

*INVESTIGATING FIBINS IN ZEBRAFISH
DEVELOPMENT AND REGENERATION*

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DECLARATION OF ORIGINALITY

I, Anna Rýdlová, declare that all work presented in this thesis is my own, and that all work from other sources, including but not limited to data, ideas and figures, have been clearly specified and appropriately referenced.

Anna Rydlova

July 2023

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ABSTRACT

Zebrafish, *Danio rerio*, is a widely accepted model of tissue regeneration. Yet, thorough understanding of the cellular and molecular pathways that govern the tightly regulated responses to injury still remain to be fully understood. Characterization of these molecular events holds a great potential for translational application in number of human disorders, including chronic fibrotic diseases. Transcript levels of the vertebrate-specific gene Fibrin were found to be significantly increased in a zebrafish model of gill tissue regeneration compared to mouse models of pulmonary fibrosis, suggesting a potential role of Fibrins in scar-less tissue regeneration. In this thesis, I used zebrafish as a model organism to investigate Fibrins in development, and in adult tissue regeneration.

Using RNA-seq analysis, flow cytometry and RNA analysis techniques I characterised the immune cell composition in the gills and WKM as well as the immune responses to viral mimetic compound, R848, in the wild-type and double-mutant (*fibina/b^{-/-}*) zebrafish. Using several microscopy techniques, I have investigated the impact of lack of functional Fibrins on the development of craniofacial cartilage and bone. I investigated the regenerative capacities and differential molecular signatures of regenerating gill and caudal fin tissues between the wild-type and *fibina/b^{-/-}* adult fish. And finally, using *in vitro* assays, I have investigated the molecular pathways that regulate FIBIN expression in human primary pulmonary fibroblasts.

The RNA-seq analysis highlighted number of differentially expressed genes between the wild-type and *fibina/b^{-/-}* fish. These genes were enriched for immune function and BMP signalling pathway components, in particular BMP-signalling antagonists. Characterization of the haematopoietic cells as well as of the ability to mount an immune response to R848 did not show significant differences between the wild type and *fibina/b^{-/-}* fish. Gill cryoinjury in the *fibina/b^{-/-}* fish was accompanied by a delay and dysregulated initiation of the regeneration process, including delayed blastema markers' expression and different dynamic of early repair and remodelling genes. Moreover, inhibition of BMP signalling in the caudal fin lead to a significantly delayed caudal fin regrowth in the *fibina/b^{-/-}* fish. Interestingly, *fibina* and *fibinb* seem to be regulated by the BMP signalling in a tissue-specific manner. *In vitro* experiments of human pulmonary fibroblasts revealed important regulation of FIBIN expression via WNT and TGF- β signalling pathways.

Abstract

Overall, this thesis highlights the importance of Fibins for timely and optimal scarless tissue regeneration. Further investigation of Fibins in zebrafish and mammalian models is needed to fully dissect Fibins' role(s) for potential therapeutic targeting in fibrotic disorders.

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LIST OF ABBREVIATIONS AND ACRONYMS

a-SMA	alpha smooth muscle actin
AEC	alveolar epithelial cells
AEC	Apical epidermal cap
AI	artificial intelligence
Akt	Protein kinase B
AMPK	5' adenosine monophosphate-activated protein kinase
ARS	Alizarin Red S
ATI	alveolar epithelial Type I cells
ATII	alveolar epithelial Type II cells
ATP	adenosine tri-phosphate
BMP	bone morphogenic protein
BrdU	Bromodeoxyuridine
CH	ceratohyal cartilage
CHT	caudal haematopoietic tissue
CNCC	cranial neural crest cells
CO ₂	carbon dioxide
COPD	Chronic Obstructive Pulmonary Disease
CPM	counts per million
CRISPR	Clustered regularly interspaced palindromic repeats
crRNA	CRISPR RNA
CT	computer tomography
DAPI	4',6-diamidino-2-phenylindole
DE	differentially expressed (genes)
DEAB	4-diethylaminobenzaldehyde
Dkk	dickkopf
DM	Dorsomorphin

DMH1	dorsomorphin homolog 1
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dpa	days post amputation
dpc	days post cryoinjury
dpf	days post fertilisation
dpt	days post treatment
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition
ER	endoplasmic reticulum
FC	fold change
FCS	foetal calf serum
FDA	Food and Drug Administration
FDR	false discovery rate
FGF	fibroblast growth factor
Fgfr1	Fibroblast growth factor receptor 1
Fibin	Fin bud initiation factor
FMT	fibroblast-to-myofibroblast transition
FVC	forced vital capacity
FZD	Frizzled receptor
GO	Gene Ontology
hpa	hours post amputation
hpc	hours post cryoinjury
hpf	hours post fertilisation
hpi	hours post injury
hpt	hours post treatment
HSC	haematopoietic stem cell

IFN	interferon
IgA	Immunoglobulin A
IGF	insulin-like growth factor
IGFR1	insulin-like growth factor receptor
IgT	Immunoglobulin T
IPF	Idiopathic Pulmonary Fibrosis
IPF LF	Idiopathic Pulmonary Fibrosis lung fibroblasts
KOH	potassium hydroxide
kPa	kilo Pascals
MF	myofibroblast
MI	myocardial infarction
microRNA	micro RNA
MO	morpholino
mRNA	mesenger RNA
NEC	neuroepithelial cell
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHLF	normal human lung fibroblasts
NMD	non-sense mediated RNA decay
NT	Nintedanib
O ₂	oxygen
OA	osteoarthritis
PBS	phosphate buffered saline
PCA	Principal component analysis
PCNA	Proliferating cell nuclear antigen
PF	pulmonary fibrosis
PFA	paraformaldehyde
PFD	Pirfenidone

PI3 kinase	phosphoinositide 3-kinase
PQ	palatoquadral cartilage
PTEN	Phosphatase and tensin homologue
qPCR	quantitative polymerase chain reaction
qRT-PCR	Real-Time Quantitative Reverse Transcription PCR
RA	retinoic acid
RIN	RNA integrity threshold
RISC	RNA-induced silencing complex (RISC)
RNA	ribonucleic acid
RNA-seq	RNA sequencing
ROS	reactive oxygen species
SAECs	small airway epithelial cells
scRNA-seq	single cell RNA sequencing
siRNA	small interfering RNA
ssRNA	single strand RNA
TFBS	transcription factor binding site
TGF- β	transforming growth factor β
TLR	Toll-like receptor
TM	Template modelling
TNF- α	tumour necrosis factor alpha
TPM	transcripts per million
TSS	Translation start site
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
WKM	Whole kidney marrow
WNT	Wingless/integrated

1 INTRODUCTION

1.1 Defining repair and regeneration

As defined by Gonzales-Rosa and colleagues in 2017, regeneration refers to ‘the structural and functional recovery of injured tissues and organs or lost body parts’ (González-Rosa, Burns and Burns, 2017). The evolution of regeneration in animals was not and is not a unidirectional process. And although there are known examples of evolutionary enhancements of regeneration capabilities of an organism, there is often a trade-off between regenerative capacity and the overall complexity of an animal (Liozner, 1995; Carlson, 2005; Maruzzo and Bortolin, 2013; Elchaninov, Sukhikh and Fatkhudinov, 2021). The regenerative capacity across the animal kingdom varies considerably from ability to regenerate entire body structure from any body part seen in planarians to very limited regeneration described in mammals (Elchaninov, Sukhikh and Fatkhudinov, 2021). In the vertebrate kingdom alone, there is a stark contrast between the scarless tissue regeneration seen in number of lower vertebrates such as zebrafish or axolotls, and mammalian vertebrate injury models that commonly result in persistent, chronic, fibrotic tissue deposition.

It is important to note that mammals do exhibit physiological regeneration including continuous replacement and turnover of cells (e.g. blood cells), surface epithelia (e.g. gut lining), general ability of epithelial wounds or bone to heal as well as the well described

remarkable regenerative capacity of the mammalian liver (Zhao, Qin and Fu, 2016). Moreover, what we generally assume has been lost in higher vertebrates, that is the regeneration of complex tissues and organs, showed not to be the case at embryonic and early foetal stages. Even in mammals, embryonic wounds, such as lost digit tips, can regenerate in a scarless manner through a blastema intermediate – the same mechanisms via which lizards regenerate their tails or axolotls their limbs. However, this ability does appear to be lost in adult individuals (Zhao, Qin and Fu, 2016).

Fibrotic (scar) tissue formation is an important physiological aspect of wound healing, particularly in mammals and higher vertebrate species. The deposition of collagens, fibronectins and other extracellular matrix (ECM) components are vital for limiting blood loss and maintaining tissue or organ structural integrity and thus preserving its, although limited, functional output. Scar tissue has significantly different mechanical properties to any and all the tissue types that it is substituting for and thus the scarred part of the organ can usually no longer perform its original function. Unless resolved, scar tissue continues to cause functional impairment. Progressive scarring of organs such as the lung, liver or kidneys can therefore have fatal consequences (Harn *et al.*, 2019; Elchaninov, Sukhikh and Fatkhudinov, 2021). Fibrotic disorders are a significant burden to individuals and healthcare systems, and the lack of thorough understanding of the processes involved, and how to reverse them, still presents an urgent unmet need.

What differentiates between successful, scarless regeneration versus chronic fibrosis outcome still remains to be fully elucidated. Several avenues of research have investigated the potential involvement of inflammation or specific regulation of regeneration-linked genes as the keys to unlock this enigma, however no general consensus on regeneration-permissive and -restricting mechanisms has been defined. Studying both the regenerative and the non-regenerative vertebrates to identify similarities and differences that shape/restrict the regenerative capacities holds the potential to design evidence-based, targeted therapies and interventions to significantly and fundamentally improve not only the quality of life but the total life expectancy of millions of people suffering from (progressive) fibrotic disorders.

In this Introduction I focus on the zebrafish as a model of scarless regeneration and laying the foundations for investigating Fibin gene(s) as potential players in the process.

1.1.1 Types of regeneration

Tissue regeneration occurs at multiple levels. The smallest injuries may possibly require only the repair of a single cell (e.g. alveolar epithelial cell death as a result of inhaled irritants) while large injuries and severe traumas may require the replacement of a whole organ or tissue, such as following limb amputation. All regeneration programs involve tightly regulated activation, highly coordinated growth and cellular (re-, de-, trans-) differentiation and reprogramming. How are resident cells activated to regenerate lost tissues, particularly in the case of whole organ/appendage loss, and what controls and mechanisms enable the process, remains a field of active research (Seifert and Muneoka, 2018).

Two particular types of regeneration are often mentioned as distinct mechanisms animals use to achieve the outcome – epimorphic and compensatory. It has been shown, however, that not only can both take place in the same organism, but to an extent, they can often happen simultaneously.

Epimorphic regeneration refers to regeneration through a blastema intermediate. Blastema refers to a group of undifferentiated, stem-like cells (current evidence, that is discussed later in this thesis, points to a lineage restricted fate of these cells) that with optimal neuronal and signalling input can regrow an entire complex structure such as the caudal fin in zebrafish or digit tip in foetal mammals (Rinkevich et al., 2015, Maden, 2018). The general overview of blastema-based regeneration includes rapid wound closure and re-epithelisation to prevent blood loss, establishment of wound epithelium that interacts with the underlying mesenchymal tissue ultimately leading to de-/trans-differentiation of cells surrounding the stump, formation of proliferating blastema and successive outgrowth of the appendage tissues, with the size and shape reflecting the original structure (Daponte, Tylzanowski and Forlino, 2021).

Compensatory regeneration on the other hand refers to processes that result in the uninjured part of the tissue/organ to undergo hypertrophy, that is, the remaining cells expand and compensate for the lost tissue. Well known examples of compensatory regeneration are found in human liver, partially in zebrafish heart (Hata, Namae and Nishina, 2007; Sallin *et al.*, 2015), and even in mammalian lung where after removal of one or more lobes the remaining tissue increases in the number of alveolar units (the basic gas-exchange unit) to compensate (Matsumoto *et al.*, 2009).

The basis for my PhD project was shaped by experiments performed in the Dallman laboratory, investigating the relevance and usefulness of zebrafish as a model of respiratory tissue inflammation and repair/regeneration, and in collaboration with an industrial partner, Boehringer Ingelheim Pharma (BI), and their research into restoration of respiratory function in patients suffering from Idiopathic Pulmonary Fibrosis. In the following paragraphs I therefore focus primarily on vertebrate respiratory tissue and its repair and regeneration.

1.2 The structure and function of mammalian lungs

The mammalian lung complex structure evolved to support the organ function, including efficient gas exchange. Gas exchange involves a passive transfer of oxygen (O₂) from environment into the organism and carbon dioxide (CO₂) from venous blood out into the environment. In simple terms, the human respiratory system is divided into airways and lung parenchyma. The lung parenchyma includes the bronchioles, alveolar ducts and the highly vascularized alveoli, and is the place of gas exchange (Fig. 1.1) (Whitsett *et al.*, 2019).

Lungs are densely innervated by sensory and sympathetic and parasympathetic nerves to maintain lung homeostasis as well as enabling the organ to fulfil other functions. Particularly chemical or mechanical activation of sensory nerves results in defensive reflexes such as altered autonomic drive, cough or altered breathing pattern to normalize alveolar pH (such as in case of excess CO₂) (Canning and Spina, 2009; Powers and Dhamoon, 2023). Apart from

gas exchange, lungs also have important immunological function such as acting as a physical barrier to prevent pathogen invasion.

Structurally, airways are supported by cartilage, smooth muscle and complex extracellular matrix (ECM), whose composition and mechanical properties can vary significantly with location. Specific requirements for ECM properties are defined by function: For example, the ECM around alveoli consists of a network of elastin fibres and collagens and co-enable the inflation/deflation of alveoli that respond to pressure gradient. In the vicinity of alveoli, the ECM thus needs to be highly flexible and adaptable to ensure that gas exchange by diffusion is not restricted due to high volumes of ECM components between the alveoli and adjacent microvasculature (Whitsett *et al.*, 2019).

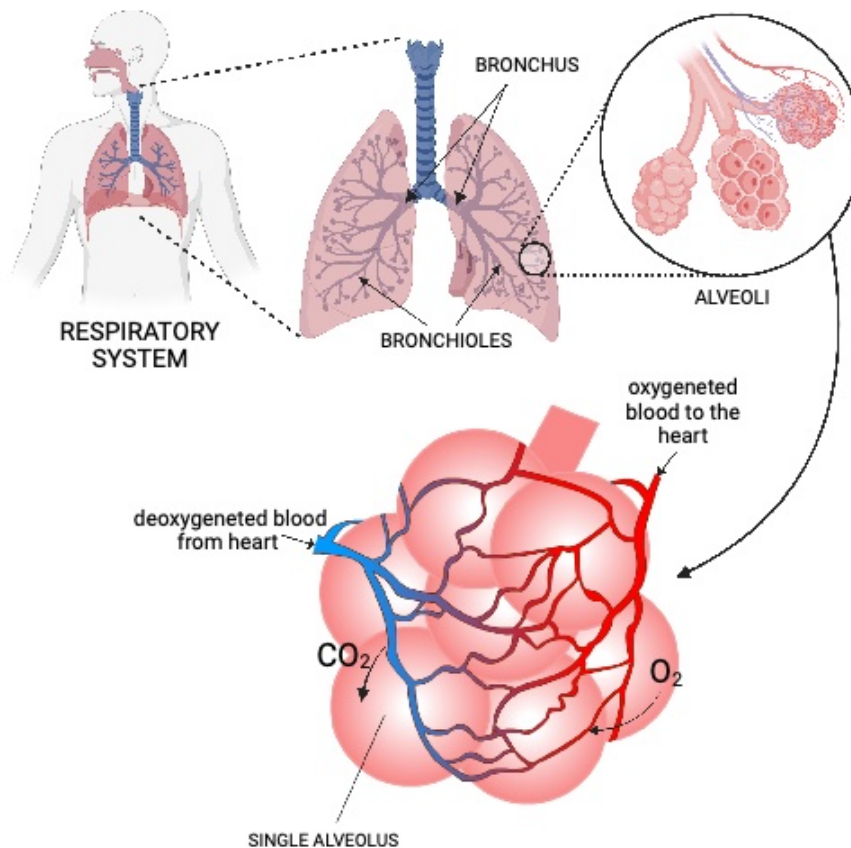


Figure 1.1 Anatomical structure of the human lungs and schematic representation of alveoli and oxygen-carbon dioxide exchange.

The human respiratory system consists of upper and lower airways. This schematic representation focusses on the structure of lower airways, including the trachea, bronchus, bronchioles and alveolar. Single alveolus schematic shows the high density of blood vessels that surround each alveolus, deoxygenated blood from the heart (blue) and oxygenated blood returning to the heart (red), and the direction of O_2 – from the alveolus to the blood vessel – and of CO_2 – from the blood vessel to the alveolus. Created with Biorender.

1.2.1 Repair and regeneration of gas exchange unit of mammalian lungs

Although previously believed to have only limited capacity for regeneration, in recent years the respiratory system has been showed to have extensive ability to respond to an insult and regenerate lost/damaged cells (Kotton and Morrissey, 2014). Lung alveoli consist primarily of Type I and Type II cells. Type I cells form around 95% of the surface area of the alveolus and are the enablers of gas exchange. These cells also have indispensable roles in fluid homeostasis, as a physical barrier to pathogens or in immune responses (Sisson *et al.*, 2010; Bagnato and Harari, 2015; Kasper and Barth, 2017). Type II cells are secretory cells that generate surfactant that reduces the tension at the air-water interface, preventing the alveoli from collapsing on themselves. Type II cells can differentiate into Type I cell during homeostatic maintenance and after injury (Sisson *et al.*, 2010). The repair/regeneration processes of the alveolar epithelial cell wall are thought to be accompanied by activation of the underlying, scarce, fibroblast cell population (Fig. 1.2). These activated fibroblasts, often referred to as myofibroblasts then secrete extracellular matrix proteins that provide structural support for newly differentiated Type I cells (from Type II cells) to replace the dead cells. The myofibroblast cell population then undergoes apoptosis and the excess extracellular matrix is digested by matrix metalloproteases (Löffek, Schilling and Franzke, 2011). Clearance of the excess extracellular matrix and the programmed death of myofibroblasts is essential for restoration of the close proximity between the pulmonary capillaries and the alveoli, leading to resumed gas exchange.

However, there are cases where the physiological repair escapes the tight control and instead of repair and regeneration, the organ stays in a state of continuous repair, involving active myofibroblasts continuously secreting extracellular matrix despite the lack of (objective) trigger. This results in excessive accumulation of scar tissue and eventual loss of function as gas exchange is halted (Strieter, 2008).

1.2.2 Pulmonary fibrosis

Scarring of the lung or pulmonary fibrosis is a serious health condition characterized by hyperplasia of alveolar epithelial cells, accumulation of inflammatory cells and most

importantly deposition of excess fibrotic tissue leading to chronic and progressive scar formation. Pulmonary fibrosis can occur as a complication of other pulmonary disorders such as Chronic Obstructive Pulmonary Diseases (COPD) (Barnes, 2019; Zantah *et al.*, 2020) or asthma (Li, Chen and Pang, 2020), or it can appear in absence of these diseases, such as the case of Idiopathic Pulmonary Fibrosis (IPF).

IPF is a progressive respiratory disease affecting over 30 000 people in the UK alone. The estimated life expectancy at the time of diagnosis is 3-5 years and apart from lung transplantation, no curative treatment is available for these patients (Sauleda *et al.*, 2018). Although the exact aetiology of IPF remains unclear, a number of risk factors has been linked to IPF development, including genetic predispositions such as associated single nucleotide polymorphisms (i.e. *MUC5B* (Xie *et al.*, 2016)), environmental factors (lifestyle-associated risk factors such as smoking (Baumgartner *et al.*, 1997) and air pollution (Johannson *et al.*, 2014)) that make the lung epithelium more susceptible to pathological damage, as well as advanced age, since IPF rarely develops in patients below 50 years of age (Raghu *et al.*, 2006).

Research into the disease origins identified a series of events with the potential to initiate and maintain IPF pathophysiology (Fig. 1.2) (Varone *et al.*, 2018):

- ☐ Repeated injury to alveolar epithelial cells (AECs),
- ☐ uncontrolled myofibroblast activation and proliferation that results in excessive extracellular matrix tissue deposition,
- ☐ irreversible changes to the tissue architecture,
- ☐ and ultimately functional decline

The molecular processes involved are themselves not different to the homeostatic ECM deposition and wound healing processes. It appears to be the imbalance in the chemical and physical stimuli as well as nature of the cells involved that leads to the excessive production of ECM components and the establishment and maintenance of the pro-fibrotic environment (Kropski and Blackwell, 2019).

1.2.2.1 Role of epithelial-mesenchymal crosstalk in IPF

Currently it is hypothesized that IPF is a disease of aberrantly activated alveolar epithelium due to repeated and sustained injury to the AECs, particularly to Type I (ATI) cells). Sustained damage to AECs leads to the over-activation of interstitial fibroblasts and their differentiation into myofibroblasts (MFs). Myofibroblasts (MFs) are cells that can originate from number of different cell types but most commonly they derive from fibroblasts through fibroblast-to-myofibroblast transition (FMT). MFs are ultimately characterized by high levels of alpha-smooth muscle actin (α -SMA) expression (giving MFs a contractile phenotype), their ability to produce and secrete high levels of ECM components, and the presence of actin stress fibres within the cell (Fuxe *et al.*, 2020). In chronic fibrosing disorders, these myofibroblasts do not undergo apoptosis once their activating trigger is gone, instead they maintain their activation status which results in excessive secretion of unstructured ECM. These events lead to formation of a non-reversible scar in the interstitial space, obstructing gas diffusion (Fig. 1.2). Moreover, the infiltration of immune cells (such as neutrophils and interstitial macrophages) and activated fibroblasts into the alveolar space during sustained AECs damage hinder the repair of the basement membrane and promote scar development (Bagnato and Harari, 2015, Jarman and Boulter, 2020).

Under homeostatic conditions, interstitial fibroblasts and myofibroblasts are primarily responsible for ECM maintenance as they are a major source of matrix metalloproteinases (such as MMP9) and their inhibitors (TIMPs) which are capable of ECM remodelling/structuring (Greenlee, Werb and Kheradmand, 2007). In IPF, these fibroblasts and myofibroblasts acquire number of differential characteristics:

- 1) Apoptosis-‘resistant’ phenotype (Thannickal and Horowitz, 2006),
- 2) senescence-resembling phenotype compared to age-matched healthy controls, manifested by mitochondrial dysfunction or shorter telomere length (Álvarez *et al.*, 2017),
- 3) increased rate of proliferation compared to matched healthy controls (Lehtonen *et al.*, 2016) and
- 4) invasive phenotype (Karvonen *et al.*, 2013).

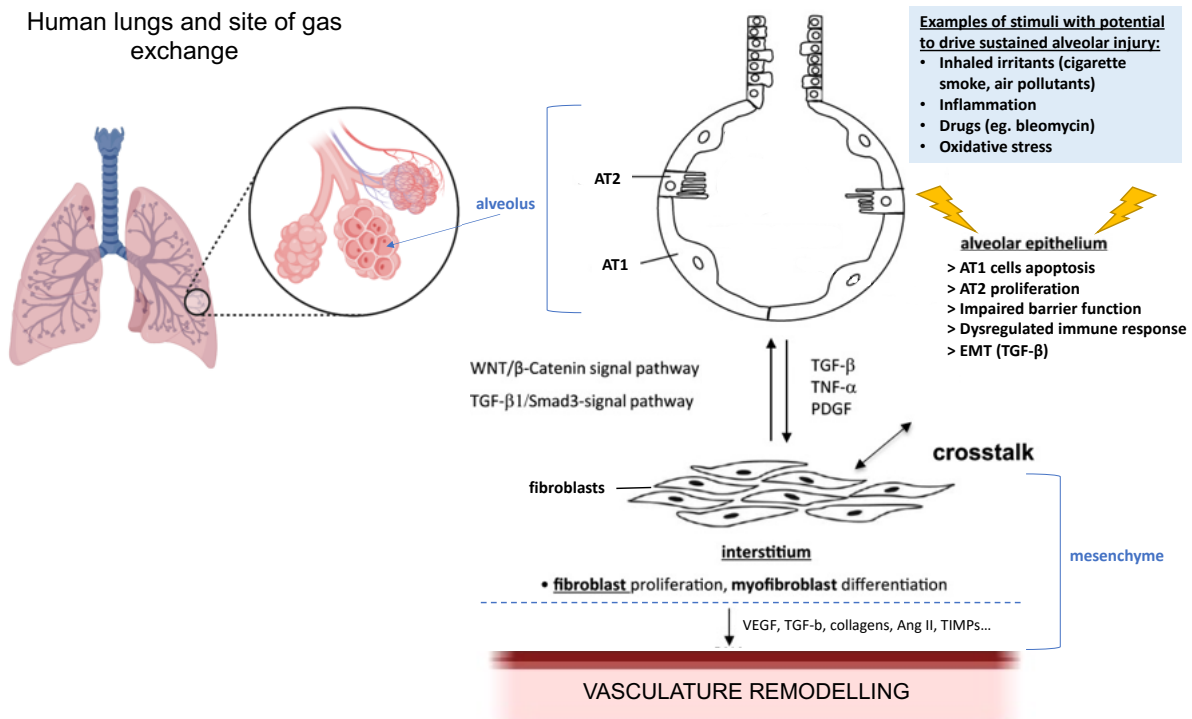


Figure 1.2 Schematic representation of the alveolar unit in Idiopathic Pulmonary Fibrosis (IPF).

Mesenchymal cells (here fibroblasts) in the interstitial space between the alveolus and the blood vessel (red boundary) are activated upon injury to the alveolar epithelium, proliferate, differentiate into myofibroblasts and secrete ECM components as well as cytokines and chemokines affecting both the alveolar epithelium and the vascular wall (vasculature remodelling). AT2: alveolar epithelial cell II/ Type II; AT1: alveolar epithelial cells I, Type I; EMT: epithelial-to-mesenchymal transition; Ang II: Angiotensin II; TIMPs: Tissue inhibitors of metalloproteinases. Adapted from (Kasper and Barth, 2017), created with Biorender.

All these characteristics support the uncontrolled, sustained activation of fibroblasts/myofibroblasts, promoting their involvement in the development and propagation of pathological fibrosis.

ECM is, under normal conditions, a vital component of the lung mesenchyme, profoundly influencing cell behaviour and phenotype. ECM composition and properties also have an important role in determining and maintaining airway architecture (Herrera, 2018). As all pulmonary fibrotic disorders show excessive ECM deposition both within the alveolar space and in the interstitium, the properties of the ECM in PF have also been investigated. As expected, the functionality and properties of ECM differ between samples from IPF and

healthy donors (Parker *et al.*, 2014; Herrera, Henke and Bitterman, 2018a). In particular, increased levels of collagens III, IV, V and VI, fibronectin, hyaluronan and versican, were found in IPF fibrotic loci (one of the hallmarks of IPF), compared to control samples from healthy lungs (Herrera *et al.*, 2019). Moreover, it was shown that the increasing stiffness of the ECM as a result of excessive deposition of ECM is capable of activating the interstitial fibroblasts as well, ultimately completing the vicious cycle of continuous fibrotic tissue deposition (Marinković, Liu and Tschumperlin, 2013; Parker *et al.*, 2014).

It has been recognized for many years, that the communication between the injured epithelium and the underlying mesenchymal tissue (including fibroblasts and myofibroblasts) is a key information-exchange loop that can drive the pro-fibrotic events. Thorough understanding of the molecular crosstalk that enables this action is yet to be achieved, but a number of potential mechanisms are already known to play a role, including TGF- β signalling, WNT signalling, or BMP signalling.

1.2.2.2 Signalling pathways involved in IPF

WNT signalling

WNT signalling is a fundamental developmental pathway that plays a vital role in lung development (Li *et al.*, 2002; Boucherat *et al.*, 2007; Loscertales *et al.*, 2008; Rajagopal *et al.*, 2008; Cornett *et al.*, 2013). The activity of the WNT signalling in humans decreases over time after fertilization and development, however, interestingly, many diseases including pulmonary fibrosis exhibit sustained re-activation of this pathway alongside TGF- β -, or PTEN/PI3kinase/Akt-mediated signalling (Selman, Pardo and Kaminski, 2008). There are two historically recognized WNT signalling strands, canonical signalling through β -catenin and non-canonical signalling, independent of β -catenin; the basic mechanisms of both strands are summarized in Fig. 1.3.

WNT signalling

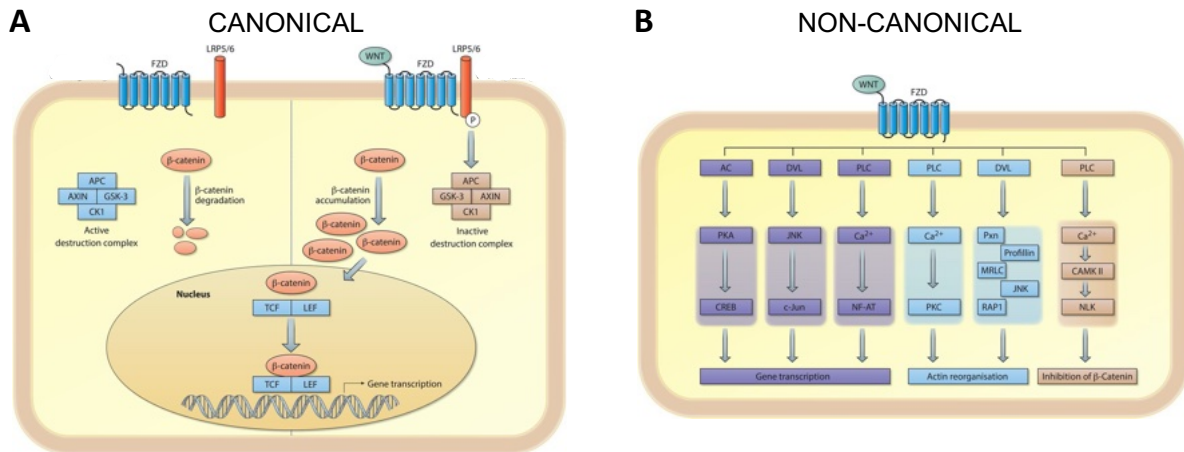


Figure 1.3 Schematic representation of canonical and non-canonical WNT signalling

(A) Canonical WNT signalling depends on WNT ligand binding to a frizzled receptor (FZD) which dimerises with low density lipoprotein receptor-related protein 5 and 6 (LRP5/6). This receptor activation results in intracellular inhibition of the b-catenin destruction complex that consists of APC, GSK-3, AXIN and CK1. The inactivation of the destruction complex enables b-catenin accumulation in the cytoplasm and translocation to the nucleus where b-catenin associates with a number of transcriptional co-activators, such as T cell factor (TCF) and lymphoid enhancer factor (LEF) and leads to induction of target gene transcription (e.g. *AXIN2*). (B) Non-canonical WNT signalling acts independently of b-catenin. Upon ligand binding to FZD receptor, without the need for LRP5/6 co-receptor, number of intracellular signalling pathways can be activated. Interestingly while some lead to induction of similar genes as in canonical WNT signalling, other (such as signalling through PLC > Ca^{2+} > CAMK II and NLK) have the opposing effect for example through inhibition of b-catenin transcription. Adapted from (Baarsma and Königshoff, 2017a). AC: adenylylcyclase; PKA: protein kinase A; CREB: cAMP responsive element binding protein; DVL: dishevelled; FZD: Frizzled receptor; JNK: c-Jun-N terminal kinase; PLC: phospholipase C; NF-AT: nuclear factor of activated T cells; PKC: protein kinase C; Pxn: paxillin; MRLC: myosin regulatory light chain; RAP1: RAS-related protein 1; CAMKII: calcium/ calmodulin-dependent kinase II; NLK: Nemo-like-kinase.

Although WNT signalling involvement in IPF was originally discovered due to upregulated WNT-related genes in gene expression profiling, the increased levels of WNT signalling ligands, effectors and target genes (including *WNT7B*, *WNT5A*, *WNT10B*, *CTNNB1* (β -catenin), *LEF1*, *WISP1* or *AXIN2*) have been confirmed by number of *in vitro* and *in vivo* studies (Königshoff et al., 2008a; Liu et al., 2009; Vuga et al., 2009; Berschneider et al., 2014; Klee et al., 2016; Baarsma and Königshoff, 2017a). Several WNT ligands, such as *WNT5A*, *WNT5B* or *WNT3A* have been investigated specifically in *in vitro* fibroblast cultures and shown to regulate lung fibroblasts proliferation, increase EMC component secretion, and increase the mRNA expression of TGF- β and connective tissue growth factor (*CTGF*), respectively (Chen et al., 2007; Spanjer et al., 2016).

TGF- β signalling

The TGF- β family of cytokines includes three mammalian isoforms, TGF- β 1, TGF- β 2 and TGF- β 3. TGF- β 1 is ubiquitously expressed by cells within the body and is the most widely studied TGF- β cytokine with respect to epithelial-mesenchymal transition (EMT) (Tatler and Jenkins, 2012), FMT, and sustained proliferation of myofibroblasts in IPF (Eickelberg and Fernandez, 2012). EMT refers to a process via which epithelial cells begin to express mesenchymal-like genes (including *ACTA2*). The epithelial cells ultimately acquire a contractile, myofibroblast-like phenotype enabling enhanced cellular migration (Hanahan and Weinberg, 2011). Increased levels of TGF- β 1 were confirmed in IPF samples compared to healthy controls (Coker *et al.*, 2001) and overactive TGF- β signalling forms the basis of animal models where experimental PF is re-created by sustained TGF- β signalling. In contrast, blocking TGF- β signalling leads to decrease in fibrotic tissue deposition in mouse bleomycin model of IPF (Bonniaud *et al.*, 2005). Fig. 1.4 shows the basics of canonical and non-canonical TGF- β signalling.

The only two FDA-approved drugs for IPF to date are Pirfenidone and Nintedanib. To some extent both exert their effects via links to TGF- β signalling. The mechanism of action of Pirfenidone (PFD), the first drug to be approved almost a decade ago, is still unclear, however, intensive research has identified several potential pathways with which PFD interacts. Conte and colleagues (Conte *et al.*, 2014a) showed that PFD significantly decreases *ACTA2* and *COL1A1* transcript levels, as well as phosphorylated p38, Akt and Smad3 proteins in TGF- β -induced primary lung fibroblasts. *In vivo* experiments in murine models also showed suppression of TNF- α , IL-6 and PDGF secretion which led to attenuation of fibrosis in the bleomycin model of IPF (Gurujeyalakshmi, Hollinger and Giri, 1999; Oku *et al.*, 2008; George and Wells, 2017).

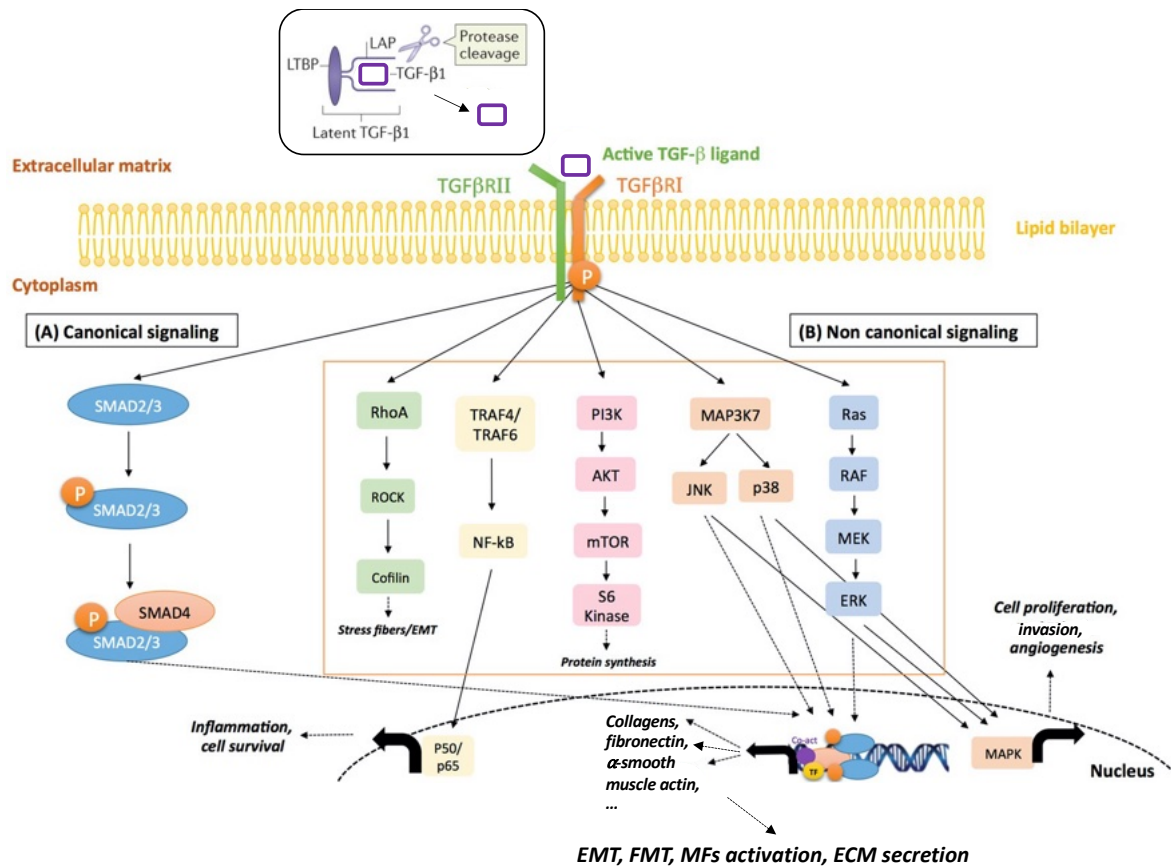


Figure 1.4 Canonical and non-canonical TGF-β signalling

TGF-β is synthesized as a pro-peptide and once secreted TGF-β is attached to the latent TGF-β binding protein (LTBP), found in the ECM, which keeps the TGF-β in an inactive form. To be able to bind its receptors, TGF-β must be cleaved from the LTBP complex by matrix metalloproteinases (MMP; eg. MMP2 or MMP9) or a number of different proteases. Once released, TGF-β ligand binds to the TGF-β receptor 2 (TGFβRII), activating TGF-β receptor 1 (TGFβRI) and inducing downstream signalling. (A) In canonical signalling, TGF-β binding to its receptors leads to phosphorylation of SMAD2/3 proteins and formation of complex with SMAD4. This complex then translocates to nucleus and leads to regulation of target gene expression. (B) The non-canonical signalling pathway refers to signalling that is mediated through SMAD-protein-independent mediators, such as Rho family of small GTPases, TNF receptor-associated factor 4/6 (TRAF4/6), phosphatidylinositide 3-kinase (PI3K), the mitogen-activated protein kinases (MAPKs), and the Ras-ERK signalling. Some of these mechanisms can act also through non-transcriptional mechanisms to promote EMT/FMT, i.e. formation of actin stress fibres via RhoA. Adapted from (Meng, Nikolic-Paterson and Lan, 2016; Costanza *et al.*, 2017).

Nintedanib (NT), marketed by Boehringer Ingelheim Pharma (BI) and approved by FDA in 2014, is a tyrosine kinase inhibitor targeting VEGFR1,2,3, FGFR1,2,3 and PDGFR α and β. NT was shown to inhibit PDGF receptor activation, fibroblast proliferation, TGF-β induced FMT and led to a decrease in total collagen and IL-1β levels *in vivo* and *in vitro* (Wollin *et al.*, 2014, 2015). Even though some efficacy in reducing fibrotic tissue load in animal models has been shown, both drugs can only slow down disease progression and neither has been

shown to improve survival rates, with all clinical trials till today having ‘change in percentage predicted forced vital capacity (FVC)’ as their primary end point (Noble *et al.*, 2011; George and Wells, 2017). Nonetheless, modulating TGF- β signalling clearly offers an opportunity to modify the processes causing pathological fibrotic tissue development.

As the versatility and similarity of the responses to induction of both the TGF- β and the WNT signalling pathways suggests, they exert their effects often using the same receptors and/or effectors. For instance, it has been shown that canonical WNT signalling is necessary for TGF- β mediated fibrosis and that TGF- β can induce canonical WNT signalling by suppressing DDK1, a WNT signalling antagonist (Akhmetshina *et al.*, 2012). TGF- β also induces the upregulation of FZD7 and FZD8 receptors in human fibroblasts, where both have been shown to interact with WNT5A, a non-canonical WNT-ligand, inducing upregulation of ECM components including *COL1A1*, *FNI* and *VCAN* (versican), as well as induce the expression of primary TGF- β target gene, *ACTA2*. Knockdown of both receptors in animal models attenuated the TGF- β 1-induced and bleomycin-induced lung fibrosis respectively (Spanjer *et al.*, 2016; Guan and Zhou, 2017). Thus, better understanding of the molecular crosstalk between TGF- β signalling and WNT signalling in the response to injury can help us design more efficient and targeted therapy for progressive lung fibrosis.

BMP signalling

Bone morphogenic protein (BMP) signalling belongs to the TGF- β superfamily of signalling molecules. BMP ligands also stimulate transmembrane receptors on target cells, however, the intracellular cascade, for canonical BMP signalling, differs from that of TGF- β . TGF- β signalling includes phosphorylation of SMAD2/3 while BMP signalling leads to phosphorylation of SMAD1/5/8(9). The notation SMAD8 or SMAD9 is used interchangeably in the existing literature – and refers to the same gene/protein (Katakawa, Funaba and Murakami, 2016). Both TGF- β and BMP pathways then compete for formation of a complex with SMAD4, a required co-factor enabling translocation to nucleus and binding to defined elements of DNA to regulate target gene expression (Fig. 1.5) (Guo and Wang, 2009; Rahman *et al.*, 2015). Although BMP signalling with respect to cartilage development or bone development (discussed later in this thesis) is often addressed on its own, in pulmonary fibrosis it is mainly the crosstalk of TGF- β and BMP signalling that is of interest.

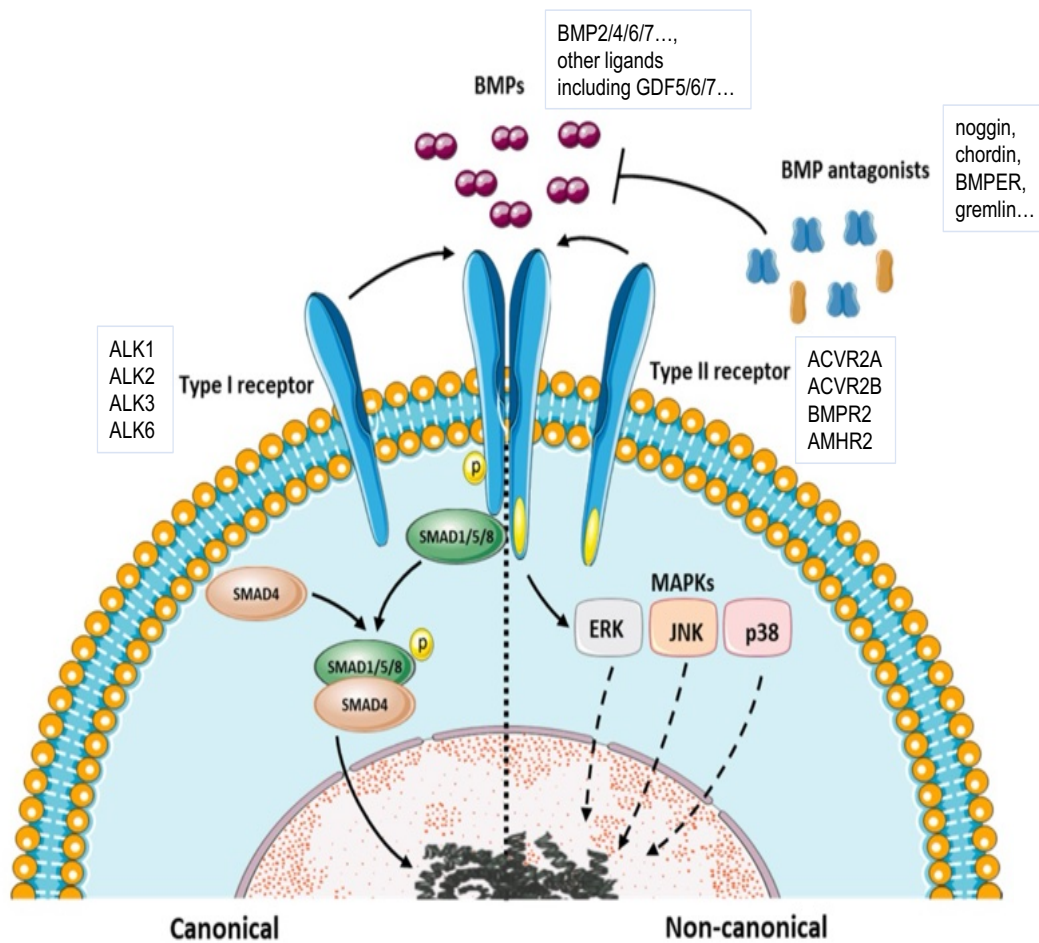


Figure 1.5 Canonical and non-canonical BMP signalling

BMP ligands are mostly secreted as mature disulfide-linked dimers. The BMP signalling cascade consists of a multimeric cell surface receptor complex which includes two Type I receptors and two Type II receptors. Upon ligand-binding Type I receptors are phosphorylated by Type II receptors (p). This leads to activation of Type I receptors, recruitment and phosphorylation of R-SMADs (SMAD1/5/8) and ultimately trimer formation with SMAD4 and translocation to the nucleus. SMADs have intrinsic DNA-binding activity and by recruiting chromatin-remodelling machinery and integrating tissue-specific transcription factors, they are able to regulate gene expression. The BMP signalling cascade can be antagonized by many mechanisms, including via extracellular antagonists noggin, gremlin, chordin or BMPER. Adapted from (Ouahoud, Hardwick and Hawinkels, 2020). ACVR, activin receptor; ALK, activin receptor-like kinase; AMHR2, anti-Müllerian hormone receptor 2; BMPR, BMP receptor; GDF, growth/differentiation factor.

In vitro studies using normal (healthy) primary human pulmonary fibroblasts have shown that BMP4 and BMP7 ligands reduced TGF- β -induced production of matrix metalloproteases involved in EMT and attenuated the secretion of ECM proteins such as collagen I, fibronectin or tenascin C (Pegorier *et al.*, 2010). Moreover, concomitant increase in TGF- β signalling with decrease in BMP signalling has been shown to be an important driver of pulmonary inflammation and subsequent fibrosis (Myllärniemi *et al.*, 2008). Using asbestos-induced

mouse model of pulmonary fibrosis, Myllärniemi and colleagues showed that increase in TGF- β signalling was accompanied with increased transcript levels and protein levels of BMP signalling antagonist gremlin (GREM1 and GREM2). The fibrosis phenotype, evaluated by measuring hydroxyproline content of the mouse lungs, was significantly reduced with BMP7 treatment and/or inhibition of TGF- β receptors (Myllärniemi *et al.*, 2008). *GREM1* can be induced by TGF- β -dependent increase in the profibrotic cytokine IL-6 (O'Reilly *et al.*, 2014). *GREM2* transcription can be directly induced by TGF- β treatment and acts to suppress BMP signalling and maintain TGF- β signalling (Huan *et al.*, 2021).

The overall anti-inflammatory effects of BMP signalling were also recently shown by Guan and colleagues. They demonstrated that BMP4 overexpression *in vivo* (mice models) and *in vitro* (primary lung-derived fibroblasts) attenuated TGF- β -induced EMT via promoting mitophagy and modulating cellular senescence, both of which are often defective in IPF fibroblasts (Dituri *et al.*, 2019; Guan *et al.*, 2022).

1.2.3 Modelling pulmonary fibrosis

Animal models have been instrumental in understanding various aspects of the IPF disease pathology, however, to date, these models cannot recapitulate all of the important aspects of the disease in particular the progressive and irreversible nature seen in humans. Mouse models of induced pulmonary fibrosis are to date the most commonly used. The fibrosis is induced by exposure to silica, bleomycin, asbestos, irradiation or even lung tissue specific transgene expression (Moore and Hogaboam, 2008; Tashiro *et al.*, 2017). Bleomycin-induced pulmonary fibrosis mouse model is the most widely used and characterized model (Moeller *et al.*, 2008). Bleomycin is an antibiotic produced by *Streptomyces verticillus*. Bleomycin was readily used as a chemotherapeutic agent, believed to cause single and double strand DNA breaks in cancerous cells thus interrupting the cell cycle. One of the unwanted side effects of bleomycin treatment however included persistent lung fibrosis from overproduction of reactive oxygen species (ROS) that lead to severe inflammatory response and activation of fibroblasts (Chaudhary, Schnapp and Park, 2006).

The TGF- β overexpression model is based on lung-tissue specific overexpression of TGF- β that drives pro-fibrotic cycle, ultimately creating an environment of persistent, progressive pulmonary fibrosis. As mentioned earlier, TGF- β induction is consistently observed in models of pulmonary fibrosis and transient overexpression of TGF- β 1 (by adenovector gene transfection) was shown to induce progressive, severe lung fibrosis after just 7 days post overexpression (Sime *et al.*, 1997). Similar to the bleomycin model, once the stimulation is removed / blocked there is a certain level or reversal of the fibrosis in the lung, however, not fully.

The current rodent models available to investigate IPF thus do not allow for exploration of the causative mechanisms behind IPF (as they have a known causative agent). Nonetheless, these models have been instrumental in establishment and testing of the only two fibrosis-halting drugs available on the market today, Nintedanib and Pirferidone (Conte *et al.*, 2014, Wollin *et al.*, 2014, 2015).

1.3 Zebrafish as a model of complex tissue regeneration

The Dallman laboratory has previously used zebrafish gill as a novel model to investigate respiratory tissue inflammation and fibrosis. Zebrafish, *Danio rerio*, is a small freshwater fish, native to southeast Asian climate. The regenerative capacity of zebrafish has been investigated for over 100 years. With the recent advances in molecular cloning and targeted genetic mutagenesis, zebrafish has become an important model of complex tissue regeneration (Beffagna, 2019).

Zebrafish has, among other tissues and organs, shown remarkable regeneration of fins, heart, liver, kidney, retina and central nervous system structures (Hata, Nanae and Nishina, 2007; Diep *et al.*, 2011; Li and Wingert, 2013; Pfefferli and Jaźwińska, 2015; Wan and Goldman, 2016; Zambusi and Ninkovic, 2020), providing us with a glimpse of the regenerative potential of almost any cell type/tissue type within the animal's body. This has led to the use of zebrafish not only as a model for regeneration but also in other fields such as toxicology,

cancer research (Letrado *et al.*, 2018; Cassar *et al.*, 2020), and, with particular continued interest, in the developmental aspects and mechanisms, and their potential re-use during regenerative processes (Cardozo *et al.*, 2017; Hou *et al.*, 2020).

1.3.1 Zebrafish gills as a model of respiratory tissue regeneration

Despite the high research interest in the development and regeneration of various zebrafish organs, very little research has focused on the regeneration of the zebrafish respiratory organ, the gills. Gills consist of highly branched primary and secondary lamellae containing blood vessels. Adult zebrafish fully depend on their gills to meet their oxygen demands, rather similarly to human or more generally mammalian lungs. The gill structures arise from pharyngeal arches around 3 days post fertilisation (dpf). Pharyngeal arches are highly conserved developmental structures acting as precursors to number of cell types including chondrocytes or neurons. While the first pharyngeal arch in all vertebrates forms the jaw and the second the hyoid structure (Graham *et al.*, 2019), only in fishes (non-amniotes) the 3rd through 6th pharyngeal arches give rise to the gills (Schoch and Witzmann, 2011; Graham and Richardson, 2012; Cadiz and Jonz, 2020).

The gills become a fully functioning respiratory organ by 14 dpf (Mierzwa *et al.*, 2020). In zebrafish, there are 4 bony gill arches on each side of the zebrafish head, with cartilaginous primary and secondary lamellae sprouting from the arches and primary filaments respectively (Fig. 1.6). Both primary and secondary lamellae are important site of the chemoreceptive neuroepithelial cells (NECs) which detect the changes in O₂ and CO₂/H⁺ and drive reflex hyperventilation during hypoxia or hypercapnia (Jonz, Fearon and Nurse, 2004; Qin, Lewis and Perry, 2010a; Abdallah, Jonz and Perry, 2015).

Arguably, there is little architectural resemblance between the zebrafish gill and the human (mammalian) lung, research has however revealed numerous shared functional aspects as well as cellular composition of the tissues (Progatzky *et al.*, 2016, 2019).

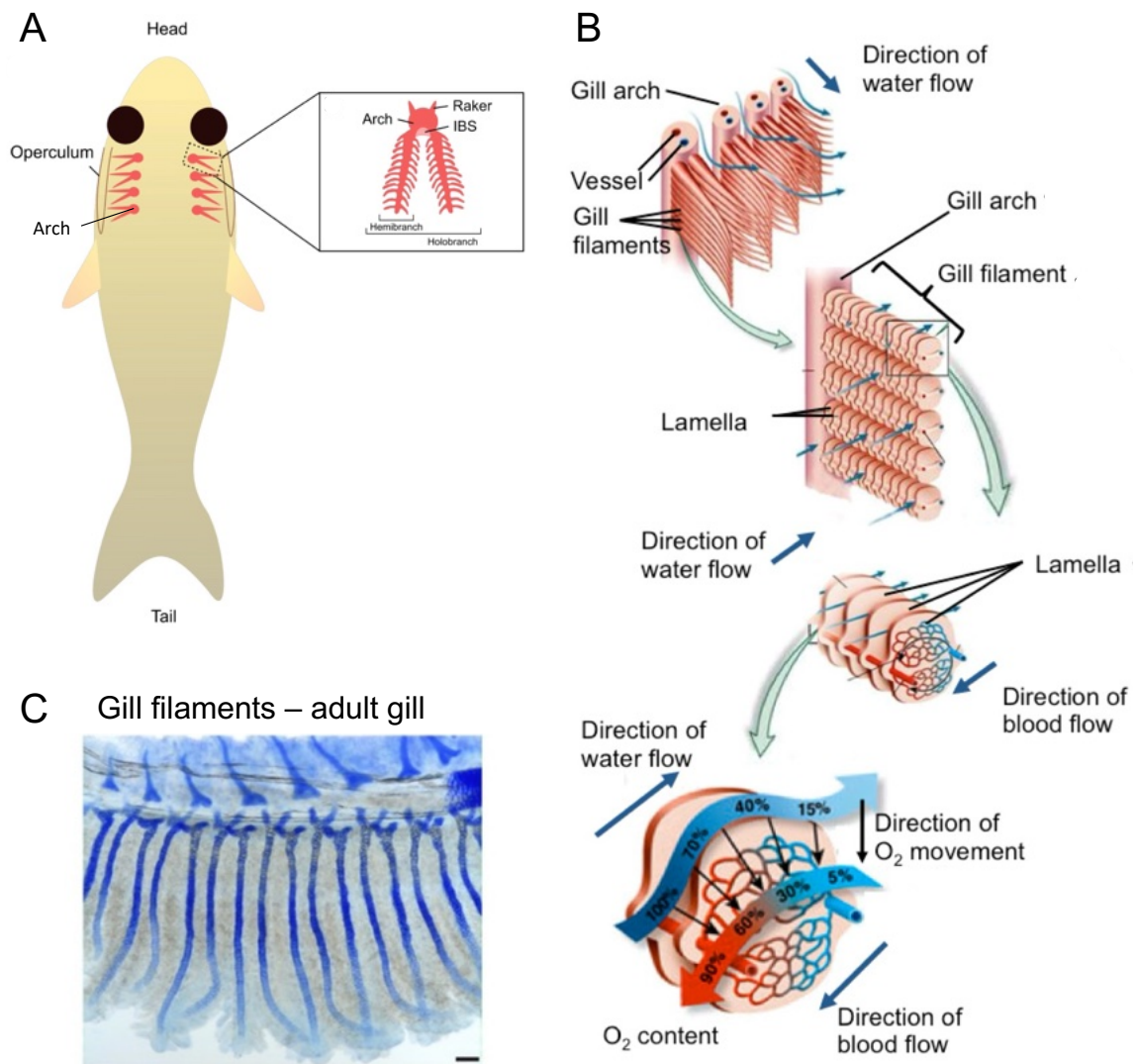


Figure 1.6 Structure of zebrafish gills and schematic representation of gas exchange at homeostasis

(A) Dorsal view of adult zebrafish showing 4 gill arches on either side of the zebrafish head. The gills are covered by operculum. The gills exist as pair structures called holobranchs that are joined by interbranchial septum (IBS) with rakers (bony tooth-like structures) projecting from the anterior part of the arch. **(B)** Overview of gas-exchange. Water and blood flow occurs in opposite directions to each other, creating a gradient of O₂. **(C)** Alcian blue staining of a single gill arch showing the cartilaginous nature of gill lamellae. Adapted from (Progatzy, 2014; Thiruppathy *et al.*, 2022).

1.3.1.1 Cellular composition of the zebrafish gill tissue

Like mammalian lung, the gill epithelium consists of specialized epithelial cells (pavement cells), mucous producing cells and progenitor cells that are required for tissue repair. Pavement cells, covering the arch as well as the filaments, are the outer most cells in direct

contact with external environment. They present thin and flat morphology with microridges that increase the overall cellular surface area, making the pavement cells well adapted for gas exchange. Unlike the mammalian lung, the epithelial cells are not ciliated. The ciliated nature of mammalian lung epithelial cells is important in pathogen and/or particle clearance from the lung to prevent infection and inflammation. Zebrafish gills must therefore possess alternative mechanisms to clear pathogens. As the gill is in direct contact with the ambient water, possibly contaminated with pathogens, the mechanism may be as simple as a continuous flow of fresh water through the gills (Derksen, Ostland and Ferguson, 1998; Evans, Piermarini and Choe, 2005; Leguen, 2018).

The gill arch, rakers and filaments' epithelium contains mucus-producing cells. Interestingly, those are not found in secondary lamellae in health, only in pathological conditions (Progatzy *et al.*, 2016; Macirella and Brunelli, 2017; Leguen, 2018). Similar to mammalian mucus-secreting cells, these zebrafish cells express the *muc5b* gene (orthologue of the mammalian *MUC5B* gene). The gill mucus was shown to bind pathogens and combined with the detection of several immunoglobulins (IgA, IgT) in the mucous as well as antimicrobial peptides and complement C3 factor. The role of these cells and ultimately of the gill appears to include immunomodulatory function beyond a simple barrier defence (Valdenegro-Vega *et al.*, 2014; Xu *et al.*, 2016; Igarashi *et al.*, 2017; Padra *et al.*, 2019).

Neuroepithelial cells (NECs) detect changes in oxygen and carbon dioxide levels, again similarly to mammalian lungs (Jonz and Nurse, 2005; Qin, Lewis and Perry, 2010b). Gills are innervated throughout the filaments and lamellae and the nerve fibres have been shown to be highly important in regeneration (discussed below) (Saito *et al.*, 2019; Mierzwa *et al.*, 2020).

The gills, like the lungs, are highly vascularized with endothelial cells lining blood and lymphatic vessels (Olson, 2002; Okuda *et al.*, 2012). In the gills, there are also specialised endothelial cells called pillar cells that are morphologically different to vascular endothelial cells and structurally support the secondary lamellae (Wilson and Laurent, 2002). And last

but not least, mitochondria-rich cells are found along the gill filaments and are involved in ion exchange and ammonia secretion (Evans, Piermarini and Choe, 2005; Leguen, 2018).

1.3.2 Gill regeneration

Mierzwa and colleagues published a very interesting paper in 2019 showing that gill tissue does regenerate after simple transection and that this event is possibly mediated through blastema intermediate. The authors reported detection of increased numbers of proliferating PCNA⁺ cells at the tip of the gill filament within 24 h after resection compared to homeostatic conditions (Mierzwa *et al.*, 2020). The authors followed the regenerating gill up till 160 days post resection, where they concluded the filament lengths of the resected filaments almost caught up with the remaining filaments. The authors further reported vascularization of the regenerating filaments (by using *fli1*:GFP zebrafish line that enables visualisation of vascular endothelial cells) as well as replacement of NECs and reestablishment of innervation (marked using serotonin and zn-12 antibody respectively). Together the data showed that upon resection, zebrafish are capable of regenerating functional gill filaments possibly through epimorphic regeneration, in a scarless manner.

Further confirmation of the regenerative potential of the gills came from another lower vertebrate model, the axolotl. Saito and colleagues in 2019 showed that axolotl gills also regenerate, that this regeneration is nerve dependent and that BMP signalling and FGF signalling are fundamental for the optimal appendage regeneration (Saito *et al.*, 2019). BMP and FGF signalling in general have been shown to act as regeneration-inducers in amphibians (Makanae, Mitogawa and Satoh, 2014; Satoh *et al.*, 2016). Research by Satoh and colleagues (Makanae, Mitogawa and Satoh, 2014; Satoh *et al.*, 2016) showed that a particular combination of BMP and FGF factors, particularly Fgf2+Fgf8+Bmp7 (or Bmp2) enables nerve-deficient regeneration. This not only corroborated work done previously that showed that regeneration is nerve-dependent, but also suggested neurons to be responsible for the secretion of the FGF and BMP factors. Saito and colleagues further showed that upon resection, blastema was formed within 10 days post amputation (dpa) and the regenerating gill filaments were vascularized by 15 dpa. Chemical inhibition of BMP signalling using Dorsomorphin, a broad BMP signalling inhibitor that blocks BMP signalling receptors

ALK2, ALK3 and ALK6, significantly slowed down gill regeneration (Saito *et al.*, 2019). Together the data from axolotl showed that gill regeneration appears to be regulated by similar mechanisms as limb and tail regeneration in this animal model. Considering the many similarities between axolotl and zebrafish when it comes to epimorphic appendage regeneration, these observations from axolotl suggest possible mechanisms to investigate further in the zebrafish model.

Previous members of the Dallman laboratory have investigated in detail the mucosal layer of the gills and its similarities to mammalian lung mucosal surfaces to study the zebrafish relevance as a model of respiratory infections. One of such studies focussed on developing parallel mouse, human and zebrafish challenge models to investigate the effects of viral RNA mimetic compound, R848, stimulation on the activation of toll-like receptors (TLRs) that are important in respiratory viral infections.

TLRs are an example of highly conserved immune defence mechanism present still today across vertebrates and invertebrates (Janeway and Medzhitov, 2002). Currently, zebrafish are known to contain orthologues for 10 out of 12 human TLRs (eg. *tlr7* or *tlr9* (zebrafish) are orthologous to *TLR7* and *TLR9* respectively (human)). Several paralogues of TLR genes have been also found in zebrafish (such as *tlr8a/b* (zebrafish) orthologous to *TLR8* (human)) and several TLRs detected in zebrafish do not have known orthologs in the human genome and are considered fish-specific (e.g. *tlr19* or *tlr21*).

All three challenge models showed fascinating similarities in the interferon and inflammatory cytokine response in terms of kinetics of transcript levels fold-change (Progatzky *et al.*, 2019). Of particular interest were the systemic and local responses of the zebrafish to stimuli and insults that have been marked as risk factors for human IPF. Progatzky and colleagues have found that acute exposure to irritants, such as cigarette smoke or silica nanoparticles, resulted in transcriptional activation of NF- κ B-dependent proinflammatory genes (*tnfa*, *il1b*, *mmp9*) (Progatzky *et al.*, 2016).

Further evidence from mammalian models shows that stimulation of fibroblasts' TLR receptors leads to cell activation and pro-inflammatory cytokine gene expression. Cheng and colleagues showed that skin fibroblasts express functional TLRs, in particular TLR1-9 whose stimulation result in synthesis of cytokines and antimicrobial peptides such as IL-6, IL-8, and MMP1. The secretion of these proinflammatory cytokines leads to neutrophil and leukocyte recruitment (Cheng et al., 2015).

Following the successful establishment of zebrafish as a model of acute mucosal inflammation, the Dallman laboratory went on to investigate zebrafish as a model of respiratory tissue fibrosis. As gills are constantly in touch with the ambient water, the Dallman laboratory used long term exposure of cigarette smoke and/or silica nanoparticle treatment to induce long term injury in order to try to mimic the known risk factors associated with pulmonary fibrosis in humans. Short term exposure to water-dissolved irritants resulted in similar cytokine and immune gene stimulation as in mammalian lungs (Progatzky, 2014; Progatzky *et al.*, 2016). However, long-term exposure of zebrafish gills did not induce chronic inflammation, did not lead to changes in collagen synthesis and/or deposition and only resulted in transient remodelling of the epithelial structures.

Moreover, a novel injury model of the gills – gill cryoinjury – was investigated (Progatzky, 2014; Ramel *et al.*, 2021). Cryoinjury is a commonly used technique particularly in zebrafish heart regeneration research. Historically, heart resection was used to study zebrafish heart regeneration and studies showed complete regrowth of the resected area, with full vascularization and electrically coupled myocardium within 60 dpr (González-Rosa and Mercader, 2012). However, the resemblance with mammalian myocardial infarction (MI) is minimal as there is very little or no ischaemia-induced cells death. Cryoinjury of the zebrafish ventricle was shown to induce both necrotic and apoptotic cell death with the associated inflammation and recruitment of immune cells more closely recapitulating the cellular and molecular changes associated with MI in humans as compared to heart resection (González-Rosa and Mercader, 2012; González-Rosa, Burns and Burns, 2017). Moreover, heart cryoinjury was found to induce scar formation, again similarly to mammalian post-MI hearts. However, in the zebrafish model, only a transient formation of fibrotic lesion is

formed that appears to be fundamental for optimal cardiomyocyte proliferation and heart regeneration (Sánchez-Iranzo *et al.*, 2018). Yet, Bise and colleagues went on to show that repeated heart cryoinjury (up to 6 times) did result in diminished capacity of the zebrafish to clear the formed fibrotic lesion and was accompanied with progressive collagen deposition as well as by reduction in the cardiomyocyte proliferation and differentiation at the early repair stages (Bise *et al.*, 2020). Overall, cryoinjury as compared to simple resection of the zebrafish heart was shown to better recapitulate certain aspects of the fibrotic response seen in mammals and therefore the Dallman laboratory hypothesized that gill cryoinjury would be a stronger insult, possibly resulting in fibrosis, whether transient or persistent, in the gills.

After cryoinjury, strong influx of innate immune cells, particularly neutrophils (within 1 dpc) and macrophages (within 3 dpc) marks the inflammatory phase of tissue regeneration. Significantly a higher number of activated caspase-3⁺ cells (marker of cellular apoptosis (Sorrells *et al.*, 2013)) was detected at 6 hpc, through to 7 days post cryoinjury (dpc) compared to control gills. Blastema markers, *sall4* and *msx1b* were significantly upregulated at all timepoints investigated (3, 7, 14 and 21 dpc) in the case of *sall4*, and at 7 and 14 dpc in the case of *msx1b*, marking the establishment and maintenance of blastema. Although a significant upregulation of *coll1a1* and other extracellular matrix coding genes was detected and peaked around 7 dpc together with a significant increase of collagen deposition at up to 21 dpc, by 6 wpc no structural changes and transcriptional differences were detected in the cryoinjured gill tissue compared to control. Thus, despite the presence of a strong fibrosis-like response to cryoinjury, the animals recover from this insult again in a scarless manner. In severe cryoinjuries, where the cartilaginous filaments were frozen all the way to the bony arch, zebrafish regenerated the filament length only partially at 91 dpc (Progatzky, 2014; Ramel *et al.*, 2021). Whether zebrafish requires more time to regenerate the lost length fully or whether the observed ‘delay’ is due to compensatory and/or homeostatic growth of the surrounding uninjured filaments remains to be investigated.

Overall, current research indicates that zebrafish possess fascinating plasticity when it comes to repair and regeneration of their body parts and that gills even after severe injury, such as the cryoinjury, regenerate with only a transient collagen deposition stage.

1.4 Discovering Fibins

The long-term and severe injuries to zebrafish gills proved not to be a suitable model for tissue fibrosis, since no longer-term collagen deposition was observed, and structural remodelling was resolved within 4-6 week following the end of injury. Still, the fascinating plasticity and regenerative potential of the gill tissue provided a new platform to investigate the differential gene regulation and cellular responses between the mammalian models of prolonged tissue fibrosis and the lower vertebrate models of complete tissue regeneration. In collaboration with Boehringer Ingelheim Pharma, the Dallman laboratory performed a comparative cross-species RNA sequencing analysis of the zebrafish gills and mouse lungs. The two mice models that were compared to the zebrafish gill cryoinjury data were bleomycin-induced pulmonary fibrosis model and TGF- β overexpression model.

Regarding timepoints, 7 dpc / 7 days post bleomycin treatment (dpt) timepoint was used to investigate differentially regulated genes. In zebrafish, 7 dpc represents a timepoint at which the strong inflammatory response is resolved and the regenerative programs are initiated (Ramel *et al.*, 2021). In mice, 7 dpt marks the transition phase from initial strong inflammatory response to fibrotic processes (Liu, De Los Santos and Phan, 2017; Ju *et al.*, 2022).

A number of genes were differentially regulated between the mouse and zebrafish models (a selection of these genes is shown in Appendix 1 (Ramel *et al.*, 2021), amongst which were two paralogues, *fibina* and *fibinb* of the human gene *FIBIN*. Table 1.1 presents the nomenclature for Fibin(s) that will be used throughout this thesis.

	GENE	PROTEIN
Human	<i>FIBIN</i>	FIBIN
Mouse	<i>Fibin</i>	FIBIN
Zebrafish	<i>fibina</i> / <i>fibinb</i>	Fibina / Fibinb
General (without referring to particular species)	Fibin(s)	

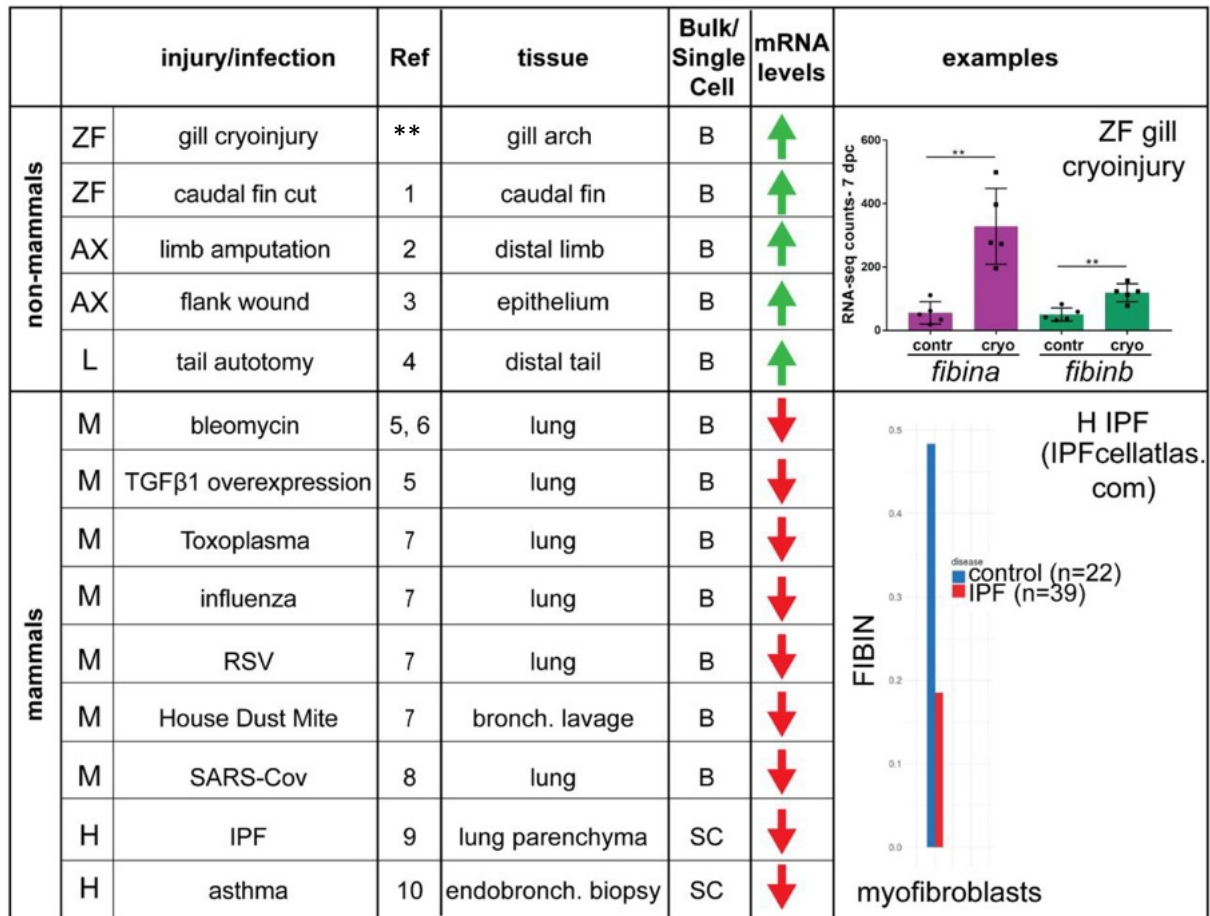
Table 1.1 Fibin(s) nomenclature (Sundberg and Schofield, 2010; Katherine, 2014).

Extensive datamining (summarized in Fig. 1.7 (Ramel *et al.*, 2021)) emphasized the differential regulation of Fibin(s) in mammalian vs non-mammalian vertebrates during tissue repair and regeneration respectively. Several other non-mammalian vertebrate models of tissue regeneration including axolotl and lizard also reported increased *fibin* levels during tissue regeneration. On the other hand, human and mice models of tissue repair (particularly of pulmonary tissue fibrosis) showed significantly decreased levels of *Fibin* (McDonough *et al.*, 2019; Reyfman *et al.*, 2019; Singhania *et al.*, 2019; Vieira Braga *et al.*, 2019; Adams *et al.*, 2020). These objective, non-Fibin(s)-focussed studies further confirmed the observation from our RNA-seq analysis of mice and zebrafish respiratory tissue injury models showing that *Fibin* was significantly downregulated in the mouse (mammal) model of pulmonary fibrosis, but significantly upregulated in the zebrafish gill cryoinjury model.

1.4.1 Proposed function(s) of Fibin(s)

Fibin received its name after its presumed function in zebrafish pectoral bud development (fin bud initiation factor) (Wakahara *et al.*, 2007). Fibin belongs to a rather poorly understood family of vertebrate-specific proteins and does not exhibit sequence homology to any known protein. In mammals there is one copy of the *FIBIN* gene, however, in lower vertebrates including the zebrafish, two paralogues are found named *fibina* and *fibinb*. Parologue is a term used for genes that have evolved through gene duplication within the same genome. The two paralogues are thought to have arisen during one of the gene duplication events in teleost

fish, approximately 300,000 years ago (Taylor *et al.*, 2001). Whole genome/gene duplication is a very interesting phenomenon particularly in terms of investigating the functions of the paralogues which in some cases can diverge, in others remain the same leaving one paralogue redundant. A significant number of well-characterized, paralogous genes have been investigated to understand possible functional variation due to the duplication.



1: GSE76564, GSE74415, GSE37165; 2: GSE67118; 3: GSE79299; 4: PRJNA545124; 5: Strobel *et al.*, in preparation, Strobel *et al.*, 2015; 6: GSE2640, GSE34814, GSE123808, GSE43695; 7: Singania *et al.*, 2019; 8: GSE68820; 9: Adams *et al.*, 2019; 10: Vieira Braga *et al.*, 2019; **: Ramel *et al.*, 2021

Figure 1.7 Datamining results showing that Fbin(s) are differentially expressed in mammalian models of fibrosis and lower vertebrate models of regeneration.

Datasets of RNA seq data from infection and/or regeneration models available online were searched for Fbin transcript levels. Non-mammalian models including zebrafish (ZF), axolotl (AX) and green anole lizard (L) showed increased Fbin transcript levels during repair/regeneration compared to uninjured tissues. Mammalian models – mouse (M) and human (H) showed decreased Fbin levels during the tissue repair/fibrosis. ZF: zebrafish; AX: axolotl; L: Lizard; H IPF: human Idiopathic Pulmonary Fibrosis.

One such example is *AXIN* and *AXIN2*, components of WNT signalling, that show functional interchangeability while having a different function. In mice, loss of *Axin* results in early embryonic lethality, while deletion of *Axin2* leads to viable animals with craniofacial defects. Yet, expressing *Axin2* under *Axin* promotor showed that *Axin2* is capable of fulfilling *Axin* functions (Chia and Costantini, 2005). It has been proposed that this interchangeability of paralogous genes that were shown to have different functions can be due to different expression patterns or temporal difference in expression, driving the diverging functions (Drobek, 2022).

Another example of well-known paralogues with diverging function are *sox9a* and *sox9b*. In zebrafish, *sox9a* and *sox9b* play important roles in development (neural crest, craniofacial or fin). Both of the paralogues are required for *col2a1* expression (major component of cartilaginous ECM) (Yan *et al.*, 2005). Yet, although both *sox9a* and *sox9b* are necessary for correct chondrogenesis, each of these genes appears to have a slightly different function: both paralogues were found to be expressed in cranial neural crest cells (cNCC) which migrate and populate pharyngeal arches. 3rd, 4th, 5th and 6th pharyngeal arches give rise to the gills in zebrafish. And while mutation of *sox9a* gives leads to completely abolished cartilage development (Yan *et al.*, 2002), mutations in *sox9b* results in the absence of ceratobranchial cartilage and mild reduction in the anterior gill arches (craniofacial development is covered in more detail in Chapter 5). Moreover, *sox9a* expression is fundamental for endochondral ossification, while expression of *sox9b* is involved in formation of dermal bone (Yan *et al.*, 2005; Dalcq *et al.*, 2012).

With the relative lack of Fibin-focussed literature, the function of mammalian FIBIN is still unclear, as are the functions of zebrafish Fibins. Research publications that specifically mention Fibin(s) in the body of the text are listed in Table 1.2, together with the study result.

Wakahara and colleagues were the first to publish a report specifically focused on Fibins in 2007 (Wakahara *et al.*, 2007). At the time, only fibinb was known (higher sequence similarity to human FIBIN) and it is the only Fibin addressed in Wakahara's study. They used

morpholino-mediated knockdown of *fibinb* to assess its function. The authors reported that *fibin* knockdown lead to complete inhibition of pectoral fin bud initiation (hence the name fin bud initiation factor). The authors went on to investigate potential molecular pathways associated with *fibin* expression and function. WNT signalling and retinoic acid signalling are well known pathways and are among the first ones activated and functional during embryonic development (Osei-Sarfo and Gudas, 2014). Using morpholinos against *wnt2b* and *tbx5* (a downstream target of *wnt2b* that is involved in the specification of the pectoral fin/forelimbs in vertebrates) the authors showed that knockdown of *wnt2b* lead to completely diminished *fibin* expression (assessed by *in situ* hybridization) while morpholino-knockdown of *tbx5* had no effect on *fibin* expression. Moreover, knockdown of *fibin* lead to abolished *tbx5* expression, confirming that *fibin* appears to be regulated by WNT signalling, lies downstream of *wnt2b* and upstream of *tbx5*. *raldh2* codes for an enzyme required for retinoic acid biosynthesis and mutations in this gene result in lack of pectoral fin formation (Gibert *et al.*, 2006). Wakahara and colleagues used DEAB, a competitive, reversable inhibitor of retinaldehyde dehydrogenases (Perz-Edwards, Hardison and Linney, 2001) to show that inhibition of RA signalling also completely abolished *fibin* expression in the presumptive pectoral fin bud regions.

Lakner and colleagues followed up Wakahara's research (Lakner *et al.*, 2011). The authors investigated *Fibin* levels in developing and adult mice and showed that *Fibin* is strongly expressed in developing mice embryos from 9 dpf as well as in multiple organs of adult mice with strongest expression in skeletal muscle and cerebellum tissues. The authors further screened several mice and human cell lines for *Fibin* expression and treated the L929 mouse fibroblast cell line (positive for *Fibin*) with manganese chloride, phorbol ester, dexamethasone, forskolin and lithium chloride to broadly investigate possible regulators of *Fibin* gene expression. Dexamethasone, phorbol ester and manganese chloride treatment for 3-8 hours and 8-24 hours respectively led to a significant increase in *Fibin* transcript levels. The authors also investigated the subcellular localisation of FIBIN by transfecting a COS-7 cell line (negative for *Fibin* expression) with GFP-tagged *Fibin*. Combined with their sequence analysis and experimental confirmation that the N-terminal of *Fibin* contains a signal sequence for guidance of FIBIN to the endoplasmic reticulum, the co-localization with endoplasmic reticulum specific marker, calnexin, suggests that FIBIN is a secreted protein.

However, experimental data showed that FIBIN was only found in the cell lysates and not in the supernatants, showing that FIBIN is most likely not a secreted protein (Lakner *et al.*, 2011).

Lastly, a fairly comprehensive investigation of Fibin was reported by Lee and colleagues (Lee *et al.*, 2021) who investigated *Fibin* in ovariectomised mice and showed possible involvement of *Fibin* in mitochondrial biogenesis and dysfunction. The authors investigated involvement of microRNAs in the mitochondrial dysfunction in muscle cells of ovariectomised mice. Firstly, the authors confirmed that ovariectomy induces mitochondrial dysfunction in the skeletal muscles and leads to upregulation of miR-141-3p. Secondly, they showed that miR-141-3p overexpression in a mouse fibroblast cell line results in significant inhibition of basal and maximal cellular respiration. And thirdly, the authors discovered that miR-141-3p targets and downregulates expression of *Fkpb5* and *Fibin* genes and inhibition of miR-141-3p by oestradiol reverses the downregulation of *Fkpb5* and *Fibin*. Overall, this study suggested novel roles for *Fibin* including function in myogenesis and optimal mitochondrial function.

FIBIN has also been reported in the differentiation of myoblasts in culture (Meyer *et al.*, 2015) with increased levels across 24 h of *in vitro* myoblast differentiation. Meyer and colleagues further reported *FIBIN* to be downregulated after TNF- α treatment and upregulated following insulin-like growth factor (IGF)-1 treatment. As TNF- α acts to inhibit myoblast differentiation and IGF-1 acts to induce myoblast differentiation, *FIBIN* appears to be positively linked to the differentiation, however, what the exact role of *FIBIN* in this setting is remains to be investigated. Kosla and colleagues (Kosla, Dvorak and Cermak, 2013) in their research investigating TGF- β controlled gene expression in chicken embryo dermal myofibroblasts, came across *FIBIN* as a gene that was strongly downregulated following a chemical inhibition of TGBR1 receptor (necessary for facilitating TGF- β signalling). The authors thus suggest that *FIBIN* is a TGF- β response gene.

	Authors/Study	Type of study	Result/Outcome
ZEBRAFISH STUDIES	Wakahara <i>et al.</i> , 2007	Investigation of <i>fibin</i> in zebrafish embryonic development	<i>fibin</i> expressed in presumptive pectoral fi buds. MO knockdown of <i>fibin</i> led to complete loss of pectoral fins. <i>fibin</i> lied upstream of <i>tbx5a</i> . Inhibition of retinaldehyde dehydrogenases (necessary for pectoral fin development) by DEAB and <i>wnt2b</i> inhibition abolished <i>fibin</i> expression.
	Lakner <i>et al.</i> , 2010	<i>in vitro</i> regulation of <i>Fibin</i> expression	<i>fibin</i> when overexpressed, was not a secreted protein. It was not possible to detect endogenous FIBIN protein in mammalian cell cultures. Manganese chloride, phorbol ester and dexamethasone treatment of L929 mouse fibroblast cell line lead to significant increase in <i>FIBIN</i> transcript levels.
HUMAN STUDIES	Rashidi-Nezhad <i>et al.</i> , 2014	Clinical case report: The clinical manifestation of deletion of <i>BBOX1</i> and <i>FIBIN</i> genes	Growth retardation Generalized hypertonion Speech delay Severe microcephaly & small frontal lobes with no evidence of gross structural abnormality Metabolites within normal ranges
	Marouli <i>et al.</i> , 2017; Macé <i>et al.</i> , 2017	Copy-number-variants (CNV) metanalysis & rare/low frequency coding variants analysis with respect to anthropometric human characteristics	Overall more then 500,000 individuals studied; CNV burden (deletion rather then duplication) within <i>FIBIN/BBOX1</i> genomic region associated with shorter height (Marouli <i>et al.</i> , 2017); rare CNVs in <i>FIBIN</i> genomic region associated with increased bone mineral density and lower higher
	Krepischi <i>et al.</i> , 2016	Analysis of genomic copy number alterations ot Wilms tumours	Down-regulation of genes within <i>BBOX1</i> and <i>FIBIN</i> region (due to deletion detected iin Wilms tumour; authors suggest that both <i>BBOX1</i> and <i>FIBIN</i> are candidate tumour suppressor genes for Wilms tumours
IN VITRO STUDIES	Meyer <i>et al.</i> , 2015	Murine myoblast cell culture	<i>Fibin</i> found significantly upregulated during differentiation with increasing FC throughout 4-, 12-, 24-hours of culture; TNF- α treatment resulted in significant downregulation of <i>Fibin</i> at 12 and 24 hours post treatment; IGF1 treatment resulted in significantly increased levels of <i>Fibin</i> at 12 & 24 hours post treatment compared to TNF- α treatment
	Kosla <i>et al.</i> , 2013	Chicken embryo dermal myofibroblasts cell culture	Inhibition of TGFBR1 lead to significant downregulation of <i>FIBIN</i> levels; the authors thus suggested that <i>FIBIN</i> is a TGF- β response gene
	Negreros <i>et al.</i> , 2019	Analysis of methylation and gene transcription in NHLF and IPF human lung fibroblasts following TGF- β treatment	Tgf- β treatemtn stimulates significant increase in <i>FIBIN</i> transcript levels at 5 dpt with a dose-dependent increase detectible at 24 hpt in NHLF; IPF LF have increased baseline levels of <i>FIBIN</i> that does not chage with TGF- β treatment at 24h or 5 dpt.
MOUSE STUDIES	Lee <i>et al.</i> , 2021	Micro-RNA analysis of ovariectomy-induced sarcopenia in mice (only skeletal muscle was harvested for experiments)	Decline in muscle mass and function mitochondrial dysfunction in ovariectomized mice possible mediated by miR-141-3p mediated downregulation of FK506 and FIBIN*

Table 1.2 Fabin(s) in literature

MO: morpholino; DEAB: competitive, reversible inhibitor of retinaldehyde dehydrogenases; FC: fold-change; NHLF: normal human lung fibroblasts; IPF LF: Idiopathic Pulmonary Fibrosis-derived lung fibroblasts; dpt: days post treatment. *The authors present Western blot results with endogenous FIBIN levels detected by an antibody. Lakner and colleagues, nor I, were able to detect endogenous FIBIN protein by Western blotting. Lee and colleagues unfortunately did not respond to email correspondence, and they do not provide details about the antibody in their paper, thus it is not possible to exactly repeat the experiment.

Very scarce evidence also links *FIBIN* with human congenital diseases, including a medical case study by Rashidi-Nezhad and colleagues, describing a young female with a deletion of *BBOX* and *FIBIN* genes, resulting in significant mental and physical impairment (Rashidi-Nezhad *et al.*, 2014). However, it is impossible to know which of the two genes, or if it is the combined deletion of both genes, is responsible for the described phenotype. Lastly, variations in the genomic region where *FIBIN/BBOX* genes are located was associated with anthropometric measures (Macé *et al.*, 2017). In this study, including over 500,000 individuals, the authors reported that increased number of rare copy number variant (CNV) genes was associated with short stature, higher BMI, and also schizophrenia.

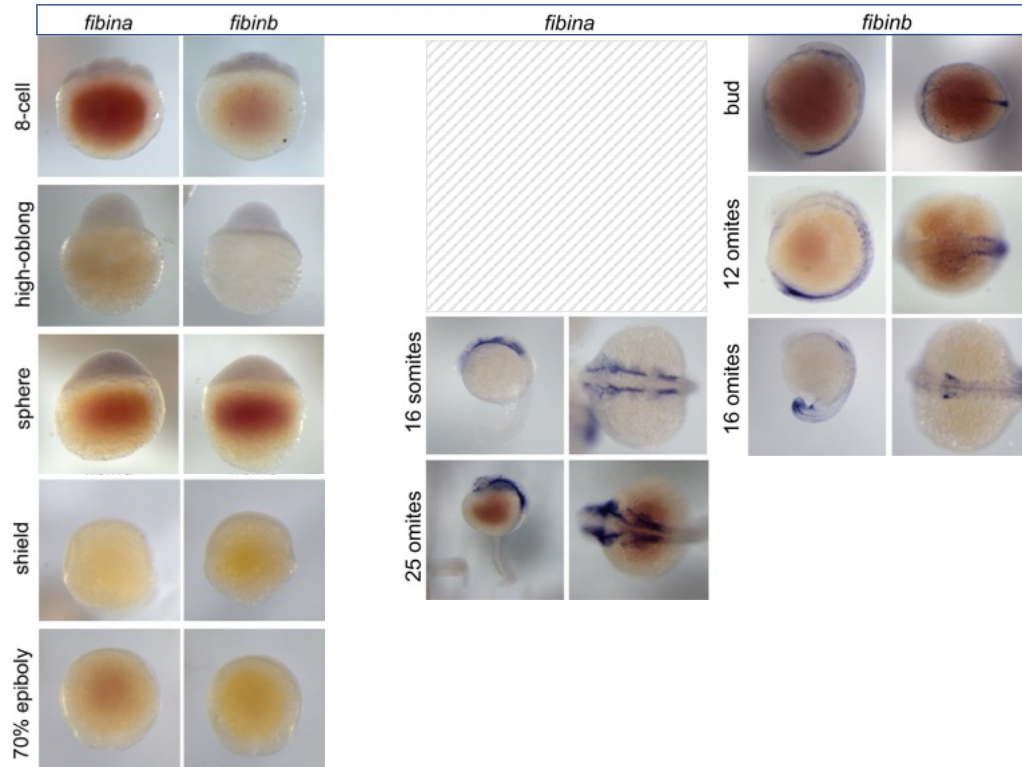
1.4.2 Preliminary experiments to investigate Fibin(s) expression

Preliminary investigations of *fibin* expression and its function from the Dallman laboratory were performed by Dr Marie-Christine Ramel, and Hannah Needham. They first attempted to replicate the results from (Wakahara *et al.*, 2007). Interestingly, using the same *fibina* and *fibinb* morpholinos and timepoints, my colleagues did not observe lack of pectoral fins in the wild-type zebrafish embryos, and thus they were not able to replicate the published results. Morpholino-mediated mRNA degradation can often be associated with significant off-target effects. Although non-specific morpholino is always used as a control, a number of off-target effects may pass unnoticed. In our laboratory, when applying the morpholino dose used by Wakahara and colleagues, we observed severe side effects and eventual death of the zebrafish embryo. It is therefore possible that the pectoral fin phenotype (or rather lack of pectoral fins) after *fibin* knockdown resulted from a generally severely affected zebrafish (due to the high morpholino dose) rather than from *fibin* knockdown itself.

Investigation of *fibina* and *fibinb* expression patterns by *in situ* hybridisation throughout development (Fig. 1.8) showed that both *fibins* show differential temporal and spatial expression patterns suggesting possibly a different function of *fibina* and *fibinb* and possibly different regulation mechanisms. *fibinb* is the first one detected at bud stage (~10 hpf) after which it is expressed in the developing notochord, somites and tail. At 16 somites (~17 hpf) stage *fibinb* expression is clear at the presumptive pectoral fin bud regions. *fibina* only

becomes expressed at 16 somite stage and is strongly expressed at the lateral side of the head region at 25 somite stage (~22 hpf).

A



B

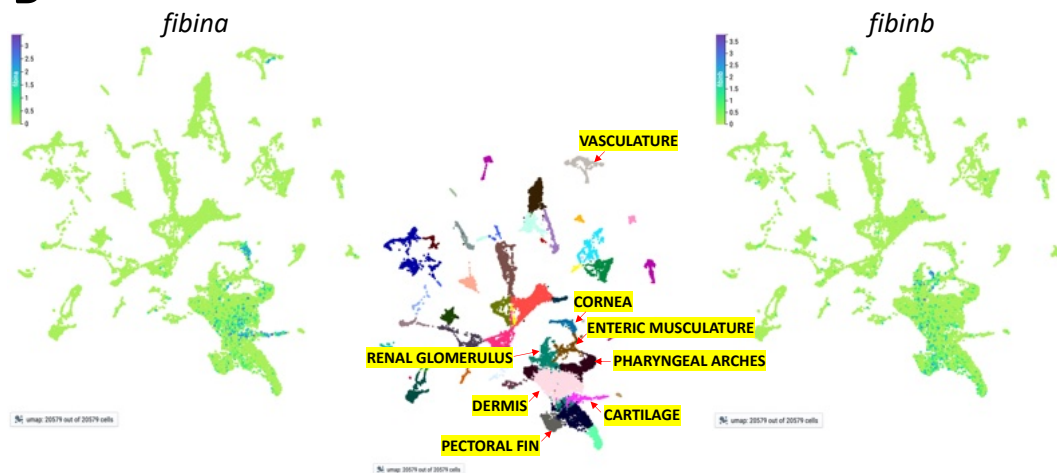


Figure 1.8 Expression pattern of *fibina* and *fibinb* in zebrafish

(A) *in situ* hybridisation of *fibina* and *fibinb* in zebrafish embryonic development; *fibina* expression visible from 16-somites stage, *fibinb* expression visible from the bud stage. Approximate timings post-fertilisation: 8-cell: 1 1/4h; high-oblong: 3 1/3h; sphere: 4h; shield: 6 h; 70% epiboly: 8h; bud: 10h; 12 somites: 14h; 16 somites: 17h; 25 somites: 22h. (Experimental data by M.-C. Ramel.) (B) Datamining results of *fibina* and *fibinb* expression in zebrafish embryos at 10 dpf. Highlighted in yellow are the tissue types where either *fibin* is expressed at this timepoint (Lange *et al.*, 2023).

1.4.3 Further datamining to investigate Fibin(s) expression

Fibin(s) expression does not appear to be organ specific, but rather cell type specific. The human protein atlas shows that in humans, both *FIBIN* gene expression and FIBIN protein are found in all tissues investigated (the lowest expression/levels are found in the eye). When it comes to cell types, *FIBIN* shows strong correlation to mesenchymal cell types, in particular fibroblasts (Uhlén *et al.*, 2015). Very similar cell-expression profile is seen in zebrafish, with mesenchymal cells (including fibroblasts, chondrocytes, osteoblasts or skeletal muscle cells) showing highest *fibina* and *fibinb* expression levels, followed by basal epithelial cells (Hou *et al.*, 2020b). Therefore, Fibin may be involved in regeneration and/or maintenance of organs and tissues that includes a cell type that expresses Fibin(s).

In our laboratory, we have also investigated the similarities and differences in the transcriptomics signature of regenerating zebrafish heart and caudal fin, alongside zebrafish gill regeneration. Our group showed that while there are common molecular pathways involved in zebrafish gill, heart, and caudal fin regeneration, the top 1000 genes that are differentially expressed only overlap by 15%. Additionally, there are more shared genes between caudal fin and gill regeneration, compared to heart regeneration. The considerable differences in the transcriptional profile of zebrafish heart regeneration compared to caudal fin and gill regeneration signatures was evident mainly through prolonged maintenance of immune-response related GO terms in zebrafish compared to caudal fin or gill regeneration (Ramel *et al.*, 2021).

Datamining of *fibina/b* expression in zebrafish gill, heart and caudal fin revealed high expression in both the gill and the caudal fin (in caudal fin *fibina* and *fibinb* show reversed proximo-distal gradient) while the zebrafish heart shows comparably very low *fibins* expression. The low expression of *fibina/b* in zebrafish heart is likely linked to the fairly low abundance of fibroblasts in the zebrafish heart – *fibina* expression is strongly linked with fibroblasts and relatively low, while *fibinb* expression is also found in endocardial/endothelial cells and smooth muscle cells (Rabinowitz *et al.*, 2017; Koth *et al.*, 2020). In this thesis I therefore also explore the potential role of Fibin(s) in caudal fin regeneration to characterize

and compare similarities and differences between regeneration of distinct organs, to possibly draw more generalized conclusions for Fibins involvement in regeneration.

1.5 Structure and cellular composition of zebrafish caudal fin

Caudal fin is a complex tissue consisting of connective tissue, lepidotrichia, actinotrichia, blood vessels, nerves and epidermis that are closely coordinated to establish, maintain and, if needed, restore the tissue structure and function. The caudal fin is a non-muscularised structure that contains 16-18 bony rays. Zebrafish retain the ability to grow throughout their lifespan and this includes the caudal fin, although there are mechanisms in place that limit the caudal fin excessive growth (Iovine, 2007). The main structural component of adult caudal fin is calcified bone matrix forming the fin rays, also called lepidotrichia. The tips of each lepidotrichia remain non-mineralized and the tissues are supported by cartilaginous matrix synthesized by non-osteoblasts (actinotrichia). The fin ray consists of two concave bones referred to as hemirays and the lepidotrichia are tightly associated with osteoblasts that deposit bone matrix into the extracellular space (Fig. 1.9(A)) (Akimenko et al., 2003).

Rabinowitz and colleagues used transcriptional, proteomics and metabolomics approaches to investigate differential gradients of Fibins (proximo-distal) within the zebrafish caudal fin. Principal component analysis of bulk RNA-seq results showed highly distinct clusters, highlighting significant positional differences within tissues, at the RNA level. In their dataset, *fibina* was highly expressed at the distal end compared to the proximal part of the fin (fold-change of 2.5) while *fibinb* showed significantly lower read counts in the distal, compared to the proximal aspect of the fin (fold-change of 0.3). As the caudal fin is capable of regeneration irrespective of where the amputation takes place, the possible implications of such expression gradient need to be further addressed taking together all relevant genes showing similar gradient patterns (Rabinowitz *et al.*, 2017).

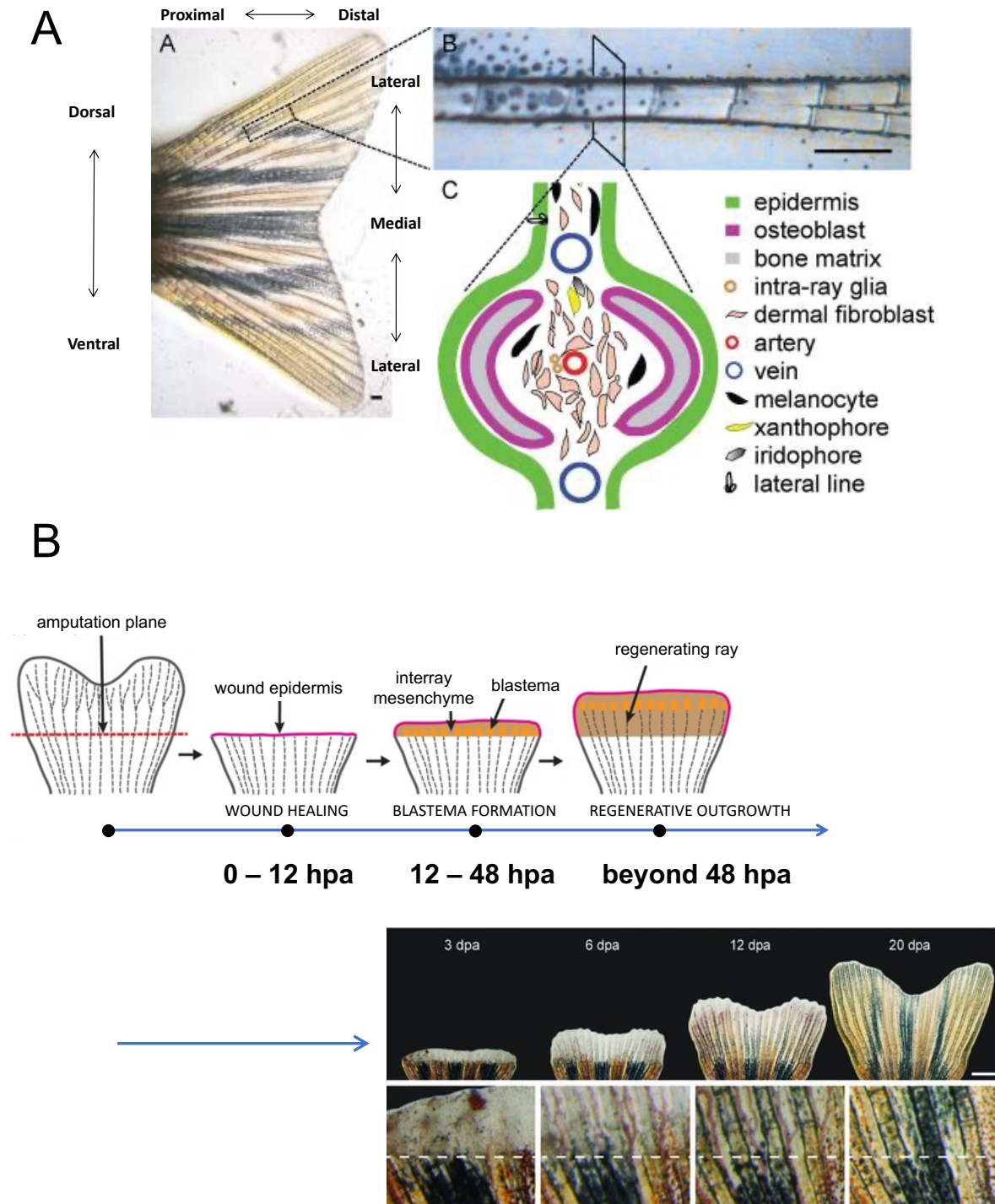


Figure 1.9 Zebrafish caudal fin structure

(A) The structure and cell types of the zebrafish caudal fin. A) Typical wild-type caudal fin with pigmented stripes and bi-lobed morphology. B) zoomed-in image of a single fin ray with bifurcation. (scale bars = 0.2 mm) C) Non-exhaustive list of cell types found within the zebrafish caudal fin. Illustration shows 10 most commonly present. (B) Events during caudal fin regeneration. After amputation, wound is closed with 12 hpa, following which a blastema begins to form (orange) distally to each ray by migrating cells. The blastema cells then proliferate and differentiate during the regenerative outgrowth, re-establishing all the lost tissues. At 20 dpa (within 3 weeks) the fin size and patterning is restored. Adapted from (Tu and Johnson, 2011; Pfefferli and Jaźwińska, 2015; Sehring and Weidinger, 2020).

1.5.1 Caudal fin regeneration

Caudal fin regenerates through epimorphic regeneration. Fig. 1.9(B) shows the timeline of zebrafish caudal fin regeneration with associated molecular pathways reported to be active during the process. Here I focus on regeneration after simple amputation (transection) which is the most commonly used technique. Other techniques such as fin cryoinjury, have also been described, and show significant differences in the extend of inflammation after insult, yet the timeline to full regeneration does not differ significantly from a simple amputation (Chassot, Pury and Jaźwińska, 2016).

1.5.1.1 Wound healing

Immediately after amputation blood coagulates at the wound site to prevent excessive blood loss. Within the following few hours, epithelial cells migrate to cover the open wound forming the wound epidermis. The organization and integrity of the wound epidermis is essential for successful regeneration (Thorimbert *et al.*, 2015a). Interestingly, zebrafish was shown to successfully form a functional wound epidermis even after 29 successive fin amputations (Azevedo *et al.*, 2011a), although experiments in very closely related salamanders and lizards show an initiation of persistent wound healing response, resulting in general decrease of regenerative capacity (Bryant *et al.*, 2017; Alibardi, 2018).

The wound epidermis gives rise to the apical epidermal cap (AEC). The cross-tissue communication between AEC and the underlying mesenchymal cells coordinates the regenerative process (Thornton, 1957; Pfefferli and Jaźwińska, 2015). Beneath the AEC, dynamic and extensive extracellular matrix remodelling takes place. The extent of the extracellular matrix remodelling depends largely on the nature of the injury. A simple tail resection/amputation is naturally associated with significantly less inflammation and cell death requiring active clearing than caudal fin cryoinjury (Chassot, Pury and Jaźwińska, 2016).

1.5.1.2 Blastema formation

The blastema is a group of linked undifferentiated cells that forms within the first 48h post tail resection in zebrafish (Iovine, 2007; Pfefferli and Jaźwińska, 2015). It is the primary source of differentiating and differentiated cells that enable the outgrowth of the appendage. In the past decade, an incredible amount of new evidence, particularly in the form of single cell RNA – sequencing datasets shed new light on the origins of the cells that form the blastema of regenerating fins. Previously, one school of thought believed that the stem-cell-like cells forming blastema are tissue resident stem cells, migrating to the wound side. These cells were thought to have the capacity to differentiate into one or more cell types (Weissman, Anderson and Gage, 2001). More recent evidence points to de-differentiation and trans-differentiation of cells that find themselves in the vicinity of the wound and re-enter the cell cycle (Lowry *et al.*, 2007; Jopling, Boue and Belmonte, 2011; Merrell and Stanger, 2016).

Osteoblasts in particular can temporarily dedifferentiate in response to amputation. In practice this includes re-entry of the terminally-differentiated cells (marked by osteocalcin) to cell-cycle, their migration to the establishing blastema and their re-differentiation through pre-osteoblastic precursors (marked by *runx2*), into maturing osteoblasts (marked by *osterix/sp7*) and mature osteoblasts (marked by *osteocalcin*) during the outgrowth of the fin (Knopf *et al.*, 2011). Sousa and colleagues (Sousa *et al.*, 2011) performed similar experiments and determined the same outcome. These experiments alone strongly support the notion of dedifferentiated cells establishing the blastema and giving rise only to the tissue type (here osteoblasts) that they were originally derived from.

Interestingly, however, Singh and colleagues (Singh, Holdway and Poss, 2012) performed an osteoblast-specific ablation immediately prior to caudal fin amputation and showed that even in the absence of osteoblasts, zebrafish were still able to fully regenerate all tissues, including bony rays, through transdifferentiation of alternative cell types (such as fibroblasts) into de novo osteoblasts. These experiments showed yet again the fascinating plasticity that zebrafish display during regeneration.

1.5.1.3 Caudal fin outgrowth

After blastema formation, the structure compartmentalises into two domains – low proliferating distal blastema and proximal blastema populated by highly proliferating and differentiating cells (Nechiporuk and Keating, 2002). The highly proliferative blastema maintains the source of de-differentiated cells throughout the regeneration process. As blastema cells re-differentiate into eg. new osteoblasts (proximally), these began migrating out of the blastema, continuing their differentiation through to the terminal stage (eg. mature osteoblasts marked by *osteocalcin*).

During the outgrowth of the amputated appendage (fin in this case), the cells within the blastema possess positional memory/information about which aspect of the caudal fin they will reconstruct. It has been proposed that positional memory exists not only along the anterior – posterior axes (that is head – caudal fin) but also dorsoventral axis (Kim and Stocum, 1986; Ludolph, Cameron and Stocum, 1990). Experiments by Uemoto and colleagues further confirmed this hypothesis, by showing that amputations at different proximo-distal positions result in varied speed of regeneration thus suggesting that blastema cells possess positional memory that expresses itself, at least partially, through rate of caudal fin outgrowth (Uemoto, Abe and Tamura, 2020). Nevertheless, full understanding of the molecular and cellular dynamic that enables full length restoration of the original appendage, including pigmentation pattern, still remains to be elucidated.

1.5.2 Molecular pathways in caudal fin regeneration

1.5.2.1 WNT signalling, FGF signalling and RA signalling

Active WNT signalling appears to be a highly conserved requirement for blastema set-up across the animals able to regenerate appendages via epimorphic regeneration. Disruption of WNT signalling leads to abnormal wound epidermis and altered blastema formation. Pharmacological inhibition of WNT signalling leads to complete inhibition of caudal fin regeneration due to absence of proliferating cells that set up the blastema (Poss *et al.*, 2000a; Boehm and Slack, 2005; Stoick-Cooper *et al.*, 2007). Particularly, *wnt10a* and *fgf20a* are

strongly expressed immediately following the amputation and mark the initiation of the wound healing phase via canonical WNT signalling dependent epithelial-mesenchymal transition of the cells surrounding the stump (Stewart *et al.*, 2014). WNT signalling is fundamental for the maintenance of highly proliferative blastema Runx⁺ preosteoblasts. Interestingly, antagonistic roles within WNT signalling have been observed, such as the expression of *wnt5b* (non-canonical WNT signalling) in the wound epithelium which, if overexpressed, inhibits regeneration (Stoick-Cooper *et al.*, 2007).

FGF signalling is also involved in the maintenance of blastema-cell proliferation (Poss *et al.*, 2000b; Lee *et al.*, 2005). *fgfr1* is expressed in the distal blastema while Fgf-ligands are expressed in the cells of the AEC. And chemical inhibition of Fgfr1 by SU5402 blocks regeneration by inhibiting blastema cell proliferation and is associated with *msx1b* downregulation in the distal blastema. Moreover, *fgf20* zebrafish mutation (*dob*) was shown to have no impact on embryonic survival but showed complete inhibition of caudal fin regeneration (Whitehead *et al.*, 2005). *Fgf20* is detectable from 6 hpa at the boundary between the forming wound epithelia and the underlying mesenchyme. From 24 hpa, the expression domain of *fgf20* overlaps with the mesenchymal blastema marker *msx1b* which is regulated by BMP signalling. FGF signalling is therefore important at the transition from WNT signalling to BMP signalling which regulates osteoblasts differentiation and caudal fin outgrowth (Wehner and Weidinger, 2015).

Similar to WNT and FGF signalling, RA signalling is also an important regulator of the cellular proliferation in the regenerating caudal fin. RA pathway is activated within hours after fin amputation (within the stump mesenchyme) and it tightly collaborates with FGF, WNT and IGF signalling in proper formation of the wound epithelium and blastema compartments. In formed blastema, RA continues to support blastema cells proliferation, among else via reduction of inhibitory non-canonical WNT signalling (Blum and Begemann, 2012).

1.5.2.2 IGF signalling

Insuline-like growth factor (IGF) signalling, a known regulator of cellular growth during development (Edgar, 2006), appears to mediate the wound-epidermis – blastema crosstalk, fundamental for blastema establishment and epimorphic regeneration (Daponte, Tylzanowski and Forlino, 2021). *igf2b* was found to be expressed in the zebrafish caudal fin blastema while the signal responsive cells expressing its receptors *igflra/b* are located in the wound epidermis. Activation of the Igflr signalling in the wound epidermis leads to sustained cell survival and wound epidermis specification. Inhibition of the IGF signalling results in downregulation of the wound epithelial marker *wnt5b* as well as of the blastema markers *msx1b* and *fgf20a* and the proliferative blastema fails to be established, inhibiting the fin regeneration.

1.5.2.3 TGF- β signalling

TGF- β signalling, particularly its subfamily, BMP signalling pathway, is only recruited in the post-blastema phase to promote patterning and morphogenesis. Several BMP members and receptors are expressed in the fin osteoblasts during caudal fin regeneration (Smith *et al.*, 2006). *bmp2* expression was confirmed in the basal epithelial cells and in the adjacent blastema, and Bmp2 and Bmp6 can regulate chondrocyte proliferation and hypertrophy during regeneration (Lozito and Tuan, 2016).

BMP signalling also acts through interaction with other signalling pathways. For example, BMP signalling activates Dkk proteins (WNT signalling inhibitors) to counteract the growth induced by the WNT signalling pathway and promote osteoblasts differentiation (Stewart *et al.*, 2014). Moreover, overexpression of chordin in the regenerating caudal fin leads to a reduction in bone matrix deposition due to defects in the maturation and function of bone-secreting cells (Smith *et al.*, 2006). Chordin is an endogenous BMP signalling antagonist acting by scavenging extracellular BMP ligands, thus preventing them from binding to their cognate receptors and initiate signalling. Interestingly, although zebrafish caudal fin rays are of dermal origin (that is not formed through an endochondral ossification), important cartilage-linked genes *sox9a* and *sox9b* are detectable and upregulated during the caudal fin

regeneration. *sox9a* and *sox9b* are known to be involved in mesenchymal condensation and chondrocyte differentiation (Lin *et al.*, 2021).

1.5.2.4 Hedgehog signalling

shh gene is expressed in the cells of the basal layer of the epidermis, in the area surrounding newly forming lepidotrichia and appears to have number of overlaps with BMP signalling. *shh* as well as *bmp2* expression in epidermis is tightly regulated by cellular interactions between basal epidermal cells and blastema (Lynda Laforest *et al.*, 1998). Ectopic expression of either *shh* or *bmp2* in the blastema results in excess bone deposition between fin rays and mispatterning of the regenerated fin (Quint *et al.*, 2002). Quint and colleagues also showed that specific inhibition of *shh* by cyclopamine leads to reduced proliferation and re-differentiation of osteoblasts precursors within the blastema, however, it does not inhibit the bone matrix secretion by already differentiated scleroblasts. Shh signalling also has a role in bone patterning and ray branching morphogenesis (Zhang *et al.*, 2012; Armstrong *et al.*, 2017)

1.5.3 Expression of Fibins during caudal fin regeneration

Analysis of available RNA seq datasets revealed that *fibina* is downregulated at blastema formation stage (24 hpa) compared to baseline (Wang *et al.*, 2020), and upregulated from 2 dpa (Nauroy *et al.*, 2019). The latest timepoint investigated was 10 dpa, with *fibina* still upregulated compared to uncut fin (Kang *et al.*, 2016; Nauroy *et al.*, 2019). *fibinb* expression follows the same trend, with significant increase in *fibinb* expression during post-blastema setup and throughout regeneration (Kang *et al.*, 2016; Thompson *et al.*, 2020). Overall, *fibins* are expressed in zebrafish caudal fin during regeneration, particularly during the outgrowth of the appendage, post blastema formation.

1.5.4 Aims

The main aim of this thesis was to provide the first comprehensive investigation into the roles of Fibin(s) in tissue development and regeneration, working on the hypothesis that Fibin(s) modulated the extracellular matrix (ECM) during these complex processes. I have primarily used zebrafish embryos and zebrafish gill and caudal fin injury as models for development and regeneration respectively. I have also used human cell culture models to investigate the molecular regulation of *FIBIN* in a mammalian system.

Aim of Chapter 3: To characterise Fibin(s) using computational methods, to characterise *fibina/b^{-/-}* double mutant fish and to investigate the transcriptional differences between wild-type and *fibina/b^{-/-}* fish.

Aim of Chapter 4: To investigate the immune system in *fibina/b^{-/-}* mutant fish as well as the response to immune stimulus.

Aim of Chapter 5: To investigate the development of craniofacial cartilage and skeletal elements in *fibina/b^{-/-}* mutant fish and to investigate the caudal fin and gill regeneration in adult *fibina/b^{-/-}* fish.

Aim of Chapter 6: To present preliminary investigation into the regulation of human *FIBIN* using *in vitro* cell cultures.

The specific background and detailed aims are described in each chapter individually.

2 METHODS

2.1 *in vivo* work

2.1.1 Zebrafish husbandary

Wild-type and mutant zebrafish strains used in this study (summarized in table 2.1) were maintained in accordance with standard practice and all procedures conformed to the requirements of UK Home Office (ASPA 1986).

Zebrafish embryos were collected within 3 hpf and placed at density of 50 zebrafish/Petri dish in system water containing 3×10^{-5} % (w/v) methylene blue, an antifungal agent (Sigma, M9140). Zebrafish were placed in an incubator at 28.5°C. Prior to any experiments that use embryos before 3 dpf (that is when majority of embryos have hatched from the chorion), the embryos were manually dechorionated, in order for the treatment to effectively reach the embryo. Dechoriation was performed under a dissecting microscope using forceps. Apart from when working on larvae up to 10 dpf, embryos at 5 dpf were transferred to 3 l tanks with system water for up to 10 days, with water changed daily, and then placed on system.

Fish were kept at 28.5°C on a 14 hours light/10 hours dark cycle and fed twice a day with brine shrimp and dry food (ZM System, ZM000, ZM100, ZM200) and standard adult food (Hikari, Hikari Tropical Micro Pellets).

Name of line	Genes affected	Original/other name
wild-type	-----	WT, AB
Tra ^{-/-} /Nac ^{-/-}	<i>transparent</i> (microphthalmia-associated transcription factor a, <i>mitfa</i>) and <i>nacre</i>	mitfa ^{w2/w2} ;tra ^{b18/b18}
fibina/b ^{-/-}	fibina and fibinb	

Table 2.1 Overview of zebrafish lines used in this study.

2.1.2 General protocols used for all animals: embryos, larvae and adult fish

2.1.2.1 Imaging

For *in vivo* imaging of zebrafish larvae or adults, the fish were first anaesthetised in 4.2% (v/v) Tricaine, equivalent to 0.17 mg/ml of Tricaine (MS-222; Sigma, E10521) and oriented in the intended position in either PBS or 0.5%-1% (w/v) low melt agarose (Lonza, NuSieve™ GTG™ Agarose, 50080). Zebrafish were then imaged either using a fluorescent stereomicroscope Leica MZ10F or using a widefield fluorescent, Zeiss Axio Observer inverted microscope. Widefield fluorescence microscopy enables to record a series of Z-plane images, providing a within-sample spatial information at high speed. Images from widefield microscope were then deconvoluted using Hyugens software with the help of FILM facility at Imperial College London. All subsequent image analysis was performed using Fiji software.

2.1.2.2 Culling

All fish were culled in 400 mg/l Tricaine (MS-222; Sigma). Fish were observed for operculum motion (suggestive of breathing). In adult fish this was followed by severing the notochord with a scalpel or full decapitation.

2.1.2.3 RNA extraction

Tissues were collected and processed using Trizol-chloroform extraction method. 200 μ l of Trizol was added to each sample and rotor and pestle were used to mechanically break the tissue apart. After 5 min, 40 μ l of chloroform was added and the sample shaken vigorously for 15 s. Samples were left for 3 min at RT and then spinned down at 12,000 x g for 15 min at 4°C. The translucent upper phase containing the RNA was collected and transferred into a new Eppendorf. RNA mini or RNA micro kit (PureLink™ RNA Mini or Micro Kit (Life Technologies)) were used for RNA extraction as per manufacturer's instructions. RNA was eluted in RNase-free water and the RNA quality and concentration determined using Thermo Scientific Nanodrop™1000. RNA was either stored at -80°C for future use or used directly for downstream processing.

2.1.2.4 cDNA synthesis

cDNA was synthesized High-Capacity cDNA reverse transcription kit (Applied Biosystems) as per manufacturer's instructions. In general, 125 ng of RNA was transcribed into cDNA (20 μ l total volume) which was subsequently diluted with 80 μ l of RNase free water to obtain concentration optimal for qPCR analysis.

2.1.2.5 qPCR

1 μ l of diluted cDNA was used for qRT-PCR using Taq fast universal 2x PCR Master Mix (Applied Biosystems) and TaqMan probes (Applied Biosystems, Table 2.2). All reactions were performed either in triplicate or duplicate using a 7500 Fast Real-time PCR system (Applied Biosystems). Cycle thresholds obtained were normalised to 18S. For relative quantification, samples were calibrated to one control sample and using the standard fold change method expressed as $2^{(-\Delta\Delta Ct)}$ relative to a control sample. Alternatively, the ΔCt method was used in for R848 challenge experiments in Chapter 4 by calculating the $2^{(-\Delta Ct)}$ between each sample and its 18S value.

Gene name	Organism	TaqMan assay identification code
18S rRNA	Zebrafish	4319413E
<i>il1b</i>	Zebrafish	Dr03114368_m1
<i>tnfa</i>	Zebrafish	Dr03126850_m1
<i>ifng1-2</i>	Zebrafish	Dr0381923_m1
<i>cxcl18b</i>	Zebrafish	Dr03436643_m1
<i>mmp9</i>	Zebrafish	Dr03139882_m1
<i>lck</i>	Zebrafish	Dr03446596_s1
<i>mxr</i>	Zebrafish	Dr03444499_s1
<i>fibina</i>	Zebrafish	Custom
<i>fibinb</i>	Zebrafish	Custom
<i>ctgf</i>	Zebrafish	Dr03135188_g1
<i>mmp2</i>	Zebrafish	Dr03106215_m1
<i>colla1a</i>	Zebrafish	Dr03150834_m1
<i>colla2</i>	Zebrafish	Dr03074431_m1
<i>coll10a1a</i>	Zebrafish	Dr03143511_m1
<i>sall4</i>	Zebrafish	Dr03143294_m1
<i>msx1b</i>	Zebrafish	Dr03074059_m1
<i>grem2b</i>	Zebrafish	Dr03423829_m1
<i>pcna</i>	Zebrafish	Dr03093294_m1
<i>spp1</i>	Zebrafish	Dr03108264_m1
<i>bmp2b</i>	Zebrafish	Dr03074090_m1
<i>FIBIN</i>	Human	Hs00831591_s1
<i>COL1A1</i>	Human	Hs00164004_m1
<i>ACTA2</i>	Human	Hs00426835_g1
<i>AXIN2</i>	Human	Hs00610344_m1
<i>GAPDH</i>	Human	Hs02786624_g1
<i>HPRT1</i>	Human	Hs02800695_m1

Table 2.2 List of qPCR probes used in this thesis.

2.1.3 Staining of zebrafish embryos and larvae

2.1.3.1 Fixation of zebrafish embryos and larvae

Zebrafish embryos and larvae were euthanised as described above and fixed in 4% (w/v) paraformaldehyde (PFA) over night at 4°C (Polysciences Inc.). Next day, fish were washed twice in phosphate buffered saline (PBS) and stored no longer than 1 month at 4°C in PBS before further processing. Fixations were performed in 1.5ml microcentrifuge tubes.

2.1.3.2 Bone and cartilage staining of embryos

Alcian blue stain: Fixed embryos were placed into 3% (v/v) hydrogen peroxide solution (H₂O₂, P4417, Sigma) for 30-60 min to remove pigmentation. Embryos were then washed in PBS and stained in Alcian blue solution consisting of 0.1% (w/v) Alcian blue 8GX (Sigma), 70% (v/v) ethanol (Fisher Scientific) and 1% (v/v) HCl (Sigma) for 1 h at room temperature in dark (under aluminium foil). Embryos were then washed with de-staining solution (70% ethanol/5% HCl solution (v/v)) for 4.5 h before another wash with PBS.

Alizarin Red S stain: Fixed embryos were immersed for 30 min in bone staining solution containing 20% (v/v) Ethylene glycol (EMD Millipore corp.) and 1% (w/v) potassium hydroxide (KOH, Sigma) followed by addition of Alizarin Red S stain at concentration of 0.05% (v/v); fish were incubated for 30 min at room temperature. Embryos were washed 3x in PBS and kept in PBS till further processing.

2.1.3.3 Fin cuts

3 dpf embryos were anaesthetised using MS-222 and placed laterally on a 1 – 2% (w/v) low melt agar. Under a dissecting microscope and using a clean blade, the caudal fin fold was amputated just posteriorly to the end of notochord. Embryos were then placed individually into 6-well plates and closely monitored.

2.1.4 Adult zebrafish work

2.1.4.1 Immune system stimulation with R848

Fish were anesthetized using 4.2% (v/v) Tricaine solution. Depth of anaesthesia was determined by gently touching the caudal fin with forceps and observing any reaction as well as by observing the gill opercular movements. After ~2 min fish were taken out of the water, placed side-ways on a petri dish with their right-side gill facing up. Using forceps, the operculum was lifted and 5 μ l of the 0.5 mg/ml resiquimod (R848, Sigma) solution or endotoxin-free water was applied to the gill tissue using 10 μ l pipette. Fish was left and closely observed for 5 min at room temperature. After 5 min of stimulation, fish were placed back into system water and fish closely monitored till tissue harvest.

2.1.4.2 Harvesting zebrafish tissues for flow cytometry

Fish were culled in 400 mg/l Tricaine (MS-222) followed by severing the notochord with a scalpel (decapitation). Operculum was removed with forceps and the gill arches were detached and collected. Any associated tissues and cells (blood or fat) were removed with forceps. Whole kidney marrow (WKM) was dissected out in the following way: the head was removed and the fish was turned with the ventral side (belly) facing up. Using scissors the body cavity was cut opened along the ventral side and all internal organs were removed, leaving only the head, trunk and tail kidney tissue. The WKM was then harvested from the dorsal side of the body cavity using forceps.

2.1.4.3 Flow cytometry

Gill tissue was first digested with collagenase (2 mg/ml) for 10 min at 37°C. The tissue was pipetted up and down several times during the 10 min to aid in the breakdown of the tissue. Digested tissue was mixed up with FCS solution (2% (v/v) in PBS). The cell suspension was then centrifuged, supernatant removed, the pellet resuspended in FCS solution (2% (v/v) in PBS), and the suspension filtered through a 40 μ m cell strainer.

WKM was dissociated into a single cell suspension by passing the harvested tissue immediately after dissection through a 40 μ m cell strainer with 2% (v/v) fetal calf serum (FCS) in phosphate buffered saline (PBS).

Immediately prior to processing, 4',6-diamidino-2-phenylindole (DAPI) was added to the samples at final concentration of 1 μ g/ml. Processing was done on 4 or 5-laser BD LSRFortessa analyzers in the Department of Life Sciences Flow Cytometry Facility (Imperial College London), using BDFACSDiva software (BD Biosciences) for data acquisition. All data were analysed with FlowJo™ software (Becton, Dickinson & Company).

2.1.4.4 Caudal fin amputation

Fish were anesthetized using 4.2% (v/v) Tricaine solution and placed laterally on a clean Petri dish. Using a clean blade, and under a dissecting microscope, a straight-line cut was made at the desired position of the zebrafish caudal fin. Fish were immediately placed back into system water and observed during recovery. Dissected fin tissue was placed in 4% (w/v) paraformaldehyde (PFA) over night at 4°C (Polysciences Inc.). Next day, fish were washed twice in phosphate buffered saline (PBS). Bone staining was performed using Alizarin Red S stain diluted at 5 μ l/ml in bone staining medium (20% (v/v) ethylene glycol, 1% (w/v) KOH). First, the fins were blocked for 30 min at room temperature in the bone staining medium, then stained with Alizarin Red S stain for 15 min at room temperature and subsequently destained in clearing solution (20% (v/v) Tween-20, 1% (w/v) KOH) for up to 3 h at room temperature. The fins were then stored in PBS at 4°C until they were imaged.

2.1.4.4.1 DMH1 treatment

For experiments using DMH1 treatment, the adult fish were kept in a 28.5°C incubator with maximum of 6 fish per 3 l tank. DMH1 at the desired concentration or DMSO (matching the volume of DMH1 added in the treatment group) was dissolved in the system water. Fish were kept in the incubator for the duration of the experiment, closely monitored, fed twice a day, with water change every day.

2.1.4.4.2 BrdU staining

Zebrafish that were used for *in vivo* BrdU labelling were subjected to caudal fin amputation and left recovered for up to 3 days. 7-8 h prior to regenerate collection, BrdU powder was

dissolved in fresh system water to final concentration of 50 mg/l and the fish were transferred to this solution and placed in an incubator. To quantify proliferating cells, amputated fins were fixed again in 4% (w/v) PFA over night as described above and washed in PBS. Purified mouse anti-BrdU primary antibody (BD Pharmingen) at concentration of 5 μ l/ml. Goat anti-mouse secondary Alexa-Fluor 488 (ThermoFisher) antibody was used for detection. Caudal fin regeneration was calculated in comparison to uncut caudal fin area.

2.1.4.5 Gill cryoinjury

Gill cryoinjury was performed as previously described (Ramel *et al.*, 2021). Briefly, adult fish were anaesthetized using 4.2-5% (v/v) MS-222 and placed with their right side facing up on a petri dish. The operculum covering the right set of gill arches was removed and the gills dried using a tissue. Metal probe was placed in liquid nitrogen and then placed directly onto the gills for 10 s. Fish were immediately transferred into a tank with fresh system water and observed for swimming pattern and operculum movement till recovered. Fish were also observed daily for the duration of the experiment. In line with ensuring as little stress for the animal as possible, lidocaine was added to the original tank with fish to be cryoinjured (at concentration of 2.12 mg/l) as well as to the recovery tank (at concentration of 2.65 mg/l). When collecting gill tissue for RNA processing, only the two outer-most arches were taken and only the cryoinjured part of the gill was cut out, using scissors and/or scalpel, for processing. The same part of the gill was then collected from the un-injured, contralateral gill tissue arches (also only the two outer most arches were used for further processing).

2.1.5 RNA-seq analysis

RNA samples for RNA-seq were sent to BI. Here the library preparation for sequencing was performed using 250 ng of total RNA input with (Illumina Inc) producing a 275 bp fragment including adapters in average size. The 24 samples sent for sequencing were sequenced on the Illumina NovaSeq platform. Raw sequencing data was processed by Praveen Baskran at BI and send to us for further analysis. The cut-off for statistically differentially expressed genes was set to 0.05 for the false discovery rate (FDR). Panther classification system (<http://pantherdb.org>) was used for functional enrichment analysis. Venn diagrams used for visualization of differentially expressed genes (DEGs) were generated using BioVenn online tool (<https://www.biovenn.nl>).

2.2 *in vitro* work

A549 cells are human lung epithelial cell line derived from a lung sample of a 58-year old male with adenocarcinoma of the lung and were obtained from ATCC (CCL-185). Cells were maintained in DMEM supplemented with 10% (v/v) FBS (Gibco). IMR-90 human pulmonary fibroblast cell line is derived a 16-week old human lung and was a gift from Andrew Innes (Imperial College London). Primary lung-derived human fibroblasts, both healthy and diseased (IPF), were provided by BI. All primary fibroblasts were studied between passages 5 and 9. One IPF and one healthy primary small airway epithelial cell (SAECs) lines were supplied by BI. Cells were only cultured in submersion in airway epithelial cell-specific media (Lonza). All cells were cultured in 37°C and 5% CO₂.

2.2.1 Monoculture experiments

Primary lung-derived fibroblasts were cultured in fibroblast-specific growth media without the addition of gentamycin (Lonza). Prior to any experiment, fibroblasts were plated in 12- or 24-well plates (no coating) till ~80% confluency was reached. Cells were starved overnight or at least for 8h in serum-free media (DMEM, Gibco). After starvation, the media was aspirated out and replaced with fresh serum-free media and treatment was added. After given timepoint the cells were harvested for downstream processing, ie, protein or RNA extraction. Treatments: TGF- β 1 (R&D systems), WNT3A (R&D systems), WNT5A (R&D Systems), CT (GSK-3 α/β inhibitor, Sigma) FH (WNT/ β -catenin signaling inhibitor, Tocris), PKF (Calphostin C – selective inhibitor of protein kinase C, Tocris).

2.2.2 Co-culture experiments

Co-culture experiments were done in 24-well plate trans-wells (Corning). Firstly, A549 cells were seeded in the upper chamber at density 30,000 cells per well in 100 μ l of supplemented media (DMEM + 10 FBS). 500 μ l of the same medium was added to the bottom compartment and cells were cultured till ~80% confluent. At the same time, in separate plate, primary fibroblasts were seeded at density of 25,000 cells per well in 500 μ l of fibroblast specific growth media without the addition of gentamycin (Lonza). After 24 h-48 h when 80% confluency was reached by both A549 cells and fibroblasts, all media was aspirated, cells washed with PBS, and starvation media (DMEM) was added at the specified volumes and left for 24 h. Prior to experiments, the starvation media was replaced by fresh one, the trans-well

inserts with A549 cells were transferred on top of the fibroblasts, and treatment was added at desired concentration. After given timepoint, media was aspirated (or collected for further analysis), cells were washed with PBS and collected for protein and RNA analysis.

2.2.3 Scratch assay

Scratch assay was used as a simple form of physical injury to the cultured cell layer. The scratch was inflicted using a 100 μ l sterilized pipette tip and gently pressing it, and pulling, across the entire well, passing as close to the well center as possible. Cells were then washed with PBS and new serum-free media was added (DMEM, Gibco).

2.2.4 siRNA transfections

For RNA interference, siRNA targeted against human FIBIN were used to knock down this gene; obtained from Ambion (ID: s51940, s51941, s51939). Three distinct silencer sequences were used to test which results in the best knockdown of FIBIN in cells (measured by fold-change decrease in RNA levels). Silencer™ Negative Control No. 1 siRNA was used as negative control for silencing experiments.

Cells were plated in 12-well plates at density of 50,000 cells per well for RNA extraction or in 6-well plates at density of 75,000 cell/well for protein extraction and transfected using Lipofectamine 3000 (Invitrogen), following the manufacturer's protocol for RNAi, unless otherwise stated.

2.2.5 RNA extraction, cDNA synthesis and qPCR

Total RNA was isolated from cells using RNeasy 96 Kit (Qiagen) or using Trizol-chloroform extraction method as described above. cDNA was generated using High-capacity cDNA Reverse Transcription Kit (ThermoFisher,). Gene transcript levels was quantified by TaqMan assay real-time PCR (ThermoFisher). TagMan probes used are listed in Table 2.2. Quantification of the fold-change ($2^{-(\Delta\Delta Ct)}$) was calculated for each biological replicate. Average Ct value was derived from technical replicates, ΔCt value calculated by subtraction of average Ct value of the target gene and average Ct value of housekeeping gene.

2.3 Computational analysis of Fibrin(s) proteins

ColabFold script, an open source software that combines fast homology search of MMSeqs2 with AlphaFold2, was used for 3D structural predictions of zebrafish FibrinA and FibrinB, mouse FIBIN and human FIBIN proteins (<https://github.com/sokrypton/ColabFold>, (Mirdita *et al.*, 2022)). SignalP 5.0 was used for prediction of signal peptides and cleavage sites (<https://services.healthtech.dtu.dk/services/SignalP-5.0/>, (Almagro Armenteros *et al.*, 2019a)). Next DeepLock was used for predictions of cellular localization of the individual proteins (<https://services.healthtech.dtu.dk/services/DeepLoc-1.0/>, (Almagro Armenteros *et al.*, 2017)). TFBS were predicted and analysed using ConSite software (<http://consite.genereg.net>, (Sandelin, Wasserman and Lenhard, 2004)).

2.4 Statistical analysis

All statistical analysis (apart from statistics for RNA-seq, which was done as described) was carried out with GraphPad Prism 9 software (GraphPad software, CA, USA). Paired or unpaired two-tailed t-tests or Mann-Whitney test were performed for parametric and non-parametric comparison of two groups, respectively. Comparisons of three or more groups was done using one-way or two-way ANOVA followed by Sidak's or Tukey's multiple comparisons test for pairwise comparison. Welch's ANOVA was used for datasets with unequal variances. Normality of data was assessed by plotting a normal QQ plot.

3 CHARACTERISATION OF FIBIN(S) AND *fibina/b*^{-/-} ZEBRAFISH

3.1 Introduction

3.1.1 Methods to modulate Fibrin(s) mRNA transcription and translation

Characterisation of the function of selected genes can be approached from multiple directions. Firstly, the Dallman laboratory has designed morpholinos to target *fibina* and *fibinb*. Several issues were encountered using this technique: The injected morpholinos at 1-cell stage are only stable for up to 6 days. Therefore, this may be a suitable method for characterisation of the role of Fibrin(s) during the early development, however, it is not useful for investigating the role of Fibrin(s) in adult tissue regeneration. It may be possible to apply the morpholinos directly to the gill tissue/caudal fin tissue prior to or following the cryoinjury or amputation respectively, however, preliminary experiments showed great variations in the gene(s) knockdown thus not providing a consistent, repeatable platform to be used. As outlined in the Introduction, our laboratory's repeats of Wakahara's experimental design, using targeted morpholinos to knockdown *fibins* during development, did not result in any

clear morphological differences or developmental delays in the affected embryos (qPCR was used to confirm the morpholino-mediated *fibina* and *fibinb* knockdown).

The second commonly used approach is to generate mutant fish, using CRISPR-Cas9, for the gene(s) of interest. Today, the choice is often to generate mutants which completely lack the coding region for the targeted genes. However, previously, CRISPR-Cas mutagenesis was used to introduce small deletions or insertions in the gene(s) of interest, several of the first-generation fish were then screened and sequenced to identify premature stop codons (discussed further below). This method was used by the Dallman laboratory as well to generate double mutant fish for both *fibina* and *fibinb* using CRISPR-Cas9 mutagenesis. The efficiency for all injected crRNAs was over 70% (expected), and the F0 generation (injected embryos) were grown to adulthood and their mosaicism was confirmed. The F0 generation was then crossed with wild-type zebrafish to generate the F1 generation which was genotyped and sequenced to identify the founder heterozygous fish for *fibina* and *fibinb* alleles. Founders were consequently outcrossed to wild-type fish once more to generate F2 generation. Heterozygous F2s were then incrossed to obtain *fibina* or *fibinb* homozygous mutants. The double mutant fish used for experiments in this thesis were *fibina*109d120^{-/-}; *fibinb*226d232^{-/-} (from now on referred to as *fibina/b*^{-/-}). Description of all mutations generated during the establishment of the double-mutant model can be found in Appendix 2. The mutant fish are fertile with no obvious delay in development. No phenotypical differences (such as lack of pectoral fins) were observed between the *fibina/b*^{-/-} fish and wild-type counterparts.

3.1.2 Validation of successful mutagenesis

CRISPR-Cas9 induced deletions and/or insertions (alterations) to the *fibin* genes was continuously confirmed by genome sequencing. Fig. 3.1 shows examples of the sequencing results from wild-type and *fibina/b*^{-/-} genotyping: *fibina* aligned with *fibina*109d120 deletions and *fibinb* aligned with *fibinb*223d232 deletion. Both of these mutations were predicted to result in premature stop codon in their respective transcripts. Appendix 3 shows aligned sequences of human and mouse *FIBIN*, zebrafish *fibina* and *fibinb*, and zebrafish *fibins* aligned with human *FIBIN* for reference. qPCR experiments were carried out to confirm that

non-sense mediated decay of *fibina/b* RNA took place in the *fibina/b*^{-/-} mutant fish. Nonsense-mediated mRNA decay (NMD) is a cellular mechanism that helps to prevent the production of abnormal or non-functional proteins by degrading mRNAs that contain premature stop codons. Premature stop codons can arise due to errors in transcription or splicing, or as a result of mutations in the DNA, like in our case. NMD is an important quality control mechanism that ensures the production of functional proteins and prevents the accumulation of potentially harmful truncated proteins. Therefore, measuring NMD activity – for example using qPCR to quantify the transcript levels between the wild-type and the mutant *fibin* genes – can be a useful indicator of the presence/absence of genetic mutations (Lykke-Andersen and Jensen, 2015). The Taqman probes for the detection of *fibina* and *fibinb* transcripts were designed such that the detection region lies outside of the deletion region, allowing us to continuously monitor NMD in all our experimental animals. This also means that although we refer to our *fibina/b*^{-/-} fish as mutant zebrafish, the transcript levels of *fibina* and *fibinb* are still detectable.

In this chapter I focus on characterisation of Fibin proteins using bioinformatics, including Fibin proteins' structures prediction and localisation as well as predicted binding sites to inform regarding possible Fibin(s) function.

3.2 Aims

- 1) To characterize *fibina* and *fibinb* protein structure, compare with human and mouse FIBIN protein structure, analyse for transcription factor binding sites (TFBS). And to compare the wild-type Fibins with mutant Fibins theoretical protein structure.
- 2) To characterise the *fibina/b*^{-/-} zebrafish response to caudal fin injury using embryo (3 dpf) and adult (4 months old) zebrafish.
- 3) To compare the transcriptome of the gill and the caudal fin between wild-type and *fibina/b*^{-/-} fish and to identify differentially expressed (DE) genes, molecular pathways and biological processes (using statistical enrichment analysis).

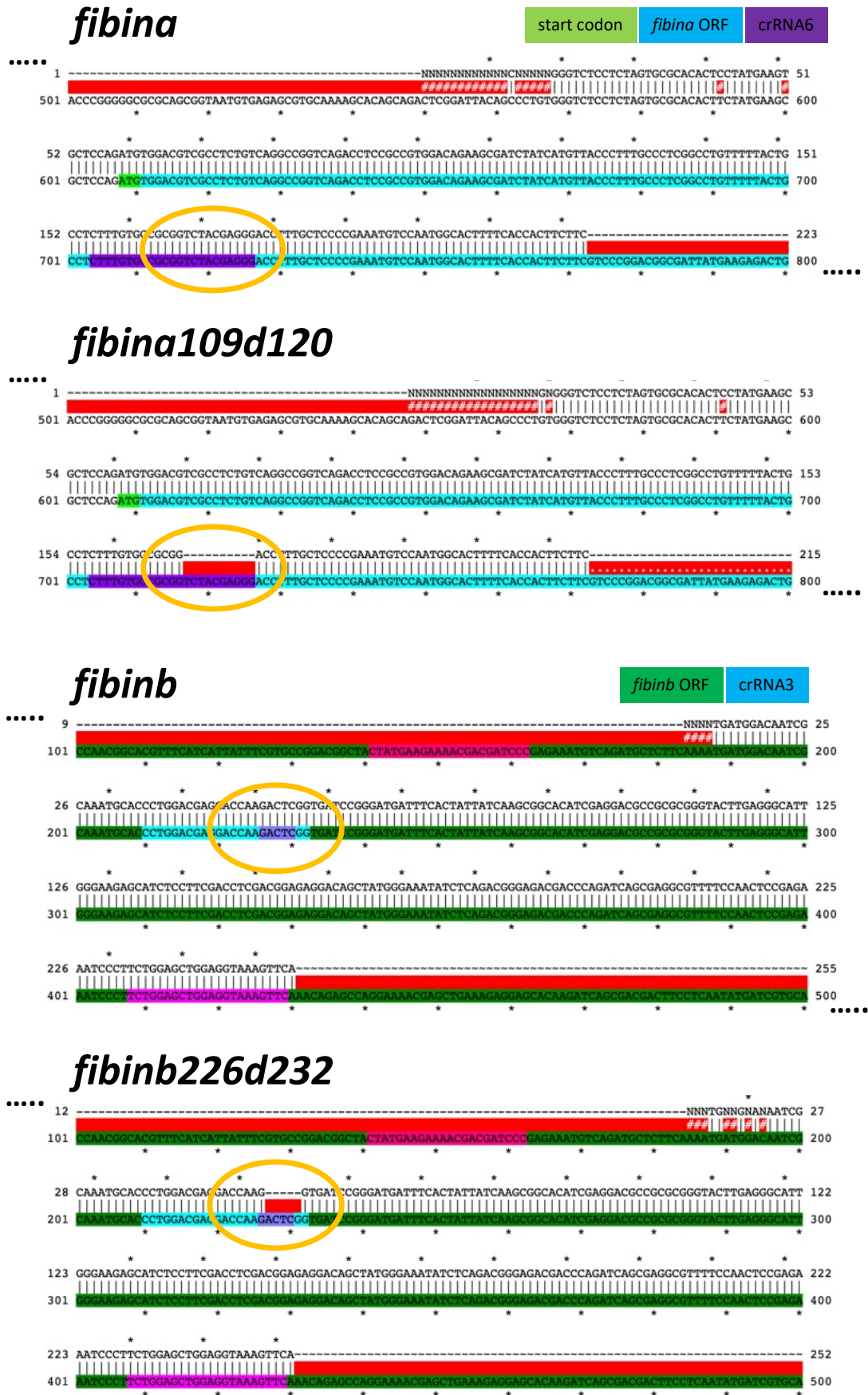


Figure 3.1 Example of genotyping results, confirming deletions at 109d120 for *fibina* and 226d232 for *fibinb* (in red).

3.3 Results

3.3.1 Computation analysis of Fibrin(s)

3.3.1.1 Fibrin protein structure prediction

Understanding protein structures and the implications of base mutations on protein structure is a fundamental aspect of protein biology. Fibrins are largely understudied proteins across the vertebrate species and have no known crystalized structure available. A protein-coding gene refers to a gene that is predicted to produce a functional protein product based on its nucleotide sequence. This typically means that the gene contains an open reading frame (ORF) which is a sequence of nucleotides starting with an initiation codon (usually AUG) and ending with a stop codon (UAA, UAG, or UGA) (Clamp *et al.*, 2007).

Prior to the year 2021, softwares used to predict protein structures (such as PsiPred) relied on a process called homology modelling. Homology modelling involves comparison of the sequence of amino acids in a protein of interest to the sequences of amino acids in known proteins with similar structures. The software then uses this comparison to generate a 3D model of the protein structure. This method has clear limitations because it requires a known protein with a similar structure to be available in order to generate an accurate prediction. Moreover, even if a similar protein is available, there may be differences in the amino acid sequence or other factors that make it difficult to accurately predict the structure.

AlphaFold, although first introduced already in 2018, became widely used in 2021. AlphaFold is an artificial intelligence (AI) system that predicts the 3D structure of proteins using deep neural networks and genetic and biochemical data to accurately predict the spatial arrangement of amino acids in a protein, without relying on known structures. I used AlphaFold to generate predicted 3D structures of human, mouse and zebrafish Fibrin(s) and to compare the predicted structures of wild-type *fibina* and *fibinb* with the mutated *fibins*. Fig. 3.2 shows the predicted structures of *fibina* and *fibinb* proteins and their mutated version.

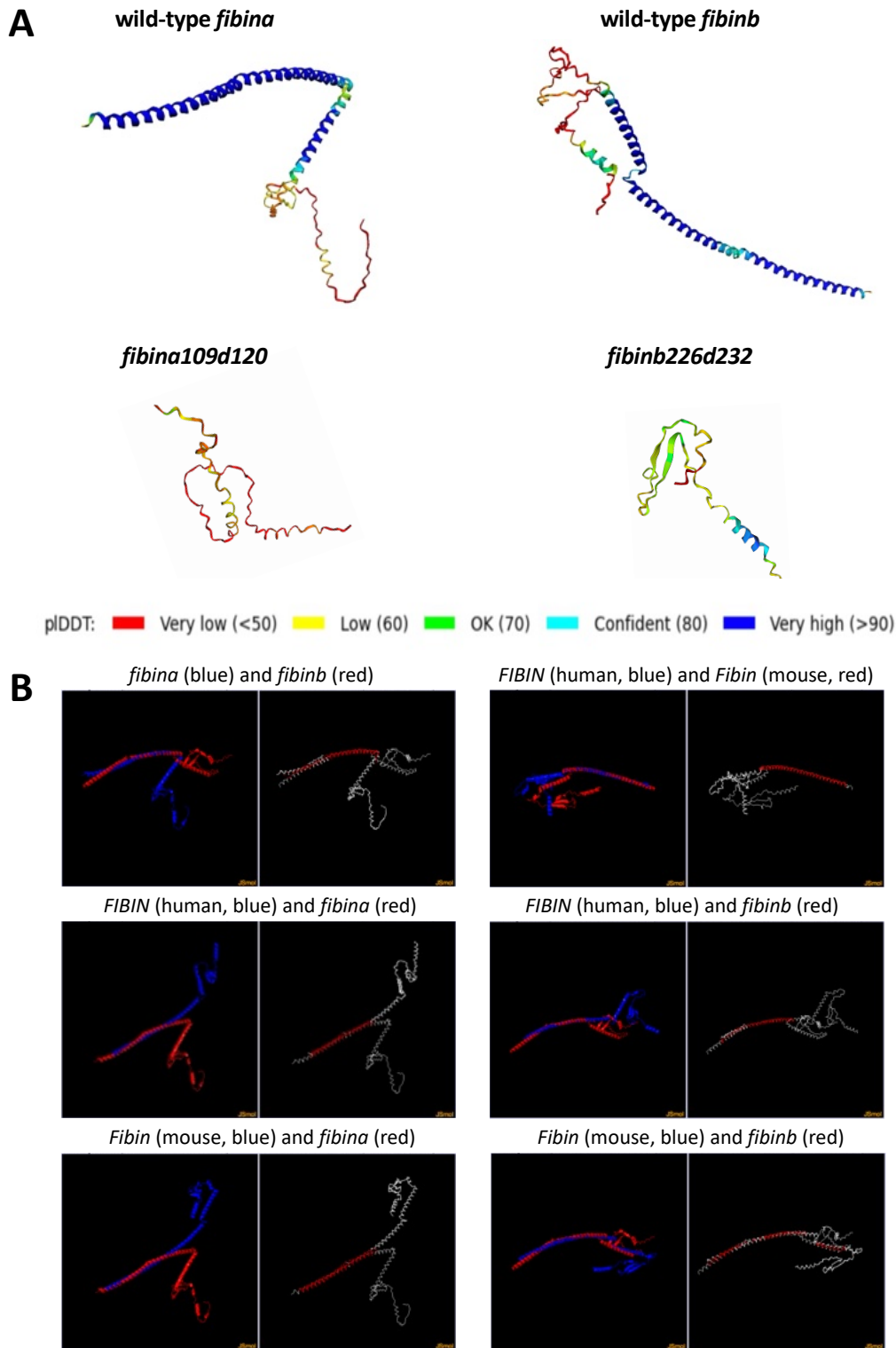


Figure 3.2 Computational prediction and structure comparison of Fbin(s) proteins

(A) 3D structure prediction of zebrafish *fibina* and *fibinb* proteins generated using AlphaFold, together with the theoretical truncated proteins generated by deletion of nucleotides 109d120 in *fibina* and 226d232 in *fibinb*. (B) Structural alignment of the 3D structures generated by AlphaFold to visualize the portions of the structures that are conserved among different species and/or paralogues. Blue and red image shows the two structures compared, while the grey and red image shows the parts of the structure in common (red).

The structural alignment of Fabin(s) showed highest structure similarity between human FIBIN and mouse FIBIN (Template Modelling (TM)-score = 0.48; with TM-score > 0.5 the proteins are considered statistically similar). Zebrafish Fibina and Fabinb showed TM score of 0.3 (Zhang and Skolnick, 2004; Xu and Zhang, 2010).

All TM scores are summarized in Table 3.1. Based on the AlphaFold 3D structure predictions and statistical assessment of structural similarity based on these 3D structures, mouse and human FIBIN were most similar, with Fabinb structure similar to human FIBIN as well as Fibina to the same degree. Fibina showed the least similarity to human FIBIN. Interestingly, Fibina and Fabinb both are predicted to have a disorganized region at their N-terminus, which is not the case for mouse or human FIBIN. Understanding of the protein structure can reveal how various regions of the protein interact with each other and with other molecules. Protein structure can thus be useful for identification of critical amino acids and regions responsible for proteins (predicted) activity, binding sites and interactions with other proteins or ligands. This analysis also confirmed that Fabin(s) do not show similarity to any other known protein.

	FIBIN (human)	FIBIN (mouse)	Fibina	Fabinb
FIBIN (human)		0.48	0.26	0.28
FIBIN (mouse)	0.48		0.28	0.35
Fibina	0.26	0.28		0.30
Fabinb	0.28	0.35	0.30	

Table 3.1 TM-scores of Fabin(s) structural comparison.

3.3.1.2 Fabin(s) localisation

Next, I used SignalP 5.0 to predict the presence of signal peptide and the position of cleavage sites (Almagro Armenteros *et al.*, 2019b). Some proteins undergo proteolytic cleavage at their N-terminus as part of their maturation process or to generate biologically active fragments. Presence of a cleavage site strongly suggests that a protein does undergo proteolytic processing. Moreover, disorganised region at the N-terminus, such as for

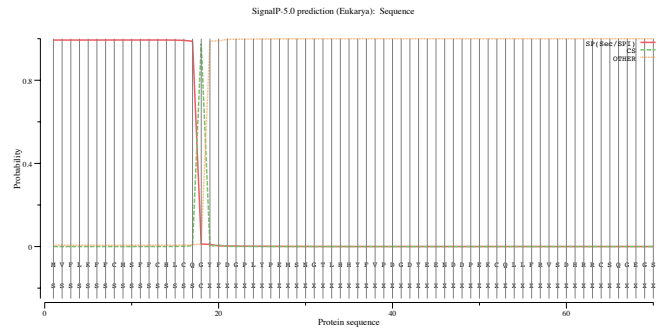
zebrafish *Fibina* and *Fibinb*, suggests that the region is susceptible to proteolytic processing, leading to the generation of functional protein fragments. Signal sequence refers to a specific sequence at the N-terminus of a protein that directs its localization to specific cellular compartments or organelles, which allows for prediction between proteins that remain intracellularly and those that are marked for extracellular localization (those that have the signal sequence). It is important to note that a disorganized region at the N-terminus could potentially impact the recognition and processing of such signal sequences, even leading to mislocalization or altered targeting of the protein. Fig. 3.3 shows the results of cleavage sites prediction (Fig. 3.3(A)) and the predicted localization of the 4 proteins (Fig. 3.3(B)).

3.3.1.3 Analysis of Fibin(s) transcription factor binding sites (TFBS)

Together with a BSc student Qiulin Huang we investigated potential TF factor binding sites on Fibin(s) from all 3 species to determine if there is a possibility of differential regulation of Fibin(s) in different species. Based on (Lakner et al, 2011), the promotor region for mouse *Fibin* lay within a 2.5 kilo-base-pairs (kbp) sequence region upstream of the translation start site (TSS). The 2.5 kbp sequence was identified in all Fibin(s) investigated and used for TFBS analysis. We used the combination of high TF specificity threshold (10 bits) and high TF binding score cut-off to find potential TFBS. Fig. 3.4 shows Venn diagrams for TFBS enrichment analysis. When we applied a high TF binding score cut off (95%), we found that only 2 TFBS were found in all Fibin(s) sequences, HFH-3 and Sox-5. Snail and HNF-3b were found to be specific to zebrafish *fibins*. Interestingly, Snail was also the only transcriptional repressor discovered during our analysis; it is known to be involved in epithelial-mesenchymal transition (EMT) in lung fibrosis by repressing the expression of E-cadherin (Wang *et al.*, 2013). Both, HFH-3, also known as FOXI1 as well as SOX-5 are involved in embryogenesis (Wright, Snopek and Koopman, 1993; Overdier *et al.*, 1997). Experimental evidence, such as Chromatin immunoprecipitation sequencing (ChIP-seq), is necessary to follow up from these theoretical predictions. Still, as Qiulin Huang has furthermore showed that even with low TF specificity threshold and low TF binding score combination, the majority of the identified TFBS were shared across the 3 species. This enrichment analysis therefore suggests that Fibin(s) are likely to show conserved regulation across species (Qiulin Huang, 2020).

A

Human FIBIN

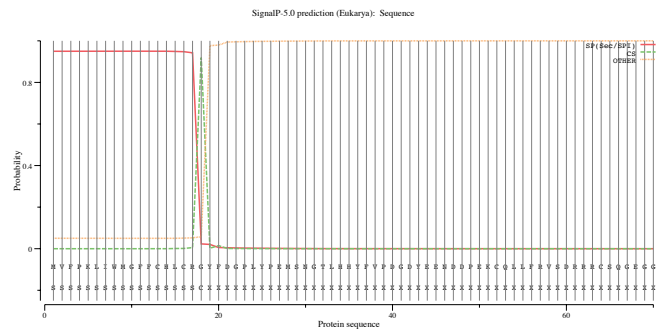


Prediction: Signal peptide (Sec/SPI)

Cleavage site between pos. 18 and 19: CQG-YF. Probability: 0.9758

Protein type	Signal Peptide (Sec/SPI)	Other
Likelihood	0.9932	0.0068

Mouse FIBIN

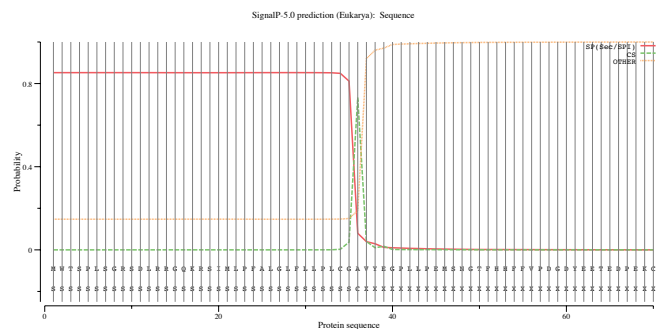


Prediction: Signal peptide (Sec/SPI)

Cleavage site between pos. 18 and 19: CRG-YF. Probability: 0.9193

Protein type	Signal Peptide (Sec/SPI)	Other
Likelihood	0.9491	0.0509

Zebrafish Fibina

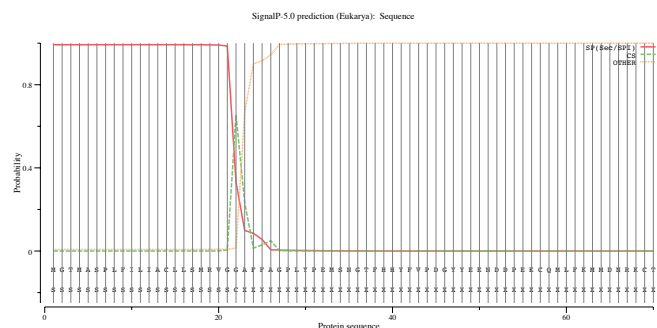


Prediction: Signal peptide (Sec/SPI)

Cleavage site between pos. 36 and 37: CGA-VY. Probability: 0.7319

Protein type	Signal Peptide (Sec/SPI)	Other
Likelihood	0.8535	0.1465

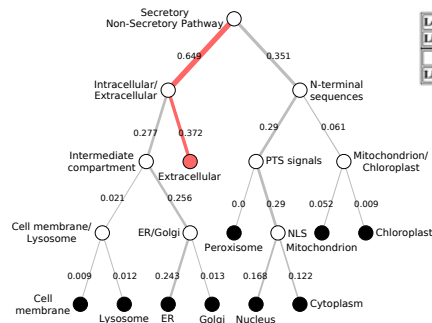
Zebrafish Fbinb



Prediction: Signal peptide (Sec/SPI)

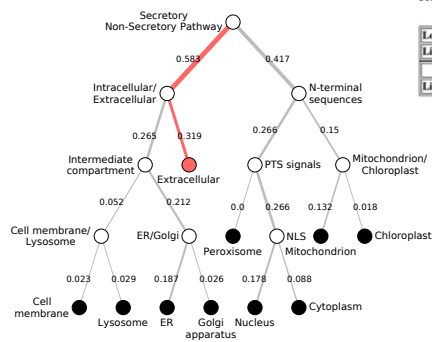
Cleavage site between pos. 22 and 23: VGG-AF. Probability: 0.6547

Protein type	Signal Peptide (Sec/SPI)	Other
Likelihood	0.9923	0.0077

B**Human FIBIN**

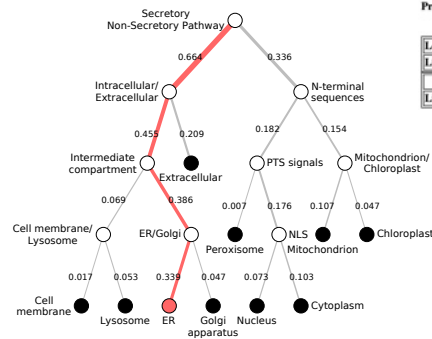
Prediction: Endoplasmic reticulum, Soluble

Localization	Endoplasmic reticulum	Extracellular	Nucleus	Cell membrane	Cytoplasm	Mitochondrion	Golgi apparatus	Plastid	Lysosome/Vacuole	Peroxisome
Likelihood	0.4106	0.2673	0.0921	0.0734	0.0562	0.0518	0.0254	0.0117	0.0113	0.0002
Type	Soluble	Membrane								
Likelihood	0.7808	0.2192								

Mouse FIBIN

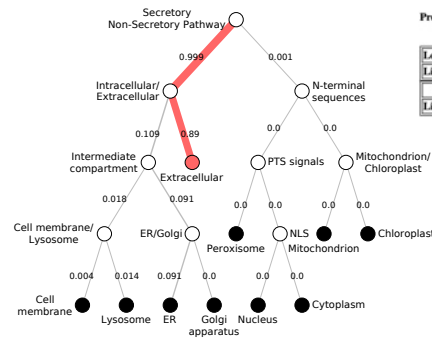
Prediction: Endoplasmic reticulum, Soluble

Localization	Endoplasmic reticulum	Extracellular	Mitochondrion	Nucleus	Cell membrane	Cytoplasm	Golgi apparatus	Lysosome/Vacuole	Plastid	Peroxisome
Likelihood	0.3111	0.1721	0.1614	0.1432	0.0844	0.0428	0.0309	0.0277	0.026	0.0004
Type	Soluble	Membrane								
Likelihood	0.7548	0.2452								

Fibina

Prediction: Extracellular, Soluble

Localization	Extracellular	Endoplasmic reticulum	Cytoplasm	Mitochondrion	Lysosome/Vacuole	Golgi apparatus	Nucleus	Plastid	Cell membrane	Peroxisome
Likelihood	0.3404	0.3031	0.1048	0.0652	0.0487	0.0439	0.0414	0.0345	0.011	0.0068
Type	Soluble	Membrane								
Likelihood	0.9143	0.0857								

Fibinb

Prediction: Extracellular, Soluble

Localization	Extracellular	Endoplasmic reticulum	Lysosome/Vacuole	Cell membrane	Mitochondrion	Cytoplasm	Golgi apparatus	Plastid	Nucleus	Peroxisome
Likelihood	0.9154	0.0678	0.014	0.0022	0.0002	0.0002	0.0001	0.0001	0	0
Type	Soluble	Membrane								
Likelihood	0.9859	0.0141								

Figure 3.3 Identification of cleavage sites and prediction of localisation of Fibin(s)

(A) SignalP 5.0 was used to predict cleavage sites in Fibin(s). (B) DeepLock was used to predict cellular localization of Fibin(s).

Moreover, mutant fish, especially females that were egg-bound, showed, in some cases, phenotypic alteration of parts of their craniofacial skeleton and of the posterior aspect of their body, anterior to the dorsal fin (highlighted with a yellow star in Fig. 3.5). This included a flattened posterior aspect of the craniofacial region with a pronounced, heightened aspect of the posterior side of the body. These phenotypical differences, however, were not found consistently in the *fibina/b^{-/-}* fish population, nor were they specifically linked to one gender or age.

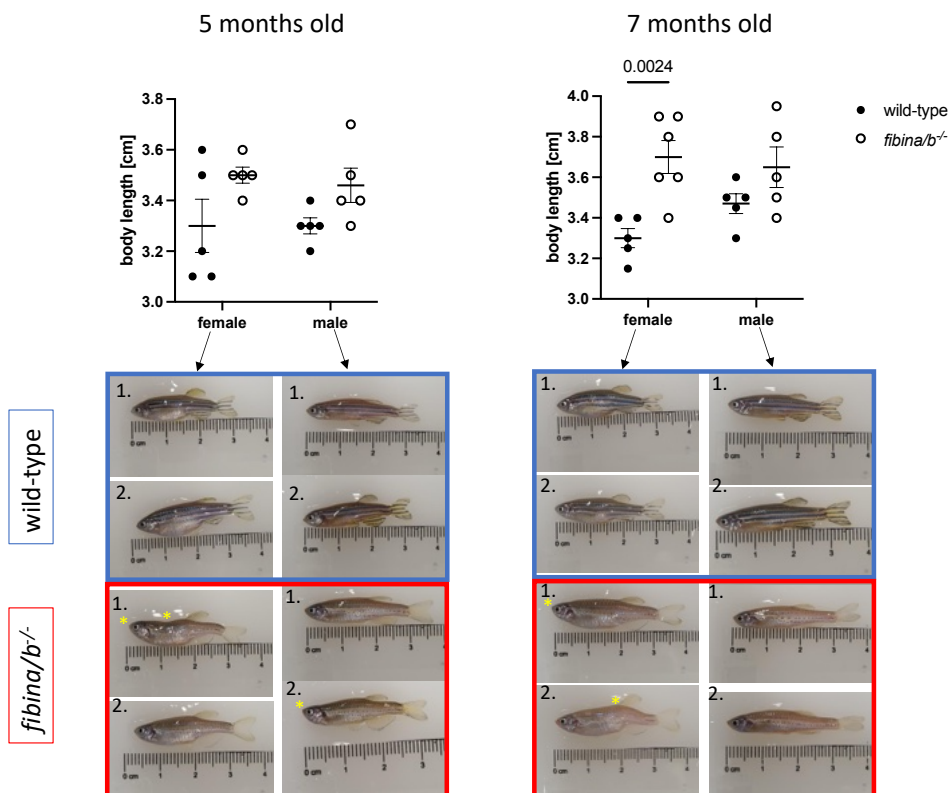


Figure 3.5 Comparison of body length of wild-type and *fibina/b^{-/-}* fish.

Each dot represents an individual fish. Mean and SEM shown. Two-way ANOVA performed followed by Sidak's multiple comparison test. p values < 0.05 shown. Yellow star indicates morphological anomalies in the craniofacial skeleton and on the dorsal side of the *fibina/b^{-/-}* body.

3.3.2.2 Response of *fibina/b^{-/-}* zebrafish to caudal fin amputation

Firstly, the caudal fin regeneration was investigated in zebrafish embryos. The reason for using the caudal fin model rather than the gill regeneration model is because at the developmental stage investigated here (3 dpf), gills are still under development and not fully

functional, and caudal fin (even at later developmental stages) allows for fast, repeated regeneration experiments due to its known high regenerative capacity (Azevedo *et al.*, 2011a). Fig. 3.6 shows the representative images of wild-type and *fibina/b^{-/-}* 3 dpf zebrafish with cut fin fold and 7 dpf zebrafish, showing the regeneration progress.

Fin fold regeneration is well established at all embryonic stages, but most commonly performed after 3 dpf. This is due to the embryos already being hatched from their chorions. The fin fold is clearly defined by 5 dpf (that is 2 days post cut) and largely regenerated, without permanent sign of the cut, by 7 dpf. The spread of data showed in Fig. 3.6(A) shows the large variance in both genotypes. The data points included in the analysis represent embryos whose caudal fin fold was cut just posteriorly to the end of their notochord, as notochord cuts result in more severe injury, with comparatively higher embryo death rate. In our experiments, no differences in the shape or length of the regenerate were found between the two genotypes, suggesting that at this developmental stage, lack of Fibins does not impact the regenerative capacity of the zebrafish fin fold.

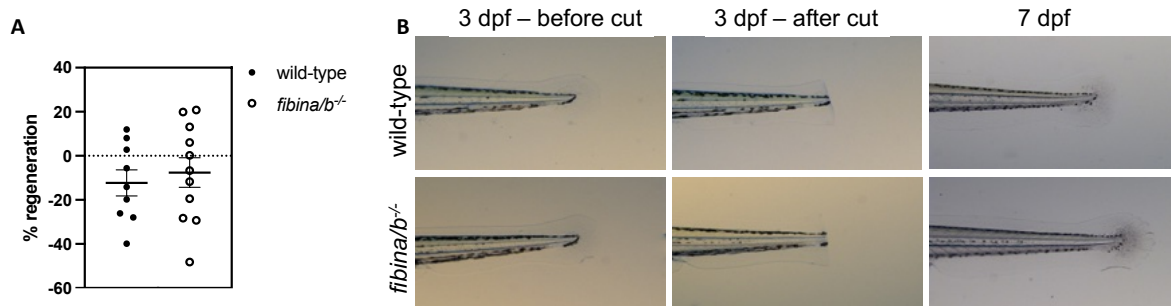


Figure 3.6 Caudal fin fold regeneration in wild-type and *fibina/b^{-/-}* embryos.

(A) percentage regeneration of the caudal fin fold cut after 4 days of regeneration (at 7 dpf). Mann-Whitney test; Mean and SEM shown. **(B)** representative images of wild-type and *fibina/b^{-/-}* embryos (n = 9 for wild-type and n = 11 for *fibina/b^{-/-}* fish).

Next, I investigated the caudal fin regeneration in adult *fibina/b^{-/-}* in comparison the wild-type fish. Caudal fin is known to regenerate fully, even after repeated amputations (Azevedo *et al.*, 2011a). Here, together with Dr M.-C. Ramel, we have subjected age-matched wild-type and

fibina/b^{-/-} fish to caudal fin amputation, left the fins to regenerate for 3 weeks, and performed another amputation, in the same plane as originally. We left the fish again, with close monitoring, for another 3 weeks and we imaged the regenerated fins. As shown in Fig. 3.7, the *fibina/b^{-/-}* fish regenerated their caudal fins successfully. Moreover, there were no obvious morphological differences during the regeneration time between wild-type and *fibina/b^{-/-}* fish. I then visualized the calcified components of the caudal fin, also referred to as lepidotrichia, using Alizarin Red S (ARS) staining. ARS staining is an easy and effective strategy of visualizing bony/calcified tissues in both embryo and adult zebrafish (Bensimon-Brito *et al.*, 2016). To properly visualize the skeleton, fish need to be cleared of pigmentation and soft tissues, for better permeability of the staining. However, as caudal fin only contains musculature at the very proximal areas, and else the lepidotrichia are covered in a thin epidermal layer with scarce mesenchymal tissue, it was possible to stain the fins directly, without prior clearing. Fig. 3.7(B) presents representative images of ARS staining, showing that the level of calcification is comparable between the two genotypes as well. Overall, these results indicate that lack of *fibins* does not itself inhibit the regenerative processes involved in caudal fin repair after amputation.

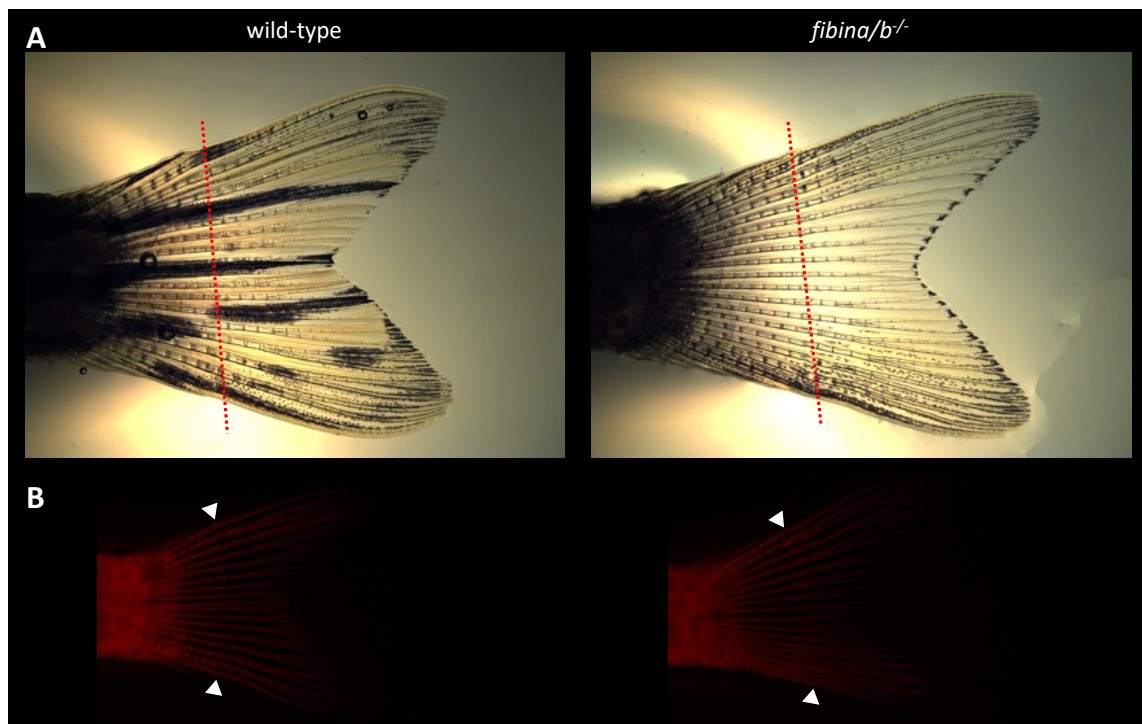


Figure 3.7 *fibina/b^{-/-}* adult fish regenerate caudal fin without any morphological alterations, as compared with wild-type fish.

(A) Representative images of wild-type and *fibina/b^{-/-}* fish at 3 weeks post repeated caudal fin amputation. Red dotted line indicates the original plane of amputation. (B) Representative images of Alizarin Red S Staining (ARS staining) of wild-type and *fibina/b^{-/-}* fish showing the calcified tissue (lepidotrichia) restoration upon injury and regeneration (n = 3 per genotype).

3.3.3 Comparative RNA-seq analysis of adult caudal fin and gill tissues between wild-type and *fibina/b*^{-/-} zebrafish

The extensive datamining done by the Dallman laboratory has indicated that Fibrin(s) genes are differentially expressed during states of injury and repair between regenerating and non-regenerating (fibrosing) vertebrate model systems (Ramel *et al.*, 2021). With no clear, consistent phenotype in the *fibina/b*^{-/-} mutant fish compared to wild-type fish and with limited understanding of Fibrin(s) and fibrosis-related molecular links, we have decided to investigate the molecular aspect of lack of Fibrin(s) in greater detail. We have therefore, in collaboration with Boehringer Ingelheim Pharma, performed a steady state RNA-seq analysis of two separate zebrafish tissues, the gill and the caudal fin, from both the wild-type and the *fibina/b*^{-/-} mutant fish in order to uncover putative genes linked to Fibrins expression.

6 wild-type and 6 *fibina/b*^{-/-} fish were used in this experiment. The tissue preparation for the RNA-seq was carried out by Dr M.-C. Ramel, the initial analysis was carried out by Praveen Baskaran (BI; read alignment and normalisation), and myself and Zhaocheng Zhao (BSc Student) carried out data normalisation, PCA analysis and Functional Enrichment analyses. Fig. 3.8 shows the process of RNA-seq, including the PCA analysis (Fig. 3.8(B)).

All but two samples passed the RNA Integrity threshold of RIN > 7.5 (minimum requirement for mRNA-seq). RIN stands for RNA integrity number and RIN allows estimation of RNA purity, integrity, and quality by taking into account possible degradation products. RIN is scored on a scale of 1 – 10, with 10 representing intact RNA (Schroeder *et al.*, 2006). Both samples were gill samples, one was male wild-type and the other female double mutant. These 2 samples were excluded from downstream analysis. Data was normalised using Log₂CPM (counts per million) method which assumes that most genes in the dataset are not differentially expressed (DE), that DE and non-DE genes behave similarly, and that the number and magnitude of up- and down-regulated genes are comparable. Compared to other normalisation methods (such as transcripts-per-million, TPM, method), CPM is useful for between-sample normalisation (Zhao *et al.*, 2021).

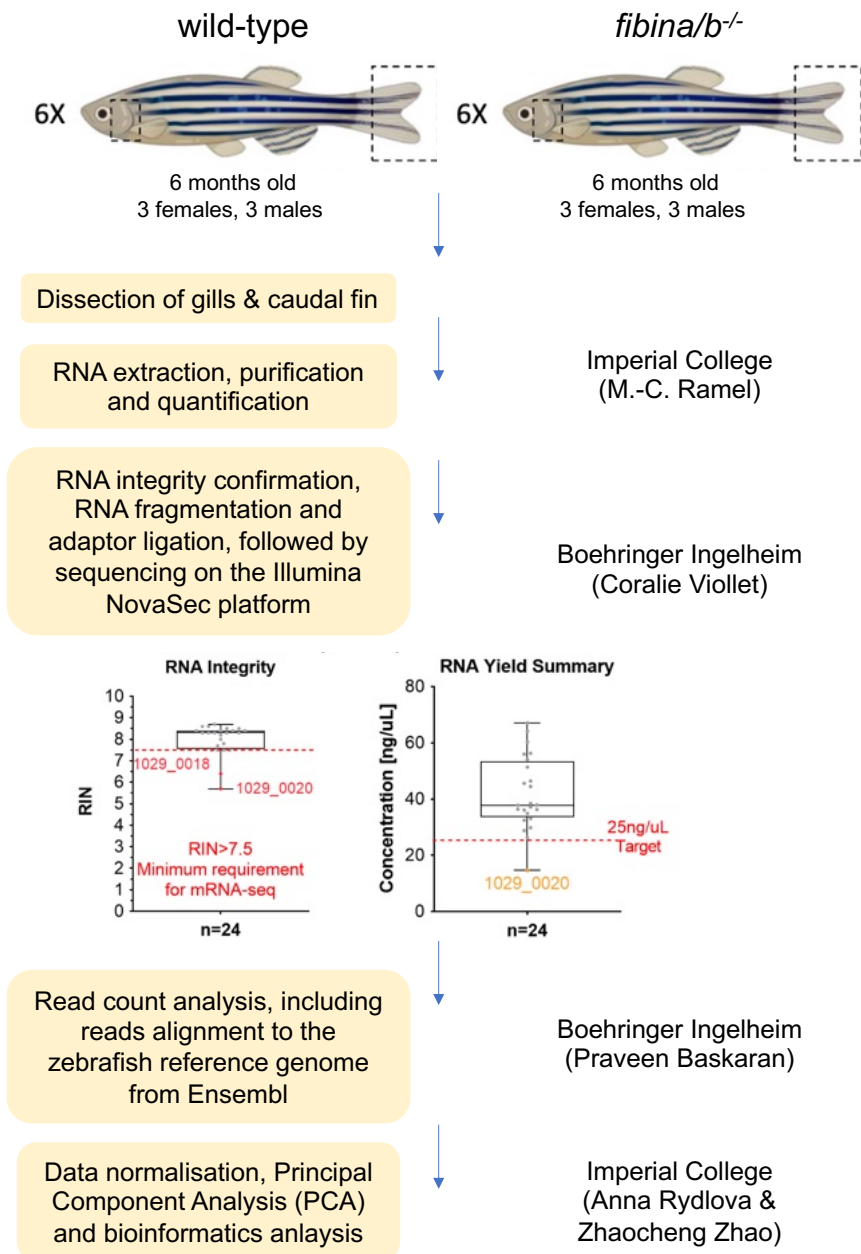
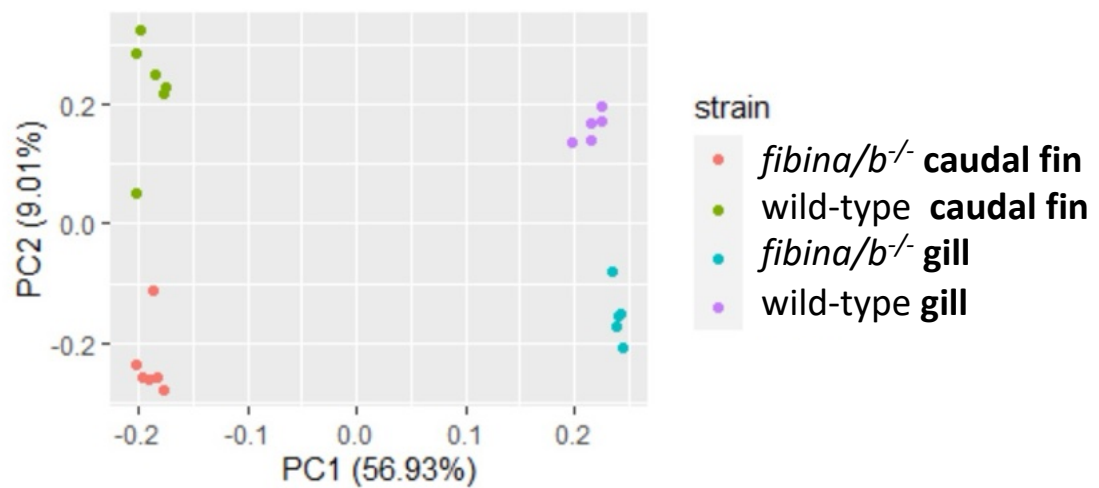
A**B**

Figure 3.8 Workflow of RNA-seq analysis and Principal Component Analysis of the samples.

(A) Experimental set up and workflow of sample preparation for subsequent RNA-seq analysis. Caudal fins and gill tissue from 6 wild-type and 6 *fibina/b^{-/-}* fish were harvested, RNA was extracted and subjected to quality control. RNA-seq was performed at Boehringer Ingelheim Pharma. (B) Principal Component Analysis (PCA) of all samples.

To determine what contributes to the most variability within our RNA-seq dataset, we carried out a Principal Component Analysis (PCA). PCA analysis is a type of unsupervised clustering of gene expression. PCA analysis revealed four clearly distinct clusters (Fig. 3.8(B)), showing that the transcriptome of caudal fin and gill of wild-type and *fibina/b^{-/-}* fish are different without overlap. The largest variance (56.93%) is accounted for by the tissue differences (that is gill vs caudal fin) for both wild-type and *fibina/b^{-/-}* fish. The lack of Fibins in *fibina/b^{-/-}* mutant fish contributes to the second largest variance (accounting for 9.01%).

Next, differentially expressed (DE) genes were selected based on FDR. For transcriptome profiling studies, such as RNA-seq, FDR is commonly used to select differentially expressed genes, rather than p-value. FDR controls for false discoveries as well as minimises false positives by estimating the expected proportion of incorrectly rejected null hypotheses. Over 1800 genes were found differentially expressed between the caudal fins of wild-type and *fibina/b^{-/-}* fish (FDR (false discovery rate – adjusted p-value) < 0.05) and over 900 in the gill tissue (Fig. 3.9). Fig. 3.9(B) shows the Venn analysis presenting the overlap of differentially regulated genes in wild-type and *fibina/b^{-/-}* fish between the caudal fin (grey) and the gill (green) tissue. The relatively small overlap (279 downregulated genes and 113 upregulated genes) suggests that Fibins may be involved to different extents or play a different role based on the tissue type (primarily bone in caudal fin vs primarily cartilage in the gill).

3.3.3.1 Statistical enrichment analysis

Statistical enrichment analysis is a method to identify classes of genes that appear in excessive numbers (over-represented) within a given set of genes, potentially indicating a correlation with a disease phenotype. This method relies on statistical approaches to

determine groups of genes that are significantly over-represented (enriched) or under-represented (depleted) (Karp *et al.*, 2021). Statistical gene enrichment analysis uses pre-defined gene sets, put together through set of selected criteria for example involvement in the same biological pathway, which allows us to overcome the potential problem of small changes in the expression of a single gene. I have analysed the differentially regulated genes from our RNA-seq against the GO biological process annotation set (Fig. 3.10).

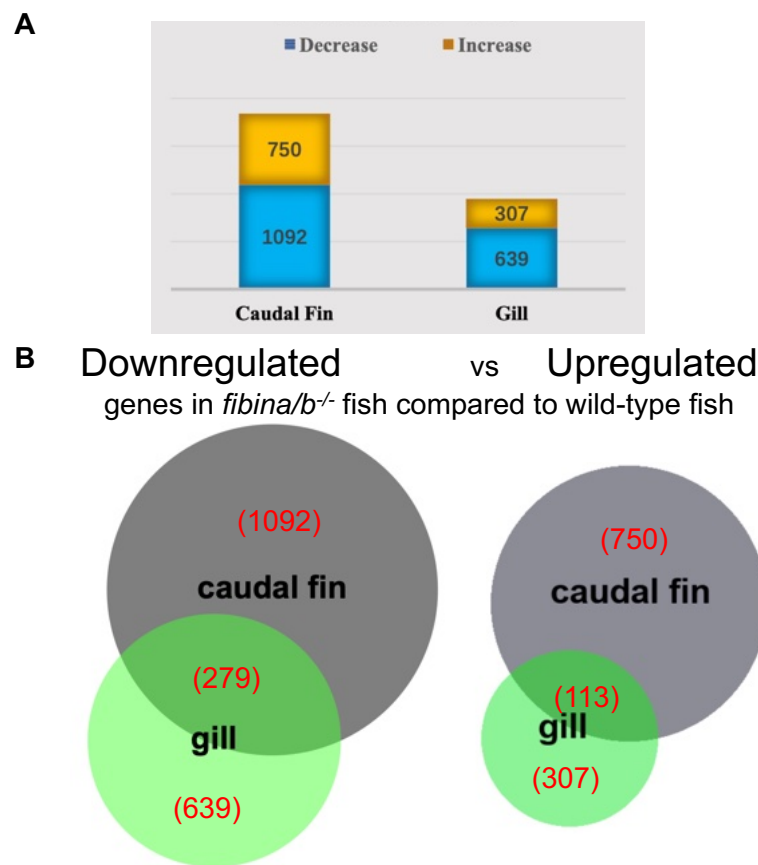


Figure 3.9 Differentially expressed genes (DEG) between wild-type and *fibina/b^{-/-}* fish (caudal fin and gill tissue).

(A) Total numbers of differentially regulated genes between wild-type and *fibina/b^{-/-}* fish. ‘Decreased’ or ‘increased’ means that the number of genes were increased/decreased in the *fibina/b^{-/-}* fish compared to wild-type fish. (B) Venn analysis of differentially expressed genes. Area-proportional Venn diagrams showing the overlap of commonly up- and down-regulated genes (in *fibina/b^{-/-}* fish compared to wild-type fish) between caudal fin (grey) and gill (green) tissues.

Among the genes that were significantly upregulated in the gill and caudal fin of *fibina/b^{-/-}* fish compared to the wild-type fish, the following GO terms were overrepresented: animal organ development (GO:0048513), haemopoiesis (GO:0030097), haematopoietic or lymphoid organ development (GO:0048534), immune system development (GO:0002520), system development (GO:0048731). Among the genes that were significantly downregulated in the gill and caudal fin of *fibina/b^{-/-}* fish compared to the wild-type fish, the following GO terms were overrepresented: immune response (GO:0006955), mitochondrion organization (GO:0007005), response to external stimulus (GO:0009605), biological process involved in interspecies interaction between organisms (GO:0044419), response to biotic stimulus (GO:0009607), response to external biotic stimulus (GO:0043207), response to other organism (GO:0051707), humoral immune response (GO:0006959), complement activation (GO:0006956), immune system process (GO:0002376), adaptive immune response (GO:0002250), immune effector process (GO:0002252), regulation of GTPase activity (GO:0043087), anatomical structure morphogenesis (GO:0009653), regulation of hydrolase activity (GO:0051336).

When looking at the gill tissue only, all statistically enriched genes (mainly upregulated in mutant compared to wild-type fish) fall in the ‘Immune system’ and ‘Immune Response’ categories of GO terms. Interestingly, when only analysing the caudal fin tissues, apart from animal organ development (GO:0048513), the gene set is statistically enriched by genes related to regulation of BMP signalling pathway (GO:0030510) (Appendix 4).

Surprisingly, the read counts of *fibina* were not found to be significantly different in the caudal fin tissue between wild-type and *fibina/b^{-/-}* fish (Fig.3.11). *fibinb* read counts were significantly decreased in the caudal fin (FDR = 0.0234) and the gill (FDR = 0.00118) of *fibina/b^{-/-}* fish compared to wild-type fish. *fibina/b^{-/-}* fish produce a premature stop codon in both *fibina* and *fibinb* genes that directs the mRNA produced for non-sense mediated decay rapidly after transcription, therefore detecting no difference in *fibina* read counts suggests that the level of *fibina* transcripts is very low in wild-type fish at steady state (homeostasis). Fig. 3.11 shows several selected genes from the RNA-seq analysis. Some that were found differentially expressed in both the gill and the caudal fin, some only in one tissue type

between the wild-type and *fibina/b*^{-/-} fish in the RNA-seq analysis and some (*colla2*) not DE. qPCR confirmation is included for each selected gene. Immune related genes and gene classes, which were found statistically enriched in the RNA-seq are not included here but are discussed in detail in the following chapter (Chapter 4).

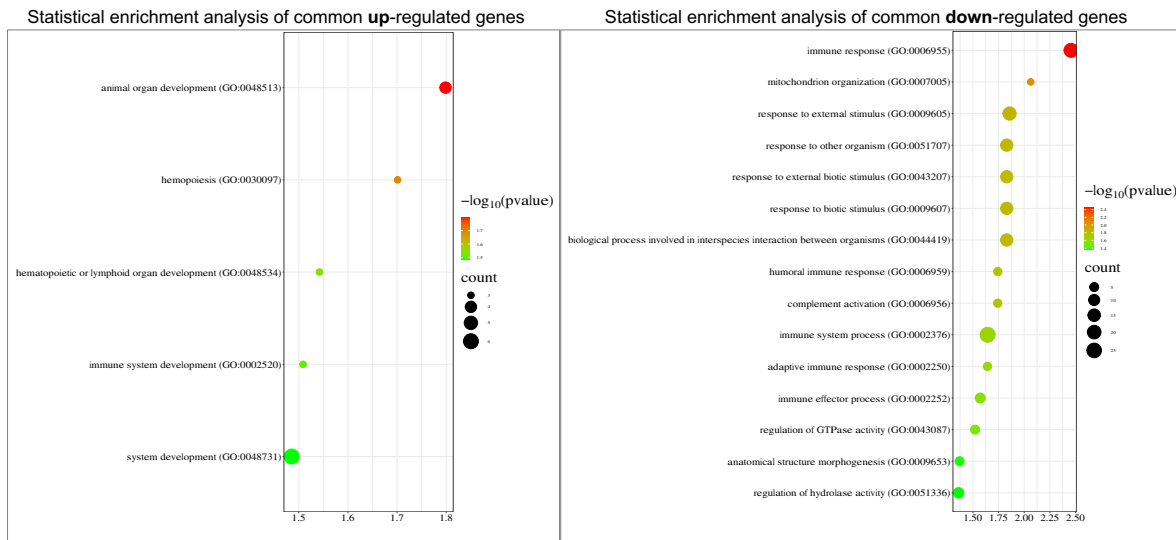


Figure 3.10 Functional enrichment analysis of the common (caudal fin and gill tissue) up- and down-regulated genes between wild-type and *fibina/b*^{-/-}.

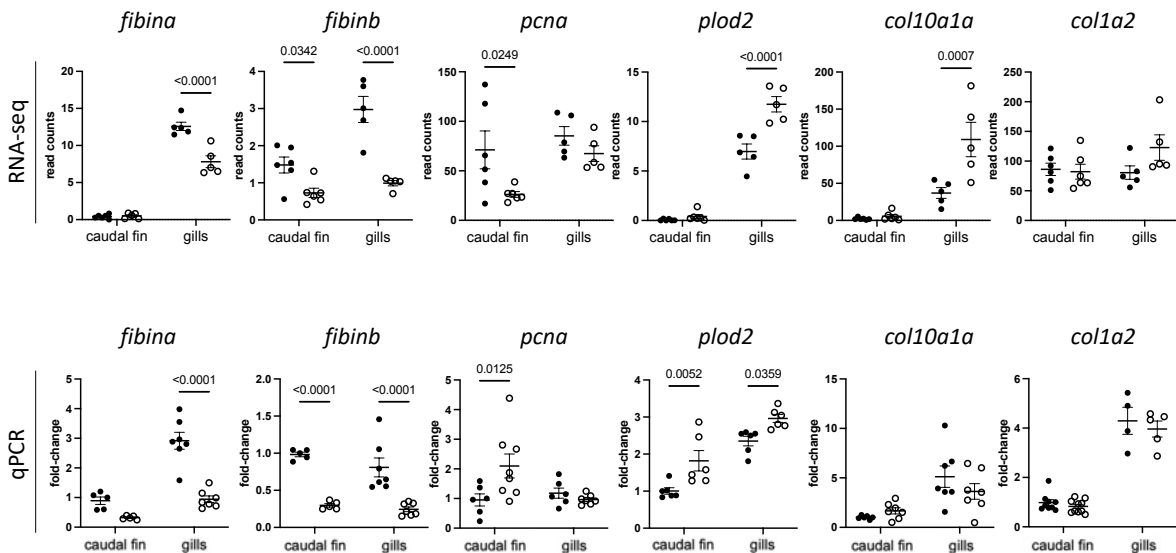


Figure 3.11 Comparison of selected genes from RNA-seq with qPCR analysis.

RNA-seq read counts of selected genes (from 6 months old wild-type and *fibina/b*^{-/-} fish) are plotted in parallel with qPCR transcript level analysis of different group of 6 months old zebrafish. Black circle = wild-type, white circle = *fibina/b*^{-/-} fish. One-way Anova; mean and SEM shown. p-values of < 0.05 are plotted. Comparisons were only made between genotypes, not between tissues.

3.4 Discussion

In this chapter, I have shown the results of computational prediction and analysis of Fibrin(s) protein structures, investigated the response of *fibina/b^{-/-}* fish to caudal fin injury, and presented the analysis of RNA-seq done in collaboration with Boehringer Ingelheim Pharma comparing the gill and caudal fin transcriptomes between wild-type and *fibina/b^{-/-}* fish.

3.4.1 Computational predictions of Fibrin(s) structures, protein localisations, and transcription factor binding sites (TFBS) analysis.

As a widely understudied gene(s) and protein(s), the structure of Fibrin(s) was unknown. I have used the state-of-the-art computational analysis, enabled by AlphaFold, to generate predicted 3D structures of human FIBIN, mouse FIBIN and zebrafish Fibina and Fibrinb. Compared to human and mouse FIBIN, zebrafish Fibrins were predicted to have a highly disorganized region at the N-terminal. There are several potentially significant outcomes of presence of a disorganized region including in protein folding and stability, post-translational modifications, proteolytic processing, signal sequences and localization or protein-protein interactions: A disorganized N-terminal can affect the stability of the 3D conformation, potentially affecting the protein function or even resulting in protein degradation. The N-terminus (as well as the C-terminus) is, particularly for eukaryotic proteins, modified by tyrosination, acetylation, arginylation and many others, which have been shown to impact normal versus disease-related processes in which the protein of interest is involved (Chen and Kashina, 2021).

Also, many proteins have specific signal sequences at their N-terminus which direct the protein localization to specific cellular or organelles, and a disorganized region at the N-terminus could potentially impact the recognition and processing of such signal sequences, possibly leading to mislocalisation and/or altered targeting of the protein. Furthermore, some proteins undergo proteolytic cleavage at their N-terminus as part of their maturation process or to generate fragments that are biologically active (Rogers and Overall, 2013). Interestingly, all four proteins were predicted to have a cleavage site at the N-terminal, including human and mouse FIBIN, that were not predicted to have a disorganized region at

the N-terminal (Fig. 3.3(A)). If one orthologous protein has a highly disorganised N-terminus while its ortholog from a different species does not, it suggests that there may be a difference in the protein function between the two species, especially if the structural features of the N-terminus are crucial for protein's function or regulation. However, it is important to note that protein function is a complex trait influenced by various factors (structural motifs, post-translational modifications, interactions with other proteins and molecules...) and thus while differences in the organisation of a part of a given protein – here the N-terminus – may provide insight into potential functional differences, experimental investigation is required to determine the actual functional implications regarding the protein's role.

Previous work by Dr Marie-Christine Ramel showed that the *fibina* and *fibinb* mutations introduced by deletions 109d120 and 226d232 respectively, lead to the respective mRNAs to undergo non-sense mediated decay (NMD). NMD is a surveillance mechanism that acts as a quality control system to ensure the removal of aberrant mRNA molecules. When mRNA undergoes NMD, the mRNA molecule is degraded and eliminated from the cell due to the presence of premature termination codons. By eliminating mRNAs with premature termination codons, NMD helps to maintain the integrity of the cellular protein production process and prevents the synthesis of truncated proteins that could have serious negative effects on the cellular function. I have, nevertheless, used AlphaFold for a computational prediction of the theoretical protein structure, to visually assess which aspects of the theoretical truncated protein remained. And as clearly visible from the side-to-side structural comparison, the only part of the theoretical proteins remaining was the highly disorganised N-terminus, which, according to cleavage site predictions, is cleaved anyway. Together, this data strongly indicated that cells with mutated *fibina* and *fibinb* genes do not produce functional Fabin(s) proteins.

I have used DeepLock to predict the localization of Fabin(s) and found, interestingly, that human and mouse FIBIN as well as zebrafish Fabinb were predicted to localize extracellularly, meaning they are secreted proteins. While Fibina was predicted to localize intracellularly, in the endoplasmic reticulum (ER). This data suggests that Fibina and Fabinb have different localization and thus possibly different function. This results also aligns with

results by Marie-Christie Ramel and Hannah Needham, who showed differential temporal and spatial tissue expression of *fibina* and *fibinb* (Introduction, Fig. 1.8(A)), further highlighting a possibly diverging roles of the paralogues *fibina* and *fibinb*.

Transcription factors play a crucial role in regulating gene expression. By identifying and characterizing the binding sites of transcription factors, TFBS analysis aids in deciphering of the regulatory elements responsible for turning genes on or off, such as in response to various cellular signals. For *Fibin(s)* the TFBS analysis revealed conserved regulation by two TFs, HFH-3 (also known as FOXI1) and SOX5.

HFH proteins (the hepatocyte nuclear factor-3)/*fork head* homolog) are a family of transcription factors that play essential roles in embryonic development tissue-specific gene expression, and organogenesis. The HFH family is a subset of the larger forkhead box (FOX) family of transcription factors. These TFs are primarily expressed in endoderm-derived tissues, such as the liver, pancreas, lung, and gastrointestinal tract. Interestingly, *FOXII* was found uniquely expressed in a rare subpopulation of alveolar epithelial cells expressing high levels of *CFTR* gene. Relevant to this project, similar expression signature was also found in ionocytes in teleost fish gills and skin (Conte, 2012) (and also in *Xenopus* larval skin (Dubaisi and Papalopulu, 2011; Quigley, Stubbs and Kintner, 2011), and Barbry and colleagues (Barbry *et al.*, 2020) termed these cells ‘pulmonary ionocytes’. Moreover Plasschaert and colleagues showed that *FOXII* overexpression in pulmonary basal cells was sufficient to drive ionocyte differentiation (Plasschaert *et al.*, 2018).

Sox-5 (alongside other *sox* genes, such as *sox6* or *sox9*) have been showed to play an important role in chondrogenesis. Sox-5 in particular was shown to be important for chondrocyte differentiation (Li *et al.*, 2021). Interestingly, binding of *Sox-5*, *Sox-6* and *Sox-9* simultaneous expression led to stimulation of high levels of the chondrocyte-specific gene, *Col2a1*, in a mouse fibroblast cell line, resulting in the trans-differentiation of fibroblasts into chondrocyte-like cells (Lefebvre, Behringer and De Crombrughe, 2001; Smits *et al.*, 2001, 2004; Kim and Im, 2011). Together, the presence of these two specific transcription factor

binding sites in the Fabin(s) promotor region indicates a possible role for Fabin(s) during development and in chondrogenic differentiation.

Overall, from the Fabin(s) proteins, mouse and human FIBIN showed the highest structural similarities. From the two zebrafish paralogues, Fabinb showed the higher structural similarity to the mammalian FIBIN. All 4 proteins had a predicted cleavage site at the N-terminal, but Fibina protein showed the least likelihood (still at 0.8535 out of 1). Fibina was also the only protein predicted to localize intracellularly, in the ER, while all other 3 Fibins were predicted to localize extracellularly. Moreover, analysis of TFBS revealed shared TFs across the 3 species suggesting a possibility of conserved regulation. Still, computational analyses and predictions are not always definitive and experimental validation is often required as a confirmation, particularly of the functional significance of these findings. Experimental techniques such as X-ray crystallography, nuclear magnetic resonance spectroscopy or cryo-electron microscopy enable to obtain high-resolution structural information and should be further explored in the future, to fully elucidate the 3D structure of Fabin(s).

3.4.2 Characterisation of *fibina/b*^{-/-} zebrafish

Building on prior work done in the Dallman laboratory, that showed that morpholino knockdown of *fibina* and/or *fibinb* resulted in no obvious morphological manifestation (such as lack of pectoral fins as suggested by Wakahara et al.), the Dallman laboratory has generated double mutant fish, *fibina109d120*^{-/-};*fibinb226d232*^{-/-} here referred to as *fibina/b*^{-/-}. These mutant fish, do not show any obvious consistent developmental or morphological differences to their wild-type counterparts. I have measured the body lengths of the *fibina/b*^{-/-} and wild-type fish, age-matched, and found that the mutant fish appear to have somewhat longer bodies, at both ages (5 and 7 months old) and for both male and female fish. It is important to note that fish body size can be affected by several factors, including diet, size of the aquarium and the rearing density (Andersson and Kettunen, 2021). Although the diet and the size of the aquarium was kept constant for the duration of these fish development, the rearing density might have not been. Therefore, to prove beyond doubt that the mutant fish

are statistically larger at any age, a larger group of age-matched fish as well as more timepoints are required.

I have also noted that some of the mutant fish showed distorted morphology of parts of their skeleton, particularly in the craniofacial region and the posterior aspect of their bodies. This was somewhat more pronounced in egg-bound female fish, however, significantly more males and females appear exactly as wild-type fish. Nonetheless, the fact that the body distortion tended to be more obvious in egg-bound females (that is fish with heavier anterior aspect of their body), this observation could potentially suggest altered (decreased) stiffness of connective tissues, including bone.

Next, I have showed that both embryos and adult *fbina/b^{-/-}* fish retained basic regenerative capacity of their caudal fins. Detailed analysis of caudal fin regeneration, as well as gill regeneration, is covered in Chapter 5. Here I showed that there was no morphological difference in the restoration of the embryo fin fold when amputated at 3 dpf and left to regenerate till 7 dpf between the two genotypes. I have further showed that repeated amputation of the adult caudal fin also did not result in any differential morphological tissue restoration between the two genotypes. Moreover, the bony aspect of the caudal fins, lepidotrichia, visualized by ARS, were not different between the wild-type and *fbina/b^{-/-}* fish.

Overall, no significant, consistent differences were found between wild-type and *fbina/b^{-/-}* fish on a structural, morphological level. It is important to remember that zebrafish show fascinating plasticity when it comes to regeneration in particular. This was shown by Singh and colleagues (Singh, Holdway and Poss, 2012) who ablated all osteoblasts in the zebrafish caudal fin, performed the fin amputation, and although the timeline was longer, the zebrafish successfully differentiated non-osteoblastic cell types into osteoblasts and regenerated their fins without any obvious morphological distortions. This means that although structurally, no differences were found, on a molecular level, there may be mechanisms in action that differentiates these two genotypes and these will be explored in detail later in this thesis (Chapter 5).

3.4.3 RNA-seq analysis uncovered several avenues for further experimental investigation

In collaboration with Boehringer Ingelheim Pharma (BI), we have performed analysis of the transcriptional profiles of gill and caudal fin tissues, at steady state, between wild-type fish and *fibina/b^{-/-}* mutants. The PCA analysis showed clear differences between the two genotypes but also between the individual tissues. As Fibrin(s) expression is limited to a specific cell type but not to a specific organ or tissue, a Venn diagram analysis was carried out to determine the commonly up- and down-regulated genes (in *fibina/b^{-/-}* fish compared to wild-type fish) between the two tissue types. Statistical enrichment analysis was then carried out on these genes to identify gene classes that appear in excess in this gene set. Genes and gene classes related to immune system, including immune system development or response to stimulus, formed the vast majority of the identified statistically enriched genes and thus this aspect is covered in detail in Chapter 4. Several other classes were identified, such as mitochondrial organisation, animal organ development or regulation of BMP signalling pathway. Quite surprisingly, *fibina* read counts were not found to be significantly different in the caudal fin tissue specifically between the wild-type and the mutant fish (*fibinb* read counts were statistically lower in both tissues in the *fibina/b^{-/-}* fish compared to wild-type fish). This was an important finding as it showed that *fibina* levels are very low in the caudal fin even in the wild-type fish and therefore, the fact that there was no statistically significant difference found can be a result of generally very low *fibina* levels at homeostasis (steady state). This brings about the biggest limitation of this RNA-seq, which is the conditions under which the wild-type and mutant fish were compared. The Dallman laboratory has previously showed that Fibrin(s) are differentially regulated between zebrafish and mammalian systems during tissue regeneration/fibrosis processes. However, this analysis only covered tissues at steady state, and as no obvious differences in the developmental processes were observed between the two genotypes, it is possible that the role of Fibrin(s) cannot be fully dissected from analysis of steady state condition. Rather, an analysis that compares timepoints during regenerative processes (of regenerating caudal fin or gill tissue for example) might be more informative on the roles of Fibrin(s) in tissue regeneration.

Nonetheless, several important takeaway points were discovered during the analysis, including the significant differences in immune-system related genes, and will be investigated

further in the following chapters. From the data presented in this chapter, it is possible to conclude that Fibrin(s) do not play a fundamental role in zebrafish development. Further analysis of the immune-related genes will shed more light on the impact of Fibrin(s) (or the lack of rather) on the ability of mutant zebrafish immune system maturation and ability to mount an immune response to a stimuli.

3.5 Conclusion

In this chapter, I have presented the first prediction of Fibrin(s) proteins 3D structures, that Fibrin(s) show conserved regulation across species by two transcription factors (HFH-3 and SOX-5). Furthermore, I have used *fibina/b^{-/-}* fish generated in the Dallman laboratory, and showed that the mutant fish do not appear to struggle with caudal fin regeneration at the larval stage as well as at in adulthood, with morphologically comparable outcomes between the wild-type and the *fibina/b^{-/-}* fish. Lastly, I have presented the analysis of RNA-seq that was performed in collaboration with BI, which showed several statistically enriched gene classes of up- and down-regulated genes between the wild-type and the mutant fish.

4 CHARACTERISATION OF *fibina/b*^{-/-} MUTANT FISH IMMUNE SYSTEM

4.1 Introduction

RNA-seq analysis (discussed in Chapter 3) uncovered a surprisingly large number of immune-related genes, differentially regulated between wild-type and *fibina/b*^{-/-} fish. The majority of these genes were found to have lower read count numbers in our mutant fish and belonged to the following categories as defined by functional enrichment analysis:

- o Response to stimulus
- o Immune response
- o Regulation of immune response
- o Regulation of immune system process
- o Positive regulation of immune response
- o Cellular response to stimulus
- o Immune system

This result suggested a possible immune deficiency in our mutant fish. Fibin(s) are strictly associated with mesenchymal-type cells and not found expressed in any haematopoietic cells. The effects of lack of Fibins on the expression of the immune-related genes (some of them strictly associated with haematopoietic cells such as *lck*) suggests that this outcome is mediated through interactions between the haematopoietic (immune) cells and mesenchymal (fibrin-expressing) cells.

To begin, I will define the terms used throughout this chapter, in particular what is meant by stromal, mesenchymal, and immune cell types. Stromal cells are non-haematopoietic cells that are part of a specific niche. An example relevant to this chapter is the bone marrow stromal cells that support haematopoiesis in mammals and other vertebrate systems. The term encompasses a range of cell types such as endothelial, epithelial, and mesenchymal cells. Mesenchymal cells are also non-haematopoietic cells, historically defined by their function to provide and maintain structural integrity of tissues and organs. This term covers cells such as fibroblasts, chondrocytes, or osteoblasts.

Immune cells, unless otherwise stated, refer to cells derived from the haematopoietic precursors that form the classical part of vertebrate immune system. The term covers all the hematopoietic lineages (innate and adaptive immune system cells).

4.1.1 Mesenchymal cells as a non-classical branch of the immune system

Fibroblasts are a subset of mesenchymal cells. Traditionally, the main function of fibroblasts was thought to be limited to providing structural support to tissues and organs via secretion of extracellular matrix (ECM) components and the ECM remodelling enzymes, such as MMP9. Over the past half a century, it became clear that not only does the generic name ‘fibroblast’ encompass truly heterogeneous cell population but also that these cells’ functions are not limited to being the tissue scaffold engineer (Parsonage *et al.*, 2005).

Technological advances including multi-omics profiling (such as scRNA sequencing), brought about new approaches of dissecting the vast roles and functions that structural cells fulfil. Several of these techniques, including 3D transcriptomics enables us to begin untangling the inter-cellular signalling crosstalk on a single cell level. In a truly landmark article in 2020, Krausgruber and colleagues used cellular phenotyping, transcriptome sequencing, chromatic accessibility profiling and epigenome mapping to investigate the regulation of immune genes across 12 different mice organs with specific focus on structural cells. One of the most intriguing results showed what the authors described as ‘epigenetically encoded immune potential in structural cells under tissue homeostasis’ (Krausgruber et al., 2020).

In this chapter I will discuss our current knowledge and understanding of how mesenchymal cells, and fibroblasts, in particular influence the haematopoietic cells.

4.1.2 The role of mesenchymal stromal cells in haematopoiesis

Mesenchymal cells are fundamental in establishing and maintaining the haematopoietic cell niche as well as directing the immune cells trafficking around the body to maintain homeostasis. Zebrafish has been long used as a model to study vertebrate haematopoiesis, even despite clear anatomical differences between zebrafish and mammalian (human) haematopoietic sites. In adult humans, the main site of haematopoiesis is the bone marrow. Bone marrow provides the environment for *de novo*, continuous generation of all haematopoietic cell lineages (Gomariz et al., 2018; Nombela-Arrieta & Manz, 2017).

In the adult zebrafish, as well as in other teleost fish, the main site of haematopoiesis is found in the whole kidney marrow, the renal tissue of the kidney (Glass *et al.*, 2011; Gore *et al.*, 2018). Datamining of online scRNA sequencing datasets showed that *fibina/b* are expressed in the mesenchymal cells surrounding the haematopoietic niche in zebrafish (Tang et al., 2017).

The role of stromal cells in supporting and defining haematopoiesis has been extensively studied both *in vitro* and *in vivo*. The establishment of long-term bone marrow cultures capable of maintaining stromal cell-dependent, haematopoietic cell production *in vitro* provided a solid argument for the study of stromal-immune crosstalk that underlies this relationship (Dorshkind, 1990; Liang & Song, 2020). Bone marrow stromal cells provide fundamental regulatory cues such as growth-promoting and differentiation-inducing molecules, necessary for haematopoiesis. These stromal cells include plethora of cells broadly referred to as endothelial, mesenchymal, or neural cells (Calvi & Link, 2014; Dorshkind, 1990; Mendelson & Frenette, 2014; Scadden, 2012). An example of such an important regulatory mechanism that demonstrates the necessity of optimal mesenchyme-haematopoietic crosstalk, fundamental for correct haematopoietic cell development involves the CXCL12 – CXCR4 axis. This axis is conserved across the vertebrate kingdom and involves mesenchymal stromal cells expressing the CXCL12 ligand and leukocytes expressing the cognate receptor, CXCR4.

CXCL12 is an essential cytokine expressed by early mesenchymal progenitors and required for the maintenance of HSC (Sugiyama *et al.*, 2006; Greenbaum *et al.*, 2013). In particular, CXCL12-abundant reticular cells (CARCs), that are cells expressing high amounts of CXCL12 appear to be key components of haematopoietic stem cells (HSCs) niche in adult bone marrow. The CXCL12/CXCR4 axis is important in promoting haematopoietic cell proliferation within the niche, migration to the niche across endothelial barriers and adhesion to the bone marrow (Becker, 2012; Möhle *et al.*, 1998). Inactivation of CXCR4 receptor on B-cells causes premature emigration of B cell precursors from the bone marrow (Nie *et al.*, 2004). T cell migration and homing is also affected by inhibition of CXCR4. Vianello and colleagues showed that inhibition of CXCR4 in thymocytes causes retention of mature T cells in the thymus (Vianello *et al.*, 2005).

The necessity for functional CXCR4-CXCL12 axis in immune cell homing, migration and adaptive immune system activation has been investigated in numerous diseases including autoimmune diseases or tumour immunity (García-Cuesta *et al.*, 2019; Zhao *et al.*, 2022).

CXCR4 signalling plays important role in stabilizing the connection between T cells and antigen-presenting cells (APCs) to enable T cell activation (Molon *et al.*, 2005).

Further experimental evidence from zebrafish emphasized the role of the CXCL12-CXCR4 beyond its role in homeostatic conditions - in tissue regeneration. In zebrafish larval fin amputation model, Isles and colleagues showed that zebrafish lacking functional *cxc4* or *cxc12* cleared neutrophils from the wound site significantly faster than wild-type fish. Aberrant neutrophil accumulation and retention in tissues is a major complication of respiratory and infectious diseases that can result in excessive tissue damage and persistent chronic inflammatory cycle. In line with this, both mutants (*cxc4* and *cxc12*) showed increased rate of fin regrowth and full regeneration compared to wild type fish (Isles *et al.*, 2019).

Haematopoiesis in zebrafish happens in two major waves (Glass *et al.*, 2011; Gore *et al.*, 2018). The site of primitive haematopoiesis is localized to the caudal haematopoietic tissue (CHT). The first immune cells to be detected during the primitive wave are myeloperoxidase, *mpx*-expressing myeloid cells and *hba1-globin*-expressing erythroid cells (Eckfeldt *et al.*, 2005). At approximated 25 hpf, the circulation begins to be established and the transition to the definitive stage of haematopoiesis takes place. Lymphoid progenitors migrate to the zebrafish thymus to give rise to *lck*- and *rag1*-expressing lymphocytes (~4 dpf) with B cells (immunoglobulin expressing cells) detected in the whole kidney marrow (WKM), the site of definitive haematopoiesis, at about 3 wpf (Langenau *et al.*, 2004, Page *et al.*, 2013).

After the primitive wave, definitive haematopoiesis brings about the HSCs which effectively generate the full range of leukocyte cell types found in adult zebrafish. The term leukocytes is a generic name for immune cells, including macrophages, neutrophils, lymphocytes (T cells and B cells), and other white blood cells. Lymphoid progenitor markers, observed also in humans, such as *rag1*, *rag2*, or *lck*, are detectable already at the end to the primitive wave (3 dpf). However, it is not till 4-6 wpf that adaptive immunity is functionally mature. During the first couple of weeks of life zebrafish therefore fully depends on its innate immune

system for defence against pathogens (Miao *et al.*, 2021). Fig. 4.1 outlines the timeline of zebrafish haematopoiesis as well as the comparison to mammalian haematopoiesis.

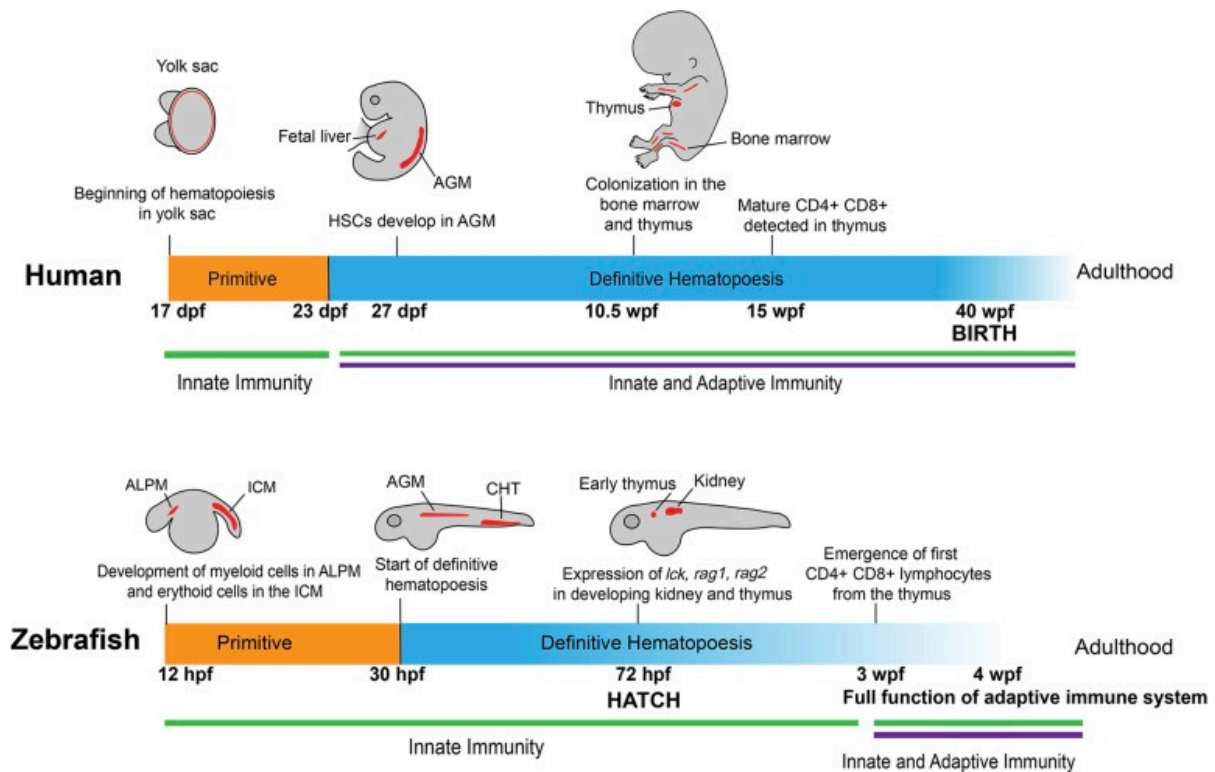


Figure 4.1 Development of human and zebrafish immune systems

Haematopoiesis in humans ~17 dpf initiates the development of the immune system. Before HSCs appear around 27 dpf, myeloerythroid progenitor cells seed in the yolk sac, followed by homing of immune cells in the bone marrow and thymus around 10.5 wpf. At 12-13 wpf, humans have detectable lymphocytes present. In contrast, the timeline for immune-system development in zebrafish starts around 12 hpf with haematopoiesis, continues with definitive haematopoiesis at 30hpf with lymphoid progenitor markers (*lck*, *rag1*, *rag2*) present by 72 hpf. The lymphocytes are, however, not fully developed at this stage. Matured lymphocytes/adaptive immunity have only been detected at 3-4 wpf, leaving the zebrafish dependent fully on their innate immune system during the early larval developmental stages (Adapted from (Miao *et al.*, 2021)).

During haematopoiesis, haematopoietic stem and progenitor cells (HSPCs) migrate through number of tissues, where they can transiently home, such as in the CHT. Once in the CHT, the HSPCs differentiate, expand, and further migrate to seed the definitive haematopoietic organs – thymus and kidney, the sites of haematopoiesis in the adult teleosts. Similar to mammals, restriction to the specified region is governed by a number of signals, specified primarily by stromal cells. Stromal cells in the CHT niche that develop through epithelial-mesenchymal transition are in direct contact with the HSCs and play a vital role in their development and maintenance (Murayama *et al.*, 2006).

Underlying the endothelial cells, mesenchymal stromal cells are distributed throughout the CHT and can be marked by high expression of *cxcl12a* (fibroblast reticular cells) (Glass et al., 2011; Tamplin et al., 2015). These cells are analogous to the CXCL12-abundant reticular cells found in the mammalian bone marrow. The *cxcl12-cxcr4* signalling axis is indeed conserved across human, mice and zebrafish systems and contributes to HSPCs guidance through tissues. Interestingly, in zebrafish, *cxcl12* expression was also detected in the gills and the skin apart from the CHT and WKM, however, it is unclear what implications this may have for the homing of the HSPCs (Glass *et al.*, 2011).

Further evidence of the importance of stromal cells for successful haematopoiesis has been demonstrated in zebrafish *oloca* mutants. *Oloca* mutants have a mutation in the *naca* gene that encodes a subunit of nascent polypeptide-associated complex (aNAC/NACA). NACA is believed to be part of a chaperon system involved in proper folding of nascent proteins. Murayama et al. found that this mutation prevents the maturation of mesenchymal progenitors in the haematopoietic niche. The CHT niche is still capable of recruiting and transiently homing HSPCs. However, the maintenance, retention, and subsequent expansion of HSPCs was significantly affected with severely compromised cellular differentiation. What was even more striking was the finding that the *oloca* mutant showed similar inability to retain haematopoietic cells in the WKM following a transplantation of adult haematopoietic cells from WKM of a transgenic zebrafish (Tg(*mpx*:GFP), labelling myeloid – neutrophil – cell population) (Murayama et al., 2015). These results indicate that a mutation affecting the crosstalk between mesenchymal and haematopoietic cells in the haematopoietic niches can significantly affect the numbers of adult haematopoietic cell population.

4.1.3 The role of mesenchymal cells in immune response to stimuli

4.1.3.1 Response to non-infectious stimuli

The interaction of mesenchymal and haematopoietic (classical immune) cells is not restricted to the generation, differentiation, and maintenance of the immune cells. During infection and inflammation, fibroblasts are instrumental in immune cell recruitment and modulation of their

tissue retention as well as survival in the damaged tissues (Pierer *et al.*, 2004; Brentano *et al.*, 2005; Bombardieri *et al.*, 2011).

In mammalian models it has been shown that fibroblasts define what Personage and colleagues called the ‘stromal address code’, which is transcriptionally encoded positional information (anatomical and intra-tissue). Based on the unique transcriptional profiles, fibroblasts signal their anatomical as well as intra-tissue position and modulate the migration and retention of haematopoietic cells (Parsonage *et al.*, 2005).

The mechanisms by which fibroblasts modulate the haematopoietic cells can be classified into the following categories:

- 1) Expression of cytokines and chemokines
 - Such as CXCL12 as discussed above, that can result in recruitment and proliferation of immune cells. Other such factors include IL1 β , IL8, PGE2 (Louault *et al.*, 2020).
- 2) Release of extracellular vesicles
 - Such as TGF- β stimulated release of PD-L1-containing extracellular vesicles capable of inhibiting the proliferation of T cells (stimulated in response to T cell receptor stimulation) (Kang *et al.*, 2020).
- 3) Modulation of ECM stiffness
 - This is discussed in further detail below.

Fibroblast-mediated increase in tissue stiffness, a hallmark of organ fibrosis as well as solid tumour microenvironment, can directly and indirectly influence the immune system. Mesenchymal cells, as the primary structural tissue components, are the masterminds behind these changes. There are numerous ways through which changes in the ECM can directly alter the behaviour of haematopoietic cells. The necessary assumption is the ability of the immune cells to *sense* the changes in the stiffness. Multiple studies have shown that immune cells indeed have conserved mechanosensing receptors which include integrins, cadherins,

selecting, cell adhesion molecules or ion channels (Huse, 2017; Zhang et al., 2020; Zhu et al., 2019).

Mechanotransduction refers to the process through which cells convert extracellular mechanical (biophysical) stimulus into intracellular (bio)chemical signalling. The intracellular signalling can cause cytoskeletal re-organization as well as genetic and epigenetic regulation (Huse, 2017; Zhu et al., 2019). The intracellular biochemical signalling is generally mediated by YAP/TAZ and TEAD. YAP/TAZ are transcriptional co-activators regulated by the Hippo signalling pathway and by cytoskeletal tension. The expression of YAP/TAZ and their co-activator TEAD (necessary for DNA binding) has been described throughout the haematopoietic cell family, including T cells, B cells or macrophages (Pan *et al.*, 2019).

In general, tissue stiffness has been shown to mediate macrophage polarization *in vitro* and *in vivo*, with stiffer matrix causing transcriptional changes indicative of pro-inflammatory phenotype with impaired phagocytotic ability (Zhang et al., 2020). Mechanistically, dendritic cells which are important for T cell activation, showed reduced migration in stiff matrix, impairing their ability to evoke an immune response (Liu *et al.*, 2014). Similarly, *in vitro* experiment showed that high collagen density matrix caused significantly reduced T cell proliferation *in vitro* while the proliferation of cancer cells was unaffected by the collagen density. Moreover, the authors observed a significant reduction in cytotoxic T cells in human mammary tumours which were marked by high collagen-density, altogether suggesting that increased ECM stiffness, mediated through fibroblasts, negatively correlates with T cell infiltration in tumours and can be implicated in the successful evasion of immunosurveillance that marks cancer progression (Kuczek et al., 2019; Nicolas-Boluda et al., 2021).

4.1.3.2 Response to a pathogen/infectious stimuli

In addition to their ability to respond to and mediate ECM stiffness, fibroblasts are also capable of pathogen recognition, through toll-like receptors (TLRs), activation of immune-

related signalling pathways and activation of an effector mechanism to deal with the invading pathogen (Bautista-Hernández *et al.*, 2017). Effective pathogen recognition and appropriate immune response appears to be necessary for long-term survival, with related signalling pathways conserved across the vertebrate kingdom. TLRs are transmembrane proteins that recognize generic pathogens-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). TLRs are conserved across vertebrates and invertebrates and thus represent a truly ancient mechanism of immune defence (Janeway & Medzhitov, 2002).

In humans, 10 TLRs have been reported to this date. TLRs found on the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6) recognize primarily cellular wall or flagellin of gram-negative bacteria (TLR5). Intracellular TLRs, TLR3, TLR7, TLR8, TLR9 are involved in detection of intracellular nucleic acids, for example viral particle (Armant & Enton, 2002; Kumagai & Akira, 2010). TLR signalling pathways are summarized in Fig. 4.2.

Evidence from mammalian models shows that stimulation of fibroblasts' TLR receptors leads to cell activation and pro-inflammatory cytokine gene expression. Cheng and colleagues showed that skin fibroblasts express functional TLRs, in particular TLR1-9 whose stimulation result in synthesis of cytokines and antimicrobial peptides such as IL-6, IL-8, and MMP1. The secretion of these proinflammatory cytokines leads to neutrophil and leukocyte recruitment (Cheng *et al.*, 2015).

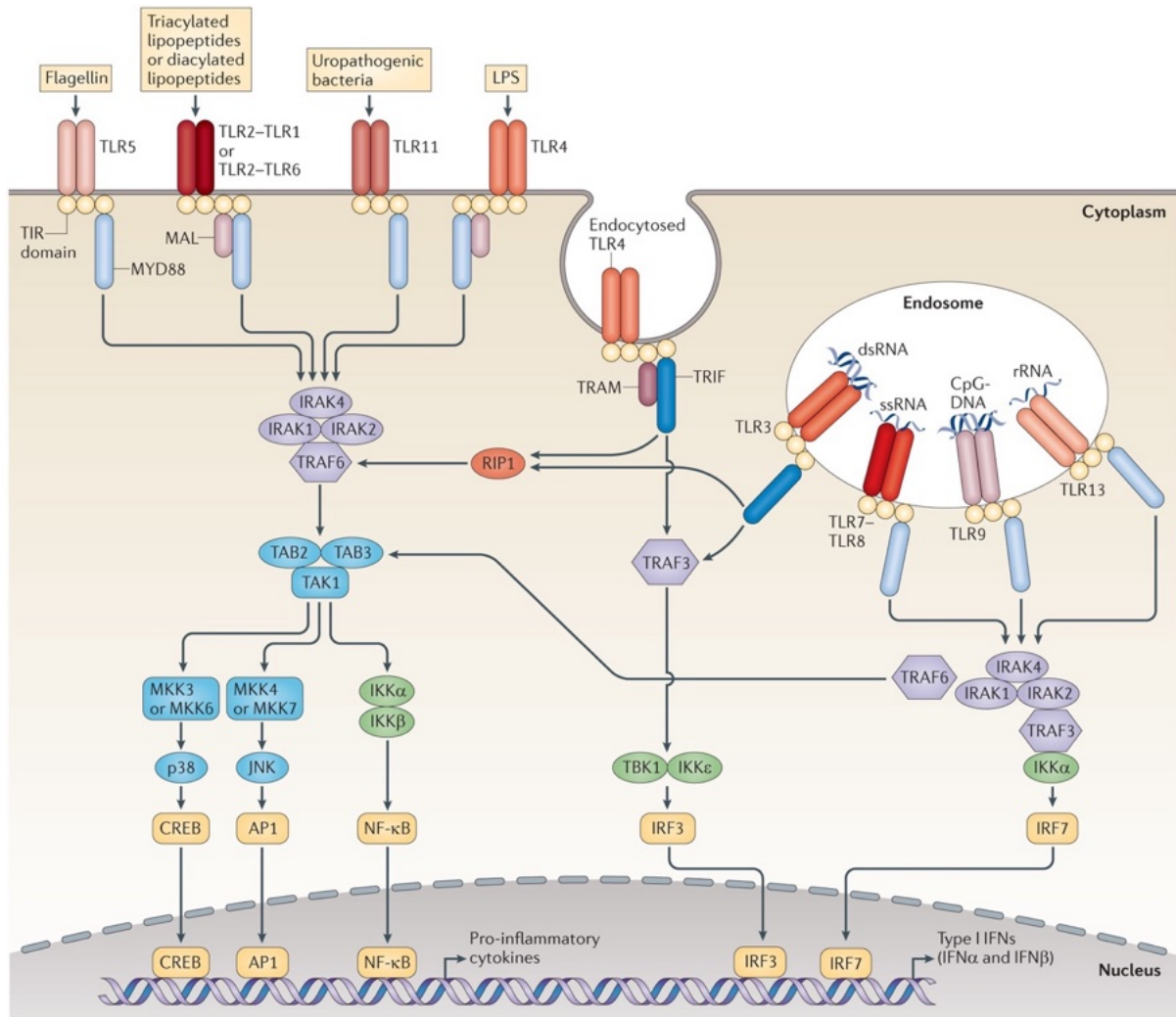


Figure 4.2 Toll-like receptor (TLR) signalling overview

The TLRs can be divided into receptors located primarily on the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6) and those located within intracellular vesicles (TLR3, TLR7, TLR8, TLR9). TLR-mediated recognition of a pathogen-associated- or danger-associated molecular pattern (PAMP, DAMP), the TLR changes conformation, leading to recruitment of adapter proteins (including TRIF, TRAM, MyD88, TIRAP). TLRs located on the cell surface recruit primarily MyD88 and TIRAP adapter proteins, followed by activation of the NF- κ B pathway. TLR8 and TLR9 also recruit MyD88 to signal through either of the two important families of transcription factors, NF- κ B and/or IRFs. Transcription factor translocation into the nucleus leads to induction of pro-inflammatory cytokines and the induction of type I interferon. dsRNA, double-stranded RNA; IKK, inhibitor of NF- κ B kinase; LPS, lipopolysaccharide; MKK, MAP kinase kinase; RIP1, receptor-interacting protein 1; rRNA, ribosomal RNA; ssRNA, single-stranded RNA; TAB, TAK1-binding protein; TAK, TGF β -activated kinase; TBK1, TANK-binding kinase 1 (adapted from O'Neill et al., 2013).

Moreover, *in vivo* stimulation of TLR9 receptors on fibroblasts by TGF- β treatment leads to the activation and transdifferentiation of these fibroblasts into myfibroblasts expressing and

secreting including IL-6 and IFN- γ . The following pro-inflammatory and pro-fibrotic microenvironment resulted in exacerbated pulmonary fibrosis in mice (Kirillov *et al.*, 2015).

Datamining of scRNA-datasets showed that zebrafish also expresses several TLRs on their mesenchymal-type cells, although the investigation of specific fibroblast-TLR stimulation and its effects on immune system is lacking (Jiang *et al.*, 2021). In our RNA sequencing, the read-counts of *tlr8a* were found to be significantly lower in *fibina/b^{-/-}* fish. Zebrafish *tlr8a* is orthologous to mammalian TLR8 which recognizes ssRNA. And although the mechanism remains unclear, this finding suggests that presence or absence of functional Fibin(s) may affect, at least on transcriptional level, genes involved in recognition of components of viral particles and thus influence response to the particular stimulus.

Previous research in the Dallman laboratory used a well characterized viral mimetic compound, R848, that is recognized by TLR7 and TLR8 receptors in mammals, to investigate zebrafish as a model of viral infection. In agreement with other studies, Progozky and colleagues showed that R848 stimulation of the human and mouse nasal mucosal tissue resulted in a robust pro-inflammatory cytokine response. Interestingly, the immune response in zebrafish gills followed a very similar kinetics and dynamics, suggesting a conserved molecular signalling through the TLR7/8 receptors in these 3 species (Progozky *et al.*, 2019). In agreement with these findings, an older study from 2012 investigating IFN responses in salmon cell lines showed that cell lines which had low or absent expression of *tlr7* and *tlr8* failed to induce proper inflammatory cytokine response upon viral stimulation (Svingerud *et al.*, 2012).

Further research by a previous laboratory colleague Dr Madina Wane demonstrated that intraperitoneal injection or topical gill application of R848 in zebrafish leads to significant transcriptional changes in the gills as well as in the WKM. Dr Wane also showed the recruitment dynamics and kinetics of immune cells into the gill tissue following R848 challenge. The recruitment of neutrophils, myeloid cells, lymphocytes, and *cd4-1*-expressing T cells was investigated using *Tg(lyz:GFP)*, *Tg(lck:GFP)* and *Tg(cd4-1:mCherry; lck:GFP)*

zebrafish transgenic lines respectively. The number of *lck*:GFP⁺ cells (lymphocytes) in the gills was significantly increased at 3- and 8-hours post R848 stimulation as compared to controls (Wane, 2021). Whether the TLR7/8 receptors stimulated are found on mesenchymal cells and/or other cell type remains unclear. Altogether, these results indicate an important role of TLR7/8-mediated response to viral infection (or viral mimetic compound) in zebrafish at the transcriptional and cellular levels. In the following experimental part of this chapter, I will therefore use R848 to challenge the wild-type and *fibina/b*^{-/-} fish gills to gain further insight into the differential regulation of immune-related genes between these fish.

4.2 Aims

In line with the results of our RNA-seq analysis, where several immune-related genes showed differential expression in *fibina/b*^{-/-} mutants compared to wild-type fish, I hypothesize that lack of Fibins leads to altered/aberrant/dysregulated interactions between the mesenchymal (fibin-expressing) and the immune cells ultimately altering the *fibina/b*^{-/-} fish responses to immunogenic stimulation.

In order to test this hypothesis, I aimed to:

- 1) Characterize the immune-cell populations in the gill and WKM at homeostasis in the *fibina/b*^{-/-} fish. To compare the flow cytometry profile to that of wild-type fish to investigate the similarities and differences in the proportion of immune cell populations in these fish.
- 2) Characterize the local and systemic immune responses of *fibina/b*^{-/-} fish to R848 stimulation by investigating the transcript levels of selected early and late immune response genes in the gill and WKM tissues at 1 and 8 hours post R848 challenge.

4.3 Results

4.3.1 Flow cytometry of adult zebrafish gills and WKM

Firstly, I investigated the hematopoietic cell populations in the gill and the WKM of wild type and *fibina/b^{-/-}* fish to explore the hypothesis that lack of fibins influences the long-term interactions between mesenchymal cells and haematopoietic cells at homeostasis, in the absence of external stimuli. The ages of the fish analysed were 3, 6 and 12 months of age, to include distinct timepoints from adult zebrafish with a fully developed immune-system and matured adaptive immune system responses (Lam *et al.*, 2004).

Flow cytometry as a tool to investigate haematopoietic cells is frequently used in both mammalian and zebrafish studies. Flow cytometry enables discrimination of cells based on their size and granularity. This discrimination is based on the light-scattering properties of the cells with forward scatter (FSC) correlates with cell size and side scatter (SSC) correlates with cell granularity, providing an indication of cell morphology.

Traver and his colleagues demonstrated that FSC/SSC scatter plot of cells from adult zebrafish WKM can distinguish the major haematopoietic cell lineages including erythrocytes, lymphocytes, myeloid and precursors (Traver *et al.*, 2003). These cellular properties were subsequently used to define the haematopoietic cells from other tissues and organs, including peripheral blood, spleen, thymus and the gill (Langenau *et al.*, 2004; Stachura and Traver, 2011).

Fig. 4.3 shows flow cytometry analysis of the immune-cell population proportions in the gills and WKM for wild-type and *fibina/b^{-/-}* fish. Statistically significant differences were found only between the lymphocyte-gated populations at 12 months of age in the gill and WKM tissue ($p = 0.005$ and $p = 0.017$) respectively). The percentage of events in the lymphocyte gated population of wild type fish were 32.2% (SD = 2.1) and 23.4% (SD = 4.4), for gill and WKM respectively. While in *fibina/b^{-/-}* fish the recorded percentage of events was 27.9% (SD = 2.9) and 17.9% (SD = 2.8) for gill and WKM respectively. Interestingly, at 6 months, the trend – although not significant ($p = 0.13$ for gills and 0.14 for WKM lymphocyte population) - is exactly opposite for both tissues investigated, meaning that while at 6 months of age it

appears there are more cells in the lymphocyte-gated population in the *fibina/b^{-/-}* fish, at 12 months there were significantly less cells in the lymphocyte gated population in these fish.

The right panels in Fig. 4.3 (B, C) show the trend in gated-population percentages across time. The changes in the percentages for both erythrocytes and lymphocytes in the gills and in the WKM show strong variation. As data is not available for fish beyond 12 months of age, it is impossible to determine if the difference seen in 12 months old fish may point to a steady decrease in lymphocyte-gated population percentage in *fibina/b^{-/-}* fish with increasing age or rather reflects potential dynamic changes in the immune cell populations across time (as at 6 months the trend was opposite with mildly increased percentages of lymphocyte-gated population in *fibina/b^{-/-}* compared to wild-type fish).

Our RNA-seq was performed with fish at 6 months of age and showed significant differences in the transcript levels of immune-related genes, in particular, significantly fewer read counts detected in the *fibina/b^{-/-}* fish. The flow cytometry results from 6 months old fish showed no significant difference in proportion of any of the investigated immune cells (erythrocytes, lymphocytes, myeloid and precursors), suggesting that the potential differences in immune-related genes between the wild-type and *fibina/b^{-/-}* are not caused by the proportions of individual gated cell population. It is important to note that the total number of cells in each gated population was not determined, however, the total number of events recorded varied greatly from fish to fish, irrespective of the genotype (Appendix 5).

My results demonstrate that, for the populations analysed, lack of *fibina* and *fibinb* is unlikely to cause significant shift in the make-up of the immune system of the affected fish especially at 6 months of age and therefore the observed differences in gene expression are likely not caused by differences in abundance of particular immune cells in the gill.

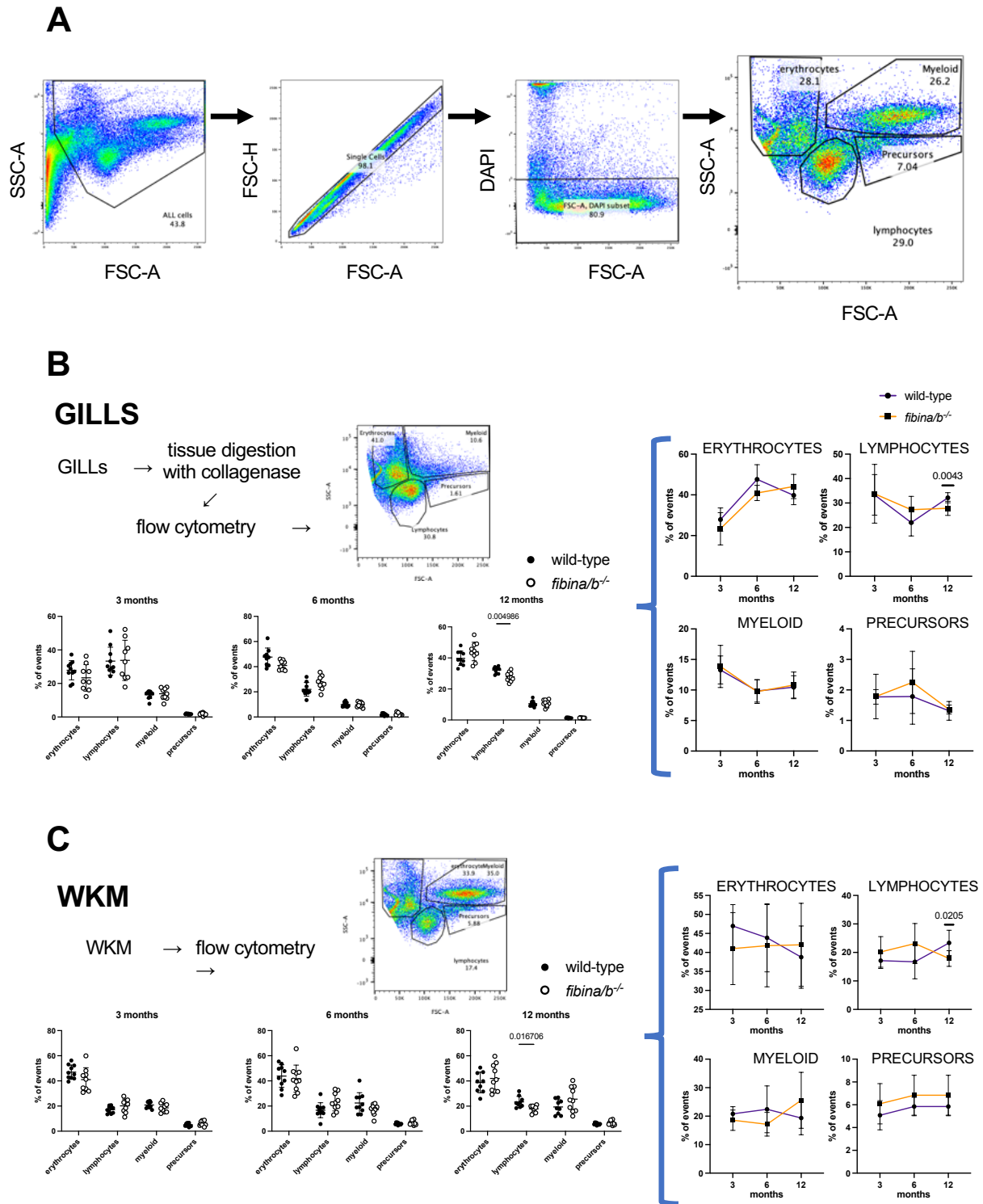


Figure 4.3 Flow cytometry of gill and WKM cells from wild-type and *fibina/b^{-/-}* adult zebrafish.

(A) Gill tissue from both wild-type and *fibina/b^{-/-}* fish was digested with collagenase and, similarly to WKM processing, was manually dissociated into a single cell suspension (tissue was passed through a 40 μ m cell strainer). Overview of the flow cytometry gating strategy and FSC/SSC profiles of gill (B) and WKM (C) tissue. Single cell suspension was then processed by flow cytometry to identify live cells. Debris was excluded on FSC-A/SSC-A, singlet cells were selected on FSC-A/FSC-H (height), and finally DAPI-cells were selected on FSC-A/DAPI. Gates are shown in black outlines.

Representative WKM sample from wild-type fish following live cell gating. Values in plots reflect percentages of events in each gate compared to total events in plot. Relative percentages of cell populations of gill tissues from 3, 6, and 12 months old, age matched, wild-type and *fibina/b^{-/-}* fish. The right panel represents the same data as shown on the left, comparing the individual population numbers across the 3 ages investigated. Relative proportion of cell populations of WKM from 3, 6, and 12 months old, age matched wild-type and *fibina/b^{-/-}* fish. The right panel represents the same data as shown on the left, comparing the individual population numbers across the 3 ages investigated. Each dot represents individual fish, n = 10, Mean and SD shown for all graphs. Two-way ANOVA performed.

4.3.2 Gill and WKM immune responses to gill tissue stimulation with R848

Genes with significantly reduced read counts in *fibina/b^{-/-}* fish were enriched for genes involved in response to stimuli and intracellular signalling. Specifically, *tlr8a*, homologue of the human *TLR8* gene that codes for the transmembrane pattern-recognition receptor TLR8 showed lower read counts in *fibina/b^{-/-}* gill and caudal fin tissues. Therefore, I have subjected fish to challenge with R848 to investigate whether the proposed differences in immune-related genes between the *fibina/b^{-/-}* fish and wild-type fish translate into differential ability to mount an immune response to stimuli.

R848 is a viral mimetic that was shown to induce a potent immune response in the zebrafish gill as well as in the WKM (Lin et al., 2020; Progatezky et al., 2019). In the following experiments, I have applied the R848 or endotoxin-free water directly onto fish gills. Both the gill and the WKM tissues were then harvested at 1 or 8 hpt and processed for qRT-PCR. The WKM tissue was collected to determine the more systemic immune response to a locally applied (gills) viral mimetic. The timepoints investigated were chosen based on previous research that suggested these two timepoints to allow for detection of both the early and late cytokine response (Progatezky et al, 2019; Wane, 2021). Thus, allowing me to detect possible difference in the dynamics or the kinetics of the immune response between *fibina/b^{-/-}* and wild-type fish. Fig. 4.4 shows the gene transcript levels of selected cytokines for both, the gill and the WKM tissues.

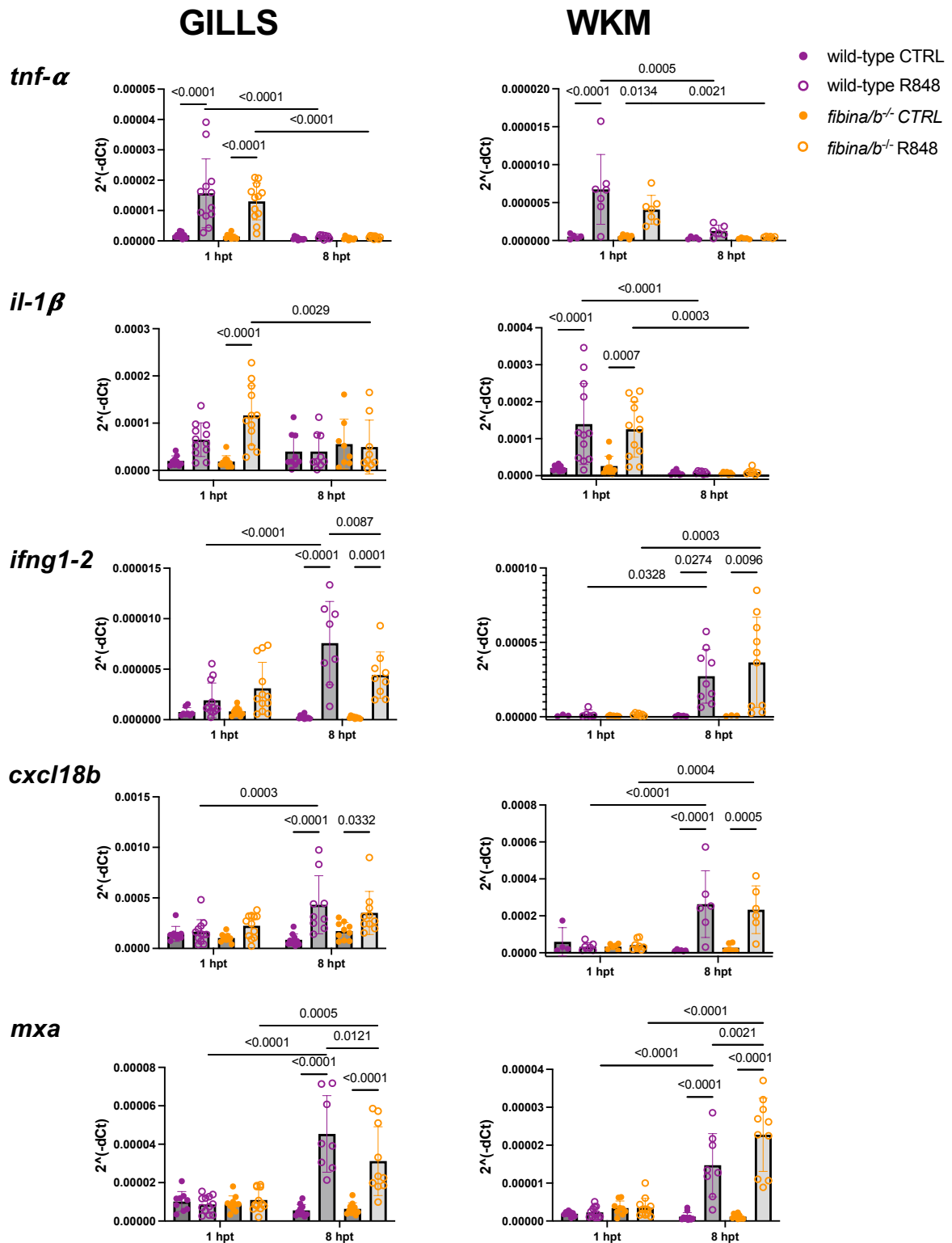


Figure 4.4 Gene transcript analysis of the gills and WKM from R848-challenged wild-type and *fibina/b*^{-/-} fish

R848 (5 μ l at 0.5mg/ml) or endotoxin-free water was topically applied to the gills of wild-type and *fibina/b*^{-/-} fish. Gill tissue and WKM tissues were collected at 1 or 8 hpt and processed. Transcript levels of selected genes were measured by qRT-PCR and are shown relative to 18S transcript levels. Each data point represents an individual fish. $n = 6-10$ fish per data point. One-way ANOVA or Welch's ANOVA was performed, followed by Didak's multiple comparison test.

R848 successfully stimulated proinflammatory cytokines, *tnfa* and *il1b*, type II interferon *ifng1-2* and chemokine *cxcl18b* in *fibina/b^{-/-}* fish. The early immune response was marked by increase in *tnfa* and *il1b* transcript levels at 1 hpt while the late response was marked by increase in *cxcl18b* and *ifng1-2* transcript levels at 8 hpt. Both the WKM and the gill showed the same kinetics of the investigated immune genes. The genes investigated were selected based on previously published observed responses to R848 stimulation in the gills (Progatzky *et al.*, 2019).

No difference was detected in *tnfa* or *il1b* fold-change between wild-type and *fibina/b^{-/-}* fish at either timepoint. Significantly lower *ifng1-2* transcript levels were detected in *fibina/b^{-/-}* fish compared to wild-type fish treated with R848 in the gill at 8 hpt but no difference ($p = 0.6$) was observed in the WKM. Although this result could suggest different kinetics or dynamics between the WKM and the gill immune response to topical gill R848 administration, the spread of the values especially for wild-type R848 treated fish is too large to provide a strong support for such notion.

Transcript levels of *mx*a, myxovirus (influenza) resistance A, were also significantly less increased upon R848 stimulation in *fibina/b^{-/-}* fish at 8 hpt compared to wild-type. Interestingly, in the WKM, the trend is exactly opposite with transcript with transcript levels of *mx*a significantly higher in *fibina/b^{-/-}* compared to wild-type fish.

Mx_a, encoded by *mx*a, is homologous to human *MXA*. Mx genes exist in nearly all vertebrates and protect the host mainly against RNA viruses by inhibiting the viral replication either in the cell cytoplasm or nucleus (Verhelst, Hulpiau and Saelens, 2013). The upregulation of Mx expression in response to viral (or in this case R848) stimulation is indirect, dependent on type I and type III interferons. It was determined that the Mx promoter in mice does not respond to either type II interferon (*ifng1-2*) or *tnfa* (Hug, *et al.*, 1988; Aebi *et al.*, 1989; Simon *et al.*, 1991; Holzinger *et al.*, 2007). As WKM tissue analysis was used as a way of investigating the more systemic effects of local R848 gill stimulation and knowing that the *mx*a transcript level induction is dependent first on the production of type I and II

interferons, it is possible that at the timepoints investigated, *mxr* regulation differs in terms of kinetics and/or dynamics between the two tissues investigated, leaving the impact of (lack of) fibins unclear.

The neutrophil chemoattractant *cxc118b* transcript levels did not show the same significant differences as the anti-viral encoding *ifng1-2* transcript levels in the gill tissue and there was also no difference in *cxc118b* transcript levels detected in the WKM of *fibina/b^{-/-}* fish compared to wild-types.

4.3.3 Gill immune responses to stimulation with R848 in wild-type fish and *fibina/b^{-/-}* fish on TraNac background

The results from our RNA seq suggested rather dramatic differential regulation of number of immune-related genes, some of which involved in response to stimulus. However, the modest transcriptional differences between the wild-type and *fibina/b^{-/-}* fish observed upon R848 stimulation do not support this interpretation of the RNA sequencing results.

One of the challenges that arose during the generation of *fibina/b^{-/-}* fish was different pigmentation pattern that all of our *fibina/b^{-/-}* mutants (with *fibina109d120^{-/-}*; *fibinb226d232^{-/-}* mutation) had. As opposed to regular wild-type zebrafish, characterized by three distinct classes of pigment cells arranged in stripes – black melanophores, reflective iridophores and yellow xanthophores, all mutants acquired a spotty pigmentation (White *et al.*, 2008). Although it is not uncommon to see spotty wild type zebrafish, there are number of mutations that affect the immune system that also cause a change in the pigmentation (Crowhurst, Layton and Lieschke, 2002; Zodrow and Tanguay, 2003).

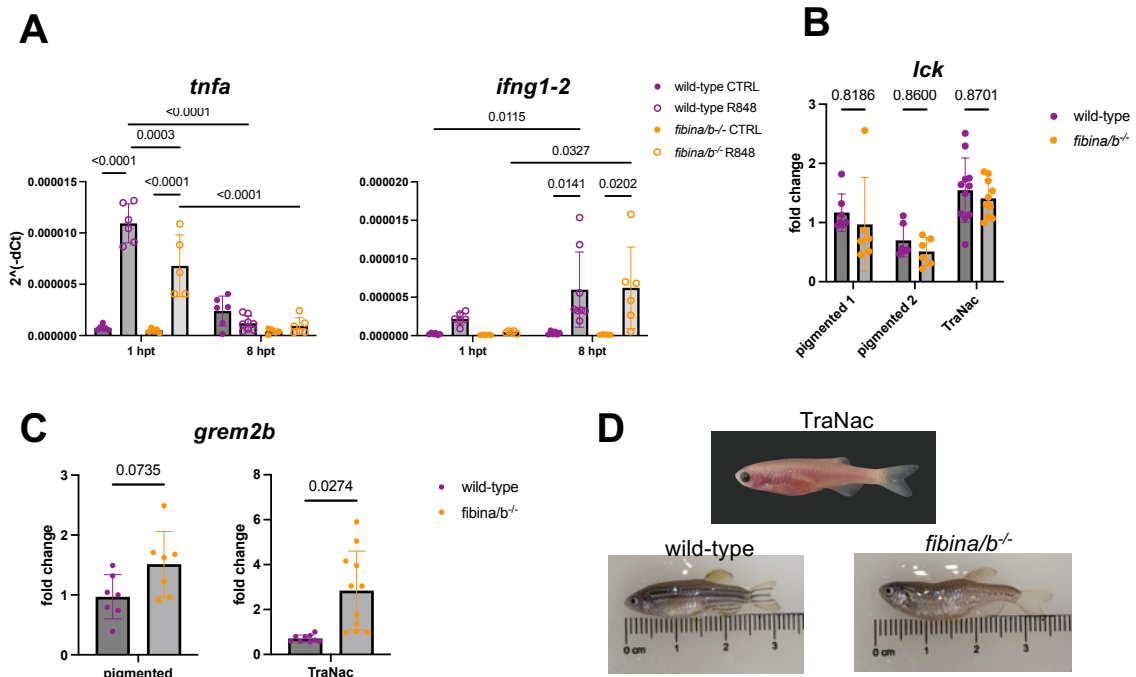


Figure 4.5 Transcript level analysis of selected genes in wild-type and *fibina/b^{-/-}* fish on TraNac- background

(A) Gene transcript analysis of the gill tissue from R848-challenged wild-type and *fibina/b^{-/-}* fish on TraNac background. R848 (5 μ l at 0.5mg/ml) or endotoxin-free water was topically applied to the gills of wild-type and *fibina/b^{-/-}* fish. Gill tissue and WKM tissues were collected at 1 or 8 hpt and processed. Transcript levels of selected genes were measured by qRT-PCR and are shown relative to 18S transcript levels. Each data point represents an individual fish. $n = 5-8$ fish per data point. (B) Transcript levels of the leukocyte specific marker *lck* in wild-type and *fibina/b^{-/-}* mutant fish on pigmented and TraNac background. Data is compared to pigmented 1 wild type fish. (C) Transcript levels of BMP-signalling pathway antagonist *grem2b* in wild-type and *fibina/b^{-/-}* mutant fish on pigmented and TraNac background. Data is compared to wild-type fish in the respective experiment. Each data point represents an individual fish. Multiple student t-tests were performed to determine statistical significance. P-values < 0.05 are plotted. (D) Comparison of wild-type and *fibina/b^{-/-}* pigmented fish and the TraNac fish (no visible difference between wild-type and *fibina/b^{-/-}* fish on TraNac background).

To investigate that the difference between the wild-type and the *fibina/b^{-/-}* fish as detected in our RNAseq is not linked to different pigmentation pattern, but rather caused by the lack of functional fibins, I have repeated the R848 gill challenge in wild-type and *fibina/b^{-/-}* zebrafish on a TraNac background (Fig. 4.5). TraNac refers to zebrafish with a double mutation in *transparent* (*tra^{b6/b6}*) and *nacre* (*nac^{w2/w2}*) genes which leads to apoptosis of melanophores and iridophores respectively (Lister *et al.*, 1999; D'Agati *et al.*, 2017). Owing to their transparent nature, TraNac fish have been widely used for intravital microscopy and *in vivo*

cell tracking among others. None of the mutations has been shown to have significantly adverse effect on the fish health, however, it is important to keep in mind that very limited literature exists that directly compares wild-type pigmented and ‘wild-type’ TraNac fish immune responses or development (Lister *et al.*, 1999; White *et al.*, 2008).

Transcript levels of *tnfa*, a representative gene for early-immune response, were significantly increased in both wild-type and *fibina/b^{-/-}* fish at 1 hpt with R848 compared to control and this increase was significantly lower in mutant fish. *Ifng1-2* transcript levels, late immune-response gene, were significantly increased at 8 hpt with R848 in both wild-type and mutant fish. There was no difference in the levels of transcripts between the two fish.

Direct comparison between the pigmented background and the TraNac background (Appendix 6) showed an overall trend where the cytokine transcript levels were lower in the TraNac fish after R848 stimulation compared to pigmented fish, irrespective of the genotype (wild-type or *fibina/b^{-/-}*). In particular, *tnfa* transcript levels in *fibina/b^{-/-}* fish were significantly lower at 1 hpt with R848 in the fish on TraNac background compared to pigmented background. And in the wild type fish, *ifng1-2* transcript levels at 8 hpt with R848 were significantly lower in TraNac-background fish. As the lower increase in cytokine levels was detected in both wild-type and *fibina/b^{-/-}* mutants on TraNac background, it is possible to conclude that the differences in pigmentation patterning (that is stripy for wild-type and spotty for *fibina/b^{-/-}* fish) are not related to the differences in immune-related genes as detected in our RNAseq.

Finally, to further confirm that pigmentation pattern-differences were unlikely to be responsible for the detected differences in our RNA-seq, *lck* transcript levels were measured in wild-type and *fibina/b^{-/-}* gill samples from both pigmented and TraNac fish. Previously (Chapter 3), *lck* transcripts were found to be significantly lower in the mutant fish subjected to the RNA seq. Fig. 4.5(B) shows that in three separate fish batches (wild-types and *fibina/b^{-/-}* mutant fish are always age-matched within one experiment) irrespective of the pigmentation, the transcript levels of *lck* do not differ between the wild-type and *fibina/b^{-/-}*

fish. To understand better if this inconsistency was associated with the immune-related genes only, I have measured the levels of another differentially expressed gene from our RNA seq, *grem2b*. *fibina/b^{-/-}* fish had significantly higher levels of *grem2b* compared to wild-type in the RNA seq, and this was replicated in subsequent, unrelated, experiments with fish on pigmented and TraNac background (Fig. 4.5 (C)).

Overall, there is a great lack of knowledge regarding our understanding of the immune and developmental differences between pigmented wild-type and TraNac fish. My data, however, suggests that such differences exist and need to be investigated further to have a better understanding of the models that we use to model human diseases.

As no consistent pattern of differential response to injury was detected between the *fibina/b^{-/-}* and wild-type fish and I was not able to replicate the immune-related gene, *lck*, differences, the data suggests that lack of *fibina* and *fibinb* does not manifest itself through modulation/regulation of the fish immune system.

4.4 Discussion

Functional enrichment analysis of DEG from our RNA sequencing analysis identified a significant number of immune-related genes in *fibina/b^{-/-}* fish, in particular genes related to response to stimulus. As Fibins are expressed both in the stromal cells of the haematopoietic niche in developing zebrafish (Tang *et al.*, 2017) and in the mesenchymal cells of adult zebrafish (Jiang *et al.*, 2021), I have used flow cytometry and qRT-PCR to investigate the potential impact of lack of Fibins on the immune-cell make-up of adult zebrafish and the immune system ability to mount a response to a viral mimetic compound, R848.

4.4.1 Immune cells in gill and WKM tissues of *fibina/b^{-/-}* mutant fish

Flow cytometry analysis of 3 and 6 months old wild-type and *fibina/b^{-/-}* fish did not reveal significant differences in the relative percentages of the erythrocyte-, lymphocyte-, myeloid-, and precursor-gated populations in the gill and whole kidney marrow between wild-type and *fibina/b^{-/-}* fish. Lymphocyte-gated population was significantly smaller in *fibina/b^{-/-}* fish at 12 months of age in both the gill and the WKM tissues. However, as no data was obtained beyond the 12 months timepoint, and the fluctuation of the percentages of individual gated-population between the 3-6-12 months old fish was substantial, it was not possible to state with certainty that there was a decrease in the *fibina/b^{-/-}* fish lymphocyte-gated population correlated with age.

Previous work by Dr Madina Wane investigated immune cell composition as well as the architectural changes in the zebrafish gills over time from larval – juvenile – adult – to aging fish (Wane, 2021). Dr Wane showed that although the gills undergo significant immunological and structural changes between 21 dpf and 1 mpf no further changes – apart from increase in size - in the gill structure or immune cell composition were observed (up to 21 months of age). In her work, Dr Wane used number of transgenic lines, including Tg(*lck*:GFP) line which allows tracking and sorting of *lck*⁺ cells. The results showed that following flow cytometry analysis, the *lck*:GFP⁺ cell population were almost entirely found in the lymphocyte gate across the ages investigated which included 2 mpf, 6-10 mpf and 18-21 mpf fish. The relative percentage of this lymphocyte-gated population remain constant

across the ages at about 20% (of all events recorded by flow cytometry from the gills). My results show a more varied percentages ranging from 20-40% of the total number of events detected in the gill tissue for both wild-type and *fibina/b^{-/-}* fish. Although the mechanisms behind such relationship remain unclear, based on the comparison of pigmented vs TraNac *lck* transcript levels (Fig. 4.5 (B)), this could be related to the different pigmentation background used in the experiments: Dr Wane used transgenic fish on TraNac background while I used fish with pigmented background. However, more research is indeed required to firstly confirm whether or not the proportion of leukocyte-gated population remains constant with increasing age and secondly to investigate in depth the similarities and differences between pigmented and TraNac zebrafish.

The exact implications of potential changes in immune cell-proportions in aging zebrafish are yet to be fully dissected. From mammalian models of aging, it is apparent that there are rather dramatic changes happening to the lung architecture and this is correlated with the changes in immune cell composition with increasing age. This includes enlargement of alveolar spaces with increased immune cell accumulation (Calvi *et al.*, 2011; Malinina *et al.*, 2020). Interestingly, in the zebrafish, the Dallman group previously showed that long-term cigarette smoke exposure did not result in chronic inflammation and observed structural changes were reversible following cessation of smoke exposure (Progatzky *et al.*, 2016). Suggesting that zebrafish gills in particular, have an ability to regulate inflammation long term. In 2020, Bottiglione and colleagues investigated the structural and transcriptional implications of lack of *il10* in *il10^{-/-}* zebrafish. The authors proposed an association between persistent inflammation, immune cell retention in the gill tissues and significant structural changes to the gill architecture in the *il10* mutant zebrafish (Bottiglione *et al.*, 2020). This means that optimal cytokine function is necessary for maintaining healthy structural and cellular environment in the gills and that dysregulated immune system can have effects on the structure of the gills, beyond the expected impact on gill mucosal tissue homeostasis.

The gills contain, next to the haematopoietic cell populations, considerable number of non-immune cells which may share some FSC/SCC properties with the immune cells (Gomariz *et al.*, 2018). Therefore, although FSC/SSC can provide the estimate of haematopoietic

populations proportions, it is impossible to say which of the gated populations may have been ‘contaminated’ with non-immune cells and to what extent. This discrepancy is potentially amplified by the gill digestion protocol where it is not possible to determine how many of the non-immune cells were digested through to the single cell suspension analysed. To this end, the results of the WKM haematopoietic cell populations provide a useful comparison, as any systemic differences in the immune cell composition are likely to be reflected in the WKM as well as in the gill tissue. The strong variation in gated populations across ages, together with the comparative trend in the immune-cell population proportions across both the gill and the WKM suggests that if there is a difference in the immune cell make-up between wild-type and the *fibina/b^{-/-}* fish, it is very subtle and it is rather unlikely that this difference can explain the differences detected in our RNA seq.

4.4.2 Immune response to R848 challenge in *fibina/b^{-/-}* mutant fish

Previous research has found that R848 induces the upregulation of inflammatory cytokines in the gills and the WKM of zebrafish, in a time-specific manner (Progatzky et al., 2019; Wane, 2021). Here, gene transcript analysis was used to compare the response to topical R848 stimulation in the gill and WKM between the wild-type and *fibina/b^{-/-}* fish to further investigate results obtained from RNA seq, suggesting a differential ability to respond to stimulus.

Results of R848 stimulation of wild-type gills replicated previously published results (Progatzky et al., 2019; Wane, 2021). Gill R848 stimulation in the mutant fish also successfully induced inflammatory mediators, including cytokines and IFN, both locally (in the gill tissue) and more systemically (in the WKM). No consistent differential regulation of either of the studied cytokines was detected across the two tissues investigated, the gill and the WKM. *Ifng1-2* transcript levels were lower in *fibina/b^{-/-}* fish upon R848 stimulation at 8 hpt compared to wild-type, however, no such trend was detected in the WKM. Similarly, *mxr* transcript levels were differentially regulated in wild-type and mutant fish at 8 hpt with R848, showing opposite trends in the gill and in the WKM. WKM responses showed that mutant fish were capable of mounting a systemic immune response that levelled up to wild-type fish immune responses. However, lack of difference between the wild-type and the *fibina/b^{-/-}* fish

does not necessarily contradict the differences observed in the gill tissue upon R848 stimulation. Several factors can contribute to the differences observed, including differences in the cellular composition of the gill and WKM as well as altered kinetics/dynamics of the immune response to topically applied R848. Previously, analysis of our RNA seq showed that *fibina/b^{-/-}* fish had detectable less *tlr8a* transcript levels in the gill tissue at homeostasis, proposing the hypothesis that *fibina/b^{-/-}* fish immune response to stimuli may be less strong compared to wild-type fish (in the gills). However, the assumptions behind such hypothesis are numerous: a) *tlr8a* is expressed in the mesenchymal cells in zebrafish gill b) the transcript levels of *tlr8a* are proportional to the number of functional receptors; c) the magnitude of the immune response to viral mimetic is proportional to the number of functional receptors. Considering that none of the other genes investigated showed any difference in the number of transcript levels between wild-type and *fibina/b^{-/-}* fish, I believe that any biologically relevant difference between wild-type and *fibina/b^{-/-}* fish's ability to mount an immune response to viral mimetic is very subtle and more research is needed to determine the molecular basis behind Fibin-related immune gene regulation as well as the immunological impact of such difference.

I was not able to replicate the results of the RNA-seq for the *lck* gene. *lck* was used as it was the only gene with known function, found to be differentially regulated between *fibina/b^{-/-}* fish and wild-type fish at homeostasis, that is a known and well described marker for a specific immune cell population – leukocytes. I tested the *lck* levels in fish from 3 different sets of experiments where *fibina/b^{-/-}* fish and wild-type fish were always age-matched, in both pigmented and TraNac background. However, replicating the results for another, non-immune-related gene, *grem2b*, was successful (Fig. 4.5 (C)). This result does lead to the question of possible immune challenge present in our fish tanks that might be desynchronised between the tanks. The *lck* transcript levels differed greatly across the 3 experiments analysed with rather smaller intra-experimental variation (with the exception of *lck* levels in 'pigmented 1' *fibina/b^{-/-}* fish which showed large variation with no statistical outliers). Unintended, tank-specific infection in our control group of fish sent for RNA-seq could cause the upregulation of number of immune genes which would manifest in our RNA-seq as downregulated genes in our *fibina/b^{-/-}* fish compared to wild-type. Regular health checks are

carried out on all of our water systems and fish are sent for routine testing several times a year. There has never been a tank-specific infection detected through these screens.

Today, it is impossible to determine if there was or was not an infection in any given tank as well as for how long a pathogen might have been present there. And because our RNA-seq was the first in depth look for Fibin-related regulatory pathways, there is no other baseline for *fibina/b^{-/-}* fish' immune system-related gene expression. Therefore, until more Fibin-related data is available both through targeted experiments and transcriptome data from online datamining, the experimental results here support the conclusion that there is no significant difference in the response to stimuli between the wild-type and *fibina/b^{-/-}* fish.

4.5 Conclusion

Overall, no consistent pattern of differences between wild-type and mutant fish was detected when investigating the immune-cell populations in the gill and WKM. And although the exact cell numbers of the individual immune subpopulations could not be determined using flow cytometry, this result indicates that the lack of Fibins in the mesenchymal cells that may interact with the haematopoietic cells, does not lead to significant differences in the haematopoietic cell make-up of adult zebrafish.

Furthermore, no clear pattern of differential response to stimuli between the wild-type and *fibina/b^{-/-}* fish was observed (levels of *cxcl18b* were not significantly different between the fish (Fig. 4.4) And lack of Fibins does not impact the ability of the zebrafish to mount an immune response to stimulus in the form of cytokines and antiviral protein. Of note, all the significant differences that were observed (including 8 hpt *ifng1-2* and *mxr* transcript levels in pigmented fish as well as 1 hpt *tnfa* levels in TraNac fish) showed lower increase in the transcript levels in *fibina/b^{-/-}* fish. This indeed does align with the result of the RNA-seq, suggesting a deficiency in the immune system of *fibina/b^{-/-}* fish. However, as I could not replicate even the differences in the levels of leukocyte-specific marker *lck*, more extensive analysis of the immune system of the *fibina/b^{-/-}* fish, including stimulation with different

pathogens stimulating range of different receptors and downstream signalling pathways, needs to be carried out in order to determine the exact differences that arise as a result of lack of Fibins as well as the possible biological relevance of this finding.

5 BMP SIGNALLING IN DEVELOPMENT AND REGENERATION OF wild-type AND *fibina/b*^{-/-} ZEBRAFISH

5.1 Introduction

Cross-species RNA sequencing (RNA-seq) analysis between regenerating (zebrafish) and non-regenerating (mouse) models of respiratory tissue damage (cryoinjury and bleomycin-induced pulmonary fibrosis respectively), revealed Fibins to be differentially regulated in the two models making them potential genes of interest for understanding scar-less tissue regeneration (Ramel et al, 2021). RNA-seq of gill and caudal fin tissue from wild-type fish and *fibina/b*^{-/-} fish generated in our laboratory revealed DEG significantly enriched in bone morphogenic protein (BMP) signalling related genes in the *fibina/b*^{-/-} fish compared to wild-types. Specifically, the number of BMP antagonists was found significantly increased in *fibina/b*^{-/-} fish, including *grem2b* or *nog2*.

5.1.1 BMP signalling overview

Bone morphogenic proteins (BMPs) are phylogenetically conserved molecules belonging to the TGF- β superfamily. BMPs were first identified by Dr Marshall R Urist as secreted molecules that promote ectopic bone formation when implanted in soft tissues (Urist 1965). Since then, more than 40 types of BMPs have been described (Matthews 2005) and many new functions characterised, ranging from development to homeostatic and regenerative processes (Nishimura *et al.*, 2012; Wang *et al.*, 2014; Windhausen *et al.*, 2015). For example, the BMP play a role in early embryonic development and dorso-ventral patterning, development of vasculature and its maintenance (Miyazono, Kamiya and Morikawa, 2010). In addition to early development, BMP signalling regulates cell proliferation, differentiation and apoptosis (Wang *et al.*, 2014), regulates ECM generation and degradation, induces cartilage and bone formation, and maintains cartilage and bone homeostasis (Shu *et al.*, 2011; Nishimura *et al.*, 2012; Windhausen *et al.*, 2015; Thielen, van der Kraan and van Caam, 2019).

Members of the BMP family and their receptors are highly conserved across the whole animal kingdom, including mice, flies or worms (Kahlem and Newfeld, 2009). Importantly, great functional similarities exist supported by cross-species experimental data. For example, the fly orthologues of BMP2 (Dpp) can induce endochondral bone formation when induced in rats or alternatively, in *Drosophila* lacking Dpp, mammalian BMP4 protein was shown to rescue the dorso-ventral body patterning (Padgett, Wozney and Gelbart, 1993; Sampath *et al.*, 1993; Schmierer and Hill, 2007; Hinck, 2012; Marques *et al.*, 2016).

The schematic of BMP signalling is showed in Chapter 1: Introduction (Fig. 1.5). BMP signalling is mediated through specific cell surface receptors, the bone morphogenic protein receptors (BMPRs). BMP signalling pathway, although following a relatively simple transduction cascade, shows striking tissue-specific complexity, redundancy and pleiotropism.

To enable signal transduction, signalling complex of 2 Type I receptors and 2 Type II receptors is required. Type I receptors possess a Gly/Ser-rich domain that needs to be phosphorylated by Type II receptors to activate kinase activity. Type II receptors are constitutively active (Salazar, Gamer and Rosen, 2016). BMP proteins' preferred receptors are ALK1, 2, 3, 6 (Type I) and ACVR2A, ACVR2B, BMPR2, AMHR2 (Type II), followed by SMAD1/5/8(9) recruitment and phosphorylation during canonical, SMAD-dependent, signalling (Ouahoud, Hardwick and Hawinkels, 2020). R-SMADS phosphorylation leads to formation of a complex with SMAD4 which is a common co-molecule. The complex then translocates to the nucleus and acts as gene expression regulator, partially through their own, weak, transcription factor ability, but mainly through recruitment of tissue specific transcription factors and chromatin-remodelling machinery (Ross *et al.*, 2006; Mullen *et al.*, 2011). Additionally, receptor assembly and phosphorylation can lead to intracellular activation of mitogen-activated protein kinases (MAPKs) such as ERK, JNK or p38, or PI3K/Akt signalling, to mediate and modulate the BMP-dependent cellular response.

5.1.2 Regulation of the BMP signalling pathway

As the complexity of the pathway suggests, the regulatory mechanisms, maintaining optimal, timely and spatially correct expression and/or secretion of BMP ligands and their receptors are multitude.

5.1.2.1 Endogenous regulation of BMP signalling

1) Pro-peptide latency

Similarly to TGF- β , the majority of BMP proteins are first synthesized as pro-peptides before undergoing posttranslational signal sequence cleavage and further modifications. The pro-peptide form of BMP4 has been shown to play a crucial role in the stabilisation of the mature BMP4 structure or intracellular trafficking while pro-BMP2 inhibits the BMP2-induced induction of a marker of osteoblasts differentiation, enzyme alkaline phosphatase (Hauburger *et al.*, 2009).

2) Antagonism by secreted receptors and ligands (Itoh and ten Dijke, 2007; Moustakas and Heldin, 2009; Brazil *et al.*, 2015; Salazar, Gamer and Rosen, 2016)

Most well characterized endogenous antagonists of BMP signalling include noggin,

gremlin and follistatin (Inoue *et al.*, 1994; Brunet *et al.*, 1998; Stafford *et al.*, 2011). All of these are secreted antagonists that act by binding to and sequestering the active ligands in the extracellular space, thus decreasing the availability of these ligands to bind their respective receptors (Balemans and van Hul, 2002; Thompson *et al.*, 2005).

3) **Negative intracellular feedback**

Intracellularly, negative feedback mechanism can be initiated by BMP-dependent SMAD6/7 transcriptional upregulation. SMAD6/7 are known as inhibitory SMADs where SMAD6 interferes with R-SMAD phosphorylation at the cytoplasmic domain of BMP receptors and marks the machinery for degradation. (Moustakas and Heldin, 2009; Brazil *et al.*, 2015; Salazar, Gamer and Rosen, 2016).

5.1.2.2 Pharmacological regulation of BMP signalling

Next to endogenous regulation of BMP signalling, a number of pharmacological agents has been developed to enable us to dissect the role(s) of BMP in various physiological scenarios. The most commonly used BMP inhibitors in zebrafish research are summarized in Table 5.1.

Dorsomorphin (also previously known as Compound C) has been used in the zebrafish research for decades and its name comes from the observed morphological changes detected in zebrafish craniofacial development (Yu *et al.*, 2008). Dorsomorphin is not a highly specific inhibitor. Next to well-studied inhibition of ALK2, ALK3 and ALK6 (inhibiting BMP signalling), dorsomorphin also inhibits VEGFR2 (also known as FLK1 or KDR), thus disrupting angiogenesis during zebrafish development (Hao *et al.*, 2010). What is more, in some research areas, dorsomorphin is known as an AMPK (AMP-activated protein kinase) inhibitor as well. AMPK is a highly evolutionary conserved, energy sensing kinase, which when activated (such as upon sensing oxygen deprivation) causes inhibition of pathways that are responsible for high ATP consumption while enhancing metabolic ATP producing pathways. However, as all BMP, VEGF and AMPK pathways are involved together in number of developmental processes, particularly in angiogenesis, the use of dorsomorphin alone does not allow for a conclusive argument to be made regarding which pathway is involved in which precise aspect of the developmental process (Dasgupta and Seibel, 2018).

About 10 years ago, a derivative or DM was created by Hao and colleagues (Hao *et al.*, 2010) named DMH1, which showed much stronger affinities for ALK1, 2, 3 receptors, while not activating non-BMP pathways, resulting in significantly fewer unwanted side-effects. The fact that DMH1 also distinguishes between ALK1,2,3 and ALK6 enabled more in-depth investigation in the redundancy of BMP signalling receptors in cartilage and bone development, homeostasis and regeneration (Hao *et al.*, 2010). In this chapter the main inhibitors used are DMH1 and dorsomorphin (DM).

Name (Ref)	Receptors targeted
Dorsomorphin ^{1,2}	ALK2, ALK3, ALK6, PDGFR- β , VEGFR2, ERK
DMH1 ³	ALK2, ALK3, (ALK1)
KO2288 ⁴	ALK1, ALK2, ALK3, ALK6
LDN193189 ⁴	ALK1, ALK2, ALK3, ALK4, ALK5, ALK6, ActRIIA and KDR

Table 5.1 Commonly used BMP inhibitors in zebrafish research

^{1,2}(Yu *et al.*, 2008; Cannon *et al.*, 2010) ³(Hao *et al.*, 2010) ⁴(Sanvitale *et al.*, 2013)

5.1.3 BMP signalling in cartilage and bone development

5.1.3.1 BMP signalling in craniofacial development

Pharyngeal arches are developmental structures that act as precursors to cartilage, neurons, glia and number of other type of cells and structures (Schilling and Kimmel, 1994). Pharyngeal arches are first populated by cranial neural crest cells (CNCCs) and BMPs play a role in number of aspects of CNCC development: induction, apoptosis, migration, skeletogenesis also proliferation, survival and differentiation (Alexander *et al.*, 2011; Zuniga *et al.*, 2011). Mouse models have shown that blocking of Bmp2/Bmp4 during this developmental window leads to depletion of CNCCs from their target destination and, among else, causes hypomorphic branchial arches which leads to failed skeletal and neural derivatives (Estrada *et al.*, 2011).

Consistent with what we know about BMPs so far, the expression of BMPs in the pharyngeal arches is also conserved across the vertebrate kingdom, including in chicken, mice and zebrafish (Francis-West, 1994; Walb and Hogawb, 1995; Holzschuh *et al.*, 2005; Liu *et al.*, 2005). In zebrafish in particular several BMPs have been reported in the developing pharyngeal arches including *bmp2a*, *bmp2b*, *bmp4* and *bmp7b*.

Dorso-ventral patterning refers to the process during which the developing embryo specifies – on a molecular level – the body orientation axis which leads to phenotypically distinct structures being formed such as craniofacial skeleton as depicted in Fig. 5.1. In zebrafish, BMPs/Chordin activator-inhibitor system controls this process (Yelon *et al.*, 2017). There are a number of theories that try to explain this BMP gradient establishment, with the most recent experimental data suggesting that Chordin does not directly modulate the distribution of BMP2b in the developing embryo rather the BMP gradient is established based on a ‘source-sink mechanism’ (Yelon *et al.*, 2017).

Although the precise mechanisms remain unclear, the progenitor cells within the pharyngeal arches expand in numbers till a critical level is reached which triggers condensation. Mesenchymal condensation in turn triggers the chondrocyte differentiation program leading to the first skeletal patterning. This process is accompanied by the upregulation of *col2a1* in the differentiating chondrocytes which is then deposited as a cartilage matrix, forming the head cartilages. Zebrafish develop a functional craniofacial skeleton within the first 5dpf and it continues growing throughout the fish’s life (Salazar, Gamer and Rosen, 2016; Raterman *et al.*, 2020).

The head cartilages can be divided into two main groups which are maintained in the initial larval stages and later, through endochondral ossification, replaced by bone (Cerny *et al.*, 2004; Raterman *et al.*, 2020):

- 1) Dorsally located neurocranial structures that support the developing brain (as well as part of the fronto-nasal process) and are derived from the dorsal condensation of cranial neural crest cells.
- 2) Ventrally located pharyngeal arch-derived structures that are necessary for feeding and gill coverage. These include both the Meckel's cartilage and palatoquadrate cartilage that derive from the ventral condensation of the neural crest cells that populated the first pharyngeal arch.

The main transcription factors governing the process of mesenchymal condensation and cartilage development are under the control of BMP signalling. These include also *runx2a/b* or *sox9a/b* (Dalcq *et al.*, 2012; Lin *et al.*, 2021). BMP ligands have been described to be fundamental for correct craniofacial development in number of vertebrate species, including Bmp5/Bmp7 double mutant mice showing underdevelopment of the branchial arches, delayed lip development/cleft lip in Bmp4 mutant mice. Dalcq and colleagues also addressed the temporal requirements of BMP signalling during craniofacial development in zebrafish. The authors used BMP inhibitors at specified times post fertilization and characterized the phenotype observed, noting significant alterations to the head cartilages happened when BMP signalling was inhibited between 27 – 45 hpf. *runx2b* expression is mainly affected by BMP signalling inhibition from 27 – 37 hpf.

5.1.3.1.1 Craniofacial bone development

Sox9+ mesenchymal cells condense to form chondrocytes which produce ECM proteins such as Col2a1 and later, hypertrophy-associated factors such as coll10a1 and Ctgf (Paul *et al.*, 2016). The centre of the condensation mass contains cells that begin to upregulate *coll10a1a* and become hypertrophic chondrocytes. With some becoming apoptotic while others transdifferentiating into osteoblasts secreting bone matrix and followed up by mineralisation (Salazar, Gamer and Rosen, 2016).

In the adult zebrafish head skeleton, there are two types of bone:

- 3) dermal – bones develop directly within a connective tissue
- 4) cartilage-replacement – bone develops indirectly through a cartilage intermediate

43 out of 74 bones in zebrafish skull are cartilage bones undergo either endochondral (ossify within cartilage) or perichondral (ossify around cartilage) bone formation. Yu and colleagues have shown that inhibiting BMP signalling using dorsomorphin after 24 hpf does not lead to embryo dorsalisation but rather causes severely delayed/inhibited vertebral and craniofacial calcification (Yu *et al.*, 2008). Similar findings were reported by (Windhausen *et al.*, 2015) as well as (Y. El Fersioui *et al.*, 2022) using DMH1 compound.

5.1.4 BMP signalling in caudal fin regeneration

BMP signalling has been widely studied in caudal fin regeneration and expression of its components characterized in the regenerating tissue. *Bmp2b* was found to be expressed in the osteoblasts, *bmp4* in the distal blastema, *bmp6* was found in the wound epidermis, and *bmp7a* in distal blastema and epidermis of the fin at 3 dpa. Moreover, BMP receptors are nearly ubiquitously expressed in the regenerating fin tissues (L. Laforest et al., 1998; Quint et al., 2002; Smith et al., 2006).

5.1.4.1 BMP signalling during wound healing

Although expression of BMPs is present, blocking BMP signalling using DMH1 does not inhibit wound healing and the formation of wound epithelia. Nonetheless, several signalling molecules necessary for the proper organization of basal epithelium, including *wnt5b*, have been found to be affected by BMP inhibition, in particular by DMH1 treatment. Interestingly though, *wnt5b* expression is also detected in the forming blastema, and it is unaffected by DMH1 treatment which suggests that BMP signalling may be playing a role in the organization of the basal wound epithelium, without affecting the wound healing processes (Thorimbert *et al.*, 2015a).

Interestingly, mammalian models of wound healing show that BMP signalling inhibits cytoskeletal organization in keratinocytes, as well as their proliferation and migration in regenerating skin epithelium. And blocking of BMPR-1B (using siRNA mediated knockdown) accelerates cell migration, indicating that different temporal and spatial BMP expression and function may take place in different vertebrate models (Lewis *et al.*, 2014).

5.1.4.2 BMP signalling during blastema formation

Studies have indicated that BMP signalling plays an important role during blastema formation in epimorphic regeneration. Next to the known expression patterns (within blastema) of BMP ligands (Schebesta et al 2006), studies that focused on the impacts of BMP inhibition on the regeneration process helped to clarify the role of BMPs during this time. Patel and colleagues (2019) found that inhibition of BMP signalling after caudal fin amputation (using LDN 193189) reduces the rate of proliferation of the cells within and around the forming blastema region, hence affecting the proper organization of blastema. Moreover, BMP signalling inhibition leads to an increase in cellular apoptosis during the blastema formation, decreased levels of *mmp2* and *mmp9* and osteoblast dedifferentiation (Rajaram *et al.*, 2017; Patel *et al.*, 2019).

5.1.4.3 BMP signalling during caudal fin outgrowth

BMP signalling is necessary for caudal fin lepidotrichia and actinotrichia outgrowth. Exogenous BMP2 protein has been showed to induce ectopic bone formation as it is a potent extracellular signalling molecule that stimulates bone formation and osteoblast differentiation. Inhibition of BMP signalling leads to significantly reduced length of the regenerating fins, diminished formation of cartilaginous lepidotrichia marked by significantly reduced *sox9* expression (structural support in regenerating fins) and improper ossification distally to the amputation site (Rajaram *et al.*, 2017). Correct mineralization of the deposited bone matrix is also regulated by BMP signalling where BMP inhibition disrupts scleroblast differentiation and function probably via disruption of the YAP/TAZ Hippo pathway effectors that normally potentiate osteoblast differentiation and bone formation, in addition to

sensing and relaying the matrix stiffness information from the cell surrounding. Disruption in osteoblasts' sensing leads to sub-optimal bone matrix deposition and mineralization ultimately affecting the size, shape, and integrity of the skeletal structures (Thorimbert *et al.*, 2015a).

Next to the skeletal components in the regenerating fins, BMP signalling is also responsible for correct vascular setup and vasculature maintenance during the regenerate outgrowth, in part through the regulation of VEGF. Disruption to BMP signalling leads to loss of patterning in the re-developing vasculature and decrease in vascular integrity resulting in severe haemorrhage, ultimately affecting the outcome of the regenerative process (Smith *et al.*, 2006).

5.1.5 BMP signalling in gill regeneration

As discussed in the Introduction, the structural basis of the gill tissue are cartilage filaments attached to a bony arch. Cartilage is well known to have very limited regeneration capacity in mammals, particularly in humans. BMP signalling plays a fundamental role in the outcome after an injury to cartilage tissue.

Generally, BMP signalling (particularly including BMP2 and BMP7 ligands) stimulated after growth plate cartilage injuries in mammals often result in premature mineralization and ossification at the injury site as well as the adjacent uninjured cartilaginous areas, often characterised by high fibrous collagen content (Su *et al.*, 2021). BMP2 and BMP7 ligands are also used clinically to enhance bone fusion in vertebrae fusion surgeries in humans (Boden *et al.*, 2000, 2002; Gothard *et al.*, 2014). On the other hand, when rats were treated systemically with noggin, also an endogenously produced BMP signalling inhibitor, the bony repair at the cartilage injury site was attenuated, possibly through delaying the process of premature hypertrophy of the chondrocytes. However, inhibition of BMP signalling in mammalian models through a knockdown of BMPRII leads to severe osteoarthritis in adulthood, even

though normal joint and cartilage development were not affected (Aigner, Soeder and Haag, 2006).

Furthermore, it was shown that the amount of remodelling of cartilaginous matrix after injury is fundamental aspect of cartilage repair: An experimental strain of mouse that is largely resistant to experimental osteoarthritis showed that after an early wave of apoptosis followed by degradation of collagen type II and aggrecan, further degradation subsided. However, in mouse strains that are not genetically modified to be resistant to OA, the degradation of collagen and aggrecan persisted and lead to a pronounced OA (Sherwood *et al.*, 2014). Altogether, the experimental evidence shows that BMP signalling is necessary for cartilage maintenance, however, optimal balance is essential during regeneration to maintain cartilage phenotype and not tilt the balance to bone/calcified phenotype.

With respect to the gill cartilage, and as mentioned in the Introduction, the mechanisms behind gill regeneration remain to be fully investigated. With particular focus on BMP signalling, Saito and colleagues (2019) based their research upon the experimental results from number of groups that showed that FGF and BMP signalling (specifically Fgf2 + Fgf8 + Bmp2 or BMP7) can fully substitute for absence of nerve tissues and induce limb regeneration in axolotl and newts. Saito and colleagues showed that BMPs are expressed in the mesenchyme and nerves of the regenerating gill in *Ambystoma mexicanum* (axolotl) and inhibition of BMP signalling (by dorsomorphin) resulted in significantly smaller outgrowth of tissue distally to the amputation plane (Saito *et al.*, 2019).

Without specific focus on BMP signalling, Mierzwa and colleagues (2020) showed that in zebrafish specifically, there was an increased number of PCNA+ cells following gill resection and together with our own data (Ramel *et al.*, 2021), that showed increased levels of the blastema marker *sall4* following gill cryoinjury. There is no data available to dissect the single cell composition of gill blastema after gill injury (resection or cryoinjury) therefore it can only be hypothesized that the blastema will be affected by the presence or absence of BMP signalling similarly as in the regenerating caudal fin. That is, the number of

proliferating cells will be lower in gills with impaired BMP signalling, which will ultimately affect the blastema spatial setup with possible phenotypical consequences for tissue outgrowth.

5.2 Aims

Results of our RNA-seq analysis showed a significantly increased expression of BMP signalling antagonists in *fibina/b^{-/-}* fish compared to wild-type fish. BMP signalling is a fundamental signalling pathway for correct development and regeneration of cartilage and bone tissue. I therefore hypothesized that lack of Fibins leads to a dysregulated BMP signalling response that affects the development as well as caudal fin and gill regeneration in *fibina/b^{-/-}* fish resulting in a mis-patterned, structurally abnormal phenotype.

In order to test this hypothesis, I have:

- 1) Characterized and compared the cartilage and bone development in the wild-type and *fibina/b^{-/-}* embryos to establish a possible role of Fibins during development.
- 2) Analyzed the caudal fin regeneration kinetics and dynamics in adult wild-type and *fibina/b^{-/-}* fish.
- 3) Investigated the transcriptional differences during the initiation of gill tissue regeneration between young and old wild-type and *fibina/b^{-/-}* fish.

5.3 Results

5.3.1 BMP signalling in the fin fold, cartilage and bone development *fibina/b^{-/-}* fish

Several BMP signalling related genes, particularly BMP pathway antagonists, were found to have significantly higher read count numbers in the *fibina/b^{-/-}* fish compared to wild-type fish. BMP signalling is fundamental for optimal development and regeneration in animals across the vertebrate kingdom. Here I, together with master students Xiangyu Sui and Serafima Davydova, used three embryonic models that allowed us to assess phenotypical implications of BMP signalling dysregulation during development:

- 1) Fin fold area/development
- 2) Cartilage development
- 3) Bone development

5.3.1.1 Fin fold development in *fibina/b^{-/-}* zebrafish

In zebrafish, the caudal fin fold can be divided into two main fin folds that begin to take shape before 22 hpf – the ventral fin fold that gives rise to unpaired fins such as the caudal fin or the anal fin, and the dorsal fin fold giving rise to the dorsal fin (van den Boogaart, Muller and Osse, 2012; Liedtke *et al.*, 2019). Previous research has shown that during development, BMP signalling acts to limit the ventral fin fold extension along the proximodistal axis. For example, overexpression of *bmp2b* in *hsp70:bmp2b* transgenic zebrafish embryos lead to significant fin-fold reduction. This is further supported by transcriptional data from *leo^{tl}/lof^{dtl}* mutant zebrafish line (long-fin and long fin-fold phenotype) showing that during the development of the long fin-fold in in these mutants, *bmp2b* is significantly downregulated (Siregar *et al.*, 2020; Castro *et al.*, 2021). Ka and colleagues showed that the molecular mechanisms include increased apoptosis of epithelial cells. They further showed that inhibition of BMP signalling using the DMH1 inhibitor increases the proliferative capacity of epithelial cells which leads to increased numbers of cells within the ventral fold and induced migration of cells distally, extending the fin fold (Ka *et al.*, 2020).

Based on our RNAseq, there are increased levels of several characterized BMP antagonists including *nog2*, *grem2b* or *bmper* in *fibina/b^{-/-}* compared to wild-type fish. Together with master student Xiangyu Sui, we have performed an in-depth characterization of the *fibina/b^{-/-}* and wild-type fish caudal fin-fold area/shape between 3 dpf and 5 dpf to investigate if the increased levels of BMP antagonist translate into phenotypically distinct fin-folds between our two genotypes. As no obvious differences were observed during any of our previous observations, albeit non-quantitative. We decided to challenge this model with exogenous BMP inhibition, to investigate whether a phenotypic difference can be uncovered. Based on Ka and colleagues we made use of the BMP specific inhibitor DMH1 that specifically blocks ALK1, ALK2 and ALK3 receptors but not *alk6* (Fig. 5.1 (A)) (Hao *et al.*, 2010; Ka *et al.*, 2020).

Fig. 5.1 (B) shows the shape of the wild-type caudal fin-fold with the typical constriction nearer to the caudal aspect of the fin-fold. The fin fold area to be measured was marked by being directly posterior to the cloaca (ventrally) and extended dorsally across the fish body and developing notochord. Fig. 5.1 (C) shows representative images of wild-type and *fibina/b^{-/-}* fish caudal fin-folds in response to range of DMH1 concentrations (0.01 μ M DMH1, 0.1 μ M DMH1, 1 μ M DMH1, 2 μ M DMH1, 10 μ M DMH1) when treated from 2 dpf – 5 dpf.

Treatment effect: For both genotypes, 0.01 μ M DMH1 and 0.1 μ M DMH1 treatment for 3 days (2 dpf to 5 dpf) did not lead to significant difference in the area size, however, a pattern can be seen showing a very mild increase in the area size with increasing DMH1 concentration. Concentrations of 1 μ M DMH1, 2 μ M DMH1 and 10 μ M DMH1 all showed significant increase in fin-fold areas for both wild-type and *fibina/b^{-/-}* fish consistent with previous literature (Ka *et al.*, 2020), and without any dose-dependent pattern visible. In terms of the shape of the fin-fold, concentrations above and including 1 μ M DMH1 treatment for 3 days resulted in clear loss of the well-defined posterior constriction as well as loss of the smooth fin-fold edges. The highest concentration used, 10 μ M DMH1, frequently resulted also in heart oedema and visible bleeding irrespective of the genotype (blue-coloured head of the fish), suggesting that this concentration is not suitable for any long-term experiments including developing embryos.

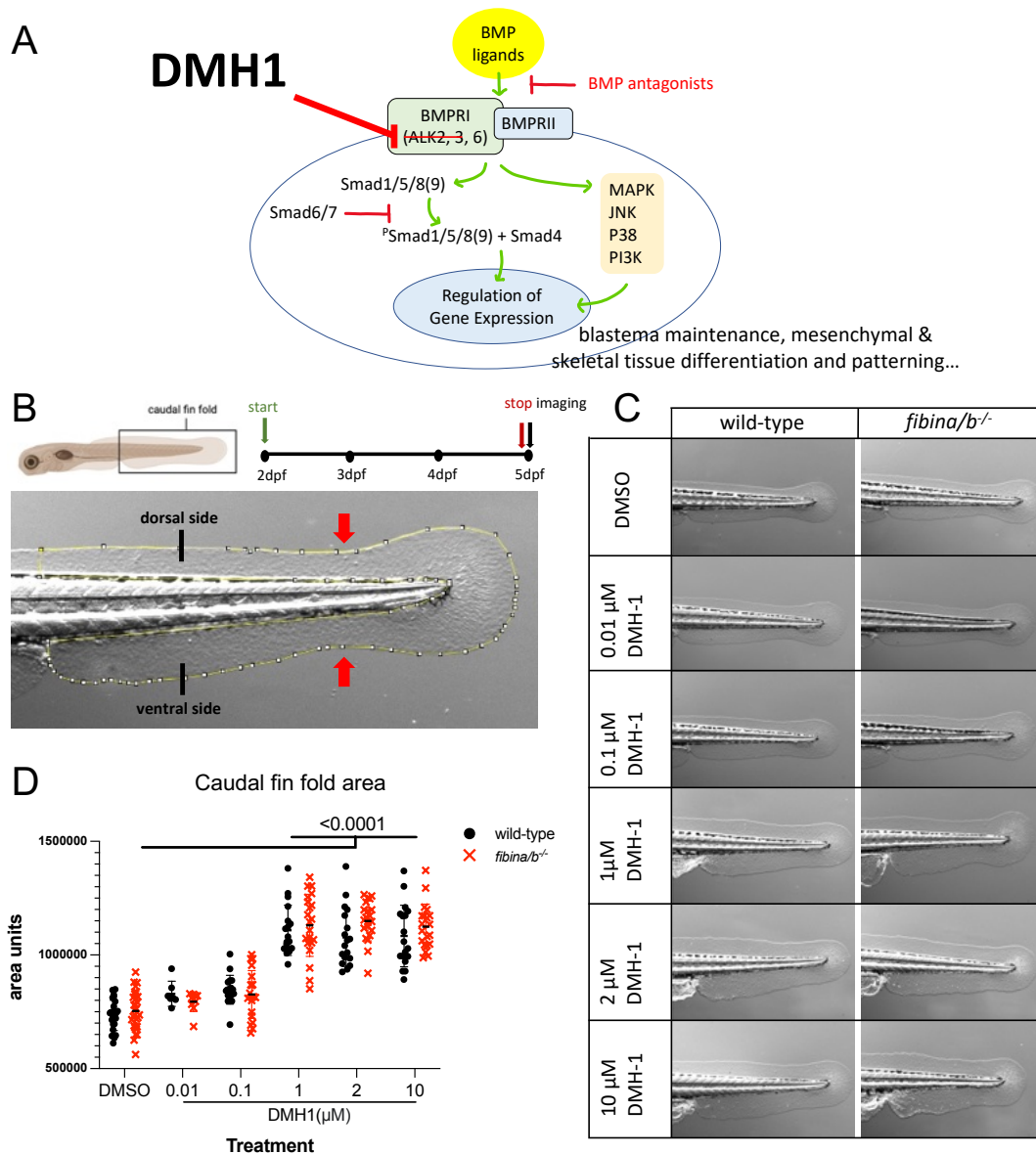


Figure 5.1 Caudal fin-fold area in wild-type and *fibina/b^{-/-}* fish with or without DMH1 treatment.

(A) Simplified schematic of BMP signalling transduction showing where does DMH1 act within the pathway. (B) Schematic of the embryonic caudal fin-fold and the timeline of the experiment. (C) Representative images of the caudal fin-folds in both wild-type and *fibina/b^{-/-}* fish, with increasing dose of DMH1. (D) Quantification of the caudal fin-fold area between the wild-type and *fibina/b^{-/-}* fish. Statistical significance was calculated to compare wild-type vs *fibina/b^{-/-}* fish at each DMH1 concentration (and DMSO control), and to compare the effect of DMH1 treatment. Multiple student t-tests were performed to determine statistical significance. P-values < 0.05 are plotted. Panels B and C were prepared by Xiangyu Sui.

We also investigated how the treatment length affects the phenotype and found that the effect of DMH1 treatment on the caudal fin fold area was cumulative (Appendix 7) in both genotypes. To determine if the treatment duration can affect the area size we treated the embryos for 1, 2 or 3 days with 0.1 μ M DMH1 and 1 μ M DMH1 and imaged at 5 dpf (Appendix 8). Treatment with 0.1 μ M DMH1 resulted in similar fold-change areas irrespective of treatment duration (for both genotypes). 1 μ M DMH1 treatment resulted in significantly increased fin fold area when fish were treated for 72 h compared to 24 h or 48 h, suggesting a concentration threshold limit for BMP-dependent limitation of fin fold size which is consistent with published literature (Ka *et al.*, 2020). This was true again for both genotypes without any difference.

Genotype effect: There was no difference in the fin-fold area quantified between the wild-type and *fibina/b^{-/-}* fish irrespective of the treatment. There was also no difference in the shape of the fin-folds observed. No difference was detected with varied treatment length either.

Overall, BMP inhibition resulted in expected changes to the zebrafish fin fold area with no significant differences observed between wild-type and *fibina/b^{-/-}* fish under any of the treatments tested. These results indicate that Fibins are not crucial in the development of caudal fin fold in embryos < 5 dpf.

5.3.1.2 Craniofacial cartilage development in *fibina/b^{-/-}* zebrafish

BMP signalling is essential for craniofacial skeleton formation – for both cartilage and bone development (Salazar et al., 2016; Yelon et al., 2017). Previous research has identified several characteristics of the developing cartilaginous craniofacial features that enable scoring and assessing impacts of BMP signalling inhibition in a quantitative, easily repeatable way. These are the ceratohyal cartilage angle, the palatoquadral cartilage – ceratohyal cartilage angle, the Meckle’s cartilage width and the palatoquadrate cartilage width (Fig. 5.2 (B)). Here we have quantified these parameters in our wild-type and *fibina/b^{-/-}*

fish to assess potential phenotypical differences between the two genotypes with or without BMP inhibition (DMH1).

DMH1 and dorsomorphin (DM) doses were tested in the range 0.1 μ M – 10 μ M DMH1 and 10-100 μ M DM based on the results of the fin-fold experiment described above and on literature research (Dalcq *et al.*, 2012; Alvarado *et al.*, 2016). Again, with focus on determining the lowest dose required to obtain a quantifiable phenotype in the wild-type fish.

Treatment effect: For treatment starting at 27.5 hpf all concentrations above and including 1 μ M DMH1 resulted in embryo death by 5 dpf thus preventing us from accurately quantifying the chosen parameters. Therefore, a concentration of 0.1 μ M DMH1 was chosen. For DM, the off-target effects (that is effect on general fish health, heart oedema etc.) were much more common than with DMH1 treatment. 50 μ M DM was the lowest DM concentration that allowed us to detect a difference in the chosen parameters, however, at the expense of severe side effects such as heart oedema (Appendix 9) any higher DM concentration resulted in premature embryo death – irrespective of the initiation time of treatment.

With treatment starting at 27.5 hpf, all four parameters quantified, CH and PQ-CH angles, and Meckel's cartilage width and PQ width (Fig. 5.2 (B)), were significantly affected by DMH1 treatment, as expected based on previous research (Ghaffari, 2017; Y. El Fersioui *et al.*, 2022). There was a significantly stronger affect seen with DMH1 treatment on Meckel's cartilage width and palatoquadral (PQ) cartilage width compared to DM treatment – significantly wider irrespective of the genotype. With treatment starting at 48 hpf, both DMH1 treatment groups (1 μ M and 10 μ M) showed significantly stronger effect on CH angle, PQ-CH angle and PQ width as compared to DM treatment.

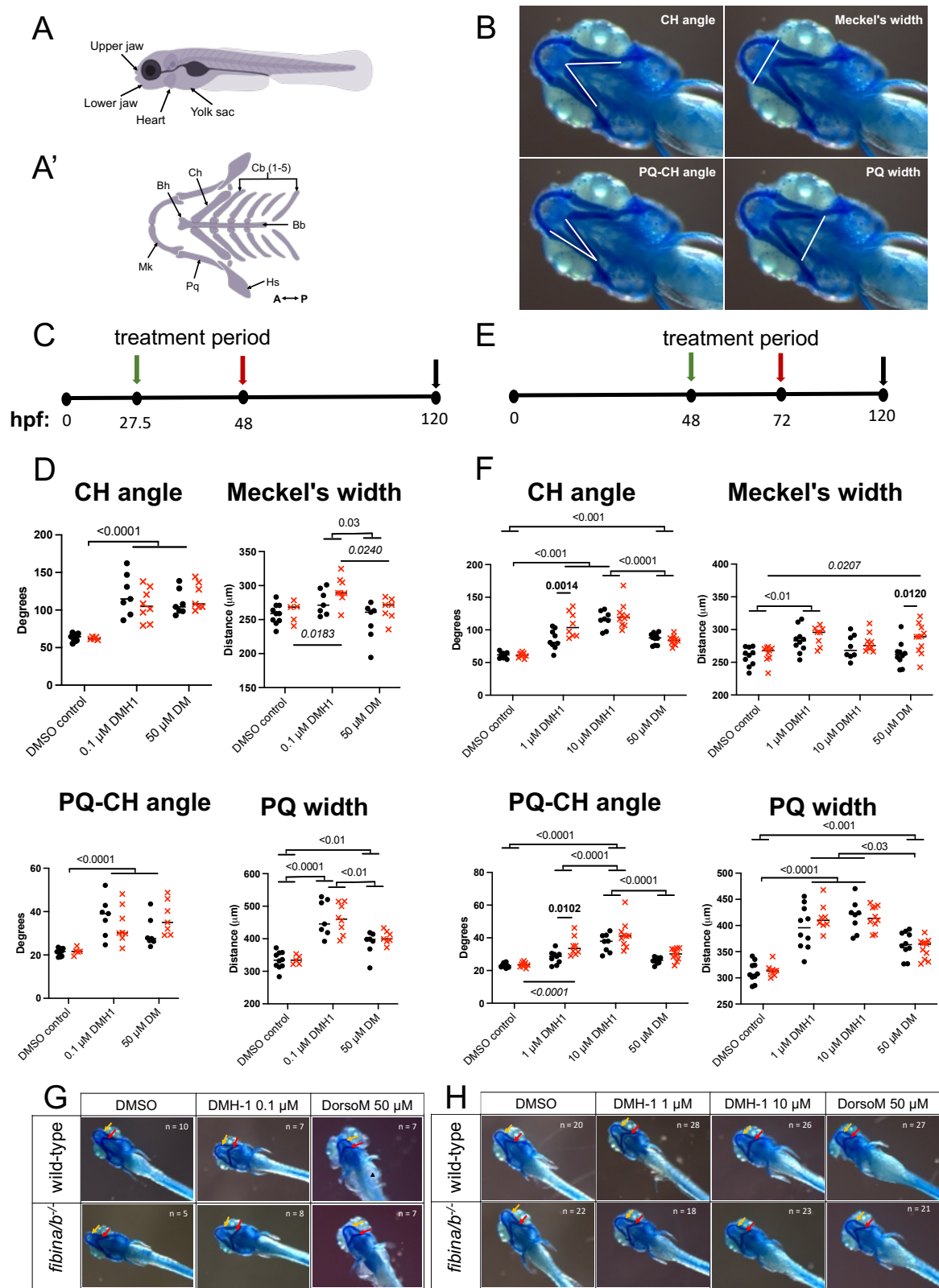


Figure 5.2 Craniofacial cartilage development in wild-type and *fibina/b^{-/-}* fish

(A) Zebrafish embryo anatomy at 5 days post fertilisation (dpf). Lateral view of zebrafish embryo. (A') Schematic of embryonic craniofacial cartilage structures from ventral view (adapted from Mork & Crump (2015)). Bb = basibranchial; Bh = basihyal; Cb = ceratobranchial; Ch = ceratohyal; Hs = hypobranchial; Mk = Meckel's; Pq = palatoquadrate. (B) Parameters measured for each Alcian blue stained embryo at 5 dpf: CH angle = ceratohyal cartilage angle, PQ-CH angle = palatoquadral cartilage – ceratohyal cartilage angle. (C, E) Schematic of the experimental timeline including the timings of DMH1 or DM (dorsomorphin) treatment. (D, F) Quantification of the four measured parameters in wild-type and *fibina/b^{-/-}* fish following either a treatment from 27.5 – 48 hpf (D) or from 48 – 72 hpf (E). Statistical significance is showed for comparison between wild-type and *fibina/b^{-/-}* fish (in bold) and to determine the impact of treatment. Two-way Anova. (G, H) Representative images for Alcian blue stained wild type and *fibina/b^{-/-}* fish at 5 dpf. All images are taken from the ventral view. (G) Treatment with DMSO control, 0.1 μ M DMH1 or 50 μ M DM (Dorsomorphin) from 27.5 – 48 hpf. (H) Treatment with 1 μ M DMH1, 10 μ M DMH1 or 50 μ M DM from 48 – 72 hpf. Yellow arrows show Meckel's cartilage – palatoquadral cartilage joint and red arrow show ceratohyal cartilage, black triangle indicates yolk sac expansion commonly observed across dorsomorphin-treated embryos. n = number of embryos per experiment (3 separate experiments combined). Panels A, A', B, G and H were prepared by Serafima Davydova.

Genotype effect: 1 μ M DMH1 treatment at 48 hp revealed significantly larger CH and PQ-CH angles in mutant fish compared to wild-type fish. Interestingly for the PQ-CH angle, wild-type fish were not significantly affected by this DMH1 concentration, suggesting that at this timepoint, with this concentration, lack of Fibins resulted in significantly altered patterning of craniofacial cartilage, possible through increased level of BMP inhibition/antagonism. Meckle's cartilage width when treated with 50 μ M DM starting at 48 hpf also showed significant difference between wild-type and *fibina/b^{-/-}* fish with wild-types not being significantly impacted with this concentration and this parameter compared to DMSO treatment control. In all cases there was an increase in the angle or in the width in the *fibina/b^{-/-}* fish (Fig. 5.2).

Overall, significant differences were found between wild-type and *fibina/b^{-/-}* fish during craniofacial cartilage development/patterning when using a low dose of BMP inhibitor. In terms of pharmacological treatment, DMH1 showed stronger BMP inhibition related craniofacial defects compared to DM that came with number of severe side effects. Based on these observations, I decided to discontinue the use of DM from further experiments.

5.3.1.3 Craniofacial bone development in *fibina/b^{-/-}* zebrafish

Bone development was investigated using Alizarin red stain that binds to calcified tissues, therefore effectively measuring the degree of calcification/ mineralisation present at a given time (Bensimon-Brito *et al.*, 2016).

Based on the results obtained when quantifying the cartilage development, we have decided to discontinue using DM due to the severe side effects present. The initiation of the treatment was at 48 hpf (being the timepoint at which we detected differences between wild-type and *fibina/b^{-/-}* fish). A dose of 0.1 μ M DMH1 and 10 dpf end-point were chosen based on the results of preliminary experiments:

- 1) Range of DMH1 concentrations was tested (0.1 – 10 μ M) with end point 5 dpf, however, the calcification was too low it was not feasible to quantify (for either genotype).
- 2) With the extended end-point (10 dpf) concentrations above and including 1 μ M DMH1 resulted in strong haemorrhaging (blue craniofacial region) in both genotypes with severely affected swimming patterns. This resulted in the need to cull (Schedule 1) number of these fish to prevent their death due to treatment. These concentrations were therefore excluded from the experiment.

Treatment effect: The calcification was quantified from lateral and ventral side (Fig. 5.3). There was no significant difference between DMSO and 0.1 μ M DMH1 treatment. This means that the calcified area in *fibina/b^{-/-}* fish was not significantly lower in the treatment group compared to control, it was only significantly different to the wild-type fish in the same treatment group. Similarly, no statistical difference was determined for wild-type fish treated with DMSO or 0.1 μ M DMH1. Currently there is a discrepancy in published literature regarding whether, and to what extent, does BMP inhibition by DMH1 at various concentrations affect craniofacial calcification. Fersioui and colleagues used 50 μ M DMH1 and found significantly decreased craniofacial calcification compared to control treatment (Y. el Fersioui *et al.*, 2022). On the other hand Shafi and colleagues used DMH1 at concentrations ranging from 3 – 30 μ M and found no difference to the calcified structures (Shafi *et al.*, 2022). Nonetheless, in both of these studies, the DMH1 concentration used were

much higher than those used in our experiments and neither of the authors mentions any significant side effects of the treatment. As our fish did not survive a long-term (up to 10 dpf) treatment with any higher concentration than 0.1 μ M DMH1, it can perhaps be hypothesized that significant baseline differences exist between wild-type fish lines across laboratories.

Genotype effect: No significant differences between wild-type and *fibina/b^{-/-}* fish were detected with DMSO treatment (control). Significant difference was detected between the wild-type and *fibina/b^{-/-}* fish, with significantly less calcified area (ARS positive) detected in *fibina/b^{-/-}* fish, when treated with 0.1 μ M DMH1, suggesting that Fibins may have a very subtle, direct or indirect, involvement in developmental bone calcification.

Although significant differences were detected in the patterning of the craniofacial cartilage, these were achieved using 10x higher DMH1 dose (1 μ M DMH1), which may explain the lack of patterning/shape differences in the calcification with 0.1 μ M DMH1.

Overall, no significant differences detected between wild-type and *fibina/b^{-/-}* fish in control groups for either of the investigated tissue development (fin-fold, cartilage, bone). Significant differences were, however, found between wild-type and *fibina/b^{-/-}* fish in craniofacial cartilage patterning (CH angle and PQ-CH angle) when fish were treated with 1 μ M DMH1 from 48 – 72 hpf. And subtle, but significant difference was also detected in craniofacial calcification between wild-type and mutant fish when treated with 0.1 μ M DMH1. Together this data demonstrates mutant fish' cartilage patterning is significantly more affected (phenotypically) when treated with low dose of BMP inhibitor DMH1 compared to their wild-type counterparts, which could point to a lower baseline BMP signalling activity. However, none or very small differential effects were found in fin-fold development or bone mineralisation respectively. Therefore, further investigation, including RNA and protein analysis, to fully understand which aspects of normal vertebrate development are affected by presence/absence of Fibins and how exactly they exert their function. And although working with zebrafish embryos provides unparalleled advantages compared to working with adult fish (ease of breeding – large numbers, not protected under UK Home Office (ASPA 1986) before

5 dpf, ease of manipulation and more), for the purpose of increasing our understanding of Fibins, embryos do not seem to provide robust enough platform to detect subtle differences between wild-type and *fibina/b^{-/-}* fish, therefore the next parts of this chapter focus on adult zebrafish tissue regeneration.

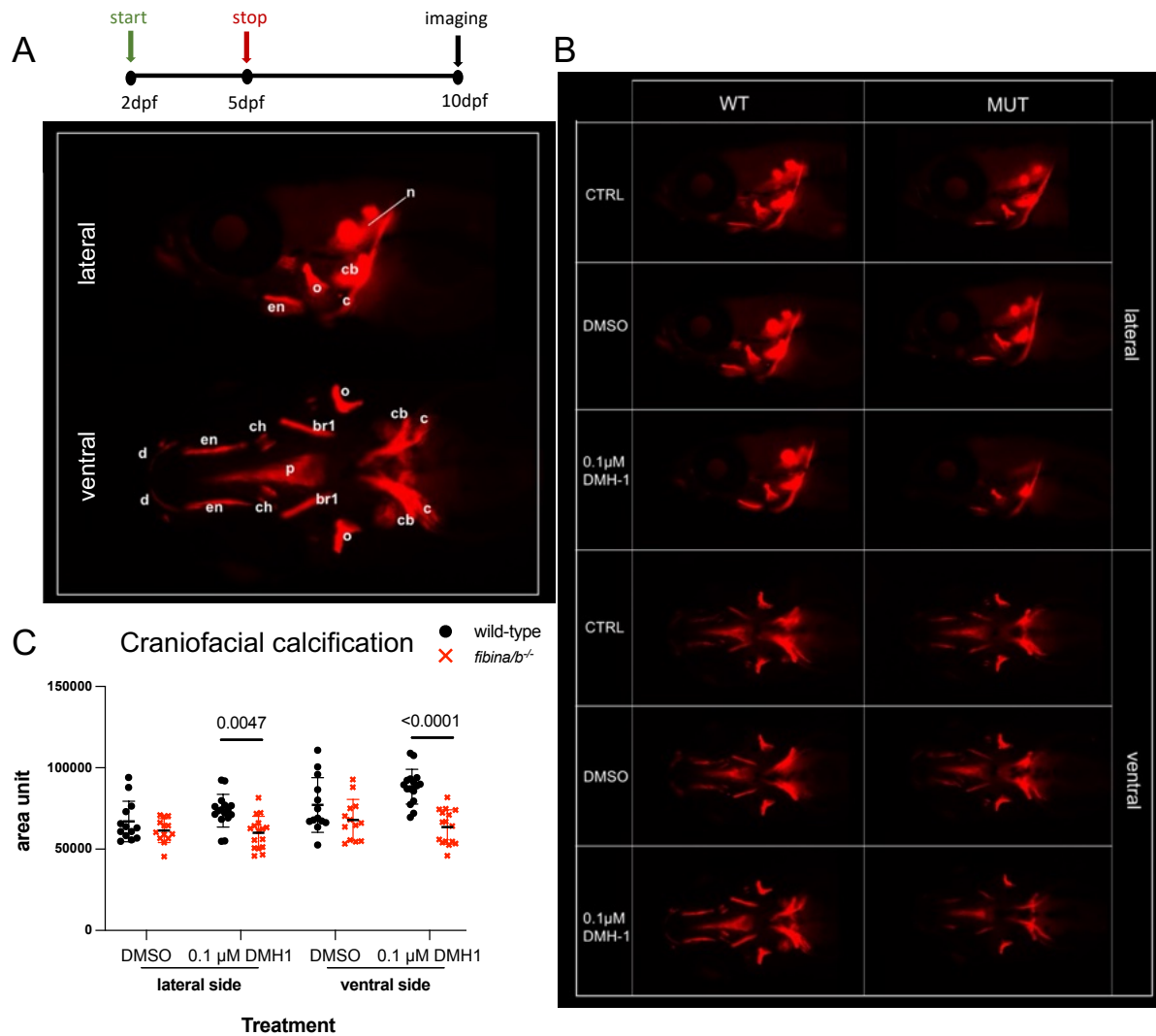


Figure 5.3 Craniofacial bone development in wild-type and *fibina/b^{-/-}* fish at 10 dpf

10 dpf larvae were euthanized, fixed in 4% PFA and stained with Alizarin Red S Stain (ARS). Fluorescent microscopy was used to collect images of the lateral and ventral view of stained (calcified) bony structures. **(A)** Lateral and ventral views of craniofacial skeleton at 10 dpf wild-type fish with names of the detectable craniofacial bones. **(B)** Representative images of craniofacial bone of wild-type and *fibina/b^{-/-}* fish at 10 dpf with no treatment (CTRL), DMSO control and 0.1μM DMH-1 treatment. **(C)** Quantification of the bone area (using Fiji, detailed description of the analysis can be found in Methods) from both lateral and ventral views. Statistical significance was calculated to compare calcified craniofacial areas between wild-type and *fibina/b^{-/-}* fish and to determine impact of DMH1 treatment. Panels A and B were prepared by Xiangyu Sui.

5.3.2 Caudal fin regeneration in *fibina/b*^{-/-} zebrafish

Caudal fin amputation has been used in this study as it is a simple, reproducible model of complex tissue regeneration involving a bone component (lepidotrichia), a cartilaginous component (actinotrichia), neuronal tissue, vasculature and stromal cells, which express *fibina* and *fibinb*. As discussed in Chapter 3, no phenotypic differences were observed between wild-type and *fibina/b*^{-/-} fish when performing single or double caudal fin amputation, both in terms of size of regenerate and bone calcification. In the experiments discussed in Chapter 3, 3 weeks were allowed for the fins to regenerate.

Investigation of the cartilage and bone development revealed that two craniofacial cartilage angles investigated in the mutant fish were significantly more affected by small dose of external BMP signalling inhibitors compared to wild-type fish. As there was a clear dose-response visible with 1 and 10 μ M DMH1 treatment (as supported by literature as well (Thorimbert *et al.*, 2015b)), these results indicate that either the mutant fish is more responsive to BMP inhibition or that there is an increased underlying BMP inhibition to start with, which is consistent with our RNA-seq results. As balanced BMP signalling and BMP signalling antagonism is necessary for optimal caudal fin regeneration, in the following experiments, I have investigated the caudal fin regeneration in the first 2.5 weeks post amputation in our wild-type and mutant fish, in the presence or absence of BMP signalling inhibitor, DMH1.

5.3.2.1 Analysis of transcript levels of selected genes at 3 dpa and 5 dpa in wild-type and *fibina/b*^{-/-} fish caudal fin

I investigated the transcript levels of selected genes during caudal fin regeneration at 3 and 5 dpa. These timepoints were selected to cover the timing of when BMP signalling plays a role in osteoblast differentiation and bone outgrowth (Thorimbert *et al.*, 2015a). *Fibina* and *fibinb* transcript levels were not significantly different at baseline, which agrees with results of our RNA-seq analysis suggesting that at homeostasis, Fibins are expressed at very low levels. Yet, at 3 and 5 dpa, significantly higher transcript levels were found in wild-type fish for both genes and clear significant difference was detected between wild-type and *fibina/b*^{-/-} fish at

these timepoints, corresponding to what we expect based on the predicted non-sense mediated RNA decay of *fibina* and *fibinb* (Chapter 3) and the presents of *fibins* during tissue regeneration.

Interestingly, the increase in transcript levels of selected blastema markers, *sall4* and *msx1b*, although not statistically significant, was noticeably lower in the *fibina/b^{-/-}* fish compared to wild-type fish at 3dpa (mean FC = 16 and 30 respectively for *msx1b* and 20 and 40 for *sall4* (Fig. 5.4)). However, no difference was detectable between the two genotypes at 5 dpa for either gene. For both genes, the wild-type levels decreased slightly from 3 to 5 dpa while the mutant transcript levels increased to match the wild-type.

In line with this, *mmp9* (gene coding for an ECM remodelling enzyme) transcript levels were significantly lower at 3 dpa in mutant fish compared to wild-type (mean FC = 60 vs 25). The increase in transcript levels in wild-type fish then decreased to mean FC = 25 and mutant fish, the mean FC levels remaining the same at 5 dpa. No obvious difference was detectable between the two genotypes at 3 and 5 dpa for the other ECM remodelling molecule-coding gene *mmp2* nor for either of the ECM component-coding genes (*coll0a1a*, *colla2*, *ctgf*). It is important to note that the baseline levels were not significantly different in either of these genes thus making it possible to compare between the increased levels directly without the need to correct for baseline levels for each genotype.

Altogether these data suggested that in the *fibina/b^{-/-}* fish there was a lower induction of genes responsible for blastema maintenance, and early matrix remodelling (*mmp9*) without a difference in matrix-coding genes and pre-osteoblast markers (such as *colla1a* or *coll0a1a*). This could reflect either a generally lower increase in the specified gene transcript levels or a delay/slower increase in the transcript levels. However, as no later timepoint was analysed it is not possible to conclude which one is responsible. *sall4* is not a BMP response gene, however, both *msx1b* and *mmp9* are. The generally lower increase of these genes' transcript levels thus also supports my hypothesis that lack of Fibins leads to a dysregulated BMP signalling during the initiation of caudal fin regeneration.

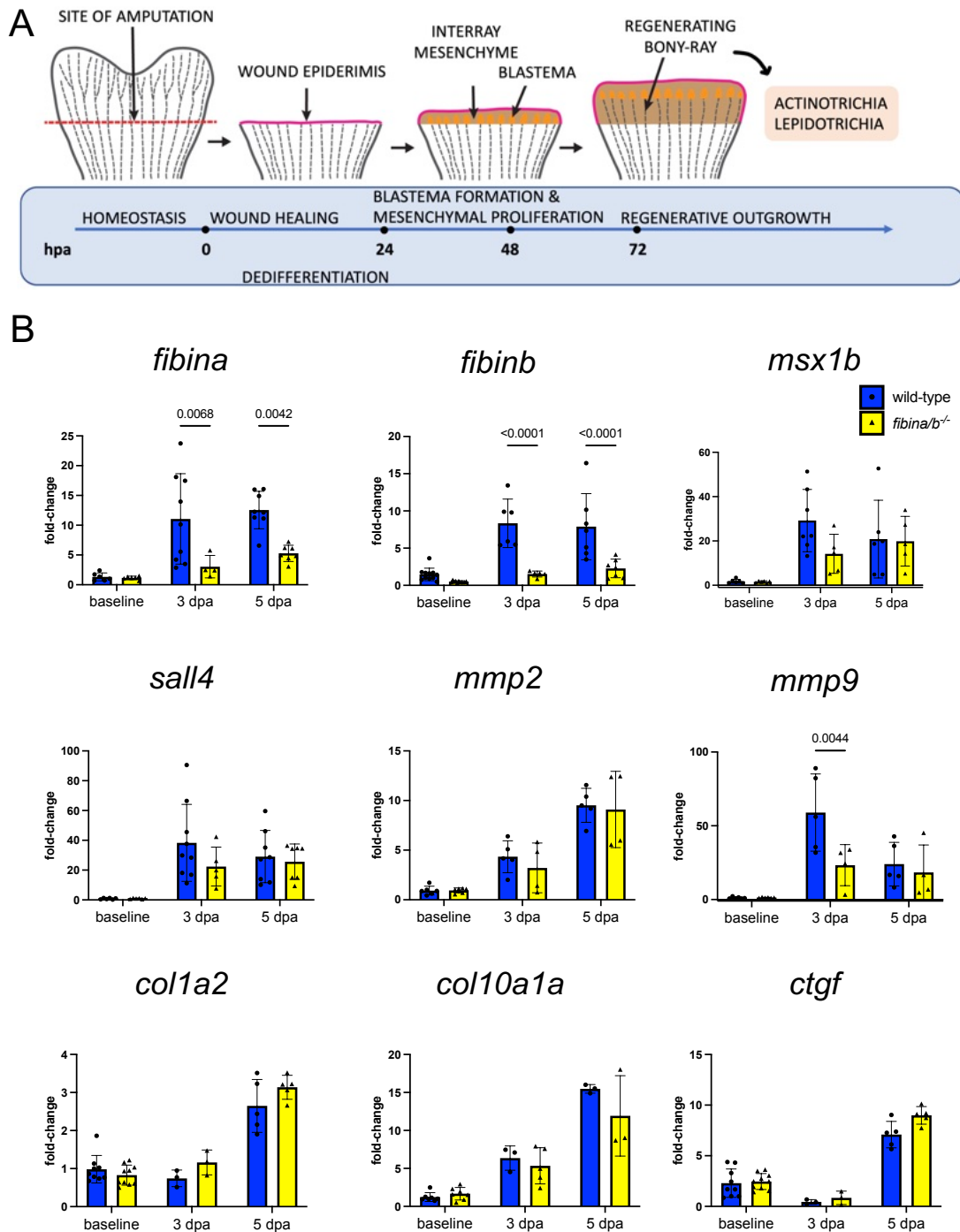


Figure 5.4 Transcript levels analysis of selected genes following caudal fin amputation of young (4-6 months old) wild-type and *fibina/b^{-/-}* fish at baseline (original fin), 3 dpa and 5 dpa

(A) Schematic outlining the stages of caudal fin regeneration in the first 5 dpa. Adapted from (Sehring & Weidinger, 2020) (B) Analysis of transcript levels of selected genes. Statistical significance is shown for comparisons between wild-type and *fibina/b^{-/-}* fish. One-way ANOVA was performed followed by Sidak's multiple comparisons test. P-values < 0.05 are plotted.

5.3.2.2 Effects of BMP signalling inhibition on caudal fin regeneration in wild-type and *fibina/b^{-/-}* zebrafish

Having determined that there are differences in the transcript levels between wild-type and *fibina/b^{-/-}* fish without observing a difference in the phenotype of the regenerating fin, I have next investigated whether low dose of BMP antagonists uncovers a phenotypical difference in the caudal fin regeneration speed and/or caudal fin structure and patterning. Based on available literature (Thorimbert *et al.*, 2015b), I have firstly investigated two possible DMH1 concentrations in wild-type fish, 1 μ M DMH1 and 10 μ M DMH1 (Appendix 10). At 3 dpa, apart from haemorrhaging which was more pronounced in the 10 μ M DMH1 group, there was no detectable difference in the size of the regenerate. At 5 dpa, however, 1 μ M DMH1 still resulted in clear bone outgrowth, albeit clearly mis-patterned, but no bone was detectable in the 10 μ M DMH1 treated group. 1 μ M DMH1 was therefore chosen as the concentration to use as it has clearly detectable effects on wild-type fish without fully blocking the caudal fin regeneration and lepidotrichia outgrowth.

Firstly, I investigated the area of the regenerates between wild-type and *fibina/b^{-/-}* fish. In the control group (DMSO treatment) only 7 dpa showed a significant difference in the size of the regenerate, which suggests possibly a slightly different kinetics of the caudal fin regeneration in the mutant fish. However, overall, the % regenerate areas were strikingly similar at all other timepoints up to 17 dpa between the wild-type and *fibina/b^{-/-}* fish.

DMH1 treatment, as expected, resulted in significantly decreased caudal fin regenerate area at all timepoints beyond 3 dpa in both wild-type and *fibina/b^{-/-}* fish. Moreover, a clear significant decrease in the regenerate areas was observed from 10 dpa onwards in the *fibina/b^{-/-}* fish compared to wild-type fish (Fig. 5.5 (A)). Fig. 5.5 (C) shows the brightfield images of the regenerating caudal fins at the selected timepoints. Striking differences can be observed at 5 dpa in the 1 μ M DMH1 treated wild-type and *fibina/b^{-/-}* fish. In the wild-type fish, clear tracks for bone matrix deposition are visible, however, no cartilaginous (actinotrichia) tracts are visible in the mutant fish. At 7 dpa, it can be seen that there is some regeneration starting to take place in the mutant fish with number of melanocytes also

migrating distally into the regenerate. However, the bone is already clearly visible (albeit mis-patterned) in the wild-type fish at the same timepoint. At 17 dpa, bone outgrowth is clearly visible in the *fibina/b^{-/-}* fish as well, however to much lower extent compared to wild-type. As this was the last timepoint I collected data for (due to health issues in the fish associated with prolonged DMH1 treatment) it is impossible to say if the mutant fish do ever catch up to the wild-type equivalent (as caudal fin outgrowth is limited in size (Uemoto, Abe and Tamura, 2020)) or not. This data showed that although not completely inhibited, small BMP inhibition in parallel with lack of Fibins resulted in significantly delayed lepidotrichia outgrowth after simple caudal fin amputation in *fibina/b^{-/-}* fish compared to wild-type, emphasizing a role of Fibins in caudal fin regeneration possibly through modulation of BMP antagonists.

Figure 5.5 (D) shows the Alizarin red staining (calcified skeletal tissue) emphasizing the lack of bone outgrowth in the *fibina/b^{-/-}* fish treated with 1 μ M DMH1 compared to clearly calcified bone outgrowth in the wild-type fish at 5 dpa. With DMSO control treatment, at 5 dpa, tissue regenerate appears the same irrespective of the genotype, and the mesenchyme seemed to continue growing. With DMH1 treatment, however, possibly due to the lack of structural support in the form of actinotrichia and regrowing lepidotrichia tracts in the *fibina/b^{-/-}* fish (in the form of collagen deposition and other ECM molecules), the mesenchyme-based regenerate folded on itself, limiting the area measurements (as depicted on the cartoon in Fig. 5.5 (D')). As outlined in the graphs, the area differences are quantifiably, statistically, different between wild-type and *fibina/b^{-/-}* fish only from 10 dpa. This could suggest a limiting feedback mechanism that is activated from lack of structural support of the tissue and causes either decrease in proliferation rates of the regenerating mesenchyme/epithelium or increase in the rate of apoptosis in the regenerate, or both. However, this is impossible to address based on available data and would need further experimental clarification.

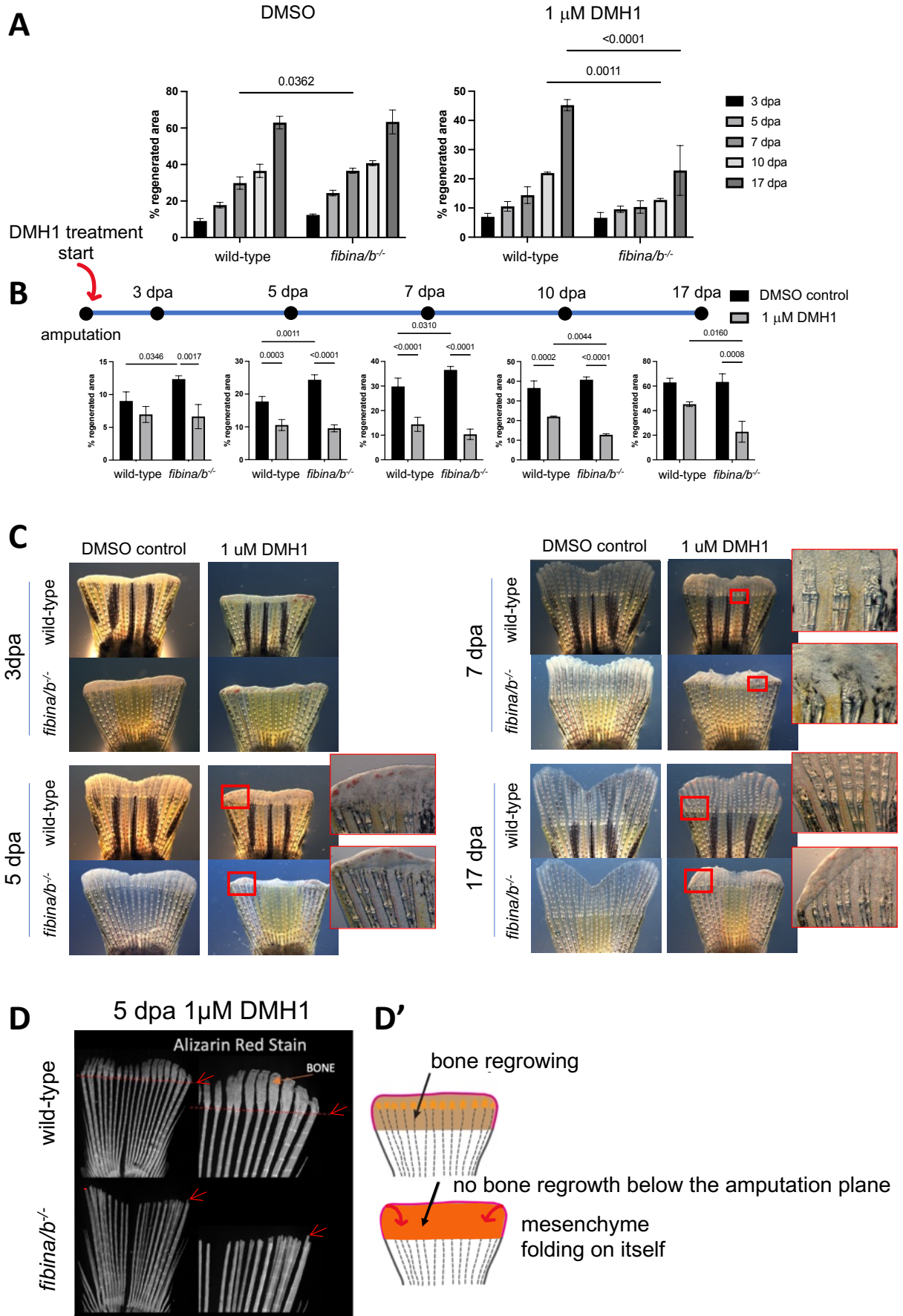


Figure 5.5 Caudal fin regeneration in wild-type and *fibina/b^{-/-}* fish

(A) Comparative time-course analysis of the regenerating caudal fin area between wild-type and *fibina/b^{-/-}* fish with or without (DMSO control) DMH1 treatment. Treatment was continuous from day 0 (amputation) to the timepoint specified. The data is re-plotted in (B) to show the direct comparison of control vs DMH1 treated regenerates areas. Statistical significance was calculated between wild-type and *fibina/b^{-/-}* fish and to determine treatment impact. Two-way Anova. (C) Bright-field microscopy images of wild-type and *fibina/b^{-/-}* fish caudal fin regeneration at 3, 5, 7 and 17 dpa (days post amputation) with or without (DMSO control) DMH1 treatment. (D) ARS staining of calcified tissues in 5 dpa regenerating caudal fins of wild-type and *fibina/b^{-/-}* fish when treated continuously with 1 μ M DMH1. Red arrow indicates amputation plane. Schematic summarizing the difference in caudal fin regeneration between wild-type and *fibina/b^{-/-}* fish when treated with DMH1.

To investigate if cellular proliferation is also involved in the difference detected, I investigated BrdU incorporation in 3 dpa fins from both genotypes when treated with 1 μ M DMH1 (Fig. 5.6). And indeed, a significant difference was detected between wild-type and *fibina/b^{-/-}* fish when treated with 1 μ M DMH1 with significantly less BrdU⁺ cells detected in the mutant caudal fin regenerates (quantified as % area). However, no significant difference is detected for DMSO (control) group, although a possible small decrease is visible. What is important to note, is that there was no significant difference in regenerate size between the treatment groups, possibly suggesting that this concentration of DMH1 (1 μ M DMH1) does not significantly affect the cellular proliferation, and, similarly to the craniofacial bone development data makes it difficult to fully appreciate the potential role of Fibins on cellular proliferation.

5.3.2.3 Analysis of caudal fin transcript levels of selected genes at 5 dpa in wild-type and *fibina/b^{-/-}* fish when treated with BMP signalling inhibitor DMH1

Phenotypically, with DMH1 treatment at 5 dpa, *fibina/b^{-/-}* fish have no calcified bone outgrowth below the amputation plane. Several genes involved in the initiation of the regeneration program after the inflammation stage (including *sall4*, *msx1b* or *mmp9*) showed lower transcript levels in *fibina/b^{-/-}* fish without any treatment at 3 dpa, but not at 5 dpa. Therefore, I decided to look at the 5 dpa timepoint whilst treating both the wild-type and *fibina/b^{-/-}* fish with 1 μ M DMH1 to understand better the significant differences in phenotype detected (Fig. 5.7).

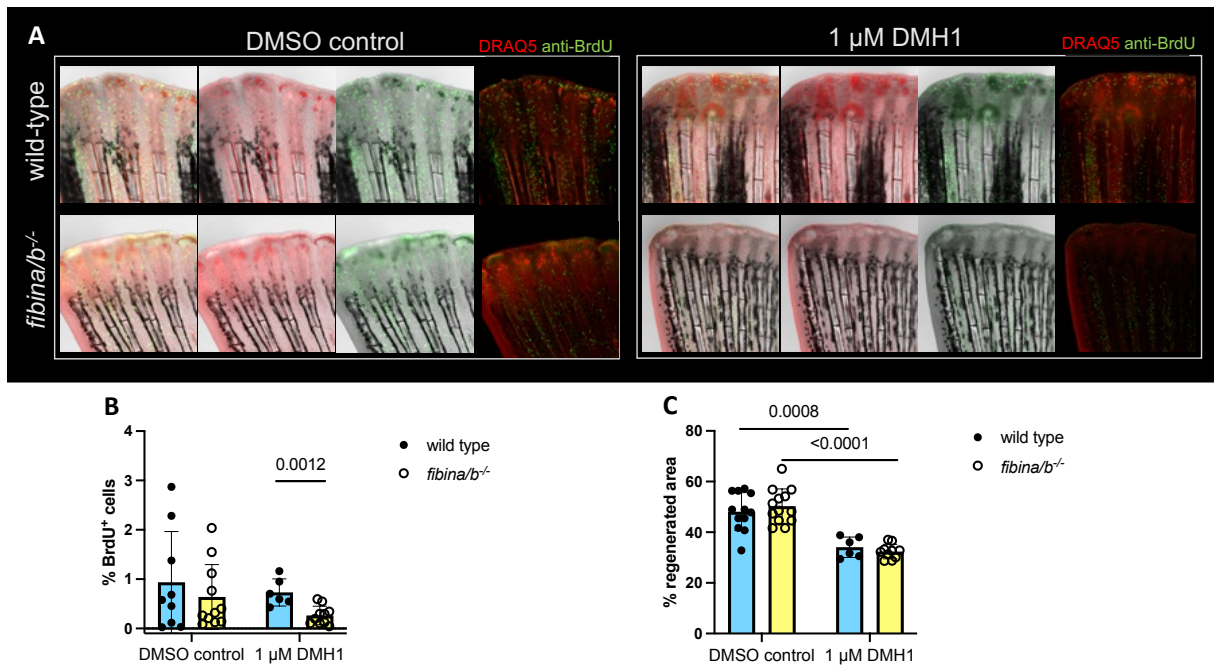


Figure 5.6 BrdU analysis of regenerating wild-type and *fibina/b^{-/-}* fish caudal fins at 3 dpa

(A) Representative maximum z-stack projections of caudal fins immunostained for BrdU (green) and and counter-stained with DRAQ5 (red, marker of cell nuclei). (B) Total average percentage of area covered by BrdU+ cells with respect to the regenerate area. (B) Average percentage regenerated area calculated with respect to original caudal fin area. One-way Anova performed with Turkey's multiple comparison test; mean and SEM shown. p-values < 0.05 shown.

Firstly, *fibina* transcript levels are significantly decreased in wild-type fish treated with DMH1, with the same pattern seen in mutant fish suggesting that BMP signalling inhibition can directly affect *fibina* transcription. *fibinb* on the other hand was not differentially regulated with DMH1 treatment. This was a very important finding, as it suggested differential regulation of *fibina* and *fibinb*, which ultimately could point to different function. From embryo *in situ* data, we know that *fibina* and *fibinb* show different spatial distribution during development, further supporting the idea that the two Fibins fulfil different roles during development and/or regeneration (Chapter 1: Introduction). As I am investigating a double mutant fish, it is not fully feasible to address these questions with this model. Future research into Fibins should thus also focus on developing single mutants for each gene to determine differential function. DMH1 treatment caused significant decrease in transcript levels of blastema marker *msx1b* as well as marker of proliferation *pcna* and BMP antagonist *gremlin2b* in wild-type fish.

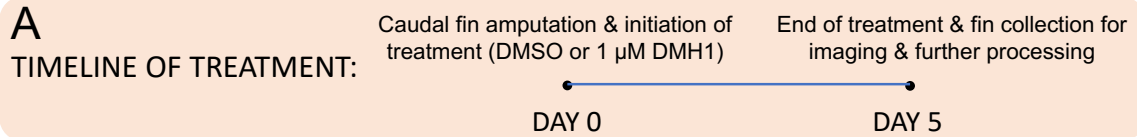
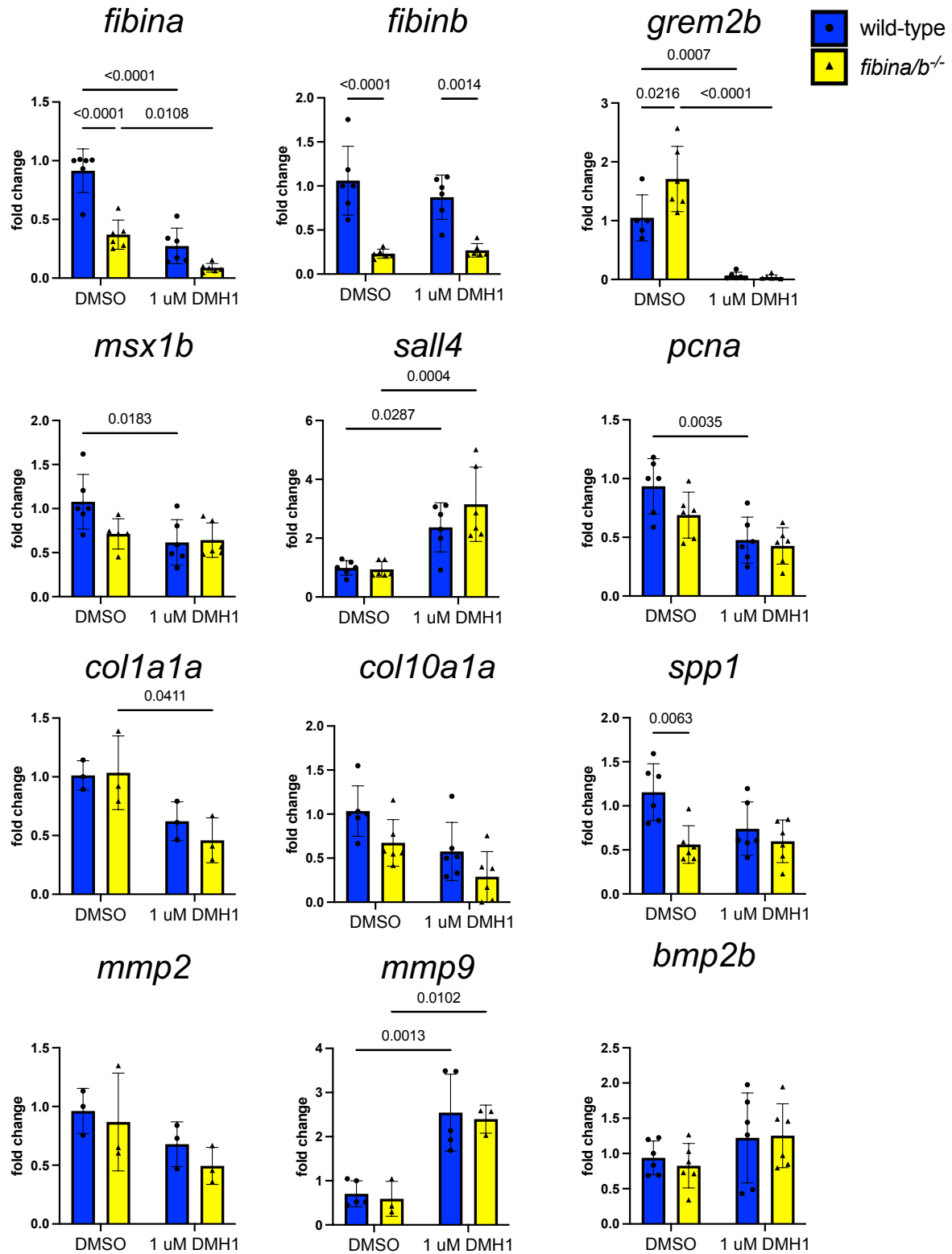
**B**

Figure 5.7 Transcript level analysis of selected genes following caudal fin amputation of young (4-6 months old) wild-type and *fibina/b^{-/-}* fish at 5 dpa with DMH1 treatment

(A) Schematic of DMH1 treatment timings. (B) Analysis of transcript levels of selected genes. Statistical significance is shown for comparisons between wild-type and *fibina/b^{-/-}* fish as well as across treatment groups. One-way Anova; mean and SD shown. p-values < 0.05 shown.

Furthermore noticeable, but not significant, decrease in transcript levels of *collala*, *coll0a1a*, *spp1* and *mmp2*. *fibina/b^{-/-}* fish showed decrease with DMH1 in transcript levels of *grem2b*, *collala*, *coll0a1a* and *mmp2*. *sall4* and *mmp9* transcript levels were significantly increased in both genotypes at 5 dpa with DMH1 treatment. *Sall4* is a BMP-independent blastema marker, which indicates a delayed blastema (marked by high *sall4* expression) assembly with DMH1 treatment. *mmp9* was previously shown to be negatively regulated by BMP signalling, which agrees with our results where decreased BMP signalling leads to increase in *mmp9* transcript levels in both wild-type and *fibina/b^{-/-}* fish (Lv *et al.*, 2013).

When comparing wild-type and *fibina/b^{-/-}* fish directly, there were no significant differences in transcript levels between any of the genes investigated with DMH1 treatment (apart from *fibina* and *fibinb* as discussed above). *grem2b* and *spp1* transcript levels were significantly different between wild-type and *fibina/b^{-/-}* fish with DMSO treatment (control) but no further difference was detected with DMH1 treatment, with *grem2b* transcript levels of both genotypes decreased almost to qPCR detection limit with DMH1 treatment, while *spp1* transcript levels of wild-type fish decreased to the level of *fibina/b^{-/-}* fish *spp1* level (which was unaffected by BMP inhibition).

msx1b, *pcna* and *spp1* show similar pattern of transcript level changes where wild-type fish have higher levels compared to mutant fish when treated with DMSO, but with DMH1 the transcript levels were lower in wild-type fish with no obvious change for *fibina/b^{-/-}*. These results can suggest that Fibins could be directly involved in the regulation of these genes or, more likely, as all these genes are co-regulated by the BMP pathway, that there is less active BMP signalling in the mutant fish with DMSO, which is then no longer impacted by more, specific BMP inhibition when using low concentration of DMH1.

Overall, transcriptionally significant differences were discovered between wild-type and *fibina/b^{-/-}* fish at 3 dpa (early caudal fin regeneration) in the regenerate tissue, specifically in genes involved in correct blastema set-up and early matrix remodelling to enable appendage outgrowth. These transcriptional differences, however, did not lead to a phenotypical difference between the two genotypes throughout the timecourse analysed (up to 17 days). BMP signalling inhibition using low dose of DMH1, resulted in significant phenotypical difference between wild-type and *fibina/b^{-/-}* fish at and after 5 dpa. Transcriptional analysis of 5 dpa regenerate tissues from both genotypes with or without DMH1 treatment revealed that *msx1b*, *pcna* and *spp1* change in transcript levels differed between wild-type and mutant fish. Neither of these genes was affected in *fibina/b^{-/-}* fish but all showed decrease in transcript levels in wild-type fish with DMH1 treatment, suggesting a baseline increased suppression of the transcript levels of these genes in mutant fish, corresponding with increased levels of BMP antagonists in the mutant fish.

5.3.3 Gill tissue regeneration

The Dallman laboratory has previously reported a new model of gill tissue regeneration – gill cryoinjury – with significantly increased severity of injury compared to other reported models of gill tissue regeneration (such as resection (Mierzwa *et al.*, 2020)). The procedure is detailed in Methods.

A previous RNA-seq done in the Dallman laboratory found *fibins* to be significantly upregulated at 7dpc in the cryoinjured gill tissue compared to the contralateral gill tissue. This up upregulation coincided with the upregulation of classical repair and matrix remodelling genes including *mmp2*, *mmp9*, collagens and more (Ramel *et al.*, 2021). Gill tissue has been particularly investigated as a model for respiratory tissue repair due to its similarities with mammalian lungs in function relating to gas exchange. The gill tissue (main structural component is cartilage) was found to regrow significant length of the lost lamellae in wild-type fish (up to day 294 post cryoinjury), with clear cartilage regrowth happening by 28 dpc as showed in Fig. 5.8. In this result section, I focussed on the structural, rather than functional, aspect of gill filament regrowth and regeneration.

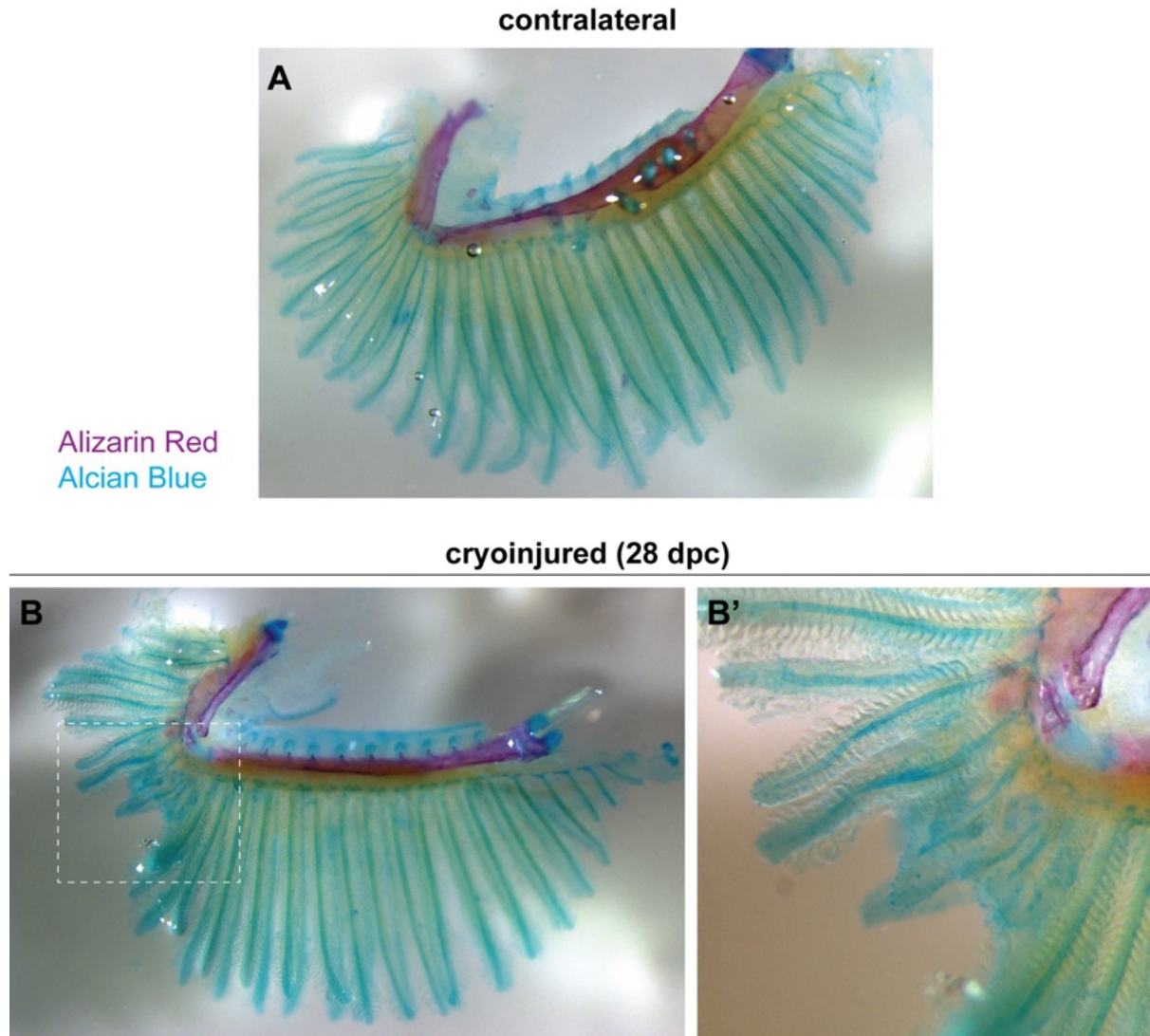


Figure 5.8 Regenerating cartilage filaments after gill cryoinjury (wild-type fish)

Representative images of Alizarin Red S Stain staining calcified tissue (the bony gill arch) and Alcian blue staining of gill filament cartilage. (A) contralateral gill arch. (B) cryoinjured gill arch. (B') zoomed-in image of cryoinjured gill arch. Figure adapted from (Ramel *et al.*, 2021).

5.3.3.1 The dynamics of selected genes' transcript levels during gill regeneration of young zebrafish

To investigate the hypothesis that Fibins play a role in the timely initiation of gill tissue regeneration after cryoinjury through interplay with BMP signalling, I have investigated a panel of genes (based on (Progatzy, 2014)) to study those involved in the early ECM remodelling ('set-up' for blastema formation and enabling blastema proliferation), blastema formation, and early ECM/cartilaginous matrix deposition in wild-type and *fibina/b^{-/-}* fish.

Importantly, all of the selected genes were found to have significantly increased read count numbers in cryoinjured versus contralateral (control) gill tissue in wild-type fish at 7 dpc (Appendix 11).

From the RNA-seq where our laboratory discovered Fibins to be differently regulated in zebrafish and mouse, both *fibina* and *fibinb* had significantly increased read count/transcript levels at 7 dpc in the cryoinjured tissue compared to contralateral tissue (*fibina* read counts were still significantly higher at 14 dpc as well). Therefore, to study the impact of lack of Fibins, 3 timepoints were selected with the earliest at 7 dpc (7 dpc, 10 dpc, 14 dpc).

A number of comparisons can be drawn from the following data including comparison of gene transcript levels in the contralateral tissues between wild-type and *fibina/b^{-/-}* fish, between the contralateral and cryoinjured tissue in a single genotype, in the cryoinjured tissues between wild-type and *fibina/b^{-/-}* fish, and even across time (post cryoinjury). This is therefore always specified in text. In general, I always compare the temporal profiles of the cryoinjured tissues between wild-type and *fibina/b^{-/-}* fish first, then focus on contralateral – cryoinjured tissue comparison for each genotype and how these changed across time.

Differential dynamics in the gene expression regulation in the cryoinjured tissue was clearly detectable from the temporal profiles of the individual genes investigated across the 7 dpc, 10 dpc and 14 dpc timepoints (Fig. 5.9). These included *sall4*, *msx1b* (blastema marker genes), *coll1a1a* and *coll10a1a* (early ECM and cartilaginous matrix genes), *mmp9*, *mmp2* (single cell RNA seq of gills showed *mmp2* to be one of the most strongly expressed genes in gill fibroblast cell population (Pan *et al.*, 2022)) and *grem2b* (BMP signalling antagonist that is positively regulated by BMP signalling (Luo *et al.*, 2021)). *coll1a1a* transcript levels were consistently lower in the *fibina/b^{-/-}* fish across the 3 timepoints, as well as *fibina* and *fibinb* (expected due to non-sense mediated decay as discussed in Chapter 3). Overall, significant differences were discovered on the transcriptional level particularly in terms of gene expression dynamics after cryoinjury.

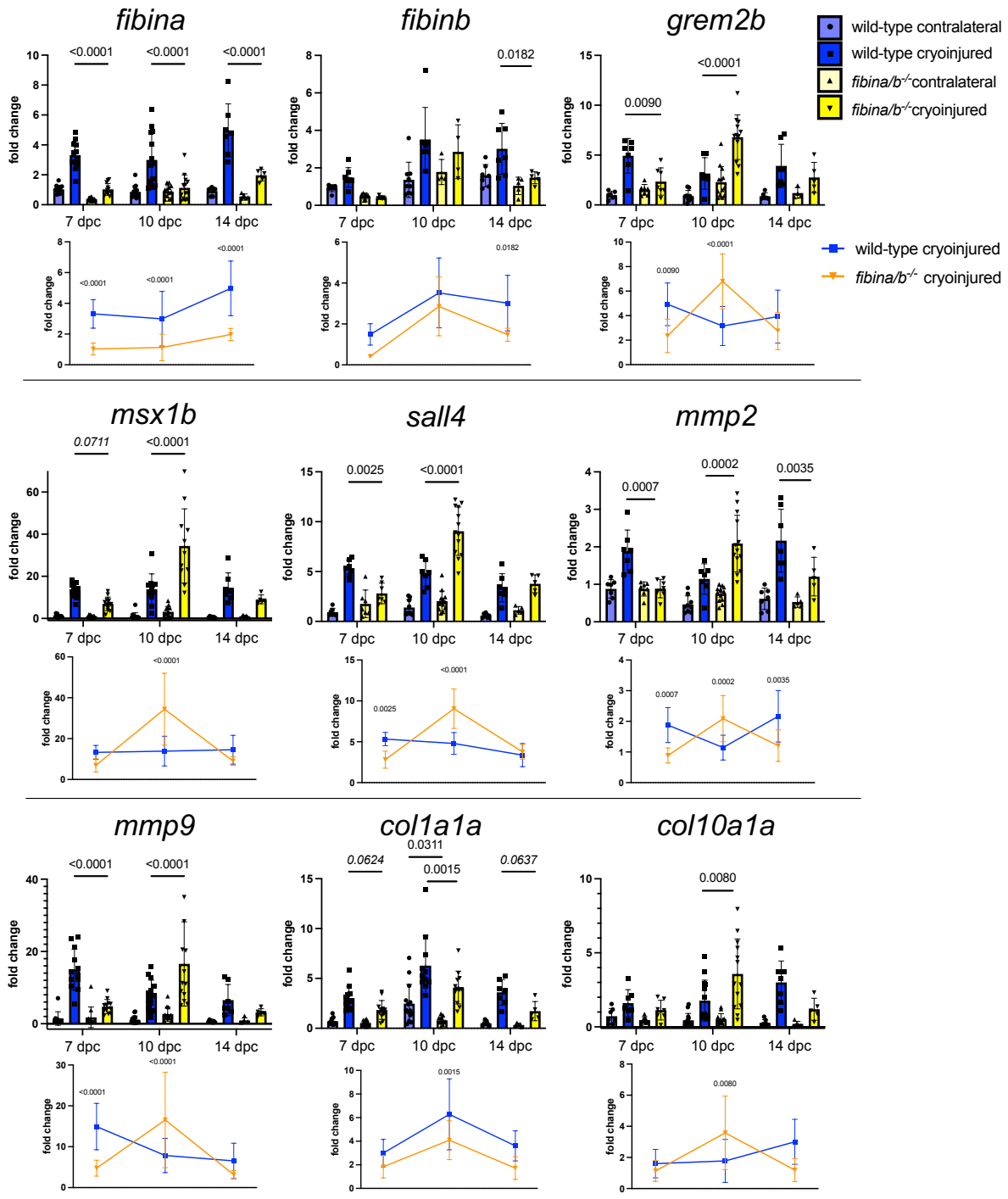


Figure 5.9 Transcript level analysis of selected genes following a gill cryoinjury in young (6 months old) wild-type and *fibina/b^{-/-}* fish, with temporal profile for each gene included.

Fish were anaesthetized and one side of the zebrafish gills were cryoinjured, fish were left to recover for the time specified as the end timepoint (7, 10 or 14 dpc). Fish were then euthanized, gill samples collected and processed for RNA. All samples for each gene are normalized to wild-type contralateral (control) gill tissue sample at 7 dpc. One-way Anova or Welch's Anova was performed; mean and SD shown. Statistically significant differences are indicated for the cryoinjured samples compared between wild-type and mutant fish. p-values <0.05 shown.

Looking closer at the individual timepoints, all the genes investigated showed lower increase in their transcript levels at 7 dpc in the cryoinjured tissue in the *fibina/b^{-/-}* mutant fish as compared to wild-types. *grem2b* as well as *sall4* showed increased transcript levels in the contralateral tissue of *fibina/b^{-/-}* compared to wild-type emphasizing the lower increase in their transcript level in response to the cryoinjury (all datapoints are first calibrated to their respective 18S and all data are plotted normalised to wild-type contralateral tissue at 7 dpc).

At 10 dpc, the situation was reversed with significantly higher increase in transcript levels of *msx1b*, *sall4*, *mmp2*, *mmp9*, *coll10a1a* and *grem2b* in cryoinjured tissue of the *fibina/b^{-/-}* fish compared to the wild-type fish. All of the genes profiled showed strong increase in transcript levels from 7 dpc to 10 dpc in the cryoinjured tissue in the *fibina/b^{-/-}* fish, stressing the difference in expression dynamics compared to wild-type fish.

At 14 dpc, in the cryoinjured tissue of *fibina/b^{-/-}* fish, we saw either a return to the levels of wild-type fish (such as *sall4*) or for all other genes, a return to lower increase in transcript levels compared to wild-type. Altogether these data suggest a delayed, dysregulated, yet robust initiation of the gene transcription cascade for gill filament regrowth in cryoinjured *fibina/b^{-/-}* fish compared to wild-type counterparts.

High *fibina* and *fibinb* levels were maintained across the 14 dpc period in the wild-type fish which is in contrast to what we saw in the original RNA seq (Ramel *et al.*, 2021). A possible explanation is a difference in the severity of the cryoinjury. The cryoinjury reported in (Ramel *et al.*, 2021) was primarily performed by a former Dallman laboratory member Dr Franze Progozky while all of the cryoinjury experiments presented in this thesis were performed by me. It is therefore possible that some differences in severity of the cryoinjury arose. However, as seen from Fig. 5.8 (Alcian blue staining of regenerating gill regeneration), tissue cryoinjury performed by me, still resulted in clear cartilage outgrowth by day 28, which is consistent with outcomes described in (Progozky, 2014) and (Ramel *et al.*, 2021).

Interestingly, *fibinb* transcript levels at 7 dpc in *fibina/b^{-/-}* fish were not increased (pattern seen in wild-type fish as well as for *fibina* in both wild-type and *fibina/b^{-/-}* fish). This could suggest *fibinb* transcription acts at least partially on a positive feedback mechanism. However, as there is a noticeable increase in *fibinb* transcript levels in cryoinjured tissue in *fibina/b^{-/-}* both at 10 dpc and 14 dpc, it is more likely that *fibinb* transcription is delayed in the mutant fish, pointing to *fibinb* transcription being governed by a complex combination of signals initiating tissue regeneration in the gills.

Comparison transcript levels of *fibina* and *fibinb* in contralateral gill tissue (controls) between wild-type and *fibina/b^{-/-}* fish (Appendix 12) showed that in wild-type fish, transcript levels of both Fibins remains similar, without any significant difference detected over time. In mutant fish, however, both *fibina* and *fibinb* transcript levels show significant increase from 7 dpc to 10 dpc, reaching the fold change of wild-type *fibina* and *fibinb* transcript levels. It is not surprising to see changes at the transcript level of Fibins in the *fibina/b^{-/-}* fish as the mutations present both lead to a premature stop codon, resulting in the mRNA to be directed for degradation (non-sense mediated decay), the fact that there appears to be a delay in the induction of *fibins* as well in the *fibina/b^{-/-}* fish, suggests the following: a) *fibina* and *fibinb* transcript levels are affected systemically in the animal, that is not only is there an increase in their transcript levels in the cryoinjured tissue but also in the contralateral tissue; b) possibility of a positive feedback mechanism: without a set baseline level of Fibins the early regenerative response to injury is delayed and dysregulated, including a delayed upregulation of Fibins. Whether Fibins directly affect intra-/extra-cellular communication and/or stimuli detection resulting in the appropriate (wild-type) pro-regenerative response or whether they exert their function through alternative mechanisms remains to be investigated.

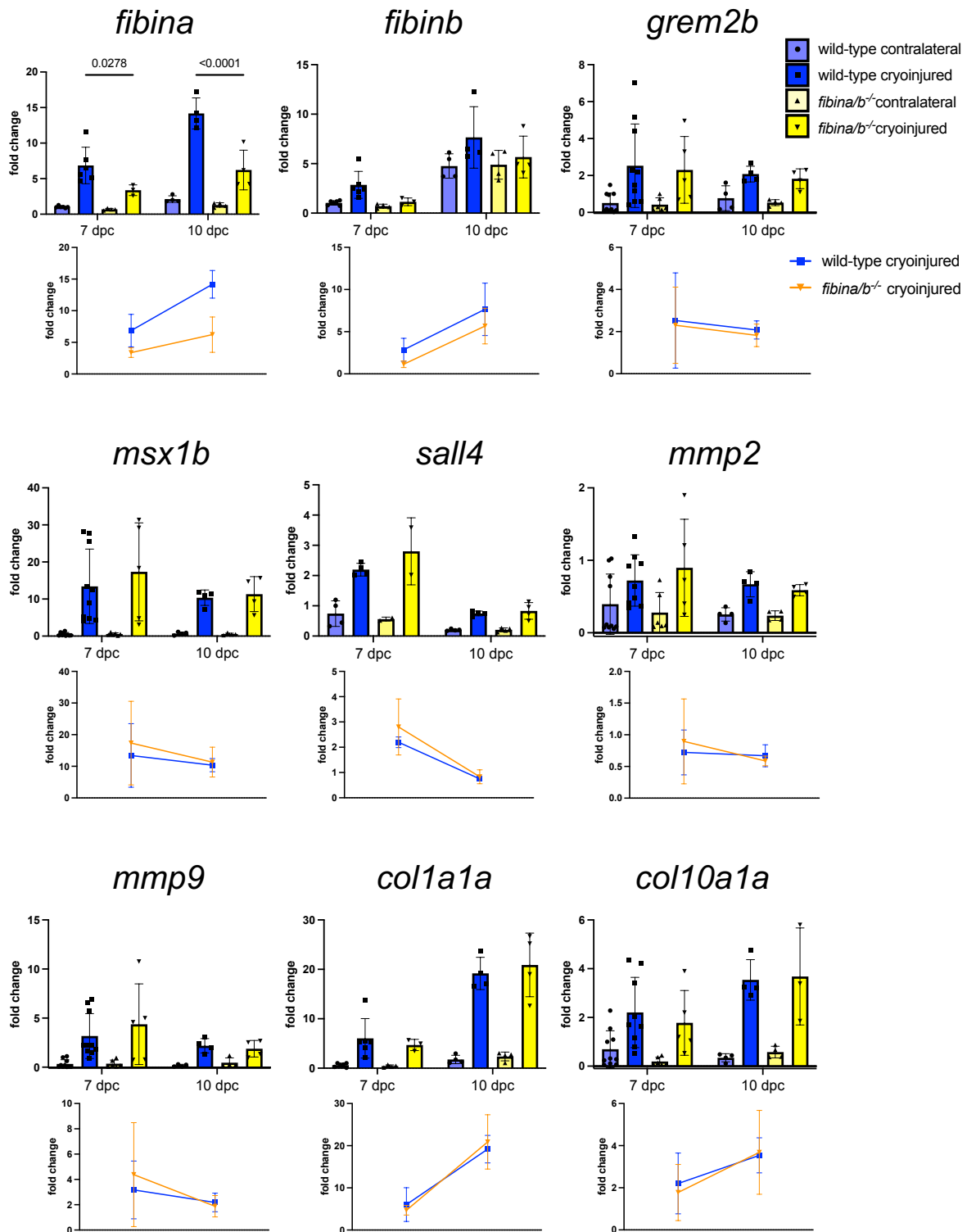


Figure 5.10 Transcript levels analysis of selected genes following a gill cryoinjury in old (15 months old) wild-type and *fibina/b*^{-/-} fish at 7 dpc and 10 dpc.

Fish were anaesthetized and one side of the zebrafish gills were cryoinjured, fish were left to recover for the time specified as the end timepoint (7 or 10 dpc). Fish were then euthanized, gill samples collected and processed for RNA. All samples for each gene are normalized to wild-type contralateral (control) gill tissue sample at 7 dpc. One-way Anova or Welch's Anova was performed; mean and SD shown. Statistically significant differences are indicated only for the cryoinjured samples compared between wild-type and mutant fish. p-values < 0.05 shown.

5.3.3.2 The dynamics of selected genes' transcript levels during gill regeneration of old zebrafish

From mouse data we know that fibin transcript levels decrease over time (Appendix 13). I have investigated this in our zebrafish (both wild-type and *fibina/b^{-/-}* fish) including ages 3, 6, 12 and 15 months old, analysing both gill and caudal fin tissues (Appendix 14). There is a difference in the trend of *fibins* transcript levels over time between the caudal fin and the gill tissues. While in the caudal fin, in the wild-type fish, the levels are highest at 3 months of age and significantly lower at 15 months of age compared to all previous timepoint (for both *fibins*), in the gill tissue there is a mild trend of increase in the *fibins* transcript levels from 3-12 months of age, with a decrease (although not significant) at 15 months of age. The variation in *fibina/b^{-/-}* fish is much greater in the gill tissue samples. Interestingly, in the mutant fish, *fibins* transcript levels are relatively higher in the gill tissue compared to the caudal fin tissue and particularly at 15 months of age, there is no significant difference between the mean FC for either *fibina* or *fibinb* in *fibina/b^{-/-}* fish compared to wild-type.

The significant differences in the dynamics and kinetics of the selected gene expression were thus investigated in 15 months old fish to determine what effect does decrease in Fibin levels with increasing age have on the differences detected between wild-type and *fibina/b^{-/-}* young fish. Old wild-type and *fibina/b^{-/-}* fish's gill were cryoinjured as discussed above and fish were let to recover for 7 or 10 dpc. At the selected timepoint the cryoinjured gill tissue and the corresponding contralateral gill tissue were collected for processing.

The temporal profiles of investigated genes from the cryo-injured tissue show the same trend for wild-type and mutant fish for all genes investigated from 7 dpc to 10 dpc, which is strikingly different to the trends observed in young fish. Large standard deviations in the data for 7 dpc indicate significant inter-fish differences (Fig. 5.10).

The mean fold change (FC) for *msx1b* at 7 dpc was 13 and 17 for wild-type and *fibina/b^{-/-}* respectively, compared to control (wild-type contralateral tissue). This fold change remained

similar at 10 dpc where it was mean FC = 10 for both wild-type and *fibina/b^{-/-}* fish. *mmp2* and *mmp9* show very similar dynamic with *mmp9* exhibiting higher mean FCs (at 7 dpc mean FC = 3 and 4 for wild-type and *fibina/b^{-/-}* fish respectively and at 10 dpc mean FC = 2 and 1.8) as compared to *mmp2*. *coll1a1* which shows strong increase of transcript levels for both wild-type and mutant fish at 10 dpc compared to 7 dpc. Of note, this increase (FC = 20 for both at 10 dpc compared to ~ 5 for both at 7 dpc) is much higher compared to the increases detected in the younger fish (at 7 dpc wild-type mean FC = 3 and mutant FC = 2; at 10 dpc wild-type mean FC = 7 and mutant mean FC = 4). Another interesting observation to note is the *grem2b* levels at both timepoints. In younger fish, *grem2b* levels were significantly upregulated in the mutants compared to wild-type at 7 dpc, however, in the old fish, although not statistically significant, the *bmp2b* transcript levels are lower in the cryoinjured tissues of mutant fish compared to the wild-type at this timepoint. At 10 dpc the increase in *grem2b* transcript levels in cryoinjured tissue is the same for both genotypes (mean FC = 3.5).

Interestingly, only *fibina* transcript levels in the cryoinjured tissue showed significant differences at both 7 dpc and 10 dpc timepoints between wild-type and mutant fish. No significant differences were detected between wild-type and *fibina/b^{-/-}* fish at either timepoint for any of the genes investigated.

The increase in *fibins* transcript levels appears to be much higher in the old fish compared to the young fish. To determine if this could be due to different baseline levels of *fibins* between young and old fish, I have normalized *fibina* and *fibinb* qPCR results to young wild-type contralateral gill tissue (Appendix 15). The results showed that the wild-type baseline (contralateral control gill samples) had significantly higher transcript levels of both *fibins* in the young compared to the old fish batches. Interestingly, the increase in transcript levels in cryoinjured tissues was therefore much ~ 2x higher for the *fibina/b^{-/-}* fish compared to wild-type fish for both genes, as the final fold-change was very similar between the two genotypes (FC ~3 for *fibina* and 1.2-1.5 for *fibinb*).

Overall, this data shows that in older wild-type fish the transcript level response of blastema markers and early matrix remodelling genes to gill cryoinjury is different, lower (in the cryoinjured tissue), compared to young wild-type fish. In fact, the levels are similar to those of *fibina/b^{-/-}* young fish at 7 dpc, without any clear increase in the transcript levels at 10 dpc for either wild-type or mutant fish. Moreover, it appears that with increasing age, and corresponding decrease in *fibina/fibinb* levels, the differences between wild-type and *fibina/b^{-/-}* fish are eliminated. Together, with the fact that *fibins*’ transcript levels increase twice as much in old fish in response to injury as compared to the response of young fish, these data suggest that Fibins do play a role during gill tissue regeneration – possibly modulating the communication and environment for kick-starting the regeneration process –, are not necessary for the regeneration to happen, and their function is attenuated in aged zebrafish. Unfortunately, it was not possible to keep the aged fish to determine how/if their gill filaments regenerate in similar timescale as their young colleagues. Future studies investigating aging and regeneration will hopefully shed light into the role of Fibins during these processes.

5.3.3.3 The effects of DMH1 treatment on the initiation and progression of gill regeneration

Next, I studied the role of BMP signalling in the initiation of the gill regeneration program to investigate the potential link between Fibins and BMP signalling pathway. (Mild inhibition of BMP receptors ALK1/2/3 by DMH1 revealed a significant phenotype difference between the regenerating caudal fins of wild-type and *fibina/b^{-/-}* fish (Fig. 5.5)).

In the regenerating gill tissue of young zebrafish, the transcript levels of all genes investigated were significantly less increased at 7 dpc in the cryoinjured tissue of mutant compared to wild-type fish. I have therefore treated both wild-type and *fibina/b^{-/-}* zebrafish with 1 μ M DMH1 for 7 days following cryoinjury to investigate the impact of BMP inhibition at the transcript levels of selected genes, and for 28 days following cryoinjury to investigate phenotypical changes in the regenerating gills between wild-type and *fibina/b^{-/-}* fish.

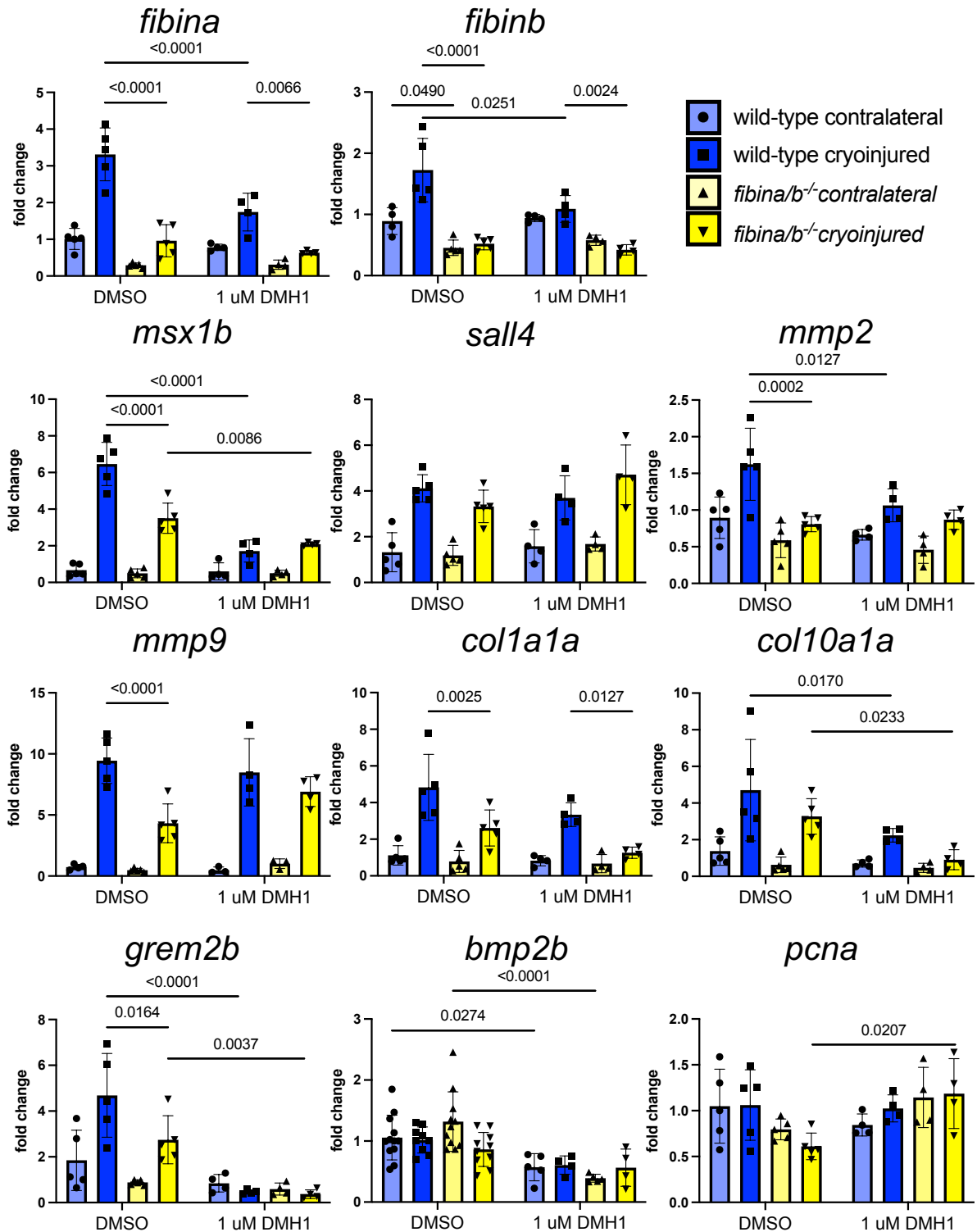


Figure 5.11 Transcript level analysis of selected genes following a gill cryoinjury in 8 months old wild-type and *fibina/b*^{-/-} fish when treated with the BMP signalling inhibitor DMH1.

Cryoinjured fish were placed either in DMSO- or 1 μ M DMH1-supplemented system water and left to recover, under close observation, for 7 days. One-way Anova or Welch's Anova was performed; mean and SD shown. Statistical significance is shown for comparisons between wild-type and *fibina/b*^{-/-} fish as well as across treatment groups to determine the impact of DMH1 treatment.

Firstly, *grem2b* and *coll0a1a* are genes directly affected by BMP inhibition (Kakoi *et al.*, 2014; Luo *et al.*, 2021), these genes are therefore also used as an internal control to determine if the low dose of DMH1 had an effect on gene transcripts. Although not significant, in the contralateral tissues of wild-type fish the mean FC for both genes was indeed halved with DMH1 treatment compared to DMSO treated controls (Fig. 5.11). There is still increase in *coll0a1a* transcript levels in DMH1 treated cryoinjured tissue, but the final mean FC is significantly lower compared to the DMSO treated cryoinjured tissue in wild-type fish. For *grem2b* 1 μ M DMH1 completely blocked the induction of the transcript levels in DMH1 treated wild-type fish.

Next looking at the transcript levels of *fibina* and *fibinb* with DMH1 treatment, both *fibina* and *fibinb* showed significantly lower increase in wild-type fish in the cryoinjured gill when treated with 1 μ M DMH1. However, the baseline levels (contralateral gill tissue) remained unaffected by the DMH1 treatment. Assuming that transcript levels directly correlate with gene transcription, this indicates that there is not much mRNA transcription of *fibina/b* taking place at homeostasis which explains the lack of change of *fibina/b* transcript levels in the contralateral tissue but transcription is downregulated during early regeneration when Fibins are detected at higher levels. Alternatively, BMP inhibition acts on *fibina/b* indirectly through the regulation of other genes which is then detected as a lower increase in *fibina/b* transcript levels at 7 dpc timepoint in the wild-type fish.

Both *fibina* and *fibinb* transcript levels follow the same dynamics in *fibina/b*^{-/-} fish irrespective of the treatment: *fibina* transcript levels are increased in cryoinjured compared to contralateral tissue, *fibinb* levels are not affected in cryoinjured tissue compared to contralateral tissue. As mentioned above (when discussing the cryoinjury timecourse in young fish), increase in *fibinb* transcript levels in appeared to be delayed from 7 dpc to 10 dpc, and therefore lack of change with DMH1 at this timepoint does not necessarily mean that *fibinb* transcript levels are not affected with DMH1 treatment, particularly in the cryoinjured tissues, rather a later timepoint (10 or 14 dpc) would need to be investigated, as at these timepoints, *fibinb* transcript levels were increased in cryoinjured tissue compared to contralateral tissue in the *fibina/b*^{-/-}.

BMP signalling inhibition during the first 7 days following gill cryoinjury showed similar pattern of transcript levels decrease in *msx1b*, *coll1a1a*, *coll10a1a* and *grem2b* in cryoinjured tissues. *mmp2* is involved in the regulation of chondrogenesis at the pre-condensation stage (Jin *et al.*, 2007). *mmp2* levels in wild-type cryoinjured tissues were significantly decreased in the DMH1 treated fish, to the level of *fibina/b^{-/-}* DMSO as well as DMH1 treated fish. As *mmp2* is known to be regulated by BMP signalling, these data suggest that the difference in increase in *mmp2* levels in wild-type fish compared to the mutant fish could be due to higher baseline levels of BMP antagonists and thus higher inhibition of BMP signalling in the *fibina/b^{-/-}* fish. Very similar pattern is seen in *msx1b* transcript levels as well where wild-type fish show substantially bigger decrease in the increase of *msx1b* transcript levels in cryoinjured tissue when treated with DMH1, compared to mutant fish. Together with the generally lower increase in other investigated genes, this data suggests a level of differential response to BMP signalling inhibition between wild-type and *fibina/b^{-/-}* fish.

Genes whose increase in cryoinjured tissues was significantly affected by DMH1 treatment included also *msx1b*, *mmp2*, *coll10a1a*, *grem2b*. Levels of *bmp2b* were significantly lower when comparing the contralateral gill tissues between treatment groups, but interestingly no significance was found for cryoinjured tissues. *Bmp2b* is not known to be directly regulated by BMP inhibition, rather, it is upregulated during chondrocyte differentiation/mesenchymal condensation, since a delay in regeneration is expected with DMH1 treatment, the general lower transcript levels with DMH1 treatment are in line with such hypothesis. *bmp2b*, together with *pcna* are the only genes that did not show clear increase in transcript levels in wild-type fish cryoinjured tissues at 7 dpc.

Brightfield images were taken of first (outer-most) gill arches from the contralateral and the cryoinjured side of both wild-type and *fibina/b^{-/-}* fish at 28 dpc (Fig. 5. 12). There was no difference observed between contralateral gills of wild-type and *fibina/b^{-/-}* fish. Clear regenerating filaments could be observed in the wild-type fish after cryoinjury, while the regrowing filaments were much less visible in the *fibina/b^{-/-}* fish.

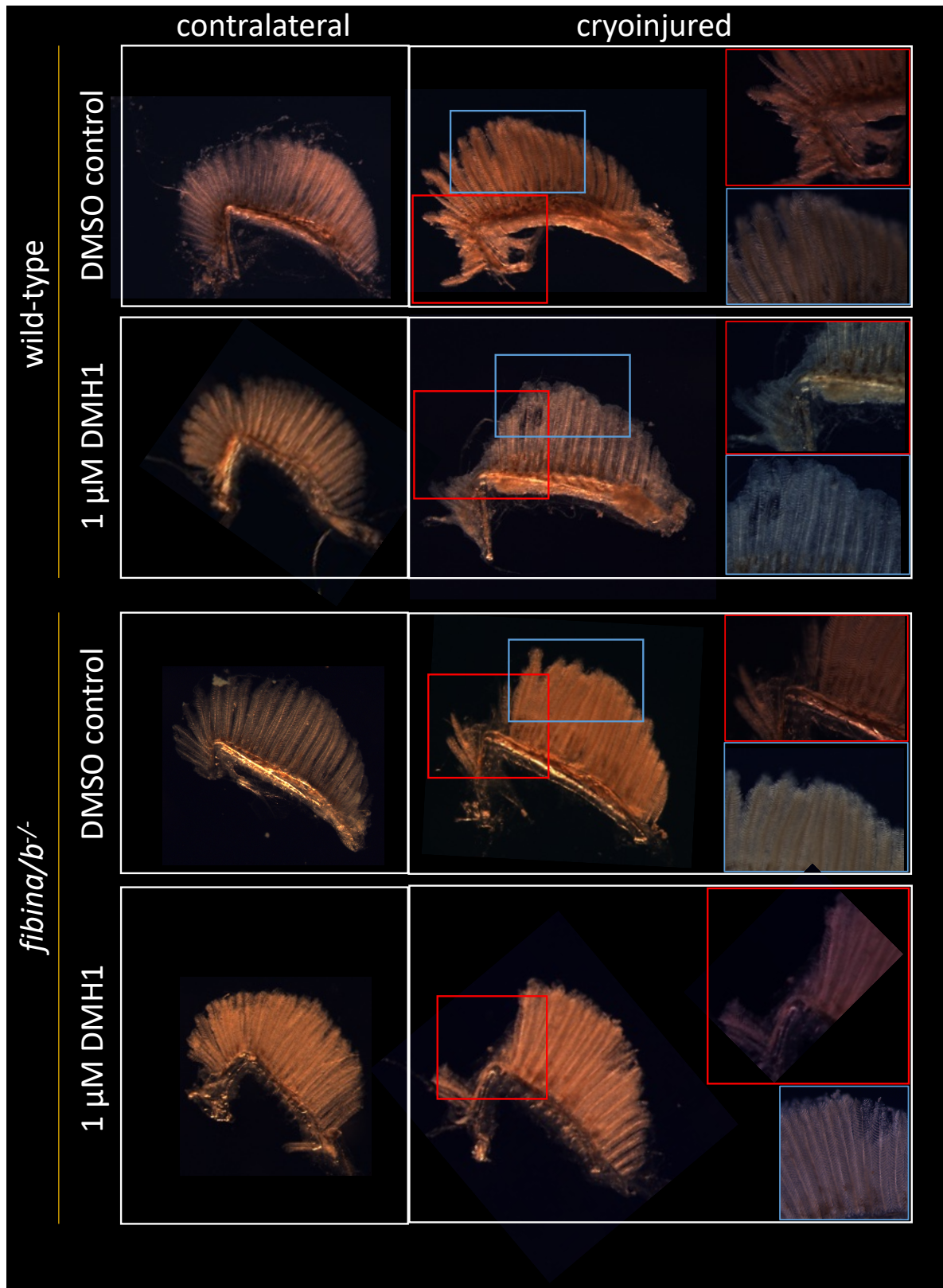


Figure 5.12 Comparison of gill filaments in wild-type and *fibina/b^{-/-}* fish at 28 days post cryoinjury, with or without DMH1 treatment

Representative images of wild-type and *fibina/b^{-/-}* zebrafish contralateral and cryoinjured gill filaments. Cryoinjured fish were placed either in DMSO- or 1 μ M DMH1-supplemented system water and left to recover, under close observation, for 7 days. Fish were then euthanized, and gills dissected.

DMH1 treatment did not seem to have a significantly different phenotypical effect on *fibina/b^{-/-}* fish compared to wild-type fish. Cryoinjured gill arches from wild-type and *fibina/b^{-/-}* fish under DMH1 treatment for 28 days showed clear differences to control cryoinjured gills. The cryoinjured area itself showed no sign of regrowing filaments, which is with agreement with (Saito *et al.*, 2019) that BMP inhibition inhibits gill tissue regeneration, yet based on previously discussed data, it is possible that the gills would still regenerate, but the regeneration is delayed. Secondly, a rather interesting observation was that the secondary lamellae at the tips of the primary lamellae appear longer compared to DMSO treated gills. It is interesting as BMP signalling in the caudal fin fold of zebrafish embryos acts to limit growth and BMP inhibition results in significant increase in caudal fin fold area (mesenchymal/cartilaginous tissue). Therefore, although 1 μ M DMH1 seems to inhibit gill tissue regeneration (at 28 dpc), it appears to, at the same time, enhance the growth of the lamellae suggesting that the continued growth of gill filaments is modulated via different molecular signals compared to the regeneration.

Overall, analysis of genes following the initial inflammatory stage of regeneration (0 – 7 dpc) significant differences were found in gill tissue regeneration between young wild-type and *fibina/b^{-/-}* fish, including differential dynamics in several key early-stage regeneration genes such as *sall4*, *msx1b*, *mmp9* or *mmp2*. Old wild-type and *fibina/b^{-/-}* fish showed interestingly no difference whatsoever in the upregulation of the selected panel of genes upon cryoinjury – a striking difference to the young fish regeneration data. BMP signalling inhibition showed several BMP-regulated genes differentially regulated between wild-type and *fibina/b^{-/-}* fish (including *msx1b*), altogether supporting my hypothesis that Fibins play a role in optimal gill tissue regeneration possibly through interaction with BMP signalling. However, the exact mechanism of Fibins interaction with BMP signalling remains to be investigated, including whether they work intracellularly, extracellularly, based on concentration gradients or possibly by direct binding to their target, and ultimately – what is the impact on tissue homeostasis and regeneration.

5.4 Discussion

In this Chapter, I have investigated the hypothesis that the lack of Fibins in the *fibina/b^{-/-}* fish leads to increased basal levels of BMP signalling antagonists and dysregulated BMP signalling during zebrafish development and/or regeneration.

5.4.1 Craniofacial cartilage and bone development

BMP signalling is necessary for cartilage and bone development and maintenance. Firstly, I have therefore investigated the phenotype of cartilage and bone development in wild-type and *fibina/b^{-/-}* fish. I found no difference between wild-type and *fibina/b^{-/-}* fish in either cartilage patterning or bone calcification (at 5 dpf and 10 dpf respectively). Together with master students Serafima Davidova and Xiangyu Sui I have then investigated if a low dose of specific BMP inhibitor DMH1 – a dose that ideally does not have a significant impact on the wild-type fish – does differentially affect the mutant fish. We have found that 1 μ M DMH1 in particular affects mutant fish to a larger extent compared to wild-type fish, with significant difference in the patterning of craniofacial cartilage. However, this was only true for treatment window from 48 hpf to 72 hpf, but not for 27.5 hpf – 48 hpf. Both genotypes are significantly affected during the earlier treatment window with images in Fig. 5.2 (G) showing the visible alteration of the patterning of the ventral head cartilages in both wild-type and *fibina/b^{-/-}* fish. In contrast the later treatment window (48 – 72 hpf) shows a lesser visible difference in cartilage patterning even with 10x higher DMH1 concentration (1 μ M DMH1) for all fish. A significant difference between the two studied genotypes was only uncovered with a DMH1 concentration that did not significantly affect the wild-type fish. My results thus further confirm the necessity of intact BMP signalling for correct development and patterning of head cartilages in zebrafish and also suggest Fibins are indeed possibly involved in the optimal signalling crosstalk during cartilage development and patterning with a role that appears to be linked to mildly increased BMP inhibition or mildly decreased efficiency of BMP signalling in the *fibina/b^{-/-}* fish, possibly due to increase in BMP antagonists. However, to properly understand the impact of increased levels of BMP antagonists on the craniofacial development and dorso-ventral skeletal patterning at steady state (with no treatment), *in situ* hybridization investigating the exact spatial localization of antagonists such as *grem2b* or *noggin* together with *fibina* and *fibinb* is necessary to estimate potential

interactions. Morpholino injections or conditional knock-in mutant fish will also be fundamental in deciphering the possible phenotypical differences arising from increased BMP signalling inhibition/decreased BMP signalling efficiency.

In terms of bone calcification, even though there was a significant difference between wild-type and mutant fish when treated with 0.1 μ M DMH1, no significant difference was detected between treatments for either genotype. Without investigating the bone calcification using ARS staining under wider range of DMH1 concentrations, different end-points or using other techniques, including protein and RNA work, it is not possible to fully confirm or disprove that Fibins play a role in the development of calcified bone in zebrafish. BMP inhibition leads to less calcified bone detectable in developing embryos or in adult regenerating limbs (Yu *et al.*, 2008; Windhausen *et al.*, 2015). Interestingly, several reports have suggested earlier timepoints and time-windows for investigation of the BMP signalling importance in chondrogenesis and bone calcification – including (Windhausen *et al.*, 2015). Windhausen and colleagues reported that BMP signalling inhibition in the 48-72 hpf window had no effect on cartilage development or patterning (using 100 μ M dorsomorphin which is 2x the dose used in my experiments) but lead to significant reduction in bone mineralization in the craniofacial region with no observed effect on chondrogenesis or osteoblast maturation (Windhausen *et al.*, 2015). However, based on the results presented in this chapter, which showed that BMP inhibition during 48 -72 hpf did significantly affect cartilage patterning in wild-type fish, it is clear that even the research baseline wild-type zebrafish does substantially differ from laboratory to laboratory.

Overall, at steady state without any additional treatment, lack of Fibins does not cause any phenotypical differences between wild-type and mutant fish. For both the cartilage development/patterning and bone calcification, other timepoints and additional specific BMP signalling inhibitors affecting other parts of the BMP signalling compared to DMH1 are necessary to determine the exact relationship between Fibins, BMP signalling, and the phenotype of wild-type and mutant fish.

5.4.2 Caudal fin regeneration

Next, I investigated caudal fin regeneration in both wild-type and mutant fish to understand, at a transcript level, possible differences between the two genotypes. Transcriptionally, there were significant differences at 3 dpa between the two genotypes. A significantly lower increase in *mmp9* transcript levels in mutant fish as well as lower increase in *msx1b* and *sall4*, blastema markers. *Mmp9* is widely studied gene in the regeneration field. *Mmp9* codes for matrix metalloprotease 9, with the most well-known role of digesting collagen fibres. *Mmp9* has number of other roles relating to tissue regeneration, including being a marker of wound epithelium (Hale *et al.*, 2017), or a marker of joint-associated mesenchymal cells (Ton and Iovine, 2013). *mmp9* is also involved in timely recruitment of inflammatory cells, including macrophages and neutrophils to the amputation site (LeBert *et al.*, 2015). Volkman and colleagues have found that inhibition of *mmp9* prior to wounding, leads to reduced inflammatory response (Volkman *et al.*, 2010). Combined with numerous research showing that optimal recruitment of macrophages and neutrophils to the injury site is necessary for complete regeneration of the lost appendage (Godwin and Rosenthal, 2014; Petrie *et al.*, 2015; Xu *et al.*, 2019), this suggests that the lower increase in *mmp9* in *fibina/b^{-/-}* fish (accompanied with lower levels of blastema markers *msx1b* and *sall4*) could indicate a delayed or improper initiation of the regeneration program in the zebrafish caudal fin.

However, no difference on the transcriptional level was detected at 5 dpa between the two genotypes and no phenotypical difference was observed at any timepoint in control fish. As it remained unclear whether the lower increase in several genes at 3 dpa in the mutant fish is simply lower at that time point or if it represents a delayed gene induction (as the mean FC are equal at 5 dpa), I used DMH1 (at a concentration that did not prevent wild-type caudal fin regeneration) and discovered a significantly delayed regeneration of the caudal fin in the mutant fish. In particular at 5 dpa, there was a significant phenotypic difference between the regenerates where in wild-type, bone tracks and early mineralization were easily visible below the amputation plane, however, no mineralization was detectable below the amputation plane in mutant fish. Investigating later timepoints, it became clear that the regeneration is not completely inhibited, only delayed. Still, the regenerated area remained significantly smaller in the mutant up to 17 dpa.

As osteoblasts begin to differentiate and secrete bone matrix from ~ 3 dpa, the lack of visible bone tracts/calcified tissue at 5 dpa suggests that in fish lacking *fibina* / *b* that are treated with DMH1, there is a significant dysregulation in the initiation of bone (lepidotrichia) and cartilage (actinotrichia) regeneration, compared to wild-type fish.

Stages of fin regeneration with respect to osteoblasts:

1) Dysregulation of the osteoblasts re-differentiation and blastema set-up

Upon caudal fin amputation, osteoblasts surrounding the stump undergo dedifferentiation from mature (non-proliferating) osteoblasts to preosteoblasts (proliferating) that form part of the establishing blastema (Knopf *et al.*, 2011). NF- κ B signalling and retinoic acid signalling have been shown to impair osteoblasts de-differentiation upon caudal fin amputation (Blum and Begemann, 2015; Mishra *et al.*, 2020). BMP signalling is not known to be involved in the pre-blastema set up, although BMP targets have been found in the wound epithelium suggesting that BMP signalling is involved in the crosstalk between the wound epithelium and the developing blastema (Smith *et al.*, 2006). Furthermore, BMP signalling is involved in optimal cell proliferation within blastema, maintaining the pool of pluripotent cells that eventually give rise to a newly formed appendage. Based on the data shown and the timeline of fin regeneration, it appears that indeed a delayed/slowed re-differentiation as well as blastema establishment and proliferation could be responsible for the significant difference in phenotype of DMH1 treated wild-type and *fibina/b*^{-/-} fish. However, as only with the DMH1 treatment, I uncovered the differential phenotype, more investigation is necessary to dissect the exact role of Fibins during the process, as well as which particular phase of bone formation with respect to osteoblasts they affect (proliferation, maturation, mineralization or apoptosis).

Firstly, better understanding of the exact timing during the fin regeneration when we begin to see a differential phenotype/transcriptome between the wild-type and *fibina/b*^{-/-} fish would enable us to narrow down the investigation to pathways relevant to the particular regeneration stage. And secondly, very useful information could be obtained by single-cell-RNA sequencing of the blastema composition of wild-type and *fibina/b*^{-/-} regenerating fins. By

assessing the proximal and distal blastema, we could compare the relative differentiation stages of the present osteoblasts, the proliferation status as well as the co-expression profiles of cells expressing *fibina* or *fibinb* or both. (Of note, Tang and colleagues (2022) performed a single cell analysis of regenerating zebrafish caudal fins at 3 dpa and from their dataset it can be determined that *fibina* and *fibinb* show no coexpression in the investigated cell pool, further supporting the evidence from Dr M-C Ramel presented in Chapter 3 showing differential expression patterns of *fibina* and *fibinb* in the developing embryos which points to a potentially differential role for each *fibin* (Tang *et al.*, 2022).)

2) Dysregulation of the osteoblasts' maturation and bone matrix secretion

Osteoblasts maturation and bone matrix secretion is regulated by BMP signalling. Analysis of immature-osteoblasts marker (*coll10a1a*), mature-osteoblasts marker (*spp1*), extracellular matrix secretion (*coll1a1a*) and matrix remodelling *mmp9* genes, revealed that while *coll10a1a* was not significantly different at 5 dpa between wild-type and mutant fish, *spp1* levels were significantly lower in the *fibina/b^{-/-}* fish compared to wild-type. This data suggests that in the *fibina/b^{-/-}* fish there may be a delay in the maturation of osteoblasts. Interestingly, when treated with DMH1, only wild-type fish *spp1* levels were affected (decreased) but not *fibina/b^{-/-}* fish levels. Similar pattern is seen with *msx1b* transcript levels which were significantly decreased with DMH1 treatment in wild-type fish but unchanged in mutant fish. This suggests that these two genes are regulated also by another pathway apart from BMP signalling and this pathway is unaffected in the mutant fish (there still is an increase in these genes following amputation, the increase is just comparatively lower to what was detected in wild-type fish). Moreover though, it shows that the mutant fish are fully not responding to the BMP-related increase in transcript levels (the difference between wild-type control and wild-type treated with low dose of DMH1). This can be due to increased BMP antagonism or possibly due to decreased sensitivity to the BMP stimulation. As *grem2b* levels were found to be significantly higher in the mutant fish at 5 dpa with DMSO treatment (control), the data does suggest that it is higher levels of BMP antagonists responsible for the transcriptional differences detected between wild-type and *fibina/b^{-/-}* fish. Yet, the lack of significant differences between the 2 genotypes at the transcript level in 5 dpa fish treated with DMH1 provides very little information to explain the obvious phenotypic difference. It is possible that the 'even-lower' levels of matrix genes *coll1a1a* and *coll10a1a* combined with the

significantly fewer BrdU⁺ cells detected was enough to result in such a strong phenotypical difference, however, more experiments including *fibina/b* rescue experiments need to be performed to fully understand the relationship between Fibins and BMP signalling in the regenerating caudal fin.

3) Dysregulation of the calcification process

Based on the images in Fig. 5.5(C,D) showing clear lack of visible bone outgrowth or actinotrichia support in *fibina/b*^{-/-} fins at 5 dpa, as well as detection of calcified bone in the *fibina/b*^{-/-} fish at later timepoints suggests that it is unlikely that a dysregulation of the calcification/mineralization process is responsible for the phenotypical differences between the two genotypes.

Based on the results from 3 and 5 dpa timecourse with no treatment, it appears that Fibins play a role in the correct set-up for the outgrowth, however, they are not vital for regeneration to happen. Yet, when combined with a small dose of BMP inhibitor, the lack of Fibins causes a significantly delayed bone outgrowth in the mutant fish compared to wild-type fish.

An interesting observation has been made by Azevedo and colleagues who performed an experiment looking at the regenerative capacity of zebrafish caudal fin after up to 29 resections. They discovered *mmp9* and *msx1b* levels in 3 dpa regenerate are substantially lower after 14+ fin amputations compared to the levels after first amputation. Yet, although *mmp9* and *msx1b* levels increase was attenuated with the increasing number of amputations, the level was still enough to allow for a successful regeneration (Azevedo *et al.*, 2011b).

Moreover, Wedler and colleagues showed fascinating data on the delay of initiation of caudal fin regeneration in old versus young short-lived killifish (*Nothobranchius furzeri*) (Wendler *et al.*, 2015). In particular the peak expression of *msx1b* was shown to be delayed by 1 full day (from 2 dpi to 3 dpi) in 36 week old fish compared to 8 weeks old fish. Furthermore, they

showed significant decrease in number of proliferating cells and increase in number of apoptotic cells in the old fish during the regenerative process.

Age-dependent analysis of *fibin* levels (Appendix 14) showed that both *fibina* and *fibinb* transcript levels significantly decrease with age, specifically at 15 months of age, compared to 3-, 6-, and 12-months old fish, in the caudal fin of wild-type zebrafish. In the mutant fish, the levels of *fibins* were significantly lower except for 15 months old fish in which the levels of wild-type fish dropped to the levels of mutant fish. It would therefore be interesting to perform these fin cut experiments in old zebrafish, to determine the levels of genes involved in the early blastema setup and initiation of the regeneration programs. Örling and colleagues recently reported significantly diminished regenerative capacity of 40 months old zebrafish with no visible bone outgrowth at 8 dpa (Örling *et al.*, 2023). It would be very interesting to investigate if lack of Fibins in zebrafish simulates the environment of an aged animal's response to regeneration.

5.4.3 Gill tissue regeneration

Compared to caudal fin regeneration, very little is known about the exact signalling pathway communications that enable successful, scar-less gill tissue regeneration. The main structural support of zebrafish gill tissue is cartilaginous filaments. I have shown that after cryoinjury, new cartilaginous matrix was detected within 28 dpc in wild-type fish.

My results indicate that there is a significant difference in the dynamics of selected gene transcript level induction between wild-type and mutant fish. In young (~6 months of age) zebrafish there was a clear, significant delay in the increase of several important genes including blastema markers *msx1b* and *sall4*, matrix remodelling enzymes *mmp9* and *mmp2* or genes coding for the matrix components themselves – *colla1a* and *coll10a1a*. This delay manifested in *fibina/b*^{-/-} fish having significantly lower transcript levels at 7 dpc in the cryoinjured tissue while having significantly higher levels of the transcript at 10 dpc compared to wild-type fish (in the cryoinjured tissue). This suggests that lack of Fibins (that

are normally significantly upregulated after 3 dpc) leads to dysregulated response to the injury at times which mark the post-inflammatory phase transitioning to regenerative/outgrowth phase.

I have next investigated the impact of DMH1 treatment – inhibition of BMP signalling – on the transcript levels of selected genes. The pattern of transcript levels of genes known to be directly regulated by BMP signalling/BMP signalling inhibition – *msx1b*, *coll1a1a*, *coll10a1a* and *grem2b* – further indicated that *fibina/b^{-/-}* fish have a higher level of BMP inhibition / lower level of active BMP signalling compared to wild-type fish during gill regeneration. This was due to the significantly lower levels (increase) of these genes in the cryoinjured tissue of the mutant fish compared to the levels in the cryoinjured tissue of the wild-types, with DMSO treatment. Under DMH1 treatment, wild-type cryoinjured gill tissue had significantly lower transcript levels of *msx1b*, *coll1a1a*, *coll10a1a* or *grem2b* compared to DMSO treated cryoinjured tissue. Moreover, the transcript levels were very similar in the cryoinjured tissue in both wild-type and *fibina/b^{-/-}* fish when treated with DMH1. However, as only one timepoint with DMH1 treatment was investigated for transcript level comparison, it is not possible to determine the impact on the gene expression dynamics throughout the early remodelling stage of gill regeneration. Investigation of phenotypical differences of wild-type and *fibina/b^{-/-}* fish treated with DMSO or DMH1 at revealed that *fibina/b^{-/-}* fish have less of the regenerating cartilaginous filaments visible at 28 dpc compared to wild-types and that BMP signalling inhibition causes complete lack of visible filament outgrowth at this timepoint in both wild-type and *fibina/b^{-/-}* fish. Again, whether this is a delay in regeneration, similarly to the caudal fin experiments, or a complete inhibition of gill regeneration remains to be determined through future experiments investigating further timepoints as well as DMH1 concentrations. Overall, based on transcript level analysis at 7 dpc and phenotype analysis at 28 dpc, significant differences have been discovered between wild-type and *fibina/b^{-/-}* fish. Based on the transcript level analysis, BMP signalling does appear to be impacted in *fibina/b^{-/-}* fish, however, further timepoints as well as other BMP signalling inhibitors or DMH1 concentrations need to be investigated, that would enable visual confirmation of differential BMP inhibition impact on the gill regeneration of wild-type and *fibina/b^{-/-}* fish.

Fascinatingly, experiments in old (15 months) wild-type and *fibina/b^{-/-}* fish showed a very different pattern and dynamics of post-cryoinjury gene expression. No significant difference was detected between the wild-type and *fibina/b^{-/-}* fish in either gene investigated at 7 or 10 dpa. I have therefore compared the levels of *fibina* and *fibinb* between 7 dpc contralateral and cryoinjured tissue of wild-type and *fibina/b^{-/-}* fish (Appendix 12) and found that the levels in the contralateral gill tissue of wild-type fish are approximately halved in the old fish compared to the young wild-types, yet the final increase in transcript levels in cryoinjured tissue is the same (mean FC = 3.2 for *fibina* and 1.4 for *fibinb*) for both young and old wild-type fish, indicating a two-times higher induction of *fibins* in old fish compared to young fish. Still, levels of blastema marker *sall4* are lower in the old wild-type fish compared to young fish at 7 dpc (mean FC of 5 vs mean FC of 2 respectively), which is comparable to *fibina/b^{-/-}* fish *sall4* transcript levels in the cryoinjured tissue at 7 dpc. It has been previously reported in axolotls, that knockdown of *sall4* at the time of limb amputation resulted in significantly increased collagen I and collagen XII expression, secretion and deposition in the vicinity of the wound resulting in a scar-like phenotype (Erickson *et al.*, 2016; Galliot *et al.*, 2017). These results suggest that *sall4* has a fundamental role in scar-less regeneration and thus attenuated increase of *sall4* in aged wild-type fish as well as in young *fibina/b^{-/-}* fish does suggest that the lack of functional Fibins mediates an environment in the young mutant fish that may mimic an environment from aged animals.

Wakahara and colleagues in their first paper describing zebrafish *fibinb* suggested that *fibin* lay upstream of the transcription factor *tbx5* (known to be involved in limb development) and that knockdown of *fibin* resulted in diminished expression of *tbx5* in the presumptive pectoral fin region (Wakahara *et al.*, 2007). As discussed in Chapter 1: Introduction, our group has not been able to replicate these experimental results and the *fibina/b^{-/-}* fish generated in our laboratory do not lack pectoral fins. However, the potential link with *tbx5* has not been yet fully dissected and considering the fact that *tbx5* lies upstream of and regulates the expression of *sall4*, it may be an interesting avenue of future research (Koshiba-Takeuchi *et al.*, 2006).

It has been generally accepted that one of the hallmarks of aging is mitochondrial dysfunction as a manifestation or cause of disbalance in energy supply and demand (López-Otín *et al.*,

2023). The other hallmarks include genomic instability, telomere attrition, epigenetic alteration, loss of proteostasis, disabled macro-autophagy, de-regulated nutrient sensing, cellular senescence, stem cell exhaustion, altered intracellular communication, chronic inflammation and dysbiosis. Lee and colleagues reported an interesting association of *FIBIN* levels and sarcopenia caused by mitochondrial dysfunction in skeletal muscle of ovariectomised mice (Lee *et al.*, 2021). The authors reported changes in the lipid metabolism of ovariectomized mice with increased total lipid content, triglycerides and cholesterol levels in the muscle as compared to control mice (sham surgery). Since alterations in lipid metabolism have been linked mitochondrial dysfunction, the authors investigated the mitochondrial function in their model. They found fewer mitochondria in general, downregulated peroxisome proliferator-activated receptor- γ coactivator 1a/b (PGC-1a/b), nuclear respiratory factor 1 and 2 (NRF-1, NRF-2) as well as mitochondrial transcription factor A (TFAM), which all are involved in mitochondrial biogenesis. In particular, the authors investigated a specific microRNA, miR-141-3p, that they found significantly upregulated in ovariectomized mice. They showed that high levels of miR-141-3p lead to decrease in mitochondrial function and inhibition of myogenic differentiation *in vitro*, and that this was mediated via inhibition of *FIBIN* and *FKBP5*. Such results would suggest that Fibin(s) play a role in the healthy maintenance and biogenesis of mitochondria and thus play a fundamental role in development and regeneration or aging. Of note, however, is the fact that Lee and colleagues showed clear western blot results of endogenous FIBIN, something that neither I personally, nor Lakner and colleagues (Lakner *et al.*, 2011) were able to do. I had further email correspondence with Lakner confirming they were not able to detect endogenous FIBIN protein; I personally tried detecting FIBIN protein from mammalian cell cultures (both primary fibroblasts and fibroblasts cell lines) as well as from zebrafish using custom-made antibodies, however, without success.

Mitochondria play a critical role in cellular energy metabolism, and disruptions in mitochondrial function have been linked to a range of developmental disorders, and optimal regeneration programs. For example, mitochondrial fusion has been shown to be important for muscle regeneration in zebrafish (Sun *et al.*, 2022) while inhibition of mitochondrial fission has been linked to impaired heart regeneration in mice (Song *et al.*, 2015). Puente and colleagues (Puente *et al.*, 2014) for example investigated the role of mitochondrial dynamics

in cardiomyocyte cell cycle arrest in response to oxygen exposure. The authors showed that exposure to high oxygen levels induced DNA damage response in cardiomyocytes which led to cell cycle arrest and decreased proliferation. Furthermore, the authors demonstrated that inhibition of mitochondrial biogenesis or oxidative phosphorylation can prevent the oxygen-induced DNA damage response and restore cardiomyocyte proliferation, with significant implications for mammalian heart regeneration (Puente *et al.*, 2014).

The role of mitochondrial dysfunction has also been investigated in the zebrafish caudal fin regeneration. Brandao and colleagues investigated the metabolic changes that occur during zebrafish caudal fin regeneration and how these changes support cell identity transitions, cell cycle re-entry (of osteoblasts), blastema formation, and bone regeneration (Brandão *et al.*, 2022). The authors found that fin amputation induces a metabolic switch from oxidative phosphorylation to glycolysis, which is necessary for blastema formation and bone regeneration. They further showed that inhibition of glycolysis during regeneration inhibits blastema formation and impairs bone regeneration, further confirming the importance of timely regulation of mitochondrial function (Lin, Sam and Evans, 2021; Brandão *et al.*, 2022).

Although it has been shown that zebrafish retain regenerative capacity throughout their lifetime, number of studies have shown that increasing age negatively impacts the regenerative capacity of caudal fin, skeletal muscle, or heart among others (Örling *et al.*, 2023; Kishi, 2004; Bise *et al.*, 2020; Reuter *et al.*, 2022; Sun *et al.*, 2022). Moreover, it has been shown that zebrafish, like humans, possess short telomeres that progressively decline with age. Telomere shortening in mammals can lead to cellular senescence, limit the replicative potential of stem and progenitor cells, and thus contributes to age-related diseases and functional decline in many tissues. The relationship between aging and mitochondrial dysfunction in zebrafish is still to be fully elucidated, including the possible role of Fibrin(s) in the processes. Still, my results support the notion that zebrafish ability to mount a regenerative response to injury changes over time particularly in the gill tissue, and show that lack of Fibrin(s) may be a contributing factor. Whether lack of Fibrin(s) causes premature

aging, possibly via modulation of BMP signalling and/or mitochondrial biogenesis remains to be fully investigated.

Together, this data can suggest that there is yet more delayed initiation of the regenerative sequence of events, particularly blastema setup in the old mutant fish or, possibly, that there is simply no ‘peak’ induction of these genes which may or may not reflect in the ability of the fish to fully regenerate the lost tissue. As there is no data to follow up the old mutant zebrafish to determine their regenerative capacity, this should be part of the future research into aging and *fibins* as it may provide novel and intriguing insights into the intra- and extra-cellular signalling networks, their activation during regeneration and possibly how these could be utilized in aging research.

6 PRELIMINARY *in vitro* INVESTIGATION OF *FIBIN* AND FUTURE RESEARCH DIRECTION

6.1 *in vitro* exploration of FIBIN regulation

Our laboratory's focus on Fibrin(s) has been brought about by its differential expression during a comparative, cross-species RNA seq of tissue repair (bleomycin- and TGF- β -induced pulmonary fibrosis in mice) and tissue regeneration (cryoinjury of the zebrafish gill). This finding has been since supported by extensive datamining that further highlighted the differences in Fibrin regulation/expression in mammalian fibrotic conditions compared to lower-vertebrate complex tissue regeneration.

The majority of my PhD was focused on characterizing *fibina* and *fibinb* during zebrafish caudal fin and gill regeneration. The findings suggested that Fibrin(s) may have a role in the optimal orchestration of the initiation of the regeneration after the initial inflammatory stage (Chapter 5), but the absence of *fibina* and *fibinb* did not impact the ability of fish to generate a robust immune response to a viral mimetic, R848 (Chapter 4).

As with any model of a human disorder/disease, ultimately, we are interested in the behaviour and regulation of *FIBIN* in humans. In this final chapter, I include preliminary results from *in vitro* characterization of *FIBIN* expression in mammalian cell culture, particularly focussing on *in vitro* modelling of Idiopathic Pulmonary Fibrosis (IPF). As the data presented in this chapter is primarily preliminary data, the structure of this chapter does not include a separate results section, rather, my results, clearly described, are included in the main text to support or oppose a known published information. The discussion part of this chapter includes the final discussion of the data presented in this thesis as well as future perspectives.

Modelling IPF, as outlined in Chapter 1: Introduction, is rather challenging. *In vivo* models such as mice or rats, do not develop spontaneous pulmonary fibrosis, and thus are intervention-based models that - to an extent and for the duration of the intervention - replicate some aspects of IPF, such as aberrant epithelial-mesenchymal crosstalk, increased ECM deposition and/or immune cell infiltration (Limjunyawong, Mitzner and Horton, 2014). *in vitro* modelling of IPF is necessarily reduced to investigation of specific components and component interactions. This includes fibroblasts/myofibroblasts responses to various biochemical or mechanical stimuli, epithelial-mesenchymal co-culture systems investigating the epithelial-mesenchymal crosstalk or various immune cells co-culture with either epithelial or mesenchymal cells or more novel 3D models such as organoid cultures, hydrogels or lung-on-a-chip cultures (Vazquez-Armendariz *et al.*, 2022). *FIBIN* in human lung is expressed in alveolar fibroblasts (primarily), adventitious fibroblasts, lipofibroblasts and myofibroblasts (Travaglini *et al.*, 2020) and therefore my investigation focuses on *FIBIN* expression in fibroblasts only. I did a screen of airway epithelial cells, both primary and cell line for *FIBIN* together with a search through the internal BI database of airway epithelial scRNA sequencing data and found either no or very low *FIBIN* expression (Appendix 16). The samples that showed some *FIBIN* expression were IPF-derived small airway epithelial cells (SAECs), therefore it is not possible to exclude the possibility that these cells were undergoing EMT, explaining the very low, but present, *FIBIN* expression.

6.1.1 Impact of TGF- β stimulation of *FIBIN* levels in cultured fibroblasts

TGF- β is an important pro-fibrotic factor and mediator of progressive fibrosis (Biernacka, Dobaczewski and Frangogiannis, 2011; Tatler and Jenkins, 2012; Conte *et al.*, 2014a). In IPF patient's lungs, TGF- β levels are consistently increased compared to healthy controls. TGF- β is produced by a variety of cells in the human lung including alveolar epithelial cells, endothelial cells, fibroblasts, myofibroblasts as well as immune cells (macrophages, neutrophils) (Ruwanpura, Thomas and Bardin, 2020). Recent research investigating the epithelial-mesenchymal crosstalk showed that particularly epithelial-derived TGF- β has profound impact on the bi-directional communication between alveolar epithelial and interstitial mesenchymal cells, and has the potential to trigger an aberrant, profibrotic phenotype (Le *et al.*, 2021).

Negreros and colleagues (Negreros *et al.*, 2019) compared cultured normal human lung fibroblasts (NHLF) from non-IPF donors and idiopathic pulmonary fibrosis lung fibroblasts (IPF LF) from IPF donors in an RNA-seq analysis. The authors reported increased *FIBIN* read counts in IPF LF compared to NHLF and increased levels of *FIBIN* in NHLF upon TGF- β 1 treatment but no change in IPF LF *FIBIN* levels upon TGF- β 1 treatment. It was striking to see higher levels of *FIBIN* reads in IPF fibroblasts compared to controls. The reason is that from all our datamining of online single-cell RNA-seq of tissue samples, *FIBIN* levels in lungs of patients diagnosed with IPF were decreased compared to healthy controls. This therefore poses an interesting question of the impact of *in vitro* culturing of cells on their transcriptomic profile and gene regulation with respect to, but not limited to, *FIBIN* expression and I discuss it later in this chapter.

Moreover, Le and colleagues (Le *et al.*, 2021) set up an epithelial-mesenchymal co-culture system to investigate the effects of epithelial injury on the transcriptome of NHLF or IPL-LF cells in the co-culture. The epithelial injury in this case was simulated by addition of recombinant TGF- β onto the epithelial layer in the apical compartment of the co-culture. The authors found that TGF- β stimulated epithelial cells lead to significantly increased in protein levels of pro-fibrotic mediators including α -SMA or collagen. Moreover, in their RNA-seq

dataset, *FIBIN* was found to be (mildly) upregulated in the mesenchymal cells in co-cultures treated with TGF- β .

As direct experiments involving TGF- β and *FIBIN* transcript levels have not been reported yet, I have first aimed to investigate this in a fibroblast cell line, IMR-90. The IMR-90 cell line is a commercially available human cell line (from ATCC) derived from normal lung tissue of a 16-week-old female. Fig. 6.1 presents preliminary time-course and concentration-response experiments showing that only 5 ng/ml TGF- β stimulation resulted in significantly increased *FIBIN* transcript levels, and only at 24 h timepoint (later timepoints did not include 5 ng/ml stimulation due to excessive cell death). I have then used this concentration and timepoint to investigate the effect of TGF- β stimulation on NHLF and IPF LF primary fibroblasts obtained through Boehringer Ingelheim Pharma collaboration.

TGF- β stimulation of primary fibroblasts for 24h lead to an increase in *FIBIN* transcript levels in both NHLF and IPF LF (Fig. 6.1(A)). There was no difference between the baseline (CTRL) *FIBIN* transcript levels of NHLF and IPF LF which is in contrast to the results reported by Negreros and colleagues (Negreros *et al.*, 2019). This shows that differences are likely to exist between individuals within non-IPF and/or IPF donors and a larger sample group of IPF as well as non-IPF primary cells will be necessary to address this question further and determine possible reasons behind the difference. Interestingly, only the IPF LF showed significant increase in *FIBIN* levels upon TGF- β treatment (mean FC = 4). Although there is a clear increasing trend in TGF- β treated NHLF (mean FC = 3 compared to CTRL mean FC = 1), this difference is not significant. *ACTA2* was used as a control in all experiments to confirm that TGF- β 1 treatment worked, as *ACTA2* is a target gene for TGF- β signalling.

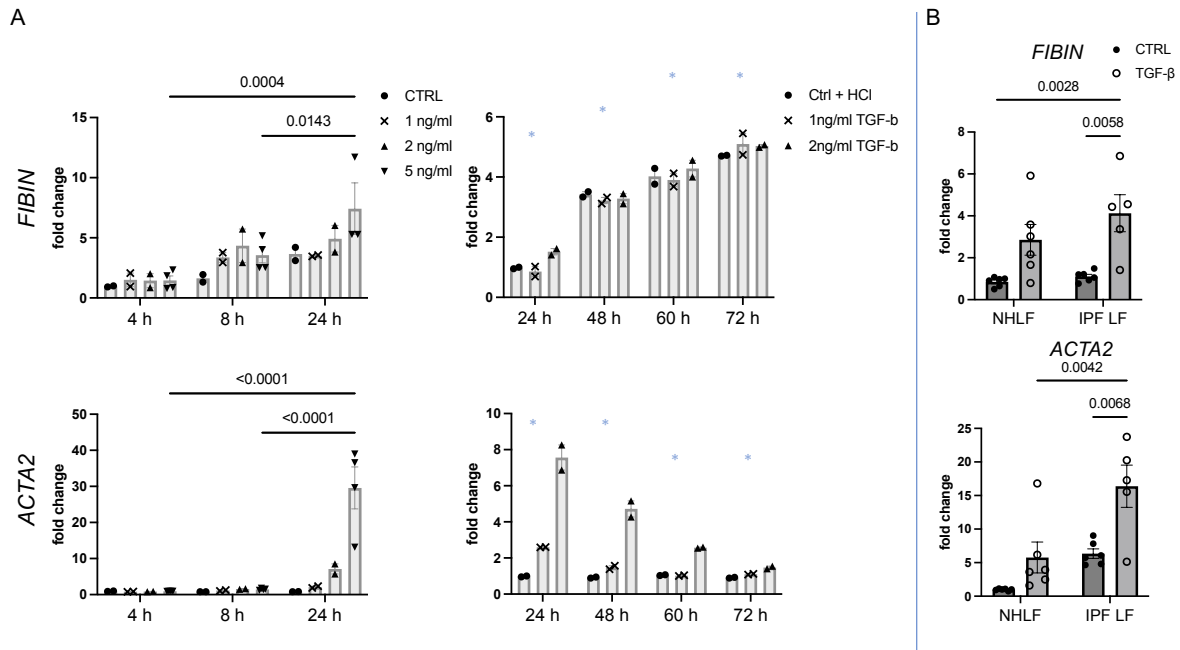


Figure 6.1 *FIBIN* transcript levels in IMR-90 fibroblast cell line (A) and primary NHLF and IPF lung fibroblasts (B) upon TGF-β1 treatment.

(A) Analysis of *FIBIN* and *ACTA2* transcript levels in time-course of TGF-β1 treatment of IMR-90 fibroblast cell line up to 72 h. Blue * specifies experiments where insufficient number of data points were available and statistical significance could not be determined. (B) Results of TGF-β1 treatment on *FIBIN* and *ACTA2* levels in primary NHLF and IPF LF after 24h. Data was normalized to *HPRT1* and is presented as fold change in mRNA levels compared to NHLF CTRL. Each dot represents a biological replicate. Lines used: NHLF 29729; IPF LF 675002; NHLF: normal human lung fibroblasts; IPF LF: IPF lung fibroblasts.

Fig. 6.1 also shows that *FIBIN* levels follow a clear trend of increase with time in culture both for the short and long time courses. It is well known that culturing cells *in vitro* in a 2D cell culture, has profound effects on the cell transcriptomic profile (J. Yu *et al.*, 2018). Not only are the cells taken from their natural, 3D environment, but the most commonly used culture system uses plastic culture plates that do not recapitulate the extracellular matrix stiffness and/or composition of the *in vivo* environment at all. Stiffness is a measure characterised by Young's modulus (elastic modulus) and its units are Pascal (Pa). As an example, the stiffness of liver tissue (considered very soft) is 0.1 to 1 kPa, of dermis – 1 – 5 kPa, of fibrotic tissues – 20 – 100 kPa, while stiffness of tissue culture plates reaches 10,000 kPa (Kanta, 2015). As all cells are capable of mechanosensing, such significant change of surrounding stiffness does lead to differential cell transcriptomic adaptation compared to *in vivo* state. This has been exemplified using cell cultured on matrices with

various stiffness (Booth *et al.*, 2012; Petersen *et al.*, 2012; Vicens-Zygmunt *et al.*, 2015a, 2015b). And, particularly relevant to my research, IPF lung samples have been shown to contain significantly stiffer ECM compared to healthy lungs. The increased stiffness is, at least in part, also implicating in the continuous and progressive nature of IPF (Jaffar *et al.*, 2018). Although I was not investigating the effect of matrix stiffness on *FIBIN* in these experiments, the results do imply that there may be a connection between increased substrate stiffness and increased *FIBIN* levels in cultured fibroblasts. And, as increased stiffness is linked to progressive pro-fibrotic signalling, elucidating this link in future Fibin(s) research may provide important insight into Fibin(s) potential role in fibrosis.

6.1.2 Impact of WNT signalling activation/suppression on *FIBIN* levels in cultured primary fibroblasts

WNT signalling plays fundamental role during development and adulthood in cellular differentiation, migration, organogenesis, self-renewal of resident stem cells as well as tissue homeostasis and tissue repair following injury. WNT signalling plays a pivotal role in lung airway formation during development (Pongracz and Stockley, 2006; Clevers, Loh and Nusse, 2014; Ota *et al.*, 2016) and is often reactivated upon injury in adulthood for lung tissue repair and regeneration. Dysregulated WNT signalling has been implicated in the pathogenesis of progressive, degenerative lung diseases such as IPF. For IPF specifically, ‘WNT signature in IPF’ refers to the over-active WNT signalling described in IPF patients’ lung samples, demonstrated by increased expression of WNT signalling target genes and increased protein levels (Chilosi *et al.*, 2003; Königshoff *et al.*, 2008b; Meuten *et al.*, 2012; Lam *et al.*, 2014; Oda *et al.*, 2016; Rydell-Törmänen *et al.*, 2016).

Canonical WNT signalling is fundamental for ATII (alveolar epithelial Type 2) cells ability to self-renew and act as progenitors for ATI cells upon alveolar epithelial injury (Guo *et al.*, 2016; Heijink *et al.*, 2016; Shi *et al.*, 2017). The pathway activation was observed *in vitro* during ATII to ATI transdifferentiation as well as upon bleomycin treatment in the mouse experimental model of pulmonary fibrosis *in vivo* (Königshoff *et al.*, 2009; Shi *et al.*, 2017). Alveolar epithelium is a major source of cytokines driving the epithelial-mesenchymal crosstalk with the underlying interstitium (such as the primarily pro-inflammatory IL-1 β and

TNF- α as well as the pro-fibrotic TGF- β) and WNT signalling ligands, such as WTN3A, activates the transcription and secretion of these cytokines. It was also shown that sustained WNT signalling may, possibly through IL-1 β and subsequently TGF- β induction, lead to progressive lung fibrosis (Shi *et al.*, 2017). Thus, although fundamental in its nature for optimal organ function, WNT signalling may cause airway epithelial cells to aberrantly produce TGF- β as well as promote epithelial-mesenchymal transition to modulate tissue repair (Chilosi *et al.*, 2003; Königshoff and Eickelberg, 2010).

Fig. 6.2 shows the preliminary results of a WNT-signalling screen of a selection of canonical and non-canonical WNT-signalling inducers and/or inhibitors that were available to me at BL. My preliminary results showed that canonical WNT signalling stimulation, either through direct addition of WTN3A ligand, or via inhibition of a component of the β -catenin destruction complex, GSK-3 α/β , led to significant decrease in *FIBIN* transcript levels in both NHLF and IPF LF with the same dynamics. On the other hand, non-canonical WNT signalling stimulation using WNT5A ligand showed no change in *FIBIN* levels, irrespective of the dose used. Increased WNT5A transcript levels have been previously found to be associated with IPF (Martin-Medina *et al.*, 2018).

AXIN2, used here as a control gene to confirm the activation/inhibition of WNT signalling, is a negative regulator of WNT signalling that is induced by canonical WNT signalling (such as WNT3A) (Dao *et al.*, 2007) and suppressed by non-canonical WNT-signalling inducers such as WNT5A. WNT5A has been also shown to directly suppress *AXIN2* levels in lung fibroblasts (Liu *et al.*, 2021) as well as to suppress WNT3A dependent increase in *AXIN2* levels (Sato *et al.*, 2010). My results however show no effect of either WNT5A or PKF/FH on *AXIN2* levels, which means it is not possible to confirm that the stimulation of non-canonical WNT signalling was successful, thus not allowing me to draw conclusions regarding *FIBIN* and non-canonical WNT signalling.

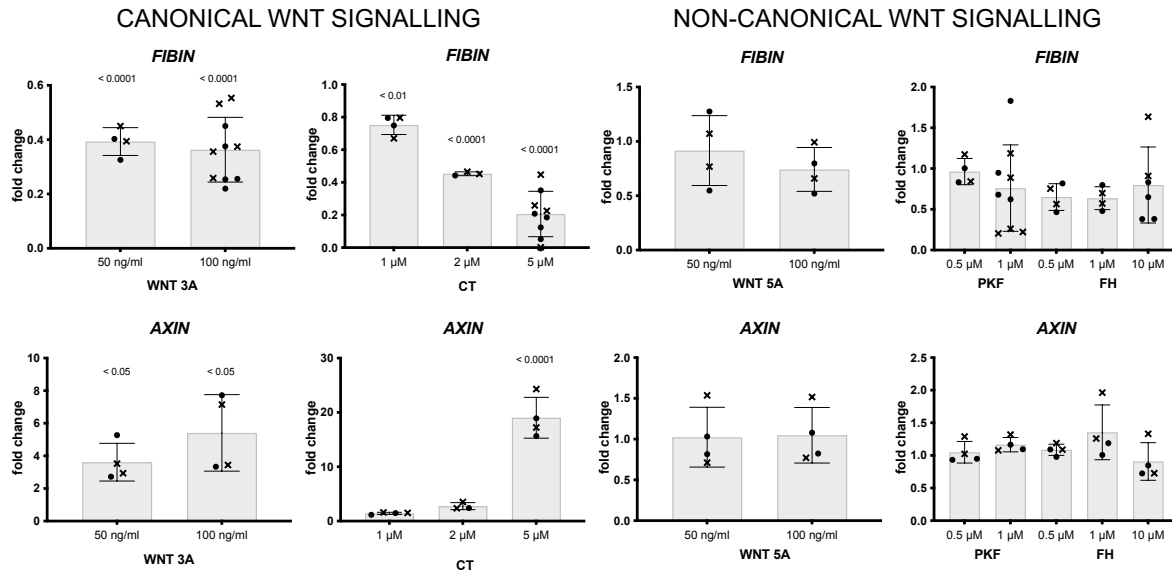


Figure 6.2 WNT signalling regulation of *FIBIN* transcript levels.

Impact of canonical and non-canonical WNT signalling stimulation by WNT3A, CT, WNT5A, PKF and FH on *FIBIN* levels in primary NHLF and IPF LF at 24h post treatment. Data was normalized to *HPRT1* and is presented as fold change in mRNA levels compared to the respective control sample. p-values are shown for samples that differed significantly from their respective controls. Data from 2 separate experiments. Lines used: NHLF 29729; NHLF 27913; IPF LF 19873; IPF LF 29542; NHLF: normal human lung fibroblasts; IPF LF: IPF lung fibroblasts; CT: GSK-3 α/β inhibitor; PKF: protein kinase C inhibitor; FH: suppressor of Tcf-mediated transcription.

6.1.3 Functional implication of *FIBIN* knockdown in primary human lung fibroblasts

To begin to understand the functional implication of *FIBIN* in fibrosis, I set up an siRNA knockdown experiment to investigate the impact of *FIBIN* knockdown on the transcription of *COL1A1* and *ACTA2*. siRNAs or small interfering RNAs are short double stranded RNA sequences that act post-transcriptionally to decrease target mRNA levels. Once introduced into the cell cytoplasm, the guide strand of the siRNA activates the RNA-induced silencing complex (RISC). siRNA-RISC then recognizes and cleaves transcribed mRNA with sequence complimentary to the siRNA guide strand (Kamola *et al.*, 2015). Three commercially available *FIBIN* targeting siRNAs (details are given in Appendix 17) were used to validate TaqMan *FIBIN* probe, only one available at the time. Fig. 6.3 presents the preliminary data.

FIBIN knockdown was successful (90.2% decrease in transcript levels), and number of interesting observations can be made. Firstly, *FIBIN* transcript levels were significantly increased upon TGF- β treatment (5 ng/ml for 24 h), further confirming my results presented in Fig. 6.1. and significantly decreased following a 24 h stimulation with TNF- α . (I will discuss TNF- α in more detail in the following paragraph). *FIBIN* knockdown resulted in attenuated increase of *COL1A1* (fold-change of 2.0 compared to 3.6 in control siRNA treated samples) and *ACTA2* (fold change of 12.4 compared to 21.6) levels in TGF- β 1 stimulated fibroblasts. Moreover, *FIBIN* knockdown, without additional treatment, caused decrease in *AXIN2* levels (mean FC = 0.01). This together with data presented in Fig. 6.2 suggests that there exists a bi-directional *FIBIN* – WNT signalling crosstalk.

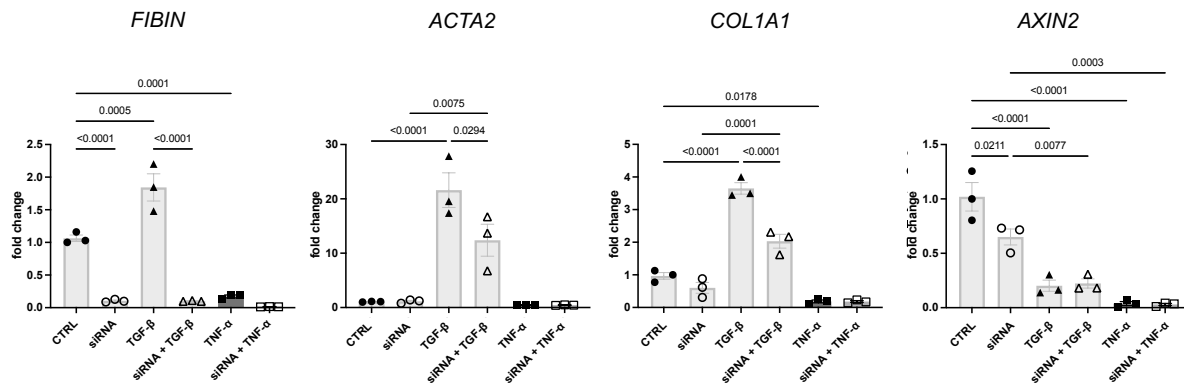


Figure 6.3 Functional knockdown of *FIBIN*.

Data showing the effects of functional *FIBIN* knockdown (by siRNA and in IPF LF only) on *FIBIN* levels (confirmation of successful knockdown), *COL1A1* levels and *ACTA2* levels after treatment with 5 ng/ml TGF- β 1. Samples transfected with non-targeting siRNA were used as controls (CTRL). Data was normalized to *HPRT1* and is presented as fold change in mRNA levels compared to the control. Each dot represents a biological replicate within a single experiment. FLs used were at passage 6. Lines used: IPF LF 675002; siRNA: *FIBIN* targeting siRNAs; IPF LF: IPF lung fibroblasts.

6.1.4 Inflammatory cytokines and *FIBIN* levels

A mouse microarray dataset from Meyer and colleagues investigating *in vitro* differentiation of murine myoblasts, showed *FIBIN* levels to increase with time in culture without any treatment (supporting my results from Fig. 6.1) and decreased upon TNF- α stimulation (Meyer *et al.*, 2015). TNF- α is a pro-inflammatory cytokine, but in contrast to TGF- β , it is primarily produced by immune cells including macrophages, T-lymphocytes or natural killer cells (Josephs *et al.*, 2018). Although, as mentioned, IPF is no longer believed to be a chronic

inflammation-driven disease, inflammation and immune cells play a significant role in the disease progression and manifestation (Heukels *et al.*, 2019; Wang *et al.*, 2022). TNF- α levels in particular were found to be strongly correlated with an IPF-progression marker CXCL13 in blood of patients with IPF (Bellamri *et al.*, 2020).

Pechkovsky and colleagues (Pechkovsky *et al.*, 2010) also reported TNF- α as a pro-inflammatory cytokine whose release, generally, decreases with increasing severity of pulmonary fibrosis (determined by CT scans). However, the authors in their previous publication also reported that increased and constant TNF- α release is indicative of disease progression in patients with SAR and pulmonary fibrosis. Thus, clear heterogeneity exists with pulmonary fibrosis patients - with a non-inflammatory, pro-fibrotic disease presentation as well as patients with some level of detectable pro-inflammatory markers and lung inflammation. Interestingly, it has been shown that although probably not involved in the pathogenesis, lung inflammation is a significant risk factor for rapid progression of IPF, with substantial innate and adaptive immunity components present in the lung tissue of these patients compared to slow progressing IPF patient samples (Balestro *et al.*, 2016).

Investigation of TNF- α driven pulmonary inflammation and remodelling using mice with TNF- α overexpression in lung epithelial cells showed higher rates of lung inflammatory markers and tissue remodelling (*Mmp2*, *Mmp9*) as well as airspace enlargement compared to control lungs. However, these mice were resistant to bleomycin induced fibrosis and showed decrease in transcript levels of genes typically associated with fibrosis (including myofibroblasts differentiation factors). Overall, the manifestation was indicative of emphysema (that is loss of elastic recoil and alveolar enlargement) rather than fibrosis (Thomson *et al.*, 2012). Moreover, anti-TNF therapy has been linked to progressive pulmonary fibrosis and interstitial lung disease (Herrinton *et al.*, 2013).

In Fig. 6.3 I showed that TNF- α treatment leads to completely abolished *FIBIN* levels, in agreement with Meyer *et al.*, as well as *AXIN2*, *COL1A1*, and *ACTA2* levels in primary human IPF-derived lung fibroblasts. TNF- α has been shown to decrease *AXIN2* levels via interfering

with b-catenin binding and/or via activation of NF- κ B pathway that leads to inhibition of WNT signalling. TNF- α has also been shown to both increase and decrease *COL1A1* and *ACTA2* levels in fibroblasts, depending on the microenvironment, however, it is known that TNF- α can inhibit TGF- β signalling by inhibiting the expression of TGF- β receptors in pulmonary fibrosis. Therefore, limiting the activation of TGF- β signalling leads to relative decrease in TGF- β response genes, *COL1A1* and *ACTA2* (Fujita *et al.*, 2003; Liu, 2008; Wu and Zhou, 2010; Orellana *et al.*, 2015).

From my *in vivo* experiments (Chapter 4), I found no association of Fibrin(s) with immune cells, or inflammation induced through activation of Tlr7/8 receptors in the zebrafish gills. Moreover, *fibina* and *fibinb* transcript levels only began to be significantly increased in zebrafish models of regeneration after the initial inflammatory phase is resolved (i.e. around 2-3 dpa in zebrafish caudal fin regeneration and around 7 dpc in zebrafish gill cryoinjury). Thus, as it has been showed that chronic inflammation does lead to chronic remodelling but not necessarily chronic fibrosis, the detected decrease of *FIBIN* levels upon TNF- α treatment, supported by data from Meyer and colleagues, may reflect the dynamics of *FIBIN* expression in the context of inflammation vs fibrosis (Meyer *et al.*, 2015; Wang *et al.*, 2018). That is, *FIBIN* role in regeneration may be dependent on permissive, non-inflammatory microenvironment conditions.

6.1.5 *in vitro* modelling of physical injury of alveolar epithelial cells and fibroblasts and the impact on *FIBIN* levels

Idiopathic pulmonary fibrosis (IPF) is believed to be an epithelial-driven disease, such that repeated physical injury to the alveolar epithelial layer ultimately results in aberrant molecular response in the underlying mesenchyme, leading to progressive fibrosis. In the past decade co-culture trans-well systems have become increasingly popular as an *in vitro* model of (not only) epithelial-mesenchymal crossover (Prasad, Hogaboam and Jarai, 2014; Epa *et al.*, 2015). Since *FIBIN* was found to be significantly increased upon physical injury in zebrafish gills and caudal fin, particularly during regeneration, here I used the transwell co-culture model system to simulate physical injury of epithelial cells and investigate the possible impacts on *FIBIN* levels in primary NHLF and IPF LF.

As outlined in Fig. 6.4(A), two separate setups were used: culturing primary human NHLF or IPF LF in monoculture and inflicting the scratch wound directly to the mesenchymal layer or culturing the fibroblasts in a transwell co-culture system with A549s used as the epithelial layer that is wounded. Two timepoints were investigated, 6 and 24 hpi (hours post injury) to assess the early and late gene transcript changes (further experimental details can be found in Methods). The results (Fig. 6.4) show that *FIBIN* transcript levels were not affected in fibroblasts either cultured in monolayer or in co-culture with A549 epithelial cells upon scratching. That is, physical wounding had no effect on *FIBIN* levels.

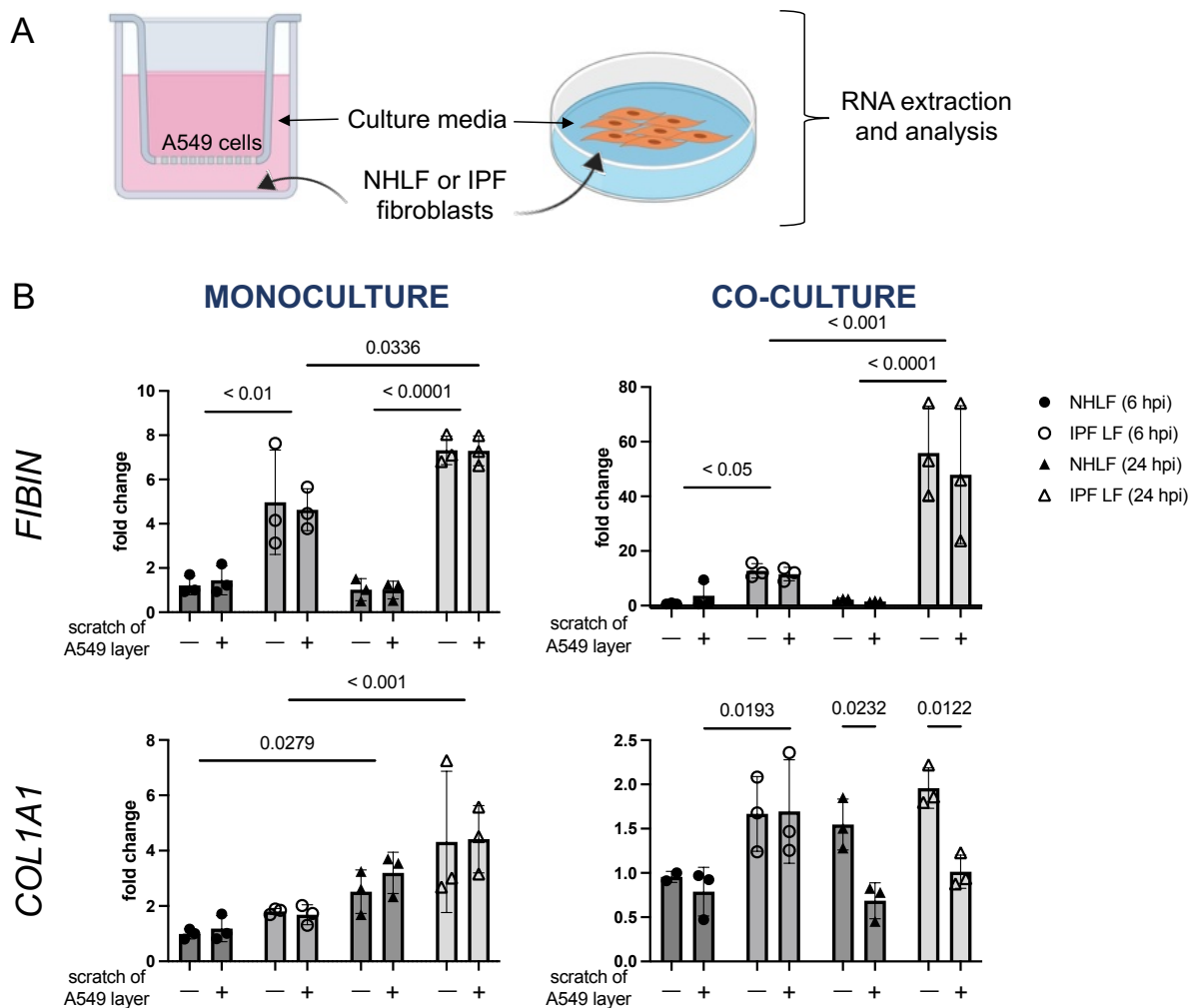


Figure 6.4 *FIBIN* transcript levels in *in vitro* model of epithelial/mesenchymal injury.

(A) Trans-well setup for co-culture of A549 cells (upper compartment) and fibroblasts (bottom compartment), compared to monolayer fibroblast culture. (B) *FIBIN* levels in NHLF and IPF LF with or without scratch to the LFs in monoculture or to the A549 cells in co-culture after 6h and 24h. The *FIBIN* levels in uninjured NHLF at 6h timepoint were used as a control for both timepoints and for both, NHLF and IPF LF samples. Data was normalized to *HPRT1* and is presented as fold change in mRNA levels compared to control ('NHLF'). Each dot represents a biological replicate. Lines used: NHLF 27913, IPF LF 19873; NHLF: normal human lung fibroblasts; IPF LF: IPF lung fibroblasts; LFs: lung fibroblasts.

Interestingly, *FIBIN* transcript levels were significantly higher in IPF LF compared to NHLF at both timepoints investigated (6 and 24 hpi) as well as in fibroblasts that were cultured in co-culture with A549 cells compared to monocultures. The primary fibroblasts used in this experiment were from different donors than the primary fibroblasts used for TGF- β stimulation experiments presented in Fig. 6.1(B). However, these results clearly show that great differences do exist even at 'homeostasis' either within NHLF donors or IPF donors or both when it comes to *FIBIN* transcript levels. This data also contrasts to online RNA seq datasets of *FIBIN* expression from lung samples of IPF and non-IPF patients (McDonough *et al.*, 2019; Vieira Braga *et al.*, 2019; Adams *et al.*, 2020), however, are in agreement with data presented by Negreros and colleagues that *FIBIN* transcript levels are increased at baseline in cultured fibroblasts from patients with IPF compared to non-IPF patients (Negreros *et al.*, 2019).

Wiegand and colleagues (Wiegand *et al.*, 2021) showed that in the scratch assay, transcript levels of proinflammatory and profibrotic cytokines (such as *CXCL8* and *TGF- β 1* respectively) are upregulated significantly as early as 3 h post scratch (hps) and, for *TGF- β 1*, are maintained at high levels through 48 hps. Collagen (*COL1A1*) on the other hand becomes significantly upregulated only after 24 hps and shows a trend of decrease in transcript levels between 3 and 6 hps. Moreover, Prasad and colleagues (Prasad, Hogaboam and Jarai, 2014), similarly to my experiments, used NHLF and IPF LF in co-culture with A549 cells and reported significant differences between the NHLF and IPF LF response to the scratch, in particular significantly faster wound healing/closure in culture with IPF LF. However, differences were only quantifiable after 48 hps. The timings used in my preliminary experiment were based on prior experiments by other members of my team at BI, specific to

the fibroblasts used (both NHLF and IPF LF). However, it is possible that both *COL1A1* and *FIBIN* transcript levels may alter following this timepoint and therefore it is necessary to further explore both the timing and the best gene-controls that would enable better characterisation of *FIBIN* transcript regulation in human fibroblasts *in vitro*.

6.2 Discussion

Tissue repair after injury follows a carefully balanced sequence of events that are, to a high degree, conserved across vertebrates. The capacity for successful regeneration, characterized by functional repair of the damaged tissues, shows, however, great variation between lower vertebrates such as zebrafish and higher vertebrates such as mice or humans. The complexity of the events that lead to either successful tissue regeneration or persistent fibrosis is reflected in the relative lack of available anti-fibrotic therapies, particularly for patients suffering from IPF, despite decades long investigation into tissue regeneration (Glass *et al.*, 2020). Novel technologies such as next generation sequencing, or single cell RNA sequencing enable us to appreciate this complexity even more. From genetic predisposition to life-style associated risk factors or immune related differences in response to injury, we know that the mechanisms of tissue repair/regeneration are multifactorial.

Fibroblasts, the main cell type expressing our gene of interest *FIBIN*, play a critical role in tissue regeneration by synthesizing and depositing ECM components. These components provide structural support and help guide cell behaviour during the repair/regeneration process. Fibroblasts are activated upon injury and migrate to the site of injury where they produce and remodel ECM. Among other main functions of fibroblasts belongs promotion of proliferation and migration of other cell types, including epithelial or immune cells and secretion of cytokines and growth factors that stimulate these processes (Parsonage *et al.*, 2005).

Fibroblasts also play a critical role in the resolution of inflammation during tissue repair/regeneration. The chemokines and cytokines that fibroblasts secrete act to attract and

recruit immune cells to the site of injury. Moreover, the ECM components secreted by fibroblasts can bind and sequester inflammatory cytokines/chemokines thus enabling the transition from the inflammatory phase of the proliferative and remodelling phases of tissue repair/regeneration (Parsonage *et al.*, 2005; Cheng *et al.*, 2015; Bautista-Hernández *et al.*, 2017; Louault, Li and Declerck, 2020).

Dysfunctional or aberrant activation of fibroblasts can lead to impaired tissue remodelling and repair and pathological fibrosis. Recent scRNA seq studies have shown that fibroblasts in pulmonary fibrosis (not only IPF) exhibit significant heterogeneity in terms of their origin (eg. resident lung fibroblasts, circulating fibrocytes or bone marrow-derived mesenchymal stem cells), phenotype, and function (eg. primarily ECM-secreting/primarily cytokine/chemokine secreting) (Willis, DuBois and Borok, 2006). This diversity in fibroblast phenotypes and functions can contribute to the heterogeneity of PF and has important implications for the development of targeted therapies.

Signalling pathways including but not limited to WNT, TGF- β or BMP signalling, fundamental for early development, as well as homeostatic maintenance and tissue repair have been subjected to great scrutiny with respect to their role in tissue repair and regeneration. Dysregulated activation/cross-talk between these pathways was shown to play an important role in the progression of IPF.

Firstly, I have shown that WNT signalling induction either through a canonical WNT3A ligand addition or via inhibition of GSK3 α/β and thus disabling the β -catenin destruction complex, allowing WTN signalling – leads to significant decrease in *FIBIN* levels in both NHLF and IPF LF upon 24 h. What is more, *FIBIN* knockdown by siRNAs lead to significant decrease in *AXIN2* transcript levels at 24 h. As *AXIN2* is a negative regulator of WNT signalling, this data suggests that positive feedback exists in lung fibroblasts where WNT signalling acts to inhibit *FIBIN* expression which in turn leads to decrease in *AXIN2* levels, effectively driving further WNT signalling.

Next, my data from *in vitro* experiments showed that in primary pulmonary fibroblasts, both NHLF and IPF LF, *FIBIN* transcription was increased upon TGF- β treatment (24 h) and knockdown of *FIBIN* lead to a lower increase in *COL1A1* and *ACTA2* levels upon TGF- β treatment. As increased collagen and other ECM components transcription and secretion is linked with pro-fibrotic niche establishment, one could argue that - based on this data - *FIBIN* knockdown is a beneficial event. Our laboratory has repeated this experiment using healthy primary lung fibroblasts (NHLF) and master students, Eleanor Graham and Elizabeth Famosa, supervised by Dr Laurence Bugeon found no difference in *COL1A1* levels or *ACTA2* levels upon *FIBIN* knockdown (Appendix 18). Furthermore, I found conflicting results on *FIBIN* transcript levels in NHLF and IPF LF. In data presented in Fig. 6.1, there was no difference between *FIBIN* transcript levels irrespective of the donor's diagnosis (IPF or non-IPF). Primary fibroblasts used for experiments presented in Fig. 6.4 on the other hand showed significant differences in *FIBIN* levels, with IPF-derived pulmonary fibroblasts exhibiting significantly higher *FIBIN* levels compared to NHLF. Differences in fibroblasts origin are likely to be important to unravel the detected inconsistencies and understand better the role and function of Fibin. This is discussed in the paragraphs below. Overall, there is substantial evidence to suggests that TGF- β signalling regulates *FIBIN* expression in human lung fibroblasts.

WNT signalling and TGF- β signalling appear to drive *FIBIN* transcription in fibroblasts in opposite directions, with both signalling pathways considered overactive in lung fibrosis. Both pathways are involved in alveolar epithelial repair upon epithelial injury, yet their roles are distinct and can have opposing effects. Canonical WNT signalling is involved in promoting the proliferation and differentiation of progenitor cells, which can replace the damaged or dead alveolar epithelial cells and is activated in response to alveolar damage (Königshoff *et al.*, 2008b; Königshoff and Eickelberg, 2010; Baarsma and Königshoff, 2017b). TGF- β signalling is activated in response to lung injury and can promote the differentiation of myofibroblasts, contributing to tissue repair, but also fibrosis (Mullen *et al.*, 2011; Conte *et al.*, 2014a). Moreover, TGF- β signalling has been shown also to inhibit WNT signalling in alveolar epithelial cells which can negatively impact the repair process, lead to

impaired proliferation and differentiation of progenitor cells, and effectively limit the regenerative capacity of the lung (Akhmetshina *et al.*, 2012; Spanjer *et al.*, 2016).

Based on my *in vivo* results of zebrafish gill and caudal fin regeneration, and transcriptional profiling of regeneration stages in anole green lizard and zebrafish (Rabinowitz *et al.*, 2017; Xu *et al.*, 2020), *FIBIN* begins to be expressed, and thus potentially involved in the regeneration process, only after the initial inflammatory process is over. With alveolar epithelial injury, WNT signalling is activated in the alveolar epithelial cells prior to TGF- β signalling in the underlying mesenchyme and the differential regulation of *FIBIN* can therefore be reflection of the regeneration/repair stages of the alveolar tissue.

6.2.1 Disease-dependent and location-dependent fibroblast heterogeneity

IPF-derived fibroblasts have been shown to respond differently *in vitro* to biochemical or physical stimulus compared to NHLF (Yanai *et al.*, 2015; Jaffar *et al.*, 2018). Negreros and colleagues (Negreros *et al.*, 2019) in their dataset showed that only NHLF responded to TGF- β treatment with an increase in *FIBIN* levels as no change in *FIBIN* levels was detected in IPF LF. However, I have found no significant difference in the *FIBIN* levels between NHLF and IPF LF upon TGF- β stimulation. Such results firstly ask for the experiments to be repeated with a range of donors for both IPF LF and NHLF, but also ideally to be repeated with the same donor's cells from different parts of the lung:

IPF is characterized by fibrotic lesions, fibroblast foci, that have very different mechanical and biochemical characteristics to the rest of the lung (Jones *et al.*, 2016). It is possible that the aberrant signalling from within the fibrotic lesion influences the rest of the cells in the organ to respond similarly to surrounding cues. It is also possible that irrespective of what the signalling/microenvironment is within and around the fibrotic lesion, fibroblasts physically distant to this locus may retain their normal, healthy signalling responses to injury. Both of these fibroblasts would be marked 'IPF-derived', yet the signalling pathways, epigenetic and genetic (pre-)programming may vary (Habiels and Hogaboam, 2014).

For instance, Pechkovsky and colleagues (Pechkovsky *et al.*, 2012) reported a fibroblast population derived from IPF donors, though not strictly from the fibroblast foci but rather near the fibrotic lesion. This population showed decreased *ACTA2* levels as well as lower rate of proliferation compared to NHLF. Which is in direct contrast to other literature showing that IPF-derived fibroblasts are characterized by high proliferation rates and high expression and secretion of *ACTA2*, *COL1A1* and other ECM molecules.

Of equal importance is to know the origin of the control, healthy fibroblasts, NHLF. Particularly when these primary fibroblasts are bought through a company, such as ATCC, it is not possible to obtain detailed description of the sample origin. Several, primarily older, publications where the authors had access to the background information, revealed that these samples often come from cancer patients – i.e. during tumour resection a biopsy is also taken from a distant, ‘healthy’ tissue or from brain dead patients whose lungs were not suitable for transplantation. Particularly the fibroblasts from cancer patients’ biopsies may not represent a healthy lung tissue and fibroblast programming as the whole microenvironment within the lungs may be affected by the tumour (Torry *et al.*, 1994; Frankel *et al.*, 2006).

This heterogeneity that exists in terms of mesenchymal-type cells in fibrotic lungs as well as the signalling tendencies of each fibroblast subpopulation further highlights the complexity of fibrotic lung disorders and the challenges they represent when we attempt to treat these. IPF is not a single gene disease, it is not a single-strike (risk-factor) disease either. IPF is likely the result of both internal (human genetics, including immune system regulation) and external (epigenetics – lifestyle, risk-factors) factors which requires multilevel approach to be understood and, possibly, cured.

Single cell RNA sequencing and -omics approaches have the potential to revolutionize the diagnosis and treatment of IPF and other currently non-curable fibrotic disorders. These approaches can help to identify the underlying genetic and molecular mechanisms that

contribute to the development and progression of IP and could lead to the development of targeted therapies and personalized treatment options. Habermann and colleagues (Habermann *et al.*, 2020), Reyfman and colleagues (Reyfman *et al.*, 2019) or Adams and colleagues (Adams *et al.*, 2020) for example have all used scRNA seq to identify possible novel cell types of interest in IPF lungs, associated with disease progression, severity, and/or potential therapeutic targets. Moreover, -omics studies including research by Herazo-Maya and colleagues (Herazo-Maya and Kaminski, 2012) and Zhang and colleagues (Zhang *et al.*, 2019) using combination of transcriptomics and proteomics to identify biomarkers for disease monitoring and therapeutic targets. However, the sheer volume of data generated using these techniques requires novel ways of analysing the data, particularly using Artificial Intelligence (AI) softwares. Maher and colleagues (Maher, Nambiar and Wells, 2022) indeed used such an approach – developing machine learning algorithms to design a predictive model for disease progression in IPF based on clinical and molecular data. The authors proposed that such model could be used to identify patients who are at high risk for disease progression and to guide personalized treatment strategies.

6.2.2 *FIBIN* and inflammation

Idiopathic pulmonary fibrosis has been for number of years thought of as a ‘chronic-inflammation-caused disease’. However, the lack of benefit to IPF patients provided by well-known anti-inflammatory medication, including steroid-based medication (such as in the PANTHER trial), lead to re-consideration of the possible disease initiation factors, and the chronic epithelial injury model of IPF pathogenesis has since been widely accepted. Inflammation, although maybe not trigger, plays a significant role in IPF, particularly its progression (Bringardner *et al.*, 2008; Glass *et al.*, 2022; Lee *et al.*, 2023). This has been suggested by genetic research which has identified specific immune-related genes, such as TLR3, TOLLIP, and IL-1RA, to be associated with increased risk or severity of the disease. In addition, several studies have demonstrated that immune modulation can affect fibroblast activation and function, further highlighting the role of the immune system in IPF (Shenderov *et al.*, 2021).

In Chapter 4 of this thesis, I investigated the consequences of lack of *fibina* and *fibinb* in zebrafish on the ability of the organism to respond to viral-mimetic, R848. The possibility that lack of *fibins* may lead to dysregulated immune response of the zebrafish to immunogenic stimulus was hypothesized based on the results of RNA sequencing analysis comparing wild-type zebrafish and *fibina/b*^{-/-} mutants. However, no difference was found in the dynamics or kinetics of cytokine/chemokine expression upon R848 stimulation between the 2 zebrafish genotypes, suggesting that lack of Fibins does not impact the zebrafish ability to mount an immune response to stimulus. *tnfa* or *ifng1-2* were amongst the genes investigated. Experiments were limited to stimulation of the inflammatory pathways by activation of intracellular Tlr7/Tlr8 receptor.

In the view of the preliminary data I presented in this chapter where I found that TNF- α stimulation appears to cause decrease in *FIBIN* transcript levels in primary fibroblasts (Fig. 6.3), I wanted to determine if *fibins* transcript levels were affected in the R848 challenge model. Interestingly, *fibinb* transcript levels were indeed significantly lower at 8 hpt in wild-type fish after R848 treatment compared to 1 hpt in wild-type fish (Appendix 19). Moreover, Amber Owen, a master student from the Dallman laboratory who did online datamining of RNA-seq datasets of lung infections, found a negative regulation of *FIBIN* in respiratory viral infections. Overall, the data suggests that Fibins expression is decreased under inflammatory conditions, including TNF- α stimulation or R848 stimulation.

How does this data link with zebrafish caudal fin and gill regeneration? From various lower vertebrate models we know that an inflammatory response upon an injury – whether sterile or pathogen-caused – is fundamental for triggering optimal repair and regeneration programs (Choi, Lee and Yu, 2023). In zebrafish caudal fin regeneration specifically, the first few hours after amputation (sterile injury) mark the inflammatory stage (Morales and Allende, 2019). The majority of studies in zebrafish investigating the role of inflammation upon injury focus on the innate immune system response, with particular focus on macrophages and neutrophils. Neutrophils are commonly the primary respondents, scavenging the apoptotic bodies and debris in the injured area. Macrophages, as the major phagocytic cells, follow through the resolution stage of inflammation, clearing the injured site, facilitating ECM

remodelling and setting stage for successful regeneration. It is only at the end of the resolution stage when cells that will set up the blastema begin to proliferate and de-differentiate (Iribarne, 2021). Petrie and colleagues have shown that, unlike in larval fin fold regeneration (Li *et al.*, 2012), in adult caudal fin regeneration, only macrophages, but not neutrophils, are in fact necessary for correct epimorphic fin regeneration (Petrie *et al.*, 2015).

Nevertheless, current research suggests that the cellular differentiation and proliferation part of the regeneration process only takes place during/after the resolution of the inflammatory phase. Meaning that the successful, scar-less regeneration seen in zebrafish caudal fin (and gill) takes place in under – largely – non-inflammatory condition. And *Fibin(s)* are upregulated – both in the caudal fin and the gill tissue – only during the post-inflammatory stage of regeneration in the zebrafish. However, such conditions are near impossible to model or find in mammalian tissue regeneration where immune cells (both innate and adaptive) and inflammatory mediators are constantly present throughout tissue repair (with varying dynamics and kinetics often specific to a given condition or disease), which could explain the consistent downregulation of *FIBIN* detected in mammalian fibrotic and infectious lung diseases.

Mouse models deficient in T and B cell populations (part of adaptive immune system), serve as an interesting model to better understand the role/necessity of adaptive immunity in mammalian tissue regeneration. For example, Bombeiro and colleagues investigated axonal regeneration in REG-KO mice and found enhanced and faster axonal regeneration in these mice compared to immunocompetent (wild-type) mice (Bombeiro *et al.*, 2016). The authors showed increased numbers of macrophages recruited to the site of the injury and suggested that the improved regeneration is due to lack of inhibitory signals to macrophages from adaptive immune cells. However, the functional, motor recovery remained dependent on the adaptive immune system presence, stressing the need for optimal balance between innate and adaptive immunity in tissue regeneration, and optimal timing of immune cell recruitment. *Rag*^{-/-} mice were also used to investigate the impact of T- and B-cell mediated immunity on pulmonary fibrosis. O'Donoghue and colleagues showed that *Rag*^{-/-} mice were protected from

bleomycin-induced pulmonary fibrosis, as confirmed not only by the lack of lymphocyte aggregates in the lungs but also by expression levels of *Colla1* gene.

Moreover, mice models of pulmonary inflammation showed that prolonged inflammation hinders the progression of the repair/regeneration programs, often causing more tissue damage due to infiltrating macrophages and neutrophils. For example, IFN- γ , a gene induced primarily upon viral infection was shown to downregulate genes relevant to wound repair including genes associated with cellular development, growth and apoptosis (Trilling *et al.*, 2013). Type I and Type III interferon signalling has also been shown to negatively impact lung repair in influenza infected mice. The authors in their dataset showed that next to decreasing epithelial cell proliferation and differentiation, *FIBIN* read counts were decreased in response to IFN- γ (Major *et al.*, 2020).

While investigation of the adaptive immune system in zebrafish caudal fin regeneration is lacking, this avenue of research is being tackled in the closely related Axolotls and their limb regeneration. At the EMBO 2022 Regeneration conference in Spain, I had the opportunity to discuss this topic with Dr Maximina H. Yun and her group (based at Centre for Regenerative Therapies TU Dresden). Lisbeth Airais Bolanos Castro, a PhD student in Dr Yun's group was presenting her preliminary PhD thesis findings about adaptive immune lineages roles in the regeneration of complex structures in axolotl. Lisbeth showed that T and B cells are recruited during tissue regeneration but that CRISPR-Cas9-mediated genetic depletion of T and B cells had no effect on the axolotl's ability to regenerate its limbs and tail. This data suggested that adaptive immunity is dispensable for limb and tail regeneration of axolotls, although whether other compensatory mechanisms exist still remains to be investigated (Bolaños-Castro and Yun, 2022).

These findings clearly show that lower-vertebrate versus higher vertebrate gap in regenerative capacity indeed may include differential participation of immune system – particularly adaptive immune system – including recruitment and function during complex tissue regeneration. Ultimately, the correct timings of steps involved in tissue repair and

regeneration, including early inflammation, appear to be crucial. In this thesis I showed that Fibins are significantly upregulated during the matrix remodelling and tissue repair/regeneration stages of zebrafish which is consistent with data from other highly regenerative lower vertebrates such as green anole lizard (Xu *et al.*, 2020). Overall, the data suggest that Fibins are temporally regulated upon tissue injury, playing a role in the post-inflammatory stages of tissue repair.

6.3 Final discussion and future perspectives: Where does this leave us with respect to Fabin(s) role in tissue regeneration?

When the Dallman laboratory first came across Fabin(s) as potential genes to focus on with respect to tissue regeneration and fibrosis research, several directions were investigated. In this thesis I have firstly presented data describing computational analysis of Fabin(s) proteins structure, their predicted localization as well as theoretical transcription factor binding sites. The analysis revealed that zebrafish Fabin(s), Fabinb more so than Fibina, showed structural similarity to human and mouse FIBIN, but not to any other known protein. Moreover, I provided further evidence that the mutated zebrafish Fibins theoretically present in *fibina/b^{-/-}* fish were unlikely to actually be detectable/present in the fish, as the only part of the protein that is/could be transcribed (due to premature stop codon) was predicted to be highly disorganized N-terminal region with high probability of proteolytic degradation. Thus, even if some mRNA molecules carrying coding information regarding the mutated Fibins would escape non-sense mediated RNA decay, the translated peptide would be quickly targeted for proteolytic degradation. Next, prediction of protein localisation revealed that zebrafish Fabinb, human FIBIN and mouse FIBIN were all predicted to localize extracellularly, while Fibina was predicted to localize intracellularly, in the endoplasmic reticulum. This was an intriguing result, as Lakner and colleagues have showed in experiments with Cos-7 cell line with exogenously expressed mouse *Fibin* (via transfection) did not indicate FIBIN to be a secreted protein (detectable in the supernatants). Furthermore, the differential localisation of Fibina and Fabinb stresses the probability of different functions of these 2 proteins, however, that is still to be further investigated.

And finally, transcription factor binding site (TFBS) prediction, showed that only 2 transcription factors were expected to potentially directly regulate Fabin(s) expression: SOX-5 and HFH-3 (FOXI1). TFBS analysis is a powerful tool that can, without the presence of crystalized protein structures and large amount of experimental evidence, provide preliminary insight into the regulation of a poorly characterized protein. In our case, this analysis revealed that based on the TFBS present, Fabin(s) may be involved in development and chondrocyte differentiation – during development and in regeneration.

In Chapter 4 I have addressed the possible immune deficiency in the *fibina/b^{-/-}* mutant fish. Immune-system related functional impairment was a rather unexpected discovery from our comparative RNA-seq dataset comparing the transcriptome of wild-type and *fibina/b^{-/-}* mutant fish gill and caudal fin tissues at homeostasis. The reason why I said unexpected is simply based on the known expression niche of Fibin(s), the mesenchymal cells. I have quantified the relative percentages of erythrocyte, lymphocyte, myeloid and precursor cell populations and found no difference between the two genotypes. The fish used were 4 months old compared to the fish used for RNA-seq which were 6 months old. As majority of the immune-related genes that were found to be downregulated in the mutant fish from our RNA-seq were linked to immune response to stimulus, I used a viral mimetic compound, R848, to investigate the possible differential response of the two genotypes both locally and systemically. The R848 was applied directly onto partially dried gill tissue, thus assessment of the early and late cytokines in the gill (at 1 and 8 hpt) provided information about local immune response of the gill tissue. Analysis of inflammatory cytokines in WKM was used as an indicator of the presence/absence of systemic immune response. *fibina/b^{-/-}* fish showed highly similar response to R848 gill stimulation both locally and systemically as the wild-type fish for all cytokines investigated. This data showed that *fibina/b^{-/-}* fish are capable of mounting an immune response to a viral stimulus, likely through Tlr8 receptors, and that this response is temporally almost identical to that of wild-type fish. This result, in stark contrast to the RNA-seq data, stressed the importance of validation (such as by qPCR) of RNA-seq data. Moreover, it suggested that although the age-matched wild-type and mutant fish were raised on the system in adjacent tanks (to limit as much as possible potential inter-system differences in water temperature changes, oxygen levels and other variables) it appears likely that the wild-type aquarium was introduced to an immune-system stimulating agent, manifesting as higher read-counts of immune-related genes compared to those from *fibina/b^{-/-}* fish in our RNA-seq. As precautions were taken to prevent such an event (as discussed in Chapter 4) it remains to suggest – in order to limit the possibility of such an event in future - that fish from various tanks per genotype should be included in the analysis, with enough n numbers to allow for statistical analysis, to enable detection of outliers. This, however, can pose extra financial strain due to need of more aquariums and introduces new variables that need to be accounted for during data analysis and interpretation.

Next, in Chapter 5, I have investigated Fibin(s) in development of cartilage and bone (with particular focus on craniofacial region) and during regeneration of those two tissues using the zebrafish gill and caudal fin as the respective models.

Our comparative RNA-seq revealed increased levels of BMP signalling antagonists present in the *fibina/b^{-/-}* mutant fish compared to wild-type fish. However, as there was no obvious morphological difference detectable between the two genotypes during development or during regeneration, I have used exogenous pharmacological inhibition of BMP signalling at concentrations that were not inhibitory (for development or regeneration) to wild-type fish. This was to uncover the possible small differences in BMP signalling, known to be present at transcriptomic level, at whole system level – phenotype.

In Chapter 5, 2 separate systems were investigated – developmental and regenerative. Together with several master students, I have found differences in the craniofacial cartilage morphology during development between wild-type and *fibina/b^{-/-}* fish. These differences were only detectable at 1 timepoint (treatment window 48 – 72 hpf) with 1 μ M DMH1. The embryos and larvae used in these experiments were kept for a maximum of 14 dpf and no differences were observed in swimming patterns, feeding or size of the wild-type and mutant fish. This data therefore suggests that although there does seem to be a difference in the BMP signalling manifesting itself in a phenotypical difference in cartilage patterning, it does not have a significant impact on the mutant fish's ability to feed and grow. Together it is therefore possible to conclude that lack of Fibin(s) does not significantly, negatively, impact the development of zebrafish. Neuhauss and colleagues (Neuhauss *et al.*, 1996) had outlined mutation categorization into 3 groups based on the effects that a given mutation has on the development of craniofacial cartilages:

- 1) 'Mutations affecting the layout of the pharyngeal skeleton', characterised by complete lack of a part of the craniofacial cartilages
- 2) 'Mutations affecting the differentiation and morphogenesis of cartilaginous skeletal elements'
- 3) 'Mutations affecting the spatial arrangement of normally formed pharyngeal skeletal elements'

The *fibina/b^{-/-}* fish, with additional low level of BMP inhibition, fall into the 3rd category, as all the cartilaginous template is laid down and detectable, only the spatial arrangement differs to wild-type fish. Which, as the authors also propose, could be due to subtle differences in the mechanical properties of the cartilage between wild-type and *fibina/b^{-/-}* mutant fish. Thus, the same mechanical stress – swimming or feeding for example – differentially impacts the morphology of the tissue. Therefore, investigation of the composition of the individual craniofacial cartilages of both genotypes as well as mechanical testing of the cartilages might provide further insightful information into the functions of Fibin(s) during development.

I have then investigated transcript-level differences between wild-type and *fibina/b^{-/-}* mutant fish during caudal fin regeneration which is summarised in Fig. 6.5. I presented data showing transcriptional differences at 3 dpa in genes marking undifferentiated and mesenchymal-lineage restricted stem cells in blastema, *sall4* and *msx1b* respectively, which both showed lower transcript levels in the *fibina/b^{-/-}* fish compared to wild-type. Similar significant trend was seen with *mmp9*, coding for ECM remodelling enzyme involved in facilitating tissue regeneration through tissue remodelling. Interestingly, low dose of pharmacological BMP signalling inhibitor, DMH1, resulted in significant phenotypical differences in the caudal fin regeneration between wild-type and *fibina/b^{-/-}* fish, with *fibina/b^{-/-}* fish presenting significantly delayed lepidotrichia outgrowth and calcification. Together my data suggest that in caudal fin regeneration, from phenotypical perspective, Fibins are dispensable for the appendage regeneration, however, are important for the optimal setup of early post-inflammatory regeneration conditions. To what level that is biologically relevant and under what circumstances, remains to be investigated.

The adult zebrafish was also used to investigate gill tissue regeneration post cryoinjury. I have presented data showing significantly altered transcriptional signature in *fibina/b^{-/-}* fish compared to wild-type fish particularly at 7 and 10 dpc. My experiments revealed possible delay in the proper transcriptional setup of post-inflammatory regeneration stage in the *fibina/b^{-/-}* fish. Moreover, investigation of aged zebrafish gill tissue regeneration in both wild-type and *fibina/b^{-/-}* fish, revealed highly different dynamics at 7 and 10 dpc, suggesting significant changes in the initiation of regeneration at different ages. As I did not collect long

term data for the phenotypical outcome of aged zebrafish gill tissue regeneration, I cannot say whether the transcriptional differences detected at the early regeneration stages translate into functional differences down the line, when comparing young and old wild-type fish as well as in comparison to age-matched *fibina/b^{-/-}* fish.

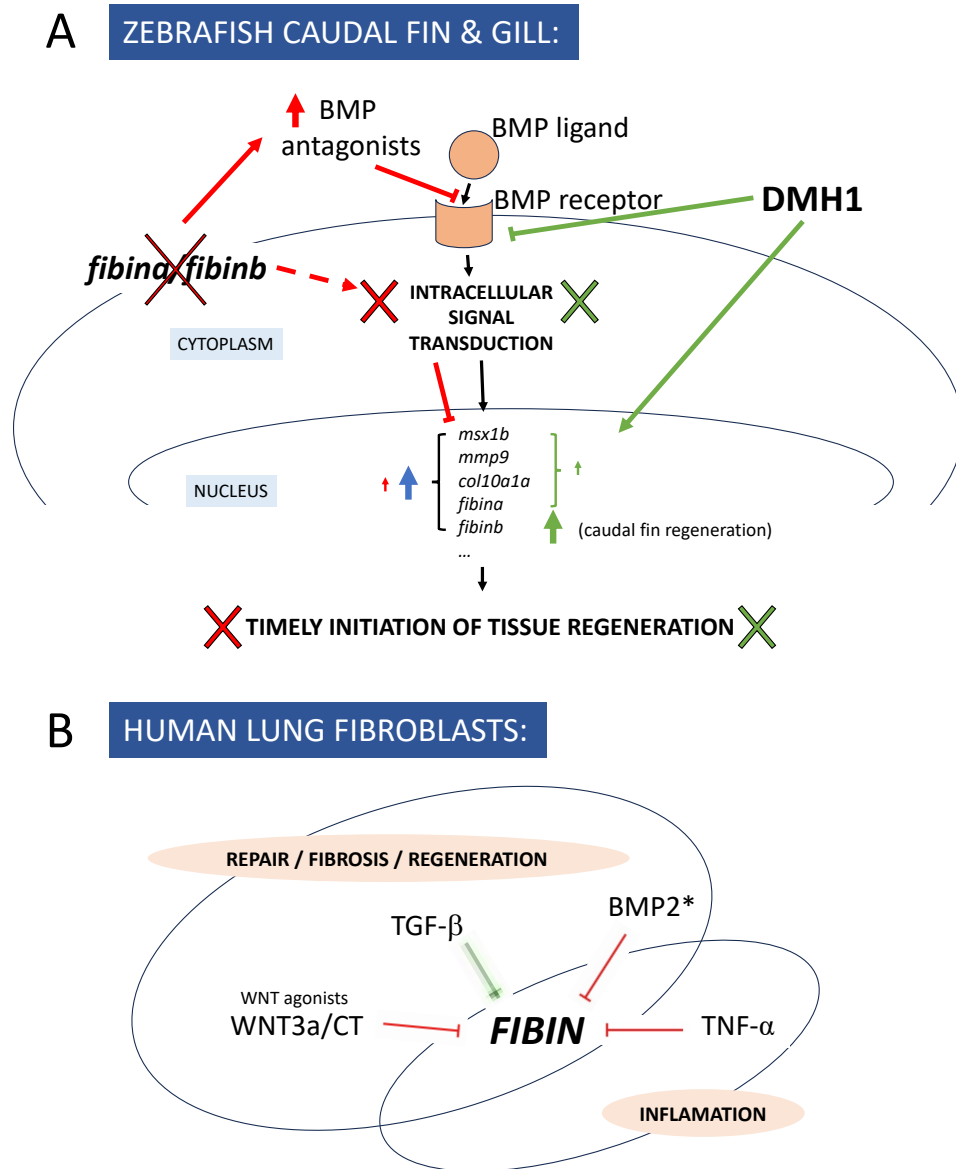


Figure 6.5 Summary of investigated signalling pathways and their effect on Fibin(s)

(A) Proposed interaction between zebrafish *fibina/fibinb* and BMP signalling pathway. Blue arrows represent signalling conditions in healthy wild-type zebrafish. Red colour (including arrows) specifies effects of *fibina/fibinb* knockout, found in the *fibina/b^{-/-}* fish. Green colour represents events following DMH1 treatment (BMP inhibition). Of note is the differential effect of DMH1 treatment on *fibina* vs *fibinb* in caudal fin regeneration where *fibina* transcript levels were found significantly lower upon DMH1 treatment in wild-type fish but *fibinb* levels remain intact the wild-type fish. (B) *FIBIN* transcript levels were investigated in human pulmonary fibroblasts upon treatment with TGF- β , canonical WNT signalling inducers WNT3a and CT, TNF- α and BMP2 (*Dr Laurence Bugeon, unpublished).

Overall, my data suggest that Fibrin(s) do not play a major role during development of zebrafish, however, are important for optimal transcriptional regulation of early tissue regeneration programs.

Several areas, including aging and/or mitochondrial dysfunction as well as matrix stiffness were considered as potential research interests related to Fibrin(s) as well. However, as I did not have the space and resources to tackle these areas during my PhD, I want to touch upon them here to lay the foundations for future investigations.

6.3.1 Matrix stiffness and Fibrin(s)

ECM stiffness is an extensively studied factor in tissue repair and regeneration. The ECM stiffness can influence lung tissue regeneration by affecting the behaviour of the cells involved in the process. Studies suggest that a stiff ECM can hinder stem cell proliferation and differentiation and limit immune cell migration to damaged areas, while a softer ECM can promote the proliferation of differentiation of stem cells, and the migration of immune cells to initiate tissue repair. Soft ECM have also been shown to support the differentiation of lung-resident stem cells, critical for lung regeneration, and overall softer ECM is considered a more permissive environment particularly for lung regeneration (Varghese, Ling and Ren, 2022).

The ECM of healthy lungs consists of collagens, elastin, proteoglycans and glycoproteins. Besides the structural support, these molecules provide mechanical stability and elastic recoil, vital for optimal lung function. In fibrotic lungs, such as in IPF, the biomechanical properties of ECM are very different. IPF lungs are characterised with significantly higher ECM stiffness which can be quantified using atomic force microscopy and Young's modulus measurements (Liu *et al.*, 2010; Froese *et al.*, 2016). The relationship between tissue stiffness and profibrotic cycle of excessive ECM deposition, mainly what drives what, still remains an area of active research as it is very difficult to measure ECM lung stiffness in patients. Currently, there is mounting evidence that suggests that the stiffening of ECM as a hallmark

of tissue fibrosis is not just a consequence of lung fibrosis rather that changes in the biochemical and biomechanical properties of lung tissue can actively drive the dysregulated response to alveolar epithelial injury, resulting in progressive pulmonary fibrosis (Liu *et al.*, 2010; Zhou *et al.*, 2013; Herrera, Henke and Bitterman, 2018b; Guo *et al.*, 2022).

How is mechanical stress transferred among cells and ECM? Outside forces, such as breathing associated stretching of the lung tissue, leads to formation of focal adhesions – clustered integrins – through which the ECM transfers the mechanical forces to the cell cytoskeleton. The integrin-activated intracellular signalling cascades (including ROCK and FAK) promotes cytosolic actin polymerisation (Janoštiak *et al.*, 2014). *in vitro* studies showed that ROCK and FAK can drive the differentiation of fibroblasts into myofibroblasts (Greenberg *et al.*, 2006; Akhmetshina *et al.*, 2008).

These mechanosensing pathways are just the first steps often leading to increased TGF- β signalling and increased ECM protein-coding genes transcription and translation. However, post-translational protein modification, as well as extracellular collagen crosslinking is an important part of the biomechanical feedback drive. Enzymes such as PLOD2, LOX, or LOXL1-4 are involved in the regulation, maturation and turnover of collagen fibrils and increased serum levels of these enzymes in patients with IPF have been associated with disease progression (Kadler, Hill and Canty-Laird, 2008; Erler and Weaver, 2009; Chien *et al.*, 2014). Moreover, results from mice experimental lung fibrosis showed that LOXL1-deficient mice were protected against the disease, due to lower degree extracellular collagen crosslinking and decreased tissue stiffness which enabled continuous matrix remodelling by MMPs (Bellaye *et al.*, 2018).

RNA seq results comparing caudal fin and gill tissue of wild-type and *fibina/b^{-/-}* fish showed significantly different, increased, read count numbers of several genes associated with extracellular matrix stiffness including but not limited to *plod2*, *fkbp10*, *lox14* or *serpinh1b*. *Plod2* for example, an enzyme that plays a key role in collagen cross-linking, important for the structural integrity and mechanical properties of the ECM was investigated in zebrafish.

A study by Gistelink and colleagues (Gistelink *et al.*, 2016) showed that *plod2* expression was upregulated during zebrafish embryonic development, and that this upregulation was associated with increased ECM stiffness. The authors also showed that knockdown of *plod2* lead to decrease in ECM stiffness. Functionally, this decrease of ECM stiffness led to increased tissue deformability and cell migration, whose optimal regulation is crucial for proper development. Another study investigated *plod2* during zebrafish caudal fin regeneration and the authors found that *plod2* transcript levels were upregulated during early stages of fin regeneration, and this upregulation was associated with increased ECM stiffness. Knockdown of *plod2* lead to decrease in ECM stiffness and impaired fin regeneration (Gistelink *et al.*, 2016; Lleras-Forero, Winkler and Schulte-Merker, 2020). Altogether, the published data suggests that by regulating ECM stiffness, *plod2* plays a crucial role in tissue mechanics and cell behaviour which are important processes in embryonic development and tissue regeneration.

With the increasing number of RNA sequencing studies offering more information on Fibrin(s) regulation in various conditions, another member of the Dallman laboratory, Dr Laurence Bugeon, has recently found new information regarding pathway interactions through the [idg.reactome](http://idg.reactome.org) database (idg.reactome.org). This search revealed Fibrin(s) to be involved in collagen degradation, degradation of extracellular matrix and extracellular matrix organisation. How Fibrin(s) influence collagen crosslinking and/or degradation, ECM composition and degradation, however, remains to be experimentally investigated/confirmed. Nevertheless, my data presented in Chapter 5 showed that Fibrin(s) seem to be involved in the early, post-inflammatory, stage of tissue repair/regeneration, and remain expressed at high levels throughout the regeneration, stages that include significant ECM remodelling to enable the repair/regeneration to take place. Thus, it is plausible to hypothesize that Fibrin(s) play a role in tissue repair/regeneration by modulating the ECM. Still, more research and experimental validation is required to fully dissect this proposed relationship and determine the possible implications for treatment of mammalian fibrotic disorders.

6.3.2 Aging, mitochondrial dysfunction and Fibrin(s)

Aging can be described as complex physiological phenomena influenced by both internal and external mechanisms as outlined in Fig. 6.6. Although many studies reported a correlation between IPF and age, the exact mechanisms that link these together remain to be discovered (López-Otín *et al.*, 2023).

6.3.2.1 Mitochondrial dynamic changes in IPF

Reduced mitochondrial function and autophagic activity is frequently observed in age-related disorders. Age-related decline in mitochondrial biogenesis, which involves growth and division, may result from a decrease in levels of PGC-1 α , a key regulator of mitochondrial biogenesis. Mouse studies showed that overexpression of PGC-1 α in aged mice was associated with reduced sarcopenia (muscle wasting) and improved mitochondrial function. An impaired balance between fission and fusion events may also contribute to the biogenesis decline with age. Fission helps to maintain mitochondrial quality and removes dysfunctional mitochondria through mitophagy, which degrades damaged mitochondria through fusion with lysosomes. However, with age, mitophagy declines, leading to an accumulation of damaged mitochondria, increased oxidative stress and increased cellular apoptosis (Sosulski *et al.*, 2015).

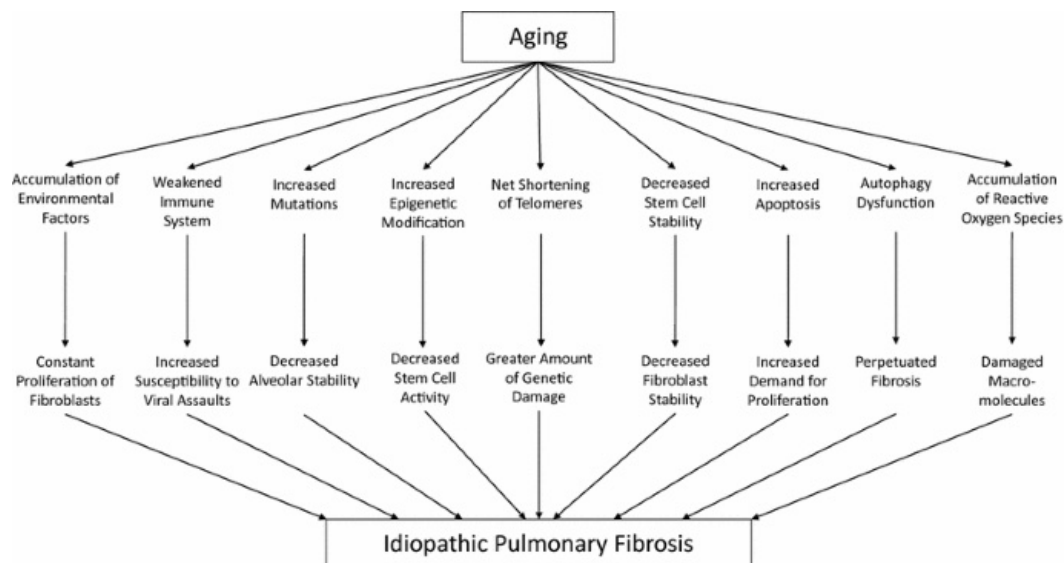


Figure 6.6 Impact of aging on specific factors that can influence the development and progression of IPF (Leung *et al.*, 2015).

6.3.2.2 Mitochondrial dynamic changes with aging

Mitochondrial dysfunction has been also implicated in the pathogenesis as well as progression of IPF. Research showed that IPF fibroblasts have increased mitochondrial fragmentation, reduced fusion events, and decreased expression of mitochondrial fusion proteins, which are associated with decreased ATP production and increased production of ROS. Moreover, decreased mitochondrial biogenesis and reduction in mediators of mitochondrial quality control in fibroblasts as well as epithelial cells of IPF origin have been documented (Kobayashi *et al.*, 2016; G. Yu *et al.*, 2018; Zank *et al.*, 2018).

Together, there are clear, overlapping aspects of mitochondrial dysfunction in aging and IPF. These include increased oxidative stress, decreased biogenesis and impaired fission/fusion events and last but not least cellular senescence characterized by irreversible cell cycle arrest (Sembrat *et al.*, 2017; Zank *et al.*, 2018). How does mitochondrial dysfunction link with our investigation of Fibin(s)? As touched upon in the Discussion part of Chapter 5, Lee and colleagues (Lee *et al.*, 2021) showed that overexpression of miR-141-3p in mice fibroblast cell line (C2C12) lead to inhibition of myogenic differentiation in these cells and significant decrease in mitochondrial function. The authors in their paper suggested that this was mediated through miR-141-3p downregulation of *FIBIN* and *FKBP5* genes. They further showed that inhibition of miR-141-3p lead to significant increase in *FIBIN* transcript levels. This study therefore offered an initial link between sarcopenia (skeletal muscle mass loss) mitochondrial dysfunction, and Fibin(s). Sarcopenia is generally characterized as age-related condition, however, it has also been described as a characteristic accompanying ovariectomy-induced obesity, as in the study by Lee and colleagues. To note, ovariectomy-induced obesity and sarcopenia, including mitochondrial dysfunction, is associated with chronic tissue inflammation, particularly increased levels of TNF- α or IFN- γ , which have been shown to cause downregulation of *FIBIN* themselves (Rogers *et al.*, 2009).

Fibin(s), as highlighted in this thesis, are strongly expressed from the early-remodelling stage of zebrafish gill and caudal fin tissue regeneration. The initial stages of tissue regeneration, including wound healing, can be significantly affected by changes to the oxygenation status, such as due to increase in reactive oxygen species (ROS). Excessive ROS production or

impairment in ROS detoxification pathways (due to mitochondrial dysfunction) leads to oxidative damage, which is one of the main causes of chronic wounds (Guo and DiPietro, 2010; Smith, Waypa and Schumacker, 2017). Mitochondria unfolded protein response is a key pathway for maintenance of mitochondrial health by detection and restoration of disrupted protein turnover in mitochondria. Studies have shown that mitochondria unfolded protein response plays a crucial role in stem cell maintenance, ageing, neurodegeneration and many others and Lin and colleagues have discovered that zebrafish depends on this pathway as well, particularly for caudal fin regeneration (Lin, Sam and Evans, 2021). This not only stresses the wide impact of mitochondrial stress on diverse aspects of tissue repair, but also indicates again the conservation of these mechanisms across lower and higher vertebrates. And therefore, further investigation into the link between Fabin(s) and mitochondria function could uncover new avenues of targeting tissue repair and regeneration.

6.4 Final conclusions

In this thesis, I have presented the first, comprehensive, investigation of the understudied gene(s) Fabin(s) and their potential role in zebrafish development, zebrafish complex tissue regeneration as well as their regulation in human pulmonary fibroblasts. While I have found no significant impact of lack of Fabin(s) on the zebrafish ability to mount an immune response to a viral-mimetic stimulus, I have shown that *fibina/b*^{-/-} fish have significantly affected dynamics of certain gene transcript involved in the early, post-inflammatory regeneration stage, and that these differences appear to be linked to BMP signalling. Furthermore, I have showed preliminary *in vitro* data demonstrating that *FIBIN* transcription is regulated by TGF- β signalling and WNT signalling, but also by TNF- α stimulation. And lastly, I have touched upon two avenues of Fabin(s)-related research that I did not have the chance to investigate in greater detail in this thesis but which show promise for future research in tissue repair and regeneration: Extracellular matrix and mitochondrial dysfunction and aging. Together, my results support the notion that Fabin(s) are positively regulated in early stages of tissue repair and regeneration (i.e. TGF- β signalling), negatively regulated by inflammatory-related signalling pathways (possibly including early, post-injury inflammation) and that Fabin(s) are possibly involved in extracellular matrix modulation, thus

playing an important role in optimal and timely scar-less tissue regeneration. Further investigation into Fbin(s) in regeneration may shed further light on the complexity and dynamics of (not just pulmonary) tissue regeneration.

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8 APPENDICES

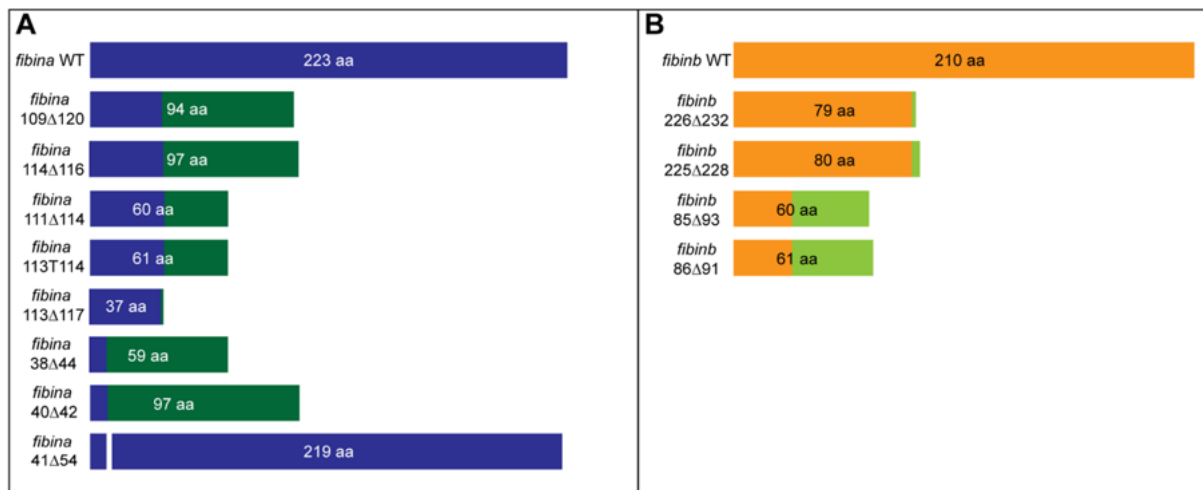
**Selection of genes up regulated in mouse PF models
and down regulated in zebrafish gills cryoinjury model**

Human gene	Mouse gene	Zebrafish gene	Description
ACKR3	ENSMUSG00000044337/Ackr3	ENSDARG00000058179/ackr3b/cxcr7b	Atypical Chemokine Receptor 3
RNF128	ENSMUSG00000031438/Rnf128	ENSDARG00000016867/rnf128a	Ring Finger Protein 128
CEMIP	ENSMUSG00000052353/Cemip	ENSDARG00000039881/cemip	Cell Migration Inducing Hyaluronidase 1
CYP7B1	ENSMUSG00000039519/Cyp7b1	ENSDARG0000008858/cyp7b1/cyp7c1	Cytochrome P450 Family 7 Subfamily B Member 1
NDRG1	ENSMUSG00000005125/Ndr1	ENSDARG00000032849/ndrg1a	N-Myc Downstream Regulated 1
CSRP2	ENSMUSG00000020186/Csrp2	ENSDARG00000011961/csrp2	Cysteine And Glycine Rich Protein 2
CXCL14	ENSMUSG00000021508/Cxcl14	ENSDARG00000056627/cxcl14	C-X-C Motif Chemokine Ligand 14

**Selection of genes down regulated in mouse PF models
and up regulated in zebrafish gills cryoinjury model**

Human gene	Mouse gene	Zebrafish Gene	Description
MMP15	ENSMUSG00000031790/Mmp15	ENSDARG00000051962/mmp15a	Matrix Metalloproteinase 15
MMP9	ENSMUSG00000017737/Mmp9	ENSDARG00000042816/mmp9	Matrix Metalloproteinase 9
FIBIN	ENSMUSG000000074971/Fibin	ENSDARG00000039949/fibina ENSDARG00000087196/fibinb	Fin Bud Initiation Factor
GDPD2	ENSMUSG00000019359/Gdpd2	ENSDARG00000073870/gdpd2	Glycerophosphodiester Phosphodiesterase Domain Containing 2
OGN	ENSMUSG00000021390/Ogn	ENSDARG00000044132/ogn	Osteoglycin
FGFBP1	ENSMUSG00000048373/Fgfbp1	ENSDARG00000091660/fgfbp1b	Fibroblast Growth Factor Binding Protein 1
SOX11	ENSMUSG00000063632/Sox11	ENSDARG00000077811/sox11a	SRY-Box Transcription Factor 11
GPT	ENSMUSG00000022546/Gpt	ENSDARG00000019541/gpt/gpt2l	Glutamic-Pyruvic Transaminase
ELAPOR1	ENSMUSG00000040412/Elapor1	ENSDARG00000042899/si:ch211-163/21.8	Endosome-Lysosome Associated Apoptosis and Autophagy Regulator 1
ITGA8	ENSMUSG00000026768/Itga8	ENSDARG00000078717/itga8	Integrin Subunit Alpha 8
ACTC1	ENSMUSG00000068614/Actc1	ENSDARG00000036371/acta1a	Actin Alpha Cardiac Muscle 1
KRT23	ENSMUSG00000006777/Krt23	ENSDARG00000019365/keratin99	Keratin 23
POF1B	ENSMUSG00000034607/Pof1b	ENSDARG00000054060/pof1b	POF1B Actin Binding Protein
GSN	ENSMUSG00000026879/Gsn	ENSDARG00000045262/gsnb	Gelsolin
SYTL2	ENSMUSG00000030616/Sytl2	ENSDARG00000075436/sytl2	Synaptotagmin Like 2
RTKN2	ENSMUSG00000037846/Rtkn2	ENSDARG00000053558/rtkn2a	Rhotekin 2

APPENDIX 1: Selection of genes found differentially regulated between mice models of pulmonary fibrosis and zebrafish model of gill regeneration (post cryoinjury).



APPENDIX 2: Details about mutations generated during the establishment of double mutant zebrafish model. (A) *fibinb* allele. (B) *fibinb* allele. Data by M.-C. Ramel

human *FIBIN* vs mouse *FIBIN*

	Score 897 bits(994)	Expect 0.0	Identities 678/803(84%)	Gaps 18/803(2%)	Strand Plus/Plus	
h. <i>FIBIN</i>	239		CGCTGGTGTGCAGAGCCACATGAAGCTGCTGGGACTGGGGCCAGGGAGCAGCAAGCC			298
m. <i>FIBIN</i>	40		CGCTGCTGCTAGAGCCACATGAAGCTGTGGAGAACAGGATCAGAGAGCGCAAGCG			99
h. <i>FIBIN</i>	299		AGCTGGGACTGAGGCGGACGTGTCTCAGGGAGAGCGTACTGCAAGACACTCCCTTC			358
m. <i>FIBIN</i>	100		AGCTGCGCTTGAAGAGGGCGCTTCTCCGAAGCGCTGACACCCGGGGAACTTTTTC			159
h. <i>FIBIN</i>	359		CTTGTGCTGGGTAAGAAAGTCTCTCTCTGGGGTCCCTGGCCATCTCGAATATCAGAAATG			418
m. <i>FIBIN</i>	160		CTTGTCTCTGACTAAACGCTTCTCCGG-----AATATCCAGAAATG			201
h. <i>FIBIN</i>	419		GTGTTCTGAAAGTCTCTGCTGAGTGTCTCTGCCACCTGTCTCAAGGCTACTTTCAT			478
m. <i>FIBIN</i>	202		GTGTTCCGAAAGTGTATCTGATGGGTTCTTTGCCACCTGTGTGAGGCTACTTTGAT			261
h. <i>FIBIN</i>	479		GGCCCCCTTACCCAGAGATGTCCAATGGGACTCTGCACCACTACTCTGTCGCCGATGG			538
m. <i>FIBIN</i>	262		GGCCCCCTTACCCAGAAATGTCTAATGGGACTTTGCTACTTACTTCTGTCCTGATGGA			321
h. <i>FIBIN</i>	539		GACTATAGAGAGAAAGATGACCCGAGAATGTCAGCTGTCTTCAAGGTGAGTGACAC			598
m. <i>FIBIN</i>	322		GACTATAGAGAGAAATGATGACCCGAGAATGTCAGCTGTCTTCAAGGTGAGTGATCGC			381
h. <i>FIBIN</i>	599		AGGCGCTGCTCCAGGGGGAGGGGAGCCAGTTGGAGCCCTGCTGAGCCTCAACCCTCGG			658
m. <i>FIBIN</i>	382		AGGCGCTGCTCCAGGGGGAGGGGGCCAAAGCAGTAGTCTGCTGAGCCTCACTCTTGA			441
h. <i>FIBIN</i>	659		GAGGAGTTACACGTGCTGGGCGCAGGTGGAGATGCTGGGCGCTGCTGGAGGGCATC			718
m. <i>FIBIN</i>	442		GAGGAGTTTCAAGTACTGGGCGCAGGTGGAGATGCTGAGCGCTCTGGAGGGCATC			501
h. <i>FIBIN</i>	719		AGCAAAAGCATCTCTACGACCTAGAGGGGGAAGAGAGCTATGGCAAGTACTCGGCGG			778
m. <i>FIBIN</i>	502		AGCAAGAGCATCTCTACGACCTGGATGGGGAAGAGAGCTATGGCAAGTACTCGAGCGG			561
h. <i>FIBIN</i>	779		GAGTCCACAGAGTGGGGTGTCTACTCAACTCGGACAAATCCTTCACTGAGCTGGAG			838
m. <i>FIBIN</i>	562		GAGTCCACAGAGTGGGGTGTCTACTCAACTCTGACAAGTCTCTCACTGAGCTGGAA			621
h. <i>FIBIN</i>	839		AGCAAGTTTCAAGCAGGGCCAGGAACAGGACGCGCGAGGAGAGCAGGCTCAACGAGGAC			898
m. <i>FIBIN</i>	622		AGCAAGTTTCAAGCAGGGCCAGGAGAGGATAGCCGGCAGGAAGACAGACTCAATGAGGAC			681
h. <i>FIBIN</i>	899		TTTCTGGGAATGCTGGTCCACACAGGTCCCTGCTGAAGGAGACACTGGACATCTCTGTG			958
m. <i>FIBIN</i>	682		TTTCTGGGAATGCTGGTCCACACAGGTCCCTGCTGAAGGAGACACTGGACATCTCTGTG			741
h. <i>FIBIN</i>	959		GGGCTCAGGGACAAATACGAGCTGTGGCCCTCACATTAGGAGCCATGGGACCCGACTA			1018
m. <i>FIBIN</i>	742		GGGCTCAGAGACAAATACGAGCTGTGGCACATACATCAGGAGCATGGGACCGCTTA			801
h. <i>FIBIN</i>	1019		GGTGGCTGAAGAAATGATTATCT	1041		
m. <i>FIBIN</i>	802		GGTGGCTGAAGAGCGACTATCT	824		

fibina vs *fibinb*

	Score 329 bits(364)	Expect 3e-94	Identities 415/566(73%)	Gaps 22/566(3%)	Strand Plus/Plus	
h. <i>FIBIN</i>	117		GGGACCTTTGCTCCCGAAATGTCCAATGGCACTTTTCAACACTTCTTCTGCCGGACGG			176
fibinb	78		GGGACCTTGTATCCAGAGATGTCCAACGGACGTTTCACTATTATTCTGTCGGACGG			137
h. <i>FIBIN</i>	177		CGATTATGAAGAGACTGAAGACCCGAGAAGTCCAGATGCTCTTCAAATGGATGCAC--			234
fibinb	138		CTACTATGAAGAAACGACGATCCCGAGAATGTAGATGCTCTTCAAATGATGGACAA			197
h. <i>FIBIN</i>	235		-CGCAGACCGTGTCACTGGAGGAAGACGGGACACGGTTATTAGGAGGATTTCTGTAT			293
fibinb	198		TGCAAA--TGACCTTGGACGAGGACCAAGACTCGGTGATCCGGATGATTTCACTAT			254
h. <i>FIBIN</i>	294		AGTAAGCAGCAGATTGAAGACGCGCGCGGTTCTGGAGACTCTCGGGAAGAGCATCTC			353
fibinb	255		TATCAAGCGGCACATCGAGGACGCGCGCGGTTCTGAGGCATTGGGAAGAGCATCTC			314
h. <i>FIBIN</i>	354		GTTTGATCTGGAGGAGGAGCAGCTACGGGAATACCTGAAGCAGAGAGATCGA-CCAGA			412
fibinb	315		CTTGACCTCGACGGAGAGGACAGCTATGGGAATATCTCAGACGGGAGA-CCAGCCAGA			373
h. <i>FIBIN</i>	413		TCGGCAGGGCTTTTCAACACGTGGAGAAATCTCTGCTGgaagctggagagcaagttcaagc			472
fibinb	374		TCAGCAGGGCTTTTCAACTCCGAGAAATCCCTCTTGGAGCTGGAGTAAGTTCAAAC			433
h. <i>FIBIN</i>	473		agaagcaggagagcggagcagagagagagaggaattcaagcagTAACCTCATCAAGCCCG			532
fibinh	434		AGAGCAGGAAAAACGAGCTGAAGAGGAGCACAAGATCAGCGACGACTTCTCAA-----			488
h. <i>FIBIN</i>	533		TGTATGAT-GT-----GAGAGAAACGCTCAGGAGACGCTGGACATCTCATCCGAGTCT			585
fibinb	489		--TATGATCTGACACGCGGACGTGCTGAAGGAGACGCTGGACATCTCACTGGGGCTG			546
h. <i>FIBIN</i>	586		AAGGATAAACACGAGCTATTCTCTGATCTGAGGAATACGGCTCCAGGCTAAGCAGG			645
fibinb	547		AAGGACAAGCAGAGCTGCTGTCACTATCATCCGAGTCAAGGGACCGCTGAGCGCG			606
h. <i>FIBIN</i>	646		CTGAAGAACGACTATCTGAAGCTTTA	671		
fibinb	607		CTGAAGAACGACTATATGAAGTGTA	632		

human *FIBIN* vs *fibina*

	Score 153 bits(169)	Expect 2e-40	Identities 378/567(67%)	Gaps 30/567(5%)	Strand Plus/Plus	
h. <i>FIBIN</i>	490		CCCAGAGATGTCATATGGGACTCTGCACACTACTTCTGTGCCGATGGGACTATGAGGA			549
fibina	129		CCCCGAAATGCTCAATGGGACTTTTCAACACTTCTTCTGCCGAGCGGATATGAAGA			188
h. <i>FIBIN</i>	559		GAAACGATGACCCCGAGAAGTGCCAGCTGCTCTTCAAGGTGAGTGAACACAGCGCTGCTC			609
fibina	189		GACTGAAGACCCCGAGAAGTGCCAGATGCTCTTCAAATGGATGACCCGAGACGCTGTCC			248
h. <i>FIBIN</i>	610		CCAGGGGAGGGGAGCCAGGTTGGCAGCTGCTGAGCCTCAACCCTCGGGAGGAGTTAC			669
fibina	249		ACTGGAGGA--AGACCG--GACACGGTTATTAG-----GAGGATTTTC			288
h. <i>FIBIN</i>	670		CGTGTGCG--GCCCAGCGTGAGGATGCTGGCGCTGCTGGAGGGCATCAGCAAA			725
fibina	289		-GTTATAGTAAGCAGC-AGATTGAAGACGCGCGCGCTTCTGGAGACTCTCGGAAGA			346
h. <i>FIBIN</i>	726		GCATCTCTTACGACCTAGACGGGGGAAGAGAGCTATGGCAAGTACTGCGCGGGAGTCCC			785
fibina	347		GCATCTGTTTATCTGGACGAGAGGACAGCTACGGGAATACCTGAAGCGAGAGATCG			406
h. <i>FIBIN</i>	786		ACCAGATCGGGATGCTACTCCAACCTGGGCAAACTCCTCACTGAGCTGGAGAGCAAGT			845
fibina	407		AGCAGATCGCGAGGCTTTCACCAACGTGGAGAAATCTGCTGGAGCTGGAGAGCAAGT			466
h. <i>FIBIN</i>	846		TCAAGCAGGGCCAGGA-ACAGGACAGCCGGCAGGAGAGCAGGCTCAACGAGGACTTCTTG			904
fibina	467		TCAAGCAGGGCCAGGAGCGGAGCAG-AGAGAGGAGAGCGAGTTGAGC-AGTAACCTCAT			524
h. <i>FIBIN</i>	905		GGAAATGC-TGGTCCACACAGGTCCTCTGCTGAAGGAGACACTGGACATCTGTGGGGCT			963
fibina	525		CAGGCCGTGTATGATGTGAGAGAAACGCTCAGAGAGCGCTGGACATCTCATCCGGACT			584
h. <i>FIBIN</i>	964		CAGGGACAATACAGAGTGTGGGCCCTCACCATTAGGAGCATGGGACCCGACTAGGTCG			1023
fibina	585		CAAGGATAAACCGAGCTCATTTTCTGATCTGAGGAATCACGGCTCAGGCTAAGCAG			644
h. <i>FIBIN</i>	1024		GCTGAAAAATGATTATCTTAAAGTATA	1050		
fibina	645		GCTGAAAAACGACTATCTGAACGTTTA	671		

human *FIBIN* vs *fibinb*

	Score 251 bits(277)	Expect 9e-70	Identities 402/575(70%)	Gaps 22/575(3%)	Strand Plus/Plus	
h. <i>FIBIN</i>	479		GGCCCCCTTACCCAGAGATGTCCAATGGGACTCTGCACCACTACTCTGTCGCCGATGGG			538
fibina	79		GGACCCCTGTATCCAGAGATGTCCAACGGACGTTTCACTATTATTCTGTCGGACGGC			138
h. <i>FIBIN</i>	539		GACTATGAGGAGAACGATGACCCCGAGAAGTGCACGCTGCTCTTCAAGGTGAGTGACAC			598
fibina	139		TACTATGAAGAAAAACGACGATCCCGGAAGATGTAGATGCTCTTCAAATGATGGACAT			198
h. <i>FIBIN</i>	599		AGGCGCTGCTCCAGGGGGAGGGGAGCCAGGTTGGCAGCTC-GCTGAGCTCACCTGCG			657
fibina	199		CGCAAAATGCACCTGGACGAGGA--CCA--AGACTGGGTATCC-----G			239
h. <i>FIBIN</i>	658		GGAGAGTTTACCGTCTGGGCGCCAGGTGGAGGATGCTGGGCGCTGCTGGAGGGCAT			717
fibina	240		GGATGATTTCACTATTATCAAGCGGCACATCGAGGACGCGCGCGGTTCTGGAGGATC			299
h. <i>FIBIN</i>	718		CAGCAAAAGCATCTCTTACGACCTAGACGGGGAAGAGACTATGGCAAGTACTGCGGCG			777
fibina	300		TGGGAAGGACATCTCTTCACTCTGACCTGACGGAGAGGACAGCTATGGGAATATCTCAGACG			359
h. <i>FIBIN</i>	778		GGAGTCCACAGATCGGGGATGCTACTTCAACTCGGCAAAATCCCTCACTGAGCTGGA			837
fibina	360		GGAGACGACCCAGATCAGCGAGGCGTTTCCAACCTCGAGAAATCCCTTCTGAGCTGGA			419
h. <i>FIBIN</i>	838		GAGCAAGTTCAAGCAGGGCCAGGAACAGGA-CAGCGCGCAGGAGAGCAGGCTCAACGAGG			896
fibina	420		GGTAAGTTCAAAACAGAGCCAGGAAAAACGAGCTGAAG-AGGAGCACAGATCAGGACG			478
h. <i>FIBIN</i>	897		ACTTTCTGGGAATGCTGGTCCACACAGGTCCCTGCTGAAGGAGACACTGGACATCTCTG			956
fibina	479		ACTTCTCAATATGATGTGTGACACGCGGACGCTGTGAAGGAGACGCTGGACATCTCAC			538
h. <i>FIBIN</i>	957		TGGGGCTCAGGGACAATACGAGCTGCTGGCCCTCACATTAGGAGCATGGGACCGAC			1016
fibina	539		TGGGGCTGAAGGACACGACGAGCTGCTGTCACTATCATTCGCGACTCAGGGACCGCC			598
h. <i>FIBIN</i>	1017		TAGTGGCTGAAAAATGATTATCTTAAAGTATAG	1051		
fibina	599		TGAGCCGCTGAAGAACGACTATATGAAGGTGTA	633		

APPENDIX 3: Nucleotide sequence alignment for human *FIBIN* and mouse *FIBIN*, *fibina* and *fibinb*, human *FIBIN* and *fibina* and human *FIBIN* and *fibinb*.

A)

GO biological process complete	number	overUnder	pvalue
tRNA processing (GO:0008033)	5	-	1.62E-03
ncRNA processing (GO:0034470)	6	-	2.84E-03
tRNA metabolic process (GO:0006399)	6	-	3.38E-03
RNA processing (GO:0006396)	13	-	3.54E-03
ncRNA metabolic process (GO:0034660)	7	-	4.92E-03
nucleic acid metabolic process (GO:0090304)	20	-	5.32E-03
RNA metabolic process (GO:0016070)	16	-	5.94E-03
gene expression (GO:0010467)	17	-	7.14E-03
RNA modification (GO:0009451)	4	-	9.31E-03
macromolecule methylation (GO:0043414)	5	-	1.36E-02
tRNA modification (GO:0006400)	3	-	1.59E-02

tRNA methylation (GO:0030488)	3	-	1.59E-02
RNA methylation (GO:0001510)	3	-	1.59E-02
cell division (GO:0051301)	2	-	1.99E-02
positive regulation of cell population proliferation (GO:0008284)	5	-	2.87E-02
methylation (GO:0032259)	8	-	3.10E-02
antigen processing and presentation (GO:0019882)	6	-	3.27E-02
cytosolic transport (GO:0016482)	2	-	3.32E-02
macromolecule metabolic process (GO:0043170)	87	-	3.73E-02
generation of precursor metabolites and energy (GO:0006091)	4	-	4.95E-02
energy derivation by oxidation of organic compounds (GO:0015980)	4	-	4.95E-02
potassium ion transmembrane transport (GO:0071805)	6	+	5.07E-03
inorganic cation import across plasma membrane (GO:0098659)	5	+	1.30E-02
inorganic ion import across plasma membrane (GO:0099587)	5	+	1.30E-02

import across plasma membrane (GO:0098739)	5	+	1.30E-02
monovalent inorganic cation homeostasis (GO:0055067)	4	+	1.57E-02
skeletal system development (GO:0001501)	10	+	1.63E-02
potassium ion import across plasma membrane (GO:1990573)	3	+	2.21E-02
cation transmembrane transport (GO:0098655)	14	+	2.23E-02
cartilage development (GO:0051216)	4	+	2.28E-02
connective tissue development (GO:0061448)	4	+	2.28E-02
sensory organ development (GO:0007423)	16	+	2.68E-02
striated muscle tissue development (GO:0014706)	5	+	2.85E-02
muscle tissue development (GO:0060537)	5	+	2.85E-02
system development (GO:0048731)	81	+	2.89E-02
ion transmembrane transport (GO:0034220)	24	+	3.08E-02
ossification (GO:0001503)	2	+	3.08E-02

skeletal muscle tissue development (GO:0007519)	2	+	3.40E-02
skeletal muscle fiber development (GO:0048741)	2	+	3.40E-02
myotube cell development (GO:0014904)	2	+	3.40E-02
myotube differentiation (GO:0014902)	2	+	3.40E-02
import into cell (GO:0098657)	6	+	3.50E-02
multicellular organism development (GO:0007275)	91	+	3.65E-02
negative regulation of signal transduction (GO:0009968)	10	+	3.78E-02
developmental process (GO:0032502)	104	+	3.87E-02
regulation of BMP signaling pathway (GO:0030510)	3	+	3.96E-02
anatomical structure development (GO:0048856)	102	+	4.46E-02
sex differentiation (GO:0007548)	2	+	4.50E-02
biological regulation (GO:0065007)	243	+	4.51E-02
potassium ion transport (GO:0006813)	7	+	4.73E-02

presynapse organization (GO:0099172)	2	+	4.81E-02
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presynapse assembly (GO:0099054)	2	+	4.81E-02
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B)

GO biological process complete	number	overUnder	pvalue
cilium organization (GO:0044782)	12	-	1.19E-02
lipid localization (GO:0010876)	10	-	1.29E-02
lipid transport (GO:0006869)	10	-	1.29E-02
cell projection assembly (GO:0030031)	13	-	1.78E-02
protein import (GO:0017038)	2	-	2.37E-02
protein localization to nucleus (GO:0034504)	2	-	2.51E-02
cilium assembly (GO:0060271)	11	-	2.54E-02
plasma membrane bounded cell projection assembly (GO:0120031)	11	-	2.54E-02
organic cyclic compound catabolic process (GO:1901361)	6	-	2.67E-02
microtubule bundle formation (GO:0001578)	2	-	3.08E-02
execution phase of apoptosis (GO:0097194)	2	-	4.24E-02
lipoprotein metabolic process (GO:0042157)	7	-	4.75E-02
cellular component organization (GO:0016043)	149	+	8.35E-04

cell cycle (GO:0007049)	94	+	8.57E-04
cellular component organization or biogenesis (GO:0071840)	150	+	1.15E-03
circulatory system development (GO:0072359)	24	+	1.91E-03
cell division (GO:0051301)	33	+	2.28E-03
extracellular matrix organization (GO:0030198)	9	+	2.41E-03
extracellular structure organization (GO:0043062)	9	+	2.41E-03
external encapsulating structure organization (GO:0045229)	9	+	2.41E-03
chemical homeostasis (GO:0048878)	19	+	2.72E-03
lipid biosynthetic process (GO:0008610)	12	+	2.90E-03
mitotic cell cycle (GO:0000278)	69	+	2.92E-03
cell cycle process (GO:0022402)	73	+	4.13E-03
mitotic cell cycle process (GO:1903047)	53	+	6.35E-03
spindle organization (GO:0007051)	17	+	7.06E-03
homeostatic process (GO:0042592)	27	+	7.51E-03
glycerolipid biosynthetic process (GO:0045017)	4	+	8.62E-03
lipid homeostasis (GO:0055088)	3	+	9.88E-03
establishment of organelle localization (GO:0051656)	16	+	1.12E-02
regulation of kinase activity (GO:0043549)	14	+	1.34E-02
stem cell differentiation (GO:0048863)	7	+	1.34E-02
heart development (GO:0007507)	10	+	1.36E-02

organelle localization (GO:0051640)	19	+	1.36E-02
anatomical structure development (GO:0048856)	118	+	1.41E-02
multicellular organism development (GO:0007275)	110	+	1.71E-02
phospholipid biosynthetic process (GO:0008654)	4	+	1.80E-02
cellular metal ion homeostasis (GO:0006875)	15	+	1.81E-02
cellular ion homeostasis (GO:0006873)	15	+	1.81E-02
cellular homeostasis (GO:0019725)	15	+	1.81E-02
cellular cation homeostasis (GO:0030003)	15	+	1.81E-02
cellular chemical homeostasis (GO:0055082)	15	+	1.81E-02
cation homeostasis (GO:0055080)	15	+	1.81E-02
metal ion homeostasis (GO:0055065)	15	+	1.81E-02
inorganic ion homeostasis (GO:0098771)	15	+	1.81E-02
tissue development (GO:0009888)	30	+	1.86E-02
tube morphogenesis (GO:0035239)	15	+	1.90E-02
developmental process (GO:0032502)	122	+	1.92E-02
regulation of phosphorus metabolic process (GO:0051174)	20	+	1.98E-02
regulation of phosphorylation (GO:0042325)	20	+	1.98E-02
regulation of phosphate metabolic process (GO:0019220)	20	+	1.98E-02
multicellular organismal process (GO:0032501)	124	+	2.12E-02
blood vessel morphogenesis (GO:0048514)	10	+	2.26E-02

organelle fission (GO:0048285)	39	+	2.27E-02
regulation of transferase activity (GO:0051338)	15	+	2.34E-02
regulation of biological quality (GO:0065008)	53	+	2.51E-02
mitotic nuclear division (GO:0140014)	25	+	2.56E-02
tube development (GO:0035295)	17	+	2.66E-02
positive regulation of cytosolic calcium ion concentration involved in phospholipase C-activating G protein-coupled signaling pathway (GO:0051482)	3	+	2.86E-02
mitochondrion organization (GO:0007005)	7	+	3.02E-02
ion homeostasis (GO:0050801)	16	+	3.04E-02
positive regulation of cellular process (GO:0048522)	61	+	3.05E-02
vesicle organization (GO:0016050)	5	+	3.12E-02
divalent inorganic cation homeostasis (GO:0072507)	13	+	3.13E-02
cellular divalent inorganic cation homeostasis (GO:0072503)	13	+	3.13E-02
cellular calcium ion homeostasis (GO:0006874)	13	+	3.13E-02
positive regulation of cytosolic calcium ion concentration (GO:0007204)	13	+	3.13E-02
regulation of cytosolic calcium ion concentration (GO:0051480)	13	+	3.13E-02
calcium ion homeostasis (GO:0055074)	13	+	3.13E-02
reproductive process (GO:0022414)	29	+	3.19E-02
reproduction (GO:0000003)	29	+	3.19E-02

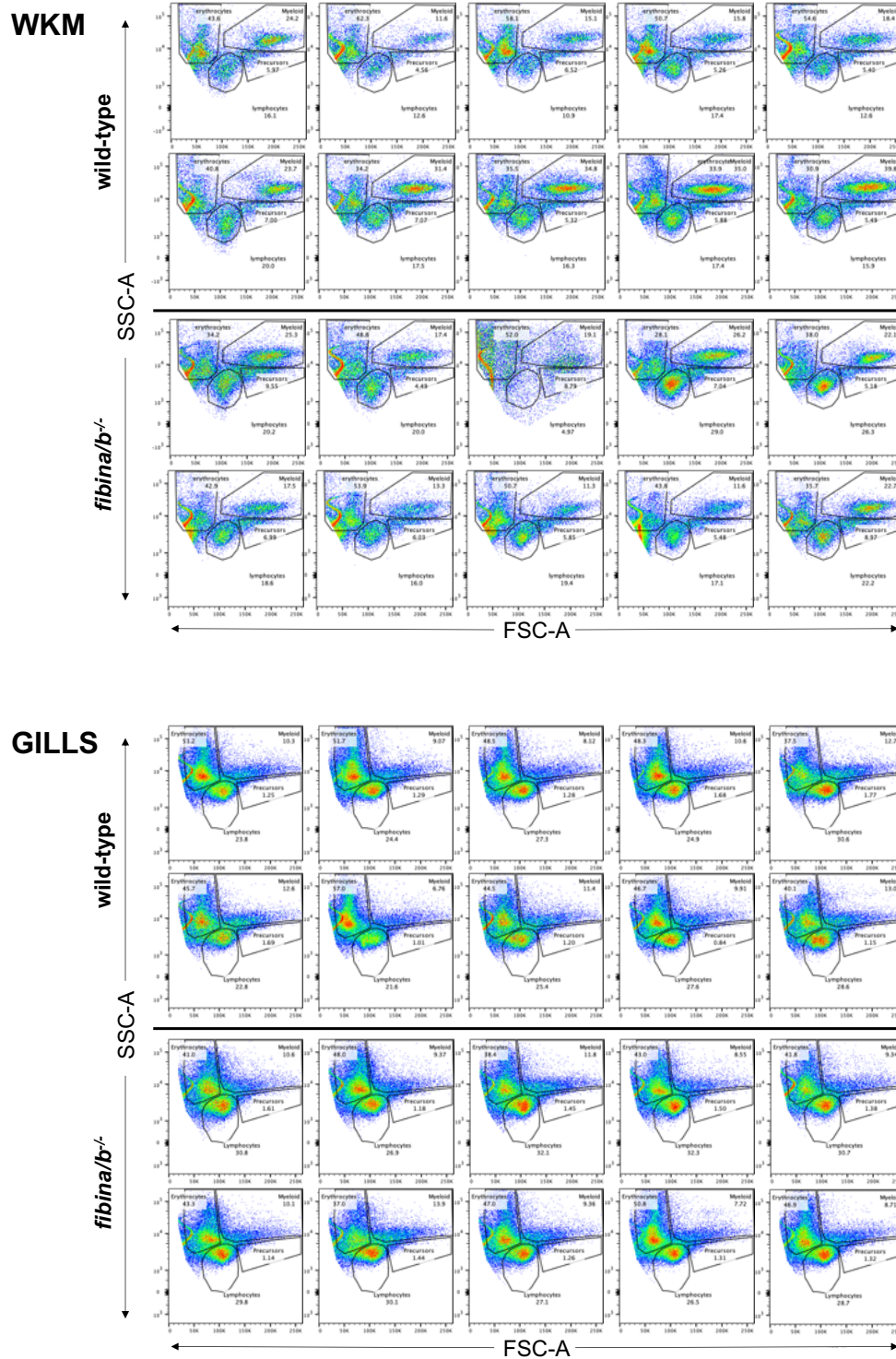
system development (GO:0048731)	97	+	3.22E-02
regulation of protein kinase activity (GO:0045859)	12	+	3.26E-02
positive regulation of cellular component movement (GO:0051272)	5	+	3.27E-02
positive regulation of cell migration (GO:0030335)	5	+	3.27E-02
positive regulation of locomotion (GO:0040017)	5	+	3.27E-02
positive regulation of cell motility (GO:2000147)	5	+	3.27E-02
mesenchyme development (GO:0060485)	4	+	3.40E-02
organelle organization (GO:0006996)	116	+	3.46E-02
positive regulation of cellular metabolic process (GO:0031325)	28	+	3.51E-02
angiogenesis (GO:0001525)	9	+	3.63E-02
positive regulation of phosphorylation (GO:0042327)	9	+	3.63E-02
positive regulation of phosphorus metabolic process (GO:0010562)	9	+	3.63E-02
positive regulation of phosphate metabolic process (GO:0045937)	9	+	3.63E-02
glycerophospholipid biosynthetic process (GO:0046474)	3	+	3.74E-02
mitotic cell cycle phase transition (GO:0044772)	8	+	3.75E-02
cell cycle phase transition (GO:0044770)	8	+	3.75E-02
organelle transport along microtubule (GO:0072384)	2	+	3.83E-02
vesicle cytoskeletal trafficking (GO:0099518)	2	+	3.83E-02

vesicle transport along microtubule (GO:0047496)	2	+	3.83E-02
animal organ development (GO:0048513)	71	+	3.87E-02
positive regulation of kinase activity (GO:0033674)	7	+	3.92E-02
negative regulation of response to external stimulus (GO:0032102)	3	+	3.94E-02
negative regulation of chemotaxis (GO:0050922)	3	+	3.94E-02
regulation of cellular component movement (GO:0051270)	11	+	3.97E-02
regulation of cell migration (GO:0030334)	11	+	3.97E-02
regulation of locomotion (GO:0040012)	11	+	3.97E-02
regulation of cell motility (GO:2000145)	11	+	3.97E-02
cell migration (GO:0016477)	19	+	3.98E-02
blood vessel development (GO:0001568)	11	+	3.99E-02
negative regulation of locomotion (GO:0040013)	4	+	4.04E-02
regulation of chemotaxis (GO:0050920)	4	+	4.04E-02
taxis (GO:0042330)	24	+	4.04E-02
chemotaxis (GO:0006935)	24	+	4.04E-02
microtubule cytoskeleton organization involved in mitosis (GO:1902850)	14	+	4.04E-02
macrophage migration (GO:1905517)	2	+	4.15E-02
macrophage chemotaxis (GO:0048246)	2	+	4.15E-02
triglyceride metabolic process (GO:0006641)	2	+	4.18E-02

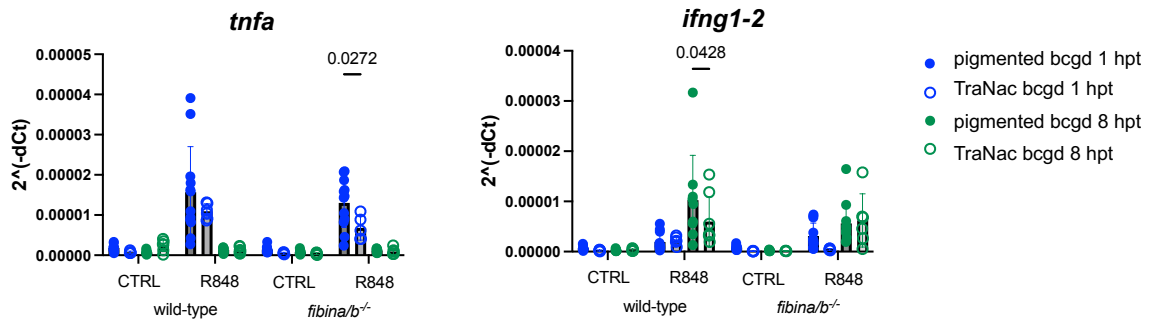
acylglycerol metabolic process (GO:0006639)	2	+	4.18E-02
neutral lipid metabolic process (GO:0006638)	2	+	4.18E-02
myeloid leukocyte activation (GO:0002274)	4	+	4.24E-02
establishment of vesicle localization (GO:0051650)	8	+	4.67E-02
vesicle localization (GO:0051648)	8	+	4.67E-02
regulation of protein modification process (GO:0031399)	18	+	4.82E-02
regulation of protein phosphorylation (GO:0001932)	17	+	4.91E-02
peptidyl-lysine modification (GO:0018205)	3	+	4.94E-02

APPENDIX 4: Results of statistical enrichment analysis of genes significantly upregulated in in caudal fin/gill (A) and genes significantly down regulated in caudal fin/gill (B) in *fibina/b*^{-/-} fish compared to wild-type.

Analysis type: Panther enrichment test.



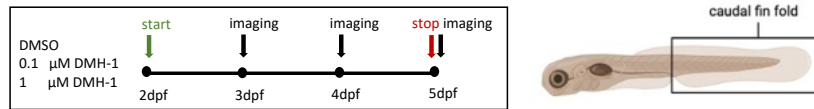
APPENDIX 5: Flow cytometry FSC/SSC plots for 12-month-old wild-type and *fibina/b^{-/-}* gill and WKM tissues.



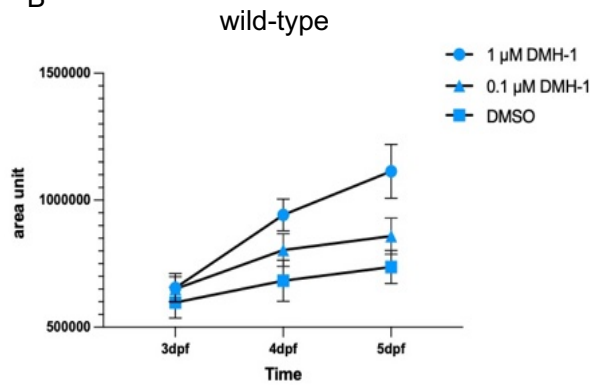
APPENDIX 6: Gene transcript analysis of the gills between wild-type and *fibina/b^{-/-}* fish on either pigmented or TraNac- background.

R848 (5 μ l at 0.5mg/ml) of endotoxin-free water were topically applied to the gills of wild-type and *fibina/b^{-/-}* fish on either pigmented or TraNac background. Gill tissues were collected and processed. Transcript levels of selected genes were measured by qRT-PCR and are shown relative to 18S transcript levels. Each data point represents an individual fish. One-way ANOVA or Welch's ANOVA (for datasets with unequal variances), was performed, followed by Sidak's multiple comparison test.

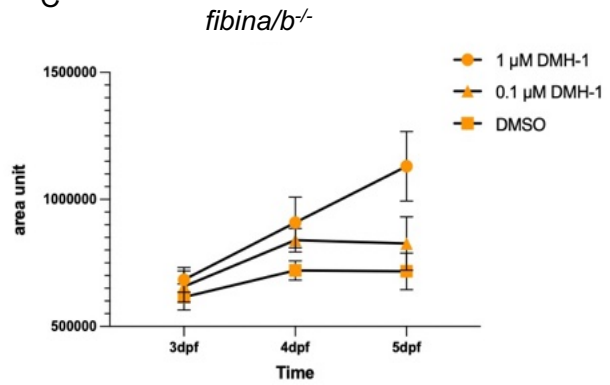
A



B

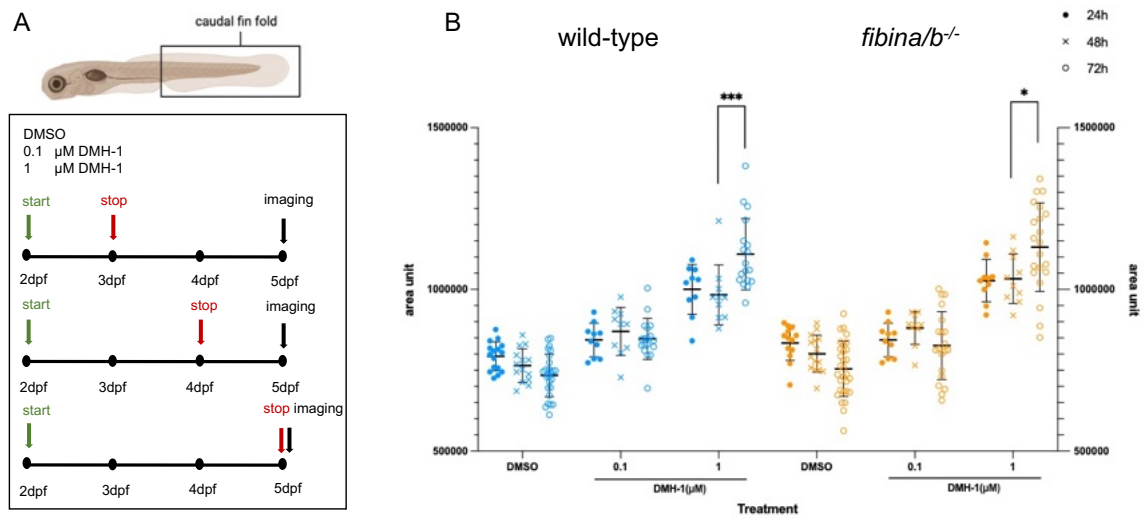


C



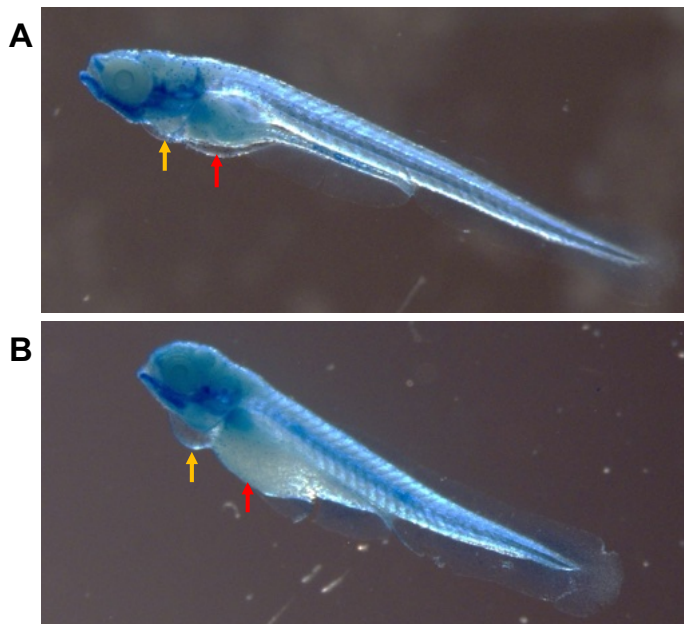
APPENDIX 7: The effect of DMH1 treatment on caudal fin fold area of wild-type and *fibina/b^{-/-}* embryos was cumulative from 3 dpf to 5 dpf.

(A) Treatment timeline and experimental setup. Both wild-type and *fibina/b^{-/-}* embryos were treated from 2 dpf to 5 dpf. Images were collected at 3 dpf, 4 dpf and 5 dpf. Quantification of the fin fold area of wild-type (B) and *fibina/b^{-/-}* fish (C). Data from 2 separate experiments. Figure by Xiangyu.



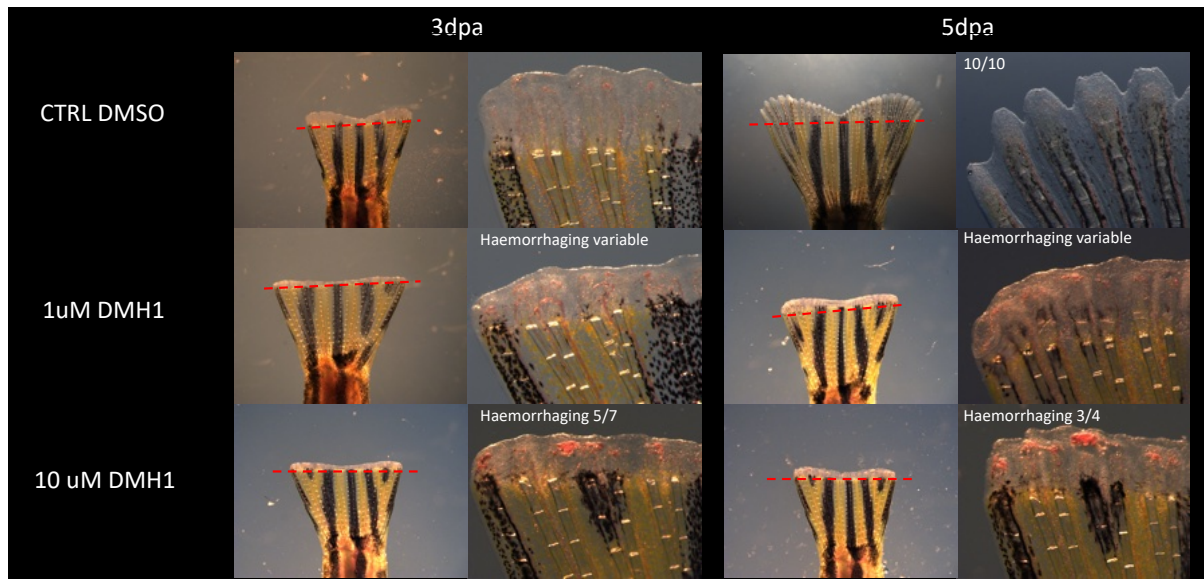
APPENDIX 8: Comparison of caudal fin fold area of wild-type and *fibina/b^{-/-}* embryos treated with DMSO, 0.1 μ M DMH1 or 1 μ M DMH1 for various treatment periods.

(A) Experimental setup with treatment timeline. All embryos were treated from 2 dpf and the treatment duration was divided into three groups: 24 h, 48 h and 72 h. All groups were imaged at 5 dpf. **(B)** quantification of the caudal fin fold area of wild-type and *fibina/b^{-/-}* embryos. Data collected from 3 separate experiments. Each dot represents a single fish. Figure by Xiangyu



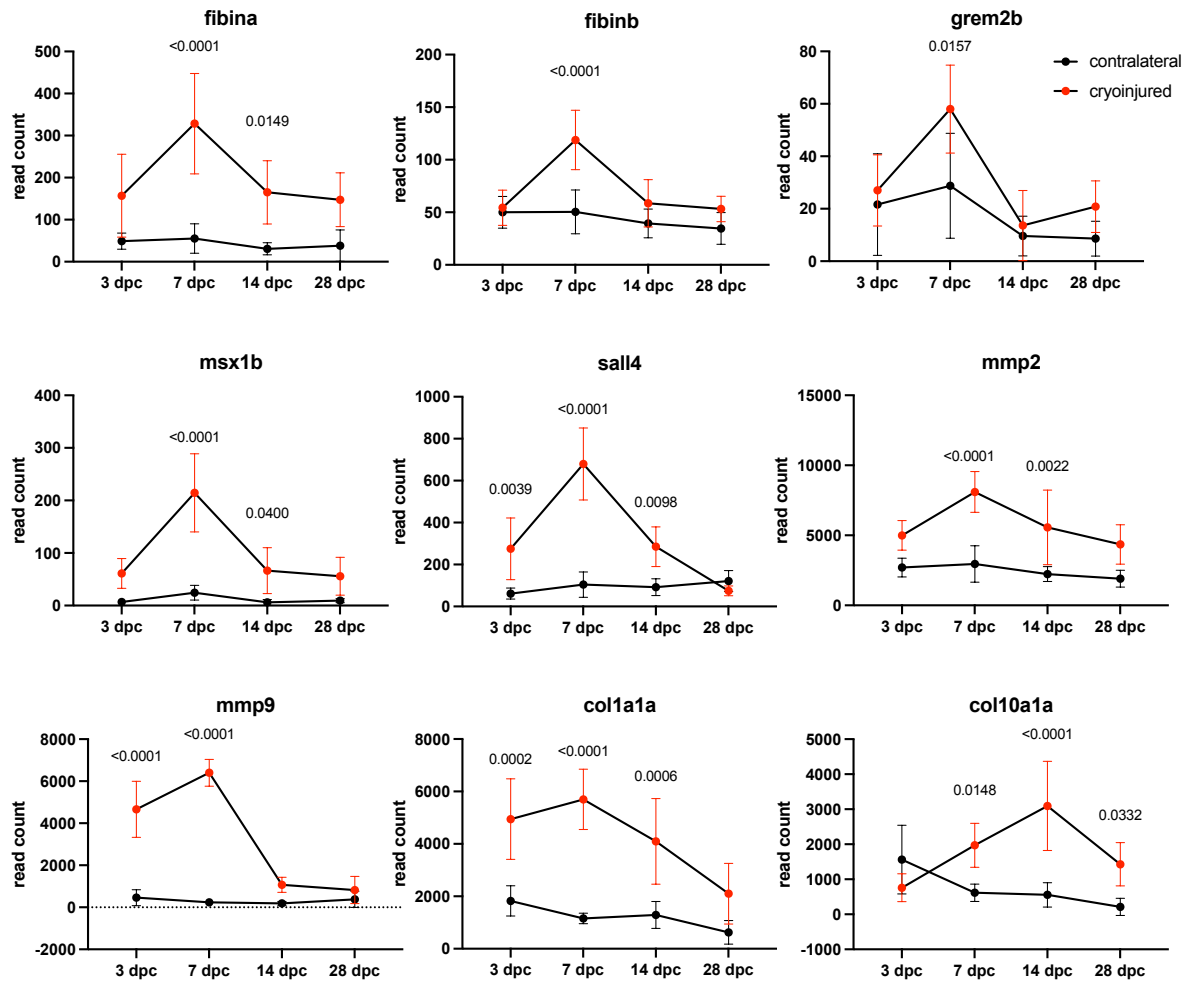
APPENDIX 9: Dorsomorphin treatment at 48 – 72 hpf led to heart oedema formation, yolk sac condensation and reduction in embryo size compared to DMSO treated fish.

Lateral view of Alcian blue stained wild-type zebrafish embryos 5 dpf after treatment at 48 – 72 hpf with A) DMSO and B) 50 μ M dorsomorphin. Yellow arrows point to the heart region and red arrows point to the yolk sac.

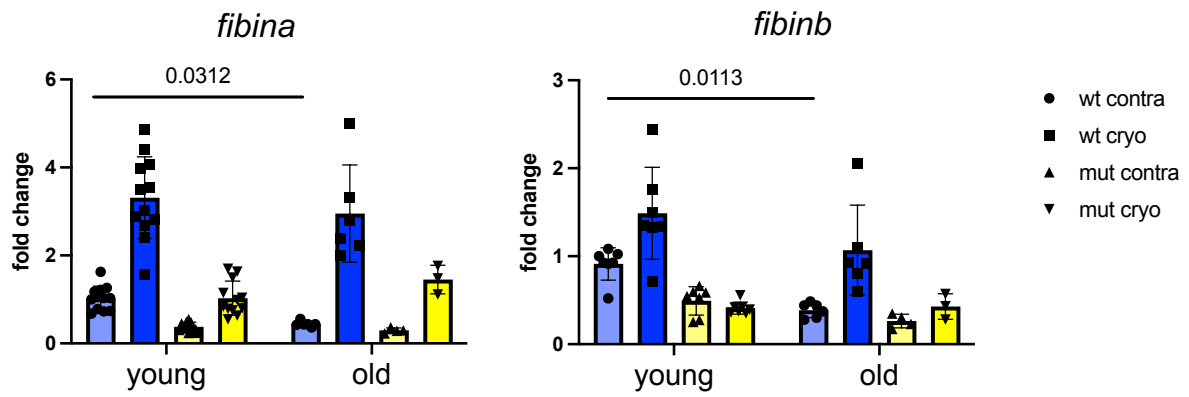


APPENDIX 10: Caudal fin dose-dependent response to DMH1 treatment during regeneration of wild-type fish.

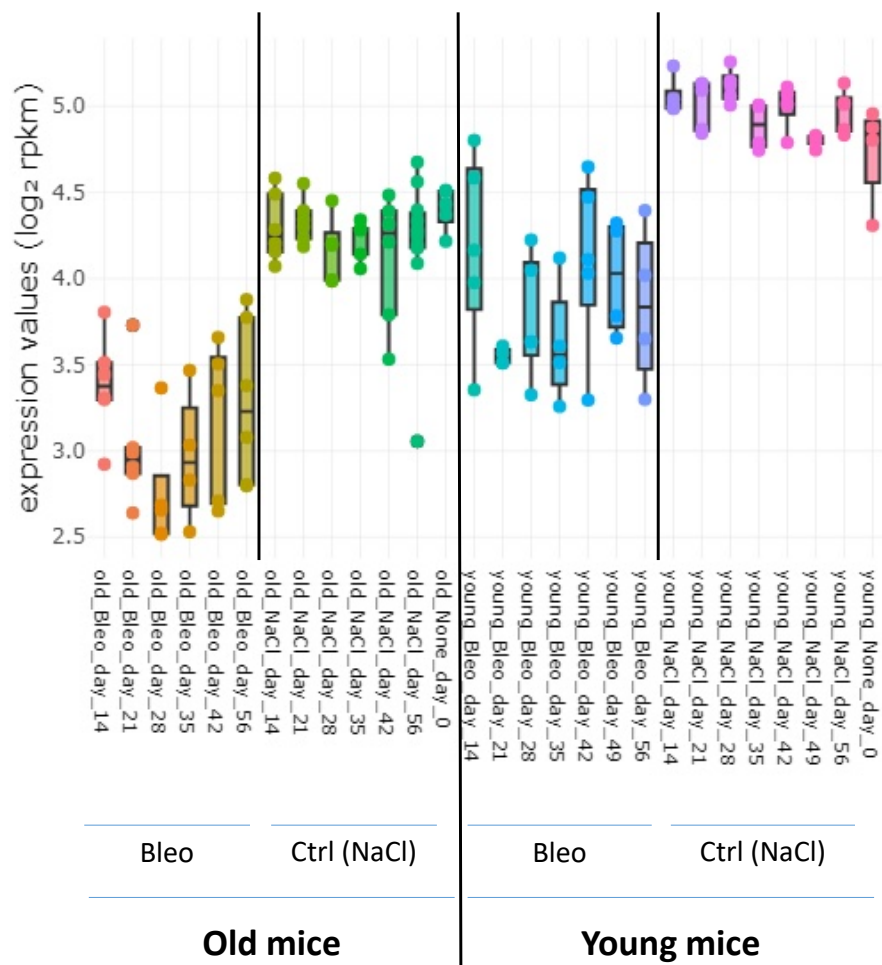
Caudal fin was amputated and fish placed in fresh system water supplemented with DMSO, 1 μ M DMH1 or 10 μ M DMH1 and placed in incubator for up to 5 days. Fins were imaged at 3 dpa and 5 dpa.



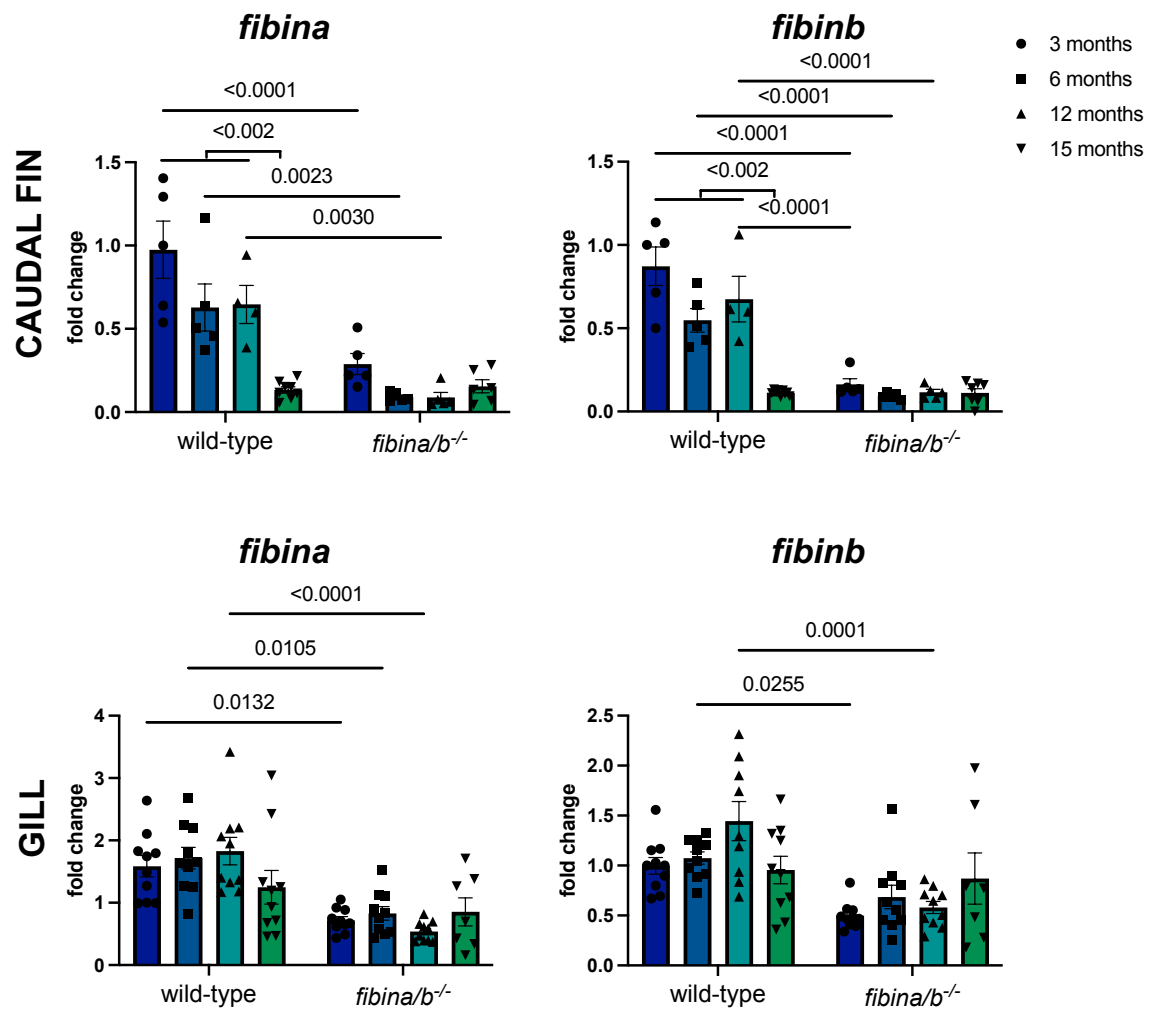
APPENDIX 11: Temporal profiles of selected genes read counts in wild-type zebrafish as determined by RNAseq analysis (graphs generated based on dataset published in (Ramel *et al.*, 2021)).



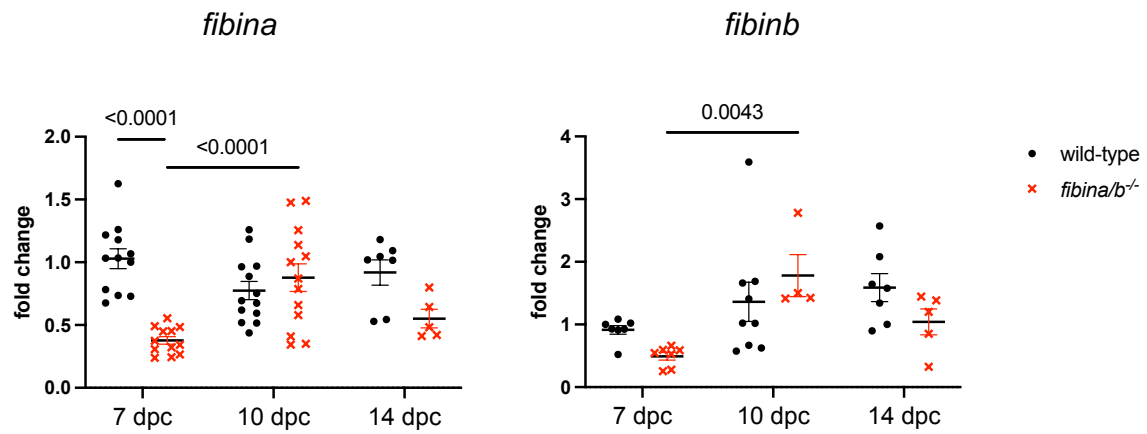
APPENDIX 12: Comparison of *fibina* and *fibinb* transcript levels in the gill tissues between young and old wild-type and *fibina/b^{-/-}* fish at 7 dpc.



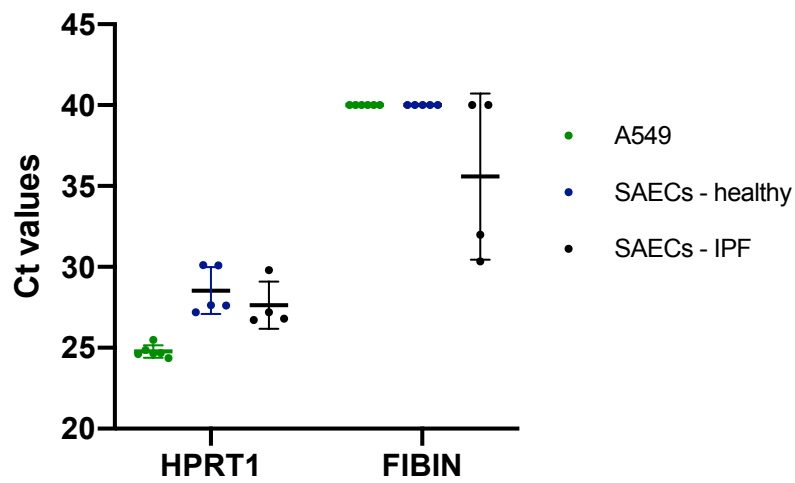
APPENDIX 13: Expression values of *FIBIN* in old (second panel) and young (4th panel) mice. Panel 1 and 3 show *FIBIN* expression upon bleomycin treatment. Data provided by Dr Stephan Klee (Boehringer Ingelheim Pharma).



APPENDIX 14: Transcript levels of *fibina* and *fibinb* in wild type and *fibina/b^{-/-}* fish across 4 ages: 3 – 15 months old.

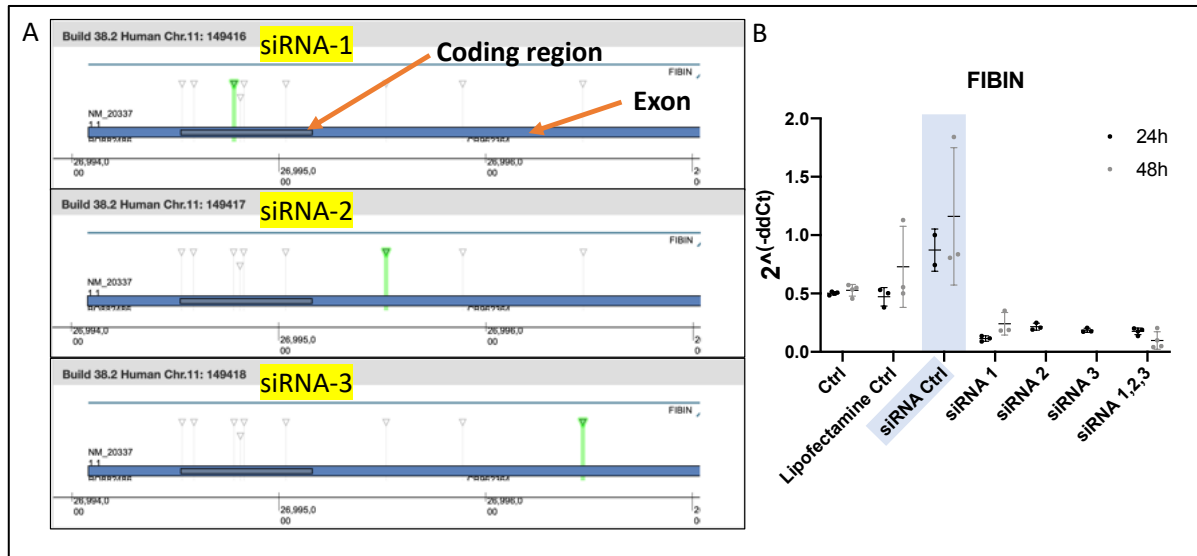


APPENDIX 15: Comparison of *fibina* and *fibinb* transcript levels in the contralateral gill tissue between wild-type and *fibina/b^{-/-}* fish.



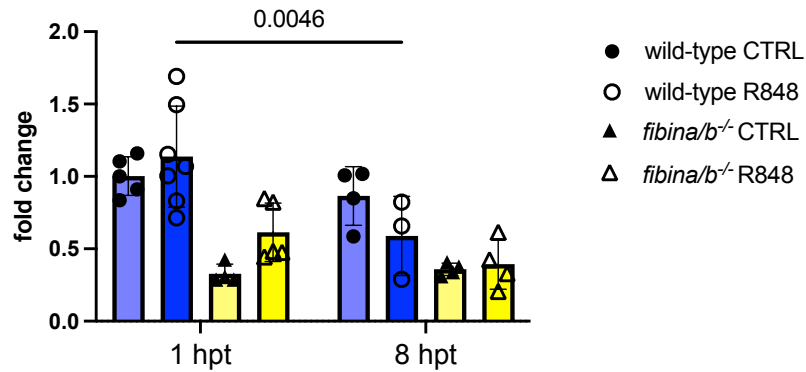
APPENDIX 16: Screening of selected lung epithelial cells for FIBIN expression.

HPRT1 was used as a housekeeping gene. Samples with HPRT1 Ct value ≥ 32 were excluded from the graph (the higher the Ct the lower the expression of the target gene with undetectable levels represented by Ct=40). Average Ct values calculated from technical triplicates; each experiment was done with 3 biological replicates. Ct: cycle threshold; SAECs: primary small airway epithelial cells.



APPENDIX 17: Details about siRNAs for targeted *FIBIN* knockdown in mammalian cell cultures.

(A) Details of *FIBIN*-targeting siRNAs used. (B) siRNA-mediated knockdown in primary IPF LF at 24h and 48h post transfection. Data was normalized to siRNA Ctrl (light blue background). IPF LF: IPF lung fibroblasts.



APPENDIX 19: Analysis of *fibinb* transcript levels in the gills between wild-type and *fibina/b*^{-/-} fish with R848 treatment.

R848 (5 μ l at 0.5mg/ml) of endotoxin-free water were topically applied to the gills of wild-type and *fibina/b*^{-/-} fish on either pigmented or TraNac background. Gill tissues were collected and processed. Transcript levels of selected genes were measured by qRT-PCR and are shown relative to 18S transcript levels. Each data point represents an individual fish. One-way ANOVA was used to calculate statistical significance (p values < 0.05 are shown).