Fgf8a mutation affects craniofacial development and skeletal gene expression in zebrafish larvae

I. G. E. Gebuijs1,2,3, S. T. Raterman1,2,3, J. R. Metz2, L. Swanenberg1,3, J. Zethof3, R. Van den Bos3, C. E. L. Carels2,4,5, F. A. D. T. G. Wagener1,2 and J. W. Von den Hoff1,2,*

ABSTRACT
Craniofacial development is tightly regulated and therefore highly vulnerable to disturbance by genetic and environmental factors. Fibroblast growth factors (FGFs) direct migration, proliferation and survival of cranial neural crest cells (CNCCs) forming the human face. In this study, we analyzed bone and cartilage formation in the head of five dpf fgf8ati282 zebrafish larvae and assessed gene expression levels for 11 genes involved in these processes. In addition, in situ hybridization was performed on 8 and 24 hours post fertilization (hpf) larvae (fgf8a, dlx2a, runx2a, col2a1a). A significant size reduction of eight out of nine craniofacial cartilage structures was found in homozygous mutant (6–36%, P<0.01) and heterozygous (7–24%, P<0.01) larvae. Also, nine mineralized structures were not observed in all or part of the homozygous (0–71%, P<0.0001) and heterozygous (33–100%, P=0.0001) larvae. In homozygote mutants, runx2a and sp7 expression was upregulated compared to wild type, presumably to compensate for the reduced bone formation. Decreased col9a1b expression may compromise cartilage formation. Upregulated dlx2a in homozygotes indicates impaired CNCC function. Dlx2a expression was reduced in the first and second stream of CNCCs in homozygous mutants at 24 hpf, as shown by in situ hybridization. This indicates an impairment of CNCC migration and survival by fgf8 mutation.

KEY WORDS: Zebrafish, FGF8, Craniofacial development, Morphology, Gene expression, Bone, Cartilage

INTRODUCTION
In the human embryo, craniofacial development starts around week 4 with the formation of five facial prominences in the pharyngeal arches by the differentiation of cranial neural crest cells (CNCCs) into chondroblasts (Sperber et al., 2010; Hall and Hördtad, 1989). These prominences give rise to the different parts of the face, including the mandible, maxilla, palate, lips and nose. Some parts of the adult craniofacial skeleton are formed by endochondral ossification that involves the replacement of a cartilage template by bone (Ornitz and Marie, 2015). Other bones such as the skull bones, mandibular body, maxilla and palate, are formed by intramembranous ossification, which is the direct deposition of bone by osteoblasts that may be also neural crest cell-derived (Ornitz and Marie, 2015). The formation of the bony and cartilaginous elements involved in morphogenesis of the mammalian head is tightly controlled by a network of signaling pathways, including bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), Wingless-int (Wnt) and Hedgehog (Hh) proteins (Salazar et al., 2016; Litsiou et al., 2005; St-Jacques et al., 1999; Monroe et al., 2012; Ornitz and Marie, 2015; Abzhanov et al., 2007). FGFs and their receptors seem to be crucial in the initial phase of craniofacial development as they direct the migration, proliferation and survival of CNCCs that form the pharyngeal arches (Crump et al., 2004; Creuzet et al., 2004). Next to that, FGFs are also crucial in the development of the lip, palate and teeth (Stanier and Pauws, 2012; Nie et al., 2006).

In humans, 22 fibroblast growth factors (FGF1-14, 16-23) can bind to and activate four distinct fibroblast growth factor receptors (FGFR1-4) (Teven et al., 2014; Ornitz and Marie, 2015). The FGFs function as autocrine, paracrine or endocrine factors. Upon binding of FGFs to their cognate receptor, specific tyrosine residues are phosphorylated leading to activation of four different intracellular signaling cascades: the RAS-MAPK, PI3K, PLCγ and STAT pathways (Ornitz and Itoh, 2015). FGF family members have key functions in early development, in particular in cell differentiation and survival and during pattern formation. Also, FGFs are essential regulators of skeletal development as they act as chemokines by recruiting and activating chondroblasts and osteoblasts (Ornitz and Marie, 2015; Horton and Degnin, 2009; Marie, 2012).

An important player in craniofacial development is FGF8 as it is involved in CNCC migration, differentiation and survival, and development of the pharyngeal arches and the palate (Trumpp et al., 2000; Kubota and Ito, 2000; Abu-Issa et al., 2002; Shao et al., 2012). In mice, Fg8 is specifically expressed at the site of fusion of the medial and lateral nasal prominences that form the lip and primary palate (Bachler and Neübuser, 2001; Wilke et al., 1997). Mice with a heterozygous Fg8 null allele display the craniofacial phenotype of the human 22q11 syndrome with an underdeveloped jaw and cleft of the bony palate (Frank et al., 2002). In both humans and mice, mutations in the Fg8 gene cause deficiency of the gonadotropin-releasing hormone, leading to idiopathic hypogonadotropic hypogonadism (IHH) with or without a defect in the sense of smell (Kallmann syndrome and normosmic IHH, respectively) (Falardeau et al., 2008). In that same study, six human IHH patients carrying different point mutations in conserved residues of the Fg8 gene were identified, three of which also had a cleft in the lip and/or palate (Falardeau et al., 2008). In a DNA sequencing study, an individual with bilateral cleft lip and palate was identified with a de novo FGF8 mutation (Riley et al., 2007). Structural analysis showed that this mutation causes a...
conformational change in the FGF8 protein that reduces the binding affinity to its cognate receptors resulting in a loss of function.

Several studies using zebrafish (Danio rerio) as a model system have investigated genes involved in craniofacial development and provided new clues on the etiology of human syndromic and non-syndromic cleft lip and/or palate (CL/P), as reviewed recently (Duncan et al., 2017). For example, variants of IRF6, a known regulator of human palatogenesis, are implicated both in syndromic (Van der Woude syndrome) and non-syndromic CL/P (Kondo et al., 2014). Zebrafish research has also investigated genes involved in craniofacial development and provided new clues on the etiology of human syndromic and non-syndromic cleft lip and/or palate (CL/P), as reviewed recently (Dougherty et al., 2013). As the irf6 gene is highly conserved, mutations in amniotes may also disrupt CNCC migration and integration during palatogenesis, eventually leading to CL/P (Dougherty et al., 2013). Alternatively, some patients with Van der Woude syndrome have a mutation in the GRHL3 gene, a downstream target of IRF6 (Peyrard-Janvid et al., 2014). Zebrafish research has revealed a key role for this gene in oral peridenum differentiation, which is indispensable for human palatogenesis (de la Garza et al., 2013).

In the present study we address the role of Fgf8 in craniofacial development using the zebrafish mutant line fgf8a<sup>ti282</sup> (also reported as acerebellar mutant, ace; Reifers et al., 1998). The mutant allele has a point mutation (G>A at position 282) in the splice donor site following exon 3, which leads to skipping of exon 3 and the reading frame to run into a stop codon. The resulting truncated protein is non-functional since it lacks the motif required for receptor activation (Reifers et al., 1998) (Fig. 1).

Previous studies using homozygous fgf8a<sup>ti282</sup> knockout zebrafish larvae at 4 and 5 days post fertilization (dpf) have reported impaired craniofacial development, showing incompletely formed or even entirely lost cartilage elements (Crump et al., 2004; Choe and Crump, 2014). These studies have only briefly described the fgf8a<sup>ti282</sup> mutant craniofacial phenotype, mainly focusing on the lower jaw region. Up to now, an extensive analysis of the effects of the lack of Fgf8a on craniofacial development is lacking. Also, effects of the mutation on downstream signaling pathways in bone and cartilage formation have not been assessed yet. In the current study, a morphometric analysis of nine cartilaginous and nine mineralized elements in the zebrafish head was performed on 5 dpf fgf8a<sup>ti282</sup> homo- and heterozygous larvae, which was compared to wild type. In addition, expression of 11 genes crucial in bone and cartilage formation was assessed in the three groups. Also, in situ hybridization was performed for selected genes at 8 and 24 hours post fertilization (hpf). We believe this research contributes to the understanding of the role of FGF8 in craniofacial development in vertebrates.

**RESULTS**

Wild-type (n=49), fgf8a<sup>ti282</sup> heterozygous (n=43) and fgf8a<sup>ti282</sup> homozygous (n=31) larvae were stained for cartilage and mineralized tissue. Fgf8a<sup>ti282</sup> homo- and heterozygous larvae showed a comparable reduction in size of the head, which seemed to affect mainly the anterior part. It was also clearly visible that a number of cartilage structures were underdeveloped and bone structures were less mineralized in homo- and heterozygous larvae (Fig. 2).

**Aberrant cartilage phenotype in fgf8a<sup>ti282</sup> homo- and heterozygous larvae**

Eight (numbers one to four, six to nine) out of nine parameters were found to be significantly smaller in size for fgf8a<sup>ti282</sup> homo- and heterozygotes compared to wild type (Fig. 3). Only parameter number five, the width between the ceratohyal-paloquadrate joints on both sides, was not significantly different for both genotypes.

The largest reduction in size in both homo- and heterozygotes was found for the length of the ethmoid plate (32 and 24% lower than in wild type) and the length of the Meckel’s cartilage (36 and 24%, respectively). First arch structures, such as Meckel’s cartilage (four, nine) and the ethmoid plate (eight), showed a much larger reduction compared to those of the superior and inferior ceratohyals, both derived from the second arch. A slightly smaller, but significant size reduction was found for the length of the anterior and posterior parts of the head, and the total head length (parameters one, two and three). Also, the width anteriorly in the head (parameter four) was significantly reduced, whereas the width more posteriorly (parameter five) was not significantly reduced, corresponding with the regions derived from respectively pharyngeal arch 1 and 2.

**Reduced bone formation in fgf8a<sup>ti282</sup> homo- and heterozygous zebrafish**

In 17 out of 43 (40%) of the fgf8a<sup>ti282</sup> heterozygous larvae and 14 out of 31 (45%) of the homozygous larvae, the parapenoid bone (one of the first bone structures that ossifies) was not mineralized, while it was mineralized in all wild-type larvae. Also, eight other mineralized structures were often absent in homo- and heterozygous larvae, with heterozygous larvae being mostly affected (Fig. 4). For instance, ceratobranchial 5 and branchiostegal ray 1 were not mineralized in...
98% of the heterozygous larvae, while both structures were found in almost all wild-type larvae (98% and 90%, respectively). In homozygotes, ceratobranchial 5 was not mineralized in 58% of the larvae, although often mineralized teeth were already present. The homozygotes never showed a complete loss of an element. The presence of mineralized structures was significantly different between the three groups for all parameters (P<0.001).

Altered gene expression in homozygous fgf8ati282 larvae
To shed light on the observed changes in formation of cartilage and bone elements, the expression of 11 different genes, related to bone and cartilage, and neural crest cells was evaluated (Fig. 5). The groups consisted of fgf8ati282 homozygous mutant (n=17), heterozygous (n=49) and wild-type (n=22) larvae. The homozygous mutants showed a significant upregulation of fgf8a compared to wild type (P<0.001) and heterozygotes (P<0.01). The expression of the osteoblast genes runx2a (an essential transcription factor early in osteoblast differentiation) and sp7 (a transcription factor that is crucial for differentiation of preosteoblasts to mature osteoblasts, as well as to increase osteoblast activity), was significantly upregulated in the mutant larvae compared to both the wild-type and heterozygous larvae. In contrast, the bone matrix gene col1a1a was significantly downregulated in mutants; col1a2 did not show any differences between the three groups. Col2a1, which encodes a chain of collagen type II, an extracellular matrix (ECM) component of cartilage, showed a reduced expression level in mutants (Fig. 5). One of the genes also encoding a component of hyaline cartilage, col9a1b, was found to be significantly lower expressed in homozygotes compared to the wild type (72%, P<0.01), although expression of the related col9a1a was not affected. The CNCC
Fig. 5. Relative expression levels for 11 genes in 5 dpf wild-type, heterozygote and mutant fgf8a^{+/+} zebrafish. Each data point represents one individual; the horizontal line depicts the median, error bars indicate the interquartile ranges. The expression was assessed for fgf8a (A), runx2a (B), runx2b (C), sp7 (D), col1a1a (E), col1a2 (F), col2a1a (G), col9a1a (H), col9a1b (I), dix2a (J) and nkx3.2 (K). Asterisks indicate the significance level: *=P<0.05, **=P<0.01, ***=P<0.001.
marker gene *dlx2a* was significantly upregulated in the homozygous mutants compared to both wild type and heterozygotes (*P*<0.05). Also the gene encoding NK homeobox 2 (*nkx3.2* or *bapx1*), involved in joint patterning, was upregulated in mutants compared to wild type (*P*<0.001) and heterozygotes (*P*<0.01). In contrast, the heterozygous larvae did not show any significant differences in gene expression compared with the wild type.

**Spatiotemporal gene expression in fgf8a^32ab2^ mutants during early development**

Wholemount in situ hybridization showed that *fgf8a* is expressed towards the vegetal pole at 8 hpf in homozygous, heterozygotes and wild-type embryos (Fig. 6A). At 24 hpf *fgf8a* was highly expressed in the dorsal diencephalon and the mid-hindbrain boundary in heterozygotes and wild-type larvae, while the structure was absent.
in the homozygous mutant larvae. Additionally, we showed abundant fgf8a expression in the posterior somites and tail end of homozygous mutant compared to heterozygous larvae.

The homeobox transcription factor dlx2a was not detected in 8 hpf homozygous mutant embryos but only in heterozygous and wild-type embryos (Fig. 6B). At 24 hpf dlx2a expression defines a subpopulation of CNCCs precursors for the pharyngeal arches. These distinct populations migrate laterally and will form the craniofacial bone and cartilage elements. In homozygous, heterozygous and wild-type larvae four well-separated populations of CNCCs were distinguished bilaterally. Our data show that populations I and II expressed more dlx2a in wild-type and heterozygous larvae, whereas in homozygous mutant larval expression was reduced in these populations and most abundant in the third (III) population.

We noted a slightly increased expression of runx2a in 8 hpf heterozygous and homozygous mutant embryos (Fig. 6C). Moreover, at 24 hpf runx2a was expressed throughout the cranial region with an increased intensity in the homozygous mutant larva.

At 8 hpf the col2a1a expression was slightly reduced in the mutant embryos as compared to wild-type and heterozygous embryos (Fig. 6D). Col2a1a positive cells in the postchordal area seemed to be located more anteriorly in the wild-type versus the heterozygous larvae at 24 hpf.

**DISCUSSION**

We analyzed the effects of the fgf8a<sup>-/-</sup> mutation on bone and cartilage formation, related gene expression in 5 dpf zebrafish larvae. Homo- and heterozygous fgf8a<sup>-/-</sup> zebrafish larvae were compared to wild-type littermates for differences in bone and cartilage development, the expression levels of 11 developmental genes. In addition, *in situ* hybridization was performed for selected genes at 8 and 24 hpf. Our data show that bone and cartilage formation is severely impaired in both fgf8a<sup>-/-</sup> homo- and heterozygous larvae in comparison to wild type. Fgf8a<sup>-/-</sup> homozygous mutant larvae also show differences in expression levels of bone, cartilage and CNCC marker genes.

All evaluated mineralized structures were significantly less identified, or even completely absent in fgf8a<sup>-/-</sup> heterozygous larvae compared to wild-type siblings. In these larvae, often the opercles and branchiostegal rays could not be identified, and all of the larvae did not show any mineralization of the entopterygoid bone. Interestingly, in 40% of the heterozygous larvae the parapophyseal bone – an intramembranous bone that is part of the roof of the mouth – was also not observed. In our study, the homozygous mutant larvae showed similar results as the heterozygotes, with absence of mineralization of certain elements. The lowest presence of mineralization was seen for the branchiostegal rays (35%) and entopterygoid bone (29%). In a previous study, nearly all fgf8a<sup>-/-</sup> homozygous mutant zebrafish larvae were also missing the branchiostegal rays (Albertson and Yelick, 2007). Interestingly, the homoygotes often had teeth, although ceratobranchial five was not mineralized, which has been reported earlier (Crum et al., 2004). A study in Fgf8 knockout mice shows that newborns are also missing skeletal elements, specifically those derived from the first pharyngeal arch, such as Meckel's cartilage and also some of the second arch (Trupp et al., 1999). The missing elements included the palate and pterygoid bones, parts of the roof of the mouth, which we take as similar to our findings in zebrafish larvae. These Fgf8 mouse mutants also show features reminiscent of agnathia and holoprosencephaly, which are also found in patients with first arch syndromes (Bixler et al., 1985). Therefore, it seems that disturbed FGF8 signaling in both zebrafish
expression between the three groups. This indicates that chondrocytes are present but the cartilage might be weaker in homozygous mutants because of a lack of col9a1b. The reduction in size of the cartilage elements might also be due to a reduced survival of chondrocytes in both hetero- and homozygotes, which was reported in a mouse study (Abu-Issa et al., 2002).

The expression of fgf8a in homozygote mutants at 5 dpf is significantly upregulated compared to the wild type and the heterozygotes. In situ hybridization for fgf8a at 24 hpf clearly showed that the mid-hindbrain barrier is missing in homozygotes. As the fgf8ati282 mutant alleles produce a non-functional protein (Reifers et al., 1998), a feedback loop might be activated to compensate for the lack of functional Fgf8 protein. Apparently, this feedback loop is not activated in heterozygotes.

We furthermore showed that the expression of dlx2a and nks3.2, two genes that are known to be inhibited by Fgf8 (Walsh and Mason, 2003; Wilson and Tucker, 2004), is enhanced in homozygote mutants. Dlx2a is involved in tooth development and is expressed in migrating CNCCs that contribute to the pharyngeal arches (Sperber et al., 2008), while nks3.2 is involved in jaw joint formation (Miller et al., 2003). Dlx2a is expressed by a subset of premigratory, migratory and condensing cranial neural crest cells (Yan et al., 2005). During the 23-somite stage three cell streams on both sides of the developing brain express dlx2a. The first stream will form the mandibular arch structures, the second stream of cells forms the structures of the hyoid arch, and the third stream separates subsequently into five distinct cell groups that will form the centrobranchial cartilages (gill arch 3–7) (Mork and Crump, 2015). In situ hybridization of dlx2a at 24 hpf showed that the third cell stream has started to form the fourth population of CNCCs. Interestingly, we observed a decrease in dlx2a in the first and second stream in homozygous mutant larvae as compared to wild type and heterozygotes. This supports the morphological defects in first and second arch structures at 5 dpf.

It is also known that FGF8 inhibits the expression of Dlx2 in mice (Thomas et al., 2000) and that of nks3.2 in zebrafish (Wilson and Tucker, 2004). Therefore, it seems that the upregulation of these two genes is caused by the loss of inhibition by Fgf8. The upregulation of dlx2a in the homozygotes might explain the presence of teeth, as it stimulates the development of the pharyngeal dentition (Borday-Biraux et al., 2006). Heterozygous fgf8a

In conclusion, bone and cartilage formation is impaired in fgf8ati282 homo- and heterozygous mutant larvae. As mainly first arch structures are affected, this points towards the impairment of CNCC function, which is supported by the gene expression and in situ hybridization data. Both the zebrafish neurocranium and the human craniofacial region are derived from migrating CNCCs. It appears that a conserved genetic network including fgf8a regulates the formation of this region in all vertebrates (Mork and Crump, 2015; Swartz et al., 2011). We have shown that a mutation in fgf8a also influences the expression of genes regulating bone and cartilage formation such as runx2a, sp7 and col1a1a, as well as dlx2a, a gene involved in the migration of CNCCs. As fgf8a is highly conserved from fish to mammals, mutations in the FGF8 gene in humans may also have negative effects on CNCCs and skeletogenesis. These mutations may lead to craniofacial malformations such as CL/P. In a human genetic study on non-syndromic cleft lip and palate, an individual with bilateral CL/P was found carrying a missense mutation in the FGF8 gene (Riley et al., 2007). This was also a loss-of-function mutation reducing the binding of FGF8 to its receptors. The current findings corroborate the value of zebrafish mutant models in the unraveling of the role of FGF8 in craniofacial development and the etiology of craniofacial malformations.

MATERIALS AND METHODS
Zebrafish breeding and husbandry
Zebrafish were raised and kept at 28°C under a 14 h light/10 h dark cycle with twice-a-day feeding at the Radboud University Zebrafish Facility. The mutant line fgf8a

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Genotyping
DNA from the tail clip biopsy and from the post-in situ samples was extracted through incubation in 15 μl NaOH (50 mM) for 30 min at 95°C. Samples were vortexed twice. After incubation, samples were placed on ice for 10 min and 1.5 μl of 1 M Tris (pH=8) was added. The DNA concentration of the samples was measured spectrophotometrically at 260 nm wavelength using a NanoDrop™ (Thermo Fisher Scientific, Wilmington, USA) and adjusted to a final quantity of 30 ng. The DNA in each individual sample was amplified by a tetra-primer amplification refractory mutation (ARMS) PCR, with a not allele-specific (outer) pair and an allele-specific (inner) pair of primers. Primer sequences used were: forward in (A) 5′ GGGAACACTGGATGGCGACGA 3′; reverse in (G) 5′ TCACAAATAGTGATGACTTTTTCACAGAC 3′; forward out 5′ AGTTGGAGATGTGCGAGAGCATTAA 3′; reverse out 5′ TTTTTTTCTTTCTTAGGTGGAGAGCATTAA 3′. For each PCR reaction, 6 pmol of both inner primers and 1.5 pmol of both outer primers was used. PCR products were loaded on a 2% agarose gel (SeaKem LE Agarose, Lonza) and separated by electrophoresis. The three possible amplicons are a 213-bp non-allele specific, a 151-bp mutant (A) allele-specific, and a 109-bp wild-type (G) allele-specific fragment.

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For genotyping after gene expression analysis a slightly different method was used. Here, 3 μl of cDNA of each individual sample was mixed with 17 μl QPCR mix and amplified by PCR using the following primers: forward: GGTGAGCCGTAGACTAATCCG and reverse: GTGTTGCCGT- GTTTGGAGC. The PCR products were loaded on a 2% agarose gel (SeaKem LE Agarose, Lonza) and separated by electrophoresis. The two possible amplicons are a 370 bp wild-type (G) fragment and a 263 bp mutant (A) fragment.

**Cartilage and bone staining**

At 5 dpf, larvae identified as wild type or heterozygous for *fgf8αβ282* through tail-clip genotyping were grouped by genotype and stained in an Eppendorf tube for cartilage and bone based on the acid-free staining protocol by Walker and Kimmel (2007) with some modifications. Larvae were euthanized with 0.1% 2-phenoxyethanol (1:500) and fixated for 30 min in 2% paraformaldehyde. After washing with water and 80% ethanol, larvae were incubated in staining solution (0.02% Alcian Blue; Sigma-Aldrich, containing 40 mM of MgCl₂) for 1.5 h to stain cartilage. Larvae were washed in ethanol and cleared through bleaching in peroxide (0.8% KOH, 1.1% H₂O₂, 0.2% Triton) followed by two washing steps: first in 0.2% Triton and then in a saturated sodium tetraborate solution. This was then followed by trypsin digestion (1 mg/ml) for 15 min and a wash in 0.2% Triton. Subsequently, bone was stained overnight with 0.003% Alizarin Red (Sigma-Aldrich). Finally, the larvae were cleared in graded series of glycerol (25%, 50% and 75%) and stored in 100% glycerol for imaging.

Homozygous *fgf8αβ282* larvae could be phenotypically identified at 30 hpf by an enlarged tectum and lack of a mid-hindbrain boundary (Brand et al., 1996). Because homozygote larvae were very fragile due to edema, they were stained individually in a 48-well plate. Larvae were euthanized with 0.1% 2-phenoxyethanol and fixated for 30 min in 2% paraformaldehyde. After washing with 100 mM Tris/40 mM MgCl₂ (pH 7.5) larvae were stained with Alcian Blue as described. Larvae were washed for 5 min in a series of 80% ethanol [100 mM Tris/40 mM MgCl₂ (pH 7.5)] and 50 and 25% ethanol [100 mM Tris (pH 7.5)] and bleached in a H₂O₂ solution (3% H₂O₂/0.5% KOH), followed by two wash steps with 25% glycerol/0.1% KOH for 10 min. Subsequently, bone was stained overnight with 0.01% Alizarin Red (pH 7.5, Sigma-Aldrich). The next day, the larvae were washed twice with 50% glycerol/0.1% KOH for 10 min and stored in 100% glycerol for imaging. Following imaging, whole larvae were washed in PBS and genotyped according to the described procedure.

**Zebrafish imaging**

Larvae stained for cartilage and bone stored in 100% glycerol were loaded into round borosilicate glass capillaries (CV6084-100, Vitrocom, USA), which were placed inside square borosilicate capillaries (CV8290-100, Vitrocom, USA) also filled with 100% glycerol. The capillaries were placed in a sample holder with an axial rotating system (adapted from Bruns et al., 2015) and images were acquired from dorsal, ventral and lateral sides of the larvae with a binocular microscope (Leica DMRE) using Leica Application Suite (LAS 3.3, Leica).

The embryos stained with *in situ* probes were cleared in a 2:1 mixture of benzyl benzoate (Merck) and benzyl alcohol (Merck) during imaging. Images were acquired with a binocular microscope (Leica DMRE) using Leica Application Suite (LAS 3.3, Leica).

**Bone and cartilage analysis**

Pictures were imported in FIJI (Schindelin et al., 2012) and the following craniofacial cartilage parameters were measured: (1) total head length, (2) length cartalohyal to anterior end of the head, (3) length cartalohyal to posterior end of the head, (4) width at Meckel’s cartilage and palatoquadrate joint, (5) width between cartalohyal and palatoquadrate joint, (6) length of superior cartalohyal, (7) length of inferior cartalohyal, (8) length of ethmoid plate, (9) length of Meckel’s cartilage from the lateral side. The straight-line tool was used to measure most parameters, but for curved elements the segment-line tool was used. For mineralized tissue stained by Alizarin Red, nine elements were scored for presence or absence of mineralization: (1) paraphenoid, (2) cleithrum, (3) notochordal sheath, (4) otoliths (all four present or not), (5) teeth (on ceratobranchial 5), (6) ceratobranchial 5, (7) opercles, (8) branchiostegal ray 1, (9) entopterygoid bone. All parameters are depicted in Fig. 7.

**Gene expression analysis**

Another 96 larvae were randomly selected from a pool of offspring from heterozygous adults to ensure a blind procedure and euthanized with 0.1% 2-phenoxyethanol. Upon euthanasia, individual larvae were transferred to 2-ml Eppendorf tubes containing a plastic grinding ball and the total RNA of each sample was isolated. To this end, the larvae were

![Figure 7](https://example.com/figure7.png)

**Fig. 7. 5 dpf zebrafish larva (wild type) stained for cartilage (blue) and bone (red).** A total of nine parameters for cartilage were assessed as shown in panels A (ventral view) and B (lateral view): (1) total head length, (2) length cartalohyal to anterior end of the head, (3) length cartalohyal to posterior end of the head, (4) width at Meckel’s cartilage and palatoquadrate joint, (5) width between cartalohyal and palatoquadrate joint, (6) length of superior cartalohyal, (7) length of inferior cartalohyal, (8) length of ethmoid plate, (9) lateral length of Meckel’s cartilage. C. The nine bone parameters scored for presence or absence (ventral view): (1) paraphenoid, (2) cleithrum, (3) notochordal sheath, (4) otoliths (four in total), (5) ceratobranchial 5, (6) teeth (on ceratobranchial 5), (7) opercles, (8) branchiostegal ray 1, (9) entopterygoid bone.
homogenized in 400 μl Trizol reagent (Invitrogen, Carlsbad, USA) using a grinding mill for 20 s at 20 Hz. Samples were incubated at room temperature for 5 min and 80 μl chloroform was then added. Tubes were shaken for 15 s, followed by incubation at room temperature for 2 min. Samples were centrifuged at 18,000 × g for 10 min in a cooled centrifuge (4°C). The supernatant was decanted and the pellet was washed and then dissolved in 100 μl DEPC-treated water. The RNA concentration and purity of the samples were determined using a NanoDrop™ spectrophotometer at 260 nm wavelength (Thermo Fisher Scientific).

The thus isolated RNA was treated with DNase to remove traces of genomic DNA. RNA (200 ng) was transferred into a PCR strip and DEPC-treated water was added to a total volume of 8 μl. 2 μl DNase mix [1 μl 10X DNase I reaction buffer and 1 μl (1 U/μl) amplification grade DNase I (both from Invitrogen)] was added and the solution was incubated for 15 min at room temperature. Following incubation, 1 μl 25 mM EDTA was added to stop the DNase reaction and the reaction mix was incubated for 10 min at 65°C and stored on ice. Samples were used to synthesize cDNA by adding 1 μl random primers (250 ng/μl), 1 μl 10 mM dNTP mix, 4 μl 5X 1st strand buffer, 1 μl 0.1 M DTT, 1 μl RNase inhibitor (10 U/μl), 0.5 μl Superscript II (reverse transcriptase 200 U/μl) (both from Invitrogen) and 0.5 μl DEPC-treated water. The resulting mix was incubated for 10 min at 25°C for annealing of the primers and then for 50 min at 42°C for reverse transcription. Enzymes were inactivated by incubation at 70°C for 15 min. Finally, the samples were diluted five times to serve as template in the qPCR reaction.

A real-time qPCR was carried out for each gene of interest. For each qPCR reaction, 4 μl of cDNA was mixed with 16 μl PCR mix (containing 10 μl SYBR green mix (2X) (Bio-Rad, Hercules, USA), 0.7 μl of each gene-specific primer (10 μM and 4.6 μl water). Primers used are listed in Table 1. The qPCR reaction (3 min 95°C, 40 cycles of 15 s 95°C and 1 min 60°C) was performed using a CFX 96 (Bio-Rad) qPCR machine. Threshold cycles (Ct values) were assessed and relative expression was calculated based on a 2-ΔΔCt method (Livak and Schmittgen, 2001).

The resulting mix was incubated for 10 min in a cooled centrifuge (4°C). The supernatant was decanted and the pellet was washed with 75% ethanol, then air-dried for 5 min and 80 μl DEPC-treated water. The resulting mix was incubated for 10 min at 25°C for annealing of the primers and then for 50 min at 42°C for reverse transcription. Enzymes were inactivated by incubation at 70°C for 15 min. Finally, the samples were diluted five times to serve as template in the qPCR reaction.

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### in situ hybridization

Wholomebium in situ hybridization was performed essentially as previously described (Thissse and Thissse, 2008). In short, primer sequences to generate

### Table 1. Forward and reverse primers used for real-time qPCR and for preparing the probes for in situ hybridization (ISH)

| Gene | Forward primer sequence | Reverse primer sequence | Accession number |
|------|-------------------------|-------------------------|------------------|
| runx2a | TGTGGCTATGGCCTTCAACA | ATCTCCACATGGTCGGGT | NM_212858.2 |
| runx2b | GGCCGACCGCAGATACAGG | CTGCTGCACTGGAGACAG | NM_212862.2 |
| sp7 | GATAACCGGTGGGCTTA | TCTGTCAGAATGCGGACAT | NM_212863.2 |
| col1a1 | TTGCTTAGCCTGCCTTCA | CCGGGGGATTATACGCTT | NM_199214.1 |
| col1a2 | GGTGCGGCTTCTGCTTCA | AACGACGCTTCTGAGGTTT | NM_182968.2 |
| col2a1a | GCACTTTCAACCCCTTATGA | TGACTAATGCTGGATCAT | NM_131292.1 |
| col9a1a | GGGGTCGTTGCTTATTTCT | TTGCTGCGGCTTCTGCGT | NM_00130624.1 |
| col9a1b | AACGATTTGTCGGCAGAGAT | TACGCACTTTGCTGATC | NM_212642.4 |
| dlx2a | GACTGCGTATGCGCTTCT | TTGGTCACAACTGGCTAC | NM_131281.2 |
| nkd3.2 | AGCGCTAAAGGCGAAATCAG | TAAAGAGAGATGATGGGCTTAC | NM_131263.1 |
| fgf8a | GCCGATGAAACTCAGGAC | AGACGAGGCAATACCTGAGG | NM_212784.1 |
| elf1a | CTGGACAGCGACATCAAT | TCAAGAGGAGATGATGGGCTTAC | NM_131281.2 |
| rpl13 | TCTGGGCGAGTCTAAGGTAGT | AGACGAGGCAATACCTGAGG | NM_212784.1 |
| ISH | Forward primer sequence in situ hybridization | Reverse primer sequence in situ hybridization | Accession number |
| fgf8a | TTAGGCTTTTGGCGTGTCT | TACCTGCTCTCACCCTGCTT | NM_131281.2 |
| dlx2a | AGGTGGCATGTTTATG | AATGGGTCGCGCTGCTAAC | NM_131311.2 |
| col2a1a | TCACTGCTCTGCTGCTA | GGAGGCTTCTTTCACCTT | NM_131292.1 |
| runx2a | CAGGACCGAAAGACAGAC | GCTAAAGGCTTGCTGGAGG | NM_212858.2 |
Chi-Square test. Also here a Bonferroni correction was applied, resulting in a significance limit of 0.05/9=0.0056. As some of the data for gene expression were not normally distributed among groups, a Kruskal–Wallis test (with gene expression level as repeated measure; genotype as independent factor) was performed, followed by Dunn’s multiple comparison test.

Acknowledgements
Dr Peter Klaren is acknowledged for advice on statistical analyses, Tom Spanings and Antoon van der Horst for animal care and Prof. Gert Flik for providing the zebrafish facilities.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: J.W.V.d.H., J.R.M., C.E.L.C., F.A.D.T.G.W.; Visualization: E.G.E.G.; Supervision: J.W.V.d.H., J.R.M., C.E.L.C., F.A.D.T.G.W.; Project administration: J.W.V.d.H.; Funding acquisition: J.W.V.d.H.

Funding
This research was in part supported by grants of the Dr Vaillant Foundation and the Radboud University Medisch Centrum.

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