Glycosaminoglycan Biosynthesis and Function in Zebrafish Development

Sugars Shaping Skeletons

JUDITH HABICHER
Heparan sulfate (HS) and chondroitin/dermatan sulfate (CS/DS) proteoglycans are glycosylated proteins with important roles in animal development and homeostasis. HS and CS/DS are long, linear glycosaminoglycan (GAG) polysaccharides and attached to a core protein they form proteoglycans. GAGs on proteoglycans are often modified by sulfate groups and mainly found in the extracellular matrix or associated to the cell membrane. They interact with different proteins, for example signaling molecules, and influence developmental processes. Cells in cartilage produce a functionally specialized dense extracellular matrix, full of proteoglycans. Using the zebrafish as a model to study GAG biosynthesis we discovered that HS production is prioritized over CS/DS production, if the availability of link structures is restricted. We also found that the effects of removing HS and CS/DS biosynthetic enzymes in zebrafish larvae typically differ from what could be hypothesized solely from knowledge of the activity of each enzyme. These findings indicated a highly complex regulation of GAG biosynthesis and we thus proceeded to identify novel GAG biosynthetic enzymes in zebrafish and characterized their expression during early development. Notably, strong expression of CS/DS glycosyltransferases was found in cartilage structures, correlating with a drastic increase of CS/DS synthesis after two days of development, and high CS/DS deposition in cartilage. Finally, to understand how different GAG biosynthetic enzymes affect zebrafish development, we decided to use the CRISPR/Cas9 technology to generate new loss of function alleles for enzymes in HS and CS/DS biosynthesis. Some mutants show disturbed larval development or adult morphology, but we found many mutants to develop into adults without major morphological abnormalities, suggesting a high redundancy for GAG biosynthetic enzymes. Many GAG glycosyltransferases and modification enzymes have multiple isoforms, suggesting that a combination of mutations in one individual will become necessary to study the loss of specific modifications. To conclude, the zebrafish model gives new insights into the GAG machinery and the CRISPR/Cas9 technology allows for swift production of new loss of function zebrafish lines with defective GAG biosynthesis.

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To Katarina

“It’s handled.”
The cover shows a \(Tg(col2a1a:mEGFP)\) 6 day old zebrafish larvae in a lateral view, where the membranes of chondrocytes, among other cells, are GFP labeled. Below, the stacking of chondrocytes within the ceratohyal, the second pharyngeal arch, in \(Tg(fli1:EGFP)\) fish, is shown in detail.
List of Papers

This doctoral thesis is based on the following papers, which are referred to in the text by their Roman numerals.


*These authors contributed equally to the work


Reprints were made with permission from the respective publishers.
Additional Publications

The following paper was published during the course of my doctoral studies, but is not included in the thesis.

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## Abbreviations

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<th>Abbreviation</th>
<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered regulatory interspaced short palindromic repeats</td>
</tr>
<tr>
<td>crRNA</td>
<td>CRISPR RNA</td>
</tr>
<tr>
<td>CS</td>
<td>Chondroitin sulfate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dpf</td>
<td>days post fertilization</td>
</tr>
<tr>
<td>DS</td>
<td>Dermatan sulfate</td>
</tr>
<tr>
<td>DSB</td>
<td>Double-strand break</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ENU</td>
<td>Ethynitrosourea</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GalNAc</td>
<td>N-acetylgalactosamine</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GlcA</td>
<td>Glucuronic acid</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>HDR</td>
<td>Homology directed repair</td>
</tr>
<tr>
<td>hpf</td>
<td>hours post fertilization</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td>IdoA</td>
<td>Iduronic acid</td>
</tr>
<tr>
<td>KS</td>
<td>Keratan sulfate</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NDST</td>
<td>N-deacetylase/N-sulfotransferase</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Nonhomologous end-joining</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>PAM</td>
<td>Protospacer adjacent motif</td>
</tr>
<tr>
<td>PAPS</td>
<td>3’phosphoadenosine-5’phosphosulfate</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>sgRNA</td>
<td>Single guide RNA</td>
</tr>
<tr>
<td>TALEN</td>
<td>Transcription activator-like effector nuclease</td>
</tr>
<tr>
<td>tracrRNA</td>
<td>Transactivating CRISPR RNA</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine (or uracil) diphosphate</td>
</tr>
<tr>
<td>ZFN</td>
<td>Zinc-finger nuclease</td>
</tr>
</tbody>
</table>

Gene symbols are not listed.
Introduction

Cell-cell communication is important for building tissues and multicellular organisms. Cells communicate to one another in a highly regulated manner in order to build, shape and organize themselves and this communication is carried out by sending and receiving signaling molecules. Everywhere where cells are not in direct cell-cell contact, signals have to travel through the extracellular matrix, the space in between cells. The extracellular matrix is mainly composed of fibrous proteins and proteoglycans. Proteoglycans consist of a core protein with long, linear sugar chains attached. The sugar chains are highly sulfated and the most anionic molecules produced by animal cells. With their negatively charged sulfate groups, proteoglycans have the ability to bind and interact with many different proteins, for example signaling molecules like growth factors and morphogens, cytokines and chemokines, and thereby regulate development and homeostasis. By docking to proteoglycans proteins can, for example, contribute in the binding of ligands to receptors or be protected from degradation. A tissue with a very dense extracellular matrix full of proteoglycans is cartilage. The dependence on proteoglycans in forming skeletal structures, specifically cartilage, during development is prominent. The main focus of this thesis is to study the complex and non-template based biosynthesis and the role of glycosaminoglycans in the extracellular matrix of cartilage during zebrafish embryonic development.
Background

Glycosaminoglycans

Glycosaminoglycans (GAGs) are linear polysaccharides, built of repeating disaccharide units of an amino sugar and an uronic acid or galactose. The amino sugar is either N-acetylglucosamine (GlcNAc) or N-acetylgalactosamine (GalNAc) and the uronic acid is either a glucuronic acid (GlcA) or iduronic acid (IdoA) (Figure 1). These sugars are then in turn modified by sulfation and epimerization.

GAGs can be divided into four major groups according to their sugar composition: heparan sulfate (HS), chondroitin/dermatan sulfate (CS/DS), keratan sulfate (KS) and hyaluronan. HS are so-called glucosaminoglycans consisting of repeating units of GlcA and GlcNAc disaccharides. In addition the polysaccharides are enzymatically modified by sulfate groups. CS/DS are galactosaminoglycans and consist of repeating disaccharides of GlcA and GalNAc. DS is the epimerized version of CS and has IdoA instead of GlcA (Thelin et al. 2013). Since there are no sugar chains found with only GlcA-GalNAc or IdoA-GalNAc disaccharides, they are referred to as CS/DS indicating the hybrid nature of these polysaccharides. CS/DS chains are also sulfated. KS is a sulfated polyN-acetyllactosamine chain and hyaluronan is the simplest and most atypical GAG, composed of GlcA and GlcNAc like HS, but it is not sulfated. In addition, it is never attached to a core protein, whereas all other GAGs occur as proteoglycans, meaning that the polysaccharides are linked to a core protein.
Figure 1. The basic structure of HS and CS/DS mono- and disaccharides with numbers indicating the C-positions.

Biosynthesis of Heparan Sulfate and Chondroitin/Dermatan Sulfate

HS and CS/DS are GAGs linked to serine residues in the core protein. Both GAGs share a common linkage region composed of four monosaccharides (Figure 2). Disaccharides, specific for the type of GAGs, are attached after completion of the linkage region. HS formation is decided by attaching a GlcNAc to the linkage region, attaching GalNAc forms CS/DS. Glycosaminoglycans are produced inside the Golgi apparatus, where a large number of enzymes are involved in polymerization and modification of the chain. In HS it has been shown that some enzymes work in physical collaboration in a complex hypothetically referred to as the “GAGosome” (Esko and Selleck 2002). UDP-sugars (Uridine diphosphate-sugars) serve as sugar donor and 3’phosphoadenosine-5’phosphosulfate (PAPS) as sulfate donor and both are produced in the cytoplasm and transported into the Golgi apparatus. PAPS is transported over special multi-transmembrane-spanning solute carrier proteins called PAPS transporter 1 and PAPS transporter 2 into the Golgi (Kamiyama et al. 2003; Kamiyama et al. 2006). After polymerization and modification in the Golgi apparatus, the proteoglycans can be secreted into the extracellular matrix, stored in the secretory granules or attached to the plasma membrane (Figure 4).
Linkage region

HS and CS/DS share a common linkage tetrasaccharide composed of one xylose, two galactose, and one glucuronic acid unit (*Figure 2*). UDP-glucose serves as a precursor for UDP-GlcA. UDP-glucose dehydrogenase (UGDH) converts UDP-glucose to UDP-GlcA, which is the template for UDP-xylose synthase (UXS1) to form xylose. The xylosyltransferases (XYLT1 or XYLTT2) attach the xylose to the serine residue in the protein. Galactosyltransferase-I (β4GALT7) adds the first galactose to the xylose, galactosyltransferase-II (β3GALT6) adds the second galactose to the first galactose, and finally glucuronyltransferase-I (β3GAT3) adds GlcA to the second galactose (*Figure 2*). Furthermore, FAM20B is a kinase that phosphorylates xylose and 2-phosphoxylose phosphatase (PXYLP1) dephosphorylates the xylose, most likely directly (Koike et al. 2014). If phosphorylation of xylose in the linkage region is not transient, it can potentially block HS and CS/DS biosynthesis, as shown *in vitro* (Koike et al. 2014).

*Figure 2.* HS and CS/DS GAGs are attached to serine residues in the core protein. Enzymes catalyzing the formation of the linkage region, common for both HS and CS/DS, are indicated.
Heparan Sulfate polymerization

HS polymerization is catalyzed by enzymes from the exostosin family. EXTL enzymes are initiating HS polymerization. All three enzymes, EXTL1, EXTL2, and EXTL3, have GlcNAc transferase activity and add a GlcNAc to the linkage region to start HS polymerization (Kim et al. 2001). However, in vivo EXTL3 seems to play the essential role in this process (Han et al. 2004) and the functional role of EXTL1 and EXTL2 remains poorly understood. EXT1 and EXT2 were both shown to have dual GlcA and GlcNAc-transferase activity (McCormick et al. 1998; Lind et al. 1998) (Figure 3). When either of the two enzymes is disrupted in mice, HS polymerization is affected, indicating that both enzymes are essential for polymerizing HS chains (Stickens et al. 2005; Lin et al. 2000). In cell lines it has been shown, that EXT1 and EXT2 form hetero-dimers in order to efficiently elongate the sugar chain (Kobayashi et al. 2000). EXT1 has both GlcA and GlcNAc-transferase activity, but it is believed that EXT2 is assisting in the transport, and maybe also the folding of EXT1 (Busse et al. 2007).

Heparan Sulfate modification

After polymerization the HS sugar chain undergoes different modifications. The enzymes catalyzing these modifications are N-Deacetylase/N-sulfotransferases (NDSTs), epimerases and sulfotransferases (Figure 3). The four NDST enzymes, NDST1, NDST2, NDST3 and NDST4, are bifunctional enzymes, which have the ability to first deacetylate the GlcNAc C-2 position and then replace it with a sulfate group (Figure 3). One C5-epimerase (GLCE) converts GlcA into IdoA. Furthermore, HS is sulfated by 2-O, 3-O and 6-O sulfotransferases. The mammalian 2-O sulfotransferase (HS2ST1) is adding a sulfate group to IdoA and it is believed that GLCE and HS2ST1 are co-localized in the Golgi and might act together. Three 6-O sulfotransferases (HS6ST1, HS6ST2, HS6ST3) and seven 3-O sulfotransferases (HS3ST1, HS3ST2, HS3ST3A1, HS3ST3A2, HS3ST4, HS3ST5, HS3ST6) are characterized in mammals creating a vast range of HS decorations essential for interaction with signaling molecules (Figure 3). HS biosynthesis has recently been reviewed (Kreuger and Kjellen 2012).

Chondroitin/Dermatan Sulfate polymerization

CS/DS chain polymerization is initiated by two CS N-acetylgalactosaminyltransferases, CSGALNACT1 and CSGALNACT2 (Figure 3). Two chondroitin sulfate synthases (CHSY1, CHSY3) and two chondroitin polymerization factors (CHPF and CHPF2) add alternating GlcA or IdoA, and GalNAc disaccharides to polymerize CS/DS (Figure 3).
Although all these polymerizing enzymes have two active sites, they cannot polymerase CS/DS chains alone; effective polymerization occurs only if any two of these enzymes are co-expressed (Izumikawa et al. 2007). In addition, a recent study shows an interaction between CSGALNACT1 and PXYP1. As CSGALNACT1 adds GalNAc to the linkage region, PXYP1 dephosphorylates the xylose. If this dephosphorylation does not occur, CS/DS cannot be polymerized further (Izumikawa et al. 2015). Exactly how all polymerizing enzymes work together is still not fully understood.

Chondroitin/Dermatan Sulfate modification

The monosaccharide GlcA can be epimerized into IdoA by repositioning the C-5 carboxyl group. This is catalyzed by DS epimerase (DSE) or DS epimerase like (DSEL) (Figure 3). Along the CS/DS polysaccharide individual saccharide units can exist in different combinations. IdoA containing disaccharides can be found in repetitive GalNAc-IdoA disaccharide domains, but also adjacent to GalNAc-GlcA or altering with GlcA containing disaccharides (Thelin et al. 2013).

After and during polymerization sulfotransferases are acting to add sulfate groups to the GAG chain at specific positions (C-positions indicated in Figure 1). Sulfation can occur at the C-4 or C-6 position of GalNAc and at the C-2 position of either GlcA or IdoA. The enzymes can be categorized according to the C-position they modify: 2-O sulfotransferases, 4-O sulfotransferases, and 6-O sulfotransferases. 4-O sulfation is a common modification in CS/DS. There are four 4-O sulfotransferases described in mammals; CHST11, CHST12 and CHST13 and CHST14. CHST14 shows substrate specificity for GalNAc residues flanked by IdoA, while the other three 4-O sulfotransferases act on GalNAc-GlcA disaccharides (Figure 3). 6-O sulfation at the GalNAc residue is catalyzed by CHST3 and CHST7 and mainly found in GalNAc-GlcA disaccharides (Figure 3). A possible existence of a 6-O sulfated dermatan sulfate was seen in bovine serum (Nadanaka et al. 1999). CHST15 is an additional enzyme adding a sulfate group to the GalNAc C-6 position, but only if GalNAc is already 4-O sulfated, resulting in a disulfated disaccharide (Figure 3). Other disulfated disaccharides are produced by UST, catalyzing 2-O sulfation in CS/DS (Figure 3). This is common on IdoA residues and occurs rarely on GlcA. The activity of UST is highest if the adjacent GalNAc monosaccharide is already 4-O sulfated, which is catalyzed by CHST14. CHST14 and DSE (maybe also DSLE) work in functional collaboration to form IdoA domains and the 4-O sulfation impedes further backepimerization to GlcA (Pacheco et al. 2009).
**Figure 3.** HS and CS/DS biosynthesis. EXTL3 and CSGALNACT enzymes initiate HS and CS/DS polymerization, respectively. EXT enzymes are polymerizing the HS chain, and CHSY and CHPF enzymes the CS/DS chain. The NDST enzymes are the first to modify the HS chain. GLCE is epimerizing GlcA to IdoA in HS, DSE and DSEL in CS/DS. A large number of sulfotransferases are then adding sulfate groups to the respective C-positions in the monosaccharides indicated.

**Proteoglycans**

Proteoglycans are proteins with one or more GAGs covalently attached. Proteoglycans are produced in the Golgi apparatus and they are after synthesis and modification secreted into the extracellular matrix (ECM), stored in secretory granules, or incorporated into the basement membrane (Figure 4). Different proteoglycan core proteins can carry different GAGs. Syndecans and glypicans are membrane bound core proteins, and while syndecans typically carry one to three HS chains and one to two CS/DS chains, glypicans are decorated only by HS. Serglycin is found in intracellular granules and it can either carry heparin, a highly sulfated form of HS, or CS/DS chains. Perlecan is only decorated by HS chains, and it is either membrane bound or secreted into the extracellular matrix. Proteins from the aggrecan family are secreted into the ECM, and have mainly chondroitin sulfate chains attached, but can also contain some KS chains. Brevican and neurocan carry up to four, versican 10-15 and aggrecan approximately 100 CS/DS chains.
The role of proteoglycans in the extracellular matrix

Proteoglycans are mainly found in the extracellular matrix (ECM). The ECM is defined as a network of macromolecules filling the extracellular space in between cells and is often described as a gel-like ground substance. It acts like a cushion, building up a turgor that keeps the tissues resistant to mechanical and compressive forces. Two different kinds of macromolecules, fibrous proteins and proteoglycans, are the main components of the ECM. Fibrous proteins like collagen, elastin, fibronectin, and laminin, form long protein filaments and are shaped like wires. The covalently cross-linked helices determine the strength of the bundles. Collagen is the most abundant protein in the ECM of cartilages, especially collagen type II. The other main molecules in the ECM are proteoglycans. The arrangement of fibrous proteins and proteoglycans organizes and strengthens the ECM (Alberts et al. 2002).

Proteoglycans in the ECM are highly negatively charged due to all the sulfate groups along the sugar chains. This specific property attracts sodium ions and via osmosis water is sucked into the ECM, which creates a swelling pressure giving the ECM its unique capacity of withstanding compressional forces (Alberts et al. 2002). Proteoglycans can associate to form huge polymeric complexes contributing to the spatial organization of the ECM. They also have regulatory functions and interact with signaling molecules such as Hedgehog (Hh), Wingless (Wnt), transforming growth factors

Figure 4: Examples of proteoglycans, which can be found in the extracellular matrix (ECM), incorporated into the membrane or in secretory granules inside the cell.
(TGFs), and fibroblast growth factors (FGFs) to generate morphogen gradients, and orchestrate cell division and tissue growth to shape developing animals (reviewed in (Hacker et al. 2005)). In FGF signaling, for example, it was found that activation of FGF receptors requires simultaneous binding to HS, thus HS is acting as a co-receptor (Olwin and Rapraeger 1992). Sulfated GAGs can also bind different chemokines and protect them against proteolysis, providing a depot essential to build up morphogen gradients during development (Hoogewerf et al. 1997).

The function of HS in interacting with different signaling pathways, for example as a co-receptor, has been studied in greater detail than other GAGs (reviewed in (Lin 2004)). Studies in the fruit fly (Baeg et al. 2001) as well as in zebrafish (Topczewski et al. 2001) show the essential role of glypicans for proper Wnt signaling. The HS fine structure has been shown to be essential for binding specific ligands, where especially 6-O sulfation is crucial for FGF signaling (reviewed in (Nakato and Kimata 2002)). CS/DS are the most abundant GAGs in the ECM and are responsible for structural and mechanical properties, but interactions with for example signaling molecules have not been demonstrated as extensively as for HS. Recently CS/DS has been shown to bind heparin-binding growth factors and axon guidance molecules in a structure dependent manner (Deepa et al. 2002; Maeda et al. 2006) and CS/DS is also involved in the development of the central nervous system, infection processes, and growth factor signaling (Beurdeley et al. 2012; Bergefall et al. 2005; Mikami and Kitagawa 2013).

Human diseases linked to Heparan Sulfate and Chondroitin/Dermatan Sulfate

A number of GAG biosynthetic enzymes are linked to human diseases with different malformations affecting the skeleton and connective tissues. Mutations in the exostosin genes, EXT1 and EXT2, cause the human autosomal dominant disease called Hereditary Multiple Exostoses (HME) (Cook et al. 1993). This disorder affects the endochondral skeleton during growth, and results in bony outgrowths, random ossification, diminished bones, and short stature (Solomon 1964). Another human disease characterized by limb malformations and short stature, as well as hearing loss, is the teotammy preaxial brachydactyly syndrome, caused by a mutation in CHSY1 (Tian et al. 2010). Mutations in DSE or CHST14 cause Ehlers-Danlos syndrome, where patients develop joint dislocations, deformations, and a hyperextensibility of the skin, among other symptoms (Mendoza-Londono et al. 2012; Muller et al. 2013; Shimizu et al. 2011). Additional genes linked to human diseases are listed in Table 1. Further details of human diseases linked to HS and CS/DS proteoglycans have recently been reviewed (Mizumoto et al. 2014).
Table 1. Genes involved in GAG biosynthesis linked to human disease.

<table>
<thead>
<tr>
<th>Human Gene</th>
<th>Disease</th>
<th>OMIM number</th>
</tr>
</thead>
<tbody>
<tr>
<td>XYLT1</td>
<td>Desbuquois dysplasia 2; Pseudoxanthoma elasticum</td>
<td>#615777, #264800</td>
</tr>
<tr>
<td>XYLT2</td>
<td>Pseudoxanthoma elasticum</td>
<td>#264800</td>
</tr>
<tr>
<td>B4GALT7</td>
<td>Ehlers-Danlos syndrome, progeroid type 1</td>
<td>#130070</td>
</tr>
<tr>
<td>B3GALT6</td>
<td>Ehlers-Danlos syndrome, progeroid type 2; Spondyloepimyskeletal dysplasia</td>
<td>#615349, #271640</td>
</tr>
<tr>
<td>B3GAT3</td>
<td>Multiple joint dislocations, short stature, craniofacial dysmorphism, and congenital heart defects</td>
<td>#245600</td>
</tr>
<tr>
<td>EXT1</td>
<td>Exostoses, multiple, type 1</td>
<td>#133700, #215300</td>
</tr>
<tr>
<td>EXT2</td>
<td>Exostoses, multiple, type 2</td>
<td>#133701</td>
</tr>
<tr>
<td>NDST1</td>
<td>Mental retardation, autosomal recessive 46</td>
<td>#616116</td>
</tr>
<tr>
<td>HS6ST1</td>
<td>Might be linked to schizophrenia and bipolar disorder</td>
<td>#614880</td>
</tr>
<tr>
<td>CSGALNACT1</td>
<td>Hereditary motor and sensory neuropathy</td>
<td>(Lencz et al. 2013)</td>
</tr>
<tr>
<td>CHSY1</td>
<td>Tentancy praxial brachydactyly syndrome; Linked to two types of peripheral neuropathies</td>
<td>#605282, #652362 (Izumikawa et al. 2013)</td>
</tr>
<tr>
<td>DSE</td>
<td>Ehlers-Danlos syndrome, Musculocontractural type 2</td>
<td>#615539</td>
</tr>
<tr>
<td>DSEL</td>
<td>Bipolar disorder</td>
<td>(Shi et al. 2011)</td>
</tr>
<tr>
<td>CHST3</td>
<td>Spondyloepiphysial dysplasia with congenital joint dislocation; possible link to schizophrenia</td>
<td>#603799</td>
</tr>
<tr>
<td>CHST14</td>
<td>Ehlers-Danlos syndrome, musculocontractural type 1</td>
<td>#608429</td>
</tr>
</tbody>
</table>

Functional studies in mice

Several studies in mice have shown the essential role of HS and CS/DS proteoglycans during development. Mice deficient of either one of the following genes EXT1, EXT2, EXT3, GLCE, NDST1 and double mutants NDST1;NDST2 are all embryonic lethal and die between E6 and E9 (Lin et al. 2000; Stickens et al. 2005; Takahashi et al. 2009; Li et al. 2003; Ledin et al. 2004; Holmborn et al. 2004; Ringvall et al. 2000; Fan et al. 2000). While the EXT1 homozygous null mutant mice fail to gastrulate, a hypomorphic mutation in EXT1 (Mitchell et al. 2001) results in an elevated range of Indian hedgehog signaling during chondrocyte differentiation and those mice die around E14.5 (Koziel et al. 2004). The longer survival might be due to the formation of some, although shorter HS chains (Yamada et al. 2004). EXT2 deficient heterozygous adult mice show abnormalities in cartilage differentiation (Stickens et al. 2005). CHSY1 mutant mice are viable, develop skeletal dysplasia with decreased bone density and fail to pattern digits properly (Wilson et al. 2012). Other mutations in genes linked to GAG biosynthesis in mice are listed in Table 2. Further details have recently been reviewed (Mizumoto et al. 2014).
Table 2. Mice with mutations in GAG biosynthetic enzymes.

<table>
<thead>
<tr>
<th>Mouse Gene</th>
<th>Symptoms of homozygous mutation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGDH</td>
<td>Developmental arrest during gastrulation with defects in endoderm and mesoderm migration</td>
<td><a href="http://www.informatics.jax.org/marker/MGI:1306785">http://www.informatics.jax.org/marker/MGI:1306785</a></td>
</tr>
<tr>
<td>UXS1</td>
<td>Die prenatally</td>
<td>(Blake et al. 2014)</td>
</tr>
<tr>
<td>XLT1</td>
<td>Shorter body length</td>
<td>(Mis et al. 2014)</td>
</tr>
<tr>
<td>XLT2</td>
<td>Poly cystic liver and kidney disease at the age of 4-5 months</td>
<td>(Condac et al. 2007)</td>
</tr>
<tr>
<td>B3GAT3</td>
<td>Embryonic lethal before 8-cell stage due to cytokinesis failure.</td>
<td>(Izumikawa et al. 2010)</td>
</tr>
<tr>
<td>EXT1</td>
<td>Fail to gastrulate and embryonic lethal (die at E7.5)</td>
<td>(Lin et al. 2000)</td>
</tr>
<tr>
<td>EXT2</td>
<td>Fail to gastrulate and die around E6-7.5.</td>
<td>(Stickens et al. 2005)</td>
</tr>
<tr>
<td>EXT1L2</td>
<td>Normal development, but have impaired liver regeneration.</td>
<td>(Nadanaka et al. 2013b; Nadanaka et al. 2013a; Nadanaka and Kitagawa 2014)</td>
</tr>
<tr>
<td>EXT1L3</td>
<td>Embryonic lethal (die at E9)</td>
<td>(Takahashi et al. 2009)</td>
</tr>
<tr>
<td>NDST1</td>
<td>Prenatal death; lung and craniofacial defects</td>
<td>(Ringwald et al. 2000; Fan et al. 2000)</td>
</tr>
<tr>
<td>NDST2</td>
<td>Reduced number of mast cell with empty storage vacuoles</td>
<td>(Forsberg et al. 1999; Humphries et al. 1999)</td>
</tr>
<tr>
<td>NDST3</td>
<td>Mild behavioral and hematological changes</td>
<td>(Palferia et al. 2008)</td>
</tr>
<tr>
<td>GECE</td>
<td>Lack of kidneys, lung failure; skeletal defects as well as aberrant chondrocyte proliferation</td>
<td>(Dierker et al. 2015; Li et al. 2003)</td>
</tr>
<tr>
<td>HS2ST1</td>
<td>Renal agenesis, over-mineralized skeletons, retardation of eye development, and neonatal death</td>
<td>(Bullock et al. 1998)</td>
</tr>
<tr>
<td>HSST1</td>
<td>Smaller, defective retinal axon guidance</td>
<td>(Habuchi et al. 2007)</td>
</tr>
<tr>
<td>HSST2</td>
<td>No abnormalities</td>
<td>(Nagai et al. 2013)</td>
</tr>
<tr>
<td>HSST3</td>
<td>Normal development</td>
<td>(HajMohammadi et al. 2003)</td>
</tr>
<tr>
<td>CSGALNACT1</td>
<td>Fertile and viable, with shorter cartilage structures</td>
<td>(Watanabe et al. 2010)</td>
</tr>
<tr>
<td>CHSY1</td>
<td>Limb patterning and skeletal defects including chondrodysplasia</td>
<td>(Wilson et al. 2012)</td>
</tr>
<tr>
<td>CHPP</td>
<td>Fertile and viable without defects</td>
<td>(Wilson et al. 2012; Saijo et al. 2011)</td>
</tr>
<tr>
<td>DSE</td>
<td>Viable, smaller, but no gross changes in major organs observed; alterations seen in the skin</td>
<td>(Maccarana et al. 2009)</td>
</tr>
<tr>
<td>DSEL</td>
<td>No morphological abnormalities</td>
<td>(Bartolini et al. 2012)</td>
</tr>
<tr>
<td>CHST3</td>
<td>Reduced cell number in spleen, brain and lymph nodes, reduced fertility</td>
<td>(Orr et al. 2013; Ichimura et al. 2002)</td>
</tr>
<tr>
<td>CHST11</td>
<td>Severe dwarfism, disrupted endochondral bone development, chondrodysplasia, disorganized cartilage growth plate, die 6 hours after birth</td>
<td>(Khupfel et al. 2005)</td>
</tr>
<tr>
<td>CHST14</td>
<td>Smaller body weight, fragile skin, less fertile, impaired proliferation of neural stem cells</td>
<td>(Bian et al. 2011; Akyuz et al. 2013)</td>
</tr>
<tr>
<td>CHST15</td>
<td>Viable and fertile</td>
<td>(Obata-Niimi et al. 2010)</td>
</tr>
</tbody>
</table>

Cartilage

The vertebrate skeleton consists predominantly of two types of skeletal tissues, cartilage and bone. Softer than bone and harder than muscle, cartilage is a type of connetive tissue functioning as a strong yet flexible support material. Neither nerves, nor blood or lymphatic vessels invade the tissue (Kimmel et al. 1998), except when cartilage is degraded to be replaced by bone during endochondral bone formation. Chondrocytes, the cartilage cells, are embedded in a dense ECM, separating each cell, or small clusters of cells, from its neighbors. Therefore the delivery of nutrients to and removal of waste products from the cells, as well as cell-cell communication, requires diffusion through the ECM. Hence, the dense ECM of cartilage has specialized characteristics.

There are three types of cartilage defined according to the composition of its ECM: hyaline, elastic and fibrous cartilage. Hyaline cartilage is the most common cartilage and its ECM is predominately composed of proteoglycans.
and collagens (type II). Elastic cartilage is rich in proteoglycans and fibrous proteins forming bundles, mainly elastin, giving this tissue great flexibility. Fibrous cartilage is the strongest type of cartilage and its ECM is the only type with collagen type I fibers (Cole 2011).

Chondrocytes of the postcranial skeleton, derive mainly from mesenchymal stem cells originating from the mesoderm, one of the three germ layers formed during early development. Hyaline cartilage is often a temporary tissue and serves as the scaffold for bone that develops via a process called endochondral ossification. Endochondral bones are one type of bone, dermal (or membrane) bone is the other. Dermal bone structures arise from intramembranous ossification, where mesenchymal stem cells differentiate into osteoblasts and form bone directly (Jabalee et al. 2013).

Craniofacial cartilage is not derived from the mesoderm. It arises from the neural crest cells, and originates therefore from the ectoderm (Knight and Schilling 2006). Neural crest cells are derived from the embryonic neuroepithelium. In the process of neurulation the neural plate forms a neural groove bringing together the neural folds at the dorsal midline and finally closing the neural tube (Huang and Saint-Jeannet 2004). Neural crest cells undergo an epithelial-to-mesenchymal transition, delaminate from the neuroepithelium and become highly migratory (Huang and Saint-Jeannet 2004; Schilling et al. 2010). Traditionally, the neural crest cells between the embryonic telencephalon and the fifth somite are defined as the cranial neural crest cells (Xia et al. 2013). They migrate ventrally, surround the pharynx and will form the skeletal, neural, and connective tissues of the pharyngeal arches, as well as the pigment cells (Schilling and Kimmel 1994; Schilling et al. 1996).

Bone and cartilage are considered vertebrate specific tissues (Hall 2005). This is true for bone, but not entirely true for cartilage (Zhang et al. 2009). Cartilage is found even in many invertebrates, showing many similarities, but also differences to vertebrate cartilages. Hemichordates, for example, show an extracellular matrix very similar to the extracellular matrix of vertebrate cartilage, but the cartilage is completely acellular (Cole and Hall 2004b). Another type of cartilage is found in sabellid polychaetes, which contains large vacuoles within the chondrocytes and is therefore called vesicular cartilage (Cole and Hall 2004a). It remains unclear if these different types of cartilages evolved independently or diversified from a single type of ancestral connective tissue (Zhang et al. 2009).

Historically, the hypothesis that chondrichthians, cartilaginous fish such as sharks, are more primitive than the now living osteichthyans, the bony fish and tetrapods, has significantly influenced research on the evolution of cartilage and bone. There is now unambiguous evidence, that dermal and perichondral bone (but not endochondral bone that actually replaces cartilage) had evolved in the gnathostome stem group prior to the divergence of chondrichthians and osteichthians (Ryll et al. 2014; Donoghue et al. 2006). Limited samples of
what could possibly be endoskeletal bone have been found in some early chondrichthians (Coates et al. 1998). The cartilage structures of chondrichthians undergo extensive mineralization, but do not become true bone. The chondrichthyan skeleton is therefore likely a derived condition that followed an evolutionary loss of bone (Zhang et al. 2009). Cartilage is also present in both living groups of jawless vertebrates, lamprey and hagfish, meaning that it is primitive for vertebrates as a whole. Living jawless vertebrates, lamprey and hagfish, show no mineralization of their cartilages.

The embryonic skeleton in all vertebrates is a collagenous cellular cartilage. Even though urochordates lack rigid endoskeletal elements (Cole and Hall 2004b), it was postulated that structures like this can arise via conserved developmental mechanisms. A recent study on the development of amphioxus (*Branchiostoma*), a representative of the cephalochordates, which is the sister group of vertebrates plus urochordates, showed that the developing oral skeleton displays conserved histological, developmental and molecular features of vertebrate embryonic cellular cartilage (Jandzik et al. 2015). They suggest that the nascent oropharyngeal skeleton of early chordates incorporated collagenous cellular cartilage that is strikingly similar to vertebrate cartilage (Jandzik et al. 2015).

**Zebradish as a Model System**

Research uses different animal model systems to study biological processes. The fruit fly (*Drosophila melanogaster*) and the nematode worm (*Caenorhabditis elegans*) are the most common invertebrates, the mouse (*Mus musculus*) and the rat (*Rattus norvegicus*) the dominating vertebrate model organisms and in the last two decades zebrafish (*Danio rerio*) has become increasingly popular. The small vertebrates (3-5 cm in length) are tropical fresh water fish, live for around 3 years, are easy to breed and handle, and cheap to keep in laboratory conditions. The zebrafish genome has been fully sequenced and annotated, and about 70 % of the human protein-coding genes are homologous to genes found in zebrafish (Howe et al. 2013). For studies in developmental biology, the zebrafish is an especially well-established model organism due to its very rapid and extrauterine development (*Figure 5*). Within 24 hours post fertilization (hpf), all main organs are formed. The heart starts to beat at 26 hpf and blood circulation shortly thereafter. Larvae hatch around 2-3 days post fertilization (dpf), but they do not require exogenous food until the yolk sack is depleted (approximately 5 dpf) (*Figure 5*). For visualization of the embryonic development it is advantageous that the chorion, a membrane initially surrounding the egg, as well as the embryo at the beginning of development, are transparent (*Figure 5*). Pigmentation appears around 30-72 hpf and can be chemically inhibited to keep the larva transparent for an extended time.
(Hill et al. 2005). For these reasons the development of the embryo can be observed and manipulated easily from the first cell stage, the fertilized egg.

Figure 5. Some stages of zebrafish development. Hours post fertilization (hpf) and days post fertilization (dpf) are indicated and true for development at an optimal temperature of 28°C.

The zebrafish embryonic development has been described and characterized in great detail, which facilitates the analysis and comparison of abnormal development (Kimmel, Ballard et al. 1995). The early embryonic development is divided into eight periods: the zygote period, the cleavage period, the blastula period, the gastrula period, the segmentation period, the pharyngula period, the hatching period, and finally the early larval period (Kimmel, Ballard et al. 1995). In our studies we focus mainly on the development of the pharyngeal arch cartilage, which is derived from cranial neural crest cells. They begin to migrate during early segmentation period (around 12 hpf) and stream down to form the seven pharyngeal arches. The cartilage structures are formed by 5 dpf, when larvae start to feed (reviewed in (Yelick and Schilling 2002)).

Pharyngeal cartilage development in zebrafish

The pharyngeal cartilage in early zebrafish development has been studied and described extensively in normal and mutant background (Kimmel et al. 1998; Kimmel et al. 1995; Schilling et al. 1996; Piotrowski et al. 1996). Pharyngeal cartilage cells derive from cranial neural crest cells, which delaminate as the neural tube closes. They migrate to form the pharyngeal arches, exhibiting a segmental organization consistent with their particular hindbrain rhombomeric origin (Schilling and Kimmel 1994). In total, seven
Pharyngeal arches are formed (Figure 6B). The first pharyngeal arch, the mandibular arch, and the second pharyngeal arch, the hyoid arch, develop first and will form the supportive structures of the jaw and the operculum. Arches three to seven, the branchial arches, will then develop and shape the skeletal supporting structures of the gills and teeth.

In fish the cartilage structures are relatively simple and therefore useful to study, especially on a cellular level. Chondrocytes are embedded in a dense ECM, and perichondrial cells are enveloping the structure (Figure 6C-D). When shaping the cartilage structures, chondrocytes start out as round disorganized cells, then intercalate and flatten. Cells line up like a neat stack of coins, thereby forming the long and slender cartilage structures (Figure 6C-D) (Kimmel et al. 1998). Most of the pharyngeal cartilages are one cell layer wide. When studying the single cells within the cartilage elements, the densely packed ECM, composed of mainly collagen fibers and proteoglycans, becomes visible (Figure 6D).

Figure 6. Pharyngeal cartilage structures in zebrafish. Ventral view of a living Tg(col2a1a:mEGFP) zebrafish larvae at 6 dpf (A) and schematic drawing indicating the seven pharyngeal arches (B). The Tg(fli1:EGFP) larvae show perichondrial cells (*) surrounding the neatly stacked chondrocytes within the ceratohyal (C). In greater detail the nucleus (arrowhead) and endoplasmatic reticulum (arrow) within the chondrocytes are shown by TEM (D). bh: basihyal, ch: ceratohyal, hs: hyosymplectic, m: Meckel’s cartilage, pq: palatoquadrate.

The specific aligned stacks of chondrocytes are found also in lamprey (Yao et al. 2008) and Branchiostoma (Jandzik et al. 2015), as well as the 380 million year old fossil jawless fish Euphaneurops (Janvier and Arsenault 2007), indicating that this cell behavior is an ancient and highly conserved
chordate characteristic. The densely packed and specialized ECM produced by chondrocytes is full of proteoglycans interacting with various signaling molecules, and they are believed to play an essential role in the morphogenesis of cartilage structures.

GAG biosynthetic enzymes in zebrafish

Zebrafish genes orthologous to human genes involved in GAG biosynthesis are listed in Table 3. Some functional studies based on zebrafish mutants in genes encoding for enzymes involved in the GAG biosynthesis have been described. Zebrafish with mutations in uss1, udgh (jekyll), ext2 (dackel), extl3 (boxer), xylt1, papst1 (pincher), fam20b (Figure 1 and 2), all develop strong abnormalities in cartilage structures, predominantly in the craniofacial regions (Eames et al. 2010; Walsh and Stainier 2001; Eames et al. 2011; Clement et al. 2008; Lee et al. 2004). Moreover, seven studies based on a technique using morpholino oligonucleotides (MO) (more details about this method see section “Gene function studies in zebrafish”) to knock down gene expression of GAG biosynthetic enzymes have been published (Table 3).

Table 3. List of zebrafish genes orthologous to human GAG biosynthetic enzymes, as well as mutant and morpholino (MO) induced phenotypes.

<table>
<thead>
<tr>
<th>Human Gene</th>
<th>Zebrafish gene</th>
<th>Mutant/Morphant</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGDH</td>
<td>udgh</td>
<td>udgh</td>
<td>(Eames et al. 2010)</td>
</tr>
<tr>
<td>UXS1</td>
<td>uss1</td>
<td>uss1</td>
<td>(Eames et al. 2010)</td>
</tr>
<tr>
<td>FAM20B</td>
<td>fam20b</td>
<td>fam20b</td>
<td>(Eames et al. 2011)</td>
</tr>
<tr>
<td>PXLYP1</td>
<td>pxlyp1</td>
<td>pxlyp1</td>
<td>(Eames et al. 2011)</td>
</tr>
<tr>
<td>XYLTI</td>
<td>xylt1</td>
<td>xylt1</td>
<td>(Eames et al. 2011)</td>
</tr>
<tr>
<td>XYLTI</td>
<td>xylt2</td>
<td>xylt2</td>
<td>(Eames et al. 2011)</td>
</tr>
<tr>
<td>B4GALT7</td>
<td>bgalt7</td>
<td>bgalt7</td>
<td>(Amsterdam et al. 2004; Nissen et al. 2006)</td>
</tr>
<tr>
<td>B4GALT6</td>
<td>bgalt6</td>
<td>bgalt6</td>
<td>(Amsterdam et al. 2004; Nissen et al. 2006)</td>
</tr>
<tr>
<td>B3GALT3</td>
<td>bgalt3</td>
<td>bgalt3</td>
<td>(Amsterdam et al. 2004; Wiwege et al. 2011)</td>
</tr>
<tr>
<td>EXT1</td>
<td>ext1a</td>
<td>ext1b</td>
<td>(Lee et al. 2004; Clement et al. 2008)</td>
</tr>
<tr>
<td>EXT1</td>
<td>ext1c</td>
<td>ext1c</td>
<td>(Lee et al. 2004; Clement et al. 2008)</td>
</tr>
<tr>
<td>EXT2</td>
<td>ext2</td>
<td>ext2</td>
<td>(Lee et al. 2004; Clement et al. 2008)</td>
</tr>
<tr>
<td>EXLT1</td>
<td>ext1l</td>
<td>ext1l</td>
<td>(Lee et al. 2004; Clement et al. 2008)</td>
</tr>
<tr>
<td>EXLT2</td>
<td>ext2l</td>
<td>ext2l</td>
<td>(Lee et al. 2004; Clement et al. 2008)</td>
</tr>
<tr>
<td>NDST1</td>
<td>ndst1a</td>
<td>ndst1b</td>
<td>(Harfouche et al. 2009)</td>
</tr>
<tr>
<td>NDST2</td>
<td>ndst2a</td>
<td>ndst2b</td>
<td>(Harfouche et al. 2009)</td>
</tr>
<tr>
<td>NDST3</td>
<td>ndst3</td>
<td>ndst3</td>
<td>(Harfouche et al. 2009)</td>
</tr>
<tr>
<td>NDST4</td>
<td>ndst4</td>
<td>ndst4</td>
<td>(Harfouche et al. 2009)</td>
</tr>
<tr>
<td>GLCE</td>
<td>glcea</td>
<td>glceb</td>
<td>(Ghiselli and Farber 2005)</td>
</tr>
<tr>
<td>HS2ST</td>
<td>hs2sta</td>
<td>hs2stb</td>
<td>(Cadwalader et al. 2012)</td>
</tr>
<tr>
<td>HS6ST1</td>
<td>hs6sta</td>
<td>hs6stb</td>
<td>(Cadwalader et al. 2012)</td>
</tr>
</tbody>
</table>
Techniques used in zebrafish

Several reasons make the zebrafish an attractive animal model to conduct large-scale experiments. Zebrafish can produce a large number of eggs. One adult female lays 100-500 eggs every five to seven days. The eggs can easily be manipulated and injected with various molecules like DNA, RNA or MOs. Embryos, larvae and adult fish can also be exposed to different compounds in the water they develop in, and the animals small size simplifies high-throughput screenings, for example in multi-well plates. This is valuable for toxicological and pharmaceutical studies, as well as for studies of gene function. In addition, researchers have started using zebrafish to study aspects of cancer biology, for example neovascularization and metastatic behavior of tumor cells (Amatruda et al. 2002; Konantz et al. 2012; Tobia et al. 2013; Marques et al. 2009).

Generation of transgenic lines

The transparent zebrafish embryo is suitable for studying tissues and organs, but in order to observe single cell behavior, labeling is needed. The discovery of the green fluorescent protein (GFP) in jellyfish paved the way for transgenic labeling of animals (Shimomura et al. 1962). The first
successful introduction of GFP in zebrafish embryos (Stuart et al. 1988) suffered unfortunately from inconsistent expression. Efficient use of a zebrafish promoter to generate a stable transgenic line expressing GFP was finally achieved in 1997 (Higashijima et al. 1997). Since then many transgenic lines have been produced, expressing different fluorescent proteins from a large number of promoters.

The most popular method to generate transgenic zebrafish is the Tol2 transposon system (Clark et al. 2011). This system is based on a construct consisting of the sequence encoding for example the fluorescent protein, and the promoter, which drives the expression, flanked by Tol2 transposable elements. This is co-injected with Tol2 transposase, which thereby incorporates the exogenous DNA into the zebrafish genome. The specific place where the exogenous DNA will be introduced is random and will result in the promoter driving the expression of the chosen protein. Typically the newly introduced protein will be transiently expressed at first, but if it is inserted in germ cells it will result in a stable transgenic line after breeding. In the following generation the introduced DNA will be present in every cell of the animal, but the protein will only show expression in the cells where the promoter is active. One common zebrafish transgenic line is the Tg(fli1:EGFP) line expressing GFP in endothelial, as well as neural crest derived cells (Lawson and Weinstein 2002) (Figure 7B). The GFP accumulates in the cytosol showing the entire cell. Another example is the Tg(col2a1a:mGFP) line (Dale and Topczewski 2011), where the GFP is expressed almost exclusively in chondrocytes and GFP is localized to the membranes, so that changes in cell shape can be studied in greater detail (Figure 7A) (see cover).

The small size, transparency, and extrauterine development of zebrafish eggs, as well as the techniques available to generate transgenic lines, provide excellent conditions to perform imaging. The overall morphology can be studied even with basic bright field microscopy. Using confocal laser scanning microscopy and the newer light sheet microscopy, it is now possible to follow specific cell populations within a whole organism while the embryo grows and develops. Recent technological advances have succeeded in monitoring and reconstructing the live, beating zebrafish heart in 3D in great detail (Mickoleit et al. 2014). All these factors make zebrafish a powerful tool for studying morphology and cell migration.
Figure 7. Transgenic zebrafish. Ventral view of the zebrafish head (larvae at 6 dpf). (A) Membrane localized GFP is expressed under the *col2a1a* promoter. Ossifying structures are stained with alizarin red. (B) GFP is expressed under the *fli1* promoter.

Studies of gene function in zebrafish

In the 90s functional studies were carried out by chemical or insertional mutagenesis. In 1996 a large number of zebrafish mutant lines generated by mutagenizing males with ethylnitrosourea (ENU) were made available for the research community (Haffter et al. 1996). In this, so called forward genetics approach, embryos were selected based on specific phenotypes and the mutation was subsequently identified. This way many mutants were established, but there was no successful method of generating a mutation in a specific gene of interest.

The morpholino oligonucleotides (MOs) based technology is a different approach to study genes loss of function in zebrafish. MOs are short antisense oligonucleotides designed to target and bind to mRNA and thereby inhibiting translation (Summerton and Weller 1997). MOs are injected into a zebrafish egg at the one-cell stage, where they will bind to the specific mRNA targeted. Both the maternally deposited, as well as zygotic mRNA will be affected. This is a way to transiently inhibit expression of a specific mRNA, without altering the DNA. The oligo structure gives MOs a high affinity to RNA and a high stability in vivo. Nevertheless, with every cell division the MO will be diluted, and the cells will be able to produce new protein. Hence, effects and phenotypes are typically only studied up to 4 to 5 dpf. MOs can be targeted to the translation start site of a gene, thereby inhibiting initialization of translation. Another possibility is to use splice-inhibiting MOs, which inhibit a splice site resulting in a loss of an exon or inhibition of pre-mRNA splicing. However, splice inhibiting MOs will not affect maternally contributed mRNA. MOs were shown to be efficient in zebrafish (Summerton and Weller 1997) and it was the method of choice to study loss of function in zebrafish for many years. Distinguishing off-target effects, including the induced activity of p53 dependent apoptosis, from
target specific effects has proven to be very difficult (Robu et al. 2007; Eisen and Smith 2008).

It was the discovery and adaptation of zinc-finger nucleases (ZFNs) that for the first time made it possible to introduce site-specific mutations in the zebrafish genome (Doyon et al. 2008; Meng et al. 2008). ZFNs are targetable nucleases, comprised of one DNA-binding domain and one FokI nuclease, able to induce a double strand break (DSB). The natural repair system of the cell ligates the two DNA ends together either by nonhomologous end-joining (NHEJ) or homology directed repair (HDR). These systems are error prone and they often imply a small insertion or deletion resulting in a mutation. The drawback of this method is that the mechanism of DNA-binding is based on a protein and therefore the engineering of this domain is technically challenging and the commercial alternative expensive. In 2009 the transcription activator-like effector nuclease (TALEN) was found in bacterial plant pathogens (Moscou and Bogdanove 2009; Boch et al. 2009). The targetable nuclease is also inducing a DSB on a specific site. The assembly of the DNA recognition domain however is less complicated and many laboratories started to engineer TALENs. Still, the workload for every new target included many cloning steps and a whole new protein with the target recognition site as well as the active nucleatic site needed to be engineered. Therefore TALENs are not suitable for large sets of targets. Finally, in 2013 the CRISPR/Cas9 system was discovered in bacteria, where a short RNA is binding to a specific sequence in the DNA, forming a complex with a nuclease, which induces a DSB. With this method, the possibility to perform targeted mutagenesis in zebrafish became available for the common researchers.

The CRISPR/Cas9 system

An adaptive immunity in prokaryotes

Prokaryotes are constantly attacked by phages and viruses and therefore in continuous need to develop ways to fight these invaders. Bacteria and archaea have established different mechanisms of defense against phage populations, one of them being the CRISPR/Cas system, which acts as a form of prokaryotic adaptive immune system.

Clustered regularly interspaced short palindromic repeats (CRISPRs) were first described in *E.coli* (Ishino et al. 1987), and later named and defined as CRISPRs (Jansen et al. 2002). For a long time researchers failed to detect these sequences because cultured bacteria lose or silence CRISPR loci. In about 90% of archaeal and 40% of bacterial genomes repetitive sequences were found to be typically 21-37 base pair (bp) long (Jansen et al. 2002). These repeated sequences are interspaced with non-repetitive, unique
spacers of extrachromosomal origin, most commonly from phage and plasmids (Figure 8) (Bolotin et al. 2005; Mojica et al. 2005; Pourcel et al. 2005). In addition a set of CRISPR associated (Cas) genes were found flanking the CRISPR locus (Figure 8) and they were predicted to participate in adaptation and degradation processes (Jansen et al. 2002).

With the CRISPR/Cas system prokaryotes established a way to generate a molecular memory of an invader as well as a way to destroy it. First, bacteria and archaea acquire short sequences of foreign DNA, so called protospacers, and upstream of this sequence the protospacer adjacent motif (PAM) has been identified (Deveau et al. 2008; Mojica et al. 2009). This motif differs between species, for *Streptococcus pyogenes* for example it is a NGG. The sequence acquisition is not yet fully understood, but every protospacer sequence in the invading genome is found next to a PAM site. In addition, two of the Cas proteins, Cas1 and Cas2, were found to be essential for efficient adaptation (Yosef et al. 2012). The leader, an A/T-rich non-coding sequence located immediately upstream of the first repeat, is determining the sequence acquisition at its end of the spacer repeats and it also directs later transcription (Figure 8).

![Figure 8](image)

*Figure 8.* The CRISPR/Cas9 system as a bacterial immune defense. Upon viral infection bacteria can acquire short sequences of viral DNA and insert them into a CRISPR cassette, flanked by CRISPR associated (Cas) genes. Subsequently, short crRNAs are transcribed and form a complex with a Cas9 nuclease, which cuts double stranded DNA and fight future viral attacks.

Once protospacers are acquired in the bacterial genome, now called spacers, transcription is initiated from the leader sequence (Figure 8). The primary transcript, the precursor CRISPR RNA (pre-crRNA) is further processed into a set of short crRNAs. In the Type-II system an additional transactivating
crRNA (tracrRNA) together with the hosts RNaseIII are involved in the maturation pathway of pre-crRNAs (Deltcheva et al. 2011). The mature crRNAs contain the spacer, the invader specific DNA, flanked by two parts of the repetitive host DNA sequence. The crRNAs are responsible for recognition of the invading DNA, by base pairing with the spacer complementary protospacers.

In order to perform sequence specific cleavage, the crRNA forms a complex with different Cas proteins, depending on the species, to ultimately degrade the invading elements (Figure 8). Of the different Cas proteins found so far, the Cas9 is the only endonuclease acting alone with crRNAs to cut double stranded DNA. The Cas9 protein “runs over” the foreign DNA, and recognizes at first PAM sites, which triggers its activity. Only after PAM site recognition, unwinding happens and complementation of the crRNA with the DNA is checked. A DSB is introduced when both PAM site recognition and perfect base pairing with crRNA has occurred. Since the PAM site is only found in the viral genome it is possible to distinguish foreign DNA from the spacer in the host genome or even the RNAs in the eliminating process. The actual DSB is performed by the two functional domains of the Cas9 protein, the RuvC and the HNH. They cut respective DNA strand (Gasiunas et al. 2012).

The food industry was first to take advantage of this system and use it to make *Streptococcus thermophilus* more resistant in dairy products (Deveau et al. 2008). The possible applications of the CRISPR/Cas system are many and this has made the field grow quickly in the last year.

**Genome editing with CRISPR/Cas9**

After the discovery of the highly efficient and specific CRISPR/Cas9 system it was used to perform genome editing. Techniques like ZFN and TALENs are based on site-specific nucleases, with both the DNA binding domain and the nuclease domain embedded in the same protein. The big advantage of CRISPR/Cas9 is that the specificity lies in the relatively short crRNA and the same nuclease, the Cas9 from the Type-II system, can be used for all targets. Hence, only a sequence of 20 nucleotides (nts) needs to be redesigned for every new target. It’s a simple, inexpensive, easily programmed and very efficient system (reviewed in (Sander and Joung 2014)). In cell culture it was first shown that the system could be modified in order to target the Cas9 endonucleases with one single guide RNA (sgRNA) molecule to induce a DSB (Jinek et al. 2012). Introducing a linker loop to connect the tracrRNA and crRNA into one single guide RNA, and co-injecting it with the Cas9, was sufficient to induce a cut on both DNA strands (Figure 9). On the complementary strand Cas9 cuts approximately 3 bp upstream of the PAM site, on the non-complementary DNA strand 3-8 bp.
Targeted mutagenesis using CRISPR/Cas9 in zebrafish

In zebrafish, targeted mutagenesis has been a big challenge, as methods from mice or yeast did not work in zebrafish. With CRISPR/Cas9, for the first time a genome editing method was found to work in zebrafish with a high specificity, requiring only a relatively short complementary RNA sequence (Hwang et al. 2013).

When T7 RNA polymerase is used to synthesize sgRNAs, target sequences need to fulfill 5’-GG-N\textsubscript{18}-NGG-3’. The first GG are preferred starting nts for T7 RNA polymerase and the NGG is the PAM site in the genome sequence. Many possible targets with these requirements can be found in the genome, and there are now tools available online to find targets, for example a TrackHub created by the Burgess Lab (http://research.nhgri.nih.gov/manuscripts/Burgess/zebrafish/download.shtml). The Burgess Lab also developed a robust system to quickly produce sgRNAs without any cloning steps (Varshney et al. 2015). In short, a first fragment, referred to as oligo A, includes the T7 promoter (18 nts), the target sequence (20 nts) and an overlap with the crRNA-tracrRNA (20 nts) (gRNA) (Figure 10). A second fragment, oligoB (80 nts), contains the seed sequence of the gRNA and can be used for all constructs (Figure 10). This way, only oligoA (58
nts) needs to be ordered for every new target. OligoA and oligoB can easily be annealed resulting in a product of 118 nts that can be transcribed into the sgRNA and used for injection.

Figure 10. Annealing strategy of oligo A and oligo B, to produce template for sgRNA, essentially as described in (Varshney et al. 2015).

In order to induce a DSB, Cas9 and sgRNA need to be co-injected into zebrafish embryos at the one-cell stage. Initially, mRNA coding for Cas9 was injected (Hwang et al. 2013), while later studies show also injection of the Cas9 protein. The Cas9 protein was shown to be active already four hours after injection, two hours faster then when injecting mRNA, which first needs to be translated into a functional protein by the cell (Sung et al. 2014). Both ways resulted in similar efficiency of mutation rates and germline transmission (Gagnon et al. 2014; Sung et al. 2014). By injecting multiple targets into one embryo, husbandry could be drastically decreased, keeping high efficiency rates for germline transmission. Also, several methods for identifying mutations have been developed, including fluorescent PCR and barcoding strategies for sequencing (Varshney et al. 2015; Sood et al. 2013).
Aims

This thesis aims to achieve a better understanding of the biosynthesis of HS and CS/DS glycosaminoglycans and their function during zebrafish development, with special focus on the chondrocyte arrangement and cartilage tissue formation.

In Paper I we set out to study four different zebrafish mutants created by forward genetics, affecting genes involved in the HS and CS/DS biosynthesis. By studying the GAG composition and correlating it to phenotypes we became more interested in how CS/DS might be able to functionally compensate for the loss of HS. In Paper II, III and IV, we aimed to identify and describe glycosyltransferases and modification enzymes for CS/DS, as well as NDST enzymes for HS, found in the zebrafish genome. Last, in Paper V, with new techniques available, we set out to perform reverse genetic experiments to generate loss of function alleles for all genes involved in HS and CS/DS biosynthesis and thereby getting insights into their function in zebrafish.
Results and discussion

Interplay between Heparan Sulfate and Chondroitin/Dermatan Sulfate biosynthesis (Paper I)

To study the roles of HS and CS/DS in zebrafish, we analyzed four different zebrafish mutants with disrupted genes involved in GAG biosynthesis: ext2, extl3, uxs1, and b3gat3 (Figure 11A). We examined the early development and focused specifically on cartilage morphology. All four mutants develop malformations and die at larval stages. Zebrafish larvae with mutations in uxs1 and b3gat3, two genes involved in the linkage region formation of HS and CS/DS, show similar phenotypes with shorter pectoral fins and smaller and rounder heads, compared to control larvae (Figure 11C). Ext2, polymerizing the HS chain, and Extl3, initiating HS synthesis, deficient zebrafish did not show similar phenotypes. While ext2 mutants completely lack pectoral fins and have small round heads, extl3 mutants develop milder phenotypes with shorter pectoral fins and an only slightly smaller head, compared to control larvae (Figure 11C). In order to explain these different phenotypes, we studied the biochemical profiles and measured the HS and CS/DS content produced by mutant larvae (Figure 11B). Interestingly, uxs1 and b3gat3 mutants, show highly reduced CS/DS content (95-89% compared to WT control), whereas HS is still produced to around 50% compared to control (Figure 11B). This indicates a preference for HS production, when formation of the linkage region is disturbed.

The total content of HS appears to relate to the severity of observed pectoral fin phenotypes. Only the ext2 mutant with the lowest HS content (20% of normal content) (Figure 11B) fail to develop pectoral fins (Figure 11C). Approximately 50% of HS content is sufficient for pectoral fin development, even if not to a full extent. However, the HS content alone cannot explain the pharyngeal cartilage phenotype, which is mildest in extl3 mutants (Figure 11C). Instead, looking at the combined content of HS and CS/DS, an increased CS/DS production can be observed. In the absence of Extl3, more CS/DS is produced, compensating for HS reduction, resulting in a milder pharyngeal cartilage phenotype. This indicates interplay between CS/DS and HS and especially highlights the significance of CS/DS in cartilage morphogenesis.
Figure 11. Biosynthesis of HS and CS/DS and the sites of action for the four genes of interest are shown schematically (A). The total content of HS and CS/DS in the different mutants is displayed relative to the control, which is set to 100% (B). Ventral views on pharyngeal cartilage elements of Tg(fli1:EGFP) zebrafish at 6 dpf, additionally stained with alizarin red, are shown for control, extl3<sup>−/−</sup>, ext2<sup>−/−</sup>, uxs1<sup>−/−</sup>, and b3gat3<sup>−/−</sup> larvae (C). ext2<sup>−/−</sup> larvae develop no pectoral fins (arrow). extl3<sup>−/−</sup> larvae have a milder cartilage phenotype compared to ext2<sup>−/−</sup>, uxs1<sup>−/−</sup>, and b3gat3<sup>−/−</sup> larvae. Schematic drawings of cartilage structures in the first five pharyngeal arches are shown below (C). bh, basihyal; ch, ceratohyal; hs, hyosymplectic; m, Meckel’s cartilage; pq, palatoquadrate.

Characterization of CS/DS glycosyltransferases and modification enzymes in zebrafish (Paper II and III)

In order to investigate further the role of CS/DS we needed to identify and study enzymes involved in polymerization, as well as modification of CS/DS during zebrafish development. Our phylogenetic analysis identified orthologous genes of all mammalian enzymes involved in CS/DS polymerization (Paper II), as well as CS/DS epimerization and sulfation (Paper III), where few zebrafish genes were retained with two copies.

The expression of these enzymes is spatially and temporally regulated during zebrafish development (Paper II and III). Notably, glycosyltransferases show early expression in the notochord, the pharyngeal cartilage structures, and the pectoral fins. This correlates with sites of massive CS/DS deposition. Furthermore, total CS/DS content in embryos
increases significantly after two dpf. Taken together, this suggests that combinatorial expression of CS/DS polymerizing enzymes regulates CS/DS synthesis in the developing embryo (Paper II). The CS/DS sulfotransferases and epimerases show large variations in their expression during early zebrafish development, indicating tissue specific CS/DS structures (Paper III). The overlap of expression of glycosyltransferases, epimerases and sulfotransferases in specific tissues supports the idea that enzymes act in collaboration to synthesize and modify GAG chains. We showed that CS/DS biosynthesis is highly dynamic compared to HS biosynthesis during zebrafish development with decreased 4-O sulfation and increased 6-O sulfation from two to four dpf (Paper III). Interestingly, the expression of sulfotransferases synthesizing disulfated disaccharides is low, in accordance with the virtually absent disulfated disaccharides, as quantified by HPLC. This in marked contrast to the numerous disulfated disaccharides in HS (Paper III).

**N-Deacetylase/N-Sulfotransferases (Paper IV)**

HS sulfotransferases and epimerases have been previously described in zebrafish (Cadwallader and Yost 2006b, a, 2007; Ghiselli and Farber 2005), with the exception of N-deacetylase/N-sulfotransferases (Ndsts). To complete the description of enzymes involved in HS biosynthesis, we identified five zebrafish ndst genes and found duplicated genes in zebrafish for both the mammalian NDST1 and NDST2. Interestingly, only a single orthologous gene, namely ndst3, equally similar to both mammalian NDST3 and NDST4 was found, indicating a local duplication of NDST3 in the tetrapod lineage. Further, we used MOs to assess the function of Ndsts during zebrafish development and found that ndst1b morphants show malformations in the craniofacial cartilage, specifically in the second pharyngeal arch. With the strong expression in the rhombomeres of the hindbrain, from where neural crest cells migrate down to form the pharyngeal arches, we hypothesize that migration of neural crest cells might be sensitive to ndst1b expression levels (Paper IV).

**CRISPR/Cas9 as a tool to generate loss of function alleles (Paper V)**

In the last study we implemented the high throughput CRISPR/Cas9 technology to generate new loss of function alleles for GAG biosynthetic enzymes in zebrafish. We started out analyzing CS/DS chain initiation in csgalnact1 and csgalnact2 mutants (Figure 12), as well as
glycosyltransferases with strong expression in cartilage structures, chpfa (Paper III) and chsy1 (Li et al. 2010). We also included acana, the gene encoding for aggrecan, because of its high expression in pharyngeal cartilages (Thisse and Thisse 2004). 6-O sulfotransferases as well as 4-O sulfotransferases for CS/DS were added to the pipeline as well as the Ust, which according to our bioinformatics studies is the only enzyme catalyzing 2-O sulfation in CS/DS. Additionally we identified mutations in Ndsts and Papss. So far, we identified 32 new alleles with null mutations for 17 different genes involved in HS and CS/DS biosynthesis. To study the role of GAG biosynthetic enzymes in zebrafish development, we crossed heterozygous mutant carriers and studied early development in the offspring. At 5 dpf we sacrificed healthy looking larvae and genotyped them, to screen for homozygous individuals. We did find larvae homozygous for 13 alleles in 9 different genes and as we raised larvae to adulthood we also found homozygous adults for all 9 genes. All of the adult mutants, except csgalnact1^{1/-1/-1}, developed normal morphology, leading to the conclusion that none of the GAG biosynthetic enzymes we analyzed were alone essential for zebrafish development. csgalnact1^{1/-1/-1} are viable and fertile, but develop major craniofacial abnormalities. To further reduce Csgalnact activity we crossed csgalnact1^{1/-1} fish into the background of csgalnact2^{4/-2} and examined double mutants, finding embryonic craniofacial malformations and lethality at larval stages. The HS and CS/DS content and disaccharide composition in the generated animals remains to be investigated, to see how the lack of enzymes alters HS and CS/DS biosynthesis.

GAG biosynthesis involves many enzymes, often with similar enzymatic activity. This redundancy requires in addition to investigations of single mutants, studies of the effects of disrupting multiple genes simultaneously. The typical crossing of mutant fish lines includes multiple generations, which is time consuming. Instead, we applied an approach to inject multiple CRISPR/Cas9 targets for different genes into the same clutch of embryos. Thereby we generated, in addition to many F1 fish with single mutated alleles, also some fish with mutations in multiple genes. This allows us to study combinatorial effects of mutating multiple genes, already in the F2 generation. A first cohort of zebrafish mutants is now available to the research community and offers new tools to shed light onto the complex GAG biosynthesis.
Conclusion and future perspective

We identified zebrafish genes involved in HS and CS/DS biosynthesis and present, together with earlier described genes, what we believe is the complete set of enzymes (Figure 12).

GAG biosynthesis - more complex than anticipated

Studying the development of zebrafish with mutations in enzymes involved in the glycosaminoglycan biosynthesis, allowed us to see the interplay between HS and CS/DS, as well as the functional compensation of CS/DS. We found that mutations in GAG biosynthetic enzymes have other effects than predicted based on their respective position in the GAG biosynthetic pathway. This was obvious with the two zebrafish lines with mutations in umps1 and b3gat3 affecting the linkage region, but resulting in a much greater...
reduction in CS/DS compared to HS. The biochemical analyses of mutant tissues should thus always be performed. Studying mutations in GAG biosynthetic enzymes and correlating possible phenotypes to enzyme activity might lead to incomplete assumptions. The large number of isoforms for most enzymes and the redundancy of individual enzymes make the GAG machinery challenging to study.

**Functional cooperation between enzymes**

It is known that HS and CS/DS biosynthetic enzymes affect each other’s activity and sometimes even interact physically in the Golgi apparatus. Studying the temporal and spatial expression of all CS/DS biosynthetic enzymes during early development we identified when and where these enzymes could function together. For example, we found that previously reported functional interactions between DSE and CHST14 could occur in cartilage structures as well as the notochord during zebrafish development since these enzymes are co-expressed. Merely expression studies are not sufficient to fully understand the role of GAG biosynthetic enzymes and future functional studies are necessary.

**Rethinking Morpholinos with new tools to perform reverse genetics**

In the zebrafish model, MOs are widely used to knock down genes and to study their function during early development. The function of ten

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*Figure 12.* HS and CS/DS biosynthesis in zebrafish. HS and CS/DS chains are attached to serine (Ser) residues in the core protein. The first four monosaccharides are attached by respective glycosyltransferases to form the linkage region, which is common for HS and CS/DS. Extl3 initiates HS polymerization, while Csgalnact1 and Csgalnact2 have this function in CS/DS. Elongation of HS is carried out by Ext enzymes, while Chsy and Chpf enzymes polymerize the CS/DS chain. The first modification on HS is carried out by Ndst enzymes, replacing an acetyl group with a sulfate group on the C-2 position of GlcNAc. Glcea and Glceb epimerize GlcA into IdoA in HS, while Dse, Dsel and Dselb is responsible for that modification in CS/DS. In HS, Hs2sts add a sulfate group to the C-2 position on IdoA, sometimes also on GlcA. Hs6sts and HS3sts add a sulfate group to the C-6 respective C-3 position of GlcNAc. In CS/DS, Ust adds a sulfate group to the C-2 position on both IdoA and GlcA. Chst11, Chst12a, Chst12b and Chst13 add a sulfate group to the C-4 position in GalNAc, if next to a GlcA, and Chst14 acts on GalNAc neighboring IdoA. Chst3a, Chst3b and Chst7 add a sulfate group to the C-6 position of GalNAc residues, whereas Chst15 does the same, but only if the C-4 position was already sulfated. Blue bars indicate two zebrafish genes orthologous to a single mammalian gene. The star indicates a single zebrafish gene orthologous to two mammalian genes. Xyl: xylose, Gal: galactose, GlcNAc: N-acetylgulosamine, IdoA: iduronic acid, GlcA: glucuronic acid, GalNAc: N-acetylgalactosamine
biosynthetic enzymes has been investigated using MO technology and several studies show strong effects on embryonic development. Zebrafish combined gleca and glecb morphants exhibit severe ventralization in early embryos (Ghiselli and Farber 2005). In contrast, Glce null-mutant mice die after birth with respiratory failure, and they lack kidneys and show skeletal abnormalities (Dierker et al. 2015; Li et al. 2003). HS 2-O-sulfotransferases were also studied by MO injections and were reported to play crucial roles during epiboly initiation in the fish embryo (Cadwalader et al. 2012). The HS2ST gene trap mice die after birth with severe eye and skeletal defects, and renal agenesis (Bullock et al. 1998). The strong and early phenotypes in the zebrafish morphants, compared to mutant mice, indicate a larger dependence of these enzymes in zebrafish. These discrepancies between two model organisms, but also the difficulty to distinguish between off-target and specific effects in MO injected zebrafish embryos, emphasize the need of a genetic method to verify morphant phenotypes. With the new techniques available, targeted mutagenesis is finally possible in zebrafish and in a recent study mutants for genes previously studied with MOs were generated, and remarkably only 20% of the mutants could recapitulate the earlier published morphant phenotype (Kok et al. 2015).

New zebrafish mutants for GAG biosynthetic enzymes
In order to better understand the GAG biosynthesis and function in vivo, we want to create organisms with null alleles for every enzyme involved in the GAG machinery. With the high throughput CRISPR/Cas9 method this is now possible (Varshney et al. 2015) and the limiting factor is not anymore the generation of new loss of function alleles, but husbandry and the phenotypic analysis of the created knockouts. For the zebrafish mutants we established, we plan to perform detailed morphological and biochemical characterization. Performing this in both whole embryos and specific organs should give us more insights into HS and CS/DS biosynthesis. Additionally, we will hopefully be able to identify more mutants in our pipeline.

Zebrafish underwent an extra round of whole genome duplication keeping two copies of certain genes compared to mammals (indicated by blue bars in Figure 12). Together with the large number of isoforms and the redundancy in GAG biosynthesis this increases the potential players in polymerization and sulfation of the polysaccharides. In order to effectively reduce specific modifications we need to analyze multiple null alleles in one organism. Generating a F1 population with both single and multiple mutations in individual fish can be achieved by injecting multiple CRISPR/Cas9 targets simultaneously.
Further improvement of CRISPR/Cas9 technologies

More sophisticated CRISPR/Cas9 tools are currently being developed, providing more potential target sites, which will enable researchers to direct mutations to evolutionary conserved functional regions. In addition, some targets work better than others and we don’t exactly know why. The cheap and efficient synthesis of CRISPR/Cas9 reagents also make it possible to design several targets for each gene, inject them and test efficiency directly in the embryos (Carrington et al. 2015). Identifying the most efficient targets increases the chance to generate mutant lines, and reduces the use of tank space and workload for husbandry and genotyping.

GAGs and cartilage development

The extracellular matrix of cartilage is particularly rich in proteoglycans and some mutants, like ext2 and extl3 mutants, show similar phenotypes, including defects in pharyngeal cartilage and pectoral fin formation. Chondrocytes depend on GAGs in order to create highly organized elements in the pharyngeal arches, but to what extent GAG chains or specific sulfation “fingerprints” regulate morphogenesis of cartilage structures remains to be investigated. Advances in microscopy, especially imaging with light sheet technology, allow for detailed observation of morphogenesis in the generated mutants and will help us further understand the mechanism of chondrocyte interaction and skeletal development.

I vårt arbete fokuserade vi på heparansulfat (HS) proteoglykaner och chondroitin/dermatansulfat (CS/DS) proteoglykaner. För att studera funktionen av HS och CS/DS biosyntes använde vi oss av zebraplanter som saknar utvalda enzymer nödvändiga för uppbyggnaden av dessa sockerkedjor. I vissa mutanter fann vi morfologiska förändringar i broskstrukturer och efter biokemiska analyser kunde vi se att HS och CS/DS samspelar i uppbyggnad av broskstrukturer (arbete I). Lite var känt om CS/DS biosyntes i zebraplanter, därför beslöt vi att identifiera zebraplantergener som motsvarar tidigare identifierade gener inblandade i CS/DS biosyntes hos däggdjur.

Vi beskrev först alla CS/DS syntetiserande enzymer och studerade dess uttrycksmönster hos unga zebraplanter (arbete II). Vi fortsatte sedan med att beskriva grupper av modifieringsenzym för HS och CS/DS som inte tidigare analyserats (arbete III och IV). Dessa studier ger oss nu en full bild av var och när GAG biosyntesenzym är uttryckta. Överlappande uttryck av vissa glykosylderivatshverar stödjer tidigare hypoteser om att dessa enzym ger tillsammans för att polymerisera sockerkedjor.

I sista delarbetet använde vi oss av den nya CRISPR/Cas9-tekniken för att skapa mutationer i HS och CS/DS biosyntesenzym hos zebraplanter (arbete V). CRISPR/Cas9-tekniken möjliggör en effektiv och riktad mutagens hos zebraplanter, vilket inte var genomförbart tidigare. Man använder sig av en kort RNA-molekyl som vägleder Cas9, ett nuklease, till en specifik sekvens i
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With a friend by your side, no road seems too long.

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Thermal stability of proteins may be affected by heparin and its derivatives. Heparin has several anti-thrombotic and pro-angiogenic properties, which are likely due to its ability to interact with proteins. In vitro studies have shown that heparin can inhibit the aggregation of platelets and the activation of coagulation factors. Additionally, heparin has been shown to promote the proliferation of endothelial cells and the growth of blood vessels in vivo.

The anti-thrombotic activity of heparin is thought to be due to its ability to bind to factors such as factor Xa and thrombin, which are involved in the initiation and propagation of the coagulation cascade. This binding inhibits the proteolytic activity of these factors, preventing the formation of fibrin and the propagation of the coagulation cascade.

The pro-angiogenic activity of heparin is likely due to its ability to interact with growth factors such as vascular endothelial growth factor (VEGF). Heparin can bind to VEGF and prevent its degradation, allowing it to exert its pro-angiogenic effects.

In conclusion, heparin is a multifunctional molecule with both anti-thrombotic and pro-angiogenic properties. These properties make it a valuable therapeutic agent in the prevention and treatment of a variety of cardiovascular and vascular diseases.


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A doctoral dissertation from the Faculty of Science and Technology, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology”.)