

Review

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

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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<sub>i</sub> generation from nucleoside triphosphates, catalyzed by NPP1 in tissues including cartilage, bone, and artery media smooth muscle cells, supports normal tissue extracellular PP<sub>i</sub> levels. Balance in PP<sub>i</sub> generation relative to PP<sub>i</sub> degradation by pyrophosphatases holds extracellular PP<sub>i</sub> levels in check. Moreover, physiologic levels of extracellular PP<sub>i</sub> suppress hydroxyapatite crystal growth, but concurrently providing a reservoir for generation of pro-mineralizing P<sub>i</sub>. Extracellular PP<sub>i</sub> 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<sub>i</sub> excess, mediated in part by upregulated NPP1 expression stimulates calcification. PP<sub>i</sub> 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<sub>i</sub> generation in the pathogenesis of certain disorders associated with pathologic calcification.

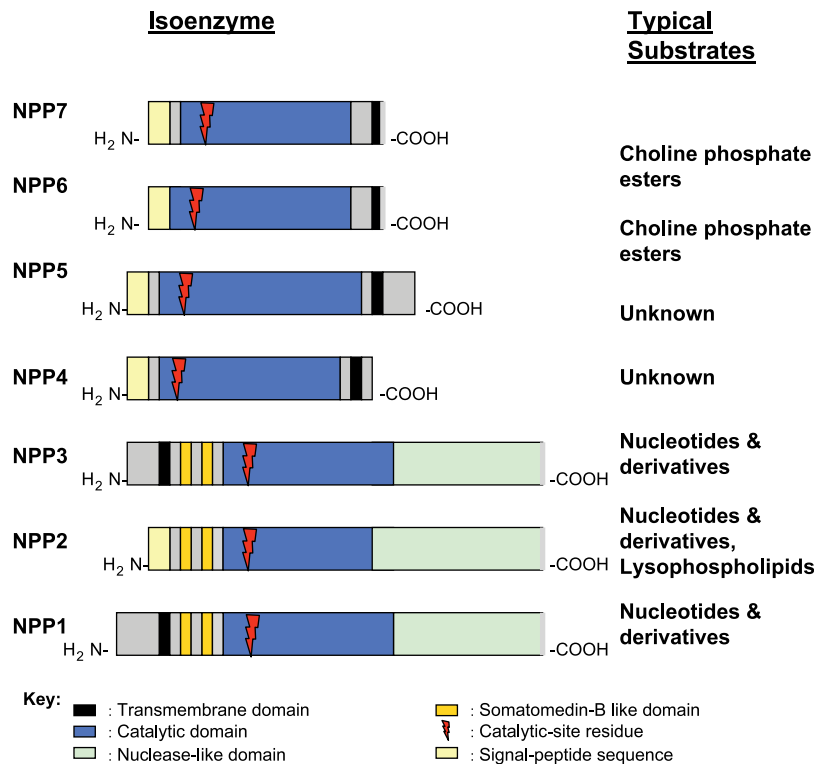
**Abbreviations:** ANK – protein product of the murine ankylosis disease susceptibility gene; CPPD – calcium pyrophosphate dihydrate; CILP – cartilage intermediate layer protein; HA – hydroxyapatite; IIAC – Idiopathic Infantile Artery Calcification; MV – matrix vesicles; NPP – nucleotide pyrophosphatase/phosphodiesterase; 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 $\beta$ , gp130<sup>RB13-6</sup>), which exist as disulfide-bonded homodimers in membranes, and whose extracellular domains can be proteolytically liberated into secreted forms, exert nucleoside triphosphate pyrophosphohydrolyase (NTPPH) activity that generates PP<sub>i</sub> 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].

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*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 $\alpha$ ), 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<sub>i</sub> 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<sub>i</sub> generation from nucleoside triphosphates in tissues (and cells) including cartilage (and chondrocytes), bone (and osteoblasts), and large arteries (and smooth muscle cells (SMCs)).

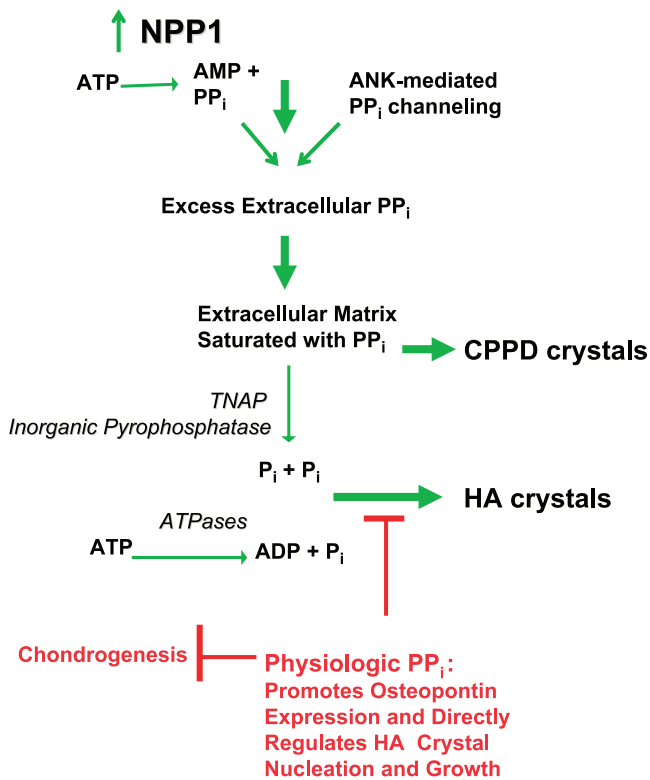
### NPP1 and ANK in PP<sub>i</sub> metabolism and calcification

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<sub>i</sub> [18].

PP<sub>i</sub> potently inhibits the nucleation and propagation of hydroxyapatite (HA) and other basic calcium phosphate crystals [19]. As such, maintenance of physiologic extracellular PP<sub>i</sub> levels by mineralization-competent cells suppresses spontaneous calcification. This has been strikingly illustrated in certain mouse models of deficient NPP1-catalyzed PP<sub>i</sub> generation [18, 20, 21], or alternately, ANK-mediated PP<sub>i</sub> 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, IIAC, 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<sub>i</sub> analogues [25]. GACI is linked to systemic (blood, urine, tissue) extracellular PP<sub>i</sub> 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 $\beta$  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 $\beta$  induces both NPP1 expression and elevation of extracellular  $PP_i$  [12, 31, 43]. The capacity of TGF $\beta$  to increase cartilage NPP activity and extracellular  $PP_i$  levels directly correlates with donor age [12, 31, 44]. The TGF $\beta$ -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 ( $\leq 8$  vs.  $\leq 4.5$  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<sup>-/-</sup> 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 $\beta$ , 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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## References

- Robson SC, Wu Y, Sun X et al. Ectonucleotidases of CD39 family modulate vascular inflammation and thrombosis in transplantation. *Semin Thromb Hemost* 2005; 31: 217–33.
- Vorhoff T, Zimmermann H, Pelletier J et al. Cloning and characterization of the ecto-nucleotidase NTPDase3 from rat brain: Predicted secondary structure and relation to other members of the E-NTPDase family and actin. *Purinergic Signalling* 2005; 1: 259–70.
- Deterre P, Gelman L, Gary-Gouy H et al. Coordinated regulation in human T cells of nucleotide-hydrolyzing ecto-enzymatic activities, including CD38 and PC-1. Possible role in the recycling of nicotinamide adenine dinucleotide metabolites. *J Immunol* 1996; 157: 1381–8.
- Stefan C, Jansen S, Bollen M. NPP-type ectophosphodiesterases: Unity in diversity. *Trends Biochem Sci* 2005; 30: 542–50.
- Bello V, Goding JW, Greengrass V et al. Characterization of a dileucine-based signal in the cytoplasmic tail of the nucleotide-pyrophosphatase NPP1 that mediates basolateral targeting but not endocytosis. *Mol Biol Cell* 2001; 12: 3004–15.
- Hickman S, Wong-Yip YP, Rebbe NF, Greco JM. Formation of lipid-linked oligosaccharides by MOPC 315 plasmacytoma cells. Decreased synthesis by a nonsecretory variant. *J Biol Chem* 1985; 260: 6098–106.
- Goding JW, Grobden B, Slegers H. Physiological and pathophysiological functions of the ecto-nucleotide pyrophosphatase/phosphodiesterase family. *Biochim Biophys Acta* 2003; 1638: 1–19.
- Goding JW. Ecto-enzymes: Physiology meets pathology. *J Leukoc Biol* 2000; 67: 285–311.
- Jansen S, Stefan C, Creemers JW et al. Proteolytic maturation and activation of autotaxin (NPP2), a secreted metastasis-enhancing lysophospholipase D. *J Cell Sci* 2005; 118: 3081–9.
- van Meeteren LA, Ruurs P, Christodoulou E et al. Inhibition of autotaxin by lysophosphatidic acid and sphingosine 1-phosphate. *J Biol Chem* 2005; 280: 21155–61.
- Cimpean A, Stefan C, Gijsbers R et al. Substrate-specifying determinants of the nucleotide pyrophosphatases/phosphodiesterases NPP1 and NPP2. *Biochem J* 2004; 381: 71–7.
- Johnson K, Hashimoto S, Lotz M et al. Up-regulated expression of the phosphodiesterase nucleotide pyrophosphatase family member PC-1 is a marker and pathogenic factor for knee meniscal cartilage matrix calcification. *Arthritis Rheum* 2001; 44: 1071–81.
- Durgam GG, Virag T, Walker MD et al. Synthesis, structure-activity relationships, and biological evaluation of fatty alcohol phosphates as lysophosphatidic acid receptor ligands, activators of PPAR-gamma, and inhibitors of autotaxin. *J Med Chem* 2005; 48: 4919–30.
- Moolenaar WH. Lysophospholipids in the limelight: Autotaxin takes center stage. *J Cell Biol* 2002; 158: 197–9.
- Koh E, Clair T, Woodhouse EC et al. Site-directed mutations in the tumor-associated cytokine, autotaxin, eliminate nucleotide phosphodiesterase, lysophospholipase D, and motogenic activities. *Cancer Res* 2003; 63: 2042–5.
- Johnson KA, Hesse L, Vaingankar S et al. Osteoblast tissue-nonspecific alkaline phosphatase antagonizes and regulates PC-1. *Am J Physiol Regul Integr Comp Physiol* 2000; 279: R1365–77.
- Vaingankar SM, Fitzpatrick TA, Johnson K et al. Subcellular targeting and function of osteoblast nucleotide pyrophosphatase phosphodiesterase 1. *Am J Physiol Cell Physiol* 2004; 286: C1177–87.
- Johnson K, Goding J, Van Etten D et al. Linked deficiencies in extracellular  $PP_i$  and osteopontin mediate pathologic calcification associated with defective PC-1 and ANK expression. *J Bone Miner Res* 2003; 18: 994–1004.
- Terkeltaub R. Inorganic pyrophosphate ( $PP_i$ ) generation and disposition in pathophysiology. *Am J Physiol: Cell Physiol* 2001; 281: C1–11.
- Okawa A, Nakamura I, Goto S et al. Mutation in Npps in a mouse model of ossification of the posterior longitudinal ligament of the spine. *Nat Genet* 1998; 19: 271–3.
- Hesse L, Johnson KA, Anderson HC et al. Tissue-nonspecific alkaline phosphatase and plasma cell membrane glycoprotein-1 are central antagonistic regulators of bone mineralization. *Proc Natl Acad Sci USA* 2002; 99: 9445–9.
- Ho AM, Johnson MD, Kingsley DM. Role of the mouse ank gene in control of tissue calcification and arthritis. *Science* 2000; 289: 265–70.
- Ruf N, Uhlenberg B, Terkeltaub R et al. The mutational spectrum of ENPP1 as arising after the analysis of 23 unrelated patients with generalized arterial calcification of infancy (GACI). *Human Mutat* 2005; 25: 98–104.
- Rutsch F, Ruf N, Vaingankar S et al. Mutations in ENPP1 are associated with ‘idiopathic’ infantile arterial calcification. *Nat Genet* 2003; 34: 379–81.
- Rutsch F, Vaingankar S, Johnson K et al. PC-1 nucleoside triphosphate pyrophosphohydrolase deficiency in idiopathic infantile arterial calcification. *Am J Pathol* 2001; 158: 543–54.
- Rutsch F, Schauerte P, Kalhoff H et al. Low levels of urinary inorganic pyrophosphate indicating systemic pyrophosphate deficiency in a boy with idiopathic infantile arterial calcification. *Acta Paediatr* 2000; 89: 1265–9.
- Anderson HC, Harmey D, Camacho NP et al. Sustained osteomalacia of long bones despite major improvement in other hypophosphatasia-related mineral deficits in tissue nonspecific alkaline phosphatase/nucleotide pyrophosphatase phosphodiesterase 1 double-deficient mice. *Am J Pathol* 2005; 166: 1711–20.
- Kobayashi Y, Goto S, Tanno T et al. Regional variations in the progression of bone loss in two different mouse osteopenia models. *Calcif Tissue Int* 1998; 62: 426–36.
- Johnson K, Terkeltaub R. Inorganic pyrophosphate ( $PP_i$ ) in pathologic calcification of articular cartilage. *Front Biosci* 2005; 10: 988–97.
- Johnson K, Pritzker K, Goding J, Terkeltaub R. The nucleoside triphosphate pyrophosphohydrolase isozyme PC-1 directly promotes cartilage calcification through chondrocyte apoptosis and increased calcium precipitation by mineralizing vesicles. *J Rheumatol* 2001; 28: 2681–91.
- Johnson K, Vaingankar S, Chen Y et al. Differential mechanisms of inorganic pyrophosphate production by plasma cell membrane glycoprotein-1 and B10 in chondrocytes. *Arthritis Rheum* 1999; 42: 1986–97.
- Derfus BA, Kurian JB, Butler JJ et al. The high prevalence of pathologic calcium crystals in pre-operative knees. *J Rheumatol* 2002; 29: 570–4.
- Harmey D, Hesse L, Narisawa S et al. Concerted regulation of inorganic pyrophosphate and osteopontin by akp2, enpp1, and ank: An integrated model of the pathogenesis of mineralization disorders. *Am J Pathol* 2004; 164: 1199–209.
- Johnson K, Terkeltaub R. Upregulated ank expression in osteoarthritis can promote both chondrocyte MMP-13 expression and calcification via chondrocyte extracellular  $PP_i$  excess. *Osteoarthritis Cartilage* 2004; 12: 321–35.
- Johnson K, Polewski M, van Etten D, Terkeltaub R. Chondrogenesis mediated by  $PP_i$  depletion promotes spontaneous aortic calcification

- in NPP1<sup>-/-</sup> mice. *Arterioscler Thromb Vasc Biol* 2005; 25: 686–91.
36. Wang W, Xu J, Du B, Kirsch T. Role of the progressive ankylosis gene (ank) in cartilage mineralization. *Mol Cell Biol* 2005; 25: 312–23.
  37. Beck GR Jr. Inorganic phosphate as a signaling molecule in osteoblast differentiation. *J Cell Biochem* 2003; 90: 234–43.
  38. Beck GR Jr, Zerler B, Moran E. Phosphate is a specific signal for induction of osteopontin gene expression. *Proc Natl Acad Sci USA* 2000; 97: 8352–7.
  39. Rogers MJ. New insights into the molecular mechanisms of action of bisphosphonates. *Curr Pharm Des* 2003; 9: 2643–58.
  40. Fujita T, Izumo N, Fukuyama R et al. Incadronate and etidronate accelerate phosphate-primed mineralization of MC4 cells via ERK1/2-Cbfa1 signaling pathway in a Ras-independent manner: Further involvement of mevalonate-pathway blockade for incadronate. *Jpn J Pharmacol* 2001; 86: 86–96.
  41. Ho AM, Johnson DM, Kingsley: Role of the mouse ank gene in control of tissue calcification and arthritis. *Science* 2000; 289: 265–70.
  42. Kalya S, Rosenthal AK. Extracellular matrix changes regulate calcium crystal formation in articular cartilage. *Curr Opin Rheumatol* 2005; 17: 325–329.
  43. Lotz M, Rosen F, McCabe G et al. Interleukin 1 beta suppresses transforming growth factor-induced inorganic pyrophosphate (PP<sub>i</sub>) production and expression of the PP<sub>i</sub>-generating enzyme PC-1 in human chondrocytes. *Proc Natl Acad Sci USA* 1995; 92: 10364–8.
  44. Rosen F, McCabe G, Quach J et al. Differential effects of aging on human chondrocyte responses to TGFβ: Increased pyrophosphate production and decreased cell proliferation. *Arthritis Rheum* 1997; 40: 1275–81.
  45. Johnson K, Jung AS, Andreyev A et al. Mitochondrial Oxidative Phosphorylation is a downstream regulator of nitric oxide effects on chondrocyte matrix synthesis and mineralization. *Arthritis Rheum* 2000; 43: 1560–70.
  46. Murshed M, Harmey D, Millan JL et al. Unique coexpression in osteoblasts of broadly expressed genes accounts for the spatial restriction of ECM mineralization to bone. *Genes Dev* 2005; 19: 1093–104.
  47. Costello JC, Ryan LM. Modulation of chondrocyte production of extracellular inorganic pyrophosphate. *Curr Opin Rheumatol* 2004; 16: 268–72.
  48. Johnson K, Svensson CI, Etten DV et al. Mediation of spontaneous knee osteoarthritis by progressive chondrocyte ATP depletion in Hartley guinea pigs. *Arthritis Rheum* 2004; 50: 1216–25.
  49. Masuda I, Halligan BD, Barbieri JT et al. Molecular cloning and expression of a porcine chondrocyte nucleotide pyrophosphohydrolase. *Gene* 1997; 197: 277–87.
  50. Masuda I, Hamada J, Haas AL et al. A unique ectonucleotide pyrophosphohydrolase associated with porcine chondrocyte-derived vesicles. *J Clin Invest* 1995; 95: 699–704.
  51. Hirose J, Masuda I, Ryan LM. Expression of cartilage intermediate layer protein/nucleotide pyrophosphohydrolase parallels the production of extracellular inorganic pyrophosphate in response to growth factors and with aging. *Arthritis Rheum* 2000; 43: 2703–11.
  52. Johnson K, Farley D, Hu SI, Terkeltaub R. One of two chondrocyte-expressed isoforms of cartilage intermediate-layer protein functions as an insulin-like growth factor 1 antagonist. *Arthritis Rheum* 2003; 48: 1302–14.
  53. Nakamura I, Ikegawa S, Okawa A et al. Association of the human NPPS gene with ossification of the posterior longitudinal ligament of the spine (OPLL). *Hum Genet* 1999; 104: 492–7.
  54. Koshizuka Y, Kawaguchi H, Ogata N et al. Nucleotide pyrophosphatase gene polymorphism associated with ossification of the posterior longitudinal ligament of the spine. *J Bone Miner Res* 2002; 17: 138–44.
  55. Tahara M, Aiba A, Yamazaki M et al. The extent of ossification of posterior longitudinal ligament of the spine associated with nucleotide pyrophosphatase gene and leptin receptor gene polymorphisms. *Spine* 2005; 3: 877–80.
  56. Mori K, Chano T, Ikeda T et al. Decrease in serum nucleotide pyrophosphatase activity in ankylosing spondylitis. *Rheumatology (Oxford)* 2003; 42: 62–5.
  57. Johnson K, Moffa A, Chen Y et al. Matrix vesicle plasma cell membrane glycoprotein-1 regulates mineralization by murine osteoblastic MC3T3 cells. *J Bone Miner Res* 1999; 14: 883–92.
  58. Lomashvili KA, Khawandi W, O'Neill WC. Reduced plasma pyrophosphate levels in hemodialysis patients. *J Am Soc Nephrol* 2005; 16: 2495–500.
  59. Williams CJ. Familial calcium pyrophosphate dihydrate deposition disease and the ANKH gene. *Curr Opin Rheumatol* 2003; 15: 326–31.
  60. Zhang Y, Johnson K, Russell RG et al. Association of sporadic chondrocalcinosis with a –4-basepair G-to-A transition in the 5'-untranslated region of ANKH that promotes enhanced expression of ANKH protein and excess generation of extracellular inorganic pyrophosphate. *Arthritis Rheum* 2005; 52: 1110–7.
  61. Reichenberger E, Tiziani V, Watanabe S et al. Autosomal dominant craniometaphyseal dysplasia is caused by mutations in the transmembrane protein ANK. *Am J Hum Genet* 2001; 68: 1321–6.
  62. Numberg P, Thiele H, Chandler D et al. Heterozygous mutations in ANKH, the human ortholog of the mouse progressive ankylosis gene, result in craniometaphyseal dysplasia. *Nat Genet* 2001; 28: 37–41.
  63. Malkin I, Dahm S, Suk A et al. Association of ANKH gene polymorphisms with radiographic hand bone size and geometry in a Chuvasha population. *Bone* 2005; 36: 365–73.
  64. Suk EK, Malkin I, Dahm S et al. Association of ENPP1 gene polymorphisms with hand osteoarthritis in a Chuvasha population. *Arthritis Res Ther* 2005; 7: R1082–90.
  65. Dong H, Maddux BA, Altomonte J et al. Increased hepatic levels of the insulin receptor inhibitor, PC-1/NPP1, induce insulin resistance and glucose intolerance. *Diabetes* 2005; 54: 367–72.
  66. Morrison JA, Gruppo R, Glueck CJ et al. Population-specific alleles: The polymorphism (K121Q) of the human glycoprotein PC-1 gene is strongly associated with race but not with insulin resistance in black and white children. *Metabolism* 2004; 53: 465–8.
  67. Gijsbers R, Ceulemans H, Bollen M. Functional characterization of the non-catalytic ectodomains of the nucleotide pyrophosphatase/phosphodiesterase NPP1. *Biochem J* 2003; 371: 321–30.
  68. Stefanovic V, Antic S. Plasma cell membrane glycoprotein 1 (PC-1): A marker of insulin resistance in obesity, uremia and diabetes mellitus. *Clin Lab* 2004; 50: 271–8.
  69. Meyre D, Bouatia-Naji N, Tounian A et al. Variants of ENPP1 are associated with childhood and adult obesity and increase the risk of glucose intolerance and type 2 diabetes. *Nat Genet* 2005; 37: 863–7.