Putting crystals in place
the regulation of biomineralization in zebrafish

für Reinhard

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Kristallen op hun plek
de regulatie van biomineralisatie in zebravissen
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op maandag 17 november 2014 des ochtends te 10.30 uur

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Chapter 1

General introduction - skeletal development & the regulation of biomineralization

The function the skeleton and its relevance to the human clinic

The endoskeleton is the determining feature distinguishing vertebrates from non-vertebrates. In humans the skeleton has a number of crucial functions: It provides protection and mechanical support to the body, is an important metabolic organ with key roles particularly in calcium and phosphate homeostasis, and represents the place of adult hematopoiesis.

There are a number of human diseases related to the muscoskeletal system; prominent examples are osteoporosis, osteoarthritis and congenital vertebral malformations (CVM). Osteoporosis is characterized by a gradual reduction of bone mass due to an imbalance between bone formation and resorption, which leads to an increased risk of fracture. In osteoarthritis, loss of joint (articular) cartilage tissue with subsequent inflammation and formation of bone spurs leads to chronic pain and loss of joint flexibility. These diseases represent a major burden to the health systems of industrialized countries as they affect huge parts of the population above 55 years.

Another as aspect related to skeletal biology is the regulation of biomineralization. Ectopic mineralizations can occur in most soft tissues and are a burden for patients with systemic mineral imbalance such as in chronic kidney disease (metastatic calcification), but are also seen as a consequence of injury as well as aging (dystrophic calcification) (Giachelli, 1999). Tissues of the cardiovascular system such as vascular smooth muscle cells (VSMCs) are particularly inclined to ectopic mineralization. Such ectopic mineralizations in the cardiovasculature correlate with severe clinical symptoms such as myocardial infarction or ischemia, and the risk of artherosclerotic plaque rupture (Giachelli, 2008).

Development of the skeleton

The two major types of tissue constituting the skeleton are cartilage and bone. Chondroblasts are building up cartilage structures and during this process turn into terminally differentiated chondrocytes, which do not proliferate anymore but are maintaining the extracellular matrix surrounding them. Comparably osteoblasts are the cells building up bone, and later on differentiate into osteocytes, which are important for mediating the remodeling bone elements upon mechanical load. Furthermore, a specialized cell type, the osteoclast, is responsible for breaking down bone for example upon increased physiological demand of calcium or during remodeling and repair of a bone element.

Studies mostly done in mouse and chick showed that the skeleton arises from three different origins. The vertebrae and part of the ribs are derived from the sclerotomal portion of the somites, the limbs from the lateral plate mesoderm, and the craniofacial skeleton is built from neural crest cells. While the somites and lateral plate mesoderm are derived from the mesoderm, the neural crest is of ectodermal origin (Gilbert, 2006).

From those precursor tissues, skeletal elements can in turn arise via two different processes: intramembranous (dermal) and chondral ossification. The crucial difference between the two is the lack of a cartilage template in intramembranous ossification. This means that mesenchymal cells proliferate and form condensations. Then cells within these condensations differentiate directly into osteoblasts (Hall and Miyake, 2000) (Fig. 1 upper panel). These osteoblasts secrete a bone-specific extracellular matrix, so-called osteoid, which becomes mineralized. Flat bones of the human skull and parts of the clavicles are examples of bones formed via intramembranous ossification.

Other bones, most prominently the long bones of the limbs, are formed via chondral ossification (Fig. 1 lower panel). This process also starts with a mesenchymal condensation; however mesenchymal cells within these condensations differentiate to chondroblasts. Thereby a cartilage element is formed, that grows to a certain size before any osteoblasts or mineralizations are detectable. After a growth phase cells surrounding this cartilage element start to form the periosteum (Fig. 1 lower panel). This is a structure containing osteoblasts, which secrete bone matrix in a directed manner (perichondral ossification) so a mineralized shaft is build up around the center the cartilage element. It will later develop into the bone collar. In parallel chondroblasts in the center of the cartilage element stop to proliferate and differentiate into hypertrophic chondrocytes (Gilbert, 2006). Hypertrophic chondrocytes start to secrete alkaline phosphatase, a factor essential for mineralization (see below) as well as angiogenic factors, which lead to an invasion of blood vessels into the forming skeletal element (Fig. 1 lower panel) (Karsenty et al., 2009). Hypertrophic chondrocytes then undergo apoptosis and are replaced by osteoblasts. The appearance of osteoblasts in the center of the cartilage element is thought to be connected with the invasion of blood vessels. These osteoblasts form the primary ossification center which leads to the gradual replacement of mineralized cartilage by trabecular bone (endochondral osteogenesis). Chondroblasts continue to proliferate in a directed manner at the edges of the primary ossification center, thereby promoting longitudinal growth. Besides constituting the primary ossification center in the shaft of the bone element cartilage growth plates are also forming in the epiphysis (the distal part of the bone element) (Fig. 1 lower panel) to promote growth towards adulthood. Until adulthood continuous bone formation and breakdown (remodeling) of bone matrix within the skeletal element occurs in a way that ensures growth, while maintaining the functionality of the bone element.

Molecular markers and regulation of skeletogenesis

The human skeleton consists of more than 200 elements. To ensure bone development occurs at the right place and the right time, an intricate network of signaling pathways based on morphogenic factors orchestrates temporal and spatial cell differentiation. Studies, mostly on focused on limb-development in mouse and chick, have revealed that epithelial-mesenchymal inductions are central to the process of bone formation, which couples a mor-

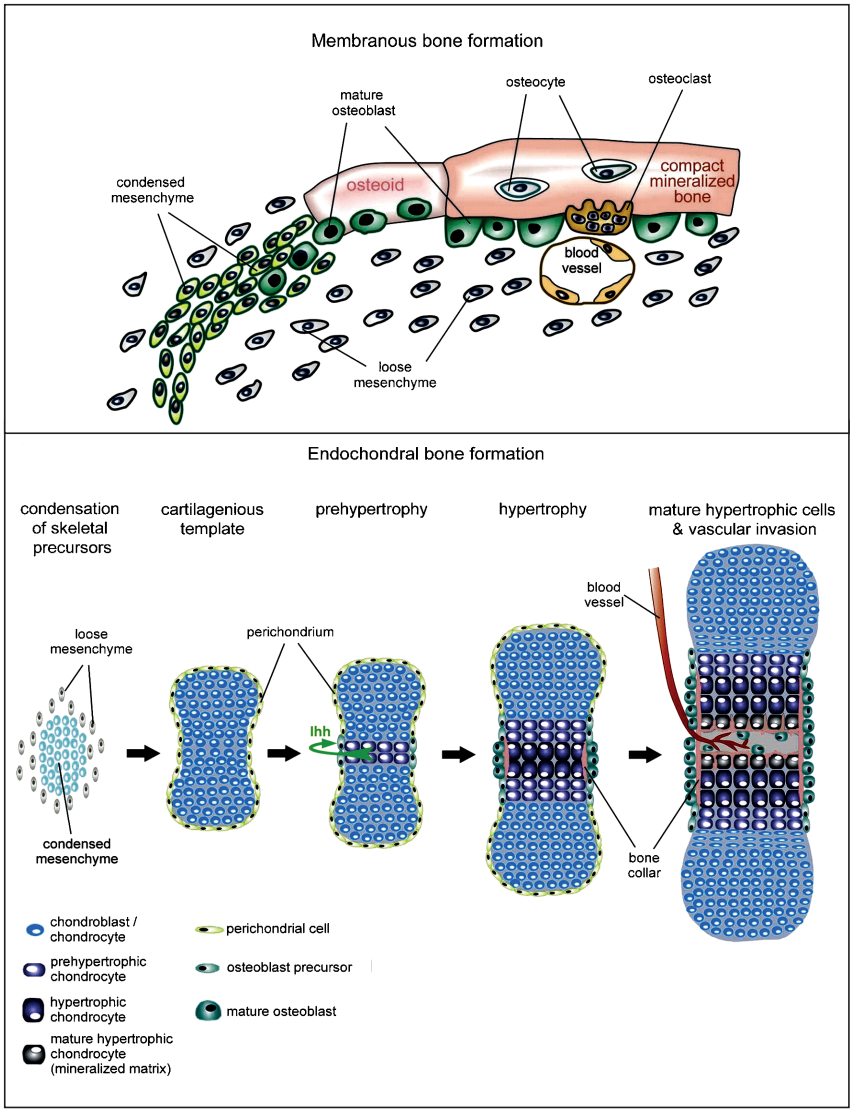

Figure 1: Scheme of intramembranous ossification (upper panel) and chrondral ossification (lower panel). In intramembranous ossification, osteoblasts differentiate directly from condensed mesenchyme. If they become enclosed within the bone matrix, they differentiate further into osteocytes. Multinucleated osteoclast are specialized in resorbing bone matrix. Chondral ossification is a multistep process. After the formation of a cartilage template osteoblasts are induced by IHH secreted from prehypertrophic chondrocytes. These osteoblasts form the bone collar around the shaft of the bone element. Centrally within the bone element, trabecular bone is replacing the cartilage after the invasion of blood vessels (primary ossification center). Modified from Hartmann 2006 (Hartmann, 2006).

phological program with the differentiation of cells in order to form a functional skeletal element. Because of the choice of the limb-model relatively more data is available regarding chondral bone formation than on the control of intramembranous bone formation.

As discussed above, the formation of a cartilage template is the first step in chondral bone formation. During this process cells undergo differentiation from mesenchymal condensations to chondroblasts, chondrocytes and hypertrophic chondrocytes. For each of these steps certain molecular markers have been identified: Cells of mesenchymal condensations typically express adhesion molecules such as N-cadherin and N-Cam (Oberlender and Tuan, 1994; Hall and Miyake, 1995). Proliferating chondroblasts express collagen type II and and aggrecan, whereas hypertrophic chondrocytes express type X collagen and alkaline phosphatase (Karsenty et al., 2009). Differentiated osteoblasts in turn express high levels of collagen type I, alkaline phosphatase and later during their commitment factors such as osteocalcin and *Spp1* (Zhang, 2010).

The spatial and temporal regulation of these differentiation processes is under control of typical developmental signaling pathways such as BMP (bone morphogenic protein), FGF (fibroblast growth factor), SHH (sonic hedgehog) and WNT (wingless type) and the expression of their respective receptors in the target cells (Karsenty et al., 2009). While *in vivo* and *in vitro* studies clearly indicate an important role for each of these pathways, the fact that they are acting often simultaneously on a cell population makes it difficult to obtain an integrated understanding, for example, where a new cartilage element will form start to form (Niswander, 2002).

In the current understanding BMPs are thought to be critical for induction of mesenchymal condensations (Hall and Miyake, 1995; Barna and Niswander, 2007). WNTs that are secreted from the ectoderm of the limb bud - and in this case are thought to signal mainly via the canonical, β-catenin dependent pathway - have been shown to have a repressive effect on early chondrocyte differentiation in the mesenchyme. In this way they can locally restrict the formation of a primary cartilage element. WNTs are also crucial for the differentiation of osteoblasts, which upon loss of β-catenin adapt a chondrocyte fate (Day et al., 2005; Hill et al., 2005; Hartmann, 2007). Signaling via FGFs, particularly FGF18, which is secreted by perichondral cells, is important for chondrocyte proliferation and differentiation (Naski et al., 1996; Liu et al., 2002). In this role, FGF signaling acts in concert with IHH that is secreted by pre-hypertrophic chondrocytes and early hypertrophic chondrocytes. IHH positively controls chondrocyte proliferation and induces PTHRP expression. PTHRP has a crucial role in inhibiting differentiation towards hypertrophic chondrocytes (Lanske et al., 1996; Vortkamp et al., 1996; St-Jacques et al., 1999). Together these factors control cartilage proliferation and differentiation in the growth plates. However, the function of these factors is not mutually exclusive for chondrocytes or osteoblasts: for example IHH is necessary for inducing osteoblast in the periosteum (St-Jacques et al., 1999) and FGF18 also plays a role in the terminal differentiation of osteoblasts (Liu et al., 2002). Besides

the factors discussed above, which are well established to play an important role, there is evidence for a number of other signals exhibiting an influence of skeletal formation and homeostasis, for example via Notch-Delta signaling, leptin or – presumably β-catenin independent – signaling via LRP5 (Karsenty et al., 2009).

The signals of different pathways are integrated within the skeletal progenitor cells and result in the expression and activity of tissue specific transcription factors for bone and chondrocyte differentiation. Members of the Sox (sex determining region Y-box9) family, particularly *Sox9* but also *Sox5* and *Sox6,* which are acting downstream of *Sox9*, are essential transcription factors within the chondrocyte lineage. In osteoblasts *Runx2* is an essential transcription factor for the early differentiation events (Komori et al., 1997). In addition to *Runx2,* osteoblasts need to express *Osterix* for becoming fully committed (Nakashima et al., 2002). Chondrocytes and osteoblasts are thought to have a common precursor, which is reflected in the fact that there is a population of skeletal precursor cells that expresses both *Sox9* and *Runx2* (Hartmann, 2009). In addition to the tissue specific transcription factors mentioned here, a number of more generally expressed transcriptional regulators are modulating gene expression in skeletal cells and are necessary for robust skeletogenesis (Hartmann, 2009).

In summary, the temporal and spatial signals mediated by different morphogens and their integration on the level of transcription factors, lead to the controlled cell differentiation and development of skeletal elements. Whereas this is relatively well understood for endochondral ossification, comparably little data is available for intramembranous ossification. A study by Abzahnov et al. 2007, showed that key factors known from endochondral ossification like *Runx2*, *Ihh* or *Spp1* are also involved in intramembranous bone formation. However, some markers that are exclusively expressed within chondral bone in either chondrocytes or osteoblasts, are co-expressed in osteoblasts of intramembranous bone. This led to the hypothesis that a chondrocyte-like osteoblast serves as a precursor for mature osteoblasts in intramembranous bone development (Abzhanov et al., 2007).

Regulation of biomineralization

Besides developmental regulation which ensures the formation of skeletal elements, also the process of biomineralization underlies rigid control. Osteoblasts are secreting a number of factors that ensure the formation of functional bone matrix. For some of them like BSP (bone sialoprotein) (Hunter and Goldberg, 1994) or DMP1 (Dentin matrix phosphoprotein 1) (Rowe, 2012) a role in modulating the mineralization process has been suggested. However, the central mechanism, that is necessary and sufficient for mineralization to occur, is removal of pyrophosphate and the presence of a fibrilar collagen matrix (Murshed et al., 2005). Physiologically these two prerequisites - coexpression of Collagen type I and the

pyrophosphatase alkaline phosphatase - exclusively coincide in mineralizing tissues like bone and teeth.

The underlying mechanisms for these requirements are defined by the biochemical properties of bone, which is a flexible yet stiff material. These attributes are achieved by generation of a composite material consisting of a flexible extracellular matrix (ECM), mostly consisting of collagen type I, onto which hydroxyapatite ($Ca_{10}[PO_4]_6$ $[OH]_2$) crystals can bind, contributing stiffness to the structure (Seeman, 2008).

The secretion of a fibrilar collagen matrix by osteoblasts is an active mechanism towards generating a mineralized matrix. In contrast the occurrence of calcium-phosphate crystals, like hydroxyapatite, in tissues of mammals is a frequent process that has to underlie constant repression to prevent certain pathologic conditions. This requirement is derived from the fact that the calcium and phosphate concentrations in body fluids and tissues are close to their precipitation threshold, ie in a metastable state (Giachelli, 1999; Giachelli, 2008; Kirsch, 2012). Due to these physiological conditions, osteoblasts do not need to "push" for mineralization to happen in their surroundings, but rather induce the nucleation of the crystal and then release the inhibition in a controlled way, that allows them to mineralize bone extracellular matrix but that does not affect neighboring soft tissues.

The main mechanism preventing mineralization is the presence of extracellular pyrophosphate (Fig. 2 A), a chemical mineralization inhibitor. Therefore to permit mineralization osteoblasts secrete enzymes that hydrolyze pyrophosphate, such as alkaline phosphatase (discussed below) (Terkeltaub, 2001). The two main sources of extracellular pyrophosphate that have so far been uncovered are the pyrophosphate channel ANK (Progressive ankylosis protein homolog) and ENPP1 (Ectonucleotide pyrophosphatase/phosphodiesterase family member 1) (Fig. 2 B). While ANK transports intracellular pyrophosphate into the extracellular environment, ENPP1 exists in a secreted form as well as a membrane bound extracellular enzyme that hydrolyses extracellular ATP to AMP, and in thereby sets free pyrophosphate (Terkeltaub, 2001; Kato et al., 2012) (Fig. 2 B). Lack of ANK and ENPP1, respectively, leads to soft tissue mineralization (Johnson et al., 2003). Of note, with ABCC6 (ATP-binding cassette sub-family C member 6) (Jansen et al., 2013) and NT5E (ecto-5'-nucleotidase) (St Hilaire et al., 2011) two genes associated with pathologic mineralizations in humans have recently been proposed to indirectly act on pyrophosphate levels, however in both cases the underlying mechanisms are not yet understood.

An important consequence follows from the fact that although ANK as well as ENPP1 are widely expressed across tissues, osteoblasts show the highest expression levels for both factors (Murshed et al., 2005). This seems somewhat counterintuitive since it implies the highest levels of a mineralization inhibitor are present in mineralizing tissues. It is likely that this represents a mechanism that ensures the protection of the surrounding soft tissues from mineralization by creating a gradient of pyrophosphate, while in their microenviro-

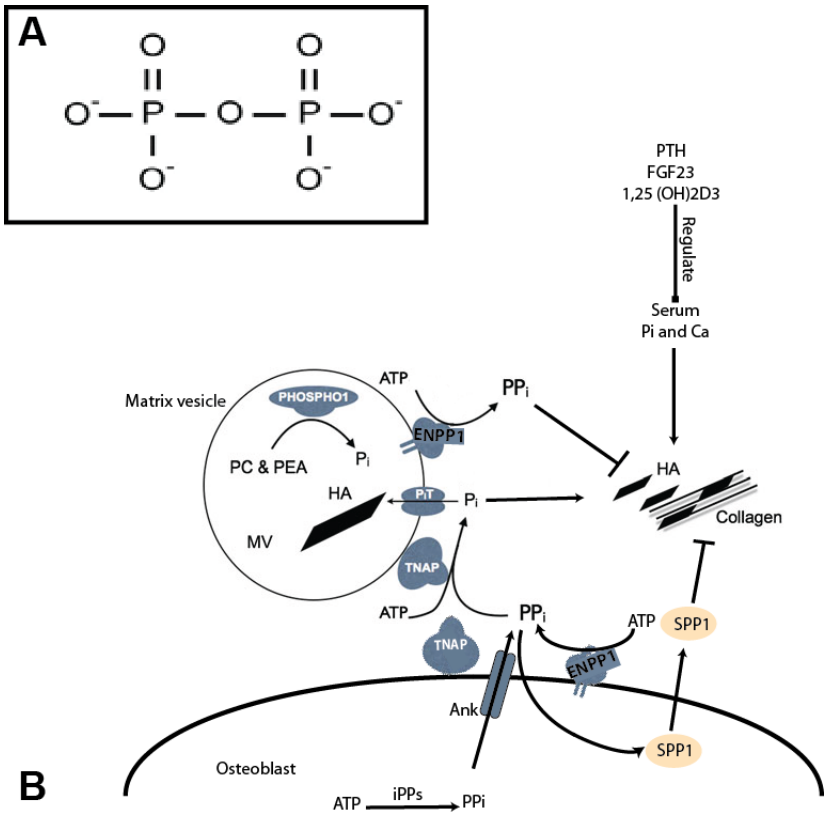

Figure 2: Regulators of biomineralization (A) Structure of the mineralization inhibitor pyrophosphate. (B) Main regulators of biomineralization. PiT is a phosphate transporter in matrix vesicles, where PHOSPHO1 mediates nucleation of hydroxyapatite (HA) crystals. Extracellularly HA crystals align with collagen fibers; crystal growth then is under control of pyrophosphate/phosphate homeostasis and SPP1. SPP1 levels are linked to extracellular pyrophosphate (Johnson et al., 2003) . iPPs – intracellular enzymes generating pyrophosphate (eg ENPP3). Modified from Millan 2012 (Millán, 2012).

ment osteoblasts degrade pyrophosphate and thereby permit mineralization (Millán, 2012) (Fig. 3). Importantly, hydrolyzing pyrophosphate increases the level of phosphate which enhances the formation of hydroxyapatite (Fig.. 3). In mammals the key enzyme identified for hydrolyzing pyrophosphate is the before mentioned alkaline phosphatase TNAP (alkaline phosphatase liver/bone/kidney (tissue-nonspecific)) (Fig. 2 B). In mice, defective bone-mineralization due to lack TNAP function can be restored by introducing a nonfunctional allele of ENPP1, thus lowering pyrophosphate levels (Hessle et al., 2002). This is of importance because TNAP can at least *in vitro*; act as a phosphatase for a wide array of substrates. Together with the fact that patients (Weiss et al., 1988) and mice (Narisawa et al., 1997) are hypophosphatemic, this has long led to the believe that the main function of TNAP is to supply phosphate rather than to break down pyrophosphate.

Systemic levels of calcium and phosphate do of course also play a role for the ability of the organism to properly mineralize bone, however humans can tolerate significant variations of calcium and phosphate levels (the normal range of the calcium x phosphate product is 1,8 – 3,8 $mmol^2/l^2$ (Jahnen-Dechent et al., 2008)). Systemic regulation of calcium and phosphate is dependent on secretion and retention via the kidney. This is a process regulated by circulating hormones such as Vitamin $1,25(OH)_2D_3$ (Calcitriol), PTH (parathyroid hormone) and FGF23. FGF23 is secreted by osteoblasts and osteocytes, thereby allowing the skeletal tissue to act on systemic calcium and phosphate levels (Sapir-Koren and Livshits, 2011).

Another important concept in bone mineralization is the compartmentation of the mineral nucleation inside matrix vesicles. It is thought that matrix vesicles which are secreted by osteoblasts provide an environment that favors crystal nucleation, thereby facilitating the initiation of mineralization in osteogenic tissue (Fig. 2 B) (Millán, 2012). Upon reaching a certain size within the vesicle the crystal is thought to penetrate the membrane and associates with the nearby collagen matrix, where further crystal growth is mediated by TNAP (Millán, 2012). PHOSPHO1 (Phosphatase, Orphan 1) has recently been identified as a crucial phosphatase for the crystal nucleation process. It is hypothesized that PHOSPHO1, by hydrolyzing its substrates phosphoethanolamine (PEA) and phosphocholine (PC), critically increases phosphate levels within the matrix vesicle to permit mineral nucleation (Yadav et al., 2011) (Fig. 2B).

Next to the regulation of phosphate and pyrophosphate levels a number of proteins are crucial for the prevention of pathologic mineralization in soft tissues. Important examples are: MGP (matrix gla protein), a calcium binding factor secreted by vascular smooth-muscle cells and chondrocytes preventing the ectopic mineralization of their respective extracellular matrix (Luo et al., 1997) as well as the blood-circulating protein FETUIN-A, which has the capacity of binding small clusters of calcium and phosphate and thereby is thought to act as a chaperon for neutralizing newly formed nanocrystals (Jahnen-Dechent et al., 2008). A particularly interesting protein involved in the regulation of biomineralization is SPP1 (secreted phospho protein 1, also known as osteopontin). SPP1 can bind strongly to hydroxyapatite and is thought to thereby inhibit crystal growth, similarly to pyrophosphate (Fig. 2B). It further serves as a ligand for osteoclasts and can thereby connect the inhibition of mineralization to its regression (Steitz et al., 2002). It has recently been discovered that SPP1 is a substrate of PHEX (Barros et al., 2013), a multifunctional, osteoblast specific protease, that also has an influence on phosphate levels via FGF23 (Barros et al., 2013).

The manifold of mechanisms involved in the regulation of biomineralization show that it is all but a trivial task to ensure controlled mineralization in bones while avoiding it in soft tissues.

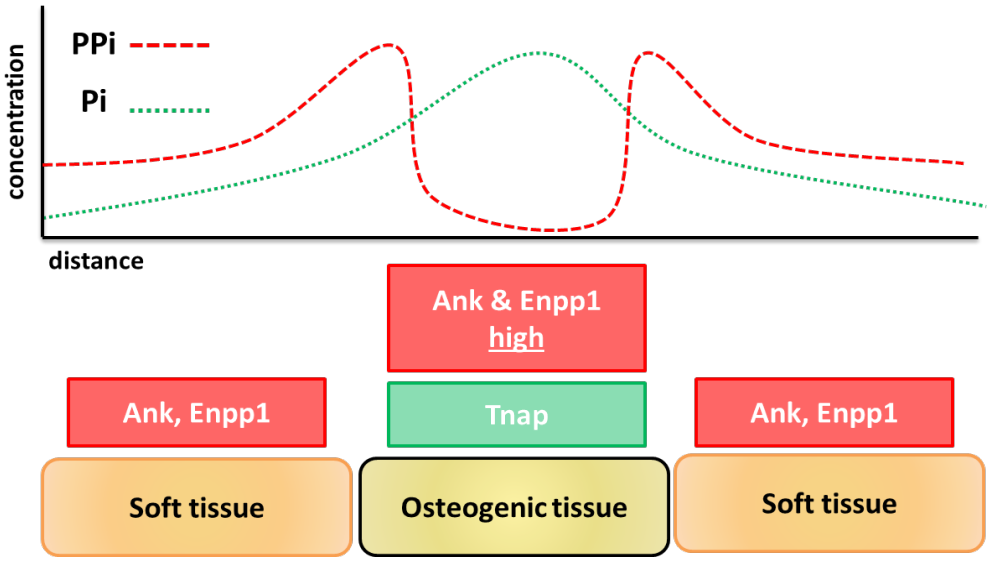

Figure 3: Phosphate and pyrophosphate gradients restricting mineralization to osteogenic tissues. The highest expression levels of Ank and Enpp1 can be found in osteogenic tisses, but both factors are also expressed in soft tissues (Murshed et al., 2005). This implies high local pyrophosphate generation, by osteoblasts, which in turn also express Tnap, locally breaking down pyrophosphate to phosphate, permitting mineralization to occur. Neighboring soft tissues are protected by the pyrophosphate gradient produced by osteoblasts in addition to their own basal pyrophosphate level.

Bone remodeling

While building up extracellular matrix and promoting mineralization, mammalian osteoblasts become entrapped within this matrix and many of them undergo apoptosis. A certain percentage however survives and differentiates into osteocytes. Osteocytes within the bone are connected via lacunae, fluid filled channels, and are thought to act as mechano-sensors influencing bone-remodeling upon mechanical stress (Seeman, 2008). As the term remodeling implies, bones are not static elements but rather are in a homeostatic equilibrium between bone generation and bone breakdown. Osteocytes are thought to be capable of breaking down bone via osteocytic osteolysis (Wysolmerski, 2012), however the main cell type responsible for the breakdown of mineralization and organic bone matrix are osteoclasts. These are polarized, often multinucleated, giant-cells, which have a sealing zone that forms a closed compartment towards the bone surface to be resorbed. Within this sealing zone the cell membrane of the osteoclast forms a ruffled border, which is thought to increase the surface for secretion and resorption. Protons and chloride (hydrochloric acid) as well as proteases are transported via the ruffled border into the sealed zone. While

the hydrochloric acid dissolves the bone mineral, proteases like cathepsinK, which is a typical osteoclast marker, break down the bone matrix (Väänänen et al., 2008). Another important marker for osteoclasts is tartrate resistant acid phosphatase (TRAP), an enzyme that has recently been postulated to regulate osteoclast attachment by dephosphorylation of the before mentioned SPP1 (Ek-Rylander and Andersson, 2010).

In contrast to chondrocytes and osteoblasts which are derived from the mesenchyme, osteoclasts derive from hematopoietic bone-marrow cells. They share a common promyeloid precursor with macrophages. A crucial factor for the differentiation of osteoclasts is the receptor activator of NF-kB ligand (RANKL) (Väänänen et al., 2008). Next to bone marrow stromal cells, osteocytes and osteoblasts are important sources of RANKL thereby creating a connection between bone generation and bone breakdown. This interaction is modulated by OPG (osteoprotegerin) a decoy receptor for RANKL (Väänänen et al., 2008). Imbalance between generation of bone matrix and breakdown results in disease. The most prominent example for this is osteoporosis which is caused by reduced bone density due to increased osteoclast activity.

Bone development in zebrafish

Zebrafish started being used as a model for skeletal development about 20 years ago. While a number of publications have investigated the mechanisms related to the pattering of craniofacial cartilages, only in recent years more work on mineralized tissues in zebrafish has been done (Javidan et al., 2009). This may in part be attributed to the fact that there are certain differences in the modes of bone formation and bone histology between tetrapods and teleosts, which are described in more detail in **chapter 2** of this thesis. Over the last years analysis of tissue specific gene expression showed that many of the key molecules identified in tetrapod skeletogenesis are also present in the zebrafish skeleton and are thus likely to have a conserved role (Li et al., 2009; DeLaurier et al., 2010; Hammond and Moro, 2012; Mitchell et al., 2013). So far, however, relatively few functional studies have been carried to confirm this assumption. The work in this thesis tries to fill this gap particularly in regard to the role of phosphate/pyryophosphate homeostasis in the regulation of biomineralization, but also describes a mutant for the zebrafish ortholog of *osterix*.

Thesis outline

Chapter 1 summarizes the current understanding of bone development and biomineralization from an anthropocentric viewpoint and should help to put the work done on zebrafish, which is described in the following chapters, into context.

Chapter 2 starts with a review of some examples where studies on skeletogenesis in zebrafish could complement knowledge derived from traditional model systems for bone development such as mouse and chick. Furthermore, chapter 2 gives an overview about evolutionary derived similarities and differences between teleost and tetrapod skeletal tissues with regard to structure and remodeling, as well as regarding the role of the notochord sheet during axial skeletogenesis, a peculiarity in teleosts.

Chapter 3 describes two novel zebrafish mutants with somewhat opposite phenotypes: *no bone* (*nobo)* does not develop mineralization it its skeletal elements, while *dragonfish* (*dgf*) develops ectopic mineralizations in multiple tissues. This chapter describes the identification of the causative mutations in *entpd5* and *enpp1*, respectively and comprises experiments that characterize *entpd5* as a novel phosphatase essential for skeletal mineralization in zebrafish.

Chapter 4 contains a detailed description of the *dgf* mutant phenotype which shows a number of parallels to human patients with mutations in *enpp1*. Further this chapter contains experiments that underline the suitability for studying pathologic mineralizations in zebrafish and provide novel insights about the cellular response to soft tissue calcifications.

Chapter 5 contains the description of a novel BAC-reporter line for a key transcription factor in osteoblasts – *osterix*. It further contains a phenotypic description of an *osterix* TILLING mutant allele. Together the data in this chapter indicate that while *osterix* is important for the development of the craniofacial skeleton in zebrafish, the first mineralized segments of axial skeleton seem to develop independently of *osterix*.

Chapter 6 contains a concluding discussion of the work presented in this thesis.

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Chapter 2

Not all bones are created equal - using zebrafish and other teleost species in osteogenesis research

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Summary

Developmental osteogenesis and pathologies of mineralized tissues are areas of intense investigations in the mammalian field, but different from other areas of organ formation and developmental biology, zebrafish have been somewhat slowin joining the area of bone research. In recent years, however, genetic screens have provided a number of exciting mutants, and transgenic lines have been developed that permit visualization of osteoblasts and osteoclasts in vivo. We here review some of the recent literature and provide examples where insights from studies in zebrafish have complemented the information available from mammalian models or clinical studies. Furthermore, we provide a comparative overview about different forms of bone within the teleost lineage, and between teleosts and mammals.

Introduction

The vertebrate skeleton serves numerous functions, most notably by providing a stable, but mobile framework against which muscles act. The endoskeleton also has protective functions for the brain and many internal organs, and serves as a store for minerals. The postcranial endoskeleton consists of the axial skeleton, supporting the main body axis, and the appendicular skeleton, supporting the extremities of the body. Bone and cartilage are the main components of the endoskeleton, being produced by osteoblasts and chondrocytes, respectively.

Given the vital roles of the skeleton it is not surprising that in the human clinic a number of diseases and pathologies affect the skeletal system. They include metabolic bone diseases such as osteoporosis and osteoarthritis as well as birth defects like cleft palate, congenital vertebral malformations or skeletal dysplasia (for reviews see McInnes and O'Dell, 2010; Ralston and Uitterlinden, 2010; Zelzer and Olsen, 2003). Particularly osteoporosis (an increased risk of bone fracture due to a decrease in bone density) and osteoarthritis (a degenerative disease affecting joints) are extremely common, and have an enormous bearing on health care costs in an ever-aging society.

In many areas of cell biology and organogenesis, zebrafish (*Danio rerio*) have provided a plethora of useful models that can be employed to study processes with relevance to human disease. In contrast to mouse and chicken, however, zebrafish has a short history as model for bone disease research, or for that matter, for studying bone formation from a developmental or cellular point of view. Part of this is certainly due to historic reasons. Initially zebrafish were mainly used to understand early developmental processes, and only gradually the use of the system has been expanded to areas of organogenesis and larval development. Another reason is that there is a diffuse notion about teleost bone being 'weird' and different from 'normal' (i.e. mammalian) bone. Within the vertebrate phylum teleost species are by far the most successful (at least in terms of species number), so one could actually argue what 'normal' is. However, these discussions are, to a degree, doomed to be pointless in an anthropocentric funding environment.

Despite a slow start, in recent years a steadily raising number of publications prove the zebrafish as a valuable complementation to the traditional model organisms. As so often before, the availability of mutants has been a driving force in the utilization of zebrafish, and forward genetic screens have already yielded hundred mutants (reviewed by Spoorendonk, 2010). Fortuitously, some zebrafish mutants survive far longer than knock outs in their murine ortologues (see below), allowing to obtain additional information even in cases where mouse mutants had been available for a number of years. An additional and exciting opportunity for studying skeletal formation and osteogenesis in fish is the ability to observe bone-forming cells (osteoblasts) *in vivo*, and transgenic lines for a number of informative markers that have been generated by now in zebrafish and medaka (*Oryzias latipes*) (Renn

and Winkler, 2009; Spoorendonk et al., 2008). This is a key advantage of using zebrafish and medaka as model organisms.

Among the bone and osteogenesis mutants that have been studied in zebrafish are some instructive examples, two of which are discussed in more detail below. Furthermore, with this review, we provide an overview about the different types of skeletal tissues that are present in zebrafish, medaka and other teleost species.

Case studies – using zebrafish for addressing biomedical questions

An attractive case for the zebrafish as a bone disease model was recently demonstrated by Clement and coworkers (Clement et al., 2008). The authors presented *in vivo* evidence in support of a loss of heterozygosity (LOH) model in osteochondroma formation, which is associated with hereditary, multiple osteochondromas syndrome (MO, also known as Hereditary Multiple Exotoses). Until recent it has been discussed in the field whether LOH or a gene dosage effect due to haploinsufficiency is responsible for osteochondroma formation as LOH could not be detected in all osteochondromas (Bovee et al., 2010).

Particularly, the majority of the patients suffering from MO carry mutations in either of the Exostosin genes EXT1 or EXT2 (Jennes et al., 2009), two genes involved in heparan sulfate (HS) biosynthesis. Clement et al. (2008) introduced the zebrafish mutant *dackel* (*dak*), harbouring a mutation in *Exostin2* as a model with an osteochondroma-like cartilage phenotype: in these mutants chondrocytes have a round shape and form clusters of cells instead of being flattened and aligned in columns.

Use of the mouse as a model for this disease has turned out to be non-trivial since both *Ext1*$^{-/-}$ and *Ext2*$^{-/-}$ mice die during early development (Lin et al., 2000; Stickens et al., 2005); and mice heterozygous for Ext1 (Hilton et al., 2005) or for *Ext2* (Stickens et al., 2005) do not form osteochondroma-like structures or, respectively, do not form those in long bones as in human patients.

Carrying out transplantation experiments of *dak*$^{-/-}$ cells into wildtype embryos Clement et al. (2008) were the first to show that in some cases *dak*$^{-/-}$ cells are not rescued by heparan sulfate secreted from the surrounding cells and can initiate outgrowths similar to human exostoses. This finding is in support of a LOH-model and has recently been independently confirmed by two new mouse models for osteochondromagenesis (Jones et al., 2010; Matsumoto et al., 2010) in which the authors by different strategies generated chondrocytes with a chimeric LOH genotype for *Ext1*.

Apparent advantages in this study were, (1) that zebrafish mutants often survive to stages that allow investigation of bone phenotypes, whereas corresponding murine models die during earlier stages of development, and (2) that transplantation experiments to create mosaic animals can be carried out with relative ease. In addition, by phenotypic comparison of *dackel* mutants with mutants isolated from a forward genetic screen the authors could introduce *papst1* (3'-phosphoadenosine 5'-phosphosulfate transporter) as a gene involved in HS synthesis. Thus *papst1* represents a potential candidate in cases of patients where no mutations in the Exostosin genes can be detected. One mechanism by which heparan sulfates are thought to act on chondrocytes is by restricting the diffusion of factors such as Hedgehog (Hh) ligands in the growth plate (Koziel et al., 2004). The importance of the hedgehog pathway is well established for skeletal development and Indian hedgehog (*Ihh*), a member of the hedgehog family of secreted ligands, is expressed in chondrocytes. There is evidence that *Ihh* controls chondrocyte proliferation and osteoblast differentiation (Lai and Mitchell, 2005; St-Jacques et al., 1999).

However, some details of the Hedgehog pathway and particularly its effect on bone mass after initial stages of development are still not well understood. Recently two studies analyzing bone homestasis in mice with reduced Hedgehog signaling resulted in contradicting findings. One group (Mak et al., 2008) found that conditional deletion of the Hedgehog receptor Patched1 (*Ptch1*) (deletion of which leads to increased Hh-signaling) in mature osteoblasts, using an *Osteocalcin*-Cre line, leads to an overall reduction in bone mass. The authors could relate this to increased osteoclastogenesis, induced via higher *PTHrP* and *RANKL* expression in osteoblasts. Another group (Ohba et al., 2008) investigated a mouse model haploinsufficient for *Ptch1*, which exhibited a high bone mass phenotype. The authors could also measure increased bone mass in patients with nevoid basal cell carcinoma (NBCCS or Gorlin Syndrome), a consequence of *Ptch1* haploinsuffiency in humans. These different findings can be attributed to the different experimental set up and to comparing a heterozygous situation to a promoter-induced knock out (Mundy and Yang, 2008).

A recent study in zebrafish (Hammond and Schulte-Merker, 2009) could contribute to a better understanding. The authors made use of a number of zebrafish mutants in the Hedgehog pathway (*ihha, ptc1, ptc2, dre (suppressor of fused)*), which in contrast to mice are viable to a stage that allows investigation of bone development. Furthermore drugs were employed acting on *smoothened*, a key mediator in the Hedgehog pathway, to either activate or suppress Hedgehog signaling. Using this experimental setup in combination with an *osterix*-reporter line the authors could show that there are two populations of osteoblasts in zebrafish. One of them, at the edge of the cartilage scaffold of forming bone elements requires Hedgehog signaling (*ihha*), but is not sensitive to high levels of Hedgehog signaling. A second population of osteoblasts arise within the cartilage template, where cells that have a chondroblast morphology and express *collagen2* start to express *osterix* and contribute to mineralization in situations, where Hedgehog signaling is increased. Importantly the latter population could be observed in mutants as well as upon titrating Smoothened activity with

small compounds. Furthermore the authors showed that increased Hh-signaling such as in *ptc2* mutants leads to an earlier onset of osteoclast activity in developing embryos whereas reduction of Hh-signaling (*ihha)* leads to a delay in comparison to wild-type embryos. This, however seemed to be independent of the number of surrounding osteoblasts or *rankl* expression.

In conclusion the study could independently and *in vivo* confirm the findings of both groups: increased Hh-signaling promotes differentiation of osteoblasts as well as osteoclasts. However, in concordance with Ohba et al. (2008) a net-increase in mineralization could be observed in zebrafish. Furthermore this study also nicely demonstrates the functional conservation of an important pathway in skeletal homeostasis and development between teleosts and mammals.

The evolution of skeletal tissues

The skeleton consists of two major subunits that evolved to a large degree independently: the dermal skeleton and the endoskeleton (Smith & Hall, 1990). The basic unit of the ancestral dermal skeleton is the odontode (Huysseune & Sire 1998, Reif 2006). Odontodes were already composed from bone, dentin and a hypermineralized layer. Their development requires epithelial-mesenchymal interaction. According to Huysseune et al. (2009, 2010), teeth are homologous to odontods and evolved when competent ectoderm migrated via the mouth and via the gill slits into the mouth cavity. Extant chondrichthyans (sharks and rays) retain odontode-like placoid scales in their dermal skeleton (Reif, 1982) and serve as examples for illustrating the homology between teeth and odontodes (Huysseune & Sire, 1998; Hall & Witten, 2007). Reviewing properties and modes of development of skeletal tissues in extant and extinct taxa, Hall & Witten (2007) conclude that skeletal tissues reflect the early evolution of highly plastic skeletogenic cells that can modulate their behavior in response to intrinsic and environmental signals.

The first jawless vertebrates had no vertebral bodies. Their skeleton was an odontode-based mineralized dermal skeleton; the vertebral column was only represented by the notochord (Donoghue 2006, Hall & Witten 2007). An endoskeleton made from 'true' *collagen type 2* based cartilage evolved only after the dermal skeleton (Cole & Hall, 2004; Hall & Witten, 2007). Compared to their common ancestors (basal osteichthyans), the postcranial dermal skeleton has been completely lost in mammals and has been largely reduced in teleost fish. Scales and scale-derived fin rays of teleost fish mainly represent a reduced dentin part of the ancestral odontodes (Sire & Akimenko 2004).

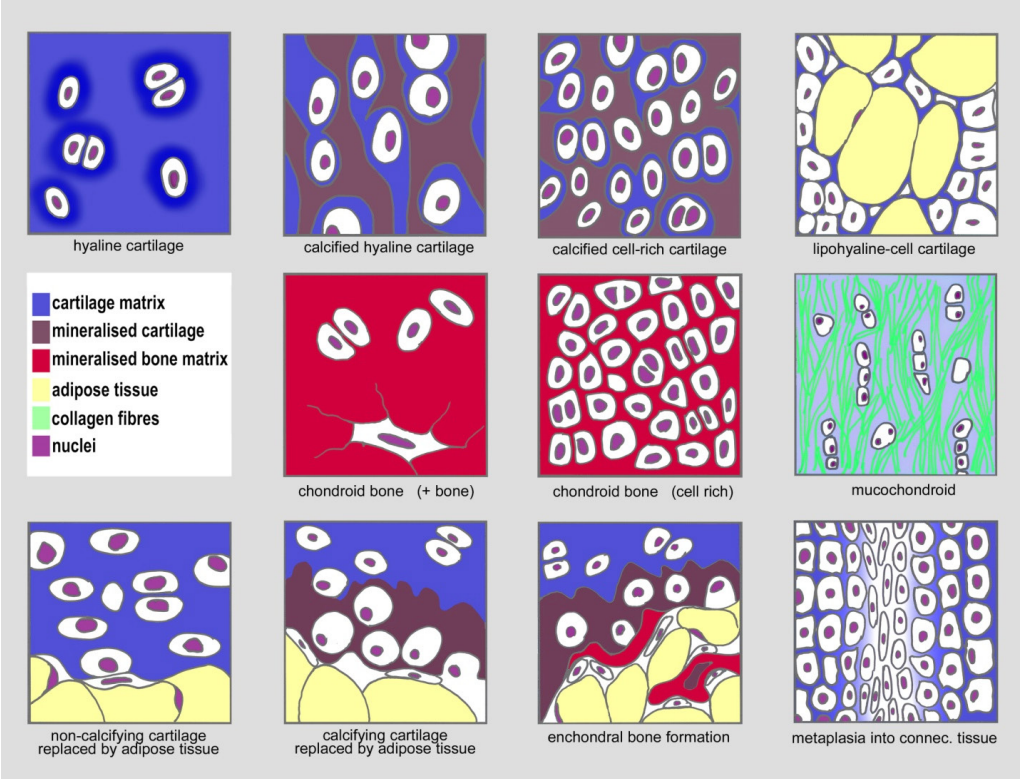

Figure 1: Hyaline cartilage and different types of tissues, that are intermediate between bone and cartilage and between cartilage and connective tissues, that typically occur in teleosts (row 1 and 2). Different modes of cartilage replacement that typically occur in teleosts (row 3). See Witten et al. (2010) for a complete list of cartilage tissues, see Hall and Witten (2007) for a more complete overview about intermediate skeletal tissues.

Given that all basic types of skeletal tissues where already present in early vertebrates (Hall & Witten 2007), the characters of skeletal tissues are conserved among vertebrates (Witten & Huysseune 2009). Consequently also transcription factors and signaling molecules that facilitate skeletal cell differentiation, and hormones that regulate the skeletal development, are conserved. Still, differences between the teleost and the mammalian skeleton exist, differences that are significant if teleosts like zebrafish or medaka are used as models in biomedical research. Teleosts evolved into the most successful group of all vertebrates with about 30.000 species. They dominate the aquatic habitats, by far the largest biosphere on the planet. In view of their enormous radiation, it would be false to assume that the mammalian skeleton is advanced and the teleost skeleton is primitive (Metscher & Ahlberg 1999, Witten & Huysseune 2009). In fact, many characters of the teleost skeleton are more advanced and/or elaborated compared to mammals. One example is the teleost skull that contains twice the number of skeletal elements compared to the mammalian skull

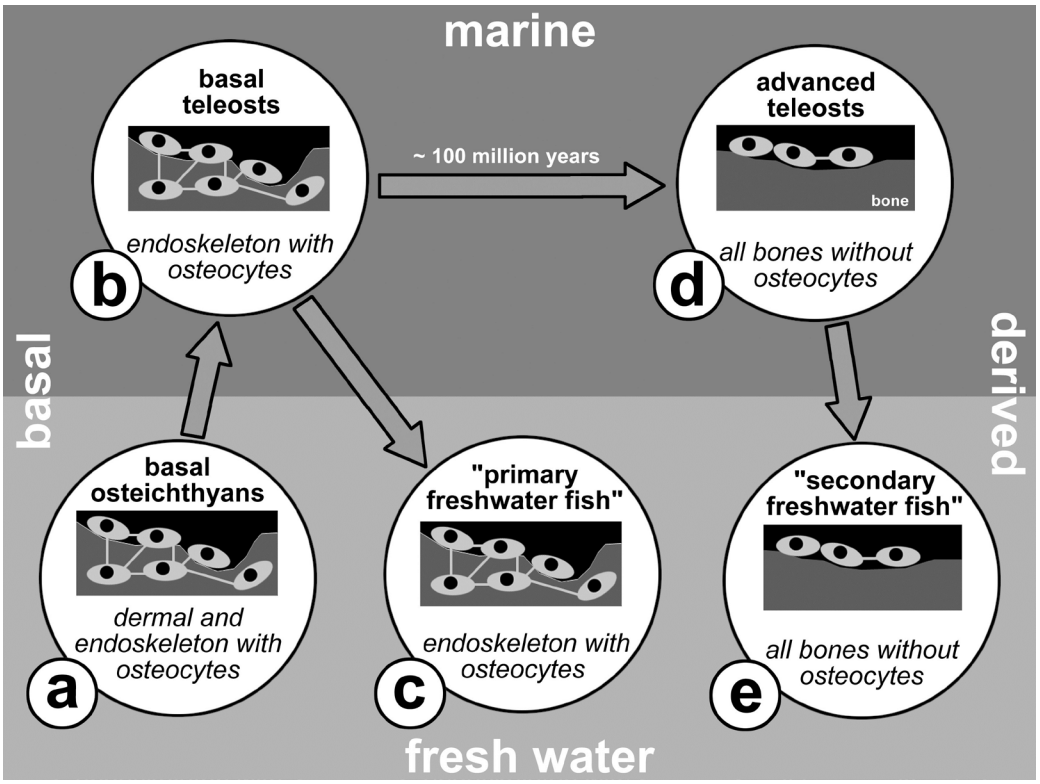

Figure 2: Relationships between phylogeny, environment, the presence of osteocytes and the type of osteoclasts in teleost fish such as zebrafish (c) and medaka (e). Basal osteichthyans, that also gave rise to tetrapods, and basal teleosts (a, b) have bone that contains osteocytes, similar to mammalian bone. These fish have mononucleated and many multinucleated osteoclasts. Osteocytes and multinucleated osteoclasts have been preserved during a 'first' wave of freshwater reinvasion by teleost fish (e; 'primary freshwater fish' such as zebrafish). During a long evolutionary period in the marine environment osteocytes disappeared (acellular bone) in almost all advanced teleosts (d). The typical osteoclast type of advanced teleosts is mononucleated. This character was maintained when advanced teleosts reinvaded the fresh water (e; 'secondary freshwater fish', e.g. medaka). Thus, teleosts that live in fresh water (or in the marine environment) can have different bone types. Modified after Witten and Huysseune (2009).

(Owen 1845). Differences between the teleost skeleton and the mammalian skeleton may also relate to adaptations to different habitats (aquatic and terrestrial), to the truncation of developmental process and to size differences (Witten & Huysseune 2009). The following section describes similarities and differences between the mammalian and the teleost skeleton.

Cartilage and bone in teleost fish

The major categories of skeletal tissues (cartilage, bone, dentine and enamel/enameloid) and the major categories of skeletal cells (chondroblasts, chondrocytes, osteoblasts, bone lining cells, osteocytes, osteoclasts, odontoblasts, ameloblasts) are present in both teleosts and in mammals (Huysseune, 2000, Witten & Huysseune 2009). Apart from dentine, enamel/enameloid and bone of attachment, that are restricted to the dermal skeleton, all skeletal tissues and respective cells occur in the teleost dermal and endoskeleton. The first step in the development of "regular" cartilage in teleosts is the condensation of mesenchymal cells (blastema stage) that develop into closely packed prechondroblasts (Huysseune & Sire, 1992a). These cells differentiate into chondroblasts and finally become separated through the secretion of extracellular cartilage matrix. Perichondral bone formation is the basic process of ossification of the cartilaginous preformed teleost endoskeleton. Different from mammals, it is often not linked to endochondral bone formation (Witten and Villwock 1997; Hall 1998; Huysseune, 2000) (Fig. 1). Perichondral bone is laid down at the surface of the cartilaginous template by cells that were formerly part of the perichondrium. The cells have now characteristics of osteoblasts and secrete bone matrix or a mixture of cartilage and bone matrix (Huysseune and Sire 1992a; Huysseune 2000; Verreijdt et al. 2002). Upon the beginning of perichondral bone formation, the former perichondrium has become a periosteum and further thickening of the bone is carried out through deposition of bone by the osteogenic cells. A typical element of the teleost endoskeleton consists of a persisting cartilage rod inside a bone tube with cartilage sticking out as a condyle. This applies especially to smaller teleost species, such as medaka and zebrafish where endochondral bone formation is uncommon (but does occur). Cartilage remains inside the bone shaft and if cartilage is removed it is replaced by adipose tissue (Huysseune, 2000; Witten et al., 2001, Witten et al. 2010a) (Fig. 1). Replacement of cartilage by spongiosa (endochondral bone formation) can more readily be observed in larger teleost species such as carp (*Cyprinus carpio*) and salmon (*Salmo salar*).

Bone formation in teleosts can be intramembranous, perichondral, or endochondral. In the endoskeleton membranous apolamellae can form from perichondral bone, a process that resembles intramembranous bone formation (Witten & Huysseune 2007). The cells involved in bone formation are osteoblasts. They derive from osteoprogenitor cells, the mesenchymal source of which has not been unequivocally identified in fish. Osteoblasts display various morphologies, depending on their secretory activity and on their position on the bone: they can be pear-shaped, spindle-shaped, or cuboidal with a pseudo-epithelial arrangement (Huysseune 2000, Witten & Hall 2002). Secretory osteoblasts have a polarized appearance, with a highly basophilic cytoplasm indicative of intensive protein production. Osteoblasts in acellular bone (see definition below) show a polarized secretion of bone matrix, continuously withdraw from the surface, and are thus never incorporated into the matrix (Weiss and Watabe 1979; Meunier 1983; Ekanayake and Hall 1987, 1988; Huysseune 2000). Structurally, bone tissue in teleost fish develops first as woven bone.

Subsequently, parallel-fibered and lamellar bone develops in more mature individuals. In larger individuals lamellar bone can also form osteons (Moss, 1961a; Smith-Vaniz et al. 1995; Witten & Hall, 2002, 2003; Meunier 2002). As fish have no haematopoietic tissue inside the bone marrow, bone marrow spaces are filled with fat tissue, besides nerves and blood vessels and some connective tissue cells (Huysseune 2000; Witten et al. 2001; Izquierdo & Witten 2010).

Intermediate skeletal tissues

Compared to mammals, additional skeletal tissue subtypes are recognized in teleost fish as part of the regular (non-pathological, non-regenerating) skeleton (Benjamin, 1988, 1990; Benjamin, et al. 1992; Meunier & Huysseune, 1992; Huysseune, 2000; Witten & Huysseune, 2007; Hall & Witten, 2007) (Fig 1). Benjamin (1990) describes seven categories of cartilage: (a) hyaline cell cartilage, (b) zellknorpel, (c) fibro/cell-rich cartilage, (d) elastic/cell-rich cartilage, (e) cell-rich hyaline cartilage, (f) matrix-rich hyaline cartilage, and (g) scleral cartilage. Secondary cartilage and chondroid cartilaginous tissues also develop on cranial dermal bones (Beresford, 1993; Benjamin, 1989; Huysseune, 2000; Witten & Hall, 2002; Gillis et al. 2006). The best studied teleost ‘’intermediate skeletal’’ tissue is chondroid bone (Fig. 1). Chondroid bone exhibits characteristics of bone and of cartilage and develops from osteogenic precursors (Huysseune, 1986; Huysseune & Verraes, 1986). This tissue contains chondrocyte-like cells (devoid of cell processes) surrounded by a bone-like matrix (Beresford, 1981; Huysseune & Verraes, 1990; Huysseune & Sire, 1990; Witten & Hall, 2002). Chondroid bone occurs in basal teleosts with osteocyte-containing bone and in advanced teleosts with acellular bone, and must not be confused with cellular bone (Beresford, 1981, 1993; Huysseune & Verraes, 1990; Huysseune & Sire, 1990; Meunier & Huysseune, 1992; Witten & Hall, 2002; Gillis et al., 2006). Chondroid bone can also be remodelled. into lamellar bone (Witten & Hall, 2002, 2003; Gillis et al., 2006).

Osteocyte-containing bone and acellular bone

Like mammalian bone, the bone of more basal teleosts such as zebrafish contains osteocytes (Fig. 2). The density of osteocytes in teleost bone can vary considerably, from one species to another and within the skeleton of one species (Moss 1961b). No data about the number of osteocytes in teleost bone are available, but in mammals, osteocytes represent 95% of all bone cells and cover 96% of all bone surfaces (Franz-Odendaal et al. 2006, Witten & Huysseune 2010). In contrast, advanced teleosts such as medaka possess acellular or anosteocytic bone, i.e. bone that has no enclosed osteocytes (Kölliker, 1859; Moss, 1961b; Ekanayake & Hall, 1987; Meunier & Huysseune, 1992; Witten et al. 2004) (Fig. 2). With few exceptions, the presence or absence of osteocytes is uniform in all elements of the teleost skeleton (for exceptions see Moss 1961a, Meunier 1989). More basal teleosts such

as salmonids and cyprinids (the group to which zebrafish belong), with cellular bone in the endoskeleton, have scales and fin rays that are acellular (Meunier 1989). Different from dentine no cell processes penetrate acellular bone. This bone bears however resemblance to atubular dentine that typifies the first-generation teeth of teleosts and to acellular mammalian cementum, both of which are also acellular and are not penetrated by cell processes (Weiss & Watabe, 1979; Ekanayake & Hall, 1987, 1988; Meunier, 1989; Sire et al., 2002; Franz-Odendaal et al. 2006). The formation of acellular bone resembles dentine formation. A polarised secretion of bone matrix ensures that cells never become entrapped in the bone matrix (Huysseune, 2000).

Development of teleost vertebral bodies, a derived process

An acellular mineralized tissue particular to all teleosts is the mineralized notochord sheath (Arratia et al. 2001; Grotmol et al., 2003; Nordvik et al., 2005; Inohaya et al. 2007, Bensimon-Brito et al. 2010). Unlike other vertebrates, formation of teleost vertebral bodies does not start with a cartilaginous anlage and also early bone is lacking. In teleosts vertebral body development starts with the mineralization of the notochord sheath. The notochord sheath consists of a cartilage-like matrix, rich in proteoglycans and collagen type II, covered by a thin layer of elastin. There is an ongoing debate weather sklerotomal derived cells from outside, or notochord cells from inside facilitate notochord sheath mineralization. Increasing evidence suggests that notochord cells facilitate mineralization of the vertebral body anlagen (Nordvik et al. 2005, Bensimion-Brito et al. 2010). Only the second phase of vertebral body development involves the apposition of bone onto the mineralized notochord sheath, similar to the intramembranous bone formation (Ekanayake & Hall, 1988, 1991; Grotmol et al., 2003; Nordvik et al., 2005). Although no cartilage contributes to the initial formation of teleost vertebral bodies (Witten & Villwock, 1997; Nordvik et al., 2005) in larger individuals cartilage at the base of the arches undergoes endochondral ossification and bone that derives from this process becomes part of the vertebral body (Zylberberg & Meunier 2008). Mineralization of the notochord sheath and the lack of cartilaginous anlagen are derived characters since basal bony fish have, similar to tetrapods (including mammals), cartilaginous vertebral body precursors (Arratia 1983).

Remodelling of the teleost skeleton

The absence of osteocytes in advanced teleosts raises questions about the regulation of bone remodelling, since cell processes from osteocytes and odontoblasts function as stress sensors in other systems. Osteocytes are believed to govern bone remodelling in response to mechanical load (Burger et al. 1995; Burger, et al. 2003; Bonewald 2004; Knothe Tate et al. 2004, Bonucci 2009). Estimates about the percentage of bone that is resorbed and replaced by new bone in humans range between four and ten per cent per year (Delling &

Vogel, 1992; Manolagas, 2000). Such estimates are lacking for teleost fish but a regular resorption and rebuilding of scales and bony skeletal elements is well documented for Atlantic salmon (Kacem et al. 1998; Persson et al. 2000; Witten & Hall, 2002, 2003). While we can expect that the cellular composition of the teleost skeleton affects if and how bone is remodelled, a number of additional factors affect the process. The main characteristics that distinguish teleosts from mammals with respect to skeletal remodelling are as follows (reviewed by Witten & Huysseune, 2009):

1. In mammals, bone resorbing cells (osteoclasts) originate from haematopoietic tissue located in the bone marrow. The haematopoietic tissue also releases factors that regulate the respective activities of osteoclasts and osteoblasts. Such an intimate spatial relationship between bone resorbing cells and haematopoietic cells does not exist in teleosts as most bone marrow spaces are filled with adipose tissue and haematopoiesis takes place in the head kidney (Field 1995, Witten et al. 2001, Song et al. 2004, Izquierdo & Witten 2010).

2. The lack of osteocytes in advanced teleosts coincides with an altered morphology of bone resorbing cells and an alternative mode of bone resorption. Osteoclasts of advanced teleosts are predominately small, mononucleated cells that can perform resorption without generating typical resorption lacunae (Witten 1997; Weiss & Watabe 1979; Kemp 2003, Witten & Huysseune 2010).

3. In mammals, endochondral bone formation is a prime cause of skeletal resorption and remodelling. Typical endochondral ossification is, however, often lacking in teleosts, especially in species with small individuals such as medaka and zebrafish. In addition, in all teleosts, vertebral bodies develop (ossify) without cartilaginous precursors and thus initially without remodelling (Arratia et al. 2001; Grotmol et al., 2003; Nordvik et al., 2005; Inohaya et al. 2007, Bensimon-Brito et al. 2010).

4. Regulation of plasma calcium content is crucial for all terrestrial vertebrates and the skeleton is tightly integrated into the animals' calcium homeostasis. Teleosts have a different approach. They obtain and release calcium from and into the water via their gills and the skeleton has not be used as a source or deposit of calcium (Guerreiro et al., 2002; Perry et al., 2003, Lall & Lewis-McCrea, 2010). In particular resorption of the endoskeleton may be used only as a last mineral resort under extreme conditions (Moss, 1962; Takagi & Yamada, 1991, 1992). When there is a need for skeletal resorption, minerals are first mobilized from the postcranial dermal skeleton (scales) and therefore minerals must not be released from the endoskeleton (Persson et al., 1998, 1999, 2000, Skonberg et al. 1997; Witten & Hall 2003; Lall & Lewis-McCrea, 2007).

5. The acellular teleost skeleton responds and adapts to mechanical load (Meyer, 1987, Huysseune et al. 1994, Kranenbarg et al., 2005; Witten et al., 2005a) but the lack of osteocytes in advanced teleosts implies that bone remodelling in response to mechanical load must be triggered by other cell types (Witten & Huysseune 2009).

6. Different from sharks, teleost are capable of repairing their endoskeleton (Moss 1962, Moss 1977, Clement et al. 1992, Ashhurst 2004) but the regenerative capacity of elements of the teleost dermal skeleton (fin rays and scales) largely exceeds the regenerative capacity of the endoskeleton (Akimenko & Smith 2007, Huysseune et al. 2009).

7. Teleosts replace their teeth throughout life, a process that requires lifelong resorption of teeth, tooth attachment bone, and dentigerous bone (Peyer, 1968; Huysseune, 1983; Huysseune & Sire, 1992b; Witten et al., 2005b; Huysseune 2000, Huysseune & Witten 2008, 2010).

8. Teleosts never stop growing, and certain skeletal elements develop rather late. Thus, growth-related skeletal modelling continues throughout life and should not be mistaken for metabolism-related skeletal remodelling (Smith-Vaniz et al., 1995; Persson et al., 1998, 1999; Kacem et al., 1998; Meunier, 2002; Reznick et al. 2002; Witten & Hall, 2002, 2003).

Conclusions

The aim of this review is to highlight similarities and differences of skeleton formation among teleosts, and between teleosts and mammals. Through the course of evolution different forms of bone have evolved, but all the basic types of skeletal tissues have already been present in ancestral jawless vertebrates. What might appear as significant differences today (e.g. osteocyte-containing bone versus acellular bone and mono- versus multi-nucleated osteoclasts) are a variation of a common scheme. Not surprisingly, there is mounting evidence that the genetic control of osteogenesis is conserved and shared among all vertebrates. In other areas, most notable vascular and endothelial cell biology, the close interaction of researchers using the full array of methodologies (from *in vitro* cell culturing systems, morpholino knock down studies, *in vivo* imaging of cell behavior in zebrafish embryos, inducible knock-outs in mice, antibody staining on histological human material,) have helped significantly to advance the field as a whole. Comparable progress can be made in the area of osteogenesis if resources are used in a complementing manner.

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Chapter 3

Entpd5 is essential for skeletal mineralization and regulates phosphate homeostasis in zebrafish

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Summary

Bone mineralization is an essential step during the embryonic development of vertebrates, and bone serves vital functions in human physiology. To systematically identify novel gene functions essential for osteogenesis, we performed a forward genetic screen in zebrafish and isolated a mutant, *no bone* (*nob*), that does not form any mineralized bone. Positional cloning of *nob* identified the causative gene to encode *ectonucleoside triphosphate/diphosphohydrolase 5* (*entpd5*), which has not been related to skeletal mineralization before. Analysis of its expression pattern demonstrates that *entpd5* is specifically expressed in osteoblasts. An additional mutant, *dragonfish* (*dgf*), exhibits ectopic mineralization in the craniofacial and axial skeleton and encodes a loss-of-function-allele of *ectonucleotides pyrophosphatase phosphodietserase-1* (*enpp1*). Intriguingly, generation of double mutant *nob/dgf* embryos restored skeletal mineralization in *nob* mutants, indicating that mechanistically, Entpd5 and Enpp-1 act as reciprocal regulators of phosphate/pyrophosphate homeostasis *in vivo.* Consistent with this, *entpd5* mutant embryos can be rescued by high levels of inorganic phosphate, and phosphate-regulating factors, such as *fgf23* and *npt2a* are significantly affected in *entpd5* mutant embryos. Our study demonstrates that Entpd5 represents a previously unappreciated essential player in phosphate homeostasis and skeletal mineralization.

Introduction

The vertebrate skeleton is composed of bone and cartilage. Bone-forming cells, osteoblasts, secrete a collagen-rich matrix which is subsequently mineralized, while bone-resorbing cells, osteoclasts, remove bone tissue and remodel it. Osteoblasts are of mesenchymal origin and Runx2 and Osterix have been identified as the major transcription factors controlling osteoblasts commitment and differentiation (Karsenty, 2008; Hartmann, 2009). Osteoclasts, on the other hand, are of hematopoietic origin and derive from the monocyte lineage (Teitelbaum, 2000). In humans the generation and remodeling of bone is a dynamic process that occurs throughout life and is dependent on age and gender. A number of human osteopathies are common, often caused by misregulation of skeletal mineral homeostasis (mainly calcium and phosphate).

Crucial in regulating biomineralization is the balance between promoters and inhibitors of biomineralization, both on an autocrine/paracrine level, as well as on a systemic level. The ratio between phosphate and pyrophosphate is central to this process. Locally, in the osteoblast and its microenviroment, phosphatases like PHOSPHO1 or alkaline phosphatase are thought to be key factors in the initiation of mineralization (Yadav et al., 2011). PHOSPHO1 is responsible for providing the phosphate necessary for nucleation of crystal growth within matrix vesicles (Stewart et al., 2006), while alkaline phosphatase can dephosphorylate various substrates, but most importantly breaks down pyrophosphate in the microenviroment of osteoblasts (Murshed et al., 2005). Pyrophosphate is a strong chemical inhibitor of bone mineral (hydroxyapatite) formation and is locally provided by the pyrophosphate channel ANK and the ectonucleotide pyrophosphatase phosphodiesterase1 (ENPP1) (Terkeltaub, 2006). On a whole-organism level, phosphate levels are regulated by controlling retention/secretion in the kidney via a hormonal network involving PTH, FGF23 and $1,25(OH)_2D_3$ (Razzaque, 2011).

Under normal conditions, calcium and phosphate concentrations of the extracellular fluid are below the level of saturation needed for spontaneous precipitation in soft tissues, but above the level sufficient to support crystal growth in skeletal tissue (Huitema and Vaandrager, 2007). For example, deficiency in the ENPP1 gene can result in pathological soft tissue mineralization, particularly in arteries (Okawa et al., 1998; Rutsch et al., 2003). On the other hand, hypophosphatemia leads to decreased mineralization of skeletal tissues, as evidenced by genetic studies in which, PHEX function is diminished (Tenenhouse, 1999).

We have taken a forward genetic approach to identify novel regulators of osteogenesis and bone mineralization, and here report the isolation and characterization of two zebrafish mutants: *no bone* (*nob*) mutants fail to form any mineralized skeleton, while *dragonfish* (*dgf*) mutants show ectopic mineralization in the craniofacial and axial skeleton. We demonstrate the causative genes to encode Entpd5 and Enpp-1, respectively, and provide evidence that

the combined activity of both factors maintains normal physiological levels of phosphate and pyrophosphate in the embryo.

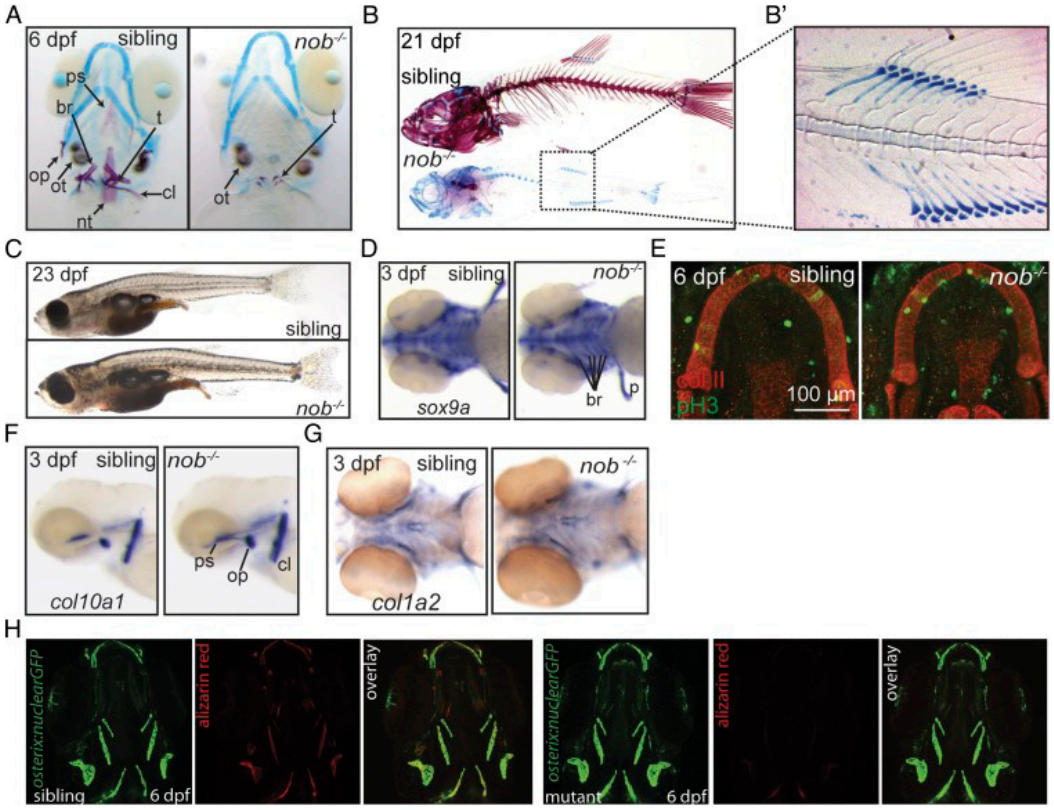

Figure 1: *Nob* mutants lack a mineralized skeleton. (A) Alizarin red/Alcian blue staining of sibling and mutant *nob* embryos. Cartilage elements appear normal in mutants. All bone is absent, but teeth and otoliths are present. (B) Skeletal staining of 21 dpf sibling and *nob* mutant individuals. (B') Enhanced contrast image highlighting the correctly patterned but unmineralized vertebra anlagen. (C) Images of sibling and mutant *nob* fish at 23 dpf, demonstrating that *nob* mutants are indistinguishable from siblings at gross morphological level. (D) Whole mount *in-situ* hybridization detecting the chondrogenic marker *sox9a* (E) Confocal projection of Meckel's cartilage of a sibling versus mutant embryo showing no difference for anti-type II collagen (red) or the proliferation marker anti-phospho-Histone H3 (green). (F, G) Whole mount *in-situ* hybridization detecting the osteoblast markers *col10a1* (F) and *col1a2* (G) in 3 dpf *nob* mutant and sibling embryos. (H) *Osterix*:GFP expression, marking early osteoblasts, in 6 dpf *nob* mutant and siblings embryos. br = 5th branchial arch, cl = cleithrum, nt = notochord tip, op = operculum, ot = otolith, ps = parasphenoid , t = teeth.

Results

Nob mutants lack a mineralized skeleton

In a forward genetic screen in zebrafish (Spoorendonk, 2010) we uncovered 14 mutant lines out of 429 families screened. One mutant, *no bone* (*nob*^{hu3718}) completely lacked a mineralized skeleton (Fig. 1A and B). Skeletal staining of mutant and sibling embryos showed that the mutant phenotype is apparent at 6 days post fertilization (dpf) (Fig. 1A). *Nob* mutant embryos maintained the ability to form mineralized teeth and otoliths (Fig. 1A), two calcified structures with a different mineral composition than bone (Kawasaki et al., 2005; Wu et al., 2011). Mutant embryos were viable when separated at 6 dpf from their siblings via alizarin-red-based *in vivo* skeletal staining (Spoorendonk et al., 2008). Except for the absence of a mineralized skeleton (Fig. 1B), we could not phenotypically distinguish mutants from siblings until 21 dpf (Fig. 1C). After about 21 dpf *nob* mutants showed slower growth and died around 35 dpf.

Dermal bone formation (which does not occur via a cartilaginous intermediate) is equally affected in *nob* mutants, indicating that the phenotype is not caused by chondrogenesis defects. Nevertheless, we asked if cartilage tissue develops normally in *nob* mutants. Alcian blue staining, labelling mucopolysacharides and glycosaminoglycans in cartilage, appeared identical in mutant versus sibling embryos (Fig. 1A and Suppl. Fig. 1A). We visualized the expression of *sox9a* and type II collagen, but could not find qualitative difference in the expression of these chondrogenic markers (Fig. 1D, E). We also analyzed the proliferation marker phospho-Histone H3, in 6 dpf *nob* mutants (n=4; average of 9.00 pH3-positive chondrocytes) versus siblings (n=4; average of 12.75 chondrocytes). Again, this did not constitute a significant difference in proliferating cells in the craniofacial elements (Fig. 1E). Together, these data suggest that chondrogenesis is unaltered in *nob* mutants.

Next, we asked whether an absence of osteoblasts might be causative for the *nob* mutant phenotype, and addressed this question using an *osterix*:GFP transgenic line (Spoorendonk et al., 2008; Hammond and Schulte-Merker, 2009) as well as other osteoblast markers. As shown in Fig. 1 F, G, H, no difference was observed in the number of *osterix* (Fig. 1H) or *type I collagen* (*col1a2*) (Fig. 1G) expressing osteoblasts between sibling and *nob* mutant embryos. In addition to this, we observed no difference in the expression of *col10a1,* which marks osteoblasts in teleosts (Avaron et al., 2006) (Fig. 1F). Together, these data demonstrate that it is not the absence of osteoblasts which is causative for the *nob* mutant phenotype.

Nob mutants encode alleles of entpd5

To identify the molecular lesion responsible for the *nob*^{hu3718} mutant phenotype, we used simple sequence length polymorphism (SSLP) and single nucleotide polymorphism (SNP) mapping. Single-embryo mapping positioned the mutation between flanking markers SNP-Z8 and CA39 (Fig. 2A) on chromosome 17. Sequencing of the zebrafish *entpd5* gene (ectonucleoside triphosphate diphosphohydrolase 5 gene, in mammals also referred to as CD39L4 or PCPH) in mutant and sibling embryos revealed a premature stop codon in the mutant allele due to a TàA transversion in the 3rd coding exon (Fig. 2B). This mutation resulted in a Leu>stop alteration at position 155, which is in the 2nd apyrase conserved domain (Fig. 2D) of the predicted protein. We also uncovered a separate, non-complementing allele (*nob*^{hu5310}). The *nob*^{hu5310} allele contained a A>G transversion in the first coding exon (Fig. 2C), resulting in a Thr>Ala alteration at position 80 (asterisk in Fig. 2D). This mutation is located in a highly conserved amino acid residue of the first apyrase conserved domain (Fig. 2D; see also Suppl. Fig. 1B).

Entpd5 expression is sufficient to rescue the nob phenotype

Next, we studied the expression pattern of *entpd5* by whole mount *in situ* hybridization. *Entpd5* and *osterix* showed an almost identical expression pattern at 3 dpf, with *osterix* expression in the region of future teeth as the single exception at this stage (Fig. 2E). To confirm that *osterix*-positive cells also express *entpd5*, we generated an *entpd5*:YFP transgenic line. As shown in Fig. 2E (middle and right panel), YFP expression was identical to the endogenous *entpd5* gene expression. We crossed the *entpd5*:YFP transgenic line with the *osterix*:mCherry transgenic line and observed that *osterix*-expressing cells also express *entpd5* (Fig. 2F), demonstrating that *entpd5* is specifically expressed in, and can serve as a marker for, osteoblasts. Of note, at all stages analyzed, we only observed *entpd5* expression in tissues associated with skeletal mineralization.

To provide independent evidence that the mutation in the two mutant *entpd5* alleles are causative for the *nob* phenotype we attempted to rescue the phenotype by injection of wild-type and *nob*^{hu5310} mutant *entpd5* cDNA under the control of a cytomegalovirus (cmv) promoter. Mosaic rescue (as expected upon plasmid DNA injection) was observed in 24 percent of the *nob*^{hu3718} mutants that were injected with wild-type cDNA (Fig. 2G and H), while the mutant *nob*^{hu5310} *entpd5* cDNA failed to rescue. Of note, rescued embryos showed only mineralization in skeletal elements, not in other parts of the embryo. Mineralization was similarly rescued when wild-type *entpd5* cDNA was expressed in the rescuing assay under the control of the *osterix* promotor (Fig. 2H).

Next, we visualized the (mosaic) location of *entpd5* positive cells of rescued *nob* mutants, and therefore injected a cmv:dendra-t2a-*entpd5* construct to mark the cells in which the *entpd5* gene was over-expressed. Surprisingly, we observed that mineralization was rescued even if osteoblasts do not inherit detectable levels of cmv:dendra-t2a- *entpd5* (Fig. 2I). This prompted us to force *entpd5* expression in a tissue distinct from osteoblasts, in order to clarify the question whether Entpd5 function needs to be provided by osteoblasts, or can be provided by other tissues. Interestingly, injections of *entpd5* under the control of an endothelial specific promoter (*kdrl*:*entpd5*) (Jin et al., 2005), resulted in rescue of *nob* mutants in a manner indistinguishable from the cases described above (Fig. 2J). These results show that while Entpd5 is essential for mineralization and is expressed by osteoblasts in the wild-type embryo, it can be provided by other cellular sources and does not need to be delivered by osteoblasts.

Dragonfish mutants encode enpp1

In the same genetic screen we also uncovered a mutant, termed *dragonfish* (*dgf*), which displayed an ectopic mineralization phenotype (Fig. 3A, B) in the axial skeleton with apparent fusion of the mineralized vertebral centra (Fig. 3A), and also displaying bone nodules at characteristic positions of the cleithrum (arrow in Fig. 3B). Single-embryo mapping positioned the mutation between two flanking markers, SSLR 210 and SSLR 961 (Fig. 3C) on chromosome 20. Sequencing (Fig. 3D, E) of two non-complementing alleles (Fig. 3G, H and Suppl. Fig. 2C) as well as a BAC-mediated rescue (Fig. 3A, F and Suppl. Fig. 2A and B) identified mutations within the *ectonucleotide pyrophosphatase/phosphodiesterase 1* gene (*enpp1*) underlying the mutant phenotype. Both alleles displayed mutations in the phosphodiesterase domain (Fig. 3H) (Stefan et al., 2005).

Phosphate homeostasis is disturbed in nob mutants

Entpd5 and Enpp-1 both hydrolyze extracellular nucleotide derivatives (Okawa et al., 1998; Mulero et al., 1999; Rutsch et al., 2003; Murphy-Piedmonte et al., 2005; Terkeltaub, 2006), with Entpd5 generating inorganic phosphate (Mulero et al., 1999; Murphy-Piedmonte et al., 2005) and Enpp-1 generating pyrophosphate (Terkeltaub, 2006). We therefore examined the epistatic relationship of both genes. Strikingly, double mutant *nob*/*dgf* embryos always formed mineralized bone and usually even exhibited signs of an ectopically mineralized skeleton (Fig. 4A). As this suggested that phosphate homeostasis is disturbed in *nob* mutants, we tested whether raising *nob* mutant embryos in excess phosphate medium would be sufficient to rescue the phenotype. Indeed, growing embryos in phosphate-rich medium resulted in partial skeletal mineralization of *nob* mutants (Fig. 4B), demonstrating that inorganic phosphate is a limiting factor for *nob* mutants to mineralize their skeleton. Of note,

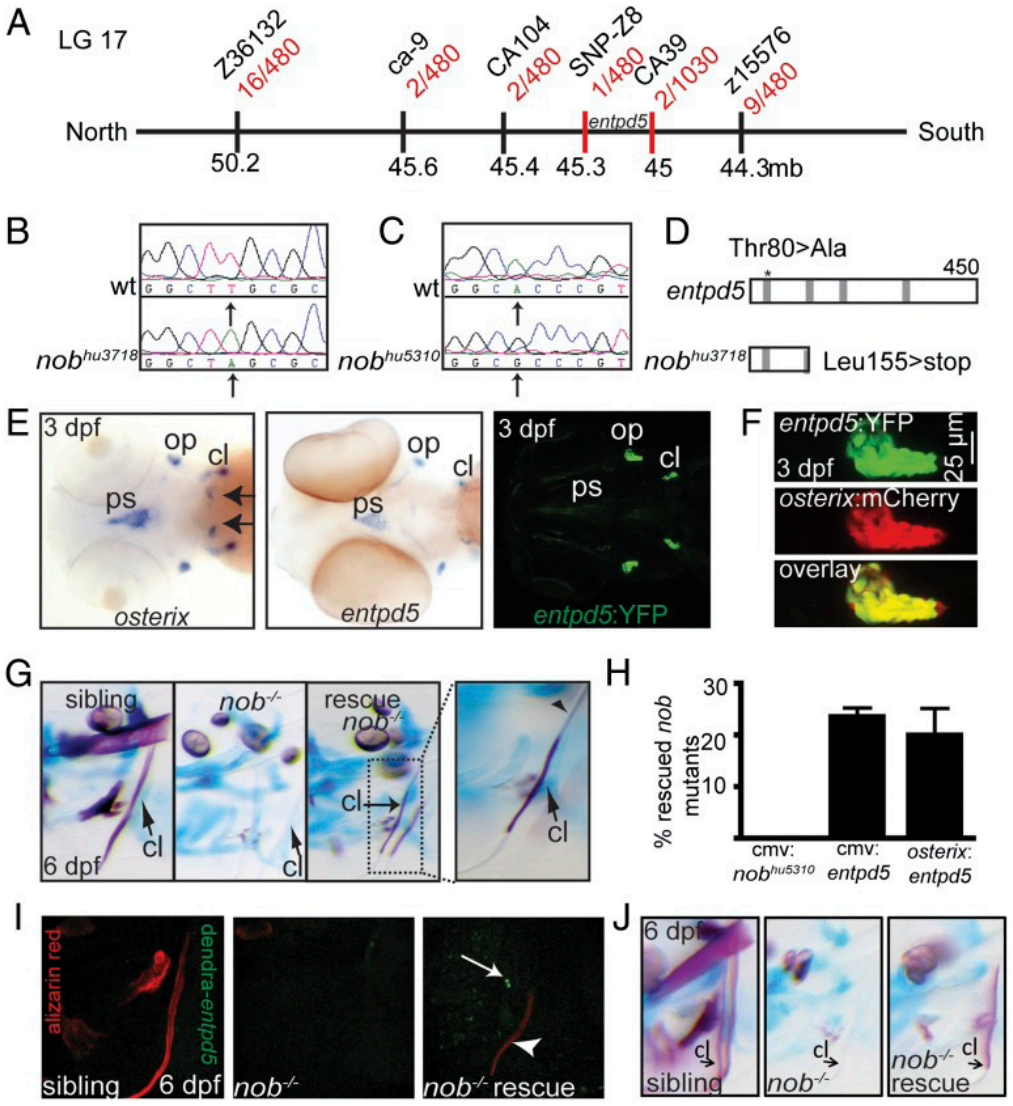

Figure 2: *Nob* mutants encode *ectonucleoside triphosphate/diphosphohydrolase 5* (*entpd5*), which is specifically expressed in osteoblasts. (A) Meiotic map of the *nob*^{hu3718} locus. Recombinants per total number of mutants tested for each polymorphic marker are depicted in red, and markers used for mapping in black. (B) Sequencing of *nob*^{hu3718} revealed a Leu155>stop mutation and (C) sequencing of *nob*^{hu5310} a Thr80>Ala mutation transversion. (D) Schematic representation of the predicted protein structures for Entpd5 and *nob*^{hu3718}. Asterisk indicates position of the mutation in *nob*^{hu5310}. Gray bars: apyrase domains within Entpd5 (Chadwick and Frischauf, 1998). See also suppl. Fig. S1. (E) Left and middle panel: *entpd5* and *osterix* are almost identically expressed in 3 dpf embryos, with *osterix* expression in the region of future teeth (arrows) being the single exception at this stage. Right panel: representative image of a 3 dpf *entpd5*:YFP transgenic embryo showing an expression pattern that matches endogenous *entpd5* transcript distribution

excess calcium had no effect on the *nob* phenotype, while exogenously supplied calcium has been shown to rescue other mutants with hypomineralized phenotypes (Vanoevelen et al., 2011; Huitema et al., 2012).

Next, we studied whether phosphate-regulating genes were affected in *nob* mutants. Two independent microarray experiments on 7 dpf *nob* sibling versus mutant embryos (Supplement) demonstrated strong down-regulation of the key phosphate homeostasis regulator, fibroblast growth factor-23 (*fgf23*), in *nob* mutants. Significant two- fold down-regulation of *fgf23* was validated by qPCR (Fig. 4C) but this difference could be ameliorated by supplying Entpd5 via injection of kdrl:entpd5 (Suppl. Fig.3). In addition to this, we studied the expression of several other phosphate regulating genes by qPCR (Fig. 4C). In concordance with FGF23 downregulation significant up-regulation of the sodium/phosphate co-transporter *npt2a* was measured in 7 dpf *nob* mutants (Fig. 4C).

Taken together, our findings show that Entpd5 is a novel and essential factor for bone mineralization, and indicate that mechanistically, Entpd5 acts on phosphate/pyrophosphate homeostasis.

DISCUSSION

We here show that Entpd5 is crucial for bone mineralization in zebrafish and that *entpd5* is specifically expressed in osteoblasts. This identifies *entpd5* as a novel key factor for bone formation. Entpd5 encodes a secreted ectonucleoside triphosphate/diphosphohydrolase that hydrolyzes preferably extracellular nucleotide diphosphates (NDPs) (Mulero et al., 1999; Murphy-Piedmonte et al., 2005) into nucleotide monophosphates (NMPs) and inorganic phosphate. Circulating extracellular nucleotides are known to be important purinergic signaling molecules that can generate a variety of physiological responses (Gartland et al., 2012; Massé and Dale, 2012). Although we do not exclude the possibility of an additional role

(middle panel) (F) Co-expression of *entpd5*:YFP and *osterix*:mCherry in the operculum of 3 dpf embryos. (G, H) *entpd5* cDNA injection (cmv-promoter) into *nob*^{hu3718} mutants results in mosaic mineralization (G). Arrows point at cleithra (cl) in (G), the arrowhead points to non-mineralized osteoid in a partially mineralized cleithrum. Bar graphs in (H) represent percentages of rescued mutant embryos at 6 dpf (expressed as mean ± SEM, 3 independent experiments). (I) Alizarin red staining of a mutant *nob* embryo that had been injected with a cmv:dendra-t2a-*entpd5* expression construct at the one-cell stage. The cleithrum is partially mineralized in the mutant (arrowhead); however, there are no dendra-positive cells immediately abutting the mineralized structure (arrow). (J) Representative example of an embryo partially rescued by endothelial-specific expression of *entpd5* (*kdr-like*:*entpd5*). cl = cleithrum.

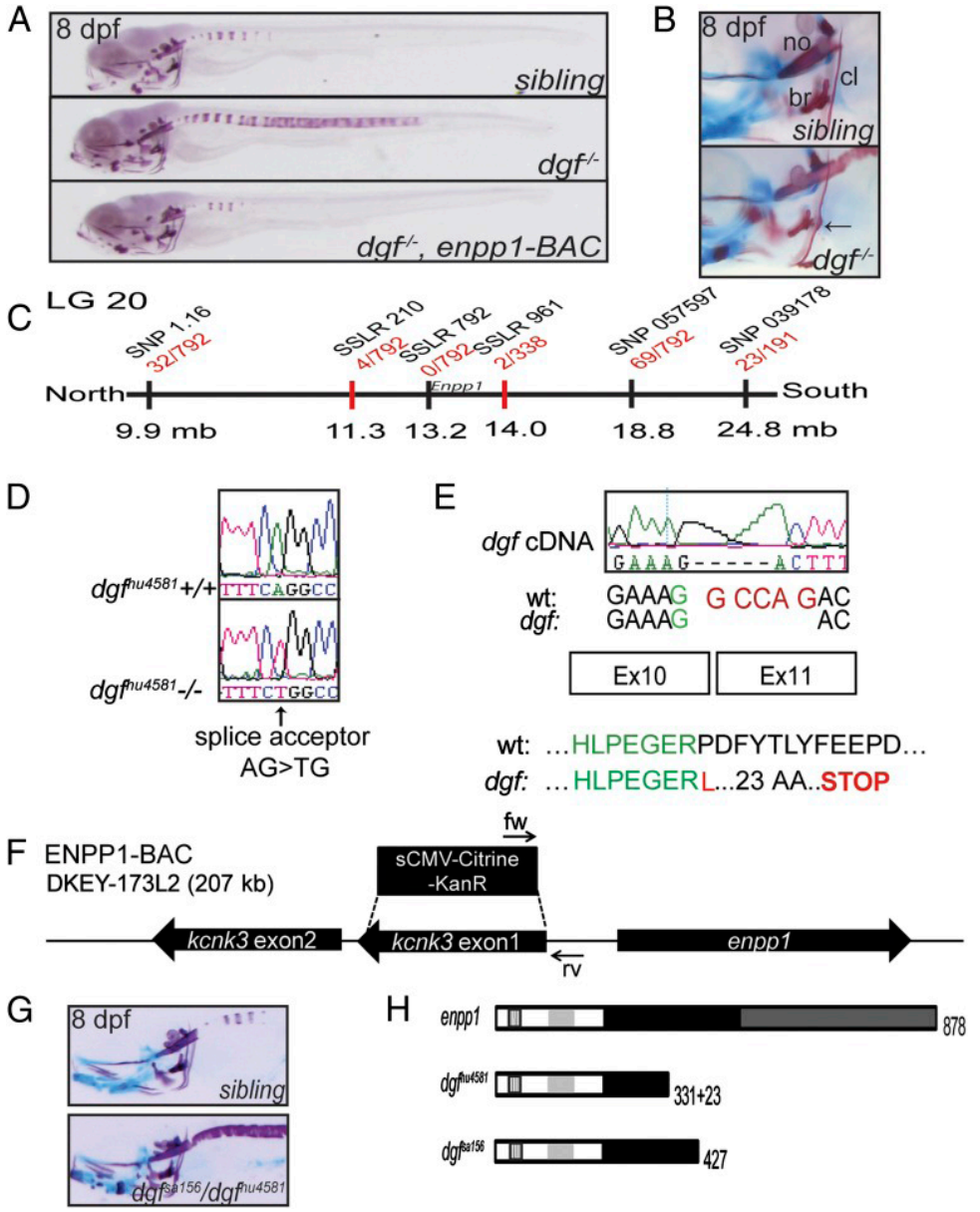

Figure 3: Ectopic mineralization phenotypes in *dgf* mutants. (A) Skeletal staining of a sibling, a *dgf* mutant and a *dgf* mutant rescued by an *enpp1* BAC transgene. *Dgf* mutants exhibit axial hyper-mineralization, resulting in partially fused vertebral centra. (B) Hyper-mineralization also affects the cleithra, which in *dgf* mutants typically exhibit nodule-like protrusions (arrow). (C) Meiotic map of the *dgf* locus. Recombinants per total number of mutants tested for each polymorphic marker are depicted in red and markers used for mapping in black. (D) Sequencing of *dgf*^{hu4581} revealed a mutation in the splice acceptor mutation before exon 11 of the *enpp1* gene. The consequence on the transcript level is a deletion of 5bp (E, upper panel) resulting in a translational frame-shift and a

of purinergic signalling in skeletal mineralization (Gartland et al., 2012), our data rather point to a role of Entpd5 in phosphate homeostasis.

The restricted expression pattern of *entpd5* in osteoblasts suggests that Entpd5 acts locally, in a microenvironment that is already permissive to allow mineralization. On one hand, this notion is supported by the rescue experiments reported here: expression of *entpd5* via an ubiquitously acting CMV promoter or an endothelial-specific *kdr-like* promoter does not lead to ectopic mineralization, but results exclusively in bone mineralization in those regions where the local microenvironment (extracellular matrix composition, pyrophosphate levels) is prone to mineralization events. On the other hand, *entpd5* does not need to be provided in a cell-autonomous manner (i.e. in osteoblasts): expression in the embryonic endothelium is sufficient to cause mineralization. In the wild-type embryo, one would still expect highest levels of Entpd5 protein at the osteoblast surface, and therefore in the immediate vicinity of a microenvironment that provides the appropriate and required composition for biomineralization (see suppl. Fig. 4 for a model).

ENTPD5 has recently been suggested to play a role in proper protein folding and glycosylation in the endoplasmic reticulum (Fang et al., 2010). However, since both the cartilage matrix and the osteoid appeared normal in *nob* mutants, and since *dgf*/*nob* double mutants can mineralize their skeleton, we consider it unlikely that a failure of proper glycosylation of extracellular matrix proteins is the limiting factor for skeletal mineralization. Rather, our data strongly suggest that a stringently controlled balance between Entpd5 and Enpp-1 activities determines the level of mineralization through controlling the ratio of inorganic phosphate versus pyrophosphate in the immediate vicinity of osteoblasts. Skeletal mineralization is a tightly controlled process, depending on the availability of inorganic phosphate release from a variety of substrates by ectoenzymes (Terkeltaub, 2006). Pyrophosphate antagonizes the ability of inorganic phosphate and calcium to form a mineral crystal. In line with this, *Enpp1* mutations in human and mice have been shown to cause ectopic mineralization due to insufficient extracellular pyrophosphate (Okawa et al., 1998; Rutsch et al., 2003). Based on the ectopic mineralization phenotype

predicted stop codon after 23 amino acids (E, lower panel). (F) Schematic representation of the BAC-construct generated for the transgenic rescue of the ***dgf*^{4581}**phenotype. Two genes are contained on the BAC, and *kcnk3* was inactivated through a recombineering approach. (G) ***Dgf*^{sa156}** fails to complement ***dgf*^{hu4581}**. (H) Schematic drawing of the predicted protein forms of ***enpp1***, ***dgf*^{hu4581}**and ***dgf*^{sa156}**, respectively. Enpp1 is a type II transmembrane protein. Striped box: transmembrane domain; light-gray: somatomedin-B-like binding domains; black: catalytic domain; dark-gray: nuclease like domain (Stefan et al., 2005). See also Suppl. Fig. 2.

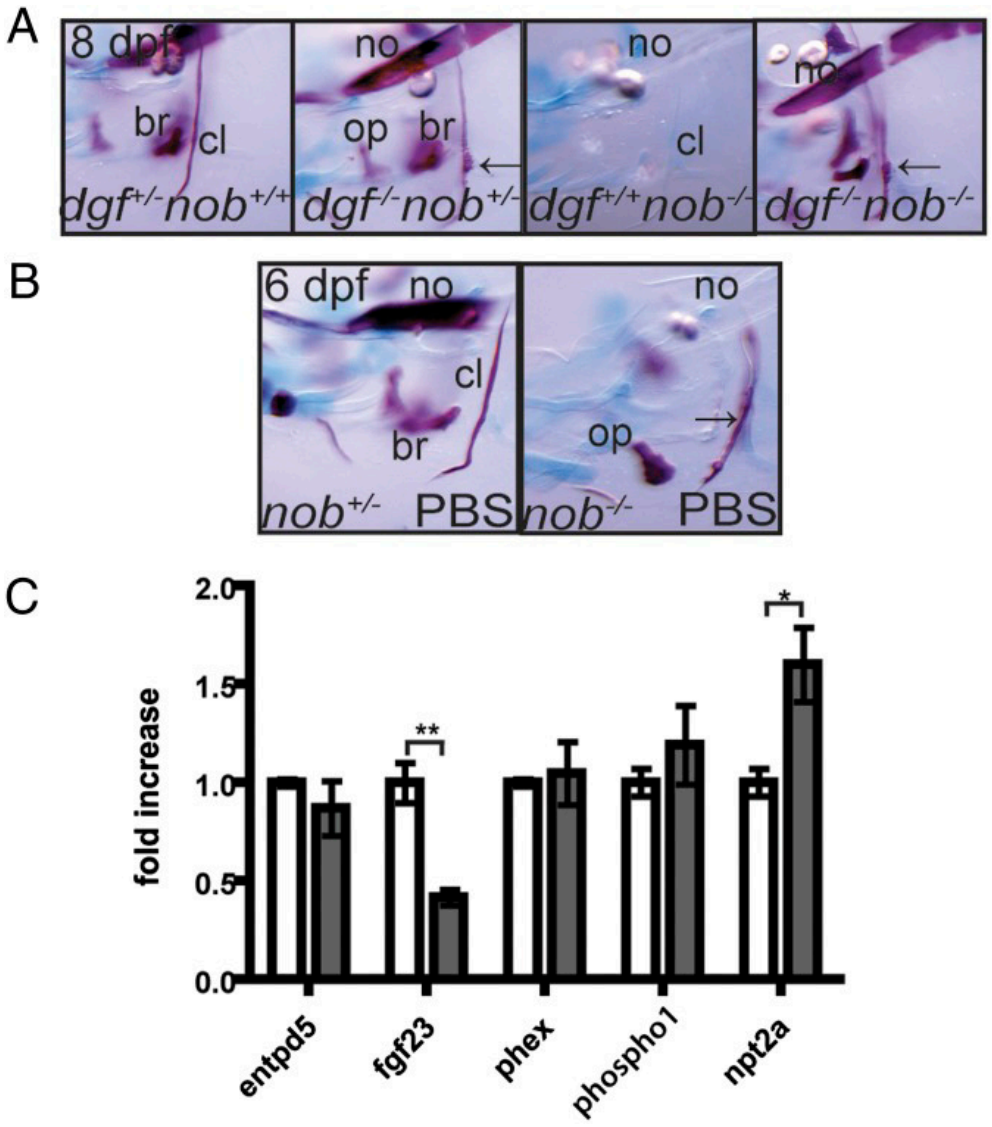

Figure 4: Phosphate homeostasis is disturbed in *nob* mutants. (A) *Nob* mutants can mineralize their skeleton in the absence of *dgf*^{hu4581} function, and will even show ectopic mineralization (rightmost panel, arrow pointing at protrusions of the cleithrum, a typical feature of *dgf* mutants). (B) Exogenous phosphate partially rescues the *nob* mutant phenotype (arrow points at the cleithrum). All images represent lateral views of the head skeleton at 6 dpf. br = 5th branchial arch, cl = cleithrum, no = notochord, op = opercule. (C) qPCR on 7 dpf embryos revealed that compared to siblings (white bars), *fgf23* expression levels are significantly down-regulated and *npt2a* expression is significantly up-regulated in *nob* mutant embryos (grey bars). Results are expressed as mean ± SEM of 3 independent experiments with *p<0.05, and **p<0.005.

of the *dgf* mutants, we show here on the phenotypic level that the function of Enpp1 is conserved between fish and mammals.

Since our study indicates that Entpd5 regulates phosphate homeostasis, we speculated that other factors regulating phosphate levels *in vivo* might be affected. Indeed, *Fgf23* (Fibroblast growth factor-23) is significantly down-regulated, and the sodium/phosphate co-transporters *npt2a* (NaPi2a) is significantly up-regulated in *nob* mutants. FGF23 is known as a key regulator of phosphate homeostasis (Sitara et al., 2008), and changes in FGF-23 activity lead to human disorders associated with either phosphate wasting or retention (Lu and Feng, 2011). FGF23 is a circulating hormone produced in the bone that mainly targets the kidneys to control the activity of NPT2A and NPT2C (Hori et al., 2011). It seems likely that the absence of skeletal mineralization in mutant *nob* zebrafish elicits compensatory mechanisms in order to regulate the low levels of inorganic phosphate. Down-regulation of *fgf23* and up-regulation of *npt2a* are consistent with this.

A murine Entpd5 knock-out has been reported, but it is unclear whether this allele (encoding an ENTPD5:lacZ fusion) represents a complete loss of function situation. These mice are viable (Read et al., 2009), but appear smaller than litter mates, a phenotype often found in hypophosphatemic mice (Sitara et al., 2008; Yadav et al., 2011). Furthermore, the mice were shown to have increased serum alkaline phosphatase (Read et al., 2009). Together with the findings of our study, we believe that the phenotype reported by Read *et al.* is likely due to disturbed phosphate homeostasis. However, we cannot exclude that the essential function of Entpd5 during osteogenesis as described here is potentially unique in basal vertebrates, and that it has shifted to other secreted paralogues in higher vertebrates, or even to completely different genes (such as alkaline phosphatase).

In summary, in this study we demonstrate that *entpd5* is essential for skeletal mineralization in zebrafish and that *entpd5* is specifically expressed in osteoblasts. We provide evidence that the combined activity of both Entpd5 and Enpp-1 maintains normal physiological levels of phosphate and pyrophosphate, and absence of the activity of either protein results in mineralization phenotypes. The *nob* mutant phenotype can be rescued by either exogenous phosphate or Entpd5 protein provided by non-osteoblast cells, suggesting that the correct systemic phosphate levels together with the appropriate extracellular microenvironment of osteogenic cells provides the basis for biomineralization.

Material and Methods

Alizarin red/ Alcian blue skeletal Staining

Skeletal staining was performed as described previously (Walker and Kimmel, 2007; Spoorendonk et al., 2008) and specimen were stored in 70% glycerol at 4°C. *In vivo* skeletal staining was performed with 0.001% calcein or 0.05 % Alizarin Red in E3 medium for 5-10 minutes and subsequent extensive washes with E3 medium.

Meiotic mapping and sequencing

Bioinformatic construction of the genomic region surrounding the *nob*^{hu3718} and the *dgf*^{hu4581}gene was performed using Ensembl databases Zv6 for *nob*^{hu3718} and Zv9 for *dgf*^{hu4581}. Meiotic mapping of the *nob*^{hu3718} and *dgf*^{hu4581}mutation was performed using standard simple sequence length polymorphisms and single nucleotide polymorphisms.

For sequencing of candidate genes, coding exons of the respective gene were amplified separately from mutant and wild-type embryos and sequenced on both strands. The Leu155>stop mutation in *nob*^{hu3718} was confirmed with primers *nob*^{3718}-ex3-fw and *nob*^{hu3718}ex3-rev. The Thr80>Ala mutation of *nob*^{hu5310} was confirmed with primers *nob*^{hu5310} -ex1-fw and *nob*^{hu5310}-ex1-rev. The splice acceptor mutation of *dgf*^{hu4581} was confirmed with primers dgfGfw and dgfGrev. Sequencing of the cDNA (Primers: dgfCfw and dgfCrv) revealed a frame-shift leading to a predicted stop-codon after a further 23 amino acids. No alternative transcripts could be detected by RT PCR. The Arg427>stop mutation of *dgf*^{sa156} was confirmed with primers saGfw and saGrv. All primer sequences are shown in the **Supplemental Experimental Procedures**. PCR conditions are available upon request.

For all experiments we have used the *nob*^{hu3718} allele and *dgf*^{hu4581} allele, respectively, unless stated otherwise.

Whole mount in situ hybridization and immunohistochemistry

All *in-situ* hybridizations were performed at least twice as previously described (Schulte-Merker, 2002; Spoorendonk et al., 2008) and embryos were subsequently genotyped. Previously described probes were *osterix* and *col10a1* (Spoorendonk et al., 2008). Probes generated for *sox9a* and *entpd5* were transcribed from the 5' part of the respective cDNA (for primers sequences, see **Supplemental Experimental Procedures**).

Immunohistochemistry was essentially done as described (Hammond and Schulte-Merker, 2009). Embryos were fixed for 1 hour in 4% PFA and stored in methanol. Embryos were rehydrated, blocked in PBS with 5% lamb serum and incubated with 1/500 anti-phospho-Histone H3 (millipore) and anti-Collagen II (1/500 Developmental Studies Hybridoma Bank) overnight at 4°C. Embryos were washed extensively and then incubated in Alexa-Fluor secondary antibodies (Molecular probes; diluted 1/500 in blocking solution) for 3 hours at room temperature. Embryos were washed extensively in the dark and mounted for analysis.

cDNA rescue experiments

TRIzol® reagent (Invitrogen) was used to extract RNA from 6 dpf embryos and mouse RNA was extracted from cultured KS483 cells (Yamashita et al., 1996). First-strand cDNA was generated using either cloning entpd5 R (fish) primers or cloning Entpd5 R (mouse) primers (**Supplemental Experimental Procedures**). Second-strand cDNA was synthesized using M-MlV reverse transcriptase (Promega) according to the manufacturer's protocol. Zebrafish *entpd5* cDNA was cloned into PCS2+ and into pBluescript containing the *osterix* promoter (Spoorendonk et al., 2008). One-cell stage embryos derived from *nob*^{hu3718} carrier fish were injected with plasmid DNA in a maximum volume of 2 nl. Alizarin red/Alcian blue staining was carried out at 6 dpf. Only injected embryos with normal size, apparently normal cartilage, without tissue malformations or general edema or apparent toxic defects were included for analysis. Each rescue experiment was performed three independent times. In total we scored 490 siblings / 131 mutant embryos injected with 100 pg *cmv*:*entpd5*; 329 siblings / 106 mutants injected with 100 pg *osterix*:*entpd5*; 500 siblings / 166 mutants with 100 pg cmv:Entpd5 (murine cDNA); 151 siblings / 63 mutants with 100 pg cmv: *nob*^{hu5310}, and 481 sibling / 129 mutants with 25 pg kdr-l:entpd5 cDNA.

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Animal procedures

All zebrafish strains were maintained at the Hubrecht Institute using standard husbandry conditions. Animal experiments were approved by the Animal Experimentation Committee (DEC) of the Royal Netherlands Academy of Arts and Sciences.

Mutagenesis and screening

ENU mutagenesis and screening was performed as previously described (Haffter et al., 1996; Spoorendonk, 2009). Embryos were grown in E3 medium until 8 dpf and subsequently fixed for skeletal staining (Alizarin red/Alcian blue) as described above.

Embryo media

Embryos were kept in E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) at 28°C. For phosphate rescue experiments, embryos were kept in phosphate buffered saline medium, PBS (137 mM NaCl, 2.7 mM KCl, 9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 80 mM $Na_2HPO_4.2H_2O$, 14.7 mM KH_2PO_4, pH 7.4). For calcium rescue experiments, embryos were kept in E3 embryo medium + 10 mM $CaCl_2$. For anaesthesia, a 0.2 % solution of 3-aminobenzoic acid ethyl ester (Sigma), containing Tris buffer, pH 7, was used (Brand, 2002).

Microarray

Analysis of the microarray data is available (Gene Expression Omnibus accession no. GSE35737; www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=npqhtqgomsaakvy&acc=GSE35737).

Transgenic lines

Transgenic lines were generated as described by Bussmann *el al.* (Bussmann and Schulte-Merker, 2011). Fluorophores were recombined into the ATG site of the *entpd5* gene (BAC clone CH211-202H12). Sequences of primers used are available on request.

BAC rescue experiment

BAC clone DKEY-173L2 used for a stable rescue of *dgf*^{hu4581}contains all exons and untranslated regions of the *enpp1* gene and 87 kb of the upstream regulatory region. The first exon of the two-exon gene *kcnk3*, which is also present on the BAC, was replaced through BAC recombineering with a cassette containing YFP under a CMV promoter (**Fig. 3F**), effectively eliminating the gene function. Transgenic lines were generated as described by Bussmann and Schulte-Merker (2011). Carriers passed on the transgene to approximately 50 % of their offspring, indicative of a single BAC integration event. Upon crossing *dgf*$^{+/-}$, *enpp1*-BAC+ with *dgf*$^{+/-}$ individuals, we observed a mutant phenotype in less than 1/8 (9.4%) of the embryos at 8 dpf. None of the embryos with a mutant phenotype tested positive for integration of the BAC construct by PCR (16/16). Primer locations are indicated in **Fig. 3F** (small arrows fw and rv). To test for the presence of rescued embryos, we performed genotyping using an SNP marker (CASCAD 039178) (Guryev et al., 2005) linked to the mutant *dgf* allele, but situated outside the genomic region covered by the BAC. This was necessary as sequencing of the *dgf* locus itself is not informative due to the presence of three copies of *enpp1* in the genome of transgenic fish. Within the transgenic population we could find approximately a quarter of embryos (6/23) with the variant of the SNP linked to the mutant *dgf* allele. These embryos were phenotypically indistinguishable from heterozygous or wild-type embryos of the same clutch (**Fig. 3A**). This shows that a BAC containing *enpp1* is sufficient to rescue the *dgf* phenotype and therefore confirms causality of the genetic lesion identified in the *dgf* mutant line.

Quantitative real-time PCR

Nob mutant and sibling embryos were separated at 6 dpf by *in vivo* skeletal (alizarin red) staining and allowed to recover for 24 hpf at 28°C. For total RNA isolation from 7 dpf embryos, a maximum of n=40 ***nob*** mutant and sibling embryos per clutch were homogenized by shredding in 600 µl of RTL lysis buffer (Qiagen RNeasy kit) containing 10% beta-mercaptoethanol. One volume of 70% ethanol was added and the homogenate was loaded onto a column for total RNA isolation according to the manufacturer's protocol, followed by DNaseI (Promega) treatment. The RNA quality and concentration were determined using a NanoDrop (Thermo scientific), and verified by gel electrophoresis. cDNA was synthesized from total RNA (1–5 µg) with random hexamers (integrated DNA technologies) using reverse transcriptase M-MLV (Promega). Primer sets were designed using Primer3 (http://frodo.wi.mit.edu/primer3/) with an optimal product size of 110–200 base pairs and, spanning two exons to avoid amplification of genomic DNA (for primer sequences, see **Table S1**). PCR efficiency and melting temperatures were determined per primer set, and we verified the specificity by gel electrophoresis. qPCR was performed using the MyIQ single color real-time PCR detection system and software (Bio-Rad). Reaction Mix: 12.5 µl SYBR Green fluorescent label (Bio-Rad), 3 µl of 1.5 µM primer mix, 4.5 µl MQ and 5 µl

cDNA (10ng/µl). Cycling conditions: 95°C for 3 minutes; 40 cycles of 95°C for 10 seconds and the optimal primer temperature for 45 seconds; followed by 95°C for 1 minute; and finally 65°C for 1 minute. Reactions were performed in triplicate. cDNA was isolated from at least three different clutches of pooled 7 dpf ***nob*** mutant and sibling embryos. Ct values were normalized for the ***ef1a*** housekeeping gene. We calculated *Nob* mutant cDNA concentrations in arbitrary units compared to the sibling average. Values are represented fold change with the sibling value set to 1. Groups were compared by paired Student's *t*-test.

Imaging

Embryos were mounted in 0.5 % low melting point agarose in a culture dish with a cover slip replacing the bottom. Imaging was performed with a Leica SP2 confocal microscope (Leica Microsystems, http://www.leica-microsystems.com/) using a 10x or 20x objective with digital zoom. Usually, z-stacks spanning approximately 5 µm were captured and were then flattened by maximum projection in ImageJ.

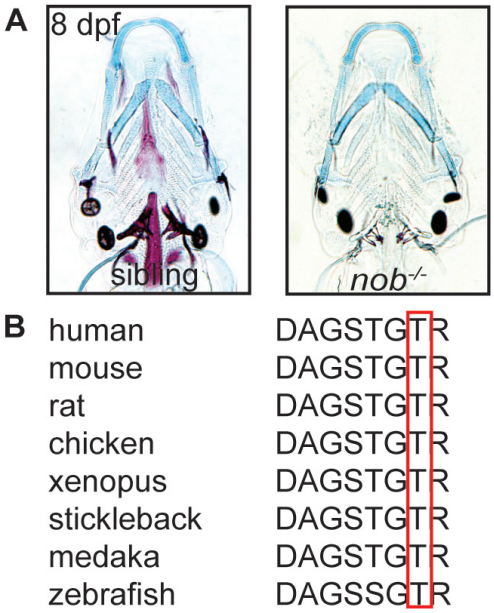

Figure S1: (A) Alcian blue staining is unaltered in nob mutants versus siblings. The images are similar to the ones presented in Figure 1A, but taken with higher contrast settings to allow an appreciation of normal chondrocyte morphology in mutant embryos. (B) Multiple sequence alignment of Entpd5 proteins demonstrating the conserved nature of zebrafish Thr80 in the first apyrase domain.

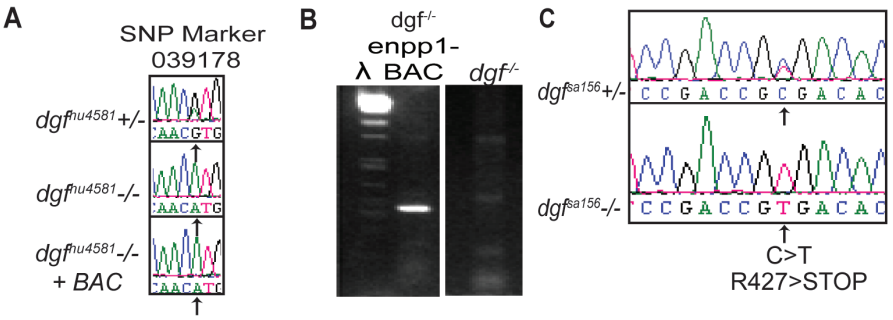

Figure S2: Dgf^{sa156} encodes a mutation in enpp1, and dgf$^{-/-}$ mutant embryos can be rescued by transgenic insertion of a BAC containing the enpp-1 gene. (A) BAC-rescued embryos exhibit a wild-type phenotype (shown in Figure 3A). The SNP variant (arrow) is linked to the dgf mutation (sequence middle panel). (B) PCR indicating the presence of the enpp1-BAC in the rescued dgf$^{-/-}$ embryos, and absence in the non-rescued dgf-/- embyro. (C) Sequencing of the dgf^{sa156} allele, which was kindly provided by the Sanger Center (Hinxton, UK) reveals an arg427>stop in the enpp1 gene.

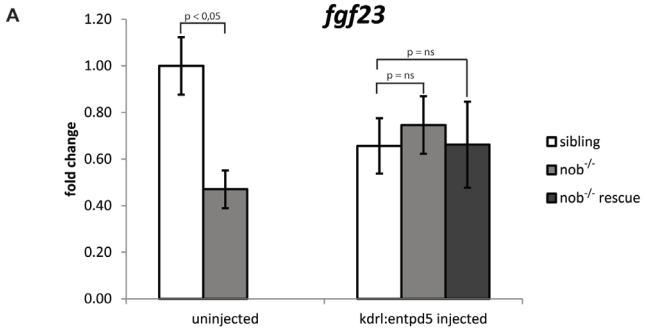

Figure S3: Quanititative real-time PCR for *fgf23* levels in 7dpf old embryos. Uninjected siblings vs. mutants are depicted in the left panel. In the right panel, embryos were injected with a *kdrl:entpd5* plasmid, stained with Alizarin Red and categorized into siblings (normal mineralization), mutants without any signs of rescue (compare Fig 2J, middle panel), and mutants with partial rescue (compare Fig 2J, right panel). Transcript levels were normalized to the level in uninjected siblings.

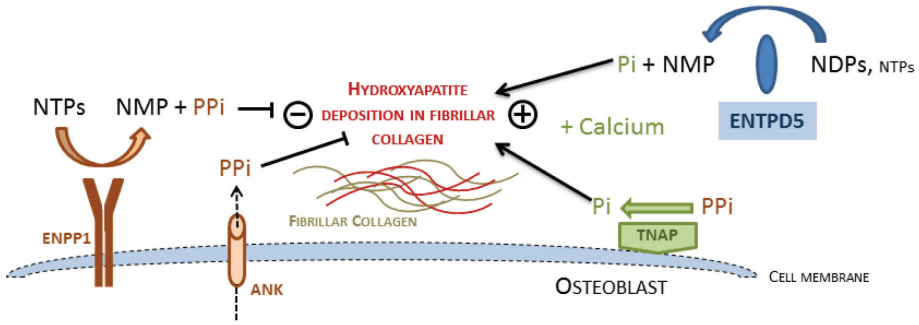

Figure S4: *entpd5* encodes an essential determinant of extracellular phosphate and bone mineralization. A graphical abstract to depict the interaction of Entpd5, Enpp1 and other factors that regulate extracellular phosphate and pyrophosphate levels. Enpp1 activity leads to generation of pyrophosphate, and in addition there is transport of pyrophosphate via the transmembrane channel ANK. Pyrophosphate inhibits the biomineralization process that occurs on fibrillar collagen matrix (osteoid). This negative effect is counterbalanced by ALP/TNAP mediated hydrolysis of pyrophosphate. This process generates free phosphate. Our data indicate that ENTPD5 is an essential contributor in generating sufficiently high levels of free phosphate necessary for proper mineralization. Biochemical studies suggest that this is achieved by hydrolyzing phosphate from NDPs (and, to a lower extent also NTPs). Please note that TNAP/alkaline phosphatase has been shown in mammals to be a key component for mineralization to occur. The respective contributions of TNAP in teleosts remain to be established, as does the role of Entpd5 in mammals.In the case of the *dgf* mutant embryos, Enpp1 activity is absent, hence there is less pyrophosphate available and the phosphate/pyrophosphate balance is shifted towards higher phosphate levels, resulting in more mineralization. In nob mutants phosphate levels are lower, tipping the balance in favor of pyrophosphate, leading to a failure to mineralize.

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Chapter 4

Zebrafish enpp1 mutants exhibit pathological mineralization, mimicking features of generalized arterial calcification (GACI) and pseudoxanthoma elasticum (PXE)

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Summary

In recent years it has become clear that, mechanistically, biomineralization is a process that has to be actively inhibited as a default state. This inhibition must be released in a rigidly controlled manner in order for mineralization to occur in skeletal elements and teeth. A central aspect of this concept is the tightly controlled balance between phosphate, a constituent of the biomineral hydroxyapatite, and pyrophosphate, a physiochemical inhibitor of mineralization. Here, we provide a detailed analysis of a zebrafish mutant, dragonfish (*dgf*), which is mutant for ectonucleoside pyrophosphatase/phosphodiesterase 1 (Enpp1), a protein that is crucial for supplying extracellular pyrophosphate. Generalized arterial calcification of infancy (GACI) is a fatal human disease, and the majority of cases are thought to be caused by mutations in ENPP1. Furthermore, some cases of pseudoxanthoma elasticum (PXE) have recently been linked to ENPP1. Similar to humans, we show here that zebrafish enpp1 mutants can develop ectopic calcifications in a variety of soft tissues – most notably in the skin, cartilage elements, the heart, intracranial space and the notochord sheet. Using transgenic reporter lines, we demonstrate that ectopic mineralizations in these tissues occur independently of the expression of typical osteoblast or cartilage markers. Intriguingly, we detect cells expressing the osteoclast markers Trap and *cathepsinK* at sites of ectopic calcification at time points when osteoclasts are not yet present in wild-type siblings. Treatment with the bisphosphonate etidronate rescues aspects of the dgf phenotype, and we detected deregulated expression of genes that are involved in phosphate homeostasis and mineralization, such as *fgf23*, *npt2a*, *entpd5* and *spp1* (also known as osteopontin). Employing a UAS-GalFF approach, we show that forced expression of enpp1 in blood vessels or the floorplate of mutant embryos is sufficient to rescue the notochord mineralization phenotype. This indicates that enpp1 can exert its function in tissues that are remote from its site of expression.

Introducion

Calcium and phosphate are the main elements in hydroxyapatite, the mineral that constitutes the vertebral skeleton and teeth. Hydroxyapatite also occurs in the form of ectopic calcifications, which can result from disease, injury or aging in a wide variety of organs and tissues. Ectopic calcifications are also often a result of imbalanced ion levels, again, specifically calcium and phosphate (in chronic kidney disease, for example) (Giachelli, 1999). Particularly when occurring in vascular tissues, ectopic calcification has been associated with increased mortality (Goodman et al., 2000; Ganesh et al., 2001).

Two key concepts have emerged from human genetic studies and animal experimental data on the control of biomineralization over the past few years. First, calcium and phosphate, which readily form an insoluble precipitate, are present in virtually all tissues and body fluids; therefore, crystallization has to be actively inhibited. Second, the balance between phosphate and pyrophosphate is a crucial determinant in the regulation of this crystallization process (Giachelli, 2008; Kirsch, 2012). Phosphate is an element that enables the formation of hydroxyapatite, whereas pyrophosphate is a strong chemical inhibitor of crystal formation (Terkeltaub, 2001).

Generalized arterial calcification of infancy (GACI; OMIM 208000) is an autosomal-recessive disorder that is characterized by the calcification of medium and large arteries in humans. It often leads to demise because of arterial stenosis and, consequently, heart failure within the first months of life. Mutations in ectonucleotide pyrophosphatase/phosphodiesterase-1 (ENPP1; formerly known as PC-1) have been identified as being causative in the majority of GACI cases investigated (Rutsch et al., 2003; Nitschke et al., 2012). Mouse models and *in vitro* data have confirmed that ENPP1 function is crucial in the regulation of biomineralization (Johnson et al., 2003; Mackenzie et al., 2012a) because ENPP1 generates extracellular pyrophosphate through the hydrolysis of extracellular ATP (Kato et al., 2012). Recently it has become clear that the spectrum of human phenotypes that are caused by mutations in ENPP1 is variable, and less severe cases present themselves with symptoms of hypophosphatemic rickets or pseudoxanthoma elasticum (PXE; OMIM 264800). PXE is predominantly characterized by mineralization in the skin and eye, as well as the vasculature, although it has a later onset than GACI (Li et al., 2012; Nitschke et al., 2012). Most cases of PXE have been associated with mutations in ABCC6 and not ENPP1; however, recently a mechanistic link between ABCC6 mutations and reduced amounts of pyrophosphate has been established (Jansen et al., 2013).

Zebrafish share many of the basic features of chondrogenesis and osteogenesis with higher vertebrates (Apschner et al., 2011; Mackay et al., 2013), and offer the opportunity to perform genetic and chemical screens (Spoorendonk et al., 2010), as well as to examine osteoblasts and osteoclasts in an *in vivo* setting. We have recently described the catalytic activity of Ectonucleoside triphosphate diphosphohydrolase 5 (Entpd5) as an essential

provider of phosphate for mineralization of the zebrafish skeleton (Huitema et al., 2012). Here, we provide a detailed analysis of the ***dragonfish*** **(***dgf***)** mutant, which displays features that are found in both GACI and PXE. Furthermore, we demonstrate the suitability of the *dgf* mutant for chemical screening of drugs that inhibit mineralization and provide evidence that Enpp1 can act at areas that are distal from its site of expression. Finally, we show that ectopic mineralizations can lead to the generation of osteoclasts, a finding that has possible consequences for the treatment of GACI.

Results

DGF^{hu4581} mutants show decreased phosphodiesterase activity and multiple ectopic calcifications

In a forward genetic screen, we have previously identified a mutant that exhibited distinct patterns of increased mineralization; furthermore, we described the positional cloning and molecular characterization of this allele, called *dgf*^{hu4581} (Huitema et al., 2012). The allele harbors a splice acceptor mutation leading to a predicted frame shift and a subsequent early stop codon within the phosphodiesterase-like catalytic domain of Enpp1 (Fig. 1A). Here, to evaluate the contribution of Enpp1 towards overall phosphodiesterase activity, we performed phosphodiesterase measurements on the lysates of *dgf*-mutant embryos. The results showed a reduction of phosphodiesterase activity by over 60%, indicating that Enpp1 accounts for the majority of phosphodiesterase activity in zebrafish embryos at this stage (Fig. 1B).

Histological examination by Alcian Blue and Alizarin Red, and van Kossa and van Gieson staining revealed multiple sites of ectopic mineralizations in *dgf* embryos. The most prominent phenotypic consequence of the *dgf* mutation was the mineralization of the notochord sheet (Fig. 1C, D; supplementary material Fig. S1A, B), which becomes apparent in all *dgf*-mutant embryos, to a variable degree, between 6 and 9 days post-fertilization (dpf). We also observed ectopic mineralization of the neural tube (Fig. 1C, D; supplementary material Fig. S1C, D), on the ceratohyal cartilage (Fig. 1E, F) and on cartilage elements of the pectoral fin (supplementary material Fig. S1E, F). Furthermore, we observed early onset of perichondral ossification in 90% of the mutants (n=20) (Fig. 1E, F). The first manifestation of the phenotype was detectable at 4 dpf in some *dgf* mutants. At this stage, the embryos showed calcifications in the inter-cranial space (Fig. 1G, H; supplementary material Fig. S1G, H) and within the myocardium (Fig. 1I, J), as well as calcifications in the area surrounding the myocardium (Fig. 1I, J, red arrowhead) and in the skin beneath the yolk sac and heart (Fig. 1K, L;supplementary material Fig. S1G, H). Occasionally, mutants survived to juvenile and young-adulthood stages; however, these showed reduced growth compared with their wild-type siblings (supplementary material Fig. S1I) and fusion in

their axial skeleton, not only of vertebral bodies but also of neural and haemal arches (Fig. 1M, N). Furthermore, we saw ectopic calcifications in the eye (Fig. 1O-Q) and the ethmoid plate cartilage (Fig. 1O, P), as well as patchy mineralization of craniofacial bone elements (Fig. 1O–P′) and mineralization of the bulbus arteriosus – the outflow tract of the heart (Fig. 1R, S; supplementary material Fig. S1J, K).

In summary, *dgf* mutants display ectopic mineralizations in a number of different soft tissues, with some variability depending on developmental timing and the site of mineralization. Ectopic mineralizations of the notochord sheath and of the pectoral fin cartilage were found relatively consistently, whereas ectopic mineralization in other tissues, such as the skin and the heart, demonstrated a higher degree of variation between clutches. Part of this variation can probably be attributed to genetic variation because zebrafish, in contrast with mice, are not maintained as inbred lines.

Soft tissue calcifications in dgf mutants probably represent passive calcium depositions

Arterial calcification, a particularly well-studied form of soft tissue calcification, has been shown to be associated with the ectopic expression of bone and cartilage markers in mouse and human, respectively (Johnson et al., 2005; Neven et al., 2007). This has been investigated less intensively in other tissues. To examine whether the *dgf* phenotype is caused by ectopic differentiation of bone or cartilage cells, we used transgenic reporter lines for the cartilage marker *collagen2a1a* (Mitchell et al., 2013), as well as for *collagen10a1* (Mitchell et al., 2013) and *osteocalcin* (Vanoevelen et al., 2011); the latter two represent osteoblast markers in zebrafish. Importantly, these genes have been shown to be expressed in calcified arteries of *Enpp1*-knockout mice (Johnson et al., 2005). In brief, we could not detect any ectopic expression of these markers at the loci of ectopic calcifications in *dgf* mutants. Examples are shown for *collagen10a1* (Fig. 2A, B) and *osteocalcin* in the heart region (Fig. 2C, D), as well as for *collagen2a1a* in the cranium (Fig. 2E, F). Within the axial skeleton of wild-type embryos, *collagen10a1* expression colocalized with mineralized vertebral bodies (Fig. 2G). Ectopically mineralized areas of the notochord, however, were devoid of *collagen10a1* expression (Fig. 2H).

Although we were unable to detect changes in any of the above markers for chondrocytes or osteoblasts, we did see changes in the appearance of *secreted phosphoprotein 1* (*spp1*; also known as Osteopontin), a calcium-binding regulator of mineralization, which is regularly detected in conjunction with ectopic calcifications (Giachelli and Steitz, 2000). Performing *in situ* hybridizations, we detected a pattern of *spp1* expression in *dgf* mutants, which correlated with loci that often develop ectopic mineralizations, such as the cranium and the skin covering the yolk sack (Fig. 2I–L, compare supplementary material Fig. S1J, K). Moreover, *spp1* showed increased expression in the skeletal elements of *dgf* embryos when compared with siblings (Fig. 2I–L).

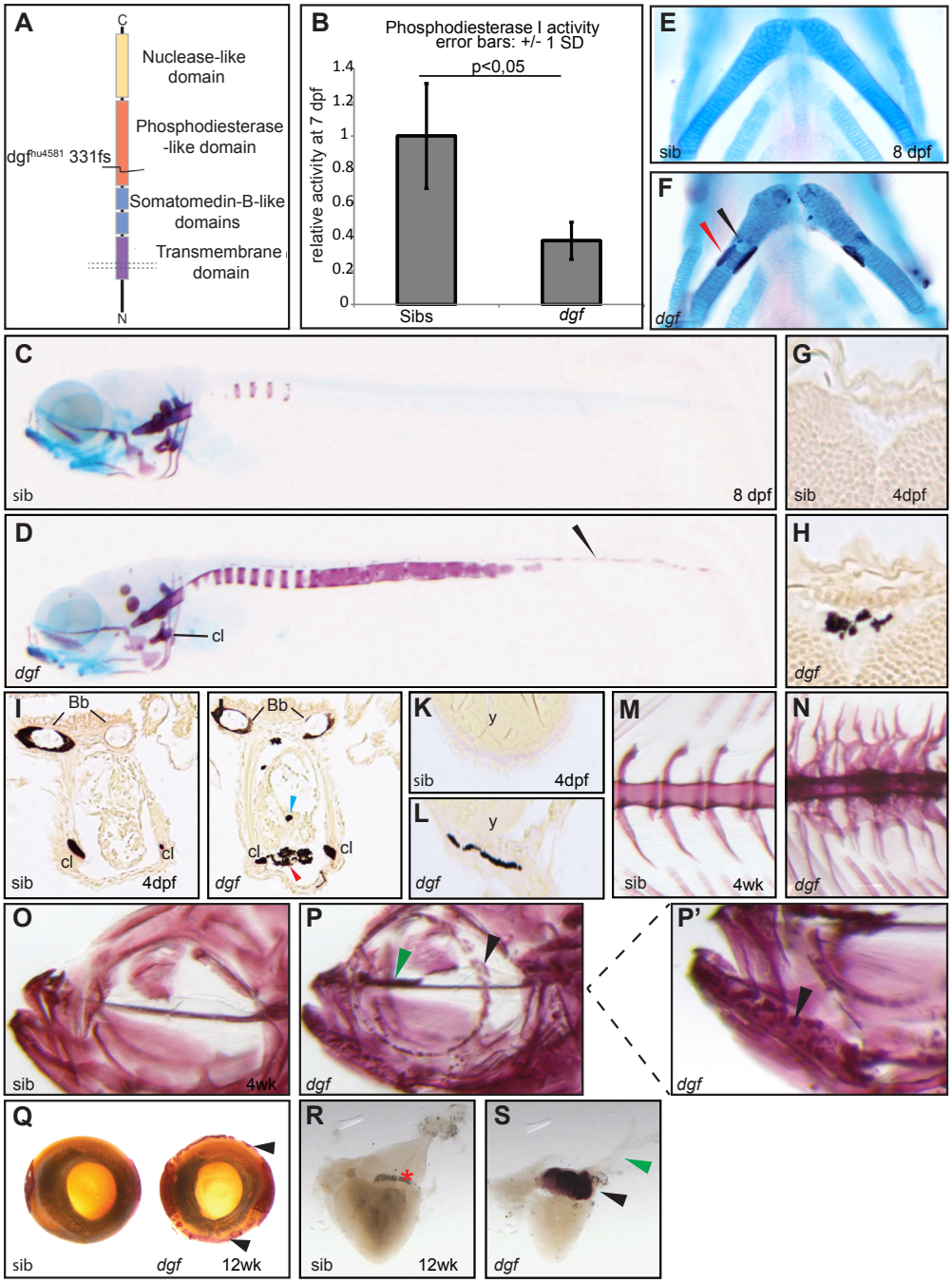

Figure 1: *dgf*^{hu4581} mutants show decreased phosphodiesterase activity and multiple ectopic calcifications. (A) Depiction of the Enpp1 protein structure; the *dgf*^{hu4581} allele represents a frame shift at amino acid 331, leading to a premature stop codon. (B) Phosphodiesterase I activity is significantly reduced in the lysate of *dgf*embryos. Means ±1 s.d. are shown. Sib(s), wild-type sibling(s). Alizarin-Red (staining mineralized tissue)

and Alcian-Blue (staining cartilage) staining of a sibling embryo (C) and *dgf* mutant (D) at 8 dpf showing extensive ectopic calcification of the notochord, as well as calcification of the neural tube (D, arrowhead). (E) Ventral view of ceratohyal cartilage element of sibling embryo; in mutant embryos, early onset of perichondral ossification (F, red arrowhead), as well as spots of ectopic cartilage calcification (F, black arrowhead), were observed. van Kossa (brown, staining mineralized tissue) and van Gieson (red, staining osteoid) staining on transverse sections of the brain of a sibling (G) and a *dgf* mutant with intracranial calcification (H). Transverse section through the heart region of sibling (I) and mutant (J) embryos, both displaying mineralized cleithra (cl) and basobranchial (bb). Mutants (J) in addition display ectopic mineralization between myocard and epicard (red arrowhead) and within the heart (blue arrowhead). (K) Transverse section at the level of the yolk sac of a sibling; (L) the mutant displays ectopic mineralization of the skin. Axial skeleton at the level of the dorsal fin of a sibling (M) and mutant (N) 4-week-old (4wk) fish. Mutants display not only fusion of vertebral bodies but also of neural and haemal arches (N). Alizarin-Red staining of juvenile sibling (O) and mutant (P and enlarged image of the indicated area in P′). Note the ectopic mineralization at the ethmoid plate cartilage element (green arrowhead in P) and nodules of mineralization at the dentary (black arrowheads in P, P′). (Q) Alizarin-Red staining showing ectopic mineralization (black arrowheads) surrounding the eye of a *dgf* adult mutant (also green arrowhead in P). (R) In the heart of adult zebrafish, no mineralization was visible in siblings. (S) In mutants extensive ectopic calcification was found upon Alizarin-Red staining in the bulbus arteriosus (black arrowhead) but not in the ventral aorta (green arrowhead). Bb, basobranchial; Cl, cleithrum; y, yolk.

Spp1 is known to be expressed by mature osteoblasts, but inflammatory cells and osteoclasts are also known to express high levels of *spp1* (Sodek et al., 2000). Given the absence of other typical osteoblast or cartilage markers at loci of ectopic calcifications, it is probable that the ectopic mineralizations we find in mutants are formed in a passive process that does not involve any osteogenic cell fate change and that expression of *spp1* is a consequence of ectopic calcification.

Treatment with the bisphosphonate etidronate is sufficient to rescue aspects of the dgf phenotype

We have previously shown by generation of double mutants that Enpp1 and Entpd5 are crucial proteins in establishing phosphate homeostasis in zebrafish (Huitema et al., 2012). Here, we wanted to test whether putatively reduced pyrophosphate levels in *dgf* mutants can be rescued by treatment with etidronate, a non-hydrolysable pyrophosphate analog, which has been used previously as a treatment for GACI in humans (Edouard et al., 2011). We applied the compound from 4 to 8 dpf, the time when vertebral bodies start to mineralize and when ectopic mineralizations around the notochord become apparent for the

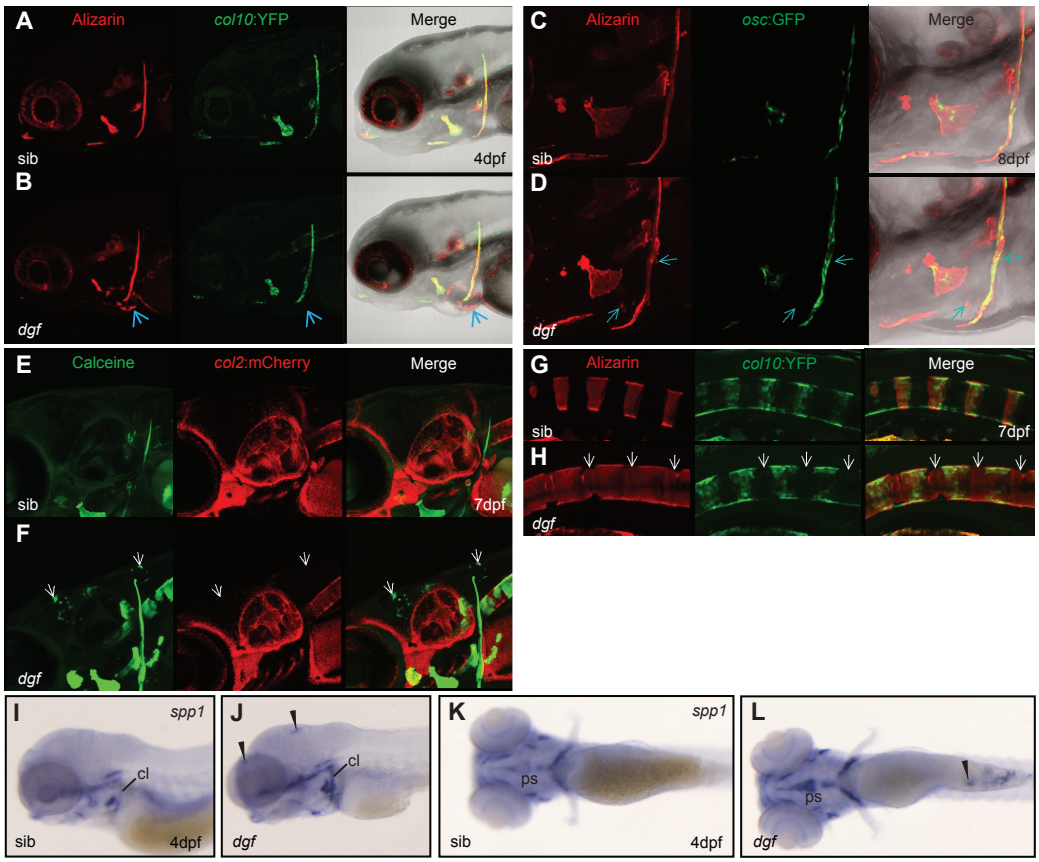

Figure 2: Soft tissue calcifications in dgf mutants probably represent passive calcium depositions. *collagen10a1*:YFP (col10) transgene in sibling (A) and mutant (B). Note that no expression of *collagen10a1*was detected at sites of ectopic mineralization (Alizarin Red) at the heart (B, blue arrow). *osteocalcin*:GFP (*osc*) combined with Alizarin staining in siblings (C) and *dgf* mutants (D), no ectopic expression of *osc* was observed to colocalize with ectopic mineralization in the heart region and pectoral fin (D, blue arrows). Calcein staining marks calcifications in *collagen2a1a*:mCherry (*col2*) transgenic line in wild-type siblings (E) and *dgf* mutants (F). The *dgf* mutant shows ectopic calcifications in the cranium (F, white arrows), however no ectopic expression of *collagen2a1a* was observed. Alizarin staining and *collagen10a1*:YFP transgene expression in the axial skeleton of a sibling (G) and *dgf* embryo (H), ectopic mineralization of the notochord sheet occurs independently of *collagen10a1* expression (white arrows, H). *In situ* hybridization for *spp1* (blue) in siblings (I, K) and dgf mutants (J, L). Note upregulation of *spp1* in mutant bone elements. Further ectopic expression occurs at loci that are frequently affected by ectopic mineralization in mutants (arrowheads in J,L; compare with supplementary material Fig. S1H). cl, cleithrum; ps, parasphenoid; sib, sibling.

first time in *dgf* mutants. At 8 dpf, we measured the mineralized area in the notochord in treated and untreated embryos (Fig. 3A′). No significant difference was detected between the treated and untreated siblings (Fig. 3A, B), but we found a significant reduction of the mineralized area in *dgf* mutants that had been treated with 100 µM etidronate as compared with that of the untreated population (Fig. 3A, B). This experiment indirectly confirms that the phenotype of *dgf* mutants is the result of decreased pyrophosphate levels. Furthermore, it underscores the suitability of the mutant for comparative testing of compounds that have putative inhibitory effects on mineralization.

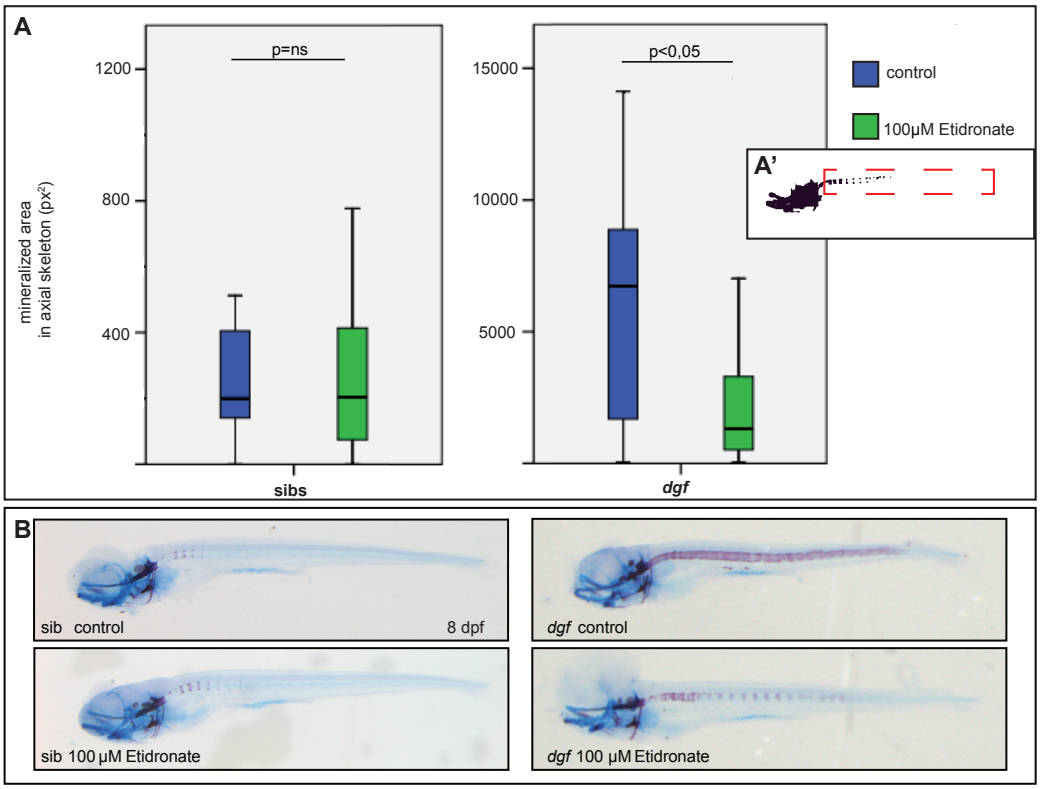

Figure 3: Treatment with the pyrophosphate analog etidronate rescues aspects of the *dgf* phenotype. (A) Measurements (in pixel area) of the mineralized area in the axial skeleton of Alizarin-Red- and Alcian-Blue-stained siblings (sibs; left panel) and mutants (right panel), which were either untreated (blue) or treated with 100 µM Etidronate (green). A′ indicates the region of interest that was measured for the analysis. No significant difference (ns) occurs in siblings (A,B left panels); in treated *dgf* mutants, the mineralized area was significantly reduced when compared with untreated *dgf* mutants (A, B right panels). *n*=50 (*dgf* 100 µM Etidronate); *n*=48 (*dgf* control); *n*= 24 (siblings 100 µM Etidronate); *n*=24 (siblings control).

Regulators of phosphate homeostasis and mineralization show altered expression levels in dgf mutants

It is known that Enpp1 loss-of-function leads to the deregulation of genes that are involved in the regulation of phosphate levels and mineralization, particularly *FGF23* (Lorenz-Depiereux et al., 2010; Mackenzie et al., 2012b) and *Spp1* (Johnson et al., 2003; Aiba et al., 2009). We performed quantitative (q)PCR analysis on RNA that had been isolated from siblings and *dgf* mutants at 7 dpf. We could indeed detect a fourfold upregulation of *fgf23* and, in concordance with this, downregulation of *npt2a*, which encodes a phosphate channel in the kidney and is negatively controlled by FGF23 (Hori et al., 2011) (Fig. 4). Furthermore, we could detect upregulation of *entpd5* and *spp1* (Fig. 4; also compare *in situ* hybridization in Fig. 2I–L), whereas *phex* and *phospho1* transcript levels remained unchanged.

Restricted expression of enpp1 is sufficient to rescue ectopic mineralizations in the notochord sheet

In mouse, it has been shown that *Enpp1* is expressed in a wide array of tissues, and high expression levels occur in bone, liver, kidney and skin (Murshed et al., 2005). Similarly, performing *in situ* hybridization for *enpp1* on zebrafish embryos revealed a ubiquitous expression pattern, and bone elements showed pronounced levels of *enpp1* expression (supplementary material Fig. S2). To our knowledge, no *in vivo* experiments have addressed whether the ubiquitous expression of *enpp1* indicates a general requirement in tissues for the protein to prevent ectopic calcification, or whether Enpp1 acts in a spatially restricted manner. To clarify this, we employed a UAS-galFF overexpression system. For this, we generated a UAS:*enpp1*-ires-TagRFP transgenic line; functionality of the Enpp1 protein was confirmed by the phosphodiesterase assay (supplementary material Fig. S3), and expression of red fluorescent protein (RFP) allowed us to identify *enpp1*-expressing cells. Because mineralization of the notochord sheet provided a reliable readout, we combined our UAS:*enpp1*-ires-TagRFP line with galFF lines that expressed *enpp1* within, or in the vicinity of, the notochord. It has recently been shown that cells within the notochord sheet contribute to the initial mineralization of vertebral centra in teleosts (Grotmol et al., 2005; Bensimon-Brito et al., 2012; Wang et al., 2013). We therefore expected that *enpp1* expression inside the notochord, driven by a transgenic *col2a1a* promoter (*col2a1a*:galFF line; Dale and Topczewski, 2011), would be sufficient to rescue the notochord phenotype of *dgf* mutants. Because the *col2a1a* promoter is also active in vacuolated notochord cells and, to a lower level, in the floorplate and hypochord, we also made use of lines that expressed *enpp1* either exclusively in vacuolated notochord cells (*sagff214a*:galFF) (Yamamoto et al., 2010) or in the floorplate (*shh*:galFF) (Ertzer et al., 2007). In addition, we tested whether expression from blood-vessel endothelial cells (*kdrl*:galFF; formerly known as *flk1*) (Beis et al., 2005) was sufficient to rescue the notochord mineralization. Fig. 5A depicts a scheme of the lines and promoters that were used. Of note, transgenic embryos were indistinguishable in length

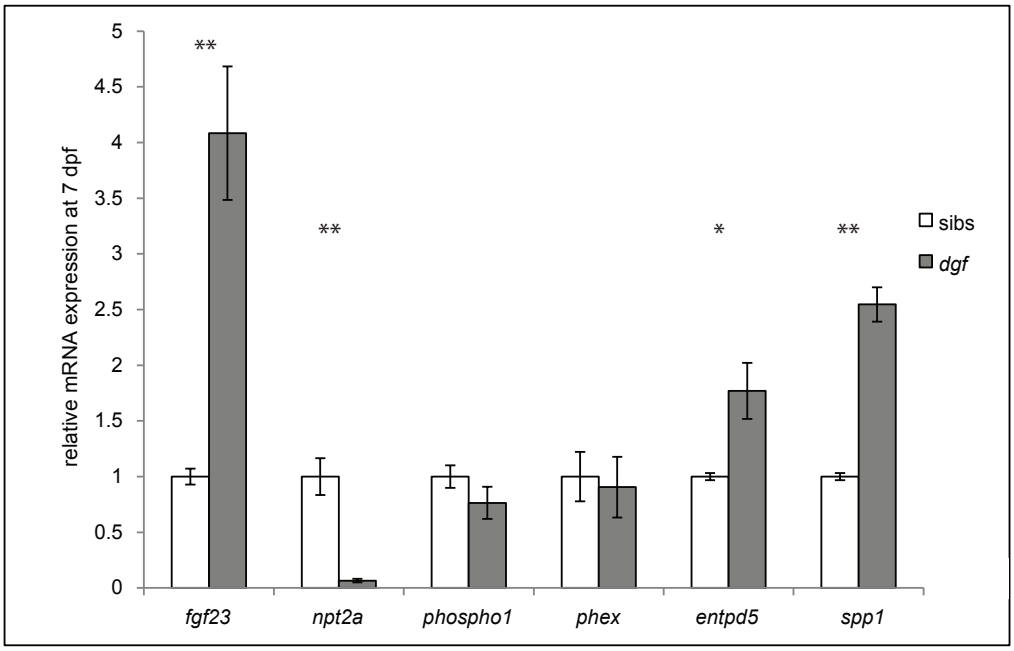

Figure 4: Expression of regulators of phosphate and biomineralization is perturbed in *dgf* mutants. qPCR analysis showing the relative gene expression levels of genes involved in phosphate homeostasis and biomineralization in siblings (sib) compared with those in dgf mutants, normalized to the expression of ef2a. *P≤0.05, **P≤0.01. Means ±s.e.m. are shown.

and morphology from their non-transgenic wild-type siblings. At 9 dpf, we performed Alizarin-Red staining of bone and scored for the presence of ectopic mineralizations in the notochord sheet.

We found that expression not only from the *col2a1a* promoter (Fig. 5B, C) but also solely from blood vessels (Fig. 5B, C‴) and the floorplate (Fig. 5B, C′) was sufficient to completely prohibit ectopic mineralizations in the notochord sheet in *dgf* mutants. Given that the expression of *enpp1* in notochord sheath cells showed substantial rescue, we were surprised to find that *enpp1* expression in vacuolated notochord cells did not prohibit ectopic mineralization of the notochord sheath (Fig. 5B, C″).

These experiments indicate that *enpp1* can act at locations that are remote from its site of expression; however, it remains to be established whether a secreted form of the protein or the diffusion of pyrophosphate are the main contributors to this effect.

Cells that express osteoclastic markers appear at ectopic mineralization sites in dgf mutants

The ectopic expression of *spp1* in the absence of other bone markers at sites of ectopic mineralization prompted us to address the possibility that osteoclasts or macrophages are present at those sites. It has been demonstrated that Spp1 is expressed by osteoclasts (Merry et al., 1993) and macrophages (Giachelli et al., 1998) and is of importance in the cellular response to ectopic calcifications (Steitz et al., 2002).

Staining of Tartrate resistant acid phosphatase (Trap) and the expression of *cathepsinK* have previously been shown to be suitable markers for osteoclasts in teleosts (Witten et al., 2001; Chatani et al., 2011; To et al., 2012). We therefore made use of Trap staining and a *cathepsinK*:YFP reporter line to investigate the presence of osteoclasts in *dgf* mutants. Trap-positive cells have only been reported to appear in zebrafish after 12 dpf (Hammond and Schulte-Merker, 2009). In *dgf* mutants, however, we found Trap staining as early as 4 dpf (Fig. 6A, B). This is the timepoint at which ectopic calcifications first became detectable in mutants, and Trap staining indeed appeared at loci that were associated with ectopic calcifications in embryos of this stage (compare supplementary material Fig. S1K). To establish a direct connection between ectopic calcifications and osteoclasts, we made use of the *cathepsinK*:YFP reporter (which was generated in our laboratory and is described in Bussmann and Schulte-Merker, 2011). Using this line, we confirmed that cells expressing *cathepsinK* colocalized with ectopic calcification sites in *dgf*-mutant embryos (Fig. 6C, D). We could not observe any cells that expressed high levels of *cathepsinK* or stained positive for Trap in sibling embryos (Fig. 6A–D). In contrast to the association of ectopic mineralizations and osteoclasts, we could hardly find any Trap- or *cathepsinK*-positive cells associated with skeletal elements, such as the cleithrum (Fig. 6B, D). This indicates that the expression of *spp1* in those loci is not derived from osteoclasts but probably osteoblasts, which are associated with these skeletal elements (compare Fig. 2A). To correlate Trap staining and *cathepsinK* expression, we combined Trap staining with the subsequent staining of YFP (using an antibody against green fluorescent protein) in *cathepsinK*:YFP-positive embryos. Indeed, we found Trap staining in close association with a subset of *cathepsinK*-positive cells (Fig. 6E–G). The lack of complete congruency of the staining patterns of *cathepsinK*:YFP and Trap can probably be attributed to the fact that YFP is active in the cytosol, whereas Trap accumulates in secretory compartments of the cell (Ljusberg et al., 2005). From 9 dpf onwards, we also found *cathepsinK*-positive cells appearing in association with vertebral elements in *dgf* mutants but, again, not in siblings (Fig. 6H, I). Of note, for reasons of comparability, we focused on observations in the skin of the heart and yolk sac area; we could, however, also observe soft tissue calcification, which was associated with cathepsinK-positive cells, in other loci, such as the brain and the heart itself (not shown). To further validate that these cells were osteoclasts, we combined the *cathepsinK*:YFP line with a reporter line for the macrophage marker *mpeg1* (Ellett et al., 2011) (*mpeg1*:gal4, UAS:RFP). We found that *cathepsinK*-

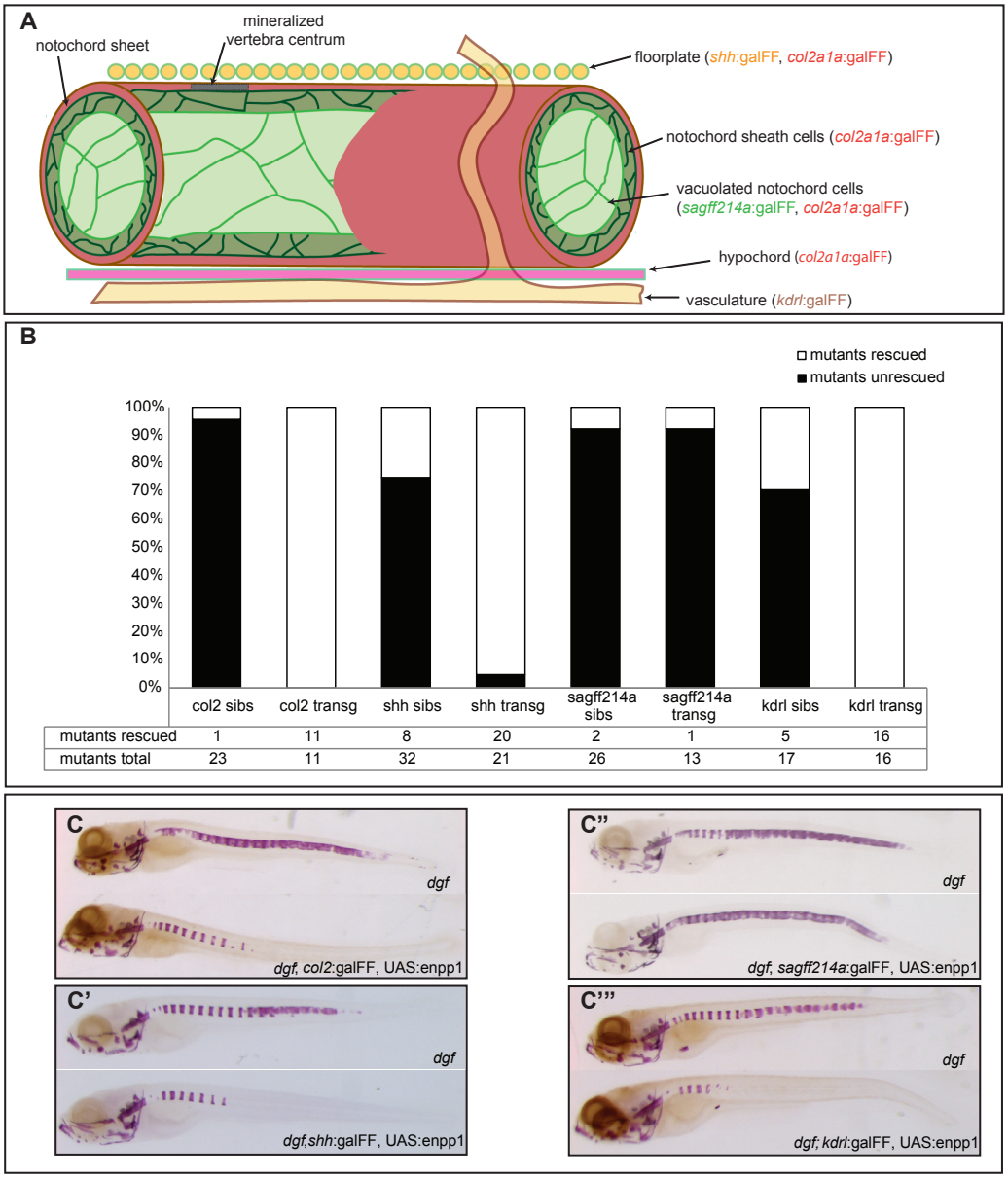

Figure 5: Distal expression of enpp1 is sufficient to prevent ectopic calcifications in the notochord. (A) Overview of the tissue-specific lines that were used in the different rescue experiments. (B) Analysis and quantification of ***dgf*** embryos and ***dgf*** transgenic lines with respect to the notochord phenotype (shown as percentages of the total number of embryos examined). Sibs, siblings; transg, transgenic line. (C–C''') Representative examples of non-transgenic ***dgf*** embryos and their transgenic ***dgf*** siblings for the respective constructs after Alizarin-Red staining.

positive cells in *dgf* mutants expressed comparable levels of RFP to macrophages, for which we could not detect any *cathepsinK*:YFP expression (Fig. 6J). The presence of this macrophage-associated marker on osteoclasts is consistent with their origin from cells of the mononuclear phagocyte lineage. Finally, we tested whether treatment with the inflammatory inhibitors ibuprofen or sulindac, between day 3 and day 6, would have an effect on the severity of the ectopic mineralization phenotype; however, we could not detect any significant results (supplementary material Fig. S4).

Discussion

Here, we provide analysis of the zebrafish mutant *dgf*, which shows ectopic mineralizations in a number of tissues. The *dgf*^{hu4581} allele represents a mutation that leads to an early stop codon within the catalytic domain of the Enpp1 protein (Huitema et al., 2012). This is reflected by a strong reduction of phosphodiesterase activity in the lysate of *dgf* mutants. Similarly, mutations in the phosphodiesterase domain, which have been detected in a number of GACI individuals, have been shown to cause loss of ENPP1 activity (Rutsch et al., 2003).

The zebrafish *dgf* phenotype shows many of the features that have been described in the clinic as a consequence of *ENPP1* mutation in the human syndromes GACI and PXE (Rutsch et al., 2003; Li et al., 2012; Nitschke et al., 2012), and that are observed in mouse upon mutation of *Enpp1* (Johnson et al., 2003; Murshed et al., 2005; Mackenzie et al., 2012b; Li et al., 2013) – most notably, ectopic calcifications in the skin and cartilaginous elements of embryos, as well as in the eye and bulbus arteriosus of juvenile to adult fish. The absence of arterial calcifications in zebrafish embryos can probably be attributed to the morphological differences in the arteries of zebrafish embryos when compared with the medium and large arteries of human and mouse, which have multiple layers of vascular smooth muscle cells. We did, however, find ectopic calcifications in the bulbus arteriosus, which is the outflow tract of the heart in juvenile and adult fish. This structure shows histological similarities to arteries, including a thick circumferential layer of smooth muscles (Hu et al., 2001). The intracranial calcifications along the midline that we observe in zebrafish embryos have, so far, not been reported in individuals with *ENPP1* mutations; however, an individual suffering from hypophosphatasia due to a *KLOTHO* mutation has been diagnosed with calcifications along the midline of the brain (Ichikawa et al., 2007).

Arterial calcification due to loss of ENPP1 function has been demonstrated to be associated with the expression of bone and cartilage markers in *Enpp1*$^{-/-}$ mice (Johnson et al., 2005). By contrast, no expression of bone markers has been observed in the calcified arteries of *Mgp* knockout mice (Luo et al., 1997). Using transgenic marker lines, which allow *in vivo* expression analysis at high resolution, we could not observe such events in any of the ectopically mineralized tissues of *dgf*-mutant embryos. This is in line with find-

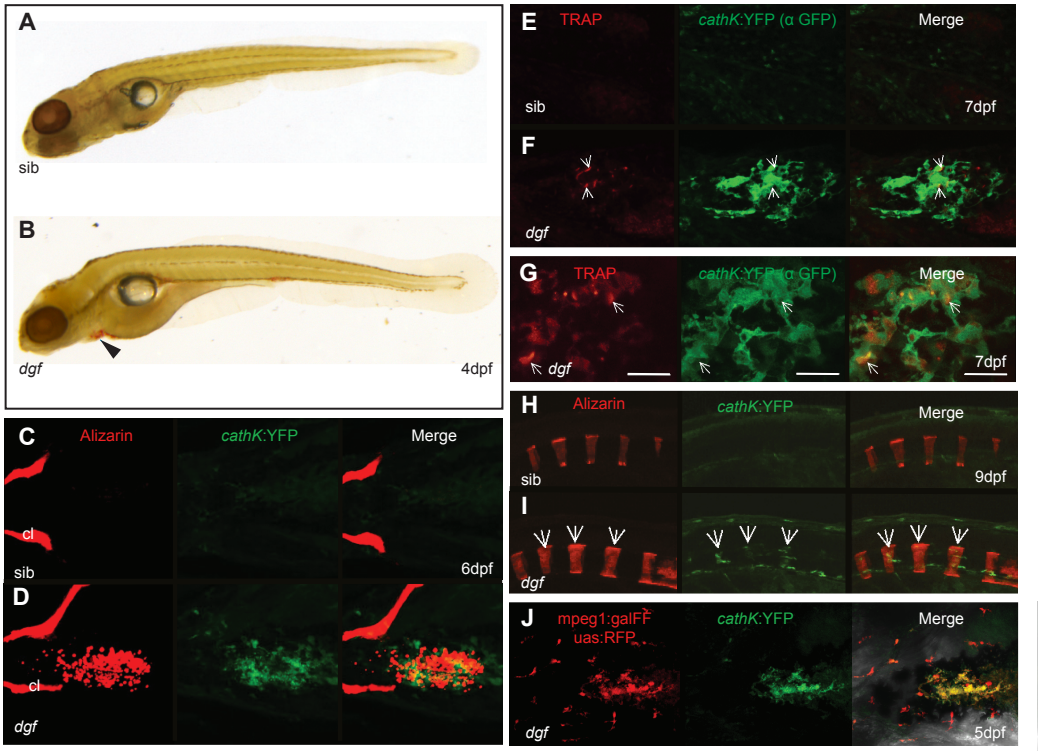

Figure 6: Cells expressing osteoclastic markers appear at ectopic mineralization sites in *dgf* mutants. Staining of Trap in a wild-type sibling (A) and *dgf* embryo (B). Trap staining (red) was visible in a *dgf* embryo at the region of the heart and yolk sac (B, arrowhead). Ventral view of sibling (C) and mutant (D) embryo with ectopic mineralization in the heart and yolk sac region. (C) No *cathepsinK*-positive cells were visible in siblings at this timepoint; (D) mutants showed colocalization of ectopic soft tissue mineralization and *cathepsinK*-positive cells, but no *cathepsinK*-positive cells were aligned to skeletal elements, such as the cleithrum (cl). (E,F) Ventral view of the yolk sac area of embryos that had been stained for Trap and with an antibody against GFP (α GFP). (F) Trap staining appeared in association with *cathepsinK*-positive cells. White arrows indicate loci of osteoclasts with high Trap activity. (G) Higher-magnification image of *cathepsinK* and Trap colocalization in a *dgf* mutant. Scale bar: 20µm. The staining of Trap in E-G is pseudo-colored for improved visibility. In the axial skeleton, *cathepsinK*-positive cells were not visible in siblings (H); however, in *dgf* mutants (I), *cathepsinK*-positive cells appeared from 9 dpf onwards and colocalized with mineralized vertebral bodies. White arrows indicate osteoclasts aligning with vertebral elements. (J) Ventral view of accumulating *cathepsinK*-positive cells in the skin of the heart region. These cells also express the macrophage marker *mpeg1*.

ings by Murshed et al. (Murshed et al., 2005) who postulated that the presence of fibrillar collagen and the removal or absence of pyrophosphate are sufficient for the occurrence of calcifications (Murshed et al., 2005). Indeed, a number of tissues where we observed ectopic calcifications, such as the skin (Le Guellec et al., 2004), cartilage elements and the notochord (Fang et al., 2010; Dale and Topczewski, 2011; Mitchell et al., 2013), as well as the bulbus arteriosus (Hu et al., 2001), are known to be rich in fibrillar collagen. Although we cannot exclude that cellular changes at the level of matrix vesicles play a role in the mineral nucleation at those loci, it is probable that the reduced levels of pyrophosphate and the presence of fibrillar collagen are the main determinants for the induction of ectopic mineralizations. In conclusion, we believe that the correlation of ectopic calcifications with the ectopic appearance of bone and/or cartilage markers might be a common event in the (mammalian) vasculature, but not necessarily other tissues. In line with these findings, Murshed et al. have also reported the absence of osteoblast markers upon the induction of calcification in the dermis of mice (Murshed et al., 2005).

The ability of the bisphosphonate etidronate to rescue the notochord mineralization of *dgf* mutants not only further supports the notion that loss of pyrophosphate is likely to be the determining factor for the *dgf* phenotype but also demonstrates the suitability of this zebrafish model to screen and evaluate other mineralization inhibitors, such as other bisphosphonates or thiosulfate.

Human genetic studies linking *ENPP1* mutations to increased FGF23 levels and subsequent hypophosphatemia (Lorenz-Depiereux et al., 2010) have recently been confirmed in an *Enpp1*$^{-/-}$ mouse model (Mackenzie et al., 2012b). In zebrafish, we observed a similar situation with strong upregulation of *fgf23* and downregulation of *npt2a*, a transporter responsible for phosphate resorption in the kidney under the control of *fgf23* (Hori et al., 2011). By contrast, we could not find differential expression of *phex1*, a regulator of *fgf23* (Rowe, 2012).

Although we cannot provide direct proof in the form of serum phosphate levels, which is a limitation inherent to the zebrafish model, it is likely that *dgf* mutants are hypophosphatemic, a notion that is supported by upregulation of *entpd5*, which we believe complements alkaline phosphatase as a local source of phosphate in the microenvironment of osteoblasts in zebrafish (Huitema et al., 2012). Upregulation of *spp1* in bone elements of *dgf* zebrafish is contradictory to observations that have been made in osteoblast cultures derived from *Enpp1*knockout mice, where *Spp1* expression was decreased (Johnson et al., 2003). Conversely, *Spp1* has been reported to be upregulated in spinal hyperostosis of *twy* mice, which represent another *Enpp1* allele (Aiba et al., 2009). Mechanistically, the upregulation of *spp1* in mutants could derive from the premature maturation of osteoblasts or a negative feedback response.

Using a UAS and galFF-based approach, we provide evidence that *enpp1*expression is crucial in the vicinity of sites of ectopic mineralizations, but does not need to be expressed in the affected tissue directly, as demonstrated by the rescue of ectopic notochord mineralization by the expression of *enpp1* in the floorplate or blood vessels. It is possible that although *enpp1* is widely expressed, it does not need to be provided locally, but can act across tissue boundaries because pyrophosphate and/or a secreted form of Enpp1 can act at loci remote from their site of expression. These factors must be readily diffusible *in vivo*, which is particularly evident from the observation that expression from the floorplate, a single line of cells dorsal to the notochord, is sufficient to completely rescue ectopic mineralizations of the notochord in embryos at 9 dpf. Surprisingly, expression from vacuolated notochord cells alone was not sufficient to rescue the phenotype. This might be explained by the epithelial nature of notochord sheath cells (Dale and Topczewski, 2011), which enclose the vacuolated notochord cells and probably function as a diffusion barrier.

Additionally, we show that early zebrafish embryos do have the potential to generate cells that express the typical osteoclast markers Trap and *cathepsinK*, which are normally only found much later in development (Hammond and Schulte-Merker, 2009). These cells co-localize with ectopic mineralizations in *dgf* embryos and represent a subpopulation of cells that express the macrophage marker *mpeg1*, indicating they are derived from the monocyte-macrophage lineage. The combination of these features strongly suggests that these cells represent a type of osteoclast that develops as a response to ectopic calcifications. In a few other instances, it has been shown previously that ectopic bone and hydroxyapatite fragments can induce osteoclast-like multinucleated giant cells (Krukowski and Kahn, 1982). Arterial calcification has previously been associated with the presence of osteoclasts (Min et al., 2000) and, more recently, Bas and colleagues have suggested an active process of mineral resorption that is mediated by $CD68^{+}$ cells in a rat model for medial artery calcification (Bas et al., 2006). Furthermore, osteoclast-like cells have been described to be associated with calcified atherosclerotic plaques (Jeziorska et al., 1998; Doherty et al., 2002). Although it is difficult to compare these findings directly, we believe it will be important to consider the existence of these soft tissue calcification-associated osteoclasts in humans because they could have important consequences for the treatment of GACI. Bisphosphonates, which are currently being used in the treatment of GACI (Rutsch et al., 2008), might not only prevent further progression of calcifications but, at the same time, also hinder their regression because bisphosphonates are widely known for their capacity to inhibit osteoclast function.

Here, we introduced the *dgf* zebrafish mutant, which represents a valuable model for investigating Enpp1 function and ectopic mineralization, and extend, through the present analysis, our understanding of Enpp1 function *in vivo*. *dgf* mutants show a number of features that are also found in GACI and PXE individuals with ENPP1 mutations; most importantly, mineralization in cartilage elements, skin and the circulatory system. Our data underline the crucial function of phosphate and pyrophosphate homeostasis in the regulation of

biomineralization across species, and we demonstrate the potential of Enpp1 to exert its function across tissues. Lastly, we show that ectopic mineralizations in soft tissue lead to a rapid osteoclastic cellular response, something which has not been fully explored in a murine or human setting.

Materials and Methods

Zebrafish maintenance

Fish were maintained and raised under standard husbandry conditions (Brand, 2002) and according to Dutch guidelines for the care and housing of laboratory animals.

Phosphodiesterase assay

Mutants and siblings (n=10) were sorted based on Alizarin live-staining (see below) or transgene expression. Embryos were sonicated to a homogenous suspension in purified water. The protein concentration was measured (by using a Thermo Scientific Pierce BCA Protein Assay Kit) and diluted to 600 µg/ml. The phosphodiesterase assay was scaled down to a microplate reader format (Biochrom Asys Expert 96) but essentially performed as described previously (Hynie et al., 1975). Results are shown for three independent biological replicates.

Skeletal stainings

Alcian-Blue and Alizarin-Red staining (Walker and Kimmel, 2007) and *in vivo*skeletal staining (Spoorendonk et al., 2008) were performed with minor modifications as previously described. For sectioning, embryos or juvenile fish were embedded in plastic and sections at 6 µm were cut on a microtome. van Kossa (Bancroft, 1996b) and van Gieson staining (Bancroft, 1996a) was performed as described elsewhere.

Trap staining

Trap staining on zebrafish embryos was performed as described previously (Witten et al., 1997; Edsall and Franz-Odendaal, 2010). Briefly, embryos were fixed in 4% paraformaldehyde, washed in H_20 and incubated for 2 hours at room temperature in tartrate buffer [0.2 M acetate buffer (pH 5.5) with 50 mM sodium tartrate dibasic dehydrate]. Embryos were then incubated in Trap staining solution {6% substrate solution [2 mM naphthol-AS-TR-

phosphate (N6000 Sigma) in N,N-dimethylformamide], 90.89% 0.2 M acetate buffer with 100 mM tartaric acid, 3% hexazotized pararosaniline (P3750 Sigma) and 0.01% of 0.1 M $MgCl_2$} for 2 hours.

Imaging

In situ hybridization and whole-mount bone staining was imaged on an Olympus SZX 16 microscope. Sections were imaged on a Zeiss Axioplan microscope. For laser confocal imaging, embryos were embedded in 0.5% low-melting-point agarose and, where applicable, anesthetized with 1.5% Tricaine mesylate. Confocal imaging was performed on a Leica SPE live-cell imaging confocal microscope using ×10 and ×20 objectives. Images were analyzed by using Leica LAS AF lite software.

In situ hybridization and immunohistochemistry

In situ hybridization and immunohistochemistry were performed as described previously (Schulte-Merker, 2002). Templates for *in vitro* transcription of *enpp1*and *spp1* were generated from cDNA. For *enpp1*, a combination of two probes was used for improved detection. Primer sequences are shown in supplementary material Table S1. Antisense digoxygenin-labeled mRNA probes were generated according to standard protocol (Promega SP6, RNA polymerase). Digoxygenin was purchased from Roche.

For detection of YFP on embryos that had been stained for Trap, a rabbit antibody against GFP (1:300, Torrey Pines TP401) and an Alexa-Fluor-488-conjugated antibody against rabbit IgG were used (1:500, Molecular Probes A11034). Embryos were fixed in 4% paraformaldehyde and permeabilized with proteinase K (Promega, 15 μg/ml) for 3 minutes.

Treatment with etidronate

Etidronate (Sigma-Aldrich, P5248) was dissolved in E3 medium (Brand, 2002). Embryos were placed into 100 μM etidronate in E3 medium at 4 dpf. The control group was placed in fresh E3 medium. At 8 dpf, embryos were analyzed by skeletal staining, as described above. Embryos were imaged and the mineralized area in the axial skeleton was measured by using ImageJ. Box plots summarize the results of three independent biological replicates. Groups were compared by using Student's *t*-test.

qPCR

Siblings were separated from *dgf* mutants at 7 dpf by using Alizarin live-staining. RNA was isolated by using the Qiagen RNeasy Kit, and a DNaseI (Promega) digest was performed on the column. RNA quality was checked on an agarose gel and measured with a Nanodrop photospectrometer (Thermo Scientific). Random hexamers were used for reverse transcription (M-MLV reverse transcriptase, Promega). The Primer 3 program was used for primer design. The primers spanned at least one intron to avoid amplification from genomic DNA. Melting temperatures and PCR efficiency were tested, and qPCR was performed using the Bio-Rad MyIQ single-color real-time PCR detection system and software. Reactions contained 12.5 µl Sybr Green (Bio-Rad), 3 µl primer mix (at 1.5 µM), 5 µl cDNA at 10 ng/µl, and 4.5 µl Millipore water. qPCR program: 3 minutes at 95°C, 10 seconds at 95°C followed by 45 seconds at the optimal primer temperature (40 cycles); 1 minute at 95°C and 1 minute at 65°C. cDNA was analyzed from three pooled clutches of embryos for the siblings and mutants. *ef1a* was used as an internal control. Groups were compared by Student's *t*-test.

Treatment with inflammatory inhibitors

Sulindac (Santa Cruz, sc-202823) and ibuprofen (Sigma-Aldrich, I4883) were dissolved in dimethylsulfoxide. From 3 dpf to 6 dpf, embryos were raised in E3 medium with DMSO only or E3 medium with either 5 µM ibuprofen or 10 µM sulindac. These compounds and concentrations have been shown previously to effectively inhibit inflammation in zebrafish embryos (d'Alencon et al., 2010).

The transgenic lines used are described in brief. Details are available upon request.

col2a1a:galFF

We recapitulated cloning of the R2 *collagen type 2a1a* enhancer element and R3 (−116 bp) promoter region, as described previously (Dale and Topczewski, 2011), and the enhancer element was placed upstream of a galFF element (Asakawa et al., 2008) in a miniTol2 vector (Balciunas et al., 2006).

shh:galFF

The sonic hedgehog (*shh*) floorplate-specific promoter construct ar-B has been described previously (Ertzer et al., 2007), and has been cloned into a miniTol2 vector (Gordon et al., 2013). A galFF element (Asakawa et al., 2008) was inserted into this vector.

sagff214a:galFF

sagff214a and its specific expression in vacuolated notochord cells has been described previously (Yamamoto et al., 2010). The line was kindly provided by the laboratory of Koichi Kawakami (National Institute of Genetics, Shizuoka, Japan).

kdrl:galFF

A galFF element was cloned in front of a 7 kb *kdrl* promoter fragment (Beis et al., 2005) within a miniTol2 vector.

UAS:enpp1-ires-tagRFP cmlc2-CFP

A cDNA clone of *enpp1* was generated by using PCR and then sequenced and placed downstream of a 5×UAS element within a Tol2 vector, described previously (Asakawa et al., 2008). An ires-tagRFP element was placed downstream of *enpp1*to enable the assessment of expression levels. Further, a *cmlc2* (Huang et al., 2003) mTurquoise (cyan fluorescent protein) cassette was included for selection purposes.

cathepsinK:YFP

cathepsinK:YFP was generated previously using a BAC CH73-114M20 (Bussmann and Schulte-Merker, 2011).

osteocalcin:GFP, col2a1a:mCherry, col10a1:YFP, mpeg1:gal4

osteocalcin:GFP (*osc*:GFP) (Vanoevelen et al., 2011), *col2a1a*BAC:mcherry (*col2*:mCherry) (Mitchell et al., 2013), *col10a1*BAC:mCitrine (*col10*:YFP) (Mitchell et al., 2013), *mpeg1*:gal4 (Ellett et al., 2011) have been described elsewhere.

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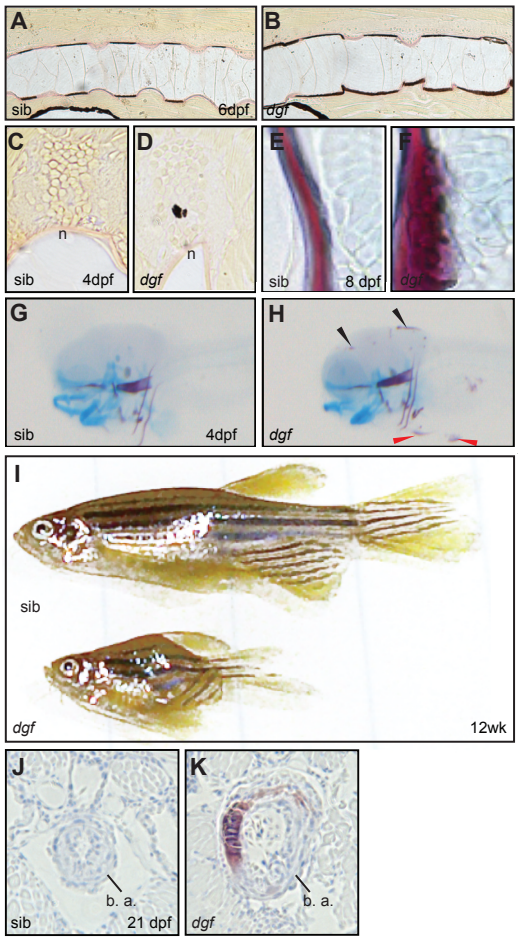
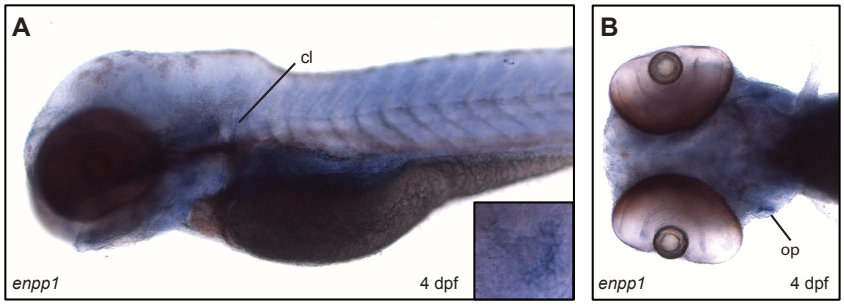
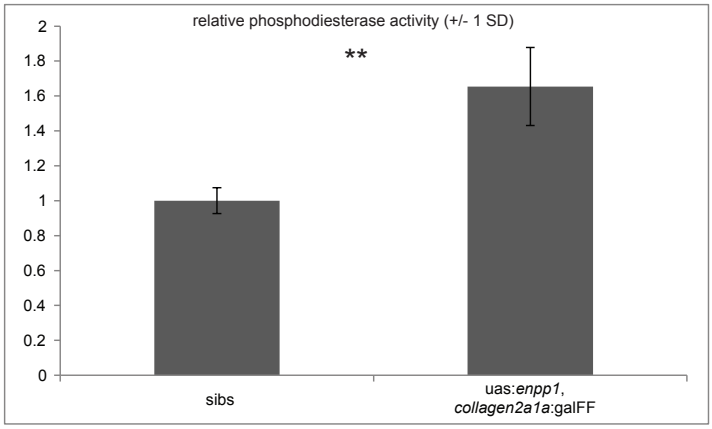


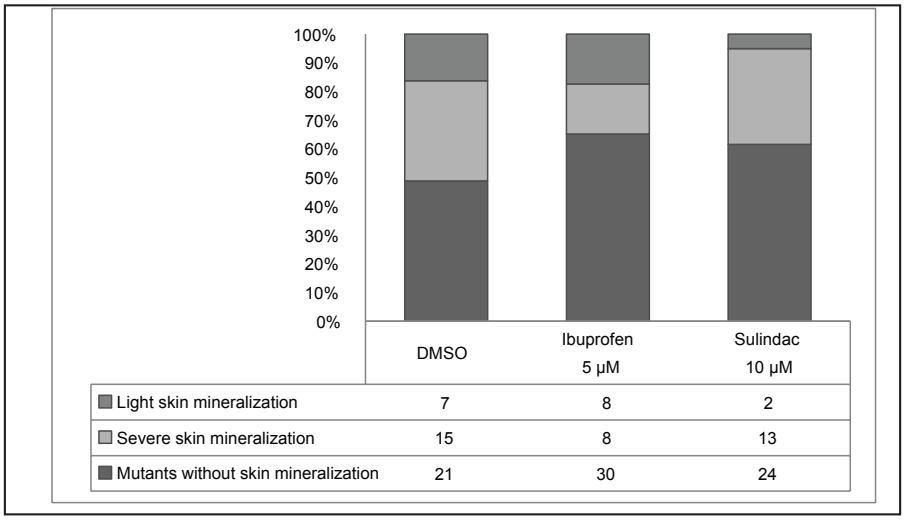
Supplemental Figure 1: Van kossa/van Gieson staining shows segmented mineralization of the notochord sheath in siblings (A), in *dgf* mutants show ectopic calcification of the intervertebral spaces (B). Transverse section through the neuraltube of sibling (C) and mutant with ectopic calcification (D). Alizarin red/Alcian blue stained cleithrum and pectoral fin cartilage of siblings (E) and *dgf* mutant (D) showing ectopic calcification. Overview of Alizarin red/Alcian blue stained sibling (G) and mutant (H). The black arrowheads indicate cranial calcifications, the red arrowheads point at mineralizations of the skin surrounding the yolk sac and heart (H). *dgf* mutants can reach adulthood in rare cases but remain smaller (I). Transverse section of alizarin stained juvenile embryos at the bulbus arteriosos (b. a.), no mineralization is visible in silbing (J), circumferential calcification in the *dgf* mutant (K).



Supplemental Figure 2: Lateral (A) and ventral (B) view of in-situ hybridisation showing the expression pattern of enpp1 at 4 dpf. Note: expression in bone elements such as cleithrum (cl) (A) and the opercle (op) (B, box in A).



Supplemental Figure 3: Phosphodiesterase activity is elevated in uas:*enpp1*, *collagen2a1a*:galFF embryos.



Supplemental Figure 4: Quantification of skin mineralizations in *dgf* mutants upon treatment with inflammatory inhibitors. Numbers summarize four biological replicates. No significance difference between the groups could be detected using a 3x3 Chi-squared test at α=0,05.

Chapter 5

The transcription factor *osterix* controls osteogenesis, but is not required for early axial skeletogenesis in zebrafish

Alexander Apschner, Josi Peterson-Maduro, Ive Logister and Stefan Schulte-Merker

Summary

In mammals *Osterix* has been identified as an osteoblast-specific transcription factor with an essential role in osteogenesis. *osterix* expression in zebrafish has been described in craniofacial bone elements, but expression was not detectable in certain aspects of the early axial skeleton. As this might have been due to technical limitations in the detection methods used in previous studies, and to conclusively investigate *osterix* expression in zebrafish, we generated a BAC-reporter line allowing detailed and reliable detection of gene expression. This approach confirmed that early in zebrafish osteogenesis, *osterix* is expressed in association with mineralized craniofacial, but not axial skeletal structures. These observations were underlined by the analysis of a zebrafish *osterix* allele derived from a TILLING approach. We could detect reduced mineralization in craniofacial bone elements; the primary pattern of the axial skeleton, however, appeared to develop completely normal at the analyzed stage. This confirms our previously stated hypothesis that the first mineralization steps of the zebrafish axial skeleton is *osterix* independent. This is in line with accumulating evidence that there are marked differences in the process of axial skeletogenesis between zebrafish and tetrapods.

Introduction

Expression of a distinct set of genes is underlying the differentiation of cells during development. Tissue specific transcription factors regulate gene expression and therefore act as switches to direct cells towards adapting a certain cell fate and expressing factors permitting them to fulfill their physiological roles. In osteoblasts, RUNX2 (Komori et al., 1997) and OSTERIX (Nakashima et al., 2002) are two key transcription factors that have been identified as crucial for osteoblast differentiation.

Full knock out of *Osterix (Osx)* in mice leads to a severe defect in osteoblast differentiation, marked by loss of expression of typical bone markers such as *Col1a1*, *Spp1*, and *Osteocalcin* (Nakashima et al., 2002; Koga et al., 2005). Mineralization is completely lost in dermal bone elements, while endochondral bone elements show some mineralization of the hypertrophic cartilage but are lacking trabecular and cortical bone. Cartilage structure, however, is normal (Nakashima et al., 2002). Postnatally *Osterix* is also required for osteocyte differentiation (Zhou et al., 2010) and *OSTERIX* mutations in humans have recently been associated to a recessive form of osteogenesis imperfecta (Lapunzina et al., 2010).

In zebrafish *osterix* is expressed in osteoblasts of the craniofacial skeleton as well as in the otic vescle (Spoorendonk et al., 2008; DeLaurier et al., 2010). A previous study in our lab which investigated the role of retinoic acid during the patterning process of the axial skeleton documented that an *osterix* promoter fragment from medaka (*Oryzias latipes*) can recapitulate *osterix* expression as observed by in situ hybridization (Spoorendonk et al., 2008). Intriguingly, while all other mineralized skeletal elements showed *osterix* expression, no signal could be detected in correlation to the first mineralized segments of the axial skeleton. *osterix* expression in the axial skeleton could only be observed much later during the growth of vertebral bodies and their extensions, the neural and haemal arches. This finding let to the hypothesis that in zebrafish there is a population of *osterix*-negative cells facilitating the first segmented mineralization of the axial skeleton (Spoorendonk et al., 2008).

This observation likely has its origin in a different morphological program for the formation of the axial skeleton between teleost fish and tetrapods: In tetrapods it is well established that vertebrae are chondral bone elements derived from the sclerotome of somites (Christ et al., 2000; Gilbert, 2006). Accordingly, knock out of *Osterix* in mice leads to defective mineralization of the vertebral column in mice (Nakashima et al., 2002). In contrast, recent studies in medaka (Willems et al., 2012; Renn et al., 2013), atlantic salmon (*Salmo salar)* (Grotmol et al., 2003; Grotmol et al., 2005; Wang et al., 2013), zebrafish (Bensimon-Brito et al., 2012) as well as other fishes (Arratia et al., 2001), indicate that in those species the first segmented pattern of mineralization occurs within the notochord sheet without a sclerotome derived cartilage template. This may be attributed to the fact that the notochord itself has very similar properties to cartilage (Stemple, 2005) and expresses high levels

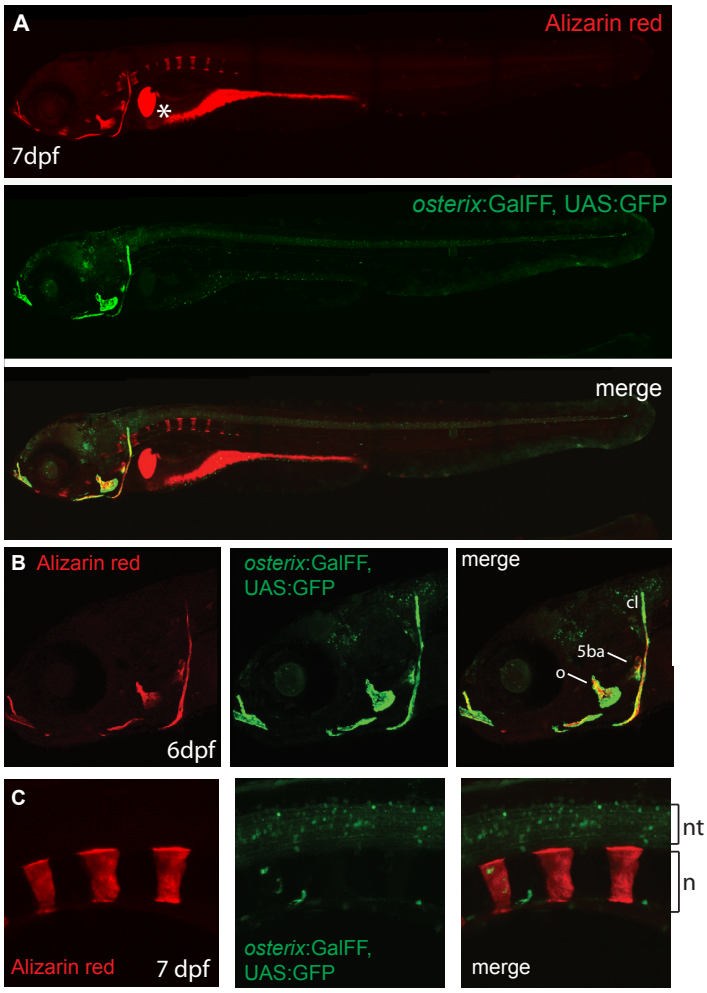


Figure 1: The osterix(BAC):GalFF transgene demarcates osterix expression in association with craniofacial bone elements, but not the axial skeleton (A) Overview of mineralized skeletal elements (red) and *osterix* expression (green); asterisk - alizarin red staining-artefact in the gut, with no correlation to mineralization. (B) osterix is expressed in elements of the head skeleton. (C) *osterix* expression can be observed in the neural tube but not in association with mineralized segments around the notochord. Note low expression of the transgene in the brain and along the neural tube (B and C). Opercle (o), 5th branchial arch (5ba), cleithrum (cl), neural tube (nt), notochord (n).

of the cartilage maker *collagen 2* (Dale and Topczewski, 2011; Apschner et al., 2014). Remarkably, not only is there no distinguished cartilage template for the 'vertebra anlagen' (the chorda centra) in teleosts, but furthermore, in salmon segmented expression of alkaline phosphatase (Grotmol et al., 2005), a marker for mineralizing tissues, has been detected in

cells positioned within the notochord. These so-called notochord sheath cells (for a scheme of these and other notochord structures see chapter 4), might have an osteo-inductive role. Although, there is some functional evidence for an active role of notochord cells in axial skeletogenesis in zebrafish (Fleming et al., 2004), the clear identification of osteo-inductive cells within the notochord of zebrafish is not trivial because in situ hybridizations cannot be used to reliably identify such cells. The notochord sheet seems to function as a diffusion barrier for some reagents used during the in situ procedure; furthermore, structures in the axial skeleton are much smaller in zebrafish than in salmon, therefore alkaline phosphatase activity cannot conclusively be attributed to cells within or outside of the notochord sheet (Bensimon-Brito et al., 2012).

The existence of *osterix*-negative osteogenic cells as postulated by Spoorendonk et al. (2008) can be challenged in two ways: One is a possible limitation in the detection of *osterix* expression – Spoorendonk et al. used a combination of in situ hybridizations and a transgenic reporter using a fragment of the medaka *osterix* promoter. In situ hybridizations are limited because of the before-mentioned reasons, while the 4,1 kb promoter of medaka could lack relevant enhancer elements necessary to drive expression in the early axial skeleton of zebrafish. The second critical point is the lack of functional data regarding the role of *osterix* in zebrafish.

To challenge these issues we have created a bacterial artificial chromosome (BAC)-reporter line (Bussmann and Schulte-Merker, 2011) for *osterix*:galFF which allows reliable detection of *osterix* expression. The BAC clone we used covers a large genomic area spanning both sides of the *osterix* transcriptional start site and thus is likely to contain all important enhancer and regulatory elements. Further, the use of the galFF/UAS system leads to enhanced expression (Asakawa and Kawakami, 2008) of GFP and thereby enables us to detect even low levels of gene expression. Additionally, we have isolated an *osterix* mutant from a TILLING screen, which introduces an early stop codon, likely to result in a non-functional protein, enabling us to assess the role of *osterix* in zebrafish skeletogenesis.

Results

The osterix(BAC):GalFF transgene reveals osterix expression in craniofacial bone elements but not the axial skeleton.

For the generation of an *osterix* reporter line we made use of a BAC covering 68kb upstream and 177kb downstream of the predicted *osterix* transcription start site. After raising injected embryos we analyzed the GFP expression pattern in stable transgenic offspring. As previously described (DeLaurier et al., 2010) we could detect early expression in the otic vesicle (not shown) as well as in association with craniofacial bone elements such

as the cleithrum, opercle and 5th branchial arch (Fig. 1 A, B). Additionally we could find expression in neurons of the brain (Fig. 1 B) and neural tube (Fig. 1 C), as well as in some vacuolated notochord cells (Suppl Fig. 1). Expression in vacuolated notochord cells was only detectable in a subset of the cells and using high laser power and high gain confocal settings. We could not detect any expression associated with mineralized vertebrae center in the axial skeleton (Fig. 1 C) at 7 dpf. In summary, the transgene reliably recapitulated expression previously described by in situ hybridization experiments and other transgenic approaches (Spoorendonk et al., 2008; DeLaurier et al., 2010), and use of the GalFF/UAS system facilitated detection of lower expression levels, such as in neurons or vacuolated notochord cells. Importantly, the absence of segmented expression in the axial skeleton makes makes it likely that *osterix* does not play a role is early axial skeletogenesis.

Generation of zebrafish osterix mutant alleles

To prove that *osterix* is indeed dispensable for the primary segmented mineralization of the axial skeleton in zebrafish, and to confirm its role in in other skeletal elements we sought to create non-functional mutant alleles for this gene. *osterix* is related to Sp1/Kruppel like transcriptional factors (Turner and Crossley, 1999), and contains three zinc finger domains in the C-terminal region and a unique N-terminal and middle region (Fig. 2 A), which has been shown to be necessary for transcriptional activation (Hatta et al., 2006). Conservation of o*sterix* between teleosts and tetrapods is remarkably high. 57 % of the amino acids are conserved and another 18% of amino acids are considered similar between zebrafish and humans (Fig. 2 A). It is therefore likely that information derived from a study on the function of the mouse *osterix* protein domains (Hatta et al., 2006) is also applicable to the zebrafish protein. Since Hatta et al. (2006) identified a transactivation domain in the central part of the protein; truncating mutations upstream of this locus are likely to abolish the transcriptional activity of *osterix* and should therefore allow investigation of *osterix* function in zebrafish. Via a TILLING approach (Wienholds et al., 2003) we could identify an allele – *osx*^{hu5666} – with an A to T conversion leading to an early stop after amino acid 134 of 440 (Fig. 2A, 2B). Additionally we generated a second allele – *osx*^{hu10293} – targeting a locus even closer to the start codon, by use of TALEN technology (Bedell et al., 2012; Zu et al., 2013). Through the TALEN approach, we could isolate a founder with a 7 bp deletion, leading to a frame-shift after amino acid 47 and a predicted premature stop after 4 amino acids (Fig. 2C); importantly both of these alleles are located upstream of the putative transactivation domain (Fig. 2A).

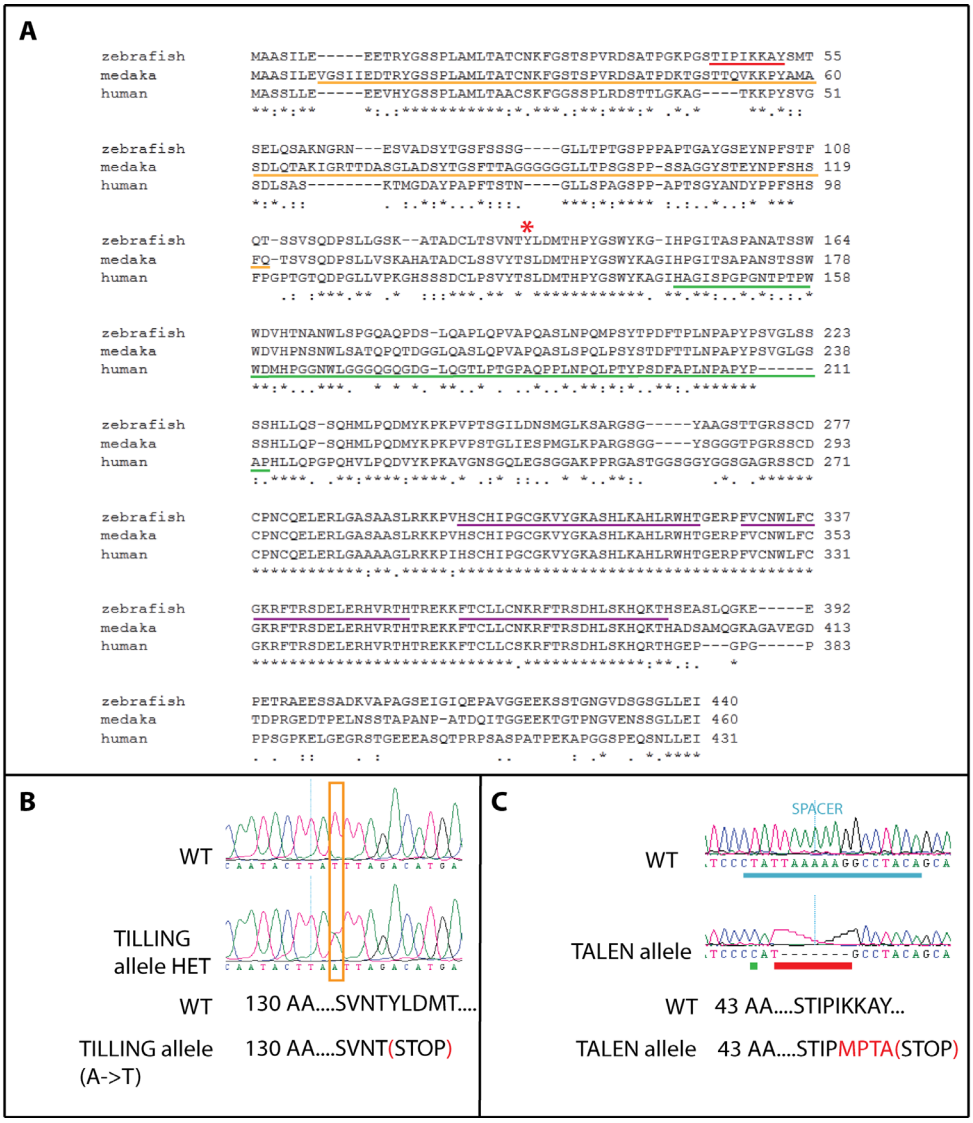


Figure 2: Generation of two zebrafish alleles in the N-terminal region of the evolutionary highly conserved Osterix protein. (A) Alignment of zebrafish, medaka and human Osterix protein sequences. Red bar – TALEN targeting region (spacer). Red asterisk indicates the position of the predicted stop codon in the TILLING allele *osx*^{hu5666}. Orange bar indicates the region lacking in a medaka osterix mRNA variant (Renn and Winkler, 2014). Green bar: transactivation domain identified by Hatta et al (Hatta et al., 2006). Purple bars indicate zinc finger domains. (B) Sequence of the *osx*^{hu5666} allele, with the orange box indicating the heterozygous peak for the base change; shown below is the predicted consequence on the protein sequence level. (C) Sequence of the *osx*^{hu10293} allele: the green bar indicates a base pair change, the red bar indicates the 7 base pair deletion; shown below is the suspected consequence on the protein level.

The transcription factor osterix controls osteogenesis, but is not required for early axial skeletogenesis in zebrafish

We carried out alizarin red stainings for the phenotypic characterization of a potential bone phenotype of *osterix* mutants. We could indeed observe a severe reduction of mineralized craniofacial bone elements in the *osx*^{hu5666} allele (Fig. 3 A, B). Dermal bone elements such as the dentary or parasphenoid were almost completely absent when compared to siblings, and bones such as the opercle and cleithrum were often smaller and misshapen (Fig. 3 A, B; suppl Fig. 2A). Interestingly, the 5th branchial arch, a chondral bone element, consistently has a normal appearance in mutants (Fig. 3 A, B and Suppl Fig. 2A, B). As we have observed in other zebrafish bone mutants, the phenotype of this *osterix* allele shows considerable variation: in embryos with a weak phenotype most bone elements are present, but deformed (suppl Fig. 2A); in embryos with a strong phenotype only remnants of the same bone elements are present (Fig. 3 A, B). This can probably be attributed to variations in the genetic background as zebrafish lines are not kept as inbred lines. A consistent feature in all mutants is the absense of mineralized teeth which arise from the 5th branchial arch (Fig. 3 B; suppl Fig. 2B), whereas the cartilage elements in mutants appear normal (suppl Fig. 2B). Remarkably, segmented mineralization of the notochord sheet and the ensuing formation of chordacentra, appears normal in mutants (Fig. 3 A). To confirm this we performed quantitative measurements of the mineralized area in the axial skeleton. We could indeed not detect a significant difference in mineralization of the axial skeleton between siblings and mutants (Fig. 3 C). The normal mineralization of the axial skeleton and the 5th branchial arch in mutants are also a strong indication that the observed phenotype is not derived from a developmental delay but represents a bone specific phenotype.

Surprisingly we could not detect any phenotype in the TALEN generated allele *osx*^{hu10293}. All bone elements as well as the teeth were present and did not show any apparent abnormalities (suppl. Fig. 2 C, D). We are currently investigating the underlying cause for this observation. One possible explanation could be existence of a transcript variant which excludes the sequence targeted by the TALEN but contains all functional domains. The existence of such a transcript variant was previously shown in medaka (Fig. 2 A) (Renn and Winkler, 2014), however we were not able to confirm a comparable variant in zebrafish.

In summary, the mutant alelle *osx*^{hu5666} shows severe malformation of most craniofacial bone elements and absence of mineralized teeth. Mineralized segments in the notochord sheath, however, appear to be unaffected. These observations are in line with its expression pattern, which suggest a role in formation of craniofacial bone elements and teeth, but not the axial skeleton.

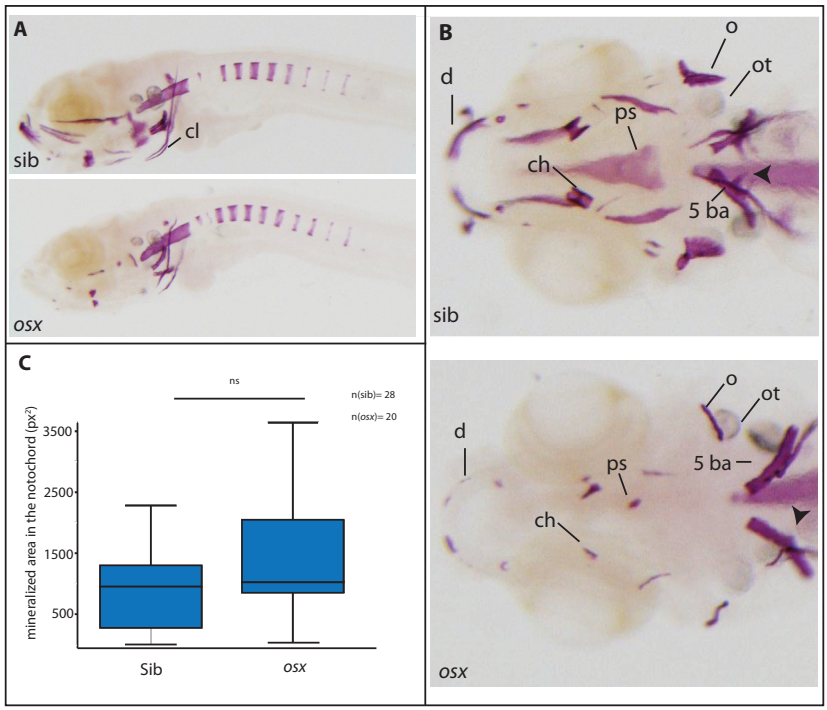


Figure 3: Phenotypic analysis of the *osx*^{hu5666} allele demonstrates a requirement for osterix in the head but not in the axial skeleton. (A) Lateral view of sibling and *osx*^{hu5666} mutant embryos, stained with alizarin red. . Note: reduced mineralization in the head skeleton of the mutant, but normal mineralization in the axial skeleton. (B) Ventral view on the head skeleton of alizarin stained embryos. Mutants show absence or reduced mineralization of bone elements and absence of mineralized teeth (arrowheads). The 5th branchial arch (5ba), appears normal. (C) Quantification of the mineralized area in the axial skeleton. No significant difference between siblings and osterix mutants could be measured. Dentary (d), ceratohyal (ch), parasphenoid (ps), opercle (o), otholits (ot).

Discussion

Osterix is considered to be a bone specific transcription factor, however recently it has been shown to be expressed also in the olfactory bulb and brain of mice (Park et al., 2011) as well as the tip of the teeth (Huitema et al., 2012) and the otic vesicle of zebrafish (DeLaurier et al., 2010). In line with this, the newly generated transgenic line *osterix*(BAC):GalFF, described above, revealed expression in all skeletal elements in the head of zebrafish embryos. Additionally, we could find expression in the otic vesicle, in neurons, and in some but not all vacuolated notochord cells. Expression levels in the latter tissues, however, were considerably lower than in osteoblasts. In contrast, we could not detect any expression in

association with mineralized vertebral centra of the notochord. This is remarkable because other genes that mark craniofacial osteoblasts in zebrafish like *collagen 10* (Apschner et al., 2014) and *enpp1* (unpublished observation) are also expressed in a pattern coinciding with the mineralized segments of the notochord sheet. Similarly, in medaka, *collagen 10* expression precedes mineralization of the notochord sheet and *osterix* only later on becomes expressed in a subset of *collagen 10* positive cells (Renn et al., 2013).

Using TILLING and TALEN approaches, respectively, we have isolated two alleles with mutations that introduce an early stop codon and are predicted to yield truncated forms of the protein. A study on the function of the OSTERIX protein domains (Hatta et al., 2006) indicated that both of our *osx* alleles are located upstream of the transactivation domain and therefore should abolish function of Osterix as a transcription factor.

In one of the two *osterix* alleles, *osx*^{hu5666}, we could indeed detect reduced mineralization of craniofacial bone elements but a normal phenotype of the axial skeleton, as expected from the *osterix* expression pattern. Additionally, we noticed absence of teeth, which normally arise from the 5th branchial arch. Comparably to zebrafish, *osterix* deletion in mice leads a severe reduction but not complete absence of skeletal mineralization (Nakashima et al., 2002). Also a delay in tooth formation has been described more recently (Cao et al., 2012). Interestingly the 5th branchial arch, a chondral bone element in zebrafish seems less effected than dermal bone elements like the parasphenoid or opercle. Also in mice dermal bone elements are more severly affected than chondral bone elements, which is attributed to the prescence of hypertrophic chondrocytes in chondral bone elements. Hypertrophic chondrocytes have the potential to mineralize their surrounding matrix (Nakashima et al., 2002). In zebrafish detailed analysis of bone markers is required in mutant versus wildtype embryos in order to determine if the molecular mechanisms underlying the mutant phenotype are comparable to the situation in mammals. It needs to be considered that over the last years evidence has accumulated that the function of some proteins involved in bone mineralization has shifted between teleosts and tretrapods; for example *collagen 10* is an osteoblast marker in zebrafish (Avaron et al., 2006), whereas in mammals it marks hypertrophic chondrocytes (Karsenty et al., 2009) and *entpd5* is a crucial source of phosphate for mineralization in zebrafish (Huitema et al., 2012), whereas a bone phenotype in *Entpd5* knockout mice has not been reported (Read et al., 2009).

In medaka, similar to our observations, a reduction in ossification of craniofacial bone elements was detected upon ablation of *osterix* positive cells (Willems et al., 2012) or upon morpholino injection targeting *osterix* (Renn and Winkler, 2014). Remarkably, upon ablation of *osterix* positive cells the authors also observed fusion of vertebral bodies which seem to initially segment normally (Willems et al., 2012), something we could not observe in zebrafish. This observeration may be caused by differences in the experimental approach (conditional ablation of cells expressing a bacterial nitroreductase versus stable mutant line), but also may be attributed to differences in the way medaka and zebrafish form their

axial skeleton: neural arches, which are *osterix*-positive bone elements in medaka as well as in zebrafish (Spoorendonk et al., 2008; Renn et al., 2013), form already at day six in medaka (Willems et al., 2012) whereas they only appear after day 12 in zebrafish, about 7 days after the first mineralized segements within the notochord sheet are visible. Generally the observation that medaka still shows segmented formation of vertebral bodies upon deletion of *osterix* positive cells indicates that also in medaka an atypical population of osteogenic cells is mediating the first segmentation of the axial skeleton. While in medaka these cells are attributed to somitic tissue (Inohaya et al., 2007; Renn et al., 2013), in zebrafish there is some evidence that these osteoinductive cells may reside within the notochord (Fleming et al., 2004; Bensimon-Brito et al., 2012), similar to the what has been proposed for the atlantic salmon (Grotmol et al., 2005; Wang et al., 2013). The finding, that *osterix* is dispensable for the early segmented mineralization of the axial skeleton, gives support to the hypothesis that an atypical population of osteogenic cells, presumambly residing inside the notochord, promotes this process in zebrafish.

Surprisingly we did not detect any specific phenotype in the *osx*^{hu10293} allele. We could up to this point in time not conclusively identify a cause for this observation. A possiblity is the existance of a transcript variant, that could contain all functional domains but excludes the region targeted by our TALEN. Such a variant has recently been described in medaka (Renn and Winkler, 2014). We could, however, not yet molecularly confirm the existense of such a transcript, which could be due to low abundance of the this variant.

In summary our data show that comparable to mammals, *osterix* in zebrafish is essential for proper skeletogenesis. Our work further confirms the hypothesis that the segmented mineralization of the notochord, which is the anlage for the future axial skeleton, is generated in an *osterix*-independent manner in zebrafish. These findings are in line with the accumulating evidence that there are marked differences in the process of axial skeletogenesis between teleosts and tetrapods and even seem to vary between teleost species like medaka and zebrafish.

Material and methods

BAC recombination

The BAC reporter line was created using a BAC CH211-51D23, covering 117kb downstream and 68kb upstream of the predicted start codon of osterix. BAC recombination was carried out as described previously (Bussmann and Schulte-Merker, 2011) with the following recombination primers

Primer fw HA1 Osx:Gal4:

5’-CAGCTCTCCTCTCCCGCTTTTGGATTGACCCTCACTGGACTGCTTCC TCCACCATGAAGCTACTGTCTTCTATCGAAC-3’

Primer rv HA2 OsxKanR:

5’-GCAGCTGTGAGATCGCAGTGAGTTTTCCGTACCTCCAGAATCGACGCGG CTCAGAAGAACTCGTCAAGAAGGCG-3’

Aligment of the protein sequences was generated on the UNIPROT website: www.uniprot.org (accessed July 8 2014).

Talens were designed using the web tool Mojo Hand on http://www.talendesign.org/ (accessed July 8 2014) and assembled using the GOLDY Talen tool kit (Bedell et al., 2012).

Sequencing and genotyping primers for the osterix alleles

osx^{HU5666} allele was genotyped with primers fw 5’- AAAAACGGACGCAATGAAAG-3’,

rv: 5-GCAGTGGTGCTTGGAGACTA-3’

The *osx*^{hu10293} allele was genotyped with primers fw 5’- TGAATGTCGCATGCATAACA-3’,

rv 5’-AACTCGGCATTTGAGGATG-3’.

For genotyping of *osx*^{hu5666} also the following KASPar (Cuppen, 2007) primers were used:

ALT: 5’-GAAGGTGACCAAGTTCATGCTGATTGCCTTACAAGCGTCAATACTTA-3’

ALA: 5’-GAAGGTCGGAGTCAACGGATTGCCTTACAAGCGTCAATACTTAA-3’

C1 : 5’-AGCCGTAAGGATGCGTCATGTCTA-3’

Skeletal stainings and quantification

Skeletal stainings was carried out as described in chapter 3. Quantification of mineralization in the axial skeleton was carried out as described in chapter 4 (Apschner et al., 2014).

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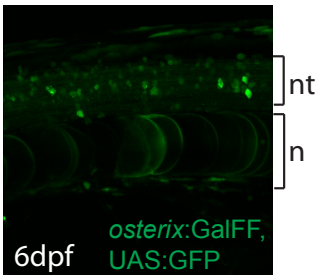
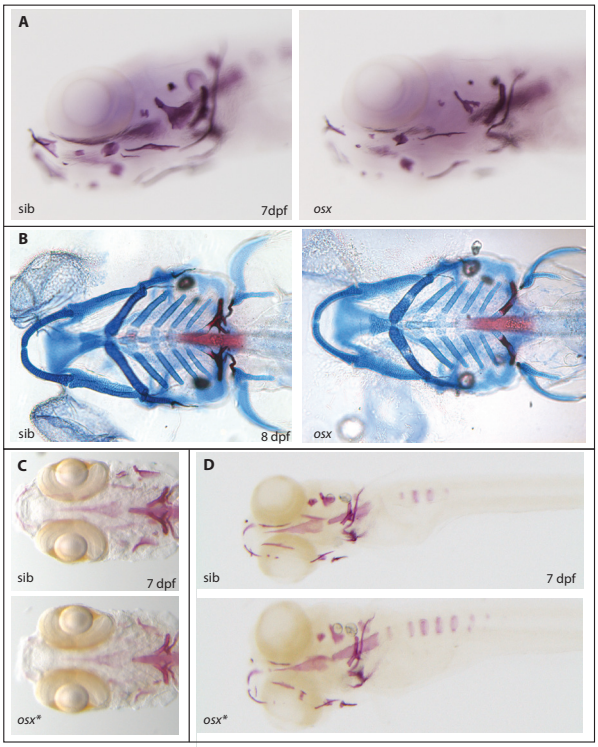


Supplementary Figure 1: Osterix expression can be found in vacuolated notochord cells. neural tube (nt), notochord (n).



Supplementary Figure 2: (A) Example of a less severe manifestation of the phenotype in the *osx*^{HU5666} allele. (B) Morphology of the head cartilage (blue) is not affected in the *osx*^{hu5666} allele. (C) and (D) no phenotype can be detected in the *osx*^{hu10293} (*osx**) allele.

Chapter 6

General discussion

General discussion

Skeletal tissues are already present in early vertebrates and consequently processes for the formation of the skeleton are evolutionary derived. There are, however, significant differences between the skeletal tissues of teleosts such as zebrafish and mammals (Apschner et al., 2011). These differences offer the opportunity to gain novel insights, but also make it necessary to (re-) investigate the key steps for bone formation and confirm if and to which degree they have been conserved over an evolutionary distance of 400 million years (Hedges et al., 2006). Only with a comprehensive understanding of the similarities and differences in skeletogenesis between zebrafish and mammals it is possible to use the potential that zebrafish offer as a model for biomedical research, and to make sensible extrapolations towards human bone biology.

The work summarized in this thesis investigated two major mechanisms regulating skeletogenesis. One is the control of biomineralization via the phosphate/pyrophosphate axis, the other the control of osteoblast differentiation via *osterix,* an osteoblast specific transcription factor. This thesis also introduces three novel zebrafish mutants: work on *no bone* (*nob*) lead to the identification of Entpd5 as a novel phosphatase critical for biomineralization in zebrafish by influencing the extracellular phosphate/pyrophosphate homeostasis; analysis of *dragonfish* (*dgf*) identified a mutant allele for Enpp1, a protein well known for its crucial role in supplying extracellular pyrophosphate; and lastly we describe two novel mutant alleles for *osterix* that have been generated by reverse genetic approaches.

Not all bones are created equal

To introduce fish as a model for skeletogenesis in **chapter 2** we discuss the variations of skeletal tissues in teleosts and how they relate to the tetrapod skeleton. Whereas in mammals mineralized skeletal elements are cellular and their matrix is either mineralized by osteoblasts or hypertrophic chondrocytes, a wider variation of skeletal tissues is present in teleosts. There are, for example, a number of cartilaginous tissues that can also be mineralized. Furthermore, bone in fish can be acellular, meaning that osteoblasts secrete their matrix in a polarized manner, so they never become engulfed by bone matrix and they never differentiate into osteocytes. This has important consequences, as for example in mammals osteocytes are thought to act as mechanosensors that direct bone remodeling upon experiencing mechanical load. Additionally, while many osteogenic factors have been shown to be present in zebrafish as well as in mammalian skeletal tissues (Li et al., 2009), there are also some differences that have been recently uncovered. For example, *collagen 10* which is a unique marker for hypertrophic chondrocytes in mammals was found to be expressed in osteoblasts in zebrafish (Avaron et al., 2006; Li et al., 2009). Another peculiar difference between zebrafish and tetrapods regards the role of the

notochord in axial skeletogenesis of zebrafish. Whereas in avians and mammals it is well accepted that vertebrae are formed via chondral ossification from somite derived tissue in a resegmentation process (Christ et al., 2000; Gilbert, 2006), in teleosts the first mineralized pattern appears within the notochord sheet and only later somite-derived tissue contributes to the vertebral body and the formation of hemal and neural arches (Arratia et al., 2001; Fleming et al., 2004; Bensimon-Brito et al., 2012). Lastly, in mammals haematopoetic bone marrow provides close spatial association of osteoclasts, which are of haematopoetic origin, and osteoblasts. This proximity facilitates reciprocal regulation between both cell types. Teleosts, however, do not have bone marrow, and while Rankl is an important factor for osteoclast differentiation in both mammals and teleosts (To et al., 2012), the origin of osteoclastic cells yet needs to be elucidated.

Phosphate/Pyrophosphate homeostasis is a key factor in vertebrate biomineralization, but different enzymes may be the major regulators

In **chapter 3** we describe the phenotypes and provide functional characterization of the "no bone" (*nob*) mutant, which completely lacks mineralization, and the "dragonfish" (*dgf*) mutant, which shows ectopic mineralization in multiple tissues. By positional cloning the respective causative mutations could be attributed to *entpd5* (*nob*) and *enpp1* (*dgf*). The well-established role of *enpp1* allowed us to characterize *entpd5,* as a novel and critical factor in the regulation of phosphate/pyrophosphate homeostasis in zebrafish. Whereas in zebrafish embryos *entpd5* expression is bone specific, in mouse preliminary data showed a much broader expression for Entpd5, which however, also included skeletal elements (unpublished observation). Interestingly, a full knock out of *Entpd5* in mice has been described without any notion of bone abnormalities (Read et al., 2009) and also preliminary analysis of knock out mice generated in our lab only revealed a subtle bone phenotype (unpublished observation), not comparable to the dramatic effect that the *entpd5* mutation causes in zebrafish. Future research will be necessary to clarify whether Entpd5 (or other Entpdases) indeed also plays a role in skeletogenesis of mammals, or if its functions have completely shifted between zebrafish and mammals as implied by some studies on mammalian *Entpd5* (Read et al., 2009; Fang et al., 2010).

Given the central and conserved role that phosphate/pyrophosphate balance has in the regulation of biomineralization (Murshed et al., 2005), this difference between the zebrafish and mouse phenotype indicates that in mice other factors than ENTPD5 are relatively more important for establishing the conditions permitting mineralization. In the induction of bone mineralization in mammals, alkaline phosphatase (TNAP) has a comparable role to *entpd5* in zebrafish (Millán, 2012). Because patients with TNAP mutations are hypophosphatemic (Weiss et al., 1988) it has long been believed that lack of phosphate is responsible for their defective bone mineralization. More recent experimental work, however, has shown that it is not only the supply of phosphate by TNAP but the local removal of pyrophosphate that

is the critical determinant for defective mineralization (Hessle et al., 2002; Murshed et al., 2005).

While our experiments regarding *entpd5* could clearly establish an important role in the phosphate/pyrophosphate homeostasis of zebrafish, future work should consider the possibility that *entpd5* could act via additional mechanisms, rather than only supplying phosphate by metabolizing nucleoside diphosphates (NDPs), for the following reasons:

- Only partial rescue can be achieved by supplying excess phosphate.

- Mineralizations are completely absent in *nob* mutants, although other enzymes with extracellular nucleotidase activity are present. For example, TNAP is also capable to metabolize NPDs (Say et al., 1991; Millan, 2006).

- Double mutants for *entpd5* and *enpp1* not only have restored bone mineralization but an ectopic mineralization phenotype, which would be unexpected in a hypophosphatemic organism. Importantly, skeletal elements in *nob*, *dgf* double mutants appear normal. This makes it likely that the main mode of function of *entpd5* is to act on the phosphate/pyrophosphate ratio, although an effect on osteoblasts function via purinergic signaling mechanisms (Gartland et al., 2012) cannot not be completely ruled out.

Although this is somewhat speculative, Entpd5 might fulfil a role in zebrafish that is comparable to TNAP in mammals – not only to provide phosphate but also be directly or indirectly involved in the removal of pyrophosphate. To our surprise, while in mammals alkaline phosphatase is an established marker for osteoblasts and hypertrophic chondrocytes (Fig. 1A), we could never detect Tnap activity in a bone specific pattern in early zebrafish embryos (unpublished observation and Fig. 1B). Only much later in development a significant association with osteoblasts was detectable (Fig. 1B). Unfortunately, little data is available regarding the role of alkaline phosphatase in zebrafish skeletal development; one study by Fleming et al. 2004 could detect *Tnap* activity at 20 dpf in elements of the head skeleton but failed to detect it in the axial skeleton (Fleming et al., 2004). Neither in fish (Huitema et al., 2012) nor in mice (Murshed et al., 2005) excessive phosphate levels are sufficient to induce mineralization. It has therefore been postulated that removal of pyrophosphate is a physiological requirement for mineralization to occur (Murshed et al., 2005). Since *enpp1* is expressed in zebrafish osteoblasts which implies the presence of pyrophosphate (Apschner et al., 2014), the question remains which enzyme provides pyrophosphatase activity in bone elements in the absence of Tnap and promotes mineralization in embryos.

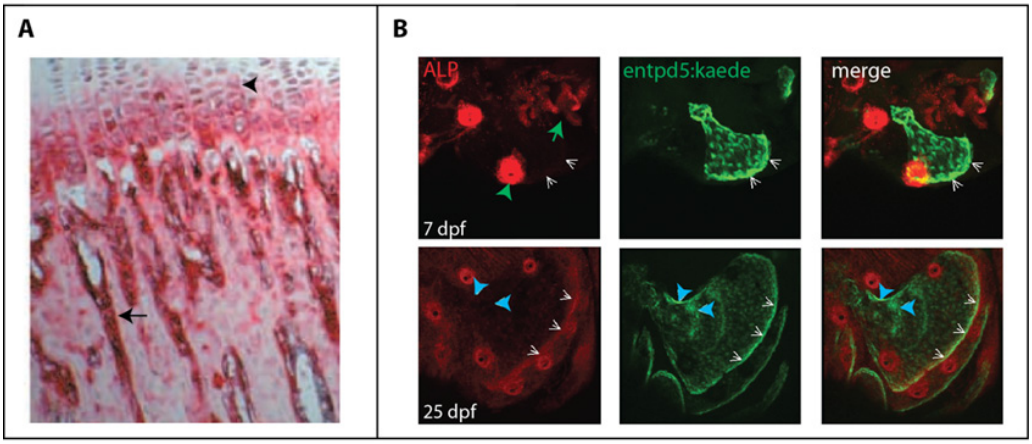


Figure 1: Alkaline phosphatase marks osteogenic cells in mammals, but is not detectable in early zebrafish skeletogenesis. (A) Staining of endogenous alkaline phosphatase activity in the growth plate of a rat tibia, showing high alkaline phosphatase in the area of maturing and hypertrophic chondrocytes (arrowhead) as well as in the ossification zone (arrow)(Miao and Scutt, 2002). (B) In zebrafish alkaline phosphatase activity can be observed in neuromasts (green arrowhead) and gills (green arrow) at 7 dpf. No activity is detectable in osteoblasts marked by entpd5:kaede (white arrows indicate the growth front of the opercle). At 25 dpf alkaline phosphatase activity correlates to the growth front of the opercle (white arrowsheads), but other osteoblasts do not show significant alkaline phosphatase activity (blue arrowheads).

In addition to the before mentioned points, *entpd5* is a good candidate to act directly on pyrophosphate, not only because it is expressed at the right place and time, but also because other apyrase proteins have been shown to use pyrophosphate as a substrate (Čurdová et al., 1982). Unfortunately, to our knowledge most studies on entpdase-substrates focused on different nucleotide derivates, and did not test pyrophosphate as a potential substrate. For this reason a biochemical study for a possible pyrophosphatase activity of zebrafish *entpd5* should be considered. Given the importance of TNAP (Millán, 2012) for bone mineralization in humans, it would also be worthwhile to investigate its role in zebrafish skeletogenesis by generating a zebrafish mutant.

In case a role for *Entpd5* in mouse bone development, can be established, it should be considered to generate *Entpd5*, *Tnap* double knock out animals. Interestingly, *Tnap* knock out mice are born with normally mineralized bones and only later develop skeletal deficiencies (Narisawa et al., 1997). Again, since the removal of pyrophosphate is an essential requirement for biomineralization, the question remains which enzyme mediates pyrophosphate breakdown in *Tnap* knock out mice and mediates bone mineralization

before birth. Other enzymes with a pyrophosphatase activity similar to *Tnap* have so far not been confirmed *in vivo*, but cannot be ruled out (Anderson et al., 2005; Murshed et al., 2005).

In summary, **chapter 3** describes a spectacular zebrafish bone mutant that is viable until juvenile stages without mineralized bones. This is an observation which would be impossible to make in a terrestrial model system due to insufficient mechanical support of the body. Importantly, this chapter shows that, while (not surprisingly) there is a conserved role for phosphate/pyrophosphate homeostasis in the regulation of biomineralization, the relative contributions of enzymes seem to be shifted from zebrafish to mammals in the sense that at knock out of *Entpd5* in mice does not lead to a comparable skeletal phenotype.

A fish model exhibiting pathologic mineralizations

In **chapter 4** we provide a detailed description of the consequences resulting from *enpp1* mutation in the *dgf* mutant. The phenotypical characterization outlines significant parallels between the *dgf* mutants and human patients affected by mutations in *ENPP1*. Also, while the population of patients harboring mutations in *ENPP1* is relatively small, ectopic mineralizations are not uncommon, particularly in patients with impaired kidney function (Giachelli, 2009). Although a mutant for *trmp7* has been described to develop kidney stones (Elizondo et al., 2010), *dgf* is the first zebrafish mutant to show ectopic mineralizations in wide array of tissues, most importantly also within the cardiovasculature, which in patients is the most critical tissue to be affected by ectopic mineralizations (Giachelli, 2009). We could provide a proof of concept for the suitability of *dgf* as a tool for screening mineralization inhibitors, using the bisphosphonate Etidronate, which may set the stage for screening larger arrays of mineralization inhibitors in the future.

The discovery of new mineralization inhibitors is a valid goal because an important insight from the work presented in this chapter was the rapid occurrence of osteoclastic cells in response to ectopic calcification. As we elaborated, this may have consequences for the treatment of GACI patients. Currently, treatments with bisphosphonates may not only suppress promotion of the pathologic calcifications but at the same time harm osteoclastic cells mediating the regression of ectopic calcifications. Interesting questions that arise from this work for future research are:

- To what degree are these osteoclastic cells also present in mice and humans?
- Are the osteoclastic cells capable to indeed reduce ectopic mineralizations?
- Which cells and molecules are mediating the first response to an ectopic soft tissue calcification?

- Are there differences between osteoclastic cells appearing at pathological calcification sites and osteoclasts associated to bone elements in wild-types?

In the *dgf* mutant a current obstacle for experiments to address the latter questions is the relatively high variation in the occurrence of ectopic mineralizations in the skin as well as the notochord. This makes experiments very time consuming because not all clutches of embryos are suitable for analysis. Possibly this can be overcome by growing embryos in conditions that promote ectopic calcifications such as high phosphate or addition of mineralization promoters like warfarin (unpublished observation). If these technical issues can be solved, *dgf* could be an interesting model for the investigation of cellular consequences of ectopic calcifications in tissues but also related processes such as foreign body reactions. An interesting opportunity also has arisen from the recent advances allowing efficient reverse genetic approaches in zebrafish like TALENs and CRIPRs (Hwang et al., 2013; Jao et al., 2013; Zu et al., 2013). These tools now allow generating mutant alleles for other mineralization inhibitors, as well as proteins involved in the directing the cellular response to ectopic mineralizations like SPP1. Zebrafish provide unique opportunities here, because ectopic calcifications can easily be visualized on a whole embryo level. This allows analysis of cellular events occurring in association with ectopic mineralizations with a temporal and spatial resolution that cannot be achieved in mice.

Segmented mineralization of the axial skeleton is promoted by an osterix-negative osteogenic cell type

In contrast to the experimental work in chapters three and four, which investigated factors that regulate bone mineralization by influencing phosphate/pyrophosphate levels, **chapter five** investigated the role of the transcription factor *osterix* in zebrafish. In mammals, *Osterix* has been shown to be a critical transcription factor regulating the differentiation of osteoblasts and thereby integrating signals from morphogenic factors that orchestrate the patterning of the skeleton. The work presented in chapter five indicated that this is also true for the craniofacial skeleton of zebrafish. Visualizing *osterix* expression by a novel *osterix*(BAC)-reporter line we confirmed that *osterix* is expressed in craniofacial skeletal elements. Not surprisingly we found that a mutation in *osterix* indeed leads to reduced mineralization or deformation of craniofacial bone elements as well as loss of mineralization in the teeth, while cartilage elements develop normally. In contrast, we could not detect any *osterix* expression associated to the mineralized segments of the notochord sheet and these mineralized segments were also not affected upon mutation of *osterix*. This finding confirms the hypothesis (Spoorendonk et al., 2008) that the primary segmentation of the axial skeleton in zebrafish is promoted by a population of osteogenic cells which does not require *osterix*. Further, it is in line with recent evidence that in contrast to birds or mammals (Christ et al., 2000; Gilbert, 2006), in zebrafish the cells promoting the primary segmented mineralized pattern of the axial skeleton are not typical osteoblasts derived

from the sclerotome, but likely to reside within the notochord sheath (Fleming et al., 2004; Bensimon-Brito et al., 2012).

These cells likely represent a form of intermediate skeletal tissue expressing the cartilage marker *collagen 2* which is present in all notochord sheath cells (Dale and Topczewski, 2011; Apschner et al., 2014) as well as *collagen 10* which is expressed in association with mineralized segments of the axial skeleton (Apschner et al., 2014) and in zebrafish is considered an osteoblast marker (Avaron et al., 2006; Li et al., 2009). While previous work in our lab has indicated that retinoic acid plays an important role for the patterning of the first segmented mineralization in the zebrafish axial skeleton (Spoorendonk et al., 2008), the precise mechanisms and a possible relation to the somite patterning still need to be established in future research.

Conclusion

This thesis has clarified a number of important issues for the future use of zebrafish as a model for skeletal research:

1. Phosphate/pyrophosphate homeostasis is a conserved mechanism underlying the regulation of biomineralization in both teleosts and tetrapods.

2. The relative contributions of enzymes to the respective phosphate and pyrophosphate levels may have shifted during evolution.

3. Zebrafish constitute a suitable model for addressing biomedical questions regarding pathologic calcification and offer unique opportunities in comparison to murine models.

4. *osterix* is functionally conserved as a key factor in skeletal development between teleosts and tetrapods.

5. A population of cells negative for *osterix*, but positive for *collagen 10* is likely to promote early segmented mineralization of the axial skeleton in zebrafish.

In summary this indicates, that while the major mechanisms in bone formation are conserved between teleosts and tetrapods, future research should also take into account that variations within those processes are not uncommon. Similar observations have been made by our lab in case of lymphangiogenesis (van Impel et al., 2014), where zebrafish mutants for a number of mammalian key transcription factors did not yield any lymph specific phenotypes, morphological processes and other molecular regulators however are

conserved (van Impel and Schulte-Merker, 2014). Such differences between model systems should not be seen as a hindrance, but as an opportunity to gain an in depth understanding of how different variations of a process can still be robust and result in a functional organism.

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Nederlandse samenvatting

Botziekten komen in onze vergrijzende samenleving steeds vaker voor. Een bekend voorbeeld is osteoporose (botontkalking), dat veroorzaakt wordt door een verstoring in de balans tussen osteoblastcellen die bot aanmaken en osteoclastcellen die het afbreken. Dit leidt tot een lagere botdichtheid en een hoger risico op botbreuken, wat ernstige gevolgen kan hebben voor de levenskwaliteit van ouderen.

Bij andere aandoeningen, zoals nierfalen en bepaalde erfelijke ziekten, kan het voorkomen dat bot wordt aangemaakt op plekken waar dit eigenlijk niet mag gebeuren. Ook hier kunnen de gevolgen ernstig zijn. Zo leidt verkalking van slagaders tot een verhoogd risico op hartfalen en beroertes. De oorzaak hiervan is wederom een verstoorde homeostase - in dit geval niet tussen de osteoblasten en osteoclasten, maar tussen ionen zoals fosfaat en calcium. Deze kleine moleculen zijn belangrijkste bestanddelen van botten.

In dit proefschrift zijn twee belangrijke mechanismen onderzocht die betrokken zijn bij de vorming van bot en de regulatie van mineralisatie (het proces dat leidt tot botvorming). Eén mechanisme bestuurt de balans tussen fosfaat en pyrofosfaat en regelt daarmee waar in een organisme mineralisatie kan optreden en waar niet. Pyrofosfaat is een sterke remmer van de vorming van botmineraal; terwijl fosfaat een essentiëel bestanddeel van botmineraal is. Verhoogde concentraties fosfaat zorgen voor de aanmaak van nieuw botmineraal.

Het andere mechanisme reguleert activiteit van zogenaamde weefselspecifieke transcriptiefactoren. Dit zijn eiwitten die de activiteit van een bepaalde set genen in een bepaald celtype controleren, waardoor deze cellen hun specifieke fysiologische functies vervullen. Zo is Osterix een weefselspecifiek transcriptiefactor in osteoblasten - de cellulaire subeenheden van bot.

De balans tussen fosfaat en pyrofosfaat en de activiteit van osteoblastspecifieke transcriptiefactoren zijn goed bestudeerd in muizen, het standaard modelorganisme voor biochemisch onderzoek naar bot en botontwikkeling. Voor zebravis, het modelorganisme van ons lab is dit echter niet het geval.

Het gebruik van de zebravis als een model voor botontwikkeling in een gezonde en zieke setting biedt unieke mogelijkheden. Door de grote aantallen nakomelingen is de zebravis een uitermate geschikt systeem voor voorwaartse genetische screens. Bovendien maakt de externe ontwikkeling van de transparante zebravisembryo in vivo beeldvorming tot op subcellulair niveau mogelijk. Nieuw onderzoek naar de belangrijkste mechanismen van botontwikkeling is nodig. Dit proefschrift presenteert nieuwe inzichten in botontwikkeling in de zebrafvis, die geextrapoleerd kunnen worden naar zoogdieren.

Hoofdstuk 1 biedt een overzicht van de huidige kennis over botontwikkeling en mineralisatie vanuit een antropocentrisch perspectief. Het plaats botonderzoek met de zebravis, dat beschreven wordt in de volgende hoofdstukken, in deze context.

Hoofdstuk 2 begint met een overzicht van enkele studies naar botontwikkeling in zebravis en hoe dit kennis uit traditionele modelsystemen zoals een muis en kip complementeert. Daarnaast geeft dit hoofdstuk een overzicht van de overeenkomsten en verschillen tussen botstructuren van beenvissen en vierpotigen.

In hoofdstuk 3 worden twee nieuwe zebravismutanten voor botontwikkeling beschreven. Mutanten missen de functie van een specifiek gen; de genen die in deze zebravissen ontbreken hebben tegenovergestelde kenmerken. De mutant no bones (nob) ontwikkelt geen botmineralisatie in zijn skeletonderdelen, terwijl in de mutant dragonfish (dgf) botmineralisatie niet alleen in skeletale weefsels plaatsvindt, maar ook in bepaalde zachte weefsels. Vervolgens wordt de identificatie van deze genetische mutaties beschreven, respectievelijk entpd5 en enpp1. De dubbele nob/dgf mutant blijkt de skeletmineralisatie in nob mutanten te herstellen. Dit wijst erop dat Entpd5 en Enpp1 als wederkerige regulatoren van de in vivo homeostase van fosfaat en pyrofosfaat fungeren. Ook laat het onderzoek zien dat de nob mutant hersteld kan worden door hoge niveau's anorganisch fosfaat toe te dienen. De conclusie hiervan is dat entpd5 een nieuwe en essentieel factor is voor de mineralisatie van botstructuren in de zebravis, door de aanmaak van fosfaat te bewerkstelligen. Tot nu toe heeft entpd5 in zoogdieren nog geen functie geassocieerd met skeletogenese.

Hoofdstuk 4 bevat een meer gedetailleerde beschrijving van de dgf mutant. Dgf heeft een aantal pathofysiologische overeenkomsten met humane mutaties in ENPP1, zoals gegeneraliseerde arteriële calcificatie in de kinderjaren (generalized arterial calcification of infancy; GACI) en pseudoxanthoma elasticum (PXE). Patiënten met GACI ontwikkelen ernstige verkalkingen in de slagaders en sterven vaak tijdens de eerste maanden na geboorte. De progressie van PXE is meestal trager, mineralisatie komt vaak voor in de huid en in de ogen. Aangetoond wordt dat dgf mutanten ectopische calcificaties in diverse zachte weefsels kunnen ontwikkelen, vergelijkbaar met de mens. Het onderzoek suggereert dat osteoclastachtige cellen aanwezig zijn op plekken waar de ectopische mineralisatie plaatsvindt. Dit fenomeen is vooralsnog nog niet in detail onderzocht in modelsystemen van zoogdieren en kan belangrijke gevolgen hebben voor de behandeling van patiënten met GACI. Als therapie worden bisfosfonaten gebruikt die de mineralisatie afremmen, maar het onderdrukken van de osteoclastfunctie zal tevens ook de afname van de verkalking in de zachte weefsels bemoeilijken. Dit is een zeer ongewenste bijwerking.

Hoofdstuk 5 beschrijft de rol van transcriptiefactor Osterix in de zebravis. In zoogdieren is Osterix geïdentificeerd als osteoblastspecifieke transcriptiefactor die een essentiële rol in botvorming speelt. De expressie van osterix in de zebravis is eerder beschreven in de craniofaciale botstructuren, maar niet in bepaalde onderdelen van het vroege axiale skelet. Dit kan het gevolg zijn geweest van beperkingen in voormalige technieken om genexpressie te detecteren. Gedetailleerde en betrouwbare detectie van genexpressie is tegenwoordig mogelijk met een BAC-reporter lijn, die voor osterix is gegenereerd. Deze studie heeft bevestigd dat in het begin van de botvorming in zebravis, osterix tot experessie komt in de craniofaciale, maar niet vroege axiale skeletstructuren. Deze waarnemingen zijn bekrachtigd door analyse van een zebravisosterixallel uit een TILLING screen. (TILLING is een methode waarmee een genmutatie geïdentificeerd kan worden.) Verminderde mineralisatie in de craniofaciale structuren is inderdaad waargenomen. De ontwikkling van het vroege axiale skelet bleek ook volkomen normaal te verlopen. Dit bevestigd de hypothese dat de mineralisatie van het axiale skelet in zebravis onafhankelijk is van osterix.

Hoofdstuk 6 bevat een afsluitende discussie over het onderzoek gepresenteerd in dit proefschrift.

Samengevat tonen deze studies dat de essentiële mechanismen in botvorming - fosfaat-pyrofosfaathomeostase en osterix-activiteit - geconserveerd zijn tussen beenvissen en viervoeters. Toekomstig onderzoek dient er echter rekening te houden dat ook variaties tussen de diverse modelorganismen kunnen voorkomen. Dergelijke verschillen moeten niet als belemmering gezien worden, maar als een kans om een diepgaand begrip te verkrijgen van hoe variaties in biologische processen robuust zijn en in de ontwikkeling van een functioneel organisme resulteren.

Curriculum Vitae

Alexander Apschner was born the 21st of April 1982 in Villach, Austria. From 1997 until 2002 he attended the Handelsakademie Villach where he obtained his highschool diploma. From 2002 until 2003 Alexander did his compulsory community service at the Red Cross Austria. After studying Food Science and Biotechnology at the University for Natural Resources and Applied Life Sciences, Vienna in 2003, Alexander enrolled in the diploma study of Molecular Biology at the University of Vienna in 2004. During his studies Alexander completed internships at the research groups of Prof. Dr. Christoph Schüller (University of Vienna), Prof. Dr. Christine Hartmann (Institute for Molecular Pathology (IMP), Vienna) and Dr. Darren Gilmour (European Molecular Biology Laboratories (EMBL), Heidelberg). He then conducted his one-year graduate student research project at the laboratory of Dr. Christine Hartmann (IMP, Vienna) and graduated with distinction in January 2010. In February 2010 he started the research described in this thesis at the research group of Prof. Dr. Stefan Schulte-Merker at the Hubrecht Institute in Utrecht. From 2012 on this research was funded by a DOC fellowship of the Austrian Academy of Sciences.

List of Publications

Apschner A., Schulte-Merker, S. and Witten, P.E. (2011). Not all bones are created equal – using zebrafish and other teleost species in osteogenesis research. Book-chapter in **Methods in Cell Biology.**

Huitema, L.F.A., Apschner, A., Logister, I., Spoorendonk, K.M., Bussmann, J., Hammond, C.L., and Schulte-Merker, S. (2012). Entpd5 is essential for skeletal mineralization and regulates phosphate homeostasis in zebrafish. **PNAS.**

Mackay E., Apschner, A., and Schulte-Merker S. (2013). A Bone to Pick with Zebrafish. **Bone Key.**

Apschner, A., Huitema L.F.A., Ponsioen B., Peterson-Maduro J., and Schulte-Merker, S. (2014). Pathological mineralization in a zebrafish enpp1 mutant exhibits features of Generalized Arterial Calcification of Infancy (GACI) and Pseudoxanthoma Elasticum (PXE). **Disease Models and Mechanisms.**

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