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Role of the co-transcriptional regulators Yap/Taz in the normal and fibrotic lung epithelia

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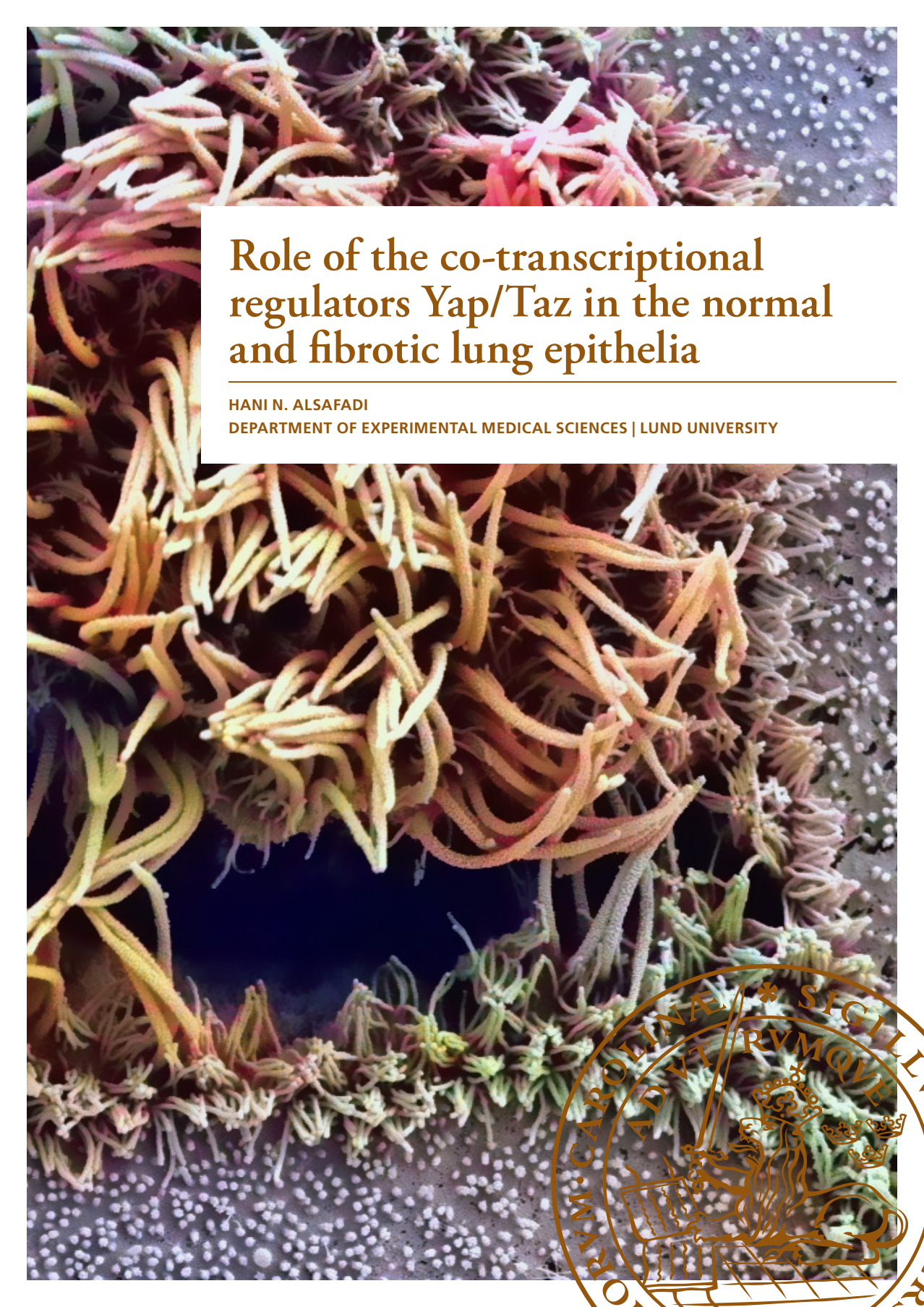
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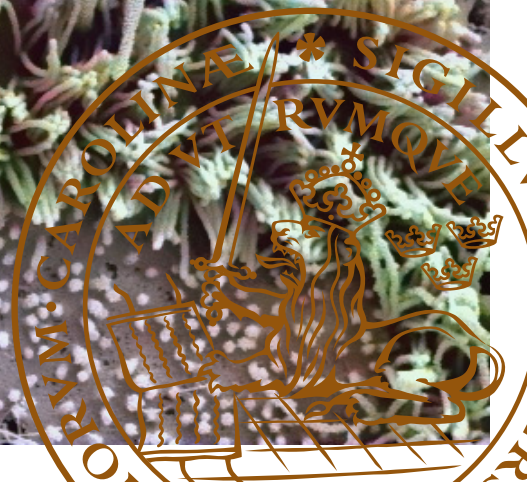
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DEPARTMENT OF EXPERIMENTAL MEDICAL SCIENCES | LUND UNIVERSITY



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Hani N. Alsafadi



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Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on 2023-02-13 at 13.00 in LUX aula, Helgonavägen 3, 223 62 Lund.

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Abstract:

Idiopathic pulmonary fibrosis (IPF) is a fatal disease that exhibits patterns of usual interstitial pneumonia with honeycombing. IPF is characterized by damaged distal lung epithelium with excessive tissue scarring and extracellular matrix remodeling. The etiology of IPF is unknown and current therapies cannot end or reverse disease progression. Aberrant reactivation of developmental pathways is evident in IPF. Among these developmental actors are the co-transcriptional regulators Yap and Taz (YT). YT modulate processes such as proliferation, differentiation, and organ size and are regulated by the Hippo pathway. YT do not have a DNA binding domain but act through interaction with other transcription factors (TFs). YT play a role in fibrotic fibroblasts, but their role is not yet known in the fibrotic lung epithelium. The aim of this thesis project is to develop the tools needed to explore the role of Hippo-YT in fibrotic lung epithelium and to identify the TFs that YT interact with to exert their various functions.

We first developed a method to simultaneously isolate proximal and distal lung progenitor cells from an individual mouse with the aid of a 3D printed surgical guide and found that the precision of dissecting the lung lobes affects the purity of the isolated distal progenitors and how they behave in organoid assays. We further found the Hippo pathway to be dysregulated in the fibrotic lung epithelium which led to increases in nuclear YT as well as known downstream targets. Interestingly, we found epithelial YT signaling to be actively involved in extracellular matrix remodeling in the fibrotic lung epithelium through modulation of lysyl oxidase expression, a collagen crosslinking enzyme. Targeting YT in vivo using an FDA approved drug ameliorated the fibrotic phenotype, indicating that YT targeting may be an option to treat fibrosis. We further used cleavage under target and release using nuclease (CUT&RUN) to identify the exact motif sequences on the genome where complexes containing YT bind in the normal and fibrotic lung epithelial. We further identified putative TFs that are known to bind the motif sequences identified. We found that YT have different interaction partners in the proximal and distal lung epithelium and further identified specific YT interactions in the human fibrotic lung epithelium.

This current research project sets the basis for the identification of exact targeting mechanisms for finding therapeutics for IPF. YT are known to be responsible for a wide range of biologic processes and targeting YT's profibrotic activity and promoting their pro-regenerative activities may result in beneficial effects for IPF patients.

Key words: Lung Epithelium, IPF, Developmental pathways, Hippo Signaling, Yap Taz, Transcription factors, 3D culture models, CUT&RUN, sequencing.

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Hani N. Alsafadi



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MADE IN SWEDEN 

To my wife and children

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Preface

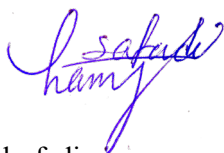
This work represents the culmination of several years of research and represents a significant contribution to the development of experimental tools, analysis methods, and generation of datasets to further our understanding of the mechanisms underlying fibrosis in the lung.

Lung fibrosis is a process that leads to the scarring of tissues and organs, and it is a major contributor to morbidity and mortality worldwide. Idiopathic pulmonary fibrosis (IPF), in specific, is lethal and has no cure; the only viable option to extend survival for IPF patients is lung transplant and this is only available for a small number of patients annually. Our understanding of the mechanisms that drive fibrosis is still incomplete, and there is a pressing need for new therapies to treat this devastating disease. The current knowledge of the disease highlights how complex it is and how many mechanisms it involves. In this thesis, I have focused on the role of the Yap/Taz transcriptional coregulators in the fibrotic lung epithelium. My work has revealed novel insights into the molecular mechanisms underlying the role of Yap/Taz in the fibrotic lung epithelium with a broader aim to set the stage for identifying new therapeutic targets to treat IPF.

This is a composite thesis book. It is divided into two main parts, the Kappa, and the research papers. The Kappa serves as a summary of the research project within the field and how the current research has contributed to the knowledge in the field. Next, all the original research papers will be sequentially placed at the end of the book. In the kappa, results from the research papers are discussed in the context of the field.

I hope that this thesis will be of interest and value to those working in the field of fibrosis and lung disease, and I look forward to continuing to contribute to this important area of research in the future.

Sincerely,



Hani N. Alsafadi

Popular summary

Lung fibrosis is a deadly disease with no available cure. Lung fibrosis can be described as an excessive injury to the lung that stiffens it preventing the bearer of the disease from breathing. If you think of the lung as a balloon, lung fibrosis would be as if you continuously damaged parts of the balloon without stopping and managed to also increase the thickness of the balloon walls which would limit how much it could move air in and out. Currently, the available drugs are only able to slow down the disease progression, offering limited relief of symptoms for these patients. These patients only survive for a few years once diagnosed. The difficulty in finding effective drugs is due to the fact that exact causes of the disease are unknown. My doctoral studies focused on identifying specific mechanisms related to lung fibrosis in order to find new effective therapies for lung fibrosis.

Although the exact causes of lung fibrosis are unknown, many molecular events have been studied and our understanding of the disease is increasing. The lung contains as many as 40 different cell types to carry out its various. Some of these cells are known as epithelial cells. Epithelial cells are located on the surface of the internal parts of the lung, and they are responsible for gas exchange (i.e., breathing). These cells are heavily damaged in patients with lung fibrosis. Our research team has found two molecules to be involved in the damage of the epithelial cells, they are called YAP and TAZ, and they are known for their ability to sense changes in stiffness, and they are responsible for a wide range of activity in the healthy lung cells such as their survival and growth. In lung fibrosis, these molecules are abnormally active and are responsible for causing some of the disease characteristics such as tissue scarring. However, in normal conditions these molecules are necessary for maintaining normal functions. We are using state-of-the-arts techniques to identify the exact roles of these molecules in the different types of cells in health and disease. While targeting the activity of YAP and TAZ seem like a viable option, a drug that stops all YAP and TAZs activities may not be beneficial for the patients as these molecules are also involved in healthy processes.

The problem with current approved drugs is that they are not specific and that is one of the reasons they are not effective. Finding out the exact roles of YAP and TAZ in the lung compartments and learning how they are involved in the progression of lung fibrosis will lead us to finding highly specific drugs. Highly specific drugs will be able to stop the harmful activity of YAP and TAZ without interfering with the healthy activity. Finally, these molecules are involved in several other organs and diseases, thus knowledge gained in this research can be easily transferable to other disciplines.

Populärvetenskaplig sammanfattning

Lungfibros är en dödlig sjukdom som vi idag inte kan bota, den kan beskrivas som en omfattande skada på lungorna som stelnar och därmed hindrar patienten från att andas. Om du tänker dig lungan som en ballong är lungfibros som om någon trycker till delar av lungan och får ballongens väggar att bli tjockare och därmed mindre töjbara. De mediciner vi har i dag kan tyvärr inte rädda patienten, de kan endast sakta ner sjukdomsförloppet något. Efter att lungfibros diagnostiserats överlever dessa patienter endast några år. En av orsakerna till att det är så svårt att hitta en fungerande behandling är att vi idag inte vet exakt vad som orsakar sjukdomen. Min avhandling fokuserar på att identifiera mekanismerna bakom lungfibros för att kunna utveckla effektiva terapier mot lungfibros.

Även om vi inte vet exakt vad som orsakar lungfibros har många molekylära mekanismer studerats och vår förståelse av sjukdomen ökar kontinuerligt. En lunga består av så mycket som 40 olika celltyper, en del av dessa är epitelceller. Epitelceller finns på ytan av lungan och är de celler som kommer i kontakt med luften när vi andas. Dessa celler sköter gasutbytet mellan luft och organism, dvs andningen. I en patient med lungfibros är många av epitelcellerna illa skadade. I mitt avhandlingsarbete har vi hittat två molekyler som är involverade i de skador som uppstår i epitelcellerna. Dessa molekyler kallas YAP och TAZ, de är delaktiga i många aktiviteter i friska lungceller, som tex utveckling och tillväxt samt att känna av förändringar i elasticitet. I frisk lungvävnad behövs dessa molekyler för att bibehålla normala funktioner. Vid lungfibros förändras dessa molekylers aktivitet och de bidrar då till vissa av de karakteristiska sjukdomsmarkörerna för lungfibros, som tex ärrvävnad. Vi använder metoder i den absoluta fronten av forskningsfältet för att identifiera vilka uppgifter dessa molekyler har i olika typer av celler, både i friska individer och vid sjukdom. Att försöka påverka YAP och TAZ aktiviteter kan verka vara en bra strategi mot lungfibros men då dessa molekyler även behövs och är aktiva i normal” vävnad och friska processer skulle en medicin som stoppar all YAP och/eller TAZ aktivitet troligen inte vara bra för patienten.

Problemet med de mediciner vi har idag för lungfibros är att de är ospecifika vilket gör dem mindre effektiva. Om vi kan förstå exakt vilken roll YAP och TAZ har i olika delar av lungan och hur de är involverade i utvecklingen av lungfibros skulle vi kunna få fram högspecifika behandlingsmetoder. Högspecifika mediciner skulle kunna stoppa de negativa effekterna av YAP och TAZ utan att påverka de bra och nödvändiga effekterna dessa molekyler har. Vidare är dessa molekyler involverade i många andra organ och sjukdomar, så ökad kunskap inom området kan även öka kunskapen inom andra forskningsområden och leda till behandlingar av andra sjukdomar där YAP och TAZ har en roll.

Abstract

Idiopathic pulmonary fibrosis (IPF) is a fatal disease that exhibits patterns of usual interstitial pneumonia with honeycombing. IPF is characterized by damaged distal lung epithelium with excessive tissue scarring and extracellular matrix remodeling. The etiology of IPF is unknown and current therapies cannot end or reverse disease progression. Aberrant reactivation of developmental pathways is evident in IPF. Among these developmental actors are the co-transcriptional regulators Yap and Taz (YT). YT modulate processes such as proliferation, differentiation, and organ size and are regulated by the Hippo pathway. YT do not have a DNA binding domain but act through interaction with other transcription factors (TFs). YT play a role in fibrotic fibroblasts, but their role is not yet known in the fibrotic lung epithelium. The aim of this thesis project is to develop the tools needed to explore the role of Hippo-YT in fibrotic lung epithelium and to identify the TFs that YT interact with to exert their various functions.

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List of papers included in the thesis

Paper I

Alsafadi HN, Stegmayr J, Ptasinski V, Silva I, Mittendorfer M, Murray L, Wagner DE. Simultaneous isolation of proximal and distal lung progenitor cells from individual mice using a 3D printed guide reduces proximal cell contamination of distal lung epithelial cell isolations. *Stem Cell Reports*. **2022**; 17(12): 2718-2731

Paper II

Wagner DE, **Alsafadi HN**, Lehmann M, Staab-Weijnitz C, Korfei M, Stein M, Mutze K, Costa R, Bölükbas D, Stegmayr J, Skronska-Wasek W, Klee S, Ota C, Baarsma H, Lindstedt S, Lindner M, Chambers R, Günther A, Kaminski N, Schiller H, Königshoff M. A Deranged Hippo-YAP/TAZ-LOX axis in distal epithelial cells modifies the ECM niche in pulmonary fibrosis. *Manuscript*

Paper III

Alsafadi HN, Johannsson M, Mínguez-Santos I, Nordin A, Wee ES, Plattiau P, Lindstedt S, Cantù C, Wagner DE. Identification of Yap/Taz co/transcriptional binding partners in the proximal and distal lung epithelia. *Manuscript*

Paper IV

Stegmayr J, **Alsafadi HN**, Langwinski W, Niroomand A, Lindstedt S, Leigh ND, Wagner DE. Isolation of high yield and quality RNA from human precision-cut lung slices for RNA-sequencing and computational integration with larger patient cohorts. *American Journal of Physiology – Lung cellular and molecular physiology*. **2021**; 320: L232-L240

List of papers outside the scope of the thesis

Gercken M, **Alsafadi HN**, Wagner DE, Lindner M, Burgstaller G, Königshoff M. Generation of human lung tissue slices for disease modeling. *JoVE*. **2019**; 144: e58437

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Langwiński W, Szczepankiewicz D, Narożna B, Stegmayr J, Wagner DE, **Alsafadi HN**, Lindstedt S, Stachowiak Z, Nowakowska J, Skrzypski M, Szczepankiewicz A. Allergic inflammation in lungs and nasal epithelium of rat model is regulated by tissue-specific miRNA expression. *Molecular Immunology*. **2022**; 147: 115-125

Abbreviations

ATI	Alveolar type I cell
ATII	Alveolar type II cell
MTEC	Mouse tracheal epithelial cell
HBEC	Human bronchial epithelial cell
IPF	Idiopathic pulmonary fibrosis
YAP	Yes-associated protein 1
TAZ	WW-domain containing transcriptional regulator 1
WW	WW refers to the two tryptophan amino acid residues
PTMs	Post-translational modifications
FDA	Food and drug administration
VP	Verteporfin
TF	Transcription factor
ChIP	Chromatin immunoprecipitation
CUT&RUN	Cleavage under target and release using nuclease
RNAseq	RNA sequencing
scRNAseq	single cell RNAseq

Introduction

The lung is a complex organ that is capable of several functions facilitated by its structure and composition. The main structural components of the lung consist of the airway branching, starting at the trachea and ending at the conducting airways, and the lung parenchyma where all the alveolar structures reside. The airways are responsible for air passage and filtration of pathogens and inhalants as the first line of defense against disease causing agents. The lung airways are divided into conducting airways and respiratory airways with a branching that can span over 23 generations in humans (i.e., a generation is defined as when an airway splits into two) (Figure 1) (Hsia et al., 2016). The main function of conducting airways is to filter and humidify the air as it passes through to the distal regions of the lung where gas exchange passively happens. The conducting airways perform particle filtration and serve as an initial barrier to pathogens through mucosal secretion and coordinated ciliary beating to expel inhaled particulates. These structural characteristics of the lung can be observed across species with a few differences. Here, the similarities and dissimilarities will be outlined where needed as both human and mouse tissues are used across the different parts of this thesis. In the human lung, the trachea, bronchi, and some of the intralobular bronchioles are surrounded by cartilage rings and smooth muscles that maintain the opening of the conducting airways. In the mouse lung, these cartilage rings are only present in the trachea (Figure 2). The respiratory airways end in the flexible alveolar sacs which are responsible for gas exchange.

The lung consists of more than 40 different cell types as recently identified by single cell RNAseq (scRNAseq) populating different parts of the lung (Adams et al., 2020). These cell types can be categorized into four main categories, epithelial, endothelial, mesenchymal, and immune cells. The presence and location of these cells varies based on the disease status and age of the lung. As an example, transitional cell types or transitional cell states have been found to exist predominantly in disease, such as the aberrant basaloid cells in idiopathic pulmonary fibrosis (IPF) (Adams et al., 2020). Moreover, the ability for the lung to regenerate in response to injury is driven by the presence and state of the various lung progenitor cells. Chronic lung diseases exhibit their pathogenesis in the disruption of the lung function by causing injury to its structural or functional components. The lung epithelium, in particular, is known to undergo repeated injury in several of the serious chronic lung diseases such as chronic obstructive

pulmonary disease and IPF. Our understanding of the lung epithelium’s role in disease is continuously increasing and its potential as a therapeutic target of interest is gaining more traction in the field. This book focuses on the lung epithelium in the context of IPF.

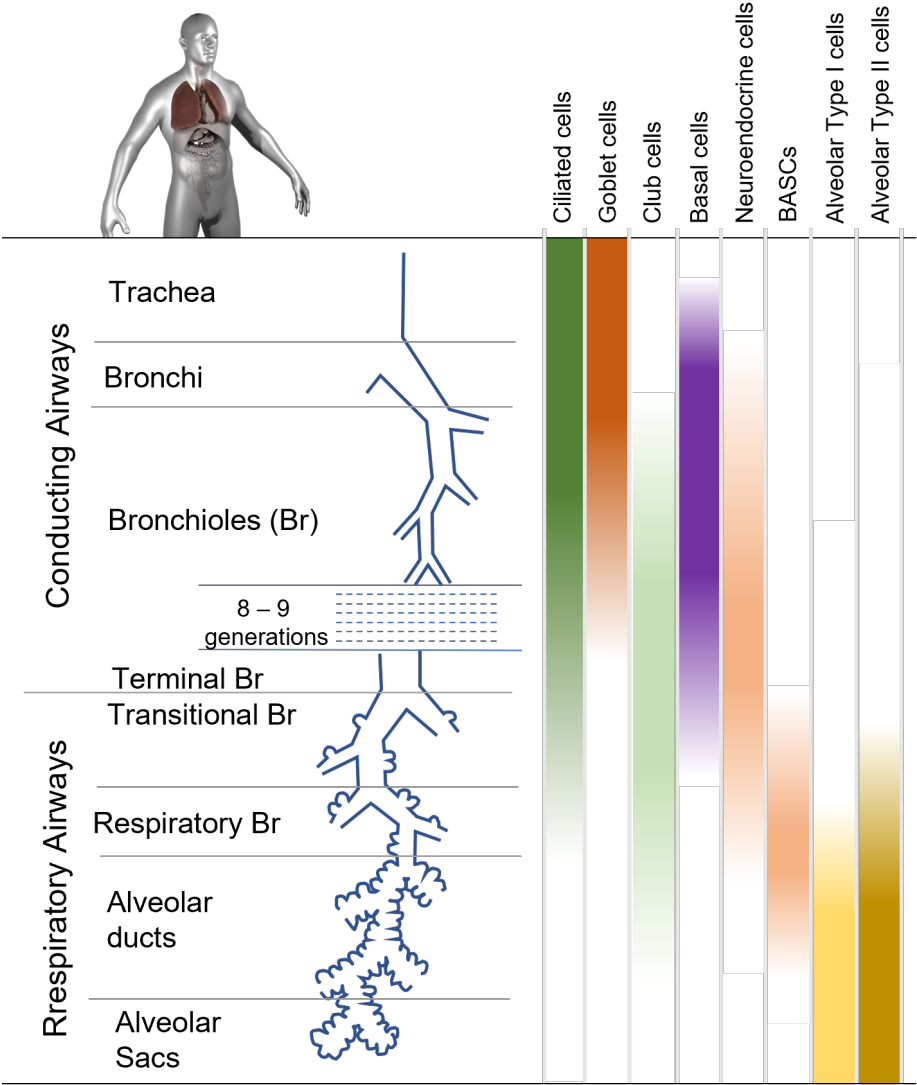


Figure 1. The airway branching structure and cellular landscape of the human lung epithelium. The human respiratory tract divides the airways dichotomously through several generations. The branching schematics are reused from (Hsia et al., 2016) with permission. On the left side the color gradients represent the approximate location of the main cell types found on the respiratory tract. These do not represent all known cell types of the lung epithelium.

The lung epithelium

The cellular makeup and function of the lung epithelium

The lung epithelium provides a continuous barrier between the external environment and the body; it covers the inner surface of the lung and consists of various cell types across the conducting and respiratory tracts based on their location and function. The respiratory tract is a branching structure where airways are divided by two in each generation for as many as 23 generations in the human lung ending at the alveoli (Hsia et al., 2016). The cellular composition of the respiratory tract changes gradually as the branching generation number increases (Figure 1). The most proximal parts of the lung epithelium in the conducting airways are populated by a pseudostratified columnar epithelium consisting mainly of ciliated cells, secretory cells, neuroendocrine cells, and basal cells (Figure 2). Later generations of the bronchial branching are less complex and have a columnar arrangement of cells and contain neuroendocrine cells in addition to ciliary, secretory, and basal cells. In the respiratory airways, the alveolar ducts contain alveolar type I and type II cells alongside the secretory cells. Finally, most of the surface of the alveolus is covered by alveolar type I cells which are responsible for gas exchange and the remainder of the alveolus consists of alveolar type II cells (Figure 2). In the normal lung, basal cells, marked by p63 and cytokeratin-5 (KRT5), and/or cytokeratin 14 (KRT14) maintain the proximal epithelium (trachea in murine epithelium and tracheal and bronchial epithelium in humans) (Rock et al., 2009) and alveolar type II (ATII) cells are known as the progenitor cell of the alveolus for their ability to regenerate and differentiate into alveolar type I cells. ATII cells are characterized by expression of pro-surfactant protein C (pro-SPC) (Barkauskas et al., 2013).

Single cell RNA sequencing (scRNAseq) studies have enhanced our understanding of the cell diversity in the lung and have revealed the presence of multiple progenitor and disease specific transitory state populations (Adams et al., 2020, Montoro et al., 2018, Strunz et al., 2020, Habermann et al., 2020). These studies have revealed new avenues for exploring the location of these newly defined cells in the normal lung and their potential roles in the maintenance of the lung epithelium. The functions of the lung epithelium are supported by its distinct structure and composition along the airway branching. Any alterations to the structures result in adverse effects on the function of the epithelium. Therefore, the type of injury and effects that are associated with injury will widely differ across the whole lung epithelium. The lung epithelium is considered the major site of injury for several chronic lung diseases such as COPD, and IPF in addition to a variety of immune diseases. The injury to the lung epithelium can be categorized into two major parts: injury to the proximal/airway epithelium and injury to the distal parenchymal epithelium. The proximal airway injuries are mainly noted to be

involved in obstructive lung disease where the airways develop defects in their function or molecular structure, such as in asthma or COPD when manifested as chronic bronchitis. On the other hand, simplistically, diseases effecting the lung parenchyma are characterized by the destruction of the alveolar spaces as observed in emphysematous lungs, or by the thickening or obliteration of the alveolar septa as observed in pulmonary fibrosis or lung cancer.

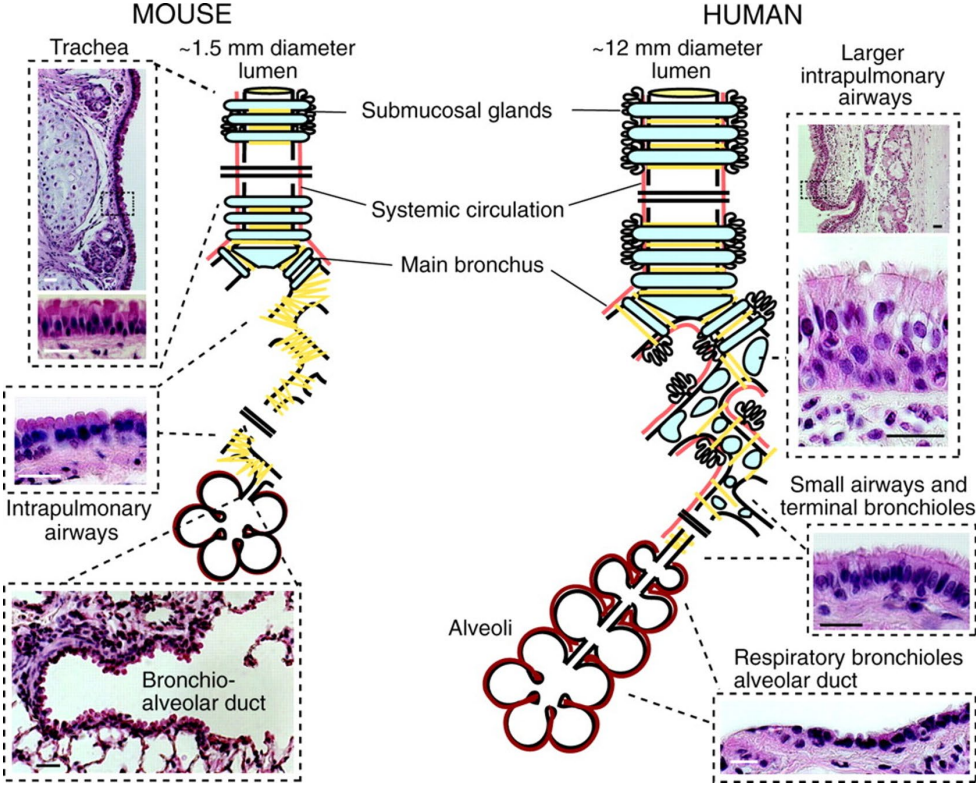


Figure 2. Comparison of the airway epithelium between the mouse and human lungs. While there are similarities between the mouse and human lung epithelia, there are a few differences of the cellular composition at different level of branching. Image is used under the Creative Commons License (Rock et al., 2010).

Idiopathic pulmonary fibrosis

Clinical characteristics and diagnosis

IPF is a chronic and progressive lung disease that is characterized by tissue scarring (fibrosing) of the lung parenchyma. This scarring, which is accompanied by damage to the lung epithelium, leads to stiffness in the lungs, making it difficult for gas exchange to occur. The exact causes of IPF are unknown and in many cases, it is detected in patients at late stages of the disease.

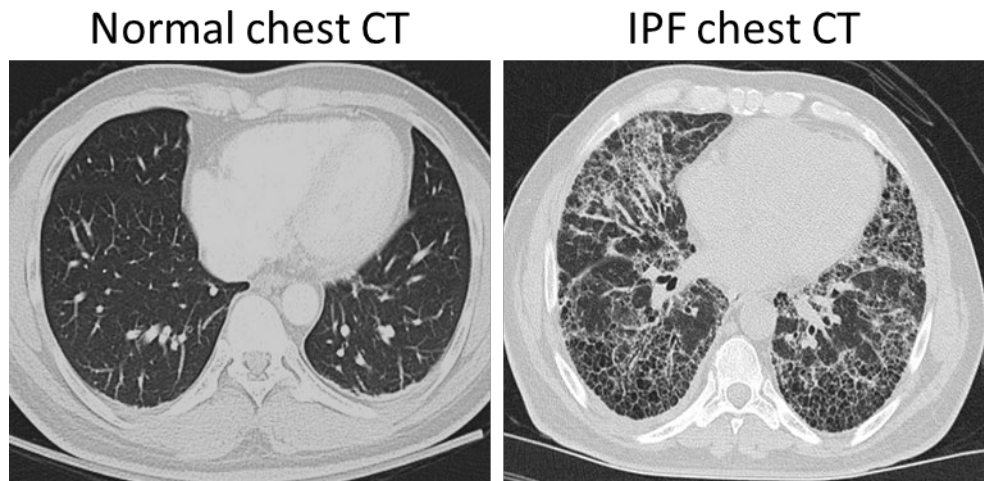


Figure 3. Radiographic representation of IPF.

Radiographic CT chest scans of a normal chest (left) and an IPF patient's chest (right). Images obtained with permission from Radiopedia.org (case reference rID: 151694 (left), rID: 129467 (right)).

IPF is categorized under the broader clinical lung disease classification of interstitial lung disease (ILD), and more specifically under the diffuse parenchymal lung diseases (DPLD). Radiographically, IPF exhibits the pattern of usual interstitial pneumonia (UIP) which represents normal lung architecture with the presence of the patches of damaged fibrosing parts heterogeneously appearing across the lung (Tanabe et al., 2020). In IPF, these patterns are also associated with characteristic honeycombing in the distal parts of the lung. The honeycombs are cysts that are randomly distributed in the fibrotic regions of the distal lung and are usually lined by fibrotic tissue (Figure 3). Histologically, IPF is also distinguished by the formation of fibrotic foci, which are regions of accumulated myofibroblasts and fibroblasts in the distal lung where excessive amounts of extracellular matrix, including newly synthesized matrix, can also be found (Figure 4).

Common symptoms of IPF include shortness of breath, a dry cough, and weight loss. A physical examination may reveal signs of decreased oxygen in the blood, such as a bluish tinge to the skin and fingernails. A diagnosis of IPF is typically made based on a combination of these symptoms, physical examination findings, and radiographic evidence. Additional tests, such as a lung biopsy or oxygen level monitoring, may also be performed to confirm the diagnosis and rule out other potential causes of lung scarring. Moreover, IPF diagnosis is made where there is an absence of other known causes of lung fibrosis.

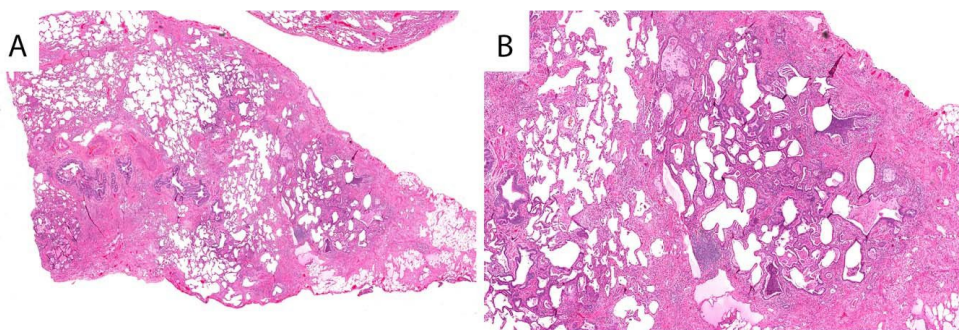


Figure 4. Histopathology of usual interstitial pneumonia/idiopathic pulmonary fibrosis.

(A) Patchy advanced pulmonary fibrosis (B) with areas of honeycomb. The use of this image is licensed under a Creative Commons Attribution 4.0 International License. The original figure contains two more panels (Smith, 2022)

The prevalence and incidence of IPF are continuously increasing at varying rates across the globe for currently unknown reasons. A recent report has examined several of the published IPF cohorts and found the incidence to range between 0.9 – 4.9 (per 100,000) in Europe, 3.5 – 13 in Asia-Pacific countries, 7.5 – 9.5 in North America (Maher et al., 2021). The prevalence was estimated to range from 3.3 – 25 (per 100,000) in Europe, 5.7 – 45.1 in Asia-Pacific countries, and 24 – 29.8 in North America. While these numbers may not push IPF out of its “rare disease” status, more epidemiological studies are needed to accurately determine the prevalence of disease. Moreover, more studies accounting for other confounders such as age, gender, and other demographics are also needed (Maher et al., 2021).

Molecular mechanisms underlying IPF

IPF mainly effects the distal lung epithelium where it is characterized by damaged alveolar epithelium and excessive extracellular matrix deposition (King et al., 2011). The exact mechanisms of how IPF is initiated are not known, however the main accepted hypothesis is that repetitive damage of the alveolar epithelium signals the (myo)fibroblasts to initiate repair mechanisms where they secrete extracellular matrix molecules such as collagens and fibronectin to mediate

epithelial repair; a process that is deranged in fibrosis. The deranged repair is thought to contribute to the pathogenesis of IPF (Hardie et al., 2010). Recent evidence suggest a more active role of IPF progression of the lung epithelium (Selman and Pardo, 2020). The scarring of the lung tissue is attributed to the excessive extracellular matrix deposition of matrix molecules such as various collagens, fibronectin, and matrix remodeling enzymes such as matrix metalloproteinases (MMPs) (Wolters et al., 2014). The extracellular matrix is heavily crosslinked which causes the increased stiffness and rigidity of the fibrotic lung (Liu et al., 2015). These processes are driven by a convoluted interaction network of several pathways such as TGF- β , Wnt, FGF, PI3k/mTOR, Notch, and other signaling pathways (Moss et al., 2022). Moreover, aging, cellular senescence, mitochondrial dysfunction, telomer shortening have all been indicated in IPF (Lehmann et al., 2017, Moss et al., 2022). The complexity of IPF mechanisms make it difficult to treat the disease.

Current approved therapies for IPF

IPF currently has no cure, and the available treatments are only able to slow down disease progression. In fact, IPF is progressive and lethal. The average life span of patients who do not receive treatment is between 3 to 4 years (Maher and Streck, 2019). Overall, 50 percent of patients die after 3 years from diagnosis (Maher and Streck, 2019). The only approved drugs for IPF are Nintedanib and Pirfenidone. These drugs, individually and in combination, have been shown to slow down IPF at various rates among patients. However, they are not able to cure the disease or reverse the fibrotic phenotype, moreover the exact molecular mechanisms of these drugs are not completely known. These drugs have been indicated to potentially act through attenuation of fibroblast activity which are mainly attributed to be the major cell type involved in the fibrotic phenotype. However, this attribution is mainly due to the fact that these drugs have been mainly tested on fibroblasts in *in vitro* studies (Knuppel et al., 2017). More recently, their molecular effects were tested in *ex vivo* models of fibrosis derived from mouse and human precision-cut lung slices (PCLS). Both drugs showed the ability to decrease fibrotic marker gene expression in the PCLS models, but only Nintedanib was able to increase markers of the alveolar epithelium in the different culture models (Lehmann et al., 2018). This effect was also observed on primary alveolar type II cells isolated from healthy and fibrotic mice and a fibrotic alveolar model of induced ATII cells derived from human induced pluripotent stem cells (iPSCs) (Ptasinski et al., 2022).

Nintedanib is a common tyrosine kinase receptor antagonist and has been shown to prevent some of the collagen fibril assembly and the reduction of fibrotic markers gene expression (Knuppel et al., 2017, Lehmann et al., 2018). The exact mechanism of Pirfenidone on the other hand is not fully understood. In addition to some evidence that it possesses anti-inflammatory functions, it has been shown to

decrease the profibrotic activity of transforming growth factor beta (TGF- β) (Stahnke et al., 2017).

The difficulty in treating IPF is in part due to the lack of complete understanding of the disease molecular mechanisms. Tremendous efforts are being undertaken spanning basic, translation and clinical scientists to try to further disease understanding to develop new therapies that target different aspects of the disease, such as small molecule drugs, nucleic acids, protein-based therapies, and others adding up to more than 50 potential therapies that are in various stages of clinical trials (Ptasinski et al., 2021). These therapies target a wide range of molecular functions such as inflammation, growth factor antagonism, chemokine inhibitors, vasodilators, and much more. However, better understanding of disease mechanisms remains critical to potentially finding new and improved therapeutics for IPF.

Activation of developmental pathways in IPF

Lung development is carried out in several stages during embryogenesis and can be mainly divided into two major events: the branching stage and the alveolarization stage (Chanda et al., 2019). Throughout these stages the formation of the lung structures is driven by several signaling pathways such as the Wnt, Notch, FGF, SHH, TGF- β signaling and much more. The molecular mechanisms of these signaling pathways become somewhat dormant in the adult lung and are only activated in specific contexts, such as in response to injury.

TGF- β signaling is mediated by several TGF- β isoforms and receptors, that when stimulated, they activate Smad signaling. The deletion of TGF- β 1 has been shown to have lethal consequences due to inflammation and loss of TGF- β II resulted in several developmental defects in mice (Kulkarni and Karlsson, 1993, Schmid et al., 1991, Sanford et al., 1997). The different TGF- β isoforms have been found to be key regulators in both branching morphogenesis and alveolarization. In the adult lung, TGF- β is considered one of the main modulators of the fibrotic injury and importantly is known to drive several of the critical and aberrant changes to extracellular matrix deposition (Westergren-Thorsson et al., 1993).

Wnt signaling is known for its role in early development across species (van Amerongen and Nusse, 2009). Canonically, Wnt ligands bind to one of the Frizzled receptors and Lrp5/6 to release β -catenin from its destruction complex to then translocate to the nucleus and activate the TCF/LEF transcription machinery. In the developing lung, Wnt2/2b and β -catenin have been found to be necessary for the specification of the lung endoderm (Goss et al., 2009). Moreover, the non-canonical Wnt ligand Wnt5a was found to be involved in alveolarization in the developing lung (Li et al., 2002). In the adult lung, Wnt has been shown to be involved in

various processes such as repair, and it is known to play an important role in the fibrotic lung epithelium (Konigshoff et al., 2008).

The Hippo signaling is another more recently described signaling pathway that controls organ size, stemness, and differentiation. The main effects of Hippo signaling is exerted through its control of the co-transcriptional regulators Yap and Taz. Yap and Taz are known to play an important role during development across several organs, but also have been shown to be important for lung development in the branching morphogenesis and alveolarization (Mahoney et al., 2014). Interestingly, Hippo signaling, and more specifically YAP/TAZ, are also known to be key mediators of a mechanosensitive signaling pathway that is regulated by mechanical cues such as stiffness. Recent reports have indicated a potential role of Hippo-YT in pulmonary fibrosis (Gokey et al., 2018, Sun et al., 2021). However, the exact mechanism of how the Hippo signaling pathway and YT contribute to fibrosis remains unclear.

Hippo signaling – YAP/TAZ

Hippo signaling was originally discovered in the fruit fly and it is named after its main kinase *Hpo* (Hippopotamus; MST1 or *STK4* in mammals), which, when deleted in the fruit fly, caused an enormous increase in the size of the fly, hence the name was chosen to describe the size change. In mammals, the Hippo signaling pathway is known for its role in differentiation, proliferation, migration, and organ size (Figure 5). While the control of organ size is not yet fully understood in all contexts, dysregulation of Hippo signaling components in specific organs results in organ enlargement (Liu-Chittenden et al., 2012). In mammals, the canonical Hippo signaling starts by the phosphorylation of the mammalian sterile 20-like kinase 1/2 (MST1/2) in association with Salvador family WW containing protein 1 (SAV1) that functions to phosphorylate the large tumor suppressors 1/2 (LAT1/2) with the aid of Mps one binder kinase activator-like 1A and 1B (MOB1). When phosphorylated, the LATS kinases will phosphorylate YT at S127(YAP) and S89(TAZ) to inhibit their nuclear activity and cause their retention in the cytoplasm through sequestration or degradation. When YT are shuttled into the nucleus, they are considered to be active and they exert their transcriptional effects through binding to other transcription factors as they do not possess a DNA binding domain (Taha et al., 2018).

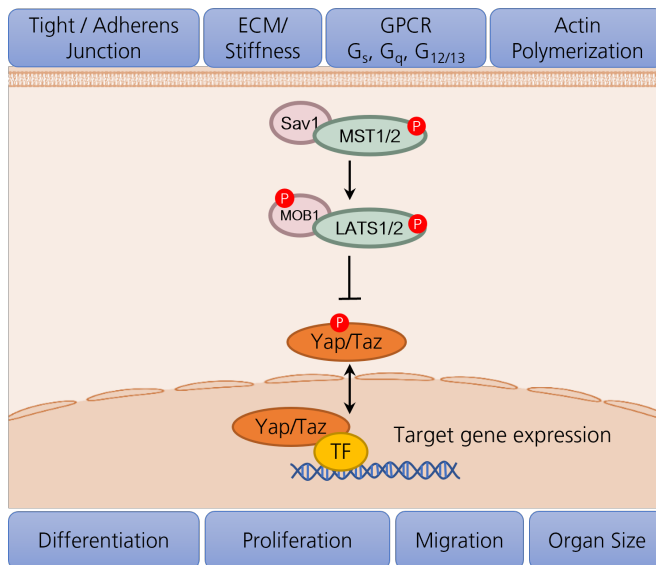


Figure 5. Overview of Hippo – YT signaling.

The Hippo kinase cascade controls YT activity by their phosphorylation to inhibit their transcriptional activity and keep them in the cytoplasm for sequestering or degradation (see Table 1 for detailed YT modifications). YT do not bind to DNA but exert their effects through binding with other TFs. They control a wide range of activities such as differentiation, proliferation, and organ size. Hippo-YT are regulated by several mechanisms including tight/Adherens junction, stiffness, GPCRs, and the cytoskeleton.

The transcriptional cofactors YAP/TAZ

Yes-associated protein (YAP) and WW domain containing transcription regulator 1 (WWTR1, or TAZ) (together referred to as YT for simplicity) are co-transcriptional regulators that control a wide range of processes involving differentiation, proliferation, migration, and organ size (Piccolo et al., 2014). YT activity is regulated by posttranslational modifications at various sites, especially their phosphorylation by LATS1 on S127 (YAP) and S89 (TAZ) that inhibits their activity by retaining them in the cytoplasm to be sequestered by the 14-3-3 complex. The state of YT activity is often indicated by, for simplicity, whether they are phosphorylated at these sites (Piccolo et al., 2014). However, the posttranslational modifications of YAP/TAZ are complex, and they are controlled by a wide variety of modifications some of which are listed in (Table 1). YAP and TAZ do not contain a DNA binding domain and, thus, they exert their transcriptional effects through interaction with other transcription factors while in the nucleus. Yap and Taz share several of the same protein domains and 40% of their amino acid sequence (Reggiani et al., 2021). Yap and Taz share the TEAD, 14-3-3, WW, STAT1, CC, TAD, and PDZ binding domains (Figure 6). The binding domains are what allow Yap/Taz to bind to other transcription factors, some of these are through known interactions and are listed in Figure 6. Some of these binding domains occur at different frequencies between Yap and Taz. For instance, YAP contains two WW domains where Taz contains only one, this may allow different binding possibilities and thus assembly of distinct transcriptional complexes. Moreover, YAP contains protein domains that do not exist in TAZ, such as the proline rich and SRC homolog (SH) domains. The Proline rich domain has been shown to interact with YAP inside the nucleus to inhibit its activity and the SH domain allows for interaction with the YES proteins and SRC homologs (Howell et al., 2004, Sudol, 1994).

Table 1. Modifications of the Yap and Taz proteins and the resulting effect of the modification.

Molecule	Modification	Site	Effect	Reference
YAP	Phosphorylation	S127	Cytoplasmic retention by the 14-3-3 complex	(Zhao et al., 2010)
YAP	Phosphorylation	S128	Blocks 14-3-3 interaction; nuclear accumulation	(Hong et al., 2017)
YAP	Phosphorylation	Y357	Nuclear retention	(Sugihara et al., 2018)
YAP	Phosphorylation	S397	Leads to degradation of YAP	(Zhao et al., 2010)
YAP	Monomethylation	K342	Block nuclear export	(Fang et al., 2018)
YAP	Monomethylation	K494	Cytoplasmic retention	(Oudhoff et al., 2013)
YAP	O-GlcNAcylation	S109	Blocks LATS1 and activates Yap	(Peng et al., 2017)
TAZ	Phosphorylation	S58, S62	Lead to Taz degradation	(Huang et al., 2012)
TAZ	Phosphorylation	S89, S117	Cytoplasmic retention by the 14-3-3 complex	(Piccolo et al., 2014)
TAZ	Phosphorylation	S311	Leads to degradation	(Huang et al., 2012)
TAZ	Phosphorylation	Y321	Altered nuclear activity	(He et al., 2016)

The structural similarity between YAP and TAZ explains the similarity in their function. However, their roles are known to not fully overlap. In fact, the full knockout of Yap is embryonic lethal while the full knockout of Taz isn't lethal but results in defects in several organs including the lung (Makita et al., 2008, Morin-Kensicki et al., 2006). Mice lacking Taz display airspace enlargement at birth, reminiscent of emphysematous changes in COPD, and heterozygous animals are resistant to the development of bleomycin induced fibrosis. However, none of these studies focused on the cell-specific roles of YT. In the current thesis, we looked at the effect of YT in the context of the adult lung epithelium, as not much is known about their role in adult lung homeostasis and their role after specific injuries.

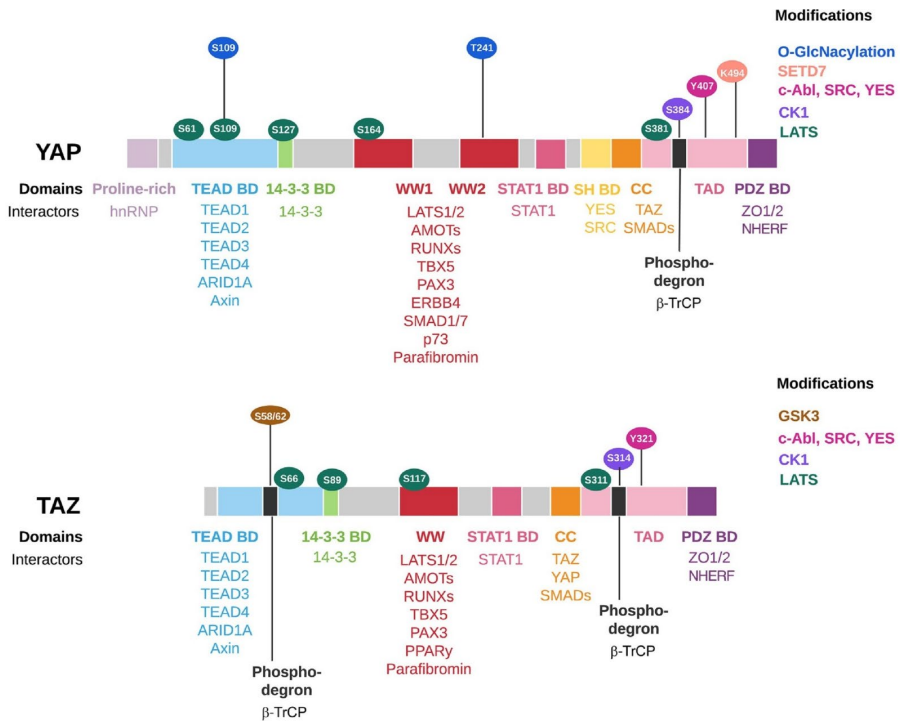


Figure 6. Protein domains of Yap and Taz: Similarities and PTMs.

Structural domains of YT are highlighted with some examples of the known TF interactors. Several of the PTMs are listed in Table 1. Abbreviations: AMOTs, angiominos; ARID1A, AT-rich interactive domain-containing protein 1A; BD, binding domain; CC, coiled coil; CK1, casein kinase 1; ERBB4, erb-b2 receptor tyrosine kinase 4; GSK3, glycogen synthase kinase 3α; hnRNP, heterogeneous nuclear ribonucleoprotein; LATS1/2, large tumor suppressor kinase 1 and 2; NHERF, Na(+)/H(+) exchange regulatory cofactor NHE-RF1; paired box gene 3; PDZ, PSD-95, Dlg1, ZO-1; PPARγ, peroxisome proliferator-activated receptor γ; RUNX, Runt-related transcription factor; SETD7, SET domain containing 7; SMAD1/7, small mother against decapentaplegic 1/7; SRC, SRC proto-oncogene, nonreceptor tyrosine kinase; STAT1, signal transducer and activator of transcription 1; TAD, trans activation domain; TAZ, WW domain containing transcription regulator 1; TBX5, T-box transcription factor 5; TEAD, TEA domain; β-TrCP, β;-transducin repeat containing E3 ubiquitin protein ligase; UTR, untranslated region; YAP, Yes-associated protein; ZO1/2, zonula occludens 1/2. YES, YES proto-oncogene 1. This illustration is re-used with permission from (Reggiani et al., 2021).

Known YT transcription factor binding partners

YT are transcriptional cofactors and can only function by interacting with other transcription factors (TFs) in the nucleus. YT can interact with TFs through several of their protein domains as highlighted in Figure 6 with some examples of their interactions. YT do not only have binding partners inside the nucleus, but also in the cytoplasm; several phosphorylation sites on both YT facilitate their degradation (Table 1). However, when they are not degraded, they are either sequestered or known to interact with complexes such as taking part in the β -catenin destruction complex (Azzolin et al., 2014). In the current research, we focused on the nuclear interactions of YT to understand their downstream effect in regulating fibrosis.

The main transcription factors that have been indicated to interact with YT are the TEAD TFs (1 – 4) due to the presence of a binding domain specific for the TEADs (Figure 6) which has led to the understanding that YT mainly exert their effects through these TFs. However, increasing evidence indicates that YT interact with a diversity of TFs in the nucleus, both in tandem and without the TEADs (Lopez-Hernandez et al., 2021). In fact, the original context of the discovery of TEAD interactions was in its role in oncogenic activity, where when it interacted with YT, its activity was considered pro-oncogenic, but when it interacted with the Vgll proteins, which compete with YT for TEAD binding, it took an active role in tumor suppression (Pobbati and Hong, 2013). Even in a tumor cell line, TEAD and Yap overlapped in approximately 90% of its coverage on the genome indicating that there are other Yap interactions independent of TEADs (Chang et al., 2021).

There are a few modes of interaction of YT to modulate transcriptional regulation that have been explored in several contexts in the presence and absence of TEADs (Figure 7) (Lopez-Hernandez et al., 2021). These modes may not cover all YT interactions as this is a growing field. While none of these interactions are validated in the lung, it is important to highlight that some of the TFs already known to interact with YT, have also been shown to be involved in IPF (Table 2). These specific YT-transcription factor interactions, however, have not yet been observed in the context of IPF.

Table 2. Yap/Taz interactions that are also known to contribute to IPF

Abbreviations: RUNX, Runt related transcription factors; ZEB1, Zinc finger E-box-binding homeobox 1; FOXO, Forkhead box protein O1; EMT, epithelial to mesenchymal transition.

TF	Role in fibrosis	Reference to IPF	Reference to YT
c-MYC	Profibrotic	(Qin et al., 2022)	(Crocì et al., 2017)
RUNX2	Profibrotic	(Mümmeler et al., 2018)	(Hong et al., 2005)
ZEB1	EMT	(Chilosi et al., 2017)	(Lehmann et al., 2016)
β -catenin	Profibrotic	(Gottardi and Königshoff, 2013)	(Heallen et al., 2011)
SMADs	Profibrotic/EMT	(Piersma et al., 2015)	(Wei et al., 2020)
TP-53	Apoptosis of Alveolar cells	(Bhandary et al., 2013)	(Di Agostino et al., 2016)
FOXO1	Anti-fibrotic	(Xin et al., 2018)	(Shao et al., 2014)

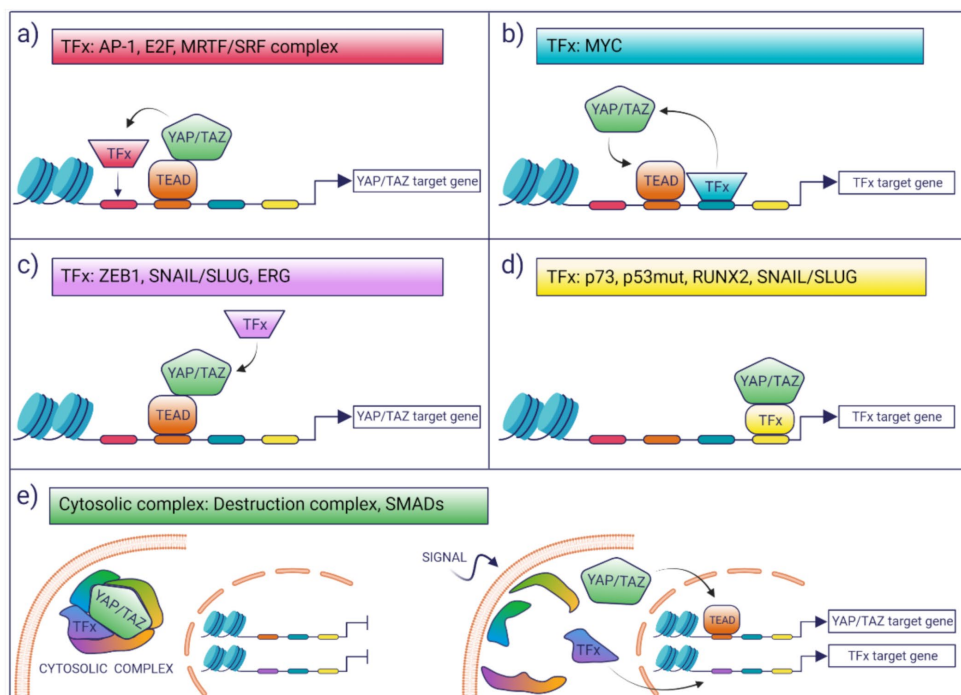


Figure 7. An overview of the general mechanisms of YT interaction with other TFs.

(a) Regulation of YAP/TAZ targets by cis interactions with other TFs; (b) regulation of other TFs targets by YAP/TAZ; (c) regulation of YAP/TAZ activity on their targets mediated only by protein–protein chromatin-associated complexes; (d) modulation of other TFs activity, on their respective targets, mediated only by protein–protein chromatin-associated complexes; (e) modulation based on non-nuclear protein–protein interaction. Image obtained under Creative Commons License (Lopez-Hernandez et al., 2021)

Upstream regulation of Hippo signaling

The regulation of Hippo signaling, and YT is multi-dimensional and highly depends on the context of the cells. For example, Hippo signaling is known to respond to mechanical cues (Dupont et al., 2011), cytoskeletal changes (Wada et al., 2011, Aragona et al., 2013), cell-cell contact (Zhao et al., 2007), cell adherence (Yang et al., 2015), and soluble factors (Yu et al., 2012). Several reviews cover the various regulation mechanisms quite well (Zhang et al., 2018, Wada et al., 2011, Ramos and Camargo, 2012, Piccolo et al., 2014, Mauviel et al., 2012, Lian et al., 2010, Kim et al., 2019, Huang et al., 2016, Meng et al., 2016). In the subsequent portion of the thesis, the focus will be restricted to mechanisms that are associated with fibrotic injury and lung homeostasis.

The lung is mechanical organ that is constantly moving with breathing and thus elasticity of the lung is key for proper function. In pulmonary fibrosis, the fibrotic

injury has been found to result in regional stiffening in the distal portions of the lung (Liu et al., 2015, Tschumperlin et al., 2018). The effect of stiffness on the Hippo signaling pathway is complex as it can be mediated through various ways. Increased ECM stiffness is caused by both increased deposition of more stiff ECM molecules (e.g., fibrillar collagens) as well as increased crosslinking of ECM components. Such examples are collagens, as well as fibronectin and other molecules that may provide different or additional integrin binding sites for the cells to attach. Increased substrate stiffness leads to increased flattening of the cells which also results in cytoskeletal rearrangement and the formation of cell stress fibers. Among the ways that stiffness related changes affect the Hippo signaling pathway is through the Rho A GTPase which has been found to inhibit LATS1/2 through modulation of F-actin fibers leading to the activation of YAP (Zhao et al., 2007). A similar mechanism has also been described where matrix stiffness causes FAK-Src-PI3K to modulate YAP in a LATS dependent manner (Kim and Gumbiner, 2015).

However, in normal cells, there appears to be a hierarchy of signals that orchestrate YAP activity. Under high cell density conditions and in the absence of added soluble factors, increased cell-cell contact and thus increased formation of Adherens junctions, results in sequestration of YAP in the cytoplasm. In high cell density conditions, other soluble factors have been shown to be capable of overcoming contact inhibition to induce YAP activation. For instance, this can be modulated by the activation of the Rho A GTPases through G-protein coupled receptors (GPCRs) that can be activated by molecules such as lysophosphatidic acid (LPA) (Cai et al., 2021, Yu et al., 2012). Interestingly, LPA has been previously implicated in pulmonary fibrosis and LPA antagonism is being intensely pursued in clinical trials for IPF. In the first-generation approach using an orally available LPA1 antagonist (BMS-986020), there was a significant decrease in the decline of FVC in patients receiving 600 mg twice daily. This same compound has also been shown to have anti-fibrotic effects in *in vitro* assays (Decato et al., 2022). However, the trial was stopped due to adverse effects associated with the liver in a subset of patients. A second-generation oral LPA antagonist therapy is currently ongoing (Corte et al., 2021).

Role of Hippo-YAP in the lung

The earlier studies of YAP, TAZ and Hippo signaling in mammals were mainly focused on their oncogenic roles. Their role in cancers across organs is well documented including their contribution to several types of lung cancer (Lin Teoh and Das, 2017, Lo Sardo et al., 2018). However, the focus of this thesis is on their role in the normal and fibrotic lung epithelia and their related mechanisms in lung development (Table 3).

Table 3. Selected YT findings in the lung epithelium

Compartment	Status	YT role	Reference
The developing lung	Development	Loss of Taz cause lung to develop emphysematous lungs	(Makita et al., 2008)
The developing lung	Development	Yap is required for TGF- β activity in formation of the airways	(Mahoney et al., 2014)
The developing lung	Development	Cytoplasmic Yap influences epithelial lineage commitment through β -catenin and Fgf10	(Volckaert et al., 2019)
The developing lung	Development	YT play a role in lung morphogenesis and alveolarization in a sequential manner	(Isago et al., 2020)
Alveolar epithelium	Fetal	Stretch induced activation of YT regulates ATI differentiation through a YT-ROCK axis	(Nguyen et al., 2021)
Alveolar epithelium	Neonatal	Lats2 inactivation reveals Yap and Taz role in driving ATI fate	(Nantie et al., 2018)
Alveolar epithelium	Postnatal	Yap regulates alveolar epithelial differentiation	(Gokey et al., 2021)
Alveolar epithelium	Neonatal	ATI to ATII plasticity is found in neonatal lungs but restricted in adult lungs	(Penkala et al., 2021)
Airway epithelium	Embryonic and Adult	Loss of Mst1/2 regulate Yap's effect on cell proliferation and differentiation	(Lange et al., 2015)
Alveolar epithelium	Adult	Lats2 loss activates YT to drive secretory cell differentiation	(Jeon et al., 2022)
Alveolar epithelium	Adult lung	YT have pro-regenerative role after lung exposure to sublethal infection	(LaCanna et al., 2019)
Airway epithelium	Adult	Wnt5a activates YT in response to inflammation	(Fan et al., 2022)
Alveolar epithelium	Adult	Yap is required for mechanical induced alveolar repair	(Liu et al., 2016)
HBEC3 cells	Fibrosis	Profibrotic activation through mTOR/PI3K/AKT	(Gokey et al., 2018)
Alveolar epithelium	Fibrosis	Taz is required for alveolar repair after bleomycin induced lung fibrosis.	(Sun et al., 2019)
Alveolar epithelium	Fibrosis	Targeting of lamellar bodies suppresses activation of Yap in ATII cells	(Kook et al., 2021)
Alveolar epithelium	Fibrosis	Epithelial Yap promotes fibrosis and epithelial Taz reduces fibrosis	(Warren et al., 2022)

Hippo-YT in lung development

In early development, YAP and TAZ have a wide range of roles across the various stages and are required for the maturation of various organs (Wu and Guan, 2021). This is reflected by the lethality of the full knock out of *Yap*, *Mst1/2*, *Sav1*, or *Lats2*, and the various defects caused by the TAZ knockout (Makita et al., 2008, Morin-Kensicki et al., 2006, Dai et al., 2017). In the lung, in specific, roles in lung morphogenesis and alveolarization have been described for Yap and Taz (Table 3). While the complete deletion of Yap was lethal, Yap conditional mice were used to result in knockout at birth; importantly, the murine lung is known to continue development for the first week postnatally and these mice showed deranged morphogenesis and the appearance of cysts in the distal parts of the lung (Mahoney et al., 2014). Lethality of *Mst1*^{-/-} mice at birth showed inhibited sacculization of the lung (Lange et al., 2015). Moreover, loss of Taz had a pronounced effect on the lung and resulted in disruption of normal alveolar epithelial development with the appearance of an emphysema-like phenotype (Makita et al., 2008). Additionally, a recent report found the expression of Yap and Taz to be sequential during lung development where Yap protein expression precedes Taz indicating a different role for each molecule during development (Isago et al., 2020). The branching morphogenesis is controlled by a feedback loop between Sonic hedgehog (SHH) and fibroblast growth factor (FGF). Yap deficiency was found to interrupt this feedback loop leading to abnormal branching morphogenesis. Moreover, cytoplasmic yap was found to modulate epithelial lineage commitment through β -catenin and Fgf10 (Volckaert et al., 2019).

Hippo-YT in the normal adult lung epithelium

Studies of YT in the recent years have shined the light on their roles in the maintenance of the proximal and distal lung epithelia where both YAP and TAZ have been shown to play a major role in proliferation, differentiation, and repair after injury (Penkala et al., 2021, Gokey et al., 2021, Hicks-Berthet et al., 2021, Jeon et al., 2022) (Table 3). In the alveolar lung epithelium, surprising findings revealed insights into the plasticity of ATI cells at the neonatal stage where ATI cells had the ability to acquire the ATII identity upon injury, a process that is mediated by YT (Penkala et al., 2021). This plasticity is later restricted from the ATII to the ATI direction in the adult lung. The plasticity of the small airway cell has also been further explored where deletion of *Lats1* and *Lats2* was shown to be sufficient to drive differentiation of small airway secretory cell into distal alveolar type I cells (Jeon et al., 2022). Reduction of YT expression on the other hand was found to promote the secretory lineage in the proximal lung epithelium. YT were also found to play a role in maintaining the homeostasis of secretory cells in the proximal lung epithelium (Hicks-Berthet et al., 2021). Moreover, YT were found to modulate alveolar epithelial repair in response to sublethal infection of

Streptococcus pneumoniae (LaCanna et al., 2019). These studies increase our awareness of YT's important roles in the maintenance and repair of the airway and alveolar epithelia. These roles need to be further explored in the context of chronic lung disease to distinguish between the pathologic versus regenerative roles of YT in these diseases.

Role in pulmonary fibrosis

The fibrotic lung has an excessive deposition of ECM with increased stiffness. These characteristics are known to influence the function of the Hippo signaling pathway. Yap and Taz were previously found to modulate the fibrotic activity of fibroblasts in IPF (Liu et al., 2015). A similar role for Taz has also been described where Taz promoted fibrotic injury and ECM deposition through increased activity in fibroblasts (Noguchi et al., 2017, Jorgenson et al., 2017). Moreover, Yap was found to play a role in the proximal fibrotic lung epithelial cell line where it modulates its fibrotic effect through the mTOR/PI3K/AKT pathway and Taz was found to be required for alveolar repair after bleomycin induced injury in mice (Gokey et al., 2018, Sun et al., 2019). However, the role of Hippo signaling, and YT are not fully understood in the context of the distal lung epithelium where the fibrotic injury mainly occurs. Recent findings allude to varying roles of alveolar epithelial Yap and Taz in the progression of lung fibrosis where Yap was found to be profibrotic while Taz was associated with repair (Warren et al., 2022). Furthermore, in a study that explored the therapeutic benefits of melatonin in bleomycin induced lung fibrosis, the beneficial effects of melatonin were found to be a result of decreased Yap nuclear localization (Zhao et al., 2018). The Hippo signaling pathway is conserved across species and it is known to cross talk with other signaling pathways. These interactions are extensively covered in the literature and are used to predict roles for the Hippo-YT in the context of pulmonary fibrosis (Knipe et al., 2015, Sun et al., 2021, Zhu et al., 2020). However, several of these crosstalk mechanisms need to be validated within the lung context. Moreover, the specific interactions of YT across the different cell types are not yet known which explains the different roles reported in different studies. The type of injury or *in vitro* setup play a prominent role of the outcome of the YT studies. Therefore, more comprehensive understanding of the role of YT in the fibrotic lung epithelium *in vivo* is needed to consider the potential of targeting YT for treatment of IPF.

The research in this thesis

Gap in knowledge

Our understanding of the molecular mechanisms driving IPF are continuously improving. The role of Hippo signaling and its effectors YAP/TAZ in the progression of the disease has been proposed in the past year and has mainly been studied in lung fibroblasts, which have been long considered the main drivers of fibrosis (Tschumperlin et al., 2018, Liu et al., 2015). The role of the lung epithelium as an active contributor for disease has only recently taken traction and more studies are exploring the lung epithelium in that capacity (Selman and Pardo, 2020). Moreover, limited knowledge exists on the role of YAP/TAZ in the normal or fibrotic lung epithelia. Moreover, evidence suggests that the lung epithelial progenitors from both the distal and proximal epithelial compartments play important roles in the pathogenesis of IPF. This is especially important since single cell studies have identified transitional cell states in the distal lung epithelium that acquire transcriptional profiles of both proximal and distal lung epithelial phenotypes simultaneously (Habermann et al., 2020, Adams et al., 2020).

Aim of the thesis

The aim of this doctoral thesis is to identify the role of YT in the fibrotic lung epithelium and define how YT exert their profibrotic activity.

The main aim of the thesis will be addressed through these specific aims:

1. Develop the methodology for simultaneous isolation of proximal and distal lung epithelial cells from the same animal.
2. Identification of the fibrotic role of YT activation in the distal lung epithelium of human disease and mouse model of fibrosis
3. Develop the bioinformatics pipelines for exploring cell identities.
4. Identify YT interaction partners in the lung epithelia

Approach and Methodology

To achieve the aims of this thesis, a myriad of methods needed to be employed for the various parts of the project. Moreover, most techniques to achieve this project needed to be developed, optimized, or implemented. The general approach for the main aim of the thesis is summarized in Figure 8. The details of all the techniques used in each of the manuscripts are included in each of these manuscripts with greater detail. This section discusses the various technologies and the challenges and reasoning for the choice of methodology in this project.

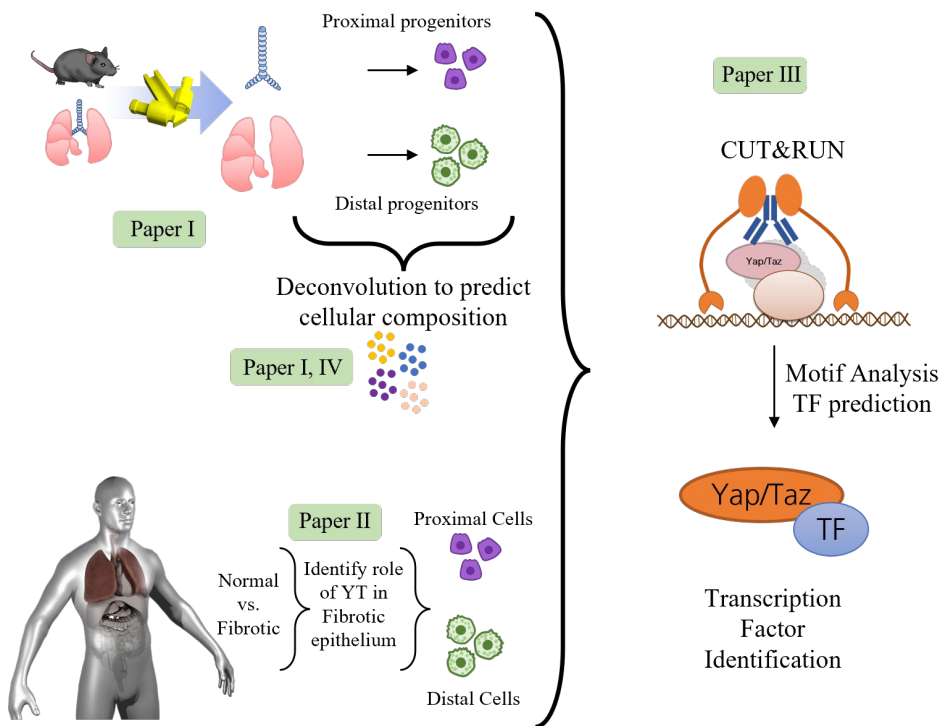


Figure 8. Overview of the approaches to achieve the aims for the thesis.

Paper I covers the method development for simultaneous isolation of proximal and distal lung epithelial progenitors and explores the implications for *in vitro* assays. **Paper II** explores the molecular mechanisms that drive YT role in fibrosis in human IPF and mouse models of fibrosis. In **Paper III**, the methodology and workflow for identification of transcription factor motifs is developed and optimized for primary mouse and human epithelial cells. **Paper IV** includes the development of computational pipelines to perform deconvolution of bulk RNAseq data using reference scRNAseq datasets to infer cell types.

Isolation and maintenance of primary cells

Immortalized cell lines, such as the MLE12 mouse epithelial cell line and the A549 human epithelial cell line, have greatly aided our understanding of epithelial response to various stimuli. However, primary cells can only recapitulate a few aspects of the cell type of interest depending on their source and the method in which they were immortalized. Thus, the use of primary cells is essential for improved understanding of the *in vivo* disease context and recapitulation of several aspects of the cell type of interest. Although isolation of primary lung epithelial cells can be difficult due to a lack of surface markers clearly distinguishing cells along the respiratory tract, there are several existing protocols that have been established for isolation of distal lung epithelial cells as well as proximal lung epithelial cells.

The isolation methods vary based on the compartment in which the cells are isolated from (i.e., proximal versus distal) as well as how the cells intend to be used in downstream assays, including the ability of the cells to be passaged or differentiated. Primary proximal epithelial progenitors (MTECs in mice or HBECs in humans) can proliferate and can be passaged several times before they become senescent. Meanwhile, distal epithelial progenitors (ATII cells) are only able to proliferate in the first passage of 2D culture after they are cultivated after cell isolation. Several of the projects in this thesis use murine and human proximal and distal lung epithelial progenitors as starting materials for use in 2D or 3D assays. Thus, optimization of cell isolation protocols designed for these downstream assays was an integral part of this work.

Proximal epithelial progenitors from human airways are somewhat straightforward to isolate and well established as the airways can be readily isolated. The human bronchial epithelial cells (HBECs) in this thesis are isolated from airway resections of excess surgical waste from lung transplants. Other previous studies have used excess surgical waste from lobectomies as well as airway brushings from bronchoscopy (Stokes et al., 2014, Langwiński et al., 2022). The location of the resected tissue piece is usually taken from the region of the main bronchi or superior to that. Thus, the isolation method of the HBECs requires no consideration of the distal progenitors and any potential for a contamination of the distal progenitors as the different airway compartments can be readily surgically resected from one another. One of the most common protocols used for HBEC isolation relies on the ability to select the basal cells using specialized media after dissociating the cells and scraping them off the bronchial tissue piece. HBEC isolation in this thesis was done as described previously and is also detailed in **Paper III** (De Santis et al., 2021). Similarly, mouse tracheal epithelial cells (MTECs) are isolated directly from surgically resected murine tracheas (Lam et al., 2011, Eenjes et al., 2018). It is generally well-accepted in the field that basal cells in the mouse lung are restricted to the trachea and beginning of the main bronchi (Mercer et al., 1994). Therefore,

isolation of these cells by dissecting the trachea also does not require any consideration for a potential contamination of the distal progenitors. MTECs have been previously isolated through several methods such as media selection or through sorting approaching by selecting for the NGFR receptor (Lam et al., 2011, Rock et al., 2009). Selecting human and mouse basal cells using a combination of the surface markers NGFR and ITGA6 has been shown to be capable of obtaining a basal cell population which can differentiate into a mature proximal epithelium when grown in air-liquid-interface cultures. However, while this method captures the majority of basal cells, the negative fraction (i.e., NGFR⁻ and ITGA6⁻) retains colony formation ability, albeit to a much lower extent. This indicates the presence of basal cells in the negative fraction even if at low capacity (Rock et al., 2009). On the one hand, for most *in vitro* studies, this is not necessarily an issue especially if the *in vitro* experiment is aimed at studying the major response of the basal cells to a specific stimulus. On the other hand, this can be problematic if a rare subtype is to be studied. Rare cell population in homeostatic conditions or disease states can sometimes be found in small numbers. Moreover, our understanding of rare populations isn't complete and thus we cannot determine whether they possess a specific cell marker without first being identified through other means such as single cell RNAseq as an example.

Several different techniques have been described to isolate distal epithelial progenitors (alveolar type II cells) from both mouse and human lungs (Jansing et al., 2018, Dobbs et al., 1986, Corti et al., 1996, Gonzalez and Dobbs, 2013, Hiemstra et al., 2019, Mao et al., 2015, Messier et al., 2012). However, the choice of isolation method may influence the outcome of the study at hand. To date, no surface marker has been identified which is effective in selecting all of the diverse pools of ATII cells that have been shown to exist in the lung. Recently, the surface marker, HTII-280, has been used to select for ATII cells in the human lung (Gonzalez et al., 2010). The cells isolated with this marker showed high purity based on SFTPC expression. The HTII-280 fragment was discovered through immunization of mice against purified human ATII cells to generate monoclonal antibodies. The function of HTII-280 as well as what it targets remains unknown. Nonetheless, this remains the state-of-the-art method to isolate and culture human ATII cells. A recent scRNAseq study showed that HTII-280⁺ contained EPCAM⁺ and EPCAM⁻ fractions, both capable of generating organoids. Moreover, the HTII-280⁻ fraction was able to generate organoids that grew faster and larger (Hoffmann et al., 2022). Therefore, HTII-280 may not be able to select all possible distal lung progenitors and its equivalent in mouse lungs is not yet known.

Protocols to isolate cell populations highly enriched for mouse ATII cells have nonetheless been developed, even in the absence of a specific marker. Many of these techniques center on negative selection steps to first remove the non-ATII cells, which results in a high purity of ATII cells following selection with general epithelial cell markers (Jansing et al., 2018). First, the lung tissue is digested,

minced, and filtered through meshes down to a 10 μm pore size. The resulting suspension is then cultured on tissue culture plastics to allow the fibroblasts and macrophages to adhere to the surface and the suspended cells are collected to be sorted. Cells that are CD31+ or CD45+ are filtered out using magnetic or flow sorting. This thesis exclusively used magnetic based sorting. Finally, the remaining fraction is considered enriched for ATII cells. The dissociation solutions are introduced to the lung using the trachea as a passage where the trachea is then manually ligated to keep the solution inside the lobes for the duration of the incubation. To our knowledge, prior to **Paper I**, there was no previous development of a protocol that differentially isolated proximal progenitors from the trachea/bronchi and distal progenitors from the lung lobes simultaneously from the same animal. This is important for clearly understanding the location of rare cell types as well as how the lung epithelium differs along the airway tree. Therefore, we developed a protocol to achieve it using a 3D printed surgical guide to mimic the ease in which such procedures are done routinely in human lungs (**Paper I**). The implications of this protocol are discussed in the discussion section.

Multicellular and 3D culture models

The use of classical *in vitro* models based on culture of an individual cell type on tissue culture plastics remains to be an efficient method for evaluating specific molecular mechanisms: However, among its drawbacks is that it lacks the ability to recapitulate several aspects of the *in vivo* context of the cells. This is important when studying the context of the cell and its interaction with other cell types or with the extracellular matrix. Thus, several models have been developed to recapitulate various aspects of the *in vivo* context. In the current thesis, we utilized several of these models.

Differentiation assays and organoid culture

In the first part of the current research, we aimed to develop a method to isolate proximal and distal progenitors from an individual mouse lung (Paper I). We then needed to validate that our method does not alter the characteristics of the isolated progenitors. Therefore, we used several differentiation assays to evaluate the functionality of the proximal and distal lung progenitors. We used air-liquid interface culture to differentiate the proximal progenitor into a muco-ciliary epithelium. We additionally differentiated proximal progenitors in organoids by seeding them in Matrigel. We found that our protocol resulted in proximal cells which were capable of forming organoids comprised of the various differentiated cell types found in proximal airway epithelium, including ciliated cells in the inner lumen of the organoids.

In this thesis, distal progenitor cell populations were validated by evaluating their ability to generate organoids *in vitro*. Unlike proximal progenitor cells which can

form organoids in the absence of stromal support cells, distal progenitors require external factors secreted by mesenchymal cells to form organoids (Barkauskas et al., 2017). It has been later shown that the cells can form organoids with the addition of specific factors secreted from the support cells (Shiraishi et al., 2019). Moreover, human distal organoids were maintained in culture long term through culturing and subculturing using media containing compounds that activate Wnt, FGF, and insulin signaling, and inhibiting TGF- β , ROCK, and p38 MAPK signaling pathways (Sachs et al., 2019). Once in culture these organoids were found to differentiate into two different trajectories. A part of them maintained their alveolar identity, while others gained proximal identity including the differentiation into basal cells (Hoffmann et al., 2022). Human alveolar type II cells cultured with mesenchymal cell support has been recently shown to push the culture towards a proximal phenotype over culture time (Kathiriya et al., 2022). Thus, culture conditions are extremely important for the generation and maintenance of alveolar organoids. In the current study, we used a cell line, CCL206 cells for mesenchymal support of the murine alveolar distal progenitors (**Paper I**). The use of a cell line instead of primary fibroblasts or endothelial cells helps to reduce variability across experiments. Thus, depending on the research question, the use of primary versus immortalized mesenchymal support cells or alternatively, identified factors, may be beneficial.

Precision-cut lung slices (PCLS)

Differentiation assays are used to explore specific mechanisms of cell behavior during and after differentiation. However, the differentiated cells in these assays do not always recapitulate the *in vivo* context fully and remain limited for some types of experiments. In particular, cells are removed from their native context and the majority of these assays lack fidelity to retaining cell-ECM interactions. Precision cut lung slices are an alternative 3D culture technique which largely retains the cellular diversity and spatial relationship of cell-cell and cell-ECM interactions. PCLS are generated by filling native lung tissue with agarose to solidify it and allow for consistent precise slicing. Different concentrations of agarose can be used to generate slices of thickness varying between 100 μm to 1.25 mm across different species. PCLS have been used to model several diseases such as IPF, COPD, infections, and other lung conditions (Alsafadi et al., 2020). In the current research, we developed a protocol to isolate high quality RNA from PCLS across several species. Furthermore, we used this protocol to perform the first RNA sequencing in human PCLS where higher concentrations of agarose are needed to reliably generate PCLS (**Paper IV**) (Stegmayr et al., 2021). Overall, PCLS can provide insights into cell-cell and cell-matrix interactions to a close degree to the *in vivo* context due to its ability to retain the diverse cell types. Furthermore, human lung tissue itself can be used to generate this model. One major limitation of the model is due to the lack of circulation and an intact immune system; thus, the immune cells cannot be recruited into the model as in the *in vivo* scenario.

Next generation sequencing (NGS)

Sequencing technologies have advanced the way we produce data from diverse experiments and in vitro models. The amount of information obtained by NGS allows for the use of advanced statistical models to extract relevant biological information. In the current research, we used several types of sequencing and performed data analysis associated with these techniques, a few of which will be discussed in the following sections.

In addition to the advantage of producing large amounts of data, the move towards open science, where such data is deposited on public servers, such as the genomic expression omnibus (GEO), has greatly accelerated the progress of science. Sharing these datasets allows other researchers to explore the data and possibly apply further analysis on these datasets. We have utilized this ability and re-analyzed and integrated several microarrays, RNAseq and scRNAseq datasets with our data.

In **paper I**, we used bulk RNAseq of mRNA to evaluate the isolated cells using the method we developed. In **Paper II**, we used microarrays to evaluate the silencing of YT in primary mouse alveolar type II cells. In **Paper III**, we used DNA sequencing to sequence the DNA fragments bound to complexes containing YT. In **Paper IV**, we used mRNA sequencing to validate the newly developed RNA isolation method for PCLS. In papers **I, II, III, and IV** we re-analyzed publicly available single cell RNAseq (scRNASeq) datasets to use for various purposes such as the use of scRNAseq datasets as a reference for deconvolution of bulk RNAseq (Papers **I, and IV**) and use to explore gene expression of specific targets or a combined signature score of a list of targets (Papers **II, III**).

RNA Sequencing

RNA sequencing (RNAseq) can be done in several ways to obtain various types of information. But simply, the RNAseq procedure includes these general steps: RNA isolation, Library preparation, and sequencing. The RNA isolation procedure and library preparation need to be adjusted based on the type of RNA that needs to be sequenced. An example, it is possible to sequence total RNA; ribosomal RNA, messenger RNA, microRNA; or targeted RNA sequencing using hybridized oligomers. The quality of the isolated RNA is the main determinant of how successful a sequencing experiment can be. Previously RNAseq was not possible to perform with PCLS due to presence of agarose which interfered with the commonly used RNA isolation protocols, resulting in low-quality and quantities of RNA. However, we optimized the RNA isolation procedure for PCLS to obtain high quality sequencing reads (**Paper IV**)(Stegmayr et al., 2021). Once RNA is selected, cDNA is synthesized to be later amplified for sequencing. Data analysis following sequencing includes alignment of the sequencing reads to the genome of interest and quantification of the genes and transcripts of these samples. There are several

algorithms for alignment, however there is not a consensus on which alignment method is superior (Corchete et al., 2020).

RNAseq has become the gold standard for quantitative analysis of the transcriptome and is considered to be the most versatile tool for gaining deep genomic understanding of biological samples (Corchete et al., 2020). Although the cost associated with running RNAseq experiments have dropped significantly overtime, it remains inaccessible for many labs due to a lack in resources or inability to perform the time consuming and advanced data analysis associated with this data.

Single cell RNA sequencing

Single cell RNAseq (scRNAseq) has become a standard in the lung and other fields for exploration of the cell types and transitional cell states during disease and disease modeling *in vitro*. Several seminal studies have generated atlases of single cell data across the various compartments of the healthy and diseased lung in the last few years (Adams et al., 2020, Habermann et al., 2020, Strunz et al., 2020, Louie et al., 2022, Montoro et al., 2018, Kathiriya et al., 2022, Hoffmann et al., 2022). These studies resulted in the discovery of several cell types and diseased cell states in the lung such as the ionocytes, aberrant basaloid cells, Krt17+/Krt5- cells, and others. Single cell studies have also furthered our understanding of the behavior of progenitor cells in the lung. For instance, lung progenitors were thought to be restricted to their regional locations such as the basal cells being exclusively in the airways and the alveolar type II (the distal progenitors) to regenerate and differentiate to alveolar type I cells (Rock et al., 2010, Barkauskas et al., 2013). While this appears to remain true in the homeostatic conditions, several studies have shown the ability of the distal progenitors to differentiate into a proximal epithelium *in vitro* using scRNAseq approaches (Kathiriya et al., 2022, Hoffmann et al., 2022). Conversely, airway cells have been shown to be capable of promoting alveolar regeneration (Kathiriya et al., 2020). Our understanding of the plasticity of the lung progenitors is being improved immensely using scRNASeq.

The processing of scRNAseq data is extensive and requires a lot of processing steps. This means that several decisions about data handling must be made for the data to take its final shape. These decisions can include library preparation, mapping methods, imputation, and data normalization. A recent report has tested various combinations of these aspects and found that the library preparation and normalization methods are the most impactful factors that affect the results of a scRNAseq data analysis (Vieth et al., 2019). Therefore, reporting the analysis pipeline for these datasets becomes immensely important for others to reproduce the results obtained by the original authors of the work. Several studies report some of the cut offs they have used for data filtration. However, this is not sufficient to reproduce the data analysis. This is important because the resulting data clusters in these datasets are usually what is used to determine if a cell type is a new one and

the expression of these selected cells will be the main driver for our understanding of this cell type. Thus, if a normalization step skews the data one way or another, this will alter the interpretation of the dataset. Finally, the field has not yet reached a standardized pipeline for this analysis. Therefore, it is crucial for every author publishing with scRNAseq datasets to take responsibility for sharing all the information necessary for data reproducibility.

For all the datasets we re-analyzed in the current research, we have used the R package Seurat (Hao et al., 2021). We have additionally shared all lines of codes used in these analyses (<https://github.com/Lung-bioengineering-regeneration-lab>). Each of the manuscripts refers to the exact repository for that manuscript.

Chromatin immunoprecipitation: Classical ChIP sequencing vs CUT&RUN

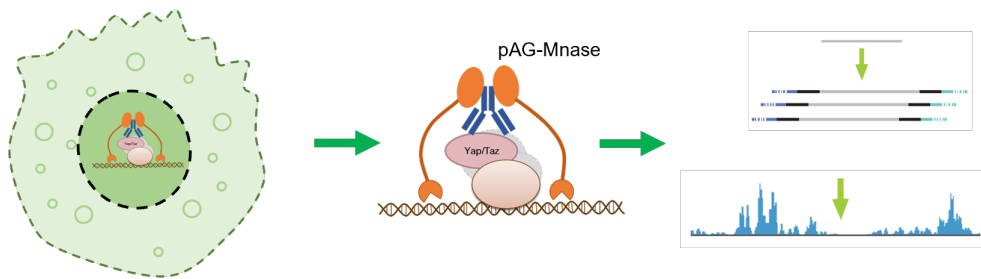


Figure 9. Overview of the CUT&RUN method.

The cells are immobilized and permeabilized and mild crosslinking is introduced. The targeting antibody is added to the cells and the MNase is then attached to the antibody. The MNase/Antibody/target is then cleaved by activating the MNase that will cleave the DNA and the whole complex is then released outside the permeable cells. Finally, DNA is purified, amplified, and sequenced

Chromatin immunoprecipitation (ChIP) methods have advanced our understanding of the dynamic processes that drive gene regulation. The main steps for doing a ChIP experiment are crosslinking, lysis, chromatin shearing, and immunoprecipitation (Schmidt et al., 2009). To obtain a high-quality ChIP experiment, a large number of cells is needed to be able to pass through these steps. Crosslinking needs to be sufficient for keeping the target molecules intact to survive the lysis process and more importantly the shearing of chromatin which requires high salt concentrations or sonication. ChIP sequencing experiments usually require extensive optimization steps. Moreover, ChIP can produce a low signal to noise ratio with a lot of background. To address several of these limitations, a recent study developed the cleavage under target and release using nuclease (CUT&RUN) to achieve the same results as ChIP with a fraction of the number of cells (Skene et al.,

2018). CUT&RUN is done through the permeabilization of intact cells followed by mild crosslinking and selecting the target using an antibody that is bound to the micrococcal nuclease (Mnase). The Mnase cleaves the DNA around the target and releases the DNA bound complexes through the permeabilized cells (Figure 9).

This method has been shown to work with a small number of cells and produces high quality peaks with high signal to noise ratio with a low background. We aimed at identifying the DNA sequences where complexes containing YT are bound in primary lung proximal and distal lung epithelial cells. The number of cells obtained from an individual or a few mouse tracheas is not nearly sufficient to run a proper ChIP experiment making it quite impossible to consider. While pooling a few mouse lungs for the alveolar type II cells may work for ChIP, there will not be a chance to compare the results with tracheal cells. Therefore, in **Paper III**, we optimized CUT&RUN to obtain sequences covered by YT. However, the method needed to first be validated for use with YT as they are not directly bound to the DNA and thus the nuclease may not be close enough to the DNA to cleave it (Figure 9).

Results and Discussion

Key Results and specific contributions to the field

A novel method to simultaneously isolate murine proximal and distal lung epithelial progenitors

Mouse models of chronic lung disease have been and remain instrumental in underlying disease mechanisms and evaluation of new targets (Moore and Hogaboam, 2008, Mercer et al., 2015, Dutt and Wong, 2006). The ability to isolate primary cells from these disease models also adds an additional advantage to the use of these models. Several protocols exist for isolation of primary cells from the murine lung. Prior to the current research, isolation methods for distal and proximal lung epithelial cells were developed independently as these cells were mainly studied in different contexts. In the current research, we developed a method simultaneously to isolate both proximal and distal lung progenitors from an individual mouse (**Paper I**) (Alsafadi et al., 2022).

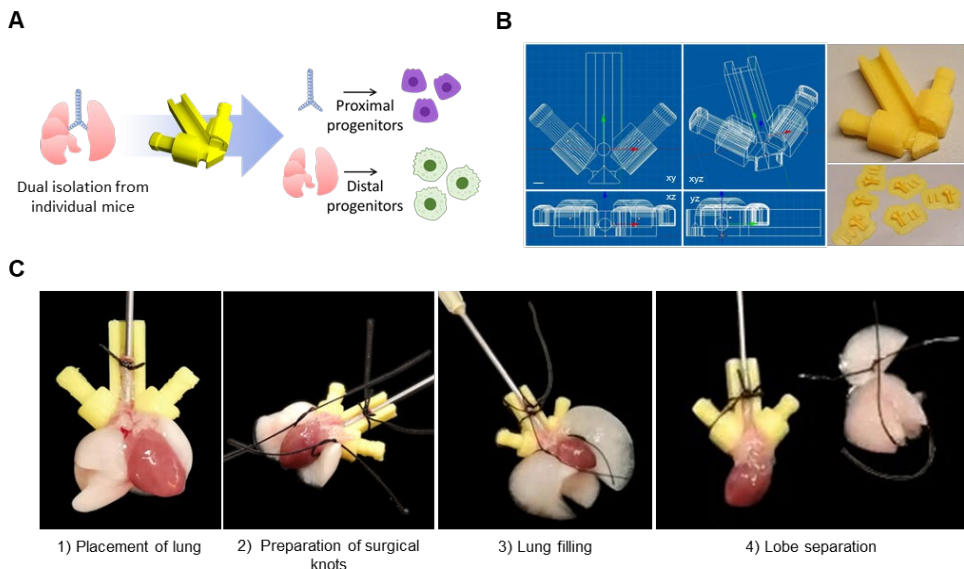


Figure 10. 3D printed lobe divider (3DLD), a surgical guide for separation of the trachea/bronchi and the lung lobes

(A) Schematics for using 3DLD to separate trachea/bronchi from the lung lobes for isolation of proximal and distal progenitors. (B) The design of the 3DLD from all direction. The 3D printable STL files can be found on the GitHub repository (github.com/Lung-bioengineering-regeneration-lab/dual_cell_isolation). (C) The process of using the device to assist in introducing dissociation solution and subsequent separation. Adapted from published Paper I (Alsafadi et al., 2022).

The proximal progenitors of the lung epithelium (i.e. basal cells) in the murine lung only extend until the main bronchi and are not present in the intralobular airways (Mercer et al., 1994). However, the exact location of where these cells are present along the respiratory tract remains unknown. This is important as isolation of the distal progenitors (i.e., ATII cells) requires the removal of the trachea. Prior to the work described in this thesis, isolation protocols for ATII cells were done by injecting the dissociation solution through the trachea followed by manual ligation of the trachea to prevent backflow of the dissociation solutions (Jansing et al., 2018). This can be problematic as the location of the ligation is at the level of the trachea and thus a small portion of the proximal airways will be in contact with the dissociation enzymes. Thus, we hypothesized that these protocols may result in co-isolation of progenitor cells which contaminate the pool of isolated distal cells. To address this, we developed a surgical guide using 3D printing. The device allows for the consistent separation of the trachea/bronchi and the lung lobes (Figure 10).

The major difference to the isolation of proximal progenitors that is introduced in this study is the brief exposure to the dissociation enzymes used for distal progenitors. We show in Paper I that the isolated and then expanded proximal progenitors using the 3DLD were able to differentiate into a mature epithelium in air-liquid-interface (ALI) culture and form organoids in Matrigel (Figure 11). After separation of the trachea, the isolation of the proximal cells was performed as previously described (Eenjes et al., 2018) with the difference that each trachea was isolated individually instead of pooling several tracheas together. The low number of cells obtained from an individual mouse trachea can be challenging for use with *in vitro* assays, thus, protocols that are able to expand the cells without losing their function are largely beneficial for generating enough cells for *in vitro* assays. We managed to perform several experiments after a single expansion of each trachea. However, some downstream experiments may require using the cells immediately after isolation. Thus, in those cases, pooling of mouse tracheas may be needed.

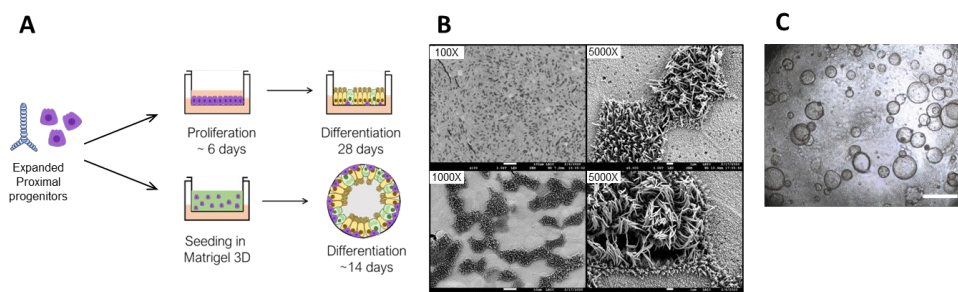


Figure 11. Differentiation of proximal progenitors isolated with 3DLD.

(A) Schematics for differentiation assays; first cells were expanded in one passage after isolation followed by culture in air-liquid-interface (ALI) or Matrigel for the indicated periods (n=4). (B) Scanning electron microscopy (SEM) of differentiated proximal epithelium in ALI. (C) Brightfield image of proximal organoids cultured in Matrigel. Adapted from published **Paper I** (Alsafadi et al., 2022).

Contamination of proximal lung epithelial cells in distal epithelial cell isolation alter the resulting organoid assay

The isolation method of distal progenitors using 3DLD differs from previous methods in that the trachea and main bronchi are removed from the lung before they are incubated with the dissociation enzymes and the dissection of the bronchi precisely occurs at the most distal part of the bronchi outside the intralobular space. We found that organoid culture of the isolated cells using classical methods produced larger organoids in comparison to those isolated with the 3DLD (**Paper I**, Figure 12). Moreover, the transcriptomic profile among the cell pellets between the two isolations showed higher variance among samples for each individual gene in comparison to those of the 3DLD isolations, indicating that the isolations from 3DLD could potentially be more consistent.

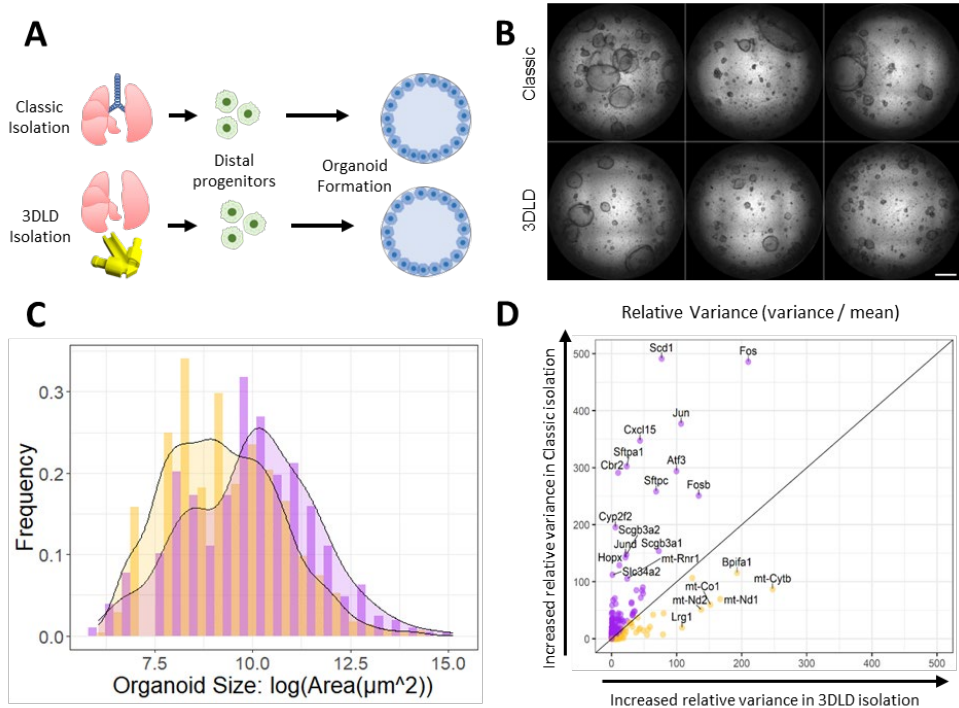


Figure 12. Distal epithelial progenitors isolated with 3DLD and classical methods give rise to organoids with larger organoids in the classic method.

(A) Schematics for distal cell isolations using classic method or 3DLD (B) Brightfield images of full transwell with cultured organoids. (C) Quantification of the organoid sizes in classic and 3DLD isolation methods. (D) Relative variance across all distal progenitor cell pellets within the same isolation in contrast to the other isolation. Adapted from Paper I (Alsafadi et al., 2022).

The transcriptomic profiles of the organoids, derived from distal progenitor of either method, were not vastly different although we did detect some statistically significant and consistent differences in a small subset of genes (**Paper I**); however, the most prominent differences were observed in the initial cell pellets directly after cell isolations.

We previously optimized the workflow for prediction of cell type proportion in bulk RNAseq data through deconvolution using a reference single cell RNAseq of cells from a similar source (**Paper IV**, Figure 13) (Stegmayr et al., 2021). In Paper IV, we were able to confirm that PCLS retained several of the cell types in the lung tissue, even when these cells were known to be associated with disease states only. Aberrant basaloid cells were discovered recently in IPF tissues through scRNAseq (Adams et al., 2020) and we found that these cells were predicted to be only in PCLS generated from IPF tissue (Figure 13B, Patient 2). This observation in PCLS, was also observed in bulk RNAseq of normal and (non)scarred IPF tissues (GSE99621) (Luzina et al., 2018).

We utilized the same analysis work flow and performed deconvolution of the bulk RNAseq data of the cell pellets, from the 3DLD and Classic isolations, using a reference single cell datasets of cells isolated in a similar way to those of the classic methods (Strunz et al., 2020). The deconvolution analysis predicted that there was a small percentage of basal cells in the classic method that were eliminated from the population of cells isolated with 3DLD (Figure 14).

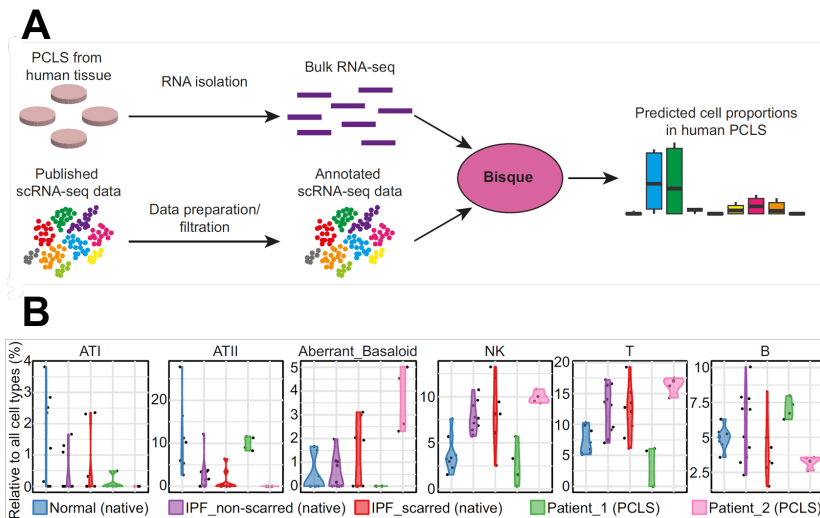


Figure 13. Reference-based deconvolution of bulk RNA-seq from human precision-cut lung slices (PCLS).

(A) schematic illustration of the workflow. (B) Comparison of Bisque-predicted cell types in native normal and idiopathic pulmonary fibrosis lung tissue (GSE99621) vs. PCLS from 2 patients. Reused with permission (Stegmayr et al., 2021).

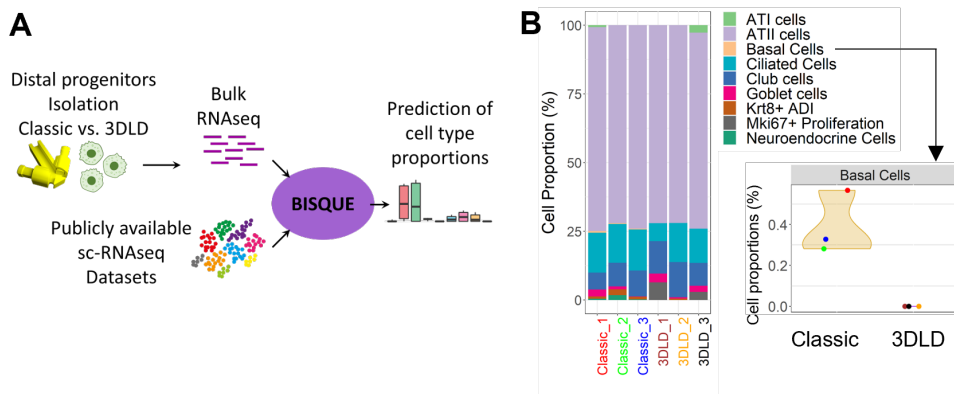


Figure 14. Deconvolution of RNAseq data from distal progenitor pellets isolated with classic or 3DLD methods reveal higher proportions of basal cells in classically isolated cells.

(A) Schematics for deconvolution of bulk RNAseq using a reference dataset (GSE141259) (Strunz et al., 2020). (B) Cell type proportions predicted by BisqueRNA for all cell pellets with basal cells highlighted on the right side.

To experimentally confirm this observation, we isolated proximal and distal progenitors using the 3DLD from an individual mouse and generated organoids from the distal progenitors while manually introducing varying concentrations of a proximal cell contamination from the same mouse (at levels predicted to be present by deconvolution) (Figure 15). However, in this set of experiments, we used the dissociation solutions for the distal cell isolation method for both the pulmonary lobes and the isolated trachea/bronchi on the same mouse (*i.e.*, to mimic what happens with tracheal ligation and incubation with distal dissociating reagents). This alteration in the protocol to isolate proximal cells is important for simulating what can happen during dissociation protocols aimed at isolating distal progenitor cells as the dissociation buffers used with proximal isolation are milder and the incubation time is shorter. Following isolation of the epithelial cells from both compartments, we seeded cells into Matrigel for organoid culture with ‘contaminating’ cells ranging from 1-3% (representative of the range of proximal cells that deconvolution predicted in Figure 14). Using high content screening, we tracked the growth of each group over time (Figure 15). Strikingly, we observed consistent and convincing differences in organoid morphology with increasing percentage of contaminating cells contributing to the presence of large organoids; this is in line with what we previously observed in Figure 12. This directly demonstrates that changes in organoid morphology can be caused by the presence of a small number of contaminating proximal cells which have survived the isolation procedure and have been co-seeded into the Matrigel for organoid formation.

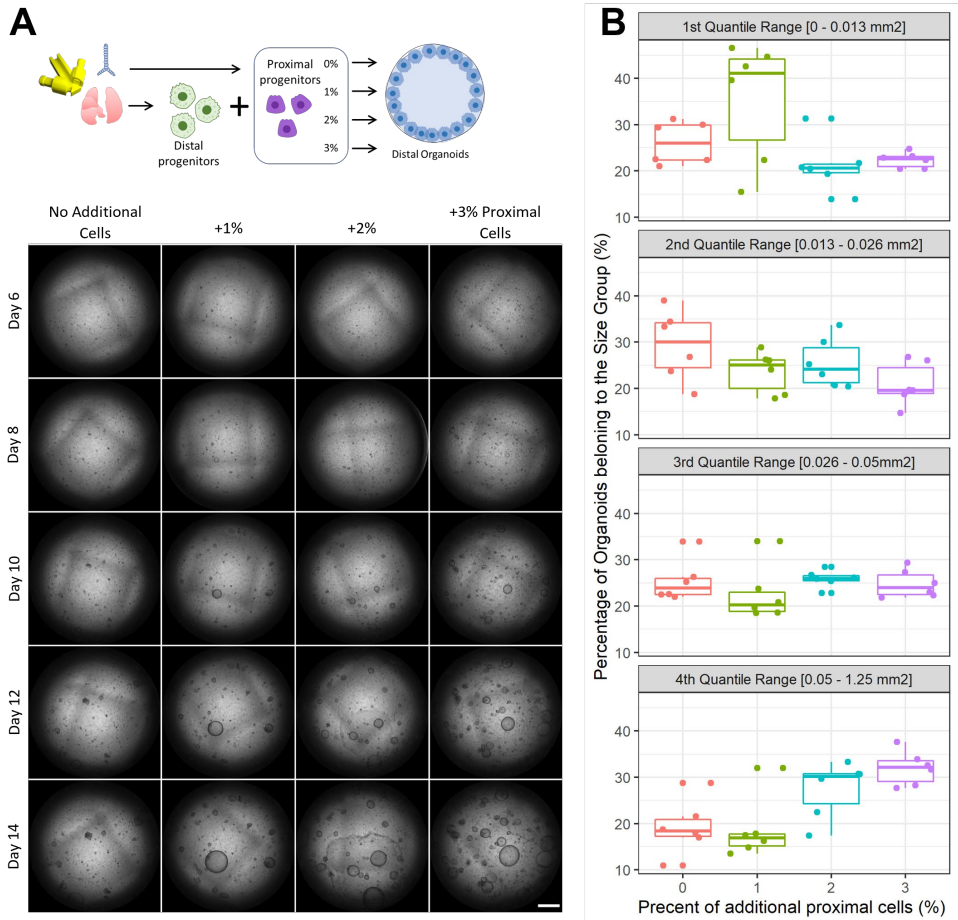


Figure 15. Contamination with proximal progenitors in organoid culture of distal progenitors results in organoids morphologically larger in size.

(A) Schematics and representative brightfield images of the high-content screening of the cultured organoids (n=6). Images are rotated to align organoids using a custom ImageJ plugin described in Paper I (Alsafadi, 2022). (B) Quantification of the organoid size distribution binned by organoid size ranges based on the quartiles of the distribution. Data is shown as percentage of each size group in relation to all organoids in the corresponding well.

The ability to isolate proximal and distal progenitors from an individual mouse can provide new insights of how these cells respond to a disease stimulus. It will offer the possibility to address heterogeneity in disease models that affect the different epithelial compartments of the lung. *e.g.* in chronic obstructive pulmonary disease (COPD), where the airway epithelium is subjected to fibrosing around airways meanwhile the alveolar epithelium is subjected to damage due to emphysema (Hogg and Timens, 2009). The role of the transitional states among cells is not fully understood and is dependent on disease context; the use of progenitors from the

same animal disease model in advanced cultures, such as air-liquid-interface and organoids culture, will aid in exploring the regenerative capacity of these cells (Kobayashi et al., 2020). Moreover, this study highlights the importance of the method of which the cells were obtained for *in vitro* differentiation assays. This is especially important when the readout of the assay is dependent on the size and morphology of the differentiated organoids (Costa et al., 2021).

Proximalization of the distal lung epithelial progenitors in vitro and in disease.

Among the main practical differences between the proximal and distal progenitors is their ability to withstand passaging in culture. The proximal progenitors (basal cells) can be cultured over several passages before they lose their ability to proliferate or differentiate into the mature proximal phenotype. On the other hand, the distal progenitors (ATII cells) are not easily passaged. Although some studies have reported the ability to passage primary ATII cells (Mao et al., 2015), they are known to differentiate into the alveolar type I phenotype when they are cultured on tissue culture plastic (Mutze et al., 2015). On the other hand, we observed that regardless of the isolation method, the organoids derived from distal lung progenitors express several markers of the proximal lung epithelium (Figure 16A). This observation was true for both classic and 3DLD isolations where we observed increases in expression of proximal epithelial markers and notable decreases of distal markers in both cell isolation (Figure 16B). However, in the classic isolation, there were 25 genes that were significantly upregulated in organoids relative to the pellet that were downregulated in the 3DLD isolation. Most of these genes were associated with proximal epithelial phenotypes (Alsafadi et al., 2022) which could be attributed to the proximal progenitor contamination in this isolation (Figure 14B). Moreover, it has recently been shown that the organoid culture of primary human ATII cells were able to transdifferentiate into proximal epithelial cell types including basal cells (Figure 16C) (Kathiriya et al., 2022). This transition occurs through transitional cell states that are very similar to the aberrant basaloid cells and Krt17+/Krt5- cell populations observed in IPF scRNAseq datasets (Adams et al., 2020, Habermann et al., 2020).

Atypical presence of basal-like cells in the alveolar regions is observed in end-stage IPF tissues (Khan et al., 2022b, Khan et al., 2022a). When these cells are isolated from IPF tissues and cultured *in vitro*, their expression profile moves towards an aberrant-basaloid like cells with persistent KRT5 expression or towards a secretory epithelial cell trajectory. This is consistent with previous reports that profiled the different subtypes of basal cells across the respiratory tract in the context of IPF (Carraro et al., 2020). We have also observed the presence of proximal markers and in the distal regions of various IPF patient tissues (**Paper II**, Figure 1A). This observation also coincides with the expression of YT, which we find to be enriched

in the fibrotic lung tissues in both alveolar and mesenchymal regions of the lung. Moreover, we found an association of YT target gene expression with the transitional cell states found in the IPF and organoid scRNAseq datasets (**Paper III**, Figure 1). IPF pathomechanisms are complex and involve a convoluted network of signaling pathways that are also involved in development, tissue homeostasis, and repair. These observations have prompted us to explore the Hippo signaling pathway and YT further to explore their role in IPF and the potential to target them for therapeutic intervention.

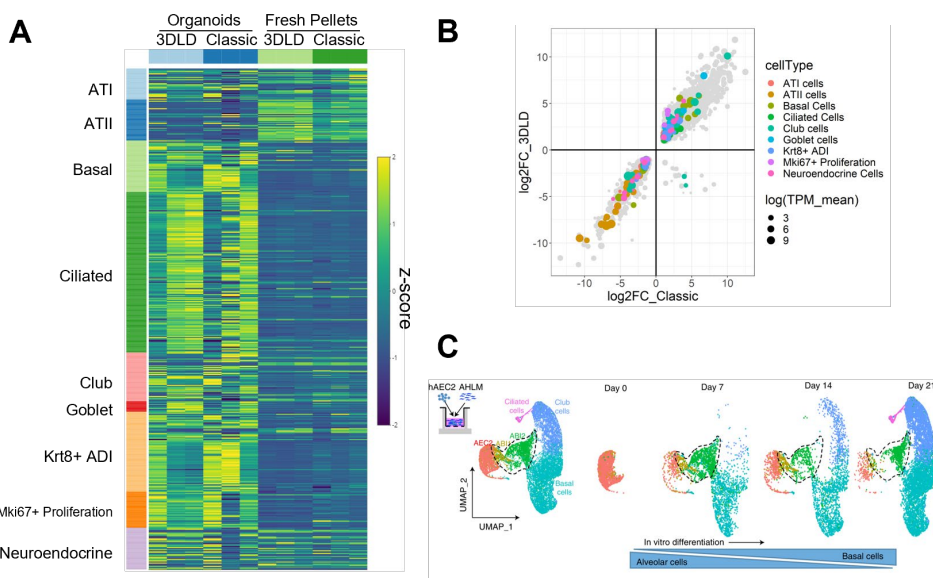


Figure 16. Proximalization of distal progenitors *in vitro*.

(A) Heatmap of gene expression z-score of distal progenitors isolated with 3DLD or classic isolations before and after culture in Matrigel for organoid generation. Genes defining each cell type are obtained from the genes defining cell type clusters in the lung epithelium single cell dataset (GEO: GSE141259). (B) significant log fold changes of gene expression between organoids and cell pellets from each isolation (Classic and 3DLD). Genes are colored based on the cell type they define in the single cell dataset. A,B: **Paper I**. (Alsafadi et al., 2022) (C) Single cell RNAseq of human Alveolar type II cells isolated by selection with HTII-280 surface marker and cultured in organoid assay for 21 days. Re-used with permission from (Kathiriyia et al., 2022).

Hippo signaling pathway components are deranged in IPF

Hippo signaling is mediated through several mechanisms such as changes in the cytoskeleton and cell to cell adherence and interactions. While the ultimate outcome of the canonical Hippo pathway is to control the phosphorylation and subcellular localization of the YT to mediate their activity, the Hippo pathway can be regulated at any step through the kinase cascade. We therefore explored whether the Hippo signaling components as a whole influence the pathogenesis of IPF. Principal component analysis (PCA) of microarray data of 100 IPF patients and 91 healthy controls based on the gene expression of Hippo signaling components and targets revealed cluster separation between IPF and controls indicating a role for the hippo pathway and YT in IPF (**Paper II**, Figure 1d). Importantly, we found that Hippo components were dysregulated in independent cohorts of human tissue samples and the bleomycin mouse model of fibrosis. Interestingly, the dysregulation was not limited to the heavily scarred regions of the lung tissue, but also in the non-scarred regions of the fibrotic subjects. An observation that we were able to validate against a published RNAseq dataset of the fibrotic scarred vs. non-scarred regions (Luzina et al., 2018). This indicates that dysregulation of Hippo pathway components may be an early event in the pathogenesis of IPF.

Targeting YT in the lung could be a therapeutic option for IPF

The enrichment of YT in the fibrotic lung epithelium can be attributed to the stiffness of the extracellular matrix and dysregulation of the hippo signaling pathway (**Paper II**) (Liu et al., 2015). However, it was not previously clear whether this enrichment and nuclear translocation of YT could be targeted for the purposes of ameliorating fibrosis. Therefore, we performed *in vivo* experiments where we targeted YT activity using the FDA approved drug, Verteporfin (VP). This is a photosensitive drug that is used to treat macular degeneration to close leaky vessels in the eye. VP was found to inhibit the interaction between YT and the TEA domain (TEAD) transcription factors (Brodowska et al., 2014). We found VP to improve the survival of mice subjected to the bleomycin fibrotic injury and to partially resolve the fibrotic phenotype as evident by histological tissue recovery and decreased collagen amounts that were induced by the fibrotic injury (**Paper II**, Figure 2). This amelioration of the fibrotic injury was observed at a protein level and not a transcriptomic level which indicated that the effects that are modulated by YT may not directly induce expression of fibrotic markers, but rather influence their production and modulation. This is consistent with previous reports of Yap's involvement in matrix remodeling and stiffness enhancement in its tumorigenic behavior (Calvo et al., 2013). Targeting YT has similarly been shown to be beneficial for treating renal fibrosis (Szeto et al., 2016).

YT in the fibrotic lung epithelium contribute directly to the remodeling of the extracellular matrix

The active role of the lung epithelium in the pathogenesis of IPF has only recently gained traction as one of the mechanisms driving disease (Selman and Pardo, 2020). To explore the profibrotic activity of epithelial YT that could lead to matrix remodeling, we developed an assay to evaluate the ability of biological fluids to remodel matrix *in vitro* (Figure 17).

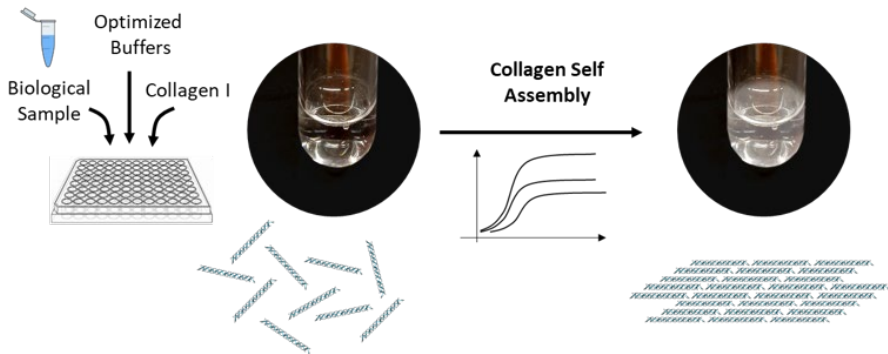


Figure 17. *In vitro* assay for evaluation of the matrix remodeling capacity of biological fluids.

The assay takes advantage of the ability of collagen I to self-assemble into an intact matrix *in vitro*. We optimized the assay condition to allow for quantifying the effects of biological fluids on the self-assembly kinetics of collagen I (**Paper II**).

We found that the secretome in the mouse bronchioalveolar lavage fluid (BALF) of fibrotic mice, which is predominantly made up of secreted factors from the lung epithelium, increased matrix remodeling and crosslinking of Collagen I (**Paper II**, Figure 3H). Moreover, secreted fluids from isolated primary mATII cells from fibrotic mice showed increased crosslinking of matrix *in vitro* compared to secretome from normal ATII cells. Using mass spectrometry (MS) proteomics, we found several profibrotic factors in the fibrotic mATII cell secretome including several matrix remodelers such lysyl oxidase (Lox), which is a well-known cross linker of collagens. We found *Lox* to be elevated in the fibrotic mouse model and human BALF; treatment of fibrotic mice with VP decreased *Lox* expression. The collagen self-assembly assay showed that this expression is also accompanied by crosslinking activity from this secretome as the addition of β -aminopropionitrile (BAPN), a small molecule Lox inhibitor, was able to reduce the crosslinking observed from these biofluids (**Paper II**). Moreover, Lox protein gene expression was elevated in primary fibrotic mATII cells, and the silencing of YT was able to abolish this increased expression indicating that YT modulate the expression of *Lox* in ATII cells. The role of Lox enzymes in IPF is well established and is known to promote the fibrotic phenotype (Chien et al., 2014, Tjin et al., 2017, Cheng et al., 2014). However, a clinical trial targeting lox like 2 proteins (LOXL2) in IPF patients

using monoclonal antibodies (Simtuzumab) was terminated due to the lack of efficiency. This does not necessarily mean that there is no effect of the Lox family proteins in matrix remodeling but indicates that several factors are at play in the pathogenesis of IPF. YT are responsible for a wide range of processes and the modulation of profibrotic factors in the lung epithelium (**Paper II**). However, the exact mechanisms of YT downstream activity are not well studied in the context of the lung epithelium. Understanding these mechanisms may lead to finding a better therapeutic or combination of therapies.

Context dependence of YT activity in the lung epithelium

YT do not directly bind to the DNA and exert their transcriptional effects through binding to other TFs. The exact TFs that interact with YT in the lung epithelium are not known. Moreover, whether these interactions differ across the different compartments of the lung epithelium are also unknown. To address this, we sought to identify the exact locations on the genome where YT containing complexes reside. Identification of transcription factors or chromatin remodelers has been traditionally analyzed using Chip sequencing whose quality is dependent on the size of the sample and the quality of the antibodies (Nakato and Sakata, 2021, Park, 2009). The number of cells isolated from an individual mouse are not sufficient for a high-quality ChIP seq experiment. Therefore we used CUT&RUN to address this limitation (Skene et al., 2018). The main advantage of CUT&RUN is its ability to obtain high quality peaks from a small sample size. As YT do not directly bind to DNA, the antibody bound nuclease could end up far from the DNA and may not be able to cleave it.

We optimized the conditions for CUT&RUN and performed it with YT antibodies on freshly isolated proximal and distal lung epithelial cells (Figure 18). We first validated that CUT&RUN can be performed on both proximal and distal lung epithelial cells by evaluating the positive control using the trimethylated histone 3 on lysine 4 (H3K4Me3), which is a chromatin tag that is associated with open chromatin on the genome and active transcription. While the association of H3K4Me3 with active transcription is not fully understood, it has been shown to control transcriptional consistency (Howe et al., 2017). We reproducibly found H3K4Me3 coverage over genomic regions associated with known cell markers of ATII cells in the isolated distal cells such as *Sftpc*, and proximal markers such as *Krt5*, and *Trp63* in the isolated proximal cells (**Paper III**, Figure 2B). These results confirm the ability of CUT&RUN to detect coverage over genomic areas using the appropriate antibodies. Further, we confirmed the possibility to detect coverage using the YT antibody which is evident by the coverage over well-known YT targets *Ctgf*, and *Cyr61* which also coincided with coverage by the H3K4Me3 which indicates active transcription at these sites (**Paper III**, Figure 2D). These

observations were also confirmed with primary human ATII cells and HBECs from normal and fibrotic tissues (**Paper III**, Figure 4).

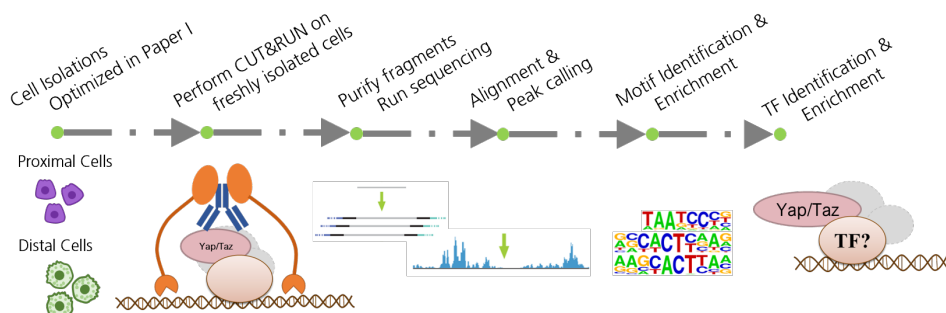


Figure 18. Schematics for CUT&RUN experiments and TF identification in Paper III.

The mouse proximal and distal cells were isolated as described in **Paper I**. CUT&RUN was performed on freshly isolated cells followed by purification of collected fragments and sequencing. Sequenced data is filtered, and TF motif binding sites are identified in the dataset and enrichment analysis of these motif sites aid in predicting which transcription factors were most likely bound to these motifs. Detailed methods are found in **Paper III**.

Interestingly, 80% (60% for human) of the genes that are covered by a YT containing complex were protein-coding genes and more than 50% of all the genomic locations occupied by YT containing complexes were intergenic compared to 5-10% promoter-TSS locations (**Paper III**, Figure 2, Figure 4). This indicates that YT are mainly involved in the regulation of genes rather than the induction of gene expression at the promoter regions.

We confirm the ability for CUT&RUN to successfully detect YT targeted sequences in primary cells. To date, only two studies have reported successful CUT&RUN experiments with Yap, both of which were done in cell lines, a pharyngeal squamous cell carcinoma cell line and an O9-1 neural crest cell line (Zhao et al., 2021, Chang et al., 2021).

Binding partners of YT: which are involved in fibrosis

The context dependence of YT can be driven by the transcription factors they may bind with, the availability of such factors based on cell type or tissue compartment, and the location of which the YT containing complex may bind. The CUT&RUN datasets offered us the unique opportunity to extract the exact motif sequences which complexes containing YT are bound to. In the murine dataset, we found that YT coverage in proximal and distal lung epithelial cells only overlaps approximately 30% percent of the targets (**Paper III**, Figure 3). Interestingly, the most enriched TF motif in the overlapped targets is the TEAD1 TF which was found

in 60% of the total sequences searched. TEAD TFs are the most studied mediators of YT activity in the nucleus (Li et al., 2010). Other TFs in the overlap were associated with proliferation, differentiation, or chromatin modulation. Moreover, TFs identified in the MTECs included Kruppel like factor 5 (Klf5) which has been shown to contribute to the developmental stages of the trachea during embryogenesis (Ohnishi et al., 2000). The YT coverage on mATII cells was on motifs of TFs associated with epithelial differentiation, stemness, and development. This data indicates that YT at baseline are involved in homeostatic processes and help maintain the proximal and distal lung epithelial progenitors.

The coverage of H3K4Me3 on the *SFTPC* gene for normal human ATII cells was quite evident confirming the success of the experiment. However, this coverage was somewhat lost for the IPF derived human ATII cells which is consistent with the alveolar damage known to occur in IPF. The same trend was evident for another ATII marker *ABCA3*, but not *LAMP2* whose coverage was present in both normal and fibrotic ATII cells (**Paper III**, Figure 4C). Moreover, the fibrotic ATII cells had H3K4Me3 coverage on markers of proximal basal cells such as *KRT5* and *TP63*. This could be explained by the concept of bronchiolization observed in IPF or by a potential transition due to proximalization of the alveolar cells (Kathiriya et al., 2022). These are important observations that will be exciting to follow-up on in future studies.

The YT coverage on the human cells had vastly different targets on the genome with a low overlap (**Paper III**, Figure 5A). This indicates that the context of the disease state and the cell type determine which regions on the genome YT complexes reside. Moreover, this could also be explained by patient heterogeneity which could lead to some differences. We performed Homer analysis to predict which TFs are represented by the motifs available in each dataset. We found that the types of TFs interacting with YT in each condition can differ greatly based on disease condition and cell type (Figure 19). The TEAD TFs seem to be most enriched in the normal ATII cells which indicates that they are involved in the homeostatic state of the alveolar epithelium.

The homeostatic YT activity in the adult cells is involved in specification of cell fate to maintain tissue homeostasis (Heng et al., 2020, Cai et al., 2021). Interestingly, runt-related transcription factor 1 (Runx1) was predicted to be interacting with YT in mouse lung epithelial cells in both proximal and distal compartments (**Paper III**, Figure 3). Inhibition of RUNX1 has been shown to be beneficial in the animal models of pulmonary fibrosis and has also been found to be elevated in the clinical samples of IPF (Dubey et al., 2022, O'Hare et al., 2021). We also find RUNX to be enriched in the fibrotic human HBEC cells which could be explained by a role for this TF in the involvement of proximal cells in pulmonary fibrosis. STAT and Smad TF families are found to interact with YT in the fibrotic alveolar epithelium in the current study. TFs belonging to these groups have been indicated in the pathogenesis of IPF, however, the current study provides first

evidence for potential mediation of these TFs with YT in the lung epithelium (Montero et al., 2021, Piersma et al., 2015). The interaction of YT with Smads has been previously reported, but the exact mechanisms of how YT interact with Smads are yet to be determined. YT interact with Foxh1 in the fibrotic ATIs, and it also interacts with Smad2 and Smad3 in the current study. Previous reports have shown that Smads interact with Foxh1 (Yoon et al., 2011). Therefore, it is possible that YT mediate this interaction between these factors.

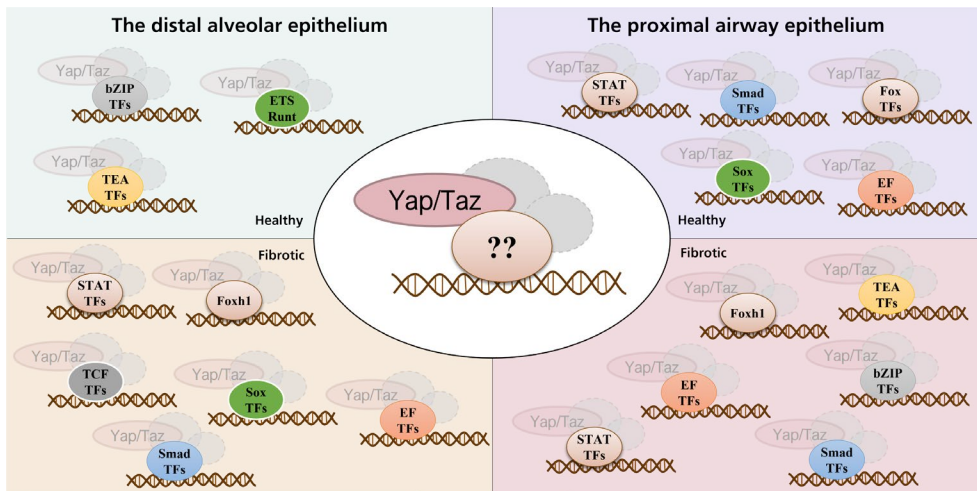


Figure 19. Overview of predicted transcription factors families interacting with YT in normal and fibrotic human lung epithelial cells.

Overview of the motif analysis results from **Paper III**. These are the main transcription factor families that were detected in the CUT&RUN datasets for each of the patient samples. Each of these TF families may have one or more TFs that were detected in the datasets.

Several TFs that were identified in the fibrotic alveolar epithelium were also present in the normal or fibrotic HBEC cells as well. Moreover, we also observed that YT have an expression signature in the transitional state observed in the single cell dataset that showed a transition of the alveolar cells to the proximal epithelial cell types (Figure 16C, **Paper III** Figure 1). These findings may indicate that YT contributes to the process of proximalization of the alveolar epithelium in IPF through these factors. In **Paper III**, we show that the TF motifs associated with TEADs are present in the normal alveolar epithelium and fibrotic proximal bronchial epithelium and the occurrence of these TF motifs is reduced in the fibrotic distal alveolar epithelium. Several studies show that the TEAD TFs are the main factors of which YT interact with in the nucleus. A previous report showed an overlap of 90% between Yap and TEAD1 targets on the genome (Chang et al., 2021). However, these studies were done mainly in the oncogenic context of YT. The current study shows that there are other TFs that can interact with YT, but it does not rule out the presence of TEAD TFs at the same time.

Concluding remarks and outlook

The difficulty in finding therapies and treatments of chronic lung diseases can be attributed, in part, to the complexity of the lung and the mechanisms that drive these diseases. The lung as an organ isn't considered a highly regenerative organ. However, it contains several distinct progenitor cell populations that have crucial roles in response to various types of injury. However, IPF is a disease of complex pathophysiology with unknown etiology. It is hypothesized that the continuous repetitive injury and impaired repair of the distal lung epithelium are the main drivers of disease progression. To date, mechanistic studies of IPF disease reveal a convoluted network of molecular mechanisms and characteristics that drive fibrotic injury making identification of therapeutic targets a very difficult task. Among the factors aspects are the excessive extracellular matrix deposition, the injured distal lung epithelium, deranged repair signaling, aberrant cell behavior, emergence of transcriptionally hyperactive cell states during disease, and the atypical reactivation of developmental signaling pathways. The current therapies for IPF, do not target any molecule or pathway in specific, but are common inhibitors that effect a wide range of processes which result in slowing down disease progression but not halting it. Several clinical trials that target specific molecules are being conducted, many of which have already failed due to lack of efficacy or adverse effects which may be due to the broad approaches taken thus far.

YT have important roles in the early lung development and are crucial for the proper growth of the lung epithelium. We and others have found active YT in the fibrotic lung epithelium in the adult lung in various compartments. Moreover, YT are mediated, in part, by Hippo signaling, which is a mechanosensitive signaling pathway that is capable of sensing stiffness. We focused on the lung epithelium in the current research as it is the main site of injury, and the role of the alveolar epithelium is not yet fully understood in the progression of lung fibrosis. The stiff matrix, reactivation of developmental pathways, and injury of the lung epithelium led us to the main hypothesis of the current research that YT are directly involved in IPF progression and pathogenesis. However, owing to their pro-regenerative and developmental roles, we also hypothesized that not all of YT activity is profibrotic. Therefore, to consider YT as viable targets for treating lung fibrosis we sought to understand the context of their actions and how they mediate their pro-fibrotic effects.

In the current research, we developed and optimized the tools to study and understand the role of YT in the fibrotic lung epithelium. First, we developed a new method to simultaneously isolate proximal and distal lung epithelial progenitors from an individual mouse lung using a surgical guide that precisely and reproducibly allow the dissection of the trachea and main bronchi from the distal lung lobes (**Paper I**). In this study, we found that the choice of isolation method of the lung epithelial progenitor cells effects the outcomes of in vitro assays that utilize

these cells. This study has important implications for the organoid culture models and the way they are used for studying the effect of chemical compounds or other molecules. Moreover, this methodology allows for exploration of effects of disease models on both proximal and distal epithelial lung progenitors simultaneously on the same lung. This model may also provide new insights into the localization and function of the newly identified transitional cell states in disease. However, this needs to be explored for each of the disease models. Second, we provide evidence for the derangement of the Hippo signaling pathway components in the lung epithelium of IPF patient tissues and the mouse model of fibrosis that leads to the activation and nuclear translocation of YT in the distal lung epithelial cells (**Paper II**). We find that YT are responsible for mediation and secretion of extracellular matrix remodeling molecules from the injured fibrotic lung epithelium. YT modulate the expression of lysyl oxidase (Lox) that contributes to the cross-linking of collagens in the extracellular matrix. Third, we optimized the conditions to study the role of YT in the nuclei of the lung epithelium by exploring their interacting partners (**Paper III**). We, for the first time, optimized the conditions to perform state-of-the art techniques such as CUT&RUN to identify exact genomic motif sequences that are bound to complexes containing YT in primary cells. In this study, we found YT to interact with TFs that are known to take part in homeostatic and maintenance activities, but we also identify TFs that are involved in modulation of the profibrotic phenotype, some of these TFs were known to be involved in fibrosis but were not known to interact with YT in the same context.

The findings in this thesis need to be further validated to draw solid conclusions about the exact TFs leading the profibrotic YT activity. While the databases used to predict TFs based on enrichment of the motif sequences have been continuously updated to include as many of the known TF factors, this remains to be a major limitation of this study. This limitation is mainly attributed to the fact that these databases are based on previous studies that confirm each of the interactions individually. Thus, it is crucial to validate the predicted TFs from the CUT&RUN datasets using other methods of different readouts. One approach is to do pulldown mass spectrometry (MS) proteomics. This approach has the potential to validate the findings in **Paper III**, however it requires extensive optimization to perform the pull-down protocols while maintaining compatibility with MS without losing the interactions between YT and other molecules in the nucleus.

Furthermore, the expression and roles of the identified TFs need to be validated within the normal and fibrotic lung tissues. First, their baseline expression at a gene and protein level as well as their co-expression with YT need to be validated within tissue sections. Once the expression of a TF is confirmed, a validation of their role needs to be validated. Several types of experiments need to be put in place to examine the various roles: an example pro-fibrotic roles vs pro-regenerative. This can be done by using a screening approach utilizing the ability of lung epithelial cells to generate organoids and combining this with a CRISPR screen to determine

whether the organoids' response to a fibrotic injury, such as the fibrosis cocktail, would be altered by the removal of any of these TFs. A similar approach has been used recently to screen for modulators of TGF- β resistance in gut organoids (Ringel et al., 2020). Furthermore, the therapeutic potential of targeting the identified TFs can be further validated in the fibrosis cocktail PCLS model we previously developed using human lung resections (Alsafadi et al., 2017). This validation can also be carried out in the organoid model of iPSC-derived ATII cells subjected to the FC injury (Ptasinski et al., 2022). This model was able to recapitulate the transitory cell types observed in IPF and thus is suitable for such screen.

This PhD thesis develops the tools and sets the basis for distinguishing between the pro-regenerative and profibrotic activity of YT. To treat fibrosis, the fibrotic phenotype needs to be targeted, but also the recovery from injury needs to be enhanced. There needs to be a balance between a single target therapeutics and common inhibitors that have no specific targets. The treatment for IPF could potentially work by simultaneously using multiple targeted therapeutics that combat the fibrotic injury and enhance repair. This idea of combing therapies has shown some effects when using both Nintedanib and Pirfenidone simultaneously, however, since both are not target-specific, their combination may not be enough to treat the disease.

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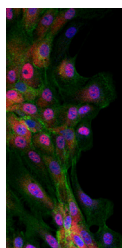
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Role of the co-transcriptional regulators Yap/Taz in the normal and fibrotic lung epithelia

This work represents the culmination of several years of research and represents a significant contribution to the development of experimental tools, analysis methods, and generation of datasets to further our understanding of the mechanisms underlying fibrosis in the lung.

