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Concerted Regulation of Inorganic Pyrophosphate and Osteopontin by *Akp2*, *Enpp1*, and *Ank*

An Integrated Model of the Pathogenesis of Mineralization Disorders

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Tissue-nonspecific alkaline phosphatase (TNAP) hydrolyzes the mineralization inhibitor inorganic pyrophosphate (PP_i). Deletion of the TNAP gene (*Akp2*) in mice results in hypophosphatasia characterized by elevated levels of PP_i and poorly mineralized bones, which are rescued by deletion of nucleotide pyrophosphatase phosphodiesterase 1 (NPP1) that generates PP_i. Mice deficient in NPP1 (*Enpp1*$^{-/-}$), or defective in the PP_i channeling function of ANK (*ank/ank*), have decreased levels of extracellular PP_i and are hypermineralized. Given the similarity in function between ANK and NPP1 we crossbred *Akp2*$^{-/-}$ mice to *ank/ank* mice and found a partial normalization of the mineralization phenotypes and PP_i levels. Examination of *Enpp1*$^{-/-}$ and *ank/ank* mice revealed that *Enpp1*$^{-/-}$ mice have a more severe hypermineralized phenotype than *ank/ank* mice and that NPP1 but not ANK localizes to matrix vesicles, suggesting that failure of ANK deficiency to correct hypomineralization in *Akp2*$^{-/-}$ mice reflects the lack of ANK activity in the matrix vesicle compartment. We also found that the mineralization inhibitor osteopontin (OPN) was increased in *Akp2*$^{-/-}$, and decreased in *ank/ank* mice. PP_i and OPN levels were normalized in [*Akp2*$^{-/-}$; *Enpp1*$^{-/-}$] and [*Akp2*$^{-/-}$; *ank/ank*] mice, at both the mRNA level and in serum. Wild-type osteoblasts treated with PP_i showed an increase in OPN, and a decrease in *Enpp1* and *Ank* expression. Thus TNAP, NPP1, and ANK coordinately regulate PP_i and OPN levels. The hypomineralization observed in *Akp2*$^{-/-}$ mice arises from the combined inhibitory effects of PP_i and OPN. In contrast, NPP1 or ANK deficiencies cause a decrease in the PP_i and OPN pools that leads to hypermineralization. *(Am J Pathol 2004, 164:1199–1209)*

Bone mineralization is a tightly controlled process, the initial stages of which begin in chondrocyte- and osteoblast-derived matrix vesicles (MVs).[1,2] MVs contain calcium and inorganic phosphate ions (P_i) and it is within the MVs that the first crystals of hydroxyapatite are formed. These crystals grow and are exposed to the extracellular environment because of protrusion through the MV membrane. Exposure of the hydroxyapatite crystals to the extracellular milieu further enables growth and proliferation of the crystals.[3–5] Inorganic pyrophosphate (PP_i) antagonizes the ability of P_i to crystallize with calcium to form hydroxyapatite and thereby suppresses hydroxyapatite crystal propagation. For normal mineral deposition to proceed, a tight balance is required between the levels of extracellular P_i and PP_i.

Three molecules have been identified as central regulators of extracellular PP_i and P_i levels (Table 1), ie, tissue-nonspecific alkaline phosphatase (TNAP), which hydrolyzes PP_i,[6–10] nucleotide pyrophosphatase phosphodiesterase 1 (NPP1), which generates PP_i from nucleoside triphosphates,[11–13] and the multiple-pass transmembrane protein ANK, which mediates intracellular to extracellular channeling of PP_i.[14,15]

TNAP is an important promoter of mineralization because it catalyzes the hydrolysis of PP_i thereby decreasing the concentrations of this calcification inhibitor, while concomitantly increasing the levels of P_i. Mice in which the TNAP gene has been inactivated (*Akp2*$^{-/-}$) mimic the most severe form of hypophosphatasia, a disease characterized by rickets, osteomalacia, spontaneous bone fractures, and increased PP_i levels.[16,17] *Akp2*$^{-/-}$ skeletal preparations show poor mineralization in the parietal bones, scapulae, vertebral bones, and ribs.[18–20]

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Table 1. Glossary of the Genes and Proteins Studied in This Article

Gene symbol	Protein symbol	Common name; synonyms
Akp2	TNAP	Tissue-nonspecific alkaline phosphatase; TNSALP
Enpp1	NPP1	Nucleotide pyrophosphatase phosphodiesterase-1; PC-1
Ank (wild-type allele) and *ank* (mutated allele)	ANK	Ankylosis protein
Opn	OPN	Osteopontin

NPP1 serves as a physiological inhibitor of calcification, at least in part by generating PP_i.[11–13,21] In human infants, severe NPP1 deficiency states were recently linked to a syndrome of spontaneous infantile arterial and periarticular calcification.[22,23] NPP1 knockout mice (*Enpp1*$^{-/-}$) also known as tiptoe walking (*ttw/ttw*) mice, spontaneously develop progressive ankylosing intervertebral and peripheral joint hyperostosis and articular cartilage calcification.[24–29] Despite the different manner in which NPP1 and ANK supply PP_i to bone matrix, a similar phenotype is associated with a naturally occurring truncation mutation of the C-terminal cytosolic domain of ANK that appears to attenuate PP_i channeling in *ank/ank* mutant mice.[14,15,30]

The TNAP-, NPP1-, and ANK-deficient mice all have altered levels of PP_i and thus, these mice are valuable tools to further understand the function of PP_i in the process of bone mineralization. Central to our understanding of this process is not only how osteoblasts and chondrocytes make and dispose of PP_i, but what downstream effects PP_i has on bone forming cells, in particular osteoblast gene expression. We have previously shown that crossbreeding the *Akp2*$^{-/-}$ and the *Enpp1*$^{-/-}$ mice rescues the PP_i levels of the single-deficient animals, resulting in a correction of the mineralization defects.[10] Given the similarity in function of NPP1 and ANK, and also the similarity in phenotype of the deficient mice, here we investigated whether simultaneously affecting TNAP and ANK function would also ameliorate the mineralization defects of the single-deficient mice as previously observed for the [*Akp2*$^{-/-}$*; Enpp1*$^{-/-}$] double deficiency. We also examined the effects of PP_i on osteoblastic gene expression. It is well established that during the process of normal bone differentiation and subsequent deposition of mineral, a number of osteoblast marker genes are expressed in a defined spatial and temporal manner. We have recently shown that osteopontin (OPN), a putative inhibitor of mineralization,[31–33] is down-regulated in the hypermineralized *Enpp1* and *ank/ank* mice.[29] Here we investigated the changes in OPN expression in the single- and double-knockout mice and the effects of TNAP-, ANK-, and NPP1-mediated alterations in PP_i levels on both OPN expression and hydroxyapatite deposition. Our data have enabled us to build a model of the concerted action of these three molecules in controlling normal mineralization that also explains the pathological abnormalities in hypo- and hypermineralization disorders.

Materials and Methods

Reagents

All routine chemicals were of an analytical grade from Sigma (St. Louis, MO), unless otherwise indicated.

Generation and Maintenance of Akp2$^{-/-}$, Enpp1$^{-/-}$, *and [*Akp2$^{-/-}$; ank/ank*] Mice*

The generation and characterization of *Akp2*$^{-/-}$, *Enpp1*$^{-/-}$, *ank/ank*, and [*Akp2*$^{-/-}$*; Enpp1*$^{-/-}$] mice has been reported previously.[10,19,20,26] Mice carrying the *ank* mutation[14,15] were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred into the *Akp2*$^{-/-}$ strain to generate [*Akp2*$^{-/-}$*; ank/ank*] double-deficient mice. To determine genotypes, genomic DNA was isolated from tails and analyzed using polymerase chain reaction (PCR) protocols.[10,29] Southern blots were used to confirm the double-knockout genotypes.

Tissue Preparation and Morphological Analysis

Whole-mount skeletal preparations were prepared by removal of skin and viscera of mice followed by a 1-week immersion in 100% ethanol, followed by 100% acetone. Samples were then transferred to a 100% ethanol solution containing 0.01% Alizarin Red S, 0.015% Alcian Blue 8GX, and 0.5% acetic acid for 3 weeks. Samples were then destained with 1% (v/v) KOH/50% glycerol solution. Cleared samples were stored in 100% glycerol. For analysis of mineral deposition, the lumbar spines from 10-day-old mice were fixed in 10% neutral buffered formalin for 5 days. After washing in phosphate-buffered saline-based sucrose solutions, samples were embedded in optimal cutting temperature (OCT) solution. Sections (16 μm) were stained using the von Kossa procedure as previously described.[34] The degree of mineralization of the spines was quantified by examining the vertebral apophyses of the von Kossa-stained lumbar sections from each respective genotype. The number of ossification centers containing visible mineral deposits (stained black/brown) *versus* those containing no deposits, was determined using low ($\times$16) magnification. Three sections, each containing a minimum of five vertebrae, were counted per mouse and the percentage of mineralized apophyses was plotted as a function of the *Akp2* and *ANK* genotypes. The number of mice examined for each genotype were: [*Akp2*$^{+/+}$*; Ank/Ank*], $n = 2$; [*Akp2*$^{+/+}$*; Ank/ank*], $n = 7$; [*Akp2*$^{+/+}$*; ank/ank*], $n = 2$; [*Akp2*$^{+/-}$*; Ank/Ank*],

$n = 8$; [*Akp2*$^{+/-}$*; Ank/ank*], $n = 9$; [*Akp2*$^{+/-}$*; ank/ank*], $n = 3$; [*Akp2*$^{-/-}$*; Ank/Ank*], $n = 2$; [*Akp2*$^{-/-}$*; Ank/ank*], $n = 9$; and [*Akp2*$^{-/-}$*; ank/ank*], $n = 2$. For immunohistochemical analysis, mouse skeletal tissues were dissected and fixed in 10% neutral buffered formalin for 2 days and then decalcified in 4% hydrochloric acid, processed for histology, and embedded in paraffin. For detection of OPN, sections were deparaffinized, blocked with 10% goat serum for 20 minutes, and incubated overnight at 4°C with rabbit polyclonal antibody to OPN (Chemicon, Temecula, CA). Washed sections were incubated for 1 hour at 22°C with biotinylated goat anti-rabbit IgG followed by a 1-hour incubation with peroxidase-conjugated avidin. Peroxidase activity was detected using the Fast DAB staining kit (Sigma), according to the manufacturer's instructions.

Isolation and Culture of Primary Calvarial Osteoblasts

Mouse calvarial cells were isolated from 3-day-old mice through sequential collagenase digestion, as previously described.[10] Calvarial cells of the same genotype were pooled and plated at a density of 4×10^4 cells/cm^2 in α-MEM (Life Technologies, Inc., Grand Island, NY), supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, and 1% L-glutamine. The cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. The medium was completely replaced every third day. For studies under mineralizing conditions, media were supplemented with β-glycerophosphate (10 mmol/L) and L-ascorbic acid (50 μg/ml).

Analysis of PP_i Levels

PP_i levels were determined by differential adsorption on activated charcoal of UDP-D-[6-^{3}H]glucose (Amersham Biosciences, Piscataway, NJ) from the reaction product of 6-phospho[6-^{3}H]gluconate, as previously described.[12,13] To determine intracellular PP_i, washed cells were heated at 65°C for 45 minutes, washed again, and lysed in 1% Triton X-100, 1.6 mmol/L $MgCl_2$, 0.2 mol/L Tris, pH 8.1 (lysis buffer).[12] Extracellular PP_i was determined from conditioned media treated in the same manner.[12] We determined cell protein, and specific activity of nucleosidetriphosphate pyrophosphohydrolase and alkaline phosphatase activities as described.[13]

Western Blot Analysis of NPP1 and ANK Localization

Primary calvarial osteoblasts were treated with mineralization media for 14 days and the cell-associated MVs were collected by collagenase digestion for 2 hours at 37°C. The supernatant was collected and initially centrifuged at 20,000 $\times$ *g* for 20 minutes at 4°C to pellet cellular debris. This was followed by centrifugation at 100,000 $\times$ *g* for 1 hour to isolate the MV fraction, which was resuspended in Hanks' balanced salt solution. Fifty μg of protein was used for Western blotting from both the MV fraction as well as the cell lysates collected in the first centrifugation. Western blot analysis was performed as previously described using rabbit anti-mouse ANK,[35] and rabbit anti-mouse NPP1.[15]

RNA Isolation and Reverse Transcriptase (RT)-PCR

Total RNA was isolated from osteoblasts using 0.5 ml of Trizol (Life Technologies)/35-mm dish and was reverse-transcribed using the Titanium One-Step RT-PCR kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions. The primers used were as follows: Mouse *Opn* (5′-CTCCCGGAGAAAGTGACTGA-3′, 5′-GACCTCAGAAGATGAACTAT-3′), mouse *Akp2* (5′-AGTCCGTGGGCATTGTGACTA-3′, 5′-TGCTGCTCCACTCACGTC GAT-3′), mouse *Ank* (5′-CTAGCAGGGTTTGTGGGAGAA-3′, 5-TTTATGAAGCAGGGG CGTGAA-3), mouse *Enpp1* (5′-GCTACAGCTTTCTAGCCATGA-3′, TTAATCCAAGCC CAGGTCCTT-3′). Mouse β-actin primers were used as a loading control. The PCR products were separated by electrophoresis on 1% agarose gels and were visualized by ethidium bromide staining with UV light illumination.

OPN Enzyme-Linked Immunosorbent Assay

To measure OPN protein levels, we developed an enzyme-linked immunosorbent assay based on a previously published method[36] using plates coated with a monoclonal antibody to native OPN (Chemicon) using a 1:2500 dilution of OPN antibody in 100 μl/well 0.1 mol/L sodium bicarbonate, pH 9.0, at 4°C overnight. Wells were blocked with 10 mmol/L Tris, 150 mmol/L NaCl, and 0.05% Tween-20, pH 8.0, for 1 hour at 22°C. Samples, diluted in 1% bovine serum albumin, 10 mmol/L Tris, and 150 mmol/L NaCl, pH 8.0, were added to the wells and incubated for 1 hour at 37°C. Recombinant murine OPN was used as standard. Washed wells were incubated sequentially for 1 hour at 37°C with rabbit anti-OPN (1:1000, Chemicon), biotinylated goat anti-rabbit IgG (1:1000), and streptavidin conjugated with AP (1:500 dilution). Color was developed using *p*-nitrophenylphosphate and read at 405 nm.

Results

Crossbreeding Akp2$^{-/-}$ Mice to ank/ank Mutant Mice Partially Rescues the Mineralization Defects Observed in the Single Mutants

We have previously shown that the simultaneous deletion of the *Akp2* and *Enpp1* genes can rescue the mineralization defects of both the *Akp2* and *Enpp1* single-knockout mice.[10] The transmembrane protein ANK acts as a transport channel for PP_i, and therefore has a similar function to NPP1 in that it also increases extracellular PP_i levels. Truncation mutation of the C-terminal intracellular domain of ANK in *ank/ank* mutant mice, results in a hyperminer-

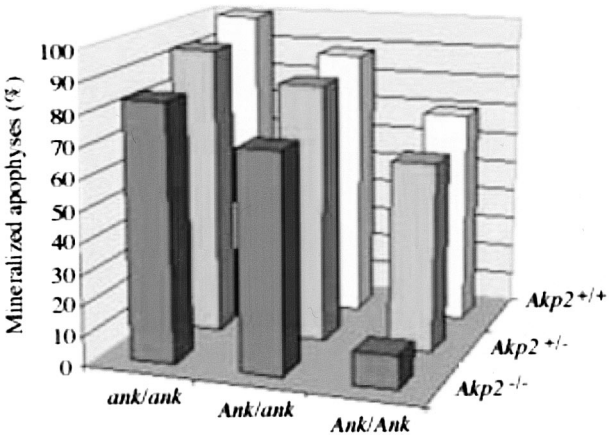

Figure 1. [*Akp2*$^{-/-}$*; ank/ank*] mice display a partial correction in mineralization defects. The degree of mineralization in the vertebral apophyses of the various single- and double-knockout animals was determined by von Kossa staining of spine sections as described in Materials and Methods. The percentage of mineralized apophyses is plotted as a function of *Akp2* and *Ank* genotypes.

alized phenotype, called murine progressive ankylosis[14,15] and these mice show remarkable similarity in phenotype to the NPP1-deficient mice.

Given this similarity in function between ANK and NPP1, we examined whether a double deficiency in both the *Ank* and *Akp2* genes could rescue the mineralization defects of both the *Akp2*$^{-/-}$ and *ank/ank* mutant mice. To this end we crossbred *ank/ank* mutant mice to *Akp2*$^{+/-}$ heterozygote mice to obtain [*Akp2*$^{-/-}$*; ank/ank*] double-mutant mice. We examined the degree of mineralization in the spines of [*Akp2*$^{-/-}$*; ank/ank*] mice using von Kossa staining to detect mineral deposition because we have previously shown[10] that the vertebral apophyses provide a sensitive indication of the extent of hypo- and hypermineralization. We calculated the percentage mineralization of the apophyses in both the single- and double-mutant animals (Figure 1). As expected, *Akp2*$^{-/-}$ mice showed a clear depression in the level of mineralization of the vertebrae, with only 10% of the vertebral apophyses mineralized. The *ank/ank* mutant animals had an increased level of mineralization in the spine, with 100% mineralization in comparison to 70% in wild-type controls. Mice doubly deficient in both *Akp2* and *Ank*, showed an improvement in the levels of abnormal mineralization (80%) in comparison to the *Akp2*$^{-/-}$ single-knockout (10%) and the *ank/ank* mutant mice (100%). In *Akp2*$^{-/-}$ animals the level of mineralization was ameliorated by mutation of just one *Ank* allele in these mice as the [*Akp2*$^{-/-}$*; Ank/ank*] mice showed normalized levels of mineralization, ~70%, ie, control levels. In the [*Akp2*$^{-/-}$*; ank/ank*] double-mutant animals there remained a higher degree of mineralization than in control mice, but the percentages were lower than in the *ank/ank* mutant animals. Thus, we conclude that crossbreeding *Akp2*$^{-/-}$ mice to *ank/ank* mice results in a partial rescue of the abnormal mineralization of both single-mutant mice.

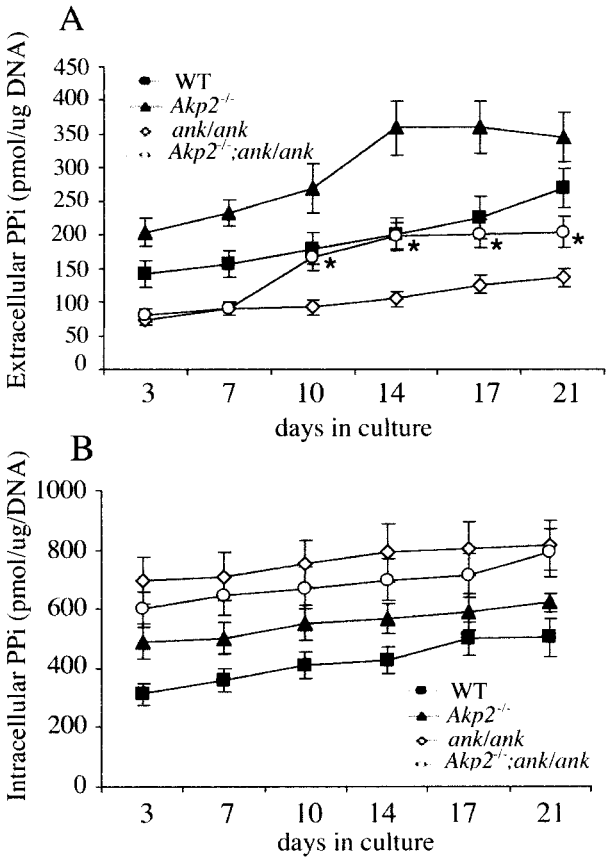

Figure 2. Intracellular levels of PP_i are normalized in [*Akp2*$^{-/-}$*; ank/ank*] mice. Primary osteoblasts were cultured in the presence of mineralizing media, ie, β-glycerophosphate (10 mmol/L) and L-ascorbic acid (50 μg/ml). At the indicated days, extracellular (**A**) and intracellular (**B**) PP_i levels were determined as described in the Materials and Methods. $n = 9$; *, $P < 0.05$ relative to *Akp2*$^{-/-}$ and *ank/ank*.

PP_i Levels Are Partially Corrected in the [Akp2$^{-/-}$; ank/ank] Double-Deficient Mice

Given the partial correction of the mineralization defects that we observed in [*Akp2*$^{-/-}$*; ank/ank*] mice, we next examined whether the levels of PP_i were also affected in these animals. In both the *Akp2*$^{-/-}$ mice and the *ank/ank* mutant animals, PP_i levels are altered (Figure 2). In *Akp2*$^{-/-}$ mice elevated levels of PP_i result from the lack of the pyrophosphatase activity of TNAP whereas in *ank/ank* mutant mice there is a decrease in extracellular levels of PP_i because of the absence of ANK-mediated transport of PP_i across the plasma membrane. The *ank/ank* mutant mice although displaying decreased levels of extracellular PP_i, have increased levels of intracellular PP_i.

We determined the profile of both intra- and extracellular PP_i levels from calvarial osteoblasts isolated from the respective knockout or mutant mice throughout the course of a 21-day bone nodule assay (Figure 2, A and B). Interestingly, we found that the extracellular levels of PP_i were significantly normalized in the [*Akp2*$^{-/-}$*; ank/ank*] osteoblasts during the latter stages of differentiation. However, for intracellular levels of PP_i, although there is a tendency toward normalization, the changes are not statistically significant.

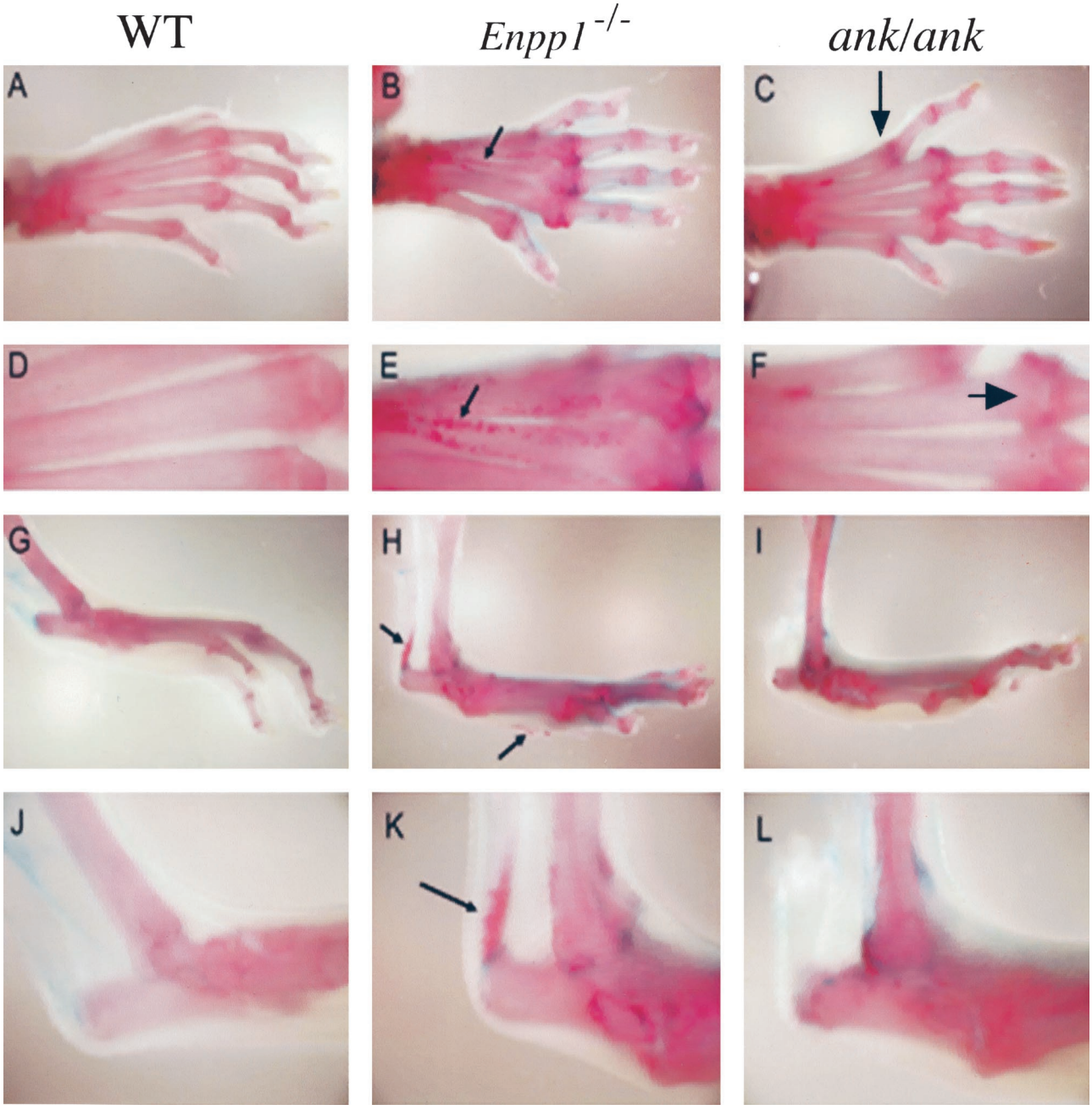

Figure 3. Whole mount skeletal preparations of wild-type (WT), *Enpp1*$^{-/-}$, and *ank/ank* mice. The specimens were stained with Alizarin Red. *Enpp1*$^{-/-}$ mice display a more severe hyperossification phenotype than *ank/ank* mice. **C: Large arrows** indicate the increased amount of mineral in the phalanges of the *ank/ank* mice. **Small arrows** indicate the areas of soft tissue mineralization in the metatarsal bones of *Enpp1*$^{-/-}$ mice (**B**, **E**), ossification of the Achilles tendon is also observed (**H**, **K**).

It seems, from the partial correction in both skeletal mineralization and PP_i concentrations, that the *Ank* and *Enpp1* deficiencies may not be identical because cross-breeding *ank/ank* mice to *Akp2*$^{-/-}$ mice does not result in the same degree of phenotypic rescue observed in the [*Akp2*$^{-/-}$*; Enpp1*$^{-/-}$] double-knockout mice. This led us to compare more closely the degree of mineralization in both the *Enpp1*$^{-/-}$ and *ank/ank* mutant animals. Whole mount examination of their Alizarin Red-stained skeletons consistently revealed subtle differences in the soft tissue ossification phenotypes of these mice. It appears that the *Enpp1*-knockout mice have a more severe phenotype than *ank/ank* mutant mice (Figure 3). The *Enpp1*$^{-/-}$ mice display soft tissue mineralization in the metatarsal bones (Figure 3, B and E) as well as ossification of the Achilles tendon (Figure 3, H and K), whereas the *ank/ank* mutants, although clearly displaying hyperossification (Figure 3C), do not have the same degree of hypermineralization as observed in the *Enpp1*$^{-/-}$ mice.

To further examine the differences between NPP1 and ANK, we crossbred *Enpp1*$^{-/-}$ and *ank/ank* mice. We surmised that if these molecules act on separate path-

Figure 4. Mineral deposition in [*Enpp1*$^{-/-}$*; ank/ank*] double-deficient mice. Nondecalcified lumbar spine sections were stained using the von Kossa technique to observe mineral deposition. Areas of excess mineral deposition are observed (**arrows**) in the *Enpp1*$^{-/-}$, *ank/ank*, and [*Enpp1*$^{-/-}$*; ank/ank*] panels. There is more severe vertebral joint ossification in the double mutant mice than that observed in single-deficient mice.

ways, [*Enpp1*$^{-/-}$*; ank/ank*] double-deficient mice would have a greater degree of hypermineralization than the single-mutant animals. Indeed, [*Enpp1*$^{-/-}$*; ank/ank*] double-deficient mice display a greater degree of paraspinal ligament ossification than the single-deficient mice as determined by von Kossa staining of the spines (Figure 4). This implies that NPP1 and ANK have distinct effects on extracellular PPi concentrations, and therefore on mineralization. This was confirmed by examination of the ANK and NPP1 localization in osteoblasts and MVs. Western blot analysis of ANK and NPP1 localization revealed that both are present in osteoblasts but only NPP1 is present in MVs (Figure 5). This suggests that the localization of NPP1 and ANK to different microenvironments

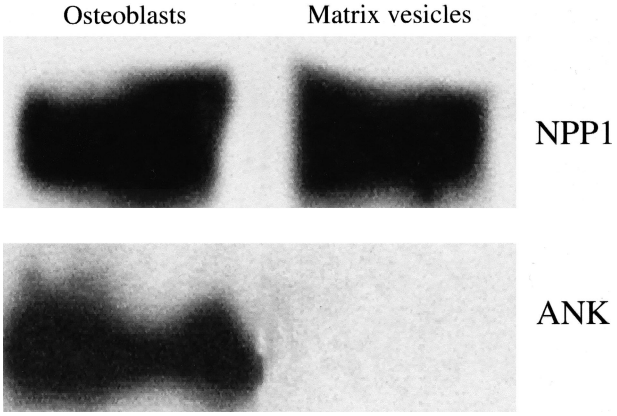

Figure 5. Localization of ANK and NPP1 in osteoblasts and MVs. Protein extracts of wild-type osteoblast lysates and osteoblast-derived MVs were used to perform Western blot analysis of ANK and NPP1 as described in Materials and Methods. NPP1 was detected in both osteoblast lysates and MVs, but ANK was only detected in osteoblast lysates.

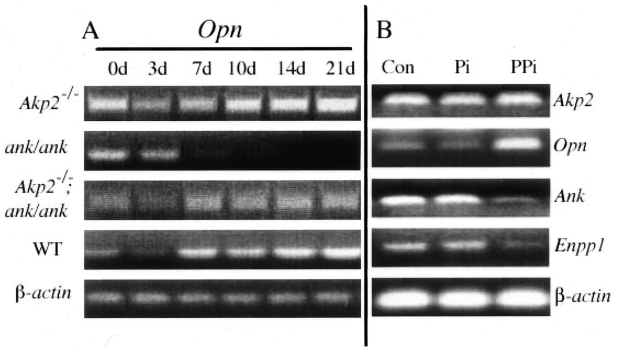

Figure 6. Expression of *Opn* in *Akp2*$^{-/-}$, *ank/ank*, and [*Akp2*$^{-/-}$*; ank/ank*] mice. **A:** Primary osteoblasts from each respective genotype were cultured in the presence of β-glycerophosphate (10 mmol/L) and L-ascorbic acid (50 μg/ml). At the indicated days total RNA was extracted and RT-PCR analysis was performed to determine the expression pattern of osteopontin (*Opn*). β-actin was used as a loading control for all genotypes, the wild-type (WT) levels of β-actin are shown. **B:** Wild-type primary osteoblasts were cultured in the presence or absence of P_i (500 pmol/L) or PP_i (500 pmol/L), after 24 hours total RNA was isolated and RT-PCR analysis was performed for *Akp2*, *Opn*, *Ank*, and *Enpp1* expression. β-actin was used as a loading control.

might account for the different mineralization abnormalities and degree of rescue observed in the *Enpp1*$^{-/-}$ and *ank/ank* models.

Osteopontin Expression Is Affected in Akp2$^{-/-}$ *and ank/ank Mutant Osteoblasts*

We next investigated the downstream events associated with either an increase or a decrease of extracellular PP_i. The mineralization defects observed in the *Akp2*-, *Enpp1*-, and *Ank*-deficient mice suggest a defect in the osteoblast compartment, because it is in the osteoblast that the process of bio-mineralization is initiated. During the process of normal bone mineralization a number of osteoblast marker genes are expressed in a well-defined spatial and temporal manner. We have previously shown that one of these markers, OPN, which is an inhibitor of mineralization,[31,37] is depressed at the mRNA level in osteoblasts isolated from *Enpp1*$^{-/-}$ mice.[29] We therefore examined the expression of *Opn* in calvarial osteoblast cells isolated from *Akp2*$^{-/-}$ and *ank/ank* mice as well as the [*Akp2*$^{-/-}$*; ank/ank*] double-deficient mice (Figure 6A). We found that throughout the 21-day period of differentiation in a bone nodule assay, *Opn* levels were altered in both the *Akp2*$^{-/-}$ and the *ank/ank* mice in comparison to wild-type controls. In *Akp2*$^{-/-}$ calvarial osteoblasts, *Opn* expression was markedly increased throughout the course of the differentiation period. In contrast, in *ank/ank* osteoblasts cells, *Opn* expression was comparable to that of wild-type cells during the initial stages of differentiation, up to day 3, but was completely shut-off during the later stages. Interestingly, we observed a normalization of *Opn* expression in [*Akp2*$^{-/-}$*; ank/ank*] mice, although the levels were not restored to those of wild-type controls. The partial normalization in *Opn* levels in the [*Akp2*$^{-/-}$*; ank/ank*] osteoblasts parallels the partial correction observed in the mineralization defects and PP_i concentrations.

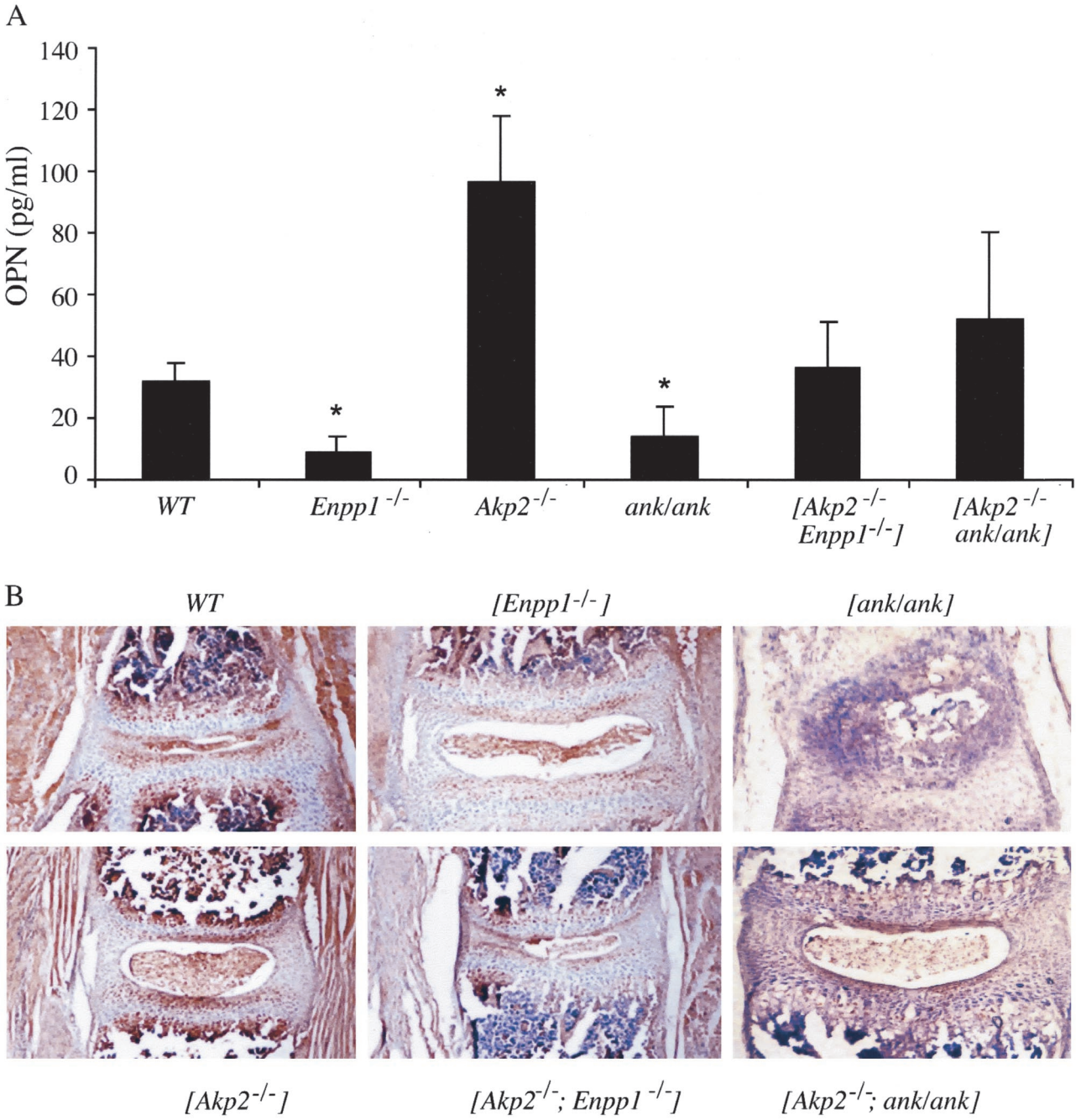

Figure 7. OPN levels in *Akp2*$^{-/-}$, *Enpp1*$^{-/-}$, and *ank/ank* mice. **A:** The serum OPN levels from the indicated mice were measured using an enzyme-linked immunosorbent assay technique as described in Materials and Methods. Serum OPN levels are depressed in *Enpp1*$^{-/-}$ and *ank/ank* mice, and are elevated in *Akp2*$^{-/-}$ mice. OPN levels are normalized in the [*Akp2*$^{-/-}$*; Enpp1*$^{-/-}$] and [*Akp2*$^{-/-}$*; ank/ank*] mice. $n = 5$; *, $P > 0.05$ relative to wild-type. **B:** Immunohistochemical analysis of the distribution of OPN in the wild-type (WT), *Enpp1*$^{-/-}$, *ank/ank*, *Akp2*$^{-/-}$, [*Akp2*$^{-/-}$*; Enpp1*$^{-/-}$] and [*Akp2*$^{-/-}$*; ank/ank*] mice vertebral apophyses using rabbit polyclonal anti-OPN as described in Materials and Methods. Original magnifications, ×20.

We next investigated whether this effect on *Opn* expression in the *Akp2*$^{-/-}$ and *ank/ank* mice was because of changes in the concentrations of PP_i given that these mice have elevated (*Akp2*$^{-/-}$), or depressed (*ank/ank*), levels of PP_i. To this end we determined whether in fact PP_i is able to modulate *Opn* expression in osteoblasts (Figure 6B). Addition of exogenous PP_i to wild-type calvarial osteoblasts caused an increase in *Opn* mRNA levels in response to PP_i. This effect is because of PP_i, and not its hydrolysis product P_i because at these concentrations P_i did not affect OPN expression in the wild-type osteoblast cells. Interestingly, PP_i levels affect not only *Opn* expression, but also *Enpp1* and *Ank* expression. In response to PP_i treatment, both *Enpp1* and *Ank* expression are depressed, thus suggesting that both NPP1 and ANK can influence *Opn* expression through their regulation of PP_i concentrations. These effects on *Opn* expression were not only observed at the mRNA level, but were

also reflected in changes in serum levels of circulating OPN in the various single- and double-mutant mouse models (Figure 7A). This analysis revealed a similar pattern of OPN changes as observed at the mRNA level in that serum levels of OPN were elevated in *Akp2*$^{-/-}$ mice and depressed in *ank/ank* mice. In the [*Akp2*$^{-/-}$*; ank/ank*] double-knockout mice we observed a normalization of serum OPN levels. Furthermore, we measured serum OPN in the *Enpp1*$^{-/-}$ mice and observed a similar reduction in concentration as that observed in the *ank/ank* mice. As with the [*Akp2*$^{-/-}$*; ank/ank*] mice, we observed a significant correction in serum OPN levels in the [*Akp2*$^{-/-}$*; Enpp1*$^{-/-}$] double-knockout mice. In addition to serum level of OPN, we also looked at the *in situ* localization of OPN in bone. Immunohistochemical analysis of spine sections from these mice revealed changes in OPN expression that paralleled the mRNA data as well as the changes in serum levels of OPN (Figure 7B). *Akp2*$^{-/-}$ mice display an increase in OPN expression in the vertebral apophyses in comparison to wild-type animals, whereas in analogous locations in *Enpp1*$^{-/-}$ and *ank/ank* mice, OPN expression is lower compared to wild-type controls. The [*Akp2*$^{-/-}$*; Enpp1*$^{-/-}$] and [*Akp2*$^{-/-}$*; ank/ank*] mice display OPN levels comparable to control animals (Figure 7B).

Thus, we conclude that the phenotype of the hypomineralized *Akp2*$^{-/-}$ mice, and the hypermineralization abnormalities observed in the *Enpp1* and *ank/ank* mutant mice are because of the effects of not one, but at least two mineralization inhibitors (Figure 8). OPN has previously been shown to inhibit hydroxyapatite crystal formation and it is thought that it physically limits crystal growth in bone. We show here that in mice lacking TNAP, there is an increase in OPN levels that results from the increased transcription of the *Opn* gene induced by elevated concentrations of extracellular PP_i. Thus, hypomineralization in the *Akp2*$^{-/-}$ mice likely ensues as a result of the combined effect of abnormally high levels of both of these mineralization inhibitors, ie, PP_i and OPN. In mice lacking NPP1, and in ANK-deficient animals, the depressed levels of PP_i lead to reduced levels of OPN, and the decrease in the levels of both mineralization inhibitors leads to an overall increase in mineralization.

Discussion

The restriction of mineral deposition to bone tissues and the regulation of the mineralization process are crucial in maintaining a healthy skeleton. Central in maintaining normal mineralization are P_i and PP_i, because mineral deposition is dependent on a balance between the intra- and extracellular levels of these compounds. Impaired hydroxyapatite deposition results in diseases such as hypophosphatasia, in which bones lack mineral because of an excess of the mineralization inhibitor PP_i. Conversely a deficiency in PP_i results in ectopic calcification and soft tissue mineralization. The *Akp2*$^{-/-}$, *Enpp1*$^{-/-}$ and *ank/ank* mice all have severe mineralization defects and associated abnormal PP_i levels and as such, are valuable tools to examine the possible roles of these genes in controlling mineralization and the effects of PP_i on this process.

We have previously shown that the simultaneous deletion of the *Akp2* and *Enpp1* genes normalizes PP_i levels thereby causing a concomitant correction in the mineralization defects of the single-knockout animals.[10] These data were significant in that they provided clear confirmation of the previously proposed pyrophosphatase role for TNAP in bone tissue[6–9] but also pointed to both NPP1 and TNAP as therapeutic targets in the treatment of mineralization diseases such as hypophosphatasia and osteoarthritis. Given that the ANK protein functions like NPP1, albeit in a different manner, to increase extracellular PP_i levels, we examined whether an analogous crossbreeding of *ank/ank* mice to *Akp2*$^{-/-}$ mice would correct the mineralization defects of the *Akp2*$^{-/-}$ and *ank/ank* single-deficient mice. We show here that the [*Akp2*$^{-/-}$*; ank/ank*] mice displayed a partial correction in the hypo- and hypermineralization phenotypes as measured by the extent of mineralization in the vertebral apophyses of these mice. This partial normalization of mineralization was paralleled by a partial correction in PP_i levels. Interestingly, we observed a normalization of extracellular PP_i levels in the [*Akp2*$^{-/-}$*; ank/ank*] mice, but the intracellular PP_i concentrations in these mice remained abnormal. Presumably this residual abnormality in PP_i levels prevented the complete normalization of mineralization in the double-knockout mice, illustrating the importance of a balance between the intra- and extracellular concentrations of PP_i in regulating hydroxyapatite deposition. In this context a question that remains unanswered is how *ank/ank* osteoblasts are able to respond to a pulse of extracellular PP_i with an increase in *Opn* expression, given the high concentration of intracellular PP_i inherent in these cells.[29]

The difference in phenotypic abnormalities between the [*Akp2*$^{-/-}$*; Enpp1*$^{-/-}$] and the [*Akp2*$^{-/-}$*; ank/ank*] mice led us to examine in detail the degree of hypermineralization in both the *Enpp1*$^{-/-}$ and *ank/ank* mutants as well as in the [*Enpp1*$^{-/-}$*; ank/ank*] double-mutant mice. We determined that there are subtle differences in the hypermineralization defects between the *ank/ank* and *Enpp1*$^{-/-}$ mice and that the ectopic calcification and soft tissue ossification of the *Enpp1*$^{-/-}$ mice is more severe than that of the *ank/ank* mice. Furthermore, the degree of soft-tissue ossification was further increased in the [*Enpp1*$^{-/-}$*; ank/ank*] double-mutants compared to the single-deficient mice, thus indicating that these two molecules act differently because the absence of both results in an additive effect on hypermineralization. The fact that NPP1 localizes to MVs but that ANK is excluded from this extracellular compartment further validates this conclusion.

It is within the membrane-limited microenvironment of the MV that initial mineral accumulation takes place, followed by propagation of the hydroxyapatite crystals that eventually burst through the MV membrane and become exposed to the extracellular matrix.[1] It is well established that MVs are enriched in TNAP[3,4] and it is now clear that TNAP is essential for the second phase of mineral deposition because MVs from hypophosphatasia patients,[38]

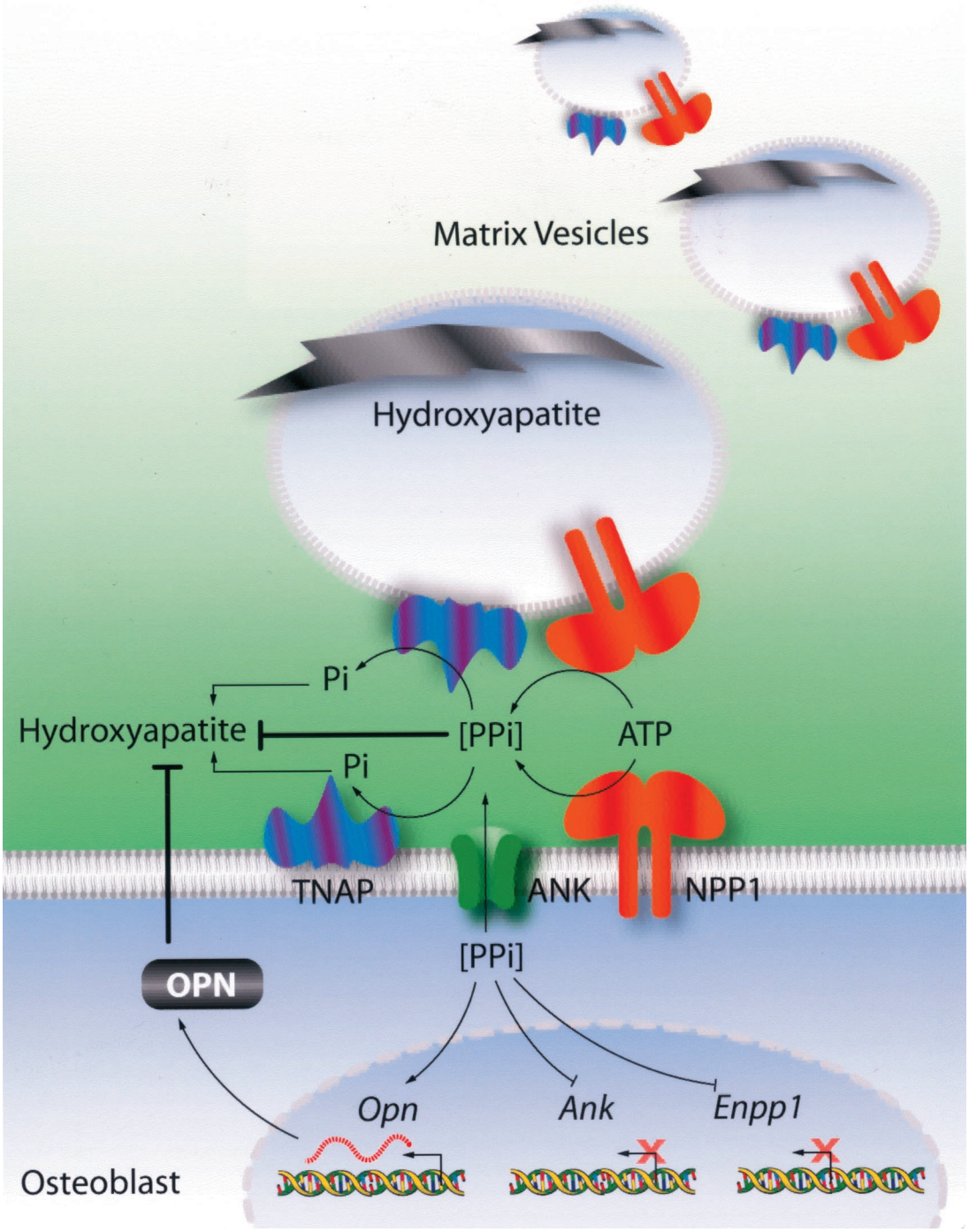

Figure 8. Diagrammatic representation of the roles of TNAP, ANK, NPP1, PP_i, and OPN in the regulation of hydroxyapatite deposition. Both NPP1 and ANK raise extracellular levels of PP_i while TNAP is required for depletion of the PP_i pool. Both TNAP and NPP1 are functional in MVs whereas ANK is not, therefore NPP1 plays a more crucial role in PP_i production than ANK. As a result, the absence of NPP1 in *Enpp1*$^{-/-}$ mice results in a more severe phenotype than in *ank/ank* mice. A negative feedback loop exists in which PP_i, produced by NPP1 and transported by the channeling action of ANK, inhibits expression of the *Enpp1* and *Ank* genes. In addition, PP_i induces expression of the *Opn* gene and production of OPN, which further inhibits mineralization. In the absence of TNAP, high levels of PP_i inhibit mineral deposition directly and also via its induction of OPN expression. The combined action of increased concentrations of PP_i and OPN causes hypomineralization. In the absence of NPP1 or ANK, low levels of PP_i, in addition to a decrease in OPN levels, leads to hypermineralization. This model clearly points to NPP1 and ANK as therapeutic targets for the treatment of hypophosphatasia. Similarly, targeting TNAP function can be useful in the treatment of hypermineralization abnormalities caused by altered PP_i metabolism.

or from *Akp2*$^{-/-}$ mice,[39] contain mineral that does not propagate. The absence of ANK in MVs suggests that its PP_i-channeling function is not required for initiation and propagation of hydroxyapatite crystals and that it is TNAP and NPP1 that are responsible for this process. Thus, the absence of NPP1 in the MVs of *Enpp1*$^{-/-}$ osteoblasts results in a greater deficit in PP_i levels than an absence of ANK, leading to the more severe hypermineralization ob-

served in *Enpp1*$^{-/-}$ mice. The deficit in extracellular PP_i production in *ank/ank* mice results only from the decreased activity of ANK in the osteoblasts. Presumably there is still a sufficient amount of PP_i within the MVs of the *ank/ank* mice (provided by NPP1) such that the hypermineralization phenotype is not as severe as in *Enpp1*$^{-/-}$ mice. Furthermore these data explain the only partial rescue in the [*Akp2*$^{-/-}$*; ank/ank*] mice because in these animals there is an excess PP_i in the MV (because of NPP1) that will not be hydrolyzed in the absence of TNAP, ie, the levels of PP_i in MVs are not normalized in the [*Akp2*$^{-/-}$*; ank/ank*] mice, and thus full rescue does not ensue. In the [*Akp2*$^{-/-}$*; Enpp1*$^{-/-}$] mice however, there is no excess of PP_i in the MVs and so mineral deposition proceeds normally.

Although the *Akp2*$^{-/-}$, *Enpp1*$^{-/-}$, and *ank/ank* mice all have severe mineralization phenotypes associated with abnormal PP_i levels, it is not known precisely how PP_i inhibits the mineralization capacity of osteoblasts. For osteoblasts to lay down mineral, a number of osteoblastic genes must be turned on and off in a well-defined sequence. Several of these characteristic matrix proteins can affect hydroxyapatite deposition and growth.[40] One of these factors is OPN, a secreted glycoprotein that has been suggested to physically limit hydroxyapatite formation and deposition both *in vivo*[31,37] and *in vitro*.[41] We have recently shown that OPN may be a potential modulator of PP_i effects.[29] In this study we examined the effects of PP_i on OPN expression in the three relevant mouse models of mineralization defects. In *ank/ank* and *Enpp1*$^{-/-}$ primary mouse osteoblasts, OPN expression is inhibited in comparison to control levels. We hypothesized that because TNAP has a crucial role in regulating PP_i levels, OPN levels might also be altered in *Akp2*$^{-/-}$ mice. In fact, we found that *Akp2*$^{-/-}$ mice showed significant elevations in OPN expression at the mRNA level. A similar pattern in serum protein levels of OPN was observed, with both *Enpp1*$^{-/-}$ and *ank/ank* mice showing a decrease in serum OPN, whereas *Akp2*$^{-/-}$ mice had increased levels. Interestingly, OPN levels appeared normal in [*Akp2*$^{-/-}$*; Enpp1*$^{-/-}$] and [*Akp2*$^{-/-}$*; ank/ank*] double homozygote mice, which paralleled either a full or partial normalization of mineralization defects and PP_i levels, thus suggesting that PP_i can regulate OPN expression. It should be noted that the range of OPN concentrations measured in this study (10 to 120 pg/ml) are lower than those reported in other studies (100 to 5000 ng/ml).[42,43] This is most likely because of our using serum rather than plasma for our measurements, because it is known that serum levels of OPN are lower than plasma levels.[36]

To confirm that the depression of OPN in *Enpp1*$^{-/-}$ and *ank/ank*, and the increase in OPN in *Akp2*$^{-/-}$ mice is because of changes in PP_i concentrations we examined the effects of exogenous addition of PP_i to wild-type osteoblasts. Exogenous PP_i resulted in an induction of *Opn* expression, and in addition, PP_i down-regulated expression of both *Enpp1* and *Ank*, although PP_i treatment had no significant effect on *Akp2* expression. We determined that this modulation of *Opn* expression is in fact because of PP_i and not its hydrolysis product P_i. It has been previously shown that P_i can induce *Opn* expression,[44] however in our study the end concentrations of P_i, resulting from the complete hydrolysis of the added PP_i, are significantly lower (500 pmol/L) than that used in the study of Beck and colleagues[44] (~10 mmol/L). We chose a PP_i concentration of 500 pmol/L because our previous data has revealed this concentration as the difference between wild-type and *Enpp1*$^{-/-}$ mice. Therefore, our data supports a direct regulation of *Opn* by PP_i, the product of ANK and NPP1 function, and further suggests a negative feedback pathway by which PP_i inhibits *Ank* and *Enpp1* expression.

We therefore conclude that under normal conditions the concerted action of TNAP, NPP1, and ANK regulate intra- and extracellular PP_i levels and that this tight regulation is required for controlled mineral deposition. Hypophosphatasia in the *Akp2* knockout mice arises from deficits in TNAP activity, resulting in an increase in extracellular PP_i levels and a concomitant increase in OPN levels; the combined effect of both these inhibitory factors leads to hypomineralization. This conclusion suggests the testable hypothesis that the simultaneous ablation of the *Opn* and the *Akp2* gene should lead to an improvement in the hypomineralization abnormalities of the *Akp2*$^{-/-}$ mice. A comparison of the [*Akp2*$^{-/-}$*; Opn*$^{-/-}$], [*Akp2*$^{-/-}$*; Enpp1*$^{-/-}$], and [*Akp2*$^{-/-}$*; ank/ank*] mice should give an indication as to the relative contributions of both OPN and PP_i in establishing the rickets/osteomalacia phenotype of the *Akp2*$^{-/-}$ mice.

In turn, an NPP1 or ANK deficiency leads to a decrease in the PP_i levels that cause a decrease in the OPN pool. Thus the simultaneous decrease of both these inhibitory factors, ie, PP_i and OPN, results in the hypermineralization abnormalities in the *Enpp1*$^{-/-}$ and *ank/ank* mice. Because the mineralization defects in *Akp2*$^{-/-}$ mice, along with elevated PP_i and OPN levels, are normalized to varying extents by ablation of either the NPP1 or ANK gene, we conclude that both NPP1 and ANK represent rational therapeutic targets for hypophosphatasia, a disease for which to date there is no treatment. In addition, our current data provide further proof that TNAP is a potential target for hypermineralization diseases such as osteoarthritis and ankylosis.

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