Review

How to build a bone: PHOSPHO1, biomineralization and beyond

Scott Dillon, M.Sci.[a*], Katherine A. Staines, Ph.D.[b], José Luis Millán, Ph.D.[c], Colin Farquharson, Ph.D.[a]

[a] The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, Midlothian, UK, EH25 9RG; [b] School of Applied Sciences, Edinburgh Napier University, Edinburgh, UK, EH11 4BN; [c] Sanford Burnham Prebys Medical Discovery Institute, La Jolla CA 92037, USA

*Correspondence: Scott Dillon, M.Sci., The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, Midlothian, UK, EH25 9RG; Tel: +44 (0)131 651 9100; Email: s1542732@sms.ed.ac.uk

Disclosures

None

Funding

This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) through an EASTBIO Doctoral Training Partnership studentship award (1803936) and Institute Strategic Programme Grant Funding (BB/J004316/1); and the National Institutes of Health (NIH) under award number (AR53102).

Abstract

Since its characterization two decades ago, the phosphatase PHOSPHO1 has been the subject of an increasing focus of research. This work has elucidated PHOSPHO1's central role in the biomineralization of bone and other hard tissues, but has also implicated the enzyme in other

biological processes in health and disease. During mineralization PHOSPHO1 liberates inorganic phosphate (P_i) to be incorporated into the mineral phase through hydrolysis of its substrates phosphocholine (PCho) and phosphoethanolamine (PEA). Localization of PHOSPHO1 within matrix vesicles allows accumulation of P_i within a protected environment where mineral crystals may nucleate and subsequently invade the organic collagenous scaffold. Here, we examine the evidence for this process, first discussing the discovery and characterization of PHOSPHO1, before considering experimental evidence for its canonical role in matrix vesicle-mediated biomineralization. We also contemplate roles for PHOSPHO1 in disorders of dysregulated mineralization such as vascular calcification, along with emerging evidence of its activity in other systems including choline synthesis and homeostasis, and energy metabolism.

Keywords: PHOSPHO1, phosphocholine, inorganic phosphate, biomineralization, matrix vesicle

1. Introduction

Biomineralization of the skeleton is a fundamental process indispensable for health and wellbeing throughout life. The vertebrate skeleton is a complex organ which performs varied and diverse functions encompassing its action as a biomechanical and protective scaffold in conjunction with the musculature, its role in calcium and phosphate ion homeostasis, and recent evidence demonstrating its capacity as an endocrine organ involved with energy homeostasis[(1)]. At the level of molecular constituents bone is composed of a combination of inorganic mineral, Type I collagen, non-collagenous proteins (NCPs) and water, arranged into an extremely ordered hierarchical structure[(2)]. The fine details of this architecture remain controversial at the nanostructural level[(3)], particularly with respect to differences between

embryonic and mature tissue, however the mineral phase has come to be regarded as a poorly-crystalline substituted hydroxyapatite phase, mainly composed of calcium phosphate.

Biomineralization of the skeleton therefore requires generation and manipulation of calcium and phosphate ions on a massive scale. The biological mechanisms through which ions are liberated, contained, and targeted to the developing collagenous framework, and ultimately nucleate mineral in a controlled manner, remain the subject of intense research. Phosphatase enzymes are essential in promoting biomineralization as one pathway through which inorganic phosphate (P_i) may be liberated from biological molecules. These include enzymes with a canonical role in bone development such as tissue non-specific alkaline phosphatase (TNAP). While TNAP activity has long been implicated in the mineralization process, it is now recognized that TNAP is only one component and the full story requires a more complex biochemical system.

Orphan phosphatase 1 (PHOSPHO1; EC 3.1.3.75; encoded by the *Phospho1* gene in the mouse and the *PHOSPHO1* gene in humans) has been an increasing focus of research in this field over the last 20 years and now holds an established function in biomineralization. Here we review the scientific literature on the characterization, localization, regulation and activity of PHOSPHO1 with respect to its role in physiological and pathological biomineralization, along with potential contributions to other biological processes.

2. Characterization, protein structure and substrate specificity

PHOSPHO1 (originally known as 3X11A) was initially cloned from chick hypertrophic growth plate chondrocytes, in which a 5-fold increase in expression was observed in comparison to resting or proliferative chondrocytes[4]. This distinct upregulation was also found be approximately 100-fold higher than in non-chondrogenic tissues. Although the specific function of this novel protein was at this stage unknown, sequencing of the 3X11A

RNA transcript derived from differential display analysis revealed two partially conserved domains which bore sequence similarity to the phosphotransferase enzyme superfamily, including the ATPases and various phosphatases catalysing P_i liberation from a range of phosphomonoester substrates[4]. Primers designed against these motifs succeeded in transcribing a 537bp fragment from RNA isolated from the human osteosarcoma SaOS-2 cell line, demonstrating 69% similarity to the chicken sequence[5]. These fragments were identified as corresponding to several previously unattributed EST transcripts from both the human and mouse *Phospho1* gene, with a high degree of conservation at both the gene and protein level between these two mammalian species. Chromosomal mapping led to the conclusion that the *Phospho1* gene was indeed conserved between the bird and mouse genomes, located within a region of conserved synteny on the HSA17 and GGA27 chromosomes respectively[5].

Stewart *et al* used homology modelling to identify three conserved peptide motifs composing the active site; certifying PHOSPHO1's membership of the Mg^{2+}-dependent haloacid dehalogenase (HAD) superfamily[6]. These included two characteristic aspartic acid residues in motif 1 which co-ordinate the catalytic Mg^{2+} ion in this family, along with other markers such as the hydrophobic amino acid residues within motif 2. Along with identification of orthology between PHOSPHO1 in rat and the previously identified species, substantial homology between the amino acid sequences of PHOSPHO1 and proteins in the fruit fly was also found, along with several plant species including the LePS2 phosphatases in the tomato plant involved with phosphate homeostasis[7-9].

A PHOSPHO1 model protein structure was developed, based upon the crystal structure of the phosphoserine phosphatases (PSPs)[6,10]. This model revealed conservation of residues complexing the substrate phosphate group, however those residues which confer substrate specificity to phosphoserine in the PSPs are absent. Roberts *et al* purified recombinant human

PHOSPHO1 by amplification of a curtailed cDNA transcript from the SaOS-2 osteosarcoma cell line and continuous spectrophotometric phosphate assays with several potential phosphomonoester substrates revealed PHOSPHO1's specific activity towards the metabolites phosphoethanolamine (PEA) and phosphocholine (PCho), with highest activity between pH6.0-7.2[11]. Disruption of the protein active site using site-directed mutagenesis decreased PEA and PCho hydrolysis dramatically to an undetectable level in some mutants[12].

3. Bone biology and mineralization

The ubiquitous phosphatase TNAP has a long-established role during skeletal biomineralization. Mutations in the *ALPL* gene are associated with several skeletal disorders in humans including various forms of hypophosphatasia[13-15]. Genetic ablation of TNAP in a mouse model induced a phenotype mimicking infantile hypophosphatasia with severe skeletal abnormalities[16,17], including those in dental and craniofacial mineralization and morphology[18,19]. Detailed ultrastructural examination of these mice revealed that although hypomineralization of the skeleton was evident, hydroxyapatite mineral crystals were generated as normal[20]. The hypomineralized phenotype was therefore attributed to the inability of the mineral phase to propagate in the absence of TNAP's hydrolysis of the potent mineralization inhibitor pyrophosphate (PP_i)[20,21].

These observations led to the hypothesis that another phosphatase was active during skeletal biomineralization with PHOSPHO1 a strong candidate to fulfil this role. Following the observation of *PHOSPHO1* upregulation in mineralizing hypertrophic chondrocytes[4], immunohistochemistry revealed localization of the protein to these cells in the chick growth plate, along with active bone surfaces at the ossification front and in the trabecular compartment[22]. Whole-mount *in situ* hybridization in the embryonic chick lower limb

furthermore demonstrated expression of *PHOSPHO1* restricted to the developing bones during ossification[23]. *In vivo* suppression of PHOSPHO1 activity using the non-competitive inhibitor lansoprazole (identified specifically as an inhibitor of PHOSPHO1[24]) during chick development induced ablation of mineralization in the lower limb bones[23]. Together these data provided strong evidence that this novel phosphatase plays a critical role in the very first steps of skeletal biomineralization.

The generation of the PHOSPHO1 knock-out mouse (*Phospho1*$^{-/-}$) enabled the detailed investigation of its phenotype and thereby allowed interrogation of the enzyme's specific function in the skeleton[25]. Huesa *et al* used a variety of compositional and biomechanical analyses to show that the mineral:matrix ratio of femora of juvenile *Phospho1*$^{-/-}$ mice was significantly lower than wild-type controls, accompanied by plastic deformation upon 3-point bending and a reduced hardness and elastic modulus[26]. A further investigation of the mechanical properties of these bones corroborated this high fracture toughness and also showed significantly higher indentation distance increases under reference point indentation compared to wild-type controls[27]. Histological examination of *Phospho1*$^{-/-}$ long bones revealed reduced mineralization in the trabecular compartment, while 10-15% of 10-day old mice exhibited a complete lack of secondary ossification centre development[25]. These bones also demonstrated osteoid accumulation; a hallmark of hypophosphatasia (Figure 1). Interestingly, micro-computed tomography (μCT) revealed no differences between 4-week *Phospho1*$^{-/-}$ and wild-type mice in bone volume relative to tissue volume (BV/TV), but rather a significantly reduced bone mineral density (BMD) which was accompanied by diverse spontaneous greenstick fractures and marked thoracic scoliosis[25] (Figure 1). Similarly, PHOSPHO1 plays a critical role in fracture healing, with induced tibial fractures in *Phospho1*$^{-/-}$ mice demonstrating osteoid accumulation and elastic deformation upon loading after four weeks post-surgery[28].

These findings have been extended to investigate the function of PHOSPHO1 in the development of bone's hydroxyapatite mineral phase at the smallest length scales. X-ray diffraction (XRD) along with thermogravimetric analysis (TGA) revealed a lower bulk mineral content with significantly lower mineral:matrix ratio in *Phospho1*$^{-/-}$ femora, also accompanied by a smaller apatite crystal size[29]. Backscattered scanning electron microscopy (BSE-SEM) furthermore revealed generalized hypomineralization relative to wild-type mice in transverse tibial cross-sections of the same animals[29]. Further analysis of the *Phospho1*$^{-/-}$ microstructure exposed diffuse regions of hypomineralization in diaphyseal cortical and trabecular bone with large areas of osteoid accumulation[30]. Interestingly, there was no discernible anatomical pattern between individual *Phospho1*$^{-/-}$ animals, but regions lacking mineral exhibited small focal areas of mineral nucleation at their borders, which failed to propagate more widely.

In the very early studies of its function, characterization of the *Phospho1*$^{-/-}$ bone phenotype was performed in young juvenile mice, and therefore to examine the bone phenotype more fully Javaheri and colleagues investigated whether PHOSPHO1 plays a persistent long-term role in the adult bone's biology where skeletal turnover is relatively slow[31]. Using digital image correlation (DIC) the expected lower stiffness of *Phospho1*$^{-/-}$ was found, which was corrected with age. µCT analysis demonstrated several differences in tibial trabecular microarchitecture, including trabecular number and connectivity, between *Phospho1*$^{-/-}$ and wild-type mice which changed transiently across four age groups, with significant differences detected in the 5- and 34-week age groups, but not in the 7- or 16-week groups. Upon examination of the cortical bone however, the authors noted a significant reduction in BV/TV in animals from 7 weeks of age which was not corrected over time. NanoCT scanning (0.6µm resolution) furthermore revealed a greater number of larger osteocyte lacunae, along with higher vascular porosity in *Phospho1*$^{-/-}$ tibiae; a surprising finding which was compounded by

an early increase in both *Pdpn* (E11) mRNA expression in primary osteoblast cell cultures, potentially indicating accelerated terminal osteocyte differentiation in these cells[31]. PHOSPHO1 may therefore play a role in regulation of osteocytogenesis, which in turn may regulate bone microarchitecture in skeletally mature animals. Further research is required however to assess whether this relationship is mediated through reduced mineralization, or whether PHOSPHO1 may act within a distinct mechanism altogether. Our data also shows that the *Phospho1*$^{-/-}$ phenotype is present during skeletal development, with murine embryos exhibiting a reduced extent of mineralization across all bones during embryogenesis (unpublished data; Figure 2).

Aged 1-year old *Phospho1*$^{-/-}$ mice showed reduced plasma concentrations of TNAP with a concomitant increase in ectonucleotide pyrophosphatase/phosphodiesterase1 (NPP1), thereby resulting in significantly higher PP_i concentrations compared with wild-type controls[25]. Intriguingly, high serum PP_i is a key characteristic of infantile hypophosphatasia in humans, with the resulting inhibition of mineralization attributed to the ensuing rickets/osteomalacia[17,32]. Yadav *et al* attempted to rescue the *Phospho1*$^{-/-}$ phenotype by cross-breeding with mice over-expressing TNAP (*ApoE-Tnap*) and thereby reduce PP_i concentration[25]. While the authors did indeed observe a ~4-fold increase in TNAP in plasma and a significant reduction in PP_i, the hypomineralized *Phospho1*$^{-/-}$ phenotype was not corrected, with animals exhibiting persistent skeletal defects at 7-months. Furthermore PHOSHO1; TNAP double knock-out (*Phospho1*$^{-/-}$*; Alpl*$^{-/-}$) mice exhibited complete ablation of skeletal mineralization and perinatal lethality[25]. These data were augmented and confirmed *in vitro* with osteoblast-like MC3T3-E1 cell lines (clones 14 and 24) along with *ex vivo* metatarsal cultures using specific PHOSPHO1 and TNAP inhibitors in culture[33].

PP_i is known to regulate the expression of other mineralization-associated proteins, particularly osteopontin (OPN; *Spp1*)[34-36]. Along with PP_i, OPN is another potent

mineralization inhibitor[37-39]. The protein's inhibitory effects are mediated through its phosphorylation status[40,41] and this has been shown to be regulated by TNAP[42]. Significantly elevated OPN, which also exhibited a greater degree of phosphorylation compared to controls, was found at the protein and gene levels in serum and spinal lysates of *Phospho1*$^{-/-}$ mice at 1- and 3-months postnatal[43]. Interestingly, no differences were observed in femoral lysates. Yadav *et al* investigated the interplay between PHOSPHO1 and OPN through the generation of *Phospho1*$^{-/-}$*; Spp1*$^{-/-}$ mice and found a partial rescue of the *Phospho1*$^{-/-}$ phenotype, with animals at 1- and 3-months of age exhibiting a reduction in the typical hyperosteoidosis and thoracic scoliosis which characterises the single knock-out animal[43]. The *Phospho1*$^{-/-}$ hypomineralized mouse phenotype is therefore partially attributable to an increased expression of OPN which may obstruct mineralization during bone formation. This is likely exacerbated by a relative hyperphosphorylation of OPN, mediated by the established reduced expression of *Alpl* in *Phospho1*$^{-/-}$ mice[25,43].

Collectively these findings establish a non-redundant role of PHOSPHO1 in mediating biomineralization of the skeleton, as well as its regulated synergy with TNAP, along with other mineralization-associated factors including PP_i and OPN, as part of this process.

4. Dental mineralization

Along with its established role in bone biomineralization, PHOSPHO1 has also been implicated in the mineralization process of the dentition. McKee *et al* studied dentin formation in perinatal mice and found localization of PHOSPHO1 to odontoblasts during the very first steps of dentin formation[44]. *Phospho1*$^{-/-}$ mice exhibited reduced mineralization in the dentin by histology, µCT and radiography; a phenotype which was aggravated by the additional deletion of one *Alpl* allele (*Phospho1*$^{-/-}$; *Alpl*$^{+/-}$). Others have examined PHOSPHO1 function in the other dental hard tissues, namely cementum and enamel.

PHOSPHO1 was found to show expression in osteoblasts and cementoblasts of alveolar bone and cementum respectively during their mineralization phases where osteoid accumulation was evident in the bone and was accompanied by delayed mineralization of the cellular cementum in *Phospho1*$^{-/-}$ animals[45]. Intriguingly, acellular cementum formation was normal, despite downregulation of *Phospho1*$^{-/-}$ in a bone sialoprotein knock-out model in this region[46]. PHOSPHO1 is therefore an enzyme critical for the integrity of the periodontal tissue interface, along with normal development of these hard tissues. In enamel, *Phospho1*$^{-/-}$ mice exhibited a 25% increase in tissue volume, with a significantly reduced level of mineralization[47]. The authors furthermore reported that ablation of PHOSPHO1 caused loss of enamel prism morphology and an impaired crystal organization compared to wild-type mice.

At the level of local cellular regulation, control of mineralization through the PHOSPHO1 pathway has been shown to be influenced by the TRPS1 transcription factor in an odontoblast-like cell line[48]. Mutations in the *Trps1* gene are associated with tricho-rhino-phalangeal syndrome in humans and manifest craniofacial and skeletal dysplasias associated with defective endochondral mineralization[49-51]. Loss of TRPS1 has also been implicated in abnormal tooth development and mineralization[52]. Kuzynski and colleagues overexpressed *Trps1* in preodontoblastic 17IIA11 cells and found reduced mineralization compared with controls[48]. Interestingly reduction of *Trps1* expression also ablated mineralization and was associated with downregulation of *Phospho1* and *Alpl* expression. The authors proposed that TRPS1 acts to repress mineralization associated genes in dentin in a biphasic manner to first inhibit ectopic mineralization, and further to prevent hypermineralization by modulation of osteogenic gene expression, including *Phospho1*.

5. The PHOSPHO1 mineralization mechanism: matrix vesicle-mediated biomineralization

Matrix vesicles (MVs) are membrane-bound nano-spherical bodies of approximately 100-300nm in diameter which are typically rich in lipids and proteins known to chelate Ca^{2+} and P_i, and which are associated with both physiological and pathological biomineralization[53]. Their function continues to be remain controversial since their discovery in 1967 in growth plate cartilage[54,55], with some authors attributing these as specimen preparation artefacts[56]. Nevertheless, many *in vitro* and *in vivo* studies have shown the first mineral crystals in diverse mineralized tissues such as bone, dentin, cartilage and mineralized vasculature are associated with these structures in the extracellular matrix (ECM)[55,57-63].

The structure and function of MVs in skeletal and vascular mineralization has recently been reviewed by several authors[53,64-67]. The biogenesis of these vesicles may occur through multiple proposed mechanisms, however the most prevalent theories include polarized budding from the parental cell membrane, or from microvilli on the cell surface, as demonstrated in hypertrophic chondrocytes and SaOS-2 osteoblast-like cells[68-71]. The theorized function of MVs in biomineralization is to facilitate a localized concentration of Ca^{2+} and P_i, protected from the ECM, from which hydroxyapatite or its precursors may form[53]. Vesicles may also assist in concentrating P_i to engender a P_i:PP_i ratio which is permissible for mineralization[72]. MVs are known to be enriched in Ca^{2+} by way of their intracellular biogenesis from mitochondria under oxidative stress, at least in growth-plate chondrocytes[73,74]. Recently published research by Chaudhary and colleagues may correlate well with these findings, showing that P_i stimulates release of MVs from osteogenic cells, although the authors did not comment on their mitochondrial origin[75].

P_i accumulation within MVs is thought to be mediated by extra- and intravesicular phosphatases, of which PHOSPHO1 plays a critical role[11,20,25,26,76-78]. Stewart and colleagues were the first to suggest PHOSPHO1 as a major intravesicular phosphatase, showing its presence in MVs isolated from embryonic chick growth plates[77]. This was more recently

confirmed in MVs isolated from the ECM of both cultured MC3T3 and SaOS-2 osteoblast-like cells using Western blotting[75] and quantitative proteomics[71,79]. Further work using both growth plate- and primary osteoblast-derived vesicles established that while TNAP does indeed liberate P_i extravesicularly, PHOSPHO1 is biochemically active intravesicularly[24,80]. Therefore it is hypothesized that accumulation of P_i inside MVs occurs via a combination of the intravesicular action of PHOSPHO1 and intravesicular trafficking of TNAP-generated P_i via a Type III Na-P_i co-transporter, P_iT1 (encoded by the *Slc20a1* gene in mice)[77,80] (Figure 3). Experimental evidence for this hypothesis comes from the work of Yadav and colleagues who generated a cartilage specific P_iT1 knock-out mouse driven by a *col2a1*-Cre on a *Phospho1*-null background (*Phospho1*$^{-/-}$*; P$_i$T1*$^{Col2/Col2}$)[81]. The authors report an exacerbation of the *Phospho1*$^{-/-}$ phenotype, including growth plate defects, extensive hyperosteoidosis, decreased BMD and impaired mechanical properties. MVs isolated from differentiating chondrocytes in these animals furthermore demonstrated a loss of their capacity to nucleate hydroxyapatite crystals compared to both *Phospho1*$^{-/-}$ and wild-type controls[81]. Interestingly, both *Phospho1*$^{-/-}$ and *Phospho1*$^{-/-}$*; P$_i$T1*$^{Col2/Col2}$ MV were reduced in numbers compared to wild-types, potentially indicating a role for PHOSPHO1 in MV biogenesis. Together these studies, along with those characterising the *Alpl*$^{-/-}$ and *Phospho1*$^{-/-}$*; Alpl*$^{-/-}$ mice[16,17,20,21,25], afford good evidence for this mechanism integrating the PHOSPHO1 and TNAP P_i generation pathways.

While this work provides a strong evidence base for the pivotal role of PHOSPHO1 in vesicle-mediated biomineralization, the specific biochemical pathway within which it achieves intravesicular P_i liberation remains unclear. Stewart *et al* proposed a mechanism through which PHOSPHO1's substrates PEA and PCho may be generated intravesicuarly by enzymatic action upon the vesicle's phospholipid membrane, as mediated by a phospholipase A_2 (PLA_2) and ectonucleotide pyrophosphatase/phosphodiesterase 6 (NPP6)[82] (Figure 3).

The PLA_2 family of enzymes catalyse cleavage of the acyl group at the *sn*-2 acyl position of glycerophospholipids resulting in a free fatty acid and lysophospholipid (LPL)[83-85]. These enzymes may therefore act to breakdown phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in the MV membrane, forming lysophosphatidylethanolamine and lysophosphocholine LPLs respectively along with arachidonic acid[82]. Indeed the MV membrane has been shown to be enriched in phospholipids containing PCho and PEA which progressively decline during mineralization[74,86,87], while PCho was also identified as an abundant metabolite in developing mouse long bones by matrix-assisted laser desorption/ionization-imaging mass spectrometry[88]. There are upwards of 30 identified mammalian PLA_2 enzymes which exhibit a huge range of localizations (including secreted, cytosolic and lysosomal groups) and have been shown to be involved with many physiological and pathological processes[83-85]. Mebarek and colleagues comprehensively reviewed the evidence for the role of phospholipases in mineralization, noting several experimental studies confirming expression of both secreted and cytosolic PLA_2s in chondrocytes and osteoblasts where they play several roles[89]. While some specific PLA_2s have been shown to have an effect on bone formation[90] it is currently unclear whether these act directly within the mineralization process. Further research is required therefore to identify specific candidate proteins which fulfil this niche.

A second enzymatic processing phase is hypothesized to convert generated LPL to PCho for direct hydrolysis by PHOSPHO1, mediated by NPP6[82]. NPP6 is a member of the nucleotide pyrophosphatase/phosphodiesterase family and has been shown to possess lysophospholipase C activity, catalysing the conversion of lysophosphocholine with a monoacylglycerol by-product[91-93]. Expression of NPP6 has been demonstrated in bone tissue lysate and was immunolocalized to hypertrophic chondrocytes and forming bone surfaces[82]. Specific localization of NPP6 to MVs has yet to be established however.

Alternative biochemical pathways through which PHOSPHO1 substrates may be generated have also been proposed (Figure 3). Neutral sphingomyelinase 2 (nSmase2) is encoded by the *Smpd3* gene in mice and is capable of catalysing the hydrolysis of another MV membrane phospholipid sphingomyelin, to produce PCho with a ceramide by-product[94]. The *fro* mutation in the *Smpd3* gene generates a distinctive phenotype with extensive musculoskeletal defects resulting in dwarfism[95,96]. Khavandgar and colleagues demonstrated that the *fro/fro* mouse exhibits delayed mineralization of the long bones and calvaria, along with impaired hypertrophy in growth plate chondrocytes during embryonic development[97]. Using tissue-specific mouse knock-out models it was also established that *Smpd3* expression is required in both osteoblasts and chondrocytes in a cell-autonomous manner for normal bone development[97,98]. These effects were moreover shown to influence mineralization during tooth development and fracture healing[99,100]. Like PC and PE, sphingomyelin is enriched in MV preparations and declines during mineralization[74,86,87], while the nSmase2 enzyme has been localized to MV isolates in conjunction with PHOSPHO1[79]. nSmase2-mediated production of PCho may therefore provide another pathway for the intravesicular generation of PHOSPHO1 substrates. Another potential alternative pathway includes generation of PCho by phosphorylation of choline through the action of the α and β choline kinases (encoded by the *Chkα* and *Chkβ* genes respectively). While mice lacking *Chkα* display lethality during embryonic development[101], the *Chkβ*$^{-/-}$ mouse exhibits forelimb deformaties and delayed mineralization, accompanied by an extended and disorganized hypertrophic zone within the growth plates at the distal radius and ulna[102]. PCho was also shown to be reduced by ~75% in primary chondrocytes isolated from these animals[102]. Intriguingly, PHOSPHO1 was upregulated in *Chkβ*$^{-/-}$ primary chondrocytes, potentially indicating compensation for a restricted substrate availability[102]. Both *Chkα* and *Chkβ* were found to be expressed in human osteoblast-like MG-63 cells, and gene silencing of *Chkα* resulted in a reduction of

~70% in cellular PCho with an accompanied inhibition of TNAP activity and mineralization in culture[103].

6. Endocrinology and regulation

As a critical effector of biomineralization it is likely that PHOSPHO1 expression and function will be stringently controlled at both the local and systemic level. As a major organ, and one which is metabolically expensive to produce and maintain, it is well established that bone formation and resorption are tightly controlled by many factors during development and in adulthood, some of the most significant of which include the sex steroids[104-106], vitamin D[107-109] and parathyroid hormone (PTH)[110-112]. Whether the observed differences in mineralization caused by regulatory influences are caused wholly or partly by control of PHOSPHO1 or other associated proteins is as yet unknown.

Some evidence for the systemic regulation of PHOSPHO1 comes from work surrounding the effect of PTH on osteoblasts, with RNA-seq in an osteocyte-like cell line (IDG-SW3 cells) stimulated with PTH in culture demonstrated an effect on both *Phospho1* and *Smpd3* expression[113]. Houston *et al* extended these analyses, examining the expression of *Phospho1*, *Smpd3* and *Alpl* specifically in a MC3T3 osteoblast-like cells and *ex vivo* calvaria culture models with continuous PTH exposure[114]. The authors report rapid and co-ordinate downregulation of *Phospho1* and *Smpd3* expression after the addition of PTH to culture media, which was likely regulated through the cAMP/PKA signalling pathway. This downregulation of both *Phospho1* and *Smpd3* was independently found in Kusa 4b10 cells and in young rats exposed to PTH and PTH-related protein 1 (PTHrP)[115,116]. The catabolic effects of continuous PTH on the skeleton are well demonstrated in human conditions such as hyperparathyroidism, however much research has associated this with the upregulation of osteoclastogenesis through the RANKL/OPG axis[117-119]. These data may indicate however a

simultaneous mechanism effecting inhibition of bone formation as mediated by PHOSPHO1. The transcription and post-translational modification (phosphorylation) of Runx2, the transcription factor and master regulator of osteoblast differentiation, is also strongly enhanced by PTH[120]. Interestingly, the overexpression of *Runx2* in mouse limb bud cultures simultaneously enhances the expression of both *Phospho1* and *Smpd3*[121].

Collectively these data surrounding PTH tentatively suggest that control of bone biomineralization may occur by modulating PHOSPHO1 expression, and thereby the MV-mediated biomineralization mechanism. This relationship is however currently far from explicit and much further research is required to establish the regulatory mechanisms behind PHOSPHO1 expression and function within the context of the MV.

7. PHOSPHO1 in pathologies of mineralization

Aside from its central function in mediating physiological biomineralization, PHOSPHO1 may also have a role to play in the onset of pathological mineral formation, for example in vascular calcification. *Phospho1* is upregulated during mineralization of vascular smooth muscle cells (VMSCs) in culture, while *Phospho1*$^{-/-}$ VSMCs exhibited a significant reduction in mineral generation[122]. Also, the PHOSPHO1 inhibitor, MLS-0263839 reduced calcification of cultured VSMCs by ~60% and when combined with the TNAP inhibitor MLS-0038949, calcification of VSMCs was reduced by ~80%[122]. *Phospho1* is also upregulated during atherosclerotic vascular calcification in a rabbit model[123]. Furthermore, Hortells *et al* induced aortic calcification in a rat nephrectomy model with a 1.2% phosphorous diet for 12 weeks post-surgery and observed significant upregulation of *Phospho1* expression in animals exhibiting calcification, along with several other mineralization-associated genes[124]. These studies suggest that PHOSPHO1 has a critical role

in VSMC mineralization and that ‘‘phosphatase inhibition’’ may offer therapeutic strategies to mitigate vascular calcification.

Several authors have investigated the process responsible for vascular calcification, with some implicating PHOSPHO1 and TNAP as part of the MV mechanism[125,126]. Scanning electron microscopy (SEM) and focussed ion beam scanning electron microscopy (FIB-SEM) of human calcified valves and vasculature revealed nanospherical particles composed of crystalline calcium phosphate[127]. Using *in vitro* models together with calcified human tissue, these particles were later implicated in the formation of mineralized atherosclerotic plaques through their initial aggregation and nucleation of the mineral phase[128]. Furthermore, particles isolated from mineralized aortae were subsequently shown to induce pathological changes in valvular endothelial cells (VECs) and valvular interstitial cells (VICs) in culture[129]. These studies demonstrate compelling similarities between calcified particles found in the vasculature and MVs. The localization of PHOSPHO1 or other MV-associated enzymes with these structures has not as yet however been confirmed and so the mechanism behind their generation remains elusive.

There is now substantial evidence that the mineralization status of the subchondral bone is altered in osteoarthritis, and that this may lead to modified mechanical integrity, engendering greater articular cartilage degeneration[130]. *Phospho1* is upregulated during chondrocyte differentiation[131], and aged hypomineralized *Phospho1*$^{-/-}$ mice exhibit increased articular cartilage degradation and osteophyte formation, when compared to the age-matched wild-type mice[132]. Further, non-invasive loading of *Phospho1*$^{-/-}$ mouse knee joints revealed diminished loading-induced changes in the subchondral bone plate thickness and epiphyseal trabecular bone microarchitecture[132]. Together, these data suggest that the hypomineralized bone phenotype in *Phospho1*$^{-/-}$ mice provokes osteoarthritis pathology. This therefore implies that local modifications in the bone matrix mineralization may underpin subchondral bone

sclerosis in osteoarthritis, however further analyses are required to fully define this relationship.

There are also more speculative roles for PHOSPHO1 in osteoarthritis due to its role as a phosphatase capable of hydrolysing PCho and PEA. Lipidomic analysis of the synovial fluid from patients with osteoarthritis suggests that alterations in the phospholipid composition and concentrations are associated with disease development, due to the lubricating function of the synovial fluid in the joint[133]. Specifically, PC concentrations are increased in the synovial fluid of both early (2.7-fold) and late (5.4-fold) osteoarthritic patients in comparison to controls[133]. Similarly, there has been reported high activity of PLA_2, thought to breakdown PC, in the synovial fluid of osteoarthritic patients[134] and a role for PLA_2, in articular cartilage chondrocyte function[135]. It has been suggested that the ratio of PC to lysophosphatidylcholine is a good diagnostic marker for rheumatoid arthritis[136]. However, whether this is the case for osteoarthritis and the potential involvement of PHOSPHO1 in this is yet to be established.

8. Other physiological roles

In the 20 years since its characterization, the vast majority of research focussing on PHOSPHO1 has concentrated on biomineralization. However, as a phosphatase capable of hydrolysing PCho and PEA with the generation of metabolites such as choline, PHOSPHO1 has the potential for activity in many other body systems. PC and PE are two of the most abundant lipids in the body, comprising 40-50% and 15-20% respectively of total cellular phospholipids of any given tissue in mammals[137]. These molecules are therefore of critical importance in a huge variety of biological systems, from the integrity of plasma membranes, and other structures such as vesicles, lipoproteins and chylomicrons, to regulating the activity of proteins at the cell membrane[137,138]. Interestingly, the breakdown products from PC in

particular have also been implicated as signalling molecules[139,140]. Although speculative, it is therefore an intriguing possibility that PHOSPHO1 may have a role to play in the biosynthesis and catalysis pathways of these phospholipids, regulating substrate availability. Choline is the breakdown product from PHOSPHO1 activity on the LPL lysophosphatidylcholine derived from PC, and is an essential dietary nutrient in mammals. Although choline is primarily used in PC biosynthesis, it may also be used to generate betaine in the liver and kidney, or acetylated to form the neurotransmitter acetylcholine in the brain[141]. PHOSPHO1 may therefore be an appealing candidate to act within a secondary mechanism of choline homeostasis in these and other tissues. Indeed, gut enteroids maintained in choline-deficient media exhibited hypomethylation at 3' CpG islands within the *Phospho1* gene, potentially regulating gene expression[142].

One system within which PHOSPHO1 plays a significant role in this regard is erythropoiesis. An expression quantitative trait locus (eQTL) analysis initially found that single nucleotide polymorphisms (SNPs) associated with β- thalassemia in human peripheral blood samples were also associated with changes in *PHOSPHO1* expression[143]. PHOSPHO1 was also found to be substantially enriched in erythroblasts undergoing differentiation[144]. Subsequently Huang *et al* investigated PHOSPHO1 in terminal erythropoiesis; a process during which the phospholipid composition of red cells is substantially altered[145]. *PHOSPHO1* expression was significantly upregulated during erythroblast differentiation, and moreover loss of PHOSPHO1 induced defective erythropoiesis accompanied by a lack of choline generation and an increased phosphocholine:choline ratio.

Several other studies have found intriguing associations between *PHOSPHO1* expression and disorders of altered energy metabolism such as diabetes and obesity. Epigenome-wide association studies (EWAS) have found significant associations between methylation at loci within the *PHOSPHO1* and the future risk of type-2 diabetes in human cohorts[146,147].

Indeed, Willmer and colleagues highlighted differential methylation at sites within the *PHOSPHO1* gene as a potentially useful biomarker for clinical application in the early detection of type-2 diabetes[148]. Moreover, increased methylation was positively correlated with high density lipoprotein (HDL) concentration in blood and was decreased in muscle tissue from diabetic patients[147]. This relationship with HDL concentration was also reproduced independently in an EWAS focussed on serum lipid profiles conducted in a separate cohort[149]. Relatedly, Wu *et al* further found an association between *PHOSPHO1* and SNPs relating to clinical measures of obesity in a Chinese population[150]. These studies provide a promising avenue of investigation for future research to consider the mechanisms through which PHOSPHO1 may act to influence lipid metabolism and disorders of its dysregulation such as diabetes and obesity. Supporting this idea, changes in *Phospho1* expression have been found in several studies examining the thermogenic brown adipose tissue (BAT) and the browning of white adipose tissue (WAT), of great relevance to obesity. *Phospho1* is expressed at a higher level in BAT than WAT[151], and suppression of fatty acid oxidation in adipose tissues causes a downregulation in expression during cold challenge[152]. Furthermore, studies which have investigated creatine metabolism as an alternative pathway for thermogenesis in beige fat have implicated PHOSPHO1 in this process, although the specific mechanism remains elusive[153,154].

9. Inhibition of PHOSPHO1 – a therapeutic target?

The LOPAC and Spectrum chemical reference libraries were used to identify potential chemical inhibitors of PHOSPHO1 and semi-automated high-throughput chemical screening used to test inhibition of PEA hydrolysis[24]. Of those identified, three molecules, Lansoprazole, Ebseleln and SCH202676 exhibited non-competitive inhibition of recombinant PHOSPHO1 activity by 80% or more[24]. This study showed that neither Lansoprazole nor SCH202676 inhibited TNAP activity but Lansoprazole has recently been shown to act as a

non-competitive inhibitor of TNAP[155]. Following this Bravo *et al* synthesized a series of benzoisothiazolone inhibitors of PHOSPHO1, the final selection from which was tested and passed medicinal chemistry criteria in deliverability, metabolic stability, solubility and permeability, whilst also demonstrating no cellular toxicity[156].

The development of these compounds opens the potential for inhibition of PHOSPHO1 activity as a therapeutic intervention. Although there has been no experimental research exploring this possibility to date, the imputation of PHOSPHO1's association with pathologies in both biomineralization and lipid metabolism may make it an attractive drug target in the future. In terms of disorders of pathological mineralization, while the PHOSPHO1-mediated mineralization mechanism is critical for the proper development of the skeleton in immature organisms as has been discussed here, there is currently no evidence to suggest that it plays an active role in maintenance of the skeleton in adulthood. Ablation of mineralization in the adult may be desirable when attempting to control soft tissue mineralization *e.g.* vascular calcification and also ectopic bone formation as is the case in several musculoskeletal disorders including osteoarthritis. In these instances pharmacological inhibition of PHOSPHO1 may prove to represent an effective intervention which also entails limited adverse off-target effects.

10. Outlook

Over the past 20 years PHOSPHO1 has been an increasing focus of research in the bone biology and biomineralization communities. Work performed in our and in other groups has succeeded in establishing its canonical role in MV-mediated biomineralization of the skeleton and dentition, while opening new fields of investigation into its function in other body systems. Many questions remain to be answered surrounding aspects of PHOSPHO1's biology, including detailed elucidation of its upstream biochemical mechanism, targeting of

the enzyme to MVs, how local and systemic regulation is integrated with other aspects of bone biology during the bone formation process, and the precise role of PHOSPHO1in musculoskeletal disease. Future studies integrating aspects of structural biology and biomineralization with cellular and molecular biology and endocrinology will give us a holistic appreciation of these processes and contribute to our understanding in this fundamental aspect of bone biology.

11. Acknowledgments

S.D., K.A.S., J.L.M. and C.F. contributed to writing and reviewing the manuscript, and approved the final version.

12. References

1. Suchacki K, Roberts F, Lovdel A, Farquharson C, Morton NM, MacRae VE, et al. Skeletal energy homeostasis: a paradigm of endocrine discovery. Journal of Endocrinology. 2017:JOE-17-0147.
2. Reznikov N, Shahar R, Weiner S. Bone hierarchical structure in three dimensions. Acta biomaterialia. 2014;10(9):3815-26.
3. Reznikov N, Shahar R, Weiner S. Three-dimensional structure of human lamellar bone: the presence of two different materials and new insights into the hierarchical organization. Bone. 2014;59:93-104.
4. Houston B, Seawright E, Jefferies D, Hoogland E, Lester D, Whitehead C, et al. Identification and cloning of a novel phosphatase expressed at high levels in differentiating growth plate chondrocytes. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research. 1999;1448(3):500-6.

5. Houston B, Paton I, Burt D, Farquharson C. Chromosomal localization of the chicken and mammalian orthologues of the orphan phosphatase PHOSPHO1 gene. Animal genetics. 2002;33(6):451-4.
6. Stewart AJ, Schmid R, Blindauer CA, Paisey SJ, Farquharson C. Comparative modelling of human PHOSPHO1 reveals a new group of phosphatases within the haloacid dehalogenase superfamily. Protein engineering. 2003;16(12):889-95.
7. Baldwin JC, Karthikeyan AS, Raghothama KG. LEPS2, a phosphorus starvation-induced novel acid phosphatase from tomato. Plant physiology. 2001;125(2):728-37.
8. Stenzel I, Ziethe K, Schurath J, Hertel SC, Bosse D, Köck M. Differential expression of the LePS2 phosphatase gene family in response to phosphate availability, pathogen infection and during development. Physiologia plantarum. 2003;118(1):138-46.
9. Baldwin JC, Karthikeyan AS, Cao A, Raghothama KG. Biochemical and molecular analysis of LePS2; 1: a phosphate starvation induced protein phosphatase gene from tomato. Planta. 2008;228(2):273.
10. Wang W, Kim R, Jancarik J, Yokota H, Kim S-H. Crystal structure of phosphoserine phosphatase from Methanococcus jannaschii, a hyperthermophile, at 1.8 Å resolution. Structure. 2001;9(1):65-71.
11. Roberts SJ, Stewart AJ, Sadler PJ, Farquharson C. Human PHOSPHO1 exhibits high specific phosphoethanolamine and phosphocholine phosphatase activities. Biochemical Journal. 2004;382(1):59-65.
12. Roberts SJ, Stewart AJ, Schmid R, Blindauer CA, Bond SR, Sadler PJ, et al. Probing the substrate specificities of human PHOSPHO1 and PHOSPHO2. Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics. 2005;1752(1):73-82.

13. Nielson CM, Zmuda JM, Carlos AS, Wagoner WJ, Larson EA, Orwoll ES, et al. Rare coding variants in ALPL are associated with low serum alkaline phosphatase and low bone mineral density. Journal of Bone and Mineral Research. 2012;27(1):93-103.

14. Ozono K, Michigami T. Hypophosphatasia now draws more attention of both clinicians and researchers: A Commentary on prevelance of c. 1559delT in ALPL, a common mutation resulting in the perinatal (lethal) form of hypophosphatasias in Japanese and effects of the mutation on heterozygous carriers. Journal of human genetics. 2011;56(3):174.

15. Ermakov S, Toliat MR, Cohen Z, Malkin I, Altmüller J, Livshits G, et al. Association of ALPL and ENPP1 gene polymorphisms with bone strength related skeletal traits in a Chuvashian population. Bone. 2010;46(5):1244-50.

16. Narisawa S, Fröhlander N, Millán JL. Inactivation of two mouse alkaline phosphatase genes and establishment of a model of infantile hypophosphatasia. Developmental Dynamics. 1997;208(3):432-46.

17. Fedde KN, Blair L, Silverstein J, Coburn SP, Ryan LM, Weinstein RS, et al. Alkaline phosphatase knock-out mice recapitulate the metabolic and skeletal defects of infantile hypophosphatasia. Journal of Bone and Mineral Research. 1999;14(12):2015-26.

18. Liu J, Nam HK, Campbell C, da Silva Gasque KC, Millán JL, Hatch NE. Tissue-nonspecific alkaline phosphatase deficiency causes abnormal craniofacial bone development in the Alpl−/− mouse model of infantile hypophosphatasia. Bone. 2014;67:81-94.

19. Foster B, Nagatomo K, Tso H, Tran A, Nociti F, Narisawa S, et al. Tooth root dentin mineralization defects in a mouse model of hypophosphatasia. Journal of Bone and Mineral Research. 2013;28(2):271-82.

20. Anderson HC, Sipe JB, Hessle L, Dhamyamraju R, Atti E, Camacho NP, et al. Impaired calcification around matrix vesicles of growth plate and bone in alkaline phosphatase-deficient mice. The American journal of pathology. 2004;164(3):841-7.

21. Anderson HC, Hsu HH, Morris DC, Fedde KN, Whyte MP. Matrix vesicles in osteomalacic hypophosphatasia bone contain apatite-like mineral crystals. The American journal of pathology. 1997;151(6):1555.

22. Houston B, Stewart AJ, Farquharson C. PHOSPHO1—a novel phosphatase specifically expressed at sites of mineralisation in bone and cartilage. Bone. 2004;34(4):629-37.

23. MacRae VE, Davey MG, McTeir L, Narisawa S, Yadav MC, Millan JL, et al. Inhibition of PHOSPHO1 activity results in impaired skeletal mineralization during limb development of the chick. Bone. 2010;46(4):1146-55.

24. Roberts S, Narisawa S, Harmey D, Millán JL, Farquharson C. Functional involvement of PHOSPHO1 in matrix vesicle–mediated skeletal mineralization. Journal of Bone and Mineral Research. 2007;22(4):617-27.

25. Yadav MC, Simao AMS, Narisawa S, Huesa C, McKee MD, Farquharson C, et al. Loss of skeletal mineralization by the simultaneous ablation of PHOSPHO1 and alkaline phosphatase function: a unified model of the mechanisms of initiation of skeletal calcification. Journal of Bone and Mineral Research. 2011;26(2):286-97.

26. Huesa C, Yadav MC, Finnilä MA, Goodyear SR, Robins SP, Tanner KE, et al. PHOSPHO1 is essential for mechanically competent mineralization and the avoidance of spontaneous fractures. Bone. 2011;48(5):1066-74.

27. Carriero A, Bruse JL, Oldknow KJ, Millán JL, Farquharson C, Shefelbine SJ. Reference point indentation is not indicative of whole mouse bone measures of stress intensity fracture toughness. Bone. 2014;69:174-9.

28. Morcos M, Al-Jallad H, Li J, Farquharson C, Millán J, Hamdy R, et al. PHOSPHO1 is essential for normal bone fracture healing: An Animal Study. Bone & Joint Research. 2018;7(6):397-405.

29. Rodriguez-Florez N, Garcia-Tunon E, Mukadam Q, Saiz E, Oldknow KJ, Farquharson C, et al. An investigation of the mineral in ductile and brittle cortical mouse bone. Journal of Bone and Mineral Research. 2015;30(5):786-95.

30. Boyde A, Staines KA, Javaheri B, Millan JL, Pitsillides AA, Farquharson C. A distinctive patchy osteomalacia characterises Phospho1-deficient mice. Journal of anatomy. 2017;231(2):298-308.

31. Javaheri B, Carriero A, Staines K, Chang Y-M, Houston D, Oldknow KJ, et al. Phospho1 deficiency transiently modifies bone architecture yet produces consistent modification in osteocyte differentiation and vascular porosity with ageing. Bone. 2015;81:277-91.

32. Whyte MP. Hypophosphatasia and the role of alkaline phosphatase in skeletal mineralization. Endocrine reviews. 1994;15(4):439-61.

33. Huesa C, Houston D, Kiffer-Moreira T, Yadav MC, Millan JL, Farquharson C. The functional co-operativity of tissue-nonspecific alkaline phosphatase (TNAP) and PHOSPHO1 during initiation of skeletal mineralization. Biochemistry and biophysics reports. 2015;4:196-201.

34. Addison WN, Azari F, Sørensen ES, Kaartinen MT, McKee MD. Pyrophosphate inhibits mineralization of osteoblast cultures by binding to mineral, up-regulating osteopontin, and inhibiting alkaline phosphatase activity. Journal of Biological Chemistry. 2007;282(21):15872-83.

35. Harmey D, Hessle L, Narisawa S, Johnson KA, Terkeltaub R, Millán JL. Concerted regulation of inorganic pyrophosphate and osteopontin by akp2, enpp1, and ank: an

integrated model of the pathogenesis of mineralization disorders. The American journal of pathology. 2004;164(4):1199-209.

36. Johnson K, Goding J, Van Etten D, Sali A, Hu SI, Farley D, et al. Linked deficiencies in extracellular PPi and osteopontin mediate pathologic calcification associated with defective PC-1 and ANK expression. Journal of bone and mineral research. 2003;18(6):994-1004.

37. Speer MY, McKee MD, Guldberg RE, Liaw L, Yang H-Y, Tung E, et al. Inactivation of the osteopontin gene enhances vascular calcification of matrix Gla protein–deficient mice: evidence for osteopontin as an inducible inhibitor of vascular calcification in vivo. Journal of Experimental Medicine. 2002;196(8):1047-55.

38. Steitz SA, Speer MY, McKee MD, Liaw L, Almeida M, Yang H, et al. Osteopontin inhibits mineral deposition and promotes regression of ectopic calcification. The American journal of pathology. 2002;161(6):2035-46.

39. Boskey A, Spevak L, Paschalis E, Doty S, McKee M. Osteopontin deficiency increases mineral content and mineral crystallinity in mouse bone. Calcified tissue international. 2002;71(2):145-54.

40. Boskey AL, Christensen B, Taleb H, Sørensen ES. Post-translational modification of osteopontin: effects on in vitro hydroxyapatite formation and growth. Biochemical and biophysical research communications. 2012;419(2):333-8.

41. George A, Veis A. Phosphorylated proteins and control over apatite nucleation, crystal growth, and inhibition. Chemical reviews. 2008;108(11):4670-93.

42. Narisawa S, Yadav MC, Millán JL. In vivo overexpression of tissue-nonspecific alkaline phosphatase increases skeletal mineralization and affects the phosphorylation status of osteopontin. Journal of bone and mineral research. 2013;28(7):1587-98.

43. Yadav MC, Huesa C, Narisawa S, Hoylaerts MF, Moreau A, Farquharson C, et al. Ablation of Osteopontin Improves the Skeletal Phenotype of Phospho1−/− Mice. Journal of Bone and Mineral Research. 2014;29(11):2369-81.

44. McKee M, Yadav M, Foster B, Somerman M, Farquharson C, Millán J. Compounded PHOSPHO1/ALPL deficiencies reduce dentin mineralization. Journal of dental research. 2013;92(8):721-7.

45. Zweifler L, Ao M, Yadav M, Kuss P, Narisawa S, Kolli T, et al. Role of PHOSPHO1 in periodontal development and function. Journal of dental research. 2016;95(7):742-51.

46. Ao M, Chavez M, Chu E, Hemstreet K, Yin Y, Yadav M, et al. Overlapping functions of bone sialoprotein and pyrophosphate regulators in directing cementogenesis. Bone. 2017;105:134-47.

47. Pandya M, Rosene L, Farquharson C, Millán JL, Diekwisch TG. Intravesicular Phosphatase PHOSPHO1 Function in Enamel Mineralization and Prism Formation. Frontiers in physiology. 2017;8:805.

48. Kuzynski M, Goss M, Bottini M, Yadav MC, Mobley C, Winters T, et al. Dual role of the Trps1 transcription factor in dentin mineralization. Journal of Biological Chemistry. 2014:jbc. M114. 550129.

49. Napierala D, Sam K, Morello R, Zheng Q, Munivez E, Shivdasani RA, et al. Uncoupling of chondrocyte differentiation and perichondrial mineralization underlies the skeletal dysplasia in tricho-rhino-phalangeal syndrome. Human molecular genetics. 2008;17(14):2244-54.

50. Fantauzzo KA, Tadin-Strapps M, You Y, Mentzer SE, Baumeister FA, Cianfarani S, et al. A position effect on TRPS1 is associated with Ambras syndrome in humans and the Koala phenotype in mice. Human molecular genetics. 2008;17(22):3539-51.

51. Momeni P, Glöckner G, Schmidt O, von Holtum D, Albrecht B, Gillessen-Kaesbach G, et al. Mutations in a new gene, encoding a zinc-finger protein, cause tricho-rhino-phalangeal syndrome type I. Nature genetics. 2000;24(1):71.

52. Kantaputra P, Miletich I, Lüdecke H-J, Suzuki E, Praphanphoj V, Shivdasani R, et al. Tricho-rhino-phalangeal syndrome with supernumerary teeth. Journal of dental research. 2008;87(11):1027-31.

53. Cui L, Houston D, Farquharson C, MacRae V. Characterisation of matrix vesicles in skeletal and soft tissue mineralisation. Bone. 2016;87:147-58.

54. Anderson HC. Electron microscopic studies of induced cartilage development and calcification. The Journal of cell biology. 1967;35(1):81.

55. Bonucci E. Fine structure of early cartilage calcification. Journal of ultrastructure research. 1967;20(1-2):33-50.

56. Landis WJ, Paine MC, Glimcher MJ. Electron microscopic observations of bone tissue prepared anhydrously in organic solvents. Journal of ultrastructure research. 1977;59(1):1-30.

57. Anderson HC. Vesicles associated with calcification in the matrix of epiphyseal cartilage. The Journal of cell biology. 1969;41(1):59-72.

58. Anderson HC, Cecil R, Sajdera SW. Calcification of rachitic rat cartilage in vitro by extracellular matrix vesicles. The American journal of pathology. 1975;79(2):237.

59. Bonucci E. The locus of initial calcification in cartilage and bone. Clinical Orthopaedics and Related Research®. 1971;78:108-39.

60. Landis WJ. A study of calcification in the leg tendons from the domestic turkey. Journal of ultrastructure and molecular structure research. 1986;94(3):217-38.

61. Reynolds JL, Joannides AJ, Skepper JN, McNair R, Schurgers LJ, Proudfoot D, et al. Human vascular smooth muscle cells undergo vesicle-mediated calcification in

response to changes in extracellular calcium and phosphate concentrations: a potential mechanism for accelerated vascular calcification in ESRD. Journal of the American Society of Nephrology. 2004;15(11):2857-67.

62. Kapustin AN, Shanahan CM. Calcium regulation of vascular smooth muscle cell–derived matrix vesicles. Trends in cardiovascular medicine. 2012;22(5):133-7.

63. Cui L, Rashdan NA, Zhu D, Milne EM, Ajuh P, Milne G, et al. End stage renal disease-induced hypercalcemia may promote aortic valve calcification via Annexin VI enrichment of valve interstitial cell derived-matrix vesicles. Journal of cellular physiology. 2017;232(11):2985-95.

64. Hasegawa T, Yamamoto T, Tsuchiya E, Hongo H, Tsuboi K, Kudo A, et al. Ultrastructural and biochemical aspects of matrix vesicle-mediated mineralization. Japanese Dental Science Review. 2017;53(2):34-45.

65. Bottini M, Mebarek S, Anderson KL, Strzelecka-Kiliszek A, Bozycki L, Simão AMS, et al. Matrix vesicles from chondrocytes and osteoblasts: Their biogenesis, properties, functions and biomimetic models. Biochimica et Biophysica Acta (BBA)-General Subjects. 2018;1862(3):532-46.

66. Murshed M. Mechanism of bone mineralization. Cold Spring Harbor perspectives in medicine. 2018:a031229.

67. Anderson HC. Matrix vesicles and calcification. Current rheumatology reports. 2003;5(3):222-6.

68. Borg TK, Runyan RB, Wuthier RE. Correlation of freeze-fracture and scanning electron microscopy of epiphyseal chondrocytes. Calcified tissue research. 1978;26(1):237-41.

69. Anderson HC. Molecular biology of matrix vesicles. Clinical orthopaedics and related research. 1995;314:266-80.

70. Fedde KN. Human osteosarcoma cells spontaneously release matrix-vesicle-like structures with the capacity to mineralize. Bone and mineral. 1992;17(2):145-51.

71. Thouverey C, Strzelecka-Kiliszek A, Balcerzak M, Buchet R, Pikula S. Matrix vesicles originate from apical membrane microvilli of mineralizing osteoblast-like Saos-2 cells. Journal of cellular biochemistry. 2009;106(1):127-38.

72. Thouverey C, Bechkoff G, Pikula S, Buchet R. Inorganic pyrophosphate as a regulator of hydroxyapatite or calcium pyrophosphate dihydrate mineral deposition by matrix vesicles. Osteoarthritis and cartilage. 2009;17(1):64-72.

73. Shapiro IM, Golub EE, Chance B, Piddington C, Oshima O, Tuncay OC, et al. Linkage between energy status of perivascular cells and mineralization of the chick growth cartilage. Developmental biology. 1988;129(2):372-9.

74. Wuthier RE, Lipscomb GF. Matrix vesicles: structure, composition, formation and function in calcification. Front Biosci. 2011;16:2812-902.

75. Chaudhary SC, Kuzynski M, Bottini M, Beniash E, Dokland T, Mobley CG, et al. Phosphate induces formation of matrix vesicles during odontoblast-initiated mineralization in vitro. Matrix Biology. 2016;52:284-300.

76. Hessle L, Johnson KA, Anderson HC, Narisawa S, Sali A, Goding JW, et al. Tissue-nonspecific alkaline phosphatase and plasma cell membrane glycoprotein-1 are central antagonistic regulators of bone mineralization. Proceedings of the National Academy of Sciences. 2002;99(14):9445-9.

77. Stewart AJ, Roberts SJ, Seawright E, Davey MG, Fleming RH, Farquharson C. The presence of PHOSPHO1 in matrix vesicles and its developmental expression prior to skeletal mineralization. Bone. 2006;39(5):1000-7.

78. Millán JL. The role of phosphatases in the initiation of skeletal mineralization. Calcified tissue international. 2013;93(4):299-306.

79. Thouverey C, Malinowska A, Balcerzak M, Strzelecka-Kiliszek A, Buchet R, Dadlez M, et al. Proteomic characterization of biogenesis and functions of matrix vesicles released from mineralizing human osteoblast-like cells. Journal of proteomics. 2011;74(7):1123-34.

80. Ciancaglini P, Yadav MC, Simao AMS, Narisawa S, Pizauro JM, Farquharson C, et al. Kinetic analysis of substrate utilization by native and TNAP-, NPP1-, or PHOSPHO1-deficient matrix vesicles. Journal of Bone and Mineral Research. 2010;25(4):716-23.

81. Yadav MC, Bottini M, Cory E, Bhattacharya K, Kuss P, Narisawa S, et al. Skeletal Mineralization Deficits and Impaired Biogenesis and Function of Chondrocyte-Derived Matrix Vesicles in Phospho1–/–and Phospho1/Pit1 Double-Knockout Mice. Journal of Bone and Mineral Research. 2016;31(6):1275-86.

82. Stewart AJ, Leong DT, Farquharson C. PLA2 and ENPP6 may act in concert to generate phosphocholine from the matrix vesicle membrane during skeletal mineralization. The FASEB Journal. 2017;32(1):20-5.

83. Burke JE, Dennis EA. Phospholipase A2 structure/function, mechanism, and signaling. Journal of lipid research. 2009;50(Supplement):S237-S42.

84. Dennis EA, Cao J, Hsu Y-H, Magrioti V, Kokotos G. Phospholipase A2 enzymes: physical structure, biological function, disease implication, chemical inhibition, and therapeutic intervention. Chemical reviews. 2011;111(10):6130-85.

85. Murakami M, Taketomi Y, Miki Y, Sato H, Hirabayashi T, Yamamoto K. Recent progress in phospholipase A2 research: from cells to animals to humans. Progress in lipid research. 2011;50(2):152-92.

86. Wu LN, Genge BR, Kang MW, Arsenault AL, Wuthier RE. Changes in phospholipid extractability and composition accompany mineralization of chicken growth plate cartilage matrix vesicles. Journal of Biological Chemistry. 2002;277(7):5126-33.

87. Wuthier RE. Lipid composition of isolated epiphyseal cartilage cells, membranes and matrix vesicles. Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism. 1975;409(1):128-43.

88. Fujino Y, Minamizaki T, Yoshioka H, Okada M, Yoshiko Y. Imaging and mapping of mouse bone using MALDI-imaging mass spectrometry. Bone reports. 2016;5:280-5.

89. Mebarek S, Abousalham A, Magne D, Do L, Bandorowicz-Pikula J, Pikula S, et al. Phospholipases of mineralization competent cells and matrix vesicles: roles in physiological and pathological mineralizations. International journal of molecular sciences. 2013;14(3):5036-129.

90. Ramanadham S, Yarasheski KE, Silva MJ, Wohltmann M, Novack DV, Christiansen B, et al. Age-related changes in bone morphology are accelerated in group VIA phospholipase A2 (iPLA2β)-null mice. The American journal of pathology. 2008;172(4):868-81.

91. Stefan C, Jansen S, Bollen M. NPP-type ectophosphodiesterases: unity in diversity. Trends in biochemical sciences. 2005;30(10):542-50.

92. Sakagami H, Aoki J, Natori Y, Nishikawa K, Kakehi Y, Natori Y, et al. Biochemical and molecular characterization of a novel choline-specific glycerophosphodiester phosphodiesterase belonging to the nucleotide pyrophosphatase/phosphodiesterase family. Journal of Biological Chemistry. 2005;280(24):23084-93.

93. Morita J, Kato K, Mihara E, Ishitani R, Takagi J, Nishimasu H, et al. Expression, purification, crystallization and preliminary X-ray crystallographic analysis of Enpp6. Acta Crystallographica Section F. 2014;70(6):794-9.

94. Khavandgar Z, Murshed M. Sphingolipid metabolism and its role in the skeletal tissues. Cellular and molecular life sciences. 2015;72(5):959-69.

95. Stoffel W, Jenke B, Blöck B, Zumbansen M, Koebke J. Neutral sphingomyelinase 2 (smpd3) in the control of postnatal growth and development. Proceedings of the National Academy of Sciences. 2005;102(12):4554-9.

96. Stoffel W, Knifka J, Koebke J, Niehoff A, Jenke B, Holz B, et al. Neutral sphingomyelinase (SMPD3) deficiency causes a novel form of chondrodysplasia and dwarfism that is rescued by Col2A1-driven smpd3 transgene expression. The American journal of pathology. 2007;171(1):153-61.

97. Khavandgar Z, Poirier C, Clarke CJ, Li J, Wang N, McKee MD, et al. A cell-autonomous requirement for neutral sphingomyelinase 2 in bone mineralization. The Journal of cell biology. 2011;194(2):277-89.

98. Li J, Manickam G, Ray S, Yasuda H, Moffatt P, Murshed M. Smpd3 expression in both chondrocytes and osteoblasts is required for normal endochondral bone development. Molecular and cellular biology. 2016:MCB. 01077-15.

99. Khavandgar Z, Alebrahim S, Eimar H, Tamimi F, McKee M, Murshed M. Local regulation of tooth mineralization by sphingomyelin phosphodiesterase 3. Journal of dental research. 2013;92(4):358-64.

100. Manickam G, Moffatt P, Murshed M. Role of SMPD3 during Bone Fracture Healing and Regulation of its Expression. Molecular and cellular biology. 2018:MCB. 00370-18.

101. Wu G, Aoyama C, Young SG, Vance DE. Early embryonic lethality caused by disruption of the gene for choline kinase α, the first enzyme in phosphatidylcholine biosynthesis. Journal of Biological Chemistry. 2008;283(3):1456-62.

102. Li Z, Wu G, Sher RB, Khavandgar Z, Hermansson M, Cox GA, et al. Choline kinase beta is required for normal endochondral bone formation. Biochimica et Biophysica Acta (BBA)-General Subjects. 2014;1840(7):2112-22.

103. Li Z, Wu G, van der Veen JN, Hermansson M, Vance DE. Phosphatidylcholine metabolism and choline kinase in human osteoblasts. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids. 2014;1841(6):859-67.

104. Compston JE. Sex steroids and bone. Physiological reviews. 2001;81(1):419-47.

105. Frenkel B, Hong A, Baniwal SK, Coetzee GA, Ohlsson C, Khalid O, et al. Regulation of adult bone turnover by sex steroids. Journal of cellular physiology. 2010;224(2):305-10.

106. Riggs BL, Khosla S, Melton III LJ. Sex steroids and the construction and conservation of the adult skeleton. Endocrine reviews. 2002;23(3):279-302.

107. Thacher TD, Clarke BL. Vitamin D insufficiency. Mayo Clinic Proceedings: Elsevier; 2011. p. 50-60.

108. Yamamoto Y, Yoshizawa T, Fukuda T, Shirode-Fukuda Y, Yu T, Sekine K, et al. Vitamin D receptor in osteoblasts is a negative regulator of bone mass control. Endocrinology. 2013;154(3):1008-20.

109. Priemel M, von Domarus C, Klatte TO, Kessler S, Schlie J, Meier S, et al. Bone mineralization defects and vitamin D deficiency: histomorphometric analysis of iliac crest bone biopsies and circulating 25-hydroxyvitamin D in 675 patients. Journal of Bone and Mineral Research. 2010;25(2):305-12.

110. Compston JE. Skeletal actions of intermittent parathyroid hormone: effects on bone remodelling and structure. Bone. 2007;40(6):1447-52.

111. Jiang Y, Zhao JJ, Mitlak BH, Wang O, Genant HK, Eriksen EF. Recombinant human parathyroid hormone (1–34)[teriparatide] improves both cortical and cancellous bone structure. Journal of Bone and Mineral Research. 2003;18(11):1932-41.

112. Hodsman AB, Bauer DC, Dempster DW, Dian L, Hanley DA, Harris ST, et al. Parathyroid hormone and teriparatide for the treatment of osteoporosis: a review of the evidence and suggested guidelines for its use. Endocrine reviews. 2005;26(5):688-703.

113. John HCS, Meyer MB, Benkusky NA, Carlson AH, Prideaux M, Bonewald LF, et al. The parathyroid hormone-regulated transcriptome in osteocytes: Parallel actions with 1, 25-dihydroxyvitamin D3 to oppose gene expression changes during differentiation and to promote mature cell function. Bone. 2015;72:81-91.

114. Houston D, Myers K, MacRae V, Staines K, Farquharson C. The expression of PHOSPHO1, nSMase2 and TNAP is coordinately regulated by continuous PTH exposure in mineralising osteoblast cultures. Calcified tissue international. 2016;99(5):510-24.

115. Allan EH, Häusler KD, Wei T, Gooi JH, Quinn JM, Crimeen-Irwin B, et al. EphrinB2 regulation by PTH and PTHrP revealed by molecular profiling in differentiating osteoblasts. Journal of Bone and Mineral Research. 2008;23(8):1170-81.

116. Gooi J, Pompolo S, Karsdal M, Kulkarni N, Kalajzic I, McAhren S, et al. Calcitonin impairs the anabolic effect of PTH in young rats and stimulates expression of sclerostin by osteocytes. Bone. 2010;46(6):1486-97.

117. Eriksen EF. Primary hyperparathyroidism: lessons from bone histomorphometry. Journal of bone and mineral research: the official journal of the American Society for Bone and Mineral Research. 2002;17:N95-7.

118. Locklin RM, Khosla S, Turner RT, Riggs BL. Mediators of the biphasic responses of bone to intermittent and continuously administered parathyroid hormone. Journal of cellular biochemistry. 2003;89(1):180-90.

119. Ma YL, Cain RL, Halladay DL, Yang X, Zeng Q, Miles RR, et al. Catabolic effects of continuous human PTH (1–38) in vivo is associated with sustained stimulation of RANKL and inhibition of osteoprotegerin and gene-associated bone formation. Endocrinology. 2001;142(9):4047-54.

120. Krishnan V, Moore TL, Ma YL, Helvering LM, Frolik CA, Valasek KM, et al. Parathyroid hormone bone anabolic action requires Cbfa1/Runx2-dependent signaling. Molecular Endocrinology. 2003;17(3):423-35.

121. Nishimura R, Wakabayashi M, Hata K, Matsubara T, Honma S, Wakisaka S, et al. Osterix regulates calcification and degradation of chondrogenic matrices through matrix metalloproteinase 13 (MMP13) expression in association with transcription factor Runx2 during endochondral ossification. Journal of Biological Chemistry. 2012;287(40):33179-90.

122. Kiffer-Moreira T, Yadav MC, Zhu D, Narisawa S, Sheen C, Stec B, et al. Pharmacological inhibition of PHOSPHO1 suppresses vascular smooth muscle cell calcification. Journal of Bone and Mineral Research. 2013;28(1):81-91.

123. Tan L, Wang Z, Li Y. Rabbit models provide insights into bone formation related biological process in atherosclerotic vascular calcification. Biochemical and biophysical research communications. 2018;496(4):1369-75.

124. Hortells L, Sosa C, Guillén N, Lucea S, Millán Á, Sorribas V. Identifying early pathogenic events during vascular calcification in uremic rats. Kidney international. 2017;92(6):1384-94.

125. V Bobryshev Y, N Orekhov A, Sobenin I, A Chistiakov D. Role of bone-type tissue-nonspecific alkaline phosphatase and PHOSPO1 in vascular calcification. Current pharmaceutical design. 2014;20(37):5821-8.

126. Bertazzo S, Gentleman E. Aortic valve calcification: a bone of contention. European heart journal. 2017;38(16):1189-93.

127. Bertazzo S, Gentleman E, Cloyd KL, Chester AH, Yacoub MH, Stevens MM. Nano-analytical electron microscopy reveals fundamental insights into human cardiovascular tissue calcification. Nature materials. 2013;12(6):576.

128. Hutcheson JD, Goettsch C, Bertazzo S, Maldonado N, Ruiz JL, Goh W, et al. Genesis and growth of extracellular-vesicle-derived microcalcification in atherosclerotic plaques. Nature materials. 2016;15(3):335.

129. van Engeland NC, Bertazzo S, Sarathchandra P, McCormack A, Bouten CV, Yacoub MH, et al. Aortic calcified particles modulate valvular endothelial and interstitial cells. Cardiovascular Pathology. 2017;28:36-45.

130. Li B, Aspden RM. Mechanical and material properties of the subchondral bone plate from the femoral head of patients with osteoarthritis or osteoporosis. Annals of the Rheumatic Diseases. 1997;56(4):247-54.

131. Chen L, Fink T, Zhang X-Y, Ebbesen P, Zachar V. Quantitative transcriptional profiling of ATDC5 mouse progenitor cells during chondrogenesis. Differentiation. 2005;73(7):350-63.

132. Staines KA, Brain F, Javaheri B, Houston D, Millan JL, Buttle DJ, et al. Hypomineralisation drives joint instability and osteoarthritis in mice. In: Committee BRS, editor. Bone Research Society Annual Meeting 2016; Liverpool, UK: Frontiers in Bone Research; 2016. p. 39.

133. Kosinska MK, Liebisch G, Lochnit G, Wilhelm J, Klein H, Kaesser U, et al. A lipidomic study of phospholipid classes and species in human synovial fluid. Arthritis & Rheumatism. 2013;65(9):2323-33.

134. Pruzanski W, Bogoch E, Wloch M, Vadas P. The role of phospholipase A2 in the physiopathology of osteoarthritis. The Journal of rheumatology Supplement. 1991;27:117-9.

135. Leistad L, Feuerherm A, Faxvaag A, Johansen B. Multiple phospholipase A2 enzymes participate in the inflammatory process in osteoarthritic cartilage. Scandinavian journal of rheumatology. 2011;40(4):308-16.

136. Fuchs B, Schiller J, Wagner U, Häntzschel H, Arnold K. The phosphatidylcholine/lysophosphatidylcholine ratio in human plasma is an indicator of the severity of rheumatoid arthritis: investigations by 31P NMR and MALDI-TOF MS. Clinical biochemistry. 2005;38(10):925-33.

137. van der Veen JN, Kennelly JP, Wan S, Vance JE, Vance DE, Jacobs RL. The critical role of phosphatidylcholine and phosphatidylethanolamine metabolism in health and disease. Elsevier; 2017.

138. Cole LK, Vance JE, Vance DE. Phosphatidylcholine biosynthesis and lipoprotein metabolism. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids. 2012;1821(5):754-61.

139. Exton J. Phosphatidylcholine breakdown and signal transduction. Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism. 1994;1212(1):26-42.

140. Exton J. Signaling through phosphatidylcholine breakdown. Journal of Biological Chemistry. 1990;265(1):1-4.

141. Li Z, Vance DE. Thematic review series: glycerolipids. Phosphatidylcholine and choline homeostasis. Journal of lipid research. 2008;49(6):1187-94.

142. Alves da Silva AV, de Castro Oliveira SB, Di Rienzi SC, Brown-Steinke K, Dehan LM, Rood JK, et al. Murine Methyl Donor Deficiency Impairs Early Growth in Association with Dysmorphic Small Intestinal Crypts and Reduced Gut Microbial Community Diversity. Current developments in nutrition. 2018;3(1):nzy070.

143. Westra H-J, Peters MJ, Esko T, Yaghootkar H, Schurmann C, Kettunen J, et al. Systematic identification of trans eQTLs as putative drivers of known disease associations. Nature genetics. 2013;45(10):1238.

144. Gautier E-F, Ducamp S, Leduc M, Salnot V, Guillonneau F, Dussiot M, et al. Comprehensive proteomic analysis of human erythropoiesis. Cell reports. 2016;16(5):1470-84.

145. Huang N-J, Lin Y-C, Lin C-Y, Pishesha N, Lewis CA, Freinkman E, et al. Enhanced phosphocholine metabolism is essential for terminal erythropoiesis. Blood. 2018:blood-2018-03-838516.

146. Chambers JC, Loh M, Lehne B, Drong A, Kriebel J, Motta V, et al. Epigenome-wide association of DNA methylation markers in peripheral blood from Indian Asians and Europeans with incident type 2 diabetes: a nested case-control study. The lancet Diabetes & endocrinology. 2015;3(7):526-34.

147. Dayeh T, Tuomi T, Almgren P, Perfilyev A, Jansson P-A, de Mello VD, et al. DNA methylation of loci within ABCG1 and PHOSPHO1 in blood DNA is associated with future type 2 diabetes risk. Epigenetics. 2016;11(7):482-8.

148. Willmer T, Johnson R, Louw J, Pheiffer C. Blood based DNA methylation biomarkers for type 2 diabetes: potential for clinical applications. Frontiers in endocrinology. 2018;9:744.

149. Sayols-Baixeras S, Subirana I, Lluis-Ganella C, Civeira F, Roquer J, Do A, et al. Identification and validation of seven new loci showing differential DNA methylation

related to serum lipid profile: an epigenome-wide approach. The REGICOR study. Human molecular genetics. 2016;25(20):4556-65.

150. Wu Y, Duan H, Tian X, Xu C, Wang W, Jiang W, et al. Genetics of Obesity Traits: A Bivariate Genome-Wide Association Analysis. Frontiers in genetics. 2018;9.

151. Wang H, Liu L, Lin JZ, Aprahamian TR, Farmer SR. Browning of white adipose tissue with roscovitine induces a distinct population of UCP1+ adipocytes. Cell metabolism. 2016;24(6):835-47.

152. Lee J, Choi J, Aja S, Scafidi S, Wolfgang MJ. Loss of adipose fatty acid oxidation does not potentiate obesity at thermoneutrality. Cell reports. 2016;14(6):1308-16.

153. Kazak L, Chouchani ET, Jedrychowski MP, Erickson BK, Shinoda K, Cohen P, et al. A creatine-driven substrate cycle enhances energy expenditure and thermogenesis in beige fat. Cell. 2015;163(3):643-55.

154. Bertholet AM, Kazak L, Chouchani ET, Bogaczyńska MG, Paranjpe I, Wainwright GL, et al. Mitochondrial patch clamp of beige adipocytes reveals UCP1-positive and UCP1-negative cells both exhibiting futile creatine cycling. Cell metabolism. 2017;25(4):811-22. e4.

155. Delomenède M, Buchet R, Mebarek S. Lansoprazole is an uncompetitive inhibitor of tissue-nonspecific alkaline phosphatase. Acta Biochimica Polonica. 2009;56(2):301.

156. Bravo Y, Teriete P, Dhanya R-P, Dahl R, San Lee P, Kiffer-Moreira T, et al. Design, synthesis and evaluation of benzoisothiazolones as selective inhibitors of PHOSPHO1. Bioorganic & medicinal chemistry letters. 2014;24(17):4308-11.

Figures

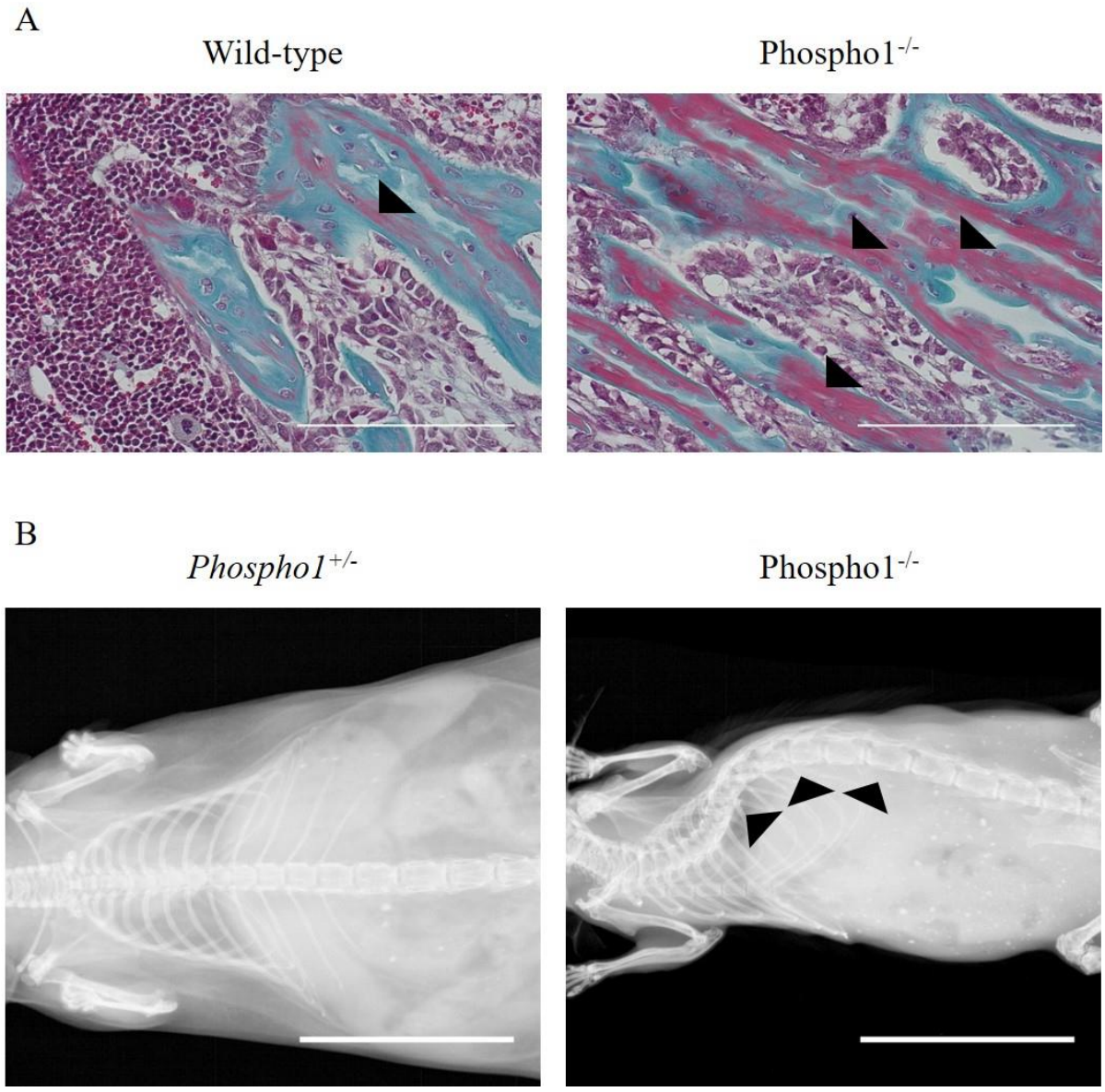

Figure 1: A) Goldner's trichrome staining of wild-type and *Phospho1*$^{-/-}$ tibial sections demonstrating osteoid accumulation in the trabeculae bone (red staining; black arrowheads). Tibiae were dissected from 21 day-old C67BL/6 mice and fixed in 4% paraformaldehyde before decalcification in 10% EDTA in PBS and standard histological processing. 3µm sections were cut using a rotary microtome and used for staining. Scale bars represent 200µm. B) Radiographic images of 1-year old *Phospho1*$^{+/-}$ and *Phospho1*$^{-/-}$ mice demonstrating thoracic scoliosis on ablation of *Phospho1* (black arrowheads). Whole-body images were acquired using a MX20 Specimen Radiograph System (Faxitron, USA). Scale bars represent 20mm.

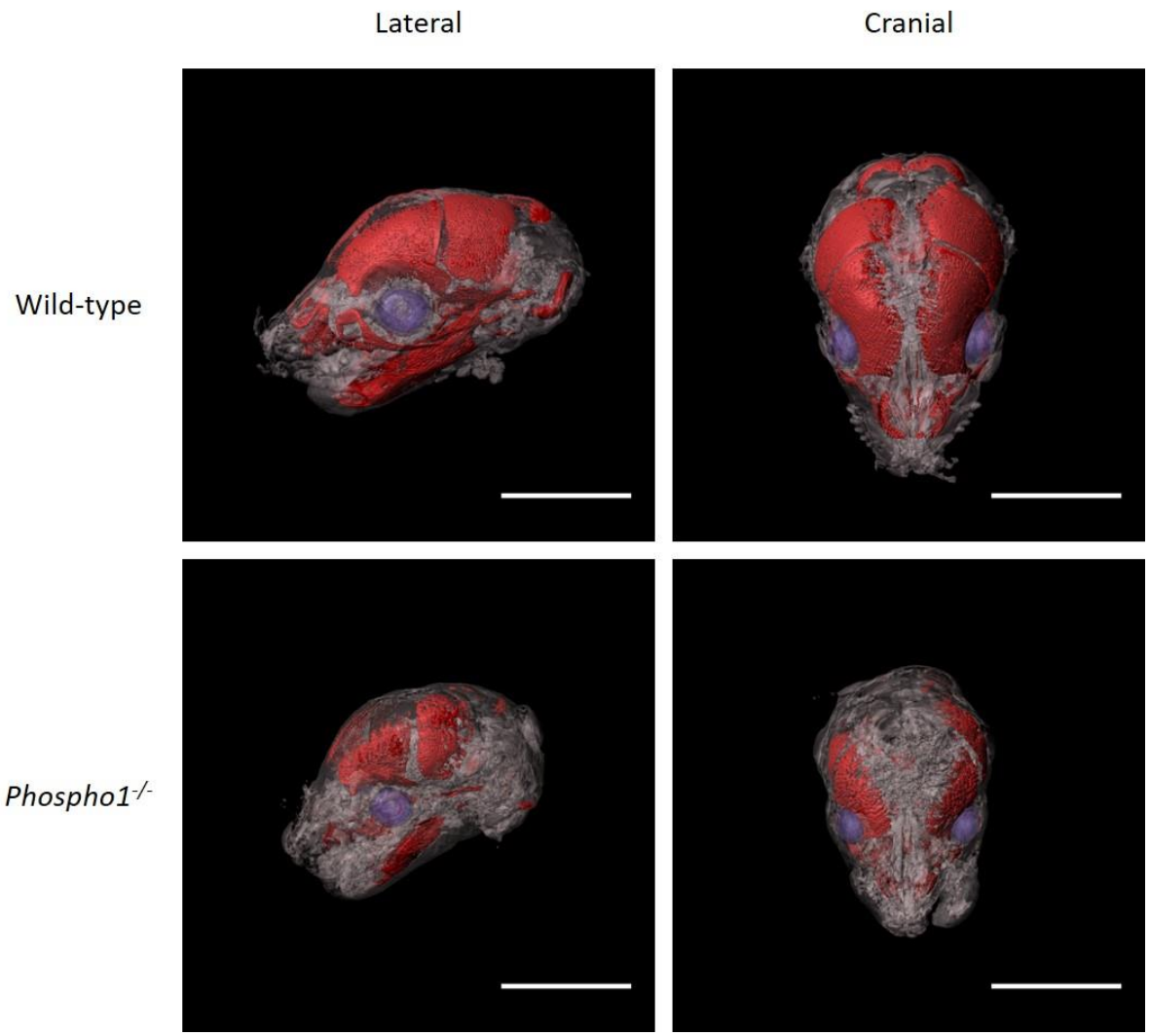

Figure 2: Optical projection tomography (OPT) reconstructions of mouse embryos at 17 days of gestation (E17) showing marked ablation of mineralization in bones of the skull in *Phospho1*$^{-/-}$ animals. E17 C67BL/6 wild-type and *Phospho1*$^{-/-}$ embryos were culled using Schedule 1 methods, fixed in 4% paraformaldehyde and whole mount stained in a 0.001% Alizarin Red solution in 1% potassium hydroxide. Samples were embedded in 1% agarose and scanned using a Bioptonics 3001 OPT Scanner (Bioptonics, UK). Data were reconstructed using NRecon (Bruker, USA) and visualised using Imaris (Bitplane, UK). Scale bars represent 10mm.

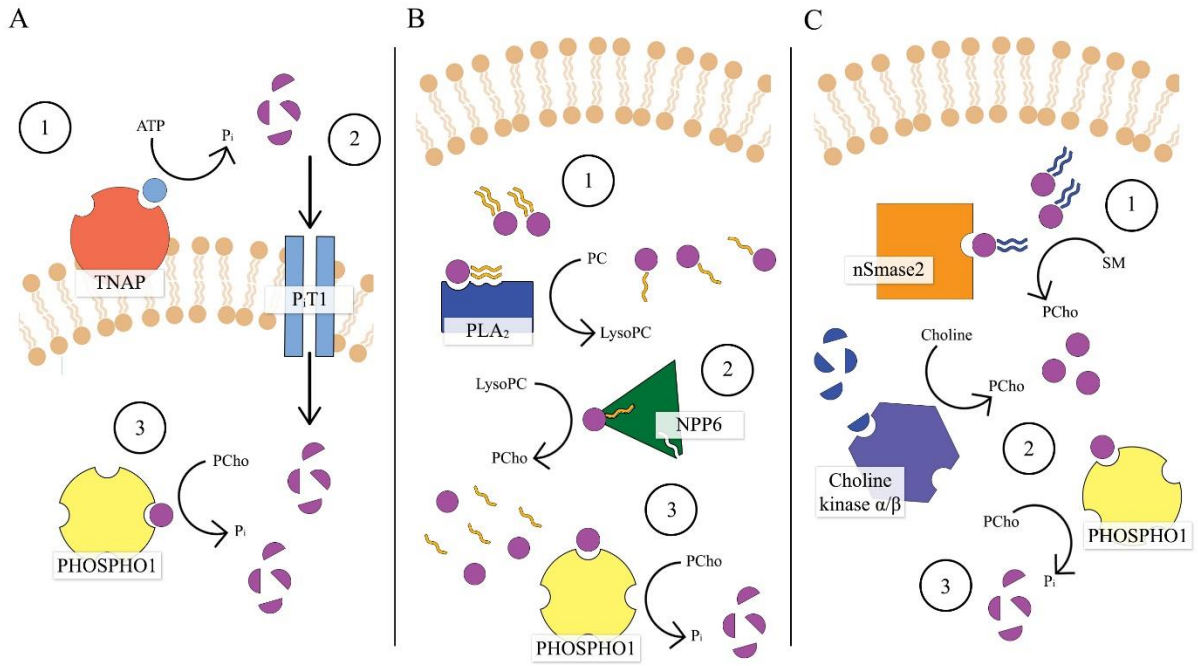

Figure 3: Schematic diagram illustrating the hypothesised mechanism of PHOSPHO1 function within MVs. A) PHOSPHO1 functions synergistically with TNAP: 1] TNAP hydrolyses its substrates to produce P_i extravesicuarly; 2] Extravesicular P_i is transported into the MV via P_iT1; 3] PHOSPHO1 hydrolyses Pcho intravesicuarly to further accumulate P_i. B) Generation of PHOSPHO1 substrates within MVs: 1] An unidentified PLA_2 converts PC from the vesicle membrane to lysoPC; 2] NPP6 subsequently catalyses the hydrolysis of lysoPC to generate Pcho; 3] PHOSPHO1 liberates P_i from PCho. C) Alternative pathways of PCho generation: 1] nSmase2 breaks down SM from the MV membrane to form Pcho; 2] The α/β choline kinases phosphorylate choline to form PCho; 3] PHOSPHO1 generates P_i from PCho.