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The Role of Phosphatases in the Initiation of Skeletal Mineralization

José Luis Millán
Sanford Children's Health Research Center, Sanford-Burnham Medical Research Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA

José Luis Millán: millan@sanfordburnham.org

Abstract

Endochondral ossification is a carefully orchestrated process mediated by promoters and inhibitors of mineralization. Phosphatases are implicated, but their identities and functions remain unclear. Mutations in the tissue-nonspecific alkaline phosphatase (TNAP) gene cause hypophosphatasia, a heritable form of rickets and osteomalacia, caused by an arrest in the propagation of hydroxyapatite (HA) crystals onto the collagenous extracellular matrix due to accumulation of extracellular inorganic pyrophosphate (PP_i), a physiological TNAP substrate and a potent calcification inhibitor. However, TNAP knockout (*Alpl*$^{-/-}$) mice are born with a mineralized skeleton and have HA crystals in their chondrocyte- and osteoblast-derived matrix vesicles (MVs). We have shown that PHOSPHO1, a soluble phosphatase with specificity for two molecules present in MVs, phosphoethanolamine and phosphocholine, is responsible for initiating HA crystal formation inside MVs and that PHOSPHO1 and TNAP have nonredundant functional roles during endochondral ossification. Double ablation of PHOSPHO1 and TNAP function leads to the complete absence of skeletal mineralization and perinatal lethality, despite normal systemic phosphate and calcium levels. This strongly suggests that the P_i needed for initiation of MV-mediated mineralization is produced locally in the perivesicular space. As both TNAP and nucleoside pyrophosphohydrolase-1 (NPP1) behave as potent ATPases and pyrophosphatases in the MV compartment, our current model of the mechanisms of skeletal mineralization implicate intravesicular PHOS-PHO1 function and P_i influx into MVs in the initiation of mineralization and the functions of TNAP and NPP1 in the extravesicular progression of mineralization.

Keywords

Biomineralization; Bone and cartilage development; Metabolic bone disease; Animal model

The System

Mineralization of cartilage, bone, and teeth occurs by a series of physicochemical and biochemical processes that together facilitate the deposition of hydroxyapatite (HA) in specific areas of the extracellular matrix (ECM). Experimental evidence has pointed to the presence of HA crystals along collagen fibrils [1] and within the lumen of chondrocyte- and osteoblast-derived matrix vesicles (MVs) [2, 3]. Investigators in the bone mineralization field are generally divided in supporting the collagen- versus the MV-mediated mechanism of mineralization. We see no incompatibility between these mechanisms. Our working

Correspondence to: José Luis Millán, millan@sanfordburnham.org.

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model considers that early mineralization takes place inside MVs, organelles that serve as a site for Ca^{2+} and inorganic phosphate (P_i) accumulation to initiate the deposition of HA crystals [3–8]. In a second step, MV membranes break down and expose preformed HA to the extracellular fluid, allowing for propagation of HA deposition onto the collagenous ECM [6, 8]. This process is orchestrated by the balanced action of promoters and inhibitors of calcification including many noncollagenous matrix proteins [9, 10].

This review article will recapitulate what we know about the functional interplay of three phosphatases, tissue-nonspecific alkaline phosphatase (TNAP), phosphatase orphan 1 (PHOSPHO1), and nucleoside pyrophosphohydrolase-1 (NPP1), during the initiation of skeletal mineralization, a process of fundamental importance to all vertebrate animal species. Alterations in the function of these phosphatases lead to soft bone, including rickets or osteomalacia, spontaneous fractures, loss of teeth, as well as inappropriate calcification of soft tissues including osteoarthritis and arterial calcification (Table 1). A key step forward has been the conceptualization of the interrelated role of these phosphatases in controlling the inorganic pyrophosphate (PP_i) to P_i ratio in MVs and the ECM, acting as a crucial determinant for initiation of skeletal mineralization.

The Key Players

Alkaline phosphatases (APs, EC 3.1.3.1) have structural similarity to a large number of other enzyme families with substantially different activities, including cofactor-independent phosphoglycerate mutase, isomerases, hydrolases, and a putative lyase, which, however, all act on similar phosphocarbohydrate (or sulfocarbohydrate) substrates [11]. In humans, the AP isozyme family is composed of TNAP, encoded by the *ALPL* gene, and the placental, germ-cell, and intestinal (IAP) isozymes, encoded by the *ALPP*, *ALPP2*, and *ALPI* genes, respectively. Mice also have four active AP loci, namely, *Akp2* (aka *Alpl*), *Akp3*, *Akp5*, and *Akp6* (aka *Alpi*), that encode TNAP, duodenum-specific IAP, embryonic AP, and global IAP, respectively [for review, 11]. Only TNAP is implicated in biomineralization, so this review will only cover the biological function of this isozyme.

PHOSPHO1 (EC 3.1.3.75), first identified in the chick [12] as a member of the haloacid dehalogenase (HAD) superfamily of Mg_2-dependent hydrolases [13], is expressed at levels 100-fold higher in mineralizing than in non-mineralizing tissues [14]. PHOSPHO1 has 42 % homology to PHOSPHO2, another member of the HAD family, that, however, has very different substrate specificity [15] and is currently not implicated in biomineralization.

The nucleotide pyrophosphatase/phosphodiesterase (NPP, EC 3.1.4.1) family, that includes NPP1, comprises seven NPPs identified to date [16]. These isoenzymes, related by 24–60 % conservation in their catalytic domains and certain other modular domains, exert generally non-redundant functions via distinctions in substrates and/or subcellular localization [17]. NPP1 (previously known as plasma cell membrane glycoprotein-1, PC-1), encoded by the *ENPP1* gene, is plasma membrane-bound, whereas autotaxin (NPP2) is secreted and B10 (NPP3) is abundant in intracellular spaces [17]. All three isozymes are expressed in a wide variety of tissues, including bone and cartilage; and all have the common ability to hydrolyze diesters of phosphoric acid into phosphomonoesters. NPPs have been implicated in various processes, including bone mineralization, signaling by insulin and by nucleotides, and the differentiation and motility of cells [18]. However, NPPs are known primarily as suppliers of intra- and extracellular PP_i using ATP as a substrate [17]. Similar to skeletal TNAP expression, NPP1 is highly abundant on the surfaces of osteoblasts and chondrocytes as well as on the membrane of their MVs [18, 19] and is the isozyme discussed here.

The PP_i/P_i Ratio

A primary inhibitor of ECM mineralization is extracellular PP_i [20]. PP_i is produced ectoplasmically by the enzymatic action of NPP1, which catabolizes extracellular ATP to produce PP_i and AMP [17]. Intracellular PP_i is also transported to the extracellular milieu by the channeling function of the ankylosis protein (ANK) [21]. TNAP plays the crucial role of restricting the concentration of extracellular PP_i to maintain a PP_i/P_i ratio that is permissive for normal bone mineralization [22–24]. Mice deficient in NPP1 (*Enpp1*$^{-/-}$) or ANK (*ank/ank*) develop soft tissue calcification, including vascular calcification, resulting from the reduced production or transport of PP_i [23–25]. In contrast, mice that completely lack TNAP function (*Alpl*$^{-/-}$) phenocopy infantile hypophosphatasia (HPP) [26, 27] in that they are born with normally calcified skeletons but begin to display hypomineralization of the skeleton at postnatal days 6–10 that worsens until their early demise at postnatal day 20 [28, 29]. The failure of HPP bones to calcify after birth results from a block in the propagation of HA in the ECM beyond the confines of the MV membrane [4, 30] because of accumulated levels of PP_i in the ECM resulting from the lack of TNAP's pyrophosphatase function [26, 27, 31]. Genetic experiments conclusively demonstrated the antagonistic roles of TNAP and NPP1 or ANK and provided conclusive evidence of the in vivo pyrophosphatase activity of TNAP. Indeed, [*Enpp1*$^{-/-}$; *Alpl*$^{-/-}$] and [*ank/ank; Alpl*$^{-/-}$] double-mutant mice showed normalized levels of extracellular PP_i and rescue of the respective mineralization phenotype of single-mutant mice [23–25]. However, it is worth pointing out that the degree of improvement of the skeletal phenotype in [*Enpp1*$^{-/-}$; *Alpl*$^{-/-}$] double-mutant mice was site-specific, with the axial skeleton showing more improvement than the appendicular skeleton, an observation that was attributed to the higher levels of expression of NPP1 in the axial skeleton [32]. Thus, TNAP's enzymatic degradation of PP_i controls the PP_i/P_i ratio to favor proliferation of HA crystals outside the MVs and along collagen fibrils. Indeed, bone mineralization is determined partly by the ability of osteoblasts to remove the physiological inhibitor of mineralization, PP_i, from their surrounding ECM via expression of TNAP and by the presence of a fibrillar collagen-rich network in the bone ECM [24]. Coexpression of TNAP and a fibrillar collagenous scaffold appears to be necessary and sufficient to cause mineralization of any ECM [24].

Interestingly, chondrocyte- and osteoblast-derived MVs in both HPP patients and *Alpl*$^{-/-}$ mice retain the ability to initiate intravesicular mineral formation and contain HA crystals [4, 30], demonstrating that TNAP is not essential for the initiation of MV-mediated ECM mineralization and suggesting that other phosphatases or another mechanism might be responsible for this first step. PHOSPHO1 is expressed at levels 120-fold higher in chondrocytes in mineralizing cartilage than in nonmineralizing tissues [14, 33], shows high phosphohydrolase activity toward phosphoethanolamine and phosphocholine [34], and is active inside chondrocyte- and osteoblast-derived MVs [35]. TNAP and PHOSPHO1 were found to be coexpressed throughout the developmental stages of limb development in the chick [36], and small-molecule compounds that inhibit PHOSPHO1 activity decreased MV-mediated calcification in vitro using mouse *Alpl*$^{-/-}$ MVs [33] and in chick embryo micromass cultures [36]. Thus, we surmised that PHOSPHO1 is involved in the first step of MV-mediated initiation of mineralization during endochondral ossification.

Our recent work clearly showed that lack of PHOS-PHO1 caused changes in the endochondral growth plate and skeletal abnormalities that included decrease or loss of secondary ossification centers, decreased bone mineral density, spontaneous fractures, osteomalacia, and scoliosis [37, 38]. *Phospho1*$^{-/-}$ mice display growth plate abnormalities, spontaneous fractures, bowed long bones, osteomalacia, and scoliosis in early life. Long bones from *Phospho1*$^{-/-}$ mice deform plastically rather than fracturing during three-point bending, and Raman microscopy revealed significantly lower mineral:matrix ratios and

lower carbonate substitutions in *Phospho1*$^{-/-}$ tibiae [37]. Primary cultures of *Phospho1*$^{-/-}$ tibial growth plate chondrocytes and chondrocyte-derived MVs showed reduced mineralizing ability, and plasma samples of *Phospho1*$^{-/-}$ mice showed reduced levels of TNAP and elevated PP_i concentrations. Transgenic overexpression of TNAP does not correct the bone phenotype in *Phospho1*$^{-/-}$ mice, suggesting that TNAP and PHOSPHO1 act on distinct pathways. In agreement with this hypothesis, double ablation of PHOSPHO1 and TNAP function led to a dramatic phenotype: complete absence of skeletal mineralization and perinatal lethality [38]. This suggests that the availability of free P_i is not sufficient to initiate mineralization and that TNAP must be involved in generating P_i in the vicinity of MVs. Calcification, both intravesicular and extravesicular, is abolished in [*Phospho1*$^{-/-}$; *Alpl*$^{-/-}$] embryos despite the availability of systemic P_i in these mice. This argues that, besides the P_i produced intravesicularly by PHOSPHO1, organic phosphates might act as an additional major source of P_i, via the putative action of TNAP and/or possibly NPP1.

Indeed, a P_i-generating function has been proposed for TNAP since the discovery of this enzyme in bone by Robison [39], and its ability to hydrolyze ATP [40] as well as polyphosphates [41] has been described. We studied phosphosubstrate catalysis by osteoblast-derived MVs at physiologic pH, analyzing the hydrolysis of ATP, ADP, and PP_i by isolated wild-type as well as TNAP-, NPP1- and PHOSPHO1-deficient MVs. We found that TNAP is an efficient ATPase, in addition to its established role as a pyrophosphatase in the MV compartment. We also found that, in contrast to its accepted role on the surface of osteoblasts and chondrocytes, NPP1 does not have a major PP_i-generating activity at the level of MVs but acts as both an ATPase and a pyrophosphatase [42]. We also showed that TNAP and NPP1 account for all the P_i-generating ability of isolated MVs. Thus, while PHOSPHO1 modifies the PP_i/P_i ratio inside MVs by releasing P_i from membrane phospholipids, TNAP has a primary role in establishing an extracellular PP_i/P_i ratio conducive for ECM mineralization, via its pyrophosphatase as well as its ATPase activity. The fact that NPP1 can act as a backup phosphatase on both PP_i and ATP helps to explain why *Alpl*$^{-/-}$ mice, which are null for TNAP activity, develop normal mineralization for the first 6 days of life. Thus, at least in mice, NPP1 can be considered a modifier of the HPP phenotype. This partial compensatory NPP1 activity, which is higher in the axial than in the appendicular skeleton [32], also explains how a single [*Phospho1*$^{-/-}$; *Alpl*$^{-/-}$] stillborn pup identified by Yadav and collaborators had residual mineralization in the axial skeleton, despite the complete absence of mineralization elsewhere [38].

Our Current Model

Analyses of [*Phospho1*$^{-/-}$; *Alpl*$^{-/-}$] mice provided unique clues to help put together a comprehensive model of the mechanisms of initiation of skeletal mineralization. Calcification, both intravesicular and extravesicular, is abolished in [*Phospho1*$^{-/-}$; *Alpl*$^{-/-}$] embryos despite the availability of systemic P_i in these mice. This argues that organic phosphates, such as ATP or ADP, might act as the major source of P_i that is required for the initiation of calcification. Chondrocytes, osteoblasts, and their derived MVs express and use phosphate transporters on their membrane for uptake of P_i [43, 44]. We must conclude that the mineralizing cells consider it efficient to invest the energy required to generate and export ATP to be used for the local generation of P_i in the immediate environment of MVs and for subsequent incorporation into MVs via P_i transporters. Thus, in the absence of both PHOSPHO1 and TNAP function there is complete lack of skeletal mineralization because there is no P_i generation from substrates attributable to the absence of TNAP's ATPase activity and the levels of ATPase provided by NPP1 in the embryonic skeleton are clearly insufficient to allow calcification to proceed. Some calcification was observed in the axial skeleton of a single stillborn [*Phospho1*$^{-/-}$; *Alpl*$^{-/-}$] double knockout pup, which was likely

attributable to P_i generation via the "ATPase" action of NPP1 and to the concomitant restriction of extracellular PP_i concentrations by the "pyrophosphatase" activity of NPP1. This provides an explanation for why complete ablation of PHOSPHO1 function only leads to a decrease in the calcification ability of MVs but not to a complete lack of calcification. Deletion of PHOSPHO1 would suppress intravesicular generation of P_i but would leave extravesicular P_i generation via TNAP's ATPase activity and influx via P_i transporters unaffected.

Integrating these data, it is now possible to propose an inclusive model for the initiation of skeletal mineralization that unifies a number of concepts and functions that have been considered contradictory in the past. This unified model starts with the MVs as the site of initiation of mineralization (Fig. 1). HA crystals appear inside the MVs favored by P_i accumulation resulting from a dual mechanism, i.e., PHOSPHO1-mediated intravesicular production and transporter-mediated influx of P_i produced extravesicularly primarily by TNAP's ATPase activity or, secondarily in the absence of TNAP, by NPP1's ATPase activity. Organophosphate compounds (ATP) and perhaps PP_i are the source of P_i for this initial step of calcification. Then, extravesicular calcification is supported primarily by TNAP's pyrophosphatase activity and secondarily by NPP1's pyrophosphatase activity and is driven by the availability of P_i and the presence of a collagenous fibrillar scaffold and guided by other ECM mineral-binding proteins. Figure 2 shows how this model is able to explain the phenotypic abnormalities observed in the *Phospho1*$^{-/-}$, *Alpl*$^{-/-}$, and [*Phospho1*$^{-/-}$; *Alpl*$^{-/-}$] knockout mice described above. This model also takes into account the roles of both organic and inorganic phosphates in skeletal calcification, incorporates the P_i-generating and PP_i-inactivating roles of TNAP, and unifies the roles of MV-mediated and collagen-mediated calcification as two separate but linked steps during endochondral ossification.

Future Challenges

We have yet to understand the intimate biochemical details of how PHOSPHO1 is implicated in intravesicular P_i generation form membrane phospholipids. Pioneering studies by Wuthier and collaborators [45] have shown that the growth and egress of nascent HA crystals from the MV lumen are accompanied by changes in the lipid composition of the MV membrane. Phosphatidylinositol, sphingomyelin, and phosphatidylethanolamine undergo rapid breakdown, while phosphatidylcholine are degraded more slowly. Given the in vitro specificity of PHOSPHO1 for phosphoethanolamine and phosphocholine [33, 34], the polar groups of phosphatidylethanolamine and phosphatidylcholine, we surmise that the physiological phospholipid changes in MVs are mediated by PHOSPHO1 action. Yet, we must elucidate if PHOSPHO1 scavenges P_i directly from these phospholipids or requires the enzymatic action of phospholipase C to release the polar groups. We are also just beginning to understand the role of NPP1 as a backup phosphatase. In this regard ENPP1 can be considered a modifier of the severity of HPP, and more work in this area will surely shed more light into the partially overlapping roles of these phosphatases/phosphodiesterases. Furthermore, the interesting suggestion that polyphosphates could be used as a readily available reservoir of P_i which, upon TNAP-mediated release, can help to concentrate P_i for either initiation or propagation of HA mineralization [41] needs to be investigated further.

The identity of the phosphate transporters implicated in the influx of P_i generated extravesicularly is yet to be determined. P_i transport in chondrocytes and osteoblasts is primarily handled by type III sodium-dependent (Na/P_i) cotransporters which have broad tissue expression [46–48]. Two related type III Na/P_i cotransporters, P_iT1/Glvr1 and P_iT2/Ram, are expressed by chondrocytes and osteoblasts; but literature reports and our own expression data [49] indicate that P_iT1 is the major mediator of P_i flux in these cell types.

There are also indications in the literature that a transporter that is not strictly Na^+-dependent but responds to increases in extracellular P_i might exist, at least in the chicken [44, 45]. Thus, quite a bit of work remains to be done to identify the transporter(s) involved in this process.

An important reason that the bone community is still skeptical of the role of MVs in bone mineralization stems from our current inability to explain how HA crystals formed in the MV environment can promote further propagation of mineralization onto the collagen matrix. In vivo, collagen molecules assemble into three-dimensional structures in which each molecule is in a staggered arrangement relative to its neighbors. Collagen is packed in three dimensions through strict and contiguous alignment of its hole and overlap zones. Extensive work by M. Glimcher, M. Nylen, W. Landis, and many others has indicated that the pattern of holes and overlap sites among collagen molecules provided channels or gaps in their assemblage where HA crystals could nucleate [50, and references therein]. It has been difficult to visualize how apatitic crystals formed within MVs could make their way to these collagen gaps. Other investigators, including K. Prostak, S. Lees, and H. Schwarcz, have found that up to 80 % of the HA can be external to the collagen fibers [51, and references therein]. Given that MVs display collagen-binding molecules on their membranes (TNAP, ANXA5), we can now begin to conceive possible mechanisms of transfer of MV-initiated HA crystals to the collagen fibers. This will undoubtedly become an area of active research in the near future.

In addition, the mineralization field needs to define if MV-mediated calcification and the enzymatic regulation of the PP_i/P_i ratio discussed in this review are universal cellular mechanisms that precede collagen-mediated propagation of matrix mineralization in all calcifying tissues or if they are restricted to certain skeletal and dental tissues. It is very clear that odontoblasts mineralize the dentin via the production of MVs and regulation of the PP_i/P_i ratio, as demonstrated through the analysis of *Alpl*$^{-/-}$ hypophosphatasia mice and the success of enzyme replacement using mineral-targeting TNAP to correct the dentin phenotype [52, 53]. Similarly, the acellular cementum is under strict regulation by the PP_i/P_i ratio [54]; and this ratio is normalized, and acellular cementum corrected, in hypophosphatasia mice treated with enzyme replacement [55]. Whether cementum mineralization proceeds via MVs has not yet been determined. Neither is it clear if enamel formation and mineralization involve the function of MVs, a tissue notoriously devoid of collagen. However, enamel defects are also present in hypophosphatasia mice, and these defects are preventable by enzyme replacement with mineral-targeting TNAP [56], arguing that the PP_i/P_i ratio is also involved in the regulation of enamel matrix mineralization. Finally, there is some evidence that dystrophic calcification of vascular smooth muscle cells, as occurs in chronic kidney disease, also involves the production and function of MVs [57]; and alterations in the PP_i/P_i ratio are crucial in the pathophysiology of medial vascular calcification in chronic kidney disease as well as in the rare condition generalized arterial calcification of infancy caused by *ENPP1* gene mutations [58, 59]. We can expect further work in this area to focus on devising pharmacological approaches to treating medial vascular calcification, a critically unmet clinical need, by targeting the functions of TNAP [58] and PHOSPHO1 [60] as a means of correcting the pathological PP_i/P_i imbalance that is central to its pathogenesis.

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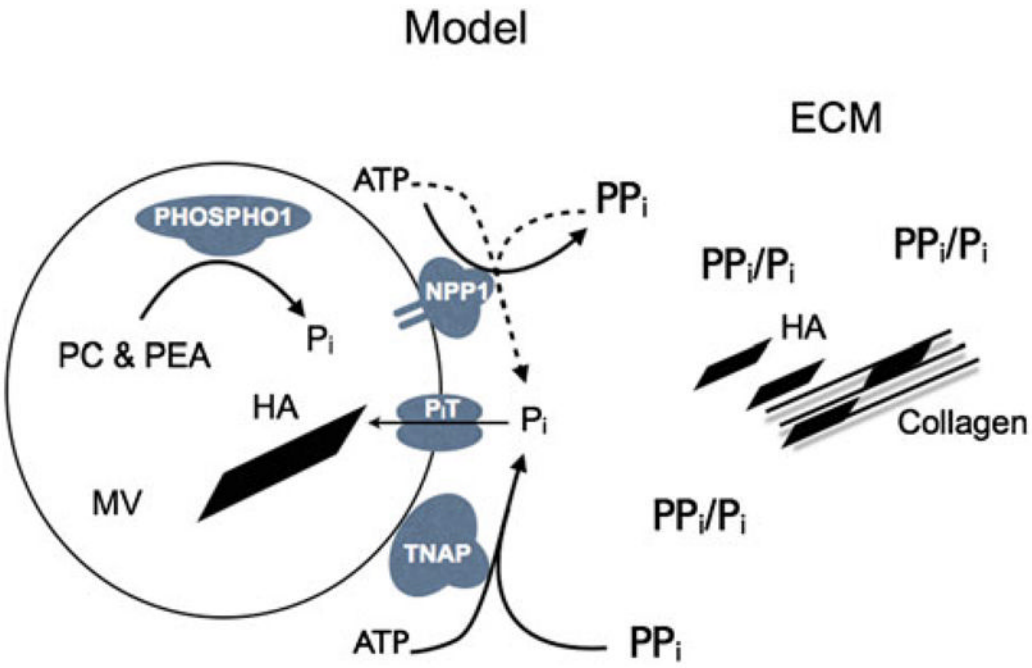

Fig. 1.

Model of initiation of skeletal mineralization including the function of PHOSPHO1, TNAP, NPP1, and phosphate transporters. The first step of MV-mediated mineralization involves the convergence of two independent biochemical pathways: intravesicular P_i generation by the enzymatic action of PHOSPHO1 and influx of P_i, generated in the perivesicular space by the activities of TNAP and NPP1, via P_i transporters. *PC* phosphocholine, *PEA* phosphoethanolamine, *MV* matrix vesicle, *PiT* phosphate transporter 1, *HA* hydroxyapatite, *ECM* extracellular matrix, *Pi* inorganic phosphate, *PPi* inorganic pyrophosphate

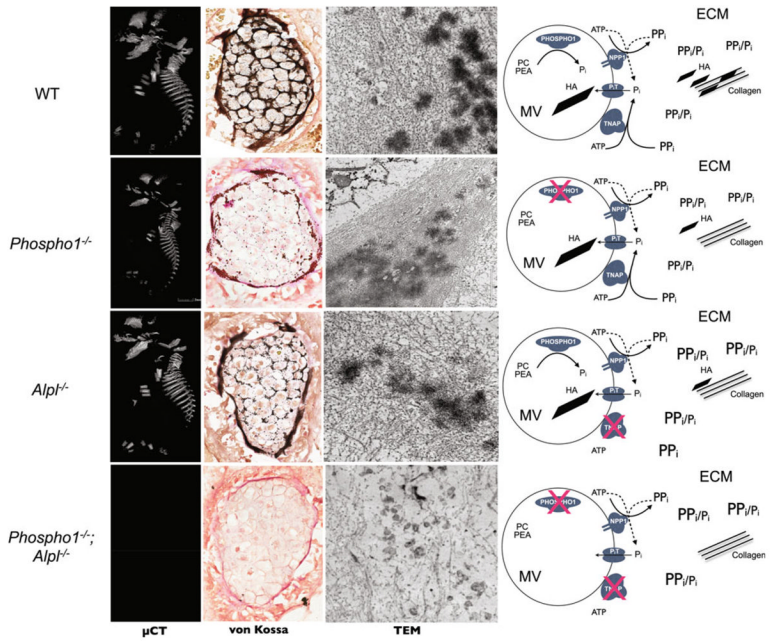

Fig. 2.

Summary of findings and how they can be explained by the unified model of initiation of skeletal mineralization. Original data were published in Yadav et al. [38]. *Wild-type* mice the first step of MV-mediated mineralization involves intravesicular P_i generation by PHOSPHO1 and influx of P_i, generated extravesicularly by TNAP and NPP1. Extravesicular propagation occurs on collagen scaffolds facilitated by the pyrophosphatase function of TNAP and NPP1. *Phospho1*$^{-/-}$ mice a growth plate and skeletal phenotype is apparent, but HA is still found inside MVs because P_iT-mediated transport of P_i, generated by TNAP and NPP1, is unaffected. *Akp2*$^{-/-}$ mice rickets and osteomalacia are prominent, due to increases in extracellular PP_i. HA crystals are still found inside MVs due to the P_i-generating ability of PHOSPHO1. P_iT-mediated influx of P_i is greatly diminished except where NPP1 activity is high (axial skeleton). [*Phospho1*$^{-/-}$; *Alpl*$^{-/-}$] mice complete absence of skeletal mineralization can be explained by the absence of intravesicular P_i generation by PHOSPHO1, the lack of extravesicular P_i generation by TNAP needed for P_iT-mediated influx, and accumulation of PP_i in the ECM. Abbreviations as in Fig. 1

Table 1

Nomenclature of the gene name(s), protein name(s), and gene knockout symbols and a brief description of the disease phenotype for each of the murine models of phosphatase deficiency

Gene names, human (mouse)	Protein names	Mouse model	Phenotype
ALPL (*Alpl*, aka *Akp2*)	TNAP (aka TNSALP)	*Alpl*$^{-/-}$	Infantile hypophosphatasia • Rickets/osteomalacia • Bowed bones • Epileptic seizures • Dentin/cementum/enamel defects
ENPP1 (*Enpp1*)	NPP1 (aka PC-1)	*Enpp1*$^{-/-}$	• Generalized arterial calcification of infancy • Osteopenia • Ossification of the posterior longitudinal ligament of the spine • Osteoarthritis
PHOSPHO1 (*Phospho1*)	PHOSPHO1	*Phospho1*$^{-/-}$ (aka *Phospho1-R74X*)	• Growth plate abnormalities • Osteomalacia • Bowed bones • Greenstick fractures • Scoliosis