

Review

Physiologic and pathologic functions of the NPP nucleotide pyrophosphatase/phosphodiesterase family focusing on NPP1 in calcification

Robert Terkeltaub

University of California School of Medicine, VA Medical Center, 3350 La Jolla Village Drive, San Diego, CA, USA

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Abstract

The catabolism of ATP and other nucleotides participates partly in the important function of nucleotide salvage by activated cells and also in removal or *de novo* generation of compounds including ATP, ADP, and adenosine that stimulate purinergic signaling. Seven nucleotide pyrophosphatase/phosphodiesterase NPP family members have been identified to date. These isoenzymes, related by up conservation of catalytic domains and certain other modular domains, exert generally non-redundant functions via distinctions in substrates and/or cellular localization. But they share the capacity to hydrolyze phosphodiester or pyrophosphate bonds, though generally acting on distinct substrates that include nucleoside triphosphates, lysophospholipids and choline phosphate esters. PP_i generation from nucleoside triphosphates, catalyzed by NPP1 in tissues including cartilage, bone, and artery media smooth muscle cells, supports normal tissue extracellular PP_i levels. Balance in PP_i generation relative to PP_i degradation by pyrophosphatases holds extracellular PP_i levels in check. Moreover, physiologic levels of extracellular PP_i suppress hydroxyapatite crystal growth, but concurrently providing a reservoir for generation of pro-mineralizing P_i. Extracellular PP_i levels must be supported by cells in mineralization-competent tissues to prevent pathologic calcification. This support mechanism becomes dysregulated in aging cartilage, where extracellular PP_i excess, mediated in part by upregulated NPP1 expression stimulates calcification. PP_i generated by NPP1 modulates not only hydroxyapatite crystal growth but also chondrogenesis and expression of the mineralization regulator osteopontin. This review pays particular attention to the role of NPP1-catalyzed PP_i generation in the pathogenesis of certain disorders associated with pathologic calcification.

Abbreviations: ANK – protein product of the murine ankylosis disease susceptibility gene; CPPD – calcium pyrophosphate dihydrate; CILP – cartilage intermediate layer protein; HA – hydroxyapatite; IIAC – Idiopathic Infantile Artery Calcification; MV – matrix vesicles; NPP – nucleotide pyrophosphatase/phosphodiesterase; OPLL – ossification of the posterior longitudinal ligament; SMC – smooth muscle cells; SNP – single nucleotide polymorphism; TNAP – tissue nonspecific alkaline phosphatase

Introduction

The extracellular catabolism of ATP and other nucleotides by coordinated ecto-enzymes mediates nucleotide salvage by activated cells and also drives removal or *de novo* generation of compounds including ATP, ADP, and adenosine that stimulate purinergic signaling [1–3]. This subject is reviewed in depth by Stefan et al. in this special issue of the journal. Among the many enzymes participating in nucleotide catabolism are certain nucleotide pyrophosphatase/phosphodiesterase (NPP) family members, including NPP1, the principal subject of this review. Seven NPPs have been identified to date (Figure 1) [4]. These isoenzymes, related by 24%–60% conservation in catalytic domains [4] and by

conservation of certain other modular domains, exert generally non-redundant functions via distinctions in substrates and/or subcellular localization. For example, the type II transmembrane ecto-enzymes NPP1 (PC-1, npps) and NPP3 (B10, CD203c, PD-1 β , gp130^{RB13-6}), which exist as disulfide-bonded homodimers in membranes, and whose extracellular domains can be proteolytically liberated into secreted forms, exert nucleoside triphosphate pyrophosphohydrolase (NTPPH) activity that generates PP_i from ATP and other nucleoside triphosphates, as discussed below, NPP1 and NPP3 both subserve other functions by alkaline pH optimum nucleotide phosphodiesterase activities [5–8]. However, the dileucine motif in the cytosolic tail of NPP1 (but not NPP3) mediates differential subcellular localization to the basolateral and apical plasma membrane, respectively, in polarized cell types [5].

Correspondence to: Robert Terkeltaub, Rheumatology Section, San Diego Veterans Affairs Medical Center, San Diego, CA 92161, USA. Tel: +1-858-5528585; Fax: +1-858-5527425; E-mail: rterkeltaub@ucsd.edu

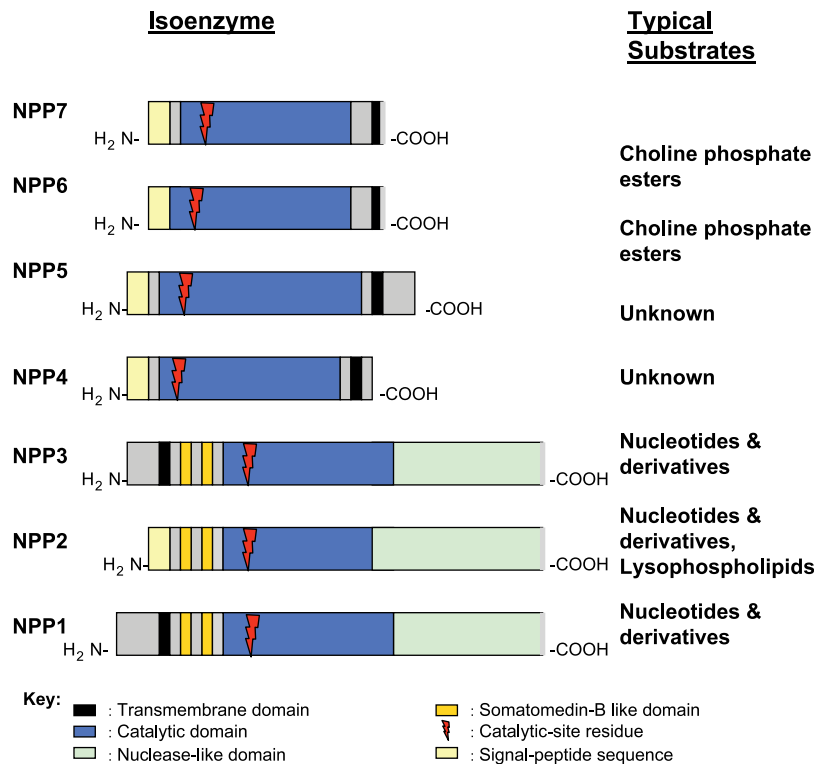


Figure 1. General Structural Features of NPP family members. The schematic highlights related structural features of NPPs 1–7, as discussed further in the text.

NPP2 (autotaxin, PD-1 α), though very similar to NPP1 and NPP3 in structural organization (Figure 1), is synthesized as a pro-enzyme and further processed to be a secretory molecule (4.9). NPP2 lysophospholipase D specific activity is much higher than that of other NPP family members and specific activity as a nucleotide pyrophosphatase/phosphodiesterase much lower than that of NPP1 and NPP3 [10, 11]. Correspondingly, we have observed that direct expression of NPP2 did not increase extracellular PP_i in chondrocytes, under conditions in which NPP2 did stimulate both alkaline phosphatase and increased calcification [12]. NPP6 and the intestinal enzyme NPP7 (Figure 1) exert lysophospholipase C or choline phosphate esterase activities [4]. The secretion of NPP2 by multiple tissues, and NPP2 accumulation in extracellular fluids, allows NPP2, in large part via lysophospholipase D activity, to exert a variety of biologically significant effects on cell growth, differentiation, adhesion, and migration, translated into functional effects in angiogenesis, tumor metastasis, and embryonic development [4, 13–15].

Comparative molecular structure-function of NPPs and their substrate specificities were recently reviewed in a thorough and lucid manner [4]. This review focuses on the functions of NPP1 in the regulation of physiologic and pathologic calcification, principally via PP_i generation from nucleoside triphosphates in tissues (and cells) including cartilage (and chondrocytes), bone (and osteoblasts), and large arteries (and smooth muscle cells (SMCs)).

NPP1 and ANK in PP_i metabolism and calcification

Subcellular trafficking mediated by the dileucine motif in the NPP1 (but not NPP3) cytosolic tail accounts for the observation that the majority of NPP activity in osteoblast plasma membranes and plasma membrane-derived mineralizing secretory vesicles (termed matrix vesicles) is accounted for by NPP1 [17]. Concordantly, cultured osteoblasts of NPP1 null mice demonstrate marked depletion (of up to 50%) in extracellular PP_i [18].

PP_i potently inhibits the nucleation and propagation of hydroxyapatite (HA) and other basic calcium phosphate crystals [19]. As such, maintenance of physiologic extracellular PP_i levels by mineralization-competent cells suppresses spontaneous calcification. This has been strikingly illustrated in certain mouse models of deficient NPP1-catalyzed PP_i generation [18, 20, 21], or alternately, ANK-mediated PP_i transport [18, 22]. In humans, 18 of 23 kindreds demonstrated homozygosity or compound heterozygosity for mutations of NPP1 in association with generalized arterial calcification of infancy (GACI, IIC, MIM# 208000) [23, 24]. This entity, described in approximately 180 individuals to date, is characterized by large artery media calcification and myointimal proliferation, commonly associated with periarticular calcification [23–25]. The disease is frequently lethal but may respond to treatment with bisphosphonates, which function in part as non-hydrolyzable PP_i analogues [25]. GACI is linked to systemic (blood, urine, tissue) extracellular PP_i deficiency [25, 26] discovered by us to be caused by mutations

widely spread through NPP1 extracellular domains [23]. Many of these NPP1 mutations, which are mostly in the nuclease-like and catalytic, domains, but also reported in the somatomedin B-like domain, have been established to impair NPP1 catalytic activity [23, 24].

Notably, PP_i serves as reservoir for alkaline phosphatase-catalyzed P_i generation that is pro-mineralizing, as

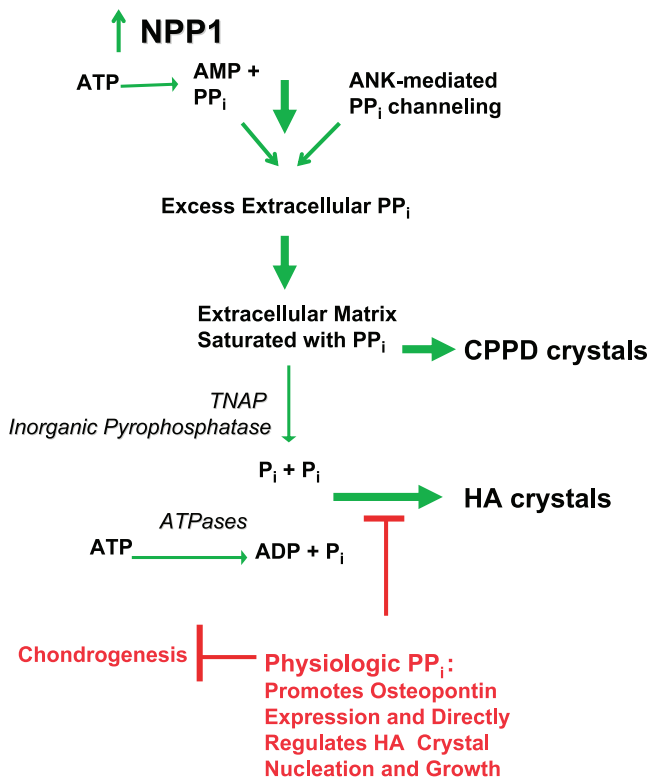


Figure 2. Proposed NPP1-mediated and PP_i -dependent mechanisms stimulating CPPD and HA crystal deposition in aging and osteoarthritis (OA): Roles of ATP and PP_i Metabolism and inorganic phosphate (P_i) generation in pathologic cartilage calcification. This model presents mechanisms underlying the common association of extracellular PP_i excess with both CPPD and HA crystal deposition in OA and chondrocalcinosis cartilages, as well as the paradoxical association of extracellular PP_i deficiency (from defective ANK or PC-1/NPP1 expression) with pathologic calcification of articular cartilage with HA crystals *in vivo*. Factors driving pathologic calcification are indicated in *green* and physiologic factors suppressing calcification in *red*. Excess PP_i generation in aging cartilages in idiopathic CPPD deposition disease of aging, and in OA cartilages, is mediated in part by marked increases in NTPPPH activity, mediated in large part by the PC-1/NPP1 isoenzyme. In idiopathic chondrocalcinosis of aging and in OA, there are substantial increases in joint fluid PP_i derived largely from cartilage. NPP1 not only directly induces elevated PP_i but also matrix calcification by chondrocytes *in vitro*. Depending on extracellular availability of substrate PP_i and the activity of pyrophosphatases, the availability of substrate ATP and the activity of ATPases, and other factors such as substantial local Mg^{++} concentrations, HA crystal deposition, as opposed to CPPD deposition, may be stimulated. In this model, excess extracellular PP_i also may result from heightened release of intracellular PP_i via increased ANK expression in OA and abnormal ANK function in familial chondrocalcinosis, as well as from deficient activity of pyrophosphatases (such as TNAP and possibly inorganic pyrophosphatase) in certain primary metabolic disorders. Also illustrated at the top of this schematic is the role in cartilage calcification in OA and aging of altered TGF β expression and responsiveness, which drives PP_i generation and release mediated via NPP1 and ANK, and diminished responsiveness to IGF-I, which normally suppresses elevation of chondrocyte extracellular PP_i .

illustrated by osteopenia in long bones of NPP1 deficient mice [27, 28]. As such, PP_i generation can both suppress and promote HA crystal deposition, depending on relative tissue levels of NPP1 and alkaline phosphatase (Figure 2) [16–21, 27, 28]. The capacity of chondrocytes to produce copious extracellular PP_i is particularly double edged, as it is directly promotes calcium pyrophosphate dihydrate (CPPD) crystal deposition (Figure 2). Depending on cartilage ATP and PP_i concentrations, and the level of activity of P_i -generating ATPases and pyrophosphatases, NPP1 excess promotes both HA and CPPD crystal formation by articular chondrocytes [12, 29–31], an event that commonly occurs in the joint in human aging and osteoarthritis (OA) [32].

PP_i appears to directly regulate expression of certain genes (including inductive effects first described by us for osteopontin and MMP-13 expression and suppressive effects on Sox9 expression)[18, 33–35]. PP_i regulates certain cellular differentiation and functions including protein synthesis [19], chondrogenesis [35], and pro-mineralizing chondrocyte maturation to terminal hypertrophic differentiation transduced partly by Pit-1 mediated P_i uptake [36]. Such effects of PP_i are analogous to effects of not only P_i [37, 38] but also bisphosphonate PP_i analogues [39, 40]. It is not clear in which subcellular compartments PP_i could act to carry out these effects and what contributions P_i derived from PP_i makes in these activities of PP_i .

Mammalian extramitochondrial mechanisms for PP_i production, degradation, and transport were recently reviewed in depth [19]. In cells such as osteoblasts and chondrocytes that normally express NPP1 relatively robustly, NPP1 and NPP3 increase intracellular PP_i , suspected to be in large part in the lumen of the ER and Golgi [12, 16, 31]. Critical to support of extracellular PP_i is apparent direct PP_i transport by the multiple-pass transmembrane protein ANK [41], which makes a major contribution to moving to the movement into the extracellular space of intracellular PP_i , including the fraction of intracellular PP_i generated by NPP1 [34].

NPP1 and PP_i metabolism in cartilage and bone

Extracellular PP_i rises markedly in articular cartilage in direct association with aging and OA, and resultant matrix supersaturation with PP_i and cartilage matrix abnormalities that alter the solubility product of PP_i and Ca^{2+} promote calcification [42]. Physiologic chondrocyte PP_i metabolism is regulated in part by growth factor and cytokine regulatory effects on chondrocyte NPP1 expression. Interruption in regulatory checks and balances on articular cartilage PP_i metabolism appears to occur in aging and diseases including OA. For example, the chondrocyte growth factor TGF β induces both NPP1 expression and elevation of extracellular PP_i [12, 31, 43]. The capacity of TGF β to increase cartilage NPP activity and extracellular PP_i levels directly correlates with donor age [12, 31, 44]. The TGF β -stimulated cellular program for chondrocyte

extracellular PP_i elevation includes substantial increases in ATP generation [45] and stimulation of NPP1 movement to the plasma membrane [12, 31].

Osteoblasts and chondrocytes have particularly high levels of both NPP1 expression and NPP specific activity [19, 46, 47]. Moreover, chondrocyte NPP activity increases in direct condordance with cartilage PP_i generation (to an average of double normal levels) in a donor age-dependent manner [47]. The age-dependent increases in NPP activity are directly linked to CPPD crystal deposition disease [47]. Upregulation of NPP1 but not NPP3 is associated with calcification by chondrocytic cells *in situ* and *in vitro* [12, 31]. Unlike NPP1, which regulates both intracellular and extracellular PP_i in chondrocytes, NPP3 appears to principally regulate only intracellular PP_i [12, 31].

Chondrocyte mitochondrial dysfunction associated with spontaneous OA in Hartley guinea pig knees promotes ATP depletion [48]. Significantly increased NPP activity and extracellular PP_i develop concurrent with the ATP-depleted state [48]. Hence, increased ATP-scavenging by energy-depleted chondrocytes likely promotes extracellular PP_i excess in human OA and aging cartilages.

A series of studies from one research group erroneously reported that cartilage intermediate layer protein (CILP), an interterritorial and pericellular matrix constituent in cartilage with a molecular weight similar to that of NPP1, was an NPP family member, even though there was no structural similarity of CILP to NPP family members [49–51]. We refuted this work [52], and in so doing, we demonstrated that increased expression of one of 2 CILP isoforms (CILP-1) in aging cartilage interferes with the regulatory effects of IGF-I on PP_i metabolism, thereby promoting increased extracellular PP_i and cartilage calcification.

NPP1 and PP_i metabolism in pathologic soft tissue calcification syndromes and pivotal role of osteopontin depletion

Consistent with the apparent co-dependent function of ANK and NPP1 to raise extracellular PP_i [34] is the remarkable similarity in the consequences of deficient ANK and PC-1 function *in vivo*. Both NPP1 deficient mice and mice homozygous for a natural C-terminal ANK mutant that appears to incapacitate ANK PP_i transport function (*ank/ank* mice) spontaneously develop a progressive phenotype of pathologic soft tissue calcification that with increasing age comes to include perispinal ligament hyperostosis, periarticular calcification leading to ossific fusion of peripheral joints, extensive articular cartilage degeneration associated with HA deposits, and large artery calcification [22, 35]. The initial implication of NPP1 deficiency in spontaneous pathologic soft tissue calcification was in ‘tiptoe walking’ *ttw/ttw* mice, which are homozygous for a spontaneous nonsense mutation that encodes for a stop codon at tyrosine 568, a position 3' of the NPP1 catalytic site [20]. It is not yet known if NPP1 expression is depressed or absent in *ttw/ttw* mice, or if the

ttw mutation, like many of the NPP1 mutations seen in humans with GACI, critically impairs catalytic activity, putatively by interfering with substrate binding.

Human ossification of the posterior longitudinal ligament (OPLL), a form of spontaneous pathologic perispinal ligament calcification common in Japanese subjects, has been linked with certain SNPs in the NPP1 gene [53–55]. It will be of interest to see if the implicated NPP1 sequence variants affect NPP1 expression and function. Interestingly, the inflammatory cytokine IL-1 depresses NPP1 expression, NPP activity, and extracellular PP_i in chondrocytes [43]. In this context, a ~30% depression in serum NPP activity is seen in males with the chronic inflammatory disease ankylosing spondylitis [56], a condition that, like OPLL and spinal alterations in NPP1-deficient mice, associated with ankylosing intervertebral soft tissue calcification.

Interestingly, periarticular and bone abnormalities are far more substantial and progressive in NPP1-deficient mice than in NPP1-deficient humans with GACI. Conversely NPP1 deficient mice [35] do not demonstrate the severe myointimal proliferative changes seen in arteries in human GACI [24, 25]. We speculate that the relatively high level of normal serum P_i in mice compared to humans (≤ 8 vs. ≤ 4.5 mg/dL, respectively) [46] plays a major role in determining these phenotypic distinctions. In this context, high dietary P_i worsens pathologic calcification in NPP1 null mice [46]. Conversely, low serum P_i induced by crossbreeding with PHEX null mice is associated with correction of pathologic artery and soft tissue calcification in both NPP1 null and *ank/ank* mice [46].

Unlike cultured cells of *ank/ank* mice, NPP1-deficient cells demonstrate low intracellular as well as extracellular PP_i levels [18]. Thus, the common basis for the remarkably similar hypermineralizing phenotypes seen in *ank/ank* mice and in NPP1 null mice (and the pathologic calcification seen in the human NPP1 deficiency state GACI) appears to rest in depression of extracellular PP_i . Furthermore, the marked depletion of extracellular PP_i and of osteopontin, the rapid, extensive calcification by both NPP1^{-/-} and *ank/ank* osteoblasts in culture are corrected by soluble NPP1, reinforcing a central role of NPP1 in skeletal PP_i and P_i metabolism and osteopontin expression [18], a notion strongly supported by *in vivo* studies [21, 33].

P_i , mediated by uptake through plasma membrane sodium-phosphate co-transport, stimulates expression of osteopontin, an inhibitor of HA crystal growth and promoter of mineral resorption [37, 38]. As cited above, exogenous PP_i also induces osteopontin expression [18, 33]. It is not yet clear whether uptake of P_i derived from extracellular PP_i is a major signaling intermediate in this process. Nevertheless, it is remarkable that one HA crystal growth inhibitor (PP_i) promotes expression of a second in the form of osteopontin. Because osteopontin knockout mice have relatively mild changes in mineralization in contrast to the marked phenotypic abnormalities in extracellular PP_i -deficient mice, PP_i clearly higher than osteopontin in the physiologic hierarchy of HA crystal growth inhibitors.

As previously reviewed [19, 29], NPP1 plays a major role in regulating nucleation of mineral in chondrocyte-, osteoblast-, and apparently artery smooth muscle cell-derived secretory bodies released by budding from the plasma membrane and termed matrix vesicles (MVs). The MVs, are enriched in NPP1 and TNAP, whose catalytic domains are predominantly exposed at the external face of MVs. The MVs provide a sheltered environment for initiation of mineral crystal formation in a manner modulated by the concentration of PP_i , though mineral propagation is mediated by other factors, including availability of fibrillar collagen in 'osteoid' to serve as a nidus for calcification with HA [46]. NPP1 is clearly the principal NPP associated with chondrocyte-derived and osteoblast-derived MVs [16, 17, 21, 30, 57]. NPP1 and TNAP exert mutually antagonistic regulatory effects on crystal deposition in MVs, and activity not shared by NPP3 [16]. Cell differentiation and a variety of calciotropic hormones and cytokines (including 1,25 dihydroxyvitamin D3, TGF β , and IL-1) can regulate the NPP1 content, NPP and alkaline phosphatase activities, PP_i content, and other compositional features of MVs [29]. However, we have not seen concentrated ANK localization in MVs [33], likely contributing to the observation that correction of pathologic calcification by TNAP deficiency is less marked in *ank/ank* than NPP1 $^{-/-}$ mice [33].

NPP1 and PP_i deficiency states are linked to accelerated chondrogenesis

Taken together, it is clear that NPP1 and PP_i physiologically function to prevent calcification of arteries and certain other soft tissues at the level of cell differentiation, and not simply at the level of mineral formation and resorption in the extracellular matrix. Most strikingly, we recently discovered that trans-differentiation of artery SMCs and accelerated intra-arterial chondrogenic differentiation mediated directly by PP_i depletion promotes spontaneous artery media calcification in NPP1 $^{-/-}$ and *ank/ank* mice [35]. Specifically, we observed that NPP1 deficiency promoted the spontaneous emergence of chondrogenesis from bone marrow stromal cells under non-calcifying conditions. Cultured NPP1 $^{-/-}$ aortic SMC preparations and NPP1 $^{-/-}$ aortic cells 023060 *in situ* expressed cbfa1, osteocalcin, and chondrocyte-specific collagens. Osteopontin expression was depressed and pro-calcifying alkaline phosphatase specific activity and calcification were markedly upregulated in cultured NPP1 $^{-/-}$ SMCs [35]. In contrast, there was no gross alteration in expression of the physiologic artery calcification inhibitors matrix gla protein and osteoprotegerin in NPP1 $^{-/-}$ mouse arterial cells [35]. The capacity of exogenous PP_i to correct spontaneous chondrogenesis in NPP1 $^{-/-}$ bone marrow stromal cells under non-calcifying conditions suggested that extracellular PP_i deficiency directly promoted chondrogenesis and trans-differentiation to chondrocytes of the SMCs, a notion supported by aortic media calcification and changes in cultured SMC differentiation and calcification

in *ank/ank* mice [35]. Therefore, acquired regional and systemic decrements in NPP1 and ANK expression and extracellular PP_i could contribute to intra-arterial chondro-osseous metaplasia and calcification in aging, diabetes mellitus, and atherosclerosis. In addition, it is noteworthy that systemic PP_i deficiency is seen in hemodialysis-dependent renal insufficiency, a condition associated with hyperphosphatemia and often extensive artery media and periarticular calcifications [58].

Conclusions and perspectives

Support of extracellular PP_i levels by NPP1 and ANK inhibits pathologic soft tissue calcification but supports hard tissue mineralization in long bones and promotes calcification of articular cartilages in aging and OA. PP_i is a central regulator of calcification in the extracellular matrix, but extracellular PP_i regulates gene expression and cellular differentiation, including major physiologic effects on chondrogenesis and expression of osteopontin. The larger significance of mutants of NPP1 and ANK in disease continues to be elucidated. For example, mutants of ANKH, concentrated mainly at the N-terminal end of the molecule, have been linked with both autosomal dominant familial and 'sporadic' CPPD crystal deposition disease of articular cartilage [59, 60]. But other ANKH mutants clustered in putative cytosolic loops well-removed the N- and C-termini are linked with the distinct phenotype of craniometaphyseal dysplasia, a disease mediated by abnormal skeletal remodeling more than pathologic calcification [61, 62]. Polymorphisms in the human homologue of ANK (ANKH) also appear to contribute to differences in hand bone size and geometry that may influence bone fragility in a homogeneous Chuvasha population [63]. In the same population, NPP1 gene polymorphisms appeared to contribute to variance in severity of hand joint OA [64].

NPP1, in a catalytic activity-independent manner, inhibits ligand-induced insulin receptor signaling [65], an effect that appears linked to NPP1 mutations associated with type II diabetes mellitus in some but not all ethnic groups studied [66, 67]. Interestingly, the K173Q SNP of NPP1, which maps to the second somatomedin-B-like domain of NPP1 and has been linked to insulin resistance, does not modulate NPP1 dimerization or catalytic activity or affect physical interaction of NPP1 with the insulin receptor [68]. Inherited states of putative 'gain-of-function' of NPP1 also have been linked to obesity [69], also likely mediated primarily via effects on insulin receptor signaling. However, it is not likely that the numerous NPP1 catalytic site-independent mutants implicated as interfering with ligand-induced insulin receptor signaling directly affect mineralization.

Last, NPP1 not only generates PP_i but also modulates N-glycosylation and secretion of glycoproteins, and proteoglycans sulfation [6–8], and NPP1 also scavenges ATP and thereby regulates purinergic receptor signaling. The potential roles in calcification of these alternative effects of NPP1, and of other NPP1 interactions with nucleotide-

hydrolyzing ecto-enzymes, remain to be determined. Nevertheless, the remarkable phenotypic similarities between NPP1-deficient and ANK-deficient mice strongly support the central role of NPP1 catalyzed PP_i generation in the regulation of calcification.

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