An adult osteopetrosis model in medaka reveals the importance of osteoclast function for bone remodeling in teleost fish

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Osteoclasts play important roles during bone growth and in maintaining bone health and bone homeostasis. Dysfunction or lack of osteoclasts leads to increased bone mass and osteopetrosis phenotypes in mouse and human. Here we report a severe osteopetrosis-like phenotype in transgenic medaka fish, in which membrane bound EGFP (mEGFP) was expressed in osteoclasts under control of the cathepsin K promoter (ctsk:mEGFP). In contrast to reporter lines with GFP expression in the cytoplasm of osteoclasts, adult fish of the mEGFP line developed bone defects indicative for an osteoclast dysfunction. Activity of tartrate-resistant acid phosphatase (TRAP) was down-regulated and excess bone was observed in most parts of the skeleton. The osteopetrotic phenotype was particularly obvious at the neural and haemal arches that failed to increase their volume in growing fish. Excess bone caused severe constriction of the spinal cord and the ventral aorta. The continuation of tooth development and the failure to shed teeth resulted in severe hyperodontia. Interestingly, at the vertebral column vertebral body arches displayed a severe osteopetrosis, while vertebral centra had no or only a mild osteopetrotic phenotype. This confirms previous reports from cichlids that, different from the arches, allometric growth of vertebral centra initially does not depend on the action of osteoclasts. Independent developmental mechanism that shapes arches and vertebral centra can also lend support to the hypothesis that vertebral centra and arches function as independent developmental modules. Together, this medaka osteopetrosis model confirms the importance of proper osteoclast function during normal skeletal development in teleost fish that requires bone modeling and remodeling.

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1. Introduction

Osteoclasts are bone resorbing cells that play important roles in modeling, remodeling and homeostasis of the mineralized skeleton. Osteoclast activity is tightly linked with that of bone forming osteoblasts in a process named bone cell coupling (Charles and Aliprantis, 2014). This is essential for keeping bone metabolism in balance and maintaining high bone quality. Excessive osteoclast numbers or increased activities lead to reduced bone mineral density and bone loss resulting in osteoporosis (Mizuno et al., 2002; Kearns et al., 2008; To et al., 2012). In contrast, a dysfunction or lack of osteoclasts results in increased bone mass and osteopetrosis. Importantly, osteopetrotic bone matrix can be brittle and may have increased fracture risk if there is insufficient remodeling of the mineralized matrix (reviewed in Sobacchi et al., 2013).

While osteoporosis and low bone mass are caused by numerous genetic and non-genetic factors (Lekamwasam et al., 2012) and affect more than 50% of adults above 50 years of age in the US (Wright et al., 2014; Jin et al., 2015), osteopetrosis is a relatively rare disease. Osteopetrosis is often caused by mutations in genes involved in osteoclast formation or function, and inherited in an autosomal recessive or autosomal dominant fashion (Cappariello et al., 2010; Crockett et al., 2011; Pangrazio et al., 2014). Depending on whether defective genes are involved in osteoclast formation or function, an osteopetrosis phenotype is described as osteoclast-poor or osteoclast-rich, respectively (Villa et al., 2009). A comprehensive list of osteoclast defects has been published by Del Fattore et al. (2008). All major osteoclast functions can be interrupted, such as the development of the ruffled border, the lysosomal storage of TRAP or the acidification of the subcellular space by H⁺–ATPase. Macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL) are expressed by osteoblasts and stromal cells and are essential for osteoclast formation. Mutations in the M-CSF or RANKL/RANK pathways therefore lead to absence or reduced numbers of osteoclasts and osteoclast-poor...
osteopetrosis (Dougall et al., 1999; Dobbins et al., 2002; Sobacchi et al., 2007; Pangrazio et al., 2012). In contrast, mutations in genes regulating osteoclast function, such as cathepsin K, encoding a collagenase (Saftig et al., 1998), or gl (Rajapurohitam et al., 2001) lead to excessive bone mass with normal or even increased numbers of dysfunctional or morphologically altered osteoclasts. A correct classification of different osteopetrosis types and phenotypes is complicated since not all types of osteopetrosis relate to primary bone disorders. It is, however, clinically relevant for current treatments for osteopetrosis (Del Fattore et al., 2008). Suggested treatments include hematopoietic stem cell transplantation (HSCT) and RANKL injection (Rappariello et al., 2010). HSCT is only effective in cases where osteoclast intrinsic functions are hampered (Crockett et al., 2011).

To better understand the genetics underlying various forms of pathological osteopetrosis, several mouse models have been established (reviewed in Sobacchi et al., 2013). However, live imaging of bone cells at high resolution is still technically limited in mouse models. Therefore, in recent years, small aquarium fish such as the zebrafish and medaka have become powerful models for bone research (Apschner et al., 2011; Bruneel and Witten, 2015). Teleost fish embryos and larvae are almost transparent and develop rapidly, making them ideally suited for live imaging of bone processes. Their skeleton develops similar to that of mammals by chondral and membranous ossification, and the genetic networks regulating bone formation are highly conserved. We previously reported an osteoclast fluorescence reporter line in medaka, which expresses membrane bound GFP (mEGFP) under control of the ctsk promoter (To et al., 2012). Using live imaging, we were able to show that transgenic activation of RANKL in embryos resulted in premature and ectopic formation of active osteoclasts. This led to increased bone resorption and an osteoporosis-like phenotype in the medaka adult stages (To et al., 2012). In the present study, we report a severe osteopetrosis-like phenotype in the same ctsk:mEGFP transgenic medaka line at adult stages. In fish with dysfunctional osteoclasts we observed excess bone particularly obvious at the endosteal bone surfaces of the neural and haemal arches of the vertebrae. These arches form protective canals for the spinal cord and the axial blood vessels, respectively. The failure to widen these canals by bone resorption during normal growth resulted in a severe constriction of the spinal cord with a curvature of the spine and the constriction, respectively, of the ventral aorta. This work establishes an adult medaka model for human osteopetrosis and underlines the importance of osteoclasts for bone remodeling in teleost fish.

2. Material and methods

2.1. Generation of transgenic fish

The generation of medaka osteoclast reporter lines expressing membrane bound EGFP (ctsk:mEGFP) and cytoplasmic mcherry (ctsk:mcherry) was described previously (To et al., 2012). The same ctsk promoter sequence was also subcloned into the KpnI and EcoRV sites in front of nuGFP into a Scel meganuclease vector to generate transgenic ctsk:nuGFP medaka lines as described previously (To et al., 2012). The nuGFP, kindly provided by Stefan Schulte-Merker (University of Muenster, Germany), localizes to the nucleus and cytoplasm in transgenic zebrafish and medaka (Knopf et al., 2011; Renn et al., 2013). All experiments were performed in accordance with approved IACUC protocols of the National University of Singapore (R14-293).

2.2. Bone staining and histological analysis

Mineralized matrix in adult fish fixed in 4% paraformaldehyde was stained with Alizarin Red as described previously (Renn and Winkler, 2009), after manually removing skin and muscles. The staining time was 3–4 days. Osteoclast activity was visualized by Tartrate-resistant acid phosphatase following the protocol described by Witten et al. (2001) for zebrafish osteoclasts. In short, specimens were decalcified with EDTA and embedded in glycol methacrylate. 5 μm sections were stained for TRAP using naphthol AS-TR phosphate as enzyme substrate and hematoxylin as color component in a solution that contained 50 mM L(+) di-sodium tartrate di-hydrate. For the histological analysis specimens were prepared following the protocol described in Bensimon-Brito et al. (2012) for zebrafish. Briefly, specimens were fixed in a mixture of buffered paraformaldehyde and glutaraldehyde, postfixed with osmium tetroxide and subsequently embedded in Epon epoxy. Sections of 1 μm were stained with toluidine blue at pH 11 for 30 s at 60 °C, rinsed in demineralized water, dried and mounted with DPX. A Carl Zeiss Axio Imager Z1 microscope was used for observations and micrography.

2.3. Imaging and statistical analysis

Adult medaka fish were anesthetized with 0.1% ethyl 3-amino benzoate methanesulfonate (Tricaine: Sigma E10521) and imaged using a Nikon SV11 stereomicroscope with the Nikon BR software. Images of single osteoclasts in live transgenic medaka were taken with a Zeiss LSM 510 Meta confocal microscope with 488 and 543 nm laser lines for GFP and mcherry, respectively. Imaging data were processed using Imaris 7.1.1 (bitplane) and ImageJ software. Alizarin red stained bone structures in analyzed fish were imaged with a Nikon SV11 stereomicroscope. For statistical quantification of the osteoporosis phenotype, vertebral 12–18 of WT (n = 4) and fish with phenotype (n = 5) were dissected from the vertebral column stained by Alizarin red. The diameter of each vertebral body was measured at its most narrow points (Fig. 3A′,B′). Also, the area of the neural canal of each vertebra was measured by ImageJ. The area differences in diameter of vertebral bodies and in the areas of neural canals of each analyzed vertebra between WT and transgenic fish were statistically calculated using one-way ANOVA with GraphPad Prism 5.

3. Results

3.1. Membrane-localized mEGFP expression in osteoclasts reduces bone resorption and leads to vertebral deformations in adult medaka

We previously reported a medaka osteoclast reporter line expressing membrane-bound mEGFP under control of the ctsk promoter. We described transgene expression from 5 dpf until 1 month, with the first vertebral osteoclasts appearing at 3 weeks post fertilization (wpf) (To et al., 2012). In addition, we also generated two ctsk reporter lines expressing GFP with a nuclear localization signal (Fig. 1A; ctsk:nuGFP) and cytoplasmic mcherry (Fig. 1B; ctsk:mcherry). Due to inefficient targeting to the nucleus, GFP fluorescence in the ctsk:nuGFP line is visible in the cytoplasm (Fig. 1A; see also Knopf et al., 2011; Renn et al., 2013). Identical temporal and spatial expression of all three reporters was observed, with mEGFP expressed at lower levels at 1.5 months post-fertilization (mpf; Fig. 1C). Confocal analysis of osteoclasts along the neural arches confirmed cytoplasmic expression (Fig. 1D,E) and membrane-localized expression (Fig. 1F) of nuEGFP, mcherry and mEGFP, respectively. The membrane-localized expression of mEGFP appeared patchy with apparent local accumulation of the reporter at the cell periphery and sparse staining in the cytoplasm (Fig. 1F). All three transgenic lines showed identical reporter expression and overall normal morphology up to 1.5 mpf (Fig. 1A–C). At 3 months, however, a significant number of ctsk:mEGFP fish developed trunk deformations with curvature of the body axis and constricted blood vessels (Fig. 1I). We analyzed the F2 progeny of three individual male ctsk:mEGFP founder fish (f0 males m1 to m3). After outcross of transgenic F1 fish to wild-type females, all of the 25 analyzed F2 offspring of founder m1 (100%), 16 of 54 analyzed offspring of m2 (29.6%), and 16 of 50 analyzed offspring of m3 (32%) showed a vertebral phenotype at 1.5 months, 5 months and 4 months,
respectively. The same phenotype was also observed in heterozygous offspring at later generations, however with reduced incidence (data not shown). Importantly, transgenic control fish expressing the cytoplasmic forms of EGFP or mcherry under control of the same ctsk promoter showed no phenotype also beyond five months. We next tested whether osteoclast functionality was impaired in the mEGFP transgenic fish showing vertebral malformations. We observed TRAP enzyme activity at the base of neural arches in control vertebra indicating the presence of pre-osteoclasts and active osteoclasts (Fig. 1J). In contrast, TRAP activity was strongly reduced in fish with malformations (Fig. 1K). Together, this suggests that membrane-bound expression of mGFP leads to dysfunctional osteoclasts in transgenic medaka, which in turn triggers vertebral malformations.

3.2. Overossification in ctsk:mEGFP medaka results in constricted spinal and haemal canals

Based on our observations of dysfunctional osteoclasts in the ctsk:mEGFP transgenic medaka, we next tested whether mineralized bone mass was increased, suggestive for reduced bone resorption. Alizarin red staining of mineralized bone revealed a curvature of the vertebral column and severe overossification of the vertebral arches (Fig. 2A,B). The amount of bone at neural and haemal arches was substantially increased. In contrast, the diameter of the vertebral body centra appeared normal (Fig. 2C–F). Importantly, intervertebral spaces were not mineralized in ctsk:mEGFP fish (Fig. 2D). There was also no excessive mineral deposition around the notochordal sheath in early developmental stages, and there was normal establishment of vertebral body identity. Also the subsequent formation of intramembranous bone around the mineralized notochordal sheath appeared normal. Instead, over-mineralization and excess bone formation appeared to be restricted to the haemal and neural arches (Fig. 2D,F).

When vertebral bodies were analyzed in transverse views, wildtype vertebra samples showed widely open and regularly shaped spinal and haemal canals, located dorsal and ventral to the notochord, respectively (Fig. 2G). In contrast, in ctsk:mEGFP fish, the spinal and haemal canals were almost completely filled with bone (Fig. 2H). Histological analysis of sagittal sections through the vertebral column showed an obstructed spinal canal in control fish, with bone surrounding the intact and non-constricted spinal cord (Figs. 2I,K). In contrast, in ctsk:mEGFP transgenic fish, bone of the neural arches was found to protrude into the spinal cord (Figs. 2J,L). The overall histology of spinal cord tissue

Fig. 1. Transgenic expression of fluorescent proteins in osteoclasts of medaka. A. Transgenic medaka expressing nuGFP under control of the ctsk promoter in osteoclasts at 3 weeks post fertilization (wpf). B. Expression of cytoplasmic mcherry under control of ctsk at 1.5 months post fertilization (mpf). C. Expression of mEGFP at 1.5 mfp. Note that expression levels are lower than in B. D,E. Confocal images of single osteoclasts positioned in neural arch of ctsk:mEGFP/cts:k:mcherry double transgenic medaka at 3 wpf. Note cytoplasmic localization of nuEGFP (D) and mcherry (E). F. Confocal image of osteoclasts in neural arch of ctsk:mEGFP single transgenic medaka at 3 wpf. Note membrane localization of mEGFP. G. ctsk:nuEGFP transgenic medaka at 3 mfp in brightfield (G) and fluorescent images (G′,G″). H. ctsk:mcherry transgenic medaka. I. ctsk:mEGFP transgenic medaka. Note malformation of vertebral column. Scales have been removed from trunk region before imaging in G′−I″. J,K. Staining of TRAP activity in osteoclasts at base of neural arches in wildtype control fish (J) and deformed ctsk:mEGFP fish (K) at 1.5 mfp.

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was strongly affected with abundant occurrence of fibrous material (Fig. 2L, yellow arrowhead), suggesting ongoing neuro-degenerative processes. Growth of the spinal cord was apparently obstructed. Also the mineralized haemal arches extended all the way to the medial surface of the centra, thereby obstructing the expansion of the dorsal aorta and its associated vessels (Fig. 2J). The obstruction of the haemal canal apparently resulted in a ventral dislocation of the dorsal aorta, as a large blood vessel could be seen along the dorsal edge of the anal fin, as well as in the dorsal trunk (Fig. 2N). Taken together, the constriction of the spinal cord and the obstruction and dislocation of blood vessels were observed in osteopetrotic transgenic fish.

3.3. Excess ossification in osteoclast-deficient ctsk:mEGFP medaka does not alter centra size but affects pharyngeal jaw bones and fin endoskeletal bones

To quantify the extent of overossification in arches versus centra, we measured the centra diameter of vertebrae 12 to 18 (Fig. 3A–C), as well as the area of the neural canals of the same vertebrae (Fig. 3D,E) in wildtype control and ctsk:mEGFP transgenic fish of the same age. Interestingly, the diameter of the centra did not change significantly in ctsk:mEGFP transgenic fish (Fig. 3F). In stark contrast, the lumen of the spinal and the haemal canal was significantly reduced in all analyzed vertebrae of transgenic fish demonstrating the requirement of osteoclasts for modeling and allometric growth of haemal and neural arches.

Similar to the failure to widen the lumina of the spinal and haemal canal, constriction respectively closure of foramina was observed on several skeletal elements. Examples are the closure of foramina in the upper and lower pharyngeal jaws (Fig. 4B,D) and an almost closed foramen in the scapula of the shoulder girdle (Fig. 4F). In upper and lower pharyngeal jaws of wildtype fish, teeth are located on bony ridges, separated by foramina. In osteopetrotic fish, these foramina are not further extended during ontogeny and are eventually absent. Likely, inadequate resorption results in a failure to shed old teeth, which causes the presence of extra teeth, whose bony bases contribute to the closure of the

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foramina (Fig. 4B,D). We also observed ectopic intramembranous ossification located at the hypurals of the caudal fin (Fig. 4H) and the pterygophora of unpaired median fins (Fig. 4K).

4. Discussion

In this study, we report a severe osteopetrotic phenotype in transgenic medaka caused by membrane-localized mEGFP expression in osteoclasts under control of the ctsk promoter. Using TRAP staining, we show that the activity of osteoclasts is strongly reduced in ctsk:mEGFP medaka. These fish are characterised by excessive bone inside the neural and haemal arches. The excess bone in the lumina of the neural and haemal canals causes a displacement of blood vessels and severely constricts the spinal cord. The affected fish develop a curvature of the vertebral column over time, and show altered swimming behavior. It is presently unclear whether these motor defects are caused by the malformation of the vertebral column or by neurological deficiencies. Considerable deposition of fibrous tissue in the spinal cord and its severe constriction likely favors the latter hypothesis. Many of the malformed fish had a shortened life expectancy and died in the first five months showing internal bleeding in the areas of displaced blood vessels (data not shown).

Osteoclasts differentiate from hematopoietic stem cell progenitors (Udagawa et al., 1990). Cell–cell contact is important for osteoclast differentiation, activation and bone resorption. Osteoclast progenitors express the membrane receptors RANK and c-FMS (reviewed by Boyle et al., 2003; Charles and Aliprantis, 2014). These get activated by RANKL and macrophage colony-stimulating factor (M-CSF), respectively, which are produced by osteoblasts and stromal cells as well as bone marrow cells. In medaka, we earlier described that overexpression of Rankl results in excessive osteoclastogenesis in the vertebral column (To et al., 2012). Using live imaging, we were able to show that medaka osteoclasts are highly dynamic and establish tight contact with osteoblasts and mineralized matrix through active cytoplasmic extensions. Prior to resorbing bone matrix, osteoclasts change their shape in order...
to attach to and establish tight contact with bone surface. Actively resorbing osteoclasts show extensive exocytosis of proteases such as cathepsin K and TRAP that catalyze degradation of the collagen matrix. Notably, mouse models for osteopetrosis are characterized by impaired ruffled border formation (Rajapurohitam et al., 2001). Teleost osteoclasts, on the other hand, show a wide range of morphologies depending on the age of the fish (Witten, et al., 2001) and the species (Witten and Huysseune, 2009). Osteoclasts can be multinucleated and have a ruffled border (Huysseune and Sire, 1992; Witten and Hall, 2003) or can be mononucleated and resorb bone without a ruffled border (Witten, 1997). Mononucleated osteoclasts without ruffled border typically perform bone resorption in young teleost fish and in teleost fish with acellular bone, such as medaka (Witten and Huysseune, 2009, 2010). Accordingly, the presence of membrane bound H+–ATPase in TRAP positive active mononucleated osteoclasts without ruffled border was shown for the cichlid Oreochromis niloticus (Witten, et al., 1999).

Also in medaka, vacuoles containing internalized bone matrix in active osteoclasts without ruffled borders were observed (Chatai, et al., 2011). Therefore, while ruffled border formation might be dispensable in medaka, membrane integrity is nevertheless essential for bone resorption. Upon contact with the bone surface, the apical cell membrane of osteoclasts develops a peripheral sealing zone that conceals the subcellular space in mammals (Li et al., 2006; Parfitt, 1988) as well as in teleosts (Witten, et al., 2000; Domon, et al., 2006). The central part of the osteoclast cell membrane, enlarged or not enlarged by a ruffled border, is the area of active bone resorption. The sealed space under the cell can be viewed as a giant extracellular phagolysosome (Baron, et al., 1993). The extracellular space is acidified by the action of a membrane bound vacuolar proton pump (H+–ATPase) (Vaanannen, et al., 1990; Stenbeck, 2002), which promotes the dissolution of bone minerals and the function of lysosomal enzymes. Enzymes such as cathepsin K and TRAP that break down bone matrix components are released through the cell membrane and dissolved products are taken up through the cell membrane (Stenbeck, 2002; Helfrich, 2003). An intact cell membrane is therefore important for cell–cell as well as cell–bone contact to allow osteoclast maturation and activity.

4.1. Osteoclast deficiency in medaka leads to an osteopetrosis phenotype

In this study, we show that transgenic expression of high levels of a membrane-localized EGFP interferes with osteoclast function and we speculate that this is due to a disintegration of the membrane structure in osteoclasts. This is similar to what has been described in osteoelastic-rich osteopetrosis, where genes such as SNX10 regulating secretory lysosome trafficking are mutated (Aker et al., 2012). This leads to the absence of a ruffled border and disrupted osteoclast activity in patients with SNX10 mutations. In humans, osteopetrosis usually affects infants and young adults with a maximum life expectancy of around 20 years (Sobacchi et al., 2013). In our medaka model, we observed the first symptoms of excess bone at around 1.5 months, which is when medaka fish normally become sexually mature. This opens the possibility that medaka osteoclasts need to accumulate a minimum level of membrane localized EGFP before an interference with osteoclast function occurs. Consistent with this, newly formed ctsk:mEGFP positive osteoclasts induced by transgenic Rankl expression in medaka are highly resorptive, as we have shown earlier (To et al., 2012). The inhibitory effect on osteoclast activity was exclusively observed with membrane-localized EGFP, but not cytoplasmic EGFP or mCherry, expressed at comparable levels. The fact that an identical phenotype was observed in the offspring of three independent ctsk:mEGFP founder fish excluded the possibility that the vertebral deformations were caused by insertional mutagenesis of a critical locus. Interestingly, Chatai et al. (2011) generated a medaka line expressing mEGFP under control of the trap promoter but did not report any obvious deformities in the fish (Chatai et al., 2011). In their study, they analysed mEGFP expression at 3 wpf. This is the stage, when osteoclasts first appear at the vertebral column and bone remodeling starts in medaka. Thus, no osteopetrosis phenotype can be expected at 3 wpf or earlier embryonic stages. Future studies need to show, whether trap:mGFP medaka develop an osteopetrosic phenotype at later stages in order to see whether promoter activity is important for inducing osteopetrotic defects in mGFP transgenic fish.

In fish with an osteopetrotic phenotype, excessive bone mass was observed at sites where endogenous osteoclasts are observed in medaka (Chatai et al., 2011; To et al., 2012). These are the sides of the neural and haemal arches in the continuously growing teleost vertebral column, where osteoclasts have to resorb bone to make space for the expanding spinal cord and blood vessels (Witten and Villwock, 1997b; Witten, et al., 2001; Witten and Huysseune, 2009). At the same time, arches grow in size due to the presence of bone-forming osteoblasts at the outside of the arches. Importantly, we did not see a reduction of overall vertebra body size, suggesting that the absence of osteoclast activity had no negative effect on osteoblast function. This would indicate an uncoupling of processes that are otherwise thought to be strictly coupled. How the action of endosteal osteoclasts and periosteal osteoblasts can be coupled during development in teleost fish with acellular bone, that lack osteocytes to provide communication, has been discussed by (Witten and Huysseune, 2009). In addition, we also observed occurrence of ectopic intramembranous bone (Fig. 4 H,K). This could indicate that osteoclasts are not only required to facilitate correct allometric growth, but also control ectopic connective tissue mineralization.

An osteopetrotic-like phenotype similar to the one described here was reported in the zebrafish panther mutant carrying deficiencies in the macrophage receptor c–Fms (Chatai et al., 2011). As in our study, also this mutant showed excess bone in neural and haemal arches. Although our phenotype appeared significantly more severe, together these studies demonstrate the importance of sufficient numbers of functional osteoclasts for remodeling the growing vertebral arches.

4.2. Vertebral bodies and vertebral arches are independent developmental units

We observed excess bone in the arches of the osteoclast-deficient medaka, but interestingly the inner diameter of the centra was not affected. This validates previous ontogenetic studies that show that osteoclasts are not involved in the growth of the centra (Witten and Villwock, 1997a). The hourglass shaped biconid centra of teleost vertebrae grow by terminal and periosteal bone apposition. Modeling through bone resorption is not required for this type of growth (Witten and Villwock, 1997b). In contrast, the lumen of the neural and haemal canal as well as the diameter of bone foramina, must be widened as the animal grows. Once the skeleton is mineralized, resorption by osteoclasts is the only way to achieve this widening (Witten and Huysseune, 2009). Osteopetrosis that affects the vertebral arches but not the vertebral centra provides further support for the idea that in teleost fish, bone formation in arches and centra is regulated by different developmental mechanisms. In teleosts, vertebral body centra are established by mineralization of the notochord sheath while arches develop as a condensation or ossification in the myosepta (Huxley, 1859; Kölliker, 1859; Laerm, 1976). This raises the question if vertebral centra and arches are separate developmental modules that are uncoupled (Fleming et al., 2004; Witten et al., 2006; Hautier et al., 2010). Examples for uncoupling of centra and arches exist in teleosts. Through remodeling, vertebrae of farmed Arctic salmon can fuse into one functional vertebral body while the arches remain separated (Witten et al., 2006). A medaka mutant phenotype with fused centra and separate arches has been reported by (Takeuchi, 1966). The opposite type of centrum–arch uncoupling is observed in zebrafish fused somite (fss) mutants where vertebral bodies are normally segmented but the patterning of neural and haemal arches is disturbed (vanEeden et al., 1996). In conclusion, these studies suggest that centra and arches are independent developmental modules.
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