The four-and-a-half-LIM-domain Protein FHL2 is a novel regulator of pulmonary fibrosis

Inauguraldissertation
zur Erlangung des Grades eines Doktors der Humanmedizin
des Fachbereichs Medizin
der Justus-Liebig-Universität Gießen

vorgelegt von Banthien, Nils
aus Hamburg, Deutschland

Gießen, 2018

The four-and-a-half-LIM-domain Protein FHL2 is a novel regulator of pulmonary fibrosis

Inauguraldissertation
zur Erlangung des Grades eines Doktors der Humanmedizin
des Fachbereichs Medizin
der Justus-Liebig-Universität Gießen

vorgelegt von Banthien, Nils
aus Hamburg, Deutschland

Gießen, 2018

Aus der Medizinischen Klinik und Poliklinik II (Innere Medizin),
unter der Leitung von Prof. Dr. Werner Seeger,
des Fachbereichs Medizin der Justus-Liebig-Universität Gießen

1. Gutachter: Prof. Dr. Werner Seeger
2. Gutachter: Prof. Dr. Christos Samakovlis

Tag der Disputation: 29.10.2019

Table of Contents

1 Introduction

1.1 Idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF), formerly known as cryptogenic fibrosing alveolitis (CPA), is a rare, grave lung disease of unknown etiology. The condition is characterized by alveolar epithelial cell damage, subsequent excessive accumulation of extracellular matrix, disruption of the alveolar epithelium and enhanced activation, proliferation and migration of fibroblasts/myofibroblasts, resulting in the destruction of the normal architecture of the lungs. IPF patients suffer from irrevocable and progressive loss of respiratory function which usually results in death (Oliver Eickelberg & Laurent, 2010; Moisés Selman, Pardo, & Kaminski, 2008). The median survival time of IPF patients is approximately 3 years, which is shorter than that of many cancer patients (Meltzer & Noble, 2008). Respiratory failure is the most common cause of death (Panos, Mortenson, Niccoli, & King, 1990). Until recently, lung transplantation was the only viable option to improve survival in affected patients. In 2014, studies on Pirfenidone, a versatile substance that integrates antifibrotic and anti-inflammative properties, and Nintedanib, an intracellular tyrosine kinase inhibitor, identified the two substances as decelerators of disease progression in IPF, which resulted in FDA approvals for both of them (King u. a., 2014; Meltzer & Noble, 2008; Richeldi u. a., 2014). Research on IPF-pathophysiology has been making headway, but the disease still presents itself as a complex medical problem. The first section of this thesis provides an outline of the contemporary understanding that we have of IPF and relates this to TGF-β-signaling, WNT-signaling and FHL2, the molecule of interest.

1.1.1 Classification

IPF belongs to the heterogeneous group of diffuse parenchymal lung diseases (DPLD). This group comprises more than 150 entities that share certain clinical, radiological, physiological, and pathological features but differ in their respective pathophysiology. DPLDs can be categorized into conditions with a known etiology, such as hypersensitivity reactions or systemic conditions like sarcoidosis and into conditions whose etiology remains unclear, like eosinophilic granuloma and the idiopathic interstitial pneumonias (IIPs) (Green, 2002). IPF belongs to the IIPs and is considered the most aggressive form in the DPLD collective (Oliver Eickelberg & Selman, 2010).

IIPs are characterized by varying degrees of fibrosis and inflammation of the lung's interstitium. They are divided into seven distinct groups: IPF, nonspecific interstitial pneumonia (NSIP), respiratory bronchiolitis interstitial lung disease (RBILD), desquamative interstitial pneumonia (DIP), acute interstitial pneumonia (AIP), cryptogenic organizing pneumonia (COP) and lymphoid interstitial pneumonia (LIP). This classification is based on histologic appearances and has evolved considerably since 1944, when Hamman and Rich where the first to describe an IIP they termed Hamman-Rich syndrome. In four patients of theirs, they observed a rapid, progressive deterioration of lung function with a fatal outcome. Autopsies revealed interstitial fibrosis and advanced honeycomb changes in the lungs of these patients. This clinical pattern would today be described as AIP. In 1969, Liebow came up with the first distinguished histological classification scheme for cases of idiopathic diffuse interstitial pneumonias. Since then, the classification of IIPs has evolved considerably. The American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias of 2001 provided the first multinational integrated clinical, radiological, and pathological approach to the classification of the IIPs. An absolute novelty at the time, this guideline provided a standardized nomenclature and diagnostic criteria which created a sophisticated framework for the

following studies (“American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias. This joint statement of the American Thoracic Society (ATS), and the European Respiratory Society (ERS) was adopted by the ATS board of directors, June 2001 and by the ERS Executive Committee, June 2001,” 2002). In order to provide clinicians with evidence based treatment recommendations and to keep the nomenclature up to speed with the scientific advances, the guideline was updated in 2011 and in 2015 (Ganesh Raghu u. a., 2011, 2015).

1.1.2 Histopathology

IPF is histologically represented by the pathologic pattern of usual interstitial pneumonia (UIP) in which the lung's parenchyma displays striking temporal and spatial heterogeneity. At a low magnification, IPF lungs have a patchy appearance with alternating areas of normal tissue, inflamed tissue, fibrosis and honeycomb cysts (cystically dilated bronchioli) (Leslie, 2005). The pathological patterns are most prominent in the lungs’ lower lobes, where they concentrate in paraseptal and subpleural areas (Harari & Caminati, 2005). The fibrotic areas are made up of dense acellular collagen, abundant smooth muscle proliferation and ECM embedded aggregates of actively proliferating and collagen-producing myofibroblasts, which are termed fibroblast foci. These fibroblast foci represent the hallmark lesions of IPF where excessive deposition of extracellular matrix gradually replaces the functioning alveolus with dense fibrosis. The foci are embedded in an ECM that primarily consists of fibronectin and collagen and are localized in subepithelial layers adjacent to areas of preserved lung. This interphase of healthy and scarred lung tissue is believed to be the site of fibrosis progression and termed the “leading edge” of fibrosis (Meltzer & Noble, 2008). Little inflammation is seen in UIP. When present, it is most likely to be encountered at sites of end-stage fibrosis. Lymphocytes and plasma cells are the typical

inflammatory agents found at these locations (Meltzer & Noble, 2008). One regular feature of IPF lungs is a substantial loss of type I alveolar epithelial cells, which leaves their underlying basement membranes exposed. Another consistent finding is the hyperplasia of type II alveolar epithelial cells (Strieter, 2005).

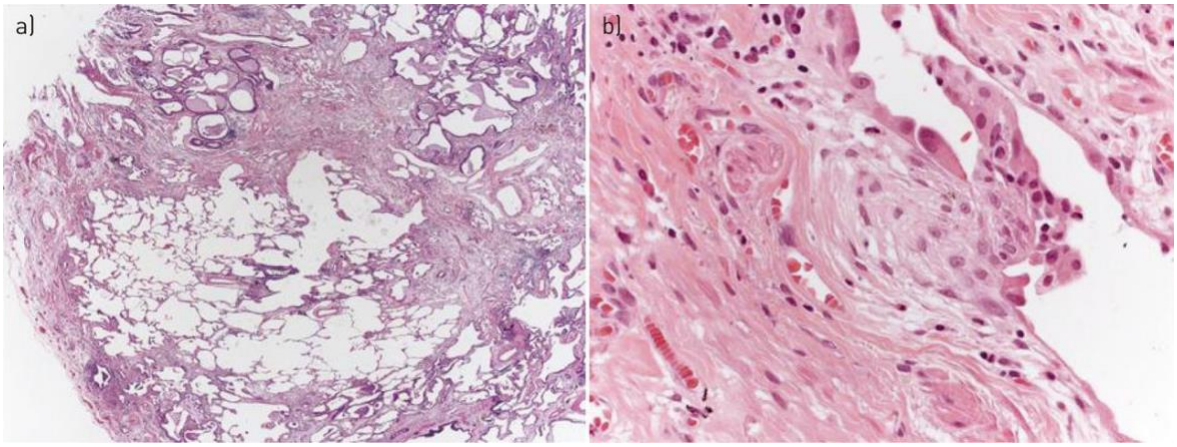


Figure 1.1: The histological pattern in IPF
A. At low magnification, the heterogeneous look of “patchy fibrosis” presents itself with alternating areas of normal tissue, inflamed tissue, fibrosis and honeycomb cysts. B. Higher magnification of a fibroblast focus, the hallmark lesions of IPF, with adjacent hyperplastic type II alveolar epithelial cells. (Wuyts u. a., 2014, Reproduced with permission of the © ERS 2018. European Respiratory Review Sep 2014, 23 (133) 308-319; DOI: 10.1183/09059180.00004914).

IPF is the only IIP to display the histological pattern of UIP with its temporal heterogeneity. The often encountered synonymous usage of the terms IPF and UIP is misleading because UIP patterns are not limited to IPF, but can occur in other conditions such as connective tissue disease, hypersensitivity pneumonitis, asbestosis, the rare Hermansky-Pudlak syndrome and drug toxicities (Meltzer & Noble, 2008; G. Raghu, Nicholson, & Lynch, 2008).

1.1.3 Pathogenesis

The pathogenesis of IPF is insufficiently understood, as the term “idiopathic” implies. However, research on the matter advances at a remarkable pace. This chapter is an introduction to the current state of knowledge about established and putative disease mechanisms.

1.1.3.1 Paradigms of IPF pathology

IPF is widely believed to represent an aberrant repair process that initiates as a response to an injury of unknown origin. The very heterogeneous appearance of the UIP pattern in lung sections prepared for microscopy, where normal lung tissue is found next to sites of end stage fibrosis and fibroblast foci, led to the popular assumption that the pathogenesis of IPF must be fueled by multiple, temporally dispersed, localized injuries. This view of a temporally dispersed disease process was, however, challenged in 2006 when Cool et al. proposed that the fibroblast foci seen on individual tissue sections do not constitute multiple isolated lesions but instead represent sections of a continuous, interconnected fibrotic reticulum that spans from the lung parenchyma to the pleura (Cool u. a., 2006). For years, a large proportion of the research community interpreted IPF as a remodeling process of the lung that stems from deregulated wound repair in response to an unresolved chronic local inflammation caused by unknown stimuli (Strieter, 2005). This model is generally referred to as the inflammatory/alveolitis hypothesis. This hypothesis’ proposition is that alveolar and interstitial inflammation prompts a reactive persistent antigenic response which in turn triggers and upholds the fibrotic process (El-Chemaly, Pacheco-Rodriguez, Ikeda, Malide, & Moss, 2009). According to this model, inflammation of the alveolar-capillary constituents and basement membrane causes the loss of type I epithelial and endothelial cells and the proliferation of type II pneumocytes. Subsequently, the integrity of the alveolar space is lost and recruitment and proliferation of stromal cells

ensues resulting in the deposition of ECM (Strieter, 2005). This focus on inflammation suffers from two main issues. First of all, the lungs of IPF patients show no significant inflammatory cellular infiltrate (see chapter 1.1.2). As a matter of fact, the histopathological pattern of UIP does not even require the presence of an inflammatory infiltrate (Bringardner, Baran, Eubank, & Marsh, 2008). Secondly, all distinctly anti-inflammatory treatment approaches failed to improve mortality or even palliate the disease's clinical course (Gomer & Lupher, 2010). Advocates of the inflammatory model retort that the failure of anti-inflammatory treatment at later stages of the disease does not rule out contribution of inflammatory mechanisms to early stage pathogenesis and that inflammation in general is the initial physiological response to any injury, hitherto maintaining that inflammation might still play a key role in the early stages of IPF. Furthermore, there are reports of IIP patients who show a histopathology where UIP resides next to NSIP, which is characterized by fibrosis and varying degrees of chronic inflammation (Flaherty u. a., 2001). This observation has prompted promoters of the inflammatory model to suggest the possibility that UIP in IPF is not a discrete disease entity, but rather the endpoint of a common IIP-spectrum where disease progression begins with inflammation-driven NSIP, which later evolves to the fibrotic UIP phenotype with only residual inflammation present (Strieter, 2002). However, this view had been controversial ever since its first mention by supporters like Strieter in 2002, and over time the failure of the inflammatory approach to yield the desired progress in the clinical field shifted the focus of the community's attention towards a different paradigm, where fibroblast dysfunction plays the dominant role in IPF-pathogenesis.

The contemporary "epithelial/fibroblastic" model relies on an ever growing body of evidence that elucidates how the proprietary components of the lung's tissue can interact without any contribution of inflammation and initiate and maintain fibrotic processes *in vitro* as *in vivo* (Oliver Eickelberg & Laurent, 2010; Strieter, 2002). Today, it is widely accepted that the contribution of inflammation to the development of IPF has been highly overrated in the past and that impaired epithelial-mesenchymal crosstalk accompanied by deregulated

proliferation of fibroblasts and alveolar epithelial cell dysfunction is the driving force behind the onset and progression of fibrosis. The observation that fibroblast foci reside in subepithelial layers in direct proximity to the sites of alveolar epithelial cell injury and repair has led to the broadly accepted assumption that defective epithelial-mesenchymal crosstalk constitutes a key mechanism in the pathogenesis of IPF. Accordingly, multiple cycles of alveolar epithelial type II (ATII) cell injury and repair via persistent or recurrent injury, in the presence or absence of local inflammation, might prompt pathologic growth factor activation and maintenance of the fibrotic response in the neighboring interstitium via paracrine communication (Oliver Eickelberg & Selman, 2010; Moisés Selman & Pardo, 2006).

1.1.3.2 Key cells in IPF: Fibroblasts

A vast array of cell types is known to be involved in the molecular mechanisms that orchestrate IPF. But the emergence of the “epithelial/fibroblastic” model of IPF has placed fibroblasts, myofibroblasts and alveolar epithelial cells (AECs) in the focus of the research communities’ efforts.

Fibroblasts stem from the mesenchyme. They are the most common and most versatile component of the connective tissue cell family in respect to their function as well as their phenotype. Their main physiological function is to synthesize the extracellular matrix and collagen that maintains the structural integrity of the connective tissues that serve as the stroma of animal tissues. Additionally, they can directly influence the proliferation, migration and apoptosis of other cell types via the secretion of growth factors, cytokines, the expression of integrins or the release of oxidants (Thannickal, Aldweib, Rajan, & Fanburg, 1998). Wound healing of the lung usually occurs through the proliferation and differentiation of type II alveolar epithelial cells, which in turn is regulated by fibroblast signaling (Uhal u. a., 1998). These attributes showcase the crucial role of fibroblasts in physiological wound healing where they produce a temporary tissue scaffold for repair processes. The timely

dissolution of this scaffold in conjunction with the controlled apoptosis of the involved fibroblasts is essential for proper tissue restoration and completion of the repair process (White, Lazar, & Thannickal, 2003). Remains of such scaffold tissue residing in the site of finished wound healing equal scarring. Fibroblasts show a great ability to adapt their phenotype to different requirements. In the healthy adult lung, they feature a phenotype that shows very limited synthetic activity. In pulmonary fibrosis however, the activated myofibroblasts encountered in the fibroblast foci are believed to synthesize and deposit the bulk of the interstitial excess collagen found in IPF lungs, making them the “leading edge” or “hot spots” of fibrosis (Visscher & Myers, 2006). Myofibroblasts are cells that combine features of fibroblasts and smooth muscle cells. Ultrastructurally, they can be distinguished from regular, quiescent fibroblasts by the presence of prominent microfilament bundles in their cytoplasm and the presence of fibronexus junctions. They characteristically express α-smooth muscle actin (α-SMA) and show features that intermediate between fibroblasts and smooth muscle cells. A key role of fibroblasts and myofibroblasts in IPF seems plausible, as these cell types are the preeminent cells in all types of fibrosis.

1.1.3.3 Key cells in IPF: AECs

AECs are the other cell types that hold a key position in the pathogenesis of IPF. This group of cells is subdivided into two types: Type I AECs, that represent about 40% of the AEC population but line 90% of the alveolar surface and type II AECs, which constitute 60% of AECs and cover merely 10% of the alveolar surface. The squamous type I AECs maintain the structural integrity of the alveolus and constitute part of the blood-air-barrier, which makes them responsible for gas exchange. Type II AECs are usually larger than their type I counterparts and solitarily reside in the alveolar-septal junction. Their main function is to produce and secrete the pulmonary surfactant, the protein-phospholipid-film that coats the alveolar surface in a monomolecular layer. This film greatly reduces the surface tension

at the air-fluid interface in order to keep the alveolus from collapsing at expiration. They are also responsible for the maintenance and the repair of the alveolar epithelium. Enabled to undergo mitotic activity, they can replicate and differentiate into type I AECs after tissue injury (Arnold, Beier, & Herrmann, 1999; Kierszenbaum, 2002).

Over the last decade, a great body of evidence has been produced that implicates aberrant AEC functioning as a key event in the pathogenesis of IPF. Biopsies of IPF lungs regularly present defects in the alveolar epithelium such as the consistent presence of hyperplastic type II AECs in direct proximity to fibroblast foci (Leslie, 2005; Pardo & Selman, 2002). Type II AECs are known to produce essential profibrotic mediators including TGF-β, the very central profibrotic cytokine (Pan u. a., 2001). AEC apoptosis is deemed to be a key profibrotic event in IPF and is thought to prompt the switch of proximate fibroblasts into the myofibroblast phenotype (Henderson u. a., 2010). Furthermore, genetic mutations in surfactant protein C (SFPC) could be linked to cases of familial IPF (also: familial pulmonary fibrosis/FPF). The resulting irregular processing of surfactant protein was identified a causative event in the development of this IPF-variant[1] (Nogee u. a., 2001; Pan u. a., 2001; Thomas u. a., 2002). Several of these *SFTPC* mutations could be directly linked to the induction of endoplasmic reticulum stress, which is able to induce AEC apoptosis (Lawson u. a., 2008; Wang u. a., 2009).

1.1.3.4 Current issues

The last two decades have overseen impressive advances in the field of IPF research that substantially deepened our insight into the nature of the disease. However, numerous important questions remain to be answered and some of these unresolved issues

[1] Familial IPF accounts for approximately two percent of total IPF cases and features an autosomal dominant inheritance pattern with incomplete penetrance. It is clinically and histologically impossible to differentiate from sporadic IPF, but starts at an earlier age and seemingly features a distinct pattern of gene transcription (Marshall, Puddicombe, Cookson, & Laurent, 2000; Meltzer & Noble, 2008; Yang u. a., 2007).

address the presumed core features of the pathogenetic process.

One issue concerns the nature of the cycle of injury and repair that is believed to initiate lung fibrosis. Different hypotheses suggest exogenous as well as endogenous stimuli. Concerning putative exogenous stimuli, the toxic exposure to a variety of non-infectious agents is a known risk factor for developing IPF such as smoking, chronic aspiration, gastroesophageal reflux, viral infections, exposures to metal dusts and wood dusts, farming, raising birds, stone cutting/polishing, exposure to livestock and even hair dressing (Marshall, Puddicombe, Cookson, & Laurent, 2000; Meltzer & Noble, 2008; Yang u. a., 2007). Disparate studies found that a major percentage of IPF patients present chronic herpesvirus infection of their lungs and that the viral antigens are expressed by type II AECs (Lawson u. a., 2008; Tang u. a., 2003). In 2011, Stoolman et al. demonstrated how latent γ-herpesvirus infection prompts a profibrotic mediator response in various cell types in the lungs of wild-type mice suggesting a similar scenario for human lung fibrosis (Stoolman u. a., 2011). However, the patients examined in the respective studies were likely to receive immunosupressive treatment, which rendered their viral infections a potential consequence of the treatment rather than a primary abnormality in IPF (Kuwano u. a., 1997). In the search for an endogenous stimulus in the initiation of IPF endogenous irregularities of AECs suggest themselves as cause of epithelial dysfunction and death by relating to insights from studies on familial IPF (Marshall u. a., 2000; Meltzer & Noble, 2008; Yang u. a., 2007)[2].

One major challenge to understanding IPF is the identification of the factors that shift the response of the injured alveolus from normal wound repair towards fibrosis. There are diseases of the lung that involve grave AEC damage, where the lung tissue shows remarkable capacity to regenerate architecturally as well as functionally, such as the acute respiratory distress syndrome (Ware & Matthay, 2000). Most animal models of IPF also present reconstitution of the lung structure over time, but in IPF disease progression and irrevocable distortion of the organ's architecture are inevitable (Moore & Hogaboam, 2008).

[2] see chapter 1.1.3.3

Approximately 15% of the families suffering from familial IPF are reported to show mutations in the telomerase complex, which indicates that an inherited impairment in AEC regeneration might be causing the respective cases of IPF (Wang u. a., 2009). It is not known whether such inherited alterations of AEC-functioning might also be of causative relevance in other cases of FIP or in sporadic IPF.

The origin of the myofibroblast in IPF is another hot topic. Three feasible scenarios have been proposed: One states that myofibroblasts in IPF are proliferated mesenchymal cells native to the lung and that their accumulation in fibroblast foci happens in response to growth factors and fibrogenic cytokines. The second scenario entertains the possibility that progenitor cells, which derive from the bone marrow, might relocate to the injured lung where they serve as fibroblast progenitors. The third scenario states that type II alveolar epithelial cells might add to the myofibroblast pool by undergoing a transformation to the fibroblast-like phenotype via epithelial to mesenchymal transition (EMT). EMT is a process that is very common in embryogenesis (G. E. M. Martin, Kolb, & Gauldie, 2006). During EMT the transformation of cell-cell and cell-extracellular matrix interactions prompts the transition from an originally polarized epithelial phenotype to an unpolarized mesenchymal one (Zhang u. a., 2010). This prompts enhanced migratory abilities, excess production of extracellular matrix components, invasiveness and higher resistance to apoptosis (Kalluri & Neilson, 2003). Epithelial cells gradually exchange their set of epithelial molecular markers, such as E-cadherin and cytokeratin for mesenchymal markers, such as α-SMA and fibroblast-specific-protein 1 (FSP1). E-cadherin is considered a master switch of EMT whose repression alone can under certain circumstances suffice to induce and complete EMT. As the transition progresses, epithelial cells detach from their epithelial layer, cross the basement membrane and locate themselves in the underlying interstitium, where they eventually lose all their epithelial characteristics (Kalluri & Weinberg, 2009). EMT was first described as a vital physiological orchestration of events in embryogenesis (Kong, Li, Wang, & Sarkar, 2011). Later, it was found to also have a physiological role in tissue repair in the

adult organism. Aberrant EMT has been identified as key event in the pathogenesis of various types of cancers, where it promotes invasiveness and various types of organ fibrosis, including that of the lung (Thiery, Acloque, Huang, & Nieto, 2009).

1.2 TGF-β signaling

The transforming growth factor-β superfamily (TGF-β) is a group of over 40 ligands that regulate a multitude of developmental and homeostatic processes in both embryos and adult organisms, among them cell growth, migration, cell-fate determination and differentiation. Aberrant TGF-β signaling is implicated in various severe human diseases such as cancer, wound healing disorders, several hereditary conditions (e.g. hereditary chondrodysplasia and persistent mullerian duct syndrome) and fibrosis in multiple organs (Blobe, Schiemann, & Lodish, 2000; Massagué, Blain, & Lo, 2000). TGF-β signaling occurs in many vertebrate and invertebrate species and has been thoroughly conserved throughout evolution, which suggests evolutionary significance (Attisano & Wrana, 2002).

1.2.1 Mechanisms of TGF-β signaling

1.2.1.1 The ligands

The TGF-β ligand superfamily contains more than 60 family members. These are the TGF-βs (TGF-β1, 2 and 3), the bone morphogenetic proteins (BMPs) and activin/inhibin/nodal. Within a subfamily, the members enact similar but non-overlapping physiological functions (Lin, Lerch, Cook, Jardetzky, & Woodruff, 2006). TGF-β is a multifunctional protein that exists in three isoforms in mammalian tissues, TGF-β1, TGF-β2 and TGF-β3 (O Eickelberg u. a., 1999). The members of each subfamily all share the characteristic trait that they form homodimers or heterodimers for signaling. This mechanism is owed to seven conserved cysteine residues within each subunit that maintain intersubunit

linkage and structural integrity by forming one intersubunit and three intrasubunit disulphide bonds respectively (Lin u. a., 2006).

1.2.1.2 The receptors

The cellular response to TGF-β signaling is transduced by transmembrane serine/threonine kinase receptors: TGFβRIs / type I, activin-like receptors 1–7 (ALK1, ALK2, ALK3, ALK4, ALK5, ALK6 and ALK7), TGFβRIIs / type II receptors (TGFβRII, bone morphogenetic protein receptor type II (BMPRII), ACTRII, ACTRIIB and anti-Mullerian hormone receptor, type II (AMHRII)) and TGFβRIII / type III receptors (betaglycan and endoglin). These are highly conserved glycoproteins that are responsible for transducing signals from all the TGF-β superfamily members except the most divergent subgroup, the glial cell-line-derived neurotrophic factor (GDNF) subfamily which signals via glycosylphosphatidylinositol (GPI)-anchored-receptors and the tyrosine kinase c-ret proto-oncogene (RET) (Böttner, Krieglstein, & Unsicker, 2000).

Activated TGF-β1 and TGF-β3 show high affinity for TGFβRIIs while activated TGF-β2 needs the presence of betaglycan in order to bind TGFβRIIs with high affinity. In order to initiate signaling, a ligand binds to two type I and type II receptors, which then go on to form a tetrameric complex on the cell surface. This enables the constitutively active kinase domain of the type II receptor to phosphorylate the type I receptors juxtamembrane domain, on whom the final biologic output depends. The activated type I receptor then consequently phosphorylates intracellular effector proteins. While each ligand binds to its specific subset of receptors, competition amongst superfamily members at the receptor level is inevitable as many ligands share receptor subunits. This way, stochiometry and the presence or absence of betaglycan act as the switches that regulate specificity on the ligand–receptor interaction as the availability of ligands and receptors at location and their affinity for each

other determine the flow of signals. Another regulating mechanism in this respect is provided by extracellular inhibitor proteins that intercept biologically activated TGF-βs before binding to their associated receptors (Bierie & Moses, 2006; Itman, Mendis, Barakat, & Loveland, 2006; Schmierer & Hill, 2007).

1.2.1.3 Smad-dependent-TGF-β signaling

Smads are the predominant intracellular mediator proteins of TGF-β signaling. There are three types of Smads: the receptor-regulated R-Smads (Smads1, -5 and -8 for BMP and Smad2 and -3 for other TGF-β ligands), the co-mediator co-Smads (Smad4) and the inhibitory I-Smads (Smad6 and -7). In TGF-β1 signaling, Smad2 and -3 cooperate. They are directly phosphorylated and activated by an activated type I receptor. This prompts them to translocate to the cells nucleus as a heteromeric complex together with the co-Smad, Smad4. Inside the nucleus they associate with various nuclear cofactors, such as MSG1 or CREB-binding protein/p300 (CBP/p300), and directly regulate the transcription of target genes via chromatin remodeling. The I-Smad, Smad7, blocks this signaling pathway by competitively binding to the type I receptor, therewith blocking Smad2/3 phosphorylation and simultaneously recruiting E3-ubiquitin ligases, such as Smad ubiquitin regulatory factor 1 (Smurf1), for ubiquitination and degradation of type I and II receptor complexes. Smad7 expression is induced by TGF-β1, preventing excessive activity of the pathway via negative feedback under physiological circumstances (Attisano & Wrana, 2002; Itman u. a., 2006; Schmierer & Hill, 2007).

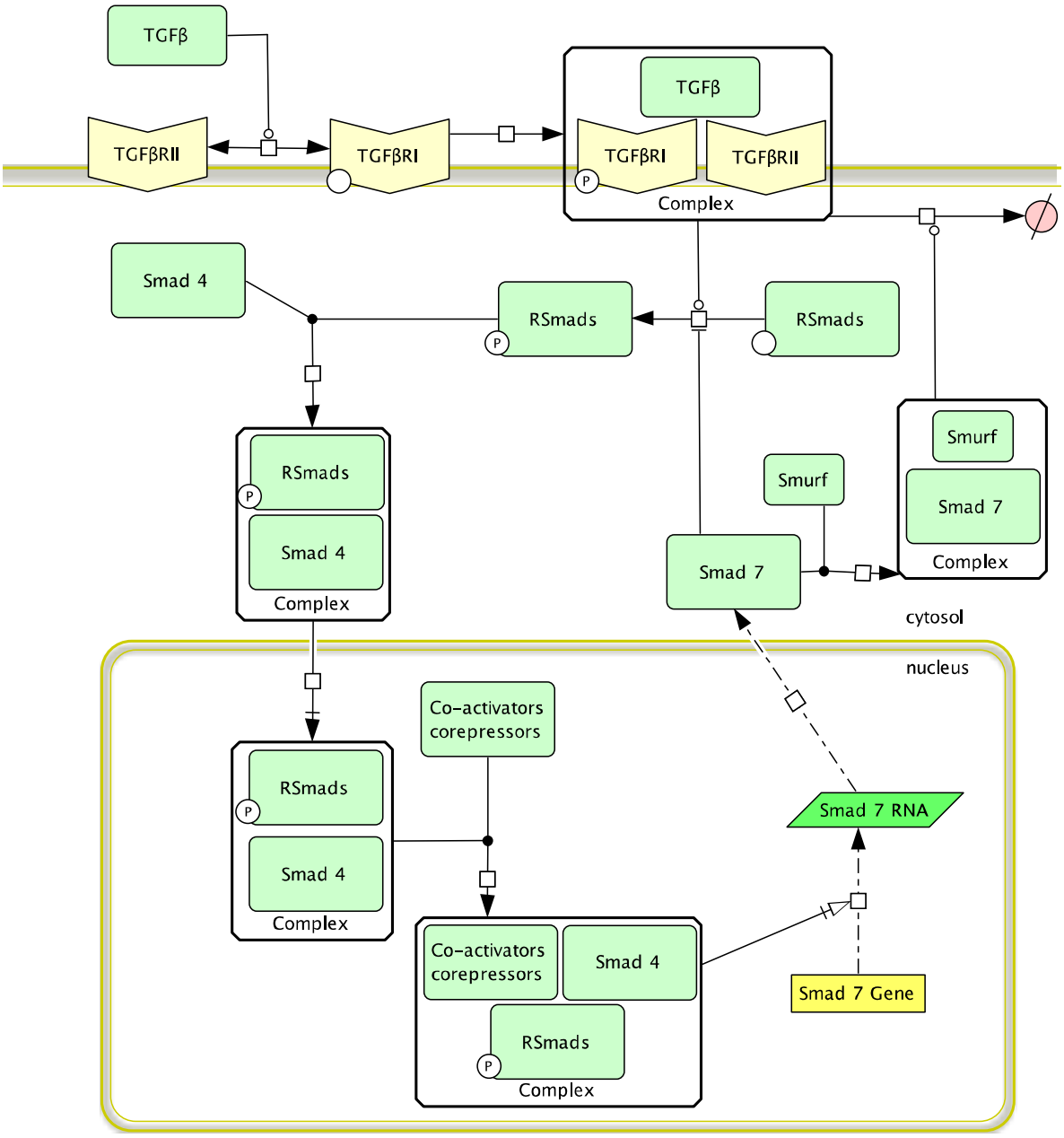


Figure 1.2: Schematic of Smad-dependent-TGF-β signaling
This schematic illustrates the flow of Smad-dependent-TGF-β signaling from the cell wall to the nucleus. The production of the I-Smad, Smad 7, is included to exhibit the pathways physiological means of self-regulation.

1.2.1.4 Smad-independent-TGF-β signaling

Even though Smads appear to be the central vehicle of signals from TGF-β superfamily members to the nucleus, TGF-βs also dispose of the ability to exert their effects via non-Smad pathways. The involved non-Smad effectors of TGF-β signaling may exert their influence via the modulation of Smad activity, the receptiveness for modulation by Smads or direct interaction with TGF-β receptors. Such molecules also serve as bridges for crosstalk of Smad-dependent-TGF-β-signaling with other major pathways (Moustakas & Heldin, 2005). The documented molecules and mechanisms of interest are so plentiful that the interested reader is kindly referred to secondary literature for further detail. Select candidates for the effectuation of Smad-independent-TGF-β-signaling comprise different branches of the mitogen-activated protein kinases (MAPK) pathway (such as Jun-N terminal kinase, p38 and PI3K kinases and ERK1/ERK2), the small GTP-binding proteins RhoA, RAS and RAC1 and members of WNT-signaling, as will be discussed in detail further on in this text (Kubiczkova, Sedlarikova, Hajek, & Sevcikova, 2012). Non-Smad effectors of TGF-β-signaling are believed to mediate the TGF-βs' effects on processes such as apoptosis, EMT, cell proliferation, matrix regulation, cell differentiation (Moustakas & Heldin, 2005). With the focus on this abundance of interdependent effectors, an enormous diversity and complexity of TGF-β signaling suggests itself.

1.2.2 TGF-β signaling in IPF

The critical role of TGF-βs in growth and development implies that aberration of the signaling pathway may lead to severe disruption of the physiological homeostasis of multiple biological functions. Deregulation of TGF-β family protein function is known to be involved in diseases such as persistent mullerian duct syndrome, hereditary chondrodysplasia, various cancer types and fibrosis (Akhurst & Hata, 2012; Jones, Spinale, & Ikonomidis, 2009; Verrecchia & Mauviel, 2007).

TGF-β1 is the family's best investigated member and its role as a central mediator of fibrosis in multiple organ systems is widely accepted (Biernacka, Dobaczewski, & Frangogiannis, 2011). In a fibrosis-setting, it induces fibroblast chemotaxis, myofibroblast differentiation, ECM synthesis, and inhibition of matrix degradation by metalloproteinases, elastases and plasminogen activators, tipping the balance between ECM production, deposition and degradation towards net accumulation (Leask & Abraham, 2004; Verrecchia & Mauviel, 2007). High levels of TGF-β1 occur in many fibrotic tissues such as fibrotic kidneys, livers and lungs (Alexakis, Maxwell, & Bou-Gharios, 2006; X. Liu, Hu, & Yin, 2006; Meltzer & Noble, 2008; Verrecchia & Mauviel, 2007).

In the healthy human lung, TGF-β1 and TGF-β3 are the predominantly expressed isoforms. They usually co-localize and their mRNA expression can be detected in bronchiolar epithelial cells and alveolar macrophages. Adding to that, TGF-β1 mRNA transcripts can also be detected in endothelial cells and fibroblasts. The pattern of expression differs in fibrotic human lungs, where TGF-β1 appears to be the predominant isoform with increased mRNA expression and protein detectable in fibroblast foci, alveolar macrophages, bronchiolar epithelial cells and hyperplastic alveolar type II cells – all of which are relevant to IPF pathology (Aubert u. a., 1994; Coker u. a., 2001).

Studies on experimental fibrosis in rats and on IPF patients have established TGF-βs, predominantly TGF-β1, as essential to the pathophysiology of the disease (Cao, Guo, Zhu, & Xu, 2000; Hiwatari u. a., 1997). In primary human fibroblasts, TGF-β1 and TGF-β3 have been shown to increase the synthesis and deposition of ECM molecules, decrease the expression of matrix metalloproteinase 1 (MMP-1) and increase the expression of TIMP metallopeptidase inhibitor 1, resulting in the employment of profibrotic properties in all the three regulatory mechanisms of ECM composition: control of ECM deposition, proteinase activity and proteinase-inhibitor activity (O Eickelberg u. a., 1999). Experiments on transgenic mice that overexpress TGF-β1 show that transient overexpression of TGF-β1 alone can induce full-fledged progressive fibrosis with minimal complementary inflammation

(Gauldie, Bonniaud, Sime, Ask, & Kolb, 2007). Smad3 null mice are resistant to TGF-β-mediated pulmonary fibrosis and they show a weakened response to bleomycin (BLM)-induced pulmonary fibrosis. Exogenous overexpression of Smad7, which inhibits the TGF-β pathway, also prevents BLM-induced lung fibrosis in mice (Bonniaud u. a., 2004, S. 3; Nakao u. a., 1999; Zhao u. a., 2002, S. 3). Administration of TGF-β1 neutralizing antibodies into a BLM mouse model inhibits collagen accumulation in the injured lung (Giri, Hyde, & Hollinger, 1993). In humans, elevated levels of TGF-β1 were observed in fibroblast foci-biopsies and bronchioalveolar lavage (BAL) fluid from IPF patients (Broekelmann, Limper, Colby, & McDonald, 1991; Cao u. a., 2000; Hiwatari u. a., 1997).

TGF-β is also known to be a key regulator in epithelial-to-mesenchymal transition in the physiological context of development as well as in the pathological contexts of cancer and fibrosis (Ozdamar u. a., 2005). It is regarded to be the archetypal cytokine for induction of EMT and there is an ever-growing amount of strong data that suggests a direct role of TGF-β1 in fibrotic EMT *in vivo* an *in vitro* (Kage & Borok, 2012). Regarding its putative role in EMT in the lung, TGF-β1 induces expression of the EMT markers α-SMA, vimentin, desmin and type-I-collagen in primary rat AECs and overexpression of TGF-β1 in transgenic mice, in a model of pulmonary fibrosis, prompts ATII cells to acquire a fibroblast-like phenotype and to express vimentin and α-SMA as well (K. K. Kim u. a., 2006; Willis u. a., 2005; Willis & Borok, 2007).

Although there is a multitude of interleukins, chemokines and growth factors active in IPF tissue, TGF-β is generally seen as the “master switch” that initiates the shift from tissue damage and respondent inflammation to tissue repair or, when repair mechanisms go awry, to fibrosis (Willis & Borok, 2007). Fibrosis can only proceed when a functioning TGF-β mechanism is at hand, as the aforementioned intervention studies have proven. To sum up the implications of the introduced data, TGF-β appears to play a pivotal role in IPF, where it seems to regulate the progression from inflammation to fibrosis, the perpetuation of fibrosis and EMT.

1.3 WNT signaling

WNT proteins are a family of 19 secreted short-range ligands with highly conserved cysteine residues that locally activate a multitude of receptor mediated signaling pathways. They play a critical role in developmental physiology and homeostatic processes. It is well recognized that aberrant WNT signaling contributes to the pathology of numerous diseases ranging from colorectal cancer to schizophrenia (Logan & Nusse, 2004; Okerlund & Cheyette, 2011). The term “WNT” is an amalgamation of the names of the two genes *wingless* and *int-1*, which where both discovered in the early 1980s and were later found to encode the same proteins (Nusse u. a., 1991; Rijsewijk u. a., 1987). WNTs occur only in metazoans and not in plants, fungi or protists (Croce & McClay, 2008).

WNT signaling is subdivided into at least three separate signaling pathways. The first and most thoroughly investigated pathway is the “canonical” β-catenin-dependent pathway, where WNTs interact with designated cell surface receptors in order to effect the cytosolic stabilization and nuclear translocation of the effector molecule β-catenin. Second is the WNT/Ca^{2+} pathway, which utilizes calmodulin kinase II and protein kinase C and third is the WNT/JNK or planar cell polarity pathway, which signals through small GTPases. The latter two signaling cascades are usually referred to as “non-canonical” WNT/β-catenin independent signaling. This effort to subdivide WNT signaling into the canonical and the non-canonical pathways was based on the observation that certain WNTs could induce altered growth characteristics and morphological transformation of mouse C57MG mammary epithelial cells and establish an ectopic axis in Xenopus embryos (McMahon & Moon, 1989; Wong, Gavin, & McMahon, 1994). These activities involved β-catenin activation and were then declared to constitute the canonical pathway. Today, the progress of WNT research gradually casts light unto an unforeseen complexity and pronounced dynamics of WNT functioning and communication, clouding the traditional picture of the uncoupled pathways. Experts like R. Nusse have begun to call for the abandonment of the established

view that downstream WNT signaling is a matter of distinct and linear pathways. A growing body of research speaks of multifaceted and plentiful crosstalk between the WNT ligands, their receptors, co-receptors and the co-factors forming “a complex network of protein interactions, with multiple outcomes, cross-talk and regulatory inputs at practically every level” of the signaling chain, i.e. extracellularly, at the plasma membrane and intracellularly (van Amerongen & Nusse, 2009). Distinct WNT signaling cascades influence each other and interact with a variety of other signaling pathways, such as that of TGF-β1, resulting in a grand signaling scheme of dizzying complexity.

In order to account for this apparent complexity, this thesis will differentiate between the functional contexts of β-catenin-dependent WNT signaling and non-β-catenin-dependent WNT signaling as opposed to the more dualistic traditional division of canonical and non-canonical WNT signaling. β-catenin-dependent WNT signaling is the signaling context of interest for this thesis, due to the comprehensive data on β-catenin-dependent WNT signaling and the growing pool of data that relate it to fibrotic processes, including IPF, as elucidated in the following sections.

1.3.1 β-catenin-dependent WNT signaling

β-catenin-dependent WNT signaling is the most thoroughly investigated of the WNT pathways. It is usually referred to as the “canonical” pathway. The characteristic trait of this pathway is that its activation results in the nuclear accumulation of β-catenin and its subsequent translocation into the nucleus where it interacts with members of the T-cell-specific transcription factor/lymphoid enhancer-binding factor (TCF/LEF1) family in order to regulate the transcription of their respective target genes (Logan & Nusse, 2004).

β-catenin is a member of the armadillo family of proteins that engages in highly promiscuous protein-protein binding. It is located in three cellular pools: The adherens junctions serve as the first pool, where β-catenin is associated with the transmembrane

receptor E-cadherin and the cytoskeletal linking protein α-catenin. The second pool is the cytoplasm and the third one the nucleus, where β-catenin associates with other transcription factors (Mulholland, Dedhar, Coetzee, & Nelson, 2005). Before the discovery of β-catenin's function as a critical co-activator of WNT signaling, the molecule was known for its function in cell adhesion as a regulator of E-cadherin (Gumbiner, 1996). The mechanism which lets β-catenin shift between these dual roles is fine-tuned via differential phosphorylation through specific binding partners such as casein kinase 1 (CK1), casein kinase 2 (CK2), glycogen synthase kinase 3 (GSK3) and CyclinD/cdk6 (Daugherty & Gottardi, 2007). The distinct phosphorylations affect the molecule's affinity to its multiple cytosolic and plasma-membrane-bound interaction partners. These properties make β-catenin a mediator in the coordination of cell adhesion and WNT signaling (Daugherty & Gottardi, 2007; K. K. Kim u. a., 2009; Y. Kim u. a., 2009).

The central regulatory mechanism in canonical WNT signaling is the management of the stability of the cytosolic β-catenin pool. When no active WNT ligand binds to the cell surface, β-catenin is first phosphorylated by CK1 and then by GSK3β, which is scaffolded by AXIN and adenomatous polyposis coli (APC), the three of them forming a so-called "destruction complex". This phosphorylation consequently leads to the ubiquitylation and 26s-proteasome-mediated degradation of β-catenin (Logan & Nusse, 2004; Moon, Kohn, De Ferrari, & Kaykas, 2004). This mechanism maintains the cytosolic and nuclear levels of β-catenin at low levels. As was mentioned before, a certain proportion of the β-catenin molecules save themselves from degradation via a relatively stable adherence to cadherins at the plasma membrane. The active destruction complex hinders β-catenin from translocating to the nucleus, resulting in the repression of β-catenin target genes by TCF and LEF1 and their associated co-repressors, Groucho and CtBP (Mulholland u. a., 2005).

Available WNTs initiate the β-catenin-dependent pathway from the outer cell membrane by forming a complex with Frizzled (FZ) serpentine receptors and the single-pass transmembrane receptors LDL-receptor-related proteins 5 and 6 (LRP5 and LRP6)

(Bhanot u. a., 1996; Tamai u. a., 2000). This provokes the membrane recruitment and activation of the phosphoprotein Dishevelled (DSH), which in turn recruits AXIN and its adherent destruction complex to the plasma membrane where AXIN binds to LRP5/6. This ultimately leads to the phosphorylation and inactivation of GSK3β, inhibiting the degradation of β-catenin (Tolwinski & Wieschaus, 2004). β-catenin then accumulates within the cytosol and translocates to the nucleus. There it allocates the basal transcription machinery to the promoters of WNT-sensitive genes through direct interaction with members of the LEF1/TCF-transcription factor family, converting them from repressors to activators by displacing their corepressors. This effects the association with coactivators such as CBP/p300, Brgl and CARM1 and the direct interaction with the TATA-binding protein. This, in turn, leads to the induction of downstream gene targets involved in cell proliferation, cell survival and cell fate like cyclin D1, PPARδ, matrilysin (MMP-7) or c-myc (Mulholland u. a., 2005).

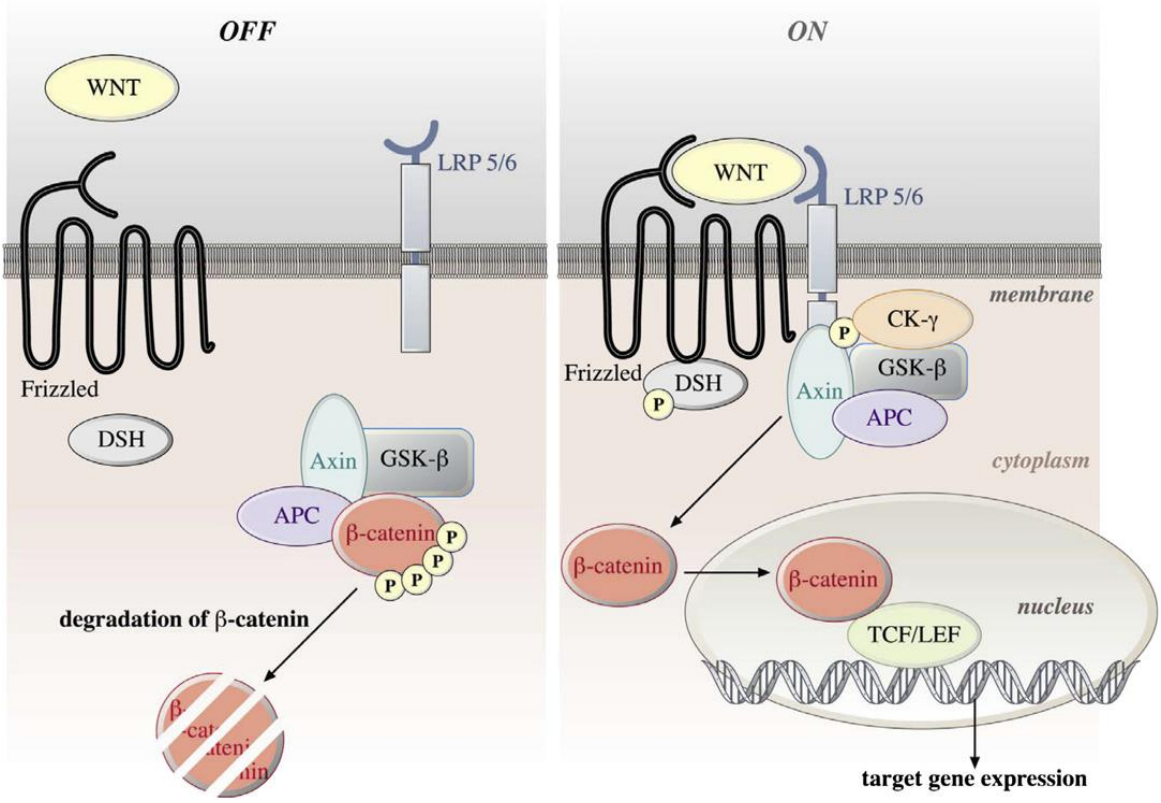


Figure 1.3: Schematic of β-Catenin-dependent WNT signaling
Left: degradation of cytosolic β-catenin in the "off-state". Right: stabilization of cytosolic β-catenin in the "on-state" with activation of the canonical signaling cascade (Königshoff & Eickelberg, 2010, Reprinted with permission of the American Thoracic Society. Copyright © 2018 American Thoracic Society. Königshoff, M., & Eickelberg, O. (2010). WNT signaling in lung disease: a failure or a regeneration signal? *American Journal of Respiratory Cell and Molecular Biology*, *42*(1), 21–31. The American Journal of Respiratory Cell and Molecular Biology is an official journal of the American Thoracic Society.)

1.3.2 WNT signaling in lung development and IPF

WNT signaling plays a crucial role in the organogenesis of the lung, where it affects epithelial as well as mesenchymal development through a tightly regulated spatiotemporal expression pattern. β-catenin regulates the differentiation of the alveolar and bronchial epithelium and modulates epithelial-mesenchymal interaction during the organ's development. It exerts its effect in an autocrine as well as paracrine manner (Cardoso & Lü, 2006). The conditional inactivation of β-catenin in embryonic lung epithelial cells in mice results in the failure of the formation of distal airways, but not in that of proximal lung

structures (Mucenski et al., 2003). The expression of activated β-catenin in respiratory epithelial cells of fetal and postnatal transgenic mice disrupts epithelial cell differentiation and prompts air space enlargement, goblet cell hyperplasia and pulmonary tumors (Mucenski et al., 2005).

It is known that defective WNT signaling is a causative factor in a multitude of diseases and several studies have linked dysregulated WNT signaling to lung development gone awry (Königshoff & Eickelberg, 2010). Its vital role during embryogenesis already suggests the need for a tight regulation of the pathway. The bulk of data concerning the involvement of WNTs in disease mainly adresses their prominent role in a variety of malignant disorders, primarily of the gastrointestinal tract. In colon cancer, for example, WNT signaling causes the formation of cancer-predisposing polyps by prolonging the proliferation of intestinal crypt cells, and more than 90% of colorectal carcinomas display mutations of either APC or β-catenin (Bienz & Clevers, 2000; Luu u. a., 2004). However, investigations into the role of WNT signaling in fibroproliferative disorders also revealed a widespread involvement of β-catenin in a multitude of abnormal fibrotic responses. For example, mice that overexpress β-catenin produce hyperplastic scars upon wounding and β-catenin levels in hyperplastic scars remain elevated for over two years while they are usually only elevated during the fibroproliferative stage of wound repair in normal granulation tissue (S. Cheon u. a., 2005; S. S. Cheon u. a., 2006). Furthermore, fibromatoses such as Dupuytren's contracture consistently display an aberrant activation of β-catenin-dependent WNT signaling (Dolmans u. a., 2011; Lacroix-Triki u. a., 2010).

Pathologic fibroproliferative responses in general constitute the aberrant activation of physiological wound healing processes. Contemporary studies that employ genomic approaches give proof of aberrantly activated embryonic signaling pathways in IPF. This indicates that that the condition might be characterized as an aberrant activation and recapitulation of distinct developmental programs (Moisés Selman et al., 2008). These studies speak of a reactivation of dormant developmental programs, such as WNT signaling,

as a causative factor in lung fibrosis. Unbiased expression microarrays have given proof of the up-regulation of several WNT-related genes, such as WNT2 and -5a, FZD7 and -10, sFRP1 and -2 and matrilysin (MMP-7), an endopeptidase that degrades extracellular matrix, in IPF lungs in comparison to healthy lungs or other ILD (Kaminski & Rosas, 2006; Moises Selman u. a., 2006; Yang u. a., 2007). The pattern of overexpression presented by these works suggests an activation of β-catenin-dependent WNT signaling (Moisés Selman et al., 2008).

In 2003, Chilosi et al. published a milestone study that demonstrated a significant nuclear accumulation of β-catenin as well as significantly increased levels of the WNT downstream targets cyclin D1, a cell cycle regulator, and MMP-7 in bronchioproliferative regions in fibroblast foci and ATII cells (Chilosi u. a., 2003). They could not detect analogous phenomena in healthy lungs or other fibroproliferative diseases such as NSIP, DIP, organizing pneumonia or diffuse alveolar damage and their deduction was that aberrant β-catenin signaling might be causally involved in the disease's pathogenesis and not just a mere epiphenomenon. Nevertheless, the group could not detect mutations of β-catenin in the examined IPF patients. Peer reviewers at the time saw this as an indication that the observed activation of β-catenin-dependent WNT signaling in IPF might simply be an epiphenomenon of the disease (Morrisey, 2003). However, normal expression and activity of a single effector molecule does not categorically rule out unknown mutations of other regulating components in the signaling chain. More studies followed up and found an up-regulation of several WNT target genes in IPF, such as osteopontin, matrilysin and WNT1-inducible signaling protein (WISP), as indication of β-catenin activation (Königshoff u. a., 2008; Pardo u. a., 2005; Zuo u. a., 2002). In 2008, Königshoff et al. reported that multiple components of β-catenin-dependent WNT signaling are overexpressed in human IPF lungs compared to healthy lungs, namely WNT1, 7b and 10b, Fzd2 and 3, β-catenin, and LEF1. By documenting increased phosphorylation of LRP6 and GSK-3β, the most sensitive indicators of WNT activity in tissue sections, they demonstrated increased functional WNT

signaling in IPF lungs. They were also able to topographically pinpoint several components of β-catenin-dependent WNT signaling to the bronchial and alveolar epithelium by immunohistochemistry and demonstrated increased WNT-activity in primary human ATII cells of IPF lungs. In functional in vitro studies, Königshoff et al. established how WNT3a induces proliferation of A549 lung epithelial cells and a significant induction of the myofibroblast activation marker α-SMA and fibroblast-specific protein and collagen synthesis in NIH-3T3 cells (Königshoff et al., 2008). In 2009, Königshoff et al. exhibited activated β-catenin-dependent WNT signaling in a BLM model of fibrosis and increased expression of WISP1 in ATII cells of BLM-treated mice as well as actual IPF patients. They showed that stimulation with WISP1 caused lung fibroblasts of humans and mice to enhance their deposition of ECM-components and primary mouse ATII cells to proliferate and undergo EMT. Finally, the inhibition of WISP1 attenuated the fibrotic response in vitro (Königshoff et al., 2009). All these results can be interpreted as signs of an activation of β-catenin-dependent WNT signaling in IPF.

WNT-dependent EMT has been established in a variety of experimental settings. In their aforementioned 2003 paper on the aberrant activation of β-catenin-dependent WNT signaling in IPF, Chilosi et al. stated that the aberrant nuclearization of β-catenin in bronchioproliferative regions might suggest a possible involvement of WNTs in EMT (Chilosi u. a., 2003). By then, a link between β-catenin-dependent signaling and EMT had already been established. In 1998, Novak et al. were able to positively correlate β-catenin/LEF-1 transcriptional activity with EMT in intestinal epithelial cells (IEC-18) and mouse mammary epithelial cells (scp2). They did so by showing how the integrin-linked kinase (ILK)-prompted down-regulation of E-cadherin in these cells, as well as the cytoplasmic stabilization and nuclear translocation of β-catenin with subsequent upregulation of β-catenin/LEF-1 transcriptional activity (as mediated via WNT1 and -3). The result of this was a switch from the cell's stationary epithelial phenotype to an invasive mesenchymal one (Novak u. a., 1998). In 2000, Eger et al. reported that estradiol treatment of mouse mammary epithelial

cells prompted loss of E-cadherin expression and nuclear translocation of β-catenin with subsequent activation of β-catenin/LEF1-signaling, which could in turn be repressed by transient expression of exogenous E-cadherin (Eger, Stockinger, Schaffhauser, Beug, & Foisner, 2000). That same year, Kim et al. gave direct evidence for a role of β-catenin-dependent WNT signaling in EMT in DLD1 colorectal carcinoma cells, by showing how overexpression of LEF1 in these cells alone resulted in the upregulation and nuclear accumulation of β-catenin with subsequent EMT (K. Kim, Lu, & Hay, 2002). The above-mentioned findings of Königshoff et al. link this well-established mechanism directly to the pathology of IPF.

In summary, functional β-catenin-dependent WNT signaling is activated in IPF in response to an unidentified stimulus. This activation may constitute a defective attempt at lung regeneration that may play a causative role in the pathogenesis of IPF via the initiation of EMT and/or fibroblast to myofibroblast activation and/or excessive ECM-deposition.

1.4 Crosstalk between TGF-β and WNT signaling

The TGF-β pathway is generally prone to engage in crosstalk, as Smads by themselves actually have a low affinity for DNA, which they regularly increase via interaction with a cofactor. It is the identity of the cofactor available at the time of TGF-β exposure that ultimately enforces target specificity (Fuxe, Vincent, & de Herreros, 2010). The β-catenin-dependent WNT and TGF-β pathways are both known to play critical roles in cell fate during development and adult tissue homeostasis. Research on crosstalk between the two speaks of a very complex and busy relationship. Mutual regulations between the two pathways may occur throughout the entire lifespan of a metazoan. Their properties predestine them to engage in crosstalk in the lungs, including the setting of IPF, because they are both essential for primordial lung specification and cell differentiation during lung morphogenesis, and because they both play a functional role in IPF with spatiotemporally overlapping sites of

action. It has been suggested that an interplay of this kind might modulate the subtleties of spatiotemporal activity of WNT signaling (Königshoff & Eickelberg, 2010). As in other crosstalk relationships, the involved mechanisms are conserved across species, which emphasizes their biological relevance (Guo & Wang, 2009).

Crosstalk between TGF-β and WNT signaling takes place in a reciprocal manner on multiple molecular levels. The best understood site of interaction is the nucleus, where Smad2 and -3 (the R-Smads), Smad4 (the Co-Smad), β-catenin and LEF/TCF-proteins may form complexes that control target genes in a mostly synergistic manner (E Labbé, Letamendia, & Attisano, 2000; Etienne Labbé & Attisano, 2006; Nishita u. a., 2000). Smad3, AXIN and Dvl1 serve as crosstalk interfaces in the cytoplasm. Smad3 appears to be part of the β-catenin destruction complex in adult human mesenchymal stem cells (MSCs) derived from bone marrow, where it promotes rapid nuclear translocation of β-catenin upon TGF-β1 mediated phosphorylation with subsequent enhanced expression of a specific set of target genes, such as B lymphoid tyrosine kinase (BLK) (Jian u. a., 2006). The scaffolding protein Axin, which is part of the β-catenin destruction complex, may promote TGF-β signaling through at least two distinct mechanisms. In one instance, it associates with Smad3 as an adaptor that directly facilitates Smad3's phosphorylation, i.e. activation, upon the triggering of the TGF-β type I receptor (Furuhashi u. a., 2001). In another instance, Axin provides a scaffold that facilitates the degradation of the inhibitory Smad7 via the ubiquitin ligase Arkadia, as illustrated in human embryonic kidney cells, and subsequently promotes TGF-β signaling. This effect can be attenuated by coexpression of WNT-1, which leads to the downregulation of Axin (W. Liu u. a., 2006). Furthermore, overexpressed Dvl1 has been shown to interact with Smad3 in murine embryonic craniofacial mesenchyme cells, although a functional consequence of this novel relationship has yet to be identified (Warner, Greene, & Pisano, 2005).

These crosstalk mechanisms suggest scenarios where multiple components of the two pathways act as integrators that bridge both signaling chains. There are also scenarios

where singular components from one pathway actively engage in the other while they are apparently functionally detached from the presumed original one. For example, Smad4 has been shown to act as a WNT-pathway component as it enhances the activation of the Xtwn promoter via β-catenin and LEF without a signal from TGF-β (Nishita u. a., 2000). Vice versa, TGF-β ligands possess the ability to activate LEF/TCF target genes in the absence of β-catenin (Etienne Labbé & Attisano, 2006). On the level of the transcription machinery, Smad3/4-transfected HepG2 (human hepatocellular carcinoma) cells that lack a nuclear β-catenin-binding cofactor still yield TGF-β dependent activation of Xtwn, which is usually characterized as a downstream WNT target (E Labbé u. a., 2000). This sharing of DNA-binding transcription factors reveals an additional level of complexity in two apparently not so distinct signaling pathways, which appear to be rather modular in their nature than monolithic. It also suggests a feasible mechanism for signal integration in cases of co-stimulation (Etienne Labbé & Attisano, 2006).

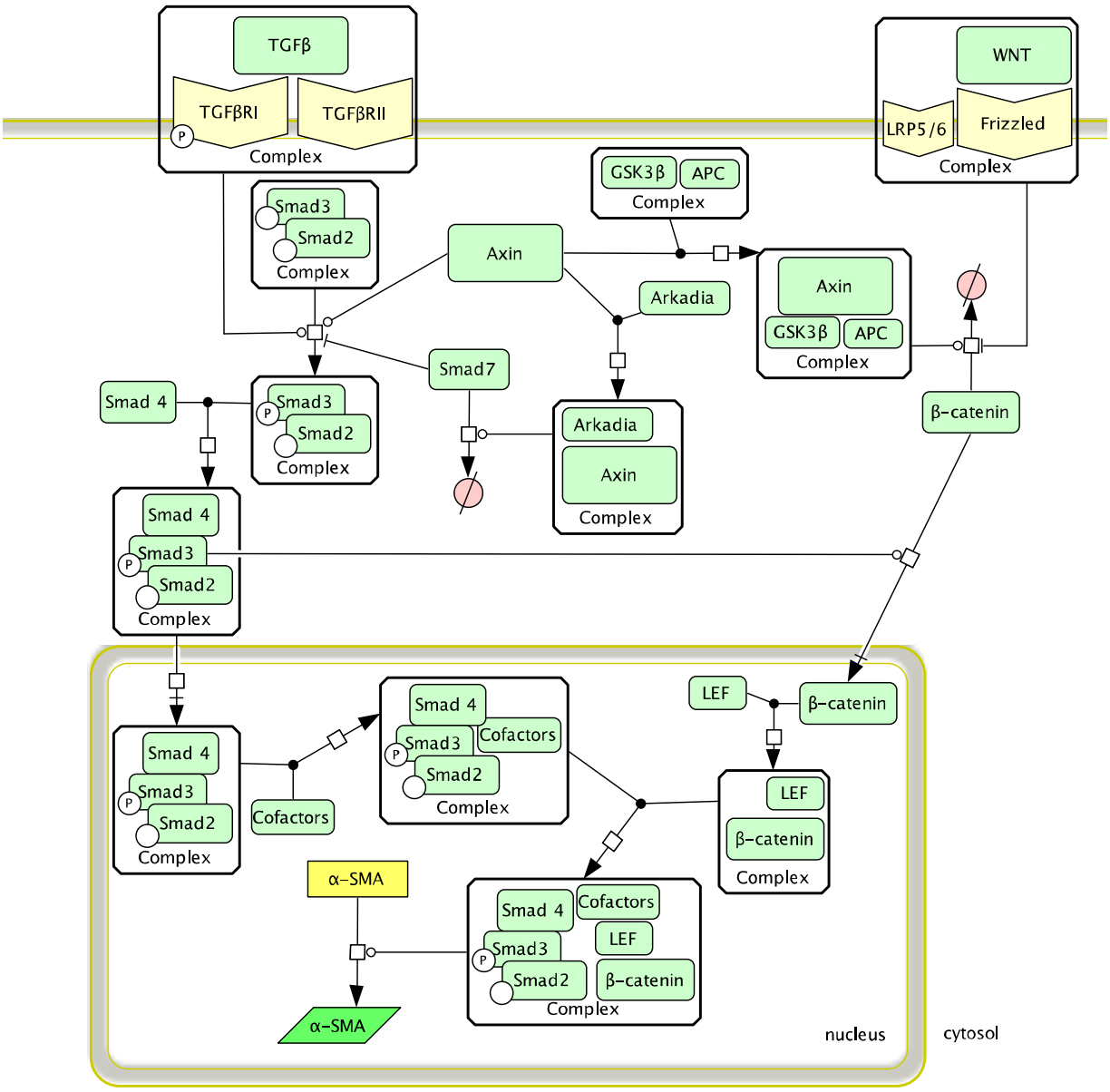


Figure 1.4: Interfaces of crosstalk between TGF-β and WNT signaling
This illustration exhibits different crosstalk-interfaces from various cellular contexts for didactic purposes. They exhibit means of reciprocal regulation of the two pathways in the cytosol as well as the nucleus. The translation of α-SMA is shown as exemplary target of synergistic signaling through a nuclear Smad/β-catenin/LEF/TCF-protein-complex.

The crosstalk occurs in physiological as well as pathological settings throughout embryogenesis and in the adult organism. Most research performed on the relevance of this crosstalk in the development of diseases concentrates on its role in carcinogenesis (Fuxe u. a., 2010; Etienne Labbé u. a., 2007). However, there is also an impressive and growing body of evidence linking faulty crosstalk between the WNT and TGF-β pathways to the pathogenesis of other conditions such as aberrant wound healing, chronic obstructive pulmonary disease and pulmonary fibrosis, particularly IPF (Baarsma u. a., 2011; S. S. Cheon u. a., 2006; Minoo & Li, 2010). There is plentiful data that relates this crosstalk to two mechanisms that have already been introduced as possible origins of the myofibroblasts in IPF's fibroblast foci: EMT and the transition of fibroblasts into myofibroblasts.

1.4.1 TGF-β1/WNT-crosstalk: EMT

TGF-β1 and WNT may jointly promote EMT in a variety of settings. For example, TGF-β can effect the formation of activator protein-1 transcription factor complex (AP1), which is also a WNT downstream target, in medial edge epithelial (MEE) cells through a non-Smad pathway. AP1 is known to induce the transcriptional factor Snail, which promotes EMT via inhibition of E-cadherin. In addition to that, TGF-β-phospho-Smad2/Smad4 complexes have been shown to functionally activate the WNT downstream target LEF-1, which consecutively serves as a crosstalk interface in order to inhibit E-Cadherin and hence also promote EMT (Minoo & Li, 2010; Nawshad, Medici, Liu, & Hay, 2007). Furthermore, pY654-β-catenin (i.e. β-catenin that is phosphorylated at Tyr-654, a tyrosine residue in the 12_{th} armadillo repeat, resulting in decreased cadherin binding), has been shown to interact with Smads in the nucleus to form so-called "EMT promoting Smad complexes" that go on to promote mesenchymal genes, such as α-SMA and PAI-1 (Daugherty & Gottardi, 2007; Fuxe u. a., 2010; Piedra u. a., 2001). Kim et al. illustrated how the laminin receptor epithelial integrin α3β1 apparently serves as a nidus in the formation of such pY654-β-

catenin/pSmad2 transcriptional complexes in primary AECs in vitro and how a deficiency of this laminin receptor lessens the EMT response of these cells to TGF-β1 accompanied by a significant decrease in the expression of the EMT-showpiece markers α-SMA and collagen I (Y. Kim u. a., 2009). K. K. Kim et al made relatable findings *in vivo* by showing how lung epithelial cell–specific loss of epithelial integrin α3β1 in mice attenuates experimental fibrosis after BLM injury via decreased accumulation of lung myofibroblasts. The group was also able to infer a pathophysiological relevance of these results by obtaining β-catenin/pSmad2 transcriptional complexes in lung tissue sections from IPF patients and by localizing pY654-β-catenin to subepithelial myofibroblasts and AECs in IPF lungs (K. K. Kim u. a., 2009). However, these findings missed out on illustrating direct transcriptional regulation of target genes by the pY654-β-catenin/pSmad2 transcriptional complexes. Zhou et al. managed to do so in 2012 and described a mechanism by which WNT/TGF-β-crosstalk might mediate EMT via the co-activator cAMP-response element-binding protein (CREB)-binding protein (CBP) in the context of pulmonary fibrosis (Zhou u. a., 2012). They illustrated that TGF-β1 prompts β-catenin dependent WNT signaling to jointly induce α-SMA expression through the formation of a complex among Smad3, β-catenin and CBP in immortalized rat AECs. Their experiments delivered examples of direct crosstalk between the two pathways by showing how TGF-β1 prevents the degradation of β-catenin as it inhibits the GSK-3β-dependent-ubiquitin/proteasome machinery with subsequent stabilization of the nuclear β-catenin pool and by showing how ICG-001, a small molecule inhibitor of CBP-dependent β-catenin signaling, prevents TGF-β1-induced EMT and α-SMA expression. These results suggest a role for β-catenin as an essential effector of TGF-β1-induced α-SMA transcription through its interaction with CBP. They give evidence of a joint transcriptional regulation of target genes related to EMT and myofibroblast differentiation by crosstalk between TGF-β1 and β-catenin-dependent WNT signaling. These findings make a case for a functional significance of EMT in the pathogenesis of IPF (Zhou u. a., 2012).

1.4.2 TGF-β1/WNT-crosstalk: fibroblast-to-myofibroblast transition

Some studies speak of a potential synergy between TGF-β signaling and WNT-signaling during the process of fibroblast-to-myofibroblast transition. Carthy et al. have published data that illustrates how WNT3a induces the differentiation of cultured mouse fibroblasts into a myofibroblast-like phenotype by the upregulation of TGF-β signaling via phosphorylation of Smad2 in a β-catenin dependent manner with subsequent α-SMA expression (Carthy, Garmaroudi, Luo, & McManus, 2011). Furthermore, the interference between TGF-β1- and WNT-signaling can also stimulate the expression of myofibroblast markers in immortalized rat cardiac fibroblasts (Shafer & Towler, 2009).

On the other hand, there is also data that ascribes β-catenin-dependent WNT signaling the potential to negatively regulate TGF-β-mediated fibroblast-to-myofibroblast transition. Michalik et al. found that Lithium (which acts as an inhibitor of GSK-3β) attenuates the TGF-β induced transition of fibroblasts to myofibroblasts in primary human fibroblasts from asthmatic patients but not in fibroblasts from healthy donors. In addition to that, Liu et al. exhibited how β-catenin-dependent WNT signaling may actually form a negative feedback loop that hinders the TGF-β induced transition of fibroblasts to myofibroblasts in cultured human skin fibroblasts and in hypertrophic scar derived fibroblasts (J. Liu u. a., 2012; Michalik u. a., 2012).

In summary, the findings presented in sections 1.4.1 and 1.4.2 illustrate that crosstalk between the TGF-β and WNT pathways might play an essential role in IPF-pathogenesis and that its course of action would very likely be via the induction of EMT and the regulation of the transition of fibroblasts to myofibroblasts. They imply that such crosstalk occurs through different interfaces, like the association of β-catenin with either Smad2 or Smad3, and that the discrimination of interfaces likely depends on cellular context and the involved cofactors.

1.5 FHL2 – a versatile LIM protein

Conserved protein interaction domains are essential parts of a cell's regulatory machinery. They facilitate the protein associations that interconnect the various regulatory processes within the cell and get them to work as a whole. Their modular architecture allows for the stunning complexity of the proteome and the communication of distinct cellular circuits (Kadrmas & Beckerle, 2004; Pawson, 2003; Pawson & Nash, 2003).

LIM protein motifs are cysteine-rich, double zinc finger modules that mediate protein-protein interaction. They were first discovered in 1988 within MEC-3, a homeodomain containing Caenorhabditis elegans transcription factor. Shortly thereafter, the same domain was identified in the C. elegance protein-lineage LIN-11 and in ISL-1, an insulin-enhancer binding protein of the rat. Hence, the name for this family of proteins is an acronym that is derived from the first letters of these three homeodomain proteins. To date, LIM domains have been identified in the proteome of every eukaryote examined but never in that of a prokaryote. LIM domains generally consist of 50 – 60 amino acids and of two zinc fingers that each coordinate a bond to one Zn^{2+}-ion. Although zinc fingers are typical DNA binding constructs, there is little evidence for direct DNA binding of the LIM domain. On the contrary, LIM domains have been shown to reduce the affinity of the DNA-binding homeodomains in LHX (LIM homeodomain) proteins (Bridwell u. a., 2001; Sánchez-García, Osada, Forster, & Rabbitts, 1993). While LIM domains display great variety in conformation as well as function, all LIM domains share the consensus sequence CX2CX16-23HX2CX2CX2CX16-21CX2(C/H/D) (X denotes any amino acid) (Schmeichel & Beckerle, 1994). Such domains can be located at the C- or N-terminus of a protein as well as internally. A LIM protein can be made up entirely of LIM domains or include them among many others, such as catalytic domains, homeodomains or cytoskeleton binding domains (Zheng & Zhao, 2007). LIM proteins have an adaptor function in the assembly of higher order protein complexes that can associate with the transcriptional machinery in the nucleus, as well as the actin

cytoskeleton of the cytoplasm. Their modular nature renders them suitable for great functional complexity (Dawid, Breen, & Toyama, 1998). They are grouped into four distinct groups, based on the assembly of their LIM domains and their general assembly. The first group comprises the LIM homeodomain (LHX) proteins and nuclear LMO (LIM-domain-only) proteins. These proteins contain two tandem N-terminal LIM domains, and they are found in the nucleus where they function as transcription factors or cofactors. The second group consists of LMO proteins that are similarly built as those of the first group but feature an additional two or more LIM domains located at their N- or C-termini. They can translocate between the nucleus and the cytoplasm. The third and fourth groups are made up of protein families that contain a heterogeneous array of additional protein-protein interaction motifs other than LIM, such as ATD (actin-target domain) and LD (leucine-aspartate repeat). Members of the fourth group also carry either an additional mono-oxygenase or a kinase catalytic motif that distinguish them from third-group members. These proteins mainly interact with the cytoskeleton, but may also translocate to the nucleus in order to facilitate target gene transcription. Research suggests that cytosolic LIM domain proteins mainly interact with the cytoskeleton and that nuclear LIM proteins participate preferentially in cell fate determination and tissue specific gene regulation (Zheng & Zhao, 2007). In summary, LIM domain proteins function as adapters in the establishment and functioning of modular protein complexes which are essential to a multitude of cellular processes (El Mourabit, Müller, Tunggal, Paulsson, & Aumailley, 2004).

The LIM protein subclass of four-and-a-half LIM (FHL) proteins consists of six members (FHL1, FHL2, FHL3, FHL4, FHL5 and ACT), which show a high degree of homology between each other and belong to the second of the aforementioned LIM domain protein groups (Coghill u. a., 2003, S. 3; Johannessen, Møller, Hansen, Moens, & Van Ghelue, 2006, S. 2). They consist of four complete and one N-terminal half LIM domains and have the ability to shuttle between the cytoplasm and the nucleus. Although they are enzymatically inactive, their function as adaptors or scaffolds for the assembly of protein

complexes makes them very versatile in nature (Kleiber, Strebhardt, & Martin, 2007). FHL proteins are known to be involved in multiple cellular processes, e.g. apoptosis, adhesion, migration cell proliferation and gene expression, but their cardinal feature is the modulation of transcription factor activity (Ding u. a., 2009; Kleiber u. a., 2007). They show cell-specific and developmentally regulated expression (Morlon & Sassone-Corsi, 2003, S.). FHL1, FHL2 and FHL3 are predominantly expressed in human muscle tissue but FHL1 and FHL2 are also expressed in various other tissues. FHL4 is solely expressed in testis (Morgan & Madgwick, 1999). ACT was detected in human tumor cell lines derived from squamous cell carcinomas, melanomas, and leukemias (Morgan & Whawell, 2000). FHL5 is the most recent addition to the family and has yet only been identified in the gills, the heart muscles and skeletal muscles of eels (Mistry u. a., 2004).

FHL2 is the most extensively studied member of the family. It was originally termed downregulated-in-rhabdomyosarcoma-cells LIM-protein (DRAL) because the first study to mention it identified it by subtractive cloning of normal myoblasts and rhabdomyosarcoma cells (Genini u. a., 1997). It is a 30-32 kDa protein that comprises 279 amino acids. Its mRNA encompasses 1416 bp and it was mapped to chromosome 2q12-q13 by fluorescent in-situ hybridization (FISH) (Chan u. a., 1998). Our understanding of the regulation of FHL2 expression remains very limited, although several factors have been implicated in the transcriptional regulation of FHL2, such as the transcription factors p53, serum response factor (SRF), Nkx2.5, MEF-2 and extracellular stimuli, such as hypertonicity, heat and ischemia, (Johannessen u. a., 2006). The expression of FHL2 is most plentiful in heart muscle cells in humans, but it is also expressed in several other organs including the brain, the liver and the lung (Chan u. a., 1998; Tanahashi & Tabira, 2000). FHL2 is known to reside in different subcellular compartments and can be encountered in the cytoplasm as well as the nucleus. The cytoplasmic form of FHL2 associates with focal adhesion complexes through binding to separate α- und β-integrin subunits (Park u. a., 2008; Samson u. a., 2004; V Wixler u. a., 2000). Its nuclear mass is below the 50-kDA cut-off for active transport

through the nuclear pores (Johannessen u. a., 2006). The shuttling of FHL2 from the cytoplasm to the nucleus is mediated via activation of the RhoA-GTPase and subsequent signaling cascades, that can be triggered by sphingosine-1-phosphate and serum (Müller u. a., 2002). This stimulus-regulated subcellular localization of FHL2 implies that the nuclear form of FHL2 mainly participates in transcriptional regulation (Fimia, De Cesare, & Sassone-Corsi, 2000). This behavior is in line with the rest of the LIM domain proteins who mainly function as cytoskeleton organizers in the cytoplasm and as tissue-specific gene regulators in the nucleus (Zheng & Zhao, 2007).

FHL2 knockout mice are viable. This points at a possible redundancy of the molecule (Chu, Bardwell, Gu, Ross, & Chen, 2000). However, continuative research has established that FHL2 can be characterized as an important adaptor protein which mediates the formation and modification of multiprotein aggregates and is involved in a multitude of signaling pathways (El Mourabit u. a., 2004). Its differential use of its LIM-domains for different types of interactions enables it to collaborate with a multitude of partners (Kleiber u. a., 2007). So far, over 50 different proteins that directly interact with FHL2 have been identified. These include receptors, signal transducers, structural proteins, transcription factors and cofactors, metabolic enzymes and many more (Johannessen u. a., 2006). Among them is the WNT effector β-catenin (B. Martin u. a., 2002). The mechanisms by which FHL2 discriminates between different interaction partners still remain to be discovered.

1.5.1 FHL2 and TGF-β

Functional cooperation between TGF-β signaling and FHL2 has been established in various instances. The first report that investigated an interaction between FHL2 and TGF-β1 was a study by Govoni et al. on the regulation of the insulin-like growth factor binding

protein-5 (IGFBP-5). The study displayed an upregulation in FHL2's expression in the LSaOS human osteosarcoma cell line after stimulation with TGF-β1 (Govoni u. a., 2006). Later, Gullotti et al. performed intriguing cell culture assays that illustrated how TGF-β1 induced the expression of FHL2 on the protein level in murine fibroblasts, which consecutively underwent a differentiation into a myofibroblast-like phenotype with altered co-expression of α-SMA (Gullotti u. a., 2011). This finding resonates with the interests of this thesis as it prompts speculation about the possibility of a functional involvement of FHL2 in the transition of lung fibroblasts to myofibroblasts.

FHL2 and TGF-β could also be associated in EMT. In a study on the impact of FHL2 on EMT in colon cancer and the invasiveness of colon cancer cells, Zhang et al. established the first direct correlation between FHL2 and EMT and of FHL2 as novel interface for crosstalk between TGFβ and β-catenin-dependent WNT signaling. They found that FHL2 is overexpressed in metastatic colon cancer cells and illustrated how TGF-β1 can induce the expression of FHL2 in a dose-dependent manner in the DLD1 and SW480 human colon cancer cell lines and that FHL2 is able to induce EMT in a TGF-β1-dependent but Smad-independent manner via the downregulation of E-cadherin and the upregulation of the EMT markers vimentin and MMP-9. In their experiments on DLD1-cells, they could directly correlate FHL2 and EMT by illustrating that overexpression of FHL2 alone downregulated the expression of E-cadherin. The inhibition of FHL2 via siRNA resulted in a complete abation of the observed effects regardless of stimulation with TGF-β1 (Zhang u. a., 2010). As was mentioned before, E-cadherin is considered to be a master switch of EMT, and the downregulation of it results in inhibition of the formation of membrane-associated E-cadherin-β-catenin complexes with subsequent stabilization of the cytosolic β-catenin-pool. This allows for enhanced nuclear shuttling of the WNT-effector. The findings of Zhang et al. can hence be summarized as EMT caused by crosstalk between TGF-β-signaling and β-catenin-dependent WNT signaling with FHL2 as an indispensable crosstalk-interface.

The point here is that under specific circumstances, FHL2 appears to be a promoter

of TGF-β-signaling in at least two specific complex transitional cellular events, namely EMT and the transition of fibroblasts to myofibroblasts, which are deemed to be key events in the pathogenesis of IPF. This alone gives way to speculations about a functional relevance of the LIM-protein in IPF. In addition to that, it is evident that FHL2 has the potential to mediate crosstalk between TGF-β signaling and β-catenin-dependent WNT signaling.

1.5.2 FHL2 and WNT signaling

FHL2 and WNT signaling were first associated in a study by Martin et al. published in 2002. The group identified FHL2 as a novel binding partner for β-catenin and illustrated how FHL2 repressed β-catenin-mediated activation of the WNT-target cyclin D1 in C2C12 mouse myoblasts. They also found that FHL2 does not compete with LEF-1 for binding to β-catenin and implied the formation of a ternary complex in physiological settings (B. Martin u. a., 2002). In 2003, Wei et al. identified FHL2 as a coactivator of β-catenin *in vivo* as well as *in vitro* and showed that it amplifies the transactivating effect of the bipartite β-catenin/TCF-transcription factor complex on WNT-responsive genes like cyclin D1 and Interleukin-8 in kidney and colon cell lines (Wei u. a., 2003). A year later, the same group illustrated how FHL2 and the crucial WNT-target-gene-coactivators CBP/p300 synergistically enhanced β-catenin/TCF-mediated transcription from WNT-responsive promoters in 293 and SW480 cells (Labalette, Renard, Neuveut, Buendia, & Wei, 2004). This elucidates how FHL2 may either enhance or repress the effects of β-catenin-mediated transcription in a cell- and promoter-specific manner, including that of WNT-responsive genes, by the selective recruitment of different coactivators and corepressors (Johannessen u. a., 2006). In addition to the direct promotion of β-catenin-mediated transcription in the nucleus, FHL2 may also thwart the phosphorylation of β-catenin in DLD1-cells and SW480-cells with subsequent stabilization and nuclear accumulation of the WNT-effector molecule (Zhang u. a., 2010).

A functional relevance of the involvement of FHL2 in β-catenin-dependent signaling has been ascribed to different physiological as well as pathological scenarios. FHL2 appears to mediate the dexamethasone-induced differentiation of murine mesenchymal stem cells into osteoblasts via activation of WNT/β-catenin-signaling dependent expression of Runx2, a major osteoblast typifying gene (Hamidouche u. a., 2008, S. 2). Furthermore, β-catenin and FHL2 form a complex together with EpICD, the intracellular domain of the epithelial cell adhesion molecule EpCAM, in different epithelial cells in order to activate target genes that drive cell proliferation (Denzel u. a., 2009). FHL2 deficiency in APC mutant mice, the showpiece animal-model for WNT-mediated intestinal carcinogenesis, was shown to reduce intestinal tumorigenesis, implying an oncogenic function of FHL2 (Labalette et al., 2010). The productive relationship between the LIM protein and the armadillo protein has also been functionally implicated in murine cardiogenesis as well as murine osteosarcoma tumorigenesis, where FHL2 also acts as an oncogene through WNT signaling (Brun u. a., 2013; Renger u. a., 2013).

In summary, these findings show that FHL2 has the potential to modulate β-catenin-dependent WNT signaling via direct interaction with β-catenin in a multitude of physiological as well as pathological circumstances.

1.5.3 FHL2 and fibrosis

Little is known about the potential pathogenicity of aberrant FHL2 signaling. Most data concerning the involvement of FHL2 in a disease context stems from cancer research (Gullotti u. a., 2011; Labalette u. a., 2010; Zhang u. a., 2010). Various studies have illustrated how the molecule may promote or suppress tumor growth, depending on the tumor cell type (Ng u. a., 2011). Various studies on colon cancer illustrate pathological contexts in which FHL2 appears as non-redundant regulator of TGF-β1-dependent/SMAD-independent EMT and as an *in vitro* regulator of the TGF-β1 mediated transition of

fibroblasts to a myofibroblast phenotype by recruitment of α-SMA into stress fibers which enhances the motility and invasiveness of peritumoural fibroblasts (Gullotti u. a., 2011; Zhang u. a., 2010).

The aforementioned studies stem from cancer research, but there is other data that connects FHL2 to fibrogenesis and fibrosis. FHL2 is a regulator of fibrogenesis in tumor stroma as well as in wound healing. It is an early response gene that is significantly and rapidly upregulated in mesenchymal cells of wounded skin. FHL2$^{-/-}$-mice display prolonged healing of skin wounds. Wixler et al. accredited this observation to reduced contractile forces and decreased mobility of the granulation-tissue's myofibroblasts, which they ascribed to reduced transcriptional activation of α-SMA in the affected cells (Viktor Wixler u. a., 2007). This implies that FHL2 non-redundantly regulates the migration and contraction of myofibroblasts in wound healing and promotes transdifferentiation of cells into a contractile, myofibroblast-like phenotype (Huss u. a., 2013; Viktor Wixler u. a., 2007). Wounded tissue can to some extent be compared to fibrotic tissue, as both share common hallmark features like an excess deposition of extracellular matrix and myofibroblasts that express α-SMA (Wight & Potter-Perigo, 2011). Therefore, findings about the role of FHL2 in wound healing can to a certain extend relate to the role of FHL2 in fibrosis. FHL2-deficiency in mesenchymal cells from FHL2-knockout mice leads to a significant impairment of extracellular matrix protein assembly, impaired collagen contraction and reduced cell migration because of its' apparent crucial role in the organization of focal adhesion structures (Park u. a., 2008). In 2008, Kirfel et al. reported impaired intestinal wound healing in FHL2 deficient mice, which they ascribed to an impaired production of collagen type III (Kirfel u. a., 2008).

These studies show how FHL2 enhances fibrogenesis via its ability to uphold the expression of α-SMA and the excessive synthesis and assembly of matrix proteins in activated myofibroblasts (Alnajar u. a., 2013). However, there are other studies that speak of scenarios where FHL2 might act as a protective factor against excessive fibrogenesis.

Huss et al. displayed aggravated experimental liver fibrosis in FHL2$^{-/-}$-mice after CCl_4-treatment with an increased expression of collagen III, TGF-β β1 and laminin in liver homogenate. From this they inferred that FHL2 acts as a protective, anti-fibrotic factor during hepatic fibrogenesis. They also studied the expression of FHL2 fibrotic human livers and found a significant upregulation of the molecule in myofibroblastic activated human hepatic stellar cells and portal fibroblasts in comparison to healthy livers. They inferred that the molecule might be of relevance in human hepatic fibrosis (Huss u. a., 2013). In a study on the role of FHL2 in the development of BLM-induced lung fibrosis, Alnajar et al. demonstrated that FHL2$^{-/-}$-mice develop more severe lung pathology than wild type mice after BLM administration. This is of special interest to this thesis as the BLM mouse model is the standard animal model for IPF (Alnajar u. a., 2013; Scotton & Chambers, 2010). The implications of their findings for the role of FHL2 in actual IPF and how they relate to the results of this thesis will be further examined in the Discussion section.

In summary, the presented studies introduce FHL2 as a central regulator in cell contraction, ECM organization, myofibroblast migration, fibroblast-to-myofibroblast transdifferentiation and EMT. These properties infer that FHL2 plays a definitive role in wound healing, fibrogenesis and fibrosis in different physiological as well as pathological contexts in various organ systems and that it may differentially enhance or repress the fibrogenic process depending on the cellular context.

1.6 Objective

TGF-β and WNT signaling both play an essential role in the pathology of IPF. Although FHL2 may modulate both signaling chains in ways that are of relevance to IPF - affecting cell contraction, ECM organization, myofibroblast migration, fibroblast-to-myofibroblast transdifferentiation and EMT - little is known about FHL2's actual role in the disease. The study by the group around Alnajar on the role of FHL2 in BLM induced lung

fibrosis shows that FHL2 moderates the pathologic changes in the BLM mouse model. This comes as a surprise, given FHL2's positive involvement in the decidedly profibrotic signaling chains of TGF-β and WNT signaling. This study's aim was to widen the scope of knowledge on the matter and investigate whether and where exactly the expression of FHL2 is regulated in IPF and whether the molecule plays a role in the development and/or upkeep of the condition. The first part of the study was designed to establish an expression profile of FHL2 in IPF patients' lungs in comparison to that of healthy people's lungs via semiquantitative PCR, real time PCR, Western Blots and Immunohistochemistry. In case that a difference in FHL2 expression levels between sick and healthy individuals should be detected, the second stage's aim was to engage functional assays in order to address the question of causality, i.e. does the observed change illustrate a causal mechanism of the pathology or is it merely an epiphenomenon. The techniques of choice for this second part were to be Cloning, Luciferase Assays and enhanced phosphorylation Western Blots.

2 Materials and Methods

2.1 Materials

2.1.1 Incidentals

Cell culture flasks: 250 ml	Greiner Bio-One, Germany
Cell culture plates: 6, 48 wells	Greiner Bio-One, Germany
Film cassette	Sigma-Aldrich, Germany
Filter tips: 10, 20, 100, 200, 1000 µl	Nerbe Plus, Germany
Glass bottles: 250, 500, 1000 ml	Fisher, Germany
MicroAmpTM 96-Well reaction plates	Applied Biosystems; USA
MicroAmpTM optical adhesive film	Applied Biosystems, USA
Parafilm	Alcan Packaging, USA
Pipette tips: 100, 200, 1000 µl	Sarstedt, Germany
Pipette tips: 10 µl	Gilson, USA
Serological pipets: 5, 10, 25, 50 ml	BD Falcon, USA
Test tubes: 15, 50 ml	Greiner Bio-One, Germany
Test tubes: 0,8, 1,5, 2 ml	Sarstedt, Germany
Trans Blot transfer medium	Bio-Rad, USA
X-Omat, scientific imaging film	Kodak, USA

2.1.2 Devices

Autoclave: 2540 EL	Systec, Germany

Balances: CP255D, CP6201	Sartorius AG, Germany
Centrifuges:	
Mini Spin Plus	Eppendorf, Germany
Spin	Promega, USA
Biofuge fresco	Heraeus, Germany
Multifuge 3SR	Heraeus, Germany
CO2 Incubator: Cytoperm 2	Heraeus, Germany
Digital camera: Kodak DC290	Kodak, USA
DNA Engine Dyad® Peltier Thermal Cycler	MJ Research, USA
Film processor: Kodak X-Omat 2000	Kodak, USA
pH-meter: pH/Cond Level 1 SET	inoLab, Germany
Freezer -20 ºC	Bosch, Germany
Freezer -80 ºC	Heraeus, Germany
Fridge +4 ºC	Bosch, Germany
Light microscopes: DMIL, DMLA	Leica, Germany
Magnetic stirrers:MR 3000, MR 3001K	Heipolph Instruments, Germany
Mobile pipettor: Pipetboy acu	Integra Biosciences, Germany
Nucleuic acid electrophoresis: Wide Mini Subcell GT	Bio-Rad Laboratories, USA
Pipettes: 2,5, 20, 100, 1000 µl	Eppendorf, Germany
Pipettes: 10, 20, 100, 200, 1000 µl	Gilson, USA
Power supply: Power Pac 200	Bio-Rad Laboratories, USA

Protein electrophoresis:	Bio-Rad Laboratories, USA
Mini Protean Electrophoresis System 3	
Spetrophotometry, Luminometry:	
FusionTM Universal Microplate Analyzer	Packard BioScience, USA
SmartSpecTM 3000	Bio-Rad Laboratories, USA
Thermal cyclers:	
Sequence Detection System 7700	PE Applied Biosystems, USA
DNA Engine Dyad	MJ Research, USA
Vortex machine	Merck, Germany
Water baths and thermostats:	
Polystat CC1-105A	Huber, Germany
E100	Lauda, Germany
HBT 130	HLC, Germany
Water purification:	Millipore, USA
Elix® 5, Milli-Q® Biocel A10	

2.1.3 Reagents

Agar	Invitrogen, USA
Agarose CE, analytical grade	Promega, USA
Ammonium persulfate	Promega, USA
Ampicillin	Sigma-Aldrich, Germany
Quick StartTM Bradford reagent	Bio-Rad, USA
Carnation milk powder	Nestlé, Switzerland

CompleteTM (Protease inhibitor cocktail tablets)	Roche, Switzerland
Dual colour precision protein standards	Bio-Rad, USA
Dulbecco's modified eagle's medium	Gibco/Invitrogen, USA
Dulbecco's phosphate buffered saline (PBS)	PAA Laboratories, Austria
ECL western blotting detection reagents	Pierce, USA
Ethanol 70%	Stockmeier Chemie, Germany
Ethanol absolute	Riedel de Haen, Germany
Ethidium bromide	Carl Roth, Germany
Fetal calf serum	PAA Laboratories, Austria
Glycin	Carl-Roth, Germany
GoTaq® Flexi DNA Polymerase	Promega, USA
Gylcerol	Promega, USA
Haematoxilin	Sigma-Aldrich, Germany
Hydrochloric acid	Sigma-Aldrich, Germany
Oligo(dT)15 Primer	Promega, USA
Opti-MEM medium	Gibco/Invitrogen, USA
Histostain® SP Kit	Zymed, USA
IPTG	Promega, USA
Isopropanol	Merck, Germany
Kanamycin	Sigma-Aldrich, Germany
Lipofectamine 2000	Invitrogen, USA
Luciferase Assay System	Promega, USA

Luria broth base (Miller's)	Invitrogen, USA
M-MLV Reverse Transcriptase	Promega, USA
Methanol	Fluka, Switzerland
PCR nucleotide mix	Promega, USA
Platinum® SYBR® Green qPCR SuperMix	Invitrogen, USA
PureYieldTM plasmid midiprep system	Promega, USA
Restriction enzymes: EcoRV, XhoI	Promega, USA
RNA purification system Roti®-Quick-Kit	Carl Roth, Germany
Rnase Zap	Sigma-Aldrich, Germany
RNeasy Mini Kit	Qiagen, Germany
Rotiphorese gel 30	Carl-Roth, Germany
Sodium dodecyl sulphate (SDS) solution 10%	Promega, USA
T4 DNA ligase	Promega, USA
TEMED	Promega, USA
TGF-β1	R&D Systems Inc., USA
Tris	Carl-Roth, Germany
Trypsin	Gibco/Invitrogen, USA
Trypsin-EDTA	PAA Laboratories, Austria
Tween 20	Promega, USA
X-Gal	Promega, USA
β-Mercaptoethanol	Sigma-Aldrich, Germany

2.2 Methods

2.2.1 Human material

All used human lung tissue samples (i.e. lung homogenates, cultured primary cells, microscope prepared slides) were taken from a pool of 400 lung samples provided by the faculty of medicine of the Justus-Liebig-University of Gießen. The probes were obtained from lung transplantations and partial lung resections that were executed either in Gießen or the University of Vienna. All probes were anonymized. The study protocol was approved by the Ethics Committee of the faculty of medicine in Gießen (AZ 31/93). Informed consent was obtained from each subject for the study protocol.

2.2.2 RNA isolation

Total RNA from lung tissue was isolated using the total RNA purification system Roti®-Quick-Kit according to the manufacturer's instructions. Total RNA from cultured human fibroblasts was isolated using the RNeasy Mini Kit according to the manufacturer's instructions.

2.2.3 Reverse transcription

Reverse transcriptase is an enzyme that uses RNA as a template for the synthesis of complementary DNA (cDNA). This feature is used in the process of Reverse transcription polymerase chain reaction (RT-PCR) in order to acquire cDNA that can be used for further PCR experiments. RT-PCR was performed as follows:

Components:	Volume/ Amount:
RNA of interest	500 ng

oligo (dT)15 (100 µg/ml)	1 µl
Nuclease free water	add up to 14 µl

This reaction batch was heated to 70 °C for 5 minutes and subsequently cooled at 4 °C for 5 minutes. Afterwards, the following components were added:

Components:	Volume/Amount:
M-MLV 5 × reaction buffer	5 µl
PCR nucleotide mix	1,25 µl
M-MLV Reverse transcriptase	200 units
Nuclease free water	add up to 25 µl

For reverse transcription and amplification, the reaction batch was incubated at 25 °C for 5 minutes and at 42 °C for 1 hour.

2.2.4 Polymerase chain reaction

The polymerase chain reaction (PCR) is a technique that allows for the millionfold amplification of a specific DNA sequence in vitro. This synthesis is accomplished via DNA-dependent DNA polymerases. The technique has been in widespread use for decades and has experienced great advancements over time. The basic PCR cycle has remained the same:

1. Denaturation: separation of the DNA double strands
2. Annealing: binding of primers to the target sequence

3. Elongation: synthesis of a new DNA strand

All primers that were used in PCR reactions are listed in the appendix.

2.2.4.1 Semiquantitative PCR

This is the basic form of PCR in which a fixed amount of cycles is used for amplification and the end products are analyzed. It is not possible, though, to analyze changes in quantity of the synthesized DNA in between cycles. This entails the possibility, that within the preset cycles the amplification processes exceed the phase of exponential DNA amplification, in which there is a linear relationship between the number of amplification cycles and the logarithm of the number of molecules. Amplification efficiency would decrease and result in a plateau effect. Therefore, extensive optimisation of the number of PCR cycles is required when performing this method. Reliable quantification of target abundance is furthermore impeded by other disruptive factors such as substrate exhaustion, competitive reactions and target reannealing. With respect to these limitations, this basic PCR technique is mainly used for the qualitative evaluation of examined material. Nevertheless, this is still a widely used technique due to its ease of use, low costs, its relative insensitivity to contamination and the possibility to quickly gain insight on what direction to take when facing a number of choices. HSC70, an ubiquitously and equally expressed gene that is free of pseudogenes, was used as the reference gene in all semiquantitative PCR reactions. PCR reaction mixes were prepared on ice as follows:

Components:	Volume/Amount:
5 × GoTaq Green buffer	10 µl
25 mM MgCl2	5 µl

10 mM dNTP mix	1 µl
10 µM forward primer	1 µl
10 µM reverse primer	1 µl
cDNA template	1 µl
GoTaq® Flexi DNA polymerase	0,25 µl
H2O (autoclaved)	30,75 µl

The DNA Engine Dyad® Peltier Thermal Cycler was set to 22 – 30 cycles, depending on the amplified sequence and the cycle-steps were programmed as follows:

Step:	Temperature:	Time:
1. Denaturation:	94 ºC	1 min
2. Annealing:	56 ºC - 60 ºC	1 min
3. Elongation:	72 ºC	2 min

The annealing temperature was optimized for each pair of primers.

2.2.4.2 Real time polymerase chain reaction

This advanced modification of the basic PCR technique allows for the simultaneous amplification and quantification of specific DNA sequences during each cycle. This is made possible by the use of either DNA binding fluorescent dyes or fluorescently labeled oligonucleotide probes in combination with thermal cyclers that detect fluorescence after each completed PCR-cycle. For the real time PCRs in this thesis, SYBR® Green I DNA binding fluorescent dye was used according to the manufacturer's instructions. HPRT, an

ubiquitously and equally expressed gene that is free of pseudogenes, was used as the reference gene in all real time PCR reactions. The reaction mixes were prepared on ice as follows:

Real time PCR mix:	
Platinum® SYBR® Green qPCR SuperMix UDG	13 µl
50 mM MgCl2	1 µl
10 µM forward primer	0,5 µl
10 µM reverse primer	0,5 µl
cDNA template	1 µl
H20 (autoclaved) to	25 µl

The Sequence Detection System 7700 was set to 45 cycles and programmed as follows:

Step:	Temperature:	Time:
1. Denaturation:	95 ºC	5 s
2. Annealing:	60 ºC	5 s
3. Elongation:	72 ºC	30 s

The relative transcript abundance of a gene was expressed in ΔCt values (ΔCt = Ctreference – Cttarget). Relative changes in transcript levels compared to controls were

expressed as ΔΔCt values (ΔΔCt = ΔCttreated – ΔCtcontrol). All ΔΔCt values correspond approximately to the binary logarithm of the fold change.

2.2.5 DNA agarose gel electrophoresis

Agarose gel electrophoresis was performed in order to separate and analyze DNA fragments obtained by PCR. The percentage of the gels varied between 1 – 2 %, depending on the size of the DNA amplicons. The gels consisted of agarose mixed with 1 × Tris-acetate-EDTA (TAE) buffer and 0,5 µg/ml ethidium bromide, a DNA intercalating dye that ultimately enables the visualization of DNA fragments via ultraviolet flourescence. Before loading the amplicons were mixed with 6 × DNA loading buffer. The electrophoresis was performed at 100 V/cm in 1 × TAE buffer.

1 × TAE buffer:	6 × DNA loading buffer:
40mM Tris-acetate, pH 8,0	0,025 % (w/v) bromophenol blue
1mM EDTA, pH 8,0	40 % (w/v) sucrose

2.2.6 Protein isolation

2.2.6.1 Protein isolation from human tissue

Lung tissue was preserved in liquid nitrogen until further processing. For protein isolation, the tissue was ground to powder under liquid nitrogen while simultaneously ice-cold lysis buffer was added. This lysate was then passed repeatedly through a 0,9 mm gauge needle fitted to a RNAse free syringe and afterwards incubated on ice for 30 min for complete lysis. Then it was centrifuged for 15 min at 15.000 g at 4 ºC. The resulting supernatant protein was quantified and stored at -20 ºC.

Tissue lysis buffer:

20 mM Tris-HCl, pH = 7,5

150 mM NaCl

1 mM EDTA

1 mM EGTA

1 % Trition X-100

2,5 mM Na3PO4

1 mM β-glycerophosphate

1 mM Na3VO4, phosphates inhibitor – added immediately prior homogenization

Complete™, protease inhibitor mix – added immediately prior homogenization

2.2.6.2 Protein isolation from cells

When confluency was reached, cell monolayers were washed twice with ice-cold 1 × PBS and cell lysis buffer was immediately applied. The cells were then detached by scraping with a rubber policeman and transferred into 1,5 ml microfuge tubes. These lysates were vortexed every 5 min for 40 min and sequentially centrifuged for 15 min at 13.000 × g at 4 ºC. The resulting supernatant protein was quantified and stored at -20 ºC.

Cell lysis buffer:

20 mM Tris-HCl, pH = 7,5

150 mM NaCl

1 mM EDTA

1 mM EGTA

0,5 % Igepal CA-630

1 mM Na3VO4, phosphates inhibitor – added immediately prior to homogenization

Complete™, protease inhibitor mix – added immediately prior to homogenization

2.2.6.3 Protein quantification

The concentrations of protein lysates were determined via spectrophotometry using Quick Start™ Bradford Dye Reagent and a FusionTM Universal Microplate Analyzer according to the manufacturer's instructions. Absorbance was measured at 570 nm.

2.2.7 SDS polyacrylamide gel electrophoresis

The analytical separation of proteins by their molecular weight was accomplished through SDS polyacrylamide gel electrophoresis (SDS-page). For loading, 30 µg of protein were mixed with 10 × SDS-loading buffer and denaturated by heating for 5 minutes at 95 °C. The gels consisted of 10 % resolving gel and 5 % stacking gel. The electrophoresis was performed in SDS running buffer.

Resolving gel:	Stacking gel:
10 % acrylaminde/bisacrylamide	5 % acrylamide/bisacrylamide
375 mM Tris-HCl, pH = 8,8	125 mM Tris-HCl, pH = 6,8
0,1 % (w/v) SDS	0,1 % (w/v) SDS

0,1 % (w/v) APS	0,1 % (w/v) APS
0,1 % (v/v) TEMED	0,1 % (v/v) TEMED

10 × SDS-loading buffer:	SDS-running buffer:
625 mM Tris-HCl, pH = 6,8	25 mM Tris-HCL, pH = 8,3
50 % (v/v) glycerol	250 mM glycine
20 % (w/v) SDS	0,1 % (w/v) SDS
9 % (v/v) b-mercaptoethanol	
0,3 % (w/v) bromophenol blue	

2.2.8 Western blot (Immunoblot)

This method allows the visualisation of a specific protein in a given sample of protein extract or tissue homogenate. After the proteins are separated by electrophoresis they are transferred to a membrane (usually nitrocellulose or polyvinylidene difluoride). Specific primary antibodies are used to target the antigen of interest and specially labeled secondary antibodies targeting the primary ones finally allow visualisation of the sought-after protein. The results are recorded on x-ray film. All antibodies used for the Western Blots of this thesis are listed in the appendix.

2.2.8.1 Blotting and analysis

The proteins separated by electrophoresis were transferred to a nitrocellulose membrane using a Mini Protean Electrophoresis System 3 containing transfer buffer. The transfer was performed at 120 V for 1 h.

For the purpose of protein detection, the blots were incubated in blocking buffer for 1 h at room temperature and thereupon incubated with the respective primary antibody in blocking buffer at 4 ºC overnight. They were then washed three times for 10 min in PBST washing buffer and then incubated with the respective horseradish peroxidase-labeled secondary antibody in blocking buffer for 1 h at room temperature. After three 10 minute washes in PBST the blots were developed using an Enhanced Chemiluminescence Immunoblotting system and visualised with scientific imaging film.

Transfer buffer:

24 mM Tris

193 mM glycine

10 % (v/v) methanol

Blocking buffer:

5 g non fat dry milk powder

PBST buffer up to 100 ml

PBST washing buffer:

PBS

0,1 % (v/v) Tween 20

2.2.8.2 Phospho-Western Blots

For phospho-Western Blots, NIH-3T3 cells were plated on 6-well plates at 150.000 cells/well and cultured in DMEM containing 10% FCS. When confluence of a maximum of 60% was reached, the cells were transfected with either 2 mg/well of empty pcDNA3.1(+) plasmid or 2 mg/well of FHL2 pcDNA3.1(+) construct using Lipofectamine 2000 at the concentration of 1 µl of Lipofectamine per 3 µg of DNA construct. Afterwards, the cells were cultured for 24 hours in DMEM containing 10% FCS and were finally treated with TGF-β1 (2

ng/ml, R&D Systems Inc., Minneapolis, MN) for 0, 0,5 and 2 hours. After the stimulation, cells were harvested and protein extraction was performed. Adjacent Western Blot analysis was performed as previously described. The experiment was repeated three times.

2.2.9 Molecular cloning

Molecular cloning is the process of creating multiple copies of a defined DNA fragment in vivo by isolating it from its source and inserting it in a self-replicating vector such as a bacterial plasmid. A great variety of specialized cloning vectors makes this method useful for a wide array of applications such as epitope tagging, creation of single-stranded DNA and RNA and protein expression. Today, molecular cloning is widespread and utilized in a multitude of biological experiments. All cloning protocols share the same basic steps:

1. Restriction: Isolation of the DNA region of interest
2. Ligation: Insertion of the DNA fragment into the vector
3. Transfection: Transfer of the construct into a host-cell
4. Selection: Identification and isolation of successfully transformed host cells for further processing

Steps 1 – 3 are generally of low efficiency, making a scrupulous selection imperative. Specialized vectors have been developed to address this problem deploying several strategies such as insertion-dependent color-screening or insertion-dependent antibiotic resistance. The amplified DNA region can then be transferred to an expression vector that is designed to actually express the recombinant protein of interest. This process is referred to as subcloning. The prepared expression vector is then transfected into a host cell where the actual experiment takes place. Plasmids that contain an insert will from now on be referred to as constructs.

2.2.9.1 DNA fragment retrieval

The DNA segment of interest was amplified by semiquantitative PCR and run on an agarose gel as previously described. Bands of the expected size were excised from the gel and purified using the Wizard® SV Gel and PCR Clean-Up system according to the manufacturer's instructions.

2.2.9.2 Ligation into the pGEM®-T Easy vector

The pGEM®-T Easy vector offers the possibility of color-screening for recombinants. It contains the lacZ gene which encodes for the enzyme β-galactosidase. When a DNA fragment is successfully inserted into the vector this lacZ gene is disrupted and expression of β-galactosidase abates. X-gal is a galactoside that yields, among others, an insoluble blue product when cleaved by β-galactosidase. X-gal and an inducer of β-galactosidase, in this case IPTG, are added for color-screening to the agar medium used for the culturing of the host strain, in this case E. coli DH5α. In result, bacterial colonies that contain no insert can be easily identified by their blue color. The pGEM®-T Easy vector also encodes ampicillin resistance of the host cell. As a result, growth of colonies that contain no vector is prevented when ampicillin is added to the culture medium. It contains 3'-T overhangs at both ends which prevent premature recircularization and are complementary to the 3'-A overhangs of a PCR product generated by the GoTaq® Flexi DNA Polymerase. This greatly enhances the efficiency of ligation. These traits combined make the pGEM®-T Easy vector an excellent candidate for the cloning of a PCR product. The ligation, positive control and background control were performed as follows:

pGEM®-T Easy-Ligation	Standard Reaction	Positive Control	Background Control
2 × Rapid Ligation Buffer	5 µl	5 µl	5 µl
pGEM®-T Easy vector	1 µl	1 µl	1 µl
PCR product	3 µl	-	-
Control Insert DNA	-	2 µl	-
T4 DNA Ligase (3 Weiss U/µl)	1 µl	1 µl	1 µl
H20 (autoclaved, deionised) to	-	10 µl	10 µl

The mixtures were incubated overnight at 4 ºC and transformed into competent E. coli DH5α for amplification.

2.2.9.3 Subcloning into expression vectors

The pGEM®-T Easy constructs served as a source of inserts which were to be subcloned into the mammalian expression vector pcDNA3.1(+). For this purpose, a fragment containing the sequence of interest was excised from the pGEM®-T Easy vector using the restriction enzymes EcoRV and XhoI and ligated into the pcDNA3.1(+) expression vector that had been previously digested by the same restriction enzymes, thus obtaining sticky ends. Restriction digestion and consecutive agarose gel electrophoresis was also done in order to analyze transformants for the presence of an insert. The digestions were performed as follows:

Digestion reaction mix:	pGEM®-T Easy	Expression vectors
Construct / Plasmid	50 µl	25 µl
EcoRV	6 µl	3 µl
XhoI	6 µl	3 µl
10 × restriction endonuclease buffer	15 µl	7 µl
H20 (autoclaved, deionised) to	150 µl	70 µl

The reaction mixtures were incubated overnight at 4 ºC and then separated by agarose gel electrophoresis. The bands of interest were excised, gel purified (see 1.2.8.1) and then ligated as follows:

pcDNA3.1(+) ligation:	
Prepared vector	1 µl
Prepared insert	3 µl
2 × Rapid Ligation Buffer	5 µl
T4 DNA ligase (3 Weiss U/µl)	1 µl
Final volume	10 µl

The mixtures were incubated overnight at 4 ºC and transformed into competent E. coli DH5α.

2.2.10 Bacterial cell culture

2.2.10.1 Preparation of agar plates

7,5 g of LB medium and 4,5 g of agar were added to 300 ml of distilled water and autoclaved. After cooling to 50 ºC, the respective antibiotic was added, and the mixture was plated in sterile petri dishes. 50 µg/ml of ampicillin were added. After solidification the dishes were stored at 4 ºC in the dark. For the culturing of bacteria transformed with pGEM®-T Easy plasmid DNA, 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-Gal were spread over the surface of ampicillin-treated plates and allowed to absorb at room temperature.

2.2.10.2 Preparation of competent bacteria

Competence is the ability of a cell to take up extracellular DNA. Bacteria of the E. coli DH5α strain were chemically treated to competency by employing the Hanahan method for high efficiency transformation and stored at -80 ºC (Sambrook & Russell, 2006).

2.2.10.3 Transformation of plasmid DNA into competent E. coli cells

An aliquot of competent E. coli DH5α was slowly thawed on ice. 60 µl of bacteria were mixed with 3 µl of construct DNA and incubated on ice for 20 min. The mixture was then heat pulsed at 42 ºC for 45 seconds and immediately returned to ice for 2 min. 950 µl of SOC medium were added, and the reaction mix was further incubated at 37 ºC for 1,5 h, shaking at 150 rpm. Then 100 µl of the mixture were plated onto duplicate agar plates and incubated overnight at 37 ºC.

SOC medium:

Bacto®-trytone	2 g
Bacto®-yeast extract	0,5 g
1 M NaCl	1 ml
1 M KCl	0,25 ml
2 M Mg2+	1 ml
2 M glucose	1 ml
H20 (autoclaved) to	100 ml

2.2.10.4 Preparation of transformed bacteria and construct retrieval

The transformed bacteria were plated onto duplicate agar plates and incubated overnight at 37 ºC. The selected colonies were then taken from the plates, inoculated into 5 ml of LB medium containing the respective antibiotic and incubated overnight at 37 ºC with shaking.

Isolation of construct DNA on a small scale was performed using the QIAprep Spin Miniprep Kit according to the manufacturer's instructions. Isolation on a larger scale was performed using a QIAprep Spin Maxiprep Kit according to the manufacturer's instructions. The resulting DNA concentrations were established by UV spectrophotometry with absorbance measured at 260 nm wavelength.

2.2.11 Mammalian Cell Culture

NIH-3T3 murine fibroblast cells were maintained in cell culture dishes in Dulbecco's modified Eagle's medium (D-MEM) containing 10% FCS. They were stored at 37 ºC in the

Cytoperm 2 incubator in 5% (v/v) CO2 and 95 % (v/v) air humidity. Each cell line was passaged before reaching 80% confluency. In order to do so, the cells were washed twice with ice cold PBS and detached from the culture dish by applying 3 ml of trypsin/EDTA solution for 2 – 3 min. The enzymatic reaction was blocked by the addition of 7 ml D-MEM containing 10% FCS and the cell suspension was split between new culture dishes at a dilution of 1/5 (cell suspension / D-MEM).

Trypsin/EDTA:

0,25% (w/v) trypsin

1,23 g/L EDTA

2.2.12 Transient Transfections

A transient transfection is the process of inoculating foreign DNA into a eukaryotic host cell. The insert is not inserted into the host's genome and therefore ignored by the cell's replication machinery. LipofectamineTM 2000 was used for all transfections carried out in this work. The respective cells were plated at a density of 20.000/cm2 and cultured in DMEM containing 10% FCS 24 h prior to the transfection, so that they would reach 90-95% confluence for the experiment. Appropriate amounts of DNA and Lipofectamine were separately diluted in 50 µl of Opti-MEM® reduced serum medium (DNA/Lipofectamine-ratio: 1 µg / 3 µl) and incubated for 5 min at room temperature. The mixtures were then combined and incubated for another 20 min at room temperature, allowing the components to form complexes. After metabolized medium was removed from the plated cells, they were covered with the transfection mixture (100 µl/well in a 48 well plate, 1 ml/well in a 6 well plate). The plates were rocked and incubated for 4 – 6 h at 37 ºC, then the transfection mixture was removed and replaced by DMEM containing 10% FCS. The cells were then incubated for 12 h at 37 ºC before further processing.

2.2.13 Luciferase Assays

Luciferase is a collective term for enzymes that enable its bearer to convert chemical energy into light, i.e. bioluminescence. This trait can be utilized in genetic engineering by linking the luminescent reaction to the process of interest, e.g. transfecting a luciferase-encoding reporter gene whose transcriptional activity is enhanced by the investigated molecule. This way, a positive correlation between the relative luminescence of the transfected cells and the amount of the investigated molecule contained within them is established.

NIH-3T3 cells were plated on 48-well plates at 15.000 cells/well and cultured overnight in DMEM containing 10% FCS. 0,15 mg/well of either FHL2 pcDNA3.1(+) plasmid or ACT pcDNA3.1(+) plasmid were then co-transfected together with 0,15 mg/well of p(CAGA)12 reporter plasmid, a reporter gene whose luciferase expression is enhanced by Smad-dependent TGF-β signaling. The transfections were performed as described above. Analysis was performed using the Luciferase Assay System according to the manufacturer's instructions. The experiment was repeated four times.

2.2.14 Immunohistochemistry

This method allows for the localization of selected proteins in tissue sections. Specific antibodies are used to bind the antigen of interest and are themselves bound by secondary antibodies, which can be prepared in different ways in order to enable visualisation.

Protein localization was assessed using a Histostain® SP Kit using the Labeled-(strept) Avidin-Biotin (LAB SA) method according to the manufacturer's instructions. Paraffin-embedded tissue sections were first de-waxed by immersion in xylene for 3 × 10 min. and subsequently rehydrated by immersing them in ethanol (2 × 5 min in 70%, 2 × 5 min in 95% and 2 × 5 min in 100%). Cooking the slides in citrate buffer for 20 min and

adjacent 10 min of warming caused antigen retrieval. The sections were then immersed in 3% (v/v) H_2O_2 for 20 min in order to block the endogenous peroxidase activity. They were blocked with reagents provided with the Histostain® SP Kit in order to prevent non-specific antibody-binding. Finally, the sections were incubated with the primary antibody overnight at 4 ºC. On the second day, the sections were treated with the secondary antibodies and other reagents from the Histostain® SP Kit according to the manufacturer's instructions. All the aforementioned steps were complemented by 2 × 5 min washes with PBS. Haematoxilin was used for 5 min of counterstaining. Finally, the sections were washed under tap water for 10 min and mounted using mounting medium.

2.2.15 Statistical analysis of data

Values are presented as mean ± SD, unless otherwise noted. Group comparisons were made using an unpaired, two-tailed Student's t-test for normally distributed data. A level of $p < 0.05$ was considered statistically significant.

3 Results

3.1 The expression of FHL2 mRNA is elevated in IPF

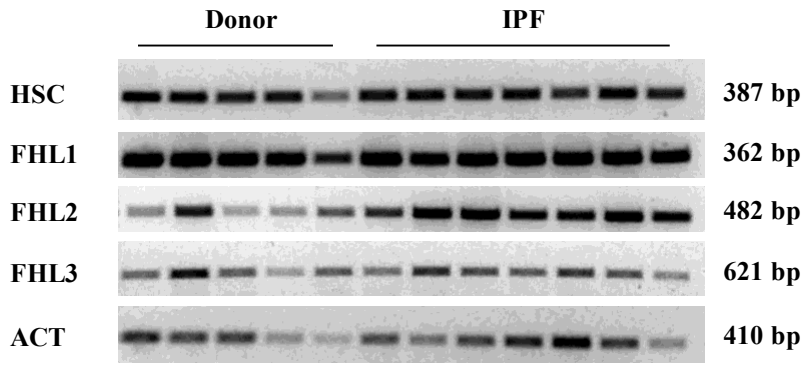
The expression of FHL1, FHL2, FHL3 and ACT in lung homogenates of IPF patients was compared to that of healthy transplant donors as controls. No attempt has been made to search for expression of FHL4 as expression studies performed by other groups have found that there is no detectable expression of FHL4 in the lung (Morgan & Madgwick, 1999).

Semi-quantitative RT-PCR results displayed a significant upregulation of FHL2 in IPF lungs accompanied by no significant change in the expression of the other examined molecules (Figure 3.1 A). Densitometric analysis was performed and ascertained the significant difference in the expression of FHL2 (Figure 3.1 B). Quantitative PCR analysis confirmed these results, and rendered the upregulation of FHL2 in IPF lungs to be 3.25 fold compared to donor lungs (Figure 3.2).

3.2 The expression of FHL2 protein is elevated in IPF

Western Blot analysis of FHL2 expression in lung homogenates of IPF patients also revealed a significant upregulation of FHL2 on the protein level, when compared with that of healthy transplant donors. Surprisingly, this effect appeared to be even more impressive on the protein level than on the mRNA level (Fig. 3.3).

A.



B.

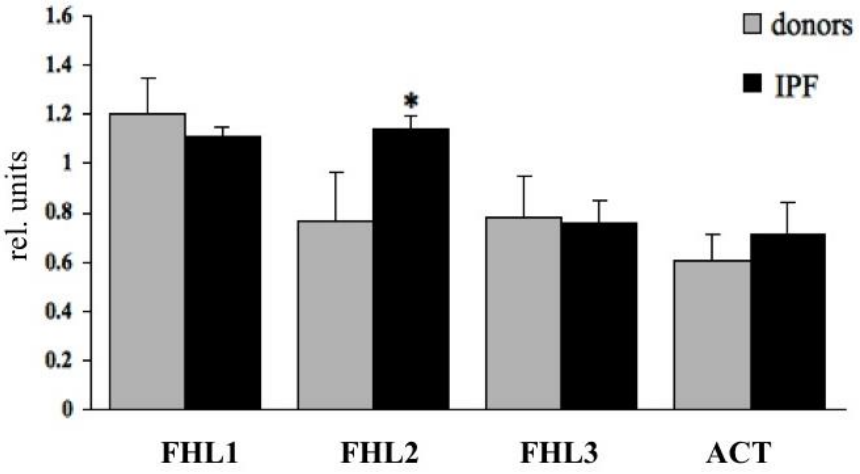


Figure 3.1: Expression of FHL1, -2, -3 and ACT in human lung homogenate from transplant donors and IPF patients
A, Semiquantitative RT-PCR analysis of FHL-1, -2, -3 and ACT expression. B, Densitometric analysis thereof (grey bar: donors, black bar: IPF). Expression of HSC served as loading control. *, $p < 0.01$. Data are representative of three independent experiments.

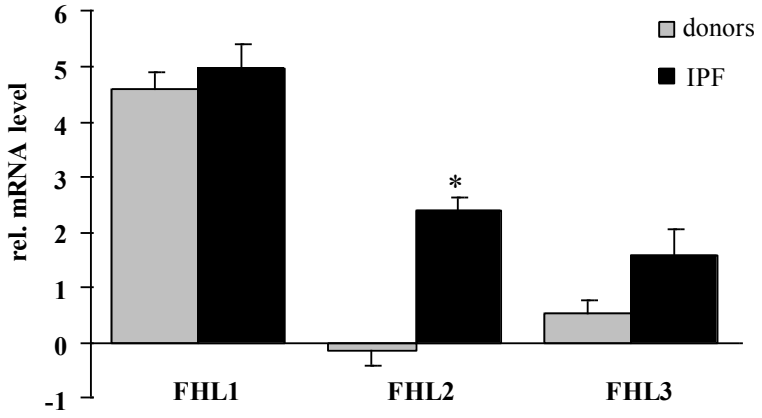


Figure 3.2: Quantitative RT-PCR analysis of FHL1, -2 and -3 expression
(n = 5 for each, grey bar: donors, black bar: IPF). *, p < 0,00005. Expression of HPRT served as loading control. Data are representative of three independent experiments.

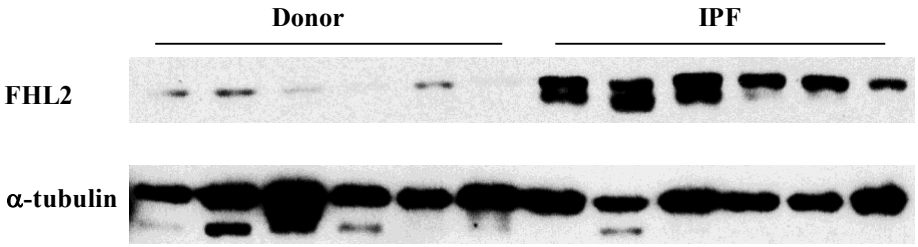


Figure 3.3: Western Blot analysis of FHL2 expression in donor lungs and IPF lungs
Expression of α-tubulin served as loading control. Data are representative of three independent experiments.

3.3 FHL2 localizes to fibroblasts and AECs in IPF

The expression analyses suggested an increase of FHL2 mRNA expression as well as of FHL2 protein amount in IPF lungs compared to lung-healthy controls. In order to verify these findings and to compare the expression pattern of FHL2 in two populations, immunohistochemistry was performed on healthy (donor) and IPF-patient lung sections. The sections were screened for specific localization of FHL2 to particular cell types. In both groups, FHL2 was found to reside in fibroblasts, as well as in bronchial and alveolar epithelial cells.

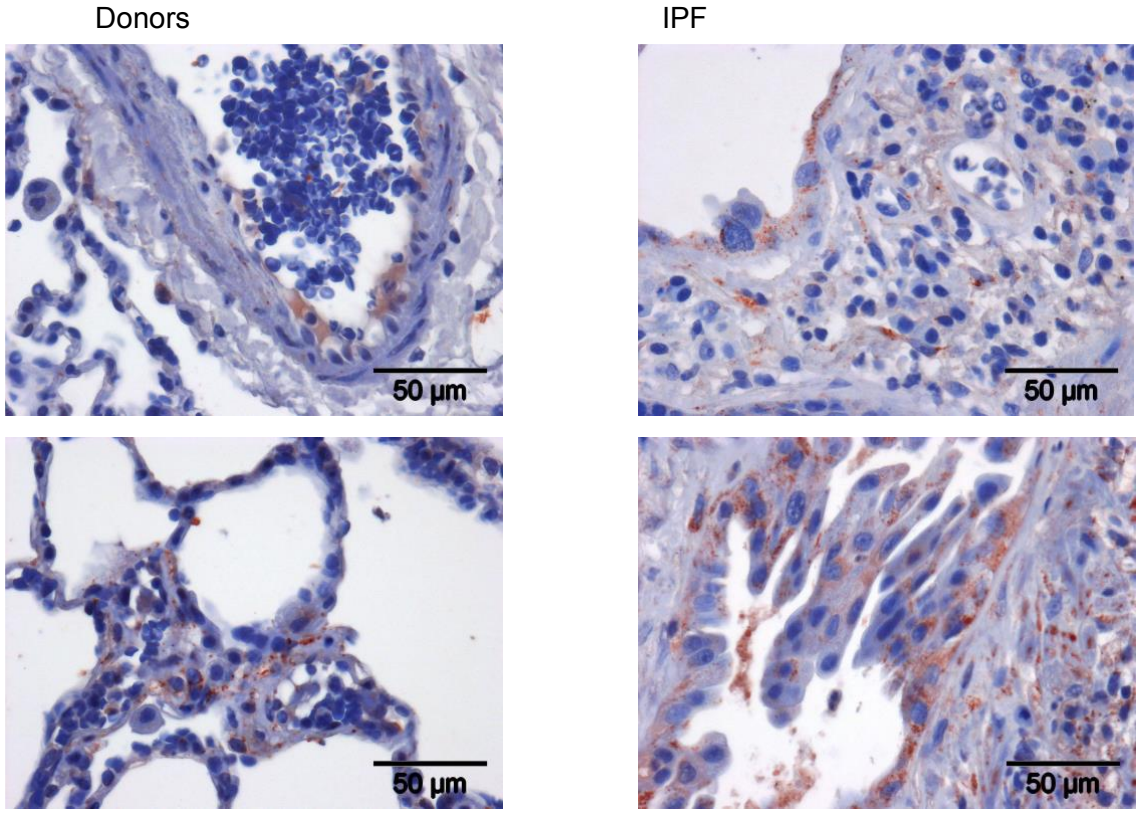
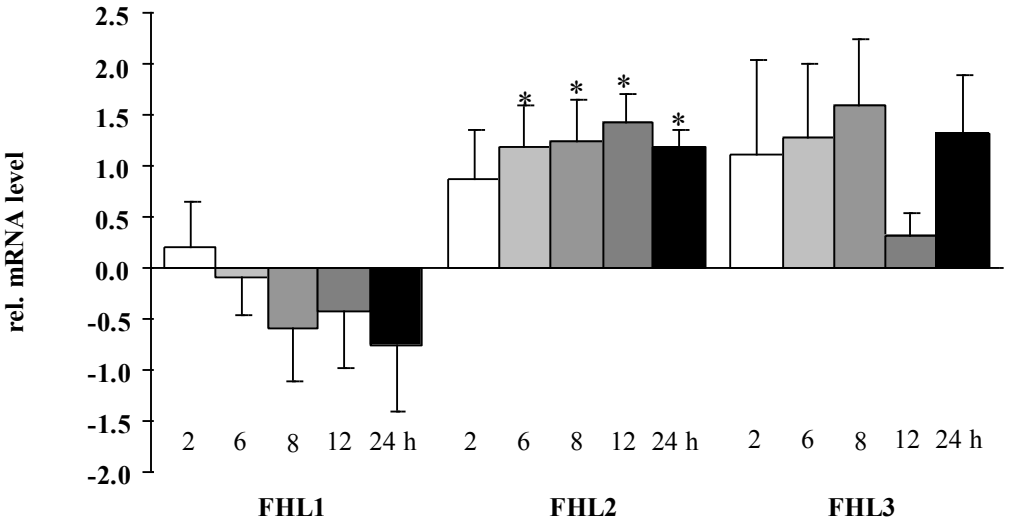


Figure 3.4: Immunohistochemical localization of FHL2 protein in lungs of donors and patients with IPF
FHL2 localized to fibroblasts and to bronchial and alveolar epithelial cells in healthy lungs as well as in IPF-lungs, as documented by immunohistochemistry.

3.4 TGF-β1 enhances the expression of FHL2 in primary human lung fibroblasts

TGF-β1 has the potential to influence FHL2 activity in various cellular contexts. Zhang et al. have published data on how TGF-β1 can dose-dependently induce FHL2 in the human colon adenocarcinoma cell lines DLD1 and SW480, and Gulotti et al displayed how TGF-β1 induces the expression of FHL2 on the protein level in murine fibroblasts (Gullotti u. a., 2011; Zhang u. a., 2010). To elucidate the regulatory mechanisms of FHL2 expression in human lung fibrosis and address the question whether TGF-β1 also affects the expression of FHL2 in primary human lung fibroblasts, primary human lung fibroblasts were stimulated with TGF-β1 (2 ng/ml) for up to 24 h, and checked for subsequent expression of FHL2, FHL1 and FHL3 via qPCR (Figure 3.4 A). As a result of this, TGF-β1 significantly increased FHL2 mRNA expression, starting with a 1,5-fold increase 6 hours after treatment. The expression profiles of FHL1 and -3 remained unaffected. Western Blots were performed with lysates of these TGF-β1-stimulated hFBs in order to analyze whether this increase in expression actually translated into the protein domain. The result was that the TGF-β1-dependent upregulation is even more impressive and more swift on the protein level, with a significant increase in FHL2 protein levels starting earlier than mRNA levels at 2 hours after treatment (Figure 3.4 B).

A.



B.

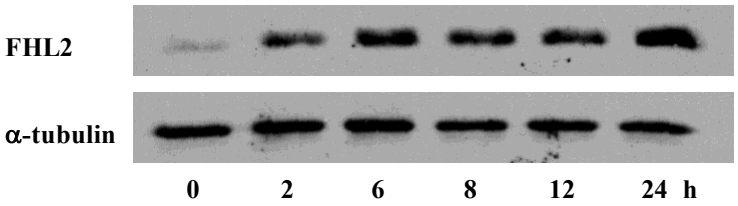
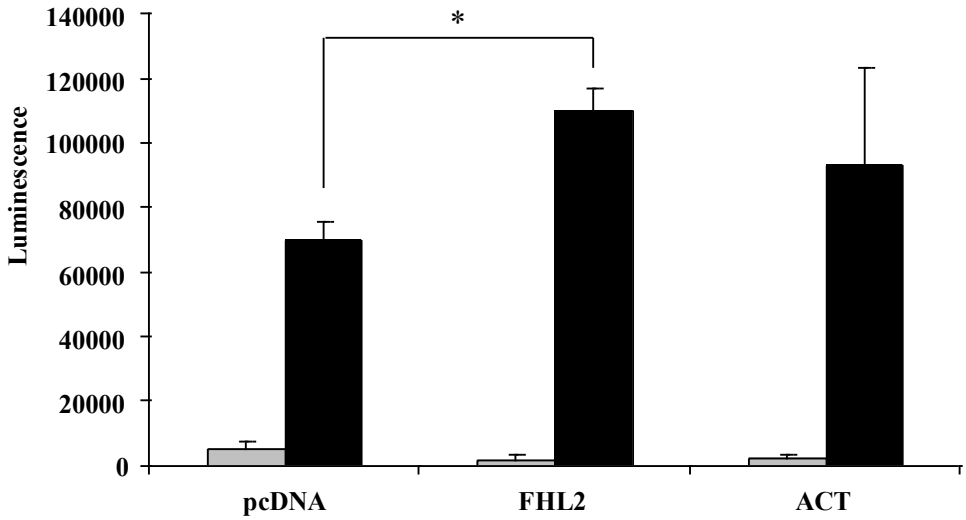


Figure 3.4: Quantitative RT-PCR analysis of FHL1, -2, -3 and ACT expression in human lung homogenates of IPF patients and healthy donors.
A, Expression of HPRT served as loading control (n = 3 for each, time points as indicated). *, $p < 0,05$ B, Western Blot Analysis of FHL2 expression in human fibroblasts; 0, 2, 6, 8, 12 and 24 hours after TGF-β1 (2 ng/ml) administration. Expression of α-tubulin served as loading control. Data are representative of three independent experiments.

3.5 FHL2 potentiates TGF-β1 signaling in NIH-3T3 cells

In order to be able to attribute pathogenetic relevance to the functioning of FHL2 in IPF, it does not suffice to just show how its expression can be modulated in fibroblasts by TGF-β1. This finding alone does not translate to a biological output of any importance to the disease process. For this purpose, it would help to show that FHL2 may itself modulate TGF-β1 and, consequently, its well-known profibrotic downstream effects. For this purpose, full length FHL2 and full length ACT (as control) were each cloned into pcDNA3.1(+) expression vectors and co-transfected into NIH-3T3 cells together with the p(CAGA)12 reporter plasmid, which reports for TGF-β1 signaling. Cells co-transfected with an empty pcDNA3.1(+) vector and p(CAGA)12 reporter plasmid served as control. As a result, Luciferase luminescence revealed a significant potentiation of TGF-β1 signaling through FHL2 overexpression whereas ACT overexpression had no significant impact (Figure 3.5 A). In order to check for this effect on the protein level, Western Blots were then performed on TGF-β1 stimulated NIH-3T3 cells that overexpressed FHL2 via pcDNA3.1(+) and equally stimulated NIH-3T3 cells containing only empty pcDNA3.1(+) as control (Figure 3.5 B). Here, the protein of interest was phosphorylated Smad2, an effector molecule of the Smad-pathway, which is activated via TGF-β1 signaling by phosphorylation. Detection of unphosphorylated Smad2 was used as loading-control. The results showed that after stimulation with TGF-β1 for 0,5 and 2 hours, the cells that overexpressed FHL2 displayed a higher accumulation of phospho-Smad2 than the cells containing only the empty vector. In summary, these findings illustrate that FHL2 has the potential to positively regulate Smad-dependent TGF-β signaling in NIH-3T3 cells on the DNA level as well as the protein level.

A.



B.

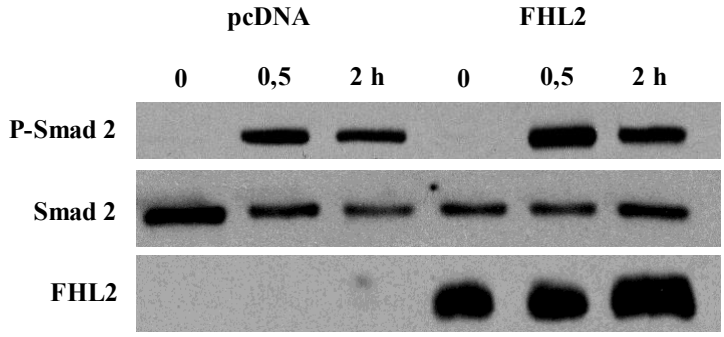


Figure 3.5: Regulation of TGF-β1 signaling by FHL2 in NIH-3T3 fibroblasts
A, Luciferase assay assessing the effect of FHL2 and ACT on baseline and TGF-β1-induced (2 ng/ml) pCAGA12-luc expression (grey bar: unstimulated, black bar: TGF-βb1 stimulated). Luciferase expression is plotted in Luciferase units. *, $p < 0{,}001$ B, Phospho-Western Blot analysis assessing the regulation of Smad2 phosphorylation in NIH-3T3 fibroblasts containing only the empty vector versus NIH-3T3 fibroblasts treated to overexpress FHL2 at 0, 0,5 and 2 hours after treatment with TGF-β1 (2 ng/ml). Data are representative of three independent experiments.

4 Discussion

The expression analyses and the immunohistochemistry experiments presented in this thesis give direct evidence that the expression of FHL2 is significantly upregulated in IPF. Does this, however, illustrate a causal mechanism in the pathophysiology of IPF or does it merely constitute an epiphenomenon?

4.1 FHL2 colocalizes with TGF-β- and WNT-signaling in IPF lungs

FHL2 is a mediator of protein-protein interaction with no proprietary enzymatic activity. In order to understand its function in a certain cellular context one needs to identify the interaction partners it connects in that context and comprehend the nature of their interaction. The presented immunohistochemical analyses localize FHL2 to fibroblasts and bronchial and alveolar epithelium in the lungs of IPF patients. In the introduction of this thesis, these cells were introduced as hot spots of IPF pathogenesis. TGF-β-signaling and β-catenin-dependent WNT-signaling are the two major developmental pathways that contribute to the pathology of IPF. FHL2's functional involvement in both of them in various contexts was highlighted. TGF-β is known to enact its effects on fibroblast foci, alveolar macrophages, bronchiolar epithelial cells and hyperplastic alveolar type II cells in the human lung, and the cellular accumulation of β-catenin in IPF lungs has been confined to bronchiolar proliferative regions, damaged alveolar structures and fibroblast foci (Aubert u. a., 1994; Chilosi u. a., 2003; Coker u. a., 2001). Therefore, the immunohistochemical analyses exhibited in this thesis give evidence of the spaciotemporal colocalization of FHL2 with TGF-β and β-catenin at prime locations of IPF pathology in the diseased human lung. In summary, this study suggests that the preconditions for a functional interplay between the parties of interest in IPF are met.

4.2 Models on the function of FHL2 in IPF

A variety of the known ways in which FHL2 can exert influence on the signaling chains of TGF-β as well as WNTs was summarized in the introduction of this thesis. With respect to the results of this thesis, different hypothetical models for a pro-fibrotic function of FHL2 in the pathophysiology of IPF can be constructed as the molecule apparently disposes of a multitude of potential points of action in the disease sequence. The following section will introduce a selection of such scenarios, which build on the original findings of this thesis in conjunction with the data from the literature that was exhibited in the introduction.

First a relatively straightforward scenario of FHL2 as pro-fibrotic coactivator of β-catenin in β-catenin-dependent WNT signaling will be illustrated. Then a variety of hypothetical scenarios for coordinated profibrotic action between TGF-β1 and FHL2 in IPF will be described, which take in account the multitude of contact points between the two molecules and the reciprocal nature of their interactions. Finally, a model of how FHL2 might act as an avid enforcer of a highly dynamic crosstalk between TGF-β1- and WNT-signaling in IPF will be introduced. Here, the untangling of possible interactions and their consequences for the disease process prove to be more challenging as FHL2 seems to have the potential to mediate both synergistic and competitive bi-directional communication between the two developmental pathways where a constant shift in balance between the interaction partners in the dynamic cellular environment might regulate a variety of biological outputs.

4.2.1 Model 1: FHL2 coactivates β-catenin in IPF

FHL2 is a known coactivator of β-catenin in β-catenin-dependent WNT signaling, which is known to play a pivotal role in the etiology of IPF[3]. Consequently, FHL2 driven

[3] see chapters 1.3.2 and 1.5.2

enhancement of WNT-signaling-dependent IPF pathology could take place where enhanced WNT-signaling and expression of FHL2 coincide in the diseased lung, namely fibroblasts/myofibroblasts and AEC Type II cells. FHL2 would supposedly act as coactivator in the expression of profibrotic β-catenin-target genes, such as α-SMA, matrilysin and cyclin D1. In this manner it might effectively enhance fibrosis-relevant events such as myofibroblast activation, excessive synthesis of protein and collagen in fibroblasts, AEC-proliferation and EMT. A schematic of this hypothetical signal flow is presented in Figure 4.1.

For future research, knockdown experiments with the experimental silencing of FHL2 in IPF fibroblasts and AEC type II cells with consecutive probing for a mitigation of the aforementioned target genes in response to stimulation with β-catenin might constitute a promising option for testing this model.

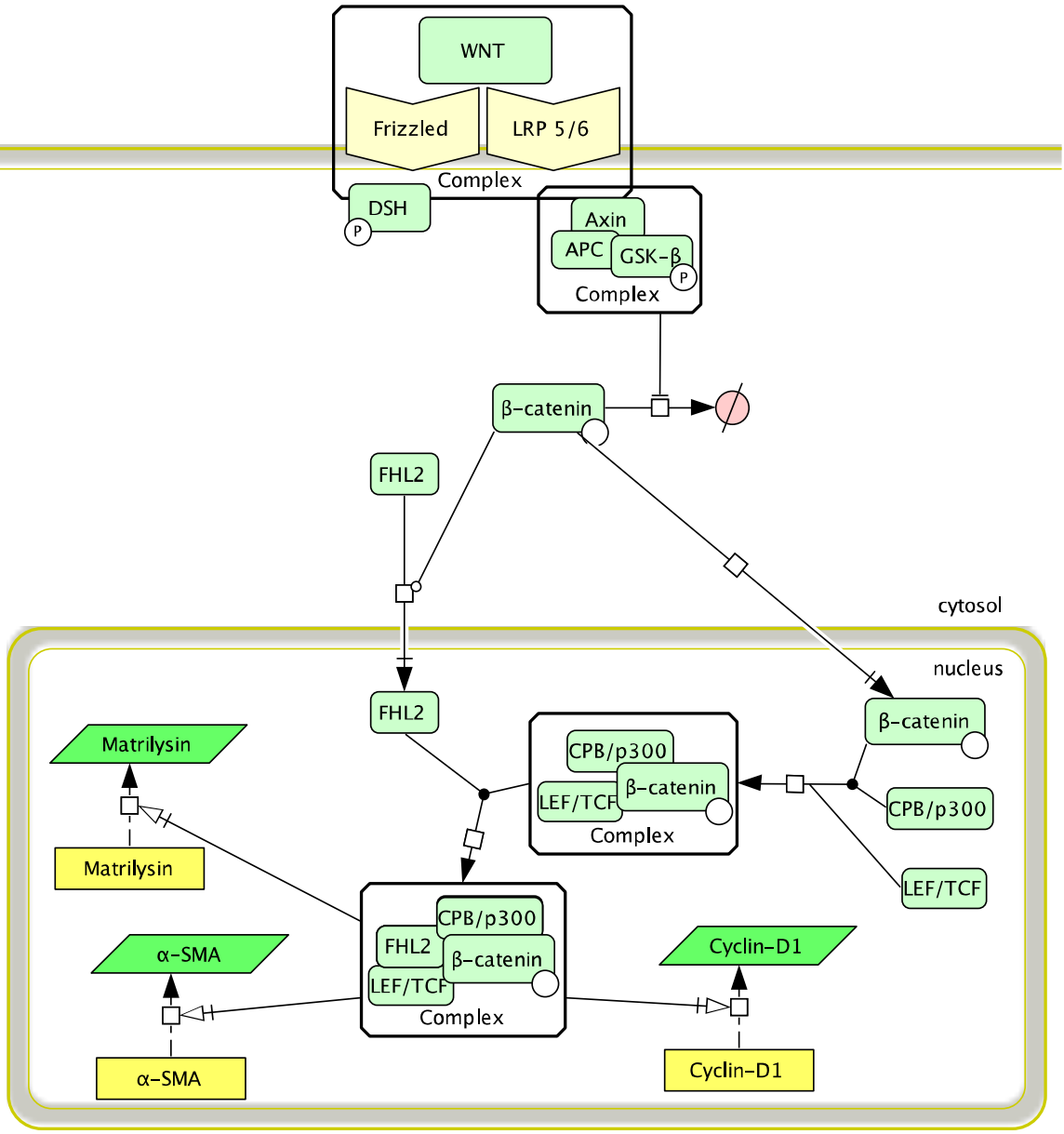


Figure 4.1: How FHL2 might regulate IPF as a coactivator of β-catenin
This schematic depicts how FHL2 might coactivate β-catenin in the expression of profibrotic target genes such as α-SMA, matrilysin and cyclin D1 in IPF-fibroblasts or AECs.

4.2.2 Model 2: FHL2 is an effector as well as enhancer of TGF-β signaling in IPF

Different modes of functional cooperation between TGF-β signaling and FHL2 from different cellular contexts were addressed in the Introduction[4]. This thesis established that TGF-β1 enhances the expression of FHL2 in primary human lung fibroblasts and that FHL2 potentiates Smad-dependent TGF-β1 signaling in NIH-3T3 cells. Hence, FHL2 might have the potential to modulate IPF-pathology through bidirectional communication with TGF-β1.

As for the presumed biological outputs of this relationship, secondary literature points to fibroblast-to-myofibroblast transition as a mechanism of interest. An aforementioned study by Gullotti et al. illustrated how TGF-β1 induces the expression of FHL2 on the protein level in murine fibroblasts, which consecutively undergo a differentiation into a myofibroblast-like phenotype with altered co-expression of α-SMA (Gullotti u. a., 2011). Although the study did focus on colon cancer, its findings relate to the fibrotic process in IPF where fibroblast-to-myofibroblast transition is also deemed to be an essential event. This resonates with the original finding of this thesis that TGF-β1 enhances the expression of FHL2 in primary human lung fibroblasts. Furthermore, this thesis demonstrates that the TGF-β1-dependent upregulation of FHL2 takes place on the mRNA level as well as the protein level. The presented experiments demonstrate a rise in FHL2's availability in response to TGF-β1 starting at 6h on the mRNA level and 2h on the protein level. In comparison, the Gulloti group produced the concerned effect only on the protein level and chose 24h as the first time point of measurement. The reason as to why the upregulation of FHL2-activity in primary human fibroblasts tends to be higher and quicker on the protein level than on the mRNA level remains unknown. Posttranscriptional mechanisms, such as decelerated degradation of FHL2 or a yet unknown regulatory mechanism of the protein's state of activity could be the cause of this, but further research would be needed for clarification. What also remains to be investigated is, whether the promotion of FHL2 via TGF-β1 occurs in a Smad-

[4] see chapter 1.5.2

dependent or Smad-independent manner. Knockdown experiments with the experimental silencing of Smad transmission in TGF-β1-stimulated primary human fibroblasts with consequent checking on the expression of FHL2 should be of service in this respect. In order to understand whether FHL2 is of relevance as a modulator of the TGF-β-driven transition of human lung fibroblasts to myofibroblasts human lungs, an emulation of the aforementioned experiments performed by Guilotti et al. with primary human lung fibroblasts as substrate should be considered. The outcome measure should check if the established upregulation of FHL2 by TGF-β prompts the expression of α-SMA and the morphological changes corresponding to fibroblast-to-myofibroblast transition in human fibroblasts.

The detection of a TGF-β1-dependent upregulation of FHL2 in human fibroblasts basically offers a logical explanation for the initial finding of this study, that FHL2 is upregulated in IPF. It might simply be a consequence of the enhanced TGF-β1-signal in IPF. Whilst the initial expression studies were performed on lung homogenates, the performed stimulation experiments indicate that at least part of this upregulation might take place in fibroblasts. This goes in accordance with the presented results from the Immunohistochemistry experiments on human IPF lungs, which depict FHL2 to reside in fibroblasts, as well as in bronchial and alveolar epithelial cells.

The presented Luciferase assays and Western blots that illustrate an upregulation of Smad-dependent TGF-β signaling in response to stabilized overexpression of FHL2 in NIH-3T3 cells prove that FHL2 has the potential to positively regulate an essential profibrotic pathway in an immortalized fibroblast cell line. With reference to the role of TGF-β1 as key fibrogenic cytokine, this links FHL2 to TGF-β1 in a functional, pro-fibrotic manner. What remains to be clarified, however, is the exact signal flow at work. With respect to secondary literature, a hypothesis of how this interaction might go about in IPF can be formulated. Accordingly, the enhancement of Smad-dependent TGF-β signaling by FHL2 might be effectuated through functional cooperation between Arkadia and FHL2. Arkadia is an E3 ubiquitin ligase that positively regulates TGF-β signaling via the ubiquitination of several

pathway components,such as Ski/SnoN and Smad 7 (W. Liu u. a., 2006; Nagano u. a., 2007). Ski/SnoN are two intranuclear transcription factors that hinder the activated heteromeric Smad complex (Smads 2, 3 and 4) in the translation of TGF-β target genes as they recruit histone deacetylases to the complex and compete with the coactivator CBP/p300 for binding to the complex (Nagano u. a., 2007). Smad 7 was already introduced as an inhibitory Smad that negatively regulates TGF-β-signaling[5]. Arkadia facilitates the translational work of the activated Smad complex by targeting Ski/SnoN in the nucleus and Smad 7 in the cellular compartment for ubiquitination with subsequent degradation, hence freeing the activated Smad complex of two functional antagonists. Xia et al. investigated the relationship between FHL2 and Arkadia in the context of Smad-dependent TGF-β signaling (Xia u. a., 2013). First, they illustrated that FHL2 enhances TGF-β-dependent gene transcription in 293t cells, something that goes in accordance with this thesis, which could produce a similar effect in NIH-3T3 cells. Then they described how Arkadia and FHL2 synergistically promote Smad dependent target gene transcription in 293t and HepG cells in a dose dependent manner. Finally, they were able to illustrate that FHL2 stabilizes the positive effect of Arkadia on TGF-β signaling as it extends Arkadia's half-life through the inhibition of its ubiquitination. They believed this to be the potential mechanism behind the observed synergy of the two molecules in 293t cells. In order to relate these discoveries to the findings of this thesis it might be of worth to extend the presented Luciferase assays by co-transfection of NIH-3T3 cells with FHL2 and Arkadia overexpressing constructs together with the p(CAGA)12 reporter plasmid with subsequent measuring of a possible dose-dependent enhancement of TGF-β signaling. In addition to that, immunohistochemical localization of Arkadia in IPF lungs could reveal whether the E3-ligase colocalizes with FHL2 in IPF.

Taken together, the results of this thesis and the knowledge accrued from secondary literature speak of a synergystic relationship between FHL2 and TGF-β in IPF. The two

[5] see chapter 1.2.1.3.

molecules might enhance each other in the disease context and initiate a derailed positive feedback loop that might effectuate and perpetuate fibrotic changes. Whether such a feedback loop is a reality in IPF cannot be clarified with the available data. Nevertheless, it might pose an incentive for further research. A schematic of this hypothetical signal flow is presented in Figure 4.2.

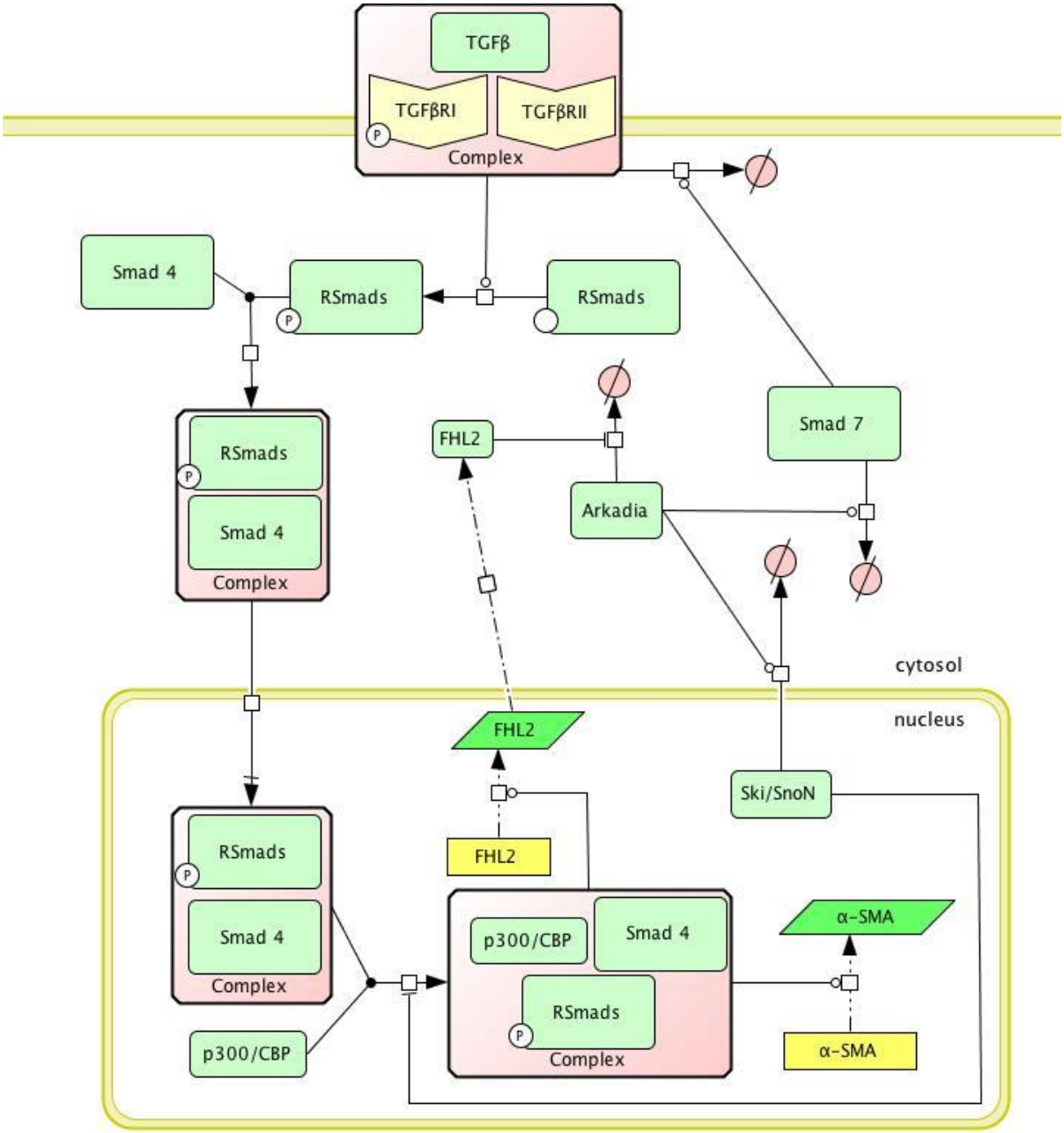


Figure 4.2: How FHL2 and TGF-β might promote each other in IPF
This schematic proposes a model for how FHL2 and TGF-β might promote each other in IPF and hence synergistically enhance the expression of disease relevant genes such as α-SMA. For means of simplification, this schematic depicts the promotion of FHL2 via TGF-β as being Smad dependent, which is a hypothetical assumption that remains to be investigated. Also, Arkadia is only depicted to reside in the cytosolic compartment, although it associates with Ski and SnoN in the nucleus in order to promote their ubiquitylation.

4.2.3 Model 3: FHL2 regulates TGF-β/WNT-crosstalk in IPF

Within the last years several studies have produced promising insights into crosstalk between the several developmental pathways involved in the pathogenesis of IPF like WNT, TGF-β β1, BMP, Hedgehog and PTEN (Moisés Selman et al., 2008). The possibility of an involvement of FHL2 in such relationships has not yet been explicitly investigated.

The finding of this thesis that TGF-β1 can promote the expression of FHL2 in human fibroblasts makes way for a discussion about the existence of a functional relevance of FHL2 as a mediator of crosstalk between TGF-β1- and WNT-signaling in IPF fibroblasts, where TGF-β could promote the coactivation of β-catenin simply by increasing the quantity of the β-catenin-coactivator FHL2. Proof of such a signal flow in the colon adenocarcinoma cell line DLD1 was illustrated by Zhang et al. in their paper on FHL2's EMT-enhancing role in colon cancer. They showed that TGF-β1 enhances β–catenin-dependent WNT signaling via the induction of FHL2 in a Smad independent manner[6] (Zhang u. a., 2010).

Furthermore, FHL2 might act as an interface for crosstalk between TGF-β and WNT signaling in IPF via the enhancement of the β-catenin-effected expression of cyclin D1 in IPF fibroblasts in a TGF-β1-triggered RhoA-mediated manner. In 2006, Watts et al. demonstrated that the expression of cyclin D1 is deregulated in IPF-fibroblasts through a RhoA dependent mechanism. They illustrated how this cascade is initiated by TGF-β1, as it induces CTGF (connective tissue growth factor), which can modulate fibroblast differentiation via a pathway that involves RhoA. They suggested that their observations illustrated a novel mechanism driving fibroblast proliferation in IPF which was experimentally prompted by TGF-β1 and could not be produced in an adult non-IPF fibroblast control cell line, implying the possibility of an IPF-specific nature for this effect (Watts, Cottrell, Hoban, & Spiteri, 2006). They did not, however, identify the mechanism via which RhoA exerts its effect on cyclin D1. The properties of FHL2 make it a hypothetical link in this matter. The

[6] see chapter 1.5.2

LIM protein is known to coactivate β-catenin together with CBP/p300 in the transcription of cyclin D1 in murine fibroblasts (Labalette et al., 2004). It can also directly associate with the cyclin D1 promoter in murine fibroblasts as another means to regulate its expression (Labalette et al., 2008). Accordingly, FHL2 siRNA inhibits the expression of cyclin D1 in the colorectal carcinoma cell lines HCT116 and SW480 (Zhang u. a., 2010). As for the missing link that connects RhoA and cyclin D1, a study by Müller et. al from 2002 identified FHL2 to be a transmitter of RhoA signals from the cell membrane to the nucleus in NIH3T3 cells, making it a recommendable option in this respect (Müller u. a., 2002). The impression at hand is that FHL2 might have a hand in linking TGF-β1-signaling, RhoA and the erratic regulation of cyclin D1 expression via β-catenin in IPF fibroblasts. A schematic of this hypothetical signal flow is presented in Figure 4.3. Knockdown experiments with the experimental silencing of FHL2 and β-catenin in serial procedures on IPF fibroblasts while observing for the expected mitigation of TGF-β1 mediated cyclin D1-expression seem to be a recommendable option for further research in this respect.

While the hitherto discussed models on FHL2-driven crosstalk between WNT signaling and TGF-β signaling in IPF suggest a synergy between the different signal-paths, FHL2's involvement in both signaling chains also holds the potential to mediate a competitive type of crosstalk between the two pathways. In the introduction of this text, the works of Liu et al. and Michalik et al. were presented, whose results speak of instances where β-catenin acts as a negative regulator of TGF-β-mediated fibroblast-to-myofibroblast transition (J. Liu u. a., 2012; Michalik u. a., 2012)[7]. While the exact mechanism of this effect could not be identified, FHL2 offers itself as a link in this respect. The LIM protein localizes to the nucleus in order to promote β-catenin-mediated gene expression, while its modulation of TGF-β-mediated target gene expression may take place in both cellular compartments. Given a limited intracellular amount of FHL2 and the necessity of nuclear shuttling of the LIM protein for the transcription of WNT target genes, the molecule hypothetically contains

[7] see chapter 1.4.2

the potential to act as modulator in the aforementioned negative feedback loop via stoichiometry. For example, its competitive recruitment by β-catenin into the nucleus might weaken its enhancement of TGF-β via the reduction of available cytosolic FHL2 molecules. The stoichiometry of FHL2 in the different cell compartments might therefore constitute a means of fine-tuning the balance between TGF-β-signaling and β-catenin-dependent WNT signaling in the given context.

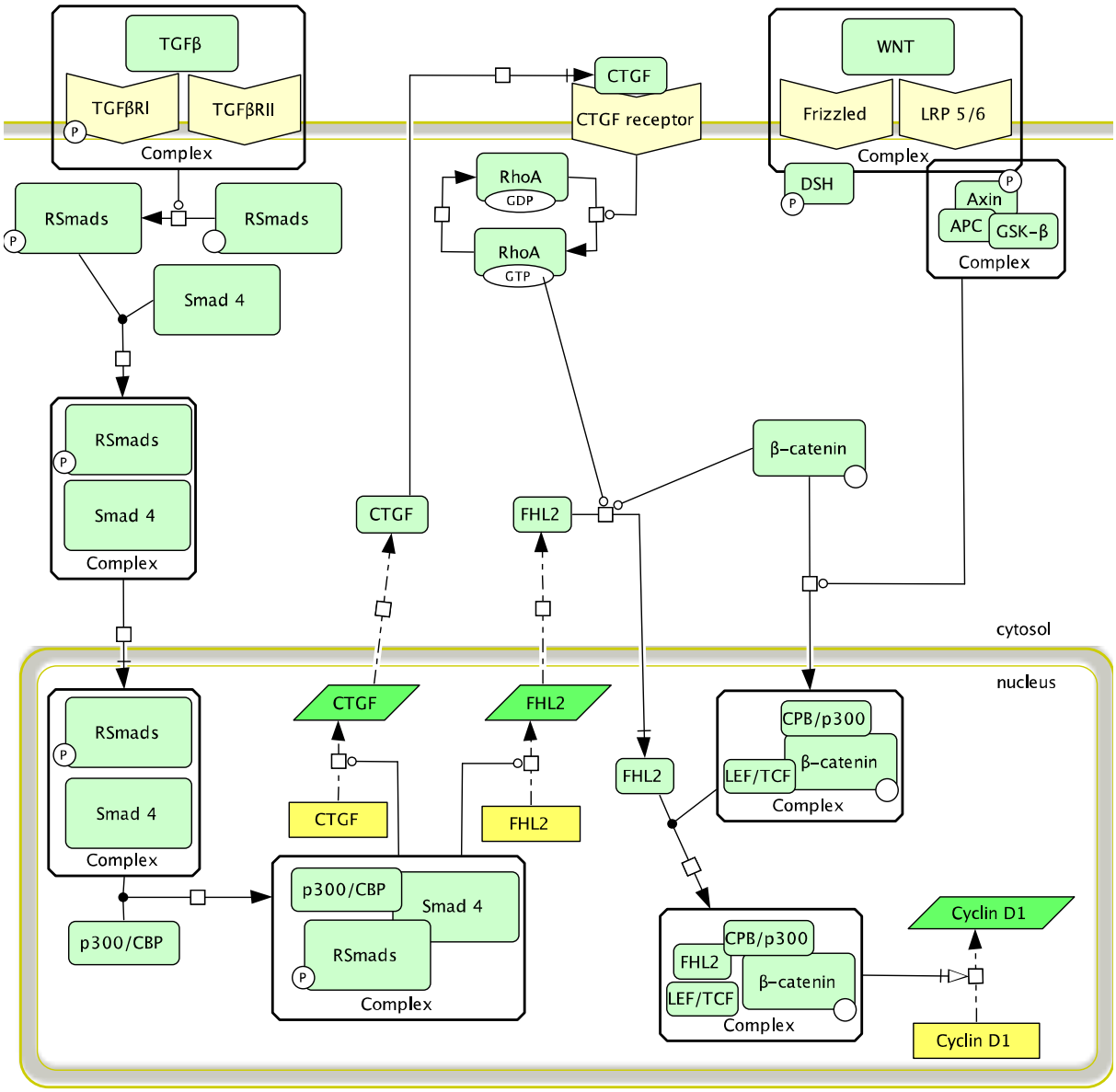


Figure 4.3: How FHL2 might translate a TGF-β mediated activation of RhoA into β-catenin-linked expression of cyclin D1 in IPF fibroblasts
This schematic integrates a variety of individual results from disparate studies that examine the cellular environments of IPF fibroblasts, murine fibroblasts and NIH3T3 cells. It is of a speculative nature. An analogous pathway in IPF fibroblasts could not be fully produced to date. Also, it must be noted again that although TGF-β does promote the expression of FHL2 in fibroblasts it is not clear whether Smad-dependent signaling is involved in this process.

4.3 FHL2 may modulate profibrotic and antifibrotic actions in IPF

The results of this thesis stand for themselves as no other work to date has been published that explicitly focuses on the role of FHL2 in IPF. As this underlines the originality of the material, it also poses a challenge to its legitimation due the lack of directly related, established data in support of the proposition of a profibrotic potential of FHL2 in IPF. The most closely related published work around, however, is an intriguing study by Alnajar et al. on the role of FHL2 in BLM-induced lung fibrosis. The study compared the degrees of lung fibrosis in FHL2-KO mice to that of wild type (WT) mice after BLM treatment. In their paper's introduction, the authors start out by stating that one might expect a loss of FHL2 to result in attenuated fibrosis owing to the seemingly profibrotic characteristics of the molecule, which were introduced in section 1.5 of this thesis (e.g. FHL2's induction of α-SMA and its promotion by key fibrotic cytokines like TGF-β in certain cellular contexts). To their surprise, the loss of FHL2 in the examined rodents instead coincided with the aggravation of BLM-mediated lung fibrosis in the model's early acute inflammation phase and the retardation of disease-resolution at later stages. They consecutively examined the expression of several ECM-genes and detected a disinhibited expression of tenascin C in FHL2-KO mice. Tenascin C is a multifunctional glycoprotein whose level of expression is thought to positively correlate with the degree of inflammation and tissue remodelling in the BLM fibrosis model. Alnajar et al. interpreted this finding as correlate of a decisive inflammatory component in the observed aggravated fibrosis in FHL2-KO mice after BLM treatment. They were able to link the observed effects of the loss of FHL2 and the disinhibition of tenascin C expression by identifying FHL2 as negative regulator of tenascin C in mouse fibroblasts. Furthermore, they displayed that FHL2 positively and non-redundantly regulates the expression of the C-type lectin receptor DC-SIGN in mouse macrophages, a macrophage-surface receptor that mediates the NF-κB-dependent transcription of proinflammatory cytokines. Loss of FHL2 inhibited the DC-SIGN mediated activation of mouse macrophages

after BLM treatment. The observed impediment of DC-sign mediated activation of macrophages was then related to the observed protraction of inflammation in FHL2-KO mice as activated macrophages also play a crucial role in the termination of inflammatory proceedings, besides their initiation and maintenance, via the restriction of the influx of polymorphonuclear leucocytes to the site of damage (Alnajar u. a., 2013; Dean u. a., 2008). Their conclusion was that the observed effects of upregulation of the pro-inflammatory tenascin C and the downregulation of DC-SIGN account for antifibrotic properties of FHL2 in the BLM model of lung fibrosis (Alnajar u. a., 2013).

The argumentation of this thesis ascribes profibrotic properties to FHL2 in the context of human IPF. In relation to this, studies from various fields that also speak of a profibrotic potential of FHL2 in different cellular contexts were highlighted in the Introduction of this text. The findings of Alnajar et al. prompt the question whether FHL2 might also maintain antifibrotic properties in IPF. Indicative of this are findings that the expression of tenascin C is also elevated in fibroblast foci of IPF lungs and that TGF-β1 can induce an upregulation of tenascin C in cultured healthy and fibrotic human fibroblasts (Estany u. a., 2014). In search of other studies that ascribe antifibrotic properties to FHL2, the work of Huss et al. comes to mind, who also identified antifibrotic properties of FHL2 in a model of hepatic fibrosis by displaying that CCl_4-treatment aggravated liver fibrosis in FHL2$^{-/-}$-mice (Huss u. a., 2013).

So, if FHL2 enacts antifibrotic effects in the human lung, does the observed upregulation of FHL2 in human IPF maybe just constitute a physiological attempt to antagonize disease mechanisms and re-establish homeostasis? Or could it be both, antifibrotic and profibrotic, in the same disease context? At first glance, the simultaneous ascription of antifibrotic and profibrotic properties to a molecule in the same disease context might seem unfeasible. These seemingly opposite properties can, however, be integrated with regard to the promiscuity and versatility of FHL2 in general and the distinctions between the disputed data sets.

First, it has to be noted that findings from research on the common “single-challenge” BLM mouse model, where mice receive a single dose of BLM, might not always easily translate to IPF, as the single-challenge model generally fails to represent central aspects of IPF. It does not produce chronic fibrosis, i.e. a pattern of UIP, and the transitory fibrotic changes it produces are apparently largely inflammation-driven (Scotton & Chambers, 2010). Alnajar et al. have employed such a “single challenge” BLM model for their study, where mice received one single intranasal dose of 2 U/kg BLM and were therefore faced with the aforementioned methodological limitations. This entails that, even though the dynamic of the development of fibrosis proved to be more dramatic in the FHL2-KO mice, they nevertheless developed similar degrees of maximal fibrosis to that of the WT mice after two weeks. Also, the fibrotic changes were transient in both groups, meaning that the FHL2-KO mice that survived treatment experienced restitution of fibrosis just like the WT mice. Alnajar et al. did identify irregularities in the functions of tenascin C and DC-SIGN in FHL2-KO mice as the alleged causes of their unfavourable outcome in BLM-induced lung fibrosis. This means that their observations evolve around inflammation-related phenomena and not around dysregulated epithelial-mesenchymal crosstalk or fibroblast to myofibroblast transition, which are currently regarded to be the driving force behind human IPF pathology, as was discussed in detail in the introduction of this thesis. This could mean that their data highlights pathophysiological mechanisms that pertain specifically to the BLM model but do not necessarily have a direct correlate in human IPF, owing to the general failure of the BLM model to emulate the human condition.

In order to address these problems of the common BLM model, Degryse et al. introduced a refined multi-challenge BLM model, which involves an 8 biweekly dosing regimen with the subsequent advantage of the development of persistent fibrosis with histopathological similarities to UIP, AEC type II hyperplasia and (albeit rare) fibroblast foci (Degryse u. a., 2010). It might be interesting to see, if a repetition of the study design of Alnajar et al. with the multi-challenge BLM model would prompt different results.

Even if the results of Alnajar et al. would translate to human IPF, and even if FHL2 would enact antifibrotic properties in the inflammation-driven stages of IPF pathogenesis, profibrotic actions at other disease stages would not be categorically impossible. The promiscuous relations of FHL2 and its modular nature allow for a myriad of functions which depend on cellular context. It is therefore plausible to suggest that in IPF FHL2 might mediate profibrotic processes in one cellular context and simultaneously mediate antifibrotic processes at a neighbouring site, e.g. it might suppress the expression of tenascin C in fibroblasts of a fibroblast focus and simultaneously enhance the epithelial to mesenchymal transition of an adjacent AEC. This would render the molecule useless as potential therapeutic target because the net effect of a modulation of FHL2's activities in IPF would not be predictable (whether profibrotic or antifibrotic).

Nevertheless, FHL2 appears to be a central regulator in IPF that serves as an interface for the execution of disease mechanisms between different effectors. Viewing IPF from the perspective of FHL2 provides a glimpse of the astounding complexity of the disease's pathophysiology.

4.4 Conclusion

There is no proven cause of IPF and treatment options are scarce. Few other diseases can claim comparable enigmatic qualities. The more we learn about IPF, the more we are challenged by its complex nature. This thesis nothing but adds to the complexity by introducing FHL2 as a novel regulator of IPF that exerts its influence at key stages of the disease process via the modulation of the signal flow within the two key signaling pathways of TGF-β1 signaling (Smad-dependent as well as Smad–independent) and β-catenin-dependent WNT signaling. This modulation comprises a variety of feasible operations that occur at a multitude of contact points in the diseased organ. On a cellular level, these interactions can be observed in the cytoplasm as well as the nucleus. Apart from the mediation of interactions that are proprietary to the distinct pathways, FHL2 also has the potential to act as a crosstalk interface between TGF-β1- and WNT signaling in IPF. This shows that the LIM protein is a significant contributor to the complex array of interconnections and feedback loops between the two pathways. The interactions that FHL2 would mediate in this respect might regulate central disease processes such as fibroblast-to-myofibroblast transition and EMT. However, FHL2 might not exclusively regulate profibrotic processes in IPF. Comprehensive literature reviews suggest that it might also have the capability to mediate antifibrotic processes in IPF, depending on the cellular context. The mechanisms that might determine whether the molecule enhances either profibrotic or antifibrotic actions are not entirely known. Considering the versatile nature of FHL2, they will probably amount to matters of selective recruitment by available interaction partners as the promiscuous LIM protein seems to link whoever it can possibly link. Another way of putting this is that the biological output of FHL2's actions is determined by its distribution within the cellular compartments, the array of compatible signals that it finds itself subjected to in these compartments and, ultimately, the balance among these signals regulating their access to the LIM protein. The sheer magnitude of FHL2's possible

downstream effects should very likely disqualify it as a direct target for future therapeutic interventions in the ongoing search for better treatments for IPF. Nevertheless, this thesis demonstrates that FHL2's profibrotic potential has to be considered in respect to the pathogenesis of IPF. The full scope of FHL2's involvement in the pathology of IPF remains to be determined by further research. This work hopes to serve as groundwork and motivation for such undertakings.

5 Zusammenfassung

Die idiopathische pulmonale Fibrose (IPF) ist ein chronisch-fortschreitendes und schweres Lungenleiden von nur unzureichend geklärter Ätiologie. Defekte in den TGF-β- und WNT-Signalwegen scheinen eine zentrale Bedeutung in der Pathogenese von IPF zu haben. Das LIM Protein FHL2 moduliert bekanntermaßen den TGF-β- als auch den WNT-Signalweg in verschiedenen zellulären Kontexten. Kontextspezifisch vermittelt es auch Crosstalk zwischen beiden Signalwegen. Neben der Möglichkeit zur Regulierung verschiedener fibrose-bezogener Prozesse, verfügt FHL2 über die Kapazität, die Transition von Fibroblasten zu Myofibroblasten und die epithelial-mesenchymale Transition (EMT) zu fördern, welche beide als zentrale Prozesse in der Entstehung von IPF gelten. Dem Molekül wird generell ein profibrotischer Charakter zugeschrieben. Es zeigt jedoch auch antifibrotische Eigenschaften im Bleomycin-Maus-Modell der IPF. Bis dato ist nichts über eine Beteiligung von FHL2 an der Entstehung und Aufrechterhaltung der humanen IPF bekannt. Diese Studie untersuchte die Expression, Lokalisation und Funktion von FHL2 in IPF. An Lungenhomogenat durchgeführte quantitative (q)RT-PCR-Untersuchungen zeigten eine 3,25-fach verstärkte Expression von FHL2-mRNA in IPF-Lungen im Vergleich zu gesunden Spenderlungen. Western Blots des gleichen Materials bestätigten eine signifikante Hochregulation von FHL2-protein in IPF-Lungen. Immunhistochemische Untersuchungen lokalisierten FHL2 in Fibroblasten als auch in bronchialen und alveolären Epithelzellen von IPF-Lungen und gesunden Spenderlungen. Die Stimulation humaner Fibroblasten mit TGF-β resultierte in einer verstärkten Expression von FHL2-mRNA (3,2-fach) und FHL2-Protein (4,2-fach). Schließlich zeigte sich in Luziferase-Assays und phospho-Western Blots, dass die stabile Überexpression von FHL2 in NIH-Zellen eine signifikante Potenzierung des Smad-abhängigen TGF-β Signalweges bewirkt. Zusammenfassend suggerieren diese Ergebnisse profibrotische Eigenschaften von FHL2 in IPF.

6 Summary

Idiopathic pulmonary fibrosis (IPF) is a progressive and grave lung disease and its etiology is insufficiently understood. Defects in TGF-β signaling and WNT signaling are deemed to be of major relevance to the pathogenetic process of IPF. The LIM protein FHL2 has been shown to modulate TGF-β signaling and WNT signaling in various cellular contexts. It may also serve as an interface for crosstalk between the two pathways. FHL2 is known to regulate fibrogenesis and to promote fibroblast to myofibroblast transition and epithelial-to-mesenchymal transition (EMT), which are deemed to constitute key events in IPF. FHL2 is often characterized as profibrotic in nature. Nevertheless, it has also been ascribed antifibrotic properties in the bleomycin-mouse-model of IPF. To date, nothing is known about a possible involvement of FHL2 in human IPF.

This study examines the expression, localization and function of FHL2 in IPF. Quantitative (q)RT-PCR performed on lung homogenates displayed a 3.25-fold upregulation of FHL2 mRNA levels in IPF lungs compared with controls (transplant donors). Western blot analysis of the same material confirmed a significant upregulation of FHL2 protein in IPF lungs. Using immunohistochemistry, FHL2 was localized to fibroblasts, as well as to bronchial and alveolar epithelial cells in IPF lungs and in healthy lungs. Stimulation of human fibroblasts with TGF-β resulted in enhanced expression of FHL2 mRNA (3.2 fold) and protein (4.2 fold). Finally, overexpression of FHL2, led to a significant potentiation of Smad-dependent TGF-β signaling in NIH-cells, as assessed by Luciferase assays and phospho Western blots. In conclusion, these results suggest that FHL2 might act as a profibrotic regulator in IPF.

7 List of Figures

All original pathway illustrations in this thesis were designed using the modeling tool CellDesigner, Ver. 4.4 (Funahashi u. a., 2008). Explanations of notation can be found under http://www.celldesigner.org/help/images/components42.png.

8 List of abbreviations

(q)RT-PCR	Quantitative reverse transcription polymerase chain reaction
AEC	Alveolar epithelial cells
AIP	Acute interstitial pneumonia
APC	Adenomatous polyposis coli
ATII-cell	Alveolar epithelial type II-cell
ATS	American Thoracic Society
BLM	Bleomycin
BMP	Bone morphogenetic protein
Co-Smad	Common Smad
DNA	Desoxyribonucleic Acid
DIP	Desquamative interstitial pneumonia
DPLD	Diffuse parenchymal lung diseases
ECM	Extracellular matrix
EMT	Epithelial-to-mesenchymal transition
ERS	European Respiratory Society
FHL	Four-and-a-half LIM
FPF	Familial pulmonary fibrosis
GSK3	Glycogen synthase kinase 3
HepG2 cells	Human hepatocellular carcinoma cells
I-Smad	Inhibitory Smad
IIPs	Idiopathic interstitial pneumonias
IPF	Idiopathic pulmonary fibrosis
LEF1	Lymphoid enhancer-binding factor
LIP	Lymphoid interstitial pneumonia
LRP	LDL-receptor-related protein
mRNA	Messenger Ribonucleic Acid
NSIP	Nonspecific interstitial pneumonia
PCR	Polymerase chain reaction
R-Smad	Receptor Smad
RNA	Ribonucleic Acid
SFPC	Surfactant protein C

siRNA	Small interfering RNA
Smurf	Smad-ubiquitination regulatory factor
TCF	T-cell-specific transcription factor
TGF-β	Transforming growth factor β
UIP	Usual interstitial pneumonia
WISP	WNT1-inducible signaling protein
WT	Wild type
α-SMA	α-Smooth muscle actin

9 References

Akhurst, R. J., & Hata, A. (2012). Targeting the TGFβ signalling pathway in disease. *Nature Reviews. Drug Discovery*, *11*(10), 790–811. http://doi.org/10.1038/nrd3810

Alexakis, C., Maxwell, P., & Bou-Gharios, G. (2006). Organ-specific collagen expression: implications for renal disease. *Nephron. Experimental Nephrology*, *102*(3-4), e71–75. http://doi.org/10.1159/000089684

Alnajar, A., Nordhoff, C., Schied, T., Chiquet-Ehrismann, R., Loser, K., Vogl, T., … Wixler, V. (2013). The LIM-only protein FHL2 attenuates lung inflammation during bleomycin-induced fibrosis. *PloS One*, *8*(11), e81356. http://doi.org/10.1371/journal.pone.0081356

American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias. This joint statement of the American Thoracic Society (ATS), and the European Respiratory Society (ERS) was adopted by the ATS board of directors, June 2001 and by the ERS Executive Committee, June 2001. (2002). *American Journal of Respiratory and Critical Care Medicine*, *165*(2), 277–304.

Arnold, G., Beier, H. M., & Herrmann, M. (1999). *Anatomie* (8., vollst. überarb. und aktual. A.). Springer-Verlag GmbH.

Attisano, L., & Wrana, J. L. (2002). Signal transduction by the TGF-beta superfamily. *Science (New York, N.Y.)*, *296*(5573), 1646–1647. http://doi.org/10.1126/science.1071809

Aubert, J. D., Dalal, B. I., Bai, T. R., Roberts, C. R., Hayashi, S., & Hogg, J. C. (1994). Transforming growth factor beta 1 gene expression in human airways. *Thorax*, *49*(3), 225–232.

Baarsma, H. A., Spanjer, A. I. R., Haitsma, G., Engelbertink, L. H. J. M., Meurs, H., Jonker, M. R., … Gosens, R. (2011). Activation of WNT/β-catenin signaling in pulmonary fibroblasts by TGF-β_1 is increased in chronic obstructive pulmonary disease. *PloS One*, *6*(9), e25450. http://doi.org/10.1371/journal.pone.0025450

Bhanot, P., Brink, M., Samos, C. H., Hsieh, J. C., Wang, Y., Macke, J. P., … Nusse, R. (1996). A new member of the frizzled family from Drosophila functions as a Wingless receptor. *Nature*, *382*(6588), 225–230. http://doi.org/10.1038/382225a0

Bienz, M., & Clevers, H. (2000). Linking colorectal cancer to Wnt signaling. *Cell*, *103*(2), 311–320.

Bierie, B., & Moses, H. L. (2006). Tumour microenvironment: TGFbeta: the molecular Jekyll and Hyde of cancer. *Nature Reviews. Cancer*, *6*(7), 506–520. http://doi.org/10.1038/nrc1926

Biernacka, A., Dobaczewski, M., & Frangogiannis, N. G. (2011). TGF-β signaling in fibrosis. *Growth Factors (Chur, Switzerland)*. http://doi.org/10.3109/08977194.2011.595714

Blobe, G. C., Schiemann, W. P., & Lodish, H. F. (2000). Role of transforming growth factor beta in human disease. *The New England Journal of Medicine*, *342*(18), 1350–1358. http://doi.org/10.1056/NEJM200005043421807

Bonniaud, P., Kolb, M., Galt, T., Robertson, J., Robbins, C., Stampfli, M., … Gauldie, J. (2004). Smad3 null mice develop airspace enlargement and are resistant to TGF-

beta-mediated pulmonary fibrosis. *Journal of Immunology (Baltimore, Md.: 1950)*, *173*(3), 2099–2108.

Böttner, M., Krieglstein, K., & Unsicker, K. (2000). The transforming growth factor-betas: structure, signaling, and roles in nervous system development and functions. *Journal of Neurochemistry*, *75*(6), 2227–2240.

Bridwell, J. A., Price, J. R., Parker, G. E., McCutchan Schiller, A., Sloop, K. W., & Rhodes, S. J. (2001). Role of the LIM domains in DNA recognition by the Lhx3 neuroendocrine transcription factor. *Gene*, *277*(1-2), 239–250.

Bringardner, B. D., Baran, C. P., Eubank, T. D., & Marsh, C. B. (2008). The role of inflammation in the pathogenesis of idiopathic pulmonary fibrosis. *Antioxidants & Redox Signaling*, *10*(2), 287–301. http://doi.org/10.1089/ars.2007.1897

Broekelmann, T. J., Limper, A. H., Colby, T. V., & McDonald, J. A. (1991). Transforming growth factor beta 1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. *Proceedings of the National Academy of Sciences of the United States of America*, *88*(15), 6642–6646.

Brun, J., Dieudonné, F.-X., Marty, C., Müller, J., Schüle, R., Patiño-García, A., … Marie, P. J. (2013). FHL2 silencing reduces Wnt signaling and osteosarcoma tumorigenesis in vitro and in vivo. *PloS One*, *8*(1), e55034. http://doi.org/10.1371/journal.pone.0055034

Cao, B., Guo, Z., Zhu, Y., & Xu, W. (2000). The potential role of PDGF, IGF-1, TGF-beta expression in idiopathic pulmonary fibrosis. *Chinese Medical Journal*, *113*(9), 776–782.

Cardoso, W. V., & Lü, J. (2006). Regulation of early lung morphogenesis: questions, facts and controversies. *Development (Cambridge, England)*, *133*(9), 1611–1624. http://doi.org/10.1242/dev.02310

Carthy, J. M., Garmaroudi, F. S., Luo, Z., & McManus, B. M. (2011). Wnt3a induces myofibroblast differentiation by upregulating TGF-β signaling through SMAD2 in a β-catenin-dependent manner. *PloS One*, *6*(5), e19809. http://doi.org/10.1371/journal.pone.0019809

Chan, K. K., Tsui, S. K., Lee, S. M., Luk, S. C., Liew, C. C., Fung, K. P., … Lee, C. Y. (1998). Molecular cloning and characterization of FHL2, a novel LIM domain protein preferentially expressed in human heart. *Gene*, *210*(2), 345–350.

Cheon, S., Poon, R., Yu, C., Khoury, M., Shenker, R., Fish, J., & Alman, B. A. (2005). Prolonged beta-catenin stabilization and tcf-dependent transcriptional activation in hyperplastic cutaneous wounds. *Laboratory Investigation; a Journal of Technical Methods and Pathology*, *85*(3), 416–425. http://doi.org/10.1038/labinvest.3700237

Cheon, S. S., Wei, Q., Gurung, A., Youn, A., Bright, T., Poon, R., … Alman, B. A. (2006). Beta-catenin regulates wound size and mediates the effect of TGF-beta in cutaneous healing. *The FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *20*(6), 692–701. http://doi.org/10.1096/fj.05-4759com

Chilosi, M., Poletti, V., Zamò, A., Lestani, M., Montagna, L., Piccoli, P., … Doglioni, C. (2003). Aberrant Wnt/beta-catenin pathway activation in idiopathic pulmonary fibrosis. *The American Journal of Pathology*, *162*(5), 1495–1502.

Chu, P.-H., Bardwell, W. M., Gu, Y., Ross, J., & Chen, J. (2000). FHL2 (SLIM3) Is Not

Essential for Cardiac Development and Function. *Molecular and Cellular Biology*, *20*(20), 7460–7462.

Coghill, I. D., Brown, S., Cottle, D. L., McGrath, M. J., Robinson, P. A., Nandurkar, H. H., … Mitchell, C. A. (2003). FHL3 is an actin-binding protein that regulates alpha-actinin-mediated actin bundling: FHL3 localizes to actin stress fibers and enhances cell spreading and stress fiber disassembly. *The Journal of Biological Chemistry*, *278*(26), 24139–24152. http://doi.org/10.1074/jbc.M213259200

Coker, R. K., Laurent, G. J., Jeffery, P. K., du Bois, R. M., Black, C. M., & McAnulty, R. J. (2001). Localisation of transforming growth factor beta1 and beta3 mRNA transcripts in normal and fibrotic human lung. *Thorax*, *56*(7), 549–556.

Cool, C. D., Groshong, S. D., Rai, P. R., Henson, P. M., Stewart, J. S., & Brown, K. K. (2006). Fibroblast foci are not discrete sites of lung injury or repair: the fibroblast reticulum. *American Journal of Respiratory and Critical Care Medicine*, *174*(6), 654–658. http://doi.org/10.1164/rccm.200602-205OC

Croce, J. C., & McClay, D. R. (2008). Evolution of the Wnt pathways. *Methods in Molecular Biology (Clifton, N.J.)*, *469*, 3–18. http://doi.org/10.1007/978-1-60327-469-2_1

Daugherty, R. L., & Gottardi, C. J. (2007). Phospho-regulation of Beta-catenin adhesion and signaling functions. *Physiology (Bethesda, Md.)*, *22*, 303–309. http://doi.org/10.1152/physiol.00020.2007

Dawid, I. B., Breen, J. J., & Toyama, R. (1998). LIM domains: multiple roles as adapters and functional modifiers in protein interactions. *Trends in Genetics: TIG*, *14*(4), 156–162.

Dean, R. A., Cox, J. H., Bellac, C. L., Doucet, A., Starr, A. E., & Overall, C. M. (2008). Macrophage-specific metalloelastase (MMP-12) truncates and inactivates ELR+ CXC chemokines and generates CCL2, -7, -8, and -13 antagonists: potential role of the macrophage in terminating polymorphonuclear leukocyte influx. *Blood*, *112*(8), 3455–3464. http://doi.org/10.1182/blood-2007-12-129080

Degryse, A. L., Tanjore, H., Xu, X. C., Polosukhin, V. V., Jones, B. R., McMahon, F. B., … Lawson, W. E. (2010). Repetitive intratracheal bleomycin models several features of idiopathic pulmonary fibrosis. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *299*(4), L442–452. http://doi.org/10.1152/ajplung.00026.2010

Denzel, S., Maetzel, D., Mack, B., Eggert, C., Bärr, G., & Gires, O. (2009). Initial activation of EpCAM cleavage via cell-to-cell contact. *BMC Cancer*, *9*, 402. http://doi.org/10.1186/1471-2407-9-402

Ding, L., Wang, Z., Yan, J., Yang, X., Liu, A., Qiu, W., … Ye, Q. (2009). Human four-and-a-half LIM family members suppress tumor cell growth through a TGF-beta-like signaling pathway. *The Journal of Clinical Investigation*, *119*(2), 349–361. http://doi.org/10.1172/JCI35930

Dolmans, G. H., Werker, P. M., Hennies, H. C., Furniss, D., Festen, E. A., Franke, L., … Wijmenga, C. (2011). Wnt signaling and Dupuytren's disease. *The New England Journal of Medicine*, *365*(4), 307–317. http://doi.org/10.1056/NEJMoa1101029

Eger, A., Stockinger, A., Schaffhauser, B., Beug, H., & Foisner, R. (2000). Epithelial mesenchymal transition by c-Fos estrogen receptor activation involves nuclear translocation of beta-catenin and upregulation of beta-catenin/lymphoid enhancer binding factor-1 transcriptional activity. *The Journal of Cell Biology*, *148*(1), 173–188.

Eickelberg, O., Köhler, E., Reichenberger, F., Bertschin, S., Woodtli, T., Erne, P., … Roth,

M. (1999). Extracellular matrix deposition by primary human lung fibroblasts in response to TGF-beta1 and TGF-beta3. *The American Journal of Physiology*, *276*(5 Pt 1), L814–824.

Eickelberg, O., & Laurent, G. J. (2010). The quest for the initial lesion in idiopathic pulmonary fibrosis: gene expression differences in IPF fibroblasts. *American Journal of Respiratory Cell and Molecular Biology*, *42*(1), 1–2. http://doi.org/10.1165/rcmb.2009-0245ED

Eickelberg, O., & Selman, M. (2010). Update in diffuse parenchymal lung disease 2009. *American Journal of Respiratory and Critical Care Medicine*, *181*(9), 883–888. http://doi.org/10.1164/rccm.201001-0124UP

El-Chemaly, S., Pacheco-Rodriguez, G., Ikeda, Y., Malide, D., & Moss, J. (2009). Lymphatics in idiopathic pulmonary fibrosis: new insights into an old disease. *Lymphatic Research and Biology*, *7*(4), 197–203. http://doi.org/10.1089/lrb.2009.0014

El Mourabit, H., Müller, S., Tunggal, L., Paulsson, M., & Aumailley, M. (2004). Analysis of the adaptor function of the LIM domain-containing protein FHL2 using an affinity chromatography approach. *Journal of Cellular Biochemistry*, *92*(3), 612–625. http://doi.org/10.1002/jcb.20096

Estany, S., Vicens-Zygmunt, V., Llatjós, R., Montes, A., Penín, R., Escobar, I., … Molina-Molina, M. (2014). Lung fibrotic tenascin-C upregulation is associated with other extracellular matrix proteins and induced by TGFβ1. *BMC Pulmonary Medicine*, *14*, 120. http://doi.org/10.1186/1471-2466-14-120

Fimia, G. M., De Cesare, D., & Sassone-Corsi, P. (2000). A family of LIM-only transcriptional coactivators: tissue-specific expression and selective activation of CREB and CREM. *Molecular and Cellular Biology*, *20*(22), 8613–8622.

Flaherty, K. R., Travis, W. D., Colby, T. V., Toews, G. B., Kazerooni, E. A., Gross, B. H., … Martinez, F. J. (2001). Histopathologic variability in usual and nonspecific interstitial pneumonias. *American Journal of Respiratory and Critical Care Medicine*, *164*(9), 1722–1727.

Funahashi, A., Matsuoka, Y., Jouraku, A., Morohashi, M., Kikuchi, N., & Kitano, H. (2008). CellDesigner 3.5: A Versatile Modeling Tool for Biochemical Networks. *Proceedings of the IEEE*, *96*(8), 1254–1265. http://doi.org/10.1109/JPROC.2008.925458

Furuhashi, M., Yagi, K., Yamamoto, H., Furukawa, Y., Shimada, S., Nakamura, Y., … Kato, M. (2001). Axin facilitates Smad3 activation in the transforming growth factor beta signaling pathway. *Molecular and Cellular Biology*, *21*(15), 5132–5141. http://doi.org/10.1128/MCB.21.15.5132-5141.2001

Fuxe, J., Vincent, T., & de Herreros, A. G. (2010). Transcriptional crosstalk between TGFbeta and stem cell pathways in tumor cell invasion: Role of EMT promoting Smad complexes. *Cell Cycle (Georgetown, Tex.)*, *9*(12). Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/20519943

Gauldie, J., Bonniaud, P., Sime, P., Ask, K., & Kolb, M. (2007). TGF-beta, Smad3 and the process of progressive fibrosis. *Biochemical Society Transactions*, *35*(Pt 4), 661–664. http://doi.org/10.1042/BST0350661

Genini, M., Schwalbe, P., Scholl, F. A., Remppis, A., Mattei, M. G., & Schäfer, B. W. (1997). Subtractive cloning and characterization of DRAL, a novel LIM-domain protein down-

regulated in rhabdomyosarcoma. *DNA and Cell Biology*, *16*(4), 433–442.

Giri, S. N., Hyde, D. M., & Hollinger, M. A. (1993). Effect of antibody to transforming growth factor beta on bleomycin induced accumulation of lung collagen in mice. *Thorax*, *48*(10), 959–966.

Gomer, R. H., & Lupher, M. L., Jr. (2010). Investigational approaches to therapies for idiopathic pulmonary fibrosis. *Expert Opinion on Investigational Drugs*, *19*(6), 737–745. http://doi.org/10.1517/13543784.2010.484018

Govoni, K. E., Amaar, Y. G., Kramer, A., Winter, E., Baylink, D. J., & Mohan, S. (2006). Regulation of insulin-like growth factor binding protein-5, four and a half lim-2, and a disintegrin and metalloprotease-9 expression in osteoblasts. *Growth Hormone & IGF Research: Official Journal of the Growth Hormone Research Society and the International IGF Research Society*, *16*(1), 49–56. http://doi.org/10.1016/j.ghir.2005.10.001

Green, F. H. Y. (2002). Overview of pulmonary fibrosis. *Chest*, *122*(6 Suppl), 334S–339S.

Gullotti, L., Czerwitzki, J., Kirfel, J., Propping, P., Rahner, N., Steinke, V., … Friedrichs, N. (2011). FHL2 expression in peritumoural fibroblasts correlates with lymphatic metastasis in sporadic but not in HNPCC-associated colon cancer. *Laboratory Investigation; a Journal of Technical Methods and Pathology*, *91*(12), 1695–1705. http://doi.org/10.1038/labinvest.2011.109

Gumbiner, B. M. (1996). Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell*, *84*(3), 345–357.

Guo, X., & Wang, X.-F. (2009). Signaling cross-talk between TGF-[beta]/BMP and other pathways. *Cell Res*, *19*(1), 71–88.

Hamidouche, Z., Haÿ, E., Vaudin, P., Charbord, P., Schüle, R., Marie, P. J., & Fromigué, O. (2008). FHL2 mediates dexamethasone-induced mesenchymal cell differentiation into osteoblasts by activating Wnt/beta-catenin signaling-dependent Runx2 expression. *The FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *22*(11), 3813–3822. http://doi.org/10.1096/fj.08-106302

Harari, S., & Caminati, A. (2005). Idiopathic pulmonary fibrosis. *Allergy*, *60*(4), 421–435. http://doi.org/10.1111/j.1398-9995.2005.00719.x

Henderson, W. R., Jr, Chi, E. Y., Ye, X., Nguyen, C., Tien, Y., Zhou, B., … Kahn, M. (2010). Inhibition of Wnt/beta-catenin/CREB binding protein (CBP) signaling reverses pulmonary fibrosis. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(32), 14309–14314. http://doi.org/10.1073/pnas.1001520107

Hiwatari, N., Shimura, S., Yamauchi, K., Nara, M., Hida, W., & Shirato, K. (1997). Significance of elevated procollagen-III-peptide and transforming growth factor-beta levels of bronchoalveolar lavage fluids from idiopathic pulmonary fibrosis patients. *The Tohoku Journal of Experimental Medicine*, *181*(2), 285–295.

Huss, S., Stellmacher, C., Goltz, D., Khlistunova, I., Adam, A. C., Trebicka, J., … Weiskirchen, R. (2013). Deficiency in four and one half LIM domain protein 2 (FHL2) aggravates liver fibrosis in mice. *BMC Gastroenterology*, *13*(1), 8. http://doi.org/10.1186/1471-230X-13-8

Itman, C., Mendis, S., Barakat, B., & Loveland, K. L. (2006). All in the family: TGF-beta family action in testis development. *Reproduction (Cambridge, England)*, *132*(2),

233–246. http://doi.org/10.1530/rep.1.01075

Jian, H., Shen, X., Liu, I., Semenov, M., He, X., & Wang, X.-F. (2006). Smad3-dependent nuclear translocation of beta-catenin is required for TGF-beta1-induced proliferation of bone marrow-derived adult human mesenchymal stem cells. *Genes & Development*, *20*(6), 666–674. http://doi.org/10.1101/gad.1388806

Johannessen, M., Møller, S., Hansen, T., Moens, U., & Van Ghelue, M. (2006). The multifunctional roles of the four-and-a-half-LIM only protein FHL2. *Cellular and Molecular Life Sciences: CMLS*, *63*(3), 268–284. http://doi.org/10.1007/s00018-005-5438-z

Jones, J. A., Spinale, F. G., & Ikonomidis, J. S. (2009). Transforming growth factor-beta signaling in thoracic aortic aneurysm development: a paradox in pathogenesis. *Journal of Vascular Research*, *46*(2), 119–137. http://doi.org/10.1159/000151766

Kadrmas, J. L., & Beckerle, M. C. (2004). The LIM domain: from the cytoskeleton to the nucleus. *Nature Reviews. Molecular Cell Biology*, *5*(11), 920–931. http://doi.org/10.1038/nrm1499

Kage, H., & Borok, Z. (2012). EMT and interstitial lung disease: a mysterious relationship. *Current Opinion in Pulmonary Medicine*, *18*(5), 517–523. http://doi.org/10.1097/MCP.0b013e3283566721

Kalluri, R., & Neilson, E. G. (2003). Epithelial-mesenchymal transition and its implications for fibrosis. *The Journal of Clinical Investigation*, *112*(12), 1776–1784. http://doi.org/10.1172/JCI20530

Kalluri, R., & Weinberg, R. A. (2009). The basics of epithelial-mesenchymal transition. *The Journal of Clinical Investigation*, *119*(6), 1420–1428. http://doi.org/10.1172/JCI39104

Kaminski, N., & Rosas, I. O. (2006). Gene expression profiling as a window into idiopathic pulmonary fibrosis pathogenesis: can we identify the right target genes? *Proceedings of the American Thoracic Society*, *3*(4), 339–344. http://doi.org/10.1513/pats.200601-011TK

Kierszenbaum, A. L. (2002). *Histology and cell biology: an introduction to pathology*. Elsevier Health Sciences.

Kim, K. K., Kugler, M. C., Wolters, P. J., Robillard, L., Galvez, M. G., Brumwell, A. N., … Chapman, H. A. (2006). Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(35), 13180–13185. http://doi.org/10.1073/pnas.0605669103

Kim, K. K., Wei, Y., Szekeres, C., Kugler, M. C., Wolters, P. J., Hill, M. L., … Chapman, H. A. (2009). Epithelial cell alpha3beta1 integrin links beta-catenin and Smad signaling to promote myofibroblast formation and pulmonary fibrosis. *The Journal of Clinical Investigation*, *119*(1), 213–224. http://doi.org/10.1172/JCI36940

Kim, K., Lu, Z., & Hay, E. D. (2002). Direct evidence for a role of beta-catenin/LEF-1 signaling pathway in induction of EMT. *Cell Biology International*, *26*(5), 463–476.

Kim, Y., Kugler, M. C., Wei, Y., Kim, K. K., Li, X., Brumwell, A. N., & Chapman, H. A. (2009). Integrin alpha3beta1-dependent beta-catenin phosphorylation links epithelial Smad signaling to cell contacts. *The Journal of Cell Biology*, *184*(2), 309–322. http://doi.org/10.1083/jcb.200806067

King, T. E., Bradford, W. Z., Castro-Bernardini, S., Fagan, E. A., Glaspole, I., Glassberg, M. K., … ASCEND Study Group. (2014). A phase 3 trial of pirfenidone in patients with idiopathic pulmonary fibrosis. *The New England Journal of Medicine*, *370*(22), 2083–2092. http://doi.org/10.1056/NEJMoa1402582

Kirfel, J., Pantelis, D., Kabba, M., Kahl, P., Röper, A., Kalff, J. C., & Buettner, R. (2008). Impaired intestinal wound healing in Fhl2-deficient mice is due to disturbed collagen metabolism. *Experimental Cell Research*, *314*(20), 3684–3691. http://doi.org/10.1016/j.yexcr.2008.09.023

Kleiber, K., Strebhardt, K., & Martin, B. T. (2007). The biological relevance of FHL2 in tumour cells and its role as a putative cancer target. *Anticancer Research*, *27*(1A), 55–61.

Kong, D., Li, Y., Wang, Z., & Sarkar, F. H. (2011). Cancer Stem Cells and Epithelial-to-Mesenchymal Transition (EMT)-Phenotypic Cells: Are They Cousins or Twins? *Cancers*, *3*(1), 716–729. http://doi.org/10.3390/cancers30100716

Königshoff, M., Balsara, N., Pfaff, E.-M., Kramer, M., Chrobak, I., Seeger, W., & Eickelberg, O. (2008). Functional Wnt signaling is increased in idiopathic pulmonary fibrosis. *PloS One*, *3*(5), e2142. http://doi.org/10.1371/journal.pone.0002142

Königshoff, M., & Eickelberg, O. (2010). WNT signaling in lung disease: a failure or a regeneration signal? *American Journal of Respiratory Cell and Molecular Biology*, *42*(1), 21–31. http://doi.org/10.1165/rcmb.2008-0485TR

Königshoff, M., Kramer, M., Balsara, N., Wilhelm, J., Amarie, O. V., Jahn, A., … Eickelberg, O. (2009). WNT1-inducible signaling protein-1 mediates pulmonary fibrosis in mice and is upregulated in humans with idiopathic pulmonary fibrosis. *The Journal of Clinical Investigation*, *119*(4), 772–787. http://doi.org/10.1172/JCI33950

Koressaar, T., & Remm, M. (2007). Enhancements and modifications of primer design program Primer3. *Bioinformatics (Oxford, England)*, *23*(10), 1289–1291. http://doi.org/10.1093/bioinformatics/btm091

Kubiczkova, L., Sedlarikova, L., Hajek, R., & Sevcikova, S. (2012). TGF-? - an excellent servant but a bad master. *Journal of Translational Medicine*, *10*, 183. http://doi.org/10.1186/1479-5876-10-183

Kuwano, K., Nomoto, Y., Kunitake, R., Hagimoto, N., Matsuba, T., Nakanishi, Y., & Hara, N. (1997). Detection of adenovirus E1A DNA in pulmonary fibrosis using nested polymerase chain reaction. *The European Respiratory Journal: Official Journal of the European Society for Clinical Respiratory Physiology*, *10*(7), 1445–1449.

Labalette, C., Nouët, Y., Levillayer, F., Colnot, S., Chen, J., Claude, V., … Wei, Y. (2010). Deficiency of the LIM-only protein FHL2 reduces intestinal tumorigenesis in Apc mutant mice. *PloS One*, *5*(4), e10371. http://doi.org/10.1371/journal.pone.0010371

Labalette, C., Nouët, Y., Sobczak-Thepot, J., Armengol, C., Levillayer, F., Gendron, M.-C., … Wei, Y. (2008). The LIM-only protein FHL2 regulates cyclin D1 expression and cell proliferation. *The Journal of Biological Chemistry*, *283*(22), 15201–15208. http://doi.org/10.1074/jbc.M800708200

Labalette, C., Renard, C.-A., Neuveut, C., Buendia, M.-A., & Wei, Y. (2004). Interaction and functional cooperation between the LIM protein FHL2, CBP/p300, and beta-catenin. *Molecular and Cellular Biology*, *24*(24), 10689–10702. http://doi.org/10.1128/MCB.24.24.10689-10702.2004

Labbé, E., & Attisano, L. (2006). Integration of Signaling Pathways Via Smad Proteins. In P.

ten Dijke & C.-H. Heldin (Eds.), *Smad Signal Transduction* (pp. 295–316). Springer Netherlands. Retrieved from http://link.springer.com/chapter/10.1007/1-4020-4709-6_15

Labbé, E., Letamendia, A., & Attisano, L. (2000). Association of Smads with lymphoid enhancer binding factor 1/T cell-specific factor mediates cooperative signaling by the transforming growth factor-beta and wnt pathways. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(15), 8358–8363. http://doi.org/10.1073/pnas.150152697

Labbé, E., Lock, L., Letamendia, A., Gorska, A. E., Gryfe, R., Gallinger, S., … Attisano, L. (2007). Transcriptional cooperation between the transforming growth factor-beta and Wnt pathways in mammary and intestinal tumorigenesis. *Cancer Research*, *67*(1), 75–84. http://doi.org/10.1158/0008-5472.CAN-06-2559

Lacroix-Triki, M., Geyer, F. C., Lambros, M. B., Savage, K., Ellis, I. O., Lee, A. H. S., & Reis-Filho, J. S. (2010). β-catenin/Wnt signalling pathway in fibromatosis, metaplastic carcinomas and phyllodes tumours of the breast. *Modern Pathology: An Official Journal of the United States and Canadian Academy of Pathology, Inc*, *23*(11), 1438–1448. http://doi.org/10.1038/modpathol.2010.141

Lawson, W. E., Crossno, P. F., Polosukhin, V. V., Roldan, J., Cheng, D.-S., Lane, K. B., … Blackwell, T. S. (2008). Endoplasmic reticulum stress in alveolar epithelial cells is prominent in IPF: association with altered surfactant protein processing and herpesvirus infection. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *294*(6), L1119–1126. http://doi.org/10.1152/ajplung.00382.2007

Leask, A., & Abraham, D. J. (2004). TGF-beta signaling and the fibrotic response. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *18*(7), 816–827. http://doi.org/10.1096/fj.03-1273rev

Leslie, K. O. (2005). Historical perspective: a pathologic approach to the classification of idiopathic interstitial pneumonias. *Chest*, *128*(5 Suppl 1), 513S–519S. http://doi.org/10.1378/chest.128.5_suppl_1.513S

Lin, S. J., Lerch, T. F., Cook, R. W., Jardetzky, T. S., & Woodruff, T. K. (2006). The structural basis of TGF-beta, bone morphogenetic protein, and activin ligand binding. *Reproduction (Cambridge, England)*, *132*(2), 179–190. http://doi.org/10.1530/rep.1.01072

Liu, J., Wang, Y., Pan, Q., Su, Y., Zhang, Z., Han, J., … Hu, D. (2012). Wnt/β-catenin pathway forms a negative feedback loop during TGF-β1 induced human normal skin fibroblast-to-myofibroblast transition. *Journal of Dermatological Science*, *65*(1), 38–49. http://doi.org/10.1016/j.jdermsci.2011.09.012

Liu, W., Rui, H., Wang, J., Lin, S., He, Y., Chen, M., … Lin, S.-C. (2006). Axin is a scaffold protein in TGF-beta signaling that promotes degradation of Smad7 by Arkadia. *The EMBO Journal*, *25*(8), 1646–1658. http://doi.org/10.1038/sj.emboj.7601057

Liu, X., Hu, H., & Yin, J. Q. (2006). Therapeutic strategies against TGF-beta signaling pathway in hepatic fibrosis. *Liver International: Official Journal of the International Association for the Study of the Liver*, *26*(1), 8–22. http://doi.org/10.1111/j.1478-3231.2005.01192.x

Logan, C. Y., & Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annual Review of Cell and Developmental Biology*, *20*, 781–810. http://doi.org/10.1146/annurev.cellbio.20.010403.113126

Luu, H. H., Zhang, R., Haydon, R. C., Rayburn, E., Kang, Q., Si, W., … He, T.-C. (2004). Wnt/beta-catenin signaling pathway as a novel cancer drug target. *Current Cancer Drug Targets*, *4*(8), 653–671.

Marshall, R. P., Puddicombe, A., Cookson, W. O., & Laurent, G. J. (2000). Adult familial cryptogenic fibrosing alveolitis in the United Kingdom. *Thorax*, *55*(2), 143–146.

Martin, B., Schneider, R., Janetzky, S., Waibler, Z., Pandur, P., Kühl, M., … Wixler, V. (2002). The LIM-only protein FHL2 interacts with beta-catenin and promotes differentiation of mouse myoblasts. *The Journal of Cell Biology*, *159*(1), 113–122. http://doi.org/10.1083/jcb.200202075

Martin, G. E. M., Kolb, M., & Gauldie, J. (2006). Targeting genes for treatment in idiopathic pulmonary fibrosis: challenges and opportunities, promises and pitfalls. *Proceedings of the American Thoracic Society*, *3*(4), 389–393. http://doi.org/10.1513/pats.200602-021TK

Massagué, J., Blain, S. W., & Lo, R. S. (2000). TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell*, *103*(2), 295–309.

McMahon, A. P., & Moon, R. T. (1989). Ectopic expression of the proto-oncogene int-1 in Xenopus embryos leads to duplication of the embryonic axis. *Cell*, *58*(6), 1075–1084.

Meltzer, E. B., & Noble, P. W. (2008). Idiopathic pulmonary fibrosis. *Orphanet Journal of Rare Diseases*, *3*, 8. http://doi.org/10.1186/1750-1172-3-8

Michalik, M., Wójcik, K. A., Jakieła, B., Szpak, K., Pierzchalska, M., Sanak, M., … Czyż, J. (2012). Lithium Attenuates TGF-β(1)-Induced Fibroblasts to Myofibroblasts Transition in Bronchial Fibroblasts Derived from Asthmatic Patients. *Journal of Allergy*, *2012*, 206109. http://doi.org/10.1155/2012/206109

Minoo, P., & Li, C. (2010). Cross-talk between transforming growth factor-beta and Wingless/Int pathways in lung development and disease. *The International Journal of Biochemistry & Cell Biology*, *42*(6), 809–812. http://doi.org/10.1016/j.biocel.2010.02.011

Mistry, A. C., Kato, A., Tran, Y. H., Honda, S., Tsukada, T., Takei, Y., & Hirose, S. (2004). FHL5, a novel actin-binding protein, is highly expressed in eel gill pillar cells and responds to wall tension. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, *287*(5), R1141–1154. http://doi.org/10.1152/ajpregu.00108.2004

Moon, R. T., Kohn, A. D., De Ferrari, G. V., & Kaykas, A. (2004). WNT and beta-catenin signalling: diseases and therapies. *Nature Reviews. Genetics*, *5*(9), 691–701. http://doi.org/10.1038/nrg1427

Moore, B. B., & Hogaboam, C. M. (2008). Murine models of pulmonary fibrosis. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *294*(2), L152–160. http://doi.org/10.1152/ajplung.00313.2007

Morgan, M. J., & Madgwick, A. J. (1999). The fourth member of the FHL family of LIM proteins is expressed exclusively in the testis. *Biochemical and Biophysical Research Communications*, *255*(2), 251–255. http://doi.org/10.1006/bbrc.1999.0180

Morgan, M. J., & Whawell, S. A. (2000). The structure of the human LIM protein ACT gene and its expression in tumor cell lines. *Biochemical and Biophysical Research Communications*, *273*(2), 776–783. http://doi.org/10.1006/bbrc.2000.3006

Morlon, A., & Sassone-Corsi, P. (2003). The LIM-only protein FHL2 is a serum-inducible transcriptional coactivator of AP-1. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(7), 3977–3982. http://doi.org/10.1073/pnas.0735923100

Morrisey, E. E. (2003). Wnt signaling and pulmonary fibrosis. *The American Journal of Pathology*, *162*(5), 1393–1397. http://doi.org/10.1016/S0002-9440(10)64271-X

Moustakas, A., & Heldin, C.-H. (2005). Non-Smad TGF-beta signals. *Journal of Cell Science*, *118*(Pt 16), 3573–3584. http://doi.org/10.1242/jcs.02554

Mucenski, M. L., Nation, J. M., Thitoff, A. R., Besnard, V., Xu, Y., Wert, S. E., … Whitsett, J. A. (2005). Beta-catenin regulates differentiation of respiratory epithelial cells in vivo. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *289*(6), L971–979. http://doi.org/10.1152/ajplung.00172.2005

Mucenski, M. L., Wert, S. E., Nation, J. M., Loudy, D. E., Huelsken, J., Birchmeier, W., … Whitsett, J. A. (2003). beta-Catenin is required for specification of proximal/distal cell fate during lung morphogenesis. *The Journal of Biological Chemistry*, *278*(41), 40231–40238. http://doi.org/10.1074/jbc.M305892200

Mulholland, D. J., Dedhar, S., Coetzee, G. A., & Nelson, C. C. (2005). Interaction of nuclear receptors with the Wnt/beta-catenin/Tcf signaling axis: Wnt you like to know? *Endocrine Reviews*, *26*(7), 898–915. http://doi.org/10.1210/er.2003-0034

Müller, J. M., Metzger, E., Greschik, H., Bosserhoff, A.-K., Mercep, L., Buettner, R., & Schüle, R. (2002). The transcriptional coactivator FHL2 transmits Rho signals from the cell membrane into the nucleus. *The EMBO Journal*, *21*(4), 736–748.

Nagano, Y., Mavrakis, K. J., Lee, K. L., Fujii, T., Koinuma, D., Sase, H., … Miyazawa, K. (2007). Arkadia induces degradation of SnoN and c-Ski to enhance transforming growth factor-beta signaling. *The Journal of Biological Chemistry*, *282*(28), 20492–20501. http://doi.org/10.1074/jbc.M701294200

Nakao, A., Fujii, M., Matsumura, R., Kumano, K., Saito, Y., Miyazono, K., & Iwamoto, I. (1999). Transient gene transfer and expression of Smad7 prevents bleomycin-induced lung fibrosis in mice. *The Journal of Clinical Investigation*, *104*(1), 5–11. http://doi.org/10.1172/JCI6094

Nawshad, A., Medici, D., Liu, C.-C., & Hay, E. D. (2007). TGFbeta3 inhibits E-cadherin gene expression in palate medial-edge epithelial cells through a Smad2-Smad4-LEF1 transcription complex. *Journal of Cell Science*, *120*(Pt 9), 1646–1653. http://doi.org/10.1242/jcs.003129

Ng, C.-F., Ng, P. K.-S., Lui, V. W.-Y., Li, J., Chan, J. Y.-W., Fung, K.-P., … Tsui, S. K. W. (2011). FHL2 exhibits anti-proliferative and anti-apoptotic activities in liver cancer cells. *Cancer Letters*, *304*(2), 97–106. http://doi.org/10.1016/j.canlet.2011.02.001

Nishita, M., Hashimoto, M. K., Ogata, S., Laurent, M. N., Ueno, N., Shibuya, H., & Cho, K. W. (2000). Interaction between Wnt and TGF-beta signalling pathways during formation of Spemann's organizer. *Nature*, *403*(6771), 781–785. http://doi.org/10.1038/35001602

Nogee, L. M., Dunbar, A. E., 3rd, Wert, S. E., Askin, F., Hamvas, A., & Whitsett, J. A. (2001). A mutation in the surfactant protein C gene associated with familial interstitial lung disease. *The New England Journal of Medicine*, *344*(8), 573–579. http://doi.org/10.1056/NEJM200102223440805

Novak, A., Hsu, S. C., Leung-Hagesteijn, C., Radeva, G., Papkoff, J., Montesano, R., … Dedhar, S. (1998). Cell adhesion and the integrin-linked kinase regulate the LEF-1 and beta-catenin signaling pathways. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(8), 4374–4379.

Nusse, R., Brown, A., Papkoff, J., Scambler, P., Shackleford, G., McMahon, A., … Varmus, H. (1991). A new nomenclature for int-1 and related genes: the Wnt gene family. *Cell*, *64*(2), 231.

Okerlund, N. D., & Cheyette, B. N. R. (2011). Synaptic Wnt signaling-a contributor to major psychiatric disorders? *Journal of Neurodevelopmental Disorders*, *3*(2), 162–174. http://doi.org/10.1007/s11689-011-9083-6

Ozdamar, B., Bose, R., Barrios-Rodiles, M., Wang, H.-R., Zhang, Y., & Wrana, J. L. (2005). Regulation of the polarity protein Par6 by TGFbeta receptors controls epithelial cell plasticity. *Science (New York, N.Y.)*, *307*(5715), 1603–1609. http://doi.org/10.1126/science.1105718

Pan, L. H., Yamauchi, K., Uzuki, M., Nakanishi, T., Takigawa, M., Inoue, H., & Sawai, T. (2001). Type II alveolar epithelial cells and interstitial fibroblasts express connective tissue growth factor in IPF. *The European Respiratory Journal: Official Journal of the European Society for Clinical Respiratory Physiology*, *17*(6), 1220–1227.

Panos, R. J., Mortenson, R. L., Niccoli, S. A., & King, T. E., Jr. (1990). Clinical deterioration in patients with idiopathic pulmonary fibrosis: causes and assessment. *The American Journal of Medicine*, *88*(4), 396–404.

Pardo, A., Gibson, K., Cisneros, J., Richards, T. J., Yang, Y., Becerril, C., … Kaminski, N. (2005). Up-regulation and profibrotic role of osteopontin in human idiopathic pulmonary fibrosis. *PLoS Medicine*, *2*(9), e251. http://doi.org/10.1371/journal.pmed.0020251

Pardo, A., & Selman, M. (2002). Molecular mechanisms of pulmonary fibrosis. *Frontiers in Bioscience: A Journal and Virtual Library*, *7*, d1743–1761.

Park, J., Will, C., Martin, B., Gullotti, L., Friedrichs, N., Buettner, R., … Wixler, V. (2008). Deficiency in the LIM-only protein FHL2 impairs assembly of extracellular matrix proteins. *The FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *22*(7), 2508–2520. http://doi.org/10.1096/fj.07-095521

Pawson, T. (2003). Organization of cell-regulatory systems through modular-protein-interaction domains. *Philosophical Transactions. Series A, Mathematical, Physical, and Engineering Sciences*, *361*(1807), 1251–1262. http://doi.org/10.1098/rsta.2003.1197

Pawson, T., & Nash, P. (2003). Assembly of cell regulatory systems through protein interaction domains. *Science (New York, N.Y.)*, *300*(5618), 445–452. http://doi.org/10.1126/science.1083653

Piedra, J., Martinez, D., Castano, J., Miravet, S., Dunach, M., & de Herreros, A. G. (2001). Regulation of beta-catenin structure and activity by tyrosine phosphorylation. *The Journal of Biological Chemistry*, *276*(23), 20436–20443. http://doi.org/10.1074/jbc.M100194200

Raghu, G., Collard, H. R., Egan, J. J., Martinez, F. J., Behr, J., Brown, K. K., … Schünemann, H. J. (2011). An official ATS/ERS/JRS/ALAT statement: idiopathic

pulmonary fibrosis: evidence-based guidelines for diagnosis and management. *American Journal of Respiratory and Critical Care Medicine*, *183*(6), 788–824. http://doi.org/10.1164/rccm.2009-040GL

Raghu, G., Nicholson, A. G., & Lynch, D. (2008). The classification, natural history and radiological/histological appearance of idiopathic pulmonary fibrosis and the other idiopathic interstitial pneumonias. *European Respiratory Review*, *17*(109), 108–115. http://doi.org/10.1183/09059180.00010902

Raghu, G., Rochwerg, B., Zhang, Y., Garcia, C. A. C., Azuma, A., Behr, J., … ATS, ERS, JRS, and ALAT. (2015). An Official ATS/ERS/JRS/ALAT Clinical Practice Guideline: Treatment of Idiopathic Pulmonary Fibrosis. An Update of the 2011 Clinical Practice Guideline. *American Journal of Respiratory and Critical Care Medicine*, *192*(2), e3–19. http://doi.org/10.1164/rccm.201506-1063ST

Renger, A., Zafiriou, M.-P., Noack, C., Pavlova, E., Becker, A., Sharkova, K., … Zelarayán, L. C. (2013). The Four and a Half LIM-domain (FHL) 2 Controls Early Cardiac Cell Commitment and Expansion via Regulating β-catenin-Dependent Transcription. *Stem Cells (Dayton, Ohio)*. http://doi.org/10.1002/stem.1332

Richeldi, L., du Bois, R. M., Raghu, G., Azuma, A., Brown, K. K., Costabel, U., … INPULSIS Trial Investigators. (2014). Efficacy and safety of nintedanib in idiopathic pulmonary fibrosis. *The New England Journal of Medicine*, *370*(22), 2071–2082. http://doi.org/10.1056/NEJMoa1402584

Rijsewijk, F., Schuermann, M., Wagenaar, E., Parren, P., Weigel, D., & Nusse, R. (1987). The Drosophila homolog of the mouse mammary oncogene int-1 is identical to the segment polarity gene wingless. *Cell*, *50*(4), 649–657.

Sambrook, J., & Russell, D. W. (2006). The Hanahan Method for Preparation and Transformation of Competent E. coli: High-efficiency Transformation. *CSH Protocols*, *2006*(1). http://doi.org/10.1101/pdb.prot3942

Samson, T., Smyth, N., Janetzky, S., Wendler, O., Müller, J. M., Schüle, R., … Wixler, V. (2004). The LIM-only proteins FHL2 and FHL3 interact with alpha- and beta-subunits of the muscle alpha7beta1 integrin receptor. *The Journal of Biological Chemistry*, *279*(27), 28641–28652. http://doi.org/10.1074/jbc.M312894200

Sánchez-García, I., Osada, H., Forster, A., & Rabbitts, T. H. (1993). The cysteine-rich LIM domains inhibit DNA binding by the associated homeodomain in Isl-1. *The EMBO Journal*, *12*(11), 4243–4250.

Schmeichel, K. L., & Beckerle, M. C. (1994). The LIM domain is a modular protein-binding interface. *Cell*, *79*(2), 211–219.

Schmierer, B., & Hill, C. S. (2007). TGFbeta-SMAD signal transduction: molecular specificity and functional flexibility. *Nature Reviews. Molecular Cell Biology*, *8*(12), 970–982. http://doi.org/10.1038/nrm2297

Scotton, C. J., & Chambers, R. C. (2010). Bleomycin revisited: towards a more representative model of IPF? *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *299*(4), L439–441. http://doi.org/10.1152/ajplung.00258.2010

Selman, M., & Pardo, A. (2006). Role of epithelial cells in idiopathic pulmonary fibrosis: from innocent targets to serial killers. *Proceedings of the American Thoracic Society*, *3*(4), 364–372. http://doi.org/10.1513/pats.200601-003TK

Selman, M., Pardo, A., Barrera, L., Estrada, A., Watson, S. R., Wilson, K., … Zlotnik, A.

(2006). Gene expression profiles distinguish idiopathic pulmonary fibrosis from hypersensitivity pneumonitis. *American Journal of Respiratory and Critical Care Medicine*, *173*(2), 188–198. http://doi.org/10.1164/rccm.200504-644OC

Selman, M., Pardo, A., & Kaminski, N. (2008). Idiopathic pulmonary fibrosis: aberrant recapitulation of developmental programs? *PLoS Medicine*, *5*(3), e62. http://doi.org/10.1371/journal.pmed.0050062

Shafer, S. L., & Towler, D. A. (2009). Transcriptional regulation of SM22alpha by Wnt3a: convergence with TGFbeta(1)/Smad signaling at a novel regulatory element. *Journal of Molecular and Cellular Cardiology*, *46*(5), 621–635. http://doi.org/10.1016/j.yjmcc.2009.01.005

Stoolman, J. S., Vannella, K. M., Coomes, S. M., Wilke, C. A., Sisson, T. H., Toews, G. B., & Moore, B. B. (2011). Latent infection by γherpesvirus stimulates profibrotic mediator release from multiple cell types. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *300*(2), L274–285. http://doi.org/10.1152/ajplung.00028.2010

Strieter, R. M. (2002). Con: Inflammatory mechanisms are not a minor component of the pathogenesis of idiopathic pulmonary fibrosis. *American Journal of Respiratory and Critical Care Medicine*, *165*(9), 1206–1207; discussion 1207–1208.

Strieter, R. M. (2005). Pathogenesis and natural history of usual interstitial pneumonia: the whole story or the last chapter of a long novel. *Chest*, *128*(5 Suppl 1), 526S–532S. http://doi.org/10.1378/chest.128.5_suppl_1.526S

Tamai, K., Semenov, M., Kato, Y., Spokony, R., Liu, C., Katsuyama, Y., … He, X. (2000). LDL-receptor-related proteins in Wnt signal transduction. *Nature*, *407*(6803), 530–535. http://doi.org/10.1038/35035117

Tanahashi, H., & Tabira, T. (2000). Alzheimer's disease-associated presenilin 2 interacts with DRAL, an LIM-domain protein. *Human Molecular Genetics*, *9*(15), 2281–2289.

Tang, Y.-W., Johnson, J. E., Browning, P. J., Cruz-Gervis, R. A., Davis, A., Graham, B. S., … Stecenko, A. A. (2003). Herpesvirus DNA is consistently detected in lungs of patients with idiopathic pulmonary fibrosis. *Journal of Clinical Microbiology*, *41*(6), 2633–2640.

Thannickal, V. J., Aldweib, K. D., Rajan, T., & Fanburg, B. L. (1998). Upregulated expression of fibroblast growth factor (FGF) receptors by transforming growth factor-beta1 (TGF-beta1) mediates enhanced mitogenic responses to FGFs in cultured human lung fibroblasts. *Biochemical and Biophysical Research Communications*, *251*(2), 437–441. http://doi.org/10.1006/bbrc.1998.9443

Thiery, J. P., Acloque, H., Huang, R. Y. J., & Nieto, M. A. (2009). Epithelial-mesenchymal transitions in development and disease. *Cell*, *139*(5), 871–890. http://doi.org/10.1016/j.cell.2009.11.007

Thomas, A. Q., Lane, K., Phillips, J., 3rd, Prince, M., Markin, C., Speer, M., … Loyd, J. E. (2002). Heterozygosity for a surfactant protein C gene mutation associated with usual interstitial pneumonitis and cellular nonspecific interstitial pneumonitis in one kindred. *American Journal of Respiratory and Critical Care Medicine*, *165*(9), 1322–1328.

Tolwinski, N. S., & Wieschaus, E. (2004). Rethinking WNT signaling. *Trends in Genetics: TIG*, *20*(4), 177–181. http://doi.org/10.1016/j.tig.2004.02.003

Uhal, B. D., Joshi, I., Hughes, W. F., Ramos, C., Pardo, A., & Selman, M. (1998). Alveolar epithelial cell death adjacent to underlying myofibroblasts in advanced fibrotic human lung. *The American Journal of Physiology*, *275*(6 Pt 1), L1192–1199.

van Amerongen, R., & Nusse, R. (2009). Towards an integrated view of Wnt signaling in development. *Development (Cambridge, England)*, *136*(19), 3205–3214. http://doi.org/10.1242/dev.033910

Verrecchia, F., & Mauviel, A. (2007). Transforming growth factor-beta and fibrosis. *World Journal of Gastroenterology: WJG*, *13*(22), 3056–3062.

Visscher, D. W., & Myers, J. L. (2006). Histologic spectrum of idiopathic interstitial pneumonias. *Proceedings of the American Thoracic Society*, *3*(4), 322–329. http://doi.org/10.1513/pats.200602-019TK

Wang, Y., Kuan, P. J., Xing, C., Cronkhite, J. T., Torres, F., Rosenblatt, R. L., … Garcia, C. K. (2009). Genetic defects in surfactant protein A2 are associated with pulmonary fibrosis and lung cancer. *American Journal of Human Genetics*, *84*(1), 52–59. http://doi.org/10.1016/j.ajhg.2008.11.010

Ware, L. B., & Matthay, M. A. (2000). The acute respiratory distress syndrome. *The New England Journal of Medicine*, *342*(18), 1334–1349. http://doi.org/10.1056/NEJM200005043421806

Warner, D. R., Greene, R. M., & Pisano, M. M. (2005). Interaction between Smad 3 and Dishevelled in murine embryonic craniofacial mesenchymal cells. *Orthodontics & Craniofacial Research*, *8*(2), 123–130. http://doi.org/10.1111/j.1601-6343.2005.00319.x

Watts, K. L., Cottrell, E., Hoban, P. R., & Spiteri, M. A. (2006). RhoA signaling modulates cyclin D1 expression in human lung fibroblasts; implications for idiopathic pulmonary fibrosis. *Respiratory Research*, *7*, 88. http://doi.org/10.1186/1465-9921-7-88

Wei, Y., Renard, C.-A., Labalette, C., Wu, Y., Lévy, L., Neuveut, C., … Buendia, M.-A. (2003). Identification of the LIM protein FHL2 as a coactivator of beta-catenin. *The Journal of Biological Chemistry*, *278*(7), 5188–5194. http://doi.org/10.1074/jbc.M207216200

White, E. S., Lazar, M. H., & Thannickal, V. J. (2003). Pathogenetic mechanisms in usual interstitial pneumonia/idiopathic pulmonary fibrosis. *The Journal of Pathology*, *201*(3), 343–354. http://doi.org/10.1002/path.1446

Wight, T. N., & Potter-Perigo, S. (2011). The extracellular matrix: an active or passive player in fibrosis? *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *301*(6), G950–955. http://doi.org/10.1152/ajpgi.00132.2011

Willis, B. C., & Borok, Z. (2007). TGF-beta-induced EMT: mechanisms and implications for fibrotic lung disease. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *293*(3), L525–534. http://doi.org/10.1152/ajplung.00163.2007

Willis, B. C., Liebler, J. M., Luby-Phelps, K., Nicholson, A. G., Crandall, E. D., du Bois, R. M., & Borok, Z. (2005). Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor-beta1: potential role in idiopathic pulmonary fibrosis. *The American Journal of Pathology*, *166*(5), 1321–1332.

Wixler, V., Geerts, D., Laplantine, E., Westhoff, D., Smyth, N., Aumailley, M., … Paulsson, M. (2000). The LIM-only protein DRAL/FHL2 binds to the cytoplasmic domain of several alpha and beta integrin chains and is recruited to adhesion complexes. *The*

Journal of Biological Chemistry, *275*(43), 33669–33678. http://doi.org/10.1074/jbc.M002519200

Wixler, V., Hirner, S., Müller, J. M., Gullotti, L., Will, C., Kirfel, J., … Buettner, R. (2007). Deficiency in the LIM-only protein Fhl2 impairs skin wound healing. *The Journal of Cell Biology*, *177*(1), 163–172. http://doi.org/10.1083/jcb.200606043

Wong, G. T., Gavin, B. J., & McMahon, A. P. (1994). Differential transformation of mammary epithelial cells by Wnt genes. *Molecular and Cellular Biology*, *14*(9), 6278–6286.

Wuyts, W. A., Cavazza, A., Rossi, G., Bonella, F., Sverzellati, N., & Spagnolo, P. (2014). Differential diagnosis of usual interstitial pneumonia: when is it truly idiopathic? *European Respiratory Review: An Official Journal of the European Respiratory Society*, *23*(133), 308–319. http://doi.org/10.1183/09059180.00004914

Xia, T., Lévy, L., Levillayer, F., Jia, B., Li, G., Neuveut, C., … Wei, Y. (2013). The four and a half LIM-only protein 2 (FHL2) activates transforming growth factor β (TGF-β) signaling by regulating ubiquitination of the E3 ligase Arkadia. *The Journal of Biological Chemistry*, *288*(3), 1785–1794. http://doi.org/10.1074/jbc.M112.439760

Yang, I. V., Burch, L. H., Steele, M. P., Savov, J. D., Hollingsworth, J. W., McElvania-Tekippe, E., … Schwartz, D. A. (2007). Gene expression profiling of familial and sporadic interstitial pneumonia. *American Journal of Respiratory and Critical Care Medicine*, *175*(1), 45–54. http://doi.org/10.1164/rccm.200601-062OC

Zhang, W., Jiang, B., Guo, Z., Sardet, C., Zou, B., Lam, C. S. C., … Wong, B. C. Y. (2010). Four-and-a-half LIM protein 2 promotes invasive potential and epithelial-mesenchymal transition in colon cancer. *Carcinogenesis*, *31*(7), 1220–1229. http://doi.org/10.1093/carcin/bgq094

Zhao, J., Shi, W., Wang, Y.-L., Chen, H., Bringas, P., Jr, Datto, M. B., … Warburton, D. (2002). Smad3 deficiency attenuates bleomycin-induced pulmonary fibrosis in mice. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *282*(3), L585–593. http://doi.org/10.1152/ajplung.00151.2001

Zheng, Q., & Zhao, Y. (2007). The diverse biofunctions of LIM domain proteins: determined by subcellular localization and protein-protein interaction. *Biology of the Cell / Under the Auspices of the European Cell Biology Organization*, *99*(9), 489–502. http://doi.org/10.1042/BC20060126

Zhou, B., Liu, Y., Kahn, M., Ann, D. K., Han, A., Wang, H., … Borok, Z. (2012). Interactions between β-catenin and transforming growth factor-β signaling pathways mediate epithelial-mesenchymal transition and are dependent on the transcriptional co-activator cAMP-response element-binding protein (CREB)-binding protein (CBP). *The Journal of Biological Chemistry*, *287*(10), 7026–7038. http://doi.org/10.1074/jbc.M111.276311

Zuo, F., Kaminski, N., Eugui, E., Allard, J., Yakhini, Z., Ben-Dor, A., … Heller, R. A. (2002). Gene expression analysis reveals matrilysin as a key regulator of pulmonary fibrosis in mice and humans. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(9), 6292–6297. http://doi.org/10.1073/pnas.092134099

10 Appendix

10.1 Primers

The primers were designed using the primer design program Primer 3 (Koressaar & Remm, 2007).

Gene	GenBank™ Ref. Sequence	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Amplicon size (bp)
Semiquantitative RT-PCR				
FHL1	NM_001449	TGGCACAAAGACTGCTTCAC	CCTTCATAGGCCACCACACT	362
FHL2	NM_001039492	GAAGCCTGTTTCCACTGCTC	CCAGCACACTTCTTGGCATA	482
FHL3	NM_004468	CTGTGCAAAATGCAACGAGT	AAACAGGCCACACAGTAGGG	621
ACT	NM_020482	GCCGACAACCTATAGGGACA	CTTGTTCTGGGTCAGGAAGC	410
Quantitative RT-PCR				
FHL1	NM_001449	CGCTGTGGAGGACCAGTATT	CCAGATTCACGGAGCATTTT	116
FHL2	NM_001039492	GAAGCCTGTTTCCACTGCTC	CTGCTGCCCTTGTACTCCAT	178
FHL3	NM_004468	CTGTGCAAAATGCAACGAGT	GGAAATGGCGGTCTTCATAG	167
Cloning				
FHL2	NM_001039492	GATATCATGACTGAGCGCTTTG	CTCGAGTCAGATGTCTTTCCC	840
ACT	NM_020482	GATATCATGACAACTGCTCACT	CTCGAGCTAGATGTCAGTGTC	855

10.2 Antibodies

A list of the antibodies used for Immunohistochemistry (IHC) and Western Blotting (WB).

Antigen	Catalogue No.	Species	Dilution	Company
Primary Antibodies				
FHL2	ab12328	rabbit	1:4000 (WB)	Abcam, UK
FHL2	hm2136	mouse	1: 10 (IHC)	Hycult, NL
Smad2	sc6200	goat	1:1000 (WB)	Santa Cruz, USA
phospho Smad2/3	sc11769	goat	1:1000 (WB)	Santa Cruz, USA
a-tubulin	sc5826	goat	1:5000 (WB)	Santa Cruz, USA
Secondary Antibodies				
Biotinylated-conjugated anti-goat IgG		goat	Ready to use (IHC)	Invitrogen, USA
Biotinylated-conjugated anti-mouse IgG		goat	Ready to use (IHC)	Invitrogen, USA
HRP-conjugated anti-mouse IgG		goat	1:3000 (WB)	Pierce, USA

10.3 Cell lines

A549 human epithelial lung carcinoma	DSMZ, Germany
NIH-3T3 murine fibroblast cell line	DSMZ, Germany
DH5α E. coli prokaryotic cell strain	Clontech, USA

10.4 Vector systems

pGEM®-T easy	Promega, USA
pcDNA3.1(+)	Invitrogen, USA
p(CAGA)12 luciferase reporter plasmid	generated as previously described

11 Publications

Nils Banthien, Izabella Chrobak, Kamila Kitowska, Melanie Königshoff, Werner Seeger, Oliver Eickelberg. „Four-and-a-half-LIM-domain proteins: Essential regulators in pulmonary fibrosis?“ 14th International Colloquium on Lung Fibrosis (ICLF), Schloss Reinhartshausen Kempinski Mainz (Germany), 7-10 September 2006, Poster

Nils Banthien, Izabella Chrobak, Kamila Kitowska, Melanie Königshoff, Werner Seeger, Oliver Eickelberg. „Four-and-a-half-LIM-domain proteins: Essential regulators in pulmonary fibrosis?“ American Thoracic Society (ATS) International Conference, San Francisco (USA), 18-23 May 2007, Poster Discussion Session

Nils Banthien, Izabella Chrobak, Kamila Kitowska, Melanie Königshoff, Werner Seeger, Oliver Eickelberg. „Four-and-a-half-LIM-domain (FHL)-2 acts as an enhancer of TGF-beta signaling in pulmonary fibrosis.“ European Respiratory Society (ERS) Annual Congress, Stockholm (Sweden), 15-19 September 2007, Electronic Poster Discussion

12 Erklärung zur Dissertation

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nichtveröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten sowie ethische, datenschutzrechtliche und tierschutzrechtliche Grundsätze befolgt. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, oder habe diese nachstehend spezifiziert. Die vorgelegte Arbeit wurde weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt und indirekt an der Entstehung der vorliegenden Arbeit beteiligt waren. Mit der Überprüfung meiner Arbeit durch eine Plagiatserkennungssoftware bzw. ein internetbasiertes Softwareprogramm erkläre ich mich einverstanden.

_____ _____

Ort, Datum Unterschrift