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# Biological stenciling of mineralization in the skeleton: Local enzymatic removal of inhibitors in the extracellular matrix

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ABSTRACT

Biomineralization is remarkably diverse and provides myriad functions across many organismal systems. Biomineralization processes typically produce hardened, hierarchically organized structures usually having nanostructured mineral assemblies that are formed through inorganic-organic (usually protein) interactions. Calcium-carbonate biomineral predominates in structures of small invertebrate organisms abundant in marine environments, particularly in shells (remarkably it is also found in the inner ear otoconia of vertebrates), whereas calcium-phosphate biomineral predominates in the skeletons and dentitions of both marine and terrestrial vertebrates, including humans. Reconciliation of the interplay between organic moieties and inorganic crystals in bones and teeth is a cornerstone of biomineralization research. Key molecular determinants of skeletal and dental mineralization have been identified in health and disease, and in pathologic ectopic calcification, ranging from small molecules such as pyrophosphate, to small membrane-bounded matrix vesicles shed from cells, and to noncollagenous extracellular matrix proteins such as osteopontin and their derived bioactive peptides. Beyond partly knowing the regulatory role of the direct actions of inhibitors on vertebrate mineralization, more recently the importance of their enzymatic removal from the extracellular matrix has become increasingly understood. Great progress has been made in deciphering the relationship between mineralization inhibitors and the enzymes that degrade them, and how adverse changes in this physiologic pathway (such as gene mutations causing disease) result in mineralization defects. Two examples of this are rare skeletal diseases having osteomalacia/odontomalacia (soft bones and teeth) – namely hypophosphatasia (HPP) and X-linked hypophosphatemia (XLH) – where inactivating mutations occur in the gene for the enzymes tissue-nonspecific alkaline phosphatase (TNAP, TNSALP, ALPL) and phosphate-regulating endopeptidase homolog X-linked (PHEX), respectively. Here, we review and provide a concept for how existing and new information now comes together to describe the dual nature of regulation of mineralization – through systemic mineral ion homeostasis involving circulating factors, coupled with molecular determinants operating at the local level in the extracellular matrix. For the local mineralization events in the extracellular matrix, we present a focused concept in skeletal mineralization biology called the *Stenciling Principle* – a principle (building upon seminal work by Neuman and Fleisch) describing how the action of enzymes to remove tissue-resident inhibitors defines with precision the location and progression of mineralization.

## 1. Introduction

Mineralized skeletons have always been a wonder of Nature. We marvel at them from empirical experimentation, from clinical knowledge, from studying the stunning variation of life forms, and also from fossil and archeological records. In a synergistic merger of geology/mineralogy with biology, biomineralization mechanisms make use of the very same mineral ions that exist outside the realm of life and biologically guide them towards the construction of composite functional assemblies having inorganic phases typically integrated with an organic extracellular matrix. In the vertebrate skeleton, this merger of organics with inorganics forms a sublime, reproducible and unique

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structure with multiple functions and with the capability of functional adaptation, growth and repair [1].

Since the most valuable commodity in biology is energy, the distinctive features of Nature's designs in biomineralization are their frugality and multifunctionality [2]. For example, think of the gradual dissolution of the calcium carbonate avian eggshell from within during fertilized egg incubation. The internal dissolution of the calcitic eggshell serves the dual purpose of providing calcium to the developing calcium-phosphate skeleton of the embryo, while at the same time thinning the shell from the inside for the moment when the bird needs to crack it for hatching (pipping) [3]. As another example of multifunctionality, consider the removal of calcium ions from the intracellular compartments of marine organisms in order to reduce cytotoxicity, while at the same time providing a mineral ion for constructing an outer protective shell [4]. As a general rule, when organisms have multiple "good-enough" strategies to procure food, survive and procreate, the energetically cheapest strategy usually wins. In this context, Nature's evolved solutions can be succinctly expressed by the energy-minimalization paradigm of establishing "maximal diversity from minimal inventory" [2].

One general principle of this maximal diversity-from-minimal-inventory paradigm is that there is a limitation in degrees of freedom in order to achieve a desired function while maximizing precision, stability and reproducibility – all at minimal metabolic cost to the organism. An interesting example illustrating this can be found for joint movement in the book *Mechanical Design in Organisms* [5]. The bones comprising any diarthrodal joint are remarkably incongruent, so much so that a dry, lifeless specimen can be effortlessly disarticulated – it is in an unstable state of having unlimited degrees of freedom. However, in its hydrated living state, a functional joint has a limited range of movement restricted by its surrounding soft connective tissue components (joint capsule, ligaments, fasciae) such that only a functionally appropriate, stable and reproducible range of movement is allowed and facilitated by the muscles. The same principle is observed in the pruning of neural connections that originally form in excess [6], or in the constructive regression of embryonic bone patterning that begins as an overconnected network of redundant elements (trabeculae), the majority of which are destined to be removed [7,8].

Given this context of Nature's optimized and energy-minimized designs, we present here a concept of skeletal mineralization where a convergence of new findings on the regulatory mechanisms underlying vertebrate mineralization allows us to present the *Stenciling Principle* for biomineralization. The stenciling principle is developed from concepts initially presented by W. Neuman, H. Fleisch and G. Russell. It invokes that instead of actively supplying requisite mineral ions with precision to a desired site of mineralization in the extracellular matrix – a process which would be environmentally dependent, metabolically expensive, and difficult to confine – there are mineral ions that *i*) are abundant and ubiquitously available both systemically and locally, *ii*) are generally prevented from precipitating as a solid mineral phase, or are stabilized as amorphous mineral precursor phases, by systemic and local mineralization inhibitors, and *iii*) are locally permitted, only in the extracellular matrix of skeletal/dental tissues, to precipitate (and crystallize) as mineral following the actions of local enzymes to degrade these inhibitors of mineralization. This we refer to as the *Stenciling Principle* for skeletal and dental mineralization. Under normal physiologic conditions, this principle assumes (and overlays onto) appropriate mineral ion abundance and homeostasis, and the requisite establishment of an appropriately assembled extracellular matrix (Fig. 1).

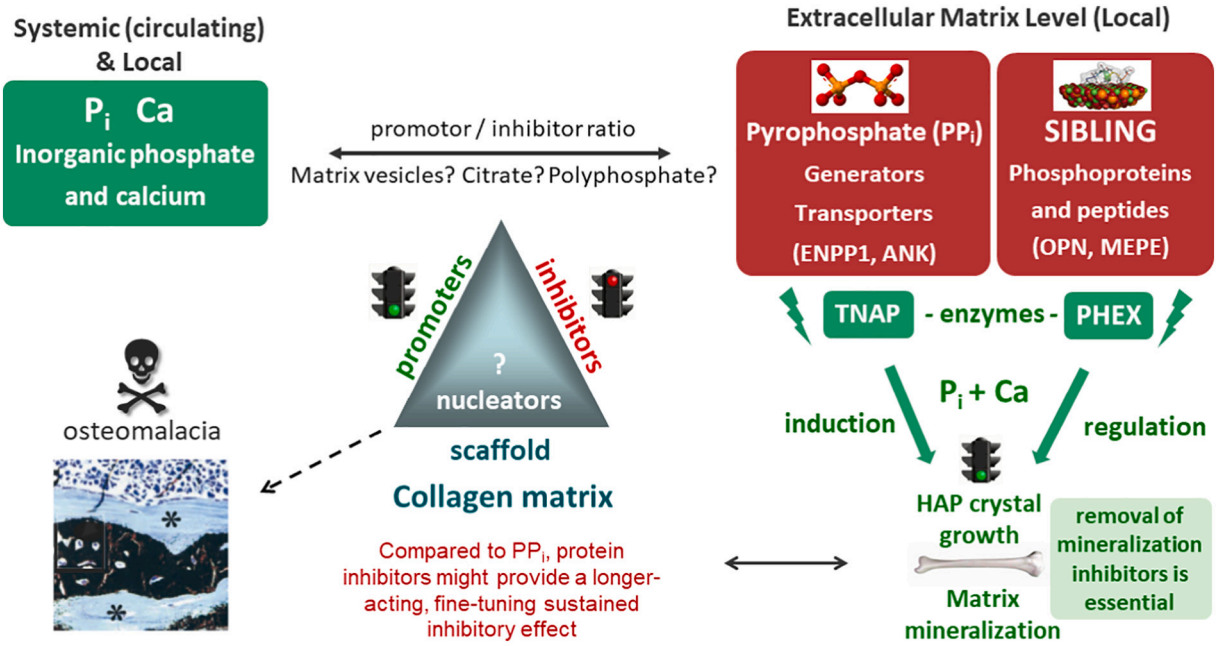


**Fig. 1.** Induction and regulation of mineralization in the skeleton. While relatively little is known about how mineral is induced/nucleated in bone tissue other than there being a requirement for appropriate levels of mineral ions (Ca and $P_i$) and a collagen fibrillar scaffold, substantial progress has been made in understanding the subsequent regulation of mineralization. Release-from-inhibition arises as a common theme, whereby inhibition of mineralization by small molecules such as pyrophosphate ($PP_i$), and by larger negatively charged and intrinsically disordered SIBLING proteins such as osteopontin (OPN), is mitigated by their enzymatic degradation. Such a normal physiologic process – to remove mineralization inhibitors to allow the propagation of mineralization in healthy bone – can be readily appreciated by the failure of these pathways in several osteomalacic diseases where inactivating mutations in the genes for these inhibitor-degrading enzymes result in debilitating accumulation of inhibitors which leads to soft (hypomineralized) bones and teeth. This enzyme-substrate relationship locally regulating mineralization in the extracellular matrix has been well-described for hypophosphatasia (substrate is $PP_i$; enzyme is tissue-nonspecific alkaline phosphatase, TNAP, TNSALP, ALPL) and X-linked hypophosphatemia (substrate is OPN; enzyme is phosphate-regulating endopeptidase homolog X-linked, PHEX). Other major potential determinants of mineralization with less defined roles include matrix vesicles, polyphosphates and citrate. ENPP1, Ectonucleotide Pyrophosphatase/Phosphodiesterase 1; ANK (ANKH), Progressive Ankylosis Protein Homolog/Human; MEPE, Matrix Extracellular Phosphoglycoprotein; SIBLING, Small Integrin-binding Ligand, N-linked Glycoproteins; asterisks, accumulation of unmineralized extracellular matrix in osteomalacic bone.

## 2. Mineral in bone

In 1690, Clopton Havers first identified the basic components of bone as “fixed salt” (mineral) and “earth” (organic matter) [9]. However, it was not until the middle of the 20th century – with the advent of X-ray diffraction and electron microscopy – that scientific debate about the ultrastructure of bone began in earnest when apparently controversial descriptions of the crystalline habit of bone mineral (apatite) were reported. At that time in the early 1950s, bone crystals were described as being either rod- or needle-shaped as based on X-ray diffraction (XRD) studies [10,11], or as larger-sized, platelet-shaped crystals with imperfections and substitutions at their periphery, as based on early transmission electron microscopy (TEM) observations [12,13]. Soon thereafter in the mid-1950s, Fernandez-Moran and Engström again reported on a needle-shaped morphology for bone mineral apatite aligned with collagen as based on TEM observations of diamond knife-cut, ultramicrotome sections of plastic-embedded bone [14]. They noticed that nano-sized needles of mineral also formed aggregated stacks referred to as flakes, with a regular spacing of several nanometers which extended cross hundreds of nanometers. This was followed by observations from Glimcher describing lathe-like, nonstoichiometric and slightly bent crystals, intimately associated with interstitial water – from this he suggested an alternative notion of bone mineral crystal morphology, this being that the apparent needles could in fact be thin platelets viewed edge-on [15]. The difficulty of visualizing platelets face-on (*en face*) was explained by their very thinness, where their electron-lucency resulted in a lack of contrast when imaged by TEM. Other TEM observations by Nylen and colleagues around this time demonstrated highly aligned mineral crystals in the gap/hole zones of collagen fibrils often in register across multiple collagen fibrils [16]. Following this, work by others provided evidence for an inherently disordered and hydrated phase of bone mineral described as amorphous calcium phosphate (ACP) [17], and also described was the mineral phase octacalcium phosphate [18], with both supposedly being stabilized by noncollagenous organic and inorganic molecules.

In the mid-1980s, Weiner and Traub developed the idea that mineral crystallites exceed the dimensions of single collagen fibrils, in accordance with observations made earlier [15,19]. Crystallites were described as being within the grooves of several collagen fibrils and aligned in register, and they developed further the notion that maintaining specimen hydration is a key requirement for a true representation of both organic and inorganic material in bone [20]. In 1989, Weiner and Traub described crystallites of mature bone growing out of their collagen fibril gap-region confinement to become confluent with adjacent mineral particles, and they also reported on a striated pattern in individual crystallites [21], which was in agreement with the earlier studies [12]. Landis et al. in 1991 presented the notion that mineral was associated with both the collagen fibril gap region and its surface using mineralized turkey leg tendon as a model for *in situ* mineralization of collagen fibrils [22]. In the mineralizing turkey leg tendon, a whisker-like crystallite morphology was reported, along with larger mineral aggregates bridging neighboring collagen fibrils, and the platelet character for the crystallites was again maintained by the idea of their limited visibility depending upon their orientation with respect to the electron beam of the microscope.

Around the same time, in the 1970s and 1980s, the concept of precise collagen crosslinking by nonreducible, aromatic covalent bonds was demonstrated and validated through studies by Eyre and colleagues who were able to locate exact sites of crosslinks in collagen using chromatographic methods [23,24]. This was an important development at the time, leading to the concept of integrated, robust arrays of collagen where crosslinked fibrillar networks of structured collagen could form extended three-dimensional assemblies creating a sturdy extracellular matrix. In 1992, Traub and colleagues conducted a TEM study of mineralization in young turkey leg tendon and described a needle-shaped habit for the crystallites that was localized to the gap regions of collagen fibrils and presumably nucleating specifically in the vicinity of the so-called *e*-band of collagen – a charged concave site of the gap region [25]. A few years later, Weiner et al. [26] published a comprehensive, integrated “rotated plywood” model for mineralized bone extracellular matrix describing mineral platelets located within collagen fibril arrays with the flat aspect of the platelets exhibiting alternating orientations – parallel or perpendicular – to the collagen array planes, in a type of coiling configuration. Meanwhile, Prockop and Fertala in 1998 demonstrated a tip-elongation pattern for collagen fibrillogenesis whereby the addition of triple helices occurs in a super-helical fashion [27]. In the 2000s, Orgel demonstrated the inherent local instability of the collagen helix by mapping electron density and by X-ray diffraction [28,29], writing that the *e*- and *d*-bands of the gap region might particularly favor interaction with noncollagenous protein species [30].

More recently, as TEM and scanning TEM (STEM) tomography used in conjunction with focused-ion beam (FIB)-prepared specimens became increasingly more available and used for bone work throughout the early 2000s, still no general consensus had been reached regarding the habit/morphology of bone mineral. One idea that emerged from looking at postmortem bone was that mineral morphology changes as the organic material decays after death, leading to larger size and confluence of the crystallites, implying that there is an optimized limit to crystallite size in living bone that is a prerequisite for normal bone structure and function *in vivo* [31]. Related to this is one of the most significant and elegant recent discoveries (in our view) regarding bone ultrastructure – the study by Bertinetti et al. in 2015 [32] highlighting the role of structural water to osmotically maintain pre-stress in bone tissue. In brief, the notion here is that the crosslinked collagen meshwork has limited extensibility and incorporates highly hydrated, noncollagenous organic molecules, including negatively charged, osmotically active small proteoglycans. Bound water effectively contributes to the tensile pre-stress exerted on the crosslinked network of collagen molecules, this being in the range up to 80 MPa. It is important to realize that bone mineral crystallites nucleate and grow within a confined and osmotically crowded environment, thus being under compression. Indeed, this pre-stress appears to be a successful strategy of Nature for providing a safe biomechanical environment in which discrete structural tissue/material components and their assemblies can each be left to perform their individual functions at optimal capacity [33] – say for example, like elements in bone that resist compression (mineral) and tension (crosslinked extracellular matrix), or similar elements in wood resisting compression (lignin) and tension (cellulose) [34]. The pervasive mineral phase presumably impregnates this pre-stressed environment as it forms, by gradually replacing some of the structural water.

## 3. Composition and structure of the extracellular matrix in bone

Discussing the composition of the extracellular matrix of bone is essentially impossible without relating it to the context of scale of observation [35]. Here, the discussion will be limited to lamellar bone – the most common type of bone in the skeleton of a mature large vertebrate such as humans. Starting at the nanometer level, it is possible to discriminate between the organic components, inorganic components and water; however, this distinction into three broad categories is an oversimplification for the following reasons. Firstly, the organic phase is an overarching umbrella term covering myriad organic components, each of which provides a unique function. Collagen is the most abundant matrix protein by mass, based on the very large size of these complex, fibrillar structural components, but in terms of molar amounts, noncollagenous proteins are roughly as equally abundant as collagen [36]. Noncollagenous proteins are often highly phosphorylated usually at serine residues (phosphoserine), as mediated mainly by the kinase FAM20C [37,38], and they bind calcium [39,40] and interface with both mineral and other organic components [41–44]. They also exist in covalently crosslinked forms (as mediated by the enzyme

tissue transglutaminase 2, TG2) that might modify monomer function and at the same time provide new polymeric functions such participating in adhesion/cohesion in a way that makes the extracellular matrix more robust [45]. In some cases they reside not only in bulk bone matrix, but at cell-matrix interfaces such as when a cement line/plane is established by osteoblasts during the reversal phase of a bone remodeling cycle, or at the osteocyte/lacuno-canalicular network interface to form a thin planar structure termed the *lamina limitans* [46,47]. At these cell-matrix interfaces, cell integrins may bind ligands in these matrix proteins – such as the RGD tripeptide – to provide outside-in signaling from the extracellular matrix to the cells. The best-studied group of noncollagenous phosphoproteins in bone is called the SIBLING protein family (small integrin-binding ligand N-linked glycoproteins) [42,48], this being part of a larger group named the SCPP proteins (secretory calcium-binding phosphoproteins) [49].

In terms of the inorganic phase of bone, mineral has been discussed in the preceding section, where mounting evidence implies that prior to carbonato-apatite crystallization there may in addition exist a combination of mineral phases including octacalcium phosphate and amorphous calcium phosphate, as will be also discussed later. With phosphate (and to some extent carbonate) being a central feature of both the organic and inorganic phases in bone, and because of the nanoscale dimensions of bone crystallites interfacing with similarly sized protein moieties – all in the presence of structural water – boundaries are blurred [50]. Indeed, there may be shared (integrated) ternary complexes involving protein/mineral, calcium and phosphate groups that define the very nanoscale interface between organics and inorganics that is so central to the extraordinary mechanical properties of bone. Overlain onto this interfacial and interphase integration complexity is the fact that SIBLING proteins are remarkably intrinsically disordered [51–53] and highly negatively charged. They have vast stretches of negative charge arising from a preponderance of carboxylate-rich, acidic Asp and Glu residues, and from the organic phosphate groups from phosphoserines, all of which can bind large amounts of calcium, either in solution or as part of the mineral's crystalline lattice surface. Likewise, electrostatic interactions occur between collagen and small proteoglycans that are also negatively charged and highly hydrated (SLRPs, small leucine-rich proteoglycans), and can interact with positively charged cations [54]. Finally, both organic and inorganic constituents have a surface layer of bound water molecules that fulfils an often-overlooked structural role, being far more than simply a diffusion and reaction medium [55–61].

At the next level – the submicrometer scale – collagen forms ordered and disordered arrays in lamellar bone [19,62,63]. The ordered collagen fibrils of the extracellular matrix form alternating 3D assemblies arranged as planar arrays of gently twisted bundles [64], to render the extracellular matrix more isotropic at the micrometer scale. Both ordered and disordered collagen arrays mineralize to a similar extent, but with the additional space between the collagen fibrils in the disordered regions, this interfibrillar compartment is likely richer in noncollagenous proteins, proteoglycans and small molecules, and collectively has been described as “interfibrillar ground matter” or historically “amorphous ground substance” because of its granular appearance by early-era light microscopy.

At the micrometer level, in all vertebrates except certain teleost fish [65], the osteocyte cellular network enters into the 3D landscape. This network is a vast cellular and dendritic system allowing direct communication to cells at the bone surface or in distant tissues, with primarily a mechanosensing [66] and endocrine function [67]. Likely it is also involved in mobilizing calcium (and phosphorous) from its enormous surface area lining the lacuno-canalicular network in which the osteocyte cells and their cell processes lie [68–70]. While the osteocyte network is naturally integrated within alternating order-disorder lamellar arrays of mineralized matrix, cellular bodies with their long dendritic projections are directly engulfed within the feltwork of disordered collagen fibrils. Such disordered “padding” around osteocytes and their processes may vary in thickness from the usual 100–200 nm to substantial volumes of up to 1 μm in the vicinity of an osteocyte cell body [71]. At the level of hundreds of microns, concentric lamellar assemblies of compact bone called osteons (also called Haversian systems) can be distinguished, as well as overlapping lamellar packets that compose individual struts of trabecular bone, all resulting from the bone remodeling activity initiated by osteoclasts. Newer osteons cut into older osteons of previous generations (then called interstitial osteons), and newer lamellar packets overlap older lamellar packets, collectively forming a characteristic patchwork appearance. The interfaces between these entities are called cement lines/planes. Cement planes are rich in noncollagenous protein – notably osteopontin [36,46] – and the regularity of their profiles varies with the state of the matrix organization at the moment osteoclastic resorption ceased. An important feature of cement planes is that biomechanically under loading, they allow for microcrack deflection, allowing for energy dissipation [72]. During their initial formation, the proteins there – including osteopontin with its integrin-binding RGD ligand and other cell adhesion ligands – likely allow for cell dynamics and cell adhesion at the early bone formation/replacement stage after resorption by osteoclasts [36]. Of note in this context, lamellar structures are generally the same in compact and in trabecular bone [73]. However, in a typical osteon of about 200 μms in diameter, there would usually be only one cement plane surrounding its entire cylindrical shape, whereas in a single trabecula of the same dimensions there would be numerous cement planes with their collective surface exceeding that of an osteon.

At the macroscopic anatomical level, the composition and structure of bone achieves another tier of intricacy as it interfaces with soft connective tissues to form entheses at ligament and tendon insertions, sutures, symphyses, growth plates and their vestiges, articular cartilage at joints and cartilaginous elements at many other locations, middle ear attachments, vascular and nerve canals, and marrow – all of which wholly make up a functional skeleton. According to a recent count of the nested tiers of bone organization from crude components to entire organismal frames, the organization of bone has 12 levels of hierarchy [74].

## 4. Mineralization mechanisms in bone

Mineralization of skeletons (including teeth) is a remarkably robust process in biology whose importance is underscored by the fact that there is almost always some degree of mineralization that occurs regardless of the severity of any particular disease and regardless of the gene mutations involved. Even in the most severe cases of osteomalacic diseases – like for example in perinatal and infantile hypophosphatasia – there is generally nevertheless some amount of skeletal mineralization. Countless spontaneous, chemically induced and transgenic mouse models having a defective mineralization phenotype still maintain some degree of mineralization. This underscores the evolutionary importance of biomineralization since Cambrian times, and indicates that Nature uses multiple ways to achieve mineralization such that a failure in one pathway often still is compatible with life, at least in higher vertebrates.

At the very core of vertebrate mineralization is the requirement for a suitable extracellular matrix – a scaffold so to speak – that is preformed and primed to be receptive to mineral deposition [75]. Not surprisingly for mineralized tissues (since indeed they are specialized connective tissues), secretory activity from resident cells creates a collagenous scaffold (with many other components) which *a priori* prescribes a fabricated template that will ultimately accrue its mineral. Heterogeneous mineral deposition events in bone occur at discrete locations within, at the surface of, and between collagen fibrils [76,77]. Thus, the beginning of the mineralization storybook in vertebrates, as we describe below for bone, starts with a collagen-based extracellular matrix whose composition and structure essentially remains the same throughout its chapters, with of course some variations depending upon the bone tissue type, location and age (Note: an exception to this is

tooth enamel, being of epithelial cell origin, which still has an extracellular matrix – but not collagen – during its development, an organic matrix which is almost entirely removed through the action of enzymes during its maturation prior to tooth eruption). Beyond systemic mineral ion homeostasis and renal phosphate wasting pathways, and other systemic factors influencing mineralization that are covered elsewhere by many fine reviews [78–84], we begin here by briefly outlining three concepts by which early mineralization in bone is thought to be regulated locally in the extracellular matrix: by matrix vesicles, by amorphous mineral precursors, and by enzymatic degradation of mineralization inhibitors in the extracellular matrix. These concepts governing the regulation of mineralization to be discussed below are depicted schematically in Fig. 2.

### 4.1. Matrix vesicles

One way to mineralize a vertebrate extracellular matrix appears to be through the cellular release (shedding/blebbing) from the plasma membrane of small (100–300 μm diameter) so-called matrix vesicles [85–88,90,91]. These osteoblast/osteocyte-shed matrix vesicles are not exosomes (which are smaller) destined for some remote location, but rather they are distinct entities that "seed" locally the extracellular matrix with phospholipid bilayer-bounded, roughly spherical packages of mineral ions and enzymes that provide the molecular machinery to induce a cascade of compartmentalized mineralization events. These events ultimately produce apatitic crystals within the interior of the vesicles. Additional growth and elongation of these crystals (at this point isolated from the extracellular matrix by the bounding membrane) occurs within the vesicle until the abundance and size of the crystals rupture the membrane, by way of biomineralization analogy much like the hatching of a chick (with its apatite-containing skeleton) from its calcitic shell. Another possibility for this rupture is that the membrane itself biochemically degrades through its own mechanisms at roughly the same time that the crystals are ready to be presented to the extracellular matrix. In either case, the net result is that calcium and phosphate ions come together within the vesicle to form crystalline

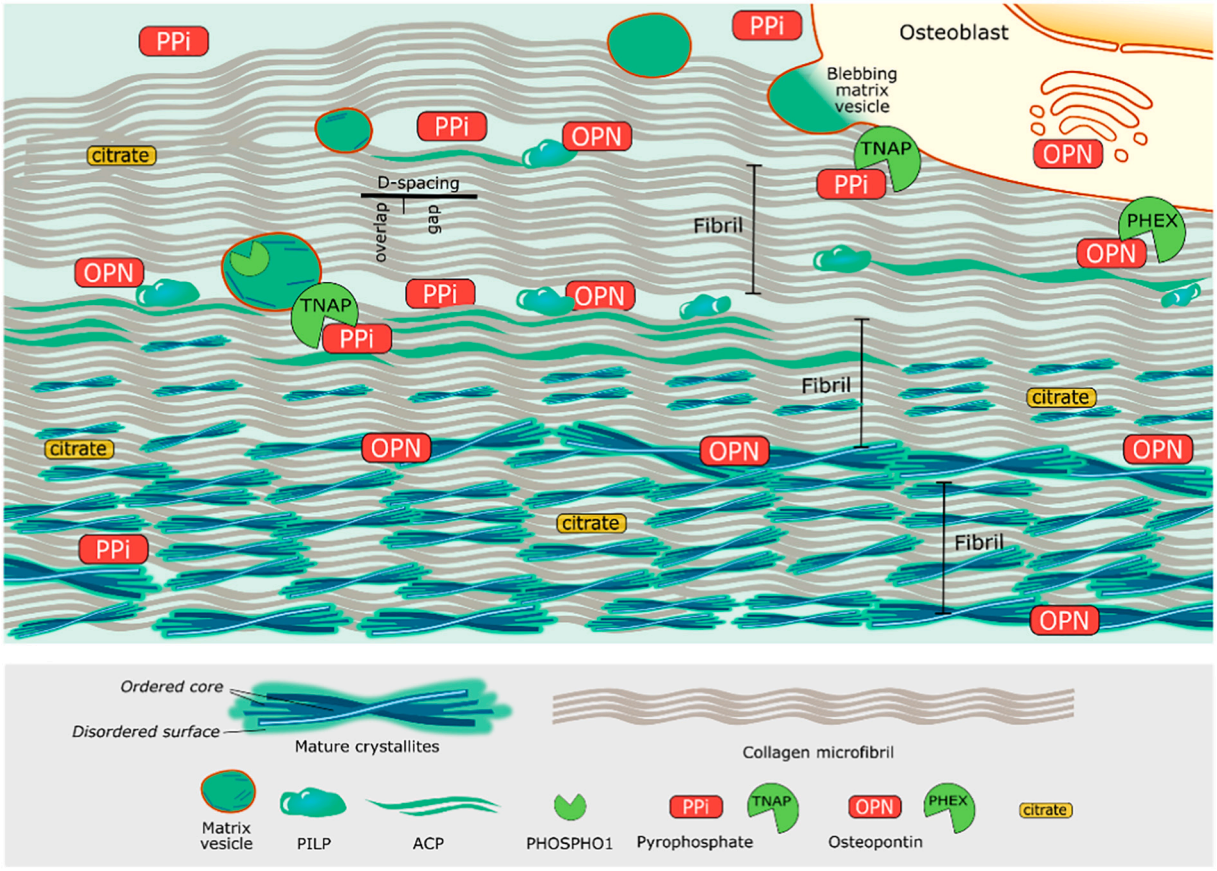


**Fig. 2.** Factors regulating extracellular matrix mineralization in the skeleton. Extracellular matrix in bone contains abundant crosslinked and branching collagen fibrils, noncollagenous proteins (notably the SIBLING protein family that includes the mineralization inhibitor osteopontin, OPN), small proteoglycans (SLRPs, small leucine-rich proteoglycans), growth factors, serum proteins, and many small bioactive molecules (such as citrate, and mineralization-inhibiting pyrophosphate, $PP_i$). With appropriate levels of mineral ions, and with tissue-specific local expression of genes by osteoblasts and osteocytes to produce mineralization inhibitor-degrading enzymes (such as TNAP to degrade $PP_i$, and PHEX to degrade OPN), incipient mineralization events and their progression are shown from top to bottom. No one mechanism accounts for this mineralization pattern – multiple pathways contribute to forming mineralized bone tissue where hierarchical and intertwining fractal organization of matrix and mineral are established. Gently twisting crossfibrillar crystallites reside in different extracellular matrix compartments, being either intra- or interfibrillar [74]. Through either broad heterogenous precipitation of mineral between, along and within collagen fibrils, or through seeded matrix vesicle depositions of mineral at specific sites in the matrix, bone mineralizes continuously and extensively throughout the organic matrix. Rather than directly nucleating and growing through classical crystal growth mechanisms, it is now widely thought that mineral forms and is sculpted at the nanoscale through the continuum of a precursor pathway. This pathway involves mineral ion accretion, sequestration, condensation, stabilization, and fluidic transport as an amorphous liquid precursor (PILP process, polymer/peptide-induced liquid precursor), finally ending with pre-stressed (often curved) carbonate-substituted apatite crystallites permeating all compartments of the extracellular matrix. Crystallites are inhomogeneous and contain an ordered, crystalline lattice-structured core several nanometers thick surrounded by a more disordered and dynamic hydrated superficial layer (~0.8-nm-thick) of mineral and counter ions, disordered mineral, and a variety of small molecules and organic moieties. This schematic is not drawn to precise scale. TNAP, Tissue-nonspecific alkaline phosphatase (also often abbreviated as TNSALP, ALPL). PHEX, Phosphate-regulating endopeptidase homolog X-linked.

apatitic mineral that is then exposed to the extracellular matrix and its constituent regulatory biomolecules. Such a seeding approach for bulk bone mineralization has the advantage of precisely placing dispersed mineral packages at exact locations with the extracellular matrix from which mineralization may subsequently propagate in all directions throughout the matrix.

In the developing tooth, during the formation of mantle dentin at the dentino-enamel junction, targeted release and placement of matrix vesicles by newly formed odontoblasts initiates at precise locations in the extracellular matrix the very first mineralization of the tooth [92–95], this mineralized mantle dentin serving as a hardened substrate upon which enamel and cementum formation and mineralization subsequently occur. Matrix vesicles are difficult to discern in the extracellular matrix of bone using conventional light and electron microscopies, and they can readily be confused with cross-sections of cell projections from osteoblasts and osteocytes which can be about the same size. To visualize their numbers and distribution in bone more clearly, Takano and colleagues used bisphosphonate (etidronate; 1-hydroxyethylidene-1,1-bisphosphonate, HEBP) loading to block bulk mineralization in rat bone, and the extent, distribution and spacing of matrix vesicles then became readily apparent by conventional transmission electron microscopy [96]. Of note from this Takano et al. study was that crystals of apatite still formed within the matrix vesicles (the inhibitory bisphosphonate apparently was excluded from the vesicular contents), but once exposed to bisphosphonate after vesicle membrane rupture, they failed to propagate additional mineralization under these experimental conditions. Under normal physiological conditions, once matrix vesicles rupture, the mineral they contain then falls under the influence of extracellular matrix molecules that will further guide mineralization events that were initiated independently within the vesicles [97,98], thus linking the two processes. Important enzymes related to mineralization and residing at the matrix vesicle membrane include PHOSPHO1 acting internally to release phosphate ions for initial mineral deposition within the vesicle, and TNAP which degrades inhibitory $PP_i$ and OPN outside the vesicle – all of which promote extracellular matrix mineralization in the vicinity of the vesicles [97,99].

### 4.2. Amorphous mineral precursors

As opposed to the idealized processes and structures represented strictly by chemical formulas, bone mineral forms, grows and matures in an exceptionally interactive and crowded aqueous environment of structural and regulatory proteins, peptides, amino acids, proteoglycans, polysaccharides, small organic and inorganic molecules, and mineral ions. In such a crowded extracellular aqueous milieu, even water is a structural component rather than just a medium. Thus, the only possible scenario for crystal formation is through heterogeneous nucleation, and once started, its trajectory is tightly controlled by various regulatory adjustments and deviations from a classic precipitation path as would otherwise occur abiotically.

The first notions on inconsistencies between the properties of the inorganic phase of bone and classic crystallization theory appeared soon after the identification of the mineral as being a carbonate-substituted apatite by McConnell in 1952 [100]. Neuman and Neuman [101] reported that bone mineralization proceeds through a metastable phase, which was quantified and identified as being amorphous calcium phosphate (ACP) [102,103] and octacalcium phosphate [104]. The very fact that bone mineral likely forms through disordered (as apposed to crystalline) precursors is presumably a direct consequence of the sequestration and stabilization of mineral ions by diverse organic moieties. Such molecules include negatively charged phosphorylated peptides and proteins (often intrinsically disordered [105,106]) with abundant Asp, Glu and phosphoserine content, often clustered together into acidic amino acid sequence stretches such as polyAsp and ASARM – the best-studied example of this being the SIBLING phosphoprotein OPN and its peptides [36,41,107–109]. Circulating proteins that infiltrate the tissue fluids of mineralized tissues also have mineral ion-binding properties and likewise may influence mineralization processes – the best-studied example being fetuin-A [110,111]. Here, an analogy can be drawn to similar features and function for the phosphoprotein casein as found in breast milk, thought to prevent phase separation (mineralization) in this calcium-rich fluid (but in this case lacking an extracellular matrix to mineralize) [112] – of note, OPN is also abundant in milk and binds calcium ions with high affinity [40,113–115]. In mineralized tissues, such organic sequestration of mineral ions can result in a spectrum of nonstoichiometric mineral polymorphs. There is evidence that the polymorphs do not necessarily appear in a unidirectional sequence according to the extent of order as described by Ostwald's ripening rule, but rather, depending on the conditions, the precursor may first dissolve altogether in order to progress towards a more-crystalline ordered phase [116].

In the crowded extracellular environment of bone, almost every component has been reported at one time or another to stabilize the amorphous phase. For example, the crosslinked collagen that forms continuous, covalently bound assemblies stabilizes ACP by template confinement [117]. Finite-element simulation of ion diffusion has shown that constricting the volume of reaction to a narrow gap less than 1-μm-thick noticeably impedes ion diffusion and results in a local depletion zone of ions around the growing crystallite [118,119]. Thus, the resulting crystal size is quite limited in the simulation model; however, the size-capping effect might be even more pronounced in reality in bone because the water available within the confined space is in fact partially structured by the charged organic moieties.

In addition to there being space limitations in the extracellular matrix of bone, the reactive agents contained therein are rich in carbonate/bicarbonate ions that make up to 5% of the weight of bone [101]. The presence of carbonate on the one hand stabilizes ACP by maintaining local depletion with respect to phosphate [120], while on the other hand, bicarbonate is a product of carbonic anhydrase, a ubiquitous and highly efficient/fast enzyme used by cells to rapidly control pH in their environment [121]. While the well-known utilization of low pH for bone mineral dissolution during resorption by osteoclasts (and as another example, during avian eggshell thinning induced by similar cell activity in the chorioallantoic membrane), new evidence points to a drop in pH as being responsible for activating available calcium ions to facilitate the transition between two hydrated phases of bone mineral – from ACP I to ACP II [122]. This apparently occurs through preferential dehydration of the hydration shell of calcium ions, and to a lesser extent of phosphate ions. Jiang et al. [123] also confirmed that in an ACP-saturated environment under constant temperature, a decrease in pH facilitates the transition of ACP to apatite crystallization by liberating calcium ions from their hydration shells. The same authors observed that under the same conditions, the addition of acidic amino acid polymer had an opposite effect – prolonging the crystallization time of the ACP-to-apatite transition. Interestingly, while calcium activity is sensitive to the presence of protons, the addition of polyanionic[2] polymer may alter ionic calcium activity and stabilize ACP by other mechanisms [124]. The polymer-induced liquid precursor (PILP) process generates a relatively long-lasting, metastable calcium- and phosphate-containing amorphous phase of sequestered ion clusters that infiltrates into the intrafibrillar confined space of a collagenous template, and eventually loses a certain proportion of associated water. In this process, the phase undergoes an amorphous-to-crystalline transformation to acquire more thermodynamically stable long-range crystalline order [125,126]. The appeal of this theory is that it explains many anomalous features of bone mineral type and organization in the mineral-collagen assembly. These include for example, *i*) co-alignment of elongated crystallites having enormous collective surface area within the

[2] The term “polyanionic polymer” is technically a more precise term than “acidic polymer” because at the pH of biological systems, or in corresponding *in vitro* models, a significant proportion of the aforementioned polymers would be deprotonated. In fact, it is the net negative charge of an anionic constituent, and not the collective activity of protons, that exerts the structuring action on ions and water dipoles.

matrix [127], *ii*) the orientation of the *c*-axis with the longest dimension of the crystallites [10,126,128], *iii*) the curvature of individual crystals [35,74,129,130], and *iv*) the fact that upon deproteinization, bone specimens do not disassemble but they retain their size and shape [126,131,132].

The PILP concept, originally described by the Gower lab using reconstituted collagen [133,134], has been demonstrated *in vitro* using cryo-TEM to capture the presence of nanoparticle clusters lining up to enter the collagen fibrils at the gap-zone regions [135]. This correlates with cryo-SEM observations captured *in situ* in forming mouse bone [136], which revealed amorphous mineral-bearing globules fusing onto collagen fibrils in the osteoid extracellular matrix. This also relates to other *in vitro* studies showing that polyelectrolyte (polycarboxylic acid) covalently bound to collagen caches chain-like aggregates of mineralization precursors along the fibrillar surface [137], and that both short- and long-range interactions involving electroneutrality and osmotic equilibrium need to be simultaneously balanced [220], all of which may provide driving forces for infiltration of mineral precursors into the water compartments within collagen fibrils. Of note, in the biogenic mineralization pathway occurring through amorphous precursors, the chronologic phase transition remains incomplete – even when precursor crystallization occurs within the core of mineral formations, their periphery remains disordered, nonstoichiometric and labile [50], and rich in bound water [61], substitutions [138], associated organic citrate [139], and inorganic molecules [140]. This labile shell structure apparently persists on the surface of the crystalline core and participates in metabolic reactions [141]. With bone maturation (and to a larger extent with diagenesis), the proportion of the labile peripheral disordered phase decreases, and, overall, mineral becomes more inert [101,141]. The stochastic bone remodeling that continues throughout life aims at recycling and renewal of bone mineral (and also matrix of course), thus keeping mineral interfaces labile and metabolically active, not only for the sake of continuity of chemical processes, but also for maintenance in the optimal range of the mechanical properties of bone [72,142].

### 4.3. Enzymatic degradation of mineralization inhibitors in the extracellular matrix

Beyond the earliest mineral nucleation mechanisms which remain poorly understood in bone, guidance/regulation of mineralization, once initiated, seems to involve the participation of a number of enzymes that degrade well-known inhibitors of mineralization. Without these enzymes, or with decreases in their activity, osteomalacia and odontomalacia set in, these terms referring to a class of disease that leads to soft bones and teeth (hypomineralization). Specific examples of osteomalacic diseases discussed below include hypophosphatasia [143–145] and X-linked hypophosphatemia [146–150]. Previous *in vivo* work done primarily in transgenic mouse models, and more recent work in humans through successful clinical trials and drug development with products now on the market, have now clearly established the importance of these enzyme-mineralization inhibitor/substrate relationships acting in the extracellular matrix. Here we highlight two of these relationships, that between the ectoenzyme tissue-nonspecific alkaline phosphatase (TNAP, TNSALP, ALPL) and the inhibitor/substrate pyrophosphate ($PP_i$), and that between the phosphate-regulating endopeptidase homolog X-linked (PHEX) and the inhibitor/substrate osteopontin (OPN, SPP1 - secreted phosphoprotein 1). Each relationship relies upon enzymatic inactivation of its substrate, a kind of "inhibiting the inhibitor" by its degradation, so to speak. Building upon foundational work by Neuman and Fleisch [151] on inhibition of mineralization in supersaturated solutions of mineral ions, we also discuss below the notion that there may be a kind of coarse- and fine-tuning of mineralization by the sequential actions of these enzyme-substrate relationship pairs, respectively (Figs. 1–3).

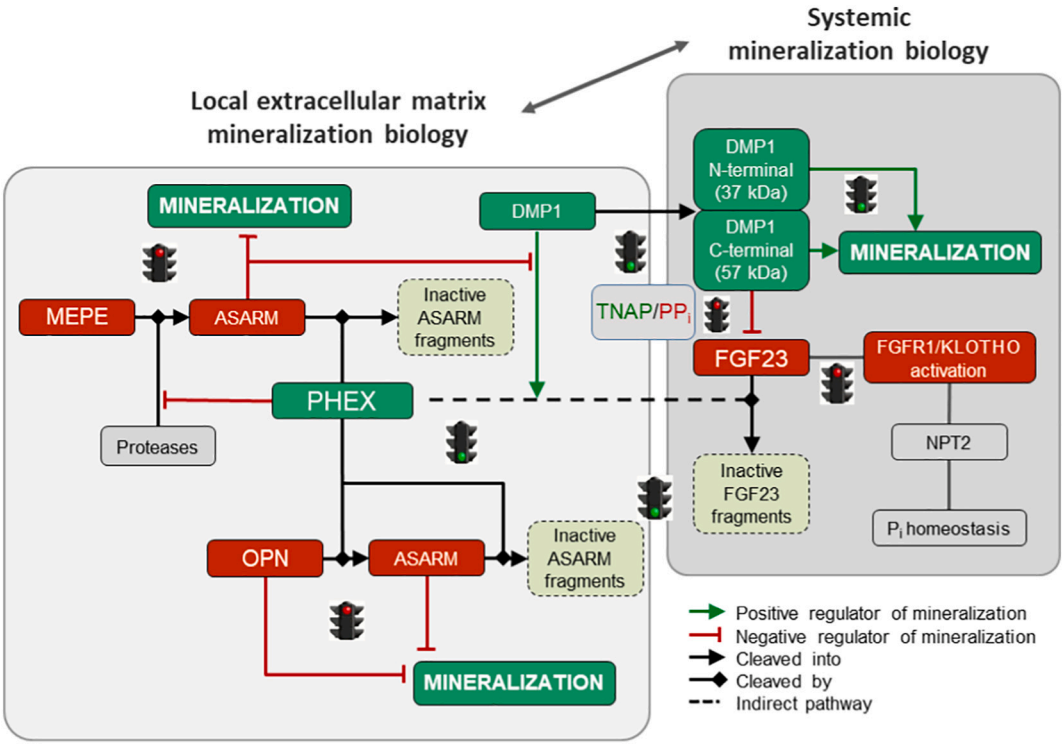


**Fig. 3.** Pathways regulating mineralization in the skeleton. Complex, interdependent, and integrated pathways converge and diverge to regulate mineralization in bone. Control of mineralization resides in the combined actions of systemic circulating factors (principally hormones called phosphatonins), and local factors in the extracellular matrix of bone (principally enyzme-substrate interactions). Together, they respectively determine mineral ion homeostasis to create appropriate circulating serum ion levels in the blood, and establish the temporospatial pattern of mineralization locally in the extracellular matrix of the skeleton. DMP1, Dentin Matrix Protein 1; MEPE, Matrix Extracellular Phosphoglycoprotein; ASARM, Acidic Serine and Aspartate-Rich Motif; PHEX, phosphate-regulating endopeptidase homolog X-linked; OPN, osteopontin; FGF23, Fibroblast Growth Factor 23; FGFR1, Fibroblast Growth Factor Receptor 1; KLOTHO; NPT2, Type II Na-Pi Co-transporter. Modified from [168].

The first-known and widely studied potent mineralization inhibitor with relevance to bone biology was pyrophosphate, with the first seminal studies being performed in the 1960s by the groups led by Neuman, Fleisch and Russell. Pyrophosphate ($PP_i$) – composed of two phosphate molecules linked by an oxygen bond – is found widely in Nature, and pivotally regulates physiologic and pathologic mineralization by acting as a potent inhibitor of crystal precipitation, growth, and dissolution [152–156]. Local tissue concentrations of $PP_i$ are controlled by a number of regulatory enzymes and transporters, notably ectonucleotide pyrophosphatase/phosphodiesterase-1 (ENPP1) and the progressive ankylosis protein (ANK) – these two proteins can increase $PP_i$ locally in tissues, although the role for ANK in this process is less clear than it is for ENPP1 [157]. ANK is a multi-pass transmembrane protein that transports intracellular $PP_i$ into the extracellular space [158–160]. ENPP1 increases extracellular $PP_i$ by hydrolysis of nucleotide triphosphates [159]. Countering this – being highly expressed by the resident cells of bones and teeth – the ectoenzyme TNAP hydrolyzes $PP_i$ to release two phosphates [161], thus providing a balancing mechanism to control the concentration of this potent mineralization inhibitor (and also the $P_i$:$PP_i$ ratio) locally in the extracellular matrix of bone. This balance is critical for proper skeletal mineralization [162]. Of note, TNAP can also remove organic phosphate side-groups from phosphorylated OPN, reducing its mineralization-inhibiting function [152,163]. Thus, in the extracellular matrix of bone, the enzymatic degradation of $PP_i$ by TNAP (together with the dephosphorylation of OPN and potentially of other matrix proteins by TNAP) controls the $P_i$:$PP_i$ ratio in favor of mineral deposition. Deficiency of TNAP activity leading to extracellular and circulating accumulations of inhibitory $PP_i$ characterizes the rare osteomalacic/odontomalacic bone and tooth disease hypophosphatasia (HPP), a heritable disorder presenting hypomineralization of the skeleton and dentition [164–166,168].

Clinical manifestations of hypophosphatasia vary from stillbirth (where there is almost no skeletal mineralization) to tooth loss being the only symptom. Besides the osteomalacia (osteoidosis) in HPP, the typical and striking oral manifestation of hypophosphatasia occurring early in life is premature and atraumatic loss of rooted primary teeth. This occurs as a result of defective mineralization caused by the accumulation of $PP_i$ in tooth cementum and alveolar jawbone leading to a weak periodontal ligament attachment of the tooth in its alveolar socket [166,169]. Derived from these findings and pre-clinical work in mice by the Millan, Whyte and McKee groups working closely with Enobia Pharma in Montreal (acquired by Alexion) in the late 2000s [170,171], additional clinical work using the same mineral-targeting (polyAsp, D10 decapeptide) form of TNAP (asfotase alfa) as an enzyme-replacement therapy in HPP patients confirmed this enzyme-substrate action in humans [144]. Many countries worldwide since 2015 have now approved the use of this mineral-binding form of TNAP for HPP therapy.

The first genetic *in vivo* demonstration of mineralization inhibition by proteins was the publication by Luo et al. in 1997, describing the fully penetrant phenotype of massive vascular mineralization in mice lacking matrix Gla protein (MGP) [172]. However, to date, most *in vivo* evidence for the direct inhibition of mineralization by proteins in vertebrates has been obtained from work done on the SIBLING phosphoprotein osteopontin (OPN) [173]. While remarkably OPN-knockout mice show generally normal skeletal mineralization [174], indeed there are some locations in these mice that show increased mineralization and crystallinity [175] as would be expected for the loss of an inhibitor. One possible explanation for the lack of a major mineralization phenotype in OPN-deficient mice may be that the members of the SIBLING protein family [176] all have many similarities – particularly conserved calcium- and mineral-binding acidic sequences with abundant Asp and Glu – and thus there may be built-in protein redundancy for inhibiting mineralization in this case of the $Opn^{-/-}$ mice. Indeed, the SIBLING proteins are believed to have arisen from gene duplication events of SPARC or a SPARC-like ancestor [42], and it would thus be reasonable to consider that collectively they could replace some of the functions of OPN in bone, including its mineralization-inhibiting function. In many other examples of transgenic mice showing osteomalacic hypomineralization phenotypes (including TNAP-deficient $Alpl^{-/-}$ mice [162,177] and *Hyp* mice [178,179], OPN is invariably upregulated in a manner that often parallels $PP_i$ levels. Thus, we consider that there is a duality in mineralization inhibition in bone, with $PP_i$ and OPN often working in tandem, and this has led us to propose the enzyme-substrate Stenciling Principle as summarized in the next section. Prior to this, it is appropriate now to review the relationship between OPN and the enzyme PHEX which degrades/inactivates it.

Inactivating mutations in the initially named *PEX* gene – later renamed as *PHEX* (phosphate-regulating endopeptidase homolog X-linked) – was identified in 1995 as the cause of the most prevalent form of inherited rickets in humans, that being X-linked hypophosphatemia (XLH) [180]. The *PHEX* gene encodes PHEX protein, an ~86 kDa membrane-bound zinc-metallopeptidase that belongs to the M13 family of peptidases and which is expressed predominantly by osteoblasts, osteocytes, odontoblasts and cementocytes [181–185]. Although assays to assess PHEX proteolytic activity *in vivo* are not yet available, it is clearly evident that the loss of PHEX (or decreased PHEX activity), underlies the XLH phenotype. In XLH and in the commonly used murine model of this disease (*Hyp* mice lacking PHEX), an increase in circulating fibroblast growth factor-23 (FGF23) leads to renal phosphate wasting [186,187], with low serum $P_i$ levels being a major cause of the extensive osteomalacia associated with this disease. In XLH, the osteomalacia (hypomineralization) causes bones to deform and pseudofracture, and the odontomalacia results in teeth becoming infected, often requiring their extraction.

Several studies have noted that full-length MEPE and MEPE peptides are increased in XLH/*Hyp* [188–191], which is consistent with our demonstration that the MEPE ASARM peptide is a substrate for PHEX [192]. Another report has described that PHEX protects MEPE from cleavage [193]. Although biochemical experiments demonstrated that full-length FGF23 and MEPE are not substrates of PHEX [188,189,194,195], some synthetic small FRET (fluorescence resonance energy transfer) peptide sequences derived from these proteins can be hydrolyzed by PHEX, with distinct catalytic efficiencies [196]. Importantly, we demonstrated that the MEPE ASARM peptide (and the OPN ASARM peptide and full-length OPN) are efficiently degraded by PHEX [41,178,192]; for degradation, there is a strict specificity for residues with negative charge (Asp and Glu) at the P1′ position [196], and this is a determinant for PHEX action.

Proteins of the SIBLING family have an acidic serine- and aspartate-rich motif (ASARM) that is highly conserved across species [197,198]. Besides having abundant Asp and Glu, the ASARM peptide contains serine residues that can be phosphorylated [199–201]. The ASARM peptide is located in the C-terminal region of all SIBLING proteins, except for osteopontin, where it is located in the middle of the protein. In most vertebrates, the ASARM peptide appears to have evolved to regulate mineralization, extending from eggshell to mammalian bone [202]. These acidic peptides are generally highly resistant to proteolysis and are potent inhibitors of bone and dentin mineralization [41,192,203]. However, the ASARM peptide can be selectively degraded by PHEX through multiple internal cleavage sites [41,192], and thus cleared from the local extracellular matrix environment where mineralization is required as dictated by high levels of PHEX expression by osteoblasts and osteocytes (and odontoblasts) [41,178,192].

Following this work showing cleavage of the mineralization-inhibiting MEPE and OPN ASARM peptides by PHEX, further attention was then given to full-length OPN which potently blocks mineralization [152]. Time-course, enzyme-substrate PHEX-OPN degradation assays analyzed by mass spectrometry, gel electrophoresis protein profiles of *Hyp* bone extracts, immunolabeling of OPN and OPN-fragment accumulation in *Hyp* mice [178], and bone and tooth dentin extracts from XLH patients [204], all demonstrated that OPN was a physiologically relevant substrate for PHEX. The work in *Hyp* mice and in XLH patient

biopsies clearly aligned with the notion that accumulated OPN in the extracellular matrices of bones and teeth in the absence of PHEX was a local contributing factor to the defective mineralization that occurs in XLH/*Hyp*. Remarkable was the finding of an extensive degradation of OPN over multiple dozens of cleavage sites (up to 5 cleavage sites in the ASARM peptide [41,178]) that essentially would inactivate such a protein inhibitor of mineralization in healthy bone where robust mineralization is required. In XLH bone – having decreased/absent PHEX activity – particularly noteworthy in addition to the generalized increase of OPN in bone was the observation of an abundant accumulation of inhibitory OPN and/or OPN fragments in the osteocyte lacuno-canalicular system. The overabundance of OPN at this site presumably functions to inhibit mineralization locally to create the hypomineralized peri-osteocytic lesions (POLs, also called halos [204]). In healthy bone, only a thin coating of OPN is normally found at the osteocyte cell-matrix interface [36,46] – at the *lamina limitans* – a matrix protein structure lining osteocyte lacunae and their canaliculi. The peri-osteocytic lesions are a hallmark characteristic of XLH [205], and such hypomineralized pliant lesions in this disease most certainly adversely affect the mechanosensing signaling output of the osteocyte network that results in aberrant bone remodeling. The altered pericellular composition and mineralization status of the POLs might also influence FGF23 production by osteocytes.

## 5. The stenciling principle – a concept for templated stenciling of mineralization in the skeleton

The concept that mineralization might be a default pathway is not new, but was suggested by earlier work on the small-molecule pyrophosphate by the Neuman, Fleisch and Russell labs [206], and by work on matrix Gla protein originally by the Karsenty and McKee labs [172], and as followed up more recently by the Murshed lab [207,208] and Hunter and Goldberg labs [209]. While the work on the small-molecule pyrophosphate extends back to the mid-20th century, genetic evidence that a *protein* could act *in vivo* in a similar inhibitory manner towards mineralization was only first demonstrated much more recently in 1997 for matrix Gla protein [172]. Whereas the matrix Gla protein work identified a key inhibitor of blood vessel mineralization, subsequent work over many years established the importance of osteopontin as an inhibitor of mineralization in bone, with major contributions on mechanisms of OPN action coming from the labs of Boskey [52,199,210], Giachelli [211,212], Hunter and Goldberg [213–215], Sorensen [200,216], Millan [162] and McKee [173] using a variety model systems. Collectively, this work, and the work of others of course, laid the foundation for the notion of what we call the *Stenciling Principle* of mineralization in the skeleton. In this concept, where calcium and phosphate ions are normally abundant, there is a specific expression

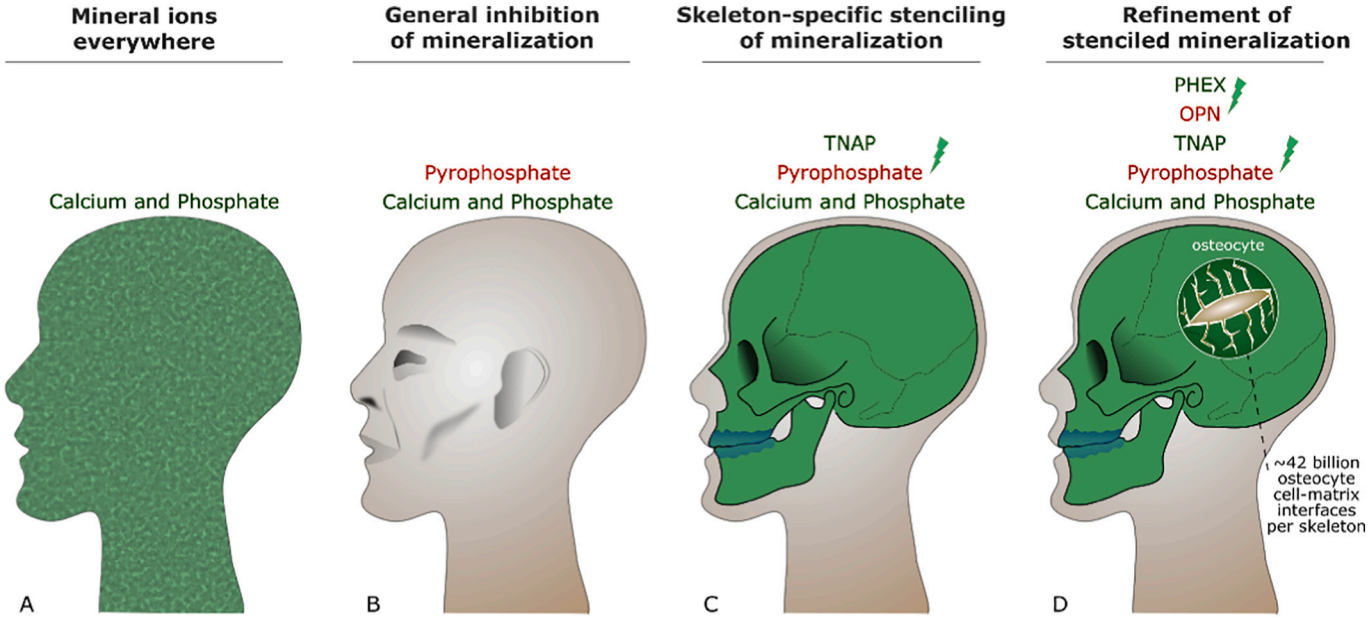


**Fig. 4.** The Stenciling Principle for mineralization of the skeleton. (A) Given adequate nutrition in a healthy individual, mineral ion homeostasis mechanisms acting across multiple organ systems results in systemic circulating and tissue fluids rich in calcium and phosphate throughout most tissue compartments. (**B**) Potential mineralization events that might readily occur throughout the body from high calcium and phosphate levels are generally inhibited everywhere in all tissues by the ubiquitous presence of abundant pyrophosphate $PP_i$ – a potent, small-molecule inhibitor of mineralization produced by many metabolic pathways. This generalized organismal inhibition prevents tissues intended to be "soft" from mineralizing as a default pathway, given the high levels of available mineral ions. (**C**) However, in the skeleton, the specific expression by cells in bones and teeth of tissue-nonspecific alkaline phosphatase (TNAP, TNSALP, ALPL) – an enzyme which degrades its inhibitory substrate pyrophosphate – results in mineralization being precisely "stenciled" at specific, connective tissue sites within the pre-formed extracellular matrix characteristic of skeletal mineralized tissues. (**D**) This so-called *Stenciling Principle* can be extended to the actions of regulatory proteins (such as osteopontin, OPN) that likewise inhibit/regulate mineralization by sequestering mineral ions (and possibly also by stabilizing an amorphous calcium-phosphate precursor phase) and by binding to (and thus slowing growth of) established mineral crystals in the extracellular matrix. Compared to the generalized inhibitory action of pyrophosphate, a refinement of mineralization patterns by the sustained action of inhibitory proteins such as OPN might provide a mechanism whereby precise control is exerted at discrete extracellular matrix locations, including at cell-matrix interfaces [219] (in the osteocyte/lacuno-canalicular system) where mineralization likely exists in a state of flux. Degradation of inhibitory OPN (thus a release from mineralization inhibition) proceeds through the enzymatic actions of PHEX (phosphate-regulating endopeptidase homolog X-linked (PHEX) expressed locally by bone and tooth cells.

pattern of enzymes by cells, at precise locations in the skeleton, that biologically "stencils" into a pre-formed extracellular matrix loaded with inhibitors a precisely patterned, inhibitor-degrading dose of enzyme to form a mineralized tissue. With such precision, neighboring connective tissue destined to be soft remains soft, whereas the enzyme-stenciled regions harden by mineralization to provide the functions of the skeleton and teeth. The Stenciling Principle does not preclude multiple levels of "release from inhibition", as multiple passes (rounds) may be required having different timings and concentrations of the active participants. Here, we describe two rounds for release of inhibition – the first round being the degradation of inhibitory pyrophosphate by the enzyme TNAP to "kickstart" the process of mineralization, and the second round being more subtle and sustained (as might be expected from a protein-processing event), as a refinement of mineralization through the gradual degradation of inhibitory OPN by the enzyme PHEX (Fig. 4). Whether dysregulation of these (or other) enzyme-substrate reactions affecting mineralization act at ectopic calcification sites remains to be determined, but calcification of ligament/tendon entheses is common in XLH [217,218], and this seems particularly worthy of further study.

As the old adage states, "the devil is in the details". For skeletons, the key to structural and functional success is in its cell-matrix-mineral interfaces. For the three major determinants of mineralization – these being 1) the ubiquitous presence of mineral ions, 2) the removal of general mineralization inhibition, and 3) the refinement of local inhibition – each has a progressively refined and specialized role. Ions such as calcium and phosphate are used in myriad key metabolic processes, and are central to life itself. However, their abundance and propensity to adversely precipitate as mineral requires a generalized inhibition. Selective removal of such inhibition ("inhibiting the inhibitor") to allow mineralization can be used to define the size, shape and layout of an organism (from *in utero* through to adulthood). As part of this process there is the third tier of mineralization regulation – the fine interfacial enzyme-stenciling control that provides refinement for mechanical resilience, metabolic responsiveness, morphological precision and sensitivity to loading, to name a few, that apparently can distinguish life from death, and health from disease.

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### *Declaration of competing interest*

Natalie Reznikov discloses that she consults for Object Research Systems Inc. in Montreal, but has no financial stake in the company. Betty Hoac discloses that she is an employee of Forbius Inc. in Montreal. All other authors have no competing interests.

### *CRediT contributions*

Natalie Reznikov: Conceptualization, Formal analysis, Investigation, Writing - Original draft, Writing - Review and editing, Visualization. Betty Hoac: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Review and editing. Daniel Buss: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Review and editing. William Addison: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Review and editing. Nilana Barros: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Review and editing. Marc McKee: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - Original draft, Writing - Review and editing, Supervision, Project administration, Funding acquisition.

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