration [220].

Previous studies have shown that PCP signalling is important for regulation of the actin cytoskeletal reorganisation across organisms, from *Drosophila* to mice. In *Drosophila*, downstream PCP genes such as multiple wing hair (*mwh*) is able to cross-link actin filaments to form bundles, as well as inhibiting the rate of actin assembly [227]. Furthermore, mutation in *Drosophila* twinstar (*tsr*), an actin cleaving protein, causes PCP defects such as abnormal patterning of the wing hairs that do not point distally [228]. Moreover, localisation of both Fmi and Fz in the wing hair cells was disrupted in *tsr* mutants, and showed an uneven and discontinuous pattern at the cell boundary [228]. These defects are conserved in mice, where cofilin (*Cfl*), the *tsr* orthologue, is necessary for the polarised localisation of PCP proteins such as Celsr1, the Fmi orthologue [229]. Furthermore, Cfl activity that enables dynamic actin rearrangement is necessary for the polarised localisation of both Vangl2 and Celsr1 for initiation of planar polarity [229]. Additionally, Yates *et al* demonstrated that PCP was required for actin organisation, and in PCP mutant mouse models such as *Vangl2*^{Lp}, *Celsr*^{Crsh}, and *Scrib*^{Crc} mice, actin organisation is perturbed resulting in a disjointed and diffuse filamentous actin distribution in both embryonic lung and kidney and did not form a continuous ring like in WT mice [14,209].

1.6.1.4. Actin interacting proteins

A key role of actin adaptor proteins is to speed up the processes that could still occur without them, but at a slower rate. Actin adaptor proteins are involved in many processes such as actin filament initiation,

elongation, and stability; some of the key proteins involved in these processes are detailed in Table 1.1. Other actin adaptor proteins are involved in specific cellular processes such as the Vasodilator-stimulated phosphoprotein (Vasp) which is involved in cell adhesion and motility [230], and the Ezrin/Radixin/Moesin (ERM) complex (discussed further in *Chapter 4, Section 4.1.1*) which is involved in tethering actin to the plasma membrane and is important in cell migration [231,232].

Proteins	Function	Reference
Arp2/3	Promotion and stabilisation of actin nucleation to initiate F-actin polymerisation	[224]
Formins		
Tropomodulins	Cap actin filaments at the minus end to stabilise F-actin to prevent disassembly	[233]
CapZ capping protein	Cap actin filaments at the plus end to prevent assembly	[234]
α-actinin	Crosslink actin filaments in aligned bundles	[235,236]
Fimbrins		
Filamins	Crosslink F-actin in antiparallel to form a mesh of actin filaments	[235,236]
Cofilin	Cleave F-actin to promote actin filament disassembly	[236]
Profilin	To bind to ADP-G-actin and promote nucleotide exchange of ADP to ATP to form ATP-G-actin which has a higher affinity for polymerisation	[236]

Table 1.1. Function of actin adaptor proteins

One important class of actin interacting proteins involved in cell migration is non-muscle myosin II (NMII). NMII is the major class of the myosin family that is present in all non-muscle eukaryotic cells and is a hexamer comprised of three pairs of peptides: two heavy chains, two regulatory light chains, and two essential light chain [237,238]. NMII works together with the actin filaments, to mediate F-actin contraction and to facilitate cell migration. The action of myosin results in the sliding of actin filaments to produce the contractile forces for cell migration [239]. In cell migration two areas of the cell exhibit particularly high actin-myosin dynamics: the leading edge where rapid actin polymerisation and adhesion to the ECM occurs, and the trailing edge which has stable actin filaments and disassembly of the adhesion complex from the ECM [239].

1.6.2. Cell junctions

The cell junctions influence a number of key cell characteristics such as morphology, migration, and interaction with surrounding cells. The main types of junctions are tight junctions, adherens junctions and gap junctions. With the exception of the gap junctions, which will not be discussed further, cell-cell junctions are intricately linked to the cell cytoskeleton via adaptor proteins, and have been shown to be regulated by the PCP pathway.

1.6.2.1. Tight junctions

Tight junctions are apically located cell junction complexes that form a continuous ring around the cell. The main function of the tight junctions is to form a selectively permeable barrier to prevent unregulated diffusion of chemicals and proteins across the two surfaces [240]. In addition, tight junctions have also been shown to have roles in cell signalling, cell polarity, and vesicle trafficking [240]. There are over 40 different types of proteins which are associated with tight junctions; of these the claudin family is the main group involved in tight junction formation [241]. In mammals, there are 24 members of the claudin family [241]. These are small four pass transmembrane proteins which have two extracellular loops, and cytoplasmic N-terminal and C-terminal domains [242]. Claudins can form either homophilic or heterophilic interactions with other claudins using their extracellular loops [242]. The other families of tight junction proteins are the occludins and tricellulin. Occludins, like the claudins, are four pass transmembrane proteins with cytoplasmic N-terminal and C-terminal domains [243]. Vietor *et al* showed that disruption of occludin resulted in perturbed tight junction organisation. Furthermore, cell polarity was also lost in these cells with cultured cells becoming multilayered [244], showing an important role for tight junction proteins in mediating cell polarity.

Tight junctions are closely linked with the actin cytoskeleton via adaptor proteins. The C-terminal end of claudin contains a PDZ binding domain which is able to bind to the cytoplasmic Zonula occludens (ZO) family of proteins which contain PDZ domains at the N-terminal end [245,246]. The C-terminal end of ZO proteins in turn directly interacts with cortical actin filaments [246]. In contrast, occludin is able to bind directly to actin filaments via its C-terminal tail [241].

1.6.2.2. Adherens junctions

Adherens junctions play an important role in mediating interactions between neighbouring cells to maintain tissue architecture. In polarised cells, adherens junctions at the zonula adherens forms a continuous structure which goes around the cell forming the “adhesion belt”, outside of this region the adherens junctions are discontinuous and have a spotted appearance [247,248]. Like tight junctions,

adherens junctions are also able to transmit extracellular signals to the actin cytoskeleton [247]. The cadherin family of adhesion proteins are one of the key adherens junction components. Classical cadherins are epithelial (E)-cadherin, which is found on many types of epithelial cells, neural (N)-cadherin, and placental (P)-cadherin. These cadherins are highly conserved in their sequence and play essential roles in tissue adhesion, but have also been shown to have roles in signalling [247].

The intracellular C-terminal portion of classical cadherins contains highly conserved domains which are able to interact with the catenin family of cytoplasmic proteins [249]. Cadherins bind directly to β -catenin (also a critical component in canonical Wnt signalling as discussed in *Section 1.3.2*). The N-terminal region of α -catenin then binds to the region preceding the armadillo (arm) repeats of β -catenin [250]. Actin filaments bind to the C-terminal end of α -catenin [249]. The central α -catenin region contains the M domain, which is a highly flexible region necessary for transmitting information about conformational changes occurring between the β -catenin binding and actin binding regions [249].

Cadherin proteins are also linked strongly with cell polarity. Celsr, a core PCP protein, is a family of non-classical cadherins [251]. Similarly Ft and Ds which are involved in global planar polarity are other members of the non-classical cadherin family [155]. Furthermore, Vangl2 has been shown to interact directly with both N-cadherin and E-cadherin to regulate its surface localisation [252,253]. Thus, these findings show a crucial link between both the classical and non-classical cadherins with cell polarity.

1.6.3. Oriented cell division

In the cell, the PCP pathway is required for a number of coordinated cell behaviour such as oriented cell division (OCD) [254]. OCD relies on the polarised orientation of mitotic spindles in response to external planar polarity cues, and is important for processes such as cell fate determination and body plan specification [255]. This process is conserved throughout evolution in both invertebrates and vertebrates [255].

In *Drosophila* pupa, the sensory organ precursor cells, also referred to as pl cells, undergo OCD into pIIa cells at the posterior end that give rise to the shaft and socket cells, and pIIb cells at the anterior end that give rise to the neurons and sheath cells [255,256]. The PCP pathway is important for the polarised orientation of the mitotic spindle in OCD. In OCD of pl and pIIa cells, the mitotic spindles are parallel to the anteroposterior body axis [256]. Furthermore, the protein Numb was localised at the anterior end, and following cell division was present in the anterior daughter cell [256]. However, pIIb cells orientate the mitotic spindles orthogonal to the anteroposterior body axis, and Numb was localised at the lateral pole [256]. In *Drosophila* PCP mutants such as in *fz⁵⁴* and dishevelled (*dsh¹*), pl cell division was randomly orientated, and the polarised Numb localisation was disrupted [256]. Other studies have highlighted other

Drosophila PCP genes such as *Vang*, *Fmi*, and *Pk* to be important for the orientated polarisation of mitotic spindles to orientate cell division of pl cells. In *Drosophila*, loss of function mutations in *Vang*, *Fmi*, and *Pk* resulted in random orientation of pl cell division [257,258]. Together these demonstrate that PCP signalling is important for OCD of pl cells [256]. However, planar polarisation was not required for the orientated division of pIIa and pIIb [256].

OCD is also highly conserved in vertebrates such as zebrafish. During gastrulation in zebrafish, cells in the dorsal epiblast undergo cell division oriented along the animal–vegetal (AV) axis [259]. Disruption of Dvl activity resulted in randomised mitotic spindle alignment and orientation of cell division [259]. This was shown to be PCP specific, as removal of the Dvl PDZ domain that is necessary for PCP signalling but not β -catenin mediated Wnt signalling, resulted in similar cell orientation disruption in cell division [259]. Furthermore, disruption of PCP signalling by *Wnt11* loss of function mutation resulted in less pronounced AV polarity in cell division [259]. Furthermore, knockdown of Vangl showed misalignment of cell division in zebrafish [259]. Thus, illustrating that PCP signalling is required for OCD, and this function is conserved throughout evolution.

In addition to the PCP pathway, the Ft-Ds pathway has also been shown to be involved in OCD. The role of the Ft-Ds pathway in OCD was first studied in the *Drosophila* wings, where the majority of cells in the wing blade (about 60 %) divide along the proximal-distal axis in mitosis [255,260]. Mutations of both *Drosophila* *ft* and *ds* genes resulted in randomised orientation during cell division [260]. The role of the Ft-Ds pathway in OCD is also conserved in vertebrates, notably in mouse postnatal kidney development. In mice, the tubular segments in the corticomedullary region of the kidney proliferate in postnatal development to lengthen the tubules, but the width of the tubules does not increase [261]. During cell division, the mitotic spindles are tightly regulated and are orientated in parallel to the tubule axis, resulting in OCD causing the lengthening of the tubules [160,261]. However, in the kidney of *Fat4*^{-/-} mutant mice OCD is lost [160]. The disruption of planar polarity leading to perturbed OCD has been linked with polycystic kidney disease (PKD) [261,262].

1.6.4. Convergent extension

Convergent extension (CE), which occurs in embryonic development drives processes such gastrulation, body axis elongation (in which CE is most studied), and neurulation. Body axis elongation relies on collective cell migration and cell intercalation, to mediate thinning along the mediolateral axis and elongation along the anterior-posterior axis [263]. In collective cell migration, cells move as a collective sheet while maintaining interaction with their neighbouring cells (Figure 1.8). In cell intercalation, the mediolateral movement causes the migrating cells to pull in between neighbouring cells (Figure 1.8) [263,264]. In embryonic development of the zebrafish, PCP is crucial for mediating both collective cell

migration and cell intercalation, resulting in shortened embryonic axes if PCP signalling is perturbed [120,265]. Mice with PCP defects also show perturbed CE in neurulation which results in the failure of the neural tubes to close [266]. However, CE defects do not affect body axis length in mice. Although mice have a shorter body axis in *Vangl2*^{Lp} mutants [214,266], this defect is not thought to arise from defective CE but as a secondary defect due to the defective neurulation. Gerrelli *et al* showed that in early development, both WT and *Vangl2*^{Lp} mice have similar rates of body axis elongation. However, following neural tube closure the rate of axis length elongation in *Vangl2*^{Lp} mutant mice reduces [266]. Thus, they hypothesised that the shortened body axis occurs as a secondary effect of the defective neural tube closure, or in a *Vangl2*^{Lp} mutation independent manner.

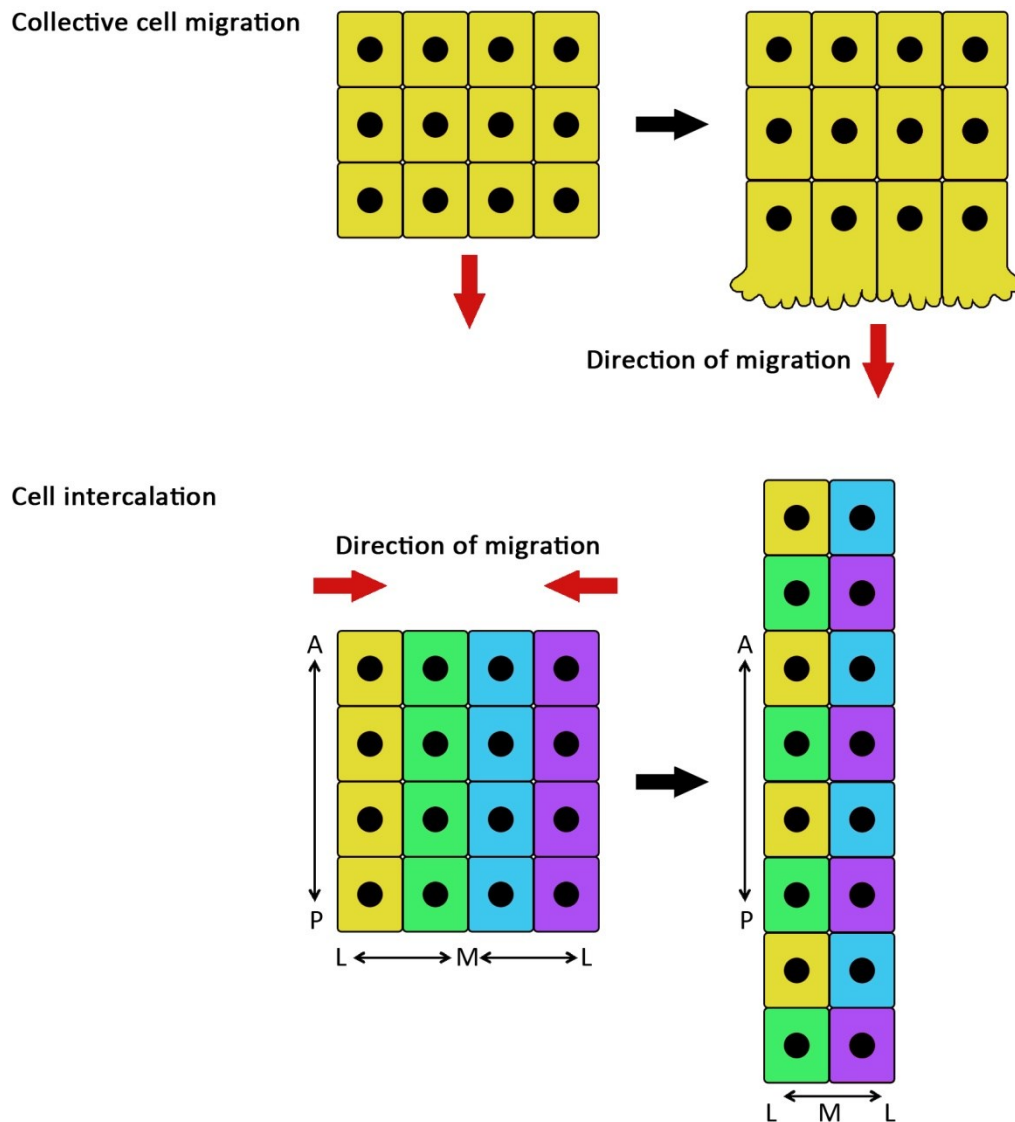


Figure 1.8. Cell migration in convergent extension

In collective cell migration, a sheet of cells moves as one unit with the cells at the leading edge protruding to form lamellipodia in the direction of migration. Contact with neighbouring cells is maintained during this process. In cell intercalation, in processes such as body axis elongation, cells migrate mediolaterally and intercalate between neighbouring cells. As a result, the body length along the A-P axis is elongated whilst narrowing along the M-L axis. Abbreviations: Anterior (A), posterior (P), medial (M), and lateral (L).

1.7. PCP mutations associated with human disease

1.7.1. PCP mutations associated with neural tube defects

In humans, NTDs are a common problem at birth, and occur at a rate of 0.5-2 per 1000 pregnancies [267,268], representing a major health risk in newborn infants. Analysis of patients and stillborn foetuses with NTDs has identified mutations in a number of PCP genes. Initial studies to look for *VANGL* mutations in patients with NTDs only identified *VANGL1* mutations [268]. However, when Lei *et al* analysed stillborn and miscarried foetuses with NTDs they identified *VANGL2* mutations [269]. Through this study they suggested that even heterozygous mutations in human *VANGL2* are likely to be embryonic lethal. In particular, they highlighted two mutations, R353C and F437S, which occurred at highly conserved sites near the PDZBD [269]. These mutations are interesting due to their proximity to the *Vangl2*^{Lp} S464N mutation in mice, and another *Vangl2* mutation, D255E, which also causes NTDs in mice. Interestingly, these *VANGL2* mutations were found to disrupt DVL association in a similar manner to the murine *Vangl2*^{Lp} mutations [269].

In another study, Iliescu *et al* identified R181Q and R274Q mutations in *VANGL1*, which are conserved residues also found in *VANGL2*, in patients with NTDs. These *VANGL1* mutations were found to cause similar defects to the murine *Vangl2*^{Lp} mutation. Both R181Q and R274Q mutations caused mislocalisation of the protein, preventing membrane localisation and cytoplasmic accumulation [270]. Additionally, both R181Q and R274Q *VANGL1* mutations lead to reduced *VANGL1* protein half-life compared with WT *VANGL1* [270]. Together, these studies illustrate that human *VANGL* mutations strongly phenocopy the murine *Vangl2*^{Lp} mutation, highlighting the usefulness of *Vangl2*^{Lp} mice to elucidate mechanisms underlying human diseases caused by *VANGL* mutations. In addition to the *VANGL* proteins, other PCP genes mutations have also been identified in patients with NTDs. A rare nonsense mutation in *CELSR1* which causes truncation of *CELSR1* was found in a cohort of patients with NTDs [271]. Recently, Yunping *et al* also reported identification of *CELSR1* mutation in another cohort of patients with NTDs (spina bifida) [272]. Functional analysis of the mutant protein found altered subcellular localisation of *CELSR1*, as well as disruption of *VANGL2* association to the plasma membrane, as seen in the murine *Celsr1*^{Crsh} model [272]. In addition to *VANGL1*, *VANGL2* and *CELSR1*, mutations in other PCP genes such as *FZ6* [273], *DVL* [274], *SCRIB* [275], and *PRICKLE1* [276] have been associated with human NTDs.

1.7.2. PCP mutations in other humans diseases

Perturbed PCP signalling and gene mutations have also been identified in many types of human cancer including breast, ovarian, uterine, and prostate cancer. In breast cancer, perturbed expression of both *VANGL1* and *VANGL2* have been identified [277]. However, *VANGL* proteins appear to both promote and suppress cancer formation in a context and cell dependent manner. For example, *VANGL2* expression has been demonstrated to inhibit Wnt/ β -catenin dependent cancers such as colon cancer, and acts as a tumour

suppressor. However, in chronic lymphoid leukaemia *VANGL2* is overexpressed and drives tumour progression [277]. *VANGL1* has also been shown to be deregulated in cancer. *VANGL1* expression is upregulated in breast cancers, and correlated with a poor prognosis in oestrogen positive breast cancer patients [277]. In addition to *VANGLs*, other PCP genes have also been shown to be implicated in cancer progression [278]. Feigin *et al* found that *SCRIB* was frequently upregulated in breast cancer but was mislocalised, and these patients often had poor clinical prognosis [278]. However, Zhan *et al*, using murine models, have shown *Scrib* to act as a tumour suppressor, whereby loss of *Scrib* resulted in promotion of breast cancer [279]. Although these studies show contradicting results, whereby loss and upregulation of *Scrib* promotes breast cancer, in both cases *Scrib* was mislocalised and so normal protein function is perturbed. Together, these studies indicate that perturbed PCP signalling has been associated with some congenital and adult human diseases. However, to date the implication of the PCP mutations in the adult lung has yet to be identified, and warrants further research.

1.8. Concluding remarks and aims

Recent mammalian studies have shown the importance of PCP signalling in the generation of a number of organs including the lungs, heart, inner ear, and kidney [14,209,211,280,281]. Together, published studies in invertebrate and vertebrate models have demonstrated that the PCP pathway can direct actin cytoskeleton reorganisation [14,188,209,217,218]. Actin cytoskeleton modification is necessary for a number of key processes such as branching morphogenesis and directed cell migration [14]. Perturbed PCP signalling disrupts embryonic development and is often incompatible with life after birth [138,202,212]. Due to the high perinatal lethality of mice with homozygous mutations in PCP genes, studies of the PCP pathway in adulthood has been hampered; however, the increasing availability of conditional alleles is beginning to enable these studies to be undertaken. For example, a conditional deletion of *Vangl2* in podocytes in the kidney was recently generated to assess whether *Vangl2* mutations alter adult kidney function and/or susceptibility to injury [211]. However, apart from this recent publication, the consequences of PCP pathway disruption in adult organs remain a key unanswered question in the field.

A recent study by Yates *et al* demonstrated an important role for PCP genes, including *Vangl2*, in embryonic lung development [14]. However, the impact of PCP disruptions in the adult lung is currently unknown. Yet research into the adult consequence of disrupted embryonic development is an important field of research in the context of lung disease, as it is known that perturbed embryonic development can increase susceptibility to adult lung disease [7,13,107–109]. Moreover, recent publications have also shown developmental pathways to regulate adult homeostasis [8]; however, aberrant signalling of these pathways can promote disease progression [9,10,108]. Elucidating the molecular mechanisms underlying lung development and disease may allow us to manipulate these signalling pathways to provide a novel

approach for treating adult lung diseases. Therefore, this thesis addressed whether the developmental defects caused by the *Vangl2*^{Lp} mutation affected adult lung homeostasis and or function, and also sought to address whether or not the *Vangl2*^{Lp} mutation increased susceptibility to injury following bacterial or viral challenge.

In summary, current publications have shown Vangl2 to play a fundamental role in embryonic lung development; however, the adult consequences were unknown. It was hypothesised that the disrupted lung architecture previously observed in *Vangl2*^{Lp} embryonic mice may persist into adulthood and increase susceptibility to lung disease. To investigate this I will:

1. Analyse the impact of the heterozygous *Vangl2*^{Lp} mutation on the adult lung structure and function.
2. Investigate the impact of *Vangl2* disruption on regulation of the actin cytoskeleton and cell junctions.
3. Examine susceptibility of adult *Vangl2*^{Lp/+} mice to tissue damage and infection.

Chapter 2: Materials and methods

2.1. Reagents and materials

All reagents were purchased from Life Technologies, unless otherwise stated.

2.2. Microscopy

Bright field and epifluorescence microscopy were performed using a Leica DM2500 upright microscope (Leica Microsystems), and images obtained using the Leica Application Suite (LAS) software (Leica Microsystems). Confocal microscopy was performed using a Leica SP5 inverted confocal microscope (Leica Microsystems) at the FILM facility (Imperial College London, South Kensington Campus), and images obtained using the LAS software. Wound healing images were taken using the Zeiss Axiovert 200 microscope (Zeiss) at the FILM facility, using the ZEN software (Zeiss).

2.3. Animals and ethics

UK Home Office guidelines, based on the Animal (Scientific Procedures) Act 1986, were observed strictly for all experiments. All murine work was performed under the project license entitled “Mechanisms of acute and chronic inflammation” (PPL number 70/7643). Surgery for lung function analysis was performed under 50 mg/ml ketamine (Kataset; Pfizer) and 5 µg/ml sodium pentobarbital anaesthesia (Sigma-Aldrich). For terminal studies mice were euthanized by either cervical dislocation or an intraperitoneal injection with a lethal dose of pentobarbital sodium (Euthatal; Merial).

The *Vangl2*^{Lp} mutation in mice is a single base pair change from G to A at position 1841. This results in an amino acid change from serine to asparagine at position 464 [197]. The *Vangl2*^{Lp} mutation was maintained on a C3H/HeH background, and mice were housed at MRC Harwell, Oxfordshire. Wild type (WT) littermates were used as controls in all experiments. For lung function studies, experimental cohorts were imported and allowed to acclimatise for at least seven days at the Imperial College Central Biomedical Services (CBS) facility (South Kensington). All mice were housed under identical conditions in a specific pathogen free environment with 12 hour light and dark cycles, controlled temperature and humidity. Mice were fed autoclaved water and irradiated chow *ad libitum*.

2.4. Human tissue samples

Paraffin wax human embryonic lung samples were provided by the Joint MRC / Wellcome Trust (grant #099175/Z/12/Z) Human Developmental Biology Resource (www.hdbr.org). Adult lung tissue was obtained from the International Institute for the Advancement of Medicine (USA).

2.5. Antibodies

Primary antibodies used for immunohistochemistry (IHC), immunofluorescence (IF), and western blots (WB) are listed in Table 2.1; and secondary antibodies used for visualisation are listed in Table 2.2. For all immunostaining IgG controls at the same concentration as the primary antibody were performed alongside the targeted antibody staining each time to assess non-specific staining and are shown in Appendix A: Figure 1 and Appendix A: Figure 2.

Target	Host	Company	Product code	Dilution
CC10	Goat	Santa Cruz	sc-9772	1:1000 (IHC)
CD68	Rat	ABD Serotec	MCA1957	1:100 (IF)
Cofilin	Rabbit	Cell signalling	5175P	1:1000 (WB) 1:400 (IF)
E-Cadherin	Rabbit	BD biosciences	610181	1:100 (IF) 1:2500 (WB)
Elastin	Rabbit	Biorbyt	orb13391	1:100 (WB)
ERM	Rabbit	Cell signalling	3142P	1:1000 (WB)
Gapdh	Rabbit	Millipore	ABS16	1:30,000 (WB)
GM130	Rabbit	Sigma	G7295	1:1500 (IF)
MMP-12	Rabbit	Abcam	Ab52897	1:1000 (WB)
P-Cofilin	Rabbit	Cell signalling	3313P	1:1000 (WB) 1:100 (IF)
Pecam1	Armenian Hamster	Abcam	Ab119341	1:500 (IF)
P-ERM	Rabbit	Cell signalling	3149P	1:1000 (WB)
PH3	Rabbit	Millipore	06-570	1:1000 (IF)
Pro-SPC	Rabbit	Millipore	AB3786	1:4000 (IHC) 1:1000 (IF)
SMA	Rabbit	Lab Vision (Thermo)	RB9010P1	1:1500 (IHC)
Vangl2	Rabbit	21st Century bio		1:1,000 (WB)
Vangl2	Rabbit	Aviva systems	OAAB15535	1:200 (IHC)
Vimentin	Rabbit	Biorbyt	orb304659	1:200 (IF)

Table 2.1. Table of primary antibodies

Abbreviations: immunohistochemistry (IHC), immunofluorescence (IF), and western blots (WB).

Target	Conjugate	Host	Company	Product code	Dilution
Anti-Armenian hamster	Cy3	Goat	Jackson	126-165-099	1:500 (IF)
Anti-Goat	AF594	Donkey	Life technologies	A11058	1:500 (IF)
Anti-Goat	AF488	Donkey	Life technologies	A11055	1:500 (IF)
Anti-Mouse	AF594	Goat	Life technologies	A11004	1:500 (IF)
Anti-Mouse	HRP	Donkey	R&D systems	HAF018	1:3000 (WB)
Anti-Rabbit	AF488	Goat	Life technologies	A11008	1:500 (IF)
Anti-Rabbit	AF594	Donkey	Life technologies	A21207	1:500 (IF)
Anti-Rabbit	HRP	Goat	Cell signalling	7074	1:2000 (WB)

Table 2.2. Table of secondary antibodies used

Abbreviations: immunofluorescence (IF), western blots (WB), Alexa Fluor (AF), horse radish peroxidase (HRP)

2.6. Tissue processing for histology and stains

Left lungs lobes were inflated with PBS, fixed in 10 % neutral buffered formalin (Sigma-Aldrich) overnight at 4 °C, and dehydrated by two changes for 1 hr each in 70 %, 80 %, 95 %, and 100 % ethanol. Tissues were then cleared by 3 x 1 hr washes in xylene, prior to embedding in paraffin wax in 2 changes of 1½ hr to allow the wax to infiltrate the tissue. Finally the embedded tissue is placed into paraffin blocks, and sectioned at 4 µm thickness. Haematoxylin and eosin (H&E), Miller's elastin staining, and Mauritius Scarlet Blue (MSB) histological staining was performed on lung sections as described below. Histological processing was performed either at the Inflammation, Repair, and Development (IRD) Section Histology service (Imperial College London) or at MRC Harwell (Oxfordshire).

2.6.1. Haematoxylin and Eosin staining

Tissue sections were dewaxed by 2 x 5 min washes in Histo-Clear (National Diagnostics), and rehydrated by 30 sec washes in 100 %, 90 %, and 70 % ethanol respectively with gentle agitation, and finally washed briefly with distilled water. Sections were stained with haematoxylin for 7 min to saturate the nuclear staining, and rinsed with running tap water. Sections were differentiated by dipping in 0.3 % acid alcohol repeatedly until only nuclear staining remained. Sections were then washed in running tap water and stained with eosin for 2 min to stain the cytoplasm. Sections were dehydrated and cleared by repeating the process of dewaxing and rehydrating in reverse and mounted using Distyrene Plasticizer Xylene (DPX; Solmedia Laboratory Supplies).

2.6.2. Miller's elastin stain

Tissue sections were dewaxed and rehydrated as described above. Sections were treated with 1 % acidified potassium permanganate for 5 minutes, prior to washing with distilled water. Sections were then bleached in 1 % oxalic acid for 1 min, washed in water, and rinsed briefly in 95 % alcohol. Sections were incubated with Miller's stain for 3 hr to stain elastin fibres, washed with 95 % alcohol to remove excess staining, and rinsed briefly in distilled water. Sections were counterstained with haematoxylin for 5 min and washed in running tap water for 5 min, and rinsed in distilled water. Sections were then stained with picro-sirius red for 5 min to stain collagen fibres; and then dehydrated, cleared, and mounted as described above.

2.6.3. Martius Scarlet Blue (MSB) stain

Tissue sections were dewaxed and rehydrated as described above. Sections were then washed with ethanol before staining the nuclei with Celestin blue for 2 min, washed briefly in water, and stained for further 2 min in haematoxylin, and differentiated using 1 % acid alcohol. Sections were washed briefly in tap water, and then rinsed in 95 % ethanol, prior to staining with Martius Yellow for 2 min, and rinsed with distilled water. Sections were then incubated in Brilliant Crystal Scarlet for 10 min and differentiated with phosphotungstic acid until only fibrin was red, and then washed in methyl blue for 2 min until collagen was stained blue. Sections were then rinsed briefly with 1 % acetic acid and dehydrated with ethanol before being cleared and mounted as described above.

2.7. DAB immunohistochemistry and quantification

2.7.1. DAB immunohistochemistry staining

Paraffin embedded sections were dewaxed and rehydrated as described in *Section 2.6.1*, with the exception of washing the sections for 2 x 5 min in phosphate buffered saline (PBS) instead of distilled water during the final wash. Endogenous peroxidase activity was quenched with 3 % hydrogen peroxide (H₂O₂; Sigma-Aldrich) in PBS for 5 min at room temperature (RT), and washed for 2 x 5 min in PBS with gentle agitation. Non-specific protein binding was blocked using 1 % bovine serum albumin (BSA; Sigma-Aldrich) and 0.2 % Triton X-100 (Sigma-Aldrich) in PBS (PBS-BT) for 45 min at RT. Sections were incubated with primary antibody diluted in blocking solution (PBS-BT) overnight at 4 °C at the concentrations shown (Table 2.1). Slides were then washed for 3 x 5 min in 0.1 % Tween20 (Sigma-Aldrich) in PBS (PBS-T) with gentle agitation. Primary antibody was detected using the Universal VECTASTAIN Elite ABC Kit (Vector Labs), for primary antibodies raised in either mouse or rabbit, or Goat VECTASTAIN Elite ABC Kit (Vector Labs) for primary antibodies raised in goat. Slides were incubated with either biotinylated rabbit anti-goat IgG (1:200) in 1.5 % normal rabbit serum in PBS or biotinylated anti-mouse/rabbit IgG (1:50) in 2 % normal horse serum in PBS for 30 min at RT. Slides were washed as previously stated. Sections were then

incubated with Elite ABC Reagent A and B (each at 1:50 dilution) in PBS for 30 min at RT. Elite ABC reagent A contains avidin for detection of the biotinylated secondary antibody, and reagent B contains biotinylated peroxidase H for visualisation. These reagents were mixed 30 min prior to use to allow complexes to form. Slides were washed as previously stated. Bound antibodies were visualised by diaminobenzidine detection (DAB; BD Bioscience) using a 1:25 dilution of DAB in the DAB buffer provided. At locations where the antibodies have bound, the peroxidase catalyses oxidation of DAB in the presence of hydrogen peroxide to form a brown precipitate. Slides are then submerged in H₂O to quench the reaction, and washed for a further 5 min with gentle agitation. Nuclei were counterstained for 20 sec in haematoxylin and then washed in running tap water for 5 min. If there was insufficient nuclear staining, haematoxylin staining was repeated. Slides were then dehydrated through 1 min washes in 70 %, 90 %, and 100 % ethanol with gentle agitation, and cleared by 2 x 10 min incubations in Histo-clear, and mounted using DPX.

2.7.2. Antigen retrieval

For Club Cell 10 (CC10), phospho-histone H3 (PH3), and Vangl2 immunostaining, antigen retrieval was performed after rehydration to reveal antibody-binding sites masked during fixation. Sections were immersed in 10 mM citrate buffer, pH 6, and heated in a microwave at full power for 3 x 3 min. Slides were then allowed to cool to RT, for 30-60 min, and washed in PBS for 2 x 5 min. Endogenous peroxidase activity blocking and the remainder of the protocol was performed as detailed above.

2.7.3. Vangl2 Immunohistochemistry

2.7.3.1. Paraffin sections

Vangl2 immunostaining was performed using the DAB immunostaining protocol described above in *Section 2.7.1*, with the following modifications. Non-specific protein binding was blocked using 1 % normal horse serum (NHS) in PBS for 20 min at RT. Endogenous avidin binding sites in the tissue and endogenous biotin receptors were blocked using an Avidin/Biotin Blocking Kit (Vector Labs). Sections were blocked with Avidin D for 15 min at RT, and washed with PBS for 5 min, then blocked with biotin for 15 min at RT, and washed with PBS for 5 min. Rabbit anti-Vangl2 was diluted in blocking solution (1 % NHS in PBS) and incubated overnight at 4 °C. Detection of the primary antibody was performed using the Universal Elite ABC Kit (Vector Labs) as detailed above.

2.7.3.2. A549 cells

A549 cells were fixed in 2 % paraformaldehyde (PFA; Sigma-Aldrich) in PBS for 15 min at RT. Cells were then washed with PBS for 4 x 5 min with gentle shaking. Endogenous peroxidase activity was blocked using 0.3 % H₂O₂ in PBS for 30 min at RT, and washed with PBS for 2 x 3 min with gentle agitation. Non-specific protein binding was blocked using 10 % foetal bovine serum (FBS) in PBS for 45 min at RT. The remainder of the procedure was performed as described for paraffin sections in Section 2.7.3.1.

2.7.4. Quantification of DAB staining

DAB intensity was quantified using the Fiji Is Just ImageJ (Fiji) open-source programme [282]. Images were captured in a TIFF format, and converted to an 8-bit image. The image threshold was adjusted to highlight areas of DAB positive staining, and the same threshold was applied for all images in the analysis. Areas of higher DAB intensity were highlighted first and so allowed for relative quantification of areas of strong DAB staining. The percentage of DAB positive area was calculated using the built-in analysis tool. Knockdown was assessed from three biological replicates, each containing three technical replicates from which two fields of view were analysed.

2.8. Immunofluorescence and phalloidin staining

2.8.1. Whole cells

A549 cells were fixed in 2 % PFA in PBS for 15 min at RT and washed with PBS for 4 x 5 min with gentle shaking. Cells were then permeabilised and non-specific protein binding blocked by incubation with PBS-BT for 1 hr at RT. Cells were incubated with primary antibody diluted in PBS-BT overnight at 4 °C at the dilutions stated in Table 2.1. Cells were washed for 4 x 5 min with PBS-T with gentle agitation prior to detection of primary antibody with the appropriate Alexa Fluor-conjugated secondary antibodies (Table 2.2) diluted in PBS-BT for 1 hr at RT in the dark. Cells were washed as previously described, and mounted using DAPI containing Prolong Gold antifade mounting media.

2.8.2. Cryosections

Cryosections of E18.5 WT and *Vangl2*^{Lp/+} whole lungs were embedded in Optimal Cutting Temperature (OCT) compound and sectioned at 10 µm at MRC Harwell (Oxfordshire). Cryosections were air dried for 1 hr at RT and washed in PBS for 2 x 5 min. Sections were then incubated in PBS-BT blocking solution for 45 min at RT, and incubated with primary antibody diluted in blocking solution overnight at 4 °C at the dilutions stated in Table 2.1. Sections were then washed for 3 x 5 min with PBS-T with gentle agitation, and

incubated with the appropriate Alexa Fluor-conjugated secondary antibodies (Table 2.2) diluted in blocking solution for 45 min in the dark. Sections were then washed and mounted as previously described.

2.8.3. Lung slices

300 µm thick lung slices were stained using a similar method to whole cell staining detailed above (*Section 2.8.1*) with the following modifications. Slices were fixed with 4 % PFA for 15 min at RT, and washed for 4 x 5 min in PBS with gentle agitation. Slices were then permeabilised in 0.5 % Triton X-100 diluted in PBS for 30 min, and washed with PBS once. Slices were then incubated with PBS-BT for 1 hr to block non-specific protein binding, and antibody staining performed as detailed above for whole cells. After washing, slices were incubated with 500 nM DAPI diluted in PBS overnight at 4 °C in the dark to stain the nuclei. Slices were transferred to slides and mounted using ProLong Gold Antifade Mountant.

2.8.4. Paraffin embedded sections

Phospho-histone H3 (PH3) staining was performed in paraffin embedded sections. Sections were dewaxed, dehydrated, and citrate buffer antigen retrieval performed as stated previously in *Section 2.7*. Once the samples had cooled they were washed with PBS for 2 x 5 min and non-specific protein binding was blocked using PBS-BT for 45 min at RT. Samples were then incubated with rabbit anti-PH3 (1:1000) diluted in blocking buffer overnight at 4 °C. Washes and detection of the primary antibody using Alex Fluor 594 conjugated donkey anti-rabbit was performed as described in *Section 2.8.2*. Sections were then mounted using ProLong Gold Antifade Mountant containing DAPI.

2.8.5. Phalloidin staining

Actin staining was performed using phalloidin toxin in cryosections to bind to filamentous actin (F-actin). Cryosections were rehydrated and blocked as described in *Section 2.8.2*. Rhodamine conjugated phalloidin toxin (Biotium #00027, 1:200) was diluted in blocking solution (PBS-BT) and incubated for 45 min at RT in the dark. Sections were then washed and mounted as described above.

2.9. Mean linear intercept

Mean linear intercept (Lm) was calculated using the Fiji software following the method detailed in Andersen *et al*, 2012 [283]. H&E stained sections were imaged at 20x and 40x magnification for adult and neonatal mice respectively. A grid of 8 horizontal lines was superimposed on each image using the Grid overlay plug-in (written by Wayne Rasband) and the number of times the alveoli intercepted the line was

counted. A minimum of three fields were imaged per lung section, and 12 sections were assessed per individual mouse. Fields of view containing blood vessels and upper airway were omitted from the analysis. L_m was calculated using the following equation:

$$Lm = NL/X$$

Where N = number of lines counted, L = length of line, and X = total number of intercepts counted.

2.10. Quantification of elastin deposition area

Sections of left lung lobes from 10 week old WT and *Vangl2^{Lp/+}* were stained with Miller's elastic staining as detailed in *Section 2.6.2*, which marks areas of elastin deposition as a dark blue/black colour. Elastin deposition at the tips of alveolar septa was quantified by drawing around the deposition area using the freehand tool in Fiji. At least 10 alveolar septa were assessed per field; three fields were assessed per lung section, and three lung sections were assessed for each individual mouse.

2.11. Cell culture

2.11.1. A549 cell line culture

The A549 cell line (kindly gifted by the Royal Brompton Hospital, Imperial College London) is an adherent line of human lung adenocarcinoma cells. A549 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % FBS, and penicillin and streptomycin, in a humidified incubator at 37 °C and 5 % CO₂. For culturing, the media was aspirated and cells washed with 10 ml PBS once. The PBS was then aspirated, 3 ml 0.05 % Trypsin-EDTA added and incubated at 37 °C until all the cells had detached. Trypsin was neutralised with 3 ml culture media and centrifuged at 190 g for 5 min to pellet the cells. The supernatant was discarded and the cells resuspended in 3 ml of culture media, of which 1 ml was transferred to a new flask containing 10 ml culture media, representing a 1:3 split for an 80-90 % confluent culture after 48 hr.

Prior to seeding, cells were counted using a haemocytometer (Immune systems) by adding 9 µl of cell suspension to the chamber. The cell suspension was then diluted to the appropriate concentration in growth media and transferred into culture plates.

2.11.2. MDCK cell line culture

The Madin-Darby Canine Kidney Epithelial (MDCK) cell line (kindly gifted by Wendy Barclay, St. Mary's Campus, Imperial College London) is an adherent epithelial cell line. MDCK cells were cultured in DMEM

supplemented with 10 % FBS, 0.1 % non-essential amino acids, and penicillin and streptomycin, in a humidified incubator at 37 °C and 5 % CO₂. For culture, the media was aspirated and 3 ml of trypsin EDTA added and incubated at 37 °C and 5 % CO₂ until all the cells had detached. Trypsin was neutralised with 9 ml of culture media, and centrifuged at 700 rpm for 5 min at 4 °C to pellet the cells. The supernatant was discarded, and cells were resuspended in 6 ml of culture media. 1 ml of cell suspension was added to a new flask with 10 ml fresh culture media, representing a 1:6 split to obtain a confluent culture after 48 hr.

2.12. Wound healing assay

Eight well chamber slides (Thermo Scientific) were coated with 300 µl of 50 µg/ml rat tail collagen type I diluted in PBS for 30 min at RT, and washed once with PBS. A594 cells were seeded at a density of 50,000 cells/well in 300 µl growth media containing antisense oligo morpholinos (MO; GeneTools) against VANGL2 (GTC CGA ACG CCG TGC CTT GTA GC), or control (CCT CTT ACC TCA GTT ACA ATT TAT A) at a final concentration of 10 µM for 72 hr. A wound was inflicted to the confluent monolayer by placing a scratch down the centre of each well using a P1000 tip. Wells were then washed with PBS to remove any dead or damaged cells, and fresh culture media containing the appropriate MO was added back onto the cells. Cells were cultured for a further 24 hr to allow cell migration and wound healing to occur. Wounds were imaged using a Zeiss Axiovert 200 (Zeiss) microscope at 0 hr and 24 hr post scratch. Wound area was calculated at each time point using the freehand tool in Fiji, and the difference in wound area between t = 0 hr and t = 24 hr was calculated to determine the area healed. The area healed was calculated from five biological replicates, each containing four technical repeats.

For Wnt5a stimulation assays, eight well chamber slides were seeded with 50,000 cells/well and cultured in growth media till confluent for 72 hr. A wound was then inflicted as described above. Cells were washed with PBS, and cultured with low serum containing (0.5 % FBS) growth media supplemented with 1 µg/ml recombinant Wnt5a (R&D Systems) or vehicle control (0.1 % BSA containing PBS). Wounds were imaged at 0 hr and 18 hr post scratch, and the area healed was calculated as described above. The area healed was calculated from three biological replicates, each containing four technical repeats.

2.13. Cell polarity quantification

Cell direction was quantified based on the Golgi direction at the leading edge 24 hr after scratch using a method adapted from Caddy *et al* [284]. The Golgi body was identified by immunofluorescent staining with anti-GM130 as described in Section 2.8.1. Images were captured along the leading edge, and a cross containing a 120 ° arc facing in the direction of migration was placed in the middle of the nucleus of all cells at the leading edge (Figure 2.1). Golgi bodies which were localised within the 120 ° arc facing the direction

of migration were classed as polarised, whereas those outside the 120° arc were classed as not polarised. The total number of correctly polarised Golgi were normalised to the total number of cells at the leading edge. For each biological replicate, three technical replicates were assessed, from which five fields of view along the leading edge were quantified for control and VANGL2 knock down.

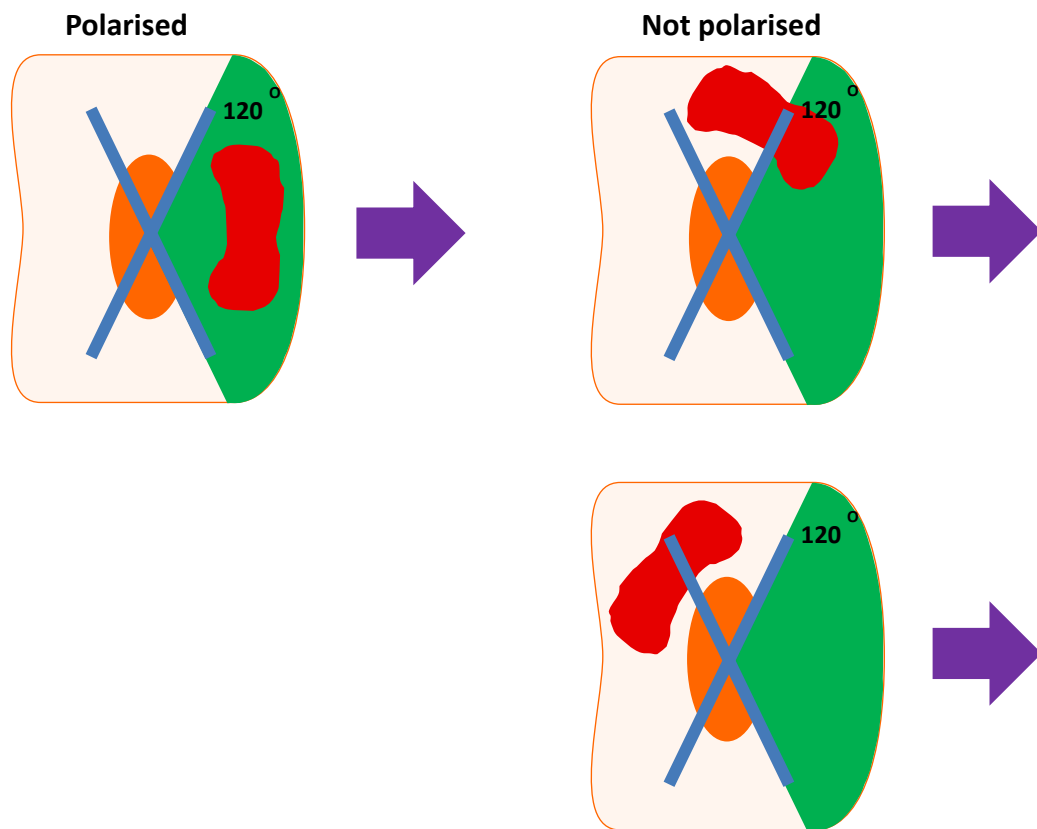


Figure 2.1. Schematic of cell polarisation quantification

Schematic illustrating the position of the 120° on the nucleus (orange) of cells at the leading edge. Purple arrow shows the direction of migration. Golgi (red) that are located within the forward facing 120° arc (green quadrant) are classed as polarised, whereas those which are outside or had majority of the Golgi body outside the area 120° arc were classed as not polarised.

2.14. iCELLigence epithelial barrier integrity measure

Epithelial barrier integrity was measured using the real-time cell analysis (RTCA) iCELLigence system (Acea Biosciences). Initially, 150 µl of growth media was added into iCELLigence 8 well E-Plates (Acea Biosciences) and left to acclimatise for 30 min at 37 °C. A single impedance reading with no cells present was measured to set the baseline value of impedance. A549s cells were seeded at a density of 50,000 cells/well in 150 µl of growth media into the wells which already contain 150 µl of growth media, bringing the total volume up to 300 µl/well. Cell impedance measures were performed hourly for 71 hr to assess cell proliferation and to determine when cells had reached confluency. Growth media was removed and the cells washed once with PBS. 300 µl of new growth media supplemented with *Alternaria alternata* extract (Greer labs) or vehicle control (PBS) was added to the cells. Impedance was measured every 10 min for 24 hr to assess epithelial barrier disruption.

2.15. Mouse alveolar type II cell isolation

Primary mouse ATII cells were isolated using a protocol modified from Mutze *et al*, 2015 [285]. To remove immune cells, 10 cm petri dishes were coated with rat anti-mouse CD45 (0.75 µg/ml; Biolegend #553076) which is expressed on all immune cells, and rat anti-mouse CD32/16 (0.75 µg/ml; Biolegend #553142) which are low affinity receptors for the mouse IgG Fc portion and are expressed on a wide range of immune cells such as B cells, monocyte, macrophages, natural killer (NK) cells, and neutrophils. Both antibodies are diluted together in 10 ml DMEM for each plate; two plates were prepared per mouse lung. To remove fibroblasts, petri dishes were coated with rat anti-mouse CD90 (0.75 µg/ml; Biolegend #553016) as described above. Coated plates were incubated overnight at 4 °C, and then washed once with DMEM.

Mice were euthanized and the thoracic cavity was exposed. Mice were then perfused using 20 ml sterile PBS through the left ventricle of the heart to remove blood from the lungs. 1.5 ml dispase (BD Biosciences) was injected into the lungs via an intratracheal cannula, followed by 0.3 ml 1 % low melting point agarose to seal the dispase inside the lungs during digestion. Once the agarose had solidified the lungs were excised and rinsed with PBS, transferred into a falcon tube containing 1.5 ml dispase, and allowed to digest for 45 min at RT. Lungs were then transferred into 10 ml DMEM supplemented with 3.6 mg/ml glucose (Applichem), 2 % GlutaMAX, 10 mM HEPES, 0.04 mg/ml DNase1 (Applichem), penicillin and streptomycin. Cells were then scraped off using forceps. The cell suspension was then passed through 100 µm and 40 µm cell strainers (Millipore) to remove tissue debris and then centrifuged at 200 g for 10 min at 15 °C. The cells were then resuspended in 20 ml of isolation media (DMEM supplemented with 3.6 mg/ml glucose, 2 % GlutaMAX, 10 mM HEPES, penicillin and streptomycin) per lung. 10 ml of the cell suspension was plated onto anti-CD45 + anti-CD16/32 coated petri dishes for 30 min at 37 °C to remove the immune cells. The cell suspension was then transferred into a new tube, and the plates washed twice

with isolation media to remove any unbound cells and pooled together. The cell suspension was then centrifuged and resuspended as before. The resuspended cells were then placed on the anti-CD90 coated plates for 1 hr at 37 °C to remove fibroblasts. Unbound cells were then collected and pooled as described above. The purified cells were then centrifuged as previously described, and resuspended in 1 ml DMEM/F12 (50:50) mix supplemented with 1x ITS, 400 mg/ml recombinant mFgf10 (R&D systems), and penicillin and streptomycin.

2.16. Preparation and culture of lung slices

Lung slices were obtained using a method adapted from Henjakovic *et al*, 2008 [286]. Mice were euthanized and the trachea exposed to insert an intratracheal cannula. The thoracic cavity was then exposed by removal of the rib cage, and the lungs were inflated using 1.2 ml of 2 % low melting point agarose (Sigma-Aldrich) dissolved in HBSS supplemented with 25 mM HEPES via the cannula, followed by 0.3 ml of air to ensure the lung parenchyma was fully inflated. Lungs were observed during the agarose inflation to ensure that all lobes were evenly inflated. A tissue dampened with ice cold HBSS was placed over the lungs and allowed to cool for the agarose to solidify. The lungs were then excised and the left lobe isolated; lung lobes were stored in HBSS on ice until ready to slice.

Lung lobes were embedded further with 2 % low melting point agarose externally to provide support during slicing. Left lung lobes were cut to obtain slices with a transverse orientation at a thickness of 300 µm using a tissue slicer (Precisionary Instruments Inc.). Slices were then cultured in DMEM supplemented with penicillin and streptomycin for 1 hr at 37 °C. Slices were then transferred into a 6-well plate containing fresh media and cultured overnight at 37 °C to remove excess agarose. Following overnight agarose removal lung slices were stained as detailed in *Section 2.8.3*.

2.17. RNA extraction and quantitative real-time PCR

2.17.1. Total RNA extraction

Total RNA was extracted from the accessory lobe of adult 10-14 week old mice or whole lung from embryonic mice by homogenising in 700 µl TRIzol reagent using a 23 gauge needle and syringe. Samples were then left for 5 min to allow the tissue to be disrupted and the cell components released whilst maintaining RNA integrity. 300 µl chloroform (Sigma-Aldrich) was then added and samples shaken vigorously by hand for 15 sec, and then centrifuged at 12,000 g for 15 min at 4 °C to separate the solution into the organic and aqueous phase. 250 µl of the top aqueous phase, which contains the total RNA, was removed and placed in a microfuge tube. Nucleic acids were precipitated by adding 250 µl of 70 % ethanol to the RNA solution.

Total RNA was isolated using the RNeasy mini kit (Qiagen). Total RNA was transferred into an RNeasy Mini Column and centrifuged at 8,000 g for 15 sec to isolate total RNA in the column, and the flow through discarded. The column was then washed with 350 µl RW1 buffer by centrifugation at 8,000 g for 15 sec to remove excess ethanol, and the flow through discarded. Carryover of genomic DNA was limited by on column digestion with 80 µl of DNase1 (Qiagen) for 15 min at RT, and washed with 350 µl RW1 buffer by centrifugation at 8,000 g for 15 sec, and the flow through discarded. The column was then washed by centrifugation with 500 µl of RPE wash for 15 sec at 8,000 g, followed by another 500 µl RPE wash for 2 min at 8,000 g, and the flow through discarded. The columns were transferred to a new collection tube and centrifuged at full speed for 1 min to remove any excess ethanol, limiting carryover of contaminants. Total RNA was then eluted with 50 µl of RNase/DNase-free water by centrifugation at 8,000 g for 15 sec. RNA concentration and purity was determined using a NanoDrop Spectrophotometer (Thermo Scientific).

2.17.2. cDNA synthesis

1 µg of total RNA was converted to cDNA using a High-Capacity cDNA reverse transcription kit. The appropriate volume containing 1 µg of total RNA was diluted in RNase/DNase free dH₂O to give a total volume of 10 µl. The total RNA sample was then mixed with 10 µl of master mix containing the components for the reverse transcription reaction. The reverse transcription master mix solution was made up as follows for each reaction: 2 µl 10x RT buffer, 0.8 µl 25x dNTPs, 2 µl 10x random primers, 1 µl reverse transcriptase enzyme, and 4.2 µl dH₂O to give a total volume of 20 µl per reaction. cDNA synthesis was performed using a PTC-200 peltier thermal cycler (MJ Research). Samples were heated to 25 °C for 10 min allowing for the random primers to bind to the RNA, and then to 37 °C for 120 min for cDNA conversion, finally the samples were heated to 85 °C for 5 min to denature the reverse transcriptase. cDNA was then diluted with 80 µl of RNase/DNase-free water, representing a 1:5 dilution.

2.17.3. Quantitative real time PCR

2.17.3.1. TaqMan detection

Quantitative real time PCR (RT-PCR) was performed on an Applied Biosystems Viia7 Real-Time PCR system. For each reaction 2.4 µl of cDNA was added to each well and mixed with 3.6 µl of master mix to give a total volume of 6 µl for each PCR reaction. The master mix solution was made up as follows for each reaction: 3 µl of TaqMan Fast Advanced Master Mix, 0.3 µl TaqMan primers (Table 2.3), and 0.3 µl dH₂O. For the PCR reaction samples were incubated at 50 °C for 2 min for Uracil N-glycosylase (UNG) incubation, 95 °C for 20 sec to activate the polymerase, followed by forty cycles of the PCR reaction consisting of 95 °C for 1 sec for denaturation, and 60 °C for 20 sec for primer annealing and extension.

Real time measurement of cDNA product following each PCR cycle was quantified by measurement of FAM fluorescence emission. TaqMan primer mix contains forward and reverse primers targeted to the gene of interest as well a TaqMan probe containing a quencher and fluorophore which binds to the region between the forward and reverse primers. The TaqMan master mix contains polymerase with exonuclease activity which is able to cleave oligonucleotides. As polymerisation occurs, the TaqMan probe is cleaved by the exonuclease activity displacing the fluorophore from the quencher allowing for the fluorescent signal to be emitted. Measurement of the fluorescent signal can therefore be related to the quantity of gene product. Relative gene expression was detected using TaqMan primers for the genes of interest and housekeeping genes. Gene expression was normalised to the average threshold cycle (CT) of the house keeping genes.

Species	Gene	Primer code
Mouse	<i>B2m</i>	Mm00437762_m1
Mouse	<i>Hprt</i>	Mm01545399_m1
Mouse	<i>Col1a1</i>	Mm00801666_g1
Mouse	<i>Col3a1</i>	Mm01254476_m1
Mouse	<i>Eln</i>	Mm00514670_m1
Mouse	<i>Fn1</i>	Mm01256744_m1
Mouse	<i>Mmp-2</i>	Mm00439498_m1
Mouse	<i>Mmp-9</i>	Mm00442991_m1
Mouse	<i>Mmp-12</i>	Mm_00500554_m1
Mouse	<i>Pecam1</i>	Mm01242584_m1
Mouse	<i>Tie2</i>	Mm00443243_m1
Mouse	<i>Vegfr1</i>	Mm00438980_m1
Mouse	<i>Nos2</i>	Mm00440502_m1
Mouse	<i>Irf5</i>	Mm00496477_m1
Mouse	<i>Fizz1</i>	Mm00445109_m1
Mouse	<i>Mrc1</i>	Mm00485148_m1

Table 2.3. Table of TaqMan primers

List of mouse TaqMan primers used for gene detection. All TaqMan primer and probe mixes were obtained from life Technologies.

2.17.3.2. Sybr green detection

Sybr green RT-PCR was performed on a LightCycler 480 Real-Time PCR system (Roche). For each reaction 2.5 µl of cDNA was added to each well and mixed with 7.5 µl of master mix to a total volume of 10 µl for each PCR reaction. The master mix solution was made up as follows for each reaction: 5 µl of LightCycler 480 SYBR Green I Master (Roche), 0.5 µl primers (200 µM forward and reverse primer mix) (Eurofins) (Table 2.4), and 2 µl dH₂O. For the PCR reaction samples were incubated at 95 °C for 5 min to activate the polymerase, followed by forty five cycles of the PCR reaction consisting of 95 °C for 5 sec for denaturation, 59 °C for 5 sec for primer annealing, and 72 °C for 10 sec for extension. Following the PCR reaction a melt curve was generated by heating the samples at 95 °C for 5 sec, followed by 65 °C for 1 min, and then continuously measuring the fluorescence signal while ramping up to 97 °C. The melt curves allow for validation that only one PCR product was amplified during the PCR reaction.

Real time measurement of cDNA product following each PCR cycle was quantified by measurement of fluorescence emission of the Sybr green dye. Although the Sybr green detection method also quantitatively assesses the PCR product at the end of each PCR cycle, the principle of the method is different from the TaqMan system. The Sybr green dye is a DNA binding dye which binds preferentially to double stranded DNA in comparison to single stranded nucleic acid. When the dye forms a complex with DNA it emits light at a wavelength of 520 nm when excited. Therefore, the fluorescence intensity increases as the double stranded PCR product increases, and so can be used to quantitatively measure the increase in PCR product. Gene expression analysis was performed by normalising the expression level of the gene of interest to the housekeeping gene.

Species	Gene	Forward primer	Reverse primer
Mouse	<i>Hprt</i>	CCTAAGATGAGCGCAAGTTGAA	CCACAGGACTAGAACACCTGCTAA
Mouse	<i>Emilin1</i>	TGCCTACGTGGTGACTCGGA	GCGAAGAAAGCTCCGGTACATGAT
Mouse	<i>Emilin2</i>	CCCTCCGGAAGTTCAGCAAT	TACCACGGTGGACTGGAGAT

Table 2.4. Table of Sybr green primers

List of forward and reverse primer sequences used for Sybr green RT-PCR. Primers were designed using the NCBI primer design tool, and validated to test the efficiency and specificity of the primer.

2.17.4. Cytoskeletal gene array

Gene expression of cytoskeletal regulators were assessed in E18.5 WT and *Vangl2*^{Lp/Lp} lungs using a Cytoskeletal Regulators RT² Profiler PCR Array (Qiagen #PAMM-088Z) following manufacturer's

instructions. For this assay, total RNA from the whole lung was extracted and quantified as described above. cDNA was synthesised using the RT² First Strand Kit (Qiagen) following manufacturer's instructions. Genomic DNA was eliminated by diluting Buffer GE (1 in 5 dilution) with 500 ng total RNA in a total volume of 10 µl for each reaction. Samples were incubated at 42 °C for 5 min in a thermocycler and stored on ice for at least 1 min. To each sample, 10 µl of reverse transcription mix containing 4 µl 5x Buffer BC3, 1 µl Control P2, 2 µl RE3 Reverse Transcriptase Mix, and 3 µl RNase-free water was added and mixed by gentle pipetting. Samples were then incubated for 15 min at 42 °C to reverse transcribe the total RNA to cDNA, and the reaction stopped by incubating at 95 °C for 5 min. 91 µl of RNase free water was then added to each sample and mixed by pipetting.

Real time PCR reactions were then performed using the RT² SYBR Green Mastermix (Qiagen). A master mix was prepared containing 650 µl 2x RT² SYBR Green Mastermix, 102 µl cDNA as prepared above, and 548 µl RNase free water and mixed by gentle pipetting. 10 µl of the master mix was added to each well of the RT² Profiler PCR Array plate, which was preloaded with primers for the detection of genes of interest. The PCR reaction was carried out in an Applied Biosystems ViiA 7 as follows: 95 °C for 10 min to activate HotStart DNA Taq Polymerase, followed by 40 cycles of 15 sec at 95 °C for denaturation, and 1 min at 60 °C for annealing and extension. SYBR green fluorescence was measured at the end of each PCR cycle for quantification of relative gene product levels.

2.18. Protein extraction and western blot

2.18.1. Protein extraction

2.18.1.1. Total protein extraction

Whole mouse lungs from E14.5 or the caudal right lobe from 10 week old lungs were lysed in cell lysis buffer (Cell Signalling) supplemented with complete EDTA-free protease inhibitors (Roche), PhosSTOP phosphatase inhibitors (Roche), 50 mM NaF (Sigma-Aldrich), and 1 µM PMSF (Sigma-Aldrich) to prevent protein degradation and dephosphorylation. Lungs were homogenised using a handheld tissue homogeniser (Ultra Group) in 300 µl lysis buffer per 50 mg tissue for adult lungs, or a pellet pestle (VWR International) in 30 µl lysis buffer per lung for embryonic lung tissue. Samples were centrifuged at 12,000 g for 10 min at 4 °C. The supernatant, containing total protein, was placed in pre-cooled microfuge tubes, and stored on ice until further use.

2.18.1.2. Protein extraction for subcellular fractionation

Membrane and cytosolic fractions were isolated from left lung lobes of 14 week old WT and *Vangl2*^{Lp/+} littermates using the Subcellular Protein Fractionation Kit for Tissue (Thermo Scientific) following

manufacturer's instructions. All extraction buffers were supplemented with Halt Protease Inhibitor Cocktail (Thermo Scientific), and 1 μ M PMSF (Sigma-Aldrich) to inhibit phosphatases. Lung lobes were homogenised with a handheld tissue homogeniser in 500 μ l cytoplasmic extract buffer per 50 mg tissue, and centrifuged at 500 g for 5 min at 4 °C. The supernatant containing the cytoplasmic extract was then collected and stored on ice till further use. The pellet was then resuspended in 325 μ l of membrane extract buffer per 50 mg tissue. Samples were vortexed for 5 sec, and incubated for 10 min at 4 °C with gentle agitation, and then centrifuged at 3,000 g for 5 min at 4 °C. The supernatant containing the membrane fraction was then removed and stored on ice until further use; the remaining pellet was then discarded.

2.18.2. Protein quantification

Protein concentration was assessed by a Bradford assay (Sigma-Aldrich). 5 μ l of sample or known BSA protein standards ranging from 1.4 mg/ml to 0 mg/ml were added to 96 microwell plates (Costar) in duplicate. 250 μ l of Bradford reagent was then added to each well. The plate was shaken for 30 sec and left at RT for 10 min to allow the colour change to occur. The Bradford assay relies on the shift in the maximum absorbance under acidic conditions from 465 nm to 595 nm of Coomassie Brilliant Blue G-250 dye upon protein binding. Thus, with increasing protein concentration there is an increase in the absorbance at 595 nm. Samples were then read using a spectrophotometer (Tecan) to measure the absorbance at 595 nm. Protein concentration of the samples was calculated by comparing the absorbance to the known BSA protein standard concentrations.

2.18.3. Western blotting

15 μ g of total protein or 5 μ g of membrane and cytosolic fraction protein were diluted in lysis buffer to a total volume of 7.5 μ l, to which 2.5 μ l of NuPage 4x LDS Sample Buffer was added, bringing the total volume to 10 μ l. Samples were heated at 95 °C for 5 min on a heat block to disrupt the protein structure. 10 μ l of each protein sample was loaded into each well of a precast 4-12 % Bis-Tris gel and resolved by gel electrophoresis at 135 V to separate proteins by molecular weight. The protein was then transferred to a nitrocellulose membrane using an iBlot Gel Transfer Device (#IB1001). To block non-specific protein binding, blots were incubated with either 5 % BSA (Sigma-Aldrich) or non-fat dried milk powder (Marvel) in Tris Buffered Saline (TBS; pH7.4; 20 mM Tris, 137 mM NaCl) containing 0.1 % Tween20 (TBS-T) for 1 hr at RT. Membranes were incubated with primary antibody overnight at 4 °C in blocking solution with gentle agitation at the appropriate concentration (Table 2.1). Blots were then washed for 4 x 15 min using TBS-T with agitation, and then incubated with the appropriate horseradish peroxidase (HRP) conjugated secondary antibody (Table 2.2) diluted in blocking solution for 1 hr at RT. Blots were washed as previously described.

Bound antibodies were visualised by incubation with 1 ml of ECL plus western blotting substrate (Thermo Scientific) following manufacturer's instructions for 5 min in the dark. During this reaction HRP catalyses the oxidation of luminol into a light emitting compound. Blots were then exposed to Amersham Hyperfilm ECL (GE Healthcare) in the dark to visualise the bound antibodies.

2.18.4. Quantification of relative protein expression

Protein bands were quantified using the Fiji software as follows. Each lane was carefully selected using the *rectangle tool* and analysed using the *Plot Lanes* tool to represent the relative density of the components within the selected rectangles as a histogram. A straight line was drawn below each peak, and the area under the curve measured using the *Wand tool*. Area under the curve for the protein of interest was normalised to loading control levels in the same lane to quantify the relative protein levels.

2.19. Sircol assay

Total protein was isolated from lung lobes as described in *Section 2.18.1*. Total collagen levels were assessed by a Sircol assay (Biocolor) following manufacturer's instructions. 100 µl of sample and collagen standards were added to 1.5 ml microcentrifuge tubes. 1 ml of Sircol Dye Reagent was added to each sample and shaken for 30 min to allow for the dye to bind to the collagen and precipitate. Tubes were then centrifuged at 12,000 rpm for 10 min to pellet the collagen, and excess unbound dye removed. The pellet was washed with 750 µl of ice-cold Acid-Salt Wash reagent to further remove any excess unbound dye, and centrifuged as before. 250 µl of Alkali reagent was added to each tube and vortexed to dissolve the bound dye. 100 µl of the collagen-bound dye from each sample was added to 96 microwell plates in duplicate, and the absorbance read at 555 nm using a spectrophotometer (Tecan). Absolute collagen concentration was calculated by comparing absorbance measurements to known reference collagen standards provided with the kit, and was then normalised to the total protein concentration within each sample (protein concentration was calculated as detailed in *Section 2.18.2*).

2.20. Lung function

Lung function was measured in LPS treated and vehicle control mice 24 hr after treatment. Animals were anaesthetised with pentobarbital sodium and ketamine. An intratracheal cannula was then inserted and fixed with a ligature of 5-0 silk. Mice were then placed on a heating pad to maintain body temperature during the experiment. Mice were then connected to a computer controlled ventilator (Flexivent, Scireq)

and ventilated based on body weight. Forced oscillation manoeuvres were then used to assess lung function parameters including elastance, tissue hysteresivity, and total theoretical lung volume.

2.21. Intranasal LPS challenge model

2.21.1. Dosing regime

10 week old male WT and *Vangl2*^{Lp/+} mice were administered a single intranasal dose of either 10 µg lipopolysaccharides (LPS) (*E. coli* K12) extract (Source BioSciences) in 50 µl PBS to induce an acute respiratory challenge [287], or 50 µl vehicle control (PBS). Lung function was measured using the Flexivent system as described in Section 2.20. Following lung function measurements, bronchoalveolar lavage fluid (BALF) and lung tissue samples were collected and processed using the methods detailed by McMillan *et al*, 2005 [288] (see Section 2.21.2 for further details).

2.21.2. BALF and tissue processing

BALF was collected by introducing 0.4 ml sterile PBS into the lungs via a syringe and then gently removing the fluid containing cells from the lungs. This was repeated three times and the BALF pooled for each mouse. BALF was then centrifuged at 190 g for 5 min. The supernatant was snap frozen and stored for further analysis, whilst the cell pellet was resuspended in 0.5 ml RPMI supplemented with 10 % FBS and penicillin and streptomycin (RPMI/FBS). The cells were counted, and 50,000 cells were loaded onto a cytospin and spun at 400 rpm for 4 min using a Cytospin3 (Shandon) and allowed to air dry. Cells were fixed in methanol for 5 min and air dried overnight.

Lung homogenate was prepared by chopping the left lung lobe with scissors and incubated with 5 ml RPMI supplemented with 10 % FBS, 100 Units/ml DNase and collagenase, penicillin, and streptomycin for 1 hr at 37 °C. Lung digest was filtered through a 70 µm cell strainer (Millipore), centrifuged at 1900 rpm for 10 min at 4 °C, and the supernatant discarded. Cell pellet was then resuspended in red blood cell lysis buffer (155 mM NH₄Cl, 9 mM KHCO₃, and 1.1 mM Na₂EDTA) for 2 min at RT, and then neutralised with 4 ml RPMI/FBS. Cells were then washed twice by centrifuging as previously described, and resuspended in 1 ml RPMI/FBS. Cells were then counted and 50,000 cells were then used for cytospin preparation as described above.

Immune cells from the BALF and lung homogenate were then stained using Wright-Giemsa stain (Sigma) by rehydrating in a modified PBS solution (3.95 mM NaH₂PO₄, 5 mM KH₂PO₄) for 30 min, and then stained in Wright-Giemsa stain for 7 min with gentle agitation. Slides were washed in modified PBS for 7 min and rinsed with running tap water to remove excess staining, left to air dry overnight, and mounted with DPX.

The immune cell populations were then assessed by counting 300 cells per sample and then calculating the percentage of macrophages and neutrophils. Immune cell populations were defined according to their characteristic morphology (Figure 2.2). Naive macrophages were defined as rounded cells with a dark purple nucleus and light purple cytoplasm. Enlarged macrophages also had a dark purple nucleus and purple cytoplasm, but were approximately two or three times larger in size and their cytoplasm appeared more granular. Neutrophils had a dark purple multi-lobed nucleus and clear cytoplasm.

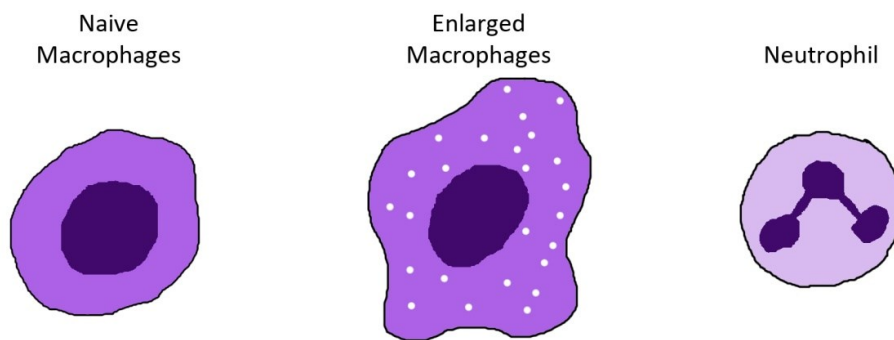


Figure 2.2. Differential immune cell staining by Wright-Giemsa

Representative images of naive macrophages, enlarged macrophages, and neutrophils when stained using Wright Giemsa.

2.22. Influenza challenge model

14 week old female WT and *Vangl2*^{lp/+} littermate mice were administered 100 plaque forming units (PFU) of influenza virus A/England/195/09 (E195) (a kind gift from Wendy Barclay) in 40 µl PBS intranasally. The E195 influenza strain is typical of the H1N1 2009 pandemic strains [289]. The virus was isolated from the nose swab of an individual in April 2009, and sequenced by the Health Protection Agency, UK (HPA), GenBank (GQ166654 to GQ166661). Weight and symptom scoring were performed daily. Symptoms were scored from 0-6 based on pilo-erection, hunching and lethargy; with 0 indicating healthy mice and 6 representing severe symptoms (Table 2.5). Mice were then analysed three and nine days post infection. BALF was processed as described above (Section 2.21.2). The left lungs lobes were snap frozen for viral load assessment, and the right lobes were processed for histology as described above (Section 2.6). Inflammation was scored from 0-5 based on lung histology using a method adapted from Matute-Bello *et al* in H&E stained slides by assessing the accumulation of immune cells around the vasculature, bronchioles, interstitium, and alveolar spaces (Table 2.6) [290].

Score	Phenotype
0	No overt symptoms
1	Pilo-erection / Slightly ruffled fur
2	Pilo-erection / ruffled fur
3	Pilo-erection / ruffled fur, hunched posture, and lethargy
4	Pilo-erection / ruffled fur, hunched posture, lethargy, period of rapid breathing
5	Pilo-erection / ruffled fur, hunched posture, lethargy, rapid shallow breathing, audible crackling
6	Absolute endpoint

Table 2.5. Symptom score for assessing mouse health

Symptom score based on observable mouse health. Mice were classed as having reached the humane endpoint if scored 4 or above on two consecutive days.

Score	Vasculature / Bronchiolar	Interstitial / Alveolar
0	None	None
1	Some nucleated cells	Few cells inside but not all alveoli
2	Single layer of nucleated cell	At least 1 cell in all alveoli
3	Two layer of nucleated cells	2 cells in each alveoli
4	3 or more layers of nucleated cells	High influx of immune cells
5	Complete obstruction	Complete obstruction

Table 2.6. Scoring criteria for histology following viral infection

2.22.1. Plaque assay

MDCK cells were cultured to confluency in T75 tissue culture flasks as detailed in *Section 2.11.2*. Following trypsinisation and centrifugation, cells were re-suspended in 36 ml culture media. 1 ml of the resuspended cell suspension was seeded into 12 well culture plates, and cultured for 24 hr to form a confluent monolayer. The culture media was aspirated from the wells, and cells washed with 1 ml PBS which was then aspirated. 100 µl of the viral suspension, prepared by homogenising one half of the left lung lobe in 250 µl of DMEM using a handheld tissue homogeniser, was placed on the cells and incubated at 37 °C for 1 hr. The viral suspension was then removed and 1 ml of flu overlay (Table 2.7) was added on top and left to solidify for 1 min. The cells were then cultured for 3 days at 37 °C. Following incubation, the flu overlay was removed and the cells were stained with 0.1 % crystal violet (Sigma-Aldrich) for 2 min and washed with distilled water. The number of visible plaques was then counted, and normalised to tissue weight.

Reagent	Concentration	Company
Minimum essential medium	1x	Life technologies
BSA Fraction V	0.21 %	Life technologies
L-glutamine	1x	Life technologies
NaHCO ₂	0.15 mM	Life technologies
HEPES	10 mM	Life technologies
Dextran	0.005 %	Sigma-Aldrich
Agarose	0.6 %	Oxoid Ltd
Trypsin	2 µg/ml	Worthington Biochemical Corporation
Penicillin and streptomycin	1x	Life technologies

Table 2.7. Flu overlay recipe

2.23. Enzyme-linked immunosorbent assay

BALF from 10 week old mice was analysed by enzyme-linked immunosorbent assay (ELISA) to measure total matrix metalloproteinase-9 (MMP-9) (R&D Systems #DY6718) and albumin (Bethyl Laboratories #E90-134) levels according to manufacturer's protocol.

2.23.1. MMP-9 ELISA

High binding affinity 96 microwell plates (Costar) were coated with 100 µl of capture antibody (2.0 µg/ml rat anti-mouse MMP-9) diluted in PBS and incubated overnight at RT. Capture antibody was aspirated from the plates and washed three times with 300 µl wash buffer (0.05 % Tween20 in PBS). Non-specific antigen binding was blocked with 300 µl block buffer per well (1 % BSA and 0.05 % NaN₃ in PBS) for 1 hr at RT, and washed as previously described. 100 µl of each sample and MMP-9 standards were incubated for 2 hr at RT, and plates were washed as before. Samples were then incubated with 100 µl of detection antibody (100 ng/ml biotinylated goat anti-mouse MMP-9) for 2 hr at RT and washed as previously described. Finally, samples were incubated with 100 µl of streptavidin-HRP for 20 min at RT in the dark and washed as before. 100 µl of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Thermo Scientific) was added to each well and left to incubate for 20 min in the dark. The reactions were stopped using 50 µl 2 N H₂SO₄. MMP-9 levels were calculated by reading the absorbance of each sample at 450 nm on a spectrophotometer and relating the absorbance to the known standard protein concentrations.

2.23.2. Albumin ELISA

High binding affinity 96 microwell plates were coated with 100 µl of capture antibody (10 µg/ml goat anti-mouse albumin) diluted in coating buffer (0.05 M carbonate bicarbonate; pH 9.6) for 1 hr at RT. Capture antibody was aspirated and the plate washed with wash buffer (0.05 % Tween20 in TBS (50 mM Tris and 140 mM NaCl; pH 8)) five times. Plates were then blocked with 200 µl of 1 % BSA in TBS for 30 min at RT, and washed as previously described. 100 µl of sample and albumin standards were diluted in sample diluent (1 % BSA and 0.05 % Tween20 in TBS) and incubated for 1 hr at RT, and the plates washed as previously described. Plates were then coated with 100 µl of detection antibody (25 ng/ml HRP conjugated goat anti-mouse albumin) diluted in sample diluent for 1 hr at RT, and washed as previously described. 100 µl of TMB substrate was added to each well and incubated for 15 min in the dark; the reaction was stopped by adding 100 µl 0.18 M H₂SO₄. Albumin levels were calculated by measuring the absorbance at 450nm using a spectrophotometer and relating the absorbance to known standard concentrations.

2.24. MMP-9 zymogram

MMP-9 activity was assessed using Novex 10 % Zymogram (Gelatin) Protein Gels according to manufacturer's protocol. 10 µl BALF and 10 µl Tris-Glycine SDS Sample Buffer (2x) were mixed; of this 10 µl was loaded into Novex Zymogram Gels, and resolved using gel electrophoresis at 125 V for 90 min. The gel was removed and washed in Zymogram Renaturing Buffer for 30 min at RT with agitation. Zymogram Renaturing buffer was removed and washed with Zymogram Developing Buffer for 30 min with gentle agitation to equilibrate the gel. This was then decanted and replaced with fresh Zymogram Developing Buffer and incubated overnight at 37 °C to allow active MMP-9 to degrade the gelatin. Gels were then stained with SimplyBlue Safestain to visualise bands and imaged using an Ettan Dige Imager (GE Healthcare). Bands were quantified using Fiji as detailed previously in *Section 2.18.4*.

2.25. Statistical tests

Statistical analysis was performed using GraphPad Prism 5 (GraphPad). Data which showed Gaussian distribution were analysed using Student's two tailed T-test or one-way ANOVA with Bonferroni correction where appropriate. Data which did not show Gaussian distribution, or where normality could not be tested, were analysed using the Mann-Whitney U test or Kruskal-Wallis with Dunn's multiple comparison test where appropriate. Figures show scatter plots of each individual repeat and the mean or median value for parametric and non-parametric data respectively. P values for each experiment is shown in the figure legend.

Chapter 3: The effect of the *Vangl2^{Lp}* mutation in postnatal lungs

3.1. Introduction

Both the canonical (β -catenin dependent) and the non-canonical (PCP) Wnt signalling pathways have been shown to be important in embryonic lung development [11,14,133,188]. Interestingly, in adult lungs, canonical β -catenin dependent Wnt signalling is not constitutively active, and only becomes activated in response to injury [291]. The role of Wnt/ β -catenin signalling in chronic lung diseases has been extensively studied, and have been shown to be perturbed in a disease specific manner [13,108,109,142]. Some studies have shown canonical Wnt signalling to be increased in lung fibroblasts isolated from patients with COPD [109,142], whereas other studies have demonstrated a reduction in Wnt signalling in epithelial cells from patients with COPD [13,107]. In comparison, relatively little focus has been paid to the non-canonical PCP signalling pathway in chronic lung disease, although some recent human studies have identified PCP genes such as *DAAM2* to be altered in COPD [189,194].

In clinical practice, lung function forms the mainstay of disease assessment. Spirometric assessment measures the flow of air during expiration against either lung volume or volume/time [24]. COPD tissue damage to lung airways and parenchyma causes air to become trapped in regions of the lungs from which it cannot be exhaled. As the disease progresses, the volume of trapped air increases, leading to a reduction in the total volume of exhaled air. Disease progression is then graded according to levels of forced expiratory volume (FEV_1), which is the volume of air exhaled in one second, in comparison to normal expected FEV_1 volumes. As the disease progresses patients show a decrease in FEV_1 . The degree of FEV_1 decline can then be graded according to GOLD emphysema staging [24]. The staging criteria and phenotype of each stage are discussed in greater detail in *Chapter 5*.

Lung function testing using FEV_1 and forced vital capacity (FVC), commonly known as spirometry, are physiological tests used to determine the ability of the lungs to inhale and exhale air. Spirometry requires the patient to perform the following manoeuvre: inspire to the lung's maximal volume, then a forced exhalation of the remaining volume [292]. This technique requires cooperation from the patient to correctly perform spirometry. As such, this measure is not suitable for very young children or animals as they are unable to perform the required manoeuvres on request.

Another method of lung function assessment includes forced oscillation techniques (FOT) that is used primarily in children [293]. FOT is a non-invasive technique which applies small pressures during normal breathing; thereby not requiring any active involvement from an individual. In FOT, an oscillatory pressure is applied, the pressure, flow, and volume signal returned by the patient is measured and fitted to various models to gain information related to lung mechanics, such as compliance, elastance, and resistance. Studies have shown that measurements by FOT are as sensitive as spirometry for measuring bronchodilation and perturbation of lung function [293]. FOT are therefore more applicable for lung function testing in young children and animals, as this test can be performed during normal breathing.

FOT measurements are interpreted from pressure-volume (P-V) loops obtained by oscillatory airwaves that are applied to the airways. This generates curves, as shown in Figure 3.1, which describe airway mechanics during inspiration and expiration. During initial inflation, alveolar spaces are collapsed, resulting in increased surface tension causing the lungs to be more difficult to inflate. Once a critical airway pressure is reached the lungs inflate more easily until it reaches total lung capacity; at which point the lung volume does not increase although pressure does [294]. During expiration, the lung mechanics occur in reverse. Lung hysteresis refers to the difference in inspiration and expiration at any given pressure; as such hysteresis is increased in emphysema. This occurs due to the increase in alveolar size which reduces the surface tension in the emphysematous lungs. Furthermore, the disorganisation of the extracellular matrix (ECM) in the emphysematous lungs results in higher energy loss, due to the increased compliance and reduced elastic recoil, causing a shift in the P-V loop [294].

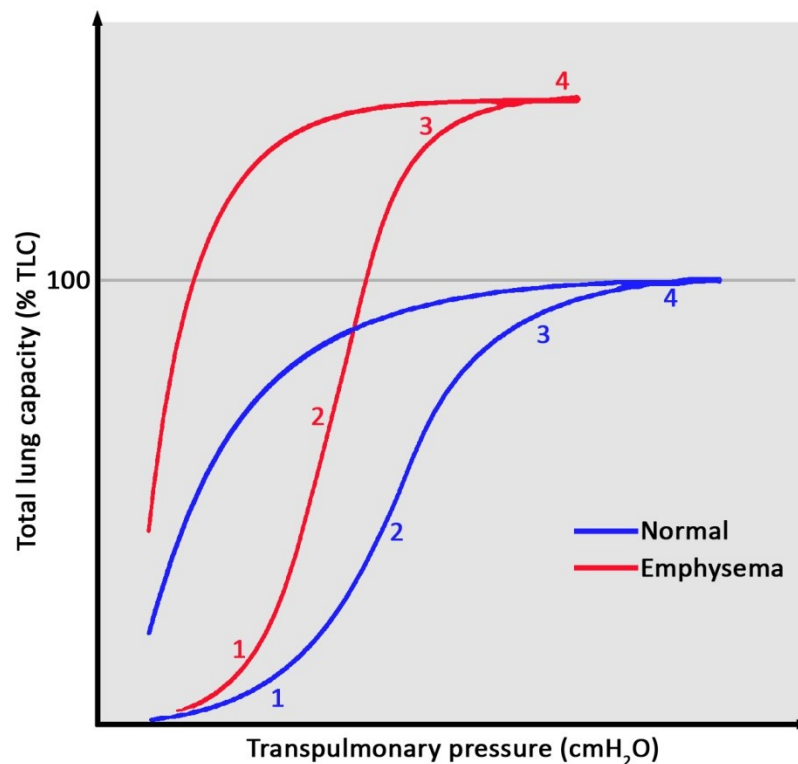


Figure 3.1. Pressure volume loops of normal and emphysema patient

Representation of characteristic pressure-volume loops in healthy and emphysema patients. In the normal lung (1) during inhalation, the lungs have a high surface tension, thus, while the pressure increases, the volume changes very little. (2) The critical airway opening pressure is reached and the lungs inflate more easily. (3) The lung is reaching the maximal volume, thus as pressure increases there is little change in volume. (4) Once the maximal volume is reached the lung volume plateaus. During the expiration this process occurs in reverse. In emphysema, the curve is shifted towards the left as the lungs are more compliant as a result of ECM remodelling. In addition, the total lung volume is also increased as elastin disruption reduces the lung recoil. (Diagram adapted from Suki *et al* [294])

A histological method of assessing disrupted alveolar architecture is by calculating the mean linear intercept (Lm). Lm is a quantitative measure of the mean alveolar airspace distance and can be used to characterise morphometric changes within the lung. The Lm can be used to assess changes to lung architecture following lung damage such as by treatment with porcine pancreatic elastase (PPE). PPE is a commonly used method of experimentally inducing emphysema, which causes a dose dependent increase in airspace size, correlating with an increase in the Lm [283]. Additionally, the Lm can also be used to assess morphological changes occurring in development. For example, *Smad3* knockout mice exhibit defective alveologenesis, resulting in an emphysema-like phenotype with increased distance between alveolar walls (increased Lm) [295]. As such, the Lm presents a sensitive measure of airspace changes occurring in the lung. However, care is required during analysis and sample preparation to avoid bias. Lm is sensitive to lung volume and so the inflation method and pressure needs to be standardised to avoid lung volume changes. In particular, regional atelectasis will adversely affect the sensitivity of the measurement, and affect the Lm. Additionally, a large number of sample areas from different regions of the lung are required to avoid bias as distal surfaces of the lungs may inflate differently to the more proximal regions.

Although adult lungs have classically been thought to have poor regenerative ability, recent studies in humans and animal models have shown that the lungs are able to undergo compensatory growth following injury such as pneumonectomy [3–6]. In addition, using murine and human *ex vivo* models Uhl *et al* demonstrated that reactivation of canonical Wnt signalling can promote repair in the adult lung [13]. These findings provide exciting new possibilities for the development of regenerative therapies for patients with chronic lung diseases.

The canonical Wnt signalling pathway has been widely studied in development, disease, and repair in the lung. However, less is known about the role of the non-canonical PCP pathway in development, and more specifically lung disease and repair. The core PCP proteins, *Celsr1* and *Vangl2* have been demonstrated to play an important role in embryonic development of the lung [14]. However, its role in adult lung disease is unknown. With the growing interest in understanding developmental pathways in greater detail as potential novel targets to promote regeneration and repair, the PCP pathway presents a novel target for promotion of lung repair. The interest in developmental pathways has been sparked by observations that a number of developmental signalling pathways become aberrantly activated in adult lung diseases such as COPD [22]. Further interest in the genetic basis for lung disease susceptibility comes from studies showing that while smoking is the major causal factor for COPD, only a small percentage (approximately 20-30 %) of smokers develop COPD [24], indicating that other factors including genetic susceptibility may play role in the onset of chronic lung disease.

In addition to signalling pathways, the innate immune system, in particular the macrophage population is recognised as essential for lung development. The lungs contain a large population of tissue-resident macrophages located close to the epithelial surface which are involved in

immunosurveillance to protect the lungs from both air-borne and blood-borne pathogens [75]. Classically, macrophages are involved in clearance of dead or dying cells, tissue surveillance, and antigen presentation leading to the recruitment of the adaptive immune system [74]. When activated, macrophages can adopt either an M1 phenotype (classical activation) which promote an inflammatory response, or an M2 phenotype (alternative activation) which is involved in remodelling and have been associated with tissue homeostasis [74]. Macrophage activation can be determined by the expression of various genes such as Nitric Oxide Synthase 2 (*Nos2*) and Interferon regulatory factor 5 (*Irf5*) for M1 macrophages or Mannose receptor 1 (*Mrc1*) and Found in inflammatory zone 1 (*Fizz1*) for M2 macrophages [73,296,297].

Interestingly, Jones *et al* demonstrated the importance of macrophages for embryonic lung development. They showed that in early lung development, M2 skewed macrophages are abundant in the lungs, particularly around regions of branching epithelium [73]. Furthermore, they reported increased macrophage numbers in alveolarisation when septation and cell migration occur [73]. The importance of macrophages in non-immunological processes is not restricted to the lungs, for example, they have also been shown to be important for remodelling of the vasculature in embryonic development [298]. Moreover, in the axolotl, an animal with an extensive regenerative capacity, macrophages have been shown to be necessary for limb regeneration. Following amputation axolotls with macrophage depletion failed to regenerate their lost limbs, and although their wounds healed, extensive fibrosis was observed [80].

Despite macrophages being vital in physiological processes such as development and regeneration, they also play key roles in disease. Macrophages can become aberrantly activated, with chronic activation of immune cells thought to contribute to pathological conditions. In COPD patients, increased macrophage numbers are found in the bronchoalveolar lavage fluid (BALF), and this is associated with poor prognosis [23,299]. Typically, these macrophages were M2 polarised [300], and contributed to disease progression by the increased expression of ECM remodelling matrix metalloproteinases (MMPs) such as MMP-2, MMP-9, and MMP-12 which can cause tissue damage [23,301–306]

MMPs are zinc dependent endopeptidases that cleave ECM components such as elastin and collagen, and are essential in many process including morphogenesis, cell migration, and adhesion. MMPs have also been shown to be involved in various diseases including COPD [13,23] and cancer [307]. MMPs can either be secreted or membrane bound, and are classified according to their substrate specificity such as collagenases, gelatinases, and elastases. Cells employ tight control of MMP activity, due to the damage that can occur if they become dysregulated. With the exception of macrophages and neutrophils, MMPs are only synthesised when required and are not stored in cells [308]. As an additional step of protection, MMPs are produced as inactive pro-MMPs which require cleavage of the pro-peptide to reveal the zinc containing catalytic site [307].

MMPs play a critical role in development; MMP-2 and MMP-14 are expressed constitutively throughout lung development from E9.5 and play a role in branching morphogenesis [309]. After the canalicular stage, MMP-2 and MMP-14 expression declines and in adult homeostasis there is very little MMP-2 and MMP-14 present [309]. MMP-9 and MMP-12 are upregulated after birth and are present in the adult lungs at homeostasis [309]. However, MMPs become dysregulated in disease and lead to pathological conditions. MMP-2, MMP-9, and MMP-12 are most commonly associated with chronic lung disease [302–306]. MMP-2 and 9 are gelatinases which typically cleave collagen, and have been shown to be increased in COPD patients [301,302,305,310]. MMP-9 has also been shown to be able to degrade elastin [311,312]. MMP-12 is an elastase and so it cleaves and remodels elastin fibres [309]. MMP-12 is also known as macrophage metalloelastase as macrophages are the predominant source of MMP-12; neutrophils are also able to secrete MMP-12 [309]. In COPD patients MMP-12 levels are increased compared with non-COPD controls [13,313].

ECM components such as collagen and elastin are essential in lung development and adult homeostasis, and play distinct functions. Collagen is the most abundant ECM protein found in the interstitium of animals, and is necessary for providing tensile strength and structural support [314,315]. Collagen is found in all major lung structures such as bronchi, blood vessels, and alveoli. In patients with chronic lung disease, the structure of collagen also becomes perturbed. However, the changes in collagen levels observed in COPD patients are very variable. Some studies have demonstrated that collagen levels are increased in COPD with thickened collagen fibres [1,316]. Whereas, other studies have shown COPD patients have reduced collagen levels [1].

Another major ECM component in the lung is elastin fibres. Elastin fibres are composed of two protein structures: elastin and microfibrils [314]. Elastin is a large, non-polar and insoluble protein complex which is synthesised by a wide range of cells including fibroblasts, smooth muscle cells, and endothelial cells [315]. Elastin enables tissues to stretch and recoil; thus it is found in tissue which repeatedly undergo stretching forces such as the lungs, heart, blood vessels, and skin [314,315]. Elastin is initially synthesised and secreted as elastin monomers (tropoelastin) into the extracellular space, where it becomes aligned with the aid of microfibrils [314,315]. Tropoelastin contains characteristic repeating unit of Ala-Ala-Ala-Lys and Ala-Ala-Lys that serve as oxidation sites for lysyl oxidase to mediate cross-linking [315]. Cross-linking of tropoelastin occurs in a head-to-tail orientation, and is then reorganised to form mature elastin fibres to suit the function of the tissue [314]. Microfibrils are a complex of many proteins which acts as scaffolds for the organisation of tropoelastin to aid in the alignment and cross-linking of elastin molecules [314]. Fibrillins are the most abundant protein family which compose the microfibrils, and contribute to the structural role of microfibrils [314,317]. Other microfibrils proteins are thought to have regulatory roles, such as microfibril-associated glycoproteins (MAGPs) which act as bridging molecules to cross-link tropoelastin and fibrillin [314].

Elastin microfibrillar interface proteins (Emilins) are a family of ECM proteins of which there are two homologues: Emilin1 and Emilin2. Emilins are found exclusively in elastin fibres and are important for mediating interaction between elastin and microfibrils, and so regulate molecular interactions to form well organised elastin fibres [318]. In embryonic mouse development, *Emilin1* is highly expressed at E8.5 in the blood vessels, and becomes highly expressed in other organs later in development such as the lungs, where it is expressed in the mesodermal cells that surround the lungs [319]. After development, *Emilin1* expression is reduced in mice, however, expression is still maintained in the adult lung [319]. *Emilin2* expression is restricted to a few organs in embryonic development, and is expressed in the neural tube, limb buds, and heart [320][321]; however, *Emilin2* expression is low in lung development [320]. In the adult lung, both Emilin1 and Emilin2 are highly expressed [320], this may be to maintain of well organised elastin structure which is essential for adult lung function.

In lung development, elastin is essential for mediating alveoli septation in postnatal alveologenesis as the mature lungs are beginning to develop [315]; disruption of the elastin network has been shown to impair alveologenesis [322]. Thus, prior to birth an extensive network of elastin fibres is pre-established in the lung [322]. Tropoelastin monomers are expressed as early as the pseudoglandular stage in smooth muscle, but do not yet form elastin fibres [323]. As development continues, tropoelastin levels continue to increase, and at the canalicular stage elastin fibres start to be formed, appearing as rings around the terminal bronchioles [323]. In the saccular phase, mesenchymal cells in the immature alveoli express high levels of tropoelastin, and elastin fibres develop further and mature. Well developed elastin fibres are also apparent at regions of branching morphogenesis [323]. As alveologenesis occurs, new elastin fibres are deposited and are thought to act as a guide along which septation occurs; pre-existing fibres are remodelled and strengthened. After development, elastin structure is stable with very little or no turn over [314]. When elastin fibres become damaged in adults, it is not properly repaired and does not show normal function [314].

In chronic lung diseases such as COPD, elastin structure in the lungs becomes altered, with up to 60 % of lung elastin sheets becoming fragmented [324]. Consequently, the elastin sheets become disorganised and enlarged, with a web like appearance [316]. Moreover, elastin fibre damage by elastases such as MMP-12 results in the formation of elastin fragments which can act as a chemoattractant in BALF to promote recruitment of monocytes, leading to a proinflammatory microenvironment which can drive the progression of COPD [306]. Houghton *et al* showed that antagonism of elastin fragments during the induction of experimental emphysema, by elastase treatment, reduced the induction of emphysema [306].

3.1.1. Aims and hypothesis

The PCP pathway is important in embryonic lung development, and perturbation of Wnt signalling by *Vangl2^{lp}* mutations have been shown to disrupt normal lung development [14]. Previous work in the Dean lab has shown that heterozygous *Vangl2^{lp}* mice have disrupted embryonic lung development, although the disruption is milder than that seen in homozygous *Vangl2^{lp}* mice [Yates L, Unpublished data]. However, the impact of the *Vangl2^{lp}* mutation on adult lung structure and function is unknown.

This chapter examines the lungs of heterozygous *Vangl2^{lp}* mutant mice to determine how disruption in lung development, caused by the *Vangl2^{lp}* mutation, impacts on the adult lungs. For this study, heterozygote *Vangl2^{lp}* mutant mice were used, as homozygote mutant mice die neonatally due to severe NTDs [201]. We hypothesised that a single allele *Vangl2^{lp}* mutation will cause altered lung structure and impaired lung function in adult mice. To test this hypothesis we:

- Determined if a single allele *Vangl2^{lp}* mutation is sufficient to alter adult lung structure.
- Performed lung function analysis to assess if *Vangl2^{lp/+}* mice have lung function perturbations.
- Determined the mechanisms by which the lung structural defects occur.
- Characterised the immunological phenotype in lungs of *Vangl2^{lp/+}* mice.

3.2. Results

3.2.1. Vangl2 is present in both embryonic and adult lungs

Previous studies have shown Vangl2 protein in both epithelial and mesenchymal cells of mouse embryonic lungs, with the highest levels in epithelial cells [14]. In wild type (WT) mice, Vangl2 localises to the cell membranes and is also present in puncta throughout the cytoplasm. Furthermore, Vangl2 is enriched at the apical (luminal) surfaces in the lung airways [14]. In homozygous *Vangl2 Looptail* (*Vangl2^{Lp}*) lungs, Vangl2 localisation is disrupted and shows reduced localisation at the cell membrane [14]. Furthermore, the *Vangl2^{Lp}* mutation also leads to increased protein instability and degradation [167,176]. Therefore, we studied Vangl2 localisation in heterozygous *Vangl2 Lp* (*Vangl2^{Lp/+}*) mice by immunohistochemistry staining in paraffin wax sections from E14.5, E17.5, and 10 week old mouse lungs.

In both WT and *Vangl2^{Lp/+}* embryonic lungs, Vangl2 was detected in lung epithelial and mesenchymal cells. In embryonic development at E14.5 and E17.5, at the pseudoglandular and saccular stage respectively, Vangl2 was strongly localised to the plasma membrane of epithelial cells of WT lungs, with weaker cytoplasmic staining (Figure 3.2A,C). In particular, Vangl2 was enriched at the apical (luminal) surface of airway epithelial cells in WT lungs (Figure 3.2A,C inserts). However, in *Vangl2^{Lp/+}* mutant lungs at both stages of embryonic development, Vangl2 was disrupted and showed reduced membrane localisation and loss of apical enrichment (Figure 3.2B,D). Although the intensity of staining was not quantified, it is notable that *Vangl2^{Lp/+}* lungs consistently showed reduced weaker Vangl2 immunostaining compared with WT littermates. No primary, rabbit IgG antibody at the same concentration as the primary antibody, controls in embryonic lungs are shown. no staining as expected (Appendix A: Figure 1).

As Vangl2 localisation was disrupted in embryonic *Vangl2^{Lp/+}* lungs, 10 week old lung sections were immunostained to investigate Vangl2 in the adult lungs. In the adult WT mouse lung, Vangl2 was localised to the alveolar epithelial cells and was enriched at the cell membrane, with weak staining in the cytoplasm (Figure 3.2E). In adult *Vangl2^{Lp/+}* lungs, Vangl2 localisation was maintained at the cell membrane and did not show any noticeable differences compared with WT littermates (Figure 3.2F).

We then quantified total Vangl2 protein levels in adult mice by western blotting, which did not show a statistically significant difference in Vangl2 levels between adult WT and *Vangl2^{Lp/+}* lungs (Figure 3.2G,H). As total Vangl2 proteins levels did not show a significant difference in the adult *Vangl2* mice, and the *Vangl2^{Lp}* mutation is known to disrupt membrane targeting of Vangl2, subcellular fractionation was performed to isolate the membrane and cytoplasmic fractions and compare the level of Vangl2 specifically in these compartments. As seen with the total Vangl2 proteins levels, there was no significant difference between Vangl2 levels in the cytoplasm of *Vangl2^{Lp/+}* lungs (Figure 3.2I,J). Western blotting did not detect a band of the correct size for Vangl2 using total adult lung membrane lysate; further optimisation of this

technique will be required to assess whether there are any differences in Vangl2 protein levels at the cell membrane in adult lungs.

Together, these results show that Vangl2 is enriched at the cell membrane in both embryonic and adult lungs. However, a single copy *Vangl2*^{Lp} mutation is sufficient to cause mislocalisation of Vangl2. The mislocalisation of Vangl2 in *Vangl2*^{Lp/+} lungs is notably more apparent in embryonic lungs compared with adult lungs. Thus, as a heterozygous *Vangl2*^{Lp} mutation results in disrupted Vangl2 localisation in embryonic development, we concluded that *Vangl2*^{Lp/+} mice would be suitable to investigate how *Vangl2* disruption impacts on adult lung homeostasis.

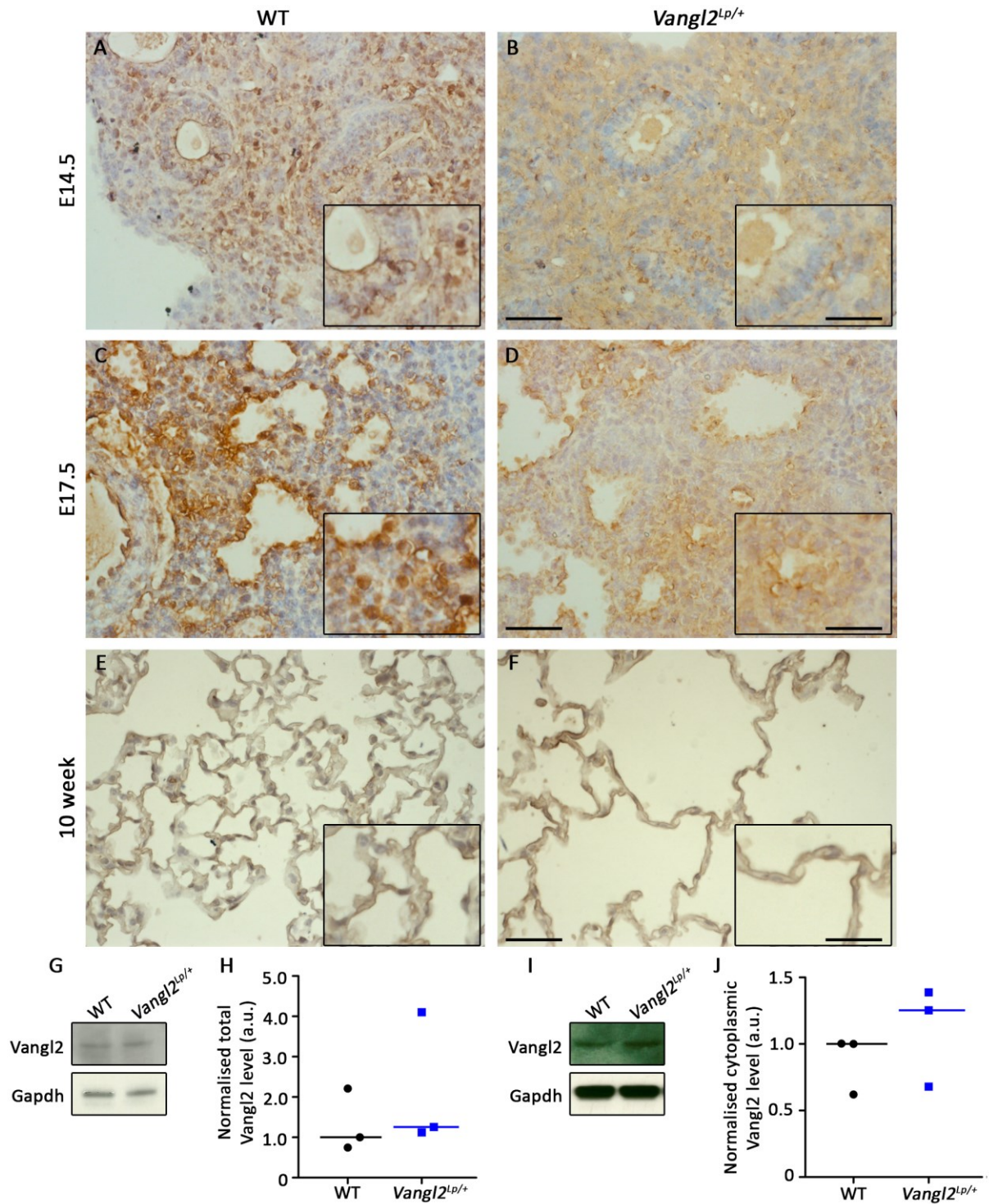


Figure 3.2. Vangl2 localisation is disrupted by a heterozygous *Vangl2*^{Lp} mutation in embryonic mice

Vangl2 localisation was assessed by immunohistochemistry in (A,B) E14.5, (C,D) E17.5, and (E,F) 10 week old mice. In WT (A) E14.5 and (C) E17.5 lungs, Vangl2 localised to cell membranes and was enriched at the apical (luminal) surface of airways. In (B) E14.5 and (D) E17.5 *Vangl2*^{Lp/+} lungs, enrichment of Vangl2 at the apical membranes was disrupted, with an increase in diffuse cytoplasmic staining observed. In adult 10 week old (E) WT and (F) *Vangl2*^{Lp/+} lungs, Vangl2 localised to the cell membranes. (G) Representative western blot of Vangl2 (75 kDa) and Gapdh loading control (37 kDa) in adult WT and *Vangl2*^{Lp/+} lungs. (H) Quantification of total Vangl2 protein in adult mouse lungs showed no significant difference between *Vangl2*^{Lp/+} and WT littermates (n=3 per group, P > 0.05). (I) Representative western blot of Vangl2 in the cytoplasmic fraction of adult WT and *Vangl2*^{Lp/+} lungs. (J) Quantification of Vangl2 in the cytoplasmic compartment showed no significant difference between *Vangl2*^{Lp/+} and WT littermates (n=3 per group, Mann-Whitney U P > 0.05). Scale bars represent (A-F) 32 μ m, and inserts 18 μ m.

3.2.2. VANGL2 localisation in human lungs is similar to that in mice

Studies in human with NTDs have identified a number of *VANGL2* mutations [269,270]. However, to date, there have been no human studies specifically investigating the role of the PCP pathway in chronic lung diseases. A recent study in smokers with and without COPD identified downregulation of some key PCP genes such as *VANGL1*, *PK1*, *PK2*, *WNT5a*, *DAAM1*, and *DAAM2* in COPD patients [194].

To determine whether *VANGL2* is expressed in the human lung, immunohistochemistry staining for *VANGL2* was performed in normal human embryonic and adult lung sections. A similar pattern of *VANGL2* localisation was found in both embryonic and adult lungs to that seen in mouse tissue (compare Figure 3.2 and Figure 3.3). *VANGL2* staining was present in both epithelial and mesenchymal cells in human embryonic lungs at 10 weeks post conception (wpc) and was particularly prominent at the apical surface of airway epithelial cells (Figure 3.3A,A'). 10 wpc in humans is the pseudoglandular stage of lung development which corresponds approximately to E14.5 in mouse lung development. In adult human lungs, *VANGL2* staining was strongest at the cell membranes of alveolar epithelial cells (Figure 3.3B,B').

Taken together, these results confirm that *VANGL2* protein is present in both murine and human lungs in development and in adult, with comparable localisation patterns. As previously identified, *VANGL2* was prominent at the plasma membranes, particularly in epithelial cells (Figure 3.2A,C,E and Figure 3.3). These findings suggest that *VANGL2* may also play an important role in both developing and adult human lungs.

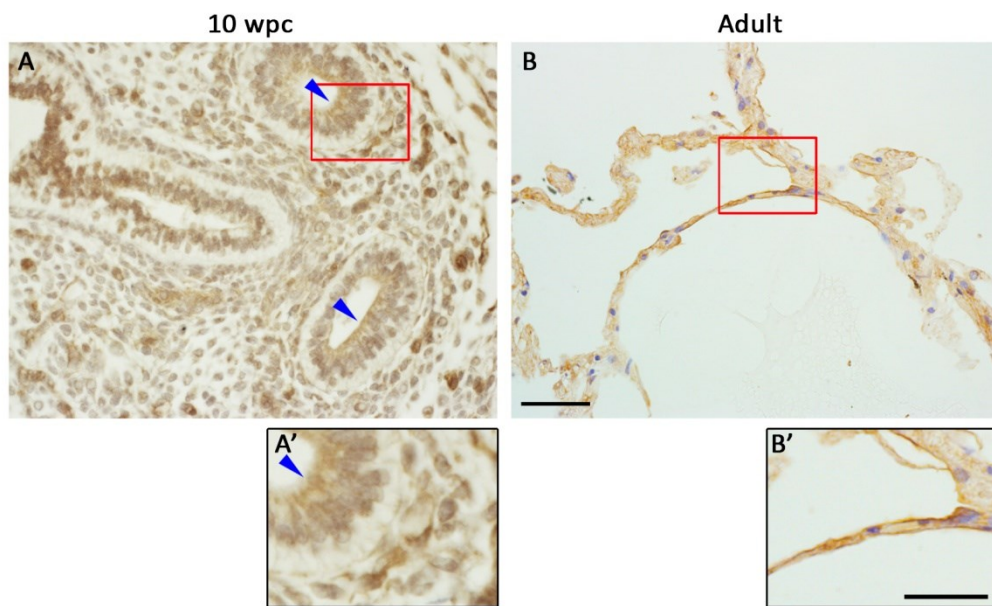


Figure 3.3. VANGL2 localisation in human embryonic and adult lung is similar to murine lungs

VANGL2 immunostaining in human (**A, A'**) embryonic lungs at the pseudoglandular stage (10 wpc), and (**B, B'**) healthy adult lung tissue showed VANGL2 was prominent at the cell membranes, and was enriched at the apical surface of airways in embryonic lung (blue arrows). (**A', B'**) Shows magnifications of the area highlighted by the red box. Scale bars represent (**A, B**) 31 μm , and (**A', B'**) 18 μm .

3.2.3. Heterozygous *Vangl2*^{Lp} mutation causes structural defects in the postnatal murine lung

Having determined that the *Vangl2*^{Lp} mutation caused mislocalisation of Vangl2 in development, the structural and functional impact of the *Vangl2*^{Lp/+} mutation in adult mouse lung was investigated. Previous studies in the Dean lab demonstrated that in embryonic development, *Vangl2*^{Lp/+} lungs display disrupted lung structure with a reduced airway number and reduced septation of the sacculi [Yates L, Unpublished data]. Furthermore, the tissue architecture of embryonic *Vangl2*^{Lp/+} lungs appeared heterogeneous, with some areas of the lung having a similar appearance to WT lungs, and other areas filled with densely compact cells with little or no airspaces [Yates L, Unpublished data]. To follow on from these findings, H&E stained sections of the lungs were examined at three distinct postnatal stages: in active alveolarisation (P7), early adulthood when the lungs are fully developed (10 weeks), as well as in aged mice (12 months).

At 7 days of age, disruption of the lung architecture was visible in *Vangl2*^{Lp/+} lungs compared with WT littermates (Figure 3.4A,B). Quantification of the Lm, a measure of the mean airspace size, showed a significant increase in *Vangl2*^{Lp/+} lungs (33.40 µm) compared with WT littermates (30.18 µm) (Figure 3.4C). In 10 week old adult mice, the structural lung defects were more pronounced with *Vangl2*^{Lp/+} lungs displaying strikingly enlarged alveolar air spaces (Figure 3.4D,E) and significantly increased Lm (57.88 µm) compared with WT littermates (28.98 µm) (Figure 3.4F). Furthermore, the abnormal architecture observed in *Vangl2*^{Lp/+} lungs persisted with aging, such that enlarged alveolar spaces were still present at 12 months of age (Figure 3.4G,H). Quantification of the airspace distance in 12 month old mice showed an increased Lm in *Vangl2*^{Lp/+} lungs (76.45 µm) compared with WT littermates (44.90 µm) (Figure 3.4I). In agreement with published data [325,326], the Lm in WT lungs increased with age as a part of the normal ageing process from 10 weeks to 12 month old lungs. The same comparison in 10 week and 12 month old *Vangl2*^{Lp/+} lungs revealed a similar increase in Lm to that of WT mice (compare Figure 3.4F and I), suggesting that *Vangl2*^{Lp/+} lungs do not undergo an accelerated progression of airspace enlargement. These results indicate that the structural defects caused by the *Vangl2*^{Lp} mutation in embryonic development persist into adulthood, but do not suggest promotion of lung degeneration with age.

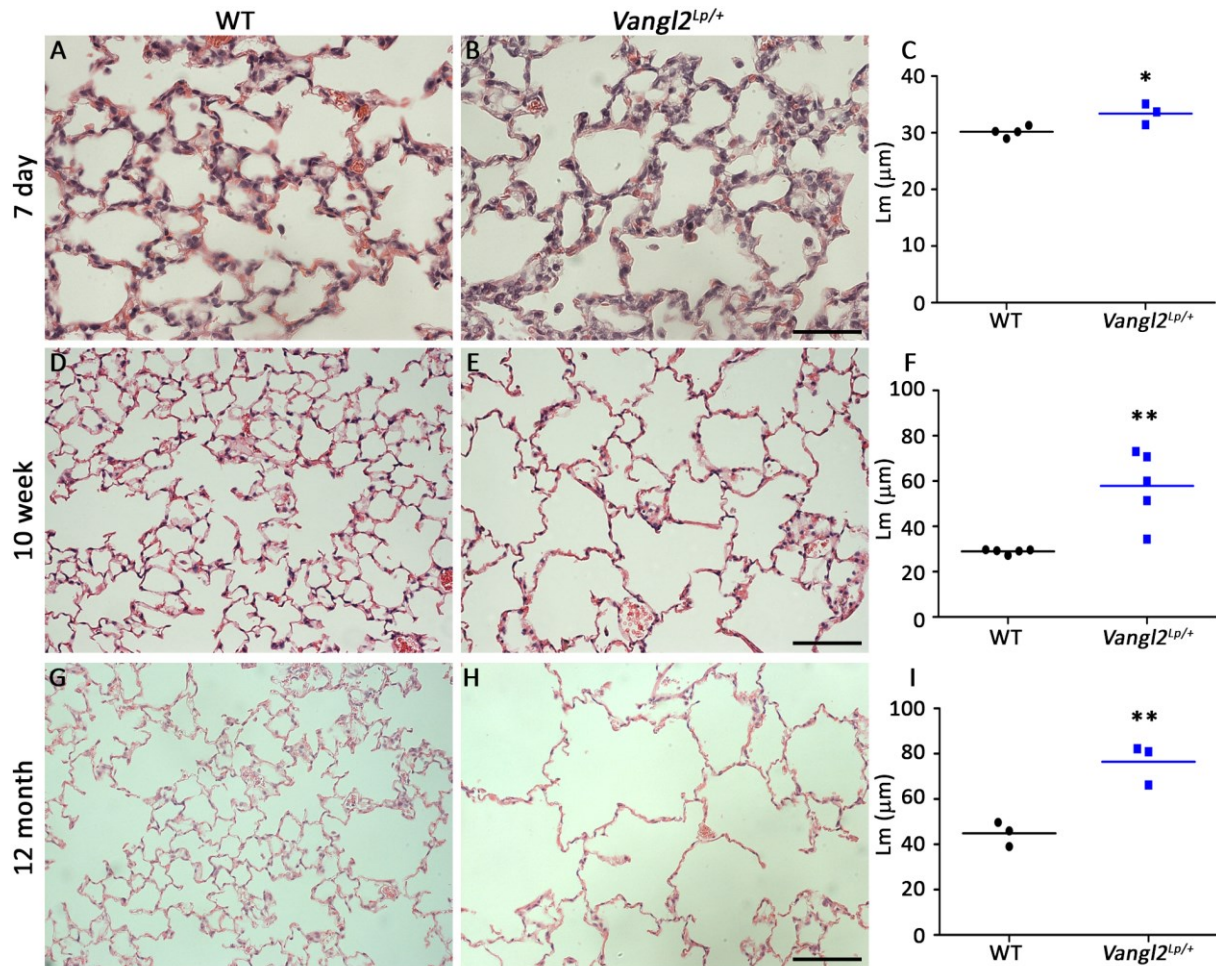


Figure 3.4. Heterozygous *Vangl2*^{Lp} mutation disrupts postnatal lung tissue architecture

Paraffin wax sections from (A,B) 7 day old, (D,E) 10 week old, and (G,H) 12 month old WT and *Vangl2*^{Lp/+} lungs were stained with H&E. Quantification of the mean alveolar airspace distance using the Lm showed enlarged alveoli in the lungs of *Vangl2*^{Lp/+} mice compared with WT littermates in (C) 7 day old (each point shows an individual mouse, student's T-test, P < 0.05 *), (F) 10 week old (each point shows an individual mouse, student's T-test P < 0.01 **), and (I) 12 month old mice (each point shows an individual mouse, student's T-test P < 0.01 **). Scale bars represent (A,B) 39 μm, and (D,E,G,H) 110 μm.

3.2.4. Lung function is perturbed in adult *Vangl2*^{Lp/+} mice

Lung function was measured in adult 10 week old mice to determine if the structural changes caused by the heterozygous *Vangl2*^{Lp} mutation resulted in a functional defect. Respiratory mechanics were measured in 10 week old *Vangl2*^{Lp/+} and WT littermates using the Flexivent system (Sireq), which uses forced oscillation techniques (FOT). *Vangl2*^{Lp/+} mice showed significantly decreased lung elastance (16.66 cmH₂O/ml) compared with WT littermates (19.67 cmH₂O/ml) (Figure 3.5A). A trend towards reduced tissue elastance was also noted in *Vangl2*^{Lp/+} mice (13.98 cmH₂O/ml) compared with WT littermates (17.16 cmH₂O/ml) (Figure 3.5B). These results indicate that the enlarged alveolar architecture in the *Vangl2*^{Lp/+} mutant lungs lead to functional defects in adult mice. In addition, *Vangl2*^{Lp/+} lungs showed a trend towards an increase in the Eta parameter (0.33 arbitrary units (a.u.)) compared with WT littermates (0.26 a.u.) (Figure 3.5C). Eta is an arbitrary measure of lung hysteresivity which compares tissue elastance and tissue dampening as a measure of the heterogeneity of the lung. Parameter B of the Salazar-Knowles equation calculates the theoretical difference in lung volume at the total lung capacity and the predicted volume at zero pressure thereby giving an indication of the total lung volume. Using this equation, *Vangl2*^{Lp/+} mice showed increased total lung volume (1.87 ml) compared with WT littermates (1.44 ml) (Figure 3.5D).

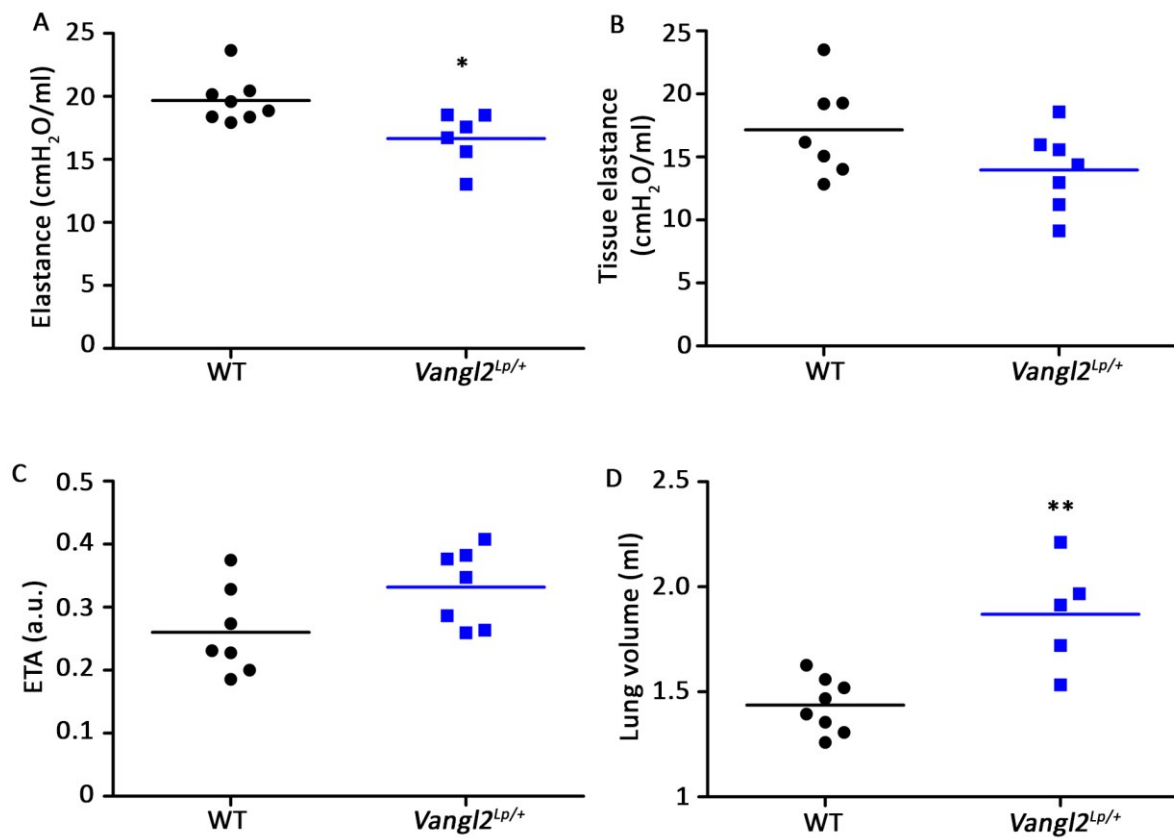


Figure 3.5. Structural defects in adult *Vangl2*^{Lp/+} lungs results in perturbed respiratory function

Scatter plots show lung function measurements of (A) elastance, (B) tissue elastance, (C) ETA, and (D) the B parameter of the Salazar-Knowles equation obtained by FOT (each point shows an individual mouse with data combined from two independent experiments, student's T-test $P > 0.05$ not significant, $P < 0.05$ *, $P < 0.01$ **). Abbreviations: arbitrary units (a.u.).

3.2.5. *Vangl2*^{Lp} mutation does not affect cell proliferation or apoptosis

To begin to elucidate mechanisms underlying the severe structural lung abnormalities in adult *Vangl2*^{Lp/+} mice, cell proliferation and apoptosis were investigated. The enlarged alveolar size in *Vangl2*^{Lp/+} lungs could have resulted from decreased proliferation, increased apoptosis, or both. However, previous studies in homozygous *Vangl2*^{Lp} embryonic mice showed that neither proliferation nor apoptosis were altered [14]. To assess if this held true in heterozygous *Vangl2*^{Lp} mice, immunostaining was performed for phosphohistone H3 (PH3), a marker of proliferating cells, and cleaved caspase 3 (CCS3), to mark apoptotic cells.

Quantification of the percentage of proliferating cells found no difference between *Vangl2*^{Lp/+} and WT littermates in either E17.5 (Figure 3.6A-C) or 10 week old adult lungs (Figure 3.6D-F). As expected, in embryonic development when the lungs are actively growing there was a higher proportion of proliferating cells compared with adult 10 week old mice for both genotypes (median value of 0.48 % and 0.042 % respectively in WT mice) (compare Figure 3.6C and Figure 3.6F). In addition, assessment of apoptosis by CCS3 staining did not show any apoptotic cells in 10 week old lungs (Figure 3.6G,H). Immunostaining of CCS3 in mouse gut sections was used as a positive control, due to the high cell turnover rate in this tissue (Figure 3.6I). These results demonstrate that the disrupted tissue architecture in adult *Vangl2*^{Lp/+} lung does not arise from an altered rate of cell proliferation. Furthermore, it is not caused by increased tissue apoptosis in the adult lungs as no apoptotic cells were detected.

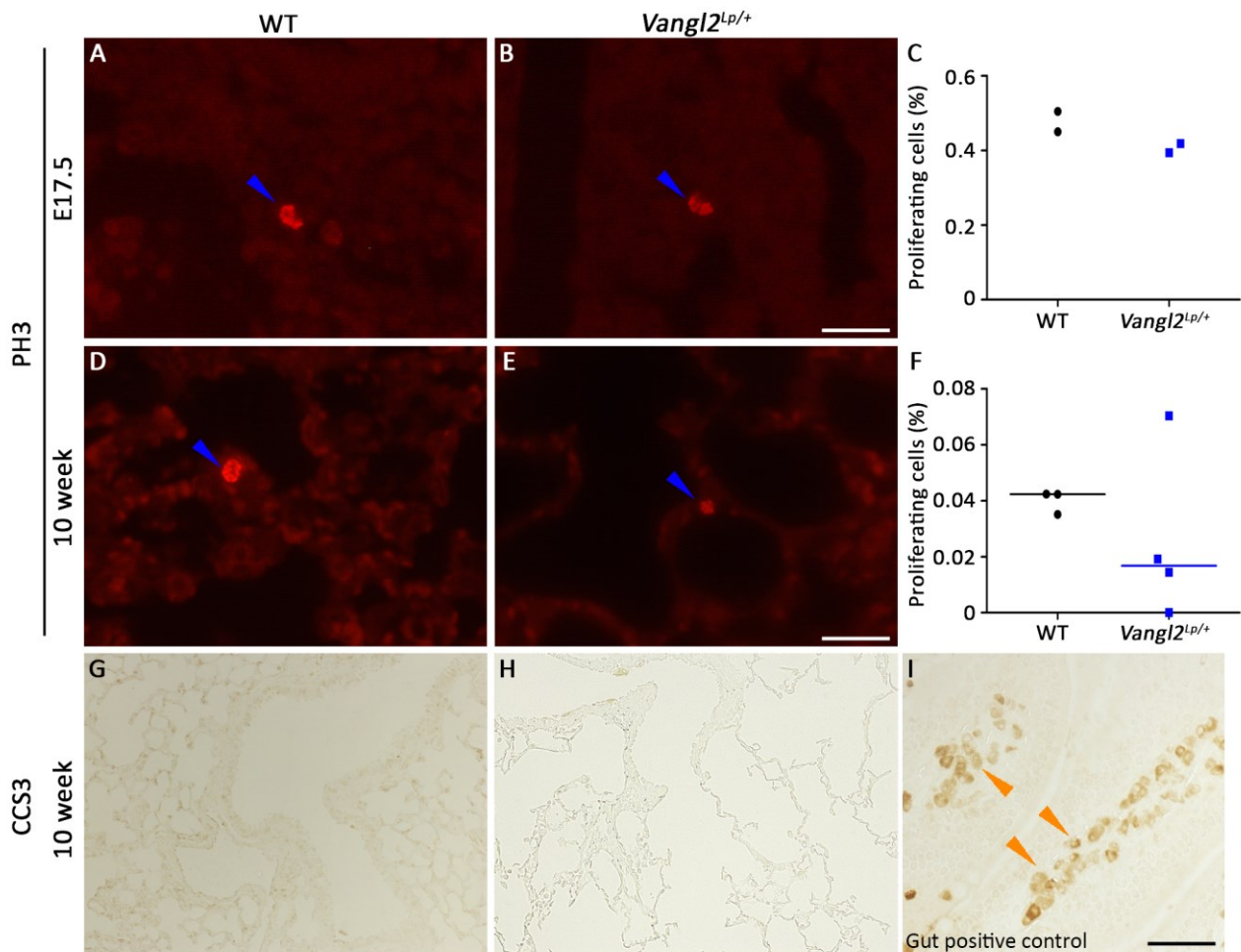


Figure 3.6. Cell proliferation is not affected by the *Vangl2^{Lp/+}* mutation

Immunofluorescence staining for PH3 in lung sections of (A,B) E17.5 and (D,E) 10 week old mice to mark proliferating cells (blue arrow). Quantification of the percentage of proliferating cells in (C) E17.5 (each point shows an individual mouse) and (F) 10 week old mice (each point shows an individual mouse, Mann-Whitney U $P > 0.05$) showed no differences between WT and *Vangl2^{Lp/+}* littermates. (G,H) Immunohistochemistry staining for CCS3 in 10 week old lungs showed no identifiable apoptotic cells. (I) Staining for CCS3 in adult mouse gut was used as a positive control and showed apoptotic cells (orange arrow). Scale bar (A,B,D,E) 18 μm and (G-I) 75 μm .

3.2.6. Cell differentiation is unaffected in adult *Vangl2*^{Lp/+} mouse lungs

Perturbed cell differentiation can also lead to altered lung structure. Previous studies have shown that differentiation was not affected in *Vangl2*^{Lp} homozygote lungs [14]. To determine whether this was also true in *Vangl2*^{Lp} heterozygotes lungs, differentiation was investigated by immunostaining for the following key markers of differentiated cell types in the lungs: ProSP-C (alveolar epithelial ATII cells), CC10 (airway secretory club cells), and α SMA to mark the smooth muscle layer surrounding proximal airways.

Adult 10 week old mice showed comparable staining for both CC10 (Figure 3.7A,B) and α SMA (Figure 3.7C,D) in the proximal airways of both WT and *Vangl2*^{Lp/+} lungs. Furthermore, no difference in the pattern of ATII cells in the distal lung was noted at 10 weeks of age (Figure 3.7E,F). Quantification of ProSP-C positive staining showed no significant difference in the percentage of ATII cells in 10 week old *Vangl2*^{Lp/+} and WT littermates (Figure 3.7G). Taken together these results indicate that the structural defects in the *Vangl2*^{Lp/+} lungs do not occur as a result of altered cell proliferation, apoptosis or differentiation.

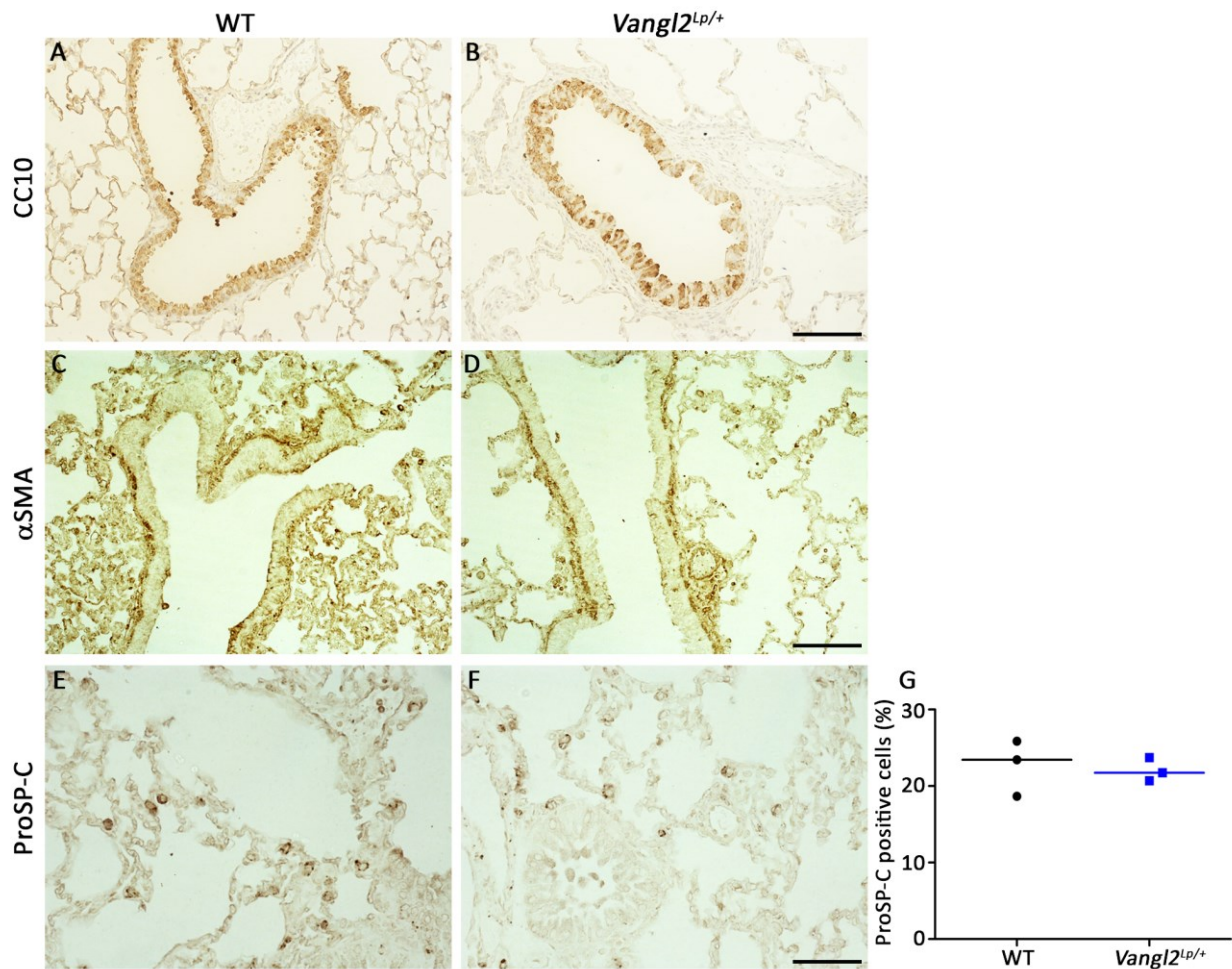


Figure 3.7. Cell differentiation is not affected by the *Vangl2*^{Lp} mutation in adult mouse lungs

Assessment of cell differentiation in adult 10 week old lungs was compared by immunohistochemistry staining for (A,B) upper airway secretory cells using anti-CC10, (C,D) mesenchymal smooth muscle cells using anti-αSMA, and (E,F) ATII cells using anti-ProSP-C showed no differences between WT and *Vangl2*^{Lp/+} littermate mice. (G) Quantification of ProSP-C positive ATII cells showed no significant difference between *Vangl2*^{Lp/+} and WT littermates (each point shows an individual mouse, Mann-Whitney U P >0.05). Scale bars represent (A,B,C,D) 77 μm, and (E,F) 39 μm.

3.2.7. Pulmonary vasculature architecture is not altered in adult *Vangl2^{Lp/+}* mice

The pulmonary vasculature develops in parallel to branching airways and the terminal acini in lung development [17]. Disruption of vasculature development can perturb lung development resulting in reduced airspace number and thickened septa [36]. As the *Vangl2^{Lp/+}* lungs have disrupted alveolar architecture with reduced airway number and septal thickening in development it was important to determine whether pulmonary vasculature was affected in *Vangl2^{Lp/+}* lungs.

To study vascular organisation, lung slices were immunostained with platelet endothelial cell adhesion molecule 1 (Pecam1) antibody, a marker for all vascular endothelial cells. Although it was not quantified, a notable difference was observed in lung slices from *Vangl2^{Lp/+}* mice, which showed increased alveolar wall thickness in some regions (compare Figure 3.8A and Figure 3.8B); this was also noticed in some paraffin embedded sections from *Vangl2^{Lp/+}* lungs but was not as pronounced. Both WT and *Vangl2^{Lp/+}* lung slices had positive staining for Pecam1 in the lungs, however some subtle differences in Pecam1 staining were consistently observed in *Vangl2^{Lp/+}* slices (Figure 3.8A,B). *Vangl2^{Lp/+}* lung slices had increased intensity of Pecam1 staining (Figure 3.8B), indicating that there was increased endothelial cells in the alveolar walls. However, this may in part be due to the altered alveolar wall structure which is thickened in the mutant mice, thereby giving the appearance of increased staining; therefore, further analysis will be required to fully validate the vascular organisation in *Vangl2^{Lp/+}* lungs. To further analyse any vascular alterations in *Vangl2^{Lp/+}* lungs, expression of key vascular genes, *Pecam1*, vascular endothelial growth factor receptor 1 (*Vegfr1*), and *Tie2* were assessed by RT-PCR but showed no significant difference between adult *Vangl2^{Lp/+}* and WT littermates (Figure 3.8C).

Vascular integrity was investigated by measuring albumin levels in the BALF as an indicator of vascular permeability. No significant difference in BALF albumin levels was detected in *Vangl2^{Lp/+}* lungs, indicating that vascular integrity was not affected by the *Vangl2^{Lp}* mutation (Figure 3.8D). As expected, albumin levels in lungs which had undergone lung function testing, which places mechanical strain on the lung, had increased BALF albumin levels (compare Figure 3.8D and Figure 3.8E). Intriguingly, *Vangl2^{Lp/+}* lung had significantly lower BALF albumin levels (176.2 µg/ml) compared with WT littermates (241.1 µg/ml) following lung function (Figure 3.8E). This suggests that *Vangl2^{Lp/+}* lungs had reduced vascular disruption following mechanical strain. Overall, these results suggest that the *Vangl2^{Lp}* mutation does not significantly affect vascular organisations in the lungs at homeostasis.

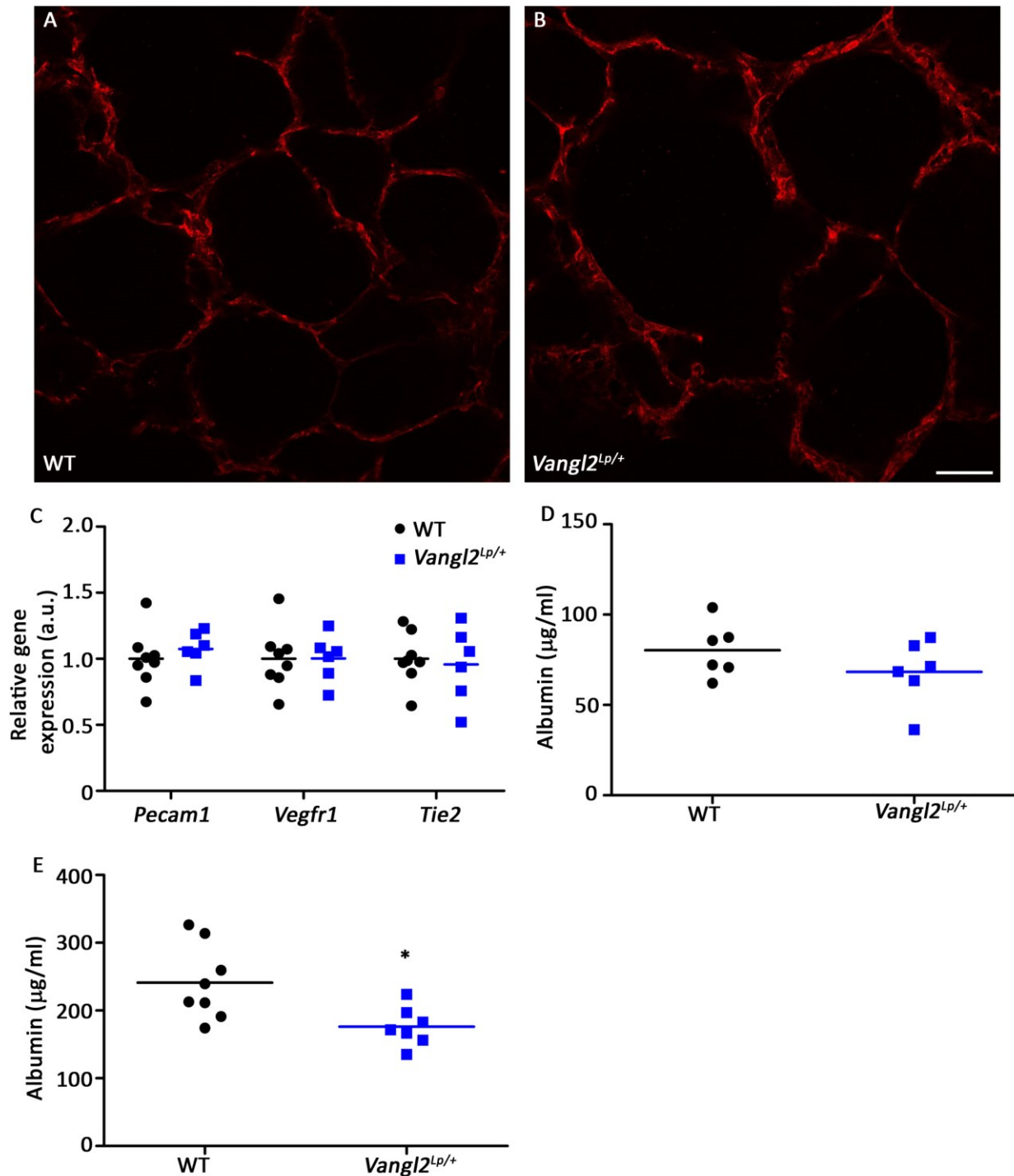


Figure 3.8. Pulmonary vasculature is not affected by the *Vangl2*^{Lp} mutation

(A,B) Immunostaining for Pecam1 in lung slices from (A) WT and (B) *Vangl2*^{Lp/+} mice showed moderately increased vasculature staining in lungs of *Vangl2*^{Lp/+} mice. (C) Gene expression of *Pecam1*, *Vegfr1*, and *Tie2* normalised to housekeeping genes *Hprt* and *B2m* expression in 10-14 week old adult WT and *Vangl2*^{Lp/+} lungs (each point shows an individual mouse, student's T-test $P > 0.05$). (D) Quantification of albumin in the BALF showed no difference, indicating that vascular permeability was not disrupted in *Vangl2*^{Lp/+} lungs (each point shows an individual mouse, student's T-test $P > 0.05$). (E) Quantification of albumin levels following mechanical ventilation showed significantly decreased albumin leakage into the BALF in *Vangl2*^{Lp/+} lungs (each point shows an individual mouse, student's T-test $P < 0.05$ *). Abbreviation: bronchoalveolar lavage fluid (BALF).

3.2.8. Elastin organisation is disrupted in *Vangl2*^{Lp/+} lungs

As lung function was altered in adult *Vangl2*^{Lp/+} mice, the underlying causes of these functional changes were investigated further. In the lungs, ECM proteins such as elastin and collagen are essential for maintaining normal physiology. However, these can become disrupted in disease. Therefore, elastin and collagen were investigated in adult *Vangl2*^{Lp/+} lungs by histological staining and RT-PCR.

Gene expression analysis of elastin (*El*) showed no significant difference between adult WT and *Vangl2*^{Lp/+} lungs (Figure 3.9A). Similarly, western blot analysis showed no significant difference in total elastin protein levels between WT and *Vangl2*^{Lp/+} lungs (Figure 3.9B,C). Despite the total levels of elastin remaining unaltered in *Vangl2*^{Lp/+} lungs, the organisation of elastin fibres was investigated to determine if elastin deposition and/or organisation was altered. Thus, Miller's elastin staining was performed to stain elastin fibres in the adult lung. Elastin fibres were visible in both WT and *Vangl2*^{Lp/+} mutant lungs in the alveolar walls and the tips of alveolar septa (Figure 3.9D,E red arrows). In WT mice, elastin fibres were tightly clustered together at the distal tips of alveolar septa (Figure 3.9D). However, in *Vangl2*^{Lp/+} lungs elastin organisation at the tips of alveolar septa was disrupted. In *Vangl2*^{Lp/+} lungs, elastin fibres formed loose, disorganised deposits which were spread over a larger area along the alveolar septa (15.74 μm^2) compared with WT littermates (9.29 μm^2) (Figure 3.9D-F).

Emilins are family of elastin interacting proteins that are highly expressed in the lungs [319,320]. The expression level of Emilin1 was similar in *Vangl2*^{Lp/+} and WT littermates. Interestingly, *Emilin2* expression was almost 40 % lower in adult *Vangl2*^{Lp/+} lungs compared with WT littermates, although this difference was not statistically significant (Figure 3.9G).

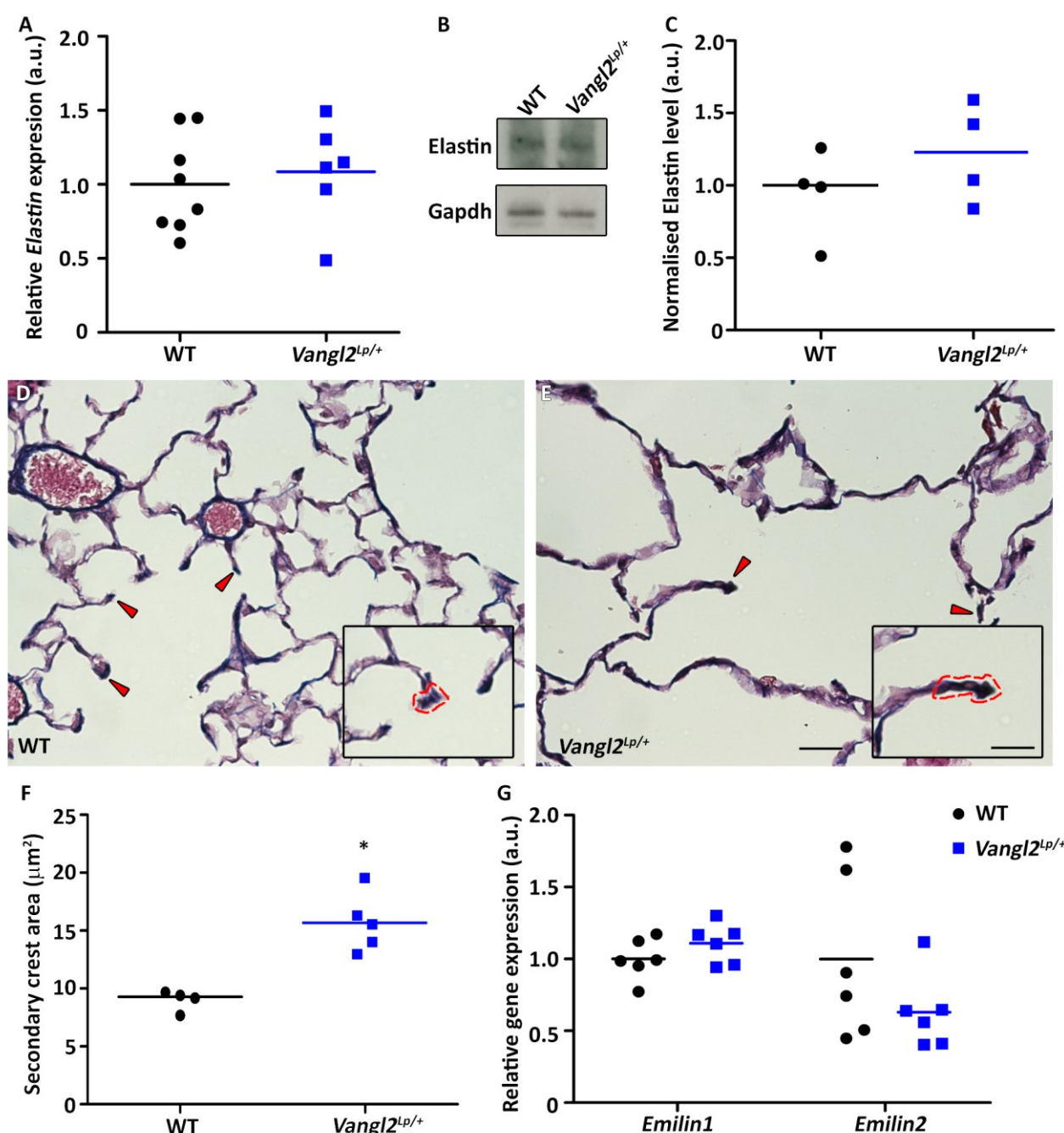


Figure 3.9. Elastin distribution was disrupted in adult *Vangl2*^{Lp/+} lungs

(A) Relative expression of *El* normalised to *Hprt* and *B2m* expression in WT and *Vangl2*^{Lp/+} lungs showed no significant difference (each point shows an individual mouse, student's T-test $P > 0.05$). (B) Representative western blot of elastin (60 kDa), and Gapdh in total protein lysate in WT and *Vangl2*^{Lp/+} lungs. (C) Quantification of total elastin protein levels normalised to Gapdh in WT and *Vangl2*^{Lp/+} littermates showed no significant difference (each point shows an individual mouse, Mann-Whitney U $P > 0.05$). (D,E) Miller's elastic staining in WT and *Vangl2*^{Lp/+} lungs showing elastin deposition as a bluish-black colour at the tips of alveolar septa (red triangles). Inserts show deposition of elastin at tips of alveolar septa (dotted red line). (F) Area of elastin deposition at the tips of alveolar septa was increased in *Vangl2*^{Lp/+} lungs compared with WT littermates (each point shows an individual mouse, Mann-Whitney U $P < 0.05$ *). (G) Relative expression of *Emilin1* and *Emilin2* normalised to *Hprt* expression in adult WT and *Vangl2*^{Lp/+} lungs showed no significant difference (each point shows an individual mouse, student's T-test $P > 0.05$). Scale bars represent (D,E) 14 μm, and inserts 19 μm.

Collagen (Col) and fibronectin 1 (Fn1), two other key ECM components, were also investigated in adult *Vangl2*^{Lp/+} lungs. No noticeable difference in collagen distribution was seen in the *Vangl2*^{Lp/+} lungs (Figure 3.10A,B). As expected, strong positive staining for collagen was present surrounding the blood vessels (red arrow) compared with the parenchyma where there was relatively little collagen deposition (Figure 3.10C purple arrow). Assessment of collagen around the vasculature and airways was similar between WT and *Vangl2*^{Lp/+} littermate lungs (data not shown). Quantification of total collagen by Sircol assay showed no significant differences in the lungs of either genotype (Figure 3.10D). Moreover, *Col1a1*, *Col3a1* and *Fn1* transcript levels were not significantly altered in *Vangl2*^{Lp/+} lungs compared with WT (Figure 3.10E).

Together these results indicate that the *Vangl2*^{Lp} mutation leads to disrupted elastin organisation. This correlates with the reduced *Emilin2* expression in *Vangl2*^{Lp/+} lungs, which is important for formation of mature elastin fibres in the lungs. In contrast, other ECM components such as collagen and fibronectin did not appear to be significantly altered by the *Vangl2*^{Lp} mutation.

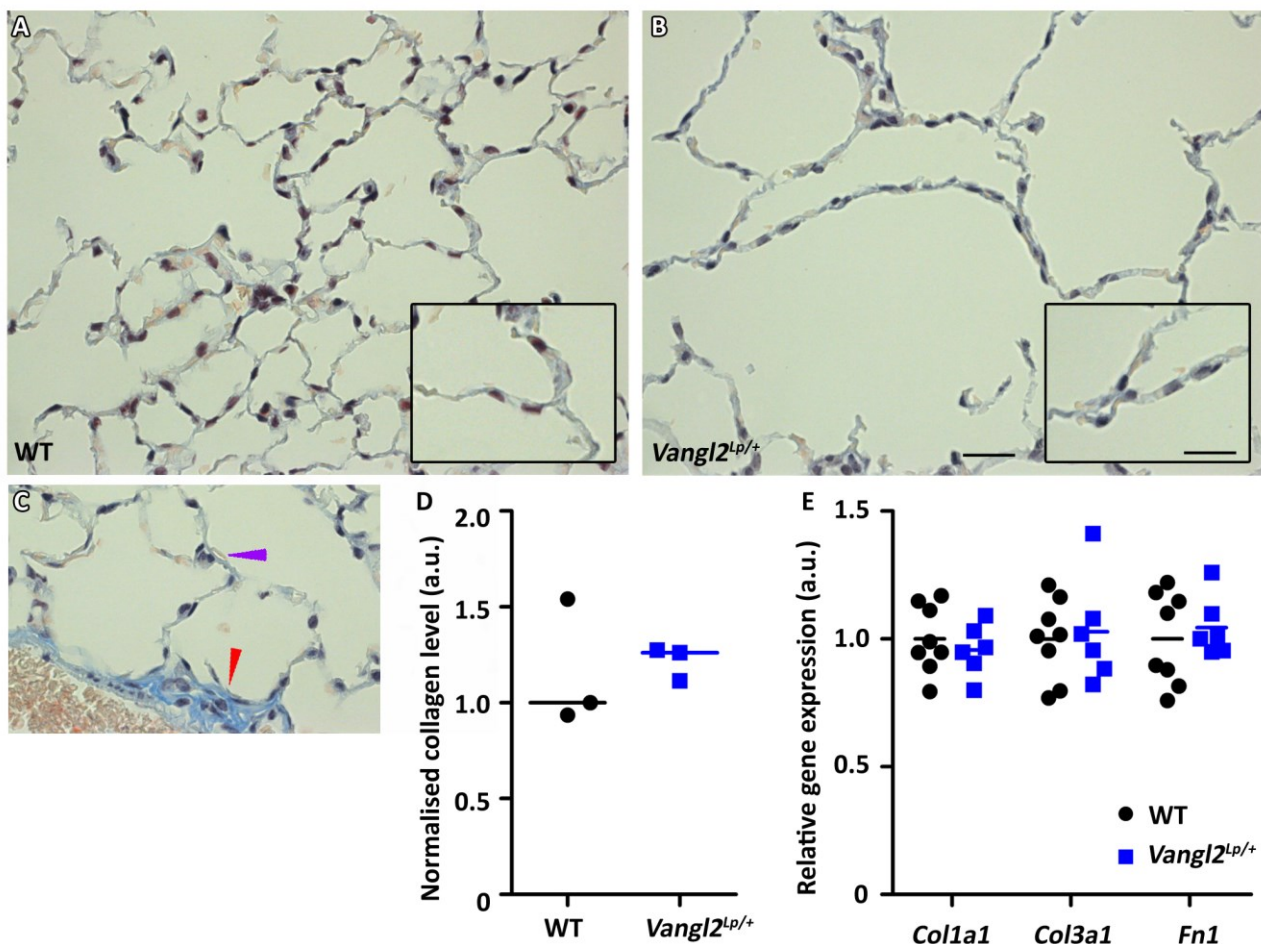


Figure 3.10. Collagen and Fibronectin1 expression is not altered in *Vangl2^{Lp/+}* mutant lungs

(A-C) MSB staining of paraffin wax sections from 10 week old WT and *Vangl2^{Lp/+}* lungs, collagen is stained pale blue. (C) Positive collagen deposition was seen around the vasculature (red arrow), and showed stronger staining in comparison to the lung parenchyma (purple arrow). (D) Quantification of total collagen levels normalised to total protein levels in lungs of *Vangl2^{Lp/+}* and WT littermates (each point shows an individual mouse, Mann-Whitney U $P > 0.05$). (E) Relative fold change in gene expression of *Col1a1*, *Col3a1*, and *Fn1* normalised to housekeeping genes *Hprt* and *B2m* expression showed no significant difference between WT and *Vangl2^{Lp/+}* lungs (each point shows an individual mouse, student's T-test $P > 0.05$). Scale bars represent (A-C) 14 μm , and insert 19 μm . Abbreviations: Collagen (Col), Fibronectin1 (Fn1), Martius Scarlet Blue (MSB).

3.2.9. *Mmp-12* expression and MMP-9 activity is increased in *Vangl2*^{Lp/+} lungs

Matrix metalloproteinases (MMPs) are an important family of proteins involved in the remodelling of ECM proteins such as elastin and collagen. As elastin organisation was disrupted in *Vangl2*^{Lp/+} lungs we assessed regulation of two key MMPs (MMP-9 and MMP-12) which are able to remodel elastin [309]. Therefore, *Mmp* expression was analysed in the lung tissue by western blotting and RT-PCR. MMP-9 activity was also assessed in the BALF by zymography.

In adult *Vangl2*^{Lp/+} lungs, *Mmp-12* transcript levels showed a 2.7-fold increase compared with WT littermates (Figure 3.11A). *Mmp-9* transcript levels were significantly decreased in *Vangl2*^{Lp/+} lungs compared with WT littermates (Figure 3.11A). However, *Mmp-2* transcript levels were unchanged between *Vangl2*^{Lp/+} and WT lungs (Figure 3.11A). Assessment of the protein levels of MMP-12 in whole lung lysate by western blotting revealed that both Pro-MMP-12 and cleaved (active) MMP-12 protein were detectable in WT and *Vangl2*^{Lp/+} lungs (Figure 3.11B). However, there was no difference in active (cleaved) MMP-12 levels in *Vangl2*^{Lp/+} lungs compared to WT littermates (Figure 3.11C).

In the BALF, *Vangl2*^{Lp/+} mice showed a trend towards increased total MMP-9 protein levels compared with WT littermates, although this did not show a statistically significant difference (Figure 3.11D). However, when MMP-9 catalytic activity was assessed in the BALF by zymography, a significant 6.5 fold increase in MMP-9 activity was observed in *Vangl2*^{Lp/+} mice compared with WT littermates (Figure 3.11E,F).

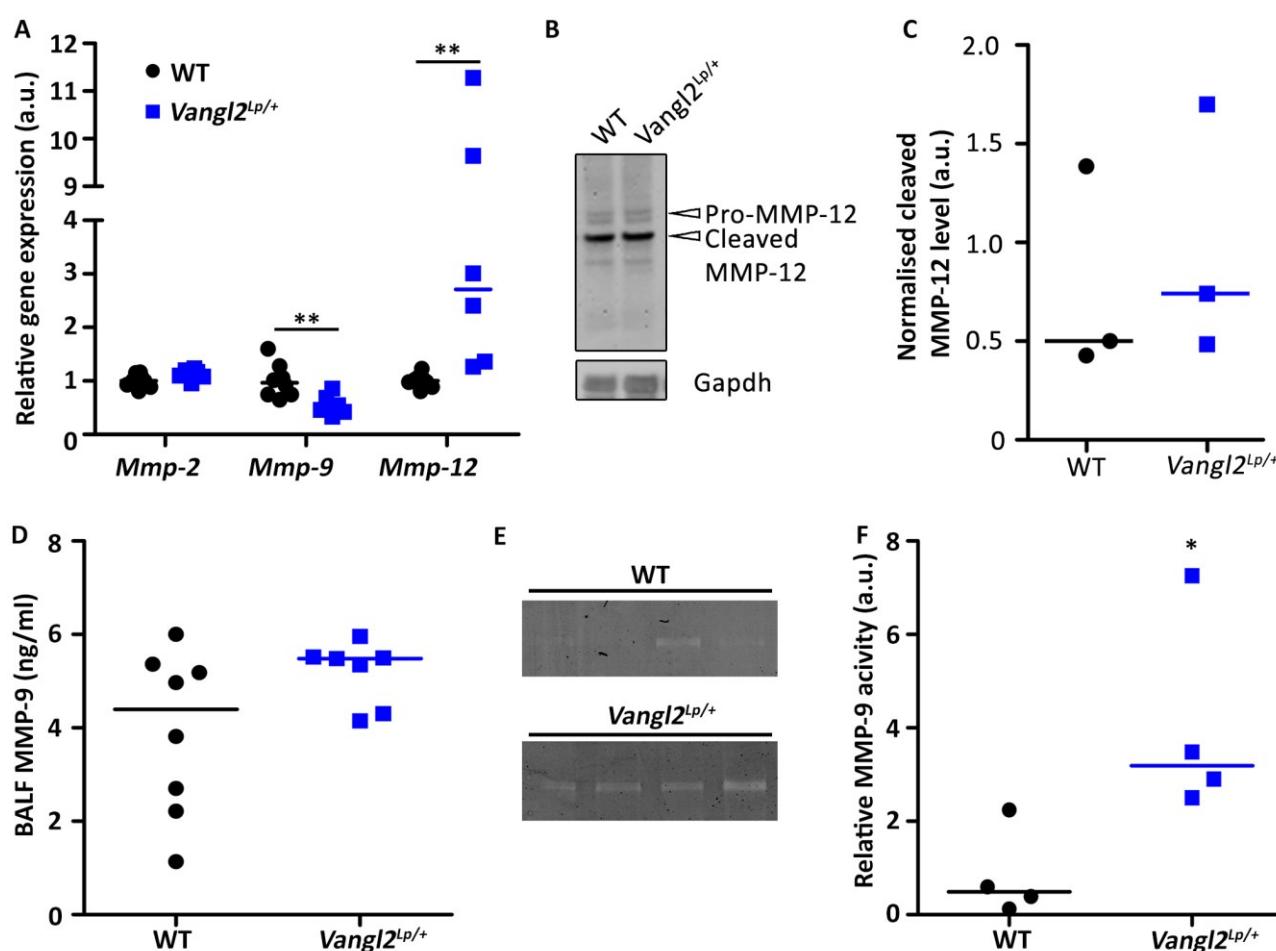


Figure 3.11. *Mmp* expression and activity is deregulated in *Vangl2^{Lp/+}* mouse lung

(A) Gene expression of *Mmp-2*, *Mmp-9*, and *Mmp-12* normalised to *Hprt* and *B2m*, showed reduced *Mmp-9* and increased *Mmp-12* expression in adult *Vangl2^{Lp/+}* lungs compared with WT controls (each point shows an individual mouse, Mann-Whitney U P >0.05 not significant, P <0.01 **). (B) Representative western blot showing Pro-MMP-12 (54 kDa) and cleaved MMP-12 (45 kDa) in 10 week old WT and *Vangl2^{Lp/+}* littermates, and Gapdh loading control. (C) Quantification of the 45 kDa cleaved (active) MMP-12 protein showed no significant difference between WT and *Vangl2^{Lp/+}* littermates (each point shows an individual mouse, Mann-Whitney U P >0.05). (D) Total MMP-9 in BALF of 10 week old mice showed no significant difference between WT and *Vangl2^{Lp/+}* mice (each point shows an individual mouse, Mann-Whitney U P >0.05). (E) Zymogram of MMP-9 activity in adult *Vangl2^{Lp/+}* and WT littermates. (F) Quantification of MMP-9 zymogram showed significantly increased MMP-9 activity in *Vangl2^{Lp/+}* mice compared with WT littermates (each point shows an individual mouse, Mann-Whitney U P <0.05 *).

3.2.10. Adult *Vangl2*^{Lp/+} lungs contain an increased population of enlarged macrophages

Two of the key lung cell types involved in MMP-9 and MMP-12 production are macrophages and neutrophils [309]. Macrophages are innate immune cells which play an essential role in immune protection in infection and disease [299]. More recently however, they have also been shown to be important for processes such as development and regeneration [71,77,80]. In chronic lung diseases, macrophage and neutrophils numbers are increased in the lungs, resulting in a proinflammatory environment which leads to disrupted tissue architecture [283,299,309,327]. Therefore, macrophage and neutrophil immune cell populations were analysed in the BALF and lung tissue of *Vangl2*^{Lp/+} and WT littermates, by performing differential cell counts using Wright Giemsa staining (Figure 3.12A,B).

The total number of immune cells in BALF was not significantly different between WT and *Vangl2*^{Lp/+} mice (Figure 3.12C). Furthermore, there was no difference in the total number of neutrophils (Figure 3.12D) or macrophages (Figure 3.12E) between both genotypes. However, in *Vangl2*^{Lp/+} BALF, a particular population of macrophages, which were morphologically distinct, were identified. This population of macrophages were approximately two to three times larger than normal and also appeared highly vacuolar (Figure 3.12B, red arrow). Quantification showed significantly more enlarged macrophages in *Vangl2*^{Lp/+} BALF (62.50 % of total macrophage population) compared with WT littermates (17.51 % of total macrophage population) (Figure 3.12F).

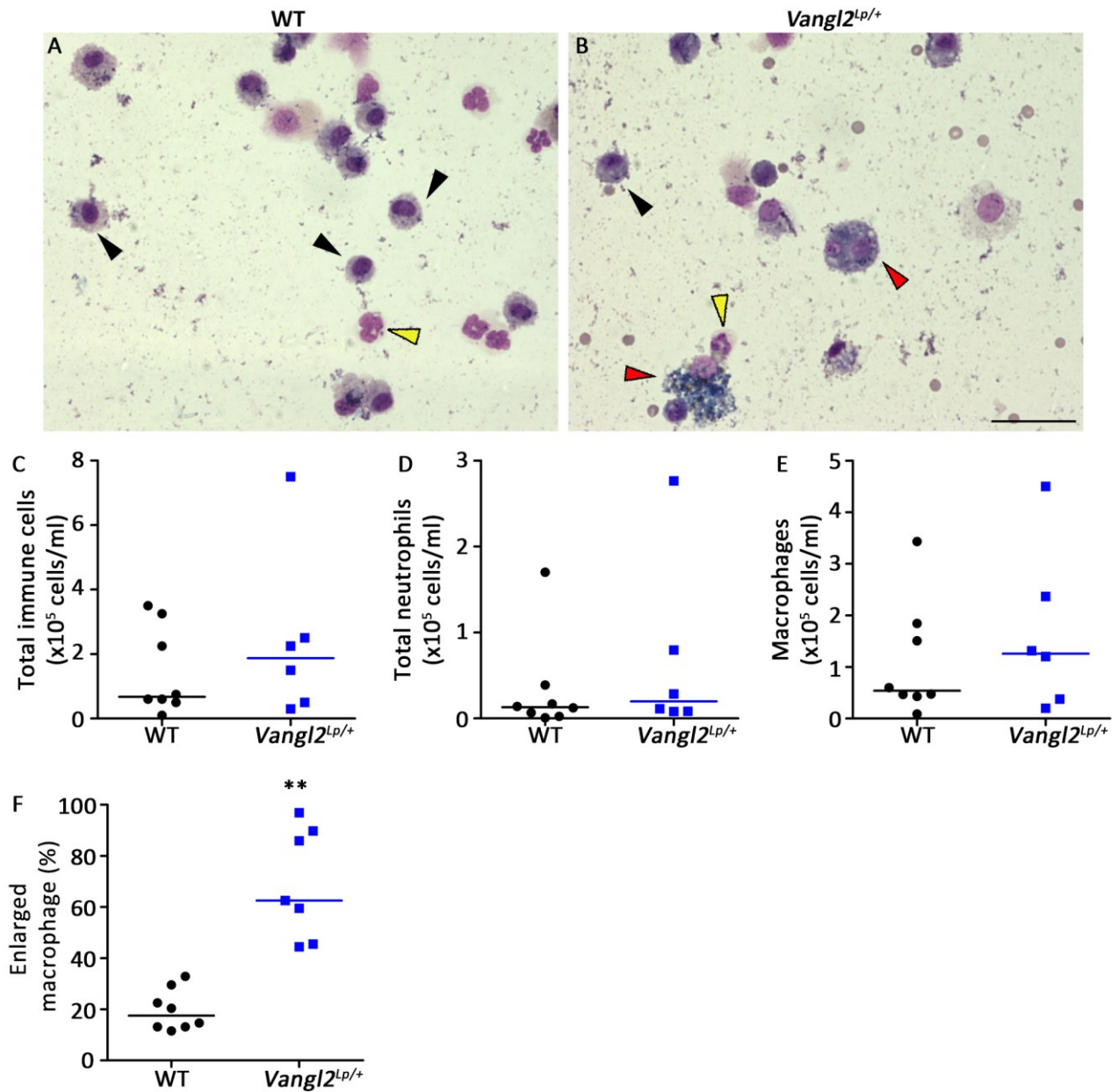


Figure 3.12. BALF of adult *Vangl2*^{Lp/+} mice have increased enlarged macrophages

(A,B) Representative cytopspins of BALF from adult (A) WT and (B) *Vangl2*^{Lp/+} mice, showing normal macrophages (black arrow), enlarged macrophages (red arrows), and neutrophils (yellow arrows). Scatter plots showing (C) total numbers of immune cells, (D) total neutrophils, and (E) total macrophage numbers in BALF of 10 week old *Vangl2*^{Lp/+} and WT littermates (each point shows an individual mouse, Mann-Whitney U P >0.05). (F) Quantification of enlarged macrophage populations in the BALF of 10 week old mice showed a significant increase in *Vangl2*^{Lp/+} mice compared with WT littermates (each point shows an individual mouse, Mann-Whitney U P <0.01 **). Scale bars represent (A,B) 30 μm.

In addition to the immune cells in the BALF, macrophages are also present in the lung interstitium that are important for immunosurveillance and the repair of tissue damage. Investigation of the immune population in the lung interstitium showed no significant difference in total immune cell (Figure 3.13A), neutrophil (Figure 3.13B), or macrophage (Figure 3.13C) numbers between *Vangl2*^{Lp/+} and WT littermates. Quantification of the proportion of enlarged macrophages in the lung interstitium did not show a significant difference in *Vangl2*^{Lp/+} lungs compared with WT controls (Figure 3.13D). Interestingly, the proportion of enlarged macrophages was greater in the lung interstitium than in BALF in both WT mice (80.87 % in lung tissue and 19.71 % in BALF) and *Vangl2*^{Lp/+} mice (90.09 % in lung tissue and 69.20 % in BALF) (compare Figure 3.12F and Figure 3.13D).

To investigate the enlarged macrophage population further, macrophage activation was investigated. Broadly speaking, activated macrophages can adopt either an inflammatory (M1) or repair (M2) phenotype. Transcript levels of M1 and M2 genes were determined in the adult lung. The expression of M2 genes (*Mrc1* and *Fizz1*) were significantly increased in *Vangl2*^{Lp/+} lungs, whereas the expression of the M1 gene *Irf5* was significantly reduced compared with WT littermates (Figure 3.13E). Expression of *Nos2*, another M1 gene, was not altered in *Vangl2*^{Lp/+} lungs compared with controls (Figure 3.13E). The localisation of macrophages was also investigated by immunostaining using the macrophage marker cluster of differentiation 68 (CD68) [328], to stain both interstitial and alveolar macrophages [74]. No difference in the localisation of macrophages in the alveolar spaces or the interstitium was detected between *Vangl2*^{Lp/+} and WT littermate lungs (Figure 3.13F,G), although the intensity of staining in the *Vangl2*^{Lp/+} macrophages was notably stronger.

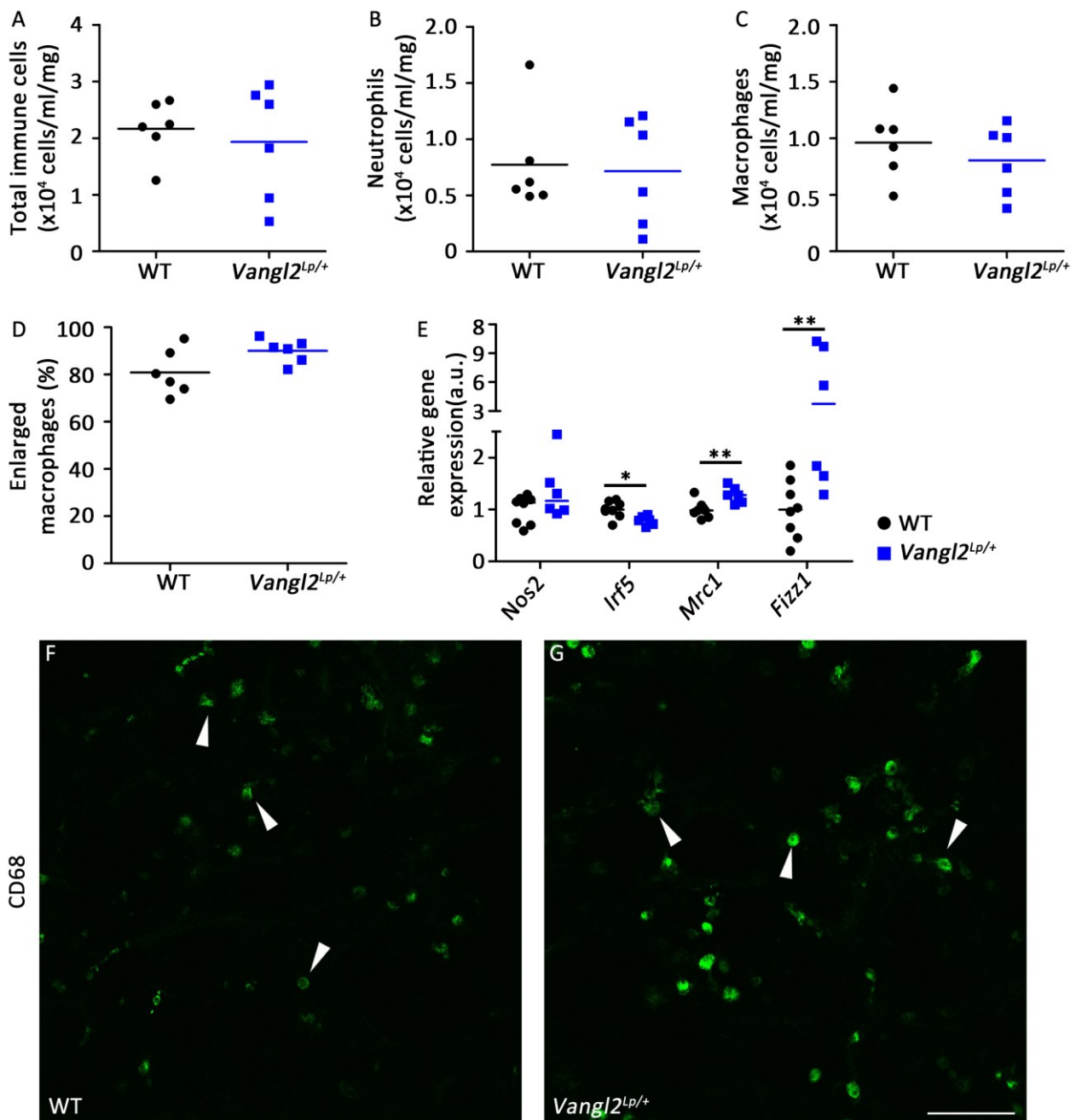


Figure 3.13. Macrophages are polarised towards an M2 phenotype in *Vangl2*^{Lp/+} lungs

Scatter plots of **(A)** total number of immune cells, **(B)** total neutrophil number, and **(C)** total macrophage numbers in lung interstitium of 10 week old *Vangl2*^{Lp/+} and WT littermates (each point shows an individual, student's T-test $P > 0.05$). **(D)** Quantification of enlarged macrophage population in the lung interstitium of 10 week old mice showed no significant difference between *Vangl2*^{Lp/+} and WT littermates (each point shows an individual mouse, student's T-test $P > 0.05$). **(E)** *Vangl2*^{Lp/+} lungs had decreased gene expression of the M1 gene, *Irf5*, and increased expression of the M2 genes, *Mrc1* and *Fizz1*, compared with WT littermates (each point shows an individual mouse, Mann-Whitney U $P < 0.05$ *). **(F,G)** Immunofluorescence staining in lung slices for CD68 showing localisation of interstitial and alveolar macrophages in the lung of 10 week old *Vangl2*^{Lp/+} and WT littermate mice (white arrow). Scale bars represent **(F,G)** 80 μm.

3.3. Discussion

3.3.1. A heterozygous *Vangl2*^{Lp} mutation results in the mislocalisation of Vangl2 in lungs

We examined the impact of PCP pathway disruption caused by a heterozygous *Vangl2*^{Lp} mutation in the adult lungs. Heterozygous *Vangl2*^{Lp} mutant mice were used in this study as homozygous *Vangl2*^{Lp} mutant mice die neonatally [201]. Initially, we assessed localisation of Vangl2 in the lungs to confirm that a single *Vangl2*^{Lp} mutation was sufficient to disrupt Vangl2 localisation. In embryonic lungs, Vangl2 was localised to the cell membranes of both epithelial and mesenchymal cells, with weaker cytoplasmic staining. Furthermore, Vangl2 was enriched at the apical membranes of epithelial cells lining the airways, correlating with previously published data [14,167,200]. In embryonic *Vangl2*^{Lp/+} lungs, Vangl2 localisation was disrupted, displaying reduced membrane localisation and increased cytoplasmic staining. Apical enrichment of Vangl2 in the airways was also decreased, correlating with previous studies of homozygous embryonic *Vangl2*^{Lp} lungs [14,200]. Together, this data indicates a single copy *Vangl2*^{Lp} mutation is sufficient to cause mislocalisation of Vangl2 in the lungs, resulting in increased cytoplasmic retention. However, these disruptions are less severe than those observed in homozygous *Vangl2*^{Lp} mice [14].

The increased cytoplasmic retention of Vangl2 may occur as a result of disrupted protein trafficking. Studies have shown that Vangl2 genetically interacts with Sec24b, which is a component of the coat protein complex II (COPII) that is necessary for the transport of Vangl2 from the endoplasmic reticulum (ER) to the Golgi. The *Vangl2*^{Lp} mutation is known to disrupt Vangl2 interaction with Sec24b, thereby preventing the transport of Vangl2 [205,206]. This results in increased retention of mutant Vangl2 in the ER, preventing it being trafficked to the plasma membranes, causing the Vangl2 mislocalisation that is observed in *Vangl2*^{Lp} mutants [205]. Likewise, *Sec24b*^{-/-} mice also show similar defects in Vangl2 localisation, but do not display mislocalisation of Fz3 another protein involved in PCP signalling [205,206]. This mechanism suggests that transport proteins are necessary for transporting PCP proteins in the cells, and different transport protein show preference for different PCP proteins.

In adult WT mice, immunostaining demonstrated strong membrane localisation of Vangl2 in epithelial cells, similar to embryonic mouse lung. Surprisingly, the pattern of Vangl2 staining did not noticeably differ between adult *Vangl2*^{Lp/+} and WT littermates, and similar membrane localisation was observed in both genotypes. However, this may have arisen due to the low sensitivity of immunostaining which is unable to detect subtle changes in protein levels. Additionally, assessment of the total Vangl2 protein levels by western blotting did not show a significant difference between WT and *Vangl2*^{Lp/+} lungs. As previous studies have shown Vangl2 localisation to be altered by the *Vangl2*^{Lp} mutation with increased cytoplasmic retention [167], subcellular fractionation was performed to assess Vangl2 localisation in the cytoplasmic and membrane compartment of the adult lung. As seen by the immunostaining and western blotting there was no significant differences in Vangl2 subcellular localisation between WT and *Vangl2*^{Lp/+} lungs. Surprisingly, Vangl2 was undetectable in the membrane fraction; this is likely due to difficulties in the

method, as there may have been insufficient levels of Vangl2 expression in adult mice to detect it efficiently by western blotting.

Together, these results showed that the *Vangl2*^{Lp} mutation caused disrupted Vangl2 localisation in embryonic lungs, but not in the adult lung; this difference in Vangl2 regulation may be linked to the requirement for PCP signalling at each stage. Taking the kidneys as example, PCP signalling is essential in embryonic development. Disruption of PCP signalling, such as in the *Vangl2*^{Lp} and conditional *Vangl2*^{-/-} mutants embryonic glomerular development is perturbed [209,211]. However, in adult kidney homeostasis Vangl2 protein is not detected, although following injury, *Vangl2* gene expression is increased and this is thought to be due to the role of PCP signalling in tissue repair [211]. Similarly, in the lung, PCP signalling is required in embryonic development when extensive branching and cell migration occurring. Disruption of PCP signalling in the *Vangl2*^{Lp} mutants perturbs embryonic lung development [14]. In adult lungs, there is limited cell turnover and migration occurring [20], thus PCP signalling may not be as important at this stage, resulting in the reduced Vangl2 regulation in adult lungs.

To the best of our knowledge, the data presented in this thesis is the first DAB immunohistochemistry staining of Vangl2 in paraffin sections. It was important to establish a protocol for immunostaining in paraffin section to assess VANGL2 localisation in human lungs as these are the only samples readily available for research. The pattern of staining in both WT and *Vangl2*^{Lp} lungs was similar to previously published immunofluorescence staining of Vangl2 in embryonic lung cryosections [14], as discussed above. Due to technical difficulties associated with preparation of cryosections from adult lungs whilst maintaining tissue architecture, we used paraffin embedded sections of adult lung tissue; as a result it was sub-optimal to perform immunofluorescence staining in wax sections due to the high level of autofluorescence. As a result, immunohistochemistry using DAB was performed. Although the pattern of pattern of Vangl2 staining was comparable there were some notable differences. For example, the punctuate Vangl2 staining observed by immunofluorescence [14] was not visible by DAB staining, and the appeared as diffuse cytoplasmic staining. One cause of this may be linked to difficulties in imaging DAB staining where punctuate staining such as Vangl2 is not difficult see, and image capture is unable to recapitulate what is visible down the microscope.

3.3.2. VANGL2 localisation in human lungs is similar to mouse lungs

VANGL2 localisation in human embryonic and adult lungs was similar to that seen in mouse lungs; this is important for potential translational applications from mice into humans. The similarities observed in VANGL2 function in both this study and previously published work [269,270], suggests that VANGL2 may play an important role in lung development in humans, and defects in PCP signalling may affect human lung development. Recent studies undertaken in the Dean lab have identified VANGL2 disruption associated

with a decline in lung function. A population study identified a single nucleotide polymorphism (SNP) in *VANGL2*, rs4656907, associated with reduced lung function specifically in smokers. Rs4656907 on its own did not show a significant association with reduced lung function. Similarly, smokers without the rs4656907 SNP also did not show a significant change in forced vital capacity (FVC). However, smokers with the rs4656907 SNP had a significant reduction in the FVC by 5.1 ml per allele and pack-year [Minelli C and Pereira M, Unpublished data]. Additionally, studies in COPD patients have shown significantly reduced expression of *VANGL2*, as well as other PCP genes including *WNT5a*, *SCRIB* and *CELSR1*, in comparison to normal donor lungs [Poobalasingam T, Unpublished data]. Together, these results indicate that PCP signalling is important in adult human lung, and become perturbed in chronic lung disease.

3.3.3. Effect of the *Vangl2*^{Lp} mutation on adult lung structure

3.3.3.1. Role of the *Vangl2*^{Lp} mutation in the alveoli

In mice, alveolarisation starts postnatally around P5 and is largely complete by P30 [17]. Analysis of the lungs near the beginning of alveolarisation, P7, showed that *Vangl2*^{Lp/+} lungs had increased alveolar size, but this difference was not as pronounced as in the adult mice by which point alveolarisation is complete. Previous studies in embryonic *Vangl2*^{Lp/+} lungs have shown that lung development was disrupted with reduced airway number and septation in the saccular stage [Yates L, Unpublished data]. This suggests that the lung structural defects in *Vangl2*^{Lp/+} mutant mice arise in embryonic development, and become exacerbated in the alveolar stage postnatally, thereby resulting in the emphysema-like architecture observed in adult mouse lung once alveolarisation is complete.

Adult *Vangl2*^{Lp/+} lungs show significantly increased alveolar size compared with WT littermates. This phenotype is similar to that observed in COPD patients with emphysema [329]. However, the overall *Vangl2*^{Lp} phenotype is distinct from emphysema in a number of aspects. In emphysema, progressive destruction of alveoli occurs, and this is associated with increased protease activity. The tissue damage caused by the proteolytic activity and initiation of repair processes results in increased proliferation and apoptosis in the lungs [329–331]. However, *Vangl2*^{Lp/+} lungs showed no difference in proliferation or apoptosis in development or adulthood. Therefore, unlike in emphysema, the airspace enlargement in the *Vangl2*^{Lp/+} mice does not arise from alveolar destruction, but originates from disrupted lung development.

As adult mice age, Lm increases in both WT and *Vangl2*^{Lp/+} lungs, as previously reported during aging [325,326]. Interestingly, the increase in Lm was similar in both genotypes, indicating that lungs of both WT and *Vangl2*^{Lp/+} mice had comparable age related changes. Thus, similar to the kidney [211], the *Vangl2*^{Lp} mutation in the lung may not cause a functional decline of the organ in normal homeostasis after completion of development. Therefore, it is likely that in the lungs, the disrupted lung structure present in

the *Vangl2*^{Lp/+} mice arises from developmental defects. However, following completion of postnatal lung development, the influence of aging in homeostasis is similar between WT and *Vangl2*^{Lp/+} lungs.

It should be noted that when performing statistical analysis for Lm in 7 day old and 12 month old mice, a Gaussian distribution was assumed although the sample size was too small to perform a test for normality. Lm measurements in 10 week old lungs, for which sample numbers were large enough to test for normality, showed a normal distribution; this was seen in mice of different litters. In addition, other published studies in mice showed a normal distribution for Lm measurements [283,332]. Therefore, it stands to reason that the mean linear intercept will be normally distributed across the different ages assessed, and so parametric statistical tests can be applied to the 7 day old and 12 month old mice.

Although *Vangl2*^{Lp/+} lungs display drastically altered lung architecture, the mice themselves do not show reduced lifespan when housed in a specific pathogen free environment [Dean C, Unpublished data]. In the kidneys, studies indicate that the *Vangl2*^{Lp} mutation does not affect glomerular function in homeostasis, but does increase susceptibility to injury [209,211]. However, our study showed that lung function was reduced in adult *Vangl2*^{Lp/+} lungs even in the absence of any challenge. The lung function parameter changes such as the decreased elastance, increased hysteresis, and increased total lung volume observed in *Vangl2*^{Lp/+} mice are similar to the lung function changes seen in patients and animal models of emphysema [294,333]. In COPD patients and animal models, protease activity damages alveolar walls leading to the disruption of elastin structure, resulting in reduced lung elastance [316,324,333]. Consequently, recoil pressure in the lung parenchyma reduces, resulting in increased total lung capacity [294]. Increased hysteresis also occurs as a result of altered tissue structure. The increase in airspace size and disorganised ECM structures results in a more compliant lung, leading to increased hysteresis [294]. Although the *Vangl2*^{Lp} mutant mice do not have destruction of the airspaces as seen in emphysema, the enlarged airspaces and perturbed elastin organisation may lead to a similar phenotype, and have similar functional defects.

As a mechanism of investigating how the disrupted lung architecture arises, we assessed cell differentiation in the adult lung. Previously published work in embryonic *Vangl2*^{Lp/Lp} lungs showed that the *Vangl2*^{Lp} mutation did not affect differentiation of specific key cell types [14]. Similarly, this study confirmed that adult heterozygous *Vangl2*^{Lp} mutant lungs had no difference in cell type differentiation, correlating with previous studies in embryonic *Vangl2*^{Lp/+} lungs in the Dean lab [Yates L, Unpublished data]. Together, these results demonstrate that lung structural defects do not result from disrupted cell proliferation, apoptosis, or differentiation, but as a result of disrupted branching morphogenesis and septation in embryonic and postnatal lung development.

3.3.3.2. Role of the *Vangl2*^{Lp} mutation in the vasculature

In addition to the alveolar structure, the vasculature was also assessed as both develop in parallel [17,31,33]. Analysis of vasculature markers did not show a significant difference in expression of vascular genes in the *Vangl2*^{Lp/+} lungs. However, in adult *Vangl2*^{Lp/+} lungs, Pecam1 staining, which was used as a marker for endothelial cells, appeared more intense indicating more endothelial cells were present. However, the lung tissue architecture of the *Vangl2*^{Lp/+} lungs also appeared thicker compared with WT littermates and was greatly altered in structure, which may have contributed to the differences seen in the appearance of the vascular organisation. Therefore, further analysis is required to fully validate this. One method would be to quantify the intensity of Pecam1 staining within in the alveolar walls. The staining intensity can then be divided by the alveolar surface area assessed to normalise the staining intensity to a given area; thereby, reducing bias as a result of altered alveolar wall thickness. Alternatively the number of endothelial cells can be counted and expressed as a percentage of total cell numbers in the lung alveoli, to quantify if endothelial cell number is altered in *Vangl2*^{Lp} mutant lungs.

Furthermore, the *Vangl2*^{Lp} mutation did not disrupt vascular integrity in the lungs at homeostasis. However, following mechanical strain by forced ventilation, adult *Vangl2*^{Lp/+} lungs had reduced disruption of vascular integrity compared with WT littermates. This result was surprising, as we hypothesised that *Vangl2*^{Lp/+} lungs would show increased disruption of vascular integrity due to their disrupted structure. One possible reason for the decreased vascular damage in *Vangl2*^{Lp/+} lungs following mechanical strain may be attributed to the forced ventilation affecting a smaller alveolar surface area due to the enlarged alveoli size. Furthermore, the altered lung structure and increased lung capacity, as determined from the lung function analysis, will cause the *Vangl2*^{Lp} mutant lungs to have different ventilation mechanism. Thus, altered ventilation mechanics in *Vangl2*^{Lp/+} lung may account for the differences in vascular disruption.

3.3.4. Influence of the *Vangl2*^{Lp} mutation on lung elastin organisation

Mature, well organised elastin fibres are essential for the maintenance of lung structure and function. In the lungs, elastin is organised as sheets which extend throughout the alveolar interstitium [316]. Histologically, elastin appears as densely packed bundles at the tips of alveolar septa [334]. In a number of adult lung diseases including COPD and IPF, extensive ECM remodelling occurs; elastin fibres becomes fragmented and web-like in COPD resulting in perturbed lung function [316]. Furthermore, in COPD patients, elastin organisation at the tips of the alveolar septa is perturbed, and has a loose appearance [334]. Elastin expression was also significantly higher in severe COPD patients compared with controls [334]. The perturbed elastin organisation in adult *Vangl2*^{Lp/+} lungs resembles the elastin disruptions observed in severe COPD [334]. In *Vangl2*^{Lp/+} lungs, elastin appeared as loose fibrils at the tips of the alveolar septa, compared with the tight bundles seen in WT littermates. However, in our study elastin

expression was not increased in *Vangl2*^{Lp/+} lungs. This difference may be due to the cause of the elastin disruption. In COPD patients, elastin structure becomes disorganised due to protease mediated alveolar damage [283]; whereas, in *Vangl2*^{Lp/+} lungs elastin disruption may occur as a result of poor elastin organisation in elastogenesis.

In line with this, *Vangl2*^{Lp/+} lungs showed a strong trend towards downregulation of *Emilin2* expression; however, the changes observed in *Emilin2* gene expression in *Vangl2*^{Lp/+} lungs was not significantly different from WT controls. Power calculations show that this test only had 17 % power, and more samples are required, with an estimated sample size of 30 individuals for an 80 % chance of seeing a statistically significant difference with a 95 % confidence level. Emilins are ECM proteins which are important for well organised elastin structure in elastogenesis [318,335]. Mice lacking *Emilin1* had fragmented elastin organisation, with a porous, irregular appearance, although the microfibril scaffold appeared normal [336]. Furthermore, in *Emilin1*^{-/-} mice, cells were no longer firmly adhered to the elastin in the basement membrane; the smooth muscle and endothelial cells in the aorta also showed increased frequency of morphological defects compared with WT control [336]. Thus, reduced *Emilin* expression in *Vangl2*^{Lp/+} lungs may result in perturbed elastin organisation, resulting in the loose, frayed appearance of elastin in the alveolar septa. The disruption of *Emilin* regulation can also directly impact on lung structure, as *Emilin1*^{-/-} mice display increased alveolar space in the lung parenchyma [337], similar to the *Vangl2*^{Lp/+} lungs. Moreover, EMILINs have also been associated with smoking injury. *EMILIN2* methylation which results in gene inactivation was increased in lungs of smokers, and was more prevalent in those with advanced stage tumours [338]. Together, these studies suggest that the perturbed *Emilin2* regulation observed in *Vangl2*^{Lp/+} lungs may result in altered elastin organisation. Furthermore, the disruption of *Emilin* regulation may in part contribute to the phenotype seen in the *Vangl2*^{Lp} lungs, as elastin disruption itself can perturb lung architecture [339]. However, further work is required to validate *Emilin2* protein levels in lungs of adult *Vangl2*^{Lp/+} mice by western blotting, and also assess its organisation in the lung tissue by immunostaining. It would also be interesting to assess *Emilin* expression in alveolarisation when elastin fibres are created and rearranged to promote cell migration. Thus, this set of experiments can elucidate if downregulation of *Emilin* expression results in perturbed elastogenesis, resulting in disrupted alveolar septation.

3.3.5. Altered MMP activity in *Vangl2*^{Lp/+} lungs may contribute to elastin remodelling

MMPs, such as MMP-9 and MMP-12, are important for elastin remodelling in tissue; therefore, we assessed MMP regulation in the lung and BALF. Previously, MMP-14 has been shown to be important in processes such as convergent extension (CE) in zebrafish [340]. Interestingly, *Vangl2* has been shown to be important for the regulation of MMP-14 activity by mediating its membrane trafficking [340]. *Vangl2* loss of

function mutation in zebrafish has been shown to increase MMP-14 activity that disrupts the ECM, causing CE defects [341].

As mentioned previously, MMP-2, MMP-9, and MMP-12 are commonly upregulated in lung diseases involving disrupted tissue repair, such as COPD [302–306]. *Vangl2*^{Lp/+} lungs showed increased expression of *Mmp-12*, which correlates strongly with the disrupted elastin distribution observed in these mice. However, neither pro-MMP-12 nor cleaved (active) MMP-12 protein levels were significantly increased in *Vangl2*^{Lp/+} lungs. MMP-12 is predominantly produced by macrophages, therefore as the whole lung homogenate was analysed in these experiments, it is possible that subtle changes in MMP-12 levels were not detected. Western blotting for MMP-12 in purified macrophages may reveal a significant difference in *Vangl2*^{Lp/+} lungs compared with WT littermates. In addition, as MMPs are released from cells before being cleaved to their active forms [307], therefore, BALF could be analysed by ELISA to assess total MMP-12 levels, and zymography used to measure MMP-12 activity.

Although, RT-PCR analysis showed that *Vangl2*^{Lp/+} lungs had significantly reduced expression of *Mmp-9*, total MMP-9 protein levels in BALF showed a trend towards upregulation in *Vangl2*^{Lp/+} mice. One possible reason for this difference may be that the RT-PCR was performed on lung tissue, whereas protein levels were studied in the BALF. Therefore, suggesting that *Mmp-9* regulation may differ between the two compartments. Interestingly, although total MMP-9 protein levels did not show a significant difference between the two genotypes, *Vangl2*^{Lp/+} mice had increased MMP-9 activity in the BALF. Although MMP-9 is classically defined as a gelatinase, it has also been shown to be involved in elastin remodelling [311]. Therefore, the increased MMP-9 activity could also contribute to the disrupted elastin fibre organisation in *Vangl2*^{Lp/+} lungs.

The altered MMP regulation in *Vangl2*^{Lp/+} lungs may arise as a direct result of the *Vangl2*^{Lp} mutation. Studies in murine models have also shown a link between Wnt signalling and *Mmp-12* expression, where Wnt7a stimulation increased *Mmp-12* expression and protein levels [342]. Lyu *et al* showed that the promoter region of *Mmp-12* contains binding sites for TCF/LEF, which is associated with canonical Wnt signalling, and AP-1 sites for c-Jun, which is involved in non-canonical Wnt signalling [342]. Suggesting that PCP signalling may directly regulate *Mmp-12* expression, leading to perturbed *Mmp* regulation in *Vangl2*^{Lp/+} lungs.

3.3.6. Altered macrophage population in lungs of *Vangl2*^{Lp/+} mice

Macrophages and neutrophils are prominent sources of MMP-12 and MMP-9 in the lungs. In response to tissue damage, immune cells are recruited into the lungs, and are a common feature of many chronic lung diseases [302,305]. In *Vangl2*^{Lp/+} lungs however, total macrophage numbers was not increased, nor any

quantitative differences in the BALF neutrophil population. However, there was an increased population of enlarged macrophages in *Vangl2*^{Lp/+} lungs which were also highly vacuolar. To characterise the phenotype of macrophages in the lungs, macrophage activation was studied. Depending on the local environment, macrophages can be activated into two major categories: classically activated (M1) macrophages involved in inflammation, and alternatively activated (M2) macrophages which are associated with immunosuppression and tissue repair [300]. In *Vangl2*^{Lp/+} lungs, we found an increase in M2 polarised macrophages, indicating that there is increased tissue remodelling occurring in *Vangl2*^{Lp/+} lungs. Interestingly, Jones *et al* reported that in postnatal lung development the macrophages in the lungs adopted an M2 polarisation [73]. The M2 skewed phenotype in *Vangl2*^{Lp/+} lungs suggest that lungs are undergoing remodelling, potentially as a result of the perturbed elastin organisation in these lungs, this in turn may drive the macrophage polarisation.

RT-PCR analysis in adult *Vangl2*^{Lp/+} lungs was performed from whole lung homogenate to determine macrophage polarisation. A limitation to this approach is that the analysis will contain gene expression from all lung cell populations, such as epithelial, mesenchymal, and endothelial cells in addition to the macrophage population. The presence of lung structural cells further limits the conclusions of this study. Although the gene expression of *Mrc1*, *Fizz1*, *Irf5*, and *Nos2* are frequently used to assess M1 and M2 polarisation [73,343,344], they can also be expressed on other cells types. *Fizz1* for example is expressed in alveolar cells and drives myofibroblast differentiation [345], and dendritic cell recruitment [346]. Therefore, to fully characterise the polarisation of alveolar macrophages, it is important to perform RT-PCR analysis from alveolar macrophages isolated from the BALF. As mentioned previously, the lung tissue also contains a large number in interstitial macrophages. The interaction of these distinct macrophage populations, and the epithelial cells, may be important in the remodelling phenotype observed, thus, further analysis of the interstitial macrophages will also be important for understanding the altered tissue environment in the *Vangl2*^{Lp/+} lungs.

Another limitation of this study is that it is still not clear how the enlarged macrophage phenotype arises. The *Vangl2*^{Lp} mutation itself may cause the altered macrophage phenotype, resulting in M2 skewed macrophages. Alternatively, the enlarged macrophages may arise as a secondary phenotype as a result of the aberrant tissue architecture in *Vangl2*^{Lp/+} lungs. The underlying tissue damage in *Vangl2*^{Lp/+} lungs may promote the activation of macrophages in the lungs. These macrophages would then be able to release mediators which contribute towards the altered immune cell phenotype. No previous connection has been shown between macrophages and PCP genes. Although interestingly, studies have shown that Scrib, a key PCP protein in lung development [188], is involved in T-cell polarity [347,348].

3.3.7. Conclusions

In this study, we identify a novel role for the PCP pathway in adult lung homeostasis. The data presented in this chapter demonstrate that a heterozygous *Vangl2*^{Lp} mutation, caused by a loss of function point mutation in *Vangl2*, is sufficient to disrupt cell migration, resulting in enlarged alveoli in adult mouse lung (Figure 3.14). These defects most likely arose from morphogenesis defects that affect both embryonic lung development and postnatal alveologenesis. Interestingly, the structural defects in *Vangl2*^{Lp/+} lungs correlated with abnormal lung function, resulting in reduced elastance and increased hysteresis in *Vangl2*^{Lp/+} lungs. In summary, the *Vangl2*^{Lp} mutation represents a novel genetic basis for adult lung disease.

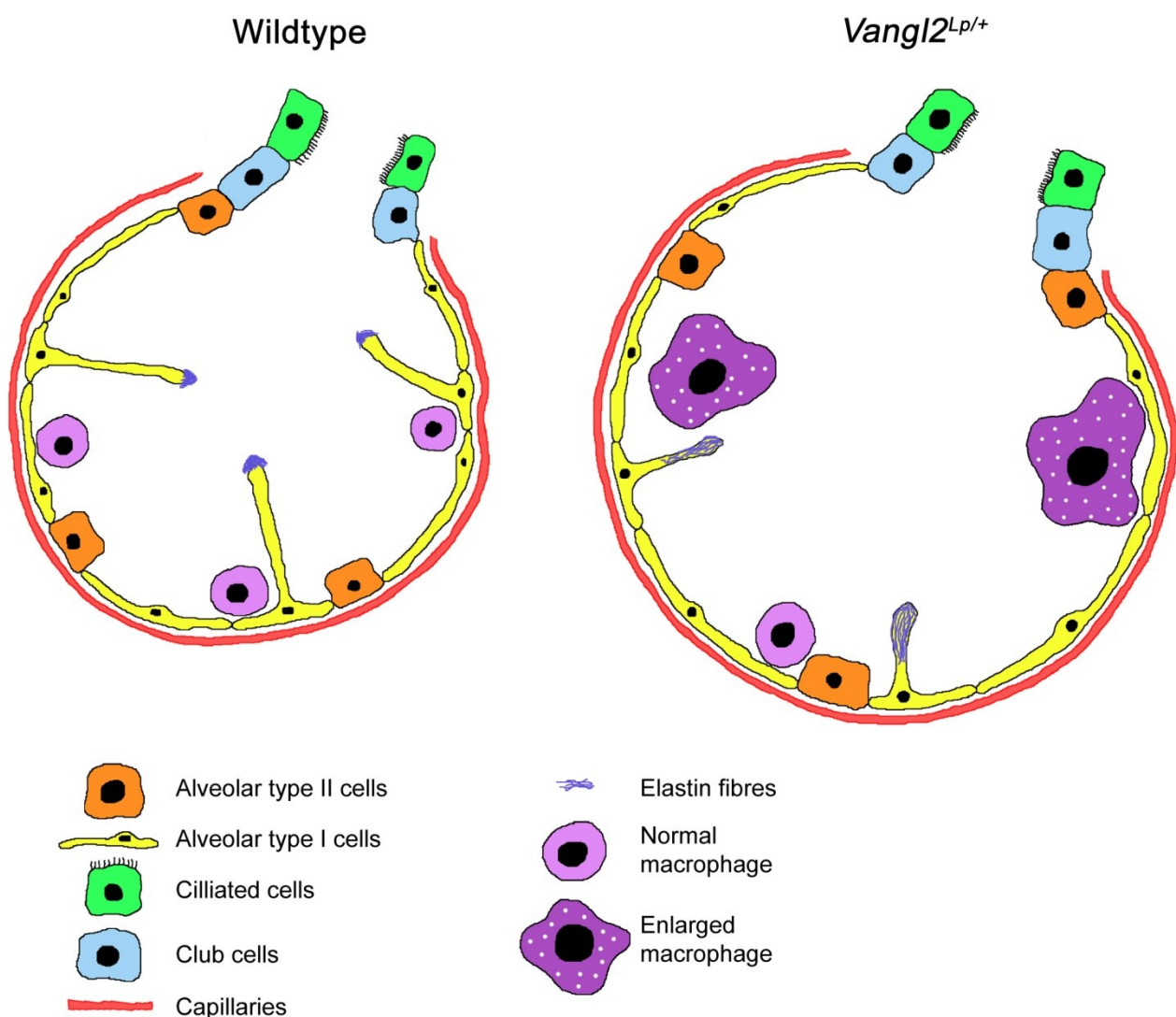


Figure 3.14. Schematic of structural alterations in *Vangl2*^{Lp/+} lungs

Comparison of alveolar architecture in WT and *Vangl2*^{Lp/+} mouse lungs showed alveolar airspace enlargement, with reduced septation in the *Vangl2*^{Lp/+} lungs. Furthermore, the elastin organisation was disrupted in the *Vangl2*^{Lp/+} mutant lungs, which had a loose elastin fibril organisation in the alveolar septa. Additionally, *Vangl2*^{Lp/+} mutant lungs had an increased proportion of enlarged macrophages, which were M2 polarised.

Chapter 4: Analysis of the actin cytoskeleton and cell-cell contacts in *Vangl2*^{Lp} mice

4.1. Introduction

One of the key downstream effects of the PCP signalling pathway is to mediate cytoskeletal reorganisation. The cell cytoskeleton is important for many functions such as cell motility, cell division, and structural support to maintain cell shape. The cytoskeleton has three main components: microtubules, intermediate filaments, and actin microfilaments (as discussed in *Chapter 1, Section 1.6.1*). Although all three structures play vital and varying roles in the cells, this study focused on the actin cytoskeleton which has previously been shown to be disrupted by PCP mutations [14,188,209]. Furthermore, PCP defects were found to perturb cell migration as a result of impaired cytoskeletal reorganisation [14,349,350].

Cell migration is a highly dynamic process; a key mechanism that enables cells to move is the continual polymerisation and de-polymerisation of actin filaments. At the leading edge of the cell, actin filaments form the lamellipodium which causes the cells to protrude [351]. The lamellipodium is formed by a mesh of cross-linked branched F-actin, which is mediated by the Arp2/3 complex, resulting in a gel-like actin network. At the barbed end, F-actin, which is pointed towards the leading edge, undergoes polymerisation causing the membrane to protrude. At the pointed end, F-actin is depolymerised primarily by cofilin (Cfl) [351]. The polarised F-actin polymerisation and depolymerisation is known as treadmilling. The lamellipodium consists of actin-myosin structures which provide the contractile force to mediate cell migration [351]. The trailing edge, which is at the back of the cell, also contains stable actin-myosin interactions which contract to push the cell forward.

The process of directed cell migration is intricately linked to the actin cytoskeleton, not only for promoting cell migration, but also for regulating the coordinated migration of cells in a uniform manner. Reffay *et al*, showed the importance of RhoA, a small GTPase which acts downstream of PCP signalling to mediate cytoskeletal dynamics in driving collective cell migration in response to mechanical and biochemical signals [352,353]. They showed that in cell migration, “leader” cells migrated forward, forming finger like projections which pull the other “follower” cells forward. This process places tension on the actin cytoskeleton in the surrounding cells to control coordinated migration of the epithelial sheet. This tension also acts to prevent the initiation of new leader cells [353]. Polarised RhoA was found to be a key regulator of this process, with high RhoA activity in “leader” cells at the migrating edge to pull cells forward, and to the rear of the “follower” cell to provide the pushing forces. Through this, they showed an important role for RhoA activity and mechanical tension in mediating direct migration of the epithelial sheet [353].

The PCP pathway has been shown to be essential in regulation of cell migration in many systems. One particular aspect in which PCP signalling has been shown to be important for is convergent extension (CE) [354]. In mice, CE is important for neurulation, the process by which vertebrate embryos form the neural tube. Mice which harbour PCP defects, such as those with the *Vangl2*^{Lp} mutation, have disrupted neural tube closure due to defective CE [266,355]; this results in an open neural tube in homozygote

Vangl2^{Lp} mutant mice leading to neonatal death. These defects in CE arise from deficiencies in both collective cell migration and cell intercalation [263]. Similarly, *in vitro* models have shown that *Vangl2* is able to regulate cell migration [253]. Lindqvist *et al* performed knockdown or overexpression of *Vangl2* in MDCK cells and showed decreased and increased cell migration respectively [253].

In addition to the core PCP proteins, mutations in PCP effector proteins, such as Fuzzy, have also been associated with defective cell migration. *Fuzzy* mutations have been shown to result in PCP defects leading to perturbed CE and neural tube closure [356]. Furthermore, Fuzzy is important in cells for the formation of the primary cilium, which is an organelle necessary for signal transduction and polarity [357]. Zilber *et al* showed that mouse embryonic fibroblasts (MEF) derived from *Fuzzy* knockout mice showed defective cell migration and polarity in wound healing *in vitro* [357]. In the skin epidermal cells, the *Vangl2*^{Lp} mutation alone is insufficient to disrupt wound healing [284]. Similarly, mice with either heterozygous or homozygous knockout of Grainyhead like 3 (*Grhl3*), a PCP mediator protein important in neural tube closure [358], also showed no defects in wound healing. However, mice which were heterozygous for both *Vangl2* and *Grhl3* had perturbed wound healing [284]. Together, these studies highlight an important role for PCP signalling in cell migration.

4.1.1. Actin interacting proteins

To facilitate the rapid F-actin dynamics necessary for cell migration many actin adaptor proteins are involved. One such actin adaptor associated with PCP signalling is cofilin (Cfl), the mammalian orthologue of *Drosophila* twinstar (tsr). Cfl is a member of the actin depolymerising factor (ADF)/Cofilin family of small actin binding proteins and has 70% homology to ADF, also known as Destrin [359]. Cfl is present in all eukaryotic cells and plays a crucial role in actin severing dynamics. Unusually, cofilin is able to bind to both actin monomers and filaments. Cfl is crucial in many cellular processes such as endocytosis, morphogenesis, neural plasticity, and inflammation, as well as in diseases such as cancer metastasis [360,361].

In mammals, there are two Cfl homologues: Cfl1 which is found in non-muscle cells and is the most abundant form, and Cfl2 which is found in muscles. The primary function of Cfl1, which from this point forward will be referred to as Cfl, is to bind to and sever uncapped actin filaments [362]. For severing, one Cfl molecule is required per actin monomer. Cfl binds to and coats GDP-F-actin with higher affinity compared with either ATP- or ADP+ Pi-F-actin [363]. This causes Cfl to bind to the “older” actin in the filament, thereby allowing rapid turnover of actin filaments which is necessary during directed actin growth for processes such as directed cell migration. Furthermore, the low affinity of Cfl for ATP-F-actin results in this portion of the filament being protected from Cfl mediated cleavage. The binding of Cfl to ADP-F-actin results in increased twisting of the F-actin, causing it to shrink. The added strain from the Cfl mediated

twisting of F-actin weakens the bonds allowing them to be more easily severed, promoting actin disassembly [363].

Cfl activity is regulated by phosphorylation and dephosphorylation of the Ser3 amino acid [364]. Slingshot (Ssh) is the major phosphatase associated with activation of Cfl by mediating dephosphorylation of Cfl Ser3 [361]. Dephosphorylation of Cfl allows it to bind to F-actin, as phosphorylated Cfl (P-Cfl) is unable to do so [364]. Conversely, deactivation of Cfl is mediated by phosphorylation of Ser3 by LIM Domain Kinase (LIMK)1 and LIMK2 [365,366]. LIMK itself is activated by various kinases such as ROCK [361,366], which are Rho proteins that acts downstream of the PCP pathway.

The role of *tsr* in PCP was first demonstrated in the *Drosophila* wing. In *Drosophila* wings, wing hairs are orientated in a uniform direction and are exclusively found at the distal edge of the cell. However, *tsr* mutants show classical PCP defects such as misaligned wing hair which do not occur at the distal edge of the cell but were more variable [228]. Furthermore, the *tsr* mutation resulted in mislocalisation of Fz and Flamingo (Fmi), the *Drosophila* homologue of Celsr, as a result of defective actin turnover [228]. In line with this, overexpression of constitutively active LIMK which inhibits *tsr* activity, thereby perturbing actin reorganisation, phenocopied the *tsr* mutation resulting in similar PCP defects, such as wing hair defects [228].

These findings are further supported by work in *Cfl* mutant (*Cfl^{CS}*) mice. Mahaffey *et al* reported that *Cfl* mutation alone did not cause PCP defects in mice, although NTD and outflow tract defects were observed [229]. However, mice double mutant for *Cfl^{CS}* and *Vangl2^{Lp}* had a more severe phenotype compared with those with a single mutation [229]. Similar to the *Drosophila* model, reduction of actin severing ability in mice with *Cfl^{CS}* and *Destrin^{Corn1/+}* double mutation resulted in mislocalisation of Vangl2 and Celsr1 [229]. However, actin dynamics seem only to be important in initiation of polarisation of Vangl2 and Celsr1 and are not required to maintain polarisation, as inhibition of actin dynamics following initiation of polarity had no effect on localisation [229]. Together, these results show a conserved role for actin reorganisation to mediate PCP signalling throughout evolution.

In addition to Cfl, other actin cytoskeletal regulators are necessary in migration. Another important family of actin cytoskeleton interacting proteins are the Ezrin/Radixin/Moesin (ERM) complex proteins. The ERM proteins have a very similar amino acid structure sharing 70-85 % homology among each other [367]. The ERM proteins are able to act as both scaffold and signalling proteins and contain three key domains. An N-terminal Four point one ERM (FERM) domain which is able to interact with membrane adhesion proteins such as CD44 and ICAM1 [231,232], central α -helical coiled-coil domain, and a C-terminal actin interacting domain [368,369]. In the inactive state ERM proteins cross-link their N- and C-terminal tails to prevent binding [370]. ERM proteins are activated by phosphorylation of a highly conserved C-terminal threonine residue (Ezrin-T567, Radixin-T564, Moesin-T558) in a Rho-dependent manner resulting in the N- and

C-terminal ends being able to bind to membrane proteins and actin respectively [371]. The ERM proteins play essential roles in cell migration, and it has been shown that perturbed activity of all three ERM proteins, even individually, results in reduced cell migration [372–374]. Recent studies have also shown a role of ERM complex proteins in planar polarity [375]. In the intestine, mice exhibit planar polarity where mitotic spindles are parallel to the apical surface of the villi, and cells are orientated along the crypt-villus axis. In *Ezrin* null (*Ez*^{-/-}) mice, the mitotic spindles remain parallel to the apical surface, however, the spindle orientation was randomly organised and fewer cells were aligned along the crypt-villus axis [375]. Demonstrating that ezrin plays a role in regulation of cell polarity in the intestine.

4.1.2. Role of Vangl2 at cell-cell junctions

The actin cytoskeleton is indirectly linked to the adherens junctions via α - and β -catenin adaptor proteins. This interaction between adherens junction proteins and the actin cytoskeleton plays an important role in mechanotransduction and transmission of signals to cells. Previous studies have shown the PCP pathway to be involved in regulation of cell junctions. These studies demonstrated that Vangl2 is involved in regulation of the adherens junctions by promoting cadherin turnover at the cell membrane by endocytosis [252,376]. Nagaoka *et al* showed that Vangl2 regulates E-cadherin surface localisation, with *Vangl2* mutations leading to mislocalisation of E-cadherins [252]. In primary epithelial cell derived from *Vangl2*^{LP/+} kidneys, E-cadherin localisation was increased at the cell membrane compared with the controls [252]. Similarly, Vangl2 has also been shown to regulate another classical cadherin, N-cadherin. Overexpression of Vangl2 in mice resulted in low or absent N-cadherin localisation at the cell membrane, thereby perturbing cell-cell adherens junctions [253]. These studies showed that Vangl2 is able to interact directly with both N- and E-cadherin to form a complex. This interaction is mediated using the Pk binding domain of Vangl2 and β -catenin binding domain of cadherins [252,376]. Furthermore, other studies have shown disruption of Vangl2 by both overexpression and knockdown results in weakened cell-cell and cell-ECM adhesion *in vitro*, as well as altered cell morphology [253]. This is thought to be mediated via interaction with Rac1 [253], which has previously been implicated in regulating cell adhesion in epithelial cells [377]. In cells, Rac1 localisation overlaps with Vangl2 at the plasma membrane, and disruption of PCP signalling by Vangl2 overexpression results in increased cytoplasmic Rac1 localisation [253]. Together, these studies demonstrate that Vangl2 is necessary for regulation of adherens junctions to maintain epithelial cell integrity.

In addition to Vangl2, Wnts have also been shown to regulate cadherin localisation. Overexpression of *Wnt7a*, which has been implicated in PCP signalling in the mammalian cochlea [378], perturbed the enrichment of F-actin at adherens junctions in the mouse neural tube; whereas in WT mice F-actin co-localised with adherens junctions [379]. This correlated with perturbed N-cadherin localisation, where

Wnt7a overexpression in mice resulted in reduced N-cadherin localisation at adherens junctions which appeared discontinuous [379]. Similarly, overexpression of another non-canonical Wnt, *Wnt11*, in zebrafish resulted in E-cadherin mislocalisation with increased punctate staining in the cytoplasm, indicating an important role for Wnt11 in the subcellular localisation of E-cadherin [380].

In addition to the adherens junctions, tight junctions are also crucial for maintenance of epithelial sheets. In the murine kidney, Vangl2 co-localises with the tight junction protein ZO-1, which is part of the tight junction complex used to connect the transmembrane claudins to the actin cytoskeleton [211]. Furthermore, in the embryonic mouse lungs, the PCP effector protein Scrib also co-localised with ZO-2 [188]. However, in embryonic *Scrib^{Crc/Crc}* lungs, ZO-2 is mislocalised and apical enrichment is lost [188]. The *Scrib^{Crc}* mutation also causes similar mislocalisation of another tight junction protein, Claudin 18 in the embryonic lungs [188], thereby, resulting in weakened epithelial integrity. Interestingly, the *Scrib^{Crc}* mutation did not disrupt apical localisation of ZO-1 [188], another member of the ZO family which also interacts with Scrib [381]. Thereby, indicating that Scrib interaction with tight junction proteins may be selective. Together, these studies have shown that PCP signalling plays an important role in mediating regulation and localisation of both adherens and tight junctions proteins in order to maintain the integrity of epithelial sheets.

An increasingly popular method for real-time analysis of key cell functions are impedance based systems which can dynamically assess cell adhesion and barrier function; one such system is iCELLigence. In this machine, electrodes at the bottom of a multi-well culture plate create an electrical flow using the cell culture media as an electrolyte (Figure 4.1). When no cells are present current is able to flow freely between electrodes with little resistance, thereby setting the baseline reading of electrical resistance (impedance). When cells are introduced, they attach to the bottom of the wells on top of the electrodes. The insulating properties of the cell plasma membrane results in disruption to the flow of the electrical current causing increased impedance. As the number of cells increase or form more mature junctions, the impedance increases proportionally. The measure of impedance is an indirect measure of changes to the cell properties, as the machine only measures the change in electrical resistance. However, within a given biological context it is possible to gather data from the cellular processes occurring via the change in impedance.

A key advantage of the iCELLigence system is that the electrical measurements are non-invasive and do not impact on the cell. Using this system, changes in cell impedance can be measured repeatedly over time, allowing measurement of cell dynamics in real time. This is useful when measuring barrier function as the dynamic changes caused by barrier disruption and recovery can be assessed in a quantitative manner.

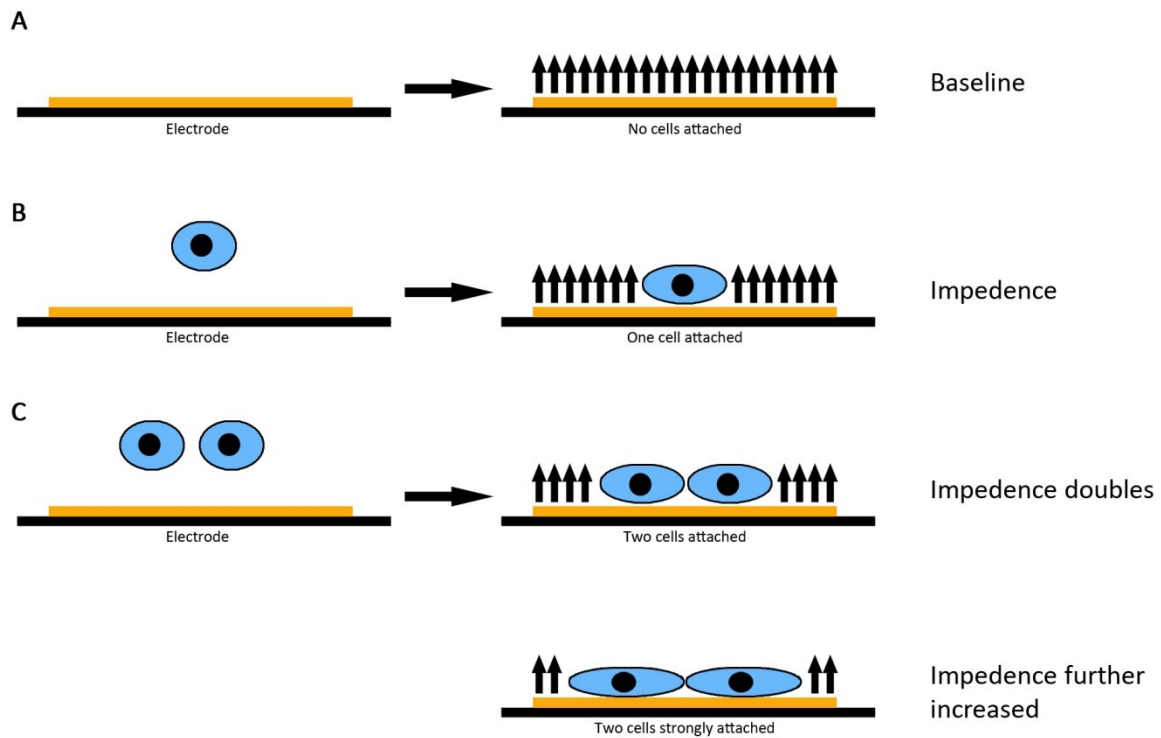


Figure 4.1. Measurement of cell barrier integrity by electrical impedance

(A) In the absence of cells there is little impedance of the electrical current, which sets the baseline measurement. (B) When cells are seeded on to the electrodes, it insulates the electrodes that are in contact with the cells resulting in increased resistance, thereby increasing impedance. (C) As the cells begin to change character e.g. proliferate (increasing in number), or form mature cell junctions and attach strongly to the base of the well, the electrical impedance increases further.

4.1.3. Aims and hypothesis

The PCP pathway is known to play a significant role in cytoskeletal regulation in processes such as directed cell migration and adherens junction integrity. We hypothesised that disruption of PCP signalling, by *Vangl2^{lp}* mutation, would perturb cell migration, by affecting actin cytoskeleton dynamics, and this in turn would also impact on epithelial integrity by affecting the adherens junctions. To test these hypotheses we:

- Evaluated the impact of the *Vangl2^{lp}* mutation on actin cytoskeleton regulation.
- Performed wound healing assays to assess the impact of VANGL2 knockdown on modulation of repair following injury.
- Assessed regulation of E-cadherin at intracellular adherens junctions in embryonic and adult *Vangl2^{lp}* mice.
- Assessed cell junction integrity by measuring cell barrier function using impedance measures.

4.2. Results

4.2.1. Cortical actin organisation is disrupted in embryonic *Vangl2*^{Lp/+} lungs

Disrupted actin organisation has previously been shown in embryonic homozygous *Vangl2*^{Lp/Lp} lungs [14]. To determine whether the actin cytoskeleton was also disrupted in heterozygous *Vangl2*^{Lp} lungs, F-actin organisation was analysed in E18.5 lungs by phalloidin staining. In WT mice, cortical actin formed a continuous ring around the cells at the plasma membrane so that individual cells were clearly defined (Figure 4.2A). However, in embryonic *Vangl2*^{Lp/+} lungs at the same age, cortical F-actin structure was disrupted, and was often discontinuous around the perimeter of the cells (Figure 4.2B). Nuclear stain using DAPI in embryonic tissue shows the tissue structure in embryonic lungs to highlight the disrupted actin structure (Appendix A: Figure 3).

To investigate the molecular mechanism underlying the altered cytoskeletal organisation in *Vangl2*^{Lp} mutant mouse lung, an RT-PCR array was performed to compare the expression levels of key cytoskeleton genes between E18.5 WT and *Vangl2*^{Lp/Lp} lungs. For this analysis, homozygous *Vangl2*^{Lp} lung were studied as these were likely to exhibit greater differences in gene expression levels from WT than heterozygous *Vangl2*^{Lp} mice. The transcript levels of 84 cytoskeletal genes were assessed. This analysis identified nine genes with a fold change greater than 1.5 between WT and *Vangl2*^{Lp/Lp} littermates (Figure 4.2C,D and Table 4.1). The heat map showed that *Cfl* expression was high in WT lungs but was downregulated in *Vangl2*^{Lp/Lp} lungs. On the other hand, *Cdc42ep2*, *Cit*, *Cyfp2*, *Fscn2*, *Mark2*, *Nck2*, *Pak1*, and *Vasp* showed low expression levels in WT lungs but were upregulated in the *Vangl2*^{Lp/Lp} lungs (Figure 4.2D).

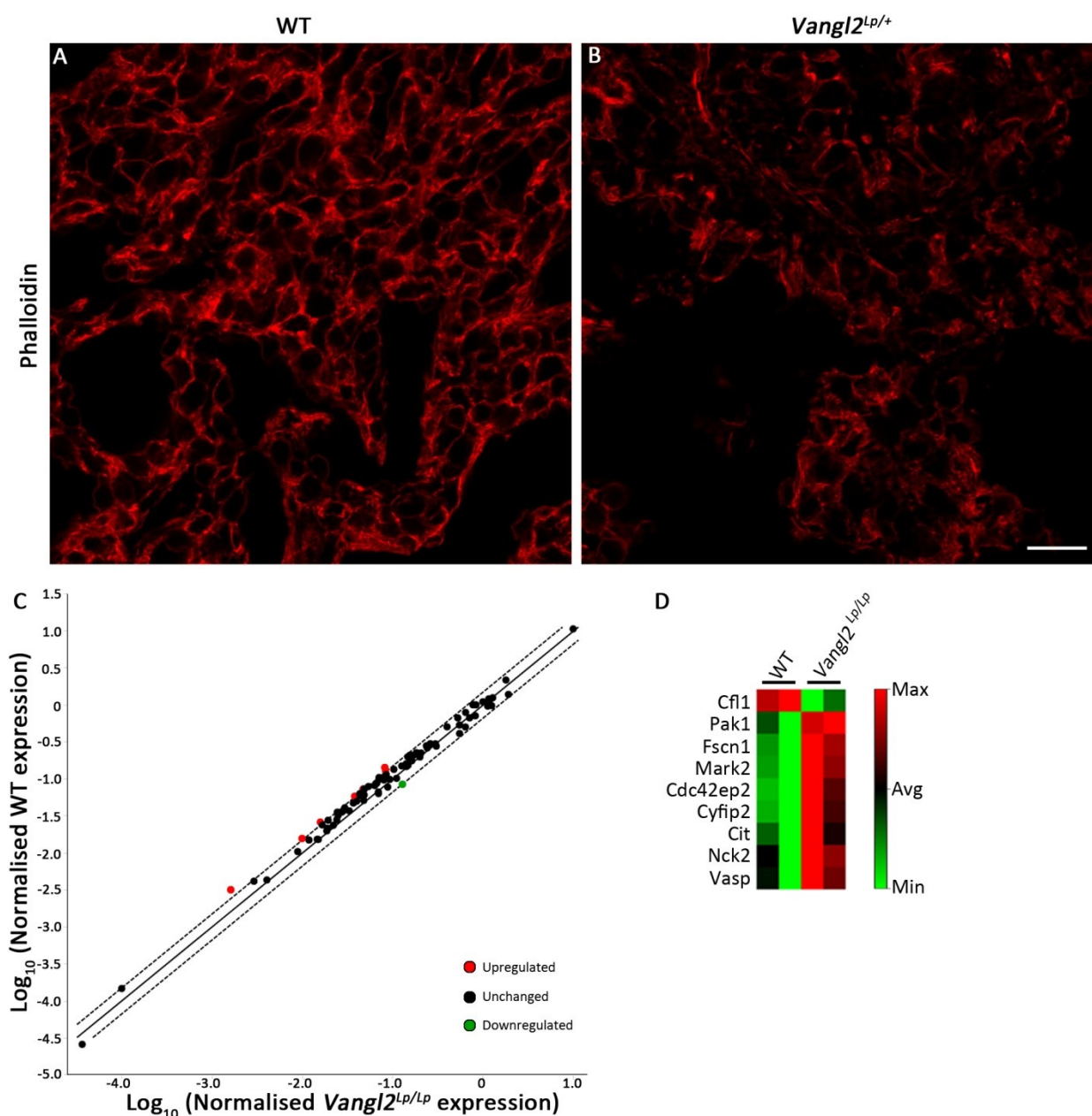


Figure 4.2. Cortical F-actin organisation is disrupted in embryonic *Vangl2*^{Lp} lungs

Phalloidin staining of cryosections showing distribution of F-actin in lungs of E18.5 **(A)** WT and **(B)** *Vangl2*^{Lp/+} mice. WT lungs show a continuous ring of cortical F-actin around the cell, whereas cortical F-actin in *Vangl2*^{Lp/+} lungs was disjointed and did not form a continuous ring. **(C)** Scatter plot comparing gene expression of cytoskeletal regulators in embryonic E18.5 WT and *Vangl2*^{Lp/Lp} lungs shows genes which were upregulated (red dots) and downregulated (green dots) by greater than 1.5 fold change or showed no change (black dots) (n=2 per group). **(D)** Heat map of expression for genes which showed greater than 1.5 fold change in WT and *Vangl2*^{Lp/Lp} lungs (n=2 per group). Scale bar **(A,B)** 14 μ m.

Down regulated genes	Up regulated genes
Cofilin1 (1.5361)	Cdc42ep2 (1.5013)
	Cit (1.6304)
	Cyfp2 (1.5154)
	Fscn2 (1.9503)
	Mark2 (1.7233)
	Nck2 (1.5355)
	Pak1 (1.5645)
	Vasp (1.5291)

Table 4.1. Table of genes showing greater than 1.5 fold change in embryonic *Vangl2*^{Lp/Lp} lungs

Genes showing greater than 1.5 fold change in embryonic *Vangl2*^{Lp/Lp} mice compared with WT mice. The fold change seen in *Vangl2*^{Lp/Lp} mice compared with WT is shown in the brackets.

4.2.2. Cofilin phosphorylation is reduced in embryonic *Vangl2*^{Lp} mutant lungs

To further analyse the actin disruption in *Vangl2*^{Lp} lungs, actin cytoskeletal regulators were studied in greater detail. Cfl regulation was studied further as *Cfl* expression was downregulated in embryonic *Vangl2*^{Lp/Lp} lungs (Table 4.1). Interestingly, previous studies have also shown Cfl to be involved in PCP signalling [228,229]. Cfl regulation was assessed by western blot in E14.5 mouse lungs as this is a period when extensive morphogenesis and cell migration occurs in the lungs.

Analysis of total Cfl levels by western blotting showed no significant difference between WT nor heterozygote and homozygote *Vangl2*^{Lp} mutant lungs (Figure 4.3A,B). However, a striking reduction in the level of phospho-Cfl (P-Cfl), the inactive form, was observed in both heterozygote (70% reduction) and homozygote (57 % reduction) *Vangl2*^{Lp} lungs compared with WT littermates (Figure 4.3A,C). Together these results indicate that regulation of Cfl activity is altered by the *Vangl2*^{Lp} mutation.

We also analysed Cfl regulation in 10 week old adult lungs, a time when very little migration occurs. Interestingly, adult *Vangl2*^{Lp/+} lungs did not show a significant difference in either total Cfl (Figure 4.3D,E) or P-Cfl levels (Figure 4.3D,F) compared with WT littermate lungs. Cfl regulation in adult WT and *Vangl2*^{Lp/+} lungs following lung function analysis by Flexivent was also assessed. The Flexivent system exerts mechanical strain on the lungs as the technique involves forced oscillation manoeuvres. The degree of strain is above that which occurs during normal breathing, therefore, lung tissue that had undergone Flexivent was used as a surrogate for injurious mechanical strain. Following Flexivent analysis, total Cfl levels in the adult lungs did not show a significant difference between *Vangl2*^{Lp/+} and WT littermates (Figure 4.3G,H). However, P-Cfl was not detectable in lungs following mechanical strain, although the Gapdh loading control was present (Figure 4.3I).

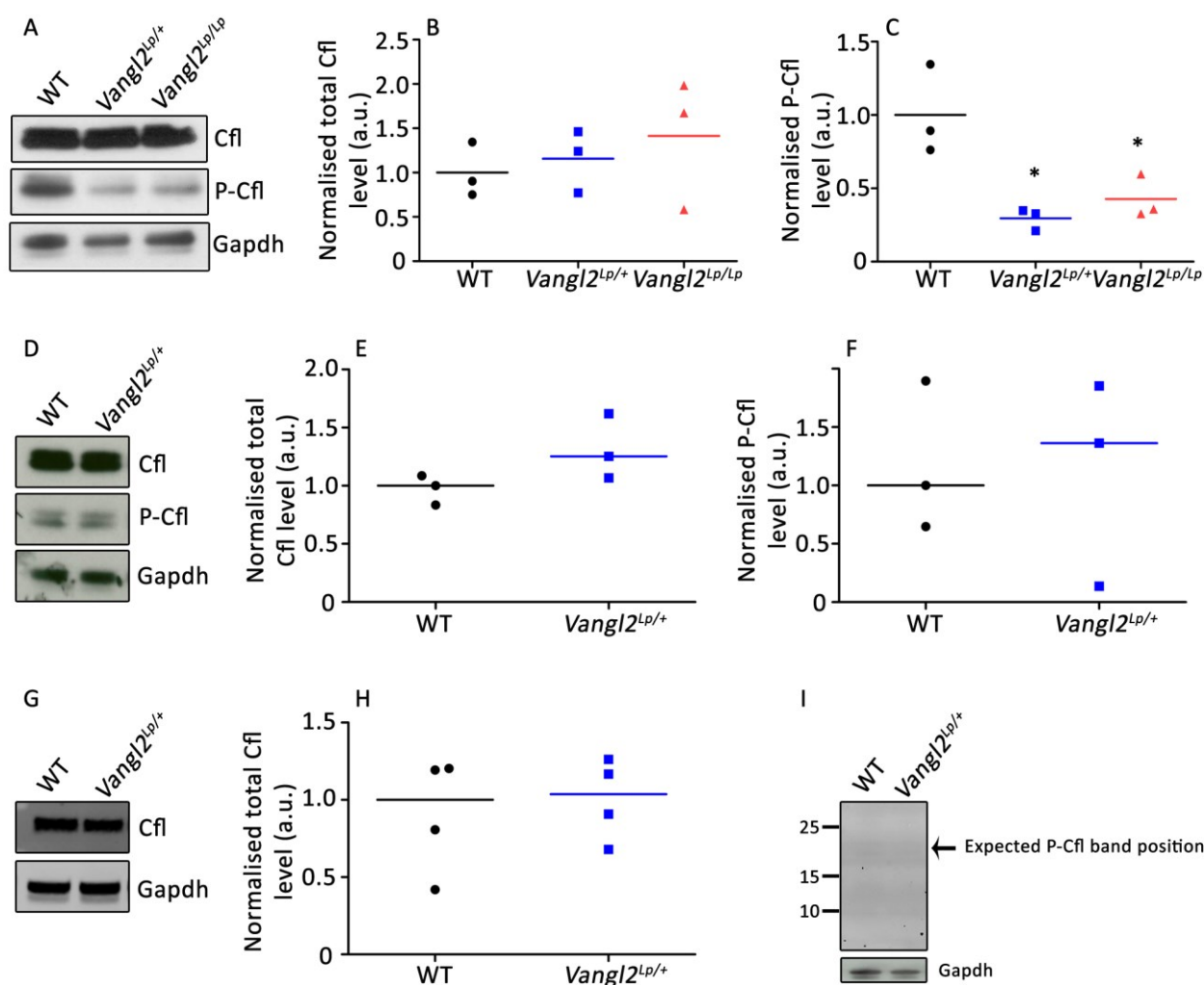


Figure 4.3. Altered Cfl protein regulation in embryonic *Vangl2*^{Lp} mutant lungs

(A) Western blot of Cfl, P-Cfl (both 19 kDa), and Gapdh in total lung protein lysate from E14.5 WT, *Vangl2*^{Lp/+} and *Vangl2*^{Lp/Lp} lungs. (B) Quantification of total Cfl levels in WT, *Vangl2*^{Lp/+}, and *Vangl2*^{Lp/Lp} littermates, revealed no significant differences (each point shows an individual replicate, with each replicate containing pooled lungs from two mice, student's T-test $P > 0.05$). (C) P-Cfl levels were significantly reduced in both *Vangl2*^{Lp/+} and *Vangl2*^{Lp/Lp} lungs compared with WT littermates (each point shows an individual replicate, with each replicate containing pooled lungs from two mice, student's T-test $P < 0.05$ *). (D) Western blot of Cfl, P-Cfl and Gapdh in total protein lysate from 10 week old WT and *Vangl2*^{Lp/+} mouse lungs. Quantification of (E) total Cfl and (F) P-Cfl levels in adult WT and *Vangl2*^{Lp/+} littermates revealed no significant differences (each point shows an individual mouse, Mann-Whitney U $P > 0.05$). (G) Following lung function in adult mouse lungs western blot analysis showed detectable levels of total Cfl. (H) Quantification of total Cfl in adult lungs following lung function showed no significant difference between *Vangl2*^{Lp/+} and WT littermate mice (each point shows an individual mouse, Mann-Whitney U $P > 0.05$). (I) P-Cfl was undetectable by western blot analysis in both adult WT and *Vangl2*^{Lp/+} mice following lung function (the black arrow shows the expected position of P-Cfl), and Gapdh loading control.

4.2.3. VANGL2 knockdown decreases rate of cell migration and perturbs cell polarity in wound healing

To test the functional impact of the altered actin organisation, cell migration was analysed during wound healing in a scratch assay, a process for which the actin cytoskeleton is critically required. Prior to conducting scratch assays, the efficiency of knockdown achieved with VANGL2 antisense morpholino oligonucleotides (MO) was validated. Human adenocarcinoma alveolar epithelial cells (A549) were cultured in the presence of control or anti-VANGL2 MO for up to 96 hr, as this was the total duration of the scratch assay. Immunohistochemistry was used to assess VANGL2 protein levels. More diffuse and weaker staining was observed in cells treated with VANGL2 MO compared with the control (Figure 4.4A,B). The level of knockdown was then quantified using a previously validated method to analyse the intensity of staining [382]. Cells treated with VANGL2 MO showed 48.8 % reduction in VANGL2 protein levels compared with control treatment (Figure 4.4C).

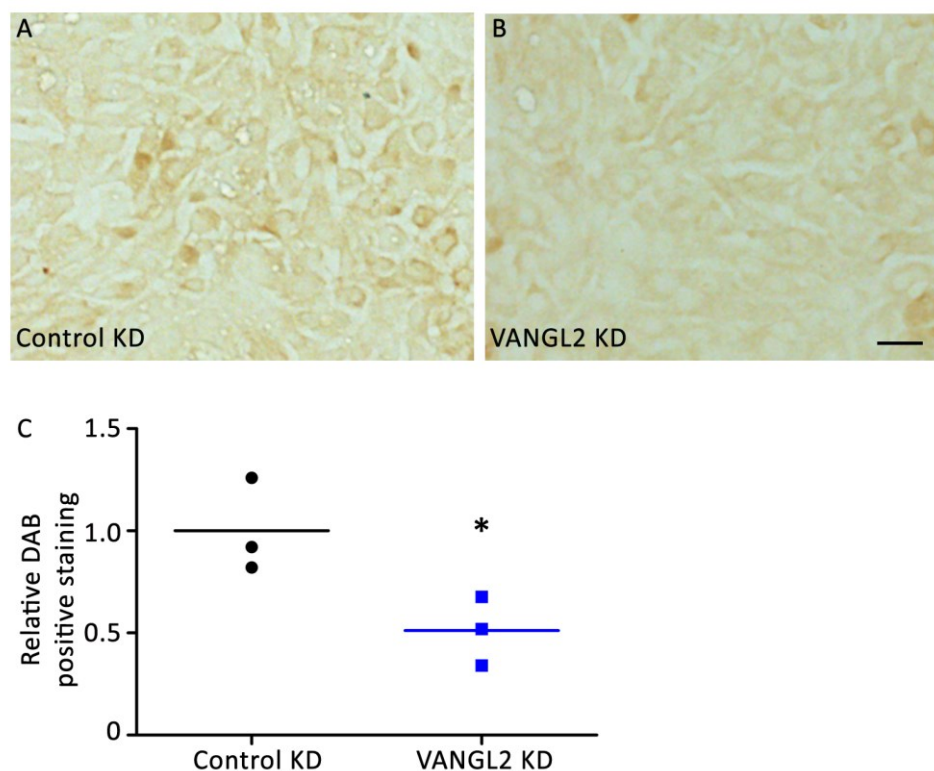


Figure 4.4. VANGL2 MO caused knockdown of VANGL2 protein levels in A549 cells

(A,B) Representative immunostaining for VANGL2 in cells treated with (A) control or (B) anti-VANGL2 MO. (C) Quantification of VANGL2 knockdown efficiency following 96 hr of MO treatment (each point shows an individual replicate, student's T-test $P < 0.05$ *). Scale bar 8 μ m. Abbreviations: knockdown (KD).

Having confirmed that MO treatment effectively reduced VANGL2 levels in A549 cells, the rate of wound healing in these cells following a scratch injury was assessed. Cells were cultured with either control or VANGL2 MO to confluency over 72 hr, following which a scratch was made down the centre of each well using a P1000 tip. The scratch was then imaged at 0 hr and 24 hr, and the area of the wound covered was quantified to assess the rate of wound healing after 24 hr. VANGL2 knockdown in A549 cells significantly reduced the rate of wound healing by 30 % compared with control cells (Figure 4.5A-E). In a complementary experiment, the PCP pathway was stimulated using Wnt5a, a non-canonical PCP Wnt ligand, during wound healing. Assessment of wound healing after 18 hr showed a 94 % increase in the area healed with Wnt5a treatment compared with control cultures (Figure 4.5F). Together, these results indicate that modulation of the PCP pathway is able to regulate the rate of cell migration in wound healing.

In scratch assay experiments, cells adopt a uniform polarity so they can migrate in the same direction (directed cell migration) to efficiently close the wound. One previously described method of assessing cell polarity in migration is to determine the position of the Golgi body of each cell in relation to the wound edge [284]. In directed cell migration, the Golgi is positioned in front of the nucleus facing in the direction of migration; however, when cell polarity is lost the Golgi is disorientated and does not face in the direction of migration. Therefore, cell polarity was assessed during migration by immunostaining cells with the GM130 Golgi marker 24 hr post scratch injury in A549s treated with either control or VANGL2 MO (Figure 4.5G,H). The number of polarised cells was quantified by placing a 120° arc on the nucleus of cells at the leading edge; cells with Golgi within the 120° arc facing the direction of migration were classed as polarised. Knockdown of VANGL2 resulted in significantly fewer polarised cells (42.4 %) compared with control (62.29 %) (Figure 4.5I).

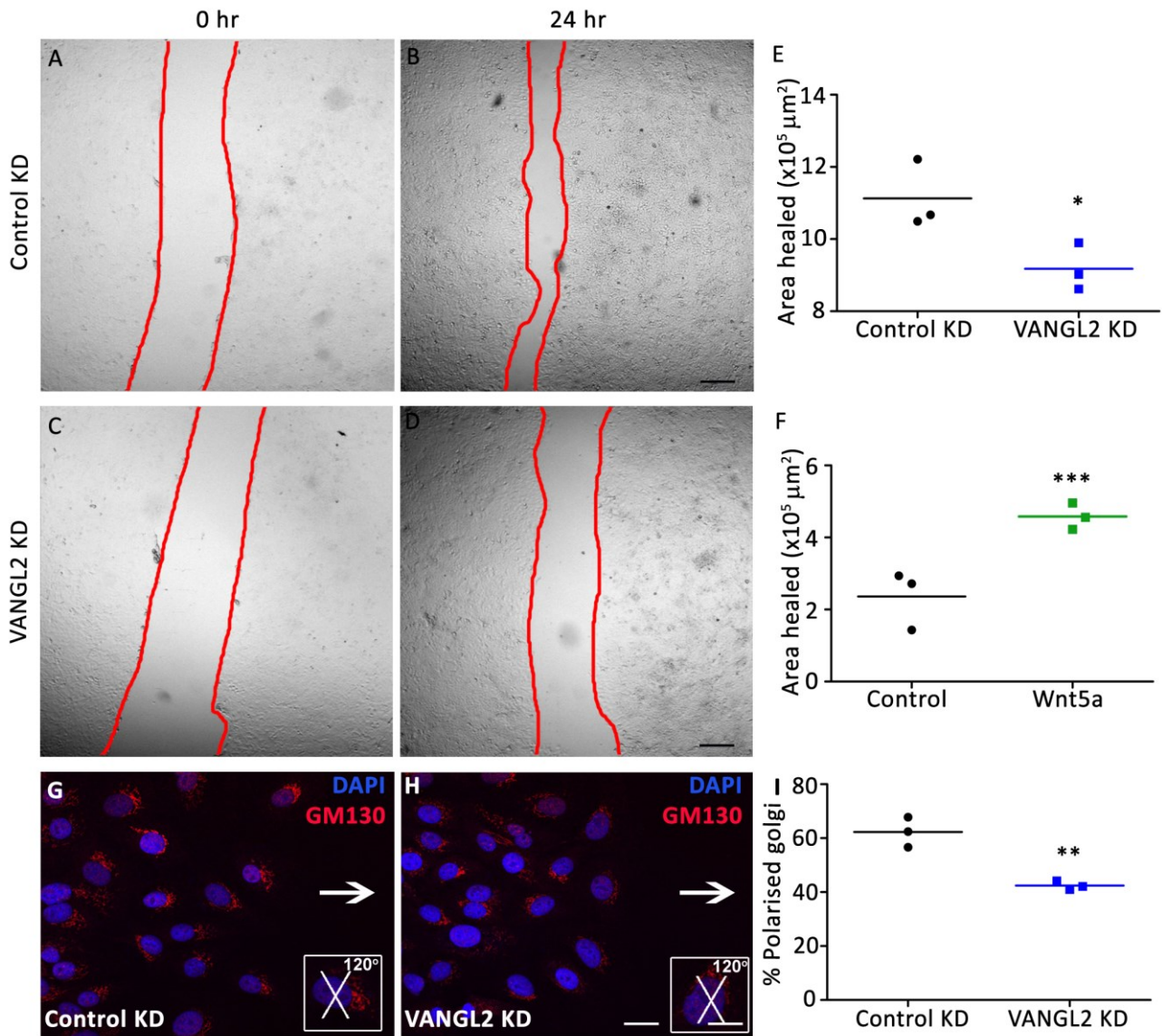


Figure 4.5. PCP signalling modulates the rate of cell migration in wound healing

(A-D) Representative images of A549 cells cultured with (A,B) control or (C,D) VANG2 MO at (A,C) 0 hr and (B,D) 24 hr post scratch, the wound edge is marked by the red line. (E) Quantification of the area of wound healed in control and VANG2 KD cells at 24 hr post scratch (each point shows an individual replicate, student's T-test $P < 0.05$ *). (F) Quantification of area of wound healed in control and Wnt5a treated cells 18 hr post scratch (each point shows an individual replicate, student's T-test $P < 0.001$ ***). (G,H) Anti-GM130 staining for Golgi body (red) localisation to determine polarity of cells at the leading edge during cell migration (white arrow signifies direction of migration). Insert in G and H show examples of crosses placed on individual cell nuclei that were used to determine cell polarisation. (I) Quantification of polarised cells with Golgi facing towards the direction of migration showed a significant reduction following VANG2 KD (each point shows an individual replicate, student's T-test $P < 0.01$ **). Scale bars; (A-D) 322 μm , (G,H) 21 μm , inserts 11 μm .

4.2.4. Embryonic mice show a trend toward increased ERM phosphorylation

Another group of cytoskeleton associated proteins important for cell migration is the ERM complex [372–374]. In a recent study, Ezrin, a ERM complex member, was shown to be important for planar polarity in the mouse intestine for aligned orientation of mitotic spindle along the crypt-villus axis [375]. In the RT-PCR cytoskeletal array we found a trend towards reduced *Moesin* (*Msn*) expression (1.19 fold) and increased *Radixin* (*Rdx*) expression (1.24 fold) in E18.5 *Vangl2*^{Lp/Lp} lungs compared with WT littermates (data not shown); *Ez* expression however, was not changed. Therefore, levels of total and P-ERM were assessed to study differences in regulation at E14.5 and 10 week old lungs of WT and *Vangl2*^{Lp} mice by western blotting. At E14.5, the levels of total ERM did not differ between WT and either heterozygote or homozygote *Vangl2*^{Lp} mice (Figure 4.6A,B). A strong trend towards increased P-ERM was observed in both *Vangl2*^{Lp/+} (4.1 fold) and *Vangl2*^{Lp/Lp} (3.7 fold) lungs compared with WT (Figure 4.6A,C); however, as only two biological replicates were used, the significance of this result could not be assessed. In adult *Vangl2*^{Lp/+} lungs there was a moderate but not significant increase in total ERM levels compared with WT lungs (Figure 4.6D,E). Additionally there was no significant difference in P-ERM levels in adult lungs (Figure 4.6D,F).

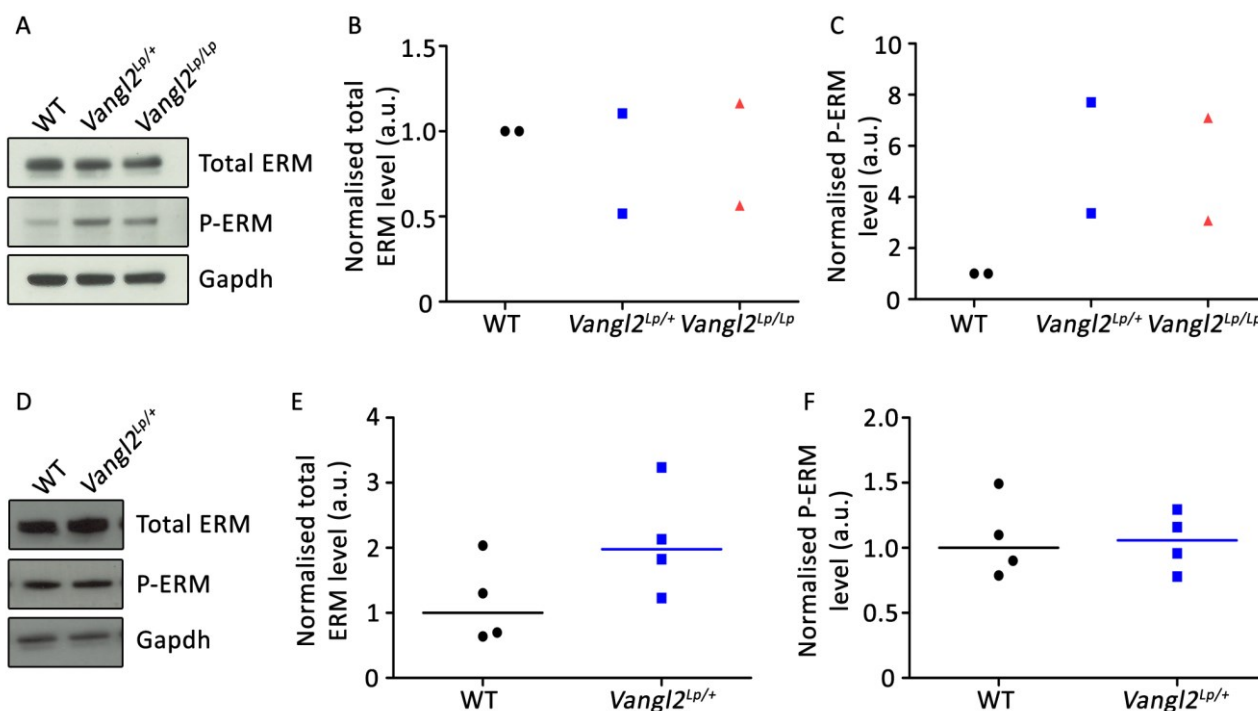


Figure 4.6. P-ERM shows a trend towards increased levels in embryonic *Vangl2*^{Lp} mutant lungs

(A) Western blot of ERM, P-ERM (both 80 kDa), and Gapdh in total protein lysate from E14.5 WT, *Vangl2*^{Lp/+} and *Vangl2*^{Lp/Lp} lungs. Quantification of (B) total ERM and (C) P-ERM levels in WT, *Vangl2*^{Lp/+}, and *Vangl2*^{Lp/Lp} littermates (each point shows an individual replicate, with each replicate containing pooled lungs from two mice). (D) Western blot of ERM, P-ERM, and Gapdh in total protein lysate from 10 week old WT and *Vangl2*^{Lp/+} mouse lungs. Quantification of (E) total ERM and (F) P-ERM levels in adult WT and *Vangl2*^{Lp/+} lungs revealed no significant differences (each point shows an individual mouse, Mann-Whitney U P > 0.05).

4.2.5. E-cadherin localisation is less discrete in *Vangl2*^{Lp/+} lungs

E-cadherin is essential for adherens junction formation and has previously been shown to be regulated by Vangl2 to control its membrane localisation [252]. In addition, E-cadherin is closely linked with the actin cytoskeleton. To assess the impact of the *Vangl2*^{Lp} mutation on E-cadherin localisation in the lungs, immunofluorescence staining was performed to analyse E-cadherin localisation in the lungs of embryonic and adult mice.

In E18.5 embryonic lungs, E-cadherin was localised around the plasma membrane of WT mice (Figure 4.7A). In *Vangl2*^{Lp/+} lungs, E-cadherin was detectable at the plasma membrane, however, the pattern of localisation was altered, and E-Cadherin appeared more diffuse and less restricted to a thin band around the edges of cells (Figure 4.7B). A similar change in E-cadherin localisation was also consistently observed in 10 week old *Vangl2*^{Lp/+} mouse lung slices (Figure 4.7C,D).

To further analyse E-cadherin regulation in the adult lungs, western blotting was performed in lungs of WT and *Vangl2*^{Lp/+} mice. Total E-cadherin levels were not significantly different between WT and *Vangl2*^{Lp/+} lungs (Figure 4.7E,F). Therefore, subcellular fractionation was performed to isolate the membrane and cytosolic compartments and assess E-cadherin levels and regulation. No significant differences were observed in levels of cytoplasmic or membrane E-cadherin between WT and *Vangl2*^{Lp/+} littermates (Figure 4.7G-I). Together, these results indicate that E-cadherin organisation is altered in embryonic, however, further analysis is required to validate this.

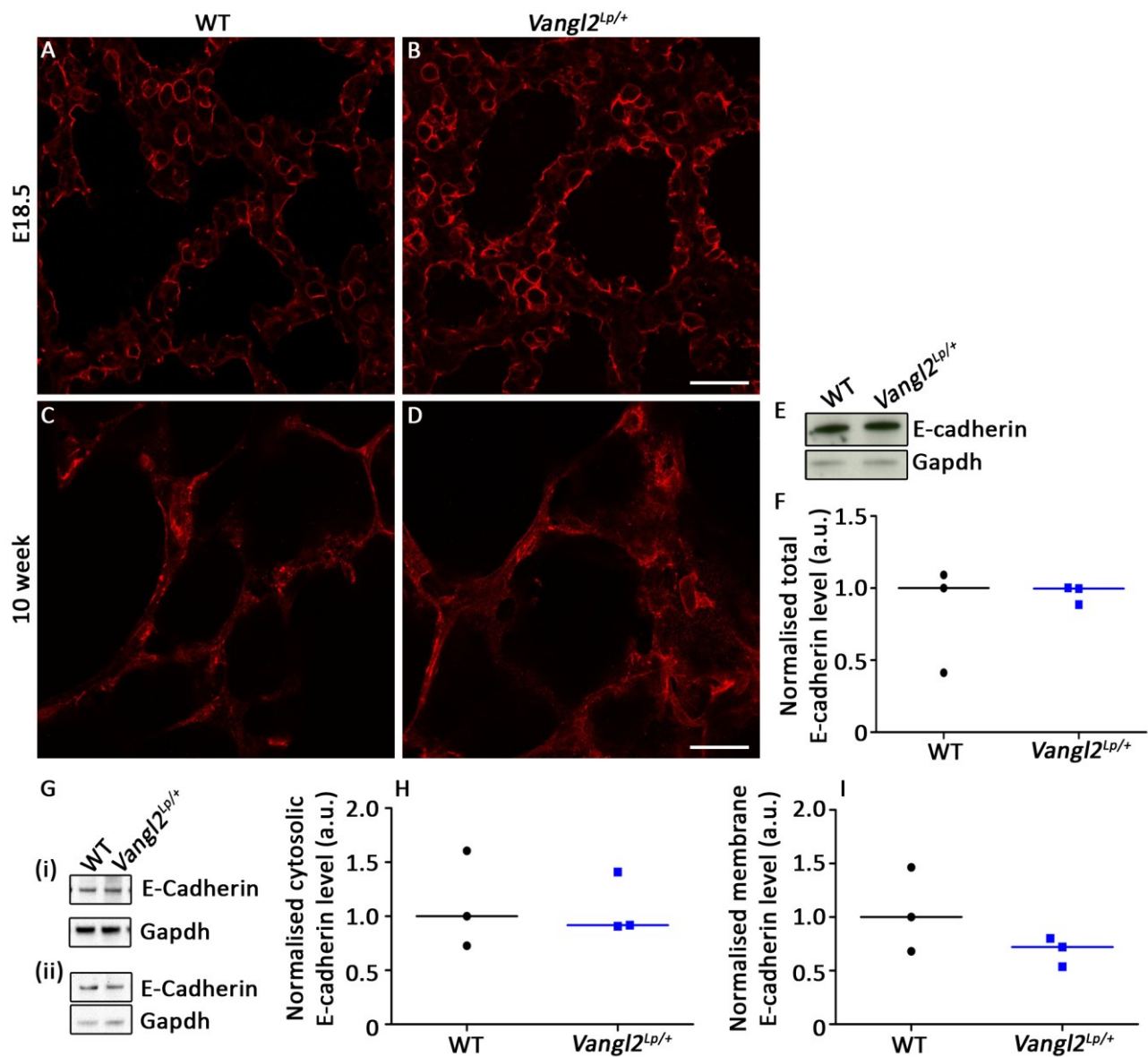


Figure 4.7. E-cadherin was less discrete in *Vangl2*^{Lp/+} embryonic and adult lungs

E-cadherin localisation in lungs of (A,B) E18.5 and (C,D) adult 10 week old mice showed localisation to the plasma membrane in both (A,C) WT and (B,D) *Vangl2*^{Lp/+} littermates. However, in both embryonic and adult lungs, the localisation of E-cadherin was less discrete at the plasma membrane of *Vangl2*^{Lp/+} mice compared with WT littermates. (E) Western blot of total E-cadherin (120 kDa) and Gapdh in total protein lysate from 10 week old WT and *Vangl2*^{Lp/+} mouse lungs. (F) Quantification of total E-cadherin levels revealed no significant differences between WT and *Vangl2*^{Lp/+} littermates (each point shows an individual mouse, Mann-Whitney U P > 0.05). (G) Western blotting of E-cadherin from the (i) cytosolic and (ii) membrane bound subcellular fraction. Quantification of E-cadherin levels in the (H) cytosolic and (I) membrane bound fraction did not show a significant difference between *Vangl2*^{Lp/+} lungs and WT controls (each point shows an individual mouse, Mann-Whitney U P > 0.05). Scale bar (A-D) 25 µm.

4.2.6. In vitro assessment of epithelial barrier integrity

Vangl2 has been linked to E-cadherin recycling, which is relatively low in lung homeostasis. Therefore, to study cell junction regulation, an enzymatic method was used to perturb cell-cell interactions to promote the recycling of E-cadherin. To dynamically assess epithelial cell barrier integrity, we aimed to isolate ATII epithelial cells from adult WT and *Vangl2*^{Lp/+} lungs and assess barrier function using the iCELLigence system to measure impedance changes following barrier disruption with *Alternaria alternata*, which from now will be referred to as *Alternaria*. *Alternaria* has previously been shown to cause severe tissue disruption *in vivo* and *in vitro* and specifically disrupts epithelial barrier function [43,383].

Prior to testing barrier function in primary epithelial cells, preliminary experiments sought to test whether changes in impedance was detectable following *Alternaria* treatment of A549 cells. The optimal density of A549 cells was determined by seeding cells at densities ranging from 1,562 - 50,000 cells/well and monitoring proliferation for 24 hr. Proliferation was assessed indirectly by measuring cell impedance (cell index). Cells seeded at a concentration of 12,500 cells/well or greater showed dose dependent increase in cell index indicating that they were proliferating (Figure 4.8A). Cells cultured at a concentration below 6,250 cells/well did not show an increase in cell index as the density was too low to cause a noticeable change in cell index (Figure 4.8A).

For on-going experiments, 50,000 cells/well was chosen as the optimal seeding density. The efficiency of *Alternaria* to disrupt barrier function *in vitro* was then tested. Therefore, cells were seeded at 50,000 cells/well and cultured to confluency for 71 hr, indicated by the plateau of the cell index (Figure 4.8B). Cultures were then treated with *Alternaria* or vehicle control (PBS) (Figure 4.8B,C red arrow). Addition of *Alternaria* caused a dose dependent decrease in the cell index by 0.15 arbitrary units (a.u.) and 0.07 a.u. following 40 µg and 20 µg *Alternaria* treatment respectively, showing disruption of the epithelial barrier integrity (Figure 4.8B,C). As the epithelial barrier begins to recover and cell junctions are reformed, the cell index increases. Cells treated with 10 µg and 20 µg of *Alternaria* showed recovery of barrier integrity and reached the control level of cell index after 8 hr and 10 hr respectively. Cells treated with 40 µg *Alternaria* took longer to recover, roughly 24 hr after treatment (Figure 4.8B,C). Interestingly, cells treated with PBS also began to show a decline in cell impedance after 77 hr in culture (Figure 4.8B,C).

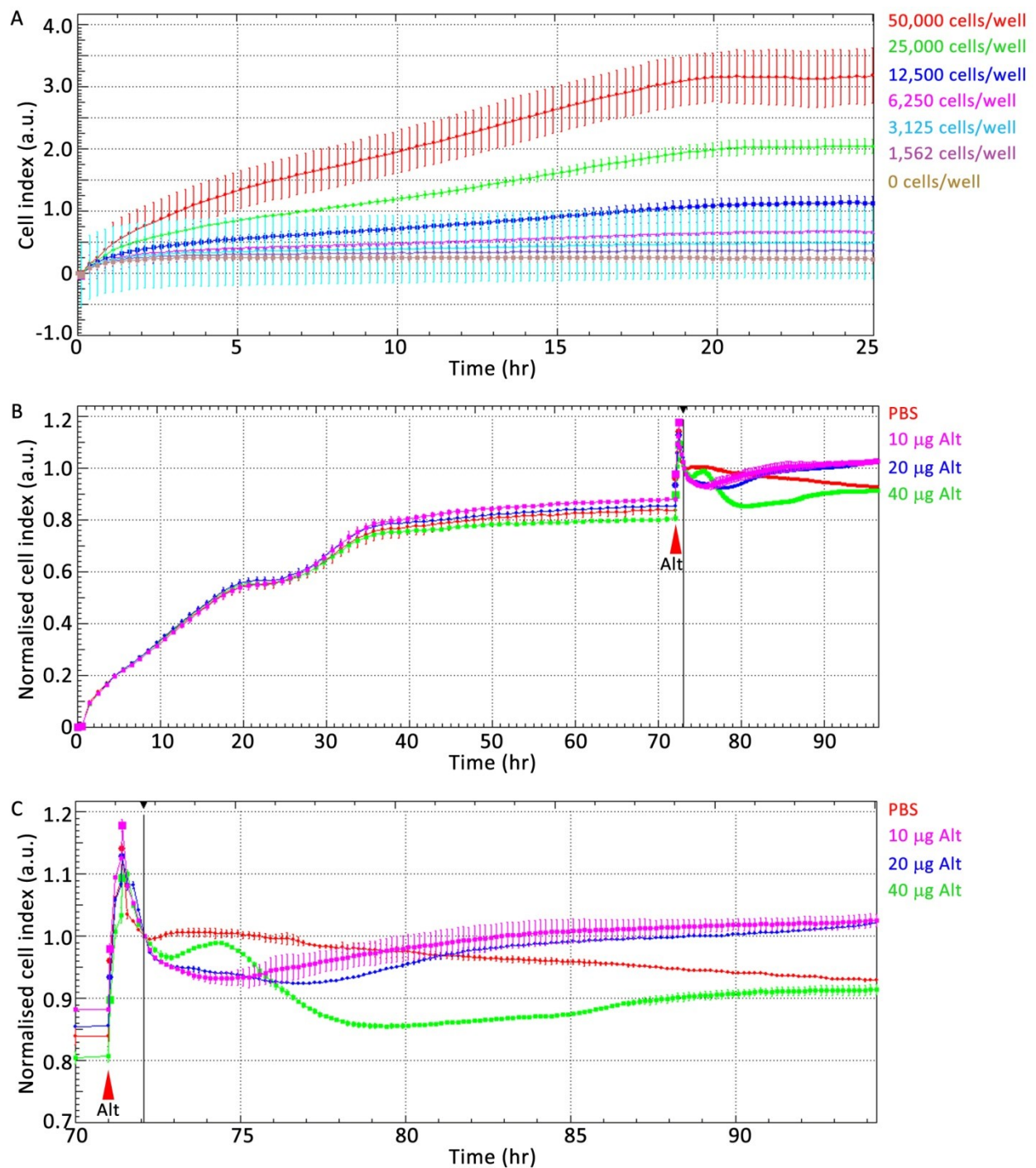


Figure 4.8. Alternaria caused epithelial barrier disruption in A549 cells

(A) A549 cells were seeded at different densities ranging from 1,562 – 50,000 cells/well, and cell proliferation was assessed over 24 hr by measuring the increase in cell impedance (n=1 containing 2 technical replicates). (B,C) A549 cells were seeded at a density of 50,000 cells/well and cultured for 71 hr; as cells proliferate the cell index increases and then begins for plateau as the cells reach confluency. Cells were then treated with 10 μ g, 20 μ g, 40 μ g *Alternaria* fungal allergen, and vehicle control (PBS) at 71 hr (red arrow). C shows an enlarged view of B, showing the cell index change during the final 24 hr period following *Alternaria* treatment. *Alternaria* caused a dose-dependent decrease in the cell index as a result of disrupted epithelial barrier integrity. As the cell junctions reform and barrier integrity is restored, the cell index rises, although this is prolonged in cells treated with higher *Alternaria* doses (n=1 containing 2 technical replicates). Abbreviations: *Alternaria* (Alt).

Having confirmed that an *in vitro* method could be used to assess barrier function in real time in cultured cells, we aimed to isolate primary ATII cells from WT and *Vangl2*^{Lp/+} lungs as a means of testing epithelial junction integrity. First, we aimed to optimise the efficiency of ATII cell isolation and culture methods using WT mice. Using a negative selection method adapted from Mutze *et al* [285], primary cells were obtained with high viability at the end of the isolation process (81.0 %). Cell purity of epithelial cells was assessed by staining for Pro-SPC which is an ATII cell marker; this showed 61.5 % cell purity of ATII cells in the isolated cells at day 0 (Figure 4.9A-C).

In initial experiments, cells were cultured in media supplemented with 10 % FBS. However, this led to excessive growth of fibroblasts after 72 hr resulting in low numbers of ATII cell in culture (Figure 4.9D-F). Therefore, the culture media was altered to limit the growth of fibroblasts by culturing the isolated cells in serum-free media supplemented with FGF10 and insulin/transferring/selenium (ITS). Using the serum-free culture media, primary ATII cells were detectable at higher frequencies (white arrows), although a confluent monolayer was not achieved (Figure 4.9G-I). Assessment of cell purity after culturing for 72 hr in the modified media showed poor ATII cell purity when assessed by Pro-SPC staining (28.4 %). Although this method reduced the growth of fibroblasts, a large number of fibroblasts were still found after 72 hr in culture (Figure 4.9J-L). As a confluent monolayer of pure alveolar epithelial cells could not be obtained, it was not possible to test epithelial barrier function in the primary ATII cells isolated from *Vangl2*^{Lp/+} lungs.

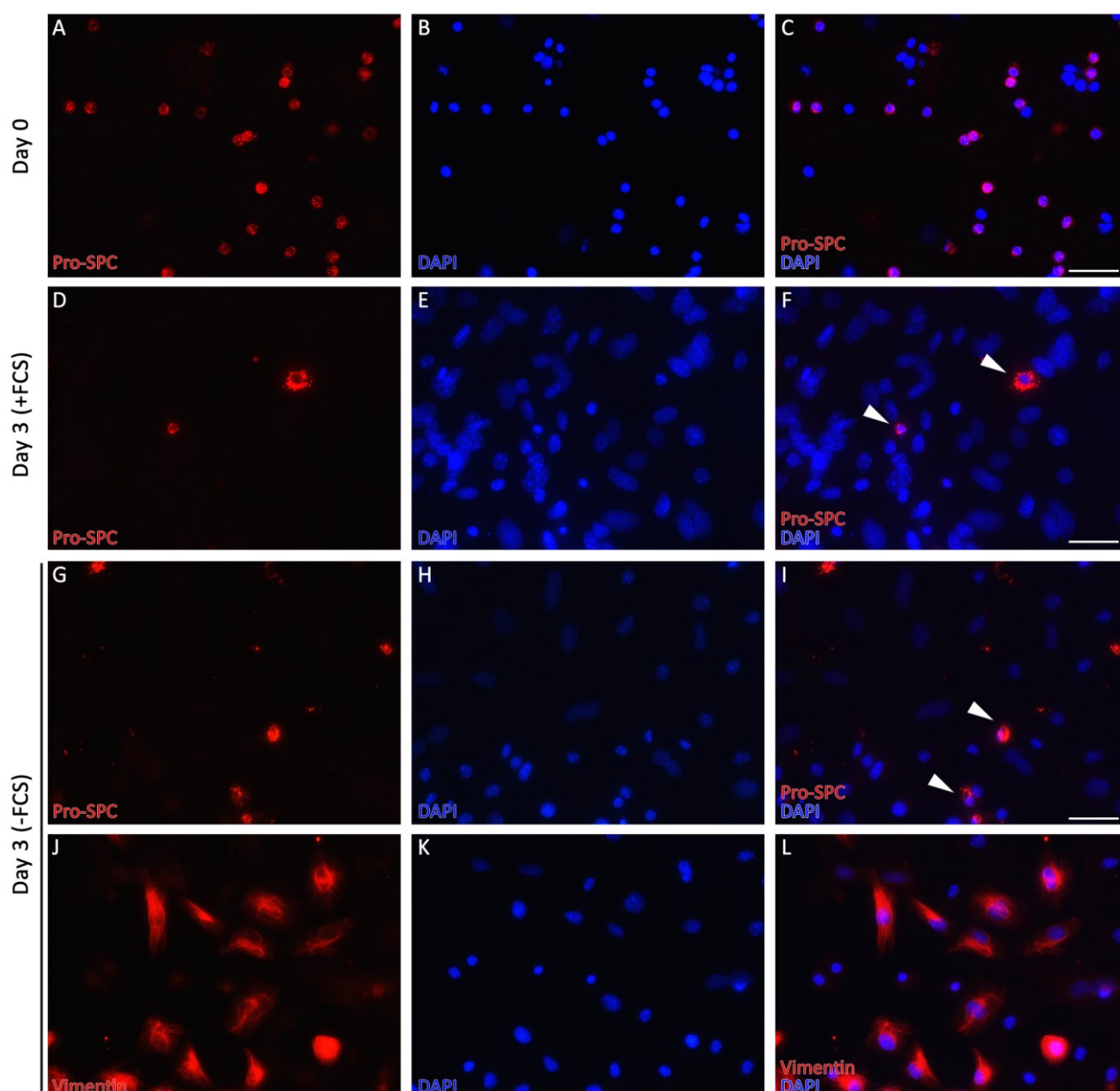


Figure 4.9. Primary epithelial ATII cells were not able to form a confluent monolayer in culture

(A-C) Immunofluorescence staining of cells prior to cell culture with (A) Pro-SPC to identify ATII cells, (B) DAPI to stain cell nuclei, and (C) merged overlay of A and B. Cell purity was assessed following culture for 72 hr with (D-F) serum containing media and (G-I) serum free media. Epithelial cells were identified by staining for (D-I) Pro-SPC (white arrow), and (J-L) fibroblasts were identified using vimentin. Scale bar 28 μ m.

4.3. Discussion

4.3.1. *Vangl2*^{Lp} mutation alters actin organisation

Previous studies have shown that one of the downstream targets of the PCP signalling pathway is cytoskeleton reorganisation. Yates *et al*, demonstrated that actin organisation was disrupted in embryonic lungs of E14.5 and E18.5 homozygous *Vangl2*^{Lp} mice, resulting in discontinuous cortical actin and a diffuse pattern of staining [14]. In this study, we show that a heterozygous *Vangl2*^{Lp} mutation in embryonic lungs is sufficient to disrupt cortical actin organisation. F-actin was discontinuous in embryonic *Vangl2*^{Lp/+} lungs and had diffuse cytoplasmic staining; demonstrating that a single allele *Vangl2*^{Lp} mutation is sufficient to disrupt actin organisation in the lung. However, actin cytoskeletal disruption was less severe in the heterozygote mutant lungs than in homozygotes [14].

Analysis of a panel of cytoskeleton genes identified *Cfl*, a key actin cleavage protein, was dysregulated in homozygous embryonic *Vangl2*^{Lp} mutant lungs. Further studies in both homozygous and heterozygote *Vangl2*^{Lp} mutant lungs showed a significant reduction in cofilin phosphorylation. As total Cfl levels remained unaffected by the *Vangl2*^{Lp} mutation, this indicates that *Vangl2*^{Lp} mutant lungs have increased levels of active Cfl present. The increased active Cfl levels may cause excessive severing of the actin filaments, resulting in the disrupted and non-continuous cortical F-actin staining in embryonic *Vangl2*^{Lp} mutant lungs. For statistical analysis of Cfl and P-Cfl levels in embryonic lung tissue a Gaussian distribution was assumed due to difficulties in quantifying western blots. Technical difficulties arose due to varying band intensities between biological replicates as a result of different exposure times which could not be controlled for. As western blots showed a consistent reduction of P-Cfl levels in *Vangl2*^{Lp} mutant lungs in all biological replicates we performed parametric statistical analysis of protein levels, as performed in previously published publications [252,384].

Regulation of Cfl activity is tightly controlled by phosphorylation and dephosphorylation by kinases and phosphatases such as LIM kinase (LIMK) and Slingshot protein phosphatases (Ssh) respectively [361,365,366]. The activity of LIMK is regulated by small GTPases such as Rac and Rho-associated kinase (ROCK) [361,366]. Furthermore, it has previously been shown that altering LIMK activity in *Drosophila* by use of constitutively active LIMK causes PCP defects in the wing hair, similar to that observed in *tsr* mutants [228]. As LIMK activity is regulated by ROCK and Rac which are known downstream targets of the PCP pathway [354], it is possible that the *Vangl2*^{Lp} mutation may disrupt LIMK regulation. This in turn would result in reduced phosphorylation of Cfl, leading to increased actin cleavage in *Vangl2*^{Lp} mutants.

ERM regulation was also altered in both heterozygote and homozygote embryonic *Vangl2*^{Lp} lungs, with a strong trend towards increased ERM phosphorylation in *Vangl2*^{Lp} mutant lungs by roughly 4 fold compared with WT littermates. However, as only two biological replicates were used for assessing embryonic ERM levels it was not possible to perform statistical analysis. Previous studies have shown that

ERM activation occurs as a result of ROCK mediated phosphorylation [371]. It is perhaps surprising that perturbed PCP signalling in this instance results in increased activation of ERM, which has been shown to promote cell migration [385]. However, studies have shown that *Vangl2* perturbations result in reduced cell migration [281]. Therefore, it is possible that, although there is increased activated ERM, it may be mislocalised and unable to efficiently promote migration. In a previous study, Valderrama *et al* showed that in cells with radixin knockdown, P-ERM was present in cells but did not localise correctly to the lamellipodia and had a more punctate appearance [373]. Therefore, further analysis by immunostaining of ERM and P-ERM is required to assess ERM localisation in *Vangl2^{Lp}* mutant lungs.

Although both Cfl and ERM were differentially regulated in embryonic lung development, no significant difference in regulation of these proteins was observed in adult lungs. However, this can be attributed to the lack of cell migration in the adult lungs, thus, there is little requirement for actin cytoskeleton reorganisation. As such in adult lungs, both Cfl and ERM likely remain in a less active state and no difference in their regulation is observed between WT and *Vangl2^{Lp/+}* lungs.

Interestingly, P-Cfl levels were undetectable by western blot in lungs of adult mice after lung function in both WT and *Vangl2^{Lp/+}* littermates. In lung function experiments, the lungs are mechanically ventilated, this may induce a stress response in the cells resulting in strain on the actin cytoskeleton by induction of mechanosensitive pathways. Previous studies have shown that the ROCK-LIMK-Cfl pathway is activated by *in vitro* sheer stress resulting in increased P-Cfl [386]. Therefore, in Flexivent analysis, mechanotransduction may lead to activation of Cfl to promote cytoskeletal reorganisation. Thus, following lung function analysis Cfl may become dephosphorylated, resulting in the inability to detect P-Cfl.

Other PCP mutants such as the *Celsr1^{Crsh}* and *Scrib^{Crc}* mice also present with similar lung disruption in embryonic development as a result of defective cell migration [14,188]. Therefore, it would be interesting to study the regulation of Cfl in these mutants to observe if the defect in Cfl regulation is specific to the *Vangl2^{Lp}* mutation or a general result of disrupted PCP signalling.

4.3.2. VANGL2 knockdown impairs wound healing and cell polarity

A key function of the cytoskeleton is to mediate cell migration. Many studies have previously highlighted a role for PCP signalling in cell migration [14,188,253,284]. In our study, knockdown of VANGL2 in human alveolar epithelial cells caused a significant decrease in cell migration. This finding complements previously published data where *Vangl2* overexpression promoted cell migration [253]. In addition to disrupted cell migration, polarisation of A549 cells during migration was also disrupted by VANGL2 knockdown, correlating with previously published work where mutations in PCP proteins such as *Grhl3* and *Fuzzy*, disrupted polarity in wound healing [284,357]. Interestingly, Caddy *et al* did not report a significant

difference in epidermal wound healing in embryonic *Vangl2^{Lp}* mice. The differences in these results may be due to tissue specific differences or the model of wound healing used; Caddy *et al* performed *in vivo* wound healing assays in *Vangl2^{Lp}* epidermal cells, whereas our study was performed *in vitro* using VANGL2 knockdown. Thus, to further validate the results of the wound healing experiments, scratch assays could be conducted using primary ATII cells isolated from *Vangl2^{Lp}* and WT lungs.

Assessment of wound healing during stimulation of PCP signalling using Wnt5a, a classical PCP pathway ligand [123], promoted wound healing *in vitro*. Previous studies in the Dean lab have shown that stimulation with Wnt4, a canonical Wnt ligand, was unable to promote wound healing in A549 cells [Hussin N, Unpublished data]. For this assay, a shorter duration of 18 hr was used, as opposed to the 24 hr used in the VANGL2 knockdown experiments, as by 24 hr, Wnt5a treated cells had fully healed. Thus, at 24 hr it was not possible to accurately quantify the area of wound healed; therefore, 18 hr was chosen as a time point when wounds were yet to fully heal and the area healed could be calculated.

Together, the results of the wound healing assays indicate that the PCP pathway is important for regulation of cell migration in wound healing. These results suggest that individuals with PCP mutations, such as in *VANGL2*, may have cell migration or repair defects which could contribute to the pathogenesis of chronic lung disease. Thus, the PCP pathway may provide a novel target for treatment of chronic lung disease, and that manipulation of the PCP pathway might be used to promote cell migration and facilitate lung epithelial repair.

For wound healing assays, normal distribution of the data was assumed, as previous publications have shown normal distribution of wound healing [357,372,387]. Furthermore, due to the small number of biological replicates, although each biological replicate contained four technical replicates, we did not observe a statistically significant difference although the spread of the data showed a clear difference. For example, in the case of wound healing during Wnt5a stimulation non-parametric statistical tests such as Mann-Whitney U did not show a significant difference although clear differences were observed in cell behaviour, where Wnt5a treated cells showed an increased rate of wound healing. This is all the more evident when taking into account that Wnt5a treated cells showed 100% wound closure by 24hrs compared to control cells where the wound did not close during preliminary experiments (data not shown), thus the end timepoint of this assay was changed to 18hr to allow for quantification of wound healing. This illustrates that Wnt5a does cause an increase in the rate of wound healing over controls but is not evident using the Mann Whitney test.

4.3.3. E-cadherin localisation is less discrete in *Vangl2*^{Lp/+} mouse lungs

The actin cytoskeleton is tightly linked with adherens junction proteins, such as the classical cadherins. E-cadherin has been shown to be important for mechanotransduction of stress signals during migration to initiate strengthening of the junctions in response to pulling forces [388]. Interestingly, *Vangl2* has been demonstrated to regulate E-cadherin turnover at the plasma membrane [252]. In the embryonic lung, E-cadherin was localised to the plasma membranes in both WT and *Vangl2*^{Lp/+} mice. However, E-cadherin localisation was less discrete at the membrane of *Vangl2*^{Lp/+} lungs, resulting in the appearance of a thicker band of E-cadherin around the cell by immunostaining. This work indicates that there may be increased retention of E-cadherin at the plasma membrane in *Vangl2*^{Lp/+} lungs compared with WT. This data correlates with previously published work showing increased retention of E-cadherin in the membrane of renal cells derived from *Vangl2*^{Lp/+} mice [252]. Furthermore, studies in embryonic *Scrib*^{Crc} lungs showed diffuse localisation of β -catenin around the membrane of luminal epithelial cells, whereas in WT lungs β -catenin was found around the cells with enriched localisation at the apical surface [188]. However, the authors did not observe a noticeable difference in E-cadherin localisation [188]. This study, together with previously published work, shows that PCP signalling is involved in regulation of adherens junctions.

In this study, the alteration of E-cadherin localisation in *Vangl2*^{Lp/+} lungs was not quantitatively assessed; thus, further quantitative analysis is required to validate these observations. One method to achieve this would be to measure the mean pixel intensity of the fluorescent signal specifically at the membrane in a defined area. The pixel intensity is proportional to the level of protein localisation; thus, higher pixel intensity indicates a greater localisation and retention of protein at the plasma membrane. Co-staining with a protein which is ectopically expressed at the membrane, such as sodium-potassium ATPase (Na^+/K^+ -ATPase), can be used for normalisation of the fluorescence intensity, as well as assessing mislocalisation of E-cadherin staining.

In the adult lung, the *Vangl2*^{Lp} mutation did not affect total E-cadherin levels, as seen previously in the embryonic *Scrib*^{Crc} mutant lungs [188]. Additionally, subcellular fractionation of cytoplasmic and membrane compartments did not show a significant difference in E-cadherin regulation. The lack of significant difference in the adult lung may be due to two reasons. First, similar to the cytoskeletal regulation, there is no need for extensive E-cadherin regulation in homeostasis as the junctions are stable. As there is no excessive stress on the cells, such as that imposed by cell migration in embryonic development, therefore there is no requirement for significant regulation of the intercellular junctions. The second reason may be due to the method of subcellular fractionation. The membrane fraction is a pool of all cellular membranes, both plasma and organelle membranes such as the Golgi and endoplasmic reticulum (ER). The Golgi and ER are essential organelles for protein synthesis and targeting and may also contain newly synthesised E-cadherin. Thus, differences in regulation of E-cadherin at the plasma membrane may be masked. Therefore, a subcellular fractionation method that specifically isolates the

plasma membrane is required to accurately determine whether E-cadherin regulation is disrupted in the adult *Vangl2*^{Lp/+} lung.

4.3.4. Methods of cell junction analysis

Because of the links between the actin cytoskeleton and adherens junctions, we wished to assess barrier function in *Vangl2*^{Lp/+} ATII cells following damage by *Alternaria*. Due to the dynamic nature of adherens junction regulation, the assessment of epithelial cell integrity by immunostaining at a single time point would not have been sufficient to give relevant information. Although assessment at multiple time points would give some information as to the dynamics of cell junction regulation, this task is laborious and difficulties still lie in accurate assessment of the junction complexes. Therefore, an *in vitro* method was used to simplify the model, and allow temporal assessment of barrier function.

As proof of principle, preliminary tests in A549 cells were performed. Cell impedance (which is used to calculate the arbitrary unit of cell index) was used as an indirect measure of cell proliferation and barrier function. These preliminary experiments demonstrated that epithelial barrier disruptions could be quantitatively assessed by cell impedance in real-time. Using the iCELLigence system alone however, it is not possible to verify if the barrier disruptions occur as a result of disrupted adherens junctions, tight junctions, or both. Therefore, immunostaining for these junctions using E-cadherin and β -catenin for adherens junctions, and ZO and claudins for tight junctions should be performed following *Alternaria* treatment to assess barrier disruption. Appropriate time points to assess the cell junction proteins by immunostaining can be identified using the iCELLigence system, such as staining at 9 hr post treatment for barrier disruption, corresponding with the maximal drop in cell index for the 40 μ g *Alternaria* treatment and a later time point to assess the epithelial barrier recovery.

Although the initial aim of this study was to assess cell junction regulation in WT and *Vangl2*^{Lp/+} lungs by isolating primary ATII cells, due to time constraints it was not possible to optimise the culture protocol to gain a confluent monolayer of epithelial cells. During the optimisation process many problems were encountered; namely excessive fibroblasts growth, poor proliferation of ATII cells, and inefficient perfusion to remove blood. A constant variable in the cell isolation process was contamination by erythrocytes. Although cardiac perfusion was performed to remove blood from the lungs, the efficiency of blood removal was very variable. Contamination of cultures by erythrocytes led to inefficient adhesion of ATII cells after seeding by physically limiting the amount of space available to attach. Although cell cultures were washed the following day to remove unattached cells and dead cell debris, high erythrocyte contamination in the initial culture prevented successful growth of ATII cells. Due to this, it was not possible to establish a robust system to test epithelial barrier function by impedance measurements using primary cells. Therefore, more selective methods of ATII cell isolation should be employed in the future, such as

fluorescence activated cell sorting (FACS). Lee *et al* generated transgenic mice which express chromatin bound green fluorescence protein (H2B-GFP) driven by the *SPC* promoter, hence forth referred to as *SPC^{GFP}* mice [389]. In *SPC^{GFP}* lungs, GFP positive cells were found to be predominantly ATII cells [389]; thus, an enriched population of ATII cells can be isolated by FACS using the GFP signal. Breeding the *Vangl2^{Lp/+}* mice with *SPC^{GFP}* mice will generate offspring which are *Vangl2^{Lp/+}/SPC^{GFP}*, which will contain the *Vangl2^{Lp}* mutation and express GFP in ATII cells. Sorting for the GFP signal in *Vangl2^{Lp/+}/SPC^{GFP}* lungs can be to isolate ATII cells which contain the *Vangl2^{Lp}* mutation. Therefore, using these mice in further studies may allow for isolation of an enriched population of ATII cells, overcoming problems faced in our study such as contamination by fibroblasts and erythrocytes.

4.3.5. Concluding remarks

In conclusion, we identified that regulation of actin cytoskeleton dynamics was perturbed in embryonic *Vangl2^{Lp}* lungs. Furthermore, *in vitro* VANGL2 knockdown in alveolar epithelial cells impaired wound healing. Together, our results suggests that disrupted actin organisation may result in cell migration defects impairing wound healing. Thus, *Vangl2* mutations may perturb repair processes in lung injury.

Chapter 5: Adult mouse lung infection models

5.1. Introduction

Several *VANGL2* mutations have been identified in patients with neural tube defects (NTDs), some of which map closely to the site of the *Vangl2*^{Lp} mutation in mice [270]. These human *VANGL2* mutations cause similar defects to those caused by the *Vangl2*^{Lp} mutation in mice such as protein mislocalisation, instability, and disrupted interaction with DVL [269,270]. Additionally, the open NTD phenotypes observed in patients with *VANGL2* and *VANGL1* mutations strongly resemble the phenotype observed in *Vangl2*^{Lp} mice such as anencephaly and cervical spina bifida [269].

Moreover, *VANGL2* protein is present in the human lungs, and the pattern of *VANGL2* localisation in embryonic and adult human lungs is similar to that in mice (Section 3.2.2). Additionally a population based study identified a *VANGL2* single nucleotide polymorphism (SNP) which in conjunction with smoking caused a significant decrease in forced vital capacity (FVC). Interestingly, this *VANGL2* SNP did not cause a significant change in FVC on its own without the additional environmental insult (smoking) [Minelli C and Pereira M, Unpublished data]. Recent studies in the Dean lab have shown a significant reduction in *VANGL2* expression in COPD patients compared with non-COPD control donors [Poobalasingam T, Unpublished data]. These findings demonstrate evidence of *VANGL2* perturbation in adult human lung disease; however, the detailed molecular mechanisms of PCP signalling in chronic lung disease remains poorly investigated.

There are a number of key chronic lung diseases including COPD and idiopathic pulmonary fibrosis (IPF). COPD is a growing concern to the health system, affecting an estimated 200 million people worldwide and was responsible for 6 % of global deaths in 2012 [1]. COPD is more prevalent in older adults and in the UK alone COPD is estimated to affect 3.7 million people, of which only 900,000 have been diagnosed and are receiving treatment. The most common cause of COPD is exposure to smoking or environmental pollutants, although genetic susceptibility is an important determinant for onset of COPD [1,2].

COPD is an umbrella term which encompasses a wide range of diseases that narrow and restrict the airways such as bronchitis, and damage the alveolar walls such as emphysema [24]. One of the key features of emphysema is destruction of the alveolar walls as a result of increased epithelial and endothelial cell apoptosis caused by both cigarette smoke and immune cells; this leads to enlarged airspaces within the lung resulting in reduced surface area for gas exchange [2]. In addition to the lung parenchyma defects, the small airways also become fibrotic in COPD and show increased mucus production [2]. As a result, this causes breathlessness, persistent cough, and frequent exacerbations due to infection in COPD patients [24].

Although the leading risk factor for COPD in patients is smoking, many other genetic and environmental factors can also lead to onset of COPD. Environmental risk factors include pathogenic infections, exposure to smoke, and dust. Host genetics have also been shown to be important for the pathogenesis of COPD. Among smokers only 20-30 % develop COPD [24], thereby indicating genetic susceptibility in smokers resulting in COPD. Mutations in the *α 1-antitrypsin* gene have been linked to COPD,

and cause increased lung function decline. These lung function defects are further accelerated in smokers with $\alpha 1$ -antitrypsin deficiency [390,391]. Other genes such as Hedgehog-interacting protein (*HHIP*), IL6 receptor, and *MMP12* have also been associated with increased risk of COPD [13,302,313,391].

The severity of COPD is classified using the Global Initiative for Chronic Obstructive Lung Disease (GOLD) staging criteria. The GOLD committee is a group organised to review published work in the field of COPD to recommend staging and clinical management of COPD; these guidelines are updated yearly [392]. GOLD staging categorises COPD into four groups of severity from Stage I (mild) to Stage IV (very severe). These staging criteria include spirometry measures of lung function, forced expiratory volume in one second (FEV₁) and FVC; as summarised in Table 5.1.

Stage	Severity	FEV ₁ /FVC	FEV ₁
I	Mild	<0.7	≥80 %
II	Moderate	<0.7	50-79 %
III	Severe	<0.7	30-49 %
IV	Very severe	<0.7	<30%

Table 5.1. GOLD staging of COPD

GOLD classifies COPD as an “important public health challenge that is both preventable and treatable” [392]. Although treatments are available for COPD patients, these treatments are not curative and are targeted towards slowing disease progression. Non-pharmacological methods of COPD treatment include cessation of smoking, pulmonary rehabilitation using exercise to improve the patient’s physical condition, and supplemental oxygen therapy for patients with low blood oxygen saturation levels [24].

Pharmacological treatments for COPD include bronchodilators such as Salbutamol, which are able to relax and open airways, providing rapid relief of bronchospasm in patients [393,394]. Another common feature of COPD patients is the proinflammatory environment with elevated levels of TNF- α , IL-6, oxygen free radicals, and MMPs [24,304,305,313], as well as high influx of immune cells such as macrophages and neutrophils [23,299,313]. Thus, corticosteroids such as prednisone are given to reduce inflammation which can lead to COPD exacerbations, it also aid to reduce relapse following severe exacerbation to improve lung function in patients [395]. Although these pharmacological and non-pharmacological therapies provide some relief from COPD symptoms and may slow disease progression, there are however, currently no

curative treatments available. One of the key problems of COPD is the lack of repair in response to injury; thus, more recent studies are investigating novel pathways to target to initiate lung repair mechanisms to promote recovery. Uhl *et al* showed that activation of the canonical Wnt pathway was able to rescue the remodelling phenotype in COPD [13]; however, this remains an experimental therapy.

Another chronic lung disease which is increasing in prevalence is IPF which has an incidence of 7.44 in 100,000 [396]. IPF is more prevalent in the older population, and is almost non-existent in people below 50 years of age. IPF leads to reduced lung function resulting in death usually 2-3 years after the initial diagnosis [396,397]. Unlike COPD, where there is a lack of tissue repair, IPF is thought to result from overactive repair processes. Although the aetiology of IPF is not fully understood, the current theory suggests that following alveolar epithelial cell (AEC) damage, ATII cells become activated resulting in the recruitment of fibroblasts. The fibroblasts in turn proliferate and undergo differentiation into myofibroblasts which produce excessive extracellular matrix (ECM) resulting in the fibrotic phenotype seen in IPF [396]. Diagnosis of IPF usually involves lung biopsies, imaging with computed tomography (CT), and lung function measurements [396]. CT scans of IPF lung show gross honeycombing caused by cystic spaces in the lungs with thickened walls as a result of the increased fibrotic tissue [113].

In both IPF and COPD, exacerbations are major clinical concerns. An acute exacerbation is rapid worsening of symptoms, such as cough and increased breathlessness often triggered by an external insult. Acute exacerbations occur in roughly 5-15 % of patients with IPF per year, greatly reducing the median survival of patients [398], and can lead to hospitalisation and death [399,400]. The molecular mechanisms underlying exacerbations are poorly understood. Acute exacerbations can be triggered by numerous causes such as infections. Studies have shown that IPF exacerbations can be caused by both bacterial [401,402], and viral infections [403,404].

Acute exacerbations caused by viral and bacterial infections lead to an increased risk of hospitalisation, and are one of the leading cause of death in COPD patients [405]. COPD exacerbations triggered by viruses are less frequent. The most common type of viral infections found in exacerbations are picornaviruses (which were predominantly rhinoviruses) and influenza A virus [406]. Bacterial infections account for a large proportion of acute exacerbations in COPD patients, and are found in roughly 40-50 % of all cases [405]. The most common species of bacteria are *Haemophilus influenza*, *Haemophilus parainfluenzae*, *Streptococcus pneumonia*, and *Moraxella catarrhalis* which together account for roughly 50 % cases of acute COPD exacerbations [407]. Bacterial exacerbations result in increased numbers of neutrophils and elevated interleukin-8 (IL-8), a neutrophil attractant, which further increase lung damage [408].

5.1.1. Aims and hypothesis

As adult *Vangl2*^{Lp/+} mice have severely altered lung structure and function with an emphysematous-like phenotype, we aimed to assess whether bacterial and/or viral infections would exacerbate the lung defects identified in these mice. We hypothesised that the *Vangl2*^{Lp/+} mice, which have underlying lung function defects, would show worse lung function compared with WT littermates in response to an infectious challenge. To investigate this hypothesis we:

- Conducted an LPS challenge to mimic a bacterial infection prior to assessing lung function.
- Used influenza A virus as viral challenge and assessed the response in *Vangl2*^{Lp/+} mice.

5.2. Results

5.2.1. LPS induced neutrophilia in adult mouse lungs

The data presented in this thesis showed adult *Vangl2*^{Lp/+} mice have severely disrupted lung structure and function (*Chapter 3*). Previously, Kobayashi *et al* demonstrated that a single dose of lipopolysaccharide (LPS), a glycolipid found in the cell walls of gram negative bacteria [409], mimics an acute exacerbation in mice with experimental emphysema [287]. Therefore, adult *Vangl2*^{Lp/+} mice were administered a single dose of LPS to investigate the effect of an acute exacerbation on lung function in these mice. We then assessed lung function 24 hr after the challenge using the Flexivent system.

Initially, the bronchoalveolar lavage fluid (BALF) was assessed 24 hr after intranasal LPS dosing to assess if a single dose of LPS was sufficient to induce an acute inflammation in the adult C3h/HeH mouse lungs. As expected, there was an influx of neutrophils into the BALF 24 hr after the LPS challenge (Figure 5.1A,B) compared with PBS treated mice in which macrophages were the predominant population (Figure 3.12). Quantification of total immune cells in the BALF showed a significant increase in immune cells following LPS injury in both WT (13.8 fold) and *Vangl2*^{Lp/+} (6.9 fold) littermates compared with PBS controls (Figure 5.1C). However, there was no significant difference in total immune cells between PBS and LPS treated lungs in both WT and *Vangl2*^{Lp/+} littermates. Differential cell counts were then conducted to analyse specific immune cell populations in the BALF. Following LPS challenge there was a significant influx of neutrophils into the BALF in both WT (68.5 fold) and *Vangl2*^{Lp/+} (53.8 fold) lungs compared with PBS treated groups (Figure 5.1D). However, there was no significant difference between WT and *Vangl2*^{Lp/+} lungs in neutrophil recruitment into the BALF (Figure 5.1D). On the other hand, LPS treatment did not induce an influx of macrophages into the BALF, and there was no significant difference between macrophage numbers between WT and *Vangl2*^{Lp/+} littermates in both PBS and LPS treated groups (Figure 5.1E).

Albumin levels in BALF were assessed as a measure of vascular integrity following LPS challenge. As expected, LPS treatment disrupted vascular permeability significantly in both WT and *Vangl2*^{Lp/+} lungs (Figure 5.1F). However, no significant differences were observed between WT and *Vangl2*^{Lp/+} littermates for either PBS or LPS treated lungs (Figure 5.1F). Together, these results indicate that a single dose of LPS was sufficient to induce an acute inflammation in both WT and *Vangl2*^{Lp/+} lungs, but no significant difference in immune cell recruitment was observed between the two genotypes.

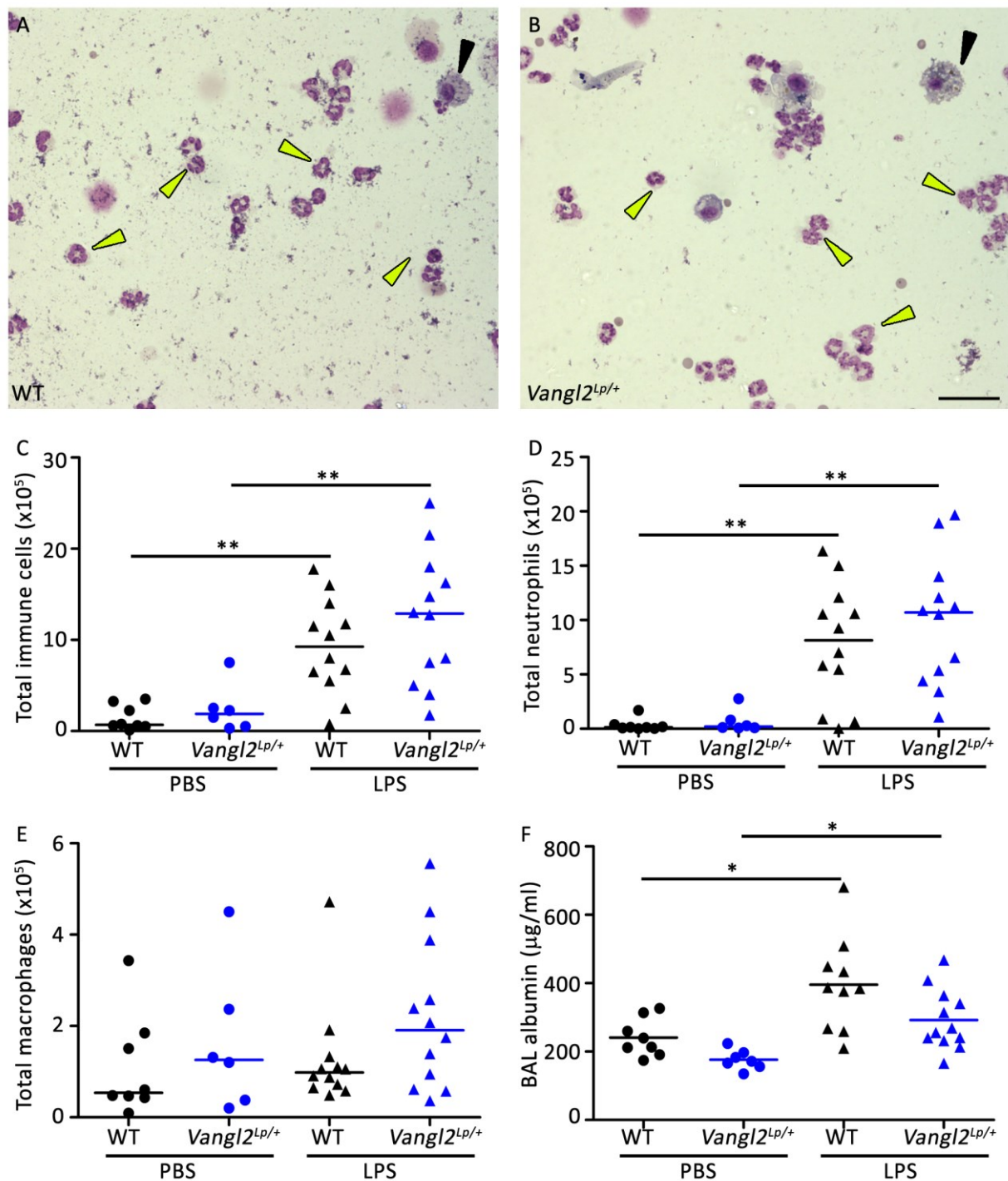


Figure 5.1. LPS induced a neutrophilic inflammation in the BALF after 24 hr

10 week old male mice were given a single intranasal dose of LPS, and immune cell recruitment into the BALF was assessed after 24 hr. (A,B) Cytopsin of cells from the BALF of 10 week old (A) WT and (B) *Vangl2*^{Lp/+} lungs showing a predominance of neutrophils (yellow arrows) compared with macrophages (black arrows) 24 hr after LPS challenge. Scatter plots represent (C) total number of immune cells, (D) total neutrophil numbers, and (E) total macrophage numbers in BALF of 10 week old *Vangl2*^{Lp/+} and WT littermates 24 hr after LPS challenge or vehicle (PBS) control. (F) Quantification of BALF albumin levels 24 hr after LPS challenge or PBS control (each point shows an individual mouse, Kruskal-Wallis with Dunn's multiple comparison test $P < 0.05$ *, $P < 0.01$ **).

5.2.2. Lung function defects in *Vangl2*^{Lp/+} mice were not exacerbated by LPS

Having confirmed that LPS induced an acute respiratory inflammation after 24 hr, lung function was tested in LPS treated mice using the Flexivent system. Lung function analysis showed significantly decreased lung elastance in *Vangl2*^{Lp/+} mice (17.10 cmH₂O/ml) compared with littermate controls (20.26 cmH₂O/ml) (Figure 5.2A). Similarly tissue elastance was also significantly reduced in *Vangl2*^{Lp/+} lungs (14.48 cmH₂O/ml) compared with WT lungs (18.19 cmH₂O/ml) (Figure 5.2B). Tissue heterogeneity, as measured by the ETA parameter, was significantly increased in *Vangl2*^{Lp/+} lungs (0.31 a.u.) compared with WT littermates (0.22 a.u.) (Figure 5.2C). Furthermore, the theoretical total lung volume (Parameter B of the Salazar-Knowles equation) was also increased in *Vangl2*^{Lp/+} lungs (1.78 ml) compared with WT littermates (1.45 ml) (Figure 5.2D).

When comparing lung function between PBS and LPS treated groups, no significant difference was found for either WT or *Vangl2*^{Lp/+} mice (compare Figure 5.2 and Figure 3.5). However, some lung function parameters which did not show a significant difference between WT and *Vangl2*^{Lp/+} lungs in control PBS treated mice such as tissue elastance and ETA, showed a significant difference following LPS challenge (compare Figure 5.2 and Figure 3.5).

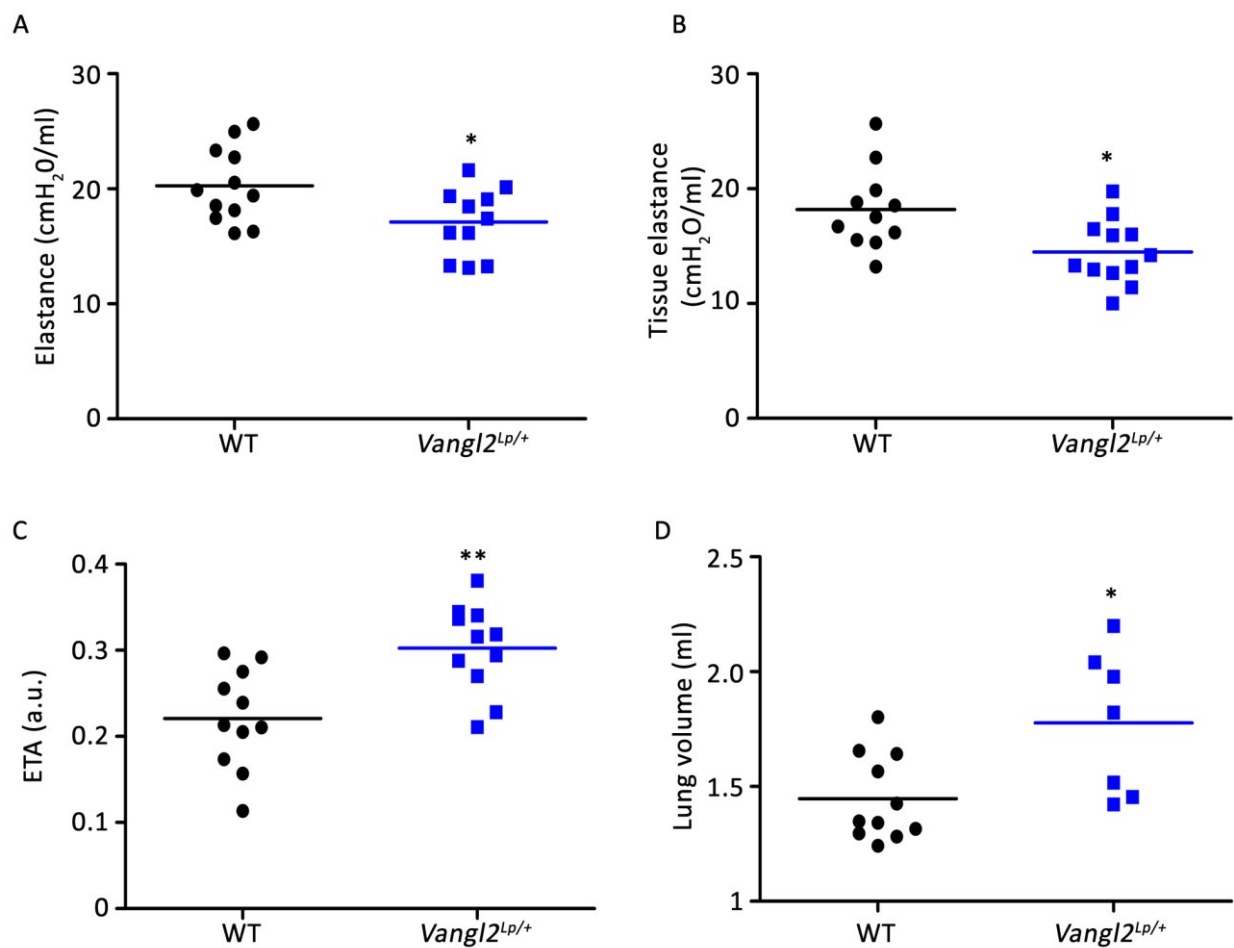


Figure 5.2. Lung function defects in *Vangl2*^{Lp/+} mice were maintained following LPS challenge

10 week old male mice were given a single intranasal dose of LPS, and lung function was assessed by the Flexivent system after 24 hr. Scatter plots showing lung function measurements of (A) elastance, (B) tissue elastance (C) tissue hysteresivity, and (D) Salazar-Knowles B parameter obtained by forced oscillation technique (each point shows an individual mouse the data is combined from two independent experiments, student's T-test P < 0.05 *, P < 0.01 **).

5.2.3. LPS did not cause structural alterations to lungs

In addition to testing lung function, lung histology was assessed to analyse if LPS caused structural alterations in the lung. The mean linear intercept (Lm) was calculated in LPS treated and PBS control mice. As expected, Lm was significantly increased in both PBS and LPS treated *Vangl2*^{Lp/+} mouse lungs (88.86 µm and 88.66 µm respectively) compared with WT littermates (45.04 µm and 56.85 µm respectively) (Figure 5.3A-E). However, LPS did not cause a significant disruption to lung structure after 24 hr in either WT or *Vangl2*^{Lp/+} mice (Figure 5.3E). These results indicate that a single dose LPS challenge was not sufficient to cause lung structural alterations after 24 hr.

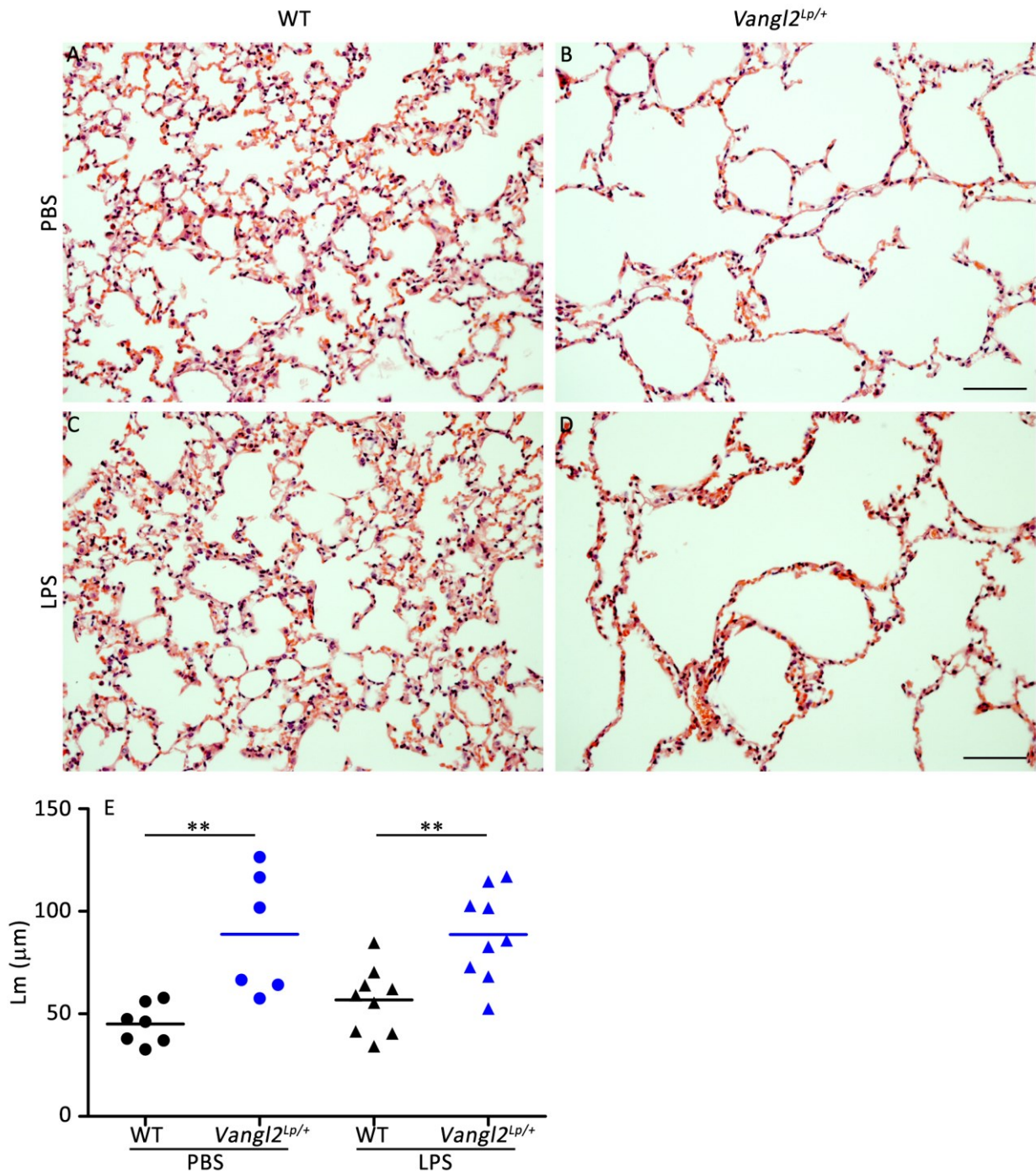


Figure 5.3. LPS did not cause structural disruption to lungs after 24 hr

Left lung lobes were isolated from WT and *Vangl2*^{Lp/+} mice 24 hr after LPS challenge to assess histology. H&E stained sections of adult lungs following (A,B) PBS and (C,D) LPS treatment in (A,C) WT and (B,D) *Vangl2*^{Lp/+} littermates. (E) Quantification of the mean linear intercept (Lm) showed increased airspace diameter in *Vangl2*^{Lp/+} mice, however Lm was unaffected by LPS treatment (each point shows an individual mouse, one-way ANOVA with Bonferroni multiple comparison, $P < 0.01$ **). Scale bar represents (A-D) 60 μm.

5.2.4. Viral dose response in C3h/HeN mice

As viruses are also known to contribute to exacerbations in COPD, we assessed the impact of a viral infection in *Vangl2*^{Lp/+} lungs. A viral infection model holds an advantage over the LPS exacerbation as this model uses a live virus which is capable of infecting a cell and replicating; thereby presenting a true infection as opposed to using a single compound to mimic an infection. In addition, this allows for a prolonged infection model, during which mice can be examined over time to assess their response to the infection, and the resolution of the infection.

To establish the optimal dose of virus to use for the infection challenge, adult C3H/HeN mice were given a range of single intranasal doses of the influenza virus A/England/195/09 (E195) virus from 1×10^5 plaque forming units (PFU) to 5×10^2 PFU, or vehicle control alone (PBS). The E195 is typical of the H1N1 pandemic strain influenza virus isolated from an individual returning to England [410]. Mouse weight and symptoms were scored daily to assess the progression of infection. The optimal dose was identified as that which was able to produce a robust weight loss correlating with onset of illness without reaching the humane endpoint of 30 % weight loss. Following weight loss, mice should then show recovery, as seen by an increase in body weight.

Following influenza infection, weight loss was observed in all mice. Mice that received a dose equal to or greater than 5×10^3 PFU had rapid weight loss, and by day 7 were near the humane endpoint (Figure 5.4A). Mice given the lowest dose of 5×10^2 PFU showed a slower weight loss and did not reach the humane endpoint by day 7 (Figure 5.4A). In addition to weight, mice infected with influenza A also showed an increase in symptoms scores. Mice given the highest dose of 1×10^5 PFU reached the humane endpoint at day 5 as they had reached a symptom score of 4 on two consecutive days (Figure 5.4B). The increase in symptom scores occurred in a dose dependent manner, with slower progression of symptoms with a lower dose of virus (Figure 5.4B).

Even at the lowest viral dose of 5×10^2 PFU the mice failed to show resolution of the infection, and continued to show weight loss until they reached the humane endpoint. Due to time constraints, further doses of virus at lower concentrations were not tested. However, based on the above investigation, a lower viral titre of 100 PFU was selected for ongoing experiments. These preliminary results demonstrated that C3h/HeN mice showed a robust dose dependent response to viral infection.

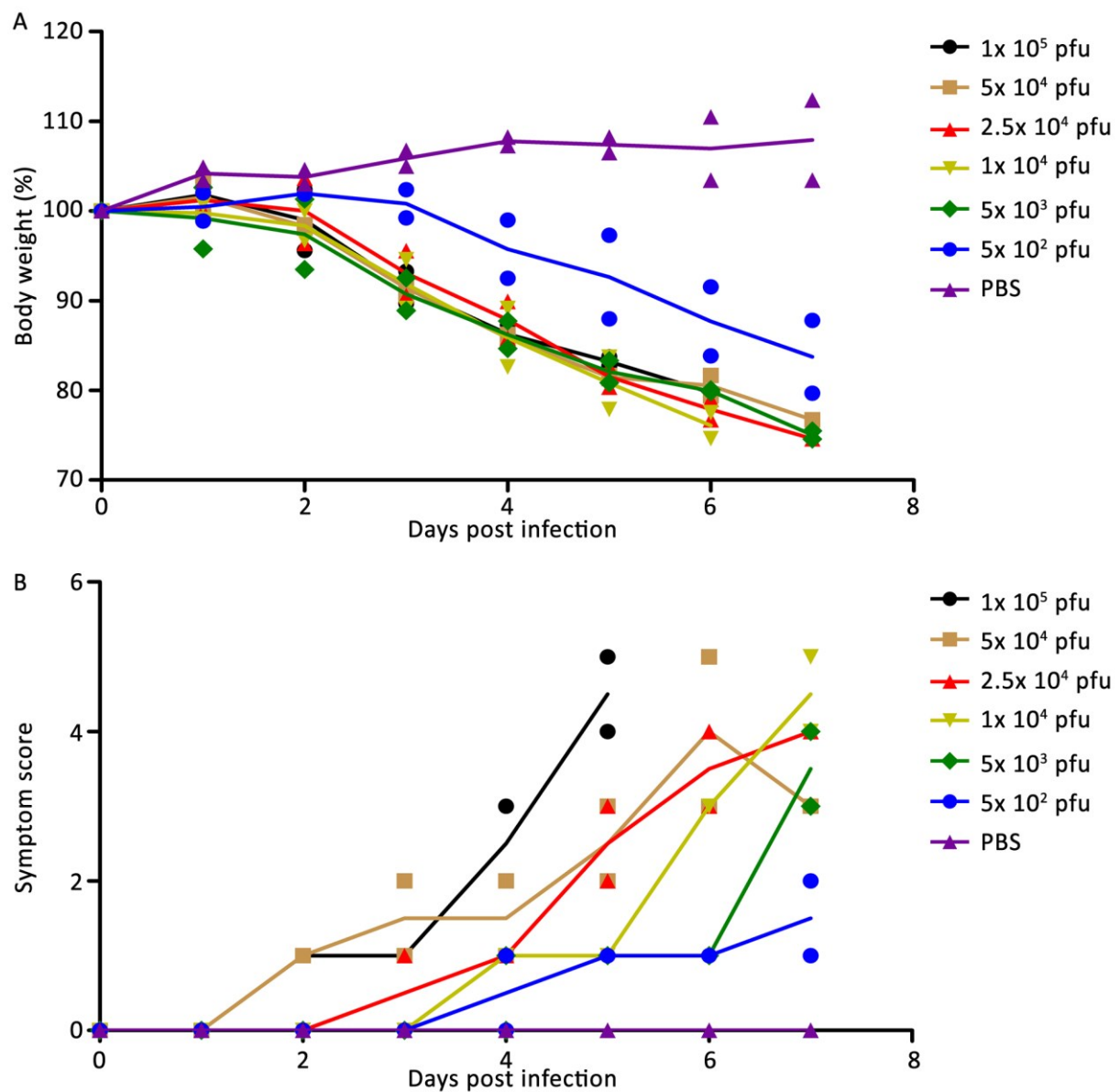


Figure 5.4. C3h/HeN mice showed robust response to E195 influenza viral infection

WT C3h/HeN mice were given a single intranasal dose of E195 influenza virus at different concentrations ranging from 5×10^2 PFU to 1×10^5 PFU, and PBS control. **(A)** Mouse weight was assessed daily for 7 days (each point shows an individual mouse and the line shows the median value). The humane endpoint was reached when mice had reached almost 30 % weight loss. **(B)** Symptom scores were assessed daily following viral infection for 7 days (each point shows an individual mouse and the line shows the median value).

5.2.5. Increased inflammation in *Vangl2*^{Lp/+} lungs in influenza infection

Having determined a suitable dose of influenza A virus, adult WT and *Vangl2*^{Lp/+} mice were infected with 100 PFU virus. Mice were analysed three and nine days post infection by assessing histology and the BALF for immune cell populations (Figure 5.5A). Weight and symptom scores were performed daily for each mouse to assess the mouse health and response to the infection.

Following flu infection both WT and *Vangl2*^{Lp/+} littermates exhibited comparable weight loss (Figure 5.5B) and increasing symptom scores (Figure 5.5C) as the disease progressed until day 7 with no significant differences between WT and *Vangl2*^{Lp/+} mice. Interestingly, at day 8 and day 9, *Vangl2*^{Lp/+} mice showed an increase in weight compared with WT mice which continued to show weight loss (Figure 5.5B). However, there was no significant difference in symptom scores between *Vangl2*^{Lp/+} mice and WT littermates at any time point (Figure 5.5C). These results indicate that *Vangl2*^{Lp/+} mice may have earlier onset of resolution of the infection compared with WT littermates as seen by the earlier increase in weight.

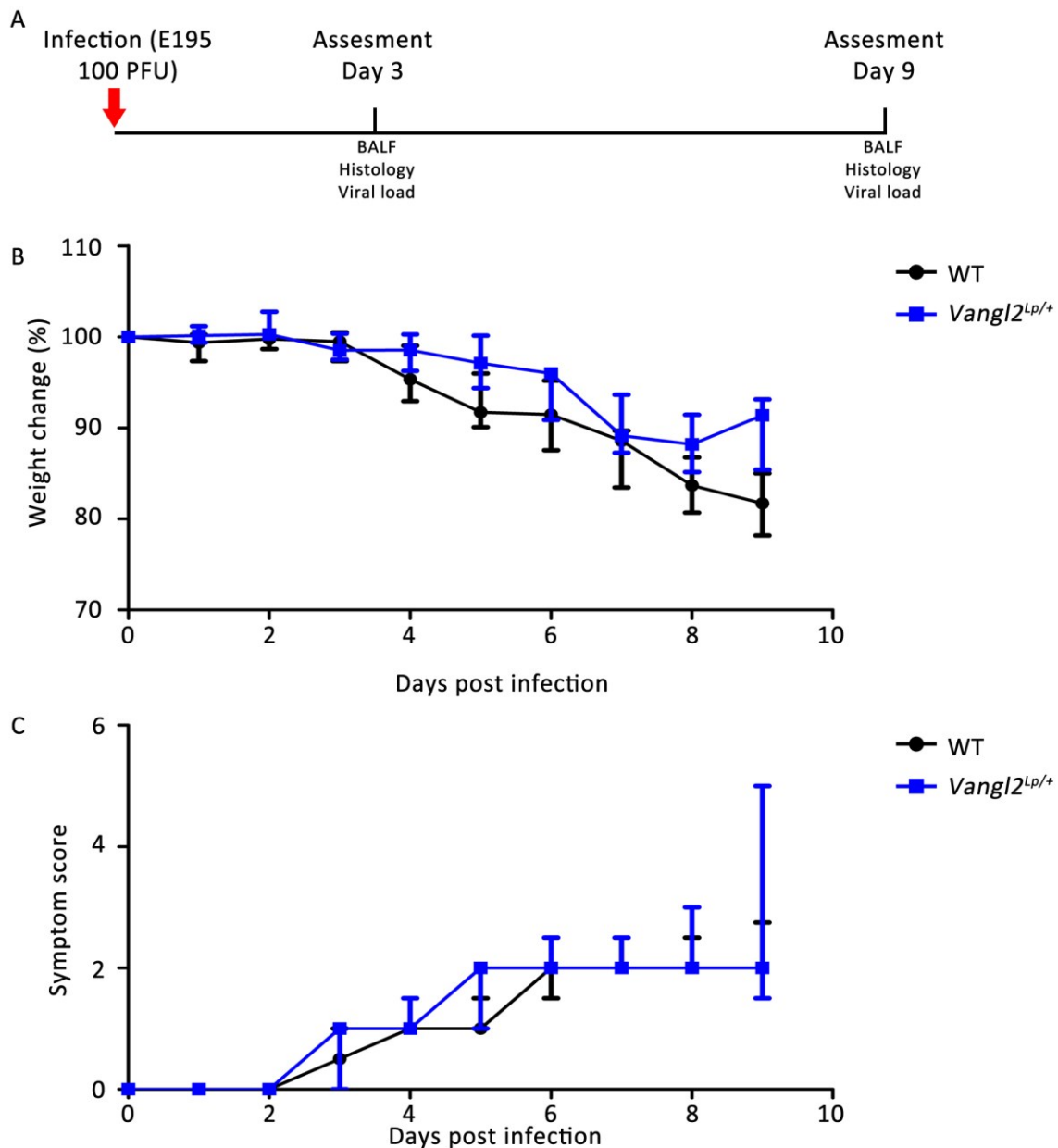


Figure 5.5. E195 Influenza A virus caused weight loss and worsened health in adult mice

(A) Timeline of viral infection and analysis of mice at day 3 and day 9 post infection (p.i). Mice were given a single intranasal dose of 100 PFU E195 virus, and mouse weight and symptom scores was assessed daily. (B) Relative weight change of WT and *Vangl2^{Lp/+}* mice following viral infection; weight change is related to the initial starting weight (n=10 for day 0-3 and n=3-5 for days 4-9 for both WT and *Vangl2^{Lp/+}*, median and range, Kruskal-Wallis with Dunn's multiple comparison test, $P > 0.05$). (C) Symptom scores of WT and *Vangl2^{Lp/+}* mice following viral infection (n=10 for day 0-3 and n=3-5 for days 4-9 for both WT and *Vangl2^{Lp/+}*, median and range, Kruskal-Wallis with Dunn's multiple comparison test, $P > 0.05$).

As mentioned above, the mice were analysed further at three and nine days post infection (p.i) to assess the viral load and inflammatory response. These time points were chosen based on the course of a typical influenza viral infection [411,412]. In a viral infection, peak infection occurs at day 3 and innate immune cells such as neutrophil are recruited into the BALF [411]. The resolution phase then occurs roughly 5 days after the initial infection, and adaptive immune cells are recruited into the lung [412]. Therefore at the day 9 the time point, the adaptive immune system will have cleared the virus from lungs. Assessment of viral load by plaque assay showed that on day 3 viral load was increased in both WT (33.41×10^3 PFU/50mg) and *Vangl2*^{Lp/+} mutant lungs (9.21×10^3 PFU/50mg). *Vangl2*^{Lp/+} lungs showed a trend towards reduced viral load although this difference was not significant (Figure 5.6A). By day 9, the viral load was below the limit of detection for both WT and *Vangl2*^{Lp/+} lungs, indicating that the virus had been cleared in both genotypes (Figure 5.6A).

Assessment of the immune cell response in the BALF showed no significant difference in total immune cell numbers between WT and *Vangl2*^{Lp/+} littermates at either time point (Figure 5.6B). Nor did we see a significant difference between day 3 and day 9 for both genotypes (Figure 5.6B). Differential cell counts of BALF immune cell populations showed no significant differences in the percentage of macrophages between WT and *Vangl2*^{Lp/+} lungs at either time point (Figure 5.6C). Furthermore, total macrophage numbers did not differ between genotypes at day 3, but were significantly increased in *Vangl2*^{Lp/+} BALF (2.90×10^5 cells/ml) at day 9 compared with WT littermates (0.39×10^5 cells/ml) (Figure 5.6D).

In the early immune response at day 3, neutrophils were the predominant immune cell population in the BALF (Figure 5.6E), and relatively few lymphocytes were present (Figure 5.6G). Later, following the recruitment of the adaptive immune system at day 9, lymphocytes became the predominant immune cell population (Figure 5.6G), and the neutrophil population was reduced (Figure 5.6E). However, at both time points there was no significant difference in the percentage of neutrophils and lymphocytes between WT and *Vangl2*^{Lp/+} littermates. Quantification of total neutrophil numbers showed a trend towards increased neutrophil numbers at day 3 compared with day 9 for both WT and *Vangl2*^{Lp/+} lungs (Figure 5.6F). Total lymphocyte numbers showed a trend to be increased at day 9 compared with day 3 although there was no significant difference (Figure 5.6H). However, at day 9 there was a significant increase in the number of lymphocytes in *Vangl2*^{Lp/+} lungs (8.42×10^5 cells/ml) compared with WT littermates (3.29×10^5 cells/ml) (Figure 5.6H).

Together, these results indicate that both WT and *Vangl2*^{Lp/+} mice showed a typical response to influenza infection, with an initial neutrophilic inflammation followed by lymphocytic inflammation in the resolution phase. However, *Vangl2*^{Lp/+} mice showed a tendency towards earlier resolution of infection based on the weight gain. Interestingly, they also showed increased macrophage and lymphocyte numbers in BALF at day 9 compared with WT littermates.

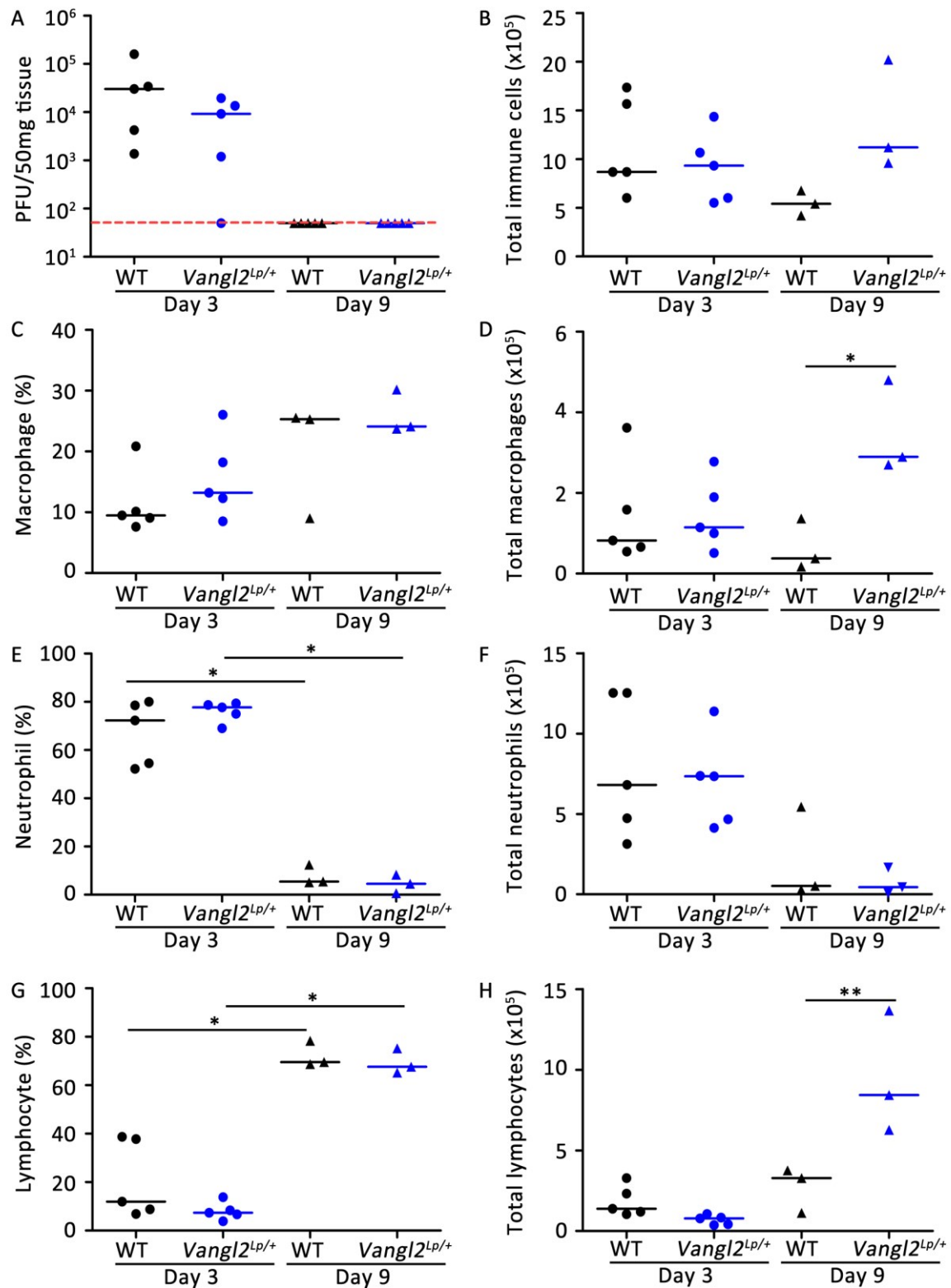


Figure 5.6. *Vangl2*^{Lp/+} mice had increased macrophage and lymphocyte numbers in the BALF

Immune response to the E195 influenza virus was assessed 3 and 9 days after the challenge. (A) Viral load in lungs of WT and *Vangl2*^{Lp/+} mice at day 3 and day 9. (B) Total immune cells in BALF of WT and *Vangl2*^{Lp/+} mice at day 3 and day 9. Percentage of (C) macrophages, (E) neutrophils and (G) lymphocytes in BALF of WT and *Vangl2*^{Lp/+} mice at day 3 and day 9. Total number of (D) macrophages, (F) neutrophils, and (H) lymphocytes in BALF of WT and *Vangl2*^{Lp/+} mice at day 3 and day 9 (each point shows an individual mouse, Kruskal-Wallis followed by Dunn's multiple comparison test, P < 0.05 *, P < 0.01 **).

5.2.6. Inflammation in the lung tissue was comparable between WT and *Vangl2*^{Lp/+} mice

In addition to the BALF, immune cells are also recruited into the lung tissue in influenza infections, resulting in inflammation around the vasculature and bronchiolar walls [413]. Therefore, H&E stained lung sections were analysed to assess the recruitment of immune cells at day 3 and day 9 p.i (Figure 5.7A-D). A modified histological scoring method adapted from Matute-Bello *et al* [290] was used to assess the inflammation in each of the following regions of the lungs: alveolar spaces, interstitium, bronchioles, and vasculature (see Section 2, Table 2.6).

Scoring of the lung histology showed increased inflammation at day 9, as shown by the increased histology score, compared with day 3 in both WT and *Vangl2*^{Lp/+} lungs (Figure 5.7E); however, no significant difference was observed between WT and *Vangl2*^{Lp/+} littermates at either time point. Thus, inflammation was studied separately in each compartment between WT and *Vangl2*^{Lp/+} lungs. However, there was no significant difference in the histological score between WT and *Vangl2*^{Lp/+} lungs in each compartment at both time points (Figure 5.7F).

Another common pathology observed in influenza infection is oedema [414]. Therefore, albumin levels were measured in the BALF as a marker of oedema resulting from disrupted vascular-epithelial integrity. Viral infection led to elevated albumin levels in BALF from both WT and *Vangl2*^{Lp/+} mice at day 3 (0.48 mg/ml and 0.47 mg/ml respectively) compared with that from naive mice (compare Figure 5.7G and Figure 3.8E). Albumin levels were further elevated at day 9 in both WT and *Vangl2*^{Lp/+} lungs (2.81 mg/ml and 1.98 mg/ml respectively) compared with day 3 (Figure 5.7G). However, no significant differences were observed between WT and *Vangl2*^{Lp/+} littermates at either time point.

Together, these results show increased inflammation and oedema in the lung tissue and alveolar space as a result of the viral infection in both genotypes at day 9; however, there was no significant difference between WT and *Vangl2*^{Lp/+} lungs.

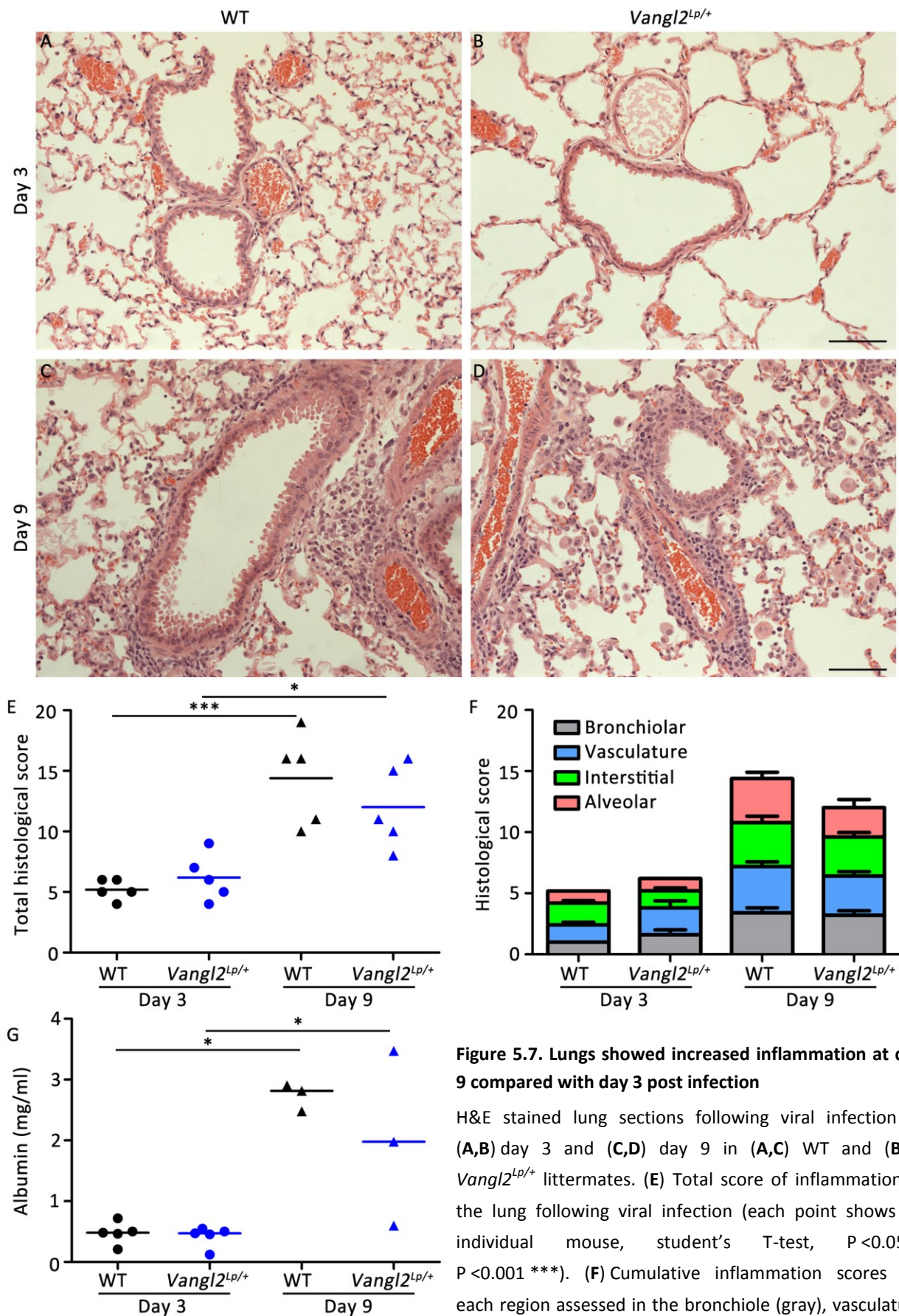


Figure 5.7. Lungs showed increased inflammation at day 9 compared with day 3 post infection

H&E stained lung sections following viral infection at (A,B) day 3 and (C,D) day 9 in (A,C) WT and (B,D) *Vangl2^{Lp/+}* littermates. (E) Total score of inflammation in the lung following viral infection (each point shows an individual mouse, student's T-test, $P < 0.05$ *, $P < 0.001$ ***). (F) Cumulative inflammation scores for each region assessed in the bronchiole (gray), vasculature (blue), interstitial space (green), and alveolar space (pink). (G) Quantification of BALF albumin levels at 3 and 9 days following viral infection (each point shows an individual mouse, Mann-Whitney U, $P < 0.05$ *).

5.3. Discussion

5.3.1. Acute infection with LPS did not worsen lung function in *Vangl2^{Lp/+}* mice

As the lungs of *Vangl2^{Lp/+}* mice structurally resembled lungs of emphysematous patients and showed a similar pattern of lung function defects, we assessed if *Vangl2^{Lp/+}* mice were more susceptible to exacerbation of lung defects by infection. Therefore, lung function was assessed following infection with LPS, a bacterial cell wall component, to mimic an acute exacerbation. LPS challenge caused an acute neutrophilic inflammation in the BALF, which is characteristic of a LPS challenge at 24 hr [287,415]. The neutrophilic inflammation did not appear to worsen lung function in either WT or *Vangl2^{Lp/+}* mice compared with PBS controls at 24hr (see Section 3.2.4). Interestingly, tissue elastance showed no significant difference between WT and *Vangl2^{Lp/+}* mice with PBS treatment, but was significantly reduced in *Vangl2^{Lp/+}* lungs following LPS challenge. This could be as a direct result of the LPS exacerbation causing the difference in tissue elastance to become more pronounced. Alternatively, it may be due to the increased sample numbers in the LPS treated group (WT n=12 and *Vangl2^{Lp/+}* n=7-12) compared with PBS controls (WT n=7-8 and *Vangl2^{Lp/+}* n=5-7) leading to higher power in statistical testing.

In a recent study, Kobayashi *et al* used a single dose of LPS as a mechanism of exacerbating lung injury in mice with underlying lung disease caused by elastase treatment [287]. Similar to the *Vangl2^{Lp/+}* mice, Kobayashi *et al* did not see a significant morphological change in the emphysematous phenotype following LPS challenge using Lm measurements. However, they found that by using a more sensitive measure by assessing the low-attenuation area % (LAA%) by micro-computed tomography (micro-CT) they were able to identify exacerbation of the emphysematous phenotype [287]. The LAA % measurement is able to more sensitively assess changes in tissue architecture, and so holds an advantage over Lm measurements. Therefore, it is possible that assessment of LPS damage in *Vangl2^{Lp/+}* lungs used in our study was not sensitive enough to pick up subtle changes in the lungs.

In addition, Lm measurements have some disadvantages when assessing milder structural changes. Firstly the Lm is a stereological method which relies on calculating the arithmetic mean, thereby centralising the data sets for each sample [416]. Consequently, by taking the mean, variation between samples is removed, which can under report subtle changes in lung morphology. Another problem is that non-standardised preparation methods such as variation in inflation volume and pressure during fixation can alter the tissue dimensions which can influence the Lm [417]. Furthermore, Lm uses analysis of 2D images of the lung to infer information about the 3D structure. In this respect, the Lm is limited as it does not take into account the volume of airspaces which are three dimensional structures. In an example used by Weibel *et al* of two airspaces with the same volume, one which is spherical and another which is an ellipsoid; taking a two dimensional cross-section of these shapes will show different lengths (Lm) although the overall volume is the same [417]. Therefore, using the Lm alone to infer the characteristic changes in 3D

may bias the data. Methods such as micro-CT that can visualise the airspaces in 3D allow for more sensitive and unbiased representation of changes occurring in the lung.

A possible cause for the lack of lung function exacerbation following LPS may be due to the 24 hr time point being insufficient to induce lung damage. Although neutrophils were recruited into the lungs, this may not be sufficient to have caused measurable damage within the lung. In the Kobayashi *et al* study, they only reported a trend towards exacerbation two weeks after LPS administration and did not notice a significant difference till 9 weeks after the challenge [287]. Therefore, it is possible that a longer time point is required to assess exacerbation of lung function by LPS in the *Vangl2*^{Lp/+} model. To this end, lung function could be assessed 9 weeks after an LPS challenge, in order to allow for lung function exacerbations to become more pronounced. In support of using a long term model, the viral challenge model did not show a difference in disease onset. However, disease resolution was altered as seen by the earlier weight gain in the *Vangl2*^{Lp/+} mice at the later time points which was not seen in WT mice. Therefore, using a long-term model, both onset and resolution of the disease could be assessed to study if the response in the *Vangl2*^{Lp/+} mice is altered from the WT littermates.

5.3.2. Influence of viral infection in lungs

As a model of viral exacerbation, mice were infected with influenza A virus. C3H/HeH mice showed the classic pattern of immune response following viral infection [411,412]. The typical response involves the rapid recruitment of innate immune cells, with a high neutrophilic inflammation immediately following infection and peaks around day 3 p.i [411,412,418]. This is followed by recruitment of the adaptive immune system, after 5 days p.i, during the resolution phase; at this time point lymphocytes such as CD8⁺ T-cell which are important for clearing the virus are recruited [412]. Compared with other mouse strains, C3H mice appear particularly susceptible to infection with E195 influenza A virus. Previous studies of E195 viral infection in BALB/C mice found doses of 1×10^4 PFU were required to induce a 20 % weight loss at day 7 [419]. However, in our model even at a low dose of 5×10^2 PFU, C3H mice showed a rapid decline in weight of nearly 20 % at day 7 in the preliminary experiment.

Furthermore, both WT and *Vangl2*^{Lp/+} mice showed a typical pattern of viral load during the infection. At day 3, which is typically the time of peak infection, the viral load increased from the initial dose of 100 PFU which was administered. At day 9 the viral load then dropped below the limit of detection as the virus was cleared by the immune system. Interestingly, there was a trend towards reduced viral load in the lungs of *Vangl2*^{Lp/+} mice compared with WT littermates at 3 days p.i. This may have been caused by the disrupted lung architecture in *Vangl2*^{Lp/+} mice resulting in reduced efficiency of viral infection as the alveolar space was enlarged. Furthermore, the altered structure may also have affected the ability of the virus to spread and infect other cells, resulting in the reduced viral load at day 3.

It is possible that the low number of viral particles used in the initial infection may also have contributed to heterogeneity in the pattern of inflammation during infection. Influenza is able to invade and replicate in both the upper respiratory tract and parenchyma [418,420]. If virus particles infect cells in the upper airways it will in turn limit the number of viruses reaching the lung parenchyma. As the lung parenchyma is the area most affected by the *Vangl2*^{Lp} mutation, reduced virus numbers reaching the lung parenchyma may dampen lung exacerbations. The low dose of viruses administered could also affect the number of initial sites of infection in the parenchyma; as a result, some areas of the lung had very little inflammation, while in other regions there was extensive inflammation. Overall, the low viral number introduces increased variability into this model, and this may account for the variability in response to the viral infection observed, potentially under representing biological differences in *Vangl2*^{Lp/+} lungs.

One aspect to note is that the initial viral dose response was undertaken in C3H/HeN mice, whereas the *Vangl2*^{Lp/+} mice are on a different sub-strain, C3H/HeH. Although both these C3H sub-strains have WT toll like receptor 4 (TLR4), which is not present in the C3H/HeJ sub-strain [421], there may be other strain differences that could affect response to the virus. Although TLR4 has typically been associated with LPS recognition in bacterial infection [422], recent work has shown it to also be involved in response to viral infections such as respiratory syncytial virus (RSV) [423], indicating that TLR4 signalling can be activated by both bacterial and viral infections. The data presented in this study suggest that C3H/HeH mice were more susceptible to E195 influenza virus compared with C3H/HeN mice, as even the lower dose of 100 PFU was fatal in a few mice at the later time point (3 of 10 mice); indicating potential difference in response to infection in the substrains. Therefore, it could be beneficial to repeat the viral infection experiment on the *Vangl2*^{Lp/+} mice using a refined viral dosing scheme tailored to the C3H/HeH strain.

As mentioned above, both WT and *Vangl2*^{Lp/+} mice showed a typical immune response to the viral infection and successfully cleared the virus. However, WT mice failed to show weight gain. Conversely, *Vangl2*^{Lp/+} showed weight gain and reduced symptom scores at day 9 p.i, and also had increased macrophage and lymphocyte numbers compared with WT littermates. Surprisingly, two *Vangl2*^{Lp/+} and one WT mouse died at day 9 although they did not show a severe weight loss or symptoms. BALF could not be obtained from these mice to analyse the inflammatory population; however, histological assessment of these mice showed severe inflammation, with airways and lung parenchyma which were obstructed by immune cells.

The data presented for the viral infection study comes from only one set of experiments with relatively low sample number (n=3-5 per genotype at each time point); thus, no final conclusion can be drawn from this data and further testing is required. As mentioned above, the C3H mouse strains appear to be highly susceptible to the E195 virus and very low viral titre was administered in the experiment which may have contributed to the heterogeneity in response to the infection. Therefore, it would be prudent to back cross the *Vangl2*^{Lp} mutation on to another mouse background such as the BALB/C strain in which the

E195 has previously been tested [419]; a higher viral dose can be given to these mice which may reduce the heterogeneity in the infection of the lung parenchyma.

5.3.3. Further work

Although lung function analysis is not classically performed following influenza virus infections, it is however, becoming more prominent in recent infection studies. Therefore, it would be interesting to test lung function in *Vangl2*^{Lp/+} mice following viral infection. Previous studies in murine models have shown altered lung function, with increased airway resistance and elastance, as well as decreased compliance, in influenza A viral infection [424]. Therefore, performing lung function analysis during viral infection in *Vangl2*^{Lp/+} mice may show exacerbated lung function defects compared with WT littermates. For this experiment, analysis of lung function throughout the course of the infection is important to assess onset of disease and resolution, as the data from this study suggests that *Vangl2*^{Lp/+} mice may show earlier onset of resolution.

Additional future work would be to study immune cell recruitment following viral infection in greater detail using specialised techniques such as flow cytometry. Wright-Giemsa staining is only able to broadly classify immune cell populations, and is not capable of distinguishing between different classes of lymphocytes such as B-cell, T-cells, or natural killer (NK) cells [425]; these populations play distinct functions in disease response. Moreover, lymphocytes populations contain further subsets, such as CD4⁺ and CD8⁺ T cell populations, which also have different functions in immune responses [426]. As an increase in the lymphocyte population was seen in *Vangl2*^{Lp/+} lungs at day 9, it would be worth investigating which subtype or subtypes of lymphocytes were altered, or whether all the populations increased similarly. Flow cytometry would allow an accurate and quantitative analysis of the immune cell populations in the lung and BALF.

In addition to infections, environmental causes can also affect lung function. One of the leading contributory factors to chronic lung disease in the modern world is smoking [1]. In line with this, cigarette smoke exposure (CSE) has been shown to cause emphysema in murine models [427,428]. Recent work performed in collaboration with the Dean lab has identified a *VANGL2* SNP in humans that is associated with a decline in lung function specifically in smokers [Minelli C and Pereira M, Unpublished data]. Based on the findings of this study, it would be interesting to assess if CSE in *Vangl2*^{Lp/+} lungs, which already have underlying lung function and structural defects, leads to exacerbation of lung function defects and increased progression of alveolar wall destruction compared with WT littermates. We hypothesise that CSE in *Vangl2*^{Lp/+} mice will show an increased rate of lung function decline compared with WT littermates, resulting in more severe airspace enlargement. In addition, it would be interesting to study whether cell proliferation and apoptosis, which are not affected in development and homeostasis in *Vangl2*^{Lp/+} lungs, are

altered in response to an injurious insult by smoking. This data could provide novel insight into the genetic susceptibility to adult lung disease, particularly in smokers.

In addition to investigating emphysema, models of other chronic lung diseases such as IPF can also be investigated. Bleomycin is often used to induce pulmonary fibrosis as a model of IPF, resulting in increased alveolar wall thickness and thickened collagen fibres with an irregular pattern [429]. Typically, IPF and COPD are characterised by excessive and lack of repair processes respectively. Therefore, it would be interesting to study if *Vangl2* defects cause different lung function perturbations in the two disease models. As *Vangl2* appears to be important for wound healing and repair processes, *Vangl2* deficiency may inhibit the excessive repair processes in IPF, thereby slowing disease progression.

5.3.4. Concluding remarks

In conclusion, the lung defects present in *Vangl2*^{Lp/+} mice were not significantly exacerbated by the bacterial or viral infection models used in our study. However, further studies will be required to reach a definite conclusion. For both models a longer time point may be required to give sufficient time for the disease to develop, and for the resolution phase to be adequately assessed. However, the gene-infection interactions studied here may not be important in progression of chronic lung diseases in *Vangl2*^{Lp} mutants. Instead, the gene-environment interactions may play a more prominent role as seen in the population study of *VANGL2* SNP.

Chapter 6: Discussion and further work

6.1. Impact of the *Vangl2* mutation in the adult mouse lung

The work presented in this thesis is the first investigation of *Vangl2*, key PCP protein, in the post-natal lungs. In this thesis, we demonstrate that a single allele *Vangl2*^{Lp} mutation caused significant structural and functional lung defects in adults; this was found to be true in both the C3H/HeH mouse strain studied in our investigation, and the C57BL/6 mouse strain [Yates LL, Unpublished data]. Together, these findings demonstrate that the lung defects arise due to the *Vangl2*^{Lp} mutation, and in both strains a single *Vangl2*^{Lp} mutant allele was sufficient to disrupt lung structure. To the best of our knowledge, this one of the few heterozygous point mutations to cause severe lung defects. Previous studies have shown that a heterozygote mutation in α 1-antitrypsin gene (*AAT*) causes an increased rate of FEV₁ decline and increased risk of COPD in humans [430]. Thus, the *Vangl2*^{Lp/+} mice present a novel tool for investigating genetic susceptibility to adult lung disease.

Previous studies of mammalian *Vangl2* have shown it to be important for normal embryonic development of many organs including the lung [14], heart [210], kidney [209], neural tube [431], and inner ear [167]. However, remarkably little research has been done to determine the consequences of these embryonic defects postnatally. Recently, Rocque *et al* demonstrated a role for *Vangl2* in the adult kidney [211]; in developing WT mice, *Vangl2* is highly expressed in the kidney [209,211], and its expression is reduced after development and is undetectable in the mature glomeruli [211]. Following kidney injury with anti-glomerular basement membrane (anti-GBM) antiserum, expression of *Vangl2* and other PCP genes such as *Wnt5a*, *Scrib*, and *Pk1* significantly increased in the adult kidney, suggesting a role for PCP signalling during podocyte repair [211].

Our study showed that *Vangl2*^{Lp} mutation disrupted adult lung structure as a result of developmental defects, however, *Vangl2* was not required in the adult lung at homeostasis, similar to the adult kidney [211]. Using the *Vangl2*^{Lp} mutant mice, we were able to study the effect of disruption of the whole PCP pathway in lungs, as previous work has shown that the mutant *Vangl2*^{Lp} isoform disrupts the function of both WT *Vangl2* and its homologue *Vangl1*, as evidenced by the increased proportion of cytoplasmic *Vangl1* in *Vangl2*^{Lp} mutants [167]. As such, *Vangl2*^{Lp} mice have a more severe phenotype than *Vangl2*^{-/-} mice [167]. Therefore, it would be interesting to study the lung architecture of *Vangl2*^{-/-} mice and compare the lungs of these mice directly with that of *Vangl2*^{Lp/+} mice. Through this investigation, we would be able to discern if *Vangl2*^{-/-} mice have similar lung disruption to the *Vangl2*^{Lp} mutant mice during embryonic development and adulthood. This would enable us to discern how important *Vangl2* alone is in lung development without simultaneous disruption of other PCP genes. Given the findings of previous studies in other organs, we hypothesise that the lung disruption of *Vangl2*^{Lp/+} mice will be more severe than that of *Vangl2*^{-/-} mice.

One disadvantage of the *Vangl2*^{Lp} mutant mouse model used in this study is that adult homozygote *Vangl2*^{Lp} mutant mice cannot be studied as they die neonatally due to severe NTDs [201]. Recent advances in gene editing such as the identification of Clustered regularly-interspaced short palindromic repeats (CRISPR) and the Cas9 enzyme have allowed greater flexibility in generating genetically modified organisms [432]. One advantage of the CRISPR/Cas9 gene editing technology is the ability to introduce single point mutations into the genome, thus, using this method it is possible to model the *Vangl2*^{Lp} mutation into mice. Studies have shown that Cre-dependent Cas9 can be expressed in a tissue type specific manner, thereby producing cell type or tissue specific genome editing. Platt *et al* demonstrated in mice that *Cas9* expression could be restricted to specific cell population by driving *Cre* expression with different promoters [432]. Therefore, using the CRISPR/Cas9 system, it is possible to introduce the *Vangl2*^{Lp} mutation specifically in the lung, allowing us to study the impact of a homozygous *Vangl2*^{Lp} mutation in the adult lung whilst circumventing the neonatal lethality. Dow *et al*, further demonstrated that *Cas9* activity could also be regulated by an inducible CRISPR (iCRISPR) system such as by the use of doxycycline (Dox) [433]. Using this system would allow for introduction of the *Vangl2*^{Lp} mutation at any stage of lung development, such as specifically during the alveolarisation stage as discussed later.

Previous studies in the Dean lab have shown that homozygous *Vangl2*^{Lp} mutations in the embryonic lungs have more severe structural defects compared with heterozygous mutant lungs [Yates L, Unpublished data]. We hypothesise that adult mouse lungs with a homozygous *Vangl2*^{Lp} mutation, introduced by the conditional CRISPR system will have exaggerated lung structural defects compared with the heterozygote *Vangl2*^{Lp} mutants used in this study. Interestingly, although *Vangl2*^{Lp/+} mice show reduced lung function compared with WT littermates, they do not have a markedly altered life span. This maybe because the mice were housed in specific pathogen free conditions and so were exposed to minimal environmental challenges. Thus, in the absence of injury or exacerbation, underlying lung function defects may not adversely affect the mouse health. Therefore, it would be valuable to analyse the lungs of adult *Vangl2*^{Lp/Lp} lungs to determine whether the lung structural and functional defects are more severe than in lungs of *Vangl2*^{Lp} heterozygote mice. The more pronounced development defects in the homozygote mutant lung in turn may affect mouse health even in the absence of lung injury.

In addition to allowing us to study homozygote *Vangl2*^{Lp} mutations in lungs, the inducible CRISPR system allow for greater flexibility to study the importance of *Vangl2* during different developmental stages. In our investigation, we examined the lungs at P7, near the beginning of alveolarisation that in mice starts around P5 [17]. Although *Vangl2*^{Lp/+} lungs showed some structural alterations at P7 compared with WT, differences were not as pronounced as in adult mice when alveolarisation is complete. This suggests that the greatly altered lung structure observed in adult *Vangl2*^{Lp/+} mice occurs during the alveolarisation phase. To investigate this, the *Vangl2*^{Lp} mutation can be induced using the iCRISPR system postnatally using Dox at the beginning of alveolarisation. This would allow us to assess the impact of the *Vangl2*^{Lp} mutation

specifically during the alveolarisation stage, once normal embryonic development has completed. Using this system we could investigate to what degree the *Vangl2*^{Lp} mutation affects cell migration and secondary crest septation at the alveolar stage.

Furthermore, conditional *Cre* expressing mice can also be used to manipulate *Vangl2* genome editing in a cell type specific manner. In embryonic lung development, interaction between the epithelium and mesenchyme is critical for branching morphogenesis. Studies in embryonic lungs have shown *Vangl2* expression in both mesenchymal and epithelial cells, although *Vangl2* localisation was stronger in epithelial cells [14]. Furthermore, PCP is not restricted to epithelial cells but is also found in mesenchymal cells that can adopt a uniform polarisation during cell migration and intercalation [162]. Thus, the *Vangl2*^{Lp} mutation can be introduced conditionally in epithelial cells by driving *Cre* expression using the *SPC* promoter to express *Cas9* in epithelial cells only [434], or by using the *Tbx4* promoter to drive genome editing in mesenchymal cells only [435]. Conditionally introducing the *Vangl2*^{Lp} mutation in epithelial and mesenchymal cells separately can highlight the importance of PCP signalling in these regions in lung development. This would allow us to investigate which cell type is more important for mediating polarity during migration, or if both cell types are equally important. Owing to the higher expression of *Vangl2* in the epithelial cells [14], we hypothesise that *Vangl2* mutation in the epithelial cells will result in more severe lung defects.

As part of our study into the phenotype of adult *Vangl2*^{Lp/+} mice, we compared lungs of “young”, 10 week old, adults with those of “aged” 12 month old mice. Although we studied the lung structure of 12 month old mice, lung function was not measured. Therefore, further work should investigate lung function of 12 month old mice to assess if lung function continues to decline with age and if this was accelerated in *Vangl2*^{Lp/+} mice. Based on the histological findings of this study, and the kidney function study by Rocque *et al* [211], we hypothesise that without a challenge lung function will decline equally in both WT and *Vangl2*^{Lp/+} mice with age.

Further work should also evaluate the ECM in the lungs of aged *Vangl2*^{Lp/+} mice. In particular elastin expression and organisation should be studied as the elastic recoil of the lungs decreases with age [436]. However, there are discrepancies in changes to total lung elastin levels during aging, with some studies showing decreased elastin levels [437], while other show increased or unchanged levels of elastin [438]. These differences may occur as a result of variations in environmental exposure or methods of elastin extraction between the different studies. Examination of the elastin organisation in the aged mice, would allow us to assess if the elastin organisation becomes further disrupted in *Vangl2*^{Lp/+} lungs. Furthermore, this would also allow us to examine if the age related reduction in lung elastic recoil occurs as a result of increasingly disrupted elastin fibre organisation during aging.

6.2. Actin cytoskeleton regulation in *Vangl2^{Lp}* lungs

One of the most well studied targets of PCP signalling is the actin cytoskeletal reorganisation. Our findings showed disrupted cortical actin organisation in lungs of embryonic *Vangl2^{Lp/+}* mice. This correlated with disrupted cofilin (Cfl) regulation in embryonic *Vangl2^{Lp}* lungs. Cfl activity is regulated by LIMK which phosphorylates and deactivates Cfl. LIMK itself is downstream of ROCK signalling [439], which is known to act downstream of PCP signalling [354]. Therefore, we hypothesise that Cfl may act as a downstream target of PCP signalling to mediate actin cytoskeletal dynamics. Thus, by investigating LIMK activity, by western blotting and immunostaining we can elucidate if the perturbed actin organisation in *Vangl2^{Lp}* lungs occurs due to disrupted LIMK/Cfl regulation.

Although immunofluorescent staining can be used to show localisation of proteins, it is not possible to assess the dynamic regulation of proteins. Actin organisation is a highly dynamic process, thus, studying a single time point by western blot or immunofluorescent staining does not represent this process. Therefore, protein interaction can be analysed in real time to investigate the dynamic interactions occurring in cell migration. One method that can be employed *in vitro* to assess protein activity and localisation is fluorescence resonance energy transfer (FRET) based biosensors that rely on the transfer of energy between two light sensitive chromophores. To the best of our knowledge, there are currently no LIMK biosensors; however, the principles of currently available biosensors can be applied to synthesising a novel LIMK biosensor. Zheng *et al*, generated a biosensor which is able to report the kinase activity of cAMP-dependent protein kinase A (PKA) [440]; it may be possible to apply the principles of this biosensor to generate a LIMK biosensor. The PKA biosensor consists of three domains: a cyan fluorescent protein (ECFP), a linker and PKA phosphorylation substrate region, and an enhanced yellow fluorescent protein (YFP) [440]. If PKA is not active, the biosensor is not phosphorylated; thus, the ECFP and YFP chromophores are separated and unable to FRET. Therefore, when the ECFP is excited it emits fluorescence at 476 nm. If the biosensor is phosphorylated by active PKA, a conformational change occurs that brings the ECFP and YFP within close proximity, allowing for FRET to occur [440]. In this instance, excitation of the donor ECFP emits light which in turn excites the YFP, resulting in an overall emission at 527 nm. Therefore, based on the wavelength of the light emitted we are able to assess levels of kinase activity in the cell. Furthermore, using this method we can also assess the localisation of kinase activity in the cell. Therefore, using a similar LIMK biosensor in cells with the *Vangl2* mutations, we will be able to study levels of LIMK activity in the cell and assess its localisation during cell migration.

6.3. Response to injury in *Vangl2^{Lp/+}* mice

Preliminary studies in the Dean lab have shown increased susceptibility to lung injury in humans associated with SNPs in *VANGL2*. Therefore, we investigated the impact of *Vangl2* disruption following injury by *in*

vitro scratch assays and *in vivo* infection models. VANGL2 knockdown resulted in reduced wound healing and loss of cell polarity during migration. The actin cytoskeleton is essential for cell migration, as *Vangl2* mutations disrupt actin organisation, this may in turn perturb cell migration, resulting in impaired wound healing and inefficient repair mechanisms. Thus, VANGL2 deficiency in patients may cause insufficient repair following injury, leading to increased susceptibility to chronic lung disease such as emphysema which are thought to arise from lack of or abnormal repair in response to injury [441].

Previously published studies of the canonical Wnt signalling pathway by Uhl *et al* showed that activation of the Wnt/ β -catenin signalling promoted repair mechanisms in *ex vivo* cultures of lungs from COPD patients [13]. Data presented in this thesis also indicates that manipulation of Wnt signalling may provide a novel avenue of therapy to promote lung repair. Thus, we performed preliminary studies to investigate the interaction of the canonical and PCP Wnt signalling in *Vangl2*^{Lp/+} lungs as previous studies have shown that the two pathways can act antagonistically [145]. In line with this, preliminary work in the Dean lab showed increased active β -catenin (ABC) levels in *Vangl2*^{Lp/+} lungs compared with WT littermates. However, this did not correlate with increased gene transcription of *Axin2* and *Nkd1*, which are targets of canonical Wnt/ β -catenin signalling [13]. Studies have shown that the ABC can function through a divergent pathway to promote actin dynamics [442]. Stamatakou *et al* demonstrated that Wnt3a promoted F-actin accumulation and increased actin dynamics in neurons; GSK3 β inhibition, which promotes canonical Wnt signalling, also promoted actin filament assembly [442]. This actin cytoskeleton remodelling was mediated by Epidermal Growth Factor Receptor Pathway Substrate 8 (Eps8), which belongs to a family of actin-binding proteins, via direct interaction with Dvl1 [442]. Therefore, we could assess Eps8 localisation by immunostaining to validate alternative pathways by which cytoskeletal dynamics are altered in *Vangl2*^{Lp} mutant lungs. Together, these studies may provide important information that could be used to target the actin cytoskeleton to promote cell migration and repair in the lungs. However, further work is required to validate the increased ABC levels observed during PCP disruption by *Vangl2*^{Lp} mutation.

In this thesis, we examined the effects of an acute LPS challenge and an influenza challenge in *Vangl2*^{Lp/+} mice to assess if the *Vangl2*^{Lp} mutation leads to increased susceptibility to injury in the lungs. A viral challenge was utilised as a long-term model of infection; the limitations of this study were discussed earlier in *Chapter 5*. Our data indicated that *Vangl2*^{Lp/+} mice were more protected from the viral infection as the viral load during peak infection was lower. *Vangl2*^{Lp/+} mice also showed earlier weight gain and reduced symptom score, demonstrating earlier onset of resolution in *Vangl2*^{Lp/+} mice compared with WT. Although the 9 day post infection time point is during the resolution period, the resolution was still ongoing as adaptive immune cells were still present in the lungs. Therefore, later time points should be studied to assess if *Vangl2*^{Lp/+} mice resolve the inflammation earlier than WT.

6.4. Macrophages in the adult *Vangl2*^{Lp/+} lungs

In addition to the disrupted lung architecture, we observed an interesting and unexpected alteration in the macrophage phenotype in adult *Vangl2*^{Lp/+} lungs. In our study, analysis of macrophage populations in the BALF of *Vangl2*^{Lp/+} mice showed an increase in a distinct population of enlarged macrophages which were highly vacuolar; studies in *Vangl2*^{Lp/+} lungs in the C57BL/6 mouse strain in the Dean lab also showed similar alterations [Yates LL, Unpublished data]. These observations indicate that the altered macrophage phenotype did not arise due to strain differences but as a result of the *Vangl2*^{Lp} mutation. In this study, we show an increase in M2 polarized macrophages with strong *Mmp-12* expression in *Vangl2*^{Lp/+} lungs. Although our data showed that the macrophage population was altered in *Vangl2*^{Lp/+} lung, it does not show how the altered macrophage population arises. The altered macrophage phenotype in *Vangl2*^{Lp/+} mice could arise by two different mechanisms; first the *Vangl2*^{Lp} mutation itself may have intrinsic effects and drives the altered macrophage phenotype, alternatively the microenvironment, be it the ECM, disrupted tissue architecture, or the cytokine profile is driving the phenotype.

To test this, we could use *in vivo* adoptive transfer of macrophages as a means of elucidating the origin of the altered macrophage phenotype in *Vangl2*^{Lp/+} lungs (Figure 6.1A). In this study, mice which ubiquitously express green fluorescent protein (GFP), such as the transgenic mouse line which expresses GFP under the control of chicken beta-actin promoter (*CBA*^{GFP}) [443], could be used to obtain cells which are identifiable by GFP expression; these cells will be used as controls. The *Vangl2*^{Lp/+} mice will also be bred with *CBA*^{GFP} mice to generate *Vangl2*^{Lp/+}/*CBA*^{GFP/+} offspring; these mice will contain the *Vangl2*^{Lp} mutation and ubiquitously express GFP.

Initially alveolar macrophages could be isolated by BALF from *CBA*^{GFP/+} and *Vangl2*^{Lp/+}/*CBA*^{GFP/+} mice; these cell populations will be identifiable by GFP expression. Following isolation, macrophages from *CBA*^{GFP/+} and *Vangl2*^{Lp/+}/*CBA*^{GFP/+} mice could be adoptively transferred by intranasal dosing into WT and *Vangl2*^{Lp/+} mice which do not express GFP (Figure 6.1A). The mice will be left for the appropriate time to enable macrophage polarisation to occur. The alveolar macrophages, consisting of a mixed population of GFP positive donor and GFP negative host cells, could be isolated and assessed by flow cytometry.

The transferred macrophages can be analysed using the proposed gating strategy in Figure 6.1B. The alveolar macrophages population will be identified as CD45⁺/CD11b⁺/CD11c⁺ [444]. GFP expression can then identify adoptively transferred (GFP⁺) and host (GFP⁻) macrophages to analyse the two populations individually. Cell morphology can be assessed using forward scatter (FSC) and side scatter (SSC); the enlarged macrophages will have a larger FSC and SSC due to their increased size and granularity compared with normal macrophages. In addition to assessing the morphology, cytokine production and expression of M1 or M2 markers can be used to assess macrophage polarisation and activation.

If the phenotype of macrophages in *Vangl2*^{Lp/+} lungs is driven by the *Vangl2*^{Lp} mutation itself, the host microenvironment will not be expected to alter the macrophage phenotype. Therefore, we would expect donor macrophages to maintain similar expression profiles when transferred into either WT or *Vangl2*^{Lp/+} lungs. If the local environment is driving the enlarged macrophage phenotype, then the transferred macrophages will adopt a similar phenotype to the macrophages in the host lungs. Houghton *et al* demonstrated that elastin fragments contain chemotactic activity which can promote disease progression during emphysema and drive macrophage recruitment [306]. Examination of the elastin structure in this thesis found that elastin organisation was disrupted in the *Vangl2*^{Lp/+} mouse lungs, which may drive the macrophage phenotype in conjunction with the disrupted tissue architecture. Based on these studies we hypothesise that the altered macrophage phenotype is driven by the perturbed microenvironment in lungs of *Vangl2*^{Lp/+} mice.

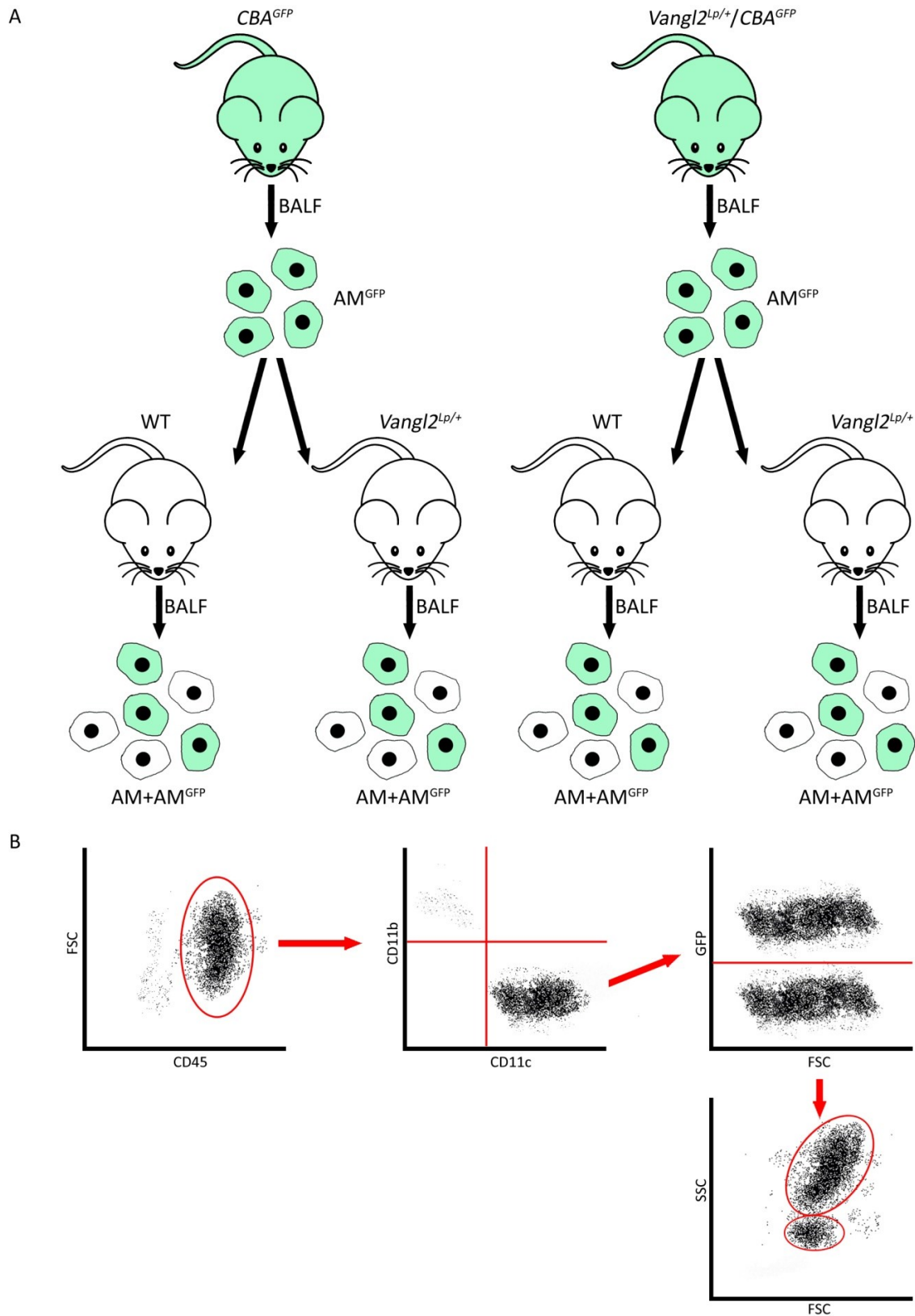


Figure 6.1. Adoptive transfer of alveolar macrophages

(A) AM isolated from the BALF of GFP expressing CBA^{GFP} and $Vangl2^{Lp/+}/CBA^{GFP}$ mice will be transferred intranasally to WT and $Vangl2^{Lp/+}$ mice. The mice will then left to rest and the AM isolated again. (B) The macrophages will be assessed by flow cytometry using the proposed gating strategy to study the macrophage phenotype. The plots show the expected clusters of immune cells. Abbreviations: Chicken β -actin driven GFP (CBA^{GFP}), alveolar macrophages expressing GFP (AM^{GFP}), forward scatter (FSC), side scatter (SSC).

6.5. PCP signalling in chronic human lung disease

As mentioned above, *VANGL* mutations have been associated with human diseases. However, the impact of *VANGL* mutations in chronic lung disease has yet to be published. Preliminary work in the Dean lab has shown that expression of *VANGL2*, and other PCP genes important for lung development such as *CELSR1* and *SCRIB* are strongly downregulated in COPD patients in comparison to non-COPD donor lung tissue. *VANGL1* expression surprisingly was not altered in COPD patients. These preliminary studies demonstrate an important role for PCP genes during human chronic lung diseases; however, further work is required to validate these findings. The preliminary studies consisted of a relatively small patient population (n=6), thus more samples should be analysed. In addition to assessing gene expression, protein levels and localisation in patient samples should also be analysed, as correct localisation of PCP proteins is essential for their proper function. Thus, if PCP disruptions cause underlying lung defects, as seen in the *Vangl2*^{Lp/+} mice in our study, this may increase susceptibility to injury as a result of ineffective repair mechanisms. Therefore, assessing *VANGL2* mutations in smokers with and without COPD may indicate if *VANGL2* status can be used as a prognostic marker for COPD susceptibility.

6.6. Overall conclusions

In summary, this thesis presents a novel role for PCP signalling in adult lung structure and function. Mice with a single point mutation in the core PCP gene *Vangl2* have disrupted adult lung architecture which arises from defects during embryogenesis and postnatal alveolarisation. The altered lung structure resulted in reduced lung function in adult mice. Humans with *VANGL2* mutations, many of which map closely to the locus of the *Vangl2*^{Lp} mutation, may have underlying lung defects that could go unnoticed. However, following injurious insults such as by smoking, the underlying lung defects may become exacerbated and cause earlier onset of disease, or increased decline in lung function. The results presented in this thesis highlight a novel pathway linked to genetic susceptibility in adult lung disease, which could be manipulated to promote intrinsic repair mechanisms.

Chapter 7: References

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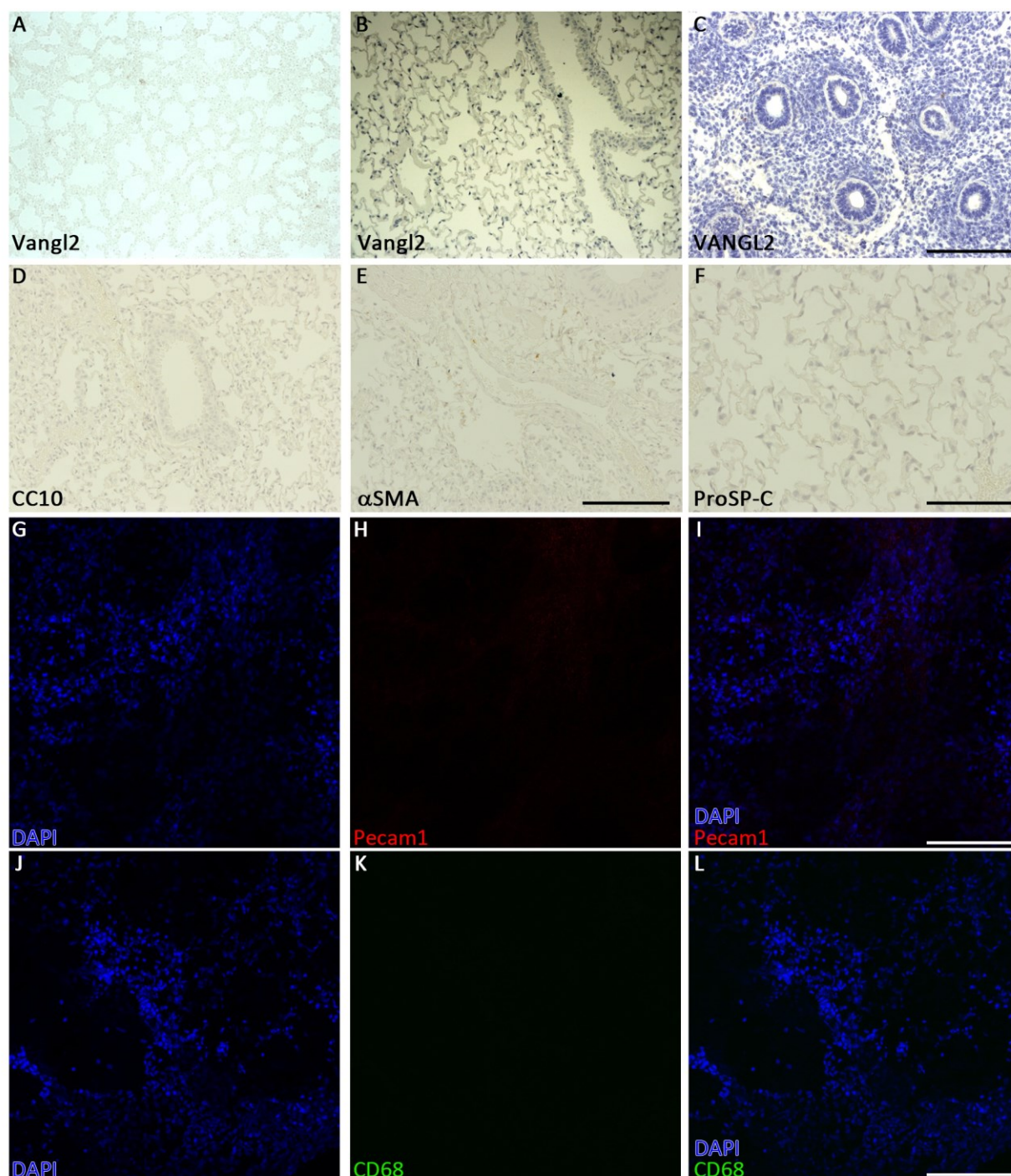
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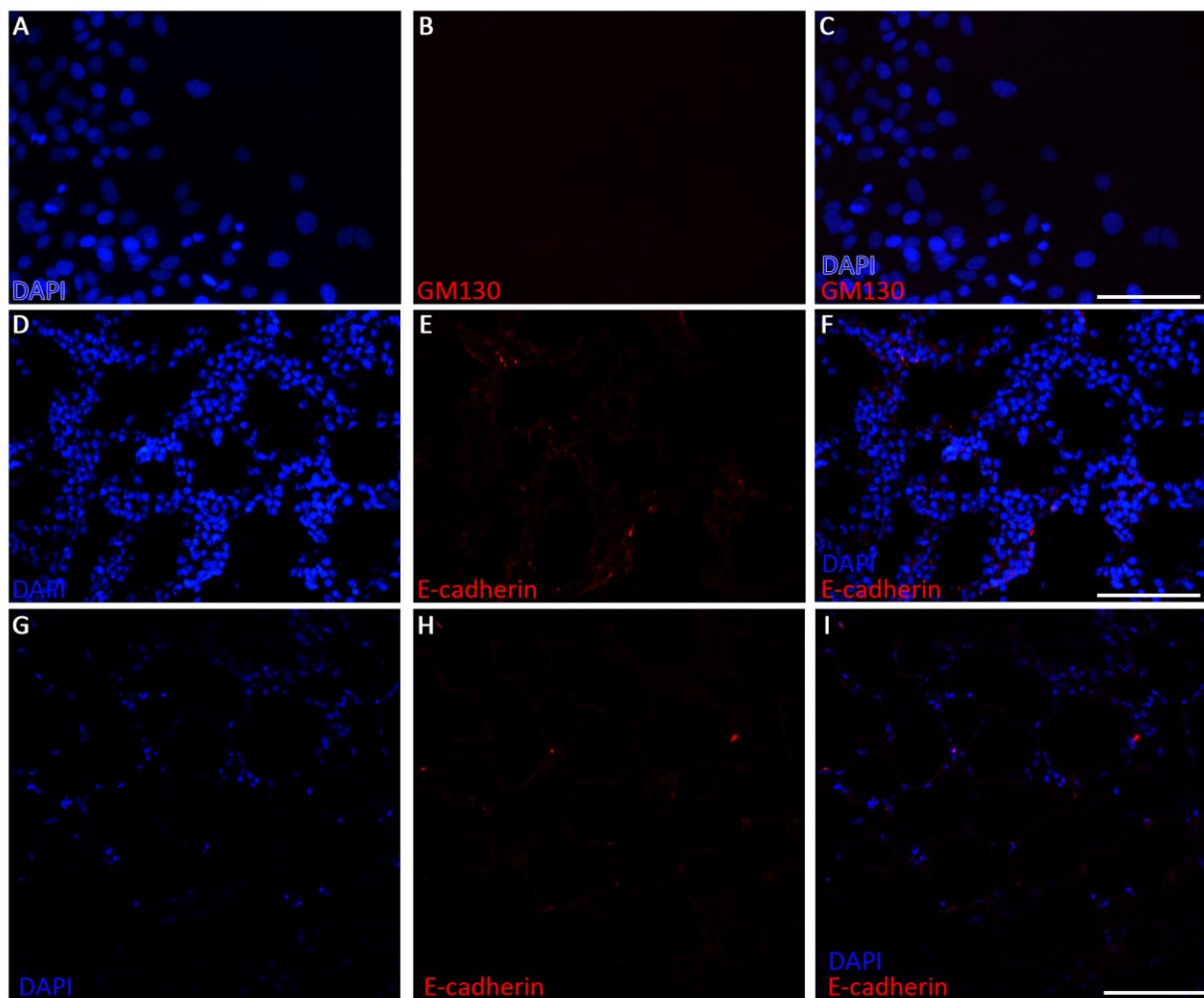
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Appendix A: Additional immunostaining



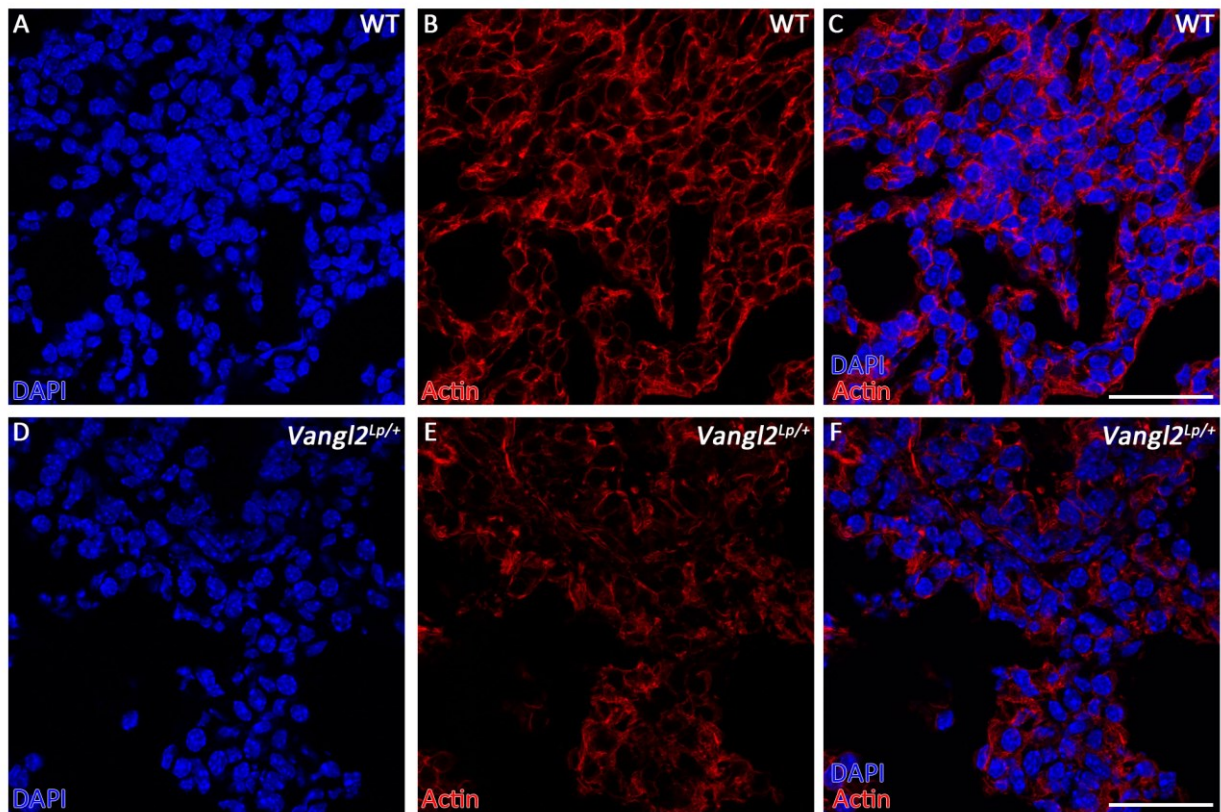
Appendix A: Figure 1. IgG control for antibody staining in Chapter 3

Immunohistochemistry of no primary rabbit IgG controls for (A-C) Vangl2 staining in (A) embryonic mouse, (B) adult mouse, and (C) embryonic human lung paraffin sections. Immunohistochemistry of (D) goat IgG controls for CC10 stain, and rabbit IgG controls for (E) αSMA and (F) ProSP-C. Immunofluorescence images of (G-I) no primary control for Pecam1, and (J-L) rat IgG control for CD68 in adult lung slices, showing the (G,J) DAPI channel, (H,K) control, and (I,L) merged image. Scale bar (A-E) 120 μm, (F) 60 μm, (G-L) 120 μm.



Appendix A: Figure 2. IgG control for antibody staining for Chapter 4

Immunofluorescence images of (A-B) rabbit IgG control for GM130 in A594 cells, and (D-I) mouse IgG control for E-cadherin in (D-F) embryonic lung sections and (G-I) adult lung slices, showing the (A,D,G) DAPI channel, (B,E,H) control, and (C,F,I) merged image. Scale bar (A-F) 60 μ m, and (G-I) 102 μ m.



Appendix A: Figure 3. Actin cytoskeleton is disrupted in embryonic *Vangl2*^{Lp/+} lungs

Staining of embryonic lung sections in E18.5 mice showing the (A,D) nucleus using DAPI, (B,E) F-actin using phalloidin dye, and (C,F) the merged image to show actin organisation in the tissue. Scale bar (A-E) 32 μ m.