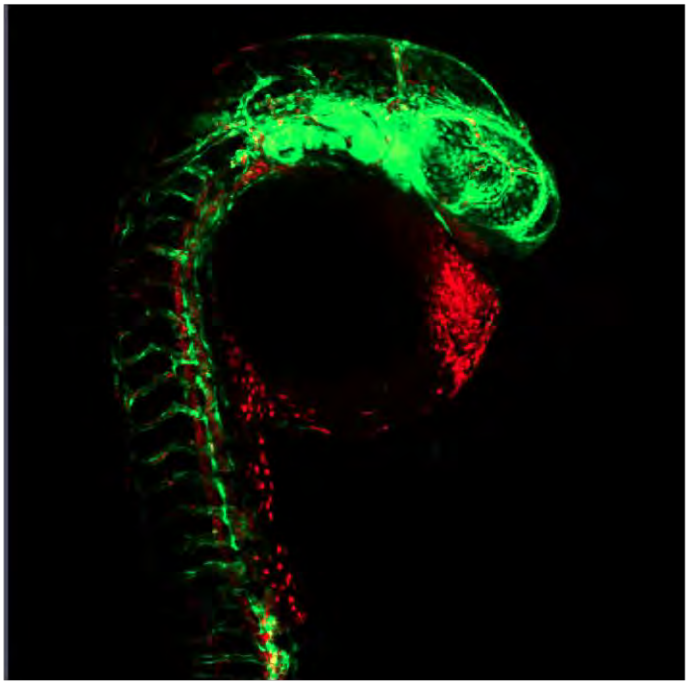
UPPSALA
UNIVERSITET

Characterization of Zebrafish glycosaminoglycan mutants by confocal microscopy



Judith Habicher

Degree project in biology, Master of science (2 years), 2010
Examensarbete i biologi 30 hp till masterexamen, 2010
Biology Education Centre and Department of Physiology and Developmental Biology and Department of Medical Biochemistry and Microbiology, Uppsala University
Supervisor: Katarina Holmborn Garpenstrand

Table of Contents

Abbreviations ____ ***2***

Summary ____ ***3***

Introduction ____ ***4***

The model organism zebrafish (*Danio rerio*) ____ 4

Glycosaminoglycan mutants ____ 5

Angiogenesis ____ 7

Pharyngeal cartilage development ____ 9

Aims ____ 11

Material and Methods ____ ***12***

Zebrafish ____ 12

Morpholino injection ____ 12

Confocal laser scanning microscopy ____ 13

Genotyping ____ 13

Results and Discussion ____ ***14***

Genotyping ____ 14

Confocal microscopy ____ 15

Angiogenesis in hi307 and hi954 mutants ____ 18

Pharyngeal cartilage development in hi307 and hi954 mutants ____ 22

Conclusion ____ ***27***

Acknowledgements ____ ***28***

References ____ ***29***

Abbreviations

B	basihyal
BMP	Bone morphogenic protein
CH	Ceratohyal
CS	Chondroitin sulphate
DA	Dorsal aorta
DLAV	Dorsal longitudinal anastomotic vessel
DLL4	Delta-like 4
DNA	Deoxyribonucleic acid
dpf	Days post fertilization
ECM	Extracellular matrix
EXT2	Exostosin 2
EXTL3	Exostosin-like 3
FLK1	Fetal liver kinase 1
FLT1	Fms-like tyrosine kinase 1
GAG	Glycosaminoglycan
GFP	Green fluorescent protein
H	Hyosymplectic
HM	Hyomandibular
hpf	Hours post fertilization
HS	Heparan sulphate
IJ	Interhyal joint
ISV	Intersegmental vessel
M	Meckel's cartilage
MO	Morpholino
P	Palatoquadrate
PA	Pharyngeal arch
PG	Proteoglycan
PI3K	Phosphoinositide-3-kinase
PTU	1-phenyl-2-thiourea
RNA	Ribonucleic acid
SY	Symplectic region
TGF	Transforming growth factor
VEGF	Vascular endothelial growth factor
WT	Wild-type

Summary

The zebrafish (*danio rerio*) has emerged over the last decades as a great model system to study developmental biology since its genome is very closely related to other vertebrates and human. The embryos are very easy to handle due to their size and they develop extrauterine in a transparent chorion. In this study we want to examine two main developmental processes, angiogenesis (the formation of new blood vessels from pre-existing ones) and pharyngeal cartilage development. We aim for a better understanding of how proteoglycans influence signalling pathways.

The two mutants used in this study, hi307 and hi954, lack glucoronysyl-transferase and UDP-glucuronic acid decarboxylase, respectively. These two enzymes are necessary for the biosynthesis of heparan sulphate (HS) and chondroitin sulphate (CS). Interactions with HS have been shown to be important for many signalling molecules in developmental processes, like vascular endothelial growth factors (VEGFs), transforming growth factors (TGFs) and bone morphogenic proteins (BMPs).

Optimizing the technique of confocal laser scanning microscope for *in vivo* studies on the zebrafish made it a strong tool to follow the embryonic development. The sprouting of the blood vessels in the process of angiogenesis did not appear to have a striking phenotype in neither of the mutants compared to the wild-type. The slight delay of the sprouts and the incomplete closure of the dorsal longitudinal anastomotic vessel (DLAV) need to be further investigated. The pharyngeal cartilage on the other hand, appeared in both mutants lacking HS, as thick and short elements, while the wild-type forms long and thin structures. The wild-type chondrocytes become oval in shape and line up to form long stacks and intercalate, whereas the chondrocytes in the mutants stay small and round-shaped and completely disorganized. The two proteoglycans HS and CS were shown to disrupt developmental processes but additional research is required to specify the principle of action.

Introduction

The model organism zebrafish (Danio rerio)

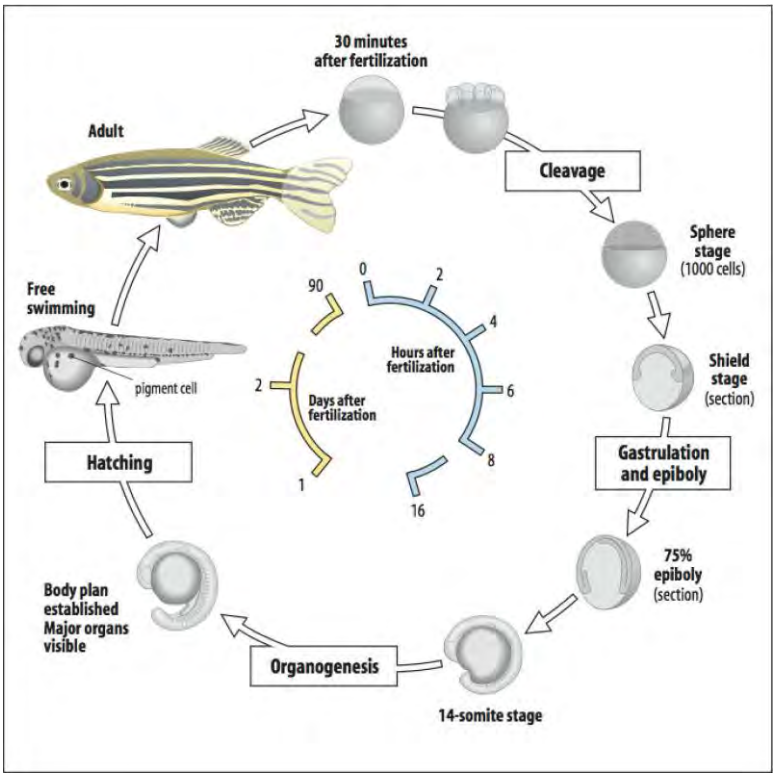


Figure 1: Life cycle of the Zebrafish *Danio rerio* (Adapted from Lewis Wolpert et al. Principles of Development, Third Edition)

Zebrafish is a model organism suitable for all studies in developmental biology mainly because of its extrauterine development in a transparent chorion, which simplifies observation and manipulation of the embryo. The embryos are with a diameter of about 0.7 mm relatively big. They first develop inside a transparent eggshell before they hatch between 48 and 72 hours post fertilization (hpf) (Kimmel et al. 1995). The first heartbeat occurs 26 hpf and it takes three days for the embryo to develop into a fish. The full life cycle is about three months (Fig.1) and the adults reach a size of about four centimetres. Zebrafish are easy to breed in large numbers and inexpensive to maintain in a laboratory system.

The zebrafish shares numerous genetic similarities with human, it has 25 chromosome pairs and many genes are closely related, often having the same or similar role in development, body function or disease (Freeman et al. 2007). Several methods are available for functional manipulation of zebrafish embryos. DNA, RNA and antisense morpholine oligonucleotides (morpholinos; MO) can easily be injected at the one cell stage allowing gain-of-function as well as loss-of-function studies. MOs are highly stable synthetic oligonucleotides. They are used to knock down genes by binding their complementary RNA sequence. MOs effectively block translation or splice sites of target genes for up to five days after injection. In addition genetic mutant lines from forward genetic screens and transgenic lines are available from stock centres.

Glycosaminoglycan mutants

In this study we use two different mutants, which both carry mutations in genes involved in the biosynthesis of heparan sulphate (HS) and chondroitin sulphate (CS) proteoglycans. Proteoglycans consist of long, unbranched polysaccharides (glycosaminoglycan, GAGs) attached to a core protein (Prydz and Dalen 2000). HS and CS differ with regard to the composition of the polysaccharide backbone but are similar in the way that they are both highly negatively charged due to abundant sulphation. Synthesis takes place in the Golgi apparatus. The first part of the chain, the linkage region, contains a tetrasaccharide formed by one xylose, two galactose and one glucuronic acid residue. The fifth saccharide determines weather the chain becomes of HS or CS origin (Fig.2) (Prydz and Dalen 2000). The attachment of a N-acetylgalactoseamine residue to the linkage region destines the chain to be of CS origin while the attachment of a N-acetylglucosamine forms a GAG chain of HS origin. Subsequently, polymerisation of the chain takes place attaching repeating disaccharide units (glucuronic acid/N-acetylglucoseamine for HS and glucuronic acid/N-acetylgalactosamine for CS) to form the backbone of the chain. Finally sulphotransferases add sulphate groups at different positions of the chain.

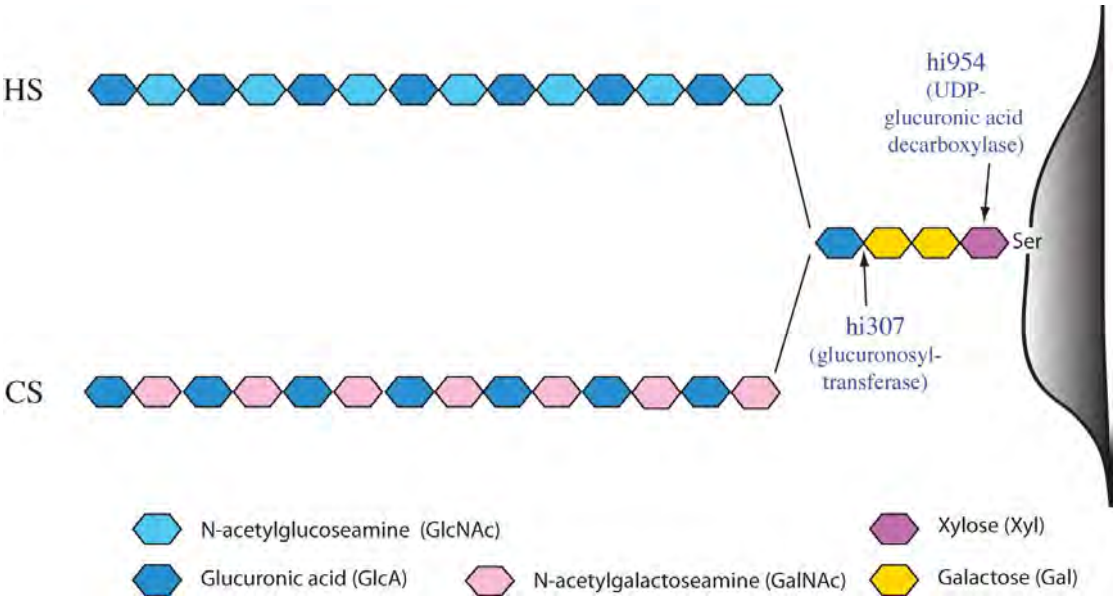


Figure 2: Biosynthesis of Heparan and Chondroitin Sulphate. Hi307 and hi954 are the two zebrafish mutants used in this study and the two arrows point out where the two enzymes glucuruonosyl-transferase and UDP-glucuronic acid decarboxylase, respectively, act. (adapted from Katarina Holmborn Garpenstrand)

Proteoglycans are produced in almost all cells and either secreted into the extracellular matrix, inserted into the plasma membrane or stored in secretory granules (Gandhi and Mancera 2008). Due to their abundant sulphation GAG chains of HS or CS origin are able to bind to many different ligands like growth factors and enzymes (Kreuger et al. 2006). Therefore they play important roles in e.g. signalling pathways of developmental processes.

The two mutants characterised in this study, hi307 and hi954, were found in a large insertional retroviral mutagenesis screen, where genes essential for embryonic development were identified (Golling et al. 2002). The hi307 insert is in the 5'UTR of the beta-1,3-glucuronyltransferase-3-like gene (Golling et al. 2002). The hi307 mutant shows a phenotype after 5 days post fertilization (dpf) (Fig.3). The head is slightly

smaller and rounded, which appears due to the malformation of the jaw, which doesn't extend anterior as compared to wild-type. In addition it displays shorter pectoral fins. RT-PCR showed a 99% reduction of the glucuronosyl-transferase transcript (Golling et al. 2002). Since the enzyme is essential for the production of the linkage region of the polysaccharide (Fig.2), both HS and CS biosynthesis should be affected by this mutation.

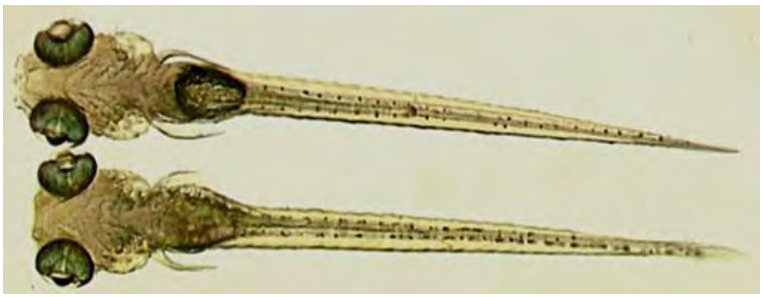


Figure 3: A ventral view of ebrafish at the age of 5dpf. Upper fish is a wild-type, fish below is a hi307 mutants (Golling et al. 2002)

The second mutant used in this study, hi954, carries the retroviral insertion in the 5'UTR of the UDP-glucuronic acid decarboxylase-1 gene (Golling et al. 2002). Also this enzyme acts in the linkage region of the polysaccharide and should affect both HS and CS biosynthesis. No transcript was detected by RT-PCR indicating a complete knock-down of the gene (Golling et al. 2002). The phenotype is similar to the hi307 mutant with a rounded and smaller head, which looks blockheaded since the jaw does not extend properly, as well as shorter pectoral fins (Fig.4).

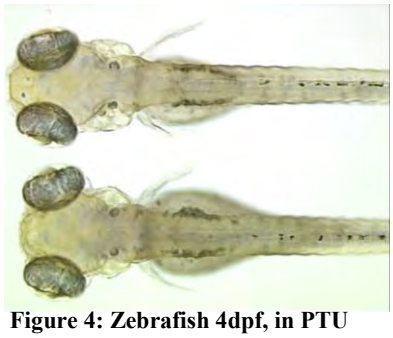


Figure 4: Zebrafish 4dpf, in PTU (treatment to prevent pigmentation), upper fish wild-type, fish below hi954 (Golling et al. 2002)

Since both mutants are relatively similar in their phenotype and both genes act in the early steps of the GAG biosynthetic pathway, it is expected that the levels of HS and CS in the mutants are similar. Quantification of HS and CS content in the mutants show that there is a 50% reduction of HS produced in both of the mutants while CS production is more heavily struck with a reduction of 90-95% (unpublished data by Katarina Holmborn Garpenstrand).

The deficiency in HS and CS leads to defective binding of many signalling molecules important for developmental processes, which has major effects on the embryo. In this study we focus on two main developmental processes in the zebrafish, angiogenesis and cartilage development.

Angiogenesis

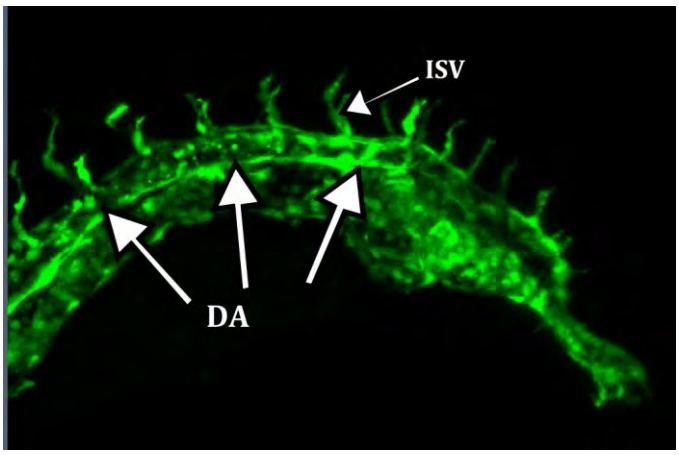


Figure 5: Sprouting angiogenesis on a zebrafish at an age of 24 hpf; DA= dorsal aorta, ISV= intersegmental vessel

Angiogenesis is the formation of blood vessels out of pre-existing vessels (Herbert et al. 2009). This stands in contrast to vasculogenesis, where blood vessels are formed *de novo* out of blood islands. There are three main mechanisms of angiogenesis: sprouting angiogenesis, intussusceptive angiogenesis and looping angiogenesis (Kilarski and Gerwins 2009).

Intussusceptive angiogenesis is the formation of a new vessel by splitting of an existing vessel. Looping angiogenesis was recently described as a biomechanical process in adult angiogenesis (Kilarski and Gerwins 2009). Sprouting angiogenesis, the more common form, occurs when a new vessel grows out from an existing one, lead by a so-called tip cell and followed by proliferating endothelial cells forming the stalk. The new vessel needs to merge with another sprout or vessel to form a new functional blood vessel.

The vascular system starts to form very early in the zebrafish embryo. At the ten-somite stage, which corresponds to 14 hpf under normal conditions at 28.5°C and 5-10 embryos/ml (Kimmel et al. 1995), individual angioblasts begin to migrate medially and form the dorsal aorta (DA) (Gering and Patient 2005). The cardinal vein is formed by selective sprouting from the aorta (Herbert et al. 2009). These vessel formations run from anterior to posterior. The first angiogenic sprouting occurs around the 21-somite-stage (19.5 hpf) from the dorsal aorta. Intersegmental vessels (ISV) sprout to the dorsal side and join to form parallel to the dorsal aorta a new vessel, the dorsal longitudinal anastomotic vessel (DLAV) (Fig.5). Every sprout occurs exactly in between the somites. The process of guidance is finely regulated in order to achieve the exact pattern of the blood vessels (Eichmann et al. 2005). The heart starts to beat around 26 hpf and blood flows through the circulatory system.

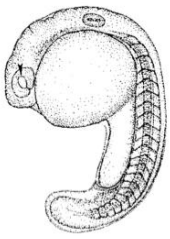


Figure 6: Zebrafish embryo at the 21-somite stage (Kimmel, Ballard et al. 1995)

Angiogenesis is a complex dynamic process with many signalling pathways involved, including signals like BMP (bone morphogenic protein), TGF-β (transforming growth factor-beta), Wnt, Notch and VEGF (vascular endothelial growth factors). Pro- and anti-angiogenic factors are involved and concentration dependent induction or

inhibition occurs. The main signalling factor in relation to vascular biology is the family of VEGFs. During embryonic development, in the process of wound healing and in tumour growth, where blood vessels are formed to provide the fast replicating cells with additional oxygen and nutrients, VEGF plays a key role (Olsson et al. 2006).

The angiogenic sprouting starts with an endothelial cell migrating out of the blood vessel becoming a tip cell. The surrounding tissues and extracellular matrix are prepared for the invasion through signalling molecules. A distinct selection of the tip cell is needed in order for the sprout to develop. If not one specific tip cell is growing out, the whole blood vessel would extend in diameter instead of a new vessel growing out (Gerhardt 2008). The tip cell leads the way through the surrounding tissue or matrix by extending filopodia following the VEGF gradient. VEGFs are directly or indirectly influencing all steps of angiogenesis: the preparation of the external tissue, the selection of the leading tip cell, migration, guidance and proliferation of the endothelial cells forming the stalk. VEGFs act by binding to tyrosine kinase receptors like FLT-1 (FMS-like tyrosine kinase) and FLK-1 (fetal liver kinase), causing dimerization. VEGF-A, a member of the VEGF-family, induces among other things an upregulation of migration and mitosis in endothelial cells. Other growth factors involved are the fibroblast growth factors (FGFs) and TGFs. They all induce additional signalling pathways like PI3K (phosphoinositide-3-kinase) and Smad signalling. VEGF also induces DLL4 (delta-like 4), which functions to pattern the endothelial population into tip and stalk cells (Gerhardt 2008). The endothelial cells following the tip cell do not adopt the tip cell phenotype but rather form a stalk in the sprouting process. They are therefore referred to as stalk cells (Gerhardt 2008). Tip cells migrate along the VEGF gradient, whereas the stalk cells proliferate in a polarized fashion to supply further endothelial cells (Gerhardt 2008).

In order to see and follow the blood vessel formation in zebrafish the gene for the retroviral green fluorescent protein (GFP) was introduced under the zebrafish fli1 promoter, a known endothelial cell marker in mouse. In the fli1:GFP transgenic zebrafish embryos GFP is expressed in all endothelial cells during vascular development (Lawson and Weinstein 2002) and the expression is persistent until day seven post-fertilization. This marker allows us to follow vascular development in the embryo by using fluorescent microscopy, in this study in particular confocal laser scanning microscopy.

Many studies have been conducted to understand what guides angiogenesis, but still there is a lack of understanding how the individual signals act together (Eichmann et al. 2005). In this study we focus on selected key regulators of angiogenesis, the proteoglycans, to study their influence on the sprouting mechanism of angiogenesis. HS proteoglycans are known regulators of angiogenesis and have been shown to bind i.e. VEGF and FGF2. By binding VEGF, HS proteoglycans are involved in the stabilization and shaping of growth factor and morphogen gradients allowing blood vessel sprouting to occur (Stringer 2006). Both mutants in this study lack HS to a great extend and we expect therefore malformed or disrupted angiogenic sprouting. The role of CS in angiogenesis has not yet been well defined. Time-lapse imaging on the confocal microscope was conducted in order to better understand the impact of both HS and CS proteoglycans on angiogenesis *in vivo*.

Pharyngeal cartilage development

Cartilage is a mesenchymal tissue including two different cell types: chondrocytes and perichondrial cells (Kimmel et al. 1998). Perichondrial cells form the so-called perichondrium and surround the chondrocytes, which produce and maintain the extracellular matrix (ECM). The ECM consists mainly of collagen, elastin fibers and proteoglycans (PGs). There are three different types of cartilage: elastic and hyaline cartilage and fibrocartilage, which are classified according to the relative amounts of the three ECM components. The hyaline cartilage is predominately composed of PGs carrying HS and CS, therefore the type we look closer at.

The vertebrate head skeleton is segmented along the anterior-posterior axis. It is divided into reiterated segments, including the pharyngeal arches (PA), which support the feeding and gill-breathing structures and the neurocranium, protecting the brain and sensory organs (Schilling and Kimmel 1997). Pharyngeal arch cartilages in zebrafish, like in other higher vertebrates, are largely derived from cranial neural crest cells (Schwend and Ahlgren 2009). Cells migrate ventrally during the first embryonic days. Most distal in the first mandibular arch, the Meckel's cartilage (M) forms the lower jaw. Proximal in the mandibular arch lies the palatoquadrate (P). In the second arch, the hyoid arch, the ceratohyal (CH) lies distal and the hyosymplectic (H) proximal (Fig.7) (Kimmel et al. 1998).

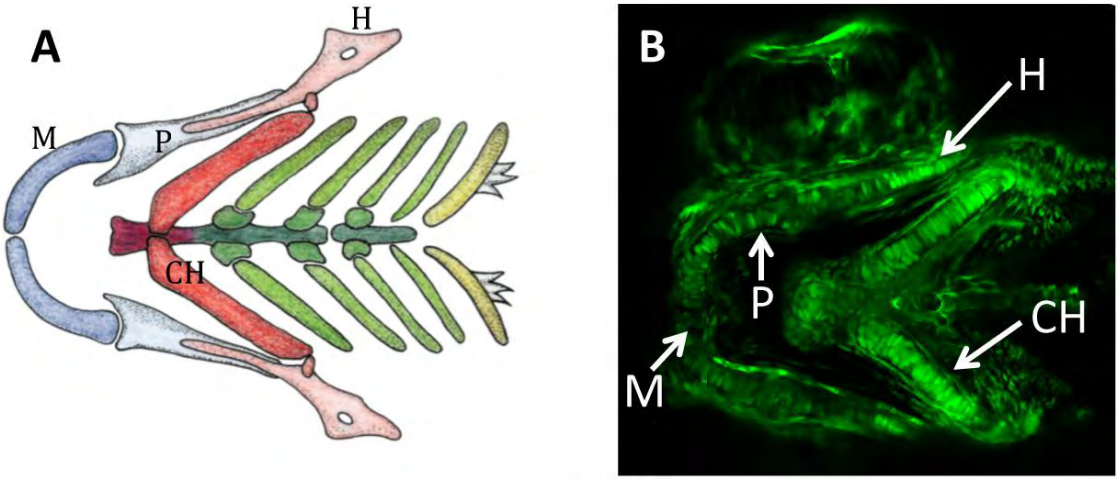


Figure 7: Diagram of pharyngeal cartilage elements in (A) (Kimmel et al. 1998). A ventral view of a 4dpf fli1:GFP zebrafish in (B). Anterior to the left; CH=ceratohyal, H=hyosymplectic, M=Meckel's cartilage, P=palatoquadrate

In cartilage tissues the chondrocytes are mostly arranged as thin single cell layer stacks, although where the cartilage forms larger regions chondrocytes are arranged as mosaic shaped sheets (Fig.8) (Kimmel et al. 1998). The stacks appear first in the symplectic (SY) region of the hyosymplectic and no mitosis could be detected at that time which suggests that the stacking is not due to the process of cell division. Cell rows are formed by orientated cellular intercalations that would elongate the developing SY in the same fashion that oriented intercalations underlie elongation of the notochord (Shih and Keller 1992). This intercalation might predominantly be driven by changes in cell adhesion. The stack building process seems to be polarized; newly added cells are added in

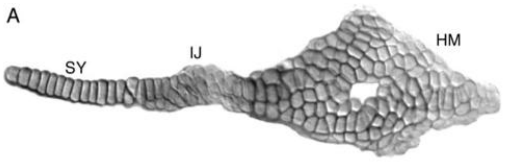


Figure 8: Chondrocyte stacks in the hyosymplectic (H) cartilage; SY=symplectic region of the H, IJ=interhyal joint region of the H, HM=hyomandibular region of the H (Kimmel et al. 1998)

the posterior region and push the stacked cells to the anterior side. The nicely stacked cells have exact two neighbour cells, without taking the perichondrial cells in consideration (Kimmel et al. 1998).

Different zebrafish mutants in jaw and branchial arch development have been described previously (Schilling et al. 1996). Among those mutants *dackel (dak*) and *boxer (box*) are affected in the biosynthesis of HS proteoglycans. *Dak* is mutant for exostosin2 (EXT2) and *box* for exostosin-like 3 (EXTL3), both acting later in the biosynthesis (Fig.9). By the two mutants, d*ak* shows the most severe defects in pharyngeal arch development. All cartilage elements are present but shorter and wider compared to the wild-type (Schilling et al. 1996). Therefore the head skeleton is strongly reduced. *Boxer* shows a less severe phenotype than *dak* but compared to the wild-type the cartilage elements are shorter and wider, single chondrocytes are smaller and disorganized (Schilling et al. 1996).

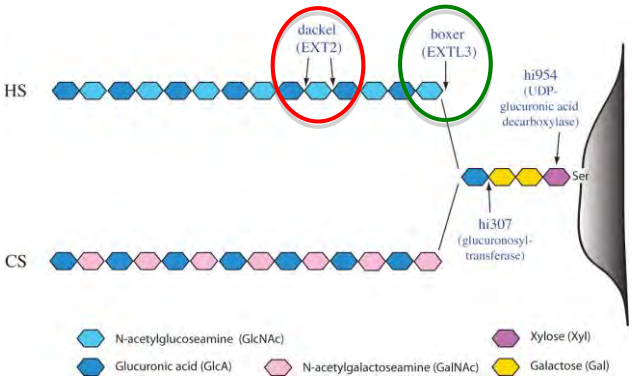


Figure 9: Biosynthesis of HS and CS. The mutant zebrafish *dackel* (mutant for EXT2) is circled in red and the arrows point out where in the GAG elongation the protein acts. The same applies for *boxer* (mutation for EXTL-3) in the green circle.

Recently a new study on *dak* has been published, where it was described to be required for morphogenesis of cartilage but not for early cartilage differentiation (Clement et al. 2008). At 60 hpf a marker for chondrocytes differentiation showed no difference in expression between wild-type and mutant.

The transgenic fli1:GFP zebrafish express GFP not exclusively in endothelial cells, expression was found as well in hematopoietic cells and in the derivatives of cranial neural crest cells (Lawson and Weinstein 2002). This makes the fli1:GFP fish an excellent model for observing the growing cartilage in the head region.

Aims

The first aim of this study is to set up and optimize the confocal microscopy technique to obtain optimal time-lapse images of the zebrafish *in vivo*. The design of the experiment needs to be optimized for the fish to survive and in the same time give strong enough signals to achieve images with a high signal-to-noise ratio.

The second aim of this study is to investigate, by confocal microscopy, the phenotype of the two mutant zebrafish strains hi307 and hi954. Both of these mutants have decreased amounts of HS and CS which are important ligands for many growth factors and therefore crucial in development. The purpose of this project is to better understand how development is affected in a GAG-deficient environment and to make that visible with the technique of confocal microscopy.

The focus lies on two main developmental processes: angiogenesis and the development of the pharyngeal cartilage. Our goal is to examine the cellular development in the zebrafish by creating time-lapse images *in vivo*. This allows us a detailed view of how cells are organizing themselves and what processes are disrupted if the fish has deficient GAG biosynthesis.

Material and Methods

Zebrafish

Zebrafish strains used in this study were the fli1:eGFP transgenic zebrafish expressing GFP under the control of the fli1 promoter (Lawson and Weinstein 2002), the Gata1:dsRed transgenic zebrafish expressing dsRed under the control of the Gata1 promoter (Tsai et al. 1991), as well as the two hi-lines hi307 and hi954, which were isolated from a retroviral insertion screen (Amsterdam et al. 2004). All zebrafish lines are purchased from ZIRC (Zebrafish International Resource Centre).

Zebrafish were kept and bred under constant conditions at a temperature of 28°C in oxygenated water on a day-night-rhythm. From around 7 dpf fish were fed twice a day, once with dry food and once with living food (*Artemia salina*). To produce embryos carrying the mutation of interest and at the same time expressing GFP under the fli1 promoter, mutants were crossed with transgenic fli1:eGFP zebrafish. To identify fish carrying the mutation heterozygous crossing of hi307 and hi954 respectively were performed. Embryos were collected from natural mating and after 4-5 days the phenotype was observed. According to Mendel's law 25% of the clutch should be homozygous mutants showing the phenotype. This confirms both parental animals to be heterozygous. Embryos carrying the fli1:eGFP transgene were sorted out under fluorescent light. With the exception of the transgenes fli1:eGFP and the Gata1:dsRed, adult fish are kept as heterozygous since homozygous fish do not survive.

Morpholino injection

Zebrafish embryos were at the one cell stage injected with a MO against VEGF-A (5'-GTATCAAAATAAACAACCAAGTTCAT-3) using a microinjector. A total concentration of 3 pM MO was injected into each embryo (see below for calculation). The injected fish were kept in an incubator at 28°C for almost 20 hours until they were analysed in the confocal microscope.

The stock concentration of the used MO was 1mM.

2 µl MO
1µl phenol red
7µl H_2O
10 µl

A drop of 0.18 mm diameter was injected.

$V = 4/3\ \pi\ r^3$ (r = 0.09 mm)
V = 3.05nl

Confocal laser scanning microscopy

The study was conducted on a ZEISS CLSM (Confocal Laser Scanning Microscope) 700 with additional heating and CO_2 chamber. Fish were kept in 28°C during the experiment.

For the angiogenesis study the zebrafish were viewed from the lateral side. Fish were taken at the 21-somite-stage and dechorionated with a pair of tweezers. With a glass pipette they were transferred into low melting agarose (1%) containing 1% of Tricane (Tricaine methanesulphonate, TMS, MS-222) to immobilize the fish. The embryos were placed in a drop of agarose in a small petri dish and positioned on their lateral side. Laser light of 488 nm wavelengths was used to illuminate the fish causing the fluorophores in the GFP expressing cells to emit the green light detected. To detect the red light laser light of 555 nm wavelengths was used. Image series were taken under different conditions trying to optimize the system first of all for the embryos to survive.

For the cartilage investigation the head region of the zebrafish was observed from ventral. Fish were taken at around 50 hpf. They were placed into a drop of low melting agarose containing Tricane as described above and positioned ventrally.

Genotyping

The examined zebrafish had to be genotyped to confirm the presence or lack of the mutation. Therefore, zebrafish larvae were lysed in a lysis mix containing 10 mM Tris pH 8, 2 mM EDTA, and 0,2 % Triton X-100. Digestion was carried out at 55°C for 2 hours followed by 5 minutes at 95°C. PCR reactions were performed by denaturing the DNA at 95°C for 5 minutes before amplification for 32 cycles, each cycle consisting of 95°C, 30 s; 56°C, 30 s; 72°C, 1 minute, with a final elongation step at 72°C for 5 minutes.
The following primers were used:
hi307 forward 5'-AAGCGACGAAGAGGACGTTA-3'
and reverse 5'-AAACCCATTACGACGAGCAC-3';
hi954 forward 5'-CCGAAAATAAACTGCCCAGA-3'
and reverse 5'-GACTCGCTTCGGGTTTACAG-3';
ß-actin, forward 5'-CTCTTCCAGCCTTCCTTCCT-3'
and reverse 5'- CTTCTGCATACGGTCAGCAA-3'.
The primers for hi307 and hi954 are flanking the retroviral insert but DNA could not be amplified from any of the mutants using the primers above. This is probably due to the considerable size of the insert. Instead primers for ß-actin were designed and run as a positive control for DNA in the sample. The PCR products were analysed on a 1% agarose-gel.

Results and Discussion

Genotyping

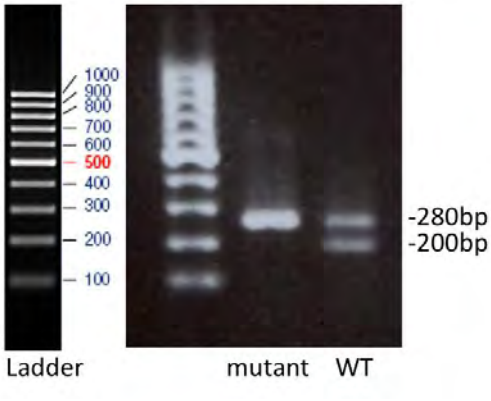


Figure 10: Agarose-gel of genotyping. In the first column of the gel a 100bp-DNA-ladder (Fermentas) is loaded. The second column shows one band at around 280bp. The third column shows two bands, one at around 280 bp and one smaller one around 200 bp.

After lysing the zebrafish and performing the PCR as described earlier, the samples were run on an agarose-gel. A 100bp marker was loaded. As expected we get a band for ß-actin in all the samples and an additional band for hi307 and hi954 respectively for the WT fish. One band on the gel indicates the mutant fish, two the WT.

Confocal microscopy

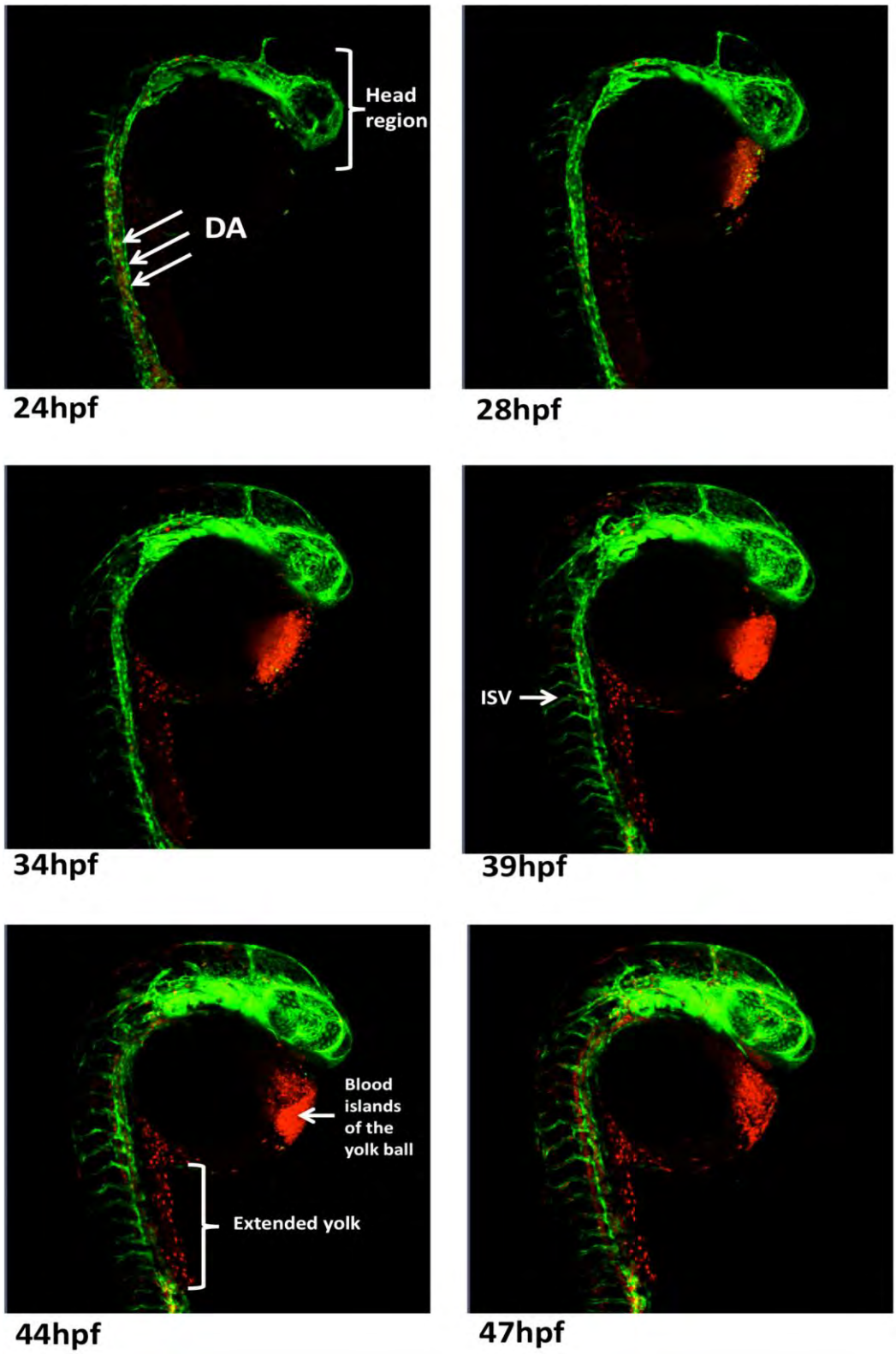


Figure 11: Zebrafish (Fli1:GFP; dsRed): A series of pictures exported from the time-lapse imaging in 10x magnification is shown. Time points are given in hours-post-fertilization (hpf). Erythrocytes are seen in red, endothelial cells in green. At 24 hpf the intersegmental vessels (ISVs) sprout out of the dorsal aorta (DA). Blood islands are seen in the yolk and single blood cells in the extended yolk. At around 2 days post fertilization erythrocytes start to flow through the vessels

In this study our focus was on confocal microscopy and optimizing this method for time-lapse imaging on zebrafish embryos. We cross-bred fli1:GFP and Gata1:dsRed transgenic zebrafish and performed time-lapse imaging with both lasers (488nm and 555nm) used. In figure 9, erythrocytes are shown in red while endothelial cells are shown in green. During vessel development the main blood vessels, the dorsal aorta (DA) and parallel to it the cardinal vein, are formed first. The first angiogenic sprouting occurs from the aorta and the intersegmental vessels (ISVs) grow out in between every somite to form the dorsal longitudinal anastomitic vessel (DLAV) at the dorsal side of the embryo. To generate a pattern of alternating arteries and veins, a second sprouting occurs from the cardinal vein with the subsequent reconnection of the ISVs. By around 28 hpf the blood cells start to flow through the blood vessels. By using the zebrafish transgenes expressing both fli1:GFP and Gata1:dsRed we are able to measure functionality of blood vessels. This will be a useful tool when studying mutants to see if the blood vessels formed are closed and functional.

VEGF is an effective signalling molecule in the guidance mechanism of angiogenic sprouting (Barkefors et al. 2008). VEGF guided sprouting starts around 20 hpf (Fig.12). The sprouting of the ISVs occurs first in an angle of 90° to the DA. After reaching the dorsal side the ISVs sprout horizontally and fuse to form the DLAV. The DLAV is a closed and intact vessel by circa 48 hpf. If VEGFA-MO is injected at the one-cell stage (shown in the right column in Fig.12) it inhibits VEGF and results in disrupted angiogenesis. ISVs sprout out slightly slower, some sprouts are completely missing and the formation of the DLAV is in parts absent. Figure 12 shows that, in contrast to results of other previous studies, ISV formation is not completely inhibited. Most probably this lack of total inhibition is due to a lower amount of MO injected.

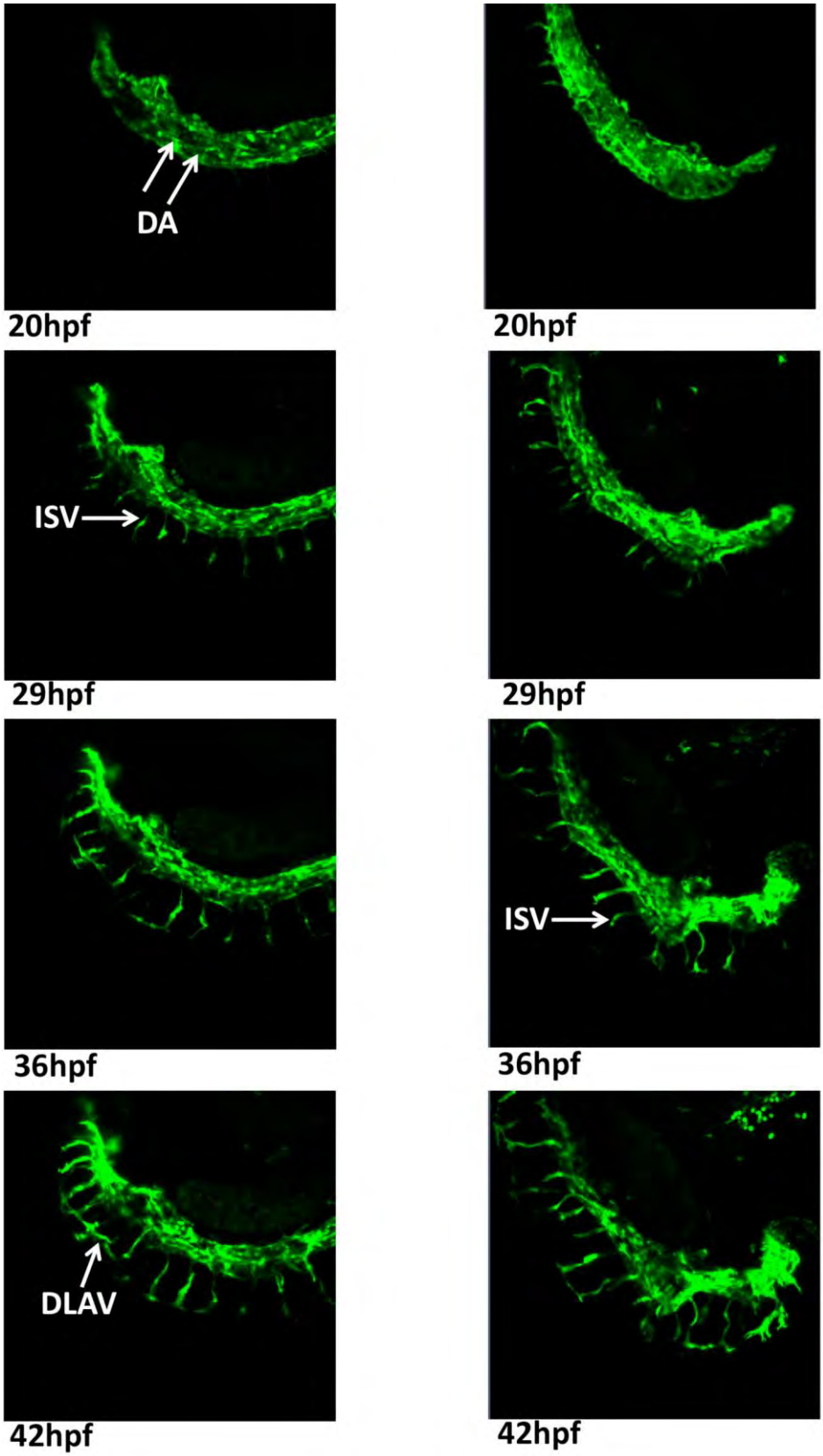


Figure 12: Zebrafish (Fli:GFP) on 20x magnification: In the left column some selected time points of the time-lapse-imaging of a control zebrafish are shown. From the dorsal aorta (DA) intersegmental vessels (ISVs) start to sprout at around 20 hpf. The ISVs sprout dorsally and form parallel to the DA the dorsal lateral anastomotic vessel (DLAV) along the whole body axis. In the right column a VEGFA-MO injected zebrafish is shown. The sprouting occurs at slightly later, not all the ISVs are formed and the fusion of the sprouts to form the DLAV is reduced.

Angiogenesis in hi307 and hi954 mutants

Several VEGFA isoforms interact with heparan sulphate PGs to form gradients in the tissue that serves to guide angiogenic sprouting by induction of directional endothelial tip cell migration (Gerhardt et al. 2003). The role of HS has previously been studied in this context (reviewed by Stringer 2006). Here we focus on the two proteoglycan mutants hi307 and hi954, which have strongly reduced levels of both HS and CS. As a result, we would expect an angiogenic phenotype with a slower growth of sprouts, an incomplete formation of the DLAV and possibly that the newly formed vessels are not closed and functional. This must be seen in comparison to the wild-type (Fig.13). Time-lapse imaging on the two mutants and the wild-type was started at 20 hpf and conducted over 24 hours. Unfortunately both mutant zebrafish display a less striking effect on angiogenic sprouting than expected.

Although a bit slower, the sprouts in the mutant hi307 zebrafish (Fig.14) grow relatively normal and form the DLAV. The mutant hi954 seem to be more affected than hi307 (Fig.15). The beginning of the angiogenic sprouting is not affected but by around 35 hpf the sprouting is definitely behind. After reaching half way the sprouts seem to loose orientation not knowing in which direction to proceed.

It is possible that the weaker phenotype in both mutants appears due to the fact that, even though severely decreased, HS and CS is available and those amounts are sufficient for proper angiogenesis to occur. Alternatively, HS and CS proteoglycans might not be absolutely essential for angiogenesis at this stage of development. The mutant embryos do however not survive for more than around two weeks due to the general effects of the mutations on multiple developmental processes. However, it is not clear if defects in vascular development are the trigger for lethality. Many mutant fish develop oedemas and they often lack swimming bladder, which could be two possible reasons for the early death. By using the Gata1:dsRed transgene, further investigations can be performed observing blood flow and vessel functionality in the mutant zebrafish.

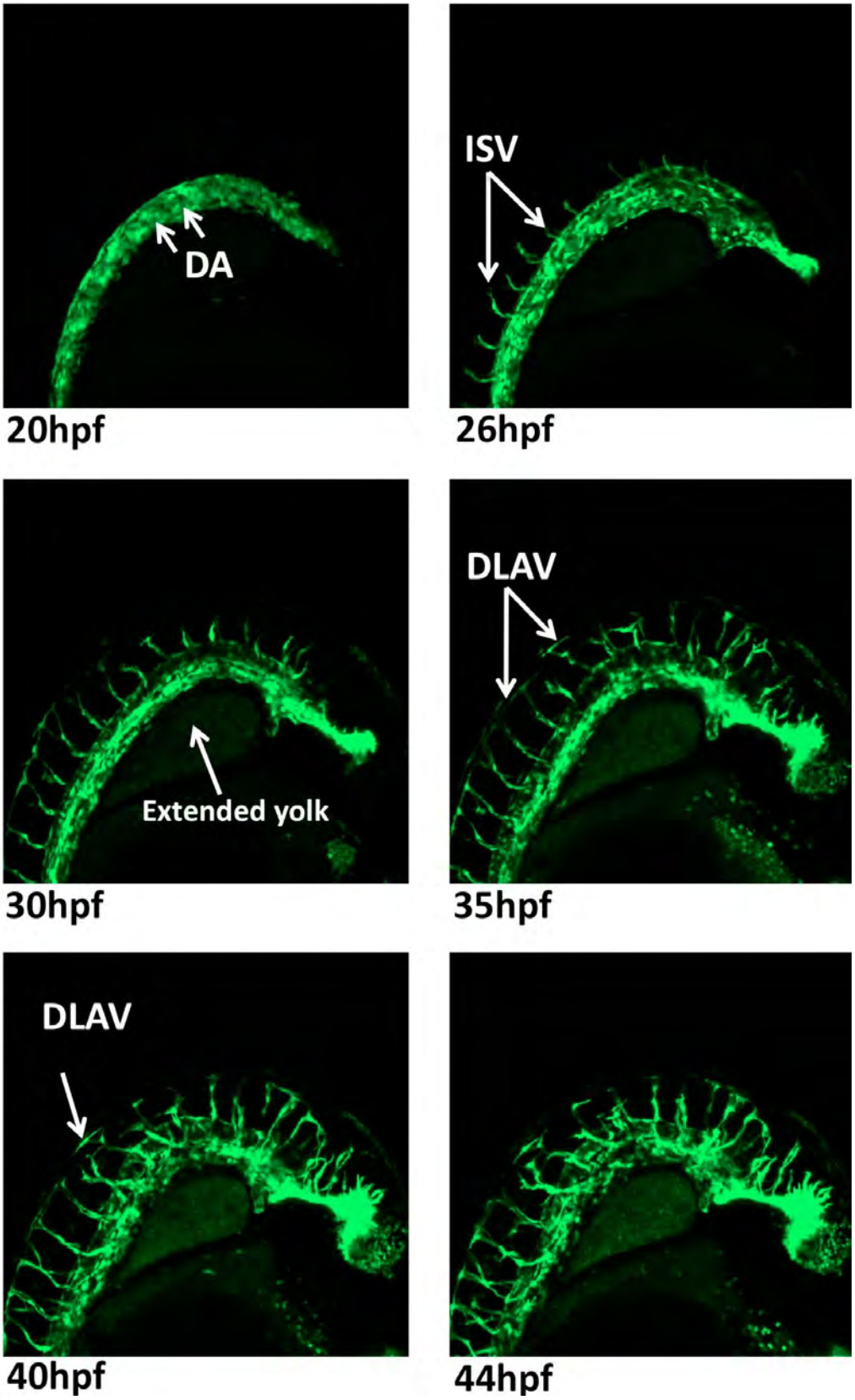


Figure 13: Zebrafish (Fli1:GFP; WT) on a 20x magnification. Angiogenic sprouting on the tail is shown over time (time points indicated in hours post fertilization (hpf)). From the dorsal aorta (DA) intersegmental vessels (ISVs) start to sprout at around 20 hpf. The ISVs sprout dorsally and form parallel to the DA the dorsal lateral anastomotic vessel (DLAV) along the whole body axis.

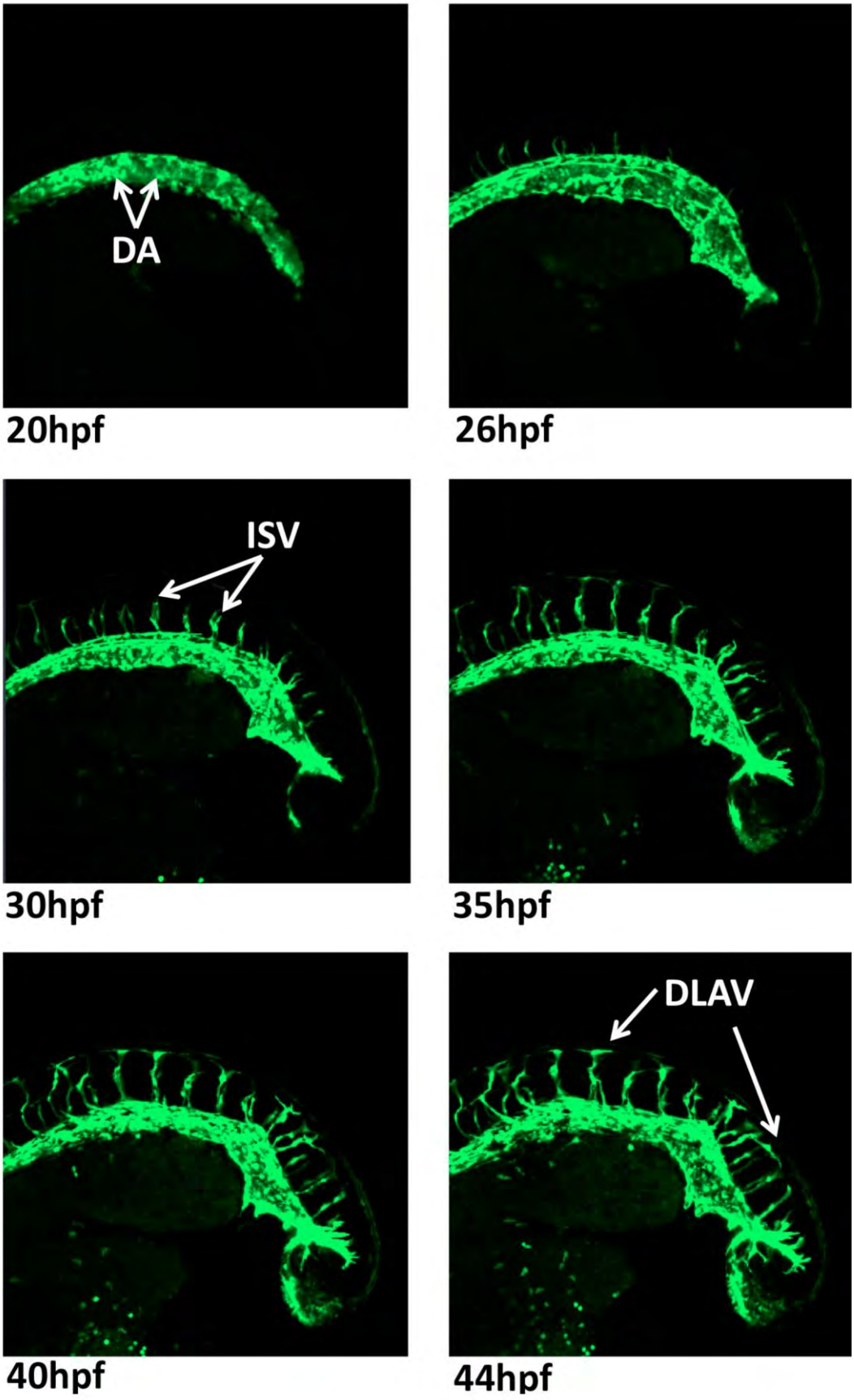


Figure 14: Mutant zebrafish (Fli1:GFP; hi307) on 20x magnification. Intersegmental vessels (ISVs) sprout out form the dorsal aorta (DA) and form the dorsal longitudinal anastomotic vessel (DLAV). This vessel is however in some parts not closed as seen in the WT (Fig.12).

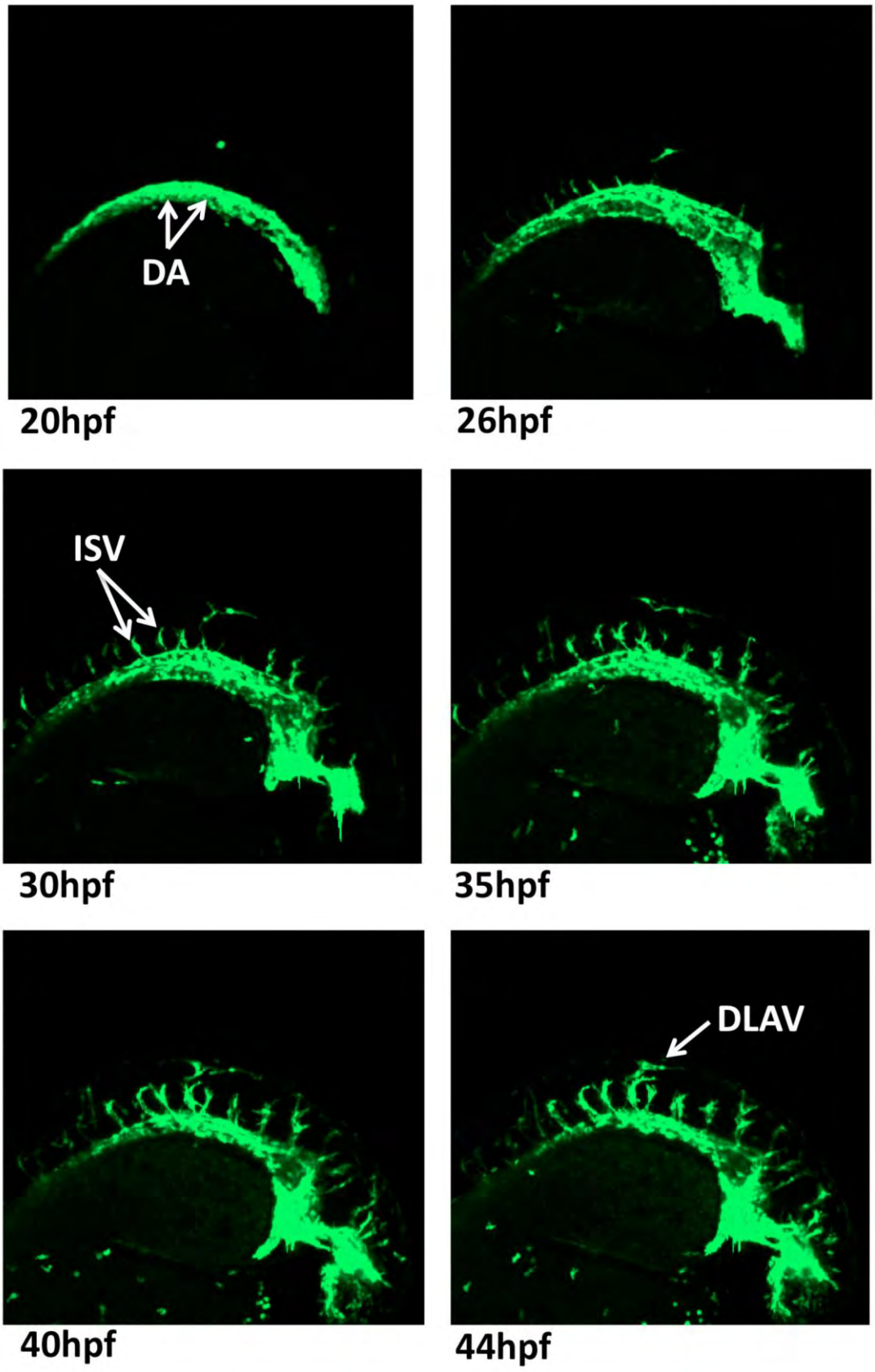


Figure 15: Mutant zebrafish (Fli1:GFP, hi954) on 20 x magnification. The intersegmental vessels (ISVs) start to sprout out normally from the dorsal aorta (DA). Half way they seem to be misguided and they almost form no dorsal longitudinal anastomotic vessel (DLAV) at this time point.

Pharyngeal cartilage development in hi307 and hi954 mutants

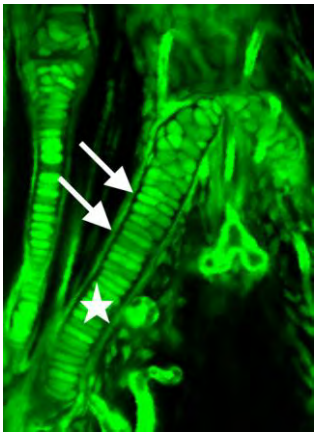


Figure 16: Detailed view on the ceratohyal of the 5dpf zebrafish (Fli1:GFP, WT). The arrows point to the perichondrial cells surrounding the chondrocytes of the ceratohyal. The star indicates the oval shaped and lined up chondrocytes in the ceratohyal. (up is anterior)

Time-lapse imaging of the development of the pharyngeal cartilage in a wild-type zebrafish was conducted from 2.5 to 5 dpf (Fig.17). The first cartilage elements stretching out anteriorly are the becoming upper jaw. The distal element of the first arch, Meckel's cartilage, appears first and moves anteriorly. Shortly after, the palatoquadrate (first arch), the hyosymplectic and the ceratohyal (both second arch) can be seen. During normal cartilage development chondrocytes begin as rounded cells and become oval shaped, form stacks and intercalate (Fig.16, star). This stacking starts around 3 dpf and progresses until about 5 dpf. Due to this, the entire cartilage stretches out anteriorly. The perichondial cells line up and surround the chondrocytes (Fig.16, arrow).

The two zebrafish mutants hi307 and hi954 display the most deviant phenotype in their head (Fig.3 and Fig.4). In particular the pharyngeal cartilage elements are affected (Fig.18). In the hi307 mutant the chondrocytes persist as small and rounded cells (red star in Fig.20). These cells fail to stack and intercalate, which leads to thicker and shorter cartilage structures and gives rise to the small and rounded head. In contrast to the wild-type, the perichondrial cells do not surround the chondrocytes. This makes the perichondrium appear random and disorganised (arrows Fig.20).

The hi307 and hi954 mutants display very similar phenotypes (Fig.18, Fig.19 and Fig.20). The phenotype of the UDP-glucuronic acid decarboxylase mutation was recently published (Eames et al. 2010). Their described *mov*^{w60} zebrafish mutant has a mutation in the same gene as the hi954 mutant described here. They conclude that the UDP-glucuronic acid decarboxylase is required for normal morphogenesis of cartilage, perichondrium and bone (Eames et al. 2010). Considering that the biosynthesis of HS and CS is affected earlier in the hi-lines compared to the previously published *dackel* and *boxer* we would expect stronger phenotypes. Surprisingly hi307 and hi954 did not show a more severe phenotype then *dackel* and *boxer*. Looking at the HS and CS profile of all four mutants some predictions can be made, but a closer comparison of the cellular development is needed.

The defects found in these two proteoglycan mutants can probably be explained by the lack of heparan sulphate. It has been shown that HS forms a complex with signalling molecules and their receptors (Pellegrini 2001), factors needed for cartilage development. Not only the disrupted signalling pathways but as well the fact that ECM is lacking to a great extent since it is built largely by proteoglycans, results in the cellular disorder. The ECM might shape the chondrocytes not only through signals but as well through mechanical forces.

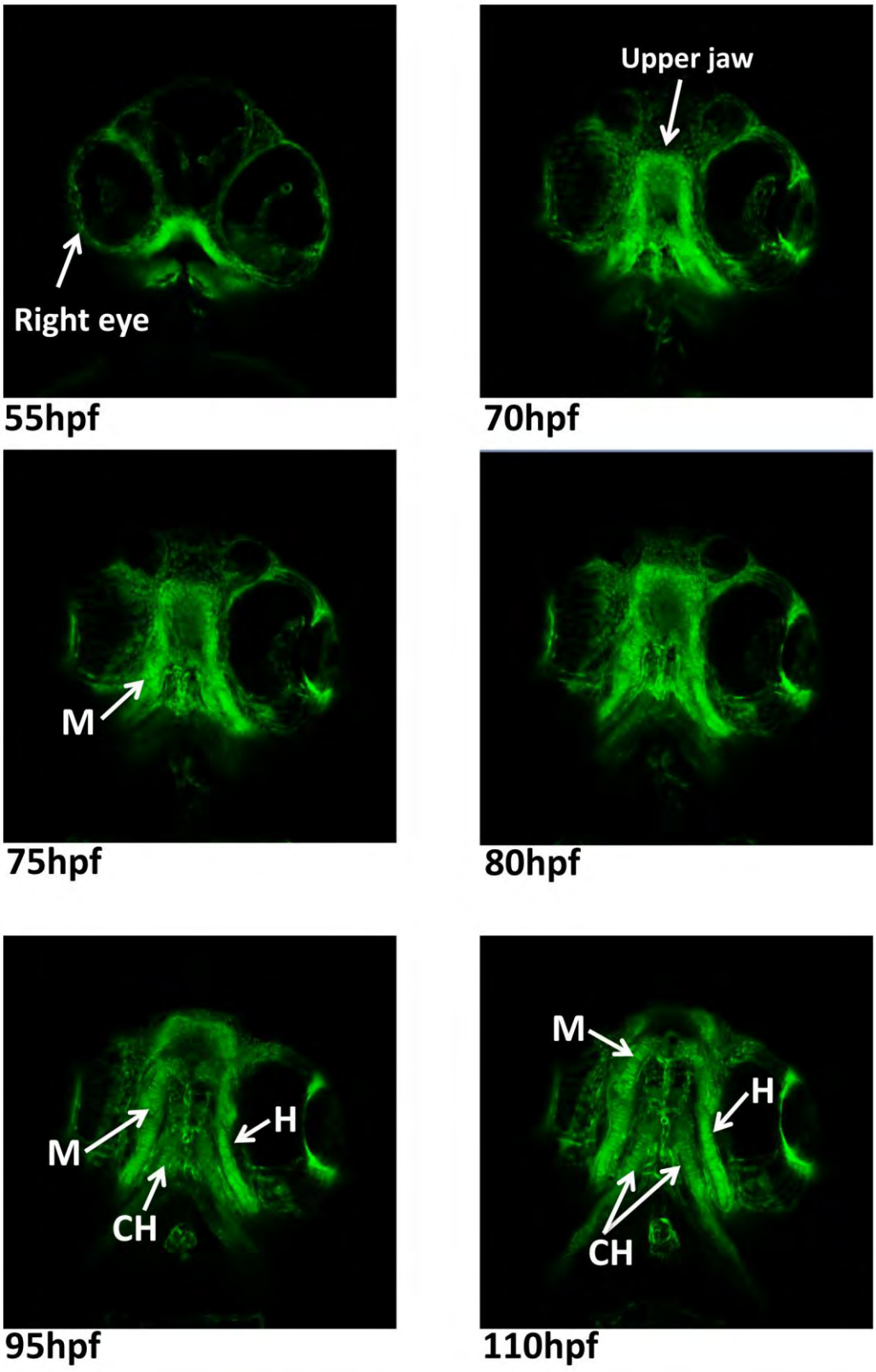


Figure 17: Ventral view of the pharyngeal cartilage development of zebrafish (Fli1:GFP; WT). First of all the upper jaw stretches out anteriorly. Meckel's cartilage (M) follows. The hyosymplectic (H) extends on both sides as a single cell layer. Chondrocytes line up and form stacks. The ceratohyal (CH) is seen most posterior.

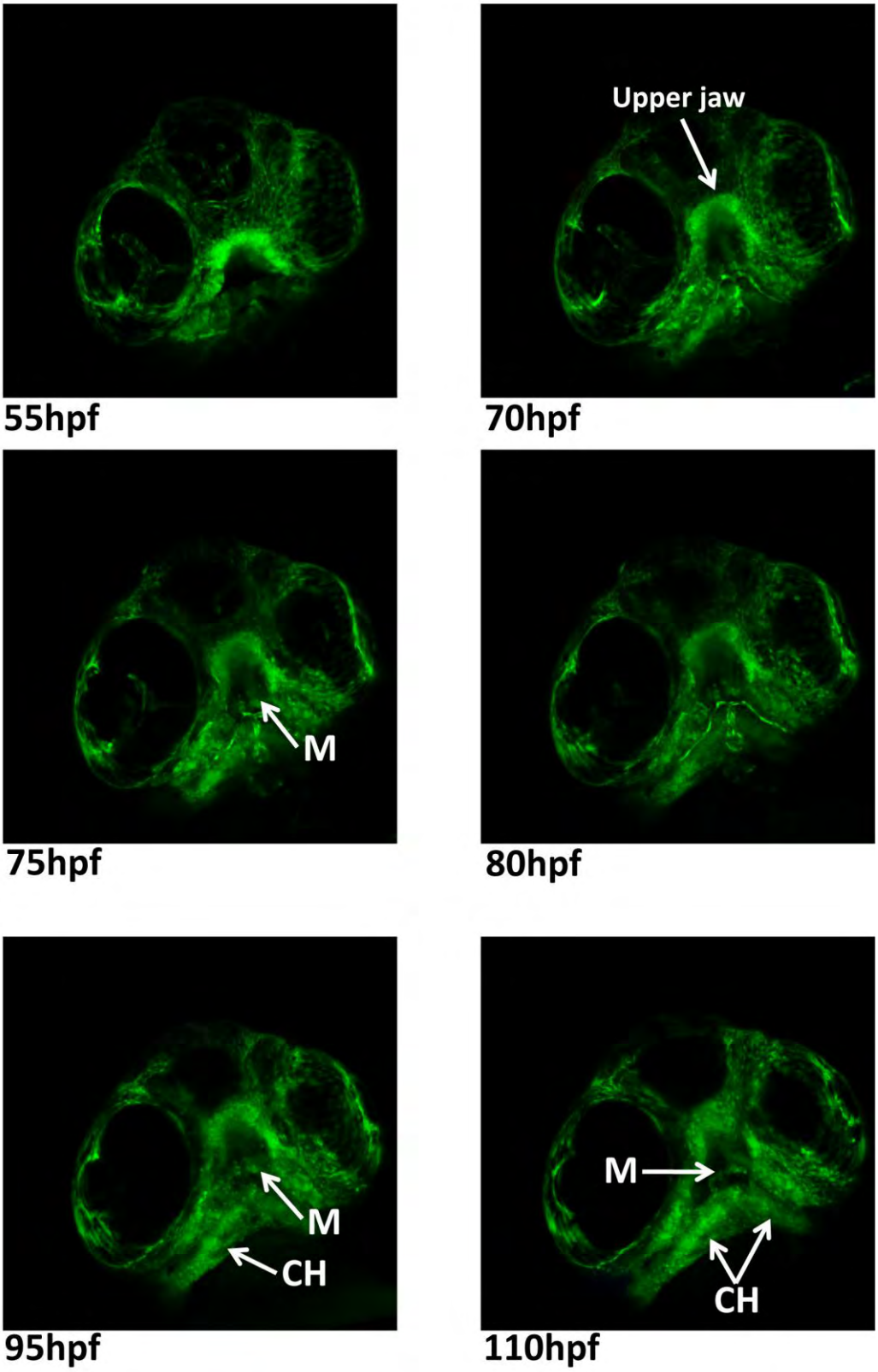


Figure 18: Ventral view of the pharyngeal cartilage development of mutant zebrafish (Fli1:GFP; hi307). The entire cartilage elements do not stretch out as seen in the WT (Fig.17). Meckel's cartilage (M) stays short and gets thicker. The hyosymplectic cannot be seen clearly. The most posterior element, the ceratohyal (CH), is short and thick. Chondrocytes are unorganized and do not stack as in the WT.

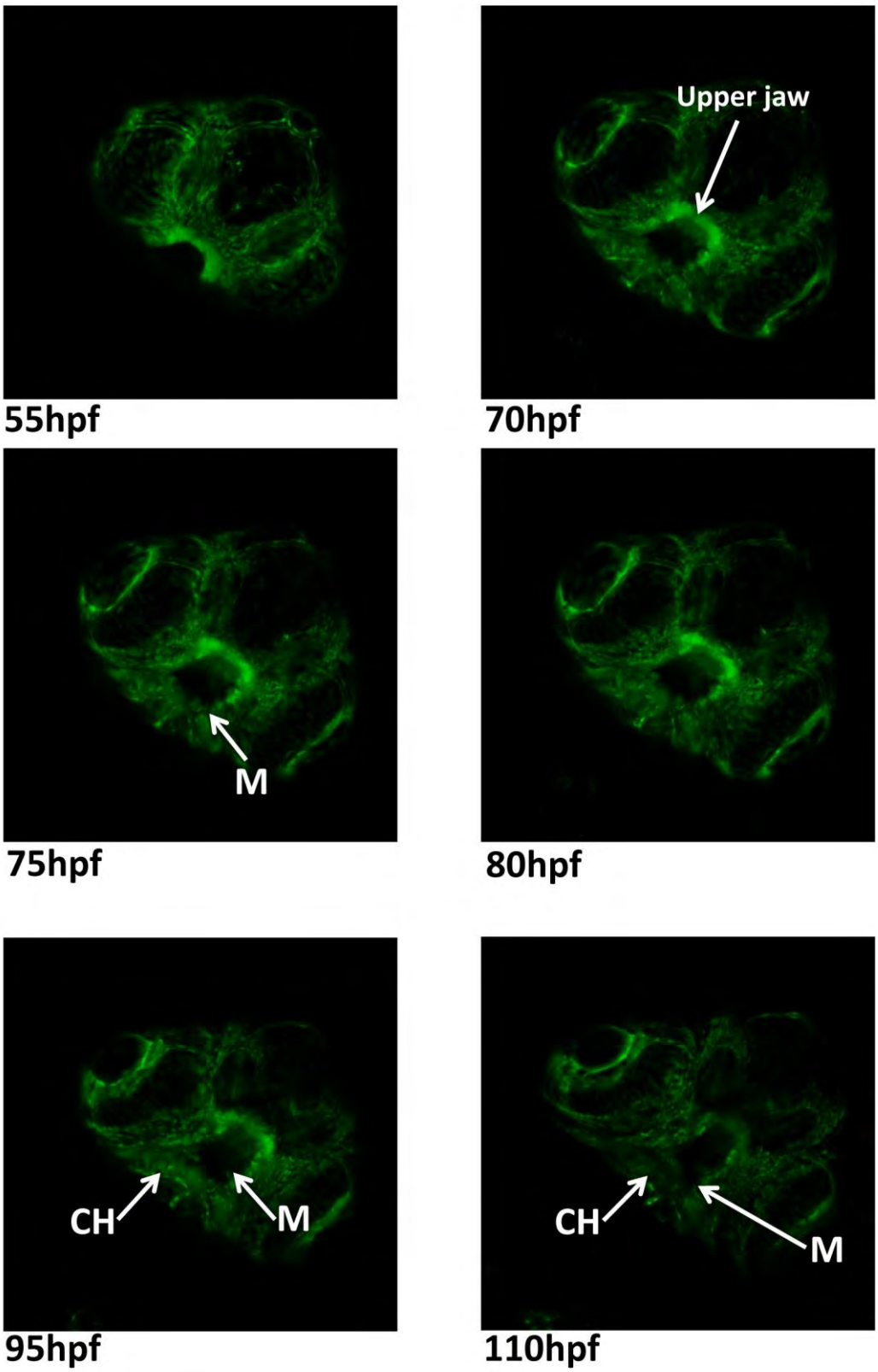


Figure 19: Ventral view of the pharyngeal cartilage development of mutant zebrafish (Fli1:GFP; hi954). The entire cartilage elements do not stretch out as seen in the WT (Fig.17). Meckel's cartilage (M) and the hyosymplectic appear very unclear. The most posterior element, the ceratohyal (CH), is short and thick. Chondrocytes are unorganized and do not stack as in the WT.

Performing long-term imaging always implies a quality compromise of the images to ensure viability of the embryos. By reducing the laser exposure one will obtain a lower resolution and single cells might not appear clearly. Therfore we produced more data at single time points. Figure 20 shows both GAG mutants with their wild-type control at 4dpf. Here the chondrocytes are clearly disorganized (red star), as well as the perichondrial cells (arrows).

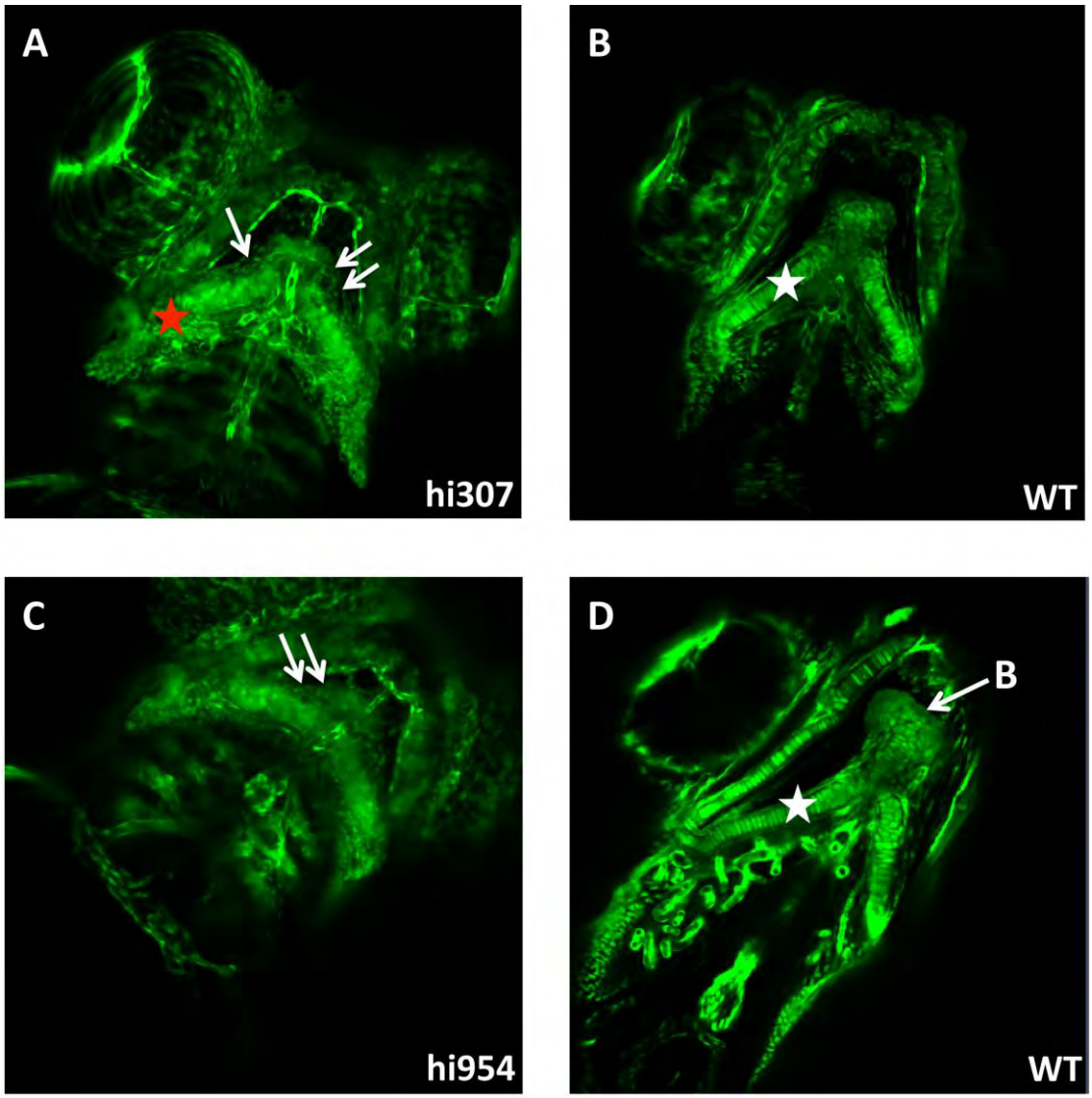


Figure 20: Ventral view of the pharyngeal cartilage development of zebrafish at 4 dpf. (A) Fli1:GFP hi307 mutant; (B) Fli1:GFP WT control; (C) Fli1:GFP hi954 mutant; (D) Fli1:GFP WT control. The red star indicates the disorganized chondrocytes in the mutants, the white stars indicate the stacking chondrocytes in the WT. B=basihyal, which is clearly affected in both mutants.

Conclusion

Confocal microscopy is an excellent way to visualize processes in developmental biology. In this study we were able to optimize the technique for live imaging of the zebrafish. Runs of up to 65 hours were conducted and the fish survived the experiment in the low melting agarose.

The study was conducted on the two different zebrafish proteoglycan mutants hi307 and hi954, with known biochemical profile. By confocal microscopy we were able to investigate the phenotypes on a cellular level. Regarding the pharyngeal cartilage development a clear phenotype could be shown. The lack of heparan and chondroitin sulphate resulted in cellular disorganization. Surprisingly, the effect on angiogenesis was not as striking as expected. Further studies have to be conducted to analyse the role of defective HS and CS production in this aspect.

When comparing the two mutants, no major differences could be seen, which was expected since the effect on the biosynthesis of HS and CS in a very similar. Although, a more detailed investigation may display more subtle differences with regard to both angiogenic and pharyngeal cartilage development. In angiogenic sprouting investigations with a higher magnification could be conducted to observe the behaviour of the filopodia. In pharyngeal cartilage development a closer look at the process of converted extension could be done. Converted extension is the process of lengthening and narrowing of a field of cells (Wallingford et al. 2002). Furthermore the exact time point and place where cellular formation starts to go wrong should be examined and if some cartilage elements are affected more than others.

To prove the hypothesis made in this study rescue experiments could be performed. It would be interesting to inject mRNA for glucoronysyl-transferase and UDP-glucuronic acid decarboxylase, in hi307 and hi954 respectively, to see if we are able to rescue the phenotypes. To clarify the role of HS and CS in detail in angiogenesis and cartilage development mutants could be crossed to create a complete knock out of HS for example. Another way to examine the role of one of the GAG chains is to knock down HS in the mutant background by the use of MOs.

Using live imaging to understand developmental processes is a strong tool because it includes all involved factors. One could even use the method to look at wound healing processes and tumour growth.

Acknowledgements

I would like to express my sincere gratitude first and foremost to you, Katarina Holmborn Garpenstrand, for your valuable supervision, guidance, and advice not only in the lab. It was a great pleasure working with you, in the good and exciting as well as in the tiring and nerve-racking moments of this project.

Big thanks go to you, Johan Ledin and Johan Kreuger for making this project possible, for your support and advice.

I want to thank as well the people sharing the office with me and the ones from the D9:4 corridor for helping me, listening to me and cheering me up when needed.

Thanks as well to Per Ahlberg for his support and input in discussions. Thank you, Bettina Ryll, for conversation and explanations about cartilage development.

Last but not least I thank my family, my parents Luis and Helene and my brother Hannes for your unconditional support even if many kilometres lie between us. A great “thank you” as well to all my friends: thank you, danke enk drhoam, grazie a tutti voi, merci beaucoup, och ett stort tack till er alla.

References

Amsterdam, A., R. M. Nissen, Z. Sun, E. C. Swindell, S. Farrington and N. Hopkins (2004). "Identification of 315 genes essential for early zebrafish development." Proc Natl Acad Sci U S A **101**(35): 12792-12797.

Barkefors, I., S. Le Jan, L. Jakobsson, E. Hejll, G. Carlson, H. Johansson, J. Jarvius, J. W. Park, N. Li Jeon and J. Kreuger (2008). "Endothelial cell migration in stable gradients of vascular endothelial growth factor A and fibroblast growth factor 2: effects on chemotaxis and chemokinesis." J Biol Chem **283**(20): 13905-13912.

Clement, A., M. Wiweger, S. von der Hardt, M. A. Rusch, S. B. Selleck, C. B. Chien and H. H. Roehl (2008). "Regulation of zebrafish skeletogenesis by ext2/dackel and papst1/pinscher." PLoS Genet **4**(7): e1000136.

Eames, B. F., A. Singer, G. A. Smith, Z. A. Wood, Y. L. Yan, X. He, S. J. Polizzi, J. M. Catchen, A. Rodriguez-Mari, T. Linbo, D. W. Raible and J. H. Postlethwait (2010). "UDP xylose synthase 1 is required for morphogenesis and histogenesis of the craniofacial skeleton." Dev Biol **341**(2): 400-415.

Eichmann, A., F. Le Noble, M. Autiero and P. Carmeliet (2005). "Guidance of vascular and neural network formation." Curr Opin Neurobiol **15**(1): 108-115.

Freeman, J. L., A. Adeniyi, R. Banerjee, S. Dallaire, S. F. Maguire, J. Chi, B. L. Ng, C. Zepeda, C. E. Scott, S. Humphray, J. Rogers, Y. Zhou, L. I. Zon, N. P. Carter, F. Yang and C. Lee (2007). "Definition of the zebrafish genome using flow cytometry and cytogenetic mapping." BMC Genomics **8**: 195.

Gandhi, N. S. and R. L. Mancera (2008). "The structure of glycosaminoglycans and their interactions with proteins." Chem Biol Drug Des **72**(6): 455-482.

Gerhardt, H. (2008). "VEGF and endothelial guidance in angiogenic sprouting." Organogenesis **4**(4): 241-246.

Gerhardt, H., M. Golding, M. Fruttiger, C. Ruhrberg, A. Lundkvist, A. Abramsson, M. Jeltsch, C. Mitchell, K. Alitalo, D. Shima and C. Betsholtz (2003). "VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia." J Cell Biol **161**(6): 1163-1177.

Gering, M. and R. Patient (2005). "Hedgehog signaling is required for adult blood stem cell formation in zebrafish embryos." Dev Cell **8**(3): 389-400.

Golling, G., A. Amsterdam, Z. Sun, M. Antonelli, E. Maldonado, W. Chen, S. Burgess, M. Haldi, K. Artzt, S. Farrington, S. Y. Lin, R. M. Nissen and N.

Hopkins (2002). "Insertional mutagenesis in zebrafish rapidly identifies genes essential for early vertebrate development." Nat Genet **31**(2): 135-140.

Herbert, S. P., J. Huisken, T. N. Kim, M. E. Feldman, B. T. Houseman, R. A. Wang, K. M. Shokat and D. Y. Stainier (2009). "Arterial-venous segregation by selective cell sprouting: an alternative mode of blood vessel formation." Science **326**(5950): 294-298.

Kilarski, W. W. and P. Gerwins (2009). "A new mechanism of blood vessel growth - hope for new treatment strategies." Discov Med **8**(40): 23-27.

Kimmel, C. B., W. W. Ballard, S. R. Kimmel, B. Ullmann and T. F. Schilling (1995). "Stages of embryonic development of the zebrafish." Dev Dyn **203**(3): 253-310.

Kimmel, C. B., C. T. Miller, G. Kruze, B. Ullmann, R. A. BreMiller, K. D. Larison and H. C. Snyder (1998). "The shaping of pharyngeal cartilages during early development of the zebrafish." Dev Biol **203**(2): 245-263.

Kreuger, J., D. Spillmann, J. P. Li and U. Lindahl (2006). "Interactions between heparan sulfate and proteins: the concept of specificity." J Cell Biol **174**(3): 323-327.

Lawson, N. D. and B. M. Weinstein (2002). "In vivo imaging of embryonic vascular development using transgenic zebrafish." Dev Biol **248**(2): 307-318.

Olsson, A. K., A. Dimberg, J. Kreuger and L. Claesson-Welsh (2006). "VEGF receptor signalling - in control of vascular function." Nat Rev Mol Cell Biol **7**(5): 359-371.

Pellegrini, L. (2001). "Role of heparan sulfate in fibroblast growth factor signalling: a structural view." Curr Opin Struct Biol **11**(5): 629-634.

Prydz, K. and K. T. Dalen (2000). "Synthesis and sorting of proteoglycans." J Cell Sci **113 Pt 2**: 193-205.

Schilling, T. F. and C. B. Kimmel (1997). "Musculoskeletal patterning in the pharyngeal segments of the zebrafish embryo." Development **124**(15): 2945-2960.

Schilling, T. F., T. Piotrowski, H. Grandel, M. Brand, C. P. Heisenberg, Y. J. Jiang, D. Beuchle, M. Hammerschmidt, D. A. Kane, M. C. Mullins, F. J. van Eeden, R. N. Kelsh, M. Furutani-Seiki, M. Granato, P. Haffter, J. Odenthal, R. M. Warga, T. Trowe and C. Nusslein-Volhard (1996). "Jaw and branchial arch mutants in zebrafish I: branchial arches." Development **123**: 329-344.

Schwend, T. and S. C. Ahlgren (2009). "Zebrafish con/disp1 reveals multiple spatiotemporal requirements for Hedgehog-signaling in craniofacial development." BMC Dev Biol **9**: 59.

Shih, J. and R. Keller (1992). "Cell motility driving mediolateral intercalation in explants of Xenopus laevis." Development **116**(4): 901-914.

Stringer, S. E. (2006). "The role of heparan sulphate proteoglycans in angiogenesis." Biochem Soc Trans **34**(Pt 3): 451-453.

Tsai, S. F., E. Strauss and S. H. Orkin (1991). "Functional analysis and in vivo footprinting implicate the erythroid transcription factor GATA-1 as a positive regulator of its own promoter." Genes Dev **5**(6): 919-931.

Wallingford, J. B., S. E. Fraser and R. M. Harland (2002). "Convergent extension: the molecular control of polarized cell movement during embryonic development." Dev Cell **2**(6): 695-706.