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


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Bone mineralization is an essential step during the embryonic development of vertebrates, and bone serves vital functions in human physiology. To systematically identify unique 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); analysis of its expression pattern demonstrates that entpd5 is specifically expressed in osteoblasts. An additional mutant, dragonfish (dfg), exhibits ectopic mineralization in the craniofacial and axial skeleton and encodes a loss-of-function allele of ectonucleotide pyrophosphatase phosphodiesterase 1 (enpp1). Intriguingly, generation of double-mutant nob/dfg embryos restored skeletal mineralization in nob mutants, indicating that mechanistically, Entpd5 and Enpp1 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.

The vertebrate skeleton is composed of bone and cartilage. Bone-forming cells, osteoblasts, secrete a collagen-rich matrix that is subsequently mineralized, whereas 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 osteoblast commitment and differentiation (1, 2). Osteoclasts, on the other hand, are of hematopoietic origin and derive from the monocytic lineage (3). In humans, the generation and remodeling of bone is a dynamic process that occurs throughout life and is dependent on age and sex. 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 biominneralization, 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 microenvironment, phosphatases such as phosphatase orphan 1 (PHOSPHO1) or tissue-nonspecific alkaline phosphatase (TNAP) are thought to be key factors in the initiation of mineralization (4). PHOSPHO1 is responsible for providing the phosphate necessary for nucleation of crystal growth within matrix vesicles (5), whereas TNAP can dephosphorylate various substrates but most importantly breaks down pyrophosphate in the microenvironment of osteoblasts (6). Pyrophosphate is a strong chemical inhibitor of bone mineral (hydroxyapatite) formation and is locally provided by the pyrophosphate channel ANK and ectonucleotide pyrophosphatase phosphodiesterase 1 (ENPP1) (7). On a whole-organism level, phosphate levels are regulated by controlling retention/secretion in the kidney via a hormonal network involving parathyroid hormone (PTH), FGF23, and 1,25(OH)2D3 (8).

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 (9). For example, deficiency in the ENPP1 gene can result in pathological soft-tissue mineralization, particularly in arteries (10, 11). On the other hand, hypophosphatemia leads to decreased mineralization of skeletal tissues, as evidenced by genetic studies in which PHOSPHO1, PHEX (phosphate regulating gene with homologies to endopeptidases on the X chromosome), or TNAP function is diminished (4, 12, 13). We have taken a forward genetic approach to identify novel regulators of osteogenesis and bone mineralization, and here we report the isolation and characterization of two zebrafish mutants: no bone (nob), mutants fail to form any mineralized skeleton, whereas dragonfish (dfg) mutants show ectopic mineralization in the craniofacial and axial skeleton. We demonstrate the causative genes to encode Entpd5 (ectonucleoside triphosphate/diphosphohydrolase 5) and Enpp1, respectively, and provide evidence that the combined activity of these factors maintains normal physiological levels of phosphate and pyrophosphate in the embryo.

Results

Nob Mutants Lack a Mineralized Skeleton. In a forward genetic screen in zebrafish (14), we uncovered 14 mutant lines out of 429 families screened. One mutant, no bone (nob) (Huitema and Bussmann), 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 d postfertilization (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 from bone (15, 16). Mutant embryos were viable when separated at 6 dpf from their siblings via alizarin red-based in vivo skeletal staining (17). 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 whether cartilage tissue develops normally in nob mutants. Alcian blue staining, labeling mucopolysaccharides and glycosaminoglycans in cartilage, appeared identical in mutant versus sibling embryos (Fig. 1A and Fig. S1A). 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 and E). We also analyzed the proliferation marker phospho-Histone H3 (pH3) 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 (17, 18) as well as other osteoblast markers. As shown in Fig. 1F–H, no difference was observed in the number of osterix (Fig. 1H)-expressing osteoblasts between sibling and nob mutant embryos. In addition, we observed no difference in the expression of type I collagen (col1a2) (Fig. 1G) or type 10 collagen (col10a1) (Fig. 1F), which marks osteoblasts in teleosts (19). Together, these data demonstrate that it is not the absence of osteoblasts that is causative for the nob mutant phenotype.

Nob Mutants Encode Alleles of entpd5. To identify the molecular lesion responsible for the nobmutant phenotype, we used simple sequence-length polymorphism and single-nucleotide polymorphism 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 (in mammals also referred to as CD39L4 or PCPH) in mutant and sibling embryos using gene specific primers (Table S1) revealed a premature stop codon in the mutant allele due to a T→A transversion in the third coding exon (Fig. 2B). This mutation resulted in a Leu>stop alteration at position 155, which is in the second apyrase conserved domain (gray bars in Fig. 2D, Upper).
of the predicted protein. We also uncovered a separate, noncomplementing allele (nob<sup>hu5310</sup>). The nob<sup>hu5310</sup> allele contained an 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 (see also Fig. S1B).

**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 (Center and Right), 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 mutations 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<sup>hu5310</sup> mutant entpd5 cDNA under the control of a cytomegalovirus (CMV) promoter. Mosaic rescue (as expected upon plasmid DNA injection) was observed in 24% of the nob<sup>hu3718</sup> mutants that were injected with wild-type cDNA (Fig. 2G and H), whereas the mutant nob<sup>hu5310</sup> 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 promoter (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 overexpressed. 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, to clarify the question of 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) (20) resulted in rescue of nob mutants in a manner indistinguishable from the cases described above (Fig. 2J). These results show that although 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 Alleles of enpp1.** In the same genetic screen we also uncovered a mutant, termed dragonfish (dgf), that displayed an ectopic mineralization phenotype (Fig. 3A and B) in the axial skeleton with apparent fusion of the mineralized vertebral centra (Fig. 3A) and also displayed 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. 3 D and E) of two noncomplementing alleles (Fig. 3 G and H and Fig. S2C) as well as a BAC-mediated rescue (Fig. 3 A and F and Fig. S2A and B) identified mutations within the *enpp1* gene underlying the mutant phenotype.

**Phosphate Homeostasis Is Disturbed in nob Mutants.** Entpd5 and Enpp1 both hydrolize extracellular nucleotide derivatives (7, 10, 11, 21, 22), with Entpd5 generating inorganic phosphate (21, 22) and Enpp1 generating pyrophosphate (7). 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/dgf 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/dgf mutants (Fig. 4B), demonstrating that inorganic phosphate is a limiting factor for nob mutants to mineralize their skeleton. Of note, excess calcium had no effect on the nob phenotype, whereas exogenously supplied calcium has been shown to rescue other mutants with hypomineralized phenotypes (23, 24).

Next, we studied whether phosphate-regulating genes were affected in nob mutants. Two independent microarray experiments on 7-dpf nob sibling versus mutant embryos (see *SI Materials and Methods*) demonstrated strong down-regulation of the key phosphate homeostasis regulator fibroblast growth factor 23 (fgf23) in nob mutants. Significant twofold down-regulation of *fgf23* was validated by quantitative (q)PCR (Fig. 4C), but this difference could be ameliorated by supplying Entpd5 via injection of *kdrl:entpd5* (Fig. S3). In addition to this, we studied the expression of several other phosphate-regulating genes (which were not present on the array chip; Fig. S1) by qPCR (Fig. 4C). In concordance with *fgf23* down-regulation, significant up-regulation of the sodium/phosphate cotransporter *npt2a* was measured in 7-dpf nob mutants (Fig. 4C).

Taken together, our findings show that Entpd5 is an 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. Taken together, our findings show that Entpd5 is a previously unappreciated and essential factor for bone formation. Entpd5 encodes a secreted ectonucleoside triphosphate/diphosphohydrolase that preferably hydrolyzes extracellular nucleotide diphosphates (21, 22) into nucleotide monophosphates and inorganic phosphate. Circulating extracellular nucleotides are known to be important purinergic signaling molecules that can...
generate a variety of physiological responses (25, 26). Although we do not exclude the possibility of an additional role of purinergic signaling in skeletal mineralization (26), 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 for mineralization. On one hand, this notion is supported by the rescue experiments reported here: Expression of entpd5 via a 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 the 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 Fig. S4 for a model).

Entpd5 has recently been suggested to play a role in 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 Enpp1 activities determines the level of mineralization through controlling the ratio of inorganic phosphate to 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 (7). Pyrophosphate antagonizes the ability of inorganic phosphate and calcium to form a mineral crystal. In line with this, Entpd5 mutations in humans and mice have been shown to cause ectopic mineralization due to insufficient extracellular pyrophosphate (10, 11). Based on the ectopic mineralization phenotype of the dgf mutants, we show how here on the phenotypic level that the function of Enpp1 is conserved between fish and mammals.

Because our study indicates that Entpd5 regulates phosphate homeostasis, we speculated that other factors regulating phosphate levels in vivo might be affected. Indeed, Fgf23 is significantly down-regulated, and the sodium/phosphate cotransporter npt2a (NaPi2a) is significantly up-regulated in nob mutants. Fgf23 is known as a key regulator of phosphate homeostasis (28), and changes in FGF23 activity lead to human disorders associated with either phosphate wasting or retention (29). Fgf23 is a circulating hormone produced in the bone that mainly targets the kidneys to control the activity of Npt2a and Npt2c (30). It seems likely that the absence of skeletal mineralization in mutant nob zebrafish elicits compensatory mechanisms to regulate the low levels of inorganic phosphate. Down-regulation of fgf23 and up-regulation of npt2a are consistent with this.

A murine Entpd5 knockout 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 (31) but appear smaller than littermates, a phenotype often found in hypophosphatemic mice (4, 28). Furthermore, the mice were shown to have increased serum alkaline phosphatase (31). Together with the findings of our study, we believe that the phenotype reported by Read et al. (31) 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 Entpd5 and Enpp1 maintains normal physiological levels of phosphate and pyrophosphate, and that the absence of 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.

Materials and Methods

Alizarin Red/Acin Blue Skeletal Staining. Skeletal staining was performed as described previously (17, 32). In vivo skeletal staining was performed with 0.001% calcein or 0.05% alizarin red in E3 medium for 5–10 min and subsequent extensive washes with E3 medium.

Meiotic Mapping and Sequencing. Bioinformatic construction of the genomic region surrounding the nobhu3718 and dgfhu4581 genes was performed using Ensembl database Zv6 (http://genome.ucsc.edu/cgi-bin/hgGateway?hgId=31290851&clade=vertebrate&org=Zebrafish&db=danRer6) for nobhu3718 and Zv8 (www.ensembl.org/Danio_rerio/Info/Index?db=core) for dgfhu4581. Meiotic mapping of the nobhu3718 and dgfhu4581 mutations 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. Additional information and all primer sequences are shown in Table S1. For all experiments, we have used the nab<sup>b<sub>nu</sub></sup> and dgf<sup>nu</sup>687 alleles, unless stated otherwise.

Whole-Mount In Situ Hybridization and Immunohistochemistry. All in situ hybridizations were performed at least twice as previously described (17, 33) and embryos were subsequently genotyped. Previously described probes were osterix and coxlα1 (17). Immunohistochemistry was essentially done as described (18) and as detailed in SI Materials and Methods.

cDNA Rescue Experiments. TRIzol reagent (Invitrogen) was used to extract RNA from 6-dpf embryos, and mouse RNA was extracted from cultured KS483 cells (34). For details, please consult SI Materials and Methods. One-cell-stage embryos derived from nob<sup>nu6</sup> carrier fish were injected with plasmid DNA in a maximum volume of 2 nL. Alizarin red/salicylic acid staining was carried out at 6 dpf. Only injected embryos with normal size, apparently normal cartilage, and 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<sup>b<sub>nu</sub></sup> and 481 siblings/129 mutants with 25 pg kdr:entpd5 cDNA.

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

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