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# The role of Nkx3.2 and Gdf5 during zebrafish skeletal development

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ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2021

ISSN 1651-6214
ISBN 978-91-513-1110-4
urn:nbn:se:uu:diva-430399

Dissertation presented at Uppsala University to be publicly examined in Ekmansalen, Evolutionsbiologiskt centrum, Norbyvägen 16, Uppsala, Friday, 26 February 2021 at 14:15 for the degree of Doctor of Philosophy. The examination will be conducted in English. Faculty examiner: Professor Abigail Tucker (King´s College London ).

**Abstract**

Waldmann, L. 2021. The role of Nkx3.2 and Gdf5 during zebrafish skeletal development. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology* 2002. 53 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-1110-4.

The vertebrate skeleton is composed of bony and cartilaginous structures that are developed under the control of numerous genetic networks. The transcription factor Nkx3.2 and the signaling molecule Gdf5 play a fundamental role during joint development and chondrogenesis, a process whereby mesenchyme cells form precartilaginous condensations followed by chondrocyte differentiation. Mutations in these genes can lead to some rare human skeletal diseases and are furthermore thought to play a role during osteoarthritis, whereby the articular cartilage in synovial joints degrades. Both genes are fairly well studied in amniotes, but their full function and regulation are not completely understood. This thesis focuses on further characterization of Nkx3.2 and Gdf5 function, by using the zebrafish *Danio rerio*, a small vertebrate, as a model organism.

We generated a CRISPR/Cas9 *nkx3.2* mutant zebrafish line and detected broad phenotypes in the axial skeleton. Nkx3.2 deficiency in knockout zebrafish confirms previously reported jaw joint loss, but also revealed new phenotypes in the occipital region, the Weberian apparatus, the vertebrae and some fins.

By identifying a cis-regulatory element of *nkx3.2* in zebrafish, we were able to generate a transgenic zebrafish line labelling the developing jaw joint and jaw joint progenitor cells. This line enables detailed documentation of jaw joint development and paves the way for a better understanding of joint development. Knockout of this *nkx3.2* enhancer sequence in zebrafish did not result in any phenotypic differences, indicating a redundant function. Besides the identification of a *nkx3.2* enhancer in the zebrafish genome, we identified homologous *nkx3.2* enhancer sequences in the genomes of multiple gnathostome species and found that they display a high degree of functional conservation.

To study the role of Gdf5, we generated a CRISPR/Cas9 *gdf5* mutant line. *gdf5* mutant zebrafish displayed abnormalities in endoskeletal elements of all median and the pectoral fins showing truncation of median fin endoskeletal elements and partial absence of pectoral fin radials.

Finally, we developed an optical projection tomography (OPT) based automated workflow to generate 3D reconstructions of *in situ* and skeletal-stained zebrafish embryos and larvae. The acquired imaging data of skeletal-stained larval zebrafish was subsequently used to quantify phenotypic differences between mutant and wild-type zebrafish groups. This technique allows for the identification of even subtle phenotypic differences at early stages of development.

To conclude, the work presented in this thesis provides further understanding of the role of Nkx3.2 and Gdf5 during skeletogenesis in zebrafish and contributes to the development of zebrafish imaging techniques.

*Keywords:* Nkx3.2, Gdf5, zebrafish, jaw joint, joints, axial skeleton, appendicular skeleton, fin, enhancer conservation, CRISPR/Cas9, OPT

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ISSN 1651-6214
ISBN 978-91-513-1110-4
urn:nbn:se:uu:diva-430399 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-430399)

*To my family*

The cover shows a 30 day old skeletal-stained zebrafish (lateral view).
Alcian blue stains cartilage in blue, and Alizarin red stains bone in red.

# List of Papers

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

I **Waldmann, L.***, Leyhr, J.*, Zhang, H., Öhman-Mägi, C., Allalou, A., Haitina, T. (2020). The broad role of Nkx3.2 in the development of the zebrafish axial skeleton.
**BioRxiv**. doi: 10.1101/2020.12.30.424496
*Submitted manuscript*
* These authors contributed equally to the work

II **Waldmann, L.**, Leyhr, J., Filipek-Górniok, B., Zhang, H., Allalou, A., Haitina, T. (2021). An evolutionarily conserved cis-regulatory element of *nkx3.2* drives jaw joint-specific expression in zebrafish.
*Manuscript*

III **Waldmann, L.***, Leyhr, J.*, Zhang, H., Allalou, A., Öhman-Mägi, C., Haitina, T. (2021). The role of Gdf5 in the development of the zebrafish fin endoskeleton.
*Submitted manuscript*
* These authors contributed equally to the work

IV Zhang, H., **Waldmann, L.**, Manuel, R., Boije, H., Haitina, T., Allalou, A. (2020). zOPT: an open source optical projection tomography system and methods for rapid 3D zebrafish imaging. *Biomedical Optics Express,* 11, 4290-4305.

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# Abbreviations

| | |
|---|---|
| bp | base pair |
| Cas | CRISPR associated |
| CRISPR | Clustered interspaced short palindromic repeats |
| crRNA | CRISPR RNA |
| dpf | days post fertilization |
| DNA | Deoxyribonucleic acid |
| DSB | Double-strand break |
| GFP | Green fluorescent protein |
| H&E | Hematoxylin-Eosin |
| hpf | hours post fertilization |
| micro CT/µCT | Micro computed tomography |
| MO | Morpholino |
| mRNA | messenger ribonucleic acid |
| NCCs | Neural crest cells |
| NHEJ | Non-homologous end joining |
| OPT | Optical Projection Tomography |
| PAM | Protospacer adjacent motif |
| RNA | Ribonucleic acid |
| sgRNA | Single guide ribonucleic acid |
| SMMD | Spondylo-megaepiphyseal-metaphyseal dysplasia |
| tracrRNA | trans-activating crRNA |
| WT | wild-type |

gene symbols are not listed

# Introduction

The vertebrate skeleton consists of numerous bones that provide support for the body and organ protection, but it also serves as the location for blood cell production and mineral storage. The development of the skeleton is complex and requires an interaction of several factors that ensure correct shaping and positioning of all skeletal elements. Bony tissue can be formed by intramembranous ossification, endochondral ossification, and perichondral ossification. Skeletal elements developing via intramembranous ossification originate from mesenchymal cell condensations, which undergo ossification without any intermediate steps. Endochondrally ossifying skeletal elements also originate from mesenchymal cell condensations, but the actual bone is formed from a cartilage template. In this case, mesenchymal cells undergo chondrogenesis and chondrocyte maturation to establish the cartilage template, which subsequently gets replaced by bony tissue. In order to provide skeletal mobility, bones require articulation in the form of joints. Joint development involves a complex interaction of both signaling molecules and transcription factors.

This work mainly focuses on two genes, one coding for the transcription factor Nkx3.2 and the other coding for the signaling molecule Gdf5. They are both involved in chondrogenesis (by repressing respectively enhancing cartilage maturation during endochondral ossification) as well as joint development during skeletogenesis. All studies presented here were carried out in the zebrafish (*Danio rerio*) at different developmental stages.

# Background

## Zebrafish anatomy and development

The zebrafish skeleton consists of both dermal and endochondral (or chondral) bones that comprise both exoskeletal and endoskeletal structures. Skeletal structures in the zebrafish include the skull, paired and median fins, and the vertebral column (Fig. 1).

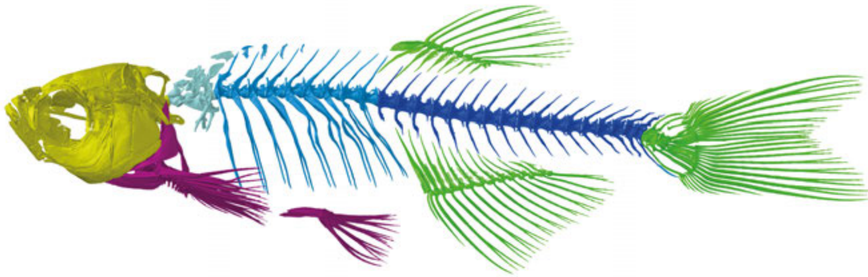

**Figure 1. Zebrafish (*Danio rerio*) skeleton.** The zebrafish skeleton comprises the skull (yellow), paired fins (magenta), median fins (green), and the vertebral column (different shades of blue). The first four vertebrae are referred to as the Weberian apparatus (light blue), followed by the rib-bearing abdominal region (medium blue) and the caudal region (dark blue).

The zebrafish skull can be divided into two main regions: the neurocranium and the viscerocranium. The viscerocranium embryonic cartilage elements originate from neural crest cells (NCCs) and form among others the pharyngeal arches, which give raise to lower and upper jaws as well as the gill bearing structures (Schilling and Kimmel, 1994). In the adult zebrafish skull, these cartilage elements are replaced by bone. Meckel´s cartilage and the palatoquadrate are derived from the first pharyngeal arch, also referred to as mandibular arch (Fig. 2). The first arch elements serve as a feeding apparatus where the U-shaped, ventrally-located Meckel´s cartilages function as the lower jaw and the dorsally-located palatoquadrate cartilages as the upper jaw. Flexibility and movement of the jaws are enabled by the articulation between Meckel´s cartilage and the palatoquadrate, which is referred to as the primary jaw joint. In the adult zebrafish, the first pharyngeal arch elements are partly replaced by the anguloarticular and quadrate bones. Another joint (hyoid) is located in the second pharyngeal arch or hyoid arch, linking the jaw supporting hyosymplectic and ceratohyal cartilages via a small element called the interhyal cartilage. The paired second arch ceratohyals and the paired ceratobranchial cartilages of the third to seventh arches connect to a ventral midline

consisting of the basihyal and posterior basibranchial (Fig. 2). Arches three to six are attached to the basibranchial via small cartilage elements called hypobranchials. In contrast, the paired ceratobranchial cartilages of arch seven do not connect to the branchial midline and are also the only elements containing dermal teeth in zebrafish and other Cypriniformes (Kimmel et al., 1995).

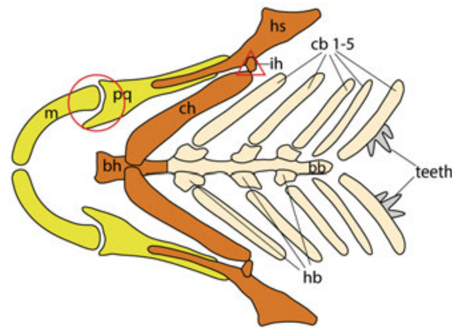


**Figure 2. Pharyngeal arches of the zebrafish larvae.** Schematic of craniofacial cartilage elements (ventral view). First pharyngeal arch in yellow, second pharyngeal arch in orange, arch 3-7 in beige.The circle marks the jaw joint, the triangle marks the hyoid joint. bb: basibranchial; bh: basihyal; cb: ceratobranchial cartilages; ch: ceratohyal; hb: hypobranchial; hs: hyosymplectic; ih: interhyal cartilage; m: Meckel´s cartilage; pq: palatoquadrate.

Skeletal structures in the neurocranium support the brain and sensory organs and derive from both NCCs and mesoderm (Kague et al., 2012). For my work, the occipital region is of special interest. The occipital region is the most posterior region of the skull and connects the skull to the vertebral column. The bones of the occipital series are the basioccipital, exoccipital, and supraoccipital (Fig. 3).

The segmented vertebral column in zebrafish can be subdivided into the Weberian, abdominal (precaudal), caudal, and caudal fin regions. The number of vertebrae can slightly vary between individual fish, with an average of 32 elements (Morin-Kensicki et al., 2002). The vertebral bodies (or centra) do not form from cartilage templates but develop from notochord secreted bone matrix (Fleming et al., 2004). The first four vertebrae, which bear modified ribs and neural arches are referred to as the Weberian ossicles/apparatus (Fig. 3). These structures connect the gas bladder to the inner ear and functions in sound transmission (von Frisch, 1938; Rosen and Greenwood, 1970).

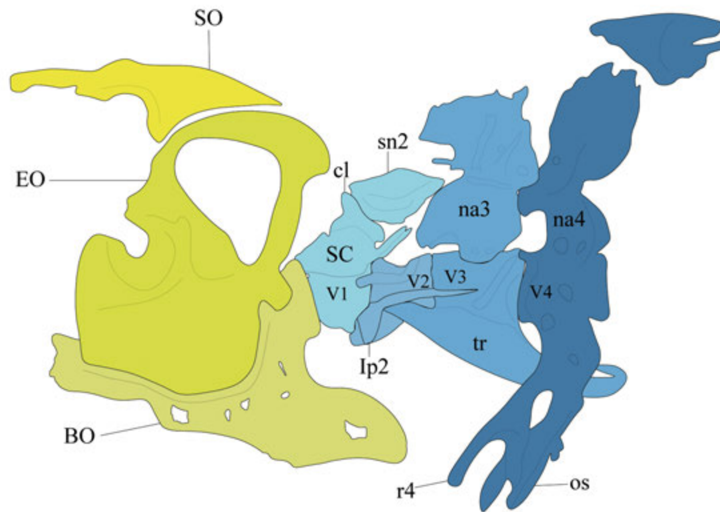


**Figure 3. Schematic drawing of the occipital bones and the Weberian apparatus in zebrafish (lateral view).** Occipital region is displayed in different shades of yellow. BO: basioccipital; EO: exoccipital; SO: supraoccipital. Weberian apparatus structures in different shades of blue. cl: claustrum ; lp2: lateral process 2 , na: neural arch; os: os suspensorium ; r4: rib 4; SC: scaphium; sn2: supraneural 2; tr: tripus; v: vertebrae.

The fins can be divided into paired and median fins. The pectoral and pelvic fins are paired fins whereas the dorsal, anal, and caudal fin are classified as median fins (Fig. 4). Here the focus will be on the pectoral and all median fins. The dorsal and anal fin are anatomically similar as they consist of proximal and distal radials connected to fin rays (lepidotrichia). The radials serve as internal support and originate from mesoderm. They initially develop from a single mesenchyme condensation and the distal radials segment away from the proximal radials later in development (Bird and Mabee, 2003).

The skeletal elements of the caudal fin that are most relevant for this thesis include the parhypural and hypurals 1-5, which all develop from individual mesoderm-derived mesenchyme condensations (Fig. 4) (Bird and Mabee, 2003).

The pectoral fin endoskeleton is composed of four proximal and six to eight distal radials (Fig. 4) (Grandel and Schulte-Merker, 1998). The proximal radials of the pectoral fin develop from a single cartilaginous disk which is subdivided by a zone of cartilage dedifferentiation that gives rise to two cartilage disk halves that then divide once more forming four proximal radials aligned along the anteroposterior axis (Dewit et al., 2011). Distal radials do not segment away from the proximal radials as in the dorsal and anal fin, but instead form independently (Grandel and Schulte-Merker, 1998). The pectoral fin endoskeleton belongs to the appendicular skeleton, homologous to the tetrapod

forelimb endoskeleton (Gehrke et al., 2015; Grandel and Schulte-Merker, 1998).

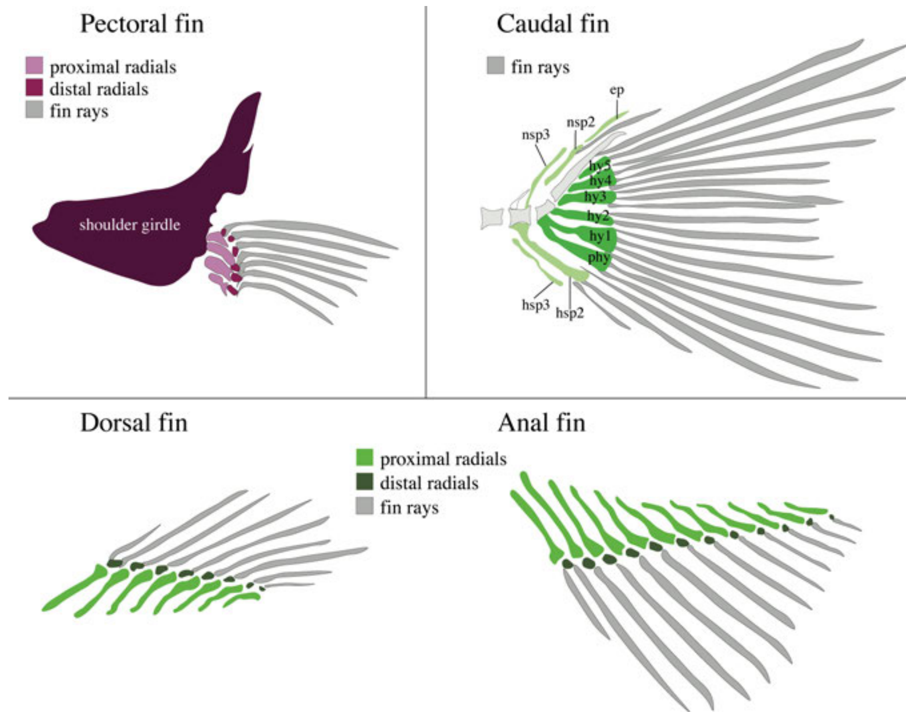


**Figure 4. Skeletal structures of zebrafish fins.** Schematic drawing of median (caudal, dorsal and anal) fins and pectoral fin (lateral view, anterior to the left). ep: epural; hsp: hemal spine; hy: hypural; nsp: neural spine; phy: parhypural.

## Chondrogenesis and chondrocyte maturation

The first stage of the development of endochondral bones is chondrogenesis and chondrocyte maturation, whereby the cartilage templates are established. Chondrogenesis begins with the formation of mesenchyme condensations which then differentiate into chondrocytes. These chondrocytes then mature into pre-hypertrophic and later hypertrophic chondrocytes. Hypertrophic chondrocytes then enter terminal maturation and get gradually replaced by endochondral bone (reviwed by Kozhemyakina et al., 2015). There are a number of genes expressed at different stages during this developmental process. Mesenchyme cells expressing *Sox9* are committed to a chondrogenic cell lineage, develop into chondroprogenitor cells and produce a high number of extracellular matrix proteins (Bi et al., 1999; Bi et al., 2001). Factors promoting chondrocyte maturation are, among others, Runx2, Runx3, and Wnt5a (Choi et al.,

2012; Yoshida et al., 2004). Indian Hedgehog (Ihh) is essential for chondrocyte hypertrophy and bone formation and positively regulates Runx2 and Wnt5a (Choi et al., 2012; Yoshida et al., 2004). In amniotes, it has been shown that Runx2 upregulates *Col10a1* in hypertrophic chondrocytes, which later undergo apoptosis or osteoblast differentiation (Fisher et al., 2003; Zheng et al., 2003).

## Patterning of the pharyngeal arches

The craniofacial skeleton of vertebrates originates from NCCs, a cell population that delaminates from the dorsal part of the developing neural tube and migrates in three streams to populate the seven pharyngeal arches (Schilling and Kimmel, 1994). Interacting signaling pathways guiding migrating NCCs are essential since the pharyngeal arches consist of complex elements located along the dorsoventral axis. The signals coordinating the migrated cells derive from endodermal and ectodermal epithelia and divide NCCs into dorsal, intermediate, and ventral subdomains.

The dorsal subdomain forms both the dorsal part of the palatoquadrate and the hyomandibula, and is controlled by Jagged-Notch signaling. Jagged-Notch is restricted to the dorsal domain by Endothelin 1 (Edn1), which is active in the ventral/intermediate domain (Zuniga et al., 2010; Barske et al. 2016). Elements of the intermediate domain include the posterior Meckel´s cartilage, the anterior palatoquadrate, the symplectic, the interhyal cartilage, and the posterior part of the ceratohyal. Distal-less (Dlx) genes in the intermediate domain are positively regulated by Edn1 and repressed by dorsal Jagged-Notch (Talbot et al., 2010; Zuniga et al., 2010). Bone morphogenetic protein (Bmp) signaling is active in the ventral domain, specifying the anterior Meckel´s cartilage and the anterior ceratohyal (Alexander et al., 2011). Bmp signaling suppresses Jagged-Notch and activates both Edn1 and Hand2, which restricts Dlx gene expression to the intermediate domain (Alexander et al. 2011; Miller et al. 2003; Zuniga et al. 2011). Both *nkx3.2* and *gdf5* are expressed within the jaw joint forming region in the intermediate domain (Bruneau et al., 1997; Miller et al., 2003).

## The transcription factor Nkx3.2

NK3 Homeobox 2 (*Nkx3.2*) is an evolutionary conserved homeobox-containing gene encoding for a transcription factor acting as a transcriptional repressor. The gene was first discovered in *Drosophila* (*bagpipe, bap*) where it plays a major role in the formation of the midgut musculature by subdividing mesoderm (Azpiazu and Frasch, 1993). In vertebrates, *Nkx3.2* (or *Bapx1*) is mainly expressed within the first mandibular arch and is necessary for primary

jaw joint development in non-mammalian vertebrates (Lukas and Olsson, 2018a; Miller et al., 2003; Square et al., 2015). Knock-down experiments in both *Xenopus* and zebrafish result in loss of the jaw joint and as a consequence fusion of Meckel´s cartilage and palatoquadrate (Lukas and Olsson, 2018a; Miller et al., 2003). Zebrafish embryos injected with *nkx3.2*-MO furthermore display loss of the retroarticular process (RAP), a cartilaginous process at the dorsoventral tip of Meckel´s cartilage (Miller et al., 2003). Upregulation of *bapx1* expression induces ectopic joint development in the first pharyngeal arch of *Xenopus* (Lukas and Olsson, 2018b). In zebrafish, *nkx3.2* is positively regulated by Edn1 signaling (Miller et al., 2003). Loss of Edn1 or its receptor Endothelin type-A receptor (EdnrA) causes transformations in ventral and intermediate arch domains including jaw joint loss (Miller et al., 2003; Nair et al., 2007). Ventrally expressed *hand2* furthermore restricts *nkx3.2* to the jaw joint forming region in the intermediate domain (Miller et al., 2003). By taking together those findings it can be concluded that Nkx3.2 displays joint promoting abilities and is important for the primary jaw joint development. Further *nkx3.2* expression in zebrafish has been described to be present in the occipital region, the vertebrae, and the median fins (Crotwell and Mabee, 2007).

The pharyngeal skeleton of extant jawless vertebrates is cartilaginous and derived, as in all vertebrates, from NCCs (McCauley and Bronner-Fraser, 2003). *nkx3.2* in lamprey, a jawless vertebrate, is expressed in ectomesenchyme surrounding the pharyngeal arches (Kuraku et al., 2010; Miyashita, 2018). A hypothesis proposed by Cerny et al. (2010) suggests that incorporation of *nkx3.2* into the intermediate domain of the first arch during evolution led to the emergence of the jaw.

In mice, *Nkx3.2* is expressed within the middle ear associated bones tympanic ring and gonium as well as in the incudomalleolar joint. Mice embryos deficient in *Nkx3.2* do not show any skeletal defects in the middle ear ossicles, but the tympanic ring is hypoplastic and the gonium absent (Tucker et al. 2004). More drastic effects of *Nkx3.2* loss in mice are prominent in the axial skeleton including the skull. The two cranial bones basioccipital and basisphenoid are reduced in size and several bones of the vertebral column are either lost or deformed in response to Nkx3.2 deficiency (Lettice et al., 1999; Tribioli and Lufkin, 1999).

## The role of Nkx3.2 during skeletogenesis

The transcription factor Nkx3.2 has been shown to act as chondrocyte maturation inhibitor (Kim et al., 2015; Provot et al., 2006). In endochondrally ossifying chicken and mouse long bones, *Nkx3.2* expression is restricted to proliferative immature chondrocytes and inhibits chondrocyte maturation via repression of the chondrocyte maturation factor Runx2 (Lengner et al., 2005; Provot et al., 2006). Overexpression of Nkx3.2 in mice resulted in cartilage

hypertrophy delay and dwarfism of endochondrally ossifying skeletal elements (Jeong et al., 2017). Nkx3.2 is directly upregulated by Sox9, a transcription factor that promotes early stages of chondrogenesis but represses hypertrophic differentiation (Akiyama et al., 2004; Yamashita et al., 2009). Inhibition of Nkx3.2 is promoted by Phosphatidylinositol-3-kinase (PI3K) (Kim et al., 2015). Inhibition of PI3K in pharmacologically treated cell cultures leads to increased *Nkx3.2* expression leading to suppression of chondrocyte maturation (Kim et al., 2015). *Nkx3.2* expression in the mandibular arch in chicken is restricted by oral epithelium expressed fibroblast growth factor 8 (Fgf8) and distal mandibular arch expressed bone and morphogenic protein 4 (Bmp4) (Wilson and Tucker, 2004). Degradation of Nkx3.2 can be induced by Indian Hedgehog (Ihh) via a Wnt5a dependent pathway promoting cartilage development (Choi et al., 2012).

### Nkx3.2 related human diseases

Nkx3.2 is linked to the rare human disease spondylo-megaepiphyseal-metaphyseal dysplasia (SMMD) (Hellemans et al., 2009; Silverman and Reiley, 1985; Simsek-Kiper et al., 2019). The disease is caused by a homozygous mutation in the first or second exon of *nkx3.2* resulting in a frameshift and a premature stop codon (Hellemans et al., 2009; Simsek-Kiper et al., 2019). Patients suffering from SMMD display the following phenotypic differences: short stature and trunk, long limbs, enlarged head, reduced neck and back mobility, lack of vertebral body ossification, large epiphyseal ossification centres, wide cartilage zones between epiphyseal ossification and metaphyses, truncated ribs and pseudoepiphyses in hands and feet (Hellemans et al., 2009). Nkx3.2 might furthermore play a role during osteoarthritis, a chronic joint disease, caused by gradual degradation of articular cartilage in synovial joints. It is suggested that Nkx3.2 might be essential to prevent articular cartilage cells from differentiation into hypertrophic chondrocytes (Caron et al., 2015).

## Growth and differentiation factor 5

Growth and differentiation factor 5 (Gdf5) belongs to the TGF-beta (transforming growth factor-beta) superfamily and is also previously known as Contact, BMP14, or CDMP1. Gdf5 binds to BMP Receptor 1B and 1A, and together with other BMPs in the TGF-beta superfamily is involved in skeletogenesis (Nishitoh et al., 1996). The expression pattern of *gdf5* in zebrafish embryos and larvae has been extensively studied and is present in the primary jaw joint, the midline of the future basihyal cartilage element, the pectoral fins and all median fins at the onset and during chondrogenesis (Bruneau et al., 1997; Crotwell et al., 2001; Miller et al., 2003; Schwend and Ahlgren, 2009).

*nxk3.2*-MO injection in zebrafish results in the loss of craniofacial *gdf5* expression*,* indicating that *gdf5* expression is regulated by Nkx3.2 (Miller et al., 2003). However, functional studies of *gdf5* in zebrafish are lacking. In amphibians, *gdf5* is expressed in the intramandibular joint as well as in the limb buds of the appendicular skeleton (Satoh et al., 2005; Square et al., 2015b). In amniotes, *gdf5* expression is restricted to the limbs and prominent in developing autopod mesenchyme, cartilage, and joints (Merino et al., 1999; Storm and Kingsley, 1996). Gdf5 loss in mice and the resulting phenotype, referred to as *brachypodism*, has been widely studied (Gruneberg and Lee, 1973; Storm and Kingsley, 1996; Storm et al., 1994). *Brachypodism* mice display shortened limb bones and severe deformations in the digits in the form of shortened phalanges, lost digit elements and occasional loss of digit joints (Gruneberg and Lee, 1973; Storm and Kingsley, 1996; Storm et al., 1994).

## The role of Gdf5 during skeletogenesis

The role and function of Gdf5 during skeletogenesis have been mainly studied in mice, chicken, and cell cultures. Overexpression and ectopic supply of Gdf5 enhances both maturation and differentiation of chondrogenic cells in a stage-dependent manner (Buxton et al., 2001; Coleman and Tuan, 2003; Merino et al., 1999; Storm and Kingsley, 1999). It has furthermore been shown that Gdf5 is able to stimulate mesenchyme cells to differentiate into cartilage in both mesenchymal cell culture and in mouse digits, at certain developmental stages (Merino et al., 1999). These results are consistent with observations in Gdf5 deficient mice, where hypertrophic cartilage zones are reduced in size and consequently lead to a delay of ossification (Storm and Kingsley, 1999).

Sox11, a factor expressed in cartilage condensations during early stages of chondrogenesis and in the joint interzone at later stages, has been suggested to positively regulate Gdf5 expression (Kan et al., 2013). Sox11 binding sites have been identified in the 5´UTR region of the Gdf5 gene and overexpression of *Sox11* both *in vivo* and *in vitro* increases levels of *Gdf5* mRNA expression (Kan et al., 2013). Wnt14, an important factor for synovial joint development is able to activate and increase *Gdf5* expression during elbow joint development and in digit regions in mouse (Guo et al., 2004). It has furthermore been suggested that Gdf5 binding to the TGF-beta superfamily antagonist Noggin might play an important role during joint development and cartilage shape regulation (Brunet et al., 1998; Guo et al., 2004; Merino et al., 1999).

## Gdf5 related human diseases

The two rare human chondroplasias, Grebe type (CGT) and Hunter-Thomson type (CHTT) are caused by mutations within the *gdf5* gene sequence (Martinez-Garcia et al., 2016; Thomas et al., 1996). Patients that possess these *gdf5* mutations display a similar phenotype to what is seen in Gdf5 deficient

mice. Both CGT and CHTT patients display phenotypes restricted to the limbs whereby long bones and digit bones (especially the phalanges) are reduced in size. In both cases, proximal elements are more affected than distally located elements (Martinez-Garcia et al., 2016; Thomas et al., 1996).

Several studies furthermore indicate that functional single-nucleotide polymorphisms in the *Gdf5* gene are a risk factor for the development of osteoarthritis (Chapman et al., 2008; Daans et al., 2011; Miyamoto et al., 2007).

## The transition of the primary jaw joint into the middle ear

A common feature for all gnathostomes is, as the name already indicates (gnathos=jaw, stoma=mouth), a jawed mouth. Elements forming the jaw within gnathostomes however differ significantly between mammalian and non-mammalian vertebrates.

The jaw in non-mammalian vertebrates is formed by the articular and quadrate bones that articulate via the primary jaw joint. By taking a closer look into mammalian evolution it becomes clear what leads to the mammalian condition where the dentary and squamosal, connected via the secondary jaw joint, function as jaw elements. The fossil record of mammalian ancestors from the Permian and Triassic display a gradual expansion of the dentary and incorporation of the primary jaw joint elements into the middle ear (reviewed by Takechi and Kuratani 2010). This transformation was accompanied by the development of the secondary jaw joint stepwise taking over the role of the primary jaw joint, allowing its integration and development into the middle ear. Fossils of some extinct synapsids such as *Diarthrognathus* (200 Mya) display a double-joint state whereby the secondary dermally ossified jaw joint was applied for feeding, enabling the medial movement of the primary endochondral jaw joint into the middle ear (Crompton, 1963). A similar transition is observable in living marsupials (Maier, 1987). During early developmental stages in marsupials, elements forming the middle ear bones incus and malleus support the feeding apparatus and enable suckling action. A few weeks after birth, the secondary jaw joint comprised of the dentary and squamosal arises leading to the state of a double jaw joint, enabling the incus and malleus to gradually move into the middle ear (Clark and Smith, 1993; Filan, 1991).

Reichert (1837) was the first to launch the theory about the homology between mammalian middle ear ossicles and non-mammalian first and second pharyngeal arch elements based on anatomy studies. Further studies confirmed homology between non-mammalian first pharyngeal arch elements Meckel´s cartilage and palatoquadrate and mammalian middle ear ossicles malleus and incus, respectively, as well as second arch element hyosymplectic

and middle ear ossicle stapes (Gaupp, 1912). As Meckel´s cartilage and palatoquadrate derive from a single mesenchymal condensation, which, later in development, is divided by the primary jaw articulation (Wilson and Tucker, 2004), the question arose whether malleus and incus also develop from a single mesenchymal condensation. Experiments in mice revealed that malleus and incus are united at the onset of cartilage development before they separate during joint development (Amin and Tucker, 2006) (Fig. 5).

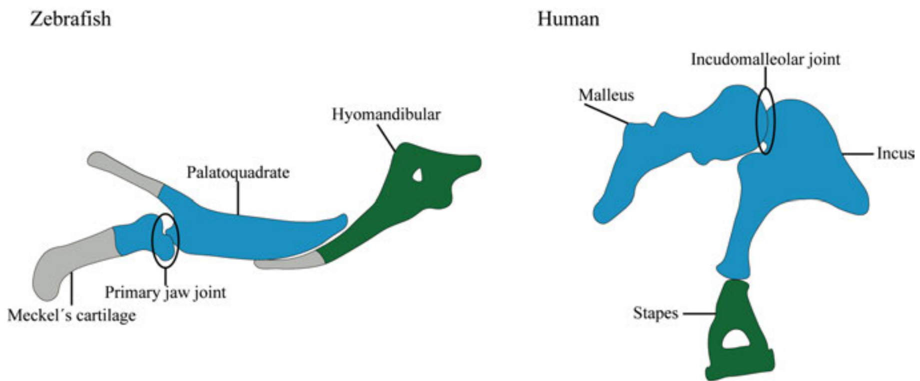


**Figure 5. Schematic representation of homology between the first and second pharyngeal arch in zebrafish to the middle ear in humans.** The posterior part of Meckel´s cartilage and the palatoquadrate are homologous to the mammalian middle ear ossicles malleus and incus (highlighted in blue). The second pharyngeal arch element hyomandibular of non-mammalian vertebrates (including zebrafish) is homologous to the middle ear ossicle stapes (highlighted in green).

## Characteristics and formation of a synovial joint

The evolution of jaw joints was a major event providing jaw flexibility and enabling active feeding. The fossil record verifies that articulated jaws did not evolve as originally suggested in Osteichthyes, but were already present in Silurian placoderms (Zhu et al., 2013). The most sophisticated joints in vertebrates are referred to as synovial joints. They enable a high degree of flexibility and enable movement of the skeleton. Synovial joints are present in the knee and elbow joints, among others, of tetrapods. They are characterized by a cavity filled with synovial fluid, allowing frictionless movement of the articulating bones. The fluid mainly consists of macromolecules like hyaluronic acid and lubricin (Tanaka et al., 2008) and is produced by synovial cells forming the capsule around the cavity, and by articular cartilage lining the surface of the articulating bones. The jaw joint of zebrafish has been shown to display synovial-like characteristics as the expression and function of the lubricin encoding gene *prg4b* has been described in joint-lining cells (Askary et al., 2016). However, whether the zebrafish jaw joints display additional synovial

joint characteristics such as synoviocytes remains unclear. At the onset of long bone formation mesenchyme cells condense before undergoing chondrogenesis. However, at the site of joint development mesenchyme cells do not undergo chondrogenesis but flatten and form an area referred to as interzone (Archer et al., 2003; Craig et al., 1987). Cells from the interzone partly give rise to joint-specific cells and structures (Archer et al., 1994; Mitrovic, 1977). A more recent study suggests that an additional continuous influx of cells into the interzone is necessary for the formation of joint components (Shwartz et al., 2016). Interzone formation is followed by joint cavitation, a process, which is suggested to be primarily facilitated by increasing extracellular matrix synthesis in order to separate the two articulating cartilage elements (Edwards et al., 1994). After cavitation, synovial-specific structures are formed, including the fluid filled cavity (Fig. 6).

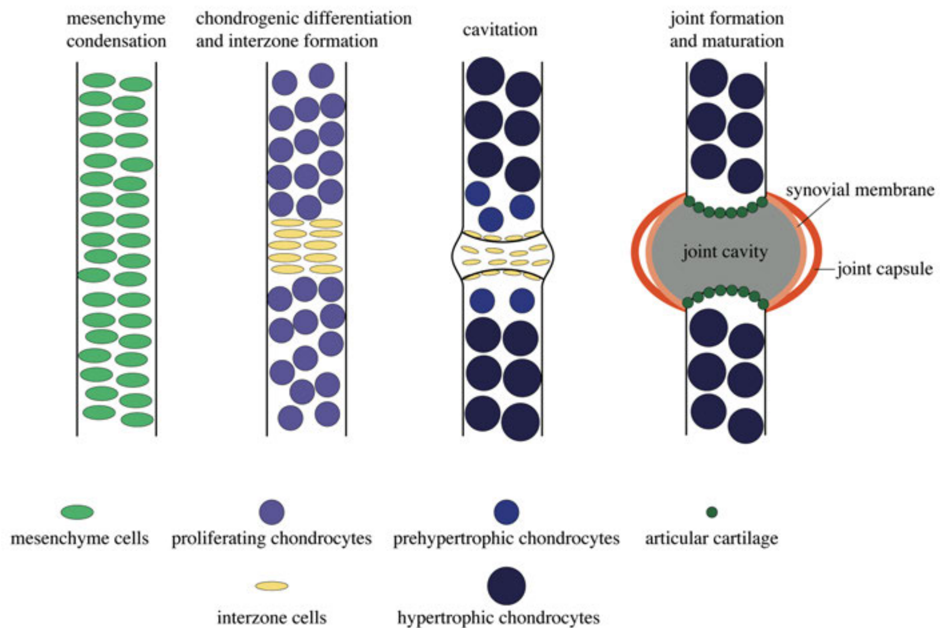


**Figure 6. Schematic drawing of synovial joint development.** Mesenchyme condensation giving rise to bony elements. Mesenchyme cells committed to the chondrogenic line undergo chondrogenesis and differentiate into chondrocytes which mature into prehypertrophic and hypertrophic chondrocytes. The interzone is formed by non-chondrogenic interzone cells which determine the position of the developing joint. Cavitation takes place, which separates the adjacent skeletal elements from each other. The mature synovial joint consists of a fluid-filled joint cavity, which is surrounded by a synovial membrane and the joint capsule. Articular cartilage is present on the cavity facing surfaces of the articulating elements.

## Transcriptional enhancers

Cis- and trans-regulatory elements are DNA sequences that control and regulate gene expression in eukaryotes. Studies have shown that these elements have contributed significantly to creating phenotypic differences and variety during evolution (Indjeian et al., 2016; Shapiro et al., 2004; Sucena and Stern, 2000). Enhancers are categorized as cis-regulatory elements and are non-coding sequences that can activate gene transcription. The cis-regulatory element can be in close proximity to the gene it is regulating but can even be located further away (Kleinjan et al., 2001; Lettice et al., 2002). Enhancers contain a variety of binding sites, also called motifs, that interact with transcription factors and other regulatory molecules (Fig. 7). For regulating transcription, the enhancer has to be in close proximity to the promoter of the gene it is regulating, which is achieved by cohesin and mediator complexes that create a DNA loop (Amano et al., 2009; Kagey et al., 2010; Schmidt et al., 2010). Transcription factors and mediator proteins interact with the enhancer sequence and recruit RNA polymerase to initiate gene transcription (Fig. 7) (Malik and Roeder, 2010).

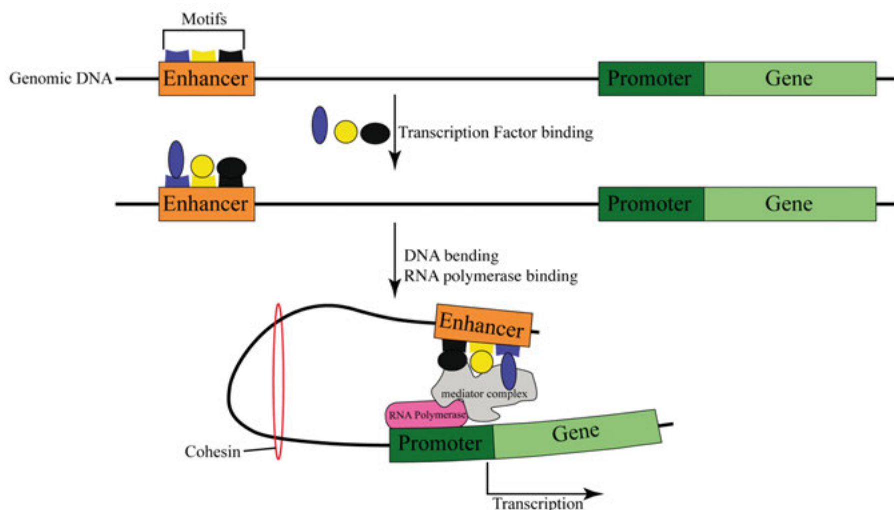


**Figure 7. Schematic drawing of enhancer function**. Enhancer sequences can be up- or downstream of the gene they are regulating and contain transcription factor binding sites (motifs). Transcription factor binding accompanied by cohesin mediated DNA looping and mediator complex binding brings the enhancer into close proximity to the regulating gene. The complex recruits RNA polymerase, which subsequently activates or enhances gene transcription.

## Zebrafish as a model organism

The zebrafish (*Danio rerio*) is a small freshwater tropical teleost fish of the family Cyprinidae. Their natural habitats are rivers and small streams in south Asia (reviewed by Engeszer et al., 2007). Starting in the 1970s when Georg Streisinger first used the zebrafish to study the nervous system, it became more and more popular and established as a model organism. The early development of the zebrafish has been extensively studied and described by Kimmel et al. (1995). They have described the development from fertilization until early larval stages at an incubation temperature of 28.5°C, which was set as the standard temperature for experimental use (Kimmel et al., 1995).

In contrast to other model organisms such as mice and rats, zebrafish are comparatively inexpensive to keep and easy to breed and handle. Other advantages include a high reproduction rate, external fertilization and development, rapid development, and transparency at early embryonic stages. The transparency of embryos is particularly convenient for *in vivo* functional studies in transgenic zebrafish lines. The high reproduction rate is especially useful to perform large-scale forward genetic screens. For studying craniofacial development in zebrafish, forward genetics techniques have been applied to identify multiple mutants displaying defects in the formation of the pharyngeal arches and cartilage differentiation (Piotrowski et al., 1996; Schilling et al., 1996).

Subsequently, to study the function and role of specific genes, reverse genetic approaches were developed and applied. One method which can be used to for generating gene knock-downs are microinjection of morpholino oligonucleotides (MOs). These are synthetic antisense oligonucleotides targeted at specific mRNAs and can inhibit both splicing and translation, resulting in a gene knock-down (reviewed by Summerton and Weller, 1997). Several negative side effects caused by MO-injections such as high off-target rates and non-specific toxicity, resulted in increased use of knock-out techniques (Nasevicius and Ekker, 2000). To generate more efficient and reliable zebrafish null mutants, new targeted mutagenesis tools such as TALENs (Christian et al., 2010), zinc-finger nucleases (Foley et al., 2009) and CRISPR-Cas9 (Cong et al., 2013) have been developed.

# Methods

## Transgenesis in zebrafish using the Tol2 system

Transgenic zebrafish lines are a great tool to label and study specific cells and tissues in the living organism. The principle of transgenesis is rather straightforward and first requires the identification of gene-specific enhancer or promoter, which is incorporated into the genome in combination with a fluorescent protein (reporter). After successful genome integration, only cells with the capability of enhancer/promoter activation are going to express the fluorescent protein, which can be visualized by using *in vivo* fluorescent microscopy. A very common method for effective genome integration is the Tol2 transposon system (Kawakami, 2007). Transposable elements are naturally occurring DNA sequences with the ability to change their location in the genome by excision and reintegration. Tol2 is an autonomous transposon which has been identified in the medaka fish *Oryzias latipes* (Koga et al., 1996). The Tol2 transposase recognizes inverted terminal repeats (ITR) and excises and reinserts DNA elements flanked by ITRs.

For generating zebrafish transgenic lines, a plasmid containing the desired enhancer or promoter sequence and a fluorescent protein is generated, flanked by Tol2 recognition sites (Fisher et al., 2006). The plasmid is injected into zebrafish embryos at the one-cell stage, together with transposase mRNA, which facilitates the excision of the Tol2 flanked DNA construct from the plasmid and integration into the zebrafish genome (Fig. 8). Since the construct is not evenly distributed after injection and subsequent cell division, the injected embryos (F0 generation) display mosaic fluorescent expression. For generating stable transgenic lines, the construct needs to be integrated into the germline of F0 fish. Germline transmission can be detected by outcrossing founder F0 with wild-type fish and analyzing F1 generation embryos (Fig. 8).

For vector assembly we used multisite Gateway-based cloning system for Tol2 transgenesis constructed by Kwan et al. (2007) in combination with a commercially available Gateway system from Invitrogen.

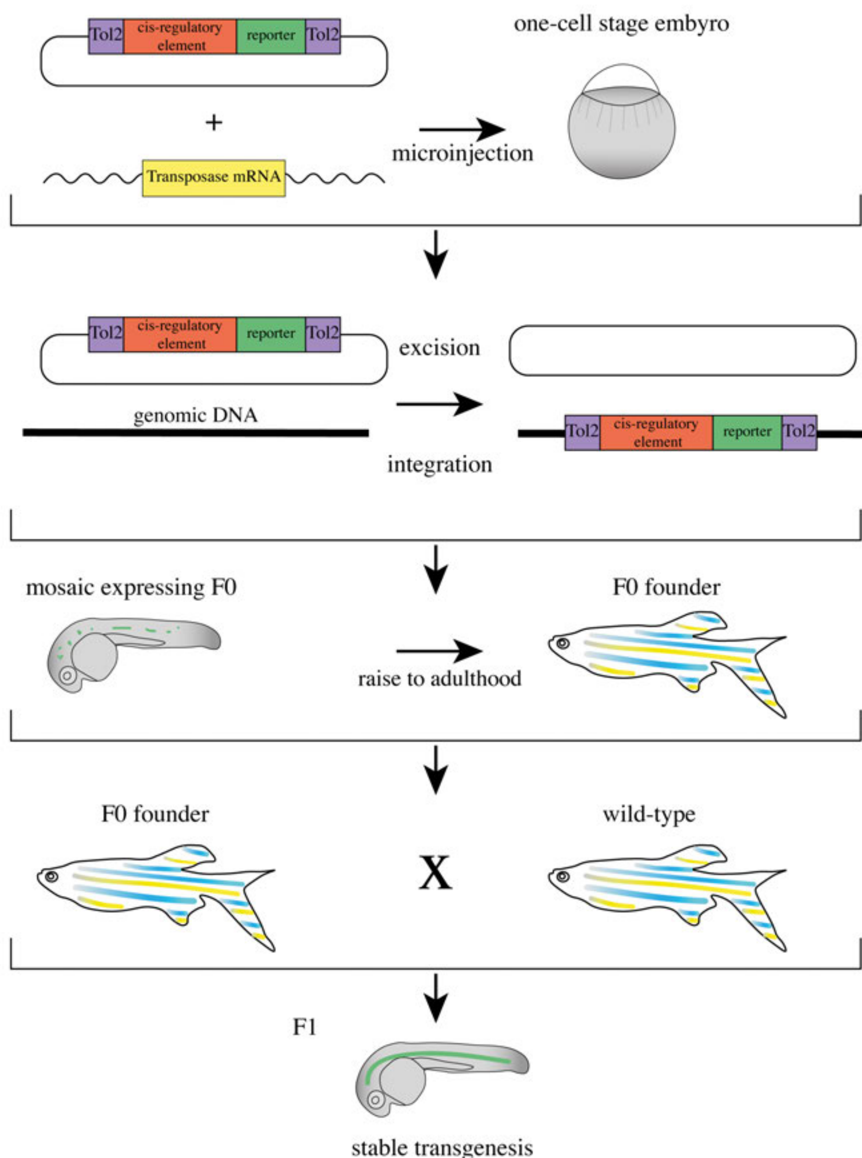


**Figure 8. Overview of the Tol2 mediated transgenesis workflow in zebrafish.** A construct containing the cis-regulatory element of interest and a fluorescent reporter gene flanked by Tol2 recognition sites is co-injected together with Tol2 transposase mRNA into a fertilized one-cell stage zebrafish embryo. After injection, Tol2 transposase mRNA is translated into Tol2 transposase which recognizes Tol2 sites on the vector and leads to the excision of the Tol2 site flanked construct and random integration into the zebrafish genome. Injected embryos display mosaic expression and are outcrossed in wild-type fish to confirm germline transmission in the F1 embryos, leading to the generation of a stable transgenic line.

# CRISPR/Cas9 genome editing

## From defense in prokaryotes to genome editing

The CRISPR/Cas system is a naturally occurring adaptive immunity response in bacteria and archaea to protect them against phages and viruses (Barrangou et al., 2007). It all started with the discovery of repeated structures separated by variable spacer sequences in the *Escherichia coli* and the halophilic Archaea *Haloferax mediterranei* genome (Ishino et al., 1987; Mojica et al., 1993). These structures, identified in more prokaryote species and named for Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR) (Jansen et al., 2002; Mojica et al., 2000) have been shown to be of viral and bacteriophage origin (Bolotin et al., 2005; Mojica et al., 2005; Pourcel et al., 2005). Prokaryotes containing spacers specific for certain viruses or phages display resistance against the invader, leading to the conclusion that CRISPRs act as adaptive immunity system (Barrangou et al., 2007; Bolotin et al., 2005; Mojica et al., 2005). Cas (CRISPR associated sequence) genes that have been detected in close proximity to CRISPRs helped to further investigate on how CRISPR is working (Haft et al., 2005; Jansen et al., 2002; Makarova et al., 2006). CRISPRs are transcribed and processed into crRNAs (CRISPR-RNAs), which are short RNA sequences (Mojica et al., 2000; Tang et al., 2002). crRNAs form a complex with Cas proteins and detect invading protospacer sequences which are subsequently degraded by the Cas-proteins (Brouns et al., 2008; Garneau et al., 2010; Marraffini and Sontheimer, 2008). Additionally, Cas proteins have been shown to be involved in integrating new spacer sequences into the CRISPR loci and in generating crRNAs (Bhaya et al., 2011; Terns and Terns, 2011). Another crucial element necessary for invader DNA recognition and detection are protospacer adjacent motifs (PAMs). PAMs are flanking protospacer sequences and are essential for Cas9 proteins to detect invading sequences. PAMs are not present in CRISPR loci spacers which protects the prokaryote from degrading their own CRISPR spacers (Bolotin et al., 2005; Horvath et al., 2008; Mojica et al., 2009).

The idea to use the CRISPR/Cas system as a genome editing tool emerged after the discovery of the CRISPR/Cas9 system. In 2020, two researchers; Emmanuelle Charpentier and Jennifer A. Doudna were awarded the Nobel Prize in Chemistry for their research leading to the use of the CRISPR/Cas9 system for genome editing. Together, they discovered the tracrRNA (trans-activating crRNA) and its function, another important puzzle piece to understand CRISPR/Cas9 system in more detail (Deltcheva et al., 2011; Jinek et al., 2012). The tracrRNA sequence is partly complementary to the repeated regions of the CRISPR locus and is involved in the maturation process of the crRNA (Deltcheva et al., 2011). tracrRNA is furthermore essential for the Cas9 endonuclease to introduce DNA double-strand breaks into the targeted protospacer sequences (Deltcheva et al., 2011; Jinek et al., 2012).

The genome editing technique using CRISPR/Cas9 is based on a synthetized chimeric sgRNA (single guide RNA) molecule consisting of the target DNA sequence (around 23 nucleotides) and the crRNA:tracrRNA. After co-injection of sgRNA and Cas9 protein or mRNA, the sgRNA forms a complex with the Cas9 protein and guides it to the target sequence. The Cas9 endonuclease subsequently introduces a double-strand break about 3 bp upstream of the PAM site (Fig. 9). Double strand breaks are repaired by the natural repair mechanism of the cell by either non-homologous end joining (NHEJ) or homology-directed repair (HDR). The DNA repair machinery however is not error-proof and often leads to the introduction of insertions and deletions (INDELS), which in most cases cause a frameshift and consequently the occurrence of a premature stop codon (Fig. 9).

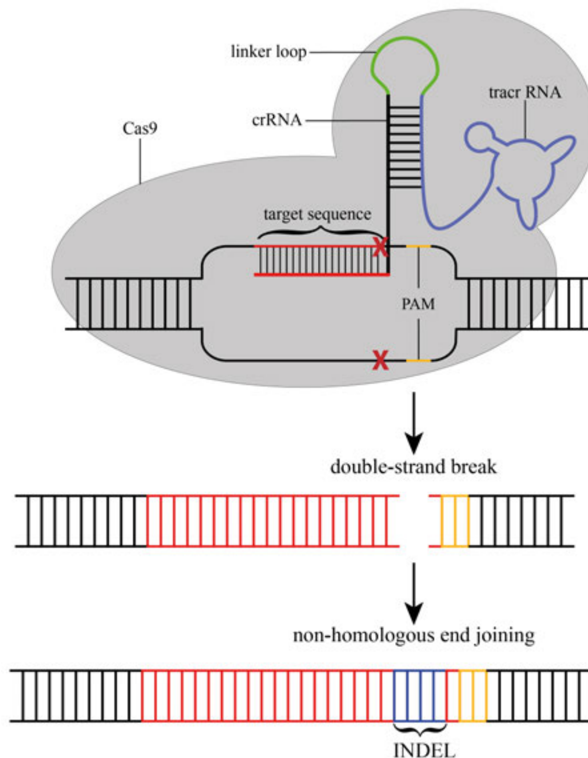


**Figure 9. Schematic drawing of CRISPR/Cas9 mediated genome-editing mechanism.** A single guide RNA, consisting of tracrRNA which is connected via a linker loop to the crRNA that recognizes the genomic region of interest. The sgRNA is designed to recognize sequences which are in close proximity to a PAM sequence (5'-NGG-3') and able to guide/recruit the Cas9 endonuclease which introduces a double-strand break (DSB). The DSB is repaired by non-homologous end joining (NHEJ), an error-prone pathway that often results base-pair insertions or deletions (INDELS). This often leads to a frameshift and the occurrence of a premature stop codon.

**CRISPR/Cas9 in zebrafish**

A number of optimization steps are required for successful targeted mutagenesis with CRISPR/Cas9 in zebrafish. In order to achieve high sgRNA activity, the target sequence should be designed with a high GC-content and a length of around 18 bp (Gagnon et al., 2014; Moreno-Mateos et al., 2015). A zebrafish codon-optimized version of the Cas9 mRNA sequence has been generated and shown to increase nuclear targeting which leads to higher mutagenesis frequencies (Jao et al., 2013).

In our experimental set up we applied a cloning-free sgRNA synthesis protocol developed by the Burgess Lab (Varshney et al., 2015). After co-injection of one to two sgRNAs and Cas9 mRNA into one-cell stage embryos, we followed a high-throughput workflow protocol for CRISPR/Cas9 in zebrafish, allowing somatic mutation efficiency verification in the F0 injected embryos (Varshney et al., 2016). Once sufficient sgRNA-Cas9 activity was detected, F0 fish were raised to adulthood and outcrossed with wild-type fish (Fig. 10). Mutation screens were performed in F1 generation to identify heterozygous mutant fish. To obtain uniform mutant fish, we performed additional outcross steps of the F1 heterozygote mutant founders. The homozygote mutant phenotypes were analyzed in the offspring of the F2 fish carrying heterozygous uniform alleles (Fig. 10).

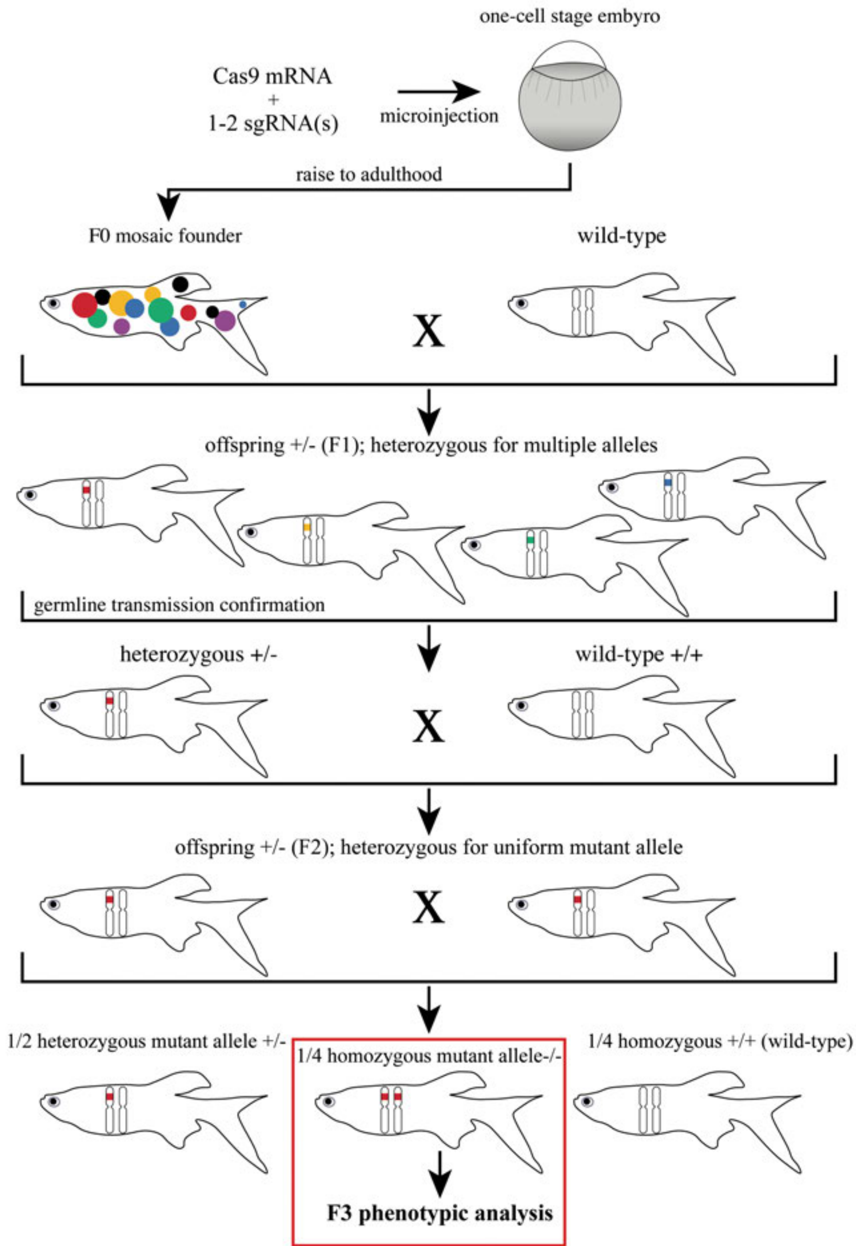


**Figure 10. Overview of the CRISPR/Cas9 workflow in zebrafish.** One to two sgRNAs targeting the same gene are co-injected together with Cas9 mRNA into a fertilized one-cell stage zebrafish embryo, resulting in chimeric embryos which are raised to adulthood. F0 mosaic founder fish are outcrossed with wild-type fish and the F1 generation is screened for germline-transmitted mutations. In order to obtain a sufficient number of heterozygous fish carrying the uniform allele of interest (here red mark in the chromosome), additional crosses with wild-type are necessary. F2 heterozygous fish carrying the uniform mutant allele are incrossed to obtain homozygote mutant fish in the F3 generation.

## Histology

For histological examination of zebrafish tissues I performed Hematoxylin-Eosin (H&E) and Nuclear Fast Red staining on deparaffinized thin sections. H&E stain is a standard stain that marks cells and tissues in blue and different shades of pink. Hematoxylin is a positively charged basic dye and stains nucleic acids blue (Chan, 2014; Fischer et al., 2008). Eosin is a negatively charged acid dye and stains cytoplasm and extracellular matrix in different shades of pink/red (Chan, 2014; Fischer et al., 2008).

In contrast to H&E staining, Nuclear Fast Red staining is faster to perform, but the obtained staining is less complex as it only stains nucleic acids in pink, making it more challenging to distinguish different tissues from one another.

## Skeletal staining

In order to analyze skeletal phenotypes in mutant zebrafish and compare these to wild-type fish, whole mount cartilage and bone staining, which is a fast and inexpensive method, can be easily performed. I performed double staining using Alcian blue to stain cartilage and Alizarin red for bone staining. Alcian blue is taken up by proteoglycans present in cartilage tissue whereas Alizarin red stain binds to calcium present in mineralized tissue such as bones and teeth (Puchtler et al., 1969; Scott, 1996). For double staining, I applied an acid-free staining protocol modified from the original developed by Walker and Kimmel (2007) whereby demineralization of bone by acid is avoided.

## Imaging with confocal microscopy and Optical Projection Tomography

Imaging of zebrafish embryos or larvae can often be quite challenging due to their small size. For 3D imaging, confocal laser scanning microscopy is frequently used. Hereby, the sample is fixed in agarose on a glass-bottomed plate and placed on the microscope stage. 2D images, that are taken by sectioning in depth acquiring Z stacks, are used for subsequent 3D reconstruction or maximum intensity projections. In this thesis, confocal microscopy was used to perform live imaging of transgenic zebrafish up to 14 dpf.

Another alternative to generate brightfield high-resolution 3D imaging is Optical Projection Tomography (OPT) (Sharpe et al., 2002). The data acquired by OPT is, in contrast to confocal imaging, obtained by rotating the sample during the acquisition, resulting in isotropic high-resolution images. OPT uses the same principle as X-ray computed tomography but utilizing visible radiation instead of X-rays. 2D images are acquired at multiple angles and by using tomographic reconstruction, a 3D image can be reconstructed. OPT was used to image and analyze cartilage and bone stained wild-type and mutant zebrafish at 5 dpf and 9 dpf.

# Aims

The overall aim of this thesis was to achieve a better understanding of genes involved in chondrogenesis and joint development in gnathostomes by using the zebrafish *Danio rerio* as a model. We furthermore aimed to generate a transgenic line labeling the jaw joint in zebrafish and to improve imaging and analysis techniques for zebrafish embryos and larvae.

Paper I

- To generate a CRISPR/Cas9 induced *nkx3.2* knockout line in zebrafish and analyze the phenotype at different developmental stages

Paper II

- To identify the regulatory elements of *nkx3.2* in gnathostome genomes and investigate whether they are evolutionary conserved
- To generate a stable transgenic zebrafish line using the *nkx3.2* enhancer element to study jaw joint development
- To generate a *nkx3.2* enhancer knockout in zebrafish by using CRISPR/Cas9 to study the resulting phenotype

Paper III

- To generate a CRISPR/Cas9 induced *gdf5* knockout line in zebrafish and analyze the phenotype at different developmental stages

Paper IV

- Using OPT to generate an automated workflow for imaging and analysis of *in situ* whole mount and skeletal stained zebrafish

# Results and Discussion

## *nkx3.2* knockout in zebrafish (Paper I)

To study the role of *nkx3.2* during zebrafish development, we generated CRISPR/Cas9-induced *nkx3.2* mutant zebrafish and analyzed the resulting phenotype at different developmental stages. The generated mutant allele has a 7 bp deletion in the first exon leading to a premature stop codon after 95 amino acids. The most obvious mutant phenotype, detectable by a simple visual inspection, was the "open-mouth" phenotype caused by jaw joint loss. Despite this severe phenotype homozygote mutant fish were able to feed and reach adulthood. OPT combined with 3D reconstruction and statistical component analysis performed on cartilage- and bone-stained larval wild-type and mutant fish revealed additional mutant phenotypes in the skull around the otic capsule. We applied cartilage and bone staining as well as µCT analysis on adult *nkx3.2* mutant fish for further investigations on the posterior skull phenotype. µCT analysis revealed severe fusions and losses of skeletal elements in the occipital region and the Weberian apparatus in *nkx3.2* mutant adult fish. The exoccipital and basioccipital were partially or completely fused in Nkx3.2 deficient fish causing partial or complete loss of the cavum sinus impar. Defects in the Weberian apparatus in response to *nkx3.2* knockout included loss of the first cervical vertebrae including the associated scaphium and claustrum. In some analyzed homozygote mutant fish, we could detect loss of cervical vertebra two and reduction of its lateral process. The third cervical vertebra displayed deformation and absence of the anterior ramus and the articulating process. The parapophyses, small bones articulating ribs 5-11 with the vertebral centra, were completely absent in *nkx3.2* mutant fish causing direct attachment of the ribs to the vertebrae. Skeletal abnormalities were detected in dorsal and anal fins, showing increased proximal radial length in the mutant line when compared to wild-type. Our results confirm the key role for *nkx3.2* during jaw joint development but more importantly reveal novel phenotypes in response to Nkx3.2 loss in zebrafish, which are reminiscent of phenotypes reported in Nkx3.2 deficient amniotes.

## Conservation of the cis-regulatory *nkx3.2* element in gnathostomes (Paper II)

In this work, we identified and functionally tested *nkx3.2* enhancer sequences from different gnathostome species. Synteny analysis revealed conserved up- and downstream regions flanking *nkx3.2*. We analyzed the regions between *Nkx3.2* upstream gene *Bod1l1* and downstream gene *Rab28* and identified the putative *Nkx3.2* enhancer sequences between *Nkx3.2* and *Rab28* for various mammalian and non-mammalian species. All sequences were cloned into Tol2 site-containing vectors and injected into zebrafish embryos to generate transgenic lines. The zebrafish *nkx3.2*:mCherry line displayed mCherry-positive cells within the zebrafish jaw joint from around 2 dpf. Expression within the notochord was detected around 1 dpf but gradually diminished as development progressed. mCherry-labelled cells in the jaw joint-forming area could be detected up to 14 dpf (later time points were not tested). Our results show that *nkx3.2*:mCherry zebrafish display mCherry expression in the jaw joint interzone and as joint development continues in joint progenitor cells. The observed mCherry activity is in accordance with endogenous *nkx3.2* expression in zebrafish (Miller et al., 2003). To further analyze mCherry-positive cells in more detail, *nkx3.2*:mCherry fish were crossed into the *fli1a*:GFP background. The double transgenic line revealed mCherry/GFP-labelled cells in the perichondrium of the first pharyngeal arch elements.

Transgenic lines generated by using identified enhancer sequences from other species displayed similar mCherry expression as in the zebrafish *nkx3.2*:mCherry line. These results confirm that all tested enhancer sequences, both from mammalian and non-mammalian species, are functionally conserved and able to activate fluorescent reporter gene expression in zebrafish.

Zebrafish *nkx3.2* enhancer knockout did not result in any phenotypic differences indicating a redundant function.

## *gdf5* knockout in zebrafish (Paper III)

In this study we investigated the effect of Gdf5 deficiency in zebrafish. We generated a CRISPR/Cas9 induced *gdf5* knockout and analyzed homozygote mutant zebrafish at different developmental stages. The *gdf5* knockout zebrafish line displays a 5 bp deletion in the second exon causing a reading frame shift which subsequently leads to a premature stop codon after 230 amino acids. By applying skeletal staining and µCT analysis we could detect skeletal abnormalities in pectoral and median fins in *gdf5* mutant fish. Dorsal and anal fin proximal and distal radials were shortened in length and the organization was disturbed in homozygote mutant fish. Caudal fin skeletal elements were truncated as well, however the more ventrally located parhypural

and hypurals 1-2 were less affected by Gdf5 loss than the more dorsally located hypurals 3-5. In order to investigate a potential joint phenotype and delay in skeletal maturation in median fins, histological analysis was performed but did not reveal any abnormalities in either fin. Phenotypic differences in the paired fins could be detected in the pectoral fins in *gdf5* mutant fish. Anteriorly located radials in the pectoral fin were truncated similar to what has been observed in median fin skeletal elements, however posteriorly located radials were completely absent. These results confirm the importance of Gdf5 during chondrogenesis in zebrafish, which leads to the hypothesis that the role of Gdf5 is highly conserved in Osteichthyes.

## Development of automated OPT workflow to quantify phenotypic differences in zebrafish mutants (Paper IV)

In this study, we developed an OPT sample stage combined with an automated workflow for rapid brightfield imaging of zebrafish embryos and larvae. The OPT setup consists of a brightfield illumination, a sample stage (customized 3D-printed) which is equipped with a rotating capillary and a stepper motor, and a lens system with a CMOS (complementary metal-oxide-semiconductor) detector. This setup allows fast loading of the sample which is subsequently rotated to generate 360 degree projections followed by specimen unloading. For reconstructing the obtained OPT data, a number of error correction steps are necessary before generating the 3D reconstruction. After the successful establishment of the automated workflow for high-quality 3D reconstruction using OPT data, we used this set up to quantify phenotypic differences in a zebrafish mutant line. We expected skeletal phenotypes in the mutant line and therefore performed cartilage- and bone-staining on a group of 5 dpf mutant larvae and a wild-type control group and created OPT 3D reconstructions. Both groups were aligned to an average reference fish, followed by a voxel-wise method to detect voxels that significantly differ between the mutant and wild-type group. The resulting maximum projection displayed statistically significant differences within the pharyngeal arch and the posterior skull region in the mutant fish. This analysis technique allowed us identification of unexpected phenotypes, which might have been undiscovered by using conventional confocal microscopy and classic brightfield microscopy.

# Conclusions and future perspectives

## Nkx3.2 deficiency in zebrafish - similar to what is seen in mammals

The role of Nkx3.2 in jaw joint development of non-mammalian vertebrates has been investigated in a number of studies before (Lukas and Olsson, 2018a; Miller et al., 2003). By generating and studying the CRISPR/Cas9 induced *nkx3.2* zebrafish mutant line **(Paper I)** we were able to detect that Nkx3.2 loss not only interferes with jaw joint development but additionally causes skeletal defects that resemble phenotypes of Nkx3.2 deficient mammals. These results confirm a consistent function of *Nkx3.2* between teleosts and amniotes. Interestingly, despite jaw joint fusion in homozygote mutant fish causing a distinctive open-mouth phenotype, the fish are still able to feed and reach adulthood.

Additional studies to analyze the effects of Nkx3.2 loss on downstream factors in all affected anatomical structures should be performed to further understand the underlying regulatory network. Future studies could also focus on *nkx3.2* expression analysis at developmental stages exceeding the embryonic and larval stages.

Since *nkx3.2* mutant fish display defects in the Weberian apparatus and the occipital region, structures that are involved in sound transmission from the swim bladder to the inner ear, behavioral studies could be performed to investigate whether these skeletal phenotypes have an impact on hearing ability.

## *nkx3.2*:mCherry transgenic zebrafish line labels jaw joint progenitor cells

In **Paper II** we identified and functionally tested an *nkx3.2* enhancer sequence in zebrafish. The generated transgenic zebrafish line (*nkx3.2*:mCherry) labels the jaw joint interzone at the onset of joint development and joint progenitor cells at later developmental stages.

This line can be useful to study zebrafish jaw joint formation in the future. Fluorescent-activated cell sorting followed by, for example, single-cell RNA sequencing could be performed to analyze *nkx3.2*:mCherry expressing cells in

more detail. This can help to achieve a better understanding of *nkx3.2* regulation by identifying the levels of expression for other genes in *nkx3.2*:mCherry positive cells.

## Conservation of the *nkx3.2* enhancer in gnathostomes

Besides identifying the *nkx3.2* enhancer sequence in the zebrafish genome, we identified homologous *nkx3.2* enhancer sequences in a variety of gnathostome genomes **(Paper II)**. By functionally testing enhancer sequences from human, mouse, frog, bichir, and elephant shark in zebrafish we showed *nkx3.2* enhancer conservation within gnathostomes. This means that throughout gnathostome evolution this conserved regulatory sequence has activated the expression of *nkx3.2* in the primary jaw joint, but it might display functional redundancy as its deletion in the zebrafish genome didn't result in phenotypes similar to Nkx3.2 gene deletion.

To better understand the regulation of *nkx3.2* by this enhancer, future studies should focus on identifying and validating transcription factors binding to this *nkx3.2* enhancer sequence.

## Gdf5 deficiency in zebrafish affects the development of the fin endoskeleton

Growth and differentiation factor 5 (Gdf5) has been shown to play an important role during chondrogenesis and chondrocyte maturation (Storm and Kingsley, 1996; Merino et al., 1999; Storm and Kingsley, 1999). Apart from documenting gene expression patterns, few studies have been carried out in zebrafish to investigate the role of Gdf5 in more detail (Crotwell et al., 2001; Miller et al., 2003; Schwend and Ahlgren, 2009). In **Paper III** we successfully generated a CRISPR/Cas9 induced *gdf5* knockout line and detected phenotypes within the fin endoskeletons. Median and pectoral fins displayed defects in endoskeletal element formation. Endoskeletal elements in the median fins displayed extreme reduction in size. Posterior radials of the pectoral fin were absent and anterior radials truncated in *gdf5* mutant fish. Our results demonstrate the importance of Gdf5 for the development of endoskeletal fin elements.

Since *gdf5* is expressed in the jaw joint in zebrafish, which did not display any phenotypic differences in the generated mutant line according to our analysis, we cannot exclude potential compensation by the closely-related *gdf6*. In order to test this possibility, a double mutant line for *gdf5* and *gdf6a* could be generated and analyzed for defects during jaw joint development.

Loss of Gdf5 has been reported previously to cause delay of chondrogenesis and chondrocyte maturation (Storm and Kingsley 1999, Merino et al., 1999). Our histological analysis in adult mutant fish however could not confirm this finding. However, we cannot exclude that there might be a certain delay of chondrogenesis in larval and juvenile mutant fish. Therefore, histological examination should be performed in younger *gdf5* mutant fish.

To investigate the effect Gdf5 deficiency has on downstream factors, *in situ* hybridization targeting associated gene transcripts could be carried out in *gdf5* mutant fish.

## Do zebrafish have true synovial joints?

The zebrafish jaw joint has been previously reported to display synovial joint characteristics (Askary et al., 2016). Additional studies are necessary to investigate whether the zebrafish jaw joint is a true synovial joint.

The *nkx3.2*:mCherry transgenic zebrafish line generated in **Paper II** could be used for fluorescent-activated cell sorting of mCherry-labelled cells and subsequent proteomic analysis to detect additional markers involved in synovial joint development.

In **Paper III** we showed in histological stained thin sections that articulations in the dorsal and anal fin between proximal and distal radials displayed similar histological properties as synovial joints. We detected both articular cartilage and a cavity enclosed by a capsule. Future studies such as *in situ* hybridization for the lubricin encoding gene *prg4b* could be done to test whether these joints possess synovial joint-characteristics. Immunohistochemistry using antibodies targeted at synovial joint-characteristic cell markers such as fibroblasts and macrophages could be performed.

## zOPT automated workflow for analyzing skeletal phenotypes in zebrafish

In the last study **(Paper IV)** a brightfield OPT setup was implemented including an automated workflow to obtain 3D reconstructions. By using this setup to image and reconstruct cartilage- and bone-stained larvae in combination with a voxel-wise method, statistically significant differences between a mutant and wild-type group could be detected.

This method allows us to detect even subtle phenotypic abnormalities in mutant fish at early stages, as shown in **Paper I**. In **Paper III** however, we could not analyze reconstructed images of mutant and wildtype groups with the voxel-wise method since the naturally occurring size variation of 9 dpf zebrafish can lead to false-positive results with small sample sizes.

# Svensk Sammanfattning

Skelettet hos ryggradsdjur är en avancerad konstruktion som består av ben, brosk och leder. Utvecklingen av skelettet styrs av ett komplext samarbete mellan diverse olika gener, som säkerställer att alla komponenter är utformade på korrekt sätt och att de växer till vid rätt tid och plats under embryonalutvecklingen. Själva benbildningen kan bli till genom flera olika processer, bland annat genom så kallad endokondral benbildning, vilket innebär att benbildningen sker inom broskvävnad. Under endokondral benbildning får mesenkymala celler signaler som leder till att de utvecklas till broskceller. Broskcellerna bygger upp en mall i vilken brosket succesivt blir ersatt av ben senare i utvecklingen.

De båda generna *Nkx3.2* och *Gdf5* har visats vara mycket viktiga för utvecklingen av leder och brosk. Mutationer i någon av dessa gener kan leda till sällsynta skelettsjukdomar och tros dessutom vara en riskfaktor för att utveckla artros. I mitt arbete har jag fokuserat på att undersöka vilka funktioner Nkx3.2 och Gdf5 har genom att använda zebrafisken, ett litet ryggradsdjur som används som modellorganism. I flera studier har jag använt mig av den nya tekniken CRISPR/Cas9 för att skapa mutationer i *nkx3.2* och *gdf5* hos zebrafiskar och sedan analysera vilken effekt dessa mutationer får hos de muterade zebrafiskarna. CRISPR/Cas9 kan liknas vid en gensax som används för att klippa isär DNA-strängar vid en bestämd position. Cellernas egen reparationsmekanism kan åtgärda skadan, men eftersom mekanismen inte är helt perfekt bildas antingen för få eller för många nukleotider där DNA-strängen skadas. Det leder till att proteinet som normalt produceras av genen blir defekt eller inte skapas alls.

I den första artikeln i avhandlingen introducerade vi en mutation i *nkx3.2*-genen hos zebrafisken. Mutationen orsakade att käkleden aldrig utvecklades, och vi hittade även tidigare okända påverkningar i form av deformationer i skallben och ryggkotor. Liknande typer av förändringar kan även ses hos däggdjur med samma genmutation.

I den andra studien lyckades vi lokalisera genens enhancer, alltså det regulatoriska elementet som förstärker gentranskriptionen. Vi använde enhancern för att genera en så kallad transgen zebrafisklinje. I de transgena zebrafiskarna är cellerna som bygger upp käkleden markerade med ett fluorescerande protein, vilket gör det möjligt att följa käkledens tillväxt under zebrafiskens tidigt utveckling. Vi fann även att *nkx3.2*-enhancern finns hos andra käkförsedda

ryggradsdjur och kunde därmed påvisa att den har ett gemensamt evolutionärt ursprung hos dessa.

I den tredje studien använde vi oss av CRISPR/Cas9 för att skapa mutationer i *gdf5*-genen. Vi kunde påvisa att *gdf5* är mycket viktigt för utvecklingen av fenornas endoskelett, vilket skapas genom endokondral benbildning. I likhet med zebrafiskarna har tidigare studier visat att broskvävnaden i extremiteterna hos *gdf5*-muterade möss och höns inte utvecklas på normalt sätt.

Eftersom zebrafiskembryon är mycket små kan det vara svårt att visualisera många fiskar på samma sätt (i samma position och vinkel) med hjälp av vanliga mikroskop. I den sista studien användes ljustomografi i kombination med en speciell *setup* för zebrafiskar för att på ett snabbt och effektivt sätt visualisera zebrafiskembryon. För att kunna visualisera skelettet hos embryonerna använde vi oss av två färgämnen för att färga brosk i blått och ben i rött. Vi visualiserade både mutant- och vildtyp-zebrafiskar och använde en speciell metod för att upptäcka små skillnader i skelettuppbyggnad, vilka annars kan vara svåra att utröna med andra tekniker.

Sammantaget bidrar min avhandling till en ökad förståelse för vilka funktioner generna *Nkx3.2* och *Gdf5* har i skelettbildning hos zebrafiskar och hur zebrafiskar kan visualiseras på ett effektivt sätt.

# Acknowledgements

I would like to thank all the wonderful colleagues, friends and family members who supported and helped me during the last few years and contributed to this work.

My first and biggest thank goes to my supervisor **Tatjana**. Thank you for giving me the opportunity and honor to be your first PhD student and all your support from the very beginning. Thank you for always listening and all the great discussions which helped me to develop as a researcher. I would not be where I am today without your guidance.

Thank you **Per** for all your support during my time as PhD student in your program and especially for your help during my last year. Thank you also for encouraging me to participate in the ISELV in China, it was definitely a high-light of my PhD.

My co supervisor **Johan,** for a lot of good advice and support during all these years.

**Jake**, all the projects we worked on together would not have become what they are today without you. Working with you has been a real pleasure and I am very grateful for all the great discussions and close collaboration. Thank you for always being picky and critical in a very good way!

**Gerrit**, danke dass du mein Interesse für die Entwicklungsbiologie während meines Masters geweckt hast. Ohne dich wäre ich vermutlich niemals hier gelandet. Vielen Dank!

To all the members of the **genome engineering zebrafish facility**. Thank you **Beata** and **Tiffany** for everything you have thought me about CRISPR/Cas9. It was great learning from the experts. Thank you **Chrysa** for all your help in the lab. Thank you **Joao**, **Therese**, **Conrad** and **Joss** for keeping excellent care of our fish.

Our collaborators **Amin** and **Hanqing**. Thank you for all the great meetings and results we achieved together. It was great fun working with you. Thank you **Caroline** for the µCT support!

To all the **administrative workers at IOB and IBG**.

**Rose-Marie** for assisting me in the beginning to find my way through the bureaucratic jungle and **Helena** and **Marie** for helping me with all laboratory related issues.

**Carina S.**, thank you for being a great PhD program coordinator.

Thanks to all the amazing former and current **colleagues at IOB**.
**Martin**, thank you for all your support and for always correcting my Swedish. **Philipp,** meine deutsche Verstärkung am Arbeitsplatz. Schee woas! **Manolis**, thank you all the unforgettable and amusing moments in the office. **Daniel,** there is nothing that can´t be solved by you. Thank you for that! **Mohammed**, great that we could "suffer" together door to door during the last few months. **Cecile, Imke, Hannah,** BBG in the secret garden was always a pleasure.
Big thanks also to **Oskar, Bianca, Anna, Sophie, Vincent, Valeria, Francois, Gregorz, Donglei, Adrianne, Jordi, Zivile, Dennis, Melanie, Daniel O-D, Ehsan, Monika, Gizem, Judith, Zsisi, GP, Elena, Josefine, Luciana, Virginia, Erik, Sara, Tara** and **Thibaut.**
**Carina Ö.,** you are such an inspiring woman and teach us all that age is just a number. **Ralf,** der Meister der PCR! Danke für die wunderbare und lustige Zeit im Labor. **Leni,** schön dass du einige Monate hier warst und Danke für die MA-Unterstützung.
**Thank you all for making this workplace special and for all the good company during fika on the red sofas.**

Without you there might not have been a life outside work **- my friends**. So glad I got to know every single each of you.

**Lucas**, thank you for everything and for always being there for me. Thanks for all the great parties in Uppsala and Stockholm and the amazing Grisslehamn weekends. Also big thanks to your family for always making me feel welcome and the great midsummer celebrations.

Thank you **Petter (+family)**, **Kanako**, **Emma** and **Stina** for making me feel like home in Uppsala, especially during my first years.

**Sofia**, the best friend in the stable (and ofc also outside the stable) I could ask for. Thank you for all the trust you have in me with **Ljomi**! I´m glad every day that I got to know both of you!

Thanks to the Uppsala- *aka* Singö-crew. **MQ,** not only a colleague but also very good friend and co-founder of the drone-bar, together with **Adrian** and **Carl**magsnus. But the crew is not complete without **Cat**, **Jonte**, **Anna**, **Fillip**,

**Elin**, **Alberto**daskleinekrokodil and **Inga**. Thank you for all the adventures on Singö and in town.

Aus den Augen aber nicht aus dem Sinn. Vielen Dank dass ihr auch trotz der weiten Entfernung immer für mich da seid und mich nicht vergessen habt. Habe mich über jeden einzelnen gefreut der sich nach Uppsala verirrt hat. Danke **Vera, Simi, Tim, Ivy, Myri, die Barthels, Hilli, Seidens und Kadda.**

Last but not least, meine Familie. **Mama**, danke dass du immer an mich glaubst und mich ermutigt hast diesen Schritt zu gehen und über meine Grenzen hinauszuwachsen. Ohne den extra Push hier und da wäre einiges vielleicht/sicherlich anders gekommen. Vielen Dank dafür! Papa wäre sicher stolz auf uns. **Caro,** die beste Schwester die man sich wünschen kann. Hier braucht es vielleicht keine grossen Worte, du weisst auch so wie wichtig du mir bist. **Michi und Günter** ihr basst scho gut nei in die Bagasch, schee dass mer eich hamm. **Emil,** die bunten Bilder sind für dich!

# References

**Akiyama, H., Lyons, J. P., Mori-Akiyama, Y., Yang, X., Zhang, R., Zhang, Z., Deng, J. M., Taketo, M. M., Nakamura, T., Behringer, R. R., et al.** (2004). Interactions between Sox9 and beta-catenin control chondrocyte differentiation. *Genes Dev.* **18**, 1072–1087.

**Alexander, C., Zuniga, E., Blitz, I. L., Wada, N., le Pabic, P., Javidan, Y., Zhang, T., Cho, K. W., Crump, J. G. and Schilling, T. F.** (2011). Combinatorial roles for BMPs and endothelin 1 in patterning the dorsal-ventral axis of the craniofacial skeleton. *Development* **138**, 5135–5146.

**Amano, T., Sagai, T., Tanabe, H., Mizushina, Y., Nakazawa, H. and Shiroishi, T.** (2009). Chromosomal Dynamics at the Shh Locus: Limb Bud-Specific Differential Regulation of Competence and Active Transcription. *Dev. Cell* **16**, 47–57.

**Amin, S. and Tucker, A. S.** (2006). Joint formation in the middle ear: Lessons from the mouse and guinea pig. *Dev. Dyn.* **235**, 1326–1333.

**Archer, C. W., Morrison, H. and Pitsillides, A. A.** (1994). The cellular aspects of the development of synovial joints and articular cartilage. *J. Anat.* **184**, 447–456.

**Archer, C. W., Dowthwaite, G. P. and Francis-West, P.** (2003). Development of synovial joints. *Birth Defects Res. Part C* **69**, 144–155.

**Askary, A., Smeeton, J., Paul, S., Schindler, S., Braasch, I., Ellis, N. A., Postlethwait, J., Miller, C. T. and Gage Crump, J.** (2016). Ancient origin of lubricated joints in bony vertebrates. *Elife* **5**, 1–13.

**Azpiazu, N. and Frasch, M.** (1993). Tinman and bagpipe: Two homeo box genes that determine cell fates in the dorsal mesoderm of Drosophila. *Genes Dev.* **7**, 1325–1340.

**Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D. A. and Horvath, P.** (2007). CRISPR provides acquired resistance against prokaryotes. *Science (80-. ).* **315**, 1709–1712.

**Barske, L., Askary, A., Zuniga, E., Balczerski, B., Bump, P., Nichols, J. T. and Crump, J. G.** (2016). Competition between Jagged-Notch and Endothelin1 Signaling Selectively Restricts Cartilage Formation in the Zebrafish Upper Face. *PLoS Genet.* **12**, e1005967.

**Bhaya, D., Davison, M. and Barrangou, R.** (2011). CRISPR-cas systems in bacteria and archaea: Versatile small RNAs for adaptive defense and regulation. *Annu. Rev. Genet.* **45**, 273–297.

**Bi, W., Deng, J. M., Zhang, Z., Behringer, R. R. and De Crombrugghe, B.** (1999). Sox9 is required for cartilage formation. *Nat. Genet.* **22**, 85–89.

**Bi, W., Huang, W., Whitworth, D. J., Deng, J. M., Zhang, Z., Behringer, R. R. and De Crombrugghe, B.** (2001). Haploinsufficiency of Sox9 results in

defective cartilage primordia and premature skeletal mineralization. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 6698–6703.

**Bird, N. C. and Mabee, P. M.** (2003). Developmental Morphology of the Axial Skeleton of the Zebrafish, Danio rerio (Ostariophysi: Cyprinidae). *Dev. Dyn.* **228**, 337–357.

**Bolotin, A., Quinquis, B., Sorokin, A. and Ehrlich, D. S.** (2005). Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology* **151**, 2551–2561.

**Brouns, S. J. J., Jore, M. M., Lundgren, M., Westra, E. R., Slijkhuis, R. J. H., Snijders, A. P. L., Dickman, M. J., Makarova, K. S., Koonin, E. V. and van der Oost, J.** (2008). Small CRISPR RNAs Guide Antiviral Defense in Prokaryotes. *Science (80-. ).* **321**, 960–964.

**Bruneau, S., Mourrain, P. and Rosa, F. M.** (1997). Expression of contact, a new zebrafish DVR member, marks mesenchymal cell lineages in the developing pectoral fins and head and is regulated by retinoic acid. *Mech. Dev.* **65**, 163–173.

**Brunet, L. J., Mcmahon, J. A., Mcmahon, A. P. and Harland, R. M.** (1998). Noggin , Cartilage Morphogenesis , and Joint Formation in the Mammalian Skeleton. *Science (80-. ).* **280**, 1455–1458.

**Buxton, P., Edwards, C., Archer, C. W. and Francis-West, P.** (2001). Growth / Differentiation Factor-5 (GDF-5) and Skeletal Development. *J. Bone Jt. Surg.* **83-A**, S1-24.

**Caron, M. M. J., Emans, P. J., Surtel, D. A. M., Van Der Kraan, P. M., Van Rhijn, L. W. and Welting, T. J. M.** (2015). BAPX-1/NKX-3.2 acts as a chondrocyte hypertrophy molecular switch in osteoarthritis. *Arthritis Rheumatol.* **67**, 2944–2956.

**Cerny, R., Cattell, M., Sauka-Spengler, T., Bronner-Fraser, M., Yu, F. Q. and Medeiros, D. M.** (2010). Evidence for the prepattern/cooption model of vertebrate jaw evolution. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 17262–17267.

**Chan, J. K. C.** (2014). The wonderful colors of the hematoxylin-eosin stain in diagnostic surgical pathology. *Int. J. Surg. Pathol.* **22**, 12–32.

**Chapman, K., Takahashi, A., Meulenbelt, I., Watson, C., Rodriguez-lopez, J., Egli, R., Tsezou, A., Malizos, K. N., Kloppenburg, M., Shi, D., et al.** (2008). A meta-analysis of European and Asian cohorts reveals a global role of a functional SNP in the 5′ UTR of GDF5 with osteoarthritis susceptibility. *Hum. Mol. Genet.* **17**, 1497–1504.

**Choi, S.-W., Jeong, D.-U., Kim, J.-A., Lee, B., Joeng, K. S., Long, F. and Kim, D.-W.** (2012). Indian Hedgehog signalling triggers Nkx3.2 protein degradation during chondrocyte maturation. *Biochem. J.* **443**, 789–798.

**Christian, M., Cermak, T., Doyle, E. L., Schmidt, C., Zhang, F., Hummel, A., Bogdanove, A. J. and Voytas, D. F.** (2010). Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics* **182**, 756–761.

**Clark, C. T. and Smith, K. K.** (1993). Cranial Osteogenesis in Monodelphis domestica (Didelphidae) and Macropus eurgenii (Macropodidea). *J. Morphol.* **215**, 119–149.

**Coleman, C. M. and Tuan, R. S.** (2003). Growth/differentiation factor 5 enhances chondrocyte maturation. *Dev. Dyn.* **228**, 208–216.

**Cong, L., Ran, A. F., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P. D., Wu, X., Jiang, W., Marraffini, L. A., et al.** (2013). References and Notes 1. *Science (80-. ).* **339**, 819–823.

**Craig, F. M., Bentley, G. and Archer, C. W.** (1987). The spatial and temporal pattern of collagens I and II and keratan sulphate in the developing chick metatarsophalangeal joint. *Development* **99**, 383–391.

**Crompton, A. W.** (1963). On the lower jaw of Diarthrognathus and the origin of the mammalian lower jaw. *Proc. Zool. Soc. London* **140**, 697–753.

**Crotwell, P. L. and Mabee, P. M.** (2007). Gene expression patterns underlying proximal-distal skeletal segmentation in late-stage zebrafish, Danio rerio. *Dev. Dyn.* **236**, 3111–3128.

**Crotwell, P. L., Clark, T. G. and Mabee, P. M.** (2001). Gdf5 is expressed in the developing skeleton of median fins of late-stage zebrafish, Danio rerio. *Dev. Genes Evol.* **211**, 555–558.

**Daans, M., Luyten, F. P. and Lories, R. J. U.** (2011). GDF5 deficiency in mice is associated with instability-driven joint damage, gait and subchondral bone changes. *Ann. Rheum. Dis.* **70**, 208–213.

**Deltcheva, E., Chylinski, K., Sharma, C. M., Gonzales, K., Chao, Y., Pirzada, Z. A., Eckert, M. R., Vogel, J. and Charpentier, E.** (2011). CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* **471**, 602–607.

**Dewit, J., Witten, P. E. and Huysseune, A.** (2011). The mechanism of cartilage subdivision in the reorganization of the zebrafish pectoral fin endoskeleton. *J. Exp. Zool. Part B Mol. Dev. Evol.* **316 B**, 584–597.

**Edwards, J. C., Wilkinson, L. S., Jones, H. M., Soothill, P., Henderson, K. J., Worrall, J. G. and Pitsillides, A. A.** (1994). The formation of human synovial joint cavities: a possible role for hyaluronan and CD44 in altered interzone cohesion. *J. Anat.* **185**, 355–67.

**Engeszer, R. E., Patterson, L. B., Rao, A. A. and Parichy, D. M.** (2007). Zebrafish in the wild: A review of natural history and new notes from the field. *Zebrafish* **4**, 21–40.

**Filan, S. L.** (1991). Development of the middle ear region in Monodelphis domestica (Marsupialia, Didelphidae): marsupial solutions to an early birth. *J. Zool.* **225**, 577–588.

**Fischer, A. H., Jacobson, K. A., Rose, J. and Zeller, R.** (2008). Hematoxylin and eosin staining of tissue and cell sections. *CSH Protoc.* **3**, 4986.

**Fisher, S., Jagadeeswaran, P. and Halpern, M. E.** (2003). Radiographic analysis of zebrafish skeletal defects. *Dev. Biol.* **264**, 64–76.

**Fisher, S., Grice, E. A., Vinton, R. M., Bessling, S. L., Urasaki, A., Kawakami, K. and McCallion, A. S.** (2006). Evaluating the biological relevance of putative enhancers using Tol2 transposon-mediated transgenesis in zebrafish. *Nat. Protoc.* **1**, 1297–1305.

**Fleming, A., Keynes, R. and Tannahill, D.** (2004). A central role for the notochord in vertebral patterning. *Development* **131**, 873–880.

**Foley, J. E., Maeder, M. L., Pearlberg, J., Joung, J. K., Peterson, R. T. and Yeh, J. R. J.** (2009). Targeted mutagenesis in zebrafish using customized zinc-finger nucleases. *Nat. Protoc.* **4**, 1855–1868.

**Gagnon, J. A., Valen, E., Thyme, S. B., Huang, P., Ahkmetova, L., Pauli, A., Montague, T. G., Zimmerman, S., Richter, C. and Schier, A. F.** (2014). Efficient mutagenesis by Cas9 protein-mediated oligonucleotide insertion and large-scale assessment of single-guide RNAs. *PLoS One* **9**, e98186.

**Garneau, J. E., Dupuis, M. È., Villion, M., Romero, D. A., Barrangou, R., Boyaval, P., Fremaux, C., Horvath, P., Magadán, A. H. and Moineau, S.** (2010). The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* **468**, 67–71.

**Gaupp, E.** (1912). Die Reichertsche Theorie. *Arch. Anat. Entwicklungsgesch.* 1–416.

**Gehrke, A. R., Schneider, I., de la Calle-Mustienes, E., Tena, J. J., Gomez-Marin, C., Chandran, M., Nakamura, T., Braasch, I., Postlethwait, J. H., Gómez-Skarmeta, J. L., et al.** (2015). Deep conservation of wrist and digit enhancers in fish. *Proc. Natl. Acad. Sci.* **112**, 803–808.

**Grandel, H. and Schulte-Merker, S.** (1998). The development of the paired fins in the zebrafish (Danio rerio). *Mech. Dev.* **79**, 99–120.

**Gruneberg, H. and Lee, A. J.** (1973). The anatomy and development of brachypodism in the mouse. *J. Embryol. Exp. Morphol.* **30**, 119–141.

**Guo, X., Day, T. F., Jiang, X., Garrett-Beal, L., Topol, L. and Yang, Y.** (2004). Wnt/beta-catenin signaling is sufficient and necessary for synovial joint formation. *Genes Dev.* **18**, 2404–2417.

**Haft, D. H., Selengut, J., Mongodin, E. F. and Nelson, K. E.** (2005). A guild of 45 CRISPR-associated (Cas) protein families and multiple CRISPR/cas subtypes exist in prokaryotic genomes. *PLoS Comput. Biol.* **1**, e60.

**Hellemans, J., Simon, M., Dheedene, A., Alanay, Y., Mihci, E., Rifai, L., Sefiani, A., van Bever, Y., Meradji, M., Superti-Furga, A., et al.** (2009). Homozygous Inactivating Mutations in the NKX3-2 Gene Result in Spondylo-Megaepiphyseal-Metaphyseal Dysplasia. *Am. J. Hum. Genet.* **85**, 916–922.

**Horvath, P., Romero, D. A., Coûté-Monvoisin, A.-C., Richards, M., Deveau, H., Moineau, S., Boyaval, P., Fremaux, C. and Barrangou, R.** (2008). Diversity, activity, and evolution of CRISPR loci in Streptococcus thermophilus. *J. Bacteriol.* **190**, 1401–1412.

**Indjeian, V. B., Kingman, G. A., Jones, F. C., Guenther, C. A., Grimwood, J., Schmutz, J., Myers, R. M. and Kingsley, D. M.** (2016). Evolving New Skeletal Traits by cis-Regulatory Changes in Bone Morphogenetic Proteins. *Cell* **164**, 45–56.

**Ishino, Y., Shinagawa, H., Makino, K., Amemura, M. and Nakata, A.** (1987). Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isoenzyme conversion in Escherichia coli, and identification of the gene product. *J. Bacteriol.* **169**, 5429–5433.

**Jansen, R., Van Embden, J. D. A., Gaastra, W. and Schouls, L. M.** (2002). Identification of genes that are associated with DNA repeats in prokaryotes. *Mol. Microbiol.* **43**, 1565–1575.

**Jao, L. E., Wente, S. R. and Chen, W.** (2013). Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 13904–13909.

**Jeong, D. U., Choi, J. Y. and Kim, D. W.** (2017). Cartilage-Specific and Cre-Dependent Nkx3.2 Overexpression In Vivo Causes Skeletal Dwarfism by Delaying Cartilage Hypertrophy. *J. Cell. Physiol.* **232**, 78–90.

**Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A. and Charpentier, E.** (2012). A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science (80-. ).* **337**, 816–821.

**Kagey, M. H., Newman, J. J., Bilodeau, S., Zhan, Y., Orlando, D. A., Van Berkum, N. L., Ebmeier, C. C., Goossens, J., Rahl, P. B., Levine, S. S., et al.** (2010). Mediator and cohesin connect gene expression and chromatin architecture. *Nature* **467**, 430–435.

**Kague, E., Gallagher, M., Burke, S., Parsons, M., Franz-Odendaal, T. and Fisher, S.** (2012). Skeletogenic Fate of Zebrafish Cranial and Trunk Neural Crest. *PLoS One* **7**, 1–13.

**Kan, A., Ikeda, T., Fukai, A., Nakagawa, T., Nakamura, K., Chung, U. Il, Kawaguchi, H. and Tabin, C. J.** (2013). SOX11 contributes to the regulation of GDF5 in joint maintenance. *BMC Dev. Biol.* **13**,.

**Kawakami, K.** (2007). Tol2: A versatile gene transfer vector in vertebrates. *Genome Biol.* **8**, S7.

**Kim, J.-A., Im, S., Cantley, L. C. and Kim, D.-W.** (2015). Suppression of Nkx3.2 by phosphatidylinositol-3-kinase signaling regulates cartilage development by modulating chondrocyte hypertrophy. *Cell. Signal.* **27**, 2389–2400.

**Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ulmann, B. and Schilling, T. F.** (1995). Stages of Embryonic Development of the Zebrafish. *Dev Dyn* **203**, 255–310.

**Kleinjan, D. A., Seawright, A., Schedl, A., Quinlan, R. A., Danes, S. and Van Heyningen, V.** (2001). Aniridia-associated translocations, DNase hypersensitivity, sequence comparison and transgenic analysis redefine the functional domain of PAX6. *Hum. Mol. Genet.* **10**, 2049–2059.

**Koga, A., Suzuki, M., Inagaki, H., Bessho, Y. and Hori, H.** (1996). Transposable element in fish. *Nature* **383**, 30.

**Kozhemyakina, E., Lassar, A. B. and Zelzer, E.** (2015). A pathway to bone: signaling molecules and transcription factors involved in chondrocyte development and maturation. *Development* **142**, 817–831.

**Kuraku, S., Takio, Y., Sugahara, F., Takechi, M. and Kuratani, S.** (2010). Evolution of oropharyngeal patterning mechanisms involving Dlx and endothelins in vertebrates. *Dev. Biol.* **341**, 315–323.

**Kwan, K. M., Fujimoto, E., Grabher, C., Mangum, B. D., Hardy, M. E., Campbell, D. S., Parant, J. M., Yost, H. J., Kanki, J. P. and Chien, C. Bin** (2007). The Tol2kit: A multisite gateway-based construction Kit for Tol2 transposon transgenesis constructs. *Dev. Dyn.* **236**, 3088–3099.

**Lengner, C. J., Hassan, M. Q., Serra, R. W., Lepper, C., Van Wijnen, A. J., Stein, J. L., Lian, J. B. and Stein, G. S.** (2005). Nkx3.2-mediated repression of Runx2 promotes chondrogenic differentiation. *J. Biol. Chem.* **280**, 15872–15879.

**Lettice, L. A., Purdie, L. A., Carlson, G. J., Kilanowski, F., Dorin, J. and Hill, R. E.** (1999). The mouse bagpipe gene controls development of axial skeleton, skull, and spleen. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 9695–9700.

**Lettice, L. A., Horikoshi, T., Heaney, S. J. H., Van Baren, M. J., Van Der Linde, H. C., Breedveld, G. J., Joosse, M., Akarsu, N., Oostra, B. A., Endo, N., et al.** (2002). Disruption of a long-range cis-acting regulator for Shh causes preaxial polydactyly. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 7548–7553.
**Lukas, P. and Olsson, L.** (2018a). Bapx1 is required for jaw joint development in amphibians. *Evol. Dev.* **20**, 192–206.
**Lukas, P. and Olsson, L.** (2018b). Bapx1 upregulation is associated with ectopic mandibular cartilage development in amphibians. *Zool. Lett.* **4**, 1–12.
**Maier, W.** (1987). Der Processus angularis bei Monodelphis domestica (Didelphidae; Marsupialia) und seine Beziehungen zum Mittelohr: Eine ontogenetische und evolutionsmorphologische Untersuchung. *Gegenbaurs Morphol. Jahrb. Leipzig* **133**, 123–161.
**Makarova, K. S., Grishin, N. V., Shabalina, S. A., Wolf, Y. I. and Koonin, E. V.** (2006). A putative RNA-interference-based immune system in prokaryotes: Computational analysis of the predicted enzymatic machinery, functional analogies with eukaryotic RNAi, and hypothetical mechanisms of action. *Biol. Direct* **1**, 1–26.
**Malik, S. and Roeder, R. G.** (2010). The metazoan Mediator co-activator complex as an integrative hub for transcriptional regulation. *Nat. Rev. Genet.* **11**, 761–772.
**Marraffini, L. A. and Sontheimer, E. J.** (2008). CRISPR Interference Limits Horizontal Targeting DNA. *Science (80-. ).* **322**, 1843–1845.
**Martinez-Garcia, M., Garcia-Canto, E., Fenollar-Cortes, M., Aytes, A. P. and Trujillo-Tiebas, M. J.** (2016). Characterization of an acromesomelic dysplasia, Grebe type case: novel mutation affecting the recognition motif at the processing site of GDF5. *J. Bone Miner. Metab.* **34**, 599–603.
**McCauley, D. W. and Bronner-Fraser, M.** (2003). Neural crest contributions to the lamprey head. *Development* **130**, 2317–2327.
**Merino, R., Macias, D., Gañan, Y., Economides, A. N., Wang, X., Wu, Q., Stahl, N., Sampath, K. T., Varona, P. and Hurle, J. M.** (1999). Expression and Function of Gdf-5 during Digit Skeletogenesis in the Embryonic Chick Leg Bud. *Dev. Biol.* 33–45.
**Miller, C. T., Yelon, D., Stainier, D. Y. R. and Kimmel, C. B.** (2003a). Two endothelin 1 effectors, hand2 and bapx1, pattern ventral pharyngeal cartilage and the jaw joint. *Development* **130**, 1353–1365.
**Miller, C. T., Yelon, D., Stainier, D. Y. R. and Kimmel, C. B.** (2003b). Two endothelin 1 effectors, hand2 and bapx1, pattern ventral pharyngeal cartilage and the jaw joint. *Development* **130**, 1353–1365.
**Mitrovic, D. R.** (1977). Development of the metatarsophalangeal joint of the chick embryo: Morphological, ultrastructural and histochemical studies. *Am. J. Anat.* **150**, 333–347.
**Miyamoto, Y., Mabuchi, A., Shi, D., Kubo, T., Takatori, Y., Saito, S., Fujioka, M., Sudo, A., Uchida, A., Yamamoto, S., et al.** (2007). A functional polymorphism in the 5′ UTR of GDF5 is associated with susceptibility to osteoarthritis. *Nat. Genet.* **39**, 529–533.
**Miyashita, T.** (2018). Development, Anatomy, and Phylogenetic Relationships of Jawless Vertebrates and Tests of Hypotheses about Early Verebrate Evolution.

**Mojica, F. J. M., Juez, G. and Rodriguez-Valera, F.** (1993). Transcription at different salinities of Haloferax mediterranei sequences adjacent to partially modified PstI sites. *Mol. Microbiol.* **9**, 613–621.

**Mojica, F. J. M., Díez-Villaseñor, C., Soria, E. and Juez, G.** (2000). Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria. *Mol. Microbiol.* **36**, 244–246.

**Mojica, F. J. M., Díez-Villaseñor, C., García-Martínez, J. and Soria, E.** (2005). Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *J. Mol. Evol.* **60**, 174–182.

**Mojica, F. J. M., Díez-Villaseñor, C., García-Martínez, J. and Almendros, C.** (2009). Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology* **155**, 733–740.

**Moreno-Mateos, M. A., Vejnar, C. E., Beaudoin, J.-D., Fernandez, J. P., Mis, E. K., Khokha, M. K. and Giraldez, A. J.** (2015). CRISPRscan: designing highly efficient sgRNAs for CRISPR/Cas9 targeting in vivo. *Nat. Methods* **12**, 982–988.

**Morin-Kensicki, E. M., Melancon, E. and Eisen, J. S.** (2002). Segmental relationship between somites and vertebral column in zebrafish. *Development* **129**, 3851–3860.

**Nair, S., Li, W., Cornell, R. and Schilling, T. F.** (2007). Requirements for Endothelin type-A receptors and Endothelin-1 signaling in the facial ectoderm for the patterning of skeletogenic neural crest cells in zebrafish. *Development* **134**, 335 LP – 345.

**Nasevicius, A. and Ekker, S. C.** (2000). Effective targeted gene “knockdown” in zebrafish. *Nat. Genet.* **26**, 216–220.

**Nishitoh, H., Ichijo, H., Kimura, M., Matsumoto, T., Makishima, F., Yamaguchi, A., Yamashita, H., Enomoto, S. and Miyazono, K.** (1996). Identification of type I and type II serine/threonine kinase receptors for growth/differentiation factor-5. *J. Biol. Chem.* **271**, 21345–21352.

**Piotrowski, T., Schilling, T. F., Brand, M., Jiang, Y. J., Heisenberg, C. P., Beuchle, D., Grandel, H., van Eeden, F. J., Furutani-Seiki, M., Granato, M., et al.** (1996). Jaw and branchial arch mutants in zebrafish II: anterior arches and cartilage differentiation. *Development* **123**, 345–56.

**Pourcel, C., Salvignol, G. and Vergnaud, G.** (2005). CRISPR elements in Yersinia pestis acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. *Microbiology* **151**, 653–663.

**Provot, S., Kempf, H., Murtaugh, C. L., Chung, U., Kim, D.-W., Chyung, J., Kronenberg, H. M. and Lassar, A. B.** (2006). Nkx3.2/Bapx1 acts as a negative regulator of chondrocyte maturation. *Development* **133**, 651–662.

**Puchtler, H., Meloan, S. N. and Terry, M. S.** (1969). On the history and mechanism of alizarin and alizarin red s stains for calcium. *J. Histochem. Cytochem.* **17**, 110–124.

**Reichert, C.** (1837). Ueber die Visceralbogen der Wirbeltiere im allgemeinen und deren Metamophose bei den Voegeln und Säugetieren. *Arch. Anat. Physiol.*

**Rosen, D. E. and Greenwood, P. H.** (1970). Origin of the Weberian Apparatus and the Relationships of the Ostariophysan and Gonorynchiform Fishes. *Am. Museum Novit.* **2428**, 1–25.

**Satoh, A., Suzuki, M., Amano, T., Tamura, K. and Ide, H.** (2005). Joint development in Xenopus laevis and induction of segmentations in regenerating froglet limb (spike). *Dev. Dyn.* **233**, 1444–1453.

**Schilling, T. F. and Kimmel, C. B.** (1994). Segment and cell type lineage restrictions during pharyngeal arch development in the zebrafish embryo. *Development* **120**, 483–494.

**Schilling, T. F., Piotrowski, T., Grandel, H., Brand, M., Heisenberg, C. P., Jiang, Y. J., Beuchle, D., Hammerschmidt, M., Kane, D. A., Mullins, M. C., et al.** (1996). Jaw and branchial arch mutants in zebrafish I: Branchial arches. *Development* **123**, 329–344.

**Schmidt, D., Schwalie, P. C., Ross-Innes, C. S., Hurtado, A., Brown, G. D., Carroll, J. S., Flicek, P. and Odom, D. T.** (2010). A CTCF-independent role for cohesin in tissue-specific transcription. *Genome Res.* **20**, 578–588.

**Schwend, T. and Ahlgren, S. C.** (2009). Zebrafish con/disp1 reveals multiple spatiotemporal requirements for Hedgehog-signaling in craniofacial development. *BMC Dev. Biol.* **9**, 1–22.

**Scott, J. E.** (1996). Alcian blue. Now you see it, now you don't. *Eur. J. Oral Sci.* **104**, 2–9.

**Shapiro, M. D., Marks, M. E., Peichel, C. L., Blackman, B. K., Nereng, K. S., Jónsson, B., Schluter, D. and Kingsley, D. M.** (2004). Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. *Nature* **428**, 717–723.

**Sharpe, J., Ahlgren, U., Perry, P., Hill, B., Ross, A., Hecksher-Sørensen, J., Baldock, R. and Davidson, D.** (2002). Optical projection tomography as a tool for 3D microscopy and gene expression studies. *Science (80-. ).* **296**, 541–545.

**Shwartz, Y., Viukov, S., Krief, S. and Zelzer, E.** (2016). Joint Development Involves a Continuous Influx of Gdf5-Positive Cells. *Cell Rep.* **15**, 2577–2587.

**Silverman, F. N. and Reiley, M. A.** (1985). Spondylo-megaepiphyseal-metaphyseal dysplasia: A new bone dysplasia resembling cleidocranial dysplasia. *Radiology* **156**, 365–371.

**Simsek-Kiper, P. O., Kosukcu, C., Akgun-Dogan, O., Gocmen, R., Utine, G. E., Soyer, T., Korkmaz-Toygar, A., Nishimura, G., Alikasifoglu, M. and Boduroglu, K.** (2019). A novel NKX3-2 mutation associated with perinatal lethal phenotype of spondylo-megaepiphyseal-metaphyseal dysplasia in a neonate. *Eur. J. Med. Genet.* **62**, 21–26.

**Square, T., Jandzik, D., Cattell, M., Coe, A., Doherty, J. and Medeiros, D. M.** (2015a). A gene expression map of the larval Xenopus laevis head reveals developmental changes underlying the evolution of new skeletal elements. *Dev. Biol.* **397**, 293–304.

**Square, T., Jandzik, D., Cattell, M., Coe, A., Doherty, J. and Medeiros, D. M.** (2015b). A gene expression map of the larval Xenopus laevis head reveals developmental changes underlying the evolution of new skeletal elements. *Dev. Biol.* **397**, 293–304.

**Storm, E. E. and Kingsley, D. M.** (1996). Joint patterning defects caused by single and double mutations in members of the bone morphogenetic protein (BMP) family. *Development* **122**, 3969–3979.

**Storm, E. E. and Kingsley, D. M.** (1999). GDF5 Coordinates Bone and Joint Formation during Digit Development. *Dev. Biol.* **209**, 11–27.
**Storm, E. E., Huynh, T. V, Copeland, N. G., Jenkins, N. A., Kingsley, D. M. and Lee, S.-J.** (1994). Limb alterations in brachypodism mice due to mutations in a new member of the TGFb-superfamily. *Nature* **368**, 639–643.
**Sucena, É. and Stern, D. L.** (2000). Divergence of larval morphology between Drosophila sechellia and its sibling species caused by cis-regulatory evolution of ovo/shaven-baby. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 4530–4534.
**Summerton, J. and Weller, D.** (1997). Morpholino anitsense oligomers: design, preparation and properties. *Antisense Nucleic Acid Drug Dev.* **7**, 187–195.
**Takechi, M. and Kuratani, S.** (2010). History of studies on mammalian middle ear evolution: A comparative morphological and developmental biology perspective. *J. Exp. Zool. (Mol. Dev. Evol.)* **314B**, 417–433.
**Talbot, J. C., Johnson, S. L. and Kimmel, C. B.** (2010). hand2 and Dlx genes specify dorsal, intermediate and ventral domains within zebrafish pharyngeal arches. *Development* **137**, 2507–2517.
**Tanaka, E., Detamore, M. S., Tanimoto, K. and Kawai, N.** (2008). Lubrication of the temporomandibular joint. *Ann. Biomed. Eng.* **36**, 14–29.
**Tang, T. H., Bachellerie, J. P., Rozhdestvensky, T., Bortolin, M. L., Huber, H., Drungowski, M., Elge, T., Brosius, J. and Hüttenhofer, A.** (2002). Identification of 86 candidates for small non-messenger RNAs from the archaeon Archaeoglobus fulgidus. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 7536–7541.
**Terns, M. P. and Terns, R. M.** (2011). CRISPR-based adaptive immune systems. *Curr. Opin. Microbiol.* **14**, 321–327.
**Thomas, J. T., Lin, K., Nandedkar, M., Camargo, M., Cervenka, J. and Luyten, F. P.** (1996). A human chondrodysplasia due to a mutation in a TGF-B superfamily member. *Nat. Genet.* **12**, 315–317.
**Tribioli, C. and Lufkin, T.** (1999). The murine Bapx1 homeobox gene plays a critical role in embryonic development of the axial skeleton and spleen. *Development* **126**, 5699–5711.
**Tucker, A. S., Watson, R. P., Lettice, L. A., Yamada, G. and Hill, R. E.** (2004). Bapx1 regulates patterning in the middle ear: altered regulatory role in the transition from the proximal jaw during vertebrate evolution. *Development* **131**, 1235–1245.
**Varshney, G. K., Pei, W., Lafave, M. C., Idol, J., Xu, L., Gallardo, V., Carrington, B., Bishop, K., Jones, M., Li, M., et al.** (2015). High-throughput gene targeting and phenotyping in zebrafish using CRISPR/Cas9. *Genome Res.* **25**, 1030–1042.
**Varshney, G. K., Carrington, B., Pei, W., Bishop, K., Chen, Z., Fan, C., Xu, L., Jones, M., LaFave, M. C., Ledin, J., et al.** (2016). A high-throughput functional genomics workflow based on CRISPR/Cas9-mediated targeted mutagenesis in zebrafish. *Nat. Protoc.* **11**, 2357–2375.
**von Frisch, K.** (1938). The sense of hearing in fish. *Nature* **141**, 8–11.
**Walker, M. B. and Kimmel, C. B.** (2007). A two-color acid-free cartilage and bone stain for zebrafish larvae. *Biotech. Histochem.* **82**, 23–28.

**Wilson, J. and Tucker, A. S.** (2004). Fgf and Bmp signals repress the expression of Bapx1 in the mandibular mesenchyme and control the position of the developing jaw joint. *Dev. Biol.* **266**, 138–150.

**Yamashita, S., Andoh, M., Ueno-Kudoh, H., Sato, T., Miyaki, S. and Asahara, H.** (2009). Sox9 directly promotes Bapx1 gene expression to repress Runx2 in chondrocytes. *Exp. Cell Res.* **315**, 2231–2240.

**Yoshida, C. A., Yamamoto, H., Fujita, T., Furuichi, T., Ito, K., Inoue, K. I., Yamana, K., Zanma, A., Takada, K., Ito, Y., et al.** (2004). Runx2 and Runx3 are essential for chondrocyte maturation, and Runx2 regulates limb growth through induction of Indian hedgehog. *Genes Dev.* **18**, 952–963.

**Zheng, Q., Zhou, G., Morello, R., Chen, Y., Garcia-Rojas, X. and Lee, B.** (2003). Type X collagen gene regulation by Runx2 contributes directly to its hypertrophic chondrocyte-specific expression in vivo. *J. Cell Biol.* **162**, 833–842.

**Zhu, M., Yu, X., Ahlberg, P. E., Choo, B., Lu, J., Qiao, T., Qu, Q., Zhao, W., Jia, L., Blom, H., et al.** (2013). A Silurian placoderm with osteichthyan-like marginal jaw bones. *Nature* **502**, 188–193.

**Zuniga, E., Stellabotte, F. and Crump, J. G.** (2010). Jagged-Notch signaling ensures dorsal skeletal identity in the vertebrate face. *Development* **137**, 1843–1852.

**Zuniga, E., Rippen, M., Alexander, C., Schilling, T. F. and Crump, J. G.** (2011). Gremlin 2 regulates distinct roles of BMP and endothelin 1 signaling in dorsoventral patterning of the facial skeleton. *Development* **138**, 5147–5156.

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Editor: The Dean of the Faculty of Science and Technology


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